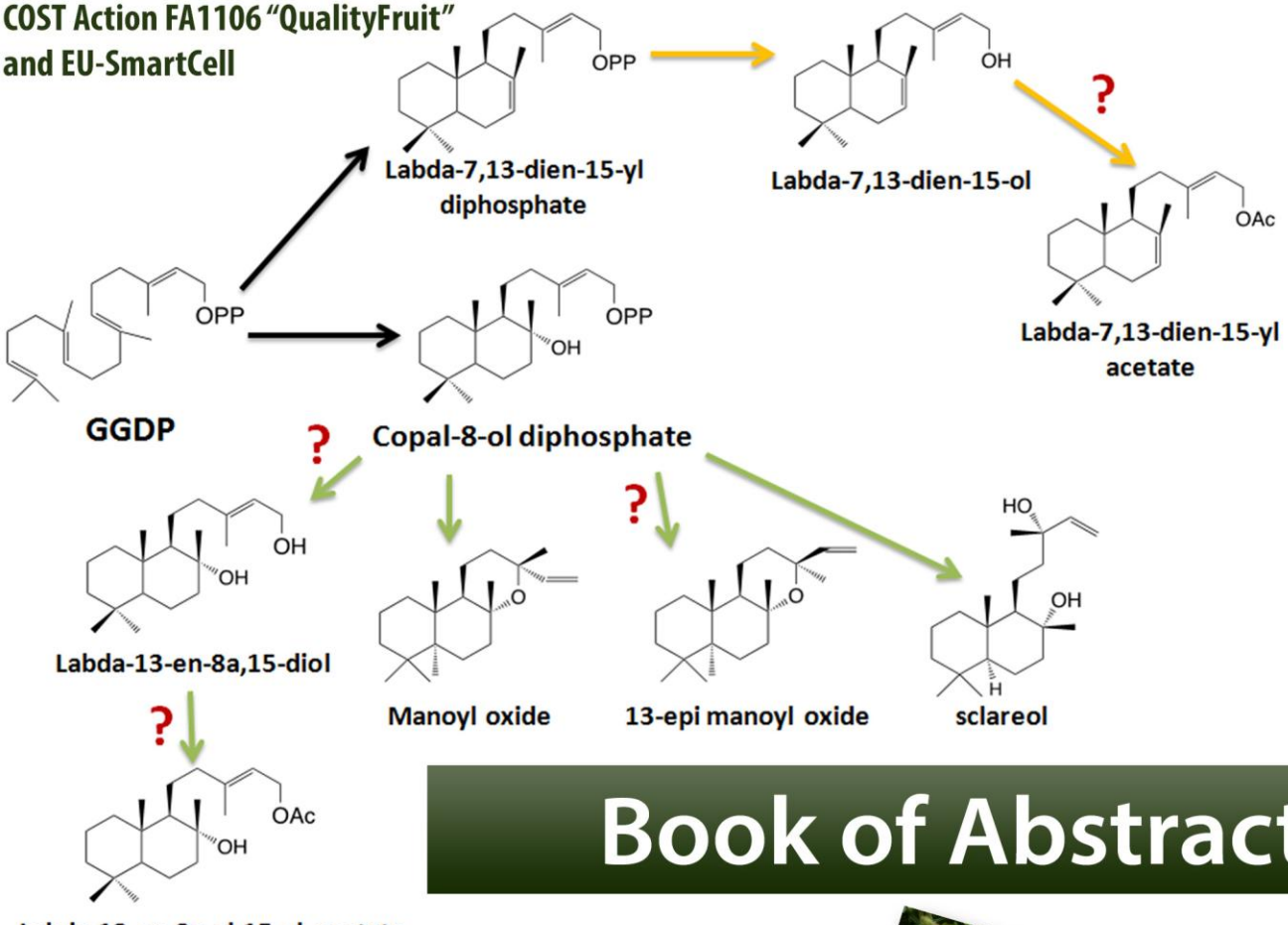
11th International Meeting on Biosynthesis, Function and Biotechnology of Isoprenoids in Terrestrial and Marine Organisms

# TERPNET2013

**Joint meeting of:**
**TERPNET 2013,**
**COST Action FA1006 "Plant Engine",**
**COST Action FA1106 "QualityFruit"**
**and EU-SmartCell**




# Book of Abstracts

# TERPNET 2013

## 11th International meeting

# Biosynthesis, Function and Biotechnology of Isoprenoids in Terrestrial and Marine Organisms

## A joint Meeting of TERPNET2013
## Cost Action "Plant Engine"
## Cost Action "QualityFruit"
## "EU-SmartCell"

**June 1-5, 2013, Kolymvari, Crete, Greece**

**Department of Pharmaceutical Science**

**Aristotle University of Thessaloniki**

**Contents**

## TERPNET 2013 - Invitation and Introduction

On behalf of TERPNET 2013, COST Action FA1006 "Plant Engine: Plant Metabolic Engineering", COST Action FA1106 "QualityFruit" and SmartCell we are pleased to welcome you in 11th TERPNET meeting taking place June 1-5, 2013 in Kolymvari, Crete, Greece.

Terpnet is an international affiliation of researchers working on all aspects of terpenes and isoprenoids, including investigations spanning from biosynthetic mechanisms to assessing the biological activities of these chemicals. This affiliation hosts bi-annual meetings that have been held in Europe, USA and Japan for the past two decades. The 10th meeting (TERPNET2011) was held in Kalmar, Sweden May 2011 and was organized by Professor Peter Brodelius, Linnaeus University.

The 2013 meeting is organized by the Local Committee headed by Prof. Angelos Kanellis in close collaboration with the Executive Committee. Exceptionally, this event will be co-organized with COST Action FA1006 "Plant Engine", COST Action FA1106 "QualityFruit" and EU project SmartCell. Cost Action FA1006 "Plant Engine" supports and enhances a Pan-European network, which combines resources, identifies target pathways and defines compounds, publicizes novel technologies and applications, sets standards for computational support, and develops synthetic approaches in plant metabolic engineering. The aim of COST action FA1106 "QualityFruit" is to foster European collaborations on fleshy fruits, such as tomato and grapevine. The main objective is to uncover new leads for improving fruit nutritional and sensory qualities by implementing integrated multidisciplinary approaches and advanced genomics and post-genomics technologies. Lastly, SmartCell is an EU Seventh Framework Program large collaborative project investigating the rational design of plant systems for the sustainable generation of value-added industrial products. In addition, prior to the main conference, the Final Meetings of two EU projects dealing with the biotechnology of plant secondary metabolites will take place, namely SmartCell and TERPMED.

The scientific program of TERPNET 2013 covers all aspects of terpene/isoprenoid research including biosynthesis and structural biology, transport and regulation, genetics, metabolic engineering and biotechnology, synthetic biology applications as well as applications in agriculture and medicine. Plants, microorganisms, parasites and marine organisms are represented.

The meeting takes place in small traditional village, Kolymvari, near Chania, Crete. The venue, the Orthodox Academy of Crete (OAC) is a modern Conference Center, situated in an

exceptionally beautiful location only a few meters from the sea, and next to the Gonia Monastery (17th Century).

We would like to thank Cost Actions FA1006 and FA1106 for sponsoring and co-organizing TERPPET2013.

We are particularly indebted to the members of the TERPNET2013 Executive Committee and the local organizing committees as well as to the staff of the OAC Conference site for their efforts for the success of this Symposium. Appreciation is also extended to a number of private firms, which contributed to the success of this important event. Lastly, we acknowledge the help of the Group of Biotechnology of Pharmaceutical Plants of the Dept. of Pharmaceutical Science, Aristotle University.

We wish you a pleasant stay in Kolymvari and an enjoyable TERPNET2013 Meeting.

Angelos K. Kanellis, Heribert Warzecha, Mondher Bouzayen and Kirsi-Marja Oksman-Caldentey.

## Terpnet Organization

Angelos K. Kanellis (Aristotle University of Thessaloniki, Greece - Chairman)
Heribert Warzecha (Technische Universitaet Darmstadt, Germany)
Mondher Bouzayen (UMR990 INRA/INP-ENSAT, France)
Kirsi-Marja Oksman-Caldentey (VTT Technical Research Centre, Finland)

**Local Organizing Committee**

Vassilios Roussis (Kapodistrinian and National University of Athens)
Kaliope Papadopoulou (University of Thessaly)
Frangiskos Kolisis (National Technical University of Athens)
Sotirios Kampranis (University of Crete)
Antonios Makris (Institute of Agrobiotechnology/CERTH)

**Executive Committee of TERPNET**

Thomas J. Bach (University of Strasbourg, France)
Jörg Bohlmann (University of British Columbia, Canada)
Albert Boronat (University of Barcelona, Spain)
Harro Bouwmeester (Wageningen University and Research Center, The Netherlands)
Peter Brodelius (Linnaeus University, Sweden)
Joe Chappell (University of Kentucky, USA)
Jonathan Gershenzon (Max Planck Institute for Chemical Ecology, Germany)
Werner Knöss (University of Bonn, Germany)
Toshiya Muranaka (Yokohama City University, Japan)
Joe Noel (Salk Institute for Biological Studies, USA)
Anne Osbourn (John Innes Centre, UK)
Reuben J. Peters (Iowa State University, USA)
Eran Pichersky (University of Michigan, USA)

## Sponsors

## Program Outline of TERPNET 2013

| Time | Fri 31 May | Sat 1 June | Sun 2 June | Mon 3 June | Tue 4 June | Wed 5 June | Thu 6 June |
|---|---|---|---|---|---|---|---|
| 8-9 | Pre-Symposium Tour Samara Gorge | Smart Cell Meeting | Official Opening | **Session V Terpenoids Roles in Fleshy Fruit Biology (sponsored by COST Action FA1106 QUALITYFRUIT)** Mondher Bouzayen Jossi Hirschberg Jules Beekwilder | **Session VIII Terpenoids and beyond sponsored by COST ACTION Plant Engine** Heribert Warzecha Paul Fraser | **Session IX Natural Roles for Terpenoids** Introduction Jonathan Gershenzon Dorothea Tholl John Pickett | Post-Symposium Tour Samaria Gorge |
| 9-10 | TERPMED FINAL PROJECT Meeting | | **Session II Terpenoids and Pharmacognocy in 21st Century** Werner Knöss Hermann Stuppner Reuben Peters | Asaph Aharoni Harry Klee | Matthias Nees Rob Verpoorte Ludger Wessjohann | Kalliopi Papdopoulou Selected Talk | |
| 10-11 | | | **Session III Exploring genomes, transcriptomes, proteomes and metabolome to understand the terpenome** Philipp Zerbe Eric Schmelz | Gianfranco Diretto Stella Grando | Coffee Break Efraim Lewinsohn | Selected Talk Selected Talk Selected Talk Coffee Break | |
| 11-12 | | | Coffee Break Joerg Bohlmann Kazuki Saito | Coffee Break **Session VI Marine terpenoids: chemical diversity, function and biotechnology** Ernesto Mollo | Elio Fantini Johann Anders Rettberg Benoit Boachon Dong Lemeng | **Session X Plant and microbial production systems for terpenoids** Alain Goossens Paul Christou | |
| 12-13 | | | Selected Talk Selected Talk Selected Talk | Vassilios Roussis Shigeru Okada Selected Talk | Lunch | Antonios Makris Anneli Ritala | |
| 13-14 | | | Lunch | Lunch | Excursion | Lunch | |
| 14-15 | | | **Session IV Terpenoids biosynthesis and regulation** Albert Ferrer Sarh O' Connor | **Session VII Understanding of structure and function of terpene synthases** Francis Mann Tariq Akhtar | | Toshiya Muranaka Selected Talk Selected Talk | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15-16 | | | Qing Liu<br>Drik Pruefer | Selected Talk<br>Selected Talk<br>Coffee Break | | Selected Talk<br>Selected Talk<br>Selected Talk<br>Coffee Break | |
| 16-17 | | Registration | Natalia Dudareva<br>Coffee Break<br>Harro Bouwmeester | Feng Chen<br>Sotirios Kampranis<br>Brigit Piechulla | | **Session XI New and Old Approaches**<br>Christina White<br>Joe Noel<br>Martin Burke<br>Selected Talk<br>Selected Talk | |
| 17-18 | | | Selected Talk<br>Selected Talk<br>Selected Talk<br>Selected Talk | Selected Talk<br>Selected Talk<br>Selected Talk | | **Session XII Terpenoids and Industrial Applications**<br>Michel Schalk<br>Richard Burlingame<br>Selected Talk<br>Selected Talk | |
| 18-19 | | | Selected Talk<br>Selected Talk<br>Selected Talk<br>Selected Talk | Selected Talk<br>Special Lecture I<br>Peter Brodersen | | General Discussion and Conclusion | |
| 19-20 | | **Session I Introduction to the Symposium**<br>Eran Pichersky<br>Thomas Calikowski | Selected Talk<br>Poster Session I | Poster Session II | | Poster Session | |
| 20-21 | | Wine Tasting and Welcome Dinner | Dinner | Dinner | Gala Dinner | Farewell Dinner | |
| 21-22 | | | | | | | |
| 22-23 | | | | | | | |
| 23-24 | | | | | | | |

**Connor**

## Program for TERPNET 2013

**A joint Meeting of TERPNET2013, Cost Action "Plant Engine", Cost Action QualityFruit" and "EU-SmartCell"**

**Biosynthesis, Function and Biotechnology of Isoprenoids in Terrestrial and Marine Organisms**

**Saturday June 1, 2013**

16:00-19:00 Registration

***Session I***

***19:00-20:10 Introduction to the Symposium***

**Discussion Leader: Joe Chappell**

19:00-19:05 Introduction by Chair

19:05-19:50 **Eran Pichersky** (invited talk)**:**

(University of Michigan, Ann Arbor, USA)

"Surely some revelation is at hand": Terpene research in the third century AD (Anno Darwini)

19:50-20:10 **Thomas Calikowski** (invited talk)

(European Commission, DG Research & Innovation E2, Project Officer "Biotechnologies")

Plant Biotechnology: a key building block of the European Bio-economy

20:10-23:00 **Wine Tasting and Welcome Reception**

**Sunday June 2, 2013 (1st Day)**

08:50-9:00 Official Opening of TERPNET 2013

***Session II***

**Terpenoids and Pharmacognocy in 21st Century**

**Discussion Leaders: Werner Knöss and Ioanna Chinou**

09:00-09:05 Introduction by Chair

09:05-09:30 **Werner Knöss** (invited talk)

(Federal Institute for Drugs and Medical Devices, Bonn, Germany)

Futuristic insights in terpenoids, medicinal plants and health - impact of biotechnology and "omic"-technologies

09:30-09:55 **Hermann Stuppner** (invited talk)

(University of Innsbruck, Innsbruck, Austria)

Plant terpenoids as source for drug leads – Challenges and research strategies

09:55-10:10 **Reuben Peters** (invited talk)

(Iowa State University, Ames, USA)

Terpene synthase logic from an enzymatic point of view

***Session III***

**"Exploring genomes, transcriptomes, proteomes and metabolome to understand the terpenome"**

**Discussion Leaders: Joerg Bohlmann and Anne Osbourn**

10:10-10:15 Introduction by Chair

10:15-10:40 **Philipp Zerbe** (invited talk)

(University of British Columbia, Vancouver, Canada)

Gene discovery of modular diterpene metabolism in non-model plant systems

10:40-11:05 **Eric Schmelz** (invited talk)

(USDA, Florida, USA)

Maize diterpenoid phytoalexins; insights into kauralexin structure, function, and biosynthesis

**11:05-11:25 Coffee Break**

11:25-11:50 **Joerg Bohlmann** (invited talk)

(University of British Columbia, Vancouver, Canada)

A modular pathway of diterpene synthases and cytochrome P450s for diterpene resin acid biosynthesis in conifers

11:50-12:15 **Kazuki Saito** (invited talk)

(Chiba University, Chiba, Japan)

Phytochemical genomics - Beyond Arabidopsis to medicinal plants

12:15-12:30 **Mark R. Wildung** (selected Talk)

(Washington State University, Pullman WA, USA)

A second generation of massively parallel DNA sequencers: the signal, the noise, and a new gold standard

12:30-12:45 **Xiaoquan Qi** (selected Talk)

(Chinese Academy of Sciences, Beijing, China)

Genome-wide investigation of rice oxidosqualene cyclases reveals divergent evolution of plant triterpenoid pathways

12:45-13:00 **Søren Bak** (selected Talk)

(University of Copenhagen, Copenhagen, Denmark)

From ecometabolomics to synthetic biology – exploring plasticity of the triterpenoid biosynthetic pathway

**13:00-14:15 Lunch**

***Session IV***

**Terpenoids biosynthesis and regulation**

**Discussion Leaders: Thomas Bach and Harro Bouwmeester**

14:10: 14:15 Introduction by Chair

14:15-14:40 **Albert Ferrer** (invited talk)

(University of Barcelona, Barcelona, Spain)

Biosynthesis of terpenoid precursors in Arabidopsis: the role of farnesyl diphosphate synthase

14:40-15:05 **Sarah O'Connor** (invited talk)

(The John Inness Centre, Norwich, UK)

Understanding 'irregular' terpenes: Iridoid biosynthesis

15:05-15:30 **Qing Liu** (invited talk)

(Wageningen University, Wageningen, the Netherlands)

Elucidation of sesquiterpene lactone biosynthesis in feverfew (*Tanacetum parthenium*)

15:30-15:55 **Dirk Pruefer** (invited talk)

(University of Muenster, Muenster, Germany)

The dandelion dilemma: With or without rubber?

15:55-16:20 **Natalia Dudareva** (invited talk)

(Purdue University, West Lafayette, USA)

Do we need to rethink the "canonical" MVA pathway?

**16:20-16:45 Coffee Break**

16:45-17:10 **Harro Bouwmeester** (invited talk)

(Wageningen University, Wageningen, the Netherlands)

Strigolactones; terpenoid signalling molecules with surprising in- and outdoor activities

17:10-17:25 **Ikuro ABE** (selected talk)

(The University of Tokyo, Tokyo, Japan)

Biosynthesis of Fungal Meroterpenoids

17:25-17:40 **Kexuan Tang** (selected Talk)

(Shanghai Jiao Tong University, Shanghai, P.R. China)

The functional studies of AP2/ERF transcription factors and the key genes of jasmonate biosynthetic pathway in *Artemisia annua* L.

17:40-17:55 **Narcisso Campos** (selected Talk)

(Centre de Recerca en Agrigenòmica, Barcelona, Spain)

Plant 3-hydroxy-3-methylglutaryl coenzyme A reductase triggers the biogenesis of endoplasmic reticulum subdomains targeted for direct delivery to the vacuole in response to stress

17:55-18:10 **Peter E. Brodelius** (selected Talk)

(Linnaeus University, Kalmar, Sweden)

Is artemisinic aldehyde □11(13) reductase a rate limiting enzyme of artemisinin biosynthesis in *Artemisia annua* L.?

18:10-18:25 **Sindy Frick** (selected Talk)

(Max Planck Institute of Chemical Ecology, Jena, Germany)

Metal ions control chain-length specificity of isoprenyl diphosphate synthases in insects

18:25-18:40 **Kathleen Brückner** (selected Talk)

(Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany)

Investigating phenolic diterpenes in rosemary (*Rosmarinus officinalis*) and sage (*Salvia fruticosa*)

18:40-18:55 **Irini Pateraki** (selected Talk)

(University of Copenhagen, Copenhagen, Denmark)

Manoyl oxide as a precursor for forskolin biosynthesis: identification and characterization of the involved biosynthetic enzymes from *Coleus forskohlii*

18:55-19:10 **Kiyoshi Ohyama** (selected Talk)

(Tokyo Institute of Technology, Tokyo, Japan)

Sterol side-chain reductase: essential for cholesterol biosynthesis in *Solanum* plants

19:10-19:25 **Myriam Seemann** (selected Talk)

(Université de Strasbourg, Strasbourg Cedex, France)

Mechanistical investigation and inhibition of IspH/LytB, a [4Fe-4S]2+ enzyme involved in the biosynthesis of isoprenoids via the MEP pathway

**19:25-20:30 Poster Session I – Posters P1 to P60.**

**20:30 Dinner**

**Monday 3, 2013 (2nd Day)**

***Session V***

**Terpenoids Roles in Fleshy Fruit Biology (organized by COST Action FA1106 QUALITYFRUIT)**

**Discussion Leaders: Mondher Bouzayen and Asaph Aharoni**

08:00-08:25 **Mondher Bouzayen** (invited talk)

(ENSAT, Auzeville Tolosane, France)

Multi-hormonal control of the developmental transitions leading to fruit ripening.

08:25-08:50 **Jossi Hirschberg** (invited talk)

(The Hebrew University of Jerusalem, Jerusalem, Israel)

Regulation of carotenoid biosynthesis: Interplay between carotenoids and phytohormones

08:50-09:15 **Jules Beekwilder** (invited talk)

(Plant Research International, Wageningen, The Netherlands)

Carotenoid derived flavour formation in fruit and other systems

09:15-09:40 **Asaph Aharoni** (invited talk)

(The Weizmann Institute of Science, Israel)

Transcriptome and Metabolome Analysis for Gene Discovery in the Steroidal Alkaloid Pathway

09:40-10:05 **Harry Klee** (invited talk)

(University of Florida, FL, USA)

Apocarotenoid volatiles in tomato fruits: amplifiers of sweet perception in humans

10:05-10:30 **Gianfranco Diretto** (invited talk)

(ENEA, Rome, Italy)

A beta-carotene/ABA regulatory loop controls tomato fruit ripening

10:30-10:55 **Stella Grando** (invited talk)

(Fondazione Edmund Mach-Istituto Agrario San Michele, San Michele, Italy)

Identification of causal mutations of metabolic QTLs associated to grape and wine flavor

**10:55-11:20 Coffee Break**

***Session VI***

**Marine terpenoids: chemical diversity, function and biotechnology**

**Discussion Leaders: Vassilios Roussis and Shigeru Okada**

11:20-11:25 Introduction by Chair

11:25-11:50 **Ernesto Mollo** (invited talk)

(Istituto di Chimica Biomolecolare/CNR, Naples, Italy)

Marine terpenoids and the conquest of land

11:50-12:15 **Vassilios Roussis** (invited talk)

(National and Kapodistrian University of Athens, Athens, Greece)

Chemodiversity and bioactivity of terpenoids from East Mediterranean algae

12:15-12:40 **Shigeru Okada** (invited talk)

(University of Tokyo, Tokyo, Japan)

The methylerythritol 4-phosphate pathway supporting biofuel production by a green microalga *Botryococcus braunii*

12:40-12:55 **Hem Thapa** (selected Talk)

(Texas A&M University, Texas, USA)

Biochemical characterization of the biosynthesis of the tetraterpenoid hydrocarbon lycopadiene from *Botryococcus braunii*

**13:00-14:15 Lunch**

***Session VII***

**Understanding of structure and function of terpene synthases**

**Discussion Leaders: Joe Chappell and Eran Pichersky**

14:15-14:20 Introduction by Chair

14:20-14:45 **Francis Mann** (invited talk)

(Winona State University, Winona, USA)

Evolution of isoprenyl diphosphate synthases: gatekeepers to greater terpenoid metabolism

14:45-15:10 **Tariq Akhtar** (invited talk)

(University of Michigan, Ann Arbor, USA)

Plant cis-prenyltransferases

15:10-15:25 **Charisse Crenshaw** (selected Talk)

(The Salk Institute/Howard Hughes Medical Institute,

CA , USA)

Decoding Terpene synthase design features using large biochemical datasets

15:25-15:40 **Tohru Dairi** (selected Talk)

(Hokkaido University, Hokkaido, Japan

Functional analysis of prenyltransferses responsible for diterpene biosynthesis in fungi

**15:40-16:00 Coffee Break**

16:00-16:25 **Feng Chen** (invited talk)

(The University of Tennessee, Knoxville, USA)

Identification and functional characterization of terpene synthase genes of microbial type from a non-seed plant *Selaginella moellendorffii*

16:25-16:50 **Sotirios Kampranis** (invited talk)

(University of Crete, Heraklion, Greece)

Reconstructing terpene biosynthesis: Protein engineering to redirect precursor fluxes

16:50-17:15 **Birgit Piechulla** (invited talk)

(University of Rostock, Rostock, Germany)

'Cineole cassette' monoterpene synthases in the genus of Nicotiana

17:15-17:30 **Andréa Hemmerlin** (selected Talk)

(NRS/Universite de Strasbourg, Strasbourg, France)

Substrate promiscuity in vivo of plant protein prenyltransferases is a result of substrate availability through the MVA or the MEP pathway

17:30-17:45 **Dan T. Major** (selected Talk)

(Ilan University, Ramat, Israel)

Electrostatically guided dynamics – the root of fidelity in a promiscuous terpene synthase?

17:45-18:00 **Ting Yang** (selected Talk)

(Wageningen University, Wageningen, The Netherlands)

Chrysanthemyl diphosphate synthase operates in planta as a bifunctional enzyme with chrysanthemol synthase activity

18:00-18:15 **Thomas D. Sharkey** (selected Talk)

(Michigan State University, East Lansing, USA)

Isoprene synthase genes form a monophyletic clade of acyclic terpene synthases in the Tps-b terpene synthase family

***Special Lecture I***

**Discussion Leader Thomas Bach**

18:20-18:25 Introduction by Chair

18:25-18:50 **Peter Brodersen** (invited talk)

(University of Copenhagen, Copenhagen, Denmark)

Unexpected links between protein farnesylation and microRNA function

**19:00-20:00 Poster session II – Posters P61 to P117**

**20:00 Dinner**

**Tuesday 4, 2013 (3rd Day)**

***Session VIII***

**"Terpenoids and beyond" (organized by COST ACTION Plant Engine)**

**Discussion Leaders: Heribert Warzecha and Kirsi-Marja Oksman-Caldentey**

08:00-08:20 **Heribert Warzecha** (invited talk)

(Technische Universität Darmstadt, Darmstadt, Germany)

Introduction to COST Action FA 1006 – PlantEngine

08:20-08:50 **Paul Fraser** (invited talk)

(Royal Holloway University of London, London, England)

Cellular and metabolomic perturbations associated with engineering isoprenoids in Solanaceae

08:50–09:20 **Matthias Nees** (invited talk)

(VTT Technical Research Centre of Finland, Finland)

Cell-based functional screening of modified betulins, abietanes and resin acids as lead compounds for anti-cancer drugs (BARC)

09:20–09:50 **Rob Verpoorte** (invited talk)

( Leiden University, The Netherlands)

Why is your plant extract like a syrup? Natural Deep Eutectic Solvents?

09:50–10:20 **Ludger Wessjohann** (invited talk)

(Leibniz Institute of Plant Biochemistry, Halle, Germany)

Beyond Terpenoids: Mechanistic and biocatalytic Insight into the formation of non-natural and mero-terpenoids

**10:10-10:45 Coffee break**

10:45–11:00 **Efraim Lewinsohn** (invited talk)

(Agricultural Research Organization (ARO), Ramat Yishay, ISRAEL)

Monoterpene biosynthesis in aphid-induced-galls and leaves of *Pistacia palaestina* Boiss

11:00–11:15 **Elio Fantini** (invited talk)

(ENEA, Rome, Italy)

Dissection of tomato lycopene biosynthesis through virus induced gene silencing

11:15–11:30 **Johann Andersen Ranberg** (invited talk)

(University of Copenhagen, Copenhagen, Denmark)

Expanding the molecular diversity through synthetic biology: Using combinatorial biochemistry for reconstruction of pathways to high-value and novel diterpenes.

11:30–11:45 **Benoit Boachon** (invited talk)

(IBMP, Strasbourg, France)

P450-dependent linalool metabolism in *Arabidopsis* flowers involved in defense against flower visiting insects

11:45–12:00 **Dong Lemeng** (invited talk)

(Wageningen University, Wageningen, The Netherlands)

Transient expression and metabolomics as tools in monoterpene indole alkaloid metabolic engineering

**12:00-13:00 Lunch**

**Excursion**

**13:30-19:30 Visit to Cretan Wineries and Virgin Olive Oil local factories**

**20:00-24:00 Gala dinner**

**Wednesday 5, 2013 (4th Day)**

***Session IX***

**Natural Roles for Terpenoids**

**Discussion Leaders: Jonathan Gershenzon and Peter Brodelius**

08:25-08:30 Introduction by Chair

08:30-08:55 **Jonathan Gershenzon** (invited talk)

(Max Planck Institute for Chemical Ecology, Jena, Germany)

Roles of sesquiterpene volatiles in plant defense and defense signaling

08:55-09:20 **John Pickett** (invited talk)

(Rothamsted Research, Harpenden, UK)

Expression of the sesquiterpene (E)-beta-farnesene, an aphid alarm pheromone, for pest control in wheat and use of other isoprenoids

09:20-09:45 **Kalliopi Papadopoulou** (invited talk)

(University of Thessaly, Larissa, Greece)

Triterpenes in legumes: A role in both the plant and their nitrogen-fixing partners

9:45-10:00 **Paal Krokene** (selected Talk)

(Norwegian Forest and Landscape Institute, Ås, Norway)

The effect of methyl jasmonate on terpene defenses in Norway spruce: defense inducer or priming agent?

10:00-10:15 **Alexandre Huchelmann** (selected Talk)

(Université de Strasbourg, Strasbourg, France)

A reevaluation of the negative effects of S-carvone on the isoprenoid biosynthesis pathway

10:15-10:30 **Raimund Nagel** (selected Talk)

(Max Planck Institute for Chemical Ecology, Jena, Germany)

Diversion products of terpenoid-based oleoresin biosynthesis function in defense

10:30-10:45 **Audrey Odom** (selected Talk)

(Washington University, Saint Louis, USA)

Scent of a parasite: identity and function of volatile organic compounds produced by malaria parasites

**10:40-11:05 Coffee Break**

***Session X***

**Plant and microbial production systems for terpenoids**

**Discussion Leaders: Paul Christou and Toshiya Muranaka**

11:10-11:15 Introduction by Chair

11:15-11:40 **Alain Goossens** (invited talk)

(Ghent University, Ghent, Belgium)

Production of plant-derived bioactive molecules, the road to synthetic biology is paved with a pleasant fragrance

11:40-12:05 **Paul Christou** (invited talk)

(University of Lleida, Lleida, Spain)

Engineering of different terpenoid biosynthetic pathways in tobacco and corn through multigene transfer

12:05-12:30 **Antonios Makris** (invited talk)

(Centre for Research and Technology Hellas, Thessaloniki, Greece)

Genetic perturbations in *Saccharomyces cerevisiae* to enhance sesquiterpenoid and diterpenoid production

12:30-12:55 **Anneli Ritala** (invited talk)

(VTT Technical Research Centre of Finland, Finland)

Hairy roots as production hosts for terpenes

**12:55-14:15 Lunch**

14:15-14:40 **Toshiya Muranaka** (invited talk)

(Osaka University, Osaka, Japan)

Production of licorice triterpenoids in both transgenic plant and yeast system

14:40-14:55 **Ajikumar Parayil** (selected Talk)

(Manus Biosynthesis, Cambridge, MA, USA)

Multivariate-modular metabolic engineering for terpenoid biosynthesis in *E. coli*

14:55-15:10 **Naoyuki Umemoto** (selected Talk)

(Central Laboratories for Key Technologies/Kirin Co., Ltd, Yokohma, Japan)

Transgenic potatoes remarkably decreasing content of glycoalkaloids

15:10-15:25 **Chao Bai** (selected Talk)

(Universitat de Lleida. Lleida, Spain)

Carotenoid biofortification of rice endosperm through combinatorial transformation for multi-gene and multi-pathway engineering

15:25-15:40 **Masao Ishimoto** (selected Talk)

(National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan)

Genetic and molecular analysis of structural diversity of saponins in soybean

15:40-15:55 **Patrícia Duarte** (selected Talk)

(Instituto de Biologia Molecular e Celular, Porto, Portugal)

A highly efficient leaf protoplast expression system for the study of anticancer alkaloid metabolism in *Catharanthus roseus*

**15:55-16:25 Coffee Break**

***Session XI***

**New and Old Approaches**

**Discussion Leaders: Joe Noel and Marc Boutry**

16:25-16:30 Introduction by Chair

16:30-16:55 **Christina White** (invited talk)

(University of Illinois, Urbana, USA)

C-H Oxidations and Synthesis

16:55-17:20 **Martin Burke** (invited talk)

(University of Illinois, Urbana, USA)

Making Molecular Prosthetics

17:20-17:45 **Joe Noel** (invited talk)

(Salk Institute for Biological Studies, San Diego, USA

Parallel and convergent metabolic evolution in terrestrial plants

17:45-18:00 **Jörg Degenhardt** (selected Talk)

(Martin Luther University Halle, Halle, Germany)

Identification of herbivore-induced terpene biosynthesis pathways and their enzymes by genetic mapping in maize (*Zea mays*)

18:00-18:15 **Marc Boutry** (selected Talk)

(University of Louvain, Belgium )

Ectopic expression of secondary metabolite transporters in suspension cells: identification of cyclic diterpenes as substrates of the *Nicotiana tabacum* NtPDR1 transporter involved in biotic stress response

***Session XII***

**Terpenoids and Industrial Applications**

**Discussion Leaders: Alain Tissier and Albert Boronat**

18:20-18:25 Introduction by Chair

18:25-18:50 **Michel Schalk** (invited talk)

(FIRMENICH SA, Geneva, Switzerland)

Toward biosynthetic routes for the production of terpenes for the favors and fragrance industry

18:50-19:15 **Richard Burlingame** (invited talk)

(Allylix, San Diego, USA)

Biosynthetic production of terpene specialty chemicals in yeast

19:15-19:30 **Jens Schrader** (selected Talk)

(DECHEMA-Forschungsinstitut, Frankfurt am Main, Germany)

*Pseudomonas putida*: from perillic acid production to a microbial platform for terpene oxyfunctionalization

19:30-19:45 **Lishan Zhao** (selected Talk)

(Amyris Inc. Emeryville, CA. USA)

Development of a terpene synthase engineering platform for the production of isoprenoids in yeast

**19:50-20:30 GENERAL DISCSUSSION AND CONCLUSION: Anne Osbourn**

**21:00 Farewell Dinner**

**Abstracts and Oral presentations**

**Contents**

## Session I - Introduction to symposium

### O1. "Surely some revelation is at hand": Terpene research in the third century AD (Anno Darwini)

Eran Pichersky

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor MI 48109, USA

By 1900 (less than 100 years after the birth of Charles Darwin in 1809),the chemistry of terpenes was a well-established field of study. The study of the biochemistry and ecology of terpenes did not start in earnest until the second half of the 20$^{th}$ century, brought about by the development of new analytical tools and the renewed interest in chemical ecology. Finally, the emergence of molecular biology in the 1980's has led to a huge accumulation of sequences of genes and proteins - mostly from plants but also from other species - involved in terpene biosynthesis, and these sequences have been used for designing both biochemical and bioinformatic experiments to probe enzyme structure and function. In addition, analysis of these sequences has identified large families of genes/proteins that can be, and have been, studied as illustrative and illuminating examples of molecular evolution.

## O2. Plant Biotechnology: a key building block of the European Bio-economy

Tomasz Calikowski

Scientific Officer, Unit "Biotechnologies", Directorate General for Research and Innovation, Directorate "Food, Agriculture and Biotechnologies", European Commission

In February 2012 the European Commission published the **Bio-economy strategy for Europe**. Its main objectives are moving to a low carbon economy and building competitive bio-based industries. Innovation for sustainable growth is reflected already now in the current EU Framework Programme 7 (FP7, 2007-2013), which is the main tool of research funding in the European Union. FP7 includes Theme 2 "Food, Agriculture and Fisheries, and Biotechnologies", with the budget of 1,935 billion euro. The specific priorities for this Theme addresses 'Grand challenges' such as primary production mitigation and adaptation to climate change, sustainable, eco-efficient and competitive bio-economy. The Theme aims to achieve production of food, feed and industrial goods such as fuels, chemicals, lubricants, pharmaceuticals in a more sophisticated and environmentally friendly manner by advances in life sciences and biotechnology. The process of building the European Bio-economy is assisted by European Technology Platforms (e.g. ETP "Plants for the Future").
The FP7 activity with the specific focus on Plant (green) Biotechnology is **Activity 2.3** "Life Sciences and biotechnology for sustainable non-food products and processes" ('biotechnologies'), and to some extent also **Activity 2.1** "Sustainable production and management of biological resources from land, forest, and aquatic environments" (focused mostly on agricultural crops, enabling-research related to agriculture, plant health and crop protection). Around 50 projects in total (ca. EUR 200M funding) and 26 projects in Activity's 2.3 portfolio (ca. EUR 112M) are related to this field. There is a significant industrial participation in many projects, both as large industry and as SMEs, as well as a strong international cooperation with partners such as the USA, Canada, India, Russia, China or Latin America.

Some **examples** of projects funded from the 2007-2012 calls include:
Energy crops: Sweet sorghum. Jatropha. Poplar. Improving plant cell walls, Plant-produced Vaccines, High-value products/Secondary Metabolites, Plant terpenoids, Carotenoids, Industrial products: Improved oils, Rubber and latex crops, forest-based composites, Biomass availability for industrial applications, Biomass from perennial grasses, India Partnership Initiative biomass/biowaste. Plant photosynthetic efficiency improvement: from a C3 to C4 system, Multipurpose crops, Fiber crops (with China), improved water stress tolerance of plants.

The last **2013 call** of FP7 has recently closed (projects under negotiation). The key feature of call 2013 was the increased support offered to SMEs, emphasis on demonstration activities and on increased impact by Bio-economy (both through scientific excellence and commitment to exploitation of results), together with full use of the discovery-innovation chain. The 'biotechnologies' call included topics on Plant High Value Products (20M euro), and on sustainable biodiversity in agriculture (EU-Latin America Partnering Initiative). The 2013 call was intended to support a transition to the next Framework Programme, i.e. **Common Strategic Framework Horizon 2020** (2014-2020).

The Commission proposal for **Horizon 2020** foresees a significant increase of funding (FP7: ca. 53 billion euro, Horizon 2020 approx. 80 billion euro), including to areas covered currently by FP7 Theme 2. Two elements of Horizon 2020 will cover biotechnology research and innovation, including plant biotechnology: **Societal Challenge** "Food security, sustainable agriculture, marine and maritime research & the bioeconomy" (with proposed 4.4 Billion euro, i.e. more than 50% increase of funding), as well as under **Leadership in enabling and industrial technologies** (ICT, nanotechnologies, materials, **biotechnology**, manufacturing, space), with overall proposed funding 4.5 Billion euro. The Commission proposal is currently under political and budgetary debate in the European Parliament and the Council.

## Session II - Terpenoids and Pharmacognocy in 21st Century

### O3. Futuristic insights in terpenoids, medical plants and health – impact of biotechnology and "omic" –technologies

Werner Knöss

Federal Institute for Drugs and Medical Devices, Bonn, Germany and
Institute of Pharmaceutical Biology, University of Bonn, Germany

Worldwide medicinal plants have been used in health care since ancient times. Even today primary health care in many developing countries of the world is still based on using complex mixtures of plant origin for therapeutic purposes. The knowledge about medicinal plants led to the development of the scientific discipline phytochemistry – initially aiming at the isolation and identification of major active constituents and characterisation of the complex mixtures of constituents. Terpenoids are an important class of natural products which considerably contribute to therapeutic effects of medicinal plants. Although there is a lot of traditional knowledge on therapeutic effects of terpenoid containing medicinal plants, sound pharmakodynamic models for the mode of action are frequently missing.

Phytochemistry always integrated new methodologies and this led to e. g. in elucidation of biosynthetic pathways of isoprenoids, modification of biosynthetic pathways and approaches to design new constituents by generating chimaeric enzymes. At the end of the last millennium biotechnological production of specific compounds seemed to be a promising strategy, although there were limitations especially with respect to production of terpenoids. Meanwhile, application of "omic"-technologies is a driving force in the process of generating new knowledge in plant research. New insights on functioning of plants and production of primary and secondary metabolites will be generated. Accordingly, further progress will be made towards producing specific patterns of terpenoids in medicinal plants and cell/tissue cultures; new terpenoids may be designed for therapeutic purposes.

With respect to the interface of phytochemistry and health a new dimension should be opened in the 21st century. There is a need to consequently apply new methodologies to research on terpenoids (and other classes of natural products) with respect to their interactions with the human body. "omic"-technologies are offering the challenge and the opportunity to monitor how complex mixtures of constituents are absorbed, distributed and metabolised, how they are effecting the metabolism, target structures and the genome. New hypotheses may be developed about multiple effects on different targets, synergisms, antagonisms – which could finally explain traditional therapeutic approaches or inspire to develop new therapeutic concepts.

## O4. Plant terpenoids as source for drug leads – challenges and research strategies

Hermann Stuppner

[1]Institute of Pharmacy/Pharmacognosy, University of Innsbruck, Innsbruck, Austria

Natural products have been the most significant source of drugs and drug leads in history. One reason for this is certainly the ability of nature to produce a fantastic array of structurally complex and diverse molecules. Since less than 15% of the globe's biodiversity have been tested for biological activity, a multitude of natural lead compounds still are awaiting discovery [1]. Terpenes represent one of the largest and most diverse classes of secondary metabolites, with over 55,000 members isolated so far [2]. Besides their usage as aromatic or flavoring substances in perfumes, cosmetics and food, they are used for medical purposes. Many of these compounds demonstrate a variety of biological effects including antibacterial, sedative, antitumor, cytotoxic, insecticidal, anti-inflammatory, molluscidal and other activities. Well known examples with established medical applications are the anti-malarial drug artemisinin and the anti-cancer drug paclitaxel [3].

Due to the abundance and the broad structural diversity combined with a wide array of biological actions terpenoids provide a rich source of potentially attractive lead compounds for the drug discovery process. But how to dig out and to recognize the respective drug leads is a challenging task. Well established strategies for the discovery of new biologically active natural products are the ethnopharmacological, chemotaxonomic and random approach. Most recently, computer-assisted approaches, such as pharmacophore modeling, virtual screening and docking have been integrated to efficiently access bioactive metabolites. All of these approaches have strengths and also limitations. Therefore, an integrative concept combining different strategies, classical and innovative ones, and gathering as many information as possible from different scientific fields is required in order to guarantee a maximum of efficacy in natural product lead finding processes.

In the course of an ongoing national research network project [4] we aim to identify and characterise compounds capable to combat inflammatory processes specifically in the cardiovascular system. The combined use of computational techniques including natural products databases with traditional knowledge about plants, high-tech chemical analysis and a broad range of in vitro, cell-based and in vivo pharmacological models delivered so far a series of promising terpenoids with interesting anti-inflammatory effects e.g. carnosol and carnosic acids from *Salvia officinalis,* ganoderic acids as farnesoid X receptor agonists, and an iridoid lactone from *Himatanthus sucuuba* as potent Nf□B inhibitor.

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Acknowledgement: We gratefully acknowledge financial support from the Austrian Science Fund (grant no S107 „DNTI")

## O5. Terpene synthase logic from an enzymatic point of view

Reuben J. Peters

Department of Biochemistry, Biophysics. & Molecular Biology, Iowa State University, USA

Construction of the terpenoid hydrocarbon backbone structure is driven by carbocationic reactions, particularly as mediated by terpene synthases, which come in two distinct classes. Class I terpene synthases catalyze heterolytic cleavage of an allylic diphosphate ester bond to initiate their reactions, while class II terpene synthases utilize protonation for the same purpose. Work from my group, which is focused on the class II diterpene cyclases and subsequently acting class I diterpene synthases, has provided some insight into certain aspects of the structure-function relationships underlying both classes of enzymes. In particular, our results have revealed the ability of single residues changes to drastically remodel reaction outcome, highlighting the role of electrostatic effects in the catalyzed complex reactions, which will be discussed.

## Session III – Exploring genomes, transcriptomes, proteomes and metabolome to understand the terpenome

### O6. Gene discovery of modular diterpene metabolism in non-model plant systems

Philipp Zerbe[1], Macaire Yuen[1], Angela Chiang[1], Harpreet K. Sandhu[1], Björn Hamberger[2], Britta Hamberger[2], Eric Schmelz[3], Alisa Huffaker[3]and Jörg Bohlmann[1]

[1]Michael Smith Laboratories, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada
[2]Department of Plant Biology and Biotechnology, University of Copenhagen, Copenhagen, Denmark
[3]Chemistry Research Unit, Center of Medical, Agricultural, and Veterinary Entomology, US Department of Agriculture, Agricultural Research Service,Gainesville, FL, USA

We established deep transcriptome resources for more than a dozen plant species that produce diterpenes of potential or known economic importance, including *Isodon rubescens*, *Rosmarinus officinalis*, *Coleus forskohlii*, *Marrubium vulgare*, *Salvia sclarea*, *Salvia divinorum*, *Grindelia robusta*, *Zea mays*, *Tripterygium wilfordii*, *Euphorbia peplus*, *Jatropha gossypiifolia*, *Abies balsamea* and *Pseudolarix amabilis*. To identify diterpene synthase (diTPS) and cytochrome P450 (P450) genes and enzymes across these different species and their diversediterpene pathways, we developeda strategy that combines (i) tissue-specific diterpeneprofiling,(ii) transcriptome analysis, (iii) phylogenetically informed candidate gene selection, and (iv) *in vitro* and *in vivo*functional characterization. Custom diTPS and P450 databases coveringthe functional space of each enzyme class were used for rapid discovery of more than50 different diTPS and over 400 P450 candidate genes. Phylogenetic interrelations revealed patterns of functionaldiversification and proved useful to guide diTPS functional elucidation. Following gene discovery, we developedproof-of-concept yeast expression platforms for several diterpenes. These and other host platforms are now being explored for combinatorial expression of diterpene pathway genes of different species to produce potentially new diterpene metabolites. The approach used in this workcan be customized to other terpene classes and plant species.

## O7. Maize kauralexins: insights into structure, function, and biosynthesis

Eric A. Schmelz, Shawn Christensen, James Sims, Alisa Huffaker, Hans Alborn, Peter Teal

Chemistry Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, US Department of Agriculture, Agricultural Research Service, Gainesville, FL 32608.

Phytoalexins are a broad category of pathogen and insect-inducible biochemicals that locally protect plant tissues. Terpenoid phytoalexins have been extensively examined numerous crop plants but until recently were considered absent in maize (*Zea mays*). We discovered a series of acidic *ent*-kaurane-related diterpenoids, termed kauralexins, present in maize stem tissues challenged with European corn borer (*Ostrinia nubilalis*) larvae and fungal pathogens. Kauralexins accumulate to levels exceeding 100 mg $g^{-1}$ FW and exhibit significant insect antifeedant and antimicrobial properties when tested *in vitro*. These activities and roles are also important to examine *in vivo*. The maize gene Anther ear 1(An1) encodes an *ent*-copalyl diphosphate synthase (*ent*-CPS) essential for normal levels of *ent*-kaurane derived gibberellin phytohormones. Mutantions in this gene, *an1*, result in a dwarf phenotype and flowers on typically pistillate ears. In contrast a gene encoding a second maize *ent*-CPS, termed An2, is highly inducible by fungal pathogens. We hypothesized that An2 is responsible for the rapid local accumulation of kauralexins following elicitation. Using a homozygous transposon insertion *an2* mutant, we demonstrate that this type I diterpene synthase is required for the inducible biosynthesis and accumulation of kauralexins in maize. Resistance assays with insects and pathogens are currently ongoing to explore the predicted alterations in plant defense phenotype.

## O8. A modular pathway of diterpene synthases and cytochrome P450s for diterpene resin acid biosynthesis in conifers

Jörg Bohlmann

University of British Columbia, Michael Smith Laboratories, Vancouver, BC Canada V6T1Z4

Conifer trees produce large amounts of oleoresin defenses for their protection against insects (e.g. bark beetles) and insect-associated fungal pathogens. Using a combination of conifer genome* and transcriptome sequencing, proteomics, and biochemical approaches, we identified and functionally characterized families of terpenoid synthases (TPS-d family) and cytochrome P450 dependent monoxygenases (CYP720B family) of conifer oleoresin biosynthesis. Both bifunctional as well as monofunctional diterpene synthases are active in conifers and together with members of the CYP720B family form a modular pathway system for diterpene resin acid biosynthesis. The many functions of members of the TPS-d and CYP720B gene families are critical for the plasticity and diversity of secondary metabolism in conifer defense and the successful evolution of long-lived conifer trees, which often survive for several hundred years in the same location defeating many generations of faster evolving insect pests and pathogens.

*Footnote: Sequence assemblies of the very large (20 Gb) genomes of two conifers, White spruce (*Picea glauca*) and Norway spruce (*P. abies*), will have been published by the time of the Terpnet 2013 meeting. These will be the first gymnosperm genomes published.

## O9. An omics perspective for the origin of metabolomic diversity in plants

Kazuki Saito[1,2]

[1]Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan
[2]RIKEN Center for Sustainable Resource Science, Yokohama, Japan

Plants as sessile organisms produce a huge variety of metabolites (metabolome), which play multiple roles in the life of plants for the interaction and adaptation to the environment, such as protective functions against herbivores, pathogenic organisms and abiotic stress. Mankind owes immense benefits to these plant metabolites for food, medicines, flavors and cosmetics. The size of metabolome in plant kingdom is estimated to be 200,000-1,000,000, which far exceeds those estimated in animals and microorganisms. An important question we should address is how and how this metabolomic diversity is originated at the levels of molecule to ecosystem. Recent advancements of genomics and related –omics definitely facilitate to address these questions. In particular, coupling deep-transcriptome analysis with metabolomics is powerful to decipher the genes' function for further understanding the origin of metabolomic diversity in plants.

In this seminar, I would like to discuss on the origin of metabolomic diversity from functional genomics perspectives with the following points:

Metabolomic analysis for understanding chemical diversity of plants

From metabolomics-based functional genomics to systems biology and structural biology in *Arabidopsis thaliana*

Origin of bioactive alkaloid and terpenoid biosyntheses in medicinal plants.

## O10. A second generation of massively parallel DNA sequencers: the signal, the noise, and a new gold standard

Mark R. Wildung

Genomics Core Lab, Washington State University, Pullman WA, USA

Exponential growth in our capacity to generate DNA sequence that was enabled by the first massively parallel sequencing (MPS) platforms, was at the expense of read length and quality. The lowcost and ease of generating large read numbers has mostly compensated for the poor quality, andpaired-end/mate-pair libraries allows some assembly of structural complexity that is greater than readlength. Single molecule real time sequencing (SMRT) has changed that landscape. Very long readlengths allowed by the absence of phase noise, are for the first time generating harvestable reads from a MPS that are longer and have higher quality than dideoxy terminator sequencing and also obviate the need for paired-end/mate pair libraries by directly providing nucleotide sequence across very long and complex regions.

We have sequenced in my lab, some of the most challenging BACs from barley and rice using SMRT and generated single contig assemblies where all previous MPS platforms and Sanger chemistry had failed. Synteny between optical mapping and our SMRT assemblies of microbial genomes is high. With SMRT assemblies we are able to detect and correct the misassemblies caused by the systemic errors of the first MPS platforms. The effective closing and validation of the human, mouse and rice genomes by a combination of SMRT sequencing and optical mapping is now expected.

In addition, observing a massively parallel array of individual DNA polymerase enzymes as they replicate DNA, provides the real-time kinetics of every nucleotide incorporation. As a modified nucleotide base in the template progresses through the polymerase active site it alters the kinetics of extension at and around the modification, leaving an identifiable kinetic footprint. By comparatively analyzing the methylomes of Salmonella, we have been able to identify a regulatory DNA methylase, which appears to be regulated by a DNA methylase, that alters the expression of genes critical tovaccine development. Similar epigenetic regulatory mechanisms are thought to be widespread, but their discovery and validation has been limited by our ability to detect modified DNA bases.

Many familiar short-read bioinformatic tools do not perform well with this rich new data. New ways to assemble, map, and scaffold with SMRT reads have developed and matured rapidly; theirtheory and application is presented.

## O11. Genome-wide investigation of rice oxidosqualene cyclases reveals divergent evolution of plant triterpenoid pathways

Zheyong Xue[1], Lixin Duan[1], Juncong Sun[1], Xia Xu[1], Huiling Wang[1], Zhenwei Tan[1], Paul ÓMáille[2], Anne Osbourn[2], Xiaoquan Qi[1]*

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The oxidosqualene cyclases (OSCs) are an important family of enzymes that cyclize 2,3-oxidosqualene to produce a diverse array of triterpenoid skeletons. Whereas the genomes of lower plants contain a single OSC that is used in the biosynthesis of sterols, the OSC family has undergone a large expansion in the genomes of higher plants. A total of 13 *OSC* genes in the *A. thaliana* genome, and these encoded enzymes produce more than 40 different triterpene skeletons (Morlacchi et al 2009). There are 12 predicted *OSC* genes in the rice genome (*Oryza sativa* L. ssp. *japonica*) (Inagaki et al. 2011). We used various methods, including yeast expression of rice *OSCs*, total gene synthesis of rice *OSCs* with a codon optimized for yeast codon preference, and GC-MS analysis of triterpenes in wild type and the transgenic rice plants, to identify the catalytic functions of all rice OSCs. A part from the predicted cycloartenol synthase (*Os02g4710/OsOSC2*), we have defined an α-/β-amyrin synthase (Os06g28820/OsOSC6), parkeol synthase (*Os11g18194/OsOSC8*), isoarborinol synthase (*Os11g35710/OsOSC11*), and a new bicyclic triterpene poaceatapetol synthase (*Os8g12730/OsOSC12*) (Xue et al 2012; Sun et al 2013). The loss-function-mutants of *OsOSC12* lack poaceatapetol biosynthetic pathway and exhibit severe sterility. These results indicated that the catalytic functions of rice OSCs are greatly different from those of *Arabidopsis thaliana*. Our phylogenetic analysis suggests that an ancient duplication has led to divergence of triterpene synthases in monocots and dicots, and the expansion of the OSC family in higher plants has occurred mainly through tandem duplication followed by positive selection for one of the duplicate copies. Most of the monocot triterpene synthases have been derived from an ancestral cycloartenol synthase, while the dicot triterpene synthases have been derived from an ancestral lanosterol synthase (Xue et al 2012).

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## O12. From ecometabolomics to synthetic biology – exploring plasticity of the triterpenoid biosynthetic pathway

Pernille Ø. Erthmann[1], Vera Kuzina[1], Bekzod Khakimov[2], Jörg M. Augustin[1], Søren Bak[1]

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[2]Department of Food Science, University of Copenhagen

Triterpenoid saponins are natural plant defense compounds rather widespread in plants. They are amphipathic molecules that may interact with sterols in membranes, and induce pore formation and cell death. The wild crucifer Barbarea vulgarisshows resistance towards flea beetles (Phyllotreta nemorum) (Agerbirk et al. 2003, Journal of chemical ecology), a severe pest in crucifer crops like oil seed rape (Brassica napus). An ecometabolomic approach identified saponins to correlate with flea beetle resistance in B. vulgaris (Kuzina et al. 2009, Plant physiology). A transcriptomic 454-dataset was used to create a quantitative trait loci map which identified specific saponins to lie in the QTL for flea beetle resistance (Kuzina et al. 2011, Phytochemistry). The proposed saponin pathway branch-off from the sterol biosynthesis with 2,3-oxidosqualene as the shared precursor. 2,3-oxidosqualene is cyclized by oxidosqualene cyclases (OSC) to a number of backbone structures, oxidized by cytochromes P450 (CYP), and glycosylated by glycosyltransferases (UGT) to create the vast structural diversity.

A 454 transcriptomic and an Illumina genomic dataset were mined for OSC, CYPs and UGTs gene candidates. The catalytic activities of five UGTs were characterized by in vitroassays in E. coli, and found to glycosylate at the C3 or/and the C28 position of selected triterpene backbones (Augustin et al. 2012, Plant physiology). An analysis of dN/dS site and branch models revealed that the UGT catalyzing the 3-O-glucosylation is under positive selection,suggesting that this UGT has evolved to become specific to the saponins. The activities of four CYPs were studied by in vitroassays in yeast, and shown to oxidize at the C28 position. To reveal the function of the OSCs the Cowpea Mosaic Virus-Hyper Translatable System (CPMV-HT) was used. This enable production of the triterpenoid backbone structures α-amyrin, β-amyrin and lupeol from the common precursor 2,3-oxidosqualene. In addition, the OSCs were found to lie within the QTL for resistance. By combinatorial expression in N.benthamianaof OSCs, CYPs and UGTs using the CPMV-HT system, anumber of known and new-to-nature saponin structures were generated. combined metabolomics, genomics, transcriptomics, and genetic approach identified saponins as determinants for flea beetle resistancein B. vulgaris, and provided the necessary molecular tools for in plantaproduction of known and new-to-naturestructures by synthetic biology. This ability to combine OSCs, CYPs and UGTs in planta by combinatorial biochemistry paves the way for a rational design triterpenes for structure-activity relationshipsfor development of bioactive triterpenes with specificity to different herbivores and diseases, and demonstrates the plasticity of the pathway.

**Session IV – Terpenoids biosynthesis and regulation**

## O13. Biosynthesis of terpenoid precursors in *Arabidopsis*: the role of fernesyl diphosphate synthase

Albert Ferrer

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Farnesyl diphosphate synthase (FPS) catalyzes the sequential head-to-tail condensation of isopentenyl diphosphate (IPP, $C_5$) with dimethylallyl diphosphate (DMAPP, $C_5$) and geranyl diphosphate (GPP, $C_{10}$) to produce farnesyl diphosphate (FPP, $C_{15}$). This short-chain prenyl diphosphate is a key branch-point intermediate in the isoprenoid biosynthetic pathway from which a variety of bioactive isoprenoids that are vital for normal plant growth and survival are produced.

The model plant *Arabidopsis thaliana* contains two genes, *FPS1* (At5g47770) and *FPS2* (At4g17190), that are differentially expressed throughout Arabidopsis development and encode three FPS isozymes (FPS1L, FPS1S and FPS2). Isozyme FPS1L contains an N-terminal sequence that has been shown to target the enzyme into mitochondria whereas isozymes FPS1S and FPS2 have been found to localize exclusivey into the cytosol. To get insight into the role of FPS isozymes in isoprenoid biosynthesis, highly purified preparations of recombinant FPS1S and FPS2 isozymes were obtained and characterized. The two short FPS isozymes display different functional and structural properties despite they share more than 90% amino acid sequence identity. Functional complementation studies of the Arabidopsis *fps2* null mutant phenotypes with chimeric *FPS* gene constructs were also undertaken. Results demonstrated that under normal conditions isozymes FPS1S and FPS2 are functionally interchangeable. Conditional *fps* weak mutants were generated using an RNA interference (RNAi)-based gene silencing strategy. Characterization of the *fps* knock-down mutants is currently under way.

## O14. Iridoid Biosynthesis

Sarah E. O'Connor

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Here we describe the biosynthesis of the iridoid compounds, a class of natural products with potent biological activities. The key biosynthetic step entails an unusual NADPH dependent reduction. The implications for enzyme discovery, engineering and mechanistic study in this pathway will be discussed.

## O15. Elucidation of sesquiterpene lactone biosynthesis in feverfew (*Tanacetum parthenium*)

Qing Liu[1], David Manzano[2,3], Nikola Tanić[4], Milica Pesic[4], Jasna Bankovic[4], Irini Pateraki[3,5], Lea Ricard[1], Albert Ferrer[2,3], Ric de Vos[6,7,8], Sander van de Krol[1], Harro Bouwmeester[1]

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Sesquiterpene lactones are a major class of plant secondary metabolites and over 4000 different structures have been elucidated. Many of these colorless, bitter tasting, lipophilic molecules are active constituents of a variety of medicinal plants. *Tanacetum parthenium* (feverfew) is a prominent example of a medicinal plant that has been used for the treatment of various diseases, such as migraine, arthritis, and cancer. In feverfew, both monocyclic germacranolide and bicyclic guaianolide sesquiterpene lactones occur, with parthenolide as the most famous and bioactive representative of the former. We showed that costunolide is the common intermediate substrate for both classes of sesquiterpene lactones and identified the genes that encode the germacranolide pathway up to 3β-hydroxyparthenolide and the cyclisation of costunolide to a guaianolide, kauniolide. The catalytic function of the individual genes was assessed through heterologous expression in yeast. Subsequently, both pathways were also completely – from the first dedicated intermediate germacrene A - reconstituted *in planta* by transient expression of up to 7 pathway genes in *Nicotiana benthamiana*. With the number of enzymatic steps introduced into *N. benthamiana* increasing, end product yield reduced, mainly due to competing conversion of pathway intermediates towards cysteine and glutathione conjugates. These conjugates seem to be attractive candidates - with improved chemical properties - for the development of new medical treatments.

## O16. The dandelion dilemma: With or without rubber?

Dirk Prüfer

Institute for Plant Biology and Biotechnology, University of Münster, Germany

Dandelion species such as *Taraxacum koksaghyz* (Russian dandelion) and *Taraxacum brevicorniculatum* are known to produce significant amounts of high-quality rubber in their laticifers, whereas others such as *Taraxacum officinale* (common dandelion) produce only small amounts. The dandelion family is therefore an excellent model system to gain insight into the biological function of natural rubber and the regulation of rubber biosynthesis in plants. Functional genomics has been used to identify several key enzymes involved in rubber biosynthesis, including cis-prenyltransferases (CPTs), small rubber particle proteins (SRRPs) and a rubber elongation factor (REF). Interestingly, the amount of rubber appears to be regulated by the relative expression of combinations of these key enzymes. Natural rubber is thought to play a major role in pathogen defence, particularly the battle against pest insects. The dandelion family must therefore have evolved different defence strategies in different species. In the common dandelion, which produces little rubber, large amounts of a latex-specific polyphenoloxidase (PPO) may provide defense functions instead of rubber. The presentation will highlight recent findings in the field of dandelion rubber, including its biosynthesis and biological function and also on its use as a valuable crop for the production of natural rubber for technical product development.

## O17. Do we need to rethink the "canonical" MVA pathway?

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In higher plants, two independent pathways localized in different cellular compartments are responsible for the biosynthesis of the universal five-carbon isoprenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In plastids, IPP is formed via the methyl-erythritol-phosphate (MEP or non-mevalonate) pathway and serves as a building block for monoterpene and diterpene biosynthesis while the classical mevalonic acid (MVA) pathway, localized in the cytosol and peroxisomes, provides IPP for the formation of sesquiterpenes, sterols, and triterpenes. The MVA pathway begins with a stepwise condensation of three molecules of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA, which undergoes reduction to the key intermediate, mevalonic acid. Mevalonic acid is then subjected to two subsequent phosphorylation reactions, catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and the final decarboxylation/elimination step catalyzed by mevalonate diphosphate decarboxylase (MVD) to form IPP. It has been recently shown that archaea contain an alternative MVA pathway which diverges at the last two reactions, possibly occurring in reverse order, phosphomevalonate is first decarboxylated to isopentenyl phosphate which then undergoes phosphorylation to IPP in reaction catalyzed by isopentenyl phosphate kinase (IPK). Remarkably, homologs of IPK were found sporadically across all three domains of life and in every green plant genome sequenced to date suggesting its universal role within the plant kingdom. Moreover, expression level of IPK in Arabidopsis was comparable to that of MVD representing the final step in the classical MVA pathway. Identification of IPK in plants raises several questions: Does the alternative MVA pathway exist in plants? If so, how is carbon partitioning between the "canonical" and alternative pathways? To what extent does each pathway contribute to the downstream products? Is IPK universally serving as a salvage pathway for IPP/DMAPP? What is the function of IPK in planta. To resolve these questions, a combination of reverse genetics in Arabidopsis, transient overexpression in tobacco and biochemistry were used.

## O18. Strigolactones. Terpenoidsignalling molecules with surprising in- and outdoor activities

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The newly identified group of plant hormones strigolactones (SLs) plays an important role in the regulation of shoot branching/tillering and root architecture in plants. In addition to this internal plant signalling function SLs are germination stimulants for root parasitic plant species of the Orobanchaceae, and chemical signals stimulating plant root colonization by symbiotic arbuscularmycorrhizal (AM) fungi. The biosynthetic pathway of SLs has been partially elucidated, using highly branched/tillered mutants of Arabidopsis (*max)*, rice (*dwarf* or *htd*), Petunia (*dad*) and pea (*rms*). Using these mutants it was shown that the carotenoid isomerase D27, the carotenoid cleavage dioxygenases CCD7 and CCD8 (in Arabidopsis called MAX3 and MAX4) and a cytochrome P450 (MAX1 in Arabidopsis) are involved in strigolactone biosynthesis. An F-box protein MAX2 and an α/β hydrolase D14 seem to be involved in strigolactone perception/downstream signaling.The role of strigolactones in the regulation of several biological processes, the regulation of their biosynthesis and the current knowledge of their biosynthetic pathway will be discussed.

## O19. Biosynthesis of Fungal Meroterpenoids

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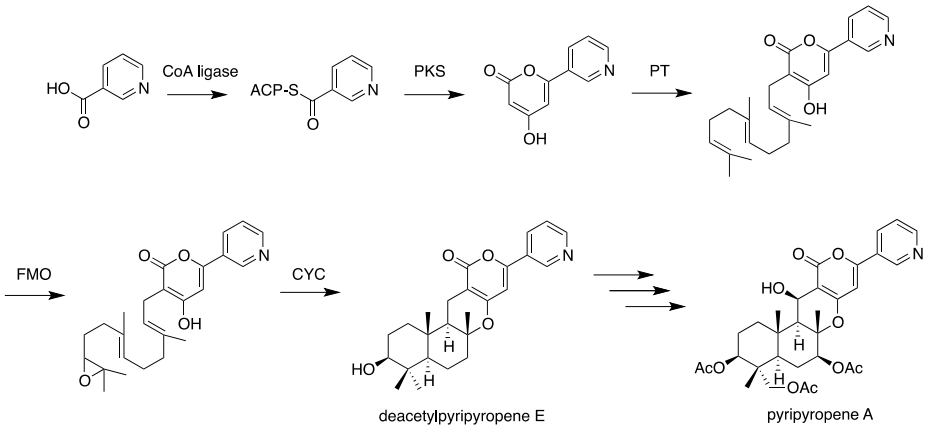
Meroterpenoids are hybrid natural products of both terpenoid and polyketide origin. We identified a biosynthetic gene cluster that is responsible for the production of the meroterpenoid pyripyropene in the fungus *Aspergillus fumigatus* through reconstituted biosynthesis of up to five steps in a heterologous fungal expression system. The cluster revealed a previously unknown terpene cyclase with an unusual sequence and protein primary structure. The wide occurrence of this sequence in other meroterpenoid and indole–diterpene biosynthetic gene clusters indicates the involvement of these enzymes in the biosynthesis of various terpenoid-bearing metabolites produced by fungi and bacteria. In addition, a novel polyketide synthase that incorporated nicotinyl-CoA as the starter unit and a prenyltransferase, similar to that in ubiquinone biosynthesis, was found to be involved in the pyripyropene biosynthesis. The successful production of a pyripyropene analogue illustrates the catalytic versatility of these enzymes for the production of novel analogues with useful biological activities.




On the other hand, terretonin is another fungal meroterpenoid isolated from *A. terreus*. Recently, we also identified the *trt* gene cluster as that responsible for terretonin biosynthesis. In this study, we expressed the polyketide synthase (*trt4*), prenyltransferase (*trt2*), methyltransferase (*trt5*), flavin-dependent monooxygenase (*trt8*) and terpene cyclase (*trt1*) genes in the *A. orzyae* NSAR1 strain and identified their product as preterretonin A. Interestingly, in the absence of *trt5*, the enzymes encoded by these genes cannot produce any cyclized products. When *trt1* was replaced with *ausL*, a terpene cyclase gene responsible for austinol and dehydroaustinol biosyntheses, AusL also cyclized only the methylated intermediate to yield protoaustinoid A. These results indicate that methylation is generally essential before cyclization in DMOA-derived meroterpenoid biosynthesis.

References: *Nature Chem.* **2**, 858 (2010); *ChemBioChem* **13**, 1132 (2012); *ChemBioChem* **13**, 1738 (2012).

# O20. The functional studies of AP2/ERF transcription factors and the key genes of jasmonate biosynthetic pathway in *Artemisia annua* L.

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*Artemisia annua* is an important medicinal plant, and the regulatory mechanism of artemisinin is always the focused research area in *A. annua*. Previous studies have shown that APETALA2/ethylene-response factor (AP2/ERF) transcription factors play important roles in the regulation of secondary metabolism. Artemisinin is produced and stored in glandular secretory trichomes present on aerial surfaces of the plant. So if there exist trichome-specific AP2/ERF transcription factors, and whether the genes directly or indirectly regulate the artemisinin biosynthetic pathway remain unknown.

In this study, six AP2/ERF transcription factors were cloned through cDNA library and RACE library screening and analyzed in *A. annua*. RT-Q-PCR showed that *AaORA* was the only transcription factor which exhibited similar expression patterns to those of amorpha-4,11-diene synthase gene (*ADS*), cytochrome P450-dependent hydroxylase gene (*CYP71AV1*) and double bond reductase 2 gene (*DBR2*) at different positions of the leaves and in different tissues of *A. annua*. The *AaORA* promoter-GUS transgenic plants showed that *AaORA* is a trichome-specific transcription factor, which is expressed in both GSTs and TSTs of *A. annua*. The up-regulation or downregulation of *AaORA* in *A. annua* positively regulated the expression levels of *ADS*, *CYP71AV1*, *DBR2* and *AaERF1*, which resulted in an increase or decrease in artemisinin and dihydroartemisinic acid in *A. annua*. Consequently, *AaORA* is an important positive regulator of the artemisinin biosynthetic pathway. The inoculation experiments with *B. cinerea* in *A. thaliana* and *A. annua* showed that *AaORA* is a positive regulator of disease resistance to *B. cinerea*. All these data demonstrate that *AaORA* is a valuable AP2/ERF transcription factor, not only for the regulation of the artemisinin biosynthetic pathway, but also for the enhancement of the disease resistance to *B. cinerea* in *A. annua*.

Jasmonic acid (JA) is an important signaling molecule in plants. However, the significance and function of endogenous JA in trichome formation and second metabolic regulation of *A. annua* remain unknown. In this study, the jasmonate biosynthetic pathway genes *AaAOC* and *AaAOS* from *A. annua* were cloned by RACE. The 2276 bp *AaAOC*-promoter was cloned by genomic walking. Several elements such as W-box, G-box and ABRE-box existed in the promoter which involved in hormone and stress responsiveness. The expression of *AaAOC* can be induced vigorously by different hormones and stresses, too. The *AaAOC* promoter-GUS fusion study showed that *AaAOC* expressed ubiquitiously in all organs of transgenic *A. annua* plants, which agreed well with the RT-PCR and RT-Q-PCR results. In *AaAOC*-overexpressing transgenic lines, the content of endogenous JA was increased and was 2 to 4.7-fold of the control, and the density of gland trichomes was also increased and was 1.5 to 1.8-fold of the control. RT-Q-PCR showed that the expression levels of *FPS*, *CYP71AV1* and *DBR2* were increased significantly in *AaAOC*-overexpressing transgenic lines, resulting in significant increase of artemisinin, dihydroartemisinic acid and artemisinic acid in *AaAOC*-overexpressing transgenic lines.

## O21. Plant 3-hydroxy-3-methylglutaryl coenzyme A reductase triggers the biogenesis of endoplasmic reticulum subdomains targeted for direct delivery to the vacuole in response to stress

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Plant 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the first committed step of the mevalonate pathway for isoprenoid biosynthesis and exerts a key regulatory role on this pathway, critical not only for normal growth and development but also for the adaptation to diverse challenging conditions[1]. We pursue the understanding of the mechanisms involved in the control of HMGR, required to fulfill its regulatory role. Plant HMGR is primarily targeted to the endoplasmic reticulum (ER), but accumulates in so far undefined structures named HMGR vesicles[2,3]. We show that these structures can be generated in different plant systems by expression of the HMGR membrane domain. Ultrastructural and immunocytochemical studies reveal that they consist on tighly stacked crystalloid-, karmellae- or whorl-shaped membrane associations. The ER hyperthrophy occurs around ER bodiespreviously characterized in *Arabidopsis thaliana*[4] and here also identified in Nicotiana benthamiana. The ER bodieshave been proposed to play a fundamental role in programmed cell death and defense against biotic and abiotic challenges[4]. We show that the HMGR-containing membrane aggregates are delivered to the vacuole in response to wounding or chemical stress. This is a fast process, occurring ina much shorter time than the previously described ER bodydelivery to the vacuole. HMGR is massively degraded in response to stress, in contrast to antimycin A-insensitive cytochrome C reductase, used as a general ER marker. Our studies uncover that plant cells have HMGR-tagged ER subdomains, which are directly and rapidly delivered to the vacuole in response to stress.

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## O22. Is artemisinic aldehyde Δ11(13) reductase a rate limiting enzyme of artemisinin biosynthesis in *Artemisia annua* L.?

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Artemisinin is a very important antimalarial drug isolated from *Artemisia annua*. The biosynthesis of this sesquiterpene endoperoxide is fairly well characterized as far as the enzymes involved (Figure 1).

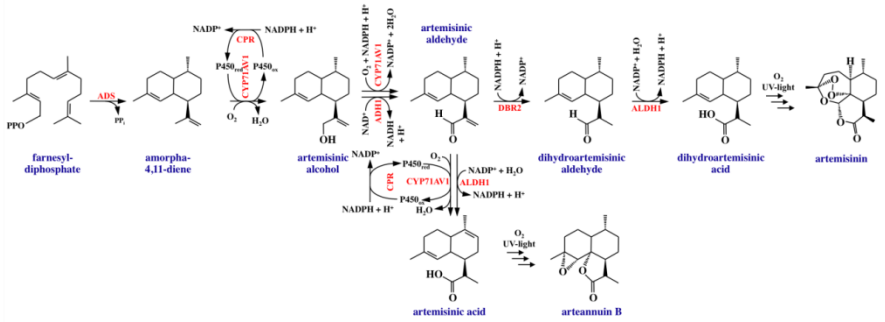


**Figure 1.** Biosynthetic pathway of artemisinin and aretannuin B in *Artemisi annua*.

The first enzyme of the pathway, *i.e.* amorpha-4,11-diene synthase (ADS), is recognized as the rate-limiting enzyme. There are two chemotypes of *A. annua*; HAP: high in dihydroartemisinic acid (DHAA) and artemisinin (ART); LAP: high in artemisinic acid (AA) and arteannuin B (AB). At the branching point in the pathway, artemisinic aldehyde (AAld) is either oxidized to AA by amorphdiene 12-hydroxylase (CYP71AV1) or reduced to dihydroartemisinic aldehyde (DHAAld) by artemisinic aldehyde Δ11(13) reductase (DBR2). The concentration of ART and AB and their precursors DHAA and AA were determined for four *A. annua* cultivars by GC-MS. The cultivar Anamed is a HAP, which accumulated DHAA and ART, while the concentrations of AB and AA were much lower than in the LAP chemotypes (cultivars #8, 14 and 47 collected in Iran). The LAP cultivars accumulated AA and AB, while the concentration of DHAA and ART were much lower than in the HAP chemotype. For instance, the ratios of DHAA to AA and ART to AB in cultivar #8 were 1:3 and 1:20 in flowers. We have also investgated by qPCR the levels of transcripts of the biosynthetic genes in the two chemotypes of *A. annua*. The expression of the majority of the genes encoding enzymes of artemisinin biosynthesis was higher in the HAP cultivar that in the LAP cultivars (up to 15 times). However, the level of DBR2 transcripts was considerably higher in the HAP cultivar than in the LAP cultivars (over 100 times). It may be suggested that DBR2 is a regulatory enzyme of artemisinin biosynthesis. A high DBR2 activity will divert the carbon flow to artemisinin formation by reduction of AAld to DHAAld which otherwise would be converted to AA by CYP71AV1.

## O23. Metal ions control chain-length specificity of isoprenyl diphosphate synthases in insects

Sindy Frick[1], R. Nagel [2], A. Schmidt[2], R.R. Bodemann[1], P. Rahfeld[1], W. Brandt[3], G. Pauls[1], J. Gershenzon[2], W. Boland[1], A. Burse[1]

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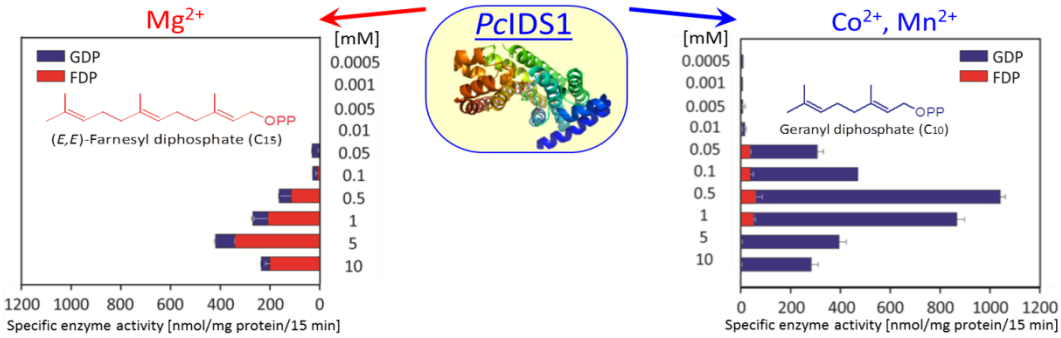
Terpenes are an extensive group of metabolites in all major divisions of life that can be divided into classes (e.g. $C_{10}$, $C_{15}$ and $C_{20}$) depending on the number of $C_5$ units included in their structures. Short chain isoprenyl diphosphate synthases (scIDS's) condense the $C_5$ units to generate the precursors of each size class, such as $C_{10}$-geranyl diphosphate (GDP), $C_{15}$-farnesyl diphosphate (FDP) or $C_{20}$-geranylgeranyl diphosphate (GGDP). The reaction requires for activation a trinuclear metal cluster, usually containing $Mg^{2+}$ or $Mn^{2+}$ (1).To understand the influence of the metal cluster on the chain-length of the products, we studied a member of the short chain IDS family (*Pc*IDS1) in juvenile horseradish leaf beetles, *Phaedon cochleariae*. In the presence of $Co^{2+}$ or $Mn^{2+}$ the *Pc*IDS1 produced 96% GDP ($C_{10}$) and 4% FDP ($C_{15}$) from isoprenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP). In contrast, with $Mg^{2+}$ only 18% GDP but 82% FDP were detectable. Using RNA interference, the *Pc*IDS1 was shown to be associated with the *de novo* synthesis of defensive monoterpenoids (chrysomelidial) in the leaf beetle larvae. As an FDP synthase the enzyme could be responsible for the biosynthesis of juvenile hormone required to control the development of the insect. The detection of $Co^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ in the fat body of *P. cochleariae* larvae suggests that the flow into $C_{10}$ vs. $C_{15}$ isoprenoids in these insects may be regulated by the metal co-factors. Consequently, metal ions may control the terpenoid metabolic flux at a branch point with both potential evolutionary and ecological implications (2).

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## O24. Investigating phenolic diterpenes in rosemary (*Rosmarinus officinalis*) and sage (*Salvia fruticosa*)

Kathleen Brückner[1], Dragana Bozic[2], David Manzano[3], Dimitra Papaefthimiou[2], Eirini Pateraki[3], Erwin Datema[4], Albert Boronat[3], Ric de Vos[4,5,6], Albert Ferrer[3], Angelos K. Kanellis[2], Alain Tissier[1]

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Plant secondary metabolites contribute substantially to the human pharmacopeae. To better exploit the medicinal plants that produce these compounds, it is necessary to understand how these compounds are synthesized within the plant. Identification of the biosynthetic pathway genes will allow both the breeding of plant varieties with improved productivity and/or metabolic profile, and the development of alternative production methods using biotechnology.

The phenolic diterpenes (PDs) carnosic acid (CA) and carnosol have high antioxidant activities and display potential for the treatment of neurodegenerative disorders. CA and carnosol are found in Rosmarinus and Salvia sp. which are known for their health promoting properties since ages.

As part of the EU-financed TERPMED project, PD biosynthesis is being investigated. To elucidate the pathway, the localization of the biosynthesis of PDs was determined. Glandular trichomes appear to contribute significantly to the biosynthesis of PDs and the transcriptome of glandular trichomes was thus determined by next generation sequencing of cDNA.

A set of gene candidates was selected according to alignment searches and analysis of trichome-specific gene expression profile. The search for terpene synthases yielded two types of candidates for the first steps of the pathway: a copalyl diphosphate synthase (CPS) and kaurene synthase-like (KSL) encoding genes. Genes encoding oxidases of cytochrome P450 clade were the most promising candidates for the downstream steps of the PDs pathway.

Finally, the first steps of the biosynthesis of PDs are reconstituted and further investigations on the role of P450 candidates in downstream steps are being carried out using N. benthamiana transient expression and heterologous expression in yeast.

Keywords: Rosemary, Greek sage, Phenolic diterpenes, Carnosic acid, Trichomes

## O25. Manoyl oxide as a precursor for forskolin biosynthesis: identification and characterization of the involved biosynthetic enzymes from *Coleus forskohlii*

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Forskolin is a labdane diterpenoid highly valued for its activity against a number of human health disorders.The majority of forskolin properties are due to its ability to act as a potent activator of the adenylyl cyclase enzyme leading to marked increase of the intracellular level of cAMP. Today forskolin derivatives have been approved as clinical drugs in Japan for treating cardiac surgery complications, heart failure, or cerebral vasospasm.Although the extensive studies that have been conducted on forskolin medical applications (more than 20,000 citations in PubMed), the biosynthetic pathway of this potent phytochemical has not yet been identified.

The only source of forskolin is the root of a plantfrom Lamiaceae(or mint) family,*Coleus forskohlii*.Gaining insight into the specific tissue, we showed that forskolin accumulates exclusivelyin specialized cells that bear oil-body like structures, localizedat the root periderm. Purification and chemical analysis of coleus root oil bodies showed that forskolin as well as its hypothetical precursor, manoyl oxide, areactually found in those structures.

Targeted mining of the deep coleus root transcriptome resulted in the identification of a small multigene family encoding diterpene synthases (CfdiTPSs), putative enzymes responsible for the formation ofthe forskolin backbone.Expression studies of the CfdiTPSs showed that CfTPS1, CfTPS2 and CfdiTPS3 transcript accumulation levels are well-correlated with the occurrence of forskolin in coleus tissues, with the highest levels of gene transcripts found in the coleus root periderm tissue. Following *in vivo* (transient expression in tobacco) and *in vitro* (coupled enzymatic assays using *E. coli* recombinant proteins) functional characterization, we showed that CfdiTPS2 (class II diTPS) in combination with CfdiTPS3 or CfdiTPS4 (class IdiTPSs) are involved in the biosynthesis of the correct stereoisomer ofmanoyl oxide, forming effectively the backbone of forskolin. Ongoing experiments, using deep cell-specific transcriptome, are carried out for the identification of the highly diverse enzyme family (cytochromes P450) putatively responsible for the conversion of manoyl oxide to forskolin. Our findings could facilitate the generation of biosustainable and cost-effective production platforms, using microorganism or moss,to supply commercially exploitable amounts of forskolin.

## O26. Sterol side-chain reductase: essential for cholesterol biosynthesis in *Solanum* plants

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Steroidal alkaloids widely occur in *Solanum* plants. These steroids are biosynthesized from cholesterol via C-26 oxidation–amination, C-22 oxidation, C-16 oxidation and glycosylation of the C-3 hydroxy group. In this study, we focused on the biosynthesis of cholesterol and successfully identified sterol $D^{24}$ reductase, a key enzyme in cholesterol production, from *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) (Fig.1).

With the amino acid sequence of human 24-dehydrocholesterol reductase as a query, BLAST analysis against potato unigene databases from the Gene Index project (http://compbio.dfci.harvard.edu/tgi/) picked up two types of cDNA sequences. We named these genes *Solanum tuberosum* Sterol Side-chain Reductases (StSSR1, StSSR2). To identify the function of StSSR1 and StSSR2, they were expressed in the yeast strains that produce 24-methylenecholesterol (MC) and desmosterol (DS). The yeast extracts were analyzed by GC-MS. Cholesterol and campesterol were detected from the DS yeast expressing *StSSR2* and the MC yeast expressing *StSSR1*, respectively. In addition, we examined the cycloartenol reductase activity using homogenates of the yeasts expressing each gene. StSSR2 reduced cycloartenol to cycloartanol. Moreover, we also identified two genes (*SlSSR1*, *SlSSR2*) from tomato. The functions of SlSSR1 and SlSSR2 were the same as that of StSSR1 and StSSR2, respectively. These results indicate that SSR2 is a key enzyme for cholesterol biosynthesis in *Solanum* plants.

This work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

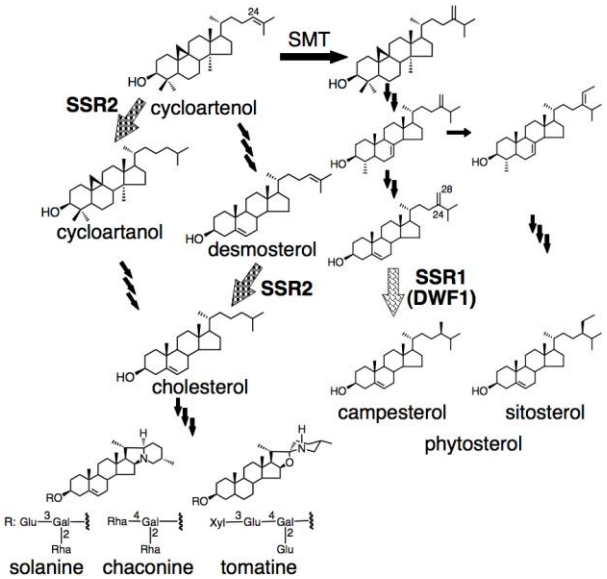


Fig.1 Biosynthetic pathway of *Solanum*

# O27. Mechanistical investigation and inhibition of IspH/LytB, a [4Fe-4S]2+ enzyme involved in the biosynthesis of isoprenoids via the MEP pathway

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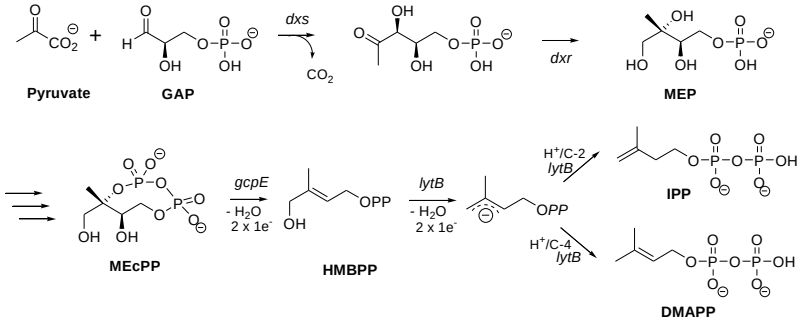
In many bacteria, including *Mycobacterium tuberculosis* responsible for tuberculosis, in the plant chloroplasts and in the malaria parasite *Plasmodium falciparum*, the biosynthesis of isoprene units occurs according to the methylerythritol phosphate (MEP) pathway, an alternative to the well-known mevalonate pathway. The MEP pathway (Scheme 1) does not exist in humans and is therefore a valuable target for the development of new specific antibacterial and antiparasitic drugs.




**Scheme 1.** MEP pathway

The last step of this pathway is catalyzed by IspH/LytB, an oxygen sensitive metalloenzyme. Mössbauer investigations performed on purified LytB and *E. coli* cells overexpressing LytB showed unequivocally that LytB contains a peculiar [4Fe-4S] cluster. Upon addition of the HMBPP substrate to LytB, one iron atom of the [4Fe-4S] changes its first coordination sphere revealing that HMBPP binds to the $[4Fe\text{-}4S]^{2+}$ *via* its OH group. This feature was used to design two new extremely potent inhibitors of *E. coli* LytB.

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**Session V – Terpenoids Roles in Fleshy Fruit Biology ( organized by COST Action FA1106 QUALITYFRUIT)**

## O28. Multi-hormonal control of the developmental transitions leading to fruit ripening

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The making of a fruit relies onsequentialdevelopmental transitions that are coordinated by a complex network of interacting genes and signalling pathways. In the case of fleshy fruit, this process involves three main stages known as fruit set, fruit enlargement, and fruit ripening. Though the role of some hormones in regulating these developmental shifts has been established, it is more likely that theircoordination is a matter of a multi-hormonal control. The co-ordinated changes in the levels of several plant hormones associated with the transition steps during fruit growth strongly suggest their dynamic involvement in theseprocesses. To address the general role of hormone cross-talk throughout fruit ontogeny,combined genome-wide transcriptomic profiling and reverse genetics approaches were applied to investigate the molecular events underlying tomato fruit development and ripening. Overall, the data emphasize the role of auxin and ethylene-dependent transcriptional control of gene expression as part of the mechanism by which the fruit developmental program switches into a ripening process. A number of transcription factors from the ERF (Ethylene Response Factors) family display an auxin-dependent regulation whereas the expression of some members of the ARF (Auxin response factors) familyshowed clear regulation by ethylene.Moreover, down-regulation of some *ARF* genes resulted in ripening phenotypes with major metabolic reorientation and structural changes, thus uncovering new roles for ARFs during fleshy fruit development and addsauxin to the list of important cues controlling fruit ripening and overall fruit quality. Likewise, some ERF genes were shown to mediate ethylene responses in ripening fruit and exploring the mechanisms by which ERFsselect their target genesshed new light on the molecular basis underlying the specificity of ethylene responses.

## O29. Regulation of carotenoid biosynthesis: Interplay between carotenoids and phytohormones

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Carotenoid pigments are indispensable components of the photosynthetic apparatus and thus are present in all green tissues of plants. In fruit and flowers they accumulate as secondary metabolites that furnish pigmentation and serve as precursors for volatiles in that attract animals. Two phytohormones, abscisic acid (ABA) and strigolactones, are produced from carotenoids.

The carotenoid biosynthesis pathway in plants has been largely deciphered at the molecular level. However, a few enzymes are still missing and the regulation of the pathway is yet unresolved. We have isolated novel mutations in tomato (*S. lycopersicum*) that alter pigmentation of flowers and fruit. Through characterization and cloning of these mutations we have identified new functions in carotenoid biosynthesis. Our results demonstrate the importance of RedOx to the biosynthesis of carotenoids and revealed a link between carotenoid biosynthesis and plastid biogenesis. We obtained evidence for the involvement of ABA in this regulation. Since impairment of carotenoid biosynthesis affects ABA, a feedback mechanism is proposed through ABA is proposed. A new type of regulation of gene expression by cis-carotenoids will be presented.

## O30. Carotenoid derived flavour formation in fruit and other systems

Jules Beekwilder[1], Harmen van Rossum[2], JeanMarc Daran[2], Frank Sonntag[3], Jens Schrader[3], Dirk Bosch[1], Robert D Hall[1],

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Fruit flavour is the perception of a blend of volatile compounds. The formation of these compounds is the result of metabolic changes occurring during fruit maturation. We are interested in the biochemical changes in the fruit underlying the production of terpenoids during fruit ripening, and the genes that operate these biochemical changes. The fruit of the raspberry is a model for the biosynthesis of apocarotenoids. Apocarotenoids, such as beta ionone, determine more than 80% of the raspberry flavour. During fruit ripening, carotenoid breakdown is accompanied by the formation of alpha and beta ionone. Both carotenoid breakdown and apocarotenoid formation in raspberry correlate with the expression level of specific raspberry CCD genes. Overexpression of raspberry CCD cDNA in tomato fruit lead to only modest increases of apocarotenoid and no concomitant loss of carotenoids was observed, in spite of the fact that tomato stores significant amounts of carotenoid. Much stronger effects on carotenoid accumulation were observed when the same CCD was expressed in carotenoid producing microbes. Plant biosynthetic genes for fruit flavour are used in synthetic biology approaches, where plant metabolic pathways are integrated into heterologous plant and/or microbial metabolism. The aim of such approaches is to understand the interaction between the metabolic pathway and the cellular chassis.

## O31. Transcriptome and metabolome analysis for gene discovery in the steroidal alkaloid pathway

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Steroidal alkaloids (SAs) are common constituents of numerous plants belonging to the *Solanaceae* family. Solanine, a major SA in potato was reported already 200 years ago and extensively studied as sprouting tubers may contain a relatively high content of SAs that is potentially dangerous for human health. Consisting of a C27 cholestane skeleton composed of several heterocyclic rings and containing nitrogen, SAs were suggested to be synthesized from cholesterol and to be further glycosylated to form steroidal glycoalkaloids (SGAs). The early stages of SA biosynthesis, predicted to start from cholesterol up to the alkamine, were not investigated at the molecular level although several possible pathways were suggested. Our main aim is to identify key genes in the biosynthesis of SGAs in the *Solaneceae* family. We recently reported that silencing of the tomato *GLYCOALKALOID METABOLISM 1* gene (*GAME1*), putatively encoding a glycoalkaloid- glycosyltransferase, resulted in up to 50% reduction in the principal tomato SGA, *α*-tomatine. This study pointed to the importance of glycosylation in avoiding toxicity to the plant cell on one hand while increasing toxicity to pathogens on the other. Through a combination of metabolic and transcript profiling we have identified a different gene, *GAME4,* that appears to play a key role in the biosynthesis of the SGA aglycone from cholesterol in both potato and tomato as its silencing resulted in a dramatic reduction of SGAs levels in potato tubers, tomato fruit and leaves (of both species). Results of experiments in which GAME4 and *Solaneaceae* plants altered in its expression were characterized in detail will be presented.

## O32. Apocarotenoid volatiles in tomato fruits: amplifiers of sweet perception in humans

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The flavor quality of commercial tomatoes has been a source of consumer dissatisfaction for many decades. The deterioration of flavor is part of a larger problem; nutrient quality of many crops has dropped as breeders have emphasized high yield. A large part of the problem is that flavor quality is poorly understood at the genetic and chemical level. A necessary first step to improving flavor is to define the foundation of what chemicals contribute to consumer liking. To accomplish that end, we took a step back, screening a large number of chemically diverse "heirloom" tomatoes. We conducted a large scale study, determining consumer preferences and correlating liking with chemical composition for ~100 varieties. The results were somewhat surprising, illustrating the weakness of traditional predictive methods and uncovering significant interactions between the taste and olfactory systems. The main driver of consumer preferences in perceived sweetness. Of particular interest is the enhancement of sweet perception by specific volatile compounds that is independent of sugar. Prominent among the volatiles that enhance sweet perception are apocarotenoids. These volatiles have the capacity to make foods taste sweeter than would be predicted from sugar content alone. Varieties that are perceived as much sweeter than would be predicted on the basis of sugar content alone are enriched for sweet-enhancing volatiles. We suggest that plants reliant upon fruit dispersal have evolved to exploit sweet-enhancing volatiles for making fruits more desirable to frugivores.

## O33. A β-carotene/ABA regulatory loop controls tomato fruit ripening

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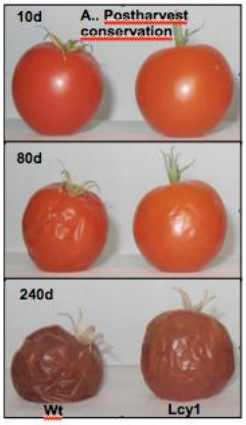
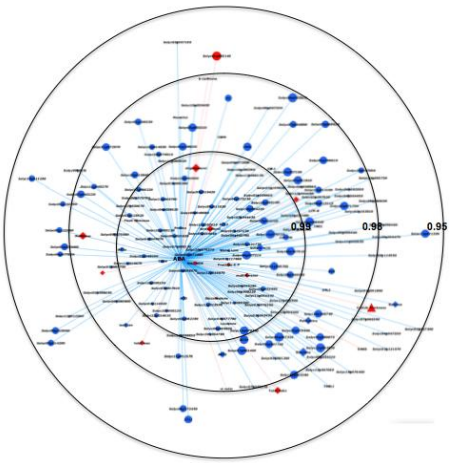
Tomato fruits accumulate, during ripening, the linear carotene lycopene, partially due to the transcriptional repression of the pathway leading to β-carotene and beta xanthophylls. Increasing evidence is accumulating for a role of ABA in controlling ripening of both climacteric and non-climacteric fruits. We generated transgenic tomato fruits overexpressing the *LYCOPENE BETA CYCLASE (LCY)* gene and accumulating β-carotene. Unexpectedly, these fruits also show enhanced shelf-life and delayed softening. Cell wall composition in ripe fruits was changed, with an increase of sugars present in the pectic backbone. LCY fruits also showed moderate changes in the levels of several metabolites, many of which can be explained in terms of delayed fruit ripening. Affymetrix microarray analyses showed changes in the expression of cell wall-remodeling enzymes, induction of ABA-regulated genes and repression of ethylene-regulated ones. In keeping with these observations, ABA and ethylene levels were, respectively, increased and decreased in LCY fruits. The transcript levels of two transcription factors, acting as master regulators of fruit ripening (Non-ripening or NOR, and Ripening Inhibitor or RIN) showed, respectively, strong direct and inverse co-regulation with ABA levels. These observations suggest that β-carotene and its downstream product, ABA, are integral parts of a regulatory loop controlling fruit ripening (Fig. 1)

**Figure 1.** Elongated shelf-life phenotype (left) and correlation network using ABA as central hub.



## O34. Identification of causal mutations of metabolic QTLs associated to grape and wine flavor

Paula Moreno-Sanz[1], Francesco Emanuelli[1], Silvia Lorenzi[1], Xiaoguang Yu[1], Laura Costantini[1],Sergio Moser[2], Roberto Larcher[2], Juri Battilana[1], M. Stella Grando[1]

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Secondary metabolites produced in grapevine berries play an essential role in high-quality wines and also contribute to the quality of table grapes. Some of the most prevalent wine odor constituents are monoterpenoids which biosynthesis via the plastidial methyl-erythritol-phosphate (MEP) pathway has been demonstratedin grapevine.

Based on a double pseudo-testcross mapping strategy, we detected a major QTL on LG5 for linalool, neroland geraniolcontent in grapevine berries at ripening time, and also an additional QTL for linalool on LG10. Further testingindicated that gain-of-function mutations in the structural gene of the MEP pathway *1-deoxy-D-xylulose-5-phosphate synthase* (*VvDXS1*)-colocalizedwith the mQTL on chromosome 5 - are the major determinants for terpenoid accumulation in Muscat grape varieties and have direct effects on theenzymatic or regulatory properties of the DXS protein.Similarly, association of genetic variants with the content of several volatile aromatic compounds were tested for candidate genes in the mQTL interval on chromosome 10. These findings may pave the way for metabolic engineering of terpenoid contents in grapevine.

**Session VI – Marine terpenoids: chemical diversity, function and biotechnology**

## O35. Marine terpenoids and the conquest of land

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Many sessile or slow-moving marine organisms, especially cnidarians, sponges, and opisthobranch mollusks, have a strong smell, but only if they are taken out of the water, because their water-insoluble molecules are volatile in air. Like their terrestrial volatile counterparts, the marine liposoluble metabolites play important roles in chemical communication, mediating key biological processes in the aquatic environment, such as sexual reproduction, nutrition, and defense. In spite of this, the notion that chemical communication in aquatic environments is mediated only by hydrophilic compounds, while odorants on land, needing to be volatile, are hydrophobic, is overemphasized in a number of research papers. Contradictory speculations on the evolution of olfactory receptor genes do not help to shed light on the issue. These incongruities call the field of chemoreception to reassess its paradigmatic foundations and the empirical and theoretical work conducted within them. Based on our most recent findings on the function and chemical diversity of marine terpenoids, a new perspective on the evolutionary history of terpenoids, and on their role in the adaptive evolution of olfaction during the transition from aquatic to terrestrial life will be presented.

## O36. Chemodiversity and bioactivity of terpenoids from East Mediterranean algae

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The marine environment is a rich source of novel and unusual secondary metabolites, many of which have already shown considerable promise for development as therapeutic agents. Chemical studies on marine algae have led to the isolation of a large number of structurally unique secondary metabolites with a wide spectrum of biological activities.

The biodiversity of the Mediterranean ecosystem hosts an immense number of indigenous species, as well as organisms that have migrated from the Atlantic Ocean, the Red and Black Seas.

As part of our studies on the chemical composition and biological activity of marine organisms, our group has investigated a significant number of algal species found along the Greek coastline. Among the investigated species red algae of the genera *Laurencia* and *Sphaerococcus* have yielded a significant number of halogenated terpenoids with cytotoxic and cytostatic activities. Brominated diterpenes from *S. coronopifolius* have exhibited settlement inhibition activity on the cyprids of *Balanus amphitrite* without toxic effects on non target organisms. The brown alga *Dilophus spirallis* was found to contain several new bicyclic and tricyclic diterpenes belonging to the classes of dolastanes and dolabellanes.

The high number of already isolated natural products, in conjunction with the complex chemical profiles and geographical and seasonal variation frequently observed for species of the genus *Laurencia*, adds an extra degree of difficulty in the detection and isolation of new metabolites.

In search of a fast and reliable screening tool for the chemical profiling of *Laurencia* algal extracts and the detection of new secondary metabolites, we have developed a high throughput fingerprinting methodology based on the complementary application of LC-MS and NMR. The preliminary results of this study point out the potential for the direct screening of crude algal extracts in order to detect new compounds, as well as to trace biomarkers and /or monitor the presence of targeted metabolites.

## O37. Early biosynthetic steps of triterpene biosynthesis in the microalga *Botryococcus braunii*, race B

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*Botryococcus braunii* is a green microalga which produces large amounts of liquid hydrocarbons. Thus, this alga has gained attention as a bio-fuel alternative to petroleum, though its practical applications have not been successful because of its very slow growth. The alga is classified into three races A, B, and L depending on the type of the hydrocarbon produced. The B race produces two types of triterpene hydrocarbons; methylsqualenes and botryococcenes. A feeding experiment using $^{14}$C-labeled sodium bicarbonate suggested that a large proportion of photosynthetically fixed carbon was incorporated into the triterpene hydrocarbon fraction [1]. The slow growth may result from such diversion of reduced carbon into energetically expensive compounds. It was recently found that the B race of *B. braunii* possesses three squalene synthase-like enzymes and its triterpenes are synthesized through a two-step reaction catalyzed by a combination of two enzymes among the three [2]. To better understand the entire process of triterpene production by *B. braunii*, it is necessary to uncover how this alga introduces photosynthetically fixed carbon into the biosynthetic pathway not for polysaccharides but rather for the pathway of triterpenes. Additionally, it is paramount to determine how the universal terpene precursor is produced and shared for the production of different types of terpenes. A feeding experiment using $^{13}$C-labeled glucose suggested that the B race of *B. braunii* uses the methylerythritol 4-phosphate (MEP) pathway for triterpene production [3]. We have been doing characterization of the MEP pathway of this alga and will present some of its unique aspects.

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## O38. Biochemical characterization of the biosynthesis of thetetraterpenoid hydrocarbon lycopadiene from *Botryococcus braunii*

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*Botryococcus braunii* isa colony-formingcosmopolitan green microalga that accumulates 30-86% of its dry weight as liquid hydrocarbons in their extracellular matrix. *B. Braunii* has received significant attention over the years as a potential source of renewable energy because the caustic hydrolysis ofthe liquid hydrocarbons results in fuels that are highly compatible with the existing petroleum infrastructure. There are three different races of *B. braunii*based on the hydrocarbons they synthesize. Race A produces fatty acid derived alkadienes and alkatrienes, race B produces thetriterpenoid hydrocarbons squalene andbotryococcenes,and race L, the focus of this study, produces the tetraterpenoid hydrocarbon knownas lycopadiene. The biosynthetic pathway for lycopadiene has not been elucidated,but it has been suggested to occur by condensation of two molecules of phytyl diphosphate, or alternatively by condensation of two molecules of geranylgeranyldiphophate (GGPP) to form lycopersene (aka lycopaoctaene), which could then be reduced to lycopadiene. To begin to study the mechanism of lycopadiene biosynthesis, we extracted crude L race hydrocarbons and analysis by GC/MS showed it contains predominantly lycopadiene plustwominor components; lycopapentaene and lycopatriene. Identification of these two minor components in the L racehas not been reported and may suggest that two molecules of GGPP are used to form lycopaoctaene by a squalene synthase (SS) or phytoene synthase (PSY)-like reaction mechanism. Lycopaoctaene may then undergo double bond reduction to form lycopapentaene, lycopatriene, and finally lycopadiene. Recently, we have developed a cell free *in vitro* assay for lycopadiene synthesis activity with assay conditions similar to that for the SS. Assays were conducted using GGPP as a substrate and algal lysate as an enzyme source. GC/MS analysis of reaction products shows the production of lycopadiene *in vitro*. This result is the first evidence of GGPP as a direct precursor for lycopadiene biosynthesis further corroborating the second proposed pathway above. We also investigated the cellular localization of lycopadiene synthesis activity in algal cells using fractionated cells components and found thatapproximately 90% of the activity was in the membrane fraction, suggesting that the enzymes responsible for lycopadiene production is associated with membranes. In order to identify genes responsible for the biosynthesis of lycopadiene, we computationally screened a *B. braunii* L race transcriptomic database for SS and PSY-like sequences. Two SS-like cDNAs and one PSY cDNAwere identified and are in the process of being analyzed for the enzyme activity of the encoded proteins. We hypothesize that one of the SS-like enzymes is responsible for the first step in lycopadiene production; condensation of two GGPP molecules.

**Session VII – Understanding of structure and function of terpene synthases**

## O39. Evolution of isoprenyldiphosphate synthases: gatekeepers to greater terpenoid metabolism

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Isoprenyldiphosphate synthases (IDS) have been studiedextensivelyin plant systems, where they catalyze production of isoprenyldiphosphates that form the chemical foundation for terpenoid metabolism. Genomic and proteomic analyses have resulted in development of models which enable researchers to trace evolutionary pathways andpredict function of plant IDS. However, bacterial IDS often fail to conform to these models, requiring further analysis of protein sequence and function. Functional characterization has revealed that bacterial systems support both vast networks of semi-redundant IDS and minimalistic networks of multi-functioning or metabolically controlled IDS, both of which lead to biosynthesis of multiple terpenoid products. Furthermore, the extensive array of sequenced bacterial genomes allows analysis into the structure-function relationship of IDS during continual genome remodeling and lends insight into the genomic and metabolic control of terpenoid metabolism in bacteria.

## O40. The Tomato cis-prenyltransferase gene family

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The sequential transfer of isopentenyl diphosphate (IPP) units to allylic diphosphate acceptors is catalyzed by enzymes known as prenyltransferases. Depending on the stereochemistry of the polyisoprenoid product, these enzymes are classified as either *trans*-prenyltransferases (TPTs) or *cis*-prenyltransferases (CPTs). While the roles of CPTs in animals and bacteria are well established, very little is known about plant CPTs. Here we describe the seven member family of CPTs from tomato (SlCPT1-SlCPT7). Three members of the tomato CPT family are involved in the synthesis of short chain (≤ C20) products - SlCPT1 produces neryl diphosphate, SlCPT6 produces *Z,Z*-FPP and SlCPT2 catalyzes the formation of nerylneryl diphosphate. Interestingly, all the short-chain CPTs from tomato are also closely linked to terpene synthase gene clusters. Enzymatic characterization of the remaining four CPT family members demonstrated that SlCPT4, SlCPT5, and SlCPT7 synthesize longer chain products (C25-C55). Phylogenetic analysis revealed the widespread occurrence of related CPTs throughout the plant kingdom, suggesting a conserved physiological function for medium chain polyisoprenoids. While no *in vitro* activity could be demonstrated for SlCPT3, its expression in the *Saccharomyces cerevisiae* dolichol biosynthesis mutant (*rer2*) complemented the temperature sensitive growth defect. We further show that dolichol biosynthesis in tomato requires a partner protein which anchors SlCPT3 to the ER membrane, the site of dolichol synthesis in all eukaryotes.

## O41. Decoding Terpene synthase design features using large biochemical datasets

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The limits of protein evolution are ultimately defined by biophysical constraints. Specifically, the maintenance of protein folding and stability is essential for the emergence novel enzyme function in the face of accumulating mutations. This fundamental requirement necessitates a viable mutational pathway towards beneficially altered function, but the frequency of such pathways has never been measured. To measure the biophysical constraints on the evolution of terpene biosynthesis, we performed a quantitative assessment of fold thermostability along all possible mutational pathways linking a wild type terpene synthase from *Nicotiana tabacum* (tobacco) to its catalytically evolved nonuple (9) mutant. We measured the thermal unfolding profiles of a 512 mutant (29=M9) library using a high throughput fluorescencebased assay.

These unfolding profiles revealed a broad range of stability phenotypes across the library. Co-variation analyses across the folding and stability landscapes of the 9 mutant combinations identified several statistically significant nonadditive effects and distinct thermal unfolding profiles. Together, these computational and experimental approaches uncovered additional levels of protein epistasis beyond the previously identified catalytic landscapes of the M9 library [1].

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# O42. Functional analysis of prenyltransferses responsible for diterpene biosynthesis in fungi

Chengwei Liu,[1] Motoyoshi Noike,[1] Atsushi Minami,[2] Hideaki Oikawa,[2] and Tohru Dairi[1]

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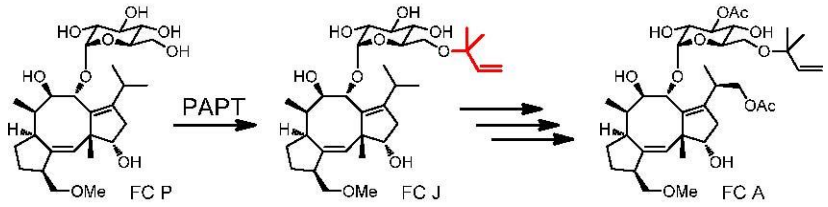
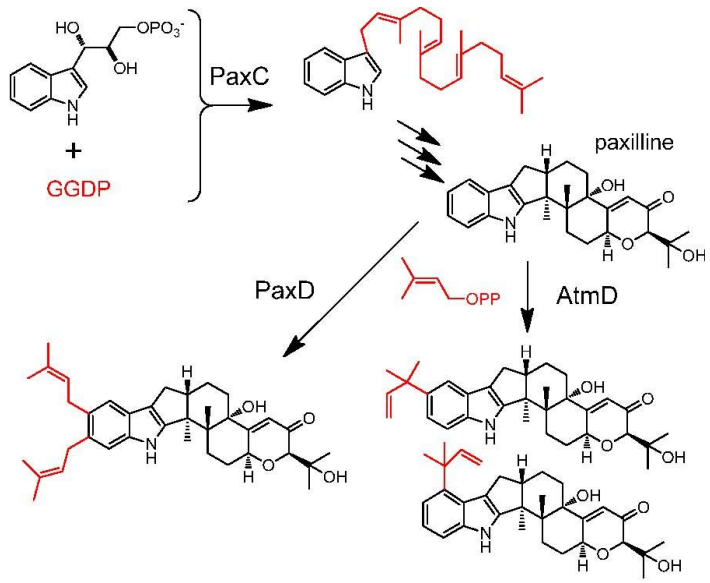
Fusicoccin A (FC) is a diterpene glycoside produced by a fungus *Phomopsis amygdali* and has a unique *O*-prenylated glucose moiety. In this study, We cloned FC biosynthetic genes clusters, which were scattered at two different loci, one contained four genes and another did nine genes, and characterized most of their products. Consequently, we identified *papt* gene encoding a prenyltransferase that reversely transferred DMAPP and geranyl diphosphate to the 6'-hydroxygroup of the D-glucose moiety of FC P, an intermediate of FC A biosynthesis.
Detailed enzymatic properties were also investigated with recombinant enzyme. This is the first report of an enzyme capable of catalyzing the prenylation of a hydroxyl group of a sugar.




We also characterized three prenyltransferases participating in indole-diterpene biosynthesis.

The *paxC* and *paxD* are located in paxilline gene cluster in *Penicillium paxilli*. The PaxC was confirmed to be a prenyltransferase that forms geranylgeranyl indole by transfer of GGDP to indole-3-glycerol phosphate and lesser to indole. The PaxD catalyzed successive transfer of two molecules of DMAPP into paxilline to yield 21,22-diprenylated paxilline: the first example of the fugal prenyltransferase catalyzing di-prenylation reaction. The *atmD* is located in aflatrem gene cluster in *Aspergillus flavus*. AtmD, which has 32% amino acid identity to PaxD, gave two major and a trace of minor products, all of which were identified as mono-prenyl paxillines. Structures of the two major products were determined to be reversely monoprenylated paxilline at 20-and 21-position. Detailed enzymatic properties of all three enzymes were also investigated.



## O43. A novel type of terpene synthase genes in a nonseed plant *Selaginella moellendorffii*

Hao Chen[1], Guanglin Li[1], Tobias G. Köllner[2], Yanbin Yin[3], Yifan Jiang[1], Ying Xu[3,4], Jonathan Gershenzon[2], Eran Pichersky[5], Feng Chen[1]

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Using *in silico* analysis, we identified a novel type of terpene synthase (TPS) genes from the genome of a nonseed plant *Selaginella moellendorffii*. Consisting of 48 members, this new type of terpene synthase genes were collectively designated as *S. moellendorffii* microbial *TPS*-like genes (*SmMTPSLs*) based on their closer evolutionary relatedness to microbial TPSs than that to classical plant TPSs. Most *SmMTPSL* genes do not contain any intron or contain only one intron, while most classical plant *TPS* genes contain six or more introns. *SmMTPSL* genes encode proteins of ~350 aa, while classical plant *TPS* genes encode proteins of 550-800 aa. Among the 48 *SmMTPSL* genes, 40 genes appear to be full-length. 34 *SmMTPSL* genes showed expression under one or multiple stress conditions tested. Full-length cDNAs for 28 *SmMTPSL* genes were cloned and expressed in *E. coli* to produce recombinant enzymes. Terpene synthase enzyme assays showed that 15 SmMTPSLs were active using geranyl diphosphate, farnesyl diphosphate or both as substrates. Some *in vitro* products of SmMTPSLs were detected in the headspace of *S. moellendorffii* plants treated with a fungal elicitor alamethicin and fall armyworm (*Spodoptera frugiperda*) caterpillars. The presence of a novel type of terpene synthase genes in the genome of *S. moellendorffii* broadens the molecular basis underlying the vast chemical diversity of terpenoids in plants.

## O44. Reconstructing terpene biosynthesis: Protein engineering to redirect precursor fluxes

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The development of heterologous systems for the production of terpenes has attracted significant attention in recent years. Reconstruction of terpene biosynthetic pathways in yeast, plant or bacterial cells can be particularly useful, not only for enzyme characterization and pathway elucidation, but also for the industrial production of compounds. The yeast *S. cerevisiae* is widely used as a host for terpene production, as it is robust, compatible with existing industrial infrastructure, and readily amenable to genetic modification. Although metabolic engineering approaches have made great strides in improving the flux through the yeast mevalonate pathway, leading to significant titres of sesquiterpenes (e.g. amorphadiene, santalene, caryophyllene), monoterpene or diterpene production has so far been less efficient. This appears to be due to the inherent design of the yeast sterol biosynthetic pathway that favours FPP synthesis. To improve monoterpene and diterpene production in yeast, a protein engineering effort to alter the product specificity of endogenous yeast enzymes and direct fluxes towards GPP or GGPP formation was undertaken. Improvements of 300-fold in sabinene and 100-fold in sclareol production are reported.

## O45. ‚Cineole cassette' monoterpene synthases in the genus of Nicotiana

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A large number of emitted plant terpenoids are known but the molecular mechanisms underlying their synthesis is often not completely understood. Many monoterpenes are synthesized by multi product terpene synthases. Flowers of the genus *Nicotiana*, especially of section *Alatae* emit a characteristic set of the so called ‚cineole cassette' monoterpenes. Typical major compounds are alpha-terpineol and 1,8-cineole, while minor compounds of the ‚cineole cassette' are limonene, sabinene, beta-myrcene, alpha-pinene and beta-pinene. We successfully isolated several monoterpene synthases of various *Nicotiana* species synthesizing the 'cineol cassette' monoterpenes. According to their major product the enzymes are named terpineol (TER) and cineole (CIN) synthases, respectively. Having these genes and enzymes in hand, a detailed study of the addition reaction of the hydroxyl group of the precursor alpha-terpineol with the double bond to introduce the second intramolecular cycle resulting in 1,8-cineole can be performed. The efficiency of this cyclization reaction is different in various TER and CIN enzymes, e.g. alpha-terpineol is synthesized in larger amounts by enzymes of *N. alata* and *N. langsdorfii*, while the *N. bonariensis, N. forgetiana, N. longiflora,* and *N. mutabilis* monoterpene synthases primarily produce 1,8-cineole. A site-directed mutagenesis approach is performed to identify amino acids with impact on the cyclization reaction. Furthermore, these ‚cineole cassette' monoterpene synthases of *Nicotiana* species may give insight in their relationships and evolution. For example, species of section *Alatae* emit 'cineole cassette' monoterpenes, while species of the sister section *Suaveolentes* don't, which may be explained by the presence/absence of terpene synthase genes, respectively. It was hypothesized that one parent of the Australian section *Suaveolentes* is a member of the present section *Alatae* and the other parent is either a member of the section *Acuminatae* (= *Petunioides*) or *N. sect. Noctiflorae*. Thus, we started to screen closely related *Nicotiana* species to search for the CIN and TER progenitor species.

# O46. Substrate promiscuity in vivo of plant protein prenyltransferases is a result of substrate availability through the MVA or the MEP pathway

Andréa Hemmerlin[1], Alexandre Huchelmann[1], Michel Rohmer[2], Thomas J. Bach[1]

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Type-I protein isoprenylation refers to a post-translational modification by a prenyl group of a cysteine residue of a so-called C-terminal CaaX motif. This zinc cation-mediated sulfur alkylation is catalyzed by protein prenyl transferases (PPTs): protein farnesyltransferase (PFT) or type I protein geranylgeranyltransferase (PGGT-I). Both the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways generate isoprenoid units used as prenyl diphosphate substrates by these enzymes. This was demonstrated by incorporation of radiolabeled MVA [1], but also of deoxyxylulose [2] into proteins. This plant-specific metabolic plasticity may guaranty the presence of continuous prenyl diphosphate pools necessary to modify essential proteins under various environmental and physiological conditions. Regulation in a cellular environment of protein isoprenylation was deciphered by using GFP-based reporter protein-substrates harboring C-terminus CaaX motifs, which are either substrate of PFT (GFP-CVIM) or PGGT-I (GFP-CVIL) [4]. The activities and substrate specificities of recombinant PPTs were compared to those observed *in vivo* using characterized *Arabidopsis* loss-of-function mutants expressing the GFP-CaaX proteins and only one active PPT: either PFT or PGGT-I. They were also compared to enzyme activities contained in plant protein extracts. Apparent inconsistencies between enzyme properties were observed in our studies. The results led us to conclude that enzyme activities and substrate specificities are tightly regulated *in vivo* and demonstrated that MEP-dependent isoprenylation is a consequence of limited PGGT-I protein substrate specificity *in vivo*. Based on this particularity, we have set up a screening system aiming at identification of metabolites or compounds that act as molecular switches for using one or the other isoprenoid pathway under particular conditions. Special attention was given to impacts of modulating isoprenoid precursor pools on PPT activities *in cellulo*. Their effects were determined to gain insight into a possible role of substrate availabilities generated either by the MVA or the MEP pathway. Interestingly, these modulations induce opposite effects on capacities to modify a farnesylated protein as compared to a geranylgeranylated protein. A model has been designed that proposes a mechanism explaining the strategy developed by plants to use either MEP or MVA pathway-derived metabolic pools to secure a continuous protein prenylation.

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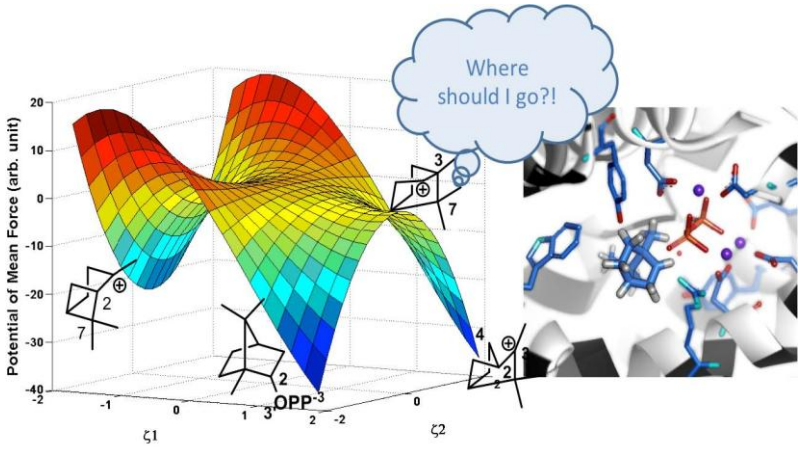
## O47. Electrostatically guided dynamics – the root of fidelity in a promiscuous terpene synthase?

Dan T. Major, Michal Weitmann

Department of Chemistry and the Lise Meitner-Minerva Center of Computational Quantum Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel

Terpene cyclases are responsible for the initial cyclization cascade in the multistep synthesis of more than 60,000 known natural products. This abundance of compounds is generated using a very limited pool of substrates based on linear isoprenoids. The astounding chemodiversity obtained by terpene cyclases suggests a tremendous catalytic challenge to these often promiscuous enzymes. In the current study we present a mechanistic view of the biosynthesis of the monoterpene bornyl diphosphate (BPP) from geranyl diphosphate by BPP synthase (BPPS) using state of the art simulation methods. We identify bornyl cation as an enzyme induced bifurcation point on the multidimensional free energy surface, connecting between the product BPP and the side product camphene. Chemical dynamics simulations suggest that the active site diphosphate moiety steers reaction trajectories towards product formation. Nonetheless, chemical dynamics is not precise enough for exclusive product formation, providing a rationale for fidelity in this promiscuous terpene cyclase.[1,2]

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## O48. Chrysanthemyl diphosphate synthase operates in planta as a bifunctional enzyme with chrysanthemol synthase activity

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Although pyrethrins represent the economically most important and widely used natural pesticide, not much is known about their biosynthesis at the genetic or enzymatic level. Only the genes involved in the first and last step of the biosynthetic pathway have been cloned, which are chrysanthemyl diphosphate synthase (CDS; (Rivera et al., 2001)) and a GDSL lipase-like protein(Kikuta et al., 2012).CDS catalyzes c1'-2-3 cyclopropanation reactions of two molecules of dimethylallyl diphosphate (DMAPP) to yield chrysanthemyl diphosphate (CPP; Fig. 1).Three proteins are known to catalyze this cyclopropanation reaction of terpene precursors. Two of them, phytoene and squalene synthase, are bifunctional enzymes with both prenyltransferase and terpene synthase activity. CDS, the other member, was reported to perform only the prenyltransferase step. Here, we show that, under lower substrate conditions prevalent in plants, CDS also catalyzes the next step converting CPP into chrysanthemol by hydrolyzing the diphosphate moiety (Fig. 1). The enzymatic hydrolysis reaction followed conventional Michaelis-Menten kinetics, with a $K_M$ value for CPP of 196 μM. For the chrysanthemol synthase activity, DMAPP competed with CPP as substrate. The DMAPP concentration required for half-maximal activity to produce chrysanthemol was ~100 μM, and significant substrate inhibition was observed at elevated DMAPP concentrations. The N-terminal peptide of CDS was identified as a plastid targeting peptide. Transgenic tobacco plants overexpressing CDS emitted chrysanthemol at a rate of 0.12 – 0.16 $\mu g \cdot h^{-1} \cdot g^{-1}$ FW. Chrysanthemol glycosides were also detected and the major one was putatively identified as chrysanthemol conjugated to malonyl-glucose. We propose that CDS should be renamed a chrysanthemol synthase (CHS) utilizing DMAPP as substrate.

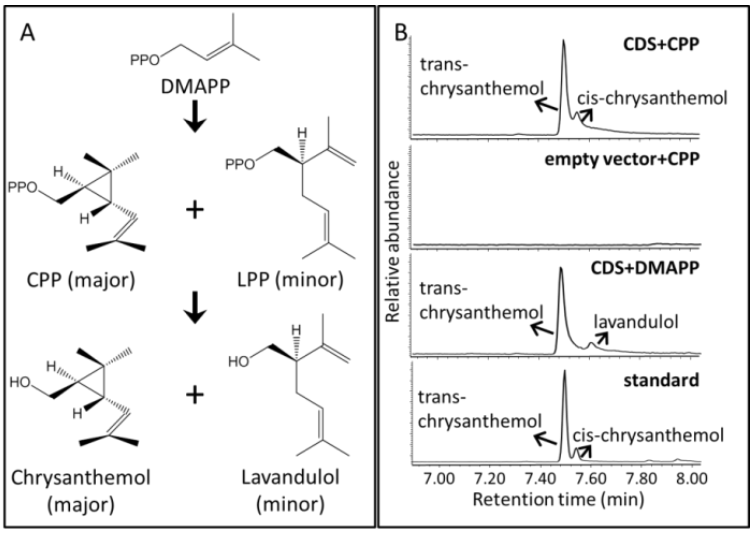


**Fig. 1.** Reactions catalyzed by CDS. A, Major, major product of the step; minor, minor product of the step.B,GC-MS chromatograms of the products of purified CDS enzyme assayed with CPP or DMAPP.

## O49. Isoprene synthase genes form a monophyletic clade of acyclic terpene synthases in the Tps-b terpene synthase family

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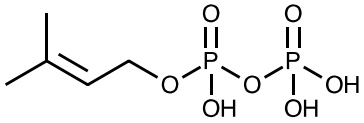
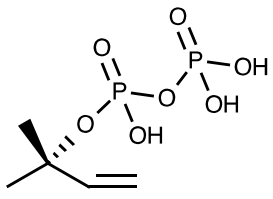
Many plants emit significant amounts of isoprene, which is hypothesized to help leaves tolerate short episodes of high temperature. Isoprene emission is found in all major groups of land plants including mosses, ferns, gymnosperms and angiosperms; however, within these groups isoprene emission is variable. The patchy distribution of isoprene emission implies an evolutionary pattern characterized by many origins or many losses. To better understand the evolution of isoprene emission we examine the phylogenetic relationships among isoprene synthase and monoterpene synthase genes in the angiosperms. In this study we identify nine new isoprene synthases within the rosid angiosperms. We also document the capacity of a myrcene synthase in *Humulus lupulus* to produce isoprene. Isoprene synthases and (*E*)-β-ocimene synthases form a monophyletic group within the Tps-b clade of terpene synthases. No asterid genes fall within this clade. The chemistry of isoprene synthase and ocimene synthase is similar and likely affects the apparent relationships among Tps-b enzymes. The chronology of rosid evolution suggests a Cretaceous origin followed by many losses of isoprene synthase over the course of evolutionary history. The phylogenetic pattern of Tps-b genes indicates that isoprene emission from non-rosid angiosperms likely arose independently.

Figure 1. Isoprene synthase does not use the linalyl diphosphate analog of DMADP suggesting it does not reattach pyrophosphate the way that cyclic monoterpene synthases do.




Dimethyl allyl diphosphate




2-methyl 3-buten 2-yl diphosphate

**Special Lecture I**

## O50. Unexpected links between protein farnesylation and microRNA function

Peter Brodersen,

University of Copenhagen

microRNAs (miRNAs) are small non-coding RNAs that play a central role in eukaryotic gene regulation. They form repressive complexes with ARGONAUTE (AGO) proteins, and bring about sequence specific mRNA repression by recruitment of AGO to complementary mRNA.

Central steps of the miRNA pathway, such as AGO loading with miRNA, and target mRNA recognition, are often viewed as reactions that take place in solution. Meanwhile, early studies identified human AGO2 as the Golgi/ER associated protein GERp95, and several recent reports on plant and animal miRNAs have confirmed the importance of membrane association for AGO function. Our studies in Arabidopsis have shown that defects in isoprenoid biosynthesis have a profound impact on miRNA function, and that defective sterol biosynthesis accounts for part of this reduced miRNA function. In addition to sterols, evidence pointing to a role of protein farnesylation in miRNA function and membrane association of AGO1 will be discussed.

**Session VIII – "Terpenoids and beyond" (Organized by COST ACTION Plant Engine)**

## O51. Introduction to COST Action FA 1006 – PlantEngine

Warzecha Heribert

Technische Universitaet Darmstadt, Biologie, Schnittspahnstrasse 3-5

A significant amount of knowledge has been gained during the last decades about the biosynthetic capacity of plants and the pathways leading to the formation of plant natural products (PNPs), many of which are of high relevance as pharmaceuticals or fine chemicals for industries. To fully exploit the capacity of engineering plants for the production of high value PNPs, COST Action FA1006, PlantEngine, will support and enhance a Pan-European network which will amalgamate resources, define target pathways and prioritize compounds, disseminate novel technologies and applications, set standards for computational support, and develop synthetic approaches in plant metabolic engineering. The current state of the Action, now in its third year, will be presented.

## O52. Cellular and Metabolomic perturbations associated with engineering isoprenoids in Solanaceae

Paul, D. Fraser[1], Peter M. Bramley, Marilise Nogueira[1], Tom Wells[1], Laura Perez[1] and Eugenia M. A. Enfissi[1]

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Isoprenoids, also known as terpenoids represent the largest and oldest class of natural products known, consisting of >40,000 different molecules, all biosynthetically related via a common precursor (isopentenyl). Many dietary derived isoprenoids confer important health properties, while other isoprenoids are high-value fine chemicals used in the feed, food, cosmetic and pharmaceutical industrial sectors. Carotenoids are a class of isoprenoids responsible for most of the orange, red and yellow colours found in nature. The carotenoid, □-carotene (provitamin A) is an essential component of the human diet. Other carotenoids such as lycopene and zeaxanthin are potent antioxidants and are believe to reduce the incidence chronic disease states, e.g. prostate cancer and age-related macular degeneration, respectively.

Over the past decade genetic/metabolic engineering of carotenoid biosynthesis and accumulation has resulted in the generation of transgenic varieties containing enhanced or altered carotenoid [1]. In achieving this important goal many fundamental lessons have been learnt. Most notably is the observation that the endogenous carotenoid pathways in higher plants appear to resist engineered changes. These mechanisms may include feedback inhibition [2], forward feed, metabolite channelling [3], and counteractive metabolic and cellular perturbations [4 and 5]. In the present presentation the progress made in the genetic engineering of isoprenoids, particularly carotenoids in tomato fruit and other *Solanaceae* will be reviewed, highlighting the limiting regulatory mechanisms that have been observed experimentally. The potential of metabolomics to characterise these changes will be illustrates and how changes in the composition of metabolites can impact on the plastids generated and their internal ultrastructure.

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## O53. Cell-based functional screening of modified betulins, abietanes and resin acids as lead compounds for anti-cancer drugs (BARC)

Matthias Nees[1], Raisa Haavikko[2], Vania Moreira[2], Sami Alakurtti[1],Jari Yli-Kauhaluoma[1,2], Heiko Rischer[1], EnkheePurev[1], Johannes Virtanen[1], Kirsi-Marja Oksman-Caldentey[1]

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In a joint project between University of Helsinki and VTT, altogether over hundred novel lead compounds based on triterpenes such as betulin and betulinic acid and diterpenes(resin acids and abietanes) from pines (Pinus sp.) and spruce (Picea sp.) weresystematically tested for novel pharmaceutical activities. Both compound classes representlarge-scale by-products of the forest industry. Chemical derivatives from betulin, abietanes and resin acids werechemically synthesized and characterized, to determine the relationship between chemical structure and their biological anti-cancer activity (structure-activity relationships SAR). In addition, an *in vitro* biotransformation approach based on a panel of native and/or genetically modified plant cell cultureswasused to generate a resource of bioactive metabolites. These terpenoid-derived compound/metabolite collections werethen combined to generate libraries for high throughput and high content cell-based functional screening. Using advanced cell culture model systems in standard plate-based(2D) and three-dimensional (3D) organotypic cultures, we specifically focused on the testing for anti-cancer properties such as inhibition of tumour cell growth and invasion. Further, the BARC project aims to explore the mechanisms of action (MOA) for the most potent candidates, and to identify synergistic effects of betulin- and resin derivatives in combination with established standard-of-care therapeutics.

Technically, the functional screening in BARC wasorganized in a hierarchical fashion. All compounds weretested for basic anti-cancer (cytotoxic, anti-proliferative, or pro-apoptotic) properties in a plate-based, high throughput-screening format, using an expanded panel of cancer cell lines. Subsequently, the same libraries were functionally tested in 3D organotypic cultures that mimic additional aspects such as tumour-specific de-differentiation, histology, and invasion. These models incorporate the extracellular matrix and tumour microenvironment as key concepts of cancer biology, with a focus on advanced, castration-resistant prostate cancers that are highly metastatic and currently incurable. Hits from these combined 2D and 3D screens were then validated by independent means, such as wound healing and cell motility/migration assays. Validated hits serve as targets for the next cycle of medicinal chemistry and SAR, to be tested in the same models as described.

Currently, the best hits are undergoing SAR/functional screening cycles, and the first MOA studies are being initiated. The most promising betulin-derived compounds show a profound inhibition of tumour-specific growth and invasion in the pharmacologically relevant range of 100 nM; which we hope to further improve. In addition to the focus on cancer biology, also other pharmacological aspects such as anti-leishmanial activities will be tested.

## O54. Why is your plant extract like a syrup? Natural Deep Eutectic Solvents?

R. Verpoorte and Y.H. Choi

Natural Products Laboratory, Institute of Biology Leiden,
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Each of us has probably a number of questions from observations we made in the past but not really know how to explain them. Like: "why is my plant extract a syrup; how are non-water soluble compounds like taxol biosynthesized; how can there be very high levels of water insoluble flavonoids and anthocyanins be present in soluble form in flowers; how do seeds and plants survive cold and drought", etc.?

Since we started with NMR profiling of our plant cell cultures (Schripsema and Verpoorte 1991), a method that now is known as NMR-based metabolomics, we have accumulated spectra of more than 28,000 plant extracts, as well as of various other organisms such as microorganisms and zebra fish. The data we collected in NMR-based metabolomics also made us to ask a further question "why are a few very simple molecules always visible in considerable amounts in the NMR spectra of any organism"? They must have a basic function in living cells. They include sugars, amino acids (e.g. proline, alanine, glutamine, asparagine), choline, and organic acids (e.g. malic, lactic, succinic acid). Sugars serve for storage and energy, the other compounds are in amounts that does not make sense to consider them only as metabolic intermediates.

This resulted in a quite exciting discovery: the Natural Deep Eutectic Solvents (NADES).

Our first idea was the possibility that the ubiquitous choline (and betaine) might form ionic liquids with the organic acids, present in similar quantities as observed from the NMR spectra. Indeed we found mixtures like choline chloride-maleic acid to form ionic liquids in certain fixed molar ratios (e.g 1:1). In further studies we found that other common neutral compounds, as well as organic acids, bases and amino acids, may form deep eutectic solvents, e.g. mixtures of sugars and also sugars with malic acid, choline, betaine or proline. These solvents are viscous and are characterized by strong hydrogen bonding between the constituents. NADES have a polarity in the range from water to ethanol, and are excellent solvents for natural products, including DNA and proteins, often with orders of magnitude higher solubility than in water. In our hypothesis many cellular and physiological functions are connected with the occurrence of NADES. For example nectar on flowers is constituted of a NADES. Both cold and drought resistant is connected with the production of compounds that form NADES, which have no vapor pressure and thus are non-volatile (liquid crystals), are liquid even at temperatures far below 0º C, miscible with water, and sometimes contain also 1M water which is strongly retained. All these characteristics fit perfectly the known chemistry of for resurrections plants and other drought resistant organisms.

Besides all implications for understanding cellular processes such as the biosynthesis of non-water soluble compounds, and the presence of anthocyanins and flavonoids in cells the NADES also have many potential applications in green chemistry for extraction and enzyme reactions. The advantage of NADES is that they are low cost, fully sustainable solvents.

References

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Dai Y, et al. (2013) Natural deep eutectic solvents as new potential media for green technology Anal Chim Acta 766: 61-68

## O55. Beyond Terpenoids: Mechanistic and miocatalytic insight into the formation of mero- and non-natural terpenoids

Ludger Wessjohann*, Wolfgang Brandt et al.

Leibniz Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle (Saale), Germany

The chemodiversity of "pure" isoprenoids is at least doubled by natural products that combine isoprenoids with other metabolic pathways, and yet can be extended further almost infinitesimally to non-natural products by using natures machinery for chemoenzymatic extensions into an artificial natural-like structural space.

The lecture will discuss selected isoprenylated compounds of commercial potential, provide insight and background of the mechanism(s) of intermolecular prenyl transfer with an emphasis on aromatic prenylation, and discuss the potential of these enzymes in biotechnology. With the background of understanding the processes involved, examples for an extension beyond the synthesis of natural products will be given.

## O56. Mono- and sesquiterpene biosynthesis in leaves and aphid-induced-galls of *Pistacia palaestina* Boiss

Karin Rand[1,2], Einat Bar[1], Rachel Davidovich-Rikanati[1], José Abramo Marchese[3], Natalia Dudareva[4], Moshe Inbar[2], Efraim Lewinsohn[1]

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The aphid *Baizongia pistaciae* L. induces large, banana-like galls on terebinth trees (*Pistacia palaestina* Boiss., Anacardiaceae). The aphids inhabit the galls for several months and the galls provide both food and protection from abiotic factors and natural enemies for the aphids. Terpenes may be part of this defense. We performed a comparative analysis of the mono- and sesquiterpene accumulation and biosynthesis in *P. palaestina* leaves and in *B. pistaciae*–induced galls in naturally growing trees in Northern Israel. Inter-tree variations were apparent in terpene composition in galls that resembled the leaf composition from the individual trees. Still, galls contained 5 to 15-fold more monoterpenes than leaves and specifically accumulated higher levels of α-thujene, α-pinene, β-pinene, sabinene, β-myrcene, α-phellandrene, and limonene. Galls have a much higher potential monoterpene biosynthetic capability than leaves as evidenced by about fivefold higher monoterpene synthase activity detected in galls as compared to leaves. Deep sequencing methodology was employed to isolate key genes in the terpene biosynthetic pathway and examine the molecular and biochemical mechanisms involved in mono- and sesquiterpene biosynthesis in galls and leaf tissues. Enhanced production and accumulation of specific plant terpenes in galls may act as an efficient chemical defense to protect the aphids inhabiting the galls from pathogens and predators.

## O57. Dissection of tomato lycopene biosynthesis through virus-induced gene silencing

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The recent sequencing of the tomato genome has uncovered thousands of novel genes, and the availability of reverse genetics tools is needed to study their function. We applied Virus Induced Gene Silencing coupled to a visual reporter system (Orzaez et al. 2009) and high resolution LC-MS, to the comprehensive study of nine candidate genes in fruit lycopene biosynthesis: phytoene synthases (*PSY)*1-3,phytoenedesaturase(*PDS*)ζ-carotene desaturase(*ZDS*), ζ-carotene and prolycopene isomerase(*ZISO* and *CrtISO*)andtwo *CrtISO*-related genes(*CrtISO-like1* and *2*)(Figure 1).The carotenoid profiles of *PSY1*- and *CrtISO*-silenced fruits are very similar to those of the existing tomato mutants, confirming that VIGS is a robust technique for studying gene function. Our data confirm already-known roles for *PSY1*, *PDS*and *CrtISO*, and suggest additional roles for *PSY3*, *ZISO*and *CrtISO-like2*.Light can partially substitute *ZISO*, but not *CrtISO* function. Silencing of the *PDS* and *ZDS* desaturasesresults in the induction of transcripts encoding the immediately downstream isomerases (*ZISO* and *CrtISO*, respectively), suggesting the existence of a regulatory loop controlling the balance between desaturation and isomerization reactions.

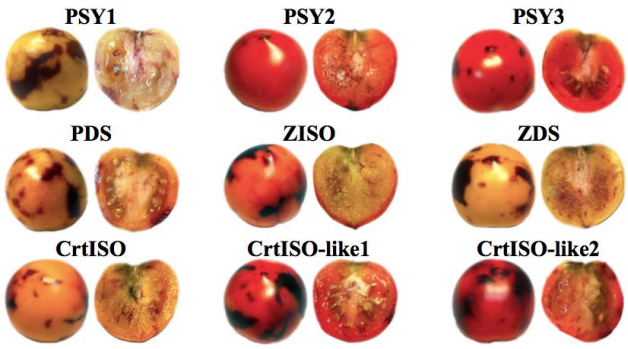


Figure 1: Visual phenotypes of ripe silenced fruits.The name of the silenced genes is shown on top. Purple sectors are sectors in which the virus-induced gene silencing has not spread.Orzaez D, et al. (2009) Plant Physiol 150: 1122-1134

## O58. Expanding the molecular diversity through Synthetic Biology: Using combinatorial biochemistry for reconstruction of pathways to high-value and novel diterpenes.

Johan Andersen-Ranberg[1,2], Søren Spanner Bach[1], Britta Hamberger[1,4], Jörg Bohlmann[3], Erik Hedenström[4], Birger Lindberg Møller[1,2,5] and Björn Hamberger[1,2,5,*]

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Diterpenes (C-20) are a subgroup of terpenoids with more than 14000 different compounds known. The main source of the structurally complex diterpene molecules is from plants, where they act as e.g. hormones or defence compounds. In plants, diterpenes are formed by cyclization governed by diterpene synthases (diTPS) which can typically be divided into two classes (type II and type I). These work in tandem by catalysing the reaction leading from the universal precursor geranylgeranyl-pyrophosphate (GGPP) into multicyclic diterpenes.

In this project we studied the plant species *Tripterygium wilfordii*, *Coleus forskohlii* and *Euphobia peplus*, accumulating the pharmacologically active diterpene compounds triptolide, forskolin and ingenol-3-angelate, respectively. From deep transcriptome sequencing data 30 candidate diTPS genes were identified and isolated. A commonly used method for functional characterization of diTPS uses coupled *in vitro* assay with purified diTPS enzymes and GGPP as substrate. However, for this study, heterologous expression of diTPS genes in *Nicotiana benthamiana* was established. This method enabled rapid functional testing of combinations of diTPS from all three plant species and correlation with already characterized diTPS. With this system it was possible to reconstruct native biosynthetic routes to high value diterpene compounds and to build novel biosynthetic routes introducing product specificity and new-to-nature diterpene compounds including stereochemical control.

Structural elucidation of novel, potentially new-to-nature diterpenoids requires purification of substantial amounts of the target molecules. We achieved medium scale production of the metabolites in the *N. benthamiana* system. Subsequent purification of amounts and purity sufficient for NMR was performed using preparative gas chromatography. This streamlined procedure allowed us to explore the biosynthetic capacities of diTPS and to isolate diterpenes with very similar properties but different structure in one simple step. Identification of diTPS involved in the biosynthesis of high value diterpenes paves the way for biotechnological production and increases our understanding of the evolution of diTPS and the molecular structure of their chemically diverse products.

## O59. P450-dependent linalool metabolism in Arabidopsis involved in defense against flower visiting insects

Benoît Boachon[1], René Höfer[1], Jean-François Ginglinger[1], Laurence Miesch[2], Lionel Allouche[2], Raphaël Lugand[1], Robert R. Junker[3], Kirsten Leiss[4] and Danièle Werck-Reichhart[1]

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[2]Laboratoire de Chimie Organique Synthétique, Institut de Chimie, Unité Mixte de Recherche 7177, CNRS-Université de Strasbourg, France
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[4]Institute of Biology, Plant Ecology, Leiden University, the Netherlands

Based on the analysis of gene expression and functional screening we identified a cytochrome P450, CYP76C1, as a potential candidate involved in the metabolism of linalool in the flowers of *Arabidopsis thaliana*. Yeast-expressed CYP76C1 was found active for the oxidation of linalool and linalool oxides. It sequentially metabolizes linalool and its oxidized derivatives leading to the production of lilac compounds and menthiafolic acid (8-COOH-linalool) *in vitro*. Headspace volatiles and metabolites were investigated in wild-type, KO mutants and over-expressing lines. Wild-type flowers were shown to emit linalool, lilac alcohols and lilac aldehydes. Conversely, the flowers of the *cyp76c1* null mutants emitted much larger amounts of linalool than the wild-type flowers, but not the lilac alcohols and aldehydes. Furthermore, the glycosylated linalool oxides derivatives including 8-OH-linalool, lilac alcohols and menthiafolic acid conjugates present in wild-type flowers did not accumulate in the *cyp76c1* mutants. The flowers of the *35S:CYP76C1* overexpressing line still emitted lilac compounds, but no linalool, and accumulated increased amount of glycosylated menthiafolic acid compared to wild-type. The bioactivity and ecological function of the flower lilac derivatives were investigated on the antagonist insect *Frankliniella occidentalis* (thrips) known as an Arabidopsis flower visitors. Thrips were shown to prefer the flowers of the *cyp76c1* mutants to wild-type. Olfactometer tests using pure compounds suggest that thrips were attracted by linalool but repelled by both lilac compounds. Our results suggest that CYP76C1 is involved in plant defense against flower visitors and provides a new tool for green chemistry and the synthesis of economically valuable compounds.

Project supported by the European Fund for Regional Development in the programme INTERREG IVA Broad Region. EU invests in your future and the COST Action FA1006 PlantEngine.

## O60. Transient expression and metabolomics as tools in monoterpene indole alkaloid metabolic engineering

Lemeng Dong[1], Miriam Goedbloed[1], Francel W.A. Verstappen[1], Sander van der Krol[1], Harro J. Bouwmeester[1]

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Monoterpene indole alkaloids (MIAs) are a large class of plant alkaloids with significant pharmaceutical interest. MIAs are produced by *Catharanthus roseus* by linking the monoterpene derived secologanin to amino acid derived tryptamine. The genes required for biosynthesis of secologanin are largely unknown. In the EU SMARTCELL project we have used gene coexpression analysis, based on comprehensive gene expression data from RNAseq from different *C. roseus* tissues, to predict the function of genes involved in secologanin biosynthesis. Candidate genes as well as known pathway genes were characterized *in planta* using agroinfiltration of *Nicotiana benthamiana*. This allowed us to almost completely reconstitute the entire secologanin pathway in *N. benthamiana*. Different options were explored to scale-up production capacity, either by alternative subcellular targeting of enzymes or by boosting of the precursor input pathway. Because the metabolic background of *N.* benthamiana is complex and the endogenous enzymes often modify the transgenic products formed, quantitative metabolomics was an essential tool to analyze the engineering results. Our analysis shows that competing endogenous enzymes and the relatively low activity of the introduced enzymes form the most important bottlenecks for a high flux through the secologanin pathway in *N. benthamiana*.

**Session IX – Natural roles for Terpenoids**

## O61. The roles of sesquiterpene volatiles in plant defense and signaling

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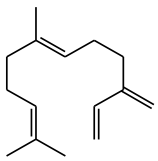
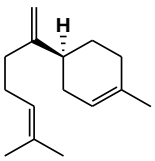
Herbivore feeding on plants usually triggers the emission of a complex blend of volatile terpenes. Although we know something about the roles of these volatiles in defense, little information is available about why such complex mixtures of terpenes are emitted that vary with plant species, organ, developmental stage and type of herbivore.

We have been investigating the complex sesquiterpene blends of maize that are emitted from its leaves, stems and roots, and the terpene synthases that catalyze their formation. To study the biological function of these sesquiterpenes, the terpene synthases have been cloned and characterized and used to transfer the formation of individual volatiles or groups of volatiles into lines of maize and Arabidopsis that do not naturally emit many volatiles.

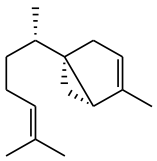
Experiments with these transformed plants have demonstrated the role of maize sesquiterpenes in attracting herbivore enemies after herbivore attack is initiated. In addition, these compounds also appear to function in direct defense not only against herbivores, but also against microbial pathogens. Finally, these substances also act as internal signals priming the induction of non-volatile anti-herbivore defenses. The various blends of maize sesquiterpenes appear to have divergent functions suggesting that different compounds mediate distinct biological activities.



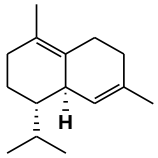
(*E*)-β-Farnesene



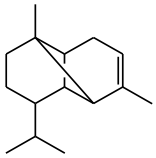

β-Bisabolene



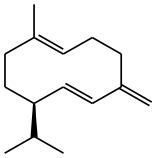
7-*epi*-Sesquithujene



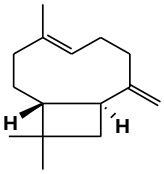

δ-Cadinene



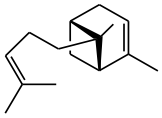
α-Copaene



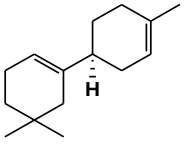
Germacrene D




(*E*)-β-Caryophyllene



(*E*)-α-Bergamotene




Macrocarpene

## O62. In defense of plant roots: Volatile terpene biochemistry and functions belowground

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Volatile terpenes have multiple activities at the plant-environment interface. Knowledge of how volatile compounds function in roots and their soil environment is still limited. We investigate the genomic and cell specific organization of terpene specialized metabolism in plant roots. *Arabidopsis* roots produce a variety of sesquiterpenes and diterpenes in an ecotype- and cell type specific manner. We show that semi-volatile terpenes called rhizathalenes, whichrepresent a so far unidentified class of tricyclic diterpenes with an unusual spiro-hydrindane structure, are biosynthesized centrally in the root stele from where they diffuse radially toward the root surface. Quantum chemical calculations supportthe formation of rhizathalenesby the type-a terpene synthase (TPS) 8 from all-*trans* GGPP in a class I type carbocationicreaction mechanism.*In vitro* and *in vivo* assays indicate thatrhizathalenesfunction as antifeedantsby reducing the consumption of outer cell layers by the root herbivore *Bradysia* (fungus gnat). In a second example, we show that the *Arabidopsis* root stele also produces the C11-homo/norterpene(*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT). However, in contrast to the known formation of DMNT from (*E*)-nerolidol in aboveground tissues, DMNT biosynthesis in roots occurs by an alternative P450-catalyzed breakdownof the triterpene precursor arabidiol. Emission of DMNT is induced by the attack of soil-borne pathogens, and we provide evidence that the arabidiol breakdown pathway contributes to belowground defense.

## O63. Expression of the sesquiterpene (E)-β-farnesene, an aphid alarm pheromone, for pest control in wheat and use of other isoprenoids

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Rothamsted Research, Harpenden, UK

Pheromonal and stress related semiochemicalisoprenoids offer opportunities for control of pests. The high volatility and instability of such isoprenoids requires delivery via plants using genetic modification (GM) or companion cropping and thereby represents a more sustainable approach than external application to crops. The elite hexaploid wheat variety Cadenza has been transformed, following successful work in the model plant *Arabidopsis thaliana*,[1] to produce highly pure (E)-β-farnesene, the sesquiterpene hydrocarbon comprising the alarm pheromone for most pest aphids and, after laboratory demonstration of efficacy, the first year of field trials accomplished. After the hoped for completion of the second year of trials in 2013, the further ecological demands for deployment of the pheromone will be reviewed, including the need for a "switched" gene expression system. *cis*-Jasmone has been identified as such a "switch"[2] and has been developed for eliciting defence by induction of tetranorisoprenoid production in crops. Other elicitors derived from herbivores transferred to plants without needing tissue damage are under investigation that cause tetranorisoprenoid based defence in non-hybrid maize[3] and also in transmission of stress signals in the rhizosphere.[4]

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## O64. Triterpenes in legumes: A role in both the plant and the nitrogen-fixing partners

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Triterpenes are one of the largest classes of plant natural product and have important functions, both in plants (by providing protection against pests and diseases) and for human use (as drugs, adjuvants, anti-microbials, anticancer agents, surfactants, preservatives etc). They are formed by cyclization of 2,3 oxidosqualene catalysed by enzymes known as oxidosqualene cyclases (OSCs). The triterpene scaffolds thus formed may be subsequently modified by downstream tailoring enzymes, such as cytochrome P450s and glucosyltansferases. Legumes are able to produce a new lateral organ *de novo*, the root nodule, by association with symbiotic soil bacteria collectively known as rhizobia. Legumes produce a variety of simple and elaborated triterpenes. Our focus is on pentacyclic triterpenes, known for their well-established pharmaceutical bioactivities. The publicly available genomic resources and biological tools (such as reverse genetics platforms) available for the two model legume plants *Lotus japonicus* and *Medicago truncatula* were exploited (a) to expand the triterpene toolkit repertoire and identify key missing biosynthetic genes and unravel regulatory mechanisms underlying triterpene production in response to developmental and environmental cues, (b) to screen for phenotypic variations in plant growth and development and (c) to identify plant targets that are subjected to the action of triterpenes during nodule organogenesis, a unique system for studying localized, inducible cell divisions in the plant root, thus illustrating novel roles for simple triterpene skeleton molecules in plant growth and development. Along these lines, we have identified homologs in *L. japonicus* of genes which encode uncharacterized proteins, for example, a U-box E3 ligase and a GSK3β, homologous to brassinosteroid hormone signaling in *Arabidopsis*, whose expression levels are induced at early stages of rhizobium interaction in *L. japonicus* and are affected by the presence of simple triterpenes.

## O65. Regulation of plant herbivory-associated volatile terpene biosynthesis by endogenous peptide signals

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A primary plant anti-herbivore defense is emission of volatiles which recruit natural enemy parasitoid and predators of insect pests. Terpenes are often the predominant components of these volatile blends and have been demonstrated to mediate attraction of beneficial insects. Recently members of a plant family of endogenous signals, the plant elicitor peptides (Peps) were discovered to induce emission of terpene and other volatiles. Treatment of maize leaves with the peptide ZmPep3 results in production of a blend that is qualitatively and quantitatively similar to that generated by herbivory. Furthermore, maize volatiles emitted after ZmPep3 treatment are sufficient to attract the parasitoid *Cotesia marginiventris*. Microarray and real-time PCR profiling of transcriptional changes revealed that ZmPep3 regulates expression of genes encoding isoprenoid biosynthetic enzymes and terpene synthases. Profiling of ZmPep3-induced changes in the maize proteome demonstrated corresponding increases of enzymes associated with terpene biosynthesis. ZmPep3 is an active signal only in maize and closely related Poaceous plants. However, Peps from other plant families, including Fabaceae and Solanaceae have been identified that regulate terpene emission associated with herbivory in their respective species. Together evidence indicates that Peps are signals that they have utility in triggering terpene emission for research purposes as well as potential field application to improve resistance to herbivores.

## O66. A reevaluation of the negative effects of S-carvone on the isoprenoid biosynthesis pathway

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*S*-carvone is a monoterpene isolated from caraway, described as down-regulating 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity [1]. This monoterpene is mainly used in food and flavor industry for its strong caraway/dill fragrance, but also in agriculture for the storage of potato tubers to prevent their sprouting. To draw a general picture of the mode of action of this molecule, we investigated its effect on HMGR activity in tobacco, a plant closely related to potato. HMGR activity measured in microsomal fractions of tobacco BY-2 cells was not inhibited by *S*-carvone. We have hypothesized that *S*-carvone may target an inducible HMGR isoform, possibly the stress-related HMGR2 isoform [2]. Elicitation of tobacco plants with cellulase leads to an increase of the HMGR activity, *HMGR2* transcription and results in the production of capsidiol, a mevalonate-derived sesquiterpenoid [3]. As expected, *S*-carvone hinders the biosynthesis of capsidiol. But instead of inhibiting, the monoterpene unpredictably stimulates not only HMGR activity, but also its transcription and protein synthesis. The question arising is: what is the link between *S*-carvone and the inhibition of capsidiol production? This sesquiterpenoid phytoalexin is produced through a signaling cascade initiated by elicitation. This cascade begins with an interaction between the elicitor and a receptor, the activation of a prenylated G protein, spiking of $Ca^{2+}$, cytoplasmic acidification, production of active oxygen species, ethylene and jasmonic acid signaling and then production of the secondary metabolite (in our case capsidiol) [4]. *S*-carvone may for this reason hit one of these early elicitation processes. We proposed a new model: as a monoterpene, *S*-carvone blocks protein isoprenylation required to initiate the signaling pathway leading to capsidiol production.

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## O67. Diversion products of terpenoid-based oleoresin biosynthesis function in plant defense

Raimund Nagel[1], Aileen Berasategui[1], Christian Paetz[2], Jonathan Gershenzon[1], Axel Schmidt[1]

[1]Department of Biochemistry,[2]Research Group Biosynthesis/NMR,Max Planck Institute for Chemical Ecology, Hans-Knoell-Str. 8, D-07745 Jena, Germany

The conifer *Picea abies* (Norway spruce) producesterpenoid-based oleoresins as part of its constitutive and induced defense responses against herbivores and pathogens like the bark beetle *Ips typographus* and its associated fungus *Ceratocystis polonica.*Resin ducts contain primarily monoterpenes ($C_{10}$) and diterpene acids ($C_{20}$). A group of enzymes at critical branch points within terpene biosynthesis are the short-chain isoprenyl diphosphate synthases (IDS), which produce geranyldiphosphate (GPP), farnesyldiphosphate (FPP), and geranylgeranyldiphosphate (GGPP),the precursors of the different terpenoid classes.

IDS1 functions as a bifunctional GPP- and GGPP synthase in *Picea abies*involved in induction of oleoresin biosynthesis within conifer defence processes (Schmidt et al., 2011).To study the importance of this particular enzyme,embryogenic tissue culture of Norway spruce was genetically modified with the *ids*1 gene under the control of a constitutive ubiquitin promoter using an *Agrobacterium tumefaciens* mediated transformation protocol(Schmidt et al., 2010). Needles of transgenic saplings overexpressing *ids*1showed a 350-fold higher transcript level and a 10-fold increase in enzyme activity compared to vector controls. But surprisingly, amountsof mono- and diterpene resin acids, sterols and carotenoids did not change. Instead, esters of geranylgeraniol with different fatty acids accumulatedin significant amounts. Although the presence of these esters has already been reported fromgymnosperm and angiosperm plant species, their function *in planta* is not understood. To test the ecological potential of geranylgeraniol fatty acid esters, behavioural assays were performed with the nun moth, *Lymantriamonacha*, an important Eurasian pest of conifers feedingonly on needles. Larvae fedon transgenic *ids*1-overexpressing seedlings showed a significantly lower survival and growth rate than larvae fed on vector control seedlings.

Our results suggest that the biosynthesis of geranylgeranylated fatty acid esters is a consequence of the abundant isoprenyldiphosphatein the transgenic spruce line. These esters function as antifeedant compounds and hence may be part of the general defence strategies of conifers against herbivorous insects.

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2. Schmidt A, Nagel R, Krekling T, Christiansen E, Gershenzon J, Krokene P (2011) Plant Mol Biol 77: 577-590

## O68. Scent of a parasite: identity and function of volatile organic compounds produced by malaria parasites

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Malaria remains a serious global health issue, causing nearly 1 million deaths each year. Severe malaria is due to infection with the apicomplexan parasite, *Plasmodium falciparum*, which is transmitted through a mosquito vector, *Anopheles gambiae*. Due to widespread parasite resistance to nearly all available antimalarials, new drug targets are desperately needed. The parasite possesses a non-mevalonate isoprenoid biosynthesis pathway that proceeds through the key intermediate methylerythritol phosphate (MEP). The MEP pathway is a well-validated antimalarial target, with convincing genetic and inhibitor-based evidence that this pathway is essential to *P. falciparum*. This provides a strong case for exploring and mining isoprenoid biology in malaria parasites to uncover additional drug targets. Since isoprenoids comprise a large and diverse group of cellular products, our long-term goal is to understand why isoprenoids are essential in malaria parasites.

In this study, we aimed to describe the identity and biological functions of isoprenoids produced by *P. falciparum*. We evaluated the nonpolar organic composition of headspace gas and parasite lysates of cultured malaria parasites, compared to headspace gas and lysates of control human erythrocytes. Both gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) were used to characterize compounds from each sample and Mass Profiler Professional (MPP, Agilent) was used to identify parasite-specific components. From over 1400 unique compounds, 21 parasite-specific compounds were identified. Surprisingly, each parasite sample contained a collection of terpenes and terpene-derived compounds, none of which were found in erythrocytes alone. Using inhibitors of parasite isoprenoid biosynthesis, we confirmed that the identified terpenes were produced in parasites through *de novo* isoprenoid biosynthesis. Since terpenes are used in other biological systems, such as plants, for insect communication, we tested the ability of these compounds to activate *A. gambiae* odorant receptors. Excitingly, these results confirm that the malaria mosquito vector appears to contain the biochemical machinery to smell malaria parasites, which may explain in part why mosquitos are preferentially attracted to humans with malaria. Ongoing efforts aim to confirm these findings in clinical samples of emanations of patients with malaria.

**Session X – Plant and microbial production systems for terpenoids**

## O69. How jasmonates can provide novel tools for the exploitation of plant metabolic circuits

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Across the plant kingdom, the jasmonate hormone steers the delicate balance between growth and the activation of defence programs, such as the production of bioactive secondary metabolites. These small organic molecules allow plants to cope with various types of stresses but often also have biological activities of high interest to human. Plant cells are capable of producing an overwhelming variety of secondary metabolites, both in terms of complexity and quantity, but this impressive metabolic machinery is still hardly exploited, mainly because of the limited molecular insight into plant metabolism.

We employ a transcriptomics based technology platform for large-scale gene discovery programs in plant metabolism. The gene collections that we establish increase our fundamental understanding of the central mechanisms that steer plant growth and metabolism and simultaneously serve as a novel resource for metabolic engineering tools. These tools are implemented in a synthetic biology platform for the sustainable production of existing or novel plant-derived molecules with superior bioactivities for the pharmaceutical, nutraceutical or agrochemical industries.

Presently, our platform focuses on the synthesis of bioactive triterpenesaponins and their building blocks in plants and yeasts, in particular *Medicagotruncatula* root cultures and *Saccharomyces cerevisiae* cells, respectively. Through combinatorial biochemistry and regulatory network engineering, we could redirect the saponin biosynthetic pathway towards the synthesis of new-to-nature molecules or extremely bioactive intermediates that otherwise do not accumulate in plant cells.

## O70. Engineering of different terpenoid biosynthetic pathways in tobacco and corn through multigene transfer

Bruna Miralpeix[1], Gemma Farré [1,2], Chao Bai[1], Uxue Zorilla-López[1], Judit Berman[1], Georgina Sanahuja[1], Teresa Capell[1], Gerhard Sandmann[3], Changfu Zhu[1], Paul Christou[1,4]

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The simultaneous transfer of multiple genes into plants enables researchers to study and modulate entire metabolic pathways, express multimeric proteins or protein complexes, and study complex genetic control circuits. Metabolic engineering in plants can be used to increase the abundance of specific metabolites or eliminate others. In many cases, it is necessary to intervene at an early bottleneck, sometimes the first committed step in the pathway, but often this only succeeds in shifting the bottleneck downstream, sometimes also causing the accumulation of an undesirable metabolic intermediate. Technical hurdles limiting the number of genes transferred to plants have introduced a significant bottleneck to the engineering of complex metabolic pathways. More recently, attempts have been made to address these limitations by developing new transformation methods that recognize the desire to introduce multiple transgenes into plants and express them in a coordinated manner. Essentially all these methods aim to achieve the creation of a SMART locus, i.e. one containing Stable Multiple Arrays of Transgenes. A SMART locus can be created by the simultaneous expression of multiple genes in a pathway, preferably representing every critical enzymatic step, therefore removing all bottlenecks and ensuring completely unrestricted metabolic flux. This approach requires the transfer of multiple enzyme-encoding genes to the recipient plant, which is achieved most efficiently if all genes are transferred at the same time. Here we discuss multi gene transfer using two examples: the early part of the terpenoid pathway in tobacco and the carotenoid pathway in corn.

Acknowledgments: Research at the Universitat de Lleida is supported by MICINN, Spain (BIO2011-23324; BIO02011-22525; BIO2012-35359; PIM2010PKB-00746;); European Union Framework 7 Program-SmartCell Integrated Project 222716; European Union Framework 7 European Research Council IDEAS Advanced Grant (to PC) Program-BIOFORCE; RecerCaixa; COST Action FA0804: Molecular farming: plants as a production platform for high value proteins; Centre CONSOLIDER on Agrigenomics funded by MICINN, Spain and German BMBF Förderkennzeichen 0315913A to G. Sandmann.

## O71. Novel genetic perturbations in Saccharomyces cerevisiae for improving terpenoid production

Fotini A. Trikka[1*], Codruta Ignea[2*], Anastasia Athanasakoglou[1], Alexandros Nikolaidis[1], Anagnostis Argiriou[1], Dragana Bozic[3], Sotirios C. Kampranis[4] and Antonios M. Makris[1§]

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[*]FAT and CI are equal authors

Due to their numerous commercial applications, there is increasing interest in the development of *S. cerevisiae* strains capable of producing high levels of terpenoids. Aiming to identify new gene targets which can be manipulated to increase sesquiterpene production, a set of HMG2 positive genetic interactors were assessed as single and digenic heterozygous deletions. Heterozygous deletions cause significant reductions in protein levels but do not lead to growth impediments frequently seen in haploid strains. Tandem heterozygous deletion of a set of three genes, the ubiquitin ligases *ubc7* and *ssm4/doa10*, and the ER resident protein *pho86*, led to an 11-fold increase in caryophyllene yields (125 mg/L in shake flasks) compared to cells lacking these modifications. The effect of the heterozygous deletions appears to be due to Hmg1p and Hmg2p stabilization. In a parallel approach, we established a carotenoid genetic screen in heterozygous deletion background and screened >4.500 strains for increased carotenoid biosynthesis. A set of 15 novel mutations were identified. The majority of these were also found to increase sesquiterpene production. By exploiting desirable haploinsufficiencies in yeast, we identified new sets of genes that can be disrupted in tandem to engineer yeast cell factory strains without negatively impacting cell growth and viability.

## O72. Plant cell is a SmartCell - Hairy roots as a platform to study terpenoidindole alkaloid pathway

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The approximately 298 000 higher terrestrial plant species offer a very diverse source of low molecular weight compounds, known as secondary metabolites,for flavour, fragrance, cosmetic, agrochemical and pharmaceutical applications either as such, as backbone structures or as leads for new semi-synthetic or synthetic forms.The current ability to modulate plant secondary metabolism is very limited because of our poor understanding of the complete metabolic pathways and their regulation at the systems level. FP-7 funded SmartCell project aims to increase the economical production of high-value terpenoids for non-food industrial use by creating a novel concept for rationally engineering plants and plant cells for improved characteristics. The monoterpeneiridoid pathway in periwinkle is applied as a target pathway, but all knowledge, tools and resources developed in SmartCell will be generic and thus have broad utility for engineering of any plant biosynthetic pathway. A systems biology approach was taken to identify, clone and screen potent pathway, transport and regulatory genes in order to create a comprehensive knowledge base. In addition, multi-gene engineering, scale-up and downstream technologies were developed with a flavor of demonstration activities to facilitate the technology transfer from science to industrial applications.

In respect of the biotechnological production systems, hairy root cultures, which are differentiated tissues, have been shown to carry excellent and stable product yields for severalsecondary metabolites. The secondary metabolite profiles of hairy roots have proven that they carry full biosynthetic capacity of the originating plant since they contain metabolites not found in the roots of the plants they are derived of e.g. essential oils. Thus hairy roots were chosen as one potent platform to study and engineer terpenoidindole alkaloid pathway in SmartCell project. The introduction of geraniol synthase (*Valerianaoffcinalis*) with chloroplast targeting in tobacco (SR1) hairy roots showed free geraniol accumulation at levels of ca. 30 µg/g (dry weight) andyielded in six different hexose and/or pentose conjugates of geraniol and hydroxy-geraniolabsent from wild type root clones. However, the central metabolic fluxes were unchanged relative to wild type lines, reflecting the stability of the central metabolism. In the presentation, the resultsfrom the engineering of the early steps of the monoterpenoid biosyntheticpathway in combination with the flux analyses, in depth non-targeted metabolomicanalyses, development of bioinformatics tools and scale-up studies are discussed.

Acknowledgements: The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement number 222716 – SMARTCELL

## O73. Production of licorice triterpenoids in both trangenic plants and yeast cutlure system

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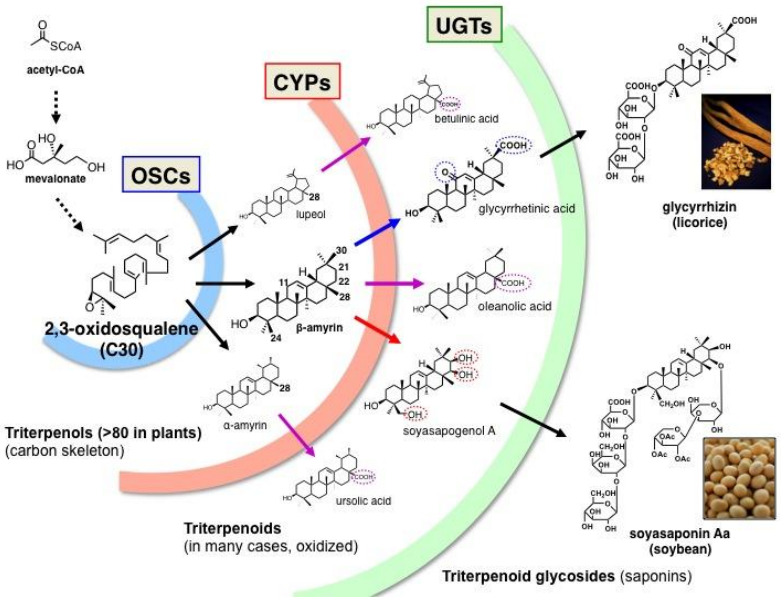
Plant triterpenoids represent a large and structurally diverse class of natural products. Among these chemicals, glycyrrhizin, a triterpenoid saponin derived from underground parts of *Glycyrrhiza* (licorice) plants, is one the most important crude drugs in the world. Glycyrrhizin and its aglycone glycyrrhetinic acid exhibit various pharmacological activities, including anti-inflammatory and hepatoprotective activities. Their production largely depends on the collection of wild licorice plants, and this has caused a decrease in licorice reserves and an increase in desertification where it is harvested. Glycyrrhizin is synthesized from □-amyrin, one of the most commonly occurring triterpenoids in plants, by series of site-specific oxidation and glycosylation. Our group isolated two genes encoding cytochrome P450 monooxygenases (CYP88D6 and CYP72A154) these perform subsequent oxidations of □-amyrin at positions C-11 and C-30, respectively. Recently we also cloned strong candidates for UDP-glucronidases for the glycosylation. Based on these results, two research projects are now on going in Japan. One is production of glycyrrhetinic acid in yeast and the other is production of glycyrrhetinic acid in soybean. Both are based on the idea of "re-direct" of common precursors, 2, 3-oxidosqualen (yeast) and □-amyrin (soybean) to the heterologous glycyrrhizin pathway by recruiting glycyrrhizin biosynthetic genes (CYPs/UGTs) not only from licorice but also appropriated plant species. In the meeting, recent progress of both projects is shown and prospects of the studies will be discussed.

This work was supported in part by Adaptable and Seamless Technology transfer Program through target-driven R&D (A-STEP) (Japan Science and Technology Agency) and the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN) in Japan.



## O74. Multivariate-modular metabolic engineering for terpenoid biosynthesis in *E. coli*

Ajikumar Parayil

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Terpenoid natural products are primarily extracted from plants in low yield, and there has been immense interest in developing cost-competitive biotechnological production of these chemicals due to limited raw material accessibility, low yields and high costs of the plant extraction process or chemical synthesis. Recently, we developed a new metabolic engineering approach, multivariate modular metabolic engineering (MMME), and a platform bacterial system capable of overproducing the terpenoids. We have demonstrated the application of our approach to engineer various diterpenoid molecules production in E. coli. MMME enabled the systematic assessment and elimination of regulatory and pathway bottlenecks by re-defining the metabolic network as modules. We have extended our approach up to three modules and established that fine balance in the pathway enzyme expression is key to the high level production of these molecules. Here, we focus on the development of MMME and its application to biosynthesis of complex terpenoids in bacteria.

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Yadav V. G., et al The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab Eng. 2012, 14, 233-41.

## O75. Transgenic potatoes remarkably decreasing content of glycoalkaloids

Naoyuki Umemoto[1], Katsunori Sasaki[1], Kiyoshi Ohyama[2], Mari Yotsu-Yamashita[3], Masaharu Mizutani[4], Hikaru Seki[5], Kazuki Saito[2], Toshiya Muranaka[5]

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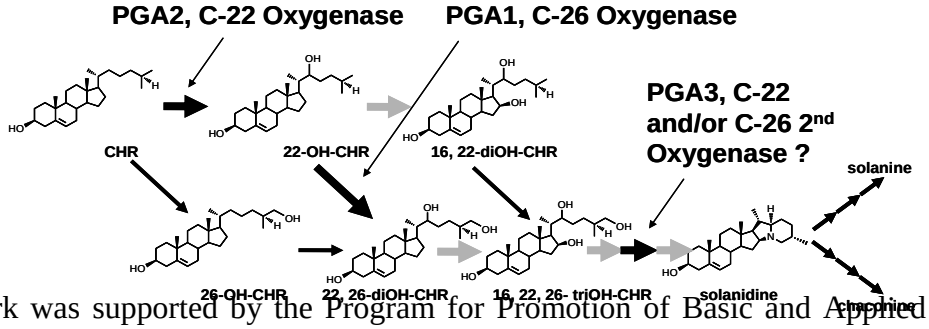
Steroidal glycoalkaloids are secondary metabolites that occur in nearly all plant organs of *Solanum* species. The principal glycoalkaloids in domesticated potato, *Solanum tuberosum* L., are solanine and chaconine (potato glycoalkaloids, PGA) that consist of solanidine aglycone and trisaccharide, solartriose and chacotriose. When present at low concentrations in the tubers, PGAs cause a bitter astringent taste of potatoes, but at high concentrations PGAs are toxic to animals and humans. PGA in tubers for consumption may not exceed food safety values of 200 mg/kg fresh weight. But high levels of PGA are found in tuber sprouts, and there are enhanced levels in greened tubers where they have exposed to sunlight.

There are some reports of the glycosylation steps of PGAs, but the biosynthesis of solanidine aglycone remains poorly understood. In the present work, using the gene expression information of Solanum tuberosum, we found three cytochrome P450 (CYP) genes which highly express in PGA-accumulated tissues. To investigate the role of the CYP genes involved in the biosynthesis, we generated transgenic potato plants in which each CYP gene expression were suppressed by the CaMV 35S promoter-expressed stem-loop RNA. The transgenic plants hardly contained PGAs. Surprisingly, they were able to grow normally and to have the same yield of tubers as the non-transgenic potato. We analyzed accumulated compounds in the gene knockdown transgenic plants to elucidate enzyme encoded by the CYP genes.




This work was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

## O76. Carotenoid biofortification of rice endosperm through combinatorial transformation for multi-gene and multi-pathway engineering

Chao Bai[1], Sol M. Rivera[2], Vicente Medina[1], Ramon Canela[2], Teresa Capell[1], Gerhard Sandmann[3], Paul Christou[1,4] and Changfu Zhu[1]

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New genetic transformation methods recognize the need to introduce multiple transgenes into plants and express them in a coordinated manner. Simultaneous multiple gene transfer enables the study and modulation of entire metabolic pathways and the elucidation of complex genetic control circuits and regulatory hierarchies. By using combinatorial genetic transformation we recreated the carotenoid biosynthetic pathway in rice (*Oryza sativa*) deficient for endosperm carotenoids, and we identified and complemented rate-limiting steps in the pathway. The following transgenes, under the control of endosperm-specific promoters were co-transformed simultaneously into rice embryogenic tissues: *Arabidopsis thaliana* orange gene (*AtOr*), *A. thaliana* 1-deoxy-D-xylulose 5-phosphate synthase gene (*Atdxs*), *Zea mays* phytoene synthase 1 gene (*Zmpsy1*), *Pantoea ananatis* phytoene desaturase gene (*PacrtI*) and chemically synthesized *Chlamydomonas reinhardtii* β-carotene ketolase gene (*sCrbkt*). A hygromycin phosphotransferase gene was used as the selectable marker. We have generated a number of independently derived transgenic plants accumulating substantial amounts of β-carotene and other nutritionally important carotenoids specifically in the endosperm. Carotenoid profile and transgene expression analysis is currently being carried out to determine the qualitative and quantitative expression of combinations of input transgenes and their influence on carotenoid profile and accumulation. Results indicate that the amount of carotenoids in plant tissues and organs does not appear to depend solely on carotenogenic enzyme activities responsible for their synthesis. The upstream precursor (MEP-derived IPP and GGPP) pathways may also positively influence their accumulation. The combinatorial transformation approach and subsequent analysis of resultant plants will contribute towards the development of a better understanding of control points and rate limiting steps in the pathway and also permit the design and implementation of more precise engineering strategies for enhancing carotenoid accumulation in cereal grains. Funding: MINECO, Spain (BIO2011-22525), and ERC Advanced grant BIOFORCE to PC.

## O77. Genetic and molecular analysis of structural diversity of saponins in soybean

Kyoko Takagi[1], Yoshitake Takada[2], Takashi Sayama[1], Hiroko Sasama[1], Masayasu Saruta[2], Akio Kikuchi[3], Shin Kato[3], Chigen Tsukamoto[4], Masao Ishimoto[1]

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Soybean is a source of physiologically active metabolites in addition to high-quality proteins and fats. Triterpene saponins, soyasaponins, are major components of these secondary metabolites and exhibit wide structural diversity. They are divided into two groups, DDMP saponins and group A saponins, according to their aglycone components. Although their chemical properties and pharmacological effects have been reported, little is known about their biosynthesis and functions in plant. The structural diversity is explained by different combinations of nine alleles at four genetic loci, *Sg-1*, *Sg-3*, *Sg-4*, and *Sg-5* (Fig.1). *Sg-1* regulates the glycosylation of the terminal sugar species on the C-22 sugar chain of group A saponins, and multiple alleles confer structural diversity of glycosylation (Takada et al. 2010). Fine mapping of *Sg-1* revealed a UDP-sugar-dependent glycosyltransferase gene; substitution of a few amino acids changed the enzyme's sugar donor specificities (Sayama et al. 2012).

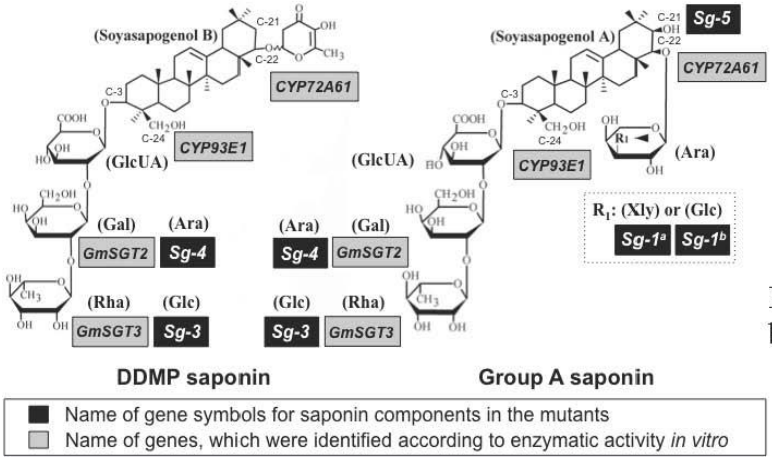


**Fig. 1** Summary of soyasaponin biosynthetic genes in soybean.

We are now focusing on the identification of the other three genes, which control sugar chain composition at the C-3 sugar moieties or the production of group A aglycon. Identification of these genes should be useful for understanding not only saponin biosynthesis but also the phytophysiological function of the highly diversified saponins.

This work was supported by the program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).

# O78. A highly efficient leaf protoplast expression system for the study of anticancer alkaloid metabolism in *Catharanthus roseus*

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**Abstract**

*Catharanthus roseus* leaves accumulate in low levels the anticancer terpenoid indole alkaloids (TIAs) vinblastine and vincristine, and intense research has uncovered a number of TIA biosynthetic steps and regulatory genes. However, there are still enormous gaps in the knowledge on the TIA biosynthetic pathway, its regulation and TIA transmembrane transport. At present, several *C. roseus* transcriptomic projects are unveiling a high number of candidate genes, urging the development of easy and efficient molecular tools for functional characterization. Leaves are the single *C. roseus* organ where the biosynthesis of the anticancer TIAs is completed, and therefore mesophyll cells are the ideal target for TIA gene functional analysis. Here, a highly efficient method for *C. roseus* mesophyll protoplast isolation and transformation is reported, enabling to reproducibly test TIA candidate genes in the precise cells where the biosynthesis and accumulation of the anticancer TIAs occur. As a proof of principle of the application of this molecular tool, the subcellular sorting determination of CroPrx1, a class III peroxidase involved in TIA biosynthesis, was investigated. Transient expression of different *CroPrx1-GFP* fusions revealed that CroPrx1 is sorted to the vacuole by a C-terminal peptide signal. After customized optimization of protoplast isolation, the transformation procedure seems to be easily adaptable to different species, suggesting that this is a plant-transferable tool that may be applied to non-model species for the functional characterization of the wealth of candidate genes emerging from next generation sequencing.

## Session XI – New and Old Appreoaches

# O79. C-H Oxidation and synthesis

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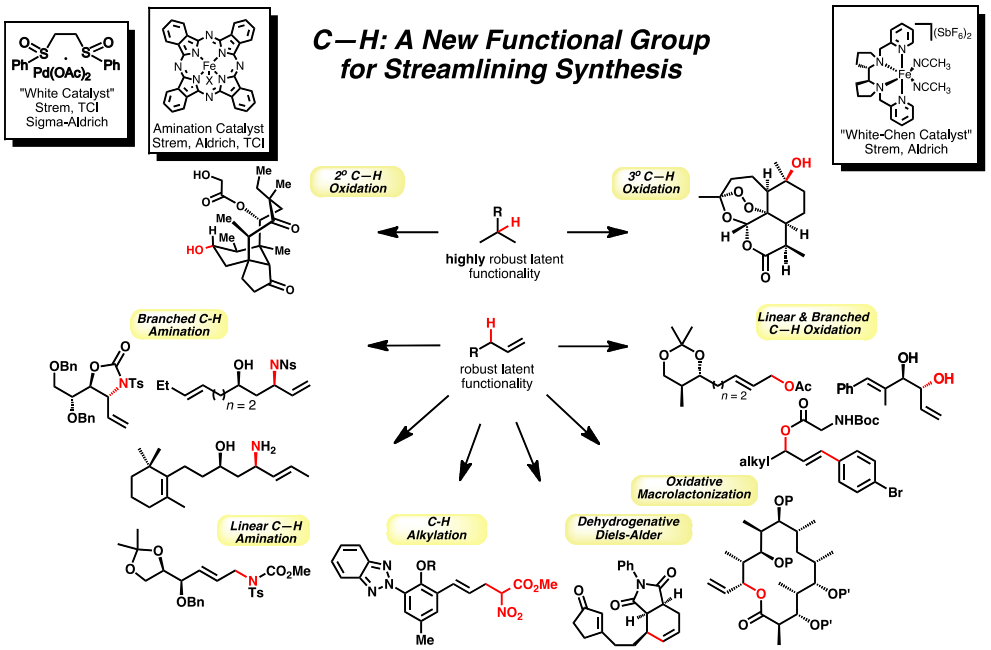
Among the frontier challenges in chemistry in the 21st century are the interconnected goals of increasing control of chemical reactivity and synthesizing stereochemically and functionally complex molecules with higher levels of efficiency. Although it has been well demonstrated that given ample time and resources, highly complex molecules can be synthesized in the laboratory, too often current reaction manifolds do not allow chemists to match the efficiency achieved in Nature. Traditional organic methods for installing oxidized functionality rely heavily on acid-base reactions that require extensive functional group manipulations (FGMs) including wasteful protection-deprotection sequences. Due to their ubiquity in complex molecules and inertness to most organic transformations, C—H bonds have typically been ignored in the context of methods development for total synthesis. Discovery and development of highly selective oxidation methods for the direct installation of oxygen, nitrogen and carbon into allylic and aliphatic C—H bonds of complex molecules and their intermediates are discussed. Unlike Nature which uses elaborate enzyme active sites, this chemistry harnesses the subtle electronic, steric, and stereoelectronic interactions between C—H bonds and small molecule transition metal complexes to achieve high regio-, chemo-, and stereoselectivities. Our current understanding of these interactions gained through empirical and mechanistic studies will be discussed. Novel strategies for streamlining the process of complex molecule synthesis enabled by these methods will be presented. Collectively, our program aims to change the way that complex molecules are constructed by defining the principles that govern reactivity of C—H bonds in complex molecule settings.




Aliphatic C—H Oxidation: *Science* 2007 (*318*) 783; *Science* 2010 (*327*) 566; *Nature Chemistry* 2011 (*3*) 216; *Science* 2012 (*335*) 807. Allylic C—H Oxidation: LAO: *JACS* 2004 (*126*) 1346; 2010 (132) 11323; *OL* 2005 (*7*) 223; *ACIE* (*45*) 8217. BAO: *JACS* 2005 (*127*) 6970; 2006 (*128*) 15076; *ACIE* 2008 (*47*) 6448; *JACS* 2011 (*133*) 12584. Macrolactonization: *JACS* 2006 (*128*) 9032; *Nature Chem.* 2009 (*1*) 547; *ACIE* 2011 (*50*) 2094. AA: *JACS* 2008 (*130*) 14090; *ACIE* 2011 (*50*) 6824. LAA: *JACS* 2008 (*130*) 3316; *JACS* 2009 (*131*) 11701. BAA: *JACS* 2007 (*129*) 7274; *JACS* 2009 (*131*) 11707. DDA: *JACS* 2011 (*133*) 14892. Allylic amination with Fe: *JACS* 2012 (*134*) 2036.

## O80. Making molecular prosthetics

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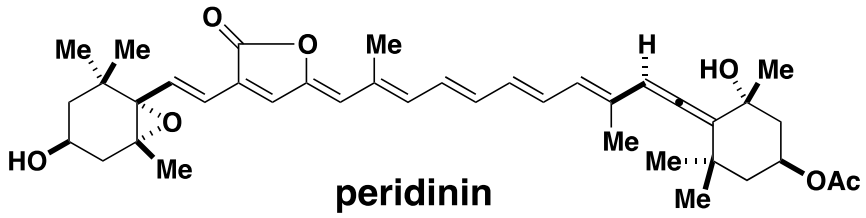
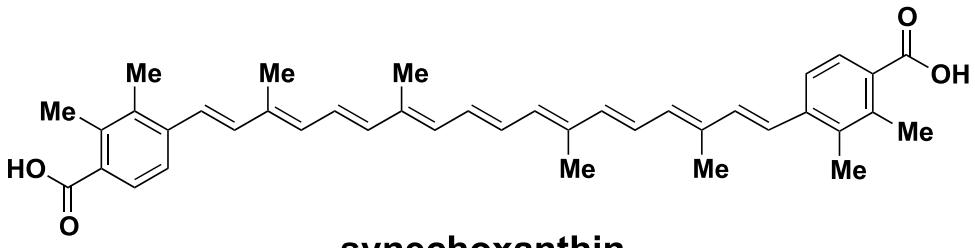
Deficiencies of human proteins that protect cells from lipid peroxidation have been linked to many prevalent diseases, including atherosclerosis, neurodegenerative disorders, and cancer. Remarkably, some species of bacteria have the ability to thrive in environments of extreme oxidative stress, which has been attributed to the presence of specialized terpenes/carotenoids in their membranes. These natural products might therefore serve as valuable prototypes for understanding and optimizing the capacity for small molecules to serve as antilipoperoxidants in human cells. This talk will describe a systematic platform for the total synthesis and biological/biophysical studies of these medicinally important compounds.




peridinin




synechoxanthin

## O81. Parallel and convergent metabolic evolution in terrestrial plants

Joseph P. Noel[1,2,3] and Jing-Ke Weng[1,2]

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During this presentation, parallel evolution, normally considered a sub-branch of convergent evolution, is separated from convergent evolution. This clarified and more specific description of protein evolution is possible as structural folds associated with specific biochemical activities are often slow to evolve and can be unequivocally established. By using homologous protein structures as precise characteristics delineating descent from a common ancestral fold, parallel and convergent evolution can be explicitly separated (Zhang and Kumar, 1997). When ancestral descendants possessing distinct biochemical activities but a shared structural lineage nevertheless contemporarily evolve to synthesize the same metabolite, the term parallel evolution is used. When distinct protein structures sharing no structural similarity result in the synthesis of the same metabolite, the term convergent evolution is employed. Functional analyses of diverse families of green plants suggest that the same chemical phenotypes arose independently more commonly than anticipated from our conventional understanding of molecular evolution. Notably, the emergence of analogous and homologous catalytic machineries through convergent and parallel evolution, respectively, seems to have occurred repeatedly in different plant lineages.

Zhang, J. and Kumar, S. (1997). Detection of convergent and parallel evolution at the amino acid sequence level. Mol. Biol. Evol. 14, 527-536.

## O82. Identification of herbivore-induced terpene biosynthesis pathways and their enzymes by genetic mapping in maize (*Zea mays*)

Annett Richter[1], Zhiwu Zhang[2], Edward Buckler[2] and Jörg Degenhardt[1]

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Plant secondary metabolites can serve as plant defensive compounds or mediators of chemical communication, e.g. as attractants for natural enemies of herbivores. Maize plants attacked by caterpillars release a mixture of mono- and sesquiterpenes that attracts parasitic wasps, which are specific enemies of the herbivores. In our effort to study the molecular base of these indirect defense mechanisms, we want to identify the genes responsible for volatile terpene biosynthesis as well as their regulatory elements.

About 5000 recombinant inbred lines of a Nested Association Mapping (NAM) population derived from 26 inbred lines were screened for herbivore-induced volatile production. The variation of volatile emission within the NAM population enabled us to identify a set of important quantitative trait loci for volatile terpene production. Genome wide association study (GWAS) utilizing a large SNP population resulted in close mapping of several QTLs. We identified a QTL for the trait '(*E*)-nerolidol emission' which is close to two putative terpene synthases, *tps2* and *tps3*. After fine mapping, we localized a SNP marker directly in the promoter of *tps2*. Biochemical characterization of TPS2 verified that this enzyme is a (*E*)-nerolidol synthase. The next step of the pathway, the conversion of (*E*)-nerolidol into the homoterpene 3,8-dimethyl-1,4,7-nonatriene (DMNT), maps to a P450 enzyme with similarity to the CYP92 group. Heterologous expression and characterization demonstrated that this P450 enzyme is indeed capable of converting nerolidol into DMNT by oxidative degradation. Thus, mapping of terpene metabolites by NAM enables us to characterize terpene biosynthetic pathways and the exact genes encoding the enzymes responsible for the pathway.

# O83. Ectopic expression of secondary metabolite transporters in suspension cells: identification of cyclic diterpenes as substrates of the *Nicotiana tabacum* NtPDR1 transporter involved in biotic stress response

Joseph Nader, Julien Roland and Marc Boutry

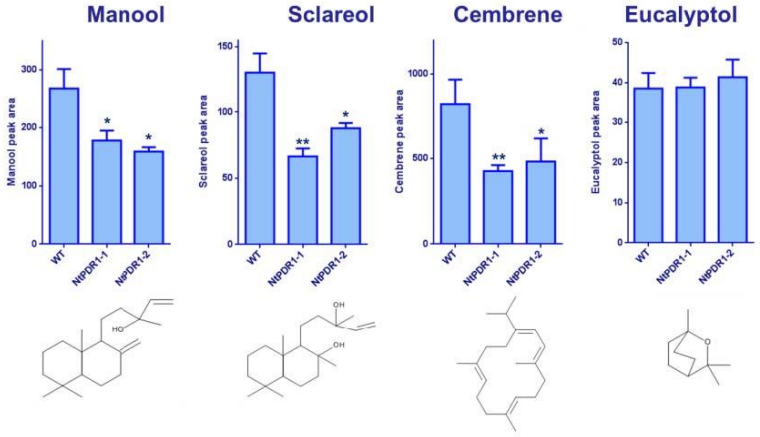
Institute of Life Sciences, University of Louvain, Belgium

While many biosynthetic pathways of the secondary metabolism are being decrypted, the proteins involved in moving intermediates or final products between cell compartments or out of the cell are still largely unknown. Our work focuses on ABC transporters belonging to the Pleiotropic Drug Resistance (PDR) subfamily. ABC transporters are powered by ATP hydrolysis and can therefore accumulate substrates against concentration gradients. Our objective is to identify the role of these transporters in the secondary metabolism and to determine whether they possess the amazing property of substrate-poly-specificity reported for some ABC transporters in yeast and animal organisms.

We identified five PDR transporters in *Nicotiana tabacum* and showed that four of them are involved in abiotic or biotic stress responses. Among them, *NtPDR1* was shown to be involved in the response to fungal pathogen infections. *NtPDR1* is expressed in the secretory cells of the long trichomes. Silencing its expression resulted in increased susceptibility of the plant to fungi. Diterpenes are likely to be NtPDR1 physiological substrates, as they are synthesized in *N. tabacum* trichomes and secreted at the leaf surface where they are involved in plant protection against biotic threats. Since they are toxic for plant cells, an efficient transport system is required to keep the intracellular concentration low. This might explain the involvement of an ABC-type transporter powered by ATP hydrolysis.

To directly identify NtPDR1 substrates, we developed a homologous expression system based on suspension cells of *N. tabacum*. NtPDR1 was localized to the plasma membrane and its activity was assessed using both toxicity and transport assays. We demonstrated that NtPDR1 transports various toxic cyclic diterpenes (cembrene, abietic acid, dehydroabietic acid, mannool and sclareol) but no linear diterpenes, nor mono-, sesqui-, or triterpenes (see figure for some examples). This demonstrates that NtPDR1 is specific for cyclic diterpenes but shows little specificity within this group of compounds. Other PDR transporters were successively expressed in *N. tabacum* cells and their transport activity is currently being tested.

In conclusion, *N. tabacum* BY2 cells are a very convenient system for expressing membrane transporters and testing their transport activity.




*Transport of terpenes by NtPDR1. The figure compares the internal concentration of the indicated terpenoids in wild-type and NtPDR1-expressing* N. tabacum *cells. NtPDR1 transports various diterpenes but not eucalyptol, a monoterpene.*

**Session XII – Terpenoids and Industrial Applications**

## O84. Toward biosynthetic routes for the production of terpenes for the flavors and fragrance industry

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Terpenoids represent a class of secondary metabolites of great economic importance for the flavor and fragrance industry. Many terpene molecules present interesting and unique olfactory properties, often difficult to replace by synthetic structural analogs. Most of these molecules are obtained from plants and products derived from plants have the disadvantage of being subject to fluctuation in price and quality due to climatic and geo-politic factors. In addition, the compounds of interest may be available only in small concentration in the raw material or only from non-sustainable resources, resulting in high prices. Chemical synthesis has been extensively developed to circumvent these problems; however, given the structural complexity, economically acceptable chemical routes are still not available for most terpene compounds important for the flavor and fragrance industry.

Recent progress in engineering isoprenoid biosynthesis in different host organisms open the possibility of developing cost effective biosynthetic routes to terpene molecules. In the last decades, extensive effort has been made to elucidate the biosynthesis of terpenoids in several plants. However, regarding plants commonly used for perfume applications, relatively little information was available for the biosynthesis of the key terpene molecules. We have thus undertaken to investigate the biosynthesis of terpene compounds which are the constituents of important perfumery ingredients. Some examples of pathway elucidation of terpene molecules of high value for the prefumery industry will be presents and the strategy for the production of these molecules will be discussed.

## O85. Biosynthetic production of terpene specialty chemicals in yeast

Richard P. Burlingame

Allylix Inc., Lexington, KY. USA

As the most diverse class of biomolecules, terpenes are found ubiquitously in nature and have been found to be effective in a wide range of high value commercial applications including flavors and fragrances, cosmetic products, food ingredients, pharmaceutical products, and insect control. In spite of their proven efficacy for these applications, there has been limited commercial development of sesquiterpenes due to their lack of availability at commercially viable prices. Allylix has developed proprietary technology for the production of terpenes using synthetic biology and metabolic engineering of *Saccharomyces cerevisiae*, bakers' or brewers' yeast. This technology provides the basis for sustainable production of sesquiterpenes and other terpenes from abundant and inexpensive raw materials, allowing reliable cost-effective production of these compounds at consistent quality. The production processes for valencene and nootkatone have been scaled to full commercial scale, and valencene and nootkatone are currently being sold. Five other terpene products are also in commercial development.

The technology platform comprises gene isolation, protein engineering, metabolic engineering, and fermentation process engineering components. Innovative and integrated use of these components has contributed to the development of strains and processes characterized by efficient carbon flux from glucose to terpenes in yeast. As bottlenecks in carbon flux, which vary from product to product and change over the course of development programs, are identified, data- and knowledge-driven strategies are used to address them. These strategies can include rational and combinatorial approaches to strain construction and screening, as well as process improvements.

Allylix technology can also serve as a platform for discovery of new products. Using straightforward chemical modification of various terpene scaffolds produced by fermentation, libraries of novel or otherwise inaccessible compounds can be generated and tested for various applications. These technologies provide mechanisms to discover and produce terpene based products that are currently unavailable or currently not commercially viable.

## O86. Pseudomonas putida: from perillic acid production to a microbial platform for terpene oxyfunctionalization

Hendrik Schewe, Jia Mi, Marco A. Mirata, Dirk Holtmann, Jens Schrader

DECHEMA Research Institute, Biochemical Engineering, Frankfurt am Main, Germany

Many *Pseudomonas putida* strains display a pronounced tolerance against organic solvents making these bacteria promising biocatalysts especially for monoterpene oxyfunctionalization reactions [1]. *P. putida* DSM 12264 grows on cymene as sole carbon and energy source. Three enzymes from the *cym* operon responsible for the oxidation of cymene to cumic acid also convert the structurally similar monoterpene limonene into perillic acid. Perillic acid, naturally found in minor concentrations in the plant *Perilla frutescens*, shows antimicrobial activity against a broad range of microorganisms. The solvent tolerance of *P. putida* allows for the establishment of a highly efficient bioprocess, as the volatile limonene, usually toxic to most microorganisms, can be used at elevated concentrations in the bioreactor. A fed-batch bioprocess with glycerol fed as the C source and limonene as the precursor and *in situ* product recovery (ISPR) by an anion exchanger fluidized bed was realized. By this means product concentrations of up to 30 g/L after 5 days were achieved [2]. This process is currently being transferred to industry and it will probably become one of the first commercial biotechnological terpene oxyfunctionalizations. The biotech perillic acid will be introduced into the cosmetics market as a natural antimicrobial enhancer for preservation purposes.

Apart from that, *P. putida* DSM 12264 is currently being used in our laboratory as starting point to develop a universal microbial platform for carrying out valuable monoterpene hydroxylation reactions under technical conditions. For a first proof-of-concept, we successfully transferred the genes encoding P450cin and its co-proteins from *Citrobacter braakii* to *P. putida.* P450cin catalyzes the regio- and stereoselective hydroxylation of 1,8-cineole to 2*S*-beta-hydroxy-1,8-cineole, a chiral synthon for the production of flavor and fragrance compounds and herbicides. Product concentrations of 1 g/L were already obtained under non-optimized cultivation conditions in shake flasks. This biotransformation is currently being optimized by process and metabolic engineering approaches. For instance, we are investigating the impact of different efflux pumps to further improve the performance of the microbial host. Finally, substrate screening experiments revealed that other monoterpenes are tolerated by *P. putida* DSM 12264 as well, allowing to expand the platform towards the production of other valuable products in the future.

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2. M.A. Mirata, D. Heerd, J. Schrader (2009) Integrated bioprocess for the oxidation of limonene to perillic acid with *Pseudomonas putida* DSM 12264. Process Biochemistry 44 (7) 764-771

## O87. Development of a terpene synthase engineering platform for the production of isoprenoids in yeast

Lishan Zhao, Svetlana Borisova, Kevin Dietzel, Michele Fleck, Jeff Kim, Andrew Main, Chris Paddon, Sarah Reisinger, Anna Tai, Patrick Westfall, Yue Yang, Kati Wu, Lan Xu

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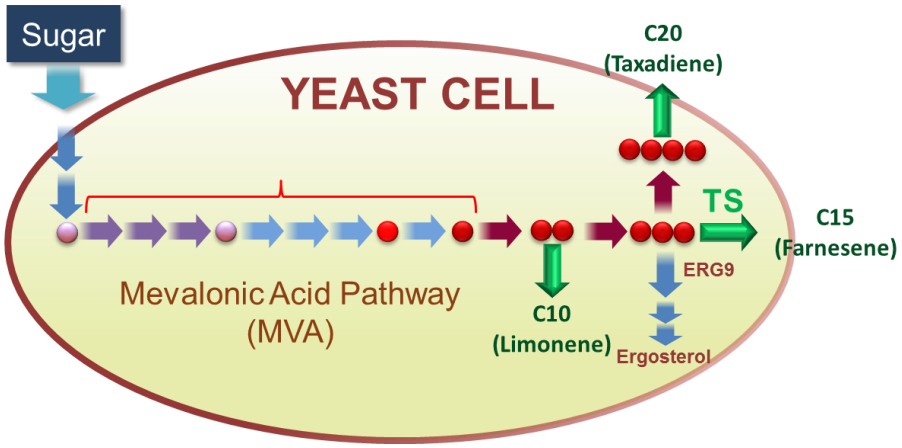
As one of the most structurally diverse classes of natural products with over 50,000 compounds, isoprenoids have traditionally been valued as commercially important products in a variety of biotechnology applications including biofuels and commodity chemicals, flavor and fragrances, pharmaceuticals, and nutraceuticals. Reliable and cost-effective production of isoprenoids is difficult to achieve via either chemical syntheses due to their complex structures or extraction directly from plant sources because of low content of these compounds. Large-scale production by microbial fermentation represents an attractive alternative source for isoprenoids. However, terpene synthases (TS), which converts prenyl diphosphate intermediates to terpenes, are slow enzymes and thus a key bottleneck for successful engineering of the isoprenoid biosynthetic pathway in microbes.

In this presentation, I will describe the development of a terpene synthase engineering platform that enabled us to overcome the limitations of terpene synthases and, as an example, how improved farnesene synthases enabled the high efficient production of farnesene, a sesquiterpene precursor for renewable chemicals and biodiesel. I will specifically focus on: 1) high throughput screens for terpene synthase engineering, 2) a competition assay for measuring terpene synthase activity and identifying improved terpene synthase variants in vivo, and 3) broad applicability of this platform for improving any terpene synthase of interest.

*Terpene synthase-enabled production of isoprenoids in Saccharomyces cerevisiae*



**Abstracts and Poster presentations**

**Poster session I, Sunday, 2nd of June**

**Posters P1 to P62**

**Poster session II, Monday, 3rd of June**

**Posters P61 to P124**

## Contents

## P1. Triterpenic content and biological evaluation of selected Cameroon propolis samples

Danai Papachroni[1], Konstantia Graikou[1], Ivan Kosalec[2], Verina Ingram[3] and Ioanna Chinou[1].

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[2]Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia
[3]CIFOR & University of Amsterdam, Netherlands

The bee-keeping product propolis (bee glue) is a resinous material that bees collect from plants to use it as a protective agent to prevent the spread of microbial in their hives and as construction material. It is claimed to improve human health and prevent diseases and is extensively used in folk medicine, in cosmetology and in food industry for health foods, beverages and nutrition supplements. It has been observed that the wide range of biological activities (antimicrobial, antiseptic, antitumor, anti-inflammatory, etc) of propolis depends on its chemical composition, more than 300 compounds have been identified in it, which in turn depends on geographical diversity. The research into African propolis is scarce and limited to North African regions, such as Tunisia [1], Algeria [2], and Egypt [3], as well as Kenya (East Africa) [4].

As a part of a systematic research on different propolis from all over the world, we report in this study the chemical analysis of three propolis samples from West Central Africa region, Cameroon (Oku in Northwest, Tekel and Ngaoundal in Adamaoua).
It is noteworthy that the Extracts of these three Propolis samples (EP) contain as abundant chemical category triterpenes followed by flavonoids and phenolics. The determined, through spectral data, triterpenes are amyrin, lupenone and ursene type. To the best of our knowledge, ursene type triterpenes have not been referred in propolis before, while amyrin and lupenone type have been found in propolis from different geographic origin (Brazil, Indonesia, Malta).
The concentration of total phenolics was also determined in these samples and they were evaluated for antimicrobial activities by diffusion method on agar medium evaluating the minimal inhibitory concentration (MIC), against six strains of human pathogenic bacteria and three fungi, showing an interest biological profile.

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## P2. *In vitro* culture of *Cistus creticus* subsp. *creticus* – a source of biological active compounds

Marijana Skorić[1*], Slađana Todorović[1], Mihailo Ristić[2], Marina Soković[1], Jasmina Glamočlija[1], Suzana Živković[1], Andreja Stojić[3], Nevena Puač[3], Angelos K. Kanellis[4]

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*Cistus creticus* subsp. *creticus* is a native plant of the Mediterranean region and it has been used since ancient times for its medicinal properties. Labdane type diterpenes are predominant compounds in leaves and stems of the *C. creticus* subsp. *creticus*, as well as in resin, while other terpenes, polyphenols and flavonoids contribute to chemical profile of this species.

*In vitro* plant tissue culture is an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemicals independent of plant availability and more consistent product quality. In order to obtain applicable production of secondary metabolites of interest we have implemented *in vitro* culture of several clones of *C. creticus,* which were previously morphogenetically described. The ethanol extracts of obtained *in vitro* cultures were analyzed by GC-FID and GC-MS, and were characterized concerning the total phenolic and flavonoid contents. Labdane diterpenes were the most abundant compounds in above ground parts extracts, but absent from the root extracts. Head-space GC-MS was used to determine the profile of volatile compounds in the atmosphere of the culture vessels. α pinene, β pinene, and camphene were the most abundant ones. PTR-MS was used for further quantification of representative volatile compounds in the atmosphere of the culture vessels.

Finally, the biological activity of obtained ethanol extract was studied. Microdilution method was used for investigation of antibacterial and antifungal activity. Almost all tested extract against human pathogenic bacteria showed better antibacterial activity than streptomycin, and much better antibacterial activity than ampicillin. All tested extracts also exhibited slightly higher or similar antifungal potential as bifonazole, and showed much better antifungal effect than ketoconazole. Antioxidant activity was determined by using DPPH (2,2-Diphenyl-1-picrylhydrazyl) test, while cytotoxic activity was previously demonstrated by sulforhodamine B (SRB) assay.

**Acknowledgements**

This work was supported by the Ministry of Education and Science of the Republic of Serbia Contracts No. 173024, 173032, 173021 and III 41011.

## P3. Headspace Solid Phase Microextraction procedure and chemical analysis of mastic gum

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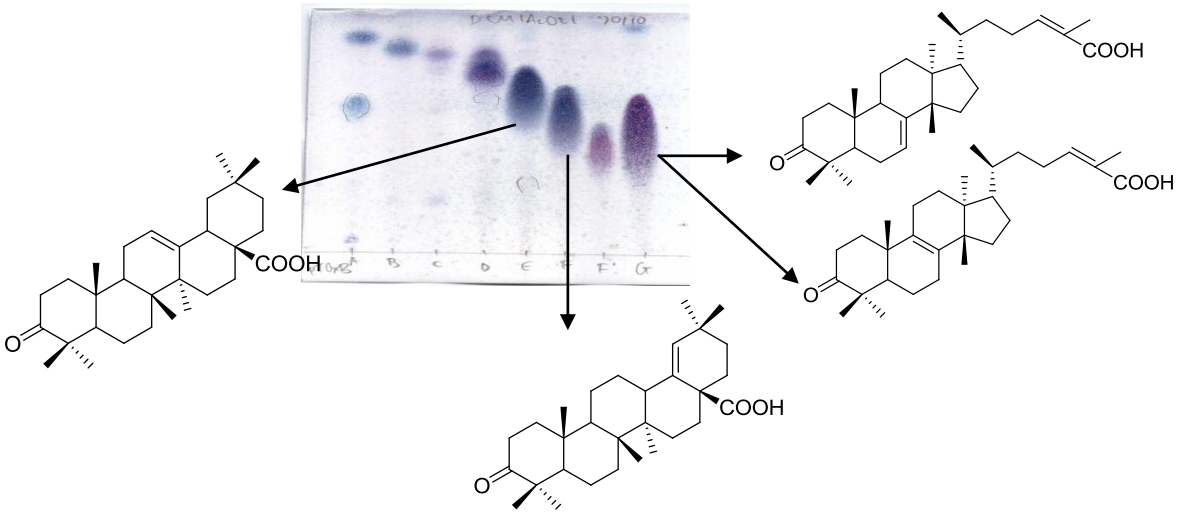
Mastic is a well-known natural resin from the trunk and branches, of *Pistacia lentiscus* var. *chia* (Anacardiaceae), which is grown as endemic only in the Greek island of Chios. It has been used in traditional Greek medicine for various gastrointestinal disorders (gastralgia, dyspepsia, peptic ulcer, etc) since antiquity. The plant has been mentioned by Hippocrates, Dioscorides, Theophrastos, and Galenos recommending its healing properties.

Recently, in several studies, mastic gum has shown very interesting antimicrobial profile against a panel of human pathogenic fungi and bacteria among which *Helicobacter pylori*.

In the framework of re-evaluation of important Greek natural products, we present the chemical analysis of mastic gum through classic chromatographic analysis but also through Headspace Solid Phase Microextraction (HSPM), which combines sampling free from organic solvents, applying to complex matrices, while it is economic, sufficiently fast and friendly to the environment and to our knowledge is presented for first time, on crude natural product

The aim was to develop a reliable analytical method based on Headspace Solid Phase Microextraction (HS-SPME) and Gas Chromatography–Mass Spectrometry (GC-MS) in order to detect volatile profile of the natural crude mastic gum. Through this analysis $\alpha$-pinene (25.6%), verbenone (14.0%), $\beta$-cymene and verbenene appeared as the most abundant constituents, representing 58% of the total, among the 27 identified volatile components of the mastic. Also 28-nor-oleanone, tirucallol, masticadienonic acid, iso-masticadienonic acid, oleanonic acid and moronic acid were isolated.



## P4. Cytotoxic Effects of Essential Oils and their components on BEND Cell Line

Ratajac Radomir[1], Stojanović Dragica[1], Petrović Tamas[1], Vasić Radica [2], Žekić- Stošić Marina[1], Stojanov Igor[1], Lako Branislav[3]

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The abundant use of anti-infective agents resulted in developing resistance of bacteria strains to certain antibiotics, presence of antibiotic residua in the food of animal origin etc. To overcome these problems, a variety of medicinal plants have been screened worldwide for their antimicrobial properties. The aim is to find new, effective antimicrobial agents with novel modes of actions. Essential oils (EOs) derived from aromatic medicinal plants have been reported to exhibit exceptionally good antimicrobial effects against bacteria, yeasts, filamentous fungi, and viruses.
The use of EOs as antimicrobial agents is not limited only by their effective concentrations *in vitro*, but also with the maximum dosage that can be administered without toxic side effects. Regarding EOs a number of investigations in cell culture systems have been carried out in order to predict their toxicity to mammalian cells *in vivo*. That means that EOs may exert cytotoxic effects to tissue cells at concentrations which do not yet show an antibacterial effect. The cytotoxic activity of EOs is based on their individual components. As in bacterial cells, the cell membrane is one of the sites of action where EOs and EO components were shown to cause permeabilization and depolarization and to reduce the activity of membrane-associated enzymes. In addition, an interaction with cellular metabolism and an induction of apoptosis have been demonstrated for EOs and oil components.
The purpose of the present work was to examine the cytotoxicity of EOs which had expressed good antibacterial activity against pathogens from cows utery. EOs ware obtained by hydrodistillation and were analyzed by gas chromatography (GC). EOs from savory (*Satureja montana*), thyme (*Thymus vulgaris*), peppermint (*Mentha piperita*) and some EOs constituents carvacrol, menthol, eugenol and thymol, which were the most effective substances used against isolates (field strains originating from cattle's uterus): *Arcanobacterium pyogenes, Escherichia coli, Staphylococcus aureus*, and *Streptococcus spp.*, were tested.

The BEND cell line derived from the uterine endometrium of a normal female cow on day 14 of the estrous cycle, morphology epithelial, was used for cytotoxicity evaluations. Different concentrations of EOs and constituents were tested by MTT assay, after an exposure time of 24h.
The EO from thyme, carvacrol and thymol present highest cytotoxic activity with $IC_{50}$ values of 0.22 - 0.34 µl/ml. The *S. montana* and *M. piperita* essential oils, eugenol and menthol showed less cytotoxicity with $IC_{50}$ values ranging from 0.52 to 1.27 µl/ml.

The results obtained have shown that EOs from savory, peppermint and EOs constituets menthol and eugenol were less toxic ageinst BEND cell lines, and their $IC_{50}$ values were equal to or slightly more higher than MICs values of these substances. However, further resarch is necessary to confirm these results and assess the toxicity of selected substances *in vivo*.

**Keywords:** essential oils; cytotoxicity; MTT; BEND cell line
***Acknowledgements:*** This work was supported by a grant from scientific project TR 031071 of Ministry of Education and Science of The Republic of Serbia

## P5. Chemical constituents and wound healing activity from the leaves of *Aquilaria sinensis*

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In recent years, new research shows that appropriately promote proMMP-9 and MMP-2 activity in specific phase of wound healing, which could help to reduce scar formation. Base on bioassay guided of wound healing activity, we found that the crude extract from leaves of *Aquilaria sinensis* has significant promoting activities for proMMP-9 and MMP-2. Therefore, the aim to find active wound healing component(s), we isolated and purified the MeOH extract from the leaves of *A. sinensis*. Twenty compounds were isolated from *A. sinensis* including aliphatic, chromones, flavonoids, ligands, glycosides, steroids, and terpenoids. All of the structures were identified by extensive spectroscopic analyses, including IR, UV, MS, 1D and 2D NMR. We measured MMP activity by Zymography method. As a result, the compound **7** has significant stimulating activities for proMMP-9 and MMP-2. Eventually, the present study explored the wound healing potential of compound **7**. In future work we will test it *in vivo* study.

## P6. A rapid and sensitive LC-MS/MS method for determination of metabolites of coenzyme Q10

Yi-Tzu Hsu, Ching-Kuo Lee

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ROC Coenzyme Q10 (CoQ10), also called ubiquinone, an essential fat-soluble substance for electron transport chain in mitochondria for synthesis of ATP. In addition, CoQ10 is widely consumed as a good compound to treat heart failure and there are many reports suggest that CoQ10 exerts a beneficial effect on a broad spectrum of pathological conditions, such as anti-inflammatory, anti-oxidant and anti-tumor functions. However, being a lipophilic substance the absorption of CoQ10 is limit and its metabolites in urine showed contained the ring structure with a short side chain and were phosphorylated. Nevertheless, the structure of vitamin K similar to CoQ10, which major urinary metabolites were conjugated with glucuronide and sulfate, therefore, we use synergi polar-RP column and TOF-MS for LC-MS/MS method to analyze serum and urine after oral administration rats in this study. The purpose of identify the metabolites of CoQ10.

## P7. Chemical constituents and melanogenesis-inhibitory activity of *Litchi chinensis*

HSU Ya-Ping[1], LEE Ching-Kuo[1, 2]

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*Litchi chinensis*, belonging to family Sapindaceae, is a king of subtropical fruit tree and famous for long time in Southeast Asia and Taiwan. The fruits of *Litchi chinensis* are called "Lychee" has dry, red pericarp and white edible aril. The seeds extract of lychee seeds extract hart which may reduce hyperpigmentation effect. In this study, the seeds of lychee was extracted with 95% ethanol and sequentially partitioned with ethyl acetate, *n*-butanol and water. Therefor, the ethyl acetate fraction with the higher inhibitory activity for tyrosinase and reduced hyperpigmentation for B16-F10 melanoma cells. The fraction was subjected to silica gel column chromatography. Ten subfractions were obtained and isolated. Compound 8, 9 isolated from lychee extracts showed activity in inhibition melanin production. The study lychee seeds were found effective anti-melanogenesis compounds and lychee seeds exacts are more important in the future.

## P8. Nepetalactones and phenolic acids as markers in the chemodiversity estimation within the genus *Nepeta* (fam. Lamiaceae)

Danijela Mišić[1], Jasmina Nestorović Živković[1], Branislav Šiler[1], Suzana Živković[1], Slavica Dmitrović[1], Aleksandra Mladenović[2], Stevan Avramov[1]

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Various biological activities of *Nepeta* species (fam. *Lamiaceae*) are closely related to their main secondary metabolites (terpenes and phenolics), and it is therefore important to understand, and be able to quantify existing chemical diversity and variability. Iridoid monoterpenes, nepetalactones, frequently appear as the main constituents of *Nepeta* species essential oils. Nepetalactone is a bicyclic monoterpenoid, i.e. it is a ten-carbon compound derived from isoprene, comprised of two fused rings: a cyclopentane and a lactone. It could exist in the form of eight stereoisomers, four diasteroisomers and their corresponding enantiomers, and only 7S diasteroisomers are found in natural sources. Among phenolic acids, rosmarinic acid, an ester of caffeic acid, has been reported as characteristic phenolic compound in genus *Nepeta*, and the whole subfamily *Nepetoidae*. Besides rosmarinic acid, a number of its related derivatives can also be found within the subfamily *Nepetoidae*. High diversity of nepetalactones and phenolic acids in *Nepeta* species candidates them as a convenient marker-system in chemodiversity evaluation within the genus.

Simple, fast and reliable UHPLC/DAD/±HESI-MS/MS method for the routine identification and quantification of nepetalactones and phenolic acids in selected *Nepeta* species was developed and validated. This method enabled rapid identification of targeted molecules in methanol extracts with a high degree of confidence, and provided high-throughput collection and quantitative analysis of analytical data. Principal component analysis was applied in search of distribution patterns and to test the significance in variation of nepetalactones and phenolic acids composition between and within the analyzed taxa. Two main groups of accessions were defined: nepetalactone-containing and nepetalactone-lacking species, which were all characterized by the prevalence of rosmarinic acid from the group of phenolic compounds. The knowledge on chemodiversity within genus *Nepeta* may allow selection of species for different groups of compounds and could significantly help in choosing the appropriate candidate species for further elucidation of the genes involved in the biosynthetic pathways of nepetalactones and phenolic acids.

## P9. The composition of essential oil *Lophanthus schrenkii* and its biological activity

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*Lophanthus schrenkii* Levin (Syn.: *Hyssopus lophanthus*) – perennial herb found in the foothills of the Eastern and Western Tien Shan, Tarbagatay and Betpakdala of Kazakhstan.
We studied by GC/MS method the composition of essential oil of *L. schrenkii* from raw material collected in the flowering stage June 30, 2008 in the mountains of Bektau-Ata in Central part of Kazakhstan. Essential oil was obtained by water distillation on the Clevenger type apparatus for 2 hours with 0.07% yield.

GC/MS analysis was performed on Agilent 7890A gas chromatograph with a mass selective detector Agilent 5975C. Injection of the sample was performed using Agilent 7693 autosampler. The system is ChemStation software (version E.02).

The main components of the volatile oil of *L. schrenkii* are spatulenol - 16.6%, 1,8-cineole - 9.7%, m-cymene – 7.6%, β-cadinol – 6.3%, ledol – 5.1%, α-kadinol - 4.7% and globulol - 4.6%. The study of the biological activity of the essential oil was carried out at the University of Mississippi, Oxford, USA. The study revealed that the essential oil of *L. shrenkii* exhibits high larvicidal activity at a dose 125 ppm, did not show anti-malaria activity, and also exhibits anti-microbial and anti-fungal activity (inhibition strains of *C. neoformans*, % Inh. - 20.45%).

Essential oil of *L. shrenkii* been studied for antioxidant activity *in vitro* with o-phenanthroline and found that the essential oil has a high coefficient of inhibition, exhibits strong antioxidant activity, which allows it to recommend further study of the activity *in vivo*.

## P10. Proteomics as a tool to identify proteins involved in the synthesis and transport of secondary metabolites in non-model plant species

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Plants produce a wide range of secondary metabolites which are generallytypical of a given species. To reveal and possibly improve the metabolic pathway leading to the synthesis of these diverse and sometimes complex compounds in non-model species, there is no alternativethan working on these species.Strikingly, proteomics of plant secondary metabolism isstill a neglected research field, most likely due to the lack of reliable sequence database andthe challenging access to low abundant proteins.However, recent progress in proteomicstoolshas been made and nucleic sequences from various species become more widely available (Proteomics (2013, 13, 663). To illustrate these advances, we extensivelyinvestigated the proteome of glandular trichomes*Menthaspicata*,*Nicotianatabacum*(Proteomics (2011) 11, 440) and*Catharanthusroseus*(Proteomics (2012) 12, 3536), known to accumulate monoterpenes, diterpenes and terpenoidindole-alkaloids, respectively. Very large sets of proteins were identified, for instance up to 1663 proteins in *C. roseus*.Among these, 63 enzymes potentially involved in secondary metabolismwere identified, of which 22 are involved in terpenoidindole alkaloid biosynthesis. Some technically challenging membrane proteins were also identified and16were predicted to be putatively involved in secondary metabolite transport. In conclusion, proteomics tools can be used to identify proteins involved in the synthesis and transport of secondary metabolites in non-model species even though 30%of the proteins identified have unclear or unknown function, indicating remaining gapsin the knowledge of the plant metabolism.

*Keywords: Catharanthusroseus, Menthaspicata, Nicotianatabacum, Non-model plant, Proteomics, Terpenoids*

## P11. Exploring function of carotenoid cleavage dioxygenase gene family in rice plant

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A family of CCDs (carotenoid cleavage dioxygenases) produces apocarotenoids including biologically active compounds such as hormones, pigments and volatiles by oxidative cleavage of carotenoids. Rice has nine putative CCD genes. Among them, three members (OsNCED1, 3 and 8) of the family are implicated in the synthesis of abscisic acid. Another three (OsCCD7, 8a and 8b) are related to control lateral shoot branching by forming novel phytohormone strigolactone. However, the function of the other three (OsCCD1, 4a and 4b) are still unclear to be elucidated. This study was performed to identify and characterize the functions of the three rice *CCD* genes(*OsCCD1, OsCCD4a* and *OsCCD4b*) and their Arabidopsis ortholog gene(*AtCCD4*) via over-expression and suppression in rice plants. Firstly, four *CCD* genes of *OsCCD1, OsCCD4a, OsCCD4b* and *AtCCD4* were constitutively over-expressed and enough number of transgenic lines($T_1$) were selected. Their leaf tissues were used for analysis of metabolites such as carotenoids and carotenoid cleavage products(CCPs) as well as microarray. Now, we are interpreting the integrated DATA of these metabolites and transcript profiles. To characterize the endogenous function of three rice *CCD* genes, the approaches by RNAi-mediated suppression were being carried out. Furthermore, we are making transgenic rice plants with ultimate aim to improve transgenic β-carotene rice, which has been already developed using bicistronic gene expression (*Ha et al.*, 2010), via engineering of the rice CCD gene functions.

## P12. Evolution and diversity of the 2-oxoglutarate-dependent dioxygenase superfamily in plants

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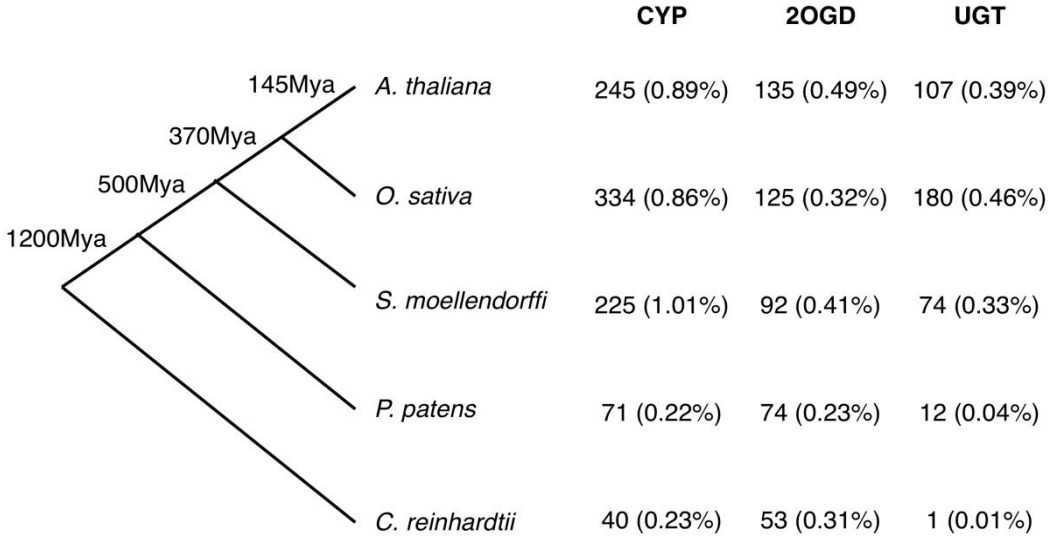
2-Oxoglutarate-dependent dioxygenase (2OGD) superfamily is the second-largest enzyme family in the plant genome and is involved in various biological aspects of oxygenation/hydroxylation reactions. Despite their biochemical significance in metabolism, a systematic analysis of plant 2OGDs remains to be accomplished. Here we present the phylogenetic classification of 479 2OGDs in model plants ranging from green algae to angiosperms. The plant 2OGDs were divided into 3 classes—AlkB, P4H, and SpM—based on a similarity search in CLANS. The AlkB class is widespread in bacteria and eukaryotes and involved in the DNA damage repair through *N*-demethylation. The P4H class is conserved in all plant taxa and involved in proline 4-hydroxylation in cell wall protein synthesis. The third SpM class involved in specialized metabolism was further divided into 19 clades (A–X) and grouped into three clans: Algal clan (A and B clades), GA clan (C–K clades), and FL clan (L–X clades). The Algal clan comprises 2OGDs from algae to angiosperms, suggesting that they play a fundamental role in plant metabolism. GA clan includes 2OGDs involved in gibberellin biosynthesis. FL clan includes 2OGDs involved in flavonoid biosynthesis and various specialized metabolisms. The most outstanding feature is the excessive multiplication of 2OGD genes of the GA and FL clans in seed plants, which is partly responsible for the diversity and complexity of these metabolisms.




| | CYP | 2OGD | UGT |
|---|---|---|---|
| *A. thaliana* | 245 (0.89%) | 135 (0.49%) | 107 (0.39%) |
| *O. sativa* | 334 (0.86%) | 125 (0.32%) | 180 (0.46%) |
| *S. moellendorffi* | 225 (1.01%) | 92 (0.41%) | 74 (0.33%) |
| *P. patens* | 71 (0.22%) | 74 (0.23%) | 12 (0.04%) |
| *C. reinhardtii* | 40 (0.23%) | 53 (0.31%) | 1 (0.01%) |

## P13. Culture media effect on α-terpineol synthase gene expression and essential oils composition in *Thymus caespititius* grown in vitro

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Keywords: Thyme, Lamiaceae, *in vitro* culture, terpene synthase, α-terpineol

*Thymus caespititius* Brot. is an aromatic plant that possess thymol-, carvacrol-, α-terpineol-sabinene- or carvacrol/thymol-rich essential oils [1]. Given the interest on this species' essential oil, *in vitro* cultures were established aiming to study the effect of using different culture media on the expression of a putative terpene synthase and on the essential oil composition.

*In vitro* cultures of two *T. caespititius* genotypes (G1, G2) were established from seeds aseptically germinated on solid Schulz medium [2]. One week after, the seedlings were transferred to MS [3] medium supplement with 0.4 $mg.L^{-1}$ BA and 0.1 $mg.L^{-1}$ IBA to obtain proliferative shoots. Five months following culture initiation, some of the shoots were transferred to SH medium [4] with the same growth regulators. Total RNA was isolated from leaves of *in vitro* shoots. cDNA was synthetized using M-MLV reverse transcriptase. The essential oils were isolated by hydrodistillation and analyzed by GC and GC-MS as in [5].

The expression of *Tctps5*, characterized as a α-terpineol synthase gene, was evaluated for both genotypes shoots grown on the two culture media. This monoterpene synthase gene expression was only observed for both genotypes grown on SH medium, while in explants grown on MS medium the expression was not detected. In terms of α-terpineol relative amount in the corresponding essential oils, shoots grown on SH medium had only 2-3% α-terpineol while in those grown on MS medium ranged from trace-1%.

The different nutrient composition of the two media altered quantitatively the composition of the essential oils of the G1 and G2 *in vitro* shoots. In addition to variation in the α-terpineol percentage, differences were also observed in others components, namely *p-cymene* (6-19% in shoots gown on MS *vs* 9-30% in shoots grown on SH), thymol (11-18% in shoots gown on MS *vs* 4-13% in those on SH medium) and carvacrol methyl ether (9-17% in shoots gown on MS medium and 4-8% in shoots grown on SH medium).

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## P14. Gene Mining for Camptothecin Production in *Ophiorrhiza pumila*

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Camptothecin (CPT) is a monoterpenoid indole alkaloid, which inhibits DNA topoisomerase I, and an important lead compound of anticancer drugs. CPT is biosynthesized in several plant species via strictosidine, the common intermediate in biosynthesis of various monoterpenoid indole alkaloids. However, catalytic reactions, intermediates and their regulatory mechanism are still unclear in the late steps after strictosidine to CPT. We conducted mining of genes and metabolites on *Ophiorrhiza pumila,* a CPT-producing Rubiaceae species. The hairy roots of *O. pumila* induced by the infection with *Agrobacterium rhizogenes* produce CPT and other specialized metabolites such as anthraquinones, while the levels of these metabolites are very low in the dedifferentiated cell suspension culture derived from the hairy roots. For the mining of biosynthetic intermediates in CPT biosynthetic pathway, the one of two genes encoding tryptophan decarboxylase (*TDC*) and secologanin synthase (*SLS*) involved in strictosidine biosynthesis was knocked down by RNAi technique in hairy roots. The accumulation amount of CPT in silenced hairy roots was reduced in positive correlation with the gene expression levels of both *TDC* and *SLS*. Untargeted metabolomic analysis of these tissues was performed by using infusion Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), LC/ FT-ICR-MS and LC/MS. Among the specific mass ion peaks detected in hairy root but not in cell suspension culture, several peaks exhibited positive or negative correlation with the gene expression levels of *TDC* and *SLS* in RNAi hairy roots as well as CPT peak. Furthermore, deep transcriptome analysis was performed on hairy roots and cell suspension cultures using the Illumina platform. A hybrid transcriptome assembly was generated using the Illumina-derived sequences and conventional Sanger-derived expressed sequence tags clones from hairy roots. Among 35,608 non-redundant unigenes, 3,649 were preferentially expressed in hairy roots compared to cell suspension culture. The candidate genes involved in the biosynthetic pathway for the monoterpenoid indole alkaloid were profiled; specifically, genes involved in post-strictosamide biosynthetic events and genes involved in the biosynthesis of anthraquinones and chlorogenic acid. Untargeted metabolomic analysis by FT-ICR-MS indicated that most of proposed intermediates in the camptothecin biosynthetic pathway accumulated in hairy roots in a preferential manner compared to cell suspension culture. These results suggest that transcriptome and metabolome datasets can facilitate the identification of genes and intermediates involved in the biosynthesis of secondary products including camptothecin in *O. pumila*.

## P15. A full transcritome analysis to isolate terpene synthases from the weed Silverleaf nightshade (*Solanum elaeagnifolium*)

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*S. elaeagnifolium* (Silverleaf nightshade) is a perennial weed of the Solanaceae family very adaptive to the mediterranean climate of warm summer with high temperatures and limited rainfalls all over the year. Due to its widespread root system and its asexual propagation by undeground parts, as well as the production of a high number of seeds, the plant quickly established and expanded in many cultivated and metropolitan areas of Greece imposing a serious threat to biodiversity and agriculture by its invasive nature. The plant is considered to be a host of several plant viruses while its fruits are toxic to many livestock animals. Inside crop cultivations it is very difficult to be eliminated or controlled. The *S. elaeagnifolium* plants are characterized by their resistance to drought and their wide adaptation to a variety of soil types.
mRNA was prepared from fully expanded leaves and open flowers of *S. elaeagnifolium* plants and sequenced by Illumina sequencing. 138.604 EST contigs of 385 bases mean length were assembled and used for the construction of 75.618 Unigenes of 1082 bases mean length. For the identification of terpene synthase putative orthologs in this EST dataset, a set consisting of the 29 functional or potentially functional genes that belong to the tomato Terpene Synthase (*TPS*) gene family was used in local BLAST searches against the *S. elaeagnifolium* EST database. Numerous Contigs/Unigenes were retrieved bearing high homology with the tomato *TPS* genes. A phylogenetic tree was constructed and it was found that *S. elaeagnifolium* ESTs deduced aminoacid sequences are placed in all the recognized phylogenetic clades of tomato TPS proteins. Putative orthologs belonging to the TPS-a, TPS-b, TPS-c, TPS-e/f, TPS-g clades were identified. Gene cloning and expression analysis of selected terpen synthases is in progress.

## P16. Insights into terpenoid biosynthesis and diversity in Apiaceae through transcriptome analysis of *Thapsia laciniata* Rouy

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*Thapsia laciniata* Rouy (Apiaceae) produces irregular and regular sesquiterpenoids with thapsane and guaiene carbon skeletons, as found in other Apiaceae species (see figure for biosynthesis). A transcriptomic analysis utilizing Illumina next-generation sequencing enabled the identification novel genes involved in the biosynthesis of terpenoids in *Thapsia*. From 66.78 million HQ paired-end reads obtained from *T. laciniata* roots, 64.58 million were assembled into 76,565 contigs (N50: 1261 bp). 17 contigs were annotated as terpene synthase, 5 of these were predicted as sesquiterpene synthases. Of the 67 contigs annotated as cytochromes P450, 18 of these are part of the CYP71 clade that primarily is perform hydroxylations of specialised metabolites. 3 contigs annotated as aldehyde dehydrogenases grouped phylogenetically with the characterized ALDH1 from *Artemisia annua* and 3 contigs annotated as alcohol dehydrogenases grouped with the recently described ADH1 from *A. annua*. ALDH1 and ADH1 were characterized as part of the artemisinin biosynthesis. We have produced a comprehensive EST dataset for *T. laciniata* roots, which contains a large sample of the *T. laciniata* transcriptome. These transcriptome data provide the foundation for future research into the molecular basis for terpenoid biosynthesis in *Thapsia* and on the evolution of terpenoids in Apiaceae.

## P17. Identification and characterization of terpene synthases involved in carrot flavor biosynthesis

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The aroma and flavor of foods, especially fruit and root crops, are due to often complex mixtures of volatile compounds. Distinctive flavor profiles vary widely from one cultivar to another and result from different proportions of key volatile and non-volatile metabolites. The characteristic flavor of carrot (*Daucus carota*) is mainly due to volatile terpenoid constituents. GC-MS analysis of the headspace of volatile compounds in freshly harvested tissue of five different commercial colored cultivars (orange "Nairobi", yellow "Yellowstone", red "Rothild", purple "Purple Haze", white "Crème de lite") identified more than 47 compounds, of which 23 were monoterpenes, 18 were sesquiterpenes, and the rest were fatty acid derivatives. However, no carrot-specific terpene biosynthesis genes have been reported to date. Transcriptome analysis of *D. carota* cultivars B6274 and B7267 resulted in the identification of at least six putative terpene synthase (*TPS*) genes, of which three are presumably monoterpene synthases and three may be sesquiterpene synthases (Iorizzo et al., 2011). cDNAs were recovered for all *TPS* candidates and subjected to *in vivo* and *in vitro* characterization. Heterologous expression in a bacterial system demonstrated that one of the terpene synthases, DcTPS_4929, is an active sesquiterpene synthase that mainly produced (*E*)-β-caryophyllene and α-humulene as the primary products. (*E*)-β-Caryophyllene is one of the most prominent terpenes in all carrot cultivars. These observations establish a framework for evaluating the role of TPS activity in contributing to the flavor of carrots.

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## P18. Towards elucidating the carnosic acid biosynthetic pathway in sage (*Salvia fruticosa*) and rosemary (*Rosmarinus officinalis*): Functional characterization of the first steps of the pathway in *E. coli, S. cerevisiae* and *N. benthamiana*

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Carnosic acid (CA) is a phenolic diterpene detected in Salvia species and rosemary, which possesses a strong antioxidant activity and has also shown to exhibit anti-tumor, anti-diabetic and antibacterial activities as well as neurodegenerative protection. One of the main objectives of the EU-TERPMED project is the elucidation of the CA biosynthetic pathway in *Salvia fruticosa* (Cretan sage) and Rosemary (*Rosmarinus officinalis*). Functional genomic approaches applied to the glandular trichomes of *S. fruticosa* and *R. officinalis* leaded to the identification of a number of terpene synthases among of which two putative diterpene synthases showing similarities to copalyl diphosphate synthase (*SfCPS and RoCPS*) and kaurene synthase-like (*SfKSL and RoKSL*) genes. Recombinant expression in *E. coli* followed by *in vitro* enzyme activity assays have shown that indeed SfCPS coupled with SfKSL resulted in the synthesis of an unknown terpene as a major product. Recombinant expression in yeast confirmed the formation of the new diterpene product produced in high amounts. The purified compound was identified as Miltiradiene, on the basis of 1D and 2D NMR data ($^{1}$H, $^{13}$C, DEPT, COSY H-H, HMQC and HMBC). Coupled transient *in vivo* assays of *SfCPS/RoCPS* and *SfKSL/RoKSL* in *N. benthamiana* further proved the product of these two enzymes to be Miltiradiene. Therefore, we show the cloning and functional characterization of a Miltiradiene synthase from the Cretan sage and Rosemary, which is responsible for the synthesis of a putative intermediate product in the carnosic acid biosynthesis.

## P19. Transriptome analysis of Lavandula angustifolia allows deciphering of terpene metabolism and functional characterization of three sesquiterpene synthases

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A 454 library of *Lavandula angustifolia* cv Diva was obtained from RNAs extracted from different plant organs including leaves, roots and inflorescences at different stages of development. More than 103,000 unigenes could be annotated with an assigned putative function. This EST collection was used to obtain full-length sequences of several key enzymes of the MEP and MEV pathways. Based on homologies to sequences present in Genbank, four *DXS*orthologs of *Salvia miltiorrhiza*could be identified. Sequences of both homomeric and heteromeric*GPPS* were also found. A sesquiterpene synthase sub-library including 37 ESTs allowed evaluation of the putative number of sesquiterpene synthase genes expressed in lavender. Partial gene sequences were used to characterize three sTPSs: a germacrene D (LaGERDS), a (*E*)-□-caryophyllene (LaCARS) and a □-cadinol synthases (LaCADS). T-cadinol synthase is reported here for the first time and its activity was studied in several biological models including transiently or stably transformed tobacco species. A molecular model of LaCADS is proposed, providing evidence of the implication of a conserved aspartate residue in the capture of a water molecule close to the cadinylcation leading to the stabilization of this carbocationin□ □-cadinol. Quantitative PCR performed from leaves and inflorescences showed two patterns of expression. *LaGERDS* and *LaCARS* were mainly expressed during early stages of flower development and, at these stages, transcript levels paralleled the accumulation of the corresponding terpene products (germacrene D and (*E*)-□-caryophyllene). By contrast, the expression level of *LaCADS* was constant in leaves and flowers.

## P20. Transcriptome analysis of *Cistus creticus* subsp. *creticus* trichomes, with focus on diterpene-related synthases

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The resin secreted from trichomes of the plant *Cistus creticus* subsp. *creticus*, endemic in the Cretan flora, is very rich in secondary metabolites. Among those the group of terpenes has been found to have many potential applications in the chemical, food and pharmaceutical industries. In this work, the tool of transcriptome profiling RNA-seq was used in trichomes of *C. creticus* to obtain sequences related to terpene biosynthesis with focus on diterpenes. A total of 385,143 contig sequences (114,239 unigenes) were obtained with mean length 207 nucleotides. Among those, 2,000 unigenes were identified as related to the biosynthesis, transport and catabolism of secondary metabolites. Using bioinformatic and expression analyses, 60 contigs were identified, potentially encoding for synthases belonging to the three major groups of terpenes, monoterpenes, sesquiterpenes and diterpenes, including labdane-type. From these, 20 partial sequences were considered as potential candidates implicated in the biosynthetic pathways of diterpenoid metabolites. Among them, two genes were selected for further characterization analyses, encoding for a 798aa class-I (DDXXD motif) and an 808aa class-II (DXDD motif) potentially labdane-type diterpene proteins. Full-length isolation of the two cDNAs was conducted, followed by expression of the gene products in *E. coli*, yeast and tobacco. Gene expression analyses in healthy and wounded tissues belonging to two leaf developmental stages rich in terpenoids, showed specific expression of terpenoid related synthases in trichomes, but no induction by wounding.

## P21. Sugar availability modulates polyisoprenoid and phytosterol profiles in *Arabidopsis thaliana* hairy root culture.

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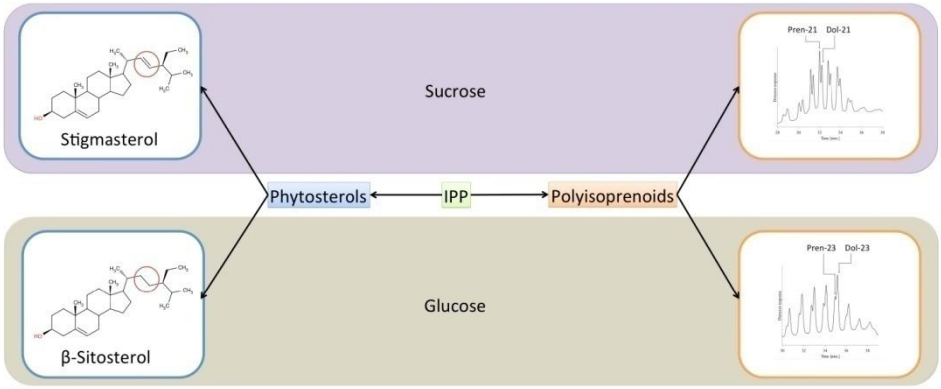
Polyisoprenoid alcohols constitute a group of hydrophobic polymers occurring as a mixture ('family') with one most abundant species and a Gaussian-like distribution of homologues in almost all living organisms. Polyisoprenoid alcohols can be divided into two groups: dolichols (hydrogenated double bond in the α-residue) and polyprenols (unsaturated ones). Dolichols have been detected in mammalian and yeast cells and in plant roots. The biological functions of dolichols and polyprenols in plants and other eukaryotes need further studies. On the other hand, the role of phosphorylated dolichols as cofactors in protein glycosylation and glycosylphosphoinositol (GPI) anchor synthesis in eukaryotic cells is well characterized.




Here, a modulatory effect of sugars on dolichol and phytosterol profiles was noted in the hairy roots of *Arabidopsis thaliana*. Arabidopsis roots contain a complex dolichol mixture comprising three groups ('families') of dolichols differing in the chain-length. These dolichols, especially the longest ones are accompanied by considerable amounts of polyprenols of the same length. The spectrum of polyisoprenoid alcohols, i.e. dolichols and polyprenols, was dependent on sugar type (glucose or sucrose) and its concentration in the medium. Moreover, the ratio of polyprenols versus respective dolichols was also modulated by sugar, with polyprenols dominating at sucrose and dolichols at glucose. Glucose concentration affected the expression level of genes encoding *cis*-prenyltransferases, enzymes responsible for elongation of the polyisoprenoid chain. Similarly to the polyisoprenoids, sterol profile responded to the sugar present in the medium, β-sitosterol dominating in roots grown on 3% or lower glucose concentrations and stigmasterol in 3% sucrose. These results indicate on involvement of sugar signalling in the regulation of *cis*-prenyltransferases and phytosterol pathway enzymes.

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## P22. Using combinatorial biochemistry for the elucidation of cytochromes P450 involved in thapsigargin biosynthesis

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Thapsigargin, a sesquiterpene lactone produced by the Mediterranean plant *Thapsia garganica*, is currently being developed as a new type of cancer drug (Genspera Ltd.). Cultivation of *T. garganica* is difficult and chemical synthesis of thapsigargin is a cumbersome and expensive procedure. Thus, we have set out to elucidate the biosynthesis of thapsigargin to enable biotechnological production. *T. garganica* belongs to the Apiaceae family and the precursor of all sesquiterpene lactones, farnesyl pyrophosphate (FPP), was found by Pickel et al. (Biochemical Journal, 2012, 448, p. 261-271) to be converted to kunzeaol in *T. garganica* by the sesquiterpene synthase, STS2 (AFV09099.1). The structure of kunzeaol makes it a probable candidate for the first step in thapsigargin biosynthesis.

Further hydroxylation and modification of kunzeaol is expected to be performed by enzymes from the cytochrome P450 family. Cytochromes P450 from Apiaceae only share low sequence identity with cytochromes P450 from other plant families. However, because so far only cytochromes P450 from the 71 clade have been found to participate in biosynthesis of sesquiterpene lactones, *Thapsia* cytochromes P450 in this clade are promising candidates for characterization. Full length sequences of eight cytochromes P450 belonging to the 71 clade found in a sequencing dataset of the transcriptome of *T. garganica* root were selected as the primary candidates to undergo expression and substrate analysis. Three expression platforms were chosen to characterize the eight cytochromes P450. In the first round *Saccharomyces cerevisiae* and the strain EPY300, which has been modified to have an enhanced production of FPP, is being utilized. The candidate cytochromes P450 are being cloned into the pESC LEU2d yeast expression vector along with one of the native cytochrome P450 reductases and the sesquiterpene synthase, STS2. In *Nicotiana benthamiana Agrobacterium tumefaciens* infiltration will be used to transiently express ST2 in combination with the eight cytochromes P450, extractions will subsequently be treated with viscozyme to avoid possible glycosylations. As a third expression platform *Physcomitrella patens* with STS2 integrated into the genome will be exploited, this type of host is interesting due to the lack of glycosylations while still belonging to the plant kingdom. A variety of sampling methods are currently being tested including the use of volatile analysis using headspace SPME fibers, hexane, diethyl ether or methanol extraction followed by analysis by GC-MS or LC-MS. This will lead to the discovery of a cytochrome P450 able to hydroxylate kunzeaol into an alcohol, an acid or facilitate formation of an epoxide.

## P23. Identification of the first bisabolene synthase of Asteraceae from linear glandular trichomes of sunflower

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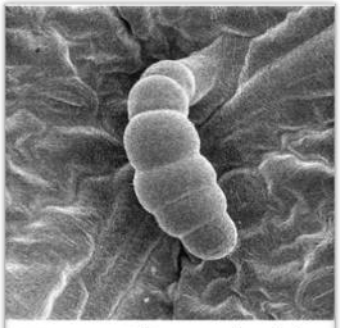

**Figure:** Linear glandular trichome of *Helianthus annuus*.

Plant glandular trichomes have a high potential to produce bioactive natural compounds. In Asteraceae, they are particularly involved in the biosynthesis of sesquiterpenes. On the epidermal surface of sunflower, two different types of glands appear -the capitate (CGT) and the linear (LGT) glandular trichomes. They differ in morphology, occurrence and chemical composition. While the CGT are known to produce sesquiterpene-lactones [1], storage of bisabolene-type sesquiterpenes was shown in LGT [2]. The biosynthesis of bisabolenes in sunflower and other Asteraceae has not been studied yet and so far only a few bisabolene synthases from other plant species
(e.g. *A. thaliana, Zea mays*) have been characterized.

To identify a candidate enzyme involved in the first step of bisabolene biosynthesis in sunflower, a screening of a cDNA pool of LGT coupled with database research for conserved amino acid sequences was carried out. The full open reading frame of the candidate gene *HaTPS12_1* was identified by means of RACE-experiments. Functional characterization of *HaTPS12_1* was carried out in the yeast strain EPY300 engineered to *de novo* synthesize an elevated level of the substrate, farnesyl pyrophosphate [3]. *HaTPS12_1* was expressed heterologously *in vivo* using high-copy plasmid [4] pESC-Leu2d. Produced compounds, enriched in the dodecane overlaid the culture, were purified and its structure was elucidated.

The product analysis with GC/MS afforded a compound with MW 204.2, for $C_{15}H_{24}$.The structure of γ-bisabolene was deduced from $^{1}$H-NMR and COSY experiments and the (Z)configuration was shown with 2D ROESY experiments. This confirmed the structure of the product of *HaTPS12_1* as (Z)-γ-bisabolene.

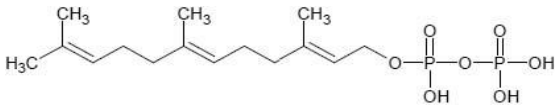


Figure: Linear glandular trichome of Helianthus annuus. Farnesyl pyrophosphate (Z)-γ-bisabolene

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[2] Spring, O., Rodon, U. and Macias, F.A. (1992) Sesquiterpenes from noncapitate glandular trichomes of Helianthus annuus L. Phytochemistry. 1992, 31: 1541 – 1544
[3] Ro, DK.; Paradise, EM.; Quellet, M.; Fisher, KJ.; Newman, KL.;, Ndungu, JM.; Ho, KA.; Eachus, RA.; Ham, TS.; Kirby, J., et al. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature; 440:940–943.
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## P24. TILLING collection of *Medicago truncatula* plants as a tool for investigation of saponin biosynthesis

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In the genus *Medicago* saponins is a complex mixture of triterpenic pentacyclic glycosides with a broad spectrum of biological properties such as antifungal, insecticidal, nematicidal, phytotoxic, allelopathic, ipocholesterolemic, anticarcinogenic and haemolytic (Tava and Avato 2006). The high potential for pharmaceutical applications ascribed to this class of phytochemicals stimulated studies on their biosynthesis for industrial exploitation and in green agriculture application.
In our Institute we have been studying the biosynthesis of saponin in *Medicago* genus using a *M. truncatula* TILLING collection (Porceddu et al., 2008). As previously proposed, the synthesis of these secondary metabolites starts from the □-amyrin, following two different pathways for hemolytic and not-hemolytic compounds and involve several oxidative steps mediated by specific cytochromes P450 (Tava et al. 2010).

Recently we have identified CYP716A12 as the key enzyme that catalized the first step in the hemolytic sapogenin pathway, from □-amyrin to oleanolic acid (Carelli et al., 2011). Further oxidative steps take place from oleanolic acid to give all the other hemolytic saponins.
Other cytochromes P450 (CYP93E2, CYP72A61, CYP72A68) have been demonstrated to be involved in the *M. truncatula* saponin biosynthesis using transgenic yeast strain (Fukushima et al., 2013).

Using our *M. truncatula* TILLING collection, we have identified three lines, mutated in P450 genes, involved in the oxidative steps of the triterpenic pentacyclic skeleton. One line founded to be involved in the hemolytic pathway is under characterization; the other two lines, currently under study, are putatively involved in the non-hemolytic saponin pathway.
An updated version of sapogenin biosynthetic pathway will be proposed.

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**Tava A**, Scotti C, Avato P. *Nat. Prod. Comm*. 2006 1(12): 1159-1180.

## P25. Terpinolene is the first olefin monoterpene intermediate to a tropolone, β-thujaplicin – Potential novel pathway to tropolone ring –

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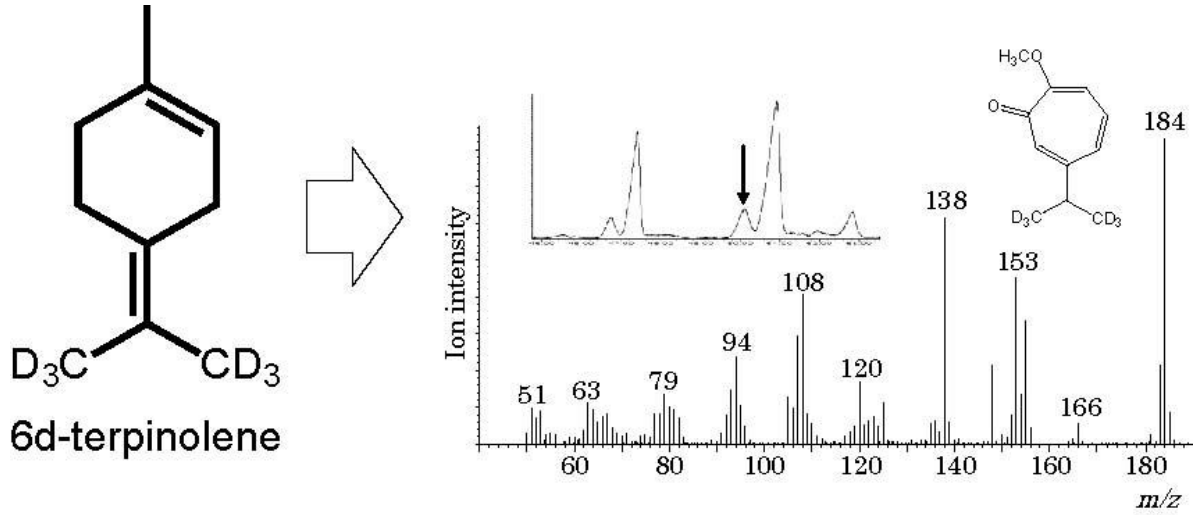
β-Thujaplicin (Hinokitiol) is a wood monoterpene and a tropolone compound, which has unique conjugated seven-membered ring. Because of its strong antifungal and antitumor activities, β-thujaplicin has been used in several fields. However, the biosynthetic pathway of it has not been elucidated. Our group had proved that geranyl pyrophosphate (GPP) was the starting material of this pathway using *Cupressus lusitanica* cell cultures with radioisotope feeding experiment. In addition, our previous study suggested that terpinolene was the next metabolite from GPP based on the results of terpene synthase assay. In this study, we performed feeding experiment of deuterium-labeled terpinolene into the cultured cell of *C. lusitanica* in order to determine whether terpinolene is intermediate of β-thujaplicin biosynthesis. GC/MS analysis of cell extract from the culture labeled-terpinolene fed revealed the existence of the peak of labeled β-thujaplicin which was not observed in treatment of non-labeled terpinolene. Identification of labeled β-thujaplicin was confirmed by mass spectrum assignment. This indicated that terpinolene was the first olefin monoterpene intermediate of β-thujaplicin biosynthesis. There has been no report that tropolone compounds are biosynthesized *via* terpene biosynthesis system, so the result suggests the existence of a novel biosynthetic pathway to produce conjugated seven-membered ring.

Fig. Structure of terpinolene fed to *C. lusitanica* cell and Mass spectrum of d-labaled β-thujaplicin extracted from the cell.



## P26. Peroxisomal localisation of isoprenoid biosynthetic enzymes

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In plants, the classical view of the isoprenoid metabolism compartmentalisation is based on the physical separation of biosynthetic enzymes between plastid, mitochondria and cytosol/RE. The role of peroxisome in isoprenoid metabolism has been brought into questioned in several reports. A recent study by Sapir-Mir et al. [1] revealed that the two short isoforms of the *Arabidopsis thaliana* isopentenyl diphosphate (IPP) isomerase (IDI), catalysing the isomerisation of IPP to dimethylallyl diphosphate are found in the peroxisome.

Following this work, we extended this type of study to investigate other isoprenoid enzymes leading us to re-evaluate the classical scheme of their cytosolic localisation. Thus, transient transformations of Madagascar periwinkle (*Catharanthus roseus*) cells with Yellow Fluorescent Protein-fused constructs revealed that the final two enzymes of the mevalonate pathway, 5-phosphomevalonate kinase and mevalonate 5-diphosphate decarboxylase are localised to peroxisomes in both *A. thaliana* and *C. roseus* [2].

Furthermore, we report on the unique triple targeting of two *Catharanthus roseus* IDI isoforms encoded by a single gene (*CrIDI1*) [3]. The triple localisation of CrIDI1 to mitochondria, plastids and peroxisomes is explained by alternative transcription initiation sites within the *CrIDI* sequence, by the specificity of a bifunctional N-terminal mitochondria/plastid transit peptide, and by the presence of a C-terminal peroxisomal targeting signal.

Recently, we provide additional data describing the peroxisomal localisation of a short isoform of farnesyl diphosphate (FPP) synthase (FPS) from *C. roseus* [4] and from *A. thaliana* (unpublished results).

On the basis of these results, we propose a new model for the subcellular distribution of the early steps leading to the biosynthesis of FPP and then to isoprenoids in plants, providing evidence for a complex compartmentalisation of IDI, FPS and the MVA pathway enzymes. Such a model highlights the existence of a potential exchange of intermediate metabolites between cytosol, ER and peroxisome but also raises the question of the specific role of individual short IDI and FPS isoforms in the biosynthesis of isoprenoid end-products. Finally, in light of these new data, one may question whether the peroxisome could be an autonomous organelle capable of synthesising isoprenoid compounds itself from its own FPP pool.

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4. Thabet (2012) J Plant Physiol 168 : 2110-16

## P27. Closing the last gap in costunolide synthesis of sunflower

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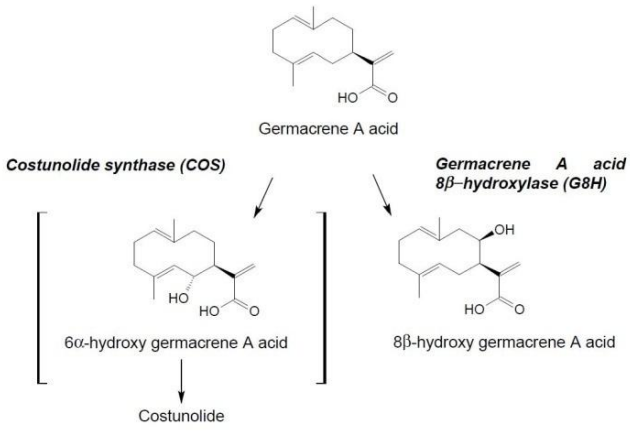
Sesquiterpene lactones (STL) are the plant metabolites of Asteraceae with the highest structural diversity [1]. Their bioactivity is of physiological, ecological as well as pharmaceutical interest and fuelled the research in biosynthesis of STL. The enzymes catalysing key steps of the STL synthesis were identified from different model plants and enabled the transformation of farnesyl diphosphate to costunolide [2, 3]. From sunflower glandular trichomes, the germacrane A synthase (HaGAS) [4], the geramcrane A oxidase (HaGAO) [5] and the germacrene A acid 8β-hydroxylase (HaG8H), a cytochrome P450 (CYP) enzyme catalysing the C-8 hydroxylation of germacrane A acid [2], were characterized recently. However, the enzyme hydroxylating C-6 as a prerequisite for the formation of the lactone ring in costunolide was not identified yet. Previously described costunolide synthases from chicory (CiCOS) [3] and lettuce (LsCOS) [2] were aligned with the two similar CYP enzymes from sunflower HaGAO [5] and HaG8H [2]. *Helianthus* EST´s similar to known costunolide synthases were also taken into account. Primers were designed on domaines conserved among costunolide synthases but significantly different in HaGAO and HaG8H. With these primers a 450 bp fragment highly similar to the previously described costunolide synthases was amplified from cDNA. Database search in the Compositae Genome Project (University of California Davis) revealed two contigs coding together for a complete ORF. After the identification of the start-and stop codon, primers were designed to amplify the full coding sequence and the complete ORF of *Helianthus annuus* cv. HA300 was identified to consist of 1497 bp. The genomic DNA sequence showed a single 561 bp intron at position 880 (+/-2 bp). This **p**utative ***H****elianthus* ***a****nnuus* **co**stunolide **s**ynthase pHaCOS has 65 %/ 64 % amino acid idententity to LsCOS/ CiCOS and 61 % identity to HaG8H. In pHaCOS the eight amino acids predicted to be in the catalytic center of LsCOS and HaG8H [2] show similarities to both enzymes. To characterise the catalytic activity of pHaCOS the coding sequence will be cloned into the pESC-Ura vector and co-expressed in the yeast strain EPY300 with a plasmid harbouring GAS, GAO and a CYP reductase [5]. Studies on the expression pattern of this enzyme and its involvement in a trichome-independent STL pathway of sunflower will be conducted.

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## P28. Large-scale synthesis of farnesyl diphosphate as substrate for recombinant sesquiterpene synthases

Thore Frister[1], Steffen Hartwig[1], Sascha Beutel[1], Thomas Scheper[1],

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Terpenes are a large and structurally diverse class of secondary metabolites, which are mainly produced by various plants as the major components in resins and essential oils. These days the majority of terpene production is still based on the isolation from plant derived raw materials using techniques such expression, solvent extraction and steam distillation. The increasing need of terpenes in various end-consumer products like costly perfumes and scented household goods especially in the emerging markets leads to higher prices and demands new, climaindependent, reliable processes for the production of terpenes. In a biotechnological approach large amounts of farnesyl diphosphate (FPP) are required. FPP is a high-value key intermediate of the terpenoid pathway and in various other biochemical processes like the prenylation of proteins and several natural products. Therefore FPP and its derivates attracted a considerable amount of attention in the pharmaceutical research [1]. After analyzing the two published major routes to FPP, the procedure according to Keller *et al*. was found to be the most effective method for a scale-up [2]. Using this synthetic approach, the renewable, plant derived starting material farnesol can be phosphorylated stepwise in a fast and cost effective reaction at low temperatures in gram scale. A first fast purification step delivers a FPP enriched solution, which contains small amounts of the mono and triphosphate esters as well. After a buffer adjustment and the exact determination of FPP concentration by reversed phase HPLC, the mixture is ready to use for the enzymatic conversion. A further purification of the ready-to-use mixture can be achieved by using fast liquid chromatography in combination with weak anion-exchange membrane adsorbers. The successful formation of FPP was confirmed by $^{1}$H-and $^{31}$P-NMR spectroscopy. For the biotransformation of FPP a recombinantly heterolog expressed patchoulol synthase from *Pogostemon cablin* was used [3]. The conversion was performed in a two-phase system consisting of an aqueous buffer and an optimized organic phase to minimize product losses. The successful formation of patchoulol and related sesquiterpenes were confirmed by GC-MS. We present a high effective large-scale synthesis of FPP to produce patchoulol in a biotechnological way, by combining straight forward organic synthesis and enzymatic transformation, using a recombinant patchoulol synthase.

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## P29. Combinatorial biosynthesis of legume natural and rare triterpenoids in engineered yeast

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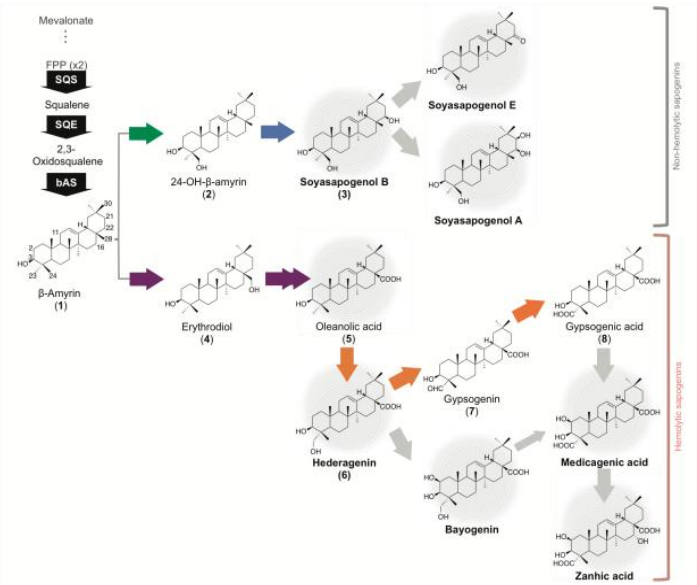
Triterpenoid saponins are a diverse group of specialized (secondary) metabolites with many biological properties. The model legume *Medicago truncatula* has an interesting profile of triterpenoid saponins from which sapogenins are differentiated into hemolytic and nonhemolytic types according to the position of their functional groups and hemolytic properties. Gene co-expression analysis confirmed the presence of candidate P450s whose gene expressions correlated highly with that of *β-amyrin synthase* (*bAS*). Among these, we identified CYP716A12 and CYP93E2 as key enzymes in hemolytic and nonhemolytic sapogenin biosynthetic pathways. The other candidate P450s showed no β-amyrin oxidation activity. However, among the remaining candidate P450s, CYP72A61v2 expression highly correlated with that of CYP93E2, and CYP72A68v2 expression highly correlated with that of CYP716A12. These correlation values were higher than occurred with bAS expression. We generated yeast strains expressing bAS, CPR, CYP93E2, and CYP72A61v2, and bAS, CPR, CYP716A12, and CYP72A68v2. These transgenic yeast strains produced soyasapogenol B and gypsogenic acid, respectively.We were therefore able to identify two CYP72A subfamily enzymes: CYP72A61v2, which modifies 24-OH-β-amyrin, and CYP72A68v2, which modifies oleanolic acid. Additionally, P450s that seemed not to work together in planta were combinatorially expressed in transgenic yeast. The yeast strains (expressing bAS, CPR, CYP72A63, and CYP93E2 or CYP716A12) produced rare triterpenoids that do not occur in M. truncatula. These results show the potential for combinatorial synthesis of diverse triterpenoid structures and enable identification of the enzymes involved in their biosynthesis



## P30. Biosynthesis of triterpenoids in the latex of *Euphorbia lathyris*: origin of isoprene units

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The latex of *Euphorbia lathyris* is known to contain important amounts of energy-rich triterpenoids. The EulaFuel project (Plant-KBBE 2009) proposes to convert this energy potential into a gasoline-type biofuel by increasing the triterpene content of the latex by genetic transformation methods.
The contribution to this project consists in investigating the biosynthesis of the triterpenes of *Euphorbia lathyris*. We focused this study on the five main triterpenoids found in the latex: lanosterol, butyrospermol, cycloartenol, 24-methylene cycloartanol and 3β-hydroxydiploptene. It is not known if isoprene units in laticifers are produced via the cytosolic mevalonate (MVA) pathway and/or the methylerythritol phosphate (MEP) pathway. This aspect was investigated using two different procedures.

On the one hand, 13C- or 2H-labeled precursors such as D-glucose, mevalonic acid or deoxyxylulose, have been used to perform labeling experiments on axenic and outdoor-grown plants. The analysis of triterpenoids by 13C NMR (or GC-MS for deuterium incorporation experiments) allows us to determine the isotopic enrichment of isoprene units. Phytol and sitosterol, respectively produced in plants via the MEP and MVA pathways, were also analyzed as references.

On the other hand, we investigated 13C/12C and 2H/1H isotope signature of triterpenoids from *Euphorbia lathyris* by GC-IRMS, by comparing the values with those of isoprenoids extracted from other plants. This second method allows to differentiate MEP and MVA pathways and to work only with natural isotopic ratios. Another advantage of this technique is the very small amount of isoprenoids required.

Through both approaches, we concluded that isoprenoid units of the five main triterpenoids in the laticifers are mainly produced via the mevalonate pathway. However, a minor contribution of MEP pathway cannot be presently excluded.

## P31. Metabolic flux analysis through the plastidic methylerythritol 4-phosphate pathway in poplar: effects of isoprene biosynthesis.

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Plants possess the plastidic methylerythritol 4-phosphate (MEP-) pathway for the synthesis and maintenance of the photosynthetic apparatus, the biosynthesis of physiologically and ecologically important volatiles, the production of some phytohormones, and for protein isoprenylation. Poplar plants, however, direct most of the C-flux through the MEP-pathway for the production of only one single volatile compound, isoprene.

Here we combined $^{13}$C-labelling technique and trasngenic poplars, modified in isoprene biosynthesis to investigate the C-fluxes through the MEP-pathway under different temperatures, light intensities and $CO_2$ concentrations. We measured the incorporation rate of $^{13}$C into the isoprene precursors dimethylallyl diphosphate (DMAPP), methylerythritol 2,4-cyclodiphosphate (MEcPP), as well as isoprene and the downstream products carotenoids, xanthophylls and chlorophylls, after feeding leaves with $^{13}CO_2$. The isotopologues different molecules were measured using proton transfer reaction (PTR-) mass spectrometry (MS), isotope ratio (IR-) MS and gas chromatography (GC-) MS.

In transgenic plants, the suppression of isoprene biosynthesis lead to a reduction of the C-fluxes through the MEP-pathway by circa 98% (compared to wild-type plants), as consequence of a much lower demand of C. Also, carotenoid and chlorophyll contents were lowered in non-isoprene emitting leaves compared to wild-type but the turnover of these compounds were higher, possibly due to the absence of protective isoprene. Using $^{13}$C, we were able to split the non-plastidic from the plastidic DMAPP pool, where the latter correlated well with the MEcPP content of leaves. In non-isoprene emitting leaves, the large amount of unlabelled DMAPP let us to suggest that either its accumulation is not plastidic, or its biosynthesis proceed at a very low rate. We further show the effects of different environmental conditions on the flux through the MEP pathway with respect to isoprene biosynthesis.

Overall the data clearly demonstrated that isoprene biosynthesis is the dominating C-sink in isoprene emitting chloroplasts, making this system highly versatile for flux control analysis of the MEP-pathway.

## P32. Autoproteolytic processing of 1-deoxy-D-xylulose 5-phosphate synthase

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The reaction catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) has been described as one of the rate-limiting steps of the methylerythrititol 4 phosphate pathway in microorganisms and plants. Although little is known about the mechanisms involved in the control of DXS, it has been proposed that protein degradation mediated by ClpP could represent a major process to adjust DXS protein levels in plastids (Flores-Perez et al. 2008, Plant Cell 20: 1303-15). Here we report a novel DXS control mechanism involving the autoproteolytic cleavage of the enzyme.

Purified recombinant DXS from different origins expressed in *E. coli* always show additional bands of lower molecular weight when analyzed by SDS-PAGE. The presence of these bands cannot be prevented even when the enzyme is extracted in the presence of different combinations of protease inhibitors. We have shown that in the case of *E. coli* DXS these additional bands correspond to degradation products of the recombinant protein. These degradation products were not detected in purified DXS electroeluted from SDS-polyacrylamide gels under denaturing conditions, indicating that the proteolytic cleavage requires the proper folding of the enzyme. To identify the proteolytic cleavage sites the N-terminal sequence of the degradation products was analyzed by Edman sequencing. Cleavage sites involved Asp, Pro and Ser residues. One of the cleavage sites was identified between residues Ser206 and Ser207 located within a non-conserved region in bacterial and plant DXS which corresponds to an external and disordered region of the enzyme extending from residues 183 to 238. Since this region could not be resolved in the crystallographic studies of bacterial DXS (Xiang et al. 2007, J Biol Chem 282(4):2676-82) its structural and/or functional role could not be established. To better characterize this cleavage site an *E. coli* DXS derivative lacking the region extending from residues 191 to 239 (EcDXS-Δ191-239) was prepared. This construct was able to complement the *E. coli* strain EcAB4-2 defective in DXS activity, thus showing that the deleted region is not essential for enzyme activity. Interestingly, immunoblot analysis of purified recombinant EcDXS-Δ191-239 showed that the presence of degradation products was strongly reduced. To check if cleavage at Ser207 was specifically dependent on the DXS protein folding, the region of EcDXS extending from residues 158 to 240 was cloned in the middle of a chimeric protein consisting of glutathione S-transferase (GST) fused to the green fluorescent protein (GFP). The chimeric constructs were expressed in *E. coli* and the recombinant proteins purified. Immunoblot analysis of the purified proteins revealed the presence of degradation fragments only in the case of the chimeric construct containing the EcDXS sequence. A second cleavage site was identified between the highly conserved residues Asp294 and Pro295. In that case the DXS processing mechanism would be similar to that described for other protein models and likely involves the posttranstlational modification of the Asp residue. The autoproteolytic processing initially described in the *E. coli* DXS has also been observed in the bacterium *Thermotoga maritima* and in the plant *Arabidopsis thaliana* enzymes, thus suggesting that autoprocessing of DXS represents a novel and general feature of this enzyme.

## P33. Production of various C50 carotenoids by metabolically engineered *C. glutamicum*

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The yellow pigmented soil bacterium *C. glutamicum* ATCC13032 is accumulating the cyclic C50 carotenoid decaprenoxanthin and its glucosides. Decaprenoxanthin is synthesized via the intermediates farnesyl pyrophosphate, geranylgeranyl pyrophosphate, lycopene and flavuxanthin1. The genome of *C. glutamicum* comprises two clusters of carotenogenic genes that are co-transcribed2. Terminal pathway engineering in a recombinant *C. glutamicum* strain led to comparable lycopene concentrations2 as in a recombinant lycopene producing *E. coli* strain3.
Here we show that *C. glutamicum* is a potential platform organism to produce alternative C50 carotenoids that are derived from lycopene. The platform strain was constructed rationally by overexpression of genes for the conversion of IPP to lycopene, by deletion of the lycopene elongase gene *crtEb* and by plasmid-borne overexpression of genes for synthesizing various C50 carotenoids from lycopene. The recombinant *C. glutamicum* strain was used for overproduction of the C50 carotenoids decaprenoxanthin, sarcinaxanthin and C.p. 450. For production of the native decaprenoxanthin the endogenous genes *crtYe/f* and *crtEb* were overexpressed, for production of sarcinaxanthin genes *crtE2 and crtYg/h* from *Micrococcus luteus* and for production of C.p. 450 the *lbtA and lbtBC* genes from *Dietzia* sp., respectively, were overexpressed. All three C50 caroenoids could also be produced in their deglucosylated forms when the respective glycosyltransferase gene was deleted.

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2Heider SA, Peters-Wendisch P, Wendisch VF: Carotenoid biosynthesis and overproduction in *Corynebacterium glutamicum***.** *BMC Microbiol* 2012, **12:**198.
3Netzer R, Stafsnes MH, Andreassen T, Goksoyr A, Bruheim P, Brautaset T: Biosynthetic pathway for gamma-cyclic sarcinaxanthin in *Micrococcus luteus*: heterologous expression and evidence for diverse and multiple catalytic functions of C(50) carotenoid cyclases. *J Bacteriol* 2010, **192:**5688-5699.

## P34. The influence of phytyl epoxide on phytosterols and polyisoprenoid alcohols accumulation in hairy roots of *Arabidopsis thaliana*

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Dolichols and phytosterols are common constituents of plant roots. In order to study the influence of phytyl epoxide on sterols and dolichol accumulation *in vitro* hairy roots of *Arabidopsis thaliana* were cultivated in the regular ½ Murashige and Skoog (MS) liquid medium supplemented with different concentration of chemically synthesized phytyl epoxide (20μM, 200μM, 1mM and 2mM) for the subsequent 14 days. Control roots were transferred to the fresh standard medium without phytyl epoxide.

Dry samples of roots were extracted with organic solvents and lipid content was analyzed by TLC, HPLC and GC. The GC-FID analysis have shown that hairy roots of *Arabidopsis thaliana* accumulated sterols i.e. campasterol, stigmasterol, β-sitosterol and traces of brasicasterol. The highest sterol accumulation (318% of the control) were obtain for 20 μM phytyl epoxide treatment whereas 1mM and 2mM treatments resulted in the decrease of the sterol content (25% and 4% of the control, respectively).

The HPLC/UV studies have shown that in control condition and in the presence of higher concentration of phytyl epoxide (1mM and 2mM) roots of Arabidopsis accumulated one-family mixture of dolichols with Dol-15 dominating. In the presence of 20 μM and 200 μM phytyl epoxide the additional family of longer dolichols was observed (Dol-19 to Dol-23 with Dol-20 dominating). Moreover for 200 μM phytyl epoxide the dominant dolichol in the second family was shifted towards longer prenolog – Dol-21. The dolichol families were accompanied by traces of polyprenols with identical chain length.

Dolichol accumulation was induced by the presence of phytyl epoxide and reached 790% of the control for 2mM phytyl epoxide.

These results indicate that phytyl epoxide might influence the metabolic pathways leading to the formation of phytosterols and dolichols in plants.

## P35. Terpenoid lipid profiles of Arabidopsis thaliana ecotypes

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Terpenoids (isoprenoids) are a very large group of natural compounds, which can be find in all living organisms. In plants and some bacteria two biosynthesis pathways responsible for formation of terpenoids can be find: the mevalonate (MVA) in cytoplasm and the methylerythritol phosphate (MEP) in plastids, although exchange of intermediates between both pathways has been reported. In contrast in animals and fungi only MVA is operating.

The aim of this study was to examine the content of selected terpenoids in 3-week-old *Arabidopsis thaliana* seedlings of a set of 123 different ecotypes collected from a wide range of geographic locations). The content of plant photosynthetic pigments (chlorophylls and carotenoids), tocopherols (vitamin E), plastoquinone, polyisoprenoids (polyprenols and dolichols) and sterols were estimated. Plastoquinone, tocopherols and pigments are representatives of the MEP pathway products sterols are derived from the MVA pathway, while polyisoprenoids are ‚mosaic' compounds of mixed MVA / MEP origin.

Plants have developed many systems to adapt to the changing environmental conditions. We plan to investigate whether variation in the biosynthesis of terpenoid lipids reflects plant adaptation to particular environmental factors. A collection of *Arabidopsis thaliana* ecotypes (NASC) was used as a model for this study. Col-0 ecotype was used to establish the analytical methods for estimation of the selected terpenoids. All plants were cultured in long day conditions on 1/2 Murashige-Skoog culture medium.

To determine the content of each of the compounds GC-FID (tocopherols, sterols), HPLC (plastoquinone, dolichols, polyprenols, chlorophylls, carotenoids) and spectrophotometric (photosynthetic pigments) methods have been used. Results of the analysis indicated considerable variation in the terpenoids accumulation among particular ecotypes).

Based on these results a mapping population has been selected for further QTL studies.

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## P36. Diterpene cyclases in the momilactone-producing moss *Hypnum plumaeforme*

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Diterpenes have various chemical structures and play important biological roles with hormonal, pharmaceutical and allelochemical activities. Momilactones (A and B) are one of the diterpenoid phytoalexins produced in rice. The biosynthesis of momilactone in rice has been well studied by research groups in Japan and USA. Two independent monofuctional diterpene cyclases (DTCs) are involved in the biosynthesis of a hydrocarbon intermediate of momilactones. OsCPS4 catalyzes the cyclization reaction of geranylgeranyl diphosphate (GGDP) to *syn*-copalyl diphosphate (*syn*-CDP). Subsequently, OsKSL4 recognizes *syn*-CDP to afford 9β-pimara-7,15-diene (Otomo et al, 2004; Peters, 2006.).

Recently, we found that a moss *Hypnum plumaeforme* produces and secretes momilactones A and B (Nozaki *et al*., 2007). In bryophytes, the biosynthesis of *ent*-kaurene are mediated by bifunctional DTCs whereas the biosynthesis in flowering plants are by monofunctional cyclases. Since rice and mosses are evolutionally distinct species, we are interesting in the biosynthetic enzyme system for momilactones between these two land plants. Here, we cloned possible two cDNAs responsible for bifunctional diterpene cyclases (HpDTC1 and HpDTC2) from *H. plumaeforme*. Both open reading frames were respectively constructed into bacterial expression vectors, and the recombinant enzymes were produced in *E. coli*. HpDTC1 enzyme catalyzed GGDP cyclization to produce a diterpene hydrocarbon, but not 9β-pimara-7,15-diene. Techniques using enzymatic total synthesis from [U-$^{13}C_6$]mevalonate, $^{13}$C-labeling and multidimensional NMR measurements (Sugai *et al*., 2011) identified the product as *ent*-pimara-9(11),15-diene. This compound was accumulated in the gametophore of *H. plumaeforme*. In contrast, HpDTC2 catalyzed the cyclization reaction of GGDP and produced 9β-pimara-7,15-diene *via syn*-CDP.

References: Otomo *et al*.: Plant J. vol.39, 886 (2004); Biosci. Biotechnol. Biochem. vol.68, 2001 (2004). Peters: Phytochem. vol.67, 2307 (2006). Nozaki et al.: Biosci. Biotechnol. Biochem. vol.71, 3127 (2007). Sugai et al.: J. Biol. Chem. vol.286, 42840 (2011).

## P37. Characterization of two isozymes of 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (Hdr) from *Burkholderia glumae* BGR1

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4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (Hdr) is the ultimate enzyme in 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway converting (*E*)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP) to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). *Burkholderia glumae*, a Gram negative rice-pathogenic bacterium, harbors 2 *hdr* genes and lacks isopentenyl diphosphate isomerase (*idi*). Molecular and genetics analysis of *hdr* gene region suggest that each *hdr* isogene was found in respective putative operon. The *Bghdr1* gene and FKBP-type peptidyl-prolyl cis-trans isomerase (*slp*) were polycistronic, as were the *Bghdr2* gene and hopanoids associated radical *S*-adenosyl methionine (SAM) domain-containing protein (*hpnH*). Hdr2 isozyme was placed in a clade different from Hdr1, and only 56.1 % identity was found between them. Nevertheless, both Hdr enzymes could complement *E. coli hdr* deletion mutant (DYTL1). Both of the recombinant Hdr proteins, BgHdr1 and BgHdr2, catalyzed reduction of HMBPP into IPP and DMAPP at a ratio of 2:1, in contrast to 5:1 ratio of other bacterial Hdrs so far characterized. The $k_{cat}$ and $K_m$ values of BgHdr1 and BgHdr2 were 187.0 $min^{-1}$ and 6.0 μM and 66.6 $min^{-1}$ and 21.2 μM respectively. Neighboring genes in the respective operon and kinetic properties of each Hdr suggests that each isozyme has separate function. (Supported by Systems and Synthetic Agrobiotech Center affiliated to Next Generation Biogreen 21 Program, Korea)

| | $k_{cat}$ ($min^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($\mu M^{-1}$ $min^{-1}$) | Specific activity (μmol $min^{-1}$ $mg^{-1}$) | Reference |
|---|---|---|---|---|---|
| **BgHdr1** | 187 ± 6.0 | 6.0 ± 1.1 | 31.1 ± 6.2 | 3.66 ± 0.11 | this study |
| **BgHdr2** | 66.6 ± 5.2 | 21.2 ± 5.5 | 3.1 ± 0.8 | 1.39 ± 0.11 | this study |
| Aa**Hdr** | 222 ± 12 | 590 ± 60 | 2.66 | 6.6 ± 0.3 | Altincicek et al., 2002 |
| **EcHdr** | 30 | - | - | 0.7~3.4 | Gräwert et al., 2004 |

## P38. WRKY transcription factors interacting with promoters of diterpenoid synthase genes in *Oryza sativa* L

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Rice produces phytoalexins in response to biotic and abiotic stresses. The phytoalexins comprise momilactones A-B, oryzalexins A-F, oryzalexin S and phytocassanes A-E, and sakuranetin. Rice phytoalexins are ditertenoids except sakuranetin, a flavonoid. In addition, momilactones A and B have allelochemical activities. To understand correlation of OsWRKY transcription factors (TFs) with diterpenoid synthase (*DTS*) gene promoters, 4 *OsWRKY* genes that were induced by UV-irradiation were selected. Interaction between the putative OsWRKY TFs and the *DTS* promoters was confirmed by using an *in planta* assay newly developed as follows. OsWRKY coding sequence was fused to the CaMV35S promoter and the fusion construct was introduced into tobacco by an infiltration method. Separately, the *DTS* promoter linked with GFP was co-infiltrated into tobacco. Interactions of OsWRKY with *DTS* promoters (*eCPS1*, *eCPS2*, *EKS*, *CSS*, *SNS*, *PMS*, and *STS*) were confirmed by expression of GFP. By using this method, interactions of OsWRKY1 and OsWRKY24 with all the aforementioned *DTS* promoters were demonstrated, whereas OsWRKY7 and OsWRKY70 did not interact with any of the *DTS* promoters. Although transcript levels of eCPS1, EKS, and SNS did not increase by UV-irradiation, their promoters could interact with OsWRKY1 and OsWRKY24 TFs. These facts suggest eCPS1, EKS, and SNS are under posttranscriptional regulation. (Supported by Systems and Synthetic Agrobiotech Center affiliated to Next Generation Biogreen 21 Program, Korea.

## P39. Molecular control of the coordinated up-regulation of carotenogenic gene expression during flower development in *Gentiana lutea*

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Over the last two decades, many carotenogenic genes have been cloned and used to generate metabolically-engineered plants producing higher levels of carotenoids. However, comparatively little is known about the regulation of endogenous carotenogenic genes in higher plants, and this restricts our ability to predict how engineered plants will perform in terms of carotenoid content and composition. During petal development in the Great Yellow Gentian (*Gentiana lutea*), carotenoid accumulation, the formation of chromoplasts and the upregulation of several carotenogenic genes are temporally coordinated (Fig. 1). We investigated the regulatory mechanisms responsible for this coordinated activity by isolating six *G. lutea* carotenogenic gene (*GlPDS*, *GlZDS*, *GlLYCB*, *GlBCH*, *GlZEP* and *GlLYCE*) promoters by inverse PCR. Each promoter was sufficient for developmentally-regulated expression of the *gusA* reporter gene following expression in tomato (*Solanum lycopersicum* cv. Micro-Tom). Interestingly, the *GlLYCB*, *GlBCH* and *GlZEP* promoters drove high levels of *gusA* expression in chromoplast-containing mature green and ripening fruits (Fig. 1), but only residual levels in chloroplast-containing immature green fruits and leaves, indicating a strict correlation between promoter activity, tomato fruit development and chromoplast differentiation. As well as core promoter elements such as TATA and CAAT boxes, all six promoters contained three common *cis*-regulatory motifs involved in the response to methyl jasmonate (CGTCA) and ethylene (ATCTA), and required for endosperm expression (Skn-1_motif, GTCAT). Our data provide insight into the regulatory basis of the coordinated upregulation of carotenogenic gene expression during flower development in *G. lutea*. Funding: ERC Advanced grant BIOFORCE to PC, MINECO, Spain (BIO2011-22525) and NSFC (31270344) to CZ.

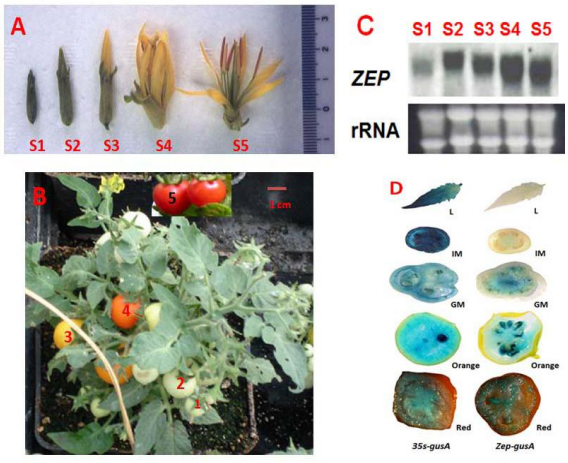


**Fig. 1**. **A**: Stages of flower development in *G. lutea*. **B**: Stages of fruit development in Micro-Tom. **C**: Expression of *ZEP* transcripts in the petals of *G. lutea* during five different stages of flower development (20µg total RNA in each lane). **D**: Histochemical GUS staining of transgenic tomato leaves and fruits carrying *Zep-gusA* and *35S-gusA* constructs, respectively. L, leaf; IM, immature green fruit; GM, mature green fruit; Orange, orange fruit; Red, red ripened fruit; *Zep-gusA*, pBI-GlZEPPro-GUS; *35sgusA*, pBI121.

## P40. In vitro culture of *Santalum Album* L. used for the elucidation of the sesquiterpene biosynthetic pathway in Sandalwood

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The world market for sandalwood oil obtained from Santalum album trees, one of the most precious plant-derived fragrance materials, is facing dramatic shortage and price increase, due to the high demand and the overexploitation of the natural resources. For these reasons, Santalum album is now registered on the IUCN Red List as an endangered species. In addition, because of the structural complexity of the sesquiterpene constituents, a cost effective chemical synthesis approach is so far not available. Therefore, a synthetic biological approach would certainly be the most appropriate solution for developing an alternative and sustainable production of sandalwood oil substitutes. Using in-vitro culture, one promising green callus type, induced from young hypocotyl segments of aseptically germinated seeds of Santalum album L., was selected for mass production. This interesting callus line was subjected to different inducing treatments (azacytidine, chitosan ...) in order to elicitate and identify the genes involved in the biosynthetic pathway leading to the sesquiterpenes molecules. The sesquiterpene profiles of the calli were analyzed by GCMS following the different treatment and interesting calli were selected and subjected to high-throughput transcriptome sequencing. This approached allowed the identification of genes encoding several sesquiterpene synthases in S. album L, and especially the gene of an alpha-santalene/beta-santalene synthase catalyzing the first committed step in the biosynthesis of the key sesquiterpene odorants of sandalwood oil.

## P41. Characterization of α-amyrin synthase from *Euphorbia tirucalli* L

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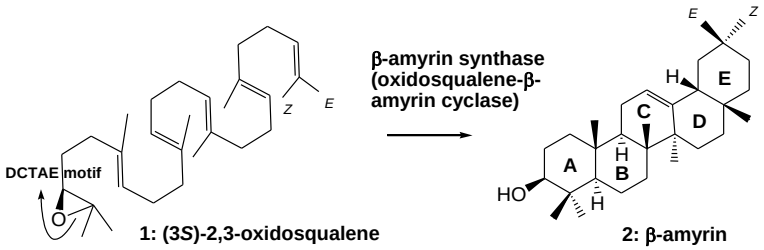
α-amyrin, a natural triterpene, is widely distributed in plant kingdom and its pentacyclic skeleton is produced by oxidosqualene cyclase (OSC). OSC enzymes are classified as membrane proteins and they catalyze the polycyclization reaction of (3*S*)-2,3-oxidosqualene to yield nearly 150 different cyclic triterpene skeletons. To date, no report has described for the successful purification and characterization of plant α-amyrin synthase. The α-amyrin synthase from *Euphorbia tirucalli* (EtAS) was expressed as a polyhistidine-tagged protein in *Saccharomyces cerevisiae* GIL77, which lacks the lanosterol synthase gene. The expression yield, determined by western blotting analysis, was 5~7 mg. By Ni-NTA affinity column chromatography and careful selection of the proper imidazole concentration during the purification processes of washing and elution, a single band was successfully obtained on an SDS-PAGE. We then tested the effects of 4 detergents on the enzyme activity. Supplementation of Triton X-100 at a concentration of 0.05 % yielded the highest activity. The optimal pH and temperature were 7.0 and 30°C, respectively. The kinetic parameters, $K_m$ and $k_{cat}$, were determined to be 27.6 □M and 42.0 $min^{-1}$, respectively. To the best of our knowledge, there is no report describing both $K_m$ and $k_{cat}$ for OSCs except for only 2 examples of rat and bovine lanosterol synthases. The α-amyrin synthase purified in this study exhibited a significantly higher catalytic efficiency ($k_{cat}/K_m$): ca $10^3$-fold than those of the 2 reported lanosterol synthases. Gel-filtration HPLC indicated that the OSC exists as a monomer, and the eluted OSC retained its activity. We also report here the circular dichroism spectrum of the α-amyrin synthase of many known OSCs for the first time. Furthermore, inhibition constants $K_i$, $IC_{50}$ and inhibitory types by iminosqualene, Ro48-8071 and U18666A were determined, indicating that iminosqualene and Ro48-8071 are potent inhibitors. Additionally, this is the first report of the kinetic data of the mutated enzymes targeted for the $D^{485}C^{486}$TAE motif, a putative initiation site for the polycyclization reaction. No activity of the D485N and the significantly decreased activity of the C564A were found, definitively demonstrating that the acidic carboxylic residue of D485 serves as a proton donor to initiate the polycyclization reaction and that the C564 is involved in hydrogen-bonding formation with the carboxyl residue of D458 to enhance the acidity.

Ref. R. Ito, Y. Masukawa and T. Hoshino, FEBS J. 2013 Mar;280(5):1267-80.




1: (3*S*)-2,3-oxidosqualene 2: β-amyrin

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## P42. The spatial distribution of terpene emission after gypsy moth feeding in poplar

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After herbivore damage many plants emit a complex blend of volatiles often dominated by terpenes. Such volatiles have been reported to function in indirect defence as attractants for natural enemies of the herbivores. Induced volatile emission can occur systemically throughout the whole plant or be restricted to the site of damage. Volatiles specifically emitted from the site of damage would seem to be better candidates for mediating indirect defense than volatiles that are also emitted from undamaged systemic parts. However, the spatial regulation of herbivore-induced volatile formation is mostly unknown.

We have started to investigate the biochemical basis and regulation of herbivore-induced volatile terpene formation in *Populus trichocarpa* at the level of a single leaf. In general, terpenes are produced from their corresponding prenyl diphosphate precursors through the action of terpene synthases. The initial products generated can be directly emitted from the plant or can be further modified to other terpenoids. A typical modification reaction is an oxidation catalyzed by P450 enzymes of the CYP71 clan.

Comparison of the terpene emission from single herbivore-damaged *P. trichocarpa* leaves with the terpene emission of undamaged adjacent leaves or undamaged leaves with direct vascular connections revealed that several volatile terpenes were exclusively released from the damaged leaves. This spatial restriction was reflected by the pattern of jasmonates found in the respective treatments- high in damaged leaves, low in undamaged ones. Two terpene synthases, TPS6 and TPS9, responsible for (*E*)-β-ocimene and (*E*)-β-caryophyllene formation, respectively, were identified and characterized. Measurement of the transcript accumulation of these *tps* genes demonstrated that induction of terpenes was controlled at the transcriptional level.

Beside terpene hydrocarbons, epoxy-ocimene, an oxidized monoterpene, was found to be exclusively emitted at the site of herbivore damage. P450 enzymes putatively catalyzing the oxidation of ocimene to epoxy-ocimene are under investigation.

## P43. Identification and quantification of steroidal saponins in *Dioscorea esculenta*

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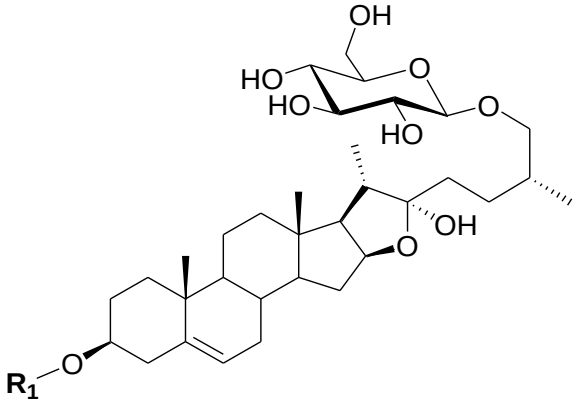
Steroidal saponins are natural surfactants with various biological activities, and in particular, the tubers of *Dioscorea* spp., known as yam, contain furostane and spirostane glycosides such as protodioscin and dioscin, respectively, which are valuable saponins required for semi-synthetic production of pharmaceutical steroidal drugs. *D. esculenta* (Togedokoro) is known as a lesser yam in Okinawa island, and contains high amounts of steroidal saponins in the tubers as the functional compounds. However, the structure and content of steroidal saponins in *D. esculenta* have not yet been fully elucidated. In this study, the structure of steroidal saponins in *D. esculenta* was identified in molecular level and the content of the identified saponins was quantified in its various organs. Firstly, for isolation and purification of steroidal saponins from *D. esculenta*, 70% (v/v) EtOH extract from the freeze-dried powder was subjected to solvent fractionation. The obtained BuOH layer was subjected to C18 solid phase extraction, and then the saponins were isolated by ODS-HPLC. The structures of three steroidal saponins obtained were determined using NMR and LC-MS, and asperoside (1), protodioscin (2), and gracilllin (3) were identified as furostanol glycosides, which were isolated from *D. esculenta* for the first time. Purification of spirostane-type steroidal saponins is in progress. Additionally, we will present the data of the quantification of the identified saponins by LC-MS analysis in different organs (tuber, tuber cortex, leaf, leaf stalk, root and stem) of *D. esculenta* as well as other *Dioscorea* spp. These data will be very useful to select the candidate genes involved in steroidal saponin biosynthesis in the tubers of *D. esculenta*.




**1** $R_1$ = Rha (4→) Rha (4→) Glu, Glu (2→) Rha

**2** $R_1$ = Rha (4→) Glu, Glu (2→) Rha

**3** $R_1$ = Glu (3→) Glu, Glu (2→) Rha

## P44. Effect of diverse light sources on phytochemical synthesis in rice seedling

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Light, one of the most important environmental factors, plays the pivotal roles not only in plant growth and development but also in phytochemical accumulation. In term of plant, phytochemicals are involved in various biological processes, including pigmentation of flowers, fruits and vegetables, plant-pathogen interactions, fertility and protection against UV light. Numerous studies have reported that phytochemical with antioxidant properties may reduce the incidence of disease and also contribute to promote human health. Understanding the effects of light quality on phytochemical production may provide useful information to adjust or enhance phytochemical synthesis in plant.

In order to investigate how light quality controls the phytochemical synthesis, rice seedling were grown under the different light sources; blue (B), red (R), white (W) and dark (D). Rice seedling under B light displayed elevated level of phytochemicals (carotenoids and phenolic compounds). Through mRNA profiling using microarray analysis, the expression of genes being related in phytochemical synthesis was indicated of being changed under B light. We are exploring novel genes in association with a two-for-one in the regulation of carotenoid and flavonoid biosynthesis and analyzing their roles in phytochemical synthesis.

## P45. Study on the cross-talk between the MVA and MEP pathways in polyisoprenoid alcohols biosynthesis

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Isoprenoids are produced by all living organism but they are exceptionally abundant and diverse in plants. Polyisoprenoid alcohols , linear polymers built of five-carbon units are divided into two groups depending on their structure : α-unsaturated polyprenols and α-saturated dolichols. Plant polyprenols are found in photosynthetic tissues, wood, seed and flowers while dolichols are present mainly in roots. Both groups are identified in cells as mixtures of prenologues. Despite extensive studies, biological role of polyisoprenoid alcohols has not yet been fully elucidated. Studies on biosynthetic origin of dolichols in plant hairy root culture have suggested the involvement of both, the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways in their synthesis.

To further elucidate the biosynthetic origin of plant dolichols *in planta* experiments have been designed. In this study hydroponically grown *Arabidopsis thaliana* plants were treated with specific inhibitors of the key enzymes in both the MVA and MEP pathways (various statins for HMG-CoA reductase and fosmidomycin for DXP reductoisomerase) to establish the involvement of those pathways in biosynthetic process leading to polyisoprenoids. Main end-products of the MVA pathway (phytosterols) and MEP pathway (chlorophylls and carotenoids) as well as dolichols and polyprenols were isolated from leaves and roots of treated plants and quantified (HPLC and GC).

Studies performed on hydroponically grown Arabidopsis plants will help to understand the cellular mechanisms and signaling processes responsible for regulation of the cross-talk of the MEP and MVA pathways leading to the formation of polyisoprenoids in particular and other isoprenoids in general.

The “Studies of nucleic acids and proteins- from basics to applied research” project is realized within International PhD Projects Programme of Foundation for Polish Science. The project is cofinanced from European Union- Regional Development Fund.

## P46. Genetic and environmental polymorphism of content and composition of DDMP-saponins in the seed of wild soybeans (*glycine soja* sieb. & zucc.)

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**[Objective]** Soybean is a traditional food material in Eastern Asia and includes various health-beneficial components. Soybean saponins are one of them and their activities depend on their chemical structures. DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one)- conjugated saponins and their degradates, group B saponins, are expected to reduce the risk of some chronic diseases. Soybean saponins in seed cotyledons are hardly studied as they are thought to have less concentration and uniform composition than those of seed hypocotyls. The purpose of this study is to estimate the factors affecting the contents and composition of DDMP-saponin components in soybean seed cotyledon.

**[Materials and Methods]** *G. soja* accessions and recombinant inbred line (RIL) between *G. soja* (high in saponin content) and *G. max* (low) were used. Saponin components were extracted with a 10-fold volume of 70% ethanol containing 0.01% EDTA-2Na at room temperature for 1 hr from cotyledon powder, and 40-fold and 12 hrs, respectively, from intact hypocotyl. The extract was analyzed by common HPLC system with an ODS C18 column.

**[Results and Discussion]** In the cotyledons of *G. soja* lines, distribution of the range of DDMP-saponin content varied 13 times difference (minimum 0.05g and maximum 0.6g per 100g dry matter). However, in the hypocotyls, the difference was about 6 times (1.6 to 10g/100g DM). As the seed cotyledon and hypocotyl occupy about 90% and 2%, respectively, saponin components in the seed cotyledon greatly influence to total saponin content and composition of a whole seed. The ability to insert an arabinose and a rhamnose at the C-3 position sugar chain were widely varied in the cotyledons. However, no relationship was observed between those abilities and total DDMP-saponin contents. Not only in the parent lines but also in some RIL lines (different generations), the distribution of saponin contents both in the seed cotyledon and hypocotyl were widely varied, and there was no relationship among individual lines. On the other hand, saponin composition, thus the ability to insert an arabinose and a rhamnose at the C-3 position sugar chain was almost constant in the parents and RILs. These results suggest that the factors affecting the contents and composition of DDMP-saponins in the seed cotyledon greatly depend on the environmental and hereditary factors, respectively.

## P47. Evolution of a complex locus for terpene biosynthesis in *Solanum*

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The existence of functional gene clusters, containing two or more genes encoding different enzymes for the same pathway in plants has recently received renewed attention. In this study, we show that a cluster of genes in*Solanum lycopersicum* (Solanaceae) contains terpene synthases (TPSs) that specify the synthesis of defensive monoterpenes and diterpenes from *cis*-prenyl diphosphates, substrates that are synthesized by enzymes encoded by *cis*-prenyl transferase (CPT) genes also locatedwithin the same cluster. The monoterpene synthases in the cluster likely evolved from a diterpene synthase in the cluster by gene duplication and divergence. In the orthologous cluster in*S. habrochaites*, a new sesquiterpene synthase gene was created by a duplication event of a monoterpene synthase followed by a localized gene conversion event directed by a diterpene synthase gene. The TPS genes in the*Solanum*cluster encoding *cis*-prenyl diphosphate-utilizing enzymes are closely related to a*Nicotiana tabacum*(Solanaceae) diterpene synthaseencoding abienol synthase (NtABS). NtABSuses the substrate copal-8-ol diphosphate, which is made from the all-*trans*geranylgeranyl diphosphate by NtCPS2. The *Solanum* gene cluster also containsan ortholog of *NtCPS2* but it appears to encode a non-functional protein. Thus, the *Solanum* functional gene cluster evolved by duplication and divergence of TPS genes, together with alterations in substrate specificity to utilize*cis*-prenyl diphosphates, and through the acquisition of CPT genes.

## P48. Sesquiterpene biosynthesis in khat (*Catha edulis*).

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Khat (*Catha edulis* Forsk., Celastraceae) is a perennial shrub common in the Middle East and Eastern Africa that was introduced to Israel by Yemenite immigrants. Its young leaves are chewed for their psycho-stimulating properties. Among the active compounds are phenylpropylamino alkaloids such as cathinone that accumulate in young leaves. In addition to the alkaloids produced, the leaves also accumulate various terpenoids including volatile monoterpenes, sesquiterpenes and non volatile terpenoids such as polyhydroxydihydrofuran sesquiterpenoids, that have been shown to have various biological activities such as anti-feedant and therapeutic anti cancer activities. In this work we aimed to characterize the process of volatile terpenoid biosynthesis in khat leaves. Leaves volatiles were sampled using solid-phase microextraction (SPME) coupled to GC-MS. The monoterpenes found were β-ocimene, α-terpinolene, limonene and traces of α-thujone. Higher amounts of sesquiterpenes were found including caryophyllene (E), α-copaene, α-cubebene, α-humulene, α-muurolene and δ- cadinene. To test the potential biosynthetic activity for sesquiterpene biosynthesis, crude protein extracts were incubated with farnesyldiphosphate as a substrate and the sesquiterpenes produced were identified using GC-MS-HS. These in vitro assays produced caryophyllene (E), elemol, α-eudesmol as well as, β-bisabolene and α-bergamotene (Z). The sesquiterpene synthase activity was higher in young leaves as compared to older leaves.

## P49. Oxidation of monoterpenes in grapes

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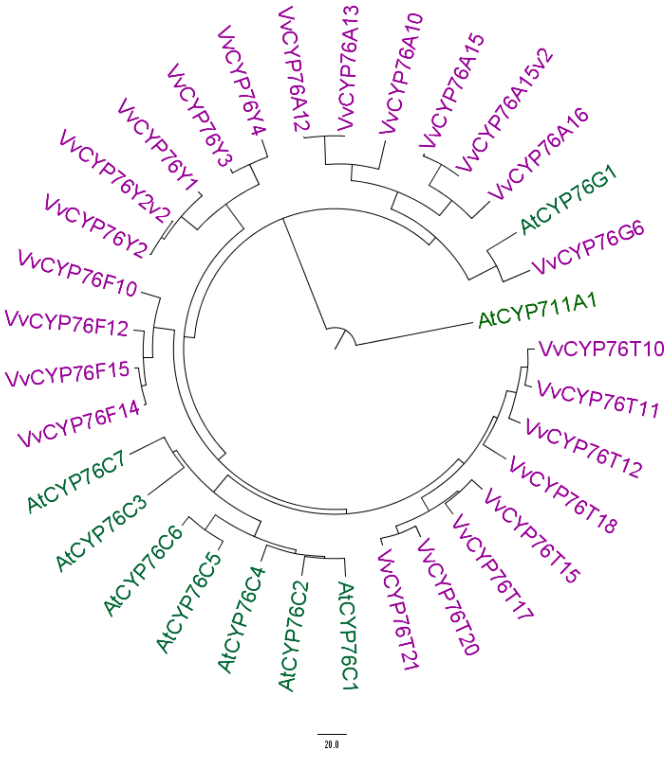
The floral aroma of wines, such as Muscat or Gewurztraminer, is a blend of monoterpenoids with different sensory properties. Many of them, for example linalool oxides, are oxygenated derivatives of simpler monoterpenols. We hypothesize that cytochromes P450 in grape berries are responsible for this oxidation step.

Some enzymes from CYP76 family in *Arabidopsis thaliana* and *Catharantus roseus* can oxidize monoterpenols, which suggests that enzymes from this family might have a similar function in grapevine (*Vitis vinifera*). Bioinformatic analysis revealed that the family CYP76 has expanded in *Vitis vinifera*: it comprises of 25 members, compared to only 9 in *Arabidopsis thaliana*.

Our primary goal was to identify members of CYP76 family that are involved in production of oxygenated terpenes in grape berries. We first selected the genes based on their expression pattern and then confirmed *in vitro* that some members of *Vv*CYP76 family readily metabolize monoterpenes, present in the grape berries.



## P50. Regulation of the iridoid pathway in Catharanthus roseus

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Plants produce terpenoid indole alkaloids (TIAs) as a response to various biotic and abiotic stresses. This requires jasmonate-dependent perception followed by coordinated gene expression through the action of specific transcription factors (TFs). TIA compounds, like vinblastine and vincristine, are exclusively produced in the plant species Catharanthus roseus and are currently used as agents against certain types of cancer. However, they accumulate in low amounts, resulting in high production costs.

In C. roseus the MYC2-dependent TF ORCA3 has been shown to regulate several steps in the TIA pathway making it a key player in the TIA regulatory network. The gene encoding geraniol-10-hydroxylase (G10H), catalysing the second committed step in the iridoid pathway branch, is owever not regulated by ORCA3 and is limiting in ORCA3-overexpressing lines. Therefore, control of G10H regulation might be essential for engineering TIA production in plants.

We conducted an elaborate RNA-Seq experiment on jasmonate-elicited C. roseus tissues aiming to identify potential regulators of G10H. A functional trans-activation assay in tobacco protoplasts identified two TFs that regulate G10H expression. We are currently functionally analysing these TFs to establish their role in the TIA regulatory network using an RNAi and overexpression strategy. In addition, we cloned all C. roseus JAZ proteins to establish their relationship with the jasmonate signalling network.

## P51. Biochemical analysis of ent-kaurene oxidase in the *Physcomitrella patens*

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Research Fellow of the Japan Society for the Promotion of Science

The moss *Physcomitrella patens* produces both *ent*-kaurene and *ent*-kaurenoic acid, which are intermediates in gibberellin (GA) biosynthesis in flowering plants. However, GAs downstream of *ent*-kaurenoic acid, such as $GA_{12}$ and $GA_4$, were not detected in the moss. A disruption mutant of bifunctional *ent*-kaurene synthase (PpCPS/KS) recovered the growth in the present of *ent*-kaurene and *ent*-kaurenoic acid, suggested that an *ent*-kaurene derivative acts as an endogenous developmental regulator (Hayashi *et al*., 2010). Recently, we showed that the biosynthetic reaction from *ent*-kaurene to *ent*-kaurenoic acid in *P. patens* is catalyzed by CYP701B1, a member of the cytochrome P450 monooxygenase superfamily. In addition, it was highly resistant to uniconazole-P, a compound that inhibits the activities of *ent*-kaurene oxidases (KOs) from higher plants (Miyazaki *et al*., 2011). To better understand the differences between CYP701B1 and higher plant KO (Pea KO, CYP701A10) activity, we investigated the kinetics of these enzymes.

Recombinant KOs were produced in the heterologous expression system using *Pichia pastoris*. Both recombinant KOs showed maximum absorption at 450nm in the CO-difference spectra and typical type I substrate binding spectra with *ent*-kaurene. Interestingly, CYP701B1 showed also substrate binding spectrum with *ent*-kaurenoic acid. In the enzyme assay of CYP701B1, radioactive spots at the polar region rather than *ent*-kaurenoic acid on TLC were observed (Miyazaki *et al*., 2011). The substrate binding spectrum supported the profile of enzyme assay. Furthermore, we are currently analyzing the gene expression of *CYP701B1* cultivated under different light conditions.

References: Hayashi *et al*., *Plant Physiol*., vol.153, 1085, 2010; Miyazaki *et al*., *FEBS Lett*., Vol.585, 1879, 2011

## P52. Feedback inhibition of deoxy-D-xylulose 5-phosphate synthase regulates the methyl erythritol 4-phosphate pathway

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The methylerythritol phosphate (MEP) pathway leads to the biosynthesis of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), the precursors for isoprene and higher isoprenoids. Isoprene has significant effects on atmospheric chemistry while other isoprenoids have diverse roles ranging from various biological processes to applications in commercial uses. Understanding the metabolic regulation of the MEP pathway is important considering the huge applications of this pathway. The deoxyxylulose 5-phosphate synthase (DXS) enzyme was cloned from *Populus trichocarpa* and the recombinant protein (*Pt*DXS) was purified from *E. coli*. The steady-state kinetic parameters were measured by a coupled enzyme assay. An LC-MS/MS-based assay involving the direct quantification of the end product of the enzymatic reaction, 1-deoxy-D-xylulose 5-phosphate (DXP), was developed. The effect of different metabolites of the MEP pathway on *Pt*DXS activity was tested. *Pt*DXS was inhibited by IDP and DMADP. Both of these metabolites compete with thiamine diphosphate for binding with the enzyme. An atomic structural model of *Pt*DXS in complex with thiamine diphosphate and $Mg^{2+}$ was built by homology modeling and refined by molecular dynamics simulations. The refined structure was used to model the binding of IDP and DMADP, showing that IDP and DMADP bind with the enzyme in a manner very similar to the binding of thiamine diphosphate. The feedback inhibition of *Pt*DXS by IDP and DMADP constitutes an important mechanism of metabolic regulation of the MEP pathway and indicates that TPP-dependent enzymes may often be affected by IDP and DMADP.

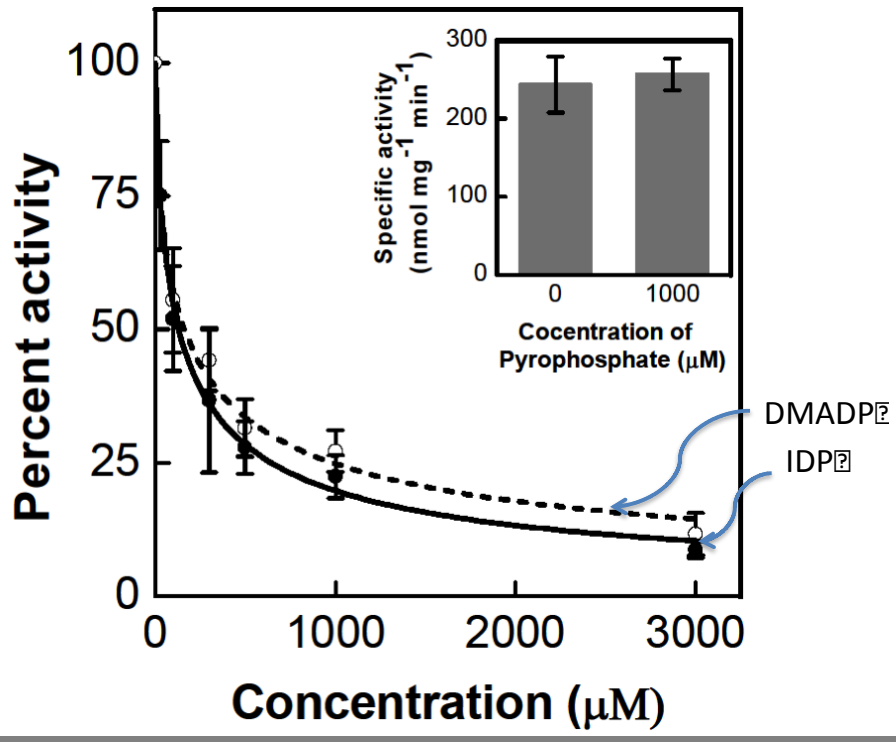


Figure 1. Inhibition of DXS by IDP and DMADP but not pyrophosphate.

## P53. Biosynthetic gene clusters for triterpenes in legumes

Afrodite Krokida, Costas Delis, Katrin Geisler, Constantine Garagounis, Daniela Tsikou, Luis M. Peña-Rodríguez, Dimitra Katsarou, Ben Field, Anne E. Osbourn, Kalliope K. Papadopoulou

Triterpenes are plant natural products that are formed by cyclization of 2,3 oxidosqualene. The triterpene scaffolds formed by enzymes known as oxidosqualene cyclases (OSCs) may be subsequently modified by enzymes such as cytochrome P450s and glucosyltansferases. Four loci of candidate metabolic gene clusters were identified in the genomes of the model legumes *Lotus japonicus* and *Medicago truncatula*, in regions flanking OSC genes. Amongst these was a *L. japonicus* cluster containing the *AMY2 OSC* gene along with genes for two different classes of cytochrome P450 and a reductase. This cluster represents a new pathway for triterpene biosynthesis in legumes. Expression of *AMY2* cluster genes in *Nicotiana benthamiana* identified a novel triterpene structure, dihydrolupeol, produced by AMY2, and allowed the biochemical characterization of a new plant cytochrome P450, CYP71D353, which catalyses the formation of 20-hydroxybetulinic acid in a sequential three-step oxidation of 20-hydroxylupeol. The cluster genes are highly coexpressed during root and nodule development, in hormone-treated plants and under different environmental stresses. A possible role for the gene cluster in plant development was revealed by the production of RNA silencing lines. These experiments also revealed an unexpected mechanism for the regulation of cluster gene expression by long distance transcriptional gene silencing.

## P54. Expression of genes involved in artemisinin biosynthesis in eight *Artemisia* species

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Artemisinin is a powerful antimalarial substance that is commercially obtained from *Artemisia annua*. We have selected eight *Artemisia* species and investigated the amount of artemisinin at three different developmental stages, *i.e.* vegetative, budding and flowering stages. All eight species produced artemisinin in various amounts (0.5 to 5 mg/g dry weight). The expression levels of eight genes involved in the artemisinin biosynthetic pathway were determined in tissues from the three developmental stages by qPCR. Four species (*A. absinthium*, *A. diffusa*, *A. sieberi* and *A. spicigeria*) showed maximum production of artemisinin at the flowering stage and three other species (*A. annua*, *A. campestris* and *A. vulgaris*) exhibited maximum production of artemisinin at the budding stage, while the highest concentration of artemisinin was found in vegetative leaves of *A. scoparia*. In *A. annua*, the higher amount of artemisinin produced was mainly due to higher expression levels of the amorpha-4,11-diene synthase (ADS) and artemisinic aldehyde Δ11(13) reductase (DBR2) genes. Furthermore, our results showed that the correlation between high expression of early genes of artemisinin biosynthesis, *i.e.* 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and farnesyl diphosphate synthase (FDS) and increased artemisinin production depends on the species. In addition, enhancement of artemisinin content is accompanied with the increased expression of HMGR, FDS, ADS, DBR2, and ALDH1 along with decreased expression of RED1.

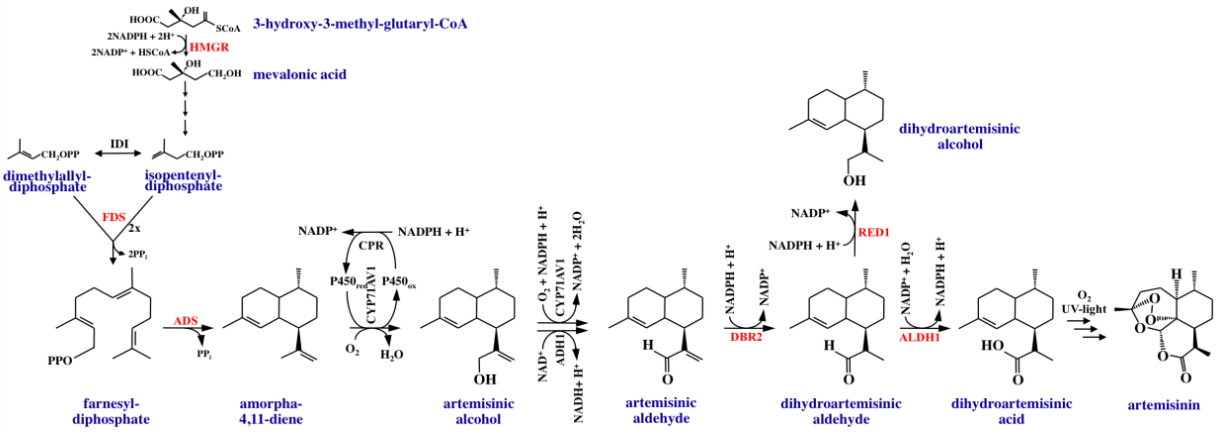


**Figure 1.** Biosynthesis of artemisinin in *Artemisia annua*. The enzymes studied are in red.

## P55. Characterization of furostanol glycoside 26-*O*-β-glucosidase involved in hydrolysis of protodioscin from *Dioscorea esculenta*

Masaru Nakayasu[1], Takashi Kawasaki[1], Hyoung-Jae Lee[1], Rie Yamamura[1], Michio Onjyo[2], Yukihiro Sugimoto[1], Masaharu Mizutani[1]

[1]Grad. School Agric. Sci., Kobe University, [2]Kagoshima University

Steroidal saponins are natural surfactants with various biological activities, and are often found in monocotyledons such as *Dioscoreaceae* and *Liliaceae*. In particular, the tubers of *Dioscorea*, known as yam, contain furostane and spirostane glycosides such as protodioscin and dioscin, respectively, which are valuable saponins required for semi-synthetic production of pharmaceutical steroidal drugs. Several P450s are likely involved in oxygenations at the C-16, C-22, and C-26 positions of cholesterol, and UGTs will function in glycosylation at C-3 and C-26. Additionally, a □-glucosidase hydrolyzes the glucosidic bond at C-26 of protodioscin to form dioscin. However, little is known about enzymes and genes for dioscin biosynthesis. To investigate steroidal saponin biosynthesis in *Dioscorea* spp., we first performed quantification of steroid saponins in *Dioscorea* plants. The results showed that *D. esculenta* accumulated high amount of steroid saponins, in particular protodioscin in the leaves, whereas *D. alata* contained little steroid saponins. Next, we perfomed comparative transcriptome analysis of the tubers with 454 pyrosequencing. We have selected several candidate unigenes of P450s, UGTs, and □-glucosidases involved in dioscin biosynthesis. In this study, we isolated *DeGH1* full-length cDNA, which is a member of glucosidase family 1. The recombinant enzyme hydrolyzed protodioscin, furostane glycoside, to form dioscin, spirostane glycoside, indicating that DeGH1 is furostanol glycoside 26-*O*-□-glucosidase (Figure 1). The expression of *DeGH1* was analyzed by quantitative RT-PCR. The DeGH1 transcript was present in all the organs of *D. esculenta* that we tested. To confirm the subcellular localization of DeGH1 in the plant, we introduced the DeGH1: GFP fusion gene into the leaves of *Nicotiana benthamiana* and we observed the localization of the GFP signals in the protoplasts isolated from the transformed leaves. The results showed that DeGH1 is localized in the chloroplasts. The results in this study suggested that DeGH1 is involved in biological defense in the plant.

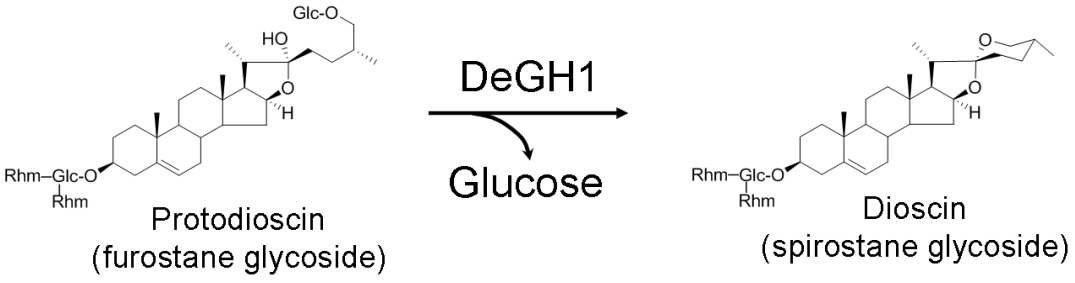


Figure 1 Catalytic Function of DeGH1

## P56. A sugar phosphatase directs isoprenoid flux in malaria parasites

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Severe malaria due to infection with *Plasmodium falciparum* causes nearly one million deaths per year, mostly in young children. New drugs are urgently needed due to widespread antimalarial drug resistance. The parasite possesses an essential non-mevalonate isoprenoid biosynthesis pathway that proceeds through the key intermediate methylerythritol phosphate (MEP). To understand why isoprenoids are essential in malaria parasites, we performed a genetic screen to identify 27 independently-derived malaria parasite strains that were resistant to a validated MEP pathway inhibitor, fosmidomycin. Surprisingly, none of the fosmidomycin-resistant parasite lines have mutations in the known target of this inhibitor. Mass spectrometric metabolic profiling of these fosmidomycin-resistant lines confirmed that fosmidomycin still inhibits MEP pathway enzymes in these strains. To identify the genetic changes that confer fosmidomycin-resistance, we performed next-generation whole genome sequencing to describe all single nucleotide polymorphisms, insertions/deletions, and copy number variations. More than half of the fosmidomycin-resistant strains, and none of the fosmidomycin-sensitive strains, contain deleterious mutations in a single locus. This locus is predicted to encode a sugar phosphatase (which we call HAD1), and localization studies reveal that HAD1 is present in the parasite cytoplasm. Characterization of recombinant HAD1 protein confirms phosphatase activity with a variety of phosphorylated sugar substrates, and $^{31}$P NMR metabolic profiling of wild-type and fosmidomycin-resistant malaria parasite strains demonstrate changes in phosphorylated sugar abundance in the resistant lines. Our studies suggest that HAD1 represents a branchpoint in sugar metabolism that controls MEP pathway flux in malaria parasites.

## P57. Cloning, expression and functional analysis of isopentenyl diphosphate isomerase from *Ficus microcarpa*

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Natural rubber (cis-1,4 polyisoprene) from Hevea brasiliensis is the main commercial rubber source owing to its high productivity and excellent physical properties. A range of synthetic polyisoprenes are produced by the polymerization of isoprene monomer derived from petroleum. Some of these polyisoprenes exhibited cis-1,4 isoprene contents > 98%. On the other hand, these synthetic rubbers are often green, and vulcanized rubber is to some extent inferior to natural rubber. Therefore, natural rubber cannot be replaced completely with synthetic rubber in many applications. Therefore, this study is attempted to provide new information on the biosynthesis of natural rubber from ***Ficus microcarpa*** which make rubber in latex like Hevea rubber, and can be grown in Japan. Isopentenyl Diphosphate (IPP) Isomerase catalyzes the interconversion between IPP and dimethyl allyl diphosphate (DMAPP). In order to examine possible participation of the IPP isomerase in the natural rubber biosynthesis, we cloned, overexpressed and characterized the cDNA clone encoding IPP isomerase from ***F. microcarpa***. The amino acid sequence of the clone contains all conserved regions of IPP isomerase. This cDNA was expressed in E. coli cells as His-tagged protein, which showed a distinct IPP isomerase activity.

## P58. Transcriptional activation of the MEP pathway gene *OsDXS3* by the bZIP transcription factor *OsTGAP1* in rice

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Plants attacked by pathogenic microorganisms respond with a variety of defensive reactions, including the production of phytoalexins. Momilactones and phytocassanes are major phytoalexins in rice and are synthesized from geranylgeranyl diphosphate that is provided by the upstream metabolic pathway, methylerythritol phosphate (MEP) pathway in plastids. We previously showed that biosynthetic genes for momilactones and phytocassanes are respectively clustered on rice chromosome 4 and chromosome 2[1)], and that an elicitor-inducible bZIP transcription factor, OsTGAP1, is involved in the regulation of the coordinated expression of biosynthetic genes for diterpenoid phytoalexins and the MEP pathway genes[2)].

In order to identify the binding sites of OsTGAP1 in rice genome, we performed ChIP-seq analysis. As the result, approximately 2700 binding sites of OsTGAP1 were found in the genome. Around the locus of momilactone biosynthetic gene cluster, OsTGAP1 bound to near the transcription start sites of two biosynthetic genes, OsKSL4 and OsCPS4. Interestingly, OsTGAP1 also predominantly bound to the intergenic regions and both ends of the biosynthetic gene cluster. Among the MEP pathway genes, *OsDXS3* gene responsible for the first committed step of the MEP pathway was the only gene bound by OsTGAP1 in the promoter region. We further focused on the transcriptional regulatory mechanism of *OsDXS3* by binding of OsTGAP1. There are two TGACG-motifs that are putative binding sequences of TGA factors in the *OsDXS3* upstream region. From the electrophoretic mobility shift assay, it was shown that the binding of OsTGAP1 to *OsDXS3* upstream region is dependent on these motifs. The promoter analysis of *OsDXS3* further indicated that these TGACG-motifs are essential for the transactivation of *OsDXS3* gene by OsTGAP1.

References

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## P59. Investigating proteins involved in the metabolic pathway of iridoids in defensive glands of *Phaedoncochleariae*

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Larvae of the mustard leaf beetle, *Phaedoncochleariae*, developed a sophisticated defense system. They produce the cyclic monoterpene chrysomelidial which deters predominantly invertebrate predators. Chrysomelidial is stored in nine pairs of exocrine glands on the back of the larvae.In case of disturbance it is discharged in droplets from the glands. The main challenge for the larvae is to develop a functional defense system without auto-intoxication. To achieve this, nontoxic precursors are used. For synthesis of chrysomelidial for example, the harmless 8-hydroxygeraniol-glucoside is produced in the tissue of the fat body. This precursor istransported into the gland reservoirand subsequently enzymatically converted into the deterrentchrysomelidial. Howeverup to date the enzyme machinery situated in the gland reservoirproducing the 8-hydroxygeraniol and proceeding via 8-oxogeranial to form the cyclicmonoterpenechrysomelidial is poorly understood.

Therefor we analyzed the proteome of the larval secretions. This revealed several sequences for candidate enzymes catalyzing steps of chrysomelidial biosynthesis. Screening *via* specific RNAi-mediated silencing of these transcripts displayed a shift in thecomposition of low molecular compounds. Therefore it was possible to assign the accumulation of chrysomelidial precursors to transcript candidates. A decreased amount of the 8-hydroxygeraniol could be attributed to the down-regulation of a β-glucosidase, which catalyzes the first step in the chrysomelidial pathway. For the second step, an oxidation, the responsible enzyme is anoxidoreductase. The cyclization to chrysomelidial, the last step, is dependent on a protein belonging to the insect specific juvenilehormone-binding protein superfamily. This is surprising, because for proteins of this family no catalytic activity was observed until now. To confirm this, the next step will be the heterologous expression and characterization of the putative cyclase.

## P60. Analysis of catalytic activity of geranylgeranyl diphosphate synthase by enzyme assay and doking simulation

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Farnesyl diphosphate synthase (FPS) and geranylgeranyl diphosphate synthase (GGPS) are both classified into a trans-type prenyltransferase family, catalysing the fundamental chain elongation reaction between allylic diphosphate and isopentenyl diphosphate. The structural difference between FPS and GGPS has not been clarified. We have studied on the substrate specificities for FPS using various artificial substrate analogs and obtained much knowledge on the enzymatic catalyst activity mechanism. However, there is little knowledge about the substrate recognition for GGPS. In this study, we carried out the enzyme reaction using artificial substrates having an oxygen atom or phenyl group in a side chain to investigate the substrate specificities from Human and Lactarius chrysorrheus. Moreover, we speculated recognition mechanism of these allylic substrates of prenyltransferases using molecular doking simulation. For simulation of the enzymatic reaction mechanism, we constructed a 3D structural model based on the crystal structure using SWISS-MODEL. Enzyme-substrate docking was carried out using the docking software GOLD. The simulation studies disclosed that some analogs with the chain length almost equal to the natural substrate (GPP) were incorporated in the same site as natural substrate. Interestingly, some analogs showing no reactivity were incorporated in completely different site for the natural substrate.

## P61. Mechanisms of chemotype formation in *Thymus vulgaris*

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Many species of essential oil-producing plants form distinct sub-populations that differ strongly in their oil composition although their morphology is nearly identical. These chemotypes have often been described in species of the Lamiaceae family including sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), majoram (*Origanum majorana*) and thyme (*Thymus vulgaris*). Only a few chemotypes of these plants produce essential oils that are valuable for their flavor, taste, and medicinal or antioxidant properties. Although the biosynthesis pathways of essential oils have been studied in many Lamiaceae species, little is known about the molecular mechanisms of chemotype formation which determine the composition of the essential oil.

In Southern France, six chemotypes of thyme were described by Granger and Passet in 1973[1]. These chemotypes are named after the most prominent monoterpene alcohol in their essential oil: geraniol (G-type), α-terpineol (A-type), sabinene hydrate (U-type), linalool (L-type), carvacrol (C-type) and thymol (T-type). A characteristic of these chemotypes is that they form an epistatic row with the geraniol type being dominant over all chemotypes: G > A > U > L > C > T. The thymol type is recessive to all others[2]. We chose these chemically distinct chemotypes of *Thymus vulgaris* to study the molecular mechanisms of chemotype determination.

We identified a group of six terpene synthase genes from the cDNA of thyme chemotypes. Expression in a bacterial system demonstrated that these terpene synthases are capable of producing almost all compounds of the thyme essential oils. The transcript levels of these genes correlate with the formation of their products in the respective chemotypes. TvTPS2, a terpene synthase producing γ-terpinene is involved in the formation of the phenolic monoterpenes carvacrol and thymol. In order to find *cis*-elements that regulate the transcription of *Tvtps2*, we sequenced the *Tvtps2* genomic region. The genomic structure of *Tvtps2* in all six chemotypes shows an intron-exon pattern typical for terpene synthases and only minor differences. The *Tvtps2* promoter region was analyzed to identify characteristic regulatory elements and sequence polymorphisms that correlate with gene activity.

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## P62. Substrate specificity of *ent*-kaurene synthases in plants

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*ent*-Kaurene synthase (KS) is a diterpene cyclase that catalyzes cyclization reaction of *ent*-copalyl diphosphate (*ent*-CDP) to *ent*-kaurene, which is a precursor of plant growth hormone gibberellin (GA). All land plants produce *ent*-kaurene by monofunctional KS or bifunctional KS. The bifunctional KS found in bryophytes and fungi catalyzes two-step reaction of geranylgeranyl diphosphate to *ent*-kaurene via *ent*-CDP. Our laboratory found that some monofunctional KS recognized not only *ent*-CDP but also *syn*-CDP and (+)-CDP to afford diterpene compounds. In this study, we investigated substrate specificity of both monofunctional and bifunctional KS, and analyzed the products from foreign substrates.

cDNAs of monofunctional KSs were cloned from *Selaginella moellendorffii*, *Lactuca sativa* L. and *Oryza sativa* (cv. Nipponbare) and the recombinant KSs were produced in *E. coli*. In addition, mutated KS of *Physcomitrella patens* showed single KS activity (no CPS activity) was used. When *ent*-CDP was used as a substrate, all KSs produced *ent*-kaurene. SmKS, LsKS and Ppcps/KS produced diterpene hydrocarbon products from *syn*-CDP and (+)-CDP. For instance, SmKS and LsKS produced pimaradiene type compound from (+)-CDP. The structure of this compound was determined by techniques of enzymatic total synthesis, 13C-labeling and NMR measurements1. Interestingly, OsKS catalyzed the *ent*-CDP cyclization but recognized no other CDP compounds unlike other KSs. These results provide following discussions:

1) OsKS strictly recognize the substrate because rice use CDP stereoisomers for diterpene secondary metabolites like phytoalexins. This may be a functional mechanism to keep homeostatic production of GA in diterpene type phytoalexin-producing plants such as rice and wheat2.
2) On the other hand, other KSs tested in this study recognize all CDP stereoisomers. It may be not necessary for these plants to keep substrate specificity of KS.

1. References: 1Sugai *et al.*, *J. Biol. Chem.*, 2012, 2Zhou *et al.*, *Phytochemistry*, 2012

## P63. Spatial and temporal patterns ofterpenoidbiosynthesis in tomato flowers

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The volatiles of tomato flowers are composed of mainly monoterpenes,sesquiterpenes and apocarotenoids. The most prominent monoterpenes are α-pinene; *p*-cymene; □ (2)-carene;β-phellandrene;□-phellandrene;and the sesquiterpene*trans*-caryophyllene, being β-phellandrene the major volatile. All these volatiles showed a diurnal pattern of emission with a peak occurring at 4:00PM corresponding to the diurnal changes in total monoterpene synthase activity in the flower. The flower organs emitting the highest level of volatiles are the petals and sepals while monoterpene synthase activity,on the basis of fresh weight,was highest in the ovary. The apocarotenoids emitted by the flowersare geranial,□-cyclocitral,geranyl acetone and □-iononebeingthe major one. Two novel members of the Carotenoid Cleavage Dioxygenase gene family (*SlCCD4a* and *SlCCD4b*) were cloned from tomatoshowing high similarity to *CCD4* genes from other plant species. Both sequences had one intron and a predicted signal peptide targeting the translated protein to the plastid.While *SlCCD4b* is primarily expressed in leaves and unripe fruits,*SlCCD4a*is expressed in anthers and showed a diurnal pattern of expression with a peak at 2:00 PM. Moreover, the expression of *CCD4a* was highest at anthesis and decreased thereafter. Our results suggest that *SlCCD4a*might be involved in the formation of □-ionone and geranyl acetonein tomato anthers.

## P64. Correlation analysis of agronomic traits and terpenoid indole alkaloids contents in different varieties of *Catharanthus roseus*

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*Catharanthus roseus* is one of the most popular herbal ornamental species in China, and it is also a highly valued medicinal plant containing over 100 pharmaceutically important terpenoid indole alkaloids such as vindoline, catharanthine and vinblastine. To investigate the agronomic traits of different *C. roseus* varieties and their correlations with the important alkaloids content is important for introduction and breeding of high alkaloids-content *C. roseus* varieties.

In this study, the main agronomic traits of 24 major *C. roseus* varieties currently grown worldwide, and their correlations with the contents of important alkaloids including diterpene indole alkaloid vinblastine and monoterpene indole alkaloids vindoline and catharanthine were investigated. It is found that significant difference existed in agronomic traits among different *C. roseus* species. China Hainan variety was superior to other varieties in most agronomic traits while European and American varieties (Pacifica series varieties, Mediterranean series varieties, Cooler series varieties, HeatWave series varieties, SunStorm series varieties) had abundant flower color, more branches and shorter plant height. Therefore, different varieties could be chosen as breeding materials with different breeding goals.

The contents of 3 important alkaloids including vindoline, catharanthine and vinblastine were found significantly different among different varieties. Vindoline content was the highest in HeatWave variety Mix Color, while catharanthine content was the highest in Pacifica variety Polka Dot. Vinblastine content was the highest in Pacifica variety Cherry Red. In *C. roseus*, vinblastine content was much lower than vindoline and catharanthine contents.

Correlation and path analyses using SPSS statistic software showed that different agronomic traits had different correlations with the alkaloids contents. The correlation of vindoline content with the first lateral branch number and stem diameter reached the significant level ($P<0.05$). Path analysis indicated that among all the agronomic traits, the positive influence of internodal distance, the first lateral branch number and whole plant leaf weight on vindoline content reached the significant level ($P<0.05$). Internodal distance significantly influenced positively catharanthine content ($P<0.05$), while the first lateral branch number and petal diameter positively affected significantly vinblastine content ($P<0.05$). In addition, flower color was also found to have certain correlations with alkaloids contents, and the contents of all the three important alkaloids (vindoline, catharanthine, vinblastine) in deep color flower *C. roseus* varieties were high. This study indicates that agronomic traits could be used as the reference for the breeding of high-alkaloids content *C. roseus* varieties.

## P65. Involvment of Arabidopsis *cis*-prenyltransferase *AtCPT6* in short-chain polyisoprenoids synthesis - studies in yeast and plants

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Polyisoprenoids, linear five-carbon unit polymers, have been detected in all living organisms, from bacteria to higher eukaryotes. They are classified into two groups: polyprenols and dolichols. Their phosphates play significant biological functions as cofactors of protein glycosylation, biosynthesis of GPI-anchor and bacterial cell wall polymers. The main enzymes involved in regulation of the prenyl chain length are *cis*-prenyltransferases (CTPs). CPTs elongate short all-*trans* precursor, oligoprenyl diphosphate, by sequential addition of the desired number of isopentenyl diphosphate (IPP) molecules which results in formation of a stretch of *cis* units.

The main goal of our project is to characterize one of the nine Arabidopsis *cis*-prenyltransferases - AtCPT6. AtCPT6 is expressed exclusively in Arabidopsis roots and the level of its transcript is increased with plant life-span. The AtCPT6 protein is localized in root microsomal fraction. HPLC analysis revealed that the major product of AtCPT6 in yeast mutant is Pren/Dol-7. Identity of these short polyisoprenoids was clearly confirmed using HPLC/ESI-MS method. This short-chain dolichol could partially functionally substitute longer chain dolichols in *S. cerevisiae* since overexpression of *AtCPT6* cDNA suppressed the defects of growth of *Δrer2* mutant at 37°C however it did not restore protein *N*-glycosylation as followed by carboxypeptidase (CPY) glycosylation status.

In Arabidopsis roots, three families of dolichols are detected: Dol-12 to Dol-14, with Dol-13 dominating, Dol-15 to Dol-18, with Dol-16 dominating and Dol-19 to Dol-30, with either Dol-21 or Dol-23 dominating. However, careful inspection of the HPLC chromatograms revealed a small amount of Dol-7 too. Moreover, we did not observed this short-chain dolichol in the roots of two insertion mutants of AtCPT6, *cpt6-1* (SALK_071255) and *cpt6-2* (SALK_064499). This suggests that Pren/Dol-7 are products of AtCPT6 both in the heterologous and homologous system.

Currently, studies on the transgenic Arabidopsis plants overexpressing *AtCPT6* are in progress.

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## P66. Genetic modification of soybean saponins showing health beneficial and undesirable taste characteristics: Changes of the composition and content in soybean processed foods

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**[Objective]** Soybean saponins are divided roughly to DDMP and group A saponins with the different chemical structures of aglycon. DDMP saponins, which exist in seed cotyledons and hypocotyls, can be decomposed to group B and E saponins. This affects to the different healthy functionality. Otherwise, group A saponins normally exist in hypocotyls as acetylated forms and they are cause of undesirable taste with bitterness and astringency in soy foods. As the different chemical structure of soy saponins affects to the health and taste characteristics, the saponin composition and content in soy products were aimed to clear. In this research, we focused on especially DDMP and deacetylated group A saponins which have not been interested until now.

**[Materials and Methods]** All soymilk products were purchased in Japanese market and freeze dried. Saponins were extracted with 80% (v/v) aquaos methanol at room temperature for 1 hr and were detected by using LC-PDA/MS/MS (ESI+) and were determined by UV absorbance at 205 nm.

**[Results and Discussion]** The 13 different soymilk products were used; No.1-6 and No.7-13 were soymilk without and with additives, respectively. Each product contained DDMP, group A, B, and E saponins but they showed different content and compositions (Fig. a). Among all products, only No.3 was prepared with squeezing at room temperature and degraded the most of DDMP saponins (Fig. b). DDMP saponins are decomposed into group B and E saponins by heat treatment. This result suggests that lipoxygenase activity also strongly affect to DDMP degradation. For group A saponins, sample No.4, 7-13 showed that almost no tetra-acetyl saponin remained (Fig. c). As heat treatment at neutral pH hardly affects to deacetylation, removal of hypocotyls seemed to reduce group A tetra-acetylsaponins. No.4 and 10 were prepared from a special var. Kinusayaka which is genetically eliminated both three lipoxygenase isozymes and all group A acetylsaponins. hese results suggest that not only genetic modification but also processing conditions would strongly affect to the composition and content of saponins in soybean processed foods.

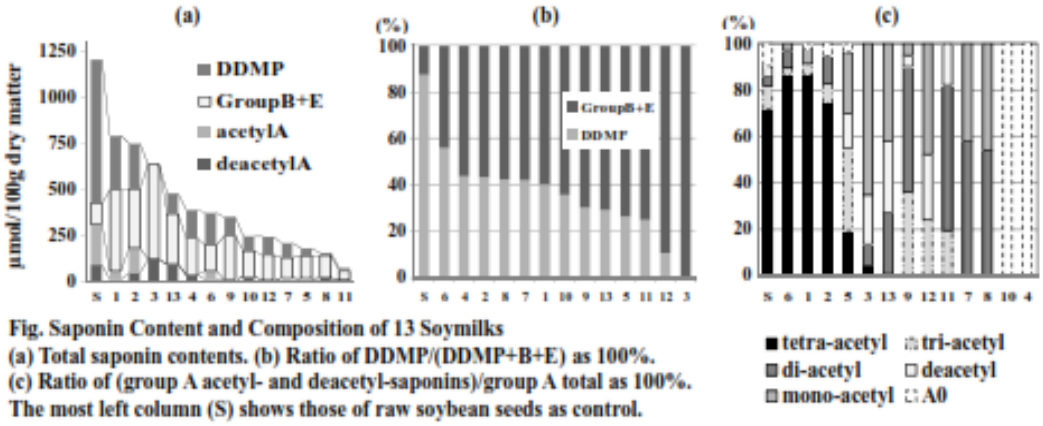


**Fig. Saponin Content and Composition of 13 Soymilks**
**(a) Total saponin contents. (b) Ratio of DDMP/(DDMP+B+E) as 100%.**
**(c) Ratio of (group A acetyl- and deacetyl-saponins)/group A total as 100%.**
**The most left column (S) shows those of raw soybean seeds as control.**

## P67. The dominant *Sg-6* synthesizes soyasaponins with a keton function at oleanane aglycone C-22 position in soybean (*Glycine max* (L.) Merr.)

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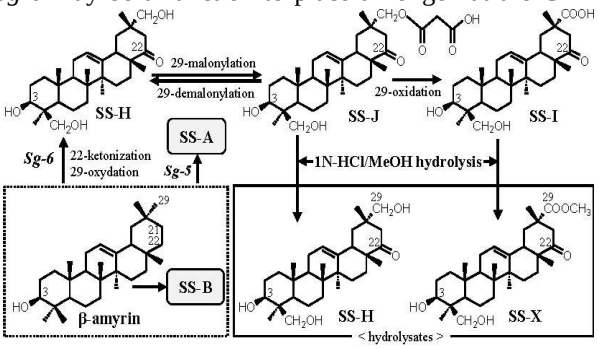
**[Objective]** Daily intake of processed soy foods appears to be one of the factors responsible for the longevity in Japanese people. Soybeans contain many functional compounds, among them, soyasaponins are a subject of increasing research interest. Soyasaponins have different tastes and health benefits depending on their chemical structures. Mutant soybeans with new saponins are expected to improve these characteristics. We have reported mutants, which contain three unidentified aglycones (Soyasapogenol (SS-) H, I and J) in the seed. Their presence is controlled by a single dominant mutation in the *Sg-6* locus. However, it is unclear why a number of saponins are made by this dominant allele and, more precisely, what the function of *Sg-6* is. In this study, we examined the saponin compounds present in the mutant.

**[Materials and Methods]** Saponins were extracted with 80% methanol and hydrolyzed with 1N-HCl. Saponins and their aglycones were analyzed by LC-PDA/MS/MS (ESI+) and purified by HPLC with an ODS column. Chemical structure was analyzed by NMR.

**[Results and Discussion]** In the mutant seeds, saponins having unidentified aglycones SS-H ($C_{30}H_{49}O_4^+$), SS-I ($C_{30}H_{47}O_5^+$) and SS-J ($C_{33}H_{51}O_7^+$) were detected, in addition to group A saponins which have SS-A and DDMP saponins which contain a SS-B residue as a part of aglycone. In the hydrolysate, SS-H was increased and SS-X ($C_{31}H_{49}O_5^+$) was newly detected, whereas SS-I was decreased and SS-J was not detected. The chemical structures of SS-H and SS-I were elucidated by NMR data of their purified substances and those of SS-X and SS-J were estimated by their MS/MS fragment pattern. All of them contained a keton function at the C-22 position and differed in a substituent at the C-29 position. It is thought that the C-29 substituent of SS-J and SS-I is modified by methanolysis to become SS-H and SS-X. *Sg-6* may be the same as *Sg-5*, CYP72A61 and CYP93E1, that are P450 family synthetizing SS-A and SS-B from β-amyrin. Thus, *Sg-6* may be a function to place an oxgen at the C-22 position 29 29 of β-amyrin. Also, the $^{29}$ substituents may be combined with the C-29 position by other enzymes. If *Sg-6* indeed catalyses keton formation at the C-22 position, this may serve as a reference to identify other genes that can ketonize β-amyrin.



## P68. Metabolic chemotype of *Nicotiana benthamiana* transiently expressing artemisinin biosynthetic pathway genes is a function of *CYP71AV1/AMO* type and relative gene dosage

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The plant *Artemisia annua* produces the anti-malaria compound artemisinin, occurs as high artemisinin production (HAP) and low artemisinin production (LAP) chemotypes. Understanding the molecular basis of *A. annua* chemotype may help optimising artemisinin biosynthesis in heterologous production platforms. We present the first systematic comparison of artemisinin biosynthesis genes to determine factors that contribute to artemisinic acid (AA) or dihydroartemisinic acid (DHAA) chemotype of agro-infiltrated leaves with ADS, *CYP71AV1*/*AMO*, *DBR2* and *ALDH1*. Results show that the enzyme activity of DBR2 and ALDH1 from the two chemotypes does not differ. The Amorphadiene Oxidase from HAP ($AMO^{HAP}$) showed reduced activity compared to that from LAP chemotype ($AMO^{LAP}$), which relates to a seven amino acid N-terminal extension in $AMO^{LAP}$ compared to $AMO^{HAP}$. The GFP fusion of both proteins show equal localization to the ER, but $AMO^{LAP}$ may be more stable. Product profile characterisation by LC-QTOF-MS/MS, UPLC-MRM-MS and GC-MS of transient expression in *Nicotiana benthamiana* show that $AMO^{LAP}$ not only displayed a higher enzyme activity but also affected the ratio of end products (e.g. leaf chemotype), which could be mimicked by reduced gene dosage of $AMO^{LAP}$ in the pathway. However, expression in combination with the *DBR2* and *ALDH1* also resulted in a qualitatively different product profile ('chemotype') when DBR2 infiltration dosage was diluted, shifting saturated (dihydro) branch toward unsaturated branch and of the pathway. Our study provides an in-depth view of a foreign terpenoid pathway in *N. benthamiana*, offering new insights relevant to the synthetic pathway engineering.

## P69. The regulation of saponin content and composition in the seedlings of wild soybean (*Glycine soja* Sieb. & Zucc.) is specific to variety and organ

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**[Objective]** Many structurally diverse groups of saponins have been reported in wild soybean (G*lycine soja*) seeds. Despite being reported the complexity of saponin composition in seed hypocotyls, the content and composition of saponins in plant is remain unknown. In order to reveal that, an array of different organs of two-week old wild soybean seedlings were analyzed. **[Materials and Methods]** Eight wild soybean accessions having different saponin phenotypes were obtained from Chung's Wild Legume Germplasm Collection at the Chonnam National University. They were germinated for 2 weeks under non-controlled conditions in triplicates. Seeds and plants were separated into 2 and 8 organs respectively as shown in Table and freeze dried. Saponins were extracted with 10-fold (v/w) volumes of 80% (v/v) aqueous methanol at room temperature for 1 hr from freeze dry powdered samples and were determined by LC-PDA/MS/MS at UV 205 nm absorbance. **[Results and Discussion]** Average total saponin amount in 1 g mature dry seeds was 17.93 μmol; where as in 2 week old seedlings, which were produced from 1 g mature seeds, it was 29.36 μmol. In seedlings, the amounts of group A and Sg-6 saponins were reduced 2.3- and 1.3-times, respectively, while DDMP+B+E saponins increased 2.5-times than those of mature seeds. Group A and Sg-6 saponins in mature seeds seemed to be simply diluted in each organ by germination; by contrast, DDMP saponins were newly synthesized. Group B+E represent 75% and 82% on the total amount of DDMP+B+E saponins in MR and LR, respectively. This degradation was not observed in any other organs. These findings strongly suggest that (i) group A and Sg-6 saponins can be used as chemotaxonomic markers to trace the origin and evolution of soybean and wild soybean, (ii) DDMP saponins may have some physiological roles in plant growth and development especially at MR and LR, and iii) the regulation of saponin content and composition in wild soybean is specific to variety and organ.

**Table. Average saponin amounts (μmol) in different organs derived from 1 g mature seeds**

Saponin In 1g mature seeds In 2 weeks old seedlings derived from 1 g mature seeds

HY; hypocotyl, CT; cotyledon, HC; hypocotyl, EC; epicotyl, AM; apical meristem, MR; main root, LR; lateral root, CL; cotyledonary leaf, UL; unifoliate leaf, TL; trifoliate leaf, -; not detected.

| groups | HY | CT | Total | HC | EC | AM | MR | LR | CL | UL | TL | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group A | 4.05 | 0.34 | 4.39 | 0.78 | 0.04 | 0.02 | 0.26 | 0.57 | 0.24 | - | 0.02 | 1.93 |
| DDMP+B+E | 7.05 | 2.75 | 9.80 | 1.30 | 0.68 | 0.64 | 1.54 | 7.07 | 8.91 | 1.51 | 2.93 | 24.58 |
| Sg-6 | 1.59 | 2.15 | 3.74 | 0.23 | 0.13 | 0.05 | 0.19 | - | 2.20 | - | 0.05 | 2.85 |
| Total | 12.69 | 5.24 | 17.93 | 2.31 | 0.85 | 0.71 | 1.99 | 7.64 | 11.35 | 1.51 | 3.00 | 29.36 |

## P70. Laticifer-specific expression of an Arabidopsis ATP citrate lyase enhances rubber biosynthesis in *Taraxacum brevicorniculatum*

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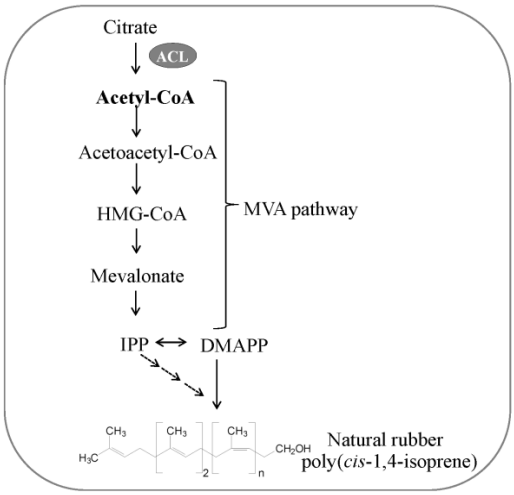
The dandelion species *Taraxacum brevicorniculatum* produces natural rubber - a unique high molecular weight biopolymer composed of more than 5,000 isoprene units - in its laticifers. The route for the provision of isopentenyl diphosphate (IPP) units for the formation of poly(*cis*-1,4-isoprene) chains in laticifers is driven by the mevalonic acid (MVA) pathway. In recent work we described the isolation and the functional characterization of the MVA pathway genes from *T. brevicorniculatum.*




In the present study we focused on the role of the central precursor of the MVA pathway –acetyl-CoA – for the rubber biosynthesis. Cytosolic acetyl-CoA is primarily synthesized by the enzyme ATP citrate lyase (ACL) and this enzyme has been shown to be a key rate-limiting step in the synthesis of acetyl-CoA in *Arabidopsis thaliana*. We analysed the endogenous *ACL* expression pattern in *T. brevicorniculatum* and – in order to examine the impact of ACL activity on the rubber formation - we expressed ACL from *A. thaliana* as fusion protein in *T. brevicorniculatum* under control of a laticifer specific promoter. We present here a tool to improve the flux of acetyl-CoA into the MVA pathway and subsequently to rubber biosynthesis by up-regulating the total ACL enzyme activity in the latex. This provides a further insight into the regulatory mechanism of the rubber formation in *T. brevicorniculatum* and confirms the importance of ACL activity and the precursor pool (acetyl-CoA, IPP) for the production of high amounts of rubber polymer. Furthermore, the knowledge about the key genes and pathway steps will support breeding programs in the future to generate Taraxacum plants with improved rubber yield.

## P71. Artemisinin metabolic engineering in heterologous plants

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Artemisinin is a sesquiterpeneendoperoxideisolated from *Artemisia annua*which is widely used for the treatment of malaria. *Artemisia annua*plants are limited in the accumulation of artemisinin and worldwide different biotechnological approaches are used to improve production. Previously we have shown that co-expression of all known biosynthesis genes in *N.benthamiana*only results in accumulation of DHAA and AA precursor molecules but not artemisinin. To investigate the role of transport activities in the accumulation of artemisinin or artemisinin related products we are testing the effect of co-expression of biosynthesis genes with GlutathionS-Transferases (GST), ABC transporters and Lipid Transfer Proteins (LTP). Candidate GST, ABC and LTP sequences were identified in *Artemisia annua*trichomecDNA libraries. Gene sequences were screened for expression profile over plant development and candidate genes were selectedof which the expression profile matches that of the biosynthesis genes. Full length gene isolation is completed or in progress. Results of co-expression of these candidate genes together with the artemisinin biosynthesis pathway genes in *N.benthamiana*will be presented.

## P72. Studies on the expression of terpene synthases using promoter-β-glucuronidase fusions in transgenic *Artemisia annua* L.

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Artemisinin, an antimalarial endoperoxide sesquiterpene, is synthesized in glandular trichomes of *Artemisia annua* L. A number of other enzymes of terpene metabolism utilize intermediates of artemisinin biosynthesis, such as isopentenyl and farnesyl diphosphate, and may thereby influence the yield of artemisinin. In order to study the expression of such enzymes, we have cloned the promoter regions of some enzymes and fused them to β-glucuronidase (GUS). In this study, we have investigated the expression of three sesquiterpene synthases and one monoterpene synthase, i.e. β-caryophyllene (CPS), *epi*-cedrol (ECS), β-farnesene (FS) and linalool (LS) synthase. The cloned promoter regions were 923, 1182, 1510 and 652 bp for CPS, ECS, FS and LS, respectively. Multiple *cis*-acting elements were predicted in the four promoters indicating that they are involved in complex regulation of gene expression. Transgenic plants carrying promoter-GUS fusions were produced. Different tissues at different stages of development were stained for GUS activity. CPS, ECS and LS showed specific expression of GUS in T-shaped trichomes (TSTs) but not in glandular secretory trichomes (GSTs), which is the site for artemisinin biosynthesis. In old leaves, GUS expression was also observed in vascular tissues. We have also observed that the LS promoter is active in guard cells. FS exhibited a more general expression as GUS staining was observed in both TSTs and GSTs as well as in leaf cells. Wounding of leaves indicated that CPS, ECS, FS and LS may be involved in the response of the plant to mechanical stress. We have also used qPCR to study the activities of the wild-type and recombinant promoters in different tissues of *A. annua*. Furthermore, all four genes appear to be activated by methyl jasmonate. In conclusion, due to the expression in T-shaped trichomes but not in glandular trichomes, it may be concluded that the terpene synthases studied will most likely have little or no effect on artemisinin production.

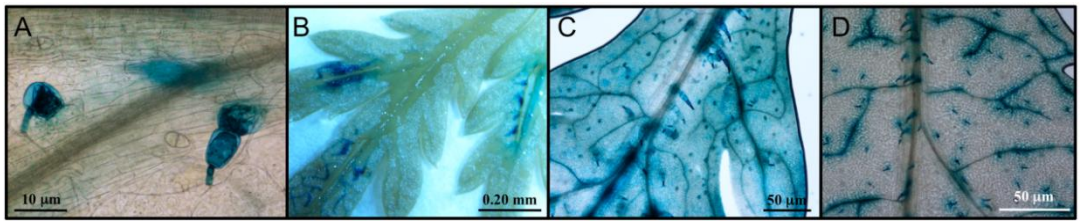


**Figure 1.** GUS staining of different transgenic *A. annua* plants. A: The CPS-promoter exhibits activity in TSTs; B: The ECS-promoter responds to wounding; C: The FS-promoter is active in different tissues; D. The LS-promoter shows activity in TSTs and vascular tissues.

## P73. Isotopic labeling of the MEP pathway in *Arabidopsis thaliana* lines overexpressing 1-deoxyxylylose phosphate synthase, shows the existence of a second methylerythritol cyclodiphosphate metabolite pool

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The chloroplast localized methyl erythritol phosphate (MEP) pathway produces the precursors of photosynthetic pigments, membrane linkage groups of photosynthesis and redox components, and many other isoprenoid-derived hormones and natural products. In this pathway, the central metabolic intermediates pyruvate and glyceraldehyde-3-phosphate are diverted towards isoprenoid biosynthesis by 1-deoxyxylulose 5-phosphate synthase (DXS). We examined the control exerted by DXS on the flux in this pathway using metabolic control analysis and a suite of transgenic, RNAi, and natural mutant lines with a range of DXS activities. Direct kinetic data were obtained from a $^{13}CO_2$ labeling system where *Arabidopsis thaliana* was labeled under natural growth conditions in a dynamic flow, climate controlled, gas exchange cuvette. HPLC-MS/MS analysis of $^{13}C$ incorporation into DXP in plant extracts coupled to measurements of DXS activity in the same leaf material showed that DXS has a high flux control coefficient (0.82) under photosynthetic steady state conditions. The maximum amount of $^{13}C$ incorporation into DXP for all lines under these conditions was about 60%. Upon examining the downstream intermediate methylerythritol cyclodiphosphate (MEcPP), we noted a four-fold increase in MEcPP concentration for all plant lines constitutively over-expressing DXS and a noticeable drop in the maximum $^{13}C$ incorporation from 60% in the wild type to 30%. However, maximum labeling of one of the pathway's end products dimethylallyl diphosphate (DMAPP) only reached 30% in all lines. We conclude that up-regulation of DXS triggers a natural efflux mechanism causing MEcPP to be diverted out of the MEP pathway. Such a second pool of MEcPP will explain the decrease of $^{13}C$ incorporation into total MEcPP. Thus, increased flux into the MEP pathway results in a substantial diversion of flux into a second, physically isolated pool of MEcPP, possibly located in the cytosol. Consequently only a limited amount of the increased flux resulting from DXS up-regulation reached downstream end products such as chlorophylls and carotenoids.

## P74. Characterization of De90B, cholesterol 22-hydroxylase involved in steroidal saponin biosynthesis in the tubers of *Dioscorea esculenta*

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Steroidal saponins, a group of steroids conjugating with sugars, are natural surfactants with various biological activities. Steroidal saponins are often found in monocotyledons such as plants of the families *Dioscoreaceae* and *Liliaceae*. In particular, the tubers of *Dioscorea* spp., known as yam, contain furostane and spirostane glycosides such as protodioscin and dioscin, respectively. These steroidal saponins are derived from cholesterol by sequential modification with oxygenation and transglycosylation reactions as shown in Figure 1. Namely, several cytochrome P450 monooxygenases (CYPs) are likely involved in oxygenations at the C-16, C-22, and C-26 positions, and UDP glycosyltransferase (UGTs) will function in transglycosylation at C-3 and C-26. But little is known about enzymes and genes for dioscin biosynthesis. To investigate steroidal saponin biosynthesis in *Dioscorea* spp., we performed comparative transcriptome analysis of the tubers of *Dioscorea* spp. with 454 pyrosequencing and selected several candidate genes for diosin biosynthesis. The full-length cDNAs of the candidate genes were isolated from a cDNA library of the tubers of *D. esculenta*. *D. esuculenta* accumulates high amounts of steroidal saponins in the tubers, which is known as a lesser yam in Okinawa island and called as Togedokoro in Japanese. One of the candidate cDNA showed significant similarity to the brassinosteroid biosynthetic gene *CYP90B1*, which is known to encode a C-22 hydroxylase of campesterol [1)], and we designated this gene as *De90B*. Recombinant De90B was expressed with a baculovirus-insect cell system, and De90B activity was measured in an in vitro assay reconstituted with NADPH-cytochrome P450 reductase. We confirmed that De90B catalyzes the C-22 hydroxylation of cholesterol. We will present biochemical analysis of De90B and its expression patterns in *Dioscorea* spp.

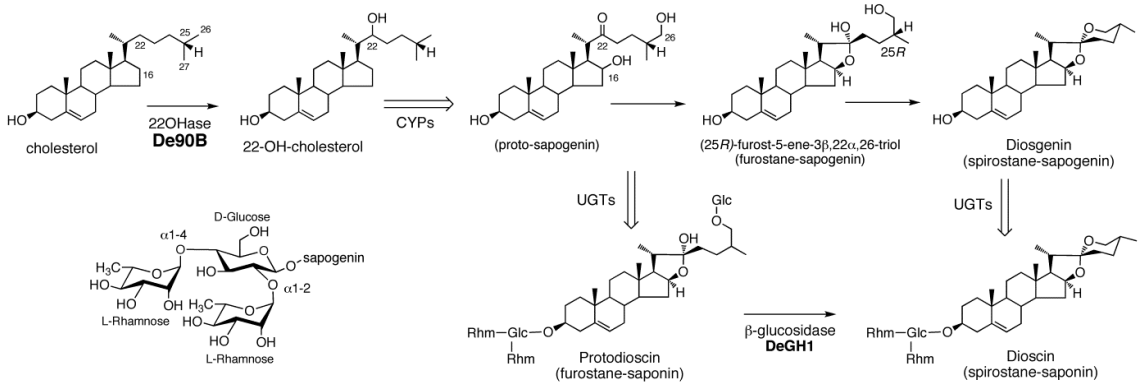


Figure. Putative biosynthetic pathways of steroidal saponins in *Dioscorea* spp.

1) Fujita, S., Ohnishi, T., Watanabe, B., Yokota, T., Takatsuto, S., Fujioka, S., Yoshida, S., Sakata, K., Mizutani, M., *Arabidopsis* CYP90B1 catalyzes the early C-22 hydroxylation of $C_{27}$, $C_{28}$, and $C_{29}$ sterols. *Plant J.* 45, 765-74 (2006)

## P75. Crucial role of the DBR2 promoter in the artemisinin biosynthetic pathway and development of a method for selection of high artemisinin yielding varieties

Ke Yang, Rashidi Sajad, Hongzhen Wang and Peter E. Brodelius

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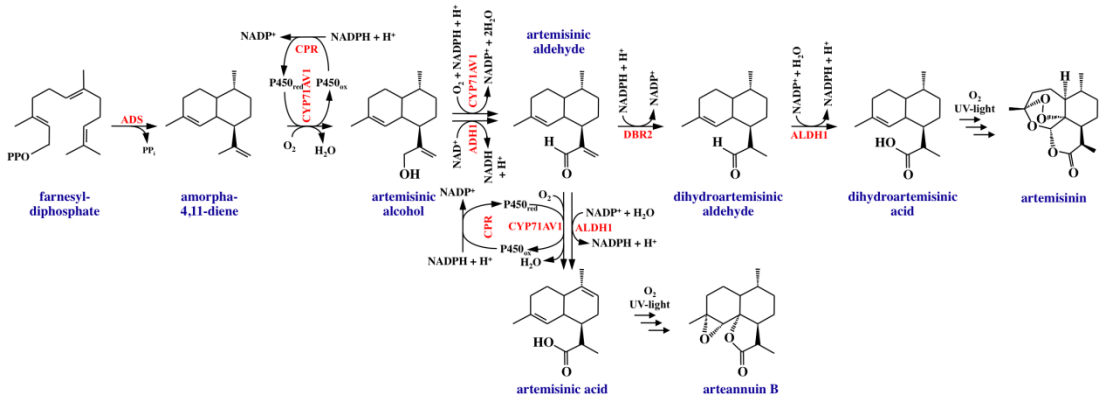
The artemisinic aldehyde double bond reductase (DBR2) gene, which expresses highly in glandular trichomes, takes up an important role in the biosynthetic pathway of the antimalarial sesquiterpenoid artemisinin in *Artemisia annua* L.. Artemisinic aldehyde is reduced to dihydroartemisinic aldehyde by DBR2, thus providing the intermediate for the final product artemisinin by competing with CYP71AV1, which oxidizes artemisinic aldehyde to artemisinic acid, the precursor of the non-antimalarial compound arteannuin B. In order to understand better if DBR2 expression level directs the artemisinic aldehyde flow for either artemisinin or arteannuin B accumulation, we first measured the dihydroartemisinic aldehyde, artemisinin, artemisinic acid and arteannuin B content of a number of *A. annua* cultivars by GC-MS. The cultivars used were classified into two major groups: the high artemisinin producer group (HAP), which includes the Anamed and ′2/39′ cultivars producing relatively high amounts of artemisinin and almost no arteannuin B; the low artemisinin producer group (LAP), which includes the ′Meise′ cultivar and a few cultivars collected in Iran producing relatively high amounts of arteannuin B and little or no artemisinin. We used quantitative PCR to determine the expression levels of DBR2 and other key genes (*i.e.* ADS, CYP71AV1 and ALDH1) of the artemisinin biosynthetic pathway during the flowering stage. Results suggest that the DBR2 expression level is significantly higher in the HAP group than in the LAP group. We cloned the promoters of the DBR2 gene from cultivars belonging to both groups and two different DBR2 promoters were found. After sequence comparison, two short fragments of approximately 100 and 400 bp, respectively, close to the ATG start codon are suggested to be crucial for the promoter activity. The relevance of promoter variation to DBR2 expression levels and artemisinin biosynthesis levels is discussed and a method to select high yielding cultivars will be presented.

**Figure 1.** Biosynthetic pathways for artemisinin and arteannuin B in *Artemisia annua*.



## P76. The bZIP Transcription Factor HY5 Modulates the Circadian Expression of the Monoterpene Synthase Gene *QH6*

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The *Artemisia annua*L. β-pinene synthase QH6 was previously determined to be circadian-regulated at the transcriptional level, showing a rhythmic fluctuation of steady-state transcript abundances. Here we isolated both the genomic sequence and upstream promoter region of *QH6*. Different regulatory elements, such as G-box (TGACACGTGGCA, -421 bp from the translation initiation site) which might have effects on rhythmic gene expression, were found. Using the yeast one-hybrid and electrophoretic mobility shift assay (EMSA), we confirmed that the bZIP transcription factor HY5 binds to this motif of *QH6*. Studies with promoter truncations before and after this motif suggested that this G-box was important for the diurnal fluctuation of the transgenic β-glucuronidase gene (*GUS*) transcript abundance in *Arabidopsis thaliana*. *GUS* gene driven by the promoter region immediately after G-box showed an arrhythmic expression in both light/dark (LD) and constant dark (DD) conditions, whereas the control with G-box retained its fluctuation in both LD and DD. We further transformed *A. thaliana* with the luciferase gene (*LUC*) driven by a 1400 bp fragment upstream *QH6* with its G-box intact or mutated, respectively. The luciferase activity assay showed that a peak in the early morning disappeared in the mutant. This suggested that other regulatory elements upstream G-box, such as a morning element at -537 bp from the translation initiation site, might function together with G-box to confer the rhythmic expression of this monoterpene synthase gene.

## P77. Farnezyl diphosphate synthase 3 (FPPS3) is responsible for the production of herbivore-induced terpene defenses.

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Volatile terpenes play an important role in the chemical defense of maize plants against a variety of biotic and abiotic stresses. Maize plants attacked by caterpillars release a mixture of mono- and sesquiterpenes which attract parasitic wasps that are specific enemies of the herbivores. In our effort to study the molecular base of these indirect defense mechanisms, we want to identify genes responsible for volatile terpene biosynthesis and its regulation.

The first step in the biosynthesis of volatile sesquiterpenes, the formation of farnesyl diphosphate (FPP), is catalyzed by prenyl transferases. In the maize genome, we found one previously characterized prenyl transferase (Li and Larkins, 1996; Cervantes-Cervantes *et al*. 2000) and two closely related genes. Heterologous expression in *E. coli* and subsequent biochemical characterization of the enzymes revealed that FPPS1 and FPPS3 produce FPP. The analysis of transcript levels indicated that the expression of these two *fpps* genes is induced by herbivory, although *fpps1* and *fpps2* are only marginally expressed. To find the FPP synthase responsible for sesquiterpene production *in planta*, we searched for quantitative trait loci (QTL) of volatile production that correspond to the FPPS locations. In a Nested Association Mapping (NAM) population screened for herbivore-induced volatile production, we identified a QTL closely related to FPPS3 that corresponded to the production of the major sesquiterpenes (*E*)-nerolidol, (*E*)-ß-farnesene and (*E*)-α-bergamotene. Within the NAM population, KY21 has a low emission of sesquiterpenes and contributes to the significance of this QTL. However, KY21 expresses the same allele of FPPS3 at a similar level but shows reduced expression of other terpene biosynthesis genes. This suggests that the QTL corresponds to a regulatory element that does not affect FPPS3 activity directly but rather other factors that regulate herbivore defense.

## P78. Early steps in the cardenolide pathway: Myth and ritual

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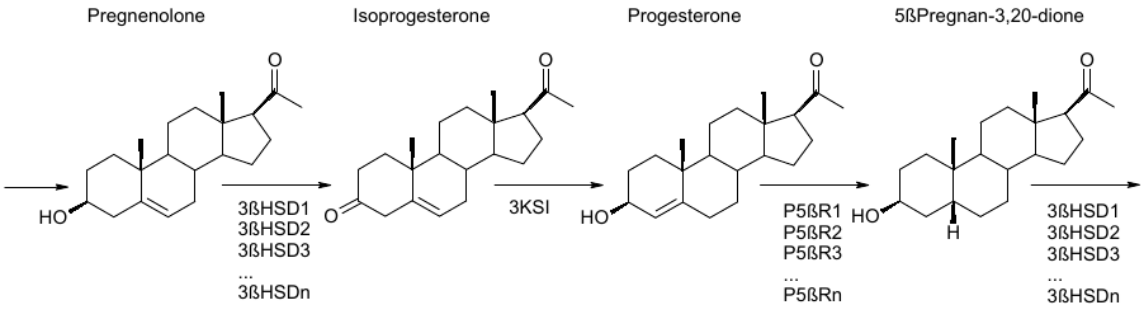
The conception of the existence of well-defined biosynthetic pathways is outdated. This holds particularly true for the formation of small natural products (SNAPs). The catalytic efficiencies described for enzymes involved in the formation of SNAPS are amazingly low. Many enzymes have been shown to catalyze not only their reported "natural" reaction but also alternative reactions. In several cases, substrate as well as product promiscuity has been demonstrated. Cardenolide biosynthesis is a good example to explain how paradigms have to be messed up. According to the text books, early steps in cardenolide biosynthesis are supposed to be catalysed by 3β-hydroxysteroid dehydrogenase (3βHSD) and progesterone 5β-reductases (P5βR). When analysing 3βHSDs and P5βRs genes and enzymes in the genera *Digitalis*, *Erysimum* and *Arabidopsis* it became clear that small gene families exist that encode for 3βHSDs or P5βRs. Some of these genes can be induced by stress. All enzymes are substrate-promiscuous enzymes and are involved probably in more biosynthetic reactions than thought before. Several catalytically active 3βHSDs were identified in *Erysimum* and in *Arabidopsis.* All of them catalyse the dehydrogenation or reduction of various $C_{17}$, $C_{19}$ and $C_{21}$ steroids. The enzymes also possess steroid 17β-dehydrogenase activity but no Δ5-3-ketosteroid isomerase (3-KSI) activity as described for animal 3βHSDs. A discrete Δ5- 3-ketosteroid isomerase was isolated from *Digitalis lanata.* Several individual P5βRs have been demonstrated to occur in *Digitalis, Erysimum, Arabidopsis* and other genera. They all possess relaxed substrate specificities and reduce C=C double bond of various large and small 1,4-enones. Cardenolide-biosynthetic enzymes may also have other functions or are operative in other biosynthetic pathways. This has recently been demonstrated in iridoid formation.




The cardenolide pathway revisited

## P79. HPS mutant library

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Henbane premnaspirodiene synthase (HPS) utilizes farnesyl diphosphate (FPP) and produces premnaspirodiene (~93% of total hydrocarbons produced) as a major product and variety of minor products. A closely related terpene synthase, tobacco 5-epi-aristolochene synthase (TEAS), is 75% identical to HPS, and produces 5-epi-aristolochene as a major product (~83% of total hydrocarbons produced) from FPP. Surrounding the enzyme active sites of these terpene synthases, 9 amino acid residues differ. An HPS mutant library, based on a combinatorial set of mutations of these nine positions encoding either HPS or TEAS residues was constructed using a new and efficient digestion/ligation method together with small DNA duplexes encoding the mutant sites. In total $2^9 = 512$ mutants were assembled and verified by whole gene sequencing. After expression of the encoded HPS mutants, each mutant was characterized to ascertain their product profiles, thermostability/solubility and kinetic properties. The results of this survey in relationship to protein epistasis and alternative product engineering will be presented and contrasted with a previous library of complementary mutants in the TEAS enzyme background.

## P80. Elucidating the transport of intermediates in vinblastine biosynthesis

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The medicinal plant *Catharanthus roseus* (Madagascan periwinkle) is currently the only commercial source of the anti-cancer microtubule inhibitors vincristine and vinblastine, two bisindole alkaloids derived from monoterpenoid indole alkaloid biosynthesis. The metabolic pathway to these two products is notable for the distinct inter-cellular and subcellular compartmentation of its biosynthetic enzymes, with a requirement for at least three cell types, namely the internal phloem and parenchyma cells, the epidermis and the laticifers, and multiple organelles; the chloroplast, the vacuole, nucleus and cytosol. The distinct localization of the biosynthetic enzymes in this pathway strongly suggests a need for transportation of intermediates both between cell types and between organelles.

Utilizing the sequenced *C.roseus* transcriptomic database, we have designed an approach to identify putative transporter protein candidates that may be involved in shuttling biosynthetic intermediates of the vinblastine pathway across membranes. This has been coupled with both virus-induced gene silencing (VIGs) in *C.roseus* and subcellular localization to demonstrate whether these transporters have a physiological role *in planta*, as well as over expression of the transport proteins in yeast to test the proteins ability to transport the biosynthetic intermediates *in vitro*.

## P81. Extracellular localization of the diterpene sclareol in *Salvia sclarea* (*Lamiaceae*)

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Sclareol is a labdane diterpene produced by solid/liquid extraction of clary sage (*Salvia sclarea* L.) inflorescences. This natural compound is used for hemisynthesis of (+/-)-norlabdane oxide, a chemical, replacing ambergris in perfumes. Because processes of excretion and accumulation of sclareol are unknown, the aim of this work was to gain knowledge on its sites of accumulation in flowers. Samples were collected in growers' fields or during different steps of the industrial process of steam-distillation and solid/liquid extraction. Samples were then analysed with a combination of complementary analytical techniques of chromatography and microscopy (gas chromatography coupled with mass spectrometry, environmental electron microscopy, second harmonic generation microscopy, polarized microscopy). According to the literature, it was hypothesized that sclareol is localized in oil pockets of secretory trichomes. This study demonstrates that this is not the case and that sclareol accumulates in a crystalline epicuticular form, mostly on calyces. This work opens questions on the biosynthesis pathway compartmentalization and the excretion process of sclareol in clary sage.

## P82. Iridoid synthase - a reductase repurposed as terpene cyclase

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Iridoids comprise a large family of cyclic monoterpenes with diverse agricultural and medicinal applications. However, the identity of the enzyme that generates the characteristic bicyclic iridoid core has hitherto remained obscure. In this poster, we present the discovery of iridoid synthase, a cyclase that generates the iridoid skeleton by a multi-stage reductive mechanism distinct from any known terpene cyclase. Iridoid synthase from the medicinal plant *Catharanthus roseus* has sequence similarity to progesterone-5β-reductases and was identified from transcriptomic data by co-expression analysis. The *in vivo* role of the enzyme was confirmed using virus-induced gene silencing and was further supported by cellular and subcellular localization studies. Insights into the evolution of this reductive cyclization emerged from substrate specificity studies and from the discovery and characterization of an analogous cyclization reaction carried out by a non-related fungal reductase. The identification of iridoid synthase highlights the power of unbiased bioinformatic strategies to mine the extraordinary chemistry harbored within plants.

## P83. Implication carotenoids and volatile terpenoids in the response of Star Ruby grapefruit to cold stress

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Storage at low temperatures is one of the most widely used technologies to maintain citrus fruit quality during their postharvest life. However, fruits of some species, as Star Ruby (SR) grapefruit, are extremely sensitive to cold, developing chilling injury (CI) symptoms in the peel. It is widely described that plant tissues synthetize volatile terpenoids in response to abiotic and biotic stresses, being recognized as an 'indirect defense' mechanism. On the other hand, carotenoids, a broad family of C40 isoprenoids, have been also recognized by their important antioxidant activity, being involved in photoprotection during photosynthesis, and in other oxidative stresses. With the aim to characterize the involvement of volatile terpenoids and carotenoids (the main pigments in the peel and pulp of grapefruit) in the response of SR grapefruit to cold stress, fruit were stored for up to 8 weeks at 2ºC (cold stress temperature) and 12ºC (non-stress temperature), evaluating the development of CI in relation to changes in carotenoid composition, and volatiles emission. Since CI has been widely associated with oxidative stress cell damage, the potential protective role of lycopene by its powerful antioxidant properties was challenged in this work. SR fruits showed two zones/areas well differentiated in color and with different susceptibility to CI: yellow areas which developed CI symptoms while red areas remained undamaged after 8 weeks of storage. Moreover, total carotenoids content in flavedo of red areas were more than twice respect to yellow areas, and lycopene and β-carotene were 10- and 3-times higher, respectively. During the storage period, at least 32 different volatiles were detected in SR fruit stored at 12ºC, but only 17 compounds were recorded in cold-stressed fruit. Under cold stress, monoterpenes emission increased contrary to sesquiterpenes which sharply decreased. Moreover, β-myrcene, β-ocimene, limonene oxide and linalool were only detected in cold stored fruit, and their appearance was coincident with the development of CI symptoms. A remarkable increment in the emission of the monoterpene limonene was registered in cold stressed fruit, although this change was not associated with CI. The emission of the sesquiterpenes nootkatone, *trans* cariophyllene, valencene and γ-selinene was substantially affected by cold stress, being reduced more than 5-times respect to non-stressed fruit. Overall, results suggest that carotenoids, mainly lycopene, could exert a protective role preventing development of CI in Star Ruby grapefruit and that the increment in the emission of specific monoterpenes appears to be a response to cold stress.

## P84. Biosynthesis of the all-round Cancer Drug candidate Thapsigargin: The first steps

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Thapsigargin is a guaianolide only found in two members of the genus *Thapsia* (Apiaceae) [1]. Covalently linked to vasculature specific peptides, thapsigargin has been converted into a efficiently targeted prodrug for the treatment of solid tumors [2]. A peptidase specific only to cancer-induced vasculature cleaves the peptide, thus unmasking the drug. The activated drug kills cancer induced vasculature development, thereby effectively strangling the tumor. The candidate marvels in being an all-round candidate applicable for many types of cancer. However, since neither wild living sources, plant cultivation or chemical synthesis can fulfill future market demands, it is necessary to develop alternative production methods, such as heterologous production.

We are investigating the enzymes involved in thapsigargin biosynthesis. The first step is a terpene synthase converting farnesyl pyrophosphate into a sesquiterpene backbone.
Next generation sequencing data, RNA-seq, enabled us to obtain two sesquiterpene synthases, TgSTS1 and TgSTS2. We have shown that TgSTS1 catalyzes d-cadinene synthesis and TgSTS2 mainly catalyzes the formation of the unstable germacrenol derivative Kunzeaol. This is thought to be an intermediate in thapsigargin biosynthesis [3]. The specificity, kinetics and promiscuity of TgSTS2 is currently being investigated further. We study the kinetics and product formation in relationship to pH, temperature, and the divalent ions involved. Ideally, such a comprehensive study of this enzyme will provide insight about how the activity and product profile can be manipulated in order to enhance the biotechnological production of Thapsigargin[4, 5]. Highly active or more specific enzymes is a key feature for any future heterologous production.

5 mutants that differ in active site residues were created by site directed mutagenesis have been created. Each mutant represents a step in the transformation of TgSTS1 towards TgSTS2, and preliminary analysis shows that the product profile from these mutants likewise shift towards that of TgSTS2. As described above, further characterization of these mutants will provide valuable information about active site residues, cofactors and the effect of manipulating abiotic factors.

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## P85. Substrate specificity and products of four sesquiterpene synthases from basil glandular trichomes.

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Four members of the *Tps* gene family previously isolated from sweet basil glandular trichomes were further examined. The genes display no apparent transit peptide and were functionally identified as sesquiterpene synthases previously by expression in *E. coli*. They included selinene synthase (SES), *α*-zingiberene synthase (ZIS), *γ*-cadinene synthase (CDS), and germacrene D synthase (GDS), named after the major compounds produced from FPP *in vitro*. *α*-Zingiberene synthase, also possess monoterpene synthase activity if GPP is provided. Here we report on the terpene synthase activities of these genes and the products formed when either farnesyldiphosphate (FPP) or geranyldiphosphate (GPP) were offered as substrates. As previously noted, all gene products produced sesquiterpenes from FPP (Fig. 1 top panels) but SES and ZIS also possess monoterpene synthase activity producing distinct monoterpenes from GPP (Fig. 1, bottom panels). 3D models will be utilized in attempts to understand substrate specificity and product formation of these enzymes.

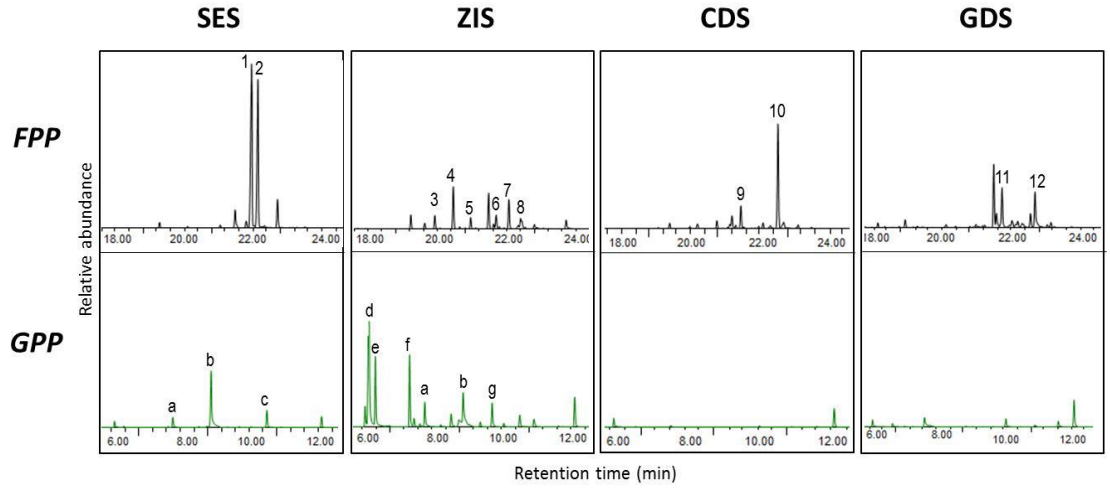


Figure 1. Identification of the products of the four basil sesquiterpene synthases: selinene synthase (SES), α-zingiberene synthase (ZIS), γ-cadinene synthase (CDS), and germacrene D synthase (GDS), SPME-Gas chromatographic separation of products synthesized from exogenously supplied FPP (top panels) or GPP (bottom panels) by *E. coli* cell-lysates overexpresssing the corresponding genes. *Sesquiterpenes*: Peak 1, *α*-selinene; 2, *β*-selinene; 3, *α*-*Z*-bergamotene; 4, *α*-*E*-bergamotene; 5, *β*-farnesene; 6, curcumene, 7, *α*-zingiberene; 8, *β*-bisabolene; 9, *γ*-cadinene; 10, *α*-cadinene; 11, germacrene D; 12, *δ*-cadinene. *Monoterpenes*: Peak a, myrcene; b, limonene; c, terpinolene; d, *α*-thujene; e, *α*-pinene; f, sabinene; g, *γ*-terpinene.

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## P86. The natural genetic variation of bornyl diphosphate synthase in Sage (*Salvia officinalis* L., Lamiaceae)

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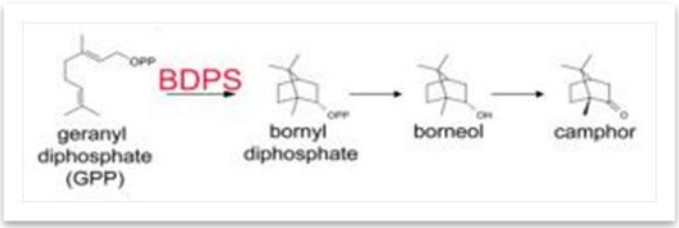
In sage (Salvia officinalis L., Lamiaceae) bornyl diphosphate synthase (BDPS) is the monoterpene synthase responsible for the first step in the biosynthesis of camphor and borneol, two of the major monoterpenes in sage.[1] Here we present results on the natural genetic variation of BDPS of 79 individual sage plants coming from populations of different West Balkan countries by a 454 sequencing approach.




The gene consists of 7 exons ranging from 142 to 407bp and 6 introns ranging from 81 to 431bp. The mean number of variations (Indels + SNPs) not differ between exons (3.3 per 100 bp) and introns (3.4 per 100 bp), but there was a remarkable difference between gene regions. While exons 1 and 6 were quite well conserved with around 1 mutation per 100bp, exons 2 and 7 were highly variable with up to 5.7 mutations per 100bp. The introns 1 and 6 were quite conserved with 1 and 1.2 variations per 100bp, respectively, while introns 3 and 5 were extremely variable with 4.6 and 7.8 variations per 100bp, respectively.

The majority of mutations affecting the coding sequence (73%) were non-synonymous leading to frame-shifts, amino acid changes or truncations.

The functional impact of the high natural genetic variation of BDPS may significantly contribute to the high degree of natural variability (chemotypes) in sage.

Acknowledgements: This work was supported by SEE-ERA.NET (grant no. SEE-ERAPLUS-064).

Reference: 1. Wise ML, Savage TJ, Katahira E, Croteau R (1998): Monoterpene synthases from common sage (Salvia officinalis). cDna isolation, characterization, and functional expression of (+)-sabinene synthase, 1,8-cineole synthase, and (+)-bornyl diphosphate synthase. J Biol Chem 273:14891-14899.

## P87. Structure-function evolution of a sesquiterpene synthase family in the *Nicotiana* genus

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5-epi-aristolchene synthase (EAS) is a member of a simple three-enzyme sesquiterpene biosynthetic pathway that produces the phytoalexin, capsidiol. Here we investigate the natural variation of EAS-like enzymes, given their wide-spread occurrence across the ecologically diverse *Solanaceae* family, in an in-depth examination of functional diversification of an enzyme family critical to pathogen resistance. This provides a starting point to understand how mutational variation affects fundamental properties associated with biological catalysis. Initially, concentrating on one genus, EAS-like genes were cloned, sequenced, enzyme expressed and then biochemically characterized from several species of *Nicotiana*. Characterization focused on three fundamental properties of enzymes, namely thermostability, kinetic properties, and product identity & diversity. From this survey, two EAS-like enzyme variants had distinct product profiles when compared to the relatively well-characterized *N. tabacum* EAS (Figure 1). A mutant library for this set of EAS-like species variants was constructed for specific residue differences surrounding the active side in otherwise highly similar sequences (~94% identity). These mutants were examined for residue-dependent catalytic effects and as a result, an unusual active site residue present in *N. tomentosa*, His404, was identified to confer EAS-like enzymes the ability to produce selinenes. The presence of a general base at this position correlates with the putative reaction mechanisms of EAS-like enzymes and the ability of a general base to shortcut the reaction trajectory to produce selinenes. Ultimately, shifts in product profiles within the *Nicotiana* genus and between neighboring genera in the larger family of *Solanaceae* may serve as the raw material for ongoing positive selection in a pathway thought to produce hydrocarbons through the eudesmyl cation.

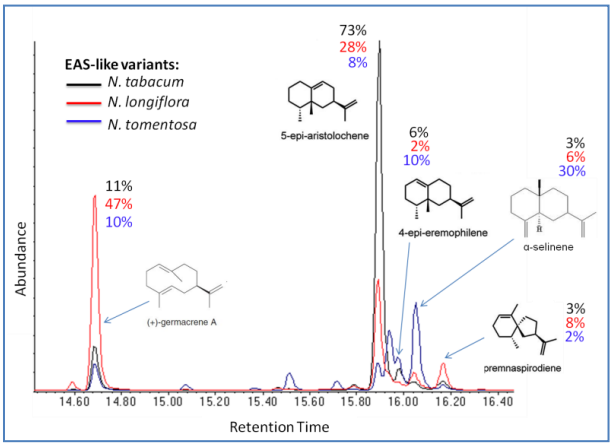


**Figure 1: GC-MS trace of product profiles from 3 EAS-like species variants**

## P88. Probing the substrate specificity of tomato santalene/bergamotene and tobacco cis-abienol synthases

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The wild tomato *Solanumhabrochaites* produces a mixture of sesquiterpenes and sesquiterpene acids which confer higher resistance against herbivores and pathogens. The biosynthesis of these compounds proceeds via the action of two enzymes which are exclusively expressed in the glandular trichomes. The enzyme zFPS (*Z,Z*-farnesyldiphosphate synthase) uses isopentenyldiphosphate (IPP) and dimethylallyldiphosphate (DMAPP) to form *Z,Z*-farnesyldiphosphate (*Z,Z*-FPP), an uncommon precursor for sesquiterpenes. ShSBS (Santalene and Bergamotene synthase), the second enzyme, generates several products using *Z,Z*-FPP as substrate, including (+)-α-santalene or (+)-endo-β-bergamotene. In tobacco (*Nicotianatabacum*) a diterpene synthase called NtABS (abienol synthase), which shares a high sequence similarity with ShSBS is also specifically expressed in glandular trichomes. Despite the fact that ShSBS is a sesquiterpene synthase, it belongs to the same class of terpene synthases, the kaurene synthase like family, which comprises mainly diterpene synthases. NtABS uses 8-hydroxy-copalyl diphosphate (8-OH-CPP) as substrate to produce only one product, *cis*-abienol. The formation of the precursor 8-OH-CPP is catalyzed by NtCPS2 (8-hydroxy-copalyl diphosphate synthase) using the precursor for diterpenes, geranylgeranyldiphosphate (GGPP). The high similarity between NtABS and ShSBS allows us to study structure-function relationships on substrate specificity. 3D structural models of ShSBS and NtABS were established using the published structure of taxadiene synthase. Based on these models, several amino acid residues were identified as potentially critical for substrate specificity. Reciprocal mutations were introduced in NtABS and ShSBS. We will describe our progress with the results of several enzyme assays of wild type and mutated enzymes with different isoprenyl precursors.

## P89. Recombinant production of plant cytochrome P450 enzymes by forisomes

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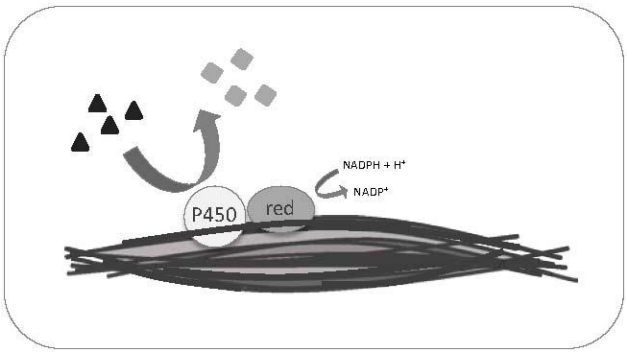
The production of functionalized terpenoids is of major interest for the pharmaceutical and chemical industry. Cytochrome P450 monooxygenases offer interesting new routes for terpenoid engineering by site-specific hydroxylation, epoxidation and dehydrogenation. However, their application in biotransformation processes is limited due to low enzyme stability and the absence of efficient expression systems for recombinant cytochrome P450 production.

Here we present the production and purification of recombinant cytochrome P450 enzymes from *Saccharomyces cerevisiae* by using a newly developed forisome technology. Forisomes are multiprotein complexes, which are involved in rapid wound sealing of the phloem in Fabaceae. By a yet unknown process, a large number of forisome subunits undergo self-assembly to form a micron-sized protein complex even when recombinant tags are translationally fused to their amino- or carboxyterminus. We utilized this capacity to generate recombinant forisomes carrying cytochrome P450s. Another major advantage of this system is the possibility to also bring other auxiliary proteins such as reductases in close proximity to the cytochrome P450 by co-expression.
The poster will highlight potential advantages of the forisome-based expression technology compared to conventional purification tags such as glutathione S-transferase with respect to quality and quantity of recombinant cytochrome P450s.



## P90. Thermo-oxidation of short-chain polyisoprenoid alcohols

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Polyisoprenoid alcohols consist of isoprene residues with a hydroxy group at one end (α-residue) and a hydrogen atom at the ω-end. These alcohols due to their molecular structure are prone to oxidation. Short polyisoprenoids and their primary oxidation products are used for the synthesis of novel compounds such new flavours, fragrances, or therapeutically active substances. Most likely oxidation of polyisoprenoids occurs in living organisms due to the initiators of oxidation, such as reactive oxygen species or ions of transition the metals etc., naturally present in the cells. Primary and secondary oxidation products of polyisoprenoids can serve as oxidation markers, or play role in cell signaling as other important oxidized lipids.

In this study short-chain isoprenoid alcohols (β-citronellol, geraniol, nerol, farnesol) were subjected to the thermo-oxidation at 50ºC and 80ºC. Hydroperoxides were measured using spectrophotometric colorimetric ferrous thiocyanate method; moreover formation of primary and secondary oxidation products was monitored by GC-FID and RP-HPLC/UV techniques. Thermo-oxidation led to decomposition of isoprenoids with simultaneous formation of hydroperoxides, followed by decomposition of the later and formation of secondary oxidation products (e.g. epoxides). The highest amount of hydroperoxides (over 220 mmol/mol of the substrate) was found after 7 days of oxidation of β-citronellol at 50ºC. Profiles of oxidation products were influenced by the chain length, number of double bonds and *cis*/*trans* configuration of the substrates.

## P91. Isolation of terpenoid and flavonoid biosynthetic genes from Fenugreek

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Collectively plants synthesize a diverse array of "Plant Natural Products" (PNPs) either as part of normal growth and development or in response to stress. PNPs represent a vast resource of complex molecules that are exploited by man for their pharmacological and other properties. Fenugreek (*Trigonella foenum-graecum* L.) is an unexploited leguminous plant with a rich PNP repertoire including terpenoids and flavonoids. Here we present the isolation of three flavonoid biosynthetic genes (CHS, IFS, VR) and one terpene synthase (oxidosqualene cyclase) gene from Fenugreek. Furthermore we have investigated the biosynthesis and involvement of the phytoalexin Medicarpin (M), in a general metal and metalloid detoxification mechanism. Our results show that, depending on copper concentrations, M was formed either both *de novo* synthesis or at the expense of its malonyl glucoside. The increases of PAL activity as well as the accumulation of CHS and VR specific transcripts are consistent with the above results. Elicitation of seedlings with three metals (copper, cadmium and aluminum) and one metalloid (selenium) significantly induced an increase in the expression of M biosynthetic genes and a concomitant increase of M and citrate exudation in the culture medium. Taken together, along with the moderate effects of the elicitors on root growth and the plasma membrane integrity, our results indicate that M as and citrate exudation may participate, as part of a non-element-specific resistance mechanism, in metal and Se detoxification.

## P92. A biocatalytic approach towards artificial terpenoid skeletons

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Terpene synthases convert prenyldiphosphates (e.g. GPP, FPP, GGPP) into a vast assortment of cyclic and acyclic compounds referred to as terpenes. Functionalization, e.g. by oxidation, results in a huge collection of compounds with very different and complex physiological properties. Thus terpenes find many applications in food, flavor, fragrance, pharmaceutical and other industries. The introduction of non-native functionalities can expand terpenoid chemical space even further. However, most classical chemical approaches lack the specificity and selectivity of enzyme catalyzed reactions and cannot modify the core structure of a given terpene scaffold, while biochemical production is mainly limited to the few natural prenyldiphosphate precursors.

To introduce new functionalities into the core terpene scaffold, we decided to utilize the prenyl converting enzymes in combination with non-natural organo-diphosphates. Thus we explored the ability of two terpene synthases, namely limonene synthase from *Cannabis sativa* (CsTPS1) and 5-*epi*-aristolochene synthase (TEAS) from *Nicotianatabaccum*, to convert a set of unusual linear alkenyldiphosphates. It could be shown, that both enzymes exhibit a remarkable promiscuity towards these new prenyldiphosphates resulting in a set of new and partially unexpected cyclic products.

## P93. The role of GA production by bacteria on plant-microbe interactions

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Gibberellin (GA) servesas an important phytohormone,playing a significant role in regulation of a wide range of growth and development processes in higher plants. In addition, GA has been found to negatively regulate basal defense against microbes. Remarkably, GA is produced not only by plants but also by plant-associated fungi and bacteria – for example, the soybean nodulating*Bradyrhizobiumjaponicum*. Our grouphas previously characterized genes from *B. japonicum* that encode the *ent*-copalyldiphosphate synthase (*CPS*) and *ent*-kaurene synthase (*KS*) expected for gibberellin production, and which define a putative GA biosynthetic operon. Interestingly, homologous operons are not only found in rhizobacteria, but also inthe rice bacterial leaf streak pathogen *Xanthomonasoryzae* pv. oryzicola(*Xoc*). Here we report biochemical characterization of the diterpene synthases from this phytopathogen, which also encode CPS and KS enzymes. Moreover, we have generated knock-out strains for both *CPS* and *KS*, and found that these exhibit reduced virulence (lesion lengths and number of bacteria), which can be restored by complementation with the relevant gene. Thus, bacterial production of GA seems to plays an important role in regulating their association withplant hosts.

## P94. Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family

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*Catharanthus roseus* or Madagascar periwinkle is the source for about 130 terpene indole alkaloids (TIAs) with a broad range of biological activities. Among these, the complex vinblastine molecule is used for various cancer chemotherapies and still considered as a lead for further drug development. Its biosynthesis is thought to result from the action of around 30 coordinately regulated enzymes in different tissues and subcellular compartments. The first step of the pathway is geraniol 8-hydroxylation (commonly misnamed geraniol 10-hydroxylation) which is a prime target for metabolic engineering of the TIA pathway. Geraniol hydroxylation was previously reported to be catalyzed by cytochrome P450 enzymes namely CYP76B6 from *Catharanthus roseus* (Collu et al., 2001) and CYP76C1 from *Arabidopsis thaliana* (Otah and Mizutani, 1998). We thus reinvestigated geraniol hydroxylase activity of a set of CYP76s from different plants in order to identify the most suitable candidate for engineering purpose and optimal production of 8-hydroxygeraniol. We identified CYP76C4 as an active geraniol 9- or 8-hydroxylase. CYP76B6 emerged as a very specialized multifunctional enzyme catalyzing with high efficiency the two sequential oxidation steps leading to the regiospecific formation of 8-oxogeraniol. CYP76B6 dual function was confirmed in *Nicotiana benthamiana* using a leaf-disc assay developed to the end. Homology modeling of CYP76C4 and CYP76B6 provides a tentative explanation to their respective regioselectivities.

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## P95. Emissions of volatile organic compounds from a boreal humic lake

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The aquatic sources of volatile organic compounds (VOCs) into atmosphere have been mainly studied in marine environments. Marine phytoplankton is known to produce volatile compounds, including monoterpenes and dimethyl sulphide, which are very effective contributors to atmospheric particle formation in marine environments. Freshwater lakes comprise large inland areas in boreal regions, but their potential contribution to atmospherically reactive compounds has not been analysed. We conducted the emission spectrum and emission rate measurements from a humic southern Finnish lake during late-summer periods in the years 2011 and 2012.

We measured the emissions of terpenoids and short-chained carbohydrates on the Lake Kuivajärvi, in southern Finland. Measurements were conducted with a floating flow-through chamber. Samples were taken from air entering and leaving enclosure to adsorbent tubes (Tenax TA-Carbopack B) or to a sampling canister (short-chained carbohydrates). Samplings were conducted three times per day; morning, afternoon and midnight. The emission measurements were combined with those of water temperature, light penetration, chlorophyll, CO2 and O2 concentrations, and phytoplankton populations. Terpenoid analyses were performed at the Finnish Meteorological Institute with TD-GC-MS, while short-chained carbohydrates in canisters were analysed with GC-FID. Phytoplankton samples were analysed later in the laboratory under microscope. Phytobiomass was analysed using chlorophyll determination with a spectrophotometer.

The VOC emissions from a humic lake were rather high, up to 9 μg m-2 h-1, which is comparable to emissions from the boreal forest soil and belowground vegetation. Fluxes consisted mainly on terpenoids, whereas the short-chained carbohydrate emissions were very low. The three most abundant compounds in emissions were monoterpenes α-pinene, Δ3-carene and β-pinene, abundant also in forest VOC fluxes. Several sesquiterpenes (most abundantly β-caryophyllene) were also seen in the emissions, although generally their emission rates were very low.

Terpenoid fluxes showed clear diurnal variation, emissions being the highest at nights and very small during days. Since the emission behaviour was opposite to terrestrial VOC emissions, which peak in the afternoon, they were most probably connected to biological or physico-chemical processes in the lake.

Our next step is to analyse the emission patterns in more detail, and especially study the seasonal variability in emission patterns starting from ice-melt in early spring to late autumn when the lake freezes over. We also aim at connecting the emissions with lake biological activity and other variables, i.e. CO2 fluxes, phytoplankton speciation and levels of dissolved organic matter.

## P96. Isoprene & Co.- Biological and ecological functions in poplar

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Plants synthesize and emit a large variety of volatile organic compounds, with terpenes and fatty-acid derivatives being the dominant classes. Whereas some volatiles are probably common to almost all plants (e.g. C6 aldehydes, alcohols, and esters, as well as acetaldehyde and methanol), others are specific to only a few related taxa. For instance, isoprene emission is a characteristic of trees such as oaks, willows and poplars. Depending on the atmospheric composition, isoprene can severely impact air quality and regional climate. For the plant itself, isoprene can enhance stress tolerance and also interfere with the attraction of herbivores and parasitoids. The presentation summarizes our current knowledge on isoprene functioning in poplar and gives an overview on the manifold molecular/biochemical changes that the removal of such a small molecule causes in poplar.

Moreover, the presentation addresses the question of whether non-isoprene emitting poplars are a chance to reduce the impact of poplar plantation on the atmosphere. In a 2-year field trial the growth performance and fitness of non-isoprene emitting poplars were tested under outdoor conditions. Neither the growth nor biomass yield of the isoprene free poplars were impaired and they were even temporarily enhanced compared with control poplars. Modelling the annual carbon balances revealed a reduced C loss of 2.2 % of the total gross primary production by absence of isoprene emission, and a 6.9 % enhanced net growth of the isoprene free poplars. However, the suppressionof isoprene biosynthesis resulted in reduced susceptibility to fungal infection whereas the attractiveness for herbivores was enhanced.

Overall the present study promises potential for the use of non- or low-isoprene-emitting poplars for more sustainable and environmentally friendly biomass production. However, the new pheno(chemo)type might reserve some surprise when tested on long-term experiments under natural condition as we are currently investigating in the contrasting climates of Oregon and Arizona.

## P97. Sesquiterpene profiles and sequence analysis of terpene synthases as used to disclose the phylogenetic ancestry of hybrid species in Asteraceae – A case study with *Helianthus x multiflorus* L.

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Hybridization is a common and frequently occurring mode of speciation in plants. In the genus *Helianthus* (Asteraceae), numerous taxa of variant ploidy levels and putative hybrid origin were recognized and interspecific crossing is an important tool in breeding of commercial sunflower. The natural triploid *H.* x *multiflorus* was assumed to have originated from hybridization of the diploid *H. annuus* with a tetraploid *H. decapetalus* [1]. However, comparison of the STL profiles from glandular trichomes with putative parents indicated that crossing of *H. annuus* with a diploid taxon of *H. decapetalus* was much more likely to explain the ancestry of the hybrid [2].

The identification of key enzymes in the STL biosynthesis of *H. annuus* now provided the possibility to compare sequences of homologous enzymes in related taxa. PCR-based amplification of the partial terpene synthase gene HaGAS1 revealed a significant length polymorphism between the diploid and the tertraploid *H. decapetalus* and the presence of two different allels of this gene in *H.* x *multiflorus*. Sequence alignment of the amplicons showed 98 % identity of one allel with *H. annuus* cv. HA300 [3] and 100 % identity of the other allel with the diploid *H. decapetalus*. This corroborates the previous results of STL profiling. Moreover, a deletion of 46bp (divided by 5bp) in the GAS1 sequence of the diploid *H. decapetalus* indicates that the origin of *H.* x *multiflorus* occurred prior to the polyploidization of *H. decapetalus* or that an additional hybridization was involved in the ancestry of this tetraploid taxon. Sequence comparison has shown to provide new insights in the phylogeny of enzymes responsible for a step in the STL biosynthesis of *Helianthus* and possibly of other Asteraceae. In future investigations, this may help to give insights in the evolution of this pathway and may explain the development of the unusual high diversity of STL.

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## P98. Terpenoid-mediated herbivore resistance in tomato

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Herbivorous insects pose serious problems in agricultural production areas. On commercial tomato, an important crop worldwide, whiteflies and spider mites are major pests. In general, wild tomatoes are more resistant to pests than cultivated tomatoes due to an elevated or qualitatively different production of an array of defense compounds. These defence metabolites are mainly produced in specialised glandular hairs (trichomes), biochemical factories on the surface of green tissues but absent from ripe tomato fruit.

Tomato breeding has been tremendously efficient in increasing fruit quality and quantity but also led to considerable loss of natural resistance. The biosynthetic pathway for the production of 7-epizingiberene in a wild tomato was re-introduced into a cultivated greenhouse variety with the aim to restore herbivore resistance. 7-Epizingiberene is a specific sesquiterpene with toxic and repellent properties that is produced and stored in glandular trichomes. We identified 7-epizingiberene synthase (ShZIS) that belongs to a new class of sesquiterpene synthases, exclusively using *Z*-*Z*-farnesyl-diphosphate (zFPP) in plastids, probably arisen through neo-functionalization of a common ancestor. Expression of the ShZIS and zFPP synthases in the glandular trichomes of cultivated tomato resulted in the production of 7-epizingiberene. These tomatoes had gained resistance to several herbivores that are pests of tomato. Hence, re-introduction of a sesquiterpene biosynthetic pathway into cultivated tomatoes restored resistance lost during breeding**.**

## P99. Fluctuating springtime photosynthesis recovery drives Scots pine to monoterpene burst mode

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In boreal regions, both the atmospheric new particle formation events and emission potential of terpenoids acting as aerosol precursors are peaking in springtime. In Finland, springtime is characterized by rather fast shift from cold winter to moderate summer conditions within a few weeks. Extreme differences between daily maximum and minimum temperatures are common, as well as large fluctuations in daily mean temperatures. Furthermore, during spring months, irradiance increases substantially and snow melt takes place. The full release of dormancy takes place in stages at about the same time when daily mean temperatures rise above zero.

We measured the gas exchange and VOC emissions of a boreal Scots pine (Pinus sylvestris L.) shoots using an automated gas exchange measurement system, based on dynamic enclosures, coupled with PTR-QMS (proton transfer reaction – quadruple mass spectrometer, Ionicon Analytik GmbH, Austria). The measurements were conducted during years 2009, 2010 and 2012 in Hyytiälä SMEAR II station (Station for Measuring forest Ecosystem – Atmosphere Relations), southern Finland.

Our results showed several types of monoterpene emission burst events (MEB events) every spring, typically in April. The MEB events continued from a couple of hours to over one week. The events were characterized by extremely high monoterpene emission potential, typically one order of magnitude higher when compared to normal springtime emission potential. When ambient conditions and other possible driving factors were investigated, no evident causes or correlations were found. However, the MEB events coincided with the early stages of photosynthesis recovery, i.e. they occurred at the time when the efficiency to use the prevailing photosynthetically active radiation was about 10-40 % when compared to summertime maximum. However, the MEB events didn't appear directly after any fixed photosynthesis recovery stage, which indicates that enhanced monoterpene emissions are not a built-in part of springtime photosynthesis recovery. Instead it seems that the MEB events were preceded by either an extremely rapid period of photosynthesis recovery due to sudden rise in temperature, or – more commonly – temporary photosynthesis downregulation, caused by a transient colder period combined with high PAR levels, followed by a warmer period. Either way, the fluctuation in photosynthesis recovery was a prerequisite for a MEB event. It also seems that, besides of the fluctuation in photosynthesis recovery, the final launch of a MEB event requires extreme transient ambient conditions such as substantial temperature changes between nighttime minimum and daytime maximum, or high PAR levels.

Although the physiological mechanisms behind the MEB events remain unexplained, they evidently are connected to photosynthesis recovery, or to the extreme changes stressing the trees in the middle of recovery process. Our findings partially explain the high springtime terpenoid emission potential, and thus also shed light on the causes for springtime peak in atmospheric new particle formation events.

## P100. Short-chain prenyltransferases involved in herbivore-induced terpene formation in western balsam poplar (*Populus trichocarpa*)

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Plant metabolites provide protection against herbivores not only by acting as toxins and feeding deterrents, but also by serving as signals for herbivore enemies. Many plant species, are reported to emit complex volatile blends upon herbivore damage that attract herbivore enemies, a response termed indirect defense. Although the qualitative and quantitative composition of herbivore-induced volatile blends differs between plant species, they are often dominated by terpenes.

One of the most important enzyme groups in terpene biosynthesis is the short-chain prenyltransferases (PTs), the enzymes that join the basic $C_5$ isoprenoid units together to form $C_{10}$, $C_{15}$ and $C_{20}$ precursors of all the major terpene end products. Here we describe the family of PT genes in western balsam poplar (*Populus trichocarpa*), a woody perennial plant species that was already established as a model plant for studying plant-insect interactions on molecular and ecological levels.

A BLAST analysis with short-chain prenyltransferase genes from other plant species revealed the presence of 17 putative PT genes in the *P. trichocarpa* genome. The characterization of recombinant PT proteins, qRT-PCR analysis of PT gene expression, RNAi-mediated knock-down of PT genes and the measurement of the enzyme products geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate in herbivore-damaged and undamaged leaves will provide insights into the role of individual short-chain prenyltransferases in herbivore-induced terpene formation in poplar.

## P101. Control of the grapevine moth *Lobesia botrana* through the manipulation of the plant terpenoid profile

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The grapevine moth Lobesia botrana is one of the key pests of grape. Damages of the vineyard are achieved both by direct larval feeding on reproductive tissue of the plant (flower buds, berries) and by secondary infections of microorganisms. Current control systems are either based on pesticides (many of which are currently being phased out) and mating disruption. Mating disruption stops the male moth from finding the female, by diffusion of the treated area with volatile sex pheromone. However, this method does not work well in non-delimited areas, or areas where pest population is high. We therefore suggest a method that instead works on the female by modifying the host-finding and the egg-laying behaviors, which in herbivore insects are mostly mediated by host plant volatiles (kairomones).

Recent wind-tunnel studies have shown that a blend of 10 synthetic grape volatiles attracts as many moth females as a bunch of green grapes or the entire headspace collection from the same grape bunch. Further investigations demonstrated that even a subset of 3 specific terpenoids(E)-□-caryophyllene, (E)-□-farnesene and (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) elicits attraction comparable to that of the complete lure in laboratory essays, and gave also promising result when tested in field conditions. In addition, it was shown that the specific ratio among compounds is crucial, since both the subtraction and the percentage variation of any of the three chemicals resulted into an almost complete loss of activity of the blend.

Recently we also undertook experiments aimed at the genetic engineering manipulation of the pathway of the three kairomonal terpenoids in Vitis vinifera cv. Chardonnay. In the creation of stable transgenic lines, two strategies are being used: the silencing of the genes responsible for the production of the three compounds (lack of the compounds) and their overexpression (alteration of the ratio between the compounds). The plants obtained will be a potential useful tool to investigate further the plant-insect interactions, and are a likely starting point of new insect control strategies based on kairomones manipulation in planta.

## P102. Terpenes for dinner? *Hylobius abietis* and its gut microbiota

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The pine weevil (Hylobius abietis) is a major pest in European conifer forests where adults feed on the bark and cambium of Norway spruce and pine seedlings. Since conifers are protected against bark-feeding herbivores by a complex mixture of secondary metabolites, includingterpenoid-based oleoresins, adult pine weevils must cope with a complex mixture of terpenesin their diet.

Many insects are known to harbor symbiotic microorganisms in their digestive system that allow them to subsist on suboptimal diets either by enhancing digestion efficiency, supplementing the diet with limiting vitamins or amino acids, or by detoxifying plant secondary metabolites. However, little is known about degradation ofthe terpenesof conifer resin by symbiotic microorganisms.

We are exploring how the pine weevil copes with the high concentrations of terpenes present in spruce bark and cambium by elucidating the metabolic fate of these compounds and their effect on the beetle. Preliminary studies have shown that antibiotic-treated weevils cannot digest terpenes as efficiently as non-treated ones. In order to understand the possible role of gut microorganisms in terpene detoxification, we are using culture-dependent and -independent methods as well as metabolic and genomic analyses to functionally characterize the pine weevil's gut microbiota. Toidentify the terpene break down products, we are planning to offer 13C labeledspruce terpenesto the weevil/microbiota system.

We hypothesize that – in addition to the direct detoxifying activity of microbial symbionts – the beetle may also benefit by consuming some of the resulting by-products after microbial transformation of plant secondary metabolites. Elucidating the fate of terpenes in this system will shed some light on how insects cope with plant induced defenses and how some symbionts allow their hosts to exploit otherwise inaccessible food sources.

## P103. Versatility and fast evolution of the cytochrome P450 enzymes in the metabolism of monoterpenols

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The sequencing of plant genomes has revealed that cytochromes P450 (CYPs) form the largest family of plant enzymes in secondary metabolism and that most of their functions were still unknown. This pointed to a very poor understanding of the plant metabolism. We therefore designed complementary strategies to track the overlooked aspects of the secondary metabolism in the model plant Arabidospsis thaliana. One of them is a predictive map of P450 functions in plant metabolism, based on the analysis of gene (co)-expression in publicly available Affymetrix ATH1 microarray data (Ehlting et al., 2008; http://www-ibmp.u-strasbg.fr/~CYPedia/). Based on CYPedia predictions, a group of four co-expressed terpene synthases (TPSs) and CYPs were predicted to be involved in common floral terpenoid metabolism. All four genes were found mainly expressed upon anthesis in the stamen filaments and petals. Investigation of the encoded enzymes in recombinant systems (yeast and Nicotiana benthamiana) and analysis of null and overexpressor mutants revealed convergent evolution and new functions of TPS, CYP76 and CYP71 enzymes in the metabolism of linalool and derived compounds. This work was extended to a more systematic exploration of the family of CYP76 enzymes in Arabidopsis, which revealed functional versatility, redundancy, specialisation and fast evolution of monoterpenol oxydases, as well as their role in herbicide metabolism.

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## P104. *M. sativa* x *M. arborea* cross (SAC) *derivatives* for saponin production in *Medicago* spp.

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In the *Medicago* genus saponins are a complex mixture of triterpenic glycosides synthetized from β-amyrin. Saponins have a wide range of biological properties such as fungicidal, molluscicidal, nematocidal, antiparasitic, insecticidal, antibacterial, antiviral and allelopathic activities. The correlation between the chemical structure of saponins (aglycone moiety and nature and position of the sugar chains) and their different biological activities is a key point for effectively making use of its (Tava and Avato, 2006).

Our aims is to evaluate hybrid derivatives originated by the interspecific cross *M. sativa x M. arborea* (SAC) (Bingham and Haas 2005) in order to obtain plant families differing in content and composition of saponins, suitable for different uses in green agriculture and pharmacology.
Ten SAC $S_2$ families were obtained by two cycles of selfing and selection for divergent sapogenin content and composition in leaves. GC analyses on 60 plants showed a wide among-families range of variation for total sapogenin content and composition. The variation observed was mainly due to the haemolytic fraction in leaves. The among-families coefficient of variation (CV) for haemolytic sapogenin content was 62% and 29% in leaves and roots respectively, compated to 21% and 20% for non-hemolytic fraction. No correlation was found between sapogenin content of leaves and roots either at family or individual plant level.

The linear regression of S2 progenies vs S1 mother plants showed a highly significant regression coefficient for medicagenic acid in leaves indicating a high heritability and genetic control of this compounds.

Expression studies by quantitative real time PCR on the *Medicago sativa* homologous of CYP716A12, the gene responsible of the first step of haemolytic saponin synthesis (Carelli et al., 2011), and on other three CYP450 genes putatively involved in saponin pathway were performed on the same plants. No correlation between expression of CYP450 genes and sapogenin content was put in evidence either in leaves or in roots suggesting a complex regulation of this biochemical pathway.

No consistent correlations between CYP450s expression in leaves and roots were found reinforcing the hypothesis that roots and leaves act as separate compartments for saponin synthesis and storage.

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## P105. *Diverse* bioconversion of wood-derived diterpene by *icotianatabacum* and *Catharanthusroseus* cells

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During recent years, concern about the environment and sustainability has increased the pressure on industry to adapt their processes and products to global trends. This has resulted in development of cleaner processes, and much attention is now paid to reusability and recycling. In this context biotransformation, i.e. the use of biological systems to induce chemical changes in synthetic or natural compounds is an attractive alternative compared to traditional chemical methods. Operating in mild conditions, biotransformationallows the minimal generation of toxic wastes. Plants contain various unique enzymes, which can be exploited in biotransformation platforms. Plant cells exhibit a vast biochemical potential, being able to transform a range of substances including pharmaceutical ingredients and industrial by-products into more valuable, more active or less toxic products.

The aim of this study was to use whole cell catalysts as tools for modification of selected resin acids in order to obtain value-added functional derivatives. The enzymatic bioconversion capacities of two plant species were tested towards dehydroabietic acid. Dehydroabietic acid (DHA) is an abundant resin acid in conifers, representing a natural wood protectant. DHA was fed to *Nicotianatabacum* and*Catharanthusroseus* cell and hairy rootcultures and bioconversion product formation was monitored using NMR analysis (Häkkinen et al. 2012). Uptake of DHA from the culture medium was observed in both species and various types of typical detoxification processes occurred in the cells. In addition, diverse responses to DHA treatment were observed, including differences in uptake kinetics, chemical modification of added substrate and changes in overall metabolism of the cells. Interestingly, the bioconversion pattern of exogenously applied DHA in *Catharanthusroseus*, a host species for pharmaceutically valuable terpenoidindole alkaloids, was very different than that of tobacco, which does not possess a terpenoidindole pathway. In tobacco, DHA is readily glycosylated in the carbonyl group, whereas in periwinkle it is proposed that a cytochrome P450–catalyzed enzymatic detoxification reaction takes place before the formation of glycosylated product.

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## P106. Identification of *Nicotiana tabacum* trichome-specific transcription promoters: the first step towards terpenoid metabolic engineering

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Plants synthesize a large range of secondary metabolites with important pharmacological applications. However their low concentration in the plant makes their commercial exploitation challenging and costly. Different approaches have been set up to overcome this problem. One consists in overproducing these molecules by up-regulating genes involved in their synthesis. However, over-expression often interferes with the cell metabolism and is counter-selected. From this point of view, metabolic engineering in trichome secretory cells might be advantageous as these cells are specialized in secondary metabolism and are not essential to the plant. For instance, *Nicotiana tabacum* tall glandular trichomes synthesize large amounts of diterpenes and might therefore represent a good host for terpenoid engineering.

A prerequisite for metabolic engineering of trichomes is to have transcription promoters that target trichome-specific expression. To uncover strong trichome-specific promoters, we used a proteomic approach to identify abundant and trichome-specific proteins. We compared the proteome of different tissues of *N. tabacum* with proteins of isolated long trichomes by differential gel electrophoresis (2D-DIGE) followed by mass spectrometry. Forty-nine protein spots were preferentially (average ratio > 2) present in trichomes. Proteins that showed the highest specificity (higher average ratio) were validated at the RNA level by performing reverse transcription-PCR on RNA extracts of the same plant tissues. Two proteins were selected and their corresponding transcription promoter regions were isolated by inverted PCR. The promoter region was fused to a hybrid reporter gene coding for a β-glucuronidase (GUS) and a yellow fluorescent protein (Venus) and introduced in the genome of *N. tabacum* via *Agrobacterium tumefaciens*. Transgenic plants are being raised and the trichome-specificity of the promoters will be confirmed by GusVenus assays.

In addition to the two promoters isolated, four others shown in the literature to be trichome-specific (pCYP71D16, pNtCPS2, pCBTS and pNtLTP1) are being used in parallel. Our objective is to perform a quantitative and qualitative comparison of the six promoters. In particular, we will compare their activity during all the different stages of the plant development as well as during the whole life cycle of trichomes.

This set of promoters will offer new tools for metabolic engineering permitting the right choice of the promoter for the expected level of expression at the appropriate development stage.

## P107. Positive genetic *interactors* of *HMG2* identify a new set of genetic perturbations for improving sesquiterpene production in *Saccharomyces cerevisiae*

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Terpenoids and isoprenoids are an important class of natural products, which includes currently used drugs, high value bioactive and industrial compounds, and fuel candidates. Due to their industrial application, there is increasing interest in the development of *S. cerevisiae* strains capable of producing high levels of terpenoids.

Aiming to identify new gene targets which can be manipulated to increase sesquiterpene production, a set of *HMG2* positive genetic interactors were assessed as single and digenic heterozygous deletions in the presence or absence of stable *HMG2*(K6R) overexpression. Upon single allele deletion, most genes examined led to increased sesquiterpene production in yeast cells. Tandem heterozygous deletion of a set of three genes, the ubiquitin ligases *ubc7* and *ssm4/doa10*, and the ER resident protein *pho86*, led to an 11-fold increase in caryophyllene yields (125 mg/L in shake flasks) compared to cells lacking these modifications. The effect of the heterozygous deletions appears to be due to Hmg1p and Hmg2p stabilization.

Heterozygous deletions cause significant reductions in protein levels but do not lead to growth impediments frequently seen in haploid strains. By exploiting desirable haploinsufficiencies in yeast, we identified a new set of genes that can be disrupted in tandem and cause significant stabilization of Hmgp and a substantial increase in sesquiterpene production. The approach presented here allows new genetic perturbations to be compiled on yeast cell factory strains without negatively impacting cell growth and viability.

## P108. *Biosynthesis* and overproduction of carotenoids in *Corynebacterium glutamicum*

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Corynebacterium glutamicumis used for the annual production of several million tons of amino acids. Here, we show that this bacterium can also serve as a model for the overproduction of carotenoids.

The natural yellow color of C. glutamicumis due tothe glycosylated C50 carotenoid pigment decaprenoxanthin. Decaprenoxanthin is synthesized from isopentenyl pyrophosphate, which is generated in the non-mevalonate pathway, via the intermediates farnesyl pyrophosphate, geranylgeranyl pyrophosphate, lycopene and flavuxanthin1.

The genes of the carotenoid gene cluster crtE-cg0722-crtBIYeYfEbwere shown to be co-transcribed. Gene deletion analysis revealed that crtI, crtEb, and crtYeYf, code for the only phytoenedesaturase, lycopene elongase, and carotenoid C45/C50 ε-cyclase, respectively. In addition, the genome of C. glutamicumpossesses a second carotenoid gene cluster comprising crtB2I2-1/2 shown here to be co-transcribed, as well. The lack of phytoene synthase CrtB in C. glutamicumΔcrtB could be compensated for byectopic expression of crtB2, thus, C. glutamicum possesses two functional phytoene synthases, namely CrtB and CrtB2. Genetic evidence for a crtI2-1/2 encoded phytoenedesaturase could not be obtained since plasmid-borne expression of crtI2-1/2 did not compensate for the lack of phytoenedesaturaseCrtI in C. glutamicumΔcrtI.

The potential of C. glutamicum to overproduce carotenoids was estimated with lycopene as example. Deletion of the gene crtEb prevented conversion of lycopene to decaprenoxanthin and accounts for the accumulation of lycopene to $0.03 \pm 0.01$ mg/g cell dry weight (CDW). Engineering conversion of geranylgeranyl pyrophosphate to lycopene by overexpression of the genes crtE, crtB and crtI in C. glutamicumΔcrtEbresulted in intensely red-pigmented cells and an 80 fold increased lycopene content of $2.4 \pm 0.3$ mg/g CDW2.

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[2]Heider S.A.E.,Peters-WendischP.,Wendisch V.F.; Carotenoid biosynthesis and overproduction in *Corynebacteriumglutamicum*. *BMC Microbiology* 2012, 12:198

## P109. Terpenoidindole *alkaloids* in hairy roots of *Rhazyastricta* (Apocynaceae)

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*Rhazyastricta*Decne., a small evergreen shrub native to the Middle East and Indian sub-continent, is a rich source of pharmacologicallyactive terpenoidindole alkaloids (TIAs).The plant has been in the focus of phytochemical, pharmacological and ethnobotanical studies due to the long use in folk medicine to treat a variety of ailments. More recently especially the antimicrobial and anticancer properties of certain constituents have been established.

In order to study TIA production and enable metabolic engineering hairy root cultures of *R. stricta* were established by co-cultivating cotyledon, hypocotyl, leaf and shoot explants with wild type *Agrobacterium rhizogenes*strain LBA 9402and*A. rhizogenes*carrying the pK2WG7-*gusA* binary vector. Hairy roots initiated from the leaf explantswithin two to six weeks.Transformation was confirmed by polymerase chain reaction (PCR) and in case of GUS clones with GUS staining assay. Transformation efficiency was 42.8% and 46.6% for wild type and GUS hairy root clones, respectively.

Alkaloids were selectively extracted and subjected to GC-MS with and without MSTFA derivatisation. Mass spectra were matched with NIST and Wiley libraries and confirmed by fragment comparison with literature data. Several validation parameters such as linearity, precision, recovery and LOD were evaluated for verification of the reliability of the developed GC–MS method. About 20 alkaloids, some of which representing new records for the species, were qualitatively determined. Furthermore, quantitative modulation of alkaloid accumulation under different conditions e.g. light vs. dark was monitored with a HPLC-UV method.

## P110. *Optimization* of Geraniol Production Heterologusly in *N. benthamiana*

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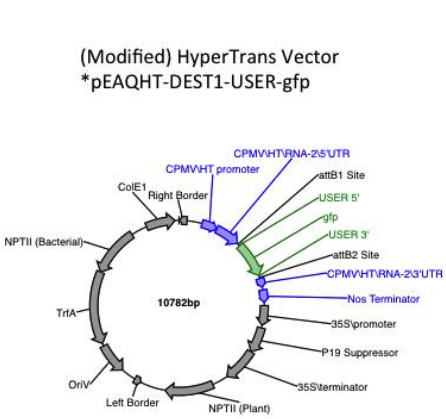
Geraniol is an important basic monoterpene that exists as a precursor for multiple secondary metabolic pathways in plants, including the iridoid and monoterpene indole alkaloid pathways, which yield a myriad of pharmacologically important natural products. Much research is being carried out globally to find economically optimal means to obtain these important geraniol derived metabolites. Strategies range from chemical synthesis to heterologous production via biological engineering. Heterologous production efforts to optimize yields of key metabolites derived from geraniol will be dependent on strategies to maximize the production of generaniol itself as to maximize the supply of precursors necessary for dependent down-stream products. Towards this end, we describe efforts to optimize the heterologous production of geraniol in *N. benthamiana* using the HyperTrans CPMV expression system.




## P111. Metatranscriptome analysis of the red algae *Laurencia microcladia* and preliminary characterization of its terpene biosynthetic pathways

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Marine organisms are characterized by high biological and chemical diversity. They produce a variety of chemical compounds of great pharmaceutical and biotechnological interest. *Laurencia* is a genus of red algae that mostly occurs in the sea near islands, recognized as important sources of secondary metabolites, mainly halogenated compounds such as sesquiterpenes, diterpenes, triterpenes and C15-acetogenins, with potential pharmacological activities. Red algae exhibit important host-microbe interactions and can be considered as ecosystems that co-evolve creating complex interactions with the seaweed-associated microbial communities. Despite this, molecular data describing the species present and their interactions are still limited.

Total RNA was prepared from *L. microcladia* algae and sequenced by Illumina sequencing. 309,845 EST contigs of 167 bases mean length were assembled and used for the construction of 40,746 Unigenes of 473 bases mean length. For the identification of the associated micro-organisms, BLAST searches against the 16S and nr databases were performed. Results indicated a high number of sequences presenting similarity with bacterial and unicellular organisms.

To illuminate the terpene biosynthetic pathways of red algae, one 283 bp contig with similarity to haloperoxidase genes was identified. Based on this information, specific primers were designed and used in 3'-RACE experiments. Sequence analysis of the isolated fragments, identified four clones of 724 bp with similarity to PAP2 haloperoxidase_like subfamily sequences. Further experiments aiming to isolate the full length gene are underway.

## P112. Optimizing recombinant expression of patchoulol-synthase from *Pogostemon cablin* and enzymatic FPP bioconversion as a model system for high level sesquiterpene production

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Terpenes constitute the largest class of flavor and fragrance molecules in nature. Important representatives of this class are used widely in various foods, beverages, exclusive perfumes and personal care products. Nowadays, terpenes are still most frequently produced by extraction and steam distillation of the relevant plant source material. Because of this often laborious, unreliable and cost demanding production route, aroma suppliers are searching for alternative ways to generate valuable terpene based flavor and fragrance compounds. Biotechnological production of terpenes with recombinant microbial strains is a promising method, but requires extensive metabolic and pathway engineering to channel endogenous farnesyl pyrophosphate (FPP) production to the corresponding terpene synthase in the microbial host.

By synthesizing large amounts of FPP in a short and reliable organic synthesis step [see "*Large scale synthesis of farnesyl diphosphate as substrate for recombinant sesquiterpene synthases*"; T. Frister, S. Hartwig, S. Beutel, T. Scheper] and conversion of this key substrate by a recombinant terpene synthase, we hereby present one possible alternative production route for sesquiterpenes.

The previously described patchoulol synthase of *Pogostemon cablin* [1, 2] was amplified from a cDNA-library of the plant. The recombinant gene was fused to various fusion tags and to the fusion protein thioredoxin. For fast and reliable cloning results, the PCR-based cloning method Gibson assembly [3] was performed. Custom peptide recognition sites for cleavage of the fusion tags and proteins could be easily attached in a one-step reaction. The resulting constructs were transformed into different *E. coli* BL21-derivative strains. DNA-sequencing of the gene-of-interest revealed a variation from the originally published sequence of about 5 percent. The resulting protein tended to form inclusion bodies in *E. coli*, and only certain strains and constructs were able to produce sufficient amounts of soluble enzyme.

The conversion of FPP to patchouli alcohol and other constituents of the native patchouli oil by the soluble synthase could be detected by GC-FID and GC-MS. This serves as a proof-of-principle for further upscale-studies with other sesquiterpene synthases in enzyme reactors and enables the development of a bioprocess strategy.

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## P113. Engineering a functional DXP pathway in *Saccharomyces cerevisiae*

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Isoprenoids and their chemical derivatives are used in many commercial and industrial products. Producing these compounds via the engineering of microbial systems is an attractive alternative to extraction from their native source because it can provide a more sustainable source, a more stable supply, and/or lower production costs. All isoprenoids are formed from the two activated hydrocarbon monomers isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Depending on the organism, these monomers are either produced from acetyl-CoA by using the mevalonate pathway or from pyruvate and glyceraldehyde-3-phosphate by using the deoxyxylulose phosphate (DXP) pathway. The baker's yeast *Saccharomyces cerevisiae* exclusively utilizes the mevalonate pathway. In this work, we describe the use of synthetic biology, proteomics, *in vitro* biochemistry and metabolomics to engineer a functional heterologous DXP pathway in *Saccharomyces cerevisiae* and demonstrate the biosynthesis of IPP and DMAPP through the DXP pathway in yeast.

## P114. Microbial conversion of (±)-linalool to linalool oxides by *Corynespora cassiicola*

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Linalool oxides are interesting for the flavor and fragrance industry because of their lavender notes. The production of pure (2R) or (2S) configured linalool oxides is preferable, because this stereocenter determines their olfactorial properties (earthy, leafy or floral, creamy).
The recently described biotransformation of (±)-linalool to linalool oxides by *Corynespora cassiicola* DSM 62475 [1] was further investigated and optimized [2].

While the use of racemic substrate (±)-linalool resulted in (2R) and (2S) configured linalool oxides, it was possible to produce nearly pure (2R) configured products (ee ≥ 90 %) from (R)-(–)-linalool, which is available from natural sources with high enantiomeric purity. In fed-batch shaking flask cultures, product concentrations above 1 g $l^{-1}$ were obtained. Substrate limitation was shown to be the key factor limiting the productivity, which was about 80 mg $l^{-1}d^{-1}$. For both linalool and linalool oxides, severe growth inhibition occurred for concentrations above 450 and 800 mg $l^{-1}$, respectively.

This led to the development of a combined *in situ* substrate feeding and product removal (SFPR) approach. The polystyrene adsorber Lewatit VP OC 1163 showed the highest loading capacity (0.55 g linalool per g resin) while maintaining a subinhibitory substrate concentration (150 mg $l^{-1}$).

The bioprocess was transferred into a bioreactor and optimized by circumventing substrate limitation via a linalool-saturated air stream. The SFPR process resulted in a more than 4-fold increase in productivity (920 mg $l^{-1}d^{-1}$) compared to the fed-batch biotransformation in a bioreactor (216 mg $l^{-1}d^{-1}$).

This way, it was possible to load the bioreactor with 10 g $l^{-1}$ substrate of which 42 % were converted to 4.8 g $l^{-1}$ product after 5 days. More than 80 % of the product was found adsorbed onto the resin as well as 35% of the substrate, which can be recycled after elution and separation from the product.

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2. Bormann, S., Etschmann, M.M.W., Mirata, M.A., Schrader, J. (2012) Integrated bioprocess for the stereospecific production of linalool oxides from linalool with *Corynespora cassiicola* DSM 62475. J Ind Microbiol Biotechnol 39 (12): 1761-1769

## P115. A novel in-silico approach to predict mediators for mediator driven bioelectrocatalysis with P450cin

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P450cin catalyzes the stereoselective hydroxylation of 1,8-cineole to (2S)-2β-hydroxy-1,8-cineole[1]. 1,8-Cineole is widely used in pharmaceutical preparations. The hydroxylated products of 1,8-cineole have a high potential for organic chemistry applications. Moreover, esters of 2-hydroxy-1,8-cineole have antimicrobial and antibacterial activities[2]. A key issue for catalytic applications of isolated P450cin is the demand for NADPH which is far too expensive to be used in equimolar concentrations during technical applications.

In our study, different mediators and prosthetic groups (e.g. phenosafranine, viologens, FAD, FMN) were tested with P450cin for the stereoselective hydroxylation of 1,8-cineole. Investigations were done in an electrochemical cell where mediators were reduced on a metal electrode, which then deliver the electrons to the heme of P450cin. Mediators which enable the electrochemically driven product formation without the need for the natural cofactor and co-proteins have been identified[3].

For bioelectrocatalysis the selection of an efficient mediator is crucial for an efficient process. To this end we have developed a computational screening method using freely available software[4]. Potential binding sites of the mediator on the enzyme are found, based on surface complementarities, using PatchDock[5]. Subsequently, the mediator-enzyme interactions are refined using FiberDock[6]. Finally, the software HARLEM is used to calculate the electron transfer path and rates between the mediators and the enzyme. The electron transfer rates were compared with measured product formation rates resulting in a good correlation. The novel *in-silico* procedure will allow a faster identification of suitable mediators for electrochemically driven P450 catalyzed reactions. It may also lead to a massive reduction of experimental effort for the development of bioelectrochemical reaction systems in the future.

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2. Miyazawa, M. and Y. Hashimoto, *Antimicrobial and bactericidal activities of esters of 2-endo-hydroxy-1,8-cineole as new aroma chemicals.* J Agric Food Chem, 2002. 50(12): 3522-3526.
3. Zengin-Çekiç, S., Holtmann, D., Güven, G., Mangold, K.-M., Schwaneberg, U., Schrader, J., *Mediated electron transfer with P450cin*, Electrochemistry Communications, 2010. 12(11): 1547-1550.
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## P116. Global metabolite profiling of glandular trichomes

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Glandular Trichomes are specialized epidermal cells which protrude from the surface of a variety of plant species. They are known as “chemical factories” producing large quantities of secondary metabolites involved in defense reactions against abiotic stresses or pathogen and herbivore attack. Among these metabolites, terpenoids play a predominant role and their intracellular concentrations can reach the molar scale. This raises the questions how glandular trichomes organize the high metabolic flux into the terpenoid pool and how precursor pathways are regulated in comparison to plant leaf tissue.

To address these questions, we developed a metabolomics platform based on GC-MS and on a novel LC-SWATH-QToF-MS2 approach. The latter allows for acquisition of high resolution MS1 and MS2 data with cycle times below 1 s, which enables the truly non-targeted analysis of thousands of precursor molecular ions simultaneously with their corresponding fragmentation spectra.

Our chromatographic platform allows for the GC-based separation of mono-, di-, and sesquiterpenes. UPLC-based separation of hydrophilic and charged primary metabolites, including intermediates of the MEP and mevalonate pathway, is achieved by ion pairing chromatography, while trichome-borne secondary metabolites such as isoprenoids, acyl sugars, flavonoids or free acids are separated in RP mode.

We analyzed the metabolic network of tomato trichomes from wild type S. habrochaites LA 1777 and cultivated tomato S. lycopersicum LA 4024 and compared central carbon and secondary metabolite profiles of both lines for trichome and leaf samples.

## P117. Air born defense signal transduction cascade with monoterpenes on *Cupressus lusitanica* culture cells

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Many experiments for plant-plant communication had been conducted widely in field or laboratory; experiments used whole plant or organs like leaf, root and shoot. However, a model experiment system, which is simple and free from the influence of environment, is favorable. Our group has used cultured cell of *Cupressus lusitanica* to investigate the chemical communication as a model. This cell line produced 10 hydrocarbon monoterpenes as well as β-thujaplicin, which is strong phytoalexin, when they were elicited. Because these monoterpenes were emitted into atmosphere, special roles as vapor were expected previously. In this work, the cells were exposed with artificial vapor monoterpenes and then headspace gases of the culture flasks were analyzed with SPME and GCMS. As results, when culture cells were exposed with artificial sabinene, *de novo* produced γ-terpinene and *p*-cymene were detected in headspace gas. When culture cells were exposed with artificial γ-terpinene as well, the products were *p*-cymene and terpinolene. In case of the *p*-cymene exposure, terpinolene was emitted into the air. However, this phenomenon was not observed with the exposure of other monoterpenes, such as α-pinene, β-pinene, limonene, myrcene and terpinolene. These results strongly suggest "signal transfer cascade" which starts from sabinene and followed by γ-terpinene, *p*-cymene and, then, end at terpinolene. Though terpinolene didn't cause air born signaling, it facilitated the starting time of β-thujaplicin production. That is, by pathogen or insect attack, *C. lusitanica* cells implement a signal cascade to prepare β-thujaplicin production of innate plant or adjacent leaves. The results led us to propose dual functions of the cascade as shown in Figure. 1. The signal can spread over longer range with the cascade of monoterpenes. 2. Tree can measure the distance from pathogen-invaded tree by depending on the kinds of monoterpenes recognized.

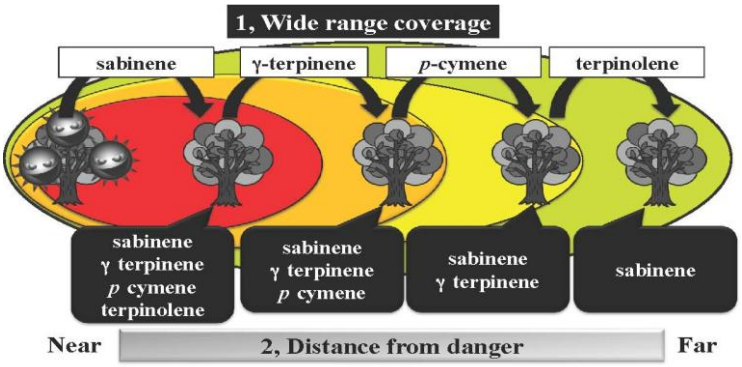


Fig. Speculated roles of air born defence signal transduction cascade.

## P118. The effect of *methyl* jasmonate on terpene defenses in Norway spruce: defense inducer or priming agent?

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Methyl jasmonate (MeJ) is a well-known inducer of defense responses in both angiosperm and gymnosperm plants. In the conifer Norway spruce (Picea abies) MeJ has been used as a tool to investigate the importance of terpenes in defense against bark beetles and their fungal associates. Application of MeJ to the outer bark of Norway spruce stems has been shown to induce traumatic resin duct formation in the sapwood, to increase terpene levels in the phloem and sapwood, and to make the trees much more resistant to insect and pathogen attack.

However, recent evidence suggests that the major role of MeJ in enhancing terpene defenses in Norway spruce may be to act as a priming agent, rather than to induce terpene levels directly. Defense priming is the process were plants that have been sensitized by e.g. prior infection, wounding or MeJ application display faster or stronger defense reactions to a subsequent challenge. Recent field experiments show that MeJ treatment alone may have very little effect on terpene levels in spruce bark or sapwood, but that wounding of MeJ treated tissues induces very strong terpene responses. For example, MeJ alone led to a ~3 fold increase in diterpene levels compared to unprimed bark, but when MeJ treated bark was mechanically wounded diterpene levels increased >26-fold within 24 hours. Interestingly, Norway spruce trees display extensive individual variation in how they respond to MeJ. In some trees terpene levels are induced 10-fold or more following MeJ treatment alone, whereas in other trees there are no noticeable direct effects of MeJ on terpene induction.

The priming effect of MeJ explains the almost complete protection against attack by the spruce bark beetle (Ips typographus) that has been observed in MeJ treated Norway spruce trees under field conditions. Bark beetles that tunnel into primed bark will quickly be met by induced defenses that are likely to place them under severe physiological stress and interfere with their ability to produce sufficient aggregation pheromones. We are currently investigating the biochemical, transcriptomic and epigenetic regulation of defense priming in Norway spruce.

## P119. Plant control on fungal symbiont organs (arbuscules) via alpha-ionone-type apocarotenoids in the AM symbiosis?

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The development and function of the arbuscular mycorrhizal (AM) symbiosis is determined by root colonization through fungal hyphae followed by the formation and maintenance of fungal arbuscules. Arbuscules, which constitute the major sites for symbiotic plant mineral nutrient acquisition, are short-lived organs undergoing degradation and turnover but why their lifespan is limited to only several days on average and whether their degradation is a regulated process is still largely unknown. There are now a number of indications that carotenoid-derived molecules (apocarotenoids) are involved in the regulation of both root colonization and arbuscule functionality. Strigolactone (SL)-apocarotenoids are exuded from roots to support early steps of extraradical proliferation of hyphae and their colonization of roots by promoting hyphal branching. SL-deficient mutants exhibit reduced colonization but there is no evidence that SLs also affect arbuscules. In contrast, suppression of the biosynthesis of another class of apocarotenoids comprising various oxygenated C13 alpha-ionone (AI) derivatives (formerly called cyclohexenone derivatives), reduces the proportion of mature arbuscules within the total population of arbuscules, while increasing the relative number of degrading arbuscules. This observation is in agreement with decreased transcript levels of the *PT4* phosphate transporter gene and other plant markers for functional arbuscules. AI derivatives accumulate concomitantly with yellow C14 mycorradicins (MRs) in and around arbusculated cells in an abundant manner, leading to a visible "yellow pigment" in some species. Interestingly, SL and AI/MR biogenesis is interconnected not only by steps of root carotenogenesis but they also share a carotenoid cleavage step catalyzed by carotenoid cleavage dioxygenase 7 (CCD7). We show that in *ccd7* mutants of pea deficient in both types of apocarotenoids colonization and arbuscules are negatively affected, whereas in *ccd8* mutants, defective in SL biosynthesis only, total numbers of arbuscules are reduced along with colonization but most are in a mature state as in wildtype plants. Supplementing the mutants with the synthetic SL analog GR24 improves colonization and associated total formation of arbuscules, but does not alter the compromized arbuscule phenotype (less mature, more degrading arbuscules) in the *ccd7* mutant. These results imply involvement of different types of apocarotenoids both in root colonization via SLs as well as in the later processes of maintaining a population of active and mature arbuscules via AI derivatives. We propose a model for a local phytoalexin-like function of moderately fungitoxic AI-type allelochemicals in attacking poorly performing fungal arbuscules thereby accelerating their degradation and removal without harming the host root cell. Preliminary results from a study of the *mtpt4* mutant of *Medicago truncatula* suggest that normal arbuscule degradation events associated with local C13 apocarotenoid synthesis and deposition can be accelerated if arbuscules are unable to deliver phosphate as is the case in the *mtpt4* mutant.

Vogel et al. (2010) *Plant J.* **61**, 300-311; Walter et al. (2011) *Nat. Prod. Rep*. **28**, 663-692

## P120. Understanding Metabolic Control of Carbon Flux through the MEP Pathway: A Systems and Synthetic Biology Approach

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Current research suggests that metabolic regulation of the plant MEP pathway is very complex, however few control mechanisms have been elucidated and it is clear that our understanding is incomplete. This is evidenced by our inability to circumvent metabolic regulation to drive high level industrial production of isoprenoids through the MEP pathway: despite many studies, the best yields to date are nowhere near theoretical maximum of the pathway. Investigation of plant MEP pathway regulation has thus far been confounded by both the relative lack of control we have over the plant system and by the complexity of the pathway regulation itself. Our approach to this problem is to reconstruct the plant MEP pathway in E. coli. In this synthetic system, it is possible to independently reguate the levels of each protein, and consequently the levels downstream metabolites. Coupling modification of protein and metabolite levels with quantitative proteomic and metabolomic analyses allows us to determine in vivo enzyme kinetic properties. Furthermore, the system can be used to examine allosteric regulation (including feed-forward and feedback regulation) in detail. This information can be used to build an in silico metabolic model to facilitate identifical of regulation control. Once constructed, the model can be calibrated using enzyme and metabolite concentrations from plant cells under specific conditions.

This approach has particular utility for examining emission of isoprene, a volatile C5 hydrocarbon which is produced by some plants in very large amounts, exerts a protective effect on plants under oxidative stress conditions, and has a major impact on atmospheric chemistry. Large changes in both MEP pathway flux and isoprene production profiles occur in very short time frames (seconds/minutes), precluding transcription and translation as mechanisms of flux control and suggesting that post-translational events such as protein modification or allosteric regulation are responsible.

For pathway reconstruction, a complete set of plant MEP pathway genes was modified for expression in E. coli and engineered as a set of synthetic operons. Individual and combinatorial integration of these operons resulted in a series of strains with altered MEP pathway flux and altered isoprenoid accumulation patterns. Using these strains, previously unidentified nodes where metabolic regulation is exerted were revealed. We are currently examining the effect of titration at each node in the pathway to identify control mechanisms, and mapping the effects of MEP pathway flux changes using systems biology approaches. This systematic study will help explain the relative contribution of each step and to describe overall metabolic flux through the pathway in an cellular context.

## P121. Combinatorial biosynthesis of triterpene saponins in plants and engineeredyeast.

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Plants synthesize an overwhelming variety of secondary metabolites, many of which possessbiological activities relevant for the pharmaceutical and chemical industry. Furthermore, there is an ever increasing demand for novel compounds, due to, among others, the growing drug tolerance and resistance in microorganisms and newly emerging diseases. In microorganisms, combinatorial biosynthesis is a widely used tool to increase structural variation in several classes of (microbial) natural products. Here, we used a functional genomics approach to identify candidate genes involved in the biosynthesis of triterpene saponins in various medicinal plants. After elicitation with methyl jasmonate and targeted metabolite profiling, genome-wide transcript profiling was carried out to identify candidate genes involved in saponin biosynthesis. The generated gene list provided the basis for a combinatorial biosynthesis platform that targets triterpene saponins in plants. Proof of concept of combinatorial biosynthesis was achieved by heterologous expression of the candidate saponin biosynthesis genes in *Medicago truncatula* hairy roots. Three of the generated transgenic hairy root lines were found to accumulate novel molecules, two of which were shown to be novel triterpene saponins, whereas the third line produced a set of novel, non-saponin compounds.

However, the low production amounts *in planta* and the complexity of the saponin mixture in the *M. truncatula* hairy roots encouraged us to develop a microbial production system for triterpene saponins. To this end, we engineered *Saccharomyces cerevisiae* to accumulate 2,3-oxidosqualene, an intermediate of the yeast sterol biosynthesis, and the precursor molecule of all plant saponins. Subsequently, plant specific oxidosqualene cyclases were used to generate yeast strains that produce high levels of triterpene backbones, such as $\alpha$-amyrin, $\beta$-amyrin, and lupeol. These yeast strains were super-transformed with the cytochrome P450s that were identified with the transcript profiling, leading to identification of cytochrome P450s that oxidize the triterpene backbones on various positions. Combining these cytochrome P450s with characterized cytochrome P450s from literature, drastically increased the diversity of triterpene saponin backbones we could produce in *S. cerevisiae*. Finally, super-transformation of the generated strains with a UDP-dependent glycosyltransferase enables us to produce an array of structurally diverse saponins in yeast.

## P122. Towards the engineering of astaxanthin biosynthesis in maize endosperm

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Astaxanthin is a high value ketocarotenoid with applications in the nutraceutical, cosmetic, food, and animal feed industries. It is not a typical plant carotenoid although its precursors, β-carotene and zeaxanthin are abundant. We had previously generated a population of transgenic maize plants with diverse carotenoid profiles. A specific plant lineage was shown to accumulate astaxanthin and other ketocarotenoids. However, astaxanthin accumulation in this specific line was shown to be rather low (4.46 μg/gDW) compared to β-carotene. It has been demonstrated that the limited astaxanthin accumulation in plants is due to a bottleneck in the conversion of adonixanthin to astaxanthin. This is in agreement with the hypothesis that the low efficiency of β-carotene ketolases in ketolating zeaxanthin to astaxanthin is the major limitation for astaxanthin accumulation in engineered plants. Our strategy to overcome this bottleneck was to screen for bacterial ketolases encoding particular enzymes with preferential substrate specificity for zeaxanthin and introduce these into plants. We have identified one promising candidate, the ketolase gene (*bkt*) from *Chlamydomonas reinhardtii*, which mediates the accumulation of substantial amounts of astaxanthin in engineered *E. coli* and transgenic Arabidopsis. In a first step to enhance astaxanthin accumulation in cereal endosperm, the *C. reinhardtii bkt* gene optimized for maize codon usage was synthesized and subsequently characterized in transgenic rice callus generated from bombardment of pGZ63-sCrbkt wherein the chemically synthesized truncated *sCrbkt* fused with pea small subunit of Rubisco (SSU) and 5´-untranslatd region (5´UTR) of rice alcohol dehydrogenase gene (*Adh*) was driven by the maize γ-zein promoter. Our data confirmed that the synthetic *bkt* gene (*sCrbkt*) product is a β-carotene/zeaxanthin ketolase with superior catalytic properties for astaxanthin production compared to *Paracoccus* β-carotene ketolase.

Plasmids p326-Zmpsy1, pGZ63-sCrbkt, pGZ63-sBrcrtZ wherein the chemically synthesized *Brevundimonas* sp. SD212 CRTZ (β-carotene hydroxylase) gene (*sBrcrtZ*) fused with pea SSU and 5´UTR of rice *Adh* was driven by the endosperm specific maize γ-zein promoter and pHorP-RNAi-Zmlyce (the 417 bp long fragment of maize lycopene ε-cyclase mRNA was chosen to construct the RNAi construct under the control of the barley D-hordein promoter) together with the selectable *bar* marker gene were co-transformed into maize (*Zea mays* L. cv. M37W) immature zygotic embryos using direct DNA transfer in order to generate maize transformants with high astaxanthin content. Four indepedent transgenic maize lines were produced and the best line with high astaxanthin production in maize endosperm was chosen to cross with high oil maize line NSL76. The hybrid NSL76 x M37W BKT overexpressing line resulted in an astaxanthin content up to 35.60μg/gDW (more than 66% of total carotenoids—53.83μg/gDW) in the T3 homozygous seed endosperm. All input transgenes involved in the target pathway were confirmed to specifically express in maize endosperm by mRNA blot analyses. Funding: Plant KBBE project CaroMaize (PIM2010PKB-00746 and BMBF 0315913A), ERC Advanced grant BIOFORCE to PC, MINECO, Spain (BIO2011-22525) and RecerCaixa.

## P123. From biochemical pathway elucidation to Metabolic Engineering of antimicrobial melleolides

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The emergence of multi-drug-resistant microbial pathogens has triggered an urgent need for novel antibiotics. Natural products traditionally represent a good source of antibiotic substances. Although isoprenoids with more than 50.000 known members represent the largest as well as structurally most diverse group of natural products (1), only two antibiotics based on a fungal diterpenoid (2) are currently in use.

Melleolides from the Armillaria species have long been known for their potent antibacterial activity. Some of the examined melleolides, like melleolide B, C and D, showed remarkable activity against *Bacillus cereus*, *Bacillus subtilis* and *Escherichia coli* (3). For armillaric acid, antimicrobial activity against *Micrococcus luteus*, *Bacillus subtilis*, *Candida albicans* and *Staphylococcus aureus* has been reported (4). Today there are approximately 50 known melleolides, produced almost exclusively by this fungal genus.
However, limited supplies of individual melleolides have prevented them from being more extensively examined and clinically developed. Metabolic pathway engineering in easily to culture microbial hosts may provide an efficient method of producing advanced melleolides. For successful metabolic engineering, the functional characterization and cloning of the underlying enzymatic steps represents a key prerequisite.

We recently cloned the biosynthetic genes (the sesquiterpenoid protoilludene synthase (5) and cytochrome P450 dependent monooxygenases) responsible for the synthesis of advanced melleolides. Using Metabolic Engineering approaches in *Saccharomyces cerevisiae* we could establish easy to culture microbial strains capable of producing advanced melleolides, which will be of value for the semisynthetic synthesis of pharmacological active melleolides and derivatives thereof.

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## P124. Expression of two early genes of the terpenoid biosynthetic pathway in tobacco results in major suppression of the pathway and profound changes in non-target metabolites in a differentiation state dependent manner

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The reconstruction of biosynthetic pathways by genetic engineering in heterologous organisms is one of the current aims of metabolic engineering and synthetic biology. Substantial advances have been reported in this context in microbes. However, many challenges remain particularly in plant systems, due to their complexity and organization at the subcellular and organelle levels. The creation of an exogenous metabolic pathway may influence the metabolite flow in plant cells. A new metabolite may be produced, or the introduced pathway might compete for one or more intermediates.

Regulation of the terpenoid pathway is complex, and control mechanisms are rather unknown. This is in part due to the involvement of two pathways, that operate simultaneously in each cell, for the biosynthesis of isopentenyl diphoshpate, IPP, the universal $C_5$ building block for all isoprenoids. The mevalonate pathway (MEV), which operates in the cytoplasm and the methyl erythritol phosphate (MEP) pathway localized mostly in plastids. The coexistence of both pathways in higher plants may produce intracellular trafficking of precursors. This metabolite interchange occurs to varying degrees between species and depends on physiological conditions. The regulatory systems controlling this cross talk are not yet fully understood and remain a major challenge in biochemistry and metabolic engineering. The engineering of any terpenoid pathway may have a direct effect on other branches of related (or even to apparently unrelated pathways).

In an effort to investigate potential pleiotropic effects that might arise following the reconstruction of a heterologous pathway in a plant host, we introduced geranyl diphosphate synthase (*Gpps*) from *Arabidopsis thaliana* and geraniol synthase (*Ges*) from *Valeriana officinalis* into tobacco plants. These two monoterpenoid enzymes are naturally localized in the plastids. We evaluated two non-native targets, cytosol and mitochondria, and performed an in depth non-targeted metabolomic analysis to obtain more insights into the overall changes induced by the modulation of the monoterpenoid pathway. Two analytical platforms, NMR and GC-MS were used as well as targeted transcript profiling of transgenic plants. Our results clearly demonstrate that the engineering of the early steps of the monoterpenoid biosynthetic pathway has direct effects on non-target pathways.

The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement number 222716 – SMARTCELL

## P125. From ecometabolomics to synthetic biology – exploring plasticity of the triterpenoid biosynthetic pathway

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Triterpenoid saponins are natural plant defense compounds rather widespread in plants. They are amphipathic molecules that may interact with sterols in membranes, and induce pore formation and cell death. The wild crucifer Barbarea vulgarisshows resistance towards flea beetles (Phyllotreta nemorum) (Agerbirk et al. 2003, Journal of chemical ecology), a severe pest in crucifer crops like oil seed rape (Brassica napus). An ecometabolomic approach identified saponins to correlate with flea beetle resistance in B. vulgaris (Kuzina et al. 2009, Plant physiology). A transcriptomic 454-dataset was used to create a quantitative trait loci map which identified specific saponins to lie in the QTL for flea beetle resistance (Kuzina et al. 2011, Phytochemistry). The proposed saponin pathway branch-off from the sterol biosynthesis with 2,3-oxidosqualene as the shared precursor. 2,3-oxidosqualene is cyclized by oxidosqualene cyclases (OSC) to a number of backbone structures, oxidized by cytochromes P450 (CYP), and glycosylated by glycosyltransferases (UGT) to create the vast structural diversity.

A 454 transcriptomic and an Illumina genomic dataset were mined for OSC, CYPs and UGTs gene candidates. The catalytic activities of five UGTs were characterized by in vitroassays in E. coli, and found to glycosylate at the C3 or/and the C28 position of selected triterpene backbones (Augustin et al. 2012, Plant physiology). An analysis of dN/dS site and branch models revealed that the UGT catalyzing the 3-O-glucosylation is under positive selection,suggesting that this UGT has evolved to become specific to the saponins. The activities of four CYPs were studied by in vitroassays in yeast, and shown to oxidize at the C28 position. To reveal the function of the OSCs the Cowpea Mosaic Virus-Hyper Translatable System (CPMV-HT) was used. This enable production of the triterpenoid backbone structures α-amyrin, β-amyrin and lupeol from the common precursor 2,3-oxidosqualene. In addition, the OSCs were found to lie within the QTL for resistance. By combinatorial expression in N.benthamianaof OSCs, CYPs and UGTs using the CPMV-HT system, anumber of known and new-to-nature saponin structures were generated. combined metabolomics, genomics, transcriptomics, and genetic approach identified saponins as determinants for flea beetle resistancein B. vulgaris, and provided the necessary molecular tools for in plantaproduction of known and new-to-naturestructures by synthetic biology. This ability to combine OSCs, CYPs and UGTs in planta by combinatorial biochemistry paves the way for a rational design triterpenes for structure-activity relationshipsfor development of bioactive triterpenes with specificity to different herbivores and diseases, and demonstrates the plasticity of the pathway.

## P126. The molecular basis of a good wine: genetic engineering as a tool for identifying novel aroma compounds

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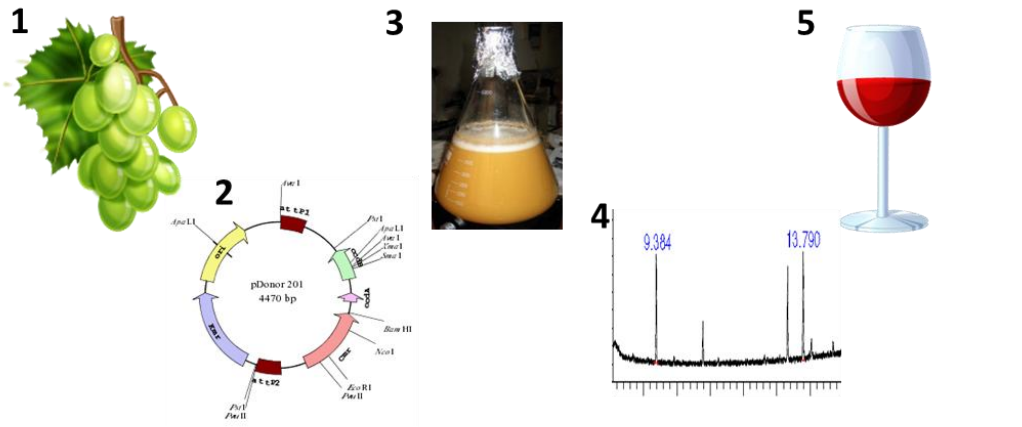
Plants produce a plethora of complex biochemicals, known as secondary metabolites, that act as insect and herbivore repellents, phytoalexins, or have antibiotic or fungicidal activities. For society however, these compounds are often important as pharmaceuticals or for their flavour and aroma characteristics. The last of these functions are particularly important in the case of wine grapes, where the complex mix of secondary metabolites is intrinsically linked to the character of the wine produced. Although the compounds in a finished wine can arise from diverse sources, including from yeast and other microbes or from the physical processing of the wine during production, many compounds are derived directly from secondary metabolites present in the harvested grapes. One particular class of chemicals, known as terpenoids, are well known as aroma active compounds, with rotundone recently being found to be responsible for the peppery aroma of Shiraz wine. The identification of aroma compounds is made difficult due to their characteristically low concentrations in both grapes and wine. Thus, we are developing an alternative method of identifying these potential compounds whereby we clone target genes that may be involved in the *in planta* biosynthesis of terpenoids from grapes themselves, and express the active enzymes in a microorganism. Using this technique, combined with targeted metabolic engineering of the microorganism, grape-specific terpenoids can be produced in much higher quantities than are generally found in the grape. The structural and sensory characteristics of compounds produced are then much more readily measured. Through a combination of phylogenetic analysis and the investigation of transcriptional regulation, we have identified numerous genes that are potentially involved in the formation of sesquiterpene hydrocarbons, alcohols or ketones, and are currently investigating the biochemical activity of these enzymes in genetically engineered yeast.




**1 – Identify target genes. 2 – Amplify and clone. 3 – Express enzymes in yeast.**
**4 – Analyse novel volatile compounds synthesised. 5 – Relate to wine aroma.**

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