

Induction of isoprenyl diphosphate synthases, plant hormones and defense signalling genes correlates with traumatic resin duct formation in Norway spruce (*Picea abies*)

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Abstract Norway spruce (*Picea abies*) defends itself against herbivores and pathogens by formation of traumatic resin ducts filled with terpenoid-based oleoresin. An important group of enzymes in terpenoid biosynthesis are the short-chain isoprenyl diphosphate synthases which produce geranyl diphosphate (C₁₀), farnesyl diphosphate (C₁₅), and geranylgeranyl diphosphate (C₂₀) as precursors of monoterpenes, sesquiterpenes, and diterpene resin acids, respectively. After treatment with methyl jasmonate (MJ) we investigated the expression of all isoprenyl diphosphate synthase genes characterized to date from Norway spruce and correlated this with formation of traumatic resin ducts and terpene accumulation. Formation of traumatic resin ducts correlated with higher amounts of monoterpenes, sesquiterpenes and diterpene resin acids and an upregulation of isoprenyl diphosphate synthase genes producing geranyl diphosphate or geranylgeranyl diphosphate. Among defense hormones, jasmonate and jasmonate-isoleucine conjugate accumulated to higher levels in trees with

extensive traumatic resin duct formation, whereas salicylate did not. Jasmonate and ethylene are likely to both be involved in formation of traumatic resin ducts based on elevated transcripts of genes encoding lipoxygenase and 1-aminocyclopropane-1-carboxylic acid oxidase associated with resin duct formation. Other genes involved in defense signalling in other systems, mitogen-activated protein kinase3 and nonexpressor of pathogenesis-related gene1, were also associated with traumatic resin duct formation. These responses were detected not only at the site of MJ treatment, but also systemically up to 60 cm above the site of treatment on the trunk.

Keywords *Picea abies* · Prenyltransferase · Isoprenyl diphosphate synthase · Methyl jasmonate · Traumatic resin ducts · Terpenes

Introduction

A characteristic feature of conifers in the pine family (Pinaceae) is the production of oleoresin, a very viscous mixture primarily composed of mono-, sesqui-, and diterpenes (Keeling and Bohlmann 2006a, b). Resin-producing structures are present constitutively in many species, but resin accumulation is also induced after herbivore or pathogen attack and probably plays an important defensive role as a physical and chemical barrier against invaders (Franceschi et al. 2005; Krokene et al. 2008a, b). Resin induction involves the differentiation of newly formed traumatic resin ducts (TRD) localized in the young sapwood (Christiansen et al. 1999; Martin et al. 2002), enhanced biosynthesis of terpenoid constituents (Martin et al. 2002; Miller et al. 2005; Zeneli et al. 2006) and induction of genes and enzymes involved in terpenoid

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biosynthesis (Martin et al. 2002, 2004; Fäldt et al. 2003; Huber et al. 2005; Byun-McKay et al. 2006; Hamberger and Bohlmann 2006; Ro and Bohlmann 2006; Schmidt and Gershenzon 2007, 2008; Schmidt et al. 2010b).

Two main classes of enzymes are involved in the biosynthesis of oleoresin. First, three different kinds of prenyltransferases, referred to collectively as isoprenyl diphosphate synthases (IDS), catalyze the sequential condensation reactions between several units of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Reaction products are geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅), and geranylgeranyl diphosphate (GGPP, C₂₀), the precursors of monoterpenes, sesquiterpenes and diterpenes, respectively. GPP, FPP, and GGPP are each formed by a specific IDS: GPP synthase (GPPS, EC 2.5.1.1), FPP synthase (FPPS, EC 2.5.1.10), and GGPP synthase (GGPPS, EC 2.5.1.30), respectively (Gershenzon and Kreis 1999). Second, terpene synthases (TPS) in combination with cytochrome P450 mono-oxygenases (Cyp P450) convert these linear diphosphates into the huge diversity of terpenes present in conifers (Martin et al. 2004; Keeling and Bohlmann 2006b) (Fig. 1).

Numerous genes and enzymes involved in induced oleoresin synthesis have been characterized recently from conifers, including some for IDS (Tholl et al. 2001; Burke and Croteau 2002; Schmidt et al. 2005, 2010b; Schmidt and Gershenzon 2007, 2008), some for TPS (Fäldt et al. 2003; Martin et al. 2004; Huber et al. 2005; Byun-McKay et al. 2006; Ro and Bohlmann 2006), and some for Cyp P450 (Hamberger and Bohlmann 2006). Due to their position at a branch point of terpene biosynthesis, IDS are

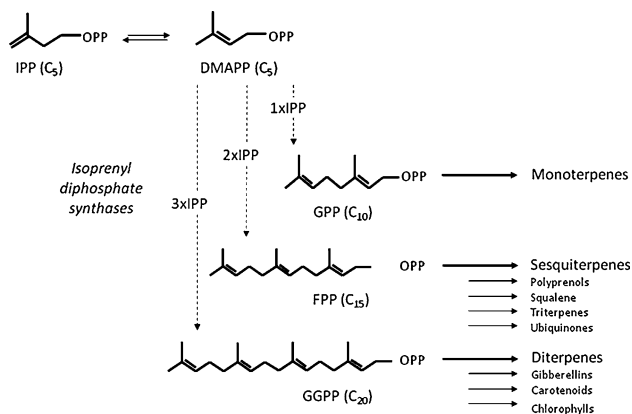


Fig. 1 Outline of terpenoid oleoresin biosynthesis in conifers showing the condensations of the two C₅ diphosphates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), to make the larger prenyl diphosphates, geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅) and geranylgeranyl diphosphate (GGPP, C₂₀). Subsequent cyclizations of these intermediates form the major terpene products in Norway spruce. Thus the isoprenyl diphosphate synthases catalyze the branch-point reactions leading to the different terpene classes

likely to have a key regulatory function in terpene biosynthesis (Martin et al. 2002; Schmidt and Gershenzon 2007, 2008; Schmidt et al. 2010b).

The ecological relevance of terpenoids has been tested in a series of studies using exogenous treatment with methyl jasmonate (MJ) or the free acid jasmonate (JA) to induce conifer defense reactions (Franceschi et al. 2002, Martin et al. 2002, 2003; Fäldt et al. 2003; Hudgins et al. 2003, 2004; Huber et al. 2005; Miller et al. 2005; Ralph et al. 2007). MJ has been shown to increase Norway spruce (*Picea abies*) resistance to colonization by the spruce bark beetle, *Ips typographus*, and its virulent fungal associate, *Ceratocystis polonica* (Erbilgin et al. 2006; Zeneli et al. 2006; Krokene et al. 2008b). Although MJ has been used successfully in several studies to induce and characterize induced defense responses in conifers, endogenous roles of octadecanoids and the putative signal transduction chain for local and systemic responses in conifer defense induction have only been demonstrated in a few studies.

Since formation of TRD results from a transient change of xylem differentiation, other hormones that regulate xylem development, like salicylate (SA) or ethylene, could also have a function in this process. Hudgins and Franceschi (2004) showed that MJ application in Douglas fir (*Pseudotsuga menziesii*) induced production of ethylene, a jasmonate-linked plant hormone which also acts as an elicitor of conifer defence responses. Ethylene has been shown to increase monoterpene synthesis after wounding and fungal inoculation in *Pinus* spp. (Peters 1977; Popp et al. 1995). Miller et al. (2005) have demonstrated that feeding by the white pine weevil (*Pissodes strobi*) results in the induction of genes leading to JA in Sitka spruce (*Picea sitchensis*) by RNA-blot experiments. Urbanek-Krajnc et al. (2011) showed that application of salicylate induces antioxidant defense responses in the phloem of *P. abies* and inhibits colonization by *I. typographus*.

However, there is still limited knowledge about the endogenous levels of hormones in conifers. We also know little about the signal transduction pathways leading to activation and/or de novo formation of resin ducts in local or systemic acquired resistance. More knowledge about the expression patterns of genes involved in octadecanoid and ethylene biosynthesis or other signal cascades could reveal important insights about the regulation of TRD formation and resin biosynthesis.

Here, we demonstrate correlated changes among the formation of TRD, terpene accumulation, and expression of *IDS* transcripts in Norway spruce. Using the same tissue we are also able to measure endogenous levels of the hormones JA, JA-Ile, and SA and to differentiate between local and systemic induction of genes putatively involved in conifer signalling processes. Our results suggest that IDS catalyze a regulatory step of induced oleoresin production

in Norway spruce and that both JA and ethylene-induced signalling pathways are involved in systemic acquired resistance in conifers.

Materials and methods

Chemicals

All chemicals and solvents were analytical grade and were obtained from Merck (<http://www.merck.de>), Roth (<http://www.carlroth.com>), Serva (<http://www.serva.de>), or Sigma (<http://www.sigmaaldrich.com>).

Accession numbers

Sequence data of *MPK3*, and *NPR1* cDNA-fragments from this article can be found in the NCBI data libraries under accession JF927723 and JF927724. Sequence data of the 13-*LOX* cDNA-fragment is shown as S1 within the supplemental material.

Plant material

The experimental trees were selected from 12 full-sib Norway spruce (*P. abies*) families planted out as 1-year-old seedlings at Hogsmark, Ås, SE Norway in 2000. The 12 families originated from controlled crosses between parent trees that were resistant or susceptible to mass-inoculation with *Ceratocystis polonica* (Brignolas et al. 1998; Christiansen et al. 1999), a virulent blue-staining fungus associated with the spruce bark beetle. We selected three families from crosses between susceptible parents (S × S) with an average of 48.0% blue-stained sapwood after fungal mass-inoculation, and three families from crosses between resistant parents (R × R) with an average of 1.7% blue-stained sapwood (E. Christiansen, unpublished data). Trees from one R × R cross (family 24) and one S × S cross (family 15) were selected for more detailed chemical and molecular analysis, and the parents of these families had an average of 2.0 and 51.0% blue-stained sapwood, respectively.

Ten trees from each of the six full-sib families were treated with an aqueous solution of 50 mM methyl jasmonate (MJ) and 0.1% Tween 20 on the uppermost 6 cm of the 2001 internode on June 22, 2005. On July 14–19 the trees were felled and 10 cm long stem sections were cut at different heights above the treated section (0, 15, 30, 45, and 60 cm above). The upper 2 cm of each section was put on fixative for later microscopy (2% paraformaldehyde and 1.25% glutaraldehyde buffered in 50 mM L-piperazine-N-N'-bis (2-ethane sulfonic) acid, pH 7.2), whereas the remaining 8 cm were frozen in liquid nitrogen. On June 26,

2006 two samples were taken from untreated control trees of family 24 and 15 using the same procedure.

All subsequent chemical and molecular analyses, including measurements of terpene content, hormone quantity and gene transcript level, were carried out on selected frozen samples based on microscopic analyses of traumatic resin duct (TRD) formation. Tree number 7 and 9 of family 24 were selected as examples of MJ-treated trees with extensive formation of TRD, while tree number 3 and 7 of family 15 and tree number 2 of family 24 were investigated as examples of MJ-treated trees with low formation of TRD. Untreated trees from both families were used as controls.

The relative levels of terpenes, hormones and gene transcripts in each sample were obtained by calibration against the mean quantity in MJ-treated tissue in trees with low formation of TRD. Data for control trees and trees with extensive and low formation of TRD are presented as values of individual biological replicates (calculated as the mean of three technical replicates per biological replicate). Combined data for all sampling distances away from the MJ treatment site are presented as boxplots in the supplemental material (Figs. S1–S4).

Microscopy preparation and image analysis

Anatomical samples from all trees were processed for light microscopy as described by Franceschi et al. (2000). Briefly, samples were rinsed with buffer, dehydrated in ethanol and embedded in acrylic resin. Cross sections (1 μm thick) were cut with an ultra-microtome, dried onto gelatin-coated slides, stained with Stevenel's blue, and mounted with immersion oil. Digital images were taken with a Leitz Aristoplan photo-microscope fitted with a Leica DC300 CCD camera (Leica, Wetzlar, Germany), using bright field optics.

To quantify the extent of TRD in the sapwood of all investigated samples, cross-sections were imaged at 16× magnification, and the percent coverage of resin ducts (including the epithelial cells lining the ducts) was measured across 870 μm in the tangential direction. Percentage data were arcsine transformed and subjected to analysis of variance using JMP (SAS Institute, NC). Data are presented as means + 95% confidence intervals, $n = 26$ –30 for resistant trees and 25–29 for susceptible trees for the different sampling positions along the stem.

Extraction of resin terpenes

Terpene extractions of bark and sapwood samples were based on the procedures of Martin et al. (2002). All steps of the sample preparation were carried out in 2 ml vials with a Teflon-coated screw cap (Hewlett-Packard, Palo Alto, CA,

USA). Sample pieces (200 mg) were submerged into 1.5 ml of *tert*-butyl methyl ether containing 150 µg/ml isobutylbenzene and 200 µg/ml pimaric acid as internal standards, and extracted for 14 h with constant shaking at room temperature.

To purify extracted terpenes from other organic acids, the ethereal extract was transferred to a fresh vial and washed with 0.3 ml of 0.1 M (NH₄)₂CO₃ (pH 8.0). The extract was then split into two equal portions. For the analysis of diterpene resin acids, one aliquot was methylated by adding 50 µl of 0.2 M *N*-trimethylsulfonium hydroxide in methanol (Macherey-Nagel, Düren, Germany) to 0.4 ml of the washed ethereal extract in a separate vial. After incubation at room temperature for 2 h, the solvent was evaporated under nitrogen to leave 100 µl of sample, which was stored at −20°C until analysis by GC-MS. For the analysis of monoterpenes and sesquiterpenes, the remainder of the original sample was prepared for GC-MS analysis by filtering through a Pasteur pipette column filled with 0.3 g of silica gel (Sigma 60 Å) overlaid with 0.2 g of anhydrous MgSO₄. The column was washed with 1 ml of diethyl ether, and the combined eluant collected in a new vial and evaporated to an approximate volume of 100 µl.

Analysis of monoterpenes, sesquiterpenes and diterpenes

GC-MS analysis of monoterpenes and sesquiterpenes was carried out with a Hewlett-Packard 6890 system, using a DB-WAX column [0.25 mm × 0.25 µm × 30 m (J&W Scientific, Folsom, CA, USA)]. Split injections (1 µl ethereal extract) were made at a ratio of 1:5 with an injector temperature of 220°C. The instrument was programmed to hold an initial temperature of 40°C for 3 min and then increased at a rate of 3°C min^{−1} to 80°C. The temperature was then increased at a rate of 5°C min^{−1} to 180°C with a 5 min hold, followed by a final ramp of 15°C min^{−1} up to 240°C. Helium was used as carrier gas at a constant flow of 1 ml min^{−1}. The MS detector was operated using the total ion mode at a temperature of 230°C. For the identification of compounds, the MS detector was operated using a mass range of 40–350 and spectra were collected under standard conditions (electron impact ionization at 70 eV).

Analysis of diterpene constituents was performed on the same GC-MS instrument fitted with an HP-5 column (0.25 mm × 0.25 µm × 30 m, Hewlett-Packard). Injections were made with 1 µl of the concentrated, derivatized ethereal extract. GC-MS split ratios were 1:10 with an injector temperature of 220°C. The instrument was programmed for an initial temperature of 120°C and increased at a rate of 1°C min^{−1} to 150°C, followed by 5°C min^{−1} up to 280°C (6 min hold). Helium was used as carrier gas at a

constant flow of 1 ml min^{−1}. For identification of compounds the MS detector was operated using a mass range of 40–550.

GC-MS generated peaks were quantified using Hewlett-Packard Chemstation software. Isobutylbenzene was used as the internal standard for quantification of monoterpenes and sesquiterpenes, while methylated pimaric acid was used as an internal standard to calculate diterpene concentrations. For quantitative analysis of monoterpenes and sesquiterpenes, the MS detector was operated in the single ion mode monitoring ions at *m/z* 91, 93 and 161. For diterpene quantification ions were monitored at *m/z* 121, 135, 239 and 241. Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards (Sigma) or mass spectra in the Wiley or National Institute of Standards and Technology libraries.

Analysis of jasmonic acid, jasmonate-isoleucine conjugate and salicylate

The analysis of jasmonic acid (JA), jasmonate-isoleucine conjugate (JA-Ile), and salicylate (SA) were based on the procedures of Schmidt et al. (2010a). Approximately 0.1 g of ground sapwood was homogenized in 1 ml methanol spiked with 40 ng of [²H₂]JA, 40 ng [²H₄]SA, and 8 ng of [¹³C₆]JA-Ile by shaking for 30 min. Homogenates were centrifuged at 20,000g for 20 min at 4°C, the methanol phase collected, and the homogenate re-extracted with 1.0 ml methanol. The organic phases were combined and the samples evaporated to dryness in a vacuum concentrator at 30°C. The dry residue was reconstituted in 0.5 ml of 70% (v/v) methanol/water and analyzed by an API 3200TM liquid chromatography–triple quadrupole mass spectrometry system (Applied Biosystems, Darmstadt, Germany) coupled to a Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany). 10 µl samples were injected onto a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm diameter, Agilent Technologies). The mobile phase, comprised of solvent A (0.05% formic acid) and solvent B (acetonitrile), was used in a gradient of (min/%B): 0/5, 0.5'/5, 4.5'/90, 4.52'/100, 5'/100, 5.1'/5, 8.5'/5) with a flow rate of 0.8 ml min^{−1}. Compounds were detected as negative ions in a multiple reaction monitoring mode. Molecular ions [M–H][−] at *m/z* 136.8, 209.0 and 322.1 generated from endogenous SA, JA, and JA-Ile and from their internal standards 140.8, 213.0 and 328.1 were fragmented under −22, −28 and −32 V CE for SA, JA, and JA-Ile, respectively. The product ions are *m/z* 93.1 and 97.1 for SA and its internal standard respectively, *m/z* 59.1 and 61.1 for JA and its internal standard, and *m/z* 130.1 and *m/z* 136.1 for JA-Ile and its internal standard. The ratio of ion intensities of the product ions was used to quantify SA, JA and JA-Ile.

Identification of genes involved in the signal transduction chain of local or systemic acquired resistance in Norway spruce

To investigate the possible roles of genes involved in the signal transduction chain of local or systemic acquired resistance in Norway spruce, cDNA sequences with similarities to those of known candidate genes from other species were identified by using an EST database developed by the NSF Genomics of Loblolly Pine Embryogenesis Project (<http://compbio.dfci.harvard.edu>). The search revealed contig TC68325 as a putative partial sequence of a lipoxygenase (13-*LOX*). Putative sequences of non-expressor of pathogenesis-related protein 1 (*NPR1*) and mitogen-activated protein kinase 3 (*MPK3*) from Sitka spruce were identified by using the database and clone bank TREENOMIX of the Conifer Forest Health EST sequencing project (<http://www.treenomix.ca>; Ralph et al. 2008).

cDNA fragments corresponding to these sequences were isolated and cloned using PCR with appropriate primers and cDNA of MJ-treated sapwood tissue of replicate 9 (family 24) as template. Comparison of the deduced amino acid sequences to the NCBI-database (<http://www.ncbi.nlm.nih.gov>) revealed significant similarities to characterized plant sequences. The 13-*LOX* homologue fragment contained 175 bp and showed 70% amino acid identity to a 13-*LOX* (CAA65269.1) cloned and characterized from *Solanum tuberosum* (Royo et al. 1996). For the *NPR1* fragment, the deduced amino acid sequence of the 309 bp cDNA fragment showed 72% amino acid identity to a *NPR1* gene from *Capsicum annuum* (DQ648785.1), whereas sequence alignment of the 366 bp putative *MPK3* fragment revealed 80% amino acid identity to the *MPK3* gene of *Gossypium barbadense* (AF531365.1).

Quantitative real-time PCR

Total RNA from bark or sapwood was isolated using the Plant RNA Isolation Kit (Invitex, Berlin, Germany) according to the manufacturer's instructions, but including an additional DNA digestion step [RNase Free DNase Set (Qiagen, Hilden, Germany)]. Using identical amounts of total RNA, template cDNA for subsequent PCR reactions was generated using SuperscriptTM III (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Quantitative PCR was performed with Brilliant[®] SYBR Green QPCR Master Mix (Agilent Stratagene, Santa Clara, CA, USA). Quantitative real-time PCR primers for *PaIDS1-6* were designed using specific criteria, including predicted melting temperature of at least 58°C, primer length of 22–24 nucleotides, guanosine-cytosine content of at least 48% and an amplicon length of 120–150 bp. Primer

sequences are listed in Schmidt and Gershenzon (2007, 2008) and Schmidt et al. (2010b). Primers used for quantitative amplification of *MPK3*, *NPR1*, 13-*LOX* or *ACO* are shown in Table S2 (see supplemental material). For *MPK3*, *NPR1*, and 13-*LOX* specific Norway spruce sequences were obtained from isolated and cloned cDNA fragments, whereas sequences for *ACO* came from the NCBI database (<http://www.ncbi.nlm.nih.gov>) under the accession number DQ480740 (Hudgins et al. 2006).

Primer specificity was confirmed by melting curve analysis, by an efficiency of product amplification of 1.0 ± 0.1 , and by sequence verification of at least eight cloned PCR amplicons for each PCR reaction per gene. Reactions with water instead of cDNA template were run with each primer pair as a control. A standard thermal profile of 95°C for 10 min, then 45 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 30 s was used. The fluorescence signal was captured at the end of each cycle, and a melting curve analysis was performed from 53 to 95°C with data capture every 0.2°C during a 1 s-hold. Quantitative real time PCR reactions were performed as described in the operator's manual using a Stratagene MX3000PTM. Transcript quantity is the average of three determinations each of three replicate samples from 2 to 3 biological replicates. All amplification plots were analyzed with the MX3000PTM software to obtain threshold cycle (Ct) values. Transcript abundance was normalized with the transcript abundance of the ubiquitin gene (Schmidt and Gershenzon 2007).

Results

Traumatic resin ducts were more abundant in offspring from parents resistant to a bark beetle-associated fungus than in offspring from susceptible parents

Most methyl jasmonate (MJ) treated Norway spruce (*P. abies*) trees in this study exhibited traumatic resin ducts (TRD) in the newly formed xylem (Fig. 2), but trees originating from crosses between parents resistant to the bark beetle-associated fungus, *Ceratocystis polonica*, showed much stronger morphological changes in response to treatment than trees from crosses between susceptible parents. MJ treatment induced significantly more TRD in offspring from resistant parents than in offspring from susceptible parents ($F_{1,56} = 7.36$, $P = 0.009$; average for all sampling positions per tree) (Fig. 3). The increase in TRD was found both at the site of MJ treatment ($F_{1,57} = 6.60$, $P = 0.01$) and at sampling positions 15–60 cm above the MJ treatment site ($F_{1,56} = 3.62$, $P = 0.06$; all distant sampling positions combined).

There was considerable variation in TRD within families, ranging from 0 to >60% coverage at a given sampling

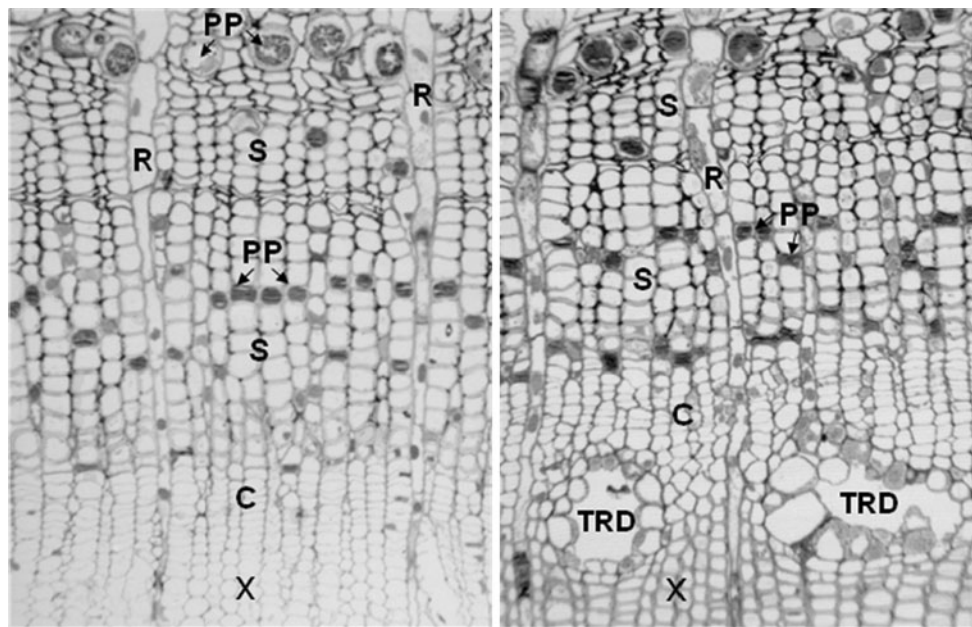


Fig. 2 Cross sections from the methyl jasmonate (MJ) treated sections of young Norway spruce stems about 25 days after MJ treatment. **a** Tree 24/2 showed no formation of traumatic resin ducts at any sampling position. **b** Tree 24/9 displayed extensive formation

of TRD covering up to 71% of the sapwood circumference in the measured cross-sections. *Abbreviations:* C cambium, PP polyphenolic parenchyma cells, R radial ray, S sieve cells, X xylem, TRD traumatic resin duct

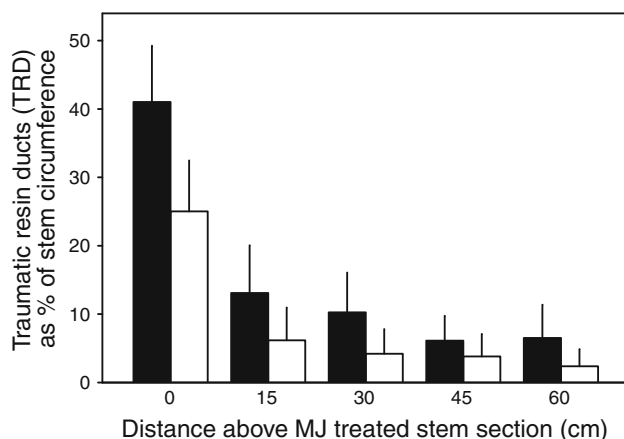


Fig. 3 Amount of traumatic resin ducts in the sapwood of Norway spruce offspring from parents resistant (*black bars*) or susceptible (*white bars*) to the bark beetle-associated fungus, *Ceratocystis polonica*, after partial treatment of the stem with methyl jasmonate. Resin ducts were quantified on sapwood samples collected at various distances above the treatment site ~4 weeks after treatment. Duct area was significantly greater in offspring from resistant versus susceptible parents (ANOVA for all sampling positions combined: $F_{1,56} = 7.36$, $P = 0.009$). Data are presented as mean + 95% confidence interval for 26–30 resistant trees and 25–29 susceptible trees per sampling position

position in both resistant and susceptible families. For this reason, the analyses in the remainder of this study were performed on a subset of trees from families 15 and 24 with contrasting expression of TRD. Trees with extensive formation of TRD after MJ treatment (70% of sapwood

circumference at treatment site, 35–53% of circumference up to 45–60 cm away from treatment site) were designated “high TRD”, while trees which showed low formation of TRD after MJ treatment (<20% of sapwood circumference, only at treatment site) were designated “low TRD” (Table S3, supplemental material). Unsprayed control trees of the same families were also analyzed.

Terpene accumulation was highest in trees with more traumatic resin ducts

To correlate terpene levels in Norway spruce with formation of TRD, we measured terpene concentrations in the bark and sapwood of high and low TRD trees following MJ treatment. High TRD trees had much higher terpene levels in the sapwood than low TRD trees or unsprayed controls, but no differences between trees were found in the bark (data not shown).

Total monoterpenes were up to 4.4-fold more abundant in high TRD trees compared to low TRD trees and untreated controls, and showed a different concentration pattern along the stem (Fig. 4; Table 1). The highest monoterpene content was detected at the treatment site itself ($1,080 \mu\text{g} \times \text{g dry weight}^{-1}$) and 15 cm above the treatment site ($1,485 \mu\text{g} \times \text{g dry weight}^{-1}$), with a gradual decrease further up the stem towards the level found in control trees. In low TRD trees or in untreated controls, no differences in monoterpene content were observed at any sampling position along the stem. The main monoterpenes

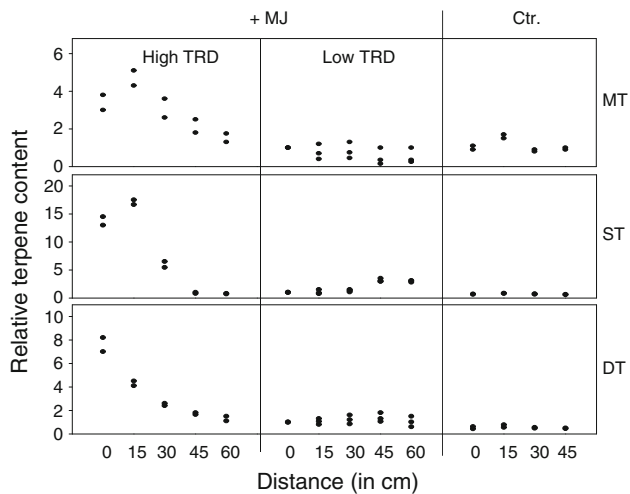


Fig. 4 Relative amounts of major monoterpenes (*MT*), sesquiterpenes (*ST*), and diterpenes (*DT*) in Norway spruce sapwood after methyl jasmonate (*MJ*) treatment in high and low traumatic resin duct (*TRD*) trees and in unsprayed control trees (*Ctr.*). Sapwood was collected at various distances above the treatment site ~4 weeks after treatment. The mean terpene content at the *MJ* treatment site in low *TRD* trees was set to 1.0. Data are presented as values of 2–3 biological replicates (each calculated as the mean of three technical replicates). The absolute levels corresponding to the 1.0 value are $246 \mu\text{g} \times \text{g dry wt}^{-1}$ for monoterpenes, $0.37 \mu\text{g} \times \text{g dry wt}^{-1}$ for sesquiterpenes, and $243 \mu\text{g} \times \text{g dry wt}^{-1}$ for diterpenes

detected in the sapwood were α -pinene and β -pinene (571 and $387 \mu\text{g} \times \text{g dry weight}^{-1}$, respectively, at the treatment site) in addition to camphene, sabinene, δ -3-carene, myrcene, limonene, and β -phellandrene (Table 1). For all sampling positions combined high *TRD* trees had much higher total monoterpene levels than low *TRD* trees and controls (median 2.6 vs. 0.7; Fig. S1).

Total sesquiterpene content was up to 13.6-fold higher in the trees with high *TRD* formation compared to low *TRD* trees and untreated controls. The concentration pattern along the stem was comparable to that found for monoterpenes. The highest sesquiterpene content was detected at the treatment site itself ($5.0 \mu\text{g} \times \text{g dry weight}^{-1}$) and 15 cm above the treatment site ($6.3 \mu\text{g} \times \text{g dry weight}^{-1}$) (Fig. 4, Table 1). However, total sesquiterpene concentration was only about 5% of that of mono- or diterpenes. The main sesquiterpenes detected in sapwood were (*E*)- β -caryophyllene and δ -cadinene (1.42 and $1.25 \mu\text{g} \times \text{g dry weight}^{-1}$, respectively, at the treatment site). Other sesquiterpenes detected were (*E*)- β -farnesene, germacrene D, and longipinene (Table 1). For all sampling positions combined high *TRD* trees had much higher total sesquiterpene levels than low *TRD* trees and controls (median 5.9 vs. 1.4 and 0.7, respectively; Fig. S1).

Total diterpene content was up to sevenfold higher in the trees with high *TRD* formation ($1,700 \mu\text{g} \times \text{g dry weight}^{-1}$) than in the other trees. The highest diterpene

Table 1 Amounts of mono-, sesqui-, and diterpenes in the sapwood of Norway spruce trees after treatment of the stem with methyl jasmonate (*MJ*)

Terpene	Low <i>TRD</i> treatment ($\mu\text{g} \times \text{g dry wt}^{-1}$)	High <i>TRD</i> treatment ($\mu\text{g} \times \text{g dry wt}^{-1}$)
<i>Monoterpenes</i>		
α -Pinene	124.0–132.2	562.6–584.4
Camphene	2.2–3.6	7.1–9.2
β -Pinene	67.3–77.2	370.1–392.6
Sabinene	1.8–2.5	6.3–8.9
Δ -3-Carene	2.8–4.2	10.5–11.4
Myrcene	8.3–10.2	29.8–32.8
Limonene	13.1–14.7	28.9–32.84
β -Phellandrene	12.9–16.2	32.6–40.2
<i>Sesquiterpenes</i>		
Longifolene	0.07–0.12	0.06–0.10
(<i>E</i>)- β -Caryophyllene	0.08–0.19	1.25–2.83
Δ -Cadinene	0.05–0.09	1.03–1.56
(<i>E</i>)- β -Farnesene	0.01–0.03	0.32–0.63
Germacrene D	0.01–0.03	0.11–0.35
Longipinene	0.04–0.09	0.86–1.07
<i>Diterpenes</i>		
Levopimarate	131.1–139.3	934.6–953.9
Abietate	25.3–34.9	169.9–190.3
Neoabietate	16.7–25.5	173.6–188.2
Isopimarate	22.4–28.2	208.7–222.6
Dehydroabietate	21.9–24.5	87.4–88.5
Sandarocopimarate	8.8–11.6	85.1–94.2

Trees with high or low traumatic resin duct (*TRD*) formation were sampled at the *MJ* treatment site. Data are presented as the range of 2–3 biological replicates (calculated as the mean of three technical replicates per biological replicate)

content ($943 \mu\text{g} \times \text{g dry weight}^{-1}$ for levopimarate) was detected at the *MJ* treatment site (Fig. 4; Table 1). Diterpenes in the sapwood included levopimarate, abietate, neoabietate, isopimarate, dehydroabietate, and sandarocopimarate. For all sampling positions combined high *TRD* trees had higher total diterpene levels than low *TRD* trees and controls (median 2.5 vs. 1.0 and 0.5, respectively; Fig. S1).

Transcripts of isoprenyl diphosphate synthases were higher in stem tissues with more traumatic resin ducts

Expression of isoprenyl diphosphate synthase (*IDS*) genes was investigated to determine if transcript abundance was correlated with the formation of *TRD*. Transcript levels of six different *IDS* genes were measured by quantitative real-time PCR in the same trees used for terpene analyses (Fig. 5).

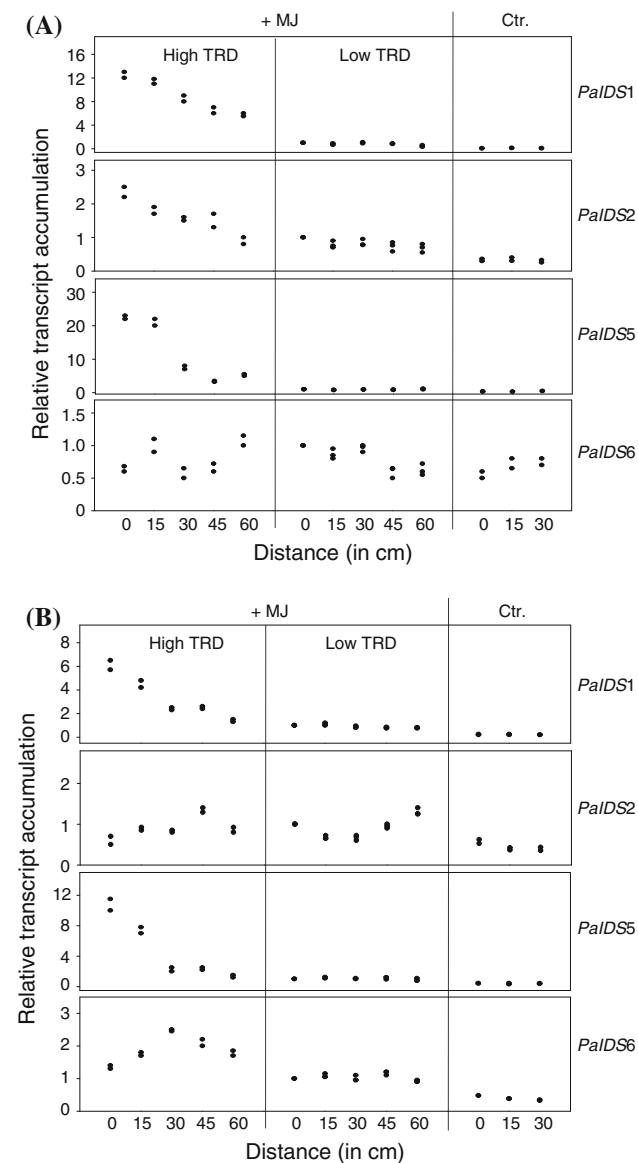


Fig. 5 Relative abundance of mRNA transcripts of *PaIDS1*, *PaIDS2*, *PaIDS5*, and *PaIDS6* genes in Norway spruce sapwood (a) and bark (b) after methyl jasmonate (MJ) treatment in high and low traumatic resin duct (TRD) trees and in unsprayed control trees (Ctr.). Samples were collected at various distances above the MJ treatment site 4 weeks after treatment. Transcript abundance of each *IDS* gene was measured by quantitative real-time PCR using SYBR-Green for detection and normalized against ubiquitin. The mean transcript abundance at the MJ treatment site in low TRD trees was set to 1.0. Data are presented as values of 2–3 biological replicates (each calculated as the mean of three technical replicates)

In the sapwood of trees with a high degree of TRD formation after MJ treatment, enhanced transcript levels were detected for *PaIDS1*, *PaIDS2*, and *PaIDS5*. At the site of MJ treatment, transcript abundance was up to 12-fold greater for *PaIDS1*, 2.2-fold for *PaIDS2* and 22-fold for *PaIDS5* relative to the expression level detected in trees with low formation of TRD after MJ treatment.

Fifteen centimeters above the MJ treated stem section, expression remained high, but sixty centimeters away expression decreased to sixfold, onefold and fourfold for *PaIDS1*, *PaIDS2*, and *PaIDS5*, respectively. In the trees with low formation of TRD, expression levels of *PaIDS1*, *PaIDS2* and *PaIDS5* were slightly higher than the constitutive levels detected in the untreated control trees. For *PaIDS6*, no changes in expression levels were measured in the sapwood, neither in trees with high nor low formation of TRD (0.5-fold to 1.0-fold changes relative to the mean expression level in trees with low formation of TRD) (Fig. 5a). For all sampling positions combined high TRD trees had much higher expression levels for *PaIDS1*, *PaIDS2*, and *PaIDS5* in the sapwood than low TRD trees and controls (median 8.4, 1.6 and 7.4 vs. 0.1–0.9; Fig. S2a).

For *PaIDS3* and *PaIDS4*, no changes in expression levels were measured in the sapwood (data not shown).

In the bark of trees with high formation of TRD, MJ treatment induced enhanced transcript levels mainly of *PaIDS1* and *PaIDS5*. The highest transcript abundances detected were 5.8-fold and 10-fold increases, respectively at the treatment site relative to the expression level at the treatment site in the bark of low TRD trees. Further up the stem, relative transcript abundance decreased almost to the constitutive expression levels found in the controls. Transcript levels of *PaIDS6* in the bark of trees with high formation of TRD increased and then decreased along the stem moving away from the treatment site, with the highest relative abundance (2.4-fold increase) 30 cm above the treatment site. For *PaIDS2* and for *PaIDS3* and *PaIDS4* (data not shown), no clear changes in expression levels were detected in the bark, neither between trees nor sampling positions within trees (0.5-fold to 1.4-fold changes relative to the mean expression level in trees with low formation of TRD) (Fig. 5b). For all sampling positions combined high TRD trees had higher expression levels for *PaIDS1*, *PaIDS2*, and *PaIDS5* in the bark than low TRD trees and controls (median 2.5, 2.4 and 1.7 vs. 0.3–0.9; Fig. S2b).

Accumulation of jasmonate and jasmonate-isoleucine conjugates, but not salicylate, was associated with formation of traumatic resin ducts

To compare resin duct formation and endogenous levels of possible defense signaling hormones in Norway spruce, we measured jasmonate (JA), jasmonate-isoleucine conjugate (JA-Ile), and salicylate (SA) concentrations in sapwood and bark from trees with high and low levels of TRD after MJ treatment. In general, absolute JA and JA-Ile levels were much higher in the bark (2.9 μg and 28.0 $\text{ng} \times \text{g}$ dry weight⁻¹, respectively) than in the sapwood (29.0 ng JA and 3.5 ng JA-Ile $\times \text{g}$ dry weight⁻¹). Trees with extensive

formation of TRD had much higher levels of JA and JA-Ile compared to trees with a low amount of TRD or unsprayed controls, both in the sapwood and in the bark. SA levels were higher at the treatment site in high TRD trees compared to low TRD trees and control trees, but only for the bark and not the sapwood. No clear absolute differences in SA were observed in the sapwood or bark for any other trees or treatments.

In the sapwood, JA and JA-Ile had a similar pattern of concentration along the stem, peaking 15 cm above the treatment site in high TRD trees at levels that were 6-fold and 12-fold higher, respectively than in low TRD trees and control trees (Fig. 6). For all sampling positions combined high TRD trees had much higher levels of JA and JA-Ile in the sapwood than low TRD trees and controls (median 4.2 and 5.0 vs. 0.1–0.8; Fig. S3). No changes were found for SA in the sapwood.

In the bark, relative JA levels were about twofold higher in high TRD trees than in low TRD trees. JA-Ile levels were up to 4.5-fold higher in trees with high formation of TRD than in other trees. The peak in JA-Ile was detected 15 cm above the treatment site. SA levels in the bark were around 2.3-fold higher in trees with high formation of TRD compared to low TRD and control trees (50.6 and 22.0 ng × g dry weight⁻¹, respectively) (Fig. 6). For all sampling positions combined high TRD trees had much higher levels of JA-Ile (but not JA) in the bark than low

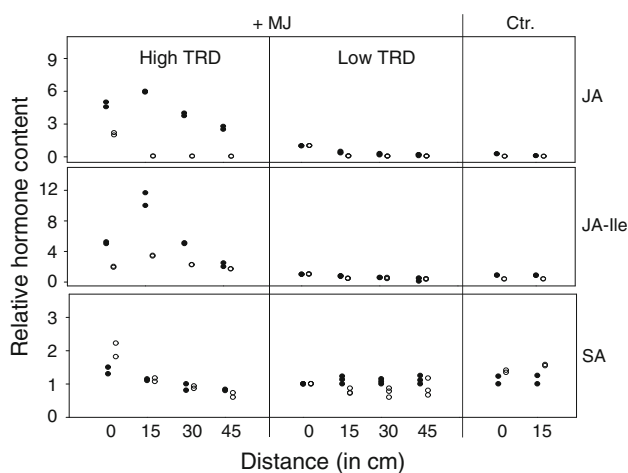


Fig. 6 Relative amounts of jasmonate (JA), jasmonate-isoleucine conjugate (JA-Ile) and salicylate (SA) in Norway spruce sapwood (black circles) and bark (white circles) after methyl jasmonate (MJ) treatment in high and low traumatic resin duct (TRD) trees and in unsprayed control trees (Ctr.). The mean hormone content at the MJ treatment site in low TRD trees was set to 1.0. Data are presented as values of 2–3 biological replicates (each calculated as the mean of three technical replicates). The absolute levels corresponding to the 1.0 value in sapwood or bark are 4.8 ng or 1.4 μg × g dry wt⁻¹ for jasmonate, 0.3 ng or 6.2 μg × g dry wt⁻¹ for the jasmonate-isoleucine conjugate, and 15 or 22 ng × g dry wt⁻¹ for salicylate

TRD trees and controls (median for JA-Ile = 2.6 vs. 0.2 and 0.05 for low TRD and controls, respectively; Fig. S3).

Genes involved in JA, SA and ethylene defense signaling were induced in trees with abundant traumatic resin ducts

The relative expression levels of four different genes thought to be involved in defense signal transduction in MJ-treated Norway spruce stems were investigated by quantitative real-time PCR. Increased transcript abundance was detected in the sapwood of trees with high formation of TRD, compared with low TRD trees and unsprayed control trees, whereas transcript levels in the bark differed only slightly among trees (Fig. 7). In sapwood, relative expression levels increased around fourfold for *non-expressor of pathogenesis-related protein 1* (NPR1), a positive regulator of systemic acquired resistance against pathogens (Dong 2004), and 20-fold for *1-aminocyclopropane-1-carboxylate oxidase* (ACO), encoding the final step in ethylene biosynthesis (Wang et al. 2002), at the MJ-treated stem section or 15 cm above it. Expression

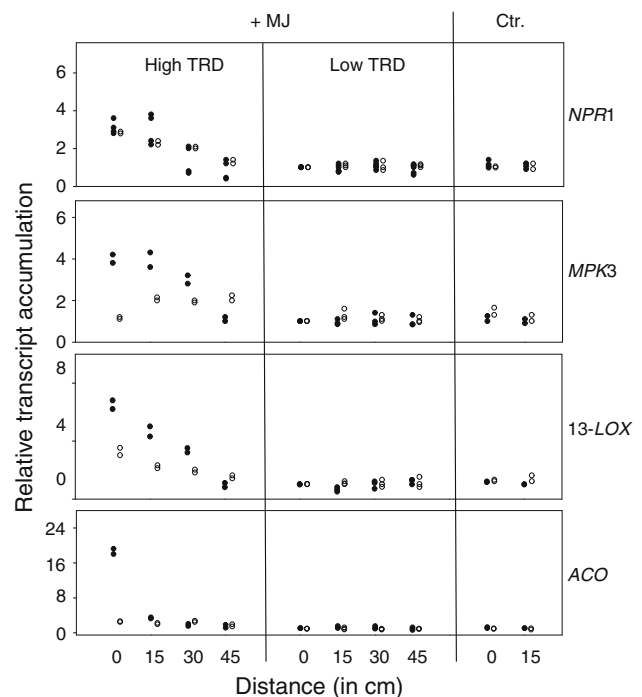


Fig. 7 Relative abundance of mRNA transcripts of *NPR1*, *MPK3*, *13-LOX*, and *ACO* genes in Norway spruce sapwood (black circles) and bark (white circles) after methyl jasmonate (MJ) treatment in high and low traumatic resin duct (TRD) trees and in unsprayed control trees (Ctr.). Transcript abundance of each gene was measured by quantitative real-time PCR using SYBR-Green for detection and normalized against ubiquitin. The mean transcript abundance at the MJ treatment site in low TRD trees was set to 1.0. Data are presented as values of 2–3 biological replicates (each calculated as the mean of three technical replicates)

increased in the same pattern sixfold for *13-lipoxygenase* (*13-LOX*) encoding an early step in JA biosynthesis (Wasternack and Kombrink 2010), and fourfold for *mitogen-activated protein kinase 3* (*MPK3*), also part of JA signaling transduction (Rodriguez et al. 2010). Further up the stem away from the treatment site, transcript levels in the sapwood decreased for all investigated genes. In the bark, expression levels of all genes increased somewhat (up to threefold) in trees with high formation of TRD (Fig. 7). No clear changes in expression were detected in the sapwood or bark of trees with low formation of TRD or in untreated controls. For all sampling positions combined high TRD trees had higher expression levels for *NPR1*, *MPK3*, *13-LOX*, and *ACO* in the sapwood than low TRD trees and controls (median 2.0, 3.4, 3.8 and 2.5 vs. 1.0–1.2; Fig. S4). In the bark median expression levels were lower and differed less between trees (around 2.0 in high TRD trees vs. 1.1 in the controls (Fig. S4).

Discussion

Traumatic resin duct formation is correlated with oleoresin accumulation in the sapwood of Norway spruce

Traumatic resin ducts (TRD) filled with oleoresin appear to be an important defensive feature against insects and pathogens in many conifers in the pine family (Huber et al. 2004; Heijari et al. 2005; Franceschi et al. 2005), including e.g. white spruce (*Picea glauca*), Sitka spruce (*Picea sitchensis*), and Norway spruce (Alfaro 1995; Tomlin et al. 1998; Nagy et al. 2000). TRD add considerably to a tree's overall oleoresin reservoir since they are much larger and more abundant than ordinary axial resin ducts in the sapwood, with a volume that is about four times greater than axial ducts per unit length in Norway spruce (Krokene et al. 2008b). After methyl jasmonate (MJ) treatment, the pattern of terpene accumulation in sapwood along the stem (Fig. 4) corresponded closely to the pattern of TRD formation (Fig. 3), suggesting that the TRDs were indeed the major repository of terpenes in the sapwood.

The oleoresin within Norway spruce TRD in this study was found to be composed primarily of mono- and diterpenes, with smaller amounts of sesquiterpenes, and had a composition similar to that reported in previous studies (Martin et al. 2002; Zeneli et al. 2006). Whereas mono- and diterpene levels in the sapwood increased fourfold to sevenfold after MJ treatment in trees with abundant TRD (relative to levels in trees with low TRD), sesquiterpene levels were up to 17 times higher in trees with high TRD. Traumatic resin ducts were also induced in the stem up to 60 cm away from the site of MJ treatment with the same

shift towards sesquiterpene production. Some of this increase may consist of volatiles that are later emitted from the stems and not stored inside TRD. Martin et al. (2003) reported that the emission of certain sesquiterpenes increased up to 100-fold in young Norway spruce plants after MJ treatment. Whatever the reason, the different ratios of terpene types found when comparing oleoresin at the MJ treatment site to that 60 cm away suggest that resin in TRDs has a terpene composition distinct from that in constitutive resin ducts.

Transcription of GPP and GGPP synthase genes is closely correlated with traumatic resin duct formation

We investigated the expression of six isoprenyl diphosphate synthase (IDS) genes that catalyze branch-point reactions leading to the different classes of terpenes in Norway spruce (Table 2) (Schmidt and Gershenzon 2007, 2008; Schmidt et al. 2010b). Of the six, the induction pattern of three genes, *PaIDS1*, *PaIDS2*, and *PaIDS5*, within the stem sapwood correlated closely with formation of TRD, suggesting that these genes and the enzymes they encode are localized in the sapwood (Figs. 3, 5a). *IDS2* produces geranyl diphosphate (GPP), the substrate for the monoterpenes; *IDS5* produces geranylgeranyl diphosphate (GGPP), the substrate for the diterpenes; while *IDS1* produces mostly GPP with some GGPP (Table 2). Within the sapwood, the transcripts most likely reside in the epithelial cells of the TRD, as was demonstrated recently for *PaIDS1* (Schmidt et al. 2010b). In the bark, *IDS* gene induction (Fig. 5b) is probably involved in terpene biosynthesis in other cell or tissue types, such as the radial resin ducts inside the radial rays and the large cortical resin ducts (Krokene et al. 2008b; Abbott et al. 2010).

The induction of *PaIDS1*, *PaIDS2*, and *PaIDS5* at the site of MJ treatment confirms the central role of these three IDS genes in induced conifer defense reactions (Schmidt and Gershenzon 2007, 2008; Schmidt et al. 2010b). The maximum induction found for *PaIDS1* in the sapwood by MJ relative to untreated sapwood was comparable to that

Table 2 Product profiles (in % of total products) for the different isoprenyl diphosphate synthases (IDS) whose transcript levels were measured in the present study

	GPP	FPP	GGPP	Reference
<i>PaIDS1</i>	90		10	Schmidt et al. (2010b)
<i>PaIDS2</i>	100			Schmidt and Gershenzon (2008)
<i>PaIDS3</i>	42	33	25	Schmidt and Gershenzon (2008)
<i>PaIDS4</i>	7.5	92.5		Schmidt and Gershenzon (2007)
<i>PaIDS5</i>			100	Schmidt and Gershenzon (2007)
<i>PaIDS6</i>			100	Schmidt and Gershenzon (2007)

previously found in MJ treated saplings (Schmidt et al. 2010b). In contrast, *PaIDS2* induction by MJ was only one third of that found previously, whereas *PaIDS5* induction by MJ was four times higher (Schmidt and Gershenzon 2007, 2008). These differences may be due to genetic variation between the spruce genotypes used or changes in susceptibility to MJ at different stages of development. In systemic induced sapwood tissue, highly elevated transcript levels for *PaIDS1*, *PaIDS2*, and *PaIDS5* were found 15 cm above the MJ-treated stem section, and transcript levels remained higher than in the unsprayed controls even further up the stem.

In bark tissues after MJ spraying, *PaIDS1*, *PaIDS5*, and to a lesser degree *PaIDS6*, also showed increased transcript levels at the site of treatment. However, maximum induction in the bark was only 50% of that in the sapwood, and well below the levels of *PaIDS1* and *PaIDS5* induction (relative to untreated bark) recently found in MJ-treated saplings (Schmidt and Gershenzon 2007; Schmidt et al. 2010b). Some of the IDS activity in the bark may be an artifact if parts of the cambium adhered to the bark during sampling, since TRD may begin to differentiate while the developing cells are still in the cambial region.

Away from the site of MJ treatment, 30–60 cm further up the trunk, slightly increased *IDS* transcript levels were detected in the bark (relative to levels in unsprayed bark tissue), but these differences were much less than those found in the sapwood. *PaIDS6* seemed to follow a different pattern than *PaIDS1* and *PaIDS5* with no or only very modest induction locally at the MJ-treated site, but slightly higher transcript levels further up the stem. However, compared with the main IDS genes involved in induced conifer defense (*PaIDS1*, *PaIDS2*, and *PaIDS5*) transcript levels of *PaIDS6* remained low, supporting a smaller role in induced defense responses (Schmidt and Gershenzon 2007).

The overall increase in mono- and diterpene concentrations in the sapwood correlates well with the induction of the IDS genes that encode the enzymes making their precursors- *PaIDS1* (GPP and GGPP), *PaIDS2* (GPP) and *PaIDS5* (GGPP). However, the observed increase in sesquiterpene levels in the sapwood is not matched by an increase in the corresponding IDS gene encoding FPP synthesis, *PaIDS4* (Schmidt and Gershenzon 2007). Because the other Norway spruce IDS monitored in this study either have no detectable FPP synthase activity (*IDS1*, 2 and 5, Schmidt and Gershenzon 2007, 2008; Schmidt et al. 2010b), or did not show an increase in transcript (*PaIDS3*), an additional FPP synthase, a bifunctional GPP/FPP synthase or a GGPP synthase also releasing FPP may be present in the sapwood after induction. Regarding *PaIDS3*, it was recently shown in *Arabidopsis thaliana* that a gene with close sequence similarity

to *PaIDS3* encodes an enzyme synthesizing C₂₅–C₄₀ long isoprenyl diphosphate products with double bonds in an (*E*)-orientation (Hsieh et al. 2011). Thus involvement of *IDS3* in induced oleoresin biosynthesis seems unlikely, as already suggested (Schmidt and Gershenzon 2008).

Jasmonate, but not salicylate signaling is involved in traumatic resin duct formation

To understand how traumatic resin duct formation is triggered, how the various aspects of the defense response (e.g. formation of TRD, terpene production) are integrated, and how a defense signal is propagated from a single site to other locations on the tree, it is necessary to identify the hormones and signaling mechanisms involved. Recent reviews suggest that jasmonic acid (JA) and ethylene are involved in defense signal transduction in conifers (Phillips et al. 2006; Zulak and Bohlmann 2010). The large number of studies that have induced conifer defence responses with exogenous MJ application (Franceschi et al. 2002; Martin et al. 2002; Hudgins et al. 2003, 2004; Huber et al. 2005; Zhao et al. 2010; Zulak et al. 2009, 2010; Hall et al. 2011) are the principal evidence for an essential role of endogenous jasmonates. However, JA has never been previously measured in intact conifers, only in cell cultures (Mueller et al. 1993; Phillips et al. 2007), and its bioactive form, the jasmonate-isoleucine conjugate (JA-Ile, Fonseca et al. 2009), has not been reported from conifers of any form. Here we detected both JA and JA-Ile in all of our samples, but in much higher levels associated with high TRD formation and greater accumulation of mono- and sesquiterpenes. All previous studies of MJ application in conifers have focused on local tissues, whereas here we also investigated systemic responses in stem parts above the treatment site. JA and JA-Ile accumulation in sapwood of high TRD trees actually increased 15 cm above the local treatment site and then declined to levels below treatment at 45 cm. This correlated very well with the pattern found for monoterpene and sesquiterpene accumulation, suggesting that JA and JA-Ile cascades are involved in triggering terpenoid biosynthesis. In bark, elevated JA levels were found only at the treatment site, possibly a residue of applied MJ that has undergone hydrolysis or an artifact caused by cambium adhering to the bark during sampling. However, the fact that we found much higher JA and JA-Ile levels in high than in low TRD trees and a much stronger response in sapwood than in bark (even though MJ was applied to the bark) clearly suggests that the JA and JA-Ile we measured are not just metabolites of the applied MJ, but reflect endogenous changes associated with defense induction.

There is considerable knowledge about the genes involved in JA biosynthesis in conifers from the sequencing of more than 200,000 ESTs from white spruce (*Picea*

glauca) and Sitka spruce (*Picea sitchensis*) in the TREE-NOMICS database (Ralph et al. 2008), and from DNA microarray experiments demonstrating gene induction following mechanical wounding or feeding by the spruce budworm (*Choristoneura occidentalis*) or the white pine weevil (*Pissodes strobi*) (Ralph et al. 2006). 13-lipoxygenase (13-LOX) is a key enzyme leading to JA formation, catalyzing the conversion of α -linolenic acid to 13(S)-hydroxyperoxylinolenic acid (Royo et al. 1996; Wasternack 2007). Another segment of the JA signal transduction cascade prior to 13-LOX involves mitogen-activated protein kinases (MPK), with MPK3 being a key gene in JA signalling in *Arabidopsis* (Takahashi et al. 2007). In high vs. low TRD trees and in sapwood vs. bark, higher endogenous JA levels correlated with the induction of 13-LOX and MPK3, suggesting a direct relationship between transcriptional activation of these two genes and JA and JA-Ile based defense signaling in *P. abies*.

In contrast to JA and JA-Ile, we found little evidence for involvement of salicylate (SA) in systemic formation of TRD in our spruce trees, neither in bark nor in wood. However, greater accumulation of SA was found in the bark at the MJ treatment site in high TRD vs. low TRD or unsprayed control trees, suggesting a possible role for SA in TRD induction. In previous studies, increases in bound and free SA have been detected in roots of several-week-old Norway spruce seedlings inoculated with *Pythium* sp. or treated with MJ (Kozłowski and Metraux 1998; Kozłowski et al. 1999). To support the involvement of SA, expression data from genes involved in SA signalling would be valuable. Because we were unable to identify a gene encoding isochorismate synthase, the key step of SA biosynthesis, among available *P. abies* sequences (due to the lack of sequence similarity to angiosperm genes), we used *NPR1* as an indicator of SA signaling. This gene encodes a central positive regulator of systemic acquired resistance signaling in many angiosperms, whose level is modulated by cross-talk between SA- and JA-dependent defence pathways (Spoel et al. 2003; Fobert and Despres 2005; Dong 2004). We detected higher induction of *NPR1* in high vs. low TRD trees and in the sapwood vs. the bark. This pattern correlates well with those for 13-LOX and MPK3, suggesting possible participation of SA-dependent signalling in spruce TRD formation.

In addition to the hormones JA, JA-Ile, and SA, ethylene has also been reported to be involved in induction of conifer defense. Ethylene induces the same anatomical and cellular defence responses as insect feeding, mechanical wounding, or fungal inoculation in Douglas fir (*Pseudotsuga menziesii*) (Hudgins and Franceschi 2004). The final step in ethylene biosynthesis is catalyzed by 1-aminocyclopropane-1-carboxylate oxidase (ACO) and the encoding gene has been used as a marker gene for involvement of

ethylene in signal transduction. Although we did not quantify ethylene accumulation, much higher transcript levels of *ACO* at the MJ treatment site in the sapwood of high TRD vs. low TRD and unsprayed control trees hint that ethylene biosynthesis may have been upregulated in high TRD trees. In addition, because of the reported link between mitogen-activated protein kinase cascades and ethylene signalling (Ouaked et al. 2003), increased expression of *MPK3* also suggests that ethylene signalling may be involved in the formation of TRD.

Despite the importance of induced terpenoid biosynthesis in conifer defense, there are still many unsolved questions about its regulation. In the present manuscript we have provided new information about the involvement of *IDS* expression in formation of TRD and the roles of various hormones in the signalling process. Further work is needed to clarify the relative importance of JA, SA and ethylene and the participation of other components in signalling cascades. Greater knowledge of the regulation of terpenoid induction in *P. abies* should contribute to a deeper understanding of conifer defence and new approaches to manage many of the destructive pests of conifer forests.

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