# Molecular analyses of *Pinus sylvestris* after induction by insect oviposition

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# 1. Summary

The Scots pine (*Pinus sylvestris*) is known to attract the egg parasitoid *Chrysonotomyia ruforum* after oviposition of the pine sawfly *Diprion pini*. Previous studies have shown that pine needles laden with eggs or treated with jasmonic acid emit higher rates of the sesquiterpene (E)-ß-farnesene that serves as an olfactory clue for the parasitic wasp. The aim of this work was to better understand underlying molecular regulation patterns that cause the ecological important volatile change in *P. sylvestris*.

By the use of degenerate primers we identified four putative sesquiterpene synthase sequences fragments from *P. sylvestris*. With cDNA from differently treated pine needles we investigated transcriptional regulation patterns of those sequences through semiquantitative RT-PCR reactions. The sequence fragment Ps*TPS4* showed a nearly two fold higher relative transcript level in cDNA sampled after sawfly egg deposition than in the artificially damaged control, suggesting an induction of the putative encoded synthase by egg deposition. Sequence comparison reveals high similarities of Ps*TPS4* to the δ-selinene synthase of *Abies grandis* and the multiple product longifolene synthase of *Picea abies*. The latter is known to synthesize minor amounts of (E)-ß-farnesene. In contrast to the sequence fragments Ps*TPS2* and Ps*TPS3* that are most similar to the γ-humulene synthase-like protein of *Picea abies*, Ps*TPS1* also has a high sequences similarity to the δ-selinene synthase of *A. grandis*. A similar but not as distinct transcript expression pattern as Ps*TPS4* was displayed by Ps*TPS1* after sawfly egg deposition. All sequence fragments showed different transcript accumulation patterns between needles treated with jasmonates and needles loaded with sawfly eggs. The reason for this may lie in different regulative responses between the treatments or might be due to concentration effects. The results furthermore hint at a possible seasonally dependent expression pattern of Ps*TPS2* and Ps*TPS3* in methyl jasmonate treated needles.

The highly significant ($p<0.001$) Ps*TPS4* transcript difference suggests an impact of the putative encoded sesquiterpene synthase on the pine volatile change after oviposition. Further identification and characterization of a full-length Ps*TPS4*-clone promise to be a rewarding future project to obtain a better understanding of the pine-sawfly interaction at the molecular genetic level.

# 2. Introduction

In contrast to the majority of other living organisms plants are sessile. They are therefore unable to avoid the stresses they encounter by shifting position, and have had to evolve effective defense mechanisms.
Stresses arise in many forms. They can be divided into abiotic stress, arising from an excess or deficit of the physical or chemical environment, and biotic stress, imposed by other organisms (Buchanan et al. 2000). Plants possess a vast array of different defense systems that help to fight off intruders (Baldwin et al. 2001). This work focuses on defense strategies plants express when attacked by herbivores.

Plant defense systems against feeding damage are generally classified by their mode of action into induced and constitutive defenses (Karban and Baldwin 1997). Constitutive defense systems are constantly active in a plant, independent of an actual attack. Many coniferous trees, for example, possess constitutive resin ducts in bark, sapwood and needles in which resin is sequestered to repel intruders on their initial attack (Funk et al. 1994, Steele 1998, Phillips et al. 2003). The constitutive defense systems of plants may change over evolutionary time and individual life spans, or within the seasons, but they are independent of actual damage (Karban and Baldwin 1997). In Douglas fir (*Pseudotsuga menziesii*), for example, a change of leaf oil among crown levels and through the growing years was reported. The fluctuating components are known to decrease the larvae growth in vitro and hence contribute to the resistance against the Western spruce budworm (*Choristoneura occidentalis*) a common Douglas fir pest (Gambliel and Cates 1995).
Induced defenses, in contrast to constitutive defenses, are triggered by damage or stress. They decrease the negative fitness consequence of attacks on a plant and are generally categorized by their mode of action (Karban and Baldwin 1997, Baldwin et al. 2001).
They may act directly by affecting the intruding species performance themselves. Grand fir (*Abies grandis*) and Norway spruce (*Picea abies*) for example, have been shown to be induced by bark beetle attack to form special resin ducts. The resin has multiple functions; it is toxic to the intruding herbivorous beetles and its associated fungi, it forms a mechanical barrier, and it serves as a wound sealing compound on the bark (Funk et

al. 1994, Bohlmann et al. 1998, Steele et al. 1998, Martin et al. 2002, Fäldt et al. 2003, Martin et al. 2003, McKay et al. 2003).
Other direct defenses are known from chicory (*Cichorium intybus*) for instance, which produces bitter components that have significant antifeedant and antifungal activity (Monde et al. 1990, Bouwmeester et al. 2002). Their production was found to be constitutive as well as induced. Kessler and Baldwin (2001) demonstrated a direct defense to herbivory in induced *Nicotiana attenuata* plants. The production of the plant volatile linalool decreased the lepidopteran oviposition rates on the tobacco plant.

In addition to direct defenses, plants express traits that facilitate an indirect defense strategy. Turlings et al. (1990) were the first to demonstrate the employment of a third trophic level by plants after herbivore attack. They could show the *de novo* synthesis of volatile organic compounds (VOCs) in corn seedling after an attack by beet armyworm (*Spodoptera exigua*). They found these VOCs to be attractive to a wasp, *Cotesia marginiventris*, parasitic to the beet armyworm. Hence, the plant attracts a natural enemy of the intruder and defends itself indirectly. Since their discovery, indirect defenses mediated by herbivore induced release of VOCs have received great scientific attention in the field of chemical ecology. Tritrophic interactions mediated by herbivores are now known for example in lima beans (*Phaseolus lunatus*), cotton (*Gossypium hirsutum*), tobacco (*N. attenuata*), the ant-associated plant *Macaranga tanarius* and *Arabidopsis thaliana* (Takabayshi and Dicke 1996, Röse et al. 1998, Heil et al. 2001, Kessler and Baldwin 2001, van Poecke et al. 2001). Recently, such interactions have been found below ground as well. The entomophathogenic nematode *Heterorhabditis megidis* was shown to be highly attracted by *Diabrotica virgifera virgifera* larvae–damaged maize plant roots. The roots reveal a dramatic increase in VOCs after larvae damage (Rasemann and Turlings 2004).
The active components in the resin and leaf oil of conifers, the bitter components of chicory and the released volatile of maize, cotton and tobacco are all terpenoids or derivates thereof. In fact, terpenoids are among the most common volatile organic signals in indirect defense together with metabolites of the shikimate lipoxygenase pathways and metabolites of the shikimic acid pathway (Pichersky and Gershenzon 2002).

**The role of terpenoids in plant defense systems and their biosynthesis**

With over 22,000 compounds identified so far (Connolly and Hill 1991), terpenoids are the largest and most wide spread class of secondary metabolites. They are distinguished according to their number of $C_5$ units as monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$), diterpenes ($C_{20}$) and so forth. Terpenes have a variety of functional roles in plants as precursors for hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone), mediators of polysaccharide assembly (polyprenyl phosphatase), farnesylated proteins, and structural components of membranes (phytosterols). Furthermore, terpenoids serve as communication and defense compounds in plants.

The mono-, sesqui- and diterpenes were mostly found to perform ecological functions (McGrarvey and Croteau 1995, Buchanan et al. 2000). They are, for example a major component of flower volatiles (Knudsen et al. 1993). (S)-linalool synthase was purified from flowers of *Clarkia breweri*, and myrcene synthase and (E)-ß-ocimene synthase were discovered in snapdragon flowers (*Antirrhinum majus*) (Pichersky et al. 1995, Pichersky and Gershenzon 2002, Duderva et al. 2003). In *A*. *thaliana*, mono- and sesquiterpenes were identified as the major components of flower volatile emissions (Chen et al. 2003). Due to the localization and emission pattern of (S)-linalool in *C*. *breweri* this terpene is believed to be an olfactory cue for pollinating insects.

As mentioned before, mono-, sesqui- and diterpenes are also the major compounds of resin produced by conifers such as grand fir (*A*. *grandis*) and Norway spruce (*P*. *abies*) (Funk et al. 1994, Bohlmann et al. 1998, Steele et al. 1998, Martin et al. 2002, Martin et al. 2003, McKay et al. 2003, Fäldt et al. 2003).

In the first step of terpene biosynthesis the $C_5$ terpenoid precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are synthesized. Three specific classes of prenyltransferases catalyze the sequential condensation of DMAPP with one to three units IPP to form geranyl diphosphate (GDP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP), which serve as the branchpoint intermediates for the monoterpenes, sesquiterpenes and diterpenes, respectively (figure 2.1). In contrast to a limited number of pathway specific prenyltransferases, the terpene synthases which act upon these substrates may number in the thousands. They are largely uncharacterized, and directly account for the broad structural diversity of terpenes.

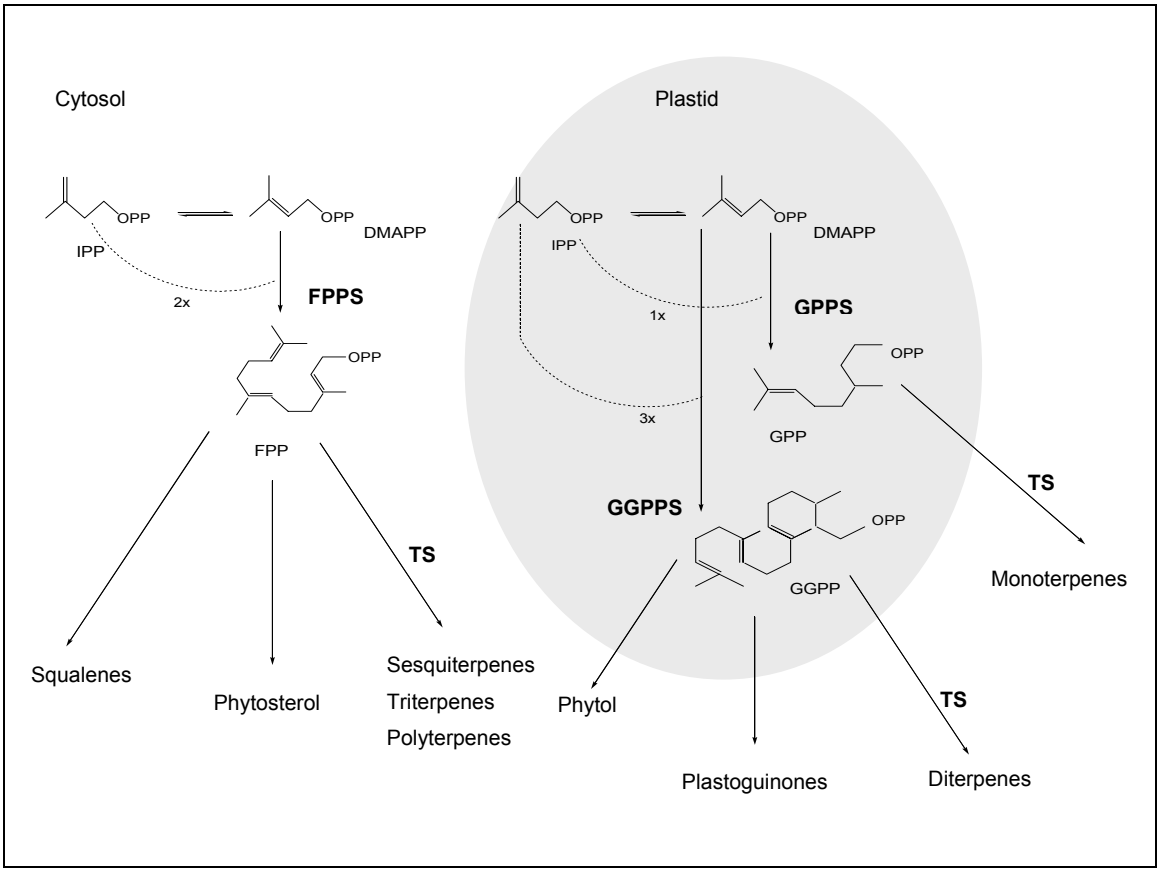



**Figure 2.1. Terpene biosynthesis.** Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the basic $C_5$ building blocks of terpenoid synthesis. Three classes of prenyltransferases catalyze the formation of the immediate precursors. Geranyl diphosphate synthase (GPPS) forms the $C_{10}$ intermediate geranyl diphosphate (GDP), farnesyl diphosphate synthase (FPPS) forms the $C_{15}$ intermediate farnesyl diphosphate (FPP) and geranylgeranyl diphosphate synthase (GGPPS) forms the $C_{20}$ intermediate geranylgeranyl diphosphate (GGPP). The synthesis of the mono-, di- and tetraterpenes is localized to the plastid, whereas the synthesis of sesqui- and triterpenes is localized to the cytosol.

Found in virtually all prenyltransferases and terpenoid synthases of bacteria, plants and animals are two aspartate-rich motifs (DxDD and DDxxD) that are involved in substrate binding by the enzyme (Wise and Croteau 1999, Trapp and Croteau 2001). They can be found in all amino acid sequences of this enzyme class and are therefore valuable tools to verify the accuracy of conducted alignments and to identify possible new terpene synthase sequences.

The two known biosynthetic pathways in higher plant cells are compartmentally separated. Monoterpenes and diterpenes are synthesized in the plastid. Sesquiterpenes are formed in the cytosol (figure 2.1). Furthermore, the biosynthesis is oftentimes restricted to specific tissue in close proximity to the site of utilization. The previously mentioned (S)-linalool for example is produced primarily in the petals of *C. breweri*. In *A.*

*grandis* and *P. abies* it was found that wounding even leads to the formation of new special cells in close proximity and / or systemically to the injury site in which specific wound induced terpenes are synthesized (Bohlmann et al. 1998, Martin et al. 2002, Pichersky and Gershenzon 2002, Byun et al. 2003)

Among the terpenes, sesquiterpenes are one of the most widely studied and best understood compound families. There are more than 300 identified distinct sesquiterpene skeletons and thousands of modified derivates (Staeks et al. 1997, for examples see figure 2.2) Sesquiterpenes display a wide range of physiological properties including immunosuppressive, antitumor and hormonal activities. Furthermore they have been found to possess antifungal and insect antifeedant properties (Cane 1999, Buchanan 2000, Kessler and Baldwin 2001).

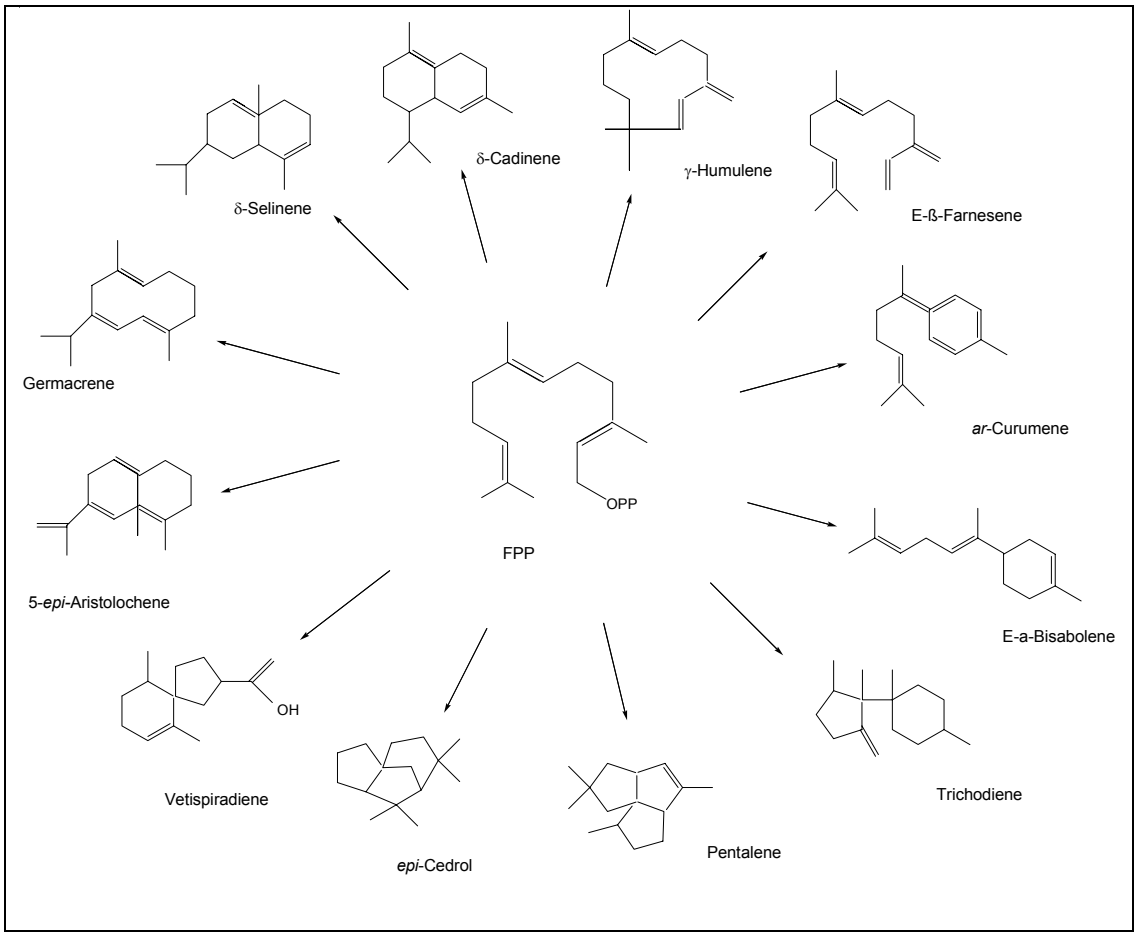



**Figure 2.2 Examples of common sesquiterpenes in plants.** Shown is the universal precursor farnesyl diphosphate (FPP) and cyclic and acyclic sesquiterpenes derived from FPP.

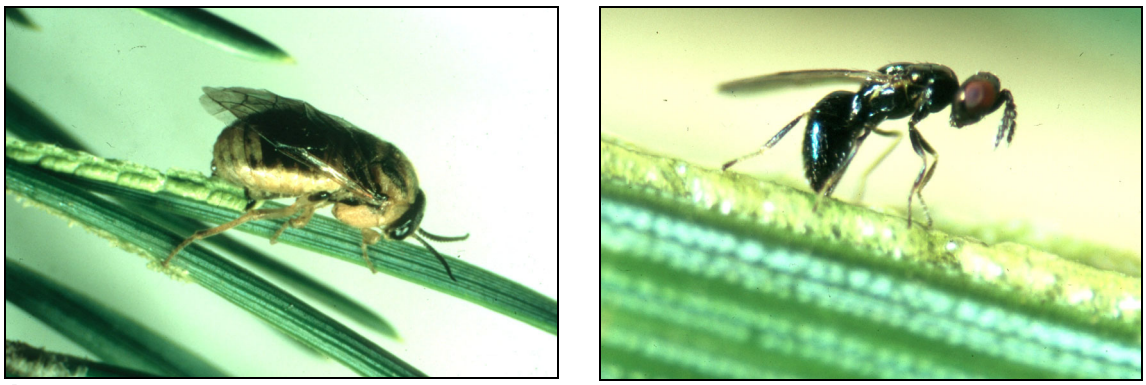
One abundant compound in both plants and animals is the acyclic sesquiterpene E-ß-farnesene (for chemical structure see figure 2.2). More than 600 papers have been published on E-ß-farnesene and its recruitment in chemical communication (Crock et al. 1997). It is used, for example, by bees and some ants as an allomone and trail pheromone and most aphid species utilize E-ß-farnesene as an alarm pheromone (Bowers et al. 1972, Crock et al. 1997). E-ß-farnesene has been found in the essential oils of hundreds of plant species, gymnosperms such as *Pinus pinaster* (Salin et al. 1995) and *Larix leptolepis* and angiosperms such as *Piper nigrum*, *Mentha x piperita*, (Crock et al. 1997), and *Zea mays* (Schnee et al. 2002). Moreover E-ß-farnesene serves as the active compound in the interspecific communication system studied in the presented work.

**Investigation of the tritrophic interaction between *P*inus *sylvestris*, *Diprion pini* and *Chrysonotomyia ruforum*: E-(ß)-farnesene as a mediator in interspecific communication**

Until now most studies on induced defense reactions of plants have been done on systems triggered by feeding damage of herbivores. Recent studies show that plants are also able to anticipate a future attack by sensing egg deposition. Changes were found for example in the plant tissue of *Pisum sativum* and the chemical plant surface of *Brassica oleracea* after egg deposition, that decreased herbivore damage or deterred future oviposition (Blakmeer et al. 1994, Dosse et al. 2000, Hilker and Meiners 2002, Hilker et al. 2002[2]).

Indirect defense reactions after insect oviposition have also been recently found in the angiosperm elm tree (*Ulmus minor*) as well as in the gymnosperm Scots pine (*Pinus sylvestris*) (Meiners and Hilker 1997, 2000, Hilker et al. 2002[1]). Local and systemic changes in volatile blends after egg deposition of potential herbivores that attract parasitoids have been found in both systems (Wegener et al. 2001, Mumm et al. 2003). The present work will focus on the pine-system and the molecular regulation reactions that may occur in the tree after insect oviposition.

The pine sawfly (*Diprion pini*) is widely distributed in northern Europe and Russia. The female sawfly lays her eggs on pine needles. Therefore she slits open a needle tangentially with her sclerotized ovipositor valves and inserts eggs into the wound (figure 2.3). The developing larvae are herbivorous and cause severe damage to foliage of their target species. The main host of *D. pini* in Europe is the Scots pine (Sharov 1993, Auger-Rozenberg et al. 1998).



A B

**Figure 2.3. Female *D. pini* laying eggs on a pine needle (A) and female *C. ruforum* laying eggs in *D. pini* eggs (B)**. A: The *D. pini* eggs are laid in a row with 10-20 eggs per needle. After laying the eggs, the female covers the eggs with a greenish secretion. B: The parasitic *C. ruforum* wasp places its eggs in *D. pini* eggs.

Hilker et al. (2002[1]) showed in olfactometer experiments that the volatile blend of *P. sylvestris* needles changes 72 hours after sawfly egg deposition in a way that is attractive to the eulophid egg parasitoid *Chrysonotomyia ruforum*. When the pine needles were artificially damaged, no such attraction could be observed. However, when the artificially damaged needles were covered with the oviduct secretion of *D. pini*, the pine needles were significantly attractive to *C. ruforum* 72 hours after application. Hence, the insertion of the oviduct secretion of *D. pini* serves as the elicitor for the attractant biosynthesis. In a systemic reaction, the pine needles change their volatile blend prior to larval hatching in order to allow the attracted parasitoids to oviposition in the hatching eggs (figure 2.3). Pine needles treated with jasmonic acid were also found to be attractive to *C. ruforum*.

In further studies, Mumm et al. (2003) found that the volatile blend from *P. sylvestris* revealed numerous mono- and sesquiterpenes after egg deposition, but they could not find a newly released compound that was not also present before egg deposition. In a quantitative comparison from oviposition-induced twigs and artificially damaged control

twigs, they showed that only the emission of E-ß-farnesene was significantly higher in the induced pine twigs. Jasmonic acid treated pine twigs showed a similar but not identical volatile spectrum. They released significantly higher amounts of (E)-ß-farnesene. Furthermore α-muurolene, γ-cadinene and δ-cadinene were significantly higher also in jasmonic acid treated twigs compared to control untreated twigs.
(E)-ß-farnesene is the single compound with a higher emission rate after both treatments. Unpublished data from Mumm et al. reveal furthermore that *C. ruforum* shows a significant attraction to undamaged pine needles with artificially added (E)-ß-farnesene. Hence, (E)-ß-farnesene is most likely the compound that serves as the interspecific signal between the tree and the eulophid egg parasitoid in this system and allows a 'communication' between the parasite and the invaded pine tree.

Recent experiments have provided a glimpse into the extensive transcriptional changes that occur after insect attack in a plant and allowed an exciting glance at the metabolic coordination underlying induced plant responses. It could be shown, for example, that the transcriptional level of terpene synthases is up regulated in response to herbivory in hybrid poplar (*Populus trichocarpa x deltoids*) as well as in Sitka Spruce (*Picea sitchensis*) (McKay et al. 2003, Arimura et al. 2004). Spatial and temporal reorganization of gene expression levels after herbivore feeding and wounding was found in *N. attenuate* and *P. abies* (Hermsmeier et al. 2001, Hietala et al. 2004). These developments involving molecular and applied ecology have bridged two previously isolated research communities. This is also the intention of the study presented here. It is the aim to identify molecular regulation systems that underlie the observed ecological effect of a higher (E)-ß-farnesene emission by *P. sylvestris* after oviposition.

**Identifying sesquiterpene synthase sequences in *P. sylvestris***

Accumulation of terpene synthase transcripts after wounding, stress, and herbivory has been demonstrated in maize, hybrid popular (*P. trichocarpa x deltoids*), Sitka spruce (*P. sitchensis*) and Norway spruce (*P. abies*), suggesting a mainly transcriptional regulation of terpenoid biosynthesis and emission (Schnee et al. 2002, Fäldt et al. 2003, McKay et al. 2003, Arimura et al. 2004). The corresponding enzymes heterologously expressed in bacteria were to be either single product synthases such as (+)-3-carene synthase in *P. abies* as well as multiple product enzymes such as tps1 in maize that catalyzes the formation of (E)-ß-farnesene, (E)-nerolidol and (E,E)-farnesol and *Pa*TPS-*Lon*, that

catalyzes the production of α-longifolene and as a minor product (E)-ß-farnesene in Norway spruce (Martin et al. 2004).

In order to test for transcriptional regulation of a (E)-ß-farnesene synthase in *P. sylvestris*, knowledge of the sequences of the encoding gene is necessary. However, there are is no sequence of an (E)-ß-farnesene synthases of *P. sylvestris* currently available in the databases. Therefore, sequences of other genes encoding terpene synthases that produce (E)-ß-farnesene and related sesquiterpenes were used to create search tools for the desired sequence as it has been done in many previous studies of terpene synthase genes (Crock et al. 1997, Bohlmann et al. 1998, Bouwmeester et al. 2001, Schnee et al. 2002). As higher emission of (E)-ß-farnesene was believed to attract the egg parasitoid, interest was focused on sesquiterpene synthase sequences, particularly other farnesene synthase sequences. Because there are nearly 200 different sesquiterpene synthase sequences from plants available in the NCBI databases (www.ncbi.nlm.nih.gov, Bethesda, USA), one has to carefully decide when choosing the query sequences in order to create sufficiently specific search tools.

From 186 published sesquiterpene synthase sequences in the NCBI databases only 5 sequences were of conifer origin and 181 were from various angiosperms. Six angiosperm sequences encode farnesene synthases, while only one conifer synthase sequences codes for a farnesene synthase (table 2.1). Therefore the question arises how useful angiosperm terpene synthase sequences are in the search for gymnosperm terpene synthase sequences.

**Table 2.1. Published sesquiterpene synthases of conifers and farnesene synthases from angiosperms in the NCBI databases.** There are only five sesquiterpene synthase sequences published from conifers. All of them are displayed. There are 181 sesquiterpene synthase sequences published from angiosperms. Only the available farnesene synthase sequences are displayed here. All farnesene synthase sequences are shown in bold frames and with a grey background. For all sequences the NCBI accession number is stated in parentheses.

| Angiosperms (**farnesene** synthases ) | Conifers (sesquiterpene sequences) |
|---|---|
| E-E-α-farnesene-synthase *Malus x domestica* (AAO22848) | α-farnesene-synthase *P. taeda* (AAO61226) |
| (E,E)-alpha-farnesene synthase *Malus x domestica* (AAS68019) | E-α-bisabolene-synthase *A. grandis* (AAK83562) |
| α-farnesene-synthase *Malus x domestica* (AAS01424) | δ-selinene-synthase *A. grandis* (AAK83561) |
| E-ß-farnesene synthase *Mentha x piperita* (AAK54279) | δ-selinene-synthase *A. grandis* (AAC05727) |
| E-ß-farnesene synthase *Citrus junos* (AAK54279) | γ-humulene-synthase *P. abies* (AAK39129) |
| tps 1 *Zea mays* (AAO18435) | |

When considering the evolutionary development of plants, one finds that the first gymnosperms appear on fossil records in the upper Devonian. The angiosperms are an evolutionary younger plant group with the earliest found fossil records in the Jurassic (Strasburger 1991). There are a diversity of opinions on the phylogenetic relationship and classification of these plant groups (Gerlderen et al. 1996, Strasburger 1991, Biswas and Johri 1997). However, the last common ancestors between the gymnosperms and angiosperms must have been at the end of carboniferous about 300 million years ago (Kirst et al. 2003), that is approximately three quarters of the time since the emergence of the first higher plants (420 million years ago). The homology of functional genes between both plant taxa can therefore be expected to be low. This is supported by the fact that gymnosperms possess a significantly larger haploid DNA content and bigger genome size than most angiosperm species. In spite of that, Kirst et al. (2003) demonstrated an apparent homology of expressed wood-forming tissue genes from *Pinus taeda* and *A*. *thaliana*. However, the sequence similarity within the conifers can be expected to be significantly higher than the sequence similarity between gymnosperms and angiosperms. This is especially true for terpene synthases involved in secondary metabolism in conifers as Trapp and Croteau (2001[1]) demonstrate by multiple sequence alignments of angiosperm and conifer terpene synthases.

This assumption is supported by the low sequence similarity an α-farnesene synthase of angiosperm (*Malus domestica*) and conifer origin (*P. taeda*) (figure 1.4). Although both synthases produce the same sesquiterpene, their amino acid identity is only 26 percent. Hence, when looking for sesquiterpene synthases of conifers, the focus should be on sequences available from conifers rather than angiosperms.

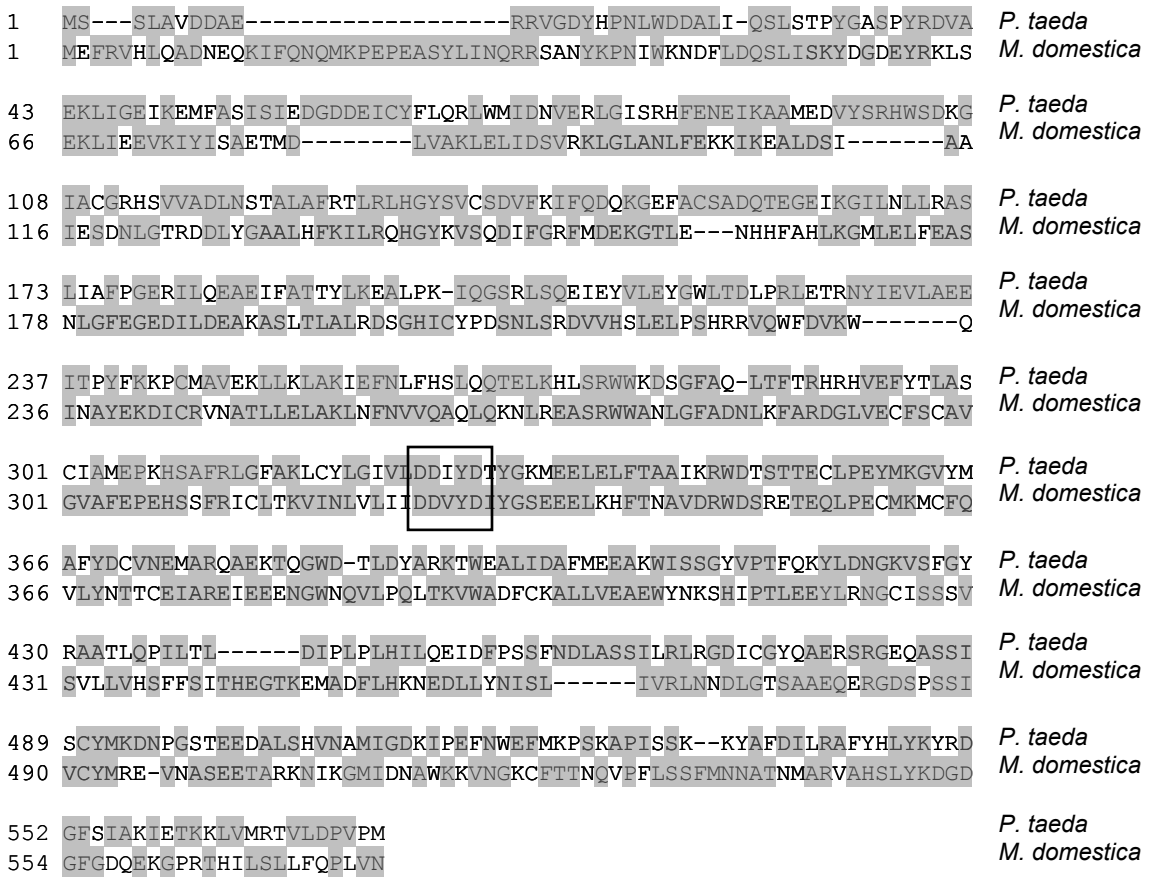



**Figure 1.4**. **Amino acid sequence alignment of the α-farnesene synthases of *Pinus taeda* and *Malus domestica* (AAS01424).** One DDxxD motif is highlighted to verify the accuracy of the alignment. The amino acid identity is 26 %. Consensus sequences are shown with a grey background.

In spite of their high chemical structural similarity, α-farnesene and ß-farnesene do not necessarily derive from a similar terpene synthase. As there is one α-farnesene synthase sequence available from *P. taeda*, it is of interest to investigate possible sequence similarity of an α- and a ß-farnesene synthase of the same plant species. Unfortunately, there is no one plant species from which both sequences are available. Interestingly enough, when comparing the amino acid sequences of monoterpene synthases and sesquiterpene synthases, it appears that the α-farnesene synthase sequences from *P. taeda* shows a strikingly high similarity to monoterpene sequences

(63-65 % amino acid identity) rather than to sesquiterpene sequences all of conifer origin (40-42 % amino acid identity). This is illustrated by a phylogenetic tree (figure 1.5).

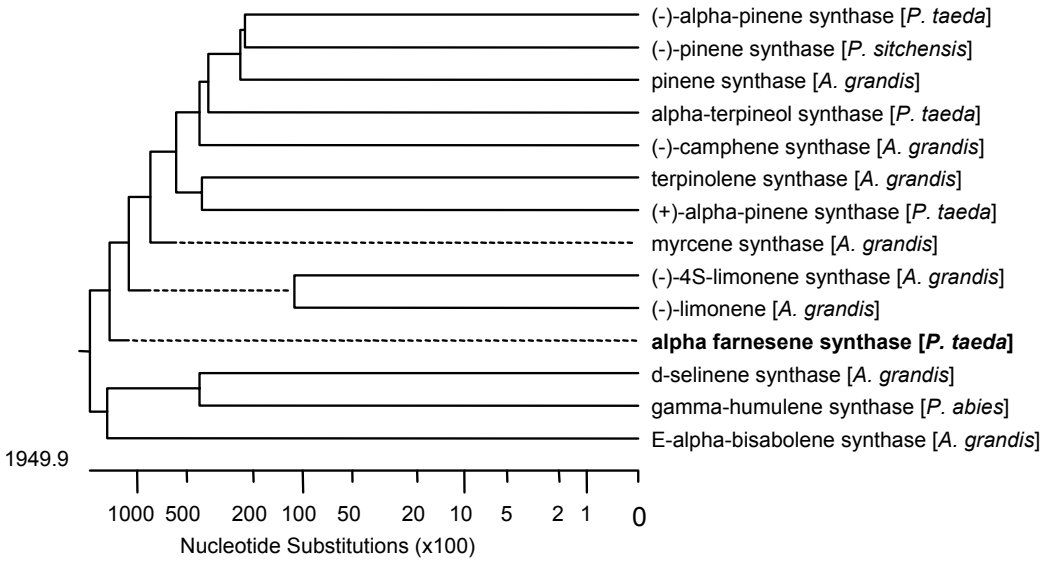



**Figure 1.5. Phylogenetic tree of monoterpene synthase sequences and sesquiterpene synthase sequences from conifers available in the NCBI databases.** The phylogenetic tree was constructed with the DNA-Star program. It visualizes the evolutionary relationships predicted from a multiple sequence alignment. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. The dotted lines on the phenogram indicate a negative branch length, a common result of averaging. Below the tree is a scale indicating the number of 'Nucleotide Substitutions' for protein sequences.

In reviewing the literature for (E)-ß-farnesene synthases that have been found to be up regulated after herbivory attack, there are to our knowledge two enzymes mentioned that produce (E)-ß-farnesene as a minor product. One in maize (Schnee et al. 2002) and one in Norway spruce (Martin et al. 2004). Hence, in screening for terpene synthase induced in *P. sylvestris* after sawfly egg deposition, the focus was put on searching for sequences similar to most of the available gymnosperm sesquiterpene sequences (δ-selinene synthase and E-α-bisabolene synthase from *A. grandis* and γ-humulene synthase from *P. abies*) rather than just on sequences similar to the α-farnesene synthase sequence from *P. taeda*, which has more similarity to monoterpene synthases. Different transcript regulation pattern of identified sequences could then indicate a regulative role of the supposed encoded sesquiterpene synthase in the induced volatile change of *P. sylvestris* resulting in a higher (E)-ß-farnesene emission. A deeper understanding of molecular regulations underlying the induced foliage change could thereby be obtained.

# 3. Materials and Methods

## 3.1 Materials used

### 3.1.1 Plant material

The tissue was harvested and treated in accordance to the earlier experiments by Hilker et al. (2002) and Mumm et al. (2003). The twigs of *Pinus sylvestris* were harvested from 30 to 40-year-old trees from a forest near Berlin. The treated and control branches were kept (with the exception of the jasmonic acid treated twigs) during the whole experiment in tap water at 10°C, 18 hours : 6 hours L:D and 2000 lx. Approximately 20-cm-long twigs were cut from the branches and treated as described below. For every treated twig a control twig was taken, that had been detached from the same branch directly opposite to the twig to be treated. Therefore there was always a paired sample taken of a treated and a control twig. After treatment the needles were separated from the stem, shock-frozen in liquid nitrogen and kept at -80°C.

### 3.1.2. Insects

A herbivorous sawflies *Diprion pini* breeding population was obtained about 15 years ago from C.Géri, INRA Orleans Research Center, France (Institut National de la Recherche Agronomique). The population was kept as described by Bombosch and Ramakers (1976) at 25°C, 18:6 L:D and 70 % relative humidity from then on at Freie Universität Berlin.

### 3.1.3. Bacteria strains and Plasmids

To clone cDNA fragments, the following bacteria strains and plasmids were used:

| Bacteria strains (INVITROGEN) | Genotype | Plasmid (INVITROGEN) |
|---|---|---|
| *Escherichia coli* DH5α | *F-, ϕ80lacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17 ($r_k$-,$m_k$+), phoA, supE44, thi-1, gyrA96, relA1, tonA* | pCR® 4 TOPO |
| *Escherichia coli* TOP 10 | *F- mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G | pCR® 4 TOPO |

### 3.1.4. Oligonucleotides

All oligonucleotide primers were produced by MWG (Germany). The three tables below contain the name, sequence and further information on the oligonucleotide primers used.

**Table 3.1. Degenerate oligonucleotide primers used to amplify cDNA fragments**. All sequences are shown in the 5'-3' direction. The melting temperature ($T_M$) is given in °C as stated by the supplier (MWG). The $T_M$ characterizes the stability of the DNA hybrid formed. At the given $T_M$ value 50 % of a given oligonucleotide is hybridized to its complementary strand. $T_M$= 69.3 + 41 (nG+nC)/s-650/s. Variable annealing temperatures were used depending on the used primer pairs. The right column contains the terpene synthases used for the creation of the degenerate primers (for abbreviation explanation see below)

| Name | sequence | $T_M$ | specific for |
|---|---|---|---|
| ts-132_for | GC(AGC)ACICC(AGC)CTICCITT(CT)(AC)GIGC(AGC) | 66.1 | terpene s. |
| ses-233_for | AA(AG)TA(CT)GCI(CT)TIGA(AG)TT(CT)CCITGGCA(CT)TG(CT) | 64.7 | sesquit. s. |
| lohu-318_for | TGGGTIGTIAT(ACT)GGIACITT(CT) | 53.6 | L, H1, H2, |
| lohu-318_revr | (AG)AAIGTICC(AGT)ATIACIACCCA | 53.6 | L, H1, H2, |
| lohu-408_rev | (CT)TCCCAIGT(CT)TTIC(GT)(AGT)AT(AG)TA | 52.7 | L, H1, H2, |
| lohu-490_rev | IGT(CT)TC(AG)AA(AG)TC(CT)TTI(GC)(AT)(AG)TCIAC | 60.4 | L, H1, H2 |
| lo-284_for | TGTGCACATCATAGAGAACTACAGCTT | 61.9 | L, H1, H2 |
| losel-471_rev | GATCTTGGATGGAGA(AG)TGTATTTGCTC | 62.7 | L1,H1,S1,S2 |

Abbreviations of the terpene synthases sequences used for the creation of the degenerate primers (with the according NCBI accession number):

L: longifolene-synthase-like-protein (*P. abies* )

S1: δ-selinene synthase (*A. grandis*) AAC05727

S2: δ-selinene synthase (*A. grandis*) AAK82561

H1: γ-humulene-synthase-like-protein (*P. abies*) AAK39129

H2: γ-humulene synthase (*A. grandis*) AAC06728

**Table 3.2. Oligonucleotide primers used for the semiquantitative RT PCR**. All sequences are shown in the 5'-3' direction. For all primer pairs 55°C was used as the annealing temperature. The maximum number of cycles where template amplification is in its linear phase at the given annealing temperature is stated

| name | sequence | cycles |
|---|---|---|
| tps1-215_for | AGGGACTCAGAAGTTGCGCAGGTG | 27 |
| tps1-734_rev | TGTTCGGGAGAGGTTGACCCATCAAG | 27 |
| tps2-215_for | TCAGAGTCATATCTGCCGCAGCTG | 27 |
| tps2-734_rev | CGTCTGGTAGAATCTGATCCATTAAC | 27 |
| tps3-223_for | GGATATAGAGAAGCAGAATTTCTA | 35 |
| tps3-690_rev | GTATACACATTCCAGAGCTGGGTT | 35 |
| tps4-30_for | CAGCGTGCCAAGATTGGAGGCA | 30 |
| tps4-227_rev | CTCCGATGTCTGACTCTGTCAT | 30 |
| tub_for | CTCGAGCATGGCATCCAGCCC | 25 |
| tub_rev | CCGTGCAGTTATCGGCTAGCTTCC | 25 |
| 18s_for | CAGTTCTTACAAGGATGCCGACCAG | 20 |
| 18s_rev | GAACCTCCGCGTAGCTAGTTAG | 20 |

**Table 3.3. The oligonucleotide primers used for RACE PCR**. All sequences are shown in 5'-3' direction. All PCR reactions were conducted with 68°C as annealing temperature.

| name | sequence |
|---|---|
| tps4-1144_for | GAAGTTGTGAAAAAGCAAGGGCGG |
| tps4-1167_rev | CCGCCCTTGCTTTTTCACAACTTC |
| tps4-1579_for | GGAGTTTATAAAGCAGGACAGTG |
| tps4-1601_rev | CACTGTCCTGCTTTATAAACTCC |
| tps4-1668_for | GGCTTCTCCATTTCTACCAAGG |
| tps4-1695_rev | CTTTGGTAGAAATGGAGAAGC |
| tps4-80_for | GAAACGACTCATGGCTCAAGTCA |
| tps4-305_rev | GGAGAAAACTGGGGCTGGAACG |

### 3.1.5. Chemicals

All chemicals were purchased from BIO-RAD, DIFCO, ROTH or SIGMA-ALDRICH, if not stated differently.

## 3.2. Treatment of pine needles

The twigs were placed into a plastic box (20 cm x 20 cm x 8 cm) and covered by a gauze lid. Probes were harvested at 48h or 72h after induction. There were two treatment occasions. In the summer of 2002, all probes were harvested after 72h and in the winter of 2003 probes were harvested at 48 and 72 hours after induction. Different treatments were applied. Table 3.4 gives an overview of all applied treatments.

### 3.2.1 Induction by insect egg deposition

Two female and two male *D. pini* were placed in a plastic box with a pine twig. The insects were placed in the box with the pine twigs approximately 12 hours prior to other applied treatments (time point zero) to allow time for the insects to mate and to lay eggs. The insects were kept in the box with the pine twig until the termination of the experiment. The needles were harvested either 48h or 72h after induction. After 72h the pine twigs had between 8 to 12 egg masses.

To mimic the cut of the needles by the ovipositor valves of the female *D. pini* eight to ten pine needles of the control twigs were slit open with a needle at time point zero. They were slit open along the midrip to create a cut of about 2 cm length and 2 mm depth. The probes taken in winter 2003 also included an undamaged control. The twigs were then just placed into a plastic box.

### 3.2.2 Induction by jasmonic acid

During the experimental treatment the twigs were supplied with a Tween 20 solution (0,05% v/v ) that contained 0.3 mM of jasmonic acid (Sigma, Germany). This solution was applied through the stem. The control twigs were supplied with a Tween 20 solution. The solutions were renewed every day.

### 3.2.3. Induction by methyl jasmonate

At time point zero, prior to placing the twigs into the plastic box, a Tween 20 solution was sprayed on the needles containing 100 µM methyl jasmonate (SIGMA). The control twigs were sprayed with a Tween 20 solution.

**Table 3.4 Applied treatments and number of samples**. Shown is the time after treatment when the needles of *P. sylvestris* twigs were harvested. The numbers indicate the amount of samples taken at that point.

| Treatment | Control | samples in summer | samples in winter | |
|---|---|---|---|---|
| | | 72h | 48h | 72h |
| egg deposition | artificially damaged | 3 | 1 | 1 |
| | undamaged (only in summer) | | 1 | 1 |
| jasmonic acid (0.3 mM in water) | Tween 20 (0.05 %) in water | 3 | 1 | 1 |
| methyl jasmonate (0.1 mM sprayed) | Tween 20 (0.05 %) sprayed | 3 | 1 | 1 |

## 3.3. Isolation of nucleic acids

### 3.3.1 Isolation of RNA and cDNA-synthesis

The harvested needles were kept at -80°C. The plant material was ground with sand in a ceramic grinder in liquid nitrogen to a fine powder. To isolate RNA the Invisorb Spin Plant RNA Mini Kit (INVITEK) was used and the listed protocol was followed. Approximately 300 mg of ground plant material was used per RNA isolation and between 100 and 1300 ng/µl RNA was isolated from the tissue. The final RNA was eluted with 30 µl of the supplied RNA-se free deionized water.

To generate cDNA from the extracted RNA the SuperScript™ III (INVITROGEN) was used. Three µg of RNA and 1 µl of oligo $(dT)_{20}$ (INVITROGEN) were employed and the following steps were done as suggested by the protocol. For further use of the cDNA in semiquantitative RT-PCR and ordinary PCR reactions, the product was diluted with 30 µl of ionized water to attain a final volume of 50 µl.

### 3.3.2 Plasmid DNA extraction from *E. coli*

Solutions

| | | |
|---|---|---|
| Lysis buffer: | 50 mM | Tris/HCl (pH 8.0) |
| | 50 mM | EDTA (pH 8.0) |
| | 15 % (w/v) | Saccharose |
| Alkaline SDS solution: | 200 mM | NaOH |
| | 1 % (w/v) | SDS |
| Potassium acetate solution; | 3 M | $CH_3COOK$ |
| | 11.5 % (v/v) | $CH_3COOH$ |

To extract larger amounts of plasmid DNA, the alkaline lysis protocol was used. For this method 2 ml of a bacterial culture (of a 5 ml overnight culture in LB-Müller, GIBCO) was centrifuged for 2 minutes at 16000 x *g*. After resuspending the pellet in 100 µl of lysis

buffer by vortexing, the cells were lysed with 200 µl of freshly-made alkaline SDS solution. For neutralization 150 µl of potassium acetate solution was added. The mixture was incubated for 10 minutes on ice and then centrifuged for 10 minutes at 16000 x *g* at 4°C. The supernatant was mixed with 0.6 volumes of isopropanol and then incubated for 10 minutes on ice to precipitate the plasmid DNA. After another centrifuge step (15 minutes at 16000 x *g* at 4°C) the DNA was washed with 70% ethanol. To solubilise the DNA-pellet 30 µl of deionized water was used.
Smaller amounts of plasmid DNA were extracted using the NucleoSpin® Plasmid Kit with the recommended protocol (MACHEREY-NAGEL). The bacterial-pellet was obtained as described above.

## 3.4. Analyzing nucleic acids

### 3.4.1 Amplification of DNA

#### 3.4.1.1 Amplification of DNA via Polymerase chain reaction (PCR)

The polymerase chain reaction was used to amplify cDNA of *P. sylvestris* with specific and degenerated primers. Taq Polymerase from PROMEGA and AMERSHAM was used.
The usual PCR consisted of:

| | |
|---|---|
| 0.2 µl | Taq DNA Polymerase ( 5 U/µl) |
| 2.5 µl | 10 x PCR-buffer for Taq Polymerase |
| 1 µl | dNTPs (10 mM each) |
| 1 µl | Primer 1 (10 pmol/µl) |
| 1 µl | Primer 2 (10 pmol/µl) |
| 0.2-3 µl | cDNA (1:2.5 dilution of the cDNA synthesis reaction) |
| ad 25 µl | $H_2O$ |

For the polymerase chain reaction a Thermocycler Tpersonal® (BIOMETRA) was used. The heat block of the thermocycler was preheated to 94°C and the lid to 110°C prior to insertion of the reaction tubes. The following temperature program was applied with a 4° temperature ramp :

| | | | |
|---|---|---|---|
| 94 °C | 130 sec | first denaturation | |
| 94 °C | 40 sec | denaturation | |
| annealing temperature | 40 sec | annealing | |
| 72 °C | 70 sec | synthesis | 30 cycles |
| 72 °C | 300 sec | final synthesis | |

### 3.4.1.2 Amplification of cDNA via semiquantitative RT PCR

When conducting a semiquantitative RT-PCR, one assumes that the documented amount of an amplified cDNA-Fragment is directly related to the amount of transcript of that fragment in the whole RNA (figure 3.1). Hence, the band thickness of a PCR product separated on an agarose gel and measured by ethidium bromide fluorescence reveals the transcript level of the amplified gene at the time of sampling.

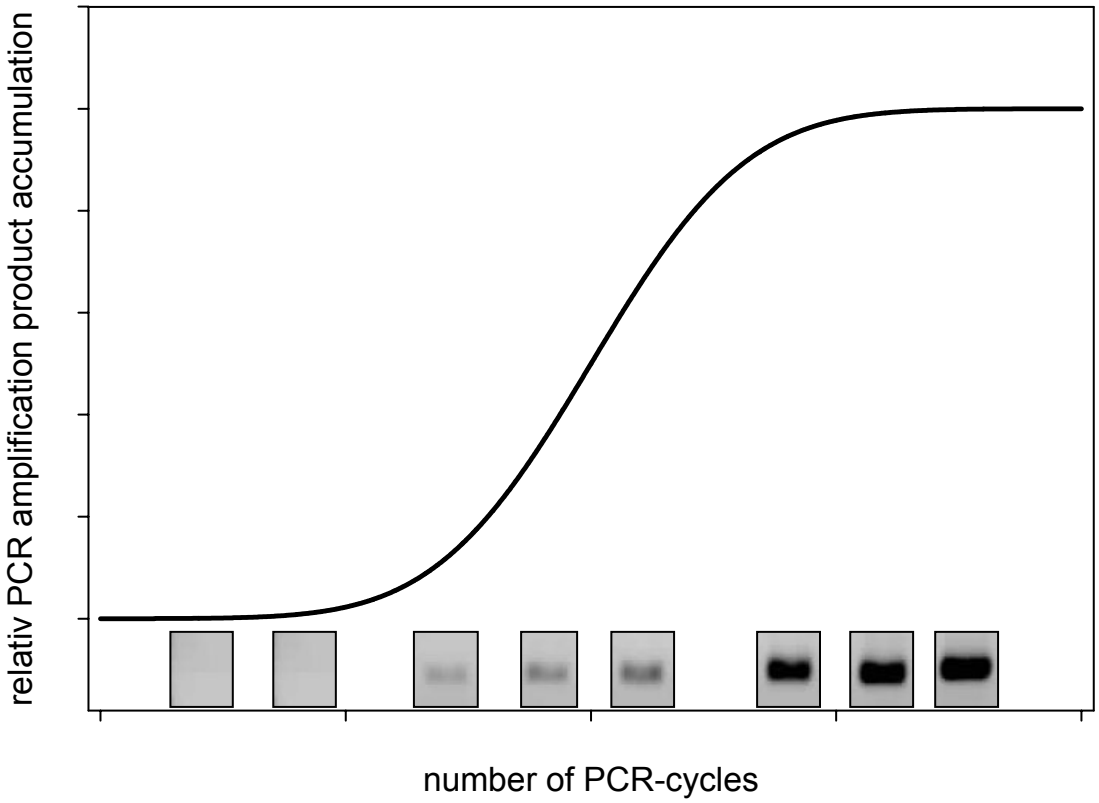



**Figure 3.1 Graphic model of a PCR reaction**. The graph shows the optimal amplification curve during a given amount of PCR cycles. Examples of agarose gel pictures of a PCR stopped at different cycle numbers are given.

To minimize possible differences in the template concentration used two pairs of so called ‘control primers’ have been used. Such primer pairs should amplify cDNA fragments whose expression rate in the test-organism is relatively even. Hence, they should be primers for so called ‘house-keeping-genes’ or other components of the RNA that are at constitutive levels. For this experiment, α-tubulin-primers and 18s rRNA primers were chosen. Both α-tubulin and ß-tubulin are the main components of the cell cytoskeleton. Thus, α-tubulin should be transcribed at a constant level throughout the life span of a tissue and should therefore be a good normalizer for the messenger RNA concentration in a given sample (Hietala et al. 2004). Ribosomal RNA is expressed constantly at the same high rates and thus can be used as an indicator and normalizer for total RNA concentration (in contrast to mRNA concentration).

After extracting RNA and the synthesis of cDNA (as described in 3.3.1), 1 µl of the cDNA was used in a PCR reaction. By documenting the photometric density units of the bands from the control primer pairs 18s rRNA and α-tubulin, the total amount of RNA and mRNA in each cDNA sample could be determined. By adjusting the used amount of cDNA for every sample accordingly, a relative even cDNA concentration for all samples could be assured.
For every tested template a separate master mix was prepared that contained all ingredients except for the primer pair. The master mix was then separated into PCR-tubes that held the primer pair to be tested. The RT-PCR reaction consisted of the components as listed in 3.4.1.1.

All semiquantitative RT-PCR-reactions have been conducted in a Thermocycler Tpersonal® (BIOMETRA). The following temperature program was always used for semiquantitative RT-PCR. The first and the final synthesis was done with a 4° C temperature ramp, all other steps were done with a 2°C temperature ramp.

| | | |
|---|---|---|
| 94 °C | 130 sec | first denaturation |
| 94 °C | 40 sec | denaturation |
| 55 °C | 40 sec | annealing |
| 72 °C | 70 sec | synthesis |
| 72 °C | 300 sec | final synthesis |

The optimal linear phase of the PCR-reaction was identified for every primer pair. All PCR-reaction were conducted with this amount of cycles from then on (for used cycles for each primer pair see table 3.2).
15 µl of the reaction volume was transferred to the gel slots of a 1.1 % agarose gel (containing 50 µg ethidium bromide / 100 ml) and was subjected to electrophoresis for 30 minutes at 100 volts. The ethidium bromide stain associated with each PCR product was detected by the Genegenius Bio Imaging system (Merck). Quantitative differences within the agarose gels were determined with the Gene Tools® (SYNGENE) computer program. The different intensity of bands was recognized as photometric density units. The data for each PCR-Fragment was then normalized relative to the detected amount of α-tubulin and / or 18s rRNA in the same template.

### 3.4.2 Cloning

For the ligation and cloning of PCR-Fragments, the TOPO® TA Cloning Kit for Sequencing (INVITROGEN) was used. The sequence was amplified by a Taq-Polymerase to assure that all DNA-Fragments had single, multiple desoxyadenosine molecules on both ends (so called 'A-overhangs'). The PCR-Fragment was cloned in the vector (pCR® 4-TOPO®) following the proposed procedure in the manual.
The plasmid DNA was transformed according to the manual in One Shot TOP 10 (INVITROGEN) chemical competent *E.coli* cells. After incubation for one hour in SOC medium, the bacteria were spread on LB medium plates (LB Lennox, IDG, with 1 % w/v agar) that contained the appropriate amount of the required antibiotic and incubated at 37 °C overnight.

| | | |
|---|---|---|
| SOC medium | 0.5 % (w/v) | yeast extract |
| | 2 % (w/v) | tryptone |
| | 10 mM | Na Cl |
| | 2.5 mM | K Cl |
| | 10 mM | $Mg\ Cl_2$ |
| | 10 mM | $Mg\ SO_4$ |
| | 20 mM | Glucose |

### 3.4.3 Restriction enzyme digests

The following enzymes were used according to their properties with the suggested buffer to digest DNA.

*Eco*RI (2000 U/ml, NEW ENGLAND BIOLABS), Spe I (10.000 U/ml, NEW ENGLAND BIOLABS) and NOT (10.000 U/ml, NEW ENGLAND BIOLABS). Incubation lasted usually 1 h at 37°C in a water bath.

### 3.4.4 DNA sequencing

To analyze the sequence of the selected PCR fragment the BigDye™Kit (PERKIN-ELMER) was used. Each stop nucleotide is here labeled with a different fluorescent marker.

The contents of a 20 µl sequencing reaction were:

1 µl of a 100 ng/µl plasmid DNA concentration

2 µl Primer (sequencing primer, 10 pmol/µl)

4 µl Big Dye™ Mix (Taq Pol fs, $MgCl_2$,dNTPs, ddNTPS: dR 110=G, dR GG=A, dTamara=T, dRot=C, reaction buffer)

10 µl $H_2O$

All sequencing reactions were done with the following temperature program in a Thermocycler Tpersonal® (BIOMETRA)

| | | |
|---|---|---|
| 96 °C | 300 sec | |
| 96 °C | 10 sec | 35 cycles |
| 55 °C | 20 sec | |
| 60 °C | 240 sec | |

To purify the DNA fragment after the sequencing reaction the DyeEx 2.0 Spin Kit was used according to the manual. The sequencing was done with an ABI PRISM® 3100 Genetic Analyzer (APPLIED BIOSYSTEMS).

The software Windows 32 SeqMan 5.00® 1989-2001 DNASTAR Inc was used to analyze the sequence data. The new sequences were compare to published sequences with the Software BLAST 2.0 of the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov, Bethesda, USA ).

### 3.4.5 Agarose gel electrophoresis

Used solution

| | | |
|---|---|---|
| 1 x TBE-buffer: | 100 mM | Tris |
| | 100 mM | Boric acid |
| | 2 mM | EDTA (pH 8.0) |
| Loading buffer: | 50 % (v/v) | Glycerol |
| | 0.25 % (v/v) | Orange G |
| 1 mM | EDTA (pH 8.0) | |

To visualize DNA- or RNA-Fragments, an 0.8 – 1.5 % agarose gel (w/v) was used. The agarose gels contained final concentration of 1 ng/ml ethidium bromide. To prepare the gels for the iMupid® Electrophoresis Unit (COSMO BIO), 0.5 % TBE buffer was used. The samples were mixed with 3 µl of the loading buffer and poured into the gel slots. The gels were usually run at 7 V/cm for about 30 minutes.
As a gel standard 0.7 µl of a 100bp DNA Ladder (1 µg/µl INVITROGEN) or a 1 kb DNA Ladder (1 µg/µl INVITROGEN) was used, depending on the expected length of the DNA-Fragment. The bands were documented by GenGenius Bio Imaging system (MERCK).

### 3.4.6 DNA purification by gel extraction

DNA-Fragments of interest were cut out of the gel with a razor blade under UV-light. For extraction the QIAquick Gel Extraction Kit (QIAGEN) was used. The following steps were done as suggested by the manual. The DNA was eluted from the membrane with 30 µl of deionized water that was heated up to 70 °C. The eluted and purified DNA was stored at -20 °C.

## 3.5 Generating full-length cDNA transcripts

### 3.5.1 First-strand cDNA synthesis

To generate the full length cDNA transcript of the isolated cDNA fragments the BD SMART™ RACE cDNA Amplification Kit (BIOSCIENCES CLONTECH) was used.
For the first strand cDNA synthesis 1 µg of total RNA (isolation as described in 3.3.1) was used.
The following modified procedure was applied:

| for the preparation of 5'RACE-Ready cDNA | |
|---|---|
| 0.8 µl | total RNA |
| 0.5 µl | dNTP (10 mM each) |
| 0.8 µl | 5' CDS primer |
| 0.8 µl | BD SMART II A oligo (only in 5'RACE) |
| ad 7 µl | $H_2O$ |

The contents were mixed and incubated for 5 minutes at 65 °C and for 1 minute at 4°C.

Then the following ingredients were added into each reaction tube:

| | |
|---|---|
| 2 µl | 5 x first-strand buffer |
| 0.5 µl | 0.1 M DTT |
| 0.5 µl | SuperScript III RT (200 U / µl) |
| 10 µl total volume | |

The contents were mixed and incubated at 50 °C for 60 minutes. The reaction was then inactivated by heating at 70 °C for 15 minutes. The synthesized cDNA was diluted with the supplied Tricine EDTA buffer and stored at -20°C.

### 3.5.2 Rapid Amplification of cDNA Ends (RACE)

For the RACE-PCR-reactions the procedure was followed as suggested in the supplied manual. The reaction was conducted for the Ps*TPS4* Fragment.

The following gene-specific primers have been used (for sequence-information see 3.1.4):

| | 5' RACE | 3' RACE |
|---|---|---|
| 1st PCR-reaction | tps4-227_rev<br>tps4-305_rev | tps4-30_for |
| 2nd nested PCR reaction | tps4-227_rev | tps4-80_for |

For the nested PCR-reaction two template-dilutions were tested: the undiluted reaction from the first RACE-PCR-reaction and a dilution of 5 µl PCR-reaction with 122.5 µl of deionized water.
The following temperature program was used for all RACE-PCR-reactions, with a 1°C temperature ramp.

| | | |
|---|---|---|
| 94 °C | 130 sec | first denaturation |
| 94 °C | 30 sec | denaturation |
| 68 °C | 30 sec | annealing |
| 72 °C | 180 sec | synthesis |
| 72 °C | 300 sec | final synthesis |

## 3.5. Statistical analysis

For statistical analysis, the Software Statistica 4.5, scientific software (StatSoft, Germany) program was used. As the sample numbers were not sufficient to undergo serious statistical analysis, only selected data was tested statistically by a One-Way-ANOVA in an effort to support the observed tendencies. In the One-Way-ANOVA summer and winter samples were tested separately. Test and control values were compared to identify relative transcript level differences. For the winter samples, the values for each treatment were compared at both sampling times. (e.g. Ja 48h versus Ja 72h) to identify a relative transcript level increase over time. In the corresponding graphs only statistical significant differences are shown.

# 4. Results

## 4.1 Screening for sesquiterpene synthase like sequences from *Pinus sylvestris* using degenerate primer pairs

To find sesquiterpene synthase-like gene fragments in *Pinus sylvestris*, degenerate primer pairs were designed (table 3.1). Sequence information from published sesquiterpene synthase sequences from conifers were used to design the degenerate primers. The purified cDNA from oviposition-induced pine needles harvested 72 hours after induction, was used for the PCR reactions with the degenerate primer pairs. The amplified fragments were isolated, cloned and sequenced. The NCBI-Database was used to find published sequences with similarities to the new sequences (table 4.1).

**Table 4.1**: **Sesquiterpene synthase gene-fragments found in *P. sylvestris*.** The names of the *P. sylvestris* sequence fragments are shown. The degenerate primer pairs used are stated as well as the name and NCBI accession number of the sequence with the highest amino acid similarity to the new sequence (using the NCBI database BLAST program). The next column shows the amino acid identity (aa identity) of both sequences in percent. The corresponding e value (stands for expect value) shows the statistical significance threshold for the reported matches against the NCBI database sequences (according to Kariln and Altschul (1990)). The default value is 10. Hence, lower expect thresholds are more stringent, leading to fewer chance matches being reported

| name | degenerate primer pair | sequence with the highest similarity | aa identity (in %) | e - value |
|---|---|---|---|---|
| Ps*TPS1* | ses-233_for losel-471_rev | δ-selinene synthase (*A. grandis*) AAK83561 | 74 | $6e^{-91}$ |
| Ps*TPS2* | ses-233_for losel-471_rev | γ-humulene synthase like protein (*P. abies*) AAK39129 | 62 | $2e^{-73}$ |
| Ps*TPS3* | lohu-284_for losel-471_rev | γ-humulene synthase like protein (*P. abies*) AAK39129 | 84 | $2e^{-94}$ |
| Ps*TPS4* | ses-233_for lohu-318_rev | δ-selinene synthase (*A. grandis*) AAK83561 | 75 | $2e^{-27}$ |

Four different cDNA sequences were identified (table 4.1), all having high similarity to known sesquiterpene synthases from conifers. They were named as *Pinus sylvestris* terpene synthase 1 to 4 (Ps*TPS1*, Ps*TPS2*, Ps*TPS3* and Ps*TPS4*). The sequences Ps*TPS1* and Ps*TPS2* were both amplified by the same primer pair.

Ps*TPS1* and Ps*TPS4* are most similar to the same δ-selinene synthase sequence from *Abies grandis*. The amino acid identity of the δ-selinene synthase to Ps*TPS1* is 74 percent and to Ps*TPS2* 75 percent.

Ps*TPS2* and Ps*TPS3*, however, are most similar to the same γ-humulene synthase like protein from *Picea abies*. The Ps*TPS2* amino acid sequence is only 62 percent identical to the amino acid sequence of the γ-humulene synthase like protein, whereas Ps*TPS3* shows a 84 percent identity to the γ-humulene synthase like amino acid sequence.

```
1   KYALEFPWHCSAPRWEARSFIEIYGENHSWLKSNFNRTVLELAKLDFNILQCIHQKEMQYITRWWRDSEVAQVNF PsTPS1
1   NA--------------------------------------------------THKEEMQLISRWWSDLYLPQLDF PsTPS2
1   CA--------------------------------------------------HHRE-LQLLSRWWSQSDIEKQNF PsTPS3
1   KYALEFPWHCXVPRLEARSFIEIYGRNXSWLKSNINQNFLELAKLDFNILQFTHQKDIQLISRWMTESDIGELNF PsTPS4

76  YRRRHMELYFWAVISIFEPEFSRSRIAFAKVTTVGTVIDDLYDTYGMLDELKTITEGVRRWDISLIDDLPEKIKI PsTPS1
26  YRKRHVELYFWAVLGTFEPEFRSSRIAFTKLSTVMTVIDDLYDTHGTLDEIKIFTEGVRRWDTSLIXRLPDHIQK PsTPS2
25  YRKRHVEFYFWMVIGTFEPEFSSSRIAFAKIATLMTIIDDLYDTHGTLEQLKIFTEAVKRWDLSLQXRLPDYIKI PsTPS3
76  YRKRHVELXFWVVIGTF                                                           PsTPS4

151 AIQFFFNTANELAAEVVSNQGPDTSAILK--DWVRYLESYLQEAEWITTGCVPTFNEYIKNAVASSGMCIVNLIP PsTPS1
101 IFEFFMKTSNEWTAEXEKKQGRDMAAYIRKNGWERYVESYLQEGEWMAAGYVPSFNEYYKNGLASSGMCVXNLIP PsTPS2
100 TLEFFFNTSNELNAEVAKMQERDMSAYIRKAGWERYXEGYMQESEWMAARHVPTFDDYMKNGKXSSGXCILNLYP PsTPS3
92                                                                              PsTPS4

224 LLLMGQPLPNNILEQI                                                          PsTPS1
176 LLLMDQILPDDILEQIHSHPD                                                    PsTPS2
175 LLLMGQLVPDNILEQIHSPSKI                                                   PsTPS3
92                                                                           PsTPS4
```

**Figure 4.1 Alignment of the four *P. sylvestris* sesquiterpene synthases sequence fragments**. The DDxxD-motif of the amino acid sequence is highlighted to verify the accuracy of the alignment. Consensus sequence parts are grey.

The amino acid sequences of Ps*TPS1*, Ps*TPS2*, Ps*TPS3* and Ps*TPS4* are shown in figure 4.1.

Table 4.2 lists the seven amino acid sequences available in the NCBI databases that show the highest similarity to the Ps*TPS4* sequence. All of them code for terpene synthases from gymnosperms. The first four are from other members of the Coniferophyta.

**Table 4.2 Sequences that produce a significant alignment with Ps*TPS4***. Listed are the seven sequences and the organism they originate from, showing the highest similarity to *PsTPS4* according to the NCBI database. The accession number and e value (for details see table 4.1) are stated.

| sequence name | accession number | e-value |
|---|---|---|
| δ-selinene synthase *A. grandis* | AAK83561 | $2e^{-19}$ |
| δ-selinene synthase *A. grandis* | AAC05727 | $1e^{-18}$ |
| γ-humulene synthase like protein *P. abies* | AAK39129 | $2e^{-17}$ |
| γ-humulene synthase *A. grandis* | AAC05728 | $7e^{-17}$ |
| limonene/borneol synthase *C. obtusa* | BAC92722 | $2e^{-13}$ |
| levopimaridiene synthase *G. biloba* | AAL09965 | $9e^{-12}$ |
| abietadiene synthase *A. grandis* | AAK83563 | $6e^{-11}$ |

## 4.2 Amplification of the Ps*TPS* sequence fragments and assessment of their relative transcript levels

To amplify the gene fragments, identified by the degenerate primers, homologous primer pairs were designed (table 3.2). The isolated corresponding sequences were compared and corrected to obtain the consensus sequences. The consensus sequences were compared with the initial sequence to control for the specificity of the used primer pairs.

The relative transcript level accumulation of Ps*TPS1*, Ps*TPS2*, Ps*TPS3* and Ps*TPS4* in the harvested samples was assessed by semiquantitative RT-PCR.
The linear amplification phase of the PCR reaction has been assessed for the primer pairs tps1-215_for-tps1-734_rev, tps2-215_for-tps2-734_rev, tps3-223_for-tps3-690_rev and tps4-30_for-tps4-227_rev (table 3.2). To correct for variations in the PCR-reactions, all experiments have been repeated at least three times. The cDNA synthesis from the harvested pine needles has been repeated at least twice so as to control for possible differences in cDNA quality. For some of the samples, RNA was isolated more than once. A summary of all conducted preparations is given in table 4.3.
It was only possible to generate PCR products visible on ethidium bromide treated agarose gels after 35 amplification cycles with undamaged control tissue cDNA using the two normalization primer pairs. This is an extremely high number of cycles and does not correspond with the number of cycles established for the normalization primer pairs for the other probes (table 3.2). Therefore it was not possible to use the cDNA from these probes in the relative transcript level analysis.

**Table 4.3 Summary of all performed RNA isolations, cDNA synthases and semiquantitative RT-PCR reactions from the needle samples**. Shown are the number of samples taken at each harvest and the RNA isolations performed from that sample. 'W': samples taken in winter, 'S': samples taken in summer. The number given in the cDNA column are the cDNA syntheses completed from each RNA sample. The given number of semiquantitative RT-PCRs reveals the total amount of RT-PCR reactions conducted with all cDNA templates and all Ps*TPS* primer pairs from all samples harvested and treated from the first column. Detailed results for all primer pairs are shown in the following graphs (starting with figure 4.4).

| treatment and time of sampling | | samples | RNA isolation per sample | cDNA synthesis per sample | semiq. RT-PCRs |
|---|---|---|---|---|---|
| egg deposition (W) | 48h | 1 | 1 | 2 | 35 |
| | 72h | 1 | 1 | 3 | 35 |
| control damaged (W) | 48h | 1 | 3 | 2 | 35 |
| | 72h | 1 | 2 | 3 | 35 |
| control undam. (W) | 48h | 1 | 2 | 2 | 2 |
| | 72h | 1 | 1 | 2 | 2 |
| Ja (W) | 48h | 1 | 3 | 3 | 20 |
| | 72h | 1 | 2 | 1 | 20 |
| Ja control (W) | 48h | 1 | 2 | 1 | 20 |
| | 72h | 1 | 2 | 2 | 20 |
| MJ (W) | 48h | 1 | 2 | 2 | 15 |
| | 72h | 1 | 2 | 2 | 15 |
| MJ control (W) | 48h | 1 | 1 | 2 | 15 |
| | 72h | 1 | 2 | 2 | 15 |
| egg deposition (S) | 72h | 2 | 1 | 2 | 21 |
| control dam. (S) | 72h | 2 | 1 | 2 | 21 |
| Ja (S) | 72h | 2 | 1 | 3 | 28 |
| Ja control (S) | 72h | 2 | 1 | 2 | 28 |
| MJ (S) | 72h | 2 | 1 | 2 | 20 |
| MJ control (S) | 72h | 2 | 1 | 2 | 20 |

### 4.2.1 Characterization of Ps*TPS1*

#### 4.2.1.1 Primer specificity of the Ps*TPS1* primer pair

To accurately amplify the DNA fragment produced by degenerate primers, specific primer sites were chosen. The primer pair tps1-215_for and tps1-734_rev was produced and used for the specific fragment amplification.

After conducting PCR reactions with this primer pair using cDNA produced from Ja and MJ treated plant samples harvested 72 hours after treatment in winter, the fragments were cloned and seven clones were sequenced. The consensus sequence was compared with alignments to the original sequence to confirm the specificity of the designed oligonucleotides (figure 4.2). The nucleotide sequences show an identity of 96 percent to each other.

```
1   AGGGACTCAGAAGTTGCGCAGGTGAATTTCTATAGGAGGCGTCACATGGAACTTTACTTTTGGGCGGTTA PsTPS1
1   AGGGACTCAGAAGTTGCGCAGGTGAATTTCTATAGGAGGCGTCACATGGAACTTTACTTTTGGGCGGTTA HP PsTPS1

71  TATCCATTTTCGAGCCCGAGTTTTCTCAAAGCAGAATCGCCTTCGCAAAAGTTACTACCGTCGGAACTGT PsTPS1
71  TATCCATTTTCGAGCCCGAGTTTTCTCAAAGCAGAATCGCCTTCGCAAAAGTTACTACCGTCGGAACTGT HP PsTPS1

141 TCTAGATGACCTTTATGATACCTACGGAATGCTAGACGAACTGAAAACCATCACAGAGGGAGTGAGACGA PsTPS1
141 TCTAGATGACCTTTATGATACCTACGGAATGCTAGACGAACTGAAAACCATCACAGAGGGAGTGAGACGA HP PsTPS1

211 TGGGATATTTCGTTGATAGACGACCTTCCGGAAAAAATAAAAATTGCAATCCAGTTTTCCTTCAATACAG PsTPS1
211 TGGGATATTTCGTTGATAGACGACCTTCCGGAAAAAATAAAAATTGCAATCCAGTTTTTCTTCAATACAG HP PsTPS1

281 CGAATGAACTGGCCGCTGAAGTCGTAAGCAAGCAAGGGCCGGACACATCAGCCATAWTAAAARATACCTG PsTPS1
281 CGAATCAACTGGCCGCTGAAGTCGTAAGCAAGCAAGGGCCGGACACATCAGCCATATTAAAAGATACCTG HP PsTPS1

351 GGTGCGATACCTTGAGTCTTATTTGCAAGAAGCGGAACTGGATAACAACTGGTTGTGTGCCGACGTTTAA PsTPS1
351 GGTGCGATACCTTGAGTCTTATTTGCAAGAAGCGGAA-TGGATAACAACTGGTTGTGTGCCGACGTTTAA HP PsTPS1

421 TGACGTACATAAAGARAGTGCCGTCGCTAGCTCAGGGATGTGCATCGTAAATTTGATTCCGCTTCTCTTG PsTPS1
420 TGA-GTACATAAAGAA--TGCCGTCGCTAGCTCAGGGATGTGCATCGTAAATTTGATTCCGCTTCTCTTG HP PsTPS1

491 ATGGGTCAACCTCTCCCGAACA                                                  PsTPS1
487 ATGGGTCAACCTCTCCCGAACA                                                  HP PsTPS1
```

**Figure 4.2 Alignment of Ps*TPS1*.** The nucleotide sequence alignment of Ps*TPS1* is compared with the amplified product from the tps1-215_for-tps1-734_rev primer pair (HP for homologous primer product). The sequences have a nucleotide identity of 96 % to each other. The letters R and W indicate contradicting nucleotide information. R stands for adenine or guanine and W stands for adenine or thymine in that position.

The specific primer-binding-sites can be recognized at the 5'-end and the 3'-end of the newly amplified sequence (figure 4.3)

Thus the tps1-215_for and tps1-734_rev primer are specific to the Ps*TPS1* sequences for the tested sample tissue

**AGGGACTCAGAAGTTGCGCAGGTGA**ATTTCTATAGGAGGCGTCACATGGAACTTTACTTTT 60

GGGCGGTTATATCCATTTTCGAGCCCGAGTTTTCTCAAAGCAGAATCGCCTTCGCAAAAGT 120

TACTACCGTCGGAACTGTTCTAGATGACCTTTATGATACCTACGGAATGCTAGACGAACTG 180

AAAACCATCACAGAGGGAGTGAGACGATGGGATATTTCGTTGATAGACGACCTTCCGGAAA 240

AAATAAAAATTGCAATCCRGTTTTTCTTCAATACAGCGAATGAACTGGCCGCTGAAGTCGT 280

RAGCAAGCAAGGGCCGGACACRTCAGCCATATTAAAAGATACCTGGGTGCGATACCTYGAG 300

TCTTATYTGCAAGAAGCSGAATGGATAACARCTGGTTGTGTGCCGACGTTTAATGAGTACA 360

TAAAGAATGCCGTCGCTAGCTCAGGGATGTGCATCGTAAATTTGATTYCGCTTCT**CTTGAT**

**GGGTCAACCTCTCCCGAACA**

**Figure 4.3 Amplified nucleotide sequence of Ps*TPS1***. The primers used to amplify the sequence are shown in bold.

#### 4.2.1.2 The relative transcript level of Ps*TPS1*

##### 4.2.1.2.1 Ps*TPS1* in oviposition induced needles

The relative transcript level of Ps*TPS1* increases in pine needles containing sawfly eggs as well as in artificially damaged pine needles after each treatment was applied (figure 4.4). When comparing the relative transcript level in both treatments 48 hours and 72 hours after application, there is a two-fold increase in relative transcript levels evident in both treatments.

At both sampling times (48h and 72h) the needles containing eggs reveal a higher relative transcript level than the corresponding control needles. 48 hours after treatment the test needles have a 13 percent higher relative transcript level compared to the control needles when the data are normalized by comparison to α-tubulin. The relative transcript level is 16 percent higher in the test needles when the raw data are normalized by 18s rRNA.

The difference in relative transcript levels between test and control twigs is somewhat greater 72 hours after the start of the experiment. The raw data normalized against α-tubulin show a 28 percent difference for the samples harvested in winter, the corresponding samples harvested in summer reveal a 32 percent difference in relative transcript level for both normalization modes. However, the relative transcript level difference of the samples taken in winter show only a 6 percent divergence between test and control needles when the data were normalized against 18s rRNA. The relative transcript accumulation difference of Ps*TPS1* between test and control needles is statistically significant for the values resulting from by α-tubulin normalization ($p<0.001$ for winter twigs, $p<0.05$ for summer twigs) but not for the values resulting from 18s rRNA normalization. The relative transcript values of the two twigs harvested in summer normalized by 18s rRNA were not homogeneously distributed, they are significantly differently distributed ($p=0.02$).

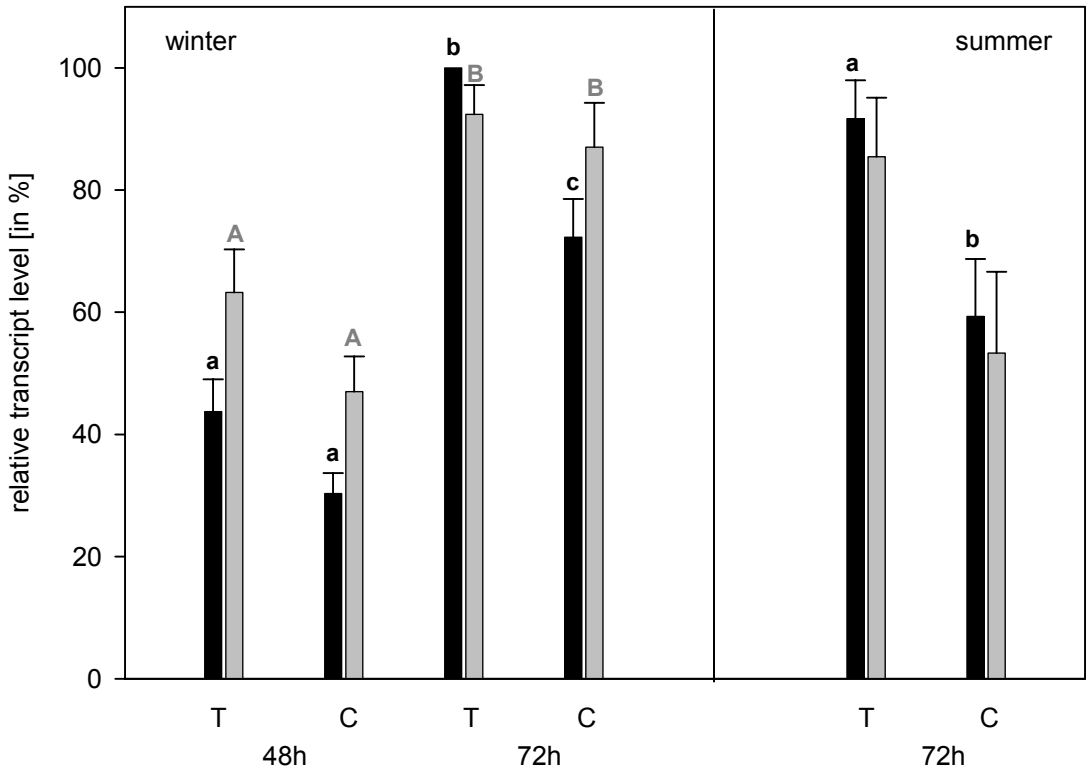



**Figure 4.4 The relative transcript level of Ps*TPS1* in oviposition induced and control pine needles**. The columns illustrate the mean relative transcript level and the standard error determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
Winter N=9, Summer α-tubulin N=10 (5/5), Summer 18s rRNA N=9 (3/4).
'T' represents test and 'C' control treatment (artificially wounded). 'N' indicates the number of semiquantitative RT-PCR reactions from which the mean and standard error were calculated. When the number of reactions was different between the two normalization modes, they are shown separately. In winter 'N' counts for semiquantitative RT-PCR of one twig. In summer 'N' shows the total amount of PCR reactions done with at least two cDNA isolations of both twigs. The numbers in parentheses show the amount of semiquantitative RT-PCR reactions conducted with each twigs.
Different letters indicate significant ($p<0.05$) differences per normalization method evaluated by a One Way ANOVA.

#### 4.2.1.2.2 Ps*TPS1* in jasmonic acid treated needles

The relative transcript level of Ps*TPS1* declines in pine needles treated with jasmonic acid, but also in the corresponding control needles (figure 4.5). There is no difference in the relative transcript accumulation visible between test and control needles 48 hours after the start of the experiment.

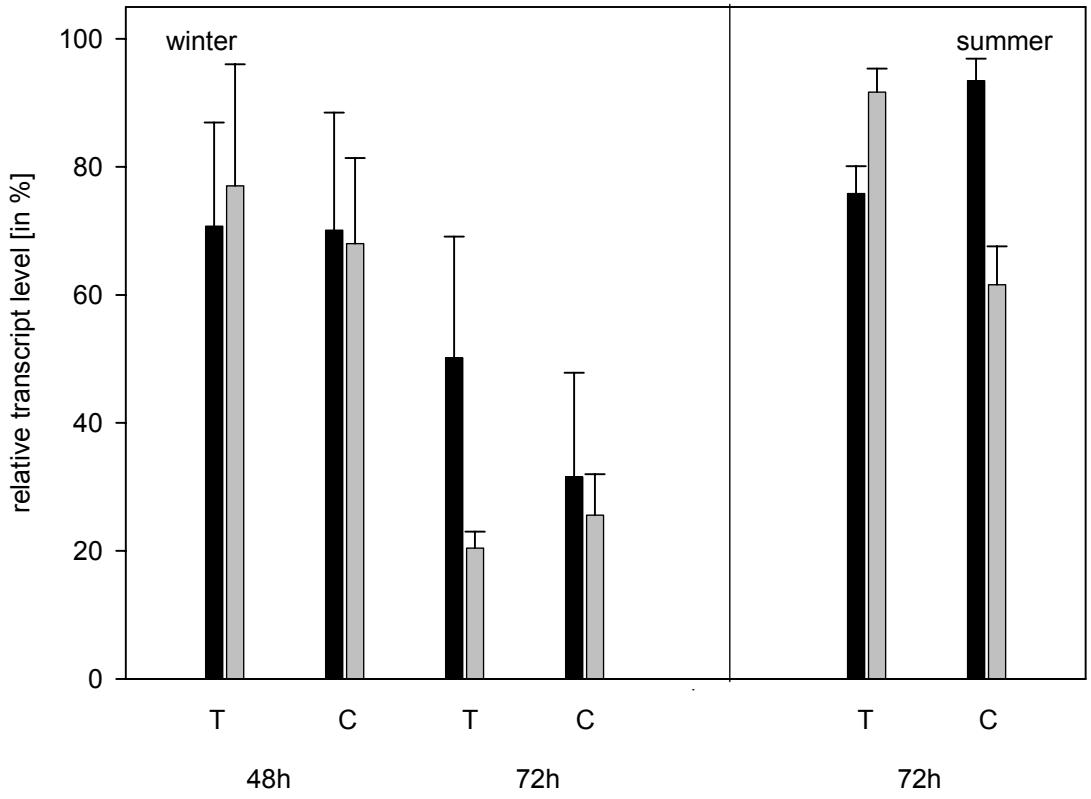



**Figure 4.5 The relative transcript level of Ps*TPS1* in pine needles induced by JA and control pine needles.** The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
T stands for test treatment (0.3 mM Ja) and C for control treatment (Tween 20).
Winter N=5, Summer N=24 (12/12). All other information is as found in the legend for figure 4.4.

The relative transcript values normalized by 18s rRNA show a two-fold decrease when comparing the levels 48 hours and 72 hours after the start of the experiment. However, the decrease in relative transcript level in the α-tubulin normalized values is only 20 percent on average between the test needles 72 hours after treatment harvested in winter compared to test and control needles sampled 48 hours after treatment. The Ps*TPS1* relative transcript level of the corresponding control needles decreases 40 percent in comparison to the 48 hour probes.

There is no consistent difference in relative transcript level evident between induced and control pine needles 72 hours after treatment. The relative transcript levels differ greatly depending on the normalization mode used, and the summer and winter samples. The α-tubulin normalized Ps*TPS1* relative transcript level is higher in the test needles than controls in the winter samples but lower in test needles than control needles in the summer samples. The opposite is true for the relative transcript level after 18s rRNA normalization.

#### 4.2.1.2.3 Ps*TPS1* in methyl jasmonate treated needles

There is no consistent trend of Ps*TPS1* relative transcript accumulation evident in the methyl jasmonate treated samples (figure 4.6).

When surveying the values obtained from the winter probes, the data normalized by 18s rRNA show a 70 percent higher relative transcript level in the methyl jasmonate treated needles at 48 and 72 hours after induction compared to the relative transcript level of the corresponding control needles.

However, the same raw data show no differences in the relative transcript level between test and control treatment 48 hours after treatment when they are normalized with α-tubulin. 72 hours after the methyl jasmonate was applied, the test needles have a 20 percent lower relative transcript level than the corresponding control needles.

Similar mixed results were obtained for the tissues harvested in summer. The control twigs display a 60 percent increase in relative transcript level when the data are normalized with α-tubulin and an 85 percent increase in relative transcript level when the data were normalized with 18s rRNA, as compared to the test needles.

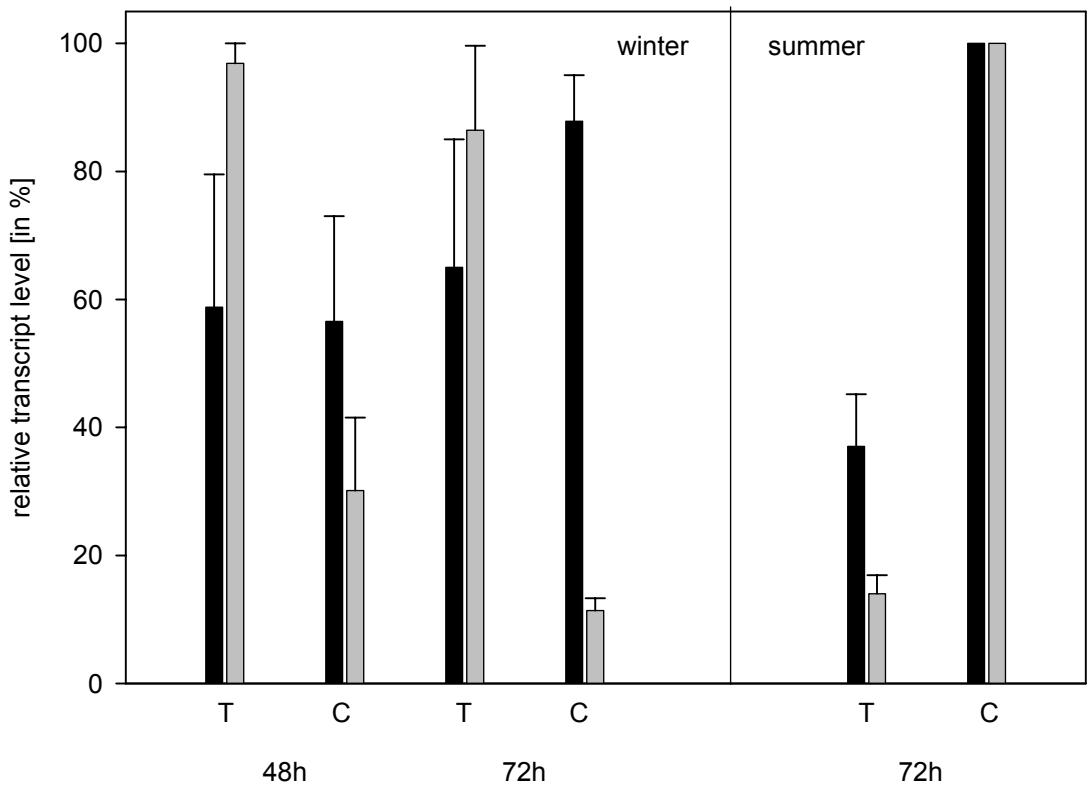



**Figure 4.6 The relative transcript level of Ps*TPS1* in MJ treated versus control pine needles**. The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
T stands for test treatment (0.1mM MJ) and C for control treatment (Tween 20).
Winter N=3, Summer N=6 (3/3), All other information is as found in the legend for figure 4.4.

## 4.2.2 Characterization of Ps*TPS2*

### 4.2.2.1 Primer specificity of the Ps*TPS2* primer pair

Two specific primer sites were chosen to amplify the Ps*TPS2* fragment. The specificity of the chosen tps-215_for-tps2-734_rev primer pair was confirmed by sequencing the PCR product from cDNA isolated from jasmonic acid treated needles harvested 72 hours after treatment in winter. The plasmid insert from ten different colonies was sequenced. The alignment of the new consensus nucleotide sequences with the original Ps*TPS2* sequence reveals a high similarity (figure 4.7). The nucleotide identity between the two sequences is 96 percent. The 5' end of the sequence does not match the original Ps*TPS2* sequence completely. Two thymine nucleotide bases are replaced by guanine or cytosine. The consensus sequence does agree with the sequence of the used tps3-215_for primer (figure 4.8). Hence the used tps3-215_for primer does not completely resemble the original Ps*TPS3* sequence.

```
1   TCAGATTTATATCTGCCGCAGCTGGATTTC-TATAGGAAACGYCACGTGGAACTTTACTTTTGGGCRGTT PsTPS2
1   TCAGAGTCATATCTGCCGCAGCTGGATTTNCTATAGGAAACGTCACGTGGAACTTTACTTTTGGGCGGTT HP PsTPS2

70  CTAGGCACATTCGAGCCCGAATTTCGTAGCAGCAGAATCGCCTTCACAAAACTTTCAACAGTGATGACGG PsTPS2
71  CTAGGCACATTCGAGCCCGAATTTCGTAGCAGCAGAATCGCCTTCACAAAACTTTCAACAGTGATGACGG HP PsTPS2

140 TTATAGATGACCTATATGATACTCACGGAACATTGGACGAAATCAAAATCTTCACTGAGGGAGTGAGAAG PsTPS2
141 TTATAGATGACCTATATGATACTCACGGAACATTGGACGAAATCAAAATCTTCACTGAGGGAGTGAGAAG HP PsTPS2

210 ATGGGATACTTCACTAATAARCCGCCTTCCAGACCACATTCAAAAAATATTCGAGTTTTTCATGAAGACA PsTPS2
211 GTGGGATACTTCACTAATAAGCCGCCTTCCRGACCACATTCAAAAAATATTCGAGTTTTTCATGAAGACA HP PsTPS2

280 TCGAATGAATGGACTGCTGAARTGGAAAARAAGCAAGGGCGCGACATGGCGGCGTATATAAGAAAAAATG PsTPS2
281 TCGAATGAATGGACTGCTGAAGTGGAAAAGAAGCAAGGGCGTGACATGGCGGCGTATATAAGAAAAAATG HP PsTPS2

350 GCTGGGAGCGATACGTTGAGTCTTATCTGCAAGAAGGGGAATGGATGGCGGCTGGATATGTCCCCTCGTT PsTPS2
351 GCTGGGAGCGATACGTTGAGTCTTATCTGCAAGAAGGGGAATGGATGGCGGCTGGATATGTCCCCTCGTT HP PsTPS2

420 TAATGAGTACTATAAGAATGGCCTCGCTAGCTCCGGGATGTGTGTGTWAAATTTGATTCCGCTTCTGTTA PsTPS2
421 TAATGAGTACTATAAGAATGGCCTCGCTAGCTCCGGGATGTGTGTGNNAAATTTGATTCCGCTTCTGTTA HP PsTPS2

490 ATGGATCAGATTCTACCAGACG                                                  PsTPS2
491 ATGGATCAGATTCTACCAGACG                                                  HP PsTPS2
```

**Figure 4.7 Alignment of Ps*TPS2*.** Shown is the nucleotide sequence alignment of *PsTPS2* with the amplified product from the tps-215_for-tps2-734_rev primer pair (HP for homologous primer product). The sequences have a nucleotide identity of 96 % to each other. R stands for adenosine or guanine, N stands for all for possible nucleotide bases and Y stands for cytosine or thymine in the sequence.

The forward and reverse primer sequence of the Ps*TPS2* primer pair can be identified in the amplified sequence (figure 4.8).

**TCAGAGTCATATCTGCCGCAGCTG**GATTTNCTATAGGAAACGTCACGTGGAACTTTACTT 60
TTGGGCGGTTCTAGGCACATTCGAGCCCGAATTTCGTAGCAGCAGAATCGCCTTCACAAA 120
ACTTTCAACAGTGATGACGGTTATAGATGACCTATATGATACTCACGGAACATTGGACGA 240
AATCAAAATCTTCACTGAGGGAGTGAGAAGGTGGGATACTTCACTAATAAGCCGCCTTCC 280
RGACCACATTCAAAAAATATTCGAGTTTTTCATGAAGACATCGAATGAATGGACTGCTGA 300
AGTGGAAAAGAAGCAAGGGCGTGACATGGCGGCGTATATAAGAAAAAATGGCTGGGAGCG 360
ATACGTTGAGTCTTATCTGCAAGAAGGGGAATGGATGGCGGCTGGATATGTCCCCTCGTT 420
TAATGAGTACTATAAGAATGGCCTCGCTAGCTCCGGGATGTGTGTGNNAAATTTGATTCC 480
GCTTCT**GTTAATGGATCAGATTCTACCAGACG**

**Figure 4.8 Amplified nucleotide sequence of Ps*TPS2***. The tps-215_for and tps2-734_rev sequence are shown in bold.

### 4.2.2.2 The relative transcript level of Ps*TPS2*

#### 4.2.2.2.1 Ps*TPS2* in oviposition induced needles

There is no significant difference in the relative transcript accumulation of Ps*TPS2* discernable, when comparing the amplification products from oviposition induced pine needles with the amplification products from artificially damaged control pine needles (figure 4.9).

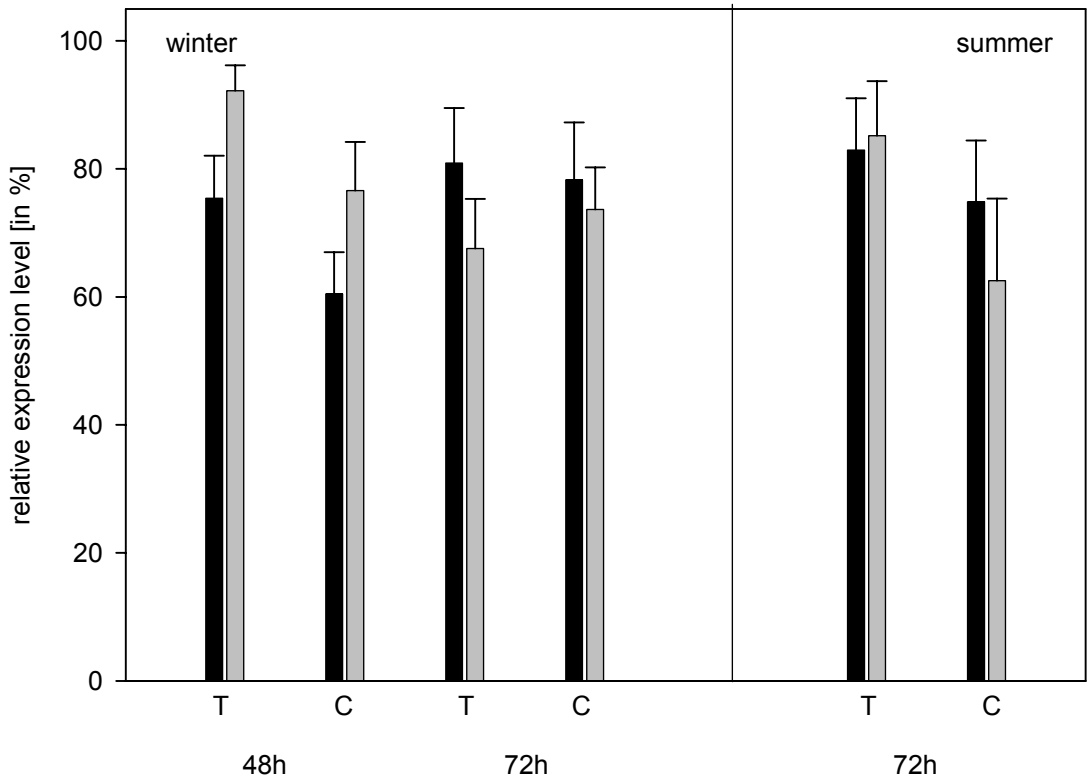



**Figure 4.9 The relative transcript level *of* Ps*TPS2* in oviposition induced and control pine needles.** The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
T stands for test treatment (egg deposition), C stands for control treatment (artificially damaged).
Winter N=9, Summer α-tubulin N=10 (5/5), Summer 18s rRNA N=9 (4/5). All other information is as found in the legend for figure 4.4.

#### 4.2.2.2.2. Ps*TPS2* in jasmonic acid treated needles

The relative transcript level of Ps*TPS2* decreases 72 hours after jasmonic acid and Tween 20 treatment as compared it to the relative transcript levels of both treatments 48 hours after the start of the experiment (figure 4.10). The relative transcript levels of the α-tubulin normalized values are 10 percent lower in the control treatments, as compared to the test treatment at both sampling times (48h and 72h) for the winter samples. The relative transcript level in same treatment 48 hours after application compared to 72 hours after application is 20 percent lower.

The same data show a slightly different pattern when they are normalized with 18s rRNA. In this case, the relative transcript level of the jasmonic acid treated needles is 40 percent higher than its corresponding control treatment and the jasmonic acid treated needles 72 hours after induction. The relative transcript accumulation of the control treatment at 72 hours is 15 percent lower than the corresponding control. However, none of the results are statistically significant.

All twigs had a higher Ps*TPS2* relative transcript level in the induced needles 72 hours after induction when compared to the corresponding Tween 20 control needles. The values normalized by α-tubulin only display a very subtle difference of relative transcript level with a 15 percent lower accumulation of relative transcript in the control needles.

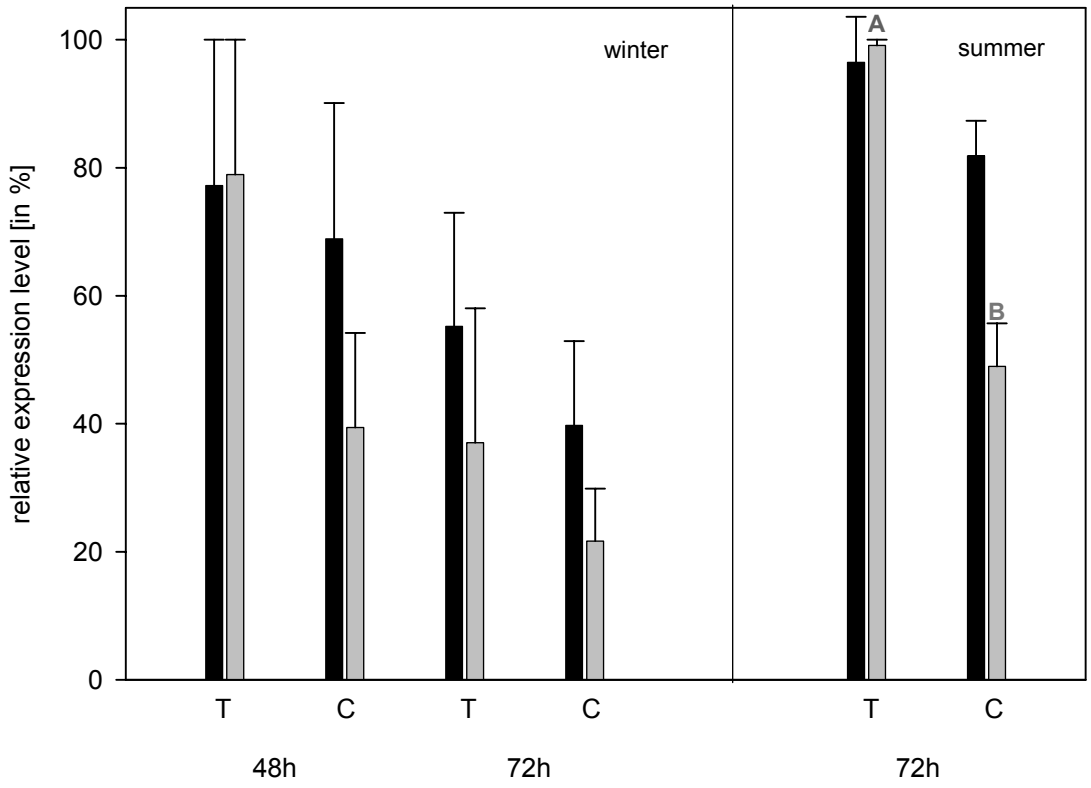



**Figure 4.10 The relative transcript level of Ps*TPS2* after Ja and control treatment.** The columns illustrate the mean relative transcript level and the standard error obtained from repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the probes taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
T stands for induction with Ja (0.3 mM),and C stands for control treatment with a Tween 20 solution. Winter N=4, Summer N=16 (8+8). All other information is as found in the legend for figure 4.4. Different numbers indicate significant ($p<0.001$) differences per normalization method evaluated by a One Way ANOVA.

The same is true for the relative transcript level resulting from 18s rRNA normalized values in winter. The relative transcript level gained by normalization with 18s rRNA in summer shows a 50 percent increase in induced needles compared to their control needles. This difference is statistically significant ($p<0.001$).

#### 4.2.2.2.3 Ps*TPS2* in methyl jasmonate treated needles

The relative transcript level of Ps*TPS2* in methyl jasmonate treated pine needles increases over time, when comparing the values normalized against α-tubulin measured at 48 hours with the corresponding values acquired 72 hours after treatment (figure 4.11). Here the relative transcript level increased by 70 percent in the methyl jasmonate treated needles and 40 percent in the control needles. However, the same raw data show a 80 percent upregulation of relative transcript in the induced needles 72 hours after treatment and no increase in the control needles, when they have been normalized against 18s rRNA.

The expression pattern in the pine needles shows a contradictory pattern 72 hours after treatment, when comparing summer and winter samples (figure 4.11). The winter probes display a higher relative transcript level in the methyl jasmonate treated probes compared to the parallel control needles. In contrast, the needles tested in summer have a higher Ps*TPS2* relative transcript level in the corresponding control pine needles.

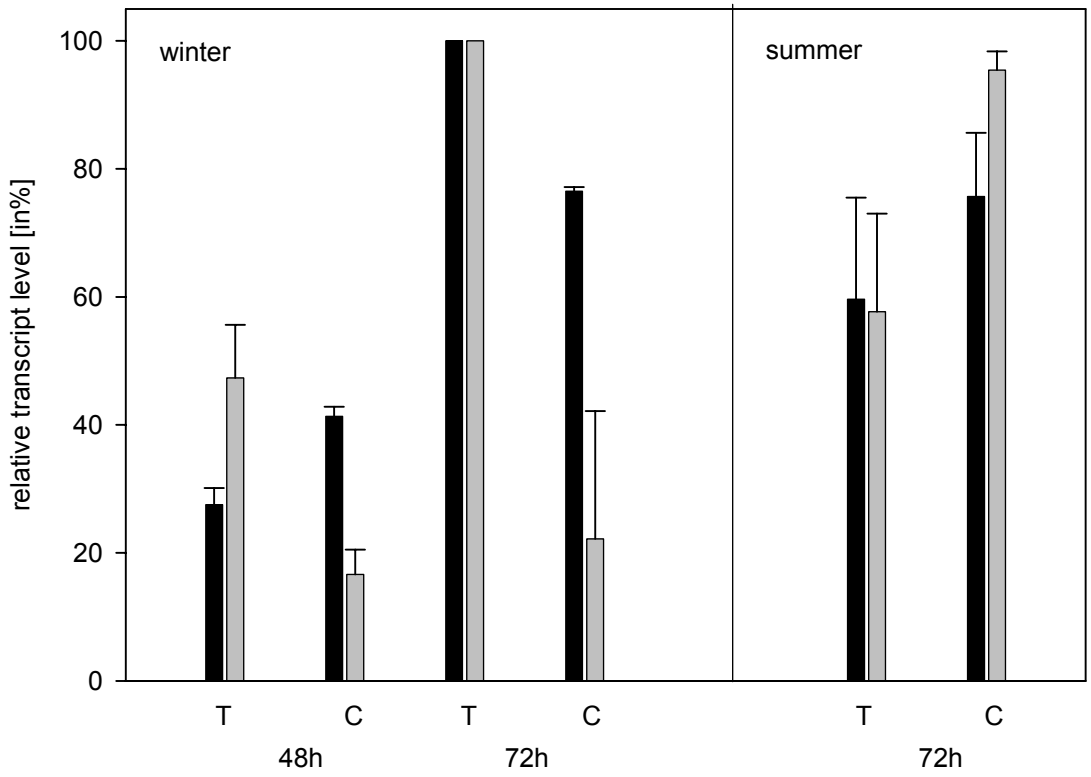



**Figure 4.11 The relative transcript level of Ps*TPS2* in MJ induced and control needles.** T stands for MJ induction (0.1 mM), C stands for Tween 20 control-treatment. The columns illustrate the mean relative transcript level and the standard error determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA.
Winter N= 2, Summer N=8 (4/4). All other information is as found in the legend for figure 4.4.

### 4.2.3 Characterization of Ps*TPS3*

#### 4.2.3.1 Primer specificity of the Ps*TPS3* primer pair

To amplify the Ps*TPS3* fragment, specific primer binding sites were chosen. The substrate for the PCR reaction was the cDNA from jasmonic acid induced probes harvested 72 hours after treatment in winter. The resulting product was cloned. The plasmid insert of five colonies were sequenced to verify the specificity of the tps3-233_for-tps3-690_rev primer pair. Figure 4.12 demonstrates the nucleotide alignment of the consensus sequence from five sequenced plasmid inserts with the original Ps*TPS3* sequence. The nucleotide sequence homology of both sequences is 98 percent.

```
1   GGATATAGAGAAGCAGAATTTCTACCGGAAGC--GTCACGTGGAATTTTACTTTTGGATGGTTATAGGSACGTTC PsTPS3
1   GGATATAGAGAAGCAGAATTTCTACCGGAAGCCCGTCACGTGGAATTTTACTTTTGGATGGTTATAGGSACGTTC HP PsTPS3

74  GAACCGGAGTTTTCGAGCAGCAGAATTGCATTCGCAAAAATTGCGACACTGATGACTATCCTAGATGATCTCTAT PsTPS3
76  GAACCGGAGTTTTCGAGCAGCAGAATTGCATTCGCAAAAATTGCGACACTGATGACTATCCTAGATGATCTCTAT HP PsTPS3

149 GATACTCACGGAACGTTGGAACAACTAAAAATCTTCACAGAAGCAGTCAAACGATGGGATCTTTCATTACAAGMC PsTPS3
151 GATACTCACGGAACGTTGGAACAACTAAAAATCTTCACAGAAGCAGTCAAACGATGGGATCYTTCATTACAAGMC HP PsTPS3

224 CGTCTTCCAGACTACATAAAGATTACTCTGGAATTCTTCTTCAACACATCCAATGAATTGAATGCTGAAGTTGCT PsTPS3
226 CGTCTTCCAGACTACATAAAGATTACTCTGGAATTCTTCTTCAACACATCCAATGAATTGAATGCTGAAGTTGCT HP PsTPS3

299 AAAATGCAAGAACGGGATATGTCAGCCTACATACGAAAAGCAGGCTGGGAACGATACMTTGAAGGGTATATGCAA PsTPS3
301 AAAATGCAAGAACGGGATATGTCAGCCTACATACGAAAAGCAGGCTGGGAACGATACMTTGAAGGGTATATGCAA HP PsTPS3

374 GAGTCCGAATGGATGGCGGCTCGACATGTCCCTACSTTTGACGATTACATGAAGAATGGCAAACCCAGCTCTGGA PsTPS3
376 GAGTCCGAATGGATGGCGGCTCGACATGTCCCTACSTTTGACGATTACATGAAGAATGGCAAACCCAGCTCTGGA HP PsTPS3

449 ATGTGTATAC                                                                  PsTPS3
451 ATGTGTATAC                                                                  HP PsTPS3
```

**Figure 4.12 Alignment of Ps*TPS3*.** The nucleotide sequence alignment of Ps*TPS3* is shown with the amplified product from the tps3-233_for-tps3-690_rev primer pair (HP for homologous primer product). The sequences have a nucleotide identity of 98 % to each other. Y indicates contradicting nucleotide information and stands for Cytosine or Thymine, M stands for Adenine or Cytosine, S stands for Guanine or Cytosine.

The tps3-233_for and tps3-690_rev oligonucleotide sequence can be recognized in the fragment amplified with this homologous primer pair (figure 4.13)

**GGATATAGAGAAGCAGAATTTCTA**CCGGAAGCCCGTCACGTGGAATTTTACTTTTGGATG 60
GTTATAGGSACGTTCGAACCGGAGTTTTCGAGCAGCAGAATTGCATTCGCAAAAATTGCG 120
ACACTGATGACTATCCTAGATGATCTCTATGATACTCACGGAACGTTGGAACAACTAAAA 180
ATCTTCACAGAAGCAGTCAAACGATGGGATCYTTCATTACAAGMCCGTCTTCCAGACTAC 240
ATAAAGATTACTCTGGAATTCTTCTTCAACACATCCAATGAATTGAATGCTGAAGTTGCT 300
AAAATGCAAGAACGGGATATGTCAGCCTACATACGAAAAGCAGGCTGGGAACGATACMTT 360
GAAGGGTATATGCAAGAGTCCGAATGGATGGCGGCTCGACATGTCCCTACSTTTGACGAT 420
TACATGAAGAATGGC**AAACCCAGCTCTGGAATGTGTATAC** 480

**Figure 4.13 Amplified nucleotide sequence of Ps*TPS3*.** The sequence is shown in 5'-3' direction. The tps3-233_for and tps3-690_rev sequence is shown in bold.

Hence, the tps3-233_for-tps3-690_rev primer pair can be considered specific for the Ps*TPS3* sequence for the tested needle tissue.

#### 4.2.3.2 The relative transcript level of Ps*TPS3*

##### 4.2.3.2.1 Ps*TPS3* in oviposition induced needles

Artificially damaged control twigs reveal a slightly higher relative transcript level than their corresponding oviposition induced needles after 48 hours (figure 4.14). The control treated needles show an 11 percent higher relative transcript level when the raw data were normalized against α-tubulin and a 15 percent higher relative transcript level when the raw data was normalized against 18s rRNA 46 hours after treatment.

There is an increase in relative transcript level of Ps*TPS3* visible in the oviposition induced pine needles compared to the corresponding control needles 72 hours after induction (figure 4.14). When the raw data are normalized against α-tubulin, the oviposition induced needles harvested in winter and in summer show a 40 percent increase in relative transcript accumulation. The relative transcript accumulation difference is somewhat lower in the needles when the data have been normalized with 18s rRNA. The oviposition induced needles harvested in winter display a 15 percent increase in relative transcript accumulation compared to the control needles harvested at the same time. The needles tested in summer show a 25 percent increase in relative transcript level when compare to the corresponding control.

The relative transcript level increase is statistically significant ($p<0.05$) when that data are normalized against α-tubulin. However, the data of the two twigs tested in summer normalized by 18s rRNA are not homogeneously distributed ($p=0.07$). Their distribution is significantly different from each other.

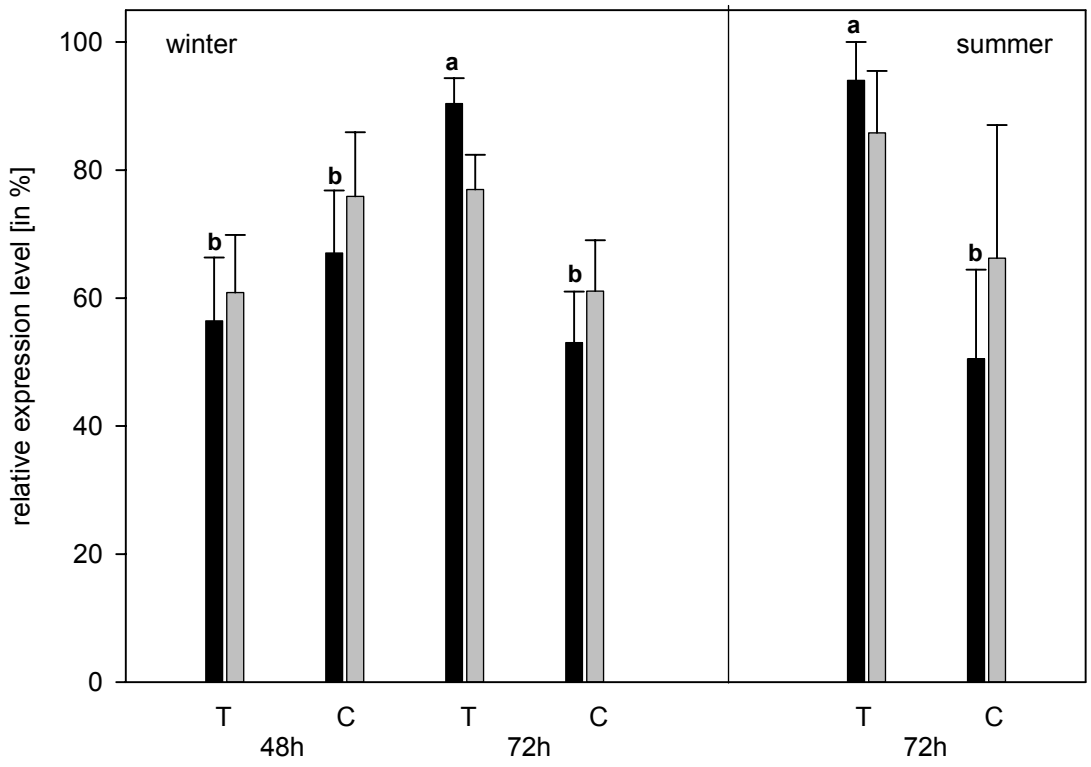



**Figure4.14 The relative transcript level of Ps*TPS3* in oviposition induced and control needles.** The columns illustrate the mean relative transcript level and the standard error determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for oviposition, C stands for artificially wounded control-treatment.
Winter N=9, Summer α-tubulin N= 6 (3/3), Summer 18s rRNA N=5 (2/3). All other information is as found in the legend for figure 4.4. Different letters indicate significant ($p<0.005$) differences per normalization method evaluated by a One Way ANOVA

#### 4.2.3.2.2 Ps*TPS3* in jasmonic acid treated needles

The jasmonic acid treated needles in all samples reveal a higher relative transcript level than the corresponding control needles (figure 4.15). The peak divergence of 60 percent can be found when comparing the relative transcript level normalized against 18s rRNA in jasmonic acid treated needles and the corresponding control treated needles harvested in summer 72 hours after treatment application. The lowest dissimilarity in relative transcript level that can be observed is 20 percent. This is the case for the α-tubulin normalized raw data 48 hours after induction in winter and 72 hours after induction in summer, as well as for the 18s rRNA normalized raw data 72 hours after induction in winter.

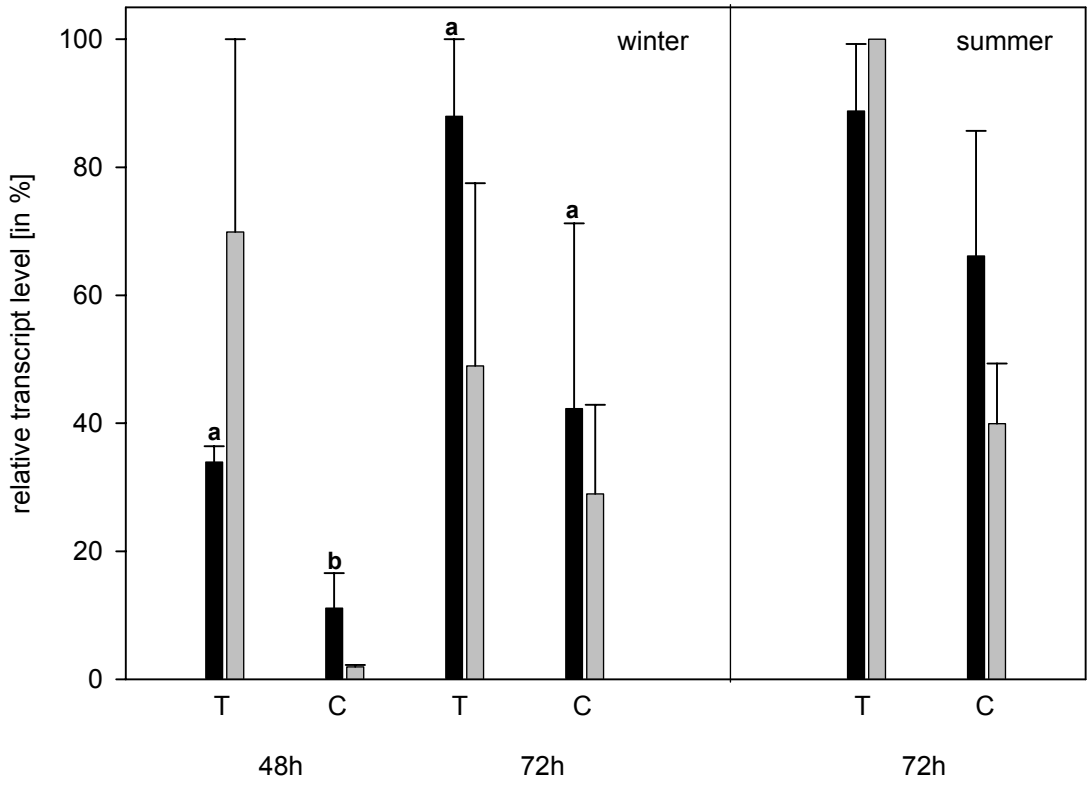



**Figure 4.15 The relative transcript level of Ps*TPS3* after Ja and control treatment.** The columns illustrate the mean relative transcript level and the standard error determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for induction with Ja (0.3 mM), and C stands for control treatment with a Tween 20 solution.
Winter N=3, Summer N=4 (2/2). All other information is as found in the legend for figure 4. Letters indicate significant ($p < 0.05$) differences per normalization method evaluated be a One Way ANOVA

However, the relative transcript level of Ps*TPS3* observed in the needles harvested in winter fluctuates greatly between the experiments. This is reflected by a high standard error for these data in figure 4.15. The stated standard error is for example 25 percent for control needles normalized against α-tubulin harvested 72 hours after the start of the experiment and 50 percent for jasmonic acid treated twigs normalized against 18s rRNA harvested 48 hours after induction.
The increase in relative transcript level in treated needles is statistically significant for the samples harvested after 48 hours in winter when the raw data are normalized against α-tubulin ($p<0.05$).

#### 4.2.3.2.3 Ps*TPS3* in methyl jasmonate treated needles

The relative transcript level of Ps*TPS3* as measured in methyl jasmonate treated samples is different when comparing the values gained from needles harvested in summer with the data gained from needles harvested in winter (figure 4.16).
The needles tested in winter reveal a higher relative transcript level of Ps*TPS3* in the methyl jasmonate treated needles compared to the corresponding control needles. This discrepancy is extremely high when the raw data is normalized by 18s rRNA. The methyl jasmonate treated needles have a 70 percent higher relative transcript level of *PsTPS3* 48 hours after the phytohormone was applied and a 95 percent higher relative transcript level 72 hours after the treatment was applied, compared to the relative transcript level in the corresponding control needles. The relative transcript upregulation is somewhat lower when the raw data is normalized against α-tubulin. 48 hours after methyl jasmonate treatment the test needles reveal a 25 percent higher relative transcript level and 72 hours after treatment the test needles show a 45 percent higher relative transcript level compared to their corresponding control.

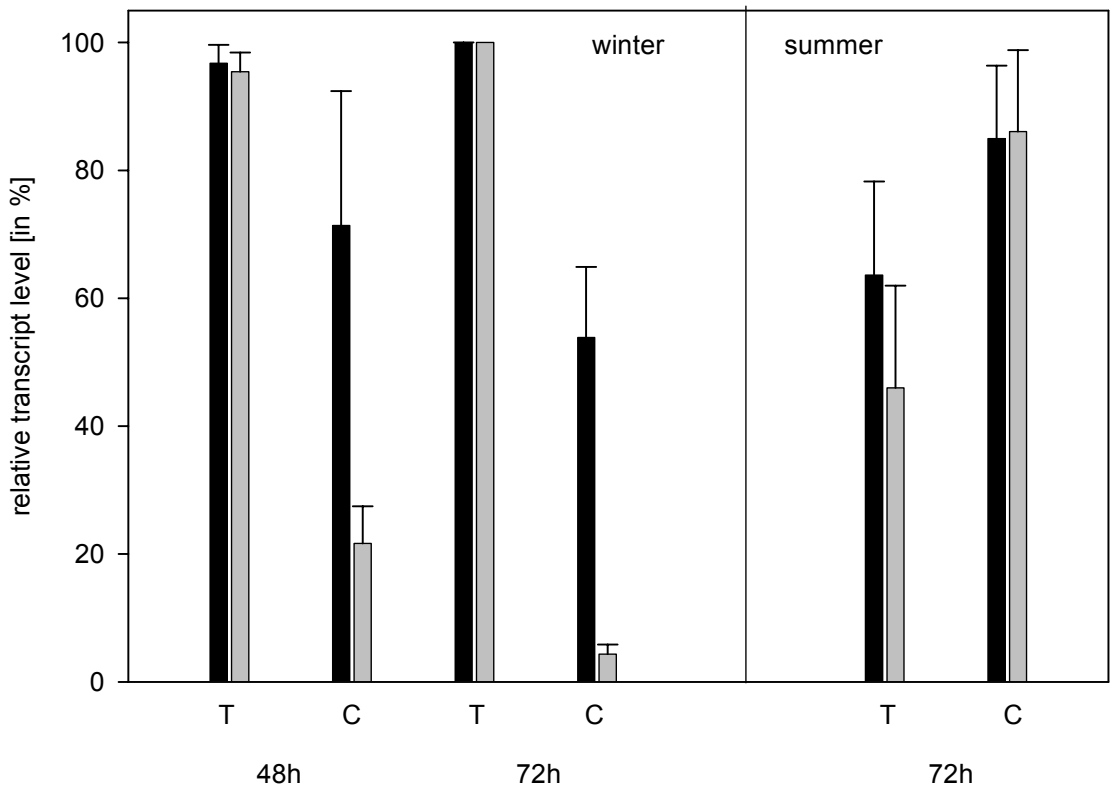



**Figure 4.16 The relative transcript level of Ps*TPS3* in MJ induced and control needles.** The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for MJ induction (0.1 mM), C stands for Tween 20 control-treatment.
Winter N=3, Summer N=7 (4/3). All other information is as found in the legend for figure 4.4.

In contrast to the pattern found in winter, the needles harvested in summer reveal a higher relative expression rate of Ps*TPS3* in the control treated needles than in the corresponding test needles. When the raw data are normalized against α-tubulin, the relative transcript level is 20 percent higher in the control needles compared to the corresponding methyl jasmonate treated needles. The same raw data reveal a 40 percent increase in relative transcript level of Ps*TPS3* in the control needles, compared to the test needles when they are normalized against 18s rRNA.

## 4.2.4. Ps*TPS4*

### 4.2.4.1 Primer specificity of the Ps*TPS4* primer pair

To amplify the Ps*TPS4* fragment the tps4-30_for-tps4-227_rev primer pair was used (table 3.2).

To test for the specificity of the amplified PCR product, fragments amplified from oviposition induced pine needle cDNA as well as from artificially damaged control tissue (both 72h in winter) were isolated and cloned. 15 plasmid inserts were sequenced. When aligning the nucleotide consensus sequences with the nucleotide sequence of Ps*TPS4*, the sequences reveal a high similarity to each other. Their nucleotide identity is 99 percent.

```
1   CAGCGTGCCAAGATTGGAGGCAAGGAGCTTTATCGAAATATATGGACGAAACGRCTCATGGCTCAAGTCAAATAT PsTPS4
1   CAGCGTGCCAAGATTGGAGGCAAGGAGCTTTATCGAAATATATGGACGAAACGACTCATGGCTCAAGTCAAATAT HP PsTPS4

76  AAACCAAAATTTTTTAGAGTTGGCGAAATTGGACTTCAATATCCTACAATTTACACATCAGAAAGACATTCAGCT PsTPS4
76  AAACCAAAATTTTTTAGAGTTGGCGAAATTGGACTTCAATATCCTACAATTTACACATCAGAAAGACATTCAGCT HP PsTPS4

151 TATCTCAAGGTGGATGACAGAGTCAGACATCGGAG                                         PsTPS4
151 TATCTCAAGGTGGATGACAGAGTCAGACATCGGAG                                         HP PsTPS4
```

**Figure 4.17 Alignment of Ps*TPS4***. The nucleotide sequence alignment of PsTPS4 is shown with the amplified product from the tps4-30_for-tps4-227_rev primer pair (PP for primer product). The sequences have a nucleotide identity of 99 % to each other. R indicates contradicting nucleotide information and stands for adenine or guanine.

The tps4-30_for sequence and the tps4-227_rev sequence can be found in the amplified PCR products (figure 4.18). A recently published longifolene synthase sequence from Norway spruce (PaTPS-Lon, AY473625, in Martin et al. 2004) has an amino acid identity of 68 percent to Ps*TPS4*.

**CAGCGTGCCAAGATTGGAGGCA**AGGAGCTTTATCGAAATATATGGACGAAACGACTCATGG 60
CTCAAGTCAAATATAAACCAAAATTTTTTAGAGTTGGCGAAATTGGACTTCAATATCCTAC 120
AATTTACACATCAGAAAGACATTCAGCTTATCTCAAGGTGG**ATGACAGAGTCAGACATCGG** 240
**AG** 230

**Figure 4.18 Amplified nucleotide sequence of Ps*TPS4***. The created primers to amplify the sequence are shown in bold.

As a result, the tps4-30_for-tps4-227_rev primer pair can be considered specific for the Ps*TPS4* sequence in the tested needle tissue.

### 4.2.4.2 The relative transcript level of Ps*TPS4*

#### 4.2.4.2.1 Ps*TPS4* in oviposition induced needles

There is an upregulation of the relative Ps*TPS4* transcript level visible 72 hours after induction, when compared to the tissue sampled 48 hours after induction (figure 4.19). The oviposition induced needles show a higher relative transcript level than the corresponding artificially damaged control needles after 72 hours (figure 4.19).The same expression pattern can be recognized on the displayed agarose gel picture (figure 4.20). The relative transcript level of the oviposition induced pine needles is 95 percent higher 72 hours after treatment compared to all treatments 48 hours after application. The artificially damaged control needles showed a 40 percent increase in relative transcript level when the raw data are normalized against α-tubulin and a 55 percent rise in relative transcript level when the raw data are normalized against 18s rRNA, 72 hours after treatment as compared to either the test or control treatments 48 hours after application. The relative transcript level of oviposition induced needles was 55 percent higher in winter needles and 30 percent higher in summer needles when the raw data are normalized against α-tubulin compared to the corresponding values of artificially damaged needles. The relative transcript level difference is slightly lower when the raw data are normalized against 18s rRNA: The needles harvested in winter show a 45 percent increase in relative transcript level and the summer needles show a 24 percent increase in relative transcript accumulation compared to the appropriate control needles. All described differences are highly significant ($p<0.001$)

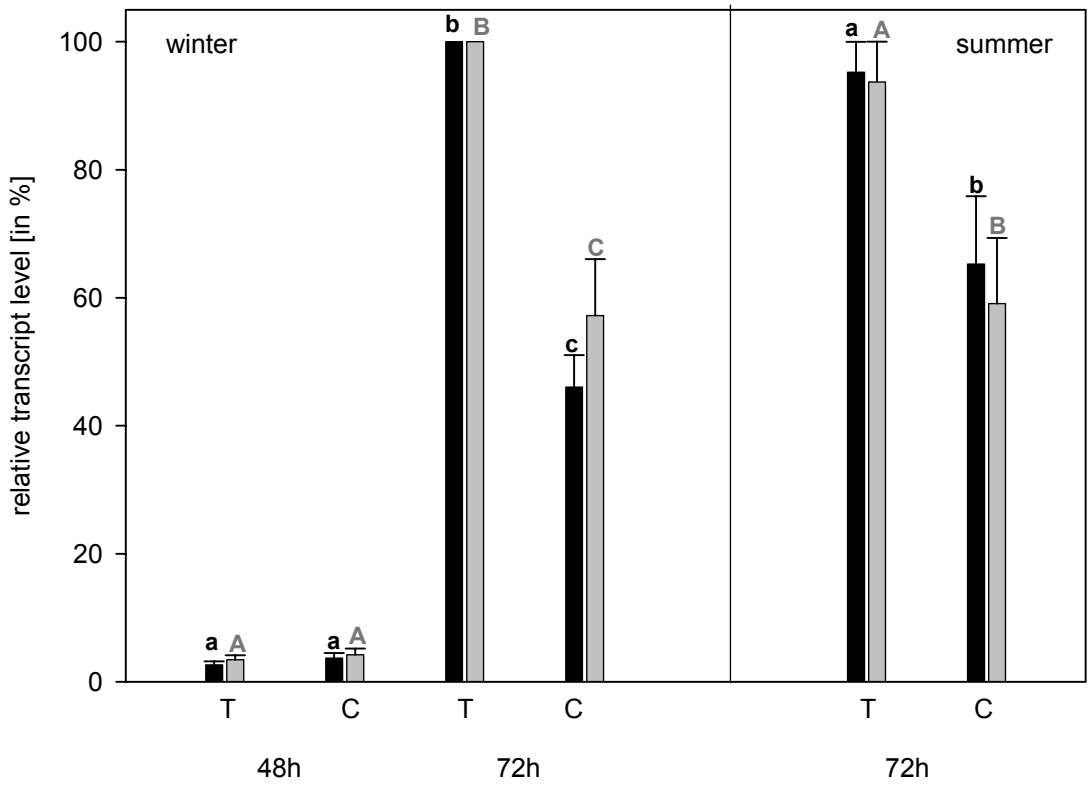



**Figure 4.19 The relative transcript level of Ps*TPS4* in oviposition induced and control needles.** The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for oviposition-induction, C stands for artificially wounded control-treatment.
Winter N=8, Summer N=8 (4/4). All other information is as found in the legend for figure 4.4. Different letters indicate significant ($p < 0.001$) differences per normalization method evaluated by a One Way ANOVA

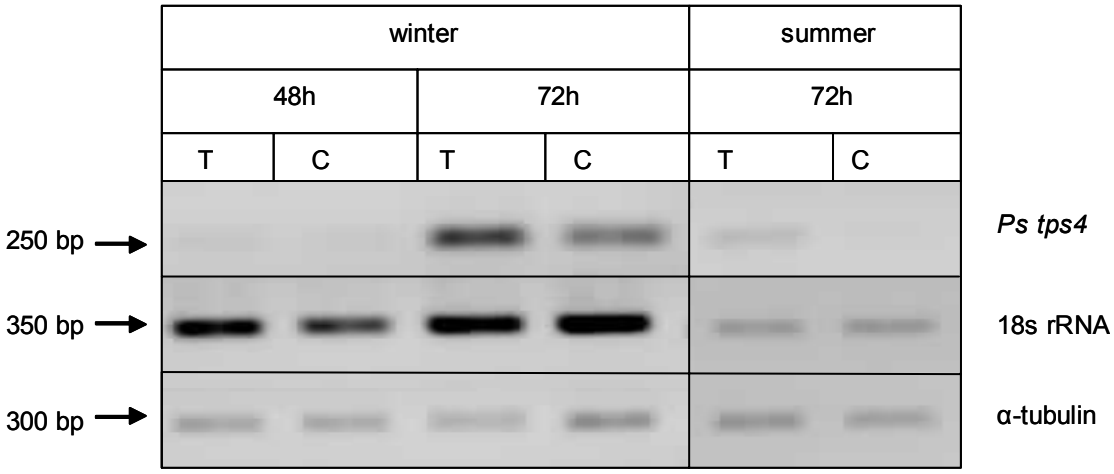



**Figure 4.20 DNA bands in an agarose gel after RT-PCR with oviposition induced pine needles.** The relative Ps*TPS4* relative transcript level was assessed by normalizing the gained photo density units against those of the 18s rRNA and α-tubulin primer pairs. T stands for oviposition induced pine needles and C for artificially damaged control pine needles. The arrows mark the expected fragment size in base pairs (bp) according to a not shown100 bp ladder.

### 4.2.4.2.2 Ps*TPS4* in jasmonic acid treated needles

The relative transcript levels of Ps*TPS4* in needles treated with jasmonic acid is variable (figure 4.21), fluctuating depending on the sample and the mode of normalization.

There was an increase in relative transcript level visible 72 hours after treatment compared to the relative transcript value of the same treatment measured 48 hours after induction, when the raw data are normalized against α-tubulin. The jasmonic acid treated needles show here a 70 percent increase in relative transcript level, whereas the control needles only show a 20 percent increase in relative transcript level. When the raw data were normalized against α-tubulin, there was a higher relative transcript level in jasmonic acid treated samples 72 hours after induction compared to all other measured relative transcript levels in the winter samples, which was not observed in the summer samples. The relative transcript level of the jasmonic acid treated samples was 30 percent higher 72 hours after induction in the needles harvested in winter, but only 4 percent higher for the needles harvested in summer compared to the relative transcript levels in the corresponding control needles.

When the data is normalized against 18s rRNA, the needles tested in winter only show a relative transcript level increase in the control probes 72 hours after application, the

relative transcript level being 50 percent higher than the level in the corresponding control treated needles, and 40 percent to 60 percent higher than the relative transcript level measured in needles harvested 48 hours after treatment application. In contrast to that, the needles harvested in summer show a 40 percent higher relative transcript level, in the jasmonic acid induced pine needles, compared to the control needles.

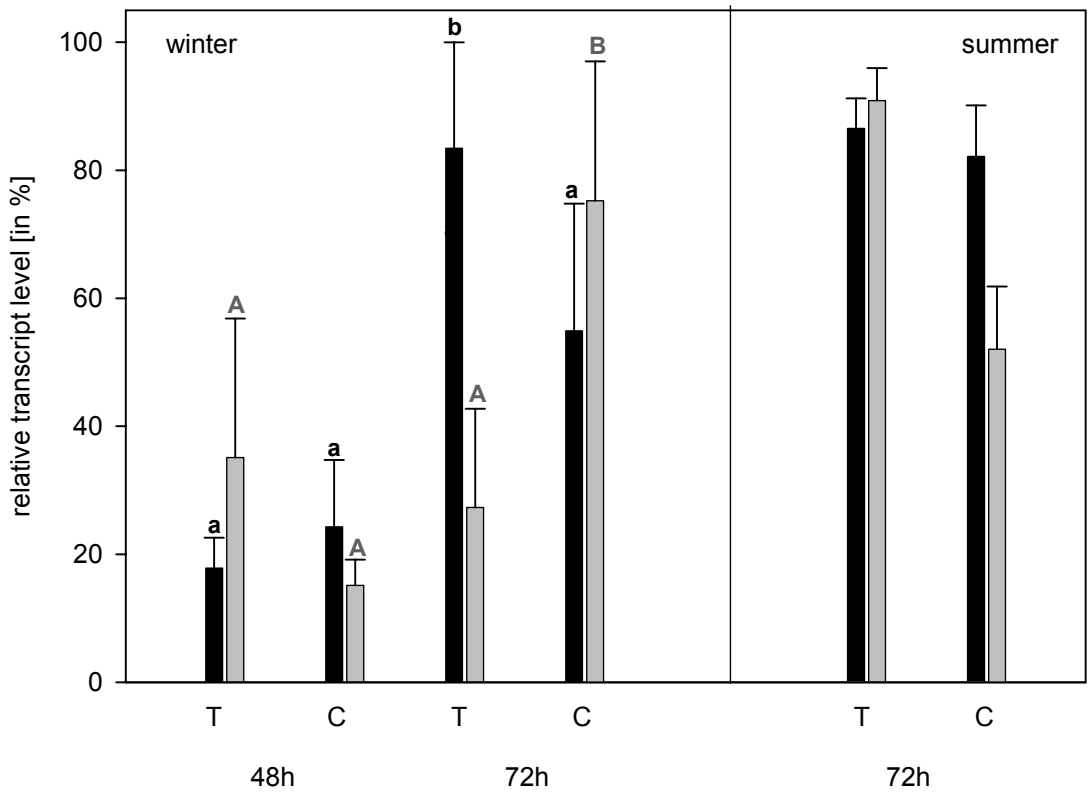



**Figure 4.21 The relative transcript level of Ps*TPS4* after Ja and control treatment.** The columns illustrate the mean relative transcript level and the standard error as determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the probes taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for induction with Ja (0.3 mM), and C stands for control treatment with a Tween 20 solution.
Winter N=4, Summer N=12 (6/6). All other information is as found in the legend for figure 4.4. Different letters indicate significant ($p < 0.05$) differences per normalization evaluated by a One Way ANOVA.

The values produced by normalizing against α-tubulin show an upregulation of the relative transcript levels 72 hours after treatment, compared to the values 48 hours after treatment. However, there is no constant difference observable between induced and control treatment.
For the sample taken 72 hours after treatment the relative transcript level differences obtained after α-tubulin and 18s rRNA normalization, are both statistical significant ($p<0.05$) for the winter needles. In summer, the 18s rRNA normalized values are not homogeneous. They show a significantly different distribution.

#### 4.2.4.2.3 Ps*TPS4* in methyl jasmonate treated needles

There is no clear trend for the changes in relative transcript level of Ps*TPS4* observable when the needles were treated with methyl jasmonate (figure 4.22).
The relative transcript values increase in the methyl jasmonate treated needles 72 hours after treatment when the raw data are normalized against α-tubulin. The relative transcript increases 50 percent compared to the same treatment 48 hours after application and 40 percent compared to the control needles harvested at the same time point. In contrast to that, the needles harvested in summer show a slightly higher (6 percent) relative transcript level in the control needles compared to the methyl jasmonate treated needles.

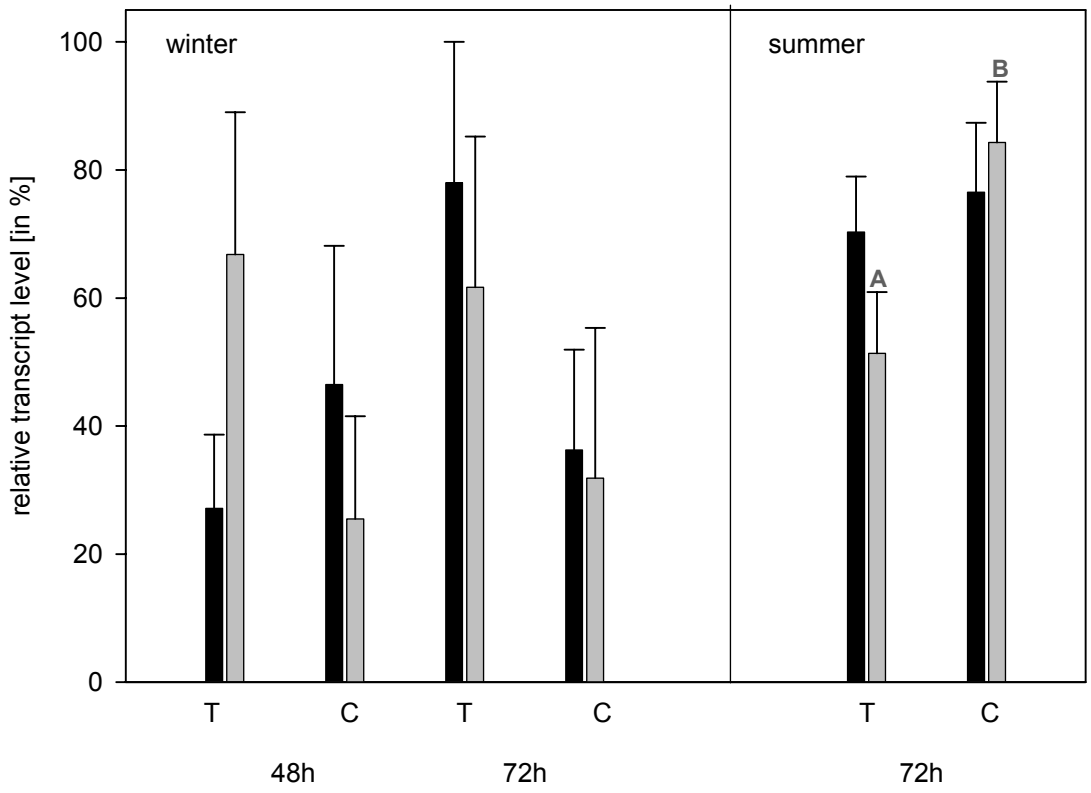



**Figure 4.22 The relative transcript level of Ps*TPS4* in MJ induced and control needles.** The columns illustrate the mean relative transcript level and the standard error determined by repetitive semiquantitative RT-PCR with at least two cDNA isolations. The data for the samples taken in summer and winter (see text) were obtained separately. The black columns represent values obtained by the normalization with α-tubulin and the lighter columns values normalized with 18s rRNA. T stands for MJ induction (0.1 M), C stands for Tween 20 control-treatment. Winter N=4, summer N=12 (6/6).
All other information is as found in the legend for figure 4.4. Different letters indicate significant ($p<0.05$) differences per normalization method evaluated by a One Way ANOVA

## 4.3 Identification of the Ps*TPS4* cDNA clone containing the complete open reading frame by RACE-PCR

In order to identify the full open reading frame of the Ps*TPS4* cDNA clone, a 5' and a 3' RACE PCR reactions (**R**apid **A**mplification of **c**DNA **E**nds) was performed. The oligonucleotides that were used are shown in table 3.2 and table 3.3. A part of the potential open reading frame of Ps*TPS4* could be identified with the 3'RACE PCR. The identified fragment is shown in figure 4.23. The nucleotide identity of the 3'RACE-fragment with the known sequence fragment of Ps*TPS4* is 100 percent in the overlapping region. This makes it highly probable, that the sequence shown is a fragment of the open reading frame of the Ps*TPS4* cDNA clone. The amino acid sequence displays at position 273 to 288 the aspartate-rich DDxxD-motif that is found in every terpene synthase.

```
1   GAAACGACTCATGGCTCAAGTCAAATATAAACCAAAATTTTTTAGAGTTGGCGAAATTGGACTTCAATATCCTAC PsTPS4
1   GAAACGACTCATGGCTCAAGTCAAATATAAACCAAAATTTTTTAGAGTTGGCGAAATTGGACTTCAATATCCTAC 3'RACE

76  AATTTACACATCAGAAAGACATTCAGCTTATCTCAAGGTGGATGACAGAGTCAGACATCGGAG             PsTPS4
76  AATTTACACATCAGAAAGACATTCAGCTTATCTCAAGGTGGATGACAGAGTCAGACATCGGAGAGCTGAATTTCT 3'RACE

138                                                                             PsTPS4
151 ACCGAAAGCGTCACGTGGAACTTTTCTTTTGGGTAGTTACAGGCACGTTCGAGCCCGAGTTTTCTCCGAGCAGAA 3'RACE

138                                                                             PsTPS4
226 TTGCATTCACGAAAATCGGTACACTGATGACTATTCTAGATGACATCTACGATACTCAGGGAACCTTTGGGGAAC 3'RACE

138                                                                             PsTPS4
301 TCAAAATCTTTACAGAGGCACTCAAACGGTAAATTTGAATTATAGGGACTCTGTCAAAATTGGCATTTGAATGAG 3'RACE

138                                                                             PsTPS4
376 GATTTTCAAATCACGTAACTCGTTGTGGAATAGAAATGTGTGTTAACTTACAGAGGCACTCAAACGGTAAATTTG 3'RACE

138                                                                             PsTPS4
451 AATTATAGGGACTCTGTCAAAATTGGCATTTGAATGAGGATTTTCAAATCAAAAAAAAAAAAAAAAAAAAGTAC 3'RACE

138                                                                             PsTPS4
526 TCTGCGTTGATACCACTGCTT                                                        3'RACE
```

**Figure 4.23 Alignment of PsTPS4 with the 3'RACE fragment**. Shown are the nucleotide sequences of both fragments in 5'-3' direction. The Ps*TPS4* fragment is shown from the tps4-80_for primer binding side, from where the 3'RACE was started. The nucleotide identity of both sequences from position 1 to 138, where both sequences overlap is 100 %.

# 5. Discussion

By screening *Pinus sylvestris* cDNA with degenerate primers, we isolated four sequence fragments, all of them with high similarities to known sesquiterpene synthase sequences of conifer origin. For one of the sequence fragments, Ps*TPS4*, we found its relative transcript level to increase in egg laden compared to artificially damaged pine needles. The highly significant ($p<0.001$) transcript level difference and the sequences similarity of Ps*TPS4* to sesquiterpene synthase sequences of conifer origin, suggests an ecological relevance of the encoded terpene synthase in the production of volatiles that attract egg parasitoids. The Ps*TPS1* sequence fragment displays a similar but not as pronounced expression regulation pattern to Ps*TPS4*. Furthermore, we found that all the Ps*TPS* transcript expression patterns varied among the three applied treatments and that the sequences most similar to each other (Ps*TPS1*and Ps*TPS4*, as well as Ps*TPS2* and Ps*TPS3*) displayed similar expression patterns.

**Validity of the degenerate primer pairs and phylogenetic implications**

In the introduction we argued that terpene synthase sequences from conifers are more closely related to each other than they are to their counterparts of angiosperm origin. As an example the amino acid alignment of α-farnesene synthases from apple and loblolly pine was shown. Our results are consistent with this presumption. The isolated sequence fragments from *P. sylvestris* reveal a high similarity only to conifer sesquiterpene synthases in the NCBI databases and not to those of angiosperm origin. Ps*TPS4* for example has a maximal amino acid identity to a conifer terpene synthase of 75 percent (δ-selinene from *Abies grandis*) but it shares only 39 percent amino acid identity to its closest angiosperm terpene synthase (terpenoid synthase from *Vitis vinifera*). Trapp and Croteau (2001[1], 2001[2]) already proposed such a common origin and a high similarity of conifer terpene synthases involved in secondary metabolism. Recently Martin et al. (2004) added to this evidence by characterizing 10 terpene synthases of *Picea abies*. All of them clustered within a conifer subfamily that branched into three distinct groups, consisting mostly of either mono-, sesqui- or diterpene synthase sequences. The α-farnesene synthase sequences from *Pinus taeda* and *P. abies* however, clustered among the monoterpene synthases, supporting a phylogenetic origin of them in the monoterpene synthases as we also suggested in the introduction.

As the degenerate primers only isolated sequences fragments that showed high similarities to sesquiterpene synthase sequences of conifer origin, they can be regarded as specific to sesquiterpene synthase sequences in conifer-cDNA.

**Validity of the normalization primer pairs**

To analyze the distinct transcript regulation of the Ps*TPS* sequences, the obtained transcript level values were normalized by transcript accumulation rate of housekeeping genes that are presumed not to fluctuate under most conditions. However, expression variability is still common in most genes used for normalization of quantitative RT-PCR reactions (Vandesomplele et al. 2002, Kim et al. 2003). Among the most commonly used sequences for normalization are the ribosomal subunit 18s and 28s rRNA, ubiquitin, histone subunits and ß-actin (Pfaffl 2004). The expression of ß-actin in conifer cDNA was tested in earlier separate experiments and was found to vary considerably between samples (Witzel 2003). The α-tubulin transcripts in contrast, showed a constant level in *P. sylvestris* cDNA as well as in earlier Real-Time PCR experiments undertaken with rice and Norway spruce tissue (Kim et al. 2003, Hietala et al. 2004) Thus, α-tubulin expression rates may be nearly constant in plants, making it a suitable gene-transcript for semiquantitative RT-PCR normalization in conifers.

In addition to α-tubulin, 18s rRNA expression was used in our experiments to normalize transcript accumulations. The relative transcript level values gained by the two normalization modes differ significantly between some samples. Jasmonic acid treated needles for example, reveal a directly contradicting pattern of relative Ps*TPS4* transcript accumulation when comparing both normalization modes. While the test needles show a 30 percent higher relative transcript level than controls by α-tubulin normalization, the same raw data reveal a 50 percent lower transcript level of the test needles compared to the control when normalized by 18s rRNA (figure 4.21). Furthermore, the 18s rRNA normalized relative transcript values varied greatly between twigs of the same treatment. This is reflected by a non homogeneous deviation of these values in statistical tests. When the 18s rRNA normalized relative transcript values of Ps*TPS1* and Ps*TPS3* in artificially damaged control needles and of Ps*TPS4* in Tween control needles (from Ja treatment) of both tested twigs in summer are compared statistically, they do not show homogeneous deviation. This was only true for 18s rRNA normalized values not for α-tubulin normalized ones. We conclude that 18s rRNA is not suitable in our experiment for transcript normalization. It can serve as no more than a control for the efficiency of

conducted RT-PCR. The fact that the amplification product of 18s rRNA primer pairs appears to be irregular between probes is most likely due to the basically 'accidental' translation into cDNA. Ribosomal RNA does not possess a poly-A tail. However, the initiators of the RT-reaction, the oligo-dTprimers, use the poly-A tail of mRNA as binding site. The primers form RNA-DNA hybrids that the reverse transcriptase uses to start transcription. In spite of that, we amplified PCR-fragments with 18s rRNA primers. This is most likely due to the complex secondary structure of the rRNA. If short double stranded RNA-parts are formed, that might allow the reverse transcriptase to bind on double strand RNA instead of on the normal RNA-DNA hybrids.

In contrast to the highly diverging relative transcript values in many probes, the oviposition induced needles reveal only a minor difference in Ps*TPS4* transcript level relative to control with both normalization modes (figure 4.19). This is an intriguing observation, as Ps*TPS4* is also the sequence that shows the most significant and consistent transcript difference among that treatment.

A surprising result of our experiments was the extremely low α-tubulin and 18s rRNA fragment amplifications in cDNA of undamaged control twigs. The experimental approach was optimized for treated needle tissue. Since the PCR-reaction cycle number for the normalization primer pairs was, for example, optimized for the treated needle cDNA, we could not obtain relative transcript values of cDNA from undamaged control tissue. This would have required a substantially higher number of cycles. This fact further emphasizes the general sensibility of the normalization methods in semiquantitative PCR-reactions and enhances the necessity of prior optimization.

**The Ps*TPS* sequences**

When comparing the identified sequences with available sequence information, Ps*TPS1* and Ps*TPS4* show high similarities to the δ-selinene synthase sequence from *Abies grandis*. At the same amino acid level Ps*TPS2* and Ps*TPS3* are most similar to the γ-humulene synthase-like sequence from *P. abies*. Ps*TPS3* is 84 percent identical and Ps*TPS2* 62 percent identical to that sequence. However, the δ-selinene sequence and the γ-humulene synthase-like sequence have a 71 percent amino acid sequence identity to each other, implying a close similarity of all for Ps*TPS* sequences to each other as well. The high similarity of the identified sequences with available sesquiterpene synthase sequences suggests strongly that the Ps*TPS* fragments are coding regions of

sesquiterpene synthase genes in *P. sylvestris*. However, the full-length cDNA clone has to be identified to verify this assumption.

The specific-primer pairs used for the amplification of the Ps*TPS* fragment might in some cases amplify products that are not identical to the original fragment obtained with the degenerate primers. This is for example most likely true for the Ps*TPS2* fragment, as the forward primer has two different nucleotide bases than the original sequence. Nevertheless, the homologous primer pairs are specific for one sequence as we showed by multiple sequencing of their products. Within each primer pair the divergences observed among different sequences obtained are due to the error rate characteristic of all Taq-Polymerases without proof reading properties. In the amplified Ps*TPS4* sequences, for example, both adenine and guanine were detected in one position (symbolized by an 'R' in figure 4.17). The original sequence possesses an adenine nucleotide base in that position and this is therefore most likely the correct nucleotide base in the amplified sequence.

When conducting multiple semiquantitative RT-PCR reactions with the specific homologous primer pairs, we found the relative Ps*TPS* transcript levels to display high standard error bars in most treatments. Only for the Ps*TPS4* sequence did we find significant transcript difference in cDNA samples for both normalization modes when comparing oviposition-induced needles and artificially damaged controls. This result is discussed in detail below, while the regulation patterns of the other Ps*TPS* fragments are discussed in an attempt to identify general trends and their possible resulting implications.

In this study jasmonic acid and methyl jasmonate (collectively referred to as jasmonates) were used as artificial stimulants of pine twig volatiles. Jasmonates are synthesized in the plant via the octadecanoid pathway and serve as regulators involved in diverse developmental processes such as seed germination, flower development and fruit ripening, and as regulatory unit of plant defense mechanisms in response to herbivory, various pathogens and environmental stress (Wasternack and Parthier 1997, Baldwin 1998, Koch et al. 1999, Chuanyou et al. 2002). Jasmonic acid is thought to be the regulatory unit within the cell that may be in equilibrium with its volatile counterpart methyl jasmonate. Methyl jasmonate can cross membranes and so could serve as a signal between cells as well as between organisms (Cheong and Choi 2003, Devoto and

Turner 2003, Stratmann 2003). Both compounds are used frequently to imitate herbivory attacks (Wegener et al. 2001, Martin et al. 2002, Martin et al. 2003, Arimura et al. 2004).

In spite of the supposed similar responses, the observed relative Ps*TPS*-transcript levels differ greatly between oviposition- and chemically treated needles. A similar transcript accumulation pattern could not be observed for any of the four Ps*TPS* sequences in oviposition-treated and in jasmonate-derivate treated needles. A reason for this might be the various plant responses to jasmonates mentioned above. Jasmonates can be expected to induce a more general stress response than an insect elicitor does in plants. In contrast to the different relative transcript levels we measured, in behavioral experiments jasmonic acid treatment of needles resulted in parasite-responses similar to oviposition-induced needles. However, the examined volatile profile of jasmonate treated plants differed from the profile emitted by egg induced plants. The compounds α-muurolene, γ-cadinene and δ-cadinene were emitted in addition to (E)-ß-farnesene by the pine needles after jasmonic acid treatment (Hilker et al. 2002[1], Mumm et al. 2003). The similar response to both volatile cues suggests that the parasitic wasp cannot distinguish between both foliage blends. Nevertheless, Mumm et al. (2004[2]) found that *C. ruforum* is attracted by pine needles induced by *Diprion pini* as well as by the diprionid species *Neodiprion sertifer* but not by *P. sylvestris* needles laden with eggs from *Gilpinia pallida*, another diprionid wasp. This might suggest that *C. ruforum* can distinguish between different emitted blends by the tree. But, if the tree emits different volatile blends in reaction to different egg parasitoids that *C. ruforum* can distinguish, it would not be expected to respond to a more general blend emitted by the needles after jasmonic acid treatment. Therefore, another explanation for the different responses of *C. ruforum* to oviposition by different wasps might lie in the sensitivity or specificity of the trigger that causes a response in the pine tree. Since *G. pallida* is only considered a marginal pest of *P. sylvestris* in Europe (Pschorn-Walcher 1982), a specific elicitor that induces an altered and parasitoid-attractive volatile blend for its egg deposition might not be sensed in *P. sylvestris*. Consequently, the volatile blend may not change after *G. pallida* oviposition, and there would be no attractive blend sensed by *C. ruforum*. A further investigation of the tree response to egg deposition by different pest species is necessary to address these questions. Expression level differences of the Ps*TPS* transcripts in response to oviposition of different insects could give some insight into the specificity of tree defense regulation. Mumm et al. (2004[1]) also found that *Pinus nigra*

needles laden with pine sawfly eggs change their volatile blend qualitatively and quantitatively from that of induced *P. sylvestris* needles. *C*. *ruforum* shows no attraction to the induced volatile blend of *P. nigra*. By screening for the *PsTPS* fragments from *P. sylvestris* in treated *P. nigra* we would be able to test for the differences of transcripts responding to insect oviposition in these two closely related species.

Although methyl jasmonate and jasmonic acid are products of the same pathway and are therefore thought to have the same regulatory actions (Liechti and Farmer 2002, Cheong and Choi 2003), the four Ps*TPS* transcripts show different regulation patterns in needles treated with jasmonic acid and methyl jasmonate. For example Ps*TPS2* and Ps*TPS3* show in contrast to the seasonal difference in transcript level 72 hours after methyl jasmonate treatment, (for discussion see below) a 10 to 50 percent increase in expression level 72 hours after jasmonate treatment independently of the season. The expression differences might be due to concentration effects, solubility and the mode of application. While jasmonic acid was dissolved in the water the twigs were supplied with at a 0.3 mM concentration, methyl jasmonate was sprayed in an 0.1 mM solution on the needle surface. The intercellular mobility of methyl jasmonate is expected to be higher. However the membrane transport and regulatory efficiency *in planta* of an artificially applied phytohormone can not be predicted in all tissues (Cheong and Choi 2003). More experiments with different concentrations of jasmonic acid and methyl jasmonate applied in both ways would need to be done to fully reveal the differences between both treatments. Here we can simply conclude that both treatments have a different effect on the pine needle terpene synthase gene expression.

**Possible seasonal regulation of Ps*TPS2* and Ps*TPS3* transcript accumulation**

Sampling was undertaken twice in this study, once in the summer and once in the winter of 2003. In needles harvested in summer both Ps*TPS2* and Ps*TPS3* show a nearly 20 percent lower expression level in the methyl jasmonate treated needles compared to the control needles. By contrast, in winter the treated needles showed the maximum expression rate of 100 percent and the corresponding control needles had a 30 or 50 percent lower rate. Although the data obtained from the winter needles originate only from one twig, the results suggest seasonal expression differences of *PsTPS2* and *Ps TPS3*.

It is known that secondary terpenoid concentrations vary greatly in conifers (Wibe et al. 1998, Sjödin et al. 2000). Gambliel and Cates (1995), for example, described changes in terpene concentrations of Douglas-fir (*Pseudotuga menziesii*) due to maturation of needles. They found primarily an increase in camphene and bornyl acetate during needle maturation. Sjödin et al. (2000) found monoterpene variations within different tissue of the same *P. sylvestris* tree. In addition to maturation dependent and tissue dependent terpene differences, the trees display seasonal changes in terpene contents as shown for example by Kylin et al. (2002) in *P. sylvestris* needles and mentioned for Douglas fir in the introduction. Besides abiotic reasons for seasonally changing terpene contents in trees (e.g. carbon availability), most herbivorous insects are only active during the summer and terpenoid based defenses of host trees could therefore be up regulated during that time. Lombardo et al. (2000) described a down regulation of the constitutive resin flow in *P. taeda* in summer but an upregulation of the induced resin flow. The lower constitutive defense in this example could be the result of a higher demand on the carbon pool by primary metabolism during the growth season. Herbivore attacks at this time could be repelled by specific induced response systems that are expressed at higher rates during this time. Similar regulation patterns could be true for *P. sylvestris* as well. Ps*TPS2* and Ps*TPS3* may code for sesquiterpene synthases that serve in the constitutive terpene defense. If so, they might show greater transcript levels in winter compared to summer and be induced to even higher levels by signals. Ps*TPS2* and Ps*TPS3*, that both have the highest amino acid similarity to the same γ-humulene synthase-like protein from *P. abies*, might have therefore also similar functions in *P. sylvestris*.

Seasonal differences were not considered in the conducted olfactometer experiments by Hilker et al. (2002[1]). *C. ruforum* was attracted by egg laden twigs in lab experiments independent of seasons. There might be two explanations for that. Firstly, the cut branches were kept in climate-chambers a couple of days prior to behavior experiments and prior to treatment for this study. The pine branches are in the climate-chambers exposed to stable conditions mimicking summer conditions in circadian rhythmic and temperature. Hence, when the tests were undertaken, the pine branches might have adapted to a volatile blend similar to the summer blend. However, this should then be true for the needles whose RNA is analyzed here as well. The found expression differences might then be just the low end of regulation differences between seasons. An additional explanation for the seasonal independent behavior of *C. ruforum* might be the

necessary prior-learning phase of the wasp. In order to recognize egg induced *P. sylvestris* volatile blends, *C. ruforum* has to be previously exposed to the sample host complex (Mumm et al. 2004[2]). Therefore, the wasps experience the changed winter volatile blend and are attracted to it in the olfactometer experiments. As Mumm et al. argue, previous learning by the wasp is necessary in order to recognize a volatile blend that is highly variable due to environmental, seasonal and geographic conditions such as the one from pine trees.

A similar trend in seasonal expression differences is visible in the Ps*TPS4* transcript accumulation in methyl jasmonate treated needles. However, the differences are in both seasons not significant for α-tubulin normalization. We did find highly significant transcript differences of Ps*TPS4* in oviposition induced needles that are consistent and deserve therefore greater attention.

**Increase of Ps*TPS1* and Ps*TPS4* transcript level by oviposition**

The Ps*TPS1* and Ps*TPS4* sequence fragments reveal a consistent transcript accumulation 72 hours after oviposition. As both sequences have the highest amino acid sequence similarity to the same δ-selinene synthase from *A. grandis*, the supposed encoded synthases could have a similar function in *P*. sylvestris.

The Ps*TPS1* relative transcript level is 30 percent higher in egg laden needles than in artificially damaged needles. Both treatments display a two fold increase in Ps*TPS1* transcript level 72 hours compared to 48 hours after treatment.

The transcript increase over time is more pronounced in the Ps*TPS4* fragment. The oviposition induced needles reveal here a 90 percent increase and the artificially damaged needles a 50 percent transcript increase 72 hours after induction, demonstrating that 40 percent of the transcript increase is due to actual oviposition. These are the greatest expression level differences that we found in this study. In spite of the small sample size, the low standard deviation (maximum 10 percent) and the high statistical significance ($p<0.001$) of this result for both normalization modes and sampling periods suggests strongly that an induction of the Ps*TPS4* sequence fragment by sawfly oviposition occurs regularly. Our results propose that insect egg deposition results in a higher expression rate of a sequence that is expressed at lower rates after artificially wounding. We detected transcriptional upregulation 72 hours after induction; a similar expression and time patterns were found by McKay et al. (2003) in a monoterpene synthase transcript (*TPS2*) from Sitka spruce (*Picea sitchensis*). A White pine weevil

(*Pissodes strobi*) attack caused here a two fold increase in transcript level 48 hours after treatment compared to an artificial wounded control. Hence, relative differences in expression patterns rather than the *de novo* expression of terpene synthases might be a more common strategy applied by conifers after insect attack when compared to artificial wound reactions.

A significant up regulation of the Ps*TPS4* and Ps*TPS1* transcripts was only detected 72 hours after oviposition, correlating with the time window in which an induced pine twig is found to be attractive for *C*. *ruforum*. One might speculate that the up regulation of the terpene synthase causing a higher (E)-ß-farnesene emission would have to be earlier in order to produce the desired compound effectively at 72 hours. In contrast to *Arabidopsis thaliana*, the signaling pathways of conifers are not fully understood (Martin et al. 2003, Martin et al. 2004). The formation of traumatic resin ducts for example, occurs about 25 days after bark beetle attack in Norway spruce (Martin et al. 2002, Martin et al. 2003). but the (+)-3-carene synthase transcript involved in induced terpene defenses, was found to be upregulated in Norway spruce only two days after methyl jasmonate treatment of stems (Fäldt et al. 2003). These results suggest a great temporal variability in transcript expression after induction in conifers. A transcript upregulation three days after induction, as we observed, is within the reaction time of terpene synthase gene expression already observed in conifers. However, the needles were only harvested 48 hours and 72 hours after induction in our study. There might be an even higher transcript accumulation of Ps*TPS1* and / or Ps*TPS4* between 48 and 72 hours after induction that was missed due to the sampling design. *C. ruforum* is attracted to induced pine needles maximally 90 hours after treatment (unpublished information given by Hilker et al.) hence, harvesting of samples between 48 and 72 hours after treatment, rather than sampling time points succeeding 72 hours would appear to be rewarding in future work to narrow down the time window of possible transcriptional regulation.

The amino acid sequence of Ps*TPS4* has, as already mentioned, highest similarities with the δ-selinene synthase from grand fir (75 percent). Recently, a new sesquiterpene synthase sequence from Norway spruce was published (*PaTPS-Lon*) that reveals high similarities to the δ-selinene synthase as well (Martin et al. 2004). *PaTPS-Lon* and the Ps*TPS4* fragment show a 68 percent identity in their amino acid sequences. *PaTPS-Lon* appears to be a multiple product synthase that forms from its substrate FPP 60 pecrent longifolene and intriguingly, 3.4 percent (E)-ß-farnesene, the crucial compound in the Scots pine attraction to egg parasitoids. While the (E)-ß-farnesene production is only

minor in *PaTPS-Lon*, the volatile pattern changes after oviposition are also subtle in the Scots pine needles volatiles. This could suggest that (E)-ß-farnesene is as well a side product of a multiple product synthase in Scots pine. Longifolene was not consistently detected by Mumm et al. (2003) when analyzing the pine volatile blend. Due to the recent *PaTPS-Lon* characterization and its similarity to our identified Ps*TPS4* sequence fragment, it appears promising to screen the *P. sylvestris* induced volatile blend again for longifolene emission. When longifolene is also a part of the *P. sylvestris* foliage blend, Ps*TPS4* and *PsTPS-Lon* might both serve in similar defense systems in *P. sylvestris* and *P. abies*.

Further characterization of the full length Ps*TPS4* clone is the next step to identify molecular regulation systems that underlie the observed ecological effect of a higher (E)-ß-farnesene emission by *P. sylvestris* after oviposition. A functional identification of the full length Ps*TPS4* clone by heterologous expression in *E. coli* and *A. thaliana* would reveal its terpene product profile and enable us to explore the ecological relevance of the observed Ps*TPS4* transcript upregulation in the induction of volatiles after sawfly oviposition. The identification and characterization of the other Ps*TPS* full-length clones is also an exciting future project to give a better understanding of the variety of secondary terpenoid synthases in conifers.

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# 7. Abbreviations

| | |
|---|---|
| bp | -base pairs |
| cDNA | -copy DNA (complementary to mRNA) |
| DNA | -deoxyribonucleic acid |
| DNAse | -deoxyribonuclease |
| dNTP | -deoxynucleotide triphosphate |
| EDTA | -ethylendiamintetra acetic acid |
| FPP | -farnesyl diphosphate |
| IPP | -isopentyl diphosphate |
| Ja | -jasmonate |
| kb | -kilo bases |
| L:D | -light : Dark |
| LB Medium | -luria-bertani (broth) medium |
| MJ | -methyl Jasmonate |
| mRNA | -messenger Ribo Nucleotide Acid |
| NCBI | -National Centre for Biotechnology Information |
| rRNA | -ribosomal Ribo Nucleotide Acids |
| RT-PCR | -Reverse Trancriptase Polymerase Chain Reaction |
| SDS | -sodiumdodecylsulfat |
| Taq | -*Thermophilus aquaticus* |
| Tris | -tris(hydroxymethyl)amoniumethane |
| TBE | -tris / borate (buffer) |
| U | -units |
| v/v | -volume per volume |
| v/w | -weight per volume |

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# Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

_____ _____

Ort, Datum Unterschrift

# Appendix

**Ps*TPS1*: The relative transcript values for oviposition induced needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| α-tubulin | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | 39.7986025 | 30.8315632 | 100 | 60.1605087 | 100 | 56.0179761 | 1 |
| | 57.6675561 | 47.871806 | 100 | 74.9683357 | 100 | 47.2065256 | 1 |
| | 47.1554454 | 34.6894025 | 100 | 83.8158013 | 100 | 12.2622052 | 1 |
| | 33.3324795 | 35.6351088 | 100 | 93.5900063 | 100 | 30.0460349 | 1 |
| | 6.27997302 | 14.2182181 | 100 | 29.3752984 | 100 | 32.1106029 | 1 |
| | 48.3374339 | 30.4020702 | 100 | 71.3225408 | 78.4735858 | 100 | 2 |
| | 51.7752465 | 14.8052039 | 100 | 87.1766741 | 100 | 78.1187823 | 2 |
| | 44.5722776 | 26.8079316 | 100 | 61.849989 | 38.5745444 | 100 | 2 |
| | 64.54444 | 37.4213879 | 100 | 88.1921154 | 100 | 60.2718413 | 2 |
| | | | | | 100 | 77.1677639 | 2 |
| **mean** | 43.7181616 | 30.2980769 | 100 | 72.2723633 | 91.704813 | 59.3201732 | |
| **st. deviation** | 15.8182788 | 10.1017747 | 0 | 18.7584688 | 19.8560705 | 29.7245871 | |
| **st. error** | 5.3 | 3.37 | 0 | 6.25 | 6.28 | 9.4 | |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | 60.0291094 | 20.7606952 | 100 | 45.5732887 | 100 | 46.1324509 | 1 |
| | 78.5845029 | 53.3230744 | 98.102463 | 100 | 100 | 35.9668766 | 1 |
| | 63.6369576 | 50.2273618 | 100 | 96.6753289 | 100 | 4.21589791 | 1 |
| | 9.50151751 | 43.0239582 | 53.482331 | 100 | 100 | 1.12325946 | 1 |
| | 74.7556856 | 73.885368 | 85.0595983 | 100 | 39.2367929 | 100 | 1 |
| | 59.3911698 | 21.6147117 | 97.7638499 | 100 | 100 | 28.0426398 | 1 |
| | 83.3347164 | 70.1894176 | 100 | 93.4993597 | 29.6727265 | 100 | 2 |
| | 59.3477576 | 43.3434737 | 97.0089097 | 100 | 100 | 65.0935886 | 2 |
| | 80.68055 | 46.7767349 | 100 | 47.2457761 | 100 | 99.2156964 | 2 |
| **mean** | 63.2513297 | 47.0160884 | 92.3796835 | 86.9993059 | 85.434391 | 53.3100455 | 2 |
| **st. deviation** | 21.0931505 | 17.234042 | 14.4608044 | 21.8006738 | 29.0014656 | 39.8912984 | |
| **st. error** | 7.03 | 5.74 | 4.82 | 7.26 | 9.67 | 13.29 | |

**Ps*TPS1*: relative transcript values for jasmonic acid treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | **48h** | | **72h** | | **48h** | | |
| | | | | | 100 | 99.5806761 | 1 |
| | | | | | 70.4019194 | 100 | 1 |
| | | | | | 93.0297519 | 100 | 1 |
| | | | | | 96.9453673 | 100 | 1 |
| | | | | | 83.7610949 | 100 | 1 |
| | | | | | 100 | 82.3300789 | 1 |
| | | | | | 100 | 51.7881914 | 1 |
| | | | | | 57.6929368 | 100 | 1 |
| | | | | | 73.6247059 | 100 | 1 |
| | | | | | 100 | 84.4263966 | 1 |
| | | | | | 100 | 26.6300979 | 1 |
| | | | | | 59.352157 | 100 | 1 |
| | | | | | 46.2384913 | 100 | 2 |
| | | | | | 61.7513553 | 100 | 2 |
| | | | | | 25.9372873 | 100 | 2 |
| | | | | | 58.2197718 | 100 | 2 |
| | | | | | 58.6025701 | 100 | 2 |
| | | | | | 68.2246346 | 100 | 2 |
| | | | | | 81.4358002 | 100 | 2 |
| | 61.5566689 | 85.0622176 | 100 | 81.1265417 | 48.3002169 | 100 | 2 |
| | 100 | 1.31301879 | 1.05977388 | 1.01909179 | 67.1622119 | 100 | 2 |
| | 12.5634178 | 100 | 10.745866 | 10.208094 | 83.5272015 | 100 | 2 |
| | 79.5590223 | 100 | 69.3831533 | 59.7248365 | 100 | 97.7111984 | 2 |
| | 100 | 64.0587893 | 69.5080861 | 5.69283825 | 85.7189038 | 100 | 2 |
| **mean** | 70.7358218 | 70.0868051 | 50.1393759 | 31.5542805 | 75.8302657 | 93.43611 | |
| **st. deviation** | 36.2592007 | 41.1704751 | 42.4034424 | 36.427534 | 21.0526276 | 17.7343526 | |
| **st. error** | 16.2 | 18.41 | 18.96 | 16.29 | 4.29 | 3.47 | |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | T | C | T | C | T | C | twig |
| | 48h | | 72h | | 48h | | |
| | | | | | | | |
| | | | | | 66.9473931 | 100 | 1 |
| | | | | | 100 | 60.7015307 | 1 |
| | | | | | 50.743501 | 100 | 1 |
| | | | | | 100 | 60.171347 | 1 |
| | | | | | 37.1213943 | 100 | 1 |
| | | | | | 100 | 90.5630868 | 1 |
| | | | | | 100 | 29.8174436 | 1 |
| | | | | | 99.6672855 | 100 | 1 |
| | | | | | 80.317861 | 100 | 1 |
| | | | | | 100 | 84.4263966 | 1 |
| | | | | | 100 | 48.8218462 | 1 |
| | | | | | 100 | 90.7231625 | 1 |
| | | | | | 100 | 25.7464324 | 2 |
| | | | | | 100 | 35.7459014 | 2 |
| | | | | | 100 | 53.3626755 | 2 |
| | | | | | 100 | 47.2348124 | 2 |
| | | | | | 100 | 23.4631348 | 2 |
| | | | | | 100 | 43.5762388 | 2 |
| | | | | | 100 | 36.3840345 | 2 |
| | 2.11014415 | 100 | 14.3117748 | 21.769965 | 66.0950337 | 100 | 2 |
| | 83.0840423 | 100 | 29.0565982 | 39.9159824 | 100 | 45.0356126 | 2 |
| | 100 | 42.4512931 | 17.5452273 | 27.0841744 | 100 | 44.6960184 | 2 |
| | 100 | 39.902491 | 23.2559095 | 35.7475463 | 100 | 17.9469548 | 2 |
| | 100 | 57.6529104 | 17.8735079 | 3.41570295 | 100 | 39.391817 | 2 |
| **mean** | 77.0388373 | 68.0013389 | 20.4086035 | 25.5866742 | 91.7038529 | 61.5753519 | |
| **st. deviation** | 42.5220488 | 29.9886174 | 5.80284235 | 14.2910299 | 17.8856455 | 29.3656341 | |
| **st. error** | 19.01 | 13.37 | 2.59 | 6.39 | 3.65 | 5.99 | |

**Ps*TPS1*: relative transcript values for methyl jasmonate treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | | | |
| | | | | | 16.7524089 | 100 | 1 |
| | | | | | 47.4989989 | 100 | 2 |
| | | | | | 31.4442573 | 100 | 1 |
| | 45.3430570 | 89.4463724 | 100 | 75.1251813 | 58.2530051 | 100 | 2 |
| | 30.9922154 | 39.8480533 | 30.7755296 | 100 | 11.7028380 | 100 | 1 |
| | 100 | 40.4385623 | 64.2935692 | 88.3638201 | 56.4990296 | 100 | 2 |
| **mean** | 58.7784241 | 56.5776626 | 65.0230329 | 87.8296671 | 37.0250896 | 100 | |
| **st. devation** | 36.4129153 | 28.4666689 | 34.6179999 | 12.4460090 | 20.11420 | 0 | |
| **st error** | 20.78 | 16.44 | 19.99 | 7.2 | 8.16 | 0 | |

| **18s rRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 8.97450475 | 100 | 1 |
| | | | | | 11.4455419 | 100 | 2 |
| | | | | | 18.8665544 | 100 | 1 |
| **mean** | 90.686114 | 52.1770506 | 100 | 13.1469067 | 2.48546155 | 100 | 2 |
| **st. devation** | 100 | 24.1076990 | 99.3008379 | 13.4442363 | 21.4552030 | 100 | 1 |
| **st error** | 100 | 14.1534968 | 60.0073312 | 7.57404172 | 21.0120358 | 100 | 2 |
| | 96.8953713 | 30.1460821 | 86.4360564 | 11.3883949 | 14.0398836 | 100 | |
| | 5.37737457 | 19.7178653 | 22.8906169 | 3.30667037 | 7.65229324 | 0 | |
| | 3.11 | 11.38 | 13.21 | 1.91 | 2.86 | 0 | |

**Ps*TPS2*: relative transcript values for oviposition induced needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 100 | 41.4716852 | 1 |
| | 77.6675311 | 51.9486793 | 100 | 94.9575372 | 100 | 16.5703506 | 1 |
| | 100 | 63.6479724 | 58.3180709 | 72.2201437 | 100 | 46.7453005 | 1 |
| | 100 | 63.7420707 | 17.5739454 | 88.2346773 | 89.0694839 | 100 | 2 |
| | 53.6046587 | 82.9691014 | 83.6542147 | 100 | 100 | 68.2702913 | 2 |
| | 43.3410612 | 20.3819383 | 100 | 8.02103106 | 100 | 92.1595508 | 2 |
| | 66.0744686 | 53.0634438 | 100 | 75.4252470 | 54.7753379 | 100 | 1 |
| | 100 | 53.6225605 | 84.0593873 | 92.0136859 | 36.6887757 | 100 | 1 |
| | 75.8528149 | 60.8228847 | 100 | 73.6929929 | 48.9424699 | 100 | 2 |
| | 61.9946195 | 94.0193016 | 84.5250403 | 100 | 100 | 83.3325445 | 2 |
| **mean .** | 75.3927949 | 60.4686614 | 80.9034065 | 78.2850350 | 82.9476067 | 74.8549723 | |
| **st. deviation** | 19.9730610 | 19.5337479 | 25.8415706 | 26.8981647 | 25.5431826 | 30.2681718 | |
| **st error** | 6.66 | 6.51 | 8.6 | 8.966 | 8.08 | 9.57 | |

| **18s rRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | 100 | 29.8591178 | 85.4682285 | 61.4022699 | 100 | 34.1531525 | 1 |
| | 100 | 52.0253052 | 42.003057 | 70.6927884 | 100 | 5.09856941 | 1 |
| | 100 | 68.389930 | 13.0224201 | 75.4374671 | 44.5347419 | 100 | 2 |
| | 100 | 94.0537114 | 81.5596948 | 41.6402230 | 100 | 60.6847034 | 2 |
| | 79.2398421 | 100 | 65.9587797 | 82.0047898 | 84.3947014 | 100 | 2 |
| | 100 | 68.2468952 | 78.8056756 | 92.0136859 | 100 | 62.7677324 | 1 |
| | 100 | 94.3609719 | 87.6366436 | 94.2573857 | 100 | 10.1896601 | 1 |
| | 84.7665721 | 82.5355061 | 81.4188671 | 100 | 37.6480538 | 100 | 2 |
| | 65.9381834 | 100 | 71.9214365 | 45.5833453 | 100 | 89.9991480 | 2 |
| **mean .** | 92.2160664 | 76.6079375 | 67.5327559 | 73.6702172 | 85.1752775 | 62.5436629 | |
| **st. deviation** | 11.9161059 | 22.7943441 | 23.3032794 | 19.7211691 | 25.5678847 | 38.4271106 | |
| **st error** | 3.97 | 7.6 | 7.77 | 6.57 | 8.52 | 12.81 | |

**Ps*TPS2*: relative transcript values for jasmonic acid treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | |
| | | | | | | | **twig** |
| | | | | | 100 | 69.8412792 | 1 |
| | | | | | 87.9361386 | 100 | 1 |
| | | | | | 100 | 79.0705956 | 1 |
| | | | | | 100 | 53.2614971 | 1 |
| | | | | | 100 | 63.7154703 | 1 |
| | | | | | 100 | 71.0821482 | 1 |
| | | | | | 100 | 31.8912676 | 1 |
| | | | | | 192.021107 | 100 | 1 |
| | | | | | 82.3636661 | 100 | 2 |
| | | | | | 94.6857696 | 100 | 2 |
| | | | | | 64.1289325 | 100 | 2 |
| | | | | | 68.3992033 | 100 | 2 |
| | 8.83614832 | 100 | 6.91335788 | 6.05972122 | 75.1754075 | 100 | 2 |
| | 100 | 95.0687282 | 54.7061151 | 40.299240 | 78.5192148 | 100 | 2 |
| | 100 | 72.6741438 | 67.9390782 | 70.4790303 | 100 | 85.6134298 | 2 |
| | 100 | 7.82779424 | 91.2545935 | 42.0530167 | 100 | 55.2475828 | 2 |
| **mean** | 77.2090371 | 68.8926666 | 55.2032862 | 39.7227520 | 96.4518399 | 81.8577044 | |
| **st. deviation** | 45.5819258 | 42.4109679 | 35.5624405 | 26.3622885 | 28.4186552 | 22.0108318 | |
| **st error** | 22.79 | 21.2 | 17.78 | 13.18 | 7.11 | 5.5 | |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | |
| | | | | | 100 | 40.2116456 | 1 |
| | | | | | 100 | 99.5040281 | 1 |
| | | | | | 100 | 72.4813793 | 1 |
| | | | | | 100 | 53.2614971 | 1 |
| | | | | | 85.6078662 | 100 | 1 |
| | | | | | 100 | 38.2750029 | 1 |
| | | | | | 100 | 42.5216902 | 1 |
| | | | | | 100 | 35.8033558 | 1 |
| | | | | | 100 | 35.974151 | 2 |
| | | | | | 100 | 77.1783589 | 2 |
| | | | | | 100 | 47.1657837 | 2 |
| | | | | | 100 | 54.5815324 | 2 |
| | 100 | 60.3581237 | 16.0491273 | 22.8595877 | 100 | 24.4326536 | 2 |
| | 100 | 30.1805486 | 14.5882974 | 19.1901143 | 100 | 43.0037843 | 2 |
| | 100 | 65.4067294 | 17.4700487 | 42.2874182 | 100 | 9.51260331 | 2 |
| | 15.8287313 | 1.71559457 | 100 | 2.3041589 | 100 | 9.50024573 | 2 |
| **mean** | 78.9571828 | 39.4152491 | 37.0268683 | 21.6603198 | 99.1004916 | 48.9629820 | |
| **st. div** | 42.0856343 | 29.5561877 | 41.9985698 | 16.4081109 | 3.59803345 | 26.9403734 | |
| **st error** | 21.04 | 14.78 | 21 | 8.2 | 0.9 | 6.735 | |

**Ps*TPS2*: relative transcript values for methyl jasmonate treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| α-tubulin | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | | | | | 13.9314086 | 100 | 1 |
| | | | | | 46.9597713 | 100 | 1 |
| | | | | | 8.14208626 | 100 | 1 |
| | | | | | 8.05482560 | 100 | 1 |
| | | | | | 100 | 37.2407073 | 2 |
| | | | | | 100 | 60.4655327 | 2 |
| | 24.9320977 | 42.8549282 | 100 | 77.151195 | 100 | 37.5487551 | 2 |
| | 30.1327449 | 39.8210643 | 100 | 75.8980302 | 100 | 70.0360299 | 2 |
| **mean** | 27.5324213 | 41.3379963 | 100 | 76.5246126 | 59.6360115 | 75.6613781 | |
| **st. deviation** | 3.67741288 | 2.14526574 | | 0.88612132 | 44.8471167 | 28.1849816 | |
| **st. error** | 2.6 | 1.5 | | 0.63 | 15.86 | 9.96 | |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | | | | | 6.26913385 | 100 | 1 |
| | | | | | 78.2662856 | 100 | 1 |
| | | | | | 4.65262072 | 100 | 1 |
| | | | | | 14.0959448 | 100 | 1 |
| | | | | | 100 | 74.4814147 | 2 |
| | | | | | 58.1568269 | 100 | 2 |
| | 55.6455514 | 20.4958352 | 100 | 9.22459940 | 100 | 93.8718878 | 2 |
| | 38.9842387 | 12.7996278 | 100 | 35.1586463 | 100 | 95.2490007 | 2 |
| **mean** | 47.3148951 | 16.6477315 | 100 | 22.1916229 | 57.6801015 | 95.4502879 | |
| **st. deviation** | 11.7813272 | 5.44204046 | | 18.3381405 | 43.3449943 | 8.82953573 | |
| **st. error** | 8.33 | 3.85 | | 19.97 | 15.32 | 2.9 | |

**Ps*TPS3*: relative transcript values for oviposition induced needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| α-tubulin | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | 35.3326902 | 50.4362055 | 100 | 64.184454 | | | |
| | 83.9535675 | 46.4010223 | 100 | 58.2795278 | | | |
| | 40.4008072 | 84.6623459 | 100 | 68.2006297 | | | |
| | 44.3698933 | 100 | 66.7465163 | 45.4163105 | | | |
| | 3.06286249 | 4.4703173 | 100 | 15.7227602 | | | |
| | 64.235039 | 60.5211085 | 100 | 52.3876928 | | | |
| | 93.2794203 | 100 | 77.3763505 | 19.4893708 | 100 | 28.6300047 | 1 |
| | 100 | 64.756528 | 89.1459874 | 53.5585618 | 100 | 48.7117861 | 1 |
| | 43.4274255 | 92.0220879 | 80.0962532 | 100 | 100 | 5.50960730 | 1 |
| **mean** | 56.4513006 | 67.0299573 | 90.3739008 | 53.0265897 | 100 | 51.0524050 | 2 |
| **st. deviation** | 29.7008828 | 29.385754 | 12.0075733 | 23.9531309 | 100 | 69.1948617 | 2 |
| **st error** | 9.9 | 9.79 | 4 | 7.98 | 64.0862893 | 100 | 2 |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | 53.2911054 | 33.9603772 | 100 | 48.6197221 | | | |
| | 100 | 45.177103 | 85.7501797 | 68.8000165 | | | |
| | 44.4768237 | 100 | 81.5766947 | 64.1717698 | | | |
| | 8.65797224 | 25.2730139 | 99.9226222 | 100 | | | |
| | 67.5414645 | 100 | 57.8310624 | 49.9390538 | | | |
| | 73.2909731 | 100 | 51.8145204 | 15.3130771 | | | |
| | 53.2433645 | 100 | 81.2897398 | 91.3697533 | | | |
| | 100 | 78.6329269 | 64.9492194 | 65.0353964 | 100 | 23.5776509 | 1 |
| | 47.1923931 | 100 | 69.6321981 | 46.5726695 | 100 | 7.49412094 | 1 |
| mean | 60.8548996 | 75.8937134 | 76.9740263 | 61.0912732 | 97.938579 | 100 | 2 |
| st. deviation | 26.9950921 | 30.1430637 | 16.233082 | 23.8082394 | 81.2921634 | 100 | 2 |
| st error | 9 | 10 | 5.41 | 7.94 | 49.8448917 | 100 | 2 |

**Ps*TPS3*: relative transcript values for jasmonic acid treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| α-tubulin | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | | | | | 100 | 33.6946410 | 1 |
| | 29.1532956 | 10.6604544 | 100 | 9.71254951 | 100 | 30.8545867 | 1 |
| | 37.2217535 | 20.8549788 | 63.8980098 | 100 | 57.4015630 | 100 | 1 |
| | 35.4659312 | 1.86232706 | 100 | 17.1690618 | 97.7750942 | 100 | 2 |
| mean | 33.9469934 | 11.1259201 | 87.9660033 | 42.2938704 | 88.7941643 | 66.1373069 | |
| st. deviation | 4.24327494 | 9.50487761 | 20.8434938 | 50.1138497 | 20.9546655 | 39.1184564 | |
| st. error | 2.45 | 5.48 | 12.03 | 28.93 | 10.475 | 19.56 | |

| 18s rRNA | winter | | | | summer | | |
|---|---|---|---|---|---|---|---|
| | 48h | | 72h | | 72h | | |
| | T | C | T | C | T | C | twig |
| | | | | | 100 | 57.7622417 | 1 |
| | 9.65466282 | 2.35360682 | 100 | 1.28659747 | 100 | 20.5697245 | 1 |
| | 100 | 1.34469616 | 1.32052659 | 40.7874879 | 100 | 27.2678532 | 2 |
| | 100 | 2.09004854 | 45.6112952 | 44.8581912 | 100 | 54.2370258 | 2 |
| mean | 69.8848876 | 1.92945051 | 48.9772739 | 28.9774255 | 100 | 39.9592113 | |
| st. deviation | 52.1609047 | 0.52327715 | 49.4257724 | 24.0671794 | 0 | 18.7778828 | |
| st. error | 30.11 | 0.3 | 28.53 | 13.9 | 0 | 9.390 | |

**Ps*TPS3*: relative transcript values for methyl jasmonate acid treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 35.8221156 | 100 | 1 |
| | | | | | 100 | 83.1034778 | 1 |
| | | | | | 35.4453334 | 100 | 2 |
| | | | | | 100 | 93.712951 | 1 |
| | 100 | 29.6308246 | 99.6239508 | 62.1147902 | 68.9071541 | 100 | 2 |
| | 99.2860120 | 96.4528721 | 100 | 31.9921496 | 100 | 18.0732875 | 1 |
| | 90.9979990 | 88.0909095 | 100 | 67.4447981 | 5.17827221 | 100 | 2 |
| **mean** | 96.7613370 | 71.3915354 | 99.8746503 | 53.8505793 | 63.6218393 | 84.9842452 | |
| **st. deviation** | 5.00394779 | 36.4067072 | 0.21711213 | 19.1166278 | 38.6877176 | 30.1626022 | |
| **st. error** | 2.890 | 21.02 | 0.130 | 11.04 | 14.620 | 11.40 | |

| **18s rRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 23.8814104 | 100 | 1 |
| | | | | | 100 | 92.6923406 | 1 |
| | | | | | 29.1158096 | 100 | 2 |
| | | | | | 64.0253021 | 100 | 1 |
| | 89.5675875 | 13.8005997 | 100 | 5.47325344 | 2.94003857 | 100 | 2 |
| | 99.2860120 | 18.0849135 | 100 | 1.33300623 | 100 | 9.85815680 | 1 |
| | 97.4978560 | 33.0340911 | 100 | 6.19391003 | 1.92580371 | 100 | 2 |
| **mean** | 95.4504852 | 21.6398681 | 100 | 4.3333899 | 45.9840521 | 86.0786425 | |
| **st. deviation** | 5.17259504 | 10.0975281 | 0 | 2.62327335 | 42.2845769 | 33.7202323 | |
| **st. error** | 2.98 | 5.83 | 0 | 1.51 | 15.98 | 12.74 | |

**Ps*TPS4*: relative transcript values for oviposition induced needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | 5.63048277 | 4.31955268 | 100 | 39.3248338 | | | |
| | 2.94614073 | 7.56230311 | 100 | 64.3514397 | 99.8439251 | 100 | 1 |
| | 2.00245937 | 3.08972628 | 100 | 52.1204142 | 100 | 43.5239963 | 1 |
| | 0.35084931 | 0.59029148 | 100 | 29.0104741 | 100 | 45.4054266 | 2 |
| | 1.79184155 | 3.37457888 | 100 | 64.4990987 | 100 | 23.9122343 | 1 |
| | 3.72139537 | 4.18805606 | 100 | 39.6905786 | 100 | 92.3934951 | 2 |
| | 1.24646013 | 0.61199425 | 100 | 25.3980737 | 100 | 73.2389694 | 2 |
| | 3.45843689 | 5.82072889 | 100 | 53.9814093 | 62.143830 | 100 | 2 |
| **mean** | 2.64350826 | 3.69465395 | 100 | 46.0470403 | 95.2484694 | 65.2768728 | |
| **st. deviation** | 1.65546113 | 2.38210865 | 0 | 15.0123123 | 13.3764058 | 29.8878677 | |
| **st. error** | 0.55 | 0.79 | 0 | 5 | 4.73 | 10.57 | |

| **18srRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | T 48h nv | C 48h nv | T 72h | C 72h nv | | | |
| | 7.7281136 | 7.19925446 | 100 | 65.5413897 | T(18s) nv | C(18s) nv | |
| | 3.68267591 | 9.45287889 | 100 | 34.4739855 | 100 | 49.7303944 | 1 |
| | 1.63837584 | 2.70351049 | 100 | 86.867357 | 49.6540274 | 100 | 1 |
| | 0.37694554 | 0.53347198 | 100 | 14.2854607 | 100 | 52.0532537 | 2 |
| | 2.55977365 | 4.01735581 | 100 | 92.1415695 | 100 | 11.5735214 | 1 |
| | 3.72139537 | 2.95627487 | 100 | 39.6905786 | 100 | 73.9147961 | 2 |
| | 3.82247772 | 1.46623622 | 100 | 64.9061883 | 100 | 67.6052025 | 2 |
| | 3.84270766 | 5.38956379 | 100 | 59.9793437 | 100 | 86.6476267 | 2 |
| **mean** | 3.42155816 | 4.21481831 | 100 | 57.2357341 | 93.7067534 | 59.0960334 | |
| **st. div** | 2.14591591 | 2.99375906 | 0 | 26.4641355 | 17.7999893 | 29.0214126 | |
| **st. error** | 0.72 | 1 | 0 | 8.82 | 6.3 | 10.26 | |

**Ps*TPS4*: relative transcript values for jasmonic acid treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 94.8738276 | 100 | 1 |
| | | | | | 100 | 12.3672839 | 2 |
| | | | | | 56.9707861 | 100 | 1 |
| | | | | | 69.5807854 | 100 | 2 |
| | | | | | 84.5985283 | 100 | 1 |
| | | | | | 100 | 75.6716687 | 1 |
| | | | | | 100 | 66.8745482 | 2 |
| | | | | | 87.670291 | 100 | 2 |
| | 26.7543908 | 22.6750030 | 100 | 19.4588314 | 100 | 49.3057612 | 1 |
| | 18.5715045 | 5.70770459 | 100 | 76.4969462 | 85.8097984 | 100 | 1 |
| | 21.4810543 | 53.8268485 | 100 | 23.7299864 | 100 | 81.4654114 | 2 |
| | 4.47315474 | 14.9057022 | 33.7550508 | 100 | 59.0078359 | 100 | 2 |
| **mean** | 17.8200261 | 24.2788146 | 83.4387627 | 54.921441 | 86.5426544 | 82.1403894 | |
| **st. deviation** | 9.52067389 | 20.8838046 | 33.1224746 | 39.6992125 | 16.2393016 | 27.72348 | |
| **st. error** | 4.76 | 10.44 | 16.55 | 19.85 | 4.69 | 8 | |

| **18s rRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 73.7907548 | 100 | 1 |
| | | | | | 100 | 6.47125318 | 2 |
| | | | | | 47.4756551 | 100 | 1 |
| | | | | | 100 | 32.8497914 | 2 |
| | | | | | 69.2169777 | 100 | 1 |
| | | | | | 100 | 11.6777267 | 1 |
| | | | | | 100 | 37.1525268 | 2 |
| | | | | | 100 | 24.9514399 | 2 |
| | 100 | 7.60203839 | 3.62444048 | 0.8815922 | 100 | 73.9586418 | 1 |
| | 18.8824577 | 24.4825534 | 72.6245404 | 100 | 100 | 40.8635561 | 1 |
| | 11.4747244 | 19.1687752 | 17.8059609 | 100 | 100 | 40.8635561 | 2 |
| | 10.0645982 | 9.31606385 | 15.1897729 | 100 | 100 | 55.765446 | 2 |
| **mean** | 35.1054451 | 15.1423577 | 27.3111787 | 75.2203981 | 90.8736156 | 52.0461615 | |
| **st. deviation** | 43.4355597 | 8.04680908 | 30.8309366 | 49.5592039 | 17.5650353 | 33.9437112 | |
| **st. error** | 21.715 | 4.025 | 15.415 | 21.78 | 5.07 | 9.8 | |

**Ps*TPS4*: relative transcript values for methyl jasmonate treated needles**. The raw data are shown, normalized against α-tubulin and 18s rRNA. All values are given in %. Mean, standard deviation and standard error are shown. Summer and winter data were obtained separately. For the summer values, numbers indicate the twig, whose cDNA was analyzed.

| **α-tubulin** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 26.7074920 | 100 | 1 |
| | | | | | 92.4993401 | 100 | 2 |
| | | | | | 35.2033659 | 100 | 1 |
| | | | | | 54.4666754 | 100 | 2 |
| | | | | | 100 | 3.50547799 | 1 |
| | | | | | 100 | 57.9174669 | 1 |
| | | | | | 100 | 48.5203166 | 2 |
| | | | | | 100 | 8.20984550 | 2 |
| | 26.7543908 | 22.675003 | 100 | 19.4588314 | 34.6643564 | 100 | 1 |
| | 57.1842971 | 61.2827047 | 100 | 66.1875032 | 77.5243553 | 100 | 1 |
| | 23.5113501 | 100 | 11.9822783 | 58.6173928 | 38.6800708 | 100 | 2 |
| | 1.11754008 | 1.93581806 | 100 | 0.78925368 | 84.0242174 | 100 | 2 |
| **mean** | 27.1418945 | 46.4733814 | 77.9955696 | 36.2632453 | 70.3141561 | 76.5127589 | |
| **st deviation** | 23.0444786 | 43.3373292 | 44.0088609 | 31.2834436 | 30.0215187 | 37.5837449 | |
| **st. error** | 11.52 | 21.67 | 22.005 | 15.64 | 8.67 | 10.85 | |

| **18s rRNA** | **winter** | | | | **summer** | | |
|---|---|---|---|---|---|---|---|
| | **48h** | | **72h** | | **72h** | | |
| | **T** | **C** | **T** | **C** | **T** | **C** | **twig** |
| | | | | | 21.3659936 | 100 | 1 |
| | | | | | 43.1663587 | 100 | 2 |
| | | | | | 20.1162091 | 100 | 1 |
| | | | | | 33.2851905 | 100 | 2 |
| | | | | | 100 | 2.02870266 | 1 |
| | | | | | 18.4451742 | 100 | 1 |
| | | | | | 100 | 78.869128 | 2 |
| | **t 48** | **c 48** | **t 72** | **c 72** | 100 | 30.4937119 | 2 |
| | 100 | 7.60203839 | 3.62444048 | 0.8815922 | 23.3984406 | 100 | 1 |
| | 73.8144243 | 21.6678135 | 100 | 26.0022334 | 58.1432665 | 100 | 1 |
| | 91.1971395 | 71.8306652 | 43.0347509 | 100 | 68.7645702 | 100 | 2 |
| | 2.11090903 | 0.81368177 | 100 | 0.51605048 | 29.4084761 | 100 | 2 |
| **mean** | 66.7806182 | 25.4785497 | 61.6647978 | 31.849969 | 51.34114 | 84.2826285 | |
| **st deviation** | 44.4647409 | 32.0986276 | 47.0989638 | 46.9733171 | 33.1158156 | 32.9070091 | |
| **st. error** | 22.23 | 16.05 | 23.55 | 23.485 | 9.56 | 9.5 | |