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ANNE HUKKANEN

Chemically Induced Resistance in Strawberry *(Fragaria × ananassa)* and Arctic Bramble *(Rubus arcticus)*

Biochemical Responses and Efficacy Against Powdery Mildew and Downy Mildew Diseases

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium, Mediteknia building, University of Kuopio, on Monday 26th May 2008, at 12 noon

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ABSTRACT

During the past decade, induced resistance has become promising means for the protection of crop plants. One objective of this Thesis was to explore the efficacy of chemically induced resistance against two important diseases of strawberry and arctic bramble. The second aim was to analyse specific defence-related biochemical and chemical changes during the development of resistance.

The model compound benzothiadiazole (BTH) decreased disease incidence and/or severity in strawberry infected with powdery mildew and in arctic bramble infected with downy mildew in the greenhouse. Phosphite-based agrochemicals, i.e. Aliette, Phosfik and Phostrol, and the control fungicide Euparen M provided moderate and high level of protection, respectively, against downy mildew.

Two tools were developed to analyse downy mildew infections in arctic bramble. Quantitative real-time PCR method proved to be specific and sensitive, enabling reliable analysis of the growth of the downy mildew pathogen, *Peronospora sparsa*, in arctic bramble. An *in vitro* plate model was developed for fast pre-evaluation of resistance to downy mildew in different genotypes or treatments of arctic bramble.

The phenolic composition in strawberry and arctic bramble leaves was analysed by mass spectrometry. BTH treatment induced the accumulation of phenolic compounds in strawberry and arctic bramble leaves and in strawberry fruits, the accumulation being weakest in arctic bramble leaves. Phosphite treatments did not affect the phenolic contents. Flavonol and ellagitannin contents varied among arctic bramble cultivars, which may partly explain the differences observed in their basal resistance to *P. sparsa*. High amount of salicylic acid was constitutively present in both plant species.

BTH treatment of arctic bramble increased or decreased the levels of 103 proteins, of which eighteen could be identified tentatively using mass spectrometry. Many of the up-regulated proteins are linked to defence reactions in other plants. Down-regulation of the proteins related to energy metabolism may explain the reduced vegetative growth of BTH-treated plants.

In conclusion, several phenolic compounds and proteins possibly involved in the development of resistance were characterised. Induction of resistance by chemicals proved to be promising means for the protection of strawberry and arctic bramble, although the efficacy against downy mildew is still unproved in the field conditions.

Universal Decimal Classification: 581.2, 582.711.712, 632.25, 632.934, 634.71, 634.75
CAB Thesaurus: induced resistance; disease resistance; defence; plant diseases; plant disease control; plant pathogens; fruit; leaves; strawberries; *Fragaria ananassa*; *Rubus arcticus*; Rosaceae; *Peronospora sparsa*; mildews; phenolic compounds; flavonols; tannins; salicylic acid; signal transduction; pathogenesis-related proteins; agricultural chemicals; HPLC; mass spectrometry; electrophoresis; western blotting; polymerase chain reaction; symptoms; yields; greenhouses

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Many warm thanks to my family and friends, particularly to my parents Rauni and Aimo, who have always been supportive and believed in me and in the accomplishment of this Thesis for all of these years. Finally yet importantly, I thank Marko for encouraging, listening and for being my dear link to real life almost throughout the whole work.

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Kuopio, May 2008

Anne Hukkanen

ABBREVIATIONS

2-DE	two-dimensional electrophoresis
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
AlaAT	alanine aminotransferase
ATP	adenosine triphosphate
avr	avirulence gene
bp	base pair
BTH	benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
CBB	Coomassie Brilliant Blue
CHAPS	3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHI	chalcone isomerase
cv.	cultivar
DSDG	dehydroshikimate dehydrogenase
DTT	dithiothreitol
dw	dry weight
DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
EDS1	*enhanced disease susceptibility1* mutant
ESI	electrospray ionisation
fw	fresh weight
GSNOR	*S*-nitrosoglutathione reductase
HCl	hydrochloric acid
HHDP	hexahydroxydiphenoyl
HPLC	high-performance liquid chromatography
HR	hypersensitive reaction
HSP	heat shock protein
ICS	isochorismate synthase
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
ITS	internal transcribed region
JA	jasmonic acid
MAPK	mitogen-activated protein kinase
MeJA	methyl jasmonate
MeSA	methyl salicylate
MS	mass spectrometry
NAC	nascent polypeptide-associated complex
NahG	salicylate hydroxylase expressing genetically modified plant
NaOH	sodium hydroxide
NIM1	*non-inducible immunity* mutant
NO	nitric oxide
NPR1	*non-expressor of PR-1 genes* mutant
PAD4	*phytoalexin deficient4* mutant
PAL	phenylalanine ammonialyase

PAMP	pathogen-associated molecular pattern
PCA	principal component analysis
PCR	polymerase chain reaction
pI	isoelectric point
POX	peroxidase
PPO	polyphenoloxidase
PR	pathogenesis-related
R gene	resistance gene
rDNA	ribosomal DNA
RGP	reversibly glycosylated protein
ROS	reactive oxygen species
SA	salicylic acid
SABP2	salicylic acid-binding protein 2
SAR	systemic acquired resistance
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
TBS	tris-buffered saline
TF	transcription factor
TMV	tobacco mosaic virus
UV	ultraviolet
vis	visible

LIST OF ORIGINAL PUBLICATIONS

This Thesis is based on the following original publications referred to in the text by their Roman numerals:

I **Hukkanen A. T.**, Kokko H. I., Buchala A. J., McDougall G. J., Stewart D., Kärenlampi S. O., Karjalainen R. O. 2007. Benzothiadiazole induces the accumulation of phenolics and improves resistance to powdery mildew in strawberries. Journal of Agricultural and Food Chemistry 55: 1862-1870.

II **Hukkanen A.**, Pietikäinen L., Kärenlampi S., Kokko H. 2006. Quantification of downy mildew (*Peronospora sparsa*) in *Rubus* species using real-time PCR. European Journal of Plant Pathology 116: 225-235.

III **Hukkanen A.**, Kostamo K., Kärenlampi S., Kokko H. 2008. Impact of agrochemicals on *Peronospora sparsa* and phenolic profiles in three *Rubus arcticus* cultivars. Journal of Agricultural and Food Chemistry 56: 1008-1016.

IV **Hukkanen A.**, Kokko H., Buchala A., Häyrinen J., Kärenlampi S. Benzothiadiazole affects the leaf proteome in arctic bramble (*Rubus arcticus*). Manuscript.

CONTENTS

1. INTRODUCTION

Plant diseases caused by micro-organisms such as bacteria, fungi and viruses have great impact in crop losses worldwide. Diseases decrease the quantity and quality of the crops, but they also cause increased costs, as growers have to invest, for example, in pesticides and resistant cultivars. It has been estimated that pre- and post-harvest yield losses caused by various pests and weeds are approximately 40-45% of all crops, of which over one-third are caused by plant diseases (Agrios 2005). The severity of the problem is well illustrated by the fact that the annual costs caused by the yield losses and plant protection activities are estimated to be around 1000 billion U.S. dollars. Because of the high economic impact of plant diseases, significant research effort has been targeted at the development of more efficient plant protection methods and resistant cultivars.

Extensive use of pesticides may have adverse effects on the environment, non-target organisms and human health. Therefore, increasingly sophisticated methods are under development for plant protection and will become available alongside with the progress made in biotechnology and knowledge of pathogen virulence and plant disease resistance mechanisms. For example, by means of genetic engineering and induced resistance, disease resistance of plants could be improved without the use of toxic chemicals. The majority of genetically modified crops commercialized today carry a trait designed to improve tolerance to various pests. Despite the promising results, safety concerns have led to restrictions in the cultivation of genetically modified crops particularly within the European Union. The application of systemic acquired resistance (SAR) in plant protection was an important milestone in plant pathology at the 1990s (e.g. Ward et al. 1991; Uknes et al. 1992). A major finding was that external exposure of plants to certain microbes or non-toxic chemicals can activate defence reactions, which leads to improved resistance against several diseases. Thus far, only few commercial products have emerged, BTH (Bion and Actigard from Syngenta) being the most thoroughly characterized one (Görlach et al. 1996; Tally et al. 1999; Oostendorp et al. 2001). The approach has shown great potential although its wide commercial breakthrough is still to come.

Today, large production units are favoured in the European agriculture policy, which has led to problems particularly in the periphery far from optimal cultivation areas. Small-scale production of high-value special crops has become an alternative to the investment on large-scale production facilities. In Finland, production of soft fruits is an important, specific sector of agriculture, which offers opportunities for small-scale farmers presuming the cultivation is profitable. Plant diseases can be the limiting factor in the production of horticultural crops; this is a particular problem because of the limited selection of plant protection products available for minor crops. Powdery mildew disease, for example, is a constant problem in the greenhouse production of many crops. Up to 50% of strawberry (*Fragaria* × *ananassa*) production has been shifted into covered systems in many European countries during the past 10 to 15 years, leading to problems with powdery mildew infections. In the field, resistant cultivars and wider selection of suitable fungicides can control the

disease, but new plant protection strategies for greenhouse use are required.

The situation is even worse for arctic bramble (*Rubus arcticus* subsp. *arcticus*). The fruits of arctic bramble have always been highly valued due to their specific aroma suitable e.g. for the manufacture of liqueur and the demand for the fruits is higher than their availability. Therefore, the cultivation area of arctic bramble increased to 20 ha in the 1990s, but sudden and wide outbreaks of downy mildew disease in the mid 1990s caused large yield losses and declining of cultivation. Similarly, downy mildew has strongly hampered the cultivation of boysenberry in other countries (McKeown 1988; Walter et al. 2004). Presently, no arctic bramble cultivars are completely resistant to downy mildew and neither enough suitable fungicides are available. Furthermore, one of the two products registered against downy mildew of arctic bramble in Finland may be out from the market in the near future. Therefore, new plant protection solutions are urgently required for arctic bramble.

The aim of this work was to explore the applicability of induced resistance against powdery mildew disease in strawberry and downy mildew disease in arctic bramble under controlled conditions in a greenhouse. The second aim was to analyse specific defence-related biochemical and chemical changes during the development of resistance to gain more insight into the resistance mechanism. Very limited information was previously available about the defence mechanisms in rosaceous plants. Externally induced resistance was selected for the study because of its promising efficacy on other crops, non-toxicity of the compounds used - keeping in mind the image of pure northern fruits - and the broad-spectrum defence response the compounds may provide without the drawback of boosting the development of resistant pathogen strains.

2. REVIEW OF THE LITERATURE

2.1 Powdery mildew of strawberry

Powdery mildew fungi are obligate biotrophic, host-specific pathogens that cannot survive outside a living plant tissue. Thus, their penetration mechanisms and retrieval of nutrients is very different from those of necrotrophs, which, in contrast to biotrophs, live on dead tissue and specifically aim at killing the plant cells by using hydrolysing enzymes (Perfect & Green 2001; Oliver & Ipcho 2004). However, this classification of plant pathogens based on their mode of nutrition is likely to be re-evaluated in the future when more information about pathogenesis factors and host defence is available. The recent approach has led to varying classifications regarding especially hemibiotrophs, which express both biotrophic and necrotrophic stages during their life cycle (Oliver & Ipcho 2004).

The fungus *Sphaerotheca macularis* f. sp. *fragariae* specifically infects strawberry, causing powdery mildew disease. Other isolates of the fungus can infect hop (*Humulus lupulus*), *Potentilla* and *Rubus* species. The disease appears in strawberry as white, powdery-like patches on the leaves (often only on the lower surface), shoots, fruits and flowers (**Figure 1**), and as upward-curling leaf edges and reddish colour of the leaves (Maas 1998). The fungus grows on the leaf surface, where it forms haustoria, specific feeding structures of biotrophic fungi, into the epidermal cells for the absorption of nutrients (Perfect & Green 2001; Oliver & Ipcho 2004). Within 4 to 6 days after the onset of infection, numerous asexual conidia emerge in chains from the epicuticular hyphae and are easily dislodged and spread to the neighbouring plants (**Figure 1**). Sexual ascospores occasionally develop in cleistothecia, but the ascospores may not be important in the normal life cycle of the fungus (Peries 1962a). *S. macularis* may overwinter in living plant material (Maas 1998).

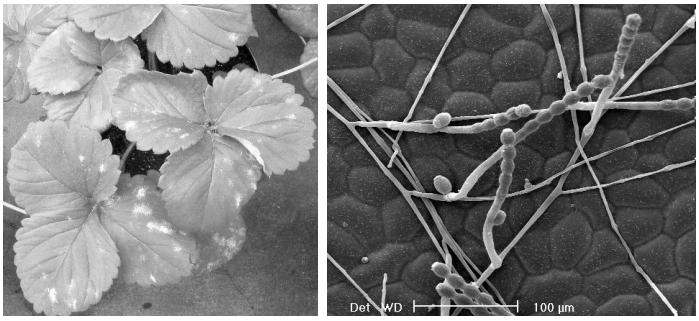

Figure 1. White patches of powdery mildew on a greenhouse-grown strawberry cv. Jonsok (left) and mycelia and conidiophores of the fungus on strawberry leaf (right; 500× magnification).

The most efficient control method is the use of resistant cultivars that are widely available, but conditions highly favourable to the fungus, i.e. high humidity and low temperature, may override the resistance (Peries 1962b; Maas 1998; Xiao et al. 2001). Breaking of resistance has become particularly problematic in the cultivation under glass due to the high infection pressure caused by powdery mildew. Furthermore, breeding for powdery mildew resistance is challenging because of the high genetic variability of the fungus and possibly several, mainly additive, genes involved in the resistance of strawberry (Davik & Honne 2005). The first resistance gene analogues were isolated from strawberry only recently and their function against pathogens is not yet resolved (Martínez Zamora et al. 2004).

Fungicides are effective, but their use is limited due to the possible development of resistant powdery mildew strains as observed in other crops (Chin et al. 2001; McRath et al. 2001). In Finland, only three fungicides against powdery mildew of strawberry are available for greenhouse use, containing azoxystrobin (Amistar and Ortiva, Syngenta) or kresoxim-methyl (Candit, BASF) as active ingredients. In some experiments, moderate control of powdery mildews has been achieved by the application of inorganic salts or silicon, but they are not widely used in the commercial production of strawberries (Reuveni et al. 2000; Ehret et al. 2002; Kanto et al. 2004). In fact, plain water may also be efficient against *S. macularis* because the conidia are easily broken in free water (Peries 1962a).

2.2 Downy mildew of arctic bramble

Downy mildew diseases are caused by host-specific oomycete species from e.g. the genera *Bremia*, *Peronospora*, *Plasmopara* and *Sclerospora*. *Peronospora sparsa* Berk. (synonym *P. rubi* Rabenh.) infection causes downy mildew disease in *Rubus* and *Rosa* species (Hall 1989; Breese et al. 1994). In *Rubus* species, the disease appears on the leaves as reddish, angular and interveinal lesions (**Figure 2**), where sporangiophores extend from the abaxial surface. The most severe consequence of *P. sparsa* infection is the "dryberry" disease, which may cause up to 100% yield losses especially in arctic bramble and boysenberry in rainy seasons (Hall 1989; Ellis et al. 1991; Lindqvist et al. 1998). In contrast to many other genera causing downy mildews, *Peronospora* sp. are obligate, highly specialised biotrophs, which do not develop motile asexual zoospores. Instead, asexual spores develop in the heads of dichotomously branched sporangiophores and spread by wind or free water to new leaves, flowers and fruits (**Figure 2**), initiating secondary infections (Hall 1989; Ellis et al. 1991). Under humid conditions, spores germinate, form haustoria and the mycelia may colonise completely the intercellular space of the plant tissue (Breese et al. 1994). New sporangiophores develop approximately in a week and grow through stomata to the abaxial leaf surface. *P. sparsa* can overwinter as mycelium in the living parts of plants such as rhizome (Ellis et al. 1991; Lindqvist et al. 1998). Sexual oospores develop in some species and may act as survival forms through winter and in transmission to new plants via soil (Hall 1989; Ellis et al. 1991). The occurrence

and significance of oospores in downy mildew infection of arctic bramble is unknown.

Unlike powdery mildews, which grow on the leaf surface, downy mildews are not visible unless the plants develop symptoms or sporulation is extensive. Therefore, diagnostic methods based on the measurement of pathogen DNA have been developed to reliably detect and quantify infections (Lindqvist et al. 1998; Aegerter et al. 2002).

P. sparsa infections can be controlled by fungicides developed against oomycetes. However, only two products, Euparen M (tolylfluanid, Bayer) and Aliette (fosetyl-Al, Bayer) are available for the control of downy mildew in arctic bramble in Finland. Furthermore, the application of Euparen M requires a long withholding period, which limits its use only to the early season.

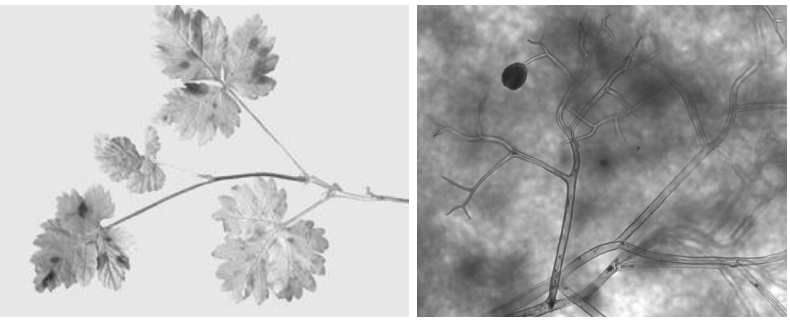

Figure 2. Lesions caused by downy mildew in arctic bramble cv. Pima (left) and part of dichotomously branched sporangiophore of the pathogen and a spore attached to one ultimate branch stained with trypan blue (right).

Thus far, arctic bramble genotypes showing complete resistance to downy mildew have not been found. A Canadian subspecies of arctic bramble (*Rubus arcticus* subsp. *stellatus*) is resistant to *P. sparsa* as is the hybrid of the Canadian and northern (*Rubus arcticus* subsp. *arcticus*) subspecies. The basis for resistance to downy mildew in *Rubus* is presently unknown. In *Arabidopsis*, approximately 30 major resistance (*R*) genes involved in cultivar-race specific resistance to downy mildew pathogen, *Hyaloperonospora parasitica*, have been discovered (Slusarenko & Schlaich 2003). In addition to possible gene-for-gene-based resistance mechanisms, other defence mechanisms may exist and be differently expressed among different genotypes of arctic bramble.

2.3 Salicylic acid in plant defence

2.3.1 Synthesis and occurrence of salicylic acid

Salicylic acid (SA, 2-hydroxybenzoic acid) is a signalling compound that is also classified as a plant hormone (Raskin 1992a). It is synthesised from either

phenylalanine or chorismic acid, both of which originate from the shikimate pathway (**Figure 3)**. The synthesis starting from phenylalanine proceeds mainly via cinnamic acid and benzoic acid/benzoyl glucoside. Only this route was previously supposed to produce SA in plants (Meuwly et al. 1995; Silverman et al. 1995; Chong et al. 2001). However, Wildermuth et al. (2001) discovered that the pathway from chorismic acid to SA, common for prokaryotes, is also active in the chloroplasts of plants, which fits well to the endosymbiotic theory of chloroplast development. According to later findings, isochorismate may in fact be the primary precursor for the synthesis of stress-related SA (Strawn et al. 2007). The exact roles and interaction of these two distinct pathways for SA synthesis are unknown.

SA is common to all plants, but its basal level varies widely among species and organs (Raskin et al. 1990). While the healthy model plants *Arabidopsis* and tobacco have the basal SA level close to zero, the plants in the other margin such as rice and potato, may contain up to 30 to 40 μg/g fresh weight (fw) of SA (Raskin et al. 1990; Silverman et al. 1995; Navarre & Mayo 2004). The study carried out on potato also showed that variable concentrations of SA are present in different plant organs, the highest levels being found in the leaves and flowers (Navarre & Mayo 2004). No conclusive explanation is available for the constitutively high SA levels in some plant species.

SA can occur in free, active form, or in inactivated, glucosylated storage form in the vacuoles (Dean et al. 2005). If in high amounts, SA is usually present as a conjugate, but exceptions also exist, as the main fraction of SA in rice is present in free form (Navarre & Mayo 2004; Silverman et al. 1995). Other types of salicylates, such as salicin and salicortin, are plentiful in *Salix* species (willows) (Julkunen-Tiitto 1989). Park et al. (2007) made recently a breakthrough by identifying methyl salicylate (MeSA) as the mobile form, by which SA signal can travel between the different tissues. MeSA is volatile and, hence, it may also participate in the signalling between the neighbouring plants (Shulaev et al. 1997). MeSA is probably for transportation use only, because it is hydrolysed to SA in the target cells by SA-binding protein 2 (SABP2) and SA is converted to MeSA by SA methyl transferase in the site of its synthesis (Park et al. 2007).

2.3.2 Functions of SA

SA is critical for the survival of plants in abiotic and biotic stresses by acting as one of the main signalling compounds required for the activation of defence reactions. The accumulation of endogenous SA is necessary in the adaptation to abiotic stresses caused by ozone, UV-C and high light radiation, heat, cold and drought (Yalpani et al. 1994; Dat et al. 1998a; Munné-Bosch & Peñuelas 2003; Scott et al. 2004; Mateo et al. 2006). External treatment with SA has also improved tolerance to e.g. cold and heat (Dat et al. 1998b; Senaratna et al. 2000). Related to developmental regulation, SA treatment has induced flowering in some plant species (Khurana & Cleland 1992; Raskin 1992b). In particular, SA may participate in the stress-induced transitions from vegetative growth to flowering or senescence (Morris et al. 2000; Martínez et al.

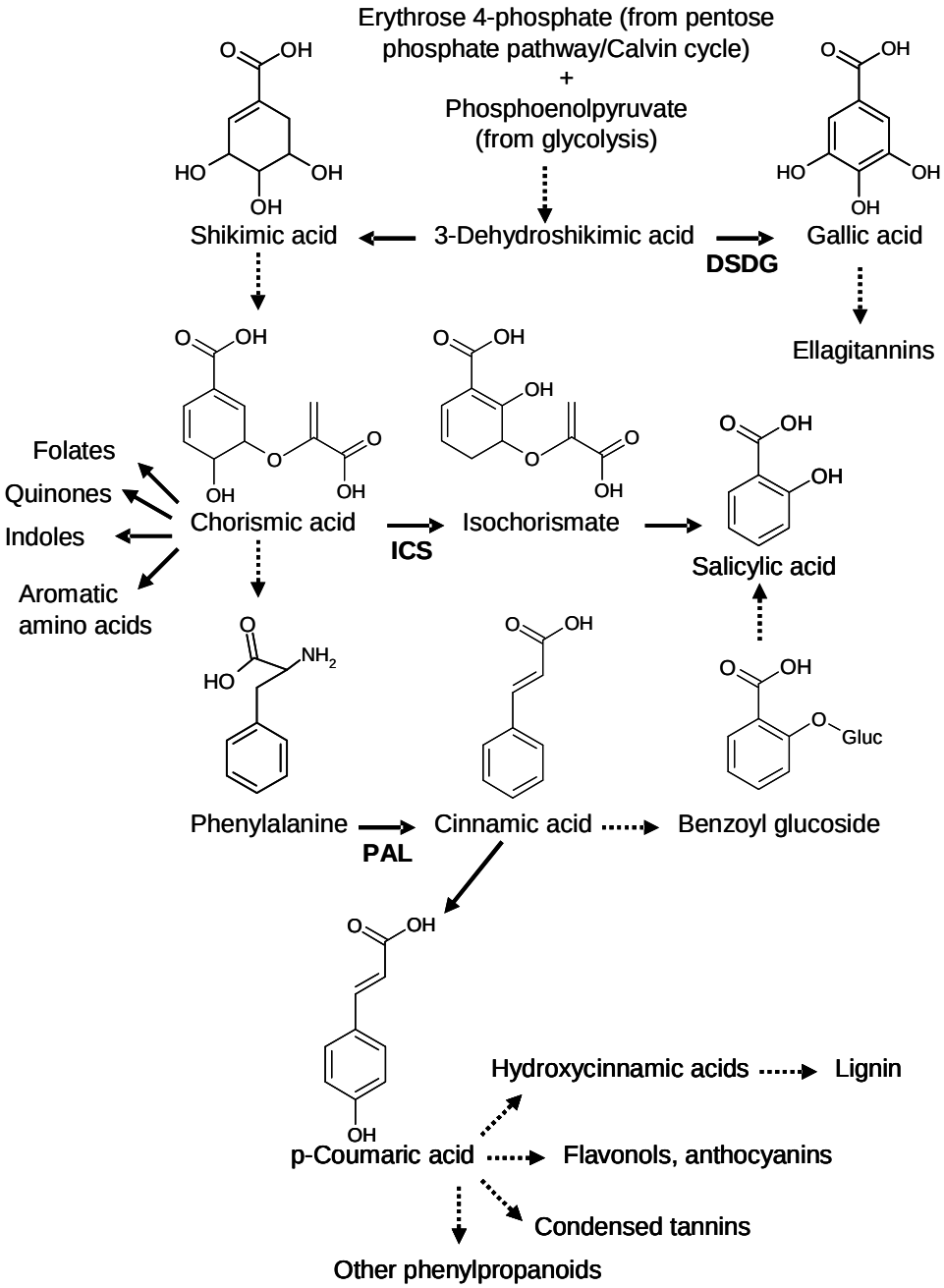

Figure 3. Branchpoints from central shikimate pathway leading to the synthesis of gallic acid derivatives, salicylic acid and phenylpropanoids (Weaver & Herrmann 1997; Dixon et al. 2001; Métraux 2002; Ossipov et al. 2003). DSDG, dehydroshikimate dehydrogenase; ICS, isochorismate synthase; PAL, phenylalanine ammonialyase.

2004). A special mode of action of SA is the control of heat production in thermogenic plants such as *Arum* lilies (Raskin 1992b).

The reason underlying the particular interest in SA is its central role in defence against pathogens, particularly biotrophs, on which numerous studies has been conducted during the past 15 years. The following evidence supports the role of SA in pathogen defence. First, external application of SA or its derivatives induces resistance to pathogens (e.g. White 1979; Uknes et al. 1992; Görlach et al. 1996). Second, the level of endogenous SA rises after pathogen challenge in resistant genotypes of *Arabidopsis*, cucumber and tobacco (Malamy et al. 1990; Métraux et al. 1990; Mauch-Mani & Slusarenko 1996). Third, the expression of bacterial isochorismate synthase in tobacco chloroplasts leads to the accumulation of SA and enhanced disease resistance (Verberne et al. 2000). Fourth, plants defective in SA synthesis, or transgenic plants (*nahG*) carrying SA degrading enzyme show higher susceptibility to pathogens in comparison to wild-type plants (Delaney et al. 1994; Nawrath & Métraux 1999). Although the evidence obtained with *nahG* plants has been challenged (Cameron 2000), since the plants also have defects in the synthesis of metabolites other than SA (Heck et al. 2003), the role of SA as a central mediator of defence signals is unquestionable.

2.3.3 SA as part of defence traffic

SA is part of complex signalling cascade, which begins by the recognition of pathogen or other external inducer and leads to the activation of ultimate defence genes, e.g. those encoding pathogenesis-related (PR) proteins. Regulatory points, which control the magnitude of SA response as well as the crosstalk with other plant hormones, exist along the whole pathway, redox balance being perhaps the most important separate factor influencing the outcome of the response (Mou et al. 2003; Bostock 2005; Noctor 2006; Mateo et al. 2006). However, many parts of the cascade are still uncharacterized or hypothetical (**Figure 4**) and most data derive from studies on *Arabidopsis* and its mutants. Although signalling is likely to be highly similar and conserved among plants, different characteristics and, in particular, fine-tuning may exist in other species such as those with high basal levels of SA.

Recognition of microbes by plants may be non-specific or plant cultivar-pathogen race-specific. In the latter case, a pathogen secretes effector molecules, which are often virulence factors of the pathogen, into plant cells and disease follows unless the plant has a specific *R* gene. The interaction between the effector and *R* gene results in the inactivation of the effector, the activation of plant defence and the arrest of infection. Thus far, a high number of effectors and *R* genes have been discovered and classified in different categories according to their function (Martin et al. 2003b). The action of at least class II and III (LZ/TIR-NB-LRR-type) *R* genes depends on the SA pathway since in transgenic *nahG Arabidopsis* plants the resistance against certain races of *Pseudomonas syringae* and *Hyaloperonospora parasitica* is lost (Delaney et al. 1994). An overview to *R* genes active against *H. parasitica* in *Arabidopsis* reveals that the function of most *R* genes is dependent on SA or other components in the SA defence pathway (Slusarenko & Schlaich 2003).

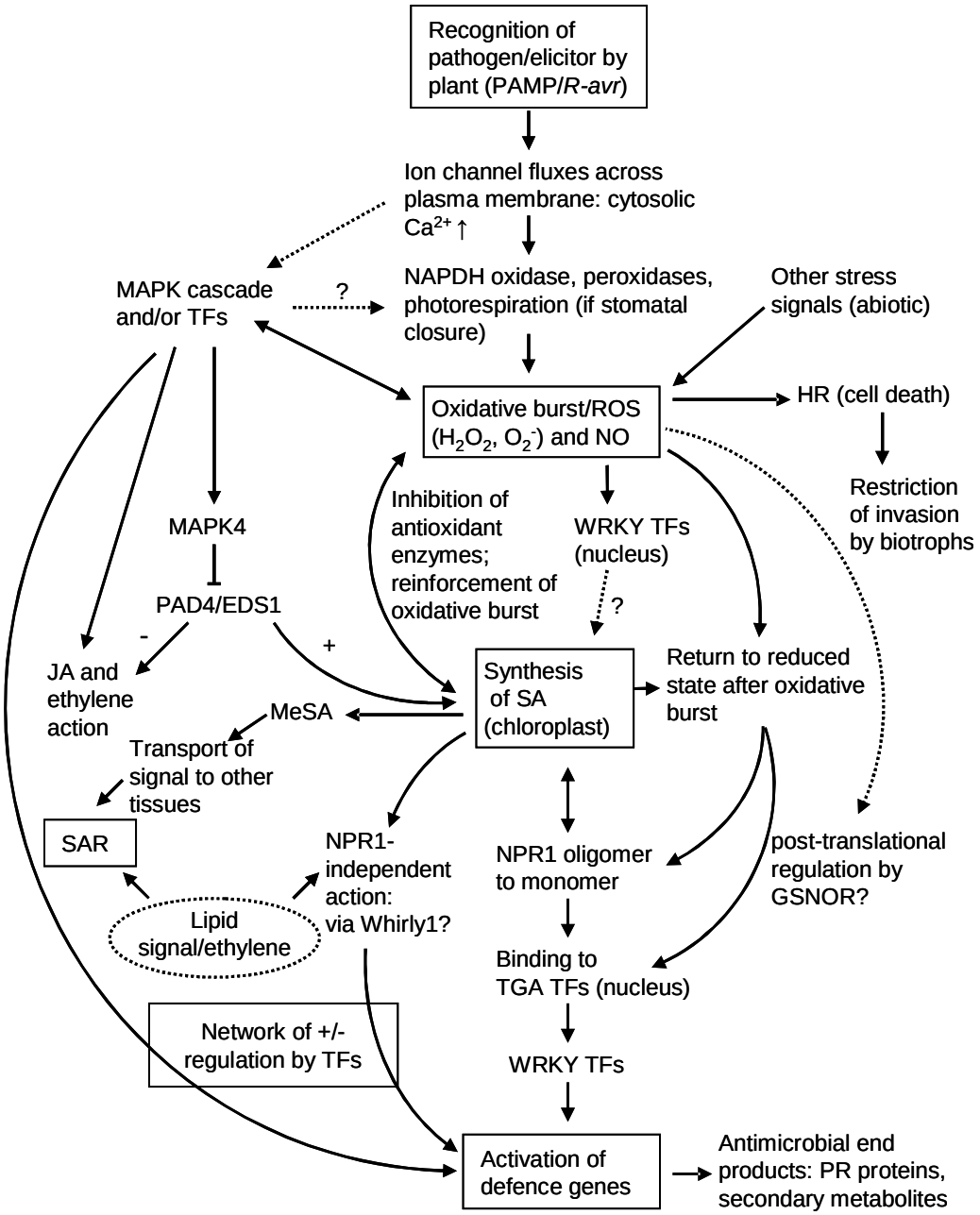

Figure 4. Current view to the signalling cascade in SA-mediated pathogen defence (modified from Shah 2003; Apel & Hirt 2004; Dong 2004; Mateo et al. 2004; Bostock 2005; Brodersen et al. 2006; Loake & Grant 2007; Eulgem & Somssich 2007). Abbreviations: EDS1, enhanced disease susceptible1 mutant; GSNOR, *S*-nitrosoglutathione reductase; JA, jasmonic acid; MAPK, mitogen activated protein kinase; NO, nitric oxide; NPR1, non-expressor of PR genes1 mutant; PAD4, phytoalexin deficient4 mutant; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species; TF, transcription factor.

Interestingly, suppression of some of the components results in partial resistance, but not in the total loss of the resistance. EDS1 and NPR1 proteins from the SA pathway (**Figure 4**) are also necessary for *R* gene-mediated resistance in tobacco (Liu et al. 2002). *R* gene-mediated resistance may involve similar responses and set of defence genes to those in the non-specific resistance, as described in the following paragraphs. It is unknown to which extent these defence pathways overlap.

Another way to activate defence is that plant receptors recognize non-self pathogen/microbe-associated molecular patterns (PAMP), such as microbial lipopolysaccharides (Mishina & Zeier 2007). The recognition leads to the opening of ion channels on plasma membrane and generation of oxidative burst by NAPDH oxidases, peroxidases or amine oxidases (Chen & Schopfer 1999; Torres et al. 2002; Apel & Hirt 2004). Additionally, increased photorespiration in the chloroplasts can quickly generate reactive oxygen species (ROS) (Mateo et al. 2004). The generation of ROS, mainly H_2O_2 and O_2^-, is necessary for the local programmed cell death to take place and for further activation of the defence cascade. Abiotic stresses also induce oxidative burst, though differently from pathogens, and abiotic stress responses are likely to intertwine via ROS generation in SA-mediated defence (Apel & Hirt 2004; Fujita et al. 2006).

Mitogen activated protein kinase (MAPK) cascades are involved especially in the early steps of defence activation, but it is unresolved, whether they activate ROS production or *vice versa* (Pedley & Martin 2005; Apel & Hirt 2004). Among many MAPK and MAPKK(K) genes found, some are inducible by SA, other signalling molecules or stresses, indicating a function in defence (Song & Goodman 2002; Pedley & Martin 2005). Like MAPKs, transcription factors may regulate the expression of early defence genes encoding proteins necessary for the activation of downstream defence responses (Garretón et al. 2002; Apel & Hirt 2004; Eulgem & Somssich 2007). ROS probably also activate the synthesis of SA, but the exact mechanism is unclear. ROS may activate transcription factors directly or via MAPKs, which then activates the enzymes of SA synthesis (Eulgem & Somssich 2007). The increased levels of SA can inhibit ROS-detoxifying enzymes catalase and ascorbate peroxidase and intensify oxidative burst, which may in turn further enhance SA synthesis (Sánchez-Casas & Klessig 1994; Shirasu et al. 1997; Wendehenne et al. 1998; Mateo et al. 2006).

However, to activate downstream signalling after oxidative burst, reduced state has to be restored, but it is still unclear how that is achieved. The levels of SA, H_2O_2 and glutathione seem to interlink in a way that SA has a dual role as both an inhibitor and inducer of ROS detoxifying enzymes (Sánchez-Casas & Klessig 1994; Rao et al. 1997; Garretón et al. 2002; Mateo et al. 2006). SA-induced accumulation of glutathione may be involved in restoring redox balance by directly detoxifying ROS, acting as a substrate for ROS-binding enzymes, or affecting thiol-groups of proteins and thus enzyme activities (Ball et al. 2004; Noctor 2006; Mateo et al. 2006). Thioredoxins, ascorbic acid or production of reducing power in pentose phosphate pathway may also participate in the redox control (Balmer et al. 2004; Dong 2004; Bostock 2005). More studies are necessary to clarify the interplay of redox status

with defence metabolism and basic metabolism as the redox balance depends on several cellular activities such as photosynthesis, photorespiration, and responses to abiotic and biotic stresses, which all affect the concentrations of oxidative radicals and reducing capacity.

Regardless of the unknown preceding steps, return to the reduced state enables the SA-induced dissociation of NPR1 oligomer to monomer and the binding to specific transcription factors in the nucleus (Mou et al. 2003). In addition to redox balance, NO via S-nitrosoglutathione reductase may control NPR1 or other components downstream of SA by adding a NO group to cysteine residues of its targets (Loake & Grant 2007; Feechan et al. 2005). Finally, a complex network of TGA- and WRKY-type transcription factors regulates positively and negatively the activation of defence genes downstream of NPR1 (Li et al. 2004; Zhang et al. 2003; Eulgem & Somssich 2007). SA also activates defence genes independently from NPR1, likely via different transcription factors such as Whirly1, which is induced by SA and capable of directly activating SA-induced defence genes, e.g. PR-10 (Desveaux et al. 2004). Recently, a lipid signal, which may be jasmonic acid (JA), has been suggested to act as part of the SA pathway, but its function in this context is unravelled (Truman et al. 2007).

Other signalling pathways mediated mainly by JA, ethylene and abscisic acid (ABA) interact antagonistically or agonistically to SA signalling (e.g. Fujita et al. 2006; Loake & Grant 2007). In a simplistic view, SA-mediated defence is active against biotrophic pathogens; JA- and ethylene-mediated pathways are active against necrotrophic pathogens and insects while ABA regulates abiotic stress responses. In reality, increasing evidence indicate that none of these hormones acts exclusively in synergistic or antagonistic way, but rather the direction of defence into certain pathway depends on the prevailing conditions and relative spatial and temporal concentrations of the hormones (Bostock 2005; Fujita et al. 2006; Loake & Grant 2007). The components of the SA signalling cascade are also shared partly by other pathways, pointing out the central role of SA as a regulator of general plant defence. MAPK4 is one of the known control points for crosstalk between SA and JA/ethylene-mediated pathways (**Figure 4**). PAD4 and EDS1 regulate positively the SA synthesis and repress JA pathway unless MAPK4 is activated, which inhibits PAD4/EDS1 and thus activates JA pathway (Brodersen et al. 2006). Besides MAPK4, at least NPR1 and WKRY transcription factors regulate the shuttling between SA- and JA/ethylene-mediated defence, but other control points are likely to be found along the pathway (Spoel et al. 2003; Li et al. 2004; Dong 2004). Interestingly, phytopathogens may also redirect plant defence to the pathway that is more favourable for their survival. For example, the phytotoxin coronatine of *Pseudomonas syringae* suppresses SA pathway by acting as a JA analogue (Laurie-Berry et al. 2006). Jasmonate-insensitive plants are more resistant to *P. syringae* and coronatine, and no typical symptoms of disease develop.

2.3.4 PR proteins are related to SA-mediated defence

Pathogenesis-related (PR) proteins are closely related to the activation of SA-mediated defence and pathogen resistance, supporting thus the function of SA as the inducer of defence. PR proteins were first thought to be proteins expressed under pathogen attack, but their induction has later also been connected to other kinds of stresses (van Loon 1994; van Loon et al. 2006). Some PR proteins or their isoforms are also involved in developmental processes, e.g. pollen development and embryogenesis (Worrall et al. 1992; Kragh et al. 1996). Thus far, 17 classes of PR proteins have been found, but the functions of some of them, e.g. PR-1, have not been characterised. However, many PR proteins show antimicrobial and/or hydrolytic activity and genetically modified plants constitutively expressing e.g. PR-1, PR-3 and PR-5 have shown higher tolerance to pathogens (Broglie et al. 1991; Alexander et al. 1993; Liu et al. 1994; Rauscher et al. 1999).

Treatments with SA or SA analogues result in the accumulation of PR proteins, the pattern of which varies among plant species (Malamy et al. 1990; Ward et al. 1991; Görlach et al. 1996; Friedrich et al. 1996). However, the expression of PR-1 is connected with the induction of SA pathway in most plant species and, therefore, it is also used as a marker for the activation of SA-mediated resistance (Delaney et al. 1994; Lawton et al. 1996; Cameron et al. 1999). Although PR-1 expression follows pathogen- or elicitor-induced accumulation of SA, and *Arabidopsis* mutants with constitutively high SA level express PR-1 constitutively (Bowling et al. 1994; Jirage et al. 2001), the amount of endogenous SA does not directly correlate with the expression of PR-1. As an example, PR-1 expression is not constitutive but rather inducible in rice, which has constitutively high levels of SA (Yang et al. 2004). SA-responsive elements have been identified in the promoters of PR-1 and PR-2 proteins (van de Rhee & Bol 1993; Shah & Klessig 1996), confirming their relation to SA pathway.

2.3.5 Systemic acquired resistance

SA has a key role in systemic acquired resistance (SAR), which is the plant counterpart to the immune system of animals. The first contact with a necrosis-inducing virulent or avirulent pathogen makes the plant more resistant to subsequent attacks and, in contrast to animals, protection is effective against a wide range of pathogens. The response is also systemic i.e. the whole plant becomes resistant after localized infection. While the first observations about SAR emerged at the early 1900th century, extensive research on the mechanisms of SAR was conducted only at the 1990s.

Functioning of SAR has been demonstrated in several plant species using viruses, bacteria or fungi as primary and secondary pathogens, covering also their combinations (Uknes et al. 1993; Mauch-Mani & Slusarenko 1994; Schneider et al. 1996). In addition, non-host bacteria, molecules of microbial origin and chemicals can induce SAR (Baillieul et al. 1995; Görlach et al. 1996; Mishina & Zeier 2007). Typically, SAR is more effective against biotrophic than necrotrophic pathogens as is SA-mediated defence in general. There are several proofs of the involvement of SA in

SAR. Endogenous SA and PR proteins accumulate systemically in untreated leaves after local treatment with a necrosis-inducing pathogen (Malamy et al. 1990; Métraux et al. 1990; Ward et al. 1991). Treatment with SA leads to the induction of similar set of PR proteins to that induced by biological SAR inducers (Ward et al. 1991; Uknes et al. 1992). Further, SAR, i.e. systemic activation of defence genes and development of resistance, is not established in mutant plants defective in SA signalling or lacking SA in the untreated/uninfected systemic tissue (Gaffney et al. 1993; Lawton et al. 1995; Vernooij et al. 1995).

SAR is initiated by the local response, where hypersensitive reaction (HR) is often involved. The signal thus generated undergoes transmission to other tissues. According to recent evidence, MeSA is necessary for the transmission of the message, but other molecules such as JA and ethylene may also be involved (Verberne et al. 2003; Park et al. 2007; Truman et al. 2007). Truman et al. (2007) suggested that JA signal precedes SA and MeSA in the activation of early systemic defence responses. MeSA is converted by SABP2 (MeSA esterase) into free SA in the target tissue and subsequent changes induced by SA in the redox balance and conformation of NPR1 can further activate defence as described for the local response (Mou et al. 2003).

Protection against the second pathogen attack develops within hours, days or weeks depending on the SAR inducer and plant species, but usually the protection lasts for weeks (Schneider et al. 1996). Defence genes such as those encoding PR proteins and enzymes for phenylpropanoid synthesis, may be directly induced by the first pathogen challenge or by biological or chemical SAR activators. Hence, the following changes in protein and metabolite profiles and cell wall structure may explain the improved resistance against the second attack (Malamy et al. 1990; Ward et al. 1991; Görlach et al. 1996). However, a new wave of gene expression, faster and stronger than the original, is active after the second challenge (Shirasu et al. 1997; Benhamou & Belanger 1998a; Kohler et al. 2002; Truman et al. 2007). Actually, some defence-related genes are expressed only after the second challenge unless a high dose of the first inducer is applied (van Hulten et al. 2006). The sensitization of the plants by SAR inducer for a quick response to the following pathogen attack, i.e. priming, might be the way to "remember" the first attack in plants, but what the primed state actually is, remains unresolved. One option is the accumulation of inactive signalling components, which are rapidly activated during the re-challenging. Consistent with this idea, a SA analogue (BTH) induces the synthesis of MAPK3 protein in *Arabidopsis*, but only a subsequent stress actually activates the protein (Beckers & Conrath 2007). The same signalling components are likely used both during the first and second wave of defence activation. Supporting this, Kohler et al. (2002) showed that potentiated gene expression also depends on NPR1. Furthermore, priming effect occurs in the expression of EDS1 and PAD4, which are required for the development of SAR (Shirasu et al. 1997; Jirage et al. 2001). Van Hulten et al. (2006) suggested that, in contrast to direct activation of the whole defence, by limiting the final systemic defence expression to conditions where it is of absolute necessity, i.e. upon a new pathogen attack, plants can compromise between the

consumption of energy in defence or active growth.

Growth-promoting non-pathogenic rhizobacteria such as *Pseudomonas fluorescens* in *Arabidopsis* may also trigger systemic protection via roots that is called induced systemic resistance (ISR). Although it largely resembles SAR, signalling is found to depend on JA rather than SA, and a different set of genes is expressed (Pieterse et al. 2001; Verhagen et al. 2004). Otherwise, large spectrum resistance and priming are also involved in ISR similarly to that in SAR although resistance is active against different pathogens (Ton et al. 2002; Pieterse et al. 2001). The recent finding about JA as part of SAR signalling may lead to new discoveries about the connections between ISR and SAR (Truman et al. 2007).

2.4 Application of SA-induced defence in plant protection

2.4.1 BTH as a model compound

Exogenously applied SA as well as the accumulation of endogenous SA induces resistance to viral, bacterial and fungal, particularly biotrophic, pathogens as described above. In addition to SA, its derivatives such as 2,6-dichloroisonicotinic acid (INA) and more recently introduced benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), can induce similar wide-spectrum, non-specific resistance and activation of PR proteins (**Figure 5**) (Ward et al. 1991; Görlach et al. 1996; Lawton et al. 1996). While exogenously applied SA and INA are phytotoxic, plants tolerate BTH at the low rates usually needed for defence activation (Tally et al. 1999). Therefore, it was released into the market as the first non-toxic plant protection product (Bion®, Actigard®) which takes advantage of SA signalling pathway to induce plant defence reactions against pathogens (Lawton et al. 1996; Görlach et al. 1996; Tally et al. 1999). During the past decade, BTH has become a model compound for induced resistance in plants, and its function and efficacy has been widely studied. As an efficient inducer of resistance, BTH could also be used to study essential components of pathogen defence in specific plant-pathogen interactions by identifying BTH-induced changes in the plant. Recently, differently substituted salicylates were tested for their ability to induce TMV resistance and PR-1 in tobacco (Silverman et al. 2005). Derivatives halogenated (Cl^-, F^-) in the 3- or 5-position to hydroxyl group were the most efficient inducers of both resistance and PR-1. These observations suggest that new compounds with higher specificity and lower active concentrations may find their way for plant protection in the near future.

BTH has been considered to act as a functional analogue of SA. This conclusion has been drawn from the following observations: BTH is able to induce disease resistance and PR genes independently from SA in *nahG* plants and no accumulation of endogenous SA has been observed after BTH treatment, indicating that BTH acts as SA or downstream of SA (Lawton et al. 1996; Friedrich et al. 1996). Since the action of BTH is suppressed in *nim1/npr1* (*nim1* is allelic with *npr1*) *Arabidopsis* mutants defective in NPR1, BTH acts upstream of NPR1 and, thus, is likely to act "as SA" in plants (Lawton et al. 1996; Kohler et al. 2002; Mou et al.

2003). BTH also inhibits catalase and ascorbate peroxidase similarly to SA and INA and, hence, reinforces the oxidative burst (Wendehenne et al. 1998). Finally, BTH activates the same SAR genes as SA or biological SAR inducer in *Arabidopsis* and tobacco though the induction is stronger with BTH (Friedrich et al. 1996; Lawton et al. 1996). BTH binds with a higher affinity to SA-binding proteins, such as SABP2 (hydrolyzing MeSA to SA) and catalase when compared with SA, which likely explains the stronger responses induced by BTH (Du & Klessig 1997; Wendehenne et al. 1998).

BTH induces systemic resistance and expression of defence genes in many plant species, including cauliflower, cucumber, grapevine and apple (Godard et al. 1999; Narusaka et al. 1999; Brisset et al. 2000; Iriti et al. 2005). Interestingly, BTH but not SA induces systemic response in cucumber, suggesting a better ability of BTH to induce systemic signal, slower turnover of BTH to metabolites or different ability to move in the plant in comparison to SA (Narusaka et al. 1999). Considering that BTH indeed is mobile

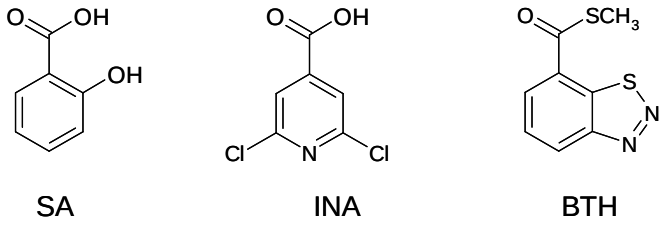

Figure 5. Structures of SA, INA and BTH.

in plants, translocation of BTH itself may be responsible for its systemic action (Friedrich et al. 1996). Friedrich et al. (1996) suggested that since BTH is a functional analogue of SA, it might also activate the signal transduction pathway similarly to SA and, thus, activate SAR. However, MeSA, if it is the systemic signal as proposed by Park et al. (2007), is generated from free SA, the level of which was shown not to be affected by BTH. Furthermore, it is still unknown whether and how the synthesis and release of MeSA is activated up- or downstream of SA in the SAR pathway.

Consequently, despite of several studies performed with BTH, its exact mode of action remains obscure and several questions unanswered. What is, for example, the role of early defence components such as MAPKs in the BTH-mediated resistance (Song & Goodman 2002)? Does BTH degrade in plants? If yes, how fast does it happen and what are the metabolites?

2.4.2 Wide spectrum resistance generated by BTH

Like SA, BTH induces resistance to a wide range of pathogens as shown by the high number of studies performed since the introduction of BTH in 1996. **Table 1** shows the plant pathogens against which BTH has shown efficacy. Most fungal species are biotrophs or hemibiotrophs, further indicating that SA pathway is more effective against biotrophic than necrotrophic pathogens. BTH has protected most plant species from powdery mildews, downy mildews and oomycete (*Phytophthora*, *Pythium*)

pathogens. However, illustrating the variations in the signalling network, potato and *Arabidopsis*, two rather different plant species, show SA-independent resistance to *Phytophthora* infection and, thus, application of BTH does not markedly prevent infection (Si-Ammour et al. 2003). On the other hand, strawberry, tobacco, cucumber and tomato, a close relative of potato, develop resistance to *Phytophthora* after BTH treatment (Tally et al. 1999; Csinos et al. 2001; Eikemo et al. 2003; Khan et al. 2004). In the same way, the efficacy of BTH against necrotrophic diseases varies among plant families. Only in some plant species BTH has provided protection to necrotrophic diseases such as grey mould (*Botrytis cinerea*), leaf spots caused by *Alternaria* sp., *Fusarium* wilts and white mould (*Sclerotinia sclerotiorum*) (Benhamou & Bélanger 1998a; Dann et al. 1998; Terry & Joyce 2000; Perez et al. 2003). The resistance has usually been only partial also in these species and often related to the accumulation of phenolic compounds. A special means to exploit BTH-induced resistance is to treat the plants prior to harvest and thus improve resistance to post-harvest pathogens (Iriti et al. 2005).

2.4.3 BTH-induced changes in plant metabolism

Overall, three types of changes in plant metabolism reflect the induced resistance: accumulation of defence-related proteins, accumulation of antimicrobial secondary compounds (phytoalexins) and alterations in primary metabolism due to the direction of energy and metabolites for defence. Similarly, BTH-induced changes belong mainly to these categories as shown in **Table 1**. BTH may cause direct or primed activation of defence genes and its concentration possibly influences the way of induction (van Hulten et al. 2006; Kohler et al. 2002).

The accumulation or activity of PR proteins has been the primary target for analysis in many BTH-related studies. In particular, chitinase (PR-3, -4, -8, -11), glucanase (PR-2) and peroxidase (POX; PR-9) activities have been determined often, probably due to simple spectrophotometric methods available. Practically all plant species show the induction of these proteins (e.g. Dann & Deverall 2000; Brisset et al. 2000; Zhu & Ma 2007). Among the proteins, POX activity is quite interesting, as peroxidases not only produce reactive oxygen species that possess antimicrobial or signalling activity but peroxidases also reinforce the cell walls by cross-linking phenolics (lignification and suberization), structural glycoproteins and polysaccharides (Hiraga et al. 2001). PR-1 is another commonly analysed protein because of its function as a marker for the SA-induced defence (Lawton et al. 1996; Cameron et al. 1999). The induction of PR-5, thaumatin-like protein, by BTH has been observed in *Arabidopsis*, tobacco, maize, rose and sugarcane (Lawton et al. 1996; Friedrich et al. 1996; Morris et al. 1998; Ramesh Sundar et al. 2001; Suo & Leung 2002) while Ziadi et al. (2001a) found the induction of PR-10 in apple. BTH has both induced (glutathione reductase, superoxide dismutase) and repressed (catalase and ascorbate peroxidase) enzymes used against oxidative stress in cells (Wendehenne et al. 1998; Liu et al. 2005; Cao & Jiang 2006). Thus, BTH may affect the activation of downstream components of the SA pathway by regulating redox balance similarly to SA.

Table 1. BTH-induced resistance and biochemical parameters measured in different plant species[a].

Plant species	Efficacy against Pathogens[b]	BTH mM[c]	BTH-induced mRNA/proteins/metabolites	Reference
apple	*Erwinia amylovora* (Bac)	0.45-0.9	β-1,3-glucanase, POX	Brisset et al. 2000
Arabidopsis	*Peronospora parasitica* (B) *Pseudomonas syringae* pv. *tabaci* (Bac) turnip crinkle virus	0.3	PR-1, PR-2, PR-5, PAL, callose	Friedrich et al. 1996; Lawton et al. 1996; Kohler et al. 2002
banana	*Colletotrichum musae* (HB) *Mycosphaerella fijiensis* (N)	n.r.	PAL, POX, PPO, catalase, β-1,3-glucanase, chitinase	Tally et al. 1999; Zhu & Ma 2007
barley	*Blumeria graminis* f. sp. *hordei* (B)	0.3	putative calcium binding EF-hand protein, homologues of receptor-like kinase, ATP-binding Clp-protease regulatory subunit, WIR1B	Wiese et al. 2003; Jansen et al. 2005
bean	*Colletotrichum lindemuthianum* (HB) *Uromyces appendiculatus* (B)	0.0001-1	H_2O_2, POX, lignin	Bigirimana & Höfte 2002; Iriti & Faoro 2003
calla lily	*Pectobacterium carotovorum* (Bac)	0.005	n.a.	Luzzatto et al. 2007
cashew	*Colletotrichum gloeosporioides* (HB)	0.07	n.a.	Lopez & Lucas 2002
cauliflower	*Peronospora parasitica* (B)	0.2	n.a.	Godard et al. 1999
chilipepper	*Colletotrichum* sp. (HB)	n.r.	n.a.	Tally et al. 1999
cocoa	n.a.	2.0	caffeine, 74 different mRNAs	Aneja & Gianfagna 2001; Verica et al. 2004
coffee	n.a.	0.3	55 genes up-regulated and 16 down-regulated	De Nardi et al. 2006
cotton	*Alternaria macrospora* (N) *Verticillium dahliae* (N) *Xanthomonas campestris* (Bac)	0.15	β-1,3-glucanase	Colson-Hanks & Deverall 2000a; Colson-Hanks et al. 2000b
cowpea	*Colletotrichum destructivum* (HB)	0.1	PAL, CHI, isoflavonoids, low molecular weight PR-proteins	Latunde-Dada & Lucas 2001
cucumber	*Cladosporium cucumerinum* (B) *Colletotrichum lagenarium* (HB) *Colletotrichum orbiculare* (HB) *Phytophthora capsici* (HB) *Pythium ultimum* (N)	0.01-1.5	PR-1a, PR-8, three other mRNAs, chitinase, POX	Benhamou & Belanger 1998b; Ishii et al. 1999; Narusaka et al. 1999; Cools & Ishii 2002; Bovie et al. 2004; Khan et al. 2004
cyclamen	*Fusarium oxysporum* f. sp. *cyclaminis* (N)	0.2	n.a.	Elmer 2006
grapevine	*Botrytis cinerea* (N)	0.3	proanthocyanidins, anthocyanins, stilbens, amino acids	Iriti et al. 2005
lettuce	*Bremia lactucae* (B)	n.r.	n.a.	Tally et al. 1999
maize	*Peronosclerospora sorghi* (B)	1.2	PR-1, PR-5	Morris et al. 1998

Table 1. Continued.

Plant species	Efficacy against Pathogens[a]	BTH mM[b]	BTH-induced mRNA/proteins/metabolites	Reference
melon	*Didymella bryoniae* (N) *Podosphaera xanthii* (B) *Pseudoperonospora cubensis* (B) *Sclerotinia sclerotiorum* (N)	0.1	chitinase, POX	Buzi et al. 2004; Bokshi et al. 2006
oilseed rape	*Leptosphaeria maculans* (N)	0.1	PR-1	Borges et al. 2003
papaya	*Phytophthora palmivora* (HB)	0.005-0.5	chitinase, β-1,3-glucanase, NPR1, PR-1b,d; PR-4, 24 other mRNAs	Zhu et al. 2003; Qiu et al. 2004
pea	*Mycosphaerella pinodes* (N) *Pseudomonas syringae* pv. *pisi* (Bac) *Uromyces viciae-fabae* (B)	0.09-0.45	β-1,3-glucanase, chitinase	Dann & Deverall 2000
peach	*Penicillium expansum* (N)	0.45	PAL, PPO, POX, SOD, total phenolics, H_2O_2	Liu et al. 2005
pear	*Alternaria alternata* (N) *Erwinia amylovora* (Bac) *Gymnosporangium asiaticum* (B) *Penicillium expansum* (N) *Venturia nashicola* (HB)	0.3-0.9	PAL, POX, PPO, chitinase, β-1,3-glucanase, glutathione reductase, H_2O_2	Ishii et al. 1999; Sparla et al. 2004; Cao & Jiang 2006
pearl millet	*Sclerospora graminicola* (B)	0.2-2.0	n.a.	Geetha & Shetty 2002
pepper	*Phytophthora capsici* (HB) *Xanthomonas* sp. (Bac)	0.1-0.3	n.a.	Romero et al. 2001; Matheron & Porchas 2002
potato	*Alternaria solani* (N) *Erysiphe cichoracearum* (B) *Fusarium semitectum* (N)	0.1-0.4	β-1,3-glucanase, PR-1	Bokshi et al. 2003; Navarre & Mayo 2004
rice	*Magnaporthe grisea* (N) *Xanthomonas oryzae* pv. *oryzae* (Bac)	0.004	lipoxygenase	Schweizer et al. 1999; Babu et al. 2003
rose	*Diplocarpon rosae* (HB)	0.05	PR-1, PR-2, PR-3, PR-5	Suo & Leung 2002
soybean	*Sclerotinia sclerotiorum* (N)	0.16-1.7	n.a.	Dann et al. 1998
spinach	*Albugo occidentalis* (B)	n.r.	n.a.	Leskovar & Kolenda 2002
strawberry	*Botrytis cinerea* (N) *Phytophthora cactorum* (HB) *Phytophthora fragariae* var. *fragariae* (HB)	1.0-9.0 0.04-4.5	n.a.	Terry & Joyce 2000; Eikemo et al. 2003
sugarcane	*Colletotrichum falcatum* (HB)	0.45	PR-5, chitinase, β-1,3-glucanase, total phenolics	Ramesh Sundar et al. 2001

Table 1. Continued.

Plant species	Efficacy against Pathogens[a]	BTH mM[b]	B BTH-induced mRNA/proteins/metabolites	Reference
sunflower	*Orobanche cumana* (weed) *Plasmopara halstedii* (B) *Puccinia helianthi* (B)	0.05-0.18	scopoletin, H_2O_2, chitinase, POX	Sauerborn et al. 2002; Roldán Serrano et al. 2007; Amzalek & Cohen 2007
tall fescue	*Bipolaris sorokiniana* (HB)	0.3	POX	Kilic-Ekici & Yuen 2004
tobacco	*Alternaria alternata* (N) *Cercospora nicotianae* (N) *Erwinia carotovora* (Bac) *Oidium neolycopersici* (B) *Peronospora tabacina* (B) *Phytophthora parasitica* var. *nicotianae* (HB) *Pseudomonas syringae* pv. *tabaci* (Bac) *Thanatephorus cucumeris* (N) tobacco mosaic virus tomato spotted wilt virus	1.0-2.2	PR-1a,b; PR-2, PR-3, PR-4, PR-5, acidic and basic chitinase, basic glucanase, POX, ribonuclease, phosphomonoesterase, phosphodiesterase, glucose-6-phosphate dehydrogenase	Friedrich et al. 1996; Cole 1999; Csinos et al. 2001; Šindelářova et al. 2002; Perez et al. 2003; Achuo et al. 2004
tomato	*Alternaria solani* (N) *Botrytis cinerea* (N) *Cladosporium fulvum* (B) *Clavibacter michiganensis* f. sp. *michiganensis* (Bac) cucumber mosaic virus *Fusarium oxysporum* f. sp. *radicis-lycopersici* (N) *Phytophthora infestans* (HB) *Pseudomonas syringae* pv. *tomato* (Bac) *Ralstonia solanacearum* (B) *Xanthomonas* sp. (Bac)	0.007-1.5	H_2O_2, POX, lycopene	Lawton et al. 1996; Tally et al. 1999; Benhamou & Bélanger 1998a; Inbar et al. 1998; Anfoka 2000; Louws et al. 2001; Baysal et a.. 2003; Achuo et al. 2004; Małolepsza 2006; Hacisalihoglu et al. 2007; Iriti et al. 2007
wheat	*Blumeria graminis* f. sp. *tritici* (B) *Puccinia recondita* (B) *Septoria tritici* (HB)	0.3	lipoxygenase, cysteine proteinase, three other proteins (WCI), PAL, POX, 24 different mRNA including PR-1a,b; PR-2, hydroxycinnamic acids	Görlach et al. 1996; Stadnik & Buchenauer 2000; Pasquer et al. 2005

[a] The table was compiled using the data published until November 2007. [b] Fungal pathogenesis behaviour: B, biotrophic; HB, hemibiotrophic; N, necrotrophic. Bac, bacterium. [c] Concentration of BTH is expressed as active ingredient (50% formulation). CHI, chalcone isomerase; PAL, phenylalanine ammonialyase; POX, peroxidase; PPO, polyphenoloxidase; SOD, superoxide dismutase; n.a., not analyzed; n.r., not reported.

The second class of changes relates to the accumulation of soluble or cell-wall-bound secondary metabolites such as phenylpropanoids (phenolics) and callose (Benhamou & Bélanger 1998b; Stadnik & Buchenauer 2000; Iriti et al. 2005). Although the level of such compounds may increase in the whole leaf or plant, more often the accumulation is located at the site of pathogen penetration (Benhamou & Bélanger 1998b; Stadnik & Buchenauer 2000). The role of phenolics in BTH-induced resistance to powdery mildew was evidenced in wheat by using an inhibitor of phenylalanine ammonialyase (PAL), the central enzyme required for the synthesis of various phenylpropanoids (**Figure 3)** (Stadnik & Buchenauer 2000). PAL-inhibited plants could not develop resistance otherwise induced by BTH. Additionally, enzymes of the phenylpropanoid pathway such as PAL and chalcone isomerase show enhanced activities after BTH treatment, demonstrating the activation of the pathway (Latunde-Dada & Lucas 2001; Zhu & Ma 2007). Accumulation of some family-specific secondary compounds also occurs after BTH treatment, e.g. resveratrol in grapevine, lycopene in tomato and caffeine in cocoa (Aneja & Gianfagna 2001; Iriti et al. 2005; Iriti et al. 2007). On the other hand, some secondary metabolites are linked to JA/ethylene-mediated rather than SA-mediated defence. For example, alkaloids and volatile terpenes are typically JA-inducible (Keinänen et al. 2001; Martin et al. 2003a). However, no clear boundaries exist in the classification of secondary compounds as SA- or JA-inducible and different signals may induce different members of the same class of compounds (Mikkelsen et al. 2003). Interestingly, many of these plant defence compounds may also have protective function against human diseases (Hooper & Cassidy 2006). This may invoke novel strategies to improve the nutritional quality of crops simply through standard plant protection practices (Fumagalli et al. 2006).

Besides secondary metabolism, activation of broad-spectrum defence by SAR inducers affects the primary metabolism. Consequently, slower vegetative growth or smaller crop yield has often, but not always, been detected as a side effect of BTH application in uninfected plants (Godard et al. 1999; Dietrich et al. 2005). Likewise, pathogen- or elicitor-induced defence redirects plant metabolism to the production of secondary compounds while nonessential primary metabolism is suppressed (Somssich & Hahlbrock 1998). In general, the trade-off between growth and defence by phenolics has been recognised e.g. in tree species, where high nitrogen levels directs the metabolism to growth and less phenolics are synthesized (Hakulinen et al. 1995). Similarly, the available nutrients, length of the growing period and BTH concentration can affect the extent of growth retardation by BTH (Dietrich et al. 2005; van Hulten et al. 2006).

When more elegant and efficient profiling methods have become available, more information about BTH- and pathogen-induced changes in plant metabolism is emerging (Pasquer et al. 2005; Curto et al. 2006). By combining these data with the analysis of resistance, it might be possible to identify the proteins critical to resistance against certain type of pathogens as well as to construct an informative general view about plant metabolism in induced resistance.

2.5 Inorganic salts in plant protection

2.5.1 Chemical inducers of resistance

A wide variety of natural or synthetic compounds can elicit defence responses in plants. Besides SA and its analogues, other organic compounds such as signalling molecules JA, MeJA and ethylene, unsaturated fatty acids, β-aminobutyric acid, chitosan, nicotinamide, probenazole, harpin and riboflavin have resistance-inducing activity against some pathogens (reviewed e.g. by Schneider et al. 1996; Oostendorp et al. 2001; Gozzo 2003). Among inorganic compounds, salts of phosphoric acid and phosphorous acid, bicarbonates, calcium chloride, silica and oxalates give a similar type of effect (Schneider et al. 1996; Oostendorpet al. 2001; Ehret et al. 2002). A common characteristic of organic inducers capable of inducing defence responses in plants is that they are either of microbial origin, plant-derived molecules released by microbes, or various signalling compounds or their derivatives whereas the basis for resistance induced by inorganic salts seems more uncertain. Nevertheless, some of these compounds are not classical SAR inducers as they induce only partial response, the response is not systemic or they also show direct antimicrobial activity. One interesting class of compounds, which has shown efficacy particularly against biotrophic diseases, is the salts of phosphorus. Benefits in using them in plant protection include their low toxicity, low price and fertilizing capacity.

2.5.2 Phosphates versus phosphites

Two types of phosphorus salts have been exploited in plant protection. Phosphoric acid (H_3PO_4) forms phosphate salts whereas phosphorous acid (H_3PO_3) forms phosphite salts (also called as phosphonates), the difference being in the oxidation state (III or V) of phosphorus (**Figure 6**). This seemingly small difference results in quite distinct properties of phosphates versus phosphites.

Phosphates are used as fertilizers and principal source of phosphorus for plants in agriculture, but in some cases, when given as a foliar spray, disease resistance of plants is improved. Mono- and dibasic potassium phosphates, KH_2PO_4 and K_2HPO_4, have shown efficacy against e.g. powdery mildews on barley, cucumber, pepper, and tomato (*Blumeria graminis* f. sp. *hordei*, *Sphaerotheca fuliginea*, *Leveillula taurica*, and *Erysiphe oronti*, respectively), anthracnose (*Colletotrichum lagenarium*) on cucumber, rust (*Puccinia sorgi*) and leaf blight (*Exserohilum turcicum*) on maize and rice blast (*Pyricularia oryzae*) (Reuveni et al. 1996; Reuveni et al. 1998; Manandhar et al. 1998; Reuveni et al. 2000; Ehret et al. 2002; Orober et al. 2002). Phosphates are not toxic to pathogens, implying that the observed enhancement of resistance is due to the activation of plant defence. This is also supported by the systemic nature of protection and SAR-related changes observed in cucumber and barley after phosphate treatment, i.e. localized cell death, oxidative burst, local and systemic accumulation of SA and increased activities of PAL, lipoxygenase and peroxidase (Reuveni et al. 1998; Reuveni et al. 2000; Orober et al. 2002; Mitchell & Walters 2004). Orober et al. (2002) suggested that the ability of phosphates and other SAR-inducing

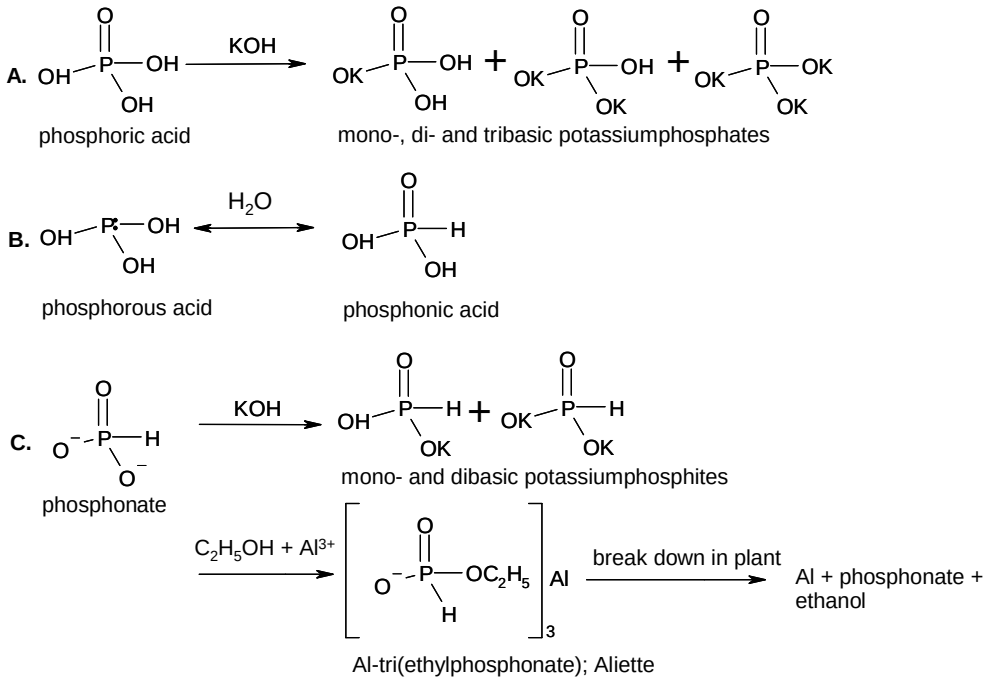

Figure 6. Formation of (A) potassium phosphate salts from phosphoric acid, (B) phosphonic acid from phosphorous acid in water (tautomeric equilibrium), and (C) potassium phosphite salts and aluminium ethylphosphonate (Aliette) from phosphonate ions. By substituting hydrogen with potassium or other alkali metal ions, pH of the solution can be elevated. For example, phosphonic acid is too acidic to be tolerated by plants.

compounds to induce SAR relates to their ability to generate HR. What triggers the initiation of HR and upstream events leading to HR remains, however, ambiguous (Orober et al. 2002). Phosphates have shown efficacy mostly against powdery mildew fungi, which are sensitive even to free water and surfactants (Peries 1962a; Ehret et al. 2002). Thus, the direct influence of phosphates on powdery mildews cannot yet be excluded.

In contrast to phosphates, plants cannot use phosphites as a phosphorus source unless soil microbes convert them to phosphates (McDonald et al. 2001). The efficacy against phytopathogens is also different from that of phosphates, since phosphites are efficient mainly against oomycete pathogens. In fact, phosphites have been used in plant protection against downy mildews and *Phytophthora* species already for thirty years (Cohen & Coffey 1986). The most studied and widely used product is Aliette (Bayer), the active ingredient of which (fosetyl-Al) also breaks down to phosphite in plants (**Figure 6**). Again, the mode of action of phosphites is not fully understood, but evidence obtained thus far indicates that two distinct mechanisms exist. Firstly, phosphorous acid and phosphites have direct antimicrobial activity on oomycetes (Fenn & Coffey 1984; Smillie et al. 1989). Earlier studies showed the accumulation of pyrophosphates and polyphosphates in *Phytophthora*

after phosphite treatment. This can deplete ATP from other processes, inhibit pyrophosphatase activity and thus lead to phosphate starvation and reduced growth of the pathogen (Niere et al. 1994). However, high levels of inorganic phosphate present may counteract the inhibitory effect of phosphites on the growth of *Phytophthora* (Smillie et al. 1989). A study by Stehmann & Grant (2000) indicates that phosphites compete with phosphates as allosteric regulators of several enzymes and thus inhibit e.g. glycolytic enzymes.

Phosphites have also augmented phosphate starvation in plants. This is supported by the finding that phosphite inhibits not only pyrophosphatases but also other enzymes and phosphate transporters (McDonald et al. 2001; Varadarajan et al. 2002). This indicates that phosphites may interfere with phosphate metabolism of plants at least under low-phosphate conditions when there is not enough phosphate to compete with phosphite in binding to plant enzymes. The observed influence of phosphate levels on the action of phosphite on pathogens and plants should guide the design of plant protection and fertilization.

Besides the direct activity on oomycetes, phosphites act indirectly by reinforcing defence reactions in plants. Phosphite treatments induce oxidative burst, HR, PR proteins and accumulation of phenolic compounds (Bécot et al. 2000; Daniel & Guest 2006; Andreu et al. 2006). Further, efficacy of phosphites has shown to depend on SA, NPR1 and the generation of ROS, but not on JA/ethylene (Molina et al. 1998; Daniel & Guest 2006). These findings suggest the activation of SA-mediated SAR pathway although phosphites are not pure SAR inducers due to their direct antimicrobial activity discussed above.

2.6 Phenolic compounds in defence against phytopathogens

2.6.1 Nature of phenolics

The synthesis of phenylpropanoids, or shortly phenolics, takes place via the shikimate pathway as presented for SA (**Figure 3**). The branch point enzyme, PAL, regulates the synthesis of the whole group of compounds including anthocyanins, flavonols, hydroxycinnamic acids and lignin, flavan-3-ols and condensed tannins, isoflavonoids, flavanones, flavones, chalcones, aurones and stilbenes (Dixon & Paiva 1995; Winkel-Shirley 2002). Most of the downstream enzymes leading to the synthesis of various phenolics have been characterized. There is an enormous variation in the form of various aglycones, the conjugates and polymerization products, but the significance of this variation is not clear. Phenolics are probably synthesised on the endoplasmic reticulum and then translocated into vacuoles, particularly in the epidermal cells, or in the cell walls and cuticular waxes (Alcerito et al. 2002; Kutchan 2005; Field et al. 2006). Phenolics are present in all plants, some classes being common to most species while the others are family- or species-specific. The wide occurrence of phenolics suggests an important role in the survival of plants.

The suggested main role of phenolics is in both constitutive and induced defence against stresses such as pathogens, insects, UV and high light radiation, low

temperatures, low nutrient levels and wounding (Dixon & Paiva 1995; Parr & Bolwell 2000). However, phenolics may also be involved in the attraction of pollinators, seed distributors and symbiotic bacteria, in the signalling, in pollen germination, and in the regulation of auxin transport, suggesting a role in the developmental regulation as well (Parr & Bolwell 2000; Taylor & Grotewold 2005). A specific characteristic of many phenylpropanoids is their high antioxidant activity, which has led to extensive research on their potential health-promoting capacity in humans (Parr & Bolwell 2000; Ross & Kasum 2002). Phenolic metabolism is not activated via any specific signalling cascade and, for example, stimuli capable of activating SA-mediated (e.g. biotrophs, UV) or JA/ethylene-mediated (e.g. wounding, insects) signalling may induce the synthesis of phenolics.

2.6.2 Phenolics in Rosaceae

Several fruit-bearing species, including small fruit plants (e.g. raspberry, arctic bramble, cloudberry and strawberry) as well as fruit trees (apple, apricot, cherry and plum) belong to the large and diverse Rosaceae family. Phenolic compounds of rosaceous fruit species have usually been analysed from edible plant parts due to the interest in their nutritional properties whereas leaf phenolics have been measured only in a few studies. Typical phenolic classes present in rosaceous plants are ellagitannins and gallic acid derivatives, anthocyanins, flavonols, hydroxycinnamic acids and some other phenolic acids such as SA, and, to a lesser extent, mono/polymeric proanthocyanidins (Määttä-Riihinen et al. 2004; Aaby et al. 2007). **Figure 7** shows structures of some typical phenolic compounds identified in strawberry fruits (Aaby et al. 2007). The significance of phenolics in the diseases of rosaceous plants is not substantially studied. In the following paragraphs, the role of soluble and cell-wall-bound phenolics in defence against plant pathogens is discussed based on the investigations done mostly on other plant species.

2.6.3 Phenolics related to defence against pathogens

Phenolics may be present constitutively in plants or synthesised only as a response to stresses such as attacks by pathogens. Constitutively present soluble secondary defence compounds, termed phytoanticipins, are usually stored as active or inactive forms in vacuoles. They can be liberated and transferred to the site of infection by vesicle trafficking or by ATP-binding cassette and multidrug/toxic compound extrusion transporter proteins (Dixon & Paiva 1995; Field et al. 2006). Attacks by necrotrophic pathogens often leads to cell death and release of defensive compounds due to the loss of cell compartmentalization. Inducible defence compounds, termed phytoalexins, are synthesised *de novo* only as a response to infection or other stress and may be directly transported to the location where they are needed (Dixon & Paiva 1995; Treutter 2006).

Although the exact mode of action of different phenolics against phytopathogens is largely unknown, evidence indicates that phenolics can disturb microbes in several ways (Dixon & Paiva 1995; Cowan 1999; Goetz et al. 1999; Grace & Logan 2000; Treutter 2006). Phenolics may crosslink with proteins and

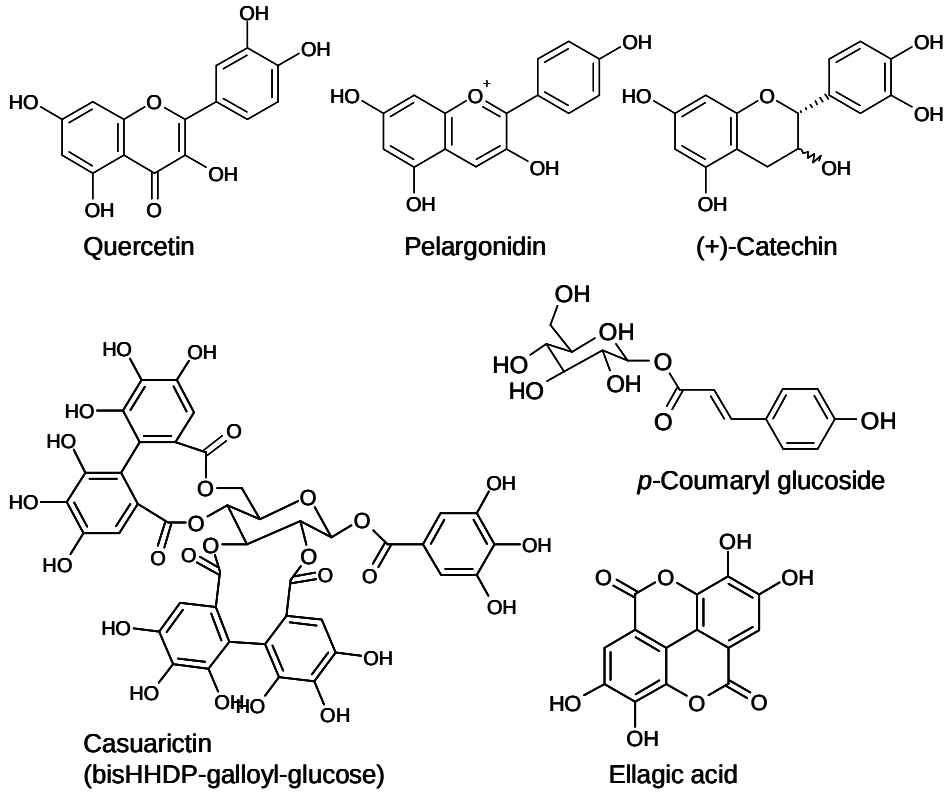

Figure 7. Representative structures of phenolic compounds found in strawberry.

polysaccharides in the cell walls to strengthen the wall against microbial hydrolysing enzymes. Phenolics may bind to proteins and, directly or via chelation of cofactors/substrates, inhibit the hydrolysing enzymes or other proteins of pathogens. By reacting with lipids and phospholipids, phenolics can disturb the membranes of pathogens, which cause increased permeability of membranes and collapse of the microbial cell. Some phenolics function as signalling molecules, SA being the best-known compound. Most phenolics are strong antioxidants and may act in the adaptation to oxidative stress caused by the infection rather than directly affecting the pathogen.

Several conditions should be met to prove the involvement of phenolics or other compounds in the defence against pathogens (Hammerschmidt 1999; Dixon 2001; Treutter 2006). Traditionally, rapid accumulation of a compound at antimicrobial concentrations in response to infection, leading to the restriction of infection and improved resistance, evidences that the compound is a phytoalexin. Additionally, the response is often faster and stronger in the resistant cultivars. Antimicrobial activity and effective concentration can also be determined for pure compounds *in vitro.* Another indication of the harmful nature to the microbe is the detoxification of the plant metabolite by the virulent pathogen (Osbourn 1999). Finally, by changing the activity of a specific metabolic pathway in the plant, its significance in the defence

can be evaluated. There are a number of mutants and genetically modified plants with increased or decreased amounts of secondary compounds, showing improved or lowered tolerance to pathogens, respectively (Dixon & Paiva 1995; Hammerschmidt 1999; Dixon 2001). However, it is difficult to draw conclusions about the functions of individual phenolics due to the very high number of variable structures present in plants. Only a small difference in the structure may change the chemical or physiological activity and a compound may have different functions in different conditions or plant species, for example, being phytoanticipin in one plant species and phytoalexin in another one (Dixon 2001).

2.6.4 Anthocyanins

Anthocyanins, i.e. glycosides of anthocyanidin aglycones, are responsible for the red and blue colours of fruits. The main anthocyanidins are cyanidin, delphinidin, pelargonidin (**Figure 7**), peonidin, petunidin and malvidin (Clifford 2000). Based on the current literature, anthocyanins appear to have a role in plant defence in the adaptation to abiotic stresses, particularly to UV and high light radiation, whereas their contribution to disease resistance may be fairly limited (Dixon & Paiva 1995; Edreva 2005). Supporting this, inoculation with non-pathogenic fungus represses the light-induced synthesis of anthocyanins in sorghum and directs the metabolism to the synthesis of deoxyanthocyanidins instead of anthocyanidins (Lo & Nicholson 1998). Deoxyanthocyanidins lacking the hydroxyl group of the central aromatic ring present in anthocyanidins are present in apple, but not in rosaceous small fruit species. Accumulation of anthocyanins in the proximity to the infection site has been detected in maize and cotton but, in both cases, the role of anthocyanins were suggested to be in the protection from oxidative stress induced during the infection rather than in restricting the growth of the pathogen (Hipskind et al. 1996; Kangatharalingam et al. 2002). This might be a common response against oxidative stress related to the generation of ROS during pathogen attack, since anthocyanins are often responsible for the purple-coloured lesions found in many plant diseases. Genetically modified rice with elevated levels of anthocyanins has higher resistance to blast infection, but other flavonoids such as flavonols may also have influenced the development of resistance (Gandikota et al. 2001). Taken together, the function of anthocyanins in plants may not lie in the direct defence against pathogens, but in the defence against oxidative and photo-oxidative damage.

2.6.5 Flavonols

Flavonols, which are synthesised prior to anthocyanins in the phenylpropanoid pathway, occur as mono- di- or triglycosides of various sugars in many plant species. Quercetin (**Figure 7**) and kaempferol are the most common aglycones, myricetin occurring in some species. The aglycones differ by the number of hydroxyl groups. There is strong evidence that flavonols, similarly to anthocyanins, function as antioxidants and photoprotectants (Lois & Buchanan 1994; Edreva 2005). In contrast to anthocyanins, flavonols may also have a role in the defence against pathogens. Flavonols often occur constitutively at certain levels in plants. The basal level may be

increased under stress conditions, but in some cases, flavonols are also synthesised *de novo* as a response to infection. Fawe et al. (1998) reported the accumulation of antifungal rhamnetin (methyl quercetin) in cucumber after infection by powdery mildew fungus *Sphaerotheca fuliginea*; this is the first report on flavonols as phytoalexins. Later findings showed that flavonoids accumulate in the epidermal cells attacked by the fungus and inside haustoria, thus likely contributing to the collapse of the pathogen (McNally et al. 2003). Further evidence about flavonols and other flavonoids as defence compounds in cucumber was achieved by Fofana et al. (2005), who reported the suppression of induced resistance to powdery mildew as a consequence of down-regulating the flavonoid pathway. The accumulation of flavonols after infection also occurs in olive infected with *Phytophthora megasperma* and *Cylindrocarpon destructans* and in carnation infected with *Fusarium oxysporum* f. sp. *dianthi* (Curir et al. 2005; Báidez et al. 2006). Another indication of the function in defence is the higher/faster accumulation of flavonols or enzymes leading to their synthesis in resistant versus susceptible cultivars, as detected e.g. in grapevine (Kortekamp 2006). Finally, flavonols have shown *in vitro* activity against microbes of both plant and animal origin although the activity varies among the aglycones (Puupponen-Pimiä et al. 2001; Báidez et al. 2006). Flavonols can also inhibit fungal enzymes (Goetz et al. 1999). In some cases, flavonols can function as attractants of beneficial symbiotic fungi or bacteria, but also of pathogenic micro-organisms (Mo et al. 1995; Lagrange et al. 2001). Despite the rather high number of studies conducted on flavonols, only few of them have proposed the mode of action for flavonols.

2.6.6 Ellagitannins

In contrast to other phenolic groups introduced in this chapter, gallic acid and its derivatives are present only in some plant families, including Rosaceae. Actually, gallic acid is, similarly to SA, not a phenylpropanoid, but hydroxylated benzoic acid (**Figure 3**). It is synthesized in the shikimate pathway from dehydroshikimate prior to PAL and can be regulated differently from the phenylpropanoids (Ossipov et al. 2003). Gallic acid may occur as bislactone, ellagic acid (**Figure 7**). Gallic acid and its oxidatively C-C coupled dimer, hexahydroxydiphenic acid (HHDP), form polymeric structures with glucose, which are called ellagitannins (Quideau & Feldman 1996). HHDP also undergoes conversion to ellagic acid once released from the polymers. Variations in the composition and number of subunits, stereochemistry of HHDP and glucose units, and the type of coupling between subunits and monomeric tannins creates an extremely high number of different gallo- and ellagitannins. Gallic acid and HHDP unit may be connected by esterification to the hydroxyl groups of glucose units (**Figure 7**) or they may be directly linked with each other via oxidative C-O coupling, as detected e.g. in dehydrodigalloyl or sanguisorboyl ester units between two gallic acids or gallic acid and HHDP, respectively (Quideau & Feldman 1996).

Very limited information is available about the function of ellagitannins in plant defence against pathogens. Antimicrobial activity of ellagitannins has been proved *in vitro* against bacteria, fungi as well as viruses, but most often animal/human pathogens have been used (Cowan 1999; Puupponen-Pimiä et al. 2001). Latté &

Kolodziej (2000) found ellagitannins ineffective against moulds, but active against yeast. A well-known property of gallo- and ellagitannins is the binding and precipitation of proteins. Gallo- and ellagitannins may inhibit herbivory by affecting the degradability of plant tissue, by inhibiting digestive enzymes or by giving rise to toxic hydrolysis products (Bhat 1998; Makkar 2003). For example, high contents of gallotannins decrease larval feeding on birch leaves (Ossipov et al. 2001). Considering phytopathogens, ellagitannins accumulate in microbe-resistant reaction zones in *Eucalyptus nitens* (Barry et al. 2001). Excluding the woody species, no evidence is available about the function of ellagitannins as phytoalexins or phytoanticipins, but there are some indications that gallic acid and its derivatives provide resistance against pathogens. The levels of gallic acid derivatives have increased in phytoplasma-infected *Catharanthus roseus* (periwinkle) (Choi et al. 2004). Gallic acid and methyl gallate have also been isolated as antifungal constituents from *Rhus javanica*, where antifungal activity was demonstrated against biotrophs (*Erysiphe graminis* and *Puccinia recondita*) and necrotrophs (*Botrytis cinerea, Magnaporthe grisea*) (Ahn et al. 2005). Furthermore, the fungi and bacteria which have a tannase to degrade gallo- and ellagitannins show higher virulence in plants, indicating a defensive role for tannins (Bhat et al. 1998). Tannase activity has not been reported, if even studied, to be present in any biotrophic phytopathogens while its presence has been demonstrated in some necrotrophic species such as *Fusarium*, *Aspergillus* and *Rhizopus* (Bhat et al. 1998). No firm conclusions can be drawn yet concerning the functions of ellagitannins in plant defence against pathogens. However, antimicrobial, antioxidant and protein-binding capacity of gallo- and ellagitannins suggest a protective role in plants. A question remains why such high numbers of different ellagitannin structures are present in plants.

2.6.7 Flavan-3-ols and proanthocyanidins

Flavan-3-ols are synthesised in the phenylpropanoid pathway from flavan-3,4-diols (leucoanthocyanidins), the precursors of anthocyanins (Winkel-Shirley 2002; Dixon et al. 2005). Flavan-3-ol monomers are (+)-catechin (**Figure 7**), (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, (+)-afzelechin and (-)-epiafzelechin, which usually occur in free form (Dixon et al. 2005). If conjugated, gallic acid and glucose are the most common conjugates. Monomers can polymerise and form proanthocyanidins, i.e. condensed tannins, the length of which varies from dimers to large polymers consisting of more than ten monomeric units. The alterations in stereochemistry, subunit composition and ways to polymerise provide for a variety of different molecules. Acid hydrolysis of polymers yield anthocyanidins, explaining the term proanthocyanidin used for the polymers. Flavan-3-ols or their polymers are commonly found in most plant species, but their amount in strawberry and *Rubus* fruits and leaves is low (Oertel et al. 2001; Määttä-Riihinen et al. 2004; Aaby et al. 2007).

The most important function of monomeric and polymeric flavan-3-ols concerns the defence against pathogens and herbivores (Dixon et al. 2005). Monomers are active against bacteria and fungi, the activity possibly deriving from

the property to bind metal ions (Cowan 1999; Gonzalez de Colmenares et al. 1998; Dixon et al. 2005). Flavan-3-ols also possess high antioxidant activity (Plumb et al. 1998). Polymers, similarly to ellagitannins, bind efficiently to proteins. High content of monomeric or polymeric catechins or epicatechins may provide resistance against fungal pathogens in e.g. avocado, cocoa, raspberry, and tea, which contains high amounts of (+)-catechin and its derivatives (Laun et al. 1994, Ardi et al. 1998; Punyasiri et al. 2005; Chaves & Gianfagna 2007). (+)-Catechin has also been identified as the antimicrobial component in strawberry leaves and its level increases during infection by *Alternaria alternata* (Yamamoto et al. 2000). Negative correlation between herbivory by insects or animals and the content of proanthocyanidins have been observed in some cases, but contradictory results have also been recorded (Grayer et al. 1992; Ward & Young 2002; Tikkanen & Julkunen-Tiitto 2003; Makkar 2003). Herbivores can affect the activity of tannins by secreting tannin-binding protein in saliva, which detoxifies tannins (Juntheikki 1996). In conclusion, flavan-3-ols as monomers, oligomers or polymers seem to be important for the defence against pathogens and herbivores in many plants, particularly if present in high concentrations.

2.6.8 Hydroxycinnamic acids

Hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinnamic acids are synthesised from *p*-coumaric acid early in the phenylpropanoid pathway. Hydroxycinnamic acids occur usually as conjugates with various other molecules such as sugars (**Figure 7**), quinic acid, amides, lipids and flavonoids, and are found in some form in most plant species (Clifford 1999). One significant role of hydroxycinnamic acids in plants is probably in the protection from UV radiation since they can act as antioxidants and absorb UV light (Grace & Logan 2000; Edreva 2005). Hydroxycinnamic acids are also among the most studied phenylpropanoids in terms of plant pathogen resistance. Hydroxycinnamic acids show antimicrobial activity *in vitro* and they accumulate as phytoalexins in many plant-pathogen interactions (Daayf et al. 1997; Sander & Heitefuss 1998; Walters et al. 2001; Puupponen-Pimiä et al. 2001; Daayf et al. 2003; Bisogno et al. 2007). Genetically modified tobacco with decreased levels of phenylpropanoids and specifically chlorogenic acid (caffeoylquinic acid) showed higher susceptibility against *Cercospora nicotianae* (Maher et al. 1994). As presented for flavonols, different hydroxycinnamic acids show distinct activities. Bisogno et al. (2007) found that only ferulic acid was antifungal against *Aspergillus* sp. while coumaric acids and caffeic acid had no effect on the fungal growth. Similarly, *p*-coumaroyl-hydroxyagmatine, but not its nonhydroxylated form, acts as an antifungal phytoalexin against powdery mildew in barley (Röpenack et al. 1998). Consequently, no generalisations about the function or activity of individual hydroxycinnamates, or other phenolics as well, can be made because their activity is highly dependent on the particular conditions. However, perhaps the most important function of hydroxycinnamic acids in disease resistance is their crosslinking to the cell walls and acting as precursors for lignin synthesis discussed below.

2.6.9 Cell-wall-bound phenolics

One of the best-established functions of phenolics in the constitutive or induced defence against pathogens is the reinforcement of plant cell walls by crosslinking with other molecules. Cell-wall-associated phenolics may also protect the epidermal cells from UV radiation (Fischbach et al. 1999). Hydroxycinnamic acids, mainly coumaric and ferulic acid, are the principal monomeric phenolics attached to the cell walls (McLusky et al. 1999; Stadnik & Buchenauer 2000; Peyron et al. 2002). Flavonol glycosides, lignin, benzoic acids and benzaldehydes can also serve as constituents of cell walls (Keller et al. 1996; Fischbach et al. 1999; Markham et al. 2000; Martens 2002). The formation of cell walls has been studied mostly on cereals and grasses, where the composition of cell wall has impact e.g. on industrial processing, on the quality of dietary fibre for humans and on the digestion in the ruminants. Ferulic acid is the main cell-wall-bound phenolic in grasses. Through the generation of H_2O_2, peroxidases may cause the formation of ferulic acid radicals, which couple to dehydrodimers (Ralph et al. 2004). Short chains of polysaccharides and lignin are linked via acylation to these ferulic acid dehydrodimers to generate a complex network. The *p*-coumaric acid is not likely to form dehydrodimers, as it undergoes fast radical transfer reactions with other phenolics, but it may crosslink to lignin and polysaccharides by photochemical dimerisation. Etherified ferulates may be more common than etherified *p*-coumarates, but more information is needed to efficiently separate ester- and ether-linked hydroxycinnamates extracted from the cell walls (Ralph et al. 2004). Type of the bond influences the resistance of cell walls and the extractability of the compounds. It is unclear how the phenolics other than hydroxycinnamates are bound to the cell walls.

Pathogen attack and induction of plant defence may lead to the modification of cell walls and the accumulation of specific appositions (papillae), which contain callose, proteins, H_2O_2 and phenolics (Hückelhoven 2007). Particularly, the rapid formation of papillae appears to be critical for the development of resistance against the penetration of biotrophic fungi (Dai et al. 1995; Stadnik & Buchenauer 2000; Hückelhoven 2007). Papillae may also form in susceptible plants, but the response may be too slow to stop the pathogen. A high number of microscopic studies have shown the accumulation of phenolic material at the site of infection and around the invading fungus, the growth of which is ceased (Dai et al. 1995; Dai et al. 1996; Benhamou & Nicole 1999; McLusky et al. 1999; Stadnik & Buchenauer 2000). Increased amounts of alkali-hydrolysable phenolics, usually hydroxycinnamic acids, have been measured in the cell walls of infected plants (Keller et al. 1996; McLusky et al. 1999; Stadnik & Buchenauer 2000). In barley, cell walls become more resistant to alkali treatments during infection by powdery mildew, indicating modification in the cell wall composition (Röpenack et al. 1998).

Lignin is a specific phenolic polymer present in the cell walls of plants, which, in addition to its structural function, has also been associated with defence. Lignin is formed by non-enzymatic oxidative polymerisation and is composed of monolignol subunits *p*-coumaryl, coniferyl and sinapyl alcohols synthesised from the corresponding hydroxycinnamic acids (Whetten et al. 1998). The proportions of

different subunits vary between plant species and conditions. Lignin is highly resistant to degradable enzymes and crosslinking with other phenolics, polysaccharides and proteins further complicate its structure. Lignification of cell walls occurs in many plants during the infection. However, the slow accumulation and contradictory results obtained indicate that lignification is not always required for defence, but might rather be one type of phenolic response to pathogens, which varies among plants (Nicholson & Hammerschmidt 1992; Dai et al. 1995; Dixon & Paiva 1995; Dai et al. 1996; Stadnik & Buchenauer 2000).

AIMS OF THE STUDY

The general aim of this Thesis was to evaluate the efficacy and applicability of induced resistance against powdery mildew of strawberry and downy mildew of arctic bramble and to follow specific defence-related biochemical and chemical changes during the development of resistance.

The aims in the individual studies were to:

- evaluate the efficacy of BTH against powdery mildew in strawberry (**I**)
- analyse the phenolic contents of leaves and fruits in strawberry after the treatment with BTH and powdery mildew conidia (**I**)
- develop quantitative real-time PCR method for detection and quantification of downy mildew (*Peronospora sparsa*) in arctic bramble (**II**)
- evaluate the efficacy of different agrochemicals against downy mildew in arctic bramble under greenhouse conditions (**III**)
- analyse the growth of *Peronospora sparsa*, the phenolic content and protein profiles in arctic bramble leaves after the treatment with agrochemicals (**III, IV**)

3. MATERIALS AND METHODS

3.1 Plant and fungal material, experimental design and treatments

3.1.1 Strawberry (I)

For the evaluation of the efficacy of BTH against powdery mildew of strawberry and to analyse possible biochemical changes caused by the BTH treatment in strawberry leaves, two experiments were carried out in the greenhouse of the University of Kuopio. Micropropagated plants of cv. Jonsok were used in the main experiment performed in 2004 while bare-root, cold-stored frigo plants of cv. Jonsok were used in the second experiment in 2005, which was established to obtain fruits for the analysis of phenolic compounds.

All plants were grown in 12–cm pots in a peat-sand mixture (3:1) and fertilised weekly with Superex-9 (Kekkilä, Finland). The growth conditions were as follows: relative humidity 50-70% (night-day), daylight 20-24 h and temperature 17–22 °C (night-day). Before starting the experiments, plants were grown until they had at least three fully developed, normal size leaves. In both experiments, the plants were divided randomly into four different groups (100 plants in each group), which were placed on separate tables to prevent spreading of contamination from the other treatments. Two groups were sprayed with distilled water and the other two groups with 0.8 g/l of BTH (Bion® 50WG, Syngenta) in distilled water until run-off (ca. 5 ml per plant).

Two days after the BTH treatment the strawberry plants were inoculated with powdery mildew (*Sphaerotheca macularis*) conidia by shaking sporulating leaves above the plants. Only one group of water- and BTH-treated plants were inoculated whereas the other two groups were left untreated. Evaluation of spore density was done by calculating conidia fallen on glass plates placed among the plants. From every plant, diseased area was measured (as mm^2) from two leaves selected prior to inoculation and the number of powdery mildew patches, infected and healthy leaves were calculated twice a week. Diseased leaves for the inoculation were from cv. Jonsok, on which powdery mildew was maintained by regularly re-inoculating new, young plants.

Strawberry leaves were sampled 0, 2, 4 and 7 days after inoculation i.e. 2, 4, 6 and 9 days after water/BTH treatment. For laboratory analyses, five different plants per treatment per sampling time were randomly selected and from each of those plants, one young, full-size leaf per plant was collected separately in liquid nitrogen. Each plant was sampled only once to avoid an eventual cutting-effect. The samples were stored at -70 °C. Fruits were sampled only in the second experiment in 2005. Ripe fruits from each plant were harvested and weighed separately and stored at -20 °C. After cropping, leaves were cut off and weighed (fresh weight).

3.1.2 Arctic bramble (II-IV)

Arctic bramble cvs Pima and Muuruska were grown *in vitro* as micropropagated plantlets in MS medium (Murashige & Skoog 1962) containing 0.5 mg/l 6-benzylaminopurine, 0.25 mg/l indole-3-butyric acid and 2 ml/l plant preservative mixture (PPM™) for the development and testing of the quantitative real-time PCR method (**II**). Mature arctic bramble leaves showing downy mildew symptoms as well as the leaves of cloudberry (*Rubus chamaemorus*), strawberry and *Rubus arcticus* subsp. *stellatus* were obtained from the germplasm of the University of Kuopio between years 2003-2005 (**II**). *Peronospora sparsa* was isolated from arctic bramble cv. Pima in 2005, and maintained on detached leaves of micropropagated arctic bramble on sterile 0.8% water agar plates. The quickest and most abundant sporulation was obtained at temperatures ≤ 22 ºC with daylight period of approximately 15-20 h. The leaves from *in vitro*-grown plants were detached and placed abaxial surface uppermost on water agar plates. The leaves were inoculated by applying a drop of conidial suspension on the leaves in a sterile hood. The development of infection was followed by calculating sporangiophores under a stereomicroscope every other day. Three parallel samples, each consisting of approximately five *in vitro* leaves, were collected from cv. Pima and Muuruska on water agar plates 4 and 7 days after inoculation. The samples were stored at -20 °C.

A greenhouse experiment was carried out with cvs Pima and Mespi and a new arctic bramble clone (12B14) in 2007 to evaluate their basal resistance and the efficacy of several agricultural chemicals against downy mildew (*Peronospora sparsa*) (**III**). All the plants were propagated vegetatively in the greenhouse and kept dormant below 4 °C until the start of the experiment. Plants were grown in 2 l pots in peat-sand-vermiculite (14:3:3) under the following conditions: relative humidity 70%, daylight 18 h and temperature 13–18 °C (night-day). Five plants per cultivar were randomly selected for each treatment at the beginning of flowering. The treatments applied for each cultivar are shown in **Table 2**. The plants were sprayed until run-off and differently treated groups were kept separated for the first 12 h, after which the plants were placed randomly on tables. Prior to inoculation, three leaves were detached from each plant and placed abaxial side up on 0.8% water agar plates for the inoculation. The inoculation on the plates was done as described above (**II**). In the greenhouse, controlled inoculation with *P. sparsa* spores was done four days after the chemical treatments. Fresh spores were suspended in sterile water (7 500 spores/ml) with 0.025% surfactant (Silwet Gold, Chemtura) and the mixture was sprayed on all plants until run-off (ca. 2 ml per plant). Relative humidity was raised to 95% after inoculation to facilitate germination of the spores. Symptoms of downy mildew were evaluated in each plant twice a week for one month by counting reddish angular lesions, which usually carried visible sporangiophores on the abaxial side of the leaf. The plants were sampled 0 and 8 days after inoculation i.e. 4 and 12 days after the chemical treatments. Two to three leaves of each plant were collected separately in liquid nitrogen at both sampling points and stored at -70 °C. Of the detached leaves inoculated on water agar plates (five plates per treatment/cultivar), one leaflet per plate was collected 2, 4 and 6 days after the inoculation and stored at -20 °C.

For protein analyses (**IV**), cvs Pima and Mespi were grown in a greenhouse as described above for study **III**. Plants were divided in two groups, one of which was sprayed with distilled water and the other one with 0.6 g/l of BTH at the beginning of flowering. The study (**IV**) was repeated twice and in the second time only cv. Mespi was used. Three parallel plants were sampled 0, 2, 4, 7 and 11 days after BTH treatment in the first experiment. In the other two studies, sampling was done only 4 days after BTH treatment, which was found to be the point of maximal induction for PR-1 protein.

Table 2. Agricultural chemicals applied on arctic bramble cultivars (**III**).

Product (manufacturer)	Active ingredient	Concentration	Applied on cvs
Distilled water, control	-	-	Pima, Mespi, 12B14
Bion 50WG (Syngenta)	benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH)	0.005% (w/v) 0.04% (w/v)	Pima Pima, 12B14
Aliette 80WG (Bayer)	fosetyl-aluminum	0.3% (w/v)	Pima, 12B14
Euparen M (Bayer)	tolylfluanid	0.15% (w/v)	Pima
Farm-Fos-44 (Farm-Fos Ltd)	potassium salt of phosphorous acid	0.5% (v/v)	Pima
Kaliumfosfiet (van Iperen)	potassium salt of phosphorous acid	0.4% (v/v)	Pima
Phosfik (Kemira Growhow Oyj)	salts of phosphorous acid	0.5% (v/v)	Pima, 12B14
Phostrol (Nufarm Americas Inc.)	potassium, sodium and ammonium salts of phosphorous acid	0.4% (v/v)	Pima

An outdoor experiment was also run with cvs Pima and Mespi in the Research Garden of the University of Kuopio for two successive years in 2004-2005 to evaluate agricultural chemicals against *P. sparsa* spreading naturally from the environment (**IV**). The plants were grown in 20 l plastic boxes, six plants in each. Four of these boxes were used for each treatment. Similarly to the greenhouse experiments, the plants were sprayed with distilled water, 0.6 g/l of BTH, 0.5% (v/v) Farm-Fos-44, 0.5% KH_2PO_4 (w/v) or 0.3% (w/v) Aliette 80WG prior to flowering, but in this experiment the treatments were repeated two more times in approximately ten-day intervals. At the end of the second season, the aerial biomass was collected from each box separately and dry weight was determined. The rhizome from each box was also washed and the fresh weight was determined.

3.1.3 Microscopy of pathogens

Fresh leaves or leaf disks were used for microscopy. For trypan blue staining, the leaves were boiled twice in ethanol-staining mixture (1:2) for 1 min and incubated in the mixture at room temperature for 4 h. The staining mixture consisted of water-glycerin-acetic acid-phenol (1:1:1:1) and 0.05% (w/v) trypan blue. Excess colour was removed by washing in water. Samples were mounted in water-glycerin (2:1).

For aniline blue staining, the leaves were cleared and fixed in ethanol overnight. The samples were washed in water and transferred in phosphate buffer (pH 9) for two hours, after which they were transferred into 0.005% aniline blue in phosphate buffer and mounted in the stain on the slides. For some samples, 1/30 vol. of fluorescence

brightener (Sigma) stock solution (0.1% in Tris-HCl, pH8.8) was added.

3.2 Extraction and analysis of phenolic compounds

3.2.1 Extraction of soluble phenolics (I, III)

Soluble phenolic compounds were extracted from the leaves with 70% acetone. Five parallel samples per treatment were prepared, each sample representing one separate plant/leaf. Frozen leaves were weighed (leaf weights varied between 0.5-1.0 g fw for strawberry and 0.1-0.2 g fw for arctic bramble) and extracted three times for 1 min with 2-5 ml of 70% acetone in water by an Ultra-Turrax homogeniser. After centrifugation at 5000 × g for 5 min, the supernatants were combined. Acetone was evaporated in a vacuum centrifuge at room temperature immediately (**I**) or, for arctic bramble extracts, the samples were stored in acetone at -20 °C and acetone was evaporated prior to HPLC after the storage (**III**). After evaporation, the concentrate was adjusted to 4 ml (**III**) or 5 ml (**I**) with distilled water. 1.25 ml of methanol was added in the strawberry extracts (**I**), which were stored in water-methanol at -20 °C then.

Phenolics and soluble solids from fruits (**I**) were extracted as previously described (Hukkanen et al. 2006). Briefly, fruits from one plant were homogenized in a food processor and a sub-sample of 5 g was weighed for the analysis of phenolics. Each sample was extracted with altogether 50 ml of 70% acetone in a shaker. After centrifugation, a 12 ml aliquot of the total extract was concentrated to less than 4 ml by vacuum evaporation, after which 1 ml of methanol was added and the extract was made up to 5 ml with distilled water. Fruits from eight plants per treatment were analysed. The phenolic extracts were analysed by HPLC immediately after the extraction. Soluble solids were measured from fresh juice, which was obtained by centrifugating the remaining fruit homogenate (Hukkanen et al. 2006).

3.2.2 Extraction of cell-wall-bound phenolics (I)

The pellet remaining from the extraction of soluble phenolics from strawberry leaves was washed once with 70% acetone, 50% methanol and 100% methanol to remove traces of residual soluble phenolics, after which the pellet was dried in a vacuum centrifuge. The pellet was hydrolysed with 1 M NaOH (1 ml/20 mg of dry material) at 70 °C for 1 h. The hydrolysate was acidified with 1/10 volume of concentrated HCl and extracted twice with an equal volume of ethyl acetate by shaking vigorously for 10 min. The combined extracts were dried and stored at -20 °C.

The method was modified from that of Röpenack et al. (1998). The aim was to release ester-bonded phenolics, but not more tightly bound ether-linked phenolics such as lignin from the cell walls. The hydrolysis time and conditions were adjusted to maximize the yield of total phenolics. The yield was found to increase rapidly during the first hour of hydrolysis at 70 °C, but after that, only small amounts of phenolics were gradually released. Thus, the compounds released after the first hour were considered to derive from ether-linked material or polymers (Martens 2002;

Peyron et al. 2002). Recovery through the hydrolysis and extraction process was determined with (+)-catechin, *p*-coumaric acid, ferulic acid, gallic acid, hydroxybenzoic acid and rutin standards by adding 0, 20 and 40 μg of each compound together with NaOH and 10 mg of dried homogenous pellet into tubes. Recovery was 91% for *p*-coumaric acid and ferulic acid and 98% for hydroxybenzoic acid while gallic acid, rutin and (+)-catechin degraded rapidly during hydrolysis or were not extracted into ethyl acetate.

3.2.3 Analysis of phenolic compounds (I, III)

Total phenolic content of all phenolic extracts was determined with the Folin-Ciocalteu method (Singleton & Rossi 1965). The reaction volume was decreased to 1/10 of the original as described by Anttonen & Karjalainen (2005). The results are expressed as mg of gallic acid equivalents per g of fresh leaves or fruits.

Individual phenolic compounds were analyzed by HPLC, the Hewlett-Packard 1090 series apparatus (Waldbronn, Germany) consisting of two pumps, an autosampler, a column oven, and a diode array detector coupled to HP Chemstation data handling software. Prior to HPLC, the samples were filtered through 0.2 μm Acrodisc syringe filters (PAL Co.).

For all kinds of strawberry extracts (**I**), a Vydac RP C18 column (250 × 4.6 mm, particle size 5 μm; The Separations Group, USA) was used. Injection volume was 25 μl. The separation conditions were the following: temperature 35 °C; flow rate 0.8 ml/min; gradient of acetonitrile (A; % v/v) in 1% formic acid (B): 0-10 min, 0-0% A; 10-60 min, 0-15% A; 60-70 min, 15-25% A; 70-80 min, 25-100% A; 80-83 min, 100% A; 83-90 min, 100-0% A; 90-97 min, 0% A.

For arctic bramble extracts (**III**), ODS Hypersil column (60 × 4.6 mm, particle size 3 μm; Agilent Technologies, USA) was used. Injection volume was 15 μl. The conditions of separation were the following: temperature 35 °C; flow rate 2 ml/min; gradient of acetonitrile (A; % v/v) in 1% formic acid (B): 0-15 min, 0-8% A; 15-30 min, 8-13% A; 30-35 min, 13-25% A; 35-40 min, 25-100% A; 40-41 min, 100% A; 41-48 min, 100-0% A; 48-55 min, 0% A.

The compounds were classified as anthocyanins, flavonols, ellagitannins, hydroxycinnamic acids or other phenolic groups according to their UV-vis spectra that were recorded at 250, 280, 320, 350 and 520 nm (**I, III**). The compounds were quantified as equivalents of the following standard compounds: ellagitannins as ellagic acid; flavonols as rutin, quercetin or kaempferol; compounds with absorbance maximum near 280 nm as gallic acid; hydroxycinnamic acids as chlorogenic acid and *p*-coumaric acid; flavan-3-ols as (+)-catechin; and anthocyanins as cyanidin chloride. The results are expressed as μg of standard equivalents per g of fresh leaves or fruits.

3.2.4 Mass spectrometry of phenolics (I, III)

The main peaks in the HPLC chromatograms were analysed by mass spectrometry (MS). The MS system consisted of a Finnigan LTQ linear ion trap mass spectrometer (San Jose, CA, USA) combined with either a Finnigan Surveoyr HPLC pump, an

autosampler and a UV detector (Uvikon 735 LC, Kontron instruments) (**I**) or a Hewlett Packard 1100 series pump, an autosampler and a diode-array UV-vis detector (**III**). The mass data were collected in negative ionisation mode and full mass spectra were scanned at *m/z* 150-2000 for leaf samples and at *m/z* 250-700 for fruit samples. Fruit extracts were also analysed in positive ionisation mode (**III**). The following ionisation conditions were used: capillary temperature 280 ˚C, capillary voltage –10 V and needle voltage –4.5 kV. The collision energy of 35 V was used to obtain MS^2 and MS^3 fragmented daughter ions from the most abundant parent ions.

3.3 Analysis of salicylic acid (I, IV)

Salicylic acid was analysed according to the method described by Meuwly & Métraux (1993). Briefly, leaf samples were extracted with 70% ethanol and 90% methanol using an Ultra-Turrax for 1 min. After centrifugation and evaporation of alcohols, 5% trichloroacetic acid was added to precipitate proteins and chlorophyll. The centrifugated samples were extracted twice with ethyl acetate-cyclohexane (1:1) and the organic fraction containing free SA was evaporated to dryness. 1/3 volume of concentrated HCl was added into the water phase, which was hydrolysed at 80 ºC for 1 h. SA released from its conjugated form was extracted into organic solvent and evaporated to dryness. Both SA fractions (free and conjugated) were analysed by HPLC at the University of Fribourg in Switzerland.

3.4 Quantification of *Peronospora sparsa* by real-time PCR (II, III)

3.4.1 Extraction of DNA

Three methods were used in the DNA extractions. DNA from micropropagated *in vitro* plantlets and *P. sparsa* spores was extracted using a DNeasy Plant Minikit from Qiagen (**II**). Leaf material was homogenized in the extraction buffer using a disposable plastic pestle. DNA from mature leaves from outdoor- or greenhouse-grown plants was extracted with the method described by Doyle & Doyle (1990) using 1% PVPP in the extraction buffer (**II**). Large leaves were homogenized in liquid nitrogen in a mortar.

In study **III**, an E.Z.N.A. SP Plant Miniprep Kit from Omega BioTek was used. The leaflets from the plate test were homogenized without buffer in FastPrep FP120 (Savant Instruments Inc., NY, USA). The pellets remaining from the extraction of phenolics were used as the samples for greenhouse-grown plants (**III**). The pellets were centrifuged under vacuum to remove acetone and DNA was extracted without further homogenisation. The quality and quantity of DNA was determined with a NanoDrop ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA) (**II, III**).

3.4.2 Design of primers

Specific primers were designed to amplify *P. sparsa* and *Rubus* DNA from a single DNA extract. Forward primer P1 (5'-CAC GTG AAC CGT ATC AAC C-3') and reverse primer P2 (5'-GAT AGG GCT TGC CCA GTA G-3') were designed to amplify a 94 bp fragment from the internal transcribed region 1 (ITS1) of *P. sparsa*.

```
                                                                  P1→
Y15816          ----------------------------------------------CTTTCCACGTGAAC 28
AF241746        ----------------------------------------------CTTTCCACGTGAAC 59
AF266772        ----------------------------------------------CTTTCCACGTGAAC 27
Z73765          ------------------------------------------------------------

Y15816          CGTATCAACCAACATAATTGGGGGTTTG-TTTGGTGGCGGCTGCTGGCATTTTGTGCTGG 87
AF241746        CGTATCAACAACTTTAATTGGGGGTGAT-CTTGACGGTAGCTACTGG--TTTTTTACTGG 116
AF266772        CGTTTCAAACCAAATAGTTGGGGGTCTTGTCTGGTGGCGGCTGCTGGC-TTTATTGTTGG 86
Z73765          ----------------TTACAGAGTTC--ATGCCCGAAAGGGTAGACCTCCCACCCTTG 41
                              **   *       **   *       *   *       * *
                                    ←P2
Y15816          CTGGCTGCTACTGGGCAAGCCCTATCG---CGAGCGTTTGGGCCTTGGCCTGAGCTAGTA 144
AF241746        C-GGTAACCATTGAGTGAACCTCATCATGACGAACCGTTGGGCTTTTGCCTGACGTAGTA 175
AF266772        C-GGCTGCTGCTGGGTGAGCCCTATCATGGCGAGCGTTTGGGCTTCGGCCTGAGCTAGTA 145
Z73765          TGTATTATTACTTTGTT-GCTTTGGCGAGCTG--CCTTCGGGCCTTG---TATGCTCGCC 95
                       *  *    *     *     *  *  * **** *     *    * *
```

Figure 8. The alignment of ITS1 sequence from *P. sparsa* (Y15816) with those from *Hyaloperonospora parasitica* (AF241746), *Phytophthora cactorum* (AF266772) and *Botrytis cinerea* (Z73765). Primers are underlined in the sequence. The primer P2 is highly specific to *P. sparsa*.

```
                R1→
AF055741        TAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG 317
AF055755        TAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG 315
AB043838        TAAACGACTCTCGGCAACGGATATCTCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTG 339
AF163494        TAWACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG 325
AJ232900        AAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAGCGAAATG 328
                 * ************************                              **

AF055741        CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG 377
AF055755        CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG 375
AB043838        CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG 399
AF163494        CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCK 385
AJ232900        CGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCG 388
                ************************************************************
                                ←R2
AF055741        CCCAAAGCCATTAGGCCGAGGGCACGCCTGC---------------------------- 437
AF055755        CCCGAAGCCATTAGGCCGAGGGCACGCCTGC---------------------------- 435
AB043838        CCCGAAGCCATTAGGCCGAGGGCACGTCTGC---------------------------- 459
AF163494        CCCGAAGCCGTTAGGCCGAGGGCACGTCTGC---------------------------- 445
AJ232900        CCCCAAGCCTTCTGGCCGAGGGCACGTCTGC---------------------------- 448
                *** ***** *  ************* ****
```

Figure 9. The alignment of 5.8S ribosomal DNA sequence from arctic bramble (AF055741) with those from *Rubus idaeus* (AF055755), *Rosa hybrida* (AB043838), *Fragaria* × *ananassa* (AF163494) and *Arabidopsis thaliana* (AJ232900). Primers are underlined in the sequence. The sequence is highly similar between different *Rubus* and *Rosa* species.

Forward primer R1 (5'-CAA ACG ACT CTC GGC AAC-3') and reverse primer R2 (5'-CCT CGG CCT AAT GGC TT-3') were designed to amplify a 140 bp fragment from the highly conserved region of 5.8S ribosomal DNA of arctic bramble. Alignments of 5.8S and ITS1 sequences from few selected species of interest are shown in **Figures 8** and **9**.

3.4.3 Control plasmids

The ITS1 and 5.8S sequences were amplified with the P and R primers, respectively, from genomic DNA of arctic bramble cv. Pima with and without *P. sparsa*. The PCR reaction mixture consisted of 1.5 mM $MgCl_2$, 2.5 µl 10 × PCR buffer, 5 mM dNTPs, 0.3 µM primers, 0.625 U Taq DNA polymerase (MBI Fermentas) and 1 µl of template DNA in a final volume of 25 µl. The following PCR protocol was used: initial denaturation at 94 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. The final extension was at 72 °C for 5 min. The purified (QIAquick PCR Purification Kit, Qiagen) PCR products were cloned into the multiple cloning site of pT-Adv plasmid with AdvanTAge™ PCR Cloning Kit (Clontech Laboratories Inc.). After purification (QIAprep Spin Miniprep Kit, Qiagen), the plasmids were multiplied in *Escherichia coli* DH5α. The inserted fragments were confirmed by sequencing.

3.4.4 Quantitative real-time PCR

The quantitative real-time PCR method was developed to be able to follow the development of *P. sparsa* infection in the field and, particularly, in symptomless plants (**II**). Real-time PCR reactions were performed using a Failsafe Green Real-time PCR kit from Epicentre Technologies. The conditions for amplification were optimised using a selection/optimisation kit from the same manufacturer. Premix K and H were selected for further analyses. Reactions were carried out in duplicate in iCycler iQ™ 96-well PCR plates (Bio-Rad Laboratories) with iCycler™ (Bio-Rad, USA). Negative (water) and positive (plasmids or DNA known to be positive) controls were included in all analyses. The reaction mixture consisted of 10 µl of Premix H or K, 0.8 µl of enzyme blend, 0.6 µM of primers and 3 µl of DNA in a final volume of 20 µl. The following PCR protocol was used: initial denaturation at 94 ˚C for 2 min followed by 38 cycles at 94 ˚C for 20 s, 57 ˚C for 20 s and 72 ˚C for 20 s, and a final extension once at 72 ˚C for 5 min. The fluorescence was measured at the end of each cycle at 72 ˚C. Melting curves of amplified products were obtained by decreasing the temperature from 95 ˚C to 50 ˚C stepwise and recording the fluorescence at each step. The amount of *P. sparsa* DNA was always normalized to plant DNA within a sample using the equation:

$$\textit{P. sparsa}\ \text{DNA}\ \% = 2^{\,\text{Ct plant} - \text{Ct P. sparsa}} \times 100.$$

Tenfold dilution series from genomic DNA (cv. Elpee and *P. sparsa* spores) and both control plasmids were prepared to determine the efficiency and sensitivity of

the method. Within-plate variation was also determined by analysing ten parallel reactions from a single stock DNA and day-to-day variation by analysing the same sample in triplicate in four different plates.

3.5 Extraction and analysis of proteins (IV)

3.5.1 Extraction of total soluble proteins

Frozen samples from three plants (per treatment) separately were homogenized in liquid nitrogen and total soluble proteins were extracted for two-dimensional electrophoresis (2-DE) and Western blot analyses as previously described (Koistinen et al. 2002). Protein pellets were stored at -70 ºC and dissolved in 2-DE sample buffer containing 10 M urea, 2% CHAPS, 1% DTT and 0.8% Bio-Lyte 3/10 ampholyte (Bio-Rad, Hercules, CA, USA) prior to analysis. Protein concentration was measured using Bio-Rad Protein Assay Dye reagent.

3.5.2 Production of antibody for PR-1

An antibody for the detection of PR-1 protein in Western blot was produced in rabbits using His-tagged PR-1 peptide for the immunisation. A 226 bp fragment was PCR-amplified from genomic DNA isolated from arctic bramble leaves using the degenerate primer pair PR1 (5'-GAC RTG GGA TGA CAA KGT AGC-3') and PR2 (5'-TGI GTR TAR TGC CCR CAC AC-3'). The PR-1 fragment of arctic bramble was sequenced (Genbank accession EU528030) and showed 78% and 76% identity to PR-1 sequences of *Malus domestica* (DQ318212) and *Pyrus communis* (AF498321), respectively, which are the closest rosaceous species to arctic bramble of which PR-1 has been characterized. On the basis of arctic bramble sequence, more specific primers were designed including restriction sites for ligation into the expression vector (PR1: 5'-GCG GGA TCC ACG TGG GAT GAC AAG-3'; PR2: 5'-GTG AAG CTT GGG TAT AGT GCC CGC A-3'). The fragment was inserted into the expression vector pQE30 (Qiagen, CA, USA) and the PR-1 peptide containing 6×His tag was produced in *E. coli*, purified by HPLC and confirmed as described by Koistinen et al. (2002). Serum containing the antibody was purified by affinity chromatography using the PR-1-His coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). The antibody bound to the PR-1-His in the column was eluted in 1 ml fractions with 50 mM diethylamine, pH 11.5, into tubes containing 100 µl of 1 M Tris-HCl, pH 7.4, and 80 mg of NaCl. The fractions showing the highest absorbance at 280 nm were combined and the antibody was dialyzed into PBS. The specificity of the antibody was confirmed by Western analysis.

3.5.3 SDS-PAGE and Western analysis

15 µg of total leaf protein samples were separated for Western analyses of PR-1 by SDS-PAGE at 150 V for 1 h. Three parallel samples from water- and BTH-treated plants were analysed. Gels were always prepared in duplicate; one gel was used for

blotting and the other for CBB staining to confirm equal loading of the samples. Blotting and detection was performed according to Koistinen et al. (2002). The final concentration of PR-1 primary antibody was 0.2 µg/ml in TBS-milk. Secondary antibody used was goat anti-rabbit IgG conjugated with alkaline phosphatase (Zymed; diluted to 1:2000 in TBS-milk).

3.5.4 2-DE and image analysis

2-DE was performed to analyse arctic bramble leaf proteome for changes caused by BTH treatment. 150 µg of total protein was loaded on three gels for both the controls and BTH-treated plants, each gel representing one plant. 2-DE, staining with SYPRO Ruby fluorescent dye and image acquirement were performed as described by Lehesranta et al. (2006). 2-DE images were analysed with PDQuest 7.1 software (Bio-Rad). Spots were detected and matched between the gels automatically, but the spots were also examined manually to confirm the matching. The quantities of spots measured as ppm were normalized to the total quantity in all valid spots detected. To identify proteins expressed differently between the control and BTH-treated plants, normalized quantities were subjected to statistical analysis, and the spots differing significantly between the controls and BTH plants were subjected to MS analysis.

3.5.5 Identification of proteins

The gels were silver-stained according to Lehesranta et al. (2006) to be able to pick the spots of interest. The excised spots were digested in-gel according to Koistinen et al. (2002). The peptide extracts were dissolved in 25 µl of 0.1% TFA and stored at -20 ºC. Tryptic peptides were analysed using HPLC-ESI-MS/MS as described previously (Lehesranta et al. 2007). MS/MS raw data were processed and analysed using Analyst QS v1.1 and ProID software (Applied Biosystems) or Mascot search (www.matrixscience.com) through Analyst QS. ABCC non-redundant protein database was used in ProID search and MSDB (Viridiplantae) database in Mascot search. The search parameters used were the following: mass tolerance 0.15 Da; MS/MS tolerance 0.8 Da; one missed cleavage allowed; variable modifications carbamidomethyl (C), carboxymethyl (C), and oxidation (M).

3.6 Statistical analyses (I-IV)

SPSS software (versions 11.5 and 14.1 for Windows; SPSS Inc., USA) was used in all statistical analyses. Statistical significance of the differences between the means from differently treated plants were analysed with the analysis of variance using Tukey's post hoc test or Dunnett's T3 test if unequal variances were found (**I, III**). In the cases where only two treatments had been applied, the independent samples t-test was used (**II, IV**). Mann-Whitney U-test was used for nonparametric measurements or if conditions for the analysis of variance were not fulfilled (**III**). Differences were considered significant at $P<0.05$. Tendencies at $0.05 \leq P<0.10$ were also taken into

consideration in the concentrations of leaf phenolics, which showed high variation between individual plants (**I, III**).

In study **III**, principal component analysis (PCA) was used to analyse the variation of phenolic profiles among different cultivars or treatments. Four main components were extracted and each compound was clustered under the component where it had the highest factor loading. Pearson correlation coefficient was determined for the correlation between the concentrations of individual phenolic compounds and the percentage of *P. sparsa* DNA among plant DNA (**III**).

4. RESULTS AND DISCUSSION

4.1 Maintenance and diagnosis of *Peronospora sparsa*

4.1.1 Maintenance of *Peronospora sparsa* culture (II, III)

Peronospora sparsa used in the inoculations (**II**, **III**) was isolated from infected, sporulating cv. Pima onto sterile *in vitro* -grown micropropagated plantlets of arctic bramble in 2005. The morphology of the pathogen was analysed by microscopy. Although thorough staining and visualisation of intercellular mycelia was not obtained, sporangiophores typical for *Peronospora* species were observed (**Figure 2**) (Hall 1989; Ellis et al. 1991). The penetration of germinated spores (**Figure 10**) and growth of sporangiophores occurred through stomata, but also through other sites. Sporangiophores grew out of the tissue occasionally at the junctures of epidermal cells whereas penetration took place directly through the epidermal cells (**Figure 10**).

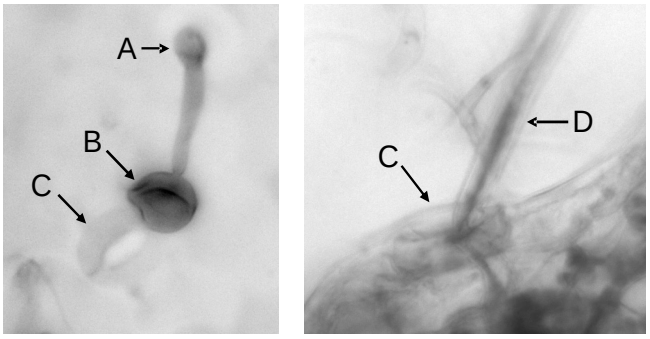

Figure 10. Germinated penetrating spore of *P. sparsa* on a leaf of arctic bramble cv. Mespi stained with aniline blue (left) and a sporangiophore growing out of the tissue through a stoma in *in vitro* –grown arctic bramble cv. Pima stained with trypan blue (right). The spore has formed a germ tube and an appressorium away from the stoma instead of penetrating through it (left). A, appressorium; B, spore; C, stoma (guard cells); D, stalk of sporangiophore.

Suspension of fresh spores into sterile water and subsequent application onto sterile micropropagated plantlets or leaves was found a useful practice in the maintenance of *P. sparsa*. After inoculation, new sporangiophores grew within 4 to 7 days, depending on the spore density of the suspension and growth conditions. Continuous light and temperatures above 23 ºC were found to inhibit sporulation, which is consistent with the observations reported in the literature for downy mildew pathogens (Breese et al. 1994; Rumbolz et al. 2002). To obtain successful infection in greenhouse-grown arctic brambles, relative humidity close to 100%, a clear day-night variation in the lightning, moderate temperature around 20 ºC and the use of a surfactant were found necessary (**III**).

4.1.2 Development of real-time PCR method for the quantification of *P. sparsa* infections (II)

Downy mildews grow inside plant tissues, sporangiophores being possibly the only visible signs of the pathogen (Breese et al. 1994; Slusarenko & Schlaich 2003). Plants do not necessarily express symptoms of the disease, particularly at the early phases of infection, and the number of symptoms (disease incidence) does not always correlate with the amount of pathogen in the plant (disease severity) (Agrios 2005). Therefore, to reliably quantify *P. sparsa* infections in arctic bramble, real-time PCR method based on the quantification of pathogen DNA in plant tissue was developed (**II**). The use of real-time PCR in the quantification of plant pathogens has become a common practice since its introduction in the 1990s (Schena et al. 2004).

The aim was to obtain highly specific amplification of *P. sparsa* because several other oomycete species also occur in *Rubus*. ITS regions vary largely between species and are often used as target sequences in PCR (e.g. Cooke et al. 2000). The primer P1 designed to amplify a fragment from ITS1 region is not fully specific to *P. sparsa*, but the much higher variability between species in the second primer P2 ensured specific amplification of *P. sparsa* (**Figure 8**). No DNA was amplified from all other oomycete or fungal species tested, namely *Phytophthora cactorum*, *Phytophthora fragariae* var. *rubi*, *Hyaloperonospora parasitica*, and *Botrytis cinerea*, as expected based on the sequence alignment (**II**: **Table 1**).

Plant primers were designed to be applicable also for plant species other than arctic bramble since *P. sparsa* occurs commonly in *Rubus* and *Rosa*. Because the plant gene selected is used as a control and, therefore, should be constantly expressed, the primers were targeted at the highly conserved 5.8S ribosomal DNA sequence (**Figure 9**). The sequence was efficiently amplified from all *Rubus* and *Rosa* species tested while clearly weaker amplification was obtained from strawberry and *Arabidopsis*. Since *P. sparsa* infects only *Rubus* and *Rosa*, amplification of 5.8S rDNA in a wider range of plant species was not primarily pursued.

Linear (R^2 > 0.980) and efficient amplification (slopes of the regression lines near the optimal, -3.3.) was achieved with both primer pairs using the control plasmids or genomic DNA from arctic bramble or *P. sparsa* spores as templates (**II**: **Figure 1**). The within-plate variation of replicate samples was 1.2% and plate-to-plate variation 2.5%. Successful amplification of *P. sparsa* DNA was achieved with as little as 37 fg of genomic DNA (**II**: **Figure 2**) and 1 fg of plasmid DNA. Thus, the method is not only quantitative but also more sensitive than the non-quantitative PCR method (250 fg) developed for *P. sparsa* by Lindqvist et al. (1998). Relative quantification approach, which was selected instead of absolute quantification, was found useful in the measurement of infection. The normalization of the amount of pathogen DNA to plant DNA in each sample eliminates most variations in, for example, the quality, extraction and loading of DNA and day-to-day performance, and a necessity for running extensive calibration curves in every plate (Livak & Schmittgen 2001; Gachon & Saindrenan 2004).

From study **II**, it was concluded that symptom expression in arctic bramble does not strictly correlate with the amount of *P. sparsa* DNA present in the plant. The

conclusion was mainly based on the analysis of cv. Mesma, which showed extensive symptoms, but contained only low amounts of pathogen DNA (**II**: **Figure 3**, **Table 2**). However, the symptoms observed in cv. Mesma may not have been caused by *P. sparsa*. This is supported by later observations made on three cultivars of arctic bramble (**III**). In study **III**, no notable growth of the pathogen was detected by PCR unless visible lesions or sporangiophores were also found (**III**: **Figures 1** and **2**, **Table 1**). Furthermore, the number of symptoms (**III**: **Figure 1**) correlated with the degree of sporulation in the plate experiment (**III**: **Table 1**) among different cultivars and treatments (described in chapter 4.2.2). Thus, the growth of the pathogen could also be evaluated based on symptom expression or sporulation, which may diminish the need for the quantitative PCR analysis in some cases. However, the method is the only objective and definite means to quantify *P. sparsa* and can be used for the evaluation of the infection in different genotypes and treatments.

4.2 Applicability of induced resistance for the protection of strawberry and arctic bramble

4.2.1 BTH improves resistance to powdery mildew in strawberry (I)

BTH improved resistance to powdery mildew in two parallel experiments conducted on strawberry under greenhouse conditions (**I**). The treatment of the plants with 0.8 g/l of BTH two days prior to inoculation almost completely prevented the development of infection compared with the corresponding water-treated plants, all of which were infected (**Figure 11A** and **B**). Two days were found sufficient for the induction of resistance in strawberry, which is consistent with the times reported in the literature (Tally et al. 1999). However, no systematic analysis was performed using longer intervals between the application of BTH and inoculation because of the high level of protection achieved using the two-day interval. In preliminary experiments, 0.6 g/l of BTH did not protect strawberry plants efficiently and, therefore, the concentration was raised to 0.8 g/l (1.2 mM active ingredient). No symptoms of phytotoxicity were observed due to BTH treatments, indicating that strawberry plants can well tolerate high concentrations of BTH. In many other plants, clearly lower concentrations of BTH have been used with high efficacy (Görlach et al. 1996; Schweizer et al. 1999; Godard et al. 1999). One reason for the good tolerance to BTH might be the high constitutive level of SA present in strawberry (**I**: **Figure 3**).

BTH showed higher efficiency in micropropagated plants than in frigo plants although the micropropagated plants were clearly more susceptible to infection (**Figure 11**). The disease severity was almost equal among the infected frigo controls and BTH-treated plants if the uninfected plants were not taken into account (**Figure 11C**). The experiment with frigo plants lasted for several weeks and only one additional BTH application was done during flowering. Therefore, powdery mildew infection also spread among BTH-treated plants and resulted in the infection of fruits

as well, demonstrating the importance of preventive treatments. However, smaller fraction of infected fruits was found in BTH-treated (42%) than in water-treated (72%) plants despite the long time between BTH treatments and harvest.

Effect of BTH may not be systemic in strawberry because newly developed leaves, which had not received BTH, seemed to be easily infected (no quantitative analysis was performed). Long-lasting efficacy of BTH has been mainly detected in monocots and, thus, a serious of treatments may be necessary in dicots in general (Tally et al. 1999). Overall, the high efficiency of BTH against powdery mildew of strawberry is in line with the results obtained on several other plants against powdery mildew fungi (Görlach et al. 1996; Lawton et al. 1996; Bokshi et al. 2003). Strawberry plants have also responded to BTH treatment in previous studies, where BTH improved resistance to *Phytophthora* diseases and grey mould (*Botrytis cinerea*) (Terry & Joyce 2000; Eikemo et al. 2003).

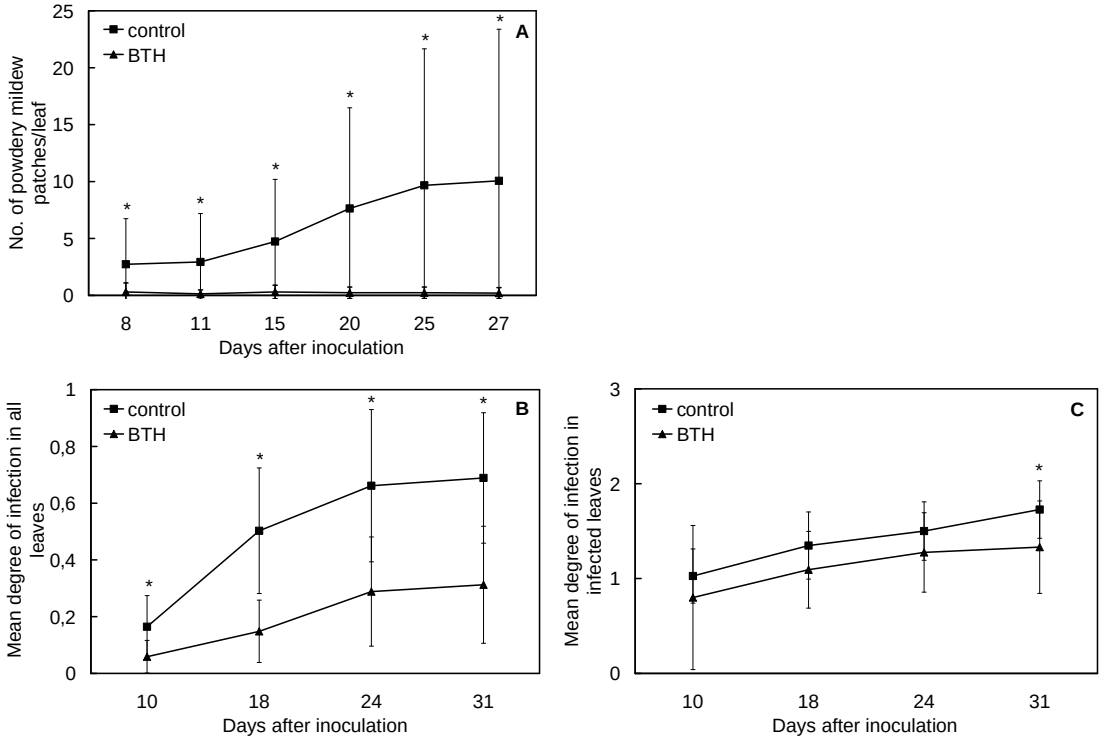

Figure 11. Development of powdery mildew infection in water- and BTH-treated (A) micropropagated cv Jonsok (n = 30), (B) frigo cv Jonsok, all leaves examined (n = 36), and (C) frigo cv Jonsok, only infected leaves examined. In B and C, the following scale was used in the evaluation of the symptoms: 0, 0%; 1, 1-20%; 2, 21-40%; 3, 41-60%; 4, 61-80%, 5, 81-100% infected of the whole leaf area. Both sides of the leaves were examined in all measurements. The bars represent standard deviations for the means. Statistical significant differences between water- and BTH-treated plants are marked with asterisks.

4.2.2 Inhibition of downy mildew by agrochemicals in arctic bramble (III)

In addition to powdery mildews, BTH has shown efficacy against other diseases caused by biotrophs, such as downy mildews of *Arabidopsis*, tobacco and cauliflower caused by *Peronospora* species (Lawton et al. 1996; Ziadi et al. 2001b, Perez et al. 2003). Consistently, BTH treatment protected the susceptible arctic bramble cv. Pima (**Figure 12A**) and a clone 12B14 (**III**: **Figure 1B**) from *P. sparsa* under greenhouse conditions. Only few symptoms were detected in cv. Pima treated with either of the two BTH concentrations tested (0.05 or 0.4 g/l) whereas symptoms rapidly increased in the controls for three weeks after inoculation (**Figure 12A**). In study **IV**, the high concentration of BTH, 0.6 g/l, was found to decrease vegetative growth in arctic bramble

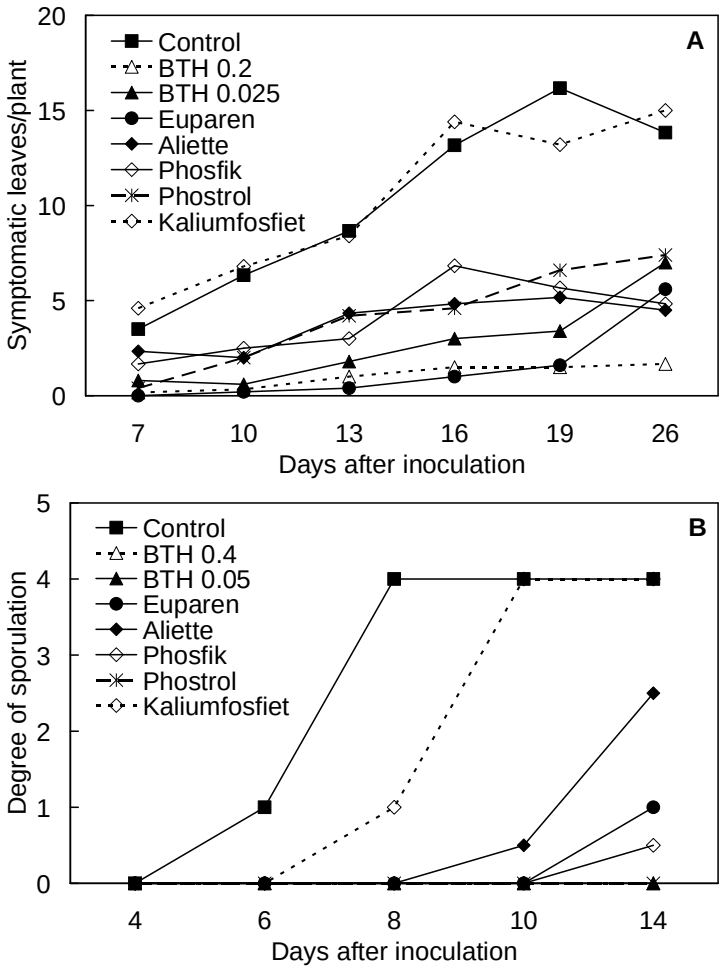

Figure 12. Development of downy mildew infection in different treatments of arctic bramble cv Pima measured (A) as the number of symptoms in the greenhouse experiment or (B) the degree of sporulation in the plate test. The following scale was used in the evaluation of sporulation: 0, 0; 1, 1-5; 2, 6-10; 3, 10-20; 4, > 20 sporangiophores per leaflet.

(**IV**: **Figure 1B**), and, therefore, lower concentrations were used in this study. Interestingly, 0.05 g/l, that is approximately ten times less than the higher concentration, was as efficient against *P. sparsa* as the high concentration. Slightly more symptoms (**Figure 12A**; **III**: **Figure 1**) but less pathogen DNA (**III**: **Figure 2**) were detected in the plants treated with 0.05 g/l of BTH than in the plants that received 0.4 g/l of BTH. Lower concentrations of BTH have been found more effective than the high concentrations also in rice (Schweizer et al. 1999). Three weeks after inoculation, symptoms started to increase also in BTH-treated plants, indicating a decline in resistance simultaneously with the growing disease pressure caused by secondary inocula. Thus, repetitive treatments with BTH may be necessary also in arctic bramble during the growing season. Despite the promising results obtained in this study, the efficacy of BTH has yet to be confirmed in the field, since the control of diseases by BTH has occasionally been weaker in the field than in a greenhouse (Wiese et al. 2003; Navarre & Mayo 2004). The reason is unclear, but varying level of organic material and microbial activity in the soil has been suggested to affect the plants' responsiveness to BTH (Wiese et al. 2003). Micro-organisms can elicit defence reactions in plants, after which the plants may not be as responsive to other stimuli as they would be in the uninduced state.

Among the fungicides registered against downy mildew of arctic bramble in Finland, Euparen M showed clearly higher efficiency against *P. sparsa* in cv. Pima than did Aliette (**Figure 12A**). No comparative, quantitative data about their efficiencies have been previously available. Based on these results, Euparen M could be used as the primary fungicide, the effect of which could be complemented by following treatments with Aliette or other phosphite-based products. However, Food Safety Authority Evira has set restrictions on use of Euparen M in Finland in 2007 due to the observation that carcinogenic nitrosamines are formed from the degradation product of tolylfluanid during ozonizing process of groundwater. Since Euparen M will possibly be recalled from the market in the near future, the requirement for new products for the control of downy mildew in arctic bramble will continue to increase.

All phosphite-containing products (including Aliette), except for Kaliumfosfiet, had similar moderate efficacy against *P. sparsa* in the greenhouse. Three weeks after inoculation, the number of symptoms was approximately 60% lower in phosphite-treated plants than in the controls (**Figure 12A**; **III**: **Figure 1**). Moderate control of *P. sparsa* has also been obtained on rose and boysenberry by Aliette and potassium salts of phosphorous acid (Aegerter et al. 2002; Walter et al. 2004). In boysenberry, late application of fungicides or phosphites during flowering was found necessary to prevent the infection of fruits (Walter et al. 2004). If phosphites also show efficacy against *P. sparsa* in arctic bramble in the field, they might be a convenient option as late season protectants due to their low toxicity and short/no withholding periods. However, some strains of *Phytophthora* and *Bremia* have shown resistance to Aliette, suggesting that phosphite-based products should not be used continuosly without alternating with other types of fungicides (Wilkinson et al. 2001; Brown et al. 2004). It is not known to which extent the efficacy of phosphites is based on their direct

antimicrobial activity and their ability to activate defence reactions in plants. This may be of significance in the risk of developing resistant pathogen strains.

4.2.3 Efficacy of agrochemicals against downy mildew in the plate model (III)

Methods using detached leaves or leaf disks instead of whole plants have been described for the testing of disease resistance in many plant-pathogen interactions. It is a particularly convenient method for testing of resistance to biotrophs, which cannot be cultured on artificial medium. Testing of resistance on agar plates has been described for e.g. downy mildew pathogens of grapevine and *Arabidopsis* (Agnola et al. 2003; Kortekamp 2006). Similarly, genotypic differences in the resistance to *P. sparsa* were seen in arctic bramble when detached mature leaves or leaves of micropropagated plantlets were inoculated on the plates (**II**: **Table 3**; **III**: **Figure 2B, Table 1**). Interestingly, the efficacy of agrochemical treatments could also be tested on the plates by using the leaves from the greenhouse-grown plants, which received agrochemicals four days prior to detachment and inoculation of the leaves (**Figure 12B**; **III**: **Table 1**). Reliability of the *in vitro* method was confirmed by the comparison of sporulation to the symptoms measured in the corresponding treatments and genotypes in the greenhouse and by quantifying pathogen DNA in the plant tissue. The trends observed among the different treatments or cultivars were similar in the plate and greenhouse experiment. The amount of *P. sparsa* DNA measured in the detached leaves was also in line with the degree of sporulation. Thus, the *in vitro* plate method is well-suited for the pre-evaluation of breeding material and agrochemicals in arctic bramble before executing larger trials in a greenhouse or in the field.

4.2.4 Resistance to downy mildew varies among arctic bramble cultivars (II, III)

Genotypic differences in the resistance to one isolate of *P. sparsa* were observed in arctic bramble in the greenhouse and plate experiments described above. Although cv. Mespi has shown downy mildew symptoms in the leaves and fruits previously in the field (Lindqvist et al. 1998), it was resistant to the isolate used in the inoculations (**III**). No symptoms, pathogen DNA (**III**: **Figure 2**; one weakly positive plant in PCR) or sporulation (**III**: **Table 1**) was found in cv. Mespi. The results obtained with naturally infected plants also indicates that cv. Mespi is not as susceptible to downy mildew as is cv. Pima; significantly less *P. sparsa* DNA was found in young leaves of cv. Mespi than in those of cv. Pima (**II**: **Table 2**). The existence of cultivar-race-specific resistance has yet to be tested by using several isolates of *P. sparsa* and different genotypes of arctic bramble. In *Arabidopsis*, cultivar-race-specific resistance to downy mildew (*Hyaloperonospora parasitica*) has been demonstrated and several *R* genes have been characterised (Slusarenko & Schlaich 2003).

The new clone 12B14 also showed lower susceptibility to *P. sparsa* than did cv. Pima. The number and development of symptoms was similar to those observed in Euparen M-treated Pima in the greenhouse and were further decreased by treatments with 0.4 g/l of BTH, Aliette and Phosfik (**III**: **Figure 1B**). However, in the plate test 12B14 had equal amount of sporangiophores compared to those in cv. Pima although

the sporulation began two days later (**III**: **Table 1**). Only BTH treatment inhibited sporulation in 12B14 in the plate experiment. Originally, the clone was selected from the breeding progeny for further testing because it showed fewer symptoms and dry fruits than other genotypes in a small scale field test performed at the University of Kuopio during 2002 to 2005. Although promising results were obtained with the clone, the level and persistence of resistance remains to be tested in larger field experiments.

4.2.5 Impact of induced resistance on plant performance (I, IV)

Activation of plant defence by BTH without subsequent infection has led to notable reductions of vegetative growth and, occasionally, of yield at least in cereals and *Brassica* (Godard et al. 1999; Heil et al. 2000; Dietrich et al. 2005). Therefore, vegetative growth and yield were compared between water- and BTH-treated plants in strawberry and arctic bramble. In strawberry (**I**), the fresh weight of leaves per plant was decreased by 17% by BTH treatment in comparison to the controls while the numbers of leaves, runners, flowers or fruits and the weight of fruits were not different between BTH-treated plants and the controls. Instead, inoculation with powdery mildew significantly decreased both the number (2.0/plant) and weight of runners (3.4 g of fw/runner) in the controls, but not in the BTH-treated plants (2.3/plant and 4.0 g/runner) compared with the uninfected control plants (2.9/plant and 5.8 g/runner), indicating slight protection by BTH.

Similarly, the dry weight of leaves was 20% lower in BTH-treated (11.7 g of dw/box containing six plants) than in water-treated arctic bramble cv. Pima (14.7 g) whereas the reduction by BTH was as high as 30% in cv. Mespi (14.7 and 20.9 g/box; **IV**: **Figure 1B**). Fresh weight of rhizome was also determined after the second season, showing reduction of 29 and 43% by BTH in comparison to the controls in cv. Pima and Mespi, respectively (**IV**: **Figure 1B**). Arctic brambles received three applications of BTH per two successive growing seasons, which may be reflected as stronger growth reduction than seen in strawberry. The high negative impact of BTH on vegetative growth was expected to result in the reduction of yield, but, surprisingly, no such effects were observed. In fact, throughout the experiments, BTH-treated arctic brambles gave slightly, but not significantly, higher yield than did the controls measured as both the numbers of flowers and fruits and the weight of fruits/plant (data not shown). The size of fruits was not significantly different between the controls and BTH-treated plants. However, evaluation of yield should be done in arctic bramble under field conditions to be able to detect long-term effects and influence of growth conditions on the plant performance.

Similar results were obtained on both plant species, giving no indications of yield losses due to the BTH treatment. Consistent with these results, BTH has not affected yield in strawberry, bean, or cyclamen, the vegetative growth of which was also decreased by BTH (Terry & Joyce 2000; Iriti & Faoro 2003; Elmer et al. 2006). SA, the action of which BTH may mimic in plants, is known to positively regulate flowering and to induce stress-related transitions from vegetative to reproductive growth (Morris et al. 2000; Martínez et al. 2004). This has also been demonstrated for

BTH in *Arabidopsis* under certain growth conditions (Dietrich et al. 2005). In arctic bramble, BTH treatment might also have caused an earlier transition to flowering and cropping compared to the control plants, which is reflected as a smaller plant size and slightly higher yields.

4.3 The role of salicylic acid in strawberry and arctic bramble

4.3.1 SA contents (I, IV)

Thus far, only a few plant species have been found to contain exceptionally high constitutive (basal) concentrations of SA (Raskin et al. 1990; Silverman et al. 1995; Navarre & Mayo 2004). Interestingly, both strawberry (**I**) and arctic bramble (**IV**) belong to that group of plants. In strawberry, basal level of total SA was ca. 20 μg/g fw in the leaves of micropropagated plants. The amount of total SA remained stable throughout the nine days sampling period, but the relative proportion of free and conjugated SA varied, free SA consisting of 2-30% of the total SA (**I**: **Figure 3**). The maximal amount of total SA in frigo plants was even higher and varied between 67-111 μg/g fw during the sampling period while the proportion of free SA was stable at 4.5-7.3% of total SA (data not shown).

In arctic bramble, basal level of total SA was 6-25 μg/g fw in the leaves of greenhouse-grown plants, the proportion of free SA varying between 9-11% of total SA. Outdoor-grown plants had 16-24 μg/g fw of total SA, of which only 0.5-6% was in free form. Slightly higher concentrations were measured in cv. Mespi than in cv. Pima. The results show a large variation in the basal SA concentrations between different types of plants, which may affect their response to external application of SA or BTH. In strawberry, the frigo plants which had clearly higher SA level did not develop as high resistance to powdery mildew by BTH as the micropropagated plants did (**Figure 11**).

The concentrations of total and free SA were highly similar in both species studied. The levels of SA found in strawberry and arctic bramble are approximately at the same level as reported for rice (35-40 μg/g in maximum; Raskin 1990; Silverman et al. 1995). No information was found about SA levels in the leaves of other rosaceous species, which might have revealed whether the high SA content is typical for rosaceous species.

4.3.2 Accumulation of SA by BTH (I, IV)

To address the influence of BTH treatment or powdery mildew infection on endogenous SA, the SA levels were determined in different treatments of strawberry (**I**) and BTH-treated arctic bramble (**IV**). BTH treatment induced similar accumulation of conjugated SA in strawberry (**I**: **Figure 3**) and arctic bramble cv. Mespi (**IV**: **Figure 1A**) 4 to 6 days after BTH treatment. No clear induction was observed in cv. Pima (data not shown). The amount of free SA was not changed by BTH or powdery mildew.

Powdery mildew infection alone did not affect the amount of conjugated SA in strawberry, but it augmented the response induced by BTH. Supporting the results, pathogen infection has not increased the level of endogenous SA in other high-SA plants either (Silverman et al. 1995; Coquoz et al. 1998). However, the lack of induction does not imply an insignificant role for SA in the defence against pathogens in high-SA plants. Results obtained on potato and rice suggest that the defence of high-SA plants also depend on SA. The high basal concentration of SA did not correlate with resistance to *Phytophthora infestans* in potato, but SA was needed to induce SAR by arachidonic acid (Yu et al. 1997). In rice, the basal level of SA correlated with the resistance to the blast pathogen (*Magnaporthe grisea*) in different cultivars and *nahG* expressing plants (Silverman et al. 1995; Yang et al. 2004). In most cases, rice and potato have also been responsive to external application of SA or BTH (Schweizer et al. 1999; Bokshi et al. 2003; Yang et al. 2004; Navarre & Mayo 2004).

BTH induced the accumulation of SA in both strawberry and arctic bramble while in other plants the levels of endogenous SA have not been affected by BTH (Friedrich et al. 1996; Lawton et al. 1996). However, the SA accumulated in strawberry and arctic bramble may be used for purposes other than signalling, which is also supported by the fact that their basal concentration of SA is already far higher than needed for the activation of defence in low-SA species (Malamy et al. 1990; Cameron et al. 1999; Verberne et al. 2000). SA-deficient rice was found to suffer from increased oxidative damage and high levels of ROS induced by abiotic or biotic stresses in comparison to control plants and, therefore, SA was suggested to act as an antioxidant in rice (Yang et al. 2004). Because the induction pattern of conjugated SA in strawberry was highly similar to that of other phenolic compounds (**Figure 13**; **I**: **Figure 3**), it is possible that SA also acts e.g. as an antioxidant in strawberry similarly to other phenolics.

4.3.3 PR-1 as a marker for the induction of SA-mediated defence pathway (IV)

The expression of PR-1 has been used as the marker for the induction of SA-mediated defence (Malamy et al. 1990; Lawton et al. 1996; Cameron et al. 1999). To address the activation of SA pathway in different treatments of arctic bramble, an antibody for PR-1 was developed (**IV**). The antibody was also tested with protein extracts from strawberry, but it recognised PR-1 of strawberry only weakly. Identity was 78% between PR-1 nucleotide sequence from arctic bramble and strawberry.

BTH strongly induced PR-1 in arctic bramble cvs Pima and Mespi, indicating the activation of SA-mediated defence pathway (**IV**: **Figure 2A-C**). The induction of PR-1 was not related to the observed accumulation of conjugated SA, since SA levels were unchanged in cv. Pima, which, however, showed the induction of PR-1. The result supports the idea that the increase in conjugated SA is not related to signalling function. Phosphite treatments did not affect the expression of PR-1 (data not shown), suggesting that they do not activate SA-mediated defence in arctic bramble. The result is in contrast with the evidence obtained from *Arabidopsis*, cauliflower and potato, where the action of phosphites was dependent on the components of SA

defence pathway and SAR-related changes were observed (Molina et al. 1998; Bécot et al. 2000; Andreu et al. 2006; Daniel & Guest 2006).

PR-1 is constitutively expressed in potato and *Arabidopsis* mutants, which have high constitutive levels of SA (Bowling et al. 1994; Jirage et al. 2001; Navarre & Mayo 2004). Interestingly, constitutive expression of PR-1 has also been found in the leaves of pear, which is a rosaceous fruit-producing species, but no information was found about its SA levels (Sparla et al. 2004). Considering the expression of PR-1, arctic bramble resembles rice more than potato since PR-1 of rice is also inducible by BTH (Yang et al. 2004). However, in some preliminary samples collected from arctic bramble for testing of the antibody, high expression of PR-1 was observed without BTH treatment. Stresses other than pathogens may have triggered the defence shown as the increased level of PR-1. It will be interesting to see if arctic bramble is responsive to BTH in the field, due to the higher concentrations of SA found in outdoor-grown plants and the expression of PR-1 without BTH treatment under uncontrolled conditions.

4.4 Phenolic compounds and their role in defence

4.4.1 Phenolic profiles in strawberry and arctic bramble

The concentrations of phenolics were measured after different treatments of strawberry (**I**) and arctic bramble (**III**) to see whether the phenolics might contribute to the resistance induced by BTH or phosphites. The compounds were tentatively identified using MS to be able to evaluate the role of specific phenolics.

In strawberry, phenolics were analysed from the leaves (soluble and cell-wall-bound phenolics) and fruits (**I**). Characteristic HPLC chromatograms were obtained from the different phenolic fractions, only ellagic acid (peak 11) being found in the all types of extracts (**I**: **Figure 1**). The phenolic composition of fruits has already been characterised extensively by others (Määttä-Riihinen et al. 2004; Aaby et al. 2005; Seeram et al. 2006) whereas only limited or no information was available about the composition of soluble and cell-wall-bound phenolics, respectively, in the leaves (Okuda et al. 1992; Oertel et al. 2001). The compounds identified for the first time in strawberry in the present study are shown in **Table 3**.

The phenolics were classified according to their UV-vis spectra based on standard compounds or literature, and were further identified by MS. Full mass spectra and MS^2 and MS^3 daughter ions formed from the most abundant parent ions in tandem MS were used to determine the masses of the whole molecules and the fragments present in the molecules. For example, the compound 20 from arctic bramble leaves (**III**: **Figure 3**), quercetin triglycoside, was detected at *m/z* 771. The daughter ions detected at *m/z* 609, 463, and 301 were obtained after the cleavage of a hexose (-162-H_2O), a deoxyhexose (-146- H_2O) and another hexose (-162- H_2O), respectively, resulting in the quercetin aglycone at *m/z* 301. Other compounds were identified similarly. Different aglycones were identified based on the fragmentation patterns of standard compounds. Ellagitannins were constantly detected as doubly

charged ions $[M-2H]^{2-}$. Typical fragments found in ellagitannins were ellagic acid/HHDP (301), gallic acid (170 or 152) and glucose (180 or 162).

In strawberry fruits, kaempferol malonylglucoside (peak 44) was identified as a new compound (**Table 3**). The same compound was later found in strawberry fruits by Aaby et al. (2007). The other compounds identified, namely flavan-3-ol compounds 33 and 35 (**I**: **Figure 1**), a flavan-3-ol dimer (34), *p*-coumaryl glucoside (36), pelargonidin 3-glucoside (38), pelargonidin 3-malonylglucoside (42), quercetin 3-glucuronide (41) and kaempferol glucuronide (43) are also reported for strawberry fruits (**I**: **Supporting information**) (Määttä-Riihinen et al. 2004; Aaby et al. 2005; Seeram et al. 2006).

In strawberry leaves, several soluble phenolic compounds were tentatively identified for the first time (**Table 3**). Most compounds were identified as ellagitannins, but ellagic acid derivatives and flavonols were also found. In addition to the newly identified compounds, casuarictin (5a), potentillin (15a), agrimoniin (the main peak 18), ellagic acid (11), ellagic acid pentose (12) and quercetin glucuronide (15b) were detected in the leaves in agreement with previous findings (**I**: **Supporting information**) (Okuda et al. 1992; Oertel et al. 2001).

In the cell-wall-bound fraction, ellagic acid (11), gallic acid (24), kaempferol hexose (27) and *p*-coumaric acid were identified (**I**: **Supporting information**). Ferulic acid, which is a common constituent of the cell walls of many plants, was not detected in strawberry (Röpenack et al. 1998; McLusky et al. 1999; Stadnik & Buchenauer 2000; Martens 2002). Although ferulic acid may actually not be present in the cell walls of strawberry, it is possible that it is more tightly bound with ether-linkages in lignin than is *p*-coumaric acid and, thus, may not have been extracted under the conditions used (Ralph et al. 2004).

In arctic bramble, soluble phenolics were measured and characterised only from the leaves (**III**). Similarly to strawberry, most compounds were ellagitannins while smaller fraction consisted of flavonols, galloylglucoses and hydroxycinnamic acids (**III**: **Figure 3** and **Supporting information**). The compounds not reported in *Rubus* species previously are listed in **Table 4**. The compounds 21, 25, 36 and 38a were identified as ellagitannins and 7, 11, 13 and 17 as hydroxycinnamates, but their detailed structures could not be determined. However, most compounds, i.e. quercetin (37) and kaempferol (peak 40) glucuronide, quercetin (34) and kaempferol (39) hexose-deoxyhexoses, caffeoyl glucose (6), sanguiin H-6 (33), lambertianin C (35), lambertianin A (30), sanguiin H-10 (18 and 27), roshenin B (or desgalloyl-sanguiin H-6; 16 and 19), casuarictin/potentillin (26), sanguiin H-5 (14), tellimagrandin I (24 and 28a) and tellimagrandin II (32) were previously identified in the leaves of *Rubus* species grown in Asia or in the fruits of raspberry or arctic bramble (Okuda et al. 1992; Yoshida et al. 1992; Tanaka et al. 1993; Mullen et al. 2002; Mullen et al. 2003; Cho et al. 2004; Määttä-Riihinen et al. 2004; Li et al. 2007). Thus, the phenolic composition of the leaves is likely highly conserved within a plant genus, as it was also concluded by Okuda et al. (1992), who analysed the applicability of ellagitannin composition as a taxonomic marker in Rosaceae. The fruits and leaves, however, have distinct phenolic profiles as presented for strawberry above.

Table 3. The newly identified phenolic compounds in the fruits and leaves of strawberry cv. Jonsok.

Compound no.[a]	Tentative identification	$[M-H]^-$	MS^2 ions[b]	MS^3 ions	UV-vis spectrum[c]
Fruits					
44	kaempferol malonylglucoside	533	489	285	kaempferol
Leaves, soluble phenolics					
1	ellagic acid derivative	481	301	257, 229,185	ellagic acid
2	quinic acid	191	111, 173	-	gallic acid
5b	ellagic acid hexose	463	301	257, 229, 185	ellagic acid
6	sanguiin H-10-like ellagitannin	783 (doubly charged)	633, 1265, 935, 897,613, 481, 301	301	ellagitannin
7	nobotanin A-like ellagitannin	858 (doubly charged)	783, 1415, 1235, 933, 915, 897, 763, 633, 481, 301	764, 481, 301	ellagitannin
9 and 19	agrimoniin-like ellagitannin (not agrimoniin)	934 (doubly charged)	1567, 1265, 1235, 858, 633, 301	-	ellagitannin
10	quercetin hexose-deoxyhexose (not rutin)	609	301	273,257, 179, 151	quercetin
14	ellagic acid deoxyhexose	447	301	300, 257, 229, 185	ellagic acid
20	lambertianin C-like ellagitannin	1402 (doubly charged)	935, 1869, 1717, 1567, 1265, 1250, 1235, 1085, 935, 897	633, 463, 301	ellagitannin
Leaves, cell-wall-bound phenolics					
24	gallic acid	169	125	-	gallic acid
26	*p*-coumaric acid	-	-	-	*p*-coumaric acid[*]
27	kaempferol hexose	447	285	255, 227, 179, 151	kaempferol

[a] Compound numbers refer to those presented in **I**: **Figure 1**. [b] The first ion listed was used in MS^3 fragmentation. [c] Spectra were compared to those of standard compounds or to the literature (e.g. Määttä-Riihinen et al. 2004; Salminen et al. 1999). [d] *p*-Coumaric acid was not ionised/detected in MS, but the compound had identical elution and spectrum with the standard.

Table 4. The phenolic compounds identified in the leaves of arctic bramble or other *Rubus* for the first time.

Compound no.[a]	Tentative identification	$[M-H]^-$	MS^2 ions[b]	MS^3 ions	UV-vis spectrum[c]
1	bis(galloyl)glucose	663	331, 483, 635	169, 271, 211, 193, 125	gallic acid
2	methyl gallate-galloylglucose	515	331	169, 271, 211, 193, 125	gallic acid
4 and 15	digalloyl glucose	483	331	169, 271, 211, 193, 125	
5	coumaryl quinic acid	355	191,209, 261, 355, 173	-	hydroxycinnamate
20	quercetin hexose-deoxyhexose-hexose	771	463, 301, 609	301, 285, 175, 227	quercetin
22 and 29a	deHHDP-lambertianin C	1250 (doubly charged)	1099, 933, 1084, 1897, 1867, 1567, 1235, 633, 469, 782	933, 1897, 1565, 783, 613, 633, 948, 1735, 1265, 469	ellagitannin
23a	kaempferol hexose-deoxyhexose-hexose	755	593, 447, 285	285, 229, 257	kaempferol
23b	trigalloylglucose	635	465, 483	313, 169, 295, 235, 447, 211, 241, 321	gallic acid
28b	ellagic acid glucuronide	477	301	301, 257, 229, 185	ellagic acid
29b	quercetin hexose-glucuronide	639	301, 337, 459, 505	179, 151, 257, 273	quercetin
38b	kaempferol hexose-glucuronide	607	285, 321, 357, 473, 589	285, 257, 241, 151, 229	-
39	kaempferol hexose-deoxyhexose	593	285	285, 257, 151, 241, 267, 229, 213, 163	kaempferol

[a] Compound numbers refer to those presented in **III**: **Figure 3**. [b] The first ion listed was used in MS^3 fragmentation and others are listed in the order of their abundance. [c] Spectra were compared to those of standard compounds or to the literature (e.g. Määttä-Riihinen et al. 2004; Salminen et al. 1999).

Several new compounds were identified in the leaves of strawberry and arctic bramble. Only few compounds identical in both species were found, namely agrimoniin/sanguiin H-6-like ellagitannins, sanguiin H-10-like ellagitannin, potentillin/casuarictin, lambertianin C-like ellagitannin, quercetin hexose-deoxyhexose, and quercetin glucuronide. Ellagic acid derivatives common in the strawberry leaves were not found in arctic bramble, except for ellagic acid glucuronide. Instead, different galloylglucoses and hydroxycinnamates were more abundant in arctic bramble than in strawberry.

4.4.2 Effect of BTH on soluble leaf phenolics in strawberry and arctic bramble

Total soluble phenolic contents in different treatments of strawberry (**I**) and arctic bramble (**III**) leaves are shown in **Figure 13A** and **D**. Although the principal aim was to analyse the concentrations of individual phenolics, the general trend among phenolics in different treatments can be illustrated by the analysis of total phenolics.

In strawberry, BTH induced the accumulation of soluble leaf phenolics and this was augmented by the following inoculation with powdery mildew (**Figure 13A**). Without inoculation, the concentrations of phenolics dropped down to the same level as in the controls nine days after the BTH treatment. Interestingly, the different concentrations of BTH had different effect on the phenolics in arctic bramble **(Figure 13D**). Whereas the treatment with 0.05 g/l of BTH increased the total phenolics 1.3-fold of that of the controls in arctic bramble four days after the treatment, the higher concentration (0.4 g/l) did not have similar influence. However, even higher concentration of BTH (0.8 g/l) was used in strawberry, where it increased the levels 1.5-fold (without infection) in comparison to the controls four days after the treatment. Thus, the concentration of BTH can be critical for the induction of specific defence responses and the same dose may have distinct effect in different species of the same plant family.

The trend observed in the concentrations of most individual compounds in strawberry was similar to that of the total phenolics (**I**: **Table 2**). Among all compounds, ellagitannins showed the highest increase by BTH in comparison to the controls, the difference being sixfold in maximum. Quercetin glycosides and ellagic acid were also increased in BTH-treated plants whereas the levels of ellagic acid derivatives were not different between the BTH-treated and control plants. Smaller increase in ellagic acid derivatives could be partly explained by competition in the synthesis between ellagitannins and other ellagic acid derivatives, with a bias toward the tannins. Only one, unidentified compound was decreased by BTH in strawberry leaves.

In arctic bramble, the higher concentration of BTH (0.4 g/l) caused the accumulation of quercetin triglycoside and unidentified compound 12 while its influence on the level of different phenolics was otherwise absent or negative (**III**: **supporting information**). The compounds decreased by BTH were unidentified ellagitannins 36 and 38a, unidentified hydroxycinnamate 3, *p*-coumaric acid derivatives 11 and 13 and, to a lesser extent, some other ellagitannins. The lower dose of BTH (0.05 g/l), on the other hand, caused the accumulation of several compounds.

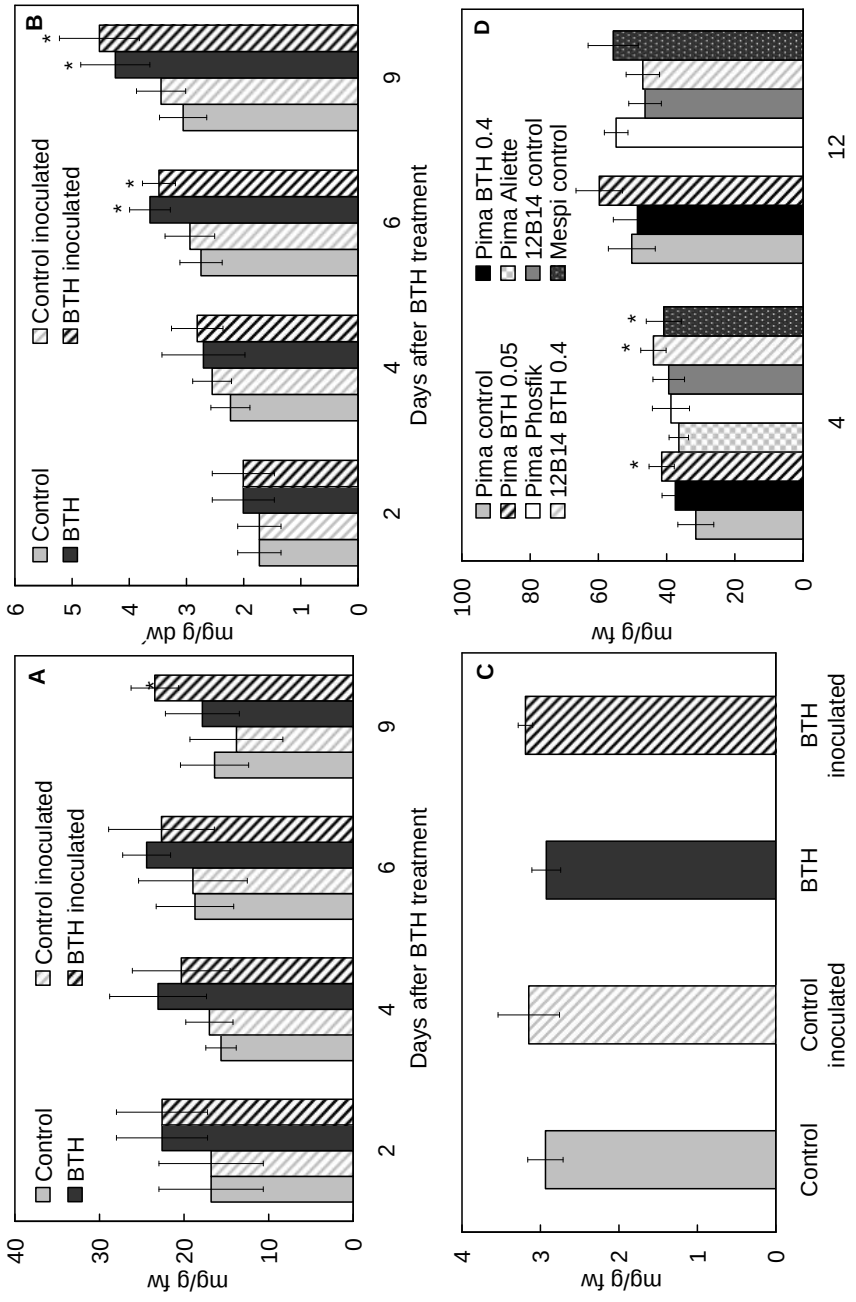

Figure 13. Level of total (A) soluble leaf phenolics in strawberry leaves, (B) cell-wall-bound phenolics in strawberry leaves, (C) soluble phenolics in strawberry fruits, and (D) soluble phenolics in arctic bramble leaves in different treatments and sampling times. Strawberry plants were inoculated 2 days and arctic brambles 4 days after BTH/agrochemical treatments. Statistical significant differences from untreated controls (from cv. Pima in arctic bramble)

Particularly, all quercetin glycosides, kaempferol glucuronide, sanguiin H-10, deHHDP- lambertianin C, tellimagrandin II, unidentified ellagitannin 25 and unidentified hydroxycinnamates 10 and 12 were increased 1.3-3.0-fold.

Increased levels of phenolic compounds have also been found in other plant species treated with BTH, such as cowpea, grapevine, peach and sugarcane, suggesting that one general action mechanism of BTH can be the enhancement of the synthesis of secondary defence metabolites (Latunde-Dadas & Lucas 2001; Ramesh Sundar et al. 2001; Iriti et al. 2005; Liu et al. 2005). In the present study (**III**), BTH concentration was found to affect the outcome of the response in arctic bramble. BTH has been shown to act dose-dependently in many plants, the response improving alongside with the higher doseuntil plateau state is reached or increased phytotoxicity of BTH at high doses start to impair the response (Godard et al. 1999; Eikemo et al. 2002). Schweizer et al. (1999) found in rice that the lowest concentration of BTH tested provided the best protection against the blast fungus, but defence responses using various doses of BTH were not studied. The results imply that the optimal dose of BTH for arctic bramble is below 0.4 g/l.

4.4.3 BTH-induced changes in cell-wall-bound phenolics in strawberry leaves

The cell-wall-bound phenolics were increased by BTH treatment with and without subsequent inoculation in the strawberry leaves. The level of total phenolics measured from the whole fraction before ethyl acetate extraction, increased steadily in the controls during the sampling period, but the highest levels were measured in the inoculated BTH-treated plants nine days after BTH treatment (**Figure 13B**). The total amount was, however, much lower than that of the soluble phenolics. The increase in cell-wall-bound phenolics in time may partly explain the higher resistance to powdery mildew in older leaves (Peries 1962b). Individual cell-wall-bound phenolics showed distinct patterns of induction (**I**: **Table 3**). The unidentified compounds 22 and 23 were significantly increased by BTH while smaller accumulation was found in gallic acid and ellagic acid. The concentrations of kaempferol hexose and *p*-coumaric acid were not different between BTH-treated plants and the controls. In contrast to the soluble phenolics, the concentrations of the cell-wall-bound phenolics remained at the high level in noninoculated BTH-treated plants. The induction of cell-wall-bound phenolics by BTH is in accordance with the results obtained on other plants (Benhamou & Bélanger 1998b; Stadnik & Buchenauer 2000).

The analysis of cell-wall-bound phenolics is not easy because of the tight and variable bonds present between the different components of the cell walls (Martens 2002; Ralph et al. 2004). Furthermore, strong alkali hydrolysis used in the extraction easily breaks the compounds, and the resulting mixture of unhydrolysed conjugates, aglycones and degradation products is complex. Organic extraction is used to separate (mainly) aglycones from other compounds, but not all aglycones are equally extractable into organic solvents. Therefore, a recovery test with standard compounds was conducted to analyse which compounds survive through the hydrolysis process. Hydroxycinnamic acids, ferulic acid and *p*-coumaric acid, which are considered as

the main phenolic group present in the cell walls, had high recoveries above 90% as also did hydroxybenzoic acid, showing that the method suits well for the analysis of those compounds. The other compounds tested, namely gallic acid, rutin and (+)-catechin, were not recovered after the hydrolysis and extraction, which indicates that they degrade in alkali or are not extractable into ethyl acetate. Despite the results of the recovery test, gallic acid and kaempferol hexose were found in the strawberry extracts, but they may have been released from conjugates more resistant to alkali than the standards used. Considering the limitations of the method, the concentrations of cell-wall-bound phenolics measured should be taken only as indicative.

4.4.4 Influence of BTH on fruit phenolics in strawberry

BTH was also able to increase the content of some individual phenolics in strawberry fruits although there were four weeks between the last BTH application and harvest (**I**: **Table 4**). The effect of BTH was not seen in the total phenolics (**Figure 13C**), but in certain individual phenolics in inoculated and noninoculated BTH-treated plants when compared to the corresponding water-treated plants. Flavan-3-ols, anthocyanins, kaempferol derivatives and unidentified compounds 28-32 and 37 were accumulated by BTH treatment. According to the present results and similar results from Iriti et al. (2004; 2005), BTH treatment can affect the quality of the edible plant parts. Surprisingly few studies have been conducted on this subject considering that e.g. the nutritional properties of the crops could be improved through standard plant protection practices. It is not known whether the slight increase in the fruit phenolics observed can explain the lower number of infected fruits found in the BTH-treated plants.

4.4.5 Effect of phosphites on phenolics in arctic bramble

Phosphite treatments had no effect on the phenolic content of artic bramble leaves as illustrated by the scattering in PCA (**III**: **Figure 5**). Regression factor scores from the controls and phosphite-treated cv. Pima were clustered together, indicating that there was no difference in the phenolic profiles of these two groups. This was supported by the closer analysis of the concentrations of individual compounds, where no significant increase or decrease was found in comparison to the controls. Similar results were obtained with all phosphite treatments analysed. A slight induction of flavonol glycosides and some ellagitannins was seen by Phosfik, particularly four days after the treatment, but its significance for the disease resistance may be small (**III**: **Supporting information**). Hence, the efficacy of phosphites against downy mildew in arctic bramble is explained by factors other than the accumulation of phenolics, e.g. by direct activity against the pathogen.

4.4.6 Variation of phenolic levels in different arctic bramble cultivars

Several previous studies have shown that phenolic content varies between cultivars (Maas et al. 1991; Anttonen et al. 2005; Hukkanen et al. 2006). The phenolic profiles from leaves of different arctic bramble cultivars also varied quantitatively, but not qualitatively (**III**: **Figure 5**). Cvs Pima, Mespi and the clone 12B14 were separated in

PC1 and PC3, indicating that the compounds clustered under those principal components mainly contribute to the differentiation of phenolic profiles between the cultivars. Cv. Pima was separated from Mespi and 12B14 in both principal components. The analysis of individual compounds shows that in cv. Pima, the level of flavonols and ellagitannins is clearly lower than in Mespi/12B14 (**III**: **supporting information**). Cv. Mespi and 12B14 are separated from each other in PC3, the compounds (flavonol triglycosides, tellimagrandin I and unidentified ellagitannin 25 and hydroxycinnamates 3, 10, 11, 13) being present at higher level in Mespi.

4.4.7 Contribution of phenolics to induced or basal resistance in strawberry and arctic bramble

The results presented above indicate that phenolic compounds, ellagitannins and flavonols in particular, may be involved in the resistance mechanisms of strawberry and arctic bramble to powdery mildew and downy mildew disease, respectively. Ellagitannins were strongly induced by BTH in strawberry at the same time as resistance to powdery mildew was significantly improved. The induction was also sufficiently rapid to be able to affect the early phases of fungal growth, i.e. germination and penetration, which are critical for the initiation of infection. The model plant *Arabidopsis* and several main crops such as cereals, tobacco, potato, maize and many horticultural crops do not contain ellagitannins, which is probably one reason for the limited information available about the role of ellagitannins in resistance to pathogens. Antimicrobial activity of ellagitannins has been proved *in vitro* and in few woody species, suggesting that ellagitannins could have activity against microbes in herbaceous plants as well (Cowan 1999; Puupponen-Pimiä et al. 2001; Barry et al. 2001). In addition to tannins, the levels of cell-wall-bound phenolics increased in BTH-treated strawberry and could have affected the penetration of the powdery mildew fungus. In other pathosystems, penetration of biotrophic fungi has been disturbed by the accumulation of phenolic material at the infection site (Dai et al. 1995; Stadnik & Buchenauer 2000; Hückelhoven 2007). An analysis of local responses around the infection sites could perhaps been more informative concerning the effects of phenolics on the penetration than is the analysis of the whole leaves done in the present study. Microscopic analyses gave some indications that phenolic material and/or callose accumulates in the proximity of germinated conidia, but systematic analysis of the deposits in the control and BTH-treated plants was not performed.

An important aspect in the present study was to analyse possible correlation between the concentration of individual phenolics and resistance to downy mildew in arctic bramble, which could reveal the key molecules contributing to the resistance. Thus, the correlation was determined between the amount of individual phenolic compounds and pathogen DNA in different cultivars and treatments. Significant negative correlation was found between the amount of *P. sparsa* DNA and quercetin diglycoside (R= -0.408*), kaempferol diglycoside (R= -0.446*), quercetin glucuronide (R= -0.424*), kaempferol glucuronide (R= -0.477*), sanguiin H-6 (R= -0.357*), sanguiin H-10 (R= -0.285*), lambertianin C (R= -0.339*), roshenin B (R= -

0.321*) and unidentified ellagitannin 21 (*R*= -0.389*) eight days after inoculation. DeHHDP-lambertianin C, which was at the highest level in BTH-treated plants, correlated negatively to pathogen DNA already at the day of inoculation. Although negative correlation was also found between the total phenolics and pathogen DNA at the day of inoculation, similar correlation was not found eight days later, indicating that the induction of specific rather than total phenolics might contribute to the resistance to *P. sparsa*.

The high concentration of flavonols, ellagitannins and some hydroxycinnamic acids in the cultivars more resistant to *P. sparsa* suggests that those compounds may be involved in the defence against downy mildew in arctic bramble. Flavonols showed the most evident negative correlation to pathogen DNA. They may inhibit the growth of *P. sparsa*, since flavonols have shown antimicrobial and antifungal activity as well as accumulated as phytoalexins in other plants (Fawe et al. 1998; Curir et al. 2005; Báidez et al. 2006). The role of ellagitannins seems more complicated, since the high total level of ellagitannins could not protect 12B14 from the infection in the plate test. Furthermore, different tannins were elevated in cv. Mespi and BTH-treated cv. Pima, both showing resistance to *P. sparsa*. The compounds showing negative correlation to the growth of *P. sparsa* should be analysed in a wider range of plant genotypes together with the analysis of resistance to several pathogen isolates to draw conclusions about the significance of these compounds in disease resistance. Factors other than the accumulation of phenolics, e.g. increase of PR proteins, may explain the resistance induced by BTH, since only the lower concentration of BTH caused the increase in phenolics.

4.5 BTH-induced changes in leaf proteome of arctic bramble

4.5.1 Identified proteins

Two-dimensional protein analysis was performed to study the effects of BTH on leaf proteome of arctic bramble cv. Mespi (**IV**: **Figure 3**). Samples were collected four days after the BTH treatment, which was shown to be the point of maximal induction of PR-1 (**IV**: **Figure 2A**) and, thus, possibly also of other proteins. Altogether 792 valid protein spots were detected, of which 103 were significantly increased or decreased by BTH treatment. Eight spots were detected only in BTH-treated plants, 27 proteins showed at least two-fold increase, 37 less than two-fold increase, 7 at least two-fold decrease and 24 less than two-fold decrease compared to the control plants. The spots significantly affected by the BTH treatment were subjected to MS analysis for identification. Consequently, 18 proteins could be tentatively identified (**IV**: **Table 1**) based on homology to proteins characterized in other species since limited data are available for protein sequences from *Rubus* or other rosaceous plants. Therefore, the majority of the proteins, including many strongly affected ones, could not be identified.

4.5.2 Changes in defence metabolism

Many of the proteins identified could be associated with defence metabolism of plants. In addition to PR-1, which was strongly induced by BTH (**IV**: **Figure 2C**), the quantity of PR-10 was increased by BTH (**IV**: **Table 1**). The function of PR-10 proteins is not fully known, but their accumulation by SA, BTH or various stresses indicate a role in active defence of plants (Ziadi et al. 2001a; McGee et al. 2001; Poupard et al. 2003). PR-10 proteins have shown ribonuclease activity and they can bind ligands such as flavonoids and cytokinins, and permeabilize membranes (Park et al. 2004; Koistinen et al. 2005; Srivastava et al. 2006; Mogensen et al. 2007). The suggested role as a carrier of flavonoid-type small hydrophobic compounds (Koistinen et al. 2005) is supported by the fact that in white-coloured mutant of strawberry, the PR-10-like allergen protein (birch Bet v 1 homologue) is down-regulated together with flavonoid synthesis (Hjernø et al. 2006). Another recent study showed that overexpression of PR-10 leads to elevated levels of cytokinins, which is in line with the ability of PR-10 proteins or their structural homologues to bind cytokinins (Srivastava et al. 2006; Pasternak et al. 2006). Cytokinins are phytohormones, which positively regulate growth, inhibit senescence and may act as antioxidants in oxidative stresses (Gidrol et al. 1994; Srivastava et al. 2006). Considering defence to pathogens, the role of PR-10 proteins seems complicated as Colditz et al. (2007) found higher resistance to oomycete pathogen *Aphanomyces euteiches* by repressing PR-10 protein in *Medicago truncatula*. Because the PR-10 family consist of several isoforms, different isoforms may be involved in the different functions.

Supporting the co-regulation with PR-10, two tentatively identified enzymes of the phenylpropanoid pathway, flavanone-3-hydroxylase and caffeoyl-CoA-3 *O*-methyltransferase, were up-regulated by BTH (**IV**: **Table 1**). Particularly, the clear increase in flavanone-3-hydroxylase (no spot detected in the controls) is highly consistent with the observed accumulation of flavonols by BTH in arctic bramble, since the enzyme catalyzes the conversion of naringenin to dihydrokaempferol or eriodictyol to dihydroquercetin in the pathway to flavonols. Caffeoyl-CoA-3 *O*-methyltransferase is involved in the synthesis of lignin (Martz et al. 1998), but the amount of lignin was not determined in this study. Supporting the present results, both enzymes were induced by BTH in wheat (Pasquer et al. 2005).

Alanine aminotransferase (AlaAT) was detected only in the BTH-treated plants (**IV**: **Table 1**). The induction of AlaAT is interesting considering some of its known functions. AlaAT has been found inducible by viral and bacterial pathogens, senescence, SA and ethylene similarly to PR-1, which suggest that it is linked to the SA-mediated defence pathway (Kim et al. 2005). AlaAT catalyzes the production of intermediates of photorespiration in peroxisomes, but may also be located in cytoplasm or mitochondria (Liepman & Olsen 2003). The role of AlaAT in photorespiration is also supported by the activation of other photorespiratory enzymes in transgenic melons carrying either alanine or serine glyoxylate aminotransferase (Taler et al. 2004). Furthermore, AlaAT was classified as enzymatic *R* protein in melon, providing resistance to downy mildew pathogen. Considering the role of

photorespiration in defence to pathogens, H_2O_2 generated in the oxidation of glycolate may be used for the development of rapid oxidative burst and activation of HR and downstream defence (Mateo et al. 2004; Taler et al. 2004). Interestingly, SA can induce stomatal closure that leads to reduced gas exchange, subsequent decrease in cellular CO_2 and activation of photorespiration (Mateo et al. 2004). Possible role of photorespiration in SA-mediated defence in general remains to be elucidated.

1-aminocyclopropane-1-carboxylate (ACC) oxidase, which catalyzes the last step in ethylene synthesis, was increased twofold by BTH in comparison to the controls (**IV**: **Table 1**). The induction of ACC oxidase by BTH has also been reported in bean (Iriti &Faoro 2003). Ethylene is suggested to act in concert with JA, as both are involved in the resistance against necrotrophs. However, ethylene released during HR may also intensify the local expression of PR proteins in SA-mediated defence and is required for TMV-induced SAR in tobacco (Verberne et al. 2003). In ethylene-insensitive mutants the local accumulation of SA is normal, but the systemic accumulation of SA and PR proteins is lower than in the control plants, indicating that ethylene is involved in the generation of systemic signal in SA-mediated defence pathway. Despite the observed induction of ethylene synthesis genes, direct increase of ethylene by BTH or SA has not been confirmed (Rao et al. 2002; Iriti & Faoro 2003). SA may prime the production of ethylene by increasing the levels of enzymes required for its synthesis and and the production of ethylene is further activated only when necessary to establish stress-induced cell death, SAR or other defence reactions. It is also possible that ethylene produced after BTH treatment causes the slow accumulation of conjugated SA observed in cv. Mespi.

4.5.3 Changes in energy metabolism

BTH both increased and decreased proteins that are involved in the production of energy. Up-regulated proteins were phosphoglycerate kinase and malate dehydrogenase whereas fructose bisphosphate aldolase, triosephosphate isomerase (chloroplastic) and ATP synthase were down-regulated (**IV**: **Table 1**). These proteins have often been found up-regulated in stressed plants, indicating higher need for energy and metabolites during defence (Curto et al. 2006; Segarra et al. 2007). In the present study, the changes observed in the proteins functioning in glycolysis, citric acid cycle and photosynthesis were relatively small (0.8/1.2) and their significance remains unclear as only a small group of proteins affected by BTH could be identified.

The most evident decrease observed in ATP synthase is consistent with the reduction of vegetative growth by BTH in arctic bramble. Cell culture studies have also shown that SA can inhibit respiration and ATP synthesis in mitochondria (Norman et al. 2004), which supports the observed decline in ATP synthase by BTH in the present study.

4.5.4 Changes in other proteins

Some of the proteins affected by BTH are involved in the degradation, folding or transport of proteins. The amount of nascent polypeptide-associated complex α chain

(αNAC) and 20S proteasome α subunit were increased by BTH (**IV**: **Table 1**). αNAC may function in the transport and targeting of nascent peptides from ribosomes or in the transport of proteins into mitochondria while 20S proteasome α subunit is a part of the large ATP/ubiquitin-dependent 26S proteolytic complex (Wang et al. 1995; Funfschilling & Rospert 1999). The activation of protein processing in the cells may be caused by the redirection of protein synthesis from basic metabolism to defence. Proteasome subunit has also shown responsiveness to SA and stresses in other studies (Etienne et al. 2000). The down-regulation of heat shock protein (HSP) 70 was quite unexpected considering that, similarly to αNAC, it is involved in the quality control of proteins together with other heat shock proteins and is often increased by stresses (Hartl & Hayer-Hartl 2002). Kanzaki et al. showed that HSP70 is also connected to the development of non-host resistance and HR in *Nicotiana benthamiana* (Kanzaki et al. 2003). The reason for the down-regulation of HSP70 by BTH in arctic bramble remains unclear.

The other proteins identified are related to diverse functions in plants. BTH increased the amount of reversibly glycosylated protein (RGP), the function of which is unclear. The protein is located in plasmodesmata, can presumably self-glycosylate and participate in cell division and the development of cell walls (Sagi et al. 2005; Drakaki et al. 2006). The other two proteins, i.e. 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and glutamine synthetase, were slightly decreased by BTH (**IV**: **Table 1**). DXR is involved in the synthesis of isoprenoid-derived molecules such as chlorophyll, plastoquinones, carotenoids and terpenes in plastids. The pathway is responsive to abiotic and biotic stresses, but it is unclear how the down-regulation of the pathway is related to SA-mediated defence, particularly considering the high number of different products coming from the pathway (Lichtenthaler 1999; Carretero-Paulet et al. 2002). The down-regulation of glutamine synthetase may be linked to the reduced vegetative growth observed in BTH-treated arctic bramble as glutamine synthetase regulates nitrogen metabolism by assimilating ammonium (Tabuchi et al. 2007).

5. SUMMARY AND CONCLUSIONS

The aim of this work was to evaluate the efficacy and applicability of induced resistance against powdery mildew and downy mildew diseases of strawberry and arctic bramble, respectively. The second aim was to analyse specific defence-related biochemical and chemical changes during the development of resistance and, thus, possibly find compounds, which are important for the resistance against the pathogens.

Quantitative real-time PCR and *in vitro* plate test for the analysis of arctic bramble-*P. sparsa* interaction

Two methods were developed for the analysis of arctic bramble-*Peronospora sparsa* interaction. Quantitative real-time PCR proved to be specific, sensitive and reproducible, enabling the quantification of low amounts of *P. sparsa* DNA from the samples showing no visible symptoms of the disease. The primers designed can also be used for the analysis of downy mildew in other *Rubus* or *Rosa* species. A simple and fast *in vitro* plate test was developed for the pre-screening of resistance in different genotypes and agrochemical treatments before executing larger experiments in the greenhouse or in the field. Both methods can be used in the evaluation of breeding material and efficacy of plant protection practices.

Resistance to pathogens was improved by BTH and other agrochemicals

BTH treatment improved resistance to both powdery and downy mildew diseases in the greenhouse when applied prior to inoculation. Induction of resistance by BTH lowered the weight of foliage in both plants, but at least under the conditions used, no reduction in fruit yield was observed. Of the two registered fungicides against *P. sparsa* in Finland, Euparen M was more efficient than Aliette. Phosphite-based products Aliette, Phosfik and Phostrol also gave moderate level of protection against *P. sparsa*. Phosphites might be a useful option for the protection of arctic bramble due to their low toxicity and short withholding periods, but their efficacy has yet to be proven under field conditions. In general, induced resistance was found as a promising means for the protection of arctic bramble and greenhouse-grown strawberry.

Differences in the basal resistance to *P. sparsa* were found in three arctic bramble cultivars. Cv. Mespi was most resistant to the pathogen isolate used, but resistance of cultivars still needs to be analysed against several pathogen isolates.

BTH activates SA-mediated defence pathway despite of high endogenous SA levels

Exceptionally high basal concentrations of SA were found in strawberry and arctic bramble leaves. BTH was shown to activate SA-mediated defence in arctic bramble, as evidenced by the accumulation of the marker protein PR-1. BTH also induced slow accumulation of SA in both species. However, the induction of PR-1 was not related to the accumulation of conjugated SA in arctic bramble, suggesting that the increase

in SA is not for signalling purpose. Because the induction of SA highly resembled that of other phenolic compounds, SA may have similar functions to those found for other phenolics.

Accumulation of phenolic compounds may be involved in the resistance to pathogens

Ellagitannins were the most abundant phenolics identified in strawberry and arctic bramble leaves, but only a few compounds identical between the species were found. Besides ellagitannins, flavonol glycosides and ellagic acid derivatives were common in strawberry whereas flavonol glycosides, hydroxycinnamates and galloyl glucoses were common in arctic bramble. Cell-wall-bound phenolics were characterised in strawberry for the first time.

BTH induced the accumulation of ellagitannins, ellagic acid and flavonol glycosides in strawberry whereas the amount of individual phenolics was increased or decreased by BTH in arctic bramble. Phosphites did not affect the phenolics in arctic bramble. Ellagitannins may be important in the resistance to powdery mildew in strawberry as their accumulation was strong and rapid enough to inhibit the initial phases of infection. The increase in cell-wall-bound phenolics may also be involved in the restriction of infection. In arctic bramble, flavonols and some ellagitannins showed negative correlation to the amount of pathogen DNA, indicating that those compounds may be effective against *P. sparsa*. However, other factors such as defence proteins are also likely to contribute to induced resistance in both plant species.

BTH affects the leaf proteome in arctic bramble

BTH increased or decreased the amount of 103 proteins in arctic bramble cv. Mespi leaves. From the 18 tentatively identified proteins, 10 were increased and 8 decreased by BTH. Among the increased proteins, PR-10, flavanone-3-hydroxylase, ACC oxidase and AlaAT were the most interesting ones in terms of defence. Decrease in ATP synthase may explain the observed reduction in vegetative growth. Since only a small fraction of the proteins affected by BTH could be identified, overview of the metabolic changes remained quite limited. However, several proteins possibly important for defence in arctic bramble were found.

Conclusion

The application of chemically induced resistance against two diseases of strawberry and arctic bramble gave promising results, which encourages evaluating further the effectiveness of the approach in the field. Several phenolic compounds and proteins possibly involved in the resistance to powdery and downy mildew pathogens were found. The identified compounds can be isolated and further tested against the pathogens. The information about the phenolic composition in the leaves or fruits can also be used for the isolation of bioactive compounds for other purposes. New information was obtained and methods developed for more efficient and targeted analysis of arctic bramble-*P. sparsa* interaction.

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