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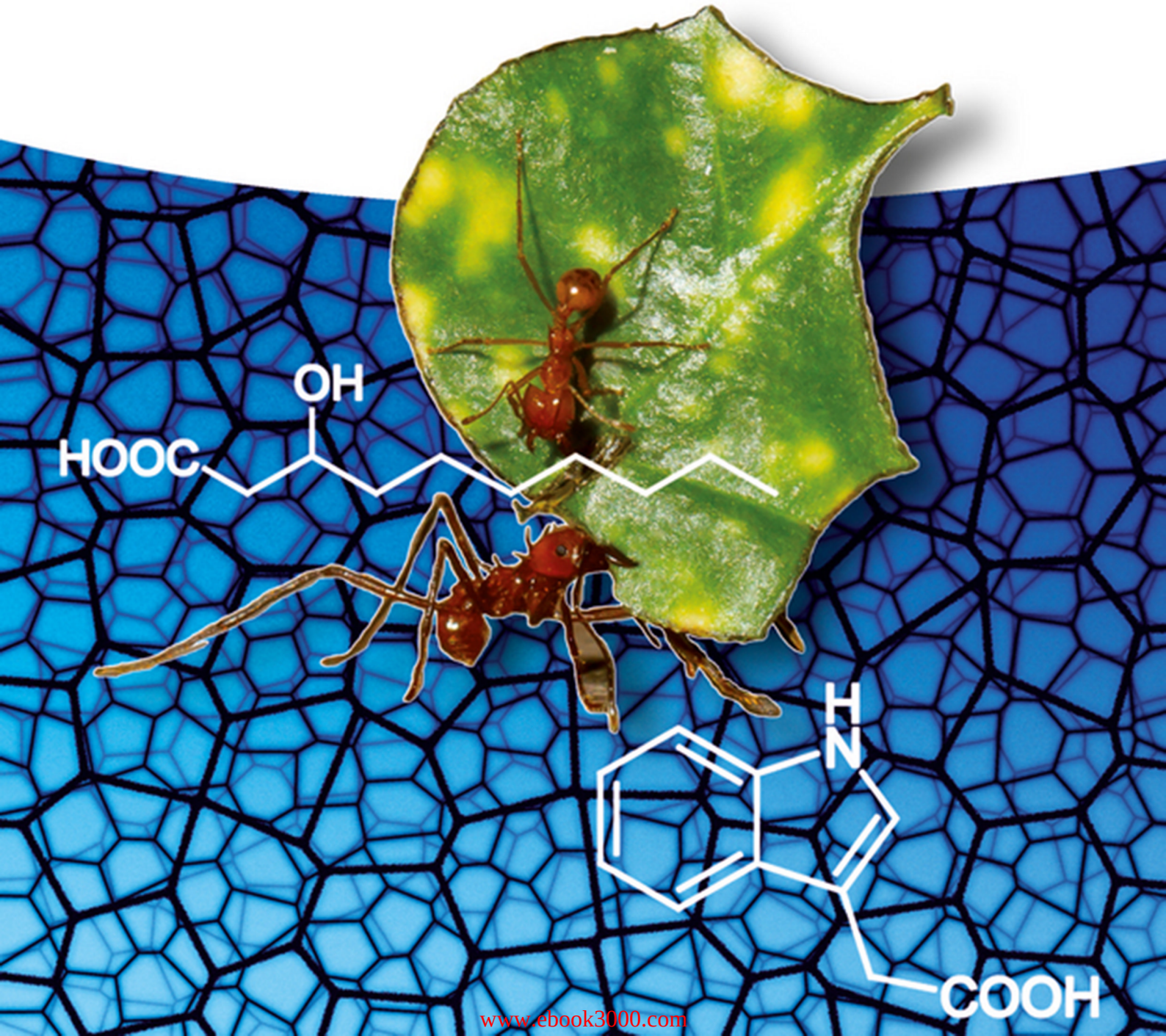
Gerd-Joachim Krauss and Dietrich H. Nies

Ecological Biochemistry

Environmental and Interspecies Interactions



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"It is interesting to contemplate an entangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent on each other in so complex a manner, have all been produced by laws acting around us."

Charles Darwin, The Origin of the Species (1859), John Murray, London

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Foreword

Ecological Biochemistry takes centre stage in modern biology. From fundamentals of secondary metabolism to resultant survival, this book gives a comprehensive view of the organisms that shape our planet, their evolution, and their biotic and abiotic interactions.

Most of the functions of organisms are expressed in their ecological biochemistry. Knowledge of their signal perception, information processing, generation of chemicals for communication, and adaptation informs about our future as organisms are exposed to environmental conditions that range from long-experienced to those that have not existed before.

Plants and microbes, which are the main focus, are experiencing a multitude of environments from soils containing salts, limiting nutrients, metal contaminants, or xenobiotics to exposure to drought, UV radiation, and temperature extremes. In natural and humanmade environments, organisms are confronted with abiotic and biotic settings, and adaptations pivot around biochemical competence.

The unifying basis of life of bacteria, Archaea, fungi, and plants is presented from a microscopic scale to a large scale and from single cells to forest ecosystems. This book provides the reader with an insight into food webs, organism interactions, and ecosystem function across biomes. Complex communities such as those experienced at the interface of soil, microbes, and roots are presented with new views of beneficial and detrimental interactions. The dialog between plants and animals, driven by biochemical signals, is presented in the context of multipartner mutualisms.

With methodological advances and new opportunities enabled by "omics" tools and latest microscopy techniques, this book bring us a modern view of biology, unifying life, and the challenges ahead.

Prof. Susanne Schmidt (PhD, MSc)
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Preface

Ecological Biochemistry refers to the interaction of organisms with their abiotic environment and other organisms by chemical means. Abiotic and biotic factors challenge the biochemical flexibility of organisms, which are usually able to adapt easily to environmental changes by alterations in their metabolism. This book covers the biochemistry behind these interactions, with a bottom-up approach from the atomic level to the systemic level.

The introductory part of the book deals with the physicochemical basis and biochemical roots of living cells, leading to secondary metabolites as crucial bridges between organisms and their respective ecosystem. These specialized compounds illustrate the heterogeneity and multitude of ecological habitats and niches that organisms have colonized so far. The metabolite diversity shows tremendous plasticity and evolutionary potential.

This book concerns the link between biochemical insights and ecological research. The study of ecosystems requires an understanding of general characteristics of ecosystem functionality. This includes knowledge about the biochemistry, biodiversity, and the dynamics of biological components (e.g., individual organisms, populations, communities) under stress, and the related capacities of ecosystems (e.g., with respect to resilience and functional redundancy) that respond to the changing environment. Furthermore, environmental research can help to maintain ecosystem health or, if necessary, to restore ecosystems. Functioning of ecosystems and communities depends highly on the interplay of its different biota in acquisition and distribution of resources required for maintenance, growth and development, adaptation to stress, and competitive and symbiotic interactions.

Our book is focused on interactions of plants, bacteria, and fungi with their environment. Plants are the fundamental constituents of terrestrial and aquatic ecosystems, which are responsible for the majority of biomass produced in our planet. Sessile plants have especially evolved intricate biochemical response mechanisms to fit into a changing environment. They employ numerous signaling molecules to perceive their environment by many sensory systems. The information is transduced toward appropriate responses via parallel signal transduction pathways, which transform environmental stimuli into the biochemical "language" of the cell.

Environmental stress factors can be classified into abiotic and biotic factors. Abiotic stress factors are variable physicochemical parameters of the surroundings, such as oxygen, light, water, minerals, and transition metals, and also xenobiotics from human impact. These parameters are interlinked with biotic stress factors, which represent influences originating from other organisms that live as coinhabitants within the habitat. Microorganisms living in biofilms or symbiotic associations may frequently alter parameters of soil and water. Specific environmental conditions may attract and favor certain microorganisms and animals in the proximity of plants. Secondary metabolites enable plants to interact with pollinators, herbivors, and animals of higher trophic level.

The last part of the book deals with methodology, which allows network-based analysis of molecular processes underlying systems phenomena. Modern techniques provide new tools for answering a range of multidisciplinary questions from the molecular basis of evolutionary adaptation to mechanisms of phenotypic plasticity, interspecies relationships, biochemical communication, and sensing of xenobiotic compounds in human-influenced ecosystems. The "omic" technologies, microscope techniques, and single cell analysis have the ambitious aim to integrate genome, transcriptome, proteome, and metabolome data, and to expand the knowledge of organisms living in and interacting with their environment.

This book is primarily designed for use by advanced undergraduate and graduate students studying biochemistry, plant physiology, ecology, microbiology, pharmacy, agriculture, and forestry. The teachers receive a compendium, allowing a feasible setup of interdisciplinary courses in life sciences. We hope that this book might be of interest to postgraduates, scientists, and those working in different disciplines in applied sciences.

We are very grateful to all contributing authors and colleagues for their excellent and timely work. We would like to thank Anke Poltermann for her extremely resourceful preparation of figures in a homogeneous design, and Dirk Dobritzsch for his excellent intention to develop various graphics of high scientific significance.

Many thanks are due to members of the editorial team of WILEY-VCH, Gregor Cicchetti, and Andreas Sendtko for their editing support, and Anne du Guerny for her patience and excellent assistance throughout the publication process.

Our aim is to enable the reader to develop an understanding of Ecological Biochemistry as an integrative scientific field. We welcome comments, suggestions, and feedback from readers of this textbook.

Halle/Saale *Gerd-Joachim Krauss*
June 2014 *Dietrich H. Nies*

Companion Website

This book spans a multitude of systems levels from atoms to ecosystems. It builds on knowledge of some essential basics in the field which might need refreshing for a better access to "Ecological Biochemistry". Professor Nies, co-editor of the book, offers a thorough presentation of these essentials, based on the latest state of knowledge, in Chapter S1 "Basic Biochemical Roots" on the companion website of the book:

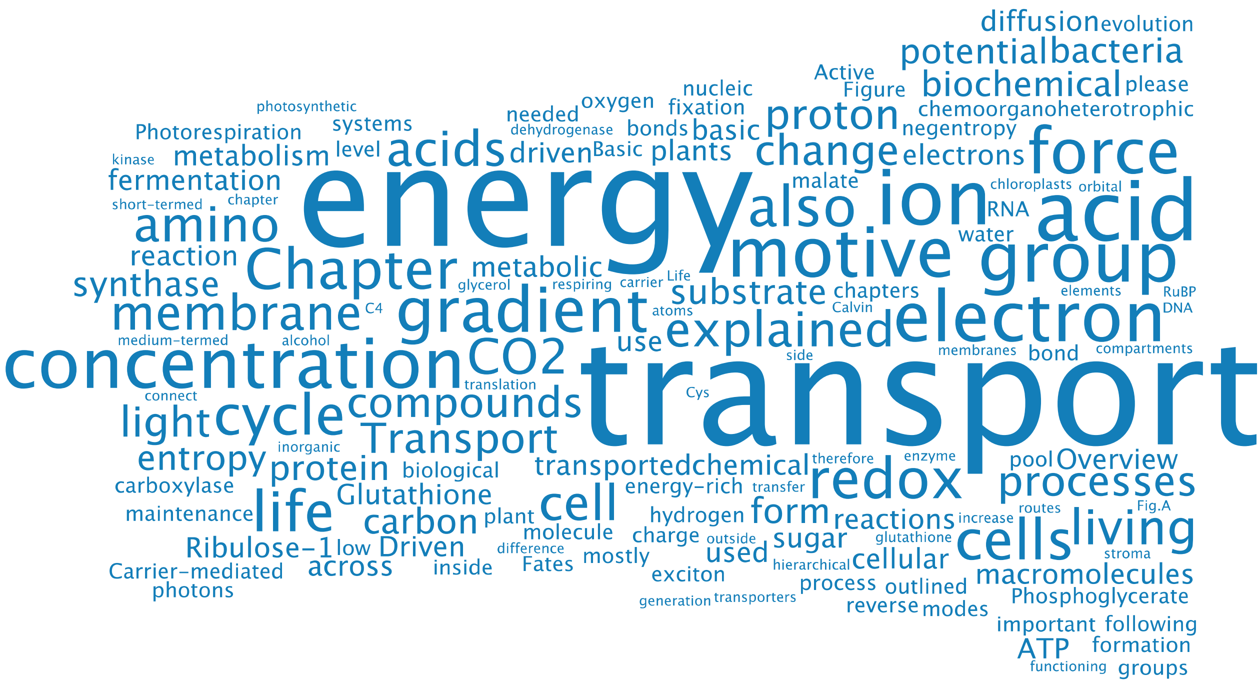
http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry 

In the print and e-book versions of the book you will find a summary of these essentials including the most important figures. All references to the website are marked by an "S".

Part I
Basics of Life

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry



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1
Basic Biochemical Roots

Dietrich H. Nies

See extended version S1 of this chapter on companion website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

Overview

A thorough presentation of the basics needed to access "Ecological Biochemistry" can be found on the companion website (see URL above). The following pages give you an idea of what exactly can be looked up on this website and at the same time reproduce the most important basics-summarizing figures often referred to in the rest of the book.

1.1
Chemistry and Physics of Life

How does life function? Chapter S1 defines "life" as a thermodynamic process, following Erwin Schrödingers' famous lecture "what is life?" delivered at Trinity College, Dublin, in February 1943. Thermodynamics, entropy, and negentropy (= order) and how a living cell manages to form "order" despite the three basic laws of thermodynamics are explained. This chapter defines life as an energy-transducing process exerted by enclosed reaction compartments (= cells). From this, maintenance energy, the allochthonous and autochthonous modes of microbial life, and the processes evolution, mutation, and selection are derived. Generating negentropy in living cells means formation of macromolecules, and the four major groups of cellular macromolecules are introduced. Major and minor bioelements are listed and their bioavailability connected to the generation of elements in stars. This leads also to water as the optimum solvent for the cellular biochemistry (See also Chapter 10) and to the necessity of a semipermeable membrane surrounding living cells (Figure 1.1).

Important terms explained: allochthonous, autochthonous, carbohydrate, carbon, cell, DNA, dry mass, dry weight, maintenance energy, entropy, evolution, element generation, information, lipid, macromolecule, semipermeable membrane, mutation, nucleic acid, maintenance power, protein, RNA, selection, thermodynamics, transport, wet mass, wet weight.

1.2
Energy and Transport

The second section of Chapter S1 introduces how a living cell functions in general. In the liquid phase of a solvent, most likely water, a cell represents a separated system that continuously uses energy from the outside to increase its negentropy inside by the synthesis of carbon-based macromolecules, thereby overcompensating the decrease in entropy inside by a higher increase in entropy outside. Further sections of this chapter show how this can be accomplished.

The chapter starts with the explanation of the lowest systems level of interest for biologists, the atoms, electrons, and photons and continues with the basic modes of energy conservation. Atoms, orbitals, and the consequences of the structure of the electron shells for the chemical features of the elements, redox energy, and electronegativity are explained in depth. Atoms form molecules at the next systems level and new features emerge by this process, which can nevertheless be deduced from the electron structure of the involved atoms. This is also true for the most important functional groups of the building blocks of the macromolecules. Which energy sources can be used by cells and how the energy from photons can be harvested and ultimately converted to an ion gradient across a biological membrane, leading to the phototrophic life style are explained (Figure 1.2).

Transport processes change chemical gradients across biological membranes and these processes are categorized in a strict hierarchical manner (Figure 1.3), which leads also to the introduction of ion motive forces such as the proton motive force, and to the chemolithoautrophic and respiring chemoorganoheterotrophic life styles (Figure 1.2).

The important F_1F_0-ATPase is needed to connect the short-termed energy pool of the proton motive force to the medium-termed energy pool of ATP and related compounds, and consequently the structure and function of this ATPase is outlined (Figure 1.4). The fourth and last life style explained here is chemoorganoheterotrophic fermentation.

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Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

In total, the basic modes of energy transformation in living beings are outlined and transport processes across biological membranes are explained in a thorough hierarchical scheme.

Important terms explained: acid anhydride group, acidocalcisomes, active transport, alcohol group, aldose, amide group, amine group, amino acid, amino sugar, antiport, *Archaea,* atom, atomic number, autotroph, bacteriorhodopsin, carbohydrate, carbonyl group, carboxyl group, charge transfer complex, chemotrophs, *Chlorobiaceae, Chloroflexaceae,* chlorophyll, chloroplast, *Chromatiaceae,* color, conjugated double bonds, cyanobacteria, diffusion (facilitated, simple), DNA, electron, pair, electron acceptor, electron donor, electronegativity, electrons (delocalized, valence), energy (concentration, conformational, light, redox), energy pool (short-termed, long-termed, medium-termed), entropy, ester group, exciton, fatty acid, fermentation, frontier orbital gap, γ-rays, glutathione, glycerol, halobacteria, heterotroph, hybridization, orbitals, hydrogen bond, ionizing radiation, isotope, ketose, methyl group, methylene group, mixed acid anhydrite, mixotroph, monosaccharide, motive force (ion, proton, sodium), neutron, nucleic acid, orbital, peptide bond, phospholipid, photon, phototroph, phycobilin, *Prochlorales,* proton, proton pump, radicals, replication, respiration, reverse electron flow, reverse electron transport, *Rhodospirillaceae,* RNA, spin, substrate-saturation, sugar acid, sugar alcohol, symport, syntrophy, thioester group, thioesters, transcription, translation, transport (carrier-mediated, membrane, primary, secondary), ultraviolet light, uniport, X-rays.

1.3 Basic Biochemistry

Starting from the physical and chemical constraints for cellular biochemistry as outlined in the first and second sections of this chapter, this part of the chapter is a concise overview of the basic biochemical pathways in living organisms including fermenting bacteria. This is a comprehensive biochemical text that covers this field in 12 sections.

1) Organization of the overall metabolism
2) Enzymes and Coenzymes
3) The backbone: fructose-1,6-bP pathway: Overview, Glucose, Hexosephosphate-isomerase, Phosphofructokinase, fructose-diphosphate-aldolase, Triosephosphate isomerase, Glyceryldehyde phosphate dehydrogenase, Phosphoglycerate kinase, Phosphoglycerate mutase, Phosphoglycerate enolase, Pyruvate kinase and phosphotransferase systems (PTS)
4) Cycles and shunts attached to the F1,6bP-pathway: Overview, 2-keto-3-desoxy-6-phosphogluconate (KDPG) pathway, Heterofermentative lactic acid fermentation and phosphoketolase, Pentosephosphate cycle (PPC), Calvin cycle, Ribulose-monophosphate cycle
5) Fates of pyruvate
6) Fates of acetyl-*S*-CoA: Overview, Tricarbonic acid cycle (TCA), Inverse TCA, Propionate fermentations, Butyric acid and butanol/acetone fermentation, Fatty acid and Polyhydroxyburyric acid (PHB) metabolism
7) Putting it together: anaerobic ecosystems
8) Assimilation of the 10 macrobioelements: Carbon, hydrogen, and oxygen, Nitrogen, Sulfur, Phosphate, Metals
9) Building blocks: Overview, E, D, Q, N, acidic amino acids and their amides, Sulfur-containing amino acids C, M, "alcohols" S and T, "conformation determinants" G and P, hydrophobic nonaromatic amino acids A, I, V, and L, Amino acids with long positively charged side chains, R and K, Aromatic amino acids, Y, F and W, Histidine and purine bases, Pyrimidine bases and pyrrol rings
10) Macromolecules in bacteria: nucleic acids and transcription, RNA and translation, Protein sorting, cellular metabolic network
11) DNA-replication and cell division in prokaryots
12) Genomes and evolution: genomes and their organization, epigenetics, phylogeny and Tree of Life.

Maps (Figures 1.5–1.13) shown on the following pages summarize fundamental metabolic routes needed to understand the consecutive chapters of this book, and to connect them to the subsequent chapters.

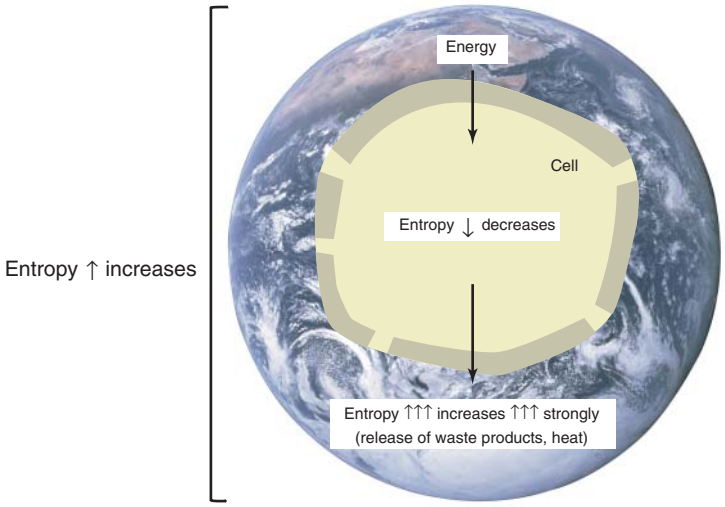


Figure 1.1 **What is Life?** To built order inside, cells continuously use energy to decrease the intracellular entropy (or increase the intracellular negentropy = order) and overcompensate this by increasing the entropy in the environment by release of waste products and heat. Thus, in the total system composed of a cell and its environment, the entropy increases steadily during the chemical reactions in a cell, and the second law of thermodynamics is kept. Energy can be light energy or chemical energy (see Section 1.2). Intracellular order means macromolecules. (Earth photo: Courtesy of NASA.)

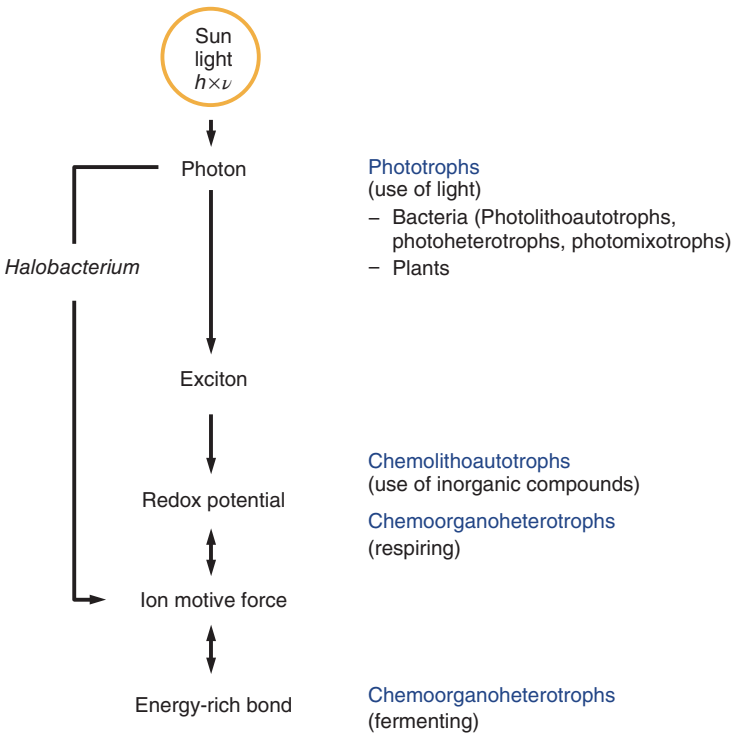
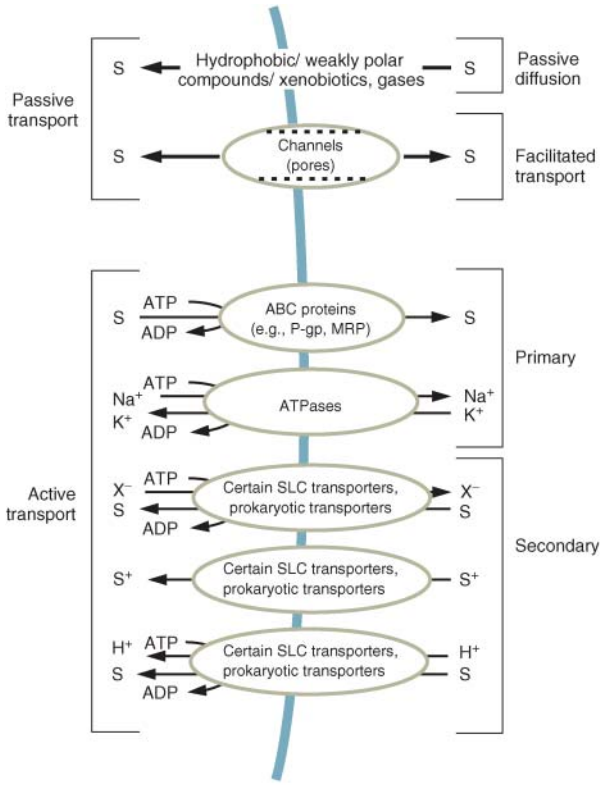


Figure 1.2 **The universal roadmap of energy conservation.** Phototrophs use light energy (photons) to create an exciton that is subsequently used to change the redox potential of a redox carrier to a lower potential (= higher energy). Electron transport from the resulting low redox potential to a more positive one drives ion transport to form an ion motive force, mostly in form of a proton motive force pmf. Finally, the ion motive force is used to generate compounds containing an energy-rich bond such as ATP. In a short cut, some archaea such as *Halobacterium* use a protein named bacteriorhodopsin to create an ion motive force directly from an exciton. Chemolithoautotrophic (cla) bacteria use the difference in redox potential of inorganic compounds to conserve energy, respiring chemoorganoheterotrophic (coh) bacteria transfer electrons from organic compounds to external electron acceptors that are mostly also inorganic compounds. Fermenting organism are also chemoorganoheterotrophs that use biochemical reactions for a direct formation of energy-rich bonds. Some phototrophs can grow photolithoautotrophically (pla), other photoheterotrophically (ph) or photomixotrophically (pm). Please note that energy-rich bonds can also be used to build an ion motive force, for example, for transport processes, and an ion motive force to drive electrons toward a low redox potential (reverse electron transport.)



I. **Simple diffusion**. Driven only by a concentration gradient. Transport rate depends on the concentration difference according to Fick's Law. *Transport of molecular hydrogen and oxygen across the cytoplasmic membrane.*

II. **Carrier-mediated transport.** Catalyzed by a carrier, mostly a trans-membrane protein. Faster than simple diffusion but showing **substrate-saturation**.

   A. **Facilitated diffusion**. Carrier-mediated transport driven exclusively by the concentration gradient of the substrate. No accumulation. *Transport through outer membrane porins, glucose and glycerol facilitators.*

   B. **Active transport.** Carrier-mediated transport driven by energy different from the concentration gradient of the substrate. **Accumulation** possible on either side of the membrane, which may lead to **concentration gradients.**

   1. **Primary transport.** Active transport driven by an energy form that is not another gradient, therefore a **gradient-forming** transport.
      a. Driven by light energy: Photons are harvested as exitons. Their energy is used to change the conformation of a transporter and the resulting conformational tension is relaxed by a transport event. Alternatively, the redox potential of a compound is decreased by the exiton energy, leading to electron transport, consecutively resulting in a transport event. *Bacteriorhodopsin, photosynthetic light reaction.*
      b. Driven by redox energy: electrons transfer from an electron donor to an electron acceptor drives the transport event. *Respiratory electron transport chain.*
      c. Driven by a chemical reaction such as ATP-hydrolysis. *Families of transport ATPases (ABC, P-type, F_1F_0), gradient-forming decarboxylases.*
   2. **Secondary transport.** Active transport driven by a concentration gradient, therefore **gradient-using** transport.
      a. **Uniport:** a **charge gradient** drives the transport reaction. Only the substrate is transported propelled by its charge. *Uptake of Mg^{2+} by CorA-like transporters, export of arsenite by ArsB- and chromate by ChrA-like transporters.*
      b. **Symport:** the substrate is transported into the **same direction** as a second ion or molecule, which forms the driving concentration gradient. *Lactose:proton symporter LacY.*
      c. **Antiport:** the substrate is transported into the **opposite direction** of the second ion or molecule, which forms the driving concentration gradient. *Sodium:proton-antiporter NhaA.*

Figure 1.3 **Hierarchy of transport processes and examples.**

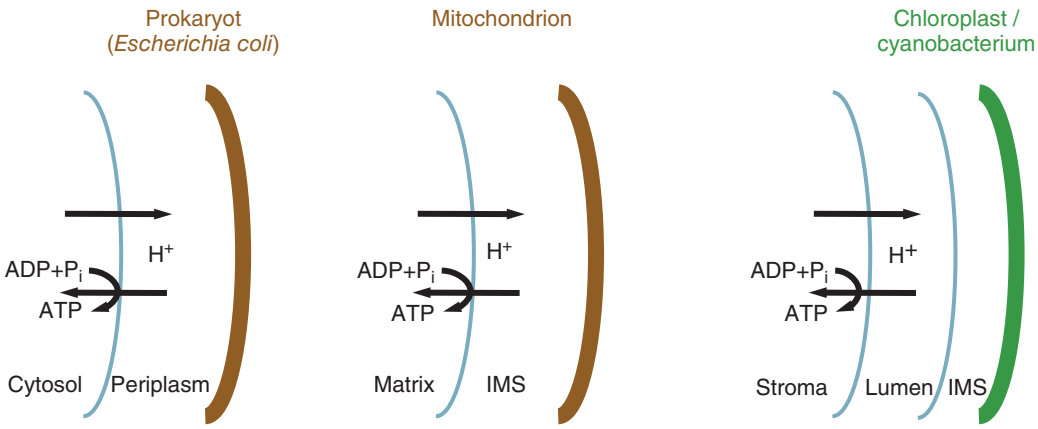


Figure 1.4 **Topology of ATP synthesis in bacteria and plant cell organelles (IMS – Intermembrane space; Lumen – Thylakoid lumen). (Graphics: D.Dobritzsch, G.-J. Krauss.)**

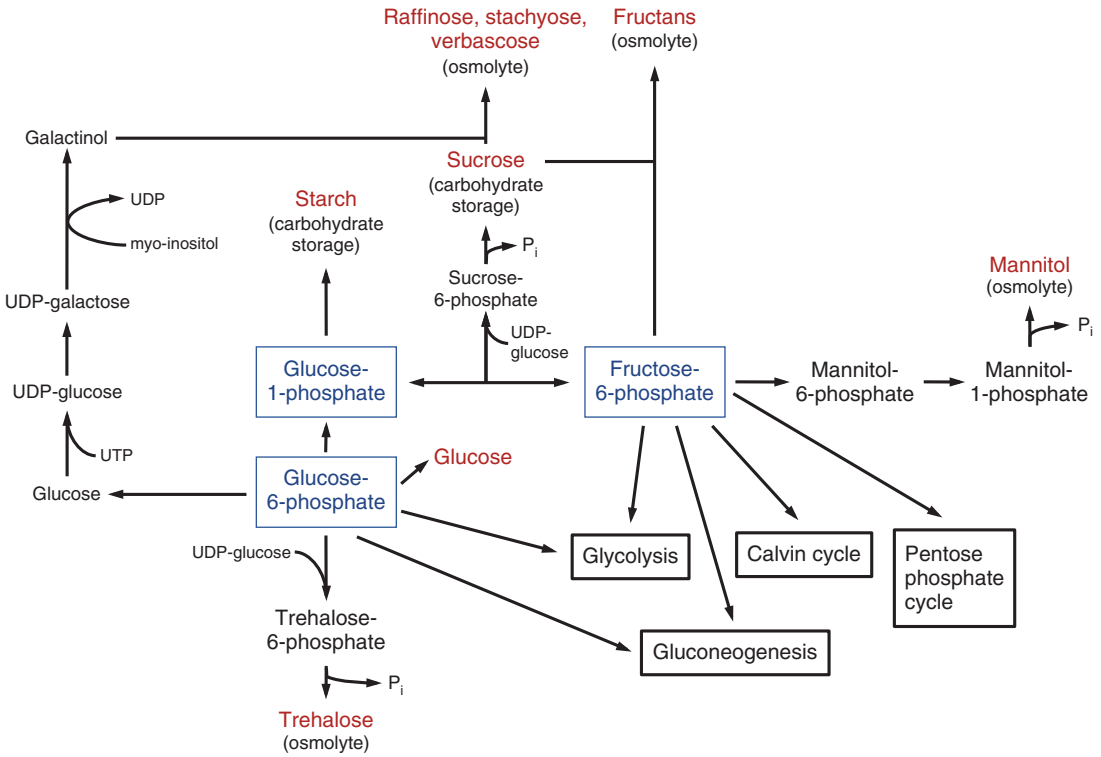


Figure 1.5 Primary sugar routes in plants. (Graphics: D. Dobritzsch, G.-J. Krauss.)

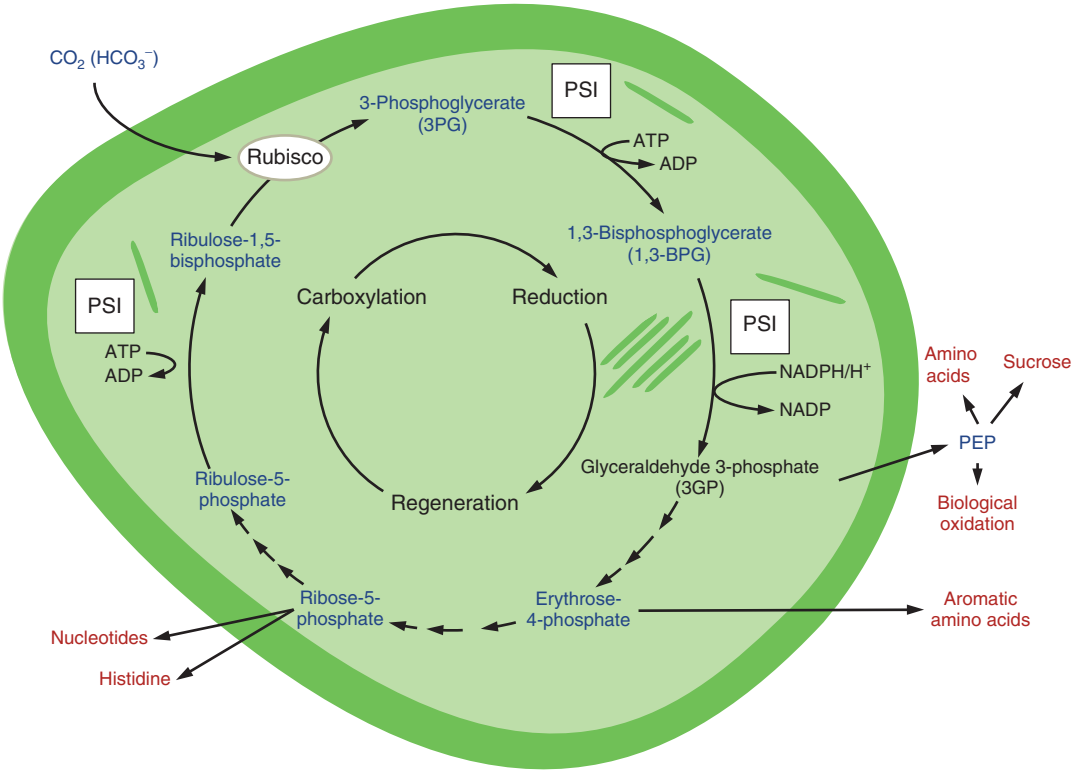


Figure 1.6 Calvin cycle in the stroma of the chloroplasts: A series of light-independent enzymatic reactions in the stroma of chloroplasts convert CO_2 and H_2O into organic compounds, using ATP and NADPH from the photosynthetic light reactions. The key enzyme for carbon fixation in this cycle is the ribulose-1,5-bisphosphate carboxylase: (Rubisco) (PSI – photosystem I). (Graphics: G.-J. Krauss, D. Dobritzsch.)

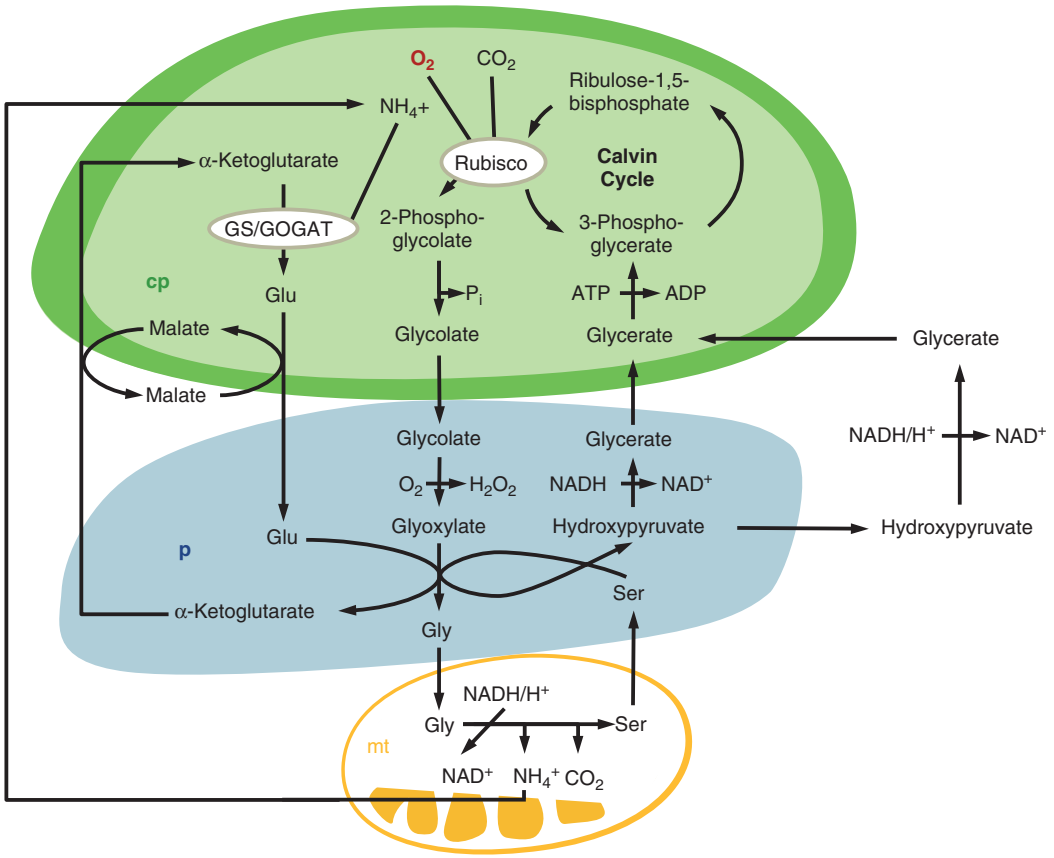


Figure 1.7 Photorespiration: Ribulose-1,5-bisphosphatecarboxylase can also oxygenate ribulose-1,5-bisphosphate (RuBP) which results in the incorporation of oxygen into the carboxyl groups of 3-phosphoglycerate and 2-phosphoglycolate. The two carbon molecule is metabolised back to RuBP via reactions in different cell compartments. Photorespiration happens when the CO_2 level inside the leaf become low and the O_2 ratio is increased relative to CO_2 concentration. Photorespiration is crucial for carbon salvage and adjustment of various metabolic functions. (GS – glutamine synthetase; GOGAT – glutamate α-ketoglutarate (oxoglutarate) aminotransferase; rubisco – ribulose 1,5,bisphosphate carboxylase-oxygenase). (Graphics: G.-J. Krauss, D. Dobritzsch.)

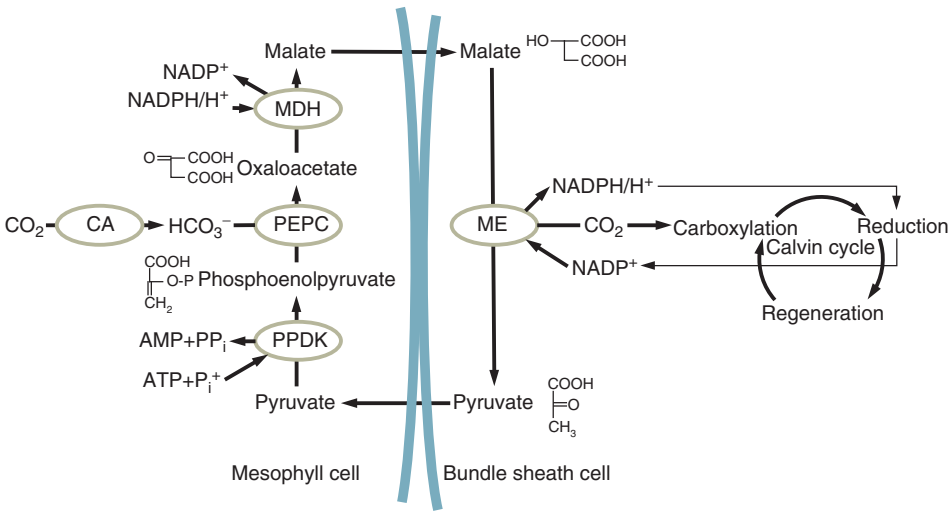


Figure 1.8 C4 photosynthesis (NADP + malic enzyme type): C4-carbon fixation: C4-plants evolved a special of CO_2 fixation in mesophyll cells via phosphoenolpyruvate (PEP) carboxylase, formation of C4 compound, usually malate, which is transported to the bundle sheet cells, where after its decarboxylation CO_2 finally is fixed by ribulose-1,5-bisphophate carboxylase. C4 carbon fixation: $NADP^+$-malic enzyme type. (CA – carboanhydrase, MDH – $NADP^+$-malate dehydrogenase, ME – NADP - malic enzyme, PEPC – phosphoenolpyruvate decarboxylase, PPDK – pyruvate orthophosphate dikinase (Graphics: D. Dobritzsch.)

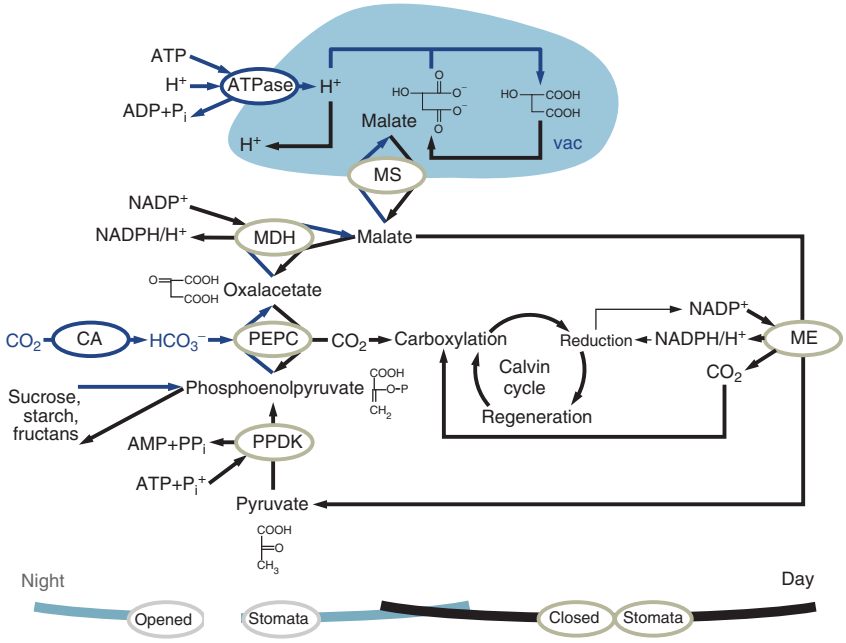


Figure 1.9 Crassulacean acid metabolism (CAM) in CAM-plants:CAM is functioning in some plants living in arid conditions. In these plants stomata open during night. Then CO_2 is transformed to malate and stored. During daytime stomata are closed to avoid loss of water and CO_2 is liberated from malate and fed into the calvin cycle.), blue arrows – reactions in the night; black arrows – reactions during day light (MDH – $NADP^+$-malate dehydrogenase; MS – malate shuttle; PEPC – phosphoenolpyruvate carboxylase; PPDK – pyruvate orthophosphate dikinase (Graphics: D. Dobritzsch.)

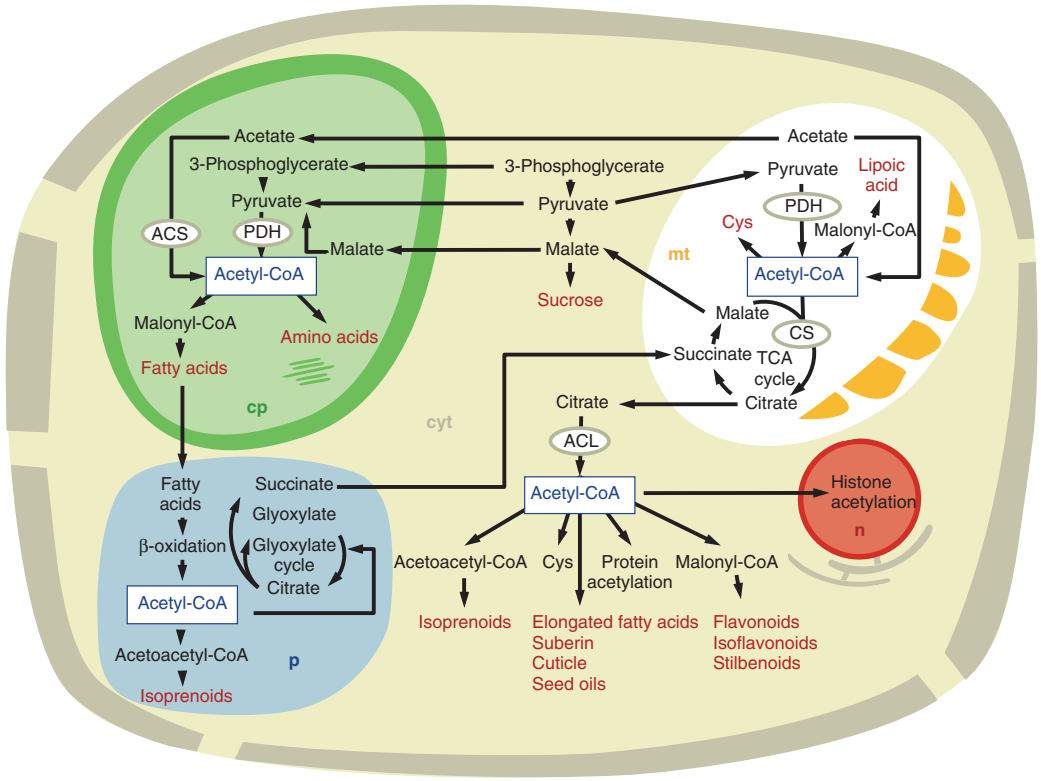


Figure 1.10 Fates of acetyl CoA in plant cells: ACS – acetyl-CoA synthase; ACL – ATP:citrate lyase; CS – citrate synthase; TCA cycle – tricarbonic acid cycle; PDH – pyruvate dehydrogenase; Leu – leucine; Cys – cysteine; Arg – arginine.) (Graphics D. Dobritzsch.)

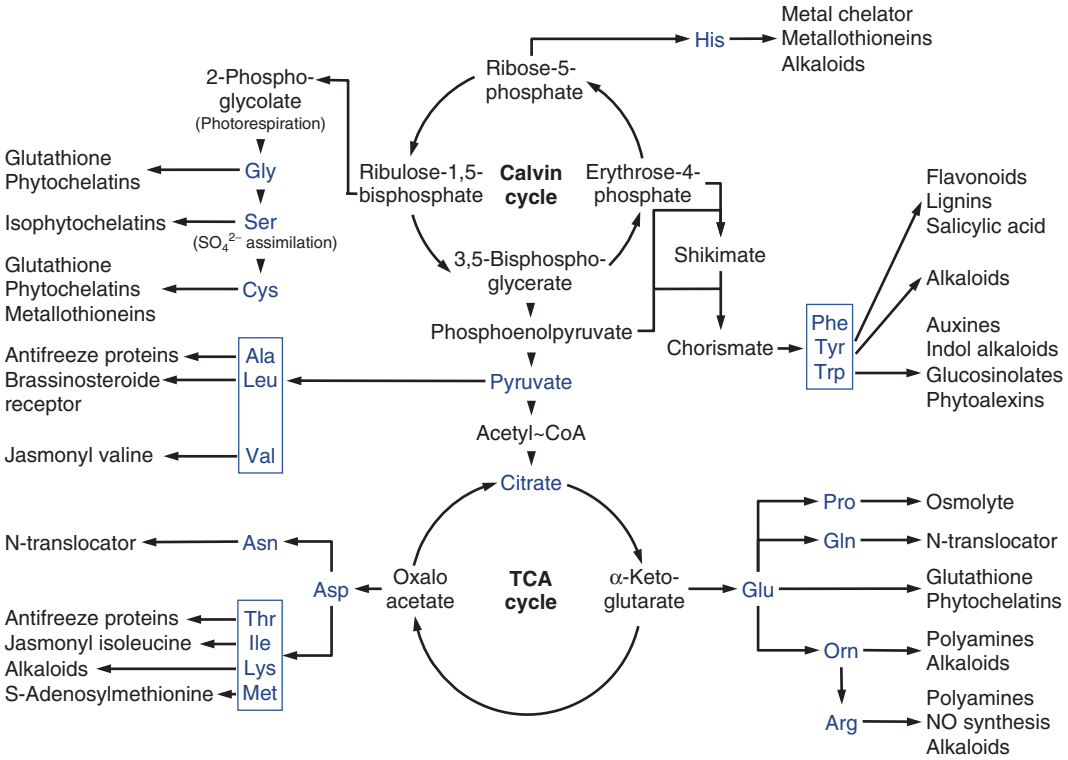


Figure 1.11 Biosynthetic origin of amino acids and their derivatives in plants. (Graphics: G-J. Krauss, D.Dobritzsch.)

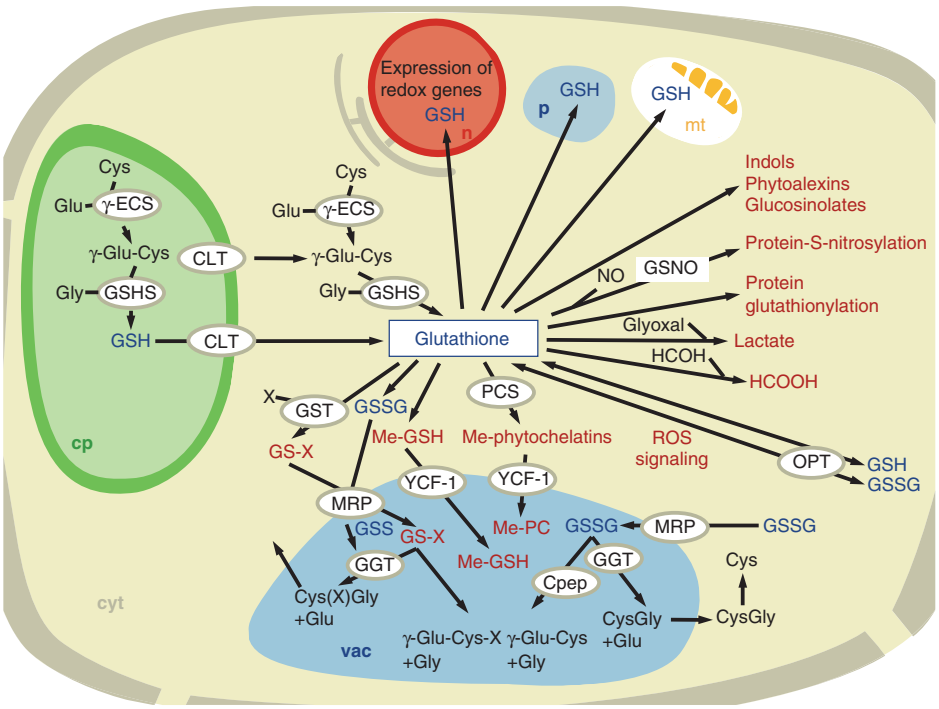


Figure 1.12 Compartmentation of glutathione metabolism in plant cells: GSSG – glutathione disulfide; GSNO – *S*-nitrosoglutathione; γ-EC – γ-glutamyl-cysteine; Glu – glutamate; Gly – glycine; Me – metal; PCS – phytochelatin synthase; GSH – glutathione; X – xenobiotic; GSX – glutathione conjugate; MRP – multridrug resistance-associated protein; Cpep – carboxypeptidase; GGT– γ-glutamyltranspeptidase; (YCF1 – yeast cadmium factor protein; CLT – chloroquinone resistance transporter; OPT – oligopeptide transporter; GST – glutathione-*S*-transferase; γ-ECS – γ-glutamyl-cysteinyl synthase; GSHS – glutathione synthase; Cys – cysteine. (Graphics: G-J. Krauss, D. Dobritzsch.)

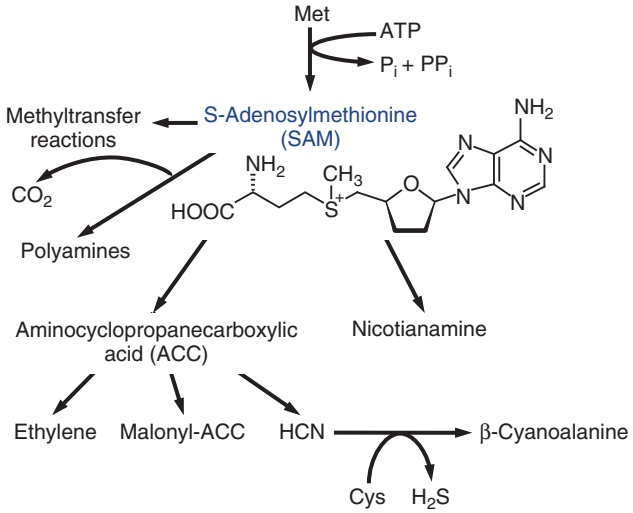
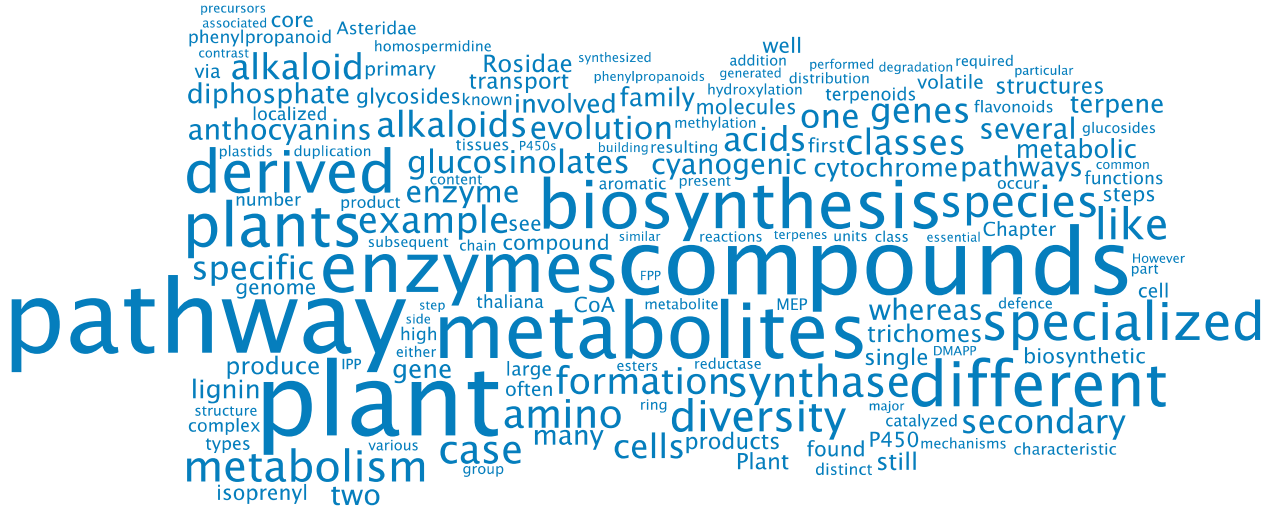


Figure 1.13 The central metabolic role of S-adenosylmethionine.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2
Specialized Plant Metabolites: Diversity and Biosynthesis

Alain Tissier, Jörg Ziegler, and Thomas Vogt

Overview

Plant secondary metabolites, also termed specialized plant metabolites, currently comprise more than 200 000 natural products that are all based on a few biosynthetic pathways and key primary metabolites. Some pathways like flavonoid and terpenoid biosynthesis are universally distributed in the plant kingdom, whereas others like alkaloid or cyanogenic glycoside biosynthesis are restricted to a limited set of taxa. Diversification is achieved by an array of mechanisms at the genetic and enzymatic level including gene duplications, substrate promiscuity of enzymes, cell-specific regulatory systems, together with modularity and combinatorial aspects. Specialized metabolites reflect adaptations to a specific environment. The observed diversity illustrates the heterogeneity and multitude of ecological habitats and niches that plants have colonized so far and constitutes a reservoir of potential new metabolites that may provide adaptive advantage in the face of environmental changes. The code that connects the observed chemical diversity to this ecological diversity is largely unknown. One way to apprehend this diversity is to realize its tremendous plasticity and evolutionary potential. This chapter presents an overview of the most widespread and popular secondary metabolites, which provide a definite advantage to adapt to or to colonize a particular environment, making the boundary between the "primary" and the "secondary" old fashioned and blurry.

2.1
Metabolite Diversity

Metabolites, or small organic molecules, which occur in a living organism, can be divided into two major classes. **Primary metabolites** are those molecules that are involved in the biosynthetic pathways of essential components of living cells, such as amino acids in proteins, nucleotides in nucleic acids, sugars as energy resource and in polysaccharides, or phospholipids as major constituents of cell membranes. Primary metabolites are common to most living cells (see Section S1.1.3). **Secondary metabolites** may first be defined as compounds that are not essential to the living cells. In contrast to primary metabolites, they are not found in every species, but are often associated with distinct taxonomic groups. Some of them may be widely distributed, that is, present in many different taxa, while others may be found in only a single species. As a result, secondary metabolites account for most of the molecular diversity of living organisms. To avoid the term "secondary" that may sound pejorative, and at the same time to better reflect the distribution of these metabolites to restricted taxa whose smallest unit is the species, the term **specialized metabolites** was recently proposed.

Plants are well known for their metabolic diversity, which has fascinated natural product chemists for many years. It is often considered that this diversity is intimately linked to the fixed mode of life of plants. Animals in comparison have a much smaller number of specialized metabolites because their mobility allows them to escape predators and to engage in communication with other individuals or organisms. Plants in contrast depend heavily on chemical communication, whether to attract beneficial organisms, such as pollinating insects, ward off pests, or signal to other plants.

The overall number of specialized metabolites produced by plants is not known but is estimated to exceed 200 000. This is, however, very likely to be a gross underestimation for several reasons. First, only a fraction of all plant species have been investigated for specialized metabolites. In addition, for each plant species that was analyzed, the compounds were often extracted from whole dried plants. It is known that different tissues within a single plant produce a different profile of specialized metabolites. Moreover, the physiological state and environmental conditions as well as the stage of development will also affect the specialized compound content of a given species. The total number of plant species is not exactly known but lies in the range of several hundred thousands, possibly around a million. Assuming each species produces at least one specialized compound that is unique (also probably

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a gross underestimate), the 200 000 mark would be easily topped.

Now that several plant genomes have been completely sequenced – and the number of sequenced plant genomes is steadily increasing following the advances in high-throughput sequencing – it has become apparent that the biosynthetic potential of plants is much larger than previously thought, as judged by the large number of genes encoding proteins of unknown function but belonging to classes of enzymes that are known to be involved in metabolic pathways. Thus, it has been estimated that 10–20% of all genes of a given plant species may be involved in specialized metabolism. For example, assuming almost equal to1.5 proteins per enzyme and 1 product per enzyme, the model plant *Arabidopsis thaliana* (*Brassicales, Rosidae*), with 26 500 genes can be estimated to produce 1750–3500 specialized metabolites. Most plant species have an even larger genome (e.g., rice has around 35 000 genes), and they would be expected to be able to produce even more specialized compounds. There are many specialized compounds that are common to many species, and each species contributes only a small fraction of unique compounds of the plant kingdom. This tremendous diversity raises issues of their function such as how they are synthesized and what molecular mechanisms have allowed this diversity to evolve. By focusing on specific examples, the issues of the evolution of specialized compound biosynthesis and their sites of biosynthesis are addressed. Specific examples of the functions of specialized metabolites are referred to in other chapters.

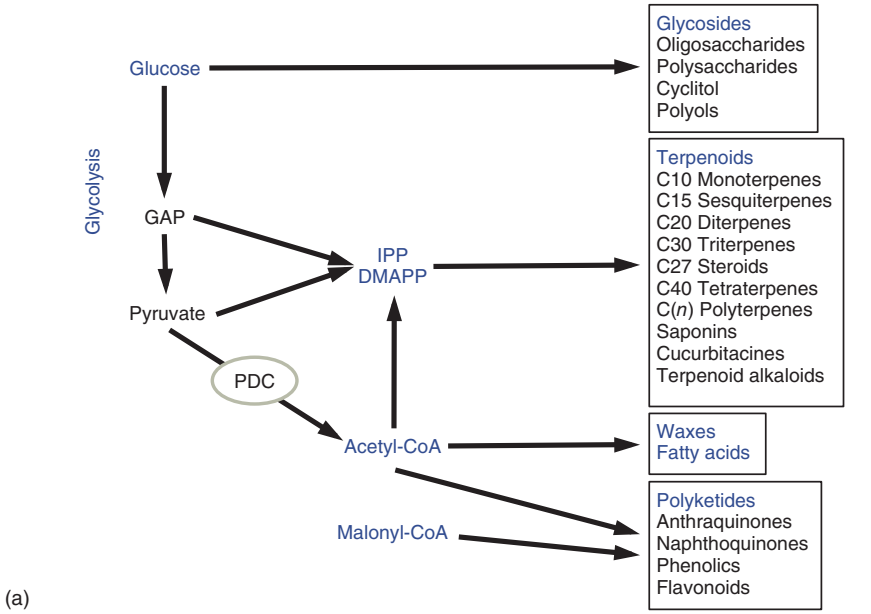
2.2 Major Classes of Plant Specialized Compounds

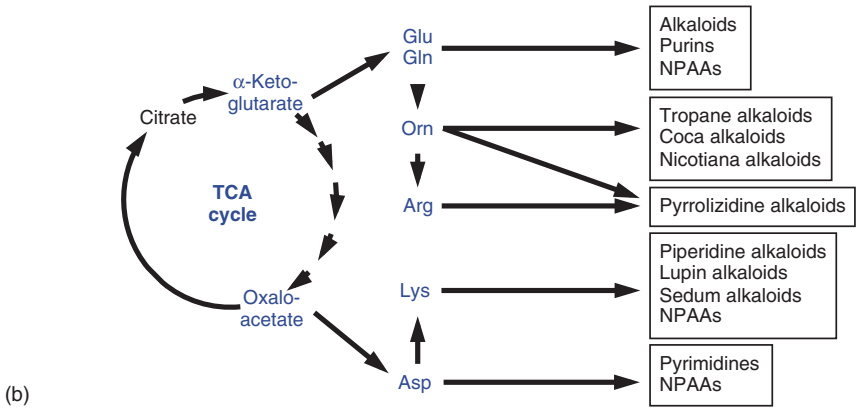
Specialized metabolites can be grouped in various classes according to their chemical nature. The most prominent classes are the **terpenoids** with over 25 000 compounds identified to date. The second largest class is represented by the **alkaloids** with 21 000 compounds, and the third is the phenolics (including **phenylpropanoids** and **flavonoids**) with an estimated 10 000 substances described to date. Each of these classes of compounds is presented here, with specific examples. There are also many other classes with smaller numbers because they occur in a small number of species or families, but they are not presented here because of space limitations. However, one of these classes, the **glucosinolates**, found in the *Brassicaceae* (*Brassicales, Rosidae*) presents a particular interest because *A. thaliana*, a *Brassicaceae*, has been extensively studied and provides an interesting case for specialized metabolism. Because of their potential role in pest control and their similarities with respect to biosynthesis, **cyanogenic glucosides** are also discussed in this chapter. Specialized metabolites are derived from precursors that are part of the primary metabolism. An overview of the connections of the major classes of secondary metabolites to central metabolism is illustrated in Figure 5.7 and Figure 2.1a–c.

2.2.1 Terpenoids

Terpenoids belong to the large family of isoprenoid compounds, which are made of variable numbers of the C5 isoprene unit. Typically, terpenoids refer to the smaller isoprenoids with a number of isoprene units lower than 8. The number of isoprene units defines to which subclass a given terpenoid belongs. Both terms terpenes and terpenoids are generally considered equivalent, although the use of terpenes is sometimes reserved for olefinic compounds (i.e., containing only C and H) and terpenoids for oxidized terpenes. Hemiterpenoids contain a single isoprene unit, whose most abundant representative is isoprene itself. It is emitted by photosynthetically active tissues of certain tree species, and up to 5×10^8 metric tons are released annually. Considering it can react with nitric oxide, it contributes to the formation of tropospheric ozone. Monoterpenoids are made of two units and frequently encountered in essential oils of aromatic and fragrant herbs and spices. Compounds such as limonene, pinene, and menthol belong to this class. Sesquiterpenoids consist of three isoprene units and are also frequently found in essential oils. Oxidated sesquiterpenes, however, are less volatile and may play roles in plant defense as phytoalexins, such as capsidiol. One sesquiterpenoid, artemisinin from wormwood (*Artemisia annua*, *Asterales, Asteridae*), is now used as a pharmaceutical ingredient for the treatment of malaria. Diterpenoids contain 20 carbon atoms and are made of four isoprene units. Diterpenes are poorly volatile at ambient temperature. The plant hormones gibberellic acids are diterpenes. Some plant diterpenes have been used as pharmaceutical ingredients, like taxol – a compound found in yew trees (*Taxus* sp., *Coniferales, Coniferophyta*) – and prescribed for the treatment of various cancers. A sample of terpenoid diversity is presented in Figure 2.2. The various classes of terpenoids and their major functions are summarized in Table 2.1.

One remarkable feature of terpenoids is that they are all derived from the same building blocks. These are the five-carbon compounds isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 2.3a). For many years, it was assumed that only one pathway, the cytosolic **mevalonate (MEV) pathway** (Figure 2.4), could supply precursors for the biosynthesis of isoprenoids and terpenoids. The MEV pathway is localized in the cytosol and starts from acetyl-CoA (see Section S1.3.6.1) onto hydroxymethylglutaryl-CoA and MEV as key intermediates. However, the low level of incorporation of label from acetyl CoA to isoprenoids in some bacteria led to the discovery of an alternative pathway. At the beginning of this pathway, glyceraldehyde-3-phosphate and pyruvate (see Section S1.3.4) are combined to 1-deoxy-D-xylulose





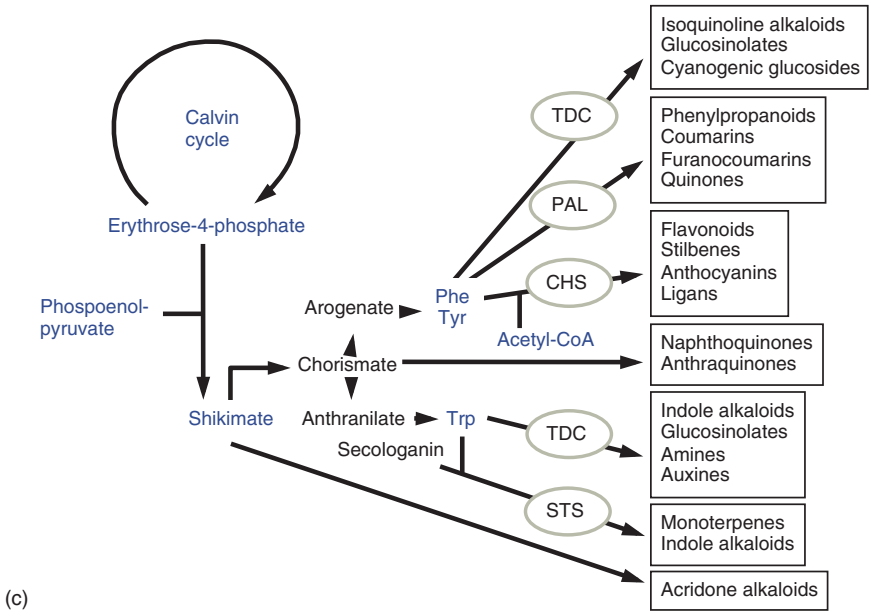


Figure 2.1 (a) Origin of plant secondary metabolites from glycolysis. (b) Origin of plant secondary metabolites from the tricarboxylic acid (TCA) cycle (see Section S1.3.6.2). (c) Origin of plant secondary metabolites from the Calvin cycle (see Section S1.3.4.5) (Courtesy of M. Wink, D. Dobritzsch.)

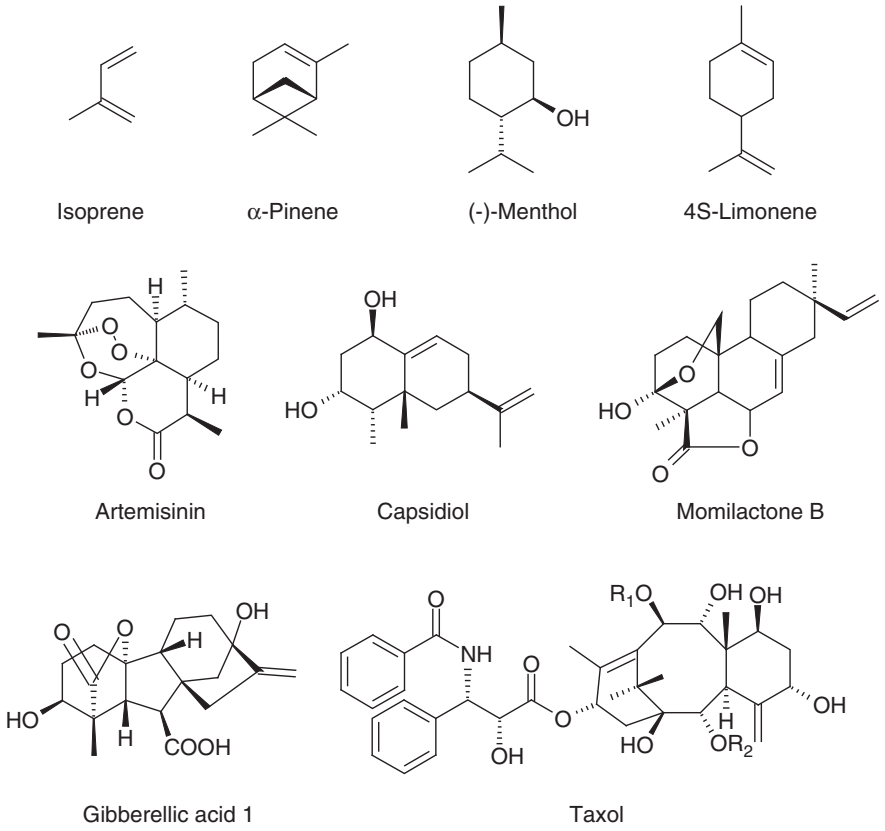
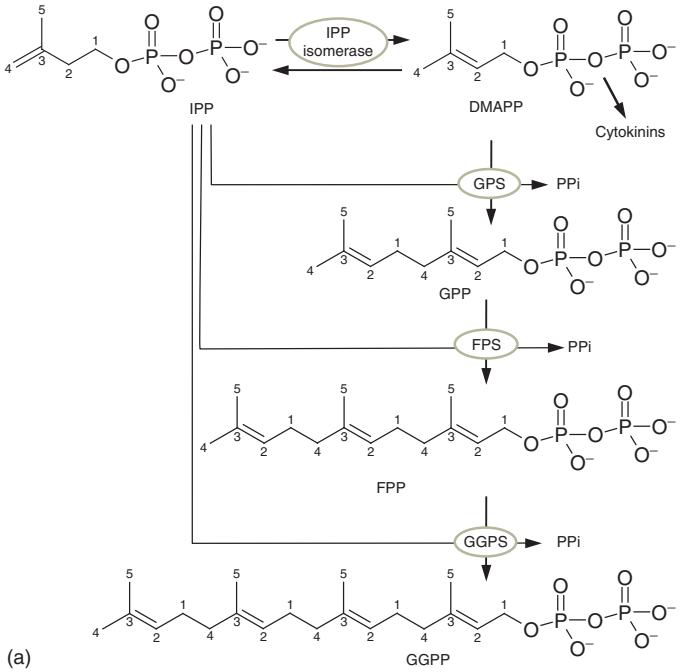


Figure 2.2 Examples of plant terpenes. Isoprene is a hemiterpene (C5). Menthol, α-pinene, and limonene are monoterpenes (C10). Artemisinin and capsidiol are sesquiterpenes (C15). Momilactone, gibberellic acid 1 (GA1), and taxol are diterpenes. Note that GA1contains only 19 carbon atoms because of the loss of one carbon atom during biosynthesis.

Table 2.1 Plant representative terpenoids, their properties, and functions.

Terpenoid class	Representative members	Features	Possible Functions
Hemiterpenes	Isoprene	Volatile	Dissipation of energy
Monoterpenes	Limonene, pinene, menthol	Volatile	Insect Attractants/deterrents, plant defense
Sesquiterpenes	Caryophyllene, artemisinin, capsidiol	±Volatile	Insect Attractants/deterrents, plant defense
Diterpenes	Gibberellic acids, conifer resin acids, phytol, taxol	Nonvolatile	Hormones, photosynthesis, plant defense
Triterpenes	Sterols, brassinosteroids, saponins, cardiac glycosides	Nonvolatile	Membrane components, hormones, defense
Tetraterpenes	Carotenoids and apocarotenoids (strigolactone)	Nonvolatile and volatile	Pigments, hormones



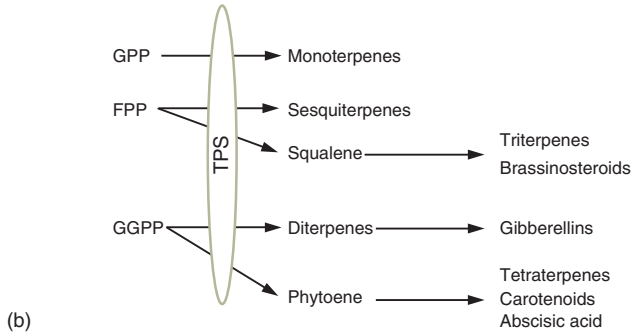


Figure 2.3 (a) Biosynthesis of terpene precursors from isoprenyl building blocks. IPP – isopentenyl diphosphate; DMAPP – dimethylallyl diphosphate; GPP – geranyl diphosphate; FPP – farnesyl diphosphate; GGPP – geranylgeranyl diphosphate. IPP and DMAPP are interconverted by IPP isomerase. DMAPP serves as the starting unit for the elongation of the isoprenyl chain. Elongation is achieved by head-to-tail linkage between an allylic diphosphate and an isopentenyl diphosphate. Each isoprenyl diphosphate is synthesized by a specific isoprenyl diphosphate synthase (GPS for GPP, FPS for FPP, and GGPS for GGPP). (b) The diversity of terpene backbones is catalyzed by enzymes belonging to the large family of terpene synthases (TPS).

5-phosphate (DXP), which is then reduced to 2C-methyl-D-erythritol 4-phosphate (MEP). MEP is then conjugated to a cytidyl nucleotide to yield 4-(cytidine-5′-diphospho)-2C-methyl-D-erythritol (CDP-ME), which is phosphorylated to 4-diphosphocytidyl-2C-methyl-D-erythritol 4-phosphate (CDP-MEP). The next two steps are a cyclization of CDP-MEP to 2C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP), and a reduction to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). In the last step, HMBPP is further reduced to IPP, with smaller quantities of DMAPP. Isomerization of IPP and DMAPP is controlled by IPP isomerase. This pathway is either called the **MEP or the DXP pathway** (Figure 2.4). After its discovery in bacteria, it was shown that the MEP pathway is also present in plants, where it is localized in the plastids and accounts for the supply of isoprenyl diphosphate precursors for plastidic isoprenoids, including the phytol chain, which is in the chlorophyll and carotenoids, and for monoterpenes and diterpenoids such as the gibberellins. IPP and DMAPP can be assembled in head-to-tail fashion to generate allylic isoprenyl diphosphate molecules of increasing length (Figure 2.3a). This gives rise to the three most important isoprenyl diphosphates, namely, geranyl (GPP) (C10), farnesyl (FPP) (C15), and geranylgeranyl diphosphates (GGPP) (C20). The enzymes that catalyze these isoprenyl chain elongations are called **isoprenyl diphosphate synthases** (Figure 2.3a). GPP, FPP, and GGPP are then metabolized by enzymes that are responsible for the structural diversity of terpene backbones. These terpene synthases (TPSs) constitute a large family of enzymes with conserved motifs required for their catalytic activity. Terpenes can be classified as acyclic or cyclic. All TPSs initiate their reactions by the formation of a carbocation, either from the dephosphorylation of the isoprenyl diphosphate, or by

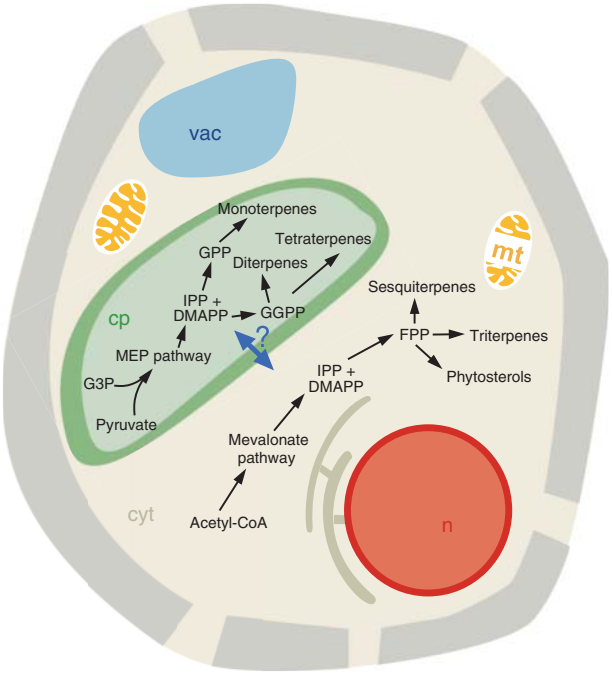


Figure 2.4 Subcellular compartmentalization of terpene biosynthetic pathways in plant cells. Abbreviations are provided in the text. The exchange of isoprenyl diphosphate precursors between cytosol and plastids occurs (represented by double arrow across the plastid envelope), but the mechanism for transport is still unknown.

proton abstraction of a C–C double bond of the isoprenyl chain. The primary carbocation can then be neutralized by hydride loss, or by a hydroxyl group. Alternatively, it can initiate a cyclization process via a succession of electron transfer and/or hydride shifts. Some TPSs produce a single main product, whereas others may yield up to 50 different terpenes. This product promiscuity is a distinctive feature of TPSs, and structural studies have allowed the identification of amino acid residues that are involved in product profile.

In plants, different subcellular compartments contribute to distinct terpene classes. Terpenes derived from FPP (sesqui- and triterpenes) are produced in the cytosol, whereas terpenes derived from GPP and GGPP are synthesized in the plastids. It follows that sesqui- and triterpenes are generally derived from the cytosolic MEV pathway, whereas mono- and diterpenes are derived from the plastidic MEP pathway. This general rule suffers some exceptions. First, it has been observed in several cases that sesquiterpenes may incorporate precursors from the MEP pathway, indicating that transport of IPP or DMAPP from the plastids to the cytosol occurs. In addition, some recently discovered sesquiterpene synthases in tomato (*Solanum lycopersicum, Solanales, Asteridae*) utilize a distinct stereoisomer of FPP (*cis,cis*-FPP as opposed to *trans, trans*-FPP) which is produced in the plastids. These sesquiterpenes are, therefore, derived from the MEP pathway.

Various enzymatic modifications can then intervene to transform the olefinic terpenes into more complex molecules. The first modifications are typically oxidations, resulting in alcohol, aldehyde, ketone, epoxyde, or carboxylic acid groups. These are electrophilic groups that can be further derivatized, for example, by acylation (acetyl), glycosylation, and malonylation, among others. To illustrate this biosynthetic framework, two examples are shown in Figures 2.5 and 2.6. The first is artemisinin, which is a sesquiterpene lactone from *A. annua* and used to treat malaria. The second, taxol, a diterpene from yew tree (*Taxus* sp.) (see Figure 4.15f), has already been mentioned for its anticancer properties. Although the elucidation of the pathways of these compounds is not yet completed, the identification of genes involved in the first steps has, in the case of artemisinin, allowed the development of alternate production methods by transposing part of the pathway in microorganisms. Typical enzymes involved in the oxidation steps are **cytochrome P450 monooxygenases**. Cytochrome P450s constitute a large family of enzymes in plants (e.g., the *Arabidopsis* genome contains 256 genes encoding P450s), whose functions still remain to be determined. A majority of these enzymes are involved in the biosynthesis of specialized metabolites, and the expansion of this gene family is a reflection of the expansion of specialized metabolites in plants. In taxol biosynthesis (Figure 2.6), the diterpene skeleton, taxadiene, is first oxidized at various positions (C5, C7, C1, C9, C10, C13). This is followed by further oxidation, which leads to the formation of the characteristic oxetane ring required for biological activity, and acylation reactions then take place bringing acetyl and benzoyl groups. The final steps link the side chain to the oxidized taxadiene core. The side chain is derived from α-phenylalanine, which is first converted to β-phenylalanine, then activated to a CoA ester before being conjugated to the oxidized taxadiene. The side chain is further modified by hydroxylation and subsequent addition of a benzoyl moiety. This still hypothetical biosynthesis scheme illustrates the sometimes highly complex pathway to plant natural products. It should be pointed out that taxol is one taxane among over 200 that *Taxus* species produce. This diversity should be viewed as an intricate **metabolic grid** that results from the multiple combinations of oxidations, acylations, side chain additions, and taxane core rearrangements. One particular oxidase could act on different substrates to oxidize the taxane skeleton at a specific position. What determines the relative abundance of a particular taxane is the combination of the affinities of all these modifying enzymes, their abundance, and spatial distribution. The concept of a **metabolic grid** is presented in Box 2.1 (Figure 2.7).

2.2.2 Alkaloid Biosynthesis

Alkaloids comprise a group of plant secondary compounds with more than 21 000 structures that have been elucidated up to now. The name alkaloid is derived from the Arabic

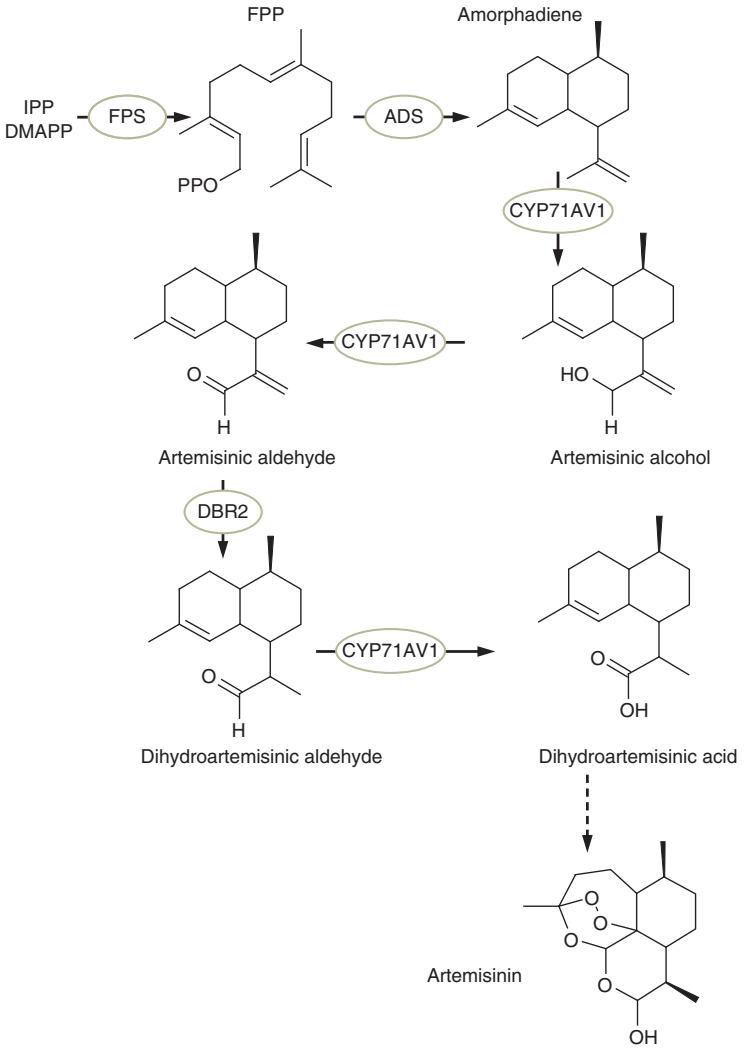


Figure 2.5 Scheme of the artemisinin pathway. Amorphadiene synthase (ADS) is the sesquiterpene synthase that transforms FPP into the first committed precursor of the pathway. Amorphadiene is then oxidized by a cytochrome P450 monooxygenase (CYP71AV1) to artemisinic alcool and aldehyde. The latter is reduced by a reductase (DBR2) to dihydroartemisinic aldehyde, and then further oxidized by CYP71AV1 to dihydroartemisinic acid. The last steps to artemisinin are currently unknown and could also occur partly through spontaneous oxidation.

name *al-quali*, the plant from which soda was first isolated. This name also reflects the basic nature of the compounds that are caused by the presence of at least one nitrogen atom. Thus, alkaloids can be defined as nitrogen-containing compounds with basic properties. In most cases, the nitrogen atom is derived from amino acids that represent the link to primary metabolism (Figure 2.8, see Section S1.3.9). The nature of the amino acid determines the class of alkaloids and constitutes the start of alkaloid biosynthesis. The amino acid is modified, mostly by decarboxylation or deamination, and undergoes further structural modifications. In some cases it may be condensed either with another modified amino acid or with compounds derived from other secondary metabolite pathways. It is the combinations of structural modifications of the alkaloid backbones and the nature of the condensation partner molecules that lead to the rich diversity in alkaloid metabolism.

2.2.2.1 Generation of Metabolic Diversity across Alkaloid Classes

To exemplify the generation of metabolic diversity in alkaloid biosynthesis, the major building blocks of certain alkaloid classes are briefly described, and how these building blocks are recruited from primary as well as specialized metabolite pathways is also illustrated. More detailed presentation of some alkaloid pathways follows in subsequent paragraphs.

Piperidine alkaloids are derived from the amino acid lysine, through its decarboxylated derivative cadaverine. After the formation of the piperidine ring (with 5 carbons and 1 nitrogen – C5N), it can be condensed to several

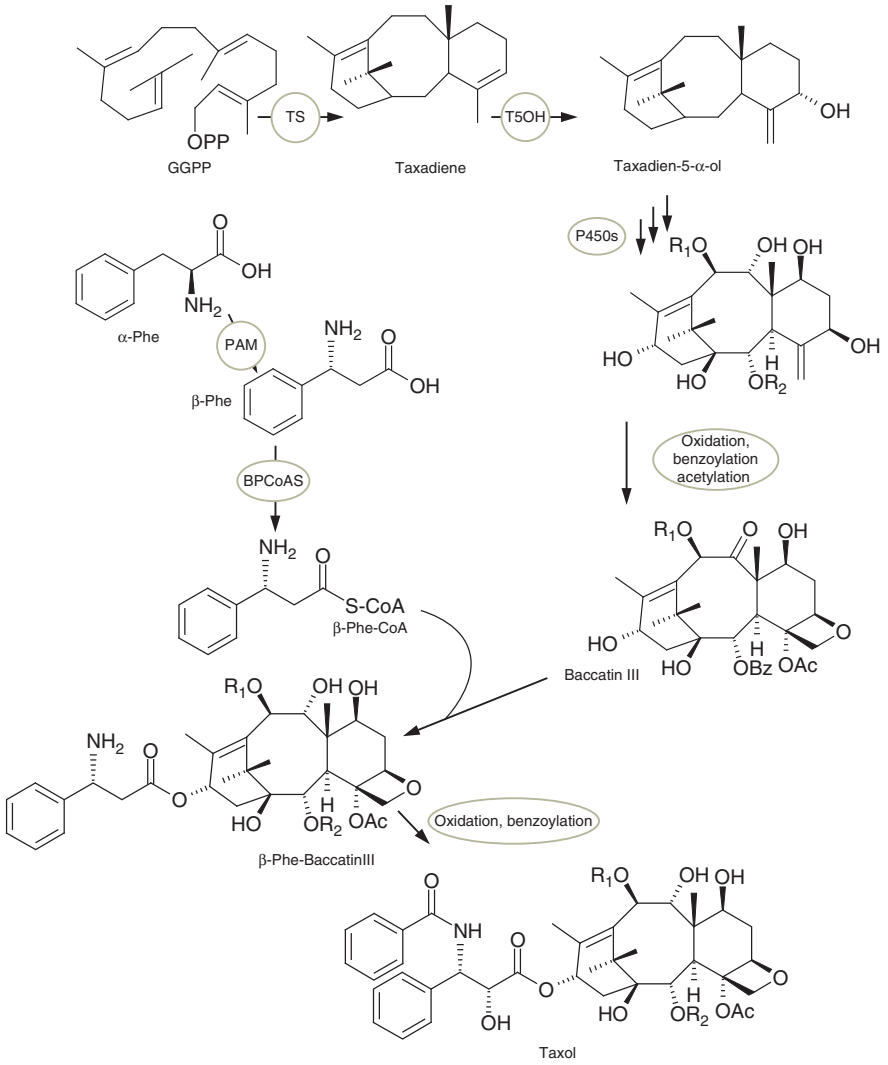


Figure 2.6 Outline of the taxol biosynthesis pathway. TS – taxadiene synthase; T5OH – taxadiene-5-hydroxylase; PAM – phenylalanine amino mutase; BPCoAS – beta-phenylalanine CoA synthase; P450 – cytochrome P450 monooxygenase. Explanations to the pathway are provided in the text.

Box 2.1: The concept of metabolic grid

Many specialized metabolites do not arise from a single linear pathway, but rather through a network of metabolites connected between them by a limited number of enzyme activities. In a theoretical scheme (see Figure 2.7), a set of 6 enzymes (represented by E1 through E6) could theoretically give rise to 27 different products. Many enzymes in speciallized metabolism show a somewhat relaxed specificity allowing them to modify in the same fashion many related metabolites. This could be, for example, a hydroxylation at a specific position of a phenylpropanoid core compound, followed by methylation. The relative amounts of each metabolite in the grid will depend on the relative expression level of the different enzymes, the availability of these enzymes in distinct tissues or cellular compartments, and the further modification or transport of the metabolites. In addition, isoforms of enzymes may coexist with slightly different affinities for molecules within a set of related metabolites. In our theoretical example, in two different tissues of the same plant, different pools of metabolites could accumulate depending on the relative expression levels of the enzymes E1 through E6. The blue path would imply strong expression of E3 and E4, and perhaps the expression of an E1 isoform with higher affinity for the $0A_02$ substrate. In the red pathway, E5 would be strongly expressed, followed by E3 and E4 isoforms with stronger affinities for the $0A_1$ series of compounds, and finally with E6. This flexibility in pathway architecture may be used to produce a distinct set of compounds with specific biological functions.

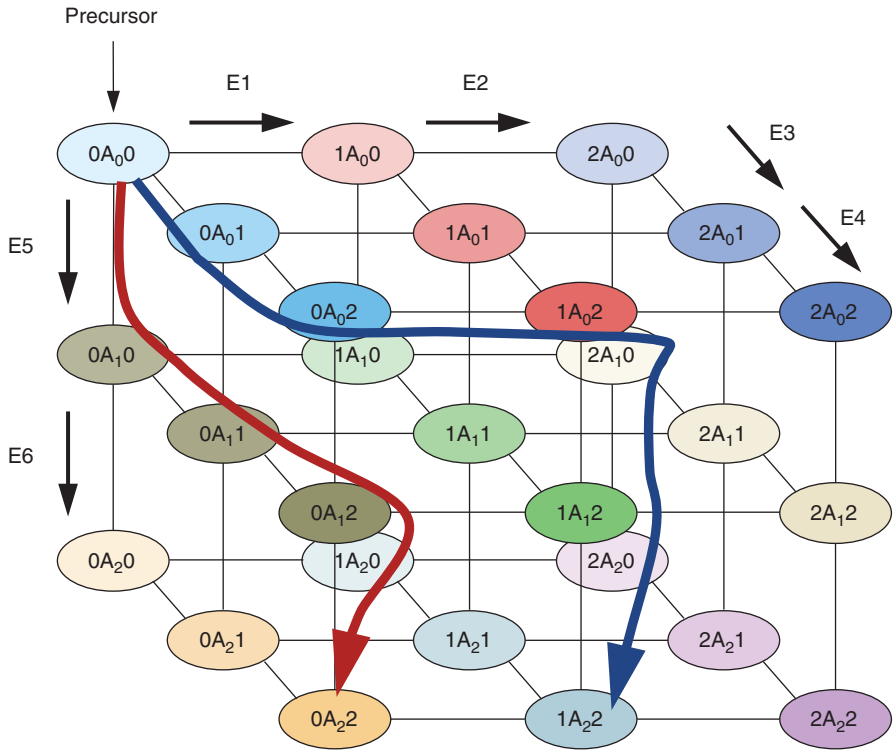


Figure 2.7 The concept of metabolic grid.

phenolic compounds, one of which is piperoyl CoA, leading to piperine, conferring its pungency to black pepper (*Piper nigrum, Piperales, Magnoliophyta*). Alternatively, the piperidine ring can be attached to nicotinic acid (a pyridine). Those alkaloids are classified as **pyridine alkaloids**. A more prominent pyridine alkaloid is nicotine, where the pyridine ring is attached to a pyrrolidine ring (C4N), which is derived from the amino acid glutamine via ornithine and, after decarboxylation, putrescine. Alternatively, the pyrrolidine ring can be modified by the sequential addition of C2 units and form the class of **tropane alkaloids**, such as the calystegines (see Figure 2.10). The tropane backbone can be further condensed to phenolic compounds leading to molecules such as cocaine or atropine. In addition, ornithine and putrescine, respectively, can also feed into the biosynthesis of **pyrrolizidine alkaloids** by the cyclization of homospermidine, which is generated from putrescine through extension by an aminobutyl moiety. The pyrrolizidine ring can be further modified by the esterification to necic acids, which resemble terpenoid structures, but they are more likely derived from amino acids such as isoleucine, valine, and, threonine, or from acetate units. A "true" metabolite from the terpene pathway is part of the **monoterpenoid indole alkaloids**. They are condensation products of secologanin, a monoterpene glucoside, and tryptamine, which is generated by the decarboxylation of tryptophan. Secologanin can also be condensed to dopamine, derived by decarboxylation and hydroxylation of tyrosine, leading to the formation of **ipecacuanha alkaloids**. Tyrosine derivatives also constitute the building blocks of the **benzylisoquinoline alkaloids** (BIAs). In this case, two tyrosine-derived molecules are condensed to form a tetrahydrobenzyliosquinoline backbone (Figure 2.9).

2.2.2.2 Generation of Metabolic Diversity within Alkaloid Classes

The backbones of the alkaloid classes described in the previous section most often undergo extensive modifications by methylations, hydroxylations, oxidations, and reductions leading to many alkaloid subclasses and to many individual compounds in these subclasses (see Figure 2.7). As an example the generation of metabolic diversity within BIA pathway is briefly described (Figure 2.9). Starting from the tetrahydrobenzylisoquinoline backbone generated by the condensation of two tyrosine-derived molecules, about 2500 structures are generated. After initial hydroxylations and methylations, the central metabolite (*S*)-reticuline is formed and serves as the precursor to most BIAs. However, hydroxylations and position-specific methylation as well as dimerizations can lead to the formation of several other BIAs before reticuline. From reticuline, the pathway bifurcates and many BIA subclasses are formed. A common theme is the generation of subclass-specific backbones by the formation of different carbon-carbon bonds. The so-called berberine bridge between the *N*-methyl group and C2′ of the benzyl moiety is formed by the berberine bridge enzyme (also called *reticuline oxidase*), and the

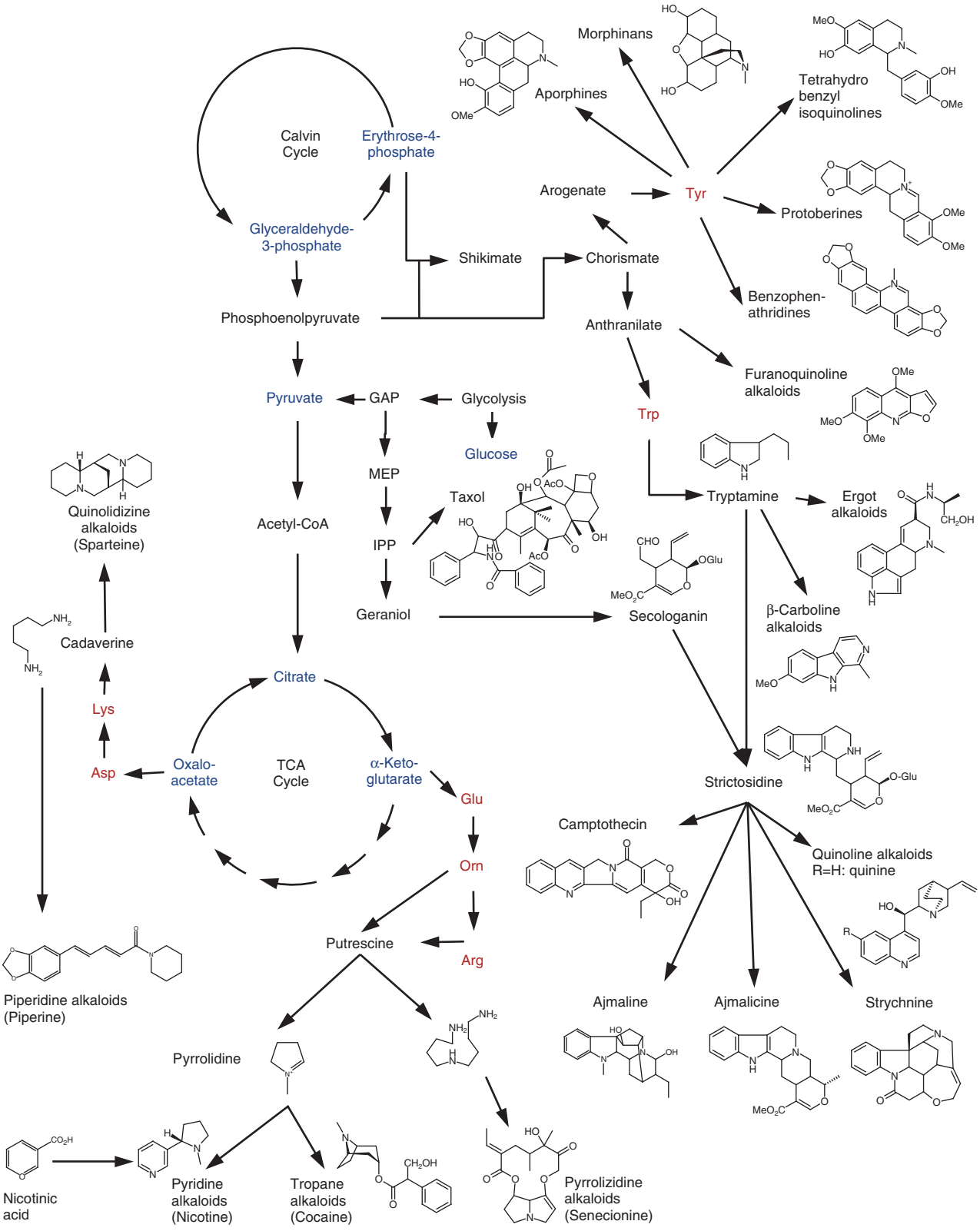


Figure 2.8 Biosynthetic origin of alkaloid biosynthesis. (Courtesy of M. Wink, D. Dobritzsch.)

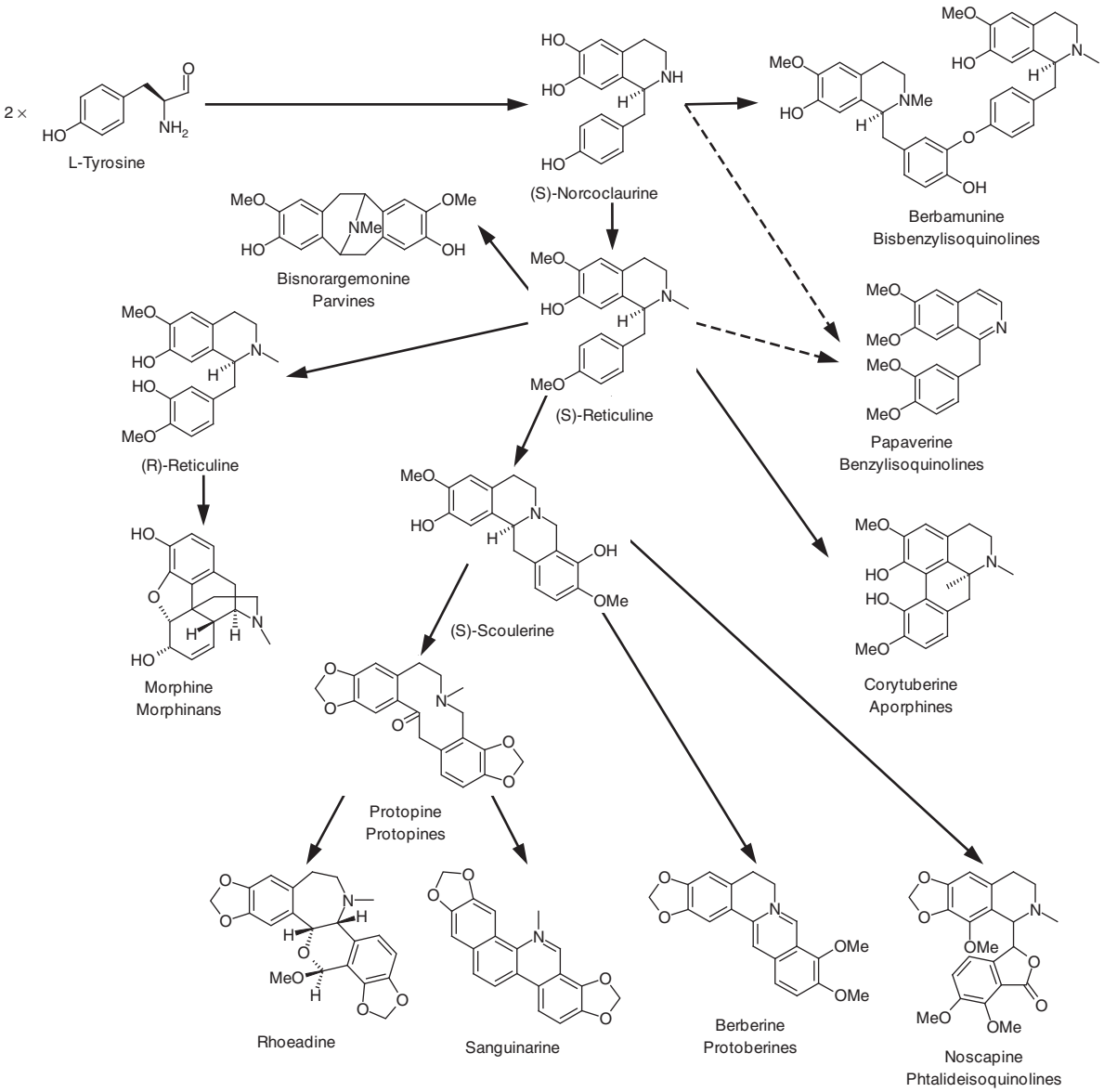


Figure 2.9 Benzylisoquinoline pathway showing a single representative compound for each benzylisoquinoline class.

resulting scoulerine compound is further modified to many different compounds. C–C phenol couplings between different carbon atoms of the isoquinoline moiety and the benzyl ring are other common reactions, which are mainly catalyzed by cytochrome P450s. Again, the position specificities of the enzymes determine the generation of individual subclasses. For example, C–C phenol coupling between C8 and C2′ leads to the aporphine alkaloids, whereas coupling between C3 and C2′ leads to the pavine alkaloids. In this way, compounds that are derived from a number of backbones generated by C–C bond formations between different positions have been isolated. Whereas most BIAs are derived from (*S*)-reticuline, the morphinane alkaloids represent an exception. Here, the backbone is generated by C–C phenol coupling between C2′ and C4a of (*R*)-reticuline, that is, the inversion of stereochemistry is necessary. The diversification of the BIA pathway into several subclasses is due to the specificities of the respective P450 monooxygenases catalyzing the C–C phenol coupling reactions. However, how these enzymes evolved and whether they have evolved from a common ancestor cannot be determined, as most of the enzymes are still unknown at the molecular level. Only the genes for two P450-dependent monooxygenases catalyzing C–C phenol coupling have been cloned. The proteins belong to two different **cytochrome P450 families** and share less than 40% identity at the amino acid level. Whether this low level of identity is also reflected in the active center and the substrate-binding pocket is currently not known.

In the case of tropinone reductase, which is involved in **tropane alkaloid biosynthesis** (Figure 2.10), modification of the reaction specificity of an enzyme leads to the

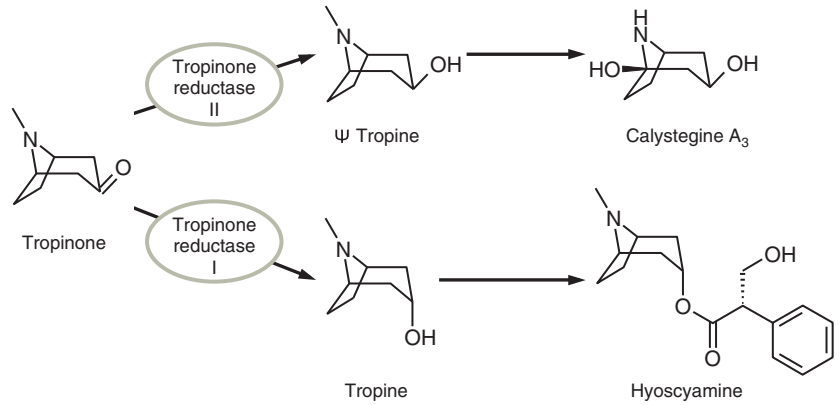


Figure 2.10 Stereospecific reduction of tropinone by tropinine reductase I and II.

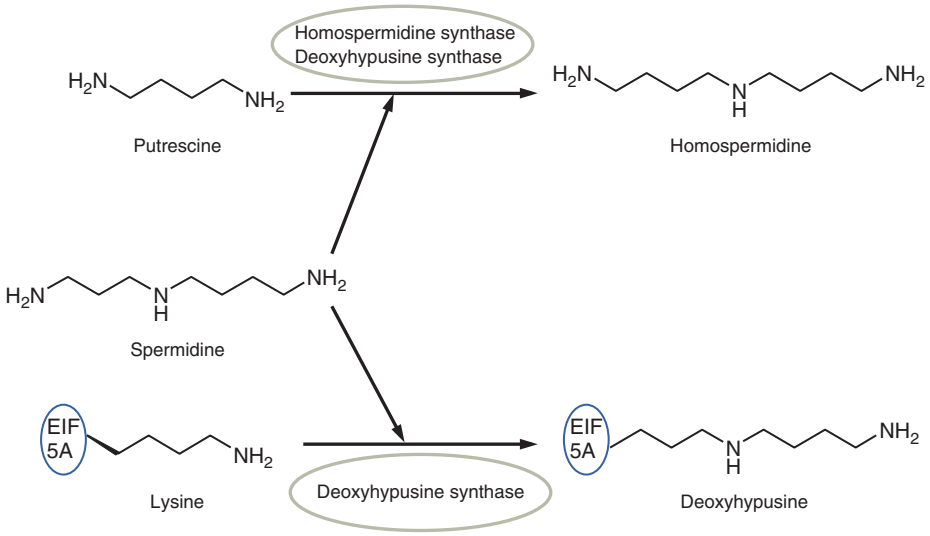


Figure 2.11 Reactions catalyzed by homospermidine synthase and deoxyhypusine synthase. EIF 5A – Elogation Initation Factor 5A.

bifurcation of a pathway. This enzyme is a member of the large short-chain dehydrogenase/reductase (SDR) enzyme family. Alcohol dehydrogenase represents the prototype of this protein family, and it is conceivable that SDRs involved in secondary metabolism evolved from alcohol dehydrogenase. Tropinone reductase (TR) catalyzes the stereospecific reduction of tropinone. TR-1 produces tropine with α configuration of the hydroxyl group, whereas TR-II generates ψ-tropine possessing the β configuration (Figure 2.10). The different products of both enzymes give rise to two different subpathways in tropane alkaloid metabolism. Whereas tropine is further esterified to compounds such as hyoscyamine or scopolamine, ψ-tropine is further modified to yield calystegines. TRI and TRII share 64% amino acid identity, and both enzymes could be interconverted by site-directed mutagenesis of the substrate-binding pockets.

A well-established example for the recruitment of an enzyme from primary metabolism to plant secondary metabolism has been elucidated in the biosynthesis for pyrrolizidine alkaloids (Figure 2.11). Here, the committed step in the generation of the bicyclic pyrrolizidine skeleton is the transfer of an aminopropyl group from spermidine to putrescine, resulting in the formation of homospermidine. The enzyme homospermidine synthase shares 79% identical amino acid residues with deoxyhypusine synthase, which transfers an aminopropyl group from spermidine to a lysine residue in order to activate the translation initiation factor 5A. Biochemical characterization of both genes revealed that deoxyhypusine synthase is able to perform both, the deoxyhypusine synthase as well as the homospermidine synthase reaction, whereas homospermidine synthase catalyzes only the generation of homospermidine (Figure 2.11). The current model for the evolution of homospermidine synthase describes that after the gene duplication of the deoxyhypusine synthase gene, one daughter gene lost the ability to modify the lysine residue of the translation initiation factor 5A, so that the former "side activity" of the gene became the exclusive activity for homospermidine synthase and was recruited for **pyrrolizidine alkaloid metabolism**.

2.2.3 Phenylpropanoid Metabolism

The general phenylpropanoid metabolism illustrates the current problem to differentiate primary and secondary metabolism. Although the key entry step into phenylpropanoid and, therefore, secondary or specialized metabolism by **phenylalanine ammonia lyase** (**PAL**) or **tyrosine ammonia lyase** and (**TAL**) to yield cinnamic acid or *p*-coumaric acid is not disputed, the direct consequence to classify all products resulting from the subsequent plethora of reactions, specifically the polymers lignin or sporopollenin as *secondary,* is heavily discussed. Lignin is a phenylpropanoid-based structure, characteristic of all vascular plants, and lignin-like material was probably available as early as 500 million years ago, soon after the earliest plant progenitors invaded the land. Its universal distribution and its requirements for structural rigidity may, therefore, be as relevant as that of cellulose. The term **ligno-cellulose** introduced by plant biologists and biotechnologists describes this close chemical and functional interaction of these essential plant polymers and precludes a clear separation between the primary and the secondary.

The structures of the phenylpropanoids are as versatile as their *in vivo* biological functions. An energy-rich thioester, 4-coumaroyl CoA, the product of coenzyme A-dependent ligases, can be perceived as the key intermediate for more than a dozen of different pathways toward chromogenic anthocyanins, flavonoids and exotic aurons, antioxidative proanthocyanidins and stilbenes, volatile phenylpropenes, complex phenolamides, or polymeric lignin, suberin and sporopollenin to name just the major classes of compounds (Figure 2.12).

The combination of the central shikimic acid pathway and subsequent general phenylpropanoid building blocks, potentially organized as individually operating **metabolons** or as loosely associated protein complexes, sometimes relies on the participation of identical proteins to catalyze the same reactions in different pathways. Cation-dependent caffeoyl CoA *O*-methyltransferase 1 (CCoAOMT1) from *Arabidopsis thaliana* catalyzes a central enzymatic methylation step in vascular bundles, required for guaiacyl lignin monomer formation, but is also recruited for the biosynthesis of the coumarin scopoletin in roots, the accumulation of phenolamides on pollen grains, or the biosynthesis of suberin in root endodermal tissues. All of these rather diverse compounds essentially use the same building block, feruloyl-coenzyme A. Logistically, this illustrates a tissue-specific and complex transcriptional and posttranscriptional organization, which involves matters of transport, organization, or degradation. The resulting variability of this pathway and diversity of individual core structures displayed in Figure 2.12 are further amplified by the combination of an array of modifying enzymes including membrane-associated cytochrome P450s as well as soluble glycosyl-, methyl- and acyltransferases. This provides another nice illustration to the concept of **metabolic grid**. Diversification of product formation coupled with tissue or cell-specific distribution as in the case of terpenoids and alkaloids enables flexible or adaptive *functional priming* toward rapidly changing and often extreme biotic and abiotic environments, which plants as sessile organisms may be or have been exposed to during evolution.

2.2.3.1 Hydroxycinnamic Acids and Lignin

PAL and TAL are evolutionary derived from a gene family involved in the degradation of histidine, histidine ammonia lyases (HALs). This provides direct evidence of a close connection of primary and specialized metabolism. The nonoxidative deamination of phenylalanine to *trans*-cinnamic acid directs a considerable part of the carbon flow from aromatic amino acid biosynthesis to the huge diversity of phenolic compounds observed in plants, microbes, and fungi. Tyrosine and tryptophan, also products of the shikimic acid pathway (see Section S1.3.9.8), to the largest part feed alkaloid or glucosinolate-derived metabolites, which are also discussed in this chapter. The shikimic acid pathway in plants is localized completely in plastids whereas large parts of the phenylpropanoid biosynthesis are considered to take place in the cytosol, likely associated with microsomal or at least membrane-like structures (Figure 2.12). Subsequent hydroxylation reactions from cinnamate to p-coumarate and caffeate are performed by cytochrome P450s. The second hydroxylation takes place at the level of energy-rich coumaroyl-shikimate or -quinate esters, both intermediates of the shikimate pathway. These unusual enzymatic steps may serve as an additional flux-control toward polymeric phenylpropanoids, allowing the production of lignin precursors only if the photosynthesis dependent energy-status of the cells or of the complete organism is high. **Feruloyl-CoA**, derived from these P450-dependent hydroxylations and a single CCoAOMT1 catalyzed methylation, is the precursor of guaiacyl lignin, the evolutionary ancient lignin polymer, characteristic of gymnosperms (Acrogymnospermae, Spermatophyta). The biosynthesis of angiosperm syringyl lignin, characterized by a higher hydroxylation and methylation pattern, evolved later independently from guaiacyl lignin formation and is characterized by an additional cytochrome P450 step, performed by coniferyl aldehyde 5-hydroxylase, followed by a subsequent methylation step, catalyzed by a cation-independent caffeic acid *O*-methyltransferase (COMT1). This *O*-methyltransferase also methylates flavonoid and anthocyanin glycosides in epidermal tissues of some plants, whereas flavonoid and anthocyanin methylations can also be performed by cation-dependent, CCoAOMT1-like enzymes. This again illustrates the plasticity of pathway as well as the occurrence of convergent evolution, an issue that is addressed later in this chapter.

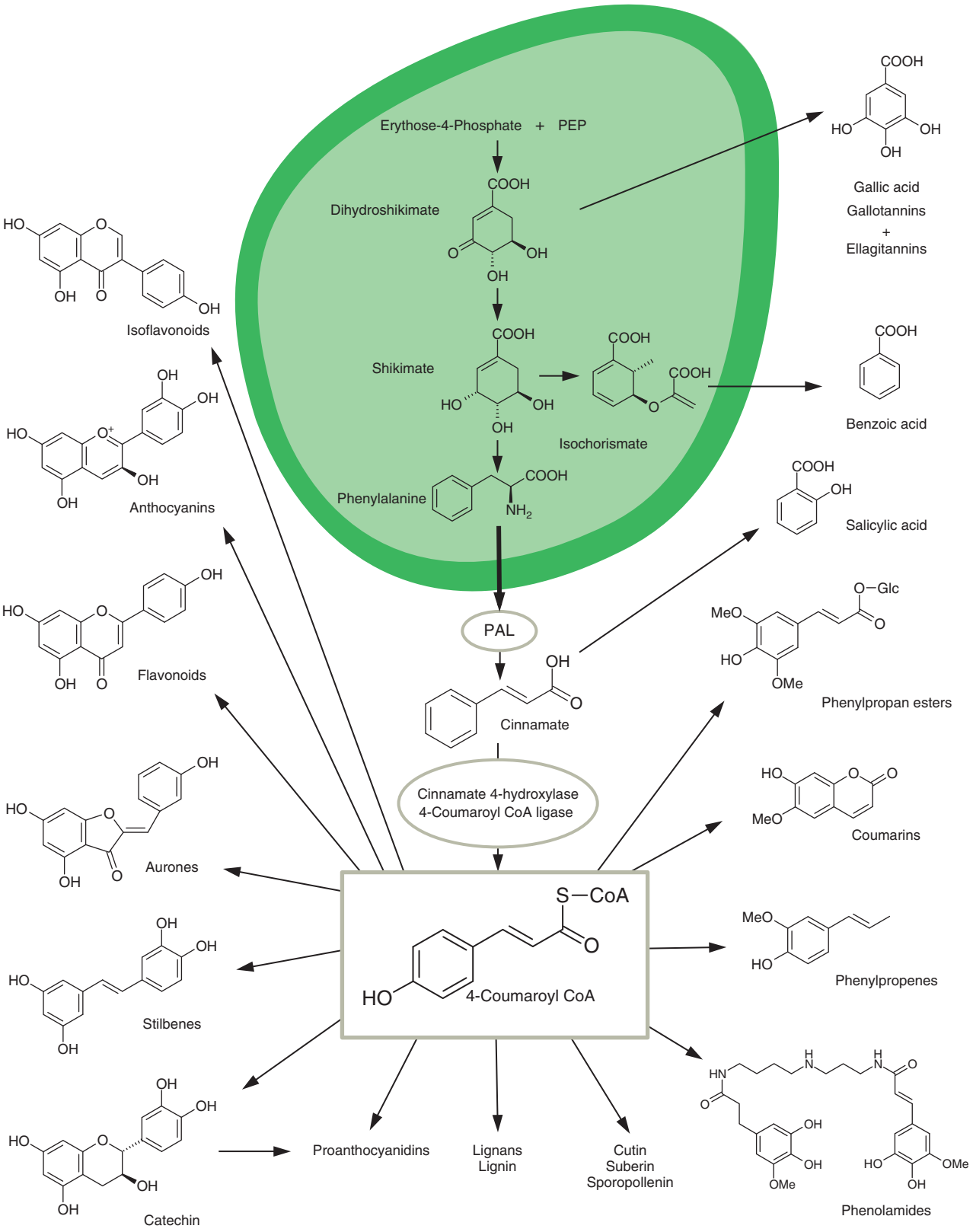


Figure 2.12 The structural diversity of phenylpropanoids originates in the shikimic acid pathway of the chloroplast and is derived to a large part from the key intermediate 4-coumaroyl coenzyme A.

The decisive steps toward the biosynthesis of soluble lignans or polymeric lignin are performed by two consecutive reductions, performed by (hydroxy)cinnamoyl CoA reductase (CCR) and (hydroxy)cinnamoyl alcohol dehydrogenase (CAD) that result in the monolignols, coumaroyl-, coniferyl-, and sinapoyl alcohol, respectively. Lignans are usually dimeric coupling products of monolignols found in the majority of plant tissues and rather abundant in seed coats and stems. Whether the required stereoselective coupling of monolignol monomer to lignans is assisted by specific proteins with monolignol binding sites providing a chiral reaction center is still disputed in the literature.

Random or assisted polymerization of these building blocks by a set of peroxidases and laccases results in a stable and rigid aromatic scaffold accompanied by cellulose and hemicellulose-like material, further impregnated with aromatic inclusions, termed ligno-cellulose, characteristic of the species-specific types of timber as we all know it. Because lignin or ligno-cellulose is the most abundant and important organic polymer on earth, lignin content and composition has been the target of genetic engineering for various purposes, including better digestibility by ruminants, improved paper, or biofuel production. Given the high metabolic control of lignin biosynthesis and the role of phenylpropanoid metabolism in defense mechanisms, successful development of such designer crops will be a major challenge.

Cutin, suberin, and sporopollenin are plant polymers where phenylpropanoids play similar structural and functional roles although these compounds do not consist of phenylpropanoid units only. Lipophilic cutin and suberin both largely consist of hydroxycinnamic acid monomers esterified with hydroxylated fatty acids and are deposited (sometimes in multiple layers as in the case of the cork oak, *Quercus suber, Fagales, Rosidae*) on plants surfaces, like leaves and stems, or in the root endodermis where they serve as water barriers or defense compounds. The structure of sporopollenin, which is part of the rigid pollen wall, is much less defined although again the contribution of fatty acids and aromatic units has been confirmed by chemical analysis as well as mutant characterization.

Soluble hydroxycinnamic acids are present in all kinds of organs usually as esters or amides in the vacuoles. Typical esters can be widespread throughout the plant kingdom like chlorogenic acid (caffeoylquinate) or be characteristic of specific plant families like sinapoyl malate, glucose, and choline esters, found in high concentrations in the leaves and seeds, respectively, of the Brassicaceae. The use of *A. thaliana* knockout mutants has improved the functional analysis of these compounds. Soluble phenolamides are found in high concentrations in flowers, specifically on the surface of pollen grains, but also occur in stems, leaves, seeds, and roots. Reported roles in abiotic stress, absorbing UV light and protecting from radicals, or biotic stress response (pathogen defence) appear plausible.

2.2.3.2 Flavonoids, (Pro)anthocyanins, and other Chromogenic Structures

Chalcone synthase (CHS), a member of the widespread polyketide synthase superfamily (see Figure 3.5), uses 4-coumaroyl CoA and three molecules of malonyl CoA as starters to build flavonoids and anthocyanins, the characteristic chromogenic phenylpropanoids in flowers of angiosperms. Anthocyanin biosynthesis is probably the best understood biosynthetic pathway because mutant phenotypes and genetic imbalances can be easily detected by a change in flower color.

Flavonoids are present also in nonflowering plants like gymnosperms, ferns, and some mosses. A combination of 2-oxoglutarate-dependent dioxygenases and cytochrome P450 monooxygenases provides a variable oxygenation pattern of the conjugated aromatic systems. The subsequent methylation, glycosylation, and acylation reactions generate thousands of either water soluble intracellular glycosides or hydrophobic extracellular aglycones found on the surface of many plant species.

A higher hydroxylation pattern is considered as *primitive* in terms of evolution, although specific hydroxylations in the A-ring (Figure 2.12), widespread in the *Asteraceae* (*Asterales, Asteridae*), are discussed as advanced features.

Color intensity is enhanced by pH, metal-ions, as well as intra- and intermolecular copigmentation, that is, stacking of acylated anthocyanins and flavonoids mediated in part by the sugar side chains and results in bathochromic color shifts of the complex. **Anthocyanins** are light-sensitive reddish flavylium cations at very low pH and may change color when the pH gradually increases from colorless, to bluish, and even yellow at high pH depending on their substitution pattern and the presence of complexing inorganic ions, like Mg^{2+}, Al^{3+}, or Fe^{3+}. Therefore, the pH as well as the ion content of the soil may significantly influence flower coloration. A multimillion dollar-worth 20 years struggle for a deep-blue rose or bluish carnation illustrates the efforts to modify and enhance specifically ornamental flower color by genetic engineering. The rose family produces only cyanidin-based anthocyanins, whereas an additional cytochrome P450-dependent flavonoid 3′,5′-hydroxylase resulting in a higher hydroxylated delphinidin-based structure is required for a bluish hue. Additional hydroxylation, methylation, and copigmentation steps were engineered to finally result in the desired lines with blue petals that are already commercially available in Japan.

The common and widespread anthocyanins should not be confused with red and purple **betacyanins** that have replaced those structures in most *Caryophyllales* (core eudicotyledons, Magnoliophyta) like in red beet or cacti. Chromogenic betacyanins contain nitrogen and, therefore, can be classified as alkaloids, which are structurally derived from two molecules of tyrosine. Their distribution in plants relies on the presence of two unique enzymes: an unusual cytochrome P450 (CYP76AD1) required for the formation of *cyclo*-DOPA, from dihydroxyphenylalanine (DOPA) and

a unique DOPA-4,5-dioxygenase (DODA), which cleaves DOPA between carbons 4 and 5 to result in betalamic acid, which is then imino-conjugated to *cyclo*-DOPA. The formation of yellow **betaxanthins** is dependent only on the presence of DODA, because conjugation of betalamic acid with various amino acids appears to be spontaneous, although the exact mechanisms why only selected amino acids are conjugated in a species or organ-specific manner are unknown. The evolutionary origin of both types of compounds characterized by the presence of CYP76D1, betalamic acid, and DODA combined with a simultaneous loss of anthocyanin synthase is still an unsolved mystery. Horizontal gene transfer has been proposed as similar reddish structures are known from some fungi, for example, in the fruit body of the fly agaric, *Amanita muscaria* (*Agaricales, Basidiomycota*), to name the most prominent one. However, the origins and reasons proposed for such an evolution are still speculative.

Like betacyanins, bright yellow aurons are of limited distribution in the plant kingdom. In contrast to betacyanins they co-occur in anthocyanin-containing plants. Their synthesis from chalcones is initiated by aureusidin synthase and results in a five-membered C-ring instead of a six-membered ring as in the case of flavonoids.

Flavonoids are not only present as flower colorants but have also important roles in signal recognition and perception. In legumes (*Fabaceae, Fabales, Rosidae*), secreted flavone aglycones initiate root colonization by rhizobia to result in the subsequent formation of nodules and establishment of a classical symbiosis (see Chapter 15). Certain flavonols like quercetin glycosides are reported to regulate the basipetal transport of the plant hormone auxin, indole acetic acid (IAA), and as a consequence may affect root and shoot development. In maize (*Zea mays, Poales, Liliopsida*) and petunia (*Petunia, Solanales, Asteridae*), flavonol aglycones are essential for pollen tube growth and reproduction.

All compounds discussed so far show at least a cell- or organ-specific distribution and a defined molecular signature or substitution pattern that is essential for the observed variety of functions. This signature is the result of substrate and position-specific glycosyl-, methyl-, or prenyltransferases and generates a compound that displays varying taste, flavor, or pharmacologic properties in humans. For example, the flavanone rhamnosido-glucoside naringin from grapefruits (*Citrus, Sapindales, Rosidae*), characterized by β-1,2-linked sugars, is extremely bitter, whereas the similar narirutin, characterized by an β-1,6-linked diglycoside at the same position, is tasteless. Likewise, the flavanone 8-prenylnaringenin is a potent phytoestrogene and very bitter, and a single methylation at position 5 eliminates both activities.

The core structure of stilbenes (Figure 2.12), with limited distribution in the plant kingdom, is also synthesized via polyketide synthase type III enzymes from 4–coumaroyl CoA and malonyl CoA, as are the flavonoids. Resveratrol, a defense compound of grape (*Vitis vinifera, Vitales, Rosidae*), synthesized by stilbene synthase, shows potent antioxidative and cancer-preventing or heart-protective activity and was long thought to be responsible for the apparent health-promoting effect of red wine, observed in Mediterranean countries, known as the *French-Paradox*. This effect, however, may also be due to oxygenated proanthocyanidin mono- and oligomers, often referred to as *condensed tannins*. These (epi)catechin-derived structures are prominent constituents in stems and seed coats. Procanthocyanidins share the common flavonoid pathway to anthocyanidins, but are not immediately glycosylated to anthocyanins, but instead reduced first by anthocyanidin reductase to (epi)catechin. Subsequent polymerization in the vacuoles and vesicle-mediated transport as complex oligomers to the cell walls is proposed for proanthocyanidins where they accumulate as characteristic compounds of seeds and fruit skins of grapes and other plants.

As in the case of proanthocyanidines, storage and intracellular transport of soluble flavonoids, anthocyanins, or betalains is still an issue of debate. The role of **MATE** (**multidrug and toxic compound extrusion**) and **ABC** (**ATP binding cassette**) transporters in the deposition and export of flavonoid conjugates are established. Further mechanisms like conjugation of certain compounds to glutathione (GSH) are still somewhat enigmatic. Availability of substrates, specificity of transporters, and vacuolar pH may determine the individual vacuolar flavonoid or betalain content of directly adjacent cell layers. Vesicle-mediated transport of compounds to the extracellular space has been suggested to explain the deposition of oligomeric proanthocyanidins in the seed coat.

2.2.3.3 Simple C_6-C_1 Phenolics

In contrast to condensed tannins, hydrolysable tannins are derived from simple phenolics like gallic acid or ellagic acid usually esterified with one or a few sugar residues. The compounds display a wide occurrence throughout the plant kingdom and are prominent, for example, in *Rosaceae* (*Rosales*), *Fagaceae* (*Fagales*), and *Anacardiaceae* (*Sapindales;* all three orders *Rosidae*). Contrary to the carbon-carbon linked condensed proanthocyanidin oligomers, the compounds can be degraded by acidic hydrolysis and release highly hydroxylated phenolic structures that rapidly interact with proteins resulting in insoluble precipitates. This has made the elucidation of the corresponding biosynthetic steps quite tedious. Nevertheless, the biosynthetic origin of gallic acid via dehydrogenation from 5-dehydroshikimate, an intermediate of the shikimate pathway, has been established by stable oxygen isotope labeling for *Rhus typhina* (*Anacardiaceae*).

The biosynthesis of simple **C_6-C_1 phenolics** is still fragmentary and not clarified in detail because there may be several parallel or species-specific routes. This is illustrated in the case of benzoic acid or salicylic acid (Figure 2.12). Benzoic acid can be synthesized from cinnamate either by

a β-oxidative pathway involving a 3-keto-acyl CoA-thiolase or by a nonoxidative pathway via benzaldehyde to benzoic acid. In contrast to microbes where ortho-hydroxylation of benzoic acid can be performed by a P450-monooxygenase encoded by the salicylic acid 4-hydroxylase (*NahG*) gene, there is evidence that in plants, salicylic acid is synthesized from isochorismate. It is striking that even in the case of such an important and structurally simple plant hormone, representing a key component in plant defense and systemic acquired resistance (SAR), the biosynthetic details are still somewhat obscure. The subsequent methyltransferase activity toward the carboxy group of salicylic acid is catalyzed by a member of the **SABATH-type family of enzymes** that modify carboxyl-groups and are generally implicated in hormone homoeostasis or phenylpropanoid volatile formation in plants. The name SABATH is derived from initials of compounds methylated by this type of enzyme (**s**alicylic **a**cid, **b**enzoic **a**cid, and **th**eobromine). This again illustrates the important role of modifying enzymes late in the biosynthesis of natural products in a constant effort to adjust to environmental conditions.

2.2.4 Glucosinolates and Cyanogenic Glucosides

Glucosinolates and **cyanogenic glucosides** are often perceived as the classical defense compounds of plants that are released only by hydrolysis on tissue and cell damage. Hydrolysis and release of toxic metabolites is initiated by thio-glucosidases, called *myrosinases* in the case of glucosinolates and by β-glucosidases in combination with nitrilases for cyanogenic glucosides. Whereas a complex mixture of thiocyanates or nitriles can be released from the various types of glucosinolates found in plants, the active principle of cyanogenic glucosides is the release of cyanide, toxic to aerobic organisms. In higher concentrations these glycosylated products are perceived as *bitter* by human taste.

Both types of compounds are essentially synthesized from amino acids. Glucosinolates, with very few exceptions, are found exclusively in the order of *Capparales* (= *Brassicales, Rosidae*). Prominent members are specifically known from the *Brassicaceae* including important vegetables like cauliflower, cabbage, or broccoli (all *Brassica* sp.), and the model species *A. thaliana*.

Glucosinolates can be divided into three different groups, based on their origin from different types of amino acids: aliphatic (allyl glucosinolates), aromatic (benzyl glucosinolates), or tryptophan (indole glucosinolates). Largely, on the basis of huge collections of T-DNA knockout lines (engineered with a defect in a single characteristic gene in each line) and existing genome sequence information from *A. thaliana*, the complex metabolic grid of all three types of glucosinolates has been elucidated in the past decade. Deamination and condensation with acetyl coenzyme A are required for some glucosinates derived from phenylalanine and specifically methionine. Incorporation of one or several additional methylene groups also involves isomerization, decarboxylation, and subsequent transamination before the formation of the glucosinolate core structure is achieved. As in the case of cyanogenic glucosides, formation of an aldoxime is performed by cytochrome P450-dependent monooxygenase. Activated aldoxime easily reacts with nucleophilic *S*-donors, likely cysteine, probably mediated by a glutathione *S*-transferase (GST) step. The resulting *S*-alkyl thiohydroximate is subsequently cleaved by a lyase. Two successive transferases, a glucosyl- and a sulfotransferase, perform the final glucosylation and sulfonation steps in glucosinolate formation. (Figure 2.13). In the *Brassicaceae*, individual species may contain up to 40 different glucosinolates. This diversity is generated not only by the different amino acids used to establish the core structure but also by a series of enzymes that are able to modify the initially formed side chains, for example, in the case of methionine-derived glucosinolates, several hydroxyalkyl or alkenyl glucosinolates are known.

Myrosinases (*S*-glucosidases) catalyze the hydrolysis of the thioglucoside-bond and thereby initiate their degradation to various stable and partly reactive metabolites like nitriles, isothiocyanates, or thiocyanates, depending on the pH value and ion content of the cells. Despite numerous investigations, it is not yet clear if myrosinases are localized in the same cells than glucosinolates, separated only by membranes, or if they occur specifically in adjacent cells and come into contact with the substrates only on cell damage. In *A. thaliana*, several links of glucosinolate metabolism to auxin metabolism are plausible. On the one hand, the indole glucosinolate degradation product indole acetonitrile can be hydrolyzed directly to IAA. Also, mutants that lack aldoxime formation have a high-auxin phenotype. Long distance transport of (desulfo)glucosinolates from parenchyma cells via phloem to seeds or floral stalks (in the latter case the concentration of glucosinolates may reach up to 100 mM) is supported by numerous experiments using wild-type or transgenic lines.

In contrast to glucosinolates, **cyanogenic glycosides** occur in diverse taxa of the Euphyllophyta (see Chapter 4), from angiosperms, mono and dicots, to gymnosperms, and ferns. High concentrations of these glycosides are found in seeds of *Rosaceae*, like almond, apple, or cherries, in leaves of bamboo and eucalyptus, or in root stocks of cassava (*Manihot esculenta*, *Euphorbiaceae, Malpighiales, Rosidae*), the most important food source for 500 million people in the tropics and subtropics. A very short biosynthetic pathway for the tyrosine-derived cyanogenic glucoside dhurrin was established for millet, *Sorghum bicolor* (*Poales, Liliopsida*), where two membrane-associated cytochrome P450-dependent monooxygenases, derived from the CYP79 and CYP71 subfamily, respectively catalyze the successive formation of *p*-hydroyphenylacetaldoxime and *p*-hydroxymandelonitrile (Figure 2.13). The latter product is stabilized by a final *O*-glucosyltransferase step. Cyanogenic glycosides are stored in vacuoles. During degradation,

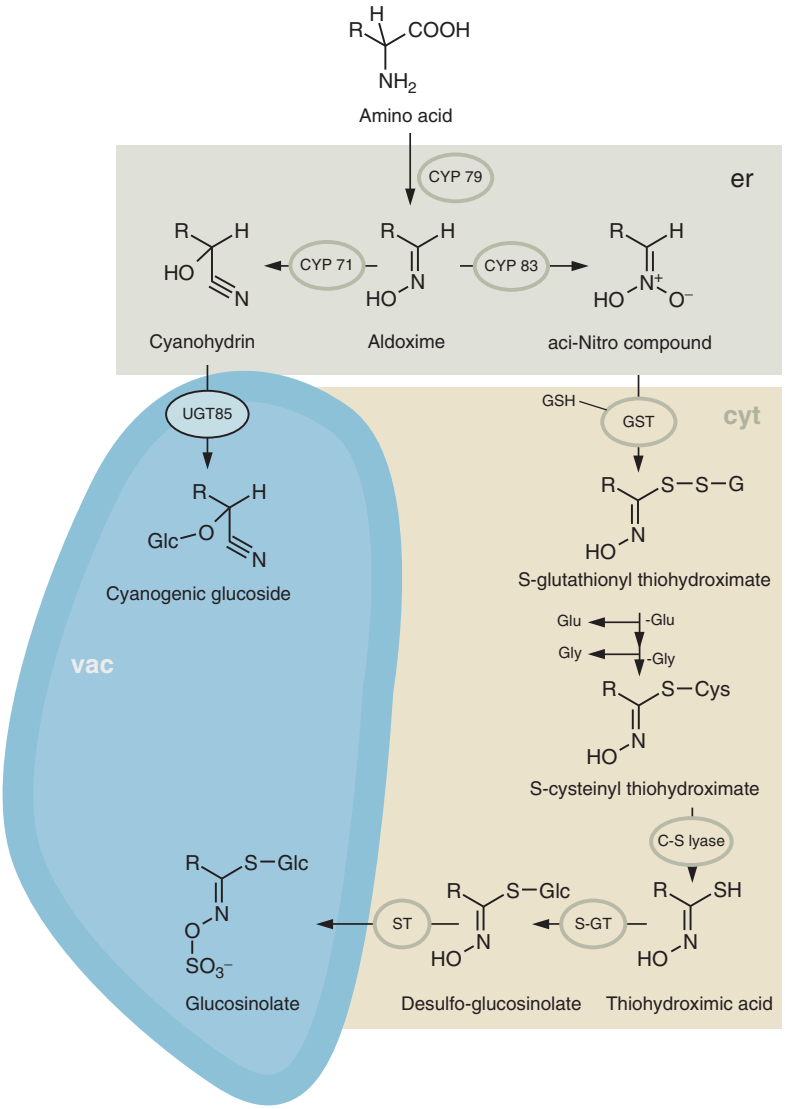


Figure 2.13 Biosynthetic steps of the glucosinolates and cyanogenic glucoside core structure both deposited in the vacuoles. GSH – glutathione; GST – glutathione-*S*-transferase; CS lyase, cysteine; S-GT – *S*-glucosyltransferase; ST – sulfotransferase; UGT – glucosyltransferase; vac – vacuoles; er – endoplasmatic reticulum; cyt – cytosol.

release of hydrogen cyanide occurs once a *ß*-glycosidase has access to the substrate via disruption of the organ or tissue on damage caused by abiotic or biotic stressors.

Direct proof of this defense potential came from *A. thaliana* plants, which contain glucosinolates but no cyanogenic glycosides. Transgenic plants expressing the whole dhurrin pathway accumulated high concentration of cyanogenic glucosides in all tissues and were less susceptible to insect attacks, for example, by the flea beetle (*Insecta, Arthropoda*). Although hydrogen cyanide is perceived as a very toxic chemical, during 400 million years of coevolution, herbivores or fungal pathogens have evolved multiple ways not only to detoxify this compound but also to make use of the cyanide group as a nitrogen source. Fungal (e.g., *Fusarium, Hypocreales, Ascomycota*) cyanide hydratases and bacterial cyanidase convert cyanide to formamide and formate and ammonia, respectively. This circumvents cyanogenesis and results in a superb source of nitrogen. Alternatively, Plant mitochondrial β-cyanoalanine synthase transfers HCN to cysteine and incorporates the resulting β-cyanoalanine into asparagine or aspartate. Specialized insects like larvae of *Zygaena* not only feed and store these compounds but also appear to be dependent on hosts with high contents of cyanogenic glycosides, in this case *Lotus corniculatus* (*Fabales, Rosidae*). Storage of high contents of the two cyanogenic glycosides linamarin and lotaustralin in males and females appear as attractant to the partner, probably because eggs with a high content of cyanogenic glycosides have a better chance to survive attacks by potential predators.

Cyanogenic glycoside and glucosinolates are strikingly similar with respect to amino acid precursors, certain

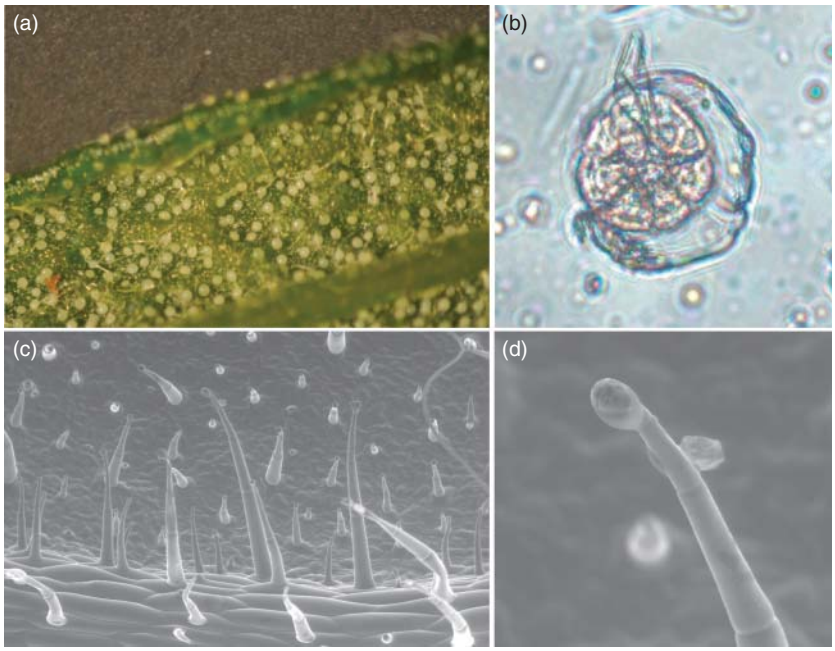


Figure 2.14 Examples of secretory tissues in plants. (a) and (b) Rosemary (*Rosmarinus officinalis, Lamiales, Asteridae*). (a) View of the abaxial side of a rosemary leaf with a high density of peltate trichomes. (b) Peltate trichome with its cuticular membrane delimiting the storage cavity. (c) and (d) Capitate trichomes of *Nicotiana sylvestris* (*Solanales, Asteridae*). (d) The exudate can be seen coming out of the glandular cells and dripping down the stem of the trichome.

biosynthetic steps like aldoxime formation, degradation, and mode of action. Glucosinolates are evolutionary younger and the corresponding pathway could have been derived from a presumed cyanogenic glucosides containing ancestor that was unable to further convert the aldoxime into a cyanohydrin and subsequently into a stable glucoside (Figure 2.13). This led to the accumulation of a single or a whole group of reactive metabolites subsequently detoxified by an established set of promiscuous transferases, including a GST, a glucosyl-, and a sulfotransferase – a scenario characteristic of many other pathways of plant secondary metabolism.

2.3 Sites of Biosynthesis and Accumulation

2.3.1 Specialized Structures and Storage

2.3.1.1 Glandular Trichomes

One of the conspicuous features of plants is the presence of hairs, or trichomes, on the surface of their aerial parts. In many plant species, these trichomes comprise glandular cells that produce and secrete large amounts of secondary metabolites. Typically, one class of metabolite is prominent, but different classes of metabolites may also be produced in single trichomes. In addition, different types of glandular trichomes are also frequently present on the same plant, allowing the production of more diverse compounds. Terpenoids are often encountered in glandular trichomes, mostly the volatile mono- and sesquiterpenoids, but also diterpenoids. For example, the volatile terpenoids, as well as volatile phenylpropanoids, are stored in the subcuticular space of the peltate trichomes of the *Lamiaceae* (*Lamiales, Asteridae*), such as mint, rosemary, basil, or lavender (Figure 2.14). When these trichomes are damaged, for example, by a foraging insect or a larger animal crushing the leaves, the content of the storage space of the peltate trichomes is released. Similar subcuticular storage spaces also occur in other plant families, for example, *Asteraceae*. Trichomes that produce nonvolatile compounds such as those of tobacco do not have such a storage space but rather exude the secretions at the tip of the trichomes (Figure 2.14). The resinous and sticky secretion often composed of diterpenoids and esters of sugars, drips then down the length of the trichome and spreads itself onto the leaf surface.

2.3.1.2 Internal Organs

Similar to external trichomes, the diversity of internal glands is remarkable, and only a few examples are mentioned here. Laticifers are elongated secretory cells that produce a milky liquid called *latex*. The composition of latex can vary between species. There are two types of laticifers, nonarticulated and articulated. The nonarticulated are made of single syncytial cells, whereas the articulated are made of a network of elongated cells that are connected between them. Rubber plants (*Hevea* sp., *Euphorbiaceae, Malpighiales, Rosidae*) produce latex with a high content of

long isoprenyl chains that when exposed to air solidify and are the basis for the manufacture of natural rubber. *Hevea*, like other plants of the *Euphorbiaceae* and *Asteraceae* (*Asterales, Asteridae*), has laticifers of the articulated type. Milkweeds in contrast have nonarticulated laticifers. Plants of the *Asteraceae* family, such as Guayule (*Parthenium argentatum*) or Dandelion (*Taraxacum* sp.), also produce latex that may provide alternatives to *Hevea*-based rubber. The biological function of latex is mostly a defensive one. When a plant is wounded, the latex solidifies, thus sealing the wound site. In addition, latex may contain toxic chemicals (e.g., the phorbol esters in several *Euphorbiaceae*) that serve as a direct defense against pathogens or pests.

Secretory resin ducts of the conifers comprise a circle of so-called epithelial cells surrounding a cavity where the secretion is delivered. There is evidence that the biosynthetic enzymes are localized to the epithelial secretory cells, much like trichome glandular cells. In conifers (*Coniferophyta*), this secretion is typically made of a mixture of terpenoids, ranging from diterpene acids like abietic acids, to more volatile compounds of the sesqui- and monoterpene classes. The secretion is often highly sticky, a characteristic feature with obvious defensive properties. The resin ducts can also be induced by wounding, and the terpene content may match or mimic that of insect pheromones, with the purpose of disrupting the life cycle of potential insect pests.

2.3.1.3 Vacuoles as Sites of Storage of Specialized Metabolites

Through the conjugation with sugars (glycosylation), or other hydrophilic groups such as malonyl, rather hydrophobic secondary metabolites may be transported and stored in the vacuoles. The release of the compounds may occur when cells are disrupted, for example, during herbivore attack, through the action of hydrolyzing enzymes, as in the case of the myrosinase involved in the degradation of glucosinolates. Anthocyanins are also typically stored in the vacuoles and as mentioned above many different glycosylation and acylation patterns may affect their color and stability. Although the localization of these compounds is well established, the transport mechanisms are still not completely elucidated (Section 2.2.3.2). In the case of anthocyanins and flavonoids, it has been shown that transfer of glutathione moieties, energy-driven pumping by ABC, or MATE transporters is required for the transport across the vacuolar membrane. Vesicular transport followed by fusion with the vacuoles is also a possibility.

2.3.2 Alkaloid Biosynthesis: Pathway Trafficking

The site of biosynthesis of specialized metabolites does not always coincide with their final localization in the plant. For example, the site of alkaloid biosynthesis and the site of accumulation can be quite different. Nicotine (Figure 2.8) is produced in the roots, but accumulates in the shoots. The sites of biosynthesis and accumulation for alkaloid classes with similar biosynthetic origin can also vary in different plants. Putrescine-*N*-methyltransferase, the first enzyme of nicotine biosynthesis, is localized to the endodermis, outer cortex, and the xylem in *Nicotiana sylvestris* roots, whereas the same enzyme is localized to the pericycle in the roots of *Atropa belladonna* and *Hyoscyamus muticus* for the biosynthesis of tropane alkaloids (all three species *Solanales, Asteridae*). However, another enzyme of the same pathway, tropinone reductase I, is localized in the endodermis and nearby cortical cells. Thus, tropane alkaloid pathway intermediates have to traffic between cell types. Interestingly, TR II (Figure 2.10), providing the intermediates to the biosynthesis of calystegines, localizes to the companion cells of sieve elements in the phloem of potato. In addition, it has been shown that on wounding or insect attack, nicotine and demethylated derivatives of nicotine (nornicotine) are secreted by the glandular trichomes of several tobacco species. Thus glandular trichomes can also serve as secretion vehicles for compounds that have been produced in other parts of the plant.

A complex trafficking of pathway intermediates is also evident for the monoterpenoid indole alkaloids (Figure 2.8) based on the localization of the participating enzymes in *Catharanthus roseus* (*Gentianales, Asteridae*) leaves. The enzymes responsible for early steps for the synthesis of the terpene part are localized to the internal phloem-associated parenchyma, whereas the later enzymes of the terpene moiety as well as the amino acid intermediate and the condensing enzymes localize to the epidermis. In addition, later enzymes of the pathway are associated with idioblasts and laticifers.

BIAs (Figure 2.9) have been reported to accumulate in laticifers in the shoots of opium poppy. But only some, if any, enzymes accumulate in this organ. Most of the enzymes are localized to different cell types surrounding the laticifers, such as the companion cells or the phloem. All this trafficking, whether between distinct intracellular compartments or between different cell types, most likely involves specific transport processes. Although in a few cases transporters of the ABC family could be shown to be involved, much remains to be discovered in this area. From the genome sequence of several plant species, the number of putative ABC transporters is quite large (over 100) and their functional assignment constitutes a major challenge in plant metabolism.

2.4 Evolution of Specialized Pathway Genes

2.4.1 Gene Duplication

Gene duplication likely plays a critical role in the emergence of new protein functions. The presence of a duplicated

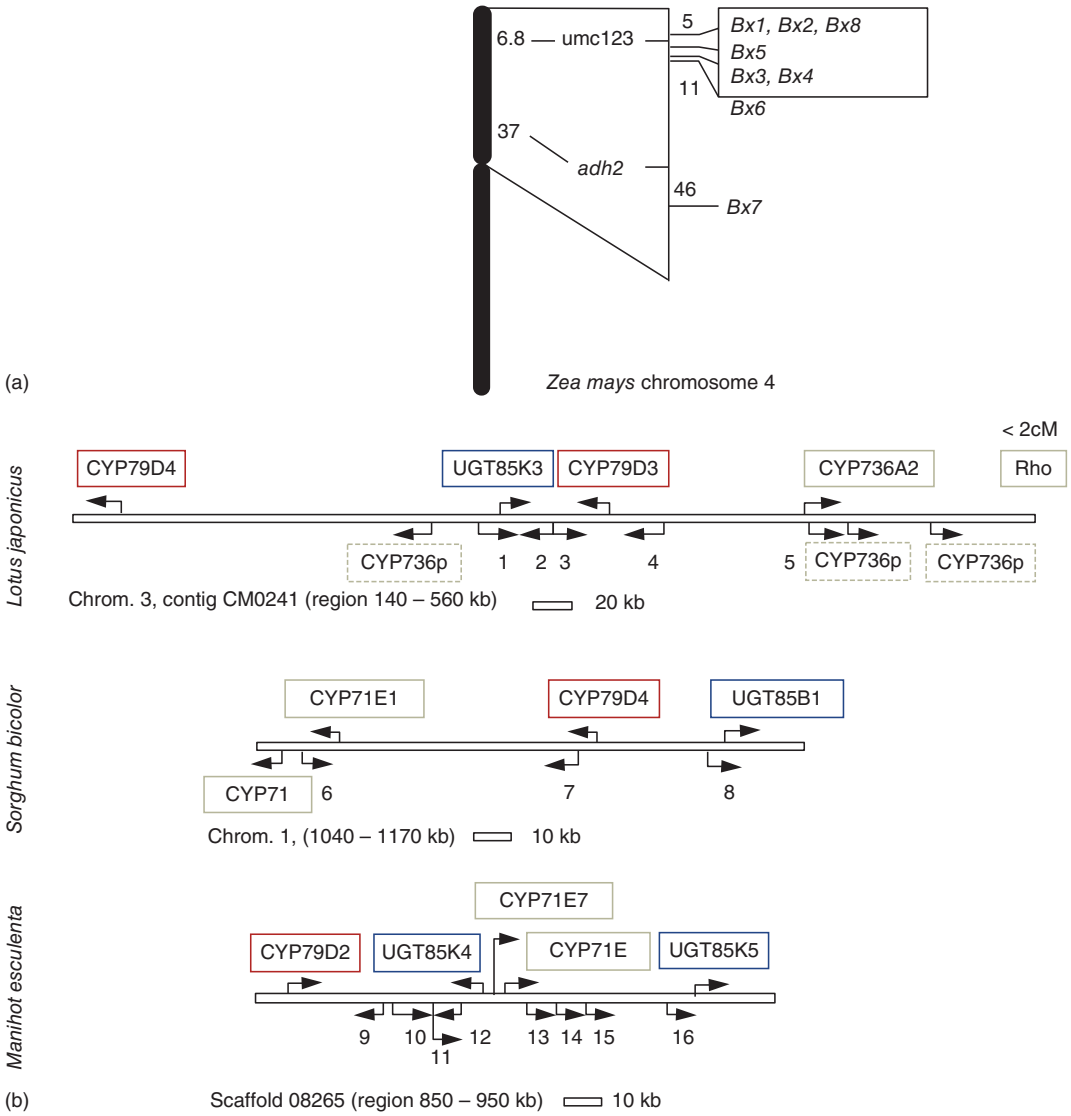


Figure 2.15 (a) Chromosome clustering of the benzoxazinoid pathway genes on chromosome 4 of maize (Frey *et al.*, 2009) (b) genomic clustering of cyanogenic glucoside pathway genes in three different species, *Lotus japonicas* (*Fabales, Rosidae*), *Sorghum bicolor* (*Poales, Liliopsida*), and *Manihot esculenta* (*Malpoghiales, Rosidae*) (Takos *et al.* 2011.)

gene allows one copy to be mutated and possibly lose its original function to gain a new one, while the other is still there to ensure that the original function is preserved. Gene duplication could occur by different mechanisms. Polyploidization either with the same genome or different related genomes through hybridization offers such opportunities for gene divergence. The sequence of plant genomes also tells us that it has undergone serial events of genome duplication followed by periods of genome contraction. The analysis of plant genome sequences also shows many chromosomal regions with several copies of highly similar genes in tandem arrays, indicating recent local duplication events. Interestingly, genes of specialized metabolism seem to be highly represented in these clusters. In *A. thaliana,* for example, on chromosome 3, there is a single cluster of 18 cytochrome P450s that all belong to the CYP71B family. Some of these are involved in indole glucosinolate biosynthesis. The molecular mechanisms by which such repeated gene duplication events may occur are not clear, but possibly involve transposons because those are also frequently found at these loci. Gene duplication allows not only the emergence of new enzyme functions but also can generate metabolic diversity by changing the expression profile of a particular enzyme, or also by changing its subcellular localization. **Retrotransposons**, which act via an RNA-intermediate, can also be involved in gene duplications. One such case could be established in *A. thaliana* for the cytochrome P450s CYP98 A8 and A9. Both genes are required the hydroxylation of spermidine hydroxycinnamic acid conjugates specifically in the tapetum of *A. thaliana*. They likely evolved from an ancestral cytochrome P450, CYP98A3, which hydroxylates coumaroyl shikimate

or quinate in the general phenylpropanoid pathway as mentioned above. During duplication, through reverse transcription and reintegration into the genome accompanied by several point mutations, the duplicated genes encoding CYP98A8 and A9 have acquired distinct substrate specificities within an evolutionary short time frame of five million years only.

In different tissues, or in different subcellular compartments, different but related substrates may be available. If the gene products or resulting enzymes show some degree of promiscuity with regard to substrates, this can lead to the appearance of new products. This, in fact, has been shown to be the case for a TPS from strawberry (*Fragaria, Rosales, Rosidae*), which through alternative splicing can be either targeted to the plastids or to the cytosol. In the plastids it acts as a monoterpene synthase, using GPP that is derived from the MEP pathway, and in the cytosol it behaves as a sesquiterpene synthase, using FPP from the MEV pathway.

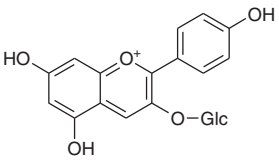
2.4.2 Chromosomal Clusters of Specialized Metabolite Pathway Genes

In prokaryotes, genes of a metabolic pathway are typically arranged in operons, ensuring a coordinated expression of all pathway genes. In higher eukaryotes, and in particular in plants, genes of a particular pathway are typically dispersed through the genome. However, recently several cases of genome clustering of genes involved in the same pathway were discovered. Examples include the benzoxazinoids from maize, triterpenoid avenacins from oat, triterpenoids from *Arabidopsis*, mono- and sesquiterpenes from tomato, or cyanogenic glucosides in alfalfa and millet (Figure 2.15). As these cases arose in completely unrelated species and concern unrelated pathways, it suggests a common underlying trend for the clustering of specialized metabolite pathway genes in plants. Evidence for molecular mechanisms explaining the recruitment of genes of a particular pathway to a single genomic locus is still lacking. The formation of these operon-like loci certainly is one of the interesting challenges linking genome evolution and specialized metabolism.

2.4.3 Convergent Evolution

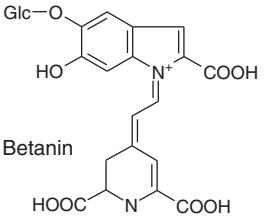
Some metabolites are present in all or almost all plant taxa. This is the case, for example, phenolics and flavonoids for which some key enzymes such as PAL and CHS can be traced to ancestral plant forms (see Chapter 3). This indicates an ancient origin of these pathways and represents a case of monophyletic evolution. On the contrary, the patchy distribution of certain secondary metabolites such as glucosinolates and certain classes of alkaloids, within phylogenetic clades (see Chapter 3), suggests that the ability to synthesize certain compounds may have evolved independently several times during evolution. **Convergent evolution** may be defined as the independent occurrence of similar or identical features in more or less distant lineages. In the area of specialized metabolism, multiple cases of convergent evolution have been reported.

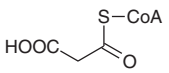
Two main classes of convergent evolution may be distinguished. The first concerns compounds that fulfill a similar function but are structurally very different. The second concerns the ability to produce identical compounds or reactions through evolutionary distinct enzymes. An example of the first type of convergent evolution is the case of betalains and anthocyanins. Both classes of compounds impart vivid colors (red, pink, magenta). Anthocyanins are phenylpropanoid-derived compounds (see above), whereas betalains can be classified as nitrogen-containing compounds derived from tyrosine. Interestingly, most plants that produce betalains, like some *Caryophyllales* (core eudicotyledons), have lost the ability to produce anthocyanins, and reciprocally plants that produce anthocyanins do not make betalains (Figure 2.16). A complex gain and loss of anthocyanin versus betalain formation may have occurred during evolution but shall not be discussed further. Overall, the reasons for this mutual exclusion are not clear. In the second type of convergent evolution, two distinct pathways



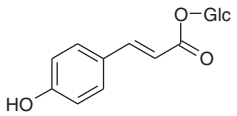
Pelargonidin-3-*O*-glucoside

(a)





(b) Malonyl-CoA



4-Coumaryl-glucoside

Figure 2.16 Convergent evolution of specialized metabolism in plants. (a) Two distinct molecules with identical function but different biosynthesis. Anthocyanins provide flower color to poppy flowers (*Papaver rhoeas, Ranunculales,* stem eudicotyledons). Betacyanins in *Bougainvillea spectabilis* and *other Caryophyllales* (core eudicotyledons) fulfill the same role, but are nitrogen-containing alkaloids. (b) In addition, acylations of anthocyanins are performed via enery-rich hydroxycinnamoyl CoA esters catalyzed by BAHD-type acyltransferases whereas betacyanins are acylated via 1-*O*-hydroxycinnamoylglucose esters catalyzed by hydroxycinnamoyl transferases.

or enzymes lead to identical products. Again anthocyanins and betacyanis may serve as an appropriate example. Aliphatic acylation of glucose residues in anthocyanins occurs via hydroxycinnamic through acyl CoA esters, like malonyl CoA catalyzed by BAHD-type acyltransferases (named after the initials of the first 4 discovered enzymes) whereas aromatic acylation of both betacyanins and anthocyanins is usually catalyzed by hydroxycinnamoyl glucose esters catalyzed by hydroxycinnamoyl transferases (Figure 2.16).

Yet a more subtle case of convergent evolution is called *repeated evolution*. In this case, the convergent enzymes do not arise from unrelated gene families but from the same large family of homologous enzymes. This happens frequently in the TPS family as well as in the case of eugenol methyltransferase. In *Clarkia breweri* (*Myrtales, Rosidae*) this enzyme belongs to the family of caffeic acid methyl transferase, whereas the basil (*Ocimum basilicum, Lamiales, Asteridae*) enzyme belongs to the isoflavone methyltransferase class.

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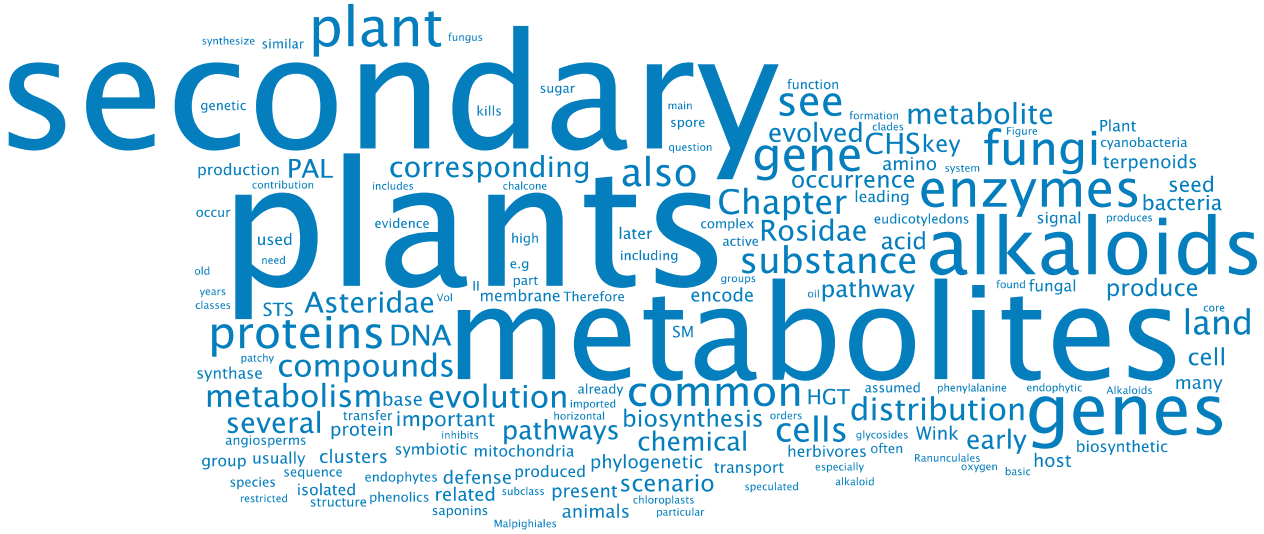
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3
Evolution of Secondary Metabolism in Plants

Michael Wink

Overview

Seed plants typically produce and store complex mixtures of secondary metabolites (SMs). The main function of secondary metabolites is defense against herbivores and microbes. However, defense is not the only function, as some SMs are signal compounds to attract pollinating and seed dispersing animals. Already the early land plants that evolved 450 million years ago had the need for chemical defense. Therefore, it can be assumed that the genes that encode the enzymes of the pathways leading to phenolics and terpenoids were present in early spore bearing plants. The occurrence and distribution of secondary metabolites in the plant kingdom of today shows an interesting, often discontinuous pattern. A specific SM is often confined to a particular systematic unit, but isolated occurrences can occur in widely unrelated taxonomic groups. The patchy occurrence could be due to convergent evolution but evidence proves that the genes that encode the biosynthesis of corresponding metabolites appear to have a much wider distribution than the actual secondary metabolite. It seems to be rather a matter of differential gene regulation, whether a pathway is active and expressed in a given taxonomic unit or not. It is speculated that the genes of some pathways derived from an early horizontal gene transfer (HGT) from bacteria, which later became mitochondria and chloroplasts. These genes/pathways should be present in most if not all land plants. About 80% of plants share relationships with symbiotic fungi (ectomycorrhiza and endophytes). Recent evidence shows that these fungi could either directly produce natural products, which were formerly considered as plant metabolites, or have transferred the corresponding pathway gene to the host plant at an earlier stage of evolution. The fungal contribution could also explain part of the patchy occurrence patterns of several secondary metabolites.

3.1
Origins of Plant Secondary Metabolism

When plants conquered the land about 450 million years ago in the Silurian and Devonian era, herbivorous animals (especially arthropods) existed and with also them bacteria and fungi. Therefore, already early plants had the need to defend themselves against herbivores and microbes. As secondary metabolites (SMs) are the main means for chemical defense, secondary metabolism is at least as old as land plants (vascular plants, Tracheophyta; see Chapter 4 for taxonomy) are. Alkaloids are especially common in angiosperms (Magnoliophyta), but some classes of them are produced by spore bearing plants (Moniliformopses; e.g., lycopods, horsetails), and some gymnosperms (Acrogymnospermae; e.g., cycads, conifers, gnetophytes). Because many alkaloids are neurotoxins, which interfere with targets of neuronal signal transduction, we may speculate that the diversification of alkaloids in angiosperms (Figure 3.1) coevolved with the rapid diversification of insect (*Insecta*, *Arthropoda*) and vertebrate (*Vertebrata*, *Chordata*) herbivores and pollinators during Cretaceous and Tertiary periods.

If we plot the distribution of terpenoids in a phylogram of plants (Figure 3.1), it is evident that all spore bearing and seed plants are able to produce them. Polyphenols have an even wider distribution and were probably already produced by multicellular algae. As a consequence, we assume that the basal pathways leading to phenolics and terpenoids must have existed 450 million years ago.

For some time botanists had assumed that secondary metabolites are being made spontaneously or with the aid of nonspecific enzymes. However, results from enzymatic studies show that biosynthetic enzymes are highly specific in most instances. Furthermore, sequence comparisons imply that most of the biosynthetic enzymes have evolved from common progenitors that had a function in primary metabolism. Because the biosynthesis is catalyzed by specific enzymes, most secondary metabolites have complex structures with a **distinct stereochemistry**. The enzymes that catalyze the **degradation or activation of secondary metabolites**, such as glucosidases, esterases, and other hydrolases, are less substrate-specific.

Despite the enormous diversity of secondary metabolites (see Chapter 2), the number of basic biosynthetic pathways

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

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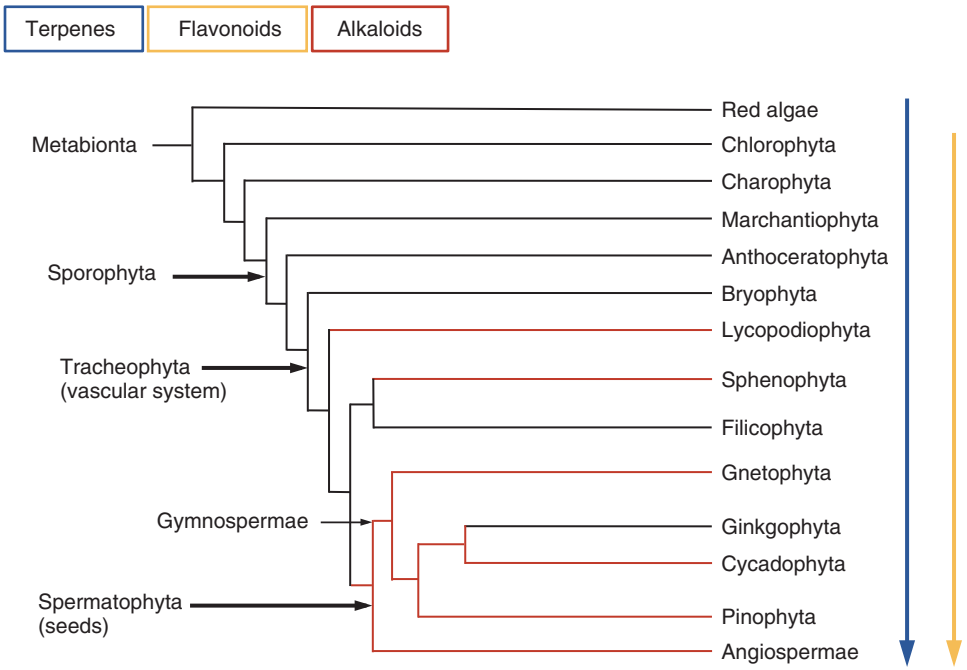

Figure 3.1 Molecular phylogeny of plants. Clades that produce alkaloids are marked in red, those with terpenes in blue, and those with phenolics in yellow.

is limited. **Precursors** for the biosynthesis of complex metabolites are usually elements of basic metabolic pathways, such as glycolysis, tricarboxyic acid cycle, or the Calvin cycle (see Chapter 2, Figure 2.1). For pathways leading to several classes of secondary metabolites, biochemists have identified and characterized the enzymes that catalyze individual steps in a pathway and the key gene that encodes the enzymes. Alkaloids are an important class of secondary metabolites, of which more than 21 000 structures have been reported. Many alkaloids are potent neurotoxins in animals because they interfere with the nervous system. Most alkaloids have amino acids as precursors (see Chapter 2). Figure 2.2 illustrates some of the important groups of alkaloids and the basic scheme of their biosynthesis.

When plants employ secondary metabolites as defense or signal compounds, it is evident that they need genes that encode the biosynthetic enzymes. In order to be useful, secondary metabolites have to be stored in high concentrations at the right site and time. These features require genes that encode transporter proteins for the import into storage compartments. Also, genes/proteins for long distance transport and turnover are essential for secondary metabolism. The question of when, where, and how the genes evolved that encode enzymes of the secondary metabolism (including those of their transport, storage, and turnover) is still a challenge.

Phylogenies can be used to discuss the question on how old secondary metabolism can be (Figure 3.1). In a similar approach, the phylogeny of angiosperms (see Chapter 4) can be used to study the evolution of pathways leading to different classes of alkaloids and other secondary metabolites (e.g., glucosinolates, cardiac glycosides) (see Chapter 2). Benzylisoquinoline alkaloids are widely distributed among *Laurales, Magnoliales* (both *Magnoliidae*), and *Ranunculales* (stem eudicotyledons) and are also present in some *Asteridae* and *Rosidae* (both core eudicotyledons). These alkaloids have in common the characteristics that they derive from the aromatic amino acids tyrosine and phenylalanine (see Section S1.3 and Chapter 2). Quinolizidine and pyrrolizidine alkaloids derive from the amino acids lysine and ornithine/arginine, respectively. Quinolizidine alkaloids are the main defense compounds in legumes (subfamily *Papilionoideae, Fabales, Rosidae*) and are also produced by some genera of *Asteridae, Caryophyllales* (core eudicotyledons), and *Ranunculales.* Pyrrolizidine alkaloids dominate in *Boraginaceae* (lamiids of incertae sedis, *Asteridae*), *Asterales* (*Asteridae*), and in the tribe *Crotalarieae* of *Fabaceae* (*Fabales*, *Rosidae*) and also occur in other genera of *Asteridae, Rosidae,* and monocots (*Liliopsida*). The disjunct distribution is not restricted to alkaloids, but is typical for most other secondary metabolites: Cardiac glycosides, for example, are found in members of the *Asparagales* (*Liliopsida*), *Ranunculales, Saxifragales* (core eudicotyledons), the orders *Geraniales, Celastrales, Fabales, Rosales, Brassicales* of the subclass *Rosidae,* the orders *Gentianales* and *Lamiales* of the subclass *Asteridae,* and thus all over the angiosperms. Only the glucosinolates have a more narrow distribution in the *Brassicales,* with a minor occurrence in the *Malpighiales* (*Rosidae*).

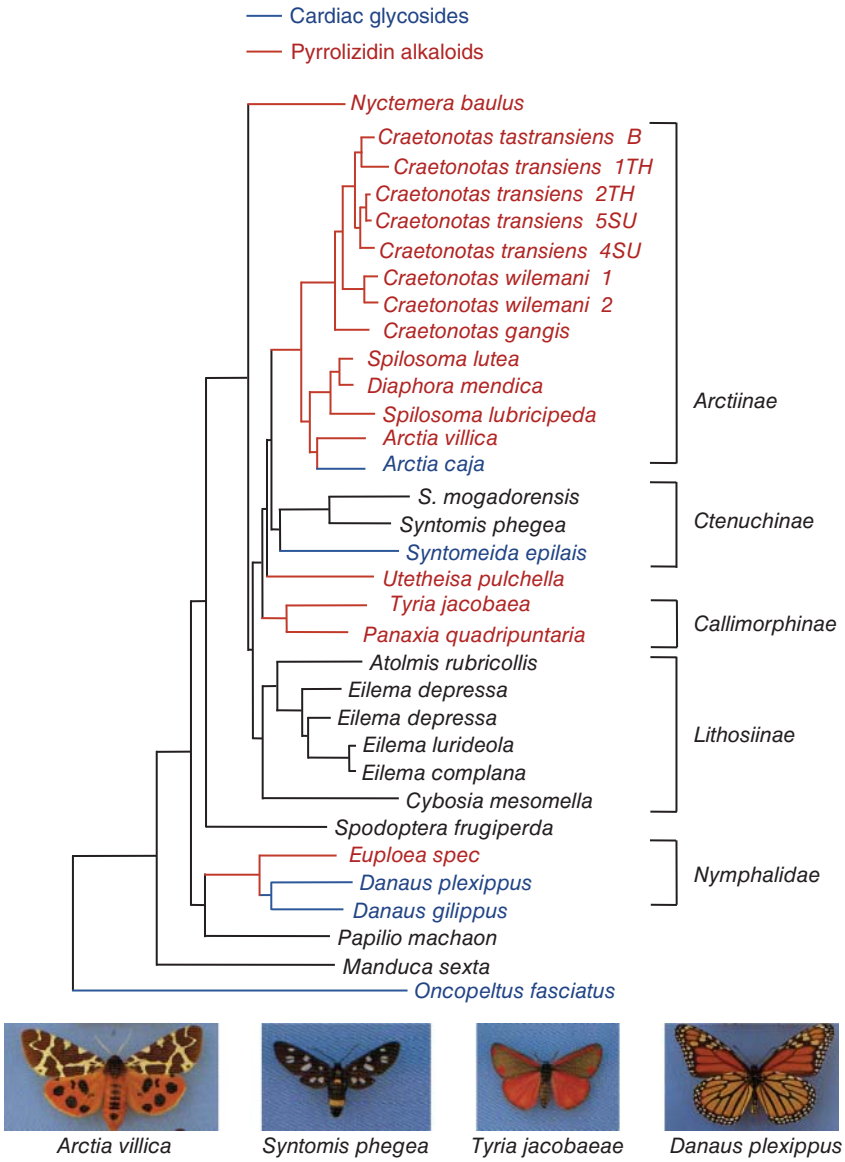

Figure 3.2 Uptake and storage of pyrrolizidine alkaloids and cardiac glycosides in *Arctiidae* and *Nymphalidae* (*Insecta, Arthropoda*). The phylogenetic tree was reconstructed using nucleotide sequences of 16S rRNA.

Looking at the distribution patterns it is obvious that secondary metabolites that share a common pathway are not restricted to a small group of related taxa but show a patchy distribution. This means that the corresponding biosynthetic pathways should also present in phylogenetic groups that are not closely related or, alternatively, the pathways evolved independently several times. This discrepancy was known for many years but had been ignored when chemosystematic relationships were inferred.

A discontinuous distribution pattern also occurs in insects that sequester chemical defense compounds of plants (Figure 3.2). The sequestration of cardenolides and pyrrolizidine alkaloids has been demonstrated in members of the *Arctiidae* and *Nymphalidae,* groups of moths (*Insecta*, *Arthropoda*) in which the larvae feed on plants that contain these toxins.

3.2 Evolutionary Alternatives

Figure 3.3 summarizes the main evolutionary alternatives: a simple situation can be considered in which a group of secondary metabolites exists, such as quinolizidine alkaloids in two phylogenetic clades that are not related (such as cytisine in *Fabaceae* and in the genera *Leontice* and *Caulophyllum* from *Berberidaceae, Ranunculales*). If scenario I (Figure 3.3) is true, then the identical alkaloids in clades A and B evolved independently and would be convergent traits. In scenario II it can be assumed that the genes encoding the enzymes for the alkaloid evolved in an ancestor common to clades A and B. If the alkaloids are not produced by all members of A and B, then this would be because the genes are inactive (maybe silenced

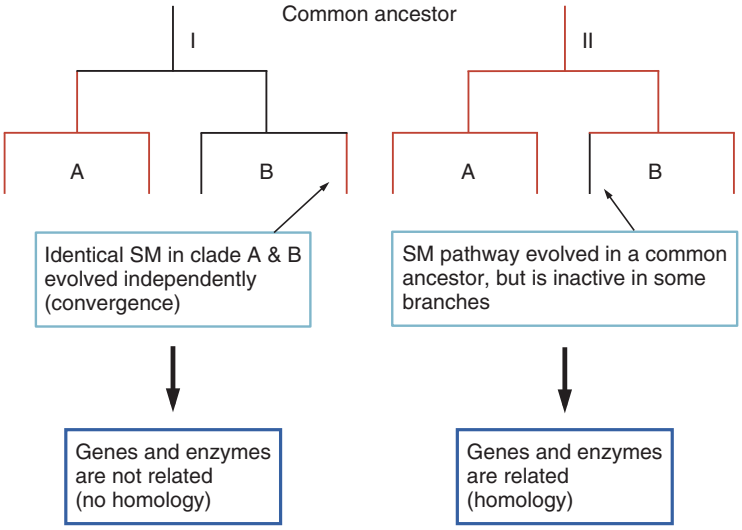

Figure 3.3 Alternatives to explain the patchy distribution of secondary metabolites. SM – secondary metabolite; A, B – clades; I, II – alternative scenarios.

epigenetically). In scenario I genes and enzymes would not be related, whereas in scenario II genes and enzymes are closely related.

Assuming that all extant taxa, ranging from spore to seed producing plants, accumulate a certain class of secondary metabolites it is very likely that all its members also possess the genetic outfit for its synthesis and storage. This in turn would suggest that the corresponding pathway must have evolved during early evolution. All lineages of land plants that are illustrated in Figure 3.1 appear to have the capacity to synthesize flavonoids and other phenolics using phenylalanine/tyrosine as a precursor. From this observation we can postulate that the genes of flavonoid biosynthesis should exist already in early land plants. A search in the protein database (NCBI) shows that the corresponding key enzymes, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), have indeed been isolated and characterized from spore forming and seed plants (Figures 3.4 and 3.5).

PAL is the key enzyme of flavonoid and coumarin formation. It catalyzes the desamination of phenylalanine to cinnamic acid (see Chapters 2 and 9). PAL genes have been detected in all land plants and also in fungi and bacteria, such as in Cyanobacteria (*Anabaena*, *Nostoc*), and in gram-positive (*Streptomyces*, *Actinobacteria*) and gram-negative bacteria (*Photorhabdus*, *Gammaproteobacteria*) (Figure 3.4). Because several sites of the PAL proteins are conserved in pro- and eukaryotes, we can assume that PAL of plants derived from a common ancestral bacterial protein (see Chapter 4). PAL has not been found in animals. Therefore, we assume that plant PAL was probably imported by horizontal gene transfer (HGT) to plant cells in early evolution by cyanobacteria, the progenitors of present-day chloroplasts. A similar picture appears if enzymes such as CHS are analyzed, which catalyze the next key step of flavonoid by combining malonyl-CoA and *p*-coumaryl-CoA to chalcone (Figure 3.5).

CHS has been detected in several seed plants as well as in the moss *Physcomitrella* (*Funariales*, *Bryopsida*), which can be regarded as a representative of early land plants (Figure 3.5). Plant CHS clusters as a sister to fungal CHS, sharing common ancestry with polyketide synthases from cyanobacteria. Plant CHS proteins have many conserved sites in common with pro- and eukaryotes, suggesting that plant CHS evolved from a common ancestral protein. It can be speculated that plant CHS have been imported from cyanobacteria by HGT during early evolution.

Many mono-, sesqui-, tri-, tetraterpenes, and steroids (see Chapter 2) occur in all land plants from mosses to seed plants. The pathways and the corresponding genes/enzymes necessary for the synthesis of active isoprene and the subsequent combination and cyclization of C5 units to mono-, sesqui-, and triterpenes already existed in the ancestors of land plants.

What about the situation of alkaloids that are more typical for angiosperms that evolved much later? A large group of alkaloids, which use tryptamine and secologanin as precursors, share a key enzyme, **strictosidine synthase** (STS) (see Chapter 2). Monoterpene indole alkaloids (MIAs) are typical for *Apocynaceae, Gelsemiaceae, Loganiaceae,* and *Rubiaceae,* all belonging to the order *Gentianales* of the subclass *Asteridae.* Theoretically, STS should, therefore, be restricted to these families. However, an amino acid sequence analysis (Figure 3.6) reveals that related genes/proteins are present in several other land plants, for example, in *Arabidopsis thaliana* (*Brassicales*, *Rosidae*), which does not synthesize MIA. Apparently, STS of plants has an older phylogenetic history. Proteins with shared amino acid sites have been isolated from animals and more importantly from bacteria (Figure 3.6). It could be speculated that the ancestor of the plant STS gene had

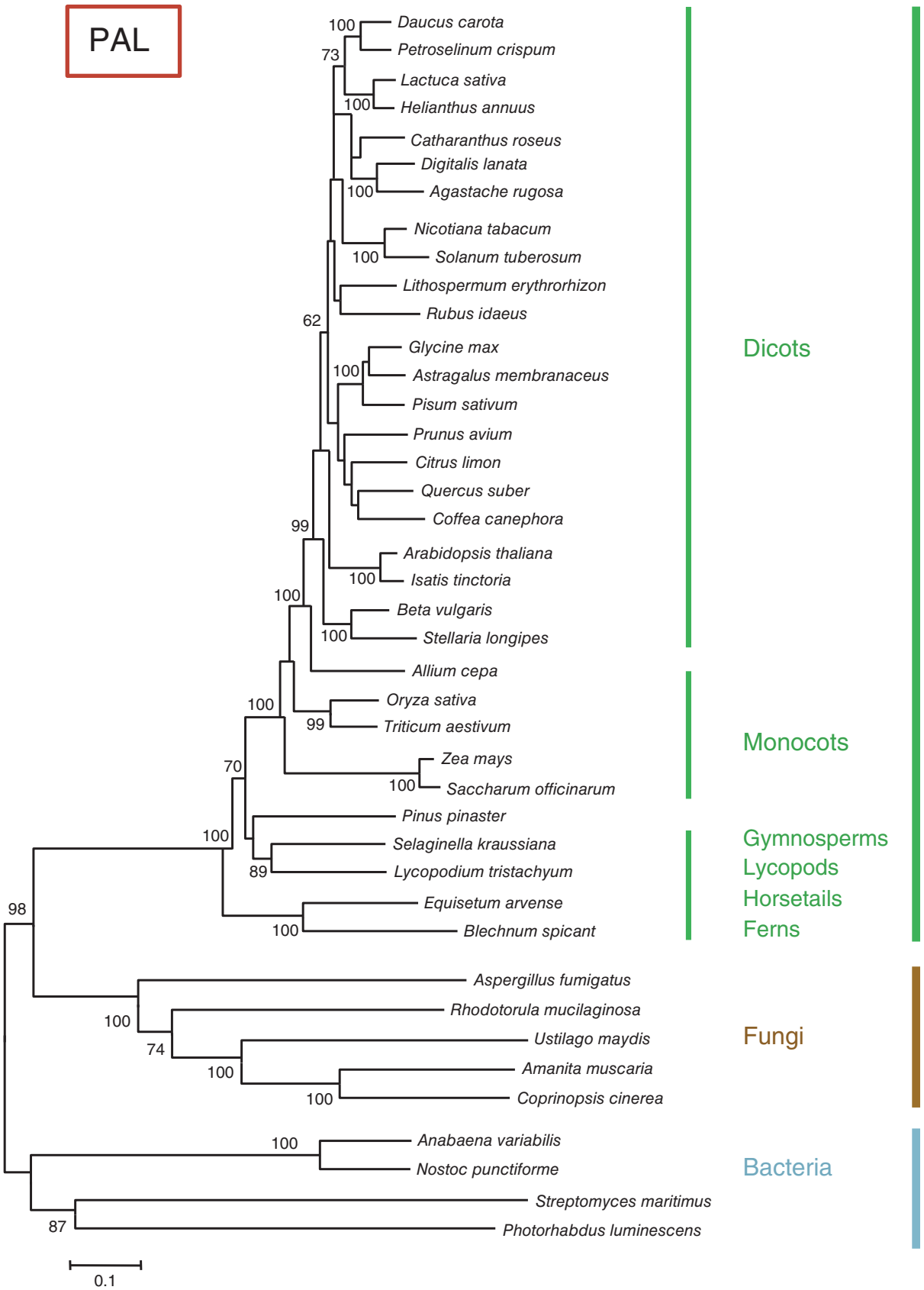

Figure 3.4 Phylogenetic relationships of PAL proteins. PAL – phenylalanine ammonia lyase.

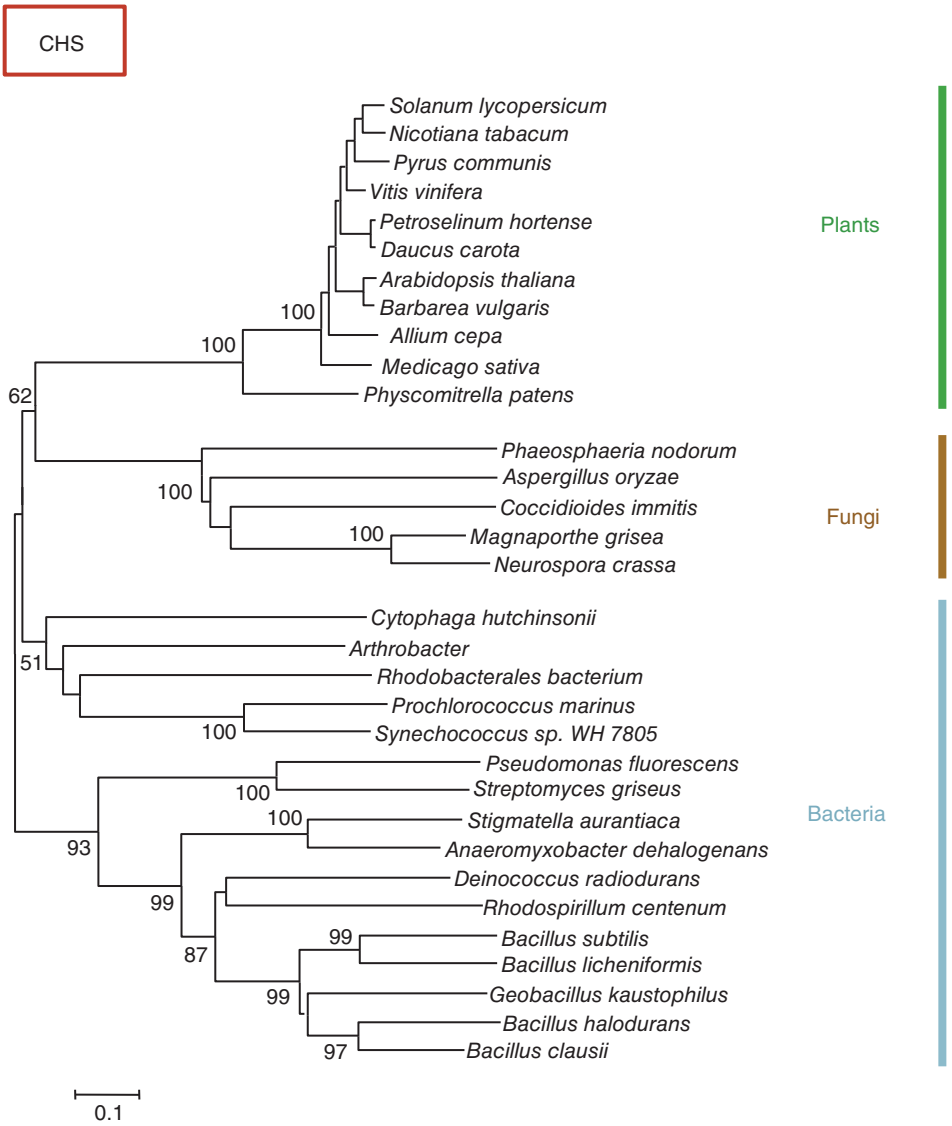

Figure 3.5 Phylogenetic relationships of CHS proteins. CHS – chalcone synthase.

been imported into plants via HGT through endosymbiotic alphaprotobacteria (the later mitochondria).

Considering these examples there is reason to speculate that the key genes of SM biosynthesis have a wide distribution among plants than previously assumed. Thus scenario II of Figure 3.3 is more likely than scenario I in some instances. Whether the genes are functional in species, which do not express the corresponding pathway, is an open question. They could exist as silent copies (regulated epigenetically) or inactive pseudogenes; this possibility can be tested by expressing the genes in a heterologous system and then to evaluate the corresponding biological activity.

Also, fungi produce a wide variety of secondary metabolites including alkaloids, terpenoids, anthraquinones, peptides, and phenolics. In contrast to plants, the genes that encode the biosynthesis genes are usually clustered. From genome analyses of several *Aspergillus* (*Pezizomycotina*, *Ascomycota*) species evidence was obtained that the genome of a single species contains several gene clusters for secondary metabolites, although most of them are silent. Thus we find a situation in fungi that is similar to that in plants. Apparently, scenario II would describe the situation in the best way and the silencing of gene clusters is regulated epigenetically. An increased acetylation of histones H3 and H4 can induce the production of secondary metabolites. Also, the downregulation of a histone deacetylase has a similar effect. It would be interesting to find out if similar mechanisms apply for silencing or activation of plant genes involved in the production of secondary metabolites.

There is also evidence for scenario I of Figure 3.3: Tropane alkaloids in *Solanaceae* (such as hyoscyamine; *Solanales, Asteridae*) and *Erythroxylaceae* (such as cocaine; *Malpighiales, Rosidae*) are apparently synthesized by enzymes of independent origin.

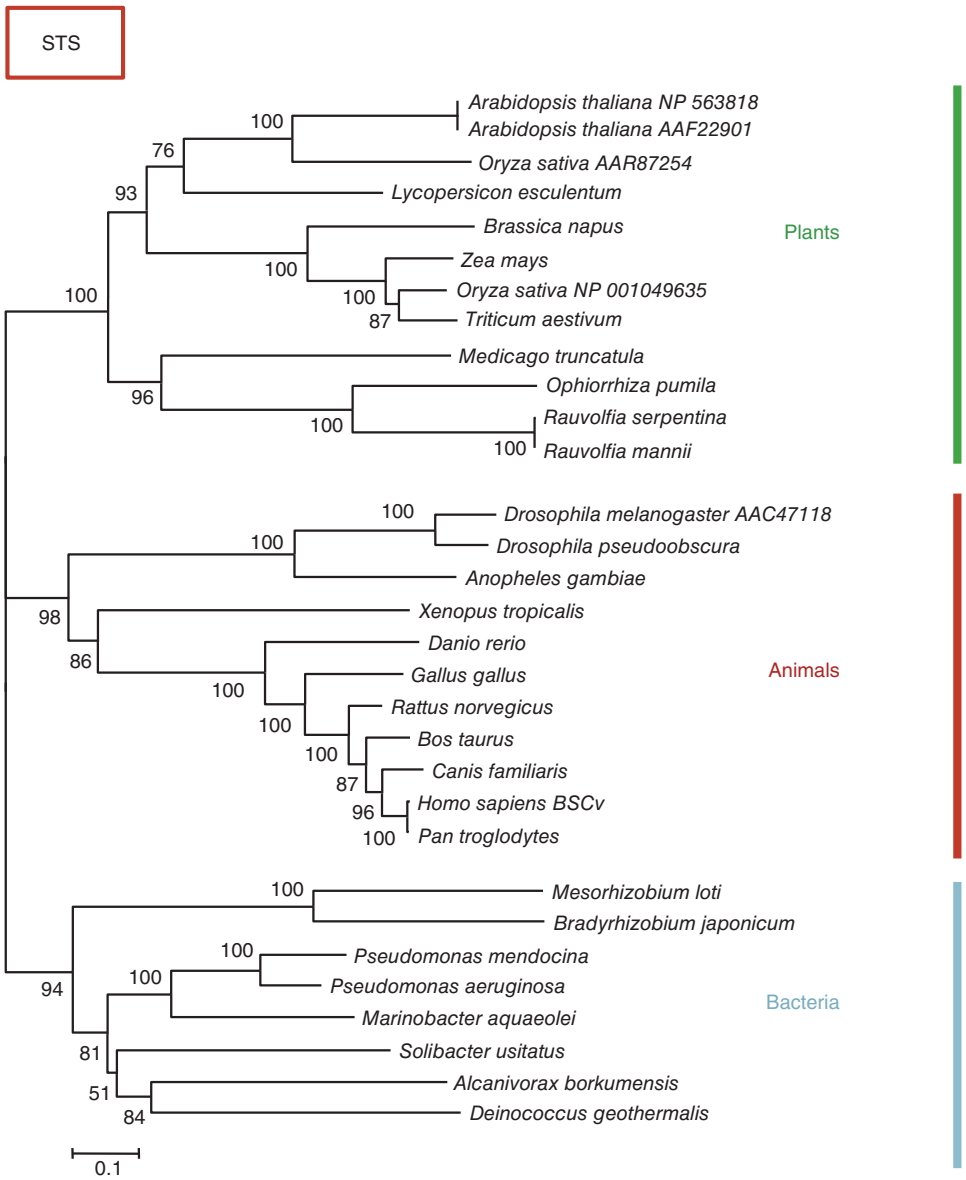

Figure 3.6 Phylogenetic relationships of STS proteins. STS – strictosidine synthase.

A phylogenetic analysis of key enzymes of the biosynthesis of secondary metabolites, such as tryptophan decarboxylase (TrDC), ornithine decarboxylase (ODC), PAL, CHS, STS, and berberine bridge enzyme (BBE), indicates that plants have probably retrieved the corresponding gene copies through early HGT from proteobacteria or cyanobacteria (the later mitochondria and chloroplasts, respectively) (Figure 3.7). These common key enzymes will generate the base structure of alkaloids, terpenoids, and flavonoids, but additional genes/enzymes are required to produce the diversification and variation observed within most classes of secondary metabolites. These additional enzymes might have evolved later during evolution from existing plant genes. Using the genome data that will be generated in the future, it will be possible to explore this scenario in more detail.

3.3 Endophytes, Symbiotic, and Ectomycorrhizal Fungi

About 80% of land plants live together with fungi (see Chapters 5 and 15). Mycorrhizal associations with land plants are an old, more than 400-million-year-old symbiosis, and are common in roots where they help to enlarge the root surface area. As a consequence the plant can obtain more water and nutrients (see Chapter 6). Common with other filamentous fungi, the plant-associated fungi can synthesize a multitude of secondary metabolites and are, therefore, important for host plants to defend them against herbivores and pathogens. Different environmental signals modulate the transcription of fungal genes involved in secondary metabolite biosynthesis (Figure 3.8).

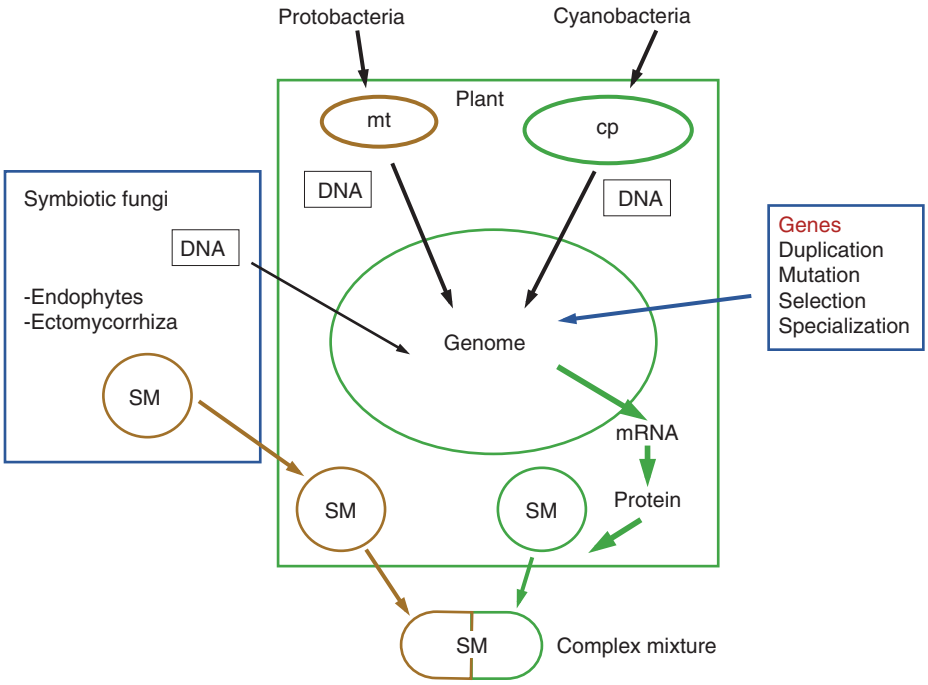

Figure 3.7 Hypothesis for the evolution of secondary metabolism in plants and the contribution of symbiotic microorganisms. SM – secondary metabolite; mt – mitochondria; cp – chloroplast.

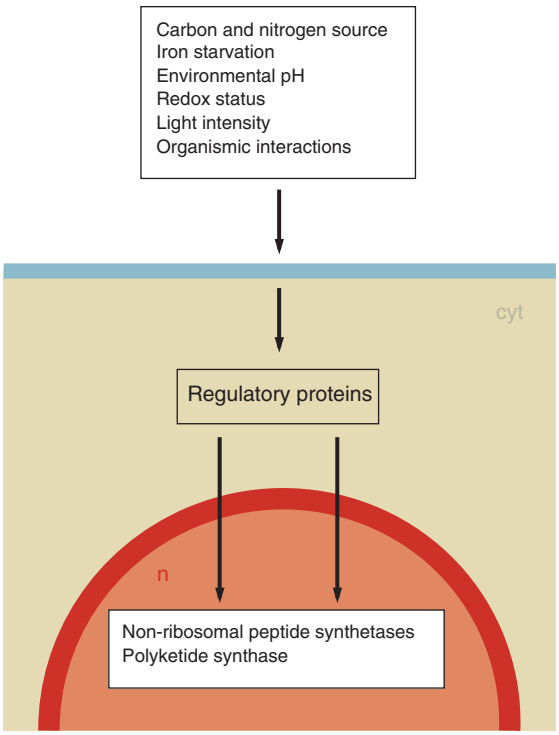

Figure 3.8 Influence of environmental signals to the regulation of secondary metabolism gene clusters in different fungi (Courtesy of G.-J. Krauss, D. Dobritzsch.)

The production of a particular natural product in plant families that are nonrelated from a phylogenetic perspective (Figure 3.3) could also be a result of the metabolic activity of endophytes and ecto- and endomycorrhizal fungi. The fungi could produce a secondary metabolite themselves or, furthermore, might have introduced the pathway genes to their host plants at an earlier stage of evolution (this would be another case of HGT) (Figure 3.7).

Ergot alkaloids, which are produced by *Claviceps* (*Pezizomycotina*, *Ascomycota*) or related fungi infesting cereals, protect their host plants against herbivores. Therefore, this interaction is rather symbiotic than parasitic. Ergot alkaloid formation has also been detected in the *Convolvulaceae* (*Solanales*, *Asteridae*). Here the occurrence of these alkaloids is linked to an endophyte. Thus, the isolated occurrence of ergot alkaloids in *Convolvulaceae* is not due to genes that occur all over the plant kingdom but due to fungal activities.

St. John's wort (*Hypericum perforatum; Clusiaceae, Malpighiales, Rosidae*) produces naphthodianthrones, such as hypericin. An endophytic fungus has been isolated from this plant that synthesizes hypericin in culture. The maytansinoid ansa antibiotics have been found in a number of angiosperms, including *Celastraceae* (*Celastrales*), *Rhamnaceae* (*Rosales*), and *Euphorbiaceae* (*Malpighiales*, all three orders belonging to the subclass *Rosidae*). It has been suggested that the occurrence of these toxins depends on infection by *Actinosynnema pretiosum* (*Actinobacteria*). *Taxus brevifolia* (*Coniferales*, *Coniferophyta*) and *Corylus avellana* (*Betulaceae*, *Fagales*, *Rosidae*) produce the diterpene alkaloid paclitaxel. An endophyte had been isolated from *Taxus brevifolia* that apparently can synthesize these alkaloids. Other examples for secondary metabolites that are produced by both endophytes and higher plants include

podophyllotoxin, camptothecin, swainsonine, and several others.

The **endophytes** are apparently able to produce hypericin, taxol, maytansine, camptothecin, and podophyllotoxin *in vitro*. It remains to be shown if they alone are responsible for the production and sequestration of the corresponding metabolites in the host plant. Alternatively, it has been speculated that the corresponding pathway genes in plants might have been imported from an endophyte at an earlier stage of evolution. As fungal genes occur in gene clusters (see preceding text) it would be a challenge to search for gene clusters in plants.

Another possibility for **HGT** would be an infection by viruses or sucking insects (aphids, cicadas, etc.).

It is a challenging question to determine the degree and contribution of endophytes to the profiles of secondary metabolites in seed plants. If the production of secondary metabolites by endophytic and ectophytic fungi is a more widespread phenomenon than usually assumed, it could offer an additional explanation for the discontinuous distribution of certain metabolites in seed plants.

The occurrence of loline, a simple pyrrolizidine alkaloid, in a grass (*Lolium pratense,* ex. *Festuca pratensis, Poales, Liliopsida*) and in its hemiparasitic root parasite (*Rhinanthus serotinus, Lamiales, Asteridae*) is due to a symbiotic endophytic fungus (*Neotyphodium uncinatum; Pezizomycotina, Ascomycota*). The fungus produces defense compounds, helpful for the plants against herbivores. Parasitic and semiparasitic plants can acquire secondary metabolites from their host plants. Therefore, detection of a particular metabolite in a parasitic plant [common in *Scrophulariaceae* (*Lamiales*, *Asteridae*), *Santalaceae* (*Santalales*, core eudicotyledons) including *Viscaceae,* and *Loranthaceae*] is evidence neither for a common genetic base nor for HGT.

Figure 3.7 tries to summarize our discussion on the evolution of secondary metabolism in plants and the possible contribution from HGT and symbiotic fungi.

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Part II
Ecological Signatures of Life

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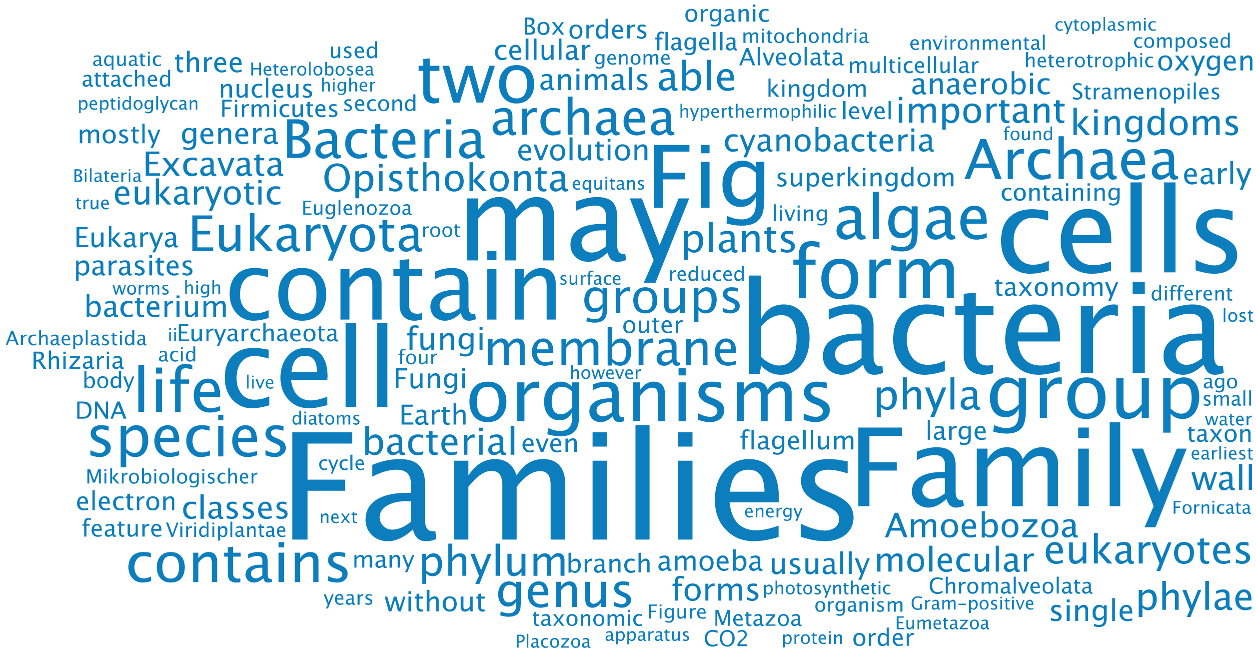

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Figure
Metazoa
Eumetazoa
Placozoa
apparatus
CO2
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order

4
Systematics of Life, Its Early Evolution, and Ecological Diversity

Dietrich H. Nies

Overview

This chapter describes the taxonomy and early evolution of the three superkingdoms *Archaea, Bacteria*, and *Eukarya*. Within the *Eukarya*, the higher plants were taxonomically resolved down to the level of orders. In the remaining chapters of this book, species mentioned in the text were always marked with a "taxonomic address," two expressions that refer to the taxonomic position of the respective species, for example, the order and class. That way it is always possible to assign this taxonomic position, and the evolutionary history behind it, to the described ecobiochemical function.

4.1
Cellular Life Forms and Subcellular Parasites

Chapter 1 describes how life functions: a cell with a semipermeable barrier to the outside takes up energy, increases the order or negentropy inside by the synthesis of macromolecules, and overcompensates this internal decrease in entropy by a stronger increase in external entropy in keeping with the second law of thermodynamics. The discussion if **viruses** are life forms is, therefore, not understandable. They are not life forms because they do not conserve their own energy, even if **mimiviruses** have larger genomes than some bacteria, and even if mimiviruses coreplicate with another virus in the same host cell. Viruses are no living entities but subcellular energy parasites and they use a host cell to "borrow" life. Viruses are composed of a protein shell and nucleic acid, which can be DNA or RNA. **Virions** are replicating RNAs that may have lost their protein shell.

All real cellular organisms on Earth belong into three basic highest level groups that are classified as **superkingdoms** (alternatively also named domains, "imperia," or "superregna"), namely *Archaea, Bacteria,* and *Eukarya* or *Eukaryota* (Box 4.1, Table 4.1, and Figure 4.1). Some authors merge the *Archaea* and *Bacteria* as kingdoms into the superkingdom *Prokarya* or *Prokaryota*, others use "kingdom" for *Eukarya* and *Prokarya*, and subkingdoms for *Archaea* and *Bacteria*; however, the expression "superkingdom" is used here (see also Tree of Life Web Project at http://tolweb.org).

Up to now there is no hint that a fourth category of cellular organism may exist on Earth. **Eukaryotes** are more closely related to the **Archaea** with respect to their transcription and translation apparatus but more closely to the **Bacteria** with respect to their cytoplasmic membrane. Overall, eukaryotes resemble mosaic organisms, a combination of Bacteria and Archaea, and are probably the result of different "marriages" between the other two groups. This leaves archaea and bacteria as the two original groups of living organisms. As the radiation of archaea starts at a shorter distance from the common archaeal/bacterial root than that of the *bacteria*, archaea may represent the oldest life forms on this planet.

Both archaea and bacteria show a **prokaryotic** cell organization. They do not posses a **nucleus** like eukaryotes, contain one or a few chromosomes, but show no mitosis or meiosis. The chromosomes may be open linear DNA molecules or rings. They posses 70S ribosomes and may transcribe genes as polycistronic mRNAs (see Section S1.3.12). This feature is more prominent in bacteria than in archaea. Neither group contains mitochondria or chloroplasts because these organelles stem from bacterial endosymbionts in eukaryotic cells.

4.2
Superkingdom Archaea

4.2.1
General Features

Archaeal cells have a **prokaryotic** cell organization. Mostly, they wrap their DNA in histone-like proteins and contain a complicated RNA polymerase reminiscent of eukaryotic proteins, which is only distantly related to the bacterial RNA polymerase. The biggest difference between **bacteria** and **eukaryotes** on one hand and archaea on the other are the components of the cytoplasmic membrane. In all groups, the phosphate residue is attached to the C1 atom of glycerol, and the phosphate group itself may contain

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Box 4.1: Taxonomy

Taxonomy of all cellular organisms is based on "species," defined for plants and later animals by Carl Linnaeus (1707–1778). Organisms belong to the same species if they are able to interbreed and produce fertile offspring. For bacteria and archaea that do not mate like plants, other criteria apply for the definition of a species, for example, bacterial strains with similar life styles and biochemical abilities. Similar species are grouped to genera and named with two words, the first indicating the genus and starting with a capital letter, the second the species within this genus. Both words are of Latin or Greek origin and, therefore, italized or underlined, for example, "*Escherichia coli.*" Other taxonomic levels combine similar genera into a family, families into orders, classes, phyla, kingdoms, and finally superkingdoms. The definitions on the highest levels of the taxonomy of life, that of kingdoms and phylae, are changing rapidly since Carl Woese and Otto Kandler redefined the Tree of Life 1990 to substitute the old animal/plant/fungi world with a more correct archaea/bacteria/eukarya tree. Moreover, the massive acquisition of genomic data, or environmental DNA and RNA sequences and metagenomics, has revealed a hidden world of unknown microorganisms of all kinds and new affiliations between old taxonomic units. From the bottom up, families, orders, and sometimes classes remain relatively constant, and from the top down, the three superkingdoms *Archaea, Bacteria,* and *Eukaryota,* too. However, the branches of the tree of life between superkingdoms and classes form a very complicated pattern, for example, with sometimes only one species with the same rank as an old-fashioned phylum. The taxonomy presented here is based on the NCBI taxonomy browser with all taxa based merely on unassigned DNA sequences (environmental or uncultures) and ribosomal data projects ignored. On the basis of this, more traditional views were assimilated into this picture. Refer to taxonomy textbooks for more details.

additional functional groups. In archaea, the C2 and C3 atoms of the glycerol are connected to a hydrophobic isoprenoid moiety via an ether bond. In bacteria and eukaryots, fatty acids are attached as esters. Moreover, in a few archaea, the compound may be very long, transverse the complete membrane, and bind to two phospho-glycerols on different sites of the membrane, thus forming an isoprenoid monolayer. Other archaea, however, possess shorter isoprenoids that form a bilayer reminiscent to the fatty-acid-containing bilayer membranes of bacteria and eukaryots. Archaea and bacteria frequently exchanged genes by horizontal transfer.

There are currently five recognized phyla in the superkingdom *Archaea* (Table 4.2). The *Euryarchaeota* comprise the methanogenic archaea, the *Crenarchaeota* many extremophiles living at the highest temperature known. *Thaumarchaeota* are important for the global nitrogen cycle on Earth. Only one genus, *Nanoarchaeum*, has been described in the *Nanoarchaeota* and no strain of the *Korarchaeota* has been cultivated up to now. This list may become longer with future environmental metagenomic sequencing projects.

4.2.2 Phylum Euryarchaeota (I. B)

Euryarchaeota comprise the crown group of the *Archaea*. They branch off from an ancestor common to most archaea except the *Nanoarchaeota*. The earliest branch of the *Euryarchaeota* is that of *Methanopyrus*, followed by *Thermococcus/Pyrococcus*, both containing hyperthermophilic organisms with maximal growth temperatures of 110 and 95 °C/106 °C, respectively. It is, therefore, likely that the *Euryarchaeota* stem from a hyperthermophilic ancestor.

Most euryarchaeots are **methanogenic** organisms (*Methanobacteria, Methanococci, Methanomicrobia, Methanopyri, Nanohaloarchaea*) while a few are not (*Archaeoglobi, Halobacteria, Thermoplasmata, Thermococci*). The methanogenic archaea play an important role in the anaerobic dissimilation of organic carbon compounds. They fancy a chemolithoautotrophic life style and oxidize molecular hydrogen with CO_2 as electron acceptor. This allows them to act as syntrophic partner of a fermenting bacterium when all other electron acceptors in an environment have been used. Together with the fermenting bacterium, for example, a *Clostridium* sp. (*Firmicutes*), they completely degrade all kinds of organic substances to acetate, CO_2, and CH_4. Moreover, some methanogenic *Euryarchaeota* such as *Methanosarcina barkeri* are able to make a living by splitting acetate to CO_2 and CH_4, which is the last step in the anaerobic degradation of organic substances (see Section S1.3.7). This shows the importance of methanogenic archaea for the **carbon cycle** on Earth. Consequently, *Euryarchaeota* occur in all anaerobic environments that allow formation of syntrophic partnerships such as swamps, rice fields, and the rumen of ruminant animals.

Methanogenesis is a complicated biochemical process that involves a variety of unusual cofactors (see Section S1.3.7). It is a very specialized process and methanogens are hardly able to degrade other organic substances than C1 compounds or acetate. Nevertheless, it is interesting that the earliest branch *Methanopyrus* is already able to perform this reaction under hyperthermophilic conditions while the other branches comprise thermophilic or mesophilic methanogens. This leads to the speculation that the earliest

Table 4.1 The living world: an overview down to the kingdom level.[a)]

Subcellular energy parasites (no life forms): Viruses and viroids
Cellular life forms
I. Archaea
II. Bacteria
III. Eukaryota
A. Amoebozoa
B. Apusozoa
C. Centroheliozoa
D. Katablepharidophyta
E. Opisthokonta
F. Oxymonadida
G. Rhizaria
Archaeplastida
H. Glaucocystophyceae
I. Rhodophyta
J. Viridiplantae
Chromalveolata
K. Alveolata
L. Cryptophyta
M. Haptophyceae
N. Stramenopiles
Excavata
Excavates
O. Fornicata
P. Malawimonadida
Q. Parabasalia
Discicristates
R. Euglenozoa
S. Heterolobosea
T. Jakobida

a) The overview is based on the information of the NCBI taxonomy browser with unidentified, uncultured organisms of uncertain assignment deleted. The three taxa that were the only known taxa a few hundred years ago, the *Metazoa* (all animals), *Fungi,* and *Streptophytina* (containing all land plants), belong to the *Opisthokonta* (*Metazoa, Fungi)* or *Viridiplantae* (Plants). Superkingdoms are listed with Latin numbers and the kingdoms within the *Eukaryota* with capital letters. *Archaea* and *Bacteria* are grouped directly in phylae without using the kingdom level. The superkingdoms are subgrouped in Tables 4.2 (*Archaea)*, 4.3 (*Bacteria*), and 4.4 (*Eukaryota*).

ancestor of the *Euryarchaeota* may have tapped an ideal energy source when living a few billion years ago. During that time, the CO_2 content of the young Earth was probably very high and the atmosphere was nearly oxygen-free and may have contained sufficient molecular hydrogen to drive successful methanogenesis. This would explain the rapid radiation of the taxon *Euryarchaeota*, and why it contains so many methanogens and maybe the decline of the overall temperature on this planet on the long run because of the sequestration of CO_2 into biomass.

This speculation also agrees with the modes of energy conservation of the other *Euryarchaeota*. *Thermococcus* and *Pyrococcus* are hyperthermophilic chemoorganotrophs that degrade proteins and sugars including starch, and they respire with elemental sulfur as electron acceptor. In addition, *Pyrococcus* is able to ferment these substances under formation of molecular hydrogen and could thus be a model for a very early syntrophic partner of *Methanopyrus.* The *Thermoplasmata* comprise three thermophilic genera that live as chemoorganoheterotrophs and chemolithoautotrophs (see Section S1.2.12). *Thermoplasma* and *Ferroplasma* contain no cell wall and are acidophilic archaea, with an optimal pH value of 2. Electron acceptors are molecular oxygen or sulfur. The third genus, *Picrophilus*, has a protein cell wall and is able to live at a pH value of −0.06 with an optimum pH of 0.7. *Archaeoglobus* is a thermophilic organism that oxidizes molecular hydrogen and organic substance using sulfate as electron acceptor. All these archaea may have occupied ecological niches other than methanogenesis when the Earth was young.

The *Halobacteria* could be the most recent branch of the *Euryarchaeota*. They are aerobic chemoorganoheterotrophs living at high sodium chloride concentrations (Figure 4.2), some of them also at high NaOH concentrations and pH values. Under limiting oxygen supply, *Halobacterium salinarium* and other species capture light energy using the protein **bacteriorhodopsin**. In contrast to the light reaction of photosynthetic bacteria, absorption of a photon does not cause an exciton being formed (see Section S1.2.6.1), which is subsequently used to create a redox-active compound, but a change in the conformation of a retinal molecule bound to bacteriorhodopsin triggers export of a proton across the membrane. Thus, light absorption forms a proton motive force directly, and not via electron transport. Maybe, a cooler Earth allowed more light to reach its surface than the hotter Earth, leading to the evolution of photosynthesis by halobacteria and many bacterial groups.

4.2.3 Phylum Crenarchaeota (I. A)

The *Crenarchaeota* are the second phylum of the *Archaea* that has been known for some time. Representative genera are *Sulfolobus, Thermoproteus, Desulfuromcoccus,* and *Pyrodictium*. These are all hyperthermophilic archaea with maximum growth temperatures of 87, 96, 95, and 110 °C, respectively. Growth is chemoorganoheterotrophic or chemolithoautotrophic with sulfur, sulfate, ferric iron, and molecular oxygen as electron acceptors. *Crenarchaeota* can be found in sulfur-rich, volcanic sites, especially in the ocean.

4.2.4 Phylum Thaumarchaeota (I. E)

Thaumarchaeota are the third phylum of the *Archaea* and comprise the former mesophilic crenarchaeota. Representative genera are *Nitrosopumilus, Nitrososphaera,* and *Crenarchaeum*. These archaea are chemolithotrophic ammonia oxidizers, which play an important role in the global **nitrogen cycle** (see Section 6.2.4.1).

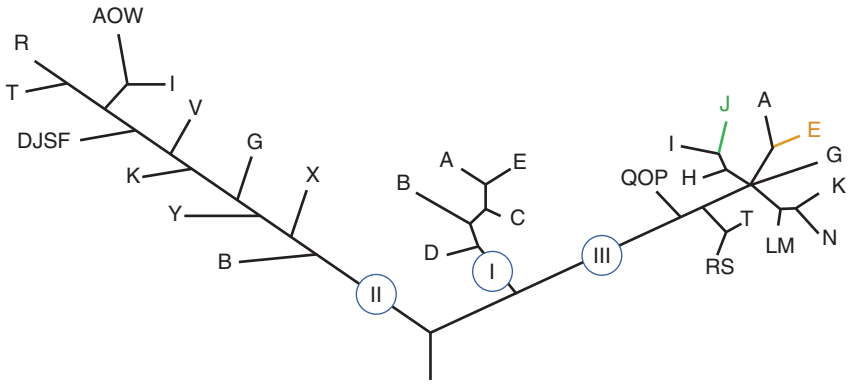

Figure 4.1 The "Tree of Life." The figure gives a rough overview of the three superkingdoms of life, the *Archaea* (I), *Bacteria* (II), and *Eukarya* (III). The letters indicate the phyla as in the tables with some bacterial and eukaryotic phyla not shown. All animals and fungi are part of IIIE (*Opisthokonta*) and all land plants of IIIJ (*Viridiplantae*). The figure was drawn not to scale and follows the ribosomal data project at *http://rdp.cme.msu.edu* and the taxonomy browser at *http://www.ncbi.nlm.nih.gov/taxonomy*.

Table 4.2 The superkingdom *Archaea* down to the level of classes.[a)]

I. *Archaea*
A. *Crenarchaeota*
1. Thermoprotei
B. *Euryarchaeota*
1. Archaeoglobi
2. Halobacteria
3. Methanobacteria
4. Methanomicrobia
5. Methanopyri
6. Nanohaloarchaea
7. Thermococci
8. Thermoplasmata
C. *Korarchaeota*
1. Korarchaea
D. *Nanoarchaeota*
1. Nanoarchaea
E. *Thaumarchaeota*
1. Thaumarchaea

a) The superkingdom *Archaea* is grouped into five phylae at the moment, labeled from IA to IE.

4.2.5 Other Phylae (I.C, I.D)

The remaining two known phylae of the *Archaea* are the *Korarchaeota* (I.C) and the *Nanoarchaeota* (I.D). No member of the *Korarchaeota* has been cultivated yet but *candidatus* "Korarchaeum cryptofilum" has been sequenced. From its genome, this organism is predicted to grow by simple peptide fermentation. Phylogenetic analysis based on the genome sequence shows that this group is an ancient archaeal lineage connected to the *Crenarchaeota* and also with some cellular features that resemble *Euryarchaeota*. Thus, this group is a remnant of a very early form of life that has appeared shortly after the separation of *Crenarchaeota* and *Euryarchaeota*.

Nanoarchaeum equitans is an even deeper branch than the *Korarchaeota* and very close to the root of all archaea.

Figure 4.2 Salt lake Chokrak (Ukraine) colored reddish by halobacteria (*Archaea, Euryarchaeota*) containing bacteriorhodopsin.

Therefore, this archaeon is a remnant of the earliest life forms on earth. *Nanoarchaeum equitans* is not a free-living organism but is associated with the crenarchaeum *Ignicoccus hospitalis* (Figure 4.3). *Ignicoccus hospitalis* contains a periplasm like gram-negative bacteria but in contrast to these, the outer membrane carries an ion motive force.

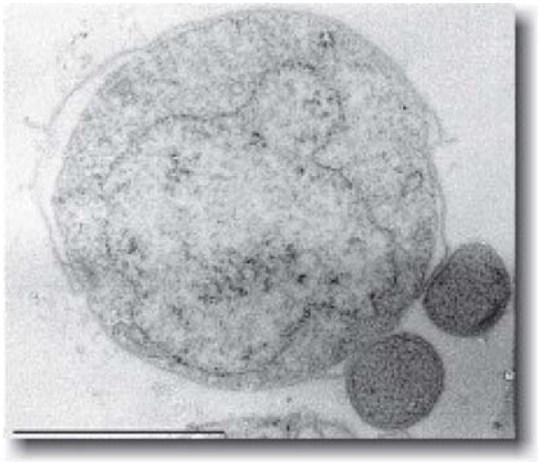

Figure 4.3 Two *Nanoarchaeum equitans* cells (*Archaea, Nanoarchaeota*) attached to a cell of *Ignicoccus hospitalis* (*Archaea, Crenarchaeota*). (Courtesy of Karl Stetter.)

Nanoarchaeum equitans is attached to this outer membrane. As *I. hospitalis* is able to live without *N. equitans*, this association is not obligate. On the other hand, *I. hospitalis* cells carrying more than two *N. equitans* cells cease growth so that the dwarf cells may be parasites. Its genome has a size of only 490 kb and is not sufficient to allow growth of *N. equitans* without its host. So, *N. equitans* may be a remnant of a very early life form but, unfortunately, it carries a reduced genome adapted to its function as an obligate parasite.

4.3 Superkingdom Bacteria

4.3.1 General Features

Like the archaea, **Bacteria** are **prokaryotes** but their cytoplasmic membrane contains phospholipids with fatty acids in an ester bond to glycerol like in the membrane of the eukaryotes. Bacteria usually contain more genes in polycistronic operons than archaea (see Sections S1.3.10 and S1.3.12). They posses one RNA polymerase, a heteromultimeric protein with the subunit composition $\alpha_2\beta\beta'\omega$. This core enzyme is not able to find the promoter sites for transcription initiation alone, because for this, a sigma factor has to bind to the core enzyme to form the holoenzyme complex. Each bacterium, therefore, contains at least one sigma factor, the housekeeping sigma factor named σ70, RpoD, or SigA. Nevertheless, the number of sigma factors may be much higher in some bacteria and exceed 100. The model bacterium *E. coli* has seven sigma factors.

Most bacteria contain a specific cell wall composed of **peptidoglycan**. This is a modified version of **cellulose**. Cellulose becomes **chitin**, used in fungi and insects, when the C2 atom of the polymerized β-D-1-4 glucose units carry an *N*-acetyl group. In peptidoglycan, the C3 atom of every second *N*-acetyl-glucosamine moiety carries a lactyl ether bound and attached to it the short amino acid chain L-Ala, D-Glu, a diamino acid such as lysine or diaminopimelate, and a D-Ala. During synthesis, two D-Ala residues are attached at this amino acid chain, the moieties are transported to the outside, the sugar backbone is polymerized, and some of the peptide chains of adjacent sugar backbones cross-link. To do this, the bond between the two D-Ala residues is transferred into a D-Ala attached to the diamino acid of the adjacent chain, directly or by small amino acid bridges such as a penta-glycine chain. Unused D-Ala–D-Ala bonds are cleaved. This transforms the murein sacculus into one large macromolecule that efficiently stabilizes the bacterial cell.

Most bacteria have a thin murein sacculus composed of three layers. Attached by a specific lipoprotein (**Braun's lipoprotein**) is an **outer membrane**, in addition to the inner or cytoplasmic membrane. The outer layer of this outer membrane is composed of **lipopolysaccharides** and the outer membrane contains **porins**, proteins that allow facilitated diffusion of selected molecules across the outer membrane. The space between inner and outer membranes is called *periplasm*. Bacteria with such a cell wall cannot be colored with the Gram stain; they are gram-negative. Another group of bacteria contains no outer membrane but a thick murein sacculus of about 40 layers instead, and in some cases other layers may be attached to it. These bacteria stain gram-positive. Some bacteria, however, contain no peptidoglycan or no cell wall at all.

The superkingdom *Bacteria* contains 30 phylae at present but only the earliest branching and the most important bacterial groups are discussed here (Table 4.3).

Table 4.3 The superkingdom *Bacteria* down to the level of phylae.[a)]

II. ***Bacteria***
A. *Actinobacteria*
B. *Aquificae*
C. *Armatimonadetes*
D. *Bacteroides/Chlorobi group*
1. Bacteroidetes
2. Chlorobi
3. Ignavibacteriae
E. *Caldiserica*
F. *Chlamydia/Verrucomicrobia group*
1. Chlamydiae
2. Lentisphaerae
3. Verrucomicrobia
G. *Chloroflexi*
H. *Chrysiogenetes*
I. *Cyanobacteri*
J. *Deferribacteres*
K. *Deinococcus-Thermus group*
L. *Dictyoglomi*
M. *Elusimicrobia*
N. *Fibrobacter/Acidobacterium group*
1. Acidobacteria
2. Fibrobacteres
O. *Firmicutes*
P. *Fusobacteria*
Q. *Gemmatimonadetes*
R. *Nitrospinae*
S. *Nitrospirae*
T. *Planctomycetes*
U. *Proteobacteria*
V. *Spirochaetes*
W. *Synergistetes*
X. *Tenericutes*
Y. *Thermodesulfobacteria*
Z. *Thermotogae*

a) The superkingdom *Bacteria* is grouped into 30 phylae at the moment, mostly labeled with IIA to IIZ; however, some of these taxonomic units are actually superphylae (underlined) and contain two or three phylae on the next level (IID, IIF, IIM).

4.3.2 Phylum Aquificae (II. B)

The earliest branching bacteria, the *Aquificae*, are hyperthermophilic organisms similar to early-branching archaea. The maximum growth temperature of *Aquifex* is 95 °C. These bacteria are obligate chemolithoautotrophs. They oxidize molecular hydrogen or reduced sulfur compounds at low levels of molecular oxygen or nitrate as electron acceptor and assimilate CO_2 with the reverse tricarbonic acid (TCA) cycle (See Section S1.3).

The genes required for this kind of relatively "modern" metabolism may have been introduced into *Aquificae* by horizontal gene transfer and evolved into varians that function under hyperthermophilic conditions. Alternatively, the *Aquificae* are models for the earliest bacteria. In this case, the *Aquificae* were adapted to use trace concentrations of molecular oxygen that should have been present even on the young Earth. Because of the high energy yield, this was an attractive life style even under microaerobic conditions.

4.3.3 Phylae Thermotogae (II. Z) and Thermodesulfobacteria (II. Y)

Thermotoga is a hyperthermophilic bacterium (up to 90 °C) containing an additional sheath, a toga, around its cell. The bacterium ferments sugars and polymeric carbohydrates to lactate, acetate, CO_2, and H_2. Moreover, *Thermotoga* is able to oxidize molecular hydrogen with ferric iron. The bacterium is thus able to form a syntrophic partnership with hyperthermophilic archaea and has acquired some genes from these organisms by horizontal gene transfer.

Thermodesulfobacterium is a thermophile and not a hyperthermophile but the sulfate-respiring bacterium with the highest growth temperature known. It contains an outstanding feature in its phospholipid acyl residues attached to glycerol in an ether bond instead of an ester bond, thus mixing a bacterial (acyl residues) with an archaeal (ether bond) feature.

With the *Aquificae*, these two groups are the three earliest lineages of the bacteria. Together with the metabolic pathways in the archaea, all chemotrophic life styles we know today are present in these organisms, including respiration with molecular oxygen. The only mode of energy capture missing is light-driven electron transport.

4.3.4 Chloroflexi (II. G)

This feature may have been evolved in the ancestors of the *Chloroflexi*. All members of the chloroflexi are thermophiles. The genus *Thermomicrobium* is exceptional because it contains no peptidoglycan or glycerol in its lipids but long-chain fatty acid diols. *Chloroflexus* is able to grow photoautotrophically with a chinon-type photosynthetic reaction center and fixation of CO_2 by the hydroxypropionate pathway (not addressed in Section S1.3). Alternatively, the bacterium can photoassimilate organic substances or can respire with molecular oxygen in the dark.

4.3.5 Crown groups of the Bacteria

After the *Chloroflexi* have branched off, the tree of the superkingdom *Bacteria* "explodes" into many branches, and the most important ones contain photosynthetic representatives at their root. It may have been the evolution of light-driven electron transport that drove the rapid radiation of the crown bacteria. So, after ancient crenarchaeotes and deep-branching bacteria, the euryarchaeotes may have been the second dominant life form on Earth and the crown bacteria the third one.

4.3.6 Phylum Proteobacteria (II.U)

Proteobacteria are probably still the dominant life form on Earth. Enterobacteria such as *E. coli* (Figure 4.4) and pseudomonads belong to this group, which is metabolically very versatile. The subphyla of the *Proteobacteria* were labeled with Greek letters, initially from α to ϵ. Later work established *Alphaproteobacteria, Betaproteobacteria*, the delta/epsilon subdivisions, *Gammaproteobacteria*, and *Zetaproteobacteria* as recognized groups. Examples for these groups are *Acetobacter, Agrobacterium, Caulobacter, Nitrobacter, Rhizobium, Zymomonas* (α), *Burkholderia, Chromobacterium, Cupriavidus, Neisseria, Nitrosomonas, Zoogloea* (β), *Escherichia,* and all enterobacteria, *Legionella, Pseudomonas, Xanthomonas* (γ), *Acinetobacter, Bdellovibrio, Desulfovibrio, Myxococcus* (δ), *Campylobacter, Helicobacter* (ϵ), and *candidatus* "Mariprofundus" (ζ).

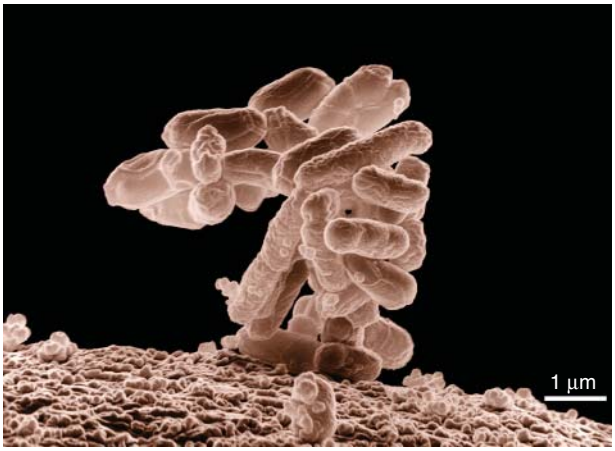

Figure 4.4 Cells of *Escherichia coli*, the most important bacterial model system (*Bacteria, Gammaproteobacteria*). 10 000-fold magnification, deep-temperature electron microscopic picture. (Source: http://emu.arsusd.)

The cellular forms, environmental functions, and life styles of proteobacteria are incredibly broad. Mainly adapted to aquatic environments, they can nevertheless be found nearly everywhere. Just a few glimpses: Chemolithoautotrophic nitrifying bacteria *Nitrosomonas* and *Nitrobacter*; sulfur- and metal-oxidizing chemolithoautotrophic *Thiobacillus, Acidithiobacillus, Beggiatoa*; hydrogen-oxidizing *Ralstonia (Cupriavidus), Paracoccus;* metal-resistant, hydrogen-oxidizing, and gold-forming *Cupriavidus metallidurans*; CO-oxidizing *Pseudomonas carboxidovorans*; methane-oxidizing *Methylomonas*; heterotrophic bacteria with broad substrate range *Pseudomonas, Burkholderia, Xanthomonas*, the last genus also containing plant-pathogens; alcohol-fermenting *Zymomonas*; acetic-acid-producing *Acetobacter*; nitrogen-fixing *Azotobacter*; enterobacteria that may be pathogens or not (*Escherichia, Salmonella, Enterobacter, Proteus, Yersinia*), light-producing *Photobacterium*; intracellular pathogenic *Rickettsia, Wolbachia*; magnetotactic *Magnetospirillum gryphiswaldense*; bacteria-eating *Bdellovibrio*; sheath-living sewage water bacterium *Sphaerotilus*; sessile authothonous *Caulobacter;* sulfate-respiring *Desulfovibrio;* and gliding fruit body-forming *Myxococcus* and *Stigmatella.*

The *Proteobacteria* contain photosynthetic purple bacteria at the root. These organisms possess a chinon-type photosynthetic reaction center and are able to assimilate CO_2 by the Calvin cycle (Section 1.3). The *Chromatiaceae* or sulfur-purple bacteria (*Chromatium, Ectothiorhodospira*) live photolithoautotrophically with H_2S as electron donor for CO_2 fixation while the nonsulfur purple bacteria or *Rhodospirillaceae* (*Rhodospirillum, Rhodobacter*) have adopted many different life styles: photolithoautotrophically with low concentrations of H_2S or H_2, photomixotrophically, chemolithoautotrophically, in the dark also anaerobic respiration and even fermentation.

4.3.7
Superphylum Bacteroidetes/Chlorobi Group (II. D)

Chlorobium and related genera of the *Chlorobiaceae* (II.D.2 *Chlorobi*) are the green sulfur bacteria, anaerobic chemolithoautotrophic bacteria using high concentrations of H_2S as electron donor for CO_2 assimilation, in this case by a reverse TCA cycle (see Section S1.3). They use chlorosomes, protein, and bacteriochlorophyll *c,d,e*-containing microcompartments to harvest light even at very low dose levels. The first member of the *Ignavibacteriae* (II.D.3) was described as moderately thermophilic anaerobic and heterotrophic bacterium in the year 2010.

Bacteroides (II.D.1 Bacteriodetes) is an important genus of sugar-fermenting, strictly anaerobic bacteria that dominate the bacterial cell population in the human gut. As a human body contains 10 times more bacterial cells than human cells, most of these are located in the gut, and most gut bacteria are *Bacteroides* species, most cells in a human body consist of these bacteria. If a human body is ruled by democracy with "one cell – one vote," *Bacteroides* would thus dominate the government. Therefore, humans can be addressed as "metaorganisms" composed of human cells that interact with a bacterial flora unique to each individual body.

4.3.8
Phylum Planctomycetes (II. T)

Planctomycetes are an exceptional bacterial phylum of autochthonous organisms living in aquatic habitats. They do not contain a peptidoglycan cell wall but a surface layer of proteins. Species of the genus *Planctomyces* contain a stalk similar to the sessile proteobacterium *Caulobacter*; however, in contrast to the *Caulobacter* stalk, that of *Planctomyces* is composed entirely of proteins and is not an extension of the cytoplasm.

All investigated *Planctomycetes* contain cellular compartments. This can be a protein microcompartment such as the anammoxosome of *Brocadia anammoxidans*, which lives by anaerobic oxidation of ammoniac to molecular nitrogen. Even more interesting is *Gemmata obscuriglobus*. This bacterium seemed to posses a "nucleus," a cellular compartment for its chromosome separated from the cytoplasm by a unit membrane; however, it was recently demonstrated that this "nuclear membrane" was an artifact.

4.3.9
Gram-Positive Bacteria (II.A, II.O, II.X)

Gram-positive bacteria are a deeply branched taxon that mainly consists of the phylae *Actinobacteria* (II.A) and the *Firmicutes* (II.O). A third minor phylum is the *Mollicutes* (II.X, *Tenericutes*) that evolved from within the *Firmicutes*. Most of the gram-positive bacteria stain positive in the Gram-stain and contain the typically 40-layered murein sacculus but there are exceptions at the root of the taxon; the mollicutes posses no cell wall at all. Gram-positive bacteria with their thick peptidoglyan are ideally adapted to soil, an environment with high sheer forces and the chance of rapidly changing water availability. Nevertheless, members of this taxon were found elsewhere, too, such as in the human gut and on the human skin.

The *Firmicutes* (II.O) are the allochthonous soil bacteria that are adapted to rapid growth. The guanine-cytosine (GC) content of their DNA is usually well below 50% in the region of 30 – 40%. Many species, even at the root of the taxon, are able to form a specific survival form, the bacterial **endospore**. These endospores survive harsh environmental conditions, temperatures of up to 100 °C, and toxic chemicals and are partly damaged by radiation. Thus, if an environmental sample is boiled and still contains bacteria that are able to grow at moderate temperature after allowing spore germination, these bacteria are usually *Firmicutes*.

The endospore is synthesized by an unequal cell division. The cytoplasm of the future mother cell grows around the

future spore cell, and both cytoplasmic membranes synthesize the complicated, multilayered, and thick spore wall in between. The spore accumulates manganese and calcium dipicolate and dehydrates. This fixes the spore proteins in a water-poor cytoplasmic environment and decreases the chance of their thermal or chemical denaturation. Induction of spore synthesis occurs under starvation conditions and spore development is regulated by a succession of mother- and spore-specific sigma factors. The spore also contains DNA repair enzymes ready to deal with mutations that occurred during endospore dormancy.

Typical endospore-forming *Firmicutes* are the aerobic *Bacillus* and the anaerobic, fermenting *Clostridium* rods plus respective related genera. Related to *Bacillus* are the genera *Sporosarcina*, urea-degrading bacteria in sarcina-type cell associations with eight cells in the corner of a cube, and *Sporolactobacillus*, fermenting lactic acid bacteria. *Desulfotomaculum* is a genus of anaerobic sulfate-respiring *Firmicutes*. Important *Firmicutes* that do not form endospore are most lactic acid bacteria (*Lactobacillus, Pediococcus, Streptococcus, Enterococcus, Lactococcus,* and *Leuconostoc*) and cocci from the genus *Staphylococcus*, an important pathogenic bacterium that has evolved multiple antibiotic resistances.

The autochthonous *Actinobacteria* (II.A) have a high GC content of more than 50% and have specialized in the slow degradation of polymers, mainly in soil. They may have evolved from allochthonous bacteria. The autochthonous *Bifidobacterium* is close to the beginning of the *Actinobacteria* line of evolution, followed by *Propionibacterium* that trails lactic acid bacteria also in the environmental succession (Section S1.3). Starting with *Propionibacterium*, the cell form of the bacteria in the *Actinobacteria* line becomes irregular, leading to the club-shaped (coryneforme) bacteria of the genera *Corynebacterium* and *Arthrobacter*. *Mycobacterium* cells contain additional cell wall layers on the peptidoglycan, which provides resistance to chemical toxins such as phenol. With *Nocardia* the cell form of the bacteria in the *Actinobacteria* line becomes mycelial, leading finally to the exospore-forming *Streptomyces* species that grow in a mycelium similar to fungi but with a smaller cellular diameter (Figure 4.5).

The *Tenericutes* or *Mollicutes* (II.X) are a special group of *Firmicutes* that no longer form a peptidoglycan cell wall. They have a reduced genome and many of them are parasites and pathogens. Representative genera are *Mycoplasma*, *Spiroplasma,* and *Ureaplasma*.

Heliobacteria (*Heliobacillus* and three other genera) reside close to the root of the gram-positive bacteria and they are phototrophs. Thus, gram-positive bacteria also seem to stem from phototrophic bacteria such as all other phyla in the crown of the *bacteria*. They form an endospore, a typical *Firmicutes* feature, are strictly anaerobic, and are able to ferment pyruvate in the dark. Their photosynthetic apparatus is composed of an iron-sulfur-type reaction center and they contain the unique bacteriochlorophyll *g*, which resembles a molecular "missing link" between the chlorophyll *a* of the cyanobacteria and the bacteriochlorophyll *a* of the other phototrophic bacteria. Because cyanobacteria and gram-positive bacteria may share a common ancestor, this may have been a *Heliobacterium*-type organism.

Figure 4.5 Colony of a *Streptomyces spec.* strain on an agar plate (*Bacteria, Actinobacteria*). (© Marco Fischer and Gary Sawers, Martin-Luther-University Halle-Wittenberg.)

4.3.10 Phylum Cyanobacteria (II.I)

Cyanobacteria are the only organisms on Earth that are able to perform oxygenic photosynthesis, using water as electron donor for assimilation of CO_2. They contain chlorophylls *a* and *b*, as most free-living cyanobacteria in addition to phycoerythrin and phycocyanin, open tetrapyrrol pigments that allow harvest of photons not used by the green plants. Associated with proteins, they form phycobilins, light-harvesting centers as component of the water-splitting photosynthetic reaction center II. This and the ability to survive low concentrations of H_2S and to assimilate molecular nitrogen provide a unique competitiveness to the cyanobacteria. In one of the five groups of free-living cyanobacteria, nitrogen fixation is mediated by specialized cells, heterocysts, that are formed by these filamentous organisms (see Chapter 6).

Free-living cyanobacteria can be sorted by morphology into (i) single cell cyanobacteria or those forming simple cell aggregates (*Gloeobacter, Synechococcus*); (ii) *Pleurocapsales* that multiply by forming "dwarf cells" (baeocytes, *Dermocarpa, Pleurocarpa, Myxosarcina*); (iii) filamentous *Oscillatoria* or *Spirulina*; (iv) filamentous but heterocyst-forming *Anabaena* or *Nostoc* (Figure 4.6); and finally (v) genera such as *Fischerella* that form branched filaments. On the taxonomic level, the phylum *Cyanobacteria* (II.I) contains one class, *Gloeobacteria,* with the genus *Gloeobacter*, the subclass *Oscillatoriophycideae* (orders *Chroococcales, Oscillatoriales*) and four orders not

Figure 4.6 *Nostoc commune* (*Bacteria, Cyanobacteria*). The heterocysts are the big "open" cells. (Mikrobiologischer Garten/Heribert Cypionka.)

sorted into a class scheme yet (*Nostocales, Pleurocapsales, Prochlorales, Stigonematales*).

Prochlorales (*Prochloron*) are cyanobacteria without phycobilins and can be cocci or filaments. The unique and important *Prochlorococcus* contains no chlorophyll *a* but instead a derivative, divinylchlorophyll *a*, in addition to chlorophyll *b* and carotins. These cyanobacteria are small, with a diameter below 1 µm, but occur in sea water with population densities of up to 100 000 cells per milliliter. Therefore, they may contribute a large part to the carbon-fixation capacity of oceans.

Chloroplasts are former cyanobacteria that became endosymbionts in eukaryotes. They miss phycobilins similar to the *prochlorales* and moved a large part of their genome to the nucleus of the host cell. Endosymbiosis of a photosynthetically active cell organelle seems to have happened over and over again in various phyla of the *eukarya*. Some organisms lost their chloroplasts during evolution and descendants of them acquired new ones. Sometimes the endoymbiont is not a cyanobacterium but algae containing a chloroplast or cyanobacterium, and this may even continue to be the reminiscent of a Russian "matryoshka" doll.

At the moment of their origination, cyanobacteria became a very successful bacterial taxon, and they changed the chemistry of the atmosphere and surface of Earth. Molecular oxygen may have been an attractive trace gas, generated by radiation, before the cyanobacteria arose. Now, O_2 was produced in large quantities, which started to oxidize all chemical compounds on the planet's surface and accumulate in the atmosphere. This development peaked with two strong increases in molecular oxygen, about 2.3 and 0.8 billion years ago. After the first major oxygenation event, eukaryotes appeared, after the second, multicellular eukaryotes. With this constant production of molecular oxygen, cyanobacteria and their endosymbiotic descendants have ruled the Earth, shaped this planet, driven evolution, and they still do!

4.4 Superkingdom Eukaryota

4.4.1 General Features

Eukarya contain a nucleus, a cellular compartment for their chromosomal DNA separated by a membrane from the cytoplasm. This DNA is in a linear form and packed by histones into chromosomes, which may become visible during mitosis and meiosis. Their genes are monocistronic. After transcription by one out of three RNA polymerases, interrupting sequences (introns) are removed and the remaining exons fused again (splicing). A poly-A tail is attached to the 3′ end of the RNA, and a specific di-guanine head structure to the 5′ end. Splicing and introns allow production of different mRNAs from one gene, the head and tail structure stabilize the RNA, which is exported for translation by an 80S ribosome through the nuclear membrane to the cytoplasm.

Eukaryotic cells (>10 µm diameter) are usually larger than bacterial or archaeal cells (<10 µm), but, as always, there are many exceptions. To keep such a huge cell organized, eukaryotic cells have a cytoskeleton more advanced than that of bacteria, and a variety of membrane-enclosed reaction compartments such as the endoplasmatic reticulum, Golgi apparatus, vacuoles, and lysosomes. They are able to import drops of liquid or solid particles of organic material by direct membrane invagination and formation of membrane-enclosed compartments (pino-/phagocytosis), and may detach such vesicles from the Golgi apparatus to release a substance to the outside (exocytosis).

The biggest difference to the bacteria and archaea are the endosymbionts, which invaded a eukaryotic host during evolution (or were taken up), and stayed there. Over time, these endosymbionts transferred most of their genome to the nucleus. As described in 4.3.10, all photosynthetic eukaryotes contain a chloroplast, a cyanobacterium, a former alga with a chloroplast, or whatever there is in a "matryoshka" series. At the end, it is a cyanobacterium or its descendant that performs photosynthesis. Such an acquisition of a photosynthetic endosymbiont has occurred independently several times in the various phyla of the *Eukaryota.*

The endosymbiont arriving before the chloroplast was the mitochondrion, a descendant of an α-proteobacterium, which performs oxidative phosphorylation and respiration for the eukaryotic cell. All eukaryotes contain a mitochondrion or a similar organelle derivative (hydrogenosome, mitosome). This is because mitochondria assemble iron-sulfur centers for the eukaryotic cell. It may well be that endosymbiosis of an α-proteobacterium with a proeukaryote formed the first eukaryotic cell, the last universal common eukaryotic ancestor LUCEA. Such an event was sensible only if the oxygen concentration was sufficiently high: at low concentrations, respiring bacteria remove

Box 4.2: Scenario for the evolution of the eukaryotic cell

Symbiosis of an early pre-eukaryotic cell with flexible spirochaeta-like bacteria may have led to the formation of the eukaryotic flagellum with its typical structure; however, there is no evidence for such a process. Nevertheless, an initial wedding between an archaeon and a bacterium must have happened long before the last universal common eukaryotic ancestor (LUCEA). This is because the transcription apparatus and other features of the eukaryotes are archaea-like but the cytoplasm with its membrane is bacteria-like. Eukaryotic cells are, therefore, microecosystems of many prokaryotes, or meta- or mosaic-organisms. The two initial partners may have been syntrophic partners with the fermenting and hydrogen-producing bacterium slowly enclosing its hydrogen-consuming archaeon for better hydrogen transfer (see Section S1.3.7). By removing the archaeal cytoplasmic membrane, a nucleus evolved such as in *Gemmata obscuriglobus* (*Bacteria, Planctomycetes*), and this nucleus also took over the genes from its bacterial partner. The resulting consortium lost the cell wall and increased in size to feed more efficiently on other prokaryotes and had to deal with the faster growing bacteria at the same time. To do so, sexual recombination individualized the cells but needed better DNA packaging for an efficient procedure, which led to meiosis and mitosis. At the end, the future mitochondrion arrived 2.3 billion years ago, possibly leading to the LUCEA.

oxygen so efficiently by respiration that the O_2 has no chance to diffuse into the center of a big eukaryotic cell to reach a mitochondrion. Thus, LUCEA should have evolved after the first oxygenation event 2.3 billion years ago. Similarly, dispersal of oxygen into the deeper layers of a multicellular eukaryote requires even higher concentrations of the gas. Consequently, multicellular animals could evolve only after the second oxygenation event, 800 million years ago. This is in agreement with the geological records of early multicellular organisms such as the Ediacara biota, which appeared 600 million years ago. There may have been earlier "weddings" between bacteria, leading to the ancestor of LUCEA (Box 4.2).

Modern taxonomy groups the *Eukaryota* into 20 kingdoms, some of them containing a huge number of phyla (Table 4.4). The most important kingdoms are discussed below: the *Amoebozoa* (III.A), *Opisthokonta* (III.E), *Rhizaria* (III.G), *Archaeplastida* {three kingdoms *Glaucocystophyceae* (III.H), *Rhodophyta* (III.I, red algae), *Chloroplastida* or *Viridiplantae* (III.J)}, *Chromalveolata* {four kingdoms *Alveolata* (III.K), *Stramenopiles* or *Heteroconta* (III.N), *Cryptophyta* (III.L), *Haptophyceae* (III.M)}, and *Excavata* {three excavate kingdoms *Fornicata* (III.O), *Parabasalia* (III.Q), *Malawimonadida* (III.P), plus three discicristate kingdoms *Jakobida* (III.T), *Heterolobosea* (III.S), *Euglenozoa* (III.R)} with the *Archaeplastida, Chromalveolata,* and *Excavata* being related groups of kingdoms. Figure 4.1 assumes the root of the *Eukarya* at the *Excavata.* Theoretically, it could also be close to the common ancestor of *Amoebozoa* and *Opisthokonta* but the *Microsporidia*, which were discussed as candidates for an early branching eukaryotic taxon, are not "primitive," but a parasitic sister group of the fungi with reduced cellular features and, therefore, true *Opisthokonta*. On the other hand, early-branching *Excavata* do not contain true mitochondria but hydrogenosomes or mitosomes. This may be a primitive feature, as if eukaryotes at the time of the LUCEA "experimented" with this endosymbiont. Some of the *Excavata* may have received the ancestor of all mitochondria, making them into the ancestors of all other *Eukaryota,* while different early *Excavata* may have "married" other endosymbionts, which allows them to live in anaerobic environments. In this case, the time point of mitochondrion entry 2.3 billion years ago should be right after the excavates branched off. The phylogeny of the superkingdom *Eukaryota* is treated differently by other authors (Box 4.3).

4.4.2 *Excavata*: Excavates (III.O, III.P, III.Q) and Discicristates (III.R, III.S, III.T)

Members of the *Excavata* are a group of kingdoms that contain mainly flagellated organisms with a suspension-feeding groove or cystostome of the excavate-type, which is used to capture and ingest small organic particles. A posteriorly directed flagellum is used to generate a feeding current to lead these particles into the cystosome, which may also have been secondarily lost in many taxa. The *Excavata* comprises two taxa of early branching eukaryots, the excavates (three kingdoms *Fornicata, Parabasalia,* and *Malawimonadida* with the sole genus *Malawimonas*) and the discicristates (three kingdoms *Euglenozoa, Heterolobosea,* and *Jakobida*) in a model that assigns the root of the *Eukaryota* close to the *Jakobida.* These are heterotrophic flagellated organisms at the root of the discicristates. With the exception of the "social" amoebae of the *Acrasiales* (order of the *Heterolobosea*), no multicellular organisms are formed within the *Excavata.* Phototrophs can be found in *Euglenozoa* (or *Euglenophyta*).

The flagellates of the kingdoms *Fornicata, Malawimonas,* and *Parabasalia* form the excavate cluster of the *Excavata* and may be the earliest branch of the eukaryotes. All *Excavata* of this excavate-type except *Malawimonas* may have evolved their mitochondrion into mitosomes or hydrogenosomes, but it is also possible that they used

Box 4.3: Other phylogenetic systems of the *Eukaryota*

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In a taxonomic–phylogenetic survey of organisms traditionally treated as fungi and plants, Jäger, Neumann, and Ohmann (2003) described the eukaryotes as kingdom separated into several subkingdoms (subregna): *Acrasiobionta* (phylum *Acrasiomycota*), *Myxobionta* (phyla *Myxomycota* and *Plasmodiophoromycota*), *Heterokontobionta* [*Chromalveolata* p.p., *Chromista*, *Stramenopila*] (phyla *Oomycota, Labyrinthulomycota, Heterokontophyta, Haptophyta,* and *Cryptophyta, Dinophyta* with question mark), *Mycobionta* (phylum *Eumycota*), *Rhodobionta* (phylum *Rhodophyta*), and *Chlorobionta* (phyla *Chlorophyta, Bryophyta, Pteridophyta,* and *Spermatophyta*). *Glaucophyta* (= *Glaucocystophyta*), *Euglenophyta,* and *Chlorarachniophyta* were listed as phyla of uncertain subkingdom affinity. Bresinsky *et al.* (2008) followed a very similar concept by dividing the kingdom *Eukarya* into subkingdoms, but in contrast to Jäger, Neumann, and Ohmann (2003), the *Glaucophyta* were treated as subkingdom *Glaucobionta*, the subkingdom *Mycobionta* split into five phyla (*Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota,* and *Basidiomycota*), and the subkingdom *Chlorobionta* (= *Viridiplantae*) separated into two phyla, *Chlorophyta* and *Streptophyta*. To consider pro- and eukaryotes or *Archaea, Bacteria* and eukaryotes as kingdoms (domains), or to drop the application of kingdom as classical higher level rank at all is also not accepted by some authors. True fungi are considered here as a kingdom (*Fungi*), comprising several phyla, in a modern survey published by a worldwide group of leading mycologists engaged in phylogenetic studies on this group of organisms. Besides *Fungi*, *Glaucophyta* + *Rhodophyta* + green plants (= *Archaeplastidia* or "green line," as well as animals (*Metazoa*) and *Stramenopiles* could be treated as kingdoms, *Plantae* and *Chromista,* respectively, but a uniform, consistent arrangement of all other groups of organisms, above all of the groups of organisms previously treated as "*Protozoa,*" is barely possible as the latter group proved to be polyphyletic.

other bacterial endosymbionts than the alphaproteobacterial premitochondrion as ancestors for their respective organelle.

The *Fornicata* (III.O) contain a single kinetid and nucleus or a pair of both. Examples for *fornicate* are *Diplomonadida*, double-organisms with a pair of kinetids and nuclei, *Retortamonadida*, and *Carpediemonas*. *Giardia lamblia* of the diplomonadids is an infectious "gut flagellate" that can cause a severe "explosive diarrhea" and may reside even in clean-looking mountain streams. *Malawimonas* is an outgroup of the *Fornicata* and resembles *Carpediemonas* but contains true mitochondria. Either this genus belongs to the discicristate-type *Excavata* or the transformation of mitochondria to mitosomes was a secondary event in the *Fornicata.*

The *Parabasalia* (III.Q) contain a parabasal apparatus. This is a specialized Golgi system connected to the flagella by two or more striated protein fibers. The kinetid is generally with four flagella and kinetosomes but additional flagella may count up to thousands. Hydrogenosomes may play the role of mitochondria in the eukaryotic biochemistry. Examples are the *Trichomonadida*, *Cristamonadida, Spirotrichonymphida,* and *Trichonymphida*. The trichomonadid *Trichomonas* is an anaerobic commensal but some species are pathogens transmitted by infected meat.

The *Jacobida* (III.T) are an early branch in the discicristate cluster of the *Excavata*, which branches further off into the *Heterolobosea* and *Euglenozoa.* Jakobids contain two flagella at the head of a large feeding groove. The posterior flagellum beats in this groove.

The *Heterolobosea* (III.S) are a group of heterotrophic amoebae with eruptive pseudopodia that are completely different from the pseudopodia of the *Amoebozoa*. The *Heterolobosea* moved on in evolution from flagellated to amoeboid forms. Losing the flagella and living on as an amoeba seems to have happened many times during the evolution of eukaryotes. One genus of the *Vahlkampfiidae* is an obligate amoeba, another one an obligate flagellate, the other genera are in-between with the amoeboid form usually dominant. The *Acrasidae* are "slime fungi," aggregating amoebae that form a fruiting body. The amoebae feed as single cells on bacteria and aggregate to form fruiting bodies when the food has been consumed. Such a social interaction also occurs in genera of the *Amoebozoa* and has also been invented more than once in evolution.

The *Euglenozoa* or *Euglenophyta* (III.R) are again flagellated, usually with two flagella in a flagellar pocket that contains a feeding apparatus close to the flagellar apparatus. The *Euglenida* contain the heterotrophic lineages *Heteronematina* and *Aphagea*, and the *Euglenea*. Species of the genus *Euglena* (Figure 4.7) can be found in standing water or sewage with high load of organic carbon, where they can live as heterotrophs or autotrophs, or both. The cells contain an eye-spot to allow phototaxis. As the plastids of *Euglena* species vary because of environmental conditions and evolutionary history, this indicates rather "recent" acquisition of these organelles. The *Diplonemea* are again heterotrophic *Euglenozoa*, as are the *Kinetoplastea* that contain a large mass of fibrillar DNA in their mitochondrion. Members of this taxon are free-living organisms such as *Bodo* and also comprise the pathogenic flagellates

Table 4.4 The superkingdom *Eukaryota* down to the level of phyla (except *Opisthokonta*).[a)]

III. *Eukaryota*
- ***A. Amoebozoa***
- ***B. Apusozoa***
- ***C. Centroheliozoa***
- ***D. Katablepharidophyta***
- ***E. Opisthokonta***
  - *1. Choanoflagellida*
  - *2. Fungi (fungi)*
  - *3. Metazoa (metazoans; Eumetazoa, Mesozoa, Placozoa, Porifera)*
    - *Eumetazoa (Bilateria, Cnidaria, Ctenophora)*
    - *Mesozoa* (2 phylae)
  - *4. Nucleariidae and Fonticula group*
- ***F. Oxymonadida***
- ***G. Rhizaria***

Archaeplastida
- ***H. Glaucocystophyceae***
- ***I. Rhodophyta***
- ***J. Viridiplantae***
  - *1. Chlorophyta (green algae)*
  - *2. Streptophyta*

Chromalveolata
- ***K. Alveolata***
  - *1. Apicomplexa (apicomplexans)*
  - *2. Chromerida*
  - *3. Ciliophora (ciliates)*
  - *4. Colpodellida*
  - *5. Dinophyceae (dinoflagellates)*
  - *6. Ellobiopsida*
  - *7. Perkinsea*
  - *8. Voromona*
- ***L. Cryptophyta***
- ***M. Haptophyceae***
- ***N. Stramonopiles* (*Heterokonta*)**
  - *1. Actinophryidae*
  - *2. Bacillariophyta (diatoms)*
  - *3. Bicosoecida (bicosoecids)*
  - *4. Blastocysta*
  - *5. Bolidophyceae*
  - *6. Chrysomerophyceae*
  - *7. Chrysophyceae (golden algae)*
  - *8. Developayella*
  - *9. Dictyochophyceae (silicoflagellates)*
  - *10. Eustigmatophyceae (eustigmatophytes)*
  - *11. Hyphochytriomycetae*
  - *12. Labyrinthulomycetae*
  - *13. Oikomonadae*
  - *14. Oomycetes*
  - *15. Pelagophyceae (pelagophytes)*
  - *16. Phaeothamniophyceae*
  - *17. Pinguiophyceae*
  - *18. Placididea*
  - *19. PX clade*
    - *a. Aurearenophyceae*
    - *b. Phaeophyceae (brown algae)*
    - *c. Xanthophyceae (yellow-green algae)*
  - *20. Raphidophyceae (raphidophytes)*
  - *21. Slopalinidae*
  - *22. Synchromophyceae*
  - *23. Synurophyceae*

Table 4.4 (*Continued*)

Excavata

Excavates
- ***O. Fornicata***
- ***P. Malawimonadida***
- ***Q. Parabasalia***

Discicristates
- ***R. Euglenozoa***
  - *1. Diplonemidales*
  - *2. Euglenida (euglenids)*
  - *3. Kinetoplastidales (kinetoplasts)*
  - *4. Symbiontidales*
- ***S. Heterolobosea***
- ***T. Jakobida***

a) The superkingdom *Eukaryota* is grouped into 20 kingdoms at the moment, labeled from IIIA to IIIT, some of these containing a huge number of phylae. *Opisthokonta* (III.E) are subgrouped in Table 4.6, the *Embryophyta* (*Streptophyta, Viridiplantae,* III.J.2) in Table 4.5.

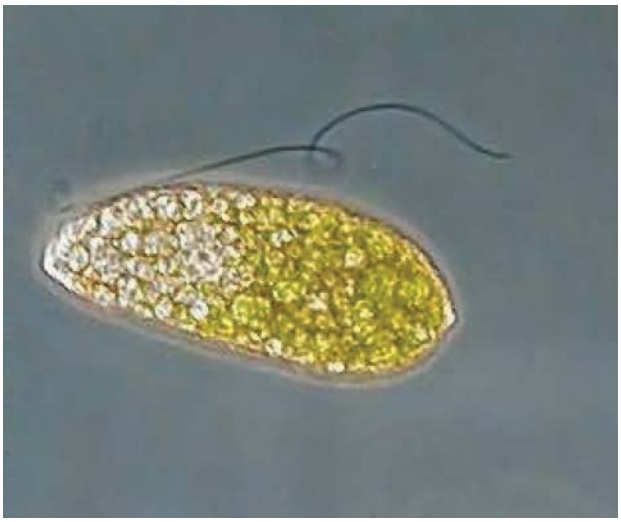

Figure 4.7 *Euglena gracilis* (*Eukaryota, Euglenozoa, Euglenida*). (Mikrobiologischer Garten/Heribert Cypionka.)

Trypanosoma and *Leishmania*, the causative agents of sleeping sickness and leishmaniasis, respectively.

4.4.3 Chromalveolata: Kingdoms *Alveolata* (III.K), *Stramenopiles* (III.N), *Cryptophyta* (III.L), and *Haptophyceae* (III.M)

The *Chromalveolata* are one of four crown branches of the *Eukarya*. The other three are the *Rhizaria* (III.G) with amoeba, foraminiferans, and radiolarians, the *Archaeplastida* with most uni- and multicellular plants (III.IJH), and the *Amoebozoa/Opisthokonta* branch (III.AE) with most amoebae, the fungi, and all animals. Within the *Chromalveolata*, the phyla *Cryptophyta* (III.L) and *Haptophyceae* (III.M) branch off early (Figure 4.1), followed by a division of the branch into the *Alveolata* (III.K) and *Stramenopiles* (also designated *Heterokonta*, III.N). The *Chromalveolata* have a complicated history of plastid gains and losses.

The ancestral organism may have possessed an ancestral archaeplastid, which was lost, a secondary endosymbiont was gained, which was again lost or reduced in some taxa. Finally, some taxa even acquired a plastid for a third time.

The *Cryptophyta* (III.L) contain photoautotrophic, mixotrophic, or heterotrophic species with two flagella. They carry a characteristic ejectosome or extrusosome, which discharges when the cells are stressed, and propels the cells away from the stressor. The *Cryptomonadales* contain chloroplasts (or leucoplasts), and these resemble cyanobacteria with chlorophylls *a*, c_2, and phycobiliproteins; however, the plastid is surrounded by four membranes and derived from a eukaryotic red alga as symbiont. The *Goniomonadales* are heterotrophs without chloroplasts.

Species of the *Haptophyta* (III.M) live as solitary cells, colonies of filaments, autotrophically, mixotrophically, or heterotrophically. If they contain a chloroplast, the major chlorophylls are *a*, c_1, c_2, and sometimes c_3. An important feature is the haptonema, an appendage situated between flagella. The *Pavlovophyceae* have two flagella, one chloroplast, no scales, and a short, tapered, and noncoiling haptonema. The *Prymnesiophyceae* possess mineralized and/or unmineralized scales that cover the cells.

The phylum *Stramenopiles* or *Heterokonta* (III.N) is a huge group of thousands of species, containing oomycetes, brown algae, and many diatoms. Motile cells are typically biflagellates with a heterokont flagellation, which means an anterior flagellum with tripartite mastigonemes (small hairs on the flagellum) in two opposite rows plus a smooth posterior flagellum. In *Blastocysta* and *Slopalinidae* (e.g., *Opalina, Protopalina, Blastocystis*), early branches of the *Stramenopiles*, the cellular surface is completely covered with cilia. These are cyst-forming organisms living as endosymbionts or parasites in the guts of fish, amphibians, reptiles, insects, or mammals. The *Bicosoecida* with the nice genus name *Cafeteria* are phagotrophic heterotrophs that are mostly attached with their smooth posterior flagellum to surfaces. *Labyrinthulomycetae* (slime nets) are mostly marine parasites of algae or invertebrates, or decomposers of organic material. *Labyrinthula algeriensis* is a parasite of the alga *Laminaria iberia,* infects the cells of its host, and multiplies in a slimy envelope as an amoeba. The *Hyphochytriales* are also slime fungi, carrying an anteriorly directed flagellum with mastigonemes. Related to these are a large group of fungi, the *Oomycetes* or *Peronosporomycetes*. These organisms have a fungus-type life style, often in aquatic environments and also include important terrestrial pathogens. In contrast to the "true" fungi of the opisthokonts, **oomycetes** rarely have chitin in their cell wall but glucan-cellulose instead. While *Saprolegnia* is a decomposer of organic material such as dead insects in water, *Phytophthora infestans* (**potato late blight** agent) was responsible for potato famines in Ireland and Scotland in the middle of the nineteenth century.

Large subtaxa of the *Stramenopiles* are algae, most important being the *Phaeophyceae* (**brown algae**), mostly multicellular algae that play an important role in marine ecosystems. *Macrocystis* may form huge underwater forests and it reaches dozens of meters in length. Many brown algae are known as *seaweeds* in the waters of the Northern hemisphere but others occur also in tropical water. The cell wall contains alginate compounds and cellulose. Their chloroplasts possess chlorophyll *a*, c_1, c_2 besides fucoxanthin and violaxanthin, which are responsible for the brown color. Smaller groups of photoautotrophic *Stramenopiles* are groups numbered as III.N.1, 5, 9, 10, 15, 16, 17, 20, and 23 in Table 4.4. All are unicellular algae, filaments, or colonial organisms. *Schizocladia* species may form branched filaments, *Chrysophyceae* (golden algae) and *Xanthophyceae* (yellow-green algae) different kinds of filaments.

Most species of the *Stramenopiles,* however, are the "true" **diatoms** of the phylum *Bacillariophyta.* These are mostly unicellular algae with the cells encased by a tightly integrated silicified element, the frustule. Diatoms are a common type of phytoplankton and an important part of the aquatic food chain (see Chapter 6). They can also be found on damp surfaces and in the top layers of soil. The chloroplasts contain four membranes, again indicating a eukaryotic endosymbiont as origin. Classes of the diatoms are the *Coscinodiscophyceae* (centric diatoms, e.g., *Paralia, Melosira, Corethron, Coscinodiscus;* Figure 4.8), *Bacillariophyceae* (pennate diatoms, e.g., *Ditylum, Eunotia*), *Fragilariophyceae* (pennate diatoms), and *Mediophyceae*.

Besides the heterokonts, the *Alveolata* (III.K) are the second major group of the *Chromalveolata*. The kingdom *Alveolata* contains the apicomplexans and the dinoflagellates. The central feature is a cortical alveola, a continuous layer of flat vesicles used to support the membrane. This alveola may form armor plates in the *Dinophyceae* and

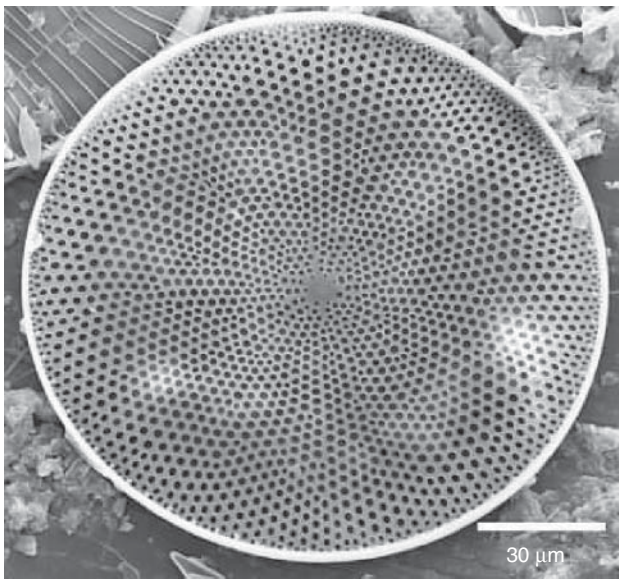

Figure 4.8 *Coscinodiscus* (*Eukaryota, Stramenopiles, Bacillariophyceae*; diatoms). Scanning electron microscope. (Mikrobiologischer Garten/Renate Kort and Erhard Rhiel.)

Figure 4.9 *Ceratium horridum*, a dinoflagellate (*Eukaryota, Alveolata*). (Mikrobiologischer Garten/Heribert Cypionka.)

Perkinsea, which contain a large group of photoautotrophic and mixotrophic or even predating or parasitic flagellates in aquatic environments. Examples are *Ceratium* (Figure 4.9) and *Noctiluca*, a bioluminescent organism. The *Apicomplexa* (classes *Aconoidasida, Conoidasida*) are all parasites except the free-living predatory flagellates of the *Colpodellida*. In at least one stage of their life, they exhibit an apicoplast, a complicated apical structure used to penetrate a host or prey cell. The genus *Plasmodium* of the *Aconoidasida* order *Hemosporida* contains over 200 species, and at least 11 infect humans as mosquito-transmitted disease: malaria. This is an important infectious disease with 225 million cases in 2010.

The last phylum of the *Alveolata* are the **ciliates**, the *Ciliophora*. These are characterized by the cilia, short flagella present in large numbers on the surface of the cell. Ciliates (Figure 4.10) are mostly heterotrophic predatory organisms feeding on bacteria and algae but some symbionts and parasites also occur. They have very complicated cell structures with two different nuclei (micro- and macronucleus for reproduction and housekeeping, respectively), contractile and digestive vacuoles, and other organelles. Some species are visible to the naked eye and reach 2 mm in length. Examples for ciliates are *Stentor* (trumpet animalcule), *Vorticella* (bell animalcule, Figure 4.11), and the best-known representative of this group, *Paramecium.*

4.4.4 Kingdom *Rhizaria* (III.G)

The *Rhizaria* are the second crown group of the *Eukaryota*. Members of this group are all unicellular heterotrophs or autotrophs. Their cells are mostly amoeboids with pseudopodia (filopodia), which may occur as simple, branching, anastomosing, or microtubula-supported axopodia. Subgroups of the *Rhizaria* are the classes *Acantharea,*

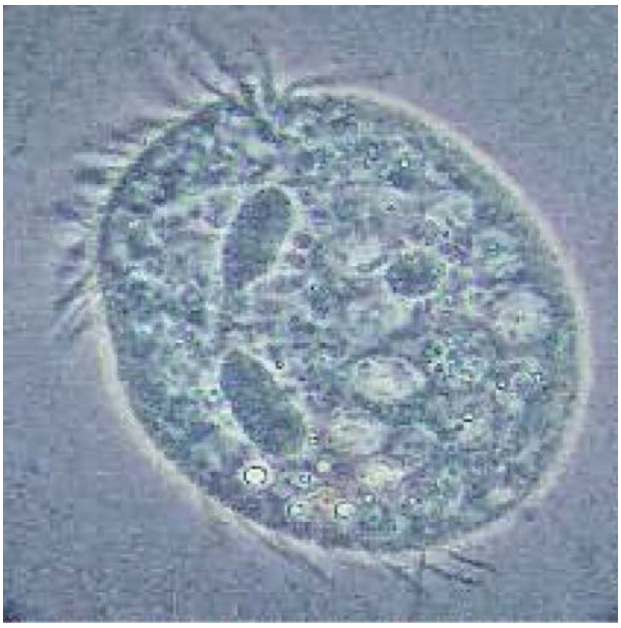

Figure 4.10 A ciliate (*Eukaryota, Alveolata, Ciliophora*) from a ditch. (Mikrobiologischer Garten/Heribert Cypionka.)

Cercozoa, Foraminifera, Gromiida with the single genus *Gramia, Haplosporidia, Polycystinea,* and *Sticholonchida*.

The *Cercozoa* are amoeboflagellates without distinctive features (in comparison to the other groups of the *Rhizaria*). Usually, they have filopodia, microbodies, extrusosomes, and may form cysts. The kinetosome connects the nucleus with the cytoskeleton. Subphyla are the *Cercomonadida*, amoeboflagellates without cell wall (*Cercomonas, Heteromita*); *Silicofilosae* with secreted silicate scales at the surface (*Allas, Euglypha, Paulinella*); *Chlorarachniophyta*, amoebes with secondary plastids surrounded by four membranes again (*Chlorarachnion*); *Phytomyxea*, parasites of plants and stramenopiles (*Phagomyxa, Pongomyxa*); *Phaeodarea*, radiolarian-like heterotrophic protists that produce a silicate hollow cytoskeleton (*Conchopsis, Haeckeliana*); and *Nucleohelea* with an amorphous centrosome adjacent to the nuclear envelope (axoplast; *Clathrulina*).

The *Haplosporidia* are plasmodial endoparasites of aquatic animals with distinctive spores containing a "lid" and intranuclear spindles designated "*kernstab*" (*Haplosporidium*). *Gromia* contains the sole genus *Gromia*, widespread in aquatic ecosystems; the *Gromia* amoeba is shelled, in size up to 5 mm, and has extensively branching pseudopodia. The cytoplasm is multinucleated. *Gromia* produces flagellated swarmer cells.

The *Foraminifera* (Figure 4.12) are the main group of the *Rhizaria* with about 270 000 species. They usually produce a multichambered shell, sometimes complicated in structure. Filopodia emerge from this chamber, which branch or merge to form a dynamic cytoplasmic network (*Ammonia*).

The *Radiolaria* classes *Acantharea* and *Polycystinea* have cells with an organic but nonliving intricate mineral skeleton (Figure 4.13). Typically, a central capsule divides the cell into

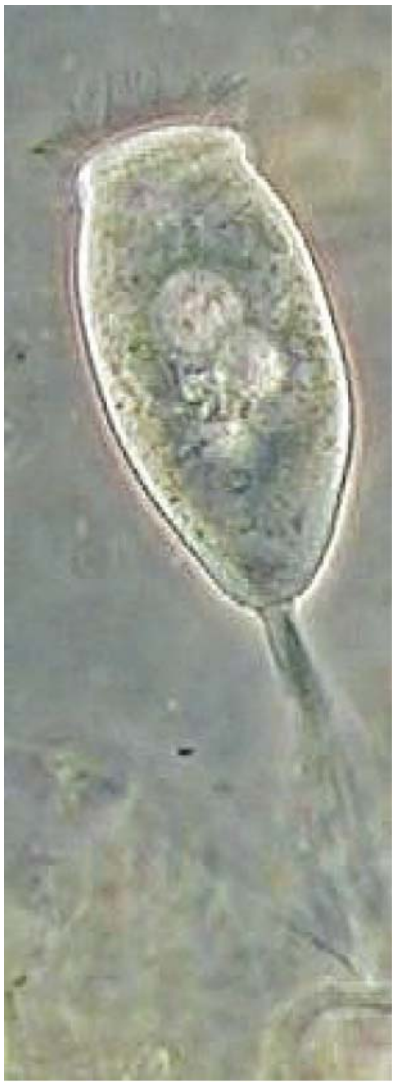

Figure 4.11 *Vorticella* (*Eukaryota, Alveolata, Ciliophora*). (Mikrobiologischer Garten/Heribert Cypionka.)

an inner and outer portion (endoplasm and ectoplasm). The endoplasm contains the nucleus and the organelles while vacuoles and lipid droplets reside in the ectoplasm. Often, members of the class *Acantharea* contain a symbiontic alga in the endoplasm (*Acanthometra*).

4.4.5 *Archaeplastida* (III.H, III.I, III.J)

Members of the third crown group of the *Eukarya*, the *Archaeplastida*, usually contain a plastid with chlorophyll *a* from an ancestral primary endosymbiosis with a cyanobacterium, a cellulose cell wall, and starch is used as storage material. Sometimes, the plastid may be lost or reduced secondarily. The three phyla of the *Archaeplastida* are the early-branching *Glaucophyta* (III.H), the *Rhodophyta* (III.I, red algae), and the *Chloroplastida* or *Viridiplantae* (III.I).

Glaucophyta (III.H) are a small group comprising only a few unicellular species. The plastids of these algae, the cyanelles, resemble cyanobacteria with phycobilisomes and still form peptidoglycan, but decreased genome size (*Cyanophoras, Glaucocystis*).

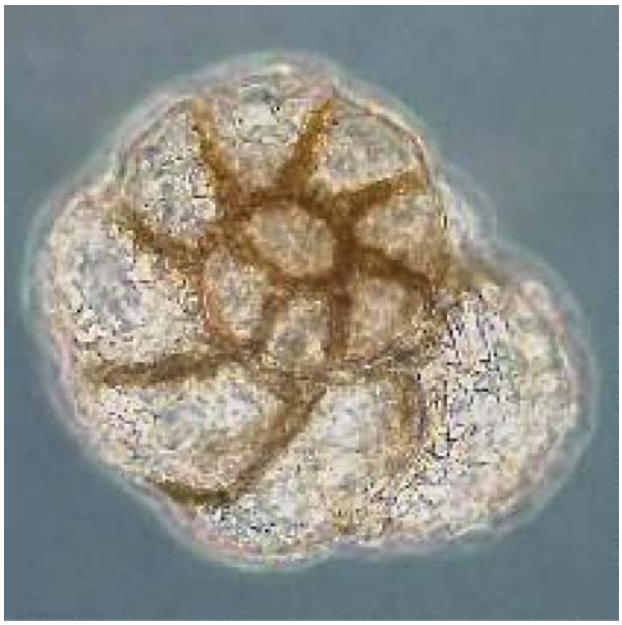

Figure 4.12 A foraminifera from the North Sea (*Eukaryota, Rhizaria*). (Mikrobiologischer Garten/Markus Bachmann.)

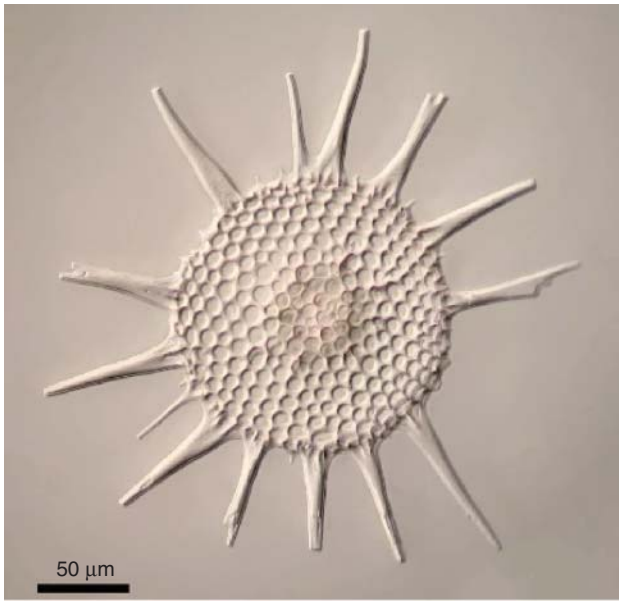

Figure 4.13 A radiolarian skeleton from the sediment of the North Sea (*Eukaryota, Rhizaria*). (Mikrobiologischer Garten/Heribert Cypionka.)

The *Rhodophyta* (III.I) contain several thousand species. Their cells lack flagella and centrioles and are colored red by phycobolisomes as accessory pigment in addition to chlorophyll *a*. Carbohydrate storage is floridean starch. Most red algae are multicellular, macroscopic, and marine organisms. Coralline red algae secrete calcium carbonate, help building

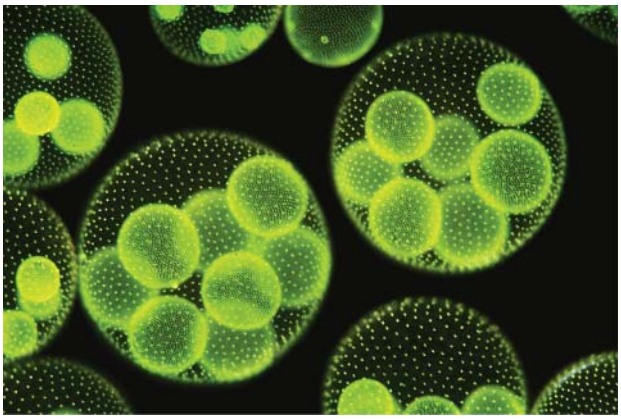

Figure 4.14 *Volvox aureus* (*Eukarya, Viridiplantae, Chlorophyta*). (© Micro_photo_fotolia.com.)

coral reefs, and are important constituents of this ecosystem (*Ceramium, Porphyra, Rhodophysema*).

Members of the kingdom *Chloroplastida* or *Viridiplantae* (III.J) have plastids with chlorophylls *a* and *b,* a cell wall usually with cellulose, and centrioles. The kingdom *Chloroplastida* has two phyla (Table 4.4), *Chlorophyta* and *Streptophyta* (III.J.2). The *Chlorophyta* (III.J.1) contain seven classes of green algae. Their cells possess flagella in pairs or multiples of two and divide without a phragmoplast. The storage material starch resides inside the plastid. Important classes of the *Chlorophytina* are the *Ulvophyceae* (*Acetabularia, Cladophora*), *Trebouxiophyceae* (*Chlorella, Nannochloris, Trebouxia*), and *Chlorophyceae* (*Chlamydomonas, Volvox,* Figure 4.14), others the *Mamiellophyceae* (*Mamiella, Micromonas*), *Nephroselmidophyceae* (*Nephroselmis*), *Pedinophyceae* (*Pedinomonas, Resultor*), and *Prasinophyceae* (*Tetraselmis*).

The phylum *Streptophyta* (III.J.2) contains the huge subphylum *Streptophytina* and four small classes, namely the *Mesostigmaphyceae* (*Mesostigma*), *Chlorokybophyceae* (*Chlorokybus*), *Klebsormidiophyceae* (*Klebsormidium*), and *Zygnemophyceae* (*Zygnematales, Desmidiales*). The *Streptophytina* contain all higher plants in the *Embryophyta* plus the two small classes *Coleochaetophyceae* (single order *Coleochaetales*) and *Charophyceae* (single order *Charales*). The *Coleochaetales* (*Coleochaete*) are algae that live in a disk-shape on stones or as epiphytes on other plants. The algae of the *Charales* are an order of freshwater "pondweeds" (*Chara*), true multicellular organisms, and a sister group of the *Embryophyta*. These two small classes are candidates for the ancestry of all higher plants.

The *Embryophyta*, finally, are the higher plants (Table 4.5). They start with the earliest branching *Marchantiophyta* (liverworts, III in Table 4.5) (Figure 4.15a), continue with the *Anthocerotophyta* (hornworts, I) and *Bryophyta* (mosses, II), and lead to the *Tracheophyta* (vascular plants, IV), containing the *Lycopodiophyta* (club mosses, IVB) and *Euphyllophyta* (IVA). The latter are divided into the *Moniliformopses* (ferns and horsetails, IVA1) and *Spermatophyta*

Table 4.5 Detailed taxonomy of the *Embryophyta* down to the level of orders.[a)]

I. Anthocerophyta (hornworts)

***A.* Anthocerotopsida**

1. Anthocerotidae

a. *Anthocerotales* (1 Family)

2. Dendrocerotidae

a. *Dendrocerotales* (1 Family)

b. *Phymatocerotales* (1 Family)

3. Notothylidae

a. *Notothyladales* (1 Family)

B. Leiosporocerotopsida

1. Leiosporocerotopsidae

a. *Leiosporocerotales* (1 Family)

II. Bryophyta (mosses)

A. Andreaeobryophytina

1. ***Andreaeobryopsid***a

a. *Andreaeobryales* (1 Family)

B. Andreaeophytina

1. Andreaeopsida

a. *Andreaeales* (1 Family)

C. Bryophytina

1. Bryopsida

- *Bryidae*
  - *Bryanae*
  - a. *Bartramiales* (1 Family)
  - b. *Bryales* (6 Families)
  - c. *Hedwigiales* (3 Families)
  - d. *Orthotrichales* (1 Family)
  - e. *Rhizogoniales* (3 Families)
  - f. *Splachnales* (2 Families)
  - *Hypnanae*
  - g. *Hookeriales* (7 Families)
  - h. *Hypnales* (43 Families)
  - i. *Hypnodendrales* (4 Families)
  - j. *Ptychomniales* (1 Family)
- *Buxbaumiidae*
  - k. *Buxbaumiales* (1 Family)
- *Dicranidae*
  - l. *Archidiales* (1 Family)
  - m. *Bryoxiphiales* (1 Family)
  - n. *Dicranales* (13 Families)
  - o. *Grimmiales* (3 Families)
  - p. *Pottiales* (4 Families)
  - q. *Scouleriales* (2 Families)
- *Diphysciidae*
  - r. *Diphysciales* (1 Family)
- *Funariidae*
  - s. *Encalyptales* (2 Families)
  - t. *Funariales* (2 Families)
  - u. *Gigaspermales* (1 Family)
- *Pleurocarpidae*
  - v. core pleurocarps (1 Family)
- *Timmiidae*
  - w. *Timmiales* (1 Family)

2. Oedipodiopsida

a. *Oedipodiales* (1 Family)

3. Polytrichopsida (hair mosses)

a. *Polytrichales* (1 Family)

4. Tetraphidopsida

a. *Tetraphidales* (1 Family)

Table 4.5 (*Continued*)

D. Sphagnophytina
1. Sphagnopsida
a. *Sphagnales* (3 Families)

E. Takakiophytina
1. Takakiopsida
a. *Takakiales* (1 Family)

III. Marchantiophyta (Liverworts)

A. Haplomitriopsida
1. Haplomitriidae
a. *Calobryales* (1 Family)
2. Treubiidae
a. *Treubiales* (1 Family)

B. Jungermanniopsida
1. Jungermanniidae
a. *Jungermanniales* (4 Families)
b. *Porellales* (3 Families)
c. *Ptilidiales* (3 Families)
2. Metzgeriidae
a. *Metzgeriales* (3 Families)
b. *Pleuroziales* (1 Family)
3. Pelliidae
a. *Fossombroniales* (3 Families)
b. *Pallaviciniales* (2 Families)
c. *Pelliales* (1 Family)

C. Marchantiopsida
1. Blasiidae
a. *Blasiales* (1 Family)
2. Marchantiidae
a. *Lunulariales* (1 Family)
b. *Marchantiales* (14 Families)
c. *Neohodgsoniales* (1 Family)
d. *Sphaerocarpales* (2 Families)

IV. Tracheophyta (vascular plants)

A. Euphyllophyta
1. Moniliformopses (ferns and horsetails)
• ***Equisetopsida***
a. *Equisetales* (horsetails. 1 Family)
• ***Marattiopsida***
b. *Marattiales* (1 Family)
• ***Ophioglossopsida***
c. *Ophioglossales* (1 Family, adder's-tongue fern)
• ***Polypodiopsida***
d. *Cyatheales* (7 Families)
e. *Polypodiales* (Ferns, 22 Familie)
f. *Salviniales* (water-ferns, 3 Families)
g. *Gleicheniales* (3 Families)
h. *Hymenophyllales* (1 Family)
i. *Osmundales* (1 Family)
j. *Schizaeales* (3 Families)
• ***Psilotopsida***
k. *Psilotales* (1 Family, whisk-ferns)
2. Spermatophyta (seed plants)
• Acrogymnospermae (Coniferophyta, Cycadophyta, Ginkgophyta, Gnetophyta)
Coniferophyta
Coniferopsida
a. Coniferales (7 Families, pine, yew, and cypresses)
Cycadophyta

Table 4.5 (*Continued*)

Cycadopsida
b. *Cycadales* (cycads, 3 Families)
Ginkgophyta
Ginkgoopsida
c. *Ginkgoales* (1 Family, maidenhair tree)
Gnetophyta
Gnetopsida
d. *Ephedrales* (1 Family)
e. *Gnetales* (1 Family)
f. *Welwitschiales* (1 Family)
• Magnoliophyta (flowering plants)
basal Magnoliophyta
g. *Amborellales* (1 Family)
h. *Austrobaileyales* (3 Families)
i. *Nymphaeales* (3 Families, water-lily and water-field)
Mesangiospermae
– Chloranthophyta
j.*Chloranthales* (1 Family)
– Ceratophyta
k. *Ceratophyllales* (1 Family)
– Eudicotyledons (Gunneridae, Pentapetalae, stem eudicotyledons)
Gunneridae
l. *Gunnerales* (2 Families)
Pentapetalae
Asteridae (following 13 orders)
Campanulinae (following 7 orders)
m. *Apiales* (4 Families)
n. *Aquifoliales* (5 Families)
o. *Asterales* (11 Families, e.g., daisies)
p. *Bruniales* (2 Families)
q. *Dipsacales* (7 Families, e.g., teasle, valerian)
r. *Escalloniales* (1 Family)
s. *Paracryphiales* (1 Family)
t. *Cornales* (7 Families, e.g., dogwood)
u. *Ericales* (25 Families, e.g., tea, ebony, phlox)
Lamiinae (following 4 orders)
v. *Garryales* (3 Families)
w. *Gentianales* (5 Families, e.g., madder)
x. *Lamiales* (23 Families, e.g., olive, mint, sesame)
y. *Solanales* (6 Families, e.g., nightshades, waterleaf)
z. *Berberidopsidales* (2 Families)
aa. *Caryophyllales* (29 Families, e.g., amaranth, sundew)
bb. *Dilleniales* (1 Family)
Rosidae (Fabinae, Malvinae, Vitales; following 17 orders)
Fabinae (following 8 orders)
cc. *Celastrales* (3 Families, e.g., bittersweet)
dd. *Cucurbitales* (8 Families, e.g., cucumber, begonia)
ee. *Fabales* (4 Families, e.g., pea)
ff. *Fagales* (8 Families, e.g., birch, beech, walnut)
gg. *Malpighiales* (40 Families, e.g., flax, willow, violet)
hh. *Oxalidales* (7 Families, e.g., wood-sorrel)
ii. *Rosales* (9 Families, e.g., rose, elm, nettle)
jj. *Zygophyllales* (2 Families)
Malvinae (following 8 orders)
kk. *Brassicales* (19 Families, e.g., mustard, papaya, horseradish)
ll. *Crossosomatales* (8 Families)
mm. *Geraniales* (3 Families, e.g., geranium)
nn. *Huerteales* (3 Families)
oo. *Malvales* (12 Families, e.g., rock-rose, mallow)

(*Continued Overleaf*)

Table 4.5 (*Continued*)

pp. *Myrtales* (12 Families, e.g., myrtle)
qq. *Picramniales* (1 Family)
rr. *Sapindales* (13 Families, e.g., mahogany)
ss. *Vitales* (1 Family, grapes)
tt. *Santalales* (19 Families, e.g., mistletoe)
uu. *Saxifragales* (15 Families, e.g., saxifrages)
Stem eudicotyledons
vv. *Buxales* (1 Family, boxwood)
ww. *Proteales* (3 Families, e.g., lotus)
xx. *Ranunculales* (8 Families, e.g., moonseed, poppy, buttercup)
yy. *Sabialales* (1 Family)
zz. *Trochodendrales* (1 Family)
– Liliopsida (monocotyledons)
aaa. *Acorales* (1 Family, sweet flag)
bbb. *Alismatales* (14 Families, e.g., pondweeds)
ccc. *Asparagales* (21 Families, e.g., agaves, iris, orchids)
Commelinids (subsequent 4 orders)
ddd. *Arecales* (1 Family, palms)
eee. *Commelinales* (5 Families, e.g., water-hyacinth)
fff. *Poales* (16 Families, e.g., bromeliads, sedges, grasses)
ggg. *Zingiberales* (8 Families, e.g., canna, banana, ginger)
hhh. *Dioscoreales* (3 Families, e.g., yam)
iii. *Liliales* (11 Families, e.g., lilies)
jjj. *Pandanales* (5 Families, e.g., screw pines)
kkk. *Petrosaviales* (1 Family)
– Magnoliidae
lll. *Canellales* (2 Families, e.g., wild cinnamon)
mmm. *Laurales* (7 Families, e.g., laurel)
nnn. *Magnoliales* (6 Families, e.g., magnolia, nutmeg)
ooo. *Piperales* (5 Families, e.g., pepper, birthwort)

B. Lycopodiophyta (club mosses)
1. Isoetopsida
a. Isoetales (quillworts, 1 Family)
b. Selaginellales (spike mosses. 1 Family)
2. Lycopodiopsida (club mosses)
a. Lycopodiales (1 Family)

a) Orders listed with small letters in italics, superorders in italics without letter, classes are italic, bold, underlined, subclasses italic and underlined, units with no rank and without italics. The taxon *Embryophyta* (higher plants) has no taxonomic rank and belongs together with the *Charophyceae* (a class of green algae) and *Coleochaetophyceae* (single order *Coleochaetales*, parenchymous charophyte algae) to the taxon *Streptophytina*. Together with four algal classes, the *Chlorokybophyceae, Klebsormidiophyceae, Mesostigmatophyceae,* and *Zygnemophyceae*, the *Streptophytina* form the phylum *Streptophyta* (Table 4.4, eukaryotic phylum III.J.2).

(seed plants, IVA2, Figure 4.15). The taxonomy of the *Embryophyta* is further detailed in Table 4.5 down to the level of taxonomic orders.

4.4.6 Kingdom *Amoebozoa* (III.A)

Together with their sister group *Opisthokonta* (III.E in Table 4.4), the *Amoebozoa* (III.A in Table 4.4) are the last crown group of the *Eukarya*. While the *Archaeplastida* have focused on a phototrophic way of life, the *Amoebozoa* and *Opisthokonta* are specialized heterotrophic organisms. Both have stemmed from flagellated ancestors and such forms are still found in some groups of the *Amoebozoa,* as are cysts as survival forms. Usually, flagellated forms of the *Amoebozoa* carry only one kinetid and a single flagellum. The most prominent feature of the *Amoebozoa*, as the name suggests, is the predominant amoeboid form with noneruptive variable pseudopodia called *lobopodia*. Subgroups of the *Amoebozoa* are the genera *Acramoeba, Multicilia, Phalansterium, Stereomyxa, Talepolella* and class-like taxa *Archamoebae, Centramoebida, Flabellinea, Himatismenida, Mycetozoa,* and *Tubulinea.*

Tubulinea are naked (*Tubulinida, Leptomyxida)* or testate (some *Arcellinida*) amoeba without flagellate stages. They are capable of changing their shapes from flattened to expanded forms and contain no centrosomes. *Tubulinida* (*Amoeba, Hartmannella*) do not change their locomotive morphology (Figure 4.16) while *Leptomyxidae* (*Leptomyxa, Rhizamoeba*) have a flattened, reticulate, or highly branched sheet as locomotive form, which may become subcylindrical in the most active form. Species of the genus *Arcella* (*Arcellinida*) possess a test, a shell outside the cytoplasm, which usually contains one distinct opening to release the lobopedia.

The *Flabellinea* (*Vanella, Thecamoeba*) resemble the *Tubulinea* by having no centrosome and no flagellated stage. They are flattened locomotive amoeba and do not change their locomotive form. In contrast to the *Tubulinea*, the cytoplasmic flow in the *Flabellinea* is polyaxial or without axis while that in the *Tubulinea* is monoaxial. Smaller groups of the *Amoebozoa* are the *Stereomyxida* with a triaminate centrosome (*Stereomyxa*) and the *Centramoebida* with a centriole-like body (*Acanthamoeba*).

Archamoebae are adapted to microaerobic and anaerobic environments and lack mitochondria. Species of the *Mastiamoebidae* are amoeboid, contain a single flagellum and kinetosome, although some stages are not flagellated, and they produce cysts (*Mastigamoeba*). *Pelomyxa palustris* as the only species of the *Polymyxidae* is anaerobic and contains multiple cilia. The *Entamoebidae* possess no flagellum, centriole, mitochondrion, peroxisome, or hydrogenosome, and a reduced Golgi apparatus. *Entamoeba coli* and *Entamoeba gingivalis* are commensals in the gut and on teeth, respectively, while *Entamoeba histolytica* is a causative agent of amebiasis, a gastrointestinal infection with amoeba, and may infect 50 million people worldwide. Through its cyst that resists harsh environmental conditions (though not the heat transpired during cooking), *E. histolytica* spreads via salad and unwashed fruits. In secondary amebiasis, organs such as the liver or brain may become infected, leading to dangerous complications.

The *Mycetozoa* (also *Eumycetozoa* or *Myxomycota*) or "true" slime moulds display the most advanced stage of development of the *Amoebozoa* in the direction of multicellular organization. The life cycle may change between bacteria-feeding uninucleate amoeba-flagellates with one or two kinetosomes, which may give rise to other stages such as nonflagellated uninucleate or multinucleate amoeba.


(a)

(b)


(c)


(d)


(e)


(f)


(g)


(h)

Figure 4.15 Plants (a–h). (a) *Marchantia polymorpha* (Marchantiophyta, Marchantiaceae) (Botanical garden, University of Halle; Courtesy of A. Fläschendräger). (b) *Euphorbia podofolia* (Malpighiales, Euphorbiaceae) (Sierra de Nipe, Cuba) (Courtesy of A. Fläschendräger). (c) *Hibiscus elatus* (Rosids, Malvaceae) (Sierra de Nipe, Cuba) (Courtesy of A. Fläschendräger). (d) *Syzygium cormiflorum*, a rainforest tree with flowers on the trunk (Rosids, Myrtaceae) (Cape Tribulation, Queensland, Australia) (Courtesy of G. Krauss). (e) *Hamamelis spec.* with freezing resistant flowers (Saxifragales, Hamamelidaceae) (Courtesy of G. Krauss). (f) *Taxus baccata* (Coniferales, Taxaceae) (© Ruud Morijn_fotolia.com) (g) *Ceratophyllum demersum*, an aquatic plant (Botanical garden, University of Halle; Courtesy of A. Fläschendräger). (h) *Arabidopsis thaliana*, the biochemist's model plant (Brassicaceae). (Courtesy of J. Heilmann.)

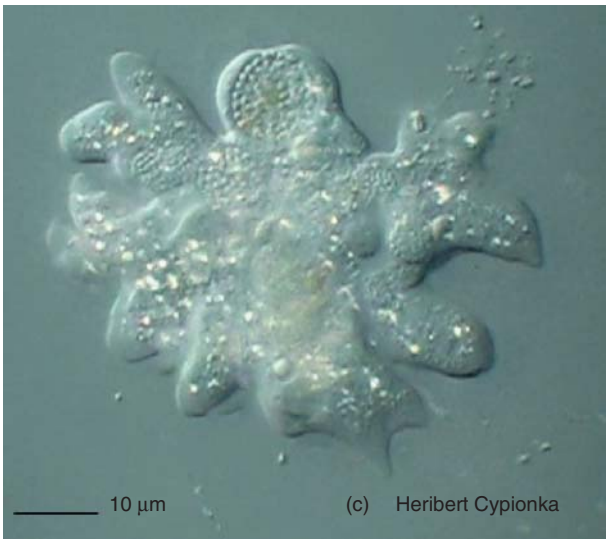

Figure 4.16 An amoeba from a sewage plant. (Mikrobiologischer Garten/Heribert Cypionka.)

When starved, the amoebae aggregate to form fruiting bodies. In the order *Protosteliales* (*Protostelium*) the envelope of the fruiting body emerges from a single amoeboid cell (sporocarp). The *Myxogastria* (plasmodial slime molds) have free-living trophic multinucleate amoebae. The fruiting body is encased by a sporocarp. The sporangium is in many cases stalked but the stalk is noncellular (*Physarum, Fuligo*). Finally, the highest stage of development is reached within the order *Dictyosteliida* (*Dictyostelium*), the cellular slime moulds. The stalk, spores, and envelope named sorocarp is produced by the aggregation of amoeba. The stalk cells forming the sorocarp die, which is a feature of multicellular organisms. *Hyperamoeba* is a *Mycetozoa* genus not assigned to one of the other three groups.

4.4.7 Kingdom Opisthokonta (III.E)

The species of the kingdom *Opisthokonta* are heterotrophs that have a single posterior cilium without mastigonemes at least in one stage of their life cycle. The flagellum is rarely lost as secondary event in some organisms. They usually contain a pair of kinetosomes or centrioles. Subkingdoms of the *Opisthokonta* are the *Choanoflagellida, Fungi, Metazoa,* and the small *Nucleariidae/Fonticula* group (Table 4.6).

The *Choanoflagellida* are the earliest branching group of the *Opisthokonta*. It contains only the order *Choanoflagellida* of solitary or colonial cells with a radial symmetry, which contain a collar of microvilli around a single flagellum (*Codonosiga, Salpingoeca, Bicosta*). *Nucleariidae* are a family of amoeba related to the genus *Fonticula*, which was previously grouped with the cellular slime molds. This indicates a close relationship of early *Opisthokonta* and *Amoebozoa*.

The previously existing taxon *Mesomycetozoa* lost its taxonomic rank and is now *incertae sedis*, with unknown

Table 4.6 Detailed phylogeny of the *Opisthokonta*.

1. Choanoflagellida
a. Choanoflagellida
2. Fungi (fungi)
a. Blastocladiomycota
a. Blastocladiomycetes
b. Chytridiomycota
a. Chytridiomycetes
b. Monoblepharidomycetes
c. Cryptomycota
a. Cryptomycetes
Dikarya
d. Ascomycota (ascomycetes)
***Saccharomyceta* (next two subphylae)**
i. Pezizomycotina
ii. Saccharomycotina
iii. Taphrinomycotina
– Archaeorhizomycetes
– Neolectomycetes
– Pneumocystidomycetes
– Schizosaccharomycetes
– Taphrinomycetes
Mitosporic Ascomycota
e. Basidiomycota (basidiomycetes)
i. Agaricomycotina
– Agaricomycetes
– Dacrymycetes
– Tremellomycetes
– Mitosporic Agaricomycotina
ii. Pucciniomycotina
– Agaricostilbomycetes
– Atractiellomycetes
– Classiculomycetes
– Cryptomycocolacomycetes
– Cystobasidiomycetes
– Microbotryomycetes
– Mixiomycetes
– Pucciniomycetes
– Tritirachiomycetes
– Mitosporic Pucciniomycotina
iii. Ustilaginomycotina
– Exobasidiomycetes
– Ustilaginomycetes
Mitosporic Basidiomycota
f. Entomophthoromycota [in-part: Zygomycota]
a. Entomophthoromycota
g. Glomeromycota
a. Archaeosporomycetes
b. Glomeromycetes
c. Paraglomeromycetes
h. Microsporidia (microsporidians)
a. Microsporidia
i. Neocallimastigomycota
a. Neocallimastigomycetes
j. Early diverging fungal lineages
a. Kickxellomycotina [in-part: Zygomycota]
b. Mortierellomycotina
c. Mucoromycotina [in-part: Zygomycota]
d. Nephridiophagidae [in-part: Zygomycota]
e. Olpidiaceae
f. Zoopagomycotina [in-part: Zygomycota]

Table 4.6 (*Continued*)

3. Metazoa (metazoans; Eumetazoa, Mesozoa, Placozoa, Porifera)[a)]
Eumetazoa (Bilateria, Cnidaria, Ctenophora)
Bilateria (Deuterostomia, Gnathostomulida, Platyhelminthes, and *Protostomia)*
Deuterostomia (deuterostomes, next 6 phylae to 3f)
a. Chaetognatha (arrow worms)
a. Chaetognatha
b. Chordata (chordates)
a. Cephalochordata
i. Cephalochordata
b. Craniata
i.** Hyperotreti **(hagfish)
– *Hyperotreti*
ii.** Vertebrata **(vertebrates)
– *Gnathostomata (jawed vertebrates)*
• *Chondrichthyes (cartilaginous fishes, sharks, and rays)*
• *Teleostomi – Euteleostomi (bony vertebrates):*
Actinopterygii (ray-finned fishes)
Sarcopterygii:
Coelacanthimorpha (fish)
Dipnotetrapodomorpha:
Dipnoi (lungfishes)
Tetrapoda (tetrapods):
⇒ *Amniota (amniotes)*
Mammalia (mammals)
Sauropsida (sauropsids)
⇒ *Amphibia (amphibians)*
– *Hyperoartia*
• *Hyperoartia (lampreys)*
c. Tunicata (tunicates)
i. Appendicularia (appendicularians)
ii. Ascidiacea (sea squirts)
iii. Thaliacea
c. Echinodermata (echinoderms)
a. Echinodermata
d. Hemichordata (hemichordates)
a. Enteropneusta (acorn worms)
b. Pterobranchia
e. Xenacoelomorpha
a. Xenacoelomorpha
f. Xenoturbellida
a. Xenoturbellida
g. Gnathostomulida
a. Gnathostomulida
h. Platyhelminthes (flatworms)
a. Catenulida
b. Cestoda (tapeworms)
c. Monogenea
d. Rhabditophora
e. Trematoda
f. Turbellaria
Protostomia
Ecdysozoa (next 10 phylae up to *Priapulida*)
i. Nematoda (roundworms)
a. Chromadorea
b. Enoplea
j. Nematomorpha (horsehair worms)
a. Gordioida
b. Nectonematoida
Panarthropoda (next 3 phylae)
k. Arthropoda (arthropods)

Table 4.6 (*Continued*)

a. Chelicerata (chelicerates)
i. Arachnida (arachnids)
ii. Merostomata (horseshoe crabs)
iii. Pycnogonida (sea spiders)
Mandibulata (mandibulates):
b. Myriapoda (myriapods)
i. Chilopoda (centipedes)
ii. Diplopoda (millipedes)
iii. Pauropoda (pauropods)
iv. Symphyla (symphylans)
c. Pancrustacea
i. Crustacea (crustaceans)
– *Branchiopoda*
– *Cephalocarida (horseshoe shrimps)*
– *Malacostraca*
– *Maxillopoda*
– *Ostracoda (mussel shrimps)*
– *Prionodiaptomus*
– *Remipedia*
ii. Hexapoda (insects)
– *Diplura (diplurans)*
– *Ellipura*
– *Insecta (true insects)*
l. Onychophora (velvet worms)
a. Onychophora
m. Tardigrada (water bears)
a. Eutardigrada
b. Heterotardigrada
Scalidophora (next 3 phylae)
n. Kinorhyncha (mud dragons)
a. Kinorhyncha
o. Loricifera (loriciferans)
a. Loricifera
p. Priapulida (priapulids)
a. Priapulida
Lophotrochozoa (next 12 phylae up to *Rotifera*)
q. Acanthocephala (thorny-headed worms)
a. Archiacanthocephala
b. Eoacanthocephala
c. Palaeacanthocephala
d. Polyacanthocephala
r. Annelida (annelid worms)
Clitellata (next 3 classes)
a. Branchiobdellae
b. Hirudinida (leeches)
c. Oligochaeta
d. Polychaeta (polychaetes)
s. Brachiopoda (lampshells)
a. Craniiformea
i. Craniata
b. Linguliformea
i. Lingulata
c. ***Phoroniformea (phoronid worms)***
i. Phoroniformea
d. Rhynchonelliformea
i. Rhynchonellata
t. Bryozoa (bryozoans)
a. Gymnolaemata
b. Phylactolaemata
c. Stenolaemata

(*Continued Overleaf*)

Table 4.6 (*Continued*)

u. Cycliophora
a. Cycliophora
v. Entoprocta (goblet worms)
a. Entoprocta
w. Gastrotricha (gastrotrichs)
a. Gastrotricha
x. Micrognathozoa (micrognathozoans)
a. Micrognathozoa
y. Mollusca (mollusks)
a. Aplacophora (solenogasters)
b. Bivalvia (bivalves)
c. Cephalopoda (cephalopods)
d. Gastropoda (gastropods)
e. Monoplacophora
f. Polyplacophora (chitons)
g. Scaphopoda (tusk shells)
z. Myzostomida
a. Myzostomida
aa. Nemertea (ribbon worms)
a. Anopla
b. Enopla
bb. Rotifera (rotifers)
a. Bdelloidea
b. Monogononta
c. Seisonidea
cc. Cnidaria (cnidarians)
a. Anthozoa (anthozoans)
b. Cubozoa (sea wasps)
c. Hydrozoa (hydrozoans)
d. Myxozoa
e. Scyphozoa (jellyfishes)
dd. Ctenophora (ctenophores)
a. Cyclocoela
b. Typhlocoela
Mesozoa (next 2 phylae)
ee. Orthonectida
a. Orthonectida
ff. Rhombozoa
a. Rhombizoa
gg. Placozoa (placozoans)
a. Placozoa
hh. Porifera (sponges)
a. Calcarea
b. Demospongiae
c. Hexactinellida
4. Nucleariidae and Fonticula group
a. Nucleariidae and Fonticula

a) The recently described genome of the Ctenophore *Mnemiopsis leidyi* confirms the close relationship of the *Cnidaria* and *Bilateria* but indicates that the *Ctenophora* might be an earlier branch of an evolutionary line that leads to *Cnidaria*, *Bilateria*, and their common sister group *Placozoa* (Ryan *et al.*, 2013). Thus, *Placozoa* might be added to the *Eumetazoa* but the *Ctenophora* removed from this taxon.

affiliation. These organisms have a spherical cell, monoflagellated or amoeboid, at least in one stage of their life cycle. Groups are the intracellular parasites of algae (*Amoeboaphelidium*); *Ichthyosporea*, parasitic or free-living saprotrophic single cells (*Rhinosporium, Ichthyophonus*) or amoeba with a huge single nucleus taking up to 50% of the cell size (*Capsaspora*); and the single genus *Corallochytrium* containing saprotrophic amoeba usually isolated from coral reefs.

The *Fungi* are one of the two major subkingdoms of the *Opisthokonta*. These are hetotrophic organisms with mitochondria and peroxisomes (one exception), often growing in hyphae usually enveloped by a chitin-containing cell wall. The *Fungi* contain 10 phyla. Members of the *Agaricomycotina* of the large phylum *Basidiomycota* grow in a mycelium or as yeast cells and produce specific basidia from terminal cells in a fertile layer. Compatible mycelia may fuse to yield a dikaryotic mycelium in which the two nuclei remain paired but do not fuse into a dikaryotic nucleus. If karyogamy happens during formation of the basidia, it is quickly followed by meiosis, one or more additional mitosis events, and migration of the nuclei into the developing basidiospores, usually four (*Agaricus*). The *Pucciniomycotina* (*Rhodotorula, Uromyces*) contain species with a multitude of life styles, from yeast stages to mycelia, and from free-living organisms to plant (rust fungi) or animal pathogens. The meiosis following karyogamy may occur in the same compartment, the probasidium or teliospore, as the nuclear fusion (holobasidia) but mostly happens in a separate one (metabasidia). The *Urediniomycotina* comprise plant parasites (rust and smut fungi) with mycelia in the parasitic phase (*Ustilago*).

The second large subphylum of the *Fungi* are the *Ascomycota*, grouped together with the *Basidiomycota* as superphylum *Dikarya*. Ascomycetes produce ascospores, mostly eight but from one to thousands, in a tube-like ascus by meiosis followed by several mitosis events. Mycelia or yeast cells can be found, also sexually and asexually replicating life stages. The *Saccharomyceta* contain the two subphylae *Saccharomycotina* with the *Saccharomycetes* (*Candida, Saccharomyces*) and the large "mold" group *Pezizomycotina* (*Aspergillus, Penicillium, Peziza, Neurospora, Nectria;* Figure 4.17), mitosporic Ascomycota, and

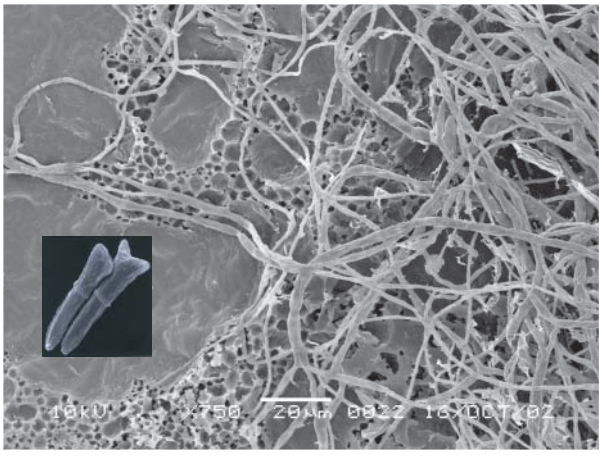

Figure 4.17 Hyphae of aquatic hyphomycetes living on the surface of alder leaves: exposed to freshwater insert: spores of *Heliscus lugdunensis* (amorph form of *Nectria lugdunensis*). (Courtesy of G. Krauss, J. Ehrman.)

Box 4.4: Taxonomy of the reader of this book

From the taxonomic point of view, the reader is placed as follows: *Eukaryota* (superkindom), *Opisthokonta* (kingdom), *Metazoa*, *Eumetazoa*, *Bilateria*, *Deuterostomia*, *Chordata* (phylum), *Craniata* (subphylum), *Vertebrata*, *Gnathostomata* (jawed vertebrates), *Teleostomi* (bony vertebrates), *Sarcopterygii* (bone fish and derivatives), *Dipnotetramorpha*, *Tetrapoda, Amniota,* class *Mammalia*, subclass *Theria*, infraclass *Eutheria*, *Boreoeutheria*, superorder *Euarchontoglires*, *Euarchonta*, order *Primates*, suborder *Haplorhini*, infraorder *Simiiformes*, parvorder *Catarrhini*, superfamily *Hominoidea*, family *Hominidae*, subfamily *Homininae*, genus *Homo*, species *Homo sapiens*, subspecies *Homo sapiens sapiens*. Good to know one's own place in evolution!

the *Taphrinomycotina* (*Schizosaccharomyces, Taphrina, Pneumocystis*).

The *Microsporidia* are obligate intracellular parasites, mostly in animals, and are exceptional among *Fungi* due to their lack of mitochondria. They are not an early branch of the Eukarya as believed some time ago but a reduced parasitic form of ascomycetes (*Nosema*). The *Glomeromycota* are filamentous primarily endomycorrhizal, arbuscular plant symbionts in the rhizosphere (*Glomus,* see Section 5.2.1). Members of the *Blastocladiomycota* and *Chytridiomycota* form ciliated cells at least in one stage of their life cycle (*Blastocladia, Chytridium*).

The *Zygomycotina* are no longer a phylum of the *Fungi* but listed as the early diverging fungal lineage *Mucoromycotina.* These are mostly filamentous fungi that reproduce sexually by a zygospore. This thick-walled compartment is formed by a junction between compatible mycelia. *Mucor* and *Rhizopus* can be found as mold on bread and fruits.

The second major subkingdom of the *Opisthokonta* are the *Metazoa*, multicellular organisms composed of wall-free cells held together by an extracellular protein matrix (usually collagen). The cells are connected by intercellular junctions. *Metazoa* reproduce sexually. An egg cell is thereby fertilized by a smaller sperm cell, often monoflagellated. The four subgroups of the *Metazoa* are the phylae *Porifera* and *Placozoa* and the superphylae *Mesozoa* and *Eumetazoa* (Table 4.6).

Porifera (sponges) are composed of cells without walls. They are sessile aquatic animals, predominantly in marine environments. Sponges do not possess a nervous or digestive system but mostly rely on a constant water flow though their body, which is composed of two cell layers with a jelly-like mesohyl in-between. Their cells may or may not specialize and are able to migrate between the different parts of the body. Egg cells may be amoeboid and sperm cells monociliated. After mating, the zygotes form ciliated larvae reminiscent of blastulae of the *Animalia*, and these swim around until they settle down to grow into a sessile adult. Old classes of the *Porifera* are the *Silicispongia* with siliceous spicules (*Farrea*, *Axinella*), now the modern classes *Demospongiae* (*Axinella)* and *Hexactinellida* (*Farrea*), and the *Calcispongiae* (now *Calcarea*) with calcium-carbonate-containing spicules (*Clathrinida*).

The *Placozoa* contain only one species, *Trichoplax adhaerens*. Reminiscent of sponges, *Trichoplax* contains two layers of epithelial cells divided by contractile fibrous cells in the middle (syncytium). This is a very flat organism gliding over surfaces with the cilia of the lower epithelial layer, and feeding by grazing biofilms or algae from this surface. There is no endoderm, ectoderm, or mesoderm; there are no nerve cells but the organism contains digestive glandular cells. It reproduces sexually by egg cells and nonciliated sperm cells but is also able to divide its body asexually simply by dividing down the middle. *Trichoplax* is an important model for the evolution of animals because its organization level is between that of sponges and the "true" animals.

The *Mesozoa* are osomotrophic endoparasites, probably reduced forms, without digestive tissues. They contain pluriciliated cells in epithelia, connected by gap, adherens, and septate junctions. They are tiny organisms consisting of an outer layer (somatoderm) and ciliated cells surrounding productive cells. The two phyla are the *Orthonectida* (*Rhopalura*) and *Rhombozoa* (*Dicyema*), which are all worm-like parasites in various marine animals. They may not be a monophylum but of different origins such as reduced *Bilateria* as a consequence of the parasitic way of life.

The *Eumetazoa* (animals) reproduce through an egg cell, mostly mating with a monoflagellated sperm cell, develop as embryos via a blastula and gastrulation, contain endoderm, mesoderm, and ectoderm (neuroderm) with contractile cells between endo- and extoderm, and connect their cells by characteristic belt desmosomes. The common cellular organization makes it highly probable that *Eumetazoa* are monophyletic. They can be subgrouped into the phylum *Cnidaria* (corals, sea anemones, jellyfish), the phylum *Ctenophora* (comp jellyfish), and the large group of *Bilateria* (*Coelomata*), the latter being subdivided into the phylae *Gnathostomulida, Platyhelminthes* (flatworms), and the lines *Protostomia* (such as *Mollusca* and *Arthropoda*) and *Deuterostomia* (*Echinodermata* and *Chordata* with fishes and tetrapods including amphibians, reptiles, birds, and mammals). This chapter ends here and refers to the taxonomy chapters in other textbooks but the reader has her or his own taxonomy in Box 4.4.

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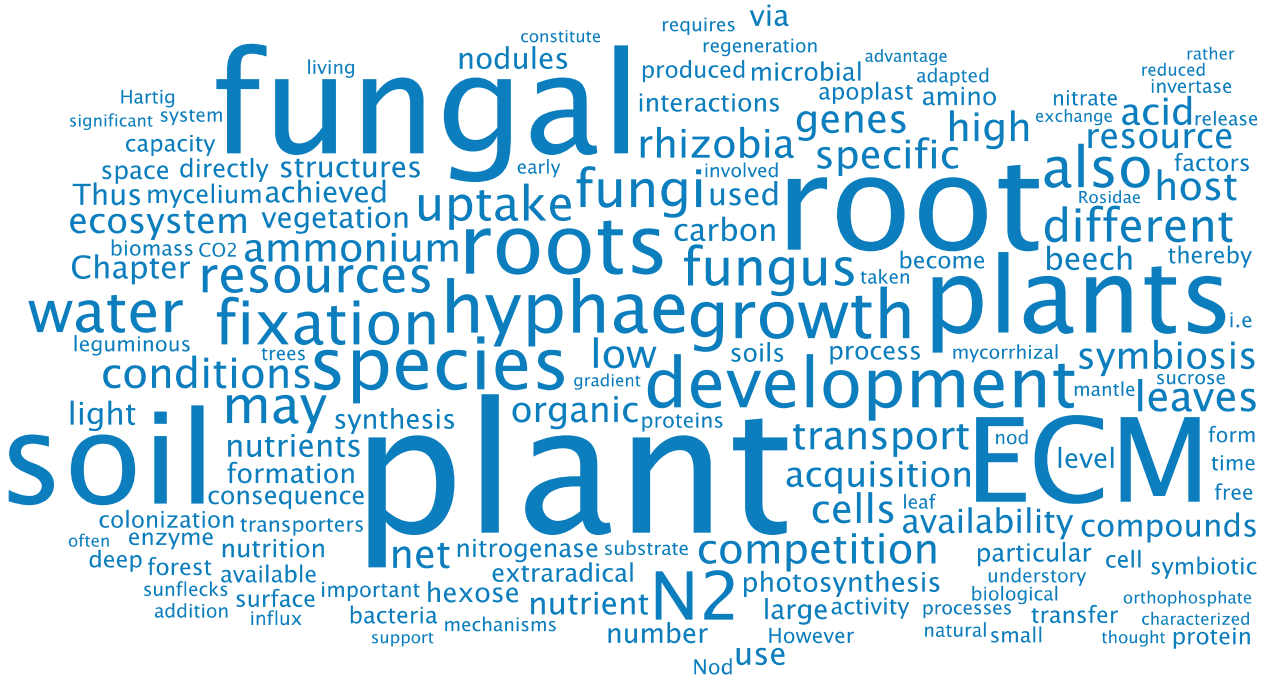
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5
Communities and Ecosystem Functioning

Heinz Rennenberg

Overview

Ecosystem and community functioning is highly dependent on the interplay of its different biota in acquisition and distribution of resources required for maintenance, growth and development, defense against abiotic and biotic stresses in the environment, and competitive and symbiotic interactions. These processes constitute the predominant "sinks" of resources in all biota, but even mutualistic coexistence may require the investment of additional resources, for example, in order to prevent a switch from coexistence to parasitism. The interplay of different biota in resource acquisition and distribution is of particular significance when availability of resources is limited. Under these conditions, different biota will compete for a limited resource, but may also avoid competition by different resource acquisition strategies in space (e.g., exploitation of different soil horizons) or/and time (e.g., preferential acquisition at different seasons). In this context, plants as primary producers play a decisive role and often control resource acquisition by other biota, in particular, by soil microbial communities.

It has been generally assumed that at a given net primary productivity (NPP), plants in their specific environment allocate different fractions of their NPP to their internal sinks and that this allocation is characterized by trade-off mechanisms. This indicates, for example, that an enhanced requirement of resources for defense or competitive interactions may result in reduced resource availability for growth and development. However, recent studies indicate that such trade-off mechanisms are not generally observed and facilitation mechanisms can be found instead, because the expansion of an existing or the generation of a new sink of resources can enhance NPP. As a consequence, the use of resources for defense and competitive interactions will, for example, not necessarily reduce resource availability for growth and development. Therefore, the investment of part of the NPP in competitive and symbiotic interactions usually will not weaken short-term plant performance and will even improve it in the long run.

Because of the immobile lifestyle of plants, successful resource acquisition requires space occupation by growth either by the plant itself or by its symbiont(s). Aboveground space occupation is needed to acquire light and CO_2, belowground space occupation to acquire nutrients and water. Occupying space and acquiring resources can be achieved by different strategies, that is, competition, symbiotic interactions, avoidance of competition, and facilitation, all of which imply the development of specific structural and physiological features. These strategies rely largely on signaling between biota often by volatile metabolites and result either in more efficient resource acquisition for a given plant species (e.g., water and nutrient acquisition by mycorrhizal roots), sharing the same resource between plant species by differential use (e.g., preferential N uptake at different seasons), or the exploitation of existing resources that are not generally available (e.g., fixation of atmospheric N_2 as N source). The latter may be defined as developing a physiological niche.

5.1
Competition for, and Distribution of, Limiting Resources as a Means of Ecosystem Functioning

Space can be considered a resource by itself, and also as a location that makes resources accessible. Thus, aboveground and belowground **space occupation** is indispensable for **resource acquisition** by plants and, hence, competition for resources is directly connected with competition for space. As a consequence, space occupation by one species can directly reduce resource availability for other species and can constitute a competitive advantage, when the availability of this resource is limited. Most prominent examples in this context are shading as a means of competition for light energy and intensive roots growth in the vicinity of hot spots of a limited nutrient in the soil, for example, by cluster roots.

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

5.1.1 Light-Capturing Strategies

Light is mostly a unidirectional resource because compared to total irradiation, diffuse irradiation is usually low. Therefore, light capture is disproportionate to the biomass involved and, hence, competition for light is size-asymmetric. This means that a small number of leaves can have a large shading effect on a competitor. As a consequence, the spatial arrangement of biomass can be more important for light capturing than the amount of biomass involved and its physiological performance such as **net primary productivity** (NPP). Thus, aboveground competition requires an integrative view on biomass architecture, biomass increment, and carbon gain.

Central European beech (*Fagus sylvatica, Fagales, Rosidae*) forests provide an interesting view on different strategies of light capturing at the ecosystem level. When fully developed, these forests are beech-dominated and also species-poor in the understory due to low light availability. Considerable understory vegetation is observed only in early spring before the leaf development of beech that takes place in late April/early May usually in a single flush during 2–3 weeks and reduces **photosynthetic active radiation** (PAR) inside the canopy to values below 100 $\mu E\, m^{-2}\, s^{-1}$. Leaves of beech natural regeneration are highly adapted to this low light availability (Figure 5.1) and exhibit light compensation of photosynthesis at extremely low irradiation and maximum photosynthesis at around 80 $\mu E\, m^{-2}\, s^{-1}$ PAR. Thus, leaves of beech natural regeneration are fully adapted to shading by the parent trees and thereby compete successfully with other understory vegetation that hardly can develop under these conditions. Compared to beech leaves developed in full sunlight, this adaptation includes (i) morphological factors such as a larger number of layers of palisade parenchyma cells and, hence, thicker leaves; (ii) cell developmental factors such as a larger number of chloroplasts per cell, a larger number of thylakoids per granum, and a lack of starch grains in the chloroplasts; and (iii) physiological factors such as a decreased chlorophyll a : b ratio, greater quantum efficiency, lower rates of dark respiration, and lower levels of Rubisco, ATP synthase, and electron transport chain constituents (e.g., cytochromes).

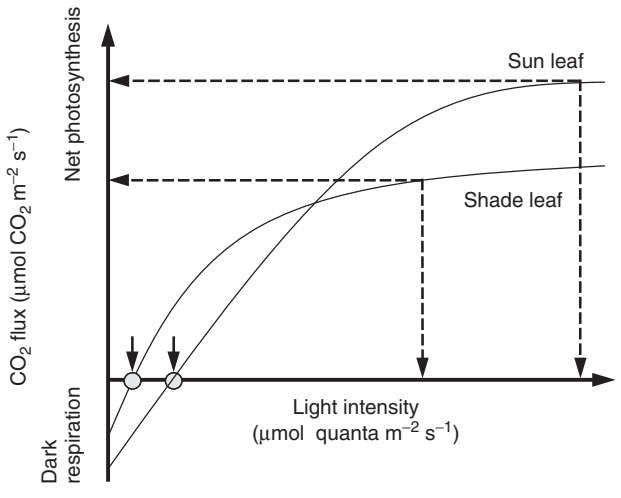

Figure 5.1 Dependency of CO_2 flux on light intensity in sun and shade leaves. → light compensation of net photosynthesis. --> maximum rate of net photosynthesis.

Such shade-adapted leaves are also developed in the inner crown of parent beech trees. Forest gap formation, for example, by storm events, has severe consequences on these light-capturing strategies, depending on as to whether this gap is formed before or subsequent to leaf development of beech. If the gap is formed before leaf development, beech natural regeneration will form light-adapted rather than shade-adapted leaves and has to compete with other woody and herbaceous understory under these conditions. Initially, this competition is strongly in favor of **early successional species** better adapted to high irradiation such as species from the genera *Acer* (maple, *Sapindales, Rosidae*) or *Rubus* (*Rosales, Rosidae*), but when the canopy closes as a consequence of space occupation by the parent trees at the gap border, beech natural regeneration takes over again. Usually, these processes require only a few years for gap closure. If the gap is formed after leaf development, natural regeneration with shade-adapted foliage will severely suffer from **photoinhibition** at high light intensities and may survive only partially depending on weather conditions. Significant development of natural regeneration will take place only in the subsequent years, when light-adapted leaves can be produced during the spring flush. This negative development can be prevented partially by a second flush of sun-adapted leaves in late summer.

A different light-capturing strategy of understory vegetation is the use of intermittent irradiance called **sunflecks**. The duration of leaf exposure in understory vegetation to sunflecks varies strongly among forest types and can range from less than 2 min in tropical forest to several hours in open coniferous forests. While plants with shade-adapted leaves respond less to sunflecks, other understory vegetation can use sunflecks as an important energy source for growth and development responsible for up to 60% of its carbon gain. The response time of photosynthesis to sunflecks varies strongly between species. Utilization of sunflecks seems to depend on a balance between substrates and intermediates of CO_2 fixation and chloroplast components such as electron transport carriers. This balance is responsible for a fast response to increased illumination followed by rapid postillumination consumption of accumulated substrates. The response time to achieve this balance together with the forest type specific duration of sunflecks will determine the competitive advantage for a given species from the utilization of sunflecks. Whether the response time of stomatal movement, a determining factor of CO_2 influx into the leaves and, hence, of substrate

availability for photosynthesis, also plays a role in the use of sunflecks is a matter of debate and seems to differ among species.

5.1.2 Competition for Nutrients

Competitive interactions in the **rhizosphere** seem to be determined by root exudation and sensing the activity of competitors. The players involved in these interactions include different annual and perennial plant species, mature plants and their regeneration, and microbial communities (see Chapter 15). It has been assumed for many years that compared to soil microorganisms, plants are poor competitors for nutrients in the soil, but more recent studies indicate that even under limiting conditions plants can successfully compete for nutrients such as nitrogen. From these studies a paradigm shift from plants being poor competitors to plants effectively competing with microorganisms for nutrients in the soil has been developed. **Root exudation** of carbohydrate is the most important carbon and energy source of rhizospheric microorganisms; the release of carbohydrate by the roots can amount to more than 20% of photosynthetic CO_2 fixation by the leaves. Apparently, carbohydrate exudation largely controls soil microbial biomass. Under unfavorable conditions, for example, on drought, when photosynthesis and, hence, carbohydrate allocation to the roots decline, bacterial biomass in the soil also declines, thereby releasing reduced N compounds from decaying microorganisms that can effectively be acquired by plant roots because of improved uptake capacities. This prevents drought-mediated disturbances in N nutrition in the short run and, at the same time, maintains microbial activity in the rhizosphere at a level fine-tuned to plant productivity under changing environmental conditions. On the other hand, bacterial populations in the rhizosphere are selectively grazed by amoeba, thereby maintaining a level of morphogenetic signaling compounds of microbial origin that support root growth.

More specific interactions with particular populations of microorganisms have recently been concluded from experiments under controlled conditions. Both **nitrifier** and **denitrifier** populations produce and release large amounts of NO during ammonium and nitrate metabolism for energy generation. These activities can result in NO concentrations of up to 250 ppb in the gas phase of the soil. Apparently, NO is sensed by plant roots and enhances the capacity for the uptake of N compounds, thereby counteracting the microbial use of these resources (Figure 5.2); in addition, NO can permeate root membranes and can be used as N source of plants after its oxidation via NO_2 to nitrate.

In addition, belowground competition for resources is characterized by the release of allelopathic compounds that inhibit growth and development of microbial and plant competitors (see Section 15.3.3).

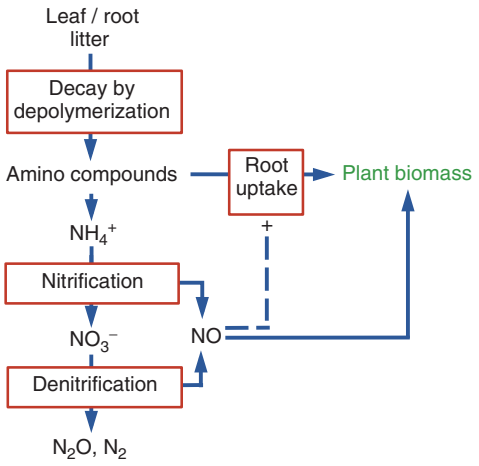

Figure 5.2 Significance of NO in plant–microbial interactions.

5.2 Joint Exploitation of Limiting Resources by Symbioses

5.2.1 Mycorrhiza

The term **mycorrhiza** originates from greek "mykes" (fungus) and "rhiza" (root) and describes a nutritional symbiosis between fungi and the root tips of plants. This symbiosis is of high significance for plant nutrition on a global scale, as about 80% of all angiosperms and gymnosperms are mycorrhizal, although it constitutes a major loss of carbohydrate produced in photosynthesis for the plant partner. In addition to improved nutrient acquisition, it can have the advantage of improved water uptake, reduced injury from chemical soil constituents such as heavy metals, and shelter from parasites. The most common mycorrhizal type is the **arbuscular mycorrhiza (AM**; Figure 5.3), a symbiosis thought to be important for land colonization by plants and, thus, probably extremely ancient. The name originates from the characteristic arbuscules formed within the cortical cells of the roots as typical structures of most AM symbioses. **Ectomycorrhizas (ECMs**; Figure 5.3) almost exclusively develop on the root tips of woody perennial plants and, therefore, are thought to have been established at a later stage of evolution. The roots of some woody plant species, for example, *Populus* (poplar, *Malpighiales, Rosidae*) or *Alnus* (alder, *Fagales, Rosidae*), can host both AM and ECM fungi, plus bacteria for symbiotic N_2 fixation (see below). ECMs are characterized by hyphal growth between the cortical cells of the roots rather than hyphal growth within these cells typical for AM symbioses. A third type of mycorrhizal symbiosis, the **ericoide mycorrhiza (ERM)**, is restricted to the *Ericaceae* (*Ericales*, *Asteridae*) and its close relatives and is characterized by exclusive colonization of the root epidermis by ERM fungi.

Some plant families contain both mycorrhizal and nonmycorrhizal species, but even in groups characterized as nonmycorrhizal (e.g., *Brassicaceae, Caryophyllaceae,*

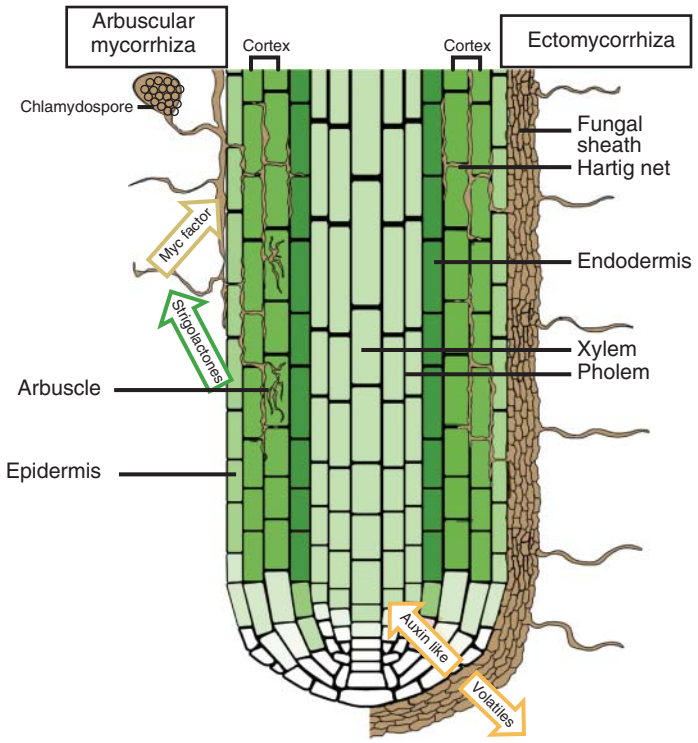

Figure 5.3 Arbuscular mycorrhiza and ectomycorrhiza. (Courtesy of R. Hampp.)

Proteaceae, and *Cyperaceae*) some mycorrhizal species do occur. The polyphyletic and, hence, independent origin of nonmycorrhizal conditions has led to the conclusion that this lifestyle is secondary, that is, caused by a loss of mycorrhizal symbiosis by environmental conditions. Such conditions include the adaptation to aquatic habitats and growth in nutrient-rich environments. In addition, the development of compensatory mechanism of effective nutrient uptake in evolution (e.g., fine roots with well-developed root hairs, proteoid and dauciform roots, root exudates increasing P solubility in soils) may have caused loss of mycorrhizal symbiosis, in particular, when these mechanisms require less input of photosynthate compared to the support of mycorrhizal fungi.

5.2.1.1 Arbuscular Mycorrhizas (AMs)

AMs are formed between autotrophic host plants and obligatory symbiotic fungi that are classified by a separate fungal subphylum, the *Glomeromycota* (*Fungi*, *Opisthokonta*). Colonization of the roots by AM fungi can take place from spores, hyphae, and infected root fragments. It is initiated by the exchange of signals as precolonization events (see Chapter 15.2.1).

Hyphae growth in the cell walls of the root cortex is facilitated by the release of fungal hydrolases such as pectinases, cellulases, and xyloglucanases. Thus, the fungal symbiont structure of AM consists of hyphae that mediate improved nutrient acquisition from the soil, hyphae that mediate allocation of these nutrients to the intraradical structures, and intraradical structures that mediate the exchange of nutrients and carbohydrates at the fungal symbiont/plant root interface (Figure 5.4).

AM plants can take up nutrients from the soil either directly by the roots or via the AM fungal symbiont, but the AM uptake pathway seems to be the major route of **nutrient absorption**. The fungal mycelium in the soil can take up nutrients beyond the area depleted by direct root uptake and can penetrate soil pores one order of magnitude smaller than those accessible by root hairs. Thereby the fungal mycelium improves the effectiveness of soil exploitation and, as a consequence, plant growth.

The largest effect of AM formation is on the **acquisition of P** that is required by both symbionts in significant amounts. P is available in the soil as inorganic P_i and in organic (P_o) forms, but is often poorly available to plant roots and a growth limiting nutrient. P_i is weakly mobile in the soil, present in the soil solution in low concentrations, immobilized in microbial biomass, and fixed as aluminum, iron, and calcium phosphate in the soil. It is absorbed by roots as orthophosphate ($H_2PO_4^-$) that is most readily available at pH 6.5. The availability of P_i can be enhanced by the release of chelating compounds such as organic acids (e.g., citrate, oxalate). AM colonization can result in downregulation of epidermal and root hair P_i transporters; as a consequence, the AM fungal contribution to P nutrition can be as high as 100%. Forms of P_o in the soil include inositol phosphates (phytate), phospholipids, and nucleic acids. Phytate can be converted by AM either directly by hydrolysis to P_i or by stimulating plant root phosphatase production and exudation; but the significance of phytate

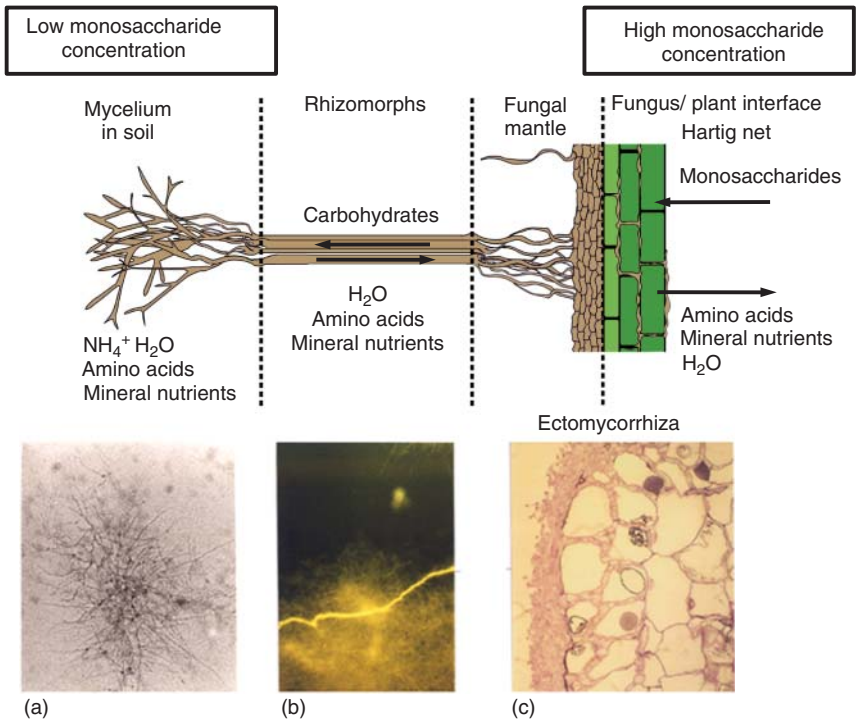

Figure 5.4 (a–c) Exchange of nutrients and carbohydrates between fungal symbiont and plant root. Light-microscopy of mycorrhizal structures are shown schematically in the upper part of the Figure. (a) Soil hyphae, responsible for nutrient uptake; (b) Rhizomorphs (highly organized hyphae for long-distance transport of nutrients towards the host, and carbohydrates for fungal growth, originating from the host; (c) fungus/plant interface. Fungal hyphae (small, round) from the root surrounding fungal mantle penetrate into the outer cortex cell walls of the plant root surrounding fungal mantle penetrate into the outer cortex cell walls of the plant root where they form a large surface area (Hartig net) for nutrient and carbohydrate exchange. (Courtesy of R. Hampp.)

hydrolysis for P nutrition is doubtful, as phytate constitutes only around 5% of the total soil P_o pool. Apparently, different from ECM and ERM, AM do not make a very significant use of soil P_o in P nutrition.

Orthophosphate absorbed from the soil solution into the fungal cytosol is used in first preference for fungal metabolism, in second for transfer into the fungal vacuoles, an acidic compartment where both orthophosphate and polyphosphate (polyP) can accumulate. PolyP constitutes a polymer P storage molecule of a variable number of orthophosphate residues, linked by energy-rich phosphoanhydride bonds. It is thought to buffer the concentration of cytoplasmic orthophosphate. **Motile vacuoles** containing polyP seem to mediate long-distance translocation of P along AM hyphae. Once intraradical structures have been reached, hydrolysis of polyP for P utilization and/or transfer to the plants probably require the combined action of specific and nonspecific fungal phosphatases. The exchange of P between the fungus and the plant preferentially takes place by active transport processes in the root cells colonized by arbuscules. These are characterized by a large surface area that can be used for membrane transport. The transfer for P involves both efflux and influx operating in series (Figure 5.5). Also, as **P influx carriers** are expressed in the fungal plasma membrane, it is assumed that the fungus controls the apoplastic P level by a coordinated

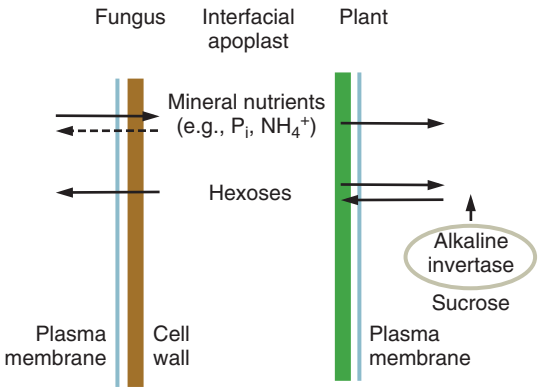

Figure 5.5 Schematic view of exchange processes at the intraradical fungal–plant interface in arbuscular mycorrhizas. (Modified from Smith and Read (2008) with permission from Elsevier.)

efflux/influx system that allows P release into the apoplast and P reabsorption. Transport of P into the plant includes transporters that are expressed only in AM roots, or are AM-inducible. In both fungal and plant plasma membranes, expression of P transporters is coupled to the expression of H^+-ATPases indicating active transport processes.

Extraradical hyphae of AM fungi can absorb inorganic N (N_i) in the form of both nitrate and ammonium. Compared to ECM and ERM, the **N acquisition** in organic forms by AM from the soil seems to be small. Although N_i is taken up

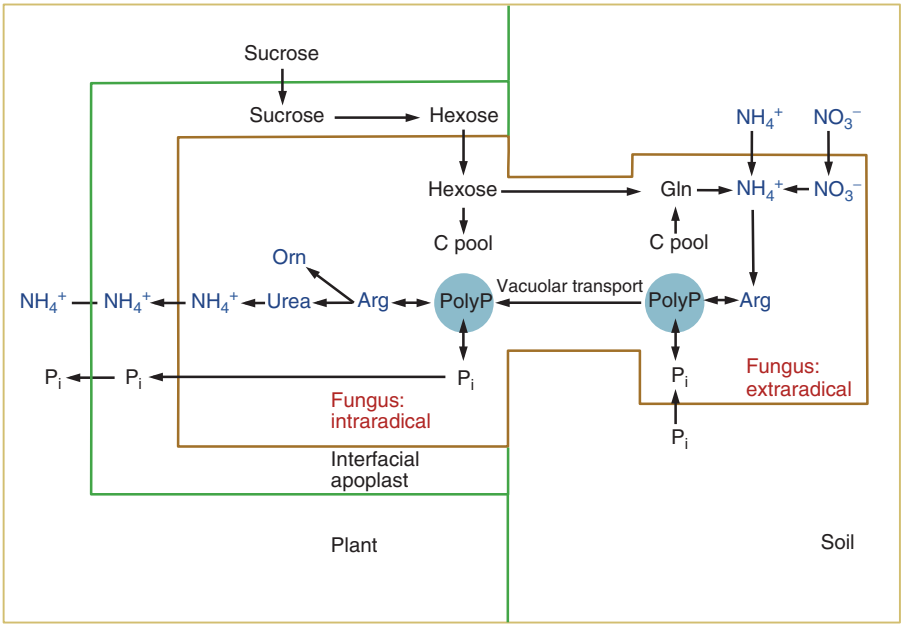

Figure 5.6 Model of nitrogen uptake, metabolism, and transfer in arbuscular mycorrhizas. (Modified from Smith and Read (2008) with permission from Elsevier.)

by AM in considerable amounts and is shown to be transferred to the plant partner, the contribution of N_i uptake by extraradical hyphae of AM fungi to plant nutrition often does not seem to be significant, but is found to be considerable in other approaches. Apparently N uptake by the roots can remain high irrespective of AM symbiosis. N_i acquired by AM can be largely used for N nutrition of the fungus.

Ammonium taken up by extraradical fungal hyphae is assimilated via the glutamine synthetase (GS)/glutamate synthase (GOGAT) system (see Section S1.3.8.2) using plant carbohydrates as backbone (Figure 5.6). The same is true for nitrate after assimilatory nitrate reduction. The Gln and Glu produced are used not only for fungal protein synthesis but also for the synthesis of the N-rich amino acid Arg, the dominant amino acid in the extraradical mycelium. Arg is transferred to the **motile vacuoles** also containing polyP and delivered from extraradical hyphae to intraradical structures via these vacuoles. The Arg delivered to the intraradical mycelium is broken down via urea to ammonium that is partially transferred to the plant partner, whereas other breakdown products seem to be recycled in the fungus (Figure 5.6).

For completing their life cycle, AM fungi are fully dependent on **organic C delivery** by the plant partner. In the presymbiotic phase, spores and germlings of AM fungi utilize stored lipids and trehalose in the absence of root exudates to support growth. Breakdown of these storage compounds is associated with considerable dark CO_2 fixation. As symbiosis develops, a high capacity to absorb hexoses is established in intraradical structures with a preference for glucose over fructose. Hexoses delivered to the fungus are thought to originate from sucrose. Sucrose is the most abundant photosynthate delivered from the leaves to the roots by phloem transport. The disaccharide cannot be taken up by AM fungal structures directly but has to be hydrolyzed first into the hexoses glucose and fructose by invertase. Plant cytoplasmic alkaline invertase activity is thought to exert plant control over hexose transfer to the fungal partner together with plant hexose influx transporters by regulating the hexose level in the apoplast (Figure 5.5). Hexoses taken up by the fungus are incorporated into trehalose and glycogen or used to synthesize lipids. This prevents hexose accumulation in the cytoplasm and, hence, **feedback inhibition** of hexose uptake by maintaining an appropriate concentration gradient of hexose between the plant and the fungus. Predominantly lipids, and also glycogen, are exported to the extraradical mycelium of the fungus to support its growth and development subsequent to its breakdown and conversion to hexose and trehalose. Lipids move to the extraradical mycelium in the cytoplasmic stream as **lipid bodies** that consist of a triacylglycerol core surrounded by a monolayer of phospholipids and protein. By these processes AM roots of a range of plants have been calculated to receive about 4–20% more photosynthate compared to nonmycorrhizal roots. The extraradical mycelium of AM fungi has little or no capacity to use organic carbon sources in the soil.

The existence of a **common AM mycelia network** (CMN) linking plants of the same or of different species is an important structural feature that allows carbon allocation within the plant community. Seedlings growing up in a community of AM plants will be rapidly linked into the CMN that can support the seedlings with inorganic nutrients and with photosynthate originating from already established plants, thereby improving seedling establishment. This nursing process may be important, for

example, for small seeded species with low seed storage capacity and when shade limits the photosynthetic capacity of the seedling. The CMN will be a "supporter" under these conditions, and the seedling will constitute a "user" that may become a "supporter" during its subsequent development. If the receiver plant is nonphotosynthetic as some achlorophyllous members of the *Polygalaceae* (*Fabales*, *Rosidae*), *Gentianaceae* (*Gentianales*, *Asteridae*), and *Burmanniaceae* (*Discoreales*, *Liliopsida*), the latter will not take place and these plants will obtain all their organic carbon via the fungus.

AM colonization also affects the water relations of plants (see Chapter 10), either directly or indirectly by changes in plant nutrition and size. Depending on plant and fungal species, the effects of AM on water relations of plants can include reduced root resistances to water transport, increased transpirational flux of water, and enhanced stomatal conductance mediated, for example, by an increase in size and branching of the root system and enhanced water uptake by and transport in the fungal hyphae. In addition, AM colonization may affect the drought tolerance of plants by mechanisms not directly connected to plant water relations, such as enhancing soil exploitation and improving soil structure.

5.2.1.2 Ectomycorrhizas (ECMs)

ECMs constitute a symbiosis between saprotrophic fungi and autotrophic plants. Thus, ECM fungi are not obligatory but optional symbiotic. ECM roots contain three structural components of fungal origin (Figure 5.3), that is, (i) an extended system of hyphae growing in the apoplast between the epidermal and the endodermal cells, the so-called Hartig net, (ii) a mantle of hyphae enclosing the root tip, and (iii) an extraradical mycelium. ECMs are clearly distinguishable from other types of mycorrhiza from the absence of intracellular penetration by the fungal hyphae. **Phylogenetic studies** indicate that ECM developed in evolution independently on several occasions over a long time span. Almost all plants containing ECM are woody perennials including the main producers of timber. ECM can be formed by a large number of different fungi predominantly in the *Basidiomycota* (*Dikarya*, *Fungi)* and is estimated in the range of 6000 species. Most prominent genera include *Amanita, Paxillus, Pisolithus, Lactaria,* and *Laccaria.* Still some of the most common ECM fungi are *Ascomycota* (*Dikarya*, *Fungi)* with *Coenococcum geophilum* as the most prominent species with a global distribution and a large range of autotrophic hosts, including European beech (*Fagus sylvatica*), in particular, on calcareous soil substrate. As tens of often distantly related fungal species can be found on the root system of one individual woody plant, the plant host is considered to possess a rather **low specificity** for the fungus in ECM symbioses. This low plant specificity may be an advantage because it opens the possibility to make use of different physiological features of different ECM fungi in nutrient acquisition and maintenance costs for the plant in a permanently changing environment. Also, many fungi of ECM symbiosis lack specificity for the plant host, but some species show clear specialization. For example, while *Alnus* spp. does not show high specificity for a particular ECM fungus, a set of ECM fungi are specialized for this tree genus.

ECMs develop on lateral roots of long roots characterized by unlimited growth. If the long root is already colonized by an ECM fungus, new lateral roots can be infected by hyphae of the existing Hartig net or the fungal mantle with the consequence that the new ECM is built by the same fungus as the parent ECM. When a portion of a long root is not colonized or a new root develops, for example, on seed germination, colonization of the lateral root can arise from different fungi originating from soil propagules. In this case, recognition, compatibility, and inoculum potential will determine the **colonization process** (see Section 15.2). When fungal hyphae get into contact with lateral roots, a series of molecular events is initiated that is under the control of genes in both partners and initially result in fungal hyphae attachment to the root epidermis. The symbiosis related (SR) proteins encoded by these genes that are unique for ECM, that is, not found in free-living mycelia or uncolonized roots, are termed **ectomycorrhizins** with different genes being responsive at early, mid, and late stages of ECM formation. In early stages of ECM formation, genes of cell wall related SR-proteins become downregulated in the fungus. Subsequently, specific fungal genes of primary metabolism and hormone synthesis become expressed, suggesting that carbohydrate exchange is initiated at the early stages of ECM development. These events coincide with the beginning of the formation of the fungal mantle and the Hartig net (Figure 5.3). Simultaneously, transcripts encoding plant defense-related proteins increase indicating some resistance to pathogenic interactions during this phase of ECM development. In the mid and later stages of ECM development, genes encoding enzymes of amino acid and protein synthesis as well as mycelium-specific genes of cell wall synthesis become transcriptionally responsive.

Fungal hyphae first get into contact with the growing root at a position directly proximal to the root cap. In addition, root hairs proliferate behind the apices of uncolonized roots and provide a large surface area for the interaction with hyphae of ECM fungi. On colonization, growth of root hairs is reoriented toward the root surface so that they can be enveloped by the fungal mantle sheath. **Recognition** of ECM fungi is thought to be achieved via fibrillar material of fungal origin that consists of glycoproteins and is found on the surface of free-living mycelia. In EMC these fibrillar polymers extend from the fungal cell wall to that of the plant root. The final morphology of an ECM is dependent on the stage of lateral root development at the time colonization takes place. If colonization is initiated after a lateral root has elongated, a typical swollen tip structure develops; colonization at an earlier stage of lateral root growth results in structures of uniform thickness.

The biomass of the overlying mantle of ECM is relatively high and can contribute 20–40% to the weight of the colonized root. The mantle provides the structures for **nutrient storage** and plays a key role in the control of the nutrient transfer from the fungus to the root. Compared to the mantle, the biomass of the Hartig net that forms the predominant interface between the fungus and the plant root is relatively small. In the zone of contact between the symbionts, the Hartig net is produced by hyphae penetrating from the inner mantle between the epidermis and the endodermis of the root axis, thereby generating a labyrinth-like structure (Figure 5.3). Typically, the hyphae of the Hartig net are densely cytoplasmic, do not contain vacuoles, but a large number of mitochondria. Lysis of the middle lamella of epidermal cells in advance of hyphal tips indicates the involvement of fungal enzymes in the penetration process. A significant increase of the surface of the colonized root with the soil is achieved by the extraradical mycelium that consists of single hyphae or aggregates of hyphae extending from the mantle, the **rhizomorphs**. The extent of the extraradical mycelium has been estimated between 300 and 8000 m per m of colonized root with an absorbing surface of 70–110 m^2 per m^2 forest surface area. Formation of rhizomorphs allows long-distance soil exploitation and transport of nutrients to the plant host (Figure 5.4).

There are numerous ecosystems in nature where P deficiency is the primary cause of productivity limitation of plants and where ECM colonization is of vital importance for **P nutrition**. ECMs show a distinct seasonality in P absorption with maximum rates in late summer or early autumn; the latter may be particularly important to prevent ecosystem P loss from fallen litter. Much higher rates of P absorption are achieved with intact compared to detached ECM at sufficient C supply indicating that the extraradical mycelium strongly contributes to P acquisition. Both P uptake velocity and substrate affinity are enhanced by ECM colonization, when orthophosphate is used as a substrate. In the fungal cytoplasm, orthophosphate taken up by ECM is rapidly incorporated into nucleotides and sugar phosphates and is also metabolized into polyP that is stored in the vacuoles of the fungal cells and can be degraded for P mobilization at low P availability. This way a relatively low cytoplasmic P_i concentration is maintained. As these vacuoles are acidic, polyP carries a strong negative charge that is balanced by positively charged cations such as K^+ and Mg^{2+} and the alkaline amino acid arginine. Orthophosphate also constitutes the form of P that passes from the fungal sheath to the plant tissues. The electrochemical potential gradient between the Hartig net (Figure 5.3) and the interfacial apoplast favors a passive efflux of orthophosphate from the fungus, but P loss from fungal hyphae is generally low. Up to 90% of the P taken up by ECM is kept in the fungal sheath and the Hartig net, and only small amounts of P are transferred to the plant. Therefore, mechanisms that control retrieval or reabsorption of P (e.g., by active influx transporters) are likely to operate in the fungal plasma membrane at the interfacial apoplast. Uptake of orthophosphate by the plant symbiont from the interfacial apoplast accumulates P against a strong electrochemical potential gradient and, therefore, also must be an active transport (see Section S1.2.7 and Section 1.2).

A large part of P in the upper horizons of forest soils, the central sites of colonization by ECM, is present in **organic P** forms such as phosphodiesters (e.g., nucleic acids and phospholipids), and the phosphomonoester inositol hexaphosphate (i.e., phytate). Some of these organic P forms may have a short half-life in soils because they are subject to degradation, for example, by soil endogeneous phosphatases and phosphomono- and phosphodiesterases. These enzymes have been identified on the surface of ECM fungal hyphae and seem to be of fungal origin. As exoenzymes they are thought to mediate immediate **recycling of organic phosphates** present in fallen litter. However, ECM fungi also have the ability to bring rock phosphate into solution. This requires acidification of the local environment that is achieved by the release of oxalate or assimilation of the ammonia (NH_3) portion of ammonium (NH_4^+), which liberates a proton (H^+). The P nutritional system of ECM plants results from mobilization of soil P, fungal P uptake, fungal P storage and mobilization, and P transfer from fungal hyphae to plant root tissue. This system seems to be regulated in a way that enables continuous P supply of the plant shoot largely independent of actual plant P requirement and actual P availability in the soil. Thus, it generates a **P nutritional steady state** in the plant that can be considered ideal for plant growth and development.

The **availability of N** is the most important factor determining productivity and development of the extensive boreal and temperate forests of the northern hemisphere dominated by ECM trees. From the beginning of its discovery ECM symbiosis has been considered as a means to improve **N nutrition** of trees growing under N limitation. In pure culture and in ECM symbioses, most ECM fungi are able to use ammonium, a few nitrate, or both inorganic N sources, but most of them grow fastest on ammonium. This is not surprising, as ammonium is the most abundant source of mineral N in most temperate forest soils. However, ammonium uptake is strongly regulated by the presence of organic N sources that may originate from free pools of these compounds in living plant or microbial cells and from the degradation of peptides and proteins by microbial depolymerization activity. Amino acids such as glutamate, glutamine, and alanine are rapidly absorbed by ECM as intact molecules and are incorporated into fungal N metabolism by various pathways. Amino acid uptake shows a distinct pH optimum (e.g., around pH 4.0 for glutamate and glutamine) and a broad substrate recognition consistent with general amino acid permeases of low substrate specificity.

Glutamine constitutes the major sink of N absorbed by ECM fungi, but other amino acids such as arginine also become rapidly labeled when ECM are supplied with

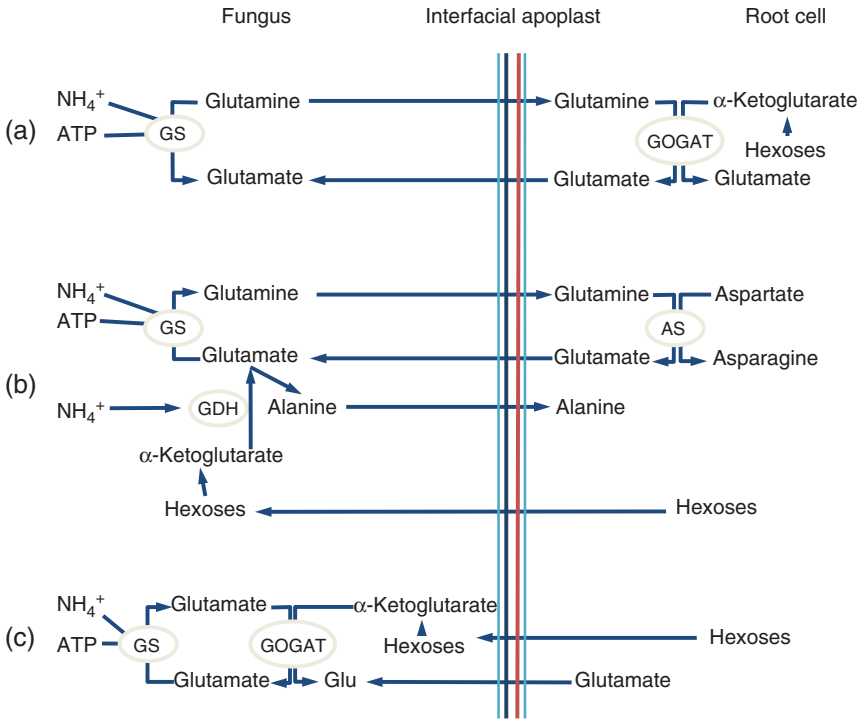

Figure 5.7 Pathways of ammonium assimilation in ectomycorrhizas. (a) *Fagus* type; (b) *Picea* type; (c) *Pisolithus* type. GS – glutamine synthetase; GOGAT – glutamate synthase; GDH – NADP-glutamate dehydrogenase; AS – asparagine synthetase. (Modified from Smith and Read (2008) with permission from Elsevier.)

^{15}N-labeled substrate. The contribution of arginine to the pool of free amino compounds in the fungal hyphae varies strongly, apparently depending on its accumulation in the vacuoles, where it stabilizes polyP (see above). Depending on both ECM fungal and plant species, a diversity of pathways has been identified for the assimilation of ammonium in ECM fungi and the transfer of assimilated N to the plant root (Figure 5.7). GS is involved in all of these pathways along with GOGAT or NADP-glutamate dehydrogenase (GDH) and asparagine synthetase (AS). In this context, glutamate synthesis by fungal GDH in the *Picea*-type pathway (Figure 5.7) is particularly interesting, because in plants this enzyme is involved predominantly in glutamate catabolism upon protein degradation rather than in the synthesis of this amino acid. In all pathways, glutamine serves as the major transport form of assimilated N exchanged between the fungal hyphae and the plant root. An alternative process is the direct exchange of N taken up as ammonium between fungal hyphae and cells of the root cortex. The significance of this process is supported by the stimulation of a high-affinity ammonium transporter in plant roots on ECM formation and the downregulation of fungal GS and other fungal enzymes of N assimilation on ECM colonization in fungal hyphae of the Hartig net that are in close contact with root cells. For this exchange, ammonia is thought to diffuse via either aquaporins or voltage-dependent anion channels through the fungal plasma membrane into the interfacial apoplast, where it is protonated to ammonium, thereby preventing its backflux by diffusion. Downregulation of the expression of ammonium importer genes also seems to contribute to an inhibition of ammonium backflux.

To support ECM fungal growth and development, carbohydrate produced in plant photosynthesis is transported into the fungus at the plant root–fungal hyphae interface via the interfacial apoplast (Figure 5.8). Reminiscent of AM, sucrose from the plant apparently cannot be taken up by the ECM fungal partner and has to be hydrolyzed first to

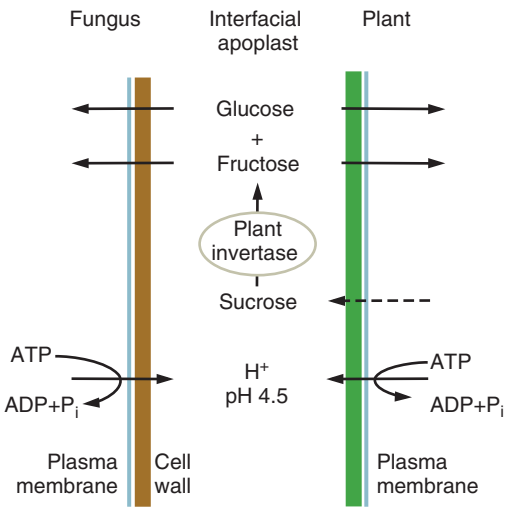

Figure 5.8 Sugar transfer in ectomycorrhizas at the plant–fungus interface. (Modified from Smith and Read (2008), with permission from Elsevier.)

Box 5.1: Chemical and biological N_2 fixation

Chemical N_2 fixation is most commonly achieved by the Haber–Bosch process that catalytically reduces N_2 with H_2 to NH_3 at high pressure and high temperature. With this industrial process, approximately 85 million metric tons of N are fixed annually worldwide and are used as fertilizer either directly as anhydrous ammonia or after conversion to ammonium salt, urea, or nitrate. Chemical N_2 fixation is an energy-demanding process that consumes large quantities of fossil fuel, that is, the energy content of 1 l oil for the production of 1 kg N fertilizer. In contrast, **biological N_2 fixation** that is estimated to produce around 150 million metric tons of NH_3 worldwide every year is ecologically innocuous.

glucose and fructose for transport into the hyphae. In ECM **hydrolysis of sucrose** is achieved by **acid invertase** activity of plant origin that often has been considered to exert plant control over the movement of hexoses into the fungus. However, acid invertase production by the plant was found to be unaffected by ECM fungal colonization. Apparently, **hexose movement** is controlled by a complex interaction of plant and fungal transporters including ATPases of both partners that control the acidic environment in the interfacial apoplast required for acid invertase activity. Subsequent to sucrose efflux from cortical root cells into the interfacial apoplast and its hydrolysis by acid invertase, glucose is preferentially taken up by fungal hexose transporters. Fructose liberated from sucrose by acid invertase activity exerts feedback inhibition on this enzyme. As a consequence of fungal glucose uptake, the apoplastic glucose level will decline and fructose will be transported by fungal hexose transporters, thereby releasing feedback inhibition of acid invertase by fructose and mediating additional hydrolysis of sucrose. Both sucrose and the hexose products of acid invertase activity can be reabsorbed by plant transporters. It is currently not understood in which way this reabsorption is regulated in order to control the net flux of carbohydrate from the root to the fungus. Such a net flux can be achieved only if a net sucrose efflux from root cortical cells and a net hexose influx into fungal hyphae are maintained. This requires a concentration gradient between the interfacial apoplast and the interior of fungal hyphae, as fungal hexose influx is mediated by passive transporters. Apparently, trehalose, glycogen, and mannitol (see Chapter 10) constitute fungal sinks into which hexoses are rapidly accumulated subsequent to its influx into the hyphae, thereby maintaining a concentration gradient of hexoses. Organic carbon can also move from the fungus into the plant, in particular, when N is exchanged between the fungus and the plant by glutamine transport (see above). In this case, the organic carbon can originate from dark CO_2 fixation by the fungus, but this movement can also provide a route for the **cycling of organic carbon** between the symbionts. Still a net flux of carbon from the plant to the fungus is achieved under these conditions, which can amount to more than 20% of photosynthetic carbon fixation by the host. This net flux does not constitute a drain on plant productivity, because photosynthetic carbon fixation is stimulated by the enhanced carbon sink strength of the roots as a consequence of ECM formation (facilitation).

5.2.2 Symbiotic N_2 Fixation

Although the largest reservoir of nitrogen (N) on the planet is present in the form of dinitrogen gas (N_2) that represents around 78% of the atmosphere, this relatively inert form of N is not directly available to plants. As plant growth is directly dependent on adequate N supply and, beside water, N is one of the most common limiting factors for plant growth and development, the invention of the **nitrogenase enzyme** is considered one of the most fundamental biochemical innovations in the evolution of life. This enzyme is used by a number of bacterial and archaeal species to convert biounavailable N_2 to bioavailable NH_3 that can then be incorporated into organic nitrogen components, such as amino acids, proteins, and nucleic acids. In this way, inert N_2 enters the biologically active part of the global biogeochemical N cycle. Thus, next to photosynthesis, N_2 fixation is probably the most important biological process on the globe.

Biological N_2 fixation by bacteria is widely distributed in the soil. Most N_2 fixers are free living and few species form symbiotic associations. The latter include rhizobia (Genus *Rhizobium* and others, *Rhizobiales, Alphaproteobacteria)* that form symbiotic associations with the roots of leguminous plants and actinomycetes (*Actinobacteria*), gram-positive, filamentous bacteria that form similar symbiotic associations with a number of plant species of at least nine plant families. Irrespective of these different life forms, N_2 fixation may proceed without the development of specific structures or may depend on the development of such structures (i.e., **heterocysts** of free-living cyanobacteria, **root nodules** of rhizobia in symbiosis with leguminous plants; *Fabales, Rosidae*). These structures can be seen as a means to efficiently generate the conditions essentially required for biological N_2 fixation because of the high sensitivity of nitrogenase to inactivation by oxygen. This view seems to be supported by the relatively low effectiveness of biological N_2 fixation by free-living bacteria in soils ($1-5\,kg\,N\,ha^{-1}\,a^{-1}$) compared to symbiotic N_2

fixation (100–200 kg N ha^{-1} a^{-1}). In nodules, gas permeability can be regulated to maintain a level of oxygen that is sufficiently high to support respiration, but sufficiently low to prevent **nitrogenase inactivation**. This is achieved by the **leg-hemoglobin**, an oxygen-binding protein present in the cytoplasm of the nodules. This protein is produced partially by the host (globin portion) and partially by the bacteria (heme portion); it exhibits an oxygen affinity 10 times higher than human hemoglobin with a K_m of around 0.01 µM and functions as a support of oxygen transport to the bacterial cells inside the nodules where oxygen is used for respiration.

However, free-living *Azotobacter* (*Gammaproteobacteria*) established in the stems of sugar cane (*Saccharum officinarum, Poales, Liliopsida*) where they are supported by the high abundance of sugar for growth and N_2 fixation can also fix as much as 150 kg N ha^{-1} a^{-1} in this crop. Thus, it may rather be the lack of abundant substrate for growth and N_2 fixation that limits N_2 fixation by free-living bacteria in the soil rather than a lack of anaerobic or microaerobic sites. In leguminous plants such substrates are produced by photosynthesis and are transported directly to the nodules.

5.2.2.1 Establishment of Symbiosis and Nodule Formation

Establishment of symbiosis between leguminous plants and rhizobia requires the **exchange of signals** between both partners, because symbiosis is not obligatory (see Section 15.13). Seedlings of leguminous plants can germinate and leguminous plants can develop throughout their entire life cycle without any association with rhizobia; similarly, rhizobia can grow and proliferate as saprophytic free-living bacteria in the soil. Therefore, formation of symbiosis between the two partners requires the migration of rhizobia to the roots of the host plant (Figure 5.9). This migration is initiated by the excretion of chemical attractants by the root hairs, in particular, flavonoids and betaines. These compounds mediate a chemotactic response by activating the bacterial NodD protein. This protein is encoded by *nodD,* one of the nodulation (*nod*) genes present on a specific plasmid (**sym plasmid**). Different from the other *nod* genes, *nodD* is constitutively expressed in the rhizobia. Once activated, the rhizobial NodD protein induces the transcription of the other *nod* genes by binding to the highly conserved *nod* box present in the promotor region of all *nod* genes except *nodD.* These *nod* genes code for nodulation proteins that are involved in the synthesis of Nod factors, that is, signaling molecules released by rhizobia that induce infection with rhizobia and nodule organogenesis (see Chapter 15). While the host-unspecific basic structure of Nod factors is synthesized by proteins encoded by the *nod* genes *nodA, nodB,* and *nodC,* host-specific modifications of the terminal *N*-acetyl-D-glucosamine are catalyzed by proteins encoded by further *nod* genes such as *nodE* and *nodF* (Figure 5.9). As a consequence, the Nod factors released by rhizobia are specific for a particular host. Specificity is achieved by the binding of specific Nod factors to specific

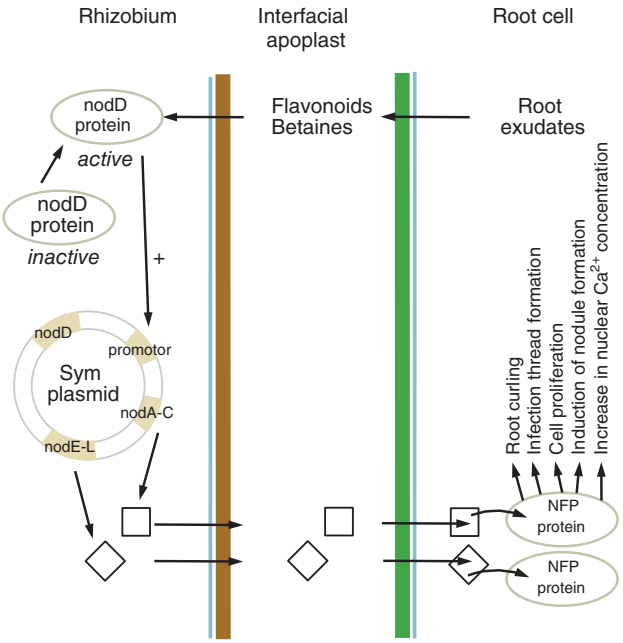

Figure 5.9 Exchange of signals between leguminous plants and rhizobia for the establishment of symbiosis. (Modified from Lüttge, Kluge, and Thiel (2010) with permission from Wiley). Nod – nodulation factor; NFP – Nod factor perception protein.

Nod factor perception (NFP) proteins (LysM (lysine motif) receptor kinases) at epidermal cell plasma membranes of the host that become activated by this binding and induce the transcription of plant *nod* genes (Figure 5.9).

As the initial step of infection, rhizobia become attached to the root hairs of the host. Attachment is achieved by specific **lectins** that recognize and bind to specific oligosaccharides sticking out of the bacterial cell wall. These oligosaccharides are specific for specific rhizobium strains and lectin specificity for these oligosaccharides are – in addition to the synthesis of specific Nod factors – part of the specificity of the host–rhizobium interaction. Rhizobia attached to the root hairs release the Nod factors that bind to NFP proteins that become activated and initiate the infection of tissue and the development of nodules.

As a first consequence, a pronounced curling of root hair tips is induced that finally forms a small compartment in which rhizobia are enclosed and proliferate. Local degradation of the root hair cell wall leads to the infection of the root hair and the formation of an **infection thread** inside the root hair in which the rhizobia are enclosed. The thread grows at its tip into the root cortex into the direction of the central cylinder and simultaneously the rhizobia enclosed proliferate. Deeper in the root cortex, cortical cells become dedifferentiated in response to specific Nod factors and start to proliferate and differentiate into the structures of the root nodules. Immediately after its formation the nodules become colonized by the bacteria via **endocytosis**, that is, the fusion of the infection thread with the plasma membrane of the host cell. Thereby the formation of a branched infection threads allows the release of rhizobia

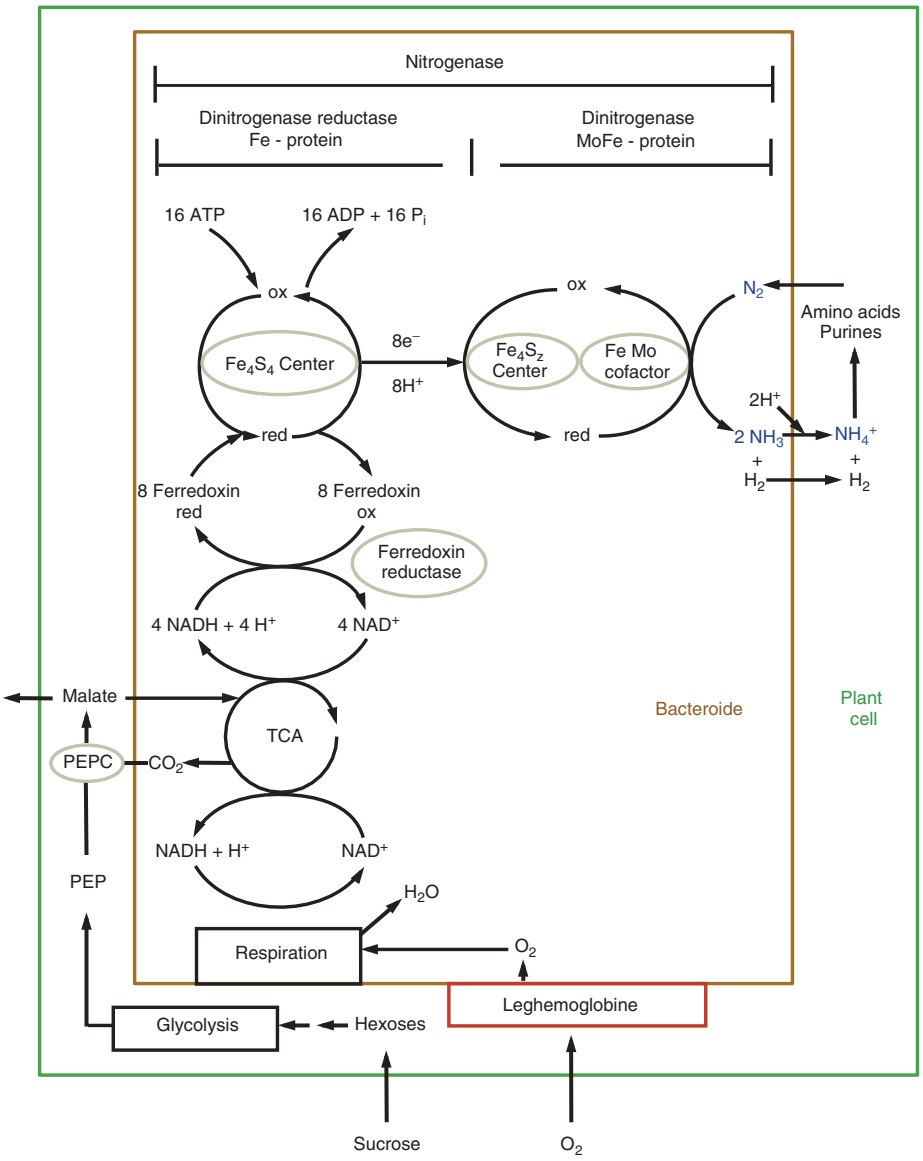

Figure 5.10 Reactions catalyzed by the nitrogenase enzyme complex. PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; TCA – tricarboxylic acid cycle (Graphics: G.-J. Krauss, D. Dobritzsch.)

into a large number of nodular cells. Inside the cells the bacteria initially continue to proliferate strongly, then stop dividing and begin to enlarge and to differentiate into N_2 fixing endosymbiotic organelles, the **bacteroids** that are surrounded by a membrane. During this process the rhizobia convert from rod- to a ball-shaped structure. On senescence of nodules at the end of the vegetation period, the membrane surrounding the bacteroids is degraded, the nodule structures deteriorate, and a large number of bacteria is released into the soil.

5.2.2.2 Mechanism of N_2 Fixation and Plant–Microbial Interactions in N Nutrition

Biological N_2 fixation by free-living bacteria and by bacteria–plant symbioses is mediated by the **nitrogenase enzyme complex** (Figure 5.10). Its formation requires a suite of *nif* genes and genes subsequently regulated by *nif* products, with all *nif* genes located on the sym plasmid of the bacteria (Figure 5.9). In a six-electron transfer reaction, the nitrogenase enzyme complex catalyzes the conversion of N_2 to NH_3. The reaction consumes 16 ATP and 8 electrons provided by reduced ferredoxins, small proteins with iron-sulfur clusters as electron carriers. While six electrons are needed for nitrogen reduction, the remaining two electrons are transferred to protons, leading to the production of molecular hydrogen in addition to ammonia:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Thus, ferredoxin reductase activity and ATP synthesis are required for biological N_2 fixation. The dinitrogenase reductase contains two identical subunits of a molecular mass of 30–72 kDa that each possess an iron-sulfur

cluster composed of 4 Fe and 4 S^{2-} involved in the redox reaction for electron transfer to the FeMo-protein. The nitrogenase has four subunits with a total molecular mass of 180–235 kDa that each possess two Mo-iron-sulfur clusters.

Both proteins of the nitrogenase enzyme complex are irreversibly inactivated by O_2, the Fe-protein with a half-life of 30–45 seconds, the MoFe-protein with a half-life of 10 min. Under natural conditions the MoFe-protein reduces N_2 and H^+, but it can also reduce a number of other substrates. In this context the reduction of acetylene to ethylene is of particular importance, because it is frequently used for the determination of nitrogenase activity. The reduction of N_2 by the nitrogenase enzyme complex requires a large investment of energy that is generated by respiratory catabolism of sucrose derived from photosynthesis and delivered by phloem transport from the leaves to the nodules. Thus, O_2 has to be available for effective respiration, but has to be kept at a low level to prevent inactivation of the nitrogenase enzyme complex. The large amount of CO_2 produced by respiration in the nodules is at least partially refixed by root **phosphoenolpyruvate (PEP) carboxylase** activity, and the organic acids produced by CO_2 refixation are supposed to be retranslocated to the leaves for further metabolism.

The ammonium produced by the nitrogenase enzyme complex is transported into the plant cell by nodulins (ammonium transporter proteins) and is rapidly assimilated into organic nitrogen compounds to avoid toxicity (Figure 5.10). This is achieved by the action of the **GS-GOGAT system** (Figure 5.7) that results in the synthesis of glutamate that may further be converted to glutamine and asparagine. In temperate environment leguminous plants (pea, clover, etc.), these amides are exported from the nodules and delivered to the shoot of the host plant by xylem transport. In leguminous plants of tropical origin, the primary products of amino acid synthesis are converted into xanthin in the infected cells. This purin is transferred to the cells that are not infected and used for the synthesis of **ureides** such as allantoic acid, allantoin, and citrulline (Figure 5.11). These are exported from the nodules and delivered to the shoot of the host plant by xylem transport in tropical species. Thus, the rhizobia–leguminous plant interaction can be defined as mutualistic, because the plant host takes advantage of the biological N_2 fixation of the rhizobia via the export of amides or ureides, and the rhizobia take advantage of host photosynthate for bacterial growth and proliferation. At the ecosystem level, biological N_2 fixation may be inhibited by nitrifyers and denitrifyers in the soil that both produce significant amounts of NO and N_2O. These gaseous N compounds are strong inhibitors of biological N_2 fixation under laboratory conditions, but their ecological significance in this context remains to be elucidated.

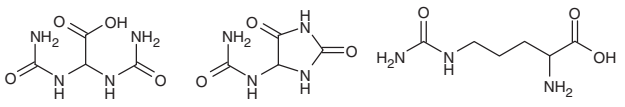

Figure 5.11 Ureide compounds used to transport nitrogen from the site of biological N_2 fixation, that is, the nodules, to the host shoot of tropical leguminous plants.

5.3 Avoidance of Competition

Defeating a competitor requires a significant resource input. In a given ecosystem it, therefore, can be more effective for growth and development to establish mechanisms of **competition avoidance**. The efficient use of low intensity irradiance rather than full sunlight by understory vegetation (see above) can be seen as such a mechanism. For the use of belowground resources, a number of mechanisms have been developed for competition avoidance by separation in space and time. At the ecosystem level, these mechanisms as a whole prevent resource leaching and, therefore, are essential for **ecosystem functioning**. Preventing leaching is of vital importance in ecosystem functioning for limiting resources such as N. In a mature temperate forest not exposed to considerable anthropogenic N input, for example, more than 95% of the annual ecosystem N use relies on recycling. Thus, already small N leaching would be a major threat to ecosystem functioning.

Exploitation of soil resources in space is largely mediated by **root growth characteristics** that are mostly determined by hereditary differences between species and are modulated by the rooting medium and competition. In mixed forests, the concurrent growth of species with shallow (e.g., pine, beech) and deep-rooting system (oak) may be a mechanism of competition avoidance by separation in space. However, deep penetrating roots are not always involved in recourse acquisition but rather in anchoring trees in their soil substrate. In beech forests, highest N uptake capacity by the roots of beech natural regeneration is observed in spring and highest N uptake capacity of mature beech trees in autumn. This can be interpreted both as a nursing effect and as a mechanism of competition avoidance by separation in time. However, this difference in the timing of highest N uptake capacity is based on **physiological requirements.** Beech natural regeneration relies partially on soil N resources for leaf growth and development in early spring because of limited N storage capacity in the small stem. In mature trees, high N storage capacity in the stems and the trunk makes leaf development in spring independent of N acquisition from the soil at this time of the season, when environmental conditions such as temperatures can be unfavorable for N uptake from the soil. Rather, stored N is used for leaf development and the N stores are filled up again during the subsequent seasons. Highest N uptake capacity of mature beech in autumn

(a) (b)

Figure 5.12 Coastal vegetation islands in the Atlantic restinga. (a) Pioneer palm *Allagoptera arenaria* with belowground trunk and leaves on the sand surface; the wind drawing circles on the sand by moving the leaves. (b) Coastal vegetation islands (front) developing into dune forest (back) (Reprinted from Lüttge (2008) with kind permission by Springer Science and Business Media.)

coincides with high N availability from leaf litter and decaying annual understory and, therefore, may prevent N leaching at the ecosystem level.

Another mechanism of **competition avoidance** is the separation by preferential use of different resources of a given nutrient. In mixed temperate forests this includes the preference for reduced (ammonium, amino compounds) versus oxidized N sources. However, the use of reduced N sources, in particular, of amino compounds, will be a competitive advantage at low N availability in forests that largely rely on N recycling for N nutrition. Reduced N will be made available from organic matter decomposition very early in the decomposition chain and, thus, may be available in larger amounts than nitrate, mainly produced by microbial nitrification. On the other hand, significant microbial consumption of nitrate will take place only under anaerobic conditions by denitrification on water saturation of the soil or in water-saturated soil microsites. Thus, nitrate may be available for plant uptake in significant amounts in well aerated soils and constitute an important alternative N source to reduced N compounds (see Section 6.2.4.1).

An extreme form of competition avoidance for soil resources has been established in carnivorous plants that grow on extremely nutrient-poor soils and acquire nutrients by catching prey with leaves transformed into different trap structures (see Section 11.2).

5.4 Facilitation Mechanisms in Communities and Ecosystem Functioning

The term **facilitation** summarizes a set of spatiotemporal processes, in which plants help their neighbors without getting a direct advantage or disadvantage for themselves. However, facilitation is often involved in communities and ecosystem functioning. The nurse-plant syndrome is one of these processes frequently found for tree seedlings. Small tree seedlings often cannot properly germinate in soil by themselves because of the limited amount of resources available. These resources are provided by mature trees via mycrorrhiza bridges (see above). Similarly, young individuals of one species may by sheltered by mature individuals of another species, thereby improving fitness, survival, and growth of the nursed plants. This can include that the nursed plants compete with or even outcompete its nurse plants during ecosystem development. Under these conditions, competition can be delayed or enforced by facilitation.

The development of **vegetation islands** on sandy coastal planes, for example, in the Atlantic restinga of South America, provide an interesting insight into facilitation processes by the nurse-plant syndrome (Figure 5.12). In these extreme environments characterized by high temperature, high irradiation, and low nutrient availability, small palms (*Arecales*, *Liliopsida*) and bromeliads (*Poales*, *Liliopsida*) constitute pioneer nurse plants that grow on the bare sand. Other plant species including species with **CAM** (**Crassulaceae acid metabolism**) photosynthesis (see Figure 1.9) such as *Clusia* spp. (*Malpighiales*, *Rosidae*) germinate and develop in the bromeliad tanks that provide water and nutrients to the seedlings, but during further development can become nurse plants on the establishment of the vegetation islands. As a consequence of this establishment, competition between species for the limited resources water and nutrients develops and may even result in the disappearance of vegetation islands, in particular, at fluctuating climatic conditions. However, vegetation islands may also develop into dense vegetation with grass land, patches of forest, or even closed dry forest ecosystems.

Hydraulic redistribution, also termed **hydraulic lift**, is another facilitation process that generates long-term positive consequences at the ecosystem level (see Section 10.1). By this process water moves particularly in semiarid or arid environments from deep soil layers to the dryer soil surface. This movement is mediated by deep-rooting plants that also posses roots in the shallow soil. During the day, water from the deep-rooting zone is lost by transpiration via the leaves. This is achieved because plant **water potential** is lower than soil water potential and water moves from

the soil into the plant and inside the plant from roots in deep soil layer, where water is most available, to the leaves and further on into the atmosphere. When transpiration ceases during the night, plant and deep soil water potential equilibrate, but a water potential gradient between the deep soil and the shallow soil remains. As roots have a much higher hydraulic conductivity than soils, this gradient drives the vertical movement of water from deep soil layers to dry shallow soils during the night. Around 50% of the water used by shallow rooted herbs is provided by this hydraulic lift. After rainfalls, when surface soils are wetter than deep soils, deep soils can be recharged with water via the roots by vertical water movement in the opposite direction. Thus, roots can provide an avenue for vertical water transport in the soil from high to low water potential, regardless of the direction of this gradient. At the ecosystem level hydraulic redistribution not only improves growth and development of shallow rooted species but also stimulates decomposition and mineralization of organic matter in the shallow soil, thereby enhancing nutrient availability for both deep rooted and shallow rooted species.

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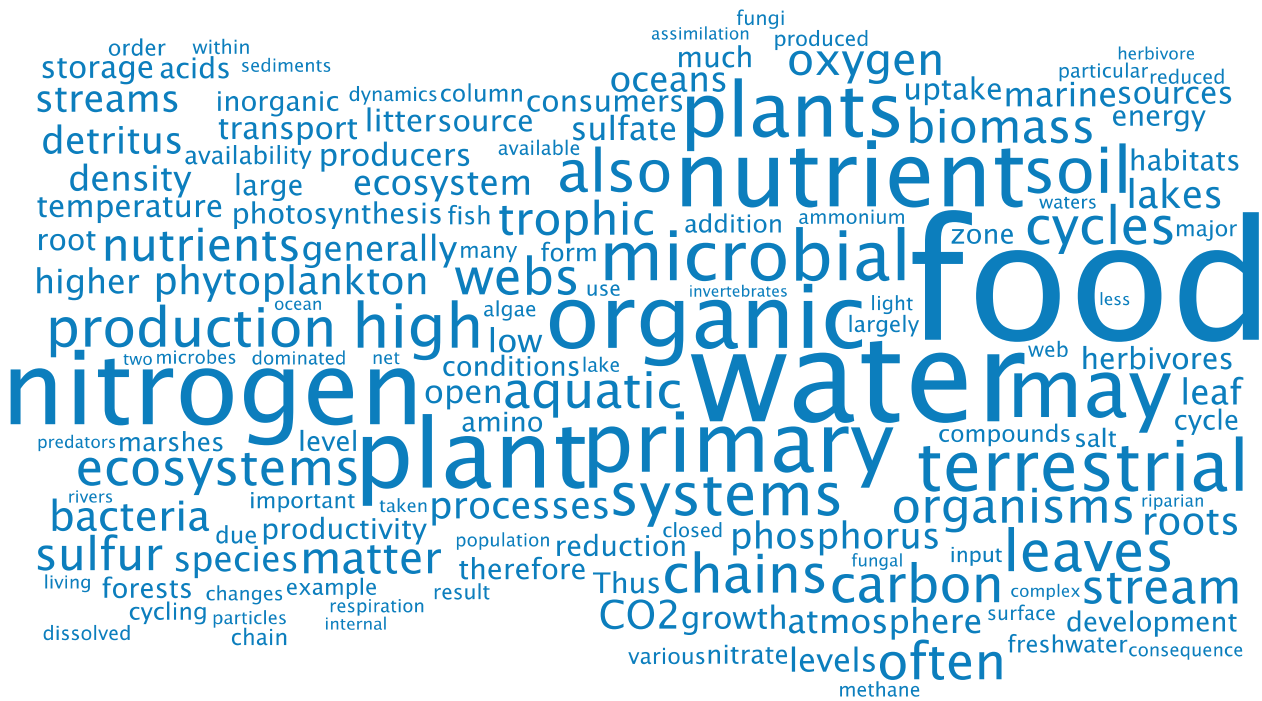
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6
Food Chains and Nutrient Cycles

Felix Bärlocher and Heinz Rennenberg

Overview

Food chains and food webs summarize feeding relationships between organisms in an ecosystem. The resulting patterns greatly affect biomagnificiation and bioaccumulation. The complexity of food webs can be expressed by connectance C (ratio of observed number of links vs. total number of theoretically possible links) and the interactive strength. Lower values of interactive strength tend to stabilize food webs.

The ultimate source of the vast majority of energy-sustaining food webs is the sun. Marine and terrestrial contributions to total primary production are fairly evenly balanced. When primary producers (plants, algae) are eaten while alive, they are said to enter the grazing food chain. Most primary producers die before being consumed and thereby fuel the detritus food chain, which is strongly influenced by microorganisms. Parasites have an essential though often underappreciated impact on food chains.

Recent approaches to food webs combine metabolic theory with ecological stoichiometry – metabolic rates of various organisms are assumed to be constrained by size, temperature, and stoichiometry. The ratio of C : N : P (Redfield ratio) in phytoplankton is remarkably stable. Energy and matter are tightly linked by chemistry and metabolic energetics. Food chains and webs result in nutrient cycles that can be examined at various scales, ranging from individuals to the entire planet. Depending on the scale, nutrient cycles are open or closed. The basic concepts of food webs and nutrient cycles can be applied regardless of habitat, but terrestrial and aquatic species experience very different environments, which has affected the evolution and dominance of species and resulted in considerable differences in nutrient cycles.

6.1
Basic Concepts

6.1.1
Food Chains and Food Webs

Food chains are linear sequences of conventionally defined or trophic species, beginning with those that do not eat any others and ending with those that are not eaten by any others. A **trophic species** is an aggregation of species belonging to the same trophic (feeding) level (Figure 6.1). The length of a food chain is the number of links between these two endpoints. Various hypotheses have been proposed and tested concerning food chain length and ecological metrics, for example, positive correlations with ecosystem size or productivity, or consistent reduction of energy available at successive levels. Food chains are also intimately connected with the concept of **biomagnification**, the increasing concentrations of certain substances (such as lipophilic pesticides) with each trophic level. The related term **bioaccumulation** refers to the fact that many nutrients or pollutants tend to be higher in a consumer than in its food or environment. Thus, herbivores generally have a greater body concentration of protein than their nitrogen-poor plant food.

In **food webs**, an attempt is made to document all trophic relationships of individual species; in other words, all existing food chains are combined into one construct. For example, *Volvox* (*Chlorophyta, Viridiplantae*) (see Figure 4.14) is consumed by *Daphnia* (*Branchiopoda, Arthropoda*) as well as by many other zooplankters, and there are several invertebrates, tadpoles, and fish that consume *Daphnia*. Traditionally, food webs have been constructed based on direct observation and identification of species and their food items. More recently, molecular analyses (barcoding) of gut contents and feces have revealed many previously unknown links, especially if we include all invertebrates and microorganisms. Not surprisingly, graphic representations of food web can be extremely complex (Figure 6.2 represents a very simple, artificial food web; examples based on actual ecosystems are shown at *www.foodwebs.org*). A standard measure of complexity in food web analyses is the **connectance C**. It is defined as the ratio of observed number of links (L) to the total number of theoretically possible links among species (S). If loops are included (e.g., A eating B and B eating A, depending on their life

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

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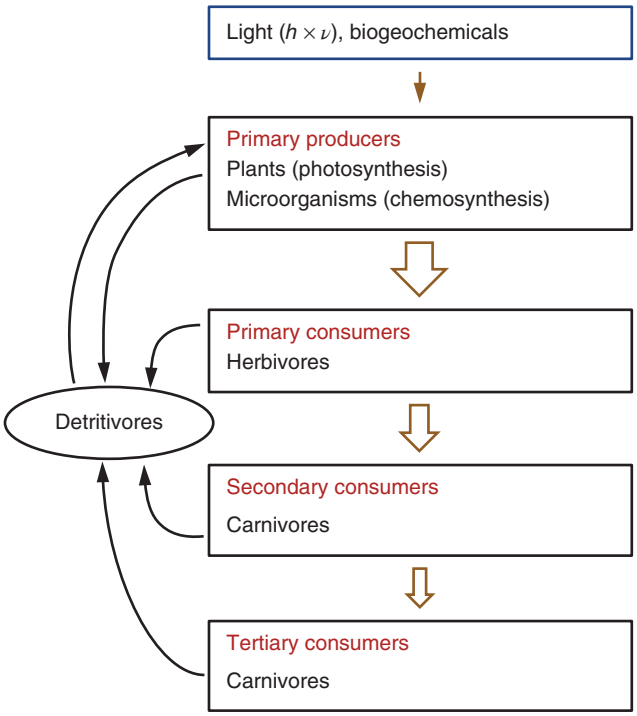

Figure 6.1 Energy flow through different trophic levels. Energy decreases in each successive trophic level (width of the arrows). (Graphics: G.-J.Krauss, D. Dobritzsch.)

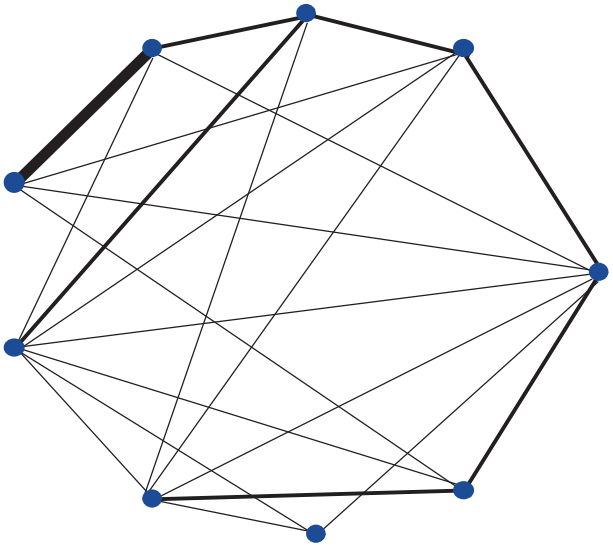

Figure 6.2 Model of a food web. Species are represented by circles. The thicknesses of the connecting lines are proportional to the amount of transferred energy or biomass.

stages), the total number of links equals L/S(S−1); if they are excluded, it is half as large. An important factor is the **interaction strength**: lower values tend to stabilize food webs.

Table 6.1 Average net primary production (NPP, in g m^{-2} year^{-1}) and biomass (kg m^{-2}) of selected ecosystems.

Continental	NPP	Biomass
Swamp and marsh	1000–2500	15
Tropical rain forest	2000	44
Tropical seasonal forest	1500	36
Temperate evergreen forest	1300	36
Temperate deciduous forest	1200	30
Boreal forest	800	20
Savannah	700	4
Cultivated land	644	1.1
Temperate grassland	500	1.6
Lakes and streams	500	0.02
Marine	NPP	Biomass
Salt marshes	Up to 8000	Up to 15
Mangroves	1000–5400	5–18
Algal beds and reefs	2000	2
Continental shelf	360	0.01
Open ocean	127	0.003

After Smith and Smith (2012) and other sources.

6.1.2 Grazing vs. Detritus Food Chains and the Microbial Loop

Ultimately, the vast majority of life on earth is sustained by the sun via photosynthetic organisms. Total net primary productivity is fairly evenly divided between marine (48.5×10^{15} g year^{-1}) and terrestrial contributions (56.4×10^{15} g year^{-1}). The productivity of selected ecosystems is listed in Table 6.1. Very high values are found in salt marshes and freshwater marshes.

It is useful to distinguish between two types of food chains. In **grazing food chains**, the primary food sources are living grasses or algae (e.g., phytoplankton). These are eaten by primary consumers such as rabbits or zooplankton, which in turn are consumed by secondary consumers such as foxes or fish. Additional links may be present.

A variable proportion of the primary production is not consumed until it has died. This process dominates with deciduous leaves: they senesce and eventually fall to the ground, or into lakes and streams (see Section 6.1.3). Dead organic matter forms the basis of **detritus food chains**. As there is no direct feedback from the consumer (microbes, invertebrates) to the food producer (trees), detritus food chains are often considered to be more stable than grazing food chains, where consumer pressure can lead to defensive adaptations by the plant, followed by counter adaptions by the consumer.

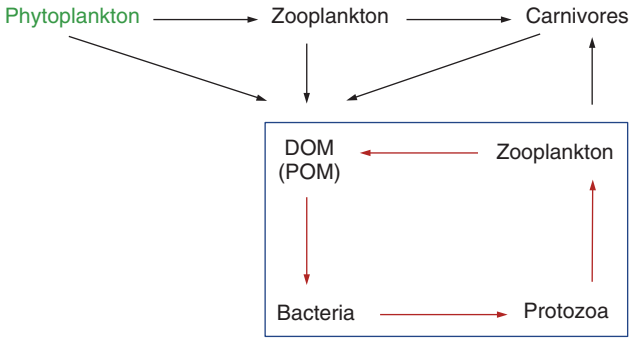

Figure 6.3 Aquatic microbial loop (in blue rectangle).

By convention, detritus is subdivided into fractions based on their size: particles greater than 1 mm are classified as **Coarse Particulate Organic Matter** (**CPOM**), particles <1 mm >0.5 µm as **Fine Particulate Organic Matter** (**FPOM**), and anything passing through 0.5 µm filters as **Dissolved Organic Matter** (**DOM**).

DOM is released by most organisms, whether they are dead or alive, and is processed in the microbial loop: bacteria (and, to a lesser extent, fungi, yeasts, and even protozoa) absorb it and thereby convert it to particulate organic matter (Figure 6.3). Bacteria may be eaten by protozoa, which may be harvested by zooplankton. The energy stored in DOM is thus reintroduced into the more conventional food chains. The **microbial loop** *sensu strictu* refers to the return of DOM to higher trophic levels via bacterial (microbial) uptake. However, DOM can also be converted to particulate forms abiotically, for example, in water exposed to strong turbulence or high winds. In oceans, this can produce conspicuous accumulations of **seafoam**; in streams, leaf leachates can form "flakes" or "clots," with or without the participation of bacteria. After heavy rains or below waterfalls, foam forms naturally in many softwater streams and traps small particles (pollen, fungal spores, arthropod parts). The further processing of foam and its contents involves microbes, protozoa, and invertebrate consumers.

6.1.3 Role of Parasites

Parasites can profoundly change the identity of food chain nodes as well as web topology in terms of chain length, connectance, and stability. For example, the fungus *Ceratocystis ulmi* (*Ascomycota*) nearly wiped out the American elm (*Ulmus americana, Rosales, Rosidae*). The infection diverts some of the energy stored in the leaves from the grazing to the detritus food chain; a substantial portion is used to produce fungal structures that have their own unique consumers. More surprising is the fact that there are at least 54 consumers that "eat" lions. In addition to the expected carnivores (other lions, leopards, hyenas), 2 arthropods, 2 bacteria (an underestimate), 31 helminths, 6 protozoans, and 10 viruses "consume" lion meat (i.e., use it for their own reproduction). As parasitism is believed by some to be the most common animal lifestyle, its impact on food webs clearly merits more attention.

6.1.4 Metabolic Theory and Ecological Stoichiometry

A recent approach to a more mechanistic understanding of food webs combines **metabolic theory** with **ecological stoichiometry**. The metabolic rate of an organism, generally estimated by its oxygen (heterotrophs) or CO_2 (autotrophs) consumption, is assumed to be constrained primarily by body size, temperature, and stoichiometry.

In a narrow sense, ecological stoichiometry concerns itself with the proportions of elements in organisms or their environment. In 1934, Redfield published an article on the observed quantities of C, N, and P in phytoplankton biomass. He found their ratio to be remarkably stable at 106:16:1 (C : N : P); this ratio is now known as the ***Redfield ratio***. Deviations can often be related to broad phylogenetic differences, which influence body size, growth rate, and presence of support structures. Thus, high P values in microorganisms reflect high growth rates because of large quantities of RNA; high P values in vertebrates are heavily influenced by its presence in bones.

Energy and matter are tightly linked by chemistry and energetics of metabolism. The ultimate goal of metabolic theory is to understand how metabolic rates control ecological processes from individuals to food webs to the entire biosphere. Combining it with ecological stoichiometry may allow predictions of how climate change (higher temperature and CO_2 concentrations) will influence communities and food webs. In quantitative terms, nearly all nonequilibrium electron transfers are driven by protein complexes associated with a small number of prosthetic groups. They originate exclusively from microbes and are highly conserved. Despite the enormous genetic diversity of life, a relatively stable set of **core genes** code for the major redox reactions essential for initiating food chains and the functioning of biogeochemical cycles.

6.1.5 Terrestrial Versus Aquatic Habitats

While the basic concepts of food chains and webs are applicable regardless of habitat, terrestrial and aquatic species experience very different environments (Figure 6.4). Terrestrial organisms are surrounded by a mixture of gases, dominated by nitrogen (78.09%) and oxygen (20.95%), followed by argon (0.92%) and CO_2 (0.039%). Air also contains a variable amount of water vapor (on average around 1%). Aquatic organisms are immersed in a liquid, water, containing variable amounts of dissolved gases, ions, molecules, and particulate matter. Air and water have very different physicochemical properties that have profoundly

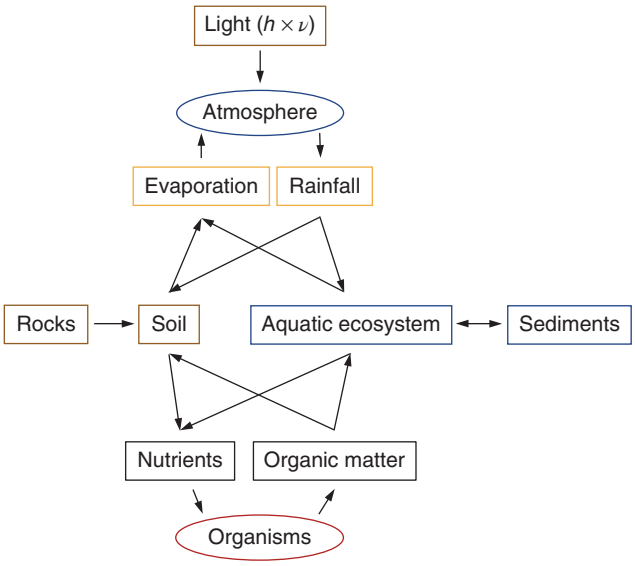

Figure 6.4 Interactions between the compartments of ecosystems. (Graphics: D. Dobritzsch, G. -J. Krauss.)

affected the evolution and types of dominant organisms and their participation in nutrient cycles and food webs. To begin with, **water has a much greater density**, which tends to counteract the effects of inertia and gravity. Large terrestrial organisms, therefore, need strong support structures. An obvious example is wood. It is essentially restricted to terrestrial plants, and a vital component is **lignin**. Lignin is present in all vascular plants but not in bryophytes. Its original function may have been linked to water transport, but its presence in red algae suggests that one of its early functions was in fact structural. Lignin is a complex structure, and as a consequence is difficult to break down and tends to accumulate. It is the second most abundant **natural polymer**, exceeded only by **cellulose**.

Systematic differences in **trophic transfer** and **biomass partitioning** between aquatic and terrestrial systems were first identified by Lindeman in the 1940s. First, primary producers in aquatic systems are dominated by unicellular organisms, such as **phytoplankton**; primary producers in terrestrial systems are dominated by multicellular, structurally complex organisms, such as vascular plants. Second, because of their different positions in the landscape, limiting nutrients, that is, nitrogen and phosphorus, are washed out of terrestrial systems and transported into aquatic systems. As a consequence, terrestrial systems are generally more nutrient-poor than aquatic systems. Implications of these differences for interactions between primary producers and consumers in terrestrial and aquatic systems have only recently been worked out at the level of size structure, growth rate, and nutrient distribution.

The size of terrestrial consumers ranges from being much larger (e.g., ruminants) to being much smaller (e.g., *Lepidoptera*, *Arthropoda*, in forests) than the plants they consume. Most aquatic food webs are much more **size-structured** with a positive correlation between trophic position and body size of a given organism. Terrestrial and aquatic primary producers perform **carbon dioxide fixation** at similar rates, but the living biomass stored in forests or grasslands is much greater than in phytoplankton. This is the consequence of a higher rate of **biomass turnover** in aquatic compared to terrestrial systems, mediated by higher abundance of aquatic consumers and their higher efficiency in removing plant material and converting it into their own biomass. These differences in abundance and consumer efficiency between terrestrial and aquatic systems are thought to be connected to differences in nutrient distribution in primary producers. The lack of vascular tissues results in phytoplankton being composed almost entirely of nutrient-rich (N and P) tissues, whereas terrestrial plants are much more variable in terms of nutrient content, depending on tissue and species. Thus, consumers, which generally have a high demand for N and P, are much more prone to face **nutritional deficits** in terrestrial compared to aquatic systems.

Net primary productivity ranges over two orders of magnitude in both terrestrial and aquatic systems, but is not consistently different between the systems. Biomass of consumers accumulates with increasing primary productivity in both systems. Thus, the entire food web expands when more resources become available to primary producers. The various components of a given food web increase at similar rates, but these rates differ between terrestrial (relatively low) and aquatic systems (relatively high), and are consistent over the entire range of primary productivity on the globe.

6.1.6 General Principles of Nutrient Cycles

Food chains and food webs result in **nutrient cycles** that can be examined at a global scale, a landscape scale, an ecosystem scale, and an individual species scale. At a **global scale**, nutrient cycles are interpreted as biogeochemical in nature, as they are characterized by fluxes between, and storage in, the biosphere, the atmosphere, the geosphere, and the marine and freshwater hydrosphere (Figure 6.4). Biogeochemical cycles are almost completely closed, because losses into space are negligible. However, current knowledge of nutrient sources and sinks is often insufficient to characterize complete global biogeochemical cycles. For some nutrients, for example, for sulfur, a nearly closed global cycle can be defined only by including not only the **troposphere** but also the **stratosphere** (with sulfate aerosols), in the atmospheric compartment.

At the **landscape** and the **ecosystem scale**, nutrient cycles can be both open or closed, depending on the particular nutrient, its availability, and environmental conditions. Closed nutrient cycles are particularly common in terrestrial systems at limiting conditions, for example, under nitrogen limitation; but natural disturbances such as weather extremes (droughts, storms) or fire

can temporarily transform nutrient cycles in a landscape and an ecosystem from a closed to an open system, with corresponding nutrient transfers to the **hydrosphere** and/or the **atmosphere**. Depending on the frequency and intensity of natural disturbances, these transfers may persist and may then result in landscape and ecosystem deterioration.

Nutrient cycles at the individual species level are particularly common in perennial plants. These cycles are characterized by storage and processes that make plant growth and development largely independent of the external availability of a limiting nutrient and, hence, of environmental factors governing this availability. Thus, storage and mobilization will help a plant to withstand unfavorable environmental conditions and can, therefore, be considered a general principle of longevity. As perennial plants have evolved on nutrient-poor soils, it has been speculated that storage and mobilization and, hence, perennial lifestyle, are evolutionary adaptations to nutrient limitation.

In aquatic habitats, the degree of openness varies considerably. For example, streams and rivers are clearly open systems. The vast majority of inorganic and organic nutrients is imported from their terrestrial surroundings, and most of the water is ultimately derived from **groundwater**. **Surface water flow** and direct **precipitation** play minor roles. These sources provide dissolved and particulate nutrients. In addition, the **riparian vegetation** makes substantial contributions to food webs, particularly in headwater streams. Whenever nutrients are released from living organisms via **mineralization**, they tend to be passively swept downstream. Instead of **cycling**, nutrients are, therefore, said to be **spiraling**; each spiral contributes to their downstream displacement (see Section 6.2.3.5). Ultimately, they end up in a lake or the ocean. Thus, running waters can be interpreted as pipes that transport nutrients from terrestrial habitats to lakes and oceans.

Lakes as a whole, especially large ones, are generally less open and tend to retain nutrients over extended periods of time. However, many are fed by large rivers, which deposit nutrients and sediments in the lake, and may eventually fill the basin. Outflows have the opposite effect. Within the lake, there may be seasonal transport processes of nutrients in connection with absence or presence of lake stratification and circulation (see Section 6.2.3.1).

Oceans, of course, are the ultimate recipients of nutrients discharged by rivers. They also receive substantial inputs via precipitation. **Mangrove forests** and **salt marshes** provide additional inputs, as do **hydrothermal vents** and **methane seeps** (see Section 6.2.2.5). Taken as a whole, oceans have to be considered **open systems**. Subsystems within oceans, for example, grazing food chains dominated by phytoplankton and zooplankton with the inclusion of the microbial loop may at least temporarily be closed. A variable proportion of this biomass will ultimately sink to the bottom and will become unavailable.

The movement of nutrients from terrestrial habitats to streams and rivers and from there to lakes and to oceans is not exclusively unidirectional, however. For example, **river floods** can carry nutrient-rich sediments onto normally dry land. Spawning salmon and other migratory fish move back from the ocean into freshwater, functioning as a nutrient "conveyor belt". Bears, eagles, and other predators disperse some of these nutrients back into terrestrial ecosystems. Humans continue to harvest huge quantities of fish, larger invertebrates, and marine mammals (whales and seals). In dry regions, the feces of seabird have formed enormous deposits of guano rich in nitrogen and phosphorus.

6.2 Aquatic Systems

Water molecules are dipolar (see Chapter 10), which profoundly affects their physicochemical properties and by extension aquatic organisms. Their structure is responsible for the density anomaly (ice is less dense than liquid water, which reaches its highest density at 4 °C), and low thermal conductivity. Together, these properties result in stratified water columns in lakes or oceans. The warm top layer (epilimnion) is easily mixed by winds; the bottom layer (hypolimnion) with the highest density is cold (in an idealized lake it reaches 4 °C). The connecting layer (metalimnion) shows a steep temperature gradient. It prevents exchange of nutrients and gases between epilimnion and hypolimnion. As a result, the hypolimnion in eutrophic lakes can become anoxic.

Water strongly absorbs light; the water column of lakes and oceans is, therefore, divided into euphotic (with photosynthesis) and aphotic (without photosynthesis) zones. In the absence of light, chemolithoautotrophic bacteria often contribute to primary production (see Section 6.2.4.4). They are particularly important near hydrothermal vents and methane seeps, where they support unique communities.

The high density of water allowed the evolution of plankton, small organisms floating or drifting in the water column. In large bodies of water, phytoplankton are the top primary producers. They are eaten by zooplankton, which in turn are consumed by larger predators. However, a considerable proportion of the primary production is diverted through DOM, which is captured by bacteria and reintroduced to higher trophic levels by nanoflagellates. More recently, the importance of the viral shunt has been documented: bacteriophages lyse bacteria, releasing bacterial debris and DOM.

Aquatic habitats can be subdivided into pelagial (open water) and benthic (on or close to the bottom of water bodies) zones. Littoral zones are located near the shore, where primary production is often dominated by emergent macrophytes (e.g., freshwater and salt marshes, mangrove forests). In these habitats, which rank among

the most productive, detritus food chains generally dominate.

Running waters are characterized by unidirectional, turbulent water flow. On mineralization, nutrients are often displaced downstream before being recaptured. Instead of nutrient cycling, we therefore talk about nutrient spiraling. The turbulence generally prevents stratification of the water column.

If we follow a stream from its source to its mouth, its physical, chemical, and biological characteristics change in a predictable manner as the stream widens, the impact of food and nutrients from the riparian zones lessens, production within the stream increases. These changes affect the numbers and types of invertebrate consumers. In headwaters, leaf-shredding invertebrates often dominate, while in downstream reaches, collectors of fine particles become more common. These predictable changes are summarized in the River Continuum Concept.

6.2.1 Important Physicochemical Properties of Water

The high density of water was instrumental in the evolution of the largest animal ever to live on earth, the blue whale. It also allowed the existence of **plankton**. For planktonic species, active movement is generally less important than passive movement. **Plankton** provides a crucial source of food to larger, actively swimming organisms such as fish and marine mammals, collectively known as **nekton**.

Water molecules are strongly bipolar (see Chapter 10). This makes them "stick" to each other through hydrogen bonds, which explains the high viscosity of water, its density anomaly and thermal features, and the fact that it is an excellent solvent (see Section S1.1.6). The **density anomaly** refers to the fact that the solid phase, ice, is lighter than liquid water, whose density peaks at 4 °C (dissolved ions lower temperature of maximum density and freezing point, but ice is always less dense than water). Natural bodies of water, therefore, freeze from the top down; if they froze from the bottom up, many lakes in temperate climates would remain largely frozen in summer because of the low thermal conductivity of water. Temperature-dependent density is responsible for the often very pronounced **stratification** of lakes and oceans. Combined with the low **thermal conductivity** of water and slow **diffusion** of ions, this can lead to steep gradients in temperature, nutrients, and other essential compounds. Thus, zones devoid of oxygen (**anoxic zones**) are not uncommon in natural waters, especially in sediments with high microbial activities. A lack of oxygen can occur in soils, especially if waterlogged, but is uncommon in the open atmosphere.

Water has a much higher **specific heat capacity** than air. Temperature fluctuations in streams, lakes, and oceans are, therefore, less extreme than in terrestrial habitats, both on a daily and a seasonal scale, and large bodies of water tend to moderate the climate of their surroundings. Extremely high temperatures are found where geothermally heated groundwater emerges through a **hydrothermal vent** (a fissure in the planet's surface) as a hot spring or, under the sea, as "black smokers." These areas can be highly productive with chemolithoautotrophic bacteria often forming the base of a unique food web including giant tube worms, clams, and limpets.

The solubility of gases in water is described by **Henry's Law**: at a constant temperature, the amount of a gas that dissolves in a given volume is proportional to the partial pressure of the gas in equilibrium with the liquid. Oxygen has a much lower solubility constant than CO_2; the maximum oxygen content of (pure) water in equilibrium with air at 0 °C is, therefore, only 14.5 mg l^{-1} (453 µM), and it declines to 9.1 (284 µM) at 20 °C. This corresponds to <1% of the oxygen concentration of air (V/V). In addition, the diffusion rate of oxygen is 3×10^5 times lower in water than in air. In aquatic systems, oxygen, therefore, often limits the kinds and abundances of organisms.

The CO_2 concentration in water at equilibrium with the air is roughly 3% of its concentration in the atmosphere. However, CO_2 concentrations in soil can be much greater (the soil atmosphere is heavily influenced by microbial decomposition processes producing CO_2). As a result, groundwater can become supersaturated with CO_2, which in calcareous soils reacts with $CaCO_3$. When reaching the surface, the excess CO_2 will tend toward an equilibrium with the atmosphere, resulting in the deposition of **travertine**. The interactions between CO_2, bicarbonate, carbonate, and pH are highly complex and driven by abiotic and biotic processes. Anthropogenic increases in atmospheric CO_2 are predicted to acidify oceans and presumably large freshwater bodies; in running waters, the influence of the soil atmosphere on groundwater will probably remain more important.

Neither oxygen (or some other gases such as methane or CO_2) nor water are biologically inert: water is essential to all organisms and often controls the rate of primary production and of decomposition in terrestrial habitats. Molecular oxygen is essential for the majority of organisms (obligate **aerobes**) and can be lethal for a minority (obligate **anaerobes**). In **aerobic respiration**, oxygen is the final acceptor of electrons initially present in organic compounds. In the absence of oxygen, this role can be taken over by a number of inorganic compounds, such as Fe^{3+} (**iron respiration**), NO_3^- (**nitrate respiration**, first step in **denitrification**), SO_4^{2-} (**sulfate respiration**), or even CO_2 (**methanogenesis**), resulting in the formation of Fe^{2+}, NO_2^-, H_2S, and CH_4. When these products are subsequently transported into oxygen-containing water, other microbes (**chemolithoautotrophs**) may recover energy from their reoxidation and use it for CO_2 fixation.

6.2.2 Marine Systems

6.2.2.1 Density Gradients and Pycnoclines

Over 70% of the earth's surface is covered by oceans, and collectively, they are responsible for nearly half of global net primary production (NPP). This indicates that productivity per area is over twice as high on land as in oceans, and large sections of the open water (**pelagial**) are indeed characterized as "biological deserts". This includes the South Pacific subtropical gyre, a vast expanse of nutrient-poor water.

The **density of ocean water** is affected primarily by salinity and temperature, and generally increases with depth. In low-latitude regions, a thin layer of low-density surface water is separated from high-density deep water by a zone of rapid density change, the **pycnocline** (density slope). Pycnoclines are primarily due to temperature differences (**thermocline**) and are an effective barrier to the movement of planktonic organisms. At higher latitudes, the water is colder and density differences due to temperature are smaller, which generally prevents the formation of a pycnocline and stable stratification. In some high latitude regions, surface waters are colder than the deep water whose higher salt concentrations result in a halocline responsible for water column stability. In these regions, the halocline may facilitate the formation of sea ice and limit the escape of carbon dioxide to the atmosphere. **Haloclines** are common in fjords and some estuaries where freshwater is deposited onto the ocean's surface water.

A stable water column may prevent nutrients mineralized in the lower regions from reaching the higher levels where there is sufficient light for photosynthesis. This potential nutrient loss is counteracted by **upwelling**, which is the wind-driven transport of dense, cool, and usually nutrient-rich water toward the ocean surface, where it replaces warmer, nutrient-depleted water. Upwelling regions usually have high primary productivity, which propagates through the food web all the way to fish as top consumers. Five upwellings, occupying 5% of the total ocean area, are responsible for 25% of the total marine fish catch.

6.2.2.2 The Euphotic Zone

Even when nutrients are not scarce, photosynthesis in the open ocean is limited by the absorption of light by water. **Photosynthetically active radiation (PAR)** is defined as the range of solar radiation (wave lengths in 400–700 nm) (see Chapter 9) that plants and bacteria can use for photosynthesis. When solar radiation reaches water, a small amount is scattered back out of the water; the rest is attenuated because of absorption by the water, algal pigments, and disssolved and particulate matter. This restricts the extent of the **euphotic zone**, where photosynthesis can occur. Typically, it ranges from a few cm down to around 200 m.

Some bacteria can exploit light with wavelengths extending into the near-infrared. Purple (*Proteobacteria*) and green sulfur bacteria (*Chlorobi*) perform anoxygenic photosynthesis, using sulfur compounds to reduce CO_2 under anaerobic conditions. They occur typically in shallow, organic-rich, anoxic environments such as subtidal marine sediments. Recently, green sulfur bacteria have been isolated from a deep-sea hydrothermal vent, where the only source of light is geothermal radiation rather than the sun.

6.2.2.3 The Pelagial Zone

In contrast to terrestrial ecosystems, the vast majority of primary production in the ocean is performed by free-living microscopic organisms called **phytoplankton**. Larger autotrophs, such as **seagrasses** and **macroalgae** (seaweeds), are generally confined to the **littoral** zone and adjacent shallow waters, where they can attach to the underlying substrate but still be within the photic zone.

Thus, in the **pelagial** (open water), the conventional picture of a marine food chain begins with phytoplankton, continues with **zooplankton**, eaten by krill or other crustaceans, on to several species of fish and may end with large fish, seabirds, or mammals (see Figure 6.3). Several modifications and additions are necessary, however; phytoplankton typically release variable amounts of dissolved organic carbon (OC). In addition, a substantial proportion of phytoplankton may not be eaten while alive. It, therefore, enters a **detrital food chain**. Approximately 10% of the biological mass generated in the euphotic zone sinks into deeper water, where it is decomposed. About 1% reaches the deep ocean floor. This process, a **biological pump**, removes CO_2 and nutrients. A permanent **thermocline** prevents the return of these nutrients to the upper zone throughout much of the subtropical gyres.

In all these transformations, bacteria play a major role. In oceans, at least 50% of the CO_2 fixed by photosynthesis each day ends up supporting microbial respiration and the production of bacterial cells. What happens to this vast bacterial biomass? An early assumption was that most of it was consumed by **nanoflagellates**, serving as food for metazoa, eventually ending up in higher trophic levels dominated by large fish or mammals. This pathway has been labeled the "**microbial loop**" (Figure 6.3). Heterotrophic nanoflagellates are, for example, the widespread marine heterotroph *Paraphysomonas* sp., the marine mixotroph *Ochromonas* sp., and the freshwater heterotroph *Spumella* (all *Chrysophyceae, Stramonopiles*).

More recently, the significance of the "**viral shunt**" has been recognized (Figure 6.5). There are up to 10 million virus-like particles (**VLPs**) per ml of seawater, corresponding to 5–10 VLP per bacterium. An estimated 5000 viral genotypes occur in 200 ml of seawater; 1 kg of marine sediment may harbor 1 million genotypes. A variable proportion of marine viruses belongs to the so-called giant viruses or **giruses** (genomes > 300 kB, capsid diameter > 0.2 mm).

Many marine viruses are **phages** (viruses attacking bacteria). They are believed to kill around 4–50% of bacteria each day, with the rest of the bacteria being eaten by protists.

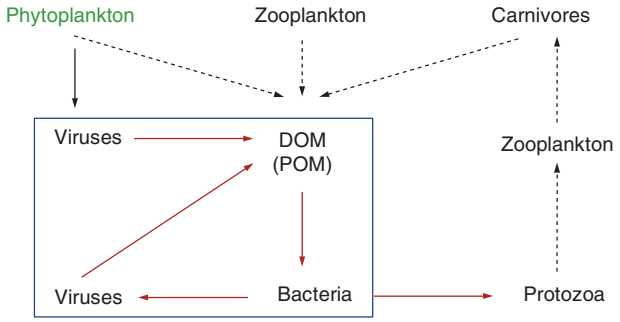

Figure 6.5 Viral shunt in an aquatic microbial loop (in blue rectangle). Rapid cycling through this shunt shortcircuits the transfer of energy and organic matter to higher trophic levels. The shunt diverts mass flow from the traditional food chain (dashed line).

Viral mass in the oceans at 2×10^{11} kg of C is exceeded only by bacterial mass. Virally mediated release of dissolved OC has been estimated at 3–20 Gt per year, which compares to an estimated phytoplankton production of approximately 50 Gt per year.

Viruses also infect photosynthetic bacteria and algae. **Cyanophages** (viruses attacking cyanobacteria) have acquired genes essential for photosynthesis, which presumably ensure continued energy acquisition during the early stages of the infection cycle. This is considered a key evolutionary step explaining the great success of viruses in the oceans.

Grazing and viral lysis can be expected to have different effects on nutrient cycling: grazing potentially transfers nutrients to higher trophic levels. Viral lysis releases substantial quantities of both organic and inorganic dissolved substances. Both are potentially taken up by bacteria, which may again be vulnerable to viral attack. The immediate consequences of this rapidly cycling bacterial/viral loop are increased respiratory losses (CO_2 release) and reduced transfer to higher trophic levels. The longer term consequences are less clear: more rapid release of nutrients may stimulate primary production by phytoplankton.

Cycling of inorganic carbon can also be affected by viral activity. *Emiliani huxleyi* is one of the most abundant marine algae (*Isochrysidales*, *Hyptophyceae*). Its cells are covered by tiny scales of $CaCO_3$, which collectively account for about a third of the total marine $CaCO_3$ production. *E. huxleyi* often forms blooms covering 100 000 km^2. A giant virus, EhV, is instrumental in terminating these blooms, leading to massive releases of organic matter and detached carbonate scales.

6.2.2.4 Hydrothermal Vents and Methane Seeps

The depth at which oxygen production (from photosynthesis) and oxygen consumption (by respiration) are equal is called the **oxygen compensation depth**, which may be 10–100 m below sea level. Below this level, the basis of food webs is organic detritus sinking down from the upper phototrophic zone. At the very bottom of the ocean floor, geothermally heated water may escape from fissures in the earth. Such hydrothermal vents are much more productive than the surrounding area and often support complex **benthic** communities with organisms such as giant tube worms, clams, limpets, and shrimps. Traditionally, life on the seafloor was assumed to depend on a constant rain of detritus from euphotic layers. However, the density of organisms around **hydrothermal vents** is 10 000–100 000 times greater than elsewhere. These food webs are not sustained by photosynthesis powered by the sun, but by various chemicals whose oxidation provides energy to various Bacteria and Archaea (**chemolithoautotrophy**). Sulfur-oxidizing organisms play a prominent role; others use methane or hydrogen as energy source. Recently, a member of the Chlorobiaceae (*Chlorobi*) has been discovered that can use the faint thermal glow from a black smoker for photosynthesis, which agrees to the general ability of Chlorobiaceae to harvest light even at very low dose levels.

Three other submarine environments depend primarily on chemosynthesis. One is a **hypersaline seep** in the Gulf of Mexico, where H_2S-oxidizing bacteria support a similar food web as hydrothermal vents. A diverse community has also been found in oil and gas seeps in the same area down to 2200 m. The third environment, off the coast of Oregon, is seep water containing methane, whose oxidation supports a diverse community.

6.2.2.5 The Benthic Habitat

The majority of marine production occurs in the pelagic or open water. The other basic habitat type is the **benthic environment**, which includes the water/sediment interface and some subsurface layers. They support the **benthos**, a general term for organisms living on or near the bottom of aquatic ecosystems. The deep-sea benthic environment includes the **geothermal vents** (see Section 6.2.2.4) with their high productivity and diversity, as well as vast areas that depend on sinking living or dead organic matter. The **littoral** zone is close to the shore and extends from the high water mark (rarely flooded) to permanently submerged areas. Some of these transitional areas, where the ocean, freshwater, and the land intermingle are among the most productive regions on earth. Three biotopes are of particular importance in terms of diversity, productivity, and as nursery grounds for fish: mangroves, salt marshes, and coral reefs.

6.2.2.6 Mangroves

Mangroves (also called ***mangrove forests***) are forested intertidal wetlands, consisting of a complex mix of terrestrial, freshwater, and marine ecosystems (Figure 6.6). They are essentially restricted to within 30° of the equator.

A dominant genus is often ***Rhizophora*** (true mangroves, *Malpighiales, Rosidae*) though many other terrestrial trees, shrubs, and grasses are also present. *Rhizophora* has characteristic stilt-roots raising the plant above the water and allowing respiration even when lower roots are submerged. A molecular pump removes excess salt from root cells.

Figure 6.6 Mangrove swamp in Cape Tribulation, Queensland, Australia. Red mangroves can survive in the most inundanted areas by propping themselves up with "stilt roots". (Courtesy of G. Krauss.)

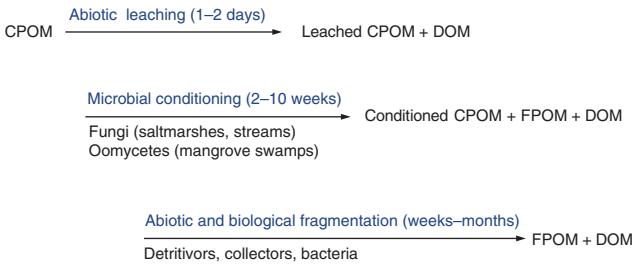

Figure 6.7 Generalized scheme of vascular plant litter processing in aquatic habitats.

Mangrove roots prevent riverborne sediments from being flushed out to sea, while mangrove tree trunks counteract erosion by waves.

Research on food webs in mangrove forests was pioneered in Florida. A generalized scheme of aquatic food webs (albeit based on terrestrial plant litter) is shown in Figure 6.7. Mangrove leaf detritus is invaded by bacteria and fungi. In contrast to salt marshes and streams, **Oomycetes** (synonym *Perenosporomycetes*, *Stramenopiles*) rather than true fungi may be the dominant colonizers. The leaf/fungus complex is consumed by detritivores (amphipods, worms, crabs). These are eaten by several levels of carnivores (fish, birds, snakes, crocodilians). The basic pattern is similar to the one found in streams where the food supply is often dominated by deciduous leaves (see Section 6.2.2). In addition to tree detritus, phytoplankton, benthic algae, and seagrasses contribute to the food web.

Anaerobic processes dominate in mangrove sediments and sulfate reduction is generally the major **diagenetic** pathway, though iron reduction has also been reported. **Denitrification** and **methanogenesis** generally play minor roles, but still are responsible for very high rates of emissions of N_2O and CH_4 from mangrove forests.

6.2.2.7 Salt Marshes

Another major habitat at the intersection of land and ocean is **salt marshes** (Figure 6.8). They occupy the intertidal zone between land and salt or brackish water and occur from the

Figure 6.8 Salt marsh in Peck's Cove, Bay of Fundy, Canada. The strong tides and winter ice scour away most of the above-ground detritus, and in spring, new shoots of *Spartina alterniflora* initiate a new cycle (picture taken on 15 June 2012).

equator to latitudes as high as 65°. When mangroves colonize the same area, they usually displace salt marshes.

Salt marshes are a challenging environment for plants: they must tolerate salt, fluctuating water levels, including complete immersion, and an anoxic sediment. Plant diversity is, therefore, low and the vegetation is dominated by halophytic plants of terrestrial origin such as the **cordgrass** (*Spartina* spp., *Poales, Liliopsida*) and **glassworts** (*Salicornia* spp., *Caryophyllales,* core eudicotyledons). These are often the first plants to colonize newly formed mudflats and initiate its conversion to a salt marsh. Their roots stabilize the mud, allowing other plants to establish themselves.

On the Atlantic coast of North America, the **smooth cordgrass** *Spartina alterniflora* dominates the area between mean sea level and mean high water. At optimal conditions, which occur around the barrier islands off the coast of Georgia, the NPP of salt marshes can be as high as 4000 g dry mass m^{-2} $year^{-1}$. This places them among the most productive ecosystems of the world. Much of the animal production in salt marshes is ultimately derived from cordgrass. Consumption of living leaves and shoots is low, and most of the energy provided by *Spartina* becomes available through the **detritus food chain**. Early decomposition studies of *Spartina* was based on dried leaves permanently submerged in litterbags, or, on ground-up leaf material incubated in flasks. Under these conditions, bacteria dominate, which contradicts visual observations of abundant fungal reproductive structures in the form of ascoma. A key clue to solving this paradox was Newell's insight that *Spartina*, like most grasses, does not abscise leaves or stems (*http://newell.myweb.uga.edu*). After senescence or death of a leaf, its decay begins while still attached to the plant, in the "**standing-dead**" condition, where it is alternately exposed to air and submerged in salt water. On such leaves, fungal to bacterial biomass was as high

as 600:1 eight weeks after senescence, and fungi captured 90% of total nitrogen within 8–10 weeks. Fungal biomass of naturally decaying *S. alterniflora* leaves can reach up to 10% of total mass. The associated fungal protein produced per year within a 1 m wide strip of salt marsh from low to high marsh in Georgia approximates the protein in a fully grown cow.

In salt marshes of the southern United States, fungal colonization is closely followed by invertebrate feeding (Figure 6.7). The **marsh periwinkle** *Littoraria irrorata* (*Gastropoda, Mollusca*), occurring at densities of 50–300 m^{-2}, selectively removes living fungal mass from standing-dead *S. alterniflora* leaves. In turn, it is a major source of food for various predators.

Further north along the Atlantic coast of North America, there is less evidence of immediate consumption of fungal-colonized *Spartina* by invertebrates. Instead, the detritus is often dislodged and may be widely dispersed within the estuary. The greatest net export occurs from exposed marshes in basins that deepen and widen toward their mouths. This is the case in the Cumberland Basin, Bay of Fundy, Canada, where salt-marsh detritus represents the largest portion of nonliving suspended organic matter. When reduced to fine particles by microbial enzymes or mechanically, it can make a substantial contribution to the diet of ***Corophium volutator***. This tube-dwelling amphipod (*Malacostrata, Arthropoda)* is abundant in intertidal mudflats. It is a major source of food for ground-feeding fish and migratory shorebirds, such as the semipalmated sandpiper. This species breeds in the southern tundra in Canada and Alaska. In late summer, the birds form flocks numbering in hundreds of thousands. On their way to coastal South America, where they overwinter, they stop in the Bay of Fundy, feeding on *Corophium* and other invertebrates to almost double their body mass. This illustrates the vast spatial spread of some food chains.

6.2.2.8 Coral Reefs

Coral reefs cover less than 0.1% of the oceans' surface but are among the most diverse and productive ecosystems on earth. They are home to 25% of all marine species. Corals are colonies of tiny polyps (*Cnidaria*, *Metazoa*) living in a mutualistic relationship with zooxanthellae (*Symbiodinium*, *Dinophyceae, Aleveolata*). These **photosynthetic algae** not only contribute to the nutrition of their coral host, but also to their host's ability to produce a calcium carbonate exoskeleton by removing CO_2 from their body fluids. When stressed, polyps expel their symbionts and take on a white appearance, a phenomenon described as coral bleaching. Corals are found throughout the oceans but reefs are restricted to regions where the average monthly temperature exceeds 18 °C throughout the year. They thrive under nutrient-poor conditions; at elevated levels, phytoplankton may take over and reduce the clarity of the water, which is essential for the corals' endosymbionts.

6.2.3 Freshwater Systems

6.2.3.1 Temperature Gradients and Circulation in Lakes

Freshwater bodies contain **lentic** and **lotic** habitats. Lotic refers to flowing water (streams and rivers), while lentic ecosystems have very slow-moving or standing water (lakes, ponds, and swamps).

Lakes share some of the properties of oceans (see Section 6.2.2.1) but strongly deviate in other aspects. In the open water (**pelagial**), **phytoplankton** is again the primary driver of food chains. Its productivity depends primarily on the availability of light and nutrients (nitrogen and phosphorus, and, for diatoms, the availability of silica).

Heat exchange between a lake and its surroundings takes place primarily at the water/air interface. During the day, radiation is absorbed near the surface; as diffusion is negligible, heat accumulation decreases rapidly and exponentially with depth. At night, surface water will cool down and sink until it encounters water of the same density. During autumn in temperate climates, this may continue until the water column has a uniform temperature of 4 °C (density maximum); any water colder than that will stay on top. At low temperatures, density differences are small, and wind can, therefore, mix the entire lake, a process called **full circulation** (Figure 6.9). Full circulation replenishes oxygen supply in deeper lake layers. With further temperature decline, ice forms on top and prevents the mixing of the water column. In spring, the top layers warm up, and a second full circulation may occur. Continued heating beyond 4 °C will result in higher density water, which will stay on top. As density changes per degree celsius increase at higher temperature, it takes more and more energy (stronger winds) to mix an entire lake. The result is typically lake **stratification** (Figure 6.9): a deep layer that may remain near 4 °C (**hypolimnion**) and an upper layer with a temperature anywhere between 8 and 24 °C (**epilimnion**). They are connected by a **thermocline** (or **metalimnion**, or *sprungschicht* or OATZ for oxic anoxic transition zone) where the temperature

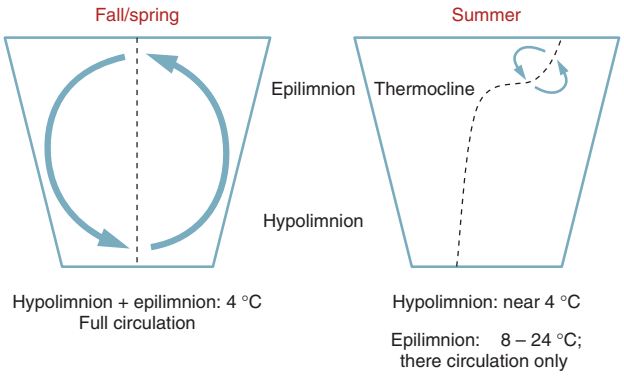

Figure 6.9 Fall/Spring and Summer temperature profile in a lake at temperate latitudes.

changes rapidly. No atmospheric oxygen reaches the hypolimnion.

6.2.3.2 Oxygenic and Anoxygenic Photosynthesis

Circulation patterns have a profound effect on primary production, decomposition, and food chains. In eutrophic (nutrient-rich) lakes, phytoplankton blooms are common during summer stratification. This releases large quantities of oxygen in the epilimnion, occasionally leading to localized **supersaturation**. While some of the phytoplankton production may be eaten by zooplankton and thereby initiate a conventional food chain, a variable proportion will end up as detritus and sink below the **thermocline** to the **hypolimnion**, where it will be attacked by microorganisms and other consumers. This uses up the oxygen, which cannot be replenished by photosynthesis below the eutrophic layer (whose depth can vary between a few cm to several m). Input from the atmosphere by the wind will be limited to the **epilimnion** until lower temperature throughout the water column once again allows complete mixing (**full circulation**; Figure 6.9).

Anaerobic conditions may allow mass development of purple and green sulfur bacteria. They require access to H_2S, absence of oxygen, and sufficient light, and are, therefore, generally restricted to the uppermost layers of the hypolimnion. Members of the genus ***Chromatium*** (*Gammaproteobacteria*) can occur in huge populations forming strikingly colored bands. Their contribution to total primary production can vary between 9 and 25% in H_2S rich and 3–5% in H_2S poor lakes. In meromictic and hypersaline lakes with an oxygen-poor hypolimnion, it may even reach 80–90%. If, on the other hand, H_2S co-occurs with oxygen, it can be oxidized by chemolithotrophs (*Beggiatoa* of the *Gammaproteobacteria*, *Thiobacillus* of the *Betaproteobacteria*) and the gained energy used for CO_2 fixation.

6.2.3.3 The Freshwater Pelagial Zone

The food chain in the pelagial of lakes starts with **phytoplankters** that are eaten by **zooplankters**. Additional links may include invertebrates and fish of increasing size and end with large fish, birds, or mammals. As in marine systems, a substantial proportion of the energy may be diverted to **detrital food chains** or **microbial loops** – phytoplankton release soluble organic compounds, some of the consumed food remains undigested and is expelled as feces, some plankters die a natural death (Figure 6.3). Bacteria are intimately involved in the fate of this detritus, but other microbes play a role as well. **Chytridiomycetes** (*Chytridiomycota, Fungi*) have a suite of exoenzymes allowing them to attack most polymers of dead phytoplankton in the pelagic zone. Their populations are often sporadic and patchy and controlled by factors such as substrate density, light, temperature, and oxygen. Fungal communities are more diverse on algae than on zooplankton, where they seem to be largely replaced by Oomycetes (*Stramenopiles*, not closely related to Fungi).

Numerous generalizations have been proposed about the **seasonal succession** of phytoplanktonic organisms in freshwater lakes. In temperate and freshwaters, a winter minimum is generally followed by a spring maximum. This may be followed by a summer minimum, which in eutrophic lakes can be quite brief and is followed by a bloom of cyanobacteria. The spring maximum is often dominated by diatoms, whose growth exhausts the supply of silica and contributes to their collapse. This suggests **bottom-up** control of productivity (see also Section 6.3.2.). On the other hand, some studies have shown that population maxima of zooplankton coincide with the decline of algal populations, suggesting a degree of **top-down** control.

Parasites can strongly modify food chains, though too little is known to allow well-founded generalizations. Infection of phytoplankton by **Chytrids** (*Chytridiomycota, Fungi*) can be very high (>70%). This may convert inedible algal cells into detrital biomass and zoospores, both of which provide food for grazers and may reduce sedimentation loss of algal carbon and thereby increase carbon path lengths and stabilize aquatic food webs. Conversely, feeding by *Daphnia* on chytrid zoospores can lower fungal parasitism of phytoplankton by significantly removing inoculum.

Viruses (bacteriophages) are very abundant in freshwater, and it has been estimated that 10–40% of prokaryotic production in freshwaters is lysed by viruses, corresponding to a mortality rate that on occasion can match grazing by protists.

6.2.3.4 The Freshwater Benthic Zone and Freshwater Marshes

Food chains of benthic lake habitats depend less on free-floating plankton. Submerged or emergent macrophytes generally dominate primary production, which is supplemented by **periphyton**, a complex biofilm consisting of algae, cyanobacteria, and heterotrophic microbes attached to submerged surfaces. The littoral is a highly structured transition zone between terrestrial and aquatic habitats. Primary production is often very high, and, per area, greatly exceeds the production of the pelagial. In marshes, most of the production is due to **emergent macrophytes** such as *Phragmites australis*, *Typha latifolia,* and *Scirpus* spp (all *Poales, Liliopsida*, Figure 6.10). Their contribution to food chains and webs depends on the ratios of lake perimeter to area and area to volume. Most lakes are small (<1 km^2) and shallow (<10 m depth), which raises the contribution of terrestrial or wetland vascular vegetation to lake metabolism. In the few documented cases, it has been estimated to range between 10% and 75%.

As in **salt marshes**, most of the biomass (≥95–99%) is not eaten before it senesces and dies (Figure 6.10). This is not immediately followed by the detachment of leaves or collapse of shoots. Terrestrial fungi play an important role in colonizing and degrading **standing-dead** shoots and leaves, which remain exposed to the air for weeks to

Figure 6.10 Freshwater marsh dominated by *Typha* (Waterfowl Park, Sackville, NB, Canada, 15 June 2012). The lighter stems and leaves were produced the previous year and have been decomposing without being submerged in water.

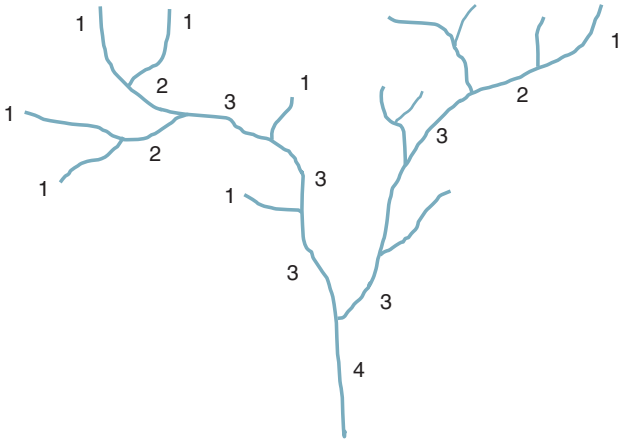

Figure 6.11 Streams and the catchments they drain in terms of nested hierarchies. The smallest permanently flowing stream is classified as a first order stream. Two first order streams join to form a second order stream; two second order streams form a third order stream, and so on. When two tributaries of unequal order fuse, the resulting stream is given the value of the higher order stream.

months. On *Phragmites australis* alone, over 600 fungal species have been recorded.

Fungal biomass and **production** on standing-dead macrophyte litter typically exceed those of bacteria by a factor of at least 9:1. Thus, much of the basis of the food chain in wetlands is dominated by plant detritus colonized and modified by fungal hyphae. Fungal biomass has been estimated to account for between 2% (direct hyphal counts) and 20% (immunoabsorbent assay, ELISA) of total organic matter.

Eventually, dead shoots and leaves topple over and fall to the sediment or into the water, where decay continues but is now dominated by bacteria. Macroinvertebrates are the primary consumers of this detrital complex. It must be emphasized, however, that the decomposition of submerged or floating macrophytes has not been well studied, and their impact on food webs of the littoral zone cannot easily be generalized.

Members of the genus *Coelomomyces* (*Blastocladiomycota, Fungi*) alternate between the littoral and the pelagial. These obligate endoparasites switch between Diptera larvae and a planktonic copepod. Its potential use as biological control of mosquitoes is under investigation.

6.2.3.5 Running Waters

The defining characteristic of running or lotic waters is the current, a gravity-induced downhill movement. The resulting high turbulence prevents stable layering. Combined with frequent contact of the water with the atmosphere, especially in waterfalls and rapids, this makes oxygen depletion less likely than in static waters. Stable thermal gradients are rare; however, the temperature of the water column may undergo daily changes of up to 4 or even 10 degrees. Turbulence also generally prevents freezing of the entire stream.

The stream habitat may extend tens of cm underneath the stream bed into the **hyporheic zone**, which is a major site of community metabolism and a refuge for many stream organisms, especially for their juvenile forms. Upwelling groundwater is often supersaturated with carbonate; when in contact with the atmosphere, it will approach a complex equilibrium with bicarbonate, CO_2, and pH.

Streams and the catchments they drain can be interpreted in terms of nested hierarchies. The smallest permanently flowing units are labeled **first order streams** (Figure 6.11). The joining of two first order streams results in a **second order stream**, two second order streams yield a **third order stream**, and so on. The stream order generally correlates with stream size and with various physical and biological features, all of which influence the structure of food webs and nutrient flows.

One of the crucial insights in stream ecology was formulated by H.B.N. Hynes: "In every respect, the valley rules the stream." This begins with the source of the water: streams are fed by surface, shallow subsurface, and deep subsurface (groundwater) flows. A longer path (determined by structure and composition of geological substrates) presents more opportunities to dissolve minerals and organic matter, which will be introduced into the stream. The riparian zone and the roots of its vegetation help stabilize the banks; fallen branches and trunks raise habitat diversity. Shading **by riparian trees** and shrubs moderates warming of the water column and influx of **PAR**; conversely, riparian vegetation is often the dominant provider of food for stream invertebrates in the form of autumn-shed leaves, needles, and branches.

Energy sources for food chains and webs are ultimately derived from primary production via photosynthesis. Production within streams (**autochthonous**) is generally

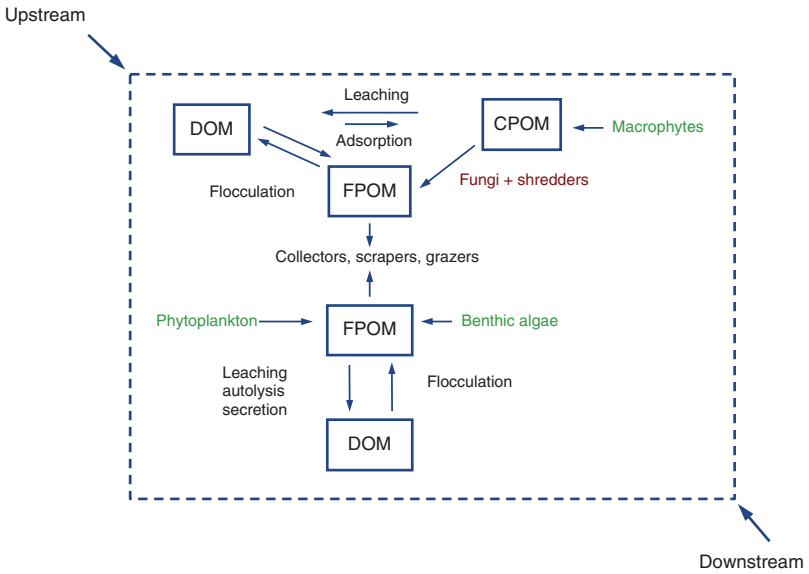

Figure 6.12 Simplified food web in a stream segment. DOM – Dissolved organic matter, CPOM – Coarse particulate organic matter; FPOM – Fine particulate organic matter.

dominated by benthic algae (a polyphyletic group assemblage of eukaryotic producers) and other microscopic producers found on stones, wood, and other surfaces. In slow-moving water, macrophytes (flowering plants, mosses, liverworts, a few lichens) may be common. True plankters are restricted to side channels with much reduced current.

The food webs of almost all running waters receive organic matter from the surrounding valley (**allochthonous** sources). This begins with dissolved (**DOM**) and particulate matter (mostly **FPOM**) introduced via surface or subsurface flows from the soil or groundwater. More obvious (though not necessarily more important in terms of quantity) are autumn-shed leaves and other debris from riparian trees (**CPOM**). A simplified overview of food webs in streams is shown in Figure 6.12.

The terms DOM, CPOM, and FPOM stand for broad categories of potential food items of heterogeneous origin. Nevertheless, it has become customary to place macroinvertebrates (insect larvae, crustaceans, mollusks, and a few other taxa) into **functional groups**, based on how they gather and eat food. **Grazers** and **scrapers** consume algae (**biofilms**, see Chapter 14) from surfaces (stones, wood, or leaves). **Shredders** consume (shred) plant detritus (primarily leaves from riparian trees and autochthonous macrophytes as well). **Collectors** feed on the abundant fine organic particles from broken-up leaves, feces of other consumers, sloughed-off biofilm particles, or flocculated dissolved matter. They may filter these particles from the water column (**collector-filterers**, or suspension feeders) or gather them from the streambed (**collector-gatherers**, or deposit feeders). **Predators** consume other animals. Broader categories emphasizing the type of food and also used for vertebrates include **algivores**, **detritivores**, **omnivores**, **invertivores**, and **piscivores**.

Research is often conducted in terms of stream segments or reaches with an emphasis on measuring what enters a segment and what leaves at the end of it. These movements are interpreted as imports and exports *from* a system rather than internal processes *within* a system. More recent approaches view streams as spatially extended transporting and processing systems. These include the **nutrient spiraling concept** and the **river continuum concept**.

In terrestrial and lentic habitats, we generally talk about **nutrient cycling**, assuming that a nutrient atom undergoes a spatially restricted cyclical movement through various compartments. In streams, this cycling through biotic and abiotic compartments is generally connected to downstream displacement (Figure 6.13). This combination of uptake and longitudinal transport processes resembles a

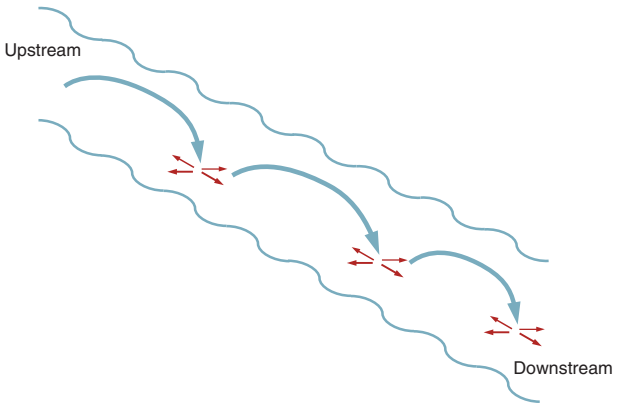

Figure 6.13 The nutrient spiraling concept: in streams and rivers, mineralized nutrient ions are generally displaced downstream before they are recaptured by algae or microorganisms. Their path, therefore, resembles a spiral rather than a cycle.

spiral; we, therefore, talk about **nutrient spiraling**. The downstream distance a nutrient molecule or ion travels during one cycle through the compartments is called the **spiraling length** (an inorganic atom is released by mineralization, travels downstream, is captured by an organism, passes through links in a food chain, and is once again released into the water column). Downstream displacement also affects larger living or dead organisms and their parts. Several species, such as blackfly and net-spinning *Trichoptera* larvae (*Insecta, Arthropoda*), depend on fine particles suspended in the water column, which they filter from the current.

The stability and productivity of food chains within a stream are strongly influenced by the degree to which it is able to retain leaves and other terrestrial plant detritus. This **retentiveness** depends on leaf size and shape as well as on stream depth, current speed, and stream bed morphology. Dams of large woody debris are particularly efficient at retaining coarse organic matter (Box 6.1).

As leaves make a major contribution, their decomposition and incorporation into food webs in streams have received considerable attention. They follow the general scheme of vascular plant litter processing in aquatic habitats (Figure 6.7). **Microbial conditioning** is largely due to a polyphyletic group of true fungi, the **aquatic hyphomycetes** (most of which are now assigned to *Ascomycota, Fungi*). When leaves are immersed in stream water, they trap the typically tetraradiate or sigmoid conidia of these fungi. The conidia germinate into hyphae that rapidly penetrate the leaf matrix and secrete exoenzymes attacking structural plant polysaccharides. The fungi take up inorganic nitrogen and phosphorus from the stream water and incorporate them into their growing mycelia. This **conditioning** makes the leaf more attractive and more nutritious to leaf-feeding invertebrates. Shredders benefit from the enrichment of the dead leaf with protein and lipid-rich fungal cells and from partial enzymatic breakdown of plant polysaccharides into more easily digested subunits.

6.2.4 Nutrient Cycles

6.2.4.1 The Nitrogen Cycle

The basic principles of nitrogen transformations are presented in Section 6.3.3.1 and apply equally to terrestrial and aquatic systems. In pristine systems, nitrogen and phosphorus often limit the growth and activity of autotrophs (phytoplankton), heterotrophic fungi, and bacteria involved in the breakdown of organic matter. Human activity has greatly increased the supply of both, resulting in eutrophication in rivers, lakes, and coastal zones. As this eventually results in an oxygen deficit, extensive "**dead zones**" in lakes and oceans are often the result.

Nitrogen in inorganic (nitrate, ammonium) or organic (amino acids) dissolved form enters streams through groundwater, surface runoff, from upstream and through precipitation; in large lakes and oceans, external inputs include large rivers and precipitation. In all habitats, **nitrogen fixation** by free-living or symbiontic (in diatoms) bacteria can add to the supply of inorganic nutrients (see Section 5.2.2.2). Its contribution is particularly large in oligotrophic oceans and lakes. Nitrogen fixation also plays a major role in coastal waters affected by large rivers. Their plumes may be characterized by an excess of phosphate and $Si(OH)_4$, which favors the development of **diazotrophs**. Nitrogen fixation, as well as breakdown of amino acids, yields **ammonium**, which is incorporated into organic molecules by various plants and microbes. Conversely, ammonium can be oxidized to **nitrate** (via nitrite) by **nitrification**, an energy-yielding process by **chemolithoautotrophic** bacteria and archaea. Nitrate (and ammonium) can be taken up and incorporated into **organic nitrogen** by algae, bacteria, and fungi. In **denitrification**, bacteria use nitrate as terminal electron acceptor during the decomposition of organic matter. This occurs in the absence of oxygen; in aquatic habitats it is generally limited to relatively eutrophic systems, below the thermocline or in sediments. It yields ammonium (nitrate ammonification), various nitrogen oxide products, and eventually **molecular**

Box 6.1: The River Continuum Concept

The **River Continuum Concept** assumes that physical variables vary in a continuous, predictable manner from a river source to its mouth, and that the resulting gradient elicits a suite of predictable responses in the biological communities and food webs. In forested regions, headwaters (stream orders 1–3) are heavily shaded. Reduced primary production in the stream is compensated for by abundant leaf litter. The ratio of primary production to community respiration is, therefore, below unity; invertebrate shredders are expected to be common. As we move downstream (orders 4–6), the stream widens, allowing greater autochthonous production (attached algae, biofilms) and becoming less dependent on riparian vegetation. In addition, fine particles may be imported from upstream sections. This is expected to increase the importance of grazers and collectors and support fewer shredders. Higher order streams (>7) may be too deep to allow much primary production and too wide to derive much benefit from the riparian vegetation. There may be some limited input from phytoplankton; imports from upstream, both in dissolved and particulate form, become increasingly important. Collectors are expected to be the dominant functional group.

nitrogen (N_2), a gas that is returned to the atmosphere and, therefore, represents a loss to the system.

Aquatic systems lack a typical, well-developed soil, which is a major nitrogen reservoir of terrestrial systems (though sediments may accumulate organic debris raining down from the water column). Similarly, they generally lack a large standing stock of vascular plant debris, which again retains a considerable amount of nitrogen in terrestrial habitats. On the other hand, water often contains a large variety of dissolved humic substances with variable amounts of nitrogen. These are generally recalcitrant and not readily used by aquatic organisms.

Organic nitrogen can enter streams via autumn-shed leaves, needles, and twigs from riparian trees and shrubs. This plant litter is generally low in nitrogen. During early stages of decomposition, fungi and bacteria take up inorganic nitrogen from the water column and use the litter's energy (stored in polysaccharides) to build up their biomass. This often results in an initial increase of nitrogen content of decaying plant litter (**nitrogen immobilization**).

Spawning salmon and a few other **anadromous fish** (are born in freshwater, migrate to ocean, then return to freshwater to reproduce) can be important local sources of nitrogen. Conversely, **catadromous fish** spend most of their lives in freshwater and migrate to the sea to breed, resulting in a net loss of nitrogen. The amounts of nutrients involved in these migrations can be very substantial. In small headwater streams in British Columbia (Canada), up to 40% of the nitrogen in the aquatic food chain is derived from the carcasses of coho salmon. Its release stimulates algal growth, which supports increased populations of Chironomids (*Insecta, Arthropoda*), mayflies, and stoneflies, providing the primary food of juvenile salmonids, which also feed directly on released eggs or decaying carcasses. Migrating salmons also enrich the surrounding terrestrial habitat; both black and brown bears, as well as various bird species, disperse their nutrients to the surrounding vegetation. Another huge transfer is based on fisheries. In 2005, an estimated 95 million tons of fish were landed commercially.

Guano is the dried excrement of seabirds, bats, and seals. It contains 11–16% nitrogen and 8–12% phosphorus. Guano produced by cormorants, pelicans, and boobies on islands and shores along the coast of Peru have long been harvested and used as fertilizers and as ingredients for explosives. At least locally it represents an important reversal of the usual movement of nitrogen and phosphorus from continents into oceans.

6.2.4.2 The Sulfur Cycle

Most of the earth's sulfur is tied up in rocks and salts or buried in marine sediments. Before the human activities of the past several centuries, sulfur became available to the biosphere primarily through the weathering of sedimentary **pyrite**. It moves through the global system by hydrologic transport. It also enters the atmosphere through natural and anthropogenic processes. Human activity, primarily through the combustion of fossil fuels, has altered the global cycle of sulfur to a greater extent than that of any other major element: one estimate suggests that some 70–100 Tg of gaseous S per year are due to anthropogenic activities, while only about 24–40 Tg S per year come from natural sources. Anthropogenically released S is mostly in the form of $\mathbf{SO_x}$ (SO_2 plus a small amount of SO_3). In the atmosphere, SO_x hydrates to $\mathbf{SO_4^{2-}}$, a nucleus for cloud formation. It dissolves in rainwater, forming dilute sulfuric acid. Damage to ecosystems results not so much from the direct effects of the acid, but rather from acid-induced **leaching** of Ca^{2+}, Al^{3+}, and various heavy metals. In Europe and North America the switch from high-S coal and oil to low-S fuels has lowered S deposition to such an extent that sulfur has become a limiting nutrient in many regions.

In aquatic ecosystems, inorganic sulfur occurs predominantly as **sulfate**, which is readily taken up by phytoplankton and other phototrophs. In the absence of oxygen (hypolimnion of eutrophic lakes, sediments), it can be used as terminal electron acceptor during the anaerobic oxidation of organic substrates. This **desulfurication** converts sulfate (elemental sulfur, or other oxidized inorganic sulfur compounds) to hydrogen sulfide.

The fate of sulfur ultimately depends on the combination of organic material, oxygen, and light. In addition to desulfurication, microbial breakdown of proteins is an important source of H_2S. In the absence of light and under aerobic conditions, **chemolithoautotrophic** bacteria oxidize hydrogen sulfide, as well as elemental sulfur and thiosulfate. Phototrophic, anoxygenic bacteria oxidize hydrogen sulfide in the presence of light and are strict **anaerobes** (*Chlorobiaceae, Chlorobium*) or **microaerobes** (*Chromatiaceae, Gammaproteobacteria*). These conditions are generally limited to a narrow band in the water column of lakes or oceans (no or very little oxygen, but sufficient light) and can result in massive blooms of sulfur bacteria.

The trace gas **dimethylsulfide** (DMS) is a breakdown product of **dimethylsulfoniopropionate** (DMSP), an osmolytically active specialized metabolite in marine algae (see Figure 10.3). It is the most abundant biological sulfur compound emitted to the atmosphere. Its release potentially returns sulfur to terrestrial ecosystems (Figure 6.14). DMS is a chemoattractant for zooplankton, seabirds, and marine mammals. In the atmosphere, it can be converted to dimethylsulfoxide (DMSO) or sulfate aerosols. Both can act as cloud condensation nuclei, leading to an increase in albedo, which reflects the incoming sunlight back into space and thereby modulates climate change. Each year, approx. 300 million tons are produced in the oceans.

6.2.4.3 The Phosphorus Cycle

Phosphorus is a major factor limiting the growth and activity of autotrophs and heterotrophic microorganisms in aquatic habitats. Higher P levels have repeatedly been

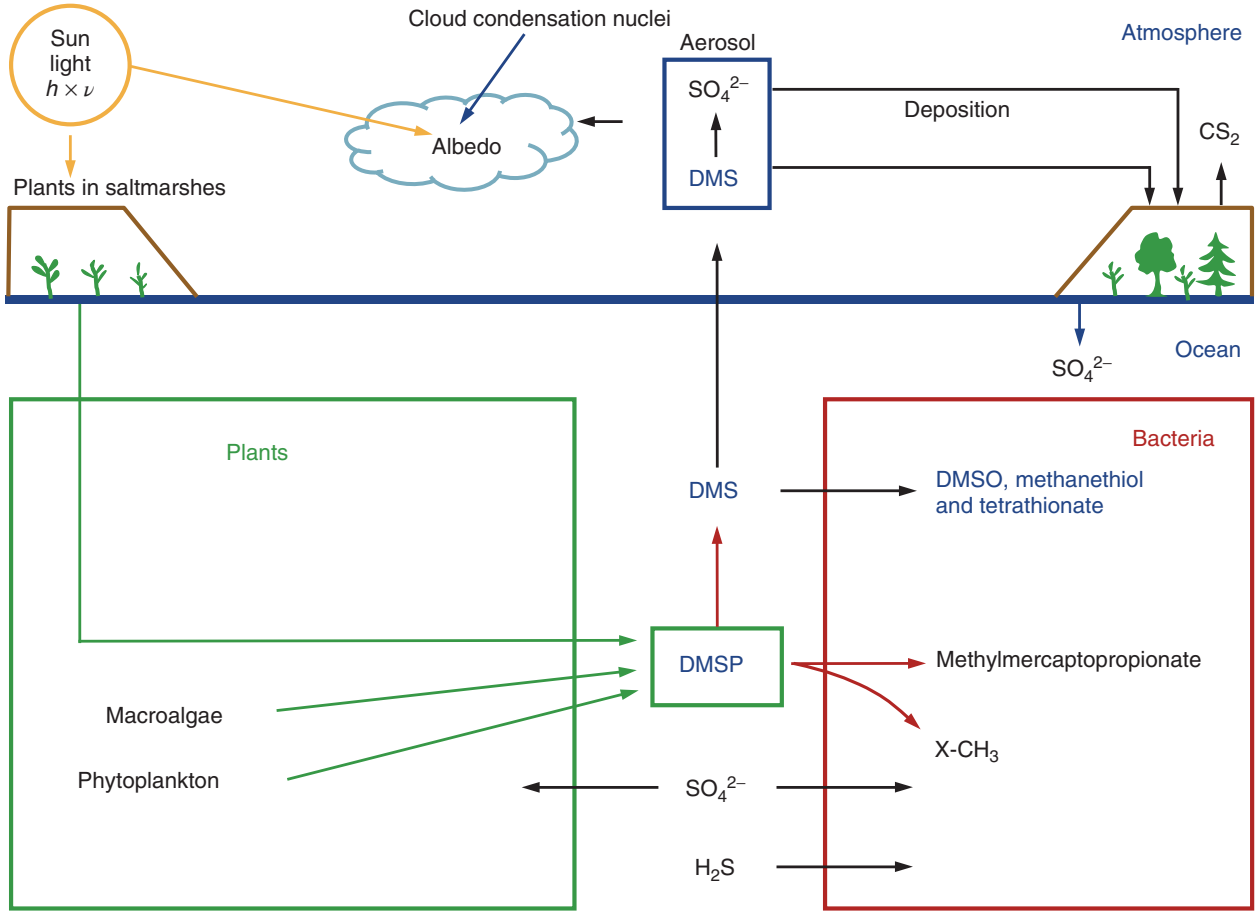

Figure 6.14 Dimethylsulfoxid (DMS) production and transformation in marine systems. (Adapted from Schäfer, Myronova, and Boden (2010) by permission of Oxford University Press.) DMSP – dimethylsulfoniopropionate; DMS – dimethylsulfide. (Graphics: G.-J. Krauss, D. Dobritzsch.)

shown to stimulate the activity and often the diversity of fungi decomposing plant litter imported from the riparian vegetation.

Phosphorus occurs as orthophosphate (PO_4^{3-}), either dissolved or attached to particles, or in soluble organic molecules, or in living or dead particulate form. Phosphorus levels are often expressed as **total phosphorus** (TP, includes all organic and inorganic forms) or **soluble reactive phosphorus** (SRP; orthophosphate and polyphosphates). SRP is considered the best indicator of phosphorus that is immediately available for microbial or plant uptake. As P cycles rapidly between organisms, TP allows more meaningful intersystem comparisons of potential productivity. On the other hand, major global transfers to the biosphere are slow and are ultimately based on weathering from rocks. P levels are usually higher in rivers draining sedimentary rocks and lower in regions of crystalline rocks. In some areas, geothermally influenced groundwater can be a significant source.

The primary source mineral for P is **apatite** [$Ca_5(PO_4)_3OH$]. Unlike other nutrients, P does not usually occur in gaseous form, except as phosphine (PH_3), which can form from organic detritus under reducing conditions (e.g., in sediments of peat bogs, mud flats, marshes). It may contribute to the appearance of will-o'-the-wisp (bluish-purple flames appearing spontaneously over stagnant waters), though the evidence is inconclusive. Despite the scarcity of gaseous forms of P, atmospheric input into aquatic systems can be significant. Smaller streams also receive P from throughfall and stemflow originating from the riparian vegetation.

Natural levels of dissolved P in freshwaters are low at around 0.01 mg P L^{-1} (PO_4^{3-}) and less severely disturbed by anthropogenic influence than N levels. Nevertheless, increased P input from agriculture and various effluents have been a primary cause for eutrophication in freshwater and marine systems. Excessive growth of phytoplankton, followed by their death and decomposition, can result in extensive anoxic zones in the water column.

In addition to dissolved P, terrestrial ecosystems also export particulate sources to rivers and lakes, in the form of plant detritus and organic and inorganic particles carried by surface runoff and subsurface pathways. Orthoposphate readily adsorbs to charged clay particles, whose import can rise dramatically during storms. Large rivers carry huge loads of sediment that they deposit in estuaries.

As with nitrogen, the movement of P in freshwater is predominantly downstream. It can be reversed by spawning salmon and other migrating fish, or by commercial fishery. The same applies to the huge guano deposits by seabirds.

6.2.4.4 The Carbon Cycle

With respect to carbon, most terrestrial ecosystems are open, with **photosynthesis** representing the main carbon input (see Section 6.3.3.4). The same process is important in aquatic systems, where it is dominated by phytoplankton rather than vascular plants, with the exception of some estuaries, marshes, and mangroves. Seaweeds, loosely defined as macroscopic, multicellular, marine algae, require a firm attachment point and, therefore, inhabit the littoral zone. They are most common on rocky shores.

Photorespiration (see Figure 1.7) in phytoplankton occurs primarily under conditions of high light and high oxygen concentrations. It promotes leakage of organics such as the photorespiratory-specific compound glycolate, which enters the bacterial loop.

Phytoplankters do not produce perennial structures, and both living and dead cells are more easily digested or broken down than the dead remains of vascular plants. Consequently, the accumulation of detritus is less pronounced in aquatic habitats than in soils, and well-defined litter layers are absent. Eutrophication in lakes and oceans results in decreased species diversity, increased biomasses of autotrophs and consumers, higher turbidity and rate of sedimentation. During the decay of this organic debris on the bottom of lakes and oceans, various electron acceptors (oxygen, ferric iron, sulfate, nitrate) are depleted, while hydrogen and carbon dioxide accumulate. At this point, only methanogenesis and fermentation are possible. **Fermentation** produces small organic compounds (ethanol, lactic acid, etc.), while **methanogenesis** removes intermediate products of decay (hydrogen, small organics) and carbon dioxide, and converts them into methane, a potent greenhouse gas that potentially returns carbon to the atmosphere. Without methanogenesis, much more carbon would accumulate in anaerobic sediments in ponds, lakes, and oceans.

Methane is relatively energy-rich. It can profitably be oxidized aerobically, for example, at the oxic–anoxic transition zone in freshwater lakes. It can also be oxidized anaerobically (e.g., with sulfate as electron acceptor, yielding HCO_3^- and HS^-). Some consortia of Archaea and Bacteria can oxidize methane with nitrate instead of sulfate. Anaerobic oxidation of methane has been estimated to remove almost 90% of the methane released from marine sediments. In addition to detrital organic matter, hydrothermal vents are another source of methane.

Various Bacteria and Archaea gain energy from the oxidation of inorganic compounds (e.g., CH_4, H_2, NH_3, H_2S, Fe^{2+}) and use it to convert carbon dioxide into organic molecules (chemolithoautotrophy). This can occur in the water column of lakes, in sediments, or near hydrothermal vents.

Freshwater ecosystems are often characterized by the relative contributions of **autochthonous** (*in situ*) and **allochthonous** (imported) carbon sources. This has been approximated by comparing daily or seasonal changes in oxygen and carbon dioxide concentrations. A net oxygen gain indicates that photosynthesis dominates, and the ecosystem is then called *autotrophic*. If there is a net release of carbon dioxide, respiration exceeds photosynthesis and the system is called *heterotrophic*. Small headwater streams are typically heterotrophic, that is, most of their OC is imported from the surrounding terrestrial ecosystem in the form of DOM and vascular plant litter. As the stream widens, photosynthesis increases and there may be a net release of oxygen. In large rivers, effluents from cities and industry can introduce large amounts of organic materials and profoundly affect the carbon budget.

6.3 Terrestrial Systems

6.3.1 Trophic Cascades

The **trophic cascades** and the central processes involved in matter and energy flow in trophic pathways of terrestrial systems are summarized in Figure 6.15, using a tree as an example of a terrestrial primary producer. As in aquatic systems (see Section 6.1), primary producers constitute the starting point of trophic cascades that include herbivorous and detrivorous food chains, both above and below ground. However, detrivorous food chains in terrestrial systems are of much greater significance below ground, because the degradation of both above- and below-ground detritus, including leaf litter, largely takes place below ground.

Grazing food chains in terrestrial systems consist of plants as primary producers, herbivores feeding on these primary producers as primary consumers, and carnivores as secondary consumers, including herbivore predators and parasitoids feeding on herbivores, and a variable and diverse cascade of higher order carnivores (Figure 6.15). Herbivory is ubiquitous in the animal kingdom. Usually, herbivores do not consume entire plants but have specialized on the consumption of specific plant organs or tissues. Caterpillars, for example, are almost exclusively leaf consumers, whereas other insects such as aphids and cicadas are phloem- and xylem suckers, respectively. Miners invade the plant and live endophytically in a particular plant organ, such as stems, leaves, or roots. The use of plants as sole food sources by herbivores poses a difficult challenge to these primary consumers, because nutrient concentrations in plant and animal tissues differ considerably, with low nitrogen and phosphorus contents prevalent in plants. This is reflected by the C : N ratio of biomass that usually approximates 40:1 in plants, and is lower than 10:1 in most animals. Herbivores play a decisive role in overcoming these

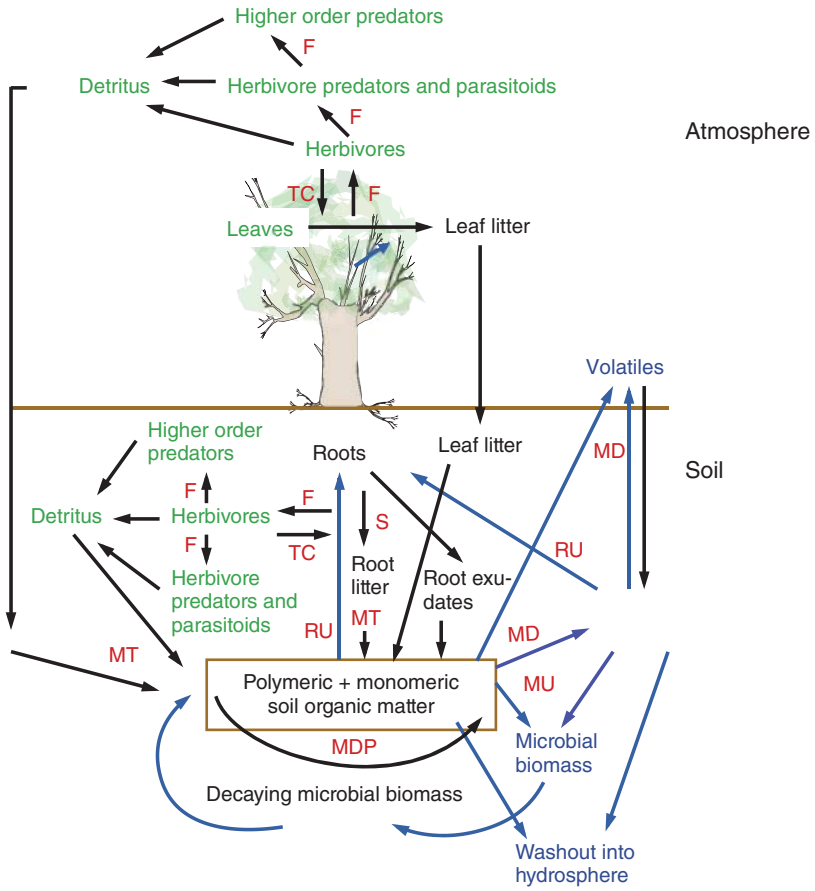

Figure 6.15 Trophic cascades in terrestrial systems. Green: herbivorous food chains; black and blue: detrivorous food chains; blue: conversion of monomeric soil organic matter; red: processes mediating trophic cascades: F – feeding; MC – microbial consumption; MD – microbial degradation; MDP – microbial depolymerisation; MT – mechanical transformation; MU – microbial uptake; RU – root uptake; S – senescence; TC – trait changes.

imbalances, a prerequisite for the development of efficient food chains and webs. Still, some of the most abundant, relatively energy-rich components of plant tissues, for example, cellulose and lignin, cannot be used directly by most herbivores and require microbial symbioses in their digestive apparatus for their use as food. This applies not only to ruminants, but also to many insects.

Carnivores usually kill their prey and need a number of prey animals during their life cycle, with the exception of parasitoides that finish their life cycle in one distinct organism. Carnivores experience a more homogeneous food composition much closer to their own requirements than herbivores. After primary producers and herbivores occupying the first and second trophic level, respectively, carnivores feeding on herbivores are third trophic level animals, carnivores feeding on carnivores higher trophic level animals. **Omnivory**, a common phenomenon in the animal kingdom, may blur the distinction between trophic levels, as omnivores feed on food from organisms at different trophic levels. This phenomenon is observed, in particular, at higher trophic positions. For example, numerous herbivores improve their nutrition by protein-rich animal food in order to enhance their nitrogen supply.

Detritus food chains start primarily with the mechanical transformation of litter of primary producers (e.g., leaf and root litter) and detritus of herbivores, herbivore predators, and higher order carnivores (Figure 6.15). This transformation includes comminution of plant litter and detritus, mixing organic matter with mineral soil, and improving soil aeration, all of which provide more favorable conditions for subsequent microbial degradation. Soil invertebrates such as earthworms (*Olgochaeta, Annelida*) are of particular significance in this mechanical transformation, but at the same time extract easily available nutrients from the detritus for their nutrition. Soil microbial degradation is initiated by the depolymerization of soil organic matter. This soil fraction is generated not only from detritus, leaf, and root litter, but also from decaying microbial biomass. The released monomeric compounds, for example, amino acids and sugars, are subject to root uptake and incorporation into plant biomass, microbial uptake, and incorporation into microbial biomass and microbial degradation to soil inorganic matter. The soil inorganic matter produced may be taken up by plant roots and microbes for incorporation into biomass or may be further converted by soil microbes

for energy production (e.g., by **nitrification** and **denitrification**). Thus, plant roots and soil microbes interact in below-ground food chains not only at different but also at the same trophic level by competing for nutrients. Constituents of both soil organic and inorganic matter may also be washed out into the hydrosphere. During microbial degradation of soil organic and inorganic matter, volatile intermediates, including the greenhouse gases methane (CH_4) and nitrous oxide (N_2O), are produced that can be released into the atmosphere. Depending on the conditions of the soil, these compounds can also be removed from the atmosphere by soil microbial consumption. Thus, the soil can be a source or a sink of atmospheric trace gases, depending on the prevailing producing or consuming microbial processes in the soil.

6.3.2 Community Dynamics and Its Regulation

The **community dynamics** in grazing (herbivore) food chains is determined by the dynamics of resources available from primary producers, herbivore-induced trait changes (defense measures) in primary producers (bottom up), and the dynamics of predator populations (top down), which are all subject to modulation by a suite of environmental factors. According to the **exploitation ecosystem hypothesis** (EEH), habitats with plant biomass limited by nutrient availability and, hence, low productivity such as tundra can only support small herbivore populations. Habitats with moderate productivity such as temperate forests and grasslands can support herbivores but cannot support an appreciable predator population, and, therefore, are characterized by plant–herbivore interactions. Highly productive habitats such as tropical forests can support both herbivore and predator populations with the results that the predators keep the herbivores sufficiently low to prevent herbivore effects on plant biomass. Recent manipulative approaches have revealed, however, that top-down regulation by the dynamics of predator populations is also relevant in habitats not characterized by high productivity. This type of regulation is reflected in the **green world hypothesis** (HHS), which predicts that nutrient-limited predator populations suppress herbivores to densities that cause negligible biomass losses to plants by herbivory. Thus, the abundance and biomass of a given population in a food web can be controlled by populations at higher trophic levels (**top-down regulation**), lower trophic levels (**bottom-up regulation**), or the same trophic level (**competitive interaction**).

Interactions within **trophic cascades** can take place at two (two-trophic cascades) or more trophic levels (tritrophic cascades, multitrophic cascades). At two-trophic levels, herbivore-plant, parasitoide-host, and predator–prey interactions have to be distinguished. An obviously negative consequence of herbivory is the loss of plant biomass and a concomitant reduction in plant growth and reproduction. The reduction in growth often exceeds the amount that can directly be attributed to biomass loss. Additional growth reduction may be because of the investment of plant resources in various defense measures. These defense measures are usually induced by insect saliva, as mechanical injury often fails to initiate similar developments. They include trait changes in allelochemistry, physiology, cell structure, morphology, allelometry, and phenology of plants. These changes are not restricted to the organs directly affected by herbivory, but rather take place at the whole plant level (**systemic defence reactions**). Thus, leaf herbivory can result in a reduction in net primary productivity because of both insect frass and plant trait changes. The reduction in net primary productivity is usually not a consequence of reduced net photosynthesis by the remaining photosynthetically active tissue. On the contrary, it is often at least partially compensated for by enhanced net photosynthetic activity of the remaining leaf tissue because of a reduction of **sink limitation** of carbohydrate production at the whole plant level. This can result in a rapid regeneration of the biomass lost by herbivory. Thus, reduction of sink limitation of photosynthetic carbon assimilation may be considered a mechanism of defense.

The vulnerability of plants to herbivory is largely determined by its tolerance or resistance. **Tolerance** will not reduce the extent of biomass consumption but will reduce or prevent damage. This can be achieved by compensation or overcompensation of the loss of biomass by herbivory and/or its consequences. Compensation or overcompensation can be mediated (i) by enhanced photosynthetic carbon assimilation by the remaining leaf tissue and/or (ii) at the expense of investment in stored reserve material. Thus, tolerance to leaf or root herbivores can best be achieved by plant species exhibiting below-ground (e.g., grasses) or stem/trunk storage (e.g., deciduous tree species) of reserve materials; plant species exhibiting leaf storage of reserves (e.g., conifer trees) will preferentially exhibit tolerance to root herbivores, as leaf herbivores will consume this storage pool. The use of reserves may, however, negatively affect future seed production based on mobilized reserves and, hence, **plant fitness**.

In contrast to tolerance, **resistance** mechanisms reduce the *frass* intensity of herbivores. This is achieved by variable food quality between plants or plant species or/and by negative consequences on the performance of a herbivore eating a particular plant or plant species. Differences in nitrogen content, in the abundance of structural components that are difficult to digest such as cellulose and lignin, and in water content strongly affect the food preference of herbivores. Generally, young plant tissues are more attractive than mature plant tissues. In addition, plants have developed specific mechanical and chemical resistance mechanisms. **Mechanical resistance** includes the formation of thorns, spikes, and trichomes, which reduce the accessibility of edible plant material, but can also harbor herbivore predators (ants, predatory mites). **Chemical resistance** due to plant

secondary metabolites includes toxins, digestion inhibitors, and repellents but also volatile organic compounds (VOCs) emitted to attract herbivore predators. Extrafloral nectaries excreting sugars can be interpreted as a combined mechanical and chemical defense measure that also will attract herbivore predators.

In **carnivory**, the general difference between predator-prey and parasitoid-host interaction, that is, the requirement of many *versus* one food source organism, causes drastic differences in population dynamics. If the infection of a host by a parasitoid population causes its death, it will also cause the death of the parasitoid and, thus, will have much more severe consequences on the parasitoid than on the host population density. By contrast, a high predator population density can have a severe impact on the prey population density. Depending on the diversity of food sources, the predator population dynamics can be uncoupled from, or dependent on, a particular prey population. The abundance of predators with a broad prey spectrum will not change significantly, when the abundance of any particular prey population changes. Conversely, the abundance of a highly specialized predator will strongly depend on the abundance of its prey.

Disturbances of trophic cascades may lead from multitrophic-level to two-trophic-level interactions. For example, plants respond to insect herbivory by the production and emission of VOCs that can attract herbivore predators and parasitoids including carnivorous insects. Thus, in plant–insect communities the dynamics of the food chain cascade can follow a top-down approach that is mediated by an **infochemical web** that follows a bottom-up approach. Disturbances in plant–herbivore interactions, for example, in the communication with predators, by transiently reduced resistance of the host plant, or temporarily high resource abundance (e.g., as a consequence of storms), can cause cyclic **mass outbreaks** of insects that result in almost complete defoliation and partial dieback at the host plant level, severe nutrient losses, disturbances of nutrient cycles, and altered vegetation structures at the ecosystem level. In North America, more than 20 million ha of forested land are affected annually by such insect mass outbreaks.

Independent of disturbances, **pulsed resource availability** of primary producers will often determine the dynamics of consumer populations in food chains. In many terrestrial systems pulsed resource availability is an inherent feature. For example, reserves stored in various organs of perennial plants are often used extensively for the spring flush of leaves. Thus, food quality and quantity available for herbivores feeding on these reserves is rapidly reduced during spring and slowly recovers during summer and fall. Availability of young leaves for herbivores can be restricted to a few weeks (e.g., European beech), to two or more flushing events (several oak species), or can be continuous (e.g., birch). Nutrient input into detritus food chains can also be pulsed (autumn leaf fall in temperate deciduous forests, mast seeding) or continuous (needle shedding in many conifer forests). However, the timing of inherently pulsed resource availability from primary producers is strongly influenced by weather and climate, for example, temperature, temperature development, and temperature extremes. Thus, the initiation of spring flushing in temperate forests and, hence, the availability of young leaves for herbivores has advanced following **global climate change**. As a consequence, the risk of damage by late frost events to primary producers and of an uncoupling between the development of primary producers and herbivores has increased.

In addition to the inherent pulsed availability of resources from primary producers, **extreme climatic events** can induce such pulses. For example, precipitation changes associated with the El Niño Southern Oscillation (**ENSO**) have a dramatic impact on terrestrial systems, in particular, on the primary productivity of arid and semiarid systems limited by precipitation, thereby altering food chains and food webs for months and years after an ENSO event.

Pulsed availability of resources from primary producers is by definition an episodic event, sometimes of relatively regular (leaf shedding), sometimes of completely unpredictable (extreme weather events) timing. It is, therefore, to be expected that few consumers have specialized on this type of food supply. As a consequence, **trophic generalists** are likely to respond to the pulsed availability of particular resources, but can switch to other resources between such pulses. The occasional high abundance of food will cause a rapid increase in the population density of these generalists, but their population will just as rapidly decline after the depletion of the resource. Predators of these generalist consumers will show a similar pattern of population density development with similarly high fluctuations, but with a temporal delay. Depending on the duration of the resource pulse and the extent of this delayed response, predators may or may not affect primary productivity. This way, pulsed resource availability elicits trophic "bottom-up" dynamics that can initiate "top-down" regulation in terrestrial systems.

6.3.3 Nutrient Cycles

Nutrient cycles in terrestrial systems are the consequence of herbivorous and detrivorous food chains/food webs and integrate nutrient movements within individual organisms. Ecosystem and landscape nutrient cycles can be open or closed, depending on nutrient availability, plant development, and environmental factors. For example, riparian forests exposed to continuous and high nutrient input from the flowing water body exhibit **open nutrient cycles**, whereas forests on limestone-derived soil in remote areas with low anthropogenic nutrient input often experience low nutrient availability and have developed largely **closed nutrient cycles** (Figure 6.16). On felling or wind break of trees in these forests, nutrient availability in the soil

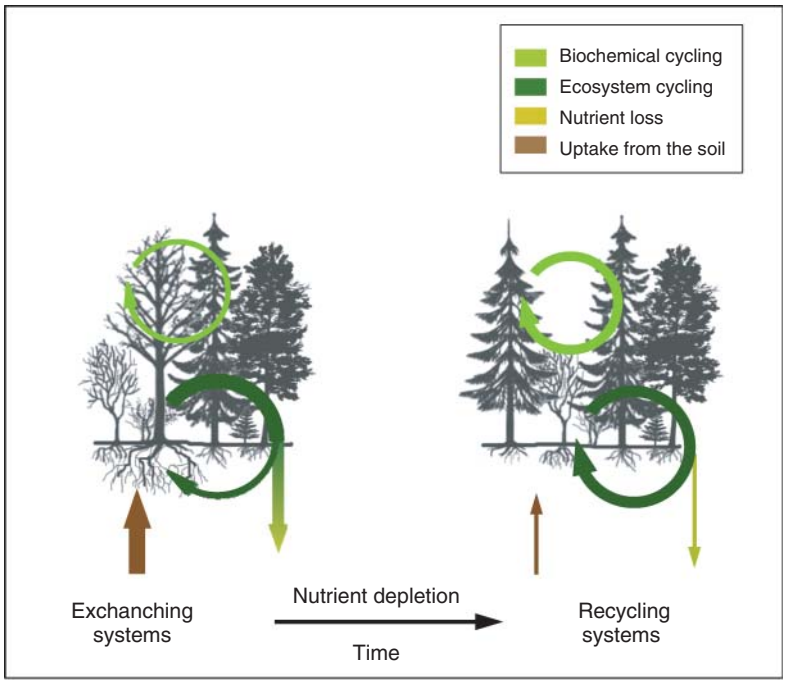

Figure 6.16 Switch from open to largely closed nutrient cycles. Arrows indicate nutrient fluxes.

increases because of decaying root biomass, resulting in a switch from largely closed to open nutrient cycles. As a consequence, naturally regenerating forests after clear cutting initially experience open nutrient cycles. However, the open cycles in such forests close again within a few years, so that young trees in a regenerating forest already experience largely closed nutrient cycles. A switch from largely closed to open nutrient cycles is also observed, when nutrient-limited ecosystems experience anthropogenic nutrient input, for example, N input from fossil fuel combustion or fertilization in agriculture. Irrespective of the causes, such a switch has severe consequences at the ecosystem level. The ecological process system changes from maintaining nutrient cycling to maximizing nutrient uptake; nutrient uptake is controlled by geochemical cycles rather than by cycling within the ecosystem. The nutrient source changes from the forest floor to the mineral soil and nutrient losses from being low to being high.

Plant internal nutrient cycles are often the consequence of seasonal storage and mobilization processes. Irrespective of whether ecosystem/landscape nutrient cycles are open or closed, **plant internal nutrient cycles** are usually open. Thus, even though a significant portion of the nutrients in leaves is retrieved and transported into storage pools before the shedding of leaves, a relatively high amount of nutrients remains in leaf litter. As a consequence, nutrient storage pools in perennial plants have to be replenished during the growing season from external sources. Thus, open plant internal nutrient cycles are a major factor in the redistribution of nutrients during ecosystem development. This redistribution is of particular importance in ecosystems that are characterized by largely closed ecosystem nutrient cycles where it is an important driver of ecosystem processes.

6.3.3.1 The Nitrogen Cycle

Nitrogen is an essential element of all living organisms. Its availability largely determines the growth and development of organisms in many ecosystems. Generally, nitrogen is available in ecosystems from internal and external sources. Internal **nitrogen sources** include the degradation of plant, microbial, and animal biomass; external sources are wet and dry deposition into plants and soil from the atmosphere, nitrogen sources in weathering rocks, and the hydrosphere. When nitrogen input from external sources is low, ecosystems rely on internal sources that may have accumulated over long periods of time and are trapped inside the ecosystem thereby generating a largely closed ecosystem internal nitrogen cycle. Within ecosystems, nitrogen is subjected to a high rate of turnover mediated by microbial and, to a lesser extent, plant processes. With these processes that are mostly located in the soil, plant and microbes compete for the growth-limiting resource nitrogen.

In the soil, decaying plant litter (leaves, roots, branches, seeds) and microbial biomass constitute the most important nitrogen sources (Figure 6.17). In addition, dinitrogen (N_2) fixation by free living and, more importantly, by symbiotic microorganisms may contribute significantly in certain ecosystems, for example, in tropical forests. Only a minor part of the nitrogen content in litter is present in easily available organic (amino acids, amino sugars) or inorganic (nitrate, ammonium) form. Most of the nitrogen is present in complex organic molecules (e.g., proteins,

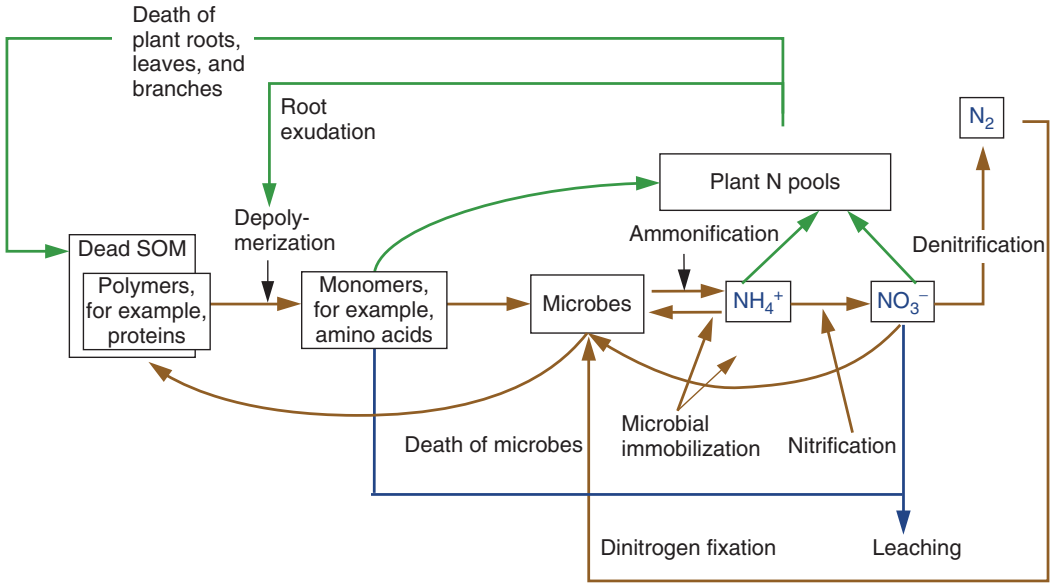

Figure 6.17 Nitrogen cycling in ecosystems. Plant processes (green); microbial processes (brown); transport in the hydrosphere (blue). (Reprinted from Rennenberg *et al.* (2009) with kind permission by John Wiley & Sons.)

nucleic acids, lignin-like compounds) that first have to be degraded by **microbial depolymerization** in order to become available for growth and development of plants and microbes. An important driver of this and other microbial processes is root exudation of carbohydrate as an essential microbial energy source. By this exudation, plants control microbial activity including microbial depolymerization in the soil.

In an increasing number of ecosystems, amino acids liberated by microbial depolymerization have recently been revealed as an important nitrogen source of plants taken up by the roots. Often this **organic nitrogen source** is preferentially taken up over **inorganic nitrogen sources** (ammonium, nitrate). Root uptake of amino acids and ammonium competes with microbial processes, such as aerobic **nitrification**. In particular, autotrophic nitrifiers exhibit a high turnover of reduced nitrogen, as they use this substrate as both a source of energy and as a source for biosynthesizing their own nitrogen compounds. Therefore, aerobic nitrifiers are strong competitors for root uptake of reduced nitrogen. The nitrate produced in nitrification can be taken up by the roots or, under anaerobic conditions, can be reduced via N_2O to N_2 by **denitrification**. In addition to microbial nitrate uptake, root uptake of nitrate will also prevent nitrate leaching into the hydrosphere, irrespective of whether it is subjected to xylem transport to the leaves, nitrate reduction and assimilation in the roots (Figure 6.17), or storage in root cell vacuoles. Competition between root nitrate uptake and denitrification is supposed to be low, because root uptake processes are inhibited under anaerobic conditions, whereas denitrification is fully dependent on anaerobic conditions. Net N_2 exchange between soil and the atmosphere is dependent on the difference between the rates of N_2 fixation and denitrification to N_2 and can thus result in either a loss or a gain of nitrogen at the ecosystem level. In addition to N_2O, NO is another gaseous intermediate of denitrification. Apparently, NO is sensed by the roots as a measure of microbial nitrogen turnover and can stimulate root uptake of reduced nitrogen by the roots in order to counteract microbial nitrogen use.

The consequences of atmospheric **nitrogen deposition** on nitrogen cycling at the ecosystem level has been studied in detail in a spruce forest (Figure 6.18). In this case study, deposition of around 40 kg N ha^{-1} y^{-1} resulted in a volatilization of 16 kg N ha^{-1} y^{-1} into the atmosphere, mostly as NO, and a leaching of 21 kg N ha^{-1} y^{-1} into the hydrosphere in the form of nitrate. Microbial N turnover was sevenfold higher than microbial storage indicating much greater dynamics of the microbial compared to the plant nitrogen pool. This is achieved by high rates of mineralization, nitrification, and immobilization. Apparently, opening the ecosystem cycling of nitrogen by nitrogen fertilization results in high nitrogen input into both atmosphere and hydrosphere.

In perennial plants, plant internal nitrogen cycling is regulated by temperature and day length (Figure 6.19). When nitrogen is needed in high amounts for flowering and leaf development in late winter/early spring, most of the nitrogen is retrieved from internal storage pools. These important developmental processes thus become independent of nitrogen uptake by the roots, which may be restricted in late winter/early spring by unfavorable soil temperatures. During the growth season, nitrogen storage pools will fill up again, in particular, at the end of the growth season, when nitrogen is retrieved from senescing leaves. Both storage and mobilization require a complex set of biochemical as well as long-distance

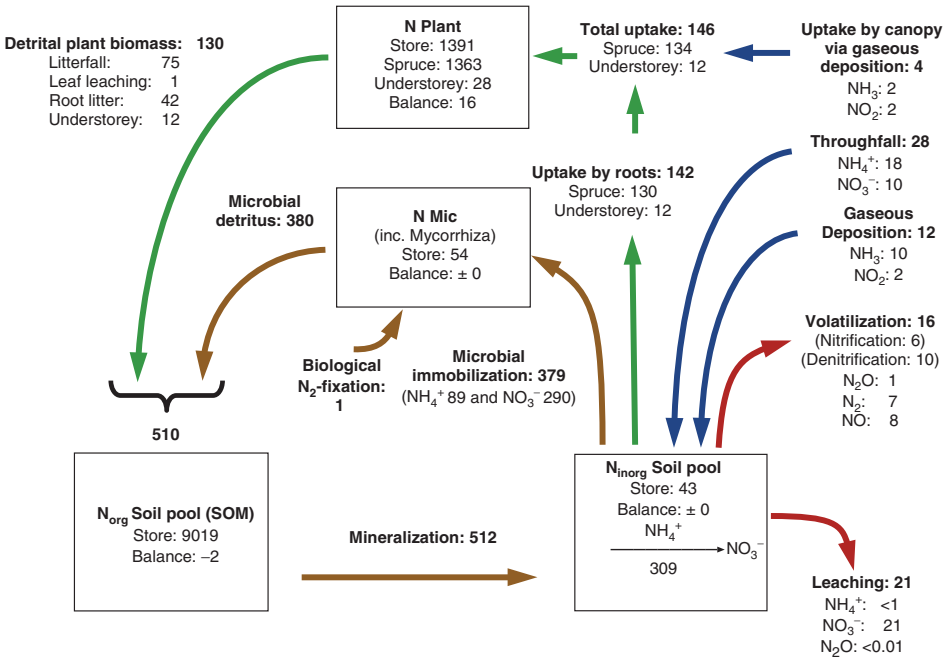

Figure 6.18 Nitrogen cycling in a mature spruce forests exposed to high atmospheric nitrogen deposition. Pools (boxes) are given in kg N ha^{-1}, fluxes (on arrows) in kg N ha^{-1} y^{-1}. Blue arrows, input into the ecosystem; red arrows, output of N into atmosphere and hydrosphere; brown arrows, internal cycling of N driven by microbial activity; green arrows, internal N cycling driven by plant N metabolism. (Modified from Kreutzer *et al.* (2009) with kind permission by John Wiley & Sons.)

transport processes. **Storage** requires (i) degradation of leaf protein on senescence, (ii) conversion of amino acids into a transportable form (e.g., glutamine, asparagine, arginine), (iii) transport of these amino acids in the phloem to the site(s) of storage, (iv) partial removal from the transport tissue and transfer into storage tissues, (v) conversion of transport amino acids into amino acids required for storage protein synthesis, and (vi) synthesis of vegetative storage proteins. For **mobilization**, the following steps are required: (i) degradation of vegetative storage proteins, (ii) conversion of the amino acids liberated into transportable amino acids (e.g., glutamine, asparagine), (iii) transport of amino acids in the xylem, but often also in the phloem, to the site(s) of use, for example, the developing leaves, (iv) removal of transport amino acids from the transport tissue and transfer into developing tissues, (v) conversion of transport amino acids into amino acids required for functional protein synthesis, and (vi) synthesis of functional proteins, for example, rubisco. The regulatory processes that control these complex sets of reactions are only partially understood (Figure 6.19).

During the growth season, the nitrogen storage pools of perennial plants that in most cases are only partially depleted will be filled up from nitrogen sources taken from the soil. In addition, soil-derived nitrogen will contribute to the nitrogen needed for growth and development not only of herbaceous plants, but also of perennial plants with continuous leaf development during the growing season. For this purpose, nitrate and ammonium have to be reduced and/or assimilated into organic nitrogen

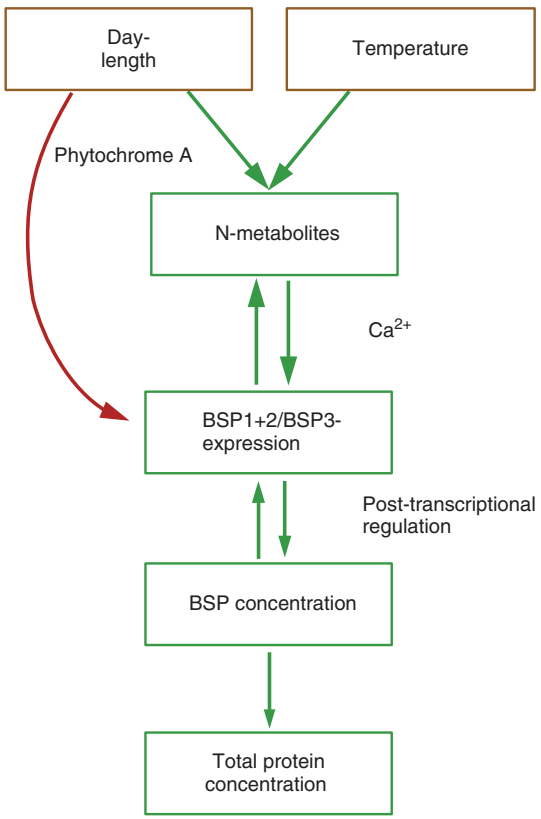

Figure 6.19 Regulation of seasonal N storage and mobilization in perennial plants. BSP, vegetative storage protein. (Modified from Rennenberg, Wildhagen, and Ehlting (2010) with kind permission by John Wiley & Sons.)

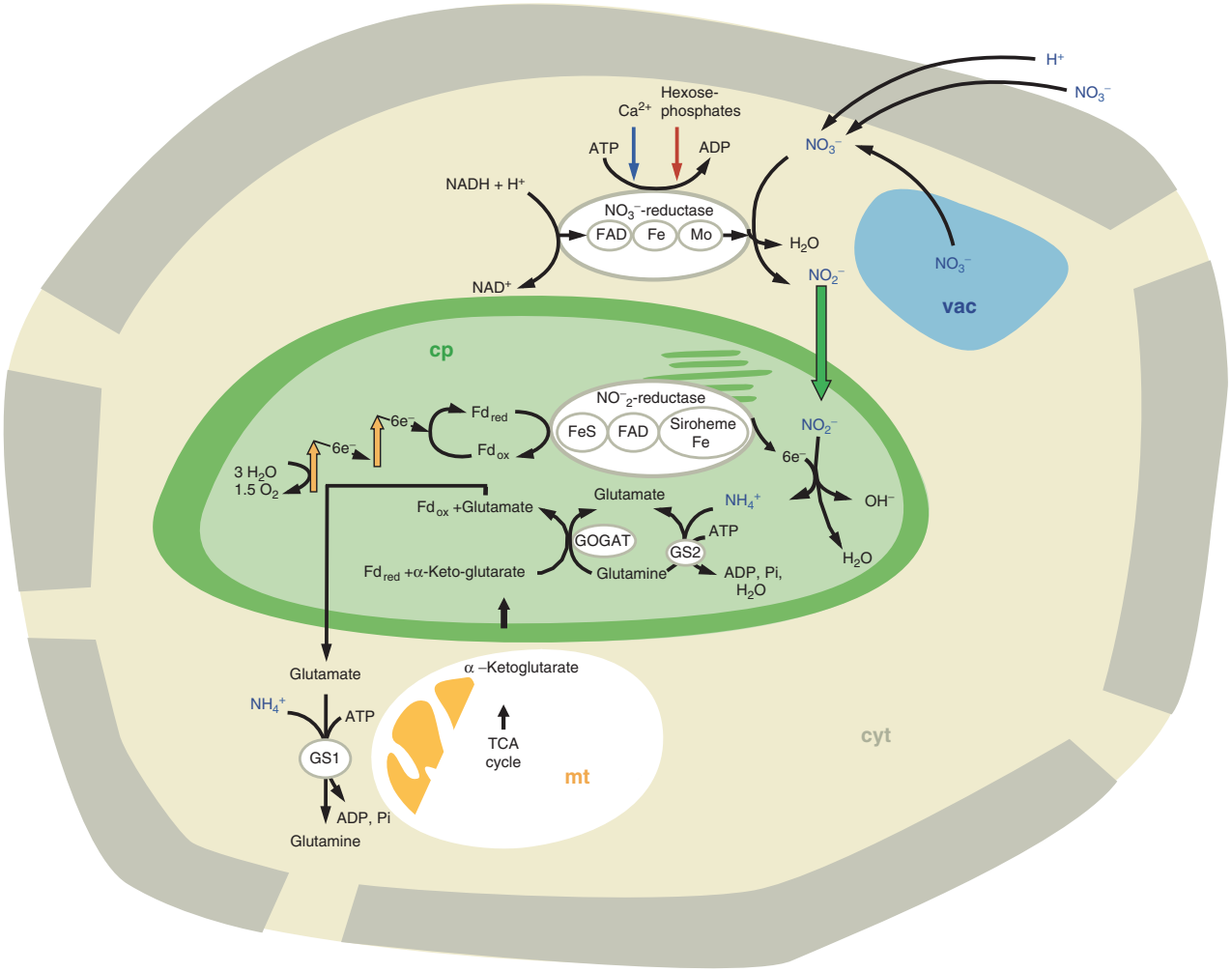

Figure 6.20 Nitrate reduction and assimilation in plants. GS1, cytosolic glutamine synthetase; GS2, plastidic glutamine synthetase; GOGAT, glutamate synthase. CYT – Cytosol; CP – Chloroplast; MT – Mitochondria; VAC – Vacuole. (Graphics: G.-J. Krauss, D. Dobritzsch.)

compounds (Figure 6.20). Depending on the species and environmental conditions, these processes can take place exclusively in the roots, exclusively in the leaves, or can be distributed between these plant organs in variable proportions. Irrespective of this distribution, **nitrogen reduction and assimilation** require a concerted action of at least three different cellular compartments, that is, the cytosol, the plastids, and the mitochondria (Figure 6.20). In addition, nitrogen sources taken up by a cell can also be stored in the vacuole. Nitrate is reduced in the cytosol to nitrite by **nitrate reductase**, a highly regulated key-enzyme of nitrogen reduction and assimilation in plants. The nitrite produced has to be transported into the plastids for further reduction to ammonium by **nitrite reductase**. The ammonium produced in this reaction as well as ammonium taken up from the soil is then incorporated in the plastids into organic nitrogen, namely, glutamate, by the **glutamine synthetase-glutamate synthase (GS-GOGAT) cycle**. In this cycle, ammonium is used for the synthesis of glutamine from glutamate in an ATP-dependent reaction catalyzed by glutamine synthetase (GS). The glutamine produced is converted into two molecules of glutamate in a reaction catalyzed by GOGAT (glutamate synthase) by transferring ammonium from glutamine to α-oxo-glutarate that originates from the TCA cycle of the mitochondria. The required reducing power for this process is generated by photosynthesis, if nitrogen assimilation takes place in the leaves, or by carbohydrate degradation, if nitrogen assimilation takes place in the roots. Thus, the initially consumed molecule of glutamate is regenerated and an additional molecule of glutamate is produced from ammonium and α-oxo-glutarate. Glutamate is the starting point for the synthesis of all other amino acids, mostly mediated by **transamination** reactions. Amino acids taken up by roots are also subjected to various transamination reactions before their use in growth and development.

6.3.3.2 The Sulfur Cycle

Sulfur is an essential constituent of amino acids and protein, many regulatory (e.g., vitamins) and secondary metabolites. It is available to plants mostly as **sulfate** in the soil. Roots

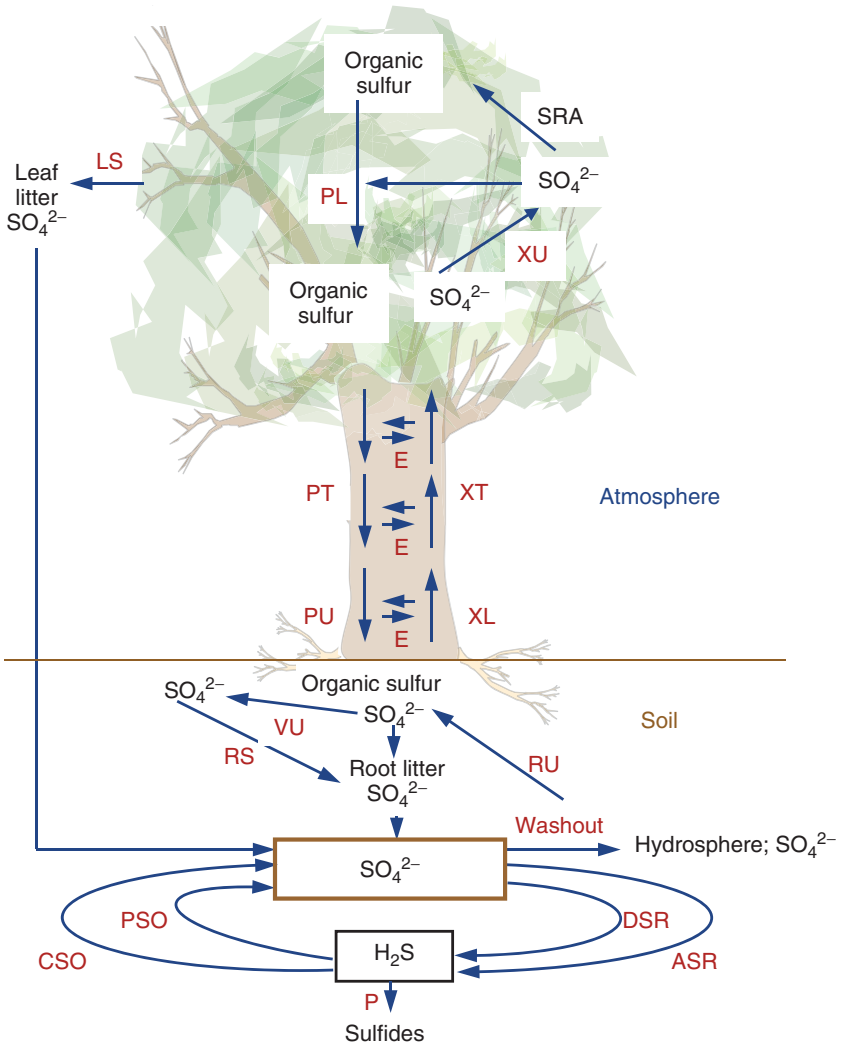

Figure 6.21 Sulfur cycling in ecosystems. LS – leaf senescence; PL – phloem loading; XU – xylem unloading; E – phloem–xylem exchange; PT – phloem transport; XT – xylem transport; PU – phloem unloading; XL – xylem loading; RS – root senescence; VU – vacuolar uptake; DSR – dissimilatory sulfate reduction; ASR – assimilatory sulfate reduction; CSO – chemolithotrophic sulfide oxidation; PSO – phototrophic sulfide oxidation; P – precipitation.

are also capable of taking up organic sulfur in the form of amino acids (cysteine, methionine) or peptides (e.g., glutathione); however, the significance of organic sulfur uptake for the nutrition of plants has not been established. Although the concentrations of plant-available sulfate in the soil solution is low, it usually exceeds the plants' sulfur requirement for growth and development. Nevertheless, sulfur deficiency is observed in intensive agriculture with crops of high sulfur demand (e.g., oilseed rape) and at low atmospheric sulfur input. Under these conditions, sulfur deficiency can be remediated by fertilization to prevent yield losses. At the ecosystem level (Figure 6.21), plant sulfate uptake competes with microbial sulfate acquisition for assimilatory (aerobic) and dissimilatory (anaerobic) **sulfate reduction to sulfide** (H_2S). The H_2S produced can be recycled by bacterial processes to sulfate either aerobically by chemolithotrophic sulfur oxidation or anaerobically by phototrophic sulfur oxidation. **Elemental sulfur** (S^o) is an important intermediate in these microbial processes that can accumulate in soils in addition to precipitation of sulfides of low solubility.

Sulfate taken up by roots is mostly stored in root vacuoles and only a minor part is subject to xylem loading and is transported to the leaves with the transpiration stream (Figure 6.21). Thus, sulfate uptake by the roots strongly exceeds the sulfur requirement of plants. Sulfate is released from this pool upon root senescence. It has therefore been assumed that roots constitute a transient storage pool of sulfate that prevents sulfate from being washed out of the soil during precipitation events. Only a minor part of the organic sulfur required for growth and development of plants seems to be produced in the roots, loaded into the xylem and transported to other tissues with the transpiration stream. Most of the plant's reduced sulfur is produced

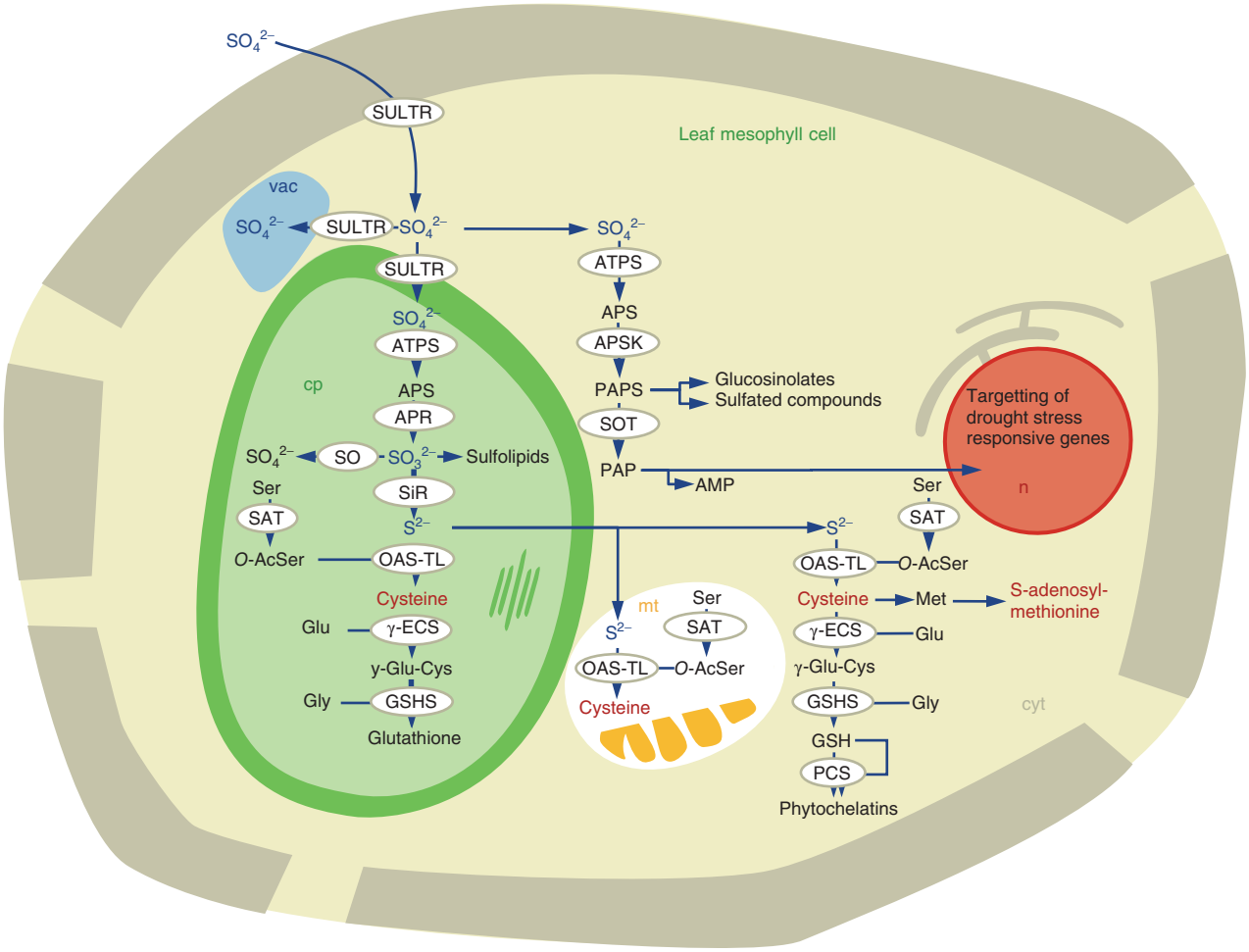

Figure 6.22 Sulfur reduction and assimilation in plants. SULTR – sulfate transporter; ATPS – ATP sulfurylase; APS – adenosine 5′-phosphosulfate; PAPS – phosphoadenosine 5′-phosphosulfate; PAP – phosphoadenosine 5′-phosphate; *O*-AcSer – *O*-acetyl serine; Ser – serine; Glu – glutamate; Gly – glycine; Met – methionine; APR – APS reductase; APSK – adenosine 5′-phosphosulfate kinase; ATPS – ATP sulfurylase; SOT – sulfotransferase; SO – sulfite oxidase; SAT – serine acetyltransferase; SiR – sulfide reductase; OAS-TL – *O*-acetylserine(thiol)lyase; γ-ECS – γ-glutamylcysteine synthetase; GSHS – glutathione synthetase; PCS – phytochelatin synthase; CYT – Cytosol; CP – Chloroplast; N – Nucleus; VAC – Vacuole. (Graphics: G.-J. Krauss, D. Dobritzsch.)

in the leaves by the **reduction and assimilation of sulfate**. Sulfate – as opposed to nitrate – and Organic sulfur are not only mobile in the xylem but also in the phloem and can be exchanged between xylem and phloem.

Organic sulfur produced in the leaves is required for the growth and development of all other plant organs and, therefore, undergoes extensive long-distance transport from the site of production (leaves: source) to the site of demand (growing parts of the trunk, root, and reproductive organs: sinks) both in the phloem and the xylem. In many plants the most abundant long-distance transport form of reduced sulfur is the tripeptide **glutathione** (γ-Glu-Cys-Gly) that – similar to sucrose in carbohydrate transport – is more chemically inert than the primary product of sulfate reduction and assimilation, cysteine. Also, glutathione can be exchanged between phloem and xylem and both sulfate and organic sulfur are stored in the wood of perennial plants. In addition, glutathione fulfills regulatory functions in the roots. Similar to the down regulation of nitrate uptake of the roots by amino compounds transported in the phloem, sulfate uptake of the roots can be down regulated by phloem allocated glutathione.

Sulfate reduction and assimilation takes place in several compartments of *Arabidopsis* cells (Figure 6.22). In contrast, the biosynthesis of cysteine is exclusively restricted to chloroplasts in the green alga *Chlamydomonas reinhardtii*. The primary product cysteine is either used for methionine synthesis, together with methionine for protein synthesis, for the synthesis of glutathione, and phytochelatins [$(\gamma\text{-Glu-Cys})_n$-Gly], and other sulfur – containing peptides, or for the synthesis of coenzymes. Part of the adenosine 5′-phosphosulfate (APS) produced in the initial reactions of this pathway is further phosphorylated to phosphoadenosine 5′-phosphosulfate (PAPS) by adenosine 5′-phosphosulfate kinase (APSK) and used for the synthesis of sulpholipids and glucosinolates in sulfotransferase (SOT)

reactions. A product of these reactions, phosphoadenosine 5′-phosphate (PAP) seems to be involved in targeting drought stress responsive genes (see Chapter 10).

6.3.3.3 The Phosphorus Cycle

Compared to other nutrients of plants, phosphorus concentrations in the soil solution and phosphorous leaching are low because of its strong adsorption to the solid phase of soils. Still, a large proportion of the phosphorus content of soils is lost during long-term soil development. In addition, during soil development phosphorus switches from preferentially binding to Ca-PO_4 minerals to preferentially binding to soil organic matter. This change is connected to phosphorus immobilization and results in a depletion of phosphorus easily available to plants. As a consequence, plants in many ecosystems have adapted to low phosphorus availability, and phosphorus primarily cycles within ecosystems. For example, in a conifer forest plantation it was estimated that 60% of the overall phosphorus supply was derived from tree internal recycling such as mobilization before the abscission of leaves, 35% was recycled from litter, and only 5% originated from the mineral pools of the soil. The high rate of internal cycling requires the development of mobile phosphorus stores different from leaves. These stores include the wood and mycorrhiza. The latter can accumulate phosphorus in, and mobilize from, polyphosphate stores found in fungal hyphae of both ectomycorrhiza and arbuscular mycorrhiza.

Several mechanisms have evolved in plants to enhance soil exploitation for phosphorus and recycling efficiency. These mechanisms include soil acidification, exudation of organic acids, and phosphatase enzymes that mobilize phosphorus from organic sources. These mechanisms are largely mediated or stimulated by mycorrhizal fungi that, in addition, improve phosphorus nutrition of plants by improved soil exploitation because of an enhanced soil adsorbing surface at reduced diameters of hyphae compared to root hairs (see Chapter 5). However, the significance of

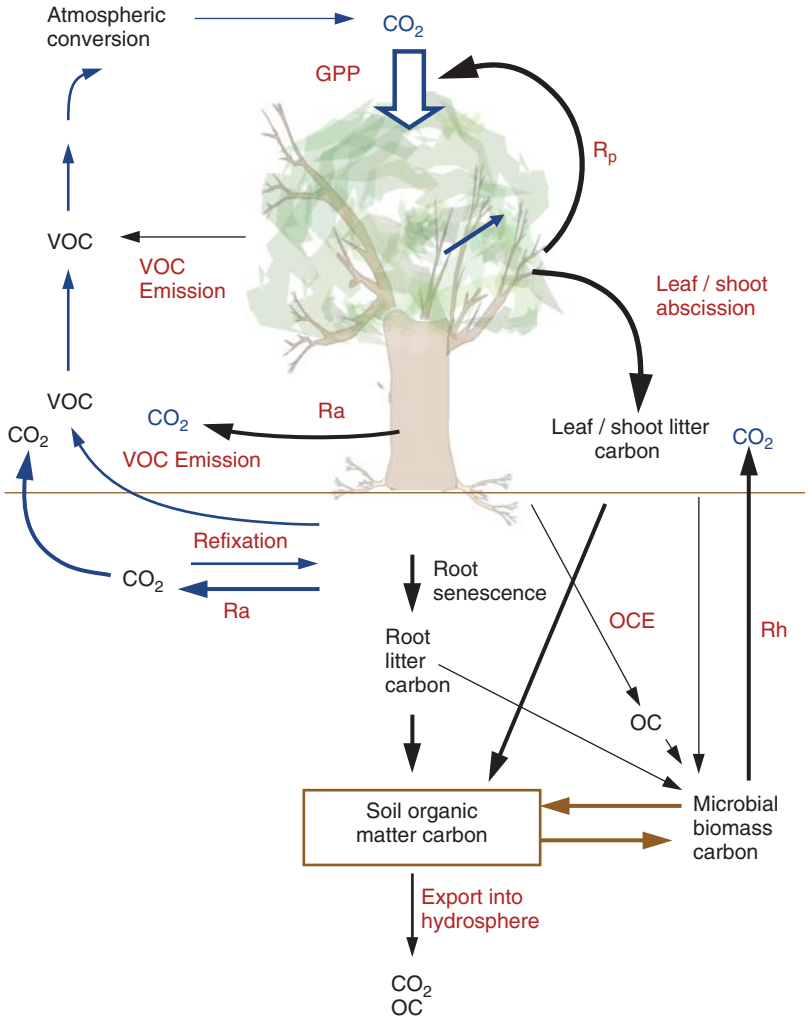

Figure 6.23 Major pools and fluxes of carbon in the ecosystem. VOCs – volatile organic compounds; OC – organic carbon; R_p – photorespiration; R_a – autotrophic respiration; R_h – heterotrophic respiration; OCE – organic carbon exudation; GPP – gross primary productivity.

organic *versus* inorganic phosphorus acquisition by plants has not yet been elucidated. In the soil, plant roots and microbes compete for growth-limiting phosphorus, in particular, in geologically old soils as found, for example, in the tropics. In contrast to nitrogen nutrition, the mechanisms of competition for phosphate and their controls are virtually unknown.

6.3.3.4 The Carbon Cycle

In most ecosystems, carbon undergoes a largely open cycle with **photosynthetic CO_2 fixation** (gross primary production, GPP) as the main process of carbon input. The major carbon pools and fluxes resulting from this carbon input are shown in Figure 6.23. Part of the GPP will be subject to immediate **photorespiration** (R_p) in the leaves; however, the released CO_2 may largely be reassimilated by photosynthesis. A major loss of carbon is due to **autotrophic respiration** (R_a) that combines the respiratory CO_2 production by photosynthetically active and inactive plant tissues, including roots and their symbionts (mycorrhiza, rhizobia, etc.). This loss can be as high as 80% of GPP in tropical forests. Further losses of carbon are due to **root exudation** of organic carbon (OCE) that may amount up to 10% of GPP and to plant volatile organic compounds (VOC) emission that is usually below 2% of GPP, but can be more than 5% of GPP when GPP is low as a consequence of unfavorable environmental conditions. In legumes, where symbiotic N_2 fixation in the roots requires a high energy input and, hence, high autotrophic respiration (R_a), a significant amount of the CO_2 respired by the roots may be recaptured by phosphoenolpyruvate carboxylase (PEPC) mediated CO_2 re-fixation in the roots.

Free-living microbes in the soil feed on the components of OCE, shoot plus root litter carbon, and soil organic matter carbon as sources of microbial biomass carbon and microbial (heterotrophic) respiration (R_h). Respiratory CO_2 and OC may also be subject to leaching into the hydrosphere. The balance of GPP and the sum of respiratory processes constitute the biomass accumulation in the ecosystem (NPP). In annual herbs the entire biomass dies at the end of vegetation period and becomes available for degradation by heterotrophic organisms in the soil. In forests, root and shoot litter may amount to around 50% of GPP.

Under anaerobic conditions in the soil, caused by water saturation after heavy rain and flooding, strictly anaerobic methanogenic archaea (*Euryarchaeota*) convert OC and CO_2 to the greenhouse gas methane that can be released into the atmosphere either directly by diffusion and ebullition of gas bubbles or indirectly after transport through the aerenchyma of plants. The most common microbial processes of methane production include:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

$$4HCOOH \rightarrow 3CO_2 + 2H_2O + CH_4$$

$$CH_3 - COOH \rightarrow CH_4 + CO_2$$

In paddy rice cultivation, plant-mediated transport is responsible for more than 90% of the release of methane into the atmosphere.

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Part III
Biochemical Response to Physiochemical Stress (Abiotic Stress)

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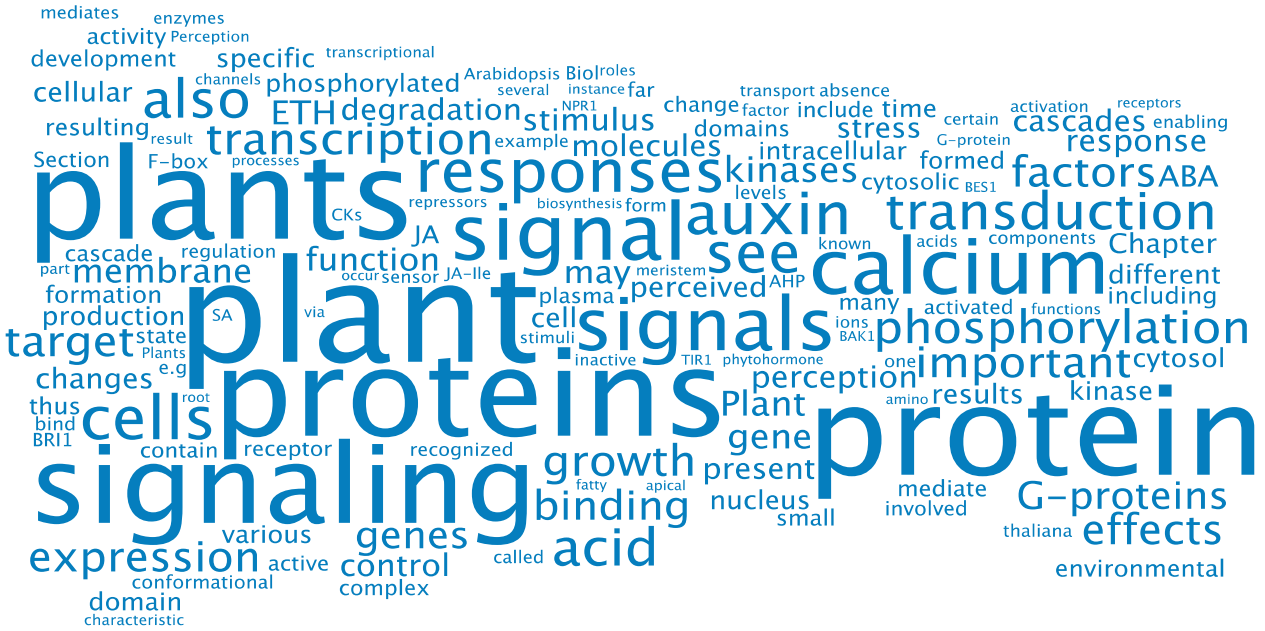
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7
Information Processing and Survival Strategies

Ingo Heilmann

Overview

Plants employ numerous **signaling molecules** in perceiving their environment and transducing the information toward appropriate responses. Many components of plant signal transduction cascades have been individually investigated. However, it is clear that no single signaling cascade acts in isolation and that there is extensive cross-talk between signaling pathways. Plants are subjected to diverse environmental inputs, which are perceived through a number of parallel sensory systems and set off various parallel signal transduction cascades. A possible advantage of the layout of plant signaling systems is the option to recognize combined stresses, such as wounding and pathogen infection, which share part of their signaling cascades. Evidently, parallel inputs must be integrated by the plant to induce appropriate **physiological responses**. The interconnections of **signal transduction cascades** serve an important function in the integration process, and influences exerted between cascades might be synergistic or antagonistic in nature. Obviously, the effects exerted by a signaling cascade on another one are inherently hard to study, and knowledge on the topic is still limited. What is known relates oftentimes to the **mutual influence (cross-talk)** between two pathways; it must be noted, however, that studies performed so far may only hint at the real *in vivo* complexity of true signaling networks that we are still far from understanding in depth.

7.1
The Stress Concept – Plants and Their Environment

Since the evolution of land plants from approximately 470 million years ago, plants can be found in a multitude of aquatic and terrestrial environments (see Chapters 5 and 6). As primary producers, plants contribute essentially to the generation of biomass on earth and have substantial impact on various ecosystems. Plants are sessile and cannot physically escape environmental factors (except possibly through reproduction) and, therefore, have evolved to cope with environmental changes in more different ways than animals. Furthermore, in their natural environments plants are in contact with diverse co-inhabitants, such as insects or microorganisms, which may have coevolved with plants to jointly occupy particular ecological niches, or which may take advantage of a plant at the plant's expense. In response to such beneficial or detrimental colonization, plants can actively aid symbiotic organisms or actively defend against parasites or pathogens. To integrate environmental inputs, plants have evolved complex, interacting signaling networks that enable appropriate responses to developmental signals and abiotic or biotic stress factors (see Parts III and IV). Part of these signaling networks are the **plant hormones (phytohormones)**, substances with signaling function that often differ in their modes of action from those of animals. It should be mentioned here that crops and horticultural plants have been bred for thousands of years by humans for desirable properties, foremost for large yield or colorful flowers, and often in monocultures. It is not surprising that the cultured plants have lost some if not most of their capabilities to interact sensibly with their surroundings and oftentimes cannot survive if human care is discontinued. Artificial growth conditions are also regularly imposed on plants grown in laboratories. In fact, much care is applied to keep environmental conditions "controlled" at nonchanging levels. This situation is clearly unnatural and may even make it difficult to interpret experimental data obtained from such stimulus-deprived plants.

The environment of a plant presents numerous stimulatory inputs that can be sensed by plants and which may or may not lead to physiological responses. A common plant response to "stress" is cessation of growth (Figure 7.1). Obviously, stress-induced growth retardation can have a detrimental impact on the yield, for example, of agricultural crops. Therefore, it is of pivotal importance to study plant stress responses and understand the underlying molecular mechanisms.

Stress factors influencing plants can be categorized in **abiotic stress factors** (see Part III), meaning those that are merely variable physical parameters of the surroundings, and **biotic stress factors** (see Part IV), which represent

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Box 7.1: *Arabidopsis thaliana* and other plant models.

Most of the work described in this chapter has been performed with the dicotyledonous herb, ***Arabidopsis thaliana***. *A. thaliana* (*Brassicales, Rosidae*) is the plant biologist's favorite pet, because of its small size, its fast generation time, and its tractability for genetic modification. Moreover, there are many genetic resources available for *A. thaliana* that facilitate the study of mutants and their associated phenotypes. The information presented in subsequent sections of this chapter was in many instances obtained by the analyses of *A. thaliana* mutants. A note on the ensuing nomenclature: Many of the presented proteins with functions in signal transduction have curious names describing the phenotypes of mutant plants defective in the production of the particular protein, whereas other proteins carry positively informative names.

A. thaliana belongs to the family of *Brassicaceae* and, thus, shares many features with important crop plants, such as rapeseed or cabbage. Other important crops, such as monocotyledonous cereal grasses, for example, corn, oat, barley and wheat, or rice, are less similar, and information gained from studies on *A. thaliana* may not immediately be applied to these species. This is even more true for yet more distantly related plants, such as trees, which obviously have biosynthetic capacities and environmental requirements that differ from those of *A. thaliana*. While in many instances concepts developed based on *A. thaliana* research have positively been confirmed for other plant species, the reader is invited to always critically question the applicability of results and, when delving deeper into the topic than this chapter can offer, consider the source of information for a certain signaling or metabolic pathway.

Evidently, wild plants growing "in nature" are more versatile in their communications – and they need to be! In this chapter the term "stress" is used to signify conditions that will prompt a response by a plant. "Stress factors," therefore, are not necessarily detrimental for the plant, because the adaptation to changing environmental conditions is an essential part of plant life.

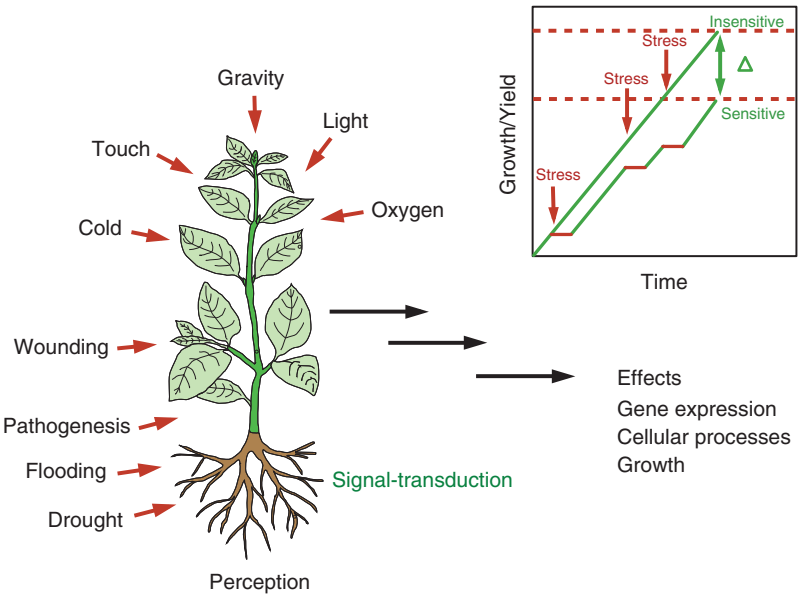

Figure 7.1 Perception of physical stimuli is followed by the generation of plant-endogenous signals. Plants are exposed to various environmental stresses. Perception of these factors results in the generation of signaling molecules by the plant. The process of signal transduction describes the translation of the physical stress into such signaling molecules and the spatial and temporal distribution of the signals within the plant. Top right, Many environmental stresses cause plants to cease growing. Perception of stresses by the plants initiates active growth inhibition that is most often not related to physical damage to the plants. Repeated exposure of plants to such stress factors can result in massive growth retardation and yield loss.

influences originating from other living organisms that are also present within the habitat of the plant. Both these aspects can be interlinked, because, for example, microorganisms may alter physical parameters of the soil, or certain physical conditions may attract and favor certain microorganisms or insects in the proximity of a plant.

7.2 Plant Signal Transduction and the Induction of Stress Responses

From the explanation of various abiotic and biotic factors described in Parts III and IV, it is evident that plants can perceive a variety of environmental stimuli and initiate

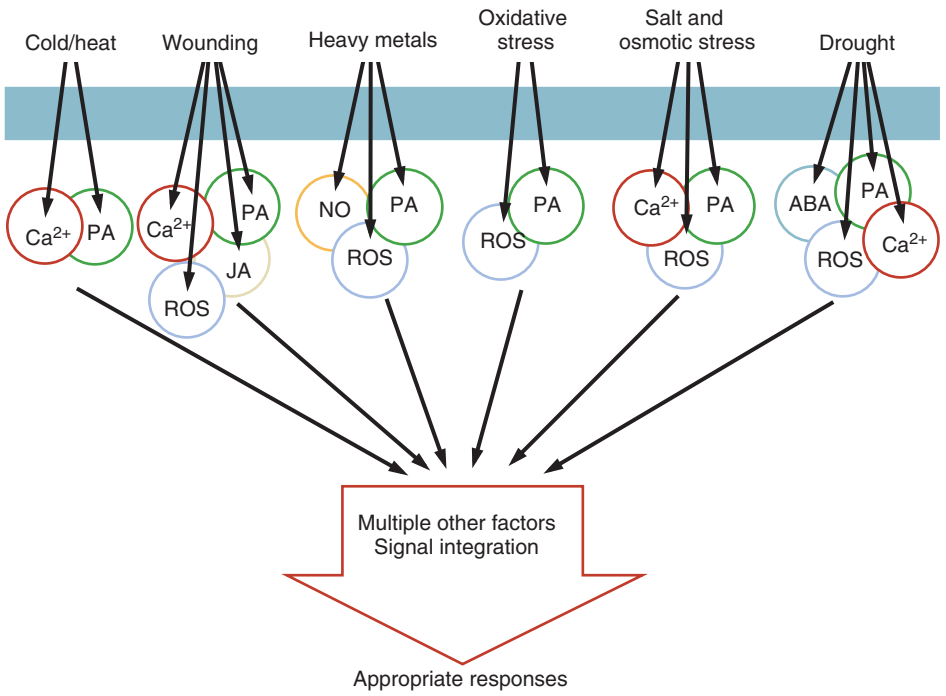

Figure 7.2 Stimulus-specific signal transduction events define appropriate responses. An environmental stimulus can cause more than one type of signal to be formed. The integration of various signals will result in the initiation of an appropriate effect in the plant, such as altered gene expression or altered patterns of growth. ABA – abscisic Acid; JA – jasmonic Acid; NO – nitric Oxide; PA – phosphatidic Acid; ROS – reactive Oxygen Species.

appropriate responses (Figure 7.2). Moreover, plants can integrate inputs from different **stimuli** perceived in parallel and modulate their responses accordingly. Obviously, plants do not "decide" by rationalizing, and the manifestation of responses that balances the inputs of different stimuli will be the result of chemical gradients of intracellular signaling molecules that are formed by the plant cells on perceiving external stimuli. In order to facilitate discussing these issues in further sections of this chapter, the term "stimulus," denoting the external physical parameter that is perceived by the plant, should be strictly distinguished from the term "signal," which denotes plant-endogenous signaling molecules with messenger function that are actively generated by the plant cells once a "stimulus" has been perceived (Figure 7.3). Intracellular signals are usually short-lived and actively degraded to avoid arrest of a cell or tissue in a stimulated state. The conversion of a stimulus to one or more intracellular signals is known as *signal transduction*.

The process of signal transduction begins with the perception of the external stimuli. The multitude of physical stimuli plants are exposed to in nature (Figure 7.1) are perceived through a diverse array of receptors and specialized cellular structures. The common theme is that perception of the stimulus must cause the formation of an intracellular signal, which can then instigate further intracellular processes (Figures 7.2 and 7.3). Signal transduction, thus, transforms the physical stimulus from the environment into the biochemical "language" of the plant cell. The intracellular signals that are formed on perception of a stimulus can be independent molecules, such as phosphatidic acid or Ca^{2+}-ions, or they can be modifications of preexisting signaling factors, such as the phosphorylation of proteins. As the first and often most localized event in signal transduction, the processes involved in the perception of various stimuli are inherently hard to study and still a matter of debate. The perception of some other stimuli is studied better.

1) The perception of **light** (see Chapter 9) requires pigment-containing structures, which can absorb light and change their conformation to mediate the formation of intracellular signaling molecules. From different plant species, various forms of light-receptors are known that can differ in the nature of the associated pigments and the perceived wavelengths. Examples include phytochrome, cryptochrome, and phototropin (see Chapter 9).
2) The perception of **temperature** is less well studied and may rely on changes in protein stability or membrane fluidity, which are largely temperature-dependent.
3) The **water status** of plants (see Chapter 10) is perceived through changes in turgor pressure and resulting effects on the plasma membrane. The force exerted by the turgor on the plasma membrane creates a steady state of ion fluxes, including Ca^{2+}, through stretch-activated ion channels that will change when turgor pressure is diminuishing as a result of water loss. Therefore, the ion transport capacity of the plasma membrane can indicate water loss. Water loss and osmotic stress are

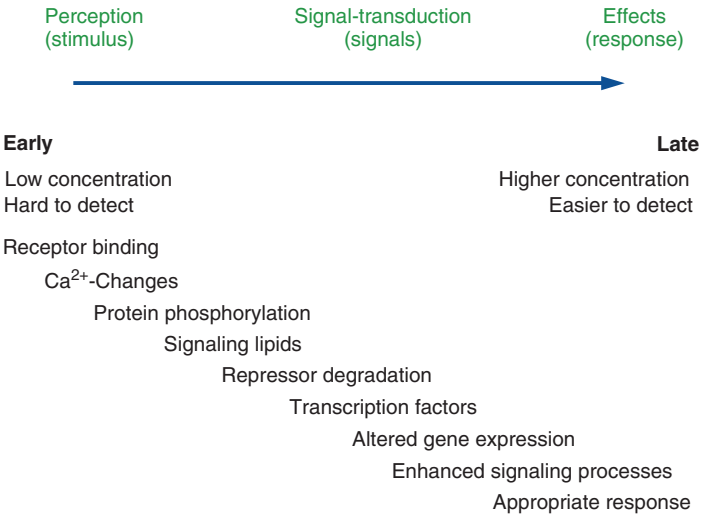

Figure 7.3 **Early and late signaling molecules.** On perception of a stress, various signaling molecules can be formed that take part in signal transduction to effect an appropriate stress response. Early signals often include changes in cytosolic Ca^{2+}-concentrations or protein phosphorylation events and occur locally and at low intensity. Later signals are already amplified and occur in higher concentrations and for longer periods of time.

related, as changes in the external osmotic potential will result in altered rates of water diffusion and effects on turgor.

4) **Touch stimuli** are perceived as a localized depression of plasma membrane area will result in the opening of stretch-activated ion channels.
5) **Salt stress** exerts both osmotic effects and specific salt effects that depend on the nature of the ions involved. The particular ionic composition of the soil surrounding plant roots can evidently be sensed by plants (see Chapter 11).
6) **Gravity** is perceived by a set of specialized cells that contain starch granules (amyloplasts). Amyloplasts are dense organelles and exert a downward force according to the vector of gravity. According to one proposed model, this downward force is perceived by stretch-activated ion channels within endomembranes of the sensitive cells, leading to the production of signaling molecules.
7) The perception of **wounding** differs from that of other stresses mentioned because wounding is a destructive stimulus. The loss of cellular integrity in a wounded tissue results in vacuolar rupture, enabling the action of vacuolar enzymes with pH optima below 5 to act on cellular compounds from which they are normally separated by compartmentation. In this fashion, cellular components are degraded, generating signaling molecules that can be perceived by surrounding non-wounded cells, which will initiate a response. In the case of wounding caused by herbivory (see Chapter 16), the chemical components of insect saliva are additionally sensed by specific receptors at the surface of plant cells, which will cause the intracellular production of signaling molecules.
8) Plants are capable of sensing **infection** by various pathogens through specific surface receptors that recognize structures that are characteristic for certain pathogens (see Chapter 15). Such characteristic structures or molecules that can be sensed by plants and elicit a defensive response are termed *pathogen-associated molecular patterns* (*PAMPs*).

The **perception** of a **stimulus** and subsequent signal transduction will generate an intracellular **signal** in the plant (see Figures 7.2 and 7.3). Although early signals are of low abundance and found only locally in a plant tissue, these early signals are amplified by means of signaling cascades (Figure 7.4). The term signaling cascade describes that a signaling molecule can induce the generation of many other signaling molecules, which each in turn set off the generation of even more signaling molecules thus exponentially amplifying the initial signal. The signaling molecules that take part in such cascades are called **second messengers**, because they appear in the second phase on perception of the external stimulus. An example for signal amplification by cascading is the stimulus-induced activation of **mitogen-activated protein kinase (MAPK) cascades**, in which a mitogen-activated protein kinase kinase kinase (e.g., a MAPKKK) may be activated, which phosphorylates several other mitogen-activated protein kinase kinases (MAPKKs), which may each phosphorylate even more protein kinases (MAPK), and so on, until a multitude of target proteins can be phosphorylated that will set off a massive response to the perceived stimulus (see Section 7.5). It should be noted, however, that the situation is likely more complex, because phosphorylation, for example, of a MAPKKK may also result in its inactivation.

Signaling cascades connect intracellular signals with mechanisms that change plant metabolism toward the

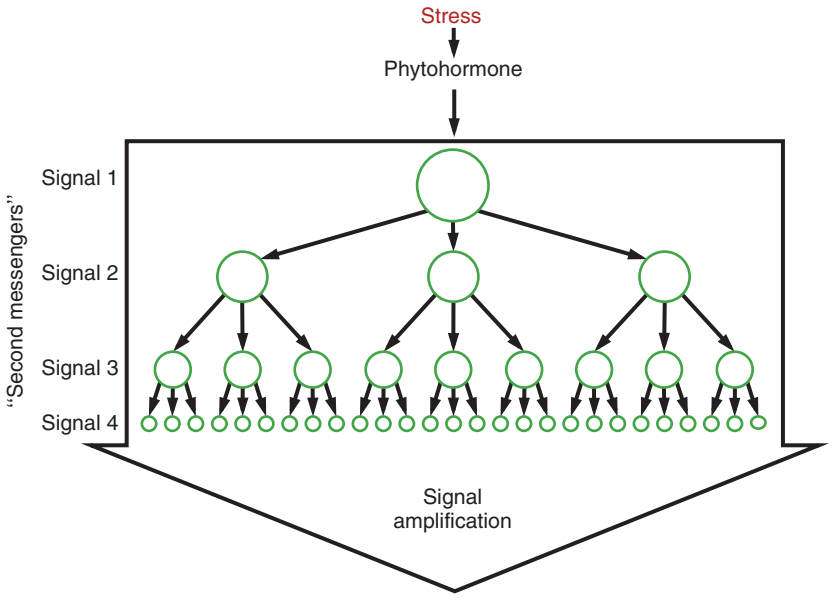

Figure 7.4 Signal amplification by hierarchical signaling cascades. To enable plants to respond to low-intensity stresses, signal transduction cascades exist that can amplify early signals of low concentrations. Amplification is achieved by signaling cascades where a signaling event of the first order (Signal 1) can set off several signaling events of the second order (Signal 2), each of which can again set off more signals of increasing order (Signal 3, Signal 4, etc.), thus resulting in an exponential amplification of signal intensity in a short time.

induction of certain responses. Responses of plants to external stimuli can be diverse. For instance, a perceived light stimulus can induce changes in metabolism to accumulate certain pigments and enhance the rate of photosynthesis. Another example is the accumulation of bitter or even toxic substances on perception of wounding and herbivory. Besides such biochemical responses, which will not become visible without detailed analysis, there can also be visible growth responses. When plants are reoriented from their usual vertical orientation, they alter their growth patterns so shoots will grow away from the vector of gravity (negative gravitropism), whereas roots will grow toward the vector of gravity (positive gravitropism), thereby righting themselves back to vertical. Similar responses can also be observed to light (phototropism), for instance, in the heliotropism of sunflowers turning toward the sun as it wanders across the sky.

Besides the induction of responses to environmental stimuli, signal transduction processes also take place without exogenous stimulation as part of the normal developmental programs of plants. Various signals that can be part of responses to exogenous stimulation also function to mediate communication between cells and tissues. An example is the signaling cascade involving the phytohormone auxin. Although growth can represent an adaptation to changed environmental conditions, oftentimes plants will cease to grow, once an external stress factor has been perceived (inset in Figure 7.1). An example is the abovementioned defense response against herbivory, which is accompanied by reduced or no growth. In other cases, such as on perception of a touch stimulus, plants respond with the cessation of growth, even though no internal metabolic changes have been discovered to date. The dwarfish growth of plants regularly exposed to strong wind or hail can limit agricultural yield. Such growth is not a result of physical damage to the plant, but rather the outcome of an active inhibition of growth on the perception of the touch stimulus.

A growth response is a process that will use a substantial portion of a plant's resources and is not reversible. Therefore, plants do not lightly alter their growth patterns in response to short-term stimulation. Instead, plants have mechanisms by which short-term transient stimulation and persisting stimulation can be distinguished (Figure 7.5). As plant cells are stimulated, intracellular signals are constantly being generated and destroyed. The overall sum of signaling molecules present at a given time in the plant represents its current stimulation status and will induce a certain set of responses. Only when a pattern of signals persists for a prolonged time, likely in consequence of a persisting combination of external stimuli, responses will be initiated. The *presentation time* of a stimulus indicates the period of time that a stimulus must be continuously present in order to induce a detectable response in the plant. For instance, a plant swaying in the wind will not respond with gravitropic curvature. However, when the plant is placed horizontally for a prolonged period of time that exceeds the presentation time, gravitropic curvature will become manifest (Figure 7.5). Once a stimulus persists for a period exceeding the presentation time, the plant will be irreversibly committed to respond and the response will manifest, even if the stimulus is now discontinued. The

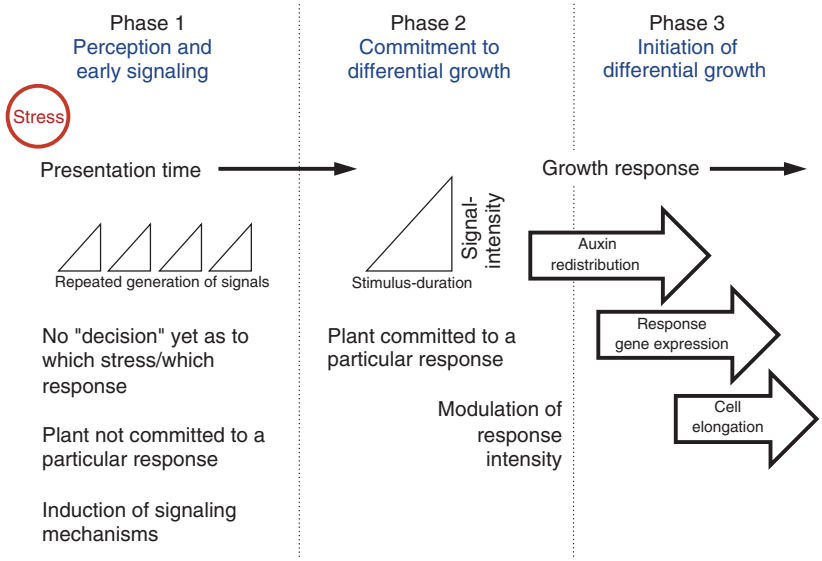

Figure 7.5 **The presentation time of a stimulus**. Plant growth responses are irreversible. Whether a stimulus is perceived as transient, thus not warranting a response, or whether it is permanent and requires a response is indicated by the presentation time of a stimulus. The presentation time is the duration a stimulus has to be present to induce a response. It is characteristic for each plant and for each stimulus. The example shown represents maize gravitropic bending. Perception of the stimulus occurs almost instantaneously. During the presentation time (Phase 1) signals are generated that do not result in a response. Only after a period of such recurring signals is the plant committed to respond (Phase 2). The accumulation of signals may still fine-tune response intensity. After this sensitive time-window has elapsed, the plant is fully committed to respond, regardless of whether the stimulus persists or not (Phase 3). Signals detectable during phases 1 and 2 are not detectable anymore during phase 3.

presentation time will depend on the plant species and plant age; for instance, the stems of mature maize plants have a presentation time of approximately 2 h before they exhibit gravitropic bending, whereas the much smaller mature oat plants will already bend after only 30 min of stimulation. The observation that plants have a presentation time for various stimuli provides us with two very important pieces of information: (i) Plants perceive stimulation immediately and (ii) they initiate a mechanism to deduce the duration for which the stimulus persists.

7.3 Phytohormones

7.3.1 Functionality of Phytohormones

Phytohormones have growth-promoting or growth-altering effects and show various molecular structures (Figure 7.6). These substances are part of a complex network of signaling cascades that ultimately underlies the control of

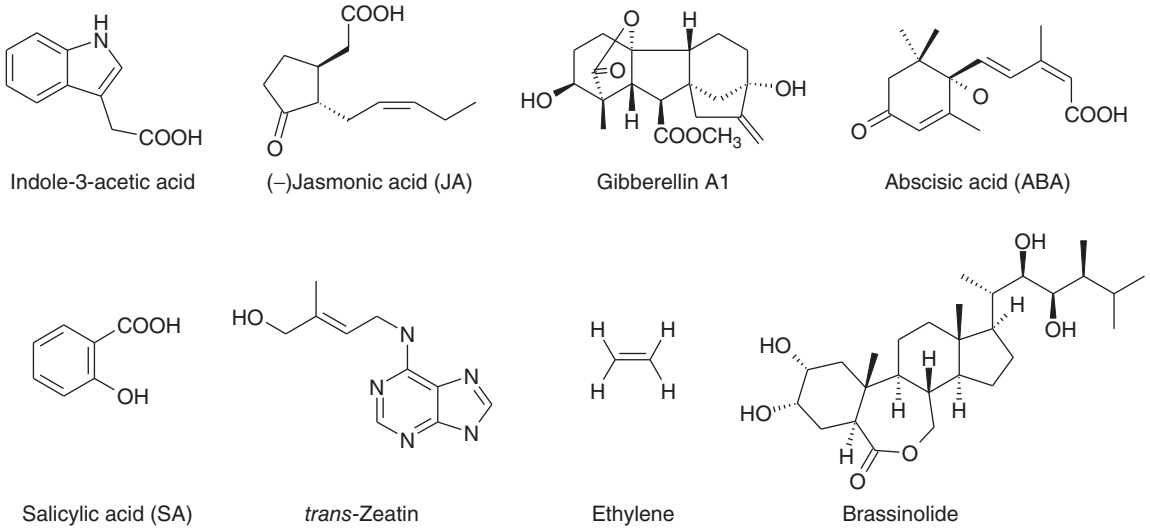

Figure 7.6 **Structures of important phytohormones.**

plant growth and development. The cross-talk between phytohormone signaling cascades is still largely unexplored and a topic of intense ongoing research.

7.3.2 Signal Transduction through Nuclear Derepression of Gene Expression

On perception of a stimulus or developmental signal, responses and developmental programs manifest through changes in the expression of specific genes that are regulated by a given signaling cascade. The phytohormones auxin, JA (jasmonic acid) and gibberellin can mediate changes in gene expression by acting in the nucleus and mediating the specific degradation of certain target proteins, such as transcriptional repressors of specific transcription factors that control the expression of relevant sets of genes (Figure 7.7). The degradation of repressors is mediated by ubiquitin-ligase complexes, which assemble only in the presence of the phytohormones. The ubiquitin-ligase complexes consist of several important protein subunits, including the proteins skip, cullin, and a so-called F-box protein (Figure 7.7). Because of these components, the core of the ubiquitin-ligase complex is also called the **SCF-complex**. Of particular interest for phytohormone signaling is the F-component of the SCF-complexes, the **F-box protein**, which mediates the specific recognition of the respective repressors to an SCF-complex. The SCF-complexes involved in the signal transduction of auxin, JA, or gibberellin contain different F-box proteins that recognize different repressors. The SCF-complexes will mark the bound repressor protein by ubiqitinylation; ubiquitinylated target proteins are subsequently recognized by the 26S-proteasome and proteolytically degraded to small peptides and amino acids (Figure 7.7). Thus, repressors are destroyed, repression is abolished, and transcription factors are released or generated that are responsible for the phytohormone-dependent activation of specific genes.

7.3.2.1 Auxins

Auxins are involved in a multitude of processes, ranging from the establishment of polarity during the development of the plant embryo or of mature plants, the control of apical dominance, shoot elongation, root development, or tropical responses. The most abundant natural auxin is **indole 3-acetic acid (IAA)**. Other substances with auxin function include 4-chloroindole 3-acetic acid, indole 3-butyric acid (IBA), 1-naphthylacetic acid (NAA), or 2,4-dichlorophenoxyacetic acid (2,4-D). IAA is synthesized from precursors originating from the shikimate pathway in the plastids that are transported to the cytosol for the last steps to form IAA (see Chapter 2). An alternative cytosolic pathway for the biosynthesis of IAA from tryptophane has so far only been reported for *Brassicaceae* (*Brassicales,*

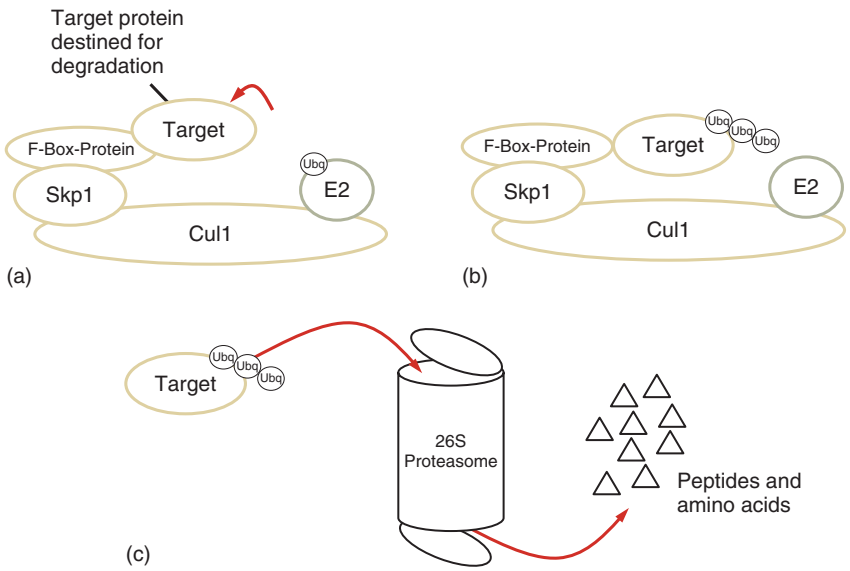

Figure 7.7 Ubiquitinylation marks proteins for degradation by the 26S-proteasome. A recurring motif in plant signal transduction is the targeted degradation of proteins that repress gene expression, resulting in its derepression. The degradation of specific proteins involves (i) the recognition and polyubiquitinylation of the target proteins by ubiquitin-ligase-complexes and (ii) the recognition and degradation of the ubiquitinylated proteins by the 26S-proteasome. Ubiquitin-ligase-complexes consist of several protein subunits, including the proteins cullin (e.g., Cul1), skip (e.g., Skp1), an F-box protein, and the ubiquitin-transferring E2-ligase subunit. Because of the components cullin, skip, and F-box-protein, such complexes are often referred to as SCF-complex. SCF-complexes covantently attach the small protein, ubiquitin (Ubq), to target proteins. The F-box-protein of an SCF-complex mediates its specificity and recruits the target protein(s) for ubiquitinylation (a). Successive transfer of various ubiquitin-units results in polyubiquitinylation of the target protein (b). The ubiquitinylated target is recognized by the 26S-proteasome, a large protein complex containing proteases and peptidases that degrades the target protein into small peptides and amino acids (c).

Rosidae), *Poaceae* (*Poales, Liliopsida*), and *Musaceae* (*Zingiberales, Liliopsida*).

Auxins can also be formed by gall-inducing bacteria, such as *Agrobacterium tumefaciens* (*Alphaproteobacteria*), which benefit from the effects of auxin on the plant. Auxin is formed in the shoot-apical meristems and transported in a basal direction throughout the plant. The ensuing auxin gradient between apical and basal organs of the plant is important as a positional cue for each cell in the plant's tissues, as the local auxin concentration will govern cell identity, differentiation, and developmental programs. In consequence, the maintenance of the auxin gradient within the plant is important for the proper formation of organs and tissues. Apoplastic auxin is protonated and can diffuse into cells. In addition, active uptake mechanisms for auxin also exist. Once inside the cells, auxin is deprotonated and cannot passively leave the cell. Directional auxin transport is achieved within plant tissues by the action of auxin-efflux carriers (Figure 7.8). These proteins, the lack of which results in the formation of nonfertile pin-like structures instead of flowers, are called **PIN-formed** (**PIN**)-**proteins**. A special feature of PIN-proteins is their localization in the plasma membrane of plant cells, which is not uniform, but highly polarized. The polar distribution of PIN-proteins results in auxin-efflux only at one side of the cells in a tissue, thus mediating directional transport of auxin. Different PIN-proteins are present in different tissues. The polarization of their localization also differs. The combination of PIN-proteins present, for example, in root tips leads to a basal transport of auxin in the center of the root toward the root tip, where auxin is transported sideways and then transported laterally in an apical direction. IAA signals are terminated by the degradation of IAA or its conversion to inactive conjugates with amino acids, such as aspartic acid.

Perception of auxin by the receptor protein T**ransport Inhibitor Response 1 (TIR1)** is one of the best-studied signaling processes in plants and the 3D-structure of the receptor has been solved using x-ray crystallography. TIR1 is an F-box protein and is a part of the SCF^{TIR1} complex (Figure 7.9). Besides TIR1, there are numerous other related F-box proteins, called **auxin-related F-box proteins (AFBs)**, which can also act as parts of SCF-complexes. In the presence of auxin, SCF^{TIR1} mediates the binding, ubiquitinylation, and degradation of AUX/IAA repressors. In the absence of auxin, these repressor proteins bind transcription factors of the auxin response factors (ARFs) family and prevent them from activating gene expression. When auxin is present, the SCF^{TIR1}-mediated degradation of AUX/IAA repressors releases the ARF transcription factors and gene expression can follow.

A second means to perceive auxin involves the A**uxin-Binding Protein 1 (ABP1**; Figure 7.10). ABP1 is an extracellular protein capable of binding IAA on the outside of the plasma membrane. IAA binding mediates the interaction of ABP1 with a transmembrane protein that possesses a cytosolic kinase domain and is involved in transducing the IAA signal to the inside of the cell. ABP1 partakes in the IAA-dependent control of potassium channels and chloride channels and may have a function in the

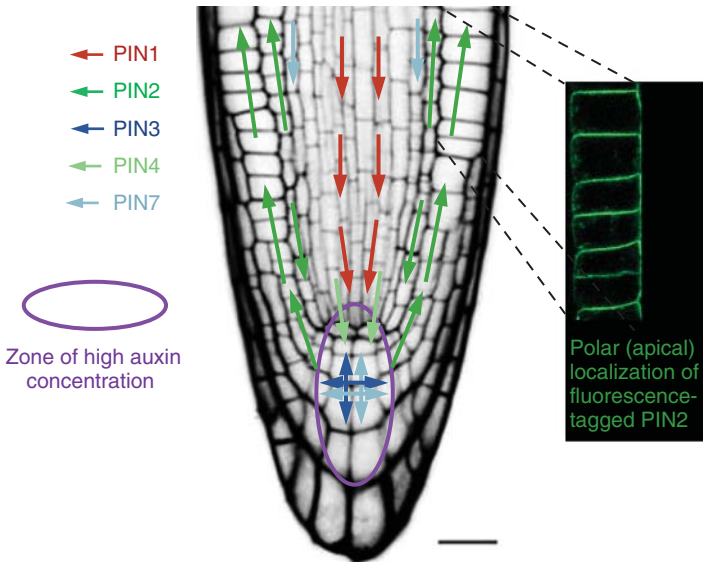

Figure 7.8 Polar transport of auxin by auxin-efflux carriers. Auxin is vectorially transported across tissues by a combination of diffusion and active polar transport. In the low-pH environment of the apoplast, auxin is protonated and can diffuse into the cells. In the cytoplasm, the higher pH results in deprotonation and prevents diffusion of auxin from the cells. Auxin-efflux-carriers of the PIN-family exhibit polar distribution in the plasma membrane and mediate the efflux of auxin into the apoplast only on one side of the cell. Thus, the polar distribution of PIN proteins results in the vectorial transport of auxin through a tissue, as indicated for an Arabidopsis root tip. Arrows indicate auxin transport by numerous PIN-proteins, signified by the colors. Inset, apical localization of fluorescence-tagged PIN2 in epidermis cells of the Arabidopsis root, mediating basal-to-apical transport of auxin through these cells.

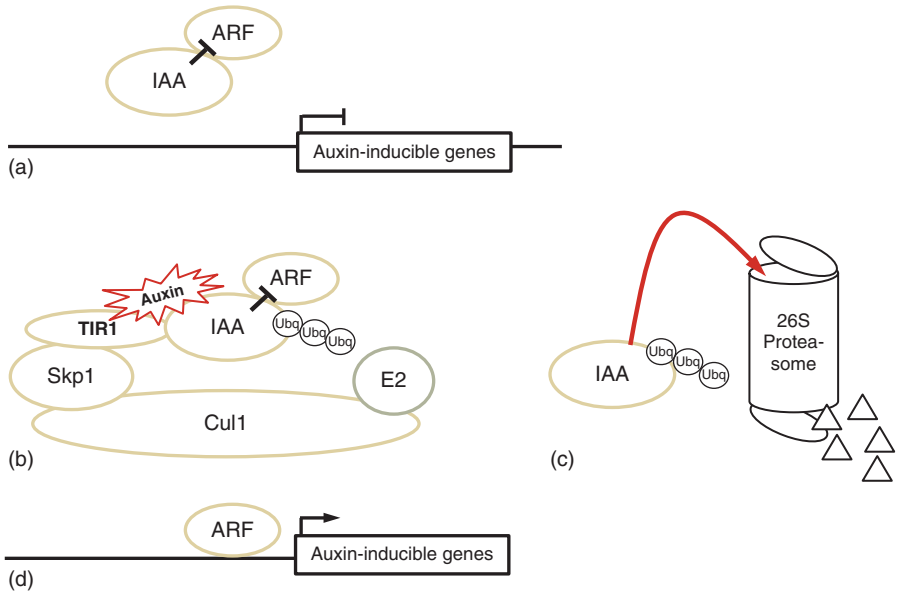

Figure 7.9 Perception of auxin by the SCF^{TIR1}-ubiquitin ligase complex and signal transduction by derepression of gene expression. Auxin is perceived through an SCF-complex containing the F-box-protein TIR1. TIR1 mediates the specific recruitment of transcriptional repressors of the AUX-IAA-family (IAA-proteins). In the absence of auxin, IAA-proteins bind and inhibit transcription factors of the auxin response factor (ARF) family (a). Auxin enables binding of IAA-proteins to SCF^{TIR1}, resulting in their ubiquitinylation (b) and subsequent proteasomal degradation (c). The derepression of ARF transcription factors results in the transcription of auxin-induced genes (d).

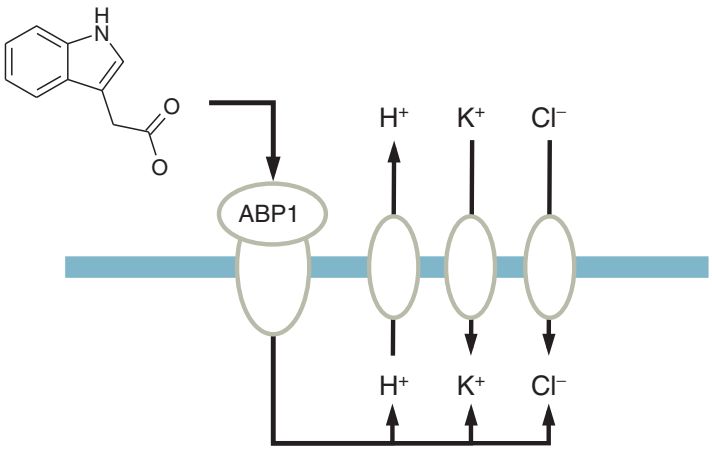

Figure 7.10 Perception of auxin by ABP1. A second, independent means to detect auxin involves the protein ABP1. ABP1 likely resides on the extracellular face of the plasma membrane. Binding of auxin to ABP1 outside the cell results in the activation of ion channels, including proton pumps and channels for potassium and chloride. Furthermore, ABP1 influences auxin-dependent formation of clathrin-coated vesicles (not depicted).

auxin-dependent relocalization of PIN-proteins; however, ABP1 is not described in more detail at this point.

7.3.2.2 Jasmonic Acid

JA has functions in the control of the development of male flower organs and in the initiation of defense responses on wounding, herbivory, or pathogen infection. The formation of JA takes place in the male flower organs; mutants deficient in JA production fail to extend their anthers. Pollen from the anthers cannot reach the pistil, resulting in male sterility. Exogenous application of JA rescues this phenotype. In addition, JA is formed in wounded tissues and serves as a wound signal. For this reason, JA is also known as the **wound hormone** of plants. On external stress, such as wounding, the biosynthesis of JA occurs within a few minutes of stimulation. A number of different forms of JA are known (Figure 7.11).

JA formation starts with the release of polyunsaturated fatty acids from plastidial galactolipids (Figure 7.12). The fatty acid precursors of JA are first oxidized to fatty acid

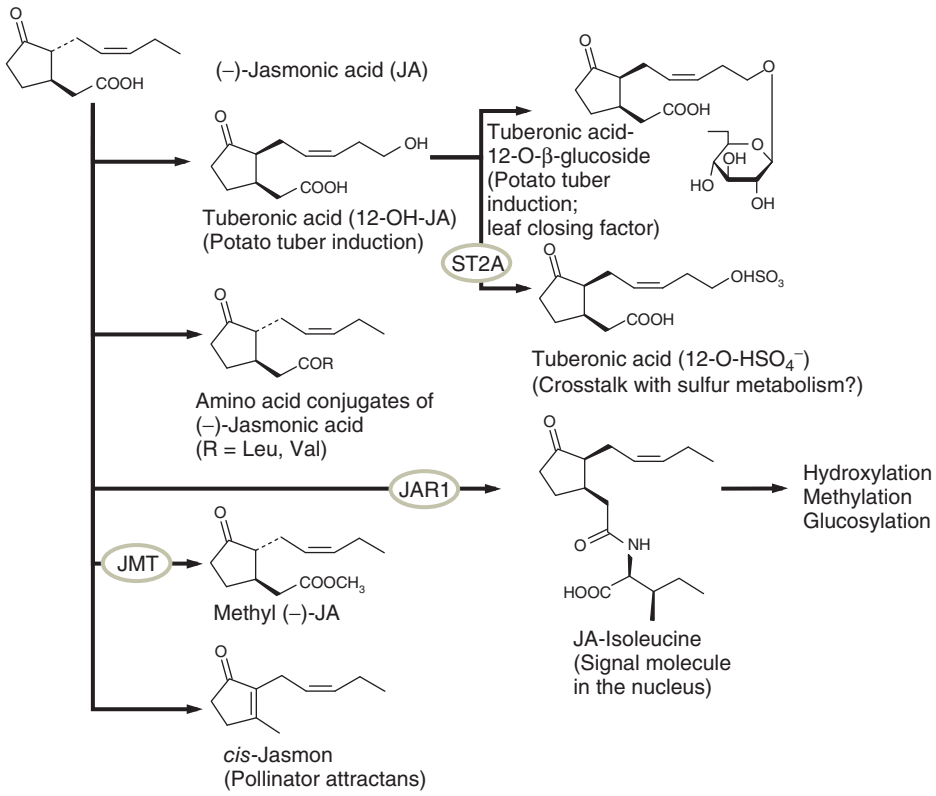

Figure 7.11 **Structures of different jasmonates**. (Graphics: D. Dobritzsch, G.-J. Krauss.)

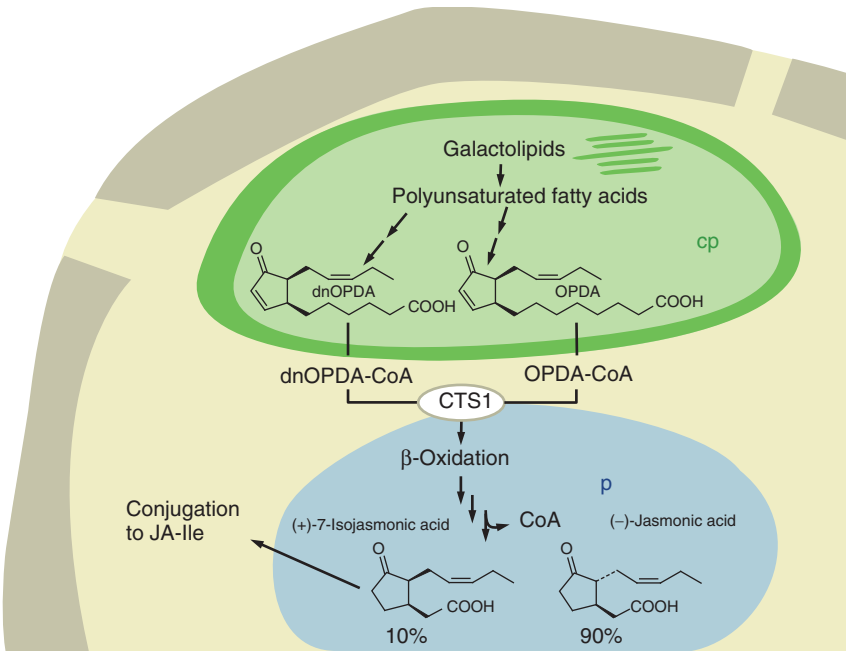

Figure 7.12 **Biosynthesis of jasmonic acid**. Jasmonic acid and all other jasmonates derive from polyunsaturated fatty acids present in plastidial galactolipids. The fatty acids are oxidized to fatty acid hydroperoxides and transformed in several steps to OPDA or, in the case of 16-carbon fatty acids, to dnOPDA. OPDA and dnOPDA are exported from the plastids to the cytosol, activated to CoA-esters and moved into peroxisomes by the transporter comatose1 (CTS1). Several rounds of peroxisomal β-oxidation yield jasmonic acid, which is exported from the peroxisomes back to the cytosol. In the cytosol, jasmonic acid can be conjugated to various amino acids. The jasmonic acid-isoleucin conjugate (JA-Ile) is a bioactive form of jasmonic acid that is recognized by the SCF^{COI1} receptor complex.

hydroperoxides. As an oxidized derivative of fatty acids, JA represents an oxylipin. Oxylipins are a diverse class of oxidized fatty acid derivatives that act as signaling molecules in many different contexts in plants, fungi, and animals. From the initial formation of fatty acid hydroperoxides, JA biosynthesis continues via several intermediate steps to the formation of the cyclic compound 2-oxo-phytodienoic acid (OPDA), which is translocated from the plastids through the cytosol into peroxisomes (Figure 7.12). In the peroxisomes, several rounds of β-oxidation result in the formation of JA, which is exported from the peroxisome. JA signals are terminated likely by degradation or chemical modification, such as sulfatylation (see Figure 7.12).

In the cytosol JA can be conjugated to amino acids, and an important bioactive signal is the jasmonic acid-isoleucine conjugate (JA-Ile, Figure 7.12). This conjugate (JA-Ile) is recognized by the receptor F-box protein **Coronatine Insensitive 1 (COI1)**. COI1 is evolutionarily related to TIR1, and the modes of receptor functions are also similar. COI1 is an F-box protein, as is TIR1, and is a part of the SCF^{COI1} complex (Figure 7.13). In the presence of JA-Ile, SCF^{COI1} mediates the binding, ubiquitinylation, and degradation of jasmonate ZIM-domain containing (JAZ)-proteins, which are the transcritional repressors in this cascade. In the absence of JA-Ile, JAZ-proteins bind transcription factors of the MYC family and prevent them from activating gene expression. In the presence of JA-Ile, SCF^{COI1}-mediated degradation of JAZ-repressors releases the MYC-transcription factors and gene expression can ensue. JA-Ile-inducible defense genes encode proteins that may inhibit the digestion of herbivorous insects or delay their development, thus limiting the propagation of herbivores in the proximity of the plant.

7.3.2.3 Gibberellins

Gibberellins are a group of phytohormones discovered first in the phytopathogenic fungus *Gibberella fujikuroi* (*Pezizomycotina, Ascomycota*), hence the name. It had been observed that infection of rice plants with *G. fujikuroi* resulted in substantially elongated stems, prompting the interest of researchers to identify the causal agent of this fascinating effect. The chemical, gibberellin A3 (GA3) was subsequently isolated. GA3 from *G. fujikuroi* is a very potent gibberellin and is used in most current studies on gibberellins. In some plants, GA3 induces the formation of flowers, delays the senescence of leaves and fruits, and aids germination. As these functions are of advantage to the storage and processing of many agricultural products, there is an applied interest in understanding gibberellin function and the associated signaling cascades. Today, more than 120 different gibberellins are known in addition to GA3, of which approximately 100 originate from plants, and at least 13 are present in all plants investigated so far. Gibberellins are formed from plastidial isoprenoid metabolism and derive from geranylgeranylpyrophosphate. The cyclic precursor ent-kaurene can be modified in various ways to enable the formation of different gibberellins. Gibberellins can be activated by 3β-hydroxylation and

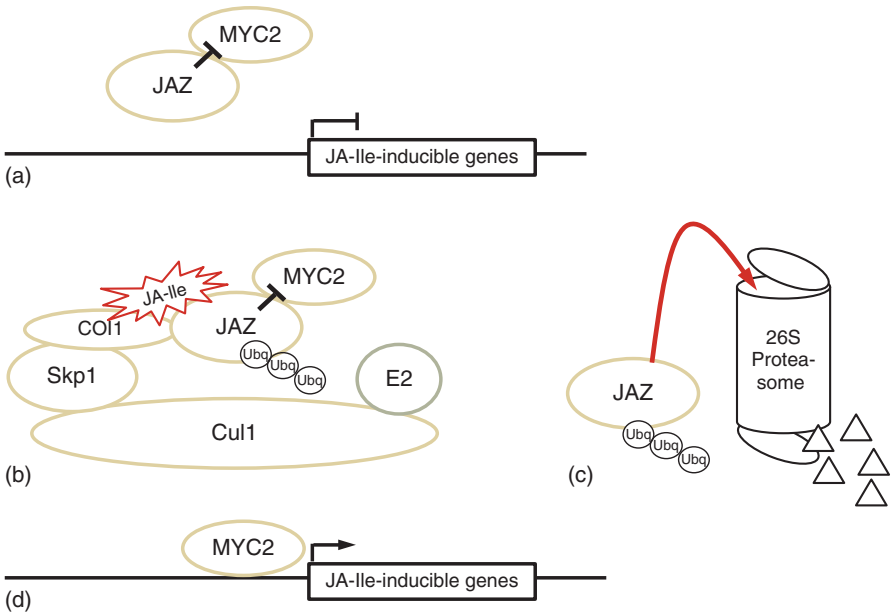

Figure 7.13 Perception of jasmonate-isoleucine (JA-Ile by) the SCF^{COI1}-ubiquitin ligase complex and signal transduction by derepression of gene expression. JA-Ile is perceived through an SCF-complex containing the F-box-protein COI1. COI1 mediates the specific recruitment of transcriptional repressors of the JAZ-family (JAZ). In the absence of JA-Ile, JAZ-proteins bind and inhibit transcription factors of the MYC family (a). JA-Ile enables binding of JAZ-proteins to SCF^{COI1}, resulting in their ubiquitinylation (b) and subsequent proteasomal degradation (c). The derepression of MYC-transcription factors results in the transcription of JA-Ile-induced genes (d).

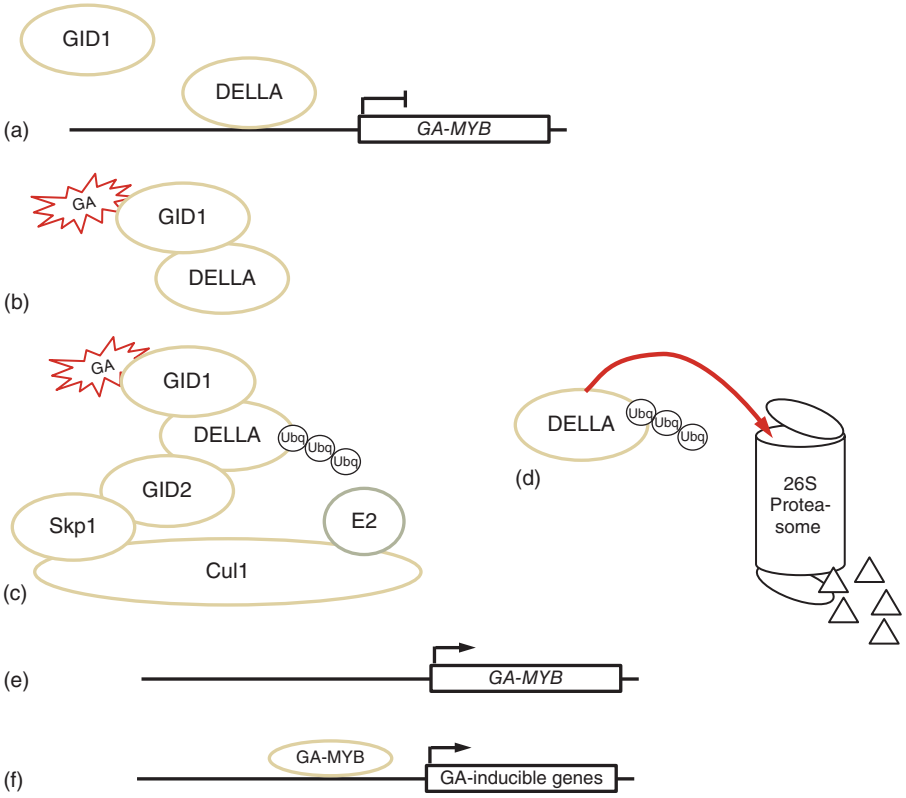

Figure 7.14 Perception of GA by binding to GID1, GID1-GA-SCF^{GID2}-mediated degradation of DELLA-repressors and derepression of gene expression. GA is perceived through high-affinity binding to GID1. The GA-GID1-complex can bind DELLA-proteins, which in the absence of GA repress genes for GA-inducible MYB-transcription factors (GA-MYB; a,b). Binding of GA-GID1-DELLA to an SCF-complex containing the F-box-protein GID2 mediates ubiquitinylation of the DELLA-proteins (c) and subsequent degradation by the 26S-proteasome (d). Removal of the DELLA-proteins delimits expression of GA-MYB (e) and enables the GA-MYB-dependent expression of GA-inducible genes (f).

deactivated by 2β-hydroxylation. The signal transduction of gibberellins acts through recognition by the F-box protein **Gibberellin-Insensitive Dwarf 1 (GID1)**.

The perception of gibberellins starts with the binding of the phytohormone to the protein GID1 (Figure 7.14). The GID1-gibberellin-complex then binds transcriptional repressors. Similar to the perception of auxin and JA-Ile by the F-box proteins TIR1 and COI1, respectively, the gibberellin-signal is further transduced through an SCF-complex containing the F-box protein, GID2 (Figure 7.14). Whereas TIR1 and COI1 are quite similar in sequence and in structural features, GID2 is less similar and only distantly related. In the presence of the GID1-gibberellin-complex, SCF^{GID2} mediates the binding, ubiquitinylation, and degradation of transcriptional repressors containing the amino acid sequence motif "DELLA" (DELLA-repressors). In the absence of gibberellin, DELLA-repressors occupy and inhibit the promoter regions for GA-MYB transcription factors. In the presence of the GID1-gibberellin-complex, SCF^{GID2}-mediated degradation of DELLA-repressors makes the promoters accessible (Figure 7.14). The subsequent expression of the GA-MYB transcription factors enables, aided by some other activating elements, the transcription of gibberellin-induced genes, thus resulting in the manifestation of gibberellin-induced cellular responses.

7.3.3 Signal Transduction through Other, Nonmembrane-Associated Receptors

7.3.3.1 Abscisic Acid

Abscisic acid (ABA) is responsible for the abscission of leaves in some plant species. ABA has widespread roles in the control of plant development and in mediating stress responses. An important function of ABA is the regulation of the dormancy of seeds and flower buds. Because of this function, ABA has also sometimes been called **dormin**. ABA inhibits the germination of potato sprouts and the germination of seeds still located in seed pods or siliques of the mother plant ("vivipary"). Stress responses regulated by ABA are related to the water equilibrium of plants. ABA serves as a signal for drought stress and is involved in the regulation of stomatal opening and closure (see Chapter 10).

ABA biosynthesis starts in the plastid with the modification of β-carotene-related pigments originating from

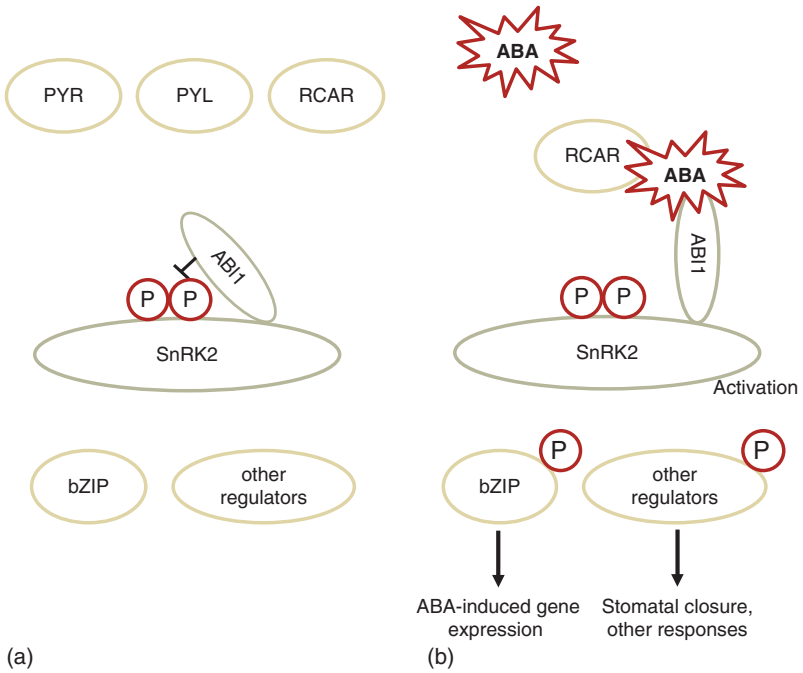

Figure 7.15 (a,b) **Perception of abscisic acid (ABA) through cytosolic receptors that activate a protein phosphorylation cascade.** ABA binds in the cytosol to the proteins PYR, PYL, or RCAR, resulting in conformational changes, which enable binding to the protein phosphatase ABI1. ABI1 regulates the protein kinase SnRK2 by maintaining it in a dephosphorylated state, inhibiting its protein kinase activity. In the presence of abscisic acid, PYR, PYL, or RCAR bind and inhibit ABI1, thereby enabling phosphorylation of SnRK2. Thus activated SnRK2 now phosphorylates target proteins controlling ABA-induced responses.

isoprenoid metabolism (see Chapter 2). ABA signals are terminated by the modification of ABA by the enzyme ABA 8′-hydroxylase.

ABA is recognized in the cytosol by binding to specific proteins, such as **Pyrabactin Resistance (PYR)**, **Pyrabactin Resistance-Like (PYL,)** or **Regulatory Component of ABA Receptor (RCAR**, Figure 7.15). On binding of ABA to PYR, PYL, or RCAR, these proteins alter their conformation, enabling binding to the protein **ABA insensitive 1** (**ABI1**). ABI1 is a protein phosphatase of the family 2C (PP2C) and is bound to a key regulator of ABA signal transduction, the protein kinase **SnRK2** (see Section 7.5 on protein phosphorylation). In the absence of ABA, ABI1 keeps SnRK2 in a dephosphorylated state, thereby inhibiting its protein kinase activity (Figure 7.15). When ABA is present and bound to PYR, PYL, or RCAR, either of these proteins can bind to ABI1 and inhibit its activity, thereby enabling SnRK2 to be phosphorylated. Thus activated SnRK2 can now phosphorylate various target proteins required for the manifestation of ABA-induced cellular responses. For instance, phosphorylation of transcription factors of the bZIP-family by SnRK2 activates transcription factors and results in the induction of ABA-induced genes. The phosphorylation of various other proteins by SnRK2 can enable stomatal closure or other cellular responses mediated by ABA.

7.3.3.2 Salicylic Acid

Salicylic acid (SA) is an important factor mediating defense responses of plants against pathogenic microorganisms or plant viruses. Precursors for the biosynthesis of SA originate from the shikimate pathway (see Chapter 2). Starting with chorismate, two alternative metabolic routes result in the formation of SA. The shorter route generates SA via an isochorismate-intermediate. In the longer route, chorismate is first converted to phenylalanine, which is then converted via transcinnamic acid to benzoic acid and finally to SA. SA signals are terminated by decarboxylation to catechol.

SA is recognized by the protein **Nonexpressor of Pathogenesis-Related Genes 1 (NPR1**, Figure 7.16). Depending on its redox-state, NPR1 aggregates to cytosolic protein-clusters that represent an inactive form of NPR1. On binding to SA, the redox-state of NPR1 changes, and the cytosolic clusters are dissolved. The release of cystine-bridges is accompanied by S-nitrosylation (see Section 7.4.3), linking SA-signaling to nitric oxide signaling. Individual NPR1 proteins can now enter the nucleus (Figure 7.16), where they serve as activators of transcription factors of the TGA-family (named thus after their preferred recognition sequence, which contains the DNA-sequence TGA). The expression of pathogenesis-related genes induced by SA is an important factor in plant defenses against pathogenic microorganisms or viruses.

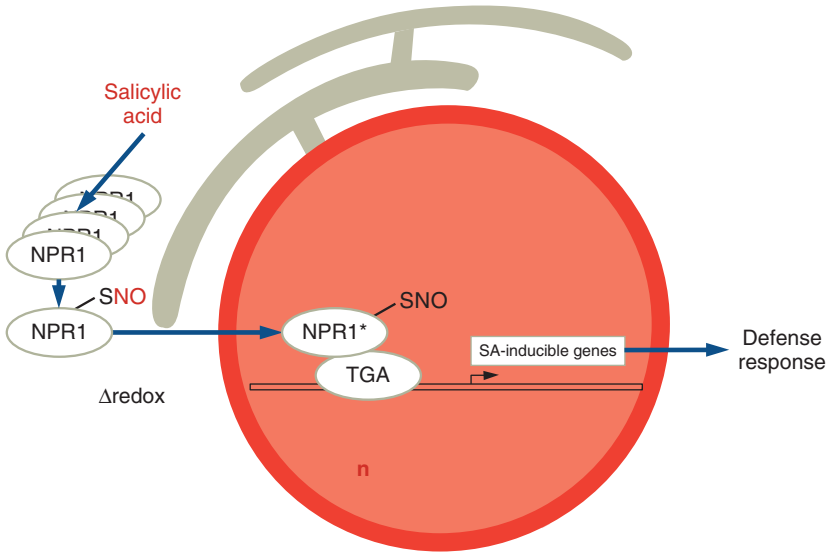

Figure 7.16 Perception of salicylic acid (SA) by redox-mediated cytosolic-to-nuclear shuttling of the regulatory protein NPR1. SA is recognized by NPR1, a redox-sensitive receptor. In the absence of SA NPR1 forms cytosolic aggregates. When SA is present, the redox-state of NPR1 changes, the cytosolic aggregates are dissolved. NPR1 enters the nucleus and activates TGA-transcription factors responsible for the expression of SA-dependent genes.

7.3.4 Signal Transduction through Transmembrane-Receptors

7.3.4.1 Cytokinins

Cytokinins (CKs) influence development and differentiation of plant cells. CKs include substances, such as trans-zeatine (tZ), cis-zeatine (cZ), or N6-isopentenyladenine (IPA). Several purine-derivatives also have CK function, such as furfuryladenine (kinetine), benzylaminopurine (BAP), or meta-topoline (mT). CKs counteract many effects of auxin, and the balance between auxin and CKs exerts substantial influence on a plant cell's developmental fate. Some CKs are formed by gall-inducing bacteria, such as *A. tumefaciens*, which use the effects of the released CKs on the plant for their benefit. CKs became a focus of biotechnology because it was discovered that genetically modified plant cells can be prompted by CKs to regenerate whole plants. This application enabled the permanent genetic modification of plants and is still a base for many plant transformation protocols used today.

The biosynthesis of CKs starts with dimethylallylpyrophosphate (DMAPP), an intermediate of terpenoid metabolism (see Chapter 2). CKs are formed in all plant cells. CK signals are terminated by the action of CK oxidases that can reside in the apoplast or within cells.

CKs are recognized by the plasma membrane-localized receptor-like histidine kinase (RHK), **Arabidopsis Histidin Kinase 4 (AHK4)**, which likely serves as a cell surface receptor (see Section 7.5). It is also contended that AHK4 may reside in the endoplasmic reticulum. AHK4 consists of several important protein domains (Figure 7.17), including the extracellular CK-binding domain, a transmembrane domain and intracellular histidine kinase and aspartate kinase domains. On binding of CK to the extracellular domain, AHK4 displays a conformational change of its intracellular domains, resulting in the ATP-dependent autophosphorylation of histidine-residues (Figure 7.17). This phosphorylation triggers the phosphorylation of aspartate residues in another portion of AHK4 (Figure 7.17), which mediates the phosphorylation of a second, soluble protein-component, the **Arabidopsis Histidine Phosphotransferprotein (AHP)**, in the cytosol (see Section 7.4.3). Phosphorylated AHP transfers to the nucleus, where it influences **Arabidopsis Response Regulators (ARRs)** of type B, which control gene expression (Figure 7.17). AHP1 is also a target for S-nitrosation by NO (see Section 7.4.2). Type B ARRs possess a DNA-binding domain called GARP (because of its similarity to proteins of the Golgi-associated retrograde protein-family). The GARP domain can bind DNA in a sequence-specific manner. In the absence of CK, little or no phosphorylated AHP is present in the nucleus, and type B ARRs are present in an inactive state autoinhibiting the transcriptional activation domain. When CK is present and the nuclear levels of phosphorylated AHP increase, type B ARRs are phosphorylated on an aspartate residue, resulting in a conformational change enabling GARP-mediated binding to DNA and interaction of the transcriptional activation domain with the promoter regions of CK-regulated genes (Figure 7.17). Phosphorylated AHP in the nucleus can also interact with type A ARRs and mediate their phosphorylation. Thus activated type A ARRs can have inhibitory effects on the DNA-interaction of type B ARRs serving as signal terminators. Active type A ARRs can also inhibit nuclear effects of light-mediated signaling cascades, such as phytochrome signals and photomorphogenesis. Although it is evident that type A ARRs

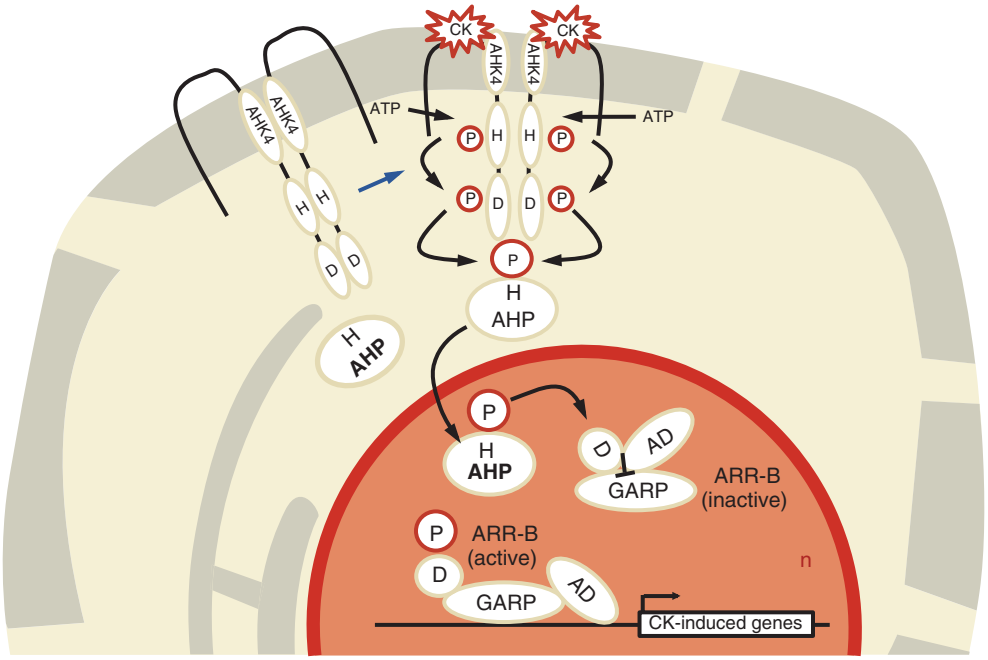

Figure 7.17 Perception of cytokinins (CKs) by a two-component system involving the membrane-bound receptor-kinase AHK4. CKs are recognized by the receptor AHK4, which is a transmembrane protein and contains extracellular and intracellular domains. On binding of CK, the conformation of AHK4 changes, resulting in autophosphorylation of histidines and aspartates at the intracellular domains. AHK4-autophosphorylation mediates phosphorylation of AHP in the cytosol. Phosphorylated AHP transfers to the nucleus, where it activates Arabidopsis response regulators (ARRs) of type B, which control CK-dependent gene expression via their GARP domain and their activation domain (AD).

exert a range of additional effects in the nucleus, these have not been well studied to date. Because AHP represents a second component that can mediate diverse effects on transfer to the nucleus, the CK signal transduction cascade is an example for a two-component signaling system.

7.3.4.2 Ethylene

Ethylene (ETH) is a gas. Its volatile nature enables ETH to pass between plants and influence whole populations. Furthermore, high ETH levels may signal that a seedling is still below ground, as above ground the ETH will quickly disperse. ETH influences the development of seedlings as well as leaf abscission, flower senescence, and fruit ripening. Seedlings respond to ETH-treatment with a characteristic growth pattern that consists of reduced root growth, reduced hypocotyl growth, and apical hook-formation. Because of these three components of the response, this ETH-induced growth pattern is called the *triple response*. ETH is also formed during fruit ripening and mediates the climacterium, that is, the transition from nonripe to ripe fruits (Figure 7.18).

ETH is derived from methionine via *S*-adenosyl-L-methionine (SAM, see Section S1.3.9.3). A key and rate-limiting step of ETH biosynthesis is the synthesis of aminocyclopropane-1-carbocylic acid (ACC). ACC is the last soluble intermediate in the sequence leading to the gaseous ETH; therefore, ACC is oftentimes measured as an indicator for ETH production. ACC is converted to ETH by the enzyme ACC-oxidase, releasing CO_2, H_2O, and HCN as by-products. The production of the highly potent respiratory toxin HCN as a by-product is noteworthy, as plants do not display limited respiratory activity during ETH production. Plant respiration is quite sensitive to HCN, and HCN generated during ETH production is very efficiently detoxified. There is no known mechanism for the termination of ETH signals, possibly because the phytohormone is released as a gas and does not accumulate.

The receptor for ETH resides in the plasma membrane and consists of two subunits, the protein **ETH Response Sensor 1 (ERS1)** and the RHK, **ETH Receptor 1 (ETR1)** (Figure 7.19, see also Section 7.5). ERS1 and ETR1 are linked by disulfide bonding and jointly coordinated copper ions. The extracellular binding site for ethylene is also formed by both partners. On the intracellular face of the plasma membrane both ERS1 and ETR1 contain binding motifs for the repressor protein Constitutive Triple Response (CTR1). In the absence of ETH, CTR1 is bound to ERS1/ETR1 and maintained in a phosphorylated form (Figure 7.19). CTR1 has similarity to MAPKKKs known from the yeast *Saccharomyces cerevisiae* (*Pezizomycotina, Ascomycota*). Phosphorylated CTR1 is active and represses a protein phosphorylation cascade of MAPKs and the endomembrane-associated protein ETH Insensitive 2 (EIN2) that ultimately controls the proteasomal degradation of the nuclear protein ETH Insensitive 3 (EIN3). Degradation of EIN3 involves an SCF-complex, in which

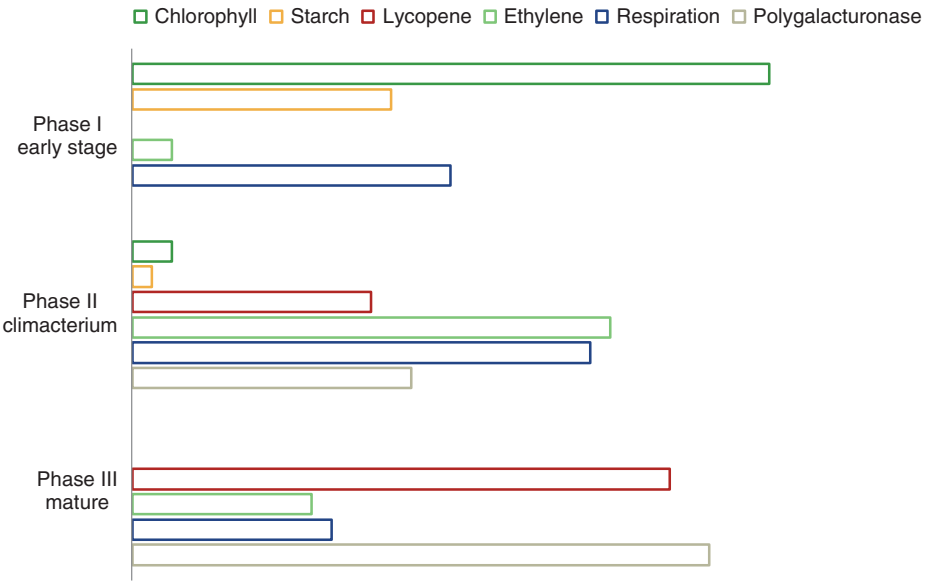

Figure 7.18 **Physiology of tomato fruit ripening.** During tomato fruit ripening chlorophyll decreases concomitant with starch. The climacterium is associated with drastically elevated levels of the phytohormone, ETH. Fruits attain their red color during ripening by accumulating the red pigment, lycopene. (Graphics: D. Dobritzsch.)

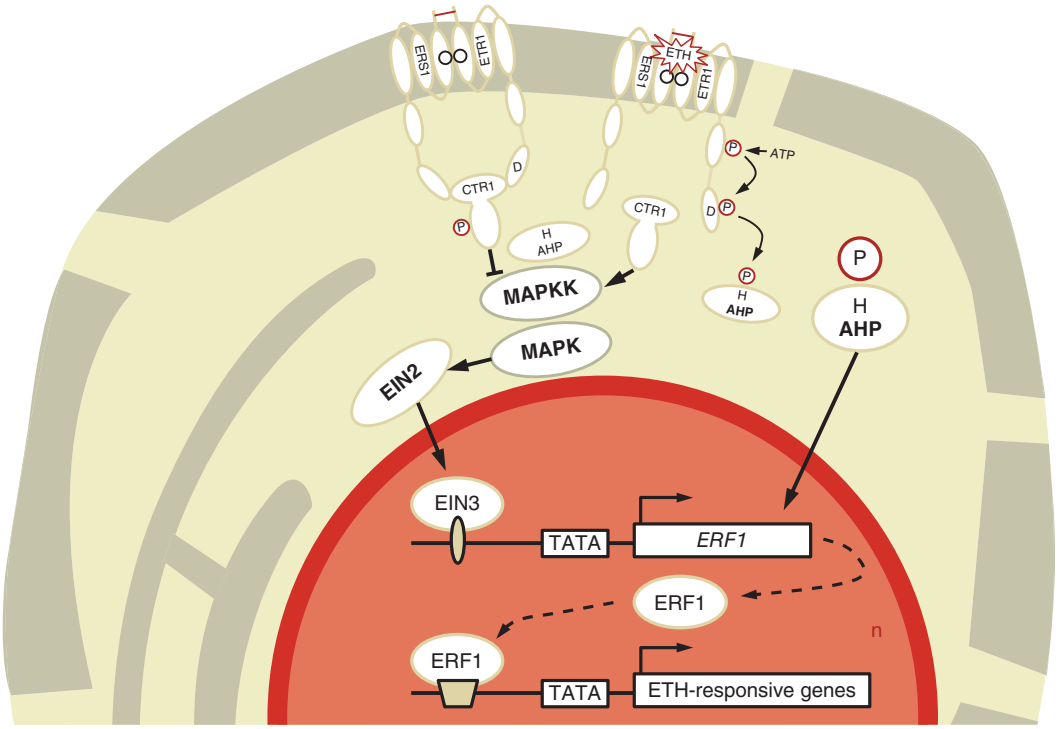

Figure 7.19 Ethylene (**ETH) signal transduction through ERS1/ETR1 and derepression of a protein phosphorylation cascade**. ETH is perceived by a plasma membrane receptor consisting of the two subunits ERS1 and ETR1. ERS1 and ETR1 are linked by disulfide bonding (red) and jointly coordinate copper ions (yellow). The intracellular domains of ERS1 and ETR1 bind the repressor protein CTR1. In the absence of ethylene, CTR1 is bound to ERS1/ETR1 and maintained in a phosphorylated form that represses a MAPK-cascade regulating the function of the endomembrane-associated protein EIN2. EIN2 controls proteasomal degradation of the nuclear protein EIN3. Degradation of EIN3 involves an SCF-complex, which contains the proteins EBF1 and EBF2 as F-box proteins. Binding of ETH to ERS1/ETR1 results in phosphorylation and a conformational change of ETR1, releasing and deactivating CTR1. Deactivation of CTR1 derepresses the MAPK-cascade regulating EIN2, resulting in reduced degradation of EIN3. EIN3 is a transcription factor enabling the production of ERF1, another transcription factor controlling the transcription of ETH-dependent genes. Besides the signal transduction cascade through CTR1, the conformational change in ERS1/ETR1 also results in phosphorylation of AHP, which can shuttle to the nucleus and phosphorylate ARR2.

the proteins EIN3-binding F-box protein 1 (EBF1) and EIN3-binding F-box protein 2 (EBF2) represent the relevant F-box proteins. When ETH is present (Figure 7.19), binding to the ERS1/ETR1 receptor results in phosphorylation and a conformational change of the intracellular portion of the receptor. In consequence, CTR1 is released from the ERS1/ETR1 receptor, is no longer phosphorylated and thus deactivated. The deactivation of CTR1 enables the progression of the MAPK-cascade and subsequent activation of EIN2 (Figure 7.19), which results in reduced degradation of nuclear EIN3. EIN3 is a transcription factor enabling the production of the protein ETH response factor 1 (ERF1). Formation of ERF1 in turn activates the transcription of genes manifesting ETH responses (Figure 7.19). The conformational change in ERS1/ETR1 additionally results in the phosphorylation of AHP, which shuttles to the nucleus and phosphorylates ARR2 (Figure 7.19). Even though it is known from other signaling contexts that ARR2 influences plant signaling, the consequences of ARR2 in the context of ETH signal transduction remain unclear.

7.3.4.3 Brassinosteroids

Brassinosteroids derive from isoprenoid metabolism (see Chapter 2) and influence various aspects of plant growth. More than 40 different brassinosteroids are known. Examples include brassinolide (BL) and castasterone (CS). Brassinosteroids enhance shoot elongation and pollen tube growth and mediate the unrolling of grass-leaves. At the molecular level, brassinosteroid effects are mediated through an activation of ETH production, through the activation of certain ion pumps, and through effects on the cytoskeleton.

The plasma membrane receptor for brassinosteroids is formed by two proteins, **Brassinosteroid-Insensitive 1 (BRI1)** and the **BRI1-Associated Receptor-Kinase 1 (BAK1**; Figure 7.20, see Section 7.5). Both proteins contain extracellular leucine-rich repeat domains. The extracellular domain of BAK1 also contains leucine-zipper motifs. Binding of brassinosteroids occurs to a specific ligand-binding-domain present only in BRI1. BRI1 and BAK1 are each anchored in the membrane by a single transmembrane domain and both proteins contain a C-terminal protein kinase domain in the cytosol. In the absence of brassinosteroids, BRI1 and BAK1 are present as separate proteins in the membrane (Figure 7.20). Other components of the brassinosteroid signal transduction cascade shuttle between the cytoplasm and the nucleus, including the transcription factor BRI1-EMS-Suppressor1 (BES1) and the transcriptional repressor Brassinazole-Resistant 1 (BZR1). In the absence of brassinosteroids, BES1 and BZR1 are maintained in a phosphorylated state by the protein kinase brassinosteroid insensitive 2 (BIN2) and are both inactive (Figure 7.20). In consequence, genes required for brassinosteroid responses are not activated by BES1, and genes underlying brassinosteroid production are not repressed by BZR1. When brassinosteroids are present and bind to BRI1, BRI1 and BAK1 associate in the plasma membrane to form a heterodimer (Figure 7.20). The association mediates a conformational change in the cytosolic protein kinase domains, resulting in autophosphorylation of both BRI1 and BAK1. So far it is unclear how the signaling cascade is further linked to BES1 and BZR1. However, it is evident that in the presence of brassinosteroids, BIN2 is inactivated. The protein phosphatase BRI1-Suppressor 1 (BSU1) now dephosphorylates BES1 and BZR1, enabling their shuttling from the cytosol to the nucleus (Figure 7.20). In the nucleus, the now active BES1 and BZR1 can bind to DNA and affect the transcription of brassinosterid-dependent genes. BES1 activates genes required for brassinosteroid responses, whereas BZR1 represses genes for brassinosteroid production, thus terminating the signal (Figure 7.20).

7.4 Other Signaling Molecules

7.4.1 Phytochromes and Cryptochromes

Plants are capable of sensing the quality of light, which represents an important environmental cue for seed germination and plastid development. For instance, sunlight reaching the earth's surface in summer and in winter differs in the proportion of red versus far-red wavelengths, and this difference can influence seasonal developmental processes in plants. **Phytochromes** and **cryptochromes** are photosensors (see Chapter 9). Light exerts important effects on plant development and morphogenesis, and some of the signal transduction elements of phytochrome or cryptochrome signaling resemble those found in phytohormone signal transduction cascades.

7.4.2 Strigolactones and Nodulation Factors

Strigolactones mediate apical dominance and appear to overall enhance auxin effects. In order to establish and maintain symbiotic interspecies-interactions between plants and fungi, plants generate and secrete strigolactones, which stimulate the branching and growth of symbiotic arbuscular mycorrhizal fungi. The enhanced branching and growth characteristics mediated by strigolactones in the fungi increase the probability of contact and establishment of a symbiotic association between plants and their respective fungal partners (see Chapters 5 and 15). Leguminous plants, such as soy, pea, bean, alfalfa, clover, and peanut, attract nitrogen-fixing bacteria of the genus *Rhizobium* (*Alphaproteobacteria*) from the soil, which act as morphogens on the plant and mediate the establishment of root nodules. Plant root-secreted flavonoids attract

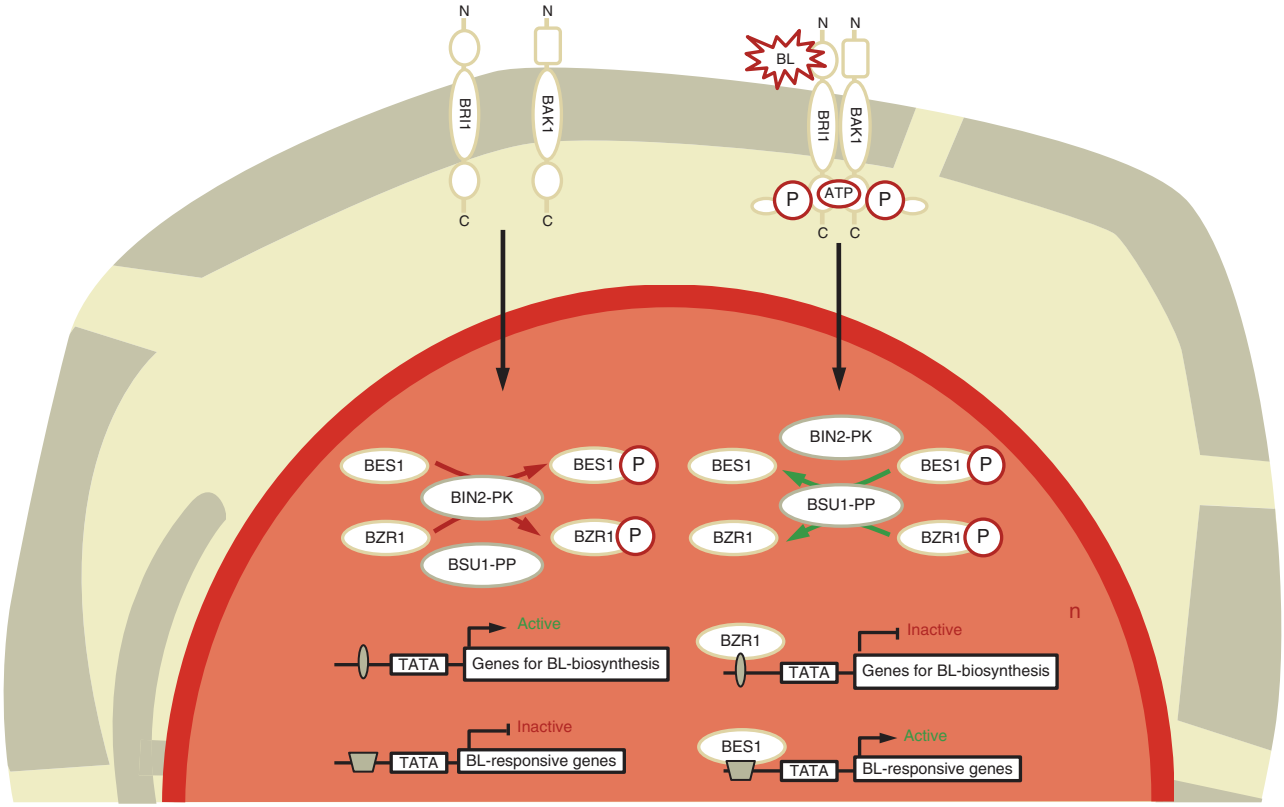

Figure 7.20 Brassinosteroid-signal transduction through the heterodimeric membrane-bound receptor-kinase BRI1/BAK1 and derepression of a protein phosphorylation cascade. Brassinolide (BL) is perceived by the combined action of the plasma membrane proteins BRI1 and BAK1. BRI1 and BAK1 are each anchored in the membrane by a single transmembrane domain and both proteins contain a C-terminal protein kinase domain in the cytosol. In the absence of BL, BRI1 and BAK1 are present as separate proteins. Other components of the brassinosteroid signal transduction cascade shuttle between the cytoplasm and the nucleus, including the transcription factor BES1 and the transcriptional repressor BZR1, which are maintained in an inactive phosphorylated state by the protein kinase BIN2 (A). Binding of BL to BRI1 causes BRI1 and BAK1 to associate. The association mediates a conformational change and autophosphorylation of the cytosolic kinase domains of both BRI1 and BAK1. Signaling events unknown so far result in the inactivation of BIN2, enabling the protein phosphatase BSU1 to dephosphorylate BES1 and BZR1. In the nucleus, the now active BES1 and BZR1 can bind to DNA and affect the transcription of BL-dependent genes. BES1 activates genes required for BL responses, whereas BZR1 represses genes for brassinosteroid production.

rhizobia, which release **nodulation factors (Nod factors)**. Nod factors exert effects on plant growth and have an important function in the establishment of the essential symbiosis of legumes with the nitrogen-fixing bacterial partners (see Chapter 15).

7.4.3 Reactive Oxygen Species and Nitric Oxide

Reactive oxygen species (ROS) are highly reactive molecules that contain oxygen (see Chapter 8). Examples include oxygen radicals, ions, and peroxides. Because of their reactivity, elevated levels of ROS are toxic. Small amounts that are locally and transiently formed serve as signaling molecules that mediate a wide range of cellular responses. In response to stimulation, ROS can be generated in different subcellular compartments. To avoid the toxicity, ROS production is tightly regulated. The control of ROS production is interlinked with protein phosphorylation (see Section 7.5), calcium signaling (see Section 7.6), and other signaling pathways. The redox modification of proteins, such as transcription factors, is a possible means for ROS to interact with other signaling pathways.

Nitric oxide (NO) is a gaseous signaling molecule involved in multiple signaling pathways. NO is a free radical and very reactive. NO can be formed by several alternative routes, including the action of arginine-dependent nitric oxide synthase, plasma membrane-associated nitrate reductase, mitochondrial electron transport processes, or via nonenzymatic reactions. In combination with redox-sensitive partners NO can exert various functions. NO mediates the reversible **S-nitrosation** of reactive thiol-groups, such as cysteins in proteins, resulting in the formation of S-nitrosothiols. S-nitrosation is an important posttranslational modification that occurs on all major protein classes and can have multiple effects on physiology. An example is the S-nitrosation of NPR1 in the context of SA-signaling (Section 7.3.3.2 and Figure 7.16). NO can also

attack transition metals, such as iron or copper that are cofactors of proteins, for example, in heme groups, acting as a nitrosyl-ligand. The nitrosylation of the metal ions typically results in the inactivation of catalytic activity, providing a means of physiological regulation. The formation of S-nitrothiols and of nitrosyl-ligands to metal ions can occur independently on the same target protein.

7.4.4 Peptide Signals

Peptides functioning as signals consist of amino acid stretches approximately between 10 and 25 residues and can serve diverse functions in plants. Functions of such signals range from the control of development to the mediation of responses to abiotic and biotic stresses. Peptide signals can also serve to convey self-incompatibility signals during sexual reproduction. In all cases the signaling peptides are produced by the processing of larger precursor proteins, and it is possible to generate more than one signal peptide from the same precursor. Peptide signals are often secreted to the apoplast, although signal sequences for secretion are not always recognizable. The secreted peptides may be present in the apoplast first as inactive forms, which must be activated by further processing.

Peptide signals have important roles in development by serving as cell-to-cell signals to control cell division and differentiation. Plant cells originate from meristems, clusters of pluripotent cells that remain in an active cell cycle and generate cells that undergo differentiation. Examples for meristems are those present at the shoot apex (the shoot-apical meristem, SAM) or at the root tip (the root apical meristem, RAM). In order to control cell division patterns underlying proper organ formation, size and activity of meristems must by strictly regulated. Peptide signals exchanged between the meristem cells and the differentiating cells in the meristem-organizing centers (MOCs) are involved in the control of meristem maintenance and are very important for plant growth. Many cells are both recipients and producers of signals, and regulatory feedback loops between cells are essential components of mechanisms preserving tissue integrity. A particular class of peptide signals involved in meristem maintenance are those engaged in the clavata (*CLV*) signaling pathway (Figure 7.21). The *Arabidopsis clavata* (*CLV*) mutants were originally named according to the characteristic club shape of their fruits (lat. "clava" means "club"). The club shape of the mutant fruits results from an increased size of floral meristems caused by the neighboring cells of the organizing center failing to limit meristem size (Figure 7.21a). Other meristems, such as the SAM, are also affected by *CLV*-like signals. The analysis of *CLV*-mutants revealed three components of a signaling pathway that act together, *CLV1*, encoding a leucine-rich repeat-receptor-like kinase (LRR-RLK) (see also Section 7.5); *CLV2*, encoding a leucine-rich repeat (LRR)-containing accessory receptor protein; and *CLV3*, encoding a peptide signal. The *CLV3* gene belongs to a family of 32 CLE genes (for *CLV*/enhancer of shoot regeneration (ESR)) in *Arabidopsis*, which encode small proteins of up to 15 kDa. The CLE proteins contain characteristic N-terminal motifs that act as signal peptides for secretion. Furthermore, CLE proteins contain a CLE-motif, a 14 amino acid signature at the C-terminus from which active signal peptides (CLEp) of 12 or 13 amino acids are processed (Figure 7.21b). The CLEp signals are recognized by cells containing CLV1 receptors, which are aided in their function by the CLV2 accessory proteins (Figure 7.21c,d).

Examples for peptide signals with roles in plant defense include systemin of *Solanaceae*, hydroxyproline-rich systemin glycopeptides (HypSys) of *Cestroideae* (both *Solanales, Asteridae*), and Arabidopsis peptides (AtPeps) of *A. thaliana*. A secreted propeptide is prosystemin, which is processed to generate mature systemin only after insect attack. Systemin and HypSys elicit the JA cascade and aid the induction of defense responses. AtPeps appear to have a broader role in plant defense, mediating defense responses also against attack by pathogenic microorganisms.

7.4.5 Volatile Signals

Plants emit volatile signals to enable long-distance signaling. Long-distance signaling can occur between individual plants of a population, or, can be used for interspecies communication to attract certain insects (see Chapter 16). A simple such interspecies signal is given by the odors generated by plants to attract pollinators. The fragrance of flowers has long been a source of human enjoyment, and the chemical basis for the volatile fragrances emitted by flowers has been determined for many examples. Floral odorants (Figure 7.22) can be categorized into diverse chemical compounds, including isoprenoid esters, such as geranylacetate (e.g., in rose flowers), isoamylacetate (e.g., in banana); linear terpenes, such as linalool (e.g., in lavender); cyclic terpenes, such as α-Ionone (e.g., in violet); or polyamines, such as putrescine or cadaverine (e.g., in the carrion flower, *Hoodia gordonii, Gentianales, Asteridae*). Obviously, the smell exuded by flowers may or may not be pleasant to human noses, as, for example, diamines emitted by *Hoodia* species resembles rotten meat and will attract flies. The odorous emissions of flowers are constant over the time of flowering; other volatile substances are emitted by plants on perception of stress.

Volatile signals are also emitted by plants on perceived **herbivory** (see Chapter 16). Plants respond to herbivory by caterpillars with the emission of characteristic mixtures of volatile aldehydes and terpenoids that can be recognized by insect predators preying on larvae feeding on the plant. The emitted signals are not generic, but rather attract predators that specifically parasite the particular herbivores currently infesting the plant. The specific nature of the plant response to herbivory indicates that the plants are capable (i) of

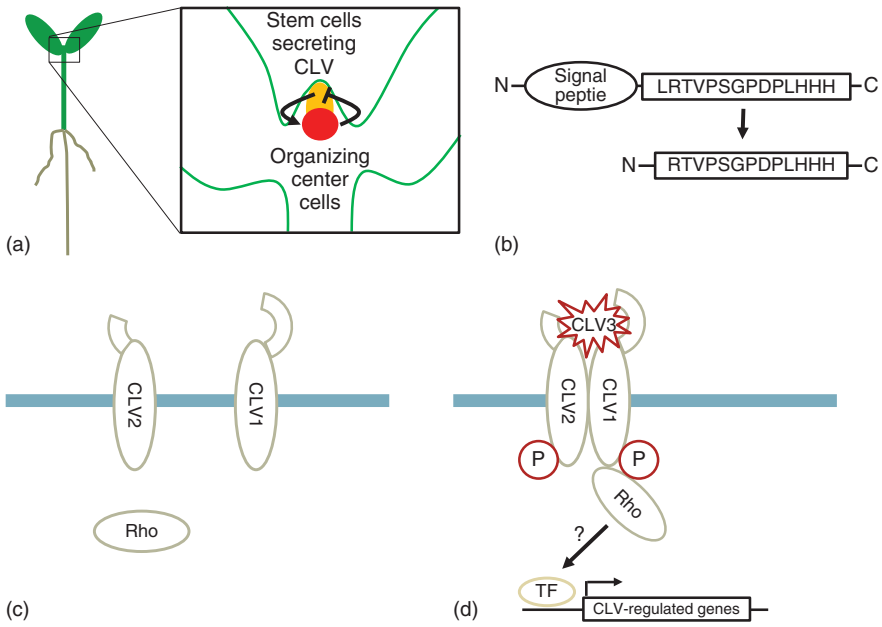

Figure 7.21 **The CLAVATA (CLV)-pathway: Peptide signals that control cell division and differentiation.** Plant cells originate from clusters of pluripotent cells (meristems) that remain in an active cell cycle and generate cells that will undergo differentiation. Examples for meristems are the shoot apical meristem (SAM) or the RAM. To ensure proper organ formation, size and activity of meristems must by strictly regulated. Peptide signals exchanged bidirectionally between the meristem cells and the differentiating cells in the MOCs are very important for plant growth (a). A particular class of peptide signals involved in limiting meristem size are those engaged in the clavata signaling pathway. The characteristic club shape of the mutant fruits (lat. *clava* = club) resulted from an increased size of floral meristems caused by the neighboring cells of the organizing center failing to limit meristem size. In the CLV-pathway three components act together, *CLV1*, *CLV2*, and *CLV3*, of which *CLV3* encodes the signal peptide itself. The *CLV3* gene belongs to a family of 32 related genes encoding small proteins of up to 15 kDa. These proteins all contain characteristic N-terminal motifs that act as signal peptides for secretion and contain a 14 amino acid signature at the C-terminus from which active signal peptides of 12 or 13 amino acids are processed (b). *CLV1* and *CLV2* encode parts of the perception machinery for the peptide signals. Recognition occurs in cells containing the CLV1 receptors, which are aided in their function by the CLV2 accessory proteins (c,d). Signal transduction furthermore involves monomeric Rho-GTPases to influence gene expression patterns.

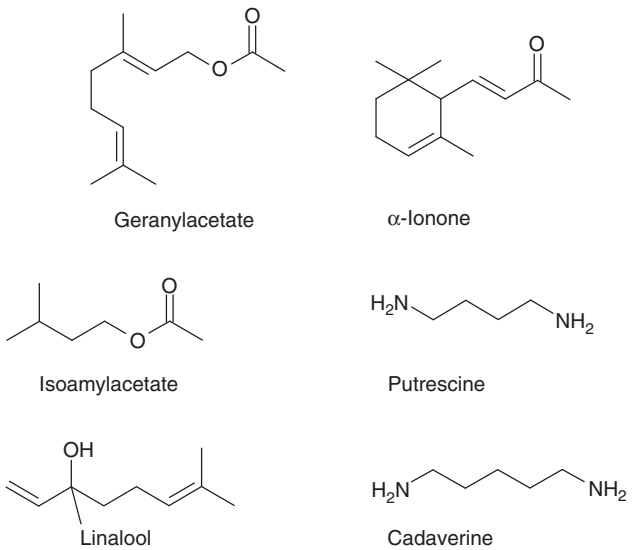

Figure 7.22 **Well-known fragrances: Floral volatiles.** Plants employ volatile signals for long-distance signaling between individual plants of a population or for interspecies communication, for example, to attract insects. Floral volatiles can be pleasant or unpleasant to the human nose and can be categorized into diverse chemical compounds. Important examples include isoprenoid esters, such as geranylacetate from rose flowers or isoamylacetate from bananas; linear terpenes, such as linalool from lavender; cyclic terpenes, such as α-ionone from violets; or amines, such as putrescine or cadaverine from the carrion flower, *Hoodia gordonii* (*Gentianales, Asteridae*).

determining which herbivore is currently feeding, and (ii) of emitting signals that specifically attract a suitable predator. Although the complex signaling events involved in the species-specific recognition are still under investigation, it is evident that the chemical composition of the herbivores' saliva contributes information, based on which the insect is recognized by the plant. The volatiles emitted on perception of the stress are not individual substances, but rather mixtures of compounds, which are called *bouquets*. Different bouquets of volatiles attract different predators and the combinations of plants, herbivores, volatile bouquets, and attracted predators are a prime example for the coevolution of different plant and insect partners in a complex ecosystem. The volatiles emitted by the plants include ketones and aldehydes that derive from fatty acid hydroperoxides, as well as isoprenoids and terpenoids (see Chapter 2).

7.4.6 Polyamines

Polyamines are involved in the control of plant growth and development and have been demonstrated to convey antisenescence and antistress effects. For instance, polyamines have demonstrated roles in defense responses of plants to environmental stresses, including metal toxicity, oxidative stress, drought, salinity, and cold stress. They occur as free polycationic molecules or are covalently linked to partners, such as phenolic compounds, resulting in soluble conjugates, or to nucleic acids or proteins, resulting in insoluble forms. Under physiological conditions, polyamines are dications, a feature important for their biological activity. It is being discussed that the enzymatic oxidation of polyamines generates ROS (see Section 7.4.3) with roles in signaling. The biosynthesis of polyamines is outlined in Figure 7.23.

7.4.7 Phosphoinositides and Inositolpolyphosphates

Phosphoinositides are minor components of cellular membranes in all eukaryotes, including plants, and account for only approximately <1% of the phospholipid mass of a leaf. All phosphoinositides are derived from the phospholipid phosphatidylinositol (PI), which can be phosphorylated at the 3-hydroxyl, 4-hydroxyl, or 5-hydroxyl positions of the inositol head group by specific phosphoinositide kinases. Phosphorylation can occur sequentially in more than one position, giving rise to a set of structurally closely related phosphatidylinositol-monophosphates and phosphatidylinositol-bisphosphates (Figure 7.24). The lipid phosphatidylinositol 3,4,5-trisphosphate has important functions in animal cells but has not been detected in any plant investigated so far. Phosphorylated phosphoinositides (PPIs) exert regulatory functions on the physiology of a cell by serving as membrane ligands to various target proteins

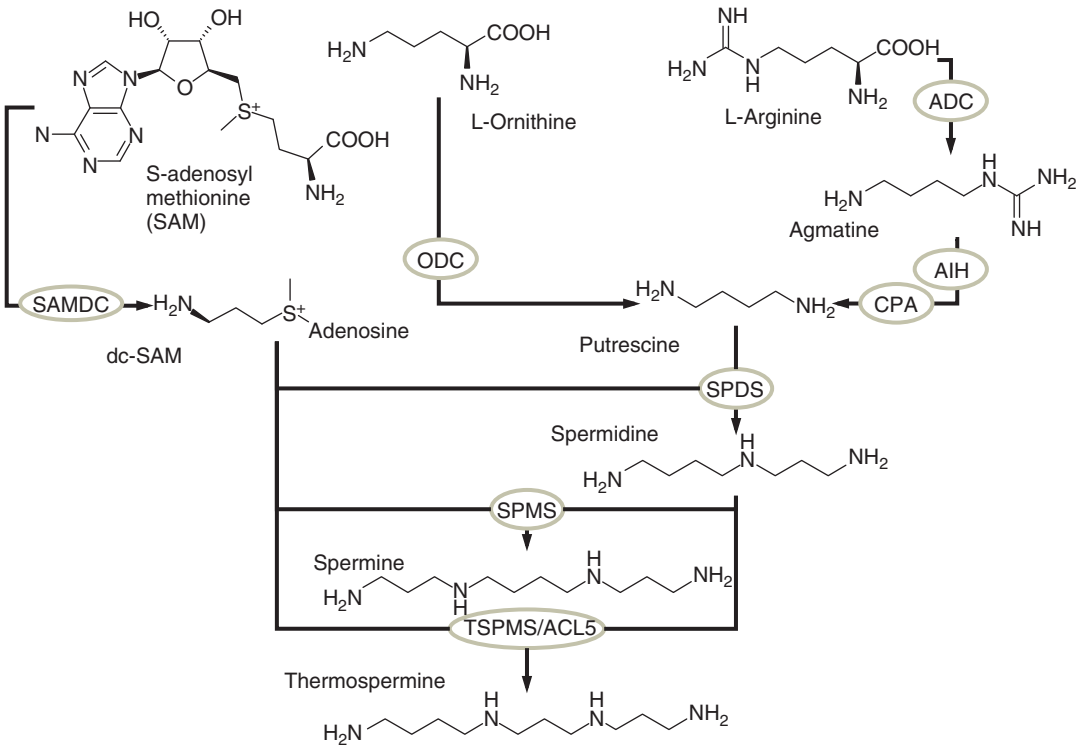

Figure 7.23 Biosynthesis of polyamines. The polyamines putrescine, spermidine, spermine, and thermospermine are synthesized from the precursors S-adenosyl methionine (SAM) or arginine, as indicated. Key enzymes of polyamine production are S-adenosyl methionine decarboxylase (SAMDC), ornithine decarboxylase (ODC), arginine decarboxylase (ADC), agmatine deaminase (AIH), carbamoyl putrescine amidase (CPA), spermidine synthase (SPDS), spermine synthase (SPMS), and thermospermine synthase (TSPMS, ACL5).

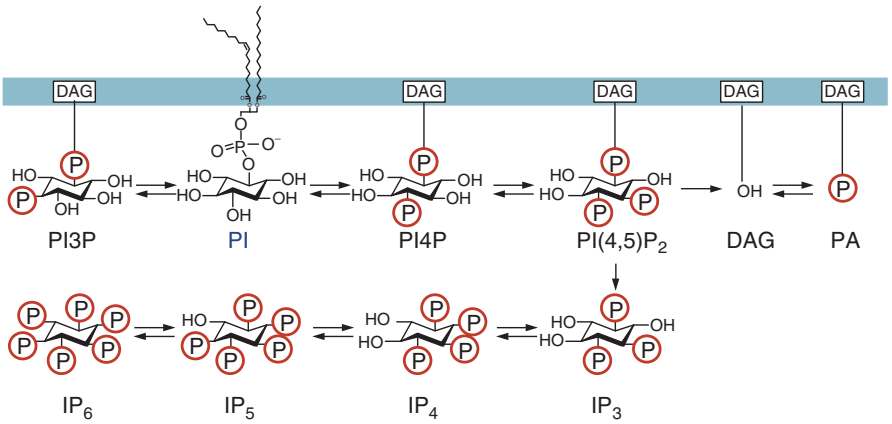

Figure 7.24 Inositol-containing phospholipids (phosphoinositides). Phosphoinositides derive from the phospholipid phosphatidylinositol (PI) by phosphorylation at the 3-hydroxyl, 4-hydroxyl, or 5-hydroxyl positions of the inositol head group. Phosphorylation can occur sequentially in more than one position, giving rise to PI-monophosphates as well as PI-bisphosphates, as indicated. The characteristic phosphorylated head groups of phosphoinositides can be recognized by highly specific binding domains in numerous proteins, enabling the recruitment of a protein to a membrane area rich in a given phosphoinositide, or the control of its biochemical activity. Phosphoinositides, such as PI4P or PI(4,5)P_2, can also be cleaved by phospholipase C into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Whereas IP_3 has known important functions in animal cells, its role in plants is still a matter of debate. IP_3 can be further phosphorylated to IP_4, IP_5, and IP_6 by soluble inositol polyphosphate-kinases. In plants, DAG is rapidly phosphorylated to phosphatidic acid (PA) by DAG-kinase; PA is being discussed as an important signaling lipid in plants. All phosphorylation reactions can be countered by dephosphorylation through phosphatases, as indicated by the arrows.

that possess specific binding domains for PPIs. Such binding domains include **Pleckstrin-homology (PH)-domains**, the **Fab1 YOTB Vac1 EEA1 (FYVE)-domain** or the **phagocytic oxidase (phox or PX)-domain**, which can all bind to different phosphoinositide species. By specifically binding to the lipids, target proteins are recruited to membranes or altered in their catalytic activity. Data on the effects of PPIs on physiological processes in plant cells are still scarce, but it is already evident that PPIs are of key importance for plant function, plant development, and for the adaptation to environmental stresses. For instance, it has recently been demonstrated that PPIs are involved in the polar localization of PIN-proteins (see Section 7.3.2.1), thus exerting effects on auxin transport and plant growth. Moreover, PPIs are essential for polar tip growth in specialized plant cells, such as pollen tubes or root hairs, where PPIs control apical secretion and exert influence on the dynamic actin cytoskeleton.

In response to a changing environment, cellular levels of PPIs change transiently, for instance, on treatment of plants with salt or heat, or on gravistimulation. So far, however, the physiological relevance of such changes remains unclear. Besides their roles as intact lipids, PPIs can be cleaved by phospholipase C, generating diacylglycerol and soluble inositolbisphosphates (IP_2) or inositoltrisphosphates (IP_3, Figure 7.24). Whereas diacylglycerol is a potent second messenger in animal cells, it is rapidly phosphorylated in plants to form phosphatidic acid (Figure 7.24), which also serves as a potent signaling molecule. Phosphatidic acid can alternatively be formed by phospholipase D acting on any given glycerophospholipid substrate. Similar to PPIs themselves, IP_3 has been shown to change transiently in plant cells on exogenous stimulation. However, the physiological relevance of such changes is still not understood. In animal cells, IP_3 is a potent activator of an endoplasmic reticulum-associated IP_3-sensitive calcium channel ("IP_3-receptor") and, thus, an elicitor and modulator of calcium release from internal stores. No IP_3-sensitive calcium channel has so far been discovered in plants. Nonetheless, microinjection of inositolpolyphosphates into plant cells have been reported to elicid transient cytosolic calcium increases; however, the origin of such inositolpolyphosphate-mediated calcium bursts and the mode of their release in plant cells remain unclear (see Section 7.6). A possible plant-specific role for inositolpolyphosphates and a link to phytohormone signaling is presented by the finding that the F-box proteins TIR1 and COI1, which act as receptors for auxin and JA-Ile, respectively, contain inositolpolyphosphates as structural cofactors (see phytohormones, Section 7.3). The inositolhexakisphosphate (IP_6) and inositolpentakisphosphate (IP_5) that are present in the TIR1 and COI1 structures, respectively, are formed from IP_2 and IP_3 precursors and may be required for receptor function.

7.4.8 Guanine Nucleotide-Binding Proteins

Numerous proteins with the ability to bind guanine nucleotides are called **G-proteins** and take part in signaling cascades in eukaryotic organisms, including plants. Depending on whether a G-protein binds GDP or GTP,

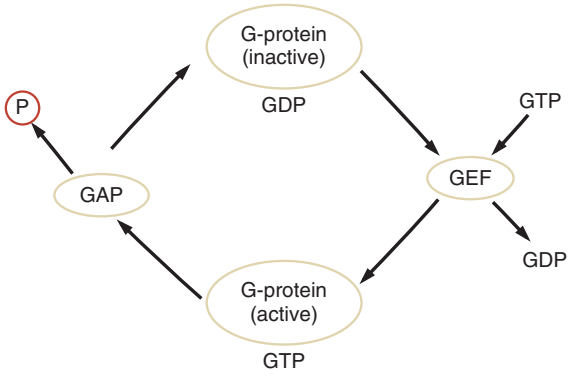

Figure 7.25 The G-protein cycle. Gunaine-nucleotide-binding proteins (G-proteins) can be present in an active, GTP-binding state or in an inactive, GDP-binding state. The two states differ through large conformational changes resulting from the binding of the GTP or GDP ligands, making G-proteins resemble "molecular switches." The inactive state can be converted to an active state by guanine-nucleotide-exchange factors (GEFs), which replace GDP with GTP. The active, GTP-binding form can be inactivated by GTPase-activating proteins (GAPs), which mediate hydrolysis of GTP to GDP and inactivate the G-protein.

its structure experiences a conformational change. These changes can be dramatic and alter the effects a G-protein can have on physiology, for instance through binding affinity of the G-protein for interacting proteins. Because of the extensive conformational change G-proteins display when bound GDP is exchanged for GTP, G-proteins are prime examples for "molecular switches" (Figure 7.25). Two enzymes mediate between the inactive, GDP-binding state and the active, GTP-binding state of a G-protein: **guanine nucleotide exchange factors (GEFs)** exchange bound GDP for GTP, activating the G-protein, whereas **GTPase-activating proteins (GAPs)** mediate the hydrolysis of bound GTP to GDP, inactivating the G-protein. The catalytic center of the GTPase hydrolyzing bound GTP is jointly formed from protein domains of both the G-protein and the GAP. The interconversion of G-proteins between active and inactive states by GEFs and GAPs is called the G-protein cycle (Figure 7.25).

Plant G-proteins include mostly small, monomeric G-proteins, fewer heterotrimeric G-proteins and possibly also other types of guanine nucleotide-binding proteins. Small G-proteins range in size between 20 and 30 kDa and function in a monomeric state. All small G-proteins share structural features, such as the presence of four guanine nucleotide-binding domains and an effector-binding domain; however, they are highly diverse in structure and function. Most small G-proteins occur in soluble and membrane-associated states, which are mediated by posttranslational modifications, such as acylation and/or isoprenylation. Because their GEFs are generally membrane-associated, the activation of small G-proteins takes place at the membrane. Binding of G-proteins to cytosolic guanine nucleotide dissociation inhibitors (GDIs) prevents the association of GDP-binding inactive G-proteins with the membrane-associated GEFs, thereby controlling the proportion of G-protein that is available for activation at the membrane. The *Arabidopsis* genome encodes at least 93 small G-proteins, and for some of these, important cellular functions have been demonstrated. The cellular functions of small G-proteins in plants include roles in phytohormone signaling, in vesicle trafficking and in cytoskeletal control. Relevant examples for signal transduction processes mediated by small G-proteins are the control of polar cell expansion during plant development, the regulation of polar tip growth of pollen tubes or during root hair formation on nutrient limitation, or the negative regulation of ABA signals. Further roles in the signal transduction cascades of auxin and brassinosteroids or have been proposed.

A much smaller group of plant G-proteins are the **heterotrimeric G-proteins**. Heterotrimeric G-proteins are composed of three dissimilar α, β, and γ-subunits (G_α, G_β and G_γ). G_α are approximately 40–45 kDa in size, G_β approximately 35 kDa, and G_γ approximately 7–10 kDa. Similar to the small, monomeric G-proteins, plant heterotrimeric G-proteins can likely also be posttranslationally modified by acylation and/or isoprenylation. Guanine nucleotide binding occurs in the α subunit, which is the largest and is related to small G-proteins. In the inactive, GDP-binding form, the α, β, and γ subunits form a heterotrimeric complex. Activation by binding to GTP releases the α subunit (G_α) from the complex, which can then interact with effector proteins and elicit further signaling events and cellular responses.

The abundance of heterotrimeric G-proteins in plants is much lower than in invertebrates or mammals. In contrast to the at least 20 mammalian G_αs, 5 G_βs, and 12 G_γs, plant genomes analyzed so far contain only much fewer recognizable G-protein subunits. For instance, there is only one recognizable G_α and one recognizable G_β encoded in the *A. thaliana* genome. Because G_γs cannot be identified based on sequence comparison alone, so far it remains unclear how many G_γs might occur in plants; for *A. thaliana*, two candidates have been identified. Whereas in mammals heterotrimeric G-proteins often associate with membrane receptors of the large class of G-protein-coupled receptors (GPCRs), so far it is not clear whether plants contain GPCRs, whether other receptor-like proteins of plants have functions similar to those of GPCRs in mammals, and whether such proteins associate with heterotrimeric G-proteins. Mammalian GPCRs can serve as GEFs for their associated heterotrimeric G-proteins, raising the question how plant heterotrimeric G-proteins are activated. It is known from mammals, however, that heterotrimeric G-proteins can also be activated in a receptor-independent manner, and it is possible that this concept applies to plants. On the basis of the analysis of *A. thaliana* mutants and genetic studies, a number of important physiological roles are attributed to plant heterotrimeric G-proteins, including roles in the control of meristem activity and the cell cycle and in the signal

transduction cascades of auxin and brassinosteroids, the mediation of salt tolerance.

7.4.9 Ion Channels

The presence of ions in a cell has multiple effects on its function. Important ions include protons (H^+) as well as many cations, such as K^+, Na^+, or anions, such as Cl^-. Because cells are surrounded by membranes, there is no free diffusion of ions, which carry large hydration spheres because of their electrical charge. Entry and exit of ions across membranes are controlled by specific membrane transporters (see Section S1.2). Ion channels allow entry of ions from a site of high concentration to a site of lower concentration, whereas ion pumps catalyze the ATP-dependent movement of ions against a concentration gradient. The interplay of channels and pumps is essential for plant cells to maintain their cytosolic ionic milieu. In nonstressed cells, the cytosol contains slightly more anions and the apoplasm more cations. This difference creates an electrochemical potential across the plasma membrane. Stress-induced activation of ion channels and subsequent influx of cations into the cytoplasm leads to reversal of the electrochemical potential and is referred to as **depolarization of the membrane**. The depolarization signal is terminated by ion pumps, which remove cations from the cytosol and reestablish the original state. Electrophysiological studies of potentials across membranes involve mathematical modeling and physics. Ion transport and ensuing electrochemical changes in membrane potential represent important elements of plant signaling and can span large distances across cellular surfaces in a very limited time. An important ion with signaling function is calcium (see Section 7.6).

7.5 Signal Transduction by Protein Phosphorylation

The biochemical activity of enzymes and proteins depends on their three-dimensional structure. The folding of a polypeptide chain into a three-dimensional molecule is influenced by many factors, including the distribution of hydrophobic and hydrophilic domains, and the distribution of surface charges and the presence of accessory proteins, such as chaperones. Folded proteins can be changed in their biochemical activity by posttranslational modifications, which can introduce hydrophobic moieties or additional surface charges, thereby fine-tuning the three-dimensional folding pattern of a target protein. An important example for the regulation by posttranslational modification is the reversible phosphorylation of target proteins by protein kinases and protein phosphatases (Figure 7.26). The addition of one or several phosphate residues on a protein introduces negative surface charges that alter the properties of the phosphorylated proteins. Phosphorylation can influence the biochemical activity or subcellular localization, the interaction with substrates, or the aggregation state of a protein.

Phosphorylation of proteins is mediated by protein kinases. These enzymes can be categorized into different groups, based on the amino acid residues they phosphorylate on a target protein or their membrane association and overall structure. According to their phosphorylation preference, protein kinases are grouped in serine/threonine kinases, tyrosine kinases, and histidine kinases. Aspartate is not phosphorylated directly by a protein kinase and is formed only by phospho-relay from a phosphorylated histidine. The most prevalent form of protein kinases in plants is serine/threonine kinases, which are mostly soluble enzymes. Tyrosine kinases have been reported only rarely for plants. An important group of protein kinases with

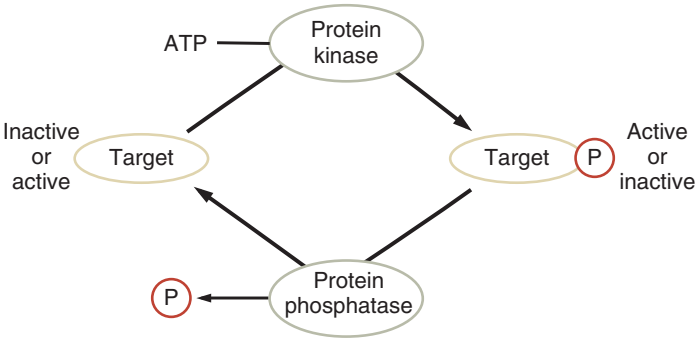

Figure 7.26 Reversible phosphorylation of proteins by protein kinases and protein phosphatases. The activity or localization of proteins can be controlled by posttranslational modifications. An important posttranslational modification is reversible phosphorylation. Protein kinases can transfer a phosphate group from ATP to a target protein, thus altering its biochemical properties. The phosphate group can be removed by protein phosphatases, reestablishing the unmodified state. The phosphorylated state can be either active or inactive, depending on the particular properties of the target protein. Phosphorylation of a protein can occur in only one, several, or multiple sites, providing a complex means to control protein activity.

particular relevance for cell signaling are transmembrane kinases consisting of an extracellular receptor domain, a transmembrane domain, and a cytosolic protein kinase domain. Transmembrane kinases can be further divided into receptor-like serine/threonine kinases (RLKs) and RHKs. Examples for RLKs include LRR-RLKs, such as BRI1 or CLV1. RHKs are represented by AHK4 and ETR1 (see Section 7.3).

Transmembrane kinases are activated by the binding of an extracellular ligand and exert their effects through different cytosolic signaling cascades initiated by the ensuing phosphorylation of a target protein. The target protein can be a domain of the kinase protein itself or an independent protein target. RLKs phosphorylate serine or threonine residues of target proteins on their activation. It remains unclear what the plant-endogenous protein targets of BRI1 or CLV1 are (cf. Figures 7.20 and 7.21). RHKs utilize histidine-to-aspartate phosphor-relay reactions to transfer a phosphate group from phosphor-histidine generated by autophosphorylation to an aspartate. The phosphor-aspartate residue can then transduce the phosphorylation signal to a phosphotransferprotein, such as AHP in CK signal transduction, and effect the translocation of AHP from the cytoplasm to the nucleus. In the nucleus, phosphorylated AHP exerts its effects on ARRs (see also Section 7.3.4, for example).

Besides acting through AHP, activated RHKs can also enable protein phosphorylation cascades involving MAPKs to induce cellular responses. MAPK-cascades represent an obvious example for hierarchically cascading signals, which is readily apparent from their nomenclature (MAPKKK, MAPKK, MAPK), enabling the amplification of an initial signal. MAPK-cascades are parts of several phytohormone signal transduction pathways, including those for SA (Figure 7.16) or ETH (Figure 7.19). The ultimate targets of protein phosphorylation cascades are often nuclear transcription factors, which are activated either directly by phosphorylation or by phosphorylation-dependent shuttling of accessory proteins from the cytosol to the nucleus. Soluble protein kinases may target numerous proteins and enzymes that can be altered in their localization or activity by the phosphorylation. Regulation by reversible phosphorylation is not restricted to signal transduction pathways, and many enzymes of primary metabolism are also targets for regulation by protein kinases (see Section S1.3). These enzymes provide an important indication that plant sensory systems and signal transduction is not limited to the adaptation to catastrophic environmental stresses. Rather, they are part of normal plant development and the continuous adjustment of plant metabolism to current needs, nutrient demands, and the available supply.

7.6 The Calcium Signaling Network

Calcium is an important and universal signaling factor in eukaryotic organisms, including plants. Under nonstress conditions, cytosolic free calcium has a concentration between 100 and 200 nM, which is 10 000-fold below the calcium concentration in the apoplastic fluid and 10 000 – 100 000-fold below the calcium concentration in organelles. Evidently, the cytoplasm maintains very low levels of free calcium, enabling calcium to serve as a potential cellular signal, once it is admitted into the cytoplasm. Despite their already-high calcium levels, calcium signals likely have signaling roles also in organelles, such as the nucleus or the plastids.

Cytosolic calcium levels can be transiently or locally elevated by enabling the influx of calcium from locations of higher calcium concentrations. The most immediate source of calcium signals is from outside the cell. The plasma membrane of plant cells contains passive calcium channels that can be activated by current or by mechanical force ("stretch-activated channels"), resulting in calcium influx and a rise in cytosolic calcium levels. Calcium is rapidly removed from the cytosol by energy-requiring export, or by deposition of calcium in cellular organelles. Different organelles, such as the nucleus, the endoplasmic reticulum, or the plastids, may serve as intracellular stores of calcium, and cytoplasmic calcium levels may be increased by the influx of calcium from these stores. So far, the molecular mechanisms of calcium release from internal stores in plants are unclear. Various signaling molecules, such as inositolpolyphosphates (see Section 7.4.6) or phytohormones (see Section 7.3), may trigger the release of calcium from intracellular stores into the cytoplasm. Elevated calcium levels in the cytosol are counteracted by calcium pumps that remove calcium from the cytosol, thus terminating the signal. The interplay of inward and outward transport of calcium gives rise to transient elevations of cytosolic calcium levels.

Elevated levels of cytosolic calcium are recognized by calcium-binding proteins. Calcium-binding proteins in plants can be categorized into sensor relays and sensor responders (Figure 7.27). Sensor relays function through their interactions with other proteins. The prime example for calcium sensor relays are **calmodulins (CaMs)**, which modulate cellular processes in a calcium-dependent fashion. CaMs and **calmodulin-like proteins (CMLs)** contain calcium-binding domains, called **EF-hand-domains** because of the importance of helices E and F of the helix-loop-helix structure for calcium binding. The large ionic radius of calcium ions (Ca^{2+}) with their associated hydration sphere enables strong effects of calcium-binding to be exerted on calcium-binding proteins, which mediate the transduction of calcium signals. Binding of calcium to the EF-hand-domains results in a dramatic conformational change of the protein. This change is required for

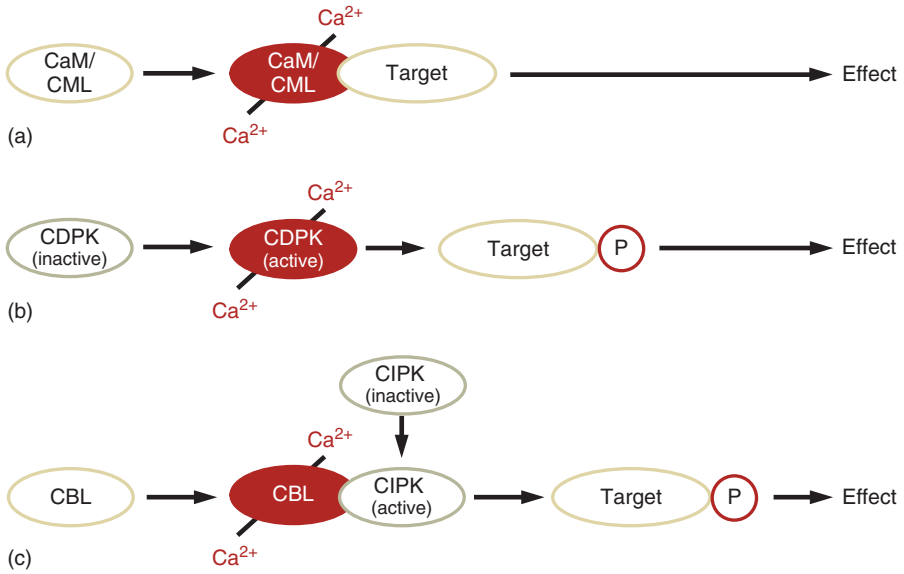

Figure 7.27 Calcium-binding proteins and the decoding of calcium signals. Calcium-binding proteins can be categorized into sensor relays and sensor responders. Sensor relays function through their interactions with other proteins, such as calmodulin (CaM) and calmodulin-like proteins (CMLs). Binding of calcium to CaM or CMLs results in conformational changes enabling interaction with partner proteins and the manifestation of calcium-dependent regulatory effects (a). Calcium sensor responders include many calcium-dependent enzymes. Examples are calcium-dependent protein kinases (CDPKs), which influence target proteins through calcium-dependent phosphorylation (b). The complexity of plant calcium-signaling is increased by the possibility of calcium sensor relays to interact with calcium responder elements. An interesting example for such complexity is the interplay between calcium sensor relay calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) (c).

the interaction with partner proteins of CaMs and their calcium-dependent regulatory effects on physiological processes. Another example for calcium sensor relays are **calcineurin B-like proteins (CBLs)**. CBLs also bind calcium via EF-hand-domains and undergo conformational changes that enable the interaction with target proteins to modulate activity or structure of these targets. The group of calcium sensor responders includes many enzymes, which may be activated or inactivated by calcium, which are components of other signaling pathways, such as phospholipases C and D or the **calcium-dependent protein kinases (CDPKs**, Figure 7.27), the latter of which also contain EF-hand-domains. Calcium sensor relays and calcium sensor responders are employed by plants to regulate biological responses that are triggered by calcium.

Calcium is not highly mobile once entering the cytosol and will not move far from its site of entry. Therefore, calcium targets should be close to the sites of calcium release to the cytosol. The emerging picture suggests that sensory proteins that respond to calcium display a very restricted spatial distribution and form localized and complex molecular networks to transduce calcium signals into cellular responses. Both the signature of a calcium increase, as modulated by the interplay of inward channels and outward pumps, and the nature of the specific calcium sensors present at the site of calcium entry into the cell will define the particular cellular responses to a perceived calcium signal. Multiple calcium sensor relays interact with multiple calcium responder elements. An interesting example for such complexity that has received increased attention in recent years is the interplay between calcium sensor relay CBLs and **CBL-interacting protein kinases (CIPKs**, Figure 7.27). Both CBLs and CIPKs occur in plants as families of multiple isoforms. A given CBL can interact with a subset of CIPKs and possibly activate the protein kinase in a calcium-dependent fashion. Assuming that CBL-isoforms have different affinities to calcium and to their target CIPKs, it might be possible for the cell to differentially set off protein phosphorylation cascades depending on intensity or duration of a perceived calcium signal.

7.7 Stress-Induced Modulation of Gene Expression by microRNAs

Various stress-signaling cascades exert effects on the physiology of plants by mediating changes in the **expression of specific sets of genes**. For instance, as part of developmental programs or gravitropic curvature, auxin induces a large group of auxin-induced genes that are required for the manifestation of the appropriate response. Similarly, wounding-induced genes are stimulated via a signal transduction cascade involving JA, and other phytohormones also mediate signaling events that will ultimately alter plant gene expression patterns, as has been described above. Changes in gene expression lead to altered abundance of certain messenger RNAs (mRNAs), which mediate altered rates of production of the encoded proteins and enzymes.

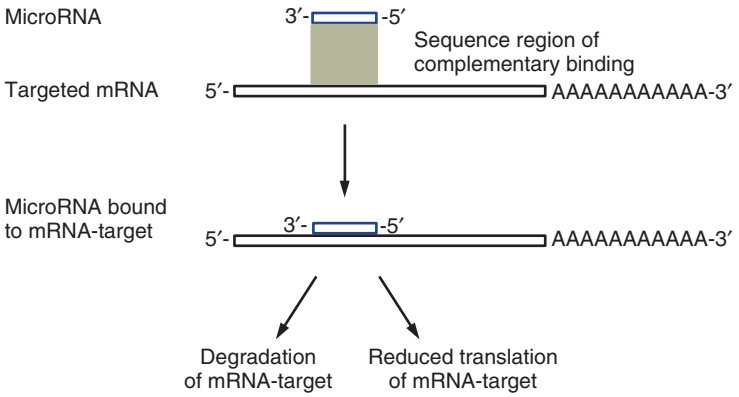

Figure 7.28 Control of mRNA stability by microRNAs. microRNAs are small RNA molecules with complementary sequences to certain mRNA targets. Specific binding to the target mRNAs results in the formation of double-stranded RNAs, which are translated to proteins with reduced efficiency or are subject to degradation, resulting in inhibitory effects of microRNAs on the expression of their target genes.

The control of transcription via DNA-dependent RNA polymerase II does not differ substantially between plants and animals. Similarities of the transcriptional machineries of plants and animals even include structure and function of transcription factors, such as those of the MYB family involved in GA signal transduction and the MYC family involved in JA signal transduction, which have both been mentioned before. Furthermore, the genomic loci of both plants and animals contain introns that separate coding regions, and similar machineries are used to process the introns and form mature mRNAs. Similarities also include the ribosomal machinery for translation, which is basically the same in all eukaryotes. A unifying element of the regulation of gene expression is the structure of mRNAs, including the characteristic sequence elements at the 5′ and the 3′ ends. Changes in gene expression patterns can be regulated at different levels, including altered production and/or altered stability of mRNAs.

Control of transcription is exerted via two possible ways. One is the interaction of a protein, called **transcription factor**, with the promoter of a gene. The protein binding to a sequence motif in the promoter is called **trans-acting factor**, while a specific DNA sequence recognized by the **transcription factor** is called **cis-element**. The binding of the protein affects the transcription of the downstream gene, either positively or negatively. Another way to regulate gene expression is via changes in chromatin structure, either by modification of the histones, which form the core of the nucleosomes, or by DNA-methylation. Chromatin can be open (euchromatin) and accessible for the transcriptional machinery, or it can be densely packed and inaccessible for transcription (heterochromatin). Only recently it was found that this kind of epigenetic regulation of gene expression also plays an important role in the stress responses of plants.

Whereas the regulation of mRNA abundance by transcriptional activation or repression is evidently a main route to control the production of proteins and enzymes that affect cellular function, there are additional means by which the production of certain proteins can be controlled and fine-tuned. An important factor contributing to the control of stress-induced gene expression in plants are **microRNAs (miRNAs)**, which exert posttranslational effects on transcript abundance and stability. miRNAs mediate cleavage or reduced translation of mRNAs (Figure 7.28). Plant miRNAs represent small regulatory RNAs of approximately 21 nucleotides in length and are processed from larger primary miRNA transcripts that display characteristic hairpin structures. On the basis of sequence complementarity, miRNAs recognize larger target mRNAs and mediate the degradation and/or the translational repression of their mRNA target. Whereas in animals a large proportion of protein-coding genes are regulated by miRNAs, in plants only a small number of target mRNAs are regulated by miRNAs. Nonetheless, plant miRNAs appear to exert major effects on plant gene expression, because many target mRNAs are transcription factors. As was mentioned before, transcription factors can control the activity of various other genes, thereby serving as signal amplifiers in developmental processes from seed germination to seed maturation. In addition to the developmental aspects, the expression profiles of certain miRNAs that target transcription factors are significantly altered in response to stress. Several miRNAs have been described to exert effects in the context of plant stress responses and during environmental adaptation, including miR398, which targets two Cu/Zn superoxide dismutases, and miR395 and miR399, which target sulfate and phosphate transporters, respectively. Furthermore, *A. thaliana* mutants compromised in miRNA biogenesis have been found to be hypersensitive to ABA. Overall, much evidence has been assembled for a role of miRNAs in the regulation of plant stress responses, but so far many questions about the contribution of miRNAs to the manifestation of plant responses to environmental stresses remain open for investigation.

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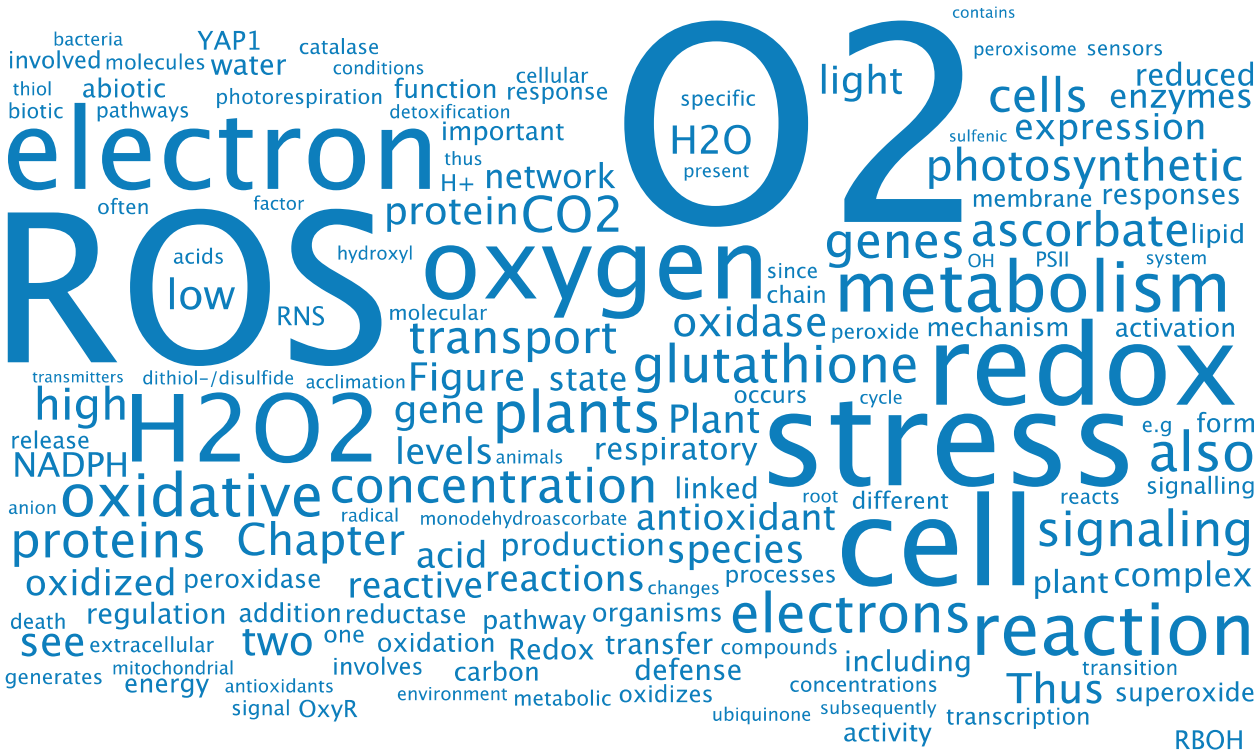
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8
Oxygen

Karl-Josef Dietz

Overview

Life is based on redox chemistry that allows the cell to generate energy for the synthesis of cellular constituents by the oxidation of reduced compounds. The evolution of organisms with oxygenic photosynthesis and their increasing abundance during earth history caused an oxygen enrichment of the atmosphere to today's concentration of 21%. Thus, currently, the predominant final electron acceptor in cell metabolism is oxygen allowing for efficient production of ATP in respiration. The important role of oxygen in the biosphere is linked to its specific chemical properties. Consecutive reduction reactions of O_2 generate the three reactive oxygen species (ROS) superoxide anion, hydrogen peroxide and hydroxyl radical, and finally water. This chapter describes properties of oxygen and its reactive derivatives, addresses the metabolic processes that consume or generate oxygen and ROS, and summarizes the relationships between abiotic, biotic stress factors and oxidative stress. Oxygen-dependent processes display specific oxygen concentration dependencies. Thus, the metabolic activities of photorespiration, mitochondrial respiration, and other O_2-dependent reactions change distinctly in response to decreasing O_2 availability as it occurs in flooded soils or in aquatic environments. ROS react with cellular constituents such as unsaturated fatty acids, proteins, and nucleic acids and may impair cell functions. ROS play a role in cell signaling and regulation of diverse processes including metabolism, transcription, and translation, in addition to disease, fitness losses, and even cell death.

8.1
Chemical Nature of Oxygen and Reactive Oxygen Species

Molecular oxygen (O_2 or dioxygen as two oxygen atoms are combined in one molecule) in its ground state is rather nonreactive because of its two unpaired π^* electrons with identical spin in two antibinding π-orbitals (triplet oxygen). Thus, even carbon-based organisms can live in an O_2-rich atmosphere without being oxidized and damaged. However, O_2 can be activated by a change in electron configuration in the outer orbital (see Section S1.2.4). Four electrons transferred to O_2 with concomitant uptake of protons liberate two molecules of water (Figure 8.1).

Physical O_2 activation is achieved by energy transfer to O_2 from excited pigments such as chlorophyll. Thereby, one electron in an antibinding π-orbital inverts its spin and pairs with the second electron. This reaction produces singlet oxygen ($^1O_2$) that has increased reactivity compared to triplet O_2 and easily reacts with unsaturated fatty acids or proteins. $^1O_2$-dependent oxidation of unsaturated lipids occurs at rather random positions of the double bonds within the lipid molecule. In contrast, enzymatic lipid peroxidation mainly occurs by lipoxygenases (LOXs), which selectively oxygenate polyenoic fatty acids (PUFAs) at specific sites and produce fatty acid hydroperoxides. These are subsequently reduced to oxilipins with precisely defined hydroxylation pattern.

Chemical activation involves electron transfer from a suitable chemical reductant. The superoxide anion radical ($O_2^{\bullet-}$) is generated after transfer of one electron to oxygen. This can also be achieved enzymatically, for example, with xanthin and oxygen as substrates catalyzed by xanthine oxidase. Photochemical $O_2^{\bullet-}$ production in cells occurs after illumination of, for example, riboflavin, which then donates its activated electron to O_2. Chemical $O_2^{\bullet-}$ synthesis requires an electron donor such as methylviologen (paraquat, 1,1′-dimethyl-4,4′-bipyridinium), which accepts electrons from photosystem I (PSI) and donates the electron to O_2. By this mechanism, methylviologen is toxic to cells and was earlier applied as herbicide to control weed.

Hydrogen peroxide (H_2O_2) withdraws electrons from transition metals such as Fe^{2+} or Cu^+ to form the hydroxyl radical ($OH^{\bullet}$) and the hydroxyl anion (OH^-) at 1 : 1 stochiometry. This reaction is called **Fenton reaction**. $OH^{\bullet}$ instantaneously oxidizes the nearby molecules within diffusion distance. Thus by reaction with $OH^{\bullet}$ cellular

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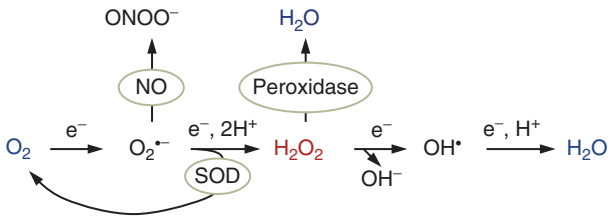

Figure 8.1 Activation of oxygen and its reduction to water. Successive transfer of 4 electrons and uptake of 4 protons from the aqueous environment generates superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and, finally, water (H_2O). The figure indicates enzymes that are involved in detoxification. $O_2^{\bullet-}$ reacts with nitric oxide (NO) to form peroxinitrite ($ONOO^-$). SOD – superoxide dismutase.

proteins, nucleic acids or lipids get oxidized and functionally damaged in an uncontrolled manner. Reduction of $OH^{\bullet}$ with concomitant uptake of a proton generates water.

Ozone (O_3) is another activated oxygen derivative that is synthesized in the atmosphere by energy transfer through UV radiation and electric discharge. NO_2 in exhaust fumes reacts, because of environmental pollution, with O_2 in the presence of UV light to release O_3 particularly during summer in stagnant air. O_3 is very reactive, oxidizes compounds preferentially in the extracellular matrix, and is toxic to cells and organisms.

Cells enzymatically produce **nitric oxide** ($NO^{\bullet}$). While animal cells express three different nitric oxide synthases, the origin of $NO^{\bullet}$ in plants can be traced to the nitrate reductase activity and other still less defined sources, possibly in the peroxisome. $NO^{\bullet}$ reacts with $O_2^{\bullet-}$ to form peroxinitrite ($ONOO^-$). $ONOO^-$ is extremely reactive, acts as oxidant and nitrating agent, and damages proteins and nucleic acids. Thus and in addition to the reactive oxygen species (ROS) $^1O_2$, $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ cells also have to deal with reactive nitrogen species (RNS) such as $NO^{\bullet}$ and $ONOO^-$ and also reactive carbonyl species and reactive sulfur species.

Progressive damage to cell components because of ROS or RNS accumulation is called **oxidative stress**. Cell metabolism makes use of oxygen as substrate for specific oxidation reactions. Safe metabolism of oxygen minimizes the release of ROS. Such "safe" reactions are the terminal electron transfer to O_2 by cytochrome a/a3 oxidase in respiration or the activity of the oxygen-evolving complex (OEC) at photosystem II (PSII) in photosynthesis. On the other hand, other reactions transfer electrons to O_2 and release ROS. In this case, detoxification mechanism must be at hand to decompose ROS and RNS. Finally, if ROS and RNS react with cell constituents in an uncontrolled manner, damaged molecules must be repaired or decomposed in order to maintain cell integrity and functionality.

8.2 Oxygen Metabolism

8.2.1 Photosynthesis and Oxygen Metabolism

Photosynthesis produces reduced compounds from oxidized substrates, in particular, sucrose from CO_2 using the energy from absorbed light quanta (see Section S1.2.6.1). Photosynthesis consists of four steps; light harvesting, charge separation in the reaction centers, photophosphorylation, and carbon fixation in the Calvin cycle. The evolution of oxygenic photosynthesis about 3.5 billion years ago enabled the cells to feed the **photosynthetic electron transport** (**PET**) with electrons from the vastly available H_2O. The enzymatic machinery for water splitting is associated with PSII and is called the oxygen-evolving complex. Time-resolved spectroscopy, protein structure analysis, and biochemical characterization have led to a detailed mechanistic understanding of the OEC that involves four Mn ions, two of which undergo redox changes. The overall reaction is $2H_2O \rightarrow O_2 + 4H^+ + 4e^-$. The electrons are transferred to a Tyr-cation in the core of PSII that has been generated as a result of light-dependent excitation of the special pair chlorophyll in the reaction center. Despite the rather complex reaction cycle with four intermediate states, ROS are not released from the OEC. However, under stressful growth conditions, the OEC is damaged and this contributes to photoinhibition, which is often defined as inhibition of the activity of PSII under strong light.

ROS can be released at different sites of the PET chain (Figure 8.2). ROS generation at increasing rates occurs if redox disequilibria build up between subsequent redox carriers within the PET. Figure 8.2 also depicts respiratory electron transport (RET) chain for comparison, which is discussed below. Singlet oxygen is produced in the PSII reaction center if the electron acceptors Q_A/Q_B of PSII are present in the reduced state and unavailable to further accept high-energy electrons from excited reaction centers. A highly reduced plastoquinone (PQ) pool that corresponds to a high ratio of plastohydroquinol (PQH_2) relative to PQ is linked to the generation of ROS. O_2 can be reduced to superoxide by plastosemiquinone, and superoxide to hydrogen peroxide by plastohydroquinone, respectively. In addition, at the PSI acceptor site, electrons are transferred from iron-containing electron carriers to O_2. Thus, in the absence of other electron acceptors such as $NADP^+$, NO_2^-, or SO_3^{2-} electrons are transferred to O_2 to produce O_2^-. This reaction is called **Mehler reaction**, but also pseudocyclic electron transport or water – water cycle as the O_2^- is subsequently reduced to H_2O by an ascorbate- or peroxiredoxin-dependent mechanism. The molecular formulas of the reaction sequence using ascorbate and monodehydroascorbate reductase are shown below. The reaction series starts with water and ends with water and the numbers on the right-hand side indicate how many times each reaction occurs:

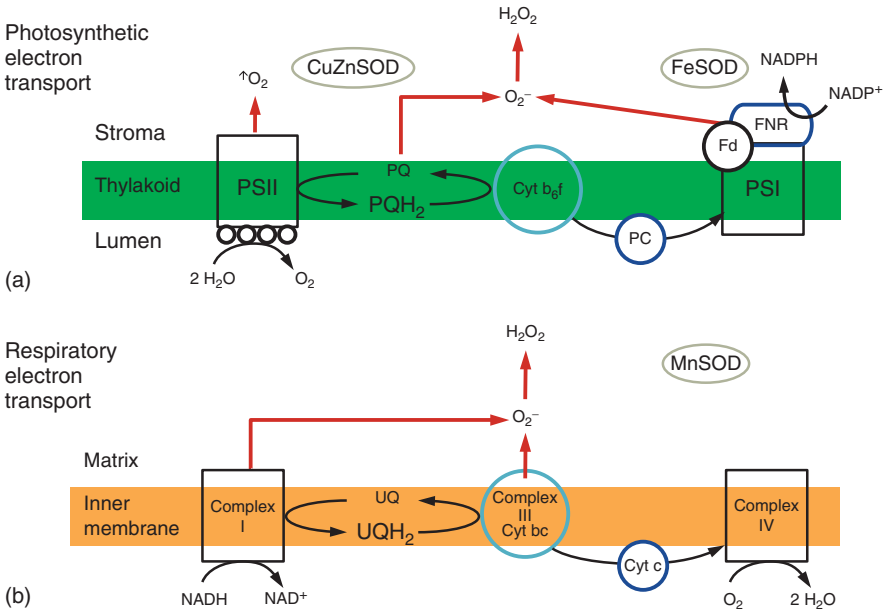

Figure 8.2 ROS generation in the photosynthetic and respiratory electron transport chains. (a) Singlet oxygen is generated at photosystem II (PSII), O_2^- in intersystem electron transport and at PSI. Chloroplast CuZnSOD and FeSOD convert O_2^- to H_2O_2. Also shown is the association of the oxygen-evolving complex with the lumen side of PSII. FNR – ferredoxin-dependent NADP reductase; Fd – ferredoxin; PC – plastocyanin; PQ – plastoquinone. (b) In mitochondrial electron transport, O_2^- is released from complex I and complex III. Mitochondrial Mn-SOD converts O_2^- to H_2O_2. Terminal oxidase reduces O_2 to H_2O. Cyt – cytochrome; UQ – ubiquinone.

$$2H_2O \xrightarrow{OEC} O_2 + 4H^+ + 4e^- \quad (1x)$$

$$O_2 + e^- \xrightarrow{PSI} O_2^- \quad (2x)$$

$$NAD(P)^- + 2H^+ + 2e^- \xrightarrow{PSI} NAD(P)H + H^+ \quad (3x)$$

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (4x)$$

$$H_2O_2 + \text{ascorbate} \xrightarrow{APX} H_2O + \text{monodehydroascorbate} \quad (5x)$$

$$2\,\text{monodehydroascorbate} + NADH + H^+ \xrightarrow{MDHAR} 2\,\text{ascorbate} + NAD^+ \quad (6x)$$

$$2H_2O \longrightarrow 2H_2O$$

ROS released by PET is linked to light absorption by both photosystems and thus part of the light reactions of photosynthesis. In addition, ROS release is also associated with the photosynthetic dark reactions by **photorespiration** (see Section S1.3 and Figure 1.7). Photorespiratory H_2O_2 production occurs in peroxisomes. The **ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)** is the CO_2-fixing enzyme in the Calvin cycle (see Section S1.3). In ambient air with at present 380 ppm CO_2 and 21% O_2, RubisCO not only catalyzes the carboxylation reaction with CO_2 as substrate but also oxygenation with O_2. While carboxylation of the substrate ribulose-1,5-bisphosphate produces two molecules of 3-phosphoglycerate, oxygenation generates one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate; O_2 competitively inhibits carboxylation (Figure 8.3). The CO_2 compensation point, that is, the CO_2 concentration where photosynthetic CO_2 fixation balances respiratory CO_2 release, depends strongly on the O_2 concentration. At ambient CO_2 and O_2 concentrations, the ratio of carboxylation to oxygenation is about 5:2. Oxygenation is suppressed with increasing CO_2 concentration. This effect explains the high productivity of C4-plants, which deploy a CO_2-concentrating mechanism using phosphoenolpyruvate carboxylase (PEPCase) to increase the CO_2 concentration at the site of RubisCO in the bundle sheath. Agriculturally important crop plants with C4-photosynthesis are maize, sorghum, and sugarcane.

The photorespiratory pathway (see Section S1.3 and Figure 1.7) involves reactions in the chloroplast, peroxisome, and mitochondrion and recycles 3 of 4 carbon atoms contained in two molecules of 2-phosphoglycolate. Following dephosphorylation in the chloroplast, glycolate is transported to the peroxisome where glycolate oxidase releases H_2O_2 and glyoxylate. Thus, H_2O_2 is released in the peroxisome in amounts stoichiometric to

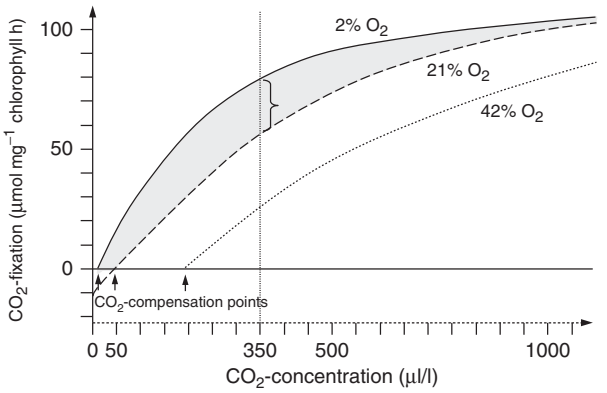

Figure 8.3 **CO_2 dependency of photosynthetic carbon assimilation at three O_2 levels.** At 21% O_2, the CO_2 compensation point of plants with C3-photosynthesis ranges around 50 µl/l. If the O_2 concentration is lowered, the CO_2compensation point concentration decreases, and it increases at elevated O_2. Stimulation of carbon assimilation by lowering O_2 concentration decreases with increasing CO_2 concentration. Because of the competitive nature of interference, O_2 inhibition of carbon assimilation is overcome at saturating CO_2 concentration.

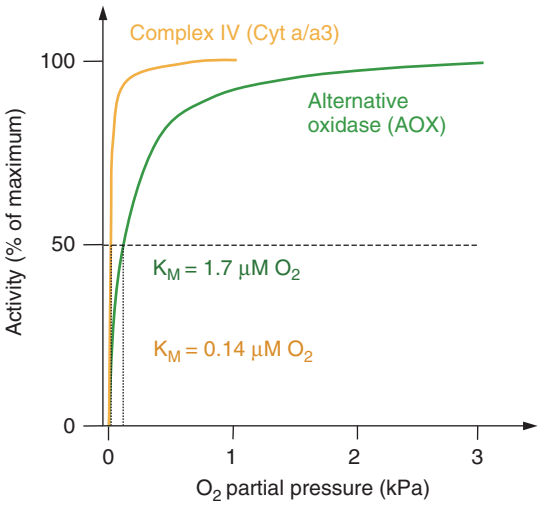

Figure 8.4 **Affinity to O_2 of mitochondrial alternative oxidase and cytochrome a/a3.** O_2 reduction activity is plotted as the function of O_2 concentration. The K_M of Cyt a/a3 for O_2 is about 0.14 µM and thus 12-fold more affine than alternative oxidase with a K_M of 1.7 µM.

RuBP oxygenation. Peroxisomal catalase and peroxidases decompose H_2O_2 to O_2 and H_2O.

8.2.2 Mitochondrion and Respiration

Mitochondria are the site of respiratory energy metabolism. The RET chain consists of four complexes that are linked by the mobile electron carriers ubiquinone (UQ) and cytochrome c. NADH is oxidized by complex I and electrons are fed into the respiratory chain. Via complex III and complex IV (other names: Cyt a/a3, cytochrome c oxidase, terminal oxidase) the electrons are ultimately transferred to O_2 to produce H_2O. Complex II oxidizes succinate and donates the electrons into the UQ pool. Mitochondrial electron transport generates ROS at low rates under physiological and at enhanced rates under pathological and stressful conditions (Figure 8.2). The RET is coupled to H^+-transfer from the matrix to the intermembrane space (see Section S1.2.9). This electrogenic H^+-transfer generates a proton motive force that is used for ATP synthesis by F_1F_0-ATP synthase. In plants, an alternative pathway without concomitant H^+-pumping and ATP synthesis is realized by transfer of electrons to O_2 catalyzed by alternative oxidase (AOX). AOX-dependent oxygen reduction is considered to function as a dissipation mechanism to reoxidize excessively formed NAD(P)H without ATP synthesis, for example, under stress and during photorespiration. Basically, this pathway converts chemical energy into heat and prevents the cell from overreduction, which would lead to ROS production and oxidative stress. In addition, an uncoupling protein also dissipates the proton motive force. Both AOX and uncoupling proteins are transcriptionally up-regulated under stressful growth conditions in plants.

RET relies on two oxidases, complex IV and AOX, that transfer electrons to O_2. The affinity of both systems to O_2 is high but slightly different (Figure 8.4). The K_M of complex IV with about 0.14 µM O_2 is 12-fold lower and thus its affinity is higher than that of AOX with about 1.7 µM. Both oxidases are usually saturated as a solution contains about 300 µM O_2 in equilibrium with air of 21% O_2 at 20 °C. The situation changes in compacted soil with high microbial activity, in flooded soil, in poorly circulating hypertrophic aquatic environment or in lakes under ice covers where O_2 levels may fall to very low levels. In lake sediments O_2 concentrations may be close to zero. Life forms in such habitats are strongly selected by their ability to acclimate to the prevailing O_2 availability.

In addition, diffusion limitations establish gradients in respiring tissues such as roots and seeds. O_2 concentrations in tissues can be measured with **O_2-sensitive microelectrodes** (Figure 8.5). A steep O_2 concentration gradient is established across the outer cortex of respiring roots. O_2 levels further drop at the endodermis and can reach very low levels in the central cylinder of the stele. Thus a severe oxygen deficit is established along the diffusion path, which can lead to hypoxic metabolism in certain areas of the root. The hypoxia is further enhanced in O_2-limited soil (see Chapter 10). Maize (*Zea mays, Poales, Liliopsida*) is the example of a hypoxia-sensitive crop species that ceases to grow in flooded fields.

A high-reduction state of electron carriers in the electron transport chain and a high electrical potential across the inner mitochondrial membrane stimulate ROS formation via complexes I and III. ROS generation also occurs by reverse electron transport from complex II. Precise figures of the rate of mitochondrial ROS production *in vivo* are missing. It has been estimated that up to 5% of the oxygen reduced in the animal respiratory chain can be converted

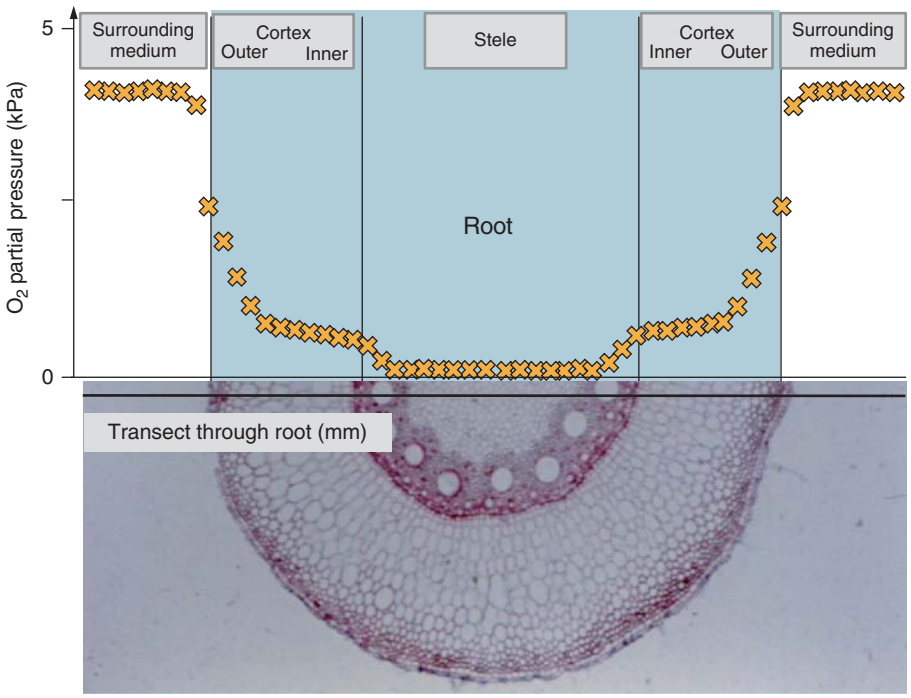

Figure 8.5 O_2 profile through the virtual transection of a maize root. The lower figure shows a cross section through a maize primary root with rhizodermis, root cortex, endodermis, and stele as imaged by light microscopy. The upper graph depicts the O_2 concentration along the root transect as measured by an O_2-sensitive impaling electrode. A steep O_2 gradient is established in the cortex and the O_2 concentration falls to extremely low levels in the stele.

to ROS. In animals, mitochondrial ROS production and their reaction with mitochondrial DNA, RNA, and proteins likely contribute to aging and cell dysfunctions.

8.2.3 Oxygen Metabolism in Peroxisomes

Peroxisomes are single membrane-bounded organelles that function in a diversity of metabolic pathways such as fatty acid oxidation and amino acid metabolism in most organisms, and some synthetic reactions for purines, pyrimidines, lipids, bile acids and cholesterol, and glyoxylate cycle in some species. Plant peroxisomes are involved in developmental processes including seed germination, photomorphogenesis, leaf senescence, fruit ripening, and in responses to abiotic and biotic stress and cell signaling by ROS and RNS. Peroxisomes contain a characteristic repertoire of **flavin-linked oxidoreductases** that transfer electrons from reductants to O_2 to form hydrogen peroxide. Thus another common feature of peroxisomes is their high contents of enzymes involved in H_2O_2 production and in antioxidant enzymes with catalase at first place. In photosynthetic plant cells, peroxisomes participate in the glyoxylate pathway of photorespiration

$$\text{glycolate} + O_2 \xrightarrow{\text{glycolate oxidase}} \text{glyoxylate} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O$$

The ambient CO_2 concentration at present is about 380 ppm. Under these conditions photorespiratory carbon flux amounts to >30% of carbon assimilation. Thus >30 µmol H_2O_2·mg chlorophyll^{-1}·h^{-1} must be detoxified in the peroxisome if the photosynthetic rate of carbon fixation proceeds at typical rates of 100 µmol CO_2 mg chlorophyll^{-1}·h^{-1}. Photorespiratory metabolite turnover increases significantly under drought stress (see Chapter 10) when stomates close and the intercellular CO_2 concentration decreases to values below 100 ppm. If stomates close tightly in wilted plants, photorespiration and photosynthetic CO_2 fixation are balanced and it has been assumed that photorespiration contributes to energy consumption and allows the cell to maintain a significant light-dependent electron flow and carbon turnover.

8.2.4 Membrane-Associated Oxygen Metabolism

The plasmamembrane of animals, plants, and filamentous fungi contains NADPH oxidases (animal NOX, plant RBOH) that catalyze the reaction:

$$\text{NADPH} + H^+ + 2O_2 \xrightarrow{\text{RBOH}} \text{NADP}^+ + 2O_2^- + 2H^+$$

NOX are heme enzymes that receive their electrons from cytosolic NADPH and transfer them to O_2 in the extracellular space. The produced superoxide anion radical functions in a variety of cell-specific reactions in the

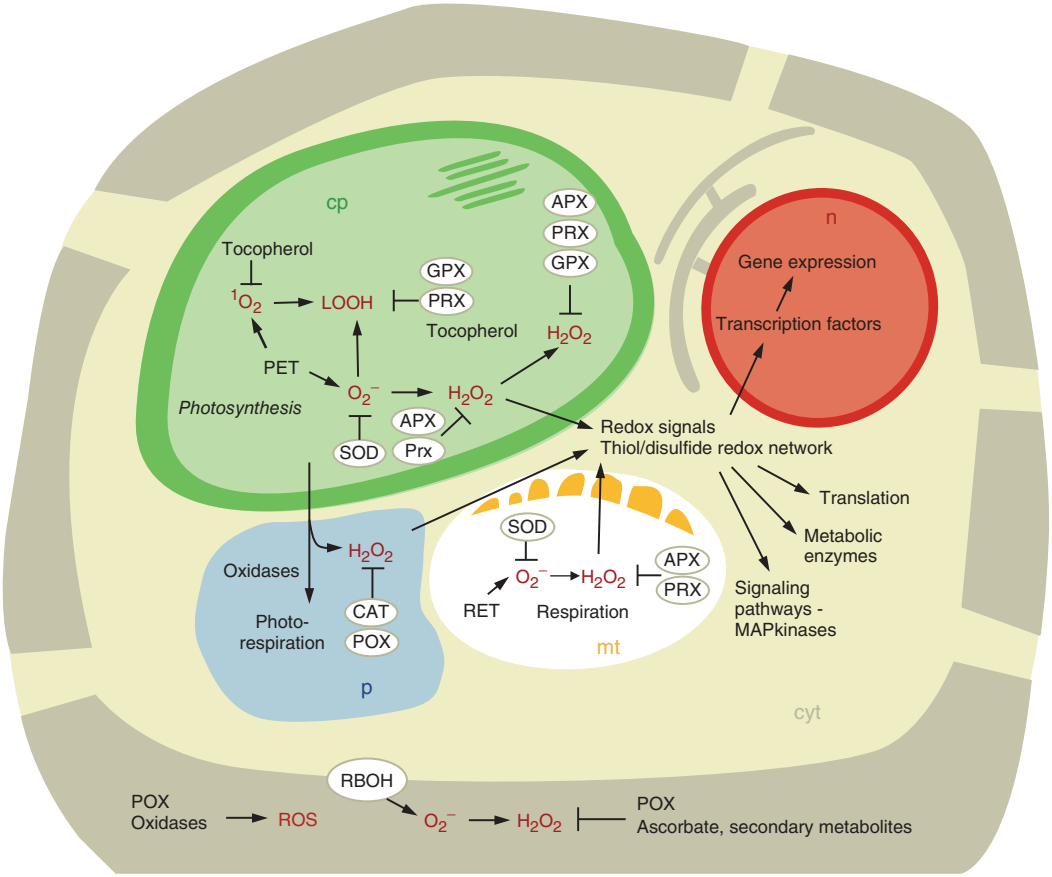

Figure 8.6 Cellular compartmentation of ROS metabolism in a plant cell. ROS are generated by photosynthetic electron transport (PET), respiratory electron transport (RET), photorespiration, membrane-bound systems, and oxidases in peroxisomes and apoplast. The scheme indicates antioxidants counteracting ROS accumulation. ROS and redox-cues take part in redox signaling, interact with other signaling pathways such as mitogen-activated protein kinases (MAP kinases) and regulate many different processes such as metabolic enzymes, translation, and gene expression. APX – ascorbate peroxidase; CAT – catalase; GPX – glutathione peroxidase; LOOH – lipid peroxide; POX – Heme peroxidase; Prx – peroxiredoxin; RBOH – plasmamembrane-bound NADPH oxidase; SOD – superoxide dismutase.

extracellular matrix such as defense against invading pathogens, oxidative cell wall metabolism, and cell-to-cell signaling. The human genome encodes six, the *Arabidopsis thaliana* (*Brassicales*, *Rosidae*) genome ten *nox*/*rboh* genes. Well-established roles of NOX in animals are their participation in phagocytosis of leucocytes, endothelial function, and regulation of vascular tone, in addition to pathophysiology such as hypertension and inflammation. In plants preferential expression of some RBOH isoforms in the root elongation zone and pollen indicates important roles in developmental processes. Stress inducibility of *rboh* gene expression and evidence from mutant plants with inactivated *rboh* genes reveal that extracellular O_2^--production functions in stress defense, cell signaling, and cell death progression.

ROS metabolism in eukaryotic cells is highly compartmentalized as summarized in Figure 8.6 for a plant cell. PET in chloroplasts, RET in mitochondria, photorespiration, and other ROS-producing pathways in peroxisomes, and plasma-membrane-associated NADPH oxidase RBOH, along with cell wall-associated oxidases, generate different types of ROS and lipid peroxides. The scheme introduces antioxidants involved in counteracting ROS accumulation and indicates the function of ROS and ROS-linked redox regulation in cell signaling, adjustment of metabolism, translation, and nuclear gene expression. Synthesis of secondary metabolites such as phenolics is induced by oxidative stress and they play a role in antioxidant defense. The antioxidant defense and signaling function of redox-cues and ROS are discussed in the following section.

8.3 Oxygen Sensing

Organisms including bacteria, yeast, mammals, and plants have evolved mechanisms to sense O_2 concentration and to adjust metabolism and developmental programs to changing O_2 availability.

Escherichia coli (*Gammaproteobacteria*) represents the best studied model bacterium and is classified as facultative

anaerobic organism. Several direct and indirect O_2-sensing mechanisms control the switch from aerobic to anaerobic metabolism. The active dimer of one of these sensors, **FNR-TR** (**fumarate and nitrate reduction transcriptional regulator**), contains a $[4Fe\text{-}4S]^{2+}$ iron sulfur cluster at low O_2, which is oxidized to $[2Fe\text{-}2S]^{2+}$ in the presence of O_2. Another sensor in *E. coli* consists of two components: the membrane-bound histidine kinase ArcB and the response regulator ArcA. Its activation is coupled to the flux and redox state of the RET chain, which in turn depends on the availability of O_2 for the terminal oxidase. In *E. coli* ArcAB controls about 170 genes including genes involved in respiration and fermentation. The low O_2 response includes the change from the less O_2-affine Cyt_{bo} terminal oxidase to the more O_2-affine Cyt_{bd}, and, finally, under anaerobiosis to oxygen-independent low-efficient alternative oxidoreductases. In addition, the respiratory chain switches from UQ as electron carrier in aerobic environment to menaquinone in hypoxic environment. Menaquinone is widely distributed in bacteria and appears ancestral to UQ and PQ. Reduced menaquinone rapidly oxidizes in high O_2 concentrations. This observation explains why during evolution, UQ and PQ are substituted for menoquinone in aerobic organisms.

Several bacteria perform **anoxygenic photosynthesis** with a single photosystem and usually utilize anaerobic metabolism (see Section S1.2.12.1). Some of these photosynthetic bacteria perform a facultative anaerobic metabolism and sense O_2. At high O_2 concentrations, these bacteria rely on respiration for energy production. Photosynthetic genes are not expressed. With a drop to low O_2 availability, the photosynthetic genes are activated and expressed. The plasma membrane expands, invaginates to form chromatophores (intracytoplasmic photosynthetic membranes), and photosynthetic metabolism is established. In *Rhodobacter capsulatus,* the redox-sensing two-component system RegB participates in gene expression control in limiting oxygen conditions. Thereby, O_2-sensing is achieved by monitoring the redox state of UQ and by dithiol/disulfide transition in RegB. glutathione (GSH) as important cellular low molecular mass thiol plays an important role in mediating the reducing signal as feeding of extracellular GSH enhances formation of intracytoplasmic photosynthetic membranes in *Rhodospirillum rubrum.* Both genera belong to the *Alphaproteobacteria.*

In multicellular animals, for example, nematodes, arthropods (*Nematoda*, *Arthropoda,* both phylae of the Protosomia), and mammals (*Mammalia*, class of the phylum *Chordata*), the hypoxia-inducible transcription factor (HIF1) consists of an α- and β-subunit and controls genes involved in anaerobic acclimation. In the presence of O_2 and 2-oxoglutarate, HIF prolyl hydroxylase oxidizes a prolin residue in the HIF1 protein to hydroxyprolin. Hydroxyprolin-HIF1 is then degraded by ubiquitin-dependent proteolysis. HIF prolyl hydroxylases acts as O_2 sensors as they are activated with increasing O_2 concentration.

$$\text{Prolyl-HIF1} + \text{2-oxoglutarate} + O_2 \rightarrow$$
$$\text{4-hydroxyprolyl-HIF1} + CO_2 + \text{succinate}$$

As a consequence of this mechanism, HIF1 is stabilized and transactivates genes for anaerobic metabolism, iron homoeostasis, and in mammalian erythropoiesis and angiogenesis. In parallel, HIF1 activity is controlled by O_2 at additional levels, one of which involves ROS as the regulator of mRNA levels for HIF1α. Tolerance to hypoxia is an important ecological adaptation. The mole rat *Spalax ehrenbergi* lives underground and serves as model organism for hypoxia-tolerant animals. Analysis of gene expression in hypoxic mole rat compared to the common brown rat *Rattus norvegicus* indicates a lower expression of genes involved in apoptosis and those known as *tumor markers in humans*.

O_2 sensor function is also attributed to **heme-containing proteins** including hemoglobin. In yeast (*Saccharomyces cerevisiae, Saccharomycetales, Ascomycota*) heme biosynthesis is under control of O_2. The cellular-free heme concentration in yeast decreases at low O_2 levels and destabilizes the transcription factor HAP1, which in turn controls repressors of anaerobiosis-related genes.

Plant roots encounter hypoxia as a consequence of soil water logging or flooding. Hypoxia induces structural changes such as aerenchyma formation, stimulated root elongation, and deposition of structural barriers to improve O_2 availability and to minimize O_2 leakage into the soil. However, the O_2 sensors still need to be identified. Plants lack HIF1-like proteins. Plant genomes encode prolyl hydroxylases, which seem critical in acclimation to hypoxia as their overexpression in transgenic *A. thaliana* induces a phenotype-like wild type in hypoxic conditions.

8.4 Antioxidant Defense

Oxidative stress defense involves three lines of responses to minimize the effects of **adverse environmental and metabolic conditions**: (i) suppression of ROS and RNS production by regulation of metabolism at the transcriptional, posttranscriptional, and metabolic level; (ii) detoxification of reactive molecules and radicals by the antioxidant defense system; and (iii) repair of oxidative damage. This paragraph addresses the antioxidant system that efficiently detoxifies ROS and RNS to avoid oxidative damage and oxidative stress.

The antioxidant system consists of low molecular weight antioxidants and enzymes. The most important hydrophilic antioxidants are ascorbic acid and GSH. They are present in mitochondria, cytosol, and plastids at millimolar concentrations. Complementary to the hydrophilic compounds are lipophilic antioxidants, in particular, carotenoids and

tocopherols. They quench singlet oxygen and terminate lipid radical chain reactions that otherwise damage lipids and biomembrane integrity. Other low molecular weight thiols substitute for GSH in some bacteria (*Firmicutes*, *Actinobacteria*), for example, bacillithiol, which consists of L-cysteinyl-D-glucosamine linked to malic acid. *Proteobacteria,* on the other hand, contain GSH, which is in all organisms an enzyme-synthesized tripeptide usually with the structure γ-glutamyl-cysteinyl-glycine (see Section S1.3).

For singlet oxygen and hydroxyl radicals, no enzymatic detoxification mechanism is known. Antioxidant enzymes detoxify the remaining ROS. The complexity of the enzymatic antioxidant system can be assessed by analyzing the completed genome sequences of plants, fungi, and animals to identify the genes coding for antioxidant enzymes. Superoxide anion is rapidly detoxified by superoxide dismutases (SODs). The catalytic centers of SODs contain essential transition metal ions, namely, iron, manganese, nickel, and copper in combination with zinc, respectively. SOD catalyze the dismutation of two O_2^- with an extremely high catalytic efficiency defined as turnover number divided by the Michaelis Menten constant $k_{cat}/K_M = 7.10^9$ l/(mol.s).

$$2\,O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

A set of different enzymes detoxify H_2O_2. Degradation of H_2O_2 is important to avoid Fenton reaction with concomitant release of extremely toxic hydroxyl radicals. Catalase contains heme as prosthetic group and catalyzes the dismutation reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O$$

Peroxidases reduce H_2O_2 to H_2O at the expense of an electron donor that is simultaneously oxidized. Protein thiols in peroxiredoxins, ascorbate as cosubstrate for ascorbate peroxidase (APX), GSH for glutathione peroxidase (GPX) and phenolics, and other secondary organic compounds for horseradish peroxidase-like heme peroxidases serve as electron donors in the reaction.

$$H_2O_2 + 2H^+ + 2e^- \xrightarrow{\text{peroxidase}} 2H_2O$$

Some of these enzymes also act on other peroxide substrates such as lipid peroxides and peroxinitrite. Lipid peroxides are converted to alcohols, peroxinitrite to nitrite. The oxidized cosubstrates need to be regenerated by metabolic reactions linked to thioredoxin (Trx) or NADPH via reductases or dehydrogenases.

The **ascorbate- and peroxiredoxin-dependent water–water cycles** exemplify the interplay between low molecular weight and enzymatic antioxidants linked to electron donors such as NADPH and ferredoxin (Fd) (Figure 8.7). The total pool of ascorbate plus monodehydroascorbate plus dehydroascorbate in plant cells often is in the range of 5–10 mM, and subcellular concentrations are estimated to be 10 mM in the chloroplast and more than 20 mM in the peroxisome of *A. thaliana.* Two monodehydroascorbate molecules spontaneously dismutate to dehydroascorbate and ascorbate. Dehydroascorbate is reduced at the expense of GSH, which is oxidized to GSSG (oxidized glutathione) (Figure 8.7). GSSG is reduced to 2 GSH again by GSH reductase using electrons from NADPH.

8.5 Reactive Oxygen Species in Abiotic Stresses

A few abiotic stressors are redox-active by themselves and directly initiate oxidative stress in the cell if present at toxic concentrations. Among these compounds are the transition metals Fe(II) and Cu(I), which donate electrons to acceptors including H_2O_2 thereby generating the extremely oxidizing hydroxyl radical in the Fenton reaction. Ozone oxidizes thiols, amines, and unsaturated carbon compounds. The two primary defense mechanisms against ozone involve stomatal closure to reduce entrance of O_3 into the intercellular space and decomposition of O_3 in the apoplast by reaction with ascorbate. Redox reactions are caused by some xenobiotics, for example, methylviologen, or photosensitizers, for example, psoralen, in the presence of UV-A or other radiation.

Besides direct oxidative stress from redox-active stressors, a common response to most abiotic stresses is the deregulation of metabolism with increased rates of ROS and RNS generation and subsequent development of oxidative stress. Thus, intensive research has established a tight linkage between cold, drought, frost, heat, heavy metals, excess light, nutrient deficiency, salt and xenobiotics, and the occurrence of oxidative stress. The well-studied example is **cadmium stress**. Cd is highly toxic to cells and causes ROS accumulation (see Section 12.6.1). Nitric oxide and antioxidant responses partially counteract Cd toxicity (see Chapter 12).

Scientists use several biochemical and genetic markers and methods to assess the level of oxidative stress in cells, some of which are listed here. ROS contents can be quantified in tissues, sections, or extracts. Nitroblue tetrazolium (NBT) stains for $O_2^{\bullet -}$, which reacts with NBT to formazan derivatives. Diaminobenzidine tetrahydrochloride (DAB) reacts with H_2O_2 in a reaction catalyzed by peroxidase and forms a brown insoluble compound detectable as precipitate. Chloromethyl-2′,7′-dichlorofluorescein diacetate is taken up by cells and deesterfied. By reaction with H_2O_2 it forms the green fluorescent dichlorofluorescein, which can be monitored by **fluorescence microscopy** (see Section 19.1.1.3). They have been widely used to assess ROS levels in disease and stress. Because of the chemical instability of ROS, the researcher has to keep in mind that all these methods determine a mixture between ROS levels and ROS production rate. In response to stress, ROS often accumulate in sensitive genotypes more than in tolerant

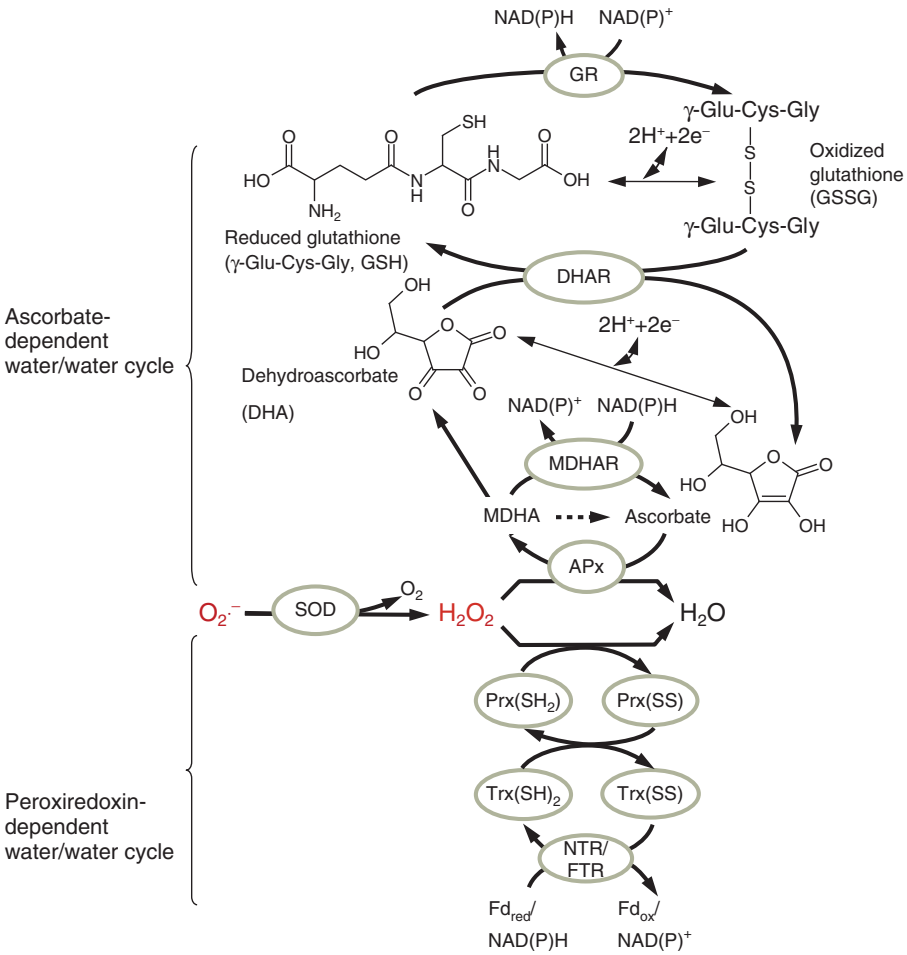

Figure 8.7 Water–water cycle for ROS detoxification and structure of the low molecular weight antioxidants ascorbate and glutathione. Superoxide is dismutated by SOD to form H_2O_2. There are two main routes of H_2O_2 detoxification in the chloroplast, the ascorbate-, and the peroxiredoxin (Prx)-dependent pathway. The ascorbate-dependent one is linked to the oxidation of ascorbate and subsequently of glutathione (GSH). Chemical structures are shown for ascorbate and dehydroascorbate, and of glutathione (GSH) and oxidized glutathione (GSSG) as partners in two electron redox reactions. Regeneration of GSSG is achieved by glutathione reductase (GR). The Prx-pathway involves reductants like thioredoxin (Trx). Oxidized thioredoxin is rereduced by NADPH-dependent Trx reductase (NTR) or ferredoxin (Fd)-dependent Trx reductase (FTR). DHA – dehydroascorbate; DHAR – dehydroascorbate reductase; MDHA – monodehydroascorbate; MDHR – monodehydroascorbate reductase.

genotypes as shown, for example, for salinity stress in wheat or cadmium stress in pea. Alternatively, the level of oxidative stress can be indirectly assessed by quantifying the consequences of toxic ROS impact. ROS react with unsaturated lipids. Following oxidation, lipids degrade and release malondialdehyde (HOCH=CH–CHO) as highly electrophilic product, which can be determined by its reaction with thiobarbituric acid. Malondialdehyde contents increase in wilted pea (Figure 8.8). Oxidative stress causes carbonylation of proteins, which is the oxidation of side chains of sensitive amino acids like lysine, arginine, proline, cysteine, and threonine residues. Other lipid degradation products are 4-hydroxynonenal and acrolein, which are reactive aldehydes and oxidize proteins (Figure 8.9). Protein carbonylation can be determined after derivatization with dinitrophenylhydrazine using antidinitrophenylhydrazine antibodies. In parallel to increased malondialdehyde levels, oxidized protein contents increased under drought stress in pea (Figure 8.8). New methods of imaging thiol redox state or H_2O_2 in cells employ redox-sensitive variants of green fluorescent proteins (roGFPs) or H_2O_2-sensing HyPer. Such probes thus allow researchers to monitor redox state and ROS levels in transgenic organisms expressing these gene-encoded sensors *in vivo*.

Many studies measured activities of **antioxidant enzymes**, which often increase on stress application. Most frequently quantified antioxidant activities are those of catalase, phenol-dependent peroxidase, SOD, and APX. These enzymes often are encoded by small gene families with different tissue and subcellular distribution. Methods such as immunoreactions, sensitivity to inhibitors, and in gel or chromatographic separation need to be used to

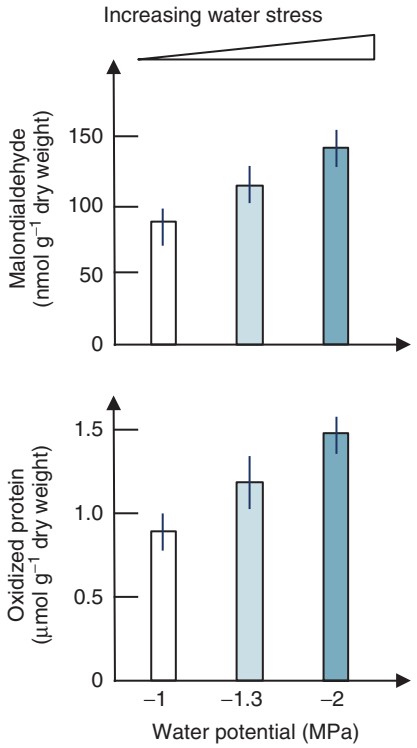

Figure 8.8 Oxidative stress in wilted pea leaves. Irrigation water was withheld from pea plants for 7 d and water potential measured, thus -1 MPa corresponds to well-watered status, −1.3 to moderate and −2 MPa to severe drought stress. The lipid peroxidation product malondialdehyde (upper figure) and the amount of oxidized protein (lower figure) increase under drought stress indicating oxidative stress. See also Moran *et al*. (1994).

distinguish the isoforms. Thus it is easier to address the distinct responses to stress at the level of transcripts as 5′- and 3′-untranslated regions differ among even highly similar gene family members. Specific transcripts have been identified whose regulation patterns serve as sensitive markers of oxidative stress. *A. thaliana* is among the best studied species in relation to abiotic stress-induced transcriptional responses. Transcripts of APX isoforms APX1 and APX2 rapidly increase on high light stress, LOX on heavy metal treatments or wounding catalase in drought-stressed tobacco (*Nicotiana* sp. *Solanales, Asteridae*). However, such transcriptional responses and thus their suitability as markers often differ between genotypes and species, as well as stress type and strength.

8.6 Reactive Oxygen Species in Biotic Interactions

Organisms in their natural environment encounter many and diverse biotic interactions, ranging from symbiosis to competition for resources, herbivorie and carnivorie, and pathogenesis, thus from favorable to neutral and negative interactions, respectively, involving all kind of organisms. Stresses often occur in combination. ROS have been described as a hallmark of successful recognition of infection and defense. Invading pathogens release a cocktail of different molecules termed **pathogen-associated molecular pattern (PAMP)** (Figure 8.10). Some PAMP compounds bind to specific receptors (**pattern recognition receptors**, **PRR**) at the plasma membrane of the plant cell that activate cell signaling pathways and cause a defense program known as **PAMP-triggered immunity (PTI)**. Receptor-mediated changes in free calcium levels in the cytoplasm (see Section 7.6) activate the NADPH oxidase RBOH, which generates O_2^- at the apoplast-facing side of the plasma membrane and thereby triggers a ROS burst. This oxidative burst initiates downstream responses including changes in gene expression, strengthening of cell wall, initiation of systemic resistance, but may also damage the pathogen or induce programmed cell death. Local cell lesions are linked to massive ROS production and are part of the hypersensitive response that impedes the spreading of the pathogen in the incompatible interaction.

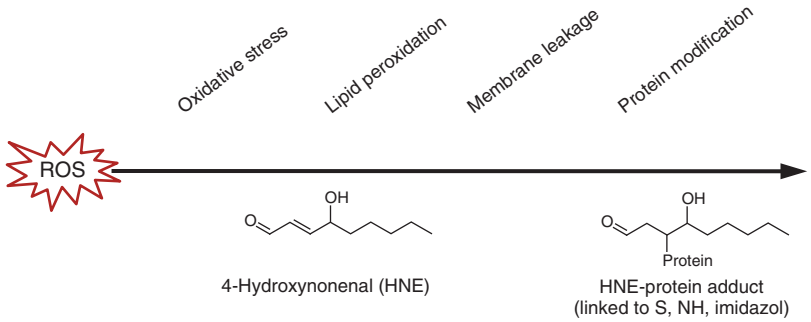

Figure 8.9 Lipid peroxidation liberating peroxidation products such as 4-hydroxynonenal and its adduct with protein. The schematics shows the time dependency of a ROS impact on lipid peroxidation, membrane leakage, and protein oxidation. 4-hydroxynonenal (HNE) is a degradation product of lipid hydroperoxides and, as reactive aldehyde, links to specific amino acid side chains. Membrane leakage is easily measured by the conductimetry of cells and tissues.

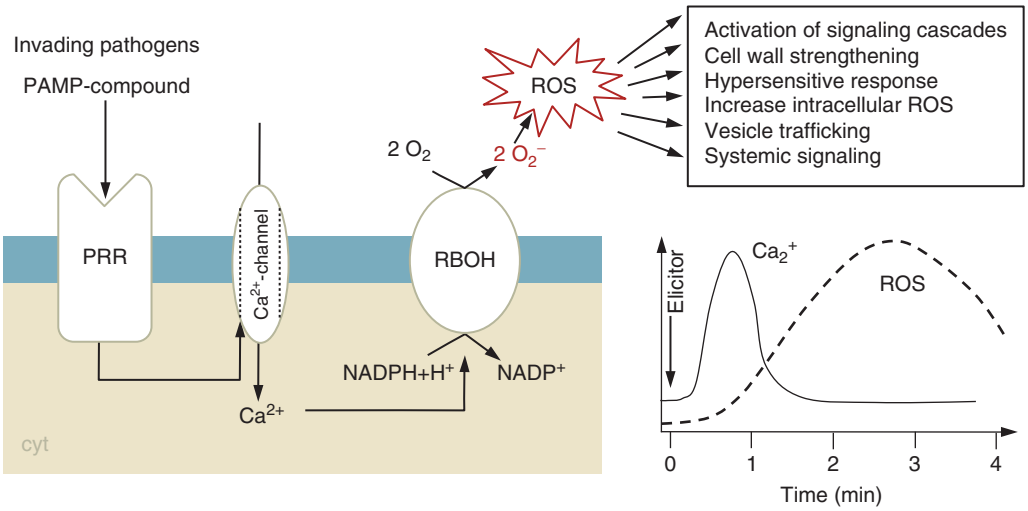

Figure 8.10 Schematics of pathogen-mediated activation of cell signaling. Compounds of the pathogen-associated molecular pattern (PAMP) bind to pattern recognition receptors (PRR), which initiate signaling cascades inside the cell. One pathway involves the elevation of free Ca^{2+} concentration and activation of NADPH oxidase (RBOH) to generate extracellular ROS. ROS mediates diverse responses as summarized in the gray-shaded box. The time kinetics shows up-regulation of free Ca^{2+} and subsequently of extracellular ROS after challenging the plant cell with appropriate elicitor.

8.7 Cell Signaling Function of Reactive Oxygen Species

The two previous paragraphs have exemplified the important role played by ROS during abiotic and biotic stress. The regulatory functions of ROS and redox state in cell signaling reach beyond stress. Oxygen and ROS decisively control the redox state of the cell, which in turn interferes with most cell processes, including cell cycle progression, metabolism, transcription, translation, and signaling. Protein activity is modulated by diverse posttranslational modifications, one of which concerns cysteinyl residues. The thiol of cysteines dissociates into the thiolate anion and H^+. As nucleophil the thiolate is easily oxidized to inter- or intramolecular disulfides (Figure 8.11a). Depending on thiol redox potential, accessibility, pK_a, and neighboring effects, dithiol proteins can reduce other proteins present in the disulfide state (Figure 8.11b). The **dithiol-/disulfide transition** induces conformational changes in the protein structures that switch or modulate the activity of enzymes or binding properties. Thus, the redox state determines the activity of the components. Specific thiol-proteins act as thiol peroxidases and decompose peroxides ranging from H_2O_2, alkylhydroperoxides, to peroxinitrite (Figure 8.11c) via a sulfenic acid intermediate (Figure 8.11d). Further oxidation of sulfenic acid to sulfinic acid (Figure 8.11e), S-nitrosylation (Figure 8.11f), and mixed disulfide formation with GSH (Figure 8.11g) represent other commonly found posttranslational modifications of cysteine with regulatory or protective function.

Using this principle of dithiol-/disulfide transition, cells maintain a redox regulatory network that is linked to energy metabolism. Redox input elements, predominantly NADPH, GSH, and FDs, represent the interface to metabolism and reduce redox transmitters (Figure 8.11h). The transmitters adjust the dithiol/disulfide state of target proteins. Intensive search using proteomics approaches has identified about 400 proteins as putative redox-regulated targets in plants. Their functional categorization assigns these proteins to diverse processes in the cell. In addition, redox sensors react with ROS and RNS, which drain electrons from the network (Figure 8.11h). In aerobic environment a continuous electron flow through the network reflects the balance between reductive and oxidative processes in the cell. Through the input element GSH, the network is linked to the predominant low molecular mass antioxidant of the cell. The dithiol-/disulfide redox network is particularly complex in plants as can be exemplified by Trxs as transmitters: vertebrate genomes code for two Trx as redox transmitters, while plant genomes encode more than 20 Trx. Both plants and mammals contain many addition proteins with Trx domains. Apparently, plants rely on an elaborated redox network required for the acclimation to their variable environment.

ROS accumulate in all aerobic organisms under stressful condition. **ROS sensing in prokaryotes** directly links to gene expression regulation of gene regulons. SoxR and OxyR were identified in mutant *E. coli* bacteria and serve as paradigms of redox-operated switches in gene transcriptional control. Depending on the type of mutation, the strains become either hypersensitive or resistant to ROS. SoxR is constitutively present in its inactive form. SoxR contains a [2Fe-2S] cluster as sensor. Activated by oxidation SoxR induces expression of SoxS which subsequently switches on the expression of genes involved in antioxidant defense including Mn-SOD. OxyR is another

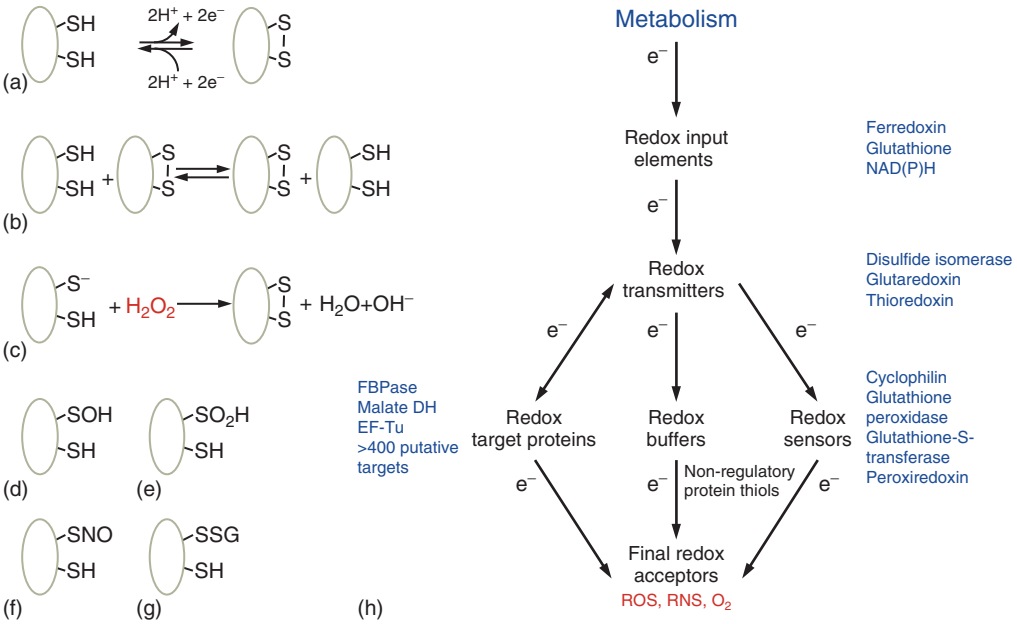

Figure 8.11 **Simplified structure of the dithiol-/disulfide redox network of the cell.** (a) Dithiol-/disulfide transition depicted as redox reaction. (b) Coupled dithiol-/disulfide transitions as principal reaction mechanism within the dithiol-/disulfide network. (c) Redox sensor with a catalytic thiol with low pK efficiently reacts with peroxide substrate and subsequently adopts the disulfide form; other posttranslational modifications of cysteines are (d) sulfenic acid, (e) sulfinic acid, (f) S-nitrosylated, and (g) glutathionylated form. (h) Simplified composite structure of the dithiol-/disulfide network consisting of input elements, transmitters, targets, sensors, buffers, and as final electron acceptors O_2, ROS, and RNS. The arrows indicate the electron flow through the network.

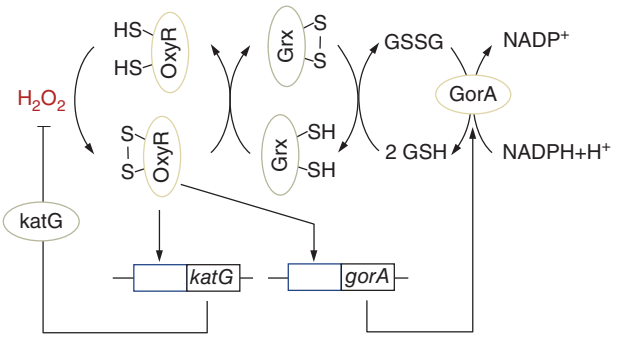

Figure 8.12 **Bacterial redox circuitry of OxyR.** The transcription factor OxyR exists in a reduced inactive and oxidized active conformation. Cellular H_2O_2 oxidizes OxyR, which then transactivates the OxyR regulon that includes catalase KatG and glutathione reductase GorA. Thus both exemplarily specified gene products participate in readjusting redox homeostasis as oxidized OxyR is rereduced via the glutaredoxin/glutathione system and catalase decomposes H_2O_2. (Based on Zheng, Aslund, and Storz (1998).)

bacterial sensor system for ROS (Figure 8.12). Conversion of the dithiol-form to the disulfide form by H_2O_2 activates the transcription factor which stimulates the expression of target genes coding for antioxidant genes, for example, catalase and GSH reductase. On reestablishment of redox homoeostasis, an electron flow from NADPH via GSH and glutaredoxin reduces OxyR and switches off the regulon.

Gene expression regulation in response to oxidative stress in yeast may be the best studied example in eukaryotes. The bZIP-transcription factor YAP1 controls transcription of about 70 genes with function in redox balance, ROS detoxification, metal and xenobiotics tolerance. Different stressors including peroxides, xenobiotics such as menadione, and the elements As, Cd, Hg, Sb, and Se activate the YAP1-dependent pathway. YAP1 protein contains two clusters of Cys that undergo thiol-/disulfide transition under ROS stress. The GPX 3 reacts with H_2O_2 to a sulfenic acid derivative and oxidizes YAP1 that subsequently releases bound YAP1-binding protein (YBP). This mechanism has been termed H_2O_2 sensor relay. Oxidized YAP1 translocates in the nucleus and activates the stress response genes. Thus the signaling pathway from ROS sensing to gene activation involves three proteins (Figure 8.13).

The pathways of ROS sensing and signal transmission often involve **signaling cascades**. Reversible phosphorylation and dephosphorylation of proteins by protein kinases and protein phosphatases, respectively, constitute an efficient mechanism to propagate and amplify signals in information processing (see Section 7.2). In eukaryotes H_2O_2 activates mitogen-activated protein kinase kinase kinase (MAPKKK) ANP1, which in turn triggers the MAPK-pathway via AtMPK3 and AtMPK6 and causes downstream activation of specific gene expression like glutathione-*S*-transferase GST6 and heat shock protein HSP18.2. Transgenic overexpression of the tobacco MAPKKK, the ANP1-homologue NPK1, enhances tolerance against salinity and heat stress of tobacco.

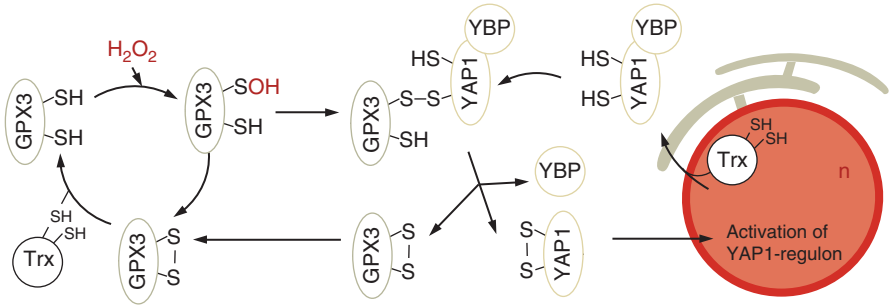

Figure 8.13 Simplifed model of H_2O_2-dependent activation of the yeast AP1-transcription factor (YAP1)-dependent regulon in yeast. GPX3 senses H_2O_2 and its catalytic thiol is converted to sulfenic acid. The sulfenic GPX3 oxidizes thiols in YAP1. Following intramolecular thiol-disulfide shuffling, YAP1 is imported in the nucleus and transactivates the target genes. Following the readjustment of cellular redox homeostasis, Trx shifts the YAP1 redox state to a more reduced condition, and YAP1 is exported from the nucleus and the YAP1 regulon switched off. GPX – glutathione peroxidase; Grx – glutaredoxin, a redox transmitter; YBP – YAP1-binding protein.

ROS and plasmamembrane-bound RBOH activity participate in systemic signaling in the high light and stress response (see Chapter 9). If some leaves or leaf sections of a plant experience high light stress, RBOH generates O_2^-, which by SOD is converted to H_2O_2 that acts as self-propagating long-distance signal to distant shaded leaves. These leaves then undergo systemic acclimation to high light without having experienced the high light condition by themselves (Figure 8.14). This type of long-distance signaling is also triggered by other abiotic and biotic stresses. Electrical signals, hormonal messengers, and microRNAs are involved in systemic signaling in addition to ROS (see Chapter 7).

If the oxidative condition prevails, redox imbalances and oxidative stress trigger cell death programs in animals. Several pathways sense the severity of the redox deviation and the accumulating ROS. Binding of ligands to specific receptors at the plasma membrane, also called **death receptors**, activates cell death signaling pathways that are antagonized by proper redox homoeostasis in the cell. ROS inhibit the antagonizing pathway and cell death proceeds. Another pathway is initiated by ROS accumulation in mitochondria, release of cytochrome c, and activation of caspase-9. Thus cell death programs intricately involve ROS and are important to shape development, remove aberrant cells, or to fight invading pathogens.

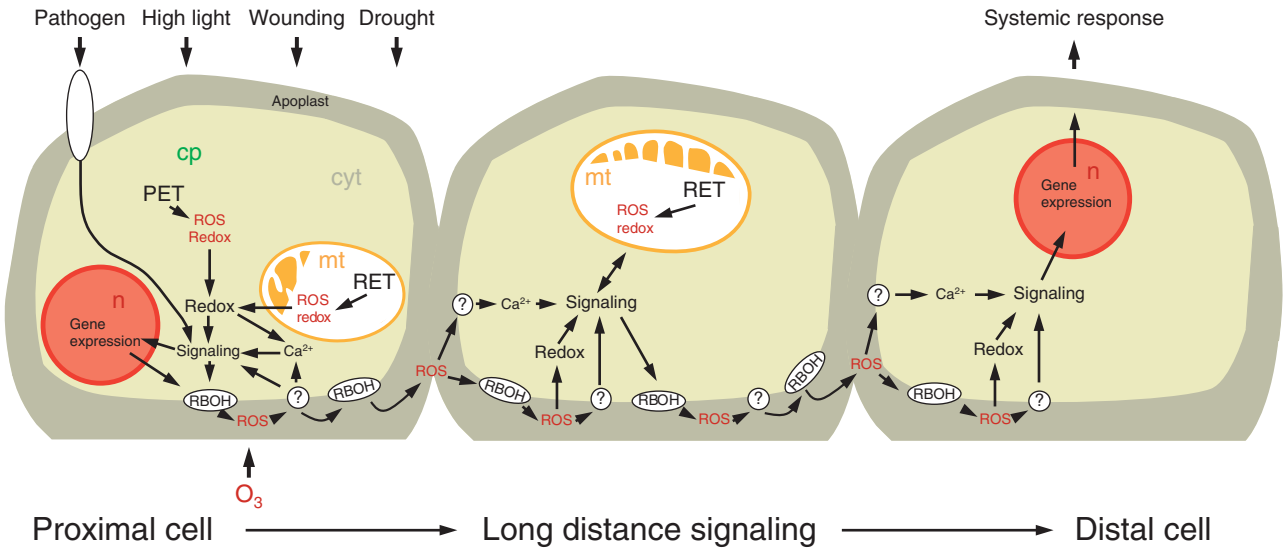

Figure 8.14 ROS-dependent long-distance stress signaling for systemic responses. Pathogens, high light, wounding, drought, and ozone initiate cell signaling by different pathways that also involve redox changes and finally activation of plasmamembrane-bound NADPH oxidase (RBOH). This occurs at the site of stress impact, that is, in the proximal cells. RBOH generates extracellular O_2^- and other ROS. The ROS stimulus activates directional signal propagation along the cell surfaces, in particular, of the vascular bundle sheaths. The signal travels through tissues and organs. This self-propagating process likely involves other signaling factors including Ca^{2+}. The long-distance signal allows the plant to activate acclimation responses in distal cell that have not encountered the stress. Cp – chloroplast; mt – mitochondrion; n – nucleus; PET – photosynthetic electron transport; RET – respiratory electron transport.

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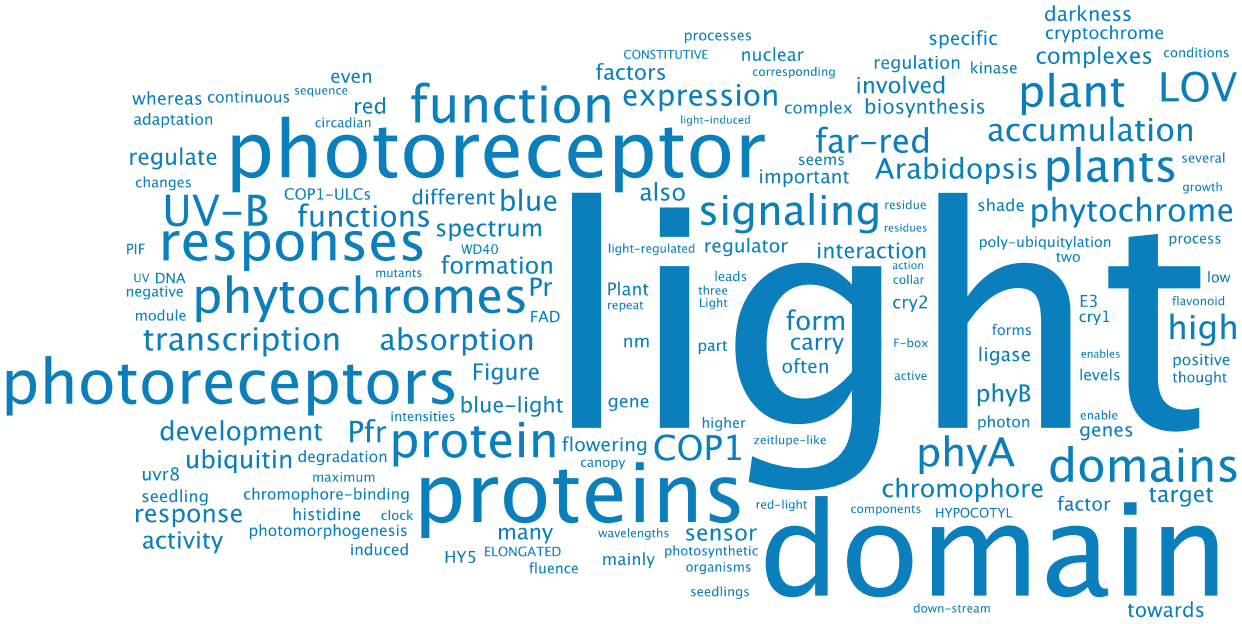
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9
Light

Thomas Kretsch

Overview

Light is often defined as part of the electromagnetic spectrum that is visible for the human eye and extends from wavelengths of about 400–740 nm. However, many biological processes are also triggered by the UV-B (280–315 nm), UV-A (315–400 nm), and the near infrared (740–800 nm) part of the spectrum that can reach Earth's surface. Light can be regarded as one of the most important external factors essential for the survival of life. It is the major source of energy for photosynthesis that propels all metabolic and physiological processes of photoautotrophic organisms, whose products are at the base of nearly all food chains. Except for this positive effects, light is a source of stress. Photons of the UV part of the spectrum carry enough energy to destroy important biological molecules, which can be deleterious to organisms. Furthermore, excess of light can also cause damage either by bleaching or creation of radicals and other reactive compounds. Finally, day length helps organisms to adapt toward seasonal changes during the year. To adapt toward surrounding light conditions, organisms evolved a multitude of photoreceptors. This chapter gives an overview about the function of selected classes of photoreceptors that sense light from the UV-B to the far-red part of the spectrum. It introduces how light is sensed by photoreceptors and transmitted by molecular signaling cascades to regulate light responses. As light is the most important for the survival of plants, many examples provided here concentrate on this class of organisms.

9.1
Principles of Light Detection and Photoreceptor Function

9.1.1
Modular Domain Structure of Photoreceptors

Photoreceptor proteins are composed of several domains, which usually are separated into **chromophore-binding** or **sensor domains** and **output domains.** With the exception of UV-B light, energy of photons is not sufficient to alter protein structures. Therefore, each photoreceptor apoprotein covalently or noncovalently binds at least one **chromophore** (greek *chromos* = colour; *phor* = carrier), a small organic cofactor that absorbs light in the visible range of the spectrum. Chromophores carry conjugated double bonds, which allow easy delocalization of electrons in an extended π system (Figure 9.1, see Section S1.2.6.1). On absorption of a photon, chromophores undergo conformational changes, which induce alterations in the structure of the chromophore-binding or sensor domain of the apoprotein. Structural changes of chromophore-binding domains are then transmitted to **output domains**. Output domains comprise a multitude of protein–protein interaction domains and sometimes possess protein enzyme activity (kinases, cyclases, etc.) or function as ion channels. Thus, these domains are mainly responsible for the translation of the light input into biochemical signals inside the cell (even though chromophore-binding domains often play a supportive role). The modular design principle of photoreceptors is thought to increase flexibility in the achievement of adaptation toward light during evolution. Separation into chromophore-binding and output domains facilitates rearrangements between these functional moduls, which can achieve novel connections between light sensing and signaling processes inside the cell. A good example of this principle is the **LOV** (**light/oxygen/voltage**) chromophore-binding domain, which forms the light detection module of many photoreceptors (see Section 9.3). An unusual combination also occurred in **neochrome** photoreceptors, which evolved from a fusion of the blue-light-sensitive phototropin photoreceptor with chromophore-binding domains of red-light-sensitive phytochromes.

9.1.2
Identification and Classification of Photoreceptor Molecules

In most cases the initial step toward identification of a photoreceptor was the determination of an **action spectrum** for specific light responses (Box 9.1; Figure 9.2). Action spectroscopy uses the observation that maxi-

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

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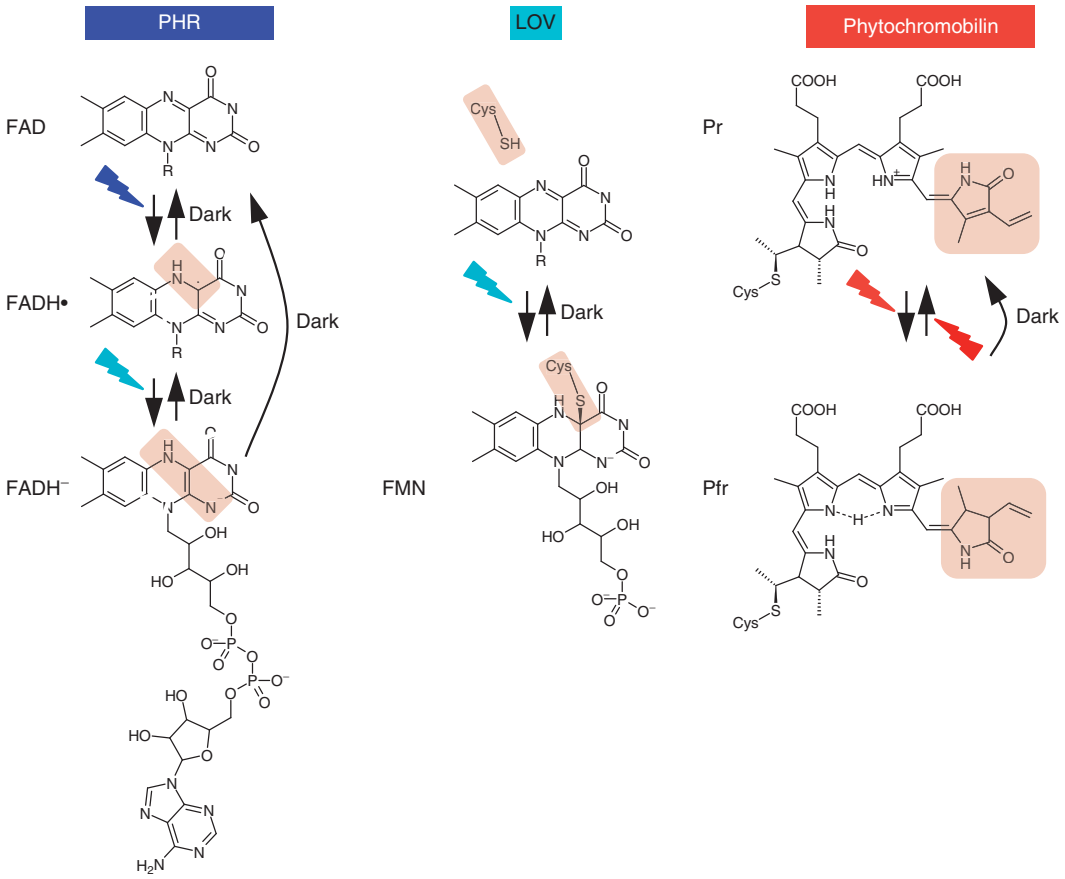

Figure 9.1 Chromophores and simplified photochemistry of chromophores in light sensor domains. Cys – cysteine residue of the apoprotein; FAD – flavin adenine dinucleotide; FADH – reduced form of flavin adenine dinucleotide; FMN – flavin mononucleotide; LOV – light/oxygen/voltage domain; Pfr – far-red-light-absorbing form of phytochrome; PHR – photolyase-related domain; Pr – red-light-absorbing form of phytochrome.

Box 9.1: Action spectroscopy

Action spectra help to identify the chromophore of an unknown photoreceptor that triggers a specific light response. Because light absorption by the chromophore propels photoreceptor activity, variations in light absorption efficiency of the chromophore are expected to cause differences in the degree of the light response under different wavelengths. Determination of an action spectrum is a multiple step process. First, the magnitude of a quantifiable response has to be determined under different wavelength, whereon either photon fluence $N(\lambda)$ (photons/surface area) or photon fluence rate $R(\lambda)$ [photons/(surface area × time)] is attenuated. Second, the magnitude of the light response is plotted against the applied values of $N(\lambda)$ or $R(\lambda)$ for the different wavelength (Figure 2a). Third, values $N_p(\lambda)$ or $R_p(\lambda)$ are determined, which are necessary to cause a fixed response level under a given wavelength, whereon p is often set to 0.5 (50%) of maximum response. Finally, the reciprocal of $N_p(\lambda)$ or $R_p(\lambda)$ is plotted against the corresponding wavelength. Alternatively, as shown in Figure 2b, relative photon efficiency is often calculated in relation to the wavelength λ_{max}, which shows highest light responsiveness $N_p(\lambda)/N_p(\lambda_{max})$ or $R_p(\lambda)/R_p(\lambda_{max})$.

mum light sensitivity is obtained under light qualities enabling highest excitation of the chromophore whereas all other wavelengths are less efficient. Reasonably, the action spectrum can provide an initial hint about the chromophore attached to an unknown photoreceptor. Actually, determination of action spectra enabled grouping into different photoreceptor classes even without the knowledge of the respective photoreceptor proteins. According to the shape of action spectra, photoreceptors were originally grouped into three major classes, namely **UV-B receptors, blue/UV-A receptors,** and the red and far-red-light-absorbing **phytochromes** (Figure 9.3). Identification and further classification of most photoreceptors was achieved by the

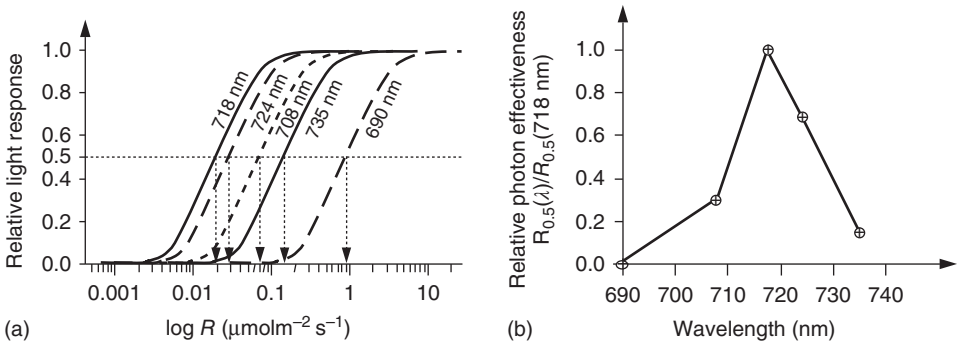

Figure 9.2 Construction of an action spectrum. (a) Exemplary photon fluence rate – response curves under different wavelengths. Values of photon fluence rates that cause 50% of maximum light responses ($R_{0.5}$-values) under different wavelengths are indicated by arrows. (b) Action spectrum calculated from $R_{0.5}$-values shown in (a). The $R_{0.5}$-value determined under 718 nm light was set to 1.

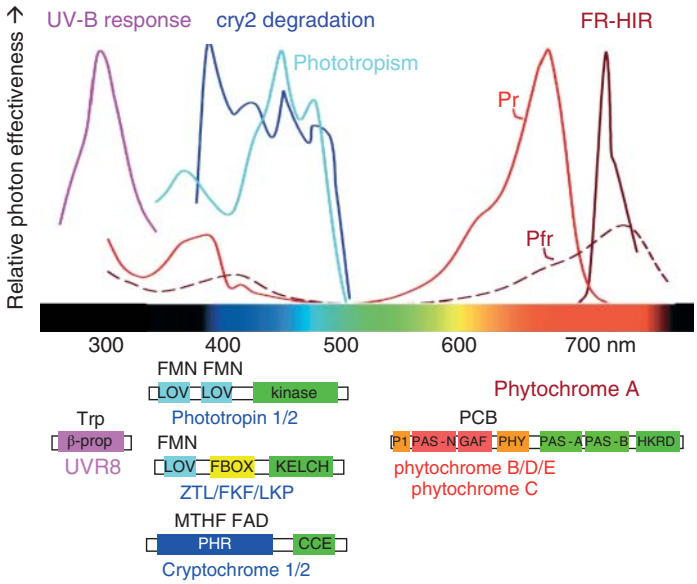

Figure 9.3 Action spectra and domain structure of plant photoreceptors. The action spectra were obtained for UV-B dependent accumulation of flavonoids in parsley cell cultures (Beggs and Wellmann, 1994), degradation of cry2 (Hoang, Bouly, and Ahmad, 2008), phototropism of avena coleoptiles (Briggs and Christie, 2002), and the inhibition of *Arabidopsis* hypocotyl elongation under continuous far-red light (FR-HIR). Photoconversion cross-sections of Pr and Pfr are drawn as values according to the maximum value of Pr. β-prop – β-propeller structure; CCE – cryptochrome c-terminal extension; cry2 – cryptochrome 2; FAD – flavin adenine dinucleotide; FMN – flavin mononucleotide; FR-HIR – far-red high irradiance response; GAF – cGMP-stimulated phosphodiesterase/*Anabena* adenylate cyclase/*E. coli* FhlA; HKRD – histidine kinase-related domain; KELCH – KELCH-repeat domain, LOV – light/oxygen/voltage domain; MTHF – methenyltetrahydrofolate; PAS – period circadian protein/Ah receptor nuclear translocator protein/single minded protein; PCB – phytochromobilin; Pfr – far-red-light absorbing form of phytochrome; PHR – photolyase-related domain; PHY – phytochrome domain; Pr – red-light-absorbing form of phytochrome; Trp – tryptophan residues; UVR8 – UV resistence locus 8.

characterization of loss-of-function mutants that do not respond to specific light qualities (Box 9.2; Figure 9.4). As photoreceptors are positive effectors in light signaling cascades, the loss-of-function phenotype was often caused by knockout mutations in corresponding genes. Action spectra conducted the gold standard to verify the molecular identity of proposed photoreceptors, because it has to be shown that the absorption spectrum of the purified photoreceptor fits to the action spectrum of corresponding light responses.

9.1.3 COP1-Containing E3 Ubiquitin Ligase Complexes: Central Components of Light Signaling in Plants

Mutants with altered light responses during plant seedling development have been a versatile tool to identify and characterize the function of photoreceptors and important components of light signaling (Box 9.2; Figure 9.4). A classical screening strategy searched for mutants with a deetiolated (Det) or Constitutive photomorphogenic (Cop) phenotype in darkness. One negative regulator in

Box 9.2: Screening for photomorphogenic mutants during plant seedling development

Light is among the most important exogenous factors for the survival of plants. A very drastic effect of light is seen during early wild type (WT) seedling development, when higher plants can switch between two developmental programs, that is, **skotomorphogenesis** or **etiolation** in the dark (Figure 9.4a) and **photomorphogenesis** in the light (Figure 9.4b). Etiolated WT seedlings show several adaptations to enable rapid growth through the soil and toward light, including elongated sprout axes (hypocotyls), small and pale leaves (cotyledons), and formation of an apical hook. In the light, elongation is arrested, the hook opens, and cotyledons start to expand and to form green chloroplasts. As changes between skoto- and photomorphogenesis are easily detectable, alterations in light-dependent seedling development were used to screen for mutants in light signaling. Screening for loss-of-function mutants helped to identify many photoreceptors and positively acting factors involved in the regulation of light responses. For example, the loss-of-function phenotype of the *phyB-5* mutant is shown in darkness (Figure 9.4a) and under continuous light (Figure 9.4b). The *phyB-5* mutant is deficient for the plant photoreceptor phytochrome B, which causes alterations in hypocotyl elongation and cotyledon development in the light. To identify negative regulators, a classical screening strategy searched for mutants with a constitutive photomorphogenic (Cop) phenotype in darkness (Figure 9.4a). The screening helped to identify CONSTITUTIVE PHOTORMORPHOGENIC 1 (COP1) as a central component of repressor complexes that block seedling photomorphogenesis and light responses downstream of several photoreceptors at all stages of plant development.

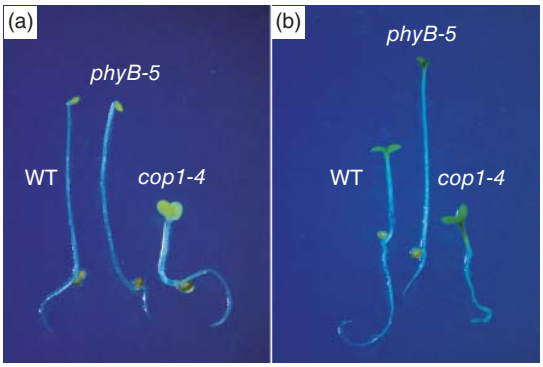

Figure 9.4 Skoto- and photomorphogenesis during plant seedling development. The seedling phenotypes are shown for wild-type (WT), the *phyB-5* knockout mutant of the phytochrome B photoreceptor, and the *cop1-4* loss-of-function allele of the CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) repressor of photomorphogenesis. Seedlings were either grown in darkness (a) and under continuous light (b) for 4 days.

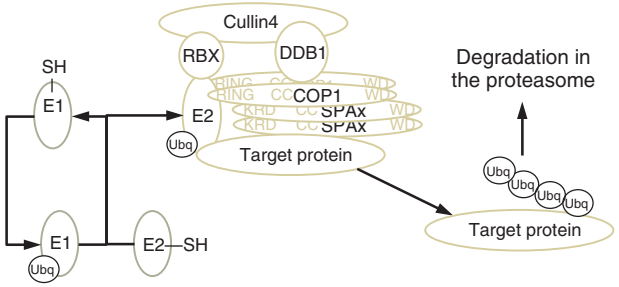

Figure 9.5 Schematic illustration of polyubiquitylation by CONSTITUTIVE PHOTOMORPHOGENIC (COP1)-containing E3 ubiquitylation complexes (COP1-ULC). E1 and E2 proteins activate ubiquitin polypeptides in an ATP-dependent process *via* thioester formation. The backbone of COP1-ULCs is formed by a Cullin 4 protein that binds to a small RBX. RING box motives (REALLY INTERSTING NEW GENE) of RBX and/or COP1 mediate interaction with activated E2-ubiquitin adducts (E2-Ubq). DAMAGED DNA-BINDING PROTEIN 1 (DDB1) function as adaptor proteins for tetramers formed by COP1 and four different SUPPRESSOR OF *phyA-105* (SPAx) proteins. COP1-SPA1 tetramers are responsible for specific interactions with target proteins that become polyubiquitylated and, as a consequence, degraded in the proteasome. CC – coiled-coil domain involved in dimer and tetramer formation; KRD – kinase-related domain; Ubq – UBIQUITIN; WD – WD40 repeat domain that forms a ß-propeller and mediates specific interactions with target proteins.

plant photomorphogenesis, **COP1** was identified as central component of repressor complexes, which blocks light response and photomorphogenic development. COP1 is composed of three major subdomains: an n-terminal RING (REALLY INTERESTING NEW GENE) Zn-finger domain, a coiled-coil domain, and the c-terminal seven propeller WD40 repeat domain (Figure 9.5). The coiled-coil domain enables dimerization of COP1 subunits and interaction with **SUPPRESSOR OF *phyA* -105 (SPA1)** proteins that compose a small subfamily of four proteins (SPA1 to SPA4) in *Arabidopsis* (*Brassicales*, *Rosidae*). SPA proteins exhibit high similarity to COP1 in their coiled-coil and WD40 domain, but carry a kinase-related domain (KRD) at their n-terminus instead of a RING finger. SPA proteins are essential for COP1 activity and both types of proteins have overlapping, though not fully redundant functions, in light signal transduction downstream of several photoreceptors.

COP1 and SPA proteins can form higher-order **COP1-containing E3 ubiquitin ligase complexes (COP1-ULCs)** with CUL4-DDB1-RBX (CULLIN4/DAMAGED DNA-BINDING PROTEIN 1/RING BOX PROTEIN). E3 ubiquitin ligases specifically interact with target proteins and catalyze their **polyubiquitylation**. Polyubiquitylated target proteins are very often rapidly degraded in the 25S proteasome, a multicomponent complex found in almost all eukaryotic organisms (see Figure 9.6).

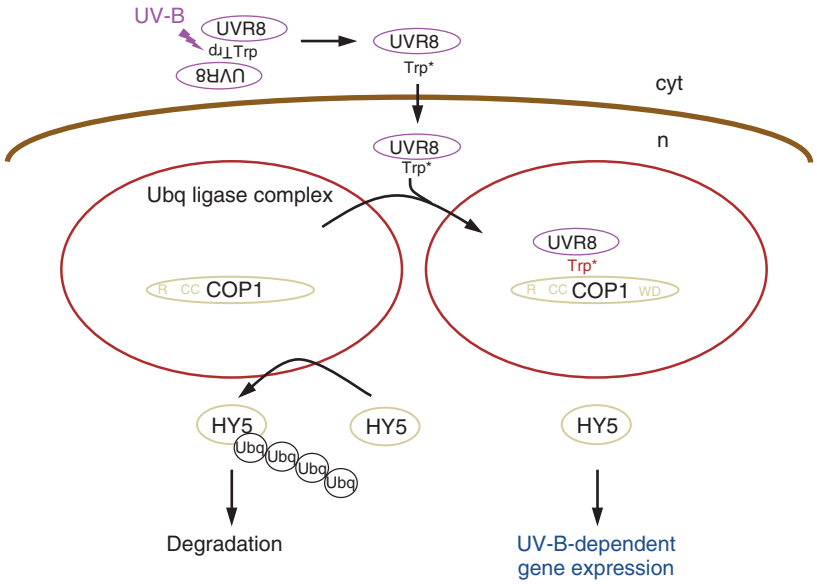

Figure 9.6 Schematic illustration of UV-B signaling. The oval represents COP1-containing ubiuqitin ligase complexes (COP1-ULCs). CC – coiled-coil domain; COP1 – CONSTITUTIVE PHOTOMORPHOGENIC 1; cyt – cytoplasm; HY5 – ELONGATED HYPOCOTYL 5 transcription factor; n – nucleus; R – RING-finger domain; Trp – tryptophan residues; Trp* – tryptophan residues excited by UV-B light; Ubq – ubiquitin; UVR8 – UV resistance locus 8; WD – WD40 repeat domain.

CULLIN4 forms the backbone of the complex. The RING domain of ring-box protein (RBX) or COP1 is responsible for the recruitment of activated **ubiquitin** polypeptides that are bound to E2 proteins in an ATP-dependent process catalyzed by E1 proteins (Figure 9.5). DDB1 functions as an adapter that recruits WD40 proteins. The WD40 repeat domains of COP1 and SPAs are responsible for specific interaction with target proteins. The heterotetrameric structure of COP1/SPA proteins is thought to increase the spectrum of specific protein–protein interactions and, as a consequence, the number of target proteins that become polyubiquitylated by COP1-ULCs.

9.2 Sensing of UV-B Light

9.2.1 Effects of UV-B Light

UV-B light comprises photons with wavelengths from 280 to 315 nm (see Figure 9.3) that can pass through the ozone layer to reach Earth's surface. UV-B photons carry high energy loads sufficient to damage biologically important macromolecules like lipids, proteins, and, most important, DNA. Therefore, most organisms have developed strategies to escape or shield against this dangerous part of the light spectrum. Because of their sessile lifestyle and exposure to sunlight, plants evolved very efficient mechanisms to protect against damaging effects of UV-B. These mechanisms include (a) efficient repair of DNA damage by photolyases, (b) development of hairs on the surface, (c) production of waxes, and (d) accumulation of phenolic compounds in the vacuoles of epidermal cells, which function as sunscreens. To control these adaptation mechanisms, plants evolved a specific UV-B photoreceptor that exhibits maximum photon effectiveness at about 280 nm.

9.2.2 UV-B Light Reception and Signal Transduction

The UV-B receptor **uv resistance locus 8** (**UVR8**) most probably forms a β-propeller structure similar to WD40 repeats that enables dimerization of the photoreceptor in its ground state. Mutant analyses suggest that UVR8, unlike other photoreceptors, does not bind a chromophore moiety to absorb light. UV-B detection seems to rely on tryptophan (Trp) residues at the surface of the proposed β-propeller structure.

Except for UVR8, the model includes two additional factors that play a central role in UV-B signaling: the basic leucine zipper (bZIP), transcription factor ELONGATED HYPOCOTYL 5 (HY5), and COP1-ULCs (Figure 9.6). HY5 is a key regulator positively regulating light responses downstream of all plant photoreceptors, including UVR8. According to the model, excitation of Trp residues in the β-propeller domain causes immediate monomerization of UVR8 dimers even under low doses of UV-B. UV-B-induced monomerization seems to be linked to nuclear accumulation of the photoreceptor, which is thought to be among the first initial steps in UVR8 light signaling. Nuclear localization of UVR8 is followed by several events, which seem to

mark the onset of the UV-B light signaling (Figure 9.6). Even though COP1-ULC activity targets HY5 for degradation in the proteasome under visible light, nuclear accumulation of the bZIP transcription factor depends on the presence of COP1 under UV-B light. UV-B light seems to convert COP1 into a positive regulator of light signaling. UV-B treatment leads to reaccumulation of COP1 in the nucleus of light-grown plants, where it can interact with UVR8. Interaction between UVR8 and COP1 results in altered substrate specificity of the corresponding COP1-ULC and blocks polyubiquitylation and degradation of HY5.

9.3 The LOV Domain: A Variable Molecular Building Block of Many Blue and UV-A Light Sensors

9.3.1 LOV Chomophor-Binding Domains

LOV chromophore-binding domains form the blue/UV-A light-sensing module for a multitude of photoreceptors. With a few exceptions, LOV domains bind flavin mononucleotide (FMN) as chromophore (Figure 9.1). On absorption of a blue-light photon by the FMN chromophore, a covalent thioether bond is formed between a cysteine residue of the apoprotein and the flavin ring. Light-induced formation of the covalent bond causes structural alterations in the LOV domain, which trigger light responses of the photoreceptor molecule. The photoreaction is able to thermally revert to the ground state in darkness, but kinetics of dark reversion varies between different LOV domains. Dark reversion is in the range of ~10–100 s for LOV1 and LOV2 domains of **phototropins**, whereas the LOV domain of members of the **zeitlupe-like protein** family shows hardly detectable reversion into the ground state.

The LOV domain forms the light sensing module in a high number of variable types of photoreceptors. LOV light sensor domains are found in phototropins, neochromes, members of the ZEITLUPE-like family, **white collar (WC)** and **vivid** photoreceptors, aureochromes, and blue-light-activated **histidine kinases**. Aureochromes carry a bZIP DNA-binding domain in front of the LOV domain. Blue light seems to increase the affinity of aureochromes to DNA and thus might directly alter gene expression. LOV histidine kinases function as **two-component sensors** (see Section 9.5.3) in some bacterial pathogens like *Brucella abortus* (*Alphaproteobacteria*) or *Pseudomonas syringae* (*Gammaproteobacteria*). The kinase function of the photoreceptor seems to regulate the virulence of *B. abortus.*

9.3.2 Phototropins and Neochromes

Phototropins are regulators of light-involved movement processes in plants. The genome of *Arabidopsis* encodes for two phototropin photoreceptor proteins, named **phototropin 1** (**phot1**) and **phototropin 2** (**phot2**). Phototropin 1 was first identified during the screening for *Arabidopsis* mutants, which did not exhibit positive phototropic curvature of hypocotyls, a process increasing the exposure of leafs toward light. Phototropins carry two LOV domains (LOV1 and LOV2) attached to a serine/threonine kinase domain (Figure 9.3). The LOV2 mainly regulates the activity of the adjacent kinase domain, whereas LOV1 is not essential for light detection but attenuates activity of LOV2. Phototropin kinase activity is enhanced in the dark and is reduced on blue-light absorption. Phototropins undergo autophosphorylation and are thought to phosphorylate downstream signaling components. They are specialized to regulate light-directed reorientation processes, but have only a weak, if any, effect at the level of transcriptional regulation. Phototropin-regulated processes mainly help to optimize photosynthesis by regulating chloroplast reorientation, stomata opening, and phototropic curvature.

Chloroplast movement optimizes exposure of the cell organelle with respect to light. Whereas the large, roundish diameter of lens-shaped chloroplasts is exposed at the cell surface to increase photosynthetic activity under weak blue light, chloroplasts move away and expose their smaller diameter to reduce photodamage under high-light intensities. Blue light-regulated stomata opening helps to optimize gas exchange in leaves. Positive phototropism (toward light) exposes plant organs to light, whereas negative phototropism helps to protect tissues from damage by high-light intensities. Both photoreceptors have overlapping functions, but differ with respect to their light sensitivity. Data show that phot1 senses lower blue light intensities compared to phot2.

Neochromes are an interesting example for evolutionary adaptation of light signaling toward specific light conditions on the molecular level. The photoreceptor is mainly found in ferns (*Moniliformopses*, *Tracheophyta*) that have adapted to grow in the canopy shade of higher plants. Fern gametophytes can form chloronema under suboptimal light conditions, which are characterized by rapid filamentous growth. In contrast to blue-light-regulated phototropism in higher plants, fern chloronema exhibit phototrophic responses toward red light. Sensing of red light seems to be achieved by the phytochrome-like domain of neochromes that transduce the red-light input to the attached phototropin part of the photoreceptor. The switch between filamentous and laminar growth of fern gametophytes is regulated by blue light, which may serve as indicator for exposure to full sun light.

9.3.3 Zeitlupe-Like Proteins

The zeitlupe-like protein family consists of three members in *Arabidopsis:* **zeitlupe** (**ztl**), **flavin-bindin/KELCH repeat/F-box** (**fkf1**), and **LOV KELCH protein 2** (**lkp2**).

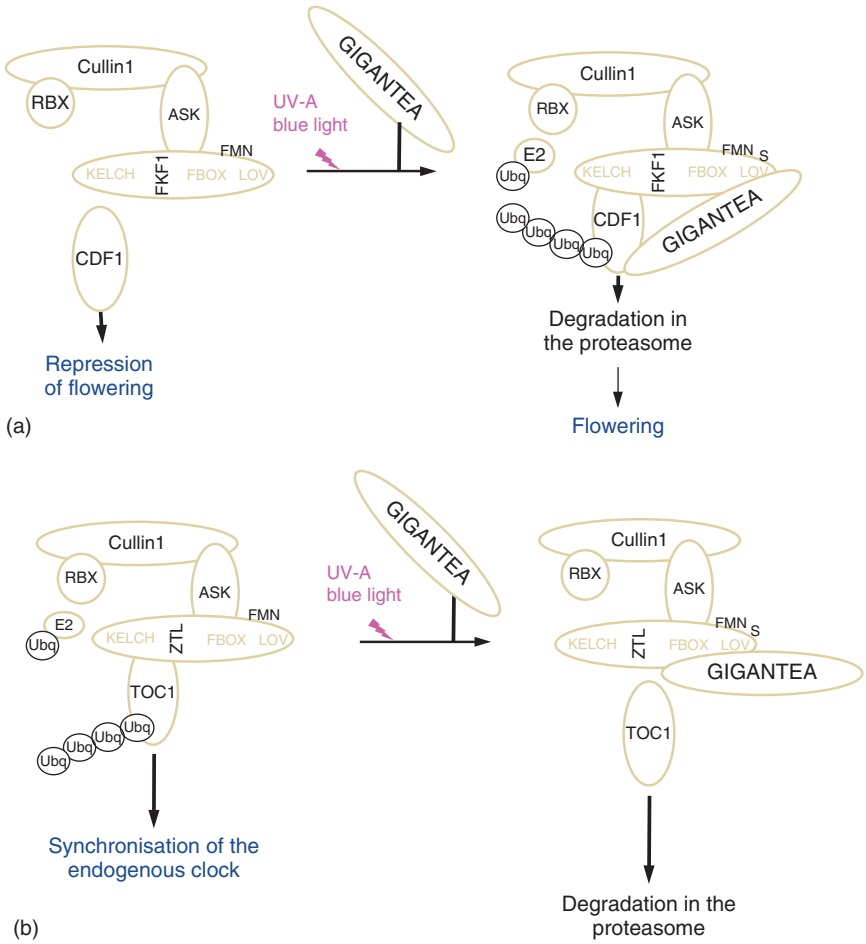

Figure 9.7 Schematic illustration of blue-light signaling mediated by members of the zeitlupe-like photoreceptor family. (a) Signaling of FLAVIN-BINDING/KELCH REPEAT/F-BOX 1 (FKF1). (b) Signaling of zeitlupe (ZTL). Both proteins are components of a light-regulated SCF (S-PHASE KINASE-RELATED PROTEIN 1/CULLIN 1/F-BOX PROTEIN) E3 ubiquitin ligase complex. ASK – ARABIDOPSIS SKP1-RELATED PROTEIN; CDF1 – CYCLING DOF FACTOR1; FMN – flavin mononucleotide; KELCH – protein–protein interaction domain named according to the sequence of the characteristic amino acid sequence; RBX – RING-BOX PROTEIN; TOC1 – TIMING OF CAB EXPRESSION 1; Ubq – ubiquitin.

The three members of the ZTL-like photoreceptor family carry three major domains: a LOV domain, an F-box domain, and a KELCH repeat domain (Figures 9.3 and 9.7). The F-box domain is a widespread sequence motif found in proteins, which are components of so-called **SCF** (S-phase kinase-related protein/Cullin1/F-box protein) **E3 ubiquitin ligase complexes** that resemble COP1-ULCs (Figure 9.5). The F-box motive links the protein to the ubiquitin ligase core complex, which consists of CULLIN1, an RBX protein, an *Arabidopsis* S-phase kinase-related protein (ASK), and an F-box protein. The ASK protein functions as an adaptor to recruit proteins to the core complex that carry an F-box domain. Whereas the core complex is more or less conserved, domain composition of F-box proteins is highly variable. Attached to the F-box domain, respective proteins carry a multitude of protein–protein interaction domains, which mediate specific recognition, polyubiquitylation, and, as a consequence, degradation of target proteins.

In photoreceptors of the ZTL-like protein family, the KELCH repeat domain is involved in recognition and interaction with specific target proteins. The LOV domain functions as photosensory domain that alters ubiquitin ligase activity by modulating interaction with target proteins in a blue light-dependent manner. On light absorption, the LOV domain mediates interaction with GIGANTEA (GI) cofactor, an important positive regulator of **photoperiodic flowering** and the endogenous clock (Figure 9.7). Members of the zeitlupe-like protein family are specifically involved in the attenuation and synchronization of the circadian clock and the regulation of flowering by the photoperiod.

Photoperiodic flowering is often observed in ecosystems with pronounced seasonal changes, whereon detection of day length helps to synchronize reproductive development to time periods offering optimum environmental conditions. Plants of the Northern Hemisphere often belong to the group of long-day plants, which start flowering when nights become shorter in spring and early summer.

In contrast, flowering of short-day plants starts under conditions with extended dark periods during the day. Interaction between FKF1 and GI enables binding and polyubiquitylation of the CYCLING DOF FACTOR1 (CDF1) (Figure 9.7a). CDF1 is a transcription factor that represses expression of the *CONSTANS* gene, a central regulator of photoperiodic flowering in plants. Activation of FKF by light leads to the accumulation of CONSTANS during the light phase, which is an important signal to induce flowering in long-day plants or repress flowering in short-day plants.

The circadian oscillators of the endogenous clock depend on transcriptional feedback loops. TOC1 (TIMING OF CAB EXPRESSION 1) and its homologue PRR5 (PSEUDE-RESPONSE REGULATOR 5) are two factors that regulate the pace of one loop of the circadian clock in *Arabidopsis*. In contrast to the positive effect of GI on ubiquitin ligase activity of FKF1, blue-light-induced formation of SCF^{ZTL}/GI complexes inhibits interaction with target proteins (Figure 9.7b). Interaction between GI and ZTL also stabilizes the F-box protein. Because expression of *GI* is clock controlled, this interaction results in a diurnal accumulation of ZTL and diurnal variations in SCF^{ZTL}/GI ubiquitin ligase activity. Diurnal variations in SCF^{ZTL}/GI activity establish rhythmic accumulation of TOC1 and its homologue PRR5, which helps to synchronize and adapt the pace of the circadian oscillator in *Arabidopsis*.

9.3.4 White Collar Light Senosors

The **white collar 1** (**WC -1**) photoreceptor is a universal blue light receptor in many fungi. The photoreceptor was identified by a mutant in the ascomycete *Neurospora crassa* (*Sordariales*, *Ascomycota*) that was impaired in carotinoid biosynthesis in the mycelium, whereas conidia were still pigmented. Thus, mutant colonies showed a colorless border or "white collar." Light responses regulated by WC1 photoreceptor include phototropism, alterations in vegetative growth, sexual and asexual spore formation, and entrainment of the **circadian clock**.

WC-1 has three PAS (PER, period circadian protein/ARNT, aryl hydrocarbon receptor nuclear translocator protein/SIM, single-minded protein) domains that are followed by a GATA-like zinc finger transcription factor domain. The first PAS domain forms a LOV domain that binds flavin adenine dinucleotide (FAD) instead of FMN. The LOV domain is responsible for blue-light absorption and activation of photoreceptor function. The third PAS domain enables dimerization with **white collar 2** (**WC-2**). WC-2 shows high similarity to WC-1, but lacks a functional LOV domain. On light absorption the WC-1/WC-2 complex (WCC) forms heterotetrameric WCC–WCC complexes that directly bind to specific promoter elements in light-regulated genes. Binding to promoter sequences induces transcriptional activation of target genes (Figure 9.8).

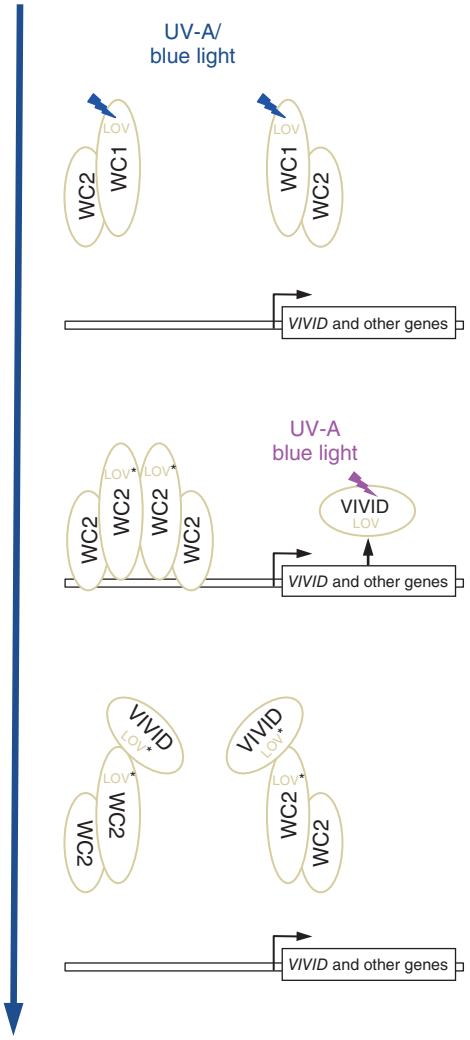

Figure 9.8 Schematic illustration of blue-light signaling mediated by white collar photoreceptors. White collar 1 (WC1) and WC2 heterodimers form heterotetrameric complexes (WCC) on light absorption by the LOV domain of WC1. WCC directly activate the transcription of light-regulated target genes. *VIVID* is among light-activated genes. Vivid functions as negative regulator of WC-dependent light signaling by its light-induced interaction with WCC heterodimers.

Vivid was also discovered in the fungus *N. crassa*. The photoreceptor mainly consists of just a LOV domain and functions as negative regulator of photoactivated WCC–WCC heterotetramers (Figure 9.7). The *VIVID* gene is up-regulated by WCC in the light, which leads to accumulation of the corresponding VIVID protein. On photoactivation of the LOV domain, VIVID interacts with WCC and disrupts the formation of transcriptionally active WCC–WCC heterotetramers. Because the expression of *VIVID* correlates with light intensities, the corresponding negative feedback loops are thought to enable photoadaptation toward strong continuous light.

9.4 Cryptochromes

9.4.1 Physiological Functions

The first cryptochrome blue-light photoreceptor, **cryptochrome 1** (**cry1**), has been isolated during the search for the mutated gene responsible for the elongated hypocotyl phenotype in the *hy4* (*elongated hypotcotyl 4*) mutant of *Arabidopsis.* The *hy4* mutant exhibited reduced light sensitivity with respect to **photomorphogenic seedling development** under continuous blue light. *Arabidopsis* **cryptochrome 2** (**cry2**) photoreceptor was identified by sequence homology to cry1. The physiological function of cry3, a third homologue, is not yet clarified. Mutant analyses revealed that cry1 and cry2 have overlapping but also distinct functions in the regulation of blue-light responses during plant development. Both photoreceptors differ with respect to light sensitivity: cry1 mainly regulates responses toward high-light intensities, whereas cry2 functions preferentially under low-photon fluence rates. cry1 and cry2 act redundantly in the control of the circadian clock, whereas cry2 has a more specific function in the regulation of photoperiodic flowering. Cryptochromes regulate blue-light-regulated gene expression and function as major blue-light receptors when the phyA photoreceptor is eliminated by a red-light pretreatment (see Section 9.6.).

Cryptochrome-like proteins could be identified in nearly all groups of eukaryotic organisms and in some bacteria. They also adapted toward additional tasks that are no longer linked to blue-light sensing. In pigeons (*Columbidae*, *Aves, Sauropsida*), cryptochrome-like proteins seem to function as magnetosensors, which enable correct navigation with respect to the terrestrial magnet field. For this purpose, proteins must be kept in an excited state by the absorption of blue light. In mammals and humans, crytopchrome-like proteins are internal components of the circadian clock, but no longer function as photoreceptors.

9.4.2 Cryptochrome Structure and Light Signaling

The n-terminal part of cry1 and cry2 shows high similarity to **photolyases**, which repair cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone adducts formed following exposure of DNA to UV light. Photolyases use light absorbed by its FAD cofactor or an additional antenna pigment like methenyltetrahydrofolate (MTHF) to enable an electron transfer and, as a consequence, reverse cross-linking between DNA bases. Photolyase-homologous domain (PHR) of cryptochromes also posseses an FAD chromophore and, most probably, MTHF as additional antenna pigment. Together, these pigments are responsible for the absorption of blue (FAD) and UV-A light (MTHF).

Despite its similarity and the presence of both chromophores, cryptochromes do not exhibit DNA repair function. On absorption of a blue-light photon the oxidized FAD ground state forms an $FADH^{\bullet}$ radical that leads to the formation of the active conformation of the photoreceptor (Figure 9.1). On absorption of green light the $FADH^{\bullet}$ radical is reduced to $FADH^{-}$. Photoconversion to $FADH^{-}$ stops light signaling and enables reoxidation to FAD in darkness. Thus, the redox-based cycling process causes accumulation of the physiologically active conformation of the photoreceptors under continuous light, but leads to rapid inactivation in darkness in the range of minutes. A major difference between photolyases and cryptochromes is the presence of a so-called cryptochrome c-terminal extension (CCE; Figures 9.3, 9.9). The CCE is highly variable in size (eighty to several hundred amino acids) and is only poorly conserved between different cryptochromes. The domain is essential for blue-light signaling and, thus, most probably functions as the output domain of the photoreceptor.

Signaling downstream of cryptochrome photoreceptors in plants shows some similarities to UVR8 (see Section 9.2.1). Cry1 modulates the activity of COP1-ULCs via its blue-light-dependent interaction with SPA1 and, most probably, SPA4. Interaction between the CCE1 domain of cry1 and the WD40 repeat domain of SPA1 connects both proteins and reduces binding of SPA1 to COP1 (Figure 9.9a). Destabilization of COP1-SPA1 interaction is thought to counteract the degradation of the HY5 transcription factor, which should result in the accumulation of this important positive transcriptional regulator of *Arabidopsis* photomorphogenesis. A similar mechanism has been proposed for the transmission of blue light sensed by cry2 toward the regulation of photoperiodic flowering. Several data demonstrated that COP1 and SPA1 are required for the degradation of CONSTANS in darkness and that cry2 counteracts this process. Cry2 also interacts with SPA1 in a blue-light-dependent manner, but, in contrast to cry1, interaction takes place between the PHR domain of the photoreceptor and the KRD of the COP1-ULC component (Figure 9.9b). These blue-light-dependent changes are thought to block CONSTANS polyubiquitylation and, thus, enable accumulation of the protein to regulate transition to flowering.

The different modes of interaction with COP1-ULCs might also be related to differences in light stability and nuclear localization between cry1 and cry2. On absorption of blue light, cry2 becomes ubiquitylated and rapidly degraded in a COP1-dependent manner, whereas cry1 is more stable. Nuclear accumulation of cry2 is light-dependant, whereas cry1 seems to be constitutively localized into the nucleus.

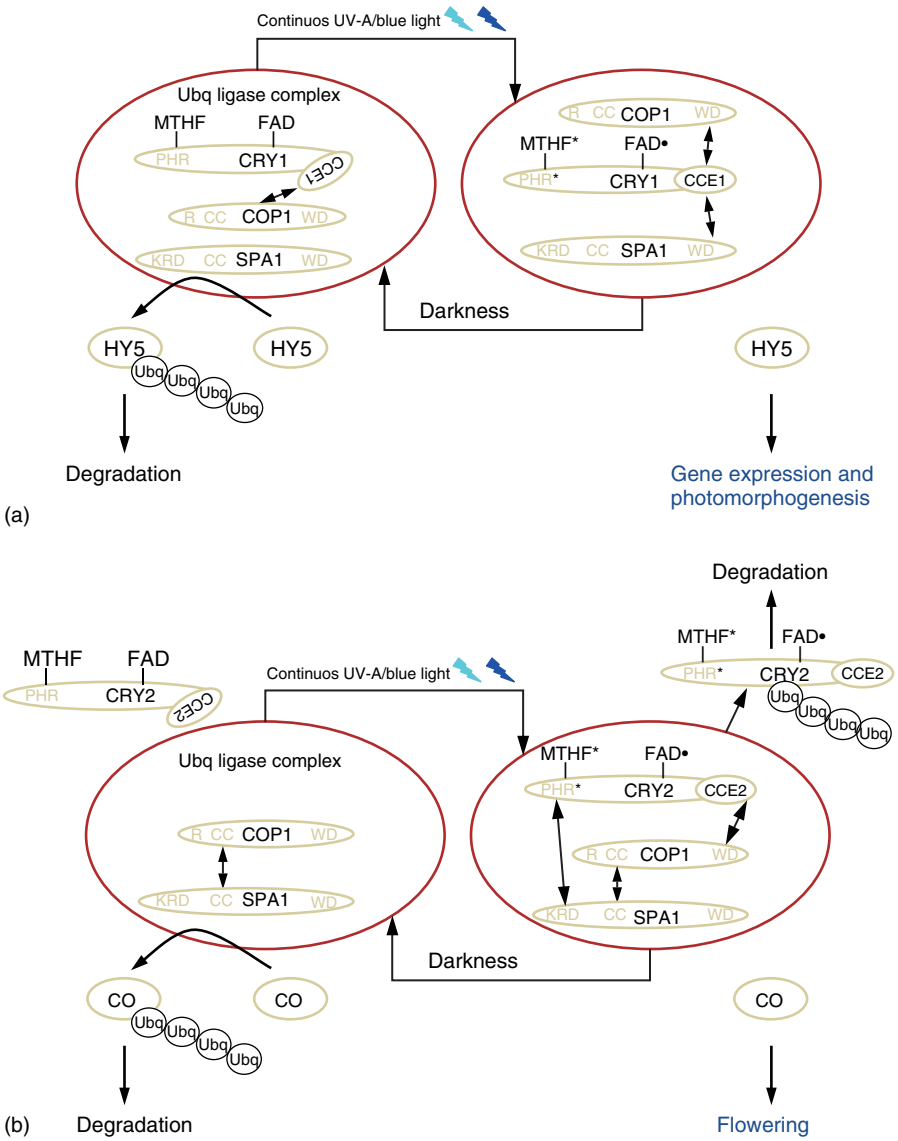

Figure 9.9 Schematic illustration of cryptochrome-dependent blue-light signaling. (a) Signaling downstream of the cryptochrome 1 (cry1) photoreceptor. (b) Signaling downstream of the cryptochrome 2 (cry2) photoreceptor. The oval represents COP1-containing ubiquitin ligase complexes. CC – coiled-coil domain; CCE – cryptochrome c-terminal extension; CO – CONSTANS transcriptional regulator; COP1 – CONSTITUTIVE PHOTOMORPHOGENIC 1; FAD – flavin adenine dinucleotide; FADH – radical-enhanced state of the FAD chromophore; HY5 – ELONGATED HYPOCOTYL 5 transcription factor; KRD – kinase-related domain; MTHF – methenyltetrahydrofolate MTHF* – MTHF excited by UV-A light; PHR – photolyase-related domain; R – RING-finger domain; SPA – SUPPRESSOR OF *phyA-105*; Trp – tryptophan residues; Ubq – UBIQUITIN; WD – WD40 repeat domain.

9.5 Phytochromes

9.5.1 The Light Sensor Module of Phytochromes

Phytochromes are photoreceptors that sense light with **linear tetrapyrrole** (**bilin**) chromophores such as phytochromobilin (PCB), phycocyanobilin, or biliverdin IXa. Phytochromes were first identified in plants because of photoreversibility of many plant light responses, which can be induced by red- and inhibited by far-red-light pulse treatments. Later on, phytochrome-related sensors were identified in a broad range of organisms, including green algae, cyanobacteria, nonoxygen-evolving photosynthetic bacteria and even in nonphotosynthetic bacteria and some fungi.

The n-terminal light sensor module of eukaryotic and many procaryotic phytochromes consist of four different domains: a variable n-terminal domain, the PAS-N domain, the GAF domain (cGMP-stimulated phosphodiesterase/*Anabaena* adenylate cyclase/*E. coli* FhlA), and the

PHY (phytochrome) domain (Figure 9.3). The corresponding light sensor module is also present in neochromes. PCB and phycocyanobilin chromophores of plant and many cyanobacterial phytochromes are linked to GAF subdomains by a conserved cysteine residue. Biliverdin chromophores found in many bacterial phytochromes and in fungi are attached to a cysteine moiety at the n-terminal subdomain. Except for the common PAS/GAF/PHY architecture, sensor domains of some prokaryotic phytochromes only consist of GAF/PHY or even one or more single GAF domains.

Phytochromes with PAS/GAF/PHY or GAF/PHY architecture exist in two stable conformers with different but overlapping absorption spectra (Figure 9.3): the **Pr-form** (P = phytochrome; r = red) with maximum absorption in the red light and the **Pfr-form** (fr = far-red) with maximum absorption in the far-red light. Most phytochromes accumulate in the Pr conformation in darkness and need light to be activated. Therefore, Pr is regarded as the physiological inactive conformation. On light absorption, the bilin chromophore undergoes a photoreversible Z/E photoisomerization at a double bond of a methin bridge linking pyrrole ring C and D of the chromophore (Figure 9.1). The isomerization causes major conformational changes in the sensor module leading to the formation of the metastable Pfr conformation. Pfr can be photoconverted into the inactive Pr-form on absorption of far-red light. Pfr/Pr photoconversion is the reason for the classical red/far-red photoreversibility of many plant light responses. The Pfr-form can fall back into the thermodynamically more stable Pr configuration, a process called *dark-reversion*. Thermodynamic relaxation to Pr competes with photoconversion at low-light intensities and inactivates the photoreceptor in darkness.

According to their photochemical properties, most phytochromes are adapted to sense red and far-red light. However, some phytochrome-mediated light responses can also be induced by blue and UV-A light because of the broad absorption spectrum of bilin chromophores. Instead of red/far-red photoreversibility, some **cyanobacterial phytochromes** (**cyanobacteriochromes**) are able to function as red/green or blue/green photoreversible photoreceptors. Red/green and blue/green photoreversible phytochromes carry one or more GAF domains that bind to PCB or phycocyanobilin chromophores but often lack adjacent PAS-N or PHY domains.

9.5.2 Physiological Functions of Phytochromes

Even though phytochrome is often described as red/far-red-controlled light switch, it should be mentioned that photoreversibility by pulse treatments are accomplished only under nonnatural laboratory conditions. Actually, the overlapping absorption spectra of Pr and Pfr are perfectly adapted to the sense enrichment of far-red light in the canopy shade or under a layer of competing photosynthetic organisms. Below leaf canopy, light is enriched in wavelengths, which are not absorbed by photosynthetic pigments such as chlorophylls and carotinoids. Whereas blue and red light are predominantly absorbed by photosynthetic antenna pigments, green light and, to an even higher extent, far-red light, can easily pass through photosynthetic tissues. Because red light is mainly absorbed by the Pr form, high levels of Pfr are formed in sun light reaching relative Pfr levels (Pfr/[Pr + Pfr]) of about 55–60%. In contrast, far-red light enriched in canopy shade converts most of Pfr back to inactive Pr. Thus, even subtle changes in the red or far-red content of light can easily be transmitted into strong changes in Pr : Pfr ratios of phytochromes.

Changes in Pr : Pfr ratios regulate **chromatic adaption** processes in algae and cyanaobacteria. Chromatic adaptation causes altered composition of photosynthetic antenna complexes mainly formed by phycobilisomes. Phycobilisomes consist of phycoreythrin, phycocyanin, and allophycocyanin antenna proteins that carry phycoerythrobilin and phycocyanonbilin. These bilin pigments can transmit energy from the green and orange part of light spectra to the photosystems of the photosynthetic machinery. It should be mentioned that some cyanobacteriochromes exhibit green/red or blue/green photoreversibility, which would perfectly fit to a regulatory function during chromatic adaptation.

An analogous adaptation process in higher plants is the **shade avoidance syndrome** (**SAS**), which becomes induced by the growth in the canopy shade of plant competitors. Among other effects, SAS is accompanied by elongated petioles, increased apical dominance, and enhanced shoot growth. Shaded leaves normally posses smaller and lower numbers of palisade cells. Chloroplasts in shaded leaves carry a higher proportion of antenna pigments and reduced levels of Calvin cycle enzymes. Their thylakoid membranes are enriched for photosystem II that functions less efficient in far-red light compared to photosystem I. Regulation of SAS is carried out mainly by PHYTOCHROME-INTERACTING FACTORS and type-II phytochromes (see Section 9.5.3).

Phytochromes became very important and potential photoreceptors in plants. Besides SAS, phytochromes regulate nearly all aspects of plant development, including light-induced germination, the switch between skotomorphogenic and photomorphogenic seedling development (Box 9.2; Figure 9.4), and transition to flowering and setting of the circadian clock. All analyzed species of higher plants posses at minimum three different **classes of phytochromes**: **phytochrome A** (phyA), **phytochrome B** (**phyB**), and phytochrome C (phyC). The phyB-type of phytochromes is often represented by two or more genes in the genome of many plant species. Because phyB- and phyC-types of photoreceptors are more stable in the light compared to phyA, these classes were subsumed as **light-stable** or **type-II phytochromes**. PhyA and phyB dominate

light responses in higher plants and have overlapping as well as different functions in light regulation.

At the transition from darkness to light, phyA and phyB are able to induce rapid, very sensitive light responses, which help to adapt plant metabolism at the onset of light. Sensitive light responses can be attributed to the accumulation of photoreceptor molecules in darkness, especially for phyA. These inductive, early light responses are subsumed as **very low-fluence responses** (**VLFRs**) in the case of phyA and **low-fluence responses** (**LFRs**) in the case of phyB. VLFR can be induced by red-light photon fluences between 0.001 and 1 µmol m^{-2}, which can be reached in the beam of a standard red-light laser pointer (λ_{max} ~650 nm; output power ~1 mW) at about 5–20 ns. LFR of phyB-type phytochromes can be induced by photon fluences between 10 and 1000 µmol m^{-2} of red light. These LFR follow the classical red/far-red photoreversible light responses in plants, whereon VLFR are sensitive enough to become induced even by far-red light.

In addition, phyA and phyB-type phytochromes are able to control so-called **high irradiance responses** (**HIRs**). Establishment of HIR needs prolonged irradiation with high-light intensities for full expression. The magnitude of HIR depends on the photon fluence rate. Thus, the underlying mechanism is adapted to determine the amount of photons that enter the tissue in a given time period. There are several indications that extent of HIR is triggered by the amount of phytochromes transferred into the nucleus by continuous light (see Section 9.5.3). As phyB is more stable under strong continuous red light, this type of photoreceptors is well adapted to trigger HIR under the corresponding wavelengths and in white light. In contrast, phyA functions as far-red light photoreceptor that controls far-red HIR with maximum light sensitivity at about 720 nm.

Phytochromes are not limited to photoautotrophic organisms. In nonphotosynthetic bacteria, photoreceptors seem to be involved in adaptation toward deleterious high-light intensities. They either induce biochemical protection mechanisms or movement processes by the alteration of flagella rotations. Regulation of flagella motility is the only known example for direct regulation of movement processes by classical phytochromes that carry a **histidine kinase domain** (**HKD**) and do not belong to the group of neochrome photoreceptors. In some fungi, phytochromes are shown to regulate the switch between asexual and sexual development.

9.5.3 Light Signal Transduction of Phytochromes

Native phytochromes normally form homodimers, which can be attributed mainly to the function of the c-terminal half of the protein. The c-terminal half of most prokaryotic phytochromes carry a functional HKD, which is involved in light signaling. The HKD transfers a phosphate from ATP to a histidine residue of its dimeric partner on light absorption leading to intrinsic autophosphorylation. The phosphate moiety of the HKD can be transferred to an aspartate residue of so-called **receiver domain proteins** or response regulators. Receiver domain proteins directly or indirectly alter the motion of flagella or regulate the transcription of target genes, which then regulate downstream light responses.

Plant phytochromes carry a histidine kinase-related domain (HKRD) at their c-terminus, but they do not posses histidine kinase activity. Analyses for kinase activities with purified plant phytochromes showed that the protein undergoes autophosphorylation and phoshorylates artificial target proteins and PHYTOCHROME KINASE SUBSTRATE 1. Plant phytochromes might function as serine/threonine kinases, but the functional relevance in light signaling is still under debate. The HKRD is dispensable for phyB function. In the case of phyA the HKRD domain seems to be necessary for the attenuation of some phyA responses.

Compared to prokaryotic phytochromes, signal transduction of plant phytochromes is more complex. Nuclear localization of phytochromes is essential for light-regulated gene expression and expression of respective light responses. Microscopic analyses revealed that *Arabidopsis* phyC, phyD, and phyE are constitutively localized to the nucleus, whereas nuclear accumulation of phyA and phyB is light-dependent (Figures 9.10, 9.11). A low amount of phyB was already detected in the nuclei of dark-grown *Arabidopsis* seedlings, but the level of the photoreceptor became strongly enhanced on red-light irradiation, though not with far-red light. Even though it cannot be excluded that phyB carries an intrinsic nuclear localization sequence (NLS), some findings indicate that the photoreceptor is cotransported on binding to **PHYTOCHROME-INTERACTING FACTORS** (**PIFs**) in its Pfr form (Figure 9.10). Thus, NLS is provided by proteins other than phyB.

PIFs belong to a subfamily of basic helix-loop-helix (bHLH) transcription factors, which specifically bind to a DNA G-box motif (–CACGTG–) often found in promoters of light-regulated genes. Interaction with phyB Pfr was obtained with PIF1, PIF3, and PIF4 to PIF8 that all carry an active phyB-binding motif (ABP) (Figure 9.10). Interaction with phyA is restricted to PIF1 and PIF3 (Figure 9.11). Interaction with phyB seems to release PIF transcription factors from binding to promoter sequences, which offers a direct mechanism to regulate gene expression. With the exception of PIF7, light-induced binding of PIFs to phyA or phyB stimulates rapid phosphorylation of the bHLH proteins followed by their rapid ubiquitylation and degradation in the 26S proteasome with half lives of ~5–20 min. Intriguingly, kinases other than phytochromes are responsible for the phosphorylation of PIFs. Further data indicate that COP1-containing E3 ubiquitin ligases are not directly involved in PIF polyubiquitylation and degradation.

The *pif1 pif3 pif4 pif5* quadrupel mutant exhibits a Cop phenotype in darkness providing clear evidences

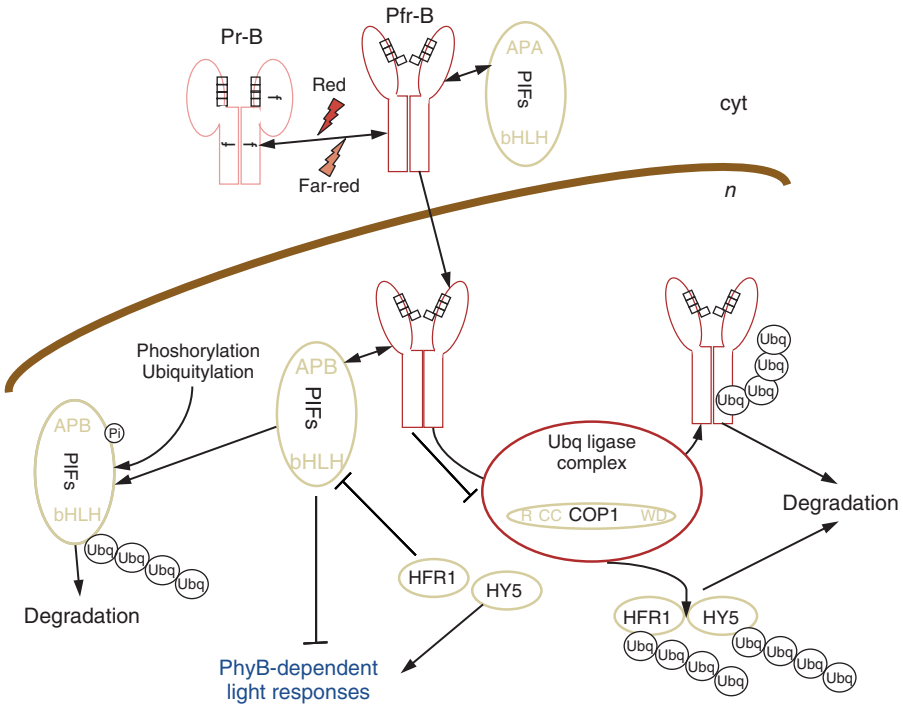

Figure 9.10 Schematic illustration of phytochrome B-dependent red-light signaling. The oval represents COP1-containing ubiquitin ligase complexes. The phytochromobilin chromophore is indicated by four rectangles. T-lines symbolize inhibitory interactions. APB – active phyB-binding motif; bHLH – basic helix-loop-helix domain; CC – coiled-coil domain; COP1 – CONSTITUTIVE PHOTOMORPHOGENIC 1; cyt – cytoplasm; HFR1 – ELONGATED HYPOCOTYL IN FAR-RED 1 transcripton factor; HY5 – ELONGATED HYPOCOTYL 5 transcription factor; KRD – kinase-related domain; n – nucleus; Pfr – far-red-light-absorbing form of phyB; PIF – PHYTOCHROME-INTERACTING FACTOR; Pi – phosphate group; Pr – red-light-absorbing form of phyB; R – RING-finger domain; Ubq – UBIQUITIN; WD – WD40 repeat domain.

that PIFs suppress photomorphogenesis and promote skotomorphogenesis of *Arabidopsis* seedlings. Mutant analyses further demonstrated that PIF proteins act as positive regulators promoting the SAS. Data also reveal a strong interference between PIF transcription factors and the function of important plant hormones (see Section 7.3), namely, abscisic acid as negative and gibberellins and auxin as positive regulators of plant photomorphogenesis and SAS.

Interestingly, the bHLH protein HFR1 (ELONGATED HYPOCOTYL IN FAR-RED 1) and related proteins do not bind to phytochromes, but form heterodimers with PIF proteins (Figure 9.10). Because heterodimers are often transcriptionally inactive, this mechanism offers an additional positively acting regulatory loop by the inhibition of PIF proteins. Furthermore, PIF transcription factors are inactivated by the binding of DELLA proteins, important negative regulators in the signaling pathway of GA. As DELLA proteins become polyubiquitylated and degraded in the presence of GA, this mechanism offers a further possibility to integrate light and hormone signaling in plants.

Phytochromes also can mediate positive light effects by the inhibition of COP1-ULCs, which are involved in polyubiquitylation and, thus, degradation of many positively acting transcription factors. As a consequence, accumulation of positively acting transcription factors like HY5, HFR1 and the MYB transcription factor LAF1 (LONG AFTER FAR-RED 1) is achieved (Figures 9.10 and 9.11). As mentioned before, HY5 seems to function as a positive effecter downstream of all photoreceptors. In contrast, LAF1 seems to be specifically involved in the regulation of phyA-dependent far-red high irradiance response (FR-HIR). HFR1 functions not only as a positive regulator of FR-HIR, but also as a negative regulator of SAS. Data show that COP1 is exported from the nucleus in a phytochrome-dependent manner, which is thought to block its function as a negative regulator of light responses. Nuclear exclusion of COP1 is a slow process that might not be prompt enough to explain the observed more rapid stabilization of the corresponding transcription factors on light treatments. PhyA and phyB directly interact with COP1, and hence it is worthwhile to speculate that phytochromes regulate COP1-ULCs in mechanisms similar to UVR8 and cryptochromes.

9.5.4 The Unique Function of Phytochrome A

The phyA photoreceptor has several unique features compared to other members of the phytochrome family in plants. It accumulates to high levels in darkness, reaching about 100–1000 × more molecules per cell compared

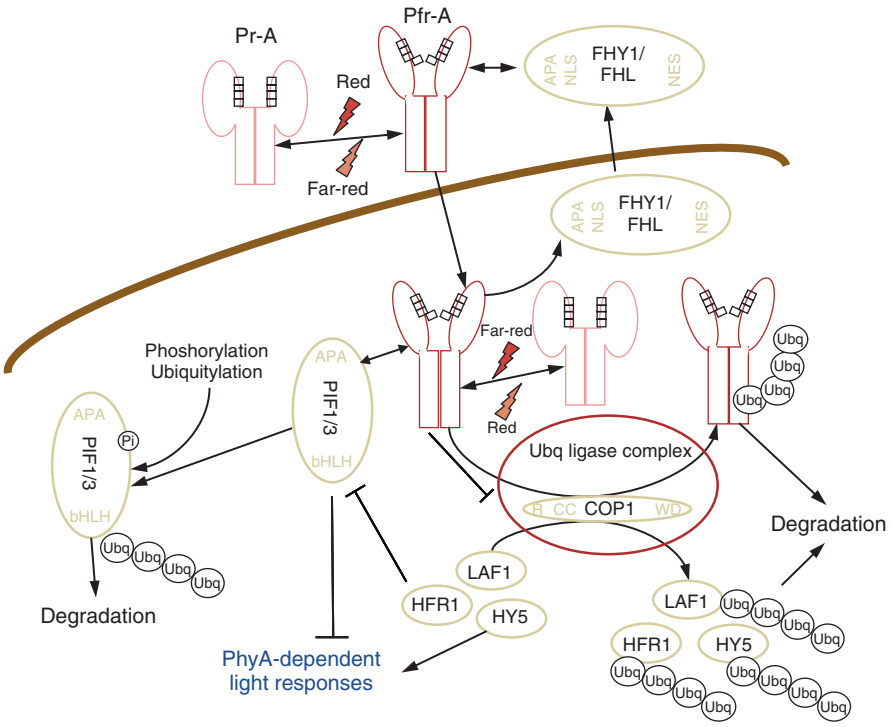

Figure 9.11 Schematic illustration of phytochrome A-dependent far-red-light signaling. The oval represents COP1-containing ubiquitin ligase complexes. The phytochromobilin chromophore is indicated by four rectangles. T-lines symbolize inhibitory interactions. APA – active phyA-binding motif; bHLH – basic helix-loop-helix domain; CC – coiled-coil domain; COP1 – CONSTITUTIVE PHOTOMORPHOGENIC 1; FHL – FHY1-like; FHY1 – FAR-RED ELONGATED HYPOCOTYL 1; HFR1 – ELONGATED HYPOCOTYL IN FAR-RED 1 transcripton factor; HY5 – ELONGATED HYPOCOTYL 5 transcription factor; KRD – kinase-related domain; LAF1 – LONG AFTER FAR-RED 1 transcription factor; NLS – nuclear localization sequence; NES – nuclear export sequence; Pfr – far-red-light-absorbing form of phyA; PIF – PHYTOCHROME-INTERACTING FACTOR; Pi – phosphate group; Pr – red-light-absorbing form of phyA; R – RING-finger domain; Ubq – UBIQUITIN; WD – WD40 repeat domain.

to phyB. High phyA levels in dark-grown seedlings and plants enable sensing of very low amounts of light-inducing VLFR. High amounts of phyA lead to a dominant effect of the photoreceptor on light-dependent gene expression at the onset of light. Nevertheless, the dominant function of phyA is most probably obstructive during late light signaling, because it would overwhelm responses downstream of other photoreceptors. Thus, phyA becomes rapidly degraded on application of red light adjusting high levels of Pfr (half life ~15 min). Compared to other plant phytochromes, degradation results in a nearly complete removal of the photoreceptor under continuous light. In addition, phyA also forms sequestered areas of phytochrome (SAP) in the cytoplasm. SAP formation occurs in the range of seconds under light conditions forming high levels of Pfr. Because phyA function is related to its nuclear accumulation, formation of these aggregates provides a further mechanism to suppress excessive red light responses.

A very unique feature of phyA is the ability to function as a far-red light receptor with maximum light sensitivity at about 720 nm. The phyA-type phytochrome induces FR-HIR even though maximum light responses of phytochromes are expected in red light that forms maximum levels of Pfr (Figure 9.3). Several complex processes have been evolved to enable the detection of far-red light. First, phyA function in continuous red light becomes inactivated by SAP formation in the cytoplasm and, second, by rapid degradation of the Pfr form. Third, as phyA degradation rates are Pfr-dependent, steady state levels of the photoreceptor remain high in far-red light and even stay constant at wavelengths above 720 nm. Consequently, absolute Pfr levels stay high in far-red light even under light conditions that only photoconvert about 1–5% of total phytochrome into the active conformation. Finally, phyA is the target of a complex import process that leads to the accumulation of extraordinary high photoreceptor levels in the nucleus under continuous far-red light by a shuttle mechanism.

Nuclear accumulation is triggered by two nuclear transport facilitators, FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and/or FHY1-LIKE (FHL). FHY1 and FHL posses a nuclear import sequence (NLS), a nuclear export sequence (NES), and a phyA-interaction domain, which specifically interacts with the Pfr form of the photoreceptor (Figure 9.11). FHY1/FHL interact with phyA-Pfr in the cytoplasm to enable the nuclear import of the active photoreceptor by a cotransport mechanism. Because phyA can cycle between the Pfr and Pr-forms and because Pr does not bind to FHY1/FHL, the photoreceptor is thought to be released in the nucleus on photoconversion back to

Pr. Whereas imported phyA remains in the nucleus, the NES of transport facilitators leads to the translocation back into the cytoplasm where they can induce the next round of nuclear import. Because cycling between Pfr and Pr is rather high at wavelengths around 710–720 nm, high levels of phyA are expected to accumulate in the nucleus under far-red light.

9.6 Other Photoreceptor Systems

In addition to photoreceptor systems described above, nature has evolved several other groups of light-detecting molecules during evolution. The BLUF domain (blue-light-utilizing FAD domain) is another light-sensing module that enables light responses toward blue light mainly in procaryotes. On light absorption, the FAD chromophore is thought to accept an electron from an adjacent tyrosine residue leading to radical formation and conformational changes of photoreceptors. The AppA (activation of photopigment and *puc* expression A) is a well-analyzed photoreceptor system that regulates the accumulation of photosystems and antenna in the purple bacterium *Rhodobacter sphaeroides* (*Alphaproteobacteria*). Blue light and oxygen reduce photosystem synthesis most probably to avoid photodamage. AppA functions both as light and oxygen sensor. Under semiaerobic conditions, light activation of AppA causes dissociation from a transcriptional repressor (PpsR), which causes downregulation of photosynthetic gene transcription. The *Escherichia coli* (*Gammaproteobacteria*) BLUF receptor antagonizes the function of a transcription regulator (BluR) by heterodimer formation. Light absorption by BluF leads to a release of BluR that positively regulates biofilm formation (see Chapter 14). Other BLUF domain proteins function by mediating second messenger accumulation. Photoavoidance response in *Euglena gracilis* (*Euglenida, Euglenozoa*) is regulated by a complex of photoactivated adenyl cyclases α and β (PAC-α/β). PAC-α/β are the only known BLUF proteins in an eucaryote. The *Rhodopseudomonas palustris* (*Alphaproteobacteria*) BLUF photoreceptor PapB enhances the activity of a cyclic dimeric GMP-specific phosphodiesterase (PapA) to negatively regulate biofilm formation.

Photoactive yellow proteins (**PYP**) are bacterial blue-light photoreceptors with a PAS domain. PYP was first described in the halophilic purple photosynthetic bacterium *Halorhodospira halophile* (*Gammaproteobacteria*). The light-induced photocycle is triggered by photoisomerization of a *trans-p*-coumaric acid chromophore. Absorption of blue light leads to the formation of *cis-p*-coumaric acid, which becomes protonated to form the active configuration of the photoreceptor. PYPs seem to be involved in the regulation of negative phototaxis and accumulation of photoprotective compounds. PYP domains are found to be fused to guanylate cyclases, phosphodiesterases, and bacteriophytochromes with histidine kinase domains (see Section 9.5).

Rhodopsins were first identified as photoreceptors in animal visual systems, and then integrated to regulate light responses in many microorganisms and algae. Rhodopsins carry a retinal chromophore that is covalently linked to a lysine residue as a protonated Schiff base. Absorption of blue light leads to isomerization from the *all-trans* to the *cis* conformation of the retinal moiety and, as a consequence, to conformational changes in the apoprotein. Rhodopsins belong to the group of seven transmembrane proteins. Thus, in contrast to most photoreceptors, rhodopsins are normally localized in membranes. Some types of rhodopsin photoreceptors function as light-gated channels that regulate ion fluxes over membranes. In the archaeal halobacteria (*Euryarchaeota*) the related bacteriorhodopsin absorbs light using a retinal, leading to proton export and formation of a proton motive force (see Section S1.2.12.1). The chromophore-binding domain is also linked to histidine kinase domains, response regulator domains, and adenylate/guanylate cyclase effector domains.

9.7 Flavonoid Biosynthesis in Plants – a Model for a Light-Regulated Adaptation Process

9.7.1 Flavonoids as Light Protectors

Flavonoids are aromatic products of the plant secondary metabolism (see Chapter 2) that are synthesized in a branched pathway starting from malonyl-CoA and 4-Cumaroyl-CoA (Figure 9.12a). Flavonoid pigments function as allelochemicals (see Chapter 15) and phytoalexins, attract animals, are involved in hormone signaling (see Chapter 5), stimulate pollen tube growth, and function in protection against light stresses. Because of their important functions, their easy detection due to their colored appearance and the strong light response of structural genes, flavonoid biosynthesis became a well-established model to study gene expression and the regulation of light responses.

Because of light absorption in the UV-A and UV-B range of the spectrum, **flavonols** most probably function as sun screens against the damaging effects of these light qualities in *Arabidopsis* and many other plant species. The hypothesis is further underlined by studies that demonstrate that *Arabidopsis* mutants with reduced flavonoid content exhibited increased sensitivity toward UV light. **Anthocyanins** absorb light mainly in the visible range of the spectrum, not in the UV. Therefore, anthocyanins are thought to protect the photosynthetic machinery against photoinhibition and photobleaching under high-light intensities. This hypothesis agrees with the formation of "juvenile" anthocyanin during the early photomorphogenic

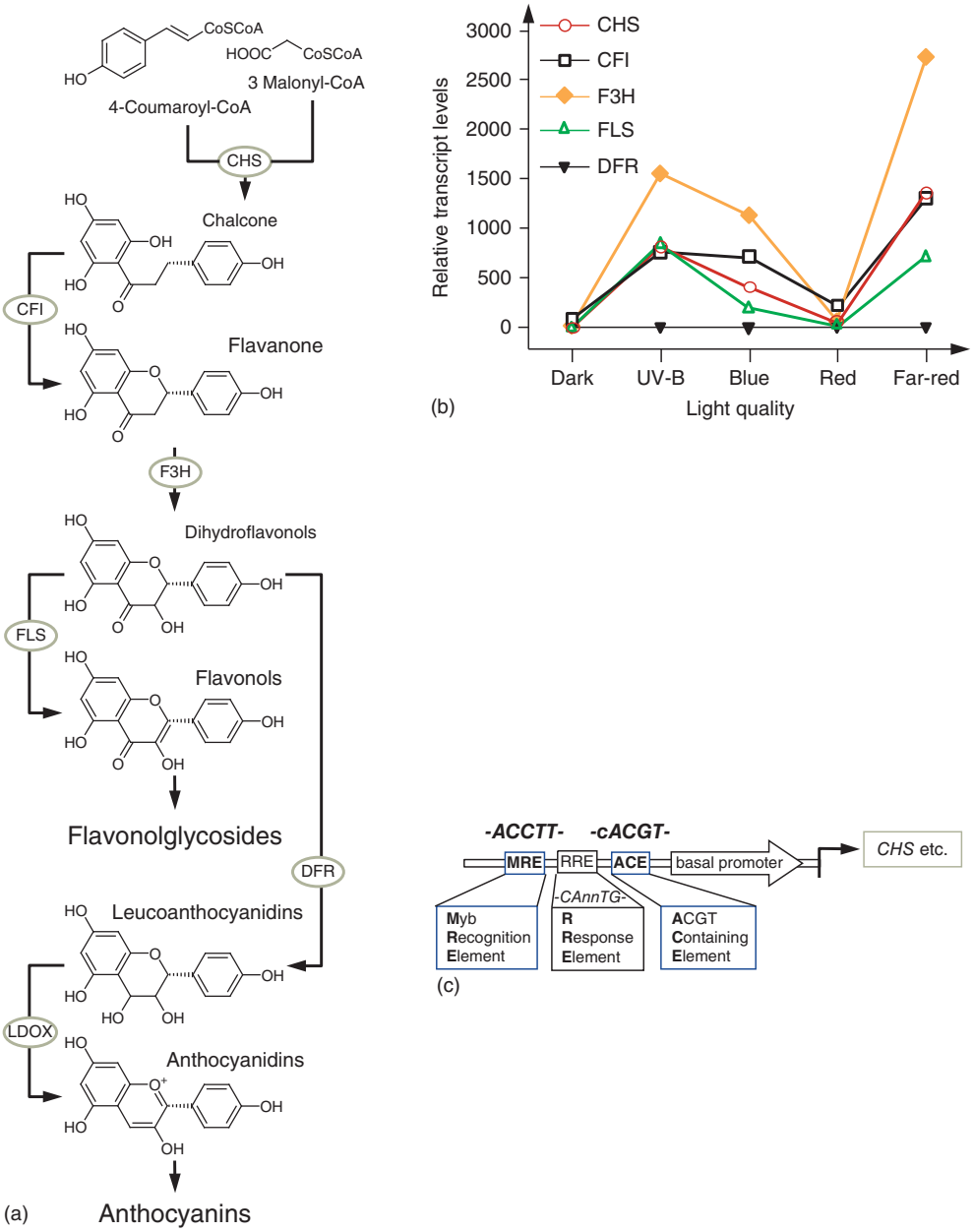

Figure 9.12 Light regulation of the flavonoid biosynthetic pathway. (a) Simplified, schematic overview of flavonoid biosynthesis in *Arabidopsis* seedlings. Enzymes involved in biosynthesis are boxed. CFI – chalcone flavone isomerase; CHS – chalcone synthase; DFR – dihydroflavonol 4-reductase; F3H – flavanone 3β-hydroxylase; FLS – flavonol synthase; LDOX – leucoanthocyanidin dioxygenase. (b) Light-induced transcript accumulation under different light qualities. *Arabidopsis* seedlings were grown in darkness for 4 days before exposure to a UV-A/B pulse (UV-B) or continuous irradiation by the indicated light qualities. Samples were harvested for total RNA extraction at 4 h after the onset of light treatment together with the dark control. Data show results of the light series of AtGenExpress microarray (*http://jsp.weigelworld.org/expviz/ expviz.jsp*). (c) Schematic representation of important promoter elements mediating light regulation of genes involved in flavonoid biosynthesis. Localization and sequences of regulatory elements are indicated.

seedling development, and in young leaves when newly formed, unbound chlorophyll molecules have a high potential to form radicals damaging developing chloroplasts. The function as protective light shields fits to the observation that glycosylated forms of flavonoids accumulate mainly in the vacuoles of leaf epidermal cells.

9.7.2 Light Regulation of the Flavonoid Biosynthesis Pathway

Many studies clearly indicate that flavonoid biosynthesis is mainly regulated on the level of gene expression. Genes for enzymes involved in flavonol biosynthesis exhibit a high degree of coregulation in *Arabidopsis* seedlings and most analyzed plant systems under diverse light regimes

(Figure 9.12b). Genes involved in flavonol biosynthesis also exhibited very similar transcript accumulation patterns toward different light qualities. Analyses with photoreceptor mutants of *Arabidopsis* demonstrated that phyA and crytochromes seem to be involved in the regulation of blue-light responses, whereas continuous far-red light and UV-B light are sensed by phyA and UVR8, respectively.

UV-B light-induced up-regulation of flavonol biosynthesis is most easily explained by the sun screen function of the pigments. Immediate response to even low doses of UV-B might not be sufficient for the full protection of plant tissues, because expression of respective enzymes takes several hours whereas exposition toward UV-B varies in minutes because of the sudden changes in clouding or shading by other plants. Presence of blue light is a good indicator for the lack of canopy shade and, thus, possible exposure to strong sun light. Blue-light-induced accumulation of flavonols should enable preadaptation to damaging doses of UV-B even in the absence of the respective light quality.

High far-red light content is normally regarded as an indicator for growth under canopy shade or in the soil and, thus, under conditions with low exposure to UV light. Thus, strong induction of flavonol biosynthesis by far-red light seems to waste the limited resources of young seedlings. Nevertheless, it has to be taken into account that young seedlings are normally first exposed to far-red light because of the growth in canopy shade or soil before reaching UV-containing sun light at the later stages. Far-red-light-induced expression of genes involved in flavonol biosynthesis enables efficient preadaptation before exposure to UV-B stress.

Flavonol synthase (FLS) and dihydroflavonol 4-reductase (DFR) are two key enzymes at a branching point of the flavonoid biosynthetic pathway (Figure 9.12a). Expression of FLS leads to the accumulation of flavonols, whereas DFR enzymatic activity is the first step in anthocyanin accumulation. Data presented in Figure 9.10b shows coexpression of *FLS* together with chalcone synthase (CHS), chalcone flavone isomerase (CFI), and F3H in 4-day-old etiolated *Arabidopsis* seedlings, which are in accord to findings that anthocyanins are not formed at this developmental stage. Analyses with younger seedlings exhibited clear light-induced accumulation of anthocyanins and coordinated expression of *DFR* and genes encoding for upstream components of the biosynthetic pathway. These observations favor a model, in which temporal and spatial competence patterns are created by endogenous factors, whereas light rather functions as a release factor that enables the expression of these patterns. Developmentally controlled factors even decide about photoreceptor usage: Phytochrome systems are most important for the expression of anthocyanin biosynthesis genes during the early stages of seedling development in many plants, whereas cryptochrome systems and UV-B receptors dominate light regulation at the later stages and in adult plants.

So far, little is known about the multiple steps that link between light perception by a specific photoreceptor and expression of a light-regulated gene in higher plants. Promoter studies helped to identify minimal DNA elements that enable light- and tissue-specific gene expression similar to full-length promoters of *CHS* (*CHALCONE SYNTHASE*) and related genes. Respective light-regulated units (LRU) in the promoters include a MYB DNA-recognition sequence (MRE; 5′-ACCTA-3′) and an ACGT-containing element (ACE; 5′-CACGT-3′) often supplemented with R response elements (RRE; –CANNTG-) or even G-box sequences (-CACGTG-) (Figure 9.12c).

The MYB-binding site of the LRU is recognized by several R2R3-MYB proteins. R2R3-MYB proteins are thought to be a prerequisite for light-regulated gene expression rather than being directly involved in light responses. Nevertheless, light seems to function more indirectly by influencing the expression of MYB transcription factors, and LAF1/MYB18 specifically functions in phyA-dependent signaling under FR-HIR conditions. ACE elements are normally recognized by bZIP transcription factors, including HY5. Analyses with *HY5* mutants demonstrated that the bZIP transcription factor is absolutely necessary for the light-induced expression of structural genes and functions as the positive regulator of the flavonoid biosynthesis pathway downstream of phyA, cryptochromes, and UVR8. RRE elements and G-boxes are shown to bind bHLH transcription factors, including PIFs. Analyses clearly show that PIF3 functions as positive regulator of genes involved in light-induced anthocyanin biosynthesis during *Arabidopsis* seedling development.

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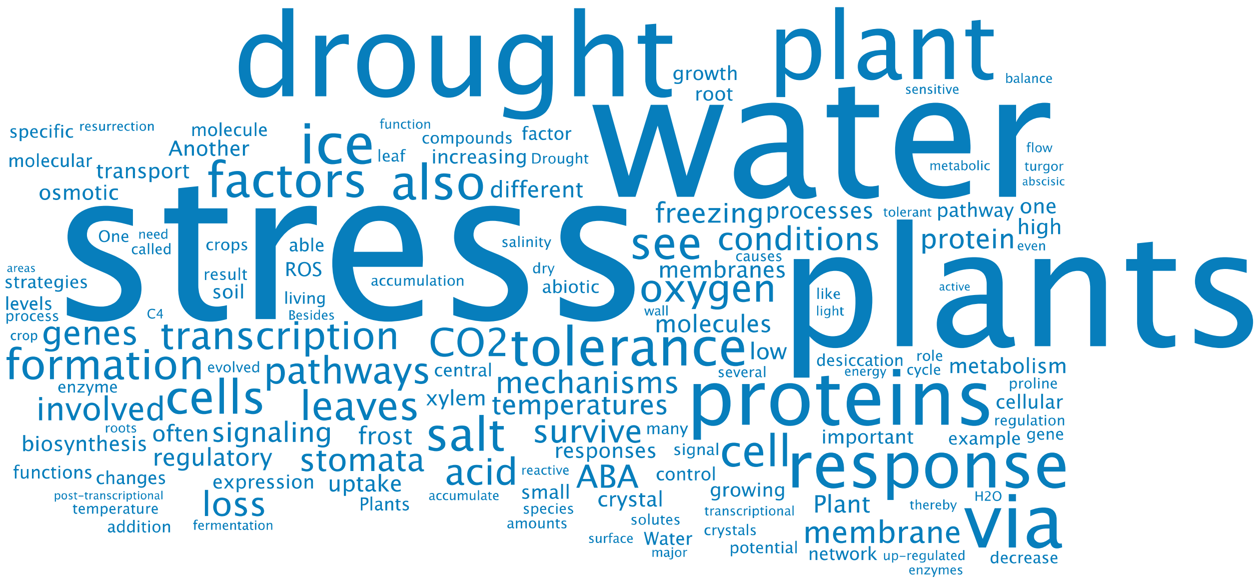
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10
Water

Wiebke Zschiesche and Klaus Humbeck

Overview

Water is essential for life. The H_2O molecule is the major component of all plants, and in order to survive, plants have to take up enormous amounts of water. Every day, there is a massive water flow from the roots where water is taken up, to the leaves where water is evaporated through stomata. Drought stress severely affects the water balance in the plant resulting in cellular disturbances of central biochemical pathways. Depending on their strategies for coping with drought stress, plants can be classified as arido-passive, arido-active, and arido-tolerant. Research in the past years has considerably increased the understanding of the molecular processes underlying the establishment of drought tolerance in plants including drought sensing, signal transduction pathways via phytohormones, and complex regulation of gene expression operated by transcription factor cascades. This knowledge about the molecular basis of drought stress is important for worldwide approaches toward drought-tolerant crops.

Low temperatures above and below the freezing point are also stress factors, limiting the yield of crop plants. While low positive temperatures disturb homeostasis of metabolic processes and membrane functions, freezing temperatures can result in extracellular ice crystal formation accompanied with water loss from the cells. Besides drought and frost, salinity also causes water shortage in the plants. Therefore, these three stress conditions induce similar molecular responses to cope with the osmotic stress. In contrast, too much water during flooding affects plants differently, and tolerance against flooding involves other metabolic pathways.

10.1
Water: the Essence of Life

On earth, the genesis of living organisms is based on the presence of water and almost all chemistry of life depends on water, as solvent or as reaction partner (see Section S1.1.6). The enormous meaning of water in biochemistry has its origin in the very special properties of the **H_2O molecule** (Figure 10.1). On the one hand, it is a small molecule with about 96 pm distance between the central oxygen atom and each hydrogen atom, which are preferentially arranged on one side of the molecule, forming an angle of about 104° between the two bonds of the oxygen atom. Because of this structure, H_2O is a strong dipole with a negative charge at the oxygen and a positive charge at the hydrogen atoms, allowing hydrogen bonds between H_2O molecules and other polar compounds. This is an important feature for its chemical reactivity and physical properties. Besides being a reaction partner in almost all chemical reactions in living organisms, water is the dominant solvent for transport processes in multicellular organisms, which are needed to reallocate resources from source to sink regions. In plants, it also ensures the maintenance of the form by turgor pressure.

Plant protoplasm under normal conditions contains 85–90% (w/w) water, whereas plastids and mitochondria contain "only" 50% water (w/w) because of their densely packed proteins, lipids, and DNA. Fruits, leaves, and roots contain more than 70% (w/w) water. In contrast to all other structures, seeds normally have a very low water content (5–15%, w/w), enabling them to survive adverse environmental conditions for a long time in a kind of "dry state." In order to keep the high water content, plants need to take up enormous amounts of water during their entire life. To synthesize 1 kg of dry weight, most plants need between 300 and 900 kg water. Some plants are genetically better adapted to arid environments than others. For example, crops needed to feed the rising population in the world show great differences in water demand (see Box 10.1). Some crops such as millet need on average only about 300 kg water per 1 kg dry weight, whereas crops like alfalfa or soy bean need 600–900 kg water to generate 1 kg dry weight. Knowledge of these differences and of the responsible mechanisms is of increasing importance in the current period of changing climate.

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

Box 10.1: Toward drought-tolerant crops

A rapidly growing global population, climate change, and increasing scarcity of fresh water are challenges humankind has to meet. On a global scale, water scarcity is the major factor limiting yield. Enormous efforts are, therefore, made worldwide to minimize yield loss by drought. These efforts include finding improved agricultural techniques and breeding drought-tolerant crops. Hence, knowledge of molecular mechanisms underlying drought responses in plants is very important and will allow genomics-based predictive plant breeding via marker-assisted generation of novel genetic variants using either natural plant resources or genetic engineering techniques.

Many examples prove that genomics-based approaches can improve drought tolerance. Transgenic crop plants (over)expressing genes involved in biosynthesis of **osmoprotectants** (e.g., glycinebetaine or proline) (Figure 10.3) and trehalose-6-phosphate, which regulates sugar utilization and starch metabolism and interacts with plant hormone signaling pathways, show clearly increased tolerance against drought. Other targeted genes were genes encoding stress proteins [e.g., proteins of the heat-shock family (chaperones) or LEA proteins]. In these attempts, drought tolerance was improved by increasing the amount of compatible solutes and protecting proteins and metabolites via expression of related enzymes. Another strategy is to manipulate the regulatory network focusing on central regulatory factors. In this approach changes in the level of this factor affect whole pathways controlling the downstream of the drought tolerance factor at multiple levels. Stress-inducible transcription factors are the targets of such an approach. Overexpression of ERF/AP2 or NAC transcription factors, for example, clearly improved tolerance against different abiotic stress conditions including drought. Quite often, first *gain-of-function* experiments are performed using the model plant *Arabidopsis thaliana.* But after identification of promising targets, experiments are transferred to crops employing the orthologous genes. A major problem in these kinds of experiments is that general overexpression of an interesting target gene can cause problems. Permanent activity of such regulatory factors in each cell is often undesirable. Therefore, state-of-the-art approaches quite often use inducible and cell-specific promoters allowing exact steering of overexpression.

Another strategy to improve drought tolerance in crop plants is to learn from a unique group of plant species termed *resurrection plants*, which have acquired vegetative desiccation tolerance (see Section 10.3.3). Several groups analyze molecular genetic mechanisms, differential proteomics, metabolic and antioxidant systems as well as macromolecular and structural stabilizing processes, which together are responsible for the plant's extraordinary capability to survive desiccation.

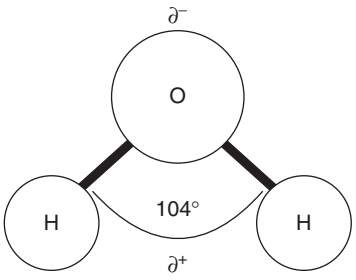

Figure 10.1 H_2O molecule.

10.2 Water Balance in Plants

10.2.1 Water Flow

Day by day, plants take up huge amounts of water from the soil. The water subsequently flows through the plant and is being transferred to the atmosphere by the leaves (Figure 10.2). The reason for this massive flow is **photosynthesis,** which needs CO_2 from the atmosphere. Most of the needed CO_2 enters the plant via **stomata** in the leaves, which form a cave at the border between plant and environment. Although required for CO_2 uptake, open stomata concurrently and unavoidably result in water loss to the dry atmosphere following the gradient in water vapor concentration. The result of this dilemma is that the balance of water in plants has to be sustained by a strong water flow from the soil to the leaf. During this journey through the plant, water passes through very different compartments and transcends different kinds of barriers. The moving forces for this process are **water potential** gradients and pressure gradients. In a more global sense, water simply follows gradients in free energy. The maintenance of water balance even under adverse environmental conditions determines the viability of a plant. Therefore, plants have evolved sophisticated mechanisms to regulate water balance at different levels in response to the environment.

Soil may be composed of different components with different capacities to hold water. **Roots** fix the plants in the soil, are the interphase between plants and mycorrhiza (see Section 5.2.1), and take up water and nutrients (see Section 11.1.3). Root hairs intimately penetrate the soil close to the roots and increase the surface area for water uptake (Figure 10.2). The size of the root system, the degree of branching, and the abundance of root hairs

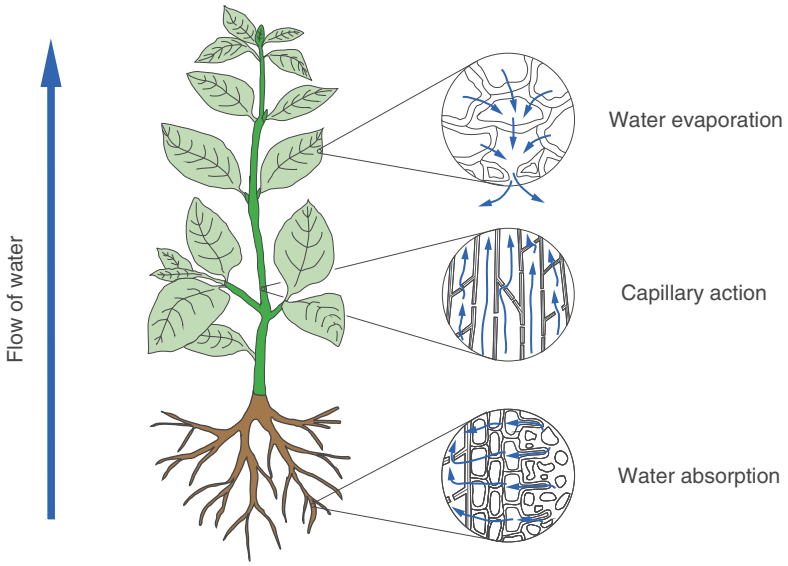

Figure 10.2 Flow of water, from the roots to the leaves. Water is adsorbed at the root cell walls, enters the xylem in the root, flows upward in the xylem system, and finally evaporates via stomata.

determine the capability for water uptake. In order to acclimate to drought conditions, plants can vary these parameters.

Once adsorbed by the cell wall, water can follow different paths to the main water pipeline, the **xylem**. Water can flow in the **apoplast pathway** through cell walls and extracellular spaces. The **symplast pathway** is based on the flow through the cytoplasm of the cells and transfer from one cell to another via plasmodesmata. Despite its polar property, the small H_2O molecule is able to cross the lipid bilayer of the membrane but this diffusion process is rather slow. Plants, in addition, have transmembrane proteins such as **aquaporins** forming a pore for the water molecules and allowing much faster water flux through the membrane. Such aquaporins can function reminiscent of regulatory valves, which can be closed in response to drought via posttranslational protein modifications, for example, phosphorylation and methylation, and via changes in the conformation. Using these different pathways, water enters the plant and moves through the outer cell layers of the root, the cortex, reaching the endodermis barrier, where the apoplast pathway is interrupted by the Casparian strip. Water then enters the xylem pipeline via symplastic and transmembrane transport.

Long-distance transport of water from the roots to the upper parts of plants takes place in the xylem, the main upstream pathway in plants (Figure 10.2). Similar to water pipes in buildings, the xylem consists of **hollow tubes** in which the water can flow easily. To build such long tubes, specialized cells undergo a differentiation process that involves programmed cell death. At the end of xylem development, the wall of the long xylem tubes contains a lignified cell wall surrounding a hollow space.

10.2.2 Loss of Water by Stomatal Transpiration

Green plants transform light energy via the complex photosynthesis process into energy-rich chemical compounds (ATP, NADPH). Plants fix atmospheric CO_2 and form carbohydrates in the **Calvin cycle** (see Section S1.3.4.5, and Section 1.3, Fig. 1.6). The first step, the fixation of CO_2, is performed by the **ribulosebisphosphate-carboxylase/oxygenase (rubisco)**. Rubisco is one of the most abundant proteins on earth. It consists of two types of subunits, eight small subunits, and eight large subunits. In addition to carboxylation of ribulose-1,5-bisphosphate, rubisco catalyzes oxygenation of ribulose-1,5-bisphosphate, yielding 2-phosphoglycolate and 3-phosphoglycerate via **photorespiration**, whereby oxygen is consumed (see Section S1.3.4.5, and Section 1.3, Figure 1.7). Which reaction takes place depends on the partial pressures of CO_2 and O_2 near the enzyme.

To assimilate carbon in photosynthesis, plants need to take up high amounts of CO_2 from the atmosphere. As the waxy cuticle that covers epidermis cells largely prevents gas exchange, most of the CO_2 enters the plant via stomata and then mingles with gas in the leaf air spaces close to the photosynthetically active mesophyll cells. On the other hand, water reaches the leaves via the xylem system, enters the cell wall of mesophyll cells, and mostly evaporates into the air spaces of the leaf. From there it diffuses through the stomata to the atmosphere. This process is called **transpiration** (Figure 10.2). Consequently, opening of stomata for the uptake of CO_2 inevitably results in loss of water. This makes stomata opening a crucial factor in water balance, which can be regulated in response to drought stress conditions.

As water loss via open stomata is a side effect of photosynthesis, which needs CO_2, plants have evolved mechanisms to more efficiently employ CO_2 by CO_2-concentrating processes (see Section S1.3.4.5, and Section 1.3), mainly the **C_4 carbon cycle** Section 1.3 and Figure 1.6, and the **crassulacean acid metabolism (CAM)** (Figure 1.9). C_4 plants like grasses use specific physically separated reactions to enrich CO_2, preventing photorespiration and allowing a reduction of stomata opening to lessen water loss. Another efficient way to minimize water loss through the stomata is realized in CAM plants. As in C_4 plants, CO_2 is fixed by the enzyme phosphoenolpyruvate carboxylase forming oxaloacetate and malate, which then is stored in the vacuole as malic acid. The somata open for the uptake of CO_2 during the night when cooler temperatures prevail. During the day, when temperatures are high, stomata are kept close to minimize water loss. During daytime the stored CO_2 is liberated via the formation of malate and its subsequent decarboxylation, and can be used by rubisco for carbon assimilation in the calvin cycle. This efficient way of temporal closure of stomata during the hot days is often found in plants growing in arid and hot environments, for example, in cacti living in deserts, and in *Welwitschia mirabilis* leaves (*Welwitschiales, Gnetophyta,* see Section 10.3.3).

10.3 Drought Stress

10.3.1 Drought Stress, a Worldwide Challenge

Human population is rapidly increasing. To feed humankind, more and more food is needed, which is mainly derived from crop plants. The arable area is limited and, in addition, adverse environmental conditions, primarily drought stress, decrease yield. At the moment and on a worldwide scale, abiotic stress conditions are responsible for more than 50% loss of yield in major crops. Because of **climate change,** this problem will become even more critical in the future.

Drought stress can be caused (i) by limited uptake of water when the soil dries out after a long period of aridity, (ii) by high soil salinity, which makes uptake of water difficult, (iii) by ground frost when water is not fully available and (iv) by increasing transpiration rates during sunny, windy, and hot days.

10.3.2 Cellular Disturbances

Water deficit affects many aspects of plant function. When the difference in water potential between soil and plant decreases during a drought period, turgor pressure in the plant declines. Processes important for plant growth like cell elongation and the connected formation of the cell wall depend on turgor pressure and, therefore, are already impaired at low stress levels. This causes a decrease or even a stop of **organ growth** of developing plants. As a result, smaller and fewer leaves result in decreased loss of water by transpiration. An extreme response can be the abscission of leaves. **Root growth** also can be retarded, but some plants are able to specifically acclimate to light drought stress via the stimulation of root growth.

At the molecular level, central biochemical pathways such as generalized protein biosynthesis and eventually photosynthesis and dissimilation are hampered. Plants react to drought stress by changes in phytohormone levels. **Abscisic acid (ABA)** accumulates while other hormones like cytokinins are down-regulated. In addition, other stress responses are initiated, for example, the accumulation of osmotically active compounds (Figure 10.3) (see Section 10.3.3). Recently, it has been shown that balancing primary and secondary sulfur metabolism (see Section 6.3.3.2, Figure 6.22) plays an important role during drought stress.

When water deficit continues and plants have no efficient strategies to cope with this situation, the turgor continues to decrease and displacement of membrane proteins disturbs membrane integrity and cellular compartmentalization.

10.3.3 Adaptations to Survive Drought Stress

To survive periods without irrigation, plants have evolved various mechanisms to cope with drought stress. The extent of drought tolerance varies considerably within the plant kingdom and there are several adaptations leading to drought tolerance (Figure 10.4). Plants living in dry areas are classified as **xerophytes**. Xerophytes have evolved three strategies to cope with drought stress:

- **Arido-passive plants** have developed two main strategies. (i) The seeds are very drought-resistant and maintain the capacity to germinate for many years. (ii) The life cycle from germination to blossom is very short. These features are the reason for a phenomenon called **flowering desert**, when after rare rainfall in a desert many plants suddenly grow and bloom simultaneously.
- **Arido-active plants** have evolved many strategies to evade water loss. One strategy realized in succulent plants, including cacti, is storage of water in specialized tissues in stems, leaves, or roots. This allows to store sometimes enormous amounts of water in specially designed parenchyma cellular tissues. The capacity for water storage is calculated by the succulent degree, which is the ratio of maximum amount of water stored (in gram) and the surface (in decimeter square). Another adaptation is to either increase the uptake of water or to decrease transpiration. The root system is the place of water uptake. Plants in arid environments often have evolved a deep and/or a widely ramified root system, enabling them to efficiently take up water.

  An example for an arido-active plant is *Welwitschia mirabilis* (*Welwitschiales, Gnetophyta, Spermatophyta*),

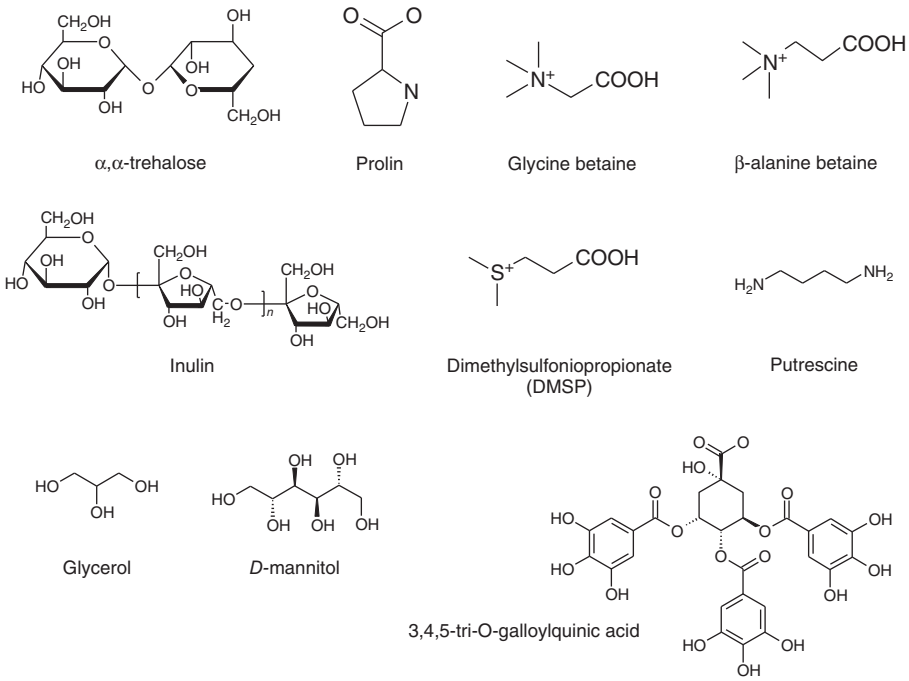

Figure 10.3 Osmolytically active substances in plants.

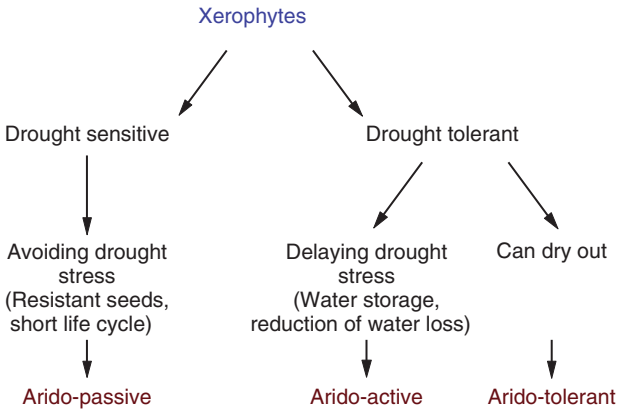

Figure 10.4 Strategies of xerophytes to survive drought stress.

a gymnosperm genus endemic to the coastal zone of the Namib Desert in southwestern Africa (Figure 10.5). Their estimated life span is 400–2000 years. *W. mirabilis* can survive drastic temperature fluctuations between 6° C at night and up to 50° C during the day. The plants are growing in habitats of extreme aridity with precipitates much less than 50 mm per year. Condensed water derived from minimal moisture of fog can be absorbed by the plants. One pair of leaves with a length of 5–7 m, the only leaves of the plant, grows continuously from a short stem. The upper leaf surface efficiently reflects light to prevent overheating. The broad leaves produce a cooler microclimate for the plant and its arthropod associates. Male and female plants both produce nectar for attracting insects (Figure 10.5). The nectar of male flowers contains 50% sugar.

Some arido-active plants, for example, maize (*Zea mays, Poales, Liliopsida*), are able to acclimate to a dry environment by increasing root growth. One way to decrease loss of water by transpiration was already discussed before, that is, the variation in CO_2 fixation in C4- and CAM plants. Other strategies are variations in the amount and size of stomata and adaptations in the shape of leaves to decrease transpiration. Plants living in dry areas and exposed to high light intensities often develop a dense pattern of trichomes to decrease light intensity at the leaf surface by refraction. Because of the many trichomes, these leaves often appear silvery and whitish.

- **Arido-tolerant plants (resurrection plants)** can survive desiccation. They live in habitats where rains are seasonal and extremely sporadic, for example, in southern parts of Africa and Australia. Only about 20 flowering plants are known that can survive in an air-dried state. This extraordinary feature was analyzed in some detail in the resurrection plant *Craterostigma plantagineum (Lamiales, Asteridae)* (Figure 10.6). This plant can lose about 90% of its water. The cells retain their chlorophyll and intact photosynthetic structures. When water becomes available again, the plant can rehydrate within hours and restore photosynthetic processes. During drying out, many cellular processes are up-regulated. Phytohormone patterns change, with very prominent accumulation of ABA. *C. plantagineum* is able to accumulate the rare C_8 sugar octulose in high amounts in fully hydrated leaves. During dehydration the octulose content decreases and sucrose is accumulated. This stress response is reversed during the rehydration of the plant. Furthermore, under

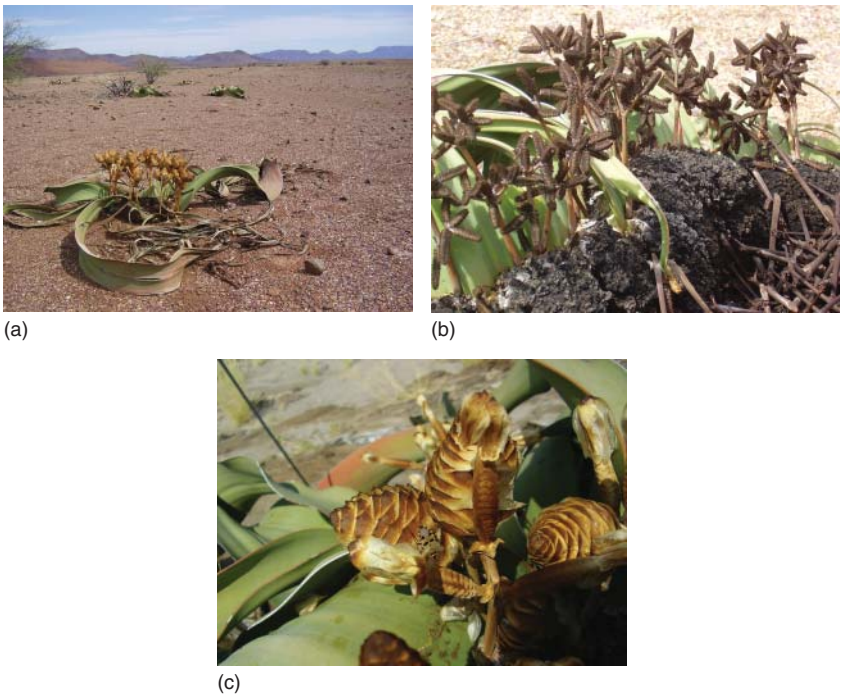

Figure 10.5 *Welwitschia mirabilis*, an arido-active plant, (a) Plants in their native habitat, (b) Male cone in flower, (c) *Welwitschia* bug (*Odontopus sexpunctatus*) on female cones. (Courtesy of P. Schreck.)

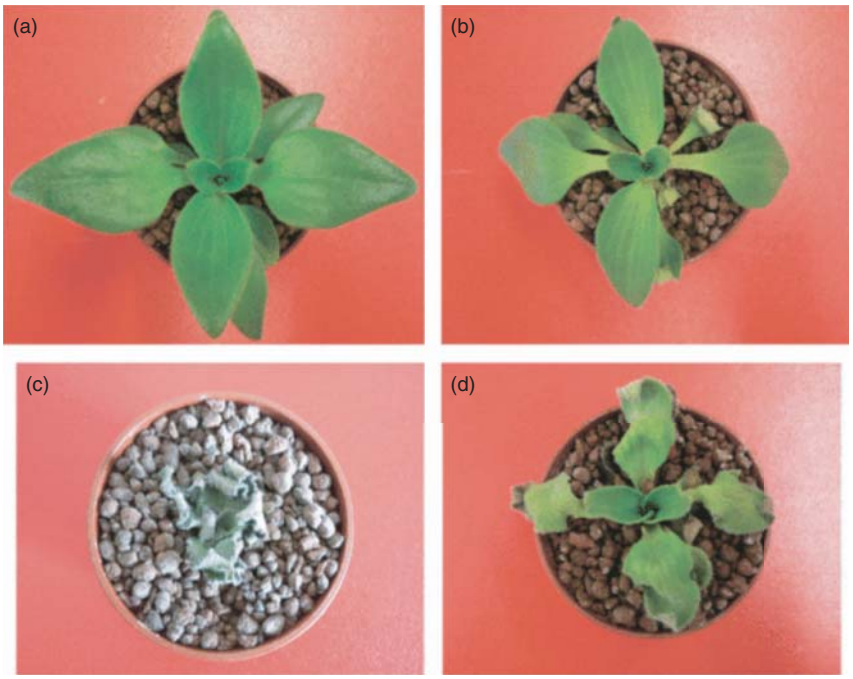

Figure 10.6 *Craterostigma plantagineum*, an arido-tolerant plant. *Craterostigma plantagineum* survives drying out. (Courtesy of J. Mundy and D. Bartels.)

drought stress **LEA (late embryogenesis abundant)** proteins accumulate. They may establish a hydrogen-bonding network in the extremely water-reduced cells. Together with sugars, LEA proteins hamper protein aggregation during desiccation.

Another resurrection plant, *Myrothamnus flabellifolius* (*Gunnerales*, core eudicotyledons), can remain in a desiccated state for 2–3 years in mountain regions of Namibia (Figure 10.7). As shown by scanning electron microscopy (see Section 19.1.3.2) the desiccation process

Figure 10.7 *Myrothamnus flabellifolius*, an arido-tolerant plant, (a) Plants in the desiccated state in a rocky habitat near Karibib, Namibia, (b) branch with rehydrated leaves. (Graphics: G. Krauss.)

is accompanied by cell wall folding of mesophyll and epidermal cells. Pectin-associated arabinanes and/or arabinogalactan proteins are responsible for keeping the cell wall flexible during desiccation. **Arabinose** is constitutively synthesized in leaf cells of *M. flabellifolius* preparing them for loss of water. In addition, vacuoles of leaf palisade and sponge mesophyll cells have a high content of **3,4,5 tri-*o*-galloyl quinic acid** (Figure 10.3). This polyphenol probably protects the cell membrane against desiccation by intercalating into the lipid membrane and acting as antioxidant against free radicals.

10.3.4 Molecular Mechanism of Drought Stress Tolerance

10.3.4.1 Downstream Signal Transduction

There is some evidence that drought, as well as cold and salinity, are sensed via changes in the function of the **plasma membrane** (Figure 10.8). The plasma membrane is very sensitive to stress conditions, which affect its fluidity and architecture. During drought and when turgor drops, the volume inside the cell decreases resulting in a shrinking of the plasma membrane. Changes in their structure may be

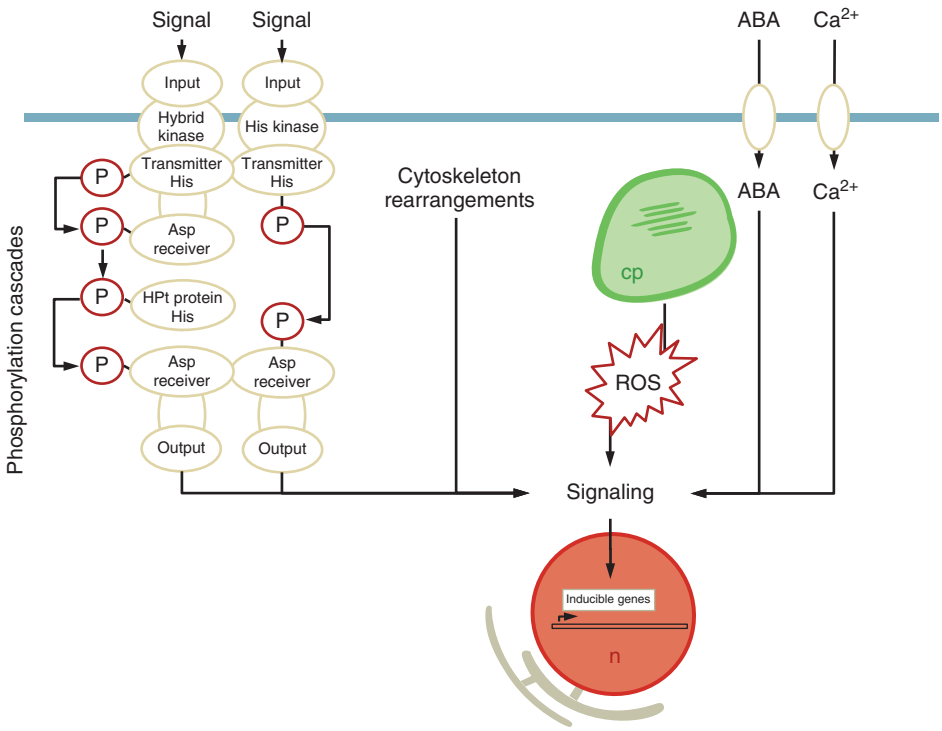

Figure 10.8 Model of drought stress signaling. Drought stress affects properties of plasma membrane and plasma membrane proteins (e.g., calcium transporter and receptor-like kinases). Signal transduction includes protein modules containing phosphorylatable Asp and His residues (HPt – histidine phosphotransferase). Calcium, kinases, ABA, and ROS are involved in downstream signaling, finally reprogramming gene expression in the nucleus. (Courtesy of D. Dobritzsch.)

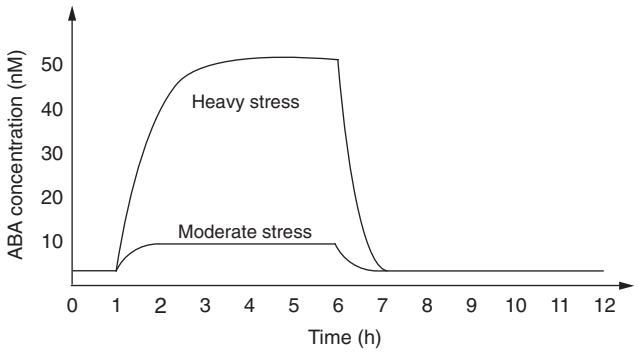

Figure 10.9 Accumulation of abscisic acid (ABA) in response to drought stress.

sensed by the rearrangement of the connected cytoskeleton. Other membrane-based sensors could be integral membrane proteins, either Ca^{2+}-permeable channels or two component regulatory systems, consisting of the sensor, a membrane-bound histidine kinase, and a corresponding cytosol-oriented response regulator that mediates the cellular response via phosphorylation cascades.

Another primary event in drought stress response is the accumulation of **reactive oxygen species (ROS)**. The sensitive chloroplast, where solar energy is absorbed and chemically conserved, is one major site of ROS production. Drought negatively affects the light and dark reactions in the chloroplast, which causes the production of ROS, for example, H_2O_2, the superoxide radical, and singlet oxygen. Such stressed plants need ROS scavenging mechanisms to effectively detoxify ROS (see Chapter 8).

When drought stress signals reach a cell via a specific sensor or phytohormones, fine-tuning and signal transduction pathways, interacting by cross talk, are turned on in the cell via second messengers, **MAPK (mitogen-activated protein kinases)** modules, transcriptional control and synthesis, and activation of proteins that confer drought tolerance (see Chapter 7). Changes in the lipid constitution of cellular membranes influence lipid-derived cellular signals such as inositol phosphates (see Section 7.4.7).

10.3.4.2 Role of Abscisic acid

Drought stress releases a hydraulic signal, possibly a rapid shift in xylem extension. The signal triggers the biosynthesis of **ABA** that can transduce the signal over long distances (Figure 10.9). ABA is accumulated in the cells in a synchronized manner, dependent on the extent of stress.

One major function of ABA is the regulation of stomata. During drought stress, the pH value is increased in the xylem where ABA is transported over long distances. Under these conditions, ABA dissociates into a proton and the ABA^- anion. Because of the resulting electric charge the phytohormone preferentially enters guard cells. When ABA reaches guard cells, changes in ion fluxes are triggered accompanied by stomatal closure and water loss reduction.

ABA functions in the plant's response to drought, salt, and low temperature via ROS production and also via NO formation linked to polyamines (putrescine, spermidine, spermine, and thermospermine) (see Section 7.4.5). Recently, MAPK cascades have also been implicated in ABA signaling (see Section 7.3.3.1).

10.3.4.3 Transcriptional Control Mechanism

Several transcription factors regulate the drought stress response. ABA-dependent and ABA-independent regulatory pathways are involved in a complex regulatory network (Figure 10.10).

The ABA-dependent drought response requires **transcription factors** of AREB/ABF, bzip, and MYB (myeloblastosis)/MYC (myelocytomatosis) type. One member of the AREB/ABF family is AREB1/ABF2, a basic domain/leucine zipper transcription factor. This transcription factor binds to the **AB**A-**r**esponsive *cis*-**e**lement (ABRE) found in the promoter of many ABA-responsive genes. Gain-of-function and loss-of-function analyses using transgenic plants demonstrated the central role of these transcription factors in drought stress tolerance. Another pathway of ABA-dependent regulation of drought stress-related genes acts via transcription factors of the MYC (MYC viral oncogene homolog) and MYB class. They accumulate in response to ABA, and among the target genes

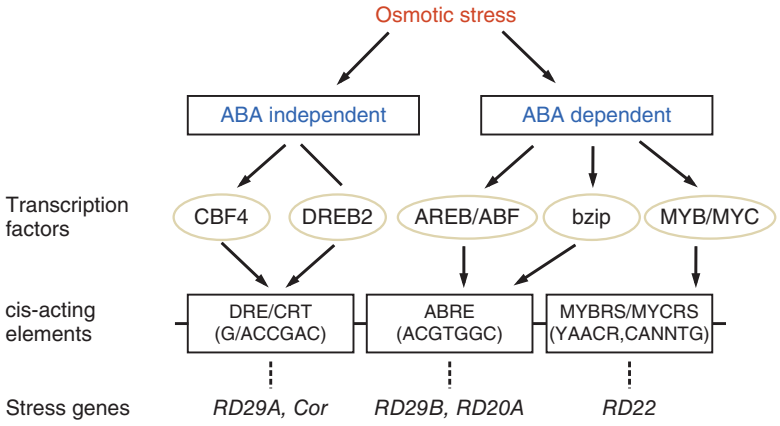

Figure 10.10 Regulatory network of osmotic stress response in plants.

of these transcription factors are many factors involved in drought tolerance.

ABA-independent factors are involved, too (Figure 10.10). One central player in this pathway is DREB2 binding to the well-known cold and drought stress *cis*-element DRE/CRT (dehydration responsive element/C-repeat). This DNA element (TACCGACAT) is present in the promoter of many stress-regulated genes. *Arabidopsis thaliana* expresses a small DREB protein family. Only two factors, DREB2A and DREB2B, seem to be involved in the drought stress signaling. Another one, DREB1A, is involved in the cold stress regulatory network (see Section 8.4). Both, DREB2 and DREB1, bind to the same *cis*-element, but not exactly at the same positions, allowing differential regulation during the different stress conditions.

The presence of different pathways of drought stress response is a prerequisite for cross talk, meaning the **interactive modulation and fine-tuning of gene expression**. The signal network can function, for example, via sophisticated interaction at promoter elements as described for the DRE or also via harmonized action of phytohormones. Additional transcription factors, for example, no apical meristem (NAC) transcription factors, zinc finger transcription factors (apetala 2/ethylene responsive factor) (AP2/ERF), and their specific *cis*-elements cooperate in the concerted interplay of the complex drought stress response pathway. Some small miRNAs are also regulated in a stress-dependent manner. As several transcription factors are targets of small RNAs, for example, NACs/miR164, transcription factor-dependent regulatory cascades may be modulated, fine-tuned, and coordinated via the action of miRNAs (see Section 7.7).

In addition to transcriptional control, **posttranscriptional cell programs** are involved in drought stress response. Farnesyltransferases are known to catalyze farnesylation of target proteins, thereby establishing a hydrophobic anchor, which is important for protein-membrane or protein–protein interactions. Mutants with altered levels of such farnesyltransferases are affected in drought tolerance indicating a role of such posttranscriptional protein modifications in the drought stress response. Another example is ubiquitination. For instance, proteolysis of the DREB2A transcription factor at the 26s proteasome is triggered via ubiquitination and this mechanism controls activity of DREB2A-dependent signaling pathways during drought stress.

Under abiotic stress, transcriptional regulation via **epigenetic mechanisms** is activated. Histones at drought stress-responsive genes like RD29, RD20, and AP2 domain-containing transcription factor are modified during drought stress (Figure 10.11). Lysine 4 at the n-terminal end of histone 3 is trimethylated, and lysine 9 is specifically acetylated. Both modifications mark a transcriptionally active, open chromatin. They are established after the onset of stress. Furthermore, analyses of mutants in epigenetic control factors like histone-modifying enzymes and chromatin remodeling factors showed clear stress phenotypes, indicating a central role of epigenetic control mechanisms in stress response in plants. This field is just emerging and exciting new findings are expected.

10.3.4.4 Drought Stress Genes

Transcriptome analyses (see Section 18.2) revealed massive reprogramming of gene expression during drought stress. These up-regulated genes encode

1) **regulatory factors**, for example, the transcription factors (see Section 10.3.4.3), which are up-regulated in the early stages of drought stress.
2) **protective chaperones**, responsible for increased stress tolerance.

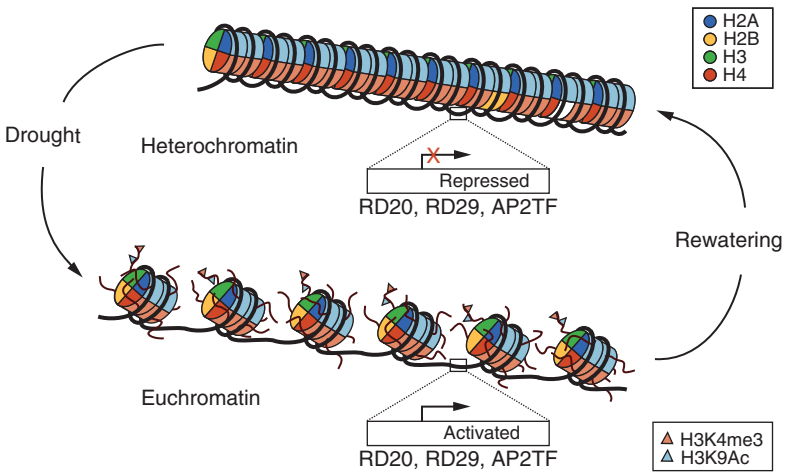

Figure 10.11 Epigenetic control of stress gene expression. Drought stress-responsive genes *RD20, RD29,* and *AP2TF* are epigenetically controlled via modifications at N-terminal ends of attached histones. Tri-methylation at lysine 4 of histone 3 (H3K4me3) and acetylation at lysine 9 of histone 3 (H3K9Ac), which are marks of transcriptionally active euchromatin, are established in response to drought stress.

3) key enzymes of the biosynthetic pathways for accumulation of **compatible solutes** like proline, betaines, or specific sugars (Figure 10.3).
4) proteins for enhanced **water transport** through membranes. These might be water channel proteins or membrane transporters.
5) factors for **detoxification of ROS** (catalase, superoxide dismutase, etc.) and protection of biomolecules such as LEA proteins.

Additional genes responding to drought stress cannot be easily divided into these specific classes and their functions are not clearly understood.

10.4 Cold Stress and Freezing

10.4.1 Lowering of Temperature

Plants need their own set of temperature requirements to live in specific habitats. In some species, lowering temperature may cause dramatic changes in cells that lead to reduced leaf growth, wilting, and chlorosis. When temperature falls below zero, the risk of ice formation within the plant increases. While some plants can withstand very low temperatures (<−40°C), others quickly die at the freezing point. This indicates that some plants have evolved efficient mechanisms to cope with such low temperatures.

Ice crystal formation does not proceed uniformly within plants. It will first occur in areas that cool down quickly and where the freezing point is highest. Normally, this is in the intercellular and the xylem vessels. In these compartments ice formation can quickly spread out. In contrast to the normal extracellular ice crystal formation, intracellular ice formation is generally lethal because the growing ice crystals mechanically destroy membranes and thereby irreversibly disable cellular functions. Plants can survive ice crystal formation outside the cell for a while. But when low temperatures persist, damaging effects become inevitable. These are mainly mechanical impact on the stability of the plasma membrane and dehydration of the cells. The reason for dehydration of the cells is that the growing extracellular ice crystal has a high affinity to water. The result is that water molecules massively flow out the cell and are bound in the growing ice crystal. Therefore, symptoms of freezing temperatures in part are similar to those observed during drought.

10.4.2 Requirements of Ice Crystal Formation

Crystallization is generally not induced exactly at 0 °C and depends on several factors. The most important ones related to plant frost stress are the following:

1) **Solute compounds**: the freezing point depends on the nature and the concentration of solute compounds in the cell. Higher concentrations of compatible solutes, for example, sugars, polyoles, and amino acids (see Figure 10.3), lower the freezing point. An increase from 0.5 osm (moles solved compound/100 g water) to 1.0 osm ensures that 100% of water is still liquid at −1°C, compared to 93% before, or that 37% of water is still liquid at −5 °C, compared to 19% before.
2) **Presence of ice nucleators**: the initiation of ice crystal formation involves H_2O molecules, which have to be arranged in a three-dimensional pattern. In this process of ice nucleation, surfaces in the immediate vicinity play an important role. Ice crystals form easily when molecular structures are present, which by their charge distribution allow H_2O molecules to arrange for ice nucleation. Such structures are called ice nucleators. Typical ice nucleators are carbohydrates and proteins of the cell wall.
3) **Kinetics of cooling down**: the form of growing ice crystals is different when cooling down occurs slowly or quickly. Rapid freezing allows the formation of only small ice crystals, while slow cooling down results in the formation of large ice crystals, which are much more dangerous than the small ones.
4) **Presence of biofilms on the leaves**: When bacteria, for example, *Pseudomonas syringae* or *Erwinia herbicola* are present on the leaves, freezing of plant tissue occurs much faster than without. The reason is that biofilms (see Chapter 14) at the surface often contains ideal ice nucleators causing initial ice formation on the leaf surface. From these focal points, ice formation spreads into the plants.

10.4.3 Strategies to Survive Low Temperatures

As only a few equatorial regions on our globe are frost-free, plants have evolved several strategies to survive such adverse low temperatures. In principle, one can distinguish between strategies to **avoid frost stress** and strategies to build up a **tolerance against frost stress**. Tolerance can be gained either via hardening primary targets of frost damage or via protection against secondary damages.

Avoiding frost stress means **thermal insulation of sensitive organs** or tissues to prevent excessive heat loss, which would result in cooling below zero degree. This can be realized by different ways. In cushion plants, for example, the sensitive buds are covered with dense vegetative tissues. Another way to minimize heat loss is the retraction below Earth's surface in winter time. These plants are called **geophytes**. The autumnal shedding of leaves or the nocturnal closing of leaves to protect the apical meristem from cooling are also efficient strategies to avoid frost stress at sensitive tissues. The other strategy is to survive temperatures below 0° within the plant, that is, gaining tolerance against

frost. Plants are able to accumulate compatible solutes in response to low temperature (Figure 10.3).

Compatible solutes substances lower the freezing point, maintaining a liquid phase within the cells at temperatures between −5 and 0°C (Figure 10.3). Measuring the accumulation of such compounds is a widely used parameter for frost stress response. Stress-related accumulation of these molecules involves on a molecular level up-regulation of genes coding for biosynthesis enzymes of such compounds. Several woody plants are able to survive even very low temperature (below −40 °C). The trick is to prevent ice crystal formation, either by changes in the composition and structure of cell walls, which, on the one hand, builds up a barrier for the growing ice crystal, or, on the other hand, to minimize putative crystallization points. Other targets of frost acclimation are the **membranes**. Changes in the lipid composition with an increase in the amount of **stabilizing lipids**, for example, phosphatidylcholine or phosphatidylethanolamin, are important for sustaining intact membranes. For this reason, enzymes involved in lipid metabolism, for example, phospholipase-D-phosphatidyl-transferase, are often regulated in response to falling temperatures. Measuring the integrity of membranes at freezing temperatures is indeed a parameter often used to determine freezing tolerance of plants. Membranes in sensitive plants get leaky already at moderate freezing temperatures, while frost-tolerant plants show intact membranes. The integrity of membranes can be detected measuring the emersion of intrinsic metabolites or proteins. One example is the measurement of plastocyanine in the cytosol (Figure 10.12).

During frost acclimation some plants including cereals accumulate **fructans** (Figure 10.3). These fructosyloligosaccharides are synthesized from sucrose. Different classes of fructans are synthesized and accumulated in plant vacuoles, for example, inulin (Figure 10.3), levan, mixed levan, inulin neoseries, and levan neoseries. Fructosyltransferases are key enzymes of fructosan biosynthesis.

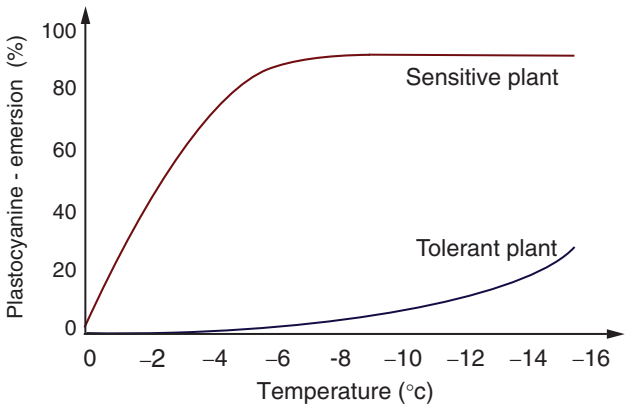

Figure 10.12 Plastocyanine emersion in the cytosol as measure for freezing tolerance. Freezing injures organelle membranes in sensitive plants, resulting in leakage of compounds, for example, plastocyanine out of chloroplasts.

1-SST (1-sucrose-sucrose-fructosyltransferase) initiates *de novo* fructan synthesis by catalyzing the transfer of a fructosyl residue from sucrose to another sucrose molecule, resulting in the formation of the trisaccharide, 1-kestose. This enzyme and other fructosyltransferases like 6G-FFT (fructan:fructan 6G-fructosyltransferase) and 6-SFT (sucrosefructan-6-fructosyltransferase) are up-regulated after low temperature exposure. Analyses of transgenic plants overexpressing such fructan biosynthesis enzymes resulted in higher amounts of fructan and increased frost tolerance. This tolerance seems to be connected with higher membrane stability. Fructans interact with membranes and stabilize them against damage through growing ice crystals.

Another strategy to prevent ice crystal formation is the production of metabolites or proteins that interact with the growing ice crystal and thereby inhibit its further growth. **Antifreeze proteins** (AFPs), which were first found in fishes living in cold water bodies below ice, also act as ice recrystallization inhibition proteins (IRIPs) in plants.. These proteins are induced in response to low temperatures. Specific NxVxG/NxVxxG repeats in the amino acid sequence form beta-roll structures that can bind to a growing ice crystal to inhibit formation of larger, dangerous ice crystals. Several AFPs are pathogenesis-related proteins. Such proteins might have dual functions. Specific characteristics of these proteins seem to enable them to either interact with pathogens or with growing ice crystals.

10.5 Salinity

10.5.1 Salinity, an Increasing Problem in Agriculture

Salinity stress is an increasing concern in agriculture. Besides natural salty habitats, for example, areas close to seashores (Figure 10.13) or close to geological salt deposits, anthropogenic influences are a major cause of salinity stress. Intensive agriculture, often combined with nearsighted and unsustainable water management, is the reason for an increasing worldwide problem of salinization of soils, decreasing crop yield far below optimal levels. Some plants are highly salt tolerant. Learning how they are able to survive even high salt concentrations is important to ensure high yields in agriculture to meet the food demands of an increasing world population in the future.

10.5.2 Salt Stress Alters Plant Functions

Uptake of water follows a downhill gradient from water in the soil to a lower water potential inside the plant. An increase in salt concentration in the soil results in a decreased water potential, thereby minimizing the water

Figure 10.13 *Salicornia europaea*, a succulent halophyte (Camarque, France). (Courtesy of G. Krauss.)

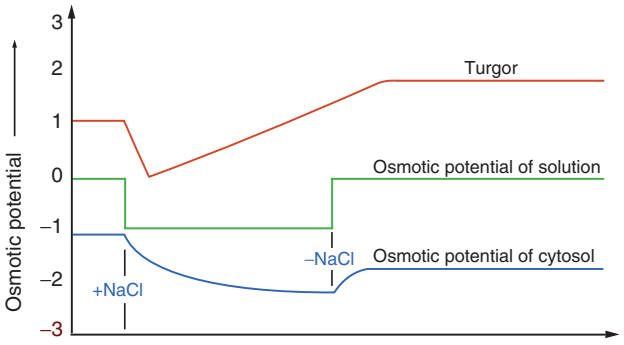

Figure 10.14 Phase response in salt stress. Addition of NaCl to the soil brings the osmotic potential outside the plant close to that inside, which stops water uptake. As a result, turgor drops immediately. After this alarm phase, plants respond to the salt-stress-inducing acclimation processes, for example, production of compatible solutes lowering the osmotic potential in the cytosol and by this creating a sufficient potential difference for new water uptake. The result is increasing turgor. After salt stress, those plants often keep a lower osmotic potential in the cytosol, being forearmed against the upcoming salt stress conditions.

potential difference. This leads to impaired **water uptake and decreasing turgor** (Figure 10.14). This type of osmotic stress, similar to drought or freezing stress, results in a water deficit within the plant cell. Therefore, damaging effects, as well as stress response mechanisms discussed already in Section 10.3.3, are partly similar under these three stress conditions.

Besides this major osmotic effect of salinity stress, higher concentrations of specific ions in the soil can also cause toxic effects. High concentrations of ions such as Na^+ or Cl^- affect **ion uptake systems** and cause aberration of ion homeostasis in the plant cells. High amounts of Na^+, for example, can cause a decrease in K^+ or can affect Ca^{2+}-flux. Ion homeostasis is important for the preservation of enzyme activities and also for signaling cascades, for example, Ca^{2+} signaling. An aberration can affect central cellular processes and cause severe damages. Impairment of enzymatic functions results in disturbances of metabolic pathways, which can lead to the accumulation of toxic products, for example, ROS.

10.5.3 Salinity Tolerance

While most plants suffer during salinity stress, there are some plants that easily survive or even show better performance under high salt concentrations. These plants are called *halophytes*. In general, there are great differences in salt tolerance. Some crop plants, for example, barley, can tolerate higher salt concentrations in the ground water without showing any loss in yield, whereas other crops like wheat are already severely injured. *Arabidopsis thaliana* (*Brassicales, Rosidae*) is rather sensitive to salt stress. Concentrations of 200 mM NaCl result in severe damages, visible as bleaching of the leaves and also growth reduction. In contrast, the halophyte *Thellungiella halophile,* which is genetically similar to *Arabidopsis thaliana,* can survive concentrations as high as 600 mM NaCl.

There are several strategies to gain salt tolerance. A prerequisite is the understanding of the underlying molecular pathways comprising signal sensing and transduction via transcriptional and posttranscriptional control, which are addressed in the next chapter. One way to elucidate this complex stress response network in plants is to use Omics techniques (see Chapter 18). The regulatory network downstream induces processes that in the end effectuate salt tolerance. Such adaptation processes are summarized in Section 8.5.5.

10.5.4 Signal Sensing and Transcriptional and Posttranscriptional Control

Salt stress causes disturbance of osmotic balances (accumulation of specific ions) and changes in metabolic pathways. **Membrane-based osmosensors** (e.g., AtHK1) are assumed to function as primary sensors during salt stress. Several investigations indicate the involvement of G-protein-coupled receptors (see Section 7.4.7). Signal transduction was shown to act via different signal molecules (see Chapter 7), such as ABA, jasmonic acid, ethylene, and salicylic acid, phospholipids, Ca^{2+}/calmodulin and includes **MAP (mitogen-activated protein)** kinase pathways and also pathways acting via 14-3-3 proteins, a family of conserved regulatory molecules expressed in all eukaryotic cells. The name of this protein family refers to a particular elution and migration pattern during their biochemical isolation.

Downstream the signaling cascades in the nucleus, the information is converted into temporal and spatial **changes in gene expression patterns**. A set of transcription factors belonging to the families of basic helix-loop-helix, DRE/CRT-binding proteins, ribosome-recycling factor,

zinc finger protein, and Ser/Arg-rich protein type transcription factors could be identified. They interact with the corresponding *cis-elements* in the promoter regions of salt stress-regulated target genes. Some of these transcription factors are known to also act in other stress signaling pathways, for example, the CRT/DRE-binding proteins involved in drought and cold stress signaling (see Sections 10.3.3 and 10.3.4). Besides these transcription factors, proteins binding to RNA or being involved in RNA synthesis and processing are involved in salt stress adaptation. Recent findings in proteomics approaches with salt stressed plants indicate the involvement of other regulatory levels, for example, **epigenetic type of control** via histone and **DNA modifications and RNA degradation** via ribonucleases. In addition to these regulators at the DNA/RNA level, proteins involved in protein biosynthesis, protein processing, transport, and degradation participate in the final realization of the salt stress response network.

MicroRNAs are intimately involved in the regulation of plant stress responses (see Section 7.7). miR159 accumulates in response to ABA (Figure 10.15). Salinity or drought stress, as well as UV stress and increased ABA levels, induce expression of its corresponding gene. The hairpin-like structure of the pri- and pre-miRNA is recognized and eventually cleaved into the small miRNA via the DCL1 system (dicer proteins), transported out of the nucleus by HST1 and then interacts with its target mRNA via AGO1 (Argonaute proteins), finally resulting in cleavage of target mRNAs or translational inhibition.

These findings elicited increasing interest in the involvement of miRNAs in plants stress response, and today we know several microRNAs (e.g., miR156, miR159, miR160, miR166, miR168, miR169, miR390, miR395, and miR398) to be involved in a complex stress-responsive regulatory network, acting downstream of stress signaling and accomplishing adaptation on physiological, metabolic, and morphological levels (see Section 7.7). Abiotic and biotic stressors induce miRNAs, which in turn mediate cross talk between different stress signaling pathways.

10.5.5 Target Processes

Transcriptional and posttranscriptional control mechanisms determine which genes are up-regulated or down-regulated in response to salt stress, which proteins are synthesized and play a role in the salt stress response, or which proteins are being degraded. The genes and proteins involved in salt stress response can be classified in several groups based on their cellular functions.

1) **Water and ion transport**: Salt stress generates water deficit in cells and also disturbs ion homeostasis. As a response, aquaporins are induced that function in water transport across membranes. Ion transporters are also regulated by salt stress involving the action of H^+-ATPases pumping protons and thereby creating more driving force for Na^+ transport and ion channel proteins.
2) **Osmotic protection**: To ensure osmotic homeostasis, plants can accumulate compatible solutes. Various osmolytes can be synthesized, such as proline, glycine betaine, or specific carbohydrates (see Figure 10.3). Proline biosynthesis is regulated in response to changes in osmotic homeostasis (Figure 10.16). Its biosynthesis starts from glutamate. Cytosol and plastid located P5CS (pyrroline-5-carboxylate synthase) and P5CR

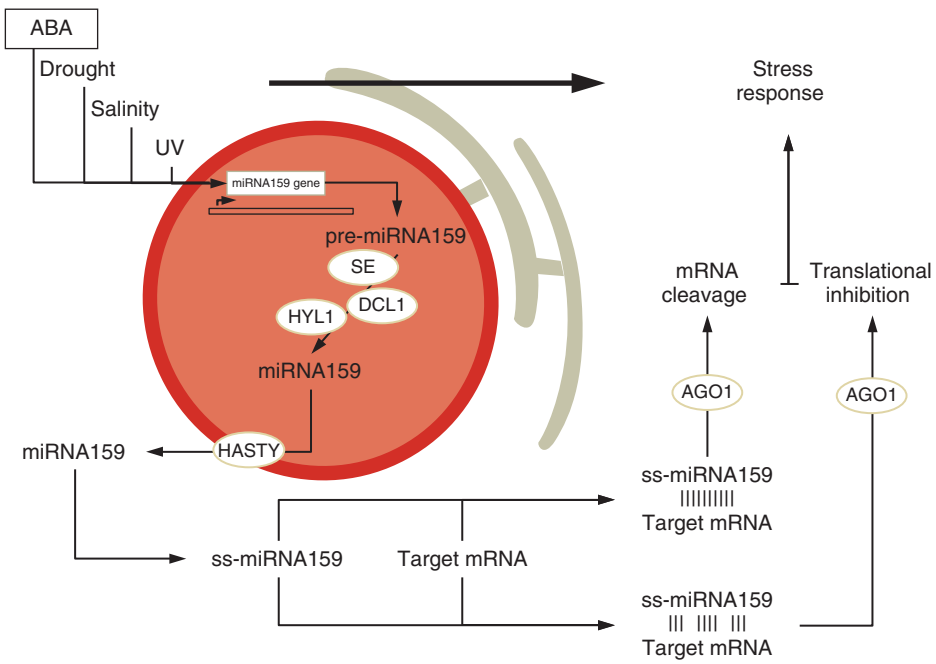

Figure 10.15 Role of miRNA 159 under drought and other abiotic stress response. (Courtesy of D. Dobritzsch.)

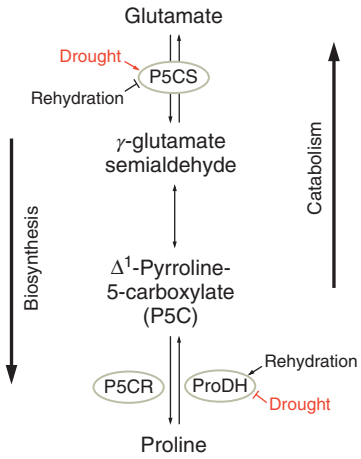

Figure 10.16 Proline biosynthesis under drought stress.

(pyrroline-5-carboxylate reductase) are involved, while the ProDH (proline dehydrogenase) catalyzes the back-reaction of the pathway, which is localized in the mitochondria. The regulation of these enzyme activities via transcriptional or posttranscriptional control sensitively adjusts the levels of proline and glutamate. During salt stress the biosynthesis is up-regulated and the catabolic pathway is repressed, causing an increase in osmotically active proline. After the stress, these processes are reversed, shifting back the balance to glutamate. In addition to its function as an osmolyte, proline maintains the structure of proteins. It operates as free radical scavenger, buffers metabolic reactions, and acts as signaling molecule modulating gene expression.

Glycine betaine (see Figure 10.3) is also induced in some plants in response to salt stress. It acts in numerous halophytic bacteria and plants. Salt stressed spinach leaves are able to accumulate this quaternary ammonium compound in chloroplasts and cytosol to more than 250 mM. ß-alanine betaine occurs only in some species of the *Plumbaginaceae* (*Caryophyllales,* core eudicotyledones). In the glycine betaine biosynthesis, the choline monooxygenase (CMO) is up-regulated (see Figure 10.17). Glycine betaine is involved in the protection of protein complexes and membranes, mainly the thylakoid membranes, under osmotic stress. Some proteins have protective functions themselves. Members of the large LEA protein family (see Section 10.3.3) have been shown to protect biomolecules during osmotic stress. These proteins are up-regulated during salinity stress.

3) Detoxification and protection: Salinity stress often causes accumulation of toxic **ROS** (see Chapter 8). Especially, electron transport chains in the chloroplasts and mitochondria are impaired by increased formation of ROS followed by fast induction of their detoxification systems (see Chapter 8). Besides proline, the osmolyte **mannitol** (see Figure 10.3) can act as scavenger for hydroxyl radicals in some plants. In addition, other protecting agents can accumulate. One example is **dimethylsulfoniopropionate**, which protects the cells of halophytic marine plants (see Section 6.2.4.2 and Figure 6.14)
4) Primary metabolism: **Photosynthetic performance** reacts very sensitively to several stress conditions including salt stress. Salinity causes water deficit in the plant, which via ABA quickly leads to stomata closure and thereby CO_2 deficiency. Another point is that chloroplasts are highly reactive compartments. Loss of water and/or disturbances in ion homeostasis rapidly causes a disorder in the serially connected electron transport enzymes. Halophytes have evolved efficient mechanisms to adjust photosynthetic performance under stress conditions. This can be via different expressions of photosynthesis related genes, and induction of quenching mechanisms, transforming light energy that has been absorbed in excess into nondestructive heat.

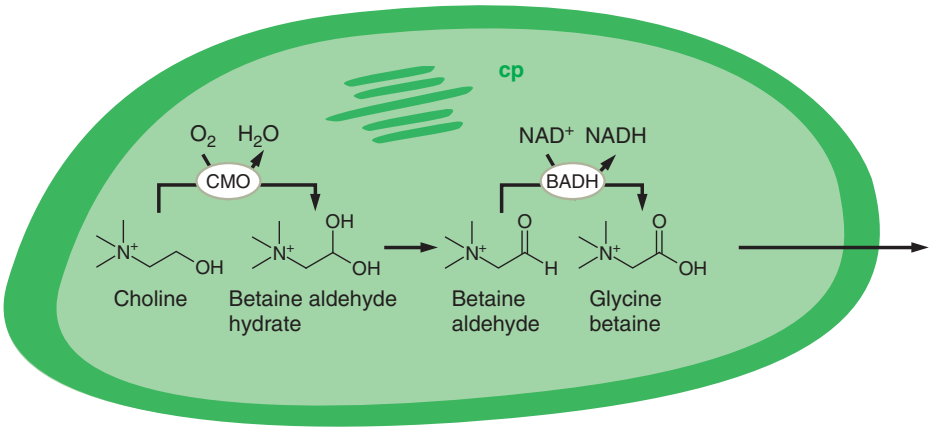

Figure 10.17 Biosynthesis of glycine betaine in *Hordeum vulgare*. CMO – Choline Monooxygenase; BADH – Betaine Aldehyde Dehydrogenase.

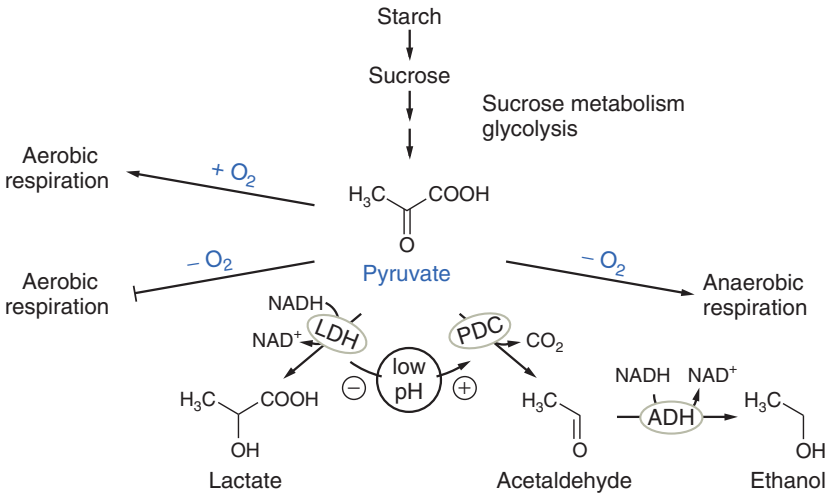

Figure 10.18 Energy metabolism under anaerobic conditions.

10.6 Flooding Stress

10.6.1 Causes of Flooding

Besides stress caused by insufficient amounts of water, some plants encounter the opposite situation: too much water. Permanent flooding is a problem for plants living in **border areas of rivers or swampland**. At these locations, only specialists can survive, for example, mangroves (*Rhizophora* sp., *Malpighiales, Rosidae*; see Section 6.2.2.6) or bald cypresses (*Taxodium distichum, Coniferales, Coniferophyta*). Rice (*Oryza sp., Poales, Liliopsida*), one of the world's leading crop plants, has been intensively studied concerning its adaptation to submerged growth. Other plants also frequently suffer from flooding, either in areas that overflow when rivers burst their banks or when soil compaction inhibits run off.

10.6.2 Effects on Cellular Energy Status

Flooding causes **oxygen deprivation**, mainly at the plant root system. Normal oxygen partial pressure in atmosphere is about 21 kPa. Some plants already show stress symptoms when it falls below 20 kPa. A decreased oxygen pressure is called *hypoxia*, while total loss of oxygen (<1 kPa) is called *anoxia*. The oxygen deprivation directly and quickly affects energy balance in the plant. Even a small drop in oxygen pressure causes a decrease in the ATP/ADP ratio, indicating impaired energy metabolism and altering plant performance at various levels. One obvious symptom is a quick stop in growth.

The reason for the **decrease in ATP/ADP level** is that oxygen is the final acceptor of the mitochondrial electron transport chain, which is the driving force for fermentation, is readily turned on (see Section S1.3.5). The first step in this pathway is the formation of acetaldehyde from pyruvate by the enzyme pyruvate decarboxylase (PDC). This enzyme has a low pH optimum at about pH 6. The second step in ethanol fermentation is the reduction of acetaldehyde to ethanol via the action of the alcohol dehydrogenase (ADH) consuming NADH again. The fermentation pathways ensure total formation of 2 molecules ATP per one molecule of hexose during glycolysis and also removal of accumulated NADH, summing up to 4 ATP per sucrose molecule. Compared to aerobic respiration where 38 ATP molecules are generated out of one molecule hexose, this is a very inefficient outcome. In many plants, expression of *ADH* genes is regulated in response to oxygen deprivation.

10.6.3 Acclimation to Hypoxia

Besides the direct effects of oxygen deprivation on energy metabolism, there are sophisticated response networks regulating additional adaptation mechanisms to hypoxia (see Chapter 8). In these networks the phytohormone **ethylene** plays a central role. A favored model plant to analyze these pathways is rice. Submergence of aerial rice tissues results in the production of ethylene, which, via the action of ethylene response factors (e.g., SNORKEL1/2), induces gibberellic acid mediated rapid shoot elongation and escape of leaves near the water surface. Another acclimation process is the formation of **aerenchyma**. Ethylene plays a central role in the formation of this specialized tissue. Via G proteins and/or ROS cell death processes are induced in specific cells. In the end, cavities are formed, which as hollow tubes allow the rapid exchange of air between shoot and root and are even used as air storage.

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organic
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concentrations
function
signalling

11
Mineral Deficiencies

11.1
Mineral Requirement and Insufficiencies

Edgar Peiter

Overview

Mineral elements perform a great diversity of functions in plants: they are essential in large molecules, such as nucleic acids, proteins, and lipids, regulate osmotic pressure, carry electrons, catalyze chemical reactions, and transmit cellular signals. Depending on the particular function, essential elements are required in very different amounts. To satisfy the plant's needs, they need to be taken up from the soil, where they may be adsorbed to soil minerals or soil organic matter. This association with the soil matrix is determined by the physicochemical characteristics of the specific mineral, which impinges on its movement toward the root. Plants are able to respond to a limited supply of mineral nutrients by improving the availability, uptake, and use of the limiting element. Morphological alterations of the plant go hand in hand with physiological adaptations. For example, root system architecture and root hair formation are regulated by the availability of nitrogen, phosphorus, and iron. When a decrease in soil pH improves the availability of a nutrient, as it is the case for phosphorus or iron, plants may release increased amounts of protons. Limited supply of these two nutrients is also counteracted by root activities that decrease the redox potential of the rhizosphere. To increase the uptake of limiting nutrients, transport proteins are induced in the outer root cell layers. In times of limited supply, some of the functions of an essential element can be taken over by other elements or by alternative mechanisms.

All these measures to optimize the acquisition and use of mineral elements are tightly regulated, either locally, that is, determined by the availability of the nutrient in the surrounding soil, or systemically, that is, by the supply status of the entire plant. The signaling networks that determine the response to nutrient deficiencies involve the full array of transcriptional, posttranscriptional, and post-translational mechanisms covered in this book. In addition, phytohormones and sugars are important factors that regulate plant responses to nutrient deficiencies. The primary sensors that detect the supply status or the external concentration of an individual nutrient are known only in a very few instances.

11.1.1
The Essentiality of Mineral Nutrients for Plants

Living organisms – including plants – consist primarily of water (see Chapter 10). The remaining 10–20%, the dry matter, is made up of more than 90% of carbon, oxygen, and hydrogen. With less than 1% of the plant weight, mineral elements contribute only a minute amount in terms of quantity (Figure 11.1). Yet none of the plant functions described in this book would be possible without mineral nutrients. Plants that lack essential mineral nutrients, therefore, employ strategies to enhance their acquisition and optimize their use. If those strategies are insufficient, the plant shows symptoms of nutrient deficiency. Plants differ widely in their ability to take up nutrients (their **nutrient uptake efficiency**). Likewise, plants differ in the amount of biomass they can produce from a given amount of nutrient taken up (their **nutrient use efficiency**). Both parameters are of great importance: they can determine the competitiveness of a wild plant species or ecotype on a given soil and the productivity of a crop species or cultivar under a given level of fertilization.

Not all chemical elements are required by plants, and some are rather toxic (see Chapter 12). Generally, an element is considered a **nutrient** if: (i) the plant cannot complete its life cycle without it; (ii) a specific function of the element is known; and (iii) a deficiency of the element causes specific symptoms. Because of their specific roles, mineral nutrients are required in different amounts (Figure 11.1). Elements (see Section S1.3.8) required in excess of 0.5 mg kg^{-1} dry matter are called *macronutrients* (Table 11.1), whereas those needed in smaller amounts are called *micronutrients* or trace elements (Table 11.2).

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

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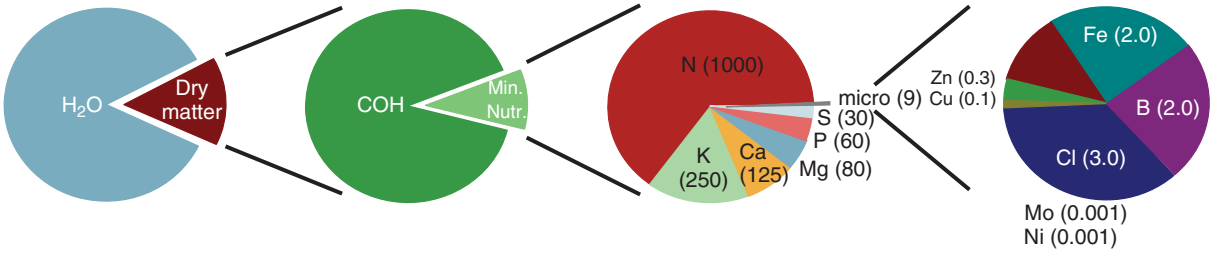


Figure 11.1 Average elemental composition of a plant. The third and fourth pie charts display the known macronutrients and micronutrients, respectively. Values are given in µmol g^{-1} dry weight. Values according to Marschner (2012).

Table 11.1 Uptake, assimilation, and important functions of the macronutrients of plants, listed in the order of their average concentration in plant tissue (Figure 11.1).

Element	Form taken up by plants	Assimilation and some functions in plants
Nitrogen (N)	**Nitrate** (NO_3^-) from aerobic soils, **ammonium** (NH_4^+) from flooded and acidic soils; **urea** ($CO(NH_2)_2$) also taken up, but quickly converted to NH_4^+ and then to NO_3^- by soil bacteria. **Amino acids** and **peptides** can be taken up if not mineralized in the soil (important in cold climates). **N_2** utilization in symbiosis with diazotrophic microorganisms; bacterial symbionts reduce N_2 and release NH_4^+ into host cytosol	NO_3^- is reduced to NH_4^+ by cytosolic nitrate reductase and plastidic nitrite reductase. NH_4^+ is assimilated into glutamate mainly by the glutamate synthase (GS)-GOGAT cycle (see Section S1.3), and transamination reactions yield further **amino acids,** providing the building blocks of **proteins**. N is also a constituent of many **heterocyclic compounds**, such as purines and pyrimidines (see Section S1.3).
Potassium (K)	**K^+**	K remains as diffusible cation in the plant and is never incorporated into organic matter. Its concentration in the cytosol is kept relatively constant at around 120–150 mM, whereas the vacuolar K concentration can vary widely. A major function of K is to create **turgor** pressure. The opening and closing of stomata depends largely on the movement of K into and out of the guard cells. Following its positive charge, K **interacts with** negatively charged **proteins** and thereby impinges on their conformation and activity. A counterflow of K against H^+ across membranes provides an **electrical equilibration**, which facilitates increased H^+-ATPase activity.
Calcium (Ca)	**Ca^{2+}**	Ca acts as **structural crosslinker** because of its divalent positive charge. In plant **cell walls**, pectins (heterogeneous, branched, and hydrated polysaccharides rich in D-galacturonic acid) are connected by intra- and intermolecular Ca bridges. Cell walls of dicotyledonous plants generally contain higher amounts of pectin thnn those of monocots, and, therefore, dicots have a higher Ca requirement. In addition, Ca stabilizes **biomembranes** by linking the negatively charged phosphate moieties of phospholipids. Ca is a ubiquitous **second messenger**, and elevations of Ca concentrations (calcium signals) in cytosol or organelles trigger specific transcriptional or posttranslational events in response to many stimuli.
Magnesium (Mg)	**Mg^{2+}**	Mg is the central atom of the chlorin ring of **chlorophyll**. It is also essential for **phosphorylation** events because it links ATP to the protein to be phosphorylated. Mg **activates** some **enzymes**, for example, rubisco by binding to its anionic carbamate. As ribosome coupling factor, Mg is required for **protein synthesis**.

Table 11.1 (*Continued*)

Element	Form taken up by plants	Assimilation and some functions in plants
Phosphorus (P)	**Orthophosphate** (HPO_4^{2-})	Phosphate ester bonds of the nucleotide **adenosine trisphosphate** (ATP) represent a general energy source, not in plants alone (see Section S1.2). By linking the nucleotides in the **nucleic acids** DNA and RNA, phosphate has also structural functions. In **phospholipids,** phosphate links glycerol and a hydrophilic group. Phosphorylation of a specific phospholipid, phosphatidyl inositol, yields phospholipids with signaling functions (see Section 7.4.7). **Phosphorylation** also activates metabolic intermediates (Section S1.3) and regulates protein function by conformational alteration (Section 7.5).
Sulfur (S)	**Sulfate** (SO_4^{2-}) and gaseous **sulfur dioxide** (SO_2)	SO_4^{2-} is activated by adenylation to yield **adenosine 5′-phosphosulfate** (APS). S is incorporated into organic matter in ester-bound (oxidized) and in reduced form. Ester-bound S is found in many **secondary metabolites**, for example, glucosinolates, and in **sulfolipids**, which are a major constituent of thylakoid membranes, especially during P deficiency. APS can be reduced to sulfite (SO_3^{2-}) by APS reductase, and further to sulfide (S^{2-}) by sulfite reductase. Transfer of sulfide to *O*-acetylserine yields the amino acid **cysteine**. The ability of cysteine to form disulfide bonds enables the stabilization of inter- and intraprotein interactions. Cysteine is also incorporated in **glutathione** (GSH), an enzymatically synthesized pseudo-tripeptide consisting of glycin, cystein, and glutamate. Because of its ability to alter its redox state by dimerization (thus forming glutathione disulfide (GSSG)), GSH is a central regulator of cellular redox status. In **Fe-S clusters**, S serves to coordinate Fe atoms within the protein structures, for example, in cytochrome b_6f (2Fe-2S) or nitrate reductase (4Fe-4S).

11.1.2 The Availability of Mineral Nutrients in the Soil

Except for some volatile compounds, such as SO_2 or NH_3, that may be absorbed by the plant's aerial parts, and minerals applied by foliar fertilization of crops, mineral nutrients are acquired from the soil. The mineral composition of a nonfertilized soil depends on its parent material. However, mineral elements incorporated into the crystal lattices of soil particles are available for uptake by the plant only after weathering. Therefore, despite very high total amounts of certain essential elements, such as Fe, in most soils, plants can suffer from deficiency because of the unavailability of the nutrient. However, as described below, plants have developed mechanisms to increase the availability of nutrients.

Clay minerals are particularly important for the supply of plants with cationic mineral nutrients. They are derived from mica, that is, sheet silicates with interlayer cations (Figure 11.2a). Layers of the so-called 2 : 1 clay minerals are composed of two sheets of SiO_2 tetrahedrons connected by a sheet of $Al(OH)_3$ octaedrons. The layers are negatively charged because of **isomorphic replacement** (i.e., similar size, but different charge) of Si^{4+} by Al^{3+} or of Al^{3+} by Fe^{2+} or Mg^{2+}. In many cases, the negative charges are initially equilibrated by nonhydrated K^+ ions, which fit into the tetrahedron gaps facing the interlayer space. Diffusion of the K^+ out of the interlayer and replacement by other, hydrated cations, causes a widening of the interlayer space, which releases K^+ and generates cation exchange sites. As this diffusion depends on a depletion of K^+ in the surrounding soil solution, high-affinity uptake of K^+ by plant roots is able to render previously nonexchangeable interlayer K^+ plant available.

A second class of soil particles able to adsorb cations are humic substances. These organic macromolecules contain functional groups carrying negative charges, such as carboxyls ($-COO^-$) and phenolic hydroxyls ($-O^-$). As the dissociation of those weakly acidic groups is pH-dependent, acidification by plant roots leads to a release of adsorbed cations.

In contrast to cation exchange, the capacity for unspecific binding of anions is very limited in soils of temperate climates. Therefore, anionic nutrients, such as NO_3^-, are prone to leaching. An exception is phosphate, which covalently binds to hydroxides of iron and aluminum by ligand exchange (Figure 11.2b). This adsorbed phosphate can be released again by a further ligand exchange reaction of the Fe/Al hydroxide matrix with organic anions, which, as discussed below, are released by some plants in response to P deficiency. In alkaline soils, P is present as poorly soluble calcium phosphates, which can be dissolved by acidification. The organic fraction of the soil may also contain considerable amounts of P, primarily as phytate (inositol hexaphosphate).

Table 11.2 Uptake and important functions of plant micronutrients, listed in the order of their average concentration in plant tissue (Figure 11.1).

Element	Form taken up by plants	Assimilation and some functions in plants
Chlorine (Cl)	**Chloride** (Cl^-)	Cl^- is incorporated with Mn and Ca in the water-splitting complex of **photosystem II**. In some plant species, Cl^- plays an essential role in **stomatal regulation.**
Boron (B)	**Boric acid** (H_3BO_3)	B deficiency affects many metabolic processes, but very few specific functions are known so far. B forms ester bonds with diols and polyols. B stabilizes **cell walls** by linking apiose moieties of the pectin rhamnogalacturonan II. B also forms **complexes with ribose** and thus binds to RNA and NAD^+.
Iron (Fe)	**Fe^{2+}** or **Fe^{3+}-phytosiderophore**	Fe is present in **Fe-S clusters**, in the porphyrin ring of **heme**, and as **nonheme Fe**. Because of its ability to reversibly alter its valency between Fe^{2+} and Fe^{3+}, it plays a role in many **redox reactions,** such as photosynthetic light reactions and respiration, where it is the central element of the cytochrome heme groups. Nitrate and nitrite reductases also contain heme-bound Fe. Fe is also the electron acceptor in the Fe_2S_2 protein **ferredoxin**, which shuttles electrons from photosystem I to nicotinamide adenine dinucleotide phosphate $(NADP)^+$ in linear photophosphorylation (or back to cytochrome b_6f in cyclic photophosphorylation), to nitrite reductase, to glutamate synthase (GOGAT), or to sulfite reductase.
Manganese (Mn)	**Mn^{2+}**	Mn is present in plants in the oxidation states Mn(II), Mn(III), and Mn(IV). It functions as the redox-reactive center of **the water-splitting complex** in photosystem II and as a constituent of the **Mn-superoxide dismutase** (SOD). In plants, Mn is a **cofactor** of over 30 enzymes.
Zinc (Zn)	**Zn^{2+}** or **Zn^{2+}-phytosiderophore**	Zn is a **cofactor** in several hundred enzymes. Many enzymes in nucleic acid metabolism (e.g., DNA and RNA polymerases, histone deacetylases) are Zn-dependent. Although Zn itself is not redox-active, it is a constituent of **CuZn-SOD**. Zn has a **structural role** in the DNA-binding domain of Zn finger transcription factors. In C_4 photosynthesis, Zn is a component of **carboanhydrase**.
Copper (Cu)	**Cu^{2+}**	Cu acts as the electron acceptor in **plastocyanin**, which transfers electrons from cytochrome b_6f to photosystem I. Cu is also essential for **lignin biosynthesis**, and thus for mechanical stability and physical pathogen resistance. As a constituent of the chloroplast-localized **CuZn-SOD,** it contributes to the detoxification of superoxide radicals.
Molybdenum (Mo)	**Molybdate** (MoO_4^{2-})	Mo is part of the FeMo cofactors of **nitrate reductase** and bacterial **nitrogenase,** and thus plays an essential role in nitrogen assimilation and N_2 fixation.
Nickel (Ni)	**Ni^{2+}**	Ni is a constituent of the urea-hydrolyzing enzyme **urease**.

11.1.3 Mineral Nutrient Movement Toward and Uptake by the Plant Root

The path mineral nutrients take toward the plant root depends on the strength of their binding to soil particles. To understand the rationale of plant responses to nutrient deficiencies, it is thus important to have a basic comprehension of nutrient mobility in the soil. As discussed above, anions (except phosphate) are generally weakly adsorbed and can be present at high (millimolar) concentrations in the soil solution. Similarly, cations that are very abundant or less competitive than K^+ for adsorption to clay minerals are also abundant in the soil solution. Those nutrients, in particular NO_3^-, Ca^{2+}, and Mg^{2+}, reach the plant root primarily by **mass flow**, that is, with the flow of water driven by plant transpiration, if the soil is not too dry. In the specific case of phosphate, adsorption to the abundant iron hydroxides diminishes its concentration in soil solution to the micromolar range, and the amounts delivered by mass flow are thus very low. In contrast, by taking up phosphate with high affinity, the plant root creates a steep concentration

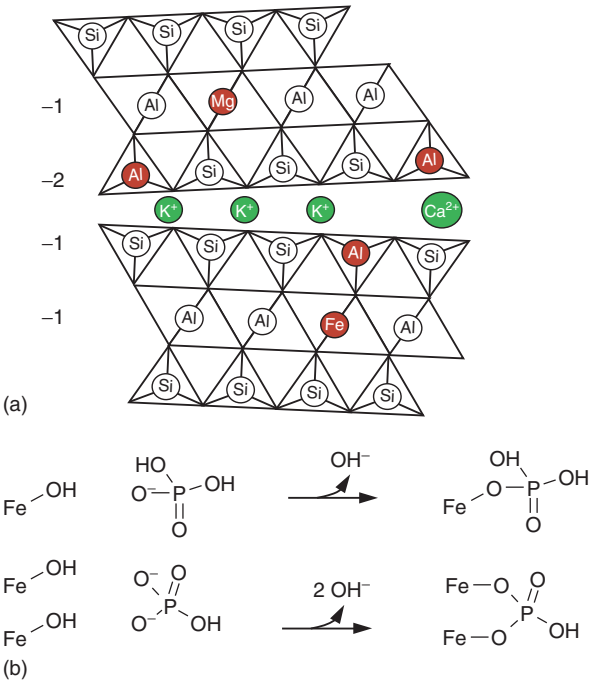


Figure 11.2 (a) Cation adsorption by 2 : 1 clay minerals. Clay minerals are negatively charged because of isomorphic replacement of Si and Al with elements of lower valency (red). The negative charge is compensated by interlayer K^+ ions, which can slowly diffuse into soil solution and be replaced by hydrated larger cations, such as Ca^{2+}, causing a widening of the interlayer space and an increased accessibility for cation exchange. (b) Phosphate adsorption to Fe and Al hydroxides. Phosphate may replace the –OH groups in a ligand exchange reaction. The adsorption may be monodentate (upper reaction) or bidentate (lower reaction).

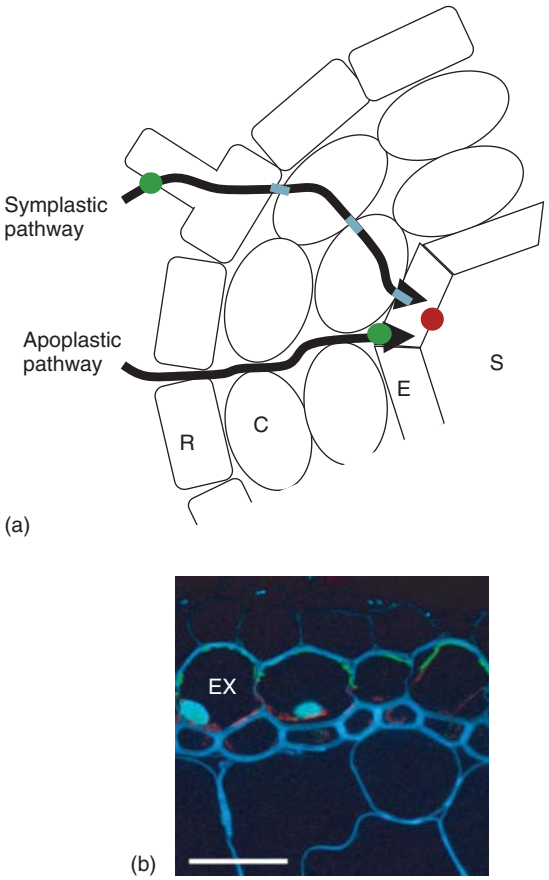


Figure 11.3 (a) Uptake and radial transport of mineral nutrients in plant roots. In the apoplastic pathway, the solute crosses the root through cell wall and water-filled intercellular spaces. The endodermis is the latest point of uptake into the cytosol through transport proteins (green). In the symplastic pathway, the nutrient is taken up by rhizodermal cells and moves from cell to cell via plasmodesmata (blue). For movement to the shoot through the xylem vessels, nutrients have to be unloaded from the symplast into the root stele apoplast through exporter proteins (red). R rhizodermis, C cortex, E endodermis, S stele. (b) The polar localization of importers and exporters guides a polar transport toward the root stele. An an example, localization of the silicon transporters Lsi1 (green) and Lsi2 (red), facilitating import into and export out of the rice exodermis (EX), respectively, is shown. Reprinted from Ma *et al.* (2007) by permission from Macmillan Publishers Ltd. Lsi – Low Silicon Rice.

gradient, which is the driving force for the **diffusion** of phosphate to the root surface. Similarly, strongly adsorbed cations, such as K^+, reach the root surface mainly by diffusion. The diffusion gradients (**depletion zones**) extend only a few millimeters from the root. Morphological plant parameters that increase the root-surface-to-soil-volume ratio are, therefore, particularly important for an efficient exploitation of diffusion-delivered nutrients. On the other hand, conditions that limit root growth and metabolism, such as soil compaction (decreasing the pore size) and waterlogging (decreasing the O_2 availability), impair the supply of diffusion-delivered nutrients. Besides mass flow and diffusion, nutrients can reach the plant root by contact exchange (**interception**), which involves the direct exchange of cations between negatively charged soil particles and cell walls within a distance of a few nanometers. This mechanism, however, is considered to be of minor importance as the soil volume in direct contact with plant roots is limited.

Once at the root surface, nutrients may enter the root by an **apoplastic pathway**, that is, movement through the cell wall, or a **symplastic pathway**, that is, uptake into the cytosol and movement from cell to cell through plasmodesmata (Figure 11.3a). The endodermis, a cell layer with radially modified walls (the **Casparian strip**), presents a barrier for apoplastic solute movement and is the latest point for a nutrient to enter the symplasm. The permeation of the plasma membrane is mediated by transport proteins (see Section S1.2). Those transporters are energized by the proton motive force across the plasma membrane. Nutrients are thus taken up by proton-coupled symport, and the induction of high-affinity transporters is one important measure of the plant to counter nutrient deficiencies. Because of the cytosol's negative charge, cations, such as K^+, Ca^{2+}, and Mg^{2+}, can be taken up by uniport (see Section S1.2) through channel proteins. In the case of K^+ this influx takes place even against its concentration gradient.

As nutrients have to cross the root radially, a polar localization of transport proteins mediating import into and export out of the endodermis has been demonstrated for some elements, such as B and Si (Figure 11.3b). A similar polarity is also expected for transporters of other nutrients.

11.1.4 Plant Responses to a Variable Mineral Nutrient Availability

To avoid or alleviate mineral nutrient deficiency, plants respond to a low supply by improving the availability, uptake, and use of the limiting nutrient. **Morphological responses** may involve the modification of the root system architecture by altering root length and branching and the formation of more and longer root hairs. In addition, plants may initiate a battery of **physiological responses**: To mobilize nutrients, roots of nutrient-deficient plants may decrease the pH and the redox potential of the rhizosphere soil and release exudates of various kinds, some examples of which are given below. Transport systems with increased substrate affinities may be induced. At a whole plant level, some nutrients can be retranslocated from older leaves via the phloem into actively growing sinks, and metabolic pathways requiring a limited mineral can be bypassed. As discussed in Chapters 5 and 15, plants also harness the power of **microorganisms** to improve their nutrient acquisition. In the rhizosphere, root-associated bacteria fix N_2, mobilize nutrients, and produce plant hormone-like compounds that alter root morphology. These microbes forage on plant-derived exudates (e.g., sugars, carboxylates, and amino acids). Plants may also enter two kinds of symbioses, which make significant, sometimes essential, contributions to the supply of nutrients. **Mycorrhizal fungi** extend the root system physically and improve the supply of diffusion-delivered minerals, in particular, phosphate, of almost all land plants. Notable exceptions include plants with specific mechanisms to mobilize phosphate, such as the *Chenopodiaceae* (*Caryophyllales,* core eudicotyledons) and the *Brassicaceae* (*Brassicales, Rosidae*). **N_2-fixing bacterial symbionts** (rhizobial bacteria, actinomycetes, cyanobacteria; see Section 5.2.2) supply the host plant directly with fixed nitrogen in the form of ammonium.

To launch these responses to mineral nutrient deficiencies, the plant needs to assess its supply status and/or the availability of the nutrient, and to subsequently transduce this information into an appropriate action. A general question is whether this information processing is a local or a systemic process. Both can occur, even for the same nutrient. With very few exceptions, the immediate sensors of internal nutrient status or external nutrient supply are unknown. However, the signaling networks that regulate deficiency responses have been unraveled to some extent, and it appears that many layers are involved: Regulatory events on transcriptional, posttranscriptional, and post-translational, as well as on hormonal and metabolic level are known and may interfere with each other, as the following examples show. The responses to a variable supply of the mineral nutrients N, P, Fe, Zn, and Cu have been studied particularly well. As already discussed, these nutrients have very different properties with respect to their requirement by the plant and their mobility in the soil. Plants have, therefore, developed specific strategies to optimize their supply and use in very different ways.

11.1.4.1 Nitrogen

To maximize the uptake ability and use efficiency of the quantitatively most important nutrient, plants respond to its availability with both morphological and physiological adaptations.

Morphological Adaptations NO_3^- promotes the elongation of already initiated lateral roots. NH_4^+ also alters root growth, but in contrast to NO_3^-, it stimulates lateral root initiation rather than elongation. The growth stimulation by NO_3^- and NH_4^+ is counteracted by a systemic repression exerted by a high N status of the plant.

Physiological Adaptations Plants operate transport systems to acquire NO_3^- with high and low affinity. The activity of both types is increased in N-deficient plants. In addition, high-affinity NO_3^- uptake, in particular, is also induced by the presence of NO_3^- in the root medium. NO_3^- uptake is mediated by the NRT1(Nitrate Transporter 1) and NRT2 protein families. Most NRT1s transport NO_3^- with low affinity, whereas NRT2s have a high affinity. An exception is the transporter AtNRT1.1 characterized in *Arabidopsis thaliana* (*Brassicales, Rosidae*). It is also called CHL1 because it was found in a screen for mutants resistant to the herbicide chlorate, which is taken up by NO_3^- transporters. Interestingly, AtNRT1.1 is a dual affinity transporter, which is switched between high- and low-affinity modes by (de-)phosphorylation. The reduction of NO_3^- by nitrate reductase (NIA) and nitrite reductase (NIR) is also regulated by the plant's NO_3^- availability: resupply of NO_3^- induces their expression within less than one hour and – with some delay – also their activity. The increased expression of NO_3^--related genes (e.g., *NIA1, NIR1, AtNRT2.1*) on NO_3^- perception is called the *primary nitrate response*. As with the described root growth responses, an ample supply of N counteracts this response. Similar to NO_3^-, the capacity for NH_4^+ uptake is increased in N-deficient plants. This correlates well with an increased expression of ammonium transporter (*AMT*) genes.

Regulation of N Availability Responses Two general N signaling pathways exist and interact in plants: direct regulation by NO_3^- and feedback repression by reduced N metabolites, for example, glutamine. The abovementioned dual-affinity NO_3^- transporter AtNRT1.1 has been identified as a key element of NO_3^- responses. Phosphorylation of the protein's T101 residue changes its transport kinetics

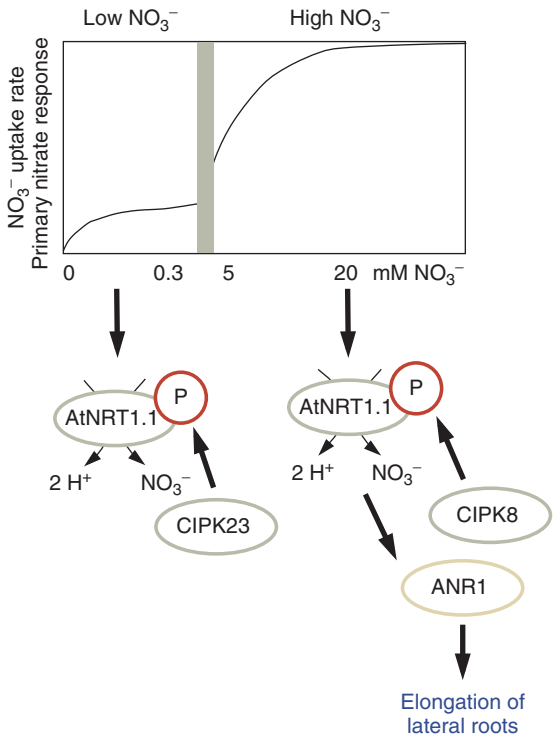


Figure 11.4 The transceptor AtNRT1.1 mediates NO_3^- uptake and regulates NO_3^- responses. At low external NO_3^- concentration, expression of the protein kinase gene *CIPK23* is upregulated. CIPK23 phosphorylates AtNRT1.1 at residue T101. At high external NO_3^- concentration, expression of another protein kinase gene, *CIPK8*, is upregulated, this time switching the protein to a low-affinity state, likely by the phosphorylation of another site. The low-affinity state of AtNRT1.1 is also required for the induction of NO_3^--induced elongation of lateral roots, which involves the transcription factor ANR1. AtNRT – *Arabidopsis thaliana* Nitrate Transporter; CIPK – CBL-interacting Protein Kinase; ANR – Arabidopsis Nitrate Regulated.

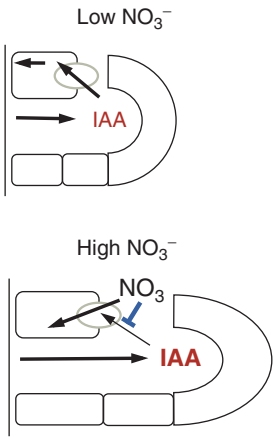


Figure 11.5 The transceptor (transporter and receptor) AtNRT1.1 (grey) directly determines lateral root growth in response to the auxin, indole 3-acetic acid (IAA). At low NO_3^- supply, the transporter moves IAA away from the root tip. At high NO_3^- supply, IAA transport is competitively blocked and IAA accumulates, resulting in an enhanced root growth rate. AtNRT – *Arabidopsis thaliana* Nitrate Transporter.

from low to high affinity (Figure 11.4). Phosphorylation is carried out by CIPK23, a calcineurin B-like interacting protein kinase (see Section 7.5). Interestingly, AtNRT1.1 is also required for the initiation of the NO_3^--triggered lateral root growth response, which suggests that the transporter may also be involved in NO_3^- sensing. This is supported by experiments on transport-defective *nrt1.1* single-point mutants, which showed that transport is not required for the sensing function, meaning uptake and signaling can be uncoupled. AtNRT1.1 was thus the first **transceptor** (transporter and receptor) identified in higher plants. Like its transport activity, the sensing function of AtNRT1.1 is regulated by phosphorylation: In response to a low NO_3^- concentration, phosphorylation by CIPK23 induces a low-level primary nitrate response. At high external NO_3^- availability, another CIPK protein, CIPK8, acts as a positive regulator for the low-affinity status of AtNRT1.1. Downstream of AtNRT1.1, the lateral root growth response to high NO_3^- depends on the MADS-box transcription factor *ANR1* (Arabidopsis Nitrate Regulated). Further complexity in the function of AtNRT1.1 has been added by findings that link the regulation of lateral root growth by AtNRT1.1 directly to auxin homeostasis (see also Section 7.3). Heterologous expression in oocytes showed that besides NO_3^-, AtNRT1.1 transports auxin, and that both substrates compete for transport. The expression of the transporter in the outermost cell layer of the lateral root tip suggests that it serves to move auxin away from the tip, thereby exerting a negative effect on growth (Figure 11.5). Low concentrations of NO_3^- competitively inhibit auxin transport by AtNRT1.1 and thus cause auxin to accumulate, resulting in enhanced lateral root growth.

N deficiency causes upregulation of the expression of the high-affinity uptake transporter *AtNRT2.1* through a systemic signal, whereas *AtNRT1.1* is downregulated. In turn, a high N status of the plant downregulates *AtNRT2.1*. This negative feedback response is dependent on the transcription factors LBD37/38/39 (Lateral Organ Boundary Domain). Interestingly, AtNRT2.1 also regulates lateral root growth under N limitation.

As for high-affinity NO_3^- transporters, expression of genes that encode AMTs are repressed by a high N status of the plant, and derepressed under N deficiency. Furthermore, AMTs were the first plant transport proteins shown to be under posttranscriptional allosteric feedback control: High NH_4^+ availability causes a phosphorylation event in the C-terminus of the protein, which functions as trimer with one pore per subunit. Phosphorylation of just one monomer causes a cooperative closure of all three pores in the complex, thereby amplifying the closure signal and accelerating the closure event.

11.1.4.2 Phosphorus

In contrast to the highly mobile NO_3^- anion, soil phosphate is firmly bound, and the zone of phosphate depletion

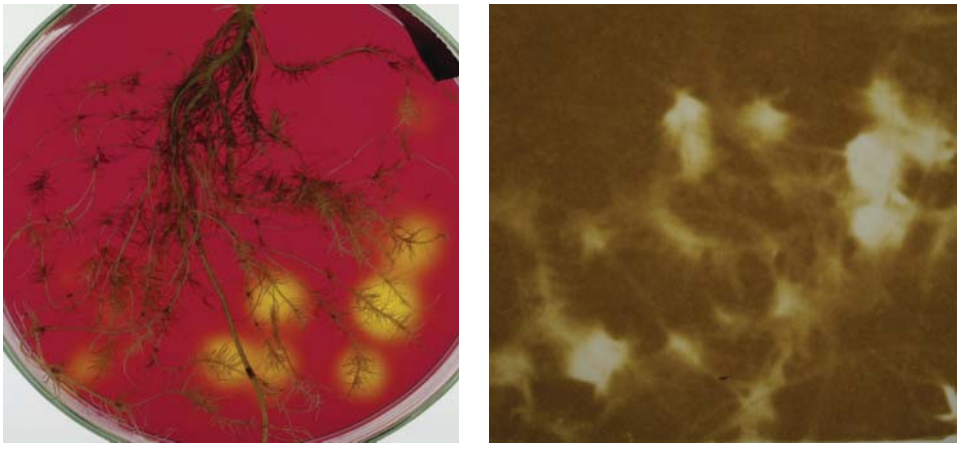
extends only a few millimeters around the root. This is reflected in the plant's mechanisms to counter P deficiency.

Morphological Adaptations P deficiency dramatically alters the shape of the plant. Generally, the proportion of root and shoot growth is shifted toward the underground organ. The root itself also undergoes morphological changes: growth and branching of lateral roots are increased, while growth of the main root is decreased. This results in a shallower and denser root system, which is optimal for an improved topsoil exploitation. In addition, root hairs grow in number and length, which has the effect of extending the P depletion zone around the root. The depletion zone is further expanded by the extraradical (i.e., outside the root) hyphae of symbiotic mycorrhizal fungi. Because of their smaller diameter, hyphae can penetrate into smaller pores than roots or root hairs. The formation of mycorrhizal symbioses (see Chapters 5 and 15) is promoted by P deficiency.

Physiological Adaptations Expression and activity of high-affinity phosphate transporters (PHTs) are strongly upregulated in the roots of P-starved plants. Split-root experiments, in which only a part of the root system is grown in low-P medium, and the other part sufficiently supplied with P, have shown that this response is regulated by the internal P status. Furthermore, the activity of the plasma membrane H^+-ATPase is increased to acidify the rhizosphere soil. This dissolves calcium phosphates (Figure 11.6a). Some plants exude high amounts of carboxylates (mainly citrate, oxalate, or malate) on P deficiency. Through a ligand exchange reaction, these organic anions render phosphates available, which are adsorbed to Fe/Al hydroxide surfaces. Phenolic compounds are also released by P-deficient plants. They reduce soil Fe^{3+}, thereby dissolving Fe^{3+}-P minerals and P-adsorbing Fe^{3+} hydroxides (Figure 11.6b). This increases the P concentration in soil solution. Because of their cytotoxicity, phenolics also inhibit the bacterial degradation of carboxylate exudates. To tap the resource of organically bound soil phosphate, P-deficient plant roots also exude acid phosphatases. Some plants, for example, *Brassica* species or the *Chenopodiaceae*, are particularly efficient in mobilizing poorly available phosphates by this array of mechanisms and thus do not rely on mycorrhizal symbioses. An extreme adaptation to soils low in P are the **proteoid roots**, dense bottle-brush-like lateral root clusters formed by the *Proteaceae* (*Proteales,* stem eudicotyledons) and by *Lupinus albus* (*Fabales, Rosidae*; Figure 11.6a). These structures, formed only under P deficiency, release high amounts of exudates and protons into a limited volume of soil and thereby extract soil phosphates with very high efficiency.

In P-limited plants, some P-containing molecules can be substituted to provide P for functions where the element is nonreplaceable. In particular, the proportion of phospholipids in biomembranes is decreased to free up phosphate. In their place, galactolipids (e.g., digalactosyl diacylglycerol, DGDG) and sulfolipids (e.g., sulfoquinovosyl diacylglycerol, SQDG) are incorporated. Accordingly, in *Arabidopsis*, expression of the genes encoding sulfolipid synthase and galactolipid synthase is induced by P starvation, and growth of the respective mutants is impaired under P-limited conditions.

Regulation of P Availability Responses Plant responses to P deficiency may be regulated locally or on a systemic level, which integrates the whole-plant P status. Generally, many morphological adaptations are determined locally, whereas physiological alterations make use of a systemic signal. In *Arabidopsis*, PHR1 (Phosphate Starvation Response),



(a) (b)

Figure 11.6 Activity of proteoid roots formed by P-deficient *Lupinus albus* (white lupin) plants. (a) Proteoid roots strongly acidify their rhizosphere, as visualized by the yellow staining of a pH indicator. (b) Proteoid roots release reducing phenolic exudates. A *L. albus* root system was placed onto filter paper containing MnO_2 (brown). Reduction of Mn(IV) to Mn^{2+} is indicated by a destaining, which is particularly intense in proteoid root zones. (Courtesy of Dagmar Rissel.)

a MYB (Myeloblastosis) transcription factor, is a central switch for the regulation of whole-plant P homeostasis and root uptake of P by PHT transporters (Figure 11.7a). *PHR1* expression itself is not altered by P starvation. Instead, PHR1 is activated by a posttranslational modification: The SUMO (Small Ubiquitin-like Modifier) E3 ligase SIZ1 covalently attaches SUMO proteins to PHR1. One target of the activated PHR1 is a micro RNA gene, miR399, the expression of which is induced by P starvation. miR399 is phloem-mobile and can be translocated to the root as a signal of P deficiency. In the root, miR399 targets the RNA of an E2 ubiquitin-conjugating enzyme, PHO2 (Phosphate). The attachment of ubiquitin triggers the degradation of a protein in the 26S-proteasome. PHO2 is, therefore, believed to negatively regulate the degradation of protein(s) involved in P uptake and translocation. Recently, this model has been confirmed by the finding that PHO1, which mediates the loading of P into the xylem, is degraded in a PHO2-dependent way. To add further complexity, miR399 is itself regulated by the mechanism of "target mimicry," whereby imperfectly fitting small nonprotein-coding RNAs (*IPS1* (Induced by Phosphate Starvation), *At4*) block the micro RNA and thus prevent it from hybridizing with *PHO2* RNA. Because of the imperfect fit of miR399 and IPS1/At4, RNA-induced gene silencing is not induced. This mechanism is believed to attenuate and fine-tune the P-deficiency response.

During P starvation, the sucrose concentration in the shoot is increased (Figure 11.7a). This causes the production of anthocyanins, a reduction of photosynthetic activity, and an upregulation of genes encoding sucrose transporters (SUCs) for phloem loading. Sucrose translocation to roots is thereby increased, and this is required for the increase in root/shoot biomass ratio observed under P limitation. Shoot-derived sucrose also appears to function as another systemic signal for P deficiency. The inhibition of sucrose translocation attenuates systemic root responses to P starvation. This was demonstrated clearly by the sensitivity of the *Arabidopsis pho3* mutant to low P supply. This mutant is defective in P-starvation-induced root phosphatase activity. The *PHO3* gene, inactivated in this mutant, turned out to encode an already known phloem-localized sucrose transporter, SUC2. Conversely, a mutant that overexpresses *SUC2* displayed an enhanced launch of plant responses to low P, even under P-sufficient conditions. The increased sucrose translocation to the root is required for the upregulation of an array of genes encoding P transporters and P-mobilizing enzymes (ribonucleases, RNases, phosphatases). The expression of miR399 in shoots and roots is also sensitive to photosynthesis and photosynthate translocation, which indicates that sucrose acts upstream of

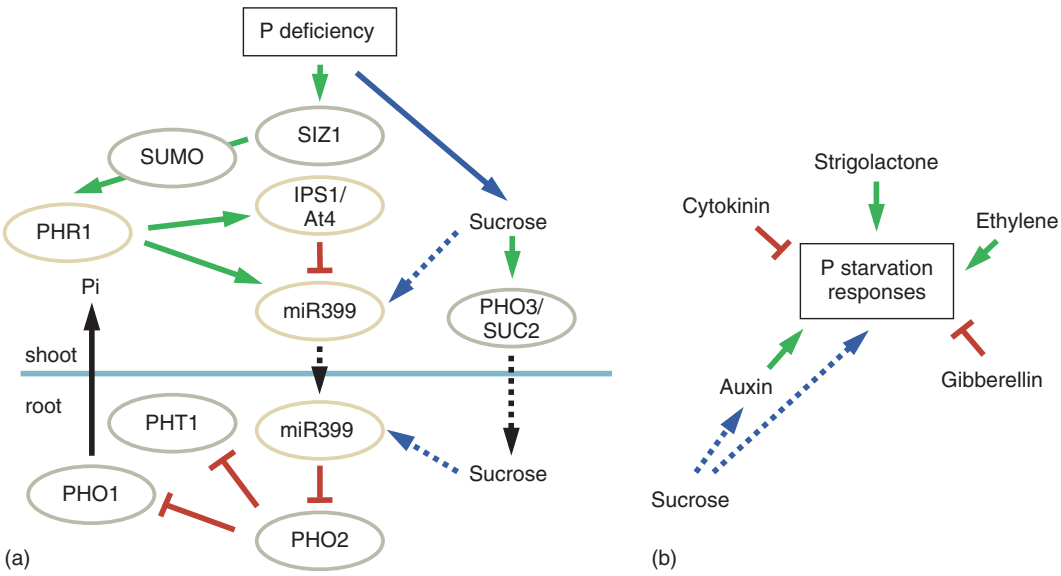


Figure 11.7 Systemic and hormonal signaling of phosphate deficiency in Arabidopsis. a) Under P deficiency, SIZ1 activates the transcription factor PHR1 by SUMOylation. One target of PHR1 is the micro RNA miR399, which is also subject to "target mimicry" regulation by IPS1/At4. miR399 is phloem-mobile and downregulates *PHO2*, which encodes a ubiquitin-conjugating enzyme. PHO2 mediates the degradation of proteins responsible for P uptake (PHT1) and xylem loading of P (PHO1). Sucrose, which accumulates in the shoot of P-deficient plants, regulates those P deficiency responses. Expression of the phloem-loading transporter PHO3/SUC2 is upregulated by sucrose, causing an increased shoot-to-root transfer. Sucrose is required for the P starvation-induced expression of miR399. The black arrows represent phloem (dotted lines) and xylem (solid line). b) P starvation responses are positively regulated by auxin, ethylene, and strigolactones. Cytokinin and gibberellins act as negative regulators. Auxin-mediated alterations of root system architecture are induced by elevated sucrose concentrations. SIZ – SAP (Scaffold Attachment Factor, Acinus, PIAS) and Miz1 (Msx2-interacting zinc finger); PHR – Phosphate Starvation Response; IPS – Induced by Phosphate Starvation; PHO – Phosphate; PHT – Phosphate Transporter; SUC – Sucrose Transporter.

miR399. Sucrose also impinges on morphological responses to P deficiency. For example, the formation of proteoid roots by *L. albus* increases when sucrose is applied exogenously, even at sufficient external P supply.

The effect of sucrose on lateral root development most likely acts through a modulation of auxin signaling. A change in auxin transport regulates the initiation of lateral root primordia. Interestingly, expression of the auxin receptor TIR1 is upregulated by P starvation. This increases auxin sensitivity and thus leads to an enhanced growth of lateral roots even without an increased auxin concentration. In contrast, the inhibition of primary root growth and the stimulation of root hair growth by P deficiency are independent of auxin. Besides auxin, ethylene, cytokinin, gibberellins, and strigolactones have been shown to be involved in P starvation responses, especially those altering the plant morphology (Figure 11.7b). Ethylene is required for the stimulation of root hair growth and lateral root elongation and for the inhibition of main root elongation during P starvation. Cytokinins decrease the lateral root initiation as well as the upregulation of P-starvation-induced genes and hence appear to act as negative regulators. Accordingly, concentrations of cytokinin and expression of the cytokinin receptor CRE1 are decreased in P-deficient roots. Sugars and cytokinins act antagonistically in the regulation of P-deficiency responses. Gibberellins also appear to be negative regulators, and the concentration of bioactive gibberellins is decreased by P starvation, causing an accumulation of DELLA proteins. Accordingly, exogeneously applied gibberellins or a mutation of DELLAs repress P starvation responses. Strigolactones, a new class of phytohormones, play a twofold role during P deficiency: exuded by roots of P-starved plants, they trigger the branching of mycorrhizal fungi and thereby stimulate the formation of this P-acquiring symbiosis. In addition, the strigolactone concentration is increased in P-deprived shoots and mediates the remodeling of shoot architecture.

Roots of plants that are sufficiently fed with phosphate via the leaves, but grow in a low-phosphate medium, still show an inhibition of primary root growth, which is typical for P-deficient plants. This demonstrates that this morphological alteration is not regulated by the systemic systems described above, but instead relies on local sensing of P availability. Some elements of this signaling network have been identified. Primary root growth inhibition is regulated by a P_5-type ATPase (phosphate deficiency response (PDR)2) and by multicopper oxidases (low phosphate root (LPR)1 and LPR2), both localized in the endoplasmic reticulum (ER) of the root meristem and with yet unknown functions. PDR2 is required to maintain the expression of *SCR* (*Scarecrow*), which is a key regulator of stem cell activity and root patterning, on P-limiting media. This local signaling network appears to be independent of phytohormones.

11.1.4.3 Iron

After oxygen, silicon, and aluminum, Fe ranks fourth in the elemental composition of the earth's crust. Despite this abundance of the metal in most mineral soils, Fe deficiency is a widespread nutritional disorder in plants. The reason for this is that in aerated soils, Fe^{3+} forms poorly soluble hydroxides and oxides, and is thus unavailable for uptake. The quantities of Fe in bulk soil solutions are thus usually far lower than the amounts required for plant growth. Iron solubility decreases with increasing redox potential and pH value, meaning that on alkaline and well-aerated soils, plants are more prone to Fe deficiency.

Morphological Adaptations Similar to P deficiency, a lack of Fe induces an increase in root hair number and length, thereby extending the rhizosphere.

Physiological Adaptations To counter the "iron problem," two general physiological strategies for uptake and mobilization have evolved in plants. The "redox strategy," also called *strategy I*, is employed by all higher plants except grasses. It makes use of the increased Fe availability at low pH and low redox potential (Figure 11.8a). To acidify their rhizosphere, strategy I plants release increased amounts of protons, in particular at the root tip, through plasma membrane-localized P-type H^+-ATPases (AHAs (Autoinhibited H^+-ATPases)). In addition, the activity of a membrane-bound reductase is strongly increased, transfering electrons from cytosolic NADH to apoplastic Fe^{3+} chelates. In the strategy I plant *A. thaliana,* the inducible Fe^{3+} reduction is carried out by the FRO2 (Ferric Reduction Oxidase) protein. The reductase has a low pH optimum and thus depends on the increased H^+-ATPase activity. The Fe^{2+} resulting from Fe^{3+} reduction is taken up into epidermal and cortical cells of the root by a H^+-coupled symporter, which is encoded by the *IRT1* (Iron-Regulated Transporter) gene in Arabidopsis. IRT1 is only poorly selective and also transports other divalent cations, such as Zn^{2+}, Cu^{2+}, and Mn^{2+}. During Fe deficiency, when IRT1 activity is strongly increased, plants may take up excessive amounts of those elements. For Zn^{2+} and Mn^{2+}, it has been demonstrated that in parallel with Fe deficiency responses, transporters are upregulated, which detoxify those metals by sequestration into root vacuoles and thereby prevent them from causing toxicity in the shoot. In addition, the synthesis of nicotianamine, a chelator of Fe and Zn, by nicotianamine synthase (NAS) (Figure 11.9), and the expression of a vacuolar nicotianamine transporter, ZIF1 (Zinc-Induced Facilitator), are increased by Fe deficiency. This increases the capacity to move Zn into the root vacuoles.

Because of the low pH optimum of the Fe^{3+}-chelate reductase, strategy I will not work if the H^+-ATPase activity is insufficient to decrease the cell wall pH value. This happens frequently if the soil solution has a high pH buffering capacity. Such conditions are often prevalent on wet, poorly aerated $CaCO_3$-containing soils. Because of the limited

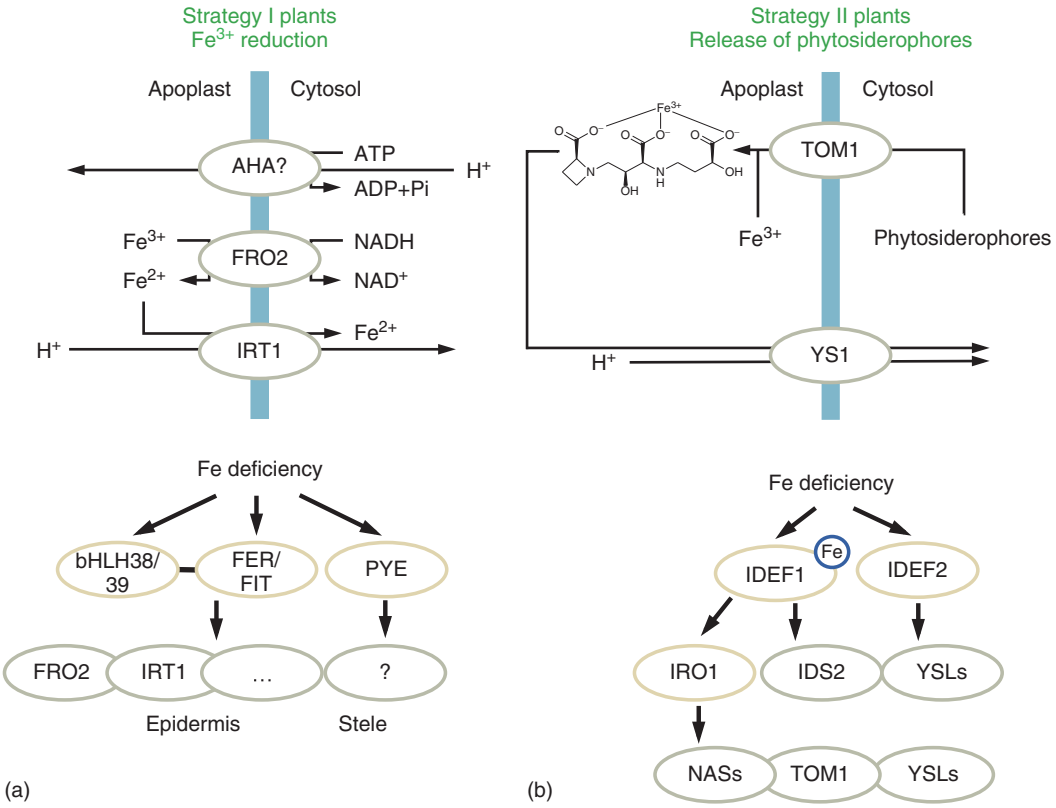


Figure 11.8 Iron deficiency responses of plants. (a). All higher plants except grasses acidify their rhizosphere through a H^+-ATPase, reduce Fe^{3+} chelates by using an NADH-dependent reductase, and take up Fe^{2+} by employing a H^+-coupled symporter. The names shown are those of the Arabidopsis proteins. Strategy I is regulated by a pair of physically interacting transcription factors. In addition, Fe deficiency responses in the root stele are initiated through a separate signaling network. (b). Strategy II plants release phytosiderophores. After chelating Fe^{3+}, the Fe^{3+}-phytosiderophore complex is reabsorbed by a H^+-coupled symporter. Strategy II is also activated on the transcriptional level, whereby a transcription factor may be the primary Fe sensor. See text for details. AHA – Autoinhibited H^+-ATPase; FRO – Ferric Reduction Oxidase; IRT – Iron-Regulated Transporter; TOM – Transporter of Mugineic Acid; YS1 – Yellow Stripe 1; bHLH – basic Helix-Loop-Helix; FIT – FER-like Iron Deficiency-Induced Transcription Factor; PYE – Popeye; IDEF1 – Iron Deficiency-responsive cis-acting Element Binding Factor; IRO – Iron-related Transcription Factor; IDS – Iron Deficiency-Specific Clone; YSL – Yellow Stripe 1-like; NAS – Nicotianamine Synthase.

diffusion of CO_2 originating from root and microbial respiration, bicarbonate (HCO_3^-) can accumulate to millimolar concentrations in the solution of such soils. Iron deficiency chlorosis is, therefore, found often on alkaline soils during wet weather.

The grasses (*Poaceae, Poales, Liliopsida*) have developed a different way of Fe acquisition based on chelation, often called *strategy II* (Figure 11.8b). Those plants release phytosiderophores, metal chelators of the mugineic acid family. Those phytosiderophores chelate the low amounts of Fe^{3+} present in soil solution and thereby cause further Fe compounds to dissolve. The Fe^{3+}-phytosiderophore complexes are subsequently taken up by epidermal and cortex cells of the root. The plant phytosiderophore deoxymugineic acid and its derivatives are synthesized from S-adenosyl methione via nicotianamine, which itself, as already mentioned, plays an important role in metal homeostasis within the plant. Three enzymes are involved in this synthesis (Figure 11.9): NAS, nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS). Phytosiderophore synthesis, release, as well as the uptake of Fe^{3+}-phytosiderophore complexes are strongly increased under Fe deficiency. Membrane transporters for both uptake and release have been identified. Mugineic acid is released by the TOM1 (Transporter Of Mugineic Acid) transporter, which is related to the nicotianamine-transporting ZIF1 protein. Both belong to the Major Facilitator Superfamily (MFS) of transporters. Reuptake of the Fe^{3+}-containing complex is mediated by the Yellow Stripe 1 (YS1) transporter, named after the chlorotic phenotype of the *ys1* mutant. YS1 mediates H^+-coupled Fe^{3+}-phytosiderophore symport, and its expression, like that of *TOM1*, is upregulated under Fe deficiency.

Interestingly, rice, a strategy II plant with a relatively poor phytosiderophore release, also contains the IRT1 transporter employed in strategy I and is thus able to take up Fe^{2+}. However, rice lacks an inducible Fe^{3+}-chelate reductase. The reason for this peculiarity is that the rice plant is adapted to flooded paddy soils, which have a low redox potential. The amount of Fe^{2+} in the bulk solution of

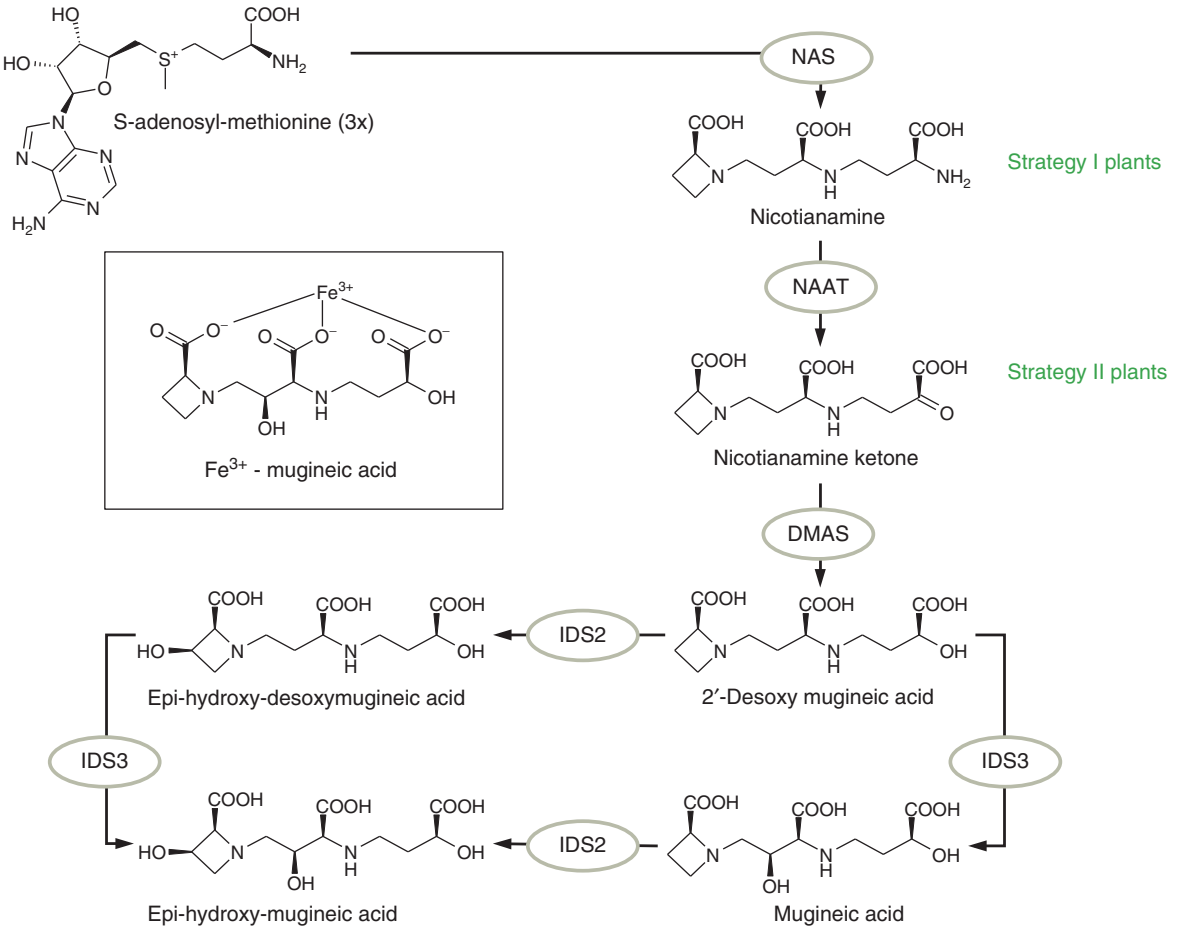


Figure 11.9 Biosynthesis of the metal chelator nicotianamine and the phytosiderophores of the mugineic acid family. Nicotianamine is synthesized from methionine via S-adenosyl methionine by S-adenosyl methionine synthase (SAMS) and nicotianamine synthase (NAS). In strategy II plants only, nicotianamine is converted to mugineic acids by nicotianamine aminotransferase (NAAT), desoxymugineic acid synthase (DMAS), and mugineic acid synthase (IDS3). Mugineic acid and 2′-desoxymugineic acid can be further metabolized by IDS2. Inset: chelation of Fe^{3+} by mugineic acid.

those soils is quite high, and hence, an active reduction of Fe^{3+} is not required. On calcareous, alkaline soils, however, rice is very prone to Fe deficiency. This problem has been addressed by plant scientists, and two biotechnological approaches have been successfully employed to improve the Fe acquisition by rice: (i) transformation with a barley NAAT gene (Figure 13.9) to increase the production of phytosiderophores and (ii) transformation with a pH-insensitive Fe^{3+}-chelate reductase from yeast. Expression of the mutated gene in rice plants strongly improved their growth and Fe uptake on a calcareous soil (Box 11.1, Figure 11.9)

Under Fe-limiting conditions, mechanisms of intracellular Fe retranslocation are also vital, in particular during plant establishment. Germinating seedlings require Fe stored in vacuoles of vascular tissues of the embryo. The loading of those vacuoles in the developing seed is mediated by the VIT1 (Vacuolar Iron Transporter) protein, and *Arabidopsis* mutants for this transporter show a severe Fe deficiency when germinating on a calcareous soil with low Fe availability. Similarly, the transporters unloading the stored vacuolar Fe, NRAMP3 (Natural Resistance-Associated Macrophage Protein) and NRAMP4, are also essential for seedling survival on calcareous soil.

In plants of both uptake strategies, Fe is translocated as Fe^{2+}-citrate complex within the xylem and as Fe^{2+}-nicotianamine complex within the phloem. Nicotianamine also plays an important role in local Fe distribution within the leaf, and, accordingly, plants that overexpress the vacuolar ZIF1 transporter, which removes the chelator from the cytosol, are impaired in intercellular Fe distribution.

Regulation of Fe Deficiency Responses The induction of strategy I and strategy II mechanisms is regulated differently. In strategy I plants, *FRO2* as well as *IRT1* are induced at the transcriptional level during Fe deficiency. This response depends on a bHLH (basic Helix-Loop-Helix)-type transcription factor, FER in tomato, or FIT (FER-like Iron Deficiency-Induced Transcription Factor) in *Arabidopsis* (Figure 11.8a). The FER/FIT protein level is itself regulated by the plant's Fe status. To induce the Fe-deficiency responses, however, a high expression of *FER/FIT* alone

Box 11.1: Improving Iron Nutrition by Optimizing the Fe^{3+} -Chelate Reductase Activity

An achilles heel of strategy I is the low pH optimum of the inducible Fe^{3+} -chelate reductase. A reduced pH dependency may thus improve iron uptake from calcareous soils. This strategy has been successfully employed in rice by introducing a mutated yeast Fe^{3+} -chelate reductase (Figure 11.10). The cDNA encoding this yeast protein, FRE1, which has a similar pH dependency than its plant counterpart, was mutagenized and selected for increased activity at high pH. Expressing this mutated protein in rice plants strongly improved their iron uptake and their growth on a calcareous soil.

As Fe deficiency chlorosis affects rice and other crops on calcareous soils, the biotechnological engineering of crops, for example, by improving Fe^{3+} -chelate reductases, can be a way to increase yields under such conditions.

is not sufficient; a second transcription factor, bHLH38 or bHLH39, is also required, which physically interacts with FER/FIT. Only the complex of both transcription factors activates the transcription of genes alleviating Fe deficiency. In addition to this transcriptional response, which primarily induces Fe mobilization and uptake mechanisms, a parallel transcriptional network in the root stele appears to be responsible for Fe homeostasis and maintenance of root growth at low Fe. Here, the bHLH transcription factor PYE (Popeye) plays a central role.

Promoter analysis of the Fe deficiency-induced gene *IDS2* (Iron Deficiency-Specific Clone) (Figure 11.8) of barley has revealed two cis-acting elements, IDE1 (Iron Deficiency-Responsive Element) and IDE2, required for strategy II Fe-deficiency responses (Figure 11.8b). IDS2 is involved in phytosiderophore biosynthesis. IDE1 and IDE2 are targets of the transcription factors IDEF1 (iron deficiency-responsive cis-acting element binding factor) and IDEF2, respectively. Rice IDEF1 is able to bind Fe and may thus be a primary Fe sensor. Both factors regulate genes related to Fe nutrition and Fe distribution. In rice, the transcription factor OsIRO2 (Iron-related Transcription Factor) is strongly induced by Fe deficiency and under the regulation of IDEF1. OsIRO2 expression upregulates strategy II enzymes and transporters on transcriptional level.

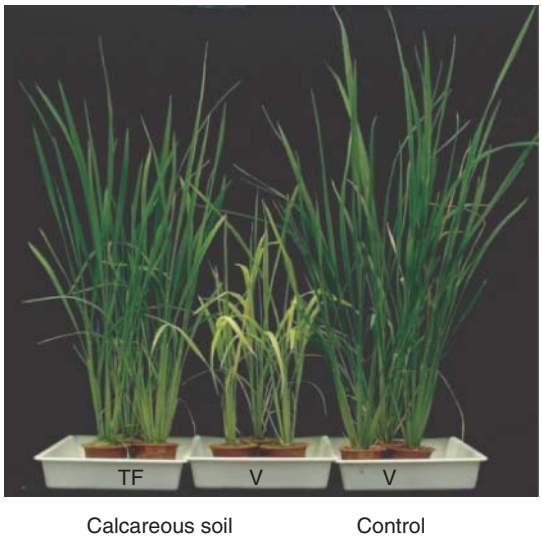


Figure 11.10 Optimizing the Fe^{3+}-chelate reductase to improve Fe nutrition. TF – transformant; V – vector control. (Ishimaru *et al.* (2007), © (2007) National Academy of Sciences, U.S.A.)

11.1.4.4 Zinc

Zinc plays a multitude of roles in plants, and over 1200 proteins transport, bind, or contain this metal. Reactions involving nucleic acids are often Zn-dependent; many transcription factors contain Zn fingers as structural element. Zn-deficient plants, therefore, display typical deficiency symptoms, such as stunted growth and poor fertility. Plant responses to Zn deficiency have been less well explored than those to iron deficiency, but is has become clear that there are interesting parallels and interactions between both elements.

Physiological Adaptations Like Fe, Zn can be taken up as the free divalent ion, Zn^{2+}. As Zn is not redox-active, a reduction prior to uptake is not required. Zn^{2+} uptake into root cells is primarily mediated by the IRT3 transporter, which, like IRT1 introduced above, is a member of the ZIP (ZRT, IRT-like Protein) family. Besides Zn^{2+}, IRT3 also transports Fe^{2+}. The expression of *IRT3* is strongly upregulated in response to Zn deficiency. In strategy II of Fe acquisition, which is utilized by grasses, Fe uptake is mediated through a complex with mugineic acid (see Section 11.1.4.3). Interestingly, this phytosiderophore also chelates Zn^{2+}, and the identified Fe^{3+}-phytosiderophore transporter, YS1, is also able to transport the Zn^{2+}-phytosiderophore complex through the root cell plasma membrane. Therefore, grasses are able to acquire Zn in the same way they acquire Fe. Once taken up into the cytosol, Zn is chelated by low molecular weight ligands, such as glutathione, phytochelatins (see Chapter 12), or nicotianamine, to avoid uncontrolled interactions with binding sites or precipitation with anions, such as phosphate. Nicotianamine plays an important role in the mobility and distribution not only of Fe, but also of Zn, and the *NAS* genes that encode the nicotianamine synthase (Figure 11.9) are also upregulated by Zn deficiency.

For transport into the shoot, Zn is unloaded into the xylem apoplast by means of a heavy metal ATPase, HMA2. Expression of this pump is upregulated during Zn deficiency. Besides this increased efficiency of Zn uptake and

translocation, Zn can also be remobilized from vacuoles and trichomes in times of limiting supply, and some redistribution of Zn via the phloem also occurs.

Regulation of Zn Deficiency Responses Zn deficiency responses are under transcriptional control. Two transcription factors, bZIP19 (Basic Leucine Zipper) and bZIP23, have been identified, which, under Zn deficiency, enhance the transcription of *NAS*s, *IRT3*, and other genes of the ZIP transporter family. A Zn-deficiency response element (ZDRE) has been identified as the target of both transcription factors. It is unknown at present how plants sense their Zn supply.

11.1.4.5 Copper

As redox-active metal, Cu has the potential to cause oxidative damage to cellular constituents. It is, therefore, bound to Cu chaperones after uptake. Cu is required in smaller amounts than either Fe or Zn (Figure 11.1), and in plants, Cu and Fe are partly interchangeable in some functions.

Physiological Adaptations Plants respond to Cu deficiency with both an improved acquisition and use efficiency. Uptake of Cu by root cells is accomplished by transporters of the COPT (Copper Transporter) family. The expression of *COPT* genes is upregulated by Cu limitation, thereby increasing Cu uptake capacity. COPTs transport Cu^{+}, whereas in aerobic soils, Cu^{2+} is the predominant form. Like Fe^{3+}, Cu^{2+}, therefore, needs to be reduced to Cu^{+} prior to uptake. Interestingly, the Fe^{3+} reductase FRO2 (see Section 11.1.4.3) is also able to reduce Cu^{2+}. Plants may also be able to take up Cu^{2+} directly by some members of the ZIP family of divalent cation transporters, which are also upregulated by Cu deficiency. To increase Cu use efficiency, plants can replace Cu by Fe in some enzymatic functions that require a redox-active metal. For example, during Cu deficiency, Cu/Zn-SODs (superoxide dismutases) are no longer produced. Instead, Fe-SODs take over their function. Other Cu-containing proteins, such as laccases, which catalyze lignin polymerization, also disappear if Cu is limiting. This sacrifice of not absolutely essential Cu proteins helps the plant to maintain vital functions where Cu cannot be replaced, such as in the photosynthetic electron transfer protein plastocyanin.

Regulation of Cu Deficiency Responses The signal transduction cascade that leads to the described Cu deficiency responses involves transcriptional and posttranscriptional mechanisms. The downregulation of the Cu-containing enzymes is brought about by the action of micro RNAs: miR398 triggers the degradation of *CSD1* (Copper/Zinc Superoxide Dismutase) and *CSD2* mRNAs, encoding CuZn-SODs; miR408, miR397, and miR857 have laccase-encoding mRNAs as their target. The expression of those four micro RNAs is upregulated by Cu deficiency. In parallel, the expression of genes encoding the proteins that take over, such as *FSD1* (Fe Superoxide Dismutase), encoding the Fe-SOD, is turned on. The central transcription factor that controls the Cu-dependent expression of uptake transporters, Cu chaperones, micro RNAs, and functional replacement genes has been identified by its homology to a regulator of Cu deficiency responses in the green alga *Chlamydomonas* (*Chlamydomonadales, Chlorophyta*), CRR1 (Copper Response Regulator). Knockout mutants of this gene, *SPL7* (Squamosa Promoter Binding Protein-Like), are hypersensitive to Cu deficiency.

(This word cloud belongs to subchapter 11.2, see next page)

11.2 Carnivorous Plants and Fungi

Gerd-Joachim Krauss and Gudrun Krauss

Overview

Carnivorous plants (CPs) grow predominantly in habitats with low nutrient content. They attract, trap, and digest animals in order to supplement nutrients such as nitrogen species, phosphates, trace elements, and small organic substances. CP evolved the ability to digest the prey in a fluid formed by different types of traps. All passive and active trapping tools originate from leaves.

Commensalism enables plants to optimize their carnivorous life style. Pitcher plants are excellent examples of coevolutionary adaptations. In open pitchers, trophic multilevel plant-commensal relationships exist, with the microbial component of crucial importance.

Two *Roridula* species (*Ericales, Asteridae*) represent protocarnivorous plants that catch insects by releasing a resinous fluid onto the leaf surface, but without the ability to digest them. Specialized mutualistic insects live there and feed on the captured insects.

Carnivorous fungi (CF) develop three-dimensional networks of specialized hyphae to trap nematodes and other microscopic animals. In the ascomycete *Arthrobotrys oligospora* (*Pezizomycotina, Ascomycota*), trap formation is triggered by multiple cellular signal transduction pathways.

11.2.1 Habitats, Diversity and Evolution

Carnivorous plants (CPs) grow in nutrient poor habitats. They are distributed worldwide and colonize very diverse environments. They occur predominantly in habitats with very insufficient nutrients (peat, rock, sand, or water), and moist to wet conditions for at least part of the year (Table 11.3). There are over 700 described CP species, but this number keeps growing. CPs have evolved special morphological, physiological, and biochemical features to attract, capture, and digest prey with the objective of supplementing macroelements such as nitrogen, phosphorus, and sulphur.

It is evident that the polyphyletic origin of CPs (independently at least six times) took place in open, **moist, and low nutrient environments**, such as white-sand savannahs and bogs. Later on, on evolution, they evolved in water bodies (swamps, marshes, oligotrophic waters), rocks, mountain slopes, and dripping rocks, and the occasional transition to an **epiphytic lifestyle** was developed. The diversity of CPs shows hotspots in the floral regions of Australia, Brazil, Mexico, India, and Southeastern North America.

The **dual use of leaves** for photosynthesis and nutrient uptake from digested prey reduces the net photosynthetic rate of terrestrial CPs. The benefits of nitrogen absorbed from prey digestion can in some cases result in increased photosynthetic carbon dioxide fixation, but in other cases the plant's photosynthetic rate does not allow survival without the uptake of organic carbon from caught organisms.

CPs benefit from the digestion of organisms or their excretions by increased growth, earlier or enhanced flowering, and higher seed production. Most CPs need insects as pollinators and source of nutrients. The so-called **pollinator – prey conflict** describes overlapping circumstances. This situation is excluded in most CPs by the temporal and spatial separation and positioning of traps and flowers as well as distinctly different signals for pollinators and prey species.

In terrestrial CPs, different types of **root systems** occur, functioning permanently or only during part of the year. Aquatic CPs never form roots. Specialized underground shoots or leaves can replace roots in terrestrial CPs and fulfill their functions. In swamps, where the action of *Sphagnum* mosses (*Sphagnales, Sphagnophytina*) lowers the pH down to 3.0, CPs need specialized physiological adaptation of roots. Adaptation of CPs to oxygen-deficient soils results in changes in the root anatomy. In some cases, special leaves can take over the function of roots to store assimilates.

Two species of the genus *Roridula* of the family *Roridulaceae* (*Ericales, Asteridae*) (Figure 11.11) represent **protocarnivorous plants**, which trap insects without the capability to digest them. The perennial shrubs are endemic to the mountainous areas of the South African Cape Region. *Roridula* is well-adapted to open habitats and a heathlike vegetation (known as *fynbos*), characterized by periodically dry conditions and outbreaks of fire in natural intervals of 10–15 years. *Roridula* can easily regenerate from seeds after fire.

The sticky fluid released from the leaves for trapping insects can be interpreted as ecological adaptation of *Roridula* to its severe habitat. The robust glistening plant surface enhances light reflection. Their hydrophobic features help to reduce water loss and protect against herbivorous insect attacks.

Two *Bromeliaceae* species (*Poales, Liliopsida*) are also considered to be **protocarnivorous**. Both are tank-bromeliads and form a phytoelm ("plant held water") in leaf axils. *Brochinia reducta* traps insects and leaf litter at low nutrient sites of Venezuela and Guiana.

Table 11.3 Carnivorous plants: trapping mechanisms, diversity, habitats, and geographical distribution.

Trap	Family/genus	No. of species	Habitat	Geographical distribution
PASSIVE TRAPS				
Pitfall traps	Nepenthaceae[a]			
	Nepenthes	120	Humid lowland forests Tropical montane forests	Subtropical regions of Asia, Australia, Seychelles, Madagascar
	Sarraceniaceae[b]			
	Sarracenia	9	Fens, swamps, coastal and grassy plains	Atlantic North America
	Darlingtonia	1	Boggy areas, near mountain streams	Oregon (USA), California (USA) (up to 2500 m)
	Cephalotaceae[c]			
	Cephalotus	1	Coastal plains	Endemic in south-west Australia
	Dioncophyllaceae[a]			
	Triphyophyllum	1	Tropical rainforests	Sierra Leone, Liberia, Ivory Coast
Sticky flypaper	Droseraceae[a]			
	Drosera	184	Marshes, fens, wet stands, boggy shorelines	All continents
	Drosophyllaceae[a]			
	Drosophyllum	1	Slightly basic soils, coastal regions	Endemic in Portugal, Spain, Morocco
	Lentibulariaceae[d]			
	Pinguicula	96	Highly humid, wet areas and montane regions (up to 1900 m)	Europe, North America, West Africa, Asia
	Byblidaceae[d]			
	Byblis	7	Sands, moderately moist heathlands	Endemic in Australia, New Guinea
Snare traps	Lentibulariaceae[d]			
	Genlisea	22	Wetlands up to 2500 m	Endemic in Guiana, Angola, Madagascar
Subterrenian adhesive leaves	Plantaginaceae[d]			
	Philcoxia	3	Rocks, white sands under seasonal rain	Grows exclusively in Central Brazilia (Cerrado)
ACTIVE TRAPS				
Snap traps	Droseraceae[a]			
	Dionaea	1	Bogs, swamps, wet savannahs	North and South Carolina (USA)
	Aldrovanda	1	Shallow and warm standing water	Central Europe, East Asia, Africa, Australia
Suction traps	Lentibulariaceae[d]			
	Ultricularia	250	Still waters, wet soils, sands, rocks, epiphytic in cloud forests	Worldwide

a) Caryophyllales.
b) Ericales.
c) Oxalidales.
d) Lamiales.
Data from: Król *et al.* (2012), Adlassnig, Peroutka, and Lendl (2012), and others.

(a) (b)

Figure 11.11 *Roridula dentata* (a), *Roridula gorgonias* (b); University of Bonn Botanical Garden. (© W. Barthlott)

Catopsis berteroniana occurs in sunny areas of Central America. The taxa do not develop glands for enzyme secretion, but they are able to resorb nutrients after bacterial decomposition.

CFs are important members of the soil biocoenosis. While the majority of fungi evolved a saprophytic or symbiotic life style, a small proportion (less than 0.5%) show a carnivorous life style. They branched off from saprophytic fungi only a few times. CFs have developed the ability to trap and consume soil organisms, such as nematodes, rhizopods, rotifers, and collembola using specific capture devices.

CFs include species within the phyla *Zygomycota*, *Basidiomycota*, and *Ascomycota*. Nematode-trapping fungi are widely distributed in ecosystems. Their adaptive life style regulates the nematode population density in different habitats and make these fungi interesting tools for controlling parasitic nematodes of plants and animals in soils.

11.2.2 Trapping Devices

All plant-trapping tools originate from leaves, which have been transformed into different specialized shapes. Capturing is supported by scent, coloration, and nectar.

1) **Passive traps** (Table 11.3):
   Small organisms are caught in containers with digestive fluid or by adhesive structures.
   A. ***Pitfall traps:***
      Pitcher-shaped leaves consist of different parts and contain a fluid to digest animals that slide down. In the genus *Nepenthes* three leaf zones can be differentiated: (i) the flat leaf base attached to the plant shoot, (ii) the rounded stalk of petiole providing attachment to the surrounding vegetation, (iii) the pitfall trap at the end of the leaf (Figure 11.12).
   B. ***Sticky flypaper traps:***
      The traps are composed of arrays of stalked glandular hairs. Small insects are attracted by colors, scent, or by the glistening of the stalked tips in the light, and immobilized immediately by secreted drops of mucilage. Such traps can move their glandular stalks by actively rolling over their prey (*Drosera* (Figure 11.13a), *Pinguicula*). In *Drosophyllum* and *Byblis* species, insects are captured by motionless digestive hairs. *Drosera glanduligera* growing in Southern Australia develop peripheral nonsticky snap tentacles. Animals touching these sensitive tentacles are catapulted within 75 ms toward the sticky glue tentacles in the trap center, where digestion takes place.
   C. ***Snare traps:***
      The trap is formed by underground leaves of *Genlisea* species (Figure 11.13b). The chlorophyll-free leaves form Y-shaped and twisted tubes. The channels contain claw-like cells and inward-directed digestive-absorptive hairs ending in bulbs. The traps are specialized for catching Protozoa and Metazoa.
   D. ***Subterranean adhesive leaves:***
      Philcoxia species develop minute leaves under the surface of white sands. Nematodes are trapped and digested by stalked glands at the leaf surface.
2) **Active traps** (Table 11.3):
   The capturing of prey is accompanied by the movement of the traps.
   A. ***Snap traps:***
      The prey is caught by closing the traps. Two symmetrical lobes of the leaf lamina snap shut over the midrip. Sessile digestive glands cover the inner surface of the trap. In *Dionaea* species (venus flytrap), three trigger hairs are located between the digestive glands on each half of the leaf lamina. *Dionaea muscipula* (Figure 11.14a) is able to emit by the rims of trap leaf under light 60 **volatile organic compounds (VOCs)** including terpenes, benzenoids, and aliphatics. Having a strong similarity to the scent of flowers and fruits, *Drosophila* flies are attracted by such smell-imitating food. Following animal contact, the sensitive hairs convert mechanosensory stimuli into electrophysiological changes causing the trap closure. The signaling process partly shows similarities to animals characterized by voltage-dependent ion channels similar to neuronal channels. The capture of insects is accompanied by calcium signaling and an increase of 12-oxo-phytodienoic acid (OPDA), a precursor to the phytohormone jasmonic acid (see Section 7.3.2.2).

      Insects activate ion channels by calcium and cause receptor potentials that create an action potential for the rapid initial closing of lobes in 100 ms, followed by

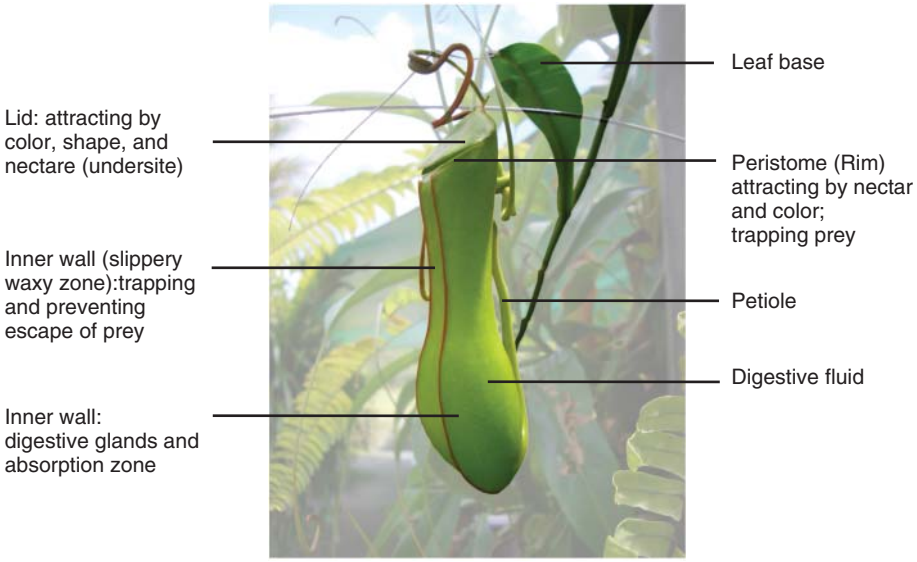
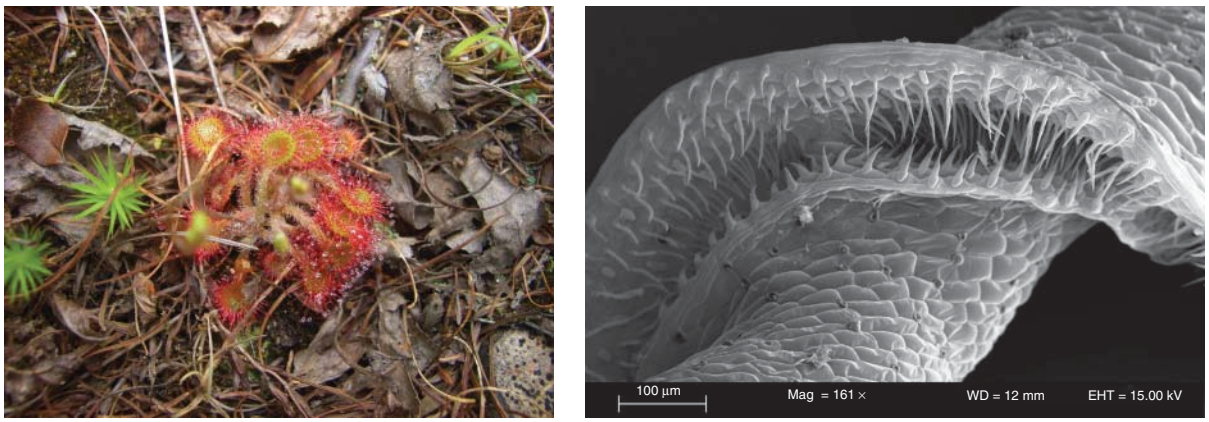
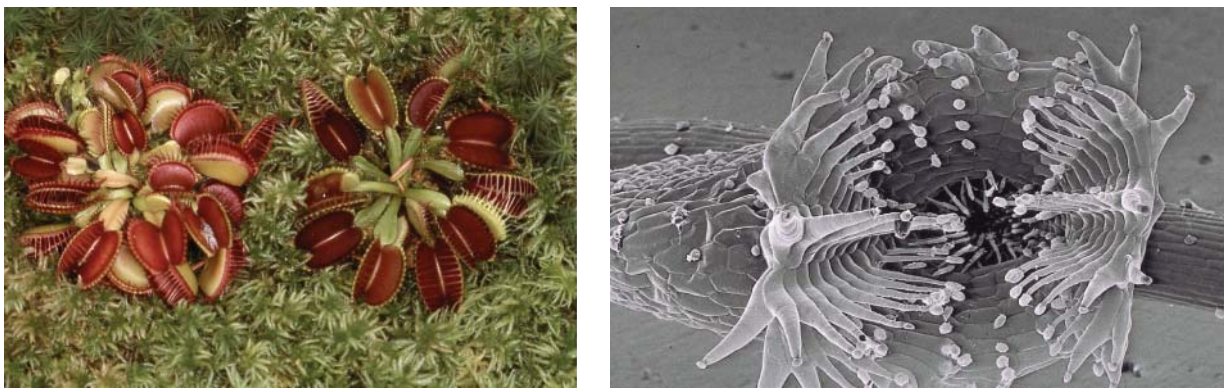


Figure 11.12 Pitcher (pitfall) trap (Nepenthales): Morphological and ecophysiological features.



(a) (b)

Figure 11.13 Passive traps: *Drosera rotundifolia* (a) (near Sackville, NB, Canada) (G. Krauss); *Genlisea aurea* (b), Scanning electron micrograph (SEM) of a door that leads into the trap interior. University of Bonn Botanical Garden (© W. Barthlott)



(a) (b)

Figure 11.14 Active traps: *Dionaea muscipula* (a) *Utricularia sandersonii* (b), SEM of the trapping door. University of Bonn Botanical Garden (© W. Barthlott)

a complete closure within 0.5–2 h. The prey initiates the acidification in the "stomach," and the production of lytic enzymes is triggered by OPDA. *Dionaea* species live on the soil surface and are specialized for trapping ground-crawling prey, such as ants (30%), spiders (30%), and beetles (10%).

In the waterwheel plant (*Aldrovanda vesiculosa*, Caryophyllales, core eudicotyledons) small traps (4–7 mm in length) are closed underwater within 100 ms after mechanical stimulation.

B. ***Suction traps:***

Bladder-like traps are found exclusively in *Utricularia* species (Figure 11.14b). The trap works in a two-phase process: (i) internal glands actively pump water out of the trap chamber; the trap is ready for catching prey, (ii) organisms (small Crustaceae, insect larvae, algae) touching sensitive hairs near the door are sucked into the trap in 300–700 µs followed by the digestion of prey. Species of the **protocarnivorous genus** *Roridula* are able to catch insects by releasing a resinous fluid onto the leaf surface. The tips of tentacle-shaped trichomes produce a water-insoluble, nonvolatile secretion containing triterpenoids as main compounds, as well as acylglycerides. In a hierarchically organized process, insects are attracted, entangled in the secretion droplets of long trichomes, and are stuck to short trichomes near the plant surface. Specialized mutualistic insects live there and feed on captured insects.

The **protocarnivorous bromeliad** species *Brocchinia reducta* capture insects in leaf rosettes forming pitfall traps (cisterns). The plant attract insects by scent. The leaf upperside is coated with wax particles forming a slippery surface. Insects fall down into the water container with a very low pH value of 3.0.

CFs develop three-dimensional networks of hyphae to trap animals. Zygomycota species form adhesive hyphae or hyphal proturbances for passively capturing small animals. In some basidiomycetes, adhesive knobs produce nematode-toxic droplets. Six different trapping structures occur in carnivorous ascomycetes, constricting rings, adhesive nets, adhesive columns, stalked adhesive knobs, unstalked adhesive knobs, and nonconstricting rings.

The ascomycete *Arthrobotrys oligospora* (teleomorph *Orbilia auricolor, Pezizomycotina, Ascomycota*) is the best studied CF. Living mainly saprophytically in diverse soils, the fungus switches to the parasitic stage in the presence of nematodes by forming three-dimensional hyphal networks to trap nematodes. When the nematode enters a constricting ring, the cells exhibit thigmonasty: pressure triggers the cells to inflate and immobilize the animal. The trapping process includes adhesion, immobilization, and penetration of the animal. As shown by genomic and proteomic studies, a distinct gene expression is involved in trap formation that triggers **multiple cellular signal transduction pathways** (Figure 11.15).

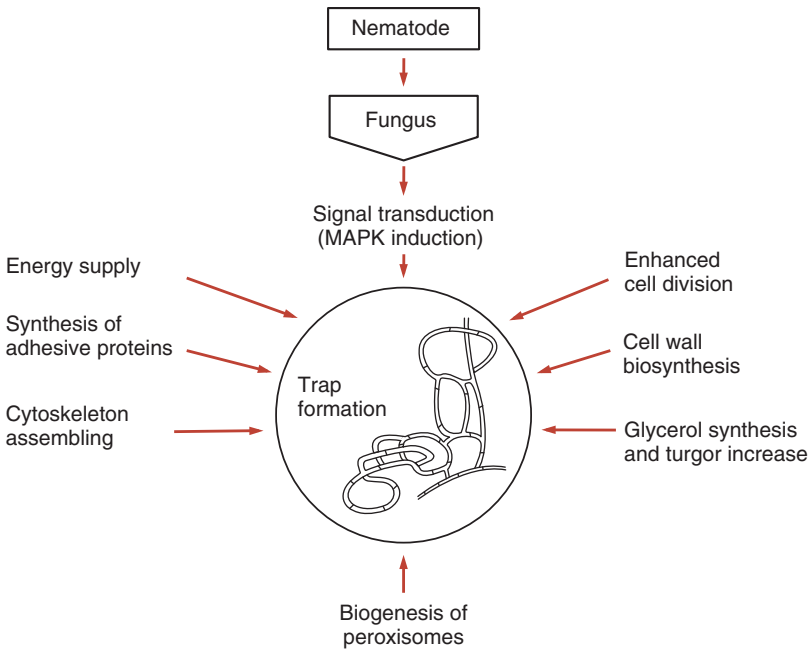


Figure 11.15 Biochemical prerequisites for nematode-induced trap formation in the fungus *Arthrobotrys oligospora*; MAPK – mitogen-activated protein kinases. (Modified from Yang *et al.* (2011).)

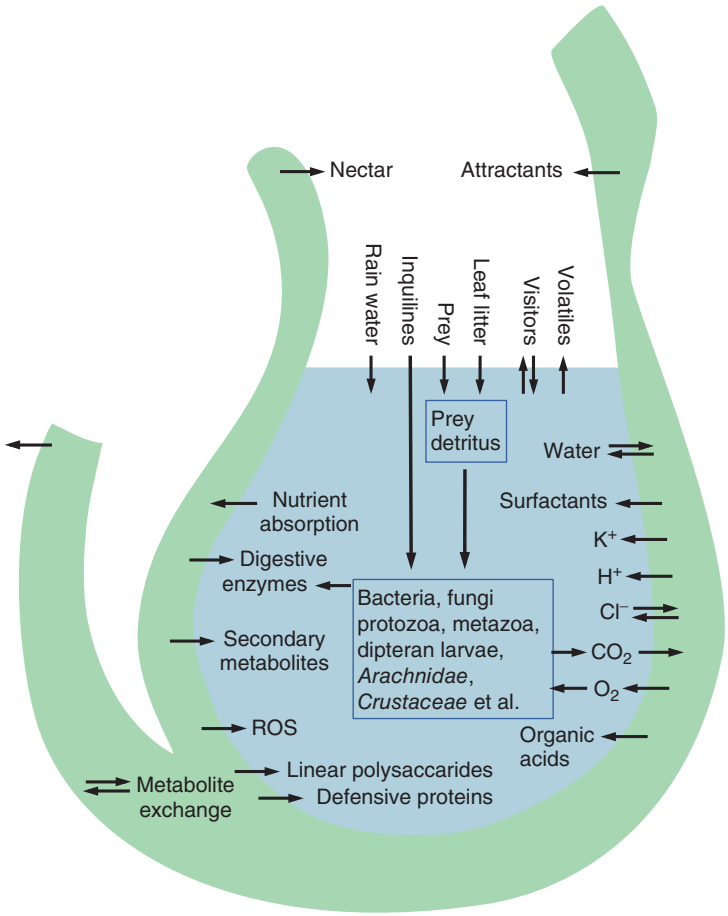


Figure 11.16 Biochemical and mutualistic interactions in a pitcher trap.

11.2.3 Prey Digestion and Nutrient Utilization

The carnivorous life style of plants and fungi evolved as a specific way to supplement nutrients such as nitrogen species, phosphate, trace elements, and small organic substances. All CPs have the ability to digest trapped animals in a fluid formed by several traps (see Section 11.2.2) expressing three different digestive processes:

1) **Sustained digestion in special compartments:** This type of passive traps can be viewed as a container filled with digestive fluid. The prey consumption is realized in pitfall traps, snare traps, and suction traps (Table 11.3).
2) **Digestion at segregated spots on the trap surface:** Using this type of passive trapping, plants catch animals with the help of mucilage-producing stalked-shaped glands, for example, in sticky flypaper and subterranean adhesive leaves (Table 11.3).
3) **Cyclic digestion in a compartment:** This type of active traps configurate a closed digestive chamber after capturing prey. The compartment is filled with digestive fluid only at the time of snap-trapping (Table 11.3).

The trap tissue is able to recognize chemical stimuli, secretion of digestive enzymes, supply of the external fluid, or slime at optimal pH using **proton pumps**, production of **reactive oxygen species** (ROS) to aid the degradation process, and assimilation of nutrients, as shown for *Nepenthes* species in Figure 11.16.

Different digestive enzymes are produced by glandular cells (Table 11.4). Some secreted enzymes are not involved in nutrient-degrading processes. In a few digestive fluids, **pathogenesis related proteins** occur. Such proteins could act as antibacterial and antifungal agents to avoid unwanted competition for released nutrients (see Section 11.2.4). Some of these proteins have hydrolytic activities. In the Caryophyllales CP lineage (Table 11.3), different classes of chitinases (Table 11.4) are likely implicated in both prey digestion and pathogenic response. In *Drosera* sp., prey increase the mucilage production. Formic acid initiates insect digestion, followed by the secretion of digestive enzymes.

Table 11.4 Digestive enzymes and pathogenesis-related (PR) proteins produced by carnivorous plants.

Proteins	*Nepenthes*	*Sarracenia*	*Darlingtonia*	*Cephalotus*	*Triphyophyllum*	*Drosera*	*Drosophyllum*	*Pinguicula*	*Byblis*	*Genlisea*	*Philcoxia*	*Dionaea*	*Aldrovanda*	*Utricularia*
Amylases	+	+	+	+				+				+		
Chitinases	+				+	+						+		+
Esterases	+	+		+	+	+	+	+		+		+		+
β-1,3-Glucanase	+											+		
β-D-Glucosaminidase	+													
β-D-Glucosidase	+													+
Lipases	+	+										+		
Nucleases	+			+				+				+		
Peroxidases	+					+	+					+		
Phosphatases	+	+		+	+	+	+	+	+	+	+	+	+	+
Phosphoamidase	+													
PR- proteins	+											+		
Proteases	+	+		+	+	+	+	+	+	+		+		+
β-D-Xylosidase	+													

Data from Barthlott *et al.* (2007), Takeuchi *et al.* (2011), Mithöfer (2011), Pereira *et al.* (2012), Schulze *et al.* (2012), and Renner and Specht (2013).

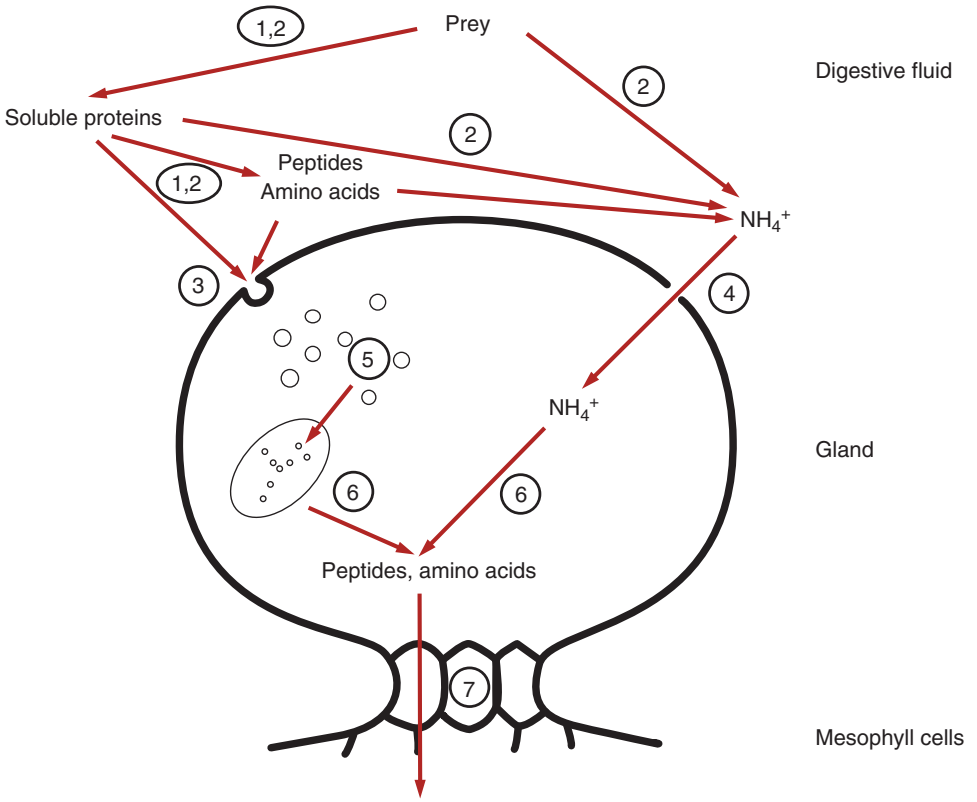


Figure 11.17 Proposed model of the uptake of nitrogen in *Nepenthes* tissues: (1) prey digestion by plant-secreted enzymes, (2) digestion by mutualistic inquilines, (3) uptake via endocytosis, (4) ammonium uptake via carriers, (5) degradation of endocytotic vesicles in secondary lysosomes, (6) incorporation of ammonium via amino acids into new peptides, (7) carrier transport of peptides and amino acids toward mesophyll cells. (Modified from Adlassnig *et al.* (2012.)

As shown for pitcher plants, proteins released from digested prey are also degraded by enzymes from **mutualistic microbial inquilines** (Figure 11.16) (see Section 11.2.4). Cellular nutrient uptake by membrane carriers is supplemented by endocytosis, which allows absorption and intracellular degradation of proteins (Figure 11.17). Amino acids and peptides are sequestered toward the mesophyll cells via distinct carrier proteins (Figure 11.17) and supported by secreted H^+ ions acting in the symport **uptake of nitrogenous compounds**.

As shown for *Dionaea muscipula*, ammonium is a major product of prey digestion. Ammonium ions produced primarily by deamination of glutamine are substrates of *Dionaea muscipula* ammonium transporter 1 (DmAMT1). This transporter expressed prey-dependent in gland cells has biochemical properties of an ion-selective channel. Interestingly, the AMT NaAMT1, localized in digestive glands in the lower part of *Nepenthes alata* pitchers, shows similar features. Obviously, ammonium in *D. muscipula* cells induces H^+ release into the trap, presumably caused by a plasma-membrane-located H^+-ATPase.

The uptake and incorporation of nitrogen from digested prey and in some cases from falling leaves depends on the CP species. In pitfall plants, 60% of prey nitrogen was found in *Nepenthes mirabilis*, but only 25% in *Cephalotus follicularis*. In ground level pitchers of *Nepenthes ampullaria*, 35% of recycled nitrogen is derived from leaf litter. Approximately, 75% of the nitrogen was absorbed during animal digestion in the suction traps of *Utricularia* spec.

The **protocarnivorous** *Roridula* species are characterized by digestive mutualism. They do not secrete degrading enzymes, but the plants benefit from bugs (e.g., *Pameridea roridulae*) and spiders (e.g., *Synaema marlothii*). These insects live omnivorously on the plant surface and feed on trapped animals. *Pameridea roridulae* can walk on the viscous plant surface without body grooming. The mirid bug delivers a thick, antiadhesive mucilage, which allows movement on a strongly adhesive surface. Insects release feces rich in nitrogen into gaps in the leaf cuticula.

No digestive glands occur in the epiphytic **protocarnivorous bromeliad** *Brocchinia reducta*. After insect trapping and bacterial prey digestion, resorption of nitrogenous organic nutrients takes place probably via specialized plant cells. Protozoa and rotifers feed on bacteria. Algae grow in bromeliad cisterns. They are the main components in the detritus-based microbial food web and the diet of mosquitoes.

Nematode trapping ascomycetes can kill animals by producing secondary metabolites. They penetrate the animals, digest them, and assimilate the nutrients. Increased

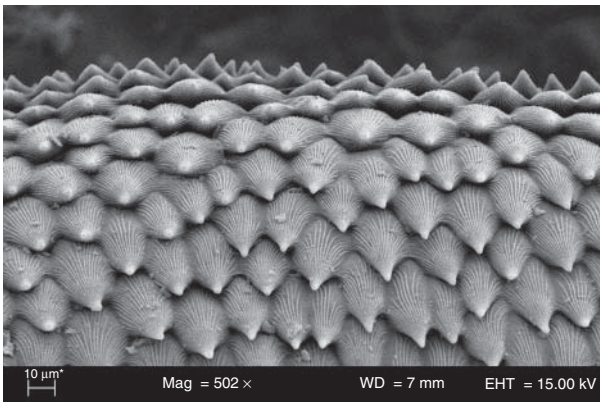


Figure 11.18 *Sarracenia flava*, SEM of the peristome. (University of Bonn Botanical Garden) (© W. Barthlott.)

biogenesis of peroxisome and accumulation of lipid droplets in hyphal cells provide evidence for digestion and storage of nutrients (Figure 11.15).

11.2.4 Coevolutionary Strategies of the Carnivorous Life Style: Pitcher Plants

Pitcher plants represent approximately 25% of all CP species and have evolved a highly adaptive plasticity to their environment. Species of the best studied genus *Nepenthes* inhabit a wide variety of tropical habitats, mostly growing as vines (up to 15 m long) or subscandent shrubs. A small number of species grow epiphytically. Some *Nepenthes* taxa produce two types of pitchers (leaf dimorphism): (i) lower pitcher rosettes developed by young plants, and (ii) upper pitchers with curled tendrils evolved by mature climbing plants (Figure 11.12).

Pitchers are divided into distinct zones (Figure 11.12). In the "attractive" zone, extrafloral nectaries secrete nectar on the tendril, at the outside of the pitcher, on the pitcher lid and the inner side of the rim (peristome).

Volatiles and distinct color patterns may contribute to the attractiveness of the traps. In *Nepenthes rafflesiana*, peristome show high contrast against the pitcher body in UV (350–370 nm), blue (430–470 nm), and green light (490–540 nm). These wavelengths correlate to the maxima of optical sensitivity in arthropods. Over 50 VOCs are emitted to support the attracting of insects.

Nepenthes species are excellent examples of convergent evolution. Both insect-pollinated flowers and pitchers attract insects of different taxa and guide them to sites of optimal benefit to the plant.

The peristome (Figures 11.12 and 11.18) shows an anisotropic surface structure, which mediates the trapping process by "aquaplaning" of insects under humid conditions. For example, capture rates of ants were 80%, of *Diptera* 10%, and of *Coleoptera* 4% (all *Insecta, Arthropoda*) in pitchers of *Nepenthes madagascariensis* (Figure 11.19). The upper internal topography of the pitcher is characterized by layered epiticular and platelet-shaped wax crystals. The formation of wax crystals and their chemical composition are highly variable between species, but they generally contain long-chain aldehydes (C_{30} to C_{32}) and primary alcohols. The wax structure maintains the inner pitcher wall's slipperiness and effectiveness. *Nepenthes gracilis* produces a coat of wax crystals at the underside of the pitcher lid. The lid secretes more nectar than the peristome and increases the prey attraction. Rain drops "flick" the insects into the pitcher trap.

(a) (b)

Figure 11.19 *Nepenthes madagascariensis* (a); near Fort Dauphin, Madagascar; *N. madagascariensis* (b); crab spider lying in wait. (University of Bonn Botanical Garden) (© W. Barthlott.)

Figure 11.20 *Sarracenia purpurea*; near Sackville, N.B., Canada.

Visiting insects lose their footing on slippery surfaces and fall into the viscoelastic pitcher fluid that collects in the lower part of the pitcher. In young *Nepenthes* pitchers, closed by the lid, a large number of digestive glands at the inner bottom part secrete a fluid, which is free of bacteria and does not contain microbial growth-promoting mineral nutrients, such as phosphate and inorganic nitrogen. Furthermore, pitcher fluids contain potentially antibacterial and antifungal substances, for example, naphthochinones, plumbagin, 7-methyl-juglone, and defensive proteins (thaumatin-like proteins).

In open pitchers, **trophic multilevel plant-commensal relationships** exist, with the microbial component of crucial importance. So-called inquilines, such as bacteria, fungi, and some algae, contribute to the prey digestion by enzyme secretion. Released nutrients can be absorbed by the plant tissues or by the inquilines (Figure 11.16). In *Sarracenia purpurea* (Figure 11.20), fungal endophytes (some ascomycetes and basidiomycetes) living in the pitcher wall tissue could influence the nutrient availability for the plant.

Pitcher fluids of different taxa are inhabited by very distinct sets of inquilines. Animal inquilines, for example, *Copepoda* (*Crustacea, Arthropoda*), assimilate organic substances mainly and excrete compounds such as ammonium, phosphate, and urea. Consumers of nutrients act as competitors for nutrients, too. As shown for *S. purpurea,* arthropods act as predators, filter feeders, and shredders. Large insect larvae frequently contribute to the digestion in the chamber by mechanically breaking up the prey in *Sarracenia* and *Nepenthes*.

Morphological, physiological, and biochemical adaptation of pitcher traps provide strong evidence for selective pressures to maximize nutrient availability and uptake under different ecological conditions.

Pitcher plants are often habitats for animals without using them as source of nutrients. *Nepenthes bicalcarata* live in a mutualistic relationship with its symbiotic ant *Camponotus schmitzii*. The plant develops swollen hollow tendrils that are inhabited by adult ants, their eggs, and pupae. In addition, the plants secrete high amount of nectar from extrafloral glands localized at two large thorn-like structures on the upper peristome edges. Using this service, the ant can walk on the slippery parts of the traps. The insects protect the plant against small herbivorous insects, such as aphids. *C. schmitzii* can swim in the pitcher fluid, haul selected prey insects to the underside of the peristome, and consume it. The plant resorbs the nutrients from their excreta.

Nepenthes gracilis live together with a crab spider commensal (*Misumenops nepenthicola, Arachnida, Arthropoda*). The animal typically captures its prey at the pitcher rim, but it can also hunt it down in the fluid.

Nepenthes lowii evolve large, tunnel-shaped aerial pitchers whose nectar attracts the mountain tree-shrew *Tupaia montana* (*Mammalia*). The plant benefits from the feces of this small animal, which belongs to a mammalian order closely related to the primates..

Older pitchers of different taxa, which contain only weakly acidic fluid, are frequently colonized by frogs, tapoles, and crabs.

Thus, commensalism enables the plant to optimize their carnivorous life style!

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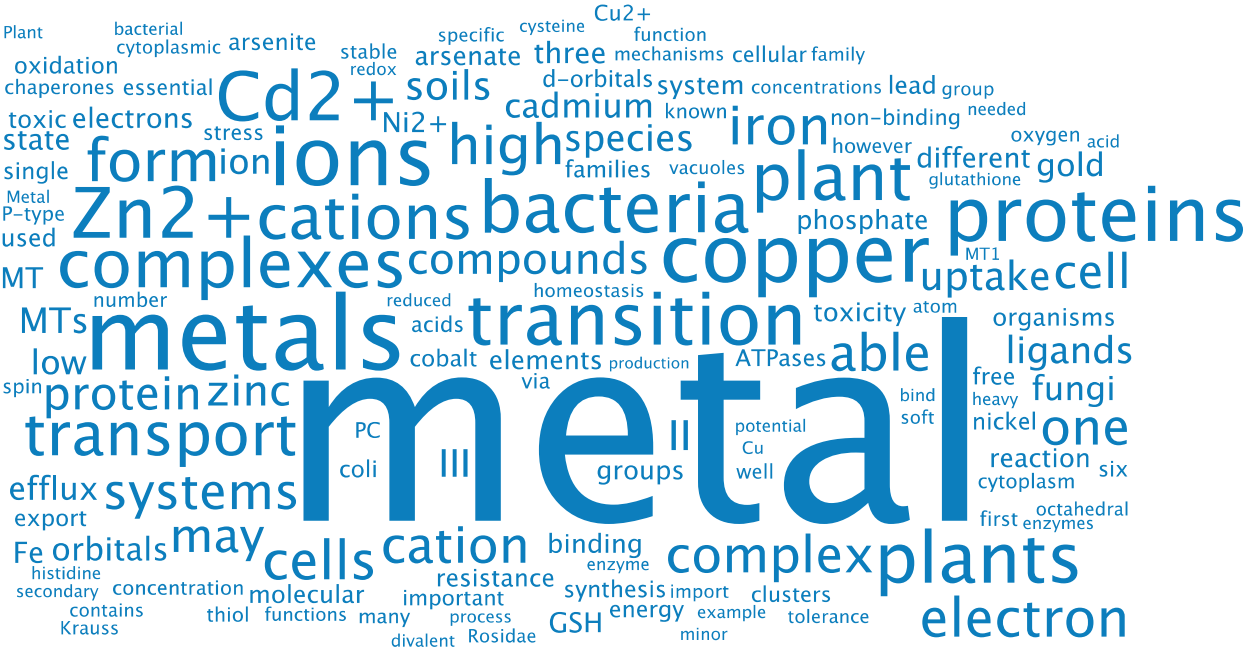
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bacteria
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proteins
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12
Excess of Metals

Dietrich H. Nies, Eva Freisinger, and Gerd-Joachim Krauss

Overview

Section S1.1.5 describes which chemical elements are available for life. The dry mass of all cells is predominantly composed of the major bioelements (Table S1.2), which are the nonmetals C, H, O, N, P, and S and the four metals Na, K, Mg, and Ca. These elements built the macromolecules and metabolites of the cell, they counteract the (mainly) negative charges of the macromolecules, they form concentration gradients, stabilize biological membranes and bridge negative charges. In addition to these major bioelements, however, minor bioelements or trace elements are needed (Table S1.3). Some of these trace elements belong to the nonmetals: the halogens Cl, Br, and I as well as B and Se. The minor nonmetallic bioelements are necessary to form halogenated or borated compounds, for Se-cysteine instead of sulfur-containing "normal" cysteine in some enzymes, and for many functions in case of Cl. Section 11.1 describes how plants that need major and minor bioelements as macro- and micronutrient take up these elements and regulate the metabolism of mineral nutrients, especially that of nitrogen, phosphorous, iron, zinc, and copper. Most minor bioelements, however, are transition metals, and these are essential at low but also toxic at higher concentrations. Other transition metals are "toxic-only." This chapter provides an introduction into the biochemistry of transition metals and how cells deal with their essential-and-toxic and toxic-only representatives.

12.1
Properties of Transition Metals

Most minor bioelements are transition metals, which are defined as those *d*-block elements that have incompletely filled **d-orbitals** (see Section S1.2.2). However, in the biological context also zinc, cadmium, and mercury are included, which feature a closed d^{10} electron shell. Transition metal ions can accommodate additional electrons in their *d, s,* and *p* orbitals, allowing them to form **complex compounds** with any organic ligand that possess a free electron pair. Some of these transition metal ions are redox-active, some are not, but usage of transition metal ions allows the cell to perform "complex biochemistry," which is required for all sophisticated biochemical reactions. So, some transition metal ions are essential. On the other hand, all transition metal ions are toxic when in surplus in the cell. **Toxicity** is based on three reasons:

1) Especially the transition metal cations of the softer elements Fe, Co, Ni, Cu, and Zn have a high **affinity for sulfur**. Binding to **thiol groups** of proteins can cause misfolding and damage leading, for instance, to inactivation of **iron-sulfur clusters**. Subsequent release of the iron may cause even more damage by production of **reactive oxygen species** (ROS). Sequestration of sulfide released intermediary during biogenesis of cysteine and iron-sulfur clusters mediates starvation of thiol compounds or again iron imbalances. Generally, this first mechanism of toxicity can be performed by all cations of the second and third transition period.
2) Some transition metal ions can cause **dangerous redox reactions**. Especially iron and copper, which may change between the oxidation states Fe(III)/Fe(II) and Cu(II)/Cu(I) at physiological redox potentials, react with ROS in a redox cycle. The reduced form of either metal, the predominant form in the cytoplasm, is able to provide an electron to H_2O_2, leading to the metal ion in the higher oxidation state, OH^- and the extremely reactive hydroxyl radical OH^*, which travels only short distances to the next macromolecule and damages or even inactivates it by a hydroxylation. Subsequently, the oxidized form of the metal is reduced again by the superoxide radical $O_2^- *$ to complete the cycle. Since both ROS are constantly produced in the aerobic cell, for instance, by flavin compounds, this **Fenton-type reaction** (first described by Haber and Weiss, 1932) is very dangerous for the cell. Therefore, a tight control of the cytoplasmic content of free iron and copper ions is essential, and each metal ion that disturbs this homoeostasis may lead to Fenton-type reactions as

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an indirect effect. Moreover, sulfur-binding transition metal cations may form bis-glutathionato-complexes with glutathione, the main cellular thiol compound in many organisms, or similar compounds with other thiols. These complexes with the general formula [X-S-Me(II)-S-Y] may react with molecular oxygen and two protons to H_2O_2, the free metal cation, and oxidized compounds X-S-S-Y that have to be reduced again by **glutathione, glutaredoxin** or **thioredoxin** (see Chapter 8). Meanwhile, the metal cation is able to bind two thiols again, releasing two protons and repeating the cycle. Since sulfur-loving transition metal cations inhibit also cysteine biosynthesis and consequently that of glutathione, increased oxidative damage cannot be repaired efficiently.

3) Some metal ions bind much tighter to metal-binding sites than other, releasing the "physiological" metal from its complex. The transition metal ions can be arranged in the **Irving-Williams series** simply due to their affinity to sulfur and oxygen ligands: Zn < Cu > Ni > Co > Cd > Fe > Mn > Mg. Hence, copper forms the most stable complexes, followed by zinc and the other metal ions down to manganese and magnesium. As an example, Co^{2+} and probably also Ni^{2+} strongly interfere with iron during synthesis of iron–sulfur clusters, with the subsequent oxidative damage caused by unbound iron. Although Zn^{2+} is binding strongly, the metal cation prefers to form tetrahedral instead of octahedral complexes because its d-orbital is completely filled and the remaining empty *s* and *p* orbitals can only accommodate the electrons of four ligands. Thus, octahedral complexes select against zinc. The same is true for copper but copper and zinc interfere in tetrahedral complexes. Basic features of transition metal ions such as **Pauling's electronegativity** (See Section S1.2.4) and polarizability can be used to predict the binding preferences of metal ions as outlined in the **hard–soft acid base (HSAB) concept** (Figure 12.1). Subsequently, the different binding preferences and abilities to form complex compounds decide which metal is able to replace another one.

As a conclusion, the cellular content of all transition metal ions has to be kept below the threshold concentration that causes damage. This is less problematic for the non-desired metal ions that are removed from the cytoplasm by binding and transport when their concentration becomes too high. These metal ions are all very soft "sulfur-lovers," which allows to identify and export them. It is more complicated for the desired but, nevertheless, also toxic cations of the first transition period. Here, an interplay of uptake, efflux, and sequestration adjusts not only the concentration of a single metal cation but also that of the cation bouquet of the cell, a process called multiple **metal cation homeostasis**.

12.2 Metal Transport through Cell Membranes

At the core of cellular metal homoeostasis are **secondary transport systems** (see Section S1.2.7, Figure S1.5), which usually have a rather broad substrate specificity and deal with groups of metals. The low substrate specificity allows a high transport rate since no time is wasted to bind and identify the substrate, and using the proton-motive force is much "cheaper" than splitting of ATP would be. Secondary uptake systems for divalent metal cations belong to various transport protein families. **MIT proteins** (metal inorganic transport, for example, CorA in *Escherichia coli, Gammaproteobacteria*) import Mg^{2+} and a selection of divalent transition metal cations including Co^{2+} and Ni^{2+}. Members of the **MgtE family** (none in *E. coli*) perform a similar action in other bacteria. **NRAMP proteins** (natural resistance-associated macrophage proteins, MntH in *E. coli*) transport Mn^{2+} but also Cd^{2+}. **ZIP proteins** (ZRT/IRT, ZupT in *E. coli*) mainly deal with divalent transition metal cations from Fe^{2+} to Zn^{2+}, and **NiCoT** (nickel cobalt transporters, not in *E. coli*) with these two cations, one or both. These proteins are all **uniporters** driven by $\Delta\Psi$, NRAMPs are probably proton-symporters energized additionally by $Z\Delta pH$ (see **Chapter 1**). Uptake of anions (sulfate, phosphate) is similarly performed by proton-symporters, and they import also the structural analogs chromate and arsenate, respectively. In case of phosphate, the respective **PitA-type proteins** accumulate phosphate:metal complexes, thus supplying essential metals together with phosphate, at the same time saving the energy of a proton plus the need to separate anion and cation of the phosphate complex before transport.

Most of these transporters belong to protein families that occur in all superkingdoms of life (see Chapter 4). Members of these families were sometimes found first in plants (e.g., the ZIP proteins), or in animals (e.g., NRAMP) and later bacterial representatives were identified, however, most of the time, the bacterial transport proteins were the archetypes of transport protein families.

Secondary efflux systems are ready to counterbalance this influx of an ion mixture and to remove surplus ions immediately, thus adjusting the bouquet. **CDF proteins** (cation diffusion facilitators) of three different subfamilies predominantly remove Zn^{2+}, Fe^{2+}, and Co^{2+}, respectively. CDF proteins occur again in all kinds of organisms. Members of other protein families export nickel, cobalt, chromate, and arsenite. In the export direction, efflux of cations needs a proton-cation antiporter to drive the reaction but $\Delta\Psi$ may be sufficient to get rid of surplus anions such as chromate or arsenite.

Secondary uptake and efflux systems may be under **flux control**. Surplus amounts of a signal compound in the cytoplasm inhibits further uptake by the importer and stimulate export by the efflux system. Together, these secondary uptake and efflux systems manage most of the

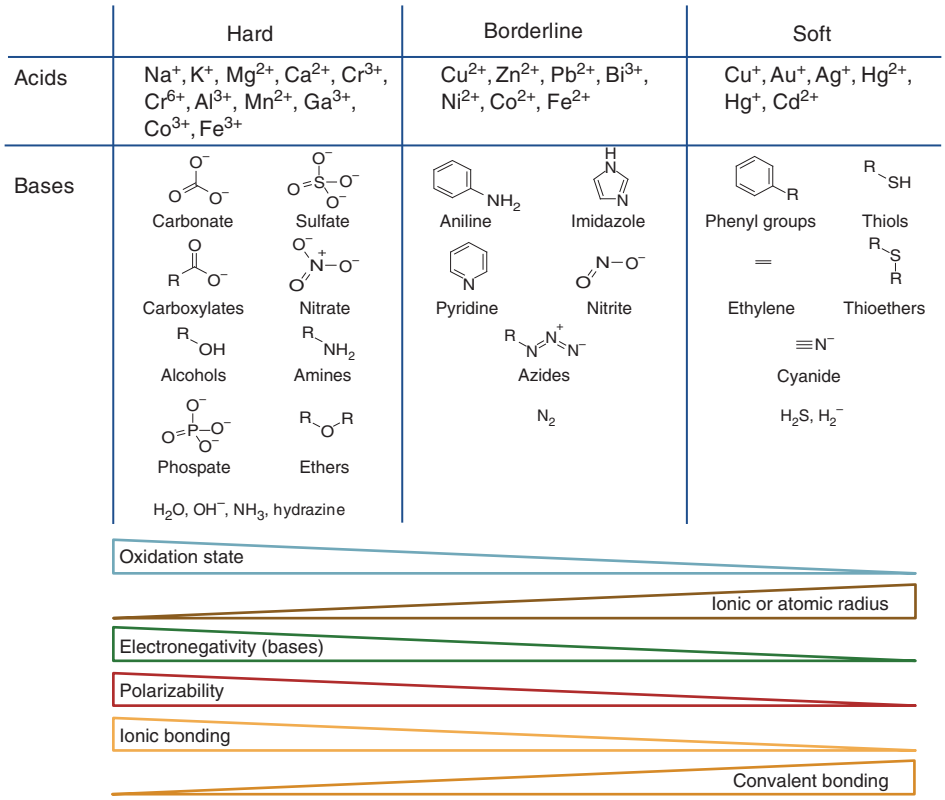

Figure 12.1 **Hard-soft acid (HSAB) theory anticipates the selectivity of metal ions for biochemical donor ligands.** Hard acids and bases tend to have a smaller ionic radius, a high oxidation state and weak polarizability. By contrast, soft species tend to have a large ionic radius, a low oxidation state and strong polarizability. Hard acids react preferentially with hard bases, and soft acids with soft bases. The affinity of a hard acid for a hard base is mostly ionic in nature, whereas the interaction between a soft acid and soft base is mostly covalent. Acids and bases that have an intermediate character between hard and soft are classified as borderline. Electronegativity describes the tendency of an atom to attract electrons toward it. By contrast, polarizability refers to the tendency of the electrons around an atom to be distorted from their regular distribution, typically toward the nucleus of another, more electronegative atom. (Reprinted from Lemire, Harrison, and Turner, (2013) by permission from Macmillan Publishers Ltd.)

necessary multiple metal homeostasis. Please note that this is not a **futile cycle**: uptake, for example, of a Zn^{2+} by a $\Delta\Psi$-driven uptake system followed by immediate removal of the same cation by a proton-cation-antiporter does not to waste energy but creates order (negentropy, see Chapter S1) because the energy is used to sustain the correct metal bouquet of the respective cellular compartment.

If uptake by the secondary systems is not sufficient to provide a metal in the desired amount to the cytoplasm (metal starvation), **primary uptake systems** are synthesized that are driven by ATP hydrolysis. These may belong to **P-type ATPases** (named by a phosphorylated intermediate during the transport cycle) or the **ABC-type** importers (ATP-binding cassette). Individual P-type ATPases may import Mg^{2+}, Zn^{2+}, or Cu^{+}. In bacteria, ABC-importers use a **periplasmic binding protein** (attached to the outer face of the cytoplasmic membrane in Gram-positive bacteria that have no periplasm) to catch the substrate and drag it to the actual ABC-importer. ABC-importers are highly specific. Individual importers are known for molybdate, tungstate, iron, manganese, nickel, zinc, sulfate, and phosphate. In case of iron, iron-complexing compounds named **siderophores** are produced by many organisms to bind Fe(III) from insoluble Fe(III) hydroxide complexes and to mobilize the metal for the cells. Some methane-degrading bacteria acquire copper needed for efficient methane oxidation by similar means.

If, on the other hand, the secondary efflux systems are not sufficient to remove excess metals, primary metal efflux systems are produced. Various groups of **P-type export ATPases** concentrate on the removal of different ions. P_{IB1}-type ATPases export monovalent transition metals such as Cu^{+}, Ag^{+}, and Au^{+}, P_{IB2}-type ATPases Zn^{2+}, Cd^{2+}, and Pb^{2+}, and P_{IB4}-type ATPases Co^{2+} and Zn^{2+} with a rate transport rate but comparable low substrate specificity. The genomes of bacteria and eukaryots alike may contain many members of the same group of P-type ATPases with slightly different functions. The bacterium *Cupriavidus metallidurans* (*Betaproteobacteria)* possesses eight P_{IB}-type ATPases. Two P_{IB1} proteins detoxify Cu^{+} by export while two different P_{IB1} proteins export this metal cation to supply it to copper-dependent proteins in the periplasm. Three P_{IB2} proteins transport *in vitro* Zn^{2+} and Cd^{2+} with equal ease but are produced only when the cell is

stressed by zinc or cadmium or lead ions, respectively. The remaining high-rate P_{IB4} protein exports Zn^{2+} at especially high zinc concentrations. The model plant *Arabidopsis thaliana* (*Brassicales*, *Rosidae*) also contains eight P_{IB}-type ATPases but their function has not yet been completely elucidated.

A specific group of **ATPases (ArsA)** is able to enhance export of arsenite through an arsenite-specific efflux system (ArsB). In Gram-negative bacteria, additional transenvelope efflux systems of the RND (resistance-nodulation-cell division protein family) are able to remove toxic cations already from the periplasm to the outside, thus preventing further uptake to the cytoplasm early on. Such systems have been described for zinc, cobalt, cadmium (CzcCBA), nickel (CnrCBA), copper and silver (CusCBA). Together with facilitated import of metal cations by the porins of the outer membrane, **RND systems** are thus mediating metal homeostasis in the periplasm.

12.3 Biochemistry of the Minor Biometals: Essential, Desired, but Also Toxic

12.3.1 Oxyanions: Molybdate and Its Neighbors

Molybdate is the most important metal for oxy-anion-catalysis. If needed, the metal is imported into bacterial and other cells by molybdate-specific ABC-importers. Inside, MoO_4^{2-} immediately is attached to a **molybdenum cofactor (MoCo), a molybdopterin**, which may be further attached to a nucleotide. MoCos are part of xanthin, sulfite, or aldehyde oxidases, or reductases for CO, formiate, arsenate, nitrate trimethyl amine N-oxide, or dimethyl sulfoxide. In photosynthetically active plant cells, MoCo acts as cofactor of the nitrate reductase (see Chapter 6, Figure 6.20).

For some (bacterial) enzymes performing similar functions, molybdenum can be substituted by tungsten residing in its specific tungsten cofactor. Again, an ABC uptake system is needed to import WO_4^{2-}, which is the trace element with the largest atomic number known. Please note that these metals only occur in their high oxidation states and hence in form of oxyanions bound to a cofactor scaffold in the cell. As low oxidation state cations, they would be extremely dangerous "soft" thiol poisons.

A cofactor completely different from the MoCos resides in addition to iron–sulfur clusters in **nitrogenase**, the enzyme needed to reduce molecular nitrogen to two molecules of ammonium at the expense of about 16 ATP (see Chapter 5, Figure 5.10). These enzymes are extremely oxygen-sensitive because the electrons needed for nitrogen reduction have to be provided at a strongly negative redox potential (see Section S1.3). Lowering this redox potential is the reason for the large amount of energy used for the process. Moreover, some of these electrons "escape" and reduce protons, leading to production of molecular hydrogen as "blind reaction" of nitrogenases. Nevertheless, Mo-containing nitrogenases are very efficient enzymes in cyanobacteria and root nodules of some plants (see Chapter 5). Some bacteria such as *Azotobacter chroococcum* (*Gammaproteobacteria*) are able to synthesize a less efficient **vanadium-containing nitrogenase** if no molybdate is available in the environment, which is one of the few examples for V as minor biometal. If neither Mo nor V are present, this bacterium is even able to synthesize a third class of nitrogenases containing only iron, which is even less efficient than the V-containing enzyme. With the exception of V, all transition metals of the groups 3, 4, 5 are rare, form insoluble hydroxide complexes in most environments and are thus not bio-available. Consequently, they have no biological importance.

Chromate enters all kinds of cells via sulfate uptake systems and can be exported again by **CHR proteins** (chromate resistance protein families) in many bacteria. CrO_4^{2-} may be reduced to Cr(III) and CHR determinants deploy superoxide dismutase-like proteins to deal with the radicals formed during this reaction, however, it is not known how the resulting Cr(III) is removed from the cell. In mammals, Cr(III) is a minor biometal and involved in **insulin response** as part of a specific chromium-binding polypeptide. Other beneficial functions of chromium are not known. Chromate pollution in soils, sediments and groundwater results in inhibition of plant growth and development, inhibition of chlorophyll biosynthesis, disturbances in water relations and mineral nutrition.

12.3.2 Manganese: the Electron Buffer

Group 7 of the periodic table of the elements contains Mn, Tc, and Re, elements featuring 5 d-electrons and 2 s-valence electrons. Tc does not possess a stable isotope and Re is extremely rare so that these metals have no biological impact, leaving Mn as only interesting biometal of this group of transition elements. Five d-electrons are a half-filled 3d shell, which represents a low state of energy. Thus, manganese is stable as divalent metal cation in the Mn(II) oxidation state, and can substitute Mg(II) in some reactions. Additionally, higher oxidation states such as (IV) exist too, which form insoluble hydroxides or oxyanions. This configuration of its electron shell gives Mn the feature of an electron buffer. Bound manganese may perform single- or dual electron transfer reaction or remain redox-inactive. With this feature, Mn is the transition metal with the lowest toxicity to bacteria and due to its relatively small, half-filled d-orbitals less likely to bind sulfur than the other transition metal cations. Mn^{2+} is imported by NRAMP, ABC, and other importers and exported in a few cases by CDF proteins, in *E. coli* by the recently identified MntP (= YebN) that does not belong to the CDF protein family.

Manganese phosphate complexes are able to perform a **superoxide dismutase** reaction, reducing one superoxide radical O_2^{-*} with a second one to generate O_2 and the less toxic H_2O_2 (see Chapter 8). It is, therefore, no surprise that manganese became also the active center of the enzyme superoxide dismutase in plant mitochondria that catalyzes the same reaction more efficiently.

The most important manganese-containing protein complex is the **photosynthetic reaction center II** of cyanobacteria and chloroplasts, which contains a cluster of 4 Mn ions as the **water-splitting complex**. This complex is able to oxidize two molecules of water to one molecule of molecular oxygen and 4 electrons without too much production of ROS. Such a reaction seems only to be possible with the "electron buffer" manganese.

12.3.3 Transition Metal Cations in Octahedral Complexes

If transition metal cations form **octahedral complexes**, six acceptor orbitals are needed to harbor the six free electron pairs from the six ligands. Four orbitals come from the single s- and the three p-orbitals of the valence shell, the two remaining orbitals are d-orbitals from the electron shell below the valence level. When these six acceptor orbitals interact with the six ligand orbitals six bonding and six antibonding molecular orbitals of different orbital energies are generated in the complex to keep the number or orbitals constant (see Section S1.2). Thus, iron and other metals in an octahedral complex possess six bonding orbitals, six antibonding orbitals of relatively high energy, plus the three nonbonding d-orbitals that do not contribute to complex formation.

The d-electrons of a transition metal cation in an octahedral complex have to be distributed to the three **nonbonding** and if required also to the two **antibonding** molecular orbitals that are lowest in energy, that is, the two molecular orbitals originating from the d-orbitals of the metal (see Section S1.2). The first three electrons {in V(II), Cr(III), Mn(IV)} each go into one of the three nonbinding orbitals, following **Hund's rule**, each in the +1/2 spin. Thus, such a complex would have a multiplicity of 4 (= 3 times 2 x ½ plus 1, quartet state).

Electron number four {moving on to Cr(II), Mn(III), both minor oxidation states) has two possibilities. If the pairing or spin-inversion energy for the nonbinding orbitals is lower than the difference in energy between non- and anti-binding orbitals, it will pair in the −1/2 spin with an electron in +1/2 spin in a nonbinding orbital, which decreases the multiplicity to 3 (low-spin complex, triplet). Alternatively, if the relationship of the two energies is vice versa, it will go to one of the anti-binding orbitals, which increases the multiplicity to 5 (quintet) and weakens the bond to one of the six ligands (high-spin complex). The kind of the central metal cation, of the ligands and their distance decides about the two energies and therefore, if a high or low-spin complex is being formed.

Electron number five {Mn(II), Fe(III)} has the same choice between a low-spin complex with a multiplicity of 2 (doublet) or a high-spin complex with 6 (sextet), which represents a state of low energy in this case because all five d-orbitals, the 3 nonbinding and the 2 anti-binding ones, each contain one electron, which is a half-filled shell state. With electron number six {Fe(II), Co(III)}, the low-spin state has a multiplicity of just one (singulet) and is again a state of low energy because now all three nonbinding d-orbitals are completely filled, with a +1/2- and a −1/2-spin electron each. In the high-spin state, one nonbinding d-orbitals harbors a pair of electrons, two nonbinding d-orbitals and the two anti-binding d-orbitals a +1/2-spin electron each, giving a multiplicity of 5 (quintet).

Electron number seven {Co(II), Ni(III)} has no choice because the three nonbinding d-orbitals are filled. It has to go to an anti-binding orbital, loosening the complex bond to one ligand. So, the multiplicity of the low-spin complex is 2 (doublet) and that of the high-spin complex 4 (quartet), with two electron pairs in nonbinding orbitals and three single electrons in the anti-binding orbitals and the remaining nonbinding orbital. With electron number eight {Ni(II)}, there is no longer a difference between low and high spin because the nonbinding orbitals are completely filled with three electron pairs and the two remaining electrons occupy the two anti-binding d-orbitals in the +1/2-spin state according to Hund's rule with a multiplicity of 3 (triplet).

With electron number nine {Cu(II)}, one anti-binding d-orbitals is fully occupied and therefore, one ligand cannot be longer accommodated. Moving finally on to ten electrons {Cu(I), Zn(II)}, the last d-orbital is fully occupied, the d-shell is complete, and octahedral complexes cannot be formed any longer.

There are other forms of complexes, too, but they will not be discussed here. Nevertheless, this simple rule of electron occupation in an octahedral complex of a transition metal cation is the key to understand the chemical features of these metals.

12.3.4 Iron: Transmitter of Single Electrons

Group 8 of the periodic system contains Fe, Ru, and Os. While the latter two are sulfur-toxic "soft" metals, albeit rare in natural ecosystems, iron is the most prominent transition metal. Because elemental synthesis in stars (see Chapter S1) is exergonic up to iron but endergonic thereafter, this process led to high amounts of iron in the universe with respect to the high atomic mass of this element. Iron was even more enriched during birth of the inner planets of our solar system because the lighter elements were blasted away by solar winds when the sun ignited its hydrogen-fusion process. Since the young Earth was anaerobic, iron existed as ferrous iron Fe(II), the soluble divalent Fe^{2+} cation. So, a lot of iron was available for early life.

This changed when the cyanobacteria started to produce molecular oxygen, allowing a Fenton reaction catalyzed by iron after the **first oxygenation event** 2.3 billion years ago (see Chapter 4), and decreasing iron availability because some iron existed now as Fe(III) that forms insoluble hydroxide complexes as all other trivalent metal cations. Subsequently, Zn(II), which became more available under these conditions, substituted Fe(II) when only the function as a redox-neutral structural metal was required.

The problem of the decreased availability of Fe(III) was solved by the evolution of **siderophores** after the oxygenation events. Iron is an ideal one-electron transfer agent. In octahedral complexes, Fe(III) has five d-electrons that may form an energetically attractive high-spin complex with all three nonbinding and the two anti-binding d-orbitals occupied by one electron each, and Fe(II) a similarly attractive low-spin complex with all three nonbinding d-orbitals harboring an electron pair (Figure 12.2). Since the ligands and their distance from the iron cation decide which kind of complex is being formed, conformational changes of an enzyme containing iron, for example, in the center of a cytochrome may favor one kind of complex or its alternative, allowing to change the redox half cell potential of iron-containing cofactors over a wide range.

Half of the cellular iron in aerobic cells resides **in iron–sulfur clusters** that usually have a negative half cell potential E_o' at pH = 7, the other half in iron-containing compounds, the cytochromes with positive half cell potentials. Thus, in a respiratory chain that transfers electrons from NADH to O_2, these two iron-containing factors are the most important electron transfer agents, supplemented by flavins, chinons, and copper.

In bacteria, fungi and plants (see Section 11.1), iron cations are imported by siderophore-dependent import routes and those transporting the unbound metal cations. Surplus iron in the cell is stored as Fe(III) in the center of plant ferritins and bacterial bacterioferritins. Since Fe^{2+} rapidly enters cells under anaerobic conditions, iron efflux system of the **CDF protein family** evolved to deal with this problem.

12.3.5 Cobalt and Nickel: between Iron and Zinc

Cobalt and nickel are not only located between iron and zinc in the periodic system of the elements, the features of these two metals are also between the redox-active iron and the non redox-active zinc cations. First of all, both cations are not able to be reduced from the +II oxidation state (the divalent metal cation) to the +III state as free cations, the half cell potentials of these reactions does not allow it under physiological conditions (Figure 12.2). Therefore, both metal cations do not perform the Fenton reaction directly. Nevertheless, at least cobalt but probably also nickel inhibit formation of iron–sulfur clusters, which inhibits synthesis of important proteins but which also disturbs iron homeostasis. It is therefore necessary to keep the cytoplasmic availability of both metals low. This is done as described in Section 12.2 by secondary uptake and efflux systems, additional ABC-uptake systems that belong to a special subgroup of these transporters in case of cobalt, and CDF as well as RND-driven export systems.

On the other hand, both oxidation states are possible in complexes. Most important cobalt-containing complex is the **corrinoid B12** and derivatives thereof. Some organisms such as *E. coli* are able to keep their cytoplasmic cobalt content especially low because they do not use cobalt cations directly but only in form of B12, which is taken up from the outside and consequently has to be synthesized by other organisms. In B12, the Co(III) is kinetically very stable because the cation is in the half-filled high-spin state (Figure 12.2), making B12 into an ideal "trading" form of cobalt between organisms. That way, free cobalt cations that may disturb iron homeostasis are avoided. Consequently,

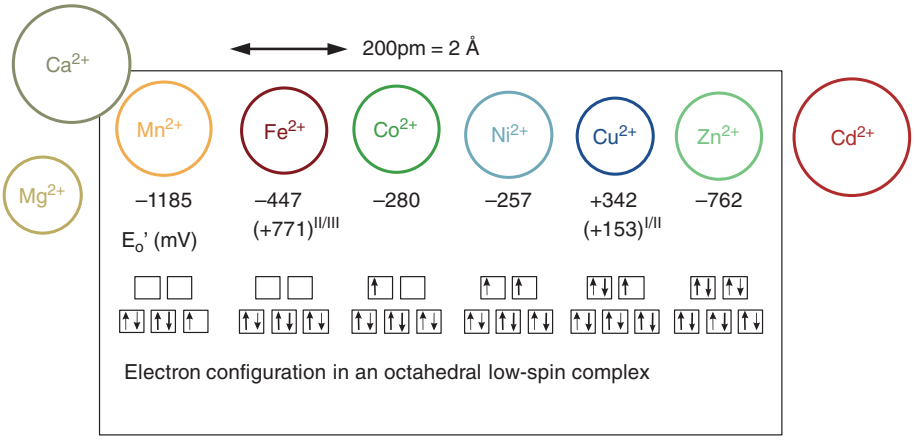

Figure 12.2 Transition metal cations and octahedral complexes. The figure shows the transition metal cations of the first transition group (colored) and a few related metals (gray) drawn to the scale. The redox potential at pH value 7 is indicated below these symbols. Further below is the occupation of the three nonbinding and two anti-binding electron orbitals in a low-spin octahedral complex. Arrows pointing up indicate a spin of +1/2 and those pointing down a spin of −1/2.

only a few cobalt-dependent enzymes use not B12-bound cobalt, such as nitrile hydratases and molinate hydrolase.

Typical **B12-dependent enzymes** are mutases that rearrange C–C and C–H bonds. During the reaction, intermediary forms such as a hydrogen atom are stored bound to the cobalt so that the remaining carbon skeleton of the substrate can rearrange. An example is the methyl-malonyl-*S*-CoA mutase (see Section S1.3.6.4) used for the reductive branch of the propionate fermentation pathway. In this important reaction, succinyl-*S*-CoA ($COOH-CH_2-CH_2-COSCoA$) is mutated to methy-malonly-*S*-CoA ($CH_3-CH(-COOH)-COSCoA$). By "parking" a hydrogen atom of C3 of succinyl-*S*-CoA at the cobalt center, the carboxy group is able to move one carbon atom up to C2, and the "parked" hydrogen atom is released back to the now terminal C3 atom. That way, the translocated carboxy group is now attached to a C–H-acidic carbon atom, can be transferred to biotin, and used to carboxylate the next pyruvate to enter this branch of the pathway. Comparable, methyl groups can be transferred, for instance, during methanogenesis to allow synthesis of Acetyl-*S*-CoA from a methyl group, carbon monoxide and HS-CoA by the acetyl-*S*-CoA synthetase (see Section S1.3.7).

Methanogenic archaea contain also a **nickel-containing tetrapyrrol ring** factor, named F_{430} for its fluorescence maximum. The nickel center in F_{430} is the last atom necessary for methanogenesis. Here, the methyl-group bound as CH3-S-CoM and factor H-S-CoB interact to form methane and the mixed heterodisulfide CoM-*S*-S-CoB, which conserves the energy gained by reduction of CO_2 to CH_4. Reduction of this compound to the two thiol compounds H-S-CoM and H-S-CoB can be subsequently used to form a protonmotive force (see Section S1.3.7). Nickel is also present in the acetyl-*S*-CoA synthetase, which is the central enzyme of chemolithoautotrophic methanogenic archaea to assimilate CO_2. Nickel-containing enzymes that exist also in aerobic organisms are the most important group of hydrogenases that assimilate molecular hydrogen as a electron pair plus two protons; urease that hydrolyzes urea ($NH_2-CO-NH_2$) to CO_2 and two NH_3, and a special nickel-containing superoxide dismutase.

Unfortunately, Ni^{2+} cannot be "prisoned" in a complex as it can be done with cobalt in B12 because there is no longer a difference between high- and low-spin complexes of Ni(II) (Figure 12.2). Instead, histidine-rich nickel chaperones sequester this metal to keep the cytoplasmic nickel availability low and provide it subsequently to the nascent active centers of hydrogenases, ureases or the anaerobic CO-dehydrogenase part of the acetyl-*S*-CoA synthetase in bacteria (see Section 12.5.3). The stomach bacterium *Helicobacter pylori* is only able to settled in the acidic environment of this organ because it is able to synthesize hydrogenase and urease, the latter used to hydrolyze urea to ammoniac that consequently buffers the direct environment of this bacterium.

12.3.6 Copper: the Oxygen-Handle

Metallic copper should have the electron configuration d^9s^2 but has $d^{10}s^1$ because a filled d-orbital is an attractive low-energy state. Consequently, copper cations may occur as monovalent cations Cu^+ with the single valence electron gone, and the divalent copper cation Cu^{2+} with the two s electrons released that actually would occupy the valence shell. The halfcell potential of the Cu^+/Cu^{2+} pair is well within the physiological range, involves a "radicalic" transfer of a single electron, thus allowing the Fenton reaction. Moreover, Cu is at the top of the Irving-Williams series (Figure 12.1) as far as transition metals of the first transition period are concerned. This makes copper an extremely dangerous minor biometal.

E. coli and maybe many other bacteria solve this problem by not taking copper cations up at all, at least voluntarily. Since the broad substrate specificity of some secondary uptake systems such as **the ZIP-family protein** ZupT leads to import of Cu^{2+} nevertheless, a **P-type APTase** can be found in nearly all free-living bacteria that removes the excess copper immediately again.

It is, nevertheless, attractive to use the redox abilities of copper, which is able to handle molecular oxygen easily because both substances feature a radicalic disposition. Thus, copper is in the active site of copper-zinc-type **superoxide dismutases**, and oxidases such as the terminal cytochrome *c* oxidase of the **respiratory chain** of mitochondria and many bacteria. Here, copper centers lead the electron from cytochrome *c* to the cytochrome aa_3 centers. An additional copper ion in the protein deals directly with the molecular oxygen. In cooperation with iron of a cytochrome and a radical-quenching tyrosine residue the copper ions prevents formation of radicalic ROS. Another important copper-containing protein is the **plastocyanin**, which transfers electrons within the photosynthetic electron transport chain in cyanobacteria and chloroplasts (see Chapter 8).

To avoid copper damage, bacteria such as *E. coli* use copper only in the periplasm and sequester it here by **copper chaperones**. If bacteria have to take copper up into the cytoplasm, other copper chaperones stand ready to sequester the metal cation (see Section 12.5.2). Eukaryots import copper by transport systems of the **CTR (for copper transport) protein family**, and sequester the metal cation immediately after transport too. P-type ATPase have a dual role in Eukaryots. At ambient copper concentrations, they transport Cu(I) into the endoplasmatic reticulum to provide it for synthesis of copper-dependent proteins. At high concentration, the proteins appear at the cytoplasmic membrane and export Cu^+. Some bacteria have two different P-type ATPases for these functions, one to provide copper for biosynthesis outside of the cytoplasm, and a second one for detoxification.

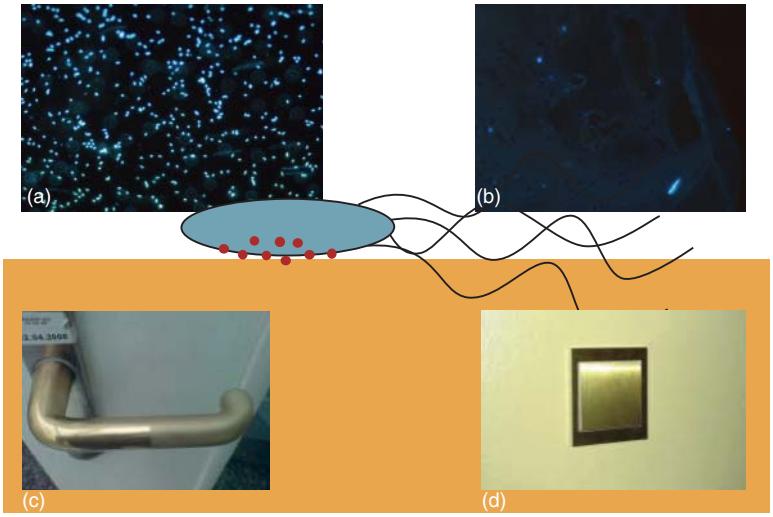

Figure 12.3 Killing of bacteria on solid copper surfaces. A bacterial cells (green ellipsoid) sitting on top of a solid copper or copper alloy surface (orange) oxidizes the copper atoms to copper ions (red). Subsequently, these are taken up into the cell by uncontrolled mechanisms and in a high local concentrations. These ions are "soft" thiol toxins and inactivate the cytoplasmic membrane and DNA by Fenton reaction. The pictures on the top show bacteria growing after 4 days on steel (a) and copper (b). Thus, copper door handles (c) and light switches (d) may help to limit the spread of multiple antibiotic-resistant bacteria in hospitals.

Calculated from the redox potential (Figure 12.2), metallic copper may also exist in organisms beside Cu(I) and Cu(II). Indeed, bacteria growing on **metallic copper surfaces** are rapidly killed because they oxidize the top layers of the metallic surface, leading to solubilization of the resulting copper ions, which subsequently inactivate their liberators. This mechanism can be used to control the spread of pathogenic bacteria in hospitals by using copper door handles, toilet seats and light switches instead of steel or plastic items (Figure 12.3).

12.3.7 Zinc (and Exceptionally Cadmium): Non Redox-Active Transition Metals

Zn^{2+} has a completely filled d-orbital and is not redox-active under physiological conditions. This makes zinc the most important trace element after iron. Since Zn^{2+} cannot be stored as oxidized zinc such as iron can as Fe(III) in ferritins or bacterioferritins, zinc homeostasis is mainly reached by controlled transport, as far as we know today. Here, a variety of secondary uptake systems (e.g., from the ZIP family) import this metal, balanced by exporters of the CDF and P-type ATPase families in all kinds of organisms. If additional zinc is needed, many bacteria possess zinc-specific ABC-importers.

Zinc, mostly located in the center of a tetrahedral complex composed of histidine, cysteine, glutamate or aspartate residues, has two functions. First, to stabilize wobbly polypeptide chains into more rigid conformations. Secondly, it can accept as **Lewis acid** a free electron pair from a Lewis base during a catalytic cycle. There are hundreds of zinc-dependent enzymes known, too many to provide a complete list here. Examples are alcohol dehydrogenase, copper-zinc-**superoxide dismutase**, and DNA and RNA polymerase. In the bacterial **RNA polymerase** the function of the zinc is to get the large RpoC or β' subunit into the correct conformation, which is controlled by the RpoZ or ω subunit. Only when RpoZ gives it OK, the β'ω heterodimer is attached to the $\alpha_2\beta$ heterotrimer, leading to the mature RNA polymerase. Eukaryotic cells possess a huge variety of regulatory proteins with a zinc finger DNA-binding motif.

There is one single example for Cd^{2+} substituting zinc in an enzyme, a **carbonic anhydrase** of the marine diatom *Thallassiosira weissflogii* (*Bacillariophyta*, *Stramenopiles*), which lives in an environment poor in zinc. This enzyme is readily able to use cadmium and zinc with equal ease at its active site, guaranteeing function of this important enzyme even when zinc is depleted. More examples for cadmium as minor biometal, however, are lacking at the moment. Due to the higher toxicity of cadmium compared to zinc and the usually above tenfold higher concentration of zinc in most environments compared to cadmium, it may well be that this diatom protein remains the only one able to perform with cadmium in its center.

12.4 Biochemistry of Chemical Elements Without Known Biological Functions

12.4.1 Cadmium and Lead

Cd(II) and Pb(II) are both not redox-active divalent metal cations. They form complexes with sulfide in anaerobic systems and other anions such as phosphate in all

environments. Especially lead phosphate $Pb_3(PO_4)_2$ has an extremely low solubility product of 10^{-54}, making the free metal cation nearly unavailable in phosphate-rich environments. Nevertheless, since PitA-type uptake systems import metal-phosphate complexes, lead is able to enter bacterial cells.

Due to the low biological value, both metals are tolerated up to a certain threshold concentration determined by the metal affinity of a regulatory **MerR-type protein**, and exported by **P-type ATPases** when the cytoplasmic concentration exceeds this value. For cadmium, CDF and RND-type export systems may also contribute to cadmium resistance, however, the P-type ATPase are central to this process because they may be able to "leach" Cd^{2+} from cytoplasmic thiol compounds. Cadmium toxicity in bacteria is well understood and is mainly based on binding to sulfur, as has been outlined above (see Chapter 12.1). Although a lead-exporting P-type ATPase is central to lead resistance, other factors, for example, a lead importer and a periplasmic component may also contribute to bacterial lead resistance but details have not been elucidated at this time.

12.4.2 The Noble Metals Silver and Gold

The transition metal cations of the second and third transition period are mostly "soft sulfur-lovers" (Figure 12.1). Thus, they act toxic by binding to thiols, intermediary sulfide, and iron–sulfur centers, with all the consequences as outlined above (see Section 12.1). Silver is only present as Ag^+ and gold as Au^+ and Au^{3+}. Both cations are unstable and can be readily reduced to the metallic forms. Moreover, gold ions are only stable in complexes. Nevertheless, mobile gold forms are produced by metabolic activities of bacteria, and other bacteria such as *C. metallidurans* transform ionic gold back to the metallic form (Figure 12.4).

Detoxification of silver and gold ions is very similar to that of Cu^+. In the cytoplasm of bacteria, copper, silver and gold occur only as monovalent cations due to the redox potential in this cellular compartment. So, Cu(II) and Au(III) are immediately reduced to the oxidation state +I. **MerR-type regulatory proteins** are ready to sense these three cations with sometimes overlapping substrate specificities, and they regulate expression genes for **P-type ATPases**, which again have overlapping substrate specificities. Sometimes, individual proteins export only one of these metals, others two, again others all three of them. In the periplasm of proteobacteria, chaperones sequester the ions and prevent re-uptake. This can be small periplasmic proteins such as CusF or SilF, or larger proteins such as SilE with a higher storage capacity. Finally, **RND-driven efflux systems** export the metal from the periplasm to the outside across the outer membrane in Gram-negative bacteria.

Mostly, silver and gold resistance in bacteria is mediated by copper resistance systems. One silver system and a gold resistance system, both from the enterobacterial genus *Salmonella* (*Gammaproteobacteria)*, however, seem to be specifically directed to the two respective metal cations. Silver has been used as an **antimicrobial substance**. So, it makes sense that silver-resistant bacteria have evolved. But is remains unclear why a gut bacterium has evolved a specific resistance against gold, which is very rare in the environment.

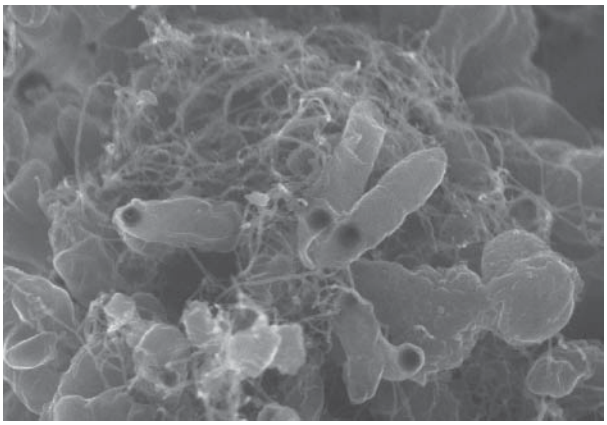

Figure 12.4 Formation of gold nano particles by *C. metallidurans* cultivated in the presence of Au(III) complexes. The bacteria were grown in a column that was percolated with mineral salts medium containing soluble Au(III) complexes. The column retained the gold while controls (dead bacteria, no bacteria) did not. Electron microscopy displayed a bacterial biofilm and the cells (ellipsoids) possessed gold nano particles (dark spots). (Courtesy of F. Reith, University of Adelaide.)

12.4.3 Mercury

Mercury is unique among the transition metals for four reasons: (i) the divalent metal cation Hg^{2+} is the most toxic one in this group; (ii) reduction of this cation to the metallic form is possible be physiological means; (iii) metallic mercury has a very low vapor pressure and is a liquid under ambient conditions, and (iv) carbon–mercury bonds are comparable stable, which allows the occurrence of organo-mercury compounds such as dimethyl mercury. These products are synthesized by many organisms such as sulfate-respiring bacteria.

The unique features of mercury directly lead to a strategy to deal with its extreme toxicity. Since Hg^{2+} threatens all thiol- and iron–sulfur-containing structures in the cytoplasm of prokaryotes and eukaryotes as well as in the periplasmic of Gram-negative bacteria, mercury has to be removed immediately. In their periplasm, **mercury chaperones** such as MerP bind the cation tightly to thiol groups. From here the cation is transferred to the thiol groups of uptake systems, for instance, MerT. In the cytoplasm, the NADPH-dependent **mercury-reductase MerA** accepts it and reduces it to the metallic, volatile form that evaporates out off the cell. Organo-mercurial compounds are even more toxic than the mercury cation, they are hydrolyzed to Hg^{2+} in the cytoplasm by MerB-type proteins.

The sensor and regulator of expression of *mer* resistance determinants, MerR, was the archetype of a whole family of regulators. MerR sits on the DNA of the *mer* determinant, preventing transcription initiation in the absence of Hg^{2+}. When the cation appears, it binds to thiol groups of the MerR dimer at the DNA, the DNA is distorted to allow a rapid transcription initiation. That way, a small concentration of the metal is sufficient to mount a rapid response of the cell and to detoxify Hg^{2+} before it can harm the cell.

12.4.4 Arsenate, Arsenite and Antimonite

Arsenic and antimony are no metals but half-metals or metalloids, with features between metals and non metals. Both are detoxified by similar systems with arsenic occurring much more frequently in the environment than antimony.

Arsenic is stable in two oxidation states, As(V) as **arsenate** $\mathbf{AsO_4^{3-}}$ and As(III) as **arsenite** $\mathbf{AsO_3^{3-}}$. In anaerobic ecosystems, arsenate can serve as electron acceptor of an anaerobic respiration process, yielding arsenite. Subsequently, arsenite can be oxidized again by chemolithoautotrophic bacteria (see Section S1.2). The result is an arsenic cycle that mobilizes the element in the environment.

Arsenate is a structural analog of **phosphate** and imported by **PitA-like** and other phosphate uptake systems in bacteria and eukaryotes including animals and man. Once inside the cell, arsenate is used instead of phosphate, however, while phosphate anhydrates and esters are stable, those with arsenate are not and hydrolyze quickly, which leads to uncoupling of cellular metabolism. This fact makes it unlikely that some bacteria contain arsenate instead of phosphate in their DNA. To deal with this problem, arsenate cannot be simply exported by efflux systems because the high concentration of cytoplasmic phosphate would competitively inhibit such a reaction, however, arsenate can be reduced to arsenite by physiological means but phosphate not to phosphite (although phosphite occurs in certain ecosystems, this compound is not stable under cytoplasmic redox conditions). Arsenite now can be readily exported by a secondary ArsB-type efflux systems. So, a basic arsenate resistance system is composed of a regulator that senses arsenite as As^{3+} cation, an arsenate reductase and an arsenite efflux system. Such as system deals also with arsenite entering the cell as glycerol-analogon by facilitated diffusion.

More efficient arsenate resistance systems in bacteria contain two additional factors. An ArsA-type ATPase binds to an ArsB-type export system and drives efflux with the additional hydrolyzation of ATP. The second component is an arsenite chaperone that sequesters the ion and drags it to the ArsAB efflux systems. In addition to efflux, arsenate can also be detoxified by methylation, leading to organo-arsenic and volatile compounds.

12.5 Metal-Binding Peptides and Proteins Involved in Transition Metal Homeostasis

12.5.1 Function of Intracellular Metal-Binding Polypeptides

Together the transport systems described above (see Section 12.2) adjust the cellular metal mélange of essential (see Section 12.3) and toxic-only metals (see Chapter 12.4) in a kinetical flow equilibrium based uptake and efflux reactions. All these transporters may be flux-controlled and directed by regulated expression of their genes. Nevertheless, the transport reactions need a counterbalance for an efficient cellular storage, sorting, allocation process, and to prevent toxic interferences between the various cations. Metal-binding polypeptides and metal chaperones are required for this function.

12.5.2 Copper Chaperones

Many bacteria seem to avoid import of copper into the cytoplasm and remove the metal immediately when it has entered accidently (see Section 12.3.6). If copper uptake is necessary, the ion is rapidly bound to **copper chaperones** in bacterial, fungal and plant cells to prevent damage. Copper chaperones are able to transport and insert the metal in specific sites of target proteins, such as **cytochrome *c* oxidase**. In the yeast *Saccharomyces cerevisiae* (*Ascomycota*, *Dikarya*), the bacterium *Salmonella enterica* (*Gammaproteobacteria*), the mammal *Homo sapiens* (*Mammalia*, *Vertebrata*) and many other organisms a copper chaperone is essential for expression of an active, Cu-containing form of the CuZn-containing **superoxide dismutase**. Other metallochaperones deliver copper to Golgi vesicles via interaction with a copper-pumping P_{IB1}-type ATPase. All these processes keep the availability of "free" copper ions in the cell in a zeptomolar (10^{-21} M) concentration range.

12.5.3 COG0523 Metal Chaperones

The cobalt and nickel levels are kept low in most organisms. Cobalt is directed to the center of cofactor B_{12} or derivatives of this corrinoid, which is a kind of non-toxic "trading form" of cobalt in the living world. The few enzymes that need nickel for synthesis of **urease** (e.g., in the pathogen *Helicobacter pylori, Epsilonproteobacteria*) or **hydrogenase** (e.g., in the model bacterium *Escherichia coli, Gammaproteobacteria*) receive it from **nickel-specific metal chaperones**, UreB and HypB, which facilitate Ni^{2+} insertion into their target protein. These proteins belong to the COG0523 group of the G3E family of P-loop GTPases and the energy of GTP-hydrolyzation is recruited for an efficient transfer process. COG0523 also contains groups

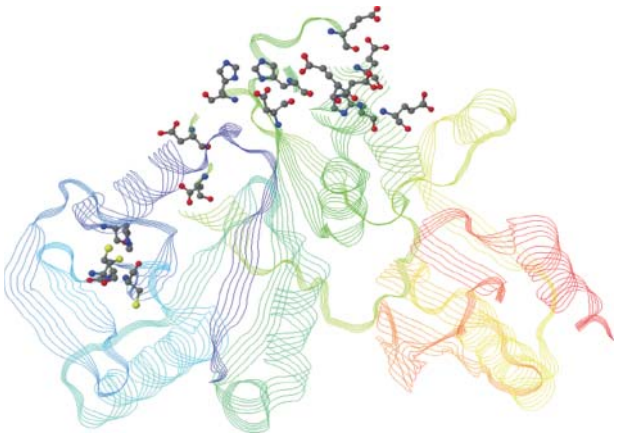

Figure 12.5 **Predicted structure of the COG0523 protein YjiA from *E. coli*.** Two protein domains are visible, one purple-blue on the left and one yellow and red on the right hand. Amino acids that might form metal-binding sites are indicated. These are in the first domain 3 cysteine and one histidine residue, which might form a site with strong metal affinity, and some aspartate, glutamate and histidine residues, which might form sites in the flexible bridge between both domains. Model created from PDB 1NIJ using Geneious (*www.geneious.com*).

of proteins needed to assemble Co^{2+} or Zn^{2+}-dependent metalloproteins.

Two COG0523 members from *E. coli* were known. YeiR binds several zinc ions, which stimulates GTPase activity. YjiA (Figure 12.5) has been crystallized, preferred Zn^{2+} over Co^{2+} and Ni^{2+}, and bound a maximum of seven Zn^{2+} per dimer, two in an internal site, one in a bridging site between the protomers, and four in surface sites. These proteins are a just emerging group that may play an important role in the **cellular metal sorting and allocation process**.

12.5.4 Glutathione and Related Compounds

Glutathione (GSH) is a crucial compound in the metabolism of plants and other organisms (see Figure 1.12). It acts as an antioxidant (see Chapter 8) and substrate for the detoxifying enzyme GSH transferase (see Chapter 13). GSH is synthesized from cysteine, glutamate and glycine via two consecutive ATP-dependent steps with **γ-glutamylcysteine** as intermediate. The respective genes for the two enzymes are *gsh1* and *gsh2* in plants and *ghsA* and *gshB* in bacteria. In plants, Cd^{2+} and Cu^{2+} up-regulate expression of both genes.

Some organisms contain additionally or solely modified glutathione, glutathione-like or other thiol compounds with functions similar to that of glutathione. In bacteria of the phylae *Firmicutes* and *Actinobacteria* a variety of thiol-rich compounds can be found instead of glutathione, but some of these bacteria import and use glutathione if the substance is available in the growth medium. In members of the pea family *Fabaceae* (*Fabales*, *Rosidae*), **homoglutathione** (γGlu-Cys-ß-Ala, hGSH) is synthesized in addition to GSH by the hGSH synthetase.

GSH is able to bind several transition metal cations and metalloids. It forms low affinity monodentate bis-glutathionato complexes with Cd^{2+}, **Cd-GS_2**, which can be

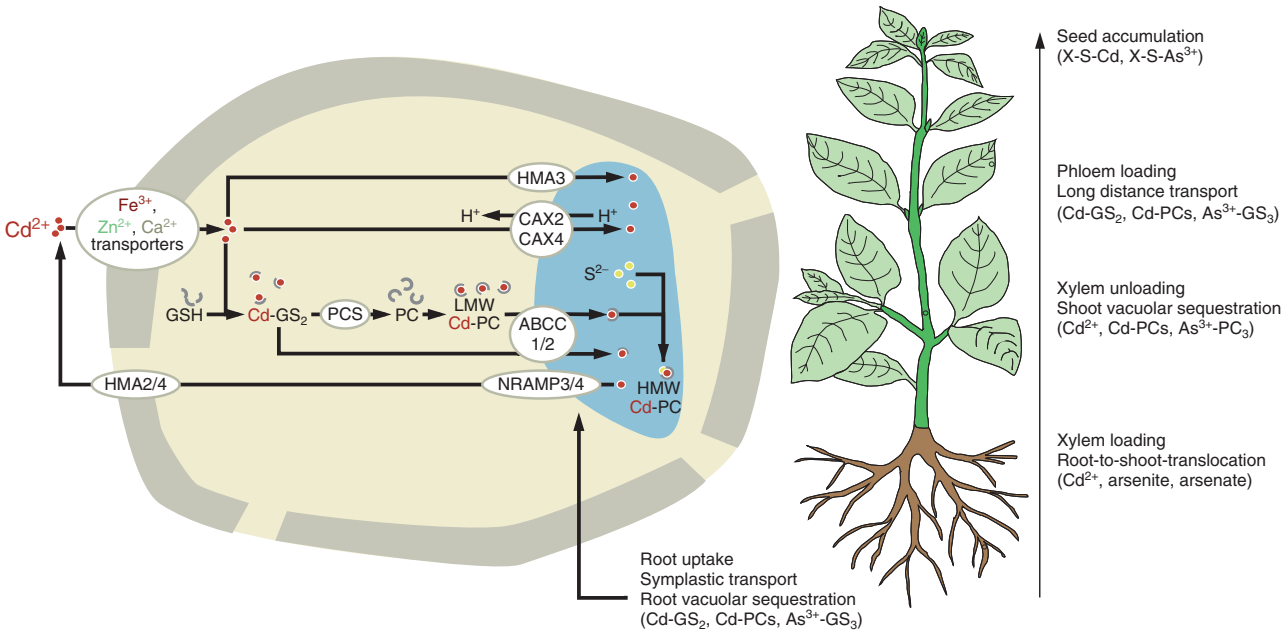

Figure 12.6 Uptake and distribution of the non-essential metal(oid)s Cd^{2+}, arsenite, arsenate and their peptide chelates in plants. ABCC – ATP-binding casette (ABC) transporter, subfamily C, corresponding to MRP – multidrug resistance associated protein; CAX2/CAX4 – cation transporter; GS_2 – glutathione complex; HMA – heavy metal transporting P-type ATPase; HMW Cd-PC – high molecular weight Cd-containing phytochelatins; LMW Cd-PC – low molecular weight Cd phytochelatins; PCS – phytochelatin synthase, X, unknown structure. NRAMP – natural resistance-associated macrophage protein.

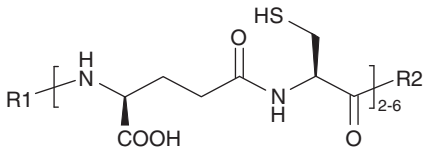

Figure 12.7 Structure of canonic phytochelatins (R1 = H, R2 = glycine) and isophytochelatines (R1 = H or cysteine, R2 = OH, glycine, serine, glutamic acid, glutamine, alanine, β-alanine).

transported into vacuoles. This process contributes to metal detoxification. During phloem loading in plants Cd-GS_2, but also complexes between arsenite and glutathione, are transported from phloem parenchym via companion cells to the sieve elements (Figure 12.6).

In the fungi *S. cerevisiae* and *Schizosaccharomyces pombe* (*Ascomycota*, *Dikarya*) vacuolar transport of Cd-GS_2 complexes is essential for Cd^{2+} tolerance, however, GSH is not involved in metal storage in metal hyperaccumulator plants (see Section 12.6.2). Nevertheless, as shown for nickel-accumulating *Noccaea* (formerly *Thlaspi; Brassicales, Rosidae*) species, a high level of GSH biosynthesis may protect against ROS. Furthermore, GSH is needed especially in most plants for metal homeostasis because it is the precursor of **phytochelatins** (PCs).

12.5.5 Phytochelatins

PCs are GSH-derived peptides of the general structure $(\gamma Glu\text{-}Cys)_{2-6}Gly$, so called **canonic PC** (Figure 12.7). They are synthesized from GSH by **PC synthases** (PCSs). PCS becomes active when two GSH molecules form metal thiolate complexes. Furthermore, activation includes the transfer of one γGlu-Cys moiety to a free GSH molecule or to a previously synthesized PC.

Canonic PCs with different lengths were first isolated from the fungus *S. pombe*. *S. cerevisiae* synthesizes upon metal exposure PC_2 only. *S. cerevisiae,* in contrast to *S. pombe,* lacks a PCS homolog but is able to produce PC_2 using a **vacuolar serine carboxypeptidase**.

In environmentally relevant concentrations (0.03 – 3 µM), cadmium and copper ions induce a strong up-regulation of PC synthesis in **phytoplankton algae** but do not influence the steady-state GSH levels. Various **PC isoforms** (Figure 12.7) occur in seed plants and algae. For example, in the green alga *Chlamydomonas reinhardtii (Chlorophyta, Chlorophyceae*) $CysPC_n$ and PC_2Ala were identified besides canonic PC by a metallothiolomics approach (see Section 18.5.3). While PC_2 and PC_3 represent the main PCs in cadmium-stressed cells of the green alga *Micrasterias denticulata* (*Streptophyta*, *Zygnematophyceae*), *C. reinhardtii* predominantly contains these $CysPC_2$ and $CysPC_3$ species. In this alga, cadmium seem to inhibit glutamate synthesis, leading to synthesis of CysGSH. Since PCS has a higher affinity to CysGSH than to GSH (Figure 18.9), this results in the production of the CysPC's instead of canonical long-chain PC's.

Plants (*Embryophyta*) are different with respect to **PC synthesis**. **Seed plants** (*Spermatophyta*, *Tracheophyta*) and some bryophytes (*Bryophyta*), in particular leafy liverworts (*Marchantiophyta*) (Figure 4.15), produce PC when exposed to transition metals, for example, Cd^{2+}, leading to detoxification by sequestration into PC-Cd complexes. These can be translocated into the vacuoles, subsequently yielding high molecular weight complexes together with sulfide. Alternatively, the complexes may be transported into the phloem sap that acts as long-distance carriers for metals (Figure 12.6). Similar to GSH, PCs are not required for metal tolerance in hyperaccumulator plants (see Section 12.6.2).

Various **Bryophytes** (*Bryophyta*) are characterized by an increase of the GSH pool in the presence of Cd^{2+} without any detectable PC synthesis. Analytical microscopic studies (see Chapter 19) support this hypothesis. The aquatic moss *Fontinalis antipyretica* (*Hypnales*, *Bryophyta*) is PC free but contains probably $CdGS_2$ in the cytosol as substitute of PC-Cd-complexes.

12.5.6 Metallothioneins and Their Ubiquitous Features

The name **metallothioneins (MTs)** was given to a superfamily of proteins that all feature extraordinarily high contents of metal ions and thiolate groups in form of cysteine residues. With usually less than 85 amino acid residues MTs are rather small proteins and can be ubiquitously found in most organisms ranging from bacteria and eukaryotic microorganisms to plants, invertebrates and vertebrates including humans.

MTs form thermodynamically extremely stable complexes with soft metal ions, preferentially with the electron configuration d^{10} (Figure 12.8).

The metal ion-binding capacity is maximised by recruiting part of the thiolate groups as bridging ligands resulting in the formation of metal-thiolate clusters. The preference of MTs to coordinate essential Zn^{2+} and Cu^{+} ions has early on suggested a homeostatic function of these proteins, while the even higher binding affinity for Cd^{2+} as well as Hg^{2+} and Ag^{+} points to a role in detoxification processes, either by directly scavenging the heavy metal ion upon entry into the cell or by rescuing the function of heavy metal poised enzymes via exchange of, for example, enzyme-bound Cd^{2+} against MT-bound Zn^{2+}. In this respect recently a new **small MT** was detected in the aquatic fungus *Heliscus lugdunensis* (*Ascomycota*, Dikarya) and found to exhibit an astonishing specificity for Cd^{2+} ions (Box 12.1)

When coordinated to Zn^{2+} or Cd^{2+} the metal-thiolate clusters of the recombinant proteins are rather inert against oxidation by atmospheric oxygen, however, incubation with a glutathione/glutathione disulfide mixture mimicking cellular oxidative stress conditions can oxidize a fraction of the thiolate groups and trigger metal ion release from the

Box 12.1: A protein designed to coordinate cadmium?

The aquatic fungus *Heliscus lugdunensis* (*Ascomycota*, Dikarya) was isolated from a heavy metal polluted spring in a former copper shale mining district in the area of *Mansfelder Land* in Central Germany (Braha *et al.*, 2007, Figures 4.17 and 12.11). The water contains among others 25 µM Cd^{2+} and 30 mM Zn^{2+}. Given the generally roughly 4 log units higher stability constants of the Cd^{2+}-thiolate clusters in MTs compared to the Zn^{2+}-thiolate clusters it is not surprising as such that **neclu_MT1** was isolated in its Cd^{2+}-bound form from the native host in this heavy metal challenged habitat (Figure 12.8.). What is, however, astonishing is the exclusive MT production in response to elevated Cd^{2+} but not to Zn^{2+}, Cu^{2+}, or As^{5+} concentrations and the different metallation of the recombinant protein with Zn^{2+} and Cd^{2+} at physiological neclu_MT1 concentrations (Loebus *et al.*, 2013). With Zn^{2+} ions a stable Zn_2neclu_MT1 species is formed, while upon incubation with excess Cd^{2+} ions Cd_3neclu_MT1 can be isolated. Usually MT-bound Zn^{2+} ions residing in tetrahedral tetrathiolate coordination sites are isostructurally replaced by Cd^{2+}. However, more recently, two MT structures were determined, in which some of the thiolate ligands are exchanged by **histidine, that is, a cyanobacterial and a plant MT** (see Figure 12.8), and exchange experiments with Cd^{2+} show some indications for different coordination modes of Zn^{2+} and Cd^{2+} in these proteins. Also neclu_MT1 contains one histidine residue next to a total of eight cysteine ligands (see Figure 12.8). Accordingly, to unravel the peculiarity of the different metallation modes of neclu_MT1 with Zn^{2+} and Cd^{2+}, investigation of the metal-binding sites and, in particular, of the third position is the key point. While the information gained by a solid state structure obtained with single **crystal X-ray crystallography** (see Section 18.3.4.1) provides by far the most conclusive insight into such a problem, to obtain single crystals, especially from MTs, is still the major bottleneck. However, a number of other **spectroscopic techniques** are available to probe the ligand sphere of a metal ion and specifically Cd^{2+}:

- **Spectrophotometric techniques**: Metal-thiolate clusters generate typical ligand-to-metal charge transfer (LMCT) bands in the UV region of the spectra centered around 230 nm for Zn^{2+}- and 250 nm for Cd^{2+}-thiolate transitions. Plotting the absorptivity increase of these bands against the equivalents of metal ions added to the respective MTs allows deduction of the number of thiolate ligands required for binding of each metal ion equivalent and hence allows to identify in a fast and efficient way if other ligands than thiolates, for example, histidine, are part of the coordination sphere.
- **Potentiometric pH titrations** are used to determine the pK_a values of coordinating groups of ligands in absence and presence of metal ions. Ultimately, the information gained can be used to calculate the thermodynamic stabilities of the respective metal complexes. However, it is also a rather straight-forward method to identify participation of potential ligands in metal ion coordination indicated by a significant drop of the respective pK_a value compared to the value of the free ligand. Evidently, data evaluation becomes more complex with increasing number of titratable groups in the peptide, nevertheless, experiments with an MT containing twelve titratable amino acids among them six cysteine and one histidine residue already showed the feasibility of the method. Given that Zn^{2+}-His and Cd^{2+}-His complexes are spectrophotometrically silent, application of potentiometric pH titrations is a very useful but undervalued and little known tool that allows the detection of metal-His coordination.
- **Nuclear magnetic resonance (NMR) spectroscopy** using the spin ½ nuclei ^{111}Cd or ^{113}Cd has become indispensible for the study of MTs. The $^{111/113}$Cd NMR chemical shifts provide qualitative information about the nature of the coordinating ligands, but an unambiguous assignment is difficult because of overlapping shift ranges. Clustered structures can be identified from two-dimensional [^{113}Cd,^{113}Cd] COSY spectra resulting in cross peaks between thiolate bridged metal ions. Usually more sensitive and robust are heteronuclear ^{113}Cd,^{1}H correlation experiments. They allow to identify the coordinating cysteine residues for each Cd(II) ion based on ^{113}Cd-(Cys)H$^{\beta}$ cross peaks and can also help to identify the bridging thiolate ligands in case of a clustered structure.
- **X-ray absorption spectroscopy (XAS)** delivers information about the structural environment around the absorbing atom or ion such as the number and nature of ligands or the coordination geometry. The sample of interest is exposed to an X-ray beam with an energy range covering the area of the absorption edge of the respective element, for example, for both Zn^{2+} and Cd^{2+} the K edge can be used. The enormous advantage of XAS compared to X-ray diffraction methods is that no crystalline material is required but measurements can be performed with amorphous samples or even on (frozen) solutions. XAS spectra are made up of an **X-ray absorption near-edge structure (XANES)** and an **extended X-ray absorption fine structure (EXAFS)** part (see Section 18.5.1.3), and information gained from the two parts of the spectra are complementary.
- **Perturbed angular correlation of γ-rays (PAC) spectroscopy**: Like XAS, PAC spectroscopy requires the application of synchrotron radiation, can be performed in solution, and provides information about the ligands and coordination geometry around a central atom or ion. Complicating matter, however, is the requirement for a radioisotope that emits two γ-rays in succession, for example, ^{111m}Cd.

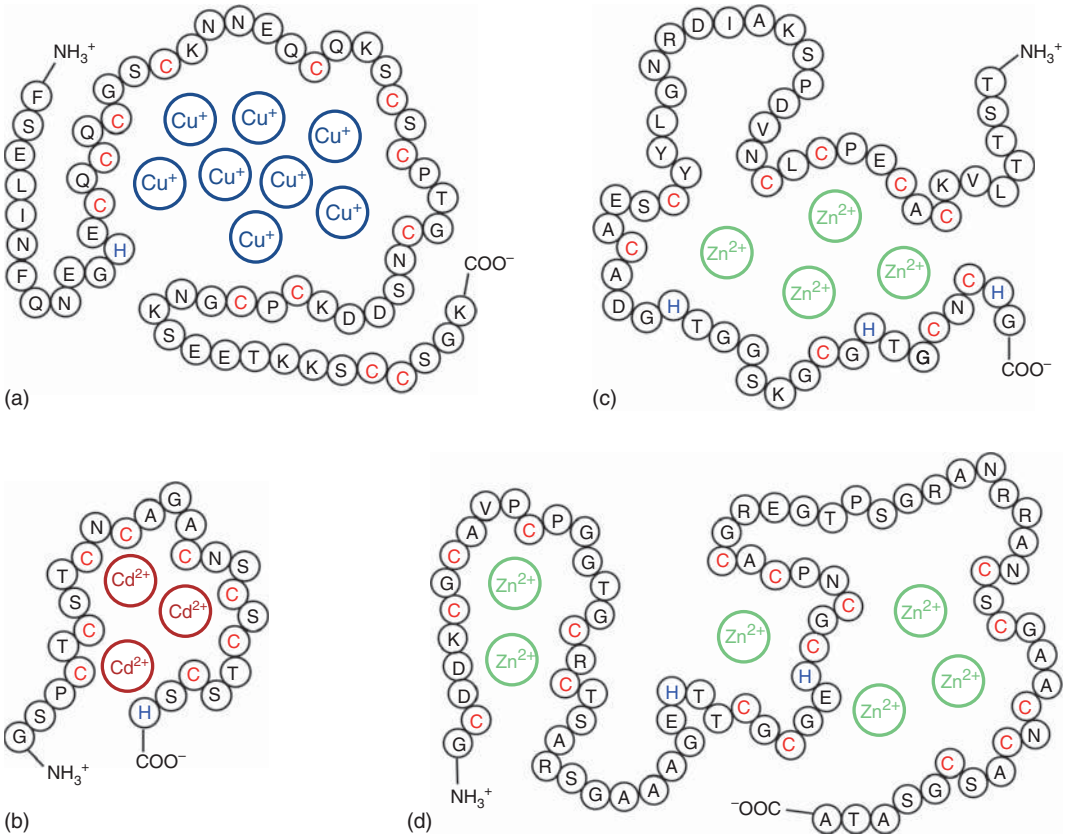

Figure 12.8 Metallothioneins: Primary amino acid sequences of (a) *Saccharomyces cerevisiae* CupI, (b) *Heliscus lugdunensis* MT1, (c) *Synechococcus elongatus* PCC 7942 MT, and (d) *Triticum aestivum* E_c-1. The Cys residues are highlighted in red, His residues in blue. In addition, the number and sort of metal ions found to be coordinated to the respective MT are depicted.

protein. This let to the perception that MTs can also exhibit a role as **ROS scavenger**.

The first MT was encountered almost 55 years ago when analyzing a naturally Cd^{2+}-rich protein fraction obtained from horse (*Mammalia*, *Vertebrata*) kidney cortex. **Mammalian MTs** have the ability to coordinate seven divalent d^{10} metal ions in two separate metal-thiolate clusters, the N-terminal β-domain hosting a $M(II)_3Cys_9$ and the C-terminal α-domain with the $M(II)_4Cys_{11}$ cluster. The two clusters are separated by just a short three amino acids long linker resulting in an overall dumbbell-like protein shape.

Only very few three-dimensional structures of MTs from other phylogenetic families are known. Structures with divalent metal ions include: (i) two **crustacean MTs** (*Arthropoda*) that contain similar α- and β-clusters as the mammalian forms but in reversed order; (ii) an MT from **sea urchin** (*Echinoida*, *Echinodermata*) featuring two β-type clusters; (iii) as well as a single-domain **cyanobacterial MT** (*Cyanobacteria*) with an α-type cluster in which two terminal coordinating Cys residues are replaced by **histidine ligands** (Figure 12.8). In addition, two Cu^+-binding **fungal** (*Fungi*, *Opisthokonta*) MTs have been structurally described, one forming a single Cu_8Cys_{10} cluster and the other one coordinating 6 Cu^+ ions in an undefined cluster arrangement. Only recently, the first structure of a **plant MT** became available. The seed specific early cysteine labeled MT from *Triticum aestivum* (*Poales*, *Liliopsida*), that is, bread **wheat E_c-1**, coordinates its six Zn^{2+} ions in a two domain arrangement consisting of the N-terminal γ-cluster, that is, Zn_2Cys_6, and the C-terminal β_E-domain, which features a Zn-finger type single metal ion-binding site of the form $ZnCys_2His_2$ as well as a Zn_3Cys_9 arrangement with β-type stoichiometry (Figure 12.8). The plant MTs show great sequence diversity which let to their further division into four subfamilies. Members of the **plant MT1, MT2, and MT3 subfamilies** are characterized by two Cys-rich regions separated by a unique, 30–45 amino acids long Cys-free linker region of presently unknown function. These linker regions are particularly interesting as they typically contain aromatic amino acids as well as β-sheet structures which distinctively differentiate them from MT sequences of other phyla.

In general, MTs contain very little secondary structural elements if any and accordingly, the three-dimensional structures of these proteins are governed by the formed metal clusters. Therefore, deciphering the structures of the linker regions in plant MTs might be a key point in understanding the functions of these proteins. Presently, no three-dimensional structure for any member of the plant MT1, MT2, and MT2 subfamilies is known, but

research results indicate that depending on the metal ion load and the origin of the protein, both single as well as two domain cluster arrangements are possible. This inherent flexibility of the proteins might contribute to the difficulties encountered during structural analyses. The fourth plant MT subfamily, the **MT4 or E_c proteins**, consist of even three Cys-rich regions, the central and C-terminal of which form together the β_E-domain described above. To a large extend, members of the four different plant MT subgroups show tissue specific expression, the MT1 forms being more abundant in roots, MT2s in shoots, **MT3s** in fleshy fruits or leaves of plants that do not develop such fruits, and occurrence of the E_c proteins is strictly restricted to the developing seeds.

Reminiscent to mammalian MTs expression pattern may vary with **developmental stages of the plant** and also responsiveness to certain internal and external triggers, such as oxidative stress, drought, heat, and wounding is observed. For example, **infection or mechanical wounding** of leaves from *Nicotiana glutinosa* (*Solanales*, *Asteridae*) leads to an increase of *mt2* RNA levels, while spraying of the leaves with a Cu^{2+} solution has a much less pronounced effect. The fact that *mt* genes are not necessarily induced by metal ions appears odd considering the high metal ion contents of MTs, but numerous examples have been described. Also production of the wheat Zn_6E_c-1 protein is not responsive to elevated Zn(II) levels but rather to the plant hormone abscisic acid (see Chapter 7). Very interesting in this respect is also the MT from *Magnaporthe grisea,* a **fungus** (*Ascomycota*, *Dikarya*) causing the rice blast disease. This highly expressed MT is also not responsive to elevated metal ion concentrations and is mostly located at the cell wall periphery. Mutants devoid of this MT were found to be non-infectious due to penetration inhibition of the rice tissue cuticle concomitant with a reduced structural integrity of the fungal cell wall. However, the lack of inducibility does not infer *per se* that MTs cannot perform a metal homeostatic function under normal growth conditions.

Supporting this view is the overexpression of **MT3 genes in ripening fruits**, for example, of *Musa acuminata* (banana, *Zingiberales, Liliopsida*) or *Citrus unshiu* Marc. (Satsuma orange, *Sapindales, Rosidae*), and hence at a developmental stage that is in general not associated with metal ion stress. Clearly metal related is the increased MT1 and MT3 production during **leaf senescence** (See Chapter 10) observed, for example, in *Brassica napus* (rape, *Brassicales, Rosidae*) and *Elaeis guineensis* (oil palm, *Arecales, Liliopsida*). Here it is feasible that MTs are needed to sequester metal ions that are released during protein degradation in order to preclude damage to the other parts of the plant and to store the precious nutrients for further plant development. Additionally, degradation of, for example, cell membranes and chlorophyll can lead to the formation of free radicals and hence oxidative stress (See Chapter 8), requiring the protective action of **MTs as reducing agent**. With the exception of the wheat E_c-1 protein that was isolated directly from wheat embryos is its Zn^{2+}-bound form, no other plant MT was isolated from its host with its natively bound metal ion so far. Accordingly, the physiologically relevant metal ion, that is, Zn^{2+} *versus* Cu^+ in the heavy metal ion unchallenged organism, remains enigmatic. This has serious implication toward structure determination as well as prediction due to the different coordination modes of the metal ions with Zn^{2+} preferring a tetrahedral coordination sphere while Cu^+ ions show diagonal or trigonal planar coordination by thiolate ligands. Hence it is obvious that research on plant MTs especially with regard to their structures and functions is still in its infancy and there is need for additional research activities to shed light onto this peculiar and exciting protein class.

12.6 Interaction of Plants and Fungi with Metals

12.6.1 Avoidance and Tolerance Mechanisms in Plants

Uptake, transport and accumulation of **mineral nutrients** are important to establish biochemical networks in cells and organs (see Section 11.1). These processes are element specific with differences between plant species and populations. Deficiency of mineral nutrients strongly affect the plant life (see Section 11.1). On the other hand, **excess of essential metals** and **presence of toxic metals** in the soil can exert selective pressure on plant population, influence plant growth and decrease crop yields. **Metalliferous soils** contain high metal concentrations caused by rock leaching, soil acidity, flooding or anthropogenic impact such as mining. This may provoke the development of natural and frequently endemic metallophyte communities.

Specific processes are used by plants to escape on the organismic level stress caused by high concentrations of transition metals:

1) **Avoidance mechanisms** diminish availability of metals to the whole plant:
   - A. Exudation of compounds by the roots to decrease metal mobilization
   - B. Precipitation of metals in the rhizosphere or root apoplast
   - C. Storage in the root cell wall
   - D. Supporting advantageous microbial associations, such as mykorrhizas (see Chapter 15). The symbiotic fungi are able to protect their plant by increasing the adsorptive capacity, and by providing controlled access to mineral nutrients (see Chapter 5).
   - E. Restriction of the apoplastic metal transport to the cortex and from the cortex to the xylem
   - F. Regulation of the abundance and activity of membrane transporters.

2) **Tolerance mechanisms** against transition metals are caused by intrinsic biochemical properties which are evolved in plants growing in soils with high metal bioavailability:
   A. Selective uptake into plant cells by transport proteins.
   B. Increase of excretion on the whole plant level, for example, by leaf shedding and guttation
   C. Preferred storage of metals (e.g., Cd^{2+} in trichomes ("leaf hairs"), e.g., in *Brassica juncea* (*Brassicales*, *Rosidae*) and *Nicotiana tabacum* (*Solanales*, *Asteridae*).
   D. Accumulation of metals in vacuoles and distinct cells
   E. Regulation of gene expression, including miRNA-dependent regulation (see Section 7.7)
   F. Binding to low-molecular intracellular chelators, such as organic acids (e.g., malate), amino acids (e.g., histidine) and peptides (e.g., glutathione; see Section 12.5.4)
   G. Controlled binding to oligopeptides (PCs) see Section 12.5.5 and proteins (MTs) see Section 12.5.6
   H. Adaptation of metabolic processes, for example, scavenging of ROS (see Chapter 8) formed under intracellular Cd^{2+} excess
   I. Increase of heat shock proteins (HSPs) as shown, for example, in roots of *Armeria maritima* (*Caryophyllales*, *Gunneridae*) grown on Cu-rich soils (HSP17), and in the aquatic fungi *H. lugdunensis* (HSP70) and *Blastocladiella emersonii* (HSP10,20,90,100; *Blastocladiales, Blastodiomycota*) under Cd^{2+} stress.

Tolerance strategies show intraspecific and interspecific differences. The distribution of metals in the plant by transport, as well as reallocation between cells and organs needs long-distance biochemical communication. The behavior and content of all metal(oid)s can be determined by use of metallomics techniques (see Section 18.5).

12.6.2 Metal Hyperaccumulators

Metal hyperaccumulators tolerate high concentrations of specific metals and are able to accumulate them in their shoots, for example, >100 ppm Cd^{2+}, >1000 ppm Cu^{2+} or Ni^{2+} or >10 000 ppm Zn^{2+}. The genetic variation both between and within plant species (ecotypes) determines accumulation rates and the resistance level. Plants are only hypertolerant to those metals that are in surplus to their natural environment.

The ability to hyperaccumulate metals is relatively seldom among angiosperms and may have evolved several times independently. Hyperaccumulation has particularly evolved in the family *Brassicaceae* (*Brassicales*, *Rosidae),* for example, in *Arabidopsis halleri* and *Noccaeae caerulescens* that both hyperaccumulate zinc and cadmium. Ni^{2+} hyperaccumulation is most common (~350 species in ~42 families). Geologically Ni^{2+}-rich soils are quite widespread worldwide. Other Taxa hyperaccumulate Cu^{2+} (~35 species in 15 families), Co^{2+} (~26 species in ~11 families), Mn^{2+} (26 species in 9 families), Zn^{2+} (about 20 species in 6 families), Pb^{2+} (14 species in 7 families), Cd^{2+} (7 species in 5 families), and As^{3+} (12 species belonging to the fern family *Pteridaceae, Polypodiales, Moniliformopses*).

Metal hyperaccumulators have developed a strongly altered pattern of **metal partitioning** in their tissues, based on following processes:

- Increased cellular uptake rates and cell-to-cell distribution
- Protection of roots against toxicity through beneficial metal shuttle systems to the shoot
- Increased translocation via symplastic and apoplastic (root-to-shoot) transport with the transpiration stream
- Increased transport to the final storage cells via overexpressed transport proteins
- Contribution of metal chelators to long-distance metal transport; for example, histidine in the Ni^{2+} hyperaccumulator *Alyssum lesbiacum* (*Brassicales*, *Rosidae*) or nicotianamine (see Section 11.1) in the Zn^{2+} hyperaccumulators *N. caerulescens* and *A. halleri.*
- High efficiency of sequestration and detoxification in (i) large epidermal cells (Zn^{2+}, *N.caerulescens*) followed by detoxification in vacuoles or (ii) exceptionally in mesophyll cells of succulent leaves {Mn^{2+}: *Gossia bidwillii* (*Myrtales*, *Rosidae*); Cd^{2+},Zn^{2+}: *Sedum alfredii* (*Saxifragales*, *Gunneridae*)}.

Metal hyperaccumulation also may function as part of a defense strategy against herbivore and/or pathogen attack. For example, trips *(Frankliniella occidentalis, Insecta, Mandibulata)* are repelled when feeding on Cd-rich *N. caerulescens* leaves.

12.6.3 Interaction of Plants with Individual Metals

Plants take up small amounts of **Co^{2+}** (see Section 11.1). Excess of this metal influences the translocation of mineral nutrients, such as P, S, Mn^{2+}, Zn^{2+}, Cu^{2+} and decreases water potential and transpiration rate.

Ni^{2+} in soils causes toxic responses, such as chlorosis and reduced growth. The transition metal disturbs the integrity of plasma membranes and inhibits germination, nutrient absorption via roots, photosynthesis and transpiration. Ni^{2+} is able to replace Co^{2+} and certain other metal ions in active sites of metalloenzyme, followed by disruption of their function (see Section 12.3.5). Since nickel hyperaccumulation in plants is the most common type of this process, various strategies are employed by these plants to detoxify high amounts of Ni^{2+} in their tissues. The tree *Sebertia acuminata* (*Ericales*, *Asteridae*) grows on natural Ni^{2+} rich soils in New Caledonia and contains in its latex ~ 25% Ni^{2+} of the dry mass. This represents the

highest content of Ni^{2+} so far found in any biological fluid. One single tree contains approximately 37 kg Ni^{2+}! In the shrub *Euphorbia helenae* (*Malpighiales*, *Rosidae*) growing in Cuba, a Ni^{2+} content of ~3 % in the dry mass was measured. To detoxify free Ni^{2+} in hyperaccumulating plants various intra-organismic chelating compounds are used, such as citrate (*S. acuminata*), histidine (*A. lesbiacum*) and nicotianamine (*N. caerulescens;* see Chapter 18, Box 18.1).

High **Cu^{2+}** exposure leads to general plant toxicity, mainly by generation of oxidative stress (see Chapter 8).

Excess of **Zn^{2+}** in soils originates from sewage sludge, fertilizers, metal mining and smelting industries. In some soils, high amounts of Zn^{2+} in the range of 150–300 mg kg^{-1} were detected. The metal inhibits numerous metabolic reactions resulting in declined growth, chlorosis and senescence. Zn^{2+} excess hampers long-distance transport of other **micronutrients** (e.g., Mn^{2+}, Cu^{2+}, Fe^{2+}) from roots to shoots. Zn^{2+} is also present in naturally occurring zinc-rich soils (calamarine soils), frequently accompanied by Cd^{2+}. Zn/Cd hyperaccumulators such as *N. caerulescens* show high translocation efficiency and decreased sequestration of Zn^{2+} into the root vacuoles, accompanied by a powerful root-to-shoot translocation. In *N. caerulescens* and *A. halleri,* citrate and malate are the most abundant ligands for Zn^{2+}.

Cd^{2+} enters plant and fungal cells via transporters (Figure 12.6) that are responsible for the uptake of essential cations. Inside the cells, Cd^{2+} induces oxidative stress by GSH depletion due to its binding to the peptide. Otherwise, Cd^{2+} can replace Zn^{2+} in Zn-binding molecules and, therefore, interfere with various Zn-dependent cellular reactions (see Sections 11.1 and 12.3.5). In the aquatic macrophyte *Ceratophyllum demersum* (*Caryophyllales*, *Ceratophyta*, Figure 4.15g) Cd^{2+} inhibits Zn^{2+} transport from the leaf veins into mesophyll cells. Such cadmium-induced Zn^{2+} starvation could be a significant contributor to Cd^{2+} toxicity in plants in general. Plants and fungi respond to Cd^{2+} stress by induction of **phytochelatin** (PC) synthesis (see Section 12.5.5). The PC precursor GSH and PCs both are able to bind Cd^{2+}. The chelates can be transported into the vacuoles via **ABC-transporters** (Figure 12.6). Cd^{2+} themselves can be transported into the vacuoles, too, and can be exported from the cells via different ABC-transporters. Cd^{2+}-thiol complexes are distributed in plants by a long-distance transport and stored in seeds (Figure 12.6). In the Cd^{2+} hyperaccumulator *N. caerulescens,* 60 % of Cd^{2+} is enriched in seeds.

Toxic amounts of **lead** in soils result from municipal sewage sludge and mining activities. Lead affects plant growth, development and photosynthesis. Pb^{2+} can inhibit enzyme activities by reacting with sulfhydryl groups, and induce the generation of ROS and expression of different genes involved in the GSH metabolism (e.g., for glutathione synthetase, -peroxidase, -reductase, and γ-glutamyl-cyteine synthase). Pb^{2+} is also known to activate the **pytochelatin synthase**. The lead hyperaccumulating **aquatic fern** *Salvinia minima* (*Salviniales*, *Polypodiopsida*, Figure 12.9b) responds to lead by synthesis of PCs, which are able to sequester the metal in the cytoplasm.

Gold, which is probably toxic to plants, was detected as nanoparticles in *Eucalyptus* trees (*Myrtales*, *Rosidae*). These plants grow in semi-arid areas of Western Australia and are able to take up **gold complexes** via a deep (~40 m) root system. Subsequently, the ions are translocated in sub-toxic concentration through the vascular system. In leaf cells, the complexes are reduced to metallic gold nanoparticles and precipitated within the cells, partly associated with Ca-oxalate crystals. The metal was measured by elemental imaging techniques (see Section 18.5.1.3)

Hg^{2+} is dominantly present in contaminated soils and it highly toxic to plants, for example, by binding to aquaporins and by causing oxidative stress. **Phytochelatins** can also contribute to mercury detoxification. Hg-PC complexes are known from rice (*Oryza sativa, Poales, Liliopsida*) and *Marrubium vulgare* (*Lamiales*, *Gunneridae*).

Arsenic occurs naturally in many soils and can be transferred into food webs. Arsenate competes with inorganic phosphate for the transport into root cells, causing **imbalances in phosphate supply** (see Section 11.1). As^{5+} as arsenate anion is the main As species in aerobic soils while As^{3+} dominates in anoxic habitats such as flooded paddy soils. Such environments increase the availability of arsenic to rice plants. Following uptake arsenate is rapidly

(a) (b)

Figure 12.9 Metal hyperaccumulating plants. (a) *Pteris vittata (Pteridophyta, Pteridaceae)*, an arsenic hyperaccumulator. (b) *Salvinia minima (Pteridophyta, Salviniaceae)*, a lead hyperaccumulator (Botanical garden, University of Halle; Courtesy of A. Fläschendräger.)

converted to arsenite (i) enzymatically by arsenate reductase, an enzyme first isolated from bacteria and yeast, and (ii) non-enzymatically by glutathione-dependent reduction. The toxicity of arsenate in plant cells is based on its high reactivity toward thiol groups in proteins and on its ability to mediate the production of ROS. As^{3+} predominantly binds to zinc-finger proteins, yielding stable As^{3+}-trithiol complexes. The formation of As^{3+}-phytochelatin-3-complexes is favored as shown in metallothiolomics studies (see Section 18.5.3). Together with As^{3+}-GS_3, As^{3+}-PC_3 is sequestered in shoots into vacuoles via **ABC-transporters** and enters symplastically the phloem stream (Figure 12.6). As^{3+} can be accumulated in seeds complexed with thiol-containing compounds. In the As^{3+} hyperaccumulating fern *Pteris vittata* (*Polypodiales*, *Polypodiopsida*, Figure 12.9a), only very small amounts of the metal were accumulated in the roots and fronds in form of As^{3+}-PC-complexes (1–3% of the total arsenic). Interestingly, the majority of accumulated arsenic (60–90% of the total arsenic) in the vacuoles of frond cells was inorganic As^{3+}.

Aluminum is the third-most abundant element in the earth's crust. Several anions chelate Al^{3+} and form stable complexes in soils. At low pH values aluminum salts are solubilized as Al^{3+}. The ion enters the plant cells via nonspecific cation transporters resulting in decreased uptake of Ca^{2+}, generation of ROS and inhibition of root growth. Endemic plants in the humid tropics have evolved mechanisms to tolerate **acidic soils** and to cope with Al^{3+} stress. Organic acids, such as **malate** and **citrate**, bind Al^{3+} in the apoplast. In the highly tolerant plant species *Camellia sinensis* (tea, *Ericales, Asteridae*) and the important economic crop *Fagopyrum esculentum* (buckwheat, *Caryophyllales, Gunneridae*, Figure 12.10) Al^{3+} can be accumulated to >1% of the dry mass in older leaves. The cation binds intracellularly to organic anions that can be stored in vacuoles. In the forage legume *Lotus pedunculatus* (*Fabales*, *Rosidae*) Al^{3+} is associated with **tannins** in root cell vacuoles. High Al^{3+} tolerance in some plants is probably mediated by the antioxidant activity of various **flavanols** (e.g., coffeic acid, catechin). Aluminum toxicity is a crucial factor limiting crop production on acid soils, which represent the majority of soils in subtropical and tropical regions.

Figure 12.10 *Fagopyrum esculentum*, a highly aluminum tolerant plant (© LianeM_Fotolia.com).

12.6.4 Avoidance and Tolerance Mechanisms in Fungi

Fungi play vital roles in ecosystems and accomplish essential activities as part of food webs (see Chapter 5). Terrestrial fungi are able to colonize metal rich soils and rhizospheres. **Mycorrhizas** represent the most widespread symbiosis between plants and fungi (see Chapter 5), can accumulate metals from soils and influence the acquisition of essential nutrients. Mykorrhiza fungi increase tolerance of their symbiotic partner to Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} or to metal mixtures from **sewage sludge** and **serpentine soils**. Ectomycorrhizal fungi have evolved metal tolerance on metalliferous soils and can improve the performance of coniferous trees. The ericoid mycorrhizal fungus *Hymenoscyphus ericae* (*Ascomycota*, *Dikarya*)

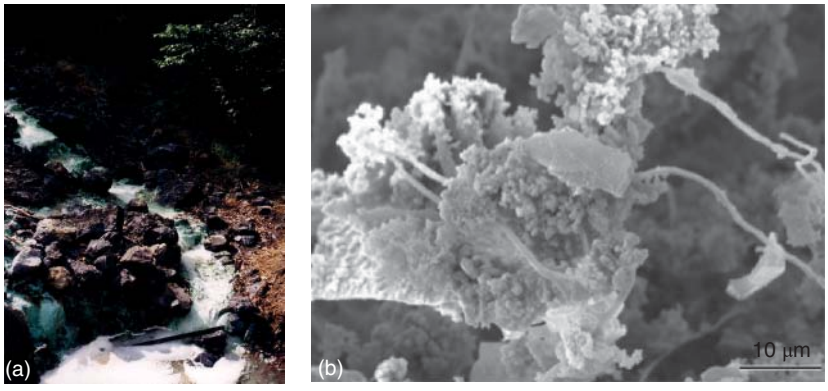

Figure 12.11 Aquatic fungi in a high heavy metal polluted stream (a) A small spring originating from a former copper smelting wastedump in the Mansfeld district (Central Germany) contains close to 2 gl^{-1} dissolved Zn^{2+}, several milligram per liter of other metals, and high levels of nitrate and sulfate (courtesy: G.Krauss). (b) Fungal hyphae covered with blue green mineral deposits (Scanning electron micrograph (SEM). Leaves exposed in the habitat (a) were colonized by fungal myzelium, and secondary minerals form a crust over the hyphae (Courtesy of G. Krauss, J. Ehrman.)

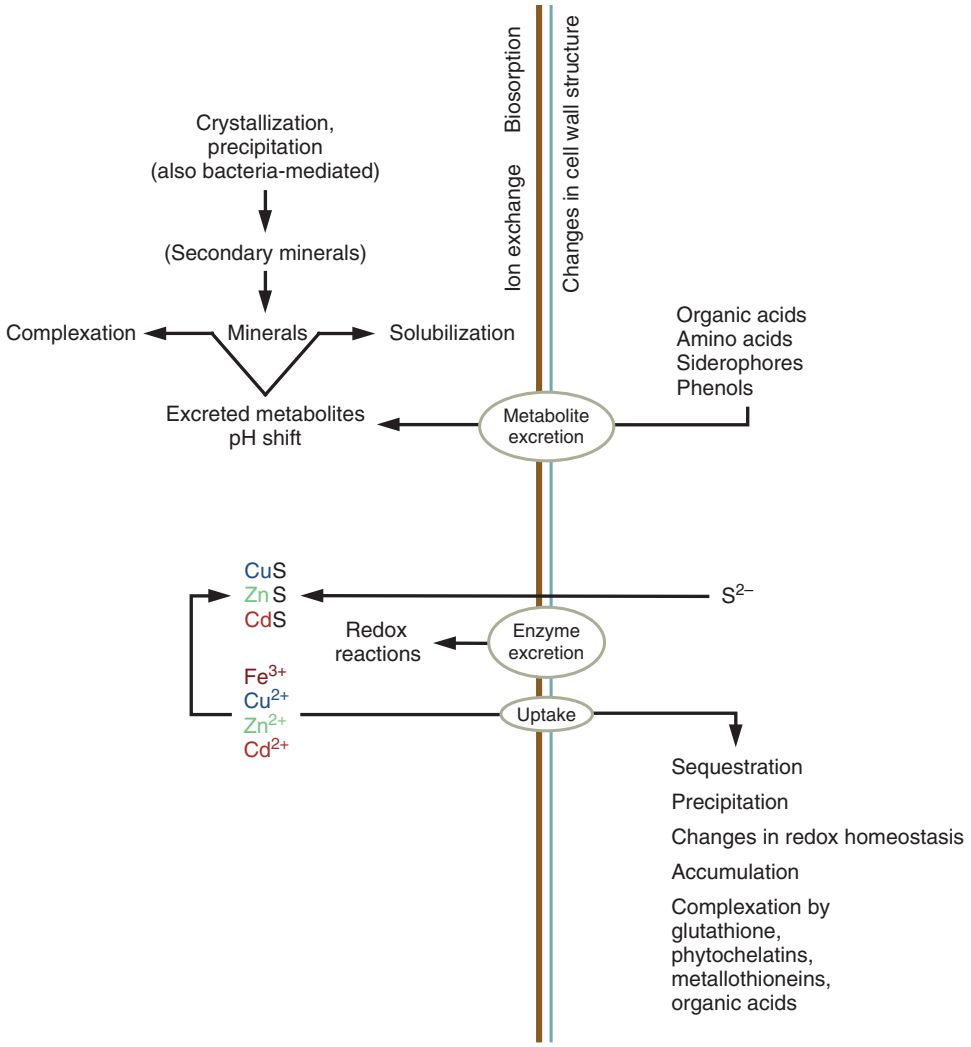

Figure 12.12 Interaction of heavy metals with fungal cells.

decreases the toxicity of As^{3+}, Cu^{2+} and Zn^{2+} in *Calluna vulgaris* (*Ericales*, *Asteridae*) growing on mine soils.

Terrestrial fungi contribute to **weathering of rocks**. They can dissolve soil components (e.g., oxides, phosphates, sulfides, and secondary minerals) by excretion of organic acids (see Section 11.1). Aquatic fungi as dominating microbial group that contributes to key processes in freshwater ecosystems (see Chapter 6) are capable to decompose leaf litter also in high metal contaminated surface water. Industrial, mining and smelting activities, for instance, have caused a Cu^{2+} excess in some ecosystems. Aquatic fungi are able to live in such high polluted areas and evolve a specific stress response (see Box 12.1; Figure 12.11). Nevertheless, pollution with mining wastewater has negative effects on richness of fungal species, their biomass formation, spore production and leaf-processing rates.

According to their high surface area-to-volume ratio, filamentous fungi exhibit **a large contact area** with metals in the habitat, which differ in speciation and bio-availability. So, different modes of adaptive metal interactions with hyphal cells have evolved (Figure 12.12).

Toxicity of metals can be avoided by **biosorption of metals** to the fungal cell wall. Metal binding is caused by carbohydrates (e.g., glucans), proteins (e.g., hydrophobins), chitosan/chitin, polyphenolics, and melanins. These mechanisms can be used as starting points for bioremediation (see Chapter 17). **Excretion of metal-chelating compounds** (e.g., organic acids, siderophores, phenolic compounds) increase metal availability in a controlled manner. Organic acids such as citric and oxalic acid increase metal cation availability by decreasing the pH value but these compounds can also chelate these metal cations.

A sophisticated adaptive network of metal homoeostasis in fungal cells is achieved by active transport and intracellular compartmention of metals, synthesis of metal-binding compounds and precipitation in vacuoles. Some fungi are able to detoxify Cu^{2+} and Cd^{2+} by the formation of **CuS and CdS crystallites** outside of the cell.

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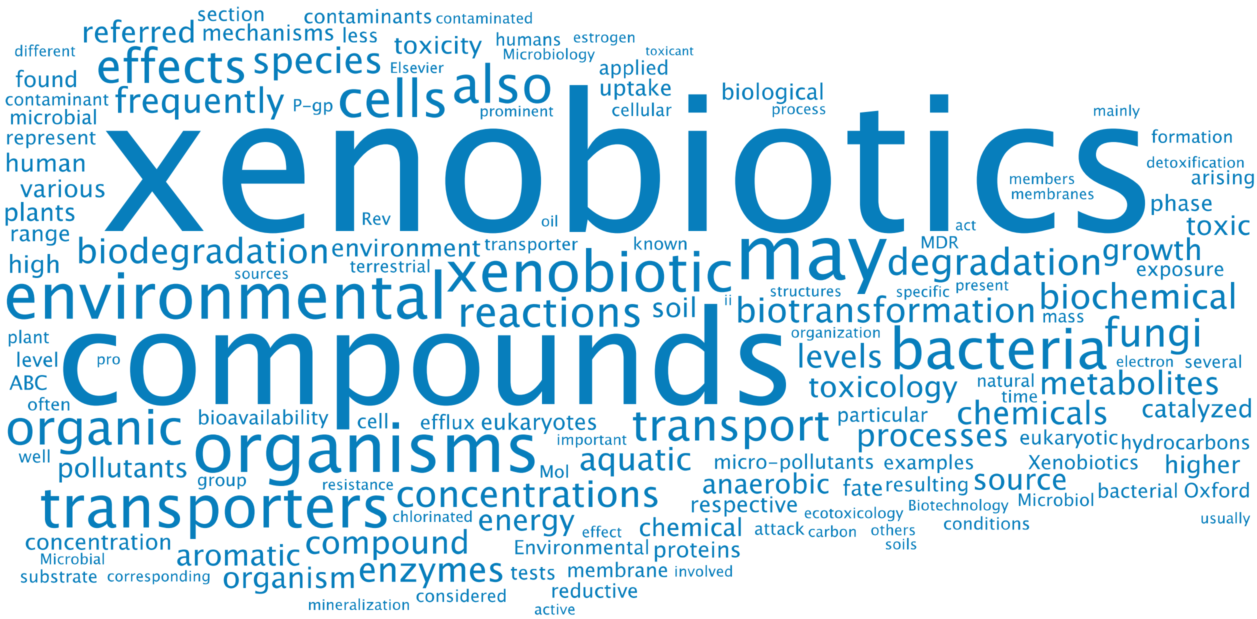
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13
Xenobiotics from Human Impacts

Magali Solé and Dietmar Schlosser

Overview

Xenobiotics are either artificial organic compounds not present in nature without prior synthesis by humans or at least not present at worryingly high concentrations without human activities. Such xenobiotic compounds have been known for a long time to contaminate environmental compartments. In addition to the more "classical" environmental mass pollutants mainly arising from industrial and agricultural activities in the past and frequently found in contaminated environmental media in high concentrations, a multitude of xenobiotics with variable origins, applications, structures, and properties altogether referred to as emerging or micro-pollutants has increasingly attracted attention during the past decade. Micro-pollutants contaminate environmental media in only minute concentrations. In this chapter, xenobiotic compounds are tracked from the respective sources of emission to the organisms where they cause biological effects and responses. The biochemical determinants of the interactions between organisms and xenobiotics are illustrated, along with their interplay with environmental factors outside the cells of organisms.

13.1
Xenobiotics: from Emission to Cellular Uptake

13.1.1
Emission, Dispersal, Fate Processes, and Bioavailability of Xenobiotics

The production of a multitude of chemicals, pesticides, and pharmaceuticals and their use and discharge were frequently associated with heavy environmental pollution in the past. In addition, accidents and wars have contributed to the contamination of environmental compartments. Prominent examples are serious contaminations of aquatic as well as terrestrial environments with various hydrocarbons from crude oil spills and leaks related to the production, storage, conveyance, use, and further processing of fuels and diverse oil products, and contamination of soils with explosives such as 2,4,6-trinitrotoluene (TNT) from military activities. "Classical" mass pollutants of xenobiotic nature can be characterized by their release into the environment in high quantities, often leading to very high environmental concentrations as exemplified for polycyclic aromatic hydrocarbons (PAHs) and TNT where soil concentrations of up to the gram per kilogram range can be found at particularly contaminated sites. **Point source pollutions** can be traced back to single, identifiable sources or origins such as, for example, municipal wastewater treatment plants (WWTPs) or industrial effluents. Well-known examples for recalcitrant point source mass pollutant mixtures contaminating wastewaters of the respective industries at high concentrations are toxic phenols and lipids contained in acidic olive oil mill wastewaters; structurally diverse, often toxic, and potentially carcinogenic dyes and pigments arising from the textile and dyestuff industries; melanoidin-type high molecular weight compounds contained in highly toxic molasses based wastewaters, and various toxic phenolic, chlorinated, and colored compounds of pulp and paper bleach plant effluents. **Nonpoint source pollutions** arise from diffuse sources, with major contributions from runoff, spray drift, and drainage in agricultural areas and impervious surfaces. PAHs from anthropogenic combustion of fossil fuels are released by smokestacks or car tailpipes, and according to the definition given earlier, they may be considered as point source pollutants. However, because of the existence of multiple point sources and long-range atmospheric transport processes, PAHs are increasingly becoming ubiquitous, nonpoint source pollutants. Xenobiotics representing environmental mass pollutants can be contrasted with the so-called **emerging contaminants** (or **micro-pollutants**) mainly on the basis of the respective amounts released and the resulting environmental concentrations. Following the fact that minute environmental concentrations of many micro-pollutants have become amenable to analysis only with the improvement of mainly mass spectroscopy-based analytical techniques during the past 10–15 years (which is one reason for referring to them as emerging contaminants), scientific, public, and

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legal awareness of micro-pollutants has increased during the past decade. There is neither a general definition nor a complete list of xenobiotics representing micro-pollutants, and the most common characteristic of this group of compounds is a concentration in the aquatic environment in the lower microgram per liter range or even lower. Emerging or micro-pollutants include both hydrophobic and polar compounds with quite diverse chemical structures and applications. They arise from, for example, urban, industrial, and agricultural activities, involve nonpoint source as well as point source emissions, and are frequently neither sufficiently degraded nor retained in municipal WWTPs. Examples of xenobiotics representing "classical" mass pollutants, and of those that may be referred to as micro-pollutants, are compiled in Table 13.1.

Following considerable variation among **dispersal routes** involving the aquatic, atmospheric, and terrestrial environment, xenobiotics can be found almost everywhere (Figure 13.1). Aquatic environments and soils act as preferred sinks. Xenobiotics not only contaminate the technical systems and facilities used for their production, further processing, and disposal together with the corresponding proximities and their areas of application, but also more distant environmental matrices (surface and groundwater and the related sediments; soils). Living organisms could, therefore, be faced with xenobiotics in nearly any environment where they are active. Workers employed in the production or processing of chemicals, plants influenced by pesticide treatment of agricultural areas, microorganisms contributing to activated sludge flocs of WWTPs, and anaerobic bacteria in aquifers contaminated with, for example, benzene-toluene-ethylbenzene-xylenes (BTEXs) compounds represent only a few places where organisms may get in contact with xenobiotics.

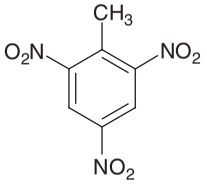
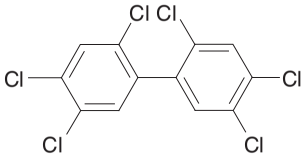
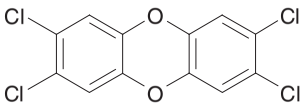
The **environmental fate** of xenobiotics and hence their concentrations in a given environmental matrix are influenced by various physicochemical and biological processes. Photolysis, hydrolysis, sorption onto sediments and soil particles, and **biodegradation** or **biotransformation** reactions (see Sections 13.3) represent prominent though incomplete examples (Figure 13.1). Together with the properties of a compound (e.g., its hydrophobicity or susceptibility to photo- or hydrolysis) and its surroundings (e.g., properties of soil particles, presence of water, climate conditions, exposure to light) such processes determine the amount of a xenobiotic that may get in contact with an organism. The term **bioavailability** integrates several dynamic processes influencing the fate of a chemical in a given environment. In this context, the amount of a substance that can cross an organism's cellular membrane at a given point in time is considered bioavailable. The bioavailability concept can be applied to any environmental compartment, but is most important when applied to xenobiotics in soils and sediments, where processes such as sorption and sequestration influence bioavailability largely. In fact, the bioavailability of an organic contaminant is considered an important factor controlling the rate of its microbial degradation in soils. Bioavailability may be distinguished from **bioaccessibility**, which is defined as the amount of a substance that is not immediately bioavailable but may become bioavailable in the future (e.g., via desorption from soil or sediment particles). A third fraction of a xenobiotic may be nonbioaccessible, because of very strong binding to particles, which practically eliminates the flux to organisms (Figure 13.2) (Katayama *et al.*, 2010; Semple *et al.*, 2007).

13.1.2 Cellular Uptake of Xenobiotics

Despite the existence of possible signaling pathways activated on recognition of xenobiotics at the cell surface, the uptake of xenobiotics by cells is frequently a prerequisite for their biological effects and corresponding cellular responses. As most xenobiotic compounds are uncharged, lipophilic organic chemicals, **passive diffusion** is the most important mechanism for cellular uptake by any organism, including those of particular relevance for the environmental fate of xenobiotics in natural terrestrial and aquatic ecosystems (microorganisms, plants, soil fauna). In addition, import into the cells occurs by **facilitated diffusion**, **primary active transport** catalyzed by **ATP-binding cassette (ABC) transporters**, or **secondary active transport** by proteins of the **solute carrier (SLC) protein family** (see Chapter 1, Section 1.2 for more details). Active transport proteins are universally distributed in both pro- and eukaryotes and can be found in, for example, bacteria, yeasts, plants, parasites, and mammals. There are transporters facilitating the cellular uptake of solutes (**influx transporters**), as well as transporters removing substances from the cytosol of cells (**efflux transporters**). A proportion of mammal members of the SLC transporter superfamily, an important group of membrane transport proteins involved in the uptake of many xenobiotics, are facilitative influx transporters. In contrast, ABC transporters are concerned mainly with the efflux of xenobiotics from cells. Apart from solute transport, solid particles may also be imported into amoeba by **phagocytosis** and into plant root cells by **endocytosis**.

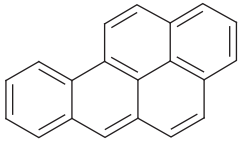
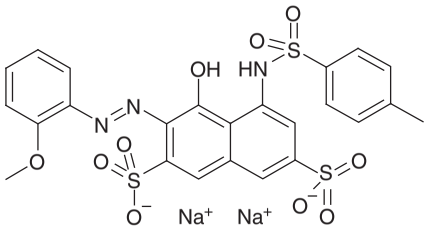
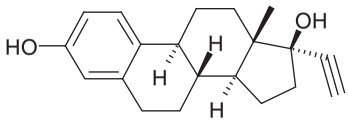
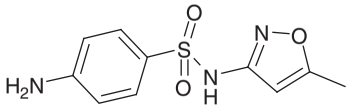
Bacterial, fungal, and plant cells are surrounded by cell walls. Bacteria and fungi are major contributors to the biodegradation of xenobiotics in terrestrial and aquatic environments. The cytoplasmic membranes of these groups of organisms are covered with cell walls composed mainly of peptidoglycan (bacteria) and chitin-glucan (fungi), which are permeable to nutrients and other chemicals and enable a concentration gradient-driven transport of compounds to the cytoplasmic membrane where the uptake mechanisms described before become operative. Whereas gram-positive bacteria have a thick cell wall composed of peptidoglycan, the cell walls of gram-negative bacteria are rather thin and, besides peptidoglycan, contain an outer membrane (lipid bilayer) of lipopolysaccharides. **Porins**, which

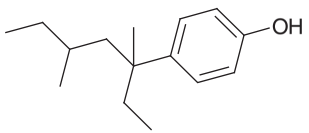
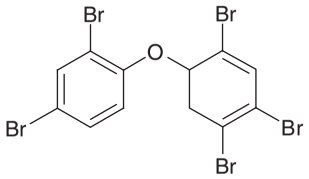
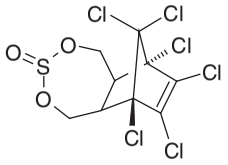
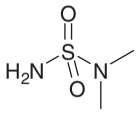
Table 13.1 Examples of "classical" environmental contaminants and micro-pollutants of xenobiotic nature, and their toxic / adverse effects on organisms.

Classes of Xenobiotics	Examples/representative(s)	Sources/uses	Contaminated environmental media	Toxic/adverse effects
"Classical" pollutants				
Benzene-toluene-ethylbenzene-xylenes (BTEX) compounds	 Benzene	occurs in petroleum products (e.g., gasoline), coal tar, and various organic chemical product formulations	Soil, surface, and groundwater contamination near petroleum and gasoline production and storage sites; easy volatilization from water and soil	Chronic benzene exposure: hematologic lesions in the bone marrow, anemia, and leukemia in humans; toluene, ethylbenzene, and xylenes: harmful effects on the nervous system
Explosives	 2,4,6-Trinitrotoluene (TNT)	Production and assembly of explosives and ammunition, war damages of production/assembly sites, use of explosives and ammunition on military testing grounds	Soil, surface, and groundwater contamination at production/assembly sites and military testing grounds	Anemia and disturbed liver functions in humans exposed to TNT over prolonged periods, adverse effects on male fertility, skin irritation from skin contact, possible human carcinogen, animals: adverse effects on blood, liver, spleen, and the immune system
Polychlorinated biphenyls (PCBs)	 Congeners with two to ten chlorine atoms attached to biphenyl	Primarily used as heat transfer, dielectric, and hydraulic fluids in, for example, transformers, capacitors, and electric motors	Accumulation in aquatic sediments, organic soil fractions, and organisms; frequently resulting from the release of PCB-contaminated media at sites related to the manufacturing or use of PCB-containing apparatus/equipment	Wide range of toxic effects in humans, depending on the respective PCB: carcinogenic (dioxin-like) effects caused by particular congeners, endocrine disruption (estrogenic effects, thyroid disorder), and neurotoxicity associated with others
Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)	 75 PCDD and 135 PCDF congeners with one to eight chlorine atoms at varying positions of the two aromatic rings, for example, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)	Formed in small amounts when organic compounds are burned in the presence of Cl, for example, in municipal waste incinerators; also produced during manufacturing of chlorophenol and chlorophenoxyacetic acid herbicides, and fiber bleaching; co-occurrence of PCDFs with PCDDs	Accumulation and high persistence in soils, sediments, animals, and humans; resulting from the accidental release at chemical or agrochemical production sites, the use of dioxin-contaminated animal feed, and the military use (herbicidal warfare) of dioxin-contaminated chlorophenoxyacetic acid herbicide mixtures (Agent Orange) during the Vietnam war	Teratogenic, mutagenic, carcinogenic (proven for PCDDs, in particular for the most toxic TCDD, and suspected in case of PCDFs), immunotoxic, hepatotoxic, and endocrine disrupting (thyroid disorder in humans) effects in humans and animals; developmental toxicity, chloracne, and nervous system pathology in humans

(*Continued overleaf*)

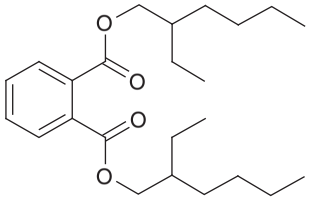
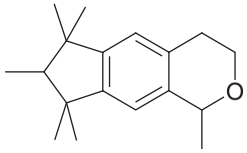
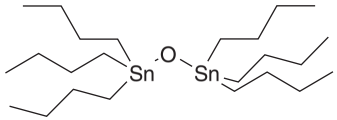
Table 13.1 (*Continued*)

Classes of Xenobiotics	Examples/representative(s)	Sources/uses	Contaminated environmental media	Toxic/adverse effects
Polycyclic aromatic hydrocarbons (PAHs)	 Benzo[*a*]pyrene	Occurs in natural oil and coal deposits, processed fossil fuels, tar, and edible oils; formed during incomplete combustion of various organic compounds: byproducts of burning of fossil fuels and wood, also found in grilled or smoked meat and in cigarette smoke	Primarily found in soil and sediments and less commonly in water, also contaminate air-suspended particulate matter; among the most widespread organic pollutants	Include carcinogenic, mutagenic, and teratogenic compounds; formation of the highly carcinogenic metabolite benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide during human metabolization of the procarcinogen benzo[*a*]pyrene
Synthetic dyes	 Acid Red 264	Dyestuff production and use in the textile, tannery, paper, plastic, cosmetics, pharmaceutical, and food industries	Primarily wastewaters related to dyestuff production and use, in case of the dyeing (textile, leather) industries resulting from excess dye in exhausted dye baths; surface and partly also groundwater contamination (in case of highly water-soluble dyes) because of poor removal of dyes in WWTPs	Biologically widespread (from bacteria to mammals) enzymatic reduction of azo dyes to yield aromatic amines, which in various cases may be carcinogenic (dependent on the respective parent azo dye)
Micro-pollutants				
Active pharmaceutical ingredients	 17α-Ethinylestradiol (EE2)	Frequently applied synthetic estrogen in oral contraceptives	Contaminant in WWTP effluents and receiving waters because of incomplete removal in WWTPs; moderate sorptive binding to WWTP sludge and aquatic sediments	Xenoestrogen, acts as endocrine disrupting chemical (EDC), contributes to the estrogenicity of WWTP effluents, can potentially impact the sustainability of aquatic wildlife populations (e.g., by fish feminization)
Active pharmaceutical ingredients	 Sulfamethoxazole	Frequently applied sulfonamide bacteriostatic antibiotic, most often used in synergistic combination with the bacteriostatic antibiotic trimethoprim (abbreviations SMX-TMP or SMZ-TMP)	Contaminant in WWTP effluents (communal and hospital wastewaters) and receiving surface waters because of incomplete removal in WWTPs	Concerns related to a possibly accelerated development of bacterial antibiotic resistance

Alkylphenols/alkylphenol ethoxylates	 Nonylphenol isomers with variously branched side chains, e.g. 4-(1-ethyl-1,3-dimethylpentyl) phenol	Release of a multitude of nonylphenol isomers with branched side chains because of incomplete degradation in WWTPs of nonylphenol ethoxylate surfactants used in various cleaning agents and contained in wastewaters; analogous release of octylphenol isomers resulting from octylphenol ethoxylates present as inpurities in nonylphenol ethoxylates; frequent use of long-chain alkylphenols other than nonylphenols as components of phenolic resins	Presence of nonylphenols and other long-chain alkylphenols in WWTP effluents and receiving waters because of their persistence in WWTPs; strong sorptive binding to WWTP sludge and aquatic sediments; may contaminate soils when WWTP sludge is used as a fertilizer	Nonylphenols (and other long-chain alkylphenols) are xenoestrogens, can act as EDCs, contribute to the estrogenicity of WWTP effluents, potentially impact the sustainability of aquatic wildlife populations (e.g., by fish feminization), cause sperm count reduction in male offspring of mammals when exposed during pregnancy and lactation
Brominated flame retardants	 Pentabromodiphenyl ether congeners (pentaBDE), for example, 2,2′,4,4′,5-penta-bromodiphenyl ether	Belongs to the group of polybrominated diphenyl ethers (PBDEs) used as flame retardants, with pentaBDE commonly used in polyurethane foam; released into the environment, for example, from pentaBDE-containing products and from emissions related to their production	Elevated concentrations in soil, water, sediments, sludge, air-suspended particulate matter, food, and organisms; strong biomagnification in carnivores and humans; highly persistent	May affect the liver, thyroid (EDC effects), and neurobehavioral development of animals
Pesticides	 Endosulfan	Worldwide use in agriculture to control insect pests	Environmentally ubiquitous (air, water, wildlife), subject to long-range atmospheric transport, semivolatile, persistent to degradation, bioaccumulation in fish	Highly acutely toxic to insects and mammals, xenoestrogen (EDC), delays sexual maturity in male children, carcinogenic effects under debate
Pesticide metabolites	 N,N-Dimethylsulfamide (DMS)	Formed by microbial transformation in soil from the pesticides (fungicides) tolylfluanid and Dichlofluanid	Contaminates surface and groundwater because of its high mobility, is not removed by riverbank filtration and hence can enter drinking water treatment processes	Formation of the carcinogenic N-nitrosodimethylamine (NDMA) from DMS during ozonation as used for disinfection of drinking water

(*Continued overleaf*)

Table 13.1 (*Continued*)

Classes of Xenobiotics	Examples/representative(s)	Sources/uses	Contaminated environmental media	Toxic/adverse effects
Plasticizers	 Phthalates, for example, di(2-ethylhexyl)phthalate (DEHP)	Used to soften plastics (plasticizers), in particular, polyvinyl chloride; phased out of many products in Europe, the USA, and Canada for health concerns	Easy release because not covalently bound to plastics, accelerated release by breaking and aging of plastics, subject to biodegradation and photodegradation when released to the outdoor environment	Anti-androgens (EDCs)
Polycyclic musk fragrances	 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[*g*]-2-benzopyrane (HHCB, Galaxolide&)	Personal care product ingredients (cleaning agents, air fresheners, and other hygiene/household products)	Presence in WWTP effluents and receiving waters because of their persistence in WWTPs; strong sorptive binding to WWTP sludge and aquatic sediments; may contaminate soils when WWTP sludge is used as a fertilizer	Inhibition of MXR transporters in aquatic organisms, suspected EDCs
Tributyltin compounds (TBT)	 Tributyltin oxide	Use of TBT in, for example, wood preservation, marine paints (antifouling agent), textiles (antifungal agent), and industrial water systems (antifouling agent in cooling towers, refrigeration water systems etc.); tributyltin oxide: most widely used compound in tributyltin formulations	Leaching of TBT from marine paints into seawater; bioconcentration up the food chain of marine predators; moderately to highly persistent	High toxicity of TBT to marine mollusks, causes sex changes (imposex) in marine gastropods such as dog whelks (EDC effects), immunosuppressive to marine mammals

Partly based on Cerniglia and Sutherland (2010), Chang (2008), Harms, Schlosser, and Wick (2011), and Kümmerer (2011).

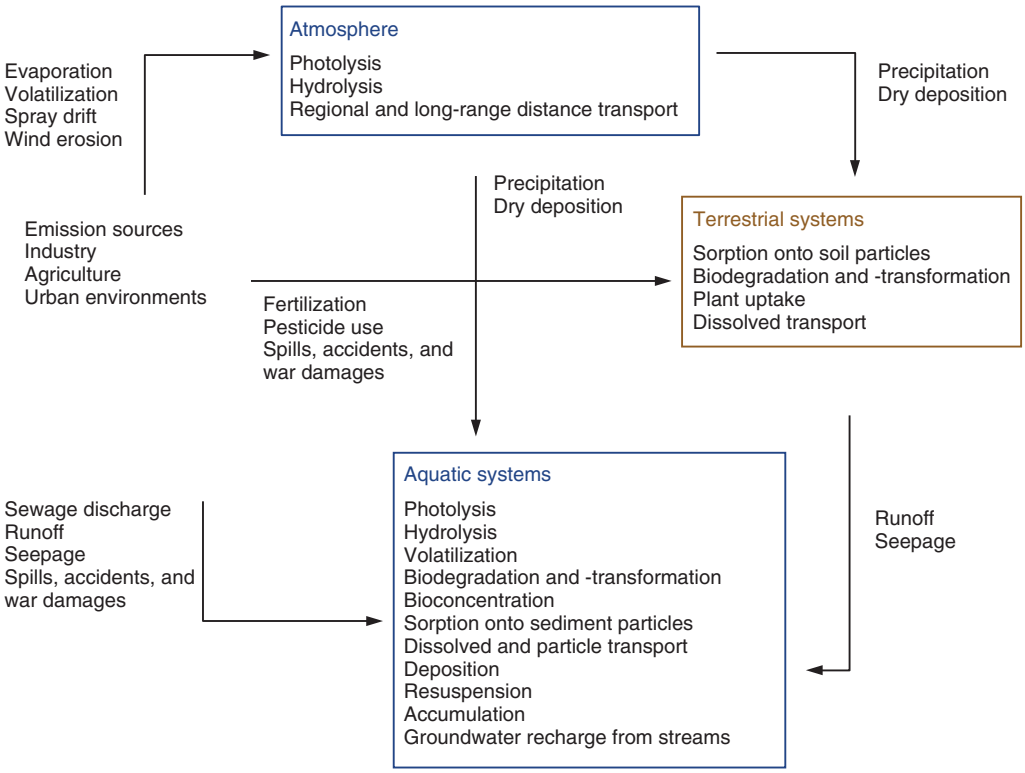


Figure 13.1 Dispersal and fate processes of xenobiotics in the environment. Partly based on Kümmerer (2011), and US Geological Survey (*http://toxics.usgs.gov/regional/emc/transport_fate.html*.)

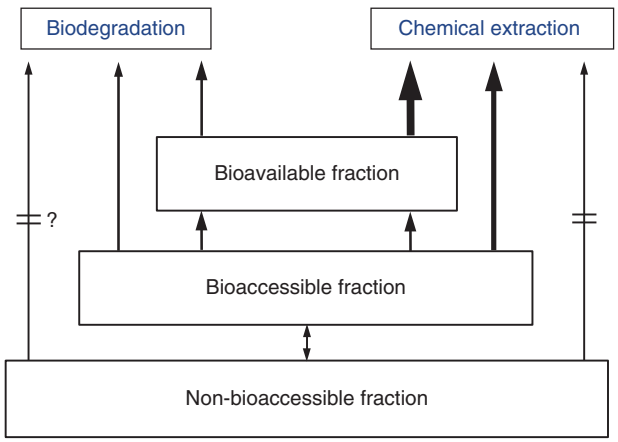


Figure 13.2 Bioavailable, bioaccessible, and nonbioaccessible fractions of an organic contaminant in a soil matrix, and associated contaminant fluxes (the width of the corresponding arrows indicates the size of the respective fluxes) when biodegradation (left) and chemical extraction (right) take place. Modified from Semple *et al.* (2007), with permission from Elsevier.

represent barrel proteins crossing the outer membrane of gram-negative bacteria, act as large pores more or less freely passed by various compounds. Plant cell walls, which may contain varying amounts of cellulose, hemicelluloses, pectin, and lignin, are permeable to xenobiotics. Such compounds can move in plants through the apoplastic system. The uptake of nonionic xenobiotic compounds by plant cells proceeds via passive diffusion and cotransport.

13.2 Adverse Effects of Xenobiotics: from Cells to Ecosystems

13.2.1 Effects at the Level of Individuals and Below

Xenobiotics (Table 13.1) as well as their metabolites may be toxic to living cells. Classic toxicology has primarily addressed toxic effects of chemicals on humans at the subcellular, organismal, and in the context of epidemiology at the population scale, with other species employed in toxicological tests mainly as human surrogates. **Environmental toxicology** has extended toxicological studies of xenobiotics to ecologically relevant organisms beyond humans (e.g., algae, aquatic and terrestrial plants, aquatic invertebrates, bees and other arthropods, birds, fish, mammals other than humans, microorganisms) while including effects at different levels of biological organization (individual, population, community, or ecosystem). Environmental toxicology is a multidisciplinary field of science aiming at the comprehensive assessment of sources, fates, and effects of harmful chemical, biological, and physical agents, and addresses social and regulatory aspects as well. Besides toxicology in its narrow sense, it also involves, for example, environmental sciences, chemistry and chemodynamics, biology with all of its relevant subdisciplines, statistics and modeling, as well as risk (i.e., exposure and hazard) assessment.

Box 13.1: Bioavailability and bioaccessibility

Bioavailability difficult to measure, and modeling of degradation (for microorganisms) or uptake kinetics (for plants or soil fauna) of a xenobiotic is often used as an estimate. The **bioaccessibility** of a xenobiotic compound is thought to be reflected by its chemical extractability from soils or sediment particles (Figure 13.2). Methods that extract only the rapid and slow desorbing domains (out of three distinct desorption domains, which are defined based on chemical extraction: "rapid," "slow," and "very slow") have reasonably been argued to measure the bioaccessible fraction. This fraction appears as a relatively defined and robust parameter and, given that it would become bioavailable within a reasonable period (i.e., within days rather than years), it could also be of toxicological relevance. Whereas bioaccessible fractions may become bioavailable over time, there is only minimal exchange between the bioaccessible and nonbioaccessible contaminant pools. The nonbioaccessible contaminant fraction resists chemical extraction and (likely) also biodegradation over extended time periods (Figure 13.2) (Katayama *et al.*, 2010; Semple *et al.*, 2007).

Acute toxicity tests address short-time effects of usually single but occasionally multiple doses (typically one application or exposure of 24 h) of xenobiotics on living organisms under controlled laboratory conditions (test duration, e.g., 96 h for algae). Frequently measured endpoints in tests with plants and algae are photosynthesis rates, chlorophyll contents, or biomass production.

Chronic toxicity tests are characterized by a repeated or continuous exposure to the toxicant of concern over a prolonged period and may include the whole life cycle (from the stage of zygote to the age of first reproduction) of an organism. Sometimes effects are monitored over several generations. Literature definitions of chronic exposure vary and depend on the specific conditions applied to quantify an effect. Exposures exceeding 10% of the life span of an individual can be defined as chronic, and exposure periods typically range from one to several months. Because of difficulties related to the often long duration and high costs of full life cycle tests, less time-consuming alternatives have been developed. The toxicity of a particular chemical can also be affected by the presence of other chemicals. The resulting interactions may be additive (the resulting toxicity is the sum of the toxicities of the concerned chemicals when applied alone), synergistic (the resulting toxicity is higher than the sum of the toxicities of the concerned chemicals when applied alone), or antagonistic (the resulting toxicity is lower than the sum of the toxicities of the concerned chemicals when applied alone).

With the exception of extreme cases (e.g., oil or chemical spills), environmental concentrations of xenobiotics commonly do not lead to severe acute toxicity in the field, and sublethal responses of organisms are most relevant under field conditions. Such responses can be investigated with a variety of chronic and subchronic toxicity tests (1–3 months exposure time) addressing, for example, growth, larval metamorphosis, metabolic rate, reproductive and developmental toxicity, and hormone and endocrine functions. Prominent examples for xenobiotics with adverse effects on normal endocrine functions in wildlife populations are **endocrine disrupting chemicals (EDCs)**, a group of hormonally active compounds with diverse structures and modes of action that include androgen, estrogen, and thyroid receptor agonists and antagonists (Table 13.1).

A considerable range of xenobiotics including representatives of classical as well as emerging pollutants (e.g., synthetic musk fragrances and various pesticides;

Box 13.2: Widely applied toxicity parameters

Toxic effects determined in **acute toxicity tests** are usually reported as the dose (LD_{50}, for terrestrial species), concentration (LC_{50}, for aquatic species), or rate (ER_{50}, for terrestrial arthropods) that is lethal to 50% of the test organisms. Nonlethal endpoints (e.g., inhibition of growth or reproduction) are commonly expressed as effective dose (ED_{50}, for terrestrial species) or concentration (EC_{50}, for aquatic species), which refers to the dose or concentration necessary to cause a 50% expression of the effect of concern. Toxic effects determined in **chronic tests** are usually reported as lowest-observed effect levels (LOELs) or lowest-observed effect concentrations (LOECs), and as no-observed-effect levels (NOELs) and no-observed-effect concentrations (NOECs). Controls are performed in the absence of toxicant. LOEL and LOEC values refer to the lowest toxicant levels or concentrations causing an effect that is significantly different from the control, whereas NOEL and NOEC values represent the highest toxicant levels or concentrations with an effect not significantly different from the control. In ecotoxicology, NOEC and LOEC values are considered as the lower and upper bounds for the maximum allowable toxicant concentration (MATC; a presumably "safe" threshold sometimes estimated as the geometric mean of the NOEC and LOEC).

Box 13.3: Measuring the effects of endocrine disrupting chemicals

Estrogen-like effects caused by xenobiotics acting as agonists of estrogen receptors (ERs) can be recorded on measuring the proliferation of estrogen-sensitive MCF-7 or T47-D human breast cancer cells after exposure to the xenobiotic of concern (E-screen assay). Another widely used assay (yeast estrogen screen = YES) for such purposes is based on recombinant yeast cells containing the human ER-α and an estrogen response element driving an expression plasmid-encoded reporter gene (*lac-Z*). An interaction of a xenoestrogen with the ER leads to the expression of the reporter gene and finally to the secretion of the enzyme ß-galactosidase whose activity can be measured easily. A drawback of the YES assay is its inability to discriminate between estrogenic and antiestrogenic compounds.

Table 13.1) and metabolites arising from microbial degradation of oil hydrocarbons, but also compounds and toxins naturally produced by invasive species, are known to inhibit **multixenobiotic resistance (MXR) mechanisms** in aquatic organisms. Such mechanisms are analogous to **multidrug resistance (MDR) mechanisms** primarily known from the resistance of human tumor cells to antitumor drugs. **P-glycoprotein (P-gp)** is a transmembrane protein belonging to the ABC transporter superfamily (P-gp is also called **ABCB1, ATP-binding cassette subfamily B member 1**, or MDR1), acts as an ATP-dependent efflux pump for a wide variety of mostly moderately hydrophobic compounds including xenobiotics, endogenous metabolites, and phospholipids, and confers MDR mechanisms in humans. P-gp-like proteins have also been found in various aquatic invertebrates and vertebrates, and are thought to contribute to MXR mechanisms of organisms in untouched and organically polluted ecosystems through pumping xenobiotics out of cells. The role of P-gps in detoxicfication of xenobiotics is further evident from their substrate overlap and coexpression with phase I detoxification enzymes such as cytochrome P450 monooxygenases (see Section 13.3.2). In addition, a few proteins termed **multidrug resistance-associated proteins (MRPs)**, which belong to the ABCC subfamily of ABC transporters and are known to confer MDR to anionic organic compounds (i.e., phase II conjugate metabolites of xenobiotics; see Section 13.3.3) in humans, likely also contribute to MXR mechanisms in aquatic organisms. The inhibition of MXR transporters by xenobiotics or natural compounds present in the environment, which are then referred to as **chemosensitizers**, may result in intracellular accumulation and toxic effects of other xenobiotics or compounds that normally would be removed without adversely affecting cells.

Bacteria are also able to export toxic xenobiotics from their cytoplasm or, in case of gram-negative bacteria, from the periplasm. Members of many transport protein families may be involved in the export of these compounds. Some of these efflux systems are located in the cytoplasmic membrane and export their substrates only across this membrane. More complicated **transenvelope efflux complexes** are composed of many subunits that are arranged in three modules and occur mostly in gram-negative bacteria. One protein complex is located in the cytoplasmic membrane and drives the transport process. These "motor devices" may belong, for example, to the ABC, the RND (resistance nodulation cell division), or the MFS (major facilitator) protein superfamily, and the energy required for the transport process is provided by ATP hydrolysis (ABC) or the proton-motive force (RND, MFS). The second protein complex is a tube-like trimeric outer membrane factor (OMF protein family) that spans the outer membrane in a barrel-like structure and extends into the periplasm. The third protein complex, the membrane fusion or periplasmic adaptor, connects the other two components. Transport by these transenvelope efflux systems may be from the periplasm or the cytoplasm, but the substrates are always exported directly to the outside of the cell.

13.2.2 Effects at Higher Levels of Biological Organization: Populations, Communities, and Ecosystems

The field of **ecotoxicology** integrates toxicology and ecology and focuses on effects of pollutants with particular emphasis on higher levels of biological organization (population, community, and ecosystem). Because of

Box 13.4: Measuring the effects of chemosensitizers

Xenobiotics with a high affinity for P-gps can act as **chemosensitizers** and competitively inhibit P-gp-dependent effluxes of other compounds normally pumped out of cells. The resulting P-gp activity can be measured with competitive transport assays, where a decreased efflux of a labeled model substrate or dye is recorded in the presence of a chemosensitizer. P-gp expression, which is known to be regulated by xenobiotics at the gene transcription level, can be followed with immunohistochemical and molecular-genetic approaches.

Box 13.5: Environmental risk assessment (ERA) framework and its key elements

An **ERA framework** covers much of practical ecotoxicology. ERA aims to characterize risks caused by environmental pollutants to ecosystems (in theory to all living organisms). In ERA, predicted environmental concentrations (PECs) of xenobiotics will be confronted with the concentrations at which toxicity effects are not expected to be triggered (predicted no effect concentration, PNEC). ERAs involve a step-wise approach:

1) PECs of pollutants are calculated for different ecosystems. Here, both short-term exposition to cover accidental spill, and long-term exposition taking into account emission, advection, diffusion, and degradation of xenobiotics is considered.
2) PNECs are estimated based on laboratory toxicity tests, which are performed using suitable indicator species. PNECs are usually derived from the lowest toxicity values observed in toxicity tests, while taking a safety factor depending on the respective endpoint and the number of tested species into account. Safety factors serve to cover the extrapolation of results from the laboratory to natural environments, from a punctual life stage to the entire life cycle, from an individual to a population or community, or from single species to an ecosystem.
3) The risk is characterized for each ecological compartment. For this, a risk quotient (RQ) is calculated as the PEC : PNEC ratio. The risk caused by a xenobiotic is judged to be acceptable if the PNEC is higher than the PEC. In case of a PEC : PNEC ratio >1, the ERA can be refined in a higher tier assessment. The exposure scenario (PEC) will be recalculated by applying a more realistic scenario (e.g., using supplementary data on the fate of a xenobiotic of concern at field scale), or more detailed ecotoxicological studies performed in mesocosms or in the field might be included to decrease the safety factor.
4) A risk statement might then be proposed as a basis for risk management.

this specific focus, ecotoxicology may be considered as a subdiscipline of environmental toxicology, although the boundaries between these disciplines are blurred. Ecotoxicology includes investigations on pollutant release, transport, and transformation, and aims at the prediction of pollutant effects, for example, using an **environmental risk assessment (ERA)** framework. If serious contaminations have already occurred, **retrospective ERAs** might be performed in order to identify the causes of the adverse effects and contribute to the development of management options. **Predictive ERAs** are intended to predict risks from future situations (e.g., in the context of the registration or authorization of a new pesticide). **Comparative ERAs** may support environmental decision making by comparing the risks of two or more alternative scenarios.

Population-level impacts of xenobiotics are often studied by monitoring vital rates (e.g., birth, death, or migration rates) or survival traits (e.g., body weight of adults and pups, eggshell thickness in case of birds). For instance, studies considering a metapopululation, which consists of subpopulations inhabiting habitat patches with different contamination levels, enable us to explore effect manifestation at a distance from the contaminated patch as may be caused by the migration of population members.

Most studies addressing the effects of xenobiotic contaminants at the **community level** are field studies. Adverse impacts of contaminants are suggested by the absence of particularly sensitive species, which are used as bioindicators. Furthermore, community metrics such as species richness or eveness may be applied to indicate the adverse effects of xenobiotics. Community ecotoxicology typically also addresses potentially hazardous effects of xenobiotics at different trophic levels, arising from xenobiotic transfer through the food web. Hereby, concentrations of xenobiotics can increase (biomagnification), decrease (trophic dilution), or remain constant with increasing trophic levels. For instance, the soil fauna feeds on bacteria, fungi, protozoa, and soil organic matter, which may result in the bioaccumulation of xenobiotics in soils.

Studies addressing impacts of xenobiotic contaminants at the **ecosystem level** have been quite variable with respect to spatial and temporal scales. Depending on the scale, the fate and movement of xenobiotics can be computer-modeled, which may be aided by measurements from extensive sampling campaigns with geographical information systems (GISs), or remote sensing technologies. Because of current global issues such as ozone depletion in the stratosphere, global warming, or global movement of persistent organic pollutants, large-scale problems are of increasing interest in ecotoxicology.

13.3 Organismal Responses: Biochemical Elimination of Xenobiotics

13.3.1 General Aspects of Biodegradation and Biotransformation Reactions

The elimination of xenobiotic compounds by organisms can occur through (i) biochemical conversion into other chemicals (further on referred to as **biotransformation** or

incomplete biodegradation), (ii) their ultimate breakdown into CO_2 and H_2O (a process frequently referred to as **biodegradation** or **mineralization**; here, we use the latter term where unambiguity is needed, to avoid confusion with biotransformation), and (iii) their **excretion** or excretion of their biotransformation metabolites. While the present section introduces the biochemical alteration of xenobiotics from a general perspective by illustrating terms, definitions, and further aspects of ecological and environmental relevance, Sections 13.3.2 and 13.3.3 deal more specifically with the enzymes involved and the corresponding biochemical reactions. Finally, Section 13.3.4 addresses the excretion of xenobiotics and their metabolites.

Biodegradation and biotransformation reactions largely influence the environmental fate of xenobiotics (Figure 13.1). Moreover, they serve as the basis for **active** (e.g., **biostimulation**) and **passive bioremediation schemes** (e.g., **monitored natural attenuation**), and are exploited in environmental biotechnologies controlling the treatment of contaminated media within confined systems (e.g., WWTPs) (compare Chapter 17, Section 17.2). There exist several principal ways to eliminate a xenobiotic compound via its biochemical alteration:

1) Organisms may utilize a xenobiotic as their sole source of carbon and energy (in other words, use it as a growth substrate) (Table 13.2). This process commonly leads to the mineralization of at least a structural part of the compound molecule, whereas other parts of the compound's carbon are used to build up biomass components. Specialized biochemical pathways for a considerable variety of environmental pollutants (e.g., BTEX compounds, a variety of chloroaromatics, EDCs such as nonylphenol and bisphenol A, methyl *tert*-butyl ether, and PAHs) have evolved in bacteria. Besides aerobic bacterial degradation of xenobiotics, where oxygen serves as the terminal electron acceptor, alternative terminal electron acceptors (e.g., nitrate, iron(III), and sulfate) can enable the anaerobic use of particular compounds as sole sources of carbon and energy. Despite a few reported exceptions, fungal

Table 13.2 Overview and examples of biodegradation and biotransformation processes of xenobiotics supporting growth of the catalyzing organisms, and of those undergoing cometabolism.

Growth support			Cometabolism			
Aerobic	Anaerobic	Anaerobic	Aerobic		Anaerobic	
Xenobiotics as e^--donor, O_2 as terminal e^--acceptor	Xenobiotics as e^--donor, alternative terminal e^--acceptors (e.g., nitrate, Fe(III), sulfate)	Xenobiotics as terminal e^--acceptor: dehalorespiration	Attack on xenobiotics by enzymes of aerobic degradation pathways/ detoxifying enzymes (initial xenobiotics functionalization: phase I reactions in eukaryotes)		Attack on xenobiotics by enzymes of anaerobic oxidation pathways employing alternative terminal e^--acceptors	Incidental reductive dehalogenation of xenobiotics
Various catabolic steps	Various catabolic steps	Successive dehalogenation steps	Further catabolic steps	Conjugate formation (phase II reactions)	One or more catabolic steps	One or more successive dehalogenation steps
Mineralization (biodegradation), energy conservation, biomass from xenobiotics	Mineralization (biodegradation), energy conservation, biomass from xenobiotics	Formation of less chlorinated metabolites (biotransformation), energy conservation	Mineralization (biodegradation)	Excretion of (conjugated) metabolites (phase III reactions) (biotransformation)	Formation of dead-end products (biotransformation)	Formation of less chlorinated metabolites (biotransformation)
Bacterial degradation of certain PAHs (example)	Bacterial growth on toluene using nitrate, Fe(III), and sulfate as terminal e^--acceptor (example)	Bacterial dehalorespiration on chlorobenzenes, PCB, PCE, and TCE (example)	Biodegradation of PAHs by white-rot fungi (example)	PAH metabolism in soil micromycetes (example)	Anaerobic bacterial cometabolism of xylenes in the presence of toluene as a growth substrate (example)	Bacterial reductive dehalogenation of diverse haloaliphatic and haloaromatic compounds

Partly extracted from Cerniglia and Sutherland (2010), Chang (2008), Futagami, Goto, and Furukawa (2008), and Harms, Schlosser, and Wick (2011).

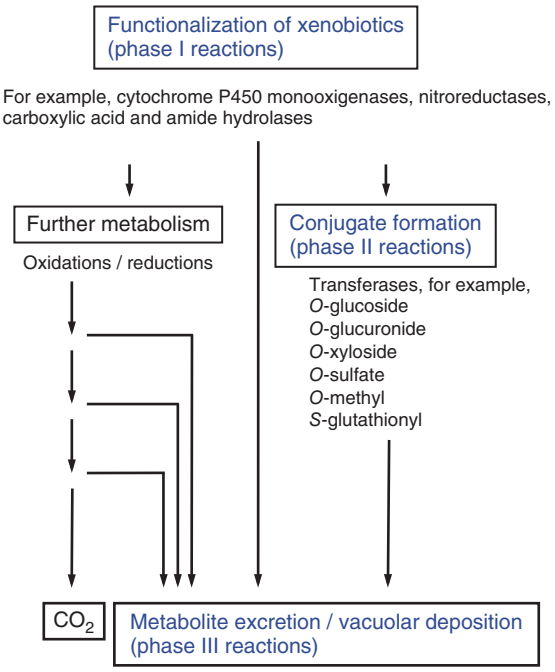


Figure 13.3 General mechanisms of cometabolic biodegradation and biotransformation of xenobiotics in eukaryotic organisms such as animals, plants, and fungi. Metabolites arising from the functionalization of xenobiotics in phase I reactions may be excreted or deposited in vacuoles in the form of usually persistent conjugates (formed in phase II reactions), or undergo further catabolism. This may finally lead to mineralization, or again to metabolite excretion at various oxidation stages if subsequent oxidation is impeded. Modified and extended from Harms, Schlosser, and Wick (2011.)

growth on xenobiotics is predominantly aerobic and limited to a much narrower range of compounds than bacterial assimilation of xenobiotics. A range of aliphatic and a few monoaromatic hydrocarbons with rather simple structures (e.g., phenol, *p*-cresol, and toluene) can serve as fungal growth substrates. The use of a xenobiotic as a source of carbon and energy commonly involves more or less compound-specific biochemical pathways. From an ecological viewpoint, the evolution of a biochemical pathway enabling growth on a compound that cannot be utilized by other species corresponds to the advantageous occupation of new ecological niche, with an accompanying detoxification of the compound perhaps providing an additional evolutionary advantage for the degrader organism in cases where bioavailable compound concentrations are high enough to be substantially toxic.

2) Xenobiotics that cannot be used as a growth substrate may be biodegraded or biotransformed in the presence of another compound referred to as cosubstrate and serve as the source of carbon and energy, which is called **cometabolism** (Figure 13.3). Cometabolism can be found among members of almost all groups of eukaryotic organisms where it represents the dominant way of biochemically altering xenobiotics, and also among bacteria where prominent examples for aerobic cometabolism include higher chlorinated dioxin congeners, Polychlorinated biphenyls (PCBs), and TNT. Cometabolic processes may result in the formation of organic metabolites when the biodegradative process is incomplete. Otherwise, it can lead to the mineralization of xenobiotics. The so-called white-rot fungi represent a particular ecophysiological group of higher fungi (*Basidiomycota*, *Dikarya*), which are capable of degrading (i.e., mineralizing) substantial amounts of the lignin component of lignocellulose in wood and plant litter. Because of the nonspecificity of the enzymatic machinery involved in lignin degradation (Table 13.3), these fungi are also able to mineralize a very broad range of xenobiotics. In other groups of organisms and fungi, cometabolic biotransformations generally predominate. Cometabolism is much less compound-specific than the use of a xenobiotic as growth substrate. Biodegradable cosubstrates often induce their degrading enzymes, which are frequently not specific enough to discriminate between the natural (co)substrates and structurally similar xenobiotics that do not support growth and energy conservation. Such effects can be found among both pro- and eukaryotes. For instance, bacteria growing on biphenyl can cometabolically convert PCBs into organic metabolites using the same enzymes. The incidental mineralization of many xenobiotics by the very unspecific lignin-degrading system of white-rot fungi, which is primarily "intended" to remove lignin from lignocellulose in order to access the polysaccharide components serving as growth substrates, has already been mentioned. Other cometabolic biotransformations, in particular, those catalyzed by intracellular enzymes of eukaryotic organisms (Table 13.3), can be considered as detoxification mechanisms seemingly designed by nature to cover a broad range of potential toxicants. In line with an enormous versatility regarding the chemical structures that can be attacked by cometabolic biotransformation reactions is the occurrence of a multitude of cytochrome P450 monooxygenase-encoding genes in the same eukaryotic species. Organic metabolites of xenobiotics that may be excreted from the cells of the organism(s) initiating attack after variable numbers of biochemical reaction steps are frequently less toxic than their parent compounds, and in case of complex (microbial) communities may be subject to further biodegradation by other community members, thereby becoming part of a trophic network. However, in some cases, organic biotransformation metabolites are more toxic than their parent compounds or may resist further degradation by other members of complex communities. These possibilities illustrate the need for a careful assessment of the further fate of organic biotransformation metabolites.

Table 13.3 Major enzyme classes and important representatives involved in the initial attack on xenobiotic compounds.

Enzyme class	Enzymes (occurence)	Localization	Reactions with xenobiotics/comments
Aerobic pathways			
Oxidoreductases: EC 1	Dioxygenases (bacteria)	Cell bound	O_2-dependent conversion of numerous aromatic xenobiotics including certain dioxin congeners and PAHs into the corresponding *cis*-dihydrodiols; O_2-dependent intra- (*ortho*) and extradiol (*meta*) ring cleavage of catechol structures arising from primary hydroxylation and subsequent dehydrogenation of xenobiotics
	Nonheme monooxygenases (bacteria, fungi)	Cell bound	O_2-dependent monohydroxylation of various aromatic xenobiocs including toluene, ethylbenzene, xylenes, (halo)phenols, and PAHs
	Cytochrome P450 monooxygenases (animals, plants, fungi, bacteria)	Cell bound	O_2-dependent epoxidation and monohydroxylation of aromatic or aliphatic structures of many pollutants including PAHs, PCDDs, alkylsubstituted aromatics, alkanes, pesticides, and pharmaceuticals
	Tyrosinases (animals, plants, bacteria, fungi)	Mainly cell bound, sometimes extracellular	O_2-dependent oxidation of various phenols including highly chlorinated ones into the corresponding *o*-diphenols (cresolase activity); Oxidation of *o*-diphenols to quinones (catecholase activity)
	Laccases (fungi, bacteria, plants)	Extracellular (fungi), cell bound (bacteria, plants)	O_2-dependent one-electron oxidation of various phenols, aromatic amines, and anthraquinone dyes; Wide range of pollutants oxidized in the presence of natural and synthetic redox mediators; Lignin-modifying enzymes
	Horseradish peroxidase and similar peroxidases	Cell bound (plants), extracellular (fungi)	H_2O_2-dependent direct oxidation of phenols and low-redox potential dyes
	Lignin peroxidases (basidiomycetous fungi)	Extracellular	H_2O_2-dependent one-electron oxidation of various high-redox potential aromatics including certain PAHs; Lignin-modifying enzymes
	Manganese peroxidases	Extracellular	H_2O_2-dependent one-electron oxidation of Mn^{2+} to Mn^{3+}, which subsequently oxidizes organic compounds such as various phenols and aromatic amines; Extended substrate range in the presence of co-oxidants (organic SH-compounds, unsaturated fatty acids, and their derivatives); Lignin-modifying enzyme
	Versatile peroxidases (basidiomycetous fungi)	Extracellular	Combines properties of lignin and manganese peroxidases; Direct oxidation of phenols and high-redox potential aromatics including dyes; Manganese-dependent reactions as for manganese peroxidase; Lignin-modifying enzymes
	Dye decolorizing peroxidases (basidiomycetous fungi)	Extracellular	H_2O_2-dependent one-electron oxidation of high-redox potential anthraquinone dyes, which can be oxidized by other peroxidases only hardly; Lignin-modifying enzymes

(*continued overleaf*)

Table 13.3 (*Continued*)

Enzyme class	Enzymes (occurence)	Localization	Reactions with xenobiotics/comments
	Heme-thiolate peroxygenases (basidiomycetous fungi)	Extracellular	H_2O_2-dependent peroxygenation of various mono- to polyaromatic pollutants including PAHs and dibenzofuran to mono- and polyhydroxylated products; Ether bond cleavage between aromatic and aliphatic parts of molecules and in alicyclic and aliphatic ethers; Functional hybrids of peroxidases and cytochrome P450 monooxygenases; Lignin-modifying enzymes
	Nitroreductases (animals, plants, bacteria, fungi)	Cell bound	NAD(P)H-dependent reduction of TNT to hydroxylamino-dinitrotoluene and amino-dinitrotoluenes; Formation of mononitroso derivatives and ring cleavage products from cyclic nitramine explosives
Hydrolases: EC3	Epoxide hydrolases (animals, plants, bacteria, fungi)	Cell bound	H_2O-dependent conversion of PAH epoxides arising from cytochrome P450 reactions into *trans*-dihydrodiols
	Amidases (animals, plants, bacteria, fungi)	Cell bound	H_2O-dependent conversion amides arising from nitrile hydratase reactions into carboxylic acids and ammonia
	Nitrilases (animals, plants, bacteria, fungi)	Cell bound	H_2O-dependent conversion of nitriles into carboxylic acids and ammonia
Lyases: EC4	Nitrile hydratases (animals, plants, bacteria, fungi)	Cell bound	H_2O-dependent conversion of nitriles into amides
Anaerobic pathways			
Oxidoreductases: EC 1	Reductive dehalogenases (bacteria)	Cell bound	Reductive dechlorination of diverse chloroaliphatic and chloroaromatic compounds including PCE, chlorobenzenes, and dioxins
	Ethylbenzene dehydrogenases (denitrifying bacteria)	Cell bound	H_2O-dependent hydroxylation of the side chain carbon atom proximal to the aromatic ring of ethylbenzene and propylbenzene
Lyases: EC4	Benzylsuccinate synthases (bacteria)	Cell bound	Addition of fumarate to the ethyl group of toluene using a radical mechanism, yielding benzyl succinate

Partly compiled from Cerniglia and Sutherland (2010), Chang (2008), Demarche *et al.* (2012), Fuchs, Boll, and Heider (2011), Futagami, Goto, and Furukawa (2008), and Harms, Schlosser, and Wick (2011).

3) Under anaerobic conditions, chlorinated alkenes (e.g., the organic solvents PCE, tetrachloroethylene and TCE, trichloroethylene) and chloroaromatic xenobiotics (e.g., PCBs, chlorophenols, and dioxins) can serve as electron acceptors and undergo a process referred to as **reductive dehalogenation** (Tables 13.2 and 13.3). Reductive dehalogenations are biotransformation reactions catalyzed by anaerobic bacteria (e.g., *Dehalococcoides* of the *Chloroflexi*, *Dehalobacter* of the *Firmicutes*). A compound- and organism-specific degree of substitution of a chlorine with a hydrogen atom takes place in case of chloroorganic compounds, eventually resulting in chloride release and a partly or sometimes fully dechlorinated organic product. H_2 originating from the fermentation of other organic compounds and delivered by H_2-producing bacteria is frequently used as an electron donor in such processes. Reductive dehalogenations of chloroorganics (chlorinated alkenes: e.g., PCE and TCE; chloroaromatic compounds: e.g., PCBs, chlorobenzenes, and dioxins) serving as terminal electron acceptors may be coupled to energy conservation in certain anaerobic bacteria, a process referred to as **dehalorespiration**. Evolutionary processes driven by naturally occurring organochlorines provide a possible explanation for the existence of dehalorespiring bacteria. In other cases, energy conservation has not been proven and corresponding reductive dechlorinations are thought to result from

incidental contacts of oxidized chloroorganics with reduced forms of electron-transfer molecules (e.g., redox-active cofactors) in a process using another electron acceptor. Reductive dehalogenations not representing dehalorespirations can be interpreted as anaerobic cometabolic biotransformations because the energy or growth benefit for the catalyzing organism(s) is not provided. Complete dehalogenation of highly chlorinated compounds is not always achieved with a single bacterial species, and microbial consortia containing several species of reductively dechlorinating bacteria differing in their preferences for the chlorine positions in a molecule can yield a higher degree of dechlorination than single bacterial species. Nevertheless, the control of reductive dechlorination processes by the redox potential is more important than control by substrate specificity. Reductive dechlorination of higher chlorinated compounds usually occurs more readily than that of already partly reduced compounds. The reductive dechlorinations catalyzed by anaerobic bacteria should not be confused with reductive dechlorinations catalyzed by fungal enzymes or in mammals. In these eukaryotic organisms, reductive dechlorinations of chloroorganic compounds are part of their basically aerobic cometabolism.

13.3.2 Initial Biochemical Attack on Xenobiotics – Phase I Reactions

Nature has created a multitude of enzymes enabling both pro- and eukaryotes to attack xenobiotic molecules. Enzymes catalyzing the initial biochemical reaction step(s) in the biotransformation or biodegradation of xenobiotics and commonly leading to the functionalization of parent compounds may be classified as oxidative or reductive (together referred to as **oxidoreductases**), **hydrolases**, **lyases**, and a particular group of lyases acting as synthases during the anaerobic catabolism of aromatic compounds (Figure 13.3, Table 13.3). Inspired by human metabolism of drugs and other xenobiotics, such enzymes (and the reactions they catalyze) are frequently referred to as **phase I enzymes (reactions)** in other eukaryotes. **Monoxygenases** of the **cytochrome P450 superfamily** present in pro- as well as eukaryotes and frequently catalyzing expoxidations and hydroxylations of xenobiotics, and other mono- (in bacteria and fungi) and **dixoxygenases** (in bacteria) are prominent examples for intracellular oxidoreductases involved in the metabolization of xenobiotics. Intracellular mono- and dioxygenases are frequently induced by their xenobiotic substrates or related compounds at the transcriptional level. Fungal extracellular oxidases (laccases) and various peroxidases, which are primarily involved in the decomposition of natural recalcitrant organic matter such as lignin and lignocellulose and referred to as **lignin-modifying enzymes**, also represent oxidoreductases meriting particular reference as such enzymes (i) enable the producing organisms to attack and thereby detoxify xenobiotics without cellular uptake, (ii) may increase the bioavailability of xenobiotics because of their extracellular localization, and (iii) are highly nonspecific and, therefore, allow attack on a broad range of xenobiotics. Fungal lignin-modifying enzymes are often differentially regulated at the level of gene transcription in response to, for example, various natural and xenobiotic organic compounds, several metals, nitrogen, carbon, and starvation conditions. Various **reductases** of pro- and eukaryotes, for example, act on nitroaromatics such as TNT, and on organochlorines. Eukaryotic (human) **carboxylic ester and amide hydrolases** catalyze the hydrolysis of organophosphate insecticides, and antiarrhythmic and anesthetic drugs, respectively. **Epoxide hydrolases** are common in all domains of life and, for instance, found in fungi as well as humans, where they convert PAH epoxides arising from cytochrome P450 monooxygenase reactions into *trans*-dihydrodiols. The widespread lyase **nitrile hydratase** converts various nitriles into amides. Under anaerobic conditions, **synthases** like the glycine radical enzyme **benzylsuccinate synthase** catalyze the initial steps in the bacterial catabolism of aromatic compounds. Benzylsuccinate synthase couples fumarate to the methyl group of toluene using a radical mechanism, which results in the formation of benzylsuccinate, The latter is subsequently converted enzymatically into benzoyl-CoA serving as a central intermediate in the anaerobic catabolism of aromatic compounds.

13.3.3 Conjugate Formation from Functionalized Xenobiotics – Phase II Reactions

The formation of water-soluble **conjugates** of functionalized xenobiotics with endogenous, highly polar molecules or moieties not exceeding a molecular mass of about 300 Da (i.e., **phase II reactions**) is a general characteristic of eukaryotic organisms and can be found in, for example, fungi, plants, and animals (Figure 13.3, Table 13.2). Nevertheless, phase II reactions are most prominent in the metabolism of pharmaceuticals and other xenobiotics in humans. Conjugate formation is catalyzed by **transferases** and produces metabolites of xenobiotics that are susceptible to **excretion**, which represents a way to eliminate hazardous compounds from cells. Typically, conjugates are not subject to further degradation by their producing organisms, whereas ambiguous data regarding their further fate under the influence of complex (microbial) communities illustrate a need for clarification. Conjugate formation can involve, for example, glucuronic acid (*O*-, *C*-, *N*-, and *S*-glucuronidation; catalyzed by respective **UDP-glucuronyltransferases**), sugars (*O*-, *C*-, *N*-, and *S*-glycosylation; catalyzed by respective

UDP-glycosyltransferases), sulfonyl groups (*O*- and *N*-sulfatation; catalyzed by respective **sulfotransferases**), glutathione (catalyzed by **glutathione S-transferases**), acetyl-coenzyme A (*N*- and *O*-acetylations; catalyzed by respective **acetyltransferases**), and methylation reactions (*O*-, *N*-, and *S*-methylation; catalyzed by respective **methyltransferases**). Plant glutathione and glycosyl transferases play a prominent role in the detoxification of herbicides, where the resulting conjugates undergo deposition in vacuoles. Not much is known about the role of conjugate formation for the metabolism of xenobiotics in prokaryotes. For instance, sulfotransferases in mycobacteria have been implicated in host–pathogen interactions.

13.3.4 Further Modification, Excretion, and Deposition of Xenobiotics and Their Metabolites – Phase III Reactions

Phase III reactions may involve further metabolism of xenobiotic conjugates arising from phase II reactions, transport (vacuolar deposition in plants, removal from cells by excretion in other organisms) of xenobiotics and their metabolites (Figure 13.3), and stable incorporation of xenobiotic metabolites into cell wall structures as a peculiarity of plants. The formation of acetyl cysteine (mercapturic acid) conjugates from glutathione conjugates in animals, and malonyl CoA conjugation of glucose and glutathione conjugates of herbicides in plants are well-known examples of further biochemical modification during phase III reactions. Eukaryotic ABC transporters of the P-gp and MRP type, which are pumping organic cationic and neutral (P-gp) and anionic compounds (MRPs) out of cells, have already been introduced in Section 13.2.1. With respect to bacteria, efflux transporters of xenobiotics have been studied intensively in the context of MDR. This process is highly relevant to patients suffering from infectious diseases caused by bacteria resistant to antibiotics, which involves active efflux. The protein complexes required for this transport process have already been mentioned (see Section 13.2.1). The prokaryotic representatives of the ABC transporter superfamily are referred to as half-transporters as they dimerize to become functional.

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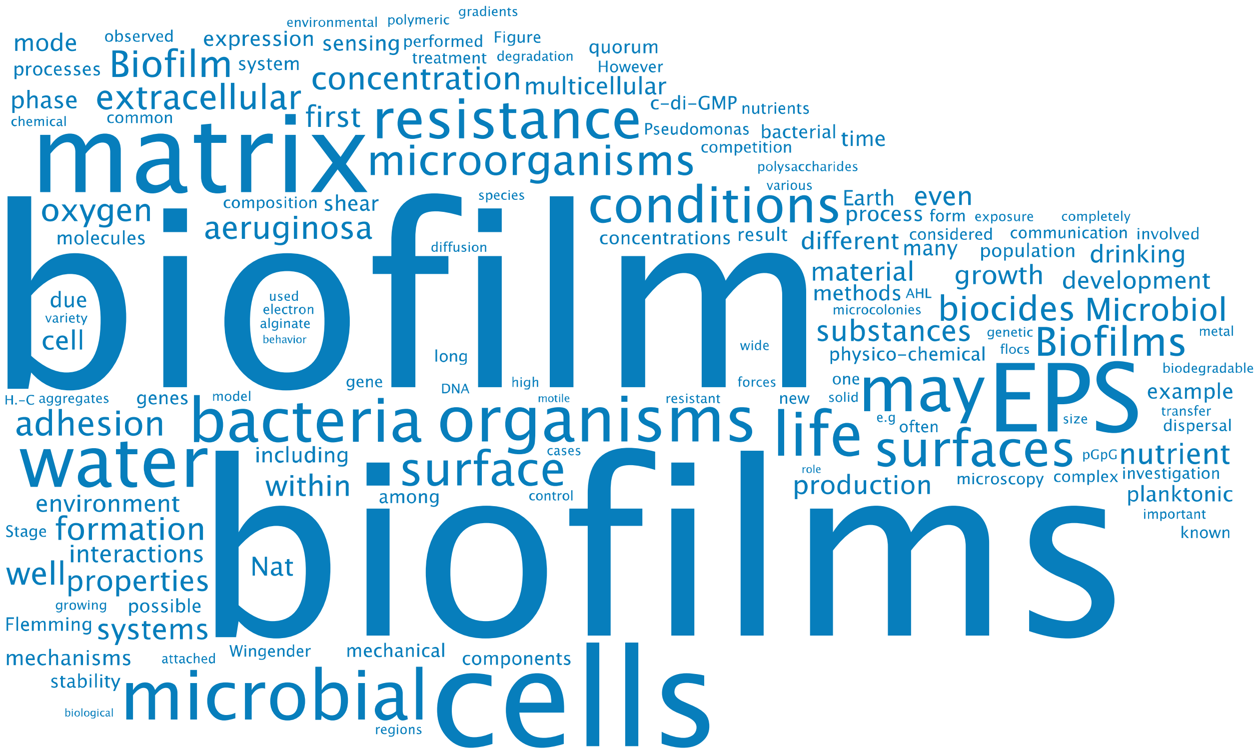
Part IV
Organismal Interactions (Biotic Stress)

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry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14
The Biofilm Mode of Life

Hans-Curt Flemming

Overview

The biofilm mode of life is common to almost all microorganisms on Earth, be it continuously or as part of their life cycle. Biofilm phenomena are very diverse. They range from films on solid surfaces, aggregates at the water-atmosphere interface to flocs as "floating biofilms," microbial mats, and sludges as very thick biofilms. Nevertheless, they all have common features. One of them is that they are embedded in a matrix of hydrated biopolymers which allows to develop emergent properties that are not shared by planktonic cells. Biofilm cells can develop synergistic multispecies relationships, because they are immobilized in the matrix. The matrix itself traps exoenzymes, turning it into an external digestion system. In biofilms, intense intercellular communication takes place, regulating the expression of numerous genes. Furthermore, biofilm cells are significantly more resistant to stress such as radiation, toxic metals, disinfectants and antibiotics. On the other side, there is fierce competition among biofilm organisms, driving the dynamic, spatially and temporally heterogeneous structure of biofilms. The biofilm mode of life is the first recorded on Earth, it is extremely flexible and successful.

14.1
What are Biofilms?

Microorganisms on Earth do not live as single cells in pure cultures – as they have been historically studied in microbiology – but rather in multispecies aggregates. Such aggregates display a huge variety of structural and functional properties. They may occur as films on interfaces ("biofilms" in the strict sense, e.g., on solid surfaces in water, soil, sediments, porous minerals, and wet atmosphere-exposed surfaces), but also as flocs ("floating biofilms"), as aggregated particles in dust and clouds, and as sludge. For all of them, the term "biofilm" has been accepted. Biofilms all have one important feature in common: the organisms live embedded in a matrix **of extracellular polymeric substances** (EPS) (Figure 14.1) and establish complex interactions. Many types of naturally occurring and engineered microbial aggregates have been subsumed under the heading of "biofilm," such as bacterial colonies, effluent treatment flocs, anaerobic digester granules, food-associated systems such as Kefir grains or the ginger beer plant, marine snow, mycelial balls, pellicles, and algal mat communities. The list is long and includes even microbial aggregates in clouds – and all of them meet the above mentioned characteristics.

Biofilms play fundamental and often deleterious roles in medicine, as habitats for pathogens in the environment, and in industrial biofouling, where they are frequently involved in **microbially influenced corrosion** and **biodeterioration** of synthetic polymers, wood, mineral materials, and pieces of art. On the other hand, biofilms are often put to beneficial use because they are the carriers of biological purification of drinking water as well as of waste water. Although these aspects will not be in the focus of this chapter, it is, nevertheless, clear that biofilms are ubiquitous and they have both very fundamental detrimental and beneficial aspects

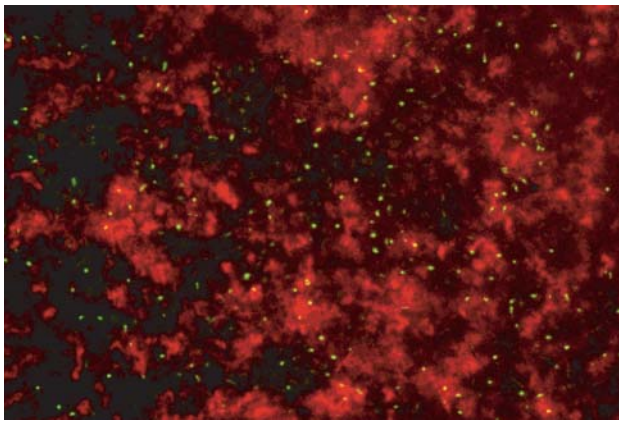

Figure 14.1 Visualization of the alginate matrix. Shown is a biofilm of *Pseudomonas aeruginosa* (*Gammaproteobacteria*) viewed by confocal scanning laser microscopy (CLSM). *P. aeruginosa* cells are stained in green with the DNA specific dye SYTO 9 and the EPS alginate in red by fluorescently labeled concanavalin A bound to alginate. The black areas represent water pores and possibly non-stained EPS polymers.

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for our life at often largely underestimated dimensions. The prerequisites of their occurrence are minimal, requiring only sufficient humidity, nutrients (even in traces), and microorganisms.

Biofilms can be considered the cradle of life; it is a common concept that life has evolved at interfaces. Essentially, the first organisms were originally located at these interfaces and multiplied there. Interfaces remained "hot spots" in evolution and biofilms are considered the oldest and most successful form of life on Earth with fossils dating back 3.5 billion years, representing the first signs of life on Earth. It is very plausible to assume that the processes of endosymbiosis, which led to eukaryotic organisms, may have evolved in such biofilms, as well as photosynthesis, which ultimatively provided the oxygen for Earth's atmosphere. Indeed, one possible origin of infectious diseases might have been the result of intense competition between biofilm microorganisms, including the emergence of predation of neighboring cells.

Aggregation and the association to surfaces of all kinds offer considerable ecological advantages for microorganisms. Practically all surfaces in non-sterile environments that provide sufficient amounts of water are colonized by biofilms, even at extreme pH-values, temperatures, salt concentrations, radiation intensities, and pressure. Some examples are listed in Table 14.1.

Table 14.1 Examples for the range of environmental conditions in which biofilms exist.

Conditions	Examples
Temperature	From −12 °C (cold saline water) to +125 °C (hot vents)
pH value	From 0 (sulfur oxidizers) to >13 (bacteria in natron lakes)
Hydrostatic pressure	From <0.1 bar (vacuum systems) to 1000 bar (barophilic bacteria at deep sea floor)
Redox potential	Entire range of redox stability of water; growth on electrodes
Salt concentration	From 0 (ultrapure water systems) to almost saturated salt solutions (hyperhalophilic bacteria in permafrost)
Nutrients	From <10 $\mu g\, l^{-1}$ to life directly on nutrients
Radiation	Biofilms on UV sources and nuclear power plants; *Deinococcus radiodurans* survives 10 000 Gy without being killed
Surface materials	Metals (including copper and silver), minerals, organic polymers, plant and animal tissue, bones, etc.
Biocides	Growth in disinfectant pipes; "persister cells"

Biofilms are also involved in the **biogeochemical cycles** of virtually all elements including most metals and they are carriers of the environmental "self-purification" processes. The basic underlying process is always the same: microorganisms on surfaces convert dissolved or particulate nutrients from the water phase and/or from their support into metabolites and new biomass. As mentioned earlier, this is the principle of **biological filtration** systems used in drinking water and **wastewater purification** as well as many other biotechnological applications.

14.2 Environmental Roles of Biofilms

Biofilms contribute strongly to primary production of biomass on earth, being especially represented in the rhizosphere and in **microbial mats**. They occur even within minerals ("endolithic biofilms") where they can survive very harsh conditions such as encountered in polar regions or the extremely dry Atacama desert. They also represent the "global cleaning company" for all biological material, which they often completely biodegrade (i.e., mineralize) to carbon dioxide and water. Most of the biodegradable biomass on Earth is not homogeneously dissolved but rather exists as heterogeneously distributed particulate matter. Particle degradation must be performed by attached microorganisms, that is, biofilms, and their **extracellular enzymes**. Thus, biological degradation is chiefly the result of the activity of microorganisms in biofilms, in most cases performed by orchestrated interactions of different microbial species.

Biofilms are driving the **mobilization of minerals** on a geological scale and, thus, providing metal ions for the biosphere. Figure 14.2a–c show a biofilm in a pore of sand stone, directly attached to the material. The acids produced by the organisms will slowly dissolve the carbonates which keep the quartz grains of the sand stone together, enhancing the weathering process which not only decomposes the mineral but also mobilizes metal ions, contributing to "**microbial weathering**". The cells are completely blanketed by EPS, which prevent **desiccation** during exposure to sun. Mineral deposition is also performed by biofilms. Particularly impressive are formations of calcium carbonate, which are attributed mainly to microbial activity.

Biofilms not only occur on solid surfaces but also at the interface between water and the atmosphere in global dimensions. The uppermost layer of surface waters is known as the **neuston**. Here, hydrophobic substances accumulate and provide nutrients for hydrophobic organisms, forming biofilms. Due to microbial products such as amphiphilic molecules they influence surface tension, and, thus, the physico-chemical conditions of the mass transfer of gases between atmosphere and water. Therefore, the biofilms in the neuston layer are essentially influencing not only the global CO_2 exchange, but also the exchange, production and

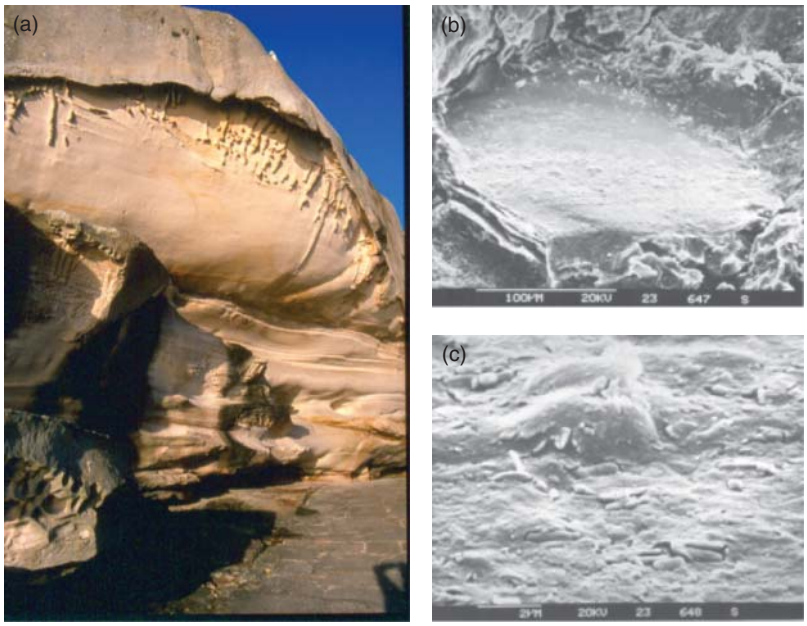

Figure 14.2 **A biofilm in a pore of sand stone**. (a) Sandstone rocks at Bondi Beach, Sydney, (b) scanning electron micrograph of biofilm in sandstone pore, (c) magnification of (b), bacterial cells visible below EPS blanket.

consumption of methane and dimethyl sulfoxide (DMSO), which is believed to be involved in cloud formation (see Section 6.2.4.2; Figure 6.14).

14.3 Life Cycle of Biofilms

The formation of biofilms begins with the first contact of a microorganism to a surface. It must be taken into consideration that most cells are surrounded by extracellular material, which will be the first to interact with the surface. The cells do not need to be viable for adhesion – the EPS and cellular appendages already present on planktonic cells are sufficient for adhesion. Biofilm development on a solid surface can be schematically depicted in six main stages (Figure 14.3).

- Stage 1: **Conditioning film**
  It is long known that waters, which are not ultrapure, always contain traces of biomolecules such as humic substances, proteins, polysaccharides, etc., which rapidly adsorb to surfaces as soon as they are exposed to the water. Within moments, the biomolecules stick irreversibly due to a multitude of weak physico-chemical interactions between groups associated with the biomolecules and the substratum. Due to their small size compared to bacteria, dissolved biomolecules rapidly "condition" substrata, effectively masking the physical–chemical makeup of the underlying surface from the bacteria. Moreover, the adsorption of such organic molecules alters many of the fundamental structural and functional characteristics of the surface. Factors affected can include surface free energy, hydrophobicity, and electrostatic charges, and the original surface properties may be strongly shielded by the presence of a conditioning film. Although the conditioning film influences bacterial adhesion, it is not a prerequisite for microbial adhesion.
- Stage 2: **Reversible and irreversible adhesion**
  The organisms adhere to a surface for a certain period of time (lag phase) until they start multiplying (log phase). The rate of multiplication is determined by nutrient availability, not by the number of cells in suspension. The first phase is dominated by physico-chemical processes, called the **race to the surface**. It has been shown that microorganisms can adhere to surfaces reversibly and irreversibly. Initial reversible adhesion has been essentially described as a physico-chemical process. Irreversible adhesion is less well characterized and can happen within less than an hour, regardless if the cells are alive or dead. A problem with the theoretical approaches to primary adhesion is clearly that reliable prediction of the adhesion behavior of certain microorganisms to certain surfaces is not yet possible derived only from the physico-chemical conditions.
- Stage 3: **Biofilm maturation I**
  Upon irreversible adhesion, microorganisms develop microcolonies. This can be observed already after few hours of contact time and represents the first stage of microbial multiplication and, thus, actual biofilm growth. Depending on the nutrient conditions, this stage can prevail for very long time, as has been shown on the inner surfaces of drinking water pipes exposed to very oligotrophic conditions.
- Stage 4**: Biofilm maturation II**
  If favorable conditions prevail, larger and thicker biofilms develop which may become confluent and multilayered.

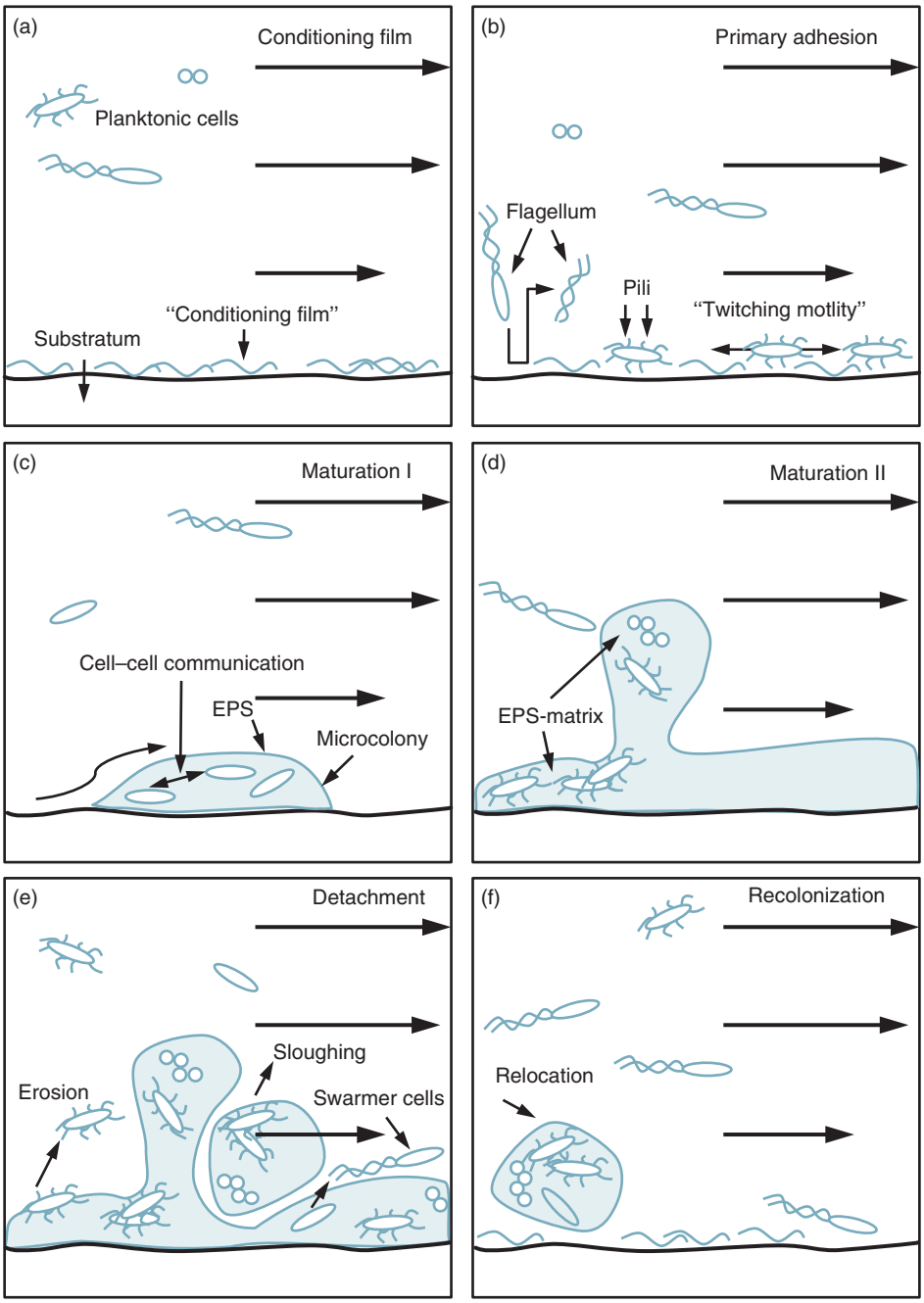

Figure 14.3 **Stages in biofilm development**. (a) Formation of a conditioning film. (b) Primary adhesion, reversible or irreversible, (c) Maturation I: formation of microcolonies, (d) Maturation II, formation of larger, thicker and more confluent biofilms, (e) erosion, sloughing off, dispersion, and (f) relocation and colonization of new surfaces.

This is observed, for example, on the inner surfaces of shower tubings if the rubber material releases biodegradable material. The physico-chemical conditions of the environment strongly influence the development of biofilms. Gene expression changes drastically beginning with primary adhesion through biofilm maturation.

- Stage 5: **Detachment**
  Sooner or later, depending on the mechanical stability of biofilms, the composition of the EPS matrix, shear forces of the water phase and physiological changes of the biofilm population, cell detachment occurs. In most systems, biofilm growth will be limited either by nutrient scarcity or by shear forces and stay in a plateau phase with large fluctuations when parts of the biofilm are detached. Interestingly, detachment can be regulated by signaling molecules, such as fatty acid messengers or nitric oxide.

  Active dispersal from biofilms is typically (but not in all species) preceded by localized death and lysis of cells in the center of mature biofilm structures. Because of the heterogeneous nature of the cells in the mature biofilm,

only a subpopulation of cells will undergo lysis. For some well-studied biofilm systems, the killing a subpopulation of cells is genetically regulated. The killed cells provide nutrients for the bacteria that will become the dispersal cells. The dispersal cells "escape" by coordinated evacuation from break-up points, leading to the characteristic hollowing of biofilm microcolonies that is observed during the dispersal stage for many biofilms. In the dispersal cells, genes that regulate features of the sessile biofilm phenotype, such as exopolysaccharides and fimbriae, are down-regulated, whereas genes that encode factors which are important for a motile lifestyle, including flagella and proteins involved in chemotaxis, are up-regulated.

- Stage 6: **Recolonization**
  Cells or patches of microorganisms and EPS eroded or actively detached from biofilms will become planktonic and can settle on other surfaces, or on preexisting biofilms. They can import new genetic material, degradation pathways and resistance genes upon immigration and, thus, compete with the "local" organisms, where they may not always be welcome.

14.4 Investigation of Biofilms

The investigation of complex natural biofilms is experimentally very demanding and, due to the large number of microorganisms and environmental variables involved, practically impossible to standardize. Thus, a critical question concerns always how well results obtained from the study of a model biofilm organism, such as *Pseudomonas* spp., can be reasonably expanded to other systems. In natural and technical systems, biofilm formation occurs in most cases in the plateau phase in approximate equilibrium with external mechanical stress and internal mechanical stability. The level of the plateau is subject to large fluctuations due to numerous sloughing events.

Biofilms are nothing new. Microbiology as a discipline originated with biofilms when in the seventeenth century Antoni van Leeuwenhoek discovered bacteria in samples scratched from the surface of his teeth – typical biofilms. However, for a long period of time, biofilms were more or less considered literally as a peripheral aspect of microbiology and not as the most common form of microbial life. But already in 1915, the stimulating effect of surfaces on microbial activity was published which was rediscovered decades later. It was J.W. Costerton who first coined 1978 the expression "biofilms" for a particular and important form of life during his investigations on the enhanced resistance of sessile microbial communities, embedded in a cohesive matrix. However, for a long period of time, the arsenal of available analytical methods was not suitable for the investigation of biofilms. The investigation of biofilms was very difficult for most of the history of microbiology because the methods available were not at all suited to investigate complex and often fragile multispecies films of microorganisms at various interfaces.

Since 1980, biofilms slowly shifted into the focus of attention of a major number of researchers. New methods such as **confocal laser scanning microscopy (CLSM)**, **fluorescence *in situ* hybridization** (FISH), a wide variety of specific fluorescent dyes, **atomic force microscopy (AFM)**, microelectrodes, microtiter plates for high-throughput analysis, and spectroscopic methods such as **NMR- and FTIR spectroscopy** as well as other spectroscopic methods were employed for the investigation of biofilms (see Chapters 18 and 19). This resulted in an impressive increase of knowledge. Particularly interesting are combined techniques such as AFM and fluorescence microscopy. Some of the most common systems for biofilm generation and investigations are listed below; they come in a wide variety of versions, adapted to the particular goal of investigation:

- **Microtiterplates**
  Very popular approaches are short-time investigations using 96 well microtiterplates which can be analyzed out by automatic readers for quantification of adhesion, detachment, gene expression and other purposes. However, they only reveal only a few hours or days of biofilm development and responses and, thus, are somewhat limited for simulation of longer term biofilm processes.
- **Coupons**
  Another option is the exposure of material samples in environments such as drinking water, sea water, process water or waste water. They are called **coupons** with the "Robins device" as a pioneering example, using metal samples adjacent to the inner surface of water pipes. Such investigations are frequently used for testing of anti-biofilm coatings or treatments. Analysis is usually performed by direct microscopy or by removal of adhering cells followed by quantification and further measurements if wished.
- **Annular reactors**
  To investigate biofilms under defined shear conditions, annular reactors are employed. Here, a cylinder rotates in a vessel which contains material samples. It is possible to operate such systems for extended periods of time and to subsequent exposure of biocides, disinfectants or cleaning substances as well as to various nutrient conditions. Biofilm analysis occurs upon removal of the materials; direct microscopy as well as any other method for biofilm investigation on a given surface can be applied.
- **Silicon tubes**
  For long-time exposure, biofilm growth on the inner surfaces of silicone tubing is suitable. The silicon material releases plasticizers and other biodegradable monomers, which support biofilm growth. Such biofilms can then be treated with oxidizing and nonoxidizing disinfectants, cleaners and other chemicals in a defined way.

14.5 The Matrix: Extracellular Polymeric Substances

14.5.1 The Extracellular Polymeric Substances (EPS)

Life in biofilms happens in a very complex microenvironment. Perhaps the most significant factor leading to this complexity are the EPS, also well known as **slime**. The EPS matrix, which has been found to be a universal property of all biofilms studied to date, consists of a highly hydrated consortium of biopolymers, such as polysaccharides, proteins, lipids, and even extracellular DNA (eDNA). The composition of the EPS matrix of a given biofilm is highly variable both in space and time. The success and ubiquitous planetary distribution of the biofilm mode of life is largely made possible by the EPS matrix, within which the biofilm cells organize and carry out their livelihoods. Figure 14.4 shows the matrix at different scales – it has to be taken into account, that the matrix is not homogeneous at all but clearly contains microdomains, which are not depicted in this figure.

The matrix represents a reservoir of a wide spectrum of extracellular enzymes, which can be retained as polysaccharide-bound complexes. An example for the interaction between exoenzymes and exopolysaccharides is the complexation of lipase by alginate in *Pseudomonas spp*. Molecular modeling (Figure 14.4d) mirrors that the positively charged amino acids of lipase bind to the negatively charged carboxyl groups of guluronate and mannuronate.

There are many more examples of extracellular enzymes in biofilms; they may be retained by similar mechanisms and, thus, kept in close proximity to the cells and activate the matrix enzymatically. With this matrix,

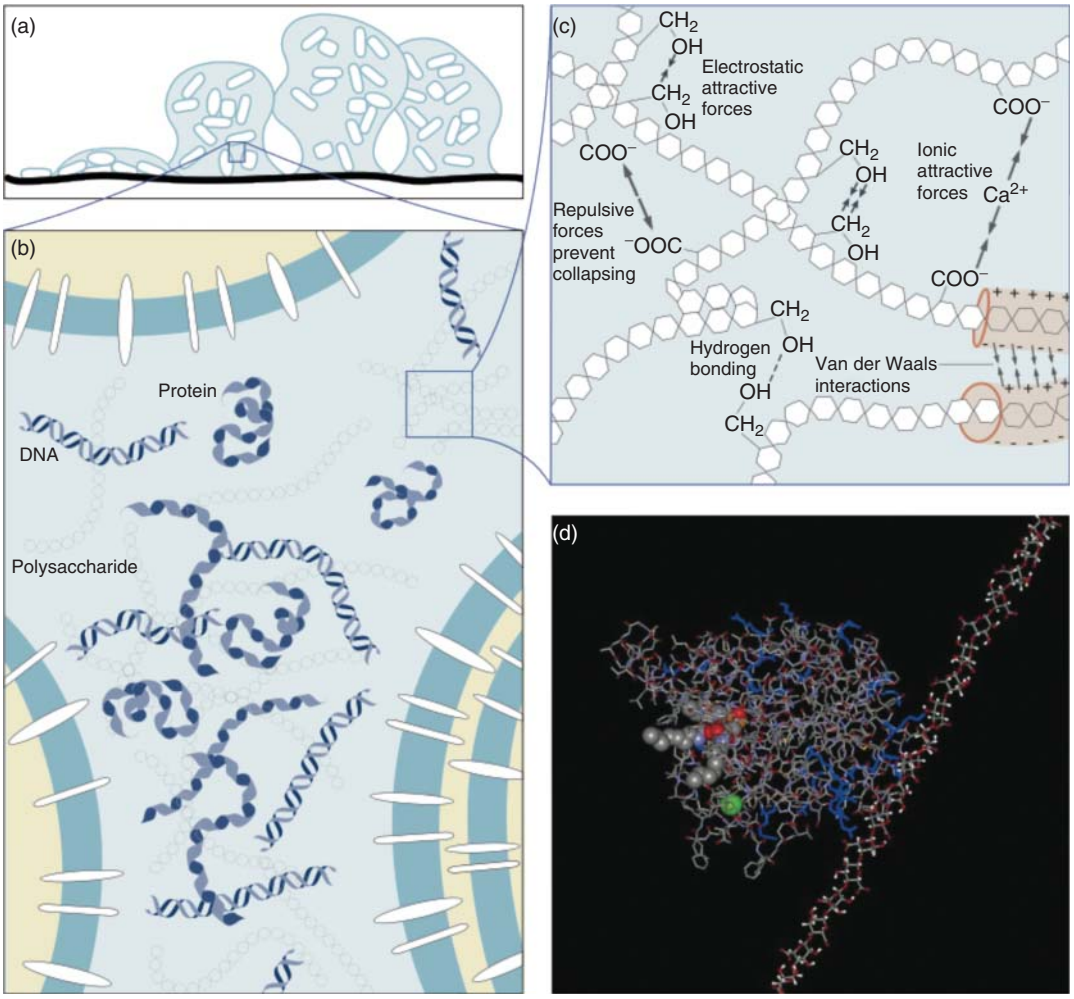

Figure 14.4 The extracellular polymeric substances matrix at different levels of resolution. (a) A model of a bacterial biofilm attached to a solid surface. (b) The major matrix components – polysaccharides, proteins, and DNA – are distributed between the cells in a nonhomogeneous pattern, setting up differences between regions of the matrix. (c) The classes of weak physico-chemical interactions and the entanglement of biopolymers that dominate the stability of the EPS matrix. (d) A molecular modeling simulation of the interaction between the exopolysaccharide alginate (right) and the extracellular enzyme lipase (left) of *Pseudomonas aeruginosa* in aqueous solution. (Reprinted with permission from Flemming and Wingender (2010).)

the biofilm organisms influence their immediate environment and establish an **external digestion system**, which enables them to degrade particles and larger solids.

EPS production is affected by the availability and composition of nutrients. In sequencing batch reactors with sludge growing on synthetic wastewater, the C : N : P ratio influences the hydrophobicity, surface charge, and EPS composition of microbial flocs. Under P-depleted conditions, for example, **uronic acids** and DNA of the EPS increases, but surface charge of microbial flocs decreases. Carbon metabolism may shift toward EPS production when the C : N and/or C : P ratio is enhanced. In successive rotating biofilm compactor (RBC) wastewater treatment stages, the composition of EPS from these biofilms may be different. Along the treatment train, extracellular protein content is highest in biofilms from the first contactor unit, while the amounts of polysaccharides and humic substances in the EPS is highest in the third unit; in addition, the hydrophobic character of EPS increases along the treatment train. The observed differences in EPSEPS composition and hydrophobicity are the result of the nutritional conditions in the system, with cells in the last unit presumably more starved than those of the first unit.

Biofilm cells are sufficiently immobilized in the EPS matrix to allow for **synergistic interactions**, for example, the orchestrated utilization of not readily biodegradable substrates. But they are not completely imprisoned, so that movement within the matrix and detachment are possible options of their life cycles. The matrix is not only a reservoir of exoenzymes but also of genetic material: eDNA is a recently acknowledged EPS component that can sometimes be present in considerable quantities. Consequently, eDNA is utilized by other organisms and **horizontal gene transfer** in biofilms is highly stimulated. Furthermore, it was suggested that viruses in biofilms may be an important carrier of genetic information and that biofilms can serve as a reservoir for viruses. Intercellular communication among resident biofilm cells is equally facilitated and intensified.

The EPS matrix and its components determine the immediate environment and conditions of life for biofilm cells. In Table 14.2, some of the functions are summarized. Electron transport is also suggested within the EPS matrix and studied in detail on the background of microbial fuel cells. Furthermore, the matrix acts as an ultimate recycling yard in which all components of lysed cells can be utilized by the remaining population. The production of EPS might also protect a colonized niche from encroachment by competitors: a recent modeling study predicts that EPS producers in a mixed-species biofilm can smother competitors and use polymer production to push themselves into the more nutrient- and oxygen-rich regions at the air–liquid interface.

14.5.2 Predation and Antagonism

It is worth considering that the matrix does not necessarily represent an advantage for *all* biofilm cells in *all* cases. In fact, biofilms are full of hot spots of antagonism including chemical warfare with antibiotics and biocins and further types of competition of all kind such parasitism and predation. Predation by protozoans and higher organisms is facilitated in most open-system biofilms due to the high concentration of easily biodegradable prey microorganisms. Interestingly, predation does not necessarily lead to the elimination of the food bacteria, but can instead contribute to their survival by entering into commensal or symbiotic predator-prey relations. This has been demonstrated for *Vibrio cholerae* and in detail also for *Legionella pneumophila* (both *Gammaproteobacteria*).

14.5.3 VBNC Forms and Persisters

Biofilm organisms experience limitations in mass transport and have adapted to that condition by formation of pores and channels, to which even parts of the population may contribute by an equivalent to eukaryotic "programmed cell death" in prokaryotes. Considerable portions of the population may find themselves in the wrong place and time and may be dormant due to starvation, leading to "dead zones" within the matrix, although we do not know how dead these zones are. Cells may actually die off but it is also possible that many of them slow down their metabolism to a point of dormancy, becoming VBNC forms ("**viable but non-cultivable**") – then, they are not so dead after all. The dormant organisms may be considered as a reserve population because they are considerably resistant to biocides and antibiotics and may come back to life after antimicrobial treatment, when the more viable and susceptible parts of the community have been eliminated. Extremely resistant subpopulations are known as **persisters**, which have special relevance in disease processes.

14.5.4 Motility within Biofilms

Although motility seems to be hampered in biofilms, it still has been shown to affect the ability of some bacteria to compete, whereas other bacteria seem to use active locomotion to avoid competition. For example, in coculture biofilms, *Pseudomonas aeruginosa* uses motility, among other traits, to blanket *Agrobacterium tumefaciens* (*Alphaproteobacteria*). Interestingly, during the initial stages of colonization in the presence of *P. aeruginosa*, a nonmotile *A. tumefaciens* mutant accumulated greater adherent biomass than the motile wild-type strain, possibly indicating that wild-type *A. tumefaciens* actively evades contact with *P. aeruginosa*, thereby reducing the extent of

Table 14.2 Functions of extracellular polymeric substances (EPS).

Function	Relevance for biofilm organism	EPS components involved
Adhesion	Initial steps in colonization of abiotic and biotic surfaces by planktonic cells, long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, amphiphilic molecules, DNA
Aggregation of bacterial cells	Bridging between cells, (temporary) immobilization of bacterial populations, basis for development of high cell densities, cell–cell recognition	Polysaccharides, proteins, DNA
Cohesion of biofilms	Structural elements forming a hydrated polymer network (biofilm matrix), mediation of mechanical stability of biofilms (frequently in conjunction with multivalent cations), determination of EPS structure (capsule, slime, sheath) and biofilm architecture, generation of matrix allowing cell–cell communication	Neutral and charged polysaccharides, proteins (e.g., amyloids, lectins), DNA
Retention of water	Maintenance of highly hydrated microenvironment around biofilm organisms, desiccation tolerance in water-deficient environments	Hydrophilic polysaccharides, Hydrophilic proteins
Protective barrier	Resistance to nonspecific and specific host defences during infection, tolerance to various antimicrobial agents (e.g., disinfectants, antibiotics), protection of cyanobacterial nitrogenase from harmful effects of oxygen; protection against some grazers	Polysaccharides, proteins
Sorption of organic compounds	Accumulation of nutrients from the environment, sorption of xenobiotics (detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotion of polysaccharide gel formation, ion exchange, mineral formation, accumulation of toxic metal ions (detoxification)	Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Digestion of exogenous macromolecules for nutrient acquisition, degradation of structural EPS allowing release of cells from biofilms	Proteins
Nutrient source	Source of C, N, and P compounds for utilization by biofilm community	Potentially all EPS components
Genetic information	Horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Redox activity in biofilm matrix, electron transport mediation to surfaces	Proteins (e.g., pili, nanowires?), humics
Export of cell components	Release of cellular material as a result of metabolic turnover	Membrane vesicles (nucleic acids, enzymes, lipopolysaccharides (LPS), phospholipids)

biofilm growth. Wild-type *P. aeruginosa* also uses motility to outcompete its own nonmotile variants for more suitable regions in biofilms. Motile *P. aeruginosa* migrates to the top of nonmotile microcolonies, forming the caps of tall, mushroom-like structures; motile cells thus access the more oxygenated and nutrient-rich regions of the culture.

14.5.5 Matrix Stability

The mechanical stability of the EPS matrix is not based on covalent bonds between the EPS components but rather on a multitude of weak physico-chemical forces such as hydrogen bonds, weak electrostatic and ionic interactions, van der Waals interactions and the **entanglement** of the long molecules. Biofilms behave like elastic bodies until a threshold pressure has been reached, after which they suddenly convert into highly viscous liquids. This observation has led to the concept of "**fluctuating binding points**" whereby the same partners interact during the viscoelastic phase. Beyond the compression threshold interactions happen with new binding partners, causing liquidity. When this point is exceeded, we slip on biofilm-coated rocks while trying to walk through a river.

It is acknowledged that the measurements of mechanical properties are challenging. Biofilms are very thin and are inherently attached to a surface. Measured parameters such as the elastic and shear modulus, adhesive strength or tensile strength are sparse but are now, even if sparsely, appearing in the literature. There is a large range of reported values for these properties, although there is general agreement that biofilms are viscoelastic. Biofilm

stability has been assessed with various experimental methods depending on the desired characteristic and available equipment. These challenges and lack of standard methods or equipment for testing attached biofilms has led to the development of many creative methods to tease out aspects of biofilm mechanical properties. In stagnant waters, biofilms can be disrupted by low shear forces while at impact points of waterfalls extremely stable biofilms with rubber-like appearance exist which serve as holdfasts for *Podostemaceae* (order *Malpighiales* of the *Rosidae* flowering plants). High shear forces may improve matrix cohesion when all those EPS components are washed out which cannot withstand the shear, leaving the biofilm with those that resist – this inevitably results in stronger matrix cohesion. Interestingly, bacterial microcolonies have been observed rolling under steady shear along surfaces without being detached. This would fit into the model of "fluctuating binding points" as mentioned above.

14.5.6 Gradients and Heterogeneity in the Biofilm Matrix

A prominent feature of biofilms is their heterogeneity. Cells growing in biofilms are not only physiologically distinct from planktonic cells, but also vary from each other, both spatially and temporally, as biofilm development proceeds. Mature biofilms contain concentration gradients of metabolic substrates and products. Oxygen is the best studied and most familiar example, and oxygen concentration profiles in biofilms are routinely measured using microelectrodes. The microscale gradients of oxygen will vary depending on the type of biofilm and the sources and sinks for the oxygen. For example, **oxygenic photosynthetic biofilms**, such as those found in microbial mat communities, establish **oxygen gradients** in diurnal cycles. Other biofilms can be completely anaerobic or contain predominantly anaerobic organisms, such as the biofilms that are associated with dental plaque. It should be emphasized that the failure of oxygen to penetrate throughout a biofilm is not a result of physical exclusion. The biofilm matrix is an aqueous solution, and solutes that are the size of oxygen diffuse in the matrix at a rate that is at least 60% or more of the diffusion rate in pure water. Oxygen fails to penetrate because it is actively respired by cells in the upper layers of the biofilm; this occurs in both mixed-species and single-species biofilms. The dynamic balance between consumption and diffusion determines the local concentration of oxygen. In one example, measurements of oxygen concentrations within and around a mixed-species heterotrophic biofilm showed that the oxygen concentration at the biofilm–fluid interface was approximately 40% of the value in well-mixed fluid. The oxygen concentration in the biofilm continued to decrease with increasing depth, and was depleted completely at a depth of approximately 175 µm into the 220-micron-thick biofilm. The concentration of any nutrient that is consumed in the biofilm will also decrease with depth into the biofilm and distance from the nutrient source.

The distribution of any solute is governed by its simultaneous production, consumption, and diffusion. Therefore, other electron acceptors, electron donors, cofactors, signaling molecules, metabolic products and antimicrobials will also establish concentration gradients that are dependent on their sources, sinks, and mobility by diffusion. As expected, the local rates of production and consumption of a solute will depend on the **microscale spatial organization** of the microorganisms that metabolize that solute. Microscale concentration profiles have been determined experimentally for a number of solutes, including nitrate, nitrite, ammonium, hydrogen sulfide, carbon dioxide, methane, hydrogen peroxide, chlorine, and chlorine dioxide; microscale pH profiles have also been determined. The measured distributions of the chemical species are generally consistent with a reaction–diffusion theory. Convective mass transport is not observed in biofilms.

14.6 Communication in Biofilms

As indicated earlier, a fascinating aspect concerning the multicellular behavior of biofilm cells is the fact that the cells can communicate among each other. Biofilms are of particular interest from an evolutionary perspective because the close proximity of individuals in a biofilm can make cooperation and communication particularly important. Microorganisms communicate and cooperate to perform a wide range of multicellular behaviors, such as dispersal, nutrient acquisition, biofilm formation and quorum sensing. Microbiologists are rapidly gaining a greater understanding of the molecular mechanisms involved in these behaviors, and their underlying genetic regulation. Such behaviors are also interesting from the perspective of social evolution – why do microorganisms engage in these behaviors given that cooperative individuals can be exploited by selfish cheaters, who gain the benefit of cooperation without paying their share of the cost? There is great potential for interdisciplinary research in this fledgling field of "**sociomicrobiology**".

A first indication for cell–cell communication in biofilms was found by the phenomenon of **quorum sensing** in Gram-negative bacteria, performed by **acyl-homoserine lactones** (AHL's) which soon was found to be part of an expanded microbial communication system which is termed **quorum sensing**. In its simplest form, this process results from the production and accumulation of signaling molecules in the surrounding environment. The signaling molecules, also referred to as **autoinducers**, bind to receptors on, or in, the bacterial cell, which leads to changes in gene expression at some threshold concentration. Quorum sensing is generally thought to act as a mechanism for the coordinated regulation of behavior at

the level of populations of cells – one view among microbiologists is that quorum sensing simply evolved because it allows bacteria to coordinate the behavior of the group and take on some of the characteristics of multicellular organisms. These chemical signals might also have a role in conflicts, both within and between species. *P. aeruginosa* possesses two separate quorum sensing systems. One is the LasR/I-system with the auto inductor N-butyryl-homoserine lactone (BHL) (see also Section 15.1) and the RhIR/I-system with N-oxododecanoyl-homoserine lactone (OdDHL) which control the expression of numerous genes. These AHLs regulate the formation of components of the secretion systems for exoenzymes such as proteases and lipases, as well as the production of other exo-products such as **rhamnolipid** and **exotoxin A**. Detachment from biofilms seems also to be influenced by AHL's, allowing for the release of cells from the matrix. An interesting aspect is the enzymatic degradation of AHL's in the EPS matrix, which may be one of the "weapons" used in competition of biofilm organisms.

Cyclic di-guanosin monophosphate (c-di-GMP) (Figure 14.5) as a secondary messenger stimulates the biosynthesis of adhesins and EPS matrix substances in biofilms and inhibits various forms of motility, essentially controlling the switching between the motile planktonic and sedentary biofilm-associated "lifestyles" of bacteria (Figure 14.6). Environmental signals trigger the synthesis and degradation by sensory which control the c-di-GMP concentration in the cell. At high concentrations, the cell becomes sessile, at low concentrations, it prefers the planktonic style of life (Figure 14.5).

However, recent research in environmental microbiology reveals that the role of quorum sensing in biofilm formation might have been overestimated and that AHL's may be not a global player in biofilm formation. Instead, biofilm development is a multifactorial process involving an interdependence between the growth environment and genetic regulation in which AHL's play a role among other factors.

14.7 Enhanced Resistance of Biofilm Organisms

Biofilms are known to exert enhanced resistance to biocides. There are different mechanisms of resistance, depending upon the biocide, the biofilm and the environmental conditions. Extreme cases have been reported such as biofilm growth in copper pipes of a central disinfection system in a hospital. In a case history, it was shown that the continuous contamination of drinking water by coliform bacteria was caused by a biofilm growing on synthetic rubber, which was employed to optimize the fitting of valves. Biodegradable additives of the rubber provided suitable nutrient conditions. This biofilm survived series of shock chlorination

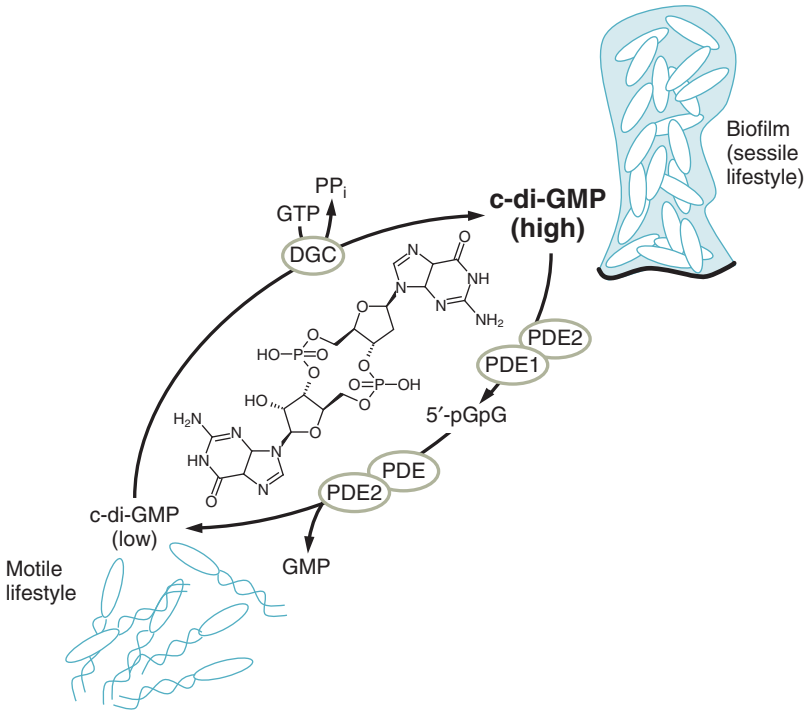

Figure 14.5 Control of biofilm formation by c-di-GMP. Concentration of c-di-GMP is regulated by DGC (Diguanylate cyclase; GGDEF domain), PDE 1 (Phosphodiesterase; EAL domain, linearizes c-di-GMP to 5′-pGpG), PDE (nonspecific cellular PDEs, further degrading 5′-pGpG to GMP), and PDE 2 (Phosphodiesterase; HD-GYP domain, metal dependent, unrelated to the EAL domain, linearizes c-di-GMP to 5′-pGpG, degrades 5′-pGpG further to GMP). pGpG – 5′-phosphoguanylyl(3′,5′)guanosine; GMP – Guanosine-5-phosphate. (Courtesy of D. Dobritzsch.)

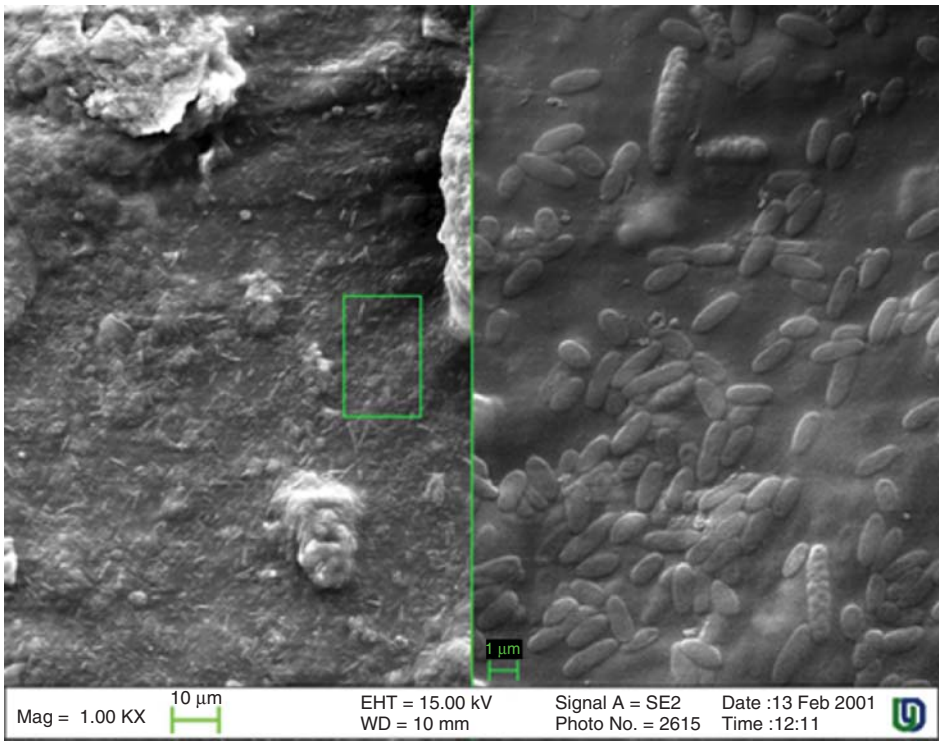

Figure 14.6 **Scanning electron micrograph of a biofilm on synthetic rubber in a drinking water distribution system after repeated shock chlorination**. The size of the bacteria exceeds 2 µm. (Courtesy of Dr. Gabriela Schaule.)

with over 50 mg l^{-1} free chlorine. Figure 14.6 shows a scanning electron micrograph of this biofilm. Normally, the size of bacteria in oligotrophic drinking water is small, mostly way below 1 µm. In this case, their size exceeded 2 µm, indicating good the nutrient conditions.

In many practical situations, biofilms are difficult to eradicate and prove recalcitrant to the application of **biocides**. Biofilms are reportedly 10–1000 times less susceptible toward a wide variety of different antimicrobial agents than are the equivalent planktonic cells. This phenomenon is thought to result from various resistance mechanisms that are associated with the biofilm mode of growth. In many cases, however, the mechanisms of biofilm resistance are still unclear and are only beginning to be elucidated. In the context of this chapter, resistance is defined as the ability of a microorganism to grow in the presence of elevated levels of an antimicrobial substance or to survive the treatment with an antimicrobial substance. Under practical conditions, elevated levels of a biocide are those which are above the concentrations usually used for the killing or control of microbial contamination in a specific application. It must be pointed out that most biofilm susceptibility studies in the laboratory and in practical situations were performed on already established biofilms, analyzing the survival and persistence of biofilm cells, while the efficacy of biocides on biofilm growth is rarely considered. From such studies, it is now widely appreciated that mature biofilms generally exhibit an increased resistance to killing by biocides. On the other hand, biocides may prevent or at least delay and control the colonization and biofilm formation on surfaces.

Two major types of microbial resistance can be distinguished: intrinsic and acquired resistance. **Intrinsic (innate) resistance** refers to a natural chromosomally controlled property, including physiological adaptation that is specific for a certain type of microorganism. **Acquired resistance** may be due to mutations with subsequent selection of resistant mutants from the population, which has been exposed to the biocide, or it may result from the uptake of plasmids or transposons which confer resistance to biocides. Formation of a biofilm can be regarded as a physiological (phenotypic) adaptation, and thus represents an intrinsic mechanism of microbial resistance to biocides. At present, it is not known if acquired resistance is of importance in biofilm resistance. It can be speculated that the high cell densities in biofilms may enhance the probability of spontaneously resistant mutants to be selected on exposure to sub lethal concentrations of biocides; in addition, high cell numbers may promote horizontal transfer of genes expressing resistance to biocides. **Quorum sensing** has been described as a mechanism leading to enhanced antibiotic tolerance of *P. aeruginosa*.

Evidence in the literature suggests that biofilm populations are protected from toxic metals by the combined action of chemical, physical, and physiological phenomena that are, in some instance, linked to phenotypic variation among the constituent biofilm cells. A multifactorial model was proposed by which biofilm populations can withstand

metal toxicity by a process of cellular diversification. Many different mechanisms of biofilm resistance are discussed in the literature, reflecting the different ways of biofilm organisms to withstand biocides. These mechanisms include physical and chemical diffusion-reaction barriers in the biofilm restricting biocide penetration of the biofilm, slow growth rate of biofilm cells due to nutrient limitation, activation of general stress response genes, the emergence of a biofilm-specific phenotype, and the presence of persister cells.

14.8 Emergent Properties of the Biofilm Mode of Life

When individual components in an environment come together and create distinct, collective and interactive properties and functions, the results are called *emergent properties*. As such, emergent properties are at the intrinsic core of the entire science of systems biology. This definition clearly applies for biofilms. Among the emergent properties of biofilms which are not achieved by individual members of suspended populations are:

- Formation of the **EPS matrix**, functional architecture and mechanical stability of biofilms
- Development of **gradients** of pH value, oxygen concentration, redox potential, substrate and product concentrations
- High variation of **different habitats** at small scale, supporting high biodiversity
- Biofilm cells are partially or wholly immobilized and, therefore, can form **synergistic microconsortia**
- **Exoenzymes** are retained in the matrix and represent an external digestion system
- **Sorption of dissolved and particulate substances** from water phase, providing possible nutrients
- Facilitated **gene transfer**
- Intensified levels of **intercellular communication**
- Enhanced **resistance to biocides** and other forms of stress

Indeed, similarities between biofilm bacteria and multicellular organisms can be perceived. For instance, bacteria (including planktonic bacteria) can sense their surroundings, and this enables them to adjust their metabolic processes to maximize the use of available substrates and to protect themselves from detrimental conditions. When bacteria are growing within a biofilm, changes in gene expression result in phenotypic heterogeneity within the biofilm, which can be interpreted as specialization or division of labor similar to cellular differentiation in multicellular organisms. In addition, bacteria secrete signal compounds, which influence gene expression and are the means by which cells communicate with one another. Thus, biofilms can be considered as interactive communities. There is even a growing body of evidence that bacteria exhibit altruistic behavior and can undergo a process similar to programmed cell death, again suggestive of multicellularity. However, there are also fundamental distinctions between bacteria and multicellular organisms. For example, while bacterial cells can react and adapt to their environmental surroundings, they do not permanently differentiate. However, recent advances in stem cell research indicate that even fully differentiated cells of multicellular organisms can be de-differentiated under the right conditions. So maybe there is no such thing as permanent differentiation after all.

Coordinated **multicellular behavior** can be observed in a variety of situations, including the development of *Escherichia coli* and *Bacillus subtilis* colonies, swarming by *Proteus* and *Serratia*, and spatially organized interspecific metabolic cooperation in anaerobic bioreactor granules (The genus *Bacillus* belongs to the *Firmicutes*, the other bacteria to the *Enterobacteriaceae* of the *Gammaproteobacteria*). The organisms benefit from multicellular cooperation by using cellular division of labor, accessing resources that cannot effectively be utilized by single cells, collectively defending against antagonists, and optimising population survival by differentiating into distinct cell types. The EPS matrix provides an **organized space** for these processes, which allows the organisms to control their immediate environment. Perhaps inevitably, anthropomorphic terms have been used to describe the biofilm mode of life, for example, "microbial landscapes," "city of microbes," and the EPS matrix as a "house of biofilm cells." All these analogies indicate how strongly the interactions in biofilms suggest complex and regulated processes. For microbial mats, the most appropriate metaphor may be biofilms as "microbial rainforests" due to their extremely complex biome of both structure and species diversity, with sunlight and water as the major limiting factors.

As a whole, biofilms are *the* success model of life in nature. They have spread not only over the entire crust of our planet, but also into its depths even in places where they were separated from any outside conditions for millennia. Literally, Earth is colonized by biofilms and microbial life is clearly not endangered and is well fit for any climate change. And if there is life on Mars, it is more than probable that it exists in the subsurface, for example, in permafrost or subsurface mineral structures, and, thus, associated with surfaces in the form of biofilms.

Biofilms seem to be a universal mode of life.

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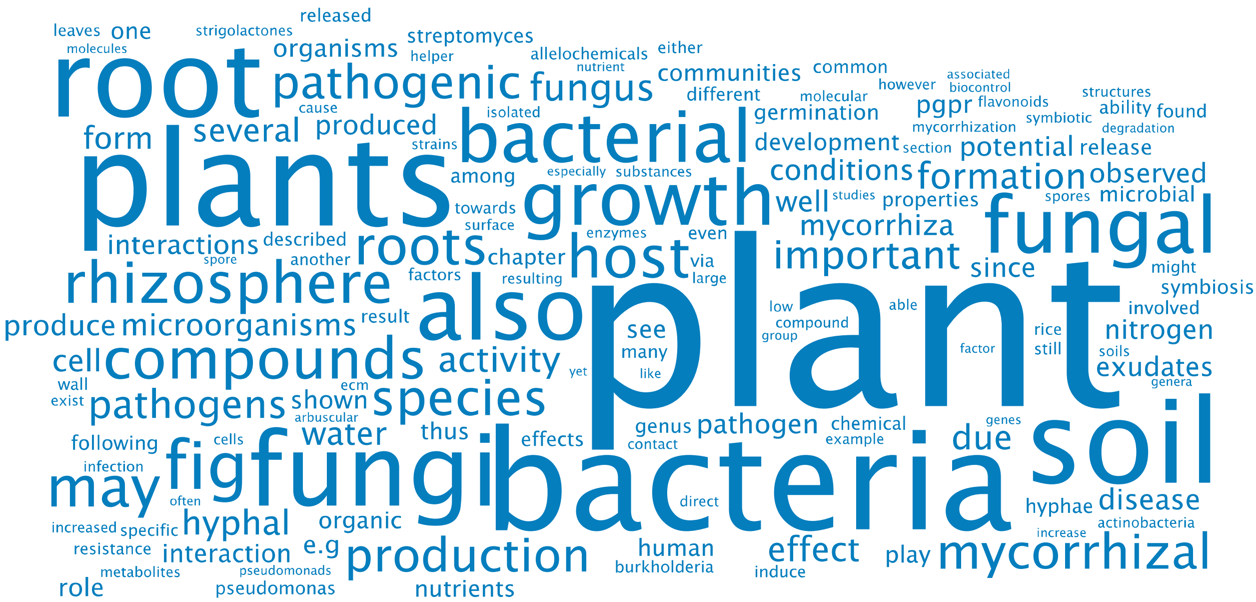

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15
Rhizosphere Interactions

Silvia D. Schrey, Anton Hartmann, and Rüdiger Hampp

Overview

Roots are important not only for water and nutrient supply of the plant, but also to release a wide range of carbon compounds of low molecular weight, such as sugars, amino, and organic acids. These can amount to between 10% and 20% of total net fixed carbon but vary based on species, nitrogen availability, and plant age. In addition, most land plants form symbioses with soil fungi, which in addition cause a considerable drain of photoassimilates. Direct (plant exudates) or indirect (via symbiotic fungi) rhizodeposition of carbon forms the basis for an environment rich in diversified microbiological populations. This was first suggested by Hiltner in 1904.

The rhizosphere is now defined as a narrow zone of soil, which is influenced by living roots. It forms a boundary layer between the root and the bulk soil. Here large fluxes of solutes and water, as well as compounds contained in the gas phase, exist. Consequently, physical soil properties can vary considerably. Depending on the demands of the plant, changes in the soil water potential can be high during the day/night cycle. In comparison to bulk soil, the soil water potential can become strongly negative during the day at high transpiration rates and less negative at night because of vertical redistribution by the root system (hydraulic lift). Special conditions also exist with regard to O_2 and pH. Following high rates of respiration by both roots and microorganisms, O_2 tension can be very low especially in wet soil where water limits diffusion rates. Uptake of solutes is often accompanied by the release of protons and organic acids, which affects the pH at the root surface.

Microorganisms of the rhizosphere establish a functional diversity that includes the decomposition of organic matter, nitrogen fixation, conversion of inorganic forms of nitrogen, solubilization of phosphate, transformation of sulfur and iron, production of siderophores (iron-binding compounds), release of plant(phyto)hormones, as well as of compounds, which are used for biotic control.

It is obvious that bacteria are an important part of the microorganisms inhabiting this ecological niche. In comparison to bulk soil, the abundance of rhizosphere bacteria is several magnitudes higher ($10^{10}-10^{12}$ microbes per gram soil versus $<10^8$ in bulk soil), but still about 100 times lower than under culture conditions. Bacteria can solubilize nutrients from the mineral soil layer, but will also sequester them. Consumption of bacteria by soil protozoa and nematodes will then liberate nutrients, which in due course will become available for plants.

Fungi form another important part of the rhizosphere. Most terrestrial plants develop symbiotic structures (mycorrhiza) with soil-borne fungi, creating another sphere, the mycorrhizosphere. In these interactions, the fungal partner provides the plant with improved access to water and soil nutrients because of more or less complex hyphal structures, which emanate from the root surface and extend far into the soil. The plant, in return, supplies carbohydrates for fungal growth and maintenance. Because of leakage and the turnover of mycorrhizal structures, these are another source for solutes released into the soil where they can be accessed by other microorganisms.

In the following interactions of bacteria, fungi and plants, and, finally those of plants with each other are addressed. With regard to soil bacteria, a wide range of bacterial activities exist such as the "good" ones (plant growth promotion, plant disease suppression, nitrogen fixation) and the "bad" ones (plant pathogens), as well as bioactive compounds of bacterial secondary metabolism, which cause the respective effects. Plant-associated bacteria act as opportunistic human pathogens. Fungi form another focus, here especially the bacterial influence on symbiotic and plant pathogenic fungi. Finally, direct (parasitic plants, plant competition) and indirect (by the help of fungi) interactions of plants themselves are described.

The examples introduced below show that the contemporary knowledge about organisms and interactions in the rhizosphere has increased recently, mainly because of the many attempts to improve plant growth and fitness.

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

The intense use of metagenomics with soil samples continuously reveals the enormous diversity of microorganisms living and thriving on plant-derived exudates. Interestingly, most of these organisms are not pathogenic in nature. This is understandable because they depend on the continuous delivery of organic compounds by the plant. This could also explain why so many rhizosphere bacteria produce toxins that mainly affect plant pathogenic microorganisms or release compounds that are plant-beneficial.

An investigation of the chemical interactions is, however, extremely limited owing to the lack of suitable experimental procedures and of set-ups that represent realistic conditions. Experimentally sound studies can most easily be performed under sterile conditions using cocultures of the respective organisms or exuded compounds. Three partite systems (host plant + two microorganisms (e.g., pathogen + antagonist)) become already very difficult to handle, and the step to field studies is enormous. Many other organisms now interfere, metabolically modifying the "identified bioactive compounds" to an unknown extend. Further, owing to different binding properties of soil particles and other soil chemical factors, the fate of any such compound remains unclear. Thus, there is still a long way to go to broaden our understanding of the processes and molecules involved in rhizosphere interactions.

15.1 Bacterial Communities in the Rhizosphere

Characterization of rhizosphere bacteria was for a long time possible only for culturable microorganisms. With the advance of sampling and molecular techniques, the ecology of rhizosphere microorganisms becomes accessible and several overviews on rhizosphere microbes of important crop plants based on 16S rDNA and 16S rRNA have been produced in recent years (Table 15.1). The structure of soil microbial communities varies largely in response to the plant cover, the soil type, and the history of the soil (arable land under rotation, monoculture, or permanent grassland).

Root exudates are an important factor for establishing microbial communities in the rhizosphere, however, exudation along roots is not homogeneous. Abundance and turnover of rhizobacteria are further regulated by microfaunal grazers such as protozoa. Consequently, beneficial effects of protozoa on plant growth have been related to nutrients released from consumed bacterial biomass (Figure 15.1). Higher spatial resolution shows that bacteria accumulate in certain areas, indicating a possibly higher exudation activity. The zone of root elongation is especially effective in attracting a large diversity of microbes. Fluorescent pseudomonads, common bacterial inhabitants of rhizospheres, preferably colonize the root base and to a lesser extent the younger root areas. Further attractive sites are the penetration points of lateral roots and around the growing root tip with the mucilage forming root cap, which offers easy access to nutrients.

The ability of rhizosphere bacteria to consume specific root exudates determines the bacterial colonization pattern of roots. By altering root exudate composition or by the production of specific exudates, plants can steer the microbial rhizosphere population. This might explain the similarity of the community structures of members of one plant species growing in different soils and of different plants in the same soil harboring very different bacterial communities. When under attack, plants may alter their root exudates composition in a way that attracts beneficial microbes. Thus, the two factors, soil type and plant species, have a major effect on rhizosphere-associated microbial communities.

Comparable to bacterial colonization of root surfaces, leaves, which are the aboveground plant surfaces termed **phyllosphere**, are commonly colonized by a diverse community of bacteria (Figure 15.2). Bacterial aggregates form at the depressions between adjunct epidermal cells, along veins and at the bases of trichomes (hairs). Usually, these bacterial aggregates form biofilms and are embedded in an EPS matrix (extracellular polymeric substances, see Section 14.5.1). The EPS might be helpful to maintain a hydrated surface because life in the phyllosphere requires adaptation to abiotic stresses. Few bacterial phyla predominate in the phyllosphere of different plants; plant factors are involved in shaping these phyllosphere communities. *Proteobacteria*, specifically *Alpha-* and *Gammaproteobacteria,* predominate the phyllosphere of distinct plant species. However, in contrast to observations of active plant recruitment of specific bacterial communities for rhizosphere colonization, such an effect has yet to be shown for the phyllosphere.

15.1.1 Plant Growth Promoting Rhizobacteria

Soil samples can contain a high proportion of plant growth promoting microorganisms, which may amount up to two-thirds of those that can be cultivated. Plant growth promotion by microbes can be direct by improved nutrient availability ("**biofertilizers**") and hormonal stimulation and increased resistance against pathogens but also indirect by controlling the growth of plant pathogenic organisms ("**biopesticides**" (Figure 15.3)).

Plant growth promoting rhizobacteria (PGPR) are usually in contact with the root surface as well as the

Table 15.1 Microbial community in the rhizosphere of agriculturally important plant species.

Plant species	Rhizosphere-dominant species
Beta vulgaris (*Caryophyllales*, core eudicotyledons)	*Proteobacteria*, *Bacteroidetes*/*Chlorobi* group
Brassica napus cv. Licosmos (*Brassicales*, *Rosidae*)	*Actinobacteria*, *Proteobacteria* (α, γ), *Firmicutes*
Brassica napus cv. Westar (*Brassicales*, *Rosidae*)	*Proteobacteria* (α, β, γ), *Bacteroidetes*/*Chlorobi* group
Dendranthema grandiflora cv. Majoor Bosshardt (*Asterales*, *Asteridae*)	*Firmicutes* (*Bacillus*), *Proteobacteria*: α (*Acetobacter*, *Azosporillum*), β (*Comamonas*, *Ralstonia*, Variovarox), γ (*Pseudomonas*),
Fragaria ananassa (*Rosales*, *Rosidae*)	α-*Proteobacteria*, *Actinobacteria*
Hordeum vulgare cv. Pastoral (*Poales*, *Liliopsida*)	*Firmicutes* (*Bacillus*), *Proteobacteria*: β (*Burkholderia*), γ (*Acinetobacter*, *Pantoea agglomerans*, *Pseudomonas*),
Lolium perenne cv. Bastion (*Poales*, *Liliopsida*)	*Firmicutes*, *Proteobacteria*: α, γ (*Pseudomonas*)
Medicago sativa (*Fabales*, *Rosidae*)	*Proteobacteria*: α, γ, *Firmicutes Bacteroidetes*/*Chlorobi* group
Persea americana (*Laurales*, *Magnoliidae*)	*Proteobacteria* γ (*Pseudomonas*), δ (*Polyangium*)
Phaseolus vulgaris (*Fabales*, *Rosidae*)	γ-*Proteobacteria*, *Bacteroidetes*/*Chlorobi* group
Pinus contorta (*Coniferales*, *Coniferophyta*)	*Proteobacteria*: α, β, γ, *Acidobacteria* (*Acidobacterium*)
Solanum tuberosum (*Solanales*, *Asteridae*)	*Proteobacteria*: α, γ, *Firmicutes* (*Bacillus megaterium*)
Trifolium pratense (*Fabales*, *Rosidae*)	*Proteobacteria*: α, γ
Zea mays (*Poales*, *Liliopsida*)	*Proteobacteria*: α (*Rhizobia*), β (*Burkholderia*), γ, *Bacteroidetes*/*Chlorobi* group

From Hawkes, DeAngelis, and Firestone (2007), modified and taxonomic information added.

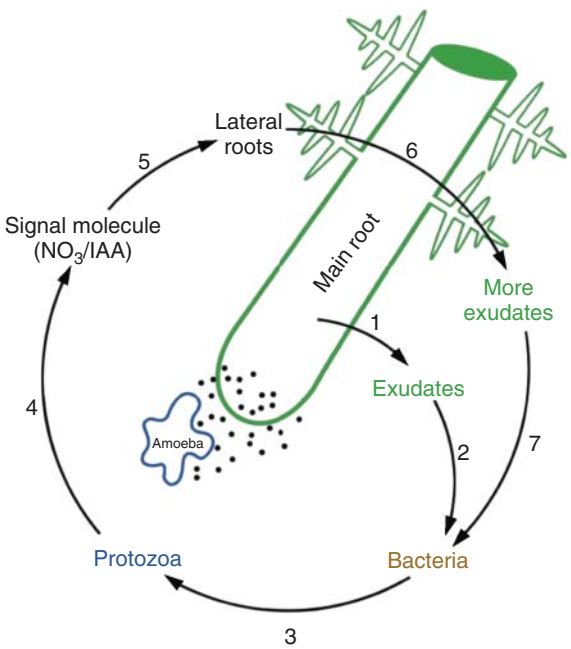


Figure 15.1 Microbial loop in soil: bacteria accumulate at sites of prolific root exudation (1,2). Protozoa feed on those bacteria (3), in return releasing nutrients and signal molecules (4). Lateral root formation is increased resulting from signaling molecules (5), resulting in even more root exudates (6) and thus an increase in the amount of bacteria. (Modified from Bonkowski (2004).)

hyphal cell walls of symbiotic fungi. PGPR may support plant growth by the mobilization of inorganic nutrients, by nitrogen fixation, or by the production or degradation of phytohormones including auxins, cytokinins, or gibberellines. Volatile substances produced by bacteria, such as acetoine or 2,3-butanediol (see Section S1.3.3.5), can also stimulate plant growth substantially (Figure 15.4). Some root-associated bacteria, among them pseudomonads and enterobacteria (*Gammaproteobacteria*), are able to stimulate plant growth by reducing inhibitory levels of ethylene in the rhizosphere through the hydrolysis of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. These root-associated or endophytic bacteria have a good potential for practical applications. In soils with low phosphate availability, bacteria release phosphate from minerals and organic phosphate sources. Although many P-solubilizing bacteria have been characterized, their relative importance in the PGPR effect is uncertain. However, if the phosphate ions are released in an area rich in mycorrhizal fungal hyphae, the hyphae may transport phosphate to the plants and the PGPR effect is detectable, emphasizing the importance of interaction in the rhizosphere between more than two partners.

Azospirillum strains (*Alphaprotobacteria*) are known mainly for their ability to fix atmospheric nitrogen. The

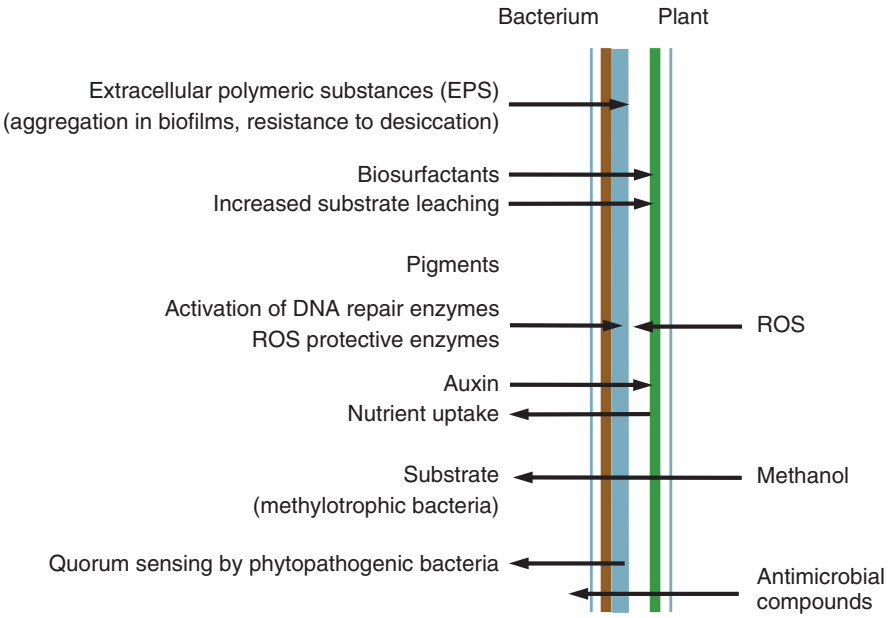


Figure 15.2 Phyllosphere: Plant–bacteria interactions. (Graphics: D. Dobritzsch, G.-J. Krauss.)

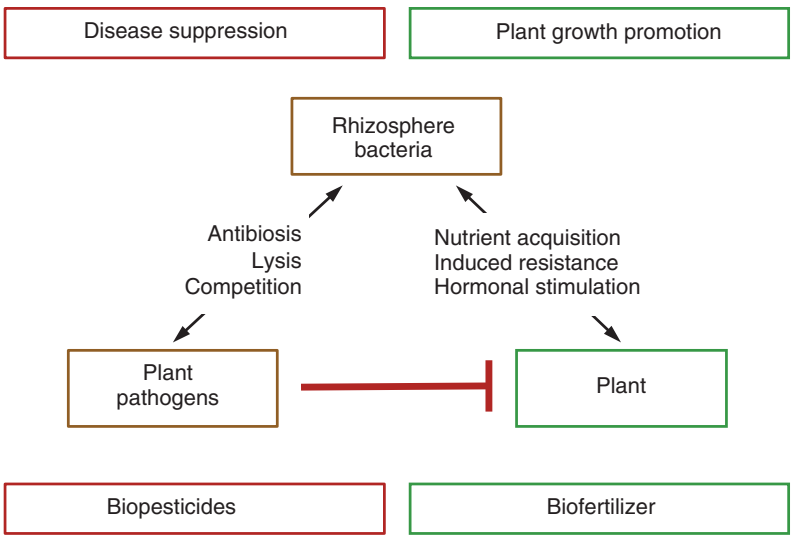


Figure 15.3 Plant growth promoting rhizobacteria can be separated into biopesticides that suppress diseases by antagonism of pathogenic microorganisms and biofertilizers that promote plant growth by exerting a direct effect toward the plant. (Modified from Berg (2009).)

2,3-butanediol Acetoine (3-hydroxybutanone)

Figure 15.4 Plant growth stimulating volatiles.

inoculation of roots with *Azospirillum*, however, often promotes plant growth not primarily through nitrogen fixation, but also because of the ability of the bacteria to produce phytohormones that stimulate root development, increase the volume of explored soil space and eventually improve nutrient uptake efficiency. The auxin-type phytohormones produced by *Azospirillum* induce root branching and thus improve plant nutrient uptake from the soil, an example for a possible trade-off of altered resource allocation. However, it has to be kept in mind that PGPR functioning can rarely be reduced to a singular bacterial trait but to a combination of many mechanisms, either by one or several microorganisms acting in concert.

Inoculation of plants with some PGPR elicits a phenomenon known as **induced systemic resistance** (ISR), Figure 15.5). ISR allows the plants to endure pathogen attacks that, without bacterial preinoculation, could be

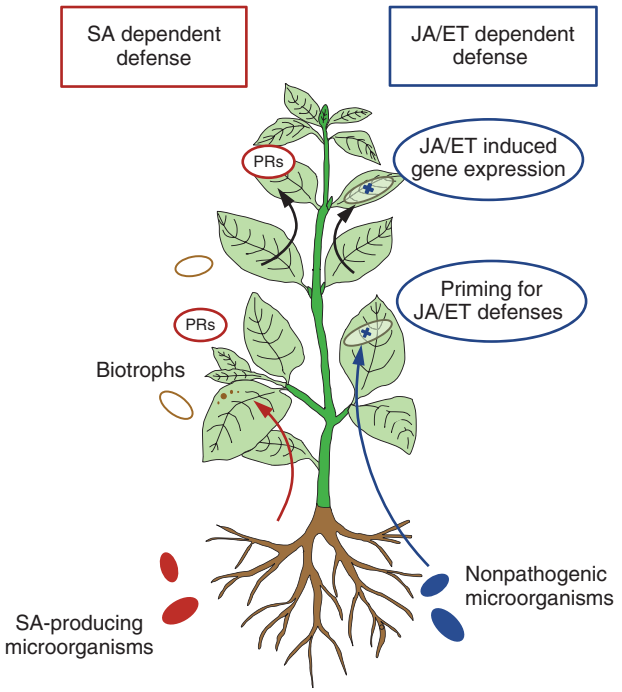


Figure 15.5 Illustration of the induction of systemic resistance in plants. Left: salicylic acid-dependent defense responses develop following infection with certain biotrophic pathogens or SA-producing soil microbes in uninfected (distal) tissues and are characterized by expression of pathogenesis-related (PR) proteins. Right: Jasmonic acid/ethylene (JA/ET)-dependent defenses can be initiated by root-associated soil bacteria and become evident following aboveground pathogen infection by the expression of JA/ET-dependent genes. JA/ET-dependent gene expression is also relevant following infection with certain necrotrophic pathogens. Both types of induced resistance are effective against a broad range of plant pathogens. (Adapted from Pieterse *et al.* (2009).)

lethal. The effect is systemic, for example, root inoculation with the biocontrol PGPR yields distal plant parts like leaves non- or less susceptible to attack by the respective pathogen. Many bacterial (and fungal) genera have thus far been shown to elicit ISR, among them *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Streptomyces*. Several molecules have been determined to induce ISR. For example, root treatment of *Phaseolus vulgaris* with a *Pseudomonas putida* strain lead to a significant reduction of the disease caused by the pathogenic fungus *Botrytis cinerea* on leaves. The isolated molecular determinant of *P. putida* mainly responsible for ISR was identified as a polyalkylated benzylamine structure (Figure 15.6). Exposure to the volatile compound 2,3-butanediol that promotes growth of *Arabidopsis* seedlings also results in a decreased disease severity following infection with the bacterial pathogen *Erwinia carotovora*. Transgenic lines of *Bacillus subtilis* that emitted reduced levels of **2,3-butanediol** (Figure 15.4), decreased *Arabidopsis thaliana* protection against pathogen infection compared with seedlings exposed to volatiles from wild-type bacterial lines. Furthermore, bacterial signaling molecules of the ***N*-acyl homoserine lactone** type (Figure 15.7), which are quorum-sensing compounds of Gram-negative bacteria, were found to exert systemic functions in plants as well.

15.1.2 Plant Disease Suppression by Rhizobacteria – Indirect Plant Growth Promotion

Infectious diseases are often caused by soil-borne organisms including both bacteria and fungi. These pathogens can infect plants directly via the roots or by emanating spores or other infectious particles that may also infect aboveground plant parts. Yet, under natural conditions **disease incidence**s are rare. This has been linked to the fact that between 1% and 35% of microbial isolates from plant-associated soil samples possess antagonistic activity against plant pathogens when tested in *in vitro* cultures.

Soils, where soil-borne diseases are infrequent despite the presence of the pathogen and susceptible host plants, are called *suppressive soils*. They show the capacity to decrease disease incidence. This phenomenon can be observed following disease severity monitoring after inoculation of sterilized soil with a pathogen compared to disease development in nonsterilized soils. Mechanisms behind this effect have been related to the activities of the soil microbial population during a time critical for the development of the pathogen. Here, effects of individual groups of microorganisms against a specific pathogen may play an important role because disease suppression is often caused by specific bacterial and fungal populations. Such naturally occurring antagonistic organisms express traits that enable them to interfere with pathogen growth, survival, and infection. Bacteria that are antagonistic to plant pathogens, represent an important part of the rhizosphere communities, and antagonistic strains amount up to 35% of the cultivable bacteria.

The most thoroughly investigated group of PGPR antanists are still the fluorescent pseudomonads. These bacteria produce several metabolites that suppress the growth of other organisms. For example, the extracellular pigment **pyoverdin** (Figure 15.6) is an efficient **siderophore** (iron carrier), and the production of pyoverdin by pseudomonads in iron-poor soils is an effective way of suppressing the growth of nonproducers by depriving the pathogens from iron. Pseudomonads also produce metal chelating agents with proposed properties other than iron scavenging. The siderophore **pyochelin** (Figure 15.6), for example, effectively binds copper and zinc, and possesses strong antimicrobial activity. The antimicrobial effect of pyochelin, and of some other siderophores, can be explained by their effective metal chelating activity. Gram-positive PGPR antagonists, like several *Bacillus* and *Streptomyces* (*Actinobacteria*) species, are also very efficient PGPR-strains with biocontrol activity, and also have effects on systemic resistance in plants. Because their

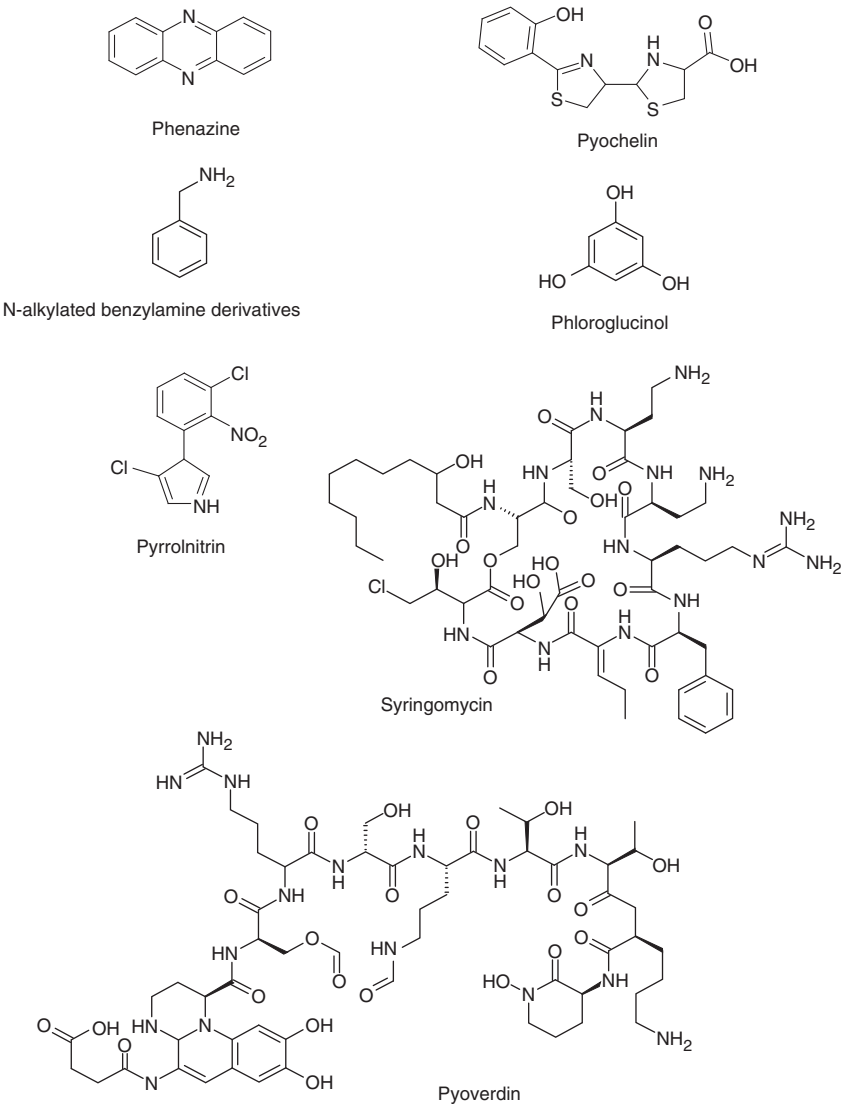


Figure 15.6 Compounds involved in plant disease suppression.

spores withstand adverse conditions, they have wide acceptance for practical application, especially following easier handling and excellent stability of inoculant preparations.

Direct **antibiosis** is used by several PGPR as a mechanism for **biocontrol**. Antibiosis by PGPR pseudomonads is often caused by the production of antimicrobial substances. These chemicals do not only suppress fungi, but are also often toxic against other bacteria. From antimicrobial compounds produced by pseudomonads, the mode of action has been partly determined for several classes of substances. These include the electron transport inhibitors **phenazines**, **phloroglucinols** (causing membrane damage in *Pythium* spp. (*Oomycetes*, *Stramenopiles*) and being phytotoxic at higher concentrations), **pyrrolnitrin** (acting as a fungicide), cyclic lipopeptides (surfactant properties against fungi and plants, chelation of cations), and **hydrogen cyanide** HCN (potent inhibitor of metalloenzymes), (Figure 15.6). Production of siderophores, lipopeptides, and antibiotics production has been observed in other PGPR isolates as well, including *Bacillus amyloliquefaciens*, *Stenotrophomonas* spp. (*Gammaproteobacteria*), and *Streptomyces* spp.

Another group of antagonistic compounds are lytic enzymes, such as cell wall hydrolases that attack pathogens. The ability to degrade fungal cell walls by chitinases is shared by many biocontrol PGPR including *Pseudomonas*, *Serratia,* and *Streptomyces* spp. In addition to chitinases, some bacterial strains produce ß-glucanases and proteases. Synergism between the action of cell wall degrading enzymes and antibiotics was observed. For example, it was shown that the pretreatment of plant pathogenic fungi with cell wall degrading enzymes rendered them more susceptible to the antifungal substance **syringomycin** (Figure 15.6).

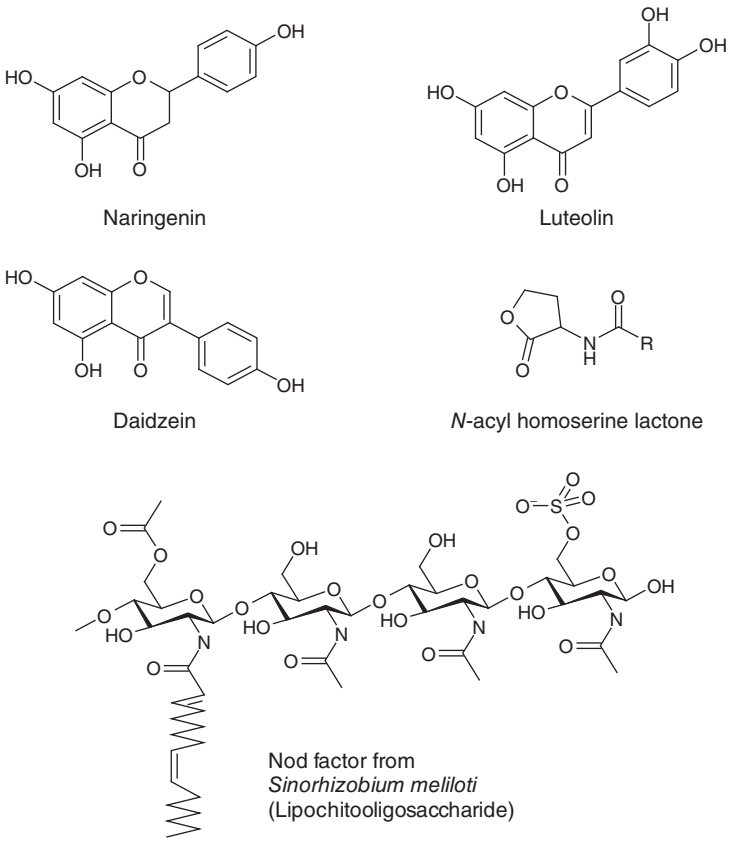


Figure 15.7 Root nodule formation related compounds. The flavonoids luteolin, naringenin, and daidzein induce *nod* gene expression in bacteria, the bacterial nod factors modulate root reorganization. *N*-acyl homoserine lactone functions in bacterial density measurement (quorum sensing).

15.1.3 Nitrogen-Fixing Plant–Bacterium Symbiosis

Certain bacteria form symbiotic structures with plants. The best-studied example is nitrogen assimilation in leguminous plants via an endophytic symbiosis with saprophytic free-living bacteria in the soil belonging to **Rhizobiales** (Genus *Rhizobium*; *Alphaproteobacteria*). They show a considerable phylogenetic diversity, which is also reflected by distinct metabolic properties. On roots of host plants, rhizobia induce the formation of organs called *nodules*, which are then colonized intracellularly. Here, they reduce nitrogen to ammonia, which can subsequently be used by the plant (see Section 5.2.2).

The establishment of this plant–bacteria interaction is regulated by chemicals. Relevant bacteria have to reach a certain population density before they are able to infect the root. Determination of this density is done following quorum-sensing (***N*-acetyl homoserine lactones**, AHLs, Figure 15.7). AHLs regulate gene expression programs in the bacteria, which are a prerequisite for symbiosis establishment. The mutual recognition of both partners of the symbiosis starts by the release of flavonoids (e.g., **naringenin**, **daidzein**, **luteolin**) by the host plant that direct bacteria toward the host root. These flavonoid signals not only attract relevant bacteria but also induce bacterial gene expression resulting in the production of so-called **nod-factors** (**lipochitooligosaccharids**) by the bacteria (Figure 15.7) (see Section 5.2.2.1).

Growth of rhizobia and nodule formation can be influenced by other soil bacteria, like streptomycetes (*Actinobacteria*). Antibiotic production by these may cause unsuccessful nodulation or even inhibit nodulation under field conditions.

Actinobacteria may not only influence root nodule formation with bacteria belonging to the genus *Rhizobia*, but may also fix nitrogen themselves. Within this bacterial phylum, members of the genus ***Frankia*** are able to fix atmospheric nitrogen either in a symbiosis with several dicotyledonous plants (e.g., *Alnus* species, *Fagales*, *Rosidae*), collectively called **actinorhizal plants** but, in contrast to rhizobia, also in nonsymbiotic state under aerobic conditions. The similarities between signaling in arbuscular mycorrhiza (AM) and legume/rhizobia symbiosis and the common ancestry of rhizobia and *Frankia* host plants indicate also a similarity in the use of signaling compounds. Even though the first sequenced genomes of *Frankia* did not reveal gene clusters homologous to rhizobial common

nod genes, it can be expected that *Frankia* Nod factor equivalents share similar features, that is, chitin-based signals (lipochitooligosaccharides) that are perceived by plant receptor kinases.

Cyanobacteria are a prokaryotic phylum with many species able to fix nitrogen. Some cyanobacteria form associations with a wide range of host plants belonging to the ferns (*Polypodiosida*), liverworts (*Marchantiophyta*), hornworts (*Anthocerophyta*), or plant families *Cycadaceae* (*Cycadales*, *Cycadophyta*) and *Gunneraceae* (*Gunnerales*, core eudicotyledons). The cyanobacterial symbionts found in these associations mainly belong to the genus **Nostoc** (*Nostocales*). These bacteria are characterized by possessing a few nitrogen-fixing cells (called **heterocysts**), resting spores called **akinetes**, and filaments that play an important role in the infection process. These so-called hormogonia are short gliding filaments that migrate rapidly into the preformed cavities of the future host plants. To trigger this event, host plants produce **hormogonia-inducing factors (HIF)**, yet the nature of these signals is still unknown. Once symbiosis is established, hormogonia production is again suppressed by as yet unidentified plant-produced hormogonia-repressing factor.

A special form of cyanobacteria symbiosis was observed in the association with the fungus *Geosiphon pyriformis*. *Geosiphon pyriformis* belongs to the *Glomeromycota* (*Fungi*, *Opisthokonta*), the same fungi that form AM and have thus attracted interest from the field of AM research (see Section 15.2.1 and Chapter 5). For *Geosiphon pyriforme* this symbiosis is obligate while the partner *Nostoc punctiforme* can be cultivated without the fungus. To establish the symbiosis, *Nostoc* and *Geosiphon* get into contact on the soil surface where the fungal hyphae surround the filaments of *N. punctiforme* and eventually incorporate them. The resulting structure is a bladder of up to 2 mm length in which the bacteria reside. Until now this is the only known fungus–cyanobacterium endosymbiosis.

15.1.4 Actinobacteria: Prolific Producers of Natural Compounds

Actinobacteria are among the most numerous bacteria in rhizospheres and play important roles in the decomposition and turnover of even the most recalcitrant organic materials such as cellulose, chitin, and lignin. Within the bacterial phylum *Actinobacteria* the order *Actinomycetales* comprises several genera that are characterized by the ability to produce a great variety of secondary metabolites. Members of the genus *Streptomyces* in particular, have developed an efficient machinery for the production and modification of secondary metabolites with antagonistic properties, probably because of their manifold interactions with other rhizosphere microorganisms. Also, the ability of streptomycetes to act as PGPR has recently attracted increased attention. Streptomycetes are generally saprophytic organisms that spend the majority of their life cycles as semidormant spores, especially under nutrient-limited conditions. When spores germinate, they produce a substrate mycelium, which uses extracellular hydrolytic enzymes to degrade organic compounds that usually resist degradation by other microbial groups, such as plant and fungal cell wall polysaccharides, and insect exoskeletons. The substrate mycelium fragments into chains of spores during maturation.

Streptomyces strains can be distinguished by their ability to produce secondary metabolites effective in organismic interactions (Figure 15.8). Several reports have shown how the biological activity of such secondary metabolites relates to their biocontrol activity. Streptomycetes produce bioactive compounds with antifungal and antibacterial activity, for example, 2-methylheptyl isonicotinate, which suppresses dominant soil-borne phytopathogenic fungi belonging to the genera *Fusarium* and *Rhizoctonia*. Such substances might exert their effect even when inoculated on seeds of cruciferous plants, resulting in resistance to fusarial wilt of crucifers. If both culture filtrate and spore suspension of such streptomycetes exhibit protective activity, they may be promising biocontrol microbes. Other compounds, like aminoglycoside antibiotic paromomycin, which inhibits the *in vitro* growth of severe oomycete plant pathogens (*Stramenopiles*) from the genera *Phytophthora* and *Pythium*, and which shows potential *in vivo* activity against red pepper and tomato late blight, have been described.

15.1.5 Plant Pathogenic Soil Bacteria

Compared to fungal plant pathogens there are relatively few soil-borne plant pathogenic members among bacteria, which belong to different subphyla of the *Proteobacteria*. Among those are the well-studied *Ralstonia solanacearum* (global distribution with unusually wide host range, causes wilt diseases, *Betaproteobacteria*), *Agrobacterium tumefaciens* (crown gall disease, *Alphaproteobacteria*), or the gammaproteobacterial genera *Xanthomonas*, *Pseudomonas*, and *Erwinia*. (*Erwinia amylovora* causes fire blight on apples, *Erwinia chrysanthemi*, *E. carotovora* soft rot). Xanthomonads cause disease symptoms in important crop plants, for example, raised circular lesions on leaves, which later on turn into brown corky cancer. They use a type III secretion system to secrete virulence effector proteins into the plant cell cytosol, which then modulate plant gene expression. *Pseudomonas* strains can be plant beneficial, but others, like *Pseudomonas syringae,* can also infect many taxonomically diverse plant species. The pathogenic ones also make use of the type III secretion system to inject proteins into the host cells. In addition, they produce the polyketide toxin, **coronatine** (Figure 15.9), which is required for full virulence and is similar to the phytohormone methyl jasmonate.

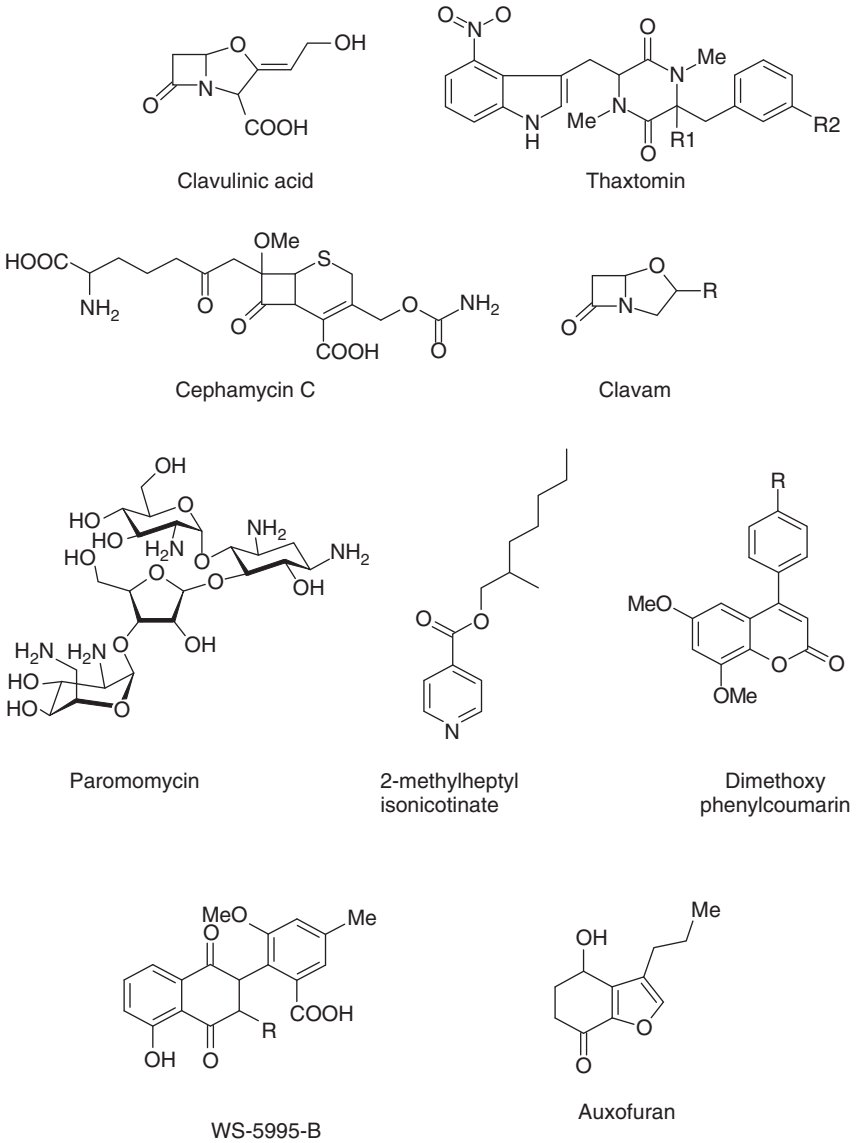


Figure 15.8 Secondary metabolites from streptomycetes.

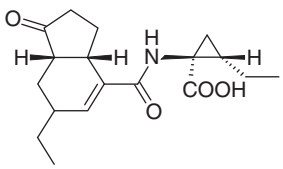


Figure 15.9 Coronatin, a polyketide produced by the plant pathogenic bacterium *Pseudomonas syringae*.

Among the hundreds of the described *Streptomyces* species, to date, only a few have been identified as plant pathogens. These species, *Streptomyces scabies*, *Streptomyces acidiscabies*, *Streptomyces turgidiscabies*, and *Streptomyces ipomoeae*, cause the common scab disease in potato and other taproot crops (reduction of root and shoot length, intense radial swelling of roots, tissue chlorosis, and necrosis). The mechanism of pathogenicity has been related to the production of a family of cyclic dipeptides, **thaxtomins**, by the streptomycete (Figure 15.8). Plant pathogenic *Streptomyces* species possess a conserved biosynthetic pathway for thaxtomin, which is essential for disease development.

15.1.6 Plant-Associated Bacteria as (Opportunistic) Human Pathogens

Recent mass infections such as **EHEC (enterohemorrhagic *E. coli*)** show the potential of plant-associated bacteria to have human hosts, or *vice versa*, that human pathogens thrive on plant surfaces. Among the rhizosphere bacteria with the potential to be opportunistic human pathogens, species of the genus *Staphylococcus* (*Firmicutes*) or the proteobacterial genera *Burkholderia* (β), *Enterobacter* (γ),

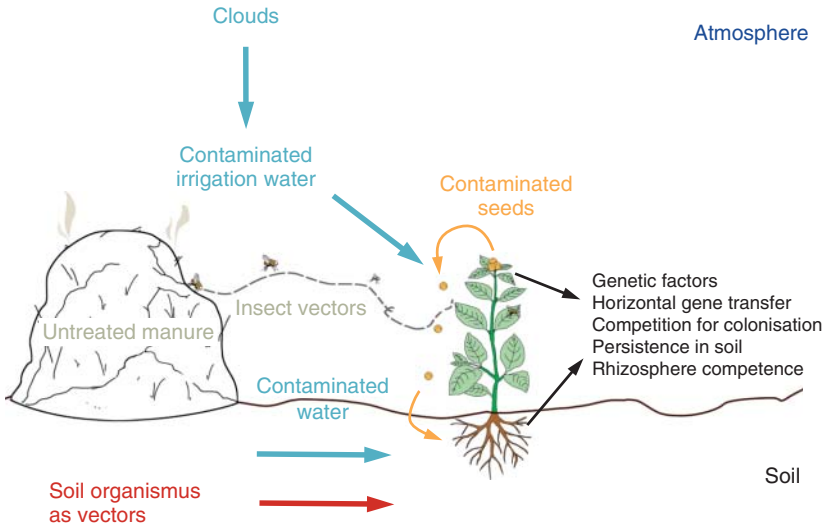


Figure 15.10 Mechanisms of spread and persistence of human pathogenic microorganism in the rhizosphere (Reproduced from Brandl (2006) with permission of Annual Review of Phytopathology.)

Herbaspirillum (β), *Ochrobactrum* (α), *Pseudomonas* (γ), *Serratia* (γ), and *Stenotrophomonas* (γ) can be found.

Animal produce was typically regarded as a main source of infection with **human pathogenic bacteria** while pathogens on fruit and vegetables are considered mostly a postharvest contamination rather than a contamination in the field. Increased reports of outbreaks associated with fresh produce have raised questions about the fitness of pathogens on plant surfaces, not only aboveground, but also belowground. Contamination may begin as early as seeds colonized with human pathogenic bacteria develop into crop plants. The bacteria may spread further via water, either by irrigation or from contaminated manure or soil, or by soil- or airborne vectors (Figure 15.10). Several human pathogenic bacteria, such as the proteobacteria *Pseudomonas aeruginosa* (γ), *Burkholderia cepacia* (β), *Erwinia* spp. (γ), and *Enterococcus faecalis* (*Firmicutes*) amplify on plant tissues. They may inhabit microsites on the plant characterized by the occurrence of sugars and other organic compounds. Competition between regular rhizosphere bacteria and **foodborne pathogens** might be because of preferential growth at root tips and sites of lateral root emergence (sites of solute release) due to easy access to root exudates. *In vitro*, it has been shown that enteric pathogens attach to these sites. Studies using the model plant *Arabidopsis thaliana* (*Brassicales, Rosidae*) have shown, however, that the whole plant can be colonized by, for example, the γ-proteobacteria *Salmonella enterica* and *E. coli* following irrigation with the bacteria. Even flowers and seeds were contaminated, implicating a further problem with spreading pathogenic bacteria. Seedborne outbreaks are frequent with raw sprouts of alfalfa, radish, and mung beens and are also potentially responsible for the recent outbreak of *E. coli* O104:H7 in Germany, resulting in the development of a **hemolytic uremic syndrome (HUS)** in a large group of patients.

Regardless of varying **rhizosphere competence** of human pathogenic and plant-associated bacteria, they have a genetic factor in common that enables both to survive in this niche. The "starvation" sigma factor RpoS involved in *in vitro* stress adaptation in *Salmonella enterica* and *E. coli* also enhances rhizosphere competence in *Pseudomonas fluorescence* and *P. putida*. Sigma factors bind to the bacterial RNA polymerase core complex to form the holoenzyme, thereby providing the ability to recognize promoter sequences on the DNA to initiate transcription (see Section S1.3.10.1). In addition, the presence of cellulose production genes, which are associated with **biofilm** production (see Chapter 14) and of UV radiation-tolerance genes is regarded as a fitness factor for the survival on plant surfaces.

From the genus *Burkholderia* (*Betaproteobacteria*) a total of 62 species have been described. Owing to their clinical importance, species belonging to the *B. cepacia* complex, comprising 17 species isolated from patients with cystic fibrosis, are intensively studied. Isolates have been described, which are able to fix nitrogen and form nodules with legume roots, constituting true endophytes. Interestingly, a phylogenetic analysis of 16S rRNA sequences of all 62 recognized *Burkholderia* species reveals two main clusters: the first one comprises the *B. cepacia* complex, together with other human pathogenic species, plant pathogens, and endosymbionts of pathogenic fungi. The second cluster contains nonpathogenic *Burkholderia* species that are associated with plants and/or the environment. This second cluster has been named "plant-beneficial *Burkholderia* cluster" and contains 31 species.

There is evidence for **horizontal gene transfer** between soil bacteria, which may increase their rhizosphere competence. A major concern is the possible acquisition of antibiotic resistance genes by potential human pathogens on agricultural crops. One other problem is that even

though pathogenic bacteria seem to exist in low numbers on plant material, they seem to be sufficient to cause a high incidence of diseases. This prompted the question whether the plant environment affects the pathogen's physiology in a way that fewer cells are required to cause a disease. One hint in this direction is that plants under microbial stress produce antimicrobial peptides. Such peptides may regulate bacterial determinants that help to overcome the first line in human defense (stomach acid). For example, the operon that confers resistance to host antimicrobial peptides is involved in the virulence in *S. enterica* and is believed to play a role in the resistance of *E. chrysanthemi* to plant antimicrobial peptides in damaged plant tissues. The same operon enables the pathogen to better resist early human host defense.

In light of these reports, bacteria that are suggested as biocontrol agents against microbial plant pathogens need to be studied thoroughly to assess possible risks to humans.

15.2 Fungi of the Rhizosphere

The rhizosphere hosts a great diversity of fungi (*Fungi, Opisthokonta*). They can broadly be separated into the decomposers, plant-beneficial mycorrhizal fungi, and plant pathogenic fungi. **Fungal decomposers** have a saprophytic lifestyle. Dead organic matter is converted into fungal biomass. They are especially important in the degradation of recalcitrant organic matter (lignocellulose), which is degraded by soil bacteria only to a lesser extent. Thus, they play an important role in the recycling of nutrients in the soil. **Mycorrhizal fungi** form a close interrelation with plant roots in which they provide the plant with improved access to water and nutrients, mainly phosphorus and nitrogen. In exchange, the fungi obtain sugars produced by the plant during photosynthesis. It is estimated that over 90% of land plants live in such an association with soil fungi. In aquatic environments, similar groups of fungal colonizers of plant roots can be found. Here, mycorrhizal fungi as well as endophytically growing fungi have been detected, which vary according to the organic matter content and pH of the surroundings. Mostly, the fungal genera involved have been described before as colonizers of terrestrial plant roots with an equally diverse ecology: arbuscular AM as well as ectomycorrhizal ECM fungi (see Section 5.2.1), ericoid and orchid mycorrhizal, and saprotrophs as well as endophytes.

The group of pathogenic soil fungi consists of those that depend on living plant tissues in order to survive. **Pathogenic fungi** are usually not dominant in soils, but infections may increase in managed areas because of the reduction in biodiversity of other soil-inhabiting organisms.

The assessment of a soil fungal community by growing isolates on pure culture media is, however, limited. Fungi of the order *Glomales* (*Glomeromycota*), for example, which are important symbionts ("endomycorrhiza"), can be cultured only in the presence of a host root. The application of molecular techniques, like rRNA-based analyses, largely improves our understanding of rhizospheric fungal communities.

In the following section, the chemical communication between plants and mycorrhizal as well as pathogenic fungi is described.

15.2.1 Mycorrhiza: Chemical Dialogue between Plants and Mycorrhizal Fungi

Most important for plant development and productivity are soil-borne fungi that develop symbiotic structures (**mycorrhiza**). They generally function as more or less mutualistic solute exchange systems. The host plant delivers carbohydrates for fungal growth and maintenance and obtains water and nutrients from the fungus. Among the different types of mycorrhizas, **endo- and ectomycorrhizas** are studied in most detail (see Section 5.2.1).

Development of **AM** is preceded by fungal spore germination, which may result in the retraction of nuclei and cytoplasm in the absence of a plant root. Because flavonoids are key signaling compounds isolated from plant root exudates, they have been proposed to play a distinct role in AM development. Several flavonoids have been shown to affect hyphal growth and differentiation and root colonization in a structure-specific manner. In turn, flavonoids also exert a fungus genus- and species-specific effect during presymbiotic growth. To widen this concept, it has also been suggested that certain soil bacteria that promote the formation of mycorrhiza (so-called **mycorrhiza helper bacteria**, see Section 15.2.3.) act by inducing flavonoid release from plants, thus facilitating root colonization by mycorrhizal plants.

The **strigolactone** 5-deoxystrigol (Figure 15.11, box 1, a) was identified as a signal molecule in the root exudates of *Lotus japonicus* and induces hyphal branching, germination of fungal spores, and alteration of fungal physiology. Because Strigolactones (a group of sesquiterpene lactones) are extremely short lived, they form a distinct gradient around the plant root, indicating to the fungus the proximity of and direction to the root. Other strigolactones, such as strigol, sorgolactone, and a synthetic analogue, GR24, mimic the activity of 5-deoxystrigol. Once the signal is perceived by the fungus, the presymbiotic phase is induced during which hyphal growth and branching and physiological activity is increased. In contrast, the fungal signaling molecules (referred to as **myc factors**) that induce a symbiosis-specific response in the host roots still remain elusive. Myc factors are diffusible molecules that may induce transcriptional changes in a putative host plant and result in enhanced lateral root growth. The involvement of strigolactones in the production of such myc factors is still unknown. Upon contact of the hyphae with the

root, the prepenetration apparatus (PPA) is formed (see Section 5.2.1.1).

Signal molecules in **ECM** formation are much less well known. The involvement of such molecules has been obvious as plant-derived compounds trigger spore germination, hyphal growth toward plant roots, and ECM formation (see Section 5.2.1.2).

Several plant-derived metabolites have been implicated in early events in mycorrhiza formation. Among those are flavonoids, diterpenes, hormones, and nutrients. The phenolic compound **rutin** (Figure 15.11, box1, c), isolated from eucalyptus root exudates has been shown to have a major effect on hyphal growth of certain *Pisolithus tinctorius* strains, whereas a tryptophan derivative, **hypaphorine** (Figure 15.11, box 1, b), which is secreted by *P. tinctorius*, can repress root hair elongation and stimulate formation of lateral roots. Hypaphorine production is increased in the presence of root exudates and during mycorrhiza formation. Antagonism of hypaphorine to plant-produced indole acetic acid (IAA) seems to be involved in the observed short root development. Phytohormones also play an important role in ECM formation. Auxin levels, in particular, play a specific role in ECM development. Plant-derived compounds in the rhizosphere enhance the biosysnthesis of hormones by ECM fungi, resulting in morphological changes and enhanced ECM formation.

15.2.2 Chemical Cross Talk between Plant Roots and Pathogenic Fungi: Signaling Involved in Recognition

Fungi (*Opisthokonta*) and *Oomycetes* (*Stramenopiles*) comprise the most plant pathogens in soil. Because the processes involved in the infection of a plant by such a

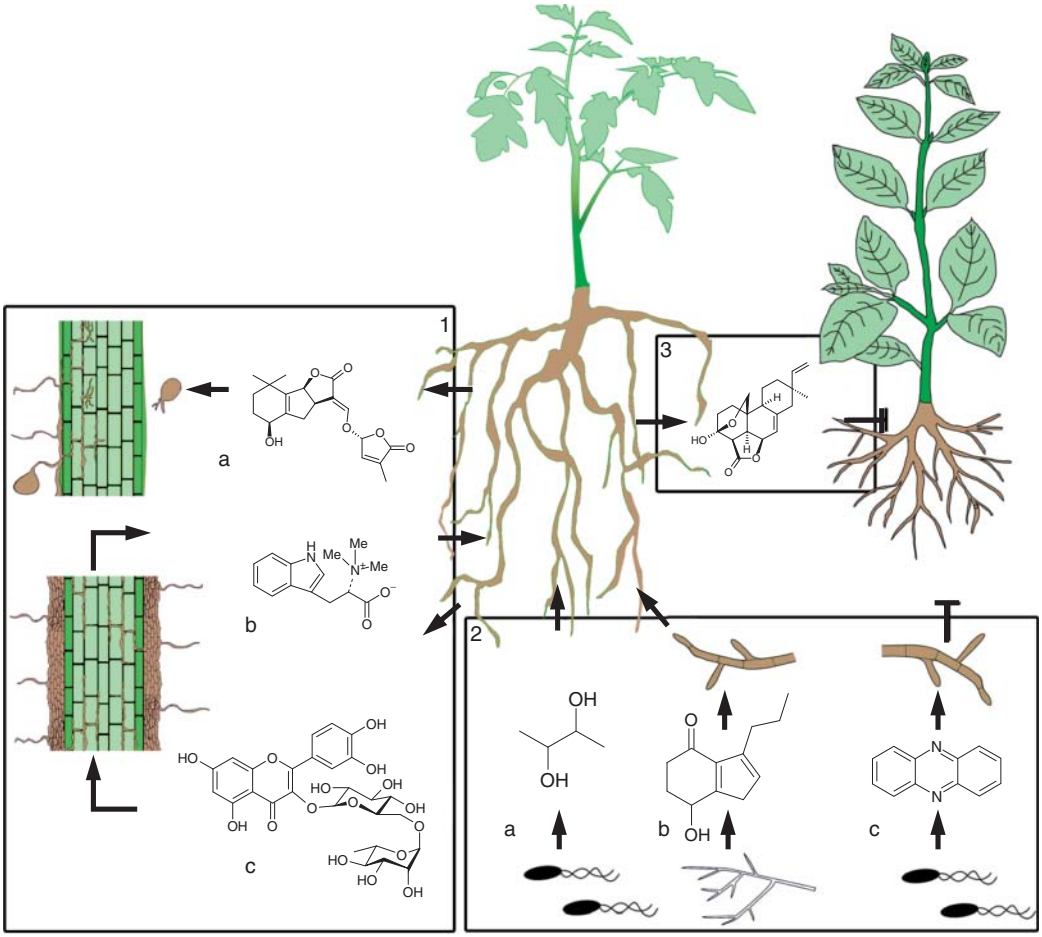


Figure 15.11 Summary of plant interaction with other soil organisms and chemical signals involved. Box 1 highlights chemical signaling in mycorrhiza formation. The strigolactone 5-deoxystrigol (Box 1, a) induces spore germination, hyphal branching, and alteration in fungal physiology in AM fungi. Rutin (Box 1, c), isolated from eucalyptus root exudates, affects the growth of the respective mycorrhizal fungus, whereas the tryptophan derivative, hypaphorine (Box1, b), secreted by the fungus, in turn affects root hair formation and lateral root growth (Section 15.2.1). Box 2 illustrates bacterial effects on plants and on the interaction between plants and fungi. 2,3 butanediol (Box 2, a) improves plant growth (Section 15.1.1). Auxofuran (Box 2, b), isolated from a mycorrhiza helper bacterium, promotes fungal growth (Section 15.2.3.1). Phenazine (c, Section 15.1.2), produced by pseudomonads, has antagonistic activity toward plant pathogenic fungi. Momilactone B (Box 3, Section 15.3.3) is released by rice roots and results in the growth inhibition of neighboring species.

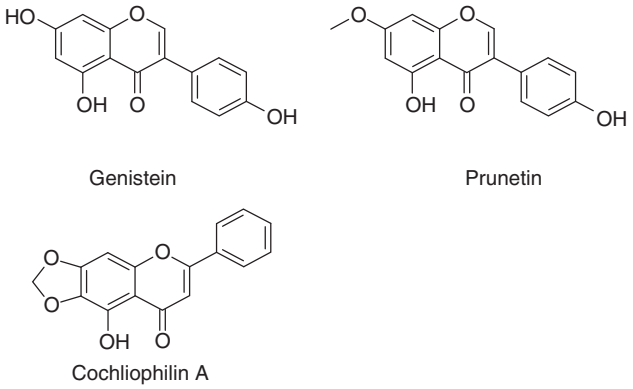


Figure 15.12 Plant-produced attractants for plant pathogenic oomycetes (*Stramenopiles*).

pathogen are very similar to those observed in the formation of arbuscular mycorrhizae (AM) formation, the involved chemical cues are used in several ways. Following contact between hypha and host plant, usually an appressorium is formed where a hyphae penetrates the host tissue. The role of flavonoids has been studied in detail in interactions of plants with Oomycetes. These are fungus-like organisms that are phylogenetically distinct from *Fungi* and contain cellulose within their cell walls as opposed to chitin in true fungi. Oomycetes produce motile zoospores that chemotactically move toward a potential infection site where they accumulate. Following morphological modifications the root tissue is penetrated. To result in an infection, zoospores of *Phytophthora sojae* are attracted by the flavonoids, **daidzein** (Figure 15.7) and **genistein** (Figure 15.12). This effect is host-specific. Zoospores of *Aphanomyces raphani*, for instance, are attracted by the host plant produced substance 3-indolcarbaldehyde, *Aphanomyces euteiches* by **prunetin** (Figure 15.12) and *Aphanomyces cochloides* by **cochliophilin A** (Figure 15.12) at low micromolar or nanomolar concentrations.

In the pathogenic interaction between legume plants and the true fungus *Fusarium* sp. (*Ascomycota, Dikarya*), plant-derived root exudates contain flavonoids that are highly stimulatory toward *Fusarium* spore germination. This stimulation is independent of that induced by nutrients. Whether this stimulation is species specific is still under debate. The specific group of compounds that induces hyphal branching in AM fungi, strigolactones, seems not to play a role in interactions with other fungi. When ECM fungi, plant-beneficial fungi, and plant pathogenic fungi were exposed to GR24 (a synthetic analogue of 5-deoxystrigol (Figure 15.11, box 1, a), no indication of an alteration of hyphal branching pattern could be observed.

15.2.3 Fungus–Bacterium Interactions

Bacteria coexist with fungal hyphae, colonizing surfaces and using compounds exuded from the hyphae. Hyphal exudates consist of low molecular weight metabolites such as complex mixtures of organic acids and iron-complexing siderophores. The bacteria have to be tolerant against antibacterial compounds, produced by the fungus, and even may have developed strategies to increase leakage from colonized fungal hyphae to improve nutrient release. In turn, effects toward the fungal "host" can be observed: bacteria induce a variety of developmental, morphological, and physiological modifications. Hyphal growth and branching, hyphal exudate composition, production of antibacterial metabolites as well as changes in fungal transcriptome have been observed.

Because of the transfer and exudation of plant-derived organic compounds to soil microsites not accessible to roots, mycorrhizal fungi can promote bacterial growth and survival, and soil bacteria can enhance the formation of mycorrhizal structures, either by promoting their establishment and functioning (**mycorrhiza helper bacteria**), or by protecting them from pathogenic microorganisms.

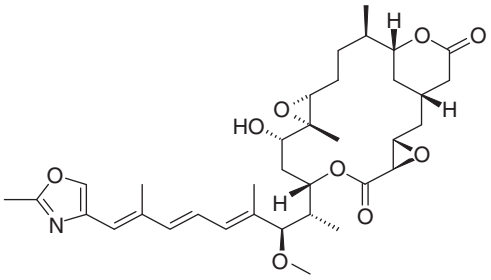
Depending on field conditions, bacteria of the mycorrhizosphere (soil zone influenced by plant root and extending hyphal network) colonize the surface of the fungal hyphae. A comparison of bacterial populations with regard to the host plant and associated (AM-forming) fungi indicate a larger influence of the latter.

With regard to plant nutrition, **bacterial mycophagy** can be of importance. Bacteria have been found to feed on both dead fungal material and surface structures of living fungi (such as the outer layers of spores). The direct antibiosis between bacteria and fungi has been discussed in Section 15.1.2.

In some fungal taxa, bacteria also live inside fungal cells as **endobacteria**. By means of 16S rRNA, endobacteria have been identified in AM-forming fungi. A model system of such an interaction has recently been developed with an isolate of the fungus *Gigaspora margarita* (*Glomeromycota*) and its β-proteobacterial endobacterium "Candidatus *Glomeribacter gigasporum,*" which is now investigated at the molecular level.

Another example is a well-investigated member of the fungal order of the *Sebacinales* (*Basidiomycota, Dikarya*), *Piriformospora indica*. This basidiomycete promotes growth and vitality of a large range of plant species, even of members of the Brassicaceae (*Brassicales, Rosidae*), which do not form mycorrhiza-like symbioses. On the basis of 16S rRNA analysis, rod-shaped bacteria detected in the cytosol of the fungal cells have been shown to be related to the α-proteobacterium *A. tumefaciens* (*Rhizobium radiobacter*). The bacterium can be kept in single culture and also shows plant growth promoting properties like its host fungus. In contrast to *A. tumefaciens*, it lacks pathogenic effects at low cell numbers. Probably there is some control of pathogenicity by the fungal host.

The complexity of such fungus–endobacterium association becomes obvious in the interaction between the rice seedling blight-causing agent *Rhizopus* (a zygomycete,



Rhizoxin

Figure 15.13 Rhizoxin, synthesized by a bacterial endophyt of the genus *Burkholderia* (*Betaproteobacteria*) of the rice pathogenic fungus *Rhizopus* sp. (*Fungi, Opisthokonta*).

Mucoromycotina) and its endofungal β-proteobacterium of the genus *Burkholderia.* The macrocyclic polyketide metabolite **rhizoxin** (Figure 15.13) that causes an abnormal swelling of the seedling roots is not produced by the fungus itself but by the endosymbiotic bacterium. Research about the interaction between rhizosphere fungi and endobacteria is still at the beginning, but opens another highly interesting aspect of rhizosphere interactions.

15.2.3.1 Mycorrhiza Helper Bacteria

Many bacterial isolates have been shown to exert a positive effect either on the formation of mycorrhiza or on the functioning of the mycorrhizal complex. Such **mycorrhiza helper bacteria** have been reported from Proteobacteria (*Agrobacterium, Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas, Klebsiella*, *Rhizobium*), Firmicutes (*Bacillus*, *Brevibacillus*, *Paenibacillus*) and Actinobacteria (*Rhodococcus*, *Streptomyces*, *Arthrobacter*). Mechanisms behind the promotion of mycorrhiza formation can broadly be separated into: (i) affecting the fungal partner (mycelial growth, spore germination); (ii) modifying the host root architecture (increase of root fungus contact sites by increasing the number of lateral roots; and (iii) controlling of soil properties (nutrient mobilization, metabolization of compounds inhibitory to mycelial growth). Numerous examples exist that show the promotion of fungal spore germination and hyphal extension of mycorrhizal fungi by mycorrhiza helper bacteria. The active substances can also be volatile and be released to the gas phase as has been shown with streptomycetes that stimulate AM fungal spore germination. The improved mycorrhization is thus often suggested to be a result of the increased potential contact areas for fungi and plant roots. In the interaction between the mycorrhizal fungus fly agaric (*Amanita muscaria, Basidiomycota, Dikarya*) and a mycorrhiza helper bacterium belonging to the genus *Streptomyces*, enhanced mycelium extension was observed due to the bacterial secondary metabolite **auxofuran** (Figures 15.8 and 15.11, box 2, b). This compound was produced by a streptomycete in single culture but even more so in dual culture with the mycorrhizal fungus itself.

An influence of the mycorrhiza helper bacteria toward potential host plants has also been described. Frequently, the increase in lateral root formation in the presence of such bacteria is observed. In combination with improved mycelial growth, this results in potential interaction points between plant root and fungus. The plant hormones auxin and ethylene are suggested to play a role in such modifications of the root system, yet this has not been connected to the production of such hormones by mycorrhiza helper bacteria.

15.2.3.2 Bacterial Mycophagy

Bacterial mycophagy describes the potential of bacteria to grow on living fungi and convert the released nutrients into bacterial biomass. In necrotrophic interactions, cell death of fungal hyphae results from the release of bacterial toxins, proteins, or cell wall degrading enzymes. Following the lysis of cells or the inhibition of fungal metabolism (i.e., killing of fungal cell), nutrients released are ingested by bacteria (Figure 15.14). Examples are numerous and can be found among the *Actinobacteria*, *Betaproteobacteria,* and *Firmicutes* bacteria of the genus *Bacillus*. Chitinase activity is commonly observed among those bacteria, yet the fungal cell wall is a difficult target comprising various polysaccharide and protein components that require a vast array of cell wall degrading enzymes for breakdown. Chitinase activity alone is thus not sufficient for the effective killing of hyphae. Instead, bacterial toxins seem to be involved. The lipodepsipeptide tolaasin produced by mushroom-infecting *Pseudomonas* isolates acts at different targets. It induces pore formation in plasma membranes and has a strong surfactant activity, reducing hydrophobicity of the hyphal surface. Possibly, cell wall degrading enzymes act in the next step to successfully lyse hyphal cells.

15.3 Plant–Plant Interactions

Plant roots do not exist solitarily under natural conditions. They are either in intimate contact with other eu- and prokaryotic organisms or are connected to them, for example, through fungal hyphal networks. This illustrates that communication between plants via neighboring roots will not be merely bidirectional (Section 15.3.1). A direct interaction of plants resulting in the **parasitism** of one plant on the other is brought about by the same chemical cues that aid in the development of AMF symbiosis, indicating the abuse of such signals for parasitic purposes (Section 15.3.2). A direct interaction without the need for physical contact can be observed in case of **allelopathy**, the production of chemicals acting as spacers to keep away the invading neighbors (Section 15.3.3).

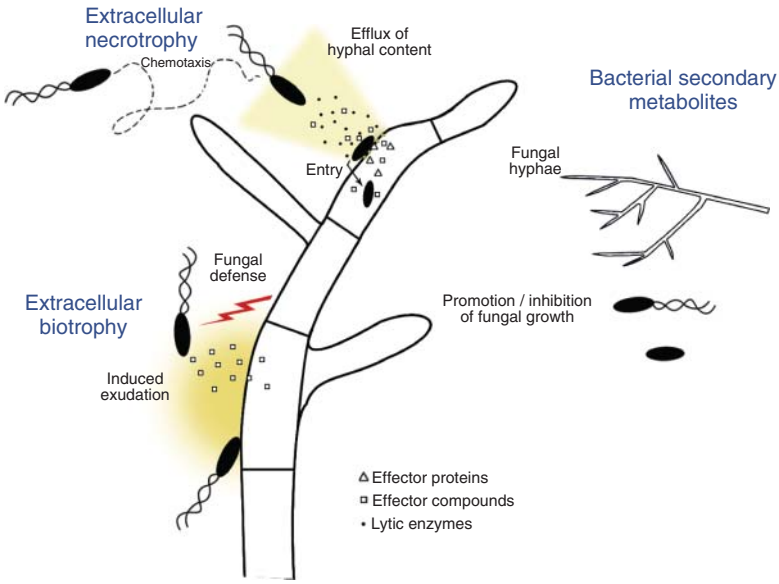


Figure 15.14 Fungus–bacterium interactions: during extracellular necrotrophy, bacteria lyse and kill hyphal cells by producing toxins and lytic enzymes. In extracellular biotrophy, bacteria make use of fungal exudates and thus need to be tolerant against antibacterial metabolites secreted by the fungus. Filamentous bacteria like Streptomycetes (top right) and other bacteria (below) are prolific producers of fungal growth modulating secondary metabolites. (Adapted from Leveau and Preston (2008).)

15.3.1 Plant–Plant Interaction via Fungal Networks

Hyphal strands connect neighboring trees and can establish a large network (common mycorrhizal network, or wood wide web, www) for assimilate transfer according to source (net producer)–sink (net consumer of assimilates) relationships. Net translocations of carbon, nitrogen, and phosphorus between plants connected by such hyphal networks have been described. **Common mycorrhizal networks** may form between the same or different plant species and also exist between fine roots of seedlings and of adult trees, possibly helping to compensate for shadowing and thus increasing seedling performance under light limitations. Molecular proof for such networks comes from DNA analysis of roots and associated fungi. *Rhizopogon* (*Basidiomycota, Dikarya*) mycorrhizas, for example, were found to colonize up to 19 trees in one plot. Although ECM is typical for trees and shrubs, some herbaceous plants as well form this type of mycorrhiza. The latter have possibly an important function in bridging forest gaps by spreading ECM fungi, as well as perpetuating fungal inocula when, for example, after fire, tree seedlings start to reestablish.

Endomycorrhiza common mycorrhizal networks have been shown to have a strong positive effect on the transfer of pathogen resistance to the host plant, e.g. in tomato plants mycorrhized with *Glomus mosseae* (*Glomeromycota*) and partly infected with the pathogenic fungus *Alternaria solani* (*Ascomycota, Dikarya*). In due course, the receiving (uninfected) plant showed increases in disease resistance and activities of potential **defense-related enzymes** such as peroxidase, polyphenol oxidase, chitinase, ß-1,3-glucanase, phenylalanine ammonia-lyase, and lipoxygenase and upregulated expression of six defense-related genes. Thus, plants may communicate via this underground communication conduit to transfer signals that induce pathogen defense.

Another aspect for which networking might be important is the **water exchange** between plant and fungus. The role of common mycorrhizal networks in the distribution of water between plants is not resolved. Because water movement is always a passive process along a water potential gradient, environmental conditions determine whether a mycorrhizal fungus can add to the plant's water supply. It was suggested that mycorrhiza help plant survival during transition from moist to dry conditions by being able to explore water reservoirs in soil pores; however, in this process, the flow of water from plant to fungus has also been observed. The **hydraulic lift (HL)** has been shown to help maintain the integrity of mycorrhizal mycelia during drought conditions. There is also evidence that water is translocated from plants performing HL through mycorrhizal hyphae to neighboring plants. This indicates that the water status of plants sharing a common mycorrhizal network might be maintained by one plant doing the HL; from there it could be shared during drought, at least as long as mycorrhizal linkages are maintained.

15.3.2 Parasitic Plants

Lifestyles of parasitic plants vary greatly across angiosperm taxa, and studies have shown that parasitism originated several times independently. Best-studied examples of parasitic plants that infect their host via their roots are ***Striga***

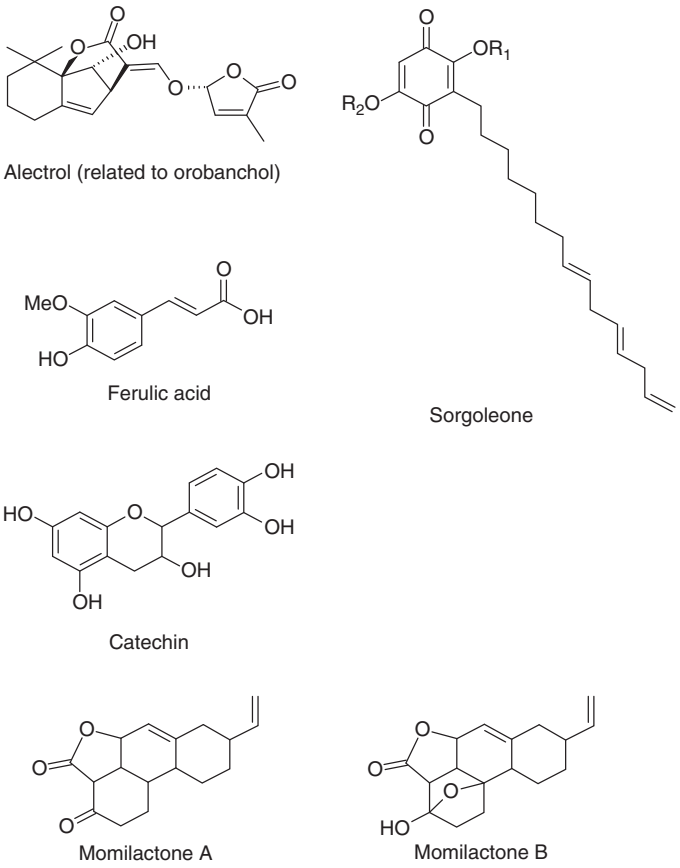


Figure 15.15 Chemicals involved in plant–plant interaction (Allelochemicals).

and ***Orobanche*** (both *Lamiales, Asteridae)*. Seedlings of both survive only briefly following germination and they germinate only in the presence of chemical cues. Strigolactones (sesquiterpene lactones, see Section 15.2.1.) are released by plant roots. They are unstable in soil and degrade quickly and induce a germination signal only within some mm distance from the root. It is assumed that a gradient in strigolactone concentration directs the growth of the emerging parasite radical toward the host root. The first such substance, strigol, was isolated from the nonhost cotton, and subsequently several true host plants (maize and millet) were found to also produce strigol. Structurally similar strigolactones were isolated from sorghum, red clover (**alectrol**, Figure 15.15), and from *Lotus japonicus* (5-desoxy-strigol, Figure 15.11, box 1, a, *Fabales, Rosidae*).

How exactly strigolactones trigger seed germination is not well understood. Several factors seem to be important: gibberellin synthesis has to be concomitant in seeds and ethylene triggers seed germination of *Striga* and *Orobanche*. Most interestingly, strigolactones also serve as important triggers in AM development (see also Section 15.2.1 and Figure 15.11), suggesting that parasitic plants may have co-opted these signals to recognize and locate a potential host plant. Following germination, such compounds result in the formation of haustoria. In addition, hydrogen peroxide is constitutively released from *Striga* germlings into the rhizosphere where it activates host peroxidases and degrades host cell wall pectins. In due course, benzoquinones are then released into the rhizosphere. These also result in haustorium formation by *Striga* roots. Studies on the molecular background indicate that benzoquinones downregulate a gene coding for one *Striga* expansin protein, while two other expansin genes are upregulated. Because expansin functions by releasing hydrogen bonds in cell walls, this regulation may play an important role in *Striga* haustorium formation.

15.3.3 Allelopathy

A distinct form of plant–plant interaction, in which one plant specifically interacts with the neighboring one, is called *allelopathy*. The expression "allelopathy" was first defined by the Austrian plant physiologist Hans Molischeme in 1937 to describe the observation that plants are able to banish surrounding plants of the same or different species. Over time, this definition was extended and now includes not only the effects of plants on other plants but also on microorganisms, which is actually a definition of chemical ecology in general.

A comparison of the chemical identities of **allelochemicals** shows that the biochemical origin cannot be

implemented to determine the "assignment" of a substance, as the production and release of allelochemicals are the result of general plant behavior impacting almost all aspects of plant ecology. This is problematic because some substances, for example, siderophores, can act as a nutritional factor but also as an allelochemical. **Ferulic acid** (Figure 15.15) is a siderophore but its action toward other organisms follows the hormesis effect (low concentrations can be favorable, higher ones toxic). Which properties does a substance need to qualify as an allelochemical? Studies have primarily focused on the phytotoxic action while other important features, such as the mode of release, bioactive concentration, persistence, and fate in the environment, are less commonly analyzed. A further problem of phytotoxic activity is that lowest doses that inhibit plant growth in field studies are a magnitude higher than what should be expected under natural conditions.

The production of allelochemicals may be constitutive or induced by biotic or abiotic factors present in the ecosystems in which plants grow. In turn, chemicals produced by plants have strong effects on **ecosystem properties**. Passage from the plant into the rhizosphere can be either passive by diffusion or by directed transport via vesicles and transporters and results in the next problem with the study of allelopathy: once the potential allelochemical has left the root, it is subject to soil physical properties that might result in oxidation or immobilization by binding to soil particles or to degradation or metabolic turnover by microorganisms.

To focus on these problems, the aforementioned ferulic acid and the vast amount of phenolic compounds serve as good examples. **Phenolics** (consisting of a hydroxyl group attached to an aromatic hydrocarbon) are abundantly produced by plants and can be found in plant decomposition products. Their biological activity ranges from changing membrane permeability, inhibiting plant nutrient uptake, affecting plant photosynthesis and respiration, and modifying protein synthesis and enzyme functioning. However, in soil they can exist either in free or bound forms. Depending on the availability, such compounds may alter their activity toward target organisms, resulting in the described effects or in its ineffectiveness.

The influential effect of allelopathic compounds on microorganisms leads to indirect effects on competing plants. For example, allelochemicals that negatively affect the growth of mycorrhizal fungi may serve as an advantage for nonmycorrhizal plants. Plant-beneficial bacteria can also be the target of such substances. The ability to inhibit symbiotic rhizobia strains would provide the producing plants with an advantage over a plant that depends on the symbiotic interaction. Further, microbial transformation of such chemicals plays a critical role in the outcome of the allelopathic effect. Microbial degradation of compounds may result in a detoxification thus disenabling a negative effect; it may also render a substance even more allelopathic.

Regardless of these difficulties, a well-studied example is the production of **momilactone** as an allelochemical released by rice roots. A large number of rice varieties have been found to inhibit the growth of neighboring species under field conditions as well as in the laboratory. In the search for allelochemicals responsible, momilactone A and B were identified (Figure 15.15). Both had been isolated from rice husks in the 1970s. In rice plants (*Oryza, Poales, Liliopsida*), momilactones are synthesized as part of a defense responses resulting in antibacterial and antifungal activities in leaves. In leaves, momilactone A and B exert antifungal activity toward the pathogenic rice blast fungus *Magnaporthe oryzae* (*Ascomycota, Dikarya*), which is the most serious fungal disease in rice, capable of causing considerable losses in rice crop yield. Inoculation of *M. oryzae* to rice leaves rapidly increased the expression of momilactone biosynthesis genes and subsequent accumulation of momilactone A in the leaves. As a result, fungal DNA in the leaves decreased.

Allelochemicals also have been suggested to play important roles in the distribution of **invasive plant species**. The spotted knapwood (*Centaurea stoebe, Asterales, Asteridae*) was introduced to North America around 100 years ago. In its natural habitats, Eastern Europe and Asia, *Centaurea* sp. is a moderately sized plant and a minor component of plant communities while it grows aggressively in North America where it dislodges the naturally occurring flora, indicating the involvement of allelochemicals. The main compound produced by *Centaurea* sp. is a racemic mixture of +/− **catechin** (a flavan-3-ol (Figure 15.15)). Catechin has been detected not only *in vitro*, but also in natural soils. Furthermore, it has a stronger effect on native North American species compared to its long-term European neighbors. These findings suggest that the strong allelopathic effects caused by catechin play a role in the weed's invasive success. Catechin also affects soil and root zone microbial communities. Native systems (including bacterial communities) adapt to allelochemicals such as catechin over time. Interestingly, when the compound was tested on isolated bacterial strains, a reversible bacteriostatic effect was observed, and removal of catechin from the system allowed normal bacterial growth kinetics to resume. This suggests that bacterial communities exposed to allelochemicals in soil might recover to their native structure and function on the removal of such compounds by degradation or transformation. This observation shows that adaptation of an invasive plant species or microorganism community to the chemistry of other species appears to be crucial to the organization and development of a community. The invading plants use compounds novel to the new environment to their advantage in the interaction with the native plants and microorganisms. Soil microorganisms that are able to detoxify specific chemicals probably evolved to do so. This could further lead to an increase in the biological activity of a thus far unknown compound that will not easily be degraded because of accumulation and prolonged persistence in the soil.

Sorgoleone, a major component of *Sorghum bicolor* (*Poales, Liliopsida*) root exudates, is another well-studied allelochemical (Figure 15.15). Again, the ability of *Sorghum* to cause "**soil sickness**" and inhibit the growth of other crops has been known for a long time. Exudation from root hairs does not exceed 20 $\mu g\, mg^{-1}$ root dry weight and it seems to be subject to a feedback inhibition mechanism, because following exudate removal, production continues. The nature of the regulation of the production is still unknown, but because it is obviously controlled by a feedback mechanism, it appears to depend on the accumulation of either sorgoleone or one of its intermediates. This could be a way to avoid autotoxicity.

Regarding the phytotoxic capabilities of sorgoleone, small seeded plants are much more prone to inhibition than large seeded ones. Large seeded weed might be less sensitive to sorgoleone by having lower absorption and translocation, faster metabolic degradation, or by having more rapid initial root growth beyond the location of *Sorghum* root exudates. Sorgoleone enters the soil directly via the root hairs and, as this happens continuously, it may be an advantage over other soil applied herbicides, possibly resulting in a greater persistence because of sustained production, as well as continuous distribution according to root growth.

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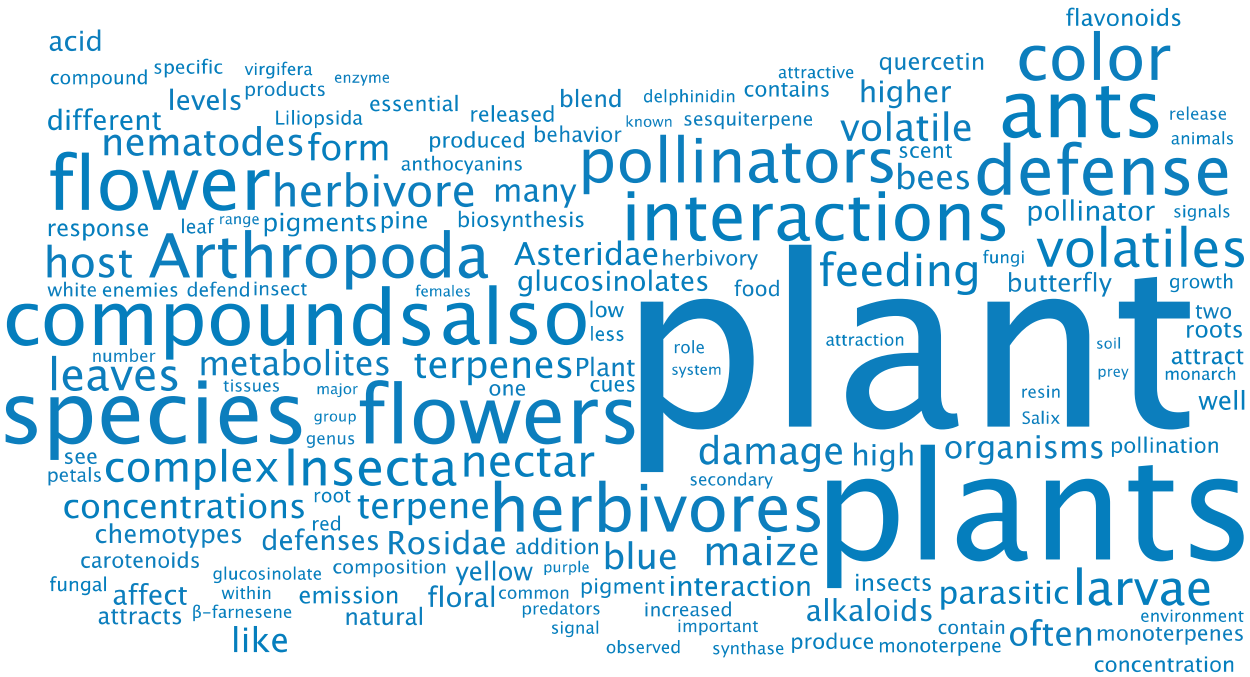
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16
Plant-Animal Dialogues

Susanne Preiß, Jörg Degenhardt, and Jonathan Gershenzon

Overview

The green plants of terrestrial ecosystems produce the largest part of the planet's biomass and thereby form the base for animal consumers. The interactions between terrestrial plants, pollinators, herbivores, and animals from higher trophic levels are so complex that only a fraction of these interdependent networks has been elucidated to date. Chemical cues guide most of these interactions where they serve numerous functions. This chapter highlights the ecology of only a few plant-animal systems and the compounds that are crucial for these interactions.

In plant interactions with other organisms, secondary metabolites often serve as attractants, deterrents, nutrients, toxins, or pigments. The extraordinarily high numbers of secondary metabolites produced by plants provides them with a large vocabulary to interact with organisms on multiple trophic levels. Without a doubt, there are many more compounds and functional roles still to be discovered. Two general strategies for metabolite production of plants can be observed in the examples shown above. First, the constitutive production of defense compounds or signals in specific tissues or developmental stages affects interacting organisms like herbivores or pollinators. Second, plant metabolites produced in response to outside cues either defend the plant directly or serve as signals to attract organisms of other trophic levels. Often, one compound can interact with several levels of a multitrophic system, which results in a high complexity. In its natural environment, the plant is embedded in a multitude of interactions on two and more trophic levels that can be organ-specific for leaves, roots, stems, flowers, and fruits.

Although our understanding of plant–animal interactions has already much improved in the past decades, it is still a major challenge to unravel these complex, interdependent networks and to elucidate their ecological impact on plants and their interacting organisms.

16.1
The Flower Pollinator System

16.1.1
General Aspects

Plant–pollinator interactions are a prerequisite for the propagation of most plant species. Therefore, they play a key role in the evolution of terrestrial plants. The diversity of flowers has been attributed to the coevolutionary relationships between plants and pollinators. These relationships are guided by flower morphology, scent, color, and nectar. The volatile and visual cues are used by the pollinator to identify the location, quality, and abundance of nectar and pollen rewards. Flowers are designed to be attractive and they often attract many different kinds of suitable pollinators. However, some interactions between plant and pollinator have coevolved so tightly that exclusive interactions have been formed, for example, between *Yucca brevifolia* (*Asparagales, Liliopsida*) and moths of the genera *Tegeticula* and *Parategeticula* (both *Insecta, Arthropoda*). Yuccas are pollinated only by these moths, and their larvae feed exclusively on yucca seeds.

In addition to pollinators that benefit the plant, nectar robbers can exploit the food rewards without pollination or herbivores can feed on the flower itself. Therefore, the plant has to adapt flowering and reward strategies in an effort to optimize protection as well as pollination.

16.1.2
Flower Color

For many pollinators, color is a reliable cue at a distance to find flowers and gather nectar or pollen. Pollinators are often attracted by a specific flower color (Table 16.1). This is especially obvious in cases where a shift in flower color is responsible for a switch from insect to hummingbird pollination. Floral scent and flower morphology often serve as additional cues for pollinator attraction.

Blue flower color is the preferred attractant of bee pollinators (Table 16.1). In temperate floras where bee pollination is dominant, convergence to blue flower color

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

Table 16.1 Flower colors, their most commonly associated pigments, and groups of pollinators.

Flower colors	Most common pigments	Pollinator attraction
Blue	Anthocyanins	Bees
Purple	Anthocyanins	Butterflies, moths, bats
Red	Anthocyanins	Birds
Yellow	Carotenoids, flavonols	Bees, butterflies
White	Flavones, flavonols	Bees, moths, bats
Dark purple, brown	Anthocyanins, chlorophyll	Beetles, bats, flies, wasps

can be observed. These blue colors are almost exclusively formed by anthocyanins (see Chapter 2), a subgroup of glucosylated flavonoids. The color of these flavonoid compounds is mostly dependent on the hydroxylation of the aromatic B-ring (Figure 16.1). The most common purple and blue pigments are cyanidin and delphinidin that have two or three hydroxylations on the B-ring, respectively. The anthocyanin pelargonidin with a single hydroxylation on the B-ring forms a red pigment. Most anthocyanins have sugars attached to the 3- or 3- and 5- positions. This glucosylation renders the anthocyanins water soluble and allows for the accumulation of the pigment in the vacuoles of epidermal cells, especially in petals. To increase their stability at low vacuole pH, anthocyanins are associated with flavone and flavonol copigments in highly structured ion-binding complexes (Figure 16.2). In association with these colorless copigments, complexes with purple cyanidin and delphinidin turn into an intense blue.

The chemical basis for the blue flower color was first discovered in *Commelina communis* (*Commelinales, Liliopsida*). A complex called *commelinin* contains a delphinidin glycoside, a flavone copigment, and two metals. Structure elucidation of the blue pigment located in the vacuoles of the petal showed a hydrogen-bonded complex with six molecules each of the anthocyanin and the flavone. Bound in this complex are two iron and magnesium cations. Although delphinidin is the most common anthocyanidin in blue flowers, there are some exceptions. In petals of the cornflower *Centaurea cyanus* (*Asterales, Asteridae*) and the morning glory *Pharbitis nil* (*Solanales, Asteridae*), cyanindin and peonidin glycosides are responsible for

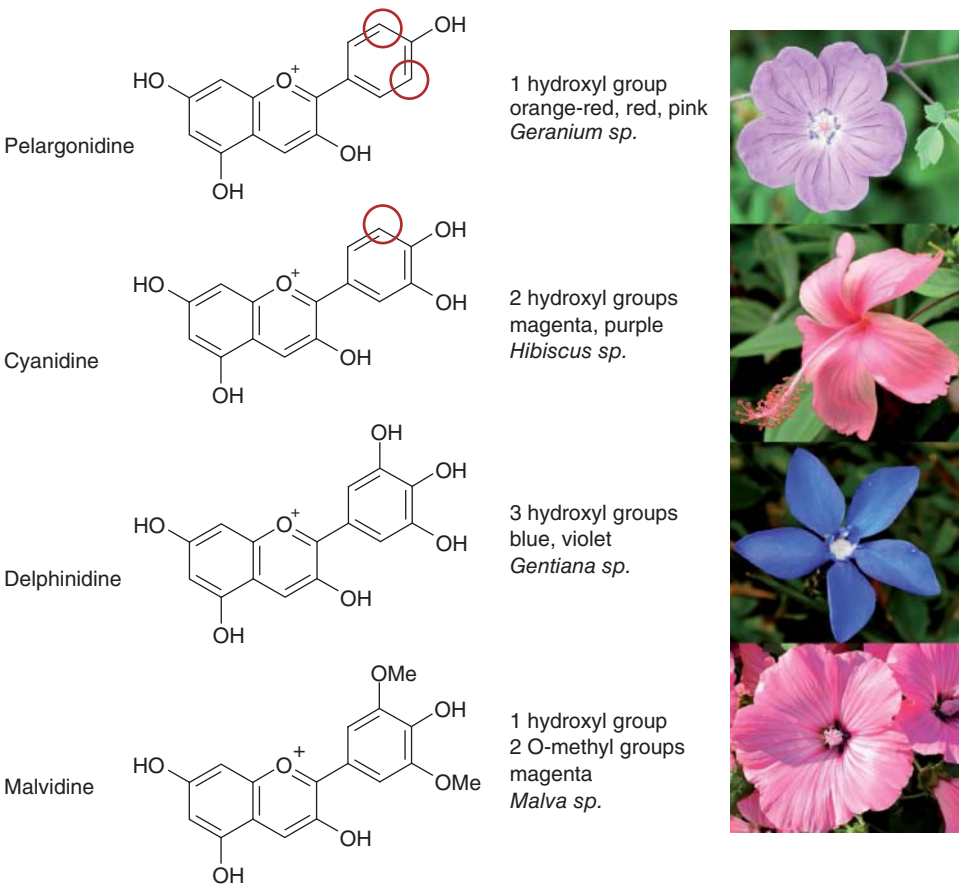

Figure 16.1 The oxidation and methylation of the B-ring of anthocyanidins determines pigment color. (First photo from above: © emer –fotolia.com, second photo from above: © thodsaph –fotolia.com, third photo from above: © RobertoC –fotolia.com, fourth photo from above: © Omika – fotolia.com.)

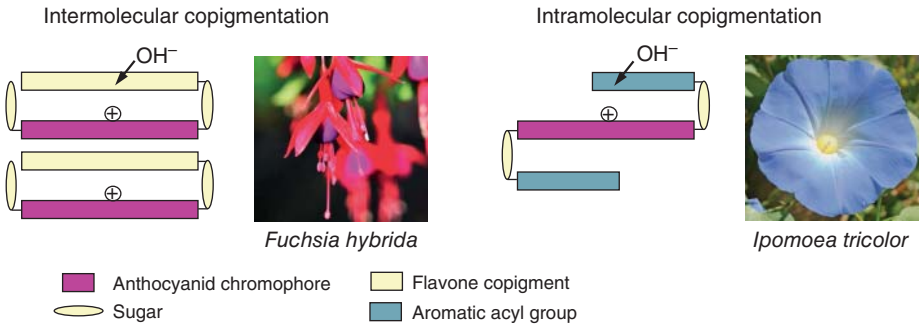

Figure 16.2 Anthocyanin pigments of flowers are protected from hydration by copigmentation. After J. Harbourne (1993). (Photo left: © drewrawcliffe – fotolia.com, photo right: © Fotolyse – fotolia.com.)

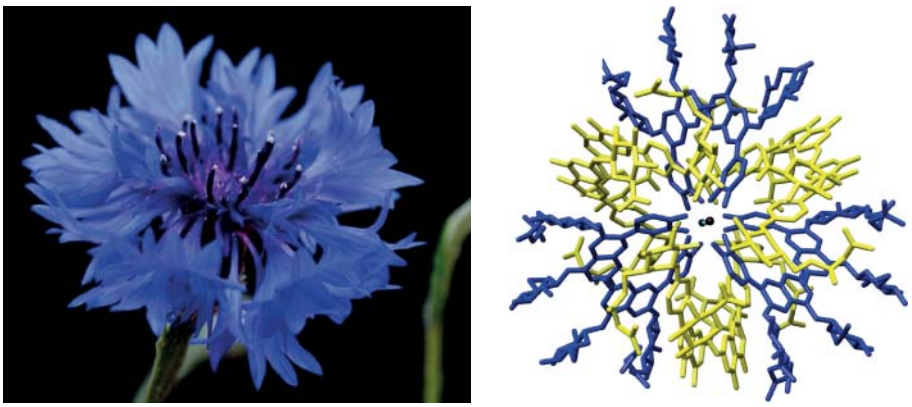

Figure 16.3 Structural analysis of pigment complexes of *Centaurea cyanus*. (a) flower of *Centaurea cyanus*. (b) The anthocyanin molecules are shown in blue and the flavone glycoside copigments in yellow. The metal ions in the center are Fe^{3+}, Mg^{2+}, Ca^{2+}. (After: *Nature*, 2005, *Vol. 436*, p.79. Photo: © Julija Sapic – fotolia.com.)

the formation of the blue color. The pigment complex of *C. cyanus* contains a very similar anthocyanin to flavone ratio and the same cations, iron and magnesium, as in *Commelina formina* (Figure 16.3). The spectral maxima of delphinidin glycosides at 535 nm (in MeOH) are closer to the wavelength of blue light at 580 nm than the maxima of cyanindin or peonidin glycosides at 525 nm (in MeOH). As a result, less flavone copigment is required in the presence of the delphinidin pigment.

The blue flower of *Hydrangea macrophylla* (*Cornales, Asteridae*) is exceptional for containing delphinidin in combination with the copigment caffeoylquinic acid, a simple phenolic, and aluminum as a metal cation. The pigment of the blue poppy *Meconopsis betonicifolia* (*Ranunculales,* stem eudicotyledons) is based on cyanidins. Because of their spectral properties, cyanidins produce a less intense blue color.

Red to purple flower color is most attractive to birds, butterflies, and moths. The pigments are most often anthocyanins like pelargonidin, peonidin, and petunidin. Their colors range from red to magenta and are widely spread throughout the plant kingdom. Plant families like the *Rosaceae* (*Rosales, Rosidae*) are considered more primitive in the course of evolution. *Rosaceae* have floral anthocyanins based only on cyanindin that prevents roses from producing blue flowers. The blue pigment delphinidin is restricted to angiosperm (*Magnoliophyta*) families that are considered more highly evolved.

In orchids (*Asparagales, Liliopsida*), the purple flower color is based on cyanidin and peonidin glycosides with acylated sugars attached at the 7- and 3- positions. These planar molecules can form stable color complexes because of intramolecular associations and do not need a copigment or metal cation. In *Penstemon* (*Lamiales, Asteridae*), the type of anthocyanidin aglycone in the flower determines which type of pollinator is attracted. Between two species of *Mimulus* (*Lamiales, Asteridae*), the red flowers of *Mimulus cardinalis* contained more anthocyanins than the pink flowers of *Mimulus lewesii*. *Mimulus cardinalis* is pollinated by hummingbirds and contained more pelargonidin-derivatives than *M. lewesii*. The pink flowers of *M. lewisii* are pollinated by bumble bees and in turn contained a higher proportion of cyanindin-derivatives.

Light yellow to orange and red flower colors are usually based on carotenoids (see Chapter 2). These tetraterpene compounds carry terminal ionone rings and their color is determined by the number and position of the double

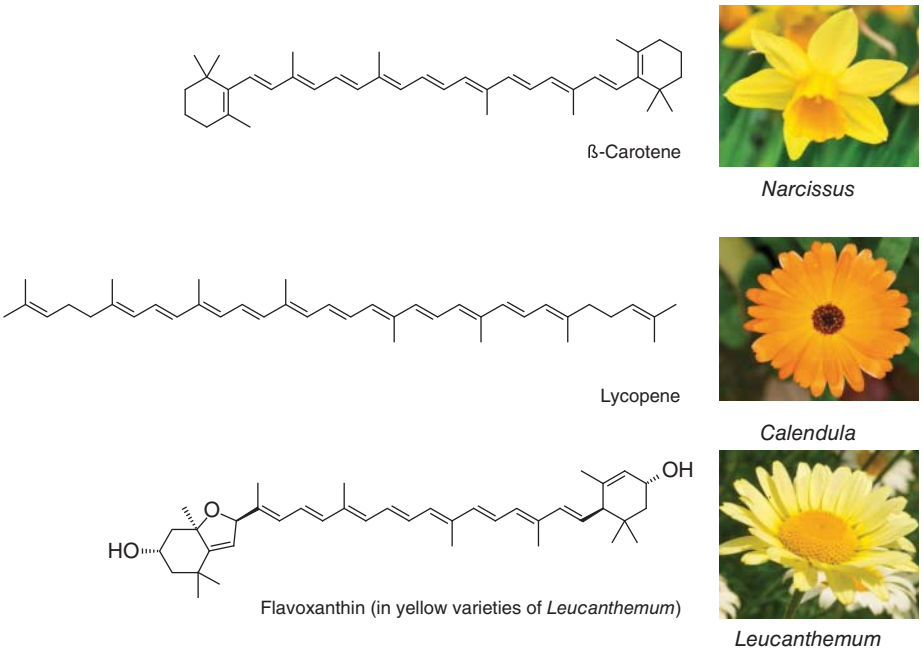

Figure 16.4 Carotenoids are pigments in many yellow flowers. (First photo from above: © Stefan Körber, second photo from above: © Bo Valentino – fotolia.com, third photo from above: © Depositphotos.com/dar19.30.)

bonds (Figure 16.4). Carotenoids are essential for the plant and are synthesized in both chloroplasts and chromoplasts. In chloroplasts, carotenoids are associated with the light-harvesting complexes of photosynthesis where they are vital for the energy transfer processes of photosynthesis and act as antioxidants (see Chapters 8 and 9). The carotenoids produced and stored in chromoplasts are not essential for the primary metabolism for the plant but provide color to flowers, fruits, and storage organs. In flowers, the carotenoids help to attract pollinators and in fruits, the yellow coloring attracts seed-distributing animals.

While the carotenoid composition of plant leaves is very similar between plant species, flowers, and roots can have very distinct carotenoid profiles. Carotenoid content ranges from low concentrations in flowers with white petals to the dark orange flowers of some marigolds (*Tagetes, Asterales, Asteridae*), which have 20-fold higher concentrations of carotenoids than leaves. The concentration of carotenoids is not only controlled by the activity of carotenoid biosynthesis genes on the level of transcription and translation, but also by the amount of lipoprotein-sequestering structures that function as carotenoid sinks.

In some plants, the biosynthetic pathway contains additional enzymes that modify carotenoids and cause significant changes in coloration. The red ketocarotenoids in fruits of red chili peppers, for example, are synthesized from antheraxanthin and violaxanthin by a capsanthin-capsorubin synthase. Carotenoid biosynthesis can also be altered by genetic engineering. Transformation of rice (*Oryza, Poales, Liliopsida*) with a phytoene synthase from daffodil (*Narcissus pseudonarcissus, Asparagales, Liliopsida*) and a carotene desaturase from the soil bacterium *Erwinia uredovora* (*Gammaproteobacteria*) resulted in plants with an increased accumulation of β-carotene, a precursor of vitamin A. Because of the yellow appearance of the endosperm, the plants were named "Golden Rice." The plant was developed to combat dietary vitamin A deficiency, which is widespread in subtropical and tropical areas.

Tomato (*Solanum lycopersicum, Solanales, Asteridae*) has often been used as a model plant to study the biosynthesis and function of carotenoids. The red color of the ripe fruits is caused by the accumulation of lycopene and attracts herbivores for seed dispersal (Figure 16.4). The intense yellow color of the flowers is based on a blend of xanthophylls, violoxanthin, zeaxanthin, and neoxanthin. The carotenoid content of tomato flowers increases at least up to 10-fold during development and attracts pollinators, especially bees like the yellow-faced bumble bee.

Marigold (*Tagetes*) flowers are notable for their high concentration of lutein. Strongly pigmented varieties have been used as a commercial source for this pigment, which can be added to poultry food to improve the quality of egg yolks. Morning glories (*Ipomoea, Solanales, Asteridae*) have been a model to study the effect of flower color and pollinator interaction on plant reproduction. In this genus, variation in flower color ranges from white to dark purple and red. The yellow and orange petals of some species are rich in carotenoids. The Japanese morning glory (*I. nil*) lacks a yellow cultivar and does not accumulate carotenoids in its petals. Another common color switch is from flowers containing anthocyanins to white or yellow morphs that lack anthocyanins. In natural habitats, pollinators discriminate against white color morphs when they are

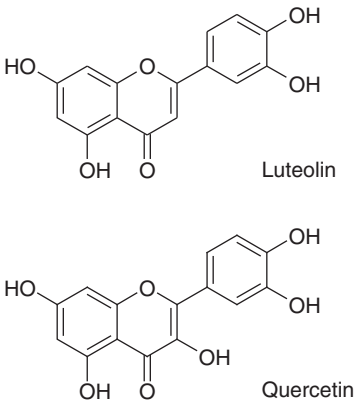

Figure 16.5 The glucosides of luteolin, a flavone, and quercetin, a flavonol, are pigments of white flowers.

rare. A consequence of lower visitation of pollinators is an increased selfing rate in white flowers. This raises the number of progeny produced by self-fertilization within a population of plants and maintains a wide range of color **polymorphisms** in *Ipomoea* species.

White flowers often contain pigments based on flavones (e.g., luteolin) and flavonols (e.g., quercetin), which are active in UV light and, therefore, attractive to bees (Figure 16.5). Flowers visited by bees often have patterns of UV pigments on their petals. These markings are called *honey guides* and direct the bees into the center of the flower toward the nectar and pollen rewards. Besides the attraction of pollinators, these flavonoids are very important for protection against UV damage, insects, and pathogens.

16.1.3 Nectar

Floral nectaries secrete an aqueous solution that contains sugars (mainly glucose, fructose, saccharose), a wide range of proteinogenic amino acids, and lipids. The composition of this nectar varies between species and attracts pollinators that are often specific for the plant. These pollinators gain nutrition from the nectar and form a mutualistic relationship with the plant. Because of its nutritional value, nectar is also exploited by nonmutualistic organisms that do not pollinate the flower but rob the flower of its nectar rewards. In addition, the metabolic activity of yeasts and fungi in the nectar can change its composition and decrease the food reward for the mutualistic animals. To defend the nectar against nonmutualistic organisms, it often contains alkaloids and phenolic compounds. Many nectar proteins have also been implicated in the defense against microbial infestation. To avoid a negative impact on pollinators, the concentration of such defense compounds needs to be finely balanced within the nectar. In addition, low concentration of volatile compounds in the nectar can serve as attractants of pollinators as well as repellents and toxins for nonmutualistic organisms.

16.1.4 Floral Scent

The ecology of floral scent has been studied only in recent years after improved techniques for the collection of volatiles were coupled with gas chromatography and electroantennographic detection. With these methods, the chemistry of floral fragrances and its effect on the behavior of pollinators can be studied in great detail. Floral fragrances differ strongly between plant species, consisting of a large number of fatty-acid derivatives, benzenoids, terpenoids, and nitrogen-containing compounds (Figure 16.6). The production and release of flower volatiles is closely regulated. Generally, in snapdragon *Antirrhinum majus* (*Lamiales, Asteridae*), methyl benzoate is one of the principal floral volatiles (Figure 16.6a). The expression of benzoic acid carboxyl methyl transferase, the enzyme responsible for the biosynthesis of this compound, is tightly correlated with the synthesis of its product and exclusively expressed in epidermal cells of the petals. The substrate of this enzyme, benzoic acid, is also correlated with product emission, indicating that earlier steps of the biosynthetic pathway are controlled as well. Enzyme expression is highest in the parts of the petal that are closer to the path that bees take to reach the nectar. Some of the petal cells have a conical shape that increases their surface area and aids in the volatilization. These areas also sport hairlike unicellular glands in the center of the basal and assist pollinators to find their way into the flower. During daylight, when bees are more active, the emission of methyl benzoate is stronger than during the night.

A striking example of the attractiveness of specific scents to pollinators is found among orchid species (Figure 16.6c). The flowers of *Ophrys sphegodes* (*Asparagales, Liliopsida*) are visited by a male solitary bee (*Andrena nigroaenea, Insecta, Arthropoda*) that mistakes them for the body of a female bee. While trying to copulate with the flower, the male bees pick up pollen and transmit it to the next flower. The compounds responsible that elicit this "pseudocopulatory" behavior are straight chain alkanes and alkenes. The floral scent of *O. sphegodes* contains these compounds in ratios that are similar to those in receptive *A. nigroaenea* female bees. After pollination, *O. sphegodes* flowers emit farnesyl hexanoate (Figure 16.6c), which is subsequently released by nonreceptive *A. nigroaenea* female bees to inhibit copulation. For the flower, it is probably advantageous to mimic the odor of nonreceptive female bees after pollination as additional floral visitors may damage the developing seeds and pollinators are redirected to unpollinated flowers.

Some plants evolved flowers that adapt to pollination by ground-dwelling mammals. Flowers of the parasitic plant *Cytinus visseri* (*Malvales, Rosida*) growing in the Mediterranean and southern Africa, release 3-hexanone (Figure 16.6c) that attracts mouse-like elephant shrews (*Macroscelidea, Mammalia, Vertebrata*).

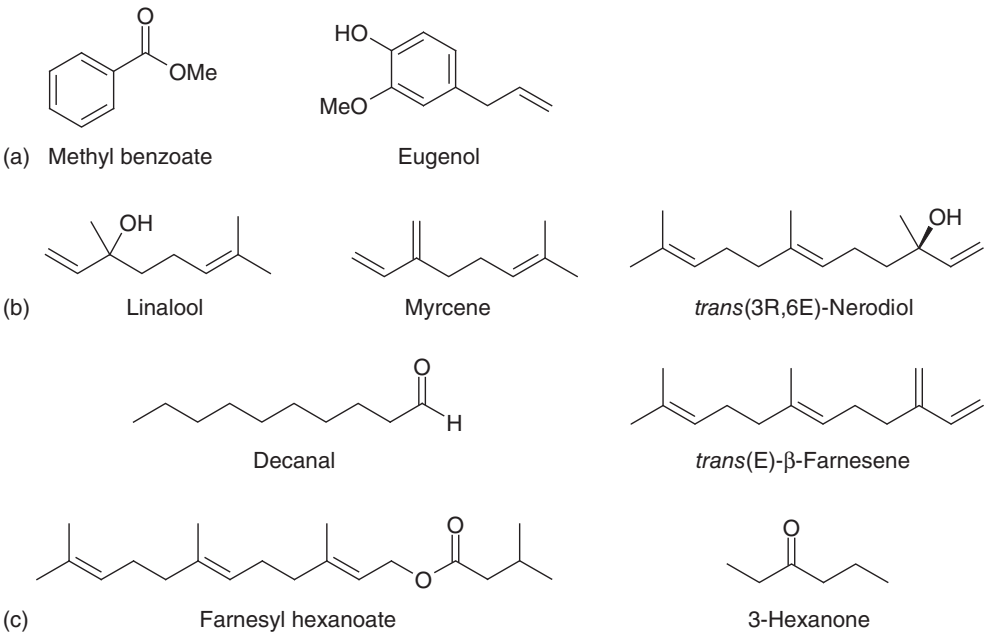

Figure 16.6 Structures of floral volatiles. (a) Aromatics, (b) Mono- and sesquiterpenes, (c) Volatile liphatic and sesquiterpene ester of *Ophrys sphegodes*.

16.1.5 Flower Pollinator Interactions Are Guided by Complex Patterns of Biochemical Cues

The nectaries of flowers attract both mutualistic pollinators and antagonistic organisms like nectar thieves or herbivores. The ratio between beneficial pollinators and detrimental visitors is influenced by many flower traits. To determine how this balance is affected by the amounts of petal pigments and a group of plant defenses known as *glucosinolates*, plants of *Raphanus sativus* (wild radish, *Brassicales, Rosidae*) with different flower color and glucosinolate content, were crossed into a similar genetic background. The anthocyanin-dominant purple and bronze morphs had a greater content of foliar glucosinolates than the anthocyanin-recessive yellow and white morphs. Pollinators preferred the yellow-flowered plants and herbivores performed better on the less-defended yellow and white genotypes with the reduced glucosinolate content. Thus, a balance between these two traits ensures an optimal pollination success.

Pollinator attraction and defense against herbivory can also be provided by the same secondary metabolites. In flowers of *Hypericum calycinum* (*Malpighiales, Rosidae*), ultraviolet pigments in the petals not only attract insect pollinators but also defend the stamens and the ovaries of the flower against herbivory.

In the genus *Dalechampia* (*Malpighiales, Rosidae*), the **plant resins** that constitute a chemical defense against herbivores and microbes have gained a secondary role in pollinator attraction. In the neotropics, most *Dalechampia* species are pollinated by resin-collecting female bees including euglossine bees and *Hypanthidium* (*Insecta, Arthropoda*). These bees use the plant resin in nest construction. Another group of *Dalechampia* species is pollinated by fragrance-collecting male euglossine bees that use the resin fragrance to attract females for mating. In contrast, a third group of Malagasy *Dalechampia* species is pollinated by pollen-feeding beetles or pollen-collecting bees that do not make use of the resin.

The interaction between flower and pollinator is not only controlled by the plant but also by the pollinator. Sometimes floral visits are necessary to stimulate nectar production. Conversely, herbivory on leaves and flowers can reduce the amount or quality of floral rewards and flower size. These alterations are mediated by plant defense pathways like the jasmonate pathway and change the reward chemistry for pollinator visitation in the damaged plants. Many pollinators can modify the pollination of the flower directly. These pollinators leave attractant or repellent scent marks that affect subsequent floral visitation. The bumblebee *Bombus lapidarus* (*Insecta, Arthropoda*) leaves a scent mark on flowers of *Melilotus officinalis* (*Fabales, Rosidae*), which deters conspecific and heterospecific (*Apis mellifera, Insecta, Arthropoda*) floral visits.

Pollinator visitation can also induce a flower color change. Flowers of *Malvaviscus arboreus var. mexicanus* (*Malvales, Rosidae*) change their color when they are robbed of nectar by orchard orioles (*Aves, Sauropsida*). These flowers are avoided by the hummingbird pollinators of *M. arboreus.*

16.2
Ant–Plant–Fungus Mutualism, a Three-Way Interaction

In both temperate and tropical communities, interactions between ants and plants are common. Plants that live in a mutualistic association with a colony of ants are called *myrmecophytes*. **Myrmecophilic** ("ant loving") **plants** often produce food rewards in the form of extrafloral nectar or food bodies. Extrafloral nectar attracts ants to the vegetative parts of the plant. As ants are often important predators of arthropods, the damage of the plant by herbivorous arthropods can be reduced significantly. Food bodies are easily removable by foraging ants and contain sugar, proteins, and lipids. Ants attracted by these structures also defend the plant against arthropod herbivores.

Myrmecophytes sometimes also provide associated ants with specialized hollow structures for nesting (domatia). Such associations play an important role in tropical ecosystems and can form obligate mutualisms. In these ecosystems, a complete dependency was observed between myrmecophytic plants of the genus *Macaranga* (*Malpighiales, Rosidae*) and ants of the genus *Crematogaster* (*Insecta, Arthropoda*).

Myrmecochory, seed dispersal by ants, was observed in over 10 000 plant species. Myrmecochorous seeds have an attachment rich in lipids, the elaiosome. The elaiosome attracts ants that carry the seeds into their colony to feed on the elaiosome. Thereby, the seeds are dispersed and stored in an underground location that provides a protected, nutrient-rich environment for germination.

Many plant–ant interactions include **fungi and bacteria as mutualistic partners**. Many ant species cultivate fungi in the domatia of American and Asian myrmecophytes. Leaf-cutter ants live in arid, semitropical, and tropical regions of South, Central, and North America. Colonies can hold millions of ants, consuming great quantities of plant material for fungal farming. Leaf-cutter ants of the genera *Atta* and *Acromyrmex* (*Insecta, Arthropoda, "Attini"*) cut leaves, flowers, and grasses, and transport the harvest to the nest (Figure 16.7). The plant material is not consumed directly, but chewed into a pulp and fertilized with feces. In subterranean gardens, this substrate is inoculated with a symbiotic leucocoprinus fungus (*Lepiota, Agaricomycetes, Basidiomycota*), the major food source of these ants. The tiniest ants tend the cultures and harvest specific protein-rich bodies (gongylidia) produced by the fungi. This material is distributed to other parts of the colony, mainly as food to the ant larvae.

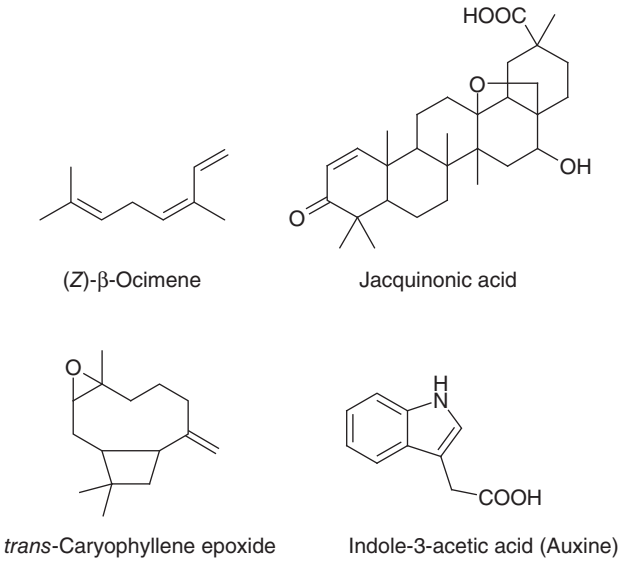

Figure 16.8 Plant metabolites affecting ant–plant mutualisms.

Leaf-cutter ants utilize a wide range of plants for fungal cultivation. Plants that produce antifungal compounds like the volatile terpenes β-ocimene and caryophyllene epoxide, or the nonvolatile triterpene jacquinonic acid, negatively affect the fungal cultures and are, therefore, avoided by ants in gathering leaves (Figure 16.8).

Acting as expert gardeners, leaf-cutter ants manage their cultures very well. Fungal growth is supported by indol-3-acetic acid, the auxin of higher plants, which is produced in the **metapleural glands** of the ants (Figure 16.8). In addition, fungal enzymes that break down plant material are produced in high concentration in hyphal tips. Ants eat the tips and defecate the still active enzymes on the plant material they have just brought into the culture to speed up decomposition.

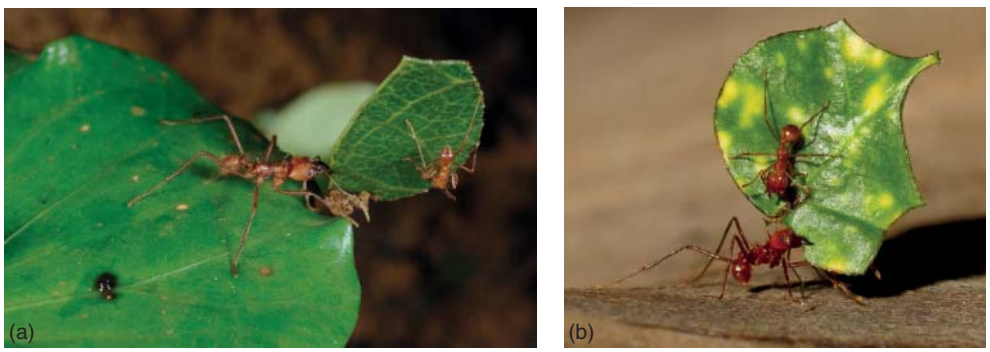

Figure 16.7 (a, b) Leaf-cutter ants. (© michaklootwijk – fotolia.com.)

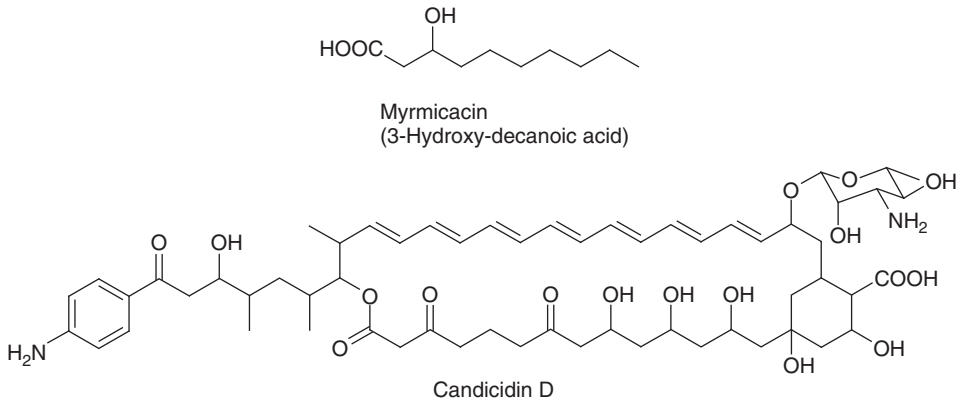

Figure 16.9 Inhibitors affecting ant–bacteria–fungi mutualism.

Leaf-cutter ants also defend their fungal culture against exploitation by pathogens or parasites. In addition to pruning, grooming, and exclusion of areas infected by other microbes, ants secrete antimicrobial and antifungal agents as well as growth hormones from their **metapleural glands**. Also, the antibacterial compound phenyl acetic acid is sprayed onto the fungal cultures to suppress bacterial growth, and myrmicacin (Figure 16.9) prevents the germination of spores of parasitic fungi.

The fungal genus *Escovopsis* (*Pezizomycotina, Ascomycota*) is a group of highly evolved obligate mycoparasites that is often encountered in the mutualistic *Lepiota* cultures. Further antibacterial compounds are formed by mutualism with bacteria in the integument and possibly in exocrine glands of leaf-cutting ants. *Pseudonocardia spec.* (Actinobacteria) live in specific crypts linked to exocrine glands on the ant's cuticula. This streptomycete species excrete the macrolide antibiotic candicidine (Figure 16.9) to protect the fungal monoculture against the pathogenic fungus *Escovopsis.* Furthermore, integumental biofilms protect the ants against ant diseases.

Recent studies suggest highly complex and diverse interactions including multiple microbial organisms that support the mutualism of leaf-cutting ants and their fungal gardens.

16.3 Phenolics in the Interaction between Plant and Animals

16.3.1 Salicin – a Defense Compound in Salix Species

Salicin is a simple benzenoid glycoside that is common in *Salix* species (*Malpighiales, Rosida*) and part of their plant defense (Figure 16.10). *Salix pentandra* contains acetylated salicin derivatives known as *salicinoids*, which, in addition to chlorogenic acid and flavonoids, defend the plant efficiently against polyphagous feeders. The plant is attacked only by specialist-feeding *Salix* insects.

No salicylates are formed by *Salix phylicifolia,* which is attacked by polyphageous insects. To study the effect of salicinoids, chlorogenic acid, and flavonoids on plant

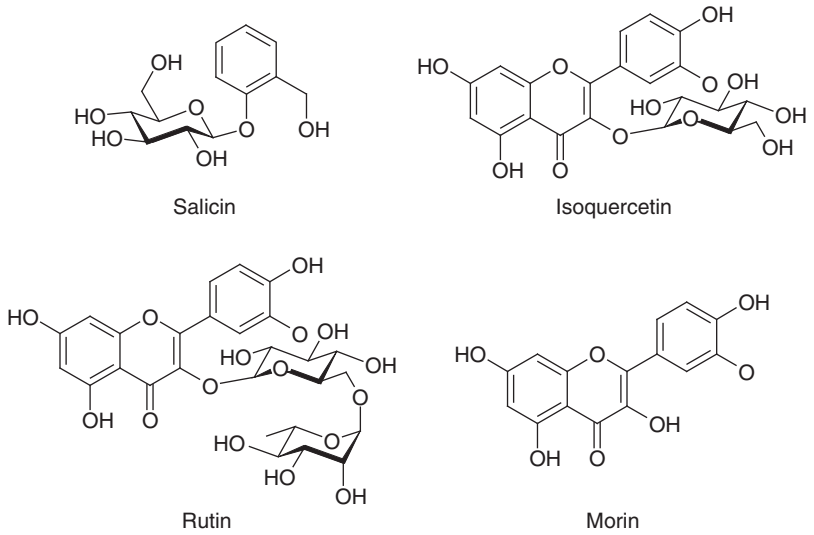

Figure 16.10 Phenolic compounds that guide interactions between plants and insects.

defense, the behavior of the polyphagous larvae of *Operophtera brumata* (*Insecta, Arthropoda*) was studied on three *Salix* species with different levels of these metabolites, *S. phylicifolia, Salix myrsinifolia*, and *S. pentandra*. A negative correlation between larval growth with the levels of salicylate and chlorogenic acid was demonstrated and, to a lesser extent, a positive correlation between larval growth and the levels of flavonoids, especially myrcetin. This indicated that salicinoids are more important than the levels of flavonoids in modulating the feeding behavior of generalist. Some specialist herbivores not only tolerate salicin but also utilize it for their own defenses. The beetle *Chrysomela aenicollis* (*Insecta, Arthropoda*) sequesters salicin and converts it to an aldehyde, which is a major component of its defensive secretion.

16.3.2 Flavonoid Signals Modulate Herbivore Behavior

Flavonoids can impact many aspects of plant–insect interactions besides providing flower color. They enable the recognition of host plants, but can also be feeding deterrents. Once flavonoids are taken up by an insect, they can often be sequestered and modified to increase its fitness.

The larvae of the blue butterfly *Polyommatus icarus* (*Insecta, Arthropoda*) sequester the flavonoids of their host plants. The predominant flavonoids of their host plants, derivatives of quercetin and kaempferol, are selectively sequestered and metabolized (Figure 16.10). Other flavonoids like myricetin derivatives, flavones, and isoflavonoids are mostly excreted. Flavonoids with simple glucose and galactose residues are preferred over those with other sugars or multiply glycosylated derivatives. Flavonoids are sequestered more efficiently in females, which results in more colorful wings. The greater color of females might serve as a visual signal to attract male mating partners.

Flavonoids play an important role in the interaction between the silkworm, *Bombyx mori* (*Insecta, Arthropoda*), and its host plant, the mulberry tree, *Morus alba* (*Rosales Rosidae*). The larvae of the silkworm moth can differentiate between the quercetin glycosides of mulberry leaves. The quercetin-3-*O*-glucosides isoquercetin and morin are feeding stimulants but the same moiety conjugated with rhamnose deters feeding (Figure 16.10). The quercetin-3-*O*-rutinoside does not affect the feeding behavior of the silkworm larvae. The cocoons of several *B. mori* strains contain the flavonoid 5-glucosides of quercetin, which are not present in mulberry leaves. Most likely, the *B. mori* larvae modify the sequestered flavonoids of their diet with a glucosyltransferase activity to produce the flavonoid 5-glucosides quercetin 5-*O*-glucoside, quercetin 5,4′-di-*O*-glucoside, and quercetin 5,7,4′-tri-*O*-glucoside.

Rutin, a derivative of quercetin, exhibits a more complex modulation of host selection behavior (Figure 16.10). This flavonoid stimulates feeding of many insects including *Heliothis virescens* and the locusts *Schistocerca americana*, *Schistocerca albolineata,* and *Melanoplus differentialis* (all *Insecata, Arthropoda*). For the larvae of *Helicoverpa zea*, *Helicoverpa armigera*, *Spodoptora littoralis*, *Spodoptora exigua,* and *Spodoptora exempta* (all *Insecata, Arthropoda*), low concentrations of rutin stimulate feeding, but higher concentrations deter the larvae. In addition, cues other than the concentration of rutin may affect the feeding behavior of these insects. For the larvae of *Trichoplusia ni* (*Insecata, Arthropoda*), a diet of soybean (*Glycine max, Fabales, Rosidae*) with a combination of rutin, quercertin 3-glucosylgalactoside, and the isoflavone genistin acted synergistically to disrupt the consumption of food. In contrast, the quercetin 3-glucoside in the pollen of sunflower *Helianthus annuus* (*Asterales, Asteridae*) promotes feeding in the western root corn worm *Diabrotica virgifera* (*Insecata, Arthropoda*). Derivates of quercetin also stimulate the probing behavior in the bean aphid *Megoura crassicauda* (*Insecata, Arthropoda*) on its host, the narrowleaf vetch (*Vicia angustifolia, Fabales, Rosidae*).

16.4 Alkaloids in the Interaction between Plants and Animals

16.4.1 The Monarch Butterfly

The monarch butterfly *Danaus plexippus* (*Insecta, Arthropoda*) is easily recognizable by the orange and black pattern on its wings. It is well known for its extensive migration between Canada and Mexico. The monarch butterfly is also able to make transatlantic crossings to the Canary Islands, the Azores, Madeira, and occasionally to Western Europe. These butterflies are also found in Australia and New Zealand. Travel over such long distances requires several generations as the normal lifespan of a butterfly is about 2 months. Studies indicate that the animals can sense the magnetic field or the earth and have photoreceptors sensitive to violet and blue light to assist them in their multigeneration migrations.

The monarch butterfly is an excellent example of a dietary specialist that exploits the chemical defenses of it host plants as an advantage. The larvae feed on a subset of the species of the genus *Asclepias* (milkweeds, *Gentianales, Asteridae*) and closely related plants that often contain cardenolides (cardiac glycosides) (Figure 16.11). These toxic steroids impair a sodium potassium ATPase in cell membranes that channels ions through the membrane to uphold the membrane potential.

The ion channel proteins of the monarch butterflies are altered such that they are not affected by the toxin. These insects can sequester cardenolides that accumulate in the wings and abdomen and make the butterfly poisonous or distasteful to many birds and mammals. Such predators

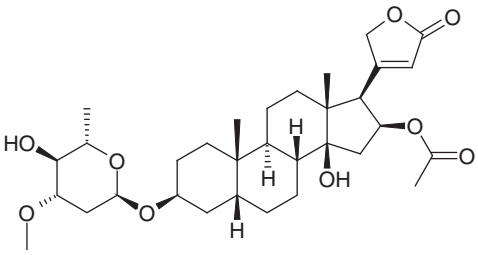

Figure 16.11 *The monarch* butterfly (*Danaus plexippus*) accumulates cardenolides-like oleandrin from its diet on milkweed *(Asclepias sp.*). (© tppix – fotolia.com.)

associate the bad taste with the bright colors and easily recognizable pattern of the butterfly to avoid it after a first experience. The bird *Pheuticus melanocephalus* (black-headed grosbeak, *Aves*, *Sauropsida*) is immune to the toxic glycosides and can, therefore, prey on the butterflies when they overwinter in Mexico. Some other birds have learned to eat only the thoracic muscles or abdominal parts that contain a lower toxin concentration. Some mice can also tolerate higher doses of cardenolides. As the concentration of cardenolides decreases over the winter, the adult butterflies become less poisonous and are more preyed on by birds and mice.

16.4.2 Optimal Defense in the Wild Ragwort Senecia jacobea

Senecio jacobeaa (*Asterales, Asteridae*), a common wild flower of the *Asteraceae* family, accumulates high concentrations of pyrrolizidine alkaloids (Figure 16.12). These metabolites are highly toxic to grazing livestock, vertebrates, nematodes, fungi, and insects. In flowering ragwort plants, a blend of 18–24 pyrrolizidine alkaloids was detected. The alkaloids are synthesized in the roots of the plant from the onset of seedling growth. During development, the metabolites are exported from the roots in the form of senecionine N-oxide and accumulate in the aboveground organs. During allocation in the aboveground tissues, the alkaloids are structurally diversified. Flowers accumulate high levels of senecionine while jaconine is the dominant alkaloid compound in leaves. An analysis of the metabolites in each organ showed differences for classes of other secondary metabolites as well. Several derivatives of flavonoid glucosides and free flavonoid structures occur in higher concentrations in the flowers than in new or old leaves. Flowers also contain higher concentrations of octadecadienoic acids and octadecenoic acids while more chlorogenic acid and oxododecanoic acid was detected in leaves. Chlorogenic acid, jacaranone, and kaempferol have been reported to affect insect performance. Kaempferol was found especially associated with resistance against thrips in hybrids of *Senecio jacobaea* and *Senecio aquaticus*.

Using a metabolomic approach (see Chapter 18) to examine the changes in many groups of secondary metabolites

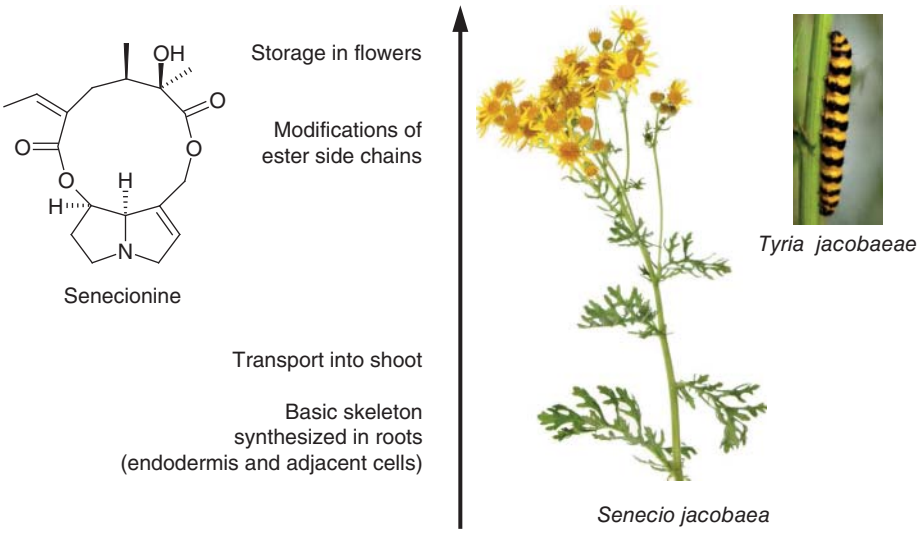

Figure 16.12 Biosynthesis, modification, and storage of the pyrolizidine alkaloid secionine in ragwort (*Senecio jacobaea*). The cinnabar moth *Tyria jacobaeae* is a specialized herbivore of *S. jacobaea* that accumulates the toxic and bitter-tasting alkaloids of the host plant to defend itself. (Ragwort © Richard Griffin – fotolia.com, *S. jacobaea* © mbz1 – fotolia.com.)

simultaneously during development results in a more complete view of the plant's defense potential compared to a targeted analysis of just one key compound. When this approach was performed in *S. jacobaea*, significant differences between young and old leaves and between leaves and flowers were found. Younger leaves were found to be better defended than older ones. From seedling to juvenile stage, terpenoids increased and alkaloids decreased. At least six pyrrolizidine alkaloids had higher levels in flowers compared to leaves, one of them being identified as senecionine. Four different pyrrolizidine alkaloids were higher in leaves, one of them being jaconine. A few compounds showed no difference in distribution between plant parts. The biological significance of different metabolite blends in plant organs is yet to be assessed, but the available data suggest that different tissues adopt different defense strategies to prevent herbivory. A theoretical framework for the prediction of organ-specific defenses was termed the *optimal defense theory*. This theory builds on the assumption that the plant distributes defenses according to the importance of the tissues, as it is too costly for the plant to allocate a maximum of defense metabolites to all tissues. For example, new leaves are more valuable than old leaves because of their higher metabolic potential and flowers are more valuable than leaves because they are necessary for reproduction.

For the specialist herbivore *Tyria jacobaeae* (*Insecta, Arthropoda*), the cinnabar moth, the pyrrolizidine alkaloids are not a feeding deterrent, but a cue for oviposition and host plant recognition (Figure 16.12). The cinnabar moth assimilates pyrrolizidine alkaloids for its own defense. Its predators associate the toxins and bitter taste with the bright colors of both the larvae and the moths and attack the insect only rarely.

16.4.3 Endophytes and Plant Parasitic Nematodes Mediate Plant–Herbivore Interactions

Plant resistance to invertebrate and mammalian herbivores can be increased by endophytes that produce secondary metabolites (see Chapter 3). Mycotoxin alkaloids were detected in many cool season agronomic grasses like *Festuca arundinacea* (Poaceae) and *Lolium perenne* (both *Poales, Liliopsida*). Infestation of the cabbage *Brassica oleracea* var. *gemmifera* (*Brassicales, Rosidae*) with the unspecialized endophyte *Acremonium alternatum* (*Pezizomycotina, Ascomcota*) lowers the growth rates and survival of the larvae of the diamondback moth *Plutella xylostella* (*Insecta, Arthorpoda*), one of the major pests of cabbage. The reduced survival of the larvae was possibly because of an altered phytosterol metabolism of the plants.

Plants can be associated with a large number of endophytic bacteria and fungi, including mycorrhizal fungi that form hyphal associations with the roots of their respective host plants and improve nutrient uptake from the soil (see Chapter 5). Interestingly, the beneficial effect of the foliar endophyte *Neotyphodium lolii* (*Pezizomycotina, Ascomcota*) on perennial ryegrass (*Lolium perenne, Poales, Liliopsida*) was reduced after infection with a mycorrhizal fungus. Because of the complexity of these interactions, the biochemical cues regulating the interactions and its consequences for plant defense toward herbivores are very difficult to predict. Further studies observed that when herbivores interacted first with the plant, the likelihood and extent of subsequent mycorrhizal colonization of the shared host was altered: in 50%, the colonization with a mycorrhizal fungus was inhibited, in 25% it had no effect, and in the rest it facilitated colonization.

Of the many species of nematodes associated with plants, only a few are plant parasites. Usually, populations of parasitic nematodes within the plant are too small to cause serious plant damage. But in larger numbers, parasitic nematodes can cause significant damage to the plant. The free-living nematode *Caenorhabditis elegans* (*Chromadorea, Nematoda*) is one of the major model species in biology. *Caenorhabditis elegans* can mediate beneficial interactions between legume plants and rhizobium bacteria. *Medicago truncatula* (*Fabales, Rosidae*) emits dimethyl sulfides, which is an attractant for *C. elegans*. It transports rhizobia (*Rhizobium,* Alphaprotobacteria) close to the legume roots and thus supports the initiation of symbiosis. Much of the biology of *C. elegans* is comparable to that of parasitic nematodes. Therefore, this model system can be utilized to understand the biochemical and molecular bases of parasitism by nematodes.

Parasitic nematodes rely on a single feeding site to obtain all the nutrients required for their development until the adult stage. After invading, both root cyst and root knot nematodes induce a permanent feeding site within cells close to the phloem and form complex biotrophic feeding structures. The changes in the plant gene expression during the formation of the feeding site affect many genes of the cell cycle and cytoskeleton. In *C. elegans* and other parasitic species, a large family of **glutathione-S-transferases** is involved in scavenging reactive oxygen species (see Chapter 8), which are released by plants on infection. The secretions of a range of parasitic nematodes contain proteins that bind fatty acids and retinol to modulate or manipulate the host plant defense. Lipid-binding proteins have the potential to interfere with the host plant lipid signal pathway and modify the acquisition of essential lipids from the host. The surface coat of parasitic nematodes forms the first contact when they migrate into host plant tissue. Compounds from the nematode surface coated together with pharyngeal and amphidial secretions are the first molecules to manipulate and interfere with the host's immune system and appear to establish the parasitic interaction.

Another type of nematodes, the **root lesion nematodes**, does not establish a biotrophic, plant-parasitiod relationship but forms necrotic lesions. The genus *Pratylenchus* (*Chromadorea, Nematoda*) infects over 350 host plants including important crops such as potato, corn, and wheat. This nematode enters the root using the stylets of its mouthpart to puncture the root. The root begins to turn brown or black forming necrotic lesions at this site of entry. This leads to the damage of the root system, which becomes manifest in secondary symptoms like stunting, leaf chlorosis, and wilt.

16.4.4 Glucosinolates Are Pungent Antiherbivore Defenses of Mustards and Related Plant Species

Besides alkaloids, another group of nitrogen-containing plant defense compounds, are the glucosinolates (see Chapter 2), which occur in the *Brassicaceae*, *Capparaceae* (both *Brassicales, Rosidae*), and a number of related plant families. Derived from amino acids, each glucosinolate molecule also contains at least two sulfur atoms and a glucose residue (Figure 16.13). Glucosinolates are responsible for the flavor and taste of vegetables, such as cabbage and broccoli (both *Brassica oleracea*), and condiments, such as mustard (*Sinapis hirta, Brassica juncea, Brassica nigra,* e.g., sinigrin, Figure 16.13) and horseradish (*Armoracia rusticana*). But, to plant herbivores, glucosinolates represent defense compounds. Many studies have shown that glucosinolates are toxins, growth inhibitors, or feeding deterrents to a wide range of insects, mammals, mollusks, and nematodes.

The basis of glucosinolate toxicity is their hydrolysis on plant damage. Plants that produce glucosinolates also produce a glucohydrolase enzyme known as *myrosinase* that cleaves the glucose from the rest of the glucosinolate skeleton (Figure 16.13). The remaining compound then is unstable and rearranges to form an isothiocyanate, nitrile, or aldehyde. In intact plants, glucosinolate hydrolysis is prevented by the spatial separation of glucosinolates from myrosinase or the inactivation of this enzyme. However, these components become mixed together on plant damage leading to the very fast formation of glucosinolate hydrolysis products. The antiherbivore activity of glucosinolates as well as most of their other biological activities is attributed to their hydrolysis products.

Glucosinolates are general toxins and deterrents for many herbivores and pathogens, but certain species have come to specialize on glucosinolate-containing plants and use these compounds as cues for feeding or egg-laying. Such specialist feeders must have some mechanism for avoiding the toxicity of the glucosinolates found in their host plants. Larvae of the diamondback moth (*P. xylostella*) and the desert locust (*Schistocerca gregaria*) cleave a sulfate group from the glucosinolate structure so that it can no longer be hydrolyzed by myrosinase. Another specialist, the cabbage white butterfly (*Pieris rapae, Insecta, Arthropoda*), produces a protein that redirects myrosinase-catalyzed hydrolysis toward the formation of less toxic hydrolysis products.

Once specialist herbivores overcome the defensive chemistry of their hosts, they may, like the monarch butterfly, be able to store plant defense compounds in their own bodies without harm and exploit them in their own protection. Several insects have been described that sequester glucosinolates, including the sawfly *Athalia rosae* and the aphid *Brevicoryne brassicae* (all *Insecta, Arthropoda*). Glucosinolate sequestration has been shown to deter predators such as ants, lizards, and birds.

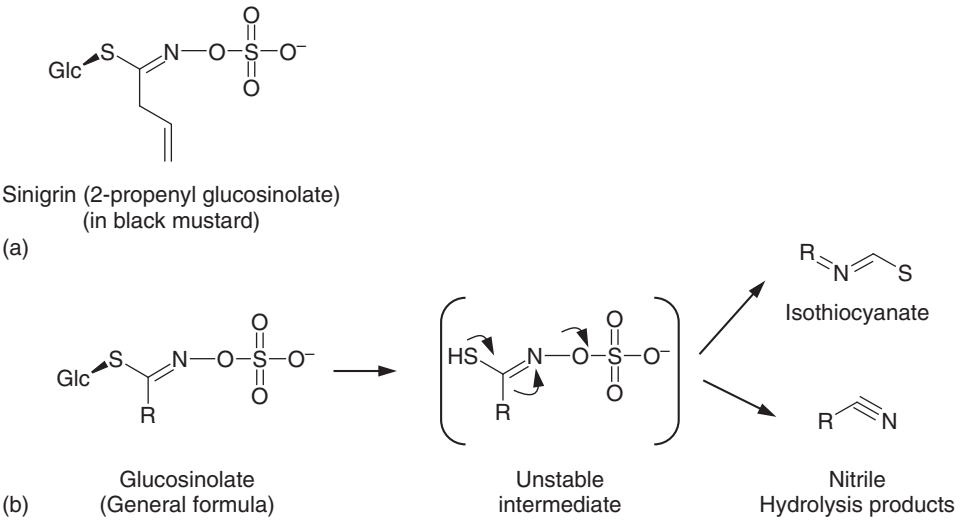

Figure 16.13 Glucosinolate defense of the plant. (a) Structure of glucosinolates. (b) The hydrolysis of glucosinolates on plant damage via myrosinase cleavage leads to biologically active products.

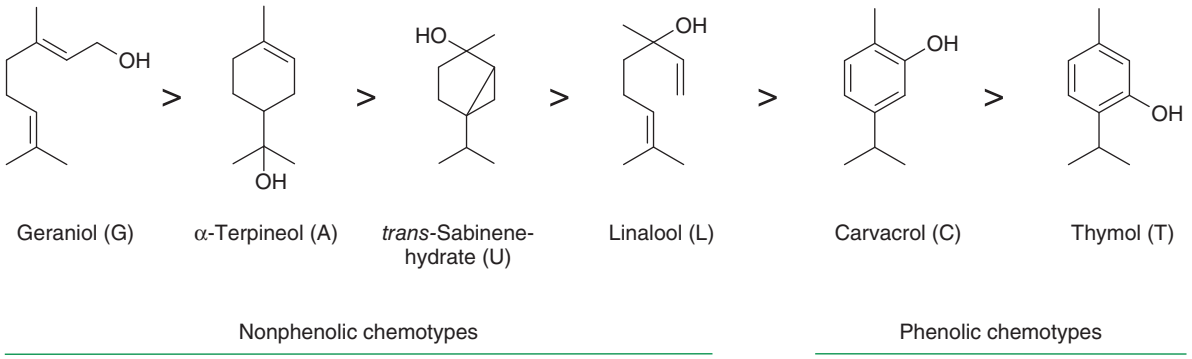

Figure 16.14 The chemotypes of thyme are morphologically similar but differ in monoterpene production. Each chemotype is characterized by its major monosesquiterpene alcohol. When crossed whith each other, the chemotypes form an epistatic row that is illustrated by the > signs. Each chemotype is dominant over those to the right but recessive to the chemotypes on its left.

16.5 Terpenes in Plant Defense

Terpenes form the largest group of metabolites among the natural products of plants (see Chapters 2 and 3). Many of the 30 000 known terpenes, especially monoterpenes, sesquiterpenes, and irregular terpenes of low molecular weight have high vapor pressures. These vapor pressures result in the emission of these compounds from the plant into the surrounding environment unless they are stored in specialized terpene storage organs, for example, the glandular trichomes of mints and the resin ducts in conifers. Although terpene volatilization from flowers has been known for many years, these compounds are also emitted from the vegetative tissues of plants. A detailed analysis of terpene production and storage organs among plant species has revealed several terpene-based defense strategies.

16.5.1 Monoterpene-Based Defenses of Thyme

The essential oil of thyme (*Thymus vulgaris, Lamiales, Asteridae*) is dominated by monoterpenes. Because of their often volatile and lipophilic nature, monoterpenes can disturb the integrity and function of biological membranes. To avoid autotoxicity, most monoterpene biosynthesis is restricted to glandular trichomes on the leaf surface. These trichomes contain a ring of secretory cells that produce the terpenes and transport them into a subcuticular space where the essential oil accumulates. Damage to the leaf will break the cuticula of the glandular trichome and expose the attacking organism to the essential oil. Depending on its composition, the monoterpenes of the essential oil exhibit antibacterial and antifungal properties as well as repellence or toxicity against nematodes, mites, mollusks, and insects.

In natural populations, thyme appears in at least seven chemotypes. Chemotypes are morphologically identical subpopulations that differ in the composition of their essential oils (Figure 16.14). Each of the chemotypes is distinguished by a dominant monoterpene that is used to name the chemotypes, and can be either geraniol (G-type), α-terpineol (A-type), sabinene hydrate (U-type), linalool (L-type), thymol (T-type), or carvacrol (C-type). The six chemotypes form an epistatic series in which the geraniol-type is dominant over all other chemotypes. The thymol and carvacrol types are recessive to all other types. The chemotypes segregate among habitats and show differences in survival depending on climatic conditions and the properties of the essential oils in defense against their enemies (Figure 16.15). The C- and T-types are relatively susceptible to cold temperatures, but well defended against snails of the genus *Helix* and *Deroceras* (both *Gastropoda, Mollusca*), which are deterred by these chemotypes. In addition, carvacrol and thymol have high antibacterial and antifungal activities that will defend the plant against microbial attack. These lipophilic monoterpenes have an aromatic system of delocalized electrons that allows the molecule to act as a proton exchanger and reduces the gradient across microbial cytoplasmic membranes. The essential oil of the L-type is dominated by linalool that has less antibacterial activity, but is highly deterrent to grasshoppers and sheep. The distribution of the chemotypes

Snail *(Helix)*	L	A	U	G	T	C
Slug *(Deroceras)*	G	A	L	U=C=T		
Cricket *(Leptophyes)*	T	C	A	G=U		L
Goat *(Capra)*	A	C	L	T	G	U
Sheep *(Ovis)*	U	T	A	G	C	L

Figure 16.15 Chemotypes of thyme differ in their deterrence against herbivore enemies. For the one-letter designation of the chemotypes, refer to Figure 16.14.

within their respective environments indicates that each of the monoterpenes provides a specific, adapted defense strategy.

16.5.2 Mammals, Wood Ants, and Scots Pine Trees

Scots pine trees (*Pinus sylvestris, Pinales, Acrogymnospermae*) are geographically widespread and characterized by substantial concentrations of monoterpenes in their tissues. The monoterpene composition is highly variable not only between tree populations but also between individuals within a population. The highest variation was observed for the monoterpene δ-3-carene, which is either absent or forms up to a quarter of the resin (Figure 16.16). This resin composition profoundly impacts the interactions with the organisms of the environment. The concentration of δ-3-carene is negatively correlated with a reduction in tree damage by red deer (*Cervus elaphus, Mammalia, Vertebrata*), but does not affect herbivory by bank voles (*Myodes glareolus, Mammalia, Vertebrata*). The young seedlings of Scots pine are attacked by slugs (*Arion ater, Gastropoda, Mollusca*). These highly impacting herbivores are deterred by increasing monoterpene concentrations in the resin and by a higher proportion of δ-3-carene to α-pinene and β-pinene.

Wood ants (*Insecta, Arthropoda*) form the third trophic level in many interactions between Scots pine and herbivores. They can both predate on or tend aphids (*Insecta, Arthropoda*) on Scots pine. In the first case, the herbivore damage is reduced. In the second, the herbivore load on the tree may be increased. But while tending the aphid colonies, it was discovered that the ants will attack other arthropods that may be herbivores or predators of herbivores. Thus, the ants can strongly affect the herbivore load on the tree. The ants show a strong preference for trees with low concentrations of δ-3-carene, a high number of aphids, and a low number of nonaphid herbivorous arthropods.

The monoterpenes of Scots pine also affect the composition of organisms in the environment belowground. Monoterpenes are introduced into the ground by needle litter. Because of their volatility, the concentrations of gaseous monoterpenes in conifer-dominated soils can be high within the first 6 months of decomposition. The decomposition of δ-3-carene is slower than that of α-pinene and β-pinene. This may contribute to the observation that the litter of trees with low δ-3-carene concentrations contains more species and decomposes faster, which in turn affects plant nutrition. A direct effect of monoterpenes on nitrifying bacteria and the immobilization of labile nitrogen by soil organisms can also affect nitrification and soil nitrogen cycling (see Section 6.3.3.1).

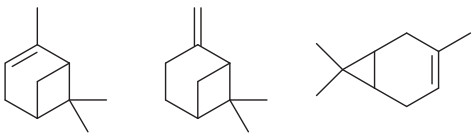

Figure 16.16 Major monterpenes in the resin of Scots pine (*Pinus sylvestris*.)

16.5.3 Ecological Role of Herbivore-Induced Plant Volatiles

Hydrophobic metabolites of low molecular weight have a high vapor pressure and will volatilize under physiological conditions. When these metabolites are released by the aboveground parts of plants, they diffuse into the atmosphere surrounding the plant. Volatiles emitted by the roots will diffuse into the rhizosphere and surrounding soil where they will be eventually absorbed by soil particles and dissolved in the aqueous phase. These physical properties make volatiles well suited as intraspecific and interspecific signaling compounds.

While low concentrations of volatile organic compounds are produced in almost every plant tissue, the highest concentrations from vegetative tissues are released in response to environmental cues. Emission increases in response to the damage of herbivory, and abiotic factors like wounding, UV-radiation, O_3 and CO_2 concentration, nutritional status of the plant, as well as temperature and light. Plant volatile blends can be very complex, sometimes consisting of hundreds of compounds. These include green leaf volatiles (e.g., hexanal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate), terpenoids (*E*)-β-ocimene, (*E*,*E*)-α-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT)) phenylpropanoids (e.g., methyl salicylate, indole), and sulfur- or nitrogen-containing compounds (e.g., isothiocyanates or nitriles, Figure 16.13). Unlike aromatic volatiles and fatty acid-derived signals, terpenes are commonly released after *de novo* synthesis and emitted in complex blends with a large structural diversity of compounds. Responsible for most of this terpene diversity are multiproduct terpene synthases, the key enzymes of terpene biosynthesis. Terpene synthases can produce blends of up to 50 different compounds from one substrate and form mixtures with defined relative ratios of products. In communication with other organisms, these mixtures might provide a more specific signal than signals consisting of single terpenes. Despite the enormous number of terpenes emitted by plants, relatively few terpenes have been clearly identified as signals between organisms to date.

16.5.4 Tritrophic Interactions with Herbivores and Herbivore Enemies

Herbivore damage to certain plants induces the emission of volatile organic compounds that attract natural enemies of the herbivores. This phenomenon has been reported in more than 15 different plant species after feeding by

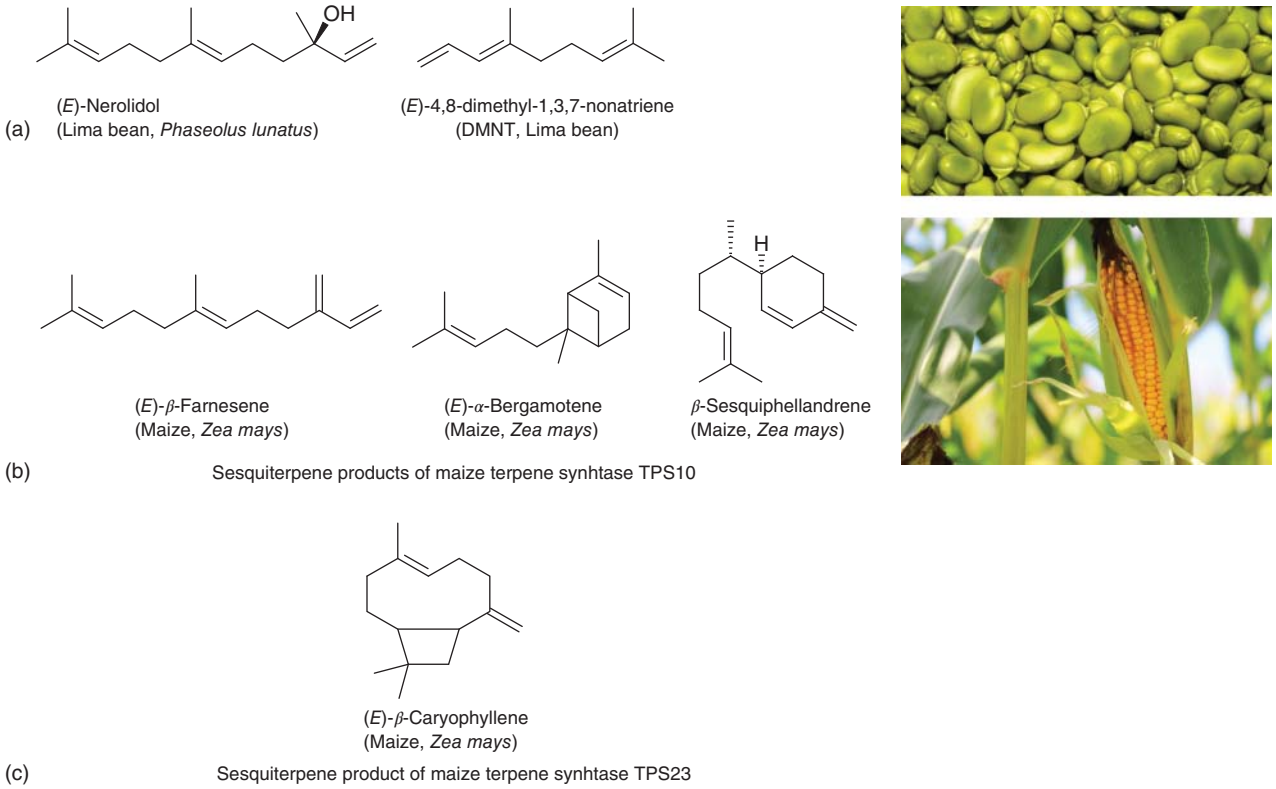

Figure 16.17 Terpene volatiles involved in volatile plant defenses of (a) lima bean, (b–c) maize. (b) Sesquiterpene products of the maize terpene synhtase TPS10. (c) Sesquiterpene product of the maize terpene *synthase* TPS23. (bean: © photopitu – Fotolia.com; maize: © siwi1 – Fotolia.com.)

an assortment of arthropod herbivores and was termed *indirect defense*. The herbivore enemies that respond to volatiles from herbivore-damaged plants include various carnivorous arthropods, both predators and parasitoids. Attraction of herbivore enemies has been shown to benefit the plant by reducing subsequent herbivory but it has been difficult to show that it increases reproductive fitness. The attraction of the predatory mite *Phytoseiulus persimilis* (*Arachnida, Arthropoda*) to plants of lima bean (*Phaseolus lunatus, Fabales, Rosidae*), infested with the spider mite *Tetranychus urticae* (*Arachnida, Arthropoda*), has been studied in detail. Olfactory assays testing the attraction of single compounds from the complex volatile blend demonstrated that the predatory mite was attracted not only to the aromatic compound methyl salicylate, but also to the sesquiterpene alcohol nerolidol.

Transgenic *Arabidopsis thaliana* (*Brassicales, Rosidae*) overexpressing a nerolidol synthase from strawberry was used as volatile source in olfactometer experiments with *P. persimilis*. The plants emitting the nerolidol were more attractive to the predator than undamaged wild type plants. In addition to an innate attraction to compounds of the plant volatile bouquet, the predatory mites also have the ability to associate odors with host presence. The homoterpene (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, an irregular C_{16} terpene olefin, is released from lima bean in response to feeding by *T. urticae* but not by the nonhost organism *Spodoptera exigua* (*Insecta, Arthropoda,* Figure 16.17). After a series of experiences in the presence and absence of the prey, the predatory mite utilized the homoterpene as an indicator for the presence of prey. The ability for associative learning may guide the predatory mites to locate their prey under natural conditions in a complex environment. Not only does this help the predator to identify plants infested with prey, but also allows it to adapt when the prey switches between different host species during the season.

Maize plants (*Zea mays, Poales, Liliopsida*) damaged by larvae of lepidopteran herbivores like *Spodoptera littoralis* emit a complex blend of volatiles dominated by mono- and sesquiterpenes. These volatiles attract females of the parasitic wasp *Cotesia marginiventris* (*Insecta, Arthropoda*), which use the lepidopteran larvae as hosts (Figure 16.6a). This parasitization might benefit the maize plants as the parasitized larvae feed less and will not procreate. As the herbivore-induced volatiles of maize consist of a complex blend compounds, it is difficult to demonstrate which of the compounds are attractive to the parasitic wasp.

The major sesquiterpenes of herbivore-induced maize are produced by the terpene synthase TPS10, which is strongly expressed after herbivory by lepidopterans (Figure 16.17). TPS10 forms (*E*)-β-farnesene, (*E*)-α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons from the substrate farnesyl diphosphate. Overexpression of *tps10*

in *A. thaliana* resulted in plants emitting high quantities of TPS10 sesquiterpene products identical to those released by maize. Using these transgenic *Arabidopsis* plants as odor sources in olfactometer assays, showed that females of the parasitoid *C. marginiventris* learn to exploit the TPS10 sesquiterpenes to locate their lepidopteran hosts after prior exposure to these volatiles in association with hosts. This gene-based dissection of the herbivore-induced volatile blend demonstrates that a single gene such as *tps10* can be sufficient to mediate the indirect defense of maize against herbivore attack. Furthermore, associative learning can also adapt parasitoids to alterations of the herbivore-induced volatile blend by plant species, age, and tissue of the plant and abiotic conditions. However, females of *C. marginiventris* are also attracted to the full blend of maize volatiles without prior association, indicating that the blend contains additional attractive compounds that elicit an innate response. So far, bioassay-guided fractionation of the maize volatiles has not yet identified such compounds. The combination of both innate and learned responses might allow this generalist parasitic wasp to locate a wide range of hosts on different plant species in a natural, complex environment. Interestingly, the emission of volatiles by herbivore damage is not only beneficial for the maize plant as larvae of lepidopteran *Spodoptera frugiperda* use these volatiles to find their food plants. Further studies are required to determine whether the benefit of the volatile signal outweighs its disadvantages under specific environmental conditions and within different ecological contexts (Figure 16.18).

The function of terpenes as defense signals under field conditions was studied on a wild tobacco species, *Nicotiana attenuata* (*Solanales, Asteridae*). The release of terpenes was mimicked by application of a lanolin paste that emitted physiological concentrations of the monoterpene linalool and the sesquiterpene (*E*)-α-bergamotene. The emission of exogenous linalool decreased lepidopteran ovipositon rates on *N. attenuata* plants while the release of (*E*)-α-bergamotene increased egg predation rates by a generalist predator. These observations provided conclusive evidence that indirect, terpene-based plant defenses can reduce the herbivore load of a plant in a natural environment (Figure 16.17).

Terpene-mediated interactions have not only been observed in response to damage of the leaves but also in response to root-feeding herbivores. Larvae of the beetle *Diabrotica virgifera virgifera* (Western corn rootworm, *Insecta, Arthropoda*) are an important pest of maize (Figure 16.18b). In response to feeding by the larvae, maize roots release a signal that strongly attracts the entomopathogenic nematode *Heterorhabditis megidis* (*Chromadorea, Nematoda*). The signal released by the maize roots was identified as (*E*)-β-caryophyllene, a sesquiterpene olefin (Figure 16.18). Most North American maize lines do not release (*E*)-β-caryophyllene, whereas European lines and the wild maize ancestor, teosinte, do so in response to *D. v. virgifera* attack. Field experiments showed a fivefold higher nematode infection rate of *D. v. virgifera* larvae on a maize variety that produces the signal than on a variety that does not. Spiking the soil near the latter variety with authentic (*E*)-β-caryophyllene decreased the emergence of adult *D. v. virgifera* to less than half.

Not only feeding, but also oviposition of the herbivore can induce terpene emission in plants. The pine sawfly (*Diprion pini, Insecta, Arthropoda*) lays its eggs on pine twigs and wounds the surface of the needles in the process. The volatiles emitted in response to oviposition attract a wasp that parasitizes sawfly eggs. The signal attracting the wasp is the sesquiterpene (*E*)-β-farnesene, which is only recognized in combination with other constitutively released pine volatiles.

16.5.5 Interference of Plant Volatile Terpenes with Insect Pheromones

Many species of aphids release a sesquiterpene alarm pheromone, (*E*)-β-farnesene, that lowers the risk on the predation of other aphids in the vicinity, either

Figure 16.18 Volatile-mediated interactions between maize, herbivores, and herbivore enemies. (a) Feeding by larvae of *Spodoptera littoralis* results in the emission of a complex volatile blend that attracts females of the braconid wasp *Cotesia marginiventris*. (b) Root damage by larvae of *Diabrotica virgifera virgifera* induces emission of the sesquiterpene (*E*)-b-caryophyllene that attracts the nematode *Heterorhabditis megidis* that is visible as small filaments. Photos by M. Held, S. Rasmann and T.C.J. Turlings, University of Neuchatel.

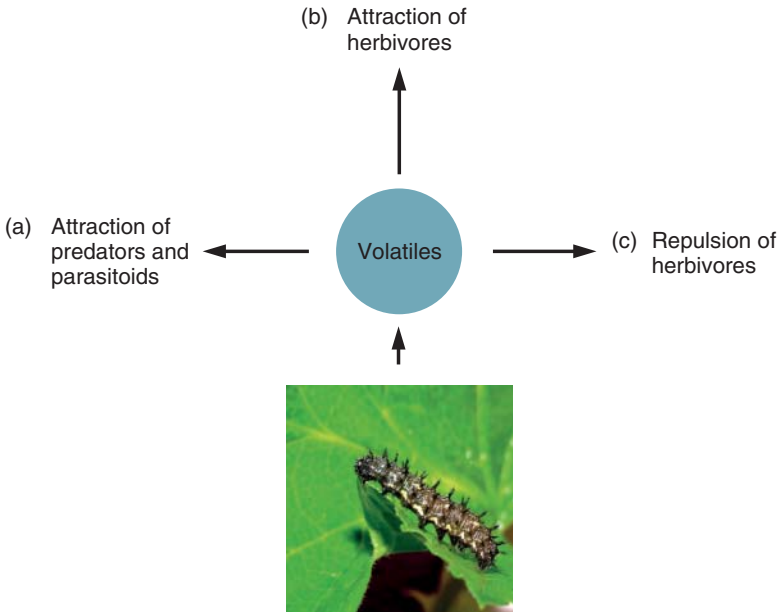

Figure 16.19 Interactions between plants, herbivores, and herbivore enemies mediated by volatile terpenes. (a) After herbivore damage triggers the release of terpenes, these substances attract predators and parasitoids that prey on the herbivores or use them as hosts for their larvae. The same terpenes can also affect other herbivores, either (b) attracting them to feed or (c) repelling them from feeding or oviposition. (Photo: © ChriSes – fotolia.com.)

by causing them to move away, or by increasing their proportion of winged progeny (Figure 16.6). To determine if plant-produced (*E*)-β-farnesene can mimic these effects, transgenic *Arabidopsis* plants overexpressing a (*E*)-β-farnesene synthase from *Mentha x piperita* (peppermint, *Lamiales, Asteridae*) were generated. These transgenic plants emitted high levels of (*E*)-β-farnesene and elicited potent alarm and repellent responses in the aphid *Myzus persicae (Insecta, Arthropoda*) and an arrestant response in the aphid parasitoid *Diaeretiella rapae (Insecta, Arthropoda*). In addition, (*E*)-β-farnesene attracted further predators and parasitoids, which are natural enemies of aphids. Thereby, the emission of (*E*)-β-farnesene after aphid-infestation provided the plant with both direct and indirect defenses. The monoterpene alcohol linalool, albeit not an alarm pheromone, also repelled *M. persicae* in experiments with transgenic *Arabidopsis* plants overexpressing a terpene synthase from strawberry.

16.5.6 Terpene-Mediated Interactions between Plants Affect Herbivores

Volatile-emitting plants can prime defense metabolism in adjacent plants. The process involves increased transcription of defense-related genes and allows the plant to respond faster and more vigorously to future herbivore attack (Figure 16.19). Most of these interactions were shown to be based on volatiles derived from the lipoxygenase pathway, the so-called green leaf volatiles. Little is known about the role of terpenes in priming and plant–plant interaction. Only one study in lima bean (*P. lunatus*) suggests a role of terpenes in this interaction. When these plants were attacked by the spider mite *T. urticae*, the neighboring plants became less susceptible to spider mites and more attractive to predatory mites like *P. persimilis*. Lima bean infested by spider mite released a volatile blend dominated by (*E*)-β-ocimene and DMNT, a monoterpene and C_{11} homoterpene, respectively (Figure 16.17). In neighboring plants, each of these compounds induced the transcript level of pathogen-related proteins and phenylalanine ammonia lyase. The volatiles also increased the transcript concentrations of two enzymes involved in terpene biosynthesis: lipoxygenase catalyzes an early step in jasmonate biosynthesis, an important regulator of terpene biosynthesis, and farnesyl diphosphate synthase, which is an enzyme of terpene biosynthesis. Interestingly, the transcript levels of these genes were induced more quickly after the exposure of lima bean to DMNT and a related homoterpene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. In other plant species, these terpenes were not effective in priming. In maize, for example, no effects were observed after the exposure of the plant to exogenous DMNT.

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Part V
The Methodological Platform

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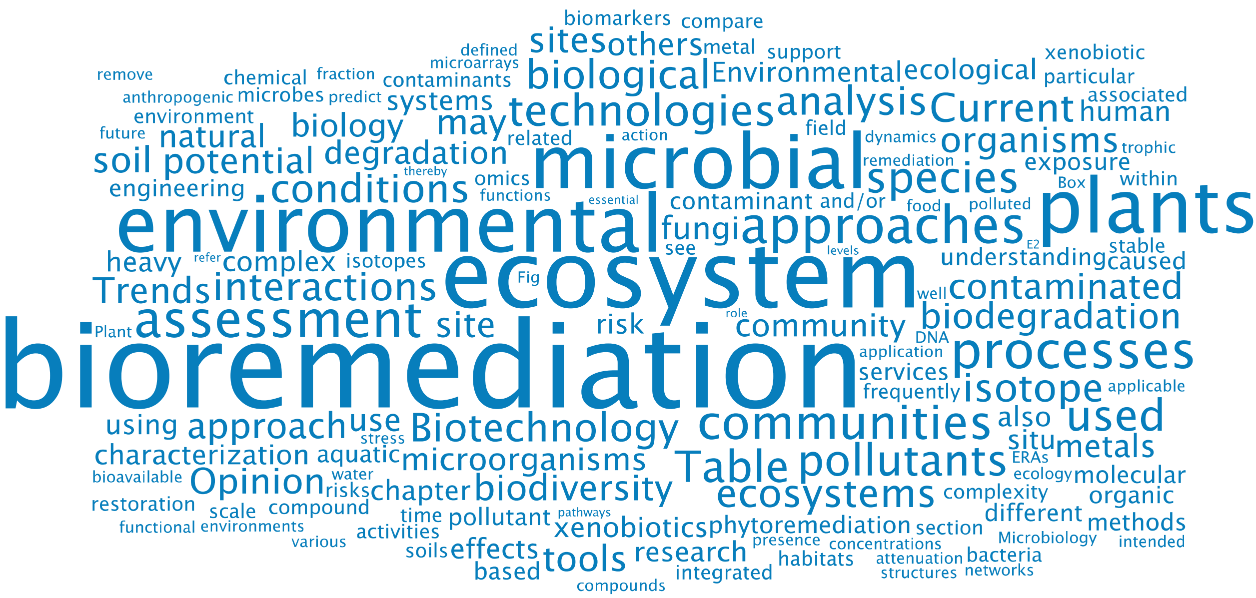
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17
Sensing of Pollutant Effects and Bioremediation

Gerd-Joachim Krauss and Dietmar Schlosser

Overview

The anthropogenic impacts on aquatic and terrestrial ecosystems are highly variable and result from numerous activities such as agricultural, aquacultural, and industrial projects, coastal engineering, urban development, and tourism. Environmental changes reduce biodiversity and ecosystem health frequently, leading to disturbances in ecosystem structure, dynamics, and functionality. Nevertheless, adaptive stress responses evolved by organisms can compensate adverse impacts on ecosystems.

Ecosystem research requires the understanding of general characteristics of ecosystem functionality. This includes the knowledge about biochemistry, physiology, biodiversity, dynamics of biological ecosystem components (individual organisms, populations, communities) under stress, and of the related capacities of ecosystems (e.g., with respect to resilience and functional redundancy) to respond to a changing environment.

A deterioration of the status of an ecosystem needs **ecosystem services**. Environmental research can help to maintain or, if necessary, to restore ecosystems. Related areas of attention include the characterization of pollutant effects and exposures, which under certain circumstances may result in the application of a suitable bioremediation scheme.

17.1
Pollutant Effect and Approaches to Characterize Exposure

Both ecological effect and exposure assessment are crucial for the characterization of risks caused by environmental pollutants to ecosystems. Methods for assessing environmental pollutant effects need to be considered when dealing with different time scales and levels of biological organization (Table 17.1).

In natural environments, levels and types of pollutants can differ tremendously. It is hence essential to explore and apply bioanalytical strategies that combine chemical analysis with biological response. Only such an integrated methodological approach will suffice the diagnosis or prediction of situations resulting from anthropogenic activities and an appropriate environmental management.

Choosing an organism as **bioindicator** for **biomonitoring** should consider the **bioavailability** (see Section 13.1.1) of the pollutant to the organism and the time course of pollutant exposure.

Biomarkers are advantageous tools for investigating modes of action of xenobiotics. Plant biomarkers indicating dangerous effects of environmental pollutants are listed in Table 17.2. The successful use of biomarkers in ecotoxicological studies needs fundamental linkages to effects through the use of suitable **bioindicator** organisms representing tools at a higher level of biological organization. For example, the content of heavy metals in metal-accumulating terrestrial or aquatic mosses serving as bioindicators can be used to detect the presence of bioavailable forms of such metals in air, soil, or water. Thiol peptides such as phytochelatins, which indicate the uptake of bioavailable metal ions, can be used as biomarkers to characterize metal ion exposure of plants and fungi using a metallomics approach (see Chapter 18). Specific cell-derived metabolites and biochemical reactions should be accessible to sensitive analysis and relatively unaffected by growth conditions. Such features make biomarkers practicable under both field and laboratory conditions. Most biomarkers are nonspecific, but they are well suited where time- and dose-dependent effects are established see section 19.3.5.

Plant biosensors are useful tools for the detection of

1) phenolic compounds using electrochemical devices,
2) cellular concentrations or fluxes of signaling molecules, for example, of phytohormones, using antibody electrochemical devices, and
3) calcium and redox potential metabolites using confocal imaging and Förster resonance energy transfer (FRET) microscopy.

Some biosensing techniques integrate microorganisms. **Molecular biosensors** involve a recombinant plasmid containing a promoter whose expression is sensitive to a target molecule. Genetically engineered organisms serve as

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Table 17.1 Time scale of toxic pollutant effects and their assessment.

Time scale	Response to environmental stress	Methodological tools
Seconds to hours	Biochemical response	Biomarker Biosensors "-omics" toolbox Microscopy
Hours to weeks	Physiological and morphological response	Biomonitoring Cell culture assays Measurement of metabolic activities, for example, photosynthesis, reactive oxygen species (ROS) assays, microbial respiration rates "-omics" toolbox Microscopy
Days to months	Changes in life cycles	Measurement of growth and reproduction rates Evaluation of particularly endangered and threatened species
Months to years	Changes in populations and communities	Behavior studies Biodiversity studies Molecular biology methods Survival rates
Years to decades	Responses in the ecosystem	Evaluation of biodiversity Measurement of nutrient flows across trophic levels Molecular biology methods

Table 17.2 Plant biomarkers for environmental pollutants (+ recommended method; – not applicable).

BIOMARKER	Pollutants						
	Heavy metals	Pesticides	PCB	PAH	Pharmaceuticals	Natural Toxins	Nanomaterials
Morphology of cells and tissues (microscopy)	+	+	+	+	+	+	+
Vitality (fluorescent cytochemistry)	+	+	+	+	+	+	+
Gene expression (genomics)	+	+	+	+	+	+	+
Stress proteins (proteomics)	+	+	+	+	+	+	+
Specific metabolites (metabolomics)	+	+	+	+	+	+	+
Membrane permeability	+	+	–	–	+	+	+
Chlorophyll fluorescence (microscopy)	+	+	+	+	–	+	–
Detoxifying enzymes (phase I and II)	–	+	+	+	+	–	–
ROS and scavenging enzymes	+	+	+	+	+	+	+
Thiolpeptides	+	+	–	+	–	–	–

bioreporters, where target molecule-responsive promoters are linked to suitable reporter genes (e.g., *lacZ* encoding for ß-galactosidase or *gfp* for green fluorescent protein) that generate a signal in the presence of the target molecule.

Contaminant exposure characterization at the community level frequently considers contaminant transfer through different trophic levels, that is, within the food web (see Chapter 6). **Staple isotope analysis** (in particular ^{13}C and ^{15}N) has been established as a useful tool to examine food web structures (Box 17.1).

Natural and anthropogenic drivers cause changes in biodiversity. The assessment of biodiversity in a qualitative and quantitative manner includes parameters such as density of species, frequency distribution of species, rarity, functional diversity, ecosystem services, and the value for the human well-being. Biodiversity is an important regulator of ecosystem processes. Effective ecosystem management requires a set of methods for identifying and analyzing all parameters that are necessary for optimizing ecosystem services and for the protection of species and their habitats.

Box 17.1: Determination of the trophic position of a species in a food web

The trophic position of species within food webs can easily be determined since the amount of the heavy nitrogen isotope (^{15}N) increases in tissues relative to that of the light nitrogen isotope (^{14}N) with each trophic level, together with the analysis of a contaminant of concern leading to a metric that allows predicting the movement of that contaminant in communities.

Ecosystem dynamics is determined by the diversity and interaction of organisms with the biosphere characterized by spatial heterogeneity and temporal variability (see Chapter 5). There is a clear need to record the birth of species and their abundances in soils (aboveground and belowground) and waters (water body and sediments). Results derived from corresponding approaches are also expected to shed light on the role of "microevolution" under environmental pressure, and on interactions among coevolving organisms in habitats as drivers of future biodiversity and ecosystem functioning.

Microorganisms are key players in the biosphere. Anthropogenic perturbations alter the composition of microbial communities. Their activities are integrated into plant community dynamics, based on stabilizing and equalizing mechanisms of coexistence.

Phenotypic microarrays are based on microbial growth (in the form of pure cultures, consortia, or communities) using multiwell plates, which enables rapid multiparallel screening of a wide variety of growth conditions and investigation of responses to various environmental factors.

Molecular biological methods are essential to unravel structures and functions particularly of complex microbial communities involving a vast majority of as yet uncultured microorganisms. They also greatly support and complement corresponding investigations involving macrobiota. The ongoing development of diverse and valuable molecular biological tools allowing linking *in situ* activities of single microbial species to taxonomic information (see also Section 17.2 (Table 17.3) has been inspired by recent advances in **DNA microarray**, **high-throughput DNA sequencing**, and **"-omics" technologies**. Table 17.3 provides an overview of important molecular biological methodologies (compare also Chapter 18) applicable to study (microbial) community structures in the environment, their functions, and responses to environmental pollutants.

17.2 Ecological Restoration and Bioremediation

17.2.1 Biological Ecosystem Components Mitigating Environmental Pollution

Environmental degradation caused by various anthropogenic disturbances including – but not restricted to – the release of chemicals and other environmental pollutants can lead to ecosystem biodiversity loss and reduction or disruption of ecosystem functions. **Restoration ecology** involves human activities intended to support or enhance the recovery of damaged ecosystems.

Bioremediation (Box 17.2) is an option to overcome environmental pollution, where biotic key players such as bacteria, fungi, and plants mitigate or completely remove environmental pollutants (Table 17.4, compare Chapter 13 for the biochemical processes involved).

Phytoremediation is an environment-friendly and cost-effective bioremediation approach to remove contaminants by using plants. **Phytoextraction** and **phytodegradation** processes make use of (i) selected plant species adapted to polluted sites, (ii) hyperaccumulating species such as those for heavy metal cations, and (iii) genetically manipulated species to accumulate higher concentrations of metals or to remediate organic xenobiotics (see Section 17.2.2 for phytoremediation approaches using transgenic plants). Using plants provides the following advantages compared to conventional methods of soil remediation: (i) the potential to treat sites polluted with more than one type of pollutant; (ii) the control of the migration of contaminants into ground water systems; (iii) the use of resulting biomass to produce electricity and heat by burning. An important disadvantage of phytoremediation is the long time periods (years to decades) necessary to remediate the soil. However, it enables the recolonization of polluted soils by adapted plants together with associated organisms, thereby having an ecosystem restoration potential going well beyond the simple removal of environmental pollutants.

Preferably, during biodegradation hazardous organic compounds are converted into compounds that can be integrated into natural **biogeochemical cycles**, thus eliminating their hazardous potential. Microorganisms like bacteria and fungi are major contributors to such biodegradation processes in many environments. Versatile, complex interactions between the members of the **microbial communities** of contaminated habitats form the basis for **metabolic networks** governing biodegradation processes under *in situ* conditions. Fungi and bacteria frequently living in close association with plants under natural conditions may support plants with respect to nutrient acquisition and protection against biotic and abiotic stress. **Mutualistic interactions** between plants and mycorrhizal fungi are known to influence plant performance and shape the composition of aboveground and belowground communities, thereby interconnecting aboveground and soil food webs (see Chapters 5, 15). Mycorrhizal associations have

Table 17.3 Molecular biological methods to investigate structures and functions of (microbial) communities (largerly based on Desai, Pathak, and Madamwar (2010), and Vilchez-Vargas, Junca, and Pieper (2010)).

Methodology	Application
Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)	Fingerprinting microbial communities through differences between taxa in denaturation of a PCR-amplified phylogenetic marker or functional (e.g., catabolic) gene; reflects relative abundances of sequences within a targeted group; provides direct access to sequence information
DNA microarrays	Hybridization of target DNA molecules of organism/strain or community genomes to probes located on the surface of the microarray (commonly a glass chip), which is detected by recording changes in fluorescence signals arising from labeling either probe or target DNA with fluorescent dyes; phylogenetic oligonucleotide arrays (POAs) or PhyloChips use rRNA gene-based probes to resolve microbial community structures in environmental samples; functional gene arrays (FGAs), for example, GeoChip, analyze functional genes or functional gene transcripts involved in biogeochemical/environmental processes including organic pollutant degradation; whole-genome arrays (WGAs) covers the complete genome of a particular microorganism and are used to contrast or correlate genomes of related microorganisms
Catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH)	Increasing the sensitivity of microbial detection using FISH probes by an *in situ* amplification of fluorescence signals based on horseradish peroxidase-labeled probes
Fluorescence *in situ* hybridization (FISH)	Selective hybridization of rRNA targeted fluorescent dye-labeled oligonucleotide probes to ribosomes of permeabilized cells, which have been prefixed on membrane filters or glass slides; fluorescent cells can be visualized or counted using fluorescence microscopy, scanning laser electron microscopy, or flow cytometry; enables phylogenetic classification and (albeit on a limited scale) quantification of physiologically active cells within complex (microbial) communities
Fluorescence *in situ* hybridization-microautoradiography (FISH-MAR)	Uptake of radioactive substrates by growing cells and visualization of the incorporated radioactivity using radiation-sensitive photographic emulsions and microscopy; enables phylogenetic and functional identification of substrate-active cells within complex (microbial) communities
Genomics	Sequencing of whole genomes of single organisms/microbial strains to support approaches targeting whole communities, for example, via identification and validation of appropriate marker genes; enables genome mining, for example, in order to elucidate genes with an unknown function of the encoded enzymes and hence to discover novel biocatalysts, to unravel the metabolism of strains with a broad catabolic diversity, or to identify genes underpinning environmental adaptations
Metagenomics	Construction of metagenomic libraries by direct cloning of DNA fragments from environmental samples and their screening for functional or taxonomic genes (e.g., using error-correcting barcoded primers) of interest; application of whole community genome amplification (WCGA) based on multiple displacement amplification (MDA) to overcome problems related to the construction of metagenome libraries from low-biomass environments and to ensure the representativeness of community microbial genomes; massive parallelization enabled by pyrosequencing, with the 454 technology (Roche/454 Life Sciences) not requiring cloning from metagenomic DNA
Metaproteomics	Extension of traditional proteomic approaches to microbial communities of environmental samples; based on two-dimensional (2D) gel electrophoresis and subsequent analysis of protein spots on 2D gels by either matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry or liquid chromatography electrospray ionization source mass spectrometry (LC-ESI-MS), followed by identification of the resulting peptide mass fingerprints through comparisons with protein data banks; enables detection of protein expression profiles and investigation of changes in the composition and abundance of proteins, hence mapping the actual functional activity of a microbial community in a given ecosystem
Metatranscriptomics	Extraction and amplification of the total mRNA from complex communities or environmental samples and subsequent cDNA synthesis, followed by either microarray hybridization of cDNA or (pyro)sequencing of the complete cDNA transcriptome; enables to monitor *on-site* expression of functional (e.g., catabolic) genes

Table 17.3 (*Continued*)

Methodology	Application
Polymerase chain reaction (PCR)	Using targeted primers and a DNA polymerase to amplify a gene or DNA region, which is used to retrieve phylogenetic information or for functional gene analysis; methodological basis for most nucleic acid-based technologies; classically performed *in vitro*
Quantitative PCR (qPCR, real-time PCR)	Enabling quantification of the relative abundance of a specific/functional group of microorganisms in the total microbial community by targeting either phylogenetic (taxon-specific) markers or functional (e.g., catabolic) genes; signature sequences unique to a particular group of microorganisms or a functional gene, which are needed to design primers/probes, have to be retrieved from database sequences
Single strand conformation polymorphism (SSCP)	Fingerprinting microbial communities through differences between taxa in the electrophoretic mobility of single stranded phylogenetic marker or functional (e.g., catabolic) gene; reflects relative abundances of sequences within a targeted group; provides direct access to sequence information
Small subunit (SSU) rRNA gene clone libraries	Direct amplification, cloning, and sequencing of SSU rRNA genes from complex communities or environmental DNA samples; enables characterization of microbial community structures, composition, and shifts
Stable isotope probing (SIP)	Provision of heavy isotope-labeled substrates (e.g., ^{13}C-labeled environmental pollutants) to complex microbial communities and subsequent analysis of nucleic acids containing the isotope label, thereby directly linking community structure to function without the need for cultivating individual microorganisms; can be applied under *in situ* conditions
Terminal-restriction fragment length polymorphism (T-RFLP)	Fingerprinting microbial communities through differences between taxa in the lengths of terminal restriction fragments of a PCR-amplified phylogenetic marker or functional (e.g., catabolic) gene; reflects relative abundances of sequences within a targeted group

Please also refer to Chapter 18 for further information.

Table 17.4 Activities of plants and microorganisms capable of metal and xenobiotic ecoremediation in soil and water/sediment.

Remediation activity of plants	⟺	Remediation activity of bacteria and fungi
Phytoextraction by uptake transporter in roots		Biofilm-mediated detoxification
Sequestration/accumulation in roots, shoots, and leaves		Plant growth promotion
		pH change
Glutathione/phytochelatin-mediated metal detoxification		Stimulation of plant cell uptake transporters
Phytovolatilization of pollutants		Mycorrhiza mediated detoxification of metals
Phytostabilization by decreasing soil erosion		Phytoprotection via microbial resistance
Rhizofiltration of pollutants from aqueous sources		Metal transformation into soluble forms
		Metal precipitation into insoluble forms
		Metal accumulation
		Metal volatilization
		Biotransformation of xenobiotics by fungal enzymes

Modified from Stout and Nüsslein (2010), with permission from Elsevier.

Box 17.2: Bioremediation technologies

Treatment of the contaminated material within the site without removing it is referred to as ***in situ* bioremediation**. ***Ex situ* bioremediation** denotes the removal of the material (e.g., excavation of contaminated soil) to be treated outside (referred to as ***on site* bioremediation** if a special facility located at the site of decontamination is used, and as ***off site* bioremediation** if this treatment facility is located at another place). More specific classifications applicable to both *in situ* as well as *ex situ* processes are, for example, **bioaugmentation** (the deliberate addition of natural or engineered microbes with the desired degradation capabilities) and **biostimulation** (the addition of nutrients and/or electron acceptors/donors to promote desired microorganisms). **Enhanced natural attenuation** uses the same principles as biostimulation but specifically refers to *in situ* treatment, as natural attenuation approaches generally do. **Natural attenuation** and **monitored natural attenuation** (human action restricted to monitoring and not aiming at the improvement of the bioremediation process itself) may be referred to as **passive bioremediation** schemes, whereas all other technologies are associated with human intervention targeting bioremediation process initiation, enhancement, or stimulation, and may be termed as **active bioremediation** technologies. **Phytoremediation** and **mycoremediation** specifically refer to the use of plants and fungi, respectively, for bioremediation purposes.

been implicated in the bioremediation of organic and metal contamination of surface soils. In aquatic habitats, biofilms composed of bacteria, fungi, and algae may cooperate with plants to remove metals and xenobiotics. Further research into this promising field will promote the understanding of bacteria–fungi–plant interactions and their exploitation in **ecoremediation** processes. For example, artificial ecosystems such as constructed wetlands are already used for water remediation.

17.2.2 Present and Future Directions

The most widely used classification of bioremediation technologies is based on the question whether the contaminated material to be treated is removed from the site of concern or not. Other classifications more or less specifically refer to the mode of human intervention associated with a particular bioremediation approach, all driven by pragmatic considerations rather than following a stringent hierarchical classification system.

From a historical perspective, bioremediation technologies have been established as "black box" engineering approaches, where the human intervention was (and still is) governed by empiricism rather than by a knowledge-based understanding of the complex processes and interactions involving both biotic and abiotic ecosystem components. While often proven to be successful, such approaches too frequently failed with respect to, for example, the degradability of the contaminant and the formation of products being more toxic and/or more susceptible to transport processes than the parent compound. Defined microbes or microbial consortia that have been derived from contaminated sites were often found promising under well-defined laboratory conditions, thus proving the microbial degradation potential available at a particular site. However, field-scale conditions of contaminated sites are much more complex than experimental systems in the laboratory. Frequently, such complex conditions are characterized by, for example, (i) the presence of more than one contaminant; (ii) the presence of poor or nonbioavailable contaminants; (iii) the lack of essential nutrients and/or electron acceptors/donors; and (iv) the presence of different types of microbes with different degradation capabilities, metabolic pathways, and nutrient and/or electron acceptor/donor

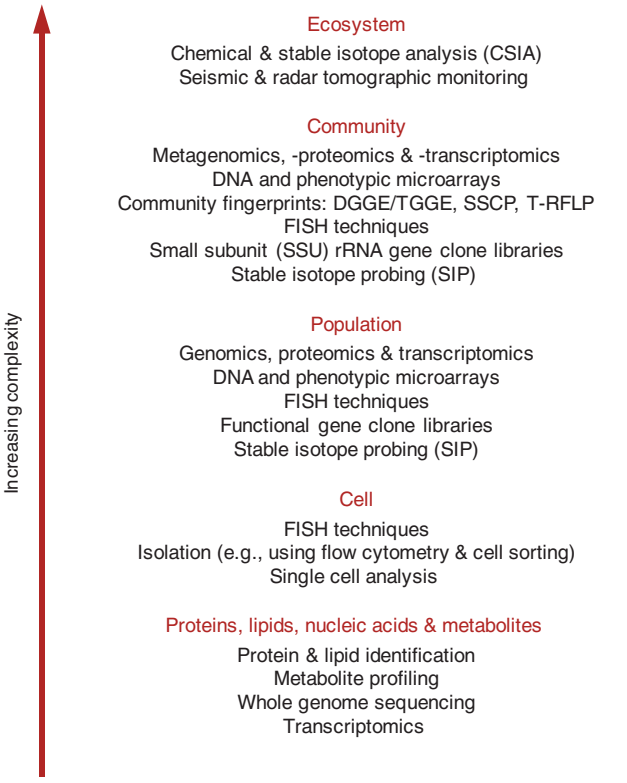

Figure 17.1 Levels of multiscale complexity associated with bioremediation and applicable methodologies and techniques related to corresponding systems biology approaches. (Modified from Chakraborty, Wu, and Hazen (2012), with permission from Elsevier).

Box 17.3: Stable isotope probing and compound specific isotope analysis

Stable isotope probing (SIP) enables tracking atoms of isotopically enriched xenobiotics (e.g., ^{13}C-labeled compounds) in complex microbial communities, thereby offering the possibility to identify catabolically active community member organisms via the analysis of labeled nucleic acids. **Compound specific isotope analysis (CSIA)** uses the fact that in biochemical reactions the lighter naturally occurring isotopes of elements (e.g., ^{12}C, ^{1}H) frequently react faster than their heavier counterparts (e.g., ^{13}C, ^{2}H). In the biodegradation or biotransformation of a xenobiotic compound, this leads to the enrichment of heavier isotopes in the residual fraction and the enrichment of lighter isotopes in reaction products (including the biomass components). Hence, decreasing concentrations of a xenobiotic compound at polluted sites accompanied by the enrichment of heavier isotopes in the residual compound fraction can be used to indicate biodegradation. Moreover, the possible determination of degradation pathway-specific isotope fractionation factors for xenobiotics may offer a way to quantify biodegradation processes at field scale.

requirements, which potentially may exhibit various antagonistic or beneficial interactions such as syntrophy and trophic networks. Moreover, complexity at field scale is a multiscale problem ranging from physicogeochemical and hydrological site characteristics to the genetic potential of the microbial community at a particular site (Figure 17.1). All in all, this complexity may result in conditions that do not favor the desired contaminant biodegradation.

Systems biology is an integrated approach to unravel the interactions and networks of complex biological systems at the ecosystem, community, cellular, and molecular level. By combining the recent advances particularly in "-omics," microarray, and high-throughput DNA sequencing technologies (Table 17.3) with sophisticated stable isotope analysis Box 17.3 and site characterization tools, the multiscale complexity related to bioremediation approaches is expected to become increasingly amenable to systems biology tools and concepts (Figure 17.1). The scale-bridging complexity associated with bioremediation and related systems biology approaches are depicted in Figure 17.1. Resulting crucial insights into the survival, metabolism, and interactions of microbes in their natural environments are expected to enable the understanding, optimization, prediction, and evaluation of microbial function and survival strategies in a particular contaminated ecosystem. Corresponding approaches have already been used in some bioremediation projects to understand the systems biology of sites contaminated with chlorinated solvents, hydrocarbons, heavy metals, and radionuclides, and to predict feasible remediation technologies for these sites.

Pathway engineering is a **synthetic biology** approach. It relies on the combined application of advanced molecular, genetic, microbiology, and protein engineering tools developed in the context of bioremediation, and aims at the construction of new catabolic pathways to remove recalcitrant pollutants from the environment. This approach is largely supported by the fact that current "-omics" technologies allow exploiting the catabolic potential of microorganisms without necessarily cultivating them. Successful pathway engineering of microbes for inorganic (heavy metals, radionuclides) and organic chemical (e.g., pesticides, nitroaromatic compounds) remediation has been demonstrated at the laboratory scale. The successful implementation at field/industrial scale after careful assessment of the biosafety of transgenic microbe species remains elusive. The expression of bacterial enzymes for the degradation of explosives has been demonstrated in plants to be used in phytoremediation approaches. Using transgenic plants to remove heavy metals from soils is another promising phytoremediation approach. As for transgenic microorganisms, the intended use of genetically engineered plants requires a careful biosafety assessment. This includes the investigation of their stability under field conditions and the elucidation of the possibility of horizontal gene transfer to rhizosphere microorganisms. Generally, the release of genetically modified organisms into the environment is restricted by current legislation that varies among different countries.

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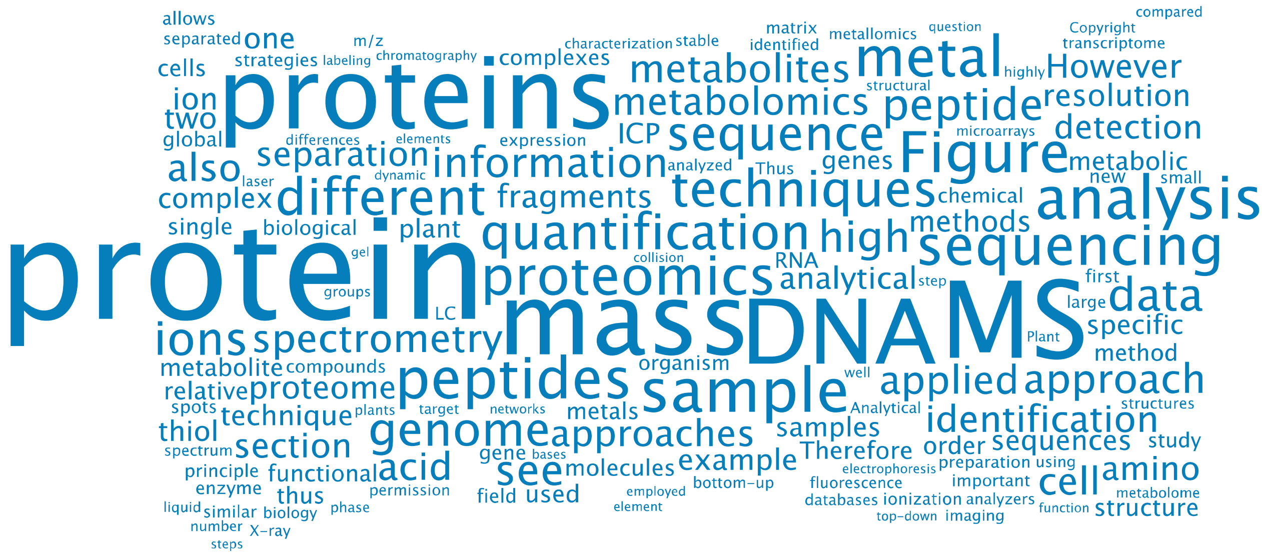

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18
The -Omics Tool Box

Dirk Schaumlöffel

Overview

The -omics tool box refers to a set of modern analytical techniques developed during the past decades that paved way for a remarkable progress of knowledge in biology, biochemistry, and life sciences. The aim of these new technologies is to acquire – ideally – complete sets of molecular data of the *genome, proteome, transcriptome, metabolome,* and related fields. The suffix "*-ome*" refers to the entirety of, for example, genes, proteins, or metabolites in a regarded system while "*-omics*" corresponds to the related analytical methods for acquiring global qualitative and quantitative information. Thus terms like *genomics*, *transcriptomics*, *proteomics*, *metabolomics*, and *metallomics* were coined. These "-omics" technologies have the ambitious aim to integrate genome, transcriptome, proteome, and metabolome data in order to expand our knowledge on organisms or ecological systems. Such integration and interpretation of large datasets improve the understanding of pathway functions and regulatory networks. An example from plant biology in Figure 18.1 demonstrates how functional interactions in a network among genes, proteins, and metabolites can be elucidated by an integrated "-omics" approach.

This chapter presents the basics of modern genomics, transcriptomics, proteomics, metabolomics, and metallomics techniques. This includes large-scale high-throughput experiments, computational, and theoretical approaches in order to advance the frontier of knowledge on biological systems.

18.1
Genomics

The **genome** is the entirety of genetic information of an organism necessary for its development and functioning. This information is encoded in the **deoxyribonucleic acid (DNA)**. The genome is usually regarded as being static; however, this paradigm is now questioned.

Genomics aims to answer the question: *What is the nucleobase sequence of the DNA?*

The tools for genomics are developed (i) to determine the whole DNA sequence of an organism and (ii) to enable genome mapping on a fine scale by assigning DNA fragments to chromosomes. These tools are analytical key techniques in molecular biology.

18.1.1
First-Generation Sequencing

Among the classic DNA sequencing methods, the **Sanger method** using the principle of chain termination became the standard method for DNA sequencing and is up to now the reference method where next-generation sequencing (NGS) is compared with. The initial Sanger method used radioactive labeling for autoradiography detection of DNA fragments after gel electrophoresis separation. Further developments include the replacement of radioactive label by fluorescence label. Recent Sanger-based approaches can sequence DNA fragments of 300–1000 nucleotides in a single reaction; however, the sequencing of the first 30–40 and after 700–900 bases is usually of lower quality.

An obstacle in DNA sequencing is that the methods allow only a sequencing of DNA fragments, for example, for Sanger-based approaches, DNA pieces reads of about 1000 bases (1 kb), while DNA of many organisms is much larger. Therefore, DNA is usually cut in small fragments before sequencing. In this way large genomes can be sequenced applying either a systematic (top-down approach) or random (shotgun approach) cut in small fragments. Then, the sequenced fragments are subsequently reassembled in order to get the whole genome sequence.

In order to have enough DNA material for sequencing, the original DNA has to be amplified by creating numerous copies. The polymerase chain reaction (PCR) method uses the natural ability of DNA to replicate itself, another principle applied in the Sanger method.

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

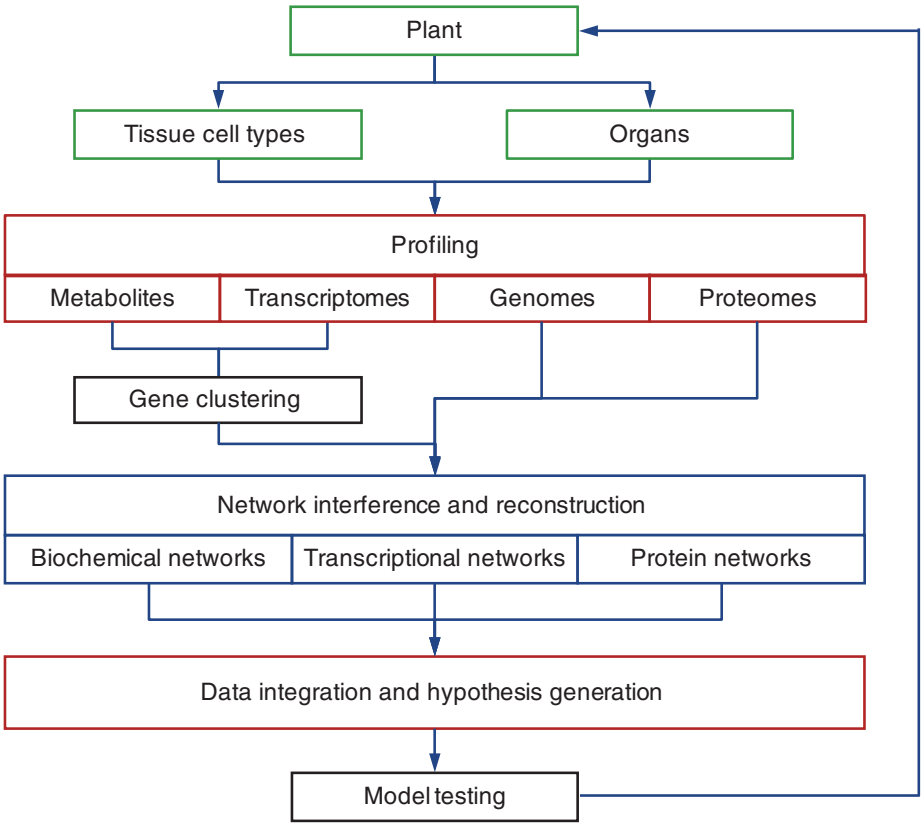

Figure 18.1 Integrated "-omics" approaches in plant biology for the elucidation of functional interactions among genes, transcripts, proteins, and metabolites. ((Reprinted from Moreno Risueno, Busch, and Benfey (2010) with permission from Elsevier.)

18.1.2 Next-Generation Sequencing (NGS)

Sanger DNA sequencing provides long sequence reads up to 1 kb with high quality and has led to important milestones such as the first sequencing of the human genome. However, its high cost and relatively low throughput stimulated the development of several rapid and cost-effective alternative approaches called **next-generation sequencing** methods or platforms. Three modern **NGS platforms** are mainly used today for high throughput genome sequencing: the 454/Roche FLX^{TM} Pyrosequencer, the Illumina Genome Analyzer^{TM}, and the Applied Biosystems SOLiD^{TM} Sequencer. These techniques are based on fragmenting the DNA followed by sequencing the fragments. Computer-processed data treatment removes poor-quality sequences and assembles the remaining high-quality data for reconstructing the full genome sequence.

18.1.2.1 Pyrosequencing: 454/Roche FLX^{TM} Pyrosequencer

In pyrosequencing, a single-strand DNA fragment and a DNA primer are incubated with several enzymes. After the incorporation of a nucleotide complementary to the base in the original DNA (template), a cascade of enzyme reactions follows. During the last reaction, visible light is emitted, which is photometrically detected. The signal intensity is proportional to the amount of nucleotides, resulting in a pyrogram from which the DNA sequence can be read.

The pyrosequencing method has been commercialized by 454 life sciences/Roche with the GS FLX^{TM} Pyrosequencer. This device includes PCR amplification in oil/water micelles of DNA strands immobilized on beads producing about one million copies of each DNA strand on the surface of the corresponding bead. Parallel high-throughput pyrosequencing is performed in hundreds of thousands of wells on a picotiter plate (3 200 000 wells per 60 × 60 mm) where each well contains a bead with copies of one specific DNA strand. Average read length is 400–700 bases; recent developments allow reads up to 1000 bases, called **Sanger-like**. This technique allows the simultaneous sequencing of small entire genomes in a typical 10 h run.

18.1.2.2 Reversible Dye Terminator Technology: Illumina Genome Analyzer^{TM}

The reversible dye terminator sequencing technology was developed by Solexa and then acquired and commercialized by Illumina. The principle is **sequencing-by-synthesis** by adding all four types of nucleotides and DNA polymerase. Each nucleotide is tagged with a different removable fluorescence dye emitting light of a specific wavelength excited by a laser. The emitted fluorescence light is detected with

high sensitivity and the data are recorded. Usually only short read lengths of 60–100 bases are realized, but this approach ensures high accuracy and avoids artifacts.

18.1.2.3 Sequencing by Ligation: Applied Biosystems SOLiD™ Sequencer

Unlike the other approaches where the enzyme DNA polymerase is employed for sequencing-by-synthesis, in the **sequencing-by-ligation** approach the enzyme DNA ligase is used. This enzyme connects ends of DNA molecules and it is sensitive for base-pairing mismatches. Sequencing by ligation uses oligonucleotides (8–9 bases) with fluorescence labels.

In the SOLiD™ system, sequencing starts with a DNA library preparation where small DNA fragments (150–180 bases) of interest are linked to adaptor sequences. DNA fragments are amplified by emulsion PCR in oil/water micelles and immobilized on beads similar to the process in the FLX™ Pyrosequencer system. DNA sequencing by ligations takes place in several cycles and ligation steps. The number of ligation steps determines the read length of sequencing. Short DNA fragments of 60–100 bases (read length) can be sequenced with high accuracy.

Besides these three dominating NGS platforms, other next-generation sequencing techniques are coming up such as Helioscope™ single molecule sequencing and nanopore DNA sequencing. The latter method aims to read the sequence of a DNA strand during its transit through nanopores. The interested reader is referred to the special literature for further information.

NGS platforms provide rapid high-throughput sequencing at lower costs than classic methods but the drawback is a shorter read length compared to Sanger sequencing. Especially, the Illumina™ and SOLiD™ platforms provide only read lengths of about 60–100 bases, which make the assembly of large and complex genome sequences error-prone. Powerful computer software and novel algorithms are required for accurate data treatment and interpretation as well as validation of the reconstructed genomes with high-quality sequence strategies.

18.1.3 Understanding the Genome: Genes and Their Functions

After DNA sequencing, further steps for understanding the genome include the prediction of genes and the search for their functions. This is realized by the integration of genomics with transcriptomics, proteomics, and metabolomics.

An important point is the comparison of new DNA sequences with known sequences in various databases. Examples for public **databases** are given in Table 18.1. The search for sequence homologies with known DNA sequences from similar organisms having annotated gene functions can lead to postulate biological functions for new DNA sequences. However, gene function prediction by homology is the first step and has to be confirmed by conventional biochemical methods, for example, complementary assays, cloning, enzyme activity tests, protein interaction tests, and gene knockouts.

18.2 Transcriptomics

The ***genome*** is defined as the genetic information of an organism necessary for its development and functioning. However, in order to function, this information has to be expressed. The first step of the expression of genes is their transcription to **ribonucleic acid (RNA)** molecules, followed by the second step, the synthesis of proteins encoded in RNA. Most RNA molecules are single-stranded

Table 18.1 Some public databases and bioinformatics resources with access to DNA and protein sequences.

Database	Organization	Link
GenBank	National Center for Biotechnology Information (NCBI), USA	*http://www.ncbi.nlm.nih.gov/genbank/*
RefSeq	National Center for Biotechnology Information (NCBI), USA	*http://www.ncbi.nlm.nih.gov/refseq/*
European Nucleotide Archive (ENA)	European Bioinformatics Institute (EMBL-EBI), UK	*http://www.ebi.ac.uk/ena/*
DNA Data Bank of Japan (DDBJ)	DNA Data Bank of Japan, Japan	*http://www.ddbj.nig.ac.jp/*
UniProt; UniProtKB/Swiss-Prot	European Bioinformatics Institute (EMBL-EBI), UK; Swiss Institute of Bioinformatics (SIB), Switzerland; Protein Information Resource (PIR), USA	*http://www.uniprot.org/*
ExPASy	Swiss Institute of Bioinformatics (SIB), Switzerland	*http://www.expasy.org/*
ProteinProspector	University of California, USA	*http://prospector.ucsf.edu/*

For more information, the reader is referred to *http://en.wikipedia.org/wiki/List_of_biological_databases#Genome_databases*.

and much shorter than double-stranded DNA. During transcription, the produced RNA molecule represents a copy of the DNA molecule in the expressed gene and carries the genetic information for the synthesis of proteins.

The **transcriptome** represents the entirety of all RNA molecules in a biological system (e.g., a cell) at a particular time. This includes the mRNA (messenger ribonucleic acid) encoding for proteins as well as rRNA (ribosomal ribonucleic acid), tRNA (transfer ribonucleic acid), snRNA (small nuclear ribonucleic acid), and other noncoding RNA. The genetic information from DNA is transcribed to mRNA. In contrast to the genome, the transcriptome is highly dynamic because genes that are actively expressed can vary with environmental conditions.

Transcriptomics aims to answer the question: *Which genes are actively expressed?*

Thus transcriptomics is the global study of gene expression at the RNA level. Usually two or more physiological states of the same organism are compared for differences in gene expression under different conditions, for example, virus-infected and noninfected plant samples, or transgenic and nontransgenic plants. The composition and quantity of mRNA in a cell indicate which genes are expressed and how frequently under given conditions at a particular time. Therefore, mRNA is extracted from the cell and reversely transcribed into **complementary deoxyribonucleic acid (cDNA)** by employing the enzyme reverse transcriptase. The most popular method to study the transcriptome is the DNA microarray approach. Also, DNA sequencing methods are used for transcriptome sequencing.

18.2.1 DNA Microarrays

DNA microarrays allow the study of changes in gene expression. After extraction of mRNA, cDNA is reconstructed and labeled with fluorescence dyes, each biological state with a different color. On the DNA microarray, all genes of the organism are arranged and attached to a surface in the form of microscopic spots containing picomolar amounts of single-stranded DNA. A prerequisite is, of course, that the genes are identified on the DNA sequence in the regarded organism. The reconstructed and labeled cDNA molecules are given on the array where they are bound (hybridized) to the matching DNA in the spots. In order to compare two samples of an organism, a laser scans the array and emitted fluorescence light is detected. The position of the spot on the array informs which gene is expressed, the intensity of the fluorescence signal shows the expression level, and the wavelength of the signal the biological state. An example is given in Figure 18.2.

Advantages of DNA microarrays are the lower cost compared to sequencing methods, and limitations are lower resolution and accuracy, as well as the need for preceding gene annotation.

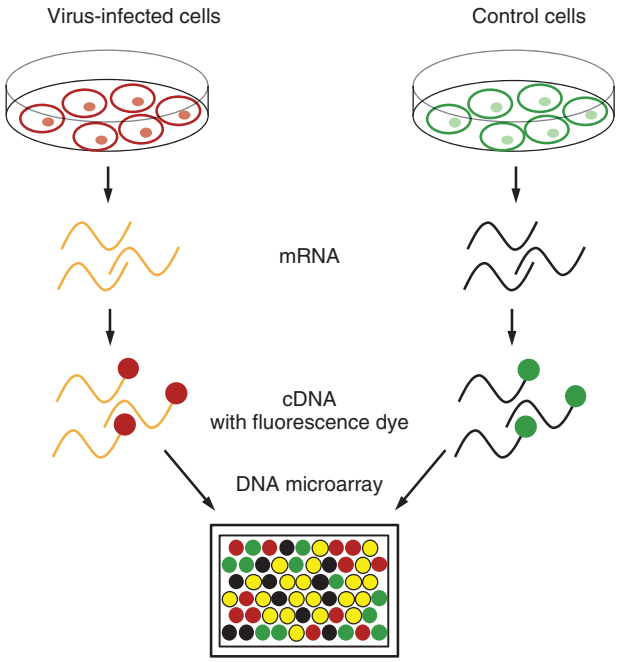

Figure 18.2 DNA microarray for the investigation of changes in DNA expression. In this example, the cDNA produced from virus-infected cells is labeled with a red, the cDNA from the control sample with a green dye. A red spot on the DNA microarray indicates that the corresponding gene is more expressed in the diseased cells; a green spot shows an expression in the control sample. By additive color mixing yellow spots indicate the same activity of the gene in both samples. The most popular fluorescence dyes used are the cyanine dyes Cy3 (green) and Cy5 (red). More information on fluorescence dyes is given in Chapter 19.

18.2.2 Transcriptome Sequencing by NGS Platforms

Transcriptome sequencing, also called ribonucleic acid sequencing (RNA seq), employs high-throughput NGS platforms (see Section 18.1.2) to sequence cDNA in order to get information about the RNA in a sample. This approach provides more precise and accurate analysis of transcripts and deeper insights than DNA microarrays. This includes transcript profiling and classification, identification of new transcripts and genes, revealing of sequence variations because of alternative splicing, and quantification of gene expression levels.

Furthermore, approaches are under development for direct sequencing of RNA (e.g., direct single molecule RNA seq) in order to avoid errors introduced by the transcription to cDNA.

18.3 Proteomics

The **proteome** is the entirety of proteins in an organism, a tissue, a cell, or a body fluid. Proteins are biopolymers that are essential parts of organism and participate in almost

every biological process within cells. They consist of a sequence of amino acids linked by peptide bonds that determine the three-dimensional structure and functions of a protein. The amino acid sequence is encoded in the genome and translated from the nucleobase sequence in the mRNA where a sequence of three bases encodes one amino acid. However, the same DNA sequence in the genome can result in hundreds of different proteins and their modifications. During transcription from the DNA, alternative splicing leads to several mRNA. Furthermore, after translation from mRNA to proteins, enzymatic reactions can result in posttranslational modifications such as phosphorylation, glycolysation, and acetylation. Moreover, several single proteins can form protein–protein complexes with new functions. All this shows that the proteome is more complex than the genome and the transcriptome. It is highly dynamic, depends on different factors like the physiological state of a cell and its environmental conditions, and varies with time.

Proteomics aims to answer the question: *Which proteins are synthesized?*

Answering this question includes the global study of identity, structure, quantity, and function of proteins. Thus, proteomics is not limited to deciphering amino acid sequences of proteins, its primary structure, but involves the study of 3D protein structures that can change in time and determine the protein function. Protein structures are subdivided in secondary (local structures such as α-helices, β-sheets, and turns), tertiary (overall shape of the protein), and quaternary (protein complexes) structures.

Global proteomics is a whole proteome analysis and refers to a systematic investigation and identification of all proteins in an organism, a tissue, a body fluid, or a cell. Selective proteomics is a targeted proteome analysis and aims to study specific proteins or groups of proteins, for example, the phosphoproteome (all phorphorylated proteins) and the glycoproteome (all glycolysated proteins). Differential proteome analysis regards the differences in protein expression levels between two or more physiological states of an organism.

Besides fundamental research in biochemistry and molecular biology, there are many applications of proteomics such as biomarker detection, drug development, and cancer research.

The analytical challenges in proteomics are manifold. The proteome is much more complex than the genome or transcriptome because of a large variety of different proteins. The difference in concentration between low-abundant and high-abundant proteins can span several orders of magnitude. Proteins cannot be amplified like DNA, thus low-abundant proteins have to be purified and concentrated before analysis. On the one hand, proteins can have big differences, for example, in size, mass, and solubility, and, on the other hand, they are rather similar, for example, regarding their isoelectric points. Also, the protein structures are more complex than DNA and RNA and contain hydrophilic and hydrophobic domains. Prediction of protein structure and function on the basis of the amino acid sequence is very difficult. Protein function depends on the 3D structure, and thus protein denaturation should be avoided if protein functions are aimed to be investigated.

18.3.1 Proteomic Strategies

In order to analyze the proteome, different strategies have been developed. The most common strategies are **bottom-up**, **shotgun,** and **top-down,** which are mainly used for deciphering the amino acid sequence for protein identification and quantification. However, all strategies employ similar experimental and computer-based methods although they apply different workflows. A typical workflow for bottom-up and top-down proteomics is shown in Figure 18.3. This includes methods for sample preparation and separation, identification and characterization, database search and bioinformatics.

18.3.1.1 Sample Preparation for Proteomics

The first step is the preparation of the sample for proteome analysis. A good knowledge of the sample is a prerequisite in order to adapt subsequent methods. Proteins have to be extracted, for example, from the tissue. This includes in general first a mechanical (e.g., by French Press or sonication) or chemical cell lysis. Following that, proteins have to be solubilized, for example, by **denaturation** with nonionic detergents or **sodium dodecyl sulfate (SDS)**. If a solubilization of the entire proteome of the cell or tissue is carried out, mainly abundant proteins can be investigated because of high sample complexity. Proteins of low concentration can be studied when the complexity of the sample is reduced by fractionation and purification steps. Proteins of interest can be enriched and purified by sample fractionation. They can be fractionated according to their localization, for example, by differential centrifugation steps in order to separate cell compartments before protein extraction. Heat denaturation or affinity chromatography is applied for protein fractionation according to their structure or function. Further purification steps are necessary when interfering substances such as lipids, polynucleotides, polysaccharides, and salts have to be removed, for example, by ultracentrifugation or dialysis.

18.3.1.2 Protein Separation

The next step is the separation of proteins. Differences in protein characteristics are the key for the choice of the protein separation technique. For example, proteins can be separated according to their function using specific enzymes or antibodies. A widely employed separation technique is the **two-dimensional gel electrophoresis** (2DE, Figure 18.4) where proteins are separated according to two basic protein properties:

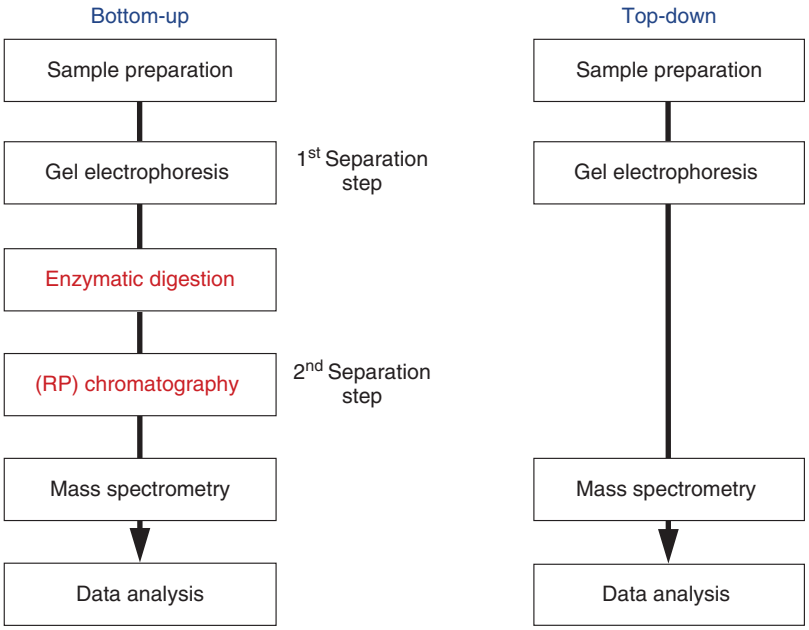

Figure 18.3 Simplified typical workflow for bottom-up and top-down proteomics.

their *charge,* depending on the amino acid composition, and their *size,* depending on the number of amino acids.

18.3.1.3 Differential Proteome Analysis

In differential analysis, two different physiological states of an organism are compared, for example, a diseased or stressed organism and a control sample. Both samples are prepared in the same way: proteins are separated by 2-DE under the same conditions and the protein map in the electropherograms are compared. Different protein spots indicating differences in protein expression between the two physiological states are identified. Subsequently, only those protein spots where differences in expression occurred are further analyzed.

18.3.1.4 Identification of Proteins

In general, protein identification is carried out via determination of the amino acid sequence and subsequent database search. Classic amino acid sequencing is carried out by Edman degradation. However, this method cannot be applied to whole proteins, but only to peptides with maximum 50–60 amino acid residues. Thus, proteins have to be fragmented to peptides before analysis. Nowadays, modern proteomic approaches rely mainly on protein sequencing by mass spectrometry (MS) (see Section 18.3.2).

18.3.1.5 Analytical Protein Microarrays

Specific proteins can be directly identified by the reaction with fluorescence-labeled antibodies in analytical protein microarrays, which is a technique similar to DNA microarrays (see Section 18.2.1). This technique allows not only protein identification but also monitoring of their expression levels (for relative protein quantification, see Section 18.3.3). Direct application of cell lysates is possible, for example, in differential analysis, without previous protein separation. However, analytical protein microarrays are limited to particular target proteins of interest such as biomarkers where antibody test were developed. In contrast, functional protein microarrays aid in protein functional analysis (see Section 18.3.4.2).

18.3.1.6 Bottom-Up Proteomics

The bottom-up strategy is the most mature and widely used approach in proteomics. Sample preparation and protein separation steps are performed as previously described.

An important step is the protein fragmentation in peptides by **enzymatic digestion**. This can be carried out directly in the protein spots in the gel of 2-DE (in-gel digestion). A widely used enzyme is trypsin that cleaves the amino acid chain only at the amino acids Arginine and Lysine, that is, the peptide bonds are hydrolyzed where the carbonyl group belongs either to an Arg or a Lys residue. This is important information for a later reconstruction of the protein sequence.

In the next step the peptides are extracted from the gel and identified by MS either by peptide mass fingerprinting or tandem MS (see Section 18.3.2). Then the peptides are either directly submitted to the MS or previously separated by reversed phase liquid chromatography (LC), which is coupled online to the MS (LC-MS). The latter approach is recommended when the sample is complex.

For protein identification, bioinformatic tools are applied. Therefore, the identified peptides are submitted to database search. This, however, is possible only if the protein sequence is known and registered in one of the

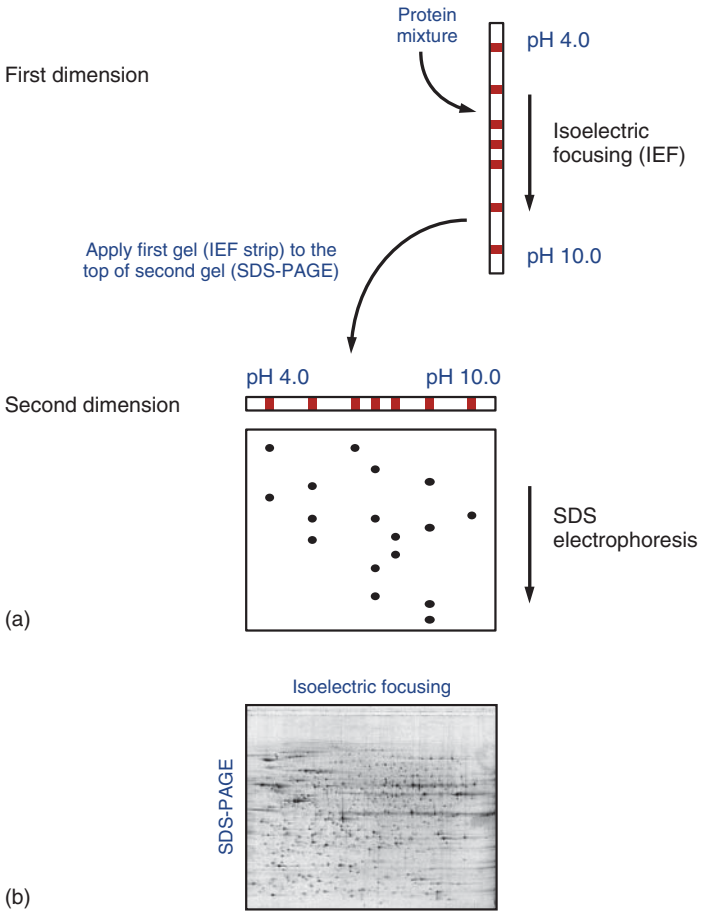

Figure 18.4 Two-dimensional gel electrophoresis (2-DE). (a) The first dimension in 2-DE is isoelectric focusing (IEF). The presepa-rated proteins by IEF are further separated in the second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (b) Example for a 2-DE protein separation and visualization of the spots with silver staining. (Reprinted from Righetti and Candiano (2011) with permission from Elsevier.)

public protein databases (Table 18.1). Hypothetical protein sequences are predicted by translation from known genome sequences. Protein sequences listed in databases are subjected to computer-simulated enzymatic digestion including the enzyme (e.g., trypsin) reaction that is also used in the experiment. This results in theoretical peptide lists for each protein in the database. During database search, experimental and theoretical peptide lists are matched producing a list of possible proteins (hits), each with a probability expressed as the percentage (score) of the protein being searched for. The accuracy of the result is higher when more peptides are identified and thus a larger part of the protein sequence is covered by the sequences of the peptide fragments. In the bottom-up approach, usually around 40% of the protein sequence is covered by identified peptides. However, if the whole protein sequence is not covered, information on, for example, posttranslational protein modifications can be lost.

18.3.1.7 Shotgun Proteomics

The shotgun proteomics approach is a special type of the bottom-up approach. The difference between both is that in the shotgun strategy proteins are not separated before enzymatic digestion, and thus no 2-DE is employed. The whole protein mixture is subjected to digestion resulting in a large number (thousands) of different peptides. This requires, however, an efficient fractionation and separation of the peptides as well as high-resolution MS. Usually, two subsequent LC steps are employed: a first fractionation of the peptide sample by cation exchange chromatography followed by reversed phase LC peptide separation in each fraction. MS results in a huge amount of data from identified peptides. As they do not belong to a single protein, bioinformatic tools are necessary for data deconvolution and protein identification by database search. The advantage of this approach is the possibility of high-throughput proteomics without time-consuming

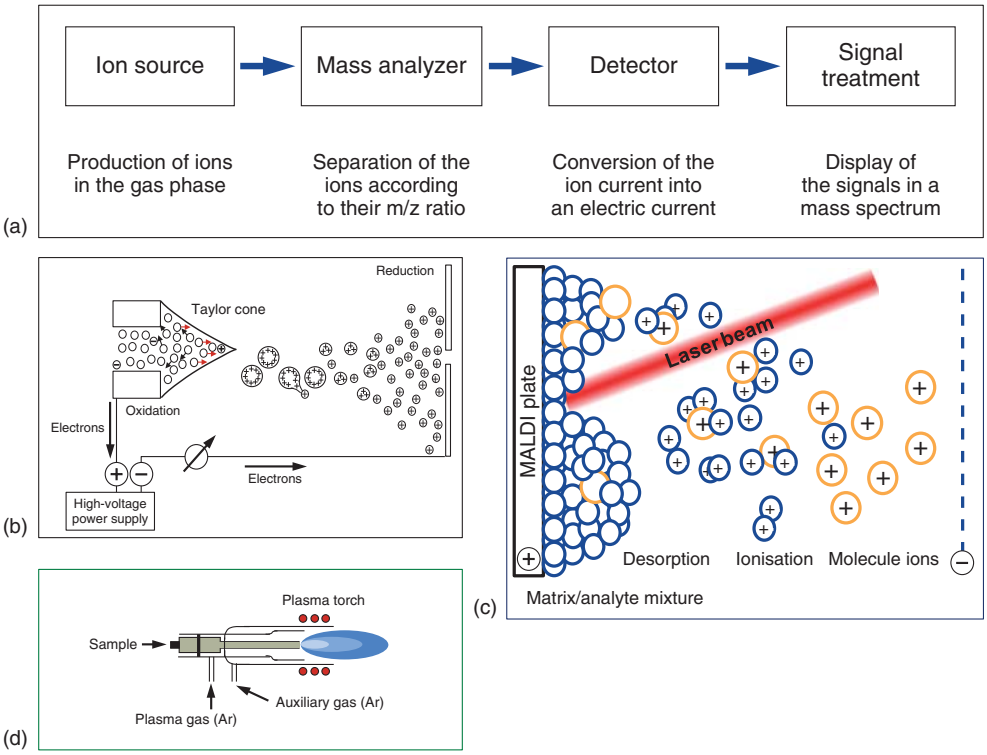

Figure 18.5 Components of a mass spectrometer (a) and three important ionization sources for proteomics: (b) Electrospray ion source (ESI) for generation of biomolecule ions (soft ionization). (Adapted with permission from Kebarle and Tang (1993), © 1993, American Chemical Society.) (c) Matrix assisted Laser Desorption/Ionization (MALDI) for generation of biomolecule ions (soft ionization). (d) Inductively coupled plasma (ICP) for generation of element ions (hard ionization).

gel electrophoresis. However, this approach does not allow protein quantification.

18.3.1.8 Top-Down Proteomics

In top-down proteomics entire proteins are analyzed by MS while time-consuming digestion to peptides as in the bottom-up approach is avoided. This requires separated and purified proteins or only simple protein mixtures. Proteins are fragmented in the gas phase of the mass spectrometer and the exact mass of the fragments is determined. Therefore, high-resolution mass spectrometers (see Section 18.3.2.2) are required. The protein sequence is reconstructed on the basis of the identified fragments. An advantage of this approach is that potentially the entire protein sequence is covered by the fragments and thus posttranslational modifications can be detected. However, gas phase fragmentation processes of proteins are complex and long, which limits the ability for couplings with LC. Moreover, the top-down approach cannot be applied to large proteins with masses higher than about 50 kDa.

18.3.1.9 *De novo* Sequencing of Proteins

The previously presented protein identification strategies rely on the entry of their sequences in databases. Thus the protein sequences had to be determined already or predicted from a genome sequence. However, it may occur that a protein has not been sequenced before and no database entry is available. In this case proteins have to be newly sequenced, which is called *de novo sequencing*. In order to do this, typically, a two-step approach is applied. In the first step, the chemical generation of polypeptide fragments of the protein is done in such a way that there is always a difference of one amino acid residue between the fragments: **ladder-generation chemistry**. In the second step, the mixture of the fragments is analyzed together by high-resolution MS (see Section 18.3.2). The resulting mass spectrum shows peaks with mass differences of one amino acid residue. In this way the amino acid sequence of the protein can be assembled.

18.3.2 Protein Mass Spectrometry

MS is the key technique for modern proteomics enabling an accurate determination of protein and peptide masses, structural analysis of protein and peptide sequences, as well as protein quantification. In general, a **mass spectrometer** is composed of different elements: a **sample introduction** system and an **ion source**, a **mass analyzer**, and a **detector** with signal treatment system (Figure 18.5a).

18.3.2.1 Ion Sources for Biomolecules

The sample introduction/ion source help the transfer of the sample to the gas phase (vaporization) followed by

an ionization of the molecules. For biomacromolecules, especially for proteins and peptides, two different ion sources have been developed: **electrospray ionization (ESI)** and **matrix-assisted laser desorption/ionization (MALDI)**. Both techniques assure a soft ionization of large biomolecules conserving, for the most part, their integrity.

During the **ESI** process (Figure 18.5b), a liquid sample solution is nebulized in an electric field of 1–5 kV between the nebulizer tip and the entrance of the MS. As a consequence, the small droplets are charged and during vaporization this charge is transferred to the biomolecules. For peptides and proteins, the polarity of the source is in the positive mode transferring a positive charge (proton) to them, which is located, for example, at the amino groups. In ESI, large molecules are often multicharged, which makes the mass spectra more complex and data deconvolution necessary. Also, it allows the analysis of very large proteins up to 200 kDa. An ESI source can be easily combined with the outlet of an LC system for an LC-ESI-MS coupling.

In contrast, **MALDI** refers to an ionization of the sample in the solid phase (Figure 18.5c). The analyte molecules such as peptides and proteins are embedded in a solid matrix of small molecules with about 5000 times molar excess of the matrix. The most commons matrices are 2,5-dihydroxybenzoic acid (DHB) and derivatives of cinnamic acid: 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and α-cyano-4-hydroxycinnamic acid. The matrix/analyte mixture is prepared in solution and then placed in the form of small drops on a metal plate where the drops leave a solid spot after drying. In vacuum, short (3–5 ns), repeated laser shots are fired on the spot. The matrix isolates the analyte molecules and stabilizes them from degradation by the laser. The matrix molecules are able to absorb photons at the laser wavelength (ultraviolet) and they are a source of protons that are transferred as positive charge to the analytes. After absorbing laser photons, matrix molecules are excited and ionized. The laser energy provokes their dissociation and passage to the gas phase where the ionized matrix molecules transfer their charge to the analytes. However, details of this process are not fully explored yet. The analytes can also already be ionized in the solid matrix before vaporization. The MALDI process produces mainly monocharged analyte ions that are accelerated in the electric field between the MALDI plate and the MS entrance, typically a Time-of-flight (TOF) MS (see Section 18.3.2.2). A coupling of LC with MALDI-MS can be realized only via a spotter where the LC eluent is continuously mixed with the matrix solution and deposited in subsequent spots on the MALDI plate.

18.3.2.2 Mass Analyzers

Mass analyzers separate ions according to their mass-to-charge ratio m/z. In order to achieve this, different physical principles are applied leading to the construction of different types of mass analyzers.

Sector field (SF) MS with magnetic and electrostatic sectors use different dispersion of ions in magnetic or electric fields. The dispersion depends on the ion kinetic energy, which in turn depends on their m/z. This principle for ion separation was historically the basis for the first mass spectrometers.

TOF MS separate ions by their different flight times in a field-free tube. This principle is based on different ion velocities acquired during acceleration in an electric field. Thus, their time of the flight to the detector is different depending on the m/z ratio.

In **quadrupole (Q) MS,** the transmission of ions traversing an electrodynamic field is measured. Only ions with an m/z ratio that is in resonance with the frequency of electrodynamic field have a stable trajectory in this field. By changing the frequency, the quadrupole can scan for ions with different m/z ratios.

On the basis of the principle of periodic ion movement in electric or magnetic fields, three different types of MS have been developed:

Ion trap (IT) MS have tridimensional or linear ITs, which are small quadrupoles with an electrodynamic field. By tuning the frequency of this field, ions with a certain m/z can be trapped in a stable periodic movement in this field. For acquiring a MS spectrum ions with different m/z are subsequently trapped and ejected to the detector.

In **Orbitrap MS** ions perform a periodic movement in an electrostatic field. In this technique, the frequency of the ion movement is measured, which is in correlation to the m/z ratio of the ion. Applying Fourier transformation the measured frequencies are converted into an MS spectrum.

The same principle is applied in **Fourier transform ion cyclotron resonance (FTICR) MS** where the ions perform a periodic movement in a magnetostatic field. The cyclotron frequency of an ion depends on its m/z ratio. The frequencies of the ions are measured and transformed into an MS spectrum.

An important parameter of all mass analyzers is their **mass resolution**, which means the ability to distinguish between two similar m/z ratios. The closer the m/z ratios that can be differentiated by an MS are, the higher their resolution. Q MS and IT MS show relative low mass resolutions, which make structural identification of peptides and proteins in complex samples difficult. In contrast, SF MS, TOF MS, Orbitrap MS, and FTICR MS show high mass resolution enabling, for example, peptide mass fingerprinting.

A typical instrument used for peptide mass fingerprinting in the bottom-up proteomics approach is MALDI TOF MS. The high mass resolution of the instrument enables the identification of peptides in a protein digest by their exact masses (m/z ratios). These masses can be calculated on the basis of the theoretical amino acid sequence obtained from databases. The experientially identified peptides are matched with the theoretical peptides in the databases for protein identification (see Section 18.3.1.5).

Orbitrap MS and FTICR MS show the highest mass resolution and thus they are powerful, but they are expensive tools for highly accurate protein and peptide sequencing and identification. Top-down proteomics approaches, especially, require these instruments for identification of entire proteins and their fragments via their exact masses.

Other parameters of mass analyzers to be considered are accuracy, mass range, and scan speed. For further reading, the interested reader is referred to special literature on MS.

18.3.2.3 Tandem Mass Spectrometry

Tandem MS refers to the coupling of two mass analyzers in one instrument. Using the **tandem-in-space** principle, two mass analyzers are arranged inline while between the two analyzers a **collision cell** is placed (Figure 18.6a). All possible combinations of mass analyzers can be envisaged. Frequent arrangements are two quadrupoles (called a **TripleQuad instrument**; the third quadrupole is not a mass analyzer but a collision cell between the two others) or a quadrupole combined with a time-of-flight (QTOF MS). Also, quadrupoles are the first mass analyzer in tandem Orbitrap and FTICR instruments.

In general, the first mass analyzer, usually a quadrupol, helps the separation and selection of ions. Then the selected ions (precursor ions) can be fragmented in the collision cell by collision-induced dissociation using an inert gas such as nitrogen (Figure 18.6b). The fragment ions (product ions) are subsequently separated by the second mass analyzer, which is often a high-resolution mass analyzer. This results in a tandem mass (or MS/MS) spectrum of the daughter ions.

Another tandem MS principle is **tandem-in-time,** which is performed in **IT** MS (Figure 18.6c). In this case the IT serves as the first mass analyzer, as collision cell and as second analyzer, all in one (Figure 18.6d). The ions are trapped, fragmented, and the fragments separated consecutively "in time." This technique enables fragmentations of fragment ions, thus several subsequent fragmentations, which is called $\mathbf{MS^n}$.

Tandem MS aids in structural analysis and thus is a key technique for peptide identification in the bottom-up and shotgun proteomics approaches. The fragmentation of peptides in the collision cell follows gas-phase chemistry, which leads to specific, predictable peptide fragments. The most common fragmentation is a cleavage of the peptide

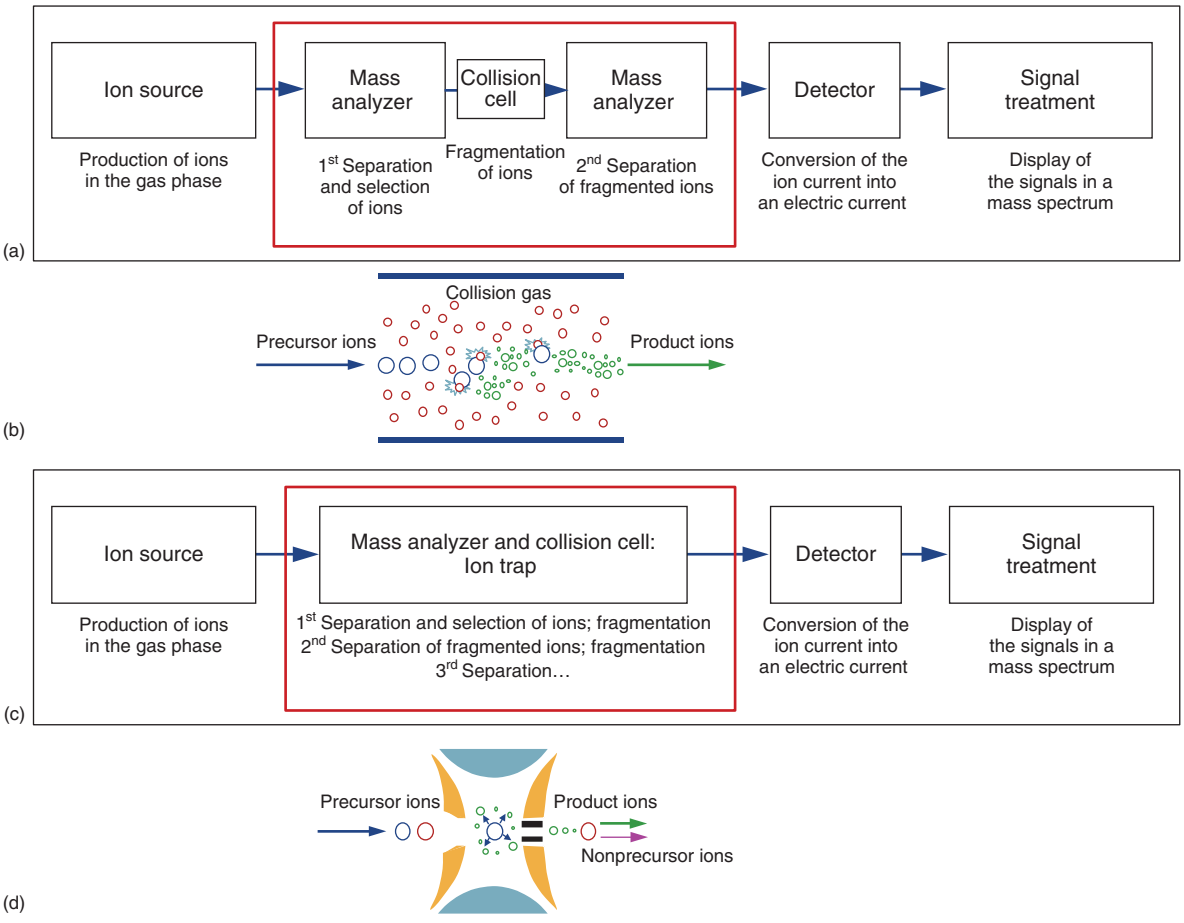

Figure 18.6 Tandem mass spectrometry: (a) Tandem-in-space principle with a collision cell between two mass analyzers. (b) Details of a collision cell. (c) Tandem-in-time principle with an ion trap as mass analyzer and collision cell in one. (d) Details of an ion trap.

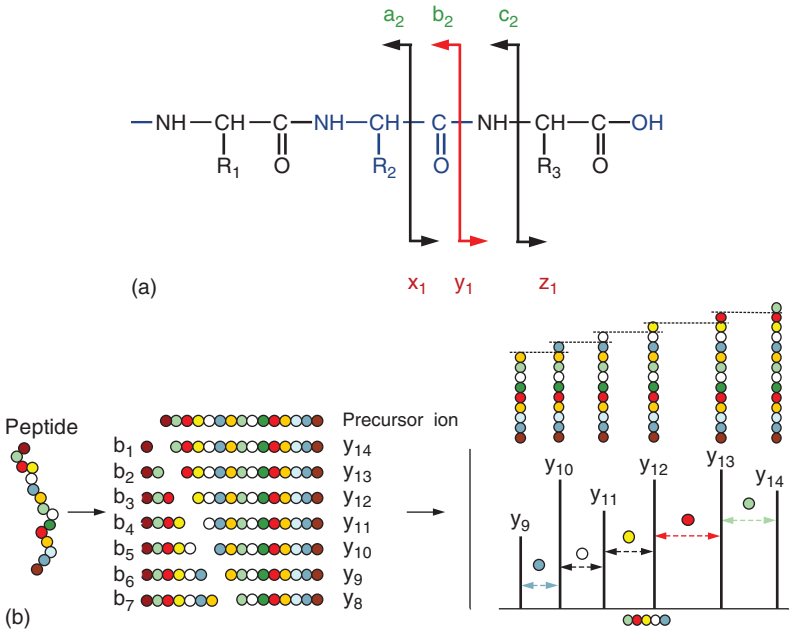

Figure 18.7 (a) Fragmentation of peptides in a collision cell. (b) Statistic fragmentation of peptides enables reconstruction of the amino acid sequence of a peptide from the MS/MS spectrum.

bond between the carbon and the nitrogen atom resulting in two types of peptide fragment ions, called *b-ions* and *y-ions* (Figure 18.7a). Each peptide fragmentation results in a series of b- and y-ions with a mass difference of one amino acid between the fragments (Figure 18.7b). In this way, the amino acid sequence of a peptide can be reconstructed from the MS/MS spectrum and, hence, the peptide be identified.

Tandem MS for top-down proteomics needs high-resolution mass analyzers. Typical instruments are FTICR MS or MALDI TOF TOF MS. In the latter instrument. two high-resolution TOF mass analyzers are combined. Fragmentation of the intact protein is performed by collisions as well as infrared photons or electrons (electron capture dissociation). For reconstruction of the protein sequence, the exact masses of fragments have to be determined with high accuracy; the mass differences between the fragments give information about the sequence.

18.3.2.4 Element Mass Spectrometry: ICP MS

Inductively coupled plasma mass spectrometry (ICP MS) is a special MS technique for ultrasensitive detection and quantification of chemical elements (see Section 18.5.1.1). In ICP MS the sample is introduced into hot argon plasma (ICP) of about 7000 K serving as ion source that destroys every chemical structure and produces simple element ions (Figure 18.5d). This technique is usually employed in inorganic and environmental chemistry. However, the application of ICP MS in proteomics is a growing field. Although all structural information is lost, ICP MS can be used as an assisting technique in proteomics complementary to other MS techniques by ultrasensitive detection of heteroelements, which occur in proteins and peptides. These elements are **sulfur** (in most proteins within the amino acids methionine and cysteine), **selenium** (in selenoproteins), **phosphorus** (in phosphorylated proteins), and **metals** (in metalloproteins). Common elements of organic molecules such as hydrogen, carbon, oxygen, and nitrogen cannot be detected by ICP MS.

For example, **LC-ICP** MS can be applied complementarily to LC-ESI MS. In this way, low-abundant selenopeptides or phosphorylated peptides can be detected sensitively in complex matrices via the selenium of phosphorus signal in ICP MS. However, in addition, LC-ESI MS has to be applied for identification of these peptides.

Another possibility is the application of **laser ablation (LA)–ICP MS** to electrophoresis gels allowing the detection of heteroelements and metals in protein spots. High-frequent laser shots ablate material from a surface such as the gel and the resulting aerosol is transferred via the gas phase into the argon plasma.

A new field is metal labeling of peptides and proteins in order to widen the scope of ICP MS also to those biomolecules that do not comprise naturally an ICP MS-detectable heteroelement. Such metal labels are, for example, highly stable metal complexes with a reactive group that can derivatize functional groups (amino groups, thiol groups) in peptides/proteins. Typically, lanthanides are used for metal labeling, which have virtually no biological background and can be detected by ICP MS with ultrahigh sensitivity.

ICP MS-based heteroelement detection in peptides and proteins can also be employed in quantification strategies (see Sections 18.3.3.2 and 18.3.3.3).

18.3.3 Quantitative Proteomics

In global proteomics the focus of investigation is a qualitative proteome analysis with the aim to sequence peptides and proteins, identify them, and discover new proteins. Targeted proteomics focus not only on the identification of specific proteins or protein groups, but in many cases their quantification also become relevant. In principle, the same proteomics strategies can be applied as for qualitative analysis with an additional step for peptide or protein quantification.

Quantitative proteomics distinguishes between relative and absolute protein quantification. Relative quantification compares the protein level in different samples without information on their absolute amount while absolute protein quantification aims to measure this.

18.3.3.1 Label-Free Relative Quantification

An example for relative quantification is the differential proteome analysis of two different physiological states by 2-DE (see Section 18.3.1.3). The relative quantitative differences in protein expression can be measured by the intensity of the protein spots after staining. Nowadays, computer software assists in protein spot recognition and relative quantification, but precision and accuracy of this approach is not very high, which allows only rough comparisons. Errors are manifold, such as incompletely separated and overlapping spots, weak spots, differences in migration between gels, and unmatched, mismatched, and undetected spots.

More advanced are mass spectrometric approaches for quantitative proteomics. A principal drawback in MS is that the intensity of a peak in the mass spectrum depends not only on the analyte concentration in the sample because analyte ionization depends also on the analyte itself and concomitant species in the sample matrix. Thus intensities of different peaks in the MS cannot be directly compared for relative quantification. However, MS intensities of the same analyte in different samples can be compared for relative differences in peptide or protein abundances in the samples under the condition that the sample matrices are similar. This approach is realized in label-free quantification strategies by MS.

18.3.3.2 Chemical and Metabolic Labeling with Stable Isotopes and Metals for Relative Quantification

The introduction of labels allows relative quantification in one sample with higher precision and accuracy as the effect of different sample matrices is avoided. Labeling can be introduced during sample preparation in bottom-up or top-down approaches for relative quantification. For example, in differential proteome analysis, the proteins (or peptides after digestion) in each sample reflecting a different physiological state are derivatized with a different label. Usually, labels contain different stable isotopes; thus they are chemically equal and are different only in mass. The differentially labeled samples are combined to one sample, which is analyzed by ESI MS or MALDI-MS. In the MS the differentially labeled proteins or peptides produce a mass shift according to the mass differences between the stable isotopes. Their peak intensities can be compared for relative quantification.

There are different methods for introducing stable isotopes. For example, proteins can be digested in ^{18}O-labeled water, which labels the resulting peptides with an oxygen isotope. Most popular methods are chemical labeling with isotope tags such as **isotope-coded affinity tags (ICAT)** containing ^{2}H or ^{13}C isotopes, and **isobaric tags for relative and absolute quantitation (iTRAQ)** employed in tandem MS. Metabolic labeling is performed, for example, by **SILAC (stable isotope labeling by amino acids in cell culture)** for differential analysis of two cell cultures. Therefore, one cell culture is grown under the presence of ^{13}C-labeled amino acids Arg or Tyr, the other culture under normal conditions.

Interesting new approaches concern the chemical labeling with rare earth metals chelated by DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or DTPA (diethylenetriamine pentaacetic acid) and applying the mass difference between two monoisotopic elements. The metal-label approach is also applied for protein and peptide quantification by ICP MS.

18.3.3.3 Absolute Quantification Strategies

While relative proteome quantification relies on the comparison of peak intensities in MS, absolute quantification requires in general a calibration of the instrument with standard substances. However, the synthesis of hundreds or thousands of standard peptides and proteins is impossible, and thus it is limited to select target peptides and proteins such as specific biomarkers. A similar approach is the **AQUA** strategy that uses a synthetic isotopically labeled standard of each target peptide, which is, however, also limited to a small number of analytes because of the high expenses for the preparation of the standards. Moreover, the **iTRAQ** method enables absolute quantification, too.

For absolute quantification of heteroatom-containing proteins and peptides, LC-ICP MS can be applied. This is relatively a simple and powerful approach as for calibration only element standards are required. If the target analyte does not comprise naturally an ICP MS-detectable heteroelement, it can be labeled with a metal tag (see Sections 18.3.2.4 and 18.3.3.2). It has to be noted that with ICP MS only elements are quantified. In LC-ICP MS approaches, all peptides and proteins relevant to quantification have to be separated before entering the MS, because all structural information is lost in the plasma. Consequently, baseline separation of the biomolecules and information on their stoichiometry (obtained by molecular MS) is mandatory to accurately quantify them via the element signal.

18.3.4 Determination of the 3D Protein Structure and Functional Evaluation

18.3.4.1 X-Ray Crystallography and Protein NMR

The tridimensional protein structure is related to the protein function. Methods for determining the 3D structure of proteins, that is, their secondary, tertiary, and quaternary structures, include X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. Both methods provide information at atomic resolution. However, they cannot be applied to proteome samples that contain many proteins at different concentration levels, but only to purified and concentrated proteins. Therefore, it is rather a single protein analysis than proteomics.

For **X-ray crystallography,** protein crystals have to be grown. The challenge is to avoid protein precipitation leading to useless amorphous material. The protein crystal is subjected to an X-ray beam and its reflection is recorded showing diffraction pattern. From the data an electron density map is calculated and an atomic model of the molecules is fitted.

NMR spectroscopy of proteins (**protein NMR**) requires aqueous solutions of highly purified protein at concentrations of 0.1–3 mM. This method is restricted to proteins smaller than 70 kDa. Multidimensional NMR experiments are performed that can last, depending on the protein concentration, hours or even several days. From the data, 3D protein models are calculated.

The obtained 3D protein models are often displayed as **Ribbon diagram** with structural parts of the secondary structure: α-helices and β-sheets. An example is presented in Figure 18.8. Protein structures are also the basis for the computer modeling of protein interactions, for example, with proteins, peptides, and other metabolites serving for their functional evaluation. Figure 18.9 shows as an example the modeling of the interaction between the enzyme phytochelatin synthase and thiol peptides in the green algae *Chlamydomonas reinhardtii*.

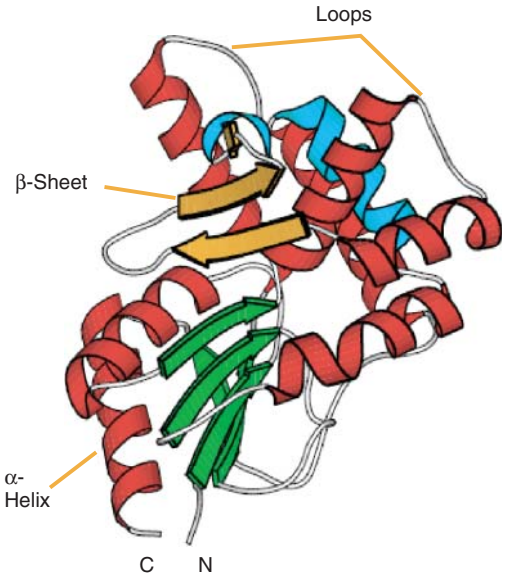

Figure 18.8 Example of a protein 3D Structure represented as Ribbon diagram showing α-helices and β-sheets: the enzyme cytidine monophosphate kinase (in complex with cytidine diphosphate) from *Escherichia coli*. The structure was obtained by a combination of X-ray crystallography and NMR spectroscopy data. (Reprinted from Briozzo *et al.* (1998), with permission from Elsevier.)

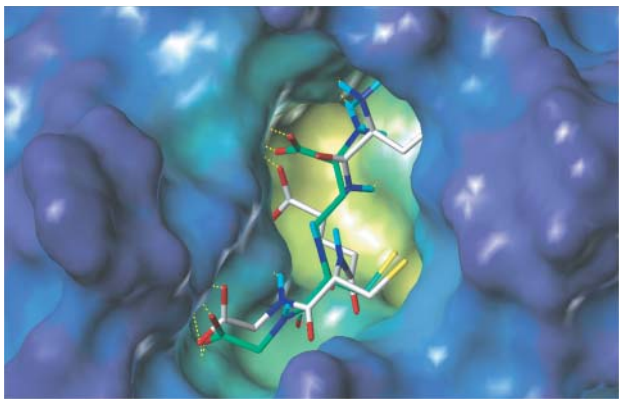

Figure 18.9 Modeling of the interaction between the enzyme phytochelatin synthase (PCS) and the two thiol peptides glutathione (GSH) and CysGSH in the green algae *C. reinhardtii*. Best docking poses generated for the complex of PCS with GSH (carbon atoms colored in green-blue) and CysGSH (carbon atoms colored in white).Yellow dashed lines indicate hydrogen bonding between the enzyme and the ligands. The calculated docking energy scores for the best poses indicate that CysGSH has a higher affinity than glutathione because of a more favorable intermolecular hydrogen bonding. This information helps the understanding of biochemical pathways for phytochelatin synthesis, that is, metal tolerance mechanisms in metal-stressed green algae. (Reprinted from Bräutigam *et al.* (2011), with permission from Wiley.)

18.3.4.2 Functional Protein Microarrays

A similar technique to DNA microarrays (see Section 18.2.1), functional protein microarrays allow the assessment of specific protein functions in a proteome sample. On the array, a large number of purified proteins are immobilized, which are either full-length functional proteins or can contain functional protein domains. This technique allows the investigation of protein activities, binding properties and posttranslational modifications as well as the identification of the substrates of enzymes of interest.

18.3.5 Meta-Omics Approaches

Meta-omics approaches aim to study the genome, transcriptome, and proteome from whole microbial communities (see Table 17.3), which can be found in environmental samples such as soil, sludge, biofilms, seawater, and freshwater but also from symbiotic communities and gut microbiota. **Metagenomics** intends further the construction of metagenomic libraries and the screening of microbial communities for functional or taxonomic genes.

Metatranscriptomics seeks to extract and amplify the total mRNA from complex communities or environmental samples followed by cDNA synthesis and sequencing. The investigated samples are rather complex but the applied techniques are those from the -omics techniques previously described. For example, **metaproteomics**, also denoted as community proteomics or environmental proteomics, comprises similar steps used in conventional proteomic studies including sample preparation, protein extraction, separation, mass spectrometric analysis, and identification by database search and bioinformatics tools (Figure 18.10). The challenge of metaproteomics is the large complexity of the samples and thus a large diversity of protein species requiring high-resolution techniques for protein separation (2D-GE and LC techniques) as well as high-resolution MS. System data from metagenomics, -transcriptomics, and -proteomics integrated with metabolomics data (see Section 18.4.3) allows a better understanding of the composition of microbial communities and their function, physiology, interaction, ecology, and evolution. More methods for studying microbial communities are listed in Chapter 17.

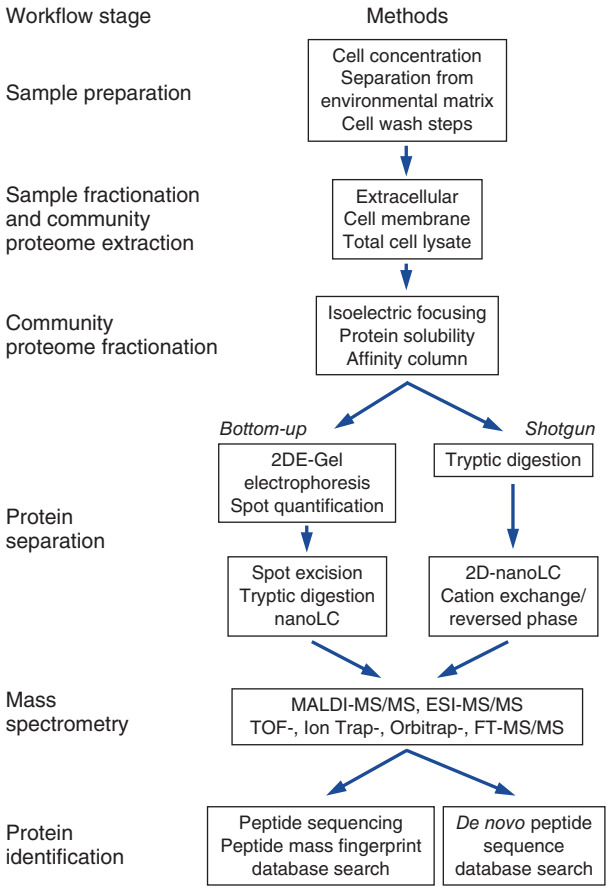

Figure 18.10 Workflow in metaproteomics (community proteomics). The different steps and methods are similar to those in classic bottom-up and shotgun proteomics approaches. (Reprinted from Wilmes and Bond (2009) with permission from Elsevier.)

18.4 Metabolomics

The **metabolome** denotes the entirety of all metabolites in an organism. Metabolites are intermediates or end products of physiological processes, usually small molecules with molecular weights below 1 kDa. Like the transcriptome and the proteome, the metabolome is highly dynamic and changes its composition each moment. The investigation of the metabolome plays an important role in plant biology as plants produce a huge number of metabolites, more than most other organisms. The structural diversity of metabolites in the plant kingdom is enormous; the total number of structures is estimated to be up to 5 000 000.

The analytic of metabolites has a long history, starting in medicine, where it is used to get information on diseases of patients. For example, ancient Chinese doctors used ants for the detection of the metabolite glucose in the body fluid – urine – for the diagnosis of diabetes. In the Middle Ages, urine was investigated for color, taste, and smell, which change depending on the containing metabolites. Nowadays, metabolomics is one of the key technologies in life sciences and biology.

Metabolomics aims to answer the question: *Which metabolites are produced in cellular processes?*

In detail, the main objective of **metabolomics** is a global identification and quantification of all metabolites in a biological system (cell, fluid, tissue) including their time- and space-resolved distribution.

Analytically, this is the most challenging approach compared to other strategies in metabolome research. **Metabolite target analysis** aims to quantify only one or a few target metabolites. **Metabolomic profiling** is intended to describe qualitatively and quantitatively the metabolic pattern for a group of related metabolites. In contrast, **metabolic fingerprinting** performs high-throughput sample screening and thus allows their rapid classification without the identification and quantification of each individual metabolite.

A further question is how the metabolome is linked to the genome, transcriptome, and proteome.

18.4.1 Analytical Strategies

In metabolome analysis, the biological question determines the experimental design, for example, whether a metabolite target analysis will be applied or a global, nontargeted metabolomics study will be undertaken. Figure 18.11 gives an overview of the analytical techniques applied.

In case of metabolite target analysis, selective sample preparation is performed with emphasis on the target metabolites. This can be specific extraction, purification, or labeling. Then an adequate separation by gas chromatography (GC) or LC or capillary electrophoresis (CE) is applied followed by sensitive selective detection. For example, if the biological question is to investigate

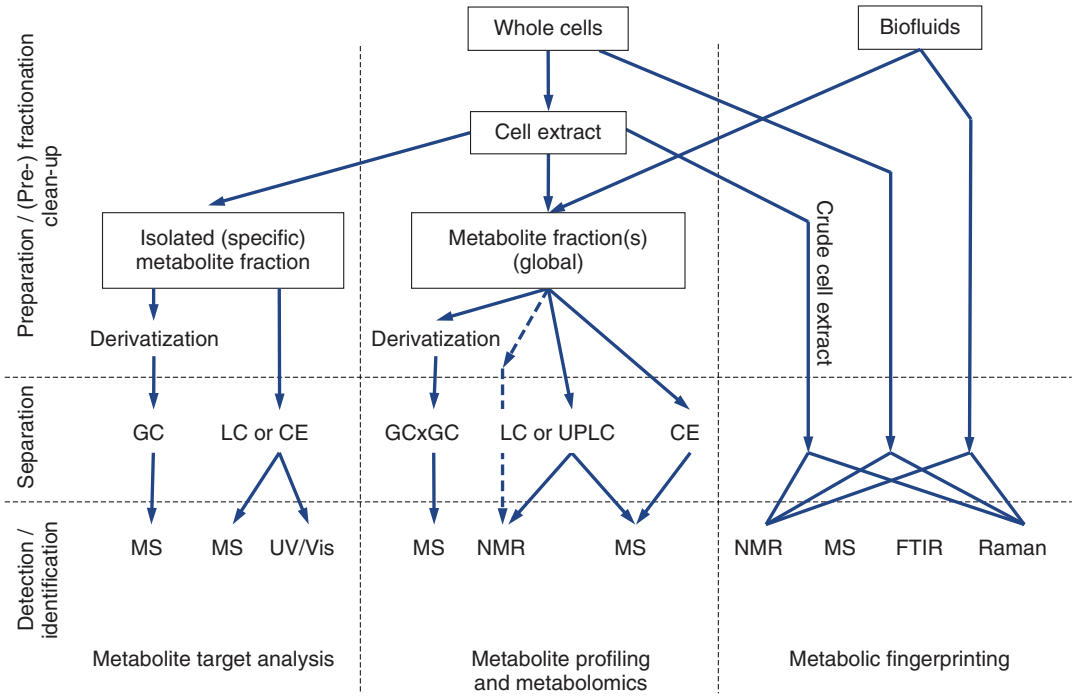

Figure 18.11 Workflows and analytical techniques applied in metabolomics studies. (Reprinted from Goodacre *et al.* (2004) with permission from Elsevier.)

the metabolomic reaction of plants to metal stress, phytochelatins are the target metabolites, which are cysteine-rich peptides with metal-chelating properties (see Section 18.5.3). In a case study, phytochelatins were selectively extracted from the metal-exposed green algae *C. reinhardtii* by hydrochloric acid and separated by reversed phase LC. A postcolumn derivatization of the phytochelatins at their thiol groups with Ellman's reagent allows their specific spectrophotometric detection (UV/Vis) (Figure 18.12).

In contrast, **nontargeted metabolomics** deals with highly complex samples containing thousands of small biological molecules of high physicochemical diversity and large variations in their relative concentrations. Their comprehensive qualitative and quantitative analysis, ideally time- and spatial-resolved, attains the limits of modern analytical techniques. Therefore, global metabolomics approaches require the combination of analytical techniques in order to cope with the high diversity of metabolites.

Generic sample pretreatment followed by high-resolution separation coupled to sensitive mass spectrometric detection is one of the important metabolomics strategies. Depending on the metabolite physicochemical properties such as hydrophilicity, size, and charge, different separation techniques are used. GC-MS, LC-MS, and CE-MS are mainly applied in metabolomics for identification and quantification of as many as possible individual compounds. The different mass spectrometers used are described in the previous section (see Section 18.3.2.2). Besides electrospray, other ion sources suitable for online couplings are employed: atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photo ionization (APPI) for LC-MS couplings; electron ionization (EI) and chemical ionization (CI) for GC-MS.

GC-MS is the most frequently used technique because it provides high separation efficiency combined with selective and sensitive mass spectrometric detection. A limitation is that, GC can separate only volatile compounds, and thus metabolites have to be derivatized before analysis. However, labile compounds can be stabilized by derivatization. The most versatile and universal derivatization for GC is silylation by introducing an R_3Si- group. Nearly all functional groups in biomolecules such as hydroxyl, carboxylic acid, amine, thiol, and phosphate groups can be derivatized by silylation. Thus it is the most suitable for global metabolite analysis by GC-MS. A new development is 2D GC combined with high-resolution MS, for example, GC×GC-TOF MS. The combination of two GC columns of different functionality, such as a hydrophobic and a polar column, provide high separation efficiency for complex metabolomic samples.

LC-MS covers a wider range of hydrophilic compounds and molecular masses, for example, peptides, and do not need derivatization. Recent LC techniques such as Ultra Performance Liquid Chromatography coupled to mass spectrometry (UPLC-MS) provide rapid and high-resolution separations for metabolite analysis. For detection, tandem MS is applied in the SRM (selected reaction monitoring) or MRM (multiple reaction monitoring) mode allowing ultrasensitive and specific detection of the metabolites by their daughter ions in MS/MS. Recent

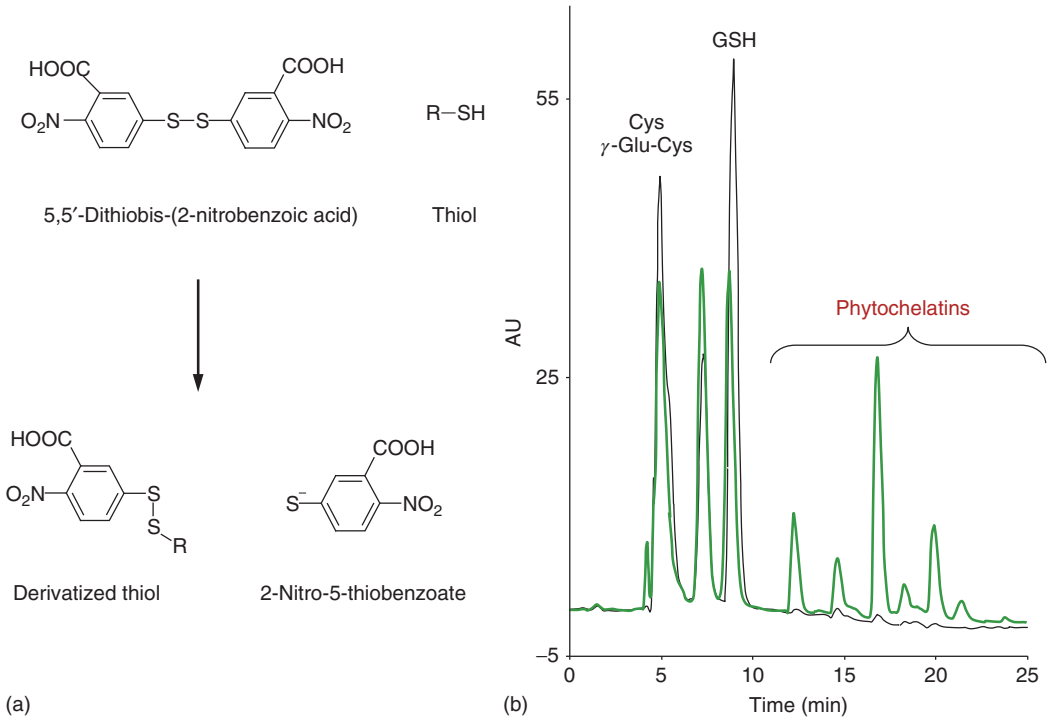

Figure 18.12 Metabolite target analysis of thiols. (a) Postcolumn derivatization of thiols with Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid); DTNB) allows their targeted analysis by the specific spectrophotometric detection of the reaction product 2-nitro-5-thiobenzoate at 412 nm. (b) Example of thiol detection in an extract of the green algae *C. reinhardtii* after cadmium stress. In addition to the thiols, cysteine (Cys), γ-glutamylcysteine (γ-Glu-Cys), and glutathione (γ-Glu-Cys-Gly, GSH), which are also present in the control sample (thin line), phytochelatins (PC) were detected, in the Cd-exposed sample, between 12 and 25 min (bold line). Phytochelatins have the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2-6$). They are synthesized in plants under metal stress by phytochelatin synthase from GSH. (Bräutigam et al. (2009).)

metabolomic platforms use complementarily GC×GC-MS and UPLC-MS for comprehensive metabolomics studies where GC and LC cover different classes of metabolites.

CE has theoretically higher separation efficiency than LC and covers a wider range of metabolite classes than GC. However, the use of **CE-MS** is less common in metabolomics but useful for specific applications, such as labile compounds (e.g., certain metal complexes), which cannot be separated by GC or LC without decomposing the metabolites.

Besides coupling techniques with MS, **NMR**, **Fourier transform infrared (FTIR),** and **Raman** spectroscopy are commonly applied in metabolomics, too. They are high-throughput methods with minimal sample preparation that do not require a prior analyte separation. Although being less sensitive than MS-based techniques, NMR and FTIR can give a global view on metabolic networks by measuring all metabolites simultaneously in crude extracts, whole cells, or body fluids. Thus they are preferred techniques for metabolic fingerprinting.

Quantification of metabolites requires standard compounds for calibration of the analytical systems. However, there are practical limitations for global **quantitative metabolomics** because for the majority of metabolites in a sample, standard compounds are not available or cannot be synthesized. Therefore, mostly relative quantification is applied in metabolomics. This means that the signal intensity of a metabolite is determined in comparison to those of another metabolite or an internal standard. In this way, unidentified metabolites present in a sample also can be quantified. The disadvantage of relative quantification is that data of different samples are difficult to compare.

Analytical platforms for metabolomics provide sometimes thousands of signals from one sample analyzed. This huge amount of data has to be carefully evaluated. For unambiguous identification, a metabolite has to be structurally characterized. Thus not every chromatographic peak, signal in the mass spectrum, or chemical shift in NMR represents necessarily a metabolite but can also be an artifact. It was estimated that only 30–50% or less of all analytical signals from a complex metabolomics sample are truly identified chemical structures. Careful method validation and regular quality controls of the analytical techniques are mandatory for obtaining reliable metabolomics data.

18.4.2 Metabolomics in Single Cells

As the cell is the structural and functional unit of all organisms, investigation of metabolic processes at cellular level is of particular interest. This requires analytical techniques with high spatial resolution. A classical method to provide

samples at cellular and subcellular level is cell fractionation. Samples from single cell can also be obtained by microsampling with microcapillaries or laser microdissection. The latter methods are described in detail in Section 19.4.

Interesting approaches are mass spectrometric-based imaging techniques. MALDI-MS imaging allows metabolite analysis at spatial resolution down to 20 µm. This is, however, not applicable to single cells but to biological tissues. Mass spectrometric imaging of single cells can be achieved with **SIMS** techniques (**secondary ion mass spectrometry**). With SIMS element mass spectra from surfaces are obtained. An energetic primary ion beam of, for example, O^- or Cs^+ ions ejects positive or negative ions from the sample surface. These secondary ions are element or small molecule ions that are measured by the MS. In particular, the **nanoSIMS** technology allows measurements at spatial resolution down to 50 nm and, therefore, it is suitable for single cell imaging. Using the Cs^+ primary ion source, negative secondary ions are generated enabling the detection of elements such as C, N (by CN^- detection), P, S, and O. This mode allows, in addition, the detection of secondary electrons. The O^- primary ion source generates positive ions for the detection of certain metals such as Ca, Mg, Mn, and Cr. NanoSIMS provides mass spectra of chemical elements and their isotopes and thus it cannot analyze chemical structures of metabolites. However, nanoSIMS was successfully applied in metabolism studies at cellular level by using stable isotopes such as ^{13}C and ^{15}N. This technique can image metabolic activities in microbial cells and, by measuring isotope ratios, cell-specific uptake rates and nutrient fluxes can be determined. For example, carbon and nitrogen fluxes were measured in the diazotrophic cyanobacterium *Aphanizomenon* sp. from the Baltic Sea by incubating the bacteria with $H^{13}CO_3^-$ and $^{15}N_2$ (Figure 18.13). High-resolution images of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios enabled the visualization of fluxes between two adjacent cells.

In another way, nanoSIMS can be regarded as a coupling of microscopy with MS. Therefore, optical microscopy is often applied as complementary technique, especially fluorescence and electron microscopy; moreover, preparation of biological materials for nanoSIMS is very similar as for electron microscopy (see Section 19.1.13). Fluorescence *in situ* hybridization (FISH) (see Chapter 17) has been combined with nanoSIMS to study microbial communities. Furthermore, nanoSIMS can be applied to image metal distribution in cells for metallomics studies (see Section 18.5.1.3).

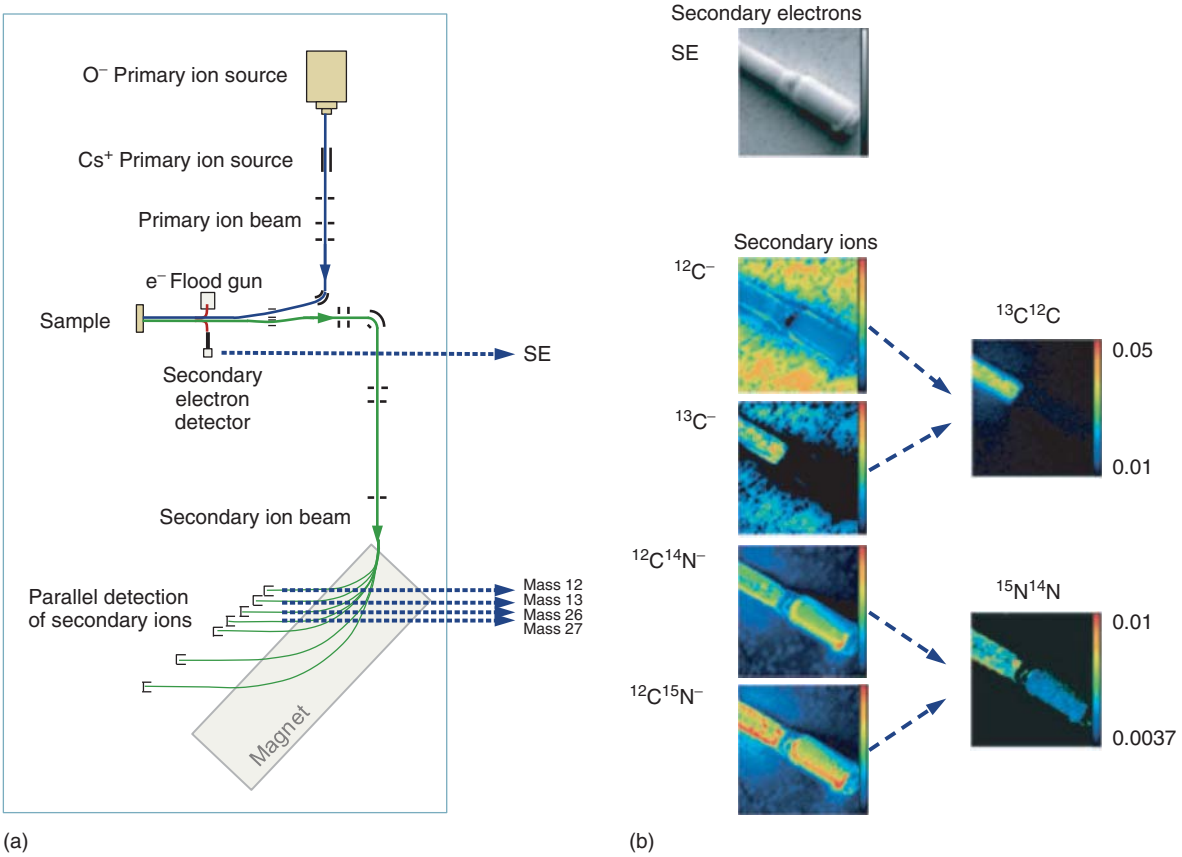

Figure 18.13 NanoSIMS imaging of metabolic activities in single cells. (a) Scheme of the nanoSIMS principle with seven parallel secondary ion detectors and one secondary electron detector. (b) Example for the visualization of carbon and nitrogen fluxes between adjacent cells of the diazotrophic cyanobacterium *Aphanizomenon* sp. using Cs^+ as primary ion beam. (Reprinted from Musat *et al.* (2012) with permission from Wiley.)

18.4.3 Integrating -Omics Techniques

Unlike the transcriptome and the proteome, the metabolome cannot be directly derived from genome data. However, it has become common nowadays to integrate genomics, transcriptomics, proteomics, and metabolomics data for a new understanding of the metabolism of organisms and their interaction with the environment.

The key question is: *How does the genotype determine the environmentally influenced phenotype?*

Linking the static **genotype** and the highly dynamic molecular **phenotype** and its physiology can be regarded as an important challenge for the future decades. For revealing the dynamic genotype-phenotype relationship the -omics techniques are combined with computer-assisted theoretical and modeling approaches as well as scientific databases, which link experimental data to search for new interpretation and knowledge. The aim of **systems biology** is integrating experimental data, reconstructing **metabolic networks,** and deriving mathematical models of metabolism. The main goal is to predict on the basis of the genome, the metabolism, the metabolic networks, and, finally, the molecular phenotype and the ecophysiology, for example, of a plant.

An approach is the *ab initio* prediction of metabolic networks from genome sequences (Figure 18.14). The genome is sequenced by NGS techniques (see Section 18.1.2) followed by a functional annotation of the genes (see Section 18.1.3) resulting in a gene list. From this gene list enzymatic reactions are postulated including their educts and products and mapping of pathways. This reaction list is the basis for building a stoichiometric matrix from which a metabolic network can be postulated. The theoretical metabolic reconstruction from genome data produces static information that does not respect the dynamic of metabolism. For example, the genome sequence contains no information on which enzyme is active or inactive, which influences thus the stoichiometric matrix. Therefore, this *ab initio* approach has to be complemented by quantitative metabolomics analysis (see Section 18.4.1), as well as by transcript and protein data, and dynamic metabolic modeling in order to predict dynamic metabolism.

For further information on this fascinating and growing field of system biology the interested reader is referred to the special literature.

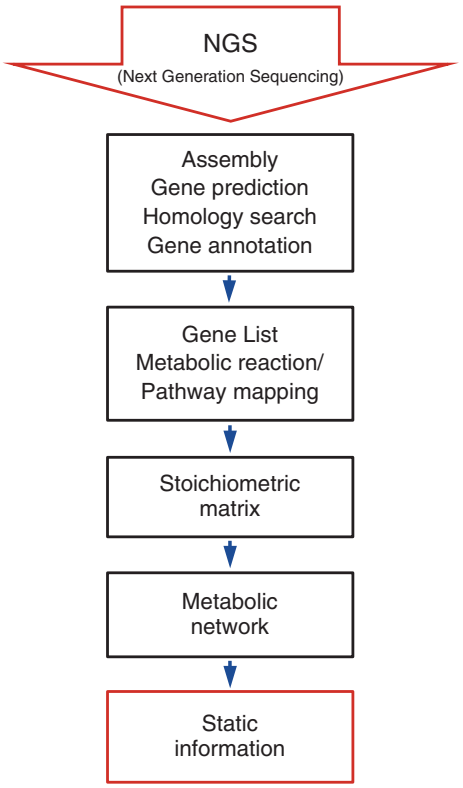

Figure 18.14 The static genotype. The complete workflow for *ab initio* prediction of metabolic networks from genome sequences. Central information is the functional annotation of genes based on homology with genes from other organisms and the corresponding stoichiometric matrix. The resulting reconstructed metabolic network provides not dynamic but static information from genome sequences. (Reprinted from Weckwerth (2011a) with kind permission from Springer Science and Business Media.)

18.5 Metallomics

Metals play an important role in many life processes: on the one hand, they can be essential; on the other hand, they can be toxic (see Chapters 11, 12). In order to describe the entirety of metal and metalloid compounds in an organism or its parts (cells, body fluids, or tissues), and in analogy to the previous described fields, the term *metallome* was coined.

Metallomics aims to answer the question: *What are the interactions and functional connections between metal ions and DNA, RNA, proteins, and metabolites?*

Most direct interactions occur between metals and proteins or metabolites. Thus, like the proteome and the metabolome, the metallome is also highly dynamic, changing each instant. Therefore, the metallome can be regarded as being mainly a subsection of the proteome and the metabolome, which includes their interactions with metals. However, metallomics require specific analytical techniques and should, therefore, be considered apart.

When regarding the already enormous number of proteins and metabolites in an organism and then adding possible metal interactions with these molecules, the metallome comprises an immense amount of diverse structures in permanent transformation. This includes not only thermodynamically stable metallobiomolecules but also lable complexes and intermediates. All this demonstrate that the analytical challenges are enormous

and that the available analytical techniques today can only approach and not fully accomplish a comprehensive metallome analysis. Therefore, it can be stated that to date global metallomics is still rather a vision than a realizable concept.

The recent focus is more on feasible targeted metallomics studies, for example, the analysis of specific metalloproteins or groups such as selenoproteins. A group of specific metallobiomolecules of particular importance in plants is the metallothiolome. This is the entirety of thiol peptides and their metal complexes involved in the thiol peptide regulated metal homeostasis.

18.5.1 Analytical Strategies

18.5.1.1 Element Mass Spectrometry (ICP MS)

As in metabolomics, the analytical goal in metallomics is the identification, quantification, and localization of compounds. The first approach in metallomics studies is the detection of all metals and metalloids present in the sample and the determination of their total concentration. The method of choice is ICP MS, a technique for ultrasensitive detection and quantification of chemical elements (see Section 18.3.2.4 and Figure 18.5d). For global trace element analysis, including biologically significant nonmetals (e.g., chlorine, bromine, iodine), the term ***ionomics*** is applied.

18.5.1.2 Coupling Techniques

For detection and identification, LC (and CE) is coupled to MS as in proteomics and metabolomics. Special attention has to be paid on the stationary phase of the LC, which should not affect the metal-biomolecule complex. Size-exclusion chromatography is often used as soft separation technique, but its chromatographic resolution is low allowing rather a fractionation of the sample than a separation of the compounds. A further specificity in metallomics is that LC is coupled additionally to ICP MS. This allows a specific, highly sensitive detection of metallobiomolecules in a sample via the metal signal in ICP MS. **LC-ICP MS** is highly selective for metal compounds while other biomolecules are not detected. Furthermore, LC-ICP MS enables quantification of metallobiomolecules by their metal signal, provided that these compounds have been structurally characterized. However, structural characterization is possible only by molecular MS. Thus the complementary application of LC-ICP MS and LC-MS is the workhorse in metallomics. Furthermore, one important limitation is that only those metallobiomolecules that are stable during the analytical procedure including sample preparation and separation can be analyzed. This, however, excludes a certain number of kinetically and thermodynamically labile metal–biomolecule complexes of the metallome from being analyzed by these methods. In certain cases, CE is applied as a separation technique in order to cope with more labile complexes. Another problem of labile compounds is the potential formation of artifacts that are detected instead of the metallobiomolecule originally present in the sample.

As described earlier (see Section 18.3.2.4), **LA-ICP MS** applied to gel electrophoresis can be employed for the detection of metals in protein spots. However, if metalloproteins are targeted to be analyzed by this method, nondenaturating gel electrophoresis, instead of SDS-PAGE, has to be used, because protein denaturation would destroy the 3D protein structure and thus release the metal cofactor.

18.5.1.3 Elemental Imaging Techniques

Techniques for imaging metals and other concomitant elements (e.g., S and P) approach the local distribution of the metallome in a cell or tissue. Third generation **synchrotron** facilities provide beamlines with high flux X-ray beams focused down to a size of about 0.5 µm. This allows **micro X-ray Fluorescence (µXRF)** and **X-ray Absorption Spectrometry (XAS)** at single cell level including **micro Extended X-Ray Absorption Fine Structure (µEXAFS)** and **micro X-ray Absorption Near Edge Structure (µXANES)**. While XRF enables elemental imaging, EXAFS and XANES provide additional information about the chemical environment of metals and thus their coordination by ligands (e.g., thiol groups). X-ray absorption of an atom results in photoelectron emission, which is scattered at the neighboring atoms. Thus the nature of these atoms influences the absorption fine structure. Single scattering is responsible for the EXAFS fine structure, multiple scattering for the XANES fine structure of the spectrum (Figure 18.15). While XANES allow identification

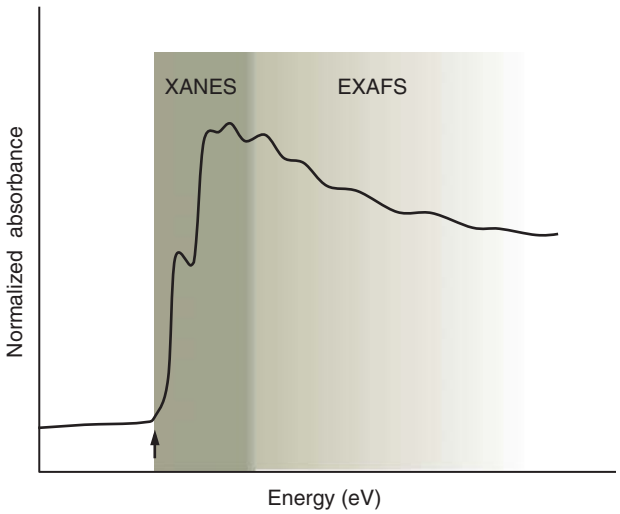

Figure 18.15 Investigation of the fine structure of an X-ray absorption spectrum provides information about the chemical environment of an element. The arrow marks the absorption edge. The energy range of the spectrum studied in XANES is near the edge (gray), the EXAFS energy range is marked in light gray.

of the neighboring atoms, EXAFS provide even information about the next but one atom. Without complex sample preparation, metals can be investigated directly in their natural environment. Therefore, biological materials, for example, cells, are frozen in liquid nitrogen (−196 °C), fixed on a sample support and analyzed as frozen hydrated samples.

NanoSIMS is another element-imaging technique described before (see Section 18.4.2). This technique is also suitable for the localization of metals at subcellular level. Unlike in X-ray techniques, the sample cannot be analyzed in frozen state but has to be dehydrated and embedded in a resin as for electron microscopy (see Section 19.1.3).

Other techniques for element-specific imaging include energy dispersive X-ray spectrometry (EDX) or electron energy loss spectroscopy (EELS) coupled to electron microscopy (see Section 19.1.3).

Although these imaging techniques provide primarily information about metal distribution with high spatial resolution and additional information on their chemical environment when using EXAFS and XANES, structural characterization of metal compounds and thus a global metallomics study is not possible.

18.5.1.4 Bioinformatic Approaches

Experimental data on the metallome acquired with the techniques described above can be supported and complemented by bioinformatic approaches. This is mainly applied for the study of metalloproteins. Genome and proteome databases can be searched for specific amino acid sequences in proteins that are prone to bind metals. For example, zinc-binding patterns can be predicted when Cys_4 or Cys_2His_2 sections are found in protein sequences. These sequences are known in Zn-binding zinc-finger proteins. Moreover, bioinformatic calculations can predict metal-binding sites from 3D protein structures, which are obtained from X-ray crystallography and protein NMR (see Section 18.3.4.1).

18.5.2 Functional Connections between DNA, Proteins, Metabolites, and Metals

As in metabolomics, in metallomics the **genotype – phenotype relation** is an issue of investigation. This aims to study which genes are responsible for the presence or absence of particular metals in organisms. These studies are carried out mainly for plants. Therefore, plant wild-type sample are compared for their metal content with mutants where specific genes are knocked out. Thousands of plant samples are analyzed for their trace element content and the metal concentrations are statistically compared. Metals are either globally analyzed in bulk samples by ICP MS (see Section 18.5.1.1) or spatially resolved by imaging techniques (see Section 18.5.1.3) where the latter method provides more information than a simple bulk analysis. In this way empirical connections between genes and metal concentrations/distributions can be established. This approach, also denoted as ionomics, does not regard the proteins and metabolites involved, which are, however, the link between genes and metals represented by metalloproteins and metallometabolites.

Other approaches try to link the genome to the metalloproteome or the metallometabolome. These methods are similar to those described for gene, protein, and metabolite functional analysis and data integration (see Sections 18.1.3, 18.3.4, and 18.4.3) extended by metal analysis. An example is given in Box 18.1.

18.5.3 Metallothiolomics

Intracellular thiol peptides are highly important for biological processes, containing the major active form of sulfur (thiol or sulfhydryl group: –SH) and can serve as ligands for metal binding. Most important are **glutathione (GSH)** and **phytochelatins (PCs)**. The latter are

Box 18.1: Example for the combination of genome and metal-metabolite analysis

The metal hyperaccumulating plant *Noccaea caerulescens* (former name *Thlaspi caerulescens, Embryophyta*, *Brassicales*) was investigated by complementary genome and metabolite analysis (Figure 18.16). Therefore, a DNA library of the plant was constructed in yeast, that is, a population of yeast cells where each cell carries different DNA fragments of the plant. Then a culture of about 400 000 yeast transformants were submitted to a nickel solution with a Ni^{2+} concentration, which is lethal for yeast. A few transformants survived the metal toxicity test suggesting that they contain a nickel-resisting gene from the plant *N. caerulescens.* These cells were submitted to DNA sequencing identifying a gene coding the enzyme nicotianamine synthase. Metal-specific LC-ICP MS demonstrated the presence of a nickel complex in the surviving yeast clones and in the original plant. Metabolite analysis by MS identified nicotianamine see Section 11.1.4.3 as ligand suggesting the presence of Ni-nicotianamine in yeast and plant. The analysis of a synthetic Ni-nicotianamine complex confirmed the results. This is one of the few examples that shows that integration of genomics and metabolomics with metal analysis leads to the specific identification of metal-metabolites Vacchina et al. (2003).

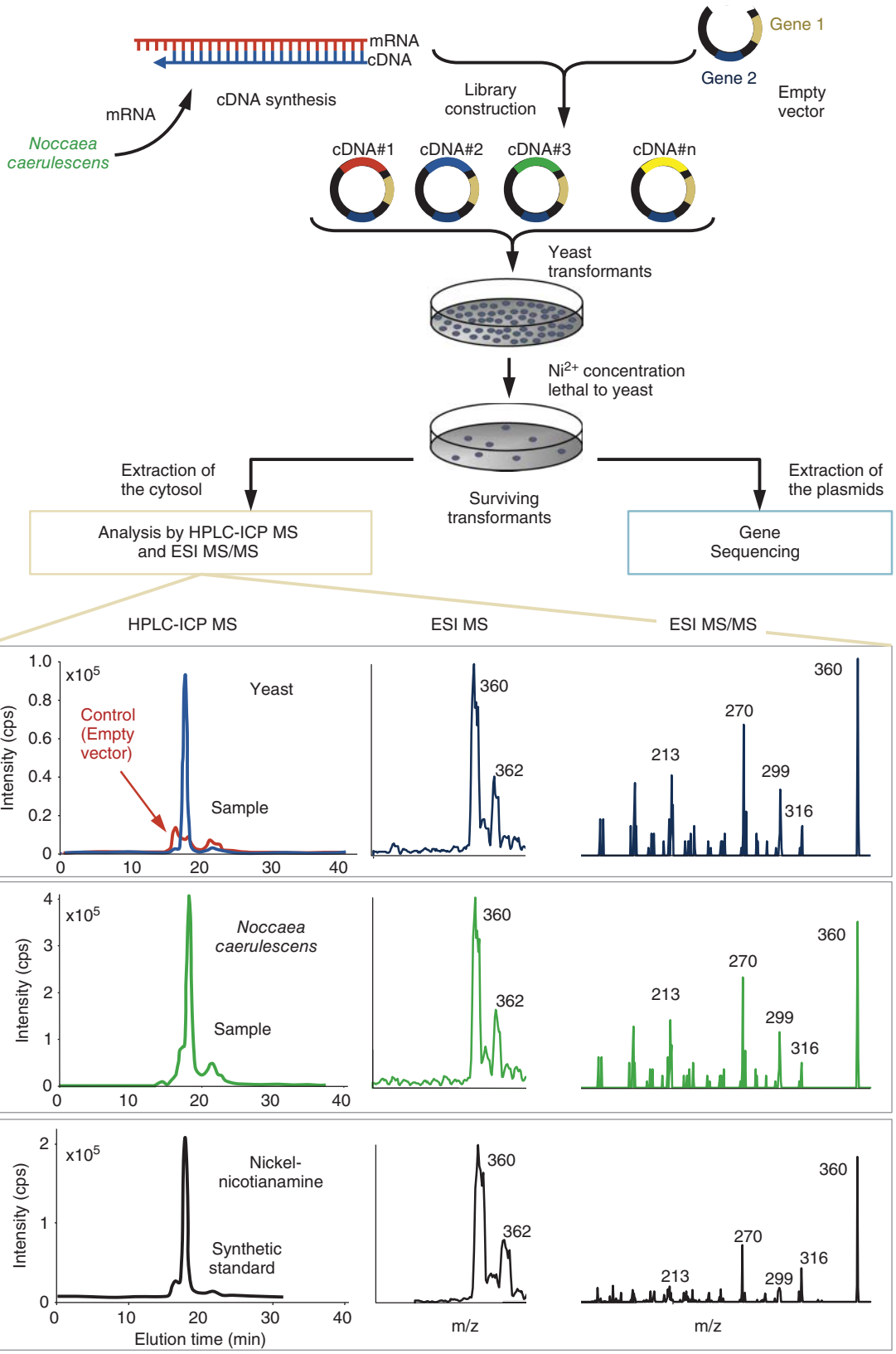

Figure 18.16 Investigation of metal resistance in the metal hyperaccumulating plant *T. caerulescence* (*N. caerulescence*) by complementary genome and metabolite analysis. (Adapted from Ref. Vacchina *et al*. (2003), © 2009 American Chemical Society.

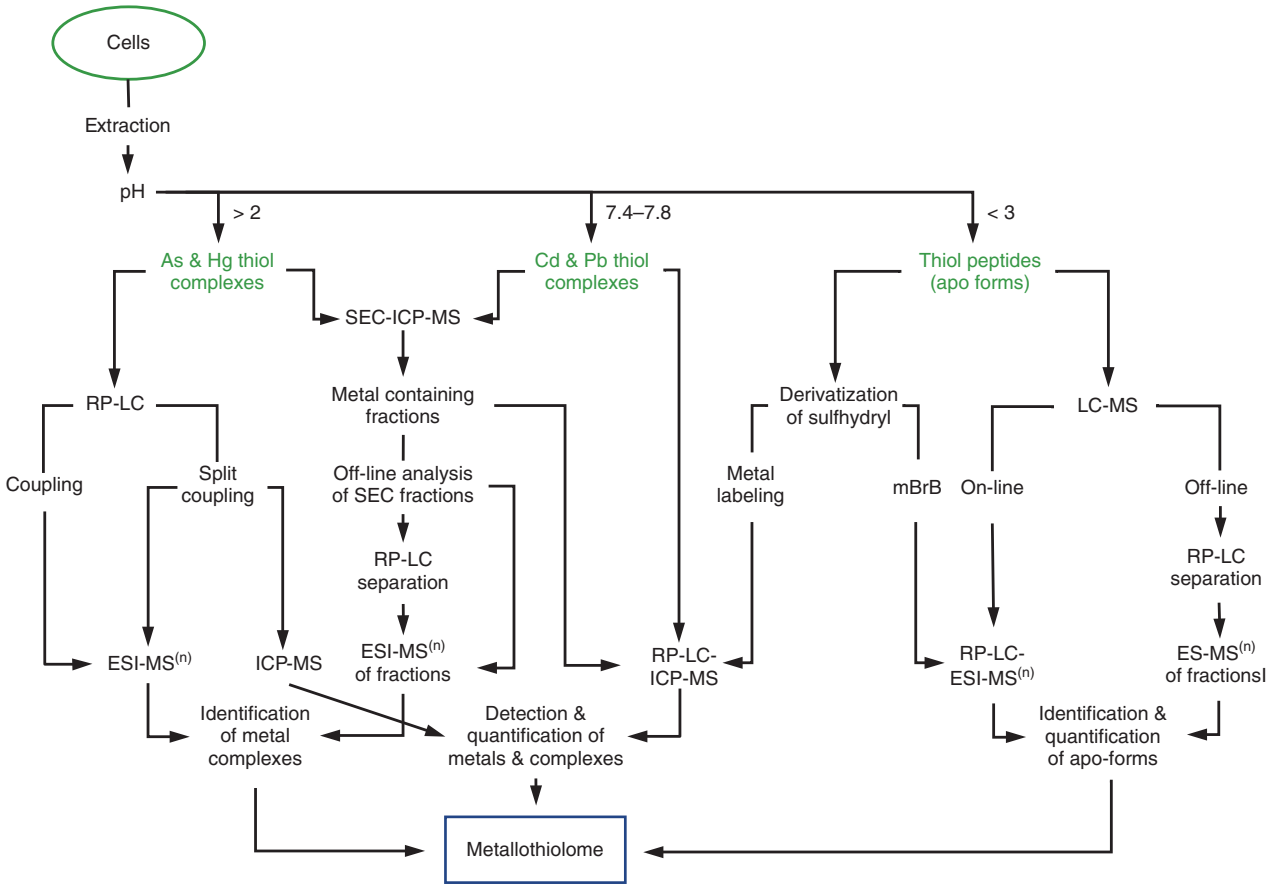

Figure 18.17 Workflow and analytical techniques in metallothiolomics for the investigation of the thiol peptide regulated metal homeostasis. (Reprinted from Wesenberg, Krauss, and Schaumlöffel (2011) with permission from Elsevier.)

synthesized in plants under metal stress (Figure 18.12b). Thiol peptides play the most relevant role in the plant and fungi **metal homeostasis**. Therefore, the entirety of thiol peptides and their metal complexes is referred to as **metallothiolome** and the concept of metallothiolomics summarizes all analytical approaches for the investigation of the thiol peptide regulated metal homeostasis.

The approaches for the characterization of the metallothiolome are based primarily on LC-MS coupling systems (Figure 18.17). In most studies thiol peptide complexes with Cd(II), Pb(II), As(III)/As(V), and Hg(II) are investigated. The fundamental difference is that As– and Hg–thiol complexes are thermodynamically more stable because of the covalent character of As–S and Hg–S bonds. Therefore, different chromatographic conditions are applied for Cd and Pb complexes in contrast to As and Hg complexes. LC-ESI MS is employed for identification of metal–thiol peptide complexes as well as for the identification, structural characterization, and quantification of the metal-free apo forms. In addition, LC-ICP MS is used for the detection and quantification of metal–thiol complexes.

The example of metallothiolomics demonstrates how progress in analytical developments contributes to revise biochemical models. Classic techniques for thiol peptide analysis use chromatography with spectrophotometric or fluorescence detection, which can lead to misleading signals. On the basis of the data from these methods, phytochelatins were formerly described with a maximum number of 11 γ-Glu-Cys units. However, only MS can unambiguously identify and accurately quantify thiol peptides eluting from a chromatography column. Therefore, with the new metallothiolomics concept based on LC-MS methods, the phytochelatin structure was revised having a maximum number of 6 γ-Glu-Cys units.

The global study of the metallothiolome demands not only the characterization of the thiol peptide apo forms but also their native metal complexes. The latter point is still a challenge for analytical chemistry. Therefore, the description of biochemical mechanisms of metal homeostasis including native metal-thiol peptide complexes is still an unsolved issue requiring novel analytical approaches for metallothiolomics.

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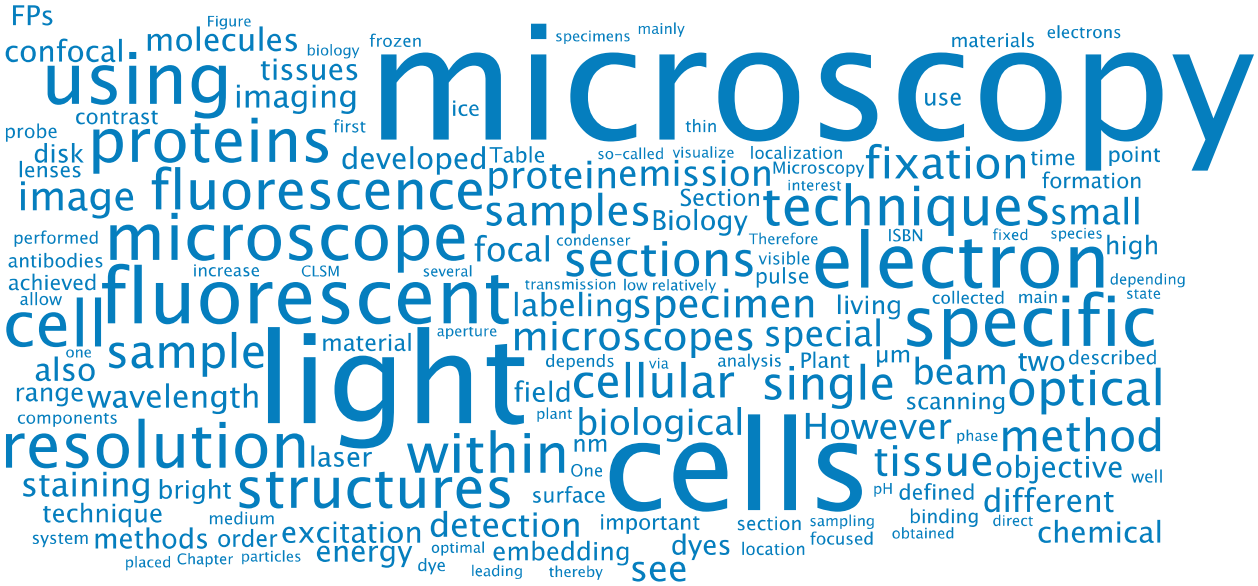
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19
Microscope Techniques and Single Cell Analysis

Bettina Hause and Gerd Hause

Overview

For centuries, progress in biological research has been connected to the development of tools and equipment that allow new insights into the living matter. The invention of and improvements in optical systems were very important because exceeding the limits of the optical resolution of the human eye delivered new insights into tissues, cells, and subcellular compartments on the one hand and cellular processes on the other. Even the very first light microscopes, developed at the beginning of the seventeenth century, enabled the discovery of "Cells as little boxes" by Robert Hooke, and of bacteria by Antoni van Leeuwenhoek. Since then, many aspects of microscopes have been improved and new illumination, staining, and detection methods have been developed in order to increase the optical resolution. In this chapter, we describe the principles and possibilities of the use of microscopes in biology, as well as specific methods of preparing biological materials in order to obtain optimum microscopic images with an appropriate scientific message. Further, emphasis is given on staining techniques used for biological materials including transgenic approaches that use the wide variance of fluorescent proteins.

19.1
Visualization Principles

19.1.1
Light Microscopy

19.1.1.1 Bright Field Microscopy

Microscopy is used to increase the optical resolution, which is defined by the distance in which two point-like structures are visible as separated structures. Several techniques were developed to get the highest possible resolution, enabling the visualization of cells, organelles, protein complexes, and molecules (Figure 19.1). The classic bright field microscope basically consists of a lamp as the light source, a condenser, an objective, and an ocular (Figure 19.2). An image of the specimen is projected onto the retina but, following the different lenses, the image is enlarged in the so-called immediate image plane. The theoretically maximal resolution of the light microscope is 0.2 µm according to the equation defined by Abbe (1884): $d = 0.61\ \lambda/A$. In this equation, the resolution (d) depends inversely on the wavelength of the light (λ) and directly on the numerical aperture of the objective (A). The numerical aperture is defined as the product of the refraction index of the medium between the front lens and the specimen and half of the aperture angle of the optical system. The best resolution of 0.2 µm can be achieved only if high-quality lenses are used in the optical system, the microscope is very well adjusted, and an immersion fluid is used to increase the refraction index. The thin sections or small cells (bacteria, yeasts, cell cultures) that are normally investigated under a bright field microscope have to be stained with a specific dye (Figure 19.3b) because most biological structures differ in terms of the refraction index rather than light absorption.

In order to investigate unstained samples, in 1933 Zernike invented a **phase contrast** principle, considering that most of the objects investigated under a microscope are so-called phase objects, that is, they do not show different levels of light absorption but differ in their refraction index. These differences in the refraction index are not visible to the human eye. Zernike introduced a phase plate into the rear focal plane of the objective. Further on, a special annulus is placed in front of the condenser. A microscope configured this way is able to enhance the differences in the phase shift of the light arising during its passage through the regions of different refraction indices. As a result, an additional phase shift of $\lambda/4$ between undeviated and diffracted light is caused and the intensity of the undeviated light is reduced in comparison to the diffracted light. Finally, the phase differences are converted into differences in brightness that can be discerned by the human eye (e.g., Figure 19.3c).

Differential interference contrast (DIC), another light microscopy technique used to investigate unstained specimens, was developed by Nomarski (in 1953). Polarized light (created by a special polarizer) is split by a beam-splitting prism placed within the condenser. Further on, a Wollaston

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

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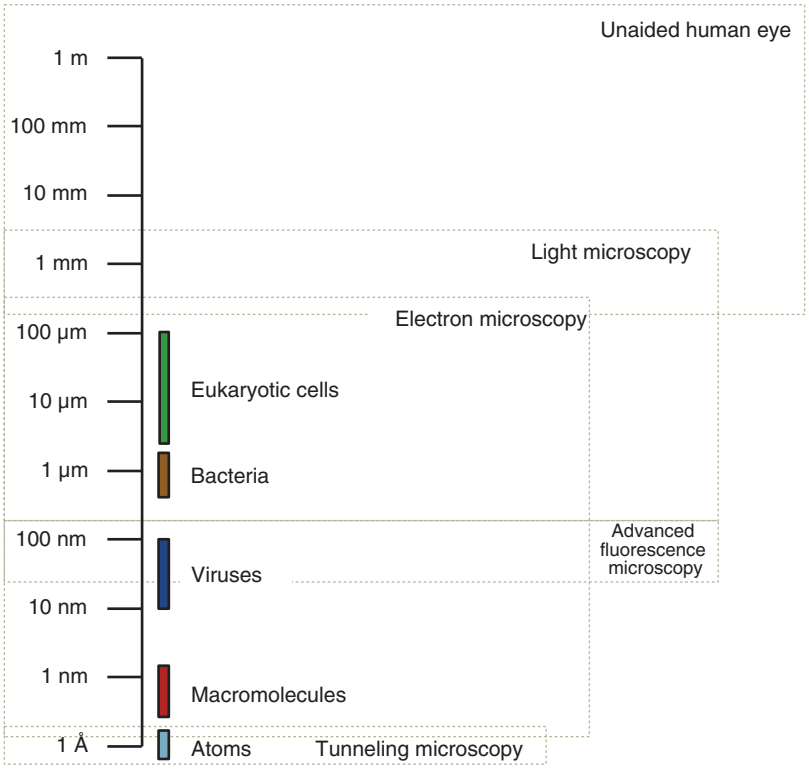

Figure 19.1 Dimensions of biological materials and the microscopy techniques required for their visualization.

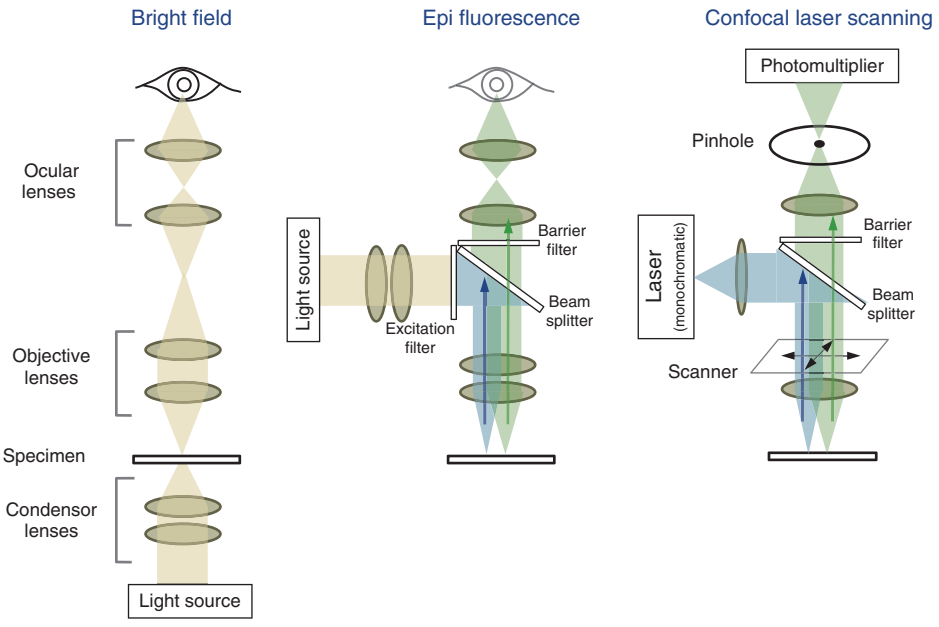

Figure 19.2 Schemes of the light path in bright field, fluorescence, and confocal laser scanning microscopy.

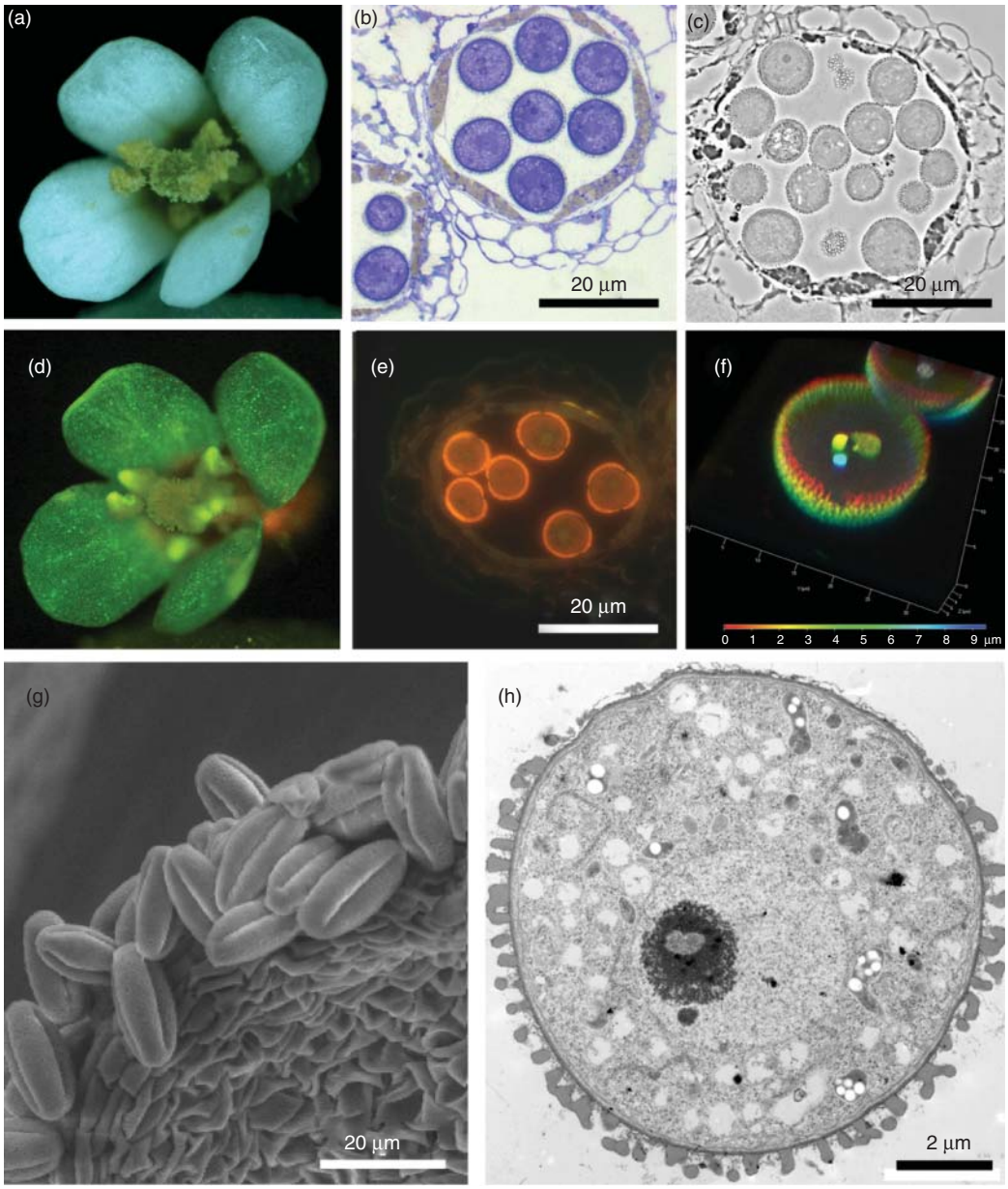

Figure 19.3 Examples of microscopy techniques used to visualize flowers (a, d) and pollen (b, c, e–h) of *Arabidopsis thaliana*. **Light microscopy**: (a). Micrograph of a flower taken by a dissecting microscope; (b). bright field image of a cross-section of stamen stained with toluidine blue; (c). phase-contrast image of an unstained cross-section of stamen. **Fluorescence microscopy**: (d). Micrograph of a flower of a transgenic plant expressing GFP taken by a dissecting microscope equipped with an epi-fluorescence device; (e). fluorescence image of a cross-section of stamen stained with acridine orange. (f). three-dimensional reconstruction of pollen grains stained with DAPI and imaged using a CLSM. The reconstruction of 24 single optical sections is shown by depth coloring. **Electron microscopy**: (g). Pollen grains during anthesis imaged using an ESEM; (h). transmission electron micrograph of a cross-section of a pollen grain. The ESEM picture shown in (g). was kindly provided by F. Syrowatka (Interdisciplinary Center of Materials Science, Halle, Germany).

(objective) prism is placed above the objective, slightly beyond the objective back focal plane. In addition, the microscope contains a second polarizer (analyzer) installed behind the objective's prism. This delivers very good images if such a system is well adjusted. The advantage of DIC compared to phase contrast is that relatively thick samples can be investigated, some spatial information can be obtained, and disturbing halo effects do not occur.

Polarization microscopy is a special branch of light microscopy that is based on the optical anisotropism of cellular components. Isotropic and anisotropic structures can be distinguished by using a polarization system consisting of crossed dichroitic filters. The sample can be turned between these crossed dichroitic filters (polarizators); isotropic substructures stay dark in all positions, whereas anisotropic structures are bright or dark depending on the

orientation within the polarizators: during a full turn they are bright four times and dark four times.

19.1.1.2 Dark Field Microscopy

This special technique was developed in order to visualize structures with a low contrast that cannot be imaged by bright field microscopy. This is achieved by placing an opaque light stop in front of the condenser, and with a special setting of the lenses in the condenser. Dark field microscopes with a highly developed setup have special objectives with an internal iris diaphragm to reduce the numerical aperture. Such systems are especially suitable for investigating fibers, unstained bacteria, yeasts, and protozoa.

19.1.1.3 Fluorescence Microscopy

A fluorescence microscope enables the detection of fluorochromes, which emit light with a certain wavelength after excitation by the light of a defined (shorter) wavelength. Generally, arc burner lamps (e.g., mercury arc lamps) are used for illumination as powerful light sources are necessary. Mercury arc lamps deliver light mainly with the highest intensities in the UV, blue, and green range. Nowadays, so-called epi-fluorescence microscopy is used (Figure 19.2), where the lamp is placed above the objective and light passes via a specific filter block through the objective to the specimen. The filter block consists of an excitation filter and a dichromatic mirror (acting as a beam splitter), which illuminate the sample with the light of a defined wavelength, causing the emission of fluorescent light. In addition, the filter block contains an emission filter that allows only the light with an appropriate wavelength to pass through, enabling detection and visualization of the fluorochrome of interest (e.g., Figure 19.3e).

19.1.2 Advanced Fluorescence Techniques/Generation of Optical Sections

Classic fluorescence microscopy is limited by an overlay of desired fluorescence signals originating from the focal plane by undesired scattered fluorescent light emitted from outside of the focal plane. This can be circumvented by sectioning after fixation and embedding (see Section 19.2). However, this does not allow working with living cells. Several techniques such as deconvolution and confocal microscopy have been invented to overcome this problem and help remove the "out of focus" light.

Deconvolution is a mathematical operation to recover an object from an image degraded by blurring and photon noise. This method is capable of calculating a three-dimensional (3D) image accompanied by increased contrast and resolution in an axial direction. Nowadays, **confocal microscopy** is widely established and uses different types of microscopes, such as confocal laser scanning microscopes, Nipkow disk confocal microscopes (also called *spinning disk microscopes*), and multiphoton microscopes. All of these microscopes represent a major advance in normal fluorescence microscopy and allow not only the visualization of structures within cells, tissues, and thick sections, but also the creation of thin optical sections. Such optical sections can be used to reconstruct 3D images when originating from different *z*-axes, or to follow specific cellular reactions over a period of time. Moreover, newly developed and advanced techniques have led to an increase in resolution that is now almost the same as the range achieved by electron microscopy.

The most important advantages of confocal microscopes are (i) their ability to create optical sections to image cells and tissues internally, (ii) the possibility of obtaining 3D reconstructions for the subcellular localization of labeled structures, (iii) excellent resolution, which is close to the theoretical limit of light microscopy (200 nm), (iv) the use of a specific wavelength of the excitation light to improve multiple labeling, (v) high sensitivity that is capable of collecting fluorescence from single molecules, and (vi) computer control of the microscope, supporting programming, and the recall of complex settings.

Confocal microscopy has a wide range of applications. Because of the overwhelming number of fluorescent dyes and proteins available (see Section 19.3.5 and Table 19.3), it is used, among other things, for immune labeling, organelle identification, protein trafficking experiments, the determination of subcellular functions, such as pH gradients, membrane potentials, and free radical formation, and the measurement of intracellular ion concentrations. All of these processes can be recorded in space by the 3D reconstruction of optical sections (see Figure 19.3f) and/or in time by obtaining time series. There are, however, several limitations that can interfere with the usage of confocal microscopy. Confocal microscopes are expensive instruments that require considerable training of personnel. The restriction to fluorescence requires the use of fluorescent dyes or proteins. Such fluorescent tags can be very bulky and can influence ion concentrations or intoxicate living cells. Moreover, photodamage to dye used or cellular constituents can occur through the high intensity of the laser focused on a fine spot within the specimen.

19.1.2.1 Confocal Laser Scanning Microscopy (CLSM)

Thin optical sections obtained by confocal laser scanning microscopy (CLSM) are generated by eliminating the light rays that originate from other focal planes by introducing a "confocal pinhole" into the light path (Figure 19.2). Only light from the focal plane is able to pass through the pinhole and is collected by a detector, usually a photomultiplier(s) (PMTs). Light rays from other focal planes are not correctly aligned with the pinhole, and are thus eliminated from the image. The focal image itself is created by scanning a finely focused laser beam across the sample in a raster pattern. The fluorescence signal out of each excited point is recorded by the detector and used to create a 2D image. Modern CLSMs

are based on either multiple PMTs arranged in a row to collect a range of specific wavelengths separated by an optical grid or on single PMTs that collect specific wavelengths obtained by a prism and transmitted by a slider. Both principles allow the simultaneous selection of single or multiple emission wavelengths to analyze multiple fluorescent signals. Moreover, it is possible to record complete fluorescent spectra via a "lamda-scan." In this case, complex multicolored samples with a significant overlap of the emission spectra can be mathematically separated followed by imaging in individual detection channels using the "linear unmixing" method.

19.1.2.2 Multiphoton Microscopy

In contrast to CLSM, as a method of single-photon microscopy, multiphoton microscopy relies on the fact that the energy of photons with longer wavelengths (700–1000 nm, low energy) can be added within the specimen to excite fluorescent dyes, which are normally excited by single photons with shorter wavelengths (350–500 nm, high energy). Using a pulsed, high-energy infrared laser, the very high level of light intensity at the focal point of the peak of each pulse results in the emission of fluorescence by the added energy from two or even three photons. However, this simultaneous absorption of multiple photons occurs only at the focal point. Therefore, multiphoton microscopy is confocal *per se*, as all emitted photons originate from the focal point and can be collected by the detector regardless of whether they have been scattered or not. Using longer wavelength helps to obtain optical sections from deeper within the tissue; the confined excitation reduces photodamage and photobleaching of the specimen. However, thermal damage can occur in a specimen if it contains chromophores that absorb the excitation wavelength. Moreover, the resolution of multiphoton microscopes is slightly lower compared to CLSM.

19.1.2.3 Spinning Disk Microscopy

In a Nipkow disk confocal microscope the image of a fluorescent specimen is produced by scanning it in a raster pattern produced by a spinning opaque wheel perforated by a series of rectangular holes: the "Nipkow disk." The disk contains up to 200 000 pinholes with a diameter of 25 µm and it spins at up to 2000 rpm. The light is focused through the moving pinholes and leads to irradiation with an array of fine light points. The fluorescence signal from each point is focused back up through the pinhole array, and the "in-focus" light can be viewed directly or detected by a sensitive charge-coupled device (CCD) camera. The speed of scanning is determined by the sensitivity of the camera; routinely, a scanning rate of about 700 frames per second can be achieved. Because of this high-speed imaging in combination with the low-light intensity necessary for illumination, Nipkow disk confocal microscopy is advantageous for live cell imaging. However, one major limitation is that the z-resolution of the microscope is not as good as that of a CLSM.

19.1.2.4 Stimulated Emission Depletion (STED), 4Pi Microscopy, and 4Pi-STED-Microscopy

The resolution of light microscopes, including confocal microscopes, is limited by diffraction, as described by Abbe (see Section 19.1.1.1). In the Stimulated Emission Depletion (STED) method, developed by S. Hell (Göttingen, Germany), the fluorescent focal spot is sharpened by the selective inhibition of its fluorescence at its outer part. After emission of the fluorescence by a very short excitation pulse, a synchronized "depletion" pulse is focused into a donut-shaped image, with the hole of the donut centered on the first pulse. The depletion pulse is tuned to an emission line of the dye causing stimulated emission, where electrons move from the excited state (from which emission occurs) to a lower energy state without emitting light. With a bright depletion pulse, almost all of the electrons excited by the excitation pulse return to the ground state, except for the very center of the focal region. Therefore, an ultrasmall focal volume is created, resulting in an increase of axial and lateral resolution, thereby overcoming the diffraction barrier.

An 4Pi-microscope is a confocal microscope with two opposing objective lenses, which are coherently illuminated and focused on the same spot. The interference of the counterpropagating wavefronts sharpens the confocal focus and narrows the main focal maximum of the excitation light in the z-direction, leading to further removal of the blur; this results in a three- to seven-times increased optical resolution compared to conventional CLSM. For the combination of STED and 4Pi, the fluorescence sample is placed in the common focus of two opposing lenses, but the excitation and detection are performed through a single lens. The excitation pulse is immediately followed by a STED pulse that enters the focal region through both lenses, stimulating emission from the excited fluorescent dye. Using this technique, a 3D resolution in the order of a few tens of nanometers can be reached.

19.1.2.5 Photo-Activated Localization Microscopy (PAL-M)/Stochastic Optical Reconstruction Microscopy (STORM)

Every point-like object is imaged as an extended spot – if two such objects come close, the overlap of the area of their spots makes it impossible to determine their precise location, and, therefore, the resolution is limited by the diffraction barrier. Both photo-activated localization microscopy (PAL-M) and Stochastic Optical Reconstruction Microscopy (STORM) are related applications of a concept distinct from optical resolution: if a point source of light is converted into a fuzzy Airy disk, repeated samplings of an individual source of light can statistically show the location of the center of its Airy disk. Here, the recording of only one spot at a time allows the localization of the center of the Airy disk leading to a much greater

precision than recording the total Airy disk of a spot itself. Therefore, statistical sampling defines the precision of the localization of the center of the Airy disk, which is not limited by the Abbe limit. The PAL-M method is based on the light-induced activation and deactivation of the fluorescence of photo-switchable fluorescent proteins (FPs) (see Section 19.3.5) by distinct light pulses. Within a large population of fluorescent molecules with an "on" and an "off" state, a low-intensity laser excitation first "activates" a few molecules so they become susceptible to a second excitation laser pulse leading to an illumination where only a few molecules are activated at the same time. The process is repeated in small areas until the location of each fluorescent molecule in the sample is recorded. A high-resolution image is then computed. A resolution in the range of 15 nm can be achieved using this method; however, because the process can take many hours to complete, PAL-M is not yet suitable for studying cellular dynamics.

19.1.2.6 Structured Illumination Microscopy (SIM)

In structured illumination microscopy (SIM), a sample is illuminated with patterned light produced by projecting light through a fine grating. The final image is reconstructed from the interference pattern created by overlaying two grids with different mesh sizes or angles (the so-called Moiré pattern). Improvements in resolution by a factor of 2.25 can be achieved using this technique compared to conventional wide-field fluorescence microscopy. One of the most important features of SIM is the enhancement of the special resolution in all three dimensions. In addition, 3D-SIM expands SIM to three or more colors and permits the imaging of conventional fluorescent reporters and dyes. Therefore, this technique is the most widely accepted super-resolution microscopy technique for biologists. However, it cannot be used for live cell imaging and is restricted to samples that must remain static during imaging.

19.1.3 Electron Microscopy

For "classic" light microscopy systems, the optical resolution cannot be better than 0.2 µm because it depends on the wavelength of the light and on the numerical aperture of the objective (see Section 19.1.1.1.). It is necessary to either minimize the wavelength or increase the numerical aperture of the objective in order to improve the resolution. Because of the fact that there are limits to increasing the numerical aperture, decreasing the wavelength is the only option. This can be achieved by using an electron beam with a wavelength of $\lambda = 4.3$ pm (using an acceleration voltage of 80 kV), which is 10 000 times shorter than the wavelength of blue light. However, correction of the electromagnetic lenses is technically very difficult and allows only an increase in the resolution of 1000 times compared to light microscopy. The electron beam is created by a cathode consisting of thermionic emitters such as tungsten or lanthanum hexaboride filaments, which is placed in front of an anode or by a field emission gun, leading to a small and precise beam.

Generally, two types of electron microscopes are available. Scanning electron microscopes (SEMs) can be used to analyze surfaces and transmission electron microscopes (TEMs) are suitable for investigating small particles or ultrathin structures.

19.1.3.1 Transmission Electron Microscopy (TEM)

The basic principle behind a transmission electron microscope is similar to that of a light microscope. The beam passes through a condenser, the sample, an objective, and finally the projector lenses. In the transmission electron microscope, the optical parts do not contain glass lenses but electronic coils generating an electromagnetic field to focus the electron beam. Electron-dense regions of the sample lead to the scattering and disappearance of electrons from the beam, whereas electrons passing through electron-translucent regions are visible as bright areas on the screen of the microscope. Finally, a dark/bright image appears on the screen showing the electron dense and electron translucent structures of the sample (see Figure 19.3h).

The point resolution of high-end TEM can be as much as 0.1 nm or even better. Such a high resolution is important for material sciences as the limit of resolution of biological specimens is determined and restricted by the preparation and properties of a sample and only secondarily by the microscope. This fact highlights the importance of optimal sample preparation, as described in Section 19.2.

Biological specimens investigated using TEM are usually negatively stained (contrasted) small particles or cells and ultrathin sections (30 – 100 nm) of embedded cells or tissues. Transmission electron microscopy is used to study the morphology and ultrastructural properties of specimens, but it is also possible to analyze the distribution of elements using energy dispersive X-ray spectrometry (EDS or EDX) or electron energy loss spectroscopy (EELS) (see Section 19.3.1), the localization of proteins (see Section 19.3.3), and the expression of genes (see Section 19.3.4).

One method used to improve the structural information obtained is **single particle analysis**. This method analyzes a large number of a single class of particles, such as negatively stained protein complexes or viruses, and frozen samples containing the particles in a thin ice layer. After the classification of particles with different orientations, it is necessary to take tens of thousands of images that then have to be compared and processed afterward using special computer software. Finally, the 3D structure of the particles can be obtained.

Using (**cryo**-) **electron tomography,** it is possible to observe thick sections (up to 400 nm) with microscopes that are able to tilt the specimen around a single axis. Images from one area are taken in defined steps at different tilt angels (maximum −70° up to 70°). Then, this set of images is used to generate a 3D image of the structure with

the help of special software. A very important prerequisite for electron tomography is perfect alignment of the microscope during the acquisition of single images. A shift in the area of interest would hinder the 3D reconstruction. The optical resolution of structures obtained using electron tomography is within the range of 5–20 nm, but it depends on the sample and the microscope.

19.1.3.2 Scanning Electron Microscopy (SEM)

SEM allows surfaces to be investigated at a high resolution. The electron beam scans the sample after passing through a system of condenser and objective lenses. As the beam interacts with the sample surface, secondary electrons exhibiting a distinct energy are emitted and back-scattered electrons and specific X-rays are also induced. Specific detectors exist for these types of beam-induced signals. The data from the beam for each point of the surface are collected during the scan. Finally, an image is created containing information about the surface topography and – depending on the detector – element composition and conductivity.

In general, biological samples for analysis using SEM have to be fixed and dehydrated as described in Section 19.2. Afterward, they have to be treated by critical point drying and surface sputter coating, both of which are necessary to remove water, which would evaporate under the high vacuum of the specimen chamber, and to obtain high conductivity of the surface, which is needed for optimal imaging. Fresh (hydrated) samples can be investigated using **environmental scanning electron microscope** (**ESEM**) only. In ESEM the samples are in a low-pressure gaseous environment. The setup of the microscope allows high humidity in the environment of the specimen. This is achieved by a secondary electron detector capable of operating in the presence of water vapor and a special differential pumping system. However, it must be noted that only fresh specimens with relatively thick outer layers (e.g., pollen, as shown in Figure 19.3g) can be analyzed using ESEM without rapid artifact formation; even relatively stable samples can start to deform and to collapse within a short period (a few seconds up to a few minutes).

19.1.4 Scanning Probe Microscopy

Several technical principles can be summarized under the generic term *scanning probe microscopy*, although all of these principles do not belong to optical techniques because a physical probe scans the surface of samples. The first system invented was **scanning tunneling microscopy**, which is based on the measurement of different tunnel currents depending on the topology of the sample. Since then several other techniques have been developed, such as atomic force microscopy, chemical force microscopy, electrostatic force microscopy, magnetic force microscopy, and scanning thermal microscopy. Because of the chemical and physical properties of biological specimens, it is difficult or impossible to use the majority of these techniques for their analysis.

19.2 Preparation of Biological Materials

A prerequisite for the optimal imaging of biological structures is the use of appropriate preparatory methods to avoid artifact formation. It is possible to analyze fresh samples without fixation only under certain circumstances, such as cells in liquid media using a light microscope or surface structures using an **ESEM** (see Section 19.1.3.2, e.g., pollen in Figure 19.3g).

Even though one or several of the following steps can be omitted depending on the experiment, the workflow for the preparation of biological materials is generally sampling – fixation – dehydration – infiltration of embedding medium – hardening – sectioning – staining.

When sampling small portions of a tissue of interest, it is important to work rapidly and precisely using optimal tools (e.g., razor blades, sharp needles, biopsy punches) to avoid wound response, dehydration, shrinkage, and/or degeneration. Single cells from liquid cultures must be concentrated and immobilized, for instance, within special sieves or in Agar. Thereafter, the material has to be fixed immediately. Fixation is a very important step for stabilizing the structures during sectioning, staining, and microscopic observation. Two general principles are used for fixation and are described in the next sections.

19.2.1 Chemical Fixation

One type of fixation is the chemical linkage of cellular molecules (mainly proteins) with appropriate substances. This method is relatively cheap and does not need special equipment or training. The fixatives that are used often for biological materials are **aldehydes**. One aldehyde group is capable of linking two proteins via amino groups. One of the most frequently used fixatives in cell biology is (para)formaldehyde. The crosslinking of four proteins can be achieved using glutaraldehyde, leading to the excellent preservation of the cellular ultrastructure, which is important in morphological investigations. In immunocytochemistry (IC), fixation with formaldehyde is preferred because the epitopes of antigens are more accessible for antibodies (see Section 19.3.3). **Metallic fixatives** such as osmium tetroxide or potassium permanganate are often used additionally to aldehydes because they give a better contrast of the lipids and thus of the cellular membranes. However, it is important to note that these fixatives influence the structure of proteins and that it is impossible to use such materials in IC. Another group of fixatives are **carbodiimides**, which are used mainly for the fixation of small acidic molecules such as phytohormones.

In order to allow infiltration with an **embedding medium,** the specimen has to be dehydrated in a stepwise manner with an organic solvent (e.g., ethanol or acetone) directly after fixation. The embedding medium for light microscopy can be relatively soft (e.g., paraffin or polyethylene glycol), whereas samples for electron microscopy must be embedded in a hard medium (e.g., epoxy resins or methacrylates). After complete infiltration of the samples, they have to hardened by cooling down (e.g., paraffin) or by polymerization (e.g., methacrylates). Depending on the properties of the biological material and the embedding medium, the whole procedure (fixation, dehydration, infiltration, and hardening) takes between 2 and 5 days.

The type of analyses required, as well as the embedding medium, determines the thickness of sections of the specimen. Samples are sectioned using steel knifes at 2–20 µm in thickness for light microscopy or with glass/diamond knifes at 30–100 nm in thickness for electron microscopy.

However, chemical fixation can cause artifacts, resulting in the appearance of structures that do not exist in living organisms. The main reason for this phenomenon is the slow transport of the fixative deep into the cell. Depending on the properties of the cells and the size of the sample, the infiltration process can last from seconds up to several minutes and it can also result in the formation of artificial structures. Some cellular borders, for instance, spore walls, are completely impermeable to chemical fixatives and prevent the preservation of cellular structures. Although slight artifacts can be interpreted and accepted in many cases, in others the artifacts can be so strong that chemical fixation is not suitable for the experiment.

19.2.2 Physical Fixation/Cryofixation

To circumvent the appearance of artifacts described above, cryofixation was developed as an alternative strategy for fixation. During cryofixation, all cellular structures are immobilized almost simultaneously within a very short time (milliseconds), caused by rapid freezing. In this case, structures related to defense processes against poisonous chemical fixatives or degradation processes will not be imaged. However, the main problem of cryofixation is the formation of crystalline ice within the cells. Ice crystals can destroy and replace cellular structures. Crystalline ice is formed when the cooling rate is too low, and forms usually in the inner parts of tissues. Only very small cells (between 5 and 10 µm, depending on the content of cytoplasmic sugars) can be frozen under atmospheric conditions without the requirement of special equipment. Therefore, a simple plunge into freezing liquid ethane or propane is applicable only for structures the size of bacteria and smaller. In most instances, however, special techniques have to be used to freeze the water in the amorphous state and to obtain vitrified ice. In contrast to chemical fixation, these techniques require specific skills, experience, and expensive equipment. Four major techniques are used for freezing fixation, which is described in the following paragraphs.

One alternative method to the apparatus-intensive cryomethods described below is **plunge freezing**, according to Tokuyasu. For this purpose, the samples have to be chemically fixed (e.g., with formaldehyde) and then infiltrated with a cryoprotectant (e.g., 2.3 M sucrose). The antifreezing compound prevents the formation of ice crystals during plunge fixation in liquid propane or liquid ethane. The advantage of this method is that it is possible to fix relatively large samples with low technological efforts and costs. However, it must be remembered that the first step in this technique is the chemical fixation of the specimen, with all the disadvantages described above.

Another method is the so-called **slam** or **metal mirror freezing** technique. Rapid freezing is achieved by the fast pressing of a small droplet of cells or of biopsies from soft organs onto a precooled metal block (mainly copper) with a very smooth surface. This can result in a thin (15–25 µm) layer of well-frozen cells that can be used for cryosectioning or freeze substitution. However, there are several disadvantages with this technique, for example, the very small volume of well-frozen cells and the restriction to soft samples. For instance, specimens from plants or fungi are not flexible enough to come into optimal contact with the metal surface, causing inadmissible results.

Thin layers of cells can be cryofixed extremely well using **spray fixation** apparatus (**propane jet**). Cells in special copper holders that form a sandwich-like structure are frozen by spraying liquid propane onto the surface of the sandwich. Because spacers restrict the cell layer within the sandwich (about 10 µm), the formation of crystalline ice can be circumvented. Such frozen materials are especially suited for freeze fracture/freeze-etching experiments; however, spray freezing is inappropriate for fixing tissues.

High-pressure freeze fixation was developed for the cryoimmobilization of tissues. The environmental pressure within a small recipient containing the sample (maximal size 6×0.2 mm) is increased up to 210 MPa for a short period of time (0.5 s). This increase in pressure increases the viscosity of cellular water (up to 1500-fold). Immediately after reaching high pressure (after about 0.2 s) the cooling process starts. The cooling rate is very high (10 000 K s^{-1} and more), which, in combination with the more viscous water, significantly reduces the growth of ice crystals. The frozen samples can be used for cryosectioning, freeze fracture, or cryosubstitution.

There are several possibilities for analyzing the cryofixed material microscopically. Plunge-frozen grids containing a very thin ice layer with small structures can be directly observed under a **cryotransmission electron microscope**. Furthermore, the frozen samples can be sectioned using a cryo(ultra)microctome and observed after staining and/or labeling. **Freeze fracturing/freeze etching** is a method suited especially for investigating membranous structures. For this purpose, the frozen material is fractured in a special

apparatus at a low temperature and within a vacuum. The cells and tissues preferentially break along cellular membranes (mainly fractures of the bilayer) rather than in a linear manner. After a short period of etching (sublimation of cellular ice along a temperature gradient), the structures are shadowed with platinum (~2 nm) and carbon (~20 nm). Afterward, the thin film (replica) can be floated away from the fractured material and observed under a transmission electron microscope.

To circumvent the generally very tricky cryosectioning, frozen samples are frequently prepared using the **cryosubstitution** technique performed by substituting cellular ice with organic solvents (acetone, methanol) containing small amounts of fixative (glutaraldehyde, osmium tetroxide) at a low temperature (−80 to −90 °C). After substitution (depending on the material, this can take 1–7 days), the temperature can be increased to infiltrate the sample with embedding media (Lowicryls at −20 to −40 °C; epoxy resins at 4 °C to room temperature). After the media has hardened, the material can be sectioned at room temperature using a conventional ultramicrotome.

19.3 Detection Methods – from Macromolecules to Ions

19.3.1 Histological Staining

Originally, the term **histological staining** was used to describe the staining of sections by the binding of a solubilized dye to specific components of a tissue. A broad range of dyes exists nowadays, enabling the specific labeling of components in sections and also in living cells, thereby adding dynamic intracellular parameters to cytological investigations. Classic histological staining is influenced by the chemical nature of the dye, the fixation and embedding techniques, and the thickness of the section. In general, the dyes can be divided into two main groups, diachromes and fluorochromes, which are observed using bright field microscopy and fluorescence microscopy, respectively. Their coloration depends on their chromophores, which can consist of chemical groups with different actions, such as carbonyl, carbimine-, azo-, nitroso-, and nitro-groups. The binding of diachromes and fluorochromes to cellular structures depends on their reactive groups and charge. The addition of auxochromes to chromophores changes the chemical features of dyes, leading to differential binding to acidic, alkaline, or neutral components of the cells. The staining itself can be direct (binding without any preprocessing or additives) or indirect (preprocessing required), progressive (staining until the desired color is reached), or regressive (destaining via the removal of excess dye after overdying, also called **differentiation**). The main examples of typical histological dyes used in bright field microscopy are toluidine and methylene blue (for complete staining of thin sections, see Figure 19.3b), sudan black (lipids), haemalm (nucleus), Giemsa (chromosomes), and iodine potassium iodide (starch).

Fluorochromes are also used in classic histological staining (Figure 19.3e). In addition, they enable the staining of diffusible components in living cells (Table 19.1). Among them are indicators specifically for the visualization and determination of ions, such as H^+ (pH determination) and Ca^{2+}, but also for the detection of reactive oxygen species (ROS). Some of these indicators not only respond to changes in the amplitude of fluorescence but also to shifts in the wavelength of the excitation or emission spectrum or both, for example, Indo-1 for Ca^{2+}. These shifts allow the so-called ratioing between signals obtained at two or more excitation or emission wavelengths, thereby omitting differences due to fluctuations in dye concentration and in overall intensity. Another option for visualizing and measuring changes in small metabolites and ions is based on FPs combined with peptides, specifically binding those molecules (see Section 19.3.5).

In order to provide qualitative, semiquantitative, and quantitative analyses for single chemical elements within a cell, **EDS, EDX,** or EELS are coupled to electron microscopy. In EDS, the sample is irradiated with a high-energy beam (electrons, protons, or X-rays) leading to the emission of X-rays by single atoms. The energy of the X-ray is characteristic of the atomic structure of the element and leads to conclusions about the elemental composition of the sample being analyzed. In EELS, the material is exposed to a beam of electrons with a known energy range, which might be lost because of inelastic scattering by components of the probe. This energy loss is indicative of the element that caused it.

19.3.2 Autoradiography

One way of visualizing specific compounds within a cell or tissue is to label them with radioactive isotopes followed by autoradiography, a photochemical detection method in which a radioactive sample is placed in close proximity to a photographic emulsion or film. This method can be used to locate specific genes or trace metabolic pathways or the organization of delicate cellular structures. A prerequisite is the pulse-labeling of living cells with a radioactively labeled precursor or compound that feeds into the biosynthetic pathway under investigation. After the incorporation of radioactivity, the cells have to be fixed and processed in order to obtain sections that can be studied under the microscope. These sections are then overlaid with a thin film of photographic emulsion. The crystals of silver halide in the emulsion respond to the radiation from gamma-rays or beta-particles and form silver grains, which become visible after film development. The length of the exposure time depends mainly on the activity of the isotope. The irradiation process is spatially accurate and cumulative.

Table 19.1 Selection of fluorescent dyes for the direct staining of cells and cellular components.

Fluorescent dye	For the labeling of	Excitation maximum (nm)	Emission maximum (nm)
Acid fuchsin	Chitin	540	630
Acridine orange	DNA//RNA	502	526//650
Aniline blue	Callose	490	515
BCECF[a)]	H^+	440/490	535
Calcofluor white	Cellulose	380	440
DAF-FM[b)]	NO	495	515
DAPI[c)]	DNA	359	441
H_2DCF-DA[d)]	ROS	490	520
Fluo-3	Ca^{2+}	480	520
FDA[e)]	Vitality test	490	525
FM1-43	Endomembranes	479	589
FM4-64	Endomembranes	506	750
FURA-2	Ca^{2+}	340/380	500/530
Hoechst 33258	DNA	365	480
Indo-1	Ca^{2+}	360	410/480
LysoTracker green[f)]	Lysosomes	504	511
MitoTracker green[f)]	Mitochondria	490	516
Monochlorobimane	Glutathione	442	477
Phalloidin, fluorophore conjugated	Actin filaments	—	—
Propidium iodide	DNA	520	610
SNARF	H^+	480	600/650

a) (2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein.
b) 4-Amino-5-methylamino-2′,7′-difluorofluorescein.
c) 4,6 Diamidino-2-phenyl indole.
d) Dihydrodichlorofluorescein diacetate.
e) Fluorescein diacetate.
f) Also available with other spectral characteristics (orange, red).

The position of the silver grains in the film layer above the sample can be observed by light or electron microscopy and provides information on the localization and distribution of radioactivity within the specimen.

19.3.3 Immunocytochemistry (IC)

The location of specific molecules (e.g., proteins, carbohydrates, or hormones) within tissues or cells can be determined with immunological techniques. These are based on a combination of immunochemistry and morphology, and, therefore, define the cellular location of biochemically defined antigens. IC can be used basically for all types of cells. Whole-mount techniques are used to immunolabel complex tissues or small organs via the infiltration of antibodies into fixed and subsequently permeabilized cells, thereby bypassing the embedding and sectioning processes. Because of problems with the penetration of antibodies and/or the occurrence of specific compounds (e.g., chlorophylls, diferulic acid, or tannins in plant cells) that have autofluorescence and may mask a specific signal, the so-called postembedding labeling is mostly favored. In this case, the specimen is fixed, embedded, and sectioned before the immunolabeling procedure is applied (see Section 19.2).

One of the main prerequisites for the use of immunocytochemical techniques is the availability of antibodies, which have to be specific against a particular antigen. The antibody should not show any irrelevant cross-reactivity with an unrelated protein that shares a common epitope with the antigen of interest. For immunolabeling, the specific antibody can be directly coupled to a marker molecule, thereby resulting in direct labeling. Most commonly,

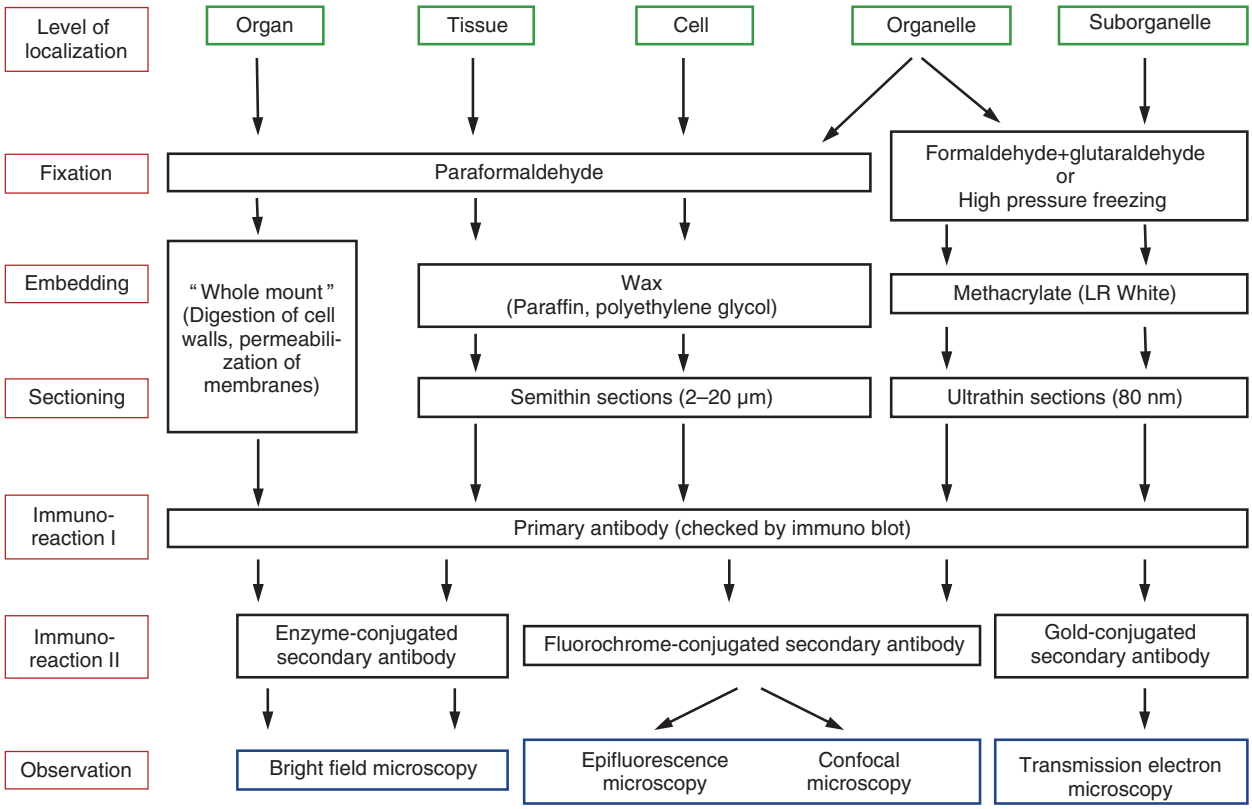

Figure 19.4 Flow chart showing the immunocytochemical process. The level of localization is defined by the nature of fixation, the embedding medium, the thickness of the sections, the reporter molecules coupled to the secondary antibody, and the type of microscope used for examination.

indirect immunolabeling consisting of two successive immunoreactions is used: in the first step the antigen is detected by the specific antibody, and this primary antibody itself is recognized in the next step by a second antibody to which a reporter molecule is attached. For both techniques, the nature of the marker depends on the level of detection (Figure 19.4); the cell- and tissue-specific occurrence of an antigen in an overview/large section is easier to visualize by bright field microscopy, whereas the location of a certain protein within a cell is better resolved by fluorescence microscopy. In the first case, secondary antibodies coupled to an enzyme that leads to the formation of a diachrome are mostly used, for example, alkaline phosphatase and horse radish peroxidase. Here, the signal is amplified by the enzymatic reaction. However, the main disadvantage of such an enzymatic reaction is that its product does not always remain strictly localized. This can be circumvented by the use of fluorescence-labeled antibodies that remain highly restricted to the binding site. A wide range of different dyes are available that are coupled to secondary antibodies (Table 19.2), which enables multiple labeling. The resolution of light microscopy is not sufficient to obtain information about the subcellular location of proteins; in this case, electron microscopy techniques utilizing ultrathin sections and colloidal gold-labeled antibodies are used. Immunolabeled ultrathin sections are usually examined in a conventional transmission electron microscope; labeled antigens are then visible as black dots of a defined size in the micrograph. For all types of immunolabeling, it is very important that negative controls are used; this is commonly done by using preimmune serum or tissues from mutants that lack the respective antigen.

19.3.4 *In situ* Hybridization (ISH)

The term *in situ* hybridization (ISH) describes the cytological detection of specific nucleic acids, such as defined regions of genomic DNA, certain mRNA species, or small RNAs. Principally, ISH is the combination of molecular-biological detection methods with morphology. It employs a labeled complementary DNA or RNA strand (called **probe**) that binds noncovalently to a specific DNA or RNA in order to visualize the target sequence within chromosomes, cells, or tissues. Usually, the specimen is fixed, thereby nucleic acids are immobilized, and the probe is able to access its target. In cases where an entire tissue or organ is small enough, ISH can be performed with permeabilized cells (whole-mount ISH). Mostly, however, tissues and cells have to be processed through embedding and sectioning to allow the penetration of the probe and the formation of hybrids between the probe and target. The detection

Table 19.2 Selection of fluorescent dyes used for immune labeling.

Fluorescent dye	Exc.max.[a] (nm)	Em.max.[b] (nm)	Fluorescent dye	Exc.max.[a] (nm)	Em.max.[b] (nm)
AlexaFluor350	346	442	Boron-dipyrromethene (BODIPY)[c]-tetramethylrhodamine (TMR)	544	570
Aminomethyl Coumarin (AMCA)	350	450	Indocarbocyanine, Cy3	547	561
Cyanine, Cy2	492	510	Tetramethyl rhodamine isothiocyanate (TRITC)	550	572
Dichloro-trazinylamino fluorescein (DTAF)	492	520	AlexaFluor546	556	573
Fluorescein isothiocyanate (FITC)	495	519	BODIPY[c] -TR	588	616
AlexaFluor488	496	519	Texas Red (TR)	596	620
BODIPY[c] -Fl	502	510	Indocarbocyanine, Cy5	647	665

a) Excitation maximum.
b) Emission maximum.
c) Boron-dipyrromethene.

of hybrids depends on the type of probe labeling used: labeling can be done by introducing radio-, fluorescent-, or antigen (digoxigenin, DIG)-labeled nucleotides that can be detected by autoradiography, fluorescence microscopy, or immunohistochemistry, respectively. **Fluorescence *in situ*-hybridization (FISH)** is mostly used for chromosomes, in order to identify regions containing specific genes. Radio- or DIG-labeled probes are used to measure and localize mRNAs and other RNAs. Afterward, the sections are either subjected to autoradiography (see Section 19.3.2) or to immunolabeling procedures. The latter is performed using antibodies directed against DIG and conjugated with alkaline phosphatase, which leads to the formation of a colored reaction product indicative of the occurrence of the hybrid.

All data described in this chapter and summarized in Figure 19.5 hint at the fact that microscopic analysis of biological materials demands the knowledge on how to combine microscopic techniques, preparation methods, and detection protocols to obtain optimal results.

19.3.5 Reporter Molecules in Transgenic Approaches

Principally, the techniques introduced above are invasive methods, that is, the tissues/cells have to be immobilized before labeling. To visualize gene expression as well as the location of proteins in living cells using noninvasive methods, the introduction of a transgene encoding a reporter molecule that is visible within a cell is mandatory. Moreover, this reduces the methods required for fluorescent labeling of proteins to the techniques of molecular biology. Next to the enzymes used because of their ability to convert nonvisible in visible dyes, the green fluorescent protein (GFP) and its variants have become the most widely used in biological sciences.

Promoter-reporter fusions are used to determine the cell and tissue specificity of the expression of the respective genes. Here, enzymes such as β-glucoronidase (GUS), β-galactosidase (LacZ), and luciferase are frequently used as reporter molecules. To visualize the enzymatic activity, the respective substrates are added to the living specimen and the staining that develops (e.g., blue color after the conversion of 5-bromo-4-chloro-3-indolyl-glucuronide by GUS) or the light emitted (e.g., bioluminescence after the conversion of luciferin to oxoluciferin by luciferase) is recorded. However, **fluorescent proteins (FPs)** are also used in promoter studies. Here, populations of cells can be marked, tissue organizations become visible, and cell lineages can be dissected.

The first FP, the **GFP**, was isolated from the jellyfish *Aequoria victoria* and is most widely used in cell biology (Fig. 19.3a,d). Nowadays, several mutant versions of this protein and FPs from other organisms are available that offer a broad range of "colors" (Table 19.3). FPs enable direct labeling of proteins via the fusion of their cDNAs followed by transformation, and they also enable direct observations of fusion proteins within living tissue. The use of FPs has revolutionized experimental designs by adding a dynamic element to cell biology, thereby enabling the visualization of specific biological structures and organelles, as well as the examination of specific chemical reactions, proteins and organelle transport, ligand binding, and interactions between different proteins. However, a couple of the pitfalls in the use of FPs are that strong overexpression can lead to mislocalization of the labeled protein within the cell and FP-fusion can affect transport processes through membranes.

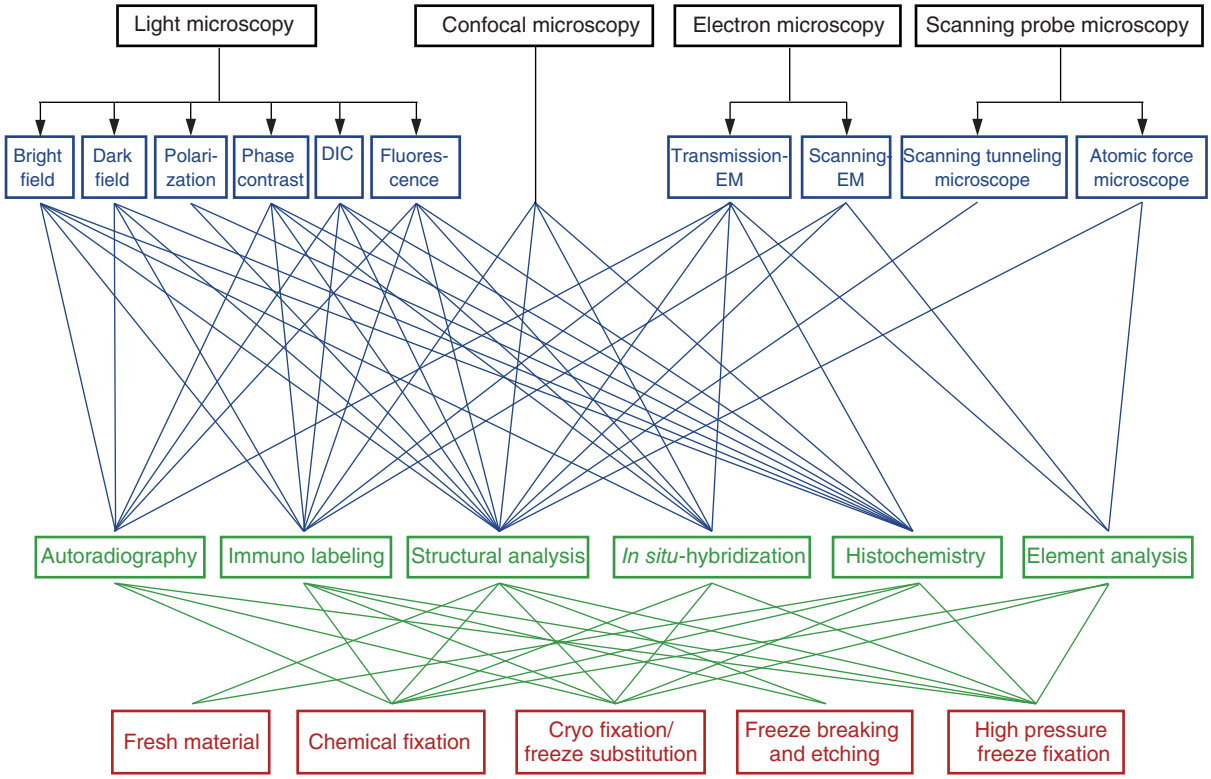

Figure 19.5 Overview of microscope techniques, preparation methods, and detection protocols used for biological research.

Table 19.3 Selection of chromophore mutants of the green fluorescent protein.

Protein	Class	Ex. max.[a] (nm)	Em. max.[b] (nm)	Oligomerization
T-Sapphire	UV-excitable green	399	511	Weak dimer
Enhanced blue fluorescent protein (EBFP)	Blue	383	448	Monomer
Enhanced cyan fluorescent protein (ECFP)	Cyan	434	477	Monomer
Cerulean	Cyan	433	475	Weak dimer
Enhanced green fluorescent protein (EGFP)	Green	489	509	Weak dimer
Emerald	Green	487	509	Weak dimer
mWasabi	Green	493	509	Monomer
Enhanced yellow fluorescent protein (EYFP)	Yellow-green	514	527	Weak dimer
Venus	Yellow-green	515	528	Weak dimer
mOrange	Orange	548	562	Monomer
tdTomato	Red	554	581	Tandem dimer
DsRed	Red	563	582	Dimer
mCherry	Red	587	610	Monomer
mPlum	Far-red	590	649	Monomer

a) Excitation maximum.
b) Emission maximum.

Table 19.4 Selection of photoactivatable, photoconvertible, and photoswitchable fluorescent proteins.

Protein	Ex. max.[a] (nm) before activation	Em. max.[b] (nm) before activation	Activation/ conversion wavelength	Ex. max.[a] (nm) after activation	Em. max.[b] (nm) after activation
Photoactivatable (from nonfluorescent to fluorescent)					
PA-GFP			405	504	517
PA-mCherry1			405	564	595
PATagRFP			405	562	595
KFP-Red			530–560	580	600
Photoconvertible (switch between colors)					
PS-CFP2	400	468	405	490	511
Dendra2	490	507	405	553	573
wtEosFP[c]	506	516	405	571	581
Kaede	508	518	380-400	572	580
wtKikGR[d]	507	517	405	583	593
ClavGR2	488	504	405	566	583
PSmOrange	548	565	490	636	662
Photoswitchable (reversible switch between on- and off-state)					
Dronpa	503	517	503-off, UV-on		
Padron	503	522	UV-off, 503-on		

a) Excitation maximum.
b) Emission maximum.
c) Tetramer, but also available as a dimer (dEos), tandem dimer (tdEos), and monomer (mEos).
d) Available as a tetramer and monomer with similar properties.

The new generation of FPs are **photoactivatable, photoconvertible, and photoswitchable FPs** (Table 19.4). They are used to optically highlight the spatiotemporal dynamics of intracellular molecules, organelles, and whole cells. Photoactivatable FPs are activated from a nonfluorescent (dark) state to a fluorescent state, whereas photoconvertible FPs undergo photoconversion from one emission wavelength to another, that is, they switch between two different fluorescent colors. In contrast, photoswitchable FPs can be reversibly photoactivated, that is, they can be reversibly switched between a fluorescent on-state and a nonfluorescent off-state by irradiation with light. These FPs show unique features that are advantageous for protein-tracking applications but vital for subdiffraction microscopy, such as PAL-M (see Section 19.1.2.5).

Other derivatives of the GFP have been identified that can be used as indicators of H^+ (pH), redox potential, or ROS. They show pH-, redox-, or ROS-dependent fluorescence properties via changes in absorbance and fluorescence emissions depending on the pH, redox potential, or occurrence of ROS within a cell or a cellular organelle. Furthermore, combinations of different variants of FPs are used as nanosensors for Ca^{2+}, different monosaccharides, phosphate, and cyclic adenosine monophosphate (cAMP). These fusion proteins utilize **fluorescence resonance energy transfer (FRET)** between emitting FPs attached to a specific binding peptide. Such peptides include the calmodulin-binding peptide M13 for Ca^{2+} detection, members of the bacterial periplasmic binding protein superfamily for the determination of maltose, ribose, glucose, glutamine, and phosphate, and cAMP-dependent protein kinase for the detection of cAMP. Binding of the respective molecule increases or decreases the interaction between the two FPs, resulting in higher or lower FRET efficiencies, respectively.

19.4 Single Cell Technologies

In contrast to histological detection methods, which visualize specific cellular components in a tissue and cell context, complex analyses of the contents of specific cells aim to get a global view of transcripts, proteins, and metabolites. However, the isolation of nucleic acids, proteins, and metabolites from single cells is hampered by the complexity of a given tissue. Therefore, this requires the **isolation of single cells or the contents of single cells**. The simplest way to collect cells of a distinct type is to isolate them by macerating tissues (animal tissue) or generating protoplasts

(plant tissue). Although such cells can easily be collected, the generation of single cells might lead to dramatic changes in gene expression and the protein pattern. Moreover, single cells separated from a complex tissue might still represent various cell types; therefore, special techniques for overcoming such limitations were developed for highly spatially resolved investigations at the single cell level.

The isolation of a certain type of cell from a complex mixture can be performed by sorting cells according to their endogenous properties; the method used is **flow cytometry**, which was developed to efficiently and reproducibly determine the relative nuclear DNA content and ploidy level in cell populations, and at a reduced cost. A flow cytometer consists of fluidics, optics, and electronics. Cells and particles are measured in suspension, and flow in single file through an illuminated volume. Scattered light and/or emitted fluorescence are recorded and converted into a graph plotting the intensity of light against the number of cells emitting light at a given time. A flow cytometer is also used to sort cells with distinct features, such as their size, viability, or chlorophyll content. The sorting of distinct cell populations is performed mainly after specific labeling; here, specific dyes (4,6 diamidino-2-phenyl indole (DAPI), fluorescein diacetate (FDA)), or immunohistochemistry can be used. GFP is often used to tag specific cells for selection. The expression of *GFP* under the control of a cell-specific promoter enables specific cells to be labeled, which can then be sorted after the generation of protoplasts and used for spatial and temporal gene expression profiling as well as for proteomics and metabolomics approaches.

An alternative method to sampling by flow cytometry is **laser microdissection** (**LMD**). A UV laser is used to cut around the cells of interest within a section or single cell layer. Because the light beam is so focused, the cut is within the range of about 1 µm, and the cells of interest are not damaged by heat. The dissected cells are then collected from the section by either catapulting them using a defocused laser beam or by gravity (Figure 19.6). LMD itself is easy to perform, reproducible, and avoids direct contact with the target cells. It is usable for all cells that can be morphologically distinguished in a section; specific labeling is not necessary. Therefore, this technique allows any cells to be harvested from any tissue from any species that can be viewed by conventional microscopy. The limitations of LMD are the tissue-processing steps that must be performed in advance of the dissection. Fixation, embedding, and sectioning of the specimen have to allow fairly good structural preservation of the morphology and optimal recovery of the molecules of

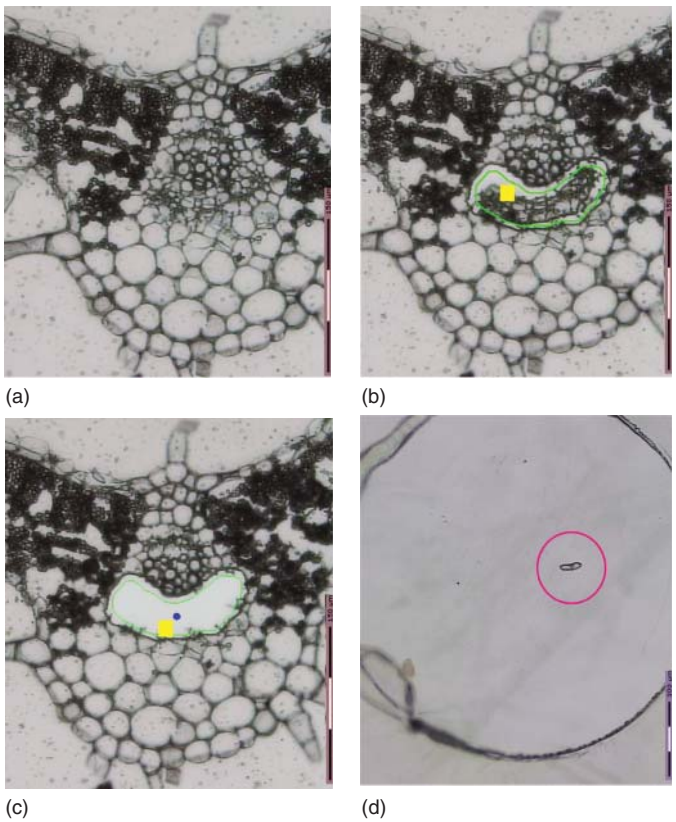

Figure 19.6 Laser microdissection (LMD) of phloem tissue from a tomato leaf section. (a). Cross-section of main vein before dissection; (b). Laser-dissected phloem tissue; the green line marks the cutting line of focused laser beam; (c). Cross-section after catapulting the dissected phloem tissue; the blue dot marks the catapulting point for defocused laser beam; (d). Dissected tissue after catapulting in the lid of a test tube. Bars represent 150 µm (a–c) and 300 µm (d). Pictures kindly provided by Dr S. Lohse (Leibniz Institute of Plant Biochemistry, Halle, Germany).

interest. Because of the collection of cells from a semithin section, the amount of RNA, protein, or metabolite that can be recovered by LMD is relatively low. To date, the use of LMD-isolated material is supported by a multitude of analytical methods that are characterized by small-sample and high-throughput strategies, as well as by highly increased sensitivity.

Up to now, only one direct method has allowed samples to be collected with the highest spatial resolution for subsequent analysis. Single cell sampling or **microsampling** performed by **microcapillaries** is the only sampling method suitable for the analysis of cell extracts from living, nontreated tissue. It is a minimally invasive sampling method that yields volumes in the pico-liter range. The material collected can be used for the isolation of RNA, proteins, and metabolites, although studies of metabolites collected from individual plant cells have been restricted to a limited number of metabolites until now. Microsampling can be successfully used for root hairs, mesophyll cells, and phloem. A highly specialized method was developed for phloem cells: the **stylectomy**. Severed stylets of phloem-feeding insects, such as aphids, scales, and mealy bugs, can be used to collect sieve-tube solutes and to determine turgor pressure and membrane potential. However, this technique is technically challenging and is restricted to certain plant-insect combinations.

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Glossary

Abiotic stress Physical environmental factors that influence plant growth and development including light, temperature, pH or available nutrients.

Abscisic acid (ABA) A phytohormone involved in the responses of plants to abiotic and biotic stress. It regulates stress-related genes and is also responsible for stomata closure during drought. Its biosynthesis starts in the plastids from xanthophylls.

Acclimation Process by which an organism is able to adjust to a change in the environment. This enables the organism to survive even under adverse conditions.

Action spectrum A graph for which relative light sensitivity of a physiological response is plotted against the wavelength of light.

Active bioremediation Approaches involve human interventions to initiate, enhance, or stimulate the bioremediation process.

Aerenchyma Tissue often be found in leaves, stems and roots of aquatic and wetland plants and refers to cavities that allow storage and transport of air.

Aerobes Organisms that use oxygen as terminal electron acceptor.

Aerobic metabolism Cell energy metabolism where electrons finally are transferred to molecular oxygen. This type of energy metabolism generates ATP with high efficiency.

Aglycone The nonsugar part of a glycoside (after removal of the sugar part).

Agonists Organisms that produce substances (secondary metabolites) that activate defense mechanisms.

Alkaloids A chemical substance containing nitrogen as part of a heterocyclic ring structure; often highly toxic or mind-altering. Alkaloids represent a diverse array of amino-acid-derived, often highly complex structures with potential effects on mammalian organisms. Used in medicine and pharmacy.

Alkylating agent Reactive secondary metabolite which introduces an alkyl substituent into DNA, proteins or other molecules.

Allochthonous Indicates that food sources are imported into an ecosystem, e.g., leaves from trees imported into a stream. Alternatively used to describe microorganisms adapted to a rapid growth rate.

Amorphous ice/vitrified ice Form of solidified water in which molecules are randomly arranged in a glass-like solid state; this is in contrast to crystalline ice where molecules are regularly arranged in a lattice needing more space.

Anaerobes Organisms that do not use oxygen as terminal electron acceptor.

Angiosperms Flowering plants.

Anoxia Complete loss of oxygen.

Anoxic Devoid of oxygen.

Antagonists Organisms that interfere with growth, survival and infection of pathogens.

Anthocyanins Red purple and blue pigments that belong to a class of plant secondary metabolites called flavonoids; anthocyanins are composed of phenolic anthocyanidin moieties and sugars.

Anthraquinones Secondary metabolites with an anthracene skeleton; anthraquinones show strong laxative effects.

Antibacterial A substance that kills or inhibits the growth of bacteria.

Antibiosis Biological interaction between two or more organisms that is detrimental to at least one of them or an antagonistic association between an organism and the metabolic substances produced by another one.

Antibiotic A substance that kills or inhibits the growth of microorganisms.

Antifreeze proteins Proteins of animals, plants, fungi, and bacteria bind to ice crystals and inhibit their growth. They protect against freezing damages.

Antifungal A substance that kills or inhibits the growth of fungi.

Antimicrobial A substance that kills or inhibits the growth of microorganisms.

Antioxidant A substance that is able to protect cells or counteract the damage caused by oxidation and free oxygen radicals (reactive oxygen species ROS).

Antioxidant defense The whole biochemical system of the cell that decomposes reactive oxygen and reactive nitrogen species. It consists of enzymes including superoxide dismutase and peroxidases and low molecular mass molecules such as ascorbic acid and glutathione.

Antiparasitic A substance that kills parasites.

Apo-form Metal-free form of a metallobiomolecule.

Ecological Biochemistry: Environmental and Interspecies Interactions, First Edition. Edited by Gerd-Joachim Krauss and Dietrich H. Nies.

Companion Website: http://www.wiley.com/go/Krauss/Nies/EcologicalBiochemistry

Apoptosis Programmed cell-death leading to a progressive fragmentation of DNA and disintegration of cells without causing inflammation.

Aquaporins Membrane-based proteins regulating water flow across the membrane.

Aquatic hyphomycetes Polyphyletic group of fungi that dominate leaf decomposition in running waters.

Arbuscular mycorrhiza Symbiosis between autotrophic plant roots and obligatory symbiotic fungi.

Attini Taxonomic tribe of two genera of leaf-cutting ants (*Atta*,15 species; *Acromyrmex*,24 species) that are able to cultivate fungi in subterranean gardens of their nests.

Autochthonous Indicates that food sources are produced within an ecosystem. Alternatively used to describe microorganisms adapted to a slow growth but maintenance energy.

Autotrophic respiration (R_a) Respiratory CO_2 production by photosynthetically active and inactive plant tissues.

Avoidance The capability of a plant to resist stress by minimizing the impacts encountered by the plant.

Bacteriocins Proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). They are typically considered to be narrow spectrum antibiotics though this has been debated.

Bacterium All bacteria consist of a single cell surrounded by a cell wall; DNA is circular; bacteria do not have internal membrane systems or a nucleus.

Base pairing Complexation of the complementary pair of polynucleotide chains of nucleic acids through hydrogen bonds between complementary purine and pyrimidine bases adenine (A) with thymine (T) or uracil (U), cytosine (C) with guanine (G).

Benthic Referring to the bottom of a water body (running waters, lakes, oceans).

Benthos Organisms found on the bottom of a water body.

Betacyanins Reddish nitrogen-containing pigments. The core structure is derived from two molecules of tyrosine.

Betalains Betacyanins and betaxanthins are referred to as betalains and have functionally replaced the anthocyanins in the Caryophyllales. Their phylogenetic origin is still a matter of debate.

Betaxanthins Yellow nitrogen-containing pigments. The core structure is a non-enzymatically formed condensation product of one molecule of betalamic acid (derived from one molecule of tyrosine) and one amino acid.

Bi-desmosidic saponins Saponins with 2 sugar chains.

Bilin Linear tetrapyrroles that are biological pigments.

Bioaccessibility The amount of a substance which is not yet directly bioavailable but may become bioavailable in the future, and is considered to be bioaccessible.

Bioaccumulation Biological enrichment of substances to higher concentration in organism than in its environment.

Bioavailability The amount of a substance available to cross an organism's cellular membrane at a given time point.

Biodegradation A designation frequently used for the ultimate biological breakdown of an organic compound to CO_2 and H_2O; however, sometimes the term is used with the understanding that incomplete biological breakdown of organics into organic metabolites is also included.

Biodeterioration Deterioration of materials (minerals, wood biological and synthetic polymers, etc.) by the action of microorganisms. Results can be the acceleration of mineral weathering, rotting of wood, impairment of synthetic polymers by degradation of plasticizers, increasing brittleness, staining, and hydrating.

Biofilms Any group of microorganisms in which cells stick to each other on an interface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS).

Bioindicators Biota that give information on the quality of environment by measuring the effect of pollutants.

Biological filtration Removal of dissolved organic substances from water by means of biofilms. The microorganisms absorb biodegradable material and convert it into metabolites (preferably CO_2 and water) and biomass. This principle is generally applied to biological drinking and waste water purification.

Biological N_2 fixation Enzymatic conversion of N_2 to ammonia by free living and symbiotic bacteria.

Biomagnification Increasing concentrations of substances with each trophic level.

Biomarker Biological parameters at the cellular and multicellular level indicating biochemical changes under environmental stress.

Biomembrane Permeation barrier around every cell or cellular compartments consisting of phospholipids, cholesterol, and membrane proteins. The integrity of its biomembrane is a requisite for any cell.

Biomonitoring Continuous observation of an area using bioindicators to assess changes in organisms or ecosystems induced by human impact.

Bioremediation The use of living organisms to remove metals and xenobiotics from the environment.

Bioreporters Living and usually genetically engineered microbial cells producing a detectable signal in reponse to a specific (chemical or physical) agent present in their environment.

Biosensors Technical device to couple a response element by a biological receptor to a transformer which alters this recognition effect into a selective and sensitive signal.

Biostimulation A kind of active bioremediation where nutrients and/or electron acceptors/donors are added to promote desired microorganisms.

Biotic stress Environmental factors that influence plant growth and development that arise from coinhabiting organisms such as other plants, soil microbes, fungi, or animals.

Biotransformation The incomplete biological breakdown of an organic compound into organic products other than the parent compound.

Bottom-up proteomics Analytical strategy in proteomics employing protein fragmentation into peptides by enzymatic digestion followed by (usually mass spectrometry) (DS) analysis of the resulting peptides and reconstruction of the protein structure.

C_4 carbon fixation The biochemical pathway of CO_2 fixation in C_4 plants. It involves fixing of CO_2 in mesophyll cells via phosphoenolpyruvate PEP, carboxylase formation of the eponymous C_4 compound, usually malate, which is transported to the bundle-sheath cells where after its decarboxylation CO_2 is fixed by Rubisco.

C6-C1-phenolics Heterogenous collection of simple phenolic compounds derived from intermediates of the shikimate pathway. Examples include gallic acid and salicylic acid.

Calvin cycle A series of light-independent enzymatic reactions in the stroma of chloroplasts converting CO_2 and water into organic compounds using ATP and NADPH from the photosynthetic light reactions. The key enzyme for carbon fixation in this cycle is Rubisco.

Cancer Various types of malignant cells that multiply out of control.

Carcinogen A substance that may cause cancer.

Cardiac glycoside A steroidal glycoside that increases the strength or rhythm of the heart beat.

Carnivorous fungi Fungi that are able to trap and consume small soil organisms.

Carnivorous plants Plants that attract, trap, and digest prey followed by nutrient absorption.

Catabolism Process of breakdown of complex molecules into simpler ones often providing biologically available energy in the form of ATP.

Chalcone synthase Key enzyme for the biosynthesis of polyketides like flavonoids and anthocyanins.

Chemical resistance Secondary metabolites mediate resistance.

Chemolithoautotrophy Oxidation of inorganic compounds to gain energy for CO_2 fixation.

Chloroplast Important compartment of plant cells; site of photosynthesis; chloroplasts have their own DNA replication, transcription, and ribosomes. Originated from cyanobacteria.

Cholesterol The most common steroid (fat-like material) found in the human body; important for membrane fluidity and as a precursor to steroid hormones; high cholesterol levels are associated with an increased risk of coronary diseases.

Chromatic adaptation of photosynthesis A process that helps to optimize light-absorption of photosynthesis; adaptation is mediated by alterations in antenna pigment composition at the thylakoid membranes of chloroplasts.

Chromatin The three-dimensional molecular structure of DNA and proteins in the nuclei of cells.

Chromophore Chemical compound that can absorb light at a certain wavelength and therefore appears colored in bright light. cofactor of a protein that is responsible for the absorption of light.

Chromophore-binding domain Part of a photoreceptor molecule that binds the chromophore; absorbs light and triggers activity of the output-domain that links photoreceptor activity to downstream signaling.

Clade Taxa that share common ancestry fall in a clade.

Common Mycorrhizal Network (CMN) Physical connection of a range of plants by fungal hyphae.

Coevolution Two or more species reciprocally influence each other's evolution.

Cometabolism Biodegradation or biotransformation of an organic compound that can be used as a growth substrate in the presence of another compound enabling growth, which is referred to as cosubstrate.

Commensalism Relationship between two species in which one organism benefits and the other species is unaffected.

Common mycelia network (CMN) Network of hyphae of mycorrhizal fungi linking plants of the same or of different species.

Compatible solutes Small molecules involved in the osmoregulation of the cell (osmoprotectants, osmolytes).

Complementary DNA (cDNA) Transcribed from mRNA by employing the enzyme reverse transcriptase.

Compound-specific isotope analysis (CSIA) Determination of the ratios of naturally occurring staple isotopes (e.g., $^{13}C/^{12}C$ $^{2}H/^{1}H$) of distinct compounds (e.g., contaminants or their degradation products) in environmental samples together with the measurement of compound concentrations enabling to gain information about the fate of compounds (e.g., to obtain indications for biodegradation to identify contaminant sources to gain information about the dispersion of contaminant plumes, etc.).

Conditioning (microbial conditioning) Changes on plant detritus because of microbial colonization that tend to make the substrate more suitable for invertebrate consumption.

Confocal microscopy Optical imaging technique increasing optical resolution and contrast of a microscopic picture based on the elimination of out-of-focus light in specimens that are thicker than the focal plane or depth of focus generated by the objective.

Connectance Ratio of observed to total number of possible links in food web.

Constitutive photomorphogenic 1 (COP1) A central component of light regulation in plants; E3 ubiquitin ligase complex that functions as a repressor of photomorphogenesis during the seedling development of higher plants; functions as negative regulator downstream of many plant photoreceptors.

Coupling techniques Also denoted as hyphenated techniques usually refers to online couplings of separation techniques with mass spectrometry. Most popular are couplings of liquid or gas chromatography and mass spectrometry (LC-MS or GC-MS).

CPOM Coarse particulate organic matter.

Crassulacean acid metabolism (CAM) Function in some plants living in arid conditions. In these plants stomata open during night. Then CO_2 is transformed to malate and stored. During daytime stomata are closed to avoid loss of water and CO_2 is liberated from malate and fed into the Calvin cycle.

Cyanobacteriochromes Phytochrome-like photoreceptors in cyanobacteria that carry a bilin chromophore.

Cyanogenic glycosides Vacuolar glycosides where the aglycone contains labile cyanide, which is released on cleavage of the glucose moiety and may serve as a defense compound.

Cytochrome P450 monooxygenase Very large superfamily of enzymes containing a heme-iron center present in all organisms and involved in the oxidation of compounds. Cytochrome P450s are one of the driving factors in metabolic diversity.

Cytoplasm Basic compartment of the cell (surrounded by the plasma membrane) in which nucleus, endoplasmic reticulum, mitochondria and other organelles are embedded.

Cytoskeleton The network of filamentous protein structures along which vesicles and organelles are moved and which are ultimately required for cell shaping.

Cytotoxic A substance that is toxic to cells, i.e., damages cell structure or function.

***De novo* sequencing** Determination of a new protein sequence not previously described in data bases.

Dehalorespiration A type of anaerobic respiration leading to energy conservation via the use of chlorinated organic compounds as terminal electron acceptors in certain anaerobic bacteria.

Denitrification Reduction of nitrate and nitrite ultimately releasing N_2.

Denitrifier Chemoorganoheterotrophic bacteria that degrade biomass and conserve energy by transferring the resulting electron to NO_3^- or other oxidized nitrogen compounds thereby liberating N_2.

Density anomaly Refers to the fact that water reaches its highest density at 4 °C.

Desiccation Drying of an organism.

Desulfurication Conversion of sulfate to hydrogen sulfide.

Detritus food chain Based on the consumption of dead organic matter.

Differentiation Process in which cells lose their omnipotence to adopt more specialized functions.

DNA Deoxyribonucleic acid, the biomolecule in cells that stores the genetic information; composed of 2 complementary nucleic acid strands bonded by G-C and A-T pairs.

DOM Dissolved organic matter.

E3 ubiquitin ligase Proteins or protein complexes that specifically link ubiquitin polypeptides to lysine residues of target proteins.

Early successional species Species abundant mainly during early ecosystem development.

Ecological restoration The process for supporting the recovery of an ecosystem that has been disturbed or destroyed; increasing the provision of ecosystem services and reversing biodiversity loss.

Ecological stoichiometry The study of the balance of chemical elements in ecological interactions.

Ecoremediation Use of multiple and multitrophic levels of ecological organization for restoring polluted terrestrial and aquatic areas.

Ecosystem services The advantages for humans obtained from ecosystems.

Ecotype A subspecies showing special adaption to distinct environmental conditions.

Ectomycorrhiza Symbiosis between autotrophic plant roots and saprotrophic fungi.

Ectomycorrhizins Symbiosis related proteins unique to ectomycorrhizae.

Emergent properties When individual components in an environment come together and create distinct, collective and interactive properties and functions.

El Nino Southern Oscillation (ENSO) Noncyclic change of circulation in the oceanographic-meteorological system of the equatorial pacific.

Electrophoresis Separation technique based on the mobility of analytes in an electric field. Important techniques are one- and two-dimensional gel electrophoresis and capillary electrophoresis.

Electrospray ionization (ESI) Ion source technique in mass spectrometers for ionization of biomacromolecules (e.g., petides and proteins) present in a liquid phase.

Endophyte Fungus that lives inside plants and often produces secondary metabolites.

Endoplasmic reticulum Cellular endomembrane system in which proteins are modified posttranslationally.

Enhanced natural attenuation Biostimulation approaches specifically addressing in-situ treatment.

Environmental risk assessment Calculation of adverse effects of human impact on ecosystems and their parts.

Enzyme Protein that catalyses a chemical reaction, e.g., the hydrolysis of acetylcholine.

Epigenetics Study of mitotically and/or meiotically heritable changes in phenotype, which are independent from genetic variation.

Epilimnion Uppermost layer of water in a lake during stratification.

Ergot A fungus (*Claviceps purpurea*) that infects grasses (especially rye) and produces pharmacologically active alkaloids.

Essential oil (=volatile oil) Mixture of volatile terpenoids and phenylpropanoids responsible for the taste and smell of many plants, especially spices.

Etiolation Developmental strategy of plants in darkness that facilitates exposure to light.

Euphotic zone Zone in lakes or oceans where photosynthesis can occur.

Exploitation ecosystem hypothesis (EEH) Nutrient limited habitats support small herbivore populations.

Extraradical mycelium Hyphae of mycorrhizal fungi reaching out into the soil beyond the rhizosphere.

Extracellular polymeric substances (EPS) Hydrated biopolymers (proteins, polysaccharides, extracellular DNA) which form the matrix in which biofilm cells are immobilized.

Facilitation Support actions between species with an advantage to one and without disadvantage to the other species.

Fixation Process to preserve biological tissue in a "near-to-life" status for histological analyses; it immobilizes cell components and terminates any biochemical reaction by the use of suitable substances (chemical fixation) or by rapid freezing (cryofixation).

Flavonoids A class of plant secondary metabolites that are composed of two aromatic rings connected by a three carbon bridge; according to the degree of oxidation of the three carbon bridge, flavonoids are classified into different subgroups including anthocyanidins and flavonols.

Flavonols A subgroup of flavonoids that absorb UV light and function as protective sunscreens in the plant epidermis.

Fluorescence Emission of light after excitation with light or electromagnetic irradiation; the emitted light has always a lower energy (longer wavelength) than the absorbed light/radiation.

Fluorescence resonance energy transfer (also Förster resonance energy transfer both abbreviated with FRET) Mechanism that describes the nonradiative energy transfer between two fluorophores; prerequisites for energy transfer from donor fluorophore to acceptor fluorophore are the small distance between both (Förster radius) and the spectral overlap of the donor emission spectrum and the acceptor absorption spectrum.

Fluorescence-in-situ-hybridization (FISH) A cytogenetic technique developed by biomedical researchers in the early 1980s that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes.

Fluorescent proteins Have intrinsic fluorescence properties by containing covalently bonded chromophores build-up by a tripeptide (Ser–Tyr–Gly); the first one (green fluorescent protein GFP) has been isolated from the jellyfish *Aequorea victoria* and exhibits bright green fluorescence when exposed to blue to ultraviolet light; nowadays a great variety of engineered GFP derivatives and fluorescent proteins of different families exist.

Fluorochrome Chemical compound that is fluorescent; it reemits light of a certain wavelength on excitation by light.

Flux control Control of the transport rate of a transporter by the substrate. Magnesium ions are, for instance, able to bind to various magnesium transporters shutting off their activity when the cytoplasmic magnesium concentration is sufficiently high. Flux control functions in addition to other regulatory processes, e.g., control of gene expression.

Food chain Linear sequence of transfer of food from one organism to another.

Food web A system of interconnected food chains.

Fourier transform ion cyclotron resonance (FTICR) MS Mass analyzer in mass spectrometry where the mass-to-charge ratio of ions is determined by the measurement of the frequency of the periodic ion movement in a magnetostatic field.

FPOM Fine particulate organic matter.

Frame-shift mutation Mutation deleting or inserting of one or two nucleotides in a gene. This shifts the normal reading frame and causes the formation of functionless proteins.

Free radical An unstable form of oxygen molecule that can damage cells.

Fructans Are polymers of fructose usually with a sucrose molecule at the end.

Functional groups 1. Classification of invertebrates into groups based on how they gather and eat food. Includes grazers, scrapers, shredders, collectors 2. specific groups within molecules that are responsible for specific chemical reactions of those.

Futile cycle A biochemical reaction cycle that is wasting energy, e.g., a simultaneous action of a kinase generating a phosphorylated intermediate that is subsequently and immediately degraded by a phosphatase.

Galls Issue deformations found in plants infected by certain microorganisms or colonized by insects.

Gas chromatography (GC) Separation technique based on the interaction of analytes in a gaseous mobile phase with a solid stationary phase.

Genome The entirety of genetic information of an organism necessary for its development and functioning. This information is encoded in deoxyribonucleic acid (DNA).

Genomics Determination of the whole DNA sequence of an organism and genome mapping on a fine scale by assigning DNA fragments to chromosomes.

Girus Giant virus.

GLC High resolution gas-liquid chromatography, a technique used to analyze volatile chemical compounds and extracts.

Glucosinolates Nitrogen and sulfur containing *S*-glycosides derived from aliphatic and aromatic amino acids. The enzyme myrosinase cleaves this glycosidic bond and releases (iso)thiocyanides or nitriles as potent defense compounds.

Glutathione (γ-Glu-Cys-Gly) Enzymatically synthesized sulphur- containing tripeptide.

Glutathione-S-transferase (GST) This enzyme catalyzes conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification. The enzyme is involved in the detoxification of reactive oxygen species.

Glycoside A chemical substance that yields at least one simple sugar on hydrolysis.

Grazing food chain Based on consumption of living primary producers.

Green world hypothesis (HHS) Predator populations suppress herbivory in nutrient limited habitats.

Gymnosperms Plants with naked seeds; includes conifers, cycads, ginkgo.

Hard-soft-acid base theory(HASB) A qualitative concept developed by Pearson to understand the factors that drive and stabilize metal complexes with organic reactants. The selectivity of metal ions for biochemical donor ligands can be anticipated.

Henry's law Describes solubility of gases in water.

Herbivore Animal that feeds on plants.

Herbivory Feeding of animals on plants.

Heterotrophic respiration (R_h) Respiratory CO_2 production by heterotrophic microbes.

High irradiance response Class of light responses that need prolonged irradiation with high light intensities for full expression; magnitude of high irradiance responses depends on light intensities.

Histidine kinase domain Part of protein kinases that often function as receptors in two-component signal transduction chains; histidine kinases transfer phosphate from ATP to a histidine residue of its dimeric interaction partners; the activated phosphate can be transposed to aspartate residues of receiver domain proteins.

Histone Conserved group of proteins that bind to negatively charged DNA and are part of the nucleosome. Modification of histone proteins are important for epigenetic regulation.

Horizontal gene transfer (HGT) Transfer of genes or genomes between unrelated organisms.

High performance liquid chromatography (HPLC) A technique used to analyze chemical compounds and extracts see Liquid Chromatography (LC).

Hydrothermal vent Fissure in ocean floor where superheated mineral-rich water emerges.

Hypersensitive response Pathogen-induced cell death, which leads to necrotic lesions and suppresses further spreading of infection. It involves activation of plasmamembrane-bound NADPH oxidase that generates superoxide on the apoplastic side of the plasmamembrane.

Hypolimnion Lowest layer of water in a lake during stratification.

Hyporheic zone Habitat below stream bed.

Hypoxia Metabolic state of insufficient oxygen supply to cells, tissues, or organisms that usually rely on oxygenic metabolism. Hypoxia induces fermentative metabolism and specific gene expression changes and may cause death in sensitive organisms.

In vitro In the laboratory or test tube.

In vivo In a living cell or organism.

Inductively coupled plasma mass spectrometry (ICP MS) Mass spectrometry technique for ultrasensitive detection and quantification of chemical elements. A hot argon plasma (ICP) of about 7000 K serves as ion source that destroys every chemical structure and produces simple element ions.

Inquilines Organisms that commensally inhabit in a structure produced by another organism.

Interaction strength Measures strength or importance of a link in a food web.

Intercalation Planar and lipophilic compounds can intercalate between base stacks of DNA; this leads to frame shift mutations (leading to inactive proteins).

Invasive methods Originally defined as a medical procedure in which breaks in the skin are created or the body cavity is entered; in cell-biology this term is used for visualization of excised, fixed, and sectioned cells/tissues/organs.

Ion channel Membrane protein that can form water-containing pores so that mineral ions can enter or leave cells.

Ion trap (IT) MS Mass analyzer in mass spectrometry separating ions by trapping them in an electrodynamic field (ion trap) and ejecting them subsequently to the detector.

Iridoids A subgroup of monoterpenoids with iridoid glucosides, secoiridoids, and secologanin.

Isobaric tags for relative and absolute quantitation (iTRAQ) Chemical labels with different masses for protein quantification in tandem mass spectrometry.

Isothiocyanate Secondary metabolites released from glucosinolates upon hydrolysis; with strong skin irritating properties.

Isotope-coded affinity tags (ICAT) Chemical labels containing ^{2}H or ^{13}C isotopes for protein quantification in mass spectrometry.

ISR (induced systemic resistance) Allows the plants to endure pathogen attacks, which, without bacterial pre-inoculation, could be lethal. The effect is systemic, e.g., root inoculation with PGPR yields the whole plant nonsusceptible. Thus far *Pseudomonas* and *Burkholderia* (*Betaproteobacteria*) and *Bacillus* spp. have been shown to elicit ISR.

Lentic Refers to ecosystems with slow-moving or standing water, e.g., lakes, ponds, swamps.

Ligand Substance that binds to a receptor in a specific way like a key in a lock.

Lipid bodies Cell organelles consisting of a triacylglycerol core surrounded by a monolayer of phospholipids and protein.

Liquid chromatography (LC) Separation technique based on the interaction of analytes in a liquid mobile phase with a solid stationary phase.

Littoral zone Shore region of lakes and oceans.

Long-distance transport Transport of metabolites that have been synthesized in a particular organ such as roots to the aerial parts via xylem or phloem.

Lotic Refers to running waters, e.g., streams, rivers.

LOV domain Light/Oxygen/Voltage chromophore-binding domains that form light-sensing modules for many photoreceptor families.

Low fluence response Class of sensitive light responses triggered by type-II phytochromes that can be induced by a single red-light pulse.

Macronutrient Mineral nutrient required in concentrations in excess of 0.5 mg kg^{-1} dry matter.

Mangrove (mangrove forest) Intertidal forested wetlands.

Mass spectrometry (MS) Separation of ions in a mass analyzer according to their mass-to-charge (m/z) ratio. A mass spectrometer is composed of a sample introduction system, an ion source, a mass analyzer, and a detector. Mass spectrometry is the key technique for modern proteomics.

Matrix assisted laser desorption/ionization (MALDI) Ion source technique in mass spectrometers for ionization of biomacromolecules (e.g., peptides and proteins) present in a solid phase.

Mechanical resistance Morphological structures mediate resistance.

MEP pathway Alternative C3 pyruvate and glycerol-3-phosphate-based pathway to isoprenoids localized in plastids.

Meristem Groups of omni- or pluripotent cells from which other cells of a tissue are generated.

Metabolic fingerprinting Rapid classification of metabolites without identification and quantification of each individual compound by high-throughput sample screening.

Metabolic theory of ecology Assumes that the metabolic rate of organisms influences patterns in ecology.

Metabolite target analysis Analysis of only one or few target metabolites.

Metabolites Intermediates or end products of physiological processes, usually small molecules with molecular weights below 1 kDa.

Metabolome Entirety of all metabolites in an organism.

Metabolomic profiling Qualitative or quantitative description of metabolic pattern for a group of related metabolites.

Metabolomics Global identification and quantification of all metabolites in a biological system (cell fluid, tissue) including their time- and space-resolved distribution.

Metabolon Array of enzymes closely associated in a specific cellular compartment discussed as a plausible platform for the biosynthesis of various specialized metabolites.

Metal hyperaccumulators Some plants are able to accumulate high concentrations of metals without suffering from any toxicity.

Metalimnion Middle layer of water in a lake during stratification.

Metallome Entirety of metal and metalloid compounds in an organism or its parts (cells, body fluids or tissues).

Metallomics An investigation of interactions and functional connections between metal ions and DNA, RNA, proteins and metabolites.

Metallophytes Plant species growing on soils contaminated with metals.

Metallothiolome Entirety of thiol peptides and their metal complexes in an organism or its parts (cells, body fluids, or tissues).

Metallothiolomics A systematic investigation of the thiol peptide regulated metal homeostasis.

Meta-omics Approaches for studying the genome, (metagenomics), transcriptome (metatranscriptomics), and proteome (metaproteomics) from whole microbial communities.

Metapleural gland Paired structure at the posterolateral end of the alitrunk that is found only in ants. The composition of gland secretions differs among ant species and can contain territorial marking pheromones, antibiotic compounds, and the plant growth hormone indole-3-acetic acid.

Methanogenesis Formation of methane by archaeal microorganisms.

MEV pathway Classical C2 Acetyl-CoA-based cytosolic pathway to isoprenoids.

Microbial loop Involves return of DOM to higher trophic levels via microbes.

Microbial mat A multilayered sheet of microorganisms, mainly bacteria and archaea. Microbial mats grow at interfaces between different types of material mostly on submerged or moist surfaces, but a few survive in deserts. They colonize environments ranging in temperature from −40°C to +120°C.

Microbially influenced corrosion Influence of parameters relevant for metal corrosion such as oxygen-content, redox potential, pH-value, ionic strength at the interface between metal and microbial biofilm. It is known to enhance the corrosion process, but inhibiting influences have also been reported.

Microconsortia Associations of microorganisms of several species which remain in spatial proximity by immobilization by the EPS matrix. They are capable of complex degradation processes.

Micronutrient Mineral nutrient required in concentrations below 0.5 mg kg^{-1} dry matter.

microRNAs Short (about 22 nucleotides) RNA molecules present in eukaryotic cells. They act as posttranscriptional

regulators mostly via degradation of target mRNAs or inhibition of their translation.

Microtubules Linear tubular structures of higher cells formed from tubulin dimers; essential for cell division and vesicular transport processes.

Mineral nutrient Element without a plant cannot complete its life cycle. A specific function is known and the deficiency of the nutrient causes specific symptoms.

Mineralization As used here indicates the ultimate biological breakdown of an organic compound to CO_2 and H_2O; may have different meanings in other biological / soil science disciplines.

Mitochondria Important organelle for compartment of eukaryotic cells; site of the TCA cycle and respiration chain (production of ATP); mitochondria have their own DNA replication transcription and ribosomes. Originated from protobacteria.

Mitosis Cell division.

Monitored natural attenuation A natural attenuation approach where human action is restricted to monitoring and not aimed at the improvement of the bioremediation process itself.

Mutant An organism carrying a genetic difference from the wild type, sometimes with detrimental effects on gene function.

Mutualism An interaction between different species that is mutually beneficial.

Mycoremediation A special form of bioremediation using fungi to remove (degrade, or sequester) contaminants from the environment.

Mycorrhiza Nutritional symbiosis between fungi and the root tips of plants.

Myrmecochory Plant seed dispersal by ants.

Myrmecophilic plants Plants that reward ants with food such as extrafloral nectar or foodbodies.

Natural attenuation In-situ reduction in mass concentration, volume toxicity mobility of environmental contaminants in soil and water caused by, or biological chemical and physical processes without human intervention.

Nekton Aquatic animals that move independently of water currents.

Neochrome Class of photoreceptors that triggers light responses in the red and blue light part of the light spectrum; evolved from a fusion between a phototropin photoreceptor with a phytochrome chromophore-binding domain.

Net primary productivity (NPP) Total amount of energy or nutrients accumulated by an ecological unit, i.e., an organism, a population, or an ecosystem.

Neurotoxin Substance with adverse effects on the central and peripheral nervous system, such as transient modulation of mood or performance of CNS.

Neurotransmitter Signal compounds in the synapses of neurons that help convert an electric signal into a chemical response; important neurotransmitters are acetylcholine, noradrenaline, adrenaline, dopamine, serotonin, histamine, glycine, GABA, glutamate, endorphins, and several other peptides.

Neuston At the interface between water and atmosphere, there is a "microlayer" of about 100 µm thickness. The neuston is the collective term for the organisms that float on the top of water (epineuston) or live right under the surface (hyponeuston). The concentration of microorganisms in this layer is several orders of magnitude higher than in the underlying water body. Mainly hydrophobic organisms accumulate there.

NGS platforms Next-generation sequencing (NGS); modern techniques for high-throughput genome sequencing based on pyrosequencing, reversible dye terminator sequencing, or sequencing by ligation.

Nitrification Oxidation of ammonia to nitrite and nitrate.

Nitrifier Chemolithoautotrophic bacteria that conserve energy by transfer of electrons from NH_4^+ or NO_2^- to O_2.

Nitrogen fixation Assimilation of atmospheric N_2 into organic compounds.

Nitrogen reduction and assimilation Metabolic reduction of nitrate and its incorporation in amino acids.

Non-invasive methods Originally defined as a medical procedure in which no break in the skin is created; in cell-biology this term is used for visualization of intact and living cells/tissues/organs by use of fluorescent proteins or dyes.

Nonpoint source pollution Environmental pollution arising from diffuse sources.

Non-protein amino acid (NPAA) Secondary metabolite, which is an analogue of a proteinogenic amino acid; if incorporated into proteins, the latter are usually inactivated.

Nucleosome The central unit of the chromatin. About 147 bp of DNA are wrapped around a histone octamer forming one nucleosome unit.

Numerical aperture Number that describes the acceptance cone of an objective.

Nutrient cycle Pathways of chemical elements or organic molecules through ecosystems.

Nutrient spiral Description of nutrient cycling in streams because of downstream displacement.

Nutrient uptake efficiency Amount of nutrient taken up compared to the amount of nutrient present in soil.

Nutrient use efficiency Amount of biomass produced per unit of nutrient acquired.

Octahedral complexes Chemical complexes composed of a central transition metal cation and six ligands that bind to this metal by donating free electron pairs. The ligands are evenly spaced and form an octaeder.

Optical resolution Ability of an optical system to separate two distinguishable radiating points.

Orbitrap MS Mass analyzer in mass spectrometry where the mass-to-charge ratio of ions is determined by the

measurement of the frequency of the periodic ion movement in an electrostatic field.

Output-domain Part of receptors that transmits input signals into biochemical signals inside the cell; output domains trigger downstream signal responses.

Oxidative stress Progressive metabolic disorder and damage to cell components as a consequence of excessive generation of reactive oxygen and reactive nitrogen species. Oxidative stress is intimately linked to metabolic disorder disease, abiotic stress, and pathogen infection.

Oxygen sensing Molecular mechanisms that allow the cell to sense changes in oxygen availability and to adjust gene expression and metabolism. Oxygen sensing often involves redox sensitive transcription factors or other posttranslational modifications catalyzed by redox-regulated enzymes.

Oxygenic photosynthesis CO_2 assimilation by cyanobacteria, eukaryotic algae and green plants that evolve O_2 by splitting water in the oxygen evolving complex of photosystem II and thus release oxygen to the environment.

PAR (Photosynthetically active radiation) defined as a range of solar radiation that can be used for photosynthesis.

Passive bioremediation Bioremediation without human intervention aiming at the improvement of the bioremediation process.

Pathway engineering Construction of new catabolic pathways in (micro)organisms to remove contaminants from the environment through the application of sophisticated molecular genetic, microbiological and protein engineering tools.

Pelagial Open water in lake or ocean.

Perception The specific recognition of a stimulus leading to a physiological response.

Persisters Multidrug tolerance is caused by a small subpopulation of microbial cells termed persisters. Persisters are not mutants but rather are dormant cells that can survive the antimicrobial treatments that kill the majority of their genetically identical siblings. Persister cells have entered a non- or extremely slow-growing physiological state, which makes them insensitive (refractory or tolerant) to the action of antimicrobial drugs.

Pharmacology The study of the nature properties and uses of drugs (see pharmacodynamics, pharmacokinetics); includes the study of endogenous active compounds.

Phase I reactions Functionalization of xenobiotics during the initial biochemical reaction step(s) of their biotransformation or biodegradation.

Phase II reactions Formation of water-soluble conjugates of functionalized xenobiotics with endogenous, highly polar molecules or moieties; most prominent in eukaryotic but also existing in prokaryotic organisms.

Phase III reactions Follow-up reactions which may involve further metabolism of xenobiotic conjugates arising from phase II reactions, transport of xenobiotics and their metabolites (vacuolar deposition in plants, removal from cells by excretion in other organisms), and incorporation of xenobiotic metabolites into plant cell wall structures.

Phenylpropanoids Aromatic heterocycles generated from aromatic amino acids and universally distributed over the plant kingdom. As soluble (usually glycosylated) form in the vacuoles or insolubly incorporated into polymeric structures like lignin suberin or sporopollenin.

Phorbolester Diterpene from Euphorbiaceae and Thymelaeaceae resembling diacylglycerol in structure and therefore activates protein kinase C.

Photoinhibition Inhibition of metabolic processes such as photosynthesis at high irradiation.

Photolyase Repair enzyme that reverse cross-linking between DNA bases by the use of blue and UV-A light.

Photomorphogenesis Developmental strategy of plants mediated by the presence of light.

Photomultiplier (PMT) Vacuum phototubes that are extremely sensitive detectors of light in a broad spectral range; PMTs multiply the current produced by light leading to the possibility to detect individual photons.

Photoperiodic flowering Induced transition to flower formation in response to the length of the light and dark periods during a day.

Photosynthetic active radiation (PAR) Fraction of the spectrum of solar radiation used for photosynthesis.

Phototropin Blue/UV-A light photoreceptor that triggers light-mediated movement processes in plants; uses two LOV domains as light sensor module.

Phototropism Growth of an organism or a part of an organism in response to light.

Physiological responses Changes in enzyme activities, gene expression, or growth that are a consequence of a perceived exogenous stimulus.

Phytochelatins [(γ-Glu-Cys)$_n$-Gly] Polypeptides of variable length enzymatically synthesized from glutathione that function as heavy metal chelators.

Phytochrome interacting factor Basic helix-loop-helix transcription factors that bind to the physiologically active Pfr-form of phytochrome photoreceptors.

Phytochromes Red/far-red light photoreceptors that use a linear tetrapyrrole as chromophore; phytochromes normally exist as two stable conformers, the red-light-absorbing Pr- and the far-red light-absorbing Pfr-form.

Phytodegradation Biochemical breakdown of contaminants caused by plant enzymes and plant catabolic processes.

Phytoextraction The removal of contaminants from soil sediment or water through uptake by plants, which can be harvested.

Phytohormones Water-soluble or volatile compounds involved in short- or long-distance signaling in plants. Are either produced within the responding tissue or are transported over long distances from the site of synthesis to that of action. Examples are cytokinins,

auxins, ethylene, gibberellic acid, oxylipins, salicylic acid and methylesters of jasmonic and salicylic acid, ethane (volatiles).

Phytoplankton Autotrophic members of plankton community.

Phytoremediation Use of plants and their associated microorganisms to remove, sequester and/or detoxify metals and xenobiotics from soils, waters, sediments, and air.

Phytosiderophore Metal chelator of the mugineic acid family released by roots of grasses to mobilize iron.

Pin-formed (PIN)-proteins A family of membrane-integral auxin efflux carriers that mediate the efflux of auxin from cells to only one side thereby causing the directional distribution of auxins through tissues.

Plankton Small organisms floating or drifting in fresh or salt water.

Plant growth-promoting rhizobacteria (PGPR) Bacteria supporting plant growth such as *Pseudomonas* spp. (*Gammaproteobacteria*) and *Bacillus* spp. (*Firmicutes*) but also members of *Azospirillum* (*Alphaproteobacteria*), *Azotobacter, Enterobacter, Pseudomonas, Serratia* and *Xanthomonas* (all *Gammaproteobacteria*).

Plant hormones (phytohormones) Signaling substances generated by plants that serve as developmental cues or are part of signal processing events to mediate adaptation to a perceived exogenous stimulus.

Plant resins Hydrocarbon secretions of plants.

Plasmodesma A channel allowing symplastic transport of molecules between two plant cells.

Point mutation Exchange of a single base pair in DNA.

Point source pollution Environmental pollution which can be traced back to a single identifiable source or origin.

Pollinator-prey-conflict Spatial separation of flowers and traps in carnivorous plants to protect pollinators against trapping.

Polymorphism The occurrence of multiple forms in the same population of a species.

Polyubiquitylation Addition of three or more ubiquitin polypeptides to lysine residues of target proteins; polyubiquitylation very often targets proteins to degradation in the proteasome.

Posttranslational modifications Modifications of proteins by enzymatic reactions after translation of the mRNA to the amino acid sequence. The most frequent modifications are phosphorylation, glycolysation, and acetylation.

Precursor Metabolite used as a starting point for a biosynthetic pathway.

Presentation time Required period a stimulus has to be continuously present to elicit a recognizable physiological response in the plant.

Primary nitrate response Increased expression of NO_3^--related genes on NO_3^- perception.

Proteasome A large protein complex containing peptidases and proteases that mediates the degradation of proteins into small peptides and amino acids.

Protein amino acid sequence Encoded in the genome, translated from the nucleobase sequence into the mRNA where a sequence of three bases is encoding one amino acid during subsequent translation at the ribosomes. All proteins are built from 20 natural amino acids linked by peptide bonds; their sequence determines the three-dimensional structure and the functions of a protein.

Protein NMR Nuclear magnetic resonance spectroscopy of proteins for the determination of the three-dimensional protein structure requiring aqueous solutions of highly purified proteins.

Proteoid roots Dense bottle-brush-like lateral root clusters formed by the *Proteaceae* and by *Lupinus albus*.

Proteome The entirety of proteins in an organism, a tissue, a cell, or a body fluid.

Proteomics A systematic investigation of the proteome including the global study of identity structure, quantity, and function of proteins.

Pycnocline Density slope in lakes or oceans because of temperature (thermocline) or salinity gradient (halocline).

Quadrupole (Q) MS Mass analyzer in mass spectrometry separating ions by their transmission, traversing an electrodynamic field.

Reactive Oxygen Species (ROS) Successive transfer of electrons onto molecular oxygen, produces partially reduced intermediates that serve as oxidants and react with cell constituents such as unsaturated fatty acids, proteins, or nucleic acids.

Receiver domain proteins Proteins that function as downstream components of two-component signaling systems; protein activity is regulated by the transfer of a phosphate residue from histidine kinase sensor proteins to an aspartate residue of the receiver domain.

Redfield ratio Atomic ratio of carbon nitrogen and phosphorus in marine phytoplankton.

Redox regulation Mechanism to control the activity of proteins. Redox regulation employs posttranslational modifications by reduction and oxidation reactions, e.g., dithiol-disulfide transitions.

Reductive dehalogenation The reductive biochemical replacement of chlorine in chloroorganics (which serve as electron acceptors) by hydrogen resulting in the dechlorination of the compound and chloride release. Reductive dehalogenation is prominent in, though not restricted to, certain anaerobic bacteria. It may be coupled to energy conservation and is then referred to as "dehalorespiration"; otherwise it represents a special type of cometabolism.

Replication Duplication of DNA prior to cell division.

Resource acquisition Uptake of nutrients and water from the soil by roots and of CO_2 and light energy from the atmosphere by leaves.

Respiration Transfer of electrons from electron donors such as NADH via an electron transport chain to molecular oxygen or other final electron acceptors and concomitant generation of ATP. In eukaryotes respiration takes place in the mitochondria.

Respiration (cellular) Process by which cells produce energy by the oxidation of organic substances subdivided by the nature of terminal electron acceptor, e.g., aerobic, nitrate, iron, sulfate respiration.

Resurrection plants Can survive extreme dehydration and are able to regain normal biochemical activities within a short period after rewatering.

Retrotransposons Specific class of transposons that are able to amplify via RNA-intermediates, reintegrate, and may rapidly increase the genome size of an organism.

Rhamnolipids A class of glycolipids produced by *Pseudomonas aeruginosa* frequently cited as the best characterized of the bacterial surfactants They have a glycosyl head group, in this case a rhamnose moiety and a 3-(hydroxyalkanoyloxy)-alkanoic acid (HAA) fatty acid tail.

Rhizosphere Space around roots containing organic compounds exuded by the plant. Forms the basis for a rich microbial community.

Rhizosphere bacteria Mainly Proteobacteria (α β γ). Composition affected by plant genotype and nutrient status.

Ribbon diagram Display of the three-dimensional protein structure with structural parts (α-helices and β-sheets) of the secondary structure.

Riparian Related to banks of watercourses.

River Continuum Concept (RCC) Describes responses of biological communities and food webs to physical and chemical changes in a river from spring to mouth.

Root lesion nematodes Plant pathogenic nematodes that cause root tissue damage.

Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase) Is an enzyme regulating the carboxylation of ribulose-1,5-bisphosphate or oxygenation. The protein complex consists of a large and a small subunit.

Sanger method Standard method in genomics for DNA sequencing using the principle of chain termination providing long sequence reads up to 1 kb with high quality.

Saponins Glycosides of triterpenes and steroids; the aglycone is usually lipophilic whereas the saponins are amphiphilic with detergent properties; distinguished are monodesmosidic saponins with 1 sugar chain and bidesmosidic saponins with 2 sugar chains.

SAR (systemic acquired resistance) Starts with a local infection and can induce resistance in not yet affected distant tissues.

SCF complex A class of E3 ubiquitin ligase complexes that consists of a CULLIN1 backbone, a small RING-box protein, an adaptor protein, and an F-box protein; the F-box protein mediates specific recognition of target proteins.

Secondary ion mass spectrometry (SIMS) A primary ion beam ejects secondary ions from a surface generating usually element mass spectra and element images of surfaces (dynamic SIMS) or enabling surface atomic monolayer and surface molecular analysis (static SIMS). NanoSIMS refers to a dynamic SIMS technique using an energetic primary ion beam of O^- or Cs^+ ions with spatial resolution down to 50 nm which allows, e.g., metabolism studies in single cells.

Secondary/specialized metabolites Compounds of high structural diversity with specific functions in the interaction of the plants with their environment. Several secondary metabolites have restricted occurence in the plant kingdom. In contrast to primary metabolites, they were not considered to play essential roles in growth development or reproduction. However, many examples have shown that this classification is blurry and the terms primary/secondary may not be appropriate any more.

Secretion The release of proteins or other molecules from the cell by the fusion of cargo-vesicles with the plasma membrane; also an important step for the insertion of integral membrane proteins into the plasma membrane.

Secretory pathway The sequential steps by which proteins and other molecules are formed wrapped in intracellular vesicles and moved toward the plasma membrane for secretion or insertion.

Sector field (SF) MS Mass analyzer in mass spectrometry with magnetic and electrostatic sectors that use the different dispersion of ions in magnetic or electric fields.

Self-incompatibility Complex recognition and rejection events to avoid self-fertilization in certain plants.

Senescence A developmental program to change the physiological status of cells and tissues before programmed cell death occurs. Includes incorporation of detrimental substances and the salvage and mobilization of valuable compounds.

Sensor domain Here used as a synonym to chromophore-binding domain of photoreceptors.

Sesquiterpene lactone Terpene with 15 C atoms; its exocyclic methylene group can bind to SH-groups of proteins or glutathione.

Shade avoidance syndrome Developmental strategy of plants that enables adaptation to the canopy shade; responses are induced by high levels of far-red light below canopy shade.

Shotgun proteomics Analytical strategy in proteomics, a special type of the bottom-up approach. Enzymatic digestion of a whole protein mixture followed by different orthogonal liquid chromatography steps for peptide separation and mass spectrometry for their identification.

Siderophores Fe(III)-complexing compounds synthesized by many organisms to capture Fe(III).

Signal Biochemical event that is generated by the plant on perception of a stimulus.

Signal compound Plants use certain metabolites as signals for other plants, animals, or microbes.

SILAC Stable isotope labeling by amino acids in cell culture for protein quantification in mass spectrometry.

Skotomorphogenesis Developmental strategy of plants in darkness that should facilitate exposition toward light.

Space occupation Growth of roots and leaves aimed at optimizing resource acquisition.

Spore plants Plants that reproduce via spores; includes mosses, ferns, lycopods, and horse tails.

Stable isotope probing (SIP) Application of substrates containing stable isotope labels (^{13}C, ^{15}N) to monitor substrate incorporation into cellular components (nucleic acids, proteins) of organisms / complex microbial communities aiming at the identification of active organisms / populations / community members.

Stimulus Any exogenous factor that can be perceived by a plant and elicits a physiological response.

Stomata Pores in the leaf or stem epidermis cell layers which allow the uptake of CO_2 from the atmosphere. Two guard cells around the pore regulate the opening.

Storage Hydrophilic secondary metabolites are stored in vacuoles whereas lipophilic compounds are sequestered in resin ducts, oil cells, trichomes lacticifers, or on the cuticle.

Stratification Refers to the separation of lake water into epilimnion, metalimnion, and hypolimnion.

Streptomycete antibiotics To date approximately 17% of biologically active secondary metabolites have been characterized from streptomycetes. Main sources are soil streptomycetes and marine actinomycetes including those from the genus *Streptomyces* (*Actinobacteria*). The most important environmental signal triggering secondary metabolism is nutrient starvation, particularly lack of phosphate. Effectors according to biological activity: antagonistic agents including antibacterials, antifungals, antiprotozoans, as well as antivirals, pharmacological agents including antitumorals, immunomodulators, neurological agents, and enzyme inhibitors, agrobiologicals including insecticides, pesticides, and herbicides, and compounds with regulatory activities such as growth factors, siderophores, or morphogenic agents.

Sulfur reduction and assimilation Metabolic reduction of sulfate and its incorporation in cysteine.

Sunflecks Allow for the use of intermittent irradiance for photosynthesis by leaves.

Synchrotron A cyclic particle accelerator typically accelerating electrons producing electromagnetic radiation such as X-rays used for i.a. X-Ray Fluorescence (XRF) X-Ray Absorption Spectrometry (XAS) Extended X-Ray Absorption Fine Structure (μEXAFS), and X-ray Absorption Near Edge Structure (μXANES) analysis.

Systemic acclimation Transmission of signals from a tissue that experiences a stress impact to distant unstressed tissue. The distant tissue activates a stress tolerance program without having been exposed to the stress.

Systems biology Integrated approach to elucidate the interrelations of complex biological systems at the ecosystem community and cellular and molecular levels.

Tandem mass spectrometry Coupling of two mass analyzers in one mass spectrometer serving for structural ana lysis; it is a key technique in proteomics approaches.

Tannins Secondary metabolites with several phenolic OH-groups that can form hydrogen- and ionic bonds with proteins thereby altering their conformation; distinguished are gallotannins and catechol tannins which derive from catechin or epicatechin.

Target Any component of the human body that can be affected by a drug.

Terpenes/Terpenoids MEP or MEV pathway derived isoprenoids associated with primary metabolism, plant communication and defense responses.

Terpenoids A very large group of secondary metabolites including monoterpenes (with 10 carbons), sesquiterpenes (15 C), diterpenes (20 C), triterpenes (30 C), steroids (27 or less), tetraterpenes (40 C).

Tetrapyrrole Chemical compounds that contain four pyrrole rings held together by methin bridges.

Time-of-flight (TOF) MS Mass analyzer in mass spectrometry separating ions by their different flight times in a field-free tube.

Top-down proteomics Analytical strategy in proteomics where entire proteins are analyzed by mass spectrometry while time-consuming digestion to peptides as in the bottom-up approach is avoided. Proteins are fragmented in the gas phase of the mass spectrometer followed by determination of the exact mass of the fragments and reconstruction of the protein sequence on the basis of the identified fragments.

Toxicology The study of toxins and their effects on cells and organisms.

Toxin A harmful biogenic substance or agent causing injury in living organisms as a result of physicochemical interactions.

Transcription Process of copying the base sequence of a gene into mRNA.

Transcription factor A protein that is able to bind to specific DNA sequences (called *cis*-elements) and thereby controls the expression of genes.

Transcriptome The entirety of all RNA molecules in a biological system (e.g., a cell) at a particular time. This includes the mRNA (messenger RNA) encoding for proteins as well as rRNA (ribosomal RNA), tRNA (transfer RNA), snRNA (small nuclear RNA), and other noncoding RNA.

Transcriptomics Global study of gene expression at the RNA level; determination of the RNA sequence by sequencing the complementary DNA (cDNA). The latter is obtained from RNA by reverse transcription using the enzyme reverse transcriptase.

Translation Process of copying the base sequence of mRNA into the amino acid sequence of proteins in the ribosome.

Transporter A membrane protein that catalyzes the transport of a molecule from one side of a biomembrane to the other side.

Tropism Directional growth in reponse to stimulation. Tropisms can be positive (growth toward the direction of the stimulus) or negative (growth away from the stumulus). Examples include gravitropisms (growth dependent on gravity) and phototropism (growth dependent on light).

Two-component sensor Histidine kinases that carry protein modules regulating phosphate transfer rates on the binding of small molecules or light-absorption by attached chromophors.

Two-dimensional gel electrophoresis Analytical method most frequently used in proteomics for separation of proteins in an electric field according to two basic protein properties, their *charge* depending on the amino acid composition and their *size* depending on the number of amino acids.

Type III secretion system Injection system for proteins used by Gram-negative bacteria to infect eukaryotic cells.

Ubiquitin A small protein that is attached to target proteins by specific E3 ubiquitin ligases; ubiquitylation is a posttranslational protein modification that helps to regulate the physiological activity of modified proteins by marking them for degradation or relocalization in the cell.

Uronic acids A class of sugar acids with both carbonyl and carboxylic acid functional groups. They are sugars in which the terminal carbon's hydroxyl group has been oxidized to a carboxylic acid.

UV light Electromagnetic radiation with wavelength between 380 and 100 nm; the UV-A (380 to 315 nm) and UV-B (315–280 nm) can reach Earth's surface and cause damage to biological molecules.

UV resistance locus 8 UV-B light photoreceptor that triggers adaptation processes toward deleterious effects of the corresponding light quality.

Very low fluence response Class of very sensitive light responses triggered by phytochrome A that can be induced by weak single light pulses.

Vesicle trafficking The complex regulatory network controlling the directional movement of vesicles between organelles and the plasma membrane.

Viable but nonculturable (VBNC) A bacterial cell in the VBNC state may be defined as one which fails to grow at the routine bacteriological cultivation conditions under which it would normally grow but which is in fact alive and has still metabolic activity. This can be understood as a stress response. Under favorable conditions such a cell can return to the culturable state. This is the definition which is commonly accepted for the behavior of hygienically relevant microorganisms.

Viral shunt Conversion of bacterial biomass to DOM and cellular debris through viral lysis.

Virus Infectious complex of macromolecules that contain their genetic information either as DNA or RNA; viruses need host cells for replication and the formation of new viral particles.

Vivid Blue/UV-A light photoreceptor that triggers light-responses in fungi; uses a LOV domain as light sensor module.

VLP Virus-like particles.

Volatile oil Blend of various terpenoids and phenylpropanoids that evaporate easily (they add taste and smell to many plants).

Water potential Measure of the relative tendency of water to move from one area to another. It is represented by the Greek letter Ψ (Psi).

Water-water cycle Metabolic pathway that dissipates excitation energy in photosynthesis by transferring electrons derived from water splitting in the oxygen-evolving complex on oxygen to produce superoxide, which is subsequently converted to hydrogen peroxide and finally water. This pathway either uses ascorbate and ascorbate peroxidase in the ascorbate-dependent water-water cycle or thiol reductases and peroxiredoxins in the thiol peroxidase-dependent water-water cycle.

White collar Blue/UV-A light photoreceptor that triggers light-responses in fungi; uses a LOV domain as light sensor module.

Xerophytes Plants living in and adapted to dry areas, e.g., deserts.

X-ray crystallography Method used for the determination of the three-dimensional protein structure by analyzing a protein crystal with an X-ray beam and recording the diffraction pattern of the X-ray reflection.

Xylem Part of the plant vascular tissue where water is transported. It can form long tubes called vessels.

Zeitlupe-like proteins Family of blue/UV-A light photoreceptors that use a LOV domain as light sensor module; function as components of SCF E3 ubiquitin ligase complexes that regulate photoperiodic flowering and entrainment of the endogenous clock.

Zooplankton Heterotrophic members of plankton community.

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