Molecular Characterization of Pine Response to Insect Egg Deposition

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Content

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Chapter 1
General introduction and outline

Plants produce a large variety of secondary metabolites that were once thought to be waste products with no specific function in the life of the plant. Today we know that these products fulfill a wide range of important purposes in an organism's life. A simplified classification of secondary metabolites distinguishes: nitrogen-containing compounds, acetylenic compounds, phenolic compounds, and terpenoids (Schoonhoven 2005). Terpenoids are probably the largest class among these secondary metabolites, and are synthesized by plants, fungi, and metazoans for many biological purposes as have already early findings shown. They fulfill purposes such as: anticompetitor adaptations, like allelopathy (Muller 1966) that e.g. reduces germination rate in neighboring plants; mate attractants e.g. sex pheromones in insects and higher animals (Riddifor & Williams 1967); trail markers e.g. in ants (Wilson 1965) insect attractants (Edgar & Culvenor 1975) and of course repellants against enemies (Yoon *et al.* 2009). Terpenoids show an immense diversity of structures and their basic units and are biosynthesized via two major pathways: the mevalonate (MVA) pathway in the cytosol or the plastidic methylerythritol phosphate (MEP) pathway in the plastids.

Terpenoids are all derived from a five-carbon precursor, namely isopentyl diphosphate (IPP) or its isomer dimethylallyl diphosphate (DMAPP). The condensation of two, three, or four of these five-carbon units results in the formation of the intermediates geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), which are substrates of the terpene synthases. The terpene synthases produce the basic carbon skeletons of the terpenoids, and their products can be classified according to the number of their constituent isoprene units. Compounds with two isoprene units (10-carbon atoms) are called monoterpenes, compounds with three isoprene units (15 carbon atoms) sesquiterpenes, and compounds with four isoprene units (20 carbon atoms) diterpenes (Fig. 1). Of these groups, monoterpenes and sesquiterpenes are often volatile (quickly vaporize in the atmosphere) and thus function in plant communication with other organisms.

Other terpene classes with a higher molecular weight are triterpenes (30 carbon atoms), tetraterpenes (40 carbon atoms), polyterpenes (> 40 carbon atoms) which will not be further discussed in this review.

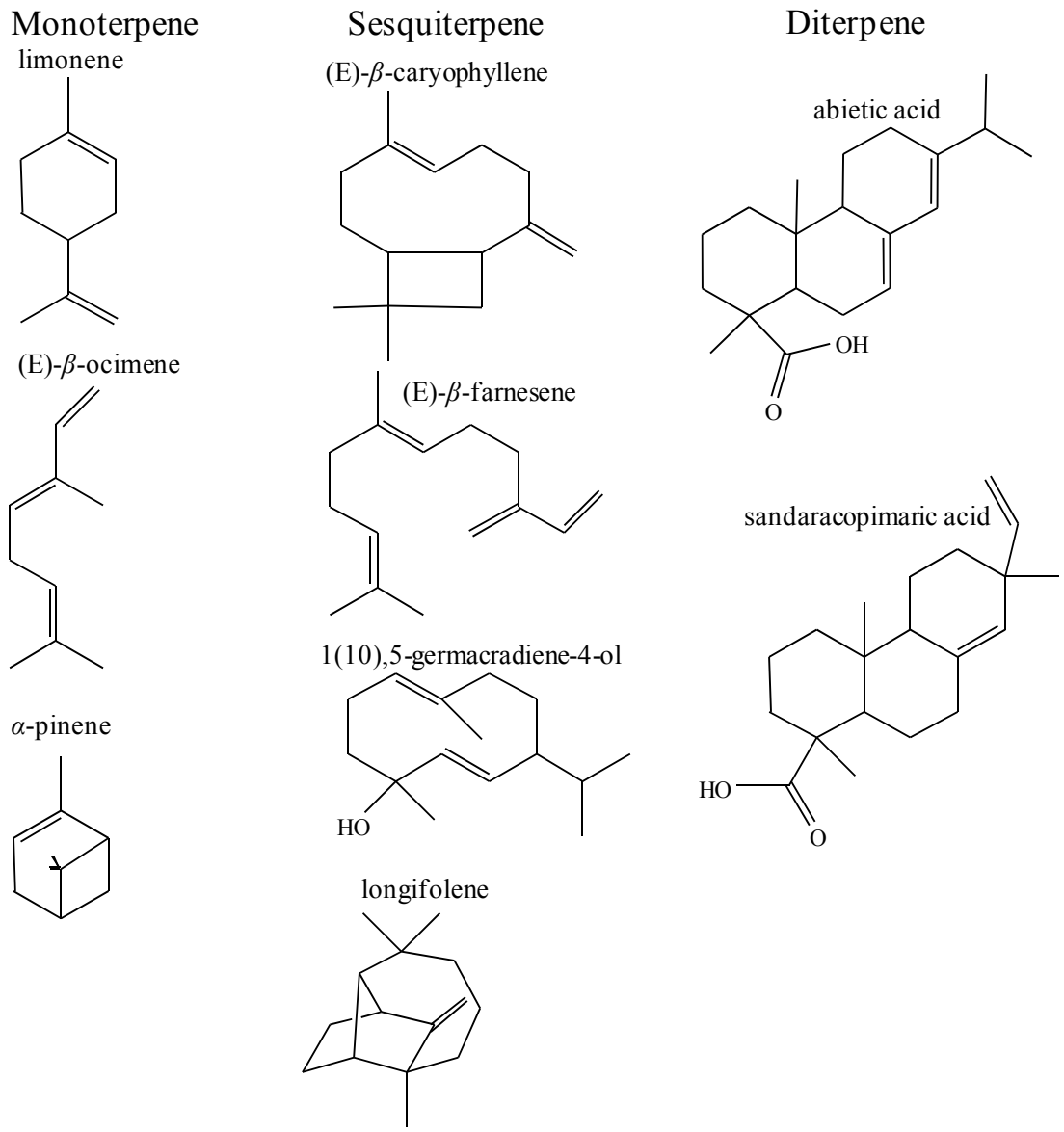

Fig. 1 Chemical structures of exemplarily monoterpenes, sesquiterpenes and diterpene resin acids.

In plants terpenes can act among others as defenses against herbivores. They can be accumulated in the plant tissue as feeding deterrent as e.g. described for the milkweed plant *Asclepias curassavica* that contains toxic cardenolides (steroidal glycosides that are derived from triterpene) in the latex canals, which shows symptoms of poisoning when consumed by the lepidopteran larvae *Trichoplusia ni* (Dussourd & Hoyle 2000). Another example is Artemisinin which is a sesquiterpene lactone that is produced by the plant *Artemisia annua*. Extracted or synthetic produced artemisinin was found to be effective against malaria and is now commercially used as an antimalarial drug (Wright *et al.* 2010).

Terpenes being released in the atmosphere can also keep insects away, as was shown for floral scents when testing the response of different insect species. Junker & Bluthgen

(2010) examined that floral scent can act not only as attractants but also as defensive cues to deter unwanted floral visitors.

Especially conifers seem to have found sophisticated defense mechanisms by using terpenes. Taking for example conifer resin, which is stored in resin ducts and specialized secretory cells, and is a complex mixture of monoterpenes, sesquiterpenes and diterpenes (Persson *et al.* 1996; Sjodin *et al.* 1996; Fäldt 2000) that helps these long-lived trees withstand many generations of pest attacks. Conifer resin can often be classified as a constitutive defense since it is stored continuously in resin ducts in needles and stems and protects the tree against insect infestation and fungal infection by acting as a toxin, an odorous repellent and a physical barrier (Martin *et al.* 2002; Franceschi *et al.* 2005). A constitutive defense provides a plant with constant protection, but requires investment of resources in defense without any knowledge about whether attack is likely or not (Wagner *et al.* 2003)

Another defense strategy is the induction of defenses that are mobilized only after initial herbivore or pathogen attack. In conifers, induced defense involves the formation of additional resin ducts (known as traumatic resin ducts). Such a flexible response to herbivore damage may allow plants to minimize their fitness cost of resistance to enemies by forming defenses only as they are needed (Heil & Baldwin 2002; Cipollini *et al.* 2003). Contrary to the constitutive defense, induced defenses require the plant to "notice" the attacker which may be accomplished by recognition of certain specific elicitors that can be located in the saliva or oviduct secretion of herbivore attackers (Hilker & Meiners 2010).

Inducible defense can be further classified into direct and indirect defenses (Dicke 1999) which is also true for the constitutive defense. The direct defense acts immediately upon the attacker as toxins, repellents or digestibility reducers (Arimura *et al.* 2009). On the other hand, indirect defenses involve the participation of a third party, a predator, parasitoid or other microorganism that attacks the plants natural enemy. So the plant promotes the effectiveness of the natural enemy.

For example, one of the well-documented indirect defenses of plants against herbivores is to emit specific blends of volatiles in response to herbivory that attract natural carnivores of the herbivore. These blends vary according to the plant and herbivore species, and mediate specific interactions of plants with herbivores and their enemies (Sabelis *et al.* 2007; Heil 2008; Arimura *et al.* 2009).

Despite the benefits of inducible defenses against herbivores or pathogens, one obvious disadvantage is the time-lag between the beginning of damage and the production of the defense (Mumm 2004). During this time the plant is unprotected (Zangerl 2003). One way to overcome this risky time-lag in the case of herbivory is to react before the actual feeding starts. For example, several studies have shown that egg deposition by an herbivore can stimulate plant defense responses well before the larvae hatch by triggering the release of

chemicals that function to attract egg parasitoids (Meiners & Hilker 2000; Hilker *et al.* 2002a; Mumm *et al.* 2003; Colazza *et al.* 2004a; Fatouros *et al.* 2005; Schnee *et al.* 2006; Bruce *et al.* 2010). For example, studies by Meiners and Hilker (2000) have shown that oviposition by the elm beetle *Xanthogaleruca luteola* induces elm leaves to emit an odor that attracts a parasitoid killing the elm beetle eggs, thereby protecting the tree from larval feeding.

Another strategy for plants to get prepared before the actual attack by feeding starts, is reaching a so called "primed state". In the early 80´s, Rhoades (1983) found that the tree *Salix sitchensis* growing close to herbivore-infested conspecifics showed higher resistance levels than trees growing further away. A similar result was found for poplar and sugar maple trees by Baldwin & Schulz (1983). Heil (2010) discovered that volatiles released from herbivore infested plants can be received by their undamaged neighbors which then raise their defense shield. Also possible is that undamaged parts of the herbivore-infested plant mount an adequate level of resistance (systemic response); therefore the signal of infestation is not only active locally but also travels in un-infested parts of the plant. Priming prepares the undamaged tissues to respond more rapidly and/or effectively to subsequent attack (Goellner & Conrath 2008). As mentioned an 'early herbivore alert' can also appear after insect egg deposition (Hilker & Meiners 2006). The resulting resistance induction, e.g. emission of parasitoid attractive volatiles, has been described so far in trees such as elm (*Ulmus minor*) and pine (*Pinus sylvestris*). However, the question whether eggs are able to induce direct defenses of elm and pine against hatching larvae has not been addressed prior to this thesis.

Much more is known about the quite effective emission of plant volatiles after herbivore damage. However, the release of volatiles to attract enemies of the herbivores has its drawbacks considering the range of odors present in a natural ecosystem. Furthermore plant volatiles may be chemically degraded through contact with ozone, hydroxyl- and nitrate radicals (McFrederick *et al.* 2008). One way to enhance the emission of volatiles is to release volatiles systemically from the whole plant and not just from the damaged portion (Meiners & Hilker 2000; Hilker *et al.* 2002a; Colazza *et al.* 2004a, b). If this systemically induced odor attracts natural enemies to the vicinity of the plant, then local cues, restricted to the oviposition or feeding site can facilitate fine-scale orientation (Fatouros *et al.* 2005).

Besides the intensity of emission, timing is also an essential factor in affecting the value of a signal to attract herbivore enemies. An induced attraction of carnivorous arthropods usually occurs within one to a few days after damage starts (Schoonhoven 2005). In the case of egg parasitoids, the age of the egg can be critical, and there may be only a short period suitable for successful parasitization due to the fast development of the egg (Peschke *et al.* 1987). This has been shown for insect eggs and egg parasitoids on bean (*Vicia faba*) by Colazza *et al*. (2004b). Timing of egg parasitation was also shown to be crucial in a conifer

system. Hilker *et al.* (2002) described the tree *Pinus sylvestris* to respond to egg deposition by the sawfly *Diprion pini* with local and systemical emission of an attractive odor for the parasitoid *Closterocerus* (former *Chrysonotomyia*) *ruforum*. Hilker *et al.* (2002a) and Mumm *et al.* (2005) showed that an increased emission of the sesquiterpene (*E*)-*β*-farnesene 72 h after oviposition is responsible for the parasitoid attraction. However, apart from (*E*)-*β*-farnesene, other volatile terpenoids within the complex blend released from *P. sylvestris* are also necessary to attract the parasitoid (Mumm & Hilker 2005; Beyaert *et al.* 2010).

Most of the indirect plant defense systems described in the literature have been studied from ecological and behavioral perspectives. However, very little is known about the biosynthesis of key substances for parasitoid attraction and how synthesis is regulated. Terpenoid formation is often thought to be regulated at the transcript level of the terpene synthase genes (McKay *et al.* 2003), but the mode of regulation can be very complex (Dudareva *et al.* 2003; Köllner *et al.* 2004) and needs to be studied individually with respect to the plant under focus, the herbivore and most important the parasitoid. A number of studies have shown that the introduction and over expression of certain terpene synthases genes into a host- as well as a non-host plant under a constitutive promoter can lead to an enhanced release of specific terpenes that attract parasitoids searching for their host (Schnee *et al.* 2006; Cheng *et al.* 2007). However, under normal conditions, gene activation is controlled by specific promoters.

Several studies have shown that plants can react to herbivore feeding by recognizing herbivore specific elicitors followed by the release of parasitoid attractive volatiles. These elicitors act by triggering various signaling pathways in the plant, such as those for jasmonic acid and salicylic acid, leading to the activation of a large array of defense-related genes which may include those for volatile formation (Kessler & Baldwin 2002). However, only few studies have been carried out to investigate gene expression in defense production after herbivore oviposition. Little *et al*. (2007) as well as Fatouros *et al.* (2008) could show that oviposition causes specific transcriptional changes in the plant, but these have not been investigated with respect to the formation of volatiles that attract enemies to herbivore eggs.

The main goal of this thesis is to use molecular tools to obtain information on the mechanisms underlying the tritrophic interaction of the conifer tree *Pinus sylvestris*, the herbivorous sawflies *Diprion pini* and *Neodiprion sertifer*, and the egg parasitoid *Closterocerus ruforum*. The parasitoid uses a volatile signal to find sawfly eggs laid on the tree, but there is no information on how the formation of this signal is regulated. The egg parasitoid is attracted by a complex blend of pine terpenoids which is characterized by enhanced quantities of the sesquiterpene (*E*)-*β*-farnesene. The pine volatile blend that attracts the egg parasitoid is released 72 h after egg deposition. There is no information about the enzyme producing the main attractant as well as the gene coding for this enzyme. We do not

know whether egg deposition changes the transcription of the gene encoding an (*E)-β*-farnesene synthase nor do we know whether transcription of other terpene synthases is affected by egg deposition

The experiments presented in *chapter 2* aimed to isolate and identify terpene synthase enzymes that might be involved in producing parasitoid attractants (Mumm & Hilker 2005). Two genes coding for pine sesquiterpene synthases were isolated and shown to be activated 72 h after oviposition by *D. pini* and *N. sertifer*. A third sesquiterpene synthase (*PsTPS*3) was not affected by oviposition at the transcriptional level.

To gain further insight into the molecular regulation of this system, *chapter 3* compares terpene synthase gene expression when the species of pine and sawfly are changed to give different combinations, some resulting in parasitoid attraction and some not. An additional terpene synthase was isolated in the course of this study that produces the major attractant previously reported for this tritrophic system, i.e. (*E*)-*β*-farnesene.

Chapter 4 explores the question of whether sawfly egg deposition prepares pine defenses against larval sawfly feeding using chemical analysis of pine foliage, molecular analysis of terpene synthase transcript levels and analysis of performance parameters of feeding sawflies.

Chapter 5 highlights the function of certain terpene synthases in plant-insect communication in gymnosperms and angiosperms.

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Chapter 2

Does egg deposition by herbivorous pine sawflies affect transcription of sesquiterpene synthases in pine?

Abstract

Scots pine (*Pinus sylvestris*; Pinaceae, Pinales) is known to defend against egg deposition by herbivorous sawflies by changing its terpenoid volatile blend. The oviposition-induced pine odor attracts egg parasitoids that kill the sawfly eggs. Here, we investigated whether sawfly egg deposition activates genes encoding pine terpene synthases by extracting mRNA from oviposition-induced *P. sylvestris*. Three new sesquiterpene synthases, *PsTPS*1, *PsTPS*2, and *PsTPS*3, were isolated that were shown on heterologous expression in *Escherichia coli* to produce (*E*)-*β*-caryophyllene and *α*-humulene (*PsTPS*1), 1(10),5-germacradiene-4-ol (*PsTPS*2), and longifolene and *α*-longipinene (*PsTPS*3) as their principal products. Quantitative RT-PCR analyses revealed that transcript levels of *PsTPS*1 and *PsTPS*2 were significantly higher in oviposition-induced twigs that were attractive to the parasitoids than in non-attractive, artificially damaged twigs. Thus, our results demonstrate a specific transcription response to egg deposition, distinct from that caused by artificial wounding. Transcripts of *PsTPS*3 did not change in response to egg deposition. The transcript levels of *PsTPS*1, *PsTPS*2, and *PsTPS*3 were also determined in relation to time after egg deposition, since pine odor is attractive to the parasitoid only 72 h after egg deposition. Transcription rates of *PsTPS*1 and *PsTPS*2 were significantly enhanced only 72 h after egg deposition, thus matching the timing of odor attractiveness, while for *PsTPS*3, enhanced transcription was not detected at any time period studied after egg deposition. The ecological significance of the oviposition-induced increase of sesquiterpene synthase transcripts is discussed.

Keywords Conifers, *Diprion pini*, Egg deposition, Induced defense, Pinus, Terpene synthase

1. Introduction

Numerous plant species were shown to change the composition of their odor in response to herbivore feeding (Karban & Baldwin 1997; Walling 2000). This attack-induced change of the plant volatile pattern is known to attract natural enemies of herbivorous arthropods, including predators and parasitoids (Dicke & van Loon 2000; Gatehouse 2002). Terpenoids are major components of plant volatile blends and play a predominant role in the attraction of enemies of the herbivores (Van Poecke & Dicke 2004).

Change in plant odor is induced not only by herbivore feeding, but also by insect egg deposition. In several plant species, oviposition-induced volatiles (among them mono- and sesquiterpenes) were shown to attract egg parasitoids (Hilker *et al*. 2002a; Hilker & Meiners 2002; Colazza *et al*. 2004; Mumm & Hilker 2006). These compounds are released locally from the site of egg deposition and also systemically from adjacent egg-free plant parts (e.g., Hilker & Meiners 2006; Hilker *et al*. 2002b). Such an indirect defensive plant strategy is considered an "early herbivore alert", since it acts even prior to the onset of larval feeding damage (Hilker & Meiners 2002, 2006).

The change of the pattern of terpenoids released by a plant under herbivore attack may be due to (1) damage of cells releasing stored terpenoids (Röse *et al*. 1996; Röse & Tumlinson 2004), or (2) changes in the de novo synthesis of terpenoids that are released immediately after production (Paré & Tumlinson 1997). This de novo synthesis in turn has often been ascribed to the activation of genes encoding terpene synthases (e.g., Bohlmann *et al*. 1999; McKay *et al*. 2003; Arimura *et al*. 2004; Kappers *et al*. 2005; Miller *et al*. 2005; Byun-McKay *et al*. 2006; Schnee *et al*. 2006).

Like feeding-induced terpenes, oviposition-induced plant terpenoid volatiles can also be expected to be a result of activation of terpene synthase genes based on the following lines of evidence: (1) while cell damage accompanying egg deposition may allow release of stored terpenes, the emission of attractive volatiles from systemically (non-damaged) oviposition-induced plant parts cannot be due to release from damaged cells, but are rather due to de novo synthesis (Meiners & Hilker 2000; Hilker *et al*. 2002b). (2) Egg deposition by *Pieris brassicae* on *Arabidopsis thaliana* does not cause any obvious leaf damage, but nevertheless induces a wide range of plant genes, including two terpene synthases (Little *et al*. 2007). It is not known whether *A. thaliana* changes its volatiles in response to insect egg deposition.

Thus, no study is available so far explaining how insect egg deposition affects the release of volatiles in those plant species that attract egg parasitoids by oviposition-induced odor. Is such a change of plant volatiles regulated on the molecular level by oviposition-induced enhanced transcription of genes encoding enzymes involved in biosynthesis of the relevant volatiles? We addressed this question by studying *Pinus sylvestris* (Scots pine), a

species that changes its odor locally and systemically in response to egg deposition by the pine sawfly *Diprion pini*. An egg parasitoid, the eulophid wasp *Chrysonotomyia ruforum* which may significantly limit sawfly populations, is attracted by the oviposition-induced odor of Scots pine (Eichhorn & Pschorn-Walcher 1976; Hilker *et al*. 2002b, 2005). The sawfly female slits a needle longitudinally with her sclerotized ovipositor valves and lays eggs into the slit. Mimicking this ovipositional wounding artificially with a scalpel does not result in release of volatiles attracting the egg parasitoid (Hilker *et al*. 2002b). These studies showed that pine responds specifically to sawfly egg deposition.

The volatile blend released from attractive, oviposition-induced *P. sylvestris* differs only slightly from the one released by a non-attractive (artificially wounded) control. Non-attractive pine twigs released the same terpenoid compounds. However, the oviposition-induced pine releases significantly larger quantities of the sesquiterpene (*E*)-*β*-farnesene (Mumm *et al*. 2003). This sesquiterpene was shown to attract the parasitoid *C. ruforum* only when offered in combination with volatiles from non-attractive control *P. sylvestris* (Mumm & Hilker 2005), indicating that the ratio of (*E*)-*β*-farnesene and other volatile terpenoids within a complex blend is responsible for parasitoid attraction. The specific role of (*E*)-*β*-farnesene for attraction of parasitoids is further supported by the following findings: (a) jasmonic acid (JA) treated pine twigs release enhanced quantities of (*E*)-*β*-farnesene, and the odor of these JA-treated pine twigs is attractive to the parasitoids (Mumm *et al*. 2003); (b) the egg parasitoid shows a very clear-cut electrophysiological antennal response to (*E*)-*β*-farnesene, (*E*)-*β*-caryophyllene, and *α*-humulene (Beyaert *et al.* 2010).

In this study, we aimed to elucidate whether egg deposition by *D. pini* affects transcription of sesquiterpene synthases. Since no sesquiterpene synthase sequence was known from *P. sylvestris*, we first identified and functionally characterized three sesquiterpene synthases in this species. Further, we compared the transcript levels of these sesquiterpene synthases in oviposition-induced pine twigs to those in artificially wounded ones to elucidate whether the transcriptional response was specific for oviposition. In previous studies (Mumm *et al*. 2003), odor from pine twigs 72 h after sawfly egg deposition was shown to attract the egg parasitoids, but it is unknown whether attractive odor is also released after a shorter or longer time. Therefore, we studied the parasitoid′s response to pine odor at different times after sawfly egg deposition. Parallel to these behavioral studies, we also studied how transcription rates of the three sesquiterpene synthases change over time after egg deposition.

2. Materials and methods

2.1. Study organisms

2.1.1. Plants

Both for the molecular studies and the behavioral bioassays with parasitoids, plant material was taken from *P. sylvestris* trees growing in forests near Berlin, Germany. In the laboratory, cut pine twigs were subjected to standardized and controlled conditions during treatment (25°C, 18:6 h light/dark cycle, and approx. 2,000 lx). Twigs kept at these standardized conditions have been proven to provide reproducible results when studying the parasitoid's response to oviposition-induced pine odor (Hilker *et al*. 2002b; Mumm *et al*. 2005). The inducibility of pine twigs by sawfly egg deposition was checked during the time when samples were taken for molecular studies. The twigs were considered inducible and used for molecular studies when the egg parasitoid *C. ruforum* responded positively to odor from samples 72 h after sawfly egg deposition. Branches of *P. sylvestris* used for analyses were cut from the middle part of 10- to 15-year-old trees and also from the lower part of 35- to 45-year-old trees. The lower part of a branch was cleaned, sterilized according to Moore and Clark (1968), and placed into water for treatment (see below). Plant material was taken in different seasons in 2004–2007.

2.1.2. Insects

The sawfly *D. pini* (Hymenoptera, Diprionidae) was reared in the laboratory on pine branches as described by Bombosch and Ramakers (1976) and Eichhorn and Pschorn-Walcher (1976) at 25 ± 1°C, 65% RH, and 18:6 h light/dark cycles. The egg parasitoid *C. ruforum* (Hymenoptera, Eulophidae) was collected in the field in southern Finland. Pine needles with parasitized host eggs were kept in Petri dishes at 5°C. To initiate parasitoid emergence from host eggs, parasitized eggs were transferred to a climate chamber with 25 ± 1°C, 65% RH, and 18:6 h light/dark cycles. Emerging adults were collected daily and transferred to 10 ± 1°C, 65% RH, and 16:8 h light/dark cycles until they were used for bioassays.

2.2. Plant treatments

2.2.1. General

All types of twigs (egg-laden, artificially wounded, and untreated) were always cut from the same larger branch to minimize possible intra-tree-variation in terpenoid metabolism. For each experiment, pine twigs with 80–100 needles were cut, placed into a glass cylinder covered by a gauze lid, and supplied with water. An oviposition-induced twig is referred to here as test twig. For controls, two types of twigs were used: (a) a twig with an artificial wounding mimicking the ovipositional wounding, and (b) an untreated twig. Test and control

twigs were always cut at the same time and kept at the same conditions. To obtain needle material for the molecular analyses, needles from control twigs were always removed at the same time points as needles from the respective test twigs and subjected to further analyses (see below).

2.2.2. Pine twig treatments

To obtain egg-laden pine twigs, three female and three male *D. pini* were added to a glass cylinder with a twig for a period of 16 h. During this time, the sawflies mated, and eggs were laid. When insects were removed from the cylinder, this was designated time zero (t0). The egg-laden twig (with about eight to ten needles carrying an egg mass) was then kept for 48, 72, or 96 h at the conditions described above. After these time periods, needles without eggs were removed from these twigs and subjected to further analyses. To obtain artificially wound-induced pine twigs, eight to ten needles of a pine twig were longitudinally slit at time point t0. The depth and length of the slit mimicked the wounding made by an ovipositing female *D. pini* with her ovipositor valves. The unwounded needles were removed from the wound-induced twigs 48, 72, or 96 h after treatment and used for further analyses. To obtain a branch-specific untreated twig control, needles from untreated control twigs were removed at the same time points as from the respective oviposition- or wound-induced twigs and subjected to further analyses. The time points when samples were collected for the molecular analyses reflected the times when behavioral studies were conducted.

2.3. Behavioral studies

A four-arm airflow olfactometer (Pettersson 1970; Vet *et al*. 1983) was used to test whether the egg parasitoid *C. ruforum* is attracted to odor of egg-laden pine twigs with an induction time other than 72 h (Hilker *et al*. 2002b). Airflows of 155 ml min^{-1} were allowed to enter a walking arena from four sides, thus establishing four distinct odor fields. One field was supplied with odor from an oviposition-induced pine twig, while the other fields were supplied with charcoal-filtered, humidified air (for further details see Hilker *et al*. 2002b). The parasitoid's response was tested to odor from twigs with 48, 72, and 96 induction time. Even though the parasitoid's response to odor from induced twigs 72 h after oviposition was known to be positive, this bioassay was included as positive control. When starting the bioassay, a single parasitoid female was introduced into the arena. The time the parasitoid spent walking in each of the four odor fields was recorded during an observation period of 600 s using a software program, The Observer 3.0 (Noldus, Wageningen, The Netherlands). Only data from active parasitoids walking at least 300 s of the observation period were used for statistical analysis. The number of parasitoids used per treatment was 34–37 (Table 1). The number of odor sources (twigs) tested was 6–9 per bioassay (Table 1). Data were analyzed by Friedman

ANOVA by comparing walking times within each of the four odor fields. Wilcoxon–Wilcox tests were used for post-hoc comparison (Köhler *et al*. 1995). The analysis was performed using StatSoft, Version 1999, STATISTIKA for Windows (Tulsa, OK, USA).

Table 1 Ecological relevance of induction time

induction time [h]*	duration of walking [s]				*N* parasitoid	*N* twig	*P*
	test field	control 1	control 2	control 3			
48	163 (84-262)	107 (8-156)	79 (37-147)	105 (44-164)	34	7	0.214
72	174[a] (116-259)	91[ab] (27-199)	65[b] (29-138)	117[ab] (41-170)	37	6	0.009
96	121 (42-182)	99 (35-156)	133 (68-245)	129 (67-177)	37	9	0.447

*Response of female egg parasitoids of *C. ruforum* to volatiles released from pine twigs carrying eggs for 48, 72, or 96 h (=induction time). Odor from these egg-laden twigs was offered in the test field of a four-arm-olfactometer with three control fields (clockwise arranged) supplied with clean air. Medians and interquartiles (parenthesis) for the time the parasitoid female spent walking over an observation period of 600 s are given for each field. The *P* value was evaluated by a Friedman ANOVA. Different letters indicate significant ($P < 0.05$) differences evaluated by Wilcoxon–Wilcox tests.

2.4. Chemicals

All chemicals and solvents were of analytical grade and were obtained from Merck, Serva, or Sigma. The substrates geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) were from Echelon Res. Lab. Inc. (Salt Lake City, UT, USA).

2.5. RNA isolation and cDNA synthesis

Needle tissue from treated *P. sylvestris* twigs (see above) harvested 48, 72, or 96 h after oviposition or artificial wounding treatments, or from respective control twigs was ground in liquid nitrogen with a sterilized mortar and pestle. To isolate RNA, the Invisorb Spin RNA Mini Kit (Invitek, Berlin, Germany) protocol was followed. Approximately, 100 mg of plant tissue was used per extraction. The RNA was eluted with 30 µl of RNAse free deionized water. For qRT-PCR, an additional DNase treatment was added using the RNase-Free DNase Kit (Qiagen, Hilden, Germany). Total RNA was checked for integrity and purity by spectrophotometer and tested additionally with RNA Nano Chips (Regent kit guide, RNA 6000 Nano assay, Agilent Technologies) by using a Bioanalyzer Agilent 2100 (Agilent Technologies). The synthesis of single stranded cDNA was carried out using Superscript III

reverse transcriptase (Invitrogen), 0.6–3 µg RNA and oligo (dT)20 primers (Invitrogen) according to the manufacturer's instructions. For qRT-PCR analysis, identical amounts of total RNA were used for reverse transcription.

2.6. Isolation of pine terpene synthase cDNA clones

Conserved regions of gymnosperm sesquiterpene synthases sequences from the following species (listed with accession numbers) were used to design degenerate primers: *Picea abies* (AAC05727, AAK39129, AAS47695) and *Abies grandis* (AAK83561, AAC06728). Using these primers, cDNA fragments were amplified from pine twigs 72 h after egg deposition by PCR under the following conditions: 0.2 µl Taq DNA Polymerase (5 U/µl), 2.5 µl 10× PCR-buffer for Taq Polymerase, 1 µl dNTPs (10 mM), 1 µl primer 1 and 2 (10 pmol/µl; see Supplemental data, Table S1), 0.2–3 µl cDNA, and H_2O (added up to 25 µl). The PCR was conducted with an initial denaturation at 94°C for 3 min, 40 cycles of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, extension at 72°C for 70 s, and a final step at 72°C for 5 min. For ligation and cloning of PCR fragments, the TOPO TA cloning™ kit for sequencing was used (Invitrogen). To generate the full-length coding cDNA sequence of the corresponding cDNA fragments, the BD SMART™ RACE cDNA Amplification Kit (Clontech) was used according to the manufacturer's instructions. The resulting cDNA amplicons were cloned into vector pCR 4-TOPO (Invitrogen) and sequenced using an ABI 3100 automatic sequencer (Applied Biosystems).

2.7. Functional expression of PsTPS1, PsTPS2, and PsTPS3

The complete open reading frames of the full length cDNA of *PsTPS*1, *PsTPS*2, and *PsTPS*3 clones and the signal peptide truncated version of *PsTPS*2 were used for functional expression. PCRs were performed with primers (see Supplemental data Table S1) using the Expand High Fidelity^{Plus} PCR System (Roche) as directed by the manufacturer. The amplification products were cloned into the pET-100D TOPO™ expression vector (Invitrogen). The expression vector was transformed into the *E. coli* strain Top 10F′ and its sequence verified. Mutation-free plasmids were transformed into the BL21 (DH3) pLysS strain of *E. coli* (Invitrogen).

For bacterial expression, a starter culture (10 ml Luria–Bertani medium with 35 µg/ml chloramphenicol and 100 µg/ml of carbenicillin) was grown for 3 days at 18°C; 5 ml of starter culture in 100 ml LB medium (with 35 µg/ml of chloramphenicol and 100 µg/ml carbenicillin) was induced with 2 mM isopropyl-*β*-galactoside (IPTG) at an OD = 0.6 and kept at 18°C for at least 15 h. The cells were centrifuged for 20 min at 9,000g. The pellets were resuspended in 3 ml of extraction buffer (Martin *et al*. 2004) and disrupted by sonication (Bandelin Sonopuls

HD 2070, Berlin, Germany) for 4 min, cycle 2, power 60%. After freezing (10 min at −20°C, 10 min at −80°C), the cell fragments were collected by centrifugation.

The supernatant containing the total bacterial crude protein extract was assayed. Each assay was performed in a 1 ml volume with 69.9 μM FPP, overlaid with 1 ml pentane, and incubated at 30°C. For control assays, substrate concentrations of 99.5 μM GPP and 37.0 μM GGPP were used. One hour after pentane addition, the assay was stopped by vigorous vortexing with the pentane overlay for 30 s and separation of the aqueous and organic fractions by centrifugation at 2,500g for 2 min. The pentane fraction was removed, and the residue was overlaid again with 1 ml pentane. In total, three consecutive pentane extractions were conducted. Finally, the pentane fractions were combined, dried over a silica/$MgSO_4$ column, and evaporated to 50–100 μl. These samples were subjected to GC–MS analyses (see below, product identification).

Enzyme concentrations were measured according to Bradford (1976) by using the BioRad reagent with bovine serum albumin (BSA) as standard. The protein concentration used in each assay was adjusted prior to a range of 0.5–2.5 μg/ml.

2.8. Sesquiterpene extraction from needle tissue

To investigate whether the expression levels of the sesquiterpene synthases in differently treated pine twigs were reflected by different amounts of the major products of these synthases, we analyzed (a) oviposition-induced pine twigs 72 h after egg deposition and (b) artificially wounded pine twigs 72 h after treatment for their sesquiterpene contents. For terpene extraction, 200 mg ground needles (see above, RNA isolation and cDNA synthesis) were used. The extraction procedure was based on a method described by Martin *et al*. (2002). All steps were carried out in 2 ml vials tightly closed with a teflon-coated screw cap (Hewlett-Packard, Palo Alto, CA, USA). The needle samples were submerged into 1.0 ml of tert-butyl methyl ether containing 150 μg/ml isobutylbenzene as internal standard and extracted 14 h overnight with constant shaking at room temperature. The ethereal supernatant was transferred to a fresh vial and washed with 0.3 ml of 0.1 M $(NH_4)2CO_3$ (pH 8.0). This sample was filtered through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous $MgSO_4$. The column was washed with 1 ml of diethyl ether. The eluate was evaporated to an approximate volume of 100 μl and used for further GC–MS analyses of terpenoids (see below, product identification).

2.9. Product identification

Products of sesquiterpene synthase assays and extracts of pine needles were analyzed on a GC system (Agilent Hewlett-Packard 6890, Agilent Technologies) coupled to a Network Mass Selective Detector (Agilent Hewlett-Packard 5973, Agilent Technologies). For analyses,

1 µl concentrated pentane phase (assays) or ether phase (needle extract) was injected at an injector temperature of 220°C on a HP-5 capillary column (30 m × 0.25 mm with a 0.25 µm phase coating; Agilent Technologies). The temperature program started with 40°C for 2 min, raised to 210°C (5°C min^{-1}), and raised further to 300°C (60°C min^{-1}, 2 min hold; helium flow: 2 ml min^{-1}). For identification of compounds, the MS detector was operated using the total ion mode at a temperature of 230°C. The products were identified by comparing mass spectra and retention times with those in the literature and in the Wiley 275.L or NIST 98.1 MS libraries. The identity of (*E*)-*β*-caryophyllene, *α*-humulene, longipinene, and longifolene was further verified by comparison with authentic standards.

Those sesquiterpenes that were the major products of the sesquiterpene synthases were quantified in pine needle extracts with 150 µg/ml isobutylbenzene as internal standard (compare above). Mean ± SE of the relative quantities of these compounds were calculated from three independent biological pine needle samples of each treatment with each biological sample analyzed three times.

2.10. QRT-PCR

Real time quantification of gene transcription was performed using SYBR green QPCR Master Mix from Stratagene (La Jolla, CA, USA) in order to address the following questions: (a) do transcript levels of *PsTPS*1-3 differ between oviposition-induced samples and artificially wounded ones?; (b) how do transcript levels of these sequences change over time after treatment?

QRT-PCRs were performed as described in the operator's manual using a Stratagene MX3000P™. Gene-specific PCR primers were designed (see Supplemental data Table S1) using criteria including predicted melting temperature of at least 58°C, primer length of 22–24 nucleotides, guanosine–cytosine content of at least 48%, and an amplicon length of 120–150 bp. Primer specificity was confirmed by melting curve analysis, by an efficiency of product amplification of 1.0 ± 0.1, and by sequence verification of at least eight cloned PCR amplicons for each gene. Reactions with water instead of cDNA template were run with each primer pair as control. The standard thermal profile of 95°C for 10 min, then 60 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 30 s was used. The fluorescence signal was captured at the end of each cycle, and a melting curve analysis was performed from the annealing temperature to 95°C with data capture every 0.2°C during a 1 s hold.

The quantity of each transcript is the average of four (48 h), five (72 h), and three (96 h) independent biological replicates, each of which is represented by at least three technical replicates. All amplification plots were analyzed with the MX3000P™ software to obtain threshold cycle (C_t) values. Transcript abundance was normalized to the transcript abundance of ubiquitin (GenBank accession number EF681766). Relative transcript values were obtained

by calibration first against the transcript abundance of the respective untreated twig control, and, second, against the transcript abundance of the artificial wounding control 72 h after treatment.

A two-way ANOVA was performed on qRT-PCR raw data to test the significance of differences in changes of *PsTPS*1, *PsTPS*2, and *PsTPS*3 transcript levels in course of time (independent samples; between-subject factor time) and due to treatment (dependent, paired samples; within-subject factor treatment). Also the combined effect of time × treatment was statistically tested. Normal distribution of qRT-PCR raw data was found for *PsTPS*1 and *PsTPS*3. *PsTPS*2 data were arctan-transformed prior to ANOVA (Sokal & Rohlf 1995). All analyses were performed using StatSoft, Version 1999, STATISTICA for Windows (Tulsa, OK, USA) (see Supplemental data Table S2).

2.11. Sequence and phylogenetic analyses

DNASTAR Lasergene program version 7.0 (Meg AlignTM) was used to align and to calculate the deduced amino acid sequences of each full-length *P. sylvestris* cDNA and of known sequences from gymno- and angiosperms. The amino acid alignment was assembled by use of ClustalW (gonnet 250 matrix, gap penalty: 10.00, gap length penalty: 0.20, delay divergent sequences: 30%, gap length 0.10, DNA transition weight 0.5). The same software was used to visualize the phylogenetic tree.

3. Results

3.1. Timing of egg parasitoid attraction to P. sylvestris foliage

Behavioral bioassays were conducted to study the parasitoid's response to pine odor after different periods of time after sawfly egg deposition. The parasitic wasps showed a significantly positive response to odor from pine twigs laden with pine sawfly eggs for 72 h, thus confirming previous results (Hilker *et al*. 2002b, 2005; Mumm *et al*. 2003). However, at shorter (48 h) or longer (96 h) times after oviposition, the odor of pine twigs did not attract the parasitoids (Table 1).

3.2. Cloning of sesquiterpene synthases from P. sylvestris

By the use of degenerate primers corresponding to conserved regions of known conifer sesquiterpene synthases and 5′-and 3′-RACE-PCR cloning strategies, three different cDNA clones containing open reading frames (ORFs) of terpene synthases were obtained from RNA isolated from oviposition-induced *P. sylvestris* twigs (72 h after egg deposition).

The clones were designated *Ps*TPS1, 1,728 bp encoding an ORF of 576 amino acids; *Ps*TPS2, 1,878 bp encoding an ORF of 626 amino acids, and *Ps*TPS3, 1,743 bp encoding an ORF of 581 amino acids. The deduced amino acid sequences of all three clones, when compared with other conifer terpene synthases, showed the two typical aspartate-rich DDxxD motifs which are involved in coordinating the bivalent metal ion for substrate binding (Bohlmann *et al*. 1999). In *Ps*TPS1 and *Ps*TPS2, an RR(x8)W motif was found near the N-terminus which might be important for catalysis (Pechous & Whitaker 2004). Furthermore, all three sequences show an RxR motif implicated in the complexation of the diphosphate function after ionization of the substrate, which prevents nucleophilic attack on any of the carbocationic intermediates formed subsequently (Starks *et al*. 1997). Analysis of the N-terminus of *Ps*TPS2 suggested the presence of a 37-amino acid plastid transit peptide (http://www.cbs.dtu.dk/services/ChloroP/) (Emanuelsson *et al*. 2000). Even though plastid targeting peptides are typical of plant monoterpene synthases (Bohlmann *et al*. 1998), the *Ps*TPS2 sequence was most similar to those of sesquiterpene synthases (Fig. 1) when disregarding the putative signal peptide.

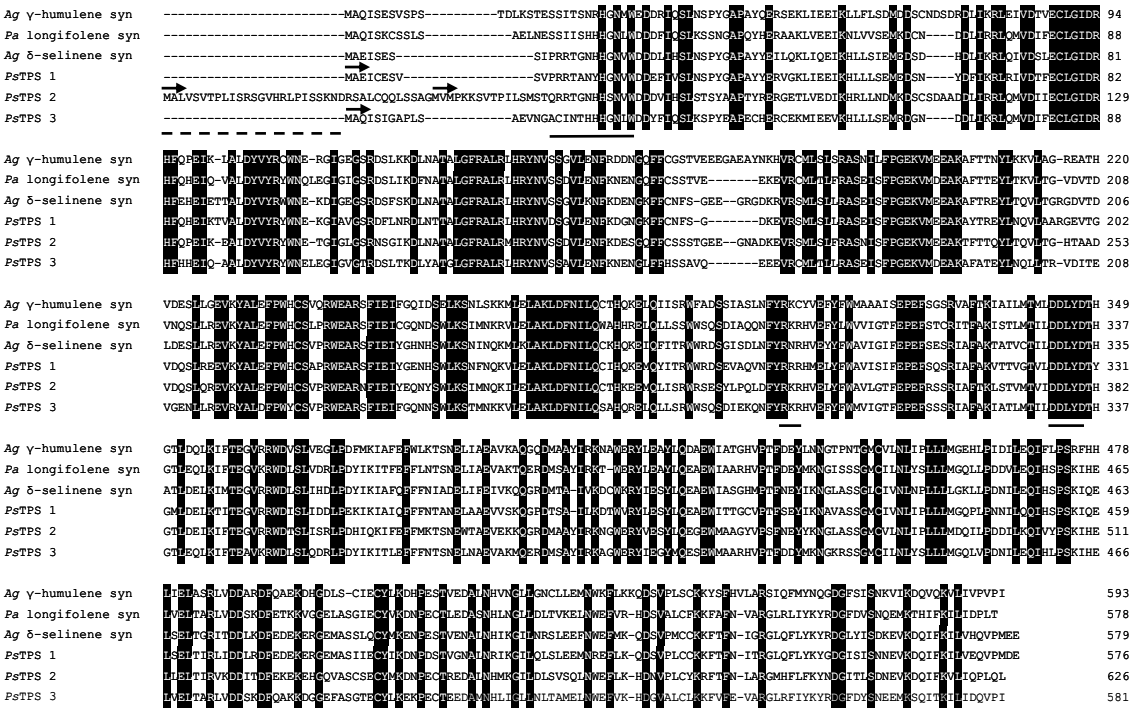

Fig. 1 Alignment of deduced amino acid sequences of three *Pinus sylvestris* (*Ps*) sesquiterpene synthases, *Ps*TPS1, *Ps*TPS2 and *Ps*TPS3, with the three other conifer sesquiterpene synthases with the highest similarity, *Abies grandis* (*Ag*) *γ*-humulene synthase (GenBank accession AAC05728), *Picea abies* (*Pa*) longifolene synthase (GenBank accession AAS47695), *A. grandis* (*Ag*) *δ*-selinene synthase (GenBank accession AAC05727). Amino acid residues that are identical in all six sequences are enclosed in black boxes. The starting codons used for expression are marked with arrows. The DDxxD,

RR(x8)W and RxR motifs (solid lines), as well as the ChloroP predicted transit peptide (dotted line) are shown.

In summary, the three *P. sylvestris* cDNAs showed greatest amino acid identity (60–80%) to three other conifer sesquiterpene synthases. In comparison with each other, the *P. sylvestris* cDNAs had identities ranging from 60 to 65%, ignoring the signal peptide of *Ps*TPS2 (Table 2).

Table 2 Sequence comparison of *P. sylvestris* and other gymnosperm sesquiterpene synthase sequences

PsTPS 1	100					
PsTPS 2	64.5	100				
PsTPS 3	61.9	61.2	100			
Ag γ-humulene syn	63.9	60.6	61.9	100		
Pa longifolene syn	63.6	65.9	79.4	64.0	100	
Ag δ-selinene syn	77.2	65.5	64.3	62.2	63.1	100
	PsTPS 1	*PsTPS* 2	*PsTPS* 3	*Ag* γ-humulene syn	*Pa* longifolene syn	*Ag* δ-selinene syn

Sequence similarities among *Ps*TPS1, *Ps*TPS2 and *Ps*TPS3, and other conifer sesquiterpene synthases genes, *A. grandis* γ-humulene synthase (*Ag*), *P. abies* longifolene synthase (*Pa*), and *A. grandis* δ-selinene synthase (*Ag*) are given in percent at the amino acid level. The analysis was performed using ClustalX and Lasergene 7. The accession numbers of the genes are given in the legend of Fig. 1.

3.3. Functional expression of P. sylvestris sesquiterpene synthases

The three *P. sylvestris* sesquiterpene synthases were heterologously expressed in *E. coli*, and the crude bacterial extracts containing recombinant protein were incubated with prenyl diphosphates. Product formation was observed for farnesyl diphosphate (FPP), but no terpenoid products were detected after incubation with geranyl diphosphate (GPP) or geranylgeranyl diphosphate (GGPP). Control bacterial extracts containing the empty vector control did not show any enzyme activity.

*Ps*TPS1 formed only two main products: (*E*)-*β*-caryophyllene and *α*-humulene, 76.3 and 23.7%, respectively, of total sesquiterpene peak area (Fig. 2a). No other terpenoid products were detectable.

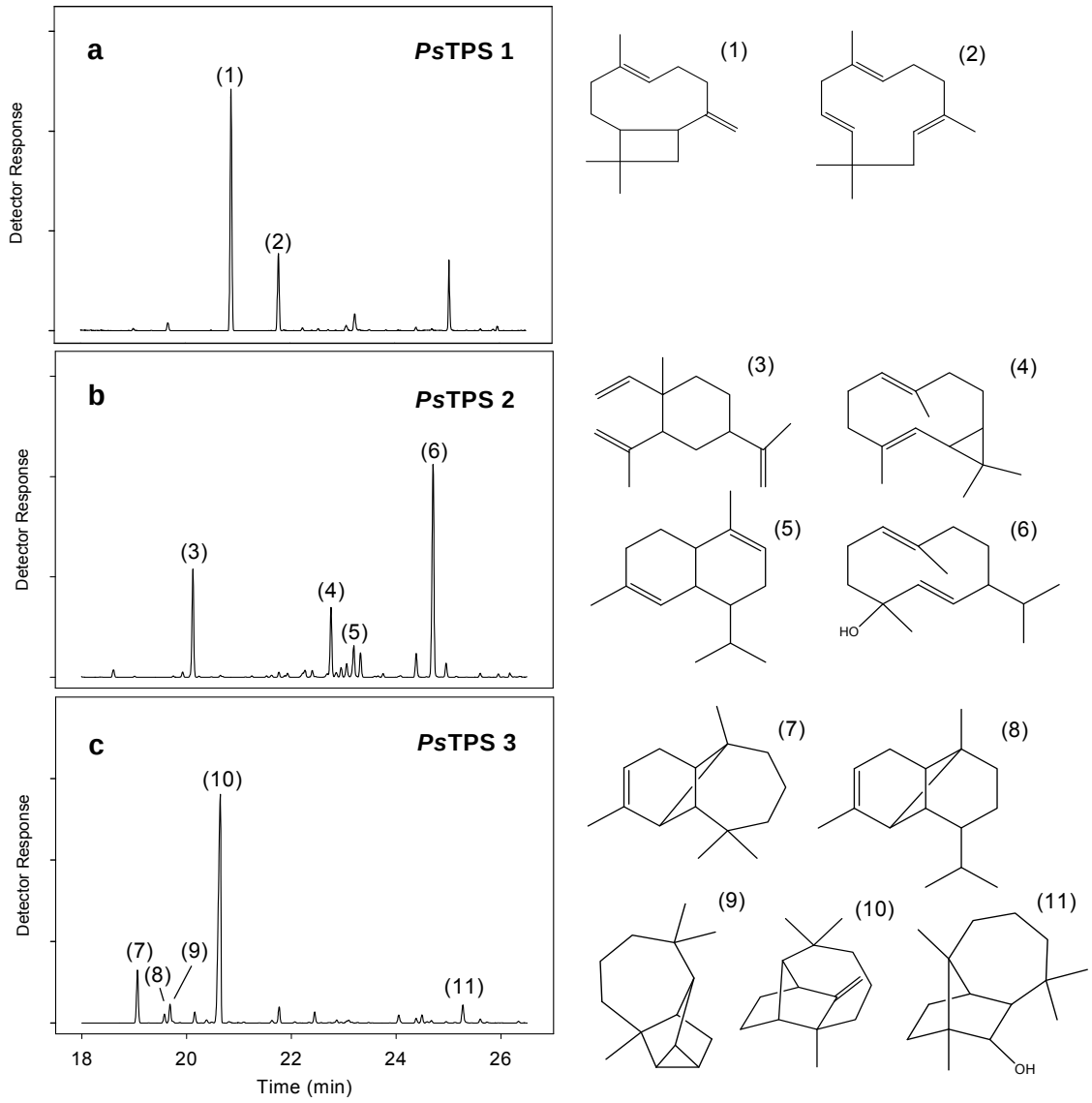

Fig. 2 GC–MS analysis of protein extracts from transformed *E. coli* expressing *P. sylvestris* terpene synthases. Assay with farnesyl diphosphate. Depicted are total ion current chromatograms in the region of sesquiterpene elution (18–26 min). a *Ps*TPS1, (1) (*E*)-*β*-caryophyllene, (2) *α*-humulene. b *Ps*TPS2, (3) *β*-elemene, (4) bicyclogermacrene, (5) *α*-amorphene, (6) 1(10),5-germacradiene-4-ol. c *Ps*TPS3, (7) *α*-longipinene, (8) *α*-ylangene, (9) longicyclene (10) longifolene, and (11) longiborneol. Minor peaks without numbers are unidentified sesquiterpenes or impurities.

For *Ps*TPS2, assays were performed with the full ORF and with a construct in which the presumptive 37-amino acid plastid targeting sequence was removed. Both forms gave one major product from FPP, 1(10),5-germacradiene-4-ol (41.5% of total sesquiterpene peak area), and two minor products, *β*-elemene (19.2%) and bicyclogermacrene (13.2%). The detected *β*-elemene likely results from the heat-induced rearrangement of germacrene A during gas chromatography (de Kraker *et al*. 1998). In addition, *Ps*TPS2 catalyzes the

production of some products below 10% abundance, one of which was tentatively identified by its mass spectrum as *α*-amorphene (Fig. 2b).

*Ps*TPS3 incubated with FPP produced longifolene as a main product with about 64.2% of total sesquiterpene peak area, *α*-longipinene in smaller amounts (11.2%), and three minor peaks tentatively identified as *α*-ylangene, longicyclene and longiborneol (Fig. 2c).

None of the three expressed terpene synthases produced (*E*)-*β*-farnesene, the terpene induced by *D. pini* oviposition for which a specific role in egg parasitoid attraction had been documented in previous studies (Mumm *et al*. 2003; Mumm & Hilker 2005).

3.4. The presence of sesquiterpene synthase products in pine needles

Both pine needles 72 h after egg deposition and the artificially wounded tissue contained the major products of the cloned sesquiterpene synthases: (*E*)-*β*-caryophyllene and *α*-humulene (the main products of recombinant *Ps*TPS1), *β*-elemene, bicyclogermacrene, and 1(10),5-germacradiene-4-ol (the major products of recombinant *Ps*TPS2), and longifolene (the dominant product of recombinant *Ps*TPS3). In oviposition-induced pine needles, the main products of *Ps*TPS1 and *Ps*TPS2 were slightly more accumulated (1.035 and 1.12 times more) when comparing with artificially wounded control samples (Fig. 3). The content of sesquiterpenes in artificially wounded needles used for calibration were 32 µg/g FW ± 3.2 *β*-elemene, 54 µg/g FW ± 4.8 (*E*)-*β*-caryophyllene, 9.2 µg/g FW ± 1.1 *α*-humulene, 41 µg/g FW ± 5.3 bicyclogermacrene, and 205 µg/g FW ± 16 1(10),5-germacradiene-4-ol. Longifolene was detected only in traces.

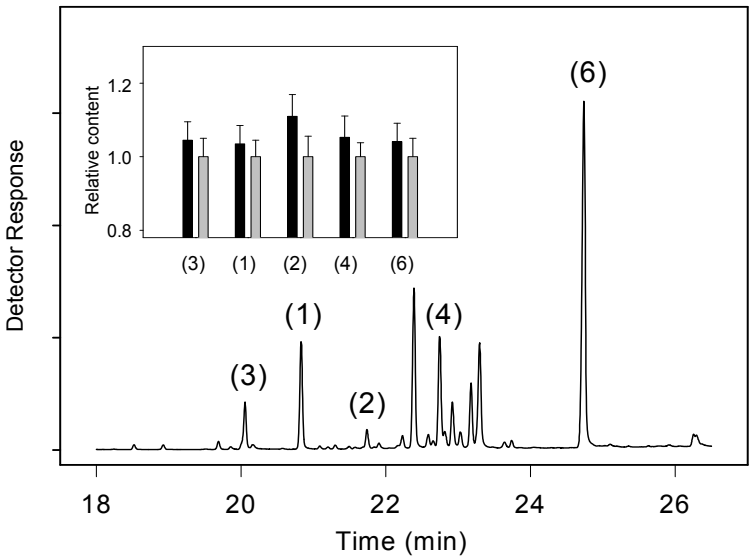

Fig. 3 GC–MS analysis of ether extracts from *P. sylvestris* needles. Depicted is a total ion current chromatogram from an extract of oviposition-induced pine needles in the region of sesquiterpene elution (18–26 min). For compound numbers see Fig. 2 Inset relative content (means ± SE) of selected sesquiterpenes in oviposition-induced pine needles (black bars) and artificially wounded ones (gray bars) 72 h after treatment. Data were calibrated against the 72-h-artificially-wounded control. Each value shows the mean of at least three technical replicates of three independent biological samples.

3.5. The effect of oviposition on sesquiterpene synthase transcript levels

Sawfly oviposition on pine significantly affected transcript levels of *PsTPS*1 and *PsTPS*2 when compared to artificially wounded pine. In contrast, no such significant treatment (oviposition) effect was detectable for *PsTPS*3. Transcript levels of *PsTPS*1 and *PsTPS*2 also changed significantly over time with a striking increase 72 h after oviposition. In contrast, no such time effect was found for *PsTPS*3 (Fig. 4, Supplemental data Table S2). The results in Table 1 show that odor from *P. sylvestris* twigs was significantly attractive to the egg parasitoid *C. ruforum* only 72 h after *D. pini* oviposition. At this time, steady state transcript levels of *PsTPS*1 and *PsTPS*2 were on average 1.9 fold and 2.6 fold higher, respectively, than those from non-attractive control twigs 72 h after artificial wounding (Fig. 4a, b).

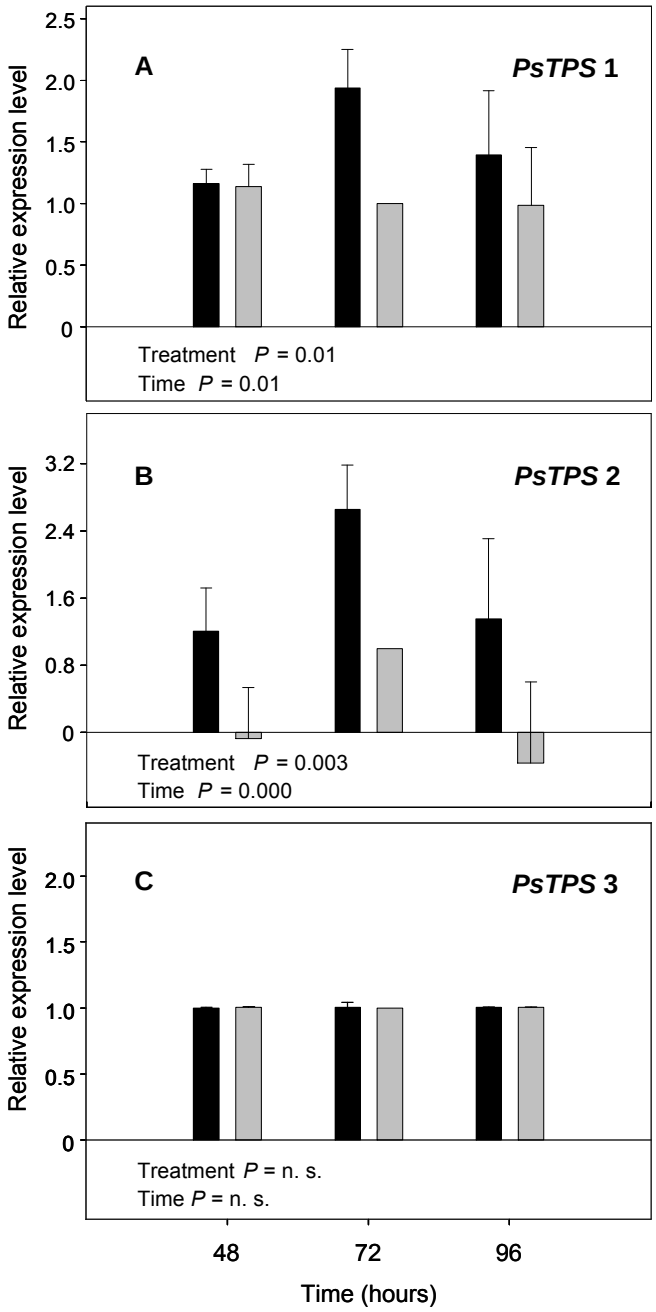

Fig. 4 Relative abundance of mRNA transcripts of genes coding for sesquiterpene synthases of *P. sylvestris* measured by quantitative RT-PCR. Transcripts of *PsTPS*1 (a), *PsTPS*2 (b) and *PsTPS*3 (c) were compared in oviposition-induced pine twigs (black bars) and artificially wounded ones (gray bars) 48, 72, and 96 h after induction (for details see text). Data were normalized to ubiquitin, and calibrated against the 72 h artificially wounded control (set to 1.0). Each value shows the mean ± SE of at least three technical replicates of four (48 h), five (72 h), and three (96 h) independent biological samples. A two-way ANOVA was performed to test the significance of differences in transcript accumulation. n.s., not significant (for details see text and supplemental data S2).

4. Discussion

We detected three new sesquiterpene synthase genes of *P. sylvestris* and identified the products of the respective enzymes. Furthermore, two of these sesquiterpene synthase genes, *PsTPS*1 and *PsTPS*2, were significantly upregulated by insect egg deposition, but not by wounding mimicking the ovipositional damage. A number of sesquiterpene synthase genes have been reported from other conifer species including grand fir (*A. grandis*), Sitka spruce (*Picea sitchensis*), and Norway spruce (*P. abies*) (Bohlmann *et al*. 1999; Martin *et al*. 2004; Miller *et al*. 2005; Byun-McKay *et al*. 2006). The expression of many of these genes is known to be inducible by different factors: mechanical wounding in grand fir (Steele *et al*. 1998a, b) and Sitka spruce (McKay *et al*. 2003; Byun-McKay *et al*. 2006), methyl jasmonate treatment in Sitka spruce (Miller *et al*. 2005), and insect attack by the stem boring pine weevil (*Pissodes strobi*) in Sitka spruce (McKay *et al*. 2003; Miller *et al*. 2005).

How much do the three new sesquiterpene synthases found in *P. sylvestris* differ from other sesquiterpene synthases in angio- and gymnosperms? The three sesquiterpene synthases isolated from *P. sylvestris* are closely related to those of other gymnosperms. A phylogenetic comparison of these three genes with other selected plant terpene synthases resulted in a clear separation of gymnosperm and angiosperm genes (Fig. 5), supporting the suggestion that terpene synthase functions have evolved independently in both groups (Bohlmann *et al*. 1998; Martin *et al*. 2004). When considering the sesquiterpene synthases found in *P. sylvestris* singly, the following similarities and differences compared to sesquiterpene synthases in other plant species are striking:

1. The sequence of the *Ps*TPS1 enzyme catalyzing the production of (*E*)-*β*-caryophyllene and *α*-humulene have 30 and 27% amino acid identity to the sequences of (*E*)-*β*-caryophyllene/*α*-humulene synthases known from *Artemisia annua* (Cai *et al*. 2002) and *A. thaliana* (Chen *et al*. 2003). Of all terpene synthases, *Ps*TPS1 share 77% amino acid sequence identity with *A. grandis* δ-selinene synthase (Steele *et al*. 1998a).
2. The *Ps*TPS2 enzyme produced 1(10),5-germacradiene-4-ol and other products with a germacrene skeleton. Unexpectedly, the amino acid sequence of this sesquiterpene synthase was found to contain an N-terminal, 37 amino acid signal peptide that may target the protein for plastid localization. However, most sesquiterpene synthases are localized in the cytosol, while monoterpene synthases are usually targeted to plastids (Keeling & Bohlmann 2006). Nevertheless, a sesquiterpene synthase with a 5′-presequence similar to the one of *Ps*TPS2 was described previously from another conifer species, *Pseudotsuga menziesii* (Huber *et al*. 2005). Without the signal peptide, the *Ps*TPS2 sequence has highest identity to longifolene synthase isolated

from Norway spruce (*P. abies*) (Martin *et al*. 2004). Expressing *Ps*TPS2 without its putative signal sequence gave the same product profile as expressing the full-length ORF.

3. The *PsTPS*3 gene encodes a longifolene synthase which has 79% amino acid identity with a sesquiterpene synthase isolated from Norway spruce (*P. abies*) (Martin *et al*. 2004), and a similar product profile. The Norway spruce enzyme also produces longifolene, as well as *α*-longipinene and longiborneol as minor products, as does the *Ps*TPS3 protein.

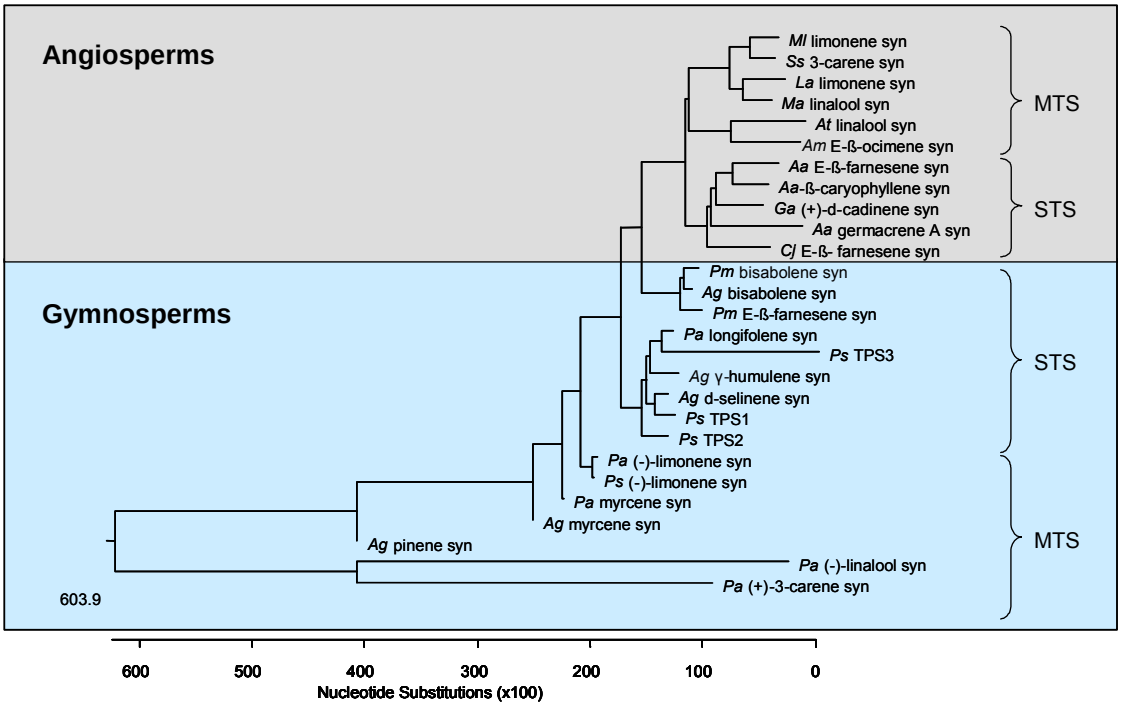

Fig. 5 Phylogenetic tree of the deduced amino acid sequences of *Ps*TPS1, *Ps*TPS2 and *Ps*TPS3 with selected monoterpene synthases (MTS) and sesquiterpene synthases (STS) of angiosperms and gymnosperms calculated by the neighbor-joining method using ClustalW and Lasergene 7 (MegAlign) software. *Aa* Artemisia annua, *Ag Abies grandis*, *Am Antirrhinum majus*, *At Arabidopsis thaliana*, *Cj Citrus junos*, Ga Gossypium arboreum, *La Lavandula angustifolia*, *Ma Mentha aquatica*, *Ml Mentha longifolia*, *Pa Picea abies*, *Pm Pseudotsuga menziesii*, *Ps Pinus sylvestris*, *Psi Picea sitchensis*, *Ss Salvia stenophylla*. GenBank protein accessions of terpene synthases are shown top to bottom: AAD50304, AAM89254, ABB73044, AAL99381, AAO85533, AAO42614, AAX39387, AAL79181, ABE3980, AAD51718, AAK54279, AAX07266, AAC24192, AAX07265, AAS47695, AAC05728, AAC05727, AAS47694, ABA86248, AAS47696, AAB71084, AAB71085, AAS47693, AAO73863

The transcript levels of the three sesquiterpene synthases studied here are not fully consistent with the profile of volatiles known to be present in the headspace of *P. sylvestris* 72 h after egg deposition by *D. pini* (Mumm *et al*. 2003). Two points are remarkable: first, some of the

products of these sesquiterpene synthases, 1(10),5-germacradiene-4-ol (*Ps*TPS2), and longifolene and *α*-longipinene (*Ps*TPS3), have not yet been detected in the headspace of oviposition-induced pine (Mumm *et al*. 2003). Second, even though *Ps*TPS1 and *Ps*TPS2 increase their transcript levels significantly 72 h after egg deposition with respect to earlier time points and artificially wounded controls, none of their products was found in enhanced quantities in the headspace of oviposition-induced pine when compared to artificially wounded samples (Mumm *et al*. 2003). Thus, the steady state transcript levels found for *Ps*TPS1 and *Ps*TPS2 are not reflected by enhanced quantities of their products in the headspace of oviposition-induced pine.

How can these inconsistencies be explained? While both 1(10),5-germacradiene-4-ol and longifolene were not detected in the headspace of oviposition-induced pine in previous work (Mumm *et al*. 2003), we did find these components in total needle extracts (Fig. 3). The absence of the major products of *Ps*TPS2 and 3 in previous headspace collections might result from these compounds being below the detection limit for the methods used (Mumm *et al*. 2003). However, the major products of *Ps*TPS1, (*E*)-*β*-caryophyllene and *α*-humulene, were indeed found in the headspace of pine 72 h after sawfly egg deposition, but not in enhanced quantities compared to artificially wounded pine (Mumm *et al*. 2003). The formation of these sesquiterpenes may not be strictly controlled at the transcript level of their respective sesquiterpene synthases. A variety of posttranscriptional controls, including gene silencing or RNA interference (Bonnet *et al*. 2006; Poethig *et al*. 2006) or controls on enzyme activity may be in operation. Alternatively, the products of *Ps*TPS1 and *Ps*TPS2 may be biosynthesized in higher quantities in oviposition-induced needles than in artificially wounded ones consistent with their enhanced transcription levels, but might remain in the egg-laden needles rather than being volatilized and serve as direct defenses against the eggs or larvae of the sawfly. Our quantitative analyses of sesquiterpenes in oviposition-induced needles indicate indeed slight accumulation of the major products of *Ps*TPS1 and *Ps*TPS2 (Fig. 3). The question of whether the products of the cloned sesquiterpene synthases have noxious or deterrent direct effects on *D. pini* eggs needs to be addressed in future work. Further molecular studies will also search for an (*E*)-*β*-farnesene synthase. This sesquiterpene is so far the only terpenoid volatile component found in significantly enhanced quantities 72 h after sawfly egg deposition (Mumm *et al*. 2003).

In conclusion, this study provides molecular evidence that insect egg deposition by *D. pini* triggers enhanced transcription of sesquiterpene synthases in *P. sylvestris*. This transcriptional response was shown to be induced specifically by egg deposition, but not by artificial damage. The enhanced transcription was observed only at that time after egg deposition (72 h) when the egg-laden pine released odor attractive to the parasitoid.

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Supplementary data (page 108-109)

Table S1. Primers used in initial screening for sesquiterpene synthase sequences, RACE-PCR, heterologous expression, and qRT-PCR.

Table S2. Two-way ANOVA: Statistical comparisons of *P. sylvestris* sesquiterpene synthase transcript levels measured by quantitative RT-PCR between samples from oviposition induced foliage and artificially wounded controls (see Fig. 4 for further details).

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Chapter 3

Species-specific responses of pine sesquiterpene synthases to sawfly oviposition

Abstract

Pinus sylvestris (Scots pine) is known to respond to eggs laid by the sawfly *Diprion pini* on its needles by releasing a blend of terpenoids, including the sesquiterpene (*E*)-*β*-farnesene. These compounds attract a wasp, *Closterocerus ruforum*, which parasitizes sawfly eggs. *D. pini* oviposition also enhances the transcription of two sesquiterpene synthases, an (*E*)-*β*-caryophyllene/*α*-humulene synthase (*PsTPS*1) and a 1(10),5-germacradiene-4-ol synthase (*PsTPS*2). To gain a better understanding of the function of these sesquiterpenes in promoting insect egg parasitism, we compared the outcome of *D. pini* oviposition on *P. sylvestris* with interactions between other pine and sawfly species: *Neodiprion sertifer* eggs on *P. sylvestris*, *Gilpinia pallida* eggs on *P. sylvestris*, *D. pini* eggs on *Pinus nigra*. The first of these attracts the parasitoid *C. ruforum*, while the latter two do not. As determined by quantitative real-time PCR, both *PsTPS*1 and *PsTPS*2 transcripts increased significantly only for those species combinations where the odor of egg-laden pine needles was attractive to *C. ruforum*. Moreover, enhanced transcription of these genes was found only at those time periods when odor was attractive, i.e. 3 days after oviposition. Thus, the *PsTPS*1 and *PsTPS*2 genes are good markers for parasitoid attraction. We also characterized a new sesquiterpene synthase from *P. sylvestris* (*PsTPS*5) which produces (*E*)-*β*-farnesene, the compound previously determined to be responsible for *C. ruforum* attraction. However, transcript levels of *PsTPS*5 were not enhanced by oviposition of sawfly species that cause *C. ruforum* attraction. More research on this experimental system is required to determine the role of oviposition-induced sesquiterpenes in attracting egg parasitoids and the role of sesquiterpene synthases in regulating sesquiterpene formation.

Keywords Gymnosperms, Conifers, *Pinus sylvestris*, Plant defense, Terpene synthases, Sesquiterpenes, Terpenoid biosynthesis, Insect eggs, Parasitoid, Herbivore

1. Introduction

Plants are well known to activate a wide range of defense mechanisms in response to attack by herbivorous arthropods (Karban *et al*. 1997; Walling 2000). One common mechanism is the emission of volatiles that attract parasitic wasps. Some parasitic wasp species infest larval stages of herbivorous insects, and their attraction to plant volatiles released during feeding has been frequently studied (D'Alessandro & Turlings 2006; Hilker & Meiners 2006; Dicke 2009). Other parasitic wasps infest herbivore eggs, and their attraction to plant volatiles released on egg laying has been shown for plant species, such as the elm *Ulmus minor* (Meiners & Hilker 2000), the pine *Pinus sylvestris* (Hilker *et al*. 2002a,b; Mumm & Hilker 2006; Hilker & Meiners 2009), and the bean plants *Vicia faba* and *Phaseolus vulgaris* (Colazza *et al*. 2004a,b). In the case of *P. sylvestris*, terpene blends are emitted both before and after oviposition by the sawfly, *Diprion pini*. However, the sesquiterpene, (*E*)-*β*-farnesene, was shown to be emitted at higher levels after oviposition (Mumm *et al*. 2003) and was demonstrated to be attractive to the egg parasitoid, *Closterocerus* (formerly *Chrysonotomyia*) *ruforum* when offered against the background of volatiles from *P. sylvestris* (Mumm & Hilker 2005). Among the necessary background odor components for this attraction are the sesquiterpenes, (*E*)-*β*-caryophyllene and *α*-humulene (Beyaert *et al*. 2010).

In order to learn more about what regulates the production of these egg parasitoid-attracting sesquiterpenes in *P. sylvestris*, we initiated an investigation of the sesquiterpene synthases of this species (Köpke *et al*. 2008). These enzymes convert (*E*,*E*)-farnesyl diphosphate, the ubiquitous, linear C_{15} intermediate of terpene metabolism, into a wide range of sesquiterpene carbon skeletons. Three *P. sylvestris* sesquiterpene synthases were characterized in earlier work (Köpke *et al*. 2008). *Ps*TPS1 catalyzes the formation of (*E*)-*β*-caryophyllene and *α*-humulene. *Ps*TPS2 catalyzes the production of 1(10),5-germacradiene-4-ol as a major product, with minor amounts of bicyclogermacrene, *α*-amorphene and germacrene A. *Ps*TPS3 forms longifolene as a major product with minor amounts of *α*-longipinene, *α*-ylangene, longiborneol and longicyclene. However, no enzyme has been discovered which forms (*E*)-*β*-farnesene, the major attractant of *C. ruforum*. In studying the expression of the isolated genes, *PsTPS*1 and *PsTPS*2 were found to have significantly higher transcript levels in *P. sylvestris* needles with *D. pini* eggs that were attractive to *C. ruforum* than in non-attractive, artificially-damaged needles.

To study the importance of chemical signals in biological interactions, it is often valuable to make comparisons among closely related species. For example, in addition to *D. pini*, the sawfly *Neodiprion sertifer* also lays its eggs and feeds on *P. sylvestris* foliage, and the volatiles attract the eulophid wasp *C. ruforum* (Mumm *et al*. 2005). Another sawfly, *Gilpinia pallida*, lays its eggs on *P. sylvestris* as well and is a host for *C. ruforum*, but the

volatiles released are not attractive to this egg parasitoid. When the host plant *P. sylvestris* is switched for *Pinus nigra*, *D. pini* will still lay eggs and feed on the needles of this pine species. However, the odor of *P. nigra* laden with *D. pini* eggs is unattractive to *C. ruforum*, possibly because the performance of this egg parasitoid is reduced compared to its performance on *D. pini* eggs laid on *P. sylvestris* (Auger *et al*. 1994; Barre *et al*. 2002). Thus, the attractiveness of pine odor induced by sawfly oviposition for the egg parasitoid *C. ruforum* is specific to certain combinations of pine and sawfly species.

In this study, we took advantage of the species specificity of interactions between pine, sawflies, and parasitoids to assess the importance of sesquiterpene synthases for the attraction of *C. ruforum*. Following our work on *P. sylvestris* with *D. pini* egg depositions, we report here the transcript levels of the pine sesquiterpene synthases, *PsTPS*1 and *PsTPS*2 in three other pairwise interactions between pine and sawfly species, some of which produce an odor attractive to the egg parasitoid *C. ruforum* and some which do not. We also describe a new terpene synthase that produces (*E*)-*β*-farnesene and examine its transcript levels during pine-sawfly interactions.

Before carrying out these experiments, it was first necessary to conduct behavioral assays with *C. ruforum* to determine the timing of its attraction to twigs upon which sawfly eggs had been laid. Previous results had shown that, when *D. pini* oviposited on *P. sylvestris*, the volatile blend was attractive 3 days after oviposition, but not before or after that time (Köpke *et al*. 2008). However, for the other combinations of pine and sawfly species, we first had to determine the timing of peak volatile attraction before conducting sesquiterpene transcript analyses since this had only been studied at 3 days after oviposition in each case (Table 1).

Table 1 Combinations of pine and sawfly species tested in previous studies for attraction of egg parasitoids to pine odor induced by sawfly egg deposition.

Pine species	**Sawfly species**	**Time after egg deposition [day]**	**Response by egg parasitoids***	**Reference**
P. sylvestris	*D. pini*	2	No attraction	Köpke et al. 2008
		3	Attraction	Hilker et al. 2002a, Köpke et al. 2008
		4	No attraction	Köpke et al. 2008
P. sylvestris	*N. sertifer*	3	Attraction	Mumm et al. 2005
P. sylvestris	*G. pallida*	3	No attraction	Mumm et al. 2005
P. nigra	*D. pini*	3	No attraction	Mumm et al. 2005

*Egg parasitoid tested: *Closterocerus* (formerly *Chrysonotomyia*) *ruforum*

2. Results and discussion

2.1. The attraction of C. ruforum to pine odor at different times after sawfly egg deposition

The olfactory response of female *C. ruforum* egg parasitoids to pine laden with eggs of various sawfly species was tested 2, 3 or 4 days after oviposition using a four-field olfactometer. We assessed whether parasitoids spent significantly longer walking in a test field supplied with odor compared with control fields. Previous studies had shown attraction or non-attraction 3 days after egg deposition, but tests had been conducted on other days for only *D. pini* on *P. sylvestris* twigs (Table 1). These results showed that the *P. sylvestris* twigs with *D. pini* eggs were attractive 3 days after oviposition, but not 2 or 4 days afterwards. Odor from *P. sylvestris* laden with *D. pini* eggs for 2 days elicited tentatively a positive response by the egg parasitoids. However, in spite of a high number of replicates, no significant attraction was recorded.

In the present work, when *P. sylvestris* twigs were tested with *N. sertifer* eggs, the pattern of response was the same with attraction evident only at 3 days, but not at 2 or 4 days after oviposition (Table 2). However, *P. sylvestris* twigs laden with *G. pallida* eggs did not show a significant attraction to *C. ruforum* at any time point tested. Finally, *P. nigra* twigs with *D. pini* eggs were also not attractive at any time tested. Whether the composition of volatile blends emitted by pine twigs changes over this time period is not known for most of these combinations besides *P. sylvestris* - *D. pini* (Mumm *et al*. 2003; Mumm & Hilker 2005). The temporal patterns of parasitoid attraction may be a result of when the sawfly egg is most suitable for infestation. For example, the eggs of the stink bug, *Nezara viridula*, laid on bean plants were suggested to be too old for successful infestation of the egg parasitoid, *Trissolcus basalis*, 96 hours after oviposition, when hatching was imminent (Colazza *et al*. 2004a).

2.2. The effect of oviposition on transcript levels of the sesquiterpene synthases TPS1 and TPS2

To investigate the role of pine sesquiterpene synthases in the attraction of egg parasitoids to sawfly eggs, we studied the transcript levels of two sesquiterpene synthase genes that were induced by oviposition in our previous studies (Köpke *et al*. 2008) in three pairwise species interactions between pine and sawflies, one attractive to *C. ruforum* and two unattractive. *P. sylvestris* needles on which *N. sertifer* eggs had been laid 3 days previously were attractive to the egg parasitoid, *C. ruforum* (Table 2). The transcript levels of *PsTPS*1 and *PsTPS*2 in these needles were significantly higher than levels of these genes in egg-free control needles not attractive to the parasitoids (Fig. 1). On average, transcript levels were 17.5-fold higher for *PsTPS*1 in egg-laden needles and 30.5-fold higher for *PsTPS*2. At 2 days after oviposition, transcript levels of these genes were not significantly different from those in

control needles, and at 4 days after oviposition transcripts were significantly reduced compared to the controls. Thus, *PsTPS*1 and *PsTPS*2 in *P. sylvestris* with *N. sertifer* eggs showed enhanced transcription only at the time when pine odor was attractive to the egg parasitoids (Table 1, 2). These results are very similar to those we obtained previously on the transcript levels of these genes in *P. sylvestris* laden with the eggs of *D. pini* (Köpke *et al*. 2008).

Table 2 Olfactory response of the female egg parasitoid *C. ruforum* to various combinations of pine and sawfly species tested at different times after sawfly egg deposition.

Pine / sawfly	**Time after oviposition [day]**	**Parasitoid walking time [s][1]**					
		Test field	**Control field 1**	**Control field 2**	**Control field 3**	***N***	**Statistics[2]**
P. sylvestris / *D. pini*	2[3]	163 (84-262)	107 (8-156)	79 (37-147)	105 (44-164)	34	n.s.
	3	152[a] (129-249)	103[b] 64-146)	73[b] (30-133)	113[ab] (74-154)	23	***P* < 0.05**
	4[3]	121 (42-182)	99 (35-156)	133 (68-245)	129 (67-177)	37	n.s.
P. nigra / *D. pini*	2	52[a] (1-83)	103[a] (8-215)	71[a] (16-197)	121[a] (31-207)	30	$P < 0.05$
	3[4]	95 (49-134)	211 (87-295)	186 (74-251)	69 (20-177)	25	n.s.
	4	94 (1-214)	87 (26-170)	124 (14-252)	117 (52-217)	23	n.s.
P. sylvestris / *G. pallida*	2	77 (37-162)	82 (36-159)	148 (75-186)	138 (105-254)	25	n.s.
	3	108 (55-132)	132 (83-225)	141 (78-167)	141 (64-184)	28	n.s.
	4	102 (2-232)	51 (2-184)	146 (43-232)	126 (15-179)	18	n.s.
P. sylvestris / *N. sertifer*	2	95 (26-145)	124 (45-169)	74 (37-180)	90 (56-200)	23	n.s.
	3	199[a] (89-293)	113[ab] (36-221)	71[b] (17-109)	67[b] 14-132	29	***P* < 0.05**
	4	61 (23-156)	96 (9-182)	115 (8-211)	92 (11-234)	27	n.s.

[1]Median values and interquartile range (parentheses) of the time parasitoid females spent in test and control fields of a four-arm olfactometer. Test field was supplied with odor of pine twig laden with eggs.

[2]Friedman analysis of variance. Different letters indicate significant ($P < 0.05$) differences evaluated by the Wilcoxon-Wilcox post-hoc test ($P < 0.05$). Bold *P*-value indicates significantly attractive pine odor. n.s., not significant ($P > 0.05$).

[3]Data taken from Köpke *et al*. 2008 for comparison

[4]Data taken from Mumm *et al*. 2005 for comparison

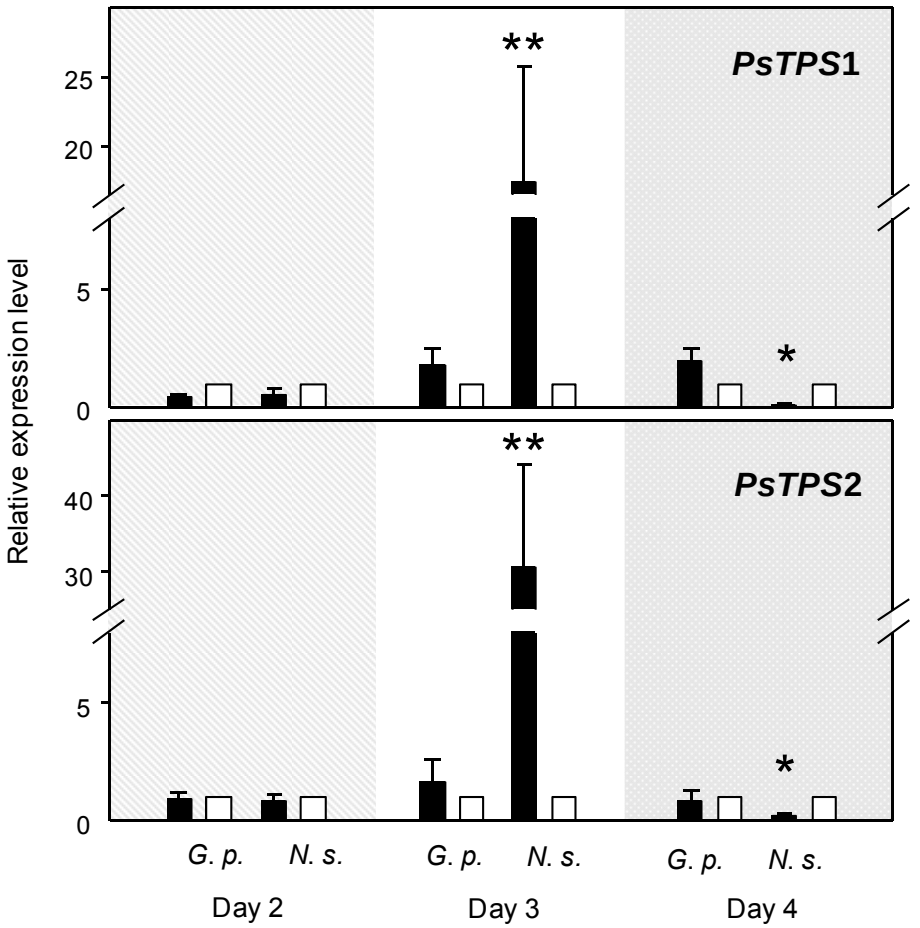

Fig. 1 Transcript levels of sesquiterpene synthases *PsTPS*1 and *PsTPS*2 in *Pinus sylvestris* needles laden with eggs of the sawflies *Gilpinia pallida* (*G.p.*) or *Neodiprion sertifer* (*N.s.*) Measurements were carried out by quantitative real-time PCR 2, 3, and 4 days after egg deposition. Oviposition-induced pine twigs: black bars; artificially wounded control twigs: white bars. Relative abundance of mRNA transcripts of genes was normalized to ubiquitin; values (mean + SE) are given relative to transcripts of artificially wounded controls (set to 1). Each value was calculated from at least 3 technical replicates of four independent biological samples. A Wilcoxon matched pairs test was performed to test the significance of differences in transcript accumulation. A * indicates a significant transcript level difference between sample and respective control at $P < 0.05$; a ** indicates $P < 0.01$. For details see text and Supplemental Table S2.

Other combinations of pine and sawfly species gave different results. Analysis of *P. sylvestris* needles on which the sawfly *G. pallida* had oviposited did not show any significant increase in transcripts of *PsTPS*1 and *PsTPS*2 at any time point tested (Fig. 1), consistent with the unattractiveness of odor released by this combination of pine and sawfly species (Table 1, 2). When *P. nigra* was substituted for *P. sylvestris* as the host tree, *D. pini* oviposition also did not lead to a significant increase in transcription of *TPS*1 and *TPS*2 at any tested time point (Fig. 2). Transcript levels of *TPS*1 even decreased slightly 4 days after egg deposition. The odor released from *P. nigra* twigs with *D. pini* eggs was not attractive to the egg parasitoids at

any time point (Table 1, 2). The *TPS*1 and *TPS*2 genes of *P. nigra* seem to be orthologs of those present in *P. sylvestris*. For *TPS*1, the nucleotide sequences of amplified quantitative real-time PCR fragments obtained from *P. sylvestris* and *P. nigra* were identical. For *TPS*2, the amplified sequence fragments obtained from both species revealed only a single nucleotide difference, albeit one which led to an amino acid difference.

Taken together, our results show that the sesquiterpene synthase transcripts *TPS*1 and *TPS*2 are induced in combinations of pine and sawfly species only when the egg-laden needles are attractive to the egg parasitoid, *C. ruforum*. Herbivore oviposition has been previously shown to cause transcriptional changes in plants (Little *et al*. 2007; Fatouros *et al*. 2008; Köpke *et al*. 2008), but this is the first documented case in which transcriptional response varies depending on the herbivore.

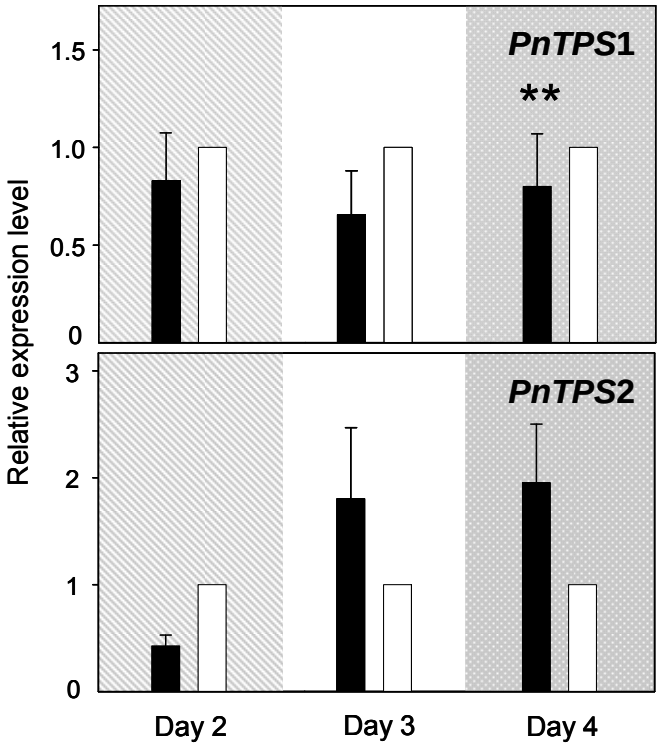

Fig. 2 Transcript levels of sesquiterpene synthases *PnTPS*1 and *PnTPS*2 in *Pinus nigra* needles laden with eggs of the sawfly *Diprion pini*. Measurements were carried out by quantitative real-time PCR 2, 3, and 4 days after egg deposition. Oviposition-induced pine twigs: black bars; artificially wounded control twigs: white bars. Relative abundance of mRNA transcripts of genes was normalized to ubiquitin; values (mean + SE) are given relative to transcripts of artificially wounded controls (set to 1). Each value was calculated from at least 3 technical replicates of four independent biological samples. A Wilcoxon matched pairs test was performed to test the significance of differences in transcript accumulation. The ** indicates a significant transcript level difference between sample and respective control at $P < 0.01$. For details see text and Supplemental Table S2.

2.3. Characterization of an (E)-β-farnesene synthase (PsTPS5) from P. sylvestris and its transcript levels after sawfly oviposition

In previous work with *P. sylvestris*, no sesquiterpene synthase gene had been isolated that encoded the formation of (*E*)-*β*-farnesene, the attractant for *C. ruforum* (Mumm *et al*. 2003). Here we used sequence information from another conifer (*E*)-*β*-farnesene synthase, that of *Pseudotsuga menziesii* (Huber *et al*. 2005), as a template for primer design. A terpene synthase sequence containing 2436 bp and coding for 812 amino acids was cloned and designated *PsTPS*5 (Fig. 4). The deduced amino acid sequence of *PsTPS*5 showed an aspartate-rich DDxxD motif (residues 555-559) typical for terpene synthases. These motifs are involved in coordinating the bivalent metal ion for substrate binding (Bohlmann *et al*. 1999). Another motif thought to be important for catalysis, the RR(x8)W sequence (residues 25-35), was found near the N-terminus (Pechous & Whitaker 2004). In addition, an RxR motif was present (residues 518-520) that is known to be involved in the complexation of the diphosphate function after ionization of the substrate, thus preventing nucleophilic attack on any of the carbocationic intermediates formed subsequently (Starks *et al*., 1997).

*PsTPS*5 shows an amino acid identity of 75-78% to two other conifer sesquiterpene synthases, (*E*)-*α*-bisabolene synthase from *Abies grandis* and (*E*)-*β*-farnesene synthase from *P. menziesii* (Figs. 3, 4). However, *PsTPS*5 shows only low amino acid similarity to the other sesquiterpene synthase sequences isolated from *P. sylvestris*, 39.0% with *PsTPS*1 and 35.4% with *PsTPS*2.

*PsTPS*5 was heterologously expressed in *Escherichia coli*, and the crude bacterial extracts containing recombinant protein were incubated with prenyl diphosphates. Production of (*E*)-*β*-farnesene was observed when using FPP as substrate; no terpenoid products were detected after incubation with GPP or GGPP. Control bacterial extracts containing the vector with a non coding control fragment did not show any enzyme activity. Besides (*E*)-*β*-farnesene, no other terpenoid products were detectable (Fig. 5). Terpene synthase enzymes producing (*E*)-*β*-farnesene as a single product have also been found in *Citrus junos* (Maruyama *et al*. 2001) and *P. menziesii* (Huber *et al*. 2005). By contrast, an (*E*)-*β*-farnesene synthase from maize functions as a multiproduct enzyme that produces (*E*)-*β*-farnesene, *α*-bergamotene and a host of minor products (Schnee *et al*. 2006). Another multiproduct (*E*)-*β*-farnesene synthase producing mainly (*E*)-*β*-caryophyllene and (*E*)-*β*-farnesene along with other side products was found in *Mentha* x *piperita* (Crock *et al*. 1997).

We analyzed the transcript level of *PsTPS*5 after sawfly oviposition in two pairwise combinations of pine and sawfly species producing an odor attractive to *C. ruforum*. However, the transcript of *PsTPS*5 did not show any significant increase with *D. pini* or *N. sertifer* oviposition, unlike for *PsTPS*1 and *PsTPS*2 (Fig. 6). Oviposition by *D. pini* on *P.*

sylvestris even led to a 0.76 reduction of *PsTPS*5 after 3 days and a 0.98-fold reduction after 4 days compared to the respective controls.

The finding that transcript levels of the (*E*)-*β*-farnesene synthase *PsTPS*5 did not increase after egg deposition by *D. pini* and *N. sertifer*, but rather decreased at just those time periods when increased (*E*)-*β*-farnesene makes pine needles attractive to *C. ruforum* (Mumm *et al*. 2003) was unexpected. The production of (*E*)-*β*-farnesene in this case may not be regulated at the transcriptional level. Recently Garms *et al*. (2008) suggested that the activity of a terpene synthase, *Mt*TPS3 from *Medicago truncatula*, is regulated post-transcriptionally by ethylene following herbivory by *Spodoptera exigua*. Alternatively, (*E*)-*β*-farnesene may be biosynthesised by more than one terpene synthase enzyme in *P. sylvestris*, and we may not have isolated the enzyme whose transcript is induced by sawfly egg deposition. Terpene synthases comprise a large gene family which appears to be formed by multiple gene duplications and subsequent neofunctionalization and subfunctionalization (Bohlmann *et al*. 1998; Trapp & Croteau 2001; Martin *et al*. 2004). Studies on other species have shown that two different terpene synthase genes from the same plant may encode proteins producing the same product. For example, two different terpene synthases of *Arabidopsis thaliana* can produce (*E*)-*β*-ocimene (Bohlmann *et al*. 2000; Fäldt *et al*. 2003). In addition, *A. grandis* has two limonene synthases, one that produces exclusively limonene, and a second one that additionally produces *α*-pinene (Bohlmann *et al*. 1997; 1999).

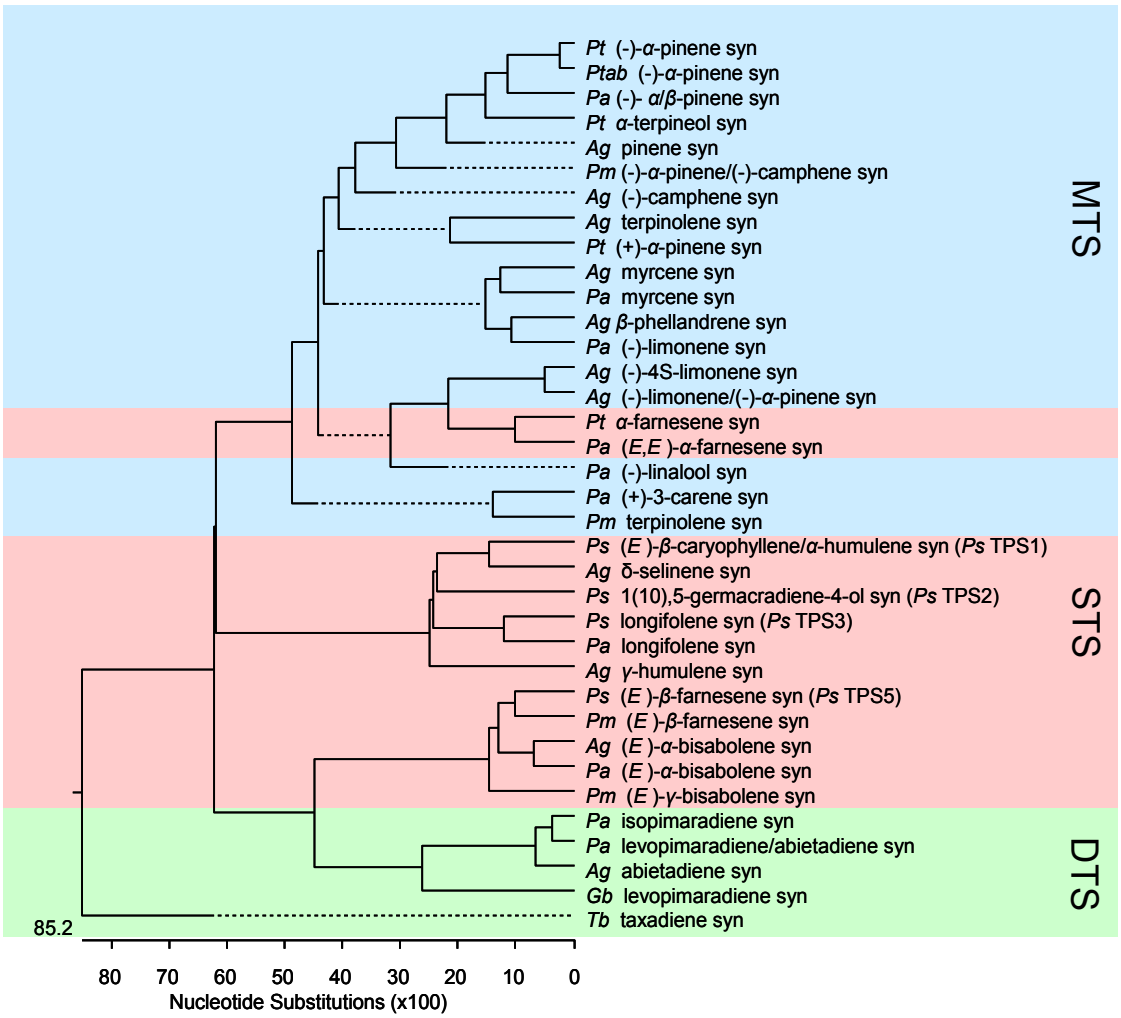

Fig. 3 Phylogenetic tree of the deduced amino acid sequences of *PsTPS*1, *PsTPS*2, *PsTPS*3 and *PsTPS*5 with selected monoterpene synthases (MTS), sesquiterpene synthases (STS) and diterpene synthases (DTS) of gymnosperms. Tree was calculated by the neighbor-joining method using ClustalW and Lasergene 7 (MegAlign) software. *Ag*, *Abies grandis*; *Gb*, *Gingko biloba*; *Pa*, *Picea abies*; *Pm*, *Pseudotsuga menziesii*; *Ps*, *Pinus sylvestris*; *Pt*, *Pinus taeda*; *Ptab*, *Pinus tabuliformis*; *Tb*, *Taxus brevifolia*. GenBank protein accessions of terpene synthases are shown top to bottom: AAO61225, ABY65904, AAS47692, AAO61227, AAB71085, AAX07267, AAB70707, AAF61454, AAO61228, AAB71084, AAS47696, AAF61453, AAS47694, AAB70907, AAF61455, AAO61226, AAS47697, AAS47693, AAO73863, AAX07264, ABV44452, AAC05727, ABV44453, ABV44454, AAS47695, AAC05728, GU248335, AAX07265, AAC24192, AAS47689, AAX07266, AAS47690, AAS47691, AAK83563, AAS89668, AAK83566

3. Conclusions

Oviposition by the sawfly *D. pini* on *P. sylvestris* needles has been shown to make them attractive to the egg parasitoid *C. ruforum*, and to increase the transcription of sesquiterpene synthase genes encoding an (*E*)-*β*-caryophyllene/*α*-humulene synthase (*PsTPS*1) and an 1(10),5-germacradiene-4-ol synthase (*PsTPS*2). Here we investigated several other combinations of pine and sawfly species and found that *TPS*1 and *TPS*2 transcripts also increased on oviposition, but only for combinations in which the egg-laden needles were attractive to *C. ruforum*. Furthermore, *P. sylvestris TPS*1 and *TPS*2 transcripts increased at just the time after egg deposition when the odor was attractive. Thus, these sesquiterpene synthase genes may be considered good markers for production of odor attractive to egg parasitoids. However, further research is necessary to find out whether the sesquiterpene products produced by the *TPS*1- and *TPS*2-encoded enzymes actually function in attracting egg parasitoids or instead play a role in direct defense against sawfly larvae, or in another process.

The key compound known to be implicated in *C. ruforum* attraction to *P. sylvestris* needles with sawfly eggs is the sesquiterpene (*E*)-*β*-farnesene (Mumm *et al*. 2003; Mumm & Hilker 2005). Here we isolated an (*E*)-*β*-farnesene synthase from *P. sylvestris*, yet its transcript level was not up-regulated during the time period of *C. ruforum* attraction. To try and better understand what regulates (*E*)-*β*-farnesene formation in sawfly oviposition-induced pine, it will be necessary to examine other levels at which (*E*)-*β*-farnesene synthase activity might be regulated as well as to search for additional (*E*)-*β*-farnesene synthase genes in *P. sylvestris*.

4. Experimental

4.1. Plant and insect material

Plant material was harvested from *P. sylvestris* and *P. nigra* trees growing in forests near Berlin, Germany. Branches of both species were cut from the middle part of 10- to 15-year-old trees and from the lower part of 35- to 45-year-old trees. The lower part of a branch was cleaned, sterilized according to Moore and Clark (1968), and placed into water for treatment (see below). Plant material was harvested in all seasons in 2007 – 2008, except for the time between May and August when new shoots were developing. In the laboratory, cut pine twigs were kept under standard conditions as previously described (Köpke *et al*. 2008).

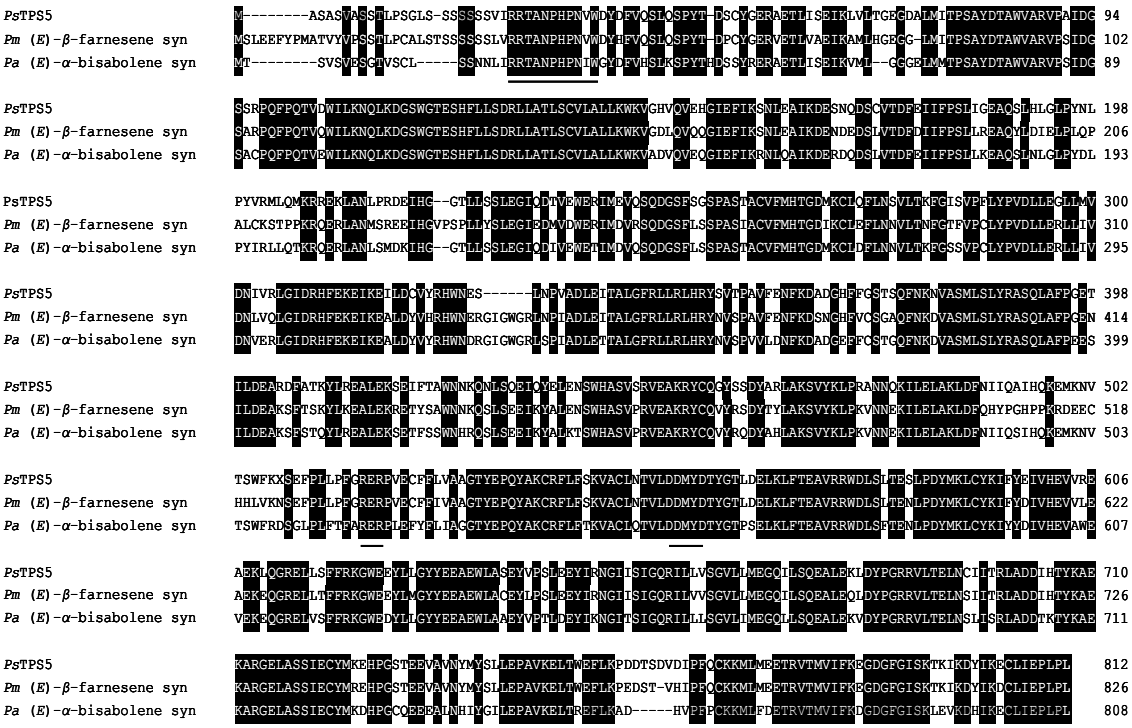

Fig. 4 Alignment of deduced amino acid sequences of the *Pinus sylvestris* (*Ps*) sesquiterpene synthase *Ps*TPS5 and two other conifer sesquiterpene synthases: *Pseudotsuga menziesii* (*Pm*) (*E*)-*β*-farnesene synthase (GenBank accession AAX07265) and *Picea abies* (*Pa*) (*E*)-*α*-bisabolene synthase (GenBank accession AAS47689). Amino acid residues that are identical in all six sequences are enclosed in black boxes. The RR(x8)W, RxR and DDxxD motifs are underlined in the sequence.

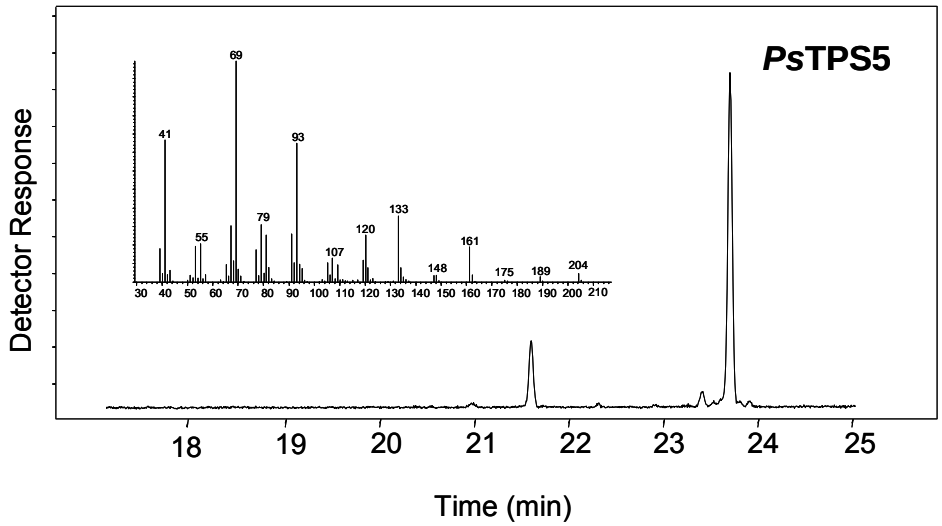

Fig. 5 Identification of (*E*)-*β*-farnesene as the product of *Ps*TPS5. Depicted is a part of the total ion current chromatogram of a GC-MS analysis of extracts from transformed bacteria expressing *PsTPS*5 and assayed using farnesyl diphosphate (FPP) as substrate. The major peak at 23.75 min is (*E*)-*β*-farnesene (with the mass spectrum given as inset). Minor peaks including that at 21.58 min (1-tetradecene) are impurities.

The sawfly *D. pini* (Hymenoptera, Diprionidae) was reared in the laboratory on pine branches at 20 °C ± 1 °C, 65% r.h., and 18:6 h light/dark cycles (Bombosch & Ramakers 1976; Eichhorn, 1976). Cocoons of the sawflies *N. sertifer* and *G. pallida* were collected in the field in southern Finland and kept at the same conditions as *D. pini*. Sawfly eggs parasitized by *C. ruforum* were also collected in southern Finland. Pine needles with parasitized sawfly eggs were supplied with water and kept at about 5 °C. About two weeks prior to bioassays, needles with parasitized eggs were transferred to 20 °C to induce parasitoid emergence (Köpke *et al*. 2008)

4.2. Plant treatments

Small pine twigs (about 20 cm long with 80-100 needles) were cut, placed into a glass cylinder covered by a gauze lid, and supplied with water. Twigs with sawfly eggs on their needles are here referred to as test twigs. For control twigs, needles were artificially wounded in a way that mimicked the ovipositional wounding conducted by sawflies prior to egg deposition (i.e. longitudinal slit through a pine needle (Hilker *et al*. 2002a). Test twigs with eggs were compared to artificially wounded control twigs to reveal transcriptional changes that can be ascribed directly to egg deposition, rather than to the wounding associated with egg laying. Test and control twigs were always cut from the same branch at the same time and kept at the same conditions to minimize possible variation in terpenoid metabolism among different trees or different parts of one tree. To obtain needle material for molecular analyses,

needles from control twigs were always removed from harvesting at the same time as needles from the respective test twigs.

For the oviposition treatment, three females and three males of a sawfly species were added to a glass cylinder containing a pine twig. The insects mated and laid eggs during the next night (4-12 egg masses, each on a different needle), and were removed from the cylinder the next morning. This was designated time zero (t=0), and twigs were then kept for 2, 3, or 4 days at standardized conditions as described (Köpke *et al*. 2008). For the artificial wounding treatment, eight to ten needles of a pine twig were longitudinally slit at time point t=0. At later time points, 2, 3 or 4 days after treatment, unwounded needles were removed for further analyses. Twigs treated in these ways were harvested for both the molecular analyses and the olfactometer behavioral studies.

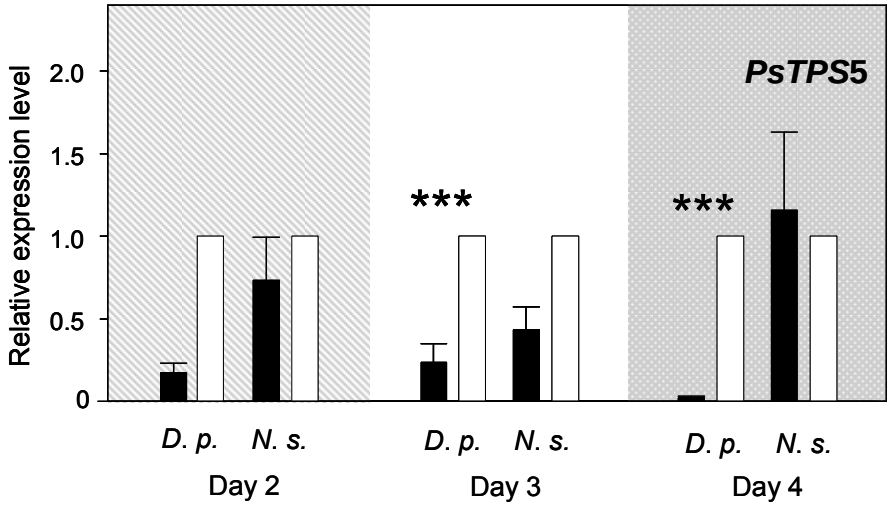

Fig. 6 Transcript levels of sesquiterpene synthase *PsTPS*5 in *Pinus sylvestris* needles laden with eggs of the sawflies *Diprion pini* (*D.p*.) or *Neodiprion sertifer* (*N.s*.). Measurements were carried out by quantitative real-time PCR 2, 3, and 4 days after egg deposition. Oviposition-induced pine twigs: black bars; artificially wounded control twigs: white bars. Relative abundance of mRNA transcripts of genes was normalized to ubiquitin; values (mean + SE) are given relative to transcripts of artificially wounded controls (set to 1). Each value was calculated from at least 3 technical replicates of four independent biological samples. A Wilcoxon matched pairs test was performed to test the significance of differences in transcript accumulation. A *** indicates a significant transcript level difference between sample and the respective control, $P < 0.001$. For details see text and Supplemental Table S2.

4.3. Olfactory responses of C. ruforum

A four-field olfactometer was used as described previously (Hilker *et al*. 2002a, 2005). Air (150 ml min^{-1}) entered the walking arena of the four-arm olfactometer from 4 sides, thus establishing four distinct odor fields. One field was supplied with odor from an oviposition-induced pine twig, while the other fields were supplied with charcoal-filtered,

humidified air. When starting a bioassay, a *C. ruforum* female was released in the center of the walking arena. The time a parasitoid spent walking in each of the four odor fields was then recorded during an observation period of 600 s using a software program, The Observer 3.0 (Noldus, Wageningen, The Netherlands). Only data from active parasitoids walking at least 300 s of the observation period were used for statistical analysis. Prior to these bioassays, parasitoids had the chance to associate the experience of host egg parasitization with odor of pine twigs as described by Hilker *et al*. (2002a) and Schröder *et al*. (2008). The combination of pine and sawfly species used for this experience was always the same species combination as the one tested later for its attractiveness in the bioassay.

The number of parasitoids used per treatment was 18 to 37. The number of odor sources (twigs) tested was 3 to 6 per bioassay. Data were analyzed by Friedman ANOVA by comparing walking times within each of the four odor fields. Wilcoxon-Wilcox tests were used for post-hoc comparisons (Köhler *et al*. 1995). If the walking time was significantly longer in the test field than in the control field C2 opposite to the test field, the response was defined as attraction. The analysis was performed using StatSoft, Version 1999, STATISTICA for Windows (Tulsa, OK, USA).

4.4. RNA isolation and cDNA synthesis

Needles from oviposition treated and artificially wounded *P. sylvestris* and *P. nigra* twigs were harvested 2, 3, or 4 days after treatment. The material was ground in liquid nitrogen with a sterilized mortar and pestle. The Invisorb Spin RNA Mini Kit (Invitek, Berlin, Germany) protocol was followed for RNA isolation. Approximately 100 mg plant tissue was used per extraction. Further handling of the RNA and evaluation of its quality and quantity was conducted as described by Köpke *et al*. (2008).

4.5. Functional expression of PsTPS5

The complete open reading frame of the full length cDNA clone of *PsTPS*5 was used for heterologous expression. PCR reactions were performed with primers (Table S1 in supplemental data) using the Expand High Fidelity^{Plus} PCR System (Roche, Mannheim, Germany) following the manufacturer's instructions. The amplification products of *PsTPS*5 were cloned into the expression vector pH9GW (Yu & Liu 2006).

Plasmids were transformed into the *E. coli* Top 10 strain (Invitrogen, Karlsruhe, Germany) and verified by sequencing. Mutation-free plasmids were transformed into the BL21 (DH3) pLysS strain of *E. coli* (Invitrogen). For bacterial expression, a starter culture (10 ml Luria-Bertani medium with 35 µg/ml chloramphenicol and 50 µg/ml kanamycin) was grown for 3 days at 18 °C. A portion of the starter culture (5 ml) was then diluted in 100 ml Overnight Express™ Instant TB medium (Novagen, Darmstadt, Germany) with 35 µg/ml

chloramphenicol and 50 µg/ml kanamycin. Cells were kept shaking for 16 h at 37 °C and later collected by centrifuging at 9,000 g for 20 min. The resulting pellets were resuspended in 3 ml of assay buffer (Martin *et al*. 2004) and disrupted by sonication (Bandelin Sonopuls HD 2070, Berlin, Germany) for 4 min, cycle 2, power 60%. After freezing (10 min at -20 °C, 10 min at -80 °C), the sample was centrifuged at 14,000 *g* for 10 min to separate the cell fragments. The supernatant containing the total bacterial crude protein extract was then assayed.

Sesquiterpene product assays were conducted with (*E*,*E*)-FPP added to the supernatant (69.9 µM FPP). The sample was overlaid with 1 ml pentane and incubated at 30 °C. Other substrates assayed included GPP at 99.5 µM and GGPP at 37.0 µM. The assay was stopped 1 h after pentane addition by vigorous vortexing with the pentane overlay for 30 s and the aqueous and organic fractions separated by centrifugation at 2,500 g for 2 min. After removal of the pentane fraction, the residue was overlaid again with 1 ml pentane. In total, three consecutive pentane extractions were conducted. The combined pentane fractions were dried over a silica/$NaSO_4$ column and evaporated under gaseous nitrogen to 50 - 100 µl. Instead of pentane, also TBME (tert-butylmethyl ether) was used as solvent. Both TBME and pentane extracts were analyzed by GC-MS (see below).

Protein concentrations were measured according to Bradford (1976) using the BioRad reagent with bovine serum albumin (BSA) as standard. Concentrations were adjusted to a range of 0.5 – 2.5 µg/ml for the measurement.

4.6. GC-MS analysis

A GC system (Agilent Hewlett-Packard 6890, Agilent Technologies) coupled to a Network Mass Selective Detector (Agilent Hewlett-Packard 5973, Agilent Technologies) was used for analyses of the pentane extracts and TBME wash of the assay of *Ps*TPS5 expressed in *E. coli*. The product (*E*)-*β*-farnesene was identified by comparison of its mass spectrum and retention time to those of an authentic standard (Sigma, St. Louis, MO, USA).

4.7. Quantitative real-time PCR

Quantitative real time PCR reactions were performed using a Stratagene MX3000P™ according to the operator's manual and the methods and standard thermal profiles as were described in Köpke *et al*. (2008). Real time quantification of gene transcription was performed using a SYBR green QPCR Master Mix from Stratagene (La Jolla, CA, USA). Primers for *PsTPS*1 and *PsTPS*2 are listed in Köpke *et al*. (2008), and those for *PsTPS*5 are given in the Supplemental Table S1. The same primers were used for amplification of transcripts of orthologous genes in *P. nigra* (*PnTPS*1, *PnTPS*2). Amplified products were verified by cloning and sequencing. Reactions with water instead of cDNA template were run with each primer pair as control.

The mean quantity of transcript was calculated from at least four independent biological replicates, each of which is represented by at least three technical replicates. Transcript abundance of the ubiquitin gene (GenBank accession number EF681766) was used for normalization. Relative normalized transcript levels were calibrated against transcript abundance determined for the appropriate artificially wounded control. All amplification plots were analyzed with the MX3000P™ software to obtain threshold cycle (C_t) values.

A Wilcoxon matched pairs test was performed on quantitative real-time PCR raw data (non-normalized) to test the significance of differences in changes of *PsTPS*1, *PsTPS*2, *PsTPS*5, *PnTPS*1, and *PnTPS*2 between egg-laden pine twigs and artificially-wounded ones. All analyses were performed using StatSoft, Version 1999, STATISTICA for Windows (Tulsa, OK, USA) (see supplemental data S2).

4.8. Sequence and phylogenetic analyses

A phylogenetic tree of the deduced amino acid sequences of each full-length *P. sylvestris* cDNA and of known terpene synthase sequences from gymnosperms was calculated by using the DNASTAR Lasergene program version 7.0 (Meg Align™). ClustalW (gonnet 250 matrix; gap penalty, 10.00; gap length penalty, 0.20; delay divergent sequences, 30%; gap length, 0.10; DNA transition weight, 0.5) was used for assembly of amino acid alignments and visualization of the phylogenetic tree.

4.9. Chemicals

All chemicals and solvents were of analytical grade and obtained from Merck (Darmstadt Germany), Serva (Heidelberg, Germany), or Sigma (St. Louis, MO, USA). The substrates geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) were from Echelon Research (Salt Lake City, UT, USA).

4.10. Accession Number

The *PsTPS*5 and *PnTPS*1-2 sequences were deposited in GenBank, accession numbers are GU248335, GU248337 and GU248336 respectively.

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Supplemental data (page 110-111)

Table S1. Primers used in initial screening for sesquiterpene synthase sequences, RACE-PCR, heterologous expression and quantitative real-time PCR.

Table S2. Wilcoxon matched pairs test: Statistical comparisons of *P. sylvestris* sesquiterpene synthase transcript levels measured by quantitative real-time PCR between samples from oviposition-induced foliage and artificially wounded controls (see Figs. 1, 2 and 6 for further details).

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Chapter 4

Can insect egg deposition "warn" a plant of future feeding damage by herbivorous larvae?

Abstract

Plant defence is inducible by both the feeding of insect herbivores and the deposition of their eggs. However, little is known about the ability of eggs to induce defences against hatching larvae. We demonstrate that oviposition by the sawfly *Diprion pini* on *Pinus sylvestris* foliage increases the plant´s defensive potential against sawfly larval feeding. Larvae that initiated their development on pine where they hatched from eggs performed worse, exhibiting decreased growth, survival and adult fecundity than those reared on egg-free twigs. Transcription of pine sesquiterpene synthases (*Ps*TPS1, *Ps*TPS2) known to be induced by egg deposition reached its highest level just before larval hatching. Concentrations of pine terpenoids and phenolic metabolites did not change significantly after oviposition and feeding treatments, but the small changes may sum up in larvae during feeding and thus affect performance. We suggest that insect egg deposition may "warn" a plant of upcoming feeding damage by larvae.

Keywords Plant defense, herbivory, insect oviposition, induced resistance, *Pinus*, Diprionidae, sesquiterpene synthases, terpenoids, phenolics.

1. Introduction

Plants respond to herbivore attack with a wide range of defensive strategies (Walling 2000; Heil 2008; Dicke 2009; Dicke *et al.* 2009; Wu & Baldwin 2009) that vary with the type of herbivore, the magnitude of attack and the physiological state of the plant (e.g. Kessler & Baldwin 2002). Studies on defence traits induced by herbivore feeding usually compare the damage-induced phenotype with those of untreated or artificially wounded controls. For example, plants damaged by herbivore feeding are well-known to release more volatiles than undamaged plants (e.g. Paré & Tumlinson 1999) and to increase their transcription of genes involved in the synthesis of defence-related compounds (e.g. Kant & Baldwin 2007). However, considering that larvae of numerous herbivore species hatch from eggs laid on the same plant where the larvae start to feed, the plant's phenotype prior to feeding damage is not necessarily that of an untreated plant, but of a plant carrying eggs. Since insect egg deposition is known to induce both direct and indirect plant defences against eggs (Hilker & Meiners 2002, 2006), the deployment of defences against larval feeding might not only be shaped by prior herbivory, but also by the presence of eggs.

A plant may assess the risk of herbivore attack by "listening" to plant damage in the vicinity. For example, an unharmed plant may perceive volatiles released from damaged or herbivore-infested neighbouring plants and be primed to respond more quickly or effectively to insect feeding on its own foliage (Engelberth *et al.* 2004; Paré *et al.* 2005; Choh & Takabayashi 2006; Heil & Kost 2006; Frost *et al.* 2008; Heil & Ton 2008). Volatiles released from damaged leaves of the same plant can also convey information about risk of attack within a plant resulting in a greater defensive response when leaves are later fed upon (Heil & Silva Bueno 2007). In addition to volatiles from damaged plants, plants can also "notice" insect egg deposition (Schröder *et al.* 2005; Hilker & Meiners 2010). Since infestation of plants by insects often starts with egg deposition, we asked whether a plant takes eggs on its leaves as warning of future larval herbivory and improves its defence against larvae.

To investigate whether insect egg deposition could influence future induced defence against larval herbivory, we studied a plant – herbivore system for which the plant is well known to be induced by insect egg deposition and the performance of the herbivore has intensively been studied with respect to the impact of plant chemicals. The pine sawfly, *Diprion pini*, feeds on the needles of Scots pine, *Pinus sylvestris*. Each *D. pini* female can lay more than 100 eggs which are deposited in a row on the edge of a needle (at maximum 30 eggs per row). About 2 weeks after egg laying, larvae hatch and feed gregariously. They may cause severe damage in

pine forests during mass outbreaks (Pschorn-Walcher & Eichhorn 1982). Egg deposition by *D. pini* on needles of Scots pine, *Pinus sylvestris*, is known to induce both locally and systemically the emission of pine terpenoid volatiles that attract egg parasitoids (Hilker *et al.* 2002; Mumm & Hilker 2006). Only volatiles released 3 days after egg deposition are attractive to the parasitoid; volatiles released earlier or a day later do not attract (Köpke *et al.* 2008). Previous work has investigated the *P. sylvestris* terpene synthase enzymes that are putatively involved in the formation of terpene volatiles (Köpke *et al*. 2008, 2010). The expression of the sesquiterpene synthase genes *PsTPS*1*,* encoding an (*E*)-*β*-caryophyllene / *α*-humulene synthase, and *PsTPS*2*,* encoding a 1(10),5-germacradiene-4-ol synthase, was induced by eggs of *D. pini* and another sawfly species coinciding with the attractiveness of egg-laden foliage to egg parasitoids (Köpke *et al.* 2010). The performance of *D. pini* larvae on pine has been studied especially with respect to the impact of needle terpenoids, phenolics and water content (reviewed by e.g. Mumm & Hilker 2006). High concentrations of mono- and diterpenes were found to negatively affect *D. pini* performance (e.g. Auger *et al.* 1994a; Barre *et al.* 2003; Heijari *et al.* 2008). Similarly, high concentrations of phenolic compounds are detrimental for *D. pini* development (e.g. Pasquier-Barre *et al.* 2001; Barre *et al.* 2003; Roitto *et al.* 2009), especially the dihydroflavonol taxifolin (Auger *et al.* 1994b). On the other hand, a high needle water content has been shown to be beneficial for *D. pini* (Pasquier-Barre *et al.* 2001).

Here we investigated the influence of *D. pini* egg deposition on *P. sylvestris* defence against feeding *D. pini* larvae by three different approaches:

i) Performance studies: We compared the growth, survival and fecundity of *D. pini* feeding on egg-laden and egg-free pine needles to determine whether egg deposition alters plant quality in a way that affects herbivore performance.

ii) Molecular analysis: We measured the expression levels of the sesquiterpene synthase genes *PsTPS*1 and *PsTPS*2 at various times after *D. pini* egg laying and larval feeding.

iii) Chemical analysis: We measured the following parameters in *P. sylvestris* needles at the same time points as in the molecular studies: concentrations of terpenoids and phenolics, and water contents, i.e. parameters known to affect performance of *D. pini*; furthermore, we determined the foliage C/N ratio which provides some general information on the nutritive value of the needle material (Stiling & Cornelissen 2007 and references therein).

2. Material and Method

2.1. Plant and insect material

Plant material of *Pinus sylvestris* was collected in forests near Berlin, Germany. Branches were taken from different trees, cleaned according to a method of Moore & Clark (1968) and kept in water at 10°C prior to needle analysis or usage for performance studies. The pine sawfly *Diprion pini* was reared in the lab according to the methods described by Bombosch & Ramakers (1976).

2.2. Performance studies

Sawflies were offered pine branches in a climate chamber (20°C, L/D: 18/6 h, 70% r.h.). Branches from 10 trees were used. A branch from each tree was divided into three twigs (about 45 cm long) which were kept in tap water. One twig was used for each of three different treatments: A, B, C. Twig A remained uninfested, while 3 males and 3 females of *D. pini* were placed on each twig B and C and left overnight to produce egg clusters. The sawflies were removed after about 24 h, and egg clusters laid on each twig were counted. Twigs were discarded when no eggs or less than 4 egg masses were laid. As soon as larvae hatched on twig C, they were gently transferred to twig A using a blunt, sterilised needle. Larvae on twig A then began to feed on twigs that had not experienced any oviposition previously. We refer to these larvae as "no oviposition" (NOP) larvae. The larvae hatching on twig B were not transferred to another twig, but instead were moved after hatching to a part of the same twig a few cm away from the hatching site. These larvae now fed on twigs that had experienced oviposition, and we refer to them as "oviposition larvae" (OP). The short-range movement of OP larvae was conducted to expose them to the same type of transfer as that experienced by NOP larvae.

When NOP and OP larvae had eaten up all the foliage on their initial twigs (after about 2 weeks), they were transferred to fresh twigs of *P. sylvestris*. These new twigs were the same for both groups of larvae and had had no prior oviposition. This transfer to fresh twigs reflects the situation in nature where, in later larval stages, *D. pini* larvae move from the twig where they fed during their first larval stages. Thus, oviposition-induced effects on plant quality are only experienced directly by young larvae. The entire larval development takes about 4 weeks at the conditions used here. The performance parameters measured were larval weight (2 days after hatching), time of larval development (from hatching to onset of pupation, i.e. spinning a cocoon), larval survival (i.e. survival from L1 to onset of pupation); cocoon weight, length of

cocoon phase and adult weight immediately after emergence. Females (< 1 day old) were dissected, and the number of eggs in their ovaries was also counted.

2.3. Molecular analysis

Five *P. sylvestris* twigs were cut from one branch and subjected to 5 different treatments as described in Table 1. In total, seven different branches were taken from 7 different trees (7 biological replicates). After treatment, pine needles were removed from the twigs, transferred to liquid nitrogen and kept at -80°C until analysis. General conditions of plant storage before and during treatments were the same as described above.

Harvested needles were ground, and RNA was extracted with the Invisorb Spin RNA Mini Kit (Invitek, Berlin, Germany). Synthesis of cDNA and measurements of transcript levels of *PsTPS*1 and *PsTPS*2 by quantitative real-time PCR followed the methods described by Köpke *et al.* (2008). Each sample (5 treatments × 7 biological replicates) was measured at least 3 times (technical replicates).

2.4. Chemical analysis

2.4.1. General

Needles from the same *P. sylvestris* twigs that had been subjected to the treatments described in Table 1 were also used for the molecular analysis.

2.4.2. Mono- and sesquiterpene extraction

Terpenes were extracted from pine needles as described by Martin *et al.* (2002). All steps were carried out in 2-ml glass vials with black, silicon-coated screw caps (VWR International, Darmstadt, Germany). Ground, frozen needles (200 mg) were extracted in *tert*-butyl methyl ether (1 ml) by constantly shaking the sample at room temperature for 14 h. Methylcaprylate (150 µg/ml) was added as an internal standard. Samples were washed with 300 µl $(NH_4)_2CO_3$ (0.1M, pH 8.0), vigorously shaken for 10 s and incubated for 2 min at RT. Samples were filtered through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous Na_2SO_4. The column was washed with 1 ml of *tert*-butyl methyl ether, and the combined eluate was collected in a clean vial, evaporated under gaseous nitrogen to an approximate volume of 250 µl and then stored at -20°C until analysed by GC-MS.

2.4.3. Diterpene extraction

Frozen and ground needle material (100 mg) was extracted in 1.5 ml *tert*-butyl methyl ether, including the internal standard tetrahydroabietic acid (34 µg/ml), by shaking the samples constantly for 14 h. Samples were washed with 300 µl of 0.1M $(NH_4)_2CO_3$. The supernatant was run through a column (Pasteur pipette filled with 0.3 g of silica gel, Sigma 60 Å), overlaid with 0.2 g anhydrous Na_2SO_4 and washed with 1 ml *tert*-butyl methyl ether. The eluate (300 ml) was transferred to a vial and methylated by adding 37.6 µl trimethylsulphonium hydroxide. Derivatised samples were subjected to GC-MS analysis.

2.4.4. Phenolic extraction

Pine needles were ground to a fine powder under liquid nitrogen and lyophilised. A sample of approx. 80 mg of the powder was extracted for 12 h at 4°C with 4 ml methanol containing 10 µg/ml chlorogenic acid as internal standard. The extract was filtered, dried under nitrogen and re-dissolved in 1 ml methanol. A 1:10 dilution in methanol was analysed using LC-MS/MS. Here we analysed three additional biological replicates for each treatment plus the 7 biological replicates we used in all other analyses (i.e. 10 branches taken from 10 different trees).

2.4.5. GC-MS analyses of terpenoids

Needle extracts were analysed on a GC system (Agilent Hewlett-Packard 6890, Agilent Technologies) coupled to a Network Mass Selective Detector (Agilent Hewlett-Packard 5973, Agilent Technologies). For analyses, 1 µl of the ether extract was injected on a HP-5 capillary column (30 m x 0.25 mm with a 0.25 µm phase coating; Agilent Technologies).

a) Mono- and sesquiterpenes. The GC-MS was set at an injector temperature of 220°C. The temperature program started with 40°C for 2 min, raised to 210°C (5°C/min), and raised further to 300°C (60°C/min 2 min hold; helium flow: 2 ml/min). The MS detector was operated using the total ion mode at a temperature of 230°C. The products were identified by comparing mass spectra and retention times with those in the literature and in the Wiley 275.L or NIST 98.1 MS libraries. The identity of most terpenes was further verified by comparison with commercially available authentic standards.

b) Diterpenes. The GC-MS was set at an injector temperature of 270°C. The temperature program started with 150°C for 3 min and afterwards rose to 280°C (3.5°C/min) and was held for 4 min. The MS detector was operated using the total ion mode at a temperature of 230°C. The products were identified by comparing mass spectra and retention times with those in the literature and in the Wiley 275.L or NIST 98.1 MS libraries. The identity of diterpenes was further verified by comparison with commercially available authentic standards.

Mono-, sesqui- and diterpenes were quantified in pine needle extracts relative to the corresponding internal standard. The relative quantities of these compounds were then averaged from the 7 independent samples of each treatment. For each sample, three technical replicates were analysed in the case of mono- and sesquiterpenes and two replicates in the case of diterpenes.

2.4.6. LC-MS/MS analyses of phenolic compounds

Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Kinetex C18 column with 100 X 4.6 mm dimensions and a particle size of 2.6 µm (Phenomenex, Aschaffenburg, Germany). Formic acid (0.05%) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-1 min, 100% A; 1-7 min, 0-65% B in A; 7-8 min 65-100% B in A; 8-9 min 100% B and 9-10 min 100% A. The total mobile phase flow rate was 1.8 ml/min. The column temperature was maintained at 25°C.

An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbospray ion source was operated in the negative ionisation mode. The instrument parameters were optimised by infusion experiments with pure standards, where available. For dimeric proanthocyanidins partially purified plant extracts were used for optimisation. The ion spray voltage was maintained at -5500 eV. The turbo gas temperature was set at 700°C. Nebulising gas was set at 70 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 10 psi. Scheduled multiple reaction monitoring (MRM) was used to monitor the conversion of the parent ion → product ion for each quantified analyte: *m/z* 300.8 →179 (collision energy (CE)-28 V; declustering potential (Tingey *et al*. 1991) -55 V) for quercetin; *m/z* 302.8 →125.1 (CE -28 V; DP -40 V) for taxifolin; *m/z* 288.9 →109.1 (CE -34 V; DP -30 V) for catechin; *m/z* 304.8 →179 (CE -28 V; DP -390 V) for gallocatechin; *m/z* 314.9 →107.1 (CE -46 V; DP -60 V) for isorhamnetin; *m/z* 352.8 →191.1 (CE -24 V; DP -25 V) for chlorogenic acid; *m/z* 430.8 →268 (CE -46 V; DP -80 V) for apigenin glucoside; *m/z* 462.9 →300 (CE -40 V; DP -390 V) for quercetin glucoside and quercetin galactoside; *m/z* 464.8 →125.1 (CE -44 V; DP -395 V) for taxifolin glucoside; *m/z* 576.9 →289.1 (CE -30 V; DP -50 V) for proanthocyanidin B1; *m/z* 592.9 →125.1 (CE -52 V; DP -400 V) for the catechin:gallocatechin dimer; *m/z* 609 →125.1 (CE -50 V; DP -45 V) for the gallocatechin dimer. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionisation efficiencies was verified by analysing a dilution series of pine needle extracts. External calibration curves for catechin, taxifolin and apigenin glucoside were created by linear regression. Flavan-3-ol concentrations were determined relative to the catechin calibration curve, flavonoids relative to taxifolin, and

flavanoid glycosides relative to apigenin glucoside. Process variability in different analyses was calculated relative to the internal standard.

2.4.7. C/N ratio measurement

Every pine needle sample (4 to 5 g) was ground in a mortar, lyophilised and later pulverised with a Retsch mill (MM200, Retsch, Düsseldorf, Germany) for 3 min (frequency: 30). The pulverised samples were poured in paper bags and kept over night in a 60°C chamber for further dehydration. The next day the samples were kept for 1 h in a desiccator to adjust to room temperature. Then each sample (18 to 19 mg) was poured into a zinc bowl, and the C/N ratio was determined using a "Vario EL II" (Elementar Analysensysteme GmbH, Hanau, Germany) at the Max Planck Institute for Biogeochemistry, Jena, Germany.

2.4.8. H_2O content

Needle samples of 100 or 200 mg fresh weight were dried in an 80°C oven for ca. 2 days. The needle water content was calculated by subtraction of dry weight from initial fresh weight.

2.5. Statistical analysis

Performance data of NOP and OP larvae were statistically compared by using the Mann-Whitney U-test, except for larval mortality data which were evaluated by a Chi-Square test. Comparison of molecular and chemical data obtained from the 5 differentially treated twigs taken from the same branch was done by a Friedman ANOVA; the Wilcoxon-Wilcox test was used for post-hoc comparisons. All statistical tests were performed using the statistical software StatSoft, Version 1999, Statistica for Windows (Tulsa, OK, USA) (see supplemental data S2).

Table 1

Diprion pini oviposition and feeding treatments applied to *Pinus sylvestris* twigs of which needles were subjected to chemical and molecular analyses after treatment

Twig	Treatment
C	Untreated control twigs
E3	Twigs with eggs of *D. pini* harvested 3 days after egg deposition
E14	Twigs with eggs of *D. pini* harvested 14 days after egg deposition, close to the time of larval hatching
E+L	Twigs with eggs of *D. pini* upon which larvae (OP larvae) hatched and fed for 2 days
L	Twigs which experienced *D. pini* larval feeding, but no eggs. Freshly hatched larvae (NOP larvae) were transferred to these twigs, and allowed to feed for 2 days.

3. Results

3.1. Performance studies

To determine if the defences of Scots pine (*P. sylvestris*) needles to feeding of the larval sawfly, *D. pini*, are affected by sawfly oviposition, comparisons were made between sawfly larvae that started their larval development on twigs which had had oviposition ("oviposition larvae", OP) and larvae that fed on twigs that had suffered no prior oviposition ("non-oviposition larvae", NOP). OP larvae performed significantly worse than NOP larvae (Fig. 1). Two day-old OP larvae gained less weight than NOP larvae (about 6 mg compared to 7 mg), and significantly fewer OP larvae (26%) survived larval development than NOP larvae (70%). Thus, larval survival from L1 to the final larval instar was reduced by over 60% when larvae started their development on egg-laden pine twigs compared to egg-free twigs. Larval developmental times and cocoon phases of OP and NOP *D. pini* did not differ. Weight of OP cocoons was lower than that of NOP cocoons. Weight of female OP cocoons averaged about 130 mg, while female NOP cocoons weighed about 142 mg. For males, weights of OP and NOP cocoons averaged 65 and 69 mg, respectively. After emergence, adult OP female weights averaged about 71 mg, while NOP females were about 14% heavier at 81 mg. OP females produced significantly less eggs than NOP females (about 100 compared to 114).

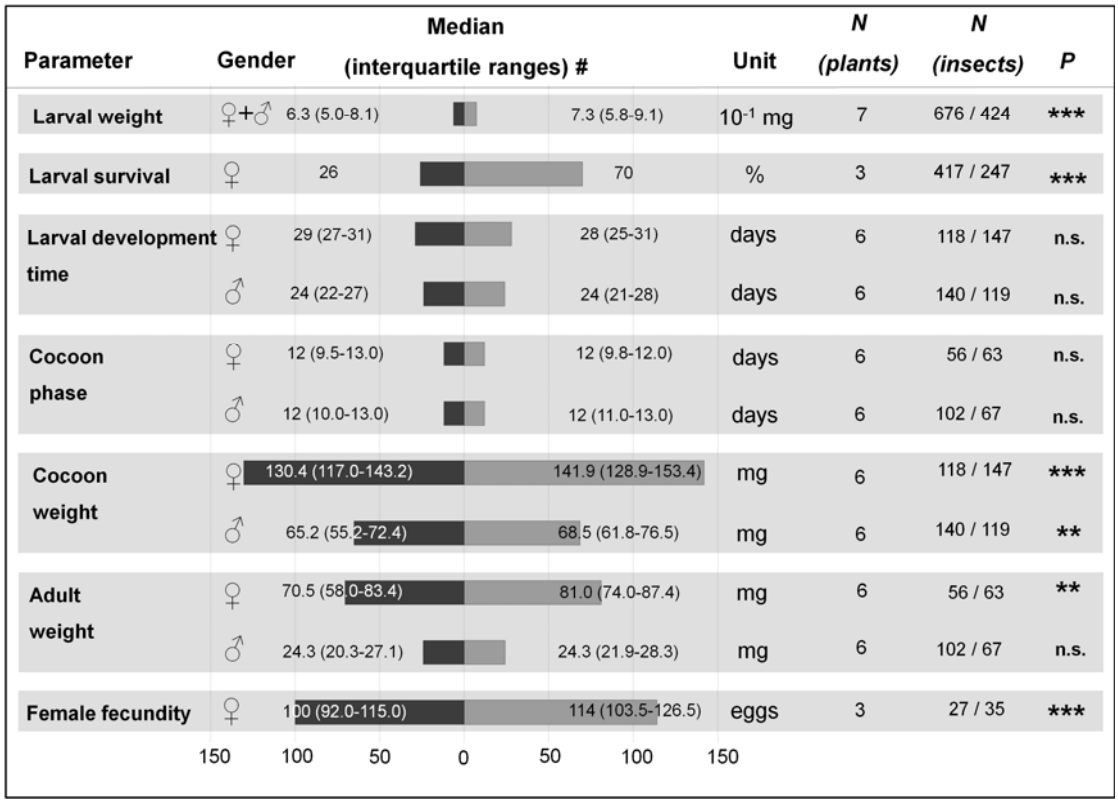

Fig. 1 Performance parameters of *Diprion pini* which started their larval development on *Pinus sylvestris* twigs which had either suffered oviposition (OP larvae, left/black) or had no oviposition (NOP larvae, right/gray). Medians were compared using the Mann-Whitney U-test. Larval survival data (onset L1 to pupation) were analysed by a χ^2-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Of the two values given for *N* (insects), those on the left are for OP and those on the right for NOP individuals. #: Medians and interquartile ranges are shown for all values except for larval survival given in % (N survival data = 100%).

3.2. Molecular analysis

The transcript levels of terpene synthase genes were studied to elucidate activation of terpene-based defences after egg deposition and feeding. Transcription of the *P. sylvestris* sesquiterpene synthases, *PsTPS*1 and *PsTPS*2, encoding an (*E*)-*β*-caryophyllene / *α*-humulene synthase and a 1(10),5-germacradiene-4-ol synthase, respectively, was significantly enhanced in twig samples of all oviposition and larval feeding treatments when compared with the untreated control (C) (Fig. 2). Increased transcription of both of these genes 3 days after egg deposition (E3) had been shown previously by Köpke *et al*. (2008). At 14 days after oviposition (E14), shortly before hatching, the transcript levels of both genes were several-fold higher than at 3 days after oviposition. But, as soon as larvae hatched from eggs and had fed for 2 days on a twig with prior eggs (E+L), transcript levels decreased significantly (*PsTPS*1), or showed a tendency to decrease (*PsTPS*2). When comparing twigs with or

without oviposition after both had experienced 2 days of larval feeding (E+L vs. L), significant differences in *PsTPS*1 but not in *PsTPS*2 transcript levels were observed.

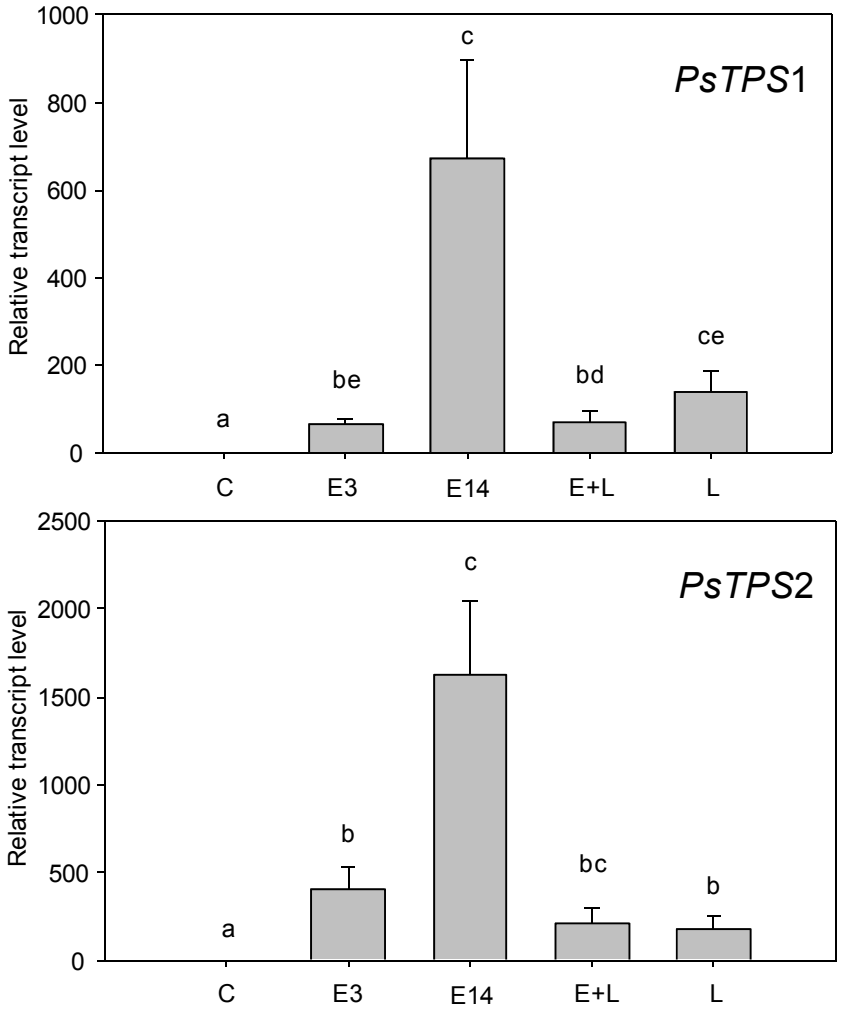

Fig. 2 Relative abundance of mRNA transcripts of *PsTPS*1 and *PsTPS*2 sesquiterpene synthases in *Pinus sylvestris* twigs after different *Diprion pini* oviposition and larval feeding treatments measured by qRT-PCR. Treatments: C, control, no treatment; E3, twigs laden with eggs of *D. pini* harvested 3 days after oviposition; E14, twigs laden with eggs harvested 14 days after oviposition, shortly before hatching; E+L, twigs on which larvae hatched from eggs and fed for 2 days; L, twigs that never carried eggs, but were fed by larvae for 2 days. Data were normalised to ubiquitin, and calibrated against values for the control. Each value shows mean + SE of 7 biological replicates with 3-5 technical replicates each. Statistical evaluation by Friedman ANOVA and Wilcoxon Wilcox as post-hoc test. Different letters above columns indicate significant ($P < 0.05$) differences between treatments according to Wilcoxon Wilcox post hoc test.

3.3. Chemical analysis

A wide range of terpene and phenolic compounds with possible roles in plant defence were analysed after the same oviposition and larval feeding treatments used for the transcript studies. The total amounts of mono- and sesquiterpenes and diterpenes did not differ significantly among treatments (Table 2). Terpenoid concentrations tended to be highest in the samples with eggs shortly before hatching (E14), where terpene synthases transcripts were highest. However, due to the high variability among replicates, no significant effects were detected. Concentrations of total phenolic compounds also did not change with respect to treatment. When considering concentrations of individual terpenoids or phenolics, no differences in concentrations were detected among samples, except for taxifolin (supplementary Table S1). Taxifolin levels decreased after egg deposition, but increased again when larvae started to feed. Neither C/N ratios nor water content differed significantly among treatments (Table 2).

Table 2 Concentrations of monoterpenes (MT), sesquiterpenes (ST), diterpenes (DT), and phenolic compounds (PC) in needles of *Pinus sylvestris* after different *Diprion pini* oviposition and feeding treatments (abbreviations: see Table 1).

Parameter measured*	**C**	**E3**	**E14**	**E+L**	**L**	***P*-Value**
Terpenoids (mg/g DW)						
Total MT & ST	17.20 (3.34)	16.88 (2.73)	17.96 (2.72)	17.92 (2.90)	17.70 (2.60)	n.s.
Total DT	0.0114 (0.0020)	0.0128 (0.0017)	0.0139 (0.0019)	0.0124 (0.0017)	0.0112 (0.0012)	n.s.
Total PC (mg/g DW)	0.776 (0.16)	0.740 (0.14)	0.760 (0.13)	0.667 (0.11)	0.845 (0.15)	n.s.
Water content (mg/100 mg FW)	56.88 (0.94)	62.42 (0.62)	63.77 (0.613)	62.09 (0.81)	59.51 (1.217)	n.s.
C/N ratio	33.58 (1.51)	33.87 (1.76)	33.16 (0.88)	35.19 (0.75)	34.24 (1.78)	n.s.

*Values are given as mean (SE) of 3 technical replicates of each of at least 7 biological samples, except for total PC and C/N measurements. Only 1 technical replicate of 7 biological replicates was analysed in case of C/N measurements. For phenolic content analyses, 1 technical replicate of 10 biological replicates was analysed. Not significant n.s. ($P > 0.05$), Friedman ANOVA. DW = dry weight; FW = fresh weight.

4. Discussion

Plants can notice egg deposition by herbivorous insects and have been suggested to take this information as a warning of future attack by hatching larvae (Hilker & Meiners 2010). This prediction was borne out in our study of pine (*P. sylvestris*) - sawfly (*D. pini*) interactions in which we found sawfly larvae performance to be worse on pine twigs where they had hatched from eggs as compared to twigs upon which larvae had been directly placed without prior oviposition. For example, the weight gain of young, 2-day-old sawfly larvae was significantly less after feeding on twigs with prior eggs than on twigs which had not suffered oviposition. This result suggests that the quality of plants where OP larvae hatched from eggs and started to feed was worse than the quality of egg-free control pine experienced by neonate NOP larvae. Such negative effects experienced by neonate OP larvae carried over to the entire period of juvenile development, even though after 2 weeks both OP and NOP larvae received egg-free twigs. Oviposition-induced plant effects on herbivore performance were found even in the next generation. Females that developed from larvae fed with needle material from egg-laden twigs during early larval development produced fewer eggs than females which spent their entire larval development on egg-free plant material.

Analysis of quality of pine material revealed no significant differences in C/N ratios and water content among pine twigs with or without *D. pini* oviposition and feeding treatments. By contrast, severe defoliation of *P. sylvestris* led to a significant change of the foliage C/N ratio (Roitto *et al.* 2003). Hence, strong damage affected this parameter, while egg deposition and a 2-day-larval-feeding period had no impact, and thus, could not have influenced performance of *D. pini* in our study. We also measured concentrations of various *P. sylvestris* terpenoids and phenolic metabolites that are thought to be anti-herbivore defences. However, concentrations of total mono- and sesquiterpenes, total diterpenes and total phenolics did not significantly change in response to *D. pini* oviposition and feeding treatment.

Nevertheless, some interesting trends were detectable in the concentrations of total mono- and sesquiterpenes and total diterpenes (Table 2). A comparison of egg-free control needles and E14 needles shows that concentrations of terpenoids tended to be higher in needles that carried eggs for 14 days. These eggs were about to hatch and thus, neonate OP larvae had to cope with the quality of these needles, whereas neonate NOP larvae faced the quality of control needles. The importance of terpenes for conifer defence is well known (Keeling & Bohlmann 2006; Gershenzon & Dudareva 2007). The performance of *D. pini* was

shown to be negatively affected by high concentrations of terpenoids, especially monoterpenes (Pasquier-Barre *et al.* 2001; Barre *et al.* 2003). High concentrations of the monoterpene 3-carene are known to impair the performance of *D. pini* (Barre *et al.* 2003). Concentrations of this monoterpene tended to be higher in E14 needles compared to the untreated control (see supplementary Table S1). These small, non-significant changes in terpenoid contents in E14 needles might have significantly impaired weight gain of neonate OP larvae by accumulation of the enhanced levels of terpenoids with every bite into a pine twig with prior eggs, whereas the chemical analysis data always just provide a snapshot in time.

In contrast to total terpenoid concentrations, total phenolic concentrations decreased in E14 needles compared to the untreated controls. Many plant phenolics are often considered as defensive compounds that deter herbivores from feeding, reduce digestibility of plant material or act as toxins (e.g. Feeny 1976; Appel 1993). However, the category of phenolics is large and heterogeneous, containing some anti-herbivore defences, but also many compounds that may play a role as anti-oxidants (Johnson & Felton 2001; Grace 2005). Moreover, the effects of plant phenolics on herbivores may depend on complex chemical interactions among the different phenolic compounds, other co-occurring plant secondary compounds as well as dietary nutrients and vitamins.

Taxifolin is a phenolic compound known to be detrimental to young larvae of *D. pini* (e.g. Auger *et al.* 1994b) and was found in our study to significantly change concentrations in response to egg deposition (lowest in E3 and E14 needles; supplementary Table S1). Neonate NOP larvae which faced the quality of control pine, took up more taxifolin than neonate OP larvae which had to cope with E14 needle quality upon hatching. When considering the previous studies showing detrimental effects of taxifolin on sawfly performance (Auger *et al.* 1994b), it seems surprising at a first glance that neonate NOP larvae showed better performance than OP larvae, in spite of the higher taxifolin levels in control needles. However, the taxifolin concentrations that were shown to impair performance of *D. pini* larvae were about 1 mg/g dry weight and more (Auger *et al.* 1994b), whereas the mean concentrations found in our needle samples ranged between 17-28 µg/g dry weight. It is unknown whether taxifolin present in such a lower range of concentrations fulfils a positive function by e.g. its antioxidative activities (Wei *et al.* 2009) rather than harming the larvae. Whether neonate NOP larvae took advantage of the enhanced levels of taxifolin (at this relatively low level of concentrations) remains to be investigated by future studies specifically

designed to address this question by e.g. determining antioxidant activities in larval midgut tissue.

The trend of having highest concentrations of terpenoids in needles laden with eggs that were about ready to hatch (E14) was supported by differences in terpene synthase transcripts. The highest levels of *PsTPS*1 and *PsTPS*2 transcripts were also found in the E14 treatments (Fig. 2). The amounts of products of these synthases, (*E*)-*β*-caryophyllene / *α*-humulene (*PsTPS*1) and 1(10),5-germacradiene-4-ol (*PsTPS*2), tended to increase 14 days after egg deposition (supplementary Table S1). In other treatments, including twigs harvested 3 days after oviposition (E3), harvested after oviposition plus larval feeding (E+L), or harvested after larval feeding without oviposition (L), transcript levels were lower, but still greater than in the untreated controls. This result is consistent with other studies showing that transcript levels of genes coding for terpene synthases are higher in tissue damaged by herbivore feeding than in undamaged controls (Arimura *et al.* 2004; Huber & Bohlmann 2004; Byun-McKay *et al.* 2006; Schnee *et al.* 2006). However, when neonate *D. pini* larvae start to damage pine tissue at the site where they hatch, transcription of *PsTPS*1 and *PsTPS*1 decreases from the high level reached after egg deposition to a lower level. Since initiation of larval feeding at the site where eggs have been laid reflects the natural situation, in nature the plant is lowering transcription of these genes in response to the onset of larval feeding rather than enhancing it. In contrast, an increase of transcript levels *PsTPS*1 and *PsTPS*1 is obvious when comparing untreated control pine twigs with those exposed to neonate feeding damage, but without prior eggs. However, this does not reflect the natural situation, since neonates do not start feeding on pine twigs without prior eggs in nature. Hence, we suggest to investigate and interpret transcript levels of defence genes in response to larval feeding with respect to the fact that in nature prior egg deposition may already have affected transcription of these genes.

In summary, our results demonstrate that performance of an herbivorous insect depends on whether larvae start to feed at the site where they hatch from eggs or at an egg-free site. Feeding at a site with prior eggs resulted in worse performance. To our knowledge, detrimental effects of egg deposition on feeding herbivores have not been shown before. In a recent study, Bruessow *et al.* (2010) studied how larvae perform on leaves of *Arabidopsis thaliana* treated with extracts of crushed eggs. While larval performance of *Pieris brassicae* was not affected by this treatment, performance of larvae of the generalist *Spodoptera littoralis* improved significantly. The authors concluded that egg deposition on a plant suppresses defence against generalist larvae. Future studies need to elucidate whether the

effects of egg deposition on larval performance differ from plant – herbivore system to system and whether extracts of crushed eggs elicit the same effects on larval performance as natural egg deposition does. For the *P. sylvestris – D. pini* system, our results indicate that deposition of herbivore eggs on a plant can indeed act as a warning leading to an increased defensive posture against the feeding larvae that hatch from those eggs. Egg deposition was shown to significantly affect transcription of terpene synthases especially shortly prior larval hatching; furthermore our data suggest that egg deposition induces finely tuned, small phytochemical changes which might sum up in larvae during feeding and thus affect performance. To designate these phytochemical changes as arising from defensive priming might be not justified by our data. Nevertheless, as long as egg laying triggers declines in larval growth, weight and survival and adult fecundity, it can certainly be said to fulfil the goal of priming by getting the plant "ready for battle" against feeding herbivores (Conrath *et al.* 2006; Frost *et al.* 2008).

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Supplementary data (page 112)

Table S1. Concentrations (µg/g dry weight) of individual mono-, sesqui- and diterpenes and phenolic compounds in needles of *Pinus sylvestris* after different *Diprion pini* oviposition and larval feeding treatments.

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Chapter 5

Ecological roles of conifer sesquiterpenes

Abstract

During their long lives, conifers have to withstand damage from a wide range of organisms, among the most serious being herbivorous insects and fungal pathogens. They have developed a distinct chemical defense shield, which consists mainly of terpenes that represent the largest and predominant group that inhere many different ecological functions. Members of the terpenoid subclasses like monoterpenes (10-carbon) and sesquiterpenes (15-carbon) are characterized by their volatile nature, whereas diterpenes (20-carbon) exhibit sticky, glue-like properties in the resin. In this review we highlight the biosynthesis and the characteristic traits (as motifs) of specific sesquiterpenes being produced by recently characterized sesquiterpene synthases of *Pinus sylvestris* (*Ps*TPS1, *Ps*TPS2, *Ps*TPS3, *Ps*TPS5). Furthermore we discuss their transcriptional kinetics and regulation in response to different types of herbivore attack and their role in ecological interactions, all with respect to already known sesquiterpene synthases from other species and their corresponding products.

Keywords Gymnosperms, Conifers, *Pinus sylvestris*, Plant defense, Sesquiterpenes, Terpenoid biosynthesis, *Ps*TPS1, *Ps*TPS2, *Ps*TPS3, *Ps*TPS5

Introduction

The majority of species in the plant kingdom belong to the angiosperms with over 240,000 known species. Compared to them gymnosperms are a small group with less than 1,000 species being distributed in four clades: cycads (300 sp.), ginkgos (1 sp.), gnetales (ca. 66-76 sp.), and conifers (414 sp.) (Bresinsky *et al.* 2008). However, the importance of gymnosperms, both economically and ecologically, is far greater than their limited species number would suggest. Moreover, gymnosperms are the ancestors of the angiosperms. The earliest gymnosperm records extend back to the late Carboniferous Period 300 million years ago. When researchers discuss the great age of gymnosperms, this is usually followed by a list of morphological features, such as their mostly evergreen needle-like shaped leaves that are often covered by a thick cuticula, and their often monoecious flowers that have been preserved over the long history of this taxon. Much less attention is paid to the chemical features, such as the defensive terpenes of conifer resin and their evolutionary history.

Among gymnosperms, the largest species group are conifers whose members are quite common and wide spread from arctic and alpine timber lines to tropical forests (Farjon 2003). All living conifers are woody plants and most are long lived trees. Conifers even hold the records for the oldest, tallest and biggest trees growing on the earth today. This indicates that despite their low species diversity, conifers must have effective defenses against enemies which have allowed them to survive the last 300 million years.

Since a tree cannot run away from its enemies, it has to fight off or tolerate attacks. This is especially a problem for evergreens like conifers since many organs, such as stems, needles, or roots are present all year around. Enemies of conifers can be found in many different biological forms. Besides mammals, birds, nematodes, bacteria, or viruses, more harmful foes include hundreds of fungi and insect species which decimate many hectares of conifer forest each year.

Conifer attackers have adapted to the different tissues and organs of their hosts to varying degrees. Some generalist herbivores, like deer or birds, feed on numerous other species and have few specific adaptations to conifers (Hohf *et al*. 1987; Danell *et al*. 1990). However, other specialist herbivores have developed a very close and specific relationship with conifers, such as the phloem feeding bark beetle with *Picea* species or the defoliating sawflies with *Pinus* species. Bark beetles are attracted to host trees by the smell of certain terpenes (Erbilgin *et al*. 2007a) and can tolerate a relatively large amount of resin components produced and released by the tree. Indeed, it seems as if bark beetles are able to turn the tree defense into their own advantage since aggregation pheromones of some species probably originated as detoxification products of host monoterpenes. In fact, many terpenes found in insects are similar to plant products which they obtained from their diet. Those terpenes either remain

unchanged or get modified, such as the iridoid glycosides (monoterpene derivates) and cardenolides (triterpenes that lost one or more carbon) stored by certain lepidopteran insects (Nishida 2002).

Plants, on the other hand developed their own adaptations to certain insects, besides repellent or feeding deterrent properties of their terpenes. For some plant species it has been shown many years ago that they produce terpenes that have juvenile hormone activity. Sláma and Williams found already in 1965 and 1966 that the conifer *Abies balsamea* produces a sesquiterpene named juvabione, identified by Bowers *et al*. (1966) that showed to have juvenile hormone activity for the bug *Pyrrhocoris apterus* which loses the ability to develop into adults and grows into supernumerary larvae after feeding.

Sawfly species like *Diprion pini* and *Neodiprion sertifer* defoliate pine forests in Scandinavian countries and have inflicted significant economic losses in the last years (Lyytikäinen-Saarenmaa 2002; Gedminas 2003). However their performance does not seem to be battered by the many different terpenes the tree produces.

Sawflies probably originated back in the Jurassic, 200 million years ago, a period dominated by gymnosperm flora, in particular by conifers (Malyshev 1959; Knerer & Atwood 1973). This might explain why this insect group can not only withstand the high resin content, but species like *N. sertifer* or *D. pini* are even able to use the resin acids for their own defense. Larvae fed needles high in resin acids produced 50% larger defense droplets (consisting mostly of resin acids) than larvae fed needles low in resin acids (Bjorkman & Larsson 1991) and so are better defended against ants and other enemies. However, accumulating high concentration of needle resin fluids also has a negative impact on the sawflies; it can reduce their growth as was demonstrated for *D. pini* (Bjorkman *et al*. 1997). This shows that even specialists that have adapted to handle the chemical defense of conifers may pay a price in reduced development when host plant defense is at high levels.

However, not only the insect can adapt to its host plant. Recent studies have shown that the host plant *Pinus sylvestris* is able to detect eggs of its enemy *D. pini*, which results in a quantitative change of volatile emission, which then attracts an egg parasitoid (*Closterocerus ruforum*), killing the herbivorous larvae (Hilker *et al.* 2002a). Headspace analyses of the emitted volatile of *D. pini* oviposition induced pine twigs have revealed that only one component - the sesquiterpene (*E*)-*β*-farnesene is emitted in higher amounts (Mumm *et al*. 2003). However, in choice tests Mumm *et al*. (2005) have shown that (*E*)-*β*-farnesene offered alone does not attract the egg parasitoid, but is does when (*E*)-*β*-farnesene was offered with the background odor of an uninfested pine twig, indicating the exigency of other volatile cues for the host finding. Previously, Beyaert and coworkers (2010) successfully identified the missing volatile cues as there are: two monoterpenes, *β*-phellandrene and (*E*/*Z*)-*β*-ocimene and two additional sesquiterpenes, (*E*)-*β*-caryophyllene and *α*-humulene. When mixed according

to ratios found in noninduced pine twigs with the respective amount of (*E*)-*β*-farnesene released by oviposition induced twigs, the odor bouquet was found to be attractive for the egg parasitoid. In our recent studies, we identified four new sesquiterpene synthases, *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 and *Ps*TPS5 that were shown to produce (*E*)-*β*-caryophyllene and *α*-humulene (*PsTPS*1), 1(10),5-germacradiene-4-ol (*PsTPS*2), longifolene and *α*-longipinene (*PsTPS*3), and (*E*)-*β*-farnesene (*PsTPS*5) as their principal products (Köpke *et al*. 2008, 2010) after heterologous expression in *Escherichia coli*.

This review focuses on this one group of conifer chemical defenses, the sesquiterpenes, and the accordant enzymes that form them. Emphasis is placed on the sesquiterpenes and sesquiterpene synthases *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 and *Ps*TPS5 that were recently isolated and identified to analyze their role in direct and indirect defenses of the Pine tree.

Terpenoids

Terpenes are the largest and most diverse class of organic compounds found in plants (Connolly & Hill 1991). They are sometimes referred to as terpenoids or isoprenoids when they have gone through chemical modifications like oxidation or rearrangement of the carbon skeleton. Terpenoids show an enormous chemical diversity but embrace a single biosynthetic origin, the fusion of five-carbon units. Nearly 15,000 terpenoids are known in plants and thousands more are waiting to be discovered (Gershenzon 1991). Their huge diversity explains their many functions within the plant and also partially in animals (Fig. 1). Although terpenes include a range of different functional groups, polarities and molecular masses, the conifer sesquiterpenes we shall discuss are non-polar hydrocarbons of low molecular weight (C-15) and hence are significantly volatile at normal biosphere temperatures.

Terpene Biosynthesis

In plants two different pathways synthesize the main starting products for terpenoid biosynthesis, isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Fig. 2). One is the recently discovered methylerythritolphosphate (MEP) pathway, forming IPP and DMAPP in the chloroplast, and the second is the mevalonic acid (MVA) pathway that produces IPP in the cytosol. The starting products formed in the chloroplasts by the MEP pathway are principally substrates for the formation of monoterpenes (C-10), diterpenes (C-20) and carotenoids (C-40), along with gibberellins, abscisic acid, isoprene and side chains of photosynthetic components like chlorophyll (C-20) and plastoquinones (C-45). The MVA

pathway on the other hand provides IPP for the biosynthesis of sesquiterpenes (C-15) and triterpenes (C-30) along with brassinosteroids, sterols, and polyterpenes.

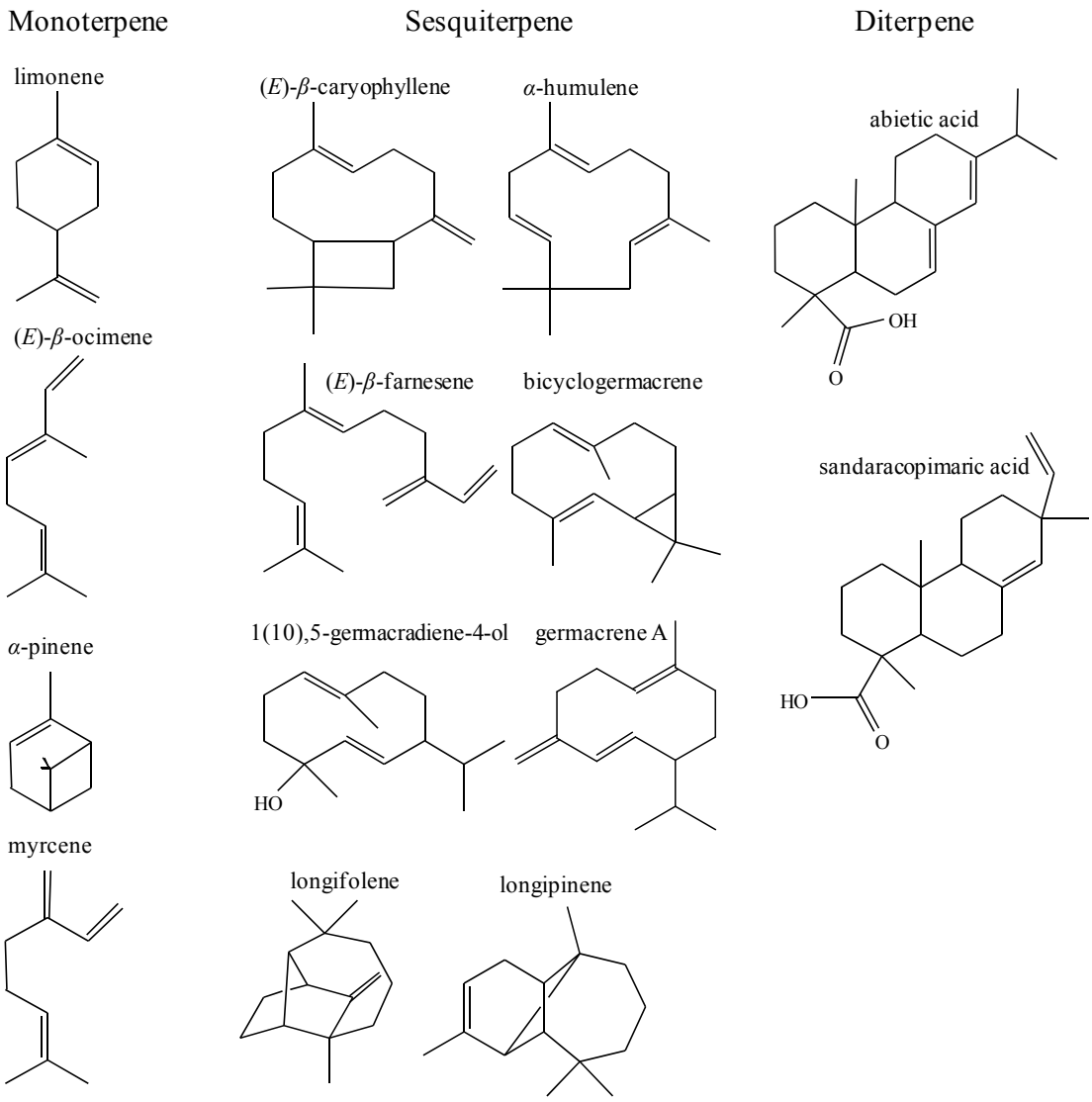

Fig. 1 Examples of mono-, sesqui- and diterpenes produced by pine.

Synthesis of all higher terpenoids proceeds from IPP (C-5) and DMAPP (C-5) *via* formation of geranyl pyrophosphate (GPP, C-10), farnesyl pyrophosphate (FPP, C-15), and geranylgeranyl pyrophosphate (GGPP, C-20). Although the two pathways, MEP and MVA, are mutually exclusive in most organisms, interactions between them have been reported so far in plants and a few bacteria species. It is believed that the MEP pathway is prevalent in the biosynthesis of mono- and diterpenes (Lange & Ghassemian 2003) which are the major components of conifer oleoresin. However, in this review the main focus is on the biosynthesis of sesquiterpenes which occurs *via* the MVA pathway in the cytosol.

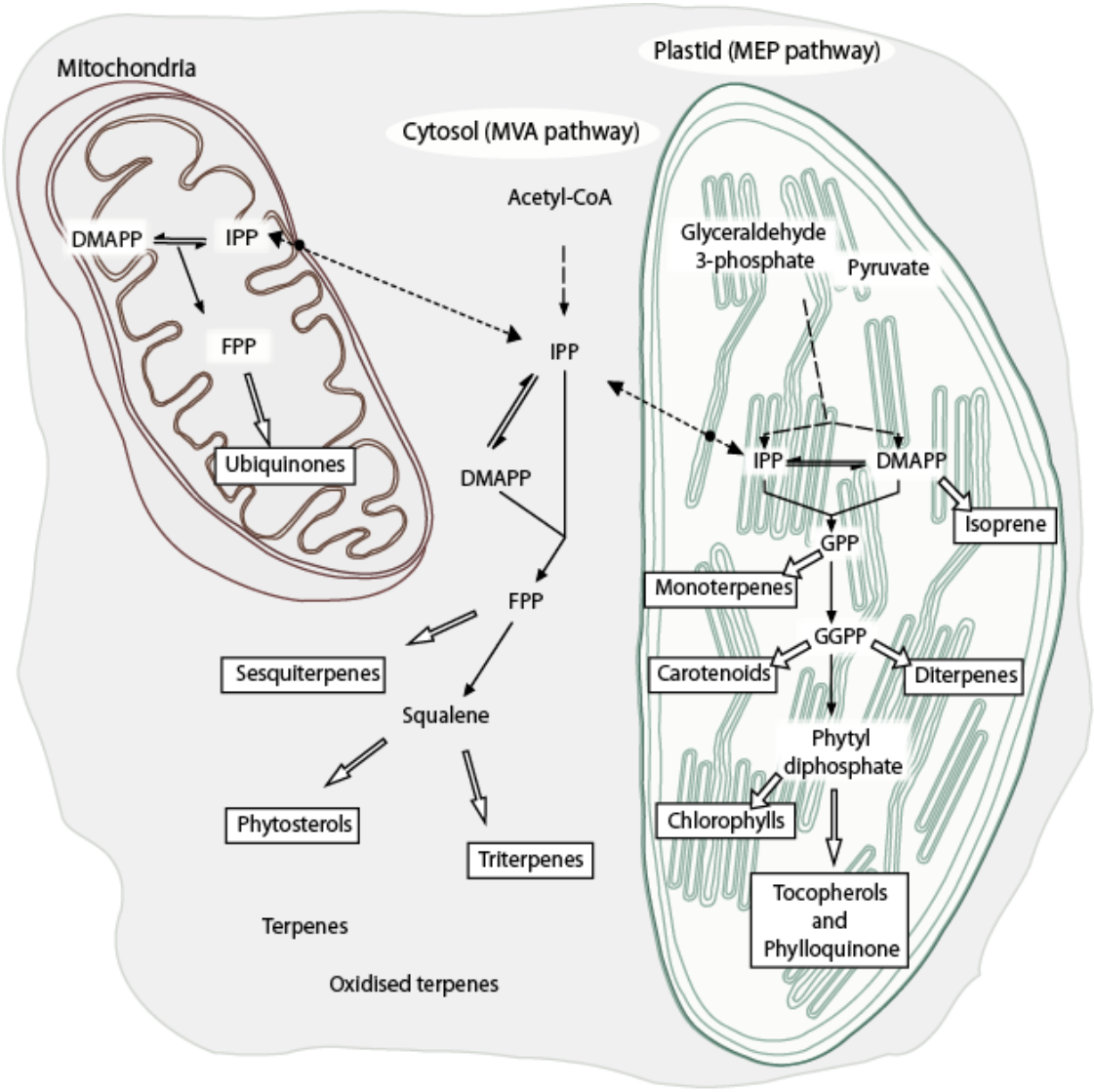

Fig. 2. Scheme of terpenoid biosynthesis in plants via the MEP and MVA pathways.

Sesquiterpene synthases

Sesquiterpene synthases use FPP (C-15) as substrate in a metal ion cofactor-dependent reaction (mainly Mg^{2+}) to form a wide range of different sesquiterpene products. The extensive sesquiterpene diversity arises not only due to the number of different sesquiterpene synthases, but also from the ability of these enzymes to form multiple products from a single substrate. Apart from the main product, nearly half of all characterized sesquiterpene synthases form significant amounts of side products when the expressed protein is assayed *in vitro* (Degenhardt *et al*. 2009). The formation of multiple products is attributed to the mechanism of the enzyme which forms reactive carbocation intermediates that can be converted to more than one cyclic or acyclic product (Zulak *et al*. 2009). Sesquiterpenes

synthases are characterized not only by their 15-carbon products but also by the occurrence of several motifs in their amino acid sequence, like the aspartate-rich DDxxD motifs involved in coordinating the bivalent metal ion cofactor for substrate binding (Bohlmann *et al*. 1999), the RR(x8)W motif which might be important for catalysis (Pechous & Whitaker 2004) and the quite common RxR motifs involved in the complexation of the diphosphate function after ionization of the substrate (Starks *et al*. 1997).

Often it is convenient to predict the biochemical function of an enzyme based on a high amino acid similarity to another characterized enzyme. However, studies with terpene synthases have shown that a few changes in the amino acid sequence can lead to a completely different product profile (Dudareva *et al*. 2003; Keeling *et al*. 2008). Köllner *et al.* (2009) identified a single amino acid in the active centre of a terpene synthase which determines the ratio of the sesquiterpenes (*E*)-*α*-bergamotene to (*E*)-*β*-farnesene, and which has changed during the evolution of maize and teosinte species. Another study showed that enzymes with the amino acid structure of a typical sesquiterpene synthase can biosynthesize monoterpenes and *vice versa* if supplied with the appropriate substrates (Köllner *et al*. 2004). Directed mutagenesis of single amino acids in terpene synthases demonstrated that alterations of the DDxxD motif dramatically altered both the kinetics and product specificity of the enzymes (Rising *et al*. 2000; Little & Croteau 2002). The nature of the divalent metal ion (Mg^{2+} vs. Mn^{2+}) can also affect terpene synthase product profiles. For example, most sesquiterpene synthases employ Mg^{2+}, but can also accept specific monoterpene metal ion cofactors like Mn^{2+} to biosynthesize sesquiterpenes although enzyme activity is mostly lower (Köllner *et al*. 2004). Some sesquiterpene synthases are also able to use GPP as substrate to form monoterpenes (Colby *et al*. 1998; Mercke *et al*. 1999; Cai *et al*. 2002; Köllner *et al*. 2004; Picaud *et al*. 2005). However, enzyme characterization has usually been carried out by heterologous expression in *Escherichia coli*; thus, most measurements were performed only *in vitro*. *In planta*, these sesquiterpene synthases probably prefer Mg^{2+} as cofactor and FPP as substrate. The sesquiterpene synthases we isolated from *P. sylvestris* exclusively used FPP as substrate. As cofactor we used Mg^{2+} in our assays since many studies have shown that Mg^{2+} is preferred by sesquiterpene synthese over Mn^{2+} which can act at a certain concentration even inhibitory (Davis & Croteau 2000). Concerning the mono- and also sesquiterpene characteristics in the amino acid sequence all four sesquiterpene synthases we identified show the specific RxR, DDxxD motives.

The kinetics of expression of terpene synthases in response to herbivory and the specificity of responses

Studies on plant defense often analyze the impact of an herbivore by comparing feeding to artificial wounding. Wounding by insect feeding or artificial damage have been intensively examined especially with respect to terpenoid biosynthesis in conifer species like Grand fir (*Abies grandis*) (Funk *et al*. 1994; Steele *et al*. 1995; Bohlmann *et al*. 1998a; Keeling & Bohlmann 2006), Sitka spruce (*Picea sitchensis*) (McKay *et al*. 2003; Miller *et al*. 2005), Douglas fir (*Pseudotsuga menziesii*) (Hudgins *et al*. 2003), and various pine (*Pinus*) species (Litvak & Monson 1998; Huber *et al*. 2004; Luchi *et al*. 2005). Wounding in general has shown to increase the activity of mono-, sesqui- and diterpene biosynthesis in conifers (Martin *et al*. 2002). Studies comparing insect feeding with artificial wounding often show that there are differences in plant response due to certain elicitors present in the insect saliva (Mattiacci *et al*. 1994; Alborn *et al*. 1997; Gomez *et al*. 2005; De Vos & Jander 2009). Scientists working in this field have long been interested in how long it takes for wounding to trigger terpenoid accumulation and addressed the mechanisms behind preparing the plant to fight against the attacker. Is the plant´s change of terpenoids in response to herbivory controlled at the level of gene transcript induction, protein formation or enzyme activity?

Steele and coworkers (1998a,b) were one of the first groups to show that monoterpene synthase genes of Grand fir were induced very early after mechanical wounding (4 h), whereas transcription of sesqui- and diterpene synthases genes did not begin until the 3rd day after wounding and reached a maximum 5 to 12 days after wounding. In comparison, the sesquiterpene synthase genes *PsTPS*1 and *PsTPS*2 in *P. sylvestris* showed significantly enhanced transcript levels 3 days after sawfly egg deposition (which includes ovipositional wounding), decreased by the 4th day (Köpke *et al*. 2008, 2010), but increased again 14 days after egg deposition, i.e. shortly before larvae hatch from eggs (unpublished data).

Steele and coworkers also showed that the individual terpene synthases of Grand fir did not respond to wounding in the same intensity or timing. We found similar results for *P. sylvestris* in response to egg deposition: While transcription of *PsTPS*1 and *PsTPS*2 was significantly affected 3 days after sawfly egg deposition, *PsTPS*3 transcript levels did not change in response to sawfly eggs. Furthermore, our studies showed that transcriptional changes of *PsTPS*1 and *PsTPS*2 were dependent on the sawfly species laying eggs (Köpke *et al*. 2010). Transcript levels of both genes were up regulated after the 3rd day after oviposition by *N. sertifer*, a close relative to *D. pini*. On the same day the plant material was found to be attractive for the parasitoid as was shown after *D. pini* oviposition. Testing the response of the parasitoid to oviposition by *G. pallida*, also a close relative of *D. pini*, at no tested time point an attraction of the parasitoid could be detected. These performance data are also supported by the transcritption data of *PsTPS*1 and *PsTPS*2 that show no significant change.

In general, the present studies show that terpene synthase responses to attack by herbivores may vary with respect to the plant species, the attacking insect species, the attacked plant tissue (bark, needles) and the type of attack (wounding, oviposition).

Jasmonic acid and the induction of sesquiterpenes

Jasmonic acid (JA) is a member of the jasmonate class of plant hormones. Its major function in regulating plant growth includes growth inhibition, senescence and leaf abscission. Jasmonic acid is also converted to a variety of derivatives including esters such as methyl jasmonate (MeJA) which can have a similar impact within the plant. It also has an important role in response in wounding response and systemic acquired resistance, resulting in an induction of many genes during plant defense by JA (Xu *et al*. 1994). Several studies show that JA and also MeJA influence the biosynthesis of terpenes in Gymnosperms, terpenes that are known to act against herbivores. Therefore many studies use JA and also MeJA to mimick natural tree defense but with the advantage of an easier and controlled application.

Both Martin *et al.* (2002) and Fäldt *et al.* (2003) have shown that MeJA induces the transient transcript accumulation of terpene snythases genes resulting in an increased biosynthesis of terpenoid resin and terpenoid accumulation in Norway spruce (*Picea abies*). Martin *et al*. (2002) also revealed that MeJA induces the formation of traumatic resin ducts (diterpene, monoterpene, sesquiterpenes) in wood of treated Norway spruce, which is normally the case after insect attack, fungal elicitation, and mechanical wounding. They specifically showed that MeJA treatment caused a 2-fold increase in monoterpene and sesquiterpene accumulation in needles without changes in terpene composition (Martin *et al*. 2003). At the same time, MeJA treatment triggered a 5-fold increase in total terpene emission from foliage. However they found a shift in composition to a blend dominated by oxygenated monoterpenes (e.g. linalool) and sesquiterpenes (e.g. (*E*)-*β*-farnesene) but no qualitative changes. These results agree with data collected by Mumm *et al*. (2003) that also showed a quantitative change of blend emitted by *P. sylvestris* after JA treatment. Furthermore, Mumm and coworkers demonstrated that the volatile blend from pine twigs treated with jasmonic acid (JA) also attracts the egg parasitoid *C. ruforum* (tritrophic system introduced earlier in this review), which is known being attracted to twigs that have been oviposition-induced (3d) by the herbivore *D. pini*. Both JA-treated and oviposition-induced pine twigs release significantly higher amounts of (*E*)-*β*-farnesene then the control twigs, which is attractive for the egg parasitoid. In another tritrophic system consisting of the angiosperm tree species *Ulmus minor*, the elm beetle *Xanthogaleruca luteola* and its corresponding parasitoid, it has been shown that the JA-treatment also induces the release of a parasitoid attractive odor. However, in contrast to the

Pine system, Wegner and coworkers (2001) found here both a qualitative and quantitative change in the emission of volatiles. In another interesting study by Erbilgin *et al.* (2006) it was tested whether terpene and phenol constituents exhibit a defensive role against bark beetles infestation. They treated Norway spruce with MeJA and found an increased accumulation of terpenes but not of phenolic compounds. Performance tests showed that bark sections treated with MeJA had significantly less bark beetle colonization than the untreated sections. They also exhibited shorter parental galleries and that fewer eggs had been deposited. The beetle number and the weight of emerged ones were also significantly lower in MeJA-treated bark. The author concluded that the increased amount of terpenoid resin present in MeJA-treated bark could be directly responsible for the observed decrease in bark beetle colonization and reproduction.

The ecological function of the sesquiterpenoid products of the identified pine sesquiterpene synthases in plant- insect and plant- pathogen interactions

As was indicated earlier in *Pinus sylvestris* (*E*)-*β*-farnesene (*Ps*TPS5), (*E*)-*β*-caryophyllene and *α*-humulene (*Ps*TPS1) were shown to be key components essential for the attraction of the egg parasoid *C. ruforum* (Beyaert *et al*. 2010). *PsTPS*1 showed significantly enhanced transcript levels at the time point when the plant material was attractive for the egg parasitoid. *PsTPS*2, producing germacrene derivatives, was found to be induced by sawfly egg deposition. In case of *PsTPS*3 there is no evidence for its function within the plant- parasitoid attraction, even though we will address in the following the possible ecological role of its sesquiterpenoid products as well of the other above mentioned sesquiterpenes. These sesquiterpenes are not exclusive in Gymnoperms; they have been also identified in Angiosperms. So, in this section we additionally include known functions of *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 and *Ps*TPS5 products in Angiosperms - insect (pathogen) interaction.

- (*E*)-*β*-farnesene (product of *Ps*TPS5)

(*E*)-*β*-farnesene occurs in a wide range of both plant and animal taxa and has a wide variety of biological activities. This acyclic sesquiterpene hydrocarbon has been frequently studied by ecologists and plant biochemists. (*E*)-*β*-farnesene synthases have been isolated from peppermint (*Mentha piperita*) (Crock *et al*. 1997), *Citrus junos* (Maruyama *et al*. 2001), *Zea mays* (Schnee *et al*. 2002), *Pseudotsuga menziesii* (Huber *et al*. 2005), and *Pinus sylvestris* (Köpke *et al*. 2010).

Jasmonic acid is involved in mediating enhanced emission of (*E*)-*β*-farnesene from plants after wounding and herbivore attack. For example, Norway spruce responds to

treatment with methyl jasmonate (MeJA) by formation of traumatic resin ducts and terpenoid accumulation in the stem; MeJA treatment of foliage results in increased terpenoid accumulation plus changes in volatile terpenoid emission (Martin *et al*. 2002). In particular, the emission of linalool and (*E*)-*β*-farnesene increases dramatically (Martin *et al*. 2002). Another example is provided a study on Scots pine which enhances the emission of (*E*)-*β*-farnesene after treatment with jasmonic acid (Mumm *et al*. 2003).

(*E*)-*β*-farnesene is known to function as pheromone, allomone, kairomone, and synomone (Mondor & Roitberg 2004; Gibson & Pickett 1983; Verheggen *et al*. 2009; Valterova *et al*. 2007). In many aphid species, (*E*)-*β*-farnesene functions as alarm pheromone in aphid colonies; the pheromone is released by the aphids upon disturbance and elicits defensive or escape responses in neighboring conspecifics (Montgomery & Nault 1977; Dawson *et al*. 1982; Xiangyu *et al*. 2002; Mondor & Roitberg 2004). The potato plant *Solanum berthaltii* produces (*E*)-*β*-farnesene which here serves as an allomone and repels herbivores like the aphid *Myzus persicae* (Gibson & Pickett 1983). Predators of aphids use (*E*)-*β*-farnesene as a foraging cue (kairomone) to find their prey (Verheggen *et al*. 2009). A synomone function of (*E*)-*β*-farnesene is known in several plants. In pine, (*E*)-*β*-farnesene is used as synomone to attract egg parasitoids after sawfly egg deposition (Mumm & Hilker 2005); the sesquiterpene is released in significantly enhanced amounts 3 days after egg deposition (Mumm *et al*. 2003). Furthermore, maize plants attacked by lepidopteran larvae use (*E*)-*β*-farnesene to attract larval parasitoids; the plants emit a volatile mixture that consists mostly of (*E*)-*α*-bergamotene and (*E*)-*β*-farnesene to attract natural enemies to the damaged plants (Schnee *et al*. 2006). A synomone function of (*E*)-*β*-farnesene is also known for interactions between *Orchis pauciflora* and pollinators (Valterova *et al*. 2007). The floral fragrance of this orchid is dominated by the sesquiterpene (*E*)-*β*-farnesene and includes other sesqui- and diterpenes that are frequent constituents of male marking pheromones of many bumble bee species. In field experiments, *O. pauciflora* inflorescences enriched with the main floral compound (*E*)-*β*-farnesene led to significantly increased pollinia export due to the enhanced attraction of pollinating visitors and led thus to increased fitness (Valterova *et al*. 2007).

Among conifers, many produce large amounts of mono- and sesquiterpenes including (*E*)-*β*-farnesene. However, not much is known about the function of (*E*)-*β*-farnesene in conifer - insect interactions besides the tritrophic system investigated in this thesis. This sesquiterpene is also a component of resin in small proportions, but does not seem to affect the physiological or chemical properties of resin. However, it does have ecological effects when released as part of the general tree bouquet. For example, feeding of the weevil *Hylobius abietis* L induces the emission of monoterpenes and sesquiterpenes, particularly linalool and (*E*)-*β*-farnesene, from branch tips (Blande *et al*. 2009) and these may serve to attract enemies of other weevils.

- (*E*)-*β*-caryophyllene (product of *Ps*TPS1)

(*E*)-*β*-caryophyllene is a bicyclic sesquiterpene hydrocarbon and a major plant volatile found in large amounts in the essential oils of many different spice and food plants, such as oregano (*Origanum vulgare*) (Mockute *et al*. 2001), cinnamon (*Cinnamomum* spp.) (Jayaprakasha *et al*. 2003), and black pepper (*Piper nigrum*) (Orav *et al*. 2004). (*E*)-*β*-Caryophyllene synthase coding genes have been isolated from several angiosperms, e.g. *Zea mays* (Köllner *et al*. 2008), *Oryza sativa* (Cheng *et al*. 2007), and *Artemisia annua* (Cai *et al*. 2002), whereas in gymnosperms only one enzyme is known, that from Scots pine, *P. sylvestris* (Köpke *et al*. 2008).

The ecological functions of (*E*)-*β*-caryophyllene cover a broad spectrum. It is commonly produced by many flowering plant species which use (*E*)-*β*-caryophyllene as an attractant for pollinators. However there are plants that do not need pollinators and reproduce by selfing like *Arabidopsis thaliana*. Interestingly *A. thaliana* flowers also emit (*E*)-*β*-caryophyllene (40% of total floral emission). So one can ask why *A. thaliana* produces (*E*)-*β*-caryophyllene in such high concentration. Huang *et al*. (unpublished data) found that here the function of (*E*)-*β*-caryophyllene is to protect the reproductive parts of the plant from pathogen infection since flowers have a high risk of pathogen attacks due to the presence of nutritive tissue. They could show that knock-out mutants unable to produce (*E*)-*β*-caryophyllene suffered more bacterial infection and produced lighter seeds, whereas plant lines with enhanced (*E*)-*β*-caryophyllene emission showed increased resistance to pathogen infection and increased seed production.

Another study demonstrating the antibacterial properties of (*E*)-*β*-caryophyllene was carried out by Juliao *et al*. (2009) who showed that the the essential oil from the leaves of two Brazilian plant species, *Lantana trifolia* and *L. fucata*, contain mainly sesquiterpenes like germacrene D, (*E*)-*β*-caryophyllene, bicyclogermacrene, and *α*-humulene. The oil of both species exhibited *in vitro* activity against *Mycobacterium tuberculosis*. Similar results were found by Sabulal *et al*. (2006, 2008) testing the oil of the two plant species *Zingiber nimmonii* and *Scheflera stellata* for antifungal and antibacterial activity. Both plant species produce an oil containing (*E*)-*β*-caryophyllene as the main constituent. The oil of *Z. nimmonii* showed significant inhibitory activity against several tested fungi, whereas that of *S. stellata* was only active against bacteria, not against fungi.

Kairomonal effects of (*E*)-*β*-caryophyllene have been shown in studies analyzing host plant location by the emerald ash borer *Agrilus planipennis*. Volatile analysis of stressed bark tissue of the ash tree *Fraxinus pennsylvanica* showed elevated levels of sesquiterpenes, among them (*E*)-*β*-caryophyllene and *α*-humulene. Both sesquiterpenes consistently elicited antennal responses by the beetle *A. planipennis*. Crook *et al*. (2008) monitored capture of adult *A. planipennis* in traps baited with several combinations of ash tree volatiles. Treatments

included two natural oil distillates that were found to contain high concentrations of (*E*)-*β*-caryophyllene and *α*-humulene. Oil-baited traps caught significantly more adult beetles than unbaited traps (Crook *et al*. 2008).

Synomonal effects of (*E*)-*β*-caryophyllene have been detected in several plants. Maize (*Zea mays*) plants emit (*E*)-*β*-caryophyllene from leaves in response to aboveground attack by lepidopteran larvae like *Spodoptera littoralis*; furthermore, roots of maize plants emit this sesquiterpene after damage by larvae of the coleopteran *Diabrotica virgifera virgifera*. Köllner *et al*. (2008) demonstrated that (*E*)-*β*-caryophyllene can attract natural enemies of both herbivores: entomopathogenic nematodes belowground (Rasmann *et al*. 2005) and parasitic wasps aboveground. Interestingly, the expression of TPS23 coding for the (*E*)-*β*-caryophyllene synthase is controlled at the transcript level and is induced independently by *D. v. virgifera* damage in roots and *S. littoralis* damage in leaves. Also, bean plants (*Vicia faba* and *Phaseolus vulgaris*) were shown to release enhanced amounts of (*E*)-*β*-caryophyllene after feeding damage and egg deposition by the bug *Trissolcus basalis.* Volatiles of feeding-damaged and egg-laden bean plants attract egg parasitoids killing the eggs (Colazza *et al*. 2004b). Another example for synomonal effects of (*E*)-*β*-caryophyllene is provided by a study of *Trifolium pratense* (red clover) which showed increased emission rates of (*E*)-*β*-caryophyllene after herbivory by *S. littoralis* caterpillars (Kigathi *et al*. 2009). Some years before Bruce *et al*. (2005) suggest that some of the emitted compounds of *T. pratense*, including (*E*)-*β*-caryophyllene, may be useful in attracting herbivore enemies.

- *α*-humulene (product of *Ps*TPS1)

α-Humulene also known as *α*-caryophyllene, is a monocyclic sesquiterpene occurring in the essential oils of e.g. *Humulus lupulus* (hops), *Cannabis sativa* and *Lindera strychnifolia*. It is an isomer of (*E*)-*β*-caryophyllene, and both are often found together as a mixture in nature, probably due to the fact that (*E*)-*β*-caryophyllene-producing sesquiterpene synthases also produce *α*-humulene (Cai *et al*. 2002; Köpke *et al*. 2008; Abel *et al*. 2009) or both are part of the minor product range of an multi-product enzyme (Arimura *et al*. 2004; Lucker *et al*. 2004; Picaud *et al*. 2006). This suggests that these compounds may have similar functions, or at least one cannot tell them apart functionally since in most cases they have not been tested individually.

As has been shown for other terpenoids (see section above for (*E*)-*β*-farnesene), jasmonic acid is also involved in the induction of *α*-humulene emission. Treatment of roots of Douglas-fir seedlings with methyl jasmonate induced traumatic resin duct formation in roots and stems as well as specific changes in the terpenoid profile of roots, stem and foliage (Huber *et al*. 2005b). While several monoterpenoids, sesquiterpenes, and diterpene acids

increased significantly in roots and stems upon treatment, *α*-humulene specifically increased in foliage.

In addition to the effects mentioned above for (*E*)-*β*-caryophyllene, *α*-humulene has repellent effects on sucking herbivores. The repellent effect was shown in e.g. tobacco producing greater amounts of *α*-humulene and caryophyllene oxide in response to herbivory by western flower thrips. In choice tests, thrips consistently preferred uninduced over induced plants (Delphia *et al*. 2007), so the enhanced *α*-humulene and caryophyllene oxide emission acts as a repellent. Another example for a repellent effect of *α*-humulene is provided by a study of Chinese red pine (*Pinus massoniana*) which contains the sesquiterpene *α*-humulene in its heartwood; *α*-humulene was shown to repel the pine nematode which feeds on the pine wood (Suga *et al*. 1993).

- germacrenes (1(10),5-germacradiene-4-ol, germacrene A (*β*-elemene) and bicyclogermacrene (product of *Ps*TPS2)

Germacrenes are typically constituents of a number of plant species and have antimicrobial and insecticidal properties, though they also play a role as insect pheromones (Maingon *et al*. 2003). For the sesquiterpene 1(10),5-germacradiene-4-ol not much is known concerning insect- or pathogen- plant interactions except for the work of Bergstrom and coworkers (1994). They determined that larvae of the pine sawfly *Neodiprion sertifer* selectively sequester selectively (*R*,*S*)-5-germacradiene-4-ol from the host trees *Pinus sylvestris* and *P. contorta*, and propose that it is part of the protective discharge of the larvae and pupa. The antiplasmodial properties of 1(10)E,5E-germacradien-4-ol from *Reneilmia cincinnata* (Zingiberaceae) were determined by Tchuendem and coworkers (1999).

For other germacrenes, various activities ranging from allelopathic over antibacterial to kairomonal activities have been found. For example, in the plant species *Mikania micrantha* three sesquiterpenoids - among them a germacradiene (2,3-epoxy1-hydroxy-4,9-germacradiene-12,8:15,6-diolide) - were isolated and identified. These sesquiterpenoids inhibited both germination and seedling growth of other tested plant species like radish, ryegrass, and white clover and also of trees like *Acacia*, *Eucalyptus* and *Pinus* species (Shao *et al*. 2005). Essential oils from *Lantana camara* and *Lantana sp*., containing high amounts of bicyclogermacrene, were examined by da Costa *et al*. (2009); the oils showed antibacterial activity and toxicity towards several bacteria strains as was also shown by Juliao 2009 for *L. trifolia*. Several other research groups have determined that plants containing bicyclogermacrene show antibacterial activity e.g. *Eugenia beaurepaireana* (Magina *et al*. 2009), *Chrysocoma ciliata* (Afolayan and Ashafa 2009), *Annona foetida* (Costa *et al*. 2009) and *Bowdichia virgilioides* (Rodrigues *et al*. 2009).

Elemanes are a group of sesquiterpenes closely related to germacrenes that represent rearrangement products or thermal artifacts of germacranes (Setzer 2008, Adio 2009). They are found in the essential oils, or volatile blends, or extracts of the leaves, buds, flowers, fruits, stem or bark, and roots of various plants. For example, sesquiterpene hydrocarbons like *β*-elemene, *β*-caryophyllene and *α*-humulene are present on the elytra of the white-spotted longicorn beetle *Anoplophora malasiaca* (Thomson). Yasui and coworkers (2008) found that the host plant of the beetle, *Citrus unshiu* emits those sesquiterpenes after mechanical wounding or beetle infestation. The authors hypothesized that the sesquiterpenes adsorb on the wax layer of the beetle elytra. Hence, the sesquiterpenes are released from both the beetles and from *Citrus* branches on which beetles feed, and act as kairomones signalling the presence of beetles to conspecifics in the field.

- longifolene (product of *Ps*TPS3)

Longifolene belongs to a rather rare sesquiterpene skeletal type but occurs frequently in higher plants, mostly in conifer resins. Its name is derived from the pine species *Pinus longifolia* from which the compound was first isolated (Naffa & Ourisson 1954). Only a few studies have examined the potential function of longifolene in plant - insect or plant-pathogen interactions.

Longifolene has kairomonal effects and attracts the pinewood nematode *Bursaphelenchus xylophilus* (Zhao *et al*. 2007) which is listed as a quarantine pest in more than 40 countries. The wood-boring cerambycid beetles *Monochamus alternatus* provides the only known means of transport for this nematode from infected to non-infected host trees. Zhao and coworkers applied the chemotactic response of pinewood nematode to develop a novel and rapid sampling method. A trap tube, baited with a blend of nematode attractive terpenes (*α*-pinene, *β*-pinene, and longifolene, 1:2.7:1.1) was shown to effectively capture third-stage dispersal juveniles of pinewood nematode from infested wood. Kairomonal effects of longifolene were also suggested for the interaction between *Abies lasiocarpa* and the Western balsam bark beetle *Dryocoetes confuses*; the tree releases (+)-longifolene and the five monoterpenes: (1S)-(-)-*α*-pinene, p-cymene, terpinolene, (R)-(-)-myrtenal and transpinocarveol after beetle attack (Camacho *et al*. 1998). In laboratory bioassays, all six compounds were attractive to both sexes of *D. confusus*. However, in field experiments none of the isolated compounds alone was attractive.

Synomonal effects of longifolene have not been proven yet. Longifolene is released from the pine *P. massoniana* along with other terpenes after damage by the lepidopteran *Dendrolimus punctatus*. All major volatiles of *P. massoniana* except for (+)-longifolene elicited electroantennogram responses by in the tachinid larval parasitoid *Carcelia*

matsukarehae, an important natural enemy of *D. punctatus* (Xu *et al*. 2006). Thus, this sesquiterpene does not function as synomone in the tritrophic interactions between pine, *Dendrolimus*, and the larval parasitoid.

- longipinene (product of *Ps*TPS3)

Only very few studies addressed the ecological functions of the sesquiterpene longipinene. This sesquiterpene is known to attract females of the spruce seed moth, *Cydia strobilella* which oviposits on seed cones of most North American spruces (*Picea spp.*) at the time of pollination; hatched larvae feed on seeds in the maturing cones (Bedard *et al*. 2002). Coupled gaschromatographic-electroantennographic detection (GC-EAD) analyses of extracts of spruce seed cone volatiles revealed that more than 17 compounds elicited antennal responses by male and female *C. strobilella*. Out of those compounds seven, including longipinene and (-)-*α*-pinene, (-)-*β*-pinene, *α*-humulene, (Z)-3-hexenol, methyl eugenol and cymen-8-ol, were more attractive to female *C. strobilella* in laboratory bioassay experiments than the complete seed cone volatile blend (Bedard *et al*. 2002). Cerda-Garcia-Rojas *et al*. (2010) found that a group of synthetic oxidized longipinane derivatives acted as antifeedants or toxins for *Spodoptera littoralis* and *Myzus persicae*.

In summary, the sesquiterpenoid products of the new *P. sylvestris* sesquiterpene synthases can fulfil a wide spectrum of ecological functions ranging from antimicrobial activities over kairomone effects on herbivores to synomonal activities in tritrophic interacitions.

Most of all living organisms, especially plants, are able to biosynthesis terpenes for many physiological and ecological purposes. Given the enormous number and diversity of terpene structures, most purposes of the vast majority of terpenes are still unknown, even though in the last years many new exciting information on the genomics, proteomics, molecular and biochemical characteristics of enzymes involved in terpenes biosynthesis were obtained. Because of their intensive terpene production, conifers in particular were and still are interesting subjects of defense related terpene biosynthesis studies. However, many terpene synthase encoding genes and their corresponding enzymes remain to be characterized for their contribution to induced direct and indirect conifer defenses in the future.

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Chapter 6

Summary

Plants unlike animals are not able to escape from enemy attack by running away. They evolved a great variety of defense strategies to withstand the manifold attacks of herbivores and pathogens. Conifers in particular have developed effective mechanisms to defend themselves directly and also indirectly by attracting enemies of their enemies. Studies of the tritrophic systems involved in indirect defense have addressed many interesting scientific questions on how plants and insects communicate.

An intensively studied indirect defense systems consists of pine (*Pinus sylvestris*), pine sawflies (*Diprion pini*) and a hymenopteran egg parasitoid specialized on eggs of sawflies on pine, i.e. *Closterocerus ruforum*. Egg deposition by the sawfly *D. pini* is known to induce *P. sylvestris* to emit higher amounts of the sesquiterpene (*E*)-*β*-farnesene on the third day after oviposition which attracts the egg parasitoid. This parasitoid lays its eggs in the *D. pini* eggs and the hatching parasitoid larva is killing the developing sawfly embryo inside the sawfly egg. Volatiles attractive to the egg parasitoid are emitted both from the site of egg deposition and adjacent tissue. Olfactometer tests showed that (*E*)-*β*-farnesene alone does not attract the parasitoid, but other volatile cues are also necessary for the parasitoid to respond. These other volatiles are the monoterpenes *β*-phellandrene and *β*-ocimene and the sesquiterpenes (*E*)-*β*-caryophyllene and *α*-humulene. Further it is known that *C. ruforum* does not only accept *D. pini* as host, but also the closely related sawfly species *Gilpinia pallida* and *Neodiprion sertifer*. While egg deposition by *N. sertifer* on *P. sylvestris* also induces volatiles that attract the egg parasitoid, egg deposition by *G. pallida* does not.

Based on this knowledge, the PhD study presented here investigated the molecular response of *P. sylvestris* to sawfly oviposition to gain new insight into the mechanisms of egg parasitoid attraction. Emphasis was placed on the identification and expression of genes encoding the terpene synthase enzymes that produce sesquiterpenes involved in attraction of the egg parasitoid.

1. Isolation and functional characterization of pine terpene synthases with potential involvement in egg parasitoid attraction

In order to investigate whether sawfly egg deposition activates genes encoding pine sesquiterpene synthases, a range of candidate genes was isolated and functionally described. The main goal was to identify an (*E*)-*β*-farnesene synthase gene since this volatile was shown to be the principal attractant of the egg parasitoid. First, mRNA was extracted from oviposition-induced *P. sylvestris* needle tissue and used for cDNA synthesis. Using cDNA as template along with sesquiterpene specific degenerate primers in PCR approaches, fragments of candidate genes were isolated. The fragments were then elongated to full coding sequences by means of RACE technology. Three new sesquiterpene synthases, named *Ps*TPS1, *Ps*TPS2 and *Ps*TPS3, were isolated. In a parallel approach using the same cDNA and a known sequence of a (*E*)-*β*-farnesene synthase from Douglas fir as template information, the fourth sesquiterpene synthase was isolated and named *Ps*TPS5. To functionally characterize the enzymes, heterologous expression was carried out in *Escherichia coli* which revealed the principal products as (*E*)-*β*-caryophyllene and *α*-humulene (*Ps*TPS1), 1(10),5-germacradiene-4-ol (*Ps*TPS2), longifolene and *α*-longipinene (*Ps*TPS3) and (*E*)-*β*-farnesene (*Ps*TPS5) (*Chapter 2 & 3*).

2. Measurement of terpene synthase transcript levels over a time course after sawfly egg deposition in comparison with parasitoid attraction bioassays

P. sylvestris is known to respond to eggs laid by the sawfly *D. pini* by releasing an attractive blend of terpenoids, with sesquiterpenes playing an important role in this process. We analysed the transcription levels of the isolated sesquiterpene synthase genes in needles oviposited upon by *D. pini* in comparison to artificially wounded needles (odor of artificially wounded, egg-free needles is not attractive to parasitoids; the artificial wounding mimicked the ovipositional wounding). We found that egg deposition enhances the transcription of the sesquiterpene synthases *PsTPS*1 ((*E*)-*β*-caryophyllene/*α*-humulene synthase) and *PsTPS*2 (1(10),5-germacradiene-4-ol synthase) in needle material that is attractive to the parasitoids (day 3 after oviposition). Transcript levels of *PsTPS*3 (longifolene synthase) did not change in response to egg deposition at all. The expression level of *PsTPS*5, which produces (*E*)-*β*-farnesene - the compound previously determined to be responsible for attraction of the egg parasitoid *C. ruforum*, was reduced in oviposition-induced needles. This result suggests that the enhanced emission of (*E*)-*β*-farnesene found in attractive, oviposition-induced pine needles is not regulated by transcriptional changes of the detected (*E*)-*β*-farnesene synthase. Alternatively, a further (*E*)-*β*-farnesene synthase than the one detected here may produce the enhanced quantities of (*E*)-*β*-farnesene in response to egg deposition. In contrast to *PsTPS*5,

transcripts of *PsTPS*1 and *PsTPS*2 were highest when the pine odor was attractive for the egg parasitoid. Since we did not observe any change in emission rate or accumulation of *PsTPS*1 and *PsTPS*2 products at this time, this could imply that production of these compounds is not determined by gene expression of *PsTPS*1 and *PsTPS*2 (*Chapter 2 & 3*).

3. Measurement of terpene synthase transcript levels after egg deposition by other sawfly-host plant combinations

To gain a better understanding of the species specificity of transcriptional changes of sesquiterpene synthases after sawfly egg deposition on pine and of its dependence on time after egg deposition, we compared effects of *D. pini* oviposition on *P. sylvestris* with those induced by other sawfly species:

N. sertifer eggs on *P. sylvestris*,

G. pallida eggs on *P. sylvestris*.

Furthermore, we determined transcription of sesquiterpene synthases of *P. nigra* after egg deposition by *D. pini.*

The herbivore-host plant combination *N. sertifer* / *P. sylvestris* showed a similar attraction pattern as that previously described for *D. pini* eggs on *P. sylvestris*, but the other two combinations (*G. pallida* / *P. sylvestris* and *D. pini* / *P. nigra*) did not lead to an induction of attractive volatiles at any of the three tested time points (tested 1, 2 and 3 d after egg deposition). As determined by quantitative real-time PCR, both *PsTPS*1 and *PsTPS*2 transcript levels, increased significantly after oviposition by *N. sertifer* on *P. sylvestris*, again only after the time point (3 days after induction) where the needle odor was attractive for the parasitoid. Expression levels of *PsTPS*5 were not elevated in any tissue attractive for the parasitoid. Thus, the *PsTPS*1 and *PsTPS*2 genes are good markers for parasitoid attraction. Hence, our results demonstrate a specific transcriptional response to egg deposition, distinct from that caused by artificial wounding. No elevated transcript levels were detected for sesquiterpene synthases in plant material that was unattractive for the egg parasitoid. Furthermore, these experiments showed that transcriptional changes of *PsTPS*1 and *PsTPS*2 depend on the sawfly species and the time after egg deposition. (*Chapter 3*)

4. Does sawfly oviposition prepare a defensive response directed at the feeding larvae?

Both oviposition and feeding by herbivorous insects are well-known to induce plant defense. In this study, we investigated if prior egg deposition affected plant defense against feeding larvae using the *P. sylvestris* - *D. pini* system. Laboratory bioassays showed that performance of sawflies which began feeding on those pine needles where they had hatched

was worse than on needles that had never had eggs. Survival rates of larvae feeding on previously egg leaden needles were significantly lower compared to survival of larvae on egg-free needles. We also could detect transgenerational effects of egg deposition on sawfly performance. Female sawflies that spent their larval development on needles with eggs were less fertile than those that developed on egg free needles. The transcription of the two sesquiterpene synthases (*PsTPS*1 and *PsTPS*2) that we had shown to be good marker genes for *D*. *pini* oviposition was clearly enhanced in needles that had had eggs as compared to those on which larvae were feeding without prior egg laying. However, the accumulation of secondary metabolites known to negatively influence larval performance did not show a similar trend. Neither terpenes (mono-, sesqui-, and diterpenes) nor phenolic compound concentrations were higher in the needles with prior oviposition. Nitrogen and water contents in feeding damaged needles with and without eggs did not differ. Nevertheless, based on the performance studies we can conclude that previous oviposition prepares the plant for defense against upcoming larval feeding although the mechanism is still unclear (*Chapter 4*).

5. Review: *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 and *Ps*TPS5: Products / Function in plant-insect interaction

In this review we highlight the biosynthesis of terpenes, their regulation within a plant and their role in plant-insect communication. Special emphasis is given to products synthesized by the sesquiterpene synthases *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 and *Ps*TPS5 identified in this PhD study. Furthermore, we compare the characteristics of the sesquiterpenes synthases identified here with those known from other sesquiterpene synthases. We also address the kinetics of expression of the sesquiterpene synthase genes in response to herbivore attack.

Chapter 7

Zusammenfassung

Pflanzen sind im Gegensatz zu Tieren nicht in der Lage, bei drohenden Gefahren von Fraßfeinden die Flucht zu ergreifen. Sie entwickelten mannigfaltige Abwehrstrategien um sich gegen Angreifer wie Herbivoren und Pathogene zu wehren. Insbesondere Koniferen mussten auf Grund ihrer langen Lebensdauer schon sehr früh wirksame Mechanismen entwickelen, um sich auf direkte als auch indirekte Art, was die Anlockung von Feinden ihrer Feinde beinhaltet, zu verteidigen. Studien zu diesen tritrophischen Systemen haben viele wissenschaftlich interessante Fragen wie z.B. zur Kommunikation zwischen Pflanzen und Insekten aufgeworfen.

Eines der umfangreich erforschten indirekten Abwehrsysteme behandelt die Interaktion zwischen der Kiefer (*Pinus sylvestris*), der Kieferblattwespe (*Diprion pini*) und dem Eiparasitoiden *Closterocerus ruforum*, der sich auf die Eier der Blattwespen auf der Kiefer, die auf den Kiefernnadeln abgelegt werden, spezialisiert hat. Es ist bekannt, dass die Eiablage der herbivoren Pflanzenwespe *D. pini* an der Wirtspflanze *P. sylvestris* eine erhöhte Abgabe des Sesquiterpenes (*E*)-*β*-Farnesen drei Tage nach der Eibablage induziert, was wiederum den Parasitoiden *C. ruforum* anlockt. Dieser Parasitoid legt seine Eier in die *D. pini* Eier, wobei dessen parasitoiden Larven die sich entwickelnden Blattwespenembryonen töten. Die für den Parasitoiden attraktiven Duftstoffe werden nicht nur direkt von den eierbelegten sondern auch systemisch von den angrenzenden eifreien Nadeln abgegeben, was so zur Verstärkung des Signals führt. Kürzlich gewonnene Erkenntnisse aus olfaktorischen Tests zum Verhalten des Parasitoiden zeigen, dass (*E*)-*β*-Farnesen nicht nur allein attraktiv auf den Parasitoiden wirkt und dass noch andere volatile Hinweise zur Anlockung benötigt werden. Als diese anderen volatilen Komponenten wurden beispielsweise die Monoterpene *β*-Phellandren and *β*-Ocimen sowie die Sesquiterpene *β*-Caryophyllen und *α*-Humulen identifiziert. Studien zum Verhalten des Eiparasitoiden zeigten weiterhin, dass *C. ruforum* neben *D. pini* auch andere Blattwespenarten, wie z.B. *Gilpinia pallida* oder *Neodiprion sertifer* als Wirt akzeptiert. Hierbei induziert nur die Eiablage von *N. sertifer* die Abgabe von für den Parasitoiden attraktiven Duftstoffen, wohingegen die Eiablage von *G. pallida* nicht diesen Effekt mit sich bringt.

Um erstmalig Erkenntnisse über die molekularen Mechanismen der Anlockung des Parasitoiden zu gewinnen, beschäftigt sich die hier vorgestellte Arbeit mit den molekularbiolgischen Grundlagen der Antwort des Baumes auf die Eiablage von Blattwespen. Eine besondere Gewichtung erfuhr hierbei die Identifizierung und Expression von Terpensynthase codierender Gene, die nach der Eiablage der Blattwespen Sesquiterpene synthetisieren, die dann wiederum anlockend für den Parasitoiden sind.

1. Isolation und funktionale Charakterisierung von Terpensynthasen aus der Kiefer (*P. sylvestris*) die vermutlich in die Anlockung des Eiparasitoiden involviert sind.

Um zu klären, ob die Eiablage von Blattwespen die Transkription von *Pinus*-Terpensynthasen induziert, wurde eine Reihe von Genkandidaten isoliert und funktional charakterisiert. Hierbei war das Hauptziel, das Gen zu identifizieren, das für die (*E*)-*β*-Farnesen Synthase codiert, da diese volatile Substanz hauptsächlich attraktiv auf den Parasitoiden wirkt. Hierzu wurde zuerst die mRNA aus Eiablage-induziertem Pflanzenmaterial isoliert und anschliessend in cDNA umgeschrieben. Diese cDNA wurde darauffolgend zusammen mit degenerierten Primern, die spezifisch für Sequenzen von Sesquiterpensynthasen sind, in PCR Versuchen verwandt, um Fragmente putativer Sesquiterpensynthase kodierender Gene zu amplifizieren. Die komplette Identifizierung der kodierenden cDNA erfolgte daraufhin durch Amplifikation der cDNA-Bereiche mithilfe 5'- und zusätzlicher 3'-RACE-PCR Technologie. Mittels dieser Methode konnten 3 Sesquiterpensynthasen identifiziert werden, die mit *PsTPS*1, *PsTPS*2 und *PsTPS*3 bezeichnet wurden. In einem parallelen Ansatz wurde unter Benutzung der gleichen cDNA und anhand der Sequenz der nahezu homologen (*E*)-*β*-Farnesen Synthase aus der Douglasie (*Pseudotsuga menzii*), der kodierende Bereich einer weiteren Sesquiterpensynthasen der *PsTPS*5 mittels PCR aus der Kiefer isoliert. Um die Funktion der Enzyme zu beschreiben, wurden diese dann heterolog in *Escherichia coli* exprimiert. Folgende Hauptprodukte konnten daraufhin identifiziert werden: (*E*)-*β*-Caryophyllen und *α*-Humulen (*Ps*TPS1), 1(10),5-Germacradien-4-ol (*Ps*TPS2), Longifolen und *α*-Longipinen (*Ps*TPS3) und (*E*)-*β*-Farnesen (*Ps*TPS5) (*Chapter 2 & 3*).

2. Trankriptmengen nach Eiablage von *D. pini* an *P. sylvestris* nach verschiedenen Zeitpunkten im Vergleich zur Attraktivität für Parasitoiden

Die Ablage von Eiern der Blattwespe *D. pini* an *P. sylvestris* verändert das pflanzliche terpenoide Duftmuster so, dass parasitische Wespen angelockt werden. Dabei spielen Sesquiterpene eine ausschlaggebende Rolle. Wir analysierten die Menge der Transkripte der

isolierten Sesquiterpensynthase Gene in *D. pini* Eiablage-induzierten Nadeln im Vergleich mit artifiziell verwundeten Nadeln, deren Duft von artifiziell verwundeten, Ei-freien Nadeln ist für den Parasitioiden nicht attraktiv ist. Es konnte 3 Tage nach Eiablage eine transkriptionelle Aktivierung der Sesquiterpensynthase Gene *PsTPS*1 ((*E*)-*β*-Caryophyllen/*α*-Humulen Synthase) und *PsTPS*2 (1(10),5-Germacradien-4-ol Synthase) in den für den Eiparasitoiden attraktiven Nadel festgestellt werden. Analysen der *PsTPS*3 Transkriptmenge (Longifolen Synthase) ergaben dahingegen zu keinem gemessenen Zeitpunkt nach Eiablage durch *D. pini* signifikant erhöhte Werte. Die *Ps*TPS5, die das für die Anlockung des Parsitoiden auschlaggebende Sesquiterpen (*E*)-*β*-Farnesen produziert, zeigt wider Erwarten eine Reduktion der Transkripte in eiinduzierten Nadeln. Dies könnte bedeuten, dass die erhöhte Synthese von (*E*)-*β*-Farnesen in den für den Parasitoiden attraktiven, Eiablage-induzierten Nadeln nicht durch die transkriptionelle Regulierung der *PsTPS*5 bestimmt wird. Eine weitere Möglichkeit der Erklärung wäre, dass eine andere (*E*)-*β*-Farnesen Synthase für die erhöhte (*E*)-*β*-Farnesen Abgabe nach Eiablage verantwortlich ist. Im Gegensatz zur *PsTPS*5, reagieren *PsTPS*1 und *PsTPS*2 auf *D. pini* Eiablage mit einer erhöhten Expression, die ausschließlich zu dem Zeitpunkt, an dem der Nadelduft attraktiv für den Parasitoiden ist, gemessen wurde. Widersprüchlicherweise und bisher ungeklärt ist die Tatsache, dass wir zu den erhöhten Transkriptmengen von *PsTPS*1 und *PsTPS*2, weder eine erhöhte Abgabe in die Atmosphäre zuordnen, noch eine erhöhte Akkumulation an Produkten in der Nadel zuordnen konnten. Dies könnte ein Indiz dafür sein, dass die Synthese dieser Produkte nicht auf der Ebene der Genexpression reguliert wird (*Chapter 2 & 3*).

3. Transkriptmengen nach Eiablage anderer Blattwespen-Wirtspflanze Kombinationen im Vergleich zur Attraktivität für Parasitoiden

Um ein besseres Verständnis über die Artenspezifität der trankriptionellen Regulierung der Sesquiterpenesynthasen nach Blattwespen-Eiablage an der Kiefer und deren Abhängigkeit von der Induktionszeit nach Eiablage zu erlangen, wurde der Einfluss von *D. pini* Eiablage an *P. sylvestris* mit den Effekten einer Eiablage durch andere Blattwespenarten verglichen:

Neodiprion sertifer an *Pinus sylvestris*,

Gilpinia pallida an *Pinus sylvestris*.

Desweiteren wurde geprüft, wie sich die Eiablage von *D. pini* an *P. nigra* auf die Transkription der Sesquiterpenesynthasen auswirkt.

Die Blattwespen-Wirtspflanze Kombinationen *N. sertifer* an *P. sylvestris* zeigte eine Induktion eines für den Eiparasitoiden *C. ruforum* attraktiven Duftmusters, während bei den beiden anderen Kombinationen (*G. pallida* / *P. sylvestris* and *D. pini* / *P. nigra*) kein attraktives

Duftmuster zu den drei gemessenen Zeitpunkten 1, 2 und 3 Tage nach Eiablage gemessen werde konnte. Quantitative PCR Analysen haben gezeigt, dass die Transkriptmengen der Terpensynthasen *Ps*TPS1 und *Ps*TPS2 nach *N. sertifer* Eiablage an *P. sylvestris* ebenfalls nur zu dem einen Zeitpunkt (nach 3 Tagen), an dem der abgegebene Duft der Nadeln attraktiv für den Parasitoiden ist, signifikant erhöht sind. Hingegen war die Expression von *PsTPS*5 in keinem für den Parasitoiden attraktiven Pflanzenmaterial erhöht. Daraus schlussfolgernd könnte man *PsTPS*1 und *PsTPS*2 als Markergene für die Parasitoiden Anlockung bezeichnen, deren Expression mit der Attraktivität des tritrophischen Systems korreliert. Unsere Ergebnisse zeigen demzufolge eine spezifische transkriptionelle Antwort nach Eiablage. Weiterhin konnte keine transkriptionelle Aktivierung der Sesquiterpensynthasen in den für den Eiparasitoiden unattraktivem Pflanzenmaterial gefunden werden. Darüber hinaus zeigen unsere Ergebnisse das die transkriptionelle Regulierung von *PsTPS*1 und *PsTPS*2 von der Blattwespenart und der Dauer der Eiablage-Induktion abhängig ist (*Chapter 3*).

4. Erfolgt nach Eiablage eine Reaktion des Baumes, die sich gegen die später schlüpfenden, dann fressenden Larven richtet?

Die pflanzliche Abwehr kann durch herbivoren Fraß oder herbivore Eiablage induziert werden. Im Falle der Blattwespen, findet zuerst eine Eiablage gefolgt vom anschließenden Fraß statt. In dieser Studie untersuchten wir, ob die herbivore Eiablage die pflanzliche Abwehr gegen später fressende Larven unter Nutzung des bekannten *P. sylvestris* - *D. pini* Systems beeinflusst. Unsere Untersuchungen zeigten eine eindeutig schlechtere Larvenentwicklung, wenn sich Larven auf Eiablage-induzierten Nadeln anstelle von auf nichtinduzierten Nadeln entwickelt haben. Die Überlebensrate von Larven, die eiinduzierte Nadeln fraßen, war signifikant kleiner als im Vergleich zu der von Larven, die nichtinduzierte Nadeln zur Verfügung hatten. Wir konnten desweiteren transgenerationale Effekte, verursacht durch die Eiablage der Blattwespen, auf die Larvenentwicklung feststellen. Weibchen deren Larvenentwicklung auf Nadeln mit Eiern erfolgte, waren weniger fruchtbar als die Weibchen die sich auf eifreien Nadeln entwickelten. Molekulare Untersuchungen zu den von uns identifizierten Sesquiterpensynthasen (*PsTPS*1und *PsTPS*2) ergaben signifikant erhöhte Transkriptmengen in Eiablage-induzierten Nadeln, während in Nadeln, die nur durch Fraß induziert wurden, ohne das eine Eiablage vorher stattfand, diese nicht gemessen wurde.

Die Untersuchungen zur Akkumulation sekundärer Metaboliten als mögliche Abwehrreaktion der Pflanze, ergaben allerdings weder für den Gehalt an Mono-, Di-, und als auch Sesquiterpenen noch nach der Analyse phenolischer Verbindungen Unterschiede. Ebenso konnten keine Veränderungen in der Stickstoff- und Wasserverfügbarkeit zwischen

Fraß beschädigten und eiinduzierten Nadeln gefunden werden. Trotzdem müssen wir basierend auf den Daten der Larvenperformanz davon ausgehen, dass die vorherige Eiablage von *D. pini* die Pflanze auf den bevorstehenden Fraß vorbereitet obzwar die genauen zugrunde liegenden Mechanismen vorerst weiter ungeklärt sind (*Chapter 4*).

5. Review: *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 und *Ps*TPS5 Produkte / Funktion in Pflanzen-Insekten Interaktion

In diesem Review wird unter spezieller Berücksichtigung der Produkte der Sesquiterpensynthasen *Ps*TPS1, *Ps*TPS2, *Ps*TPS3 und *Ps*TPS5, die im Zuge dieser Arbeit identifiziert wurden, insbesondere auf die Biosynthese der Terpene, ihrer Regulation innerhalb der Pflanze und ihrer spezifische Rolle in der Pflanzen-Insekten Kommunikation eingegangen. Weiterhin vergleichen wir die Eigenschaften dieser Sesquiterpensynthasen mit denen anderer bereits bekannter Sesquiterpensynthasen. Ferner gehen wir hier auf die Induzierbarkeit sowie die Expressionskinetik der Sesquiterpensynthase codierenden Gene als Antwort auf den Angriff von Herbivoren ein.

Supplementary data

Chapter 2

Table S1 Primers used in initial screening for sesquiterpene synthase sequences, RACE-PCR, heterologous expression, and qRT-PCR.

Research purpose	Name	Sequence 5' – 3'
Degenerate primer	Ses-233_for	AA(AG) TA(CT) GC5(CT)T5 GA(AG) TT(CT) CC5 TGG CA CA(CT) TG(CT)
	Losel-471_rev	GAT CTT GGA TGG AGA (AG)TG TAT TTG CTC
	Lo-284_for	TGT GCA CAT CAT AGA GAA CTA CAG CTT
RACE-PCR primer	PsTPS1 for	AGG GAC TCA GAA GTT GCG CAG GTG A
	PsTPS1 rev	TGT TCG GGA GAG GTT GAC CCA TCA AG
	PsTPS2 for	TCA GAG TCA TAT CTG CCG CAG CTG G
	PsTPS2 rev	CGT CTG GTA GAA TCT GAT CCA TTA AC
	PsTPS3 for	GGA TAT AGA GAA GCA GAA TTT CTA CC
	PsTPS3 rev	GTA TAC ACA TTC CAG AGC TGG GTT T
Nested RACE-PCR primer	PsTPS1nested_3'-for	CCG TCG GAA CTG TAG ATG ACC
	PsTPS1nested_5'-rev	GAT GCA CAT CCC TGA GCT AGC
	PsTPS2nested_3'-for	CGT GGA ACT TTA CTT TTG GGC GG
	PsTPS2nested_5'-rev	CCG GAG CTA GCG AGG CC
	PsTPS3nested_3'-for	CGT TCG AAC CGG AGT TTT CGA GC
	PsTPS3nested_5'-rev	CGC CAT CCA TTC GGA CTC TTG C
Amplification of the full-length coding region	PsTPS1_for	CAC CAT GGC TGA GAT TTG TGA ATC AG
	PsTPS1_rev	TTA TTC GTC CAT AGG TAC TTG CTC
	PsTPS2_for	CAC CAT GGC TCT TGT TTC TGT AAC G
	PsTPS2_rev	TCA CAG CTG CAG TGG CTG GAT AAG
	PsTPS2-T_for	CAC CAT GCTAATGCCAAAGAAATCTGTG
	PsTPS3_for	CAC CAT GGC TCA AAT TTC TAT AGG TGC
	PsTPS3_rev	TCA GAT GGG CAC TTG ATC GAT AAG
qRT-PCR primer	PsTPS1_QRT_for	TAC GGA ATG CTA GAC GAA CTG
	PsTPS1_QRT_rev	GCG GCC AGT TCA GAC GAA CTG
	PsTPS2_QRT_for	GGA TCA GAT TCT ACC AGA CG
	PsTPS2_QRT_rev	GTT ATG TCG TCC TTG ACT CG
	PsTPS3_QRT_ for	AGG GTA TAT GCA AGA GTC GC
	PsTPS3_QRT_rev	GTA TAC ACA TTC CAG AGC TGC
	Ubi-QRT-for	GTT GAT TTT TGC TGG CAA
	Ubi-QRT-rev	CAC CTC TCA GAC GAA GTA

Chapter 2

Table S2 Two-way ANOVA: Statistical comparisons of *P. sylvestris* sesquiterpene synthase transcript levels measured by quantitative RT-PCR between samples from oviposition induced foliage and artificially wounded controls (see Fig. 4 for further details).

Measurement	Source	df	*MS*	*F*	*P*
PsTPS 1	Time	2	4.603	4.873	**0.011**
	Treatment	1	4.295	7.125	**0.010**
	Treatment x time	2	1.517	2.517	0.090
PsTPS 2	Time	2	5.487	6.217	**0.003**
	Treatment	1	6.901	33.670	**0.000**
	Treatment x time	2	0.215	1.052	0.355
PsTPS 3	Time	2	1.60E-05	0.100	0.904
	Treatment	1	0	0.017	0.896
	Treatment x time	2	5.1E-05	1.792	0.177

Chapter 3

Table S1. Primers used in initial screening for sesquiterpene synthase sequences, RACE-PCR, heterologous expression and quantitative real-time PCR.

Research purpose	Name	Sequence 5' – 3'
RACE-PCR primer	*Ps*TPS5-Doug 2.2-144_rev	GTG CGA CTC AGT TCC CCA TG
Nested RACE-PCR primer	*Ps*TPS5-Doug 2.2-30_rev-nested	CTA CCC ATG CTG TGT CAT AAG
Amplification of the full-length coding region	*Ps*TPS5_farn1_start *Ps*TPS5-doug-stopneu1-2	ATGGCTAGCGCTTCTGTTGCTTCTTC TTA CAG AGG CAG TGG TTC AAT TAG pH9GW vector
qRT-PCR primer	*Ps*TPS1_QRT_for *Ps*TPS1_QRT_rev *Ps*TPS2_QRT_for *Ps*TPS2_QRT_rev *Pn*TPS1_QRT_for *Pn*TPS1_QRT_rev *Ps*TPS5_QRT_for *Ps*TPS5_QRT_rev Ubi-QRT_for Ubi-QRT_rev	please see Köpke et al., 2008, *Ps*TPS2 primer were also used for *Pn*TPS2 amplification GC AAA AGT TAC TAC CGT CGG CAG TTC GTC TAG CAT TCC GTA G GAT GTG GAC ATC CCG TTT CAG TG GAA ATG CCA AAA CCA TCT CCT TCC GTT GAT TTT TGC TGG CAA CAC CTC TCA GAC GAA GTA

Chapter 3

Table S2. Wilcoxon matched pairs test: Statistical comparisons of *P. sylvestris* sesquiterpene synthase transcript levels measured by quantitative real-time PCR between samples from oviposition-induced foliage and artificially wounded controls (see Figs. 1, 2 and 6 for further details).

Gene	Pine / sawfly species combination		*N*	T	Z	*P*
*PnTPS*1	*P. nigra* / *D. pini*	Day 2	12	33	0.47	0.637
		Day 3	12	16	1.80	0.071
		Day 4	12	3	2.82	**0.005**
*PsTPS*1	*P. sylvestris* / *G. pallida*	Day 2	14	39	0.85	0.39
		Day 3	9	12	1.24	0.21
		Day 4	9	12	1.24	0.21
	P. sylvestris / *N. sertifer*	Day 2	20	67	1.41	0.156
		Day 3	14	2	3.17	**0.0015**
		Day 4	15	23	2.10	**0.035**
*PnTPS*2	*P. nigra* / *D. pini*	Day 2	9	12	1.24	0.213
		Day 3	12	22	1.33	0.182
		Day 4	12	25	1.09	0.270
*PsTPS*2	*P. sylvestris* / *G. pallida*	Day 2	15	43	0.96	0.334
		Day 3	9	16	0.77	0.441
		Day 4	16	67	0.05	0.270
	P. sylvestris / *N. sertifer*	Day 2	19	75	0.80	0.420
		Day 3	15	11	2.78	**0.005**
		Day 4	15	15	2.55	**0.011**
*PsTPS*5	*P. sylvestris* / *D. pini*	Day 2	20	87	0.67	0.501
		Day 3	24	34	3.31	**0.0009**
		Day 4	16	0.0	3.51	**0.000**
	P. sylvestris / *N. sertifer*	Day 2	14	17	1.99	0.062
		Day 3	15	28	1.81	0.069
		Day 4	12	28	0.86	0.388

N=number of measurements; T, *Z*=test coefficients; $P < 0.05$

Chapter 4

Table S1. Concentrations (µg/g dry weight) of individual mono-, sesqui- and diterpenes and phenolic compounds in needles of *Pinus sylvestris* after different *Diprion pini* oviposition and larval feeding treatments.

	Treatments*						
Compound	**C**	**E3**	**E14**	**E+L**	**L**	**Chi Sqare**	***P*-Value**
monoterpene							
a-pinene	7613 ± 454	7319 ± 589	7804 ± 335	7671 ± 276	7401 ± 471	2.266	0.686
camphene	976 ± 41	932 ± 71	933 ± 42	922 ± 44	885 ± 51	2.266	0.735
sabinene	77 ± 11	85 ± 12	89 ± 8	93 ± 9	93 ± 10	0.800	0.935
ß-pinene	380 ± 29	379 ± 30	368 ± 16	380 ± 19	411 ± 21	2.400	0.622
myrcene	363 ± 11	381 ± 18	385 ± 14	381 ± 15	374 ± 21	1.200	0.878
d-3-carene	2259 ± 93	2519 ± 137	2618 ± 109	2576 ± 117	2698 ± 154	3.200	0.525
limonene	162 ± 32	182 ± 41	151 ± 20	144 ± 11	161 ± 8	1.466	0.832
ß-ocimene	239 ± 7	244 ± 19	270 ± 8	270 ± 8	257 ± 18	1.200	0.878
a-terpinolene	313 ± 31	314 ± 42	339 ± 14	309 ± 18	309 ± 22	0.666	0.955
sesquiterpene							
ß-elemene	289 ± 56	315 ± 56	298 ± 53	315 ± 48	298 ± 65	0.333	0.503
ß-caryophyllene	549 ± 45	582 ± 79	638 ± 64	634 ± 25	656 ± 60	3.060	0.546
a-humulene	110 ± 18	114 ± 15	125 ± 15	127 ± 13	156 ± 27	2.933	0.569
germacreneD	355 ± 39	361 ± 53	406 ± 26	411 ± 25	554 ± 106	5.200	0.267
ß-selinene	150 ± 27	124 ± 27	150 ± 38	161 ± 45	188 ± 51	4.130	0.388
bicyclogermacrene	764 ± 49	713 ± 95	733 ± 68	777 ± 52	729 ± 82	0.933	0.919
?-cadinene	588 ± 49	485 ± 66	555 ± 56	582 ± 74	600 ± 45	2.133	0.711
d-cadinene	933 ± 76	841 ± 122	894 ± 88	872 ± 92	832 ± 56	1.733	0.785
1(10),5-germacradiene-4-ol	1390 ± 281	1304 ± 405	1539 ± 258	1609 ± 259	1412 ± 326	1.066	0.899
diterpene							
sandaracopimaric acid	0.17 ± 0.016	0.18 ± 0.024	0.18 ± 0.020	0.17 ± 0.013	0.17 ± 0.017	6.971	0.137
levopimaric acid	1.11 ± 0.078	1.21 ± 0.085	1.26 ± 0.196	1.16 ± 0.132	1.12 ± 0.115	3.542	0.741
dihydroabietic acid	0.61 ± 0.055	0.70 ± 0.188	0.51 ± 0.091	0.46 ± 0.063	0.59 ± 0.086	3.066	0.546
abietic acid	0.38 ± 0.037	0.51 ± 0.070	0.49 ± 0.073	0.45 ± 0.041	0.42 ± 0.042	8.343	0.079
dimethyl pinifolate	9.18 ± 1.93	10.16 ± 1.620	11.43 ± 1.779	10.21 ± 1.662	8.86 ± 1.281	4.781	0.310
phenolic compounds							
catechin	340 ± 68	282 ± 35	295 ± 57	255 ± 26	389 ± 75	6.560	0.161
gallocatechin	27 ± 3	29 ± 3	28 ± 4	28 ± 2	28 ± 3	1.680	0.794
catechin:catechin dimer	39 ± 10	37 ± 9	38 ± 8	35 ± 7	47 ± 10	7.040	0.133
catechin:gallocat. dimer	1.88 ± 0.36	1.96 ± 0.38	2.00 ± 0.32	1.92 ±0.29	1.92 ± 0.31	1.520	0.823
gallocatechin:gallocat. dimer	23 ± 4	22 ± 3	21 ± 2	22 ± 21	21 ± 2	1.120	0.891
taxifolin	25 ± 3^{ab}	17 ± 2^{a}	20 ± 2^{ab}	28 ± 4^{b}	26 ± 3^{ab}	13.840	**0.008**
taxifolin glucoside	216 ± 110	247 ± 111	212 ± 103	199 ± 92	228 ± 109	16.720	0.151
apigenin glucoside	100 ± 16	100 ± 16	95 ± 18	95 ± 14	99 ±13	2.800	0.591
isorhamnetin	0.29 ± 0.053	0.30 ± 0.054	0.32 ± 0.060	0.32 ± 0.057	0.30 ± 0.045	2.000	0.731
quercetin	0.71 ± 0.12	0.74 ± 0.143	0.73 ± 0.12	0.68 ± 0.083	0.85 ± 0.110	4.160	0.384
quercetin galactoside	0.82 ± 0.086	0.93 ± 0.099	0.93 ± 0.105	0.96 ± 0.109	0.91 ± 0.124	3.520	0.475
quercetin glucoside	0.70 ± 0.073	0.84 ± 0.082	0.83 ± 0.087	0.83 ± 0.095	0.78 ± 0.109	0.560	0.967

*Abbreviations for treatments: C: untreated control; E3: twig with 3 day-old eggs of *D. pini*. E14: twig with 14 day-old eggs, shortly before larval hatching. E+L: twig that had been laden with eggs and on which *D. pini* larvae hatched and fed for 2 days; L: twig that experienced larval feeding, but did not carry eggs. Values are means ± SE. Statistical analyses. Friedman ANOVA. In case of significance a Wilcoxon Wilcox post hoc test was performed and significant differences ($P < 0.05$) are indicated by different letters.

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