

Functional identification and differential expression of 1-deoxy-D-xylulose 5-phosphate synthase in induced terpenoid resin formation of Norway spruce (*Picea abies*)

Michael A. Phillips · Michael H. Walter · Steven G. Ralph · Paulina Dabrowska ·
Katrin Luck · Eva Maria Urós · Wilhelm Boland · Dieter Strack ·
Manuel Rodríguez-Concepción · Jörg Bohlmann · Jonathan Gershenzon

Received: 26 April 2007 / Accepted: 16 July 2007 / Published online: 9 August 2007
© Springer Science+Business Media B.V. 2007

Abstract Conifers produce terpenoid-based oleoresins as constitutive and inducible defenses against herbivores and pathogens. Much information is available about the genes and enzymes of the late steps of oleoresin terpenoid biosynthesis in conifers, but almost nothing is known about the early steps which proceed via the methylerythritol phosphate (MEP) pathway. Here we report the cDNA

cloning and functional identification of three Norway spruce (*Picea abies*) genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step of the MEP pathway, and their differential expression in the stems of young saplings. Among them are representatives of both types of plant DXS genes. A single type I DXS gene is constitutively expressed in bark tissue and not affected by wounding or fungal application. In contrast, two distinct type II DXS genes, *PaDXS2A* and *PaDXS2B*, showed increased transcript abundance after these treatments as did two other genes of the MEP pathway tested, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 4-hydroxyl 3-methylbutenyl diphosphate reductase (HDR). We also measured gene expression in a Norway spruce cell suspension culture system that, like intact trees, accumulates monoterpenes after treatment with methyl jasmonate. These cell cultures were characterized by an up-regulation of monoterpene synthase gene transcripts and enzyme activity after elicitor treatment, as well as induced formation of octadecanoids, including jasmonic acid and 12-oxophytodienoic acid. Among the Type II DXS genes in cell cultures, *PaDXS2A* was induced by treatment with chitosan, methyl salicylate, and *Ceratocystis polonica* (a bark beetle-associated, blue-staining fungal pathogen of Norway spruce). However, *PaDXS2B* was induced by treatment with methyl jasmonate and chitosan, but was not affected by methyl salicylate or *C. polonica*. Our results suggest distinct functions of the three DXS genes in primary and defensive terpenoid metabolism in Norway spruce.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9212-5) contains supplementary material, which is available to authorized users.

M. A. Phillips (✉) · K. Luck · J. Gershenzon
Max Planck Institut für Chemische Ökologie, Abteilung
Biochemie, Hans Knöll Str. 8, Jena 07745, Germany
e-mail: mphilips@ice.mpg.de

M. H. Walter · D. Strack
Leibniz-Institut für Pflanzenbiochemie, Abteilung
Sekundärstoffwechsel, Weinberg 3, Halle (Saale) 06120,
Germany

S. G. Ralph · J. Bohlmann
Michael Smith Laboratories, University of British Columbia,
Vancouver, Canada

P. Dabrowska · W. Boland
Max Planck Institut für Chemische Ökologie, Abteilung
Bioorganische Chemie, Hans Knöll Str. 8, Jena 07745, Germany

E. M. Urós · M. Rodríguez-Concepción
Departament de Bioquímica i Biologia Molecular, Facultat de
Biologia, Universitat de Barcelona, Av. Diagonal 645, 08282
Barcelona, Spain

E. M. Urós · M. Rodríguez-Concepción
Consorci CSIC-IRTA de Genetica Molecular Vegetal, Jordi
Girona 18-26, 08034 Barcelona, Spain

Keywords *Ceratocystis polonica* · Conifer defense ·
Fungal elicitor · Isoprenoid biosynthesis ·
Methyl jasmonate · Terpenoid synthase · Oleoresin ·
Conifer genomics

Introduction

One of the most characteristic features of conifers is their production of a viscous and pungent oleoresin which is thought to serve as a defense against herbivores and pathogens (Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and Bohlmann 2006a,b). Composed of mono-, sesqui-, and diterpenes, oleoresin is present constitutively in many conifer species, but accumulation is also induced upon herbivore or pathogen attack. Induction involves the differentiation of traumatic resin ducts or resin blisters (Phillips and Croteau 1999; Martin et al. 2002), enhanced biosynthesis of terpenoid constituents (Martin et al. 2002; Miller et al. 2005; Zeneli et al. 2006), and emission of terpenoid volatiles from needles (Martin et al. 2003).

Picea spp. are a well-established system for investigating the molecular, biochemical and ecological aspects of constitutive and induced oleoresin defenses in conifers (Martin et al. 2002; Byun-McKay et al. 2003; Martin et al. 2003, 2004; Miller et al. 2005; Schmidt et al. 2005; Erbilgin et al. 2006; Keeling and Bohlmann 2006a, b; Ralph et al. 2006; Zeneli et al. 2006). Both the octadecanoid and ethylene signaling pathways are known to be involved in the insect and wound-induced defense response in spruce (Franceschi et al. 2002; Martin et al. 2002; Hudgins et al. 2003; Hudgins and Franceschi 2004; Zhao et al. 2004; Huber et al. 2005; Hudgins et al. 2006; Ralph et al. 2007). Treatment of Sitka (*P. sitchensis*) or Norway spruce (*P. abies*) with methyl jasmonate induces oleoresin accumulation (Franceschi et al. 2002; Martin et al. 2002; Miller et al. 2005), and this has been used to study the ecological roles of terpenoid defenses. For example, methyl jasmonate-treated trees were more resistant to colonization by the bark beetle, *Ips typographus* (Erbilgin et al. 2006), and the bark beetle-associated fungus, *Ceratocystis polonica* (Zeneli et al. 2006). Further understanding of the defensive function of conifer oleoresin would benefit greatly from more knowledge of the pathway of oleoresin terpenoid biosynthesis and how this is regulated.

The biosynthesis of oleoresin terpenes in conifers involves three stages: (1) the production of the central C₅ intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), by the methylerythritol phosphate (MEP) and mevalonate pathways, (2) the condensation of IPP and DMAPP into C₁₀, C₁₅, and C₂₀ prenyl diphosphates, the precursors of mono-, sesqui- and diterpenes, respectively, and (3) the late cyclization and oxidation steps catalyzed by terpenoid synthases (TPS) and cytochrome P450 dependent monooxygenases (CYP450) which determine the particular carbon skeleton and oxidation pattern of the product (reviewed in Croteau 1987). The third stage has been well studied, starting with

pioneering work on the molecular biology and enzymology of TPS and CYP450 activities in grand fir (*Abies grandis*) (Steele et al. 1995; Bohlmann et al. 1997; Steele et al. 1998a). More recently, inducible TPS gene expression was found to be responsible for much of the chemical diversity of traumatic oleoresin in Norway (Fäldt et al. 2003; Martin et al. 2004) and Sitka spruce (Miller et al. 2005). In addition, CYP450 gene expression was demonstrated to be involved in traumatic diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*) (Ro et al. 2005; Ro and Bohlmann 2006). In contrast, only limited knowledge is available on the role of second stage enzymes, the isoprenyl diphosphate synthases, in conifer oleoresin formation (Martin et al. 2002; Schmidt et al. 2005). To the best of our knowledge, there are no reports on the possible regulatory function of genes and enzymes of the first stage. However, recent microarray gene expression analysis of all known steps of the terpenoid oleoresin pathway in Sitka spruce revealed substantial induction of transcript for some of the early pathway steps upon insect attack (Ralph, et al. 2006; S. Ralph and J. Bohlmann, unpublished results).

To learn more about the first stage of terpenoid biosynthesis in conifer oleoresin production, we began by investigating the first step in the MEP pathway of terpenoid biosynthesis, which is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998; Rodriguez-Concepcion and Boronat 2002). The MEP pathway is thought to be much more important than the mevalonate pathway in providing precursors for both monoterpenes and diterpenes (Lange and Ghassemian 2003), the major components of spruce oleoresin. In addition, previous studies have shown that DXS is a highly regulated enzyme that is a significant rate-determining step of the MEP pathway in other plant species (Lois et al. 2000; Walter et al. 2000; Estevez et al. 2001). Two types of DXS genes have been characterized based on sequence properties and expression pattern: one type (type I) which is constitutively expressed in photosynthetic tissues and is probably involved in supplying substrate for primary isoprenoids, such as carotenoids and phytol, and a second type (type II) which appears to be involved in supplying substrate for specialized terpenoids involved in ecological interactions (Walter et al. 2002). Here we report the cDNA cloning, functional identification, and expression analysis of three DXS genes from Norway spruce and their different roles in terpenoid biosynthesis.

Oleoresin formation in conifers has been a difficult process to study at the molecular and biochemical levels because it typically occurs in large, slow-growing trees and is restricted to specific tissues and stages of development, or only occurs after initial herbivore or pathogen attack (Martin et al. 2002; Franceschi et al. 2005). To overcome these problems, we established a Norway spruce cell

culture system to study terpene formation and examined the expression of *DXS* and other genes of terpene biosynthesis after induction.

Materials and methods

Plant growth conditions and treatments

Norway spruce (*P. abies* L. Karst) saplings of clonal lines 3166-728 and 1015-903 were propagated from lateral branches at the Niedersächsische Forstliche Versuchsanstalt (Escherode, Germany) and grown in a walk-in growth chamber under lighting conditions as reported previously (Martin et al. 2002). After 6 months, saplings were transferred to 2 l pots with a 2:3 (v/v) mixture of peat:universal planting mix and grown in a greenhouse at 24°C with mechanical misters supplying 10 s of mist per hour. Saplings were 2–3 years old (60–90 cm tall) when treatments were carried out as follows. Methyl jasmonate (MeJA, Aldrich, Steinheim, Germany) was sprayed onto saplings as a 25 mM solution containing 0.2% Tween 20 as described previously (Martin et al. 2002). Control saplings were sprayed with a Tween 20 solution only. Mechanical wounding consisted of a series of horizontal razor blade scores along the entire length of the trunk, approximately 3–4 per cm. After 2 h, saplings were wounded a second time. For combined treatments, the freshly wounded trunks were treated with either a chitosan solution (10 mg/ml) or a *C. polonica* spore culture using a fine tipped paint brush. Saplings used for time course measurements were harvested at 1, 3, 7, and 10 days post-treatment, while those used for single time point treatments were harvested at day 3. A minimum of six saplings was harvested from each group. After harvesting of stems, the outer bark and cambium were separated from the xylem (Martin et al. 2002) and the former frozen in liquid nitrogen prior to RNA extraction.

Norway spruce cell culture: establishment, treatments, and analysis

Somatic embryogenic cultures of Norway spruce were initiated from mature seeds, propagated as callus on solid EDM6 medium (Bishop-Hurley et al. 2001) and subcultured every 7 days. Actively growing calli were then transferred to 30 ml liquid EDM6 media and grown at 24°C in darkness with gentle shaking and sub-culturing at 10–12 day intervals. For induction experiments with defined elicitors, MeJA, chitosan (Sigma Chemicals, St. Louis, MO, USA), or methyl salicylate (MeSA) (Sigma Chemicals) was added to a final concentration of 50 µM, 50 µg/ml, and 50 µM, respectively, 10 days after

subculture. For induction experiments with a fungal pathogen, *C. polonica* spore cultures (isolate 93–208/115, courtesy of the Culture Collection of the Norwegian Forest Research Institute, Ås, Norway) were diluted 1:100 into spruce liquid cultures for induction. *C. polonica* spore cultures were prepared by transferring a 5 mm disk of mycelium onto a 9 cm sterile Petri dish containing 4% malt extract (w/v), 0.4% yeast extract (w/v), and 1.5% agar (w/v) at pH 7.0, incubating the sealed plate at 22°C in darkness for 8 days, and then gently washing the plate surface with 2 ml solution of 0.9% NaCl and 0.5% Tween 20, followed by 10 ml 0.9% NaCl to collect the spores. Induced tissue from liquid Norway spruce cell cultures was harvested by filtration on a side-arm flask equipped with a Buchner funnel and No. 2 Whatman filter paper. For monoterpene analysis of cell cultures, a single 30 ml culture was used to inoculate 200 ml fresh liquid EDM6 and grown under the same conditions for 3 days. Two grams of sterile XAD4 Amberlite resin (Sigma Chemicals) were then added to the culture along with isobutylbenzene (Acros, Geel, Belgium) as internal standard (1 µg/ml final concentration), and either MeJA (50 µM final concentration), MeSA (50 µM final concentration), chitosan (50 µg/ml final concentration), or 2 ml *C. polonica* spore culture were added as elicitors. After 1 week, the cells were separated from the XAD4 resin by centrifugation, and the resin was vacuum filtered on Whatman No. 2 paper. The dried resin was extracted with 1 ml pentane with vigorous shaking for 2 h, and the solvent then passed over a Pasteur pipette column containing 100 mg each silica gel and MgSO₄ and concentrated to 100 µl under a gentle nitrogen stream. The concentrated extracts were transferred to a GC vial and analyzed by electron impact GC-MS using an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a 5973 mass selective detector as previously described (Phillips et al. 2003). For monoterpene synthase enzyme assays, triplicate cultures were induced with 50 µM MeJA in 0.2% Tween 20 (Sigma Chemicals) or 0.2% Tween 20 alone (control) and then harvested at the indicated time points by vacuum filtration for extraction and assay as described below.

RNA isolation and cDNA synthesis

RNA from cultured cells was obtained with the Plant RNeasy kit (QIAGEN, Hilden, Germany) using 100 mg tissue homogenized in a chilled 2 ml glass Tenbroeck homogenizer containing 450 µl RLT buffer (QIAGEN) and 0.14 M β-mercaptoethanol. Total RNA from spruce bark and needles was isolated with the Invisorb Spin Plant RNA kit (Invitek, Berlin, Germany). Contaminating DNA was removed via on-column Dnase I digestion (QIAGEN) in both cases. Total RNA quality and concentration were

determined using an Agilent Bioanalyzer 2100 (Palo Alto, California, USA) and RNA Nano 6000 LabChips.

cDNA library construction and screening

PolyA+ RNA was purified from total RNA isolated from MeJA-treated Norway spruce liquid culture using poly(dT)₂₅ coated magnetic Dynabeads (DynaL Biotech, Oslo, Norway). Residual rRNA contamination was determined using an RNA Pico6000 LabChip (Agilent). Five micrograms purified mRNA was used to construct a λ ZAPII-cDNA library (Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. cDNAs were separated on a CL-2B Sepharose (Stratagene) size exclusion column, ligated into the lambda ZAP vector (Stratagene), and packaged using Gigapack Gold III packaging extract (Stratagene). Average insert size in the amplified and titered library was judged by T3 and T7 primed PCR amplification of randomly selected phage plaques. The library was screened by replica filter hybridization (Bohlmann et al. 1997) using a mixture of three putative spruce *DXS* cDNA fragments, 600, 800, and 1,400 bp in length, identified in the database and clone bank of TREENOMIX:Conifer Forest Health EST sequencing project (<http://www.treenomix.ca>; Ralph et al. 2006). PCR amplified and gel purified *DXS* fragments were diluted to 20 ng/ μ l and labeled with 5 μ l [α -³²P]dCTP (10 μ Ci/ μ l, Hartmann Analytic, Braunschweig, Germany) using a MegaPrimer DNA Labeling System (Amersham Pharmacia Biotech, Uppsala, Sweden). λ phage plaques hybridizing with the *DXS* probes were isolated in two rounds of screening under high stringency conditions (overnight hybridization at 65°C in 5 \times SSC followed by three washes for 15 min at 65°C and 1 \times , 0.5 \times , and 0.1 \times SSC, or until radioactivity as judged by a hand held Geiger counter was below 10 times background) starting with 300,000 phage plaques. The in vivo excised phagemids were transfected into *E. coli* SOLR cells to obtain pBlue-script plasmids, which were sequenced using M13 and M13R on an ABI Prism[®] 3100 sequencing system.

Functional expression and characterization of recombinant *DXS* proteins

Based on TargetP and ChloroP predictions of plastid transit peptide truncation sites, pseudomature forms of each *DXS* cDNA lacking the sequence for the predicted transit peptide were PCR amplified with primers bearing attB sites for cloning into Gateway[®] entry clones with BP Clonase II and pDONR207 (Invitrogen, Carlsbad, CA, USA). Sequences of the attB primers used are provided in Supplement Table S1. Entry clones were obtained using manufacturer's protocols

and then transferred into a Gateway-compatible pET32 vector using LR Clonase II (Invitrogen). Single colonies carrying each spruce *DXS* expression construct were used to start cultures in 5 ml LB medium containing 50 μ g/ml carbenicillin for plasmid preparations. Purified destination vectors were fully sequenced and then used in a bacterial complementation assay with a *DXS*-deficient strain of *E. coli* engineered to utilize exogenously supplied mevalonate as a source for isoprenoid biosynthesis (Campos et al. 2001). *DXS* activity was confirmed by transforming the *DXS*-deficient strain with the plasmids and then growing transformed cells on mevalonate-free media.

Quantitative real-time PCR

For measurements of transcript abundance, 1 μ g total RNA from culture or saplings was converted to cDNA in a 20 μ l reverse transcription reaction using SuperScript III RT (Invitrogen) and 50 pmol anchored poly dT primer (Supplement Table S1), then diluted 1:20 with sterile water. Quantitative real-time PCR was performed using a Stratagene Mx3000P and Brilliant SYBR[®] Green assays in 20 μ l containing 1 μ l diluted template, 10 pmol each forward and reverse primer, and Taq SYBR Green mix prepared according to manufacturer's instructions (Stratagene). All primers used for quantitative PCR for Norway spruce *DXS* genes and reference genes are shown in Supplement Table S1. Primer design was performed with BeaconDesigner 5.0 (PremierBiosoft, Palo Alto, USA). All primers were HPLC purified. Six amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Primer efficiencies and fold change calculations were performed according to the Pfaffl method (Pfaffl 2001). Possible primer cross-hybridization between the three similar *PaDXS* sequences was tested by a real-time PCR competition assay using dilute, purified plasmids encoding each *DXS* cDNA as template in all possible combinations with each *DXS* primer pair. In addition to non-template (water) controls, non-RT controls were used as templates in real-time PCR assays to detect the presence of genomic DNA contamination. Reference genes used for relative quantification were Norway spruce tubulin or Norway spruce ubiquitin. Significance for fold change expression between treatment and control was tested using a one-sample *t*-test. *P*-values were calculated using Origins v.7.03.

Analysis and quantification of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA)

Following the method described by Schulze et al. (2006), 3 g frozen cell culture material were mixed with 3 ml of a

pentafluorobenzylhydroxylamine solution, 0.05 M in methanol (Sigma-Aldrich, Taufkirchen, Germany) followed by addition of 9,10- $^{2}\text{H}_2$ -dihydrojasmonic acid (250 ng) and $^{2}\text{H}_2$ -dihydrodicranenone B (250 ng) as internal standards. Tissue was homogenized for 5 min with a high performance polytron disrupter at 24,000 rpm (Ultra-Turrax T-25, IKA-Werk, Germany). Samples were derivatized while shaking for 2 h at room temperature and then transferred to 10 ml glass centrifuge tubes. Water (3 ml) was added, and the solutions were adjusted to pH 3 with HCl. The methanol–water mixture was extracted with hexane (3×10 ml), and the organic phase was collected following phase separation by centrifugation. Combined hexane layers were subsequently passed through Chromabond aminopropyl cartridges (0.5 g, Macherey-Nagel, Düren, Germany) preconditioned with 5 ml each methanol and hexane. The cartridges were washed with *i*-propanol:dichloromethane (5 ml, 2:1, (v/v)) and eluted with diethyl ether:formic acid (10 ml, 98:2, (v/v)). The samples were dried under a gentle stream of argon and the residue treated with an ethereal solution of diazomethane. After removal of diazomethane, the obtained residue was redissolved in 30 μl of dichloromethane. Derivatized samples were analyzed on a Finnigan GCQ instrument (Thermoelectron, Bremen, Germany) running in negative CI mode, as previously described (Schulze et al. 2006). Characteristic fragment ions of the perfluorobenzyl oximes of JA and OPDA were used for quantification (JA, m/z 399; 9,10- $^{2}\text{H}_2$ -dihydrojasmonic acid, m/z 403; OPDA, m/z 481; $^{2}\text{H}_2$ -dihydrodicraneone B, m/z 483). Tissue matrix effects were measured with a calibration curve based on known amounts of JA and OPDA added to non-elicited cell culture material.

Monoterpene synthase assays

Assays were performed as previously described (Bohlmann et al. 1997; Martin et al. 2002; Phillips et al. 2003) with minor modifications. In brief, approximately 200 mg cells were homogenized in a chilled 2.0 ml glass Tenbroek homogenizer in 1.0 ml cold assay buffer (100 mM HEPES pH 8.0, 10% (v/v) glycerol, 2 mM dithiothreitol, 0.5 mM MnCl_2 , 100 mM KCl), gently mixed at 4°C for 30 min, and finally centrifuged for 30 min at 16,000g in a microcentrifuge at 4°C. Supernatants were filtered through a 0.2 μm syringe filter. A 10 μl aliquot was diluted 1:10 into fresh cold assay buffer containing 0.5 μl [$1\text{-}^3\text{H}$] geranyl pyrophosphate (20 Ci/mmol, 1 mCi/ml, American Radiolabeled Chemicals, St. Louis, MO, USA) and a 1.0 ml hexane overlay. Incubations were carried out at 30°C for 20 min, then vortexed and centrifuged for 1 min to separate phases. The organic (upper) phase was aspirated and passed over a

glass column containing glass wool, silica gel 60 (Merck, Darmstadt, Germany), and MgSO_4 into a scintillation vial containing 2 ml Lumasafe LSC cocktail (Lumac B.V., Groningen, Netherlands). The extraction was repeated once, and organic phases were pooled and counted by scintillation counting. Protein concentrations were determined by Bradford assay (Biorad, Hercules, CA, USA).

Results

Norway spruce contains both type I and type II *DXS* genes

To investigate a possible role of *DXS* and other MEP pathway genes in Norway spruce terpenoid oleoresin formation, we first mined the nearly 200,000 Sitka spruce, white spruce (*P. glauca*), and interior hybrid spruce (*P. glauca* \times *engelmannii*) sequences in the EST database developed by the TREENOMIX:Conifer Forest Health Project (<http://www.treenomix.ca>; Ralph et al. 2006) for sequences with similarities to known genes of the plant MEP pathway. This search revealed three distinct contigs of ESTs for candidate spruce *DXS* genes. Partial cDNA clones corresponding to the three *DXS* candidates were then used to screen a cDNA library made from methyl jasmonate (MeJA)-treated Norway spruce cell cultures yielding full length cDNAs for three distinct genes (Fig. 1). All three *P. abies* *DXS* genes (*PaDXSs*) showed features similar to known *DXS* genes from other plant species, including the presence of an N-terminal targeting sequence, a highly conserved His at position 127 (relative to *PaDXS1*) thought to be involved in proton transfer (Lois et al. 1998), and a conserved thiamine pyrophosphate binding domain typical of transketolase-like sequences (positions 212–244). Comparison of their deduced amino acid sequences to known *DXS* sequences of plant origin indicated that one sequence, *PaDXS1*, grouped with type I *DXS* genes, while the other two, *PaDXS2A* and *PaDXS2B*, represented two distinct subclasses of type II *DXS* genes (Fig. 2). Specifically, mature *PaDXS1* exhibits 83% identity (88% similarity) to *MtDXS1* from *Medicago truncatula* (Walter et al. 2002) but only 73% identity (80% similarity) to *MtDXS2*. Mature *PaDXS2A* is 78% identical (86% similar) to *MtDXS2* and only 73% identical (81% similar) to *MtDXS1*. Likewise, *PaDXS2B* has a 80% identity score (85% similarity) to *MtDXS2* and displays only 76% identity (82% similarity) to *MtDXS1*. As for the relationships within spruce *DXS* sequences, the mature form of *PaDXS1* shows 74% and 75% amino acid identity (87% and 85% similarity) with those of *PaDXS2A* and *PaDXS2B*, respectively, while the two type II *PaDXS* share 80% identity (89% similarity).

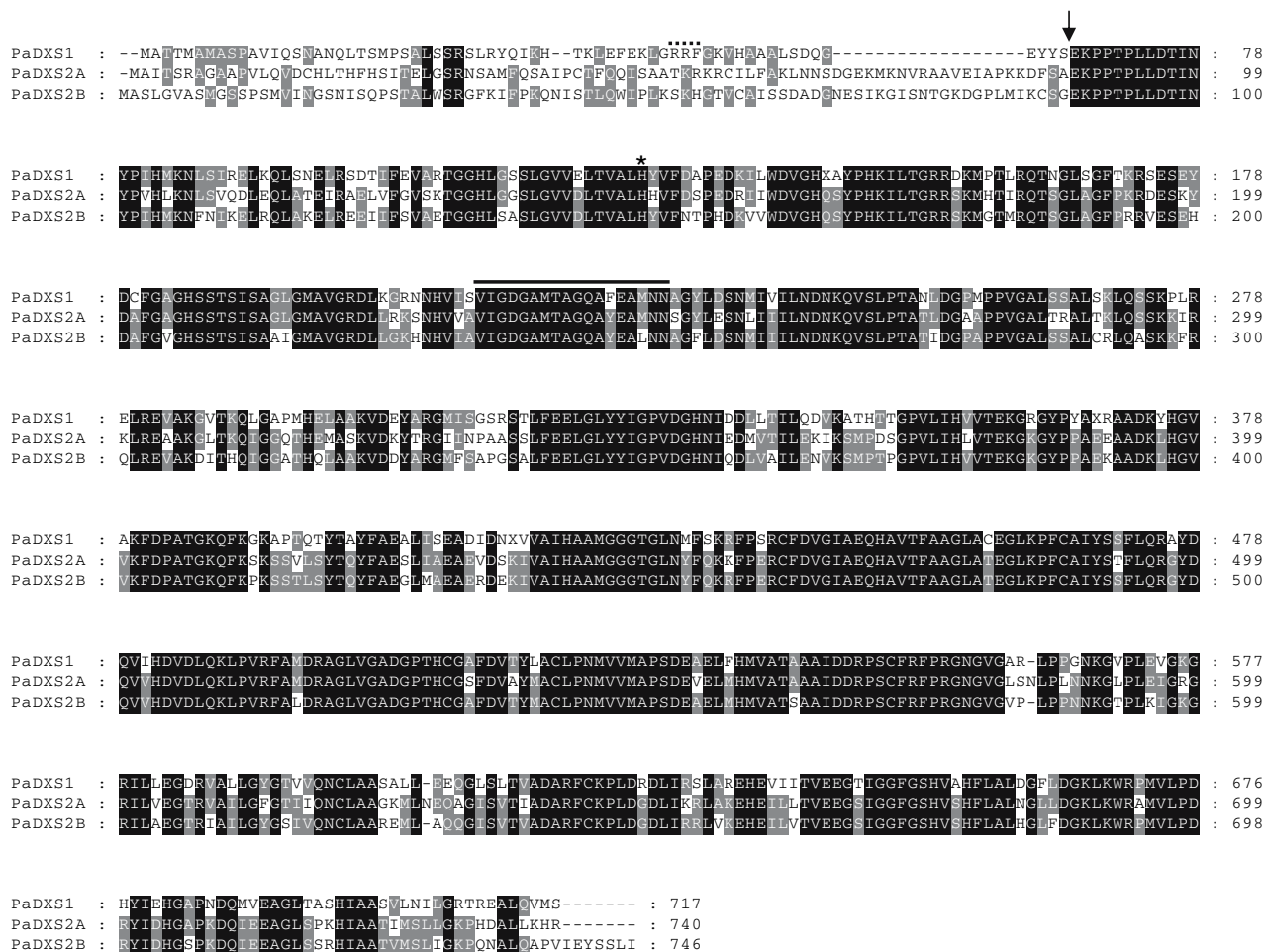


Fig. 1 Alignment of amino acid sequences of Norway spruce DXS proteins deduced from cDNAs. Alignments were performed with VectorNTI 10.0 (gap opening penalty 10, gap extension penalty 0.1). Residues conserved among all three genes are shown in black, while residues conserved in two out of the three are shown in grey. The asterisk indicates the conserved His residue thought to be involved in

Predictive algorithms suggested plastid localization for PaDXS1 (Predotar, 0.96; TargetP, 0.73) and PaDXS2B (Predotar, 0.92; TargetP, 0.87), while predictions for PaDXS2A were less clear (Predotar: 0.14 plastid, 0.82 elsewhere; TargetP: 0.22 plastid, 0.24 mitochondria). Because the targeting peptides of all known DXS sequences are bipartite, it is likely their ultimate destination is the thylakoid lumen (Krushkal et al. 2003). The amino acid sequence of PaDXS1 shows the presence of tandem Arg residues at positions 48–49 of the transit peptide N-domain (Fig. 1), followed by moderately hydrophobic residues in the H-domain and a Lys at position 68 before a conserved PPT motif, which suggests participation of the Δ pH/tat targeting pathway (Mori and Cline 2001). PaDXS2A and PaDXS2B lack the tandem Arg sequences and have instead KR and KH in the same positions (Fig. 1). They both contain numerous basic residues in the H-C domain,

proton transfer. The horizontal line denotes part of the TPP binding motif conserved among DXS proteins. The dashed line denotes the N domain of the presumed transit peptide and the arrow indicates the presumed transit peptide cleavage site. This last was the truncation site used for bacterial expression and complementation

making these sequences incompatible with the sec targeting pathway (Mori and Cline 2001). Thus, all three spruce DXS genes encode transit peptides with features consistent with targeting to the thylakoid lumen via the Δ pH/tat translocation pathway.

All three Norway spruce DXS genes encode functional proteins

Functional identification of proteins encoded by the three PaDXS cDNAs was accomplished via complementation of a DXS-deficient *Escherichia coli* strain engineered to utilize mevalonate for isoprenoid biosynthesis (Campos et al. 2001). In the absence of mevalonate, only cells transformed with a plasmid bearing a functional DXS gene are viable. Since *E. coli* lacks subcellular compartments typical

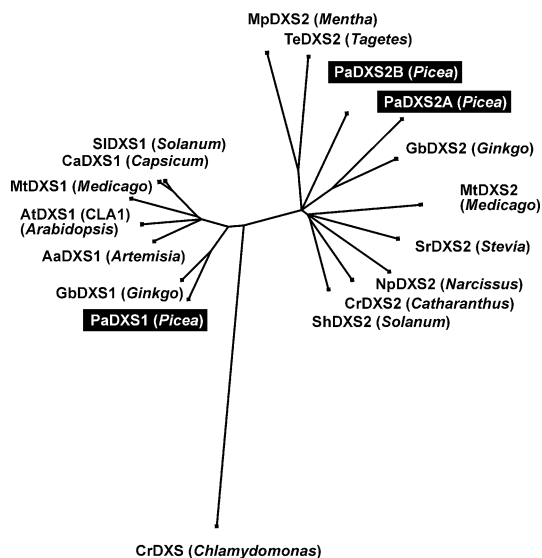


Fig. 2 Similarity tree of amino acid sequences of mature DXS proteins excluding transit peptides. The tree was generated using the programs Distances and Splits of the HUSAR analysis package using default values. The DXS of the green alga *Chlamydomonas reinhardtii* (CrDXS, AJ007559) was used as an outgroup. Novel sequences resulting from this work are highlighted. The following plant sequences were included: CaDXS1 (TKT2), *Capsicum annuum*, accession number Y15782; SIDXS1, *Solanum lycopersicum*, AF143812; MtDXS1, *Medicago truncatula*, AJ430047; AtDXS1 (CLA1), *Arabidopsis thaliana*, U27099; AaDXS1, *Artemisia annua*, AF182286; GbDXS1, *Ginkgo biloba*, AY505128; PaDXS1, *Picea abies*, EF688331; MpDXS2, *Mentha piperita*, AF019383; TeDXS2, *Tagetes erecta*, AF251020; PaDXS2B, *Picea abies*, EF688333; PaDXS2A, *Picea abies*, EF688332; GbDXS2, *Ginkgo biloba*, AY494185; MtDXS2, *Medicago truncatula*, AJ430048; SrDXS2, *Stevia rebaudiana*, AJ429232; NpDXS2, *Narcissus pseudonarcissus*, AJ279018; CrDXS2, *Catharanthus roseus*, AJ011840; ShDXS2, *Solanum habrochaites*, AY687353

of plant cells, we used truncated forms of the three *PaDXS* cDNAs lacking the putative transit peptide for complementation tests. All three *PaDXS* cDNAs, *PaDXS1*, *PaDXS2A*, and *PaDXS2B*, complemented the *E. coli* *dxs*⁻ strain, allowing growth in the absence of mevalonate, while vector-transformed controls required addition of mevalonate for growth (Fig. 3). These results clearly prove that *PaDXS1*, *PaDXS2A*, and *PaDXS2B* encode functional DXS proteins. Although we cannot exclude the possibility that additional DXS genes may exist in the Norway spruce genome, the fact that no additional contigs of ESTs for DXS-like genes were found in the TREENOMIX database of more than 200,000 spruce ESTs, which covers a diverse array of tissues and treatments (Ralph et al. 2006), and the fact that no additional DXS genes were found during screening of 300,000 phage plaques from our original cDNA library, make it unlikely that DXS genes other than those represented by *PaDXS1*, *PaDXS2A*, and *PaDXS2B* contribute much to constitutive or induced terpenoid biosynthesis in spruce.

The DXS genes are differentially expressed in sapling stems

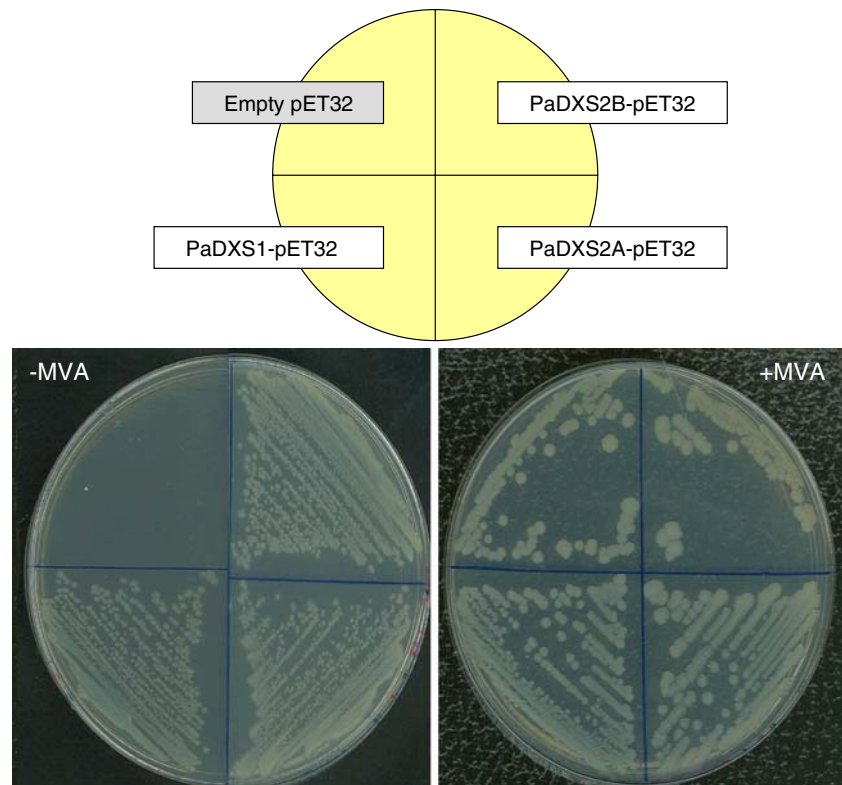
It has previously been shown that mechanical wounding or treatment of conifer trees with MeJA results in increased transcript levels of late genes of terpenoid biosynthesis, specifically members of the large *TPS* gene family and CYP720-type *CYP450* genes, leading to induced oleoresin formation (Steele et al. 1998b; Fäldt et al. 2003; Miller et al. 2005; Ro et al. 2005). To investigate the role of each of the three *PaDXS* genes in induced (traumatic) oleoresin formation in Norway spruce, we analyzed transcript levels via quantitative real-time PCR (qRT-PCR) using gene-specific primers for *PaDXS1*, *PaDXS2A*, and *PaDXS2B*. The specificity of each pair of DXS primers was confirmed in qRT-PCR SYBR Green assays in which each primer pair was individually tested using plasmids encoding each of the three cloned *PaDXS* cDNAs as template in separate reactions. In each case, DXS primers produced a C_t value at least 15 C_t s lower for the intended target than for the other two DXS cDNAs at similar concentrations. Cloning and sequencing of multiple independent qRT-PCR products obtained with each primer pair and total cDNA templates further confirmed these primer specificities.

In an initial time course analysis, we measured gene-specific changes of DXS transcript levels in RNA samples isolated from whole bark peels of Norway spruce saplings treated by mechanical wounding (Fig. 4A) or MeJA (Fig. 4B). Trees were harvested for RNA isolation at 1, 3, 7, and 10 days after treatment. *PaDXS1* transcript levels responded only slightly and transiently to wounding or MeJA (Fig. 4). In contrast, transcripts for both *PaDXS2A* and *PaDXS2B* in wounded and MeJA-treated trees had increased rapidly by the first time point measured and maintained levels well above those found in non-treated control trees over the ten-day time course (Fig. 4). Treatment with MeJA produced greater effects than mechanical wounding, and the timing of *PaDXS2A* and *PaDXS2B* transcript changes also appeared to be somewhat different when comparing MeJA treatment with wounding.

Transcripts of DXS type II and other MEP pathway genes are induced by wounding and fungal treatments in Norway spruce stems

Given the strong induction of *PaDXS2A* and *PaDXS2B* transcripts in stems 3 d after mechanical wounding (Fig. 4A), we chose this time point to compare the effects of wounding and fungal infection on the expression of the DXS genes using three different treatments. The first treatment consisted of mechanical wounding of the stems with a razor blade. The second simulated fungal infection

Fig. 3 Functional expression of Norway spruce *DXS* genes in *E. coli*. A *DXS* deficient strain of *E. coli* engineered to utilize mevalonate as a source of isoprenoids was transformed either with plasmids bearing individual spruce *DXS* genes as indicated or empty vector (negative control). Growth on media lacking mevalonate indicates an active *DXS* gene



by mechanical wounding followed by application of a chitosan solution (Miller et al. 1986; Croteau et al. 1987). The third treatment involved actual fungal inoculation accomplished by wounding followed by application of a *C. polonica* spore solution. *C. polonica* is a blue-stain pathogen of Norway spruce that is nearly always associated with the attack of the bark beetle *I. typographus*.

Wounding and fungal infection led to an increase in steady-state transcript levels from *PaDXS2A* and *PaDXS2B*, but not for *PaDXS1* (Fig. 5A), demonstrating that the lack of response of *PaDXS1* previously observed in the time course assays with wounded or MeJA-treated trees (Fig. 4) was not altered by addition of fungal elicitors. *PaDXS2A* showed a three- to fivefold increase in transcript abundance in response to mechanical wounding and fungal treatment, while induction of *PaDXS2B* transcripts was nearly twice this amount. Although treatment of wounded trees with chitosan did not increase transcript abundance beyond that of wounding alone, *C. polonica* inoculation of wounded stems caused a slight, yet reproducible increase in both *PaDXS2A* and *PaDXS2B* beyond that observed for wounding alone ($P < 0.01$).

We extended this experiment by analyzing the transcript levels of putative genes encoding two subsequent steps of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 4-hydroxyl 3-methylbutenyl diphosphate reductase (HDR). The sequence for DXR was obtained from the TREENOMIX:Conifer Forest Health

EST database (<http://www.treenomix.ca>), while a full length HDR was obtained by homology-based cDNA library screening (M. Phillips, unpublished results). Mechanical wounding and fungal treatment caused an up-regulation of transcript levels for genes encoding both DXR and HDR (Fig. 5A).

Elicitor treatment triggers monoterpene accumulation in Norway spruce cell suspension cultures and increases in jasmonic acid and OPDA

To distinguish between the effects of wounding and fungal infection on *DXS* expression, we sought an experimental system that would allow application of fungal elicitors without mechanical wounding, a difficult challenge with intact trees and their protective bark covering. However, an inducible cell culture system would meet these requirements and allow the regulation of terpene formation to be investigated apart from specific cell types. We established a Norway spruce cell suspension from calli of an embryogenic culture that had been initiated from mature seeds. Despite the lack of specialized, resin forming cells typical of conifer stems in the culture (Keeling and Bohlmann 2006b), we detected small amounts of monoterpenes resembling the oleoresin compounds of intact trees. Using a sterilized XAD4 resin overlay to trap and concentrate monoterpenes, we reproducibly measured α -pinene,

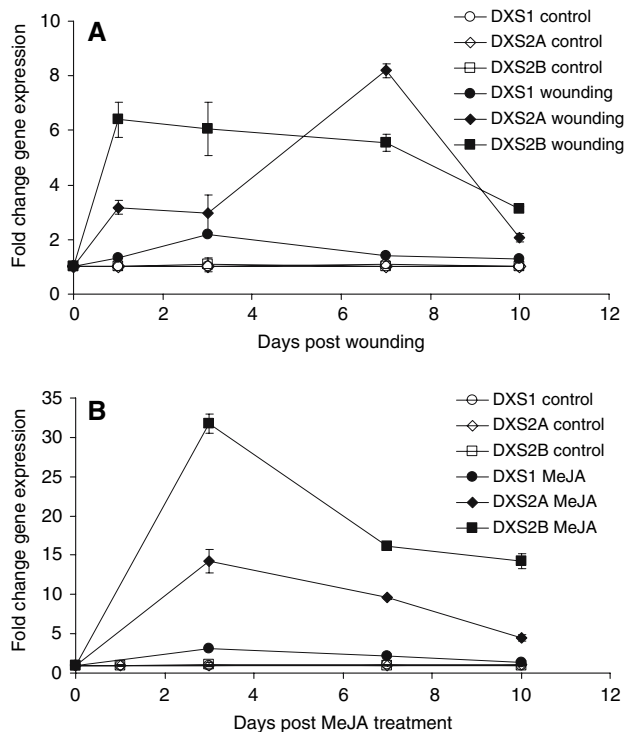


Fig. 4 Changes in steady-state transcript levels of different *DXS* genes in Norway spruce saplings following mechanical wounding (**A**) or MeJA treatment (**B**). A minimum of six saplings per replicate were harvested and pooled, used in RNA extractions, and analyzed by qRT-PCR in triplicate ($n = 12$). Control saplings for the MeJA treatment were sprayed with 0.2% (v/v) Tween 20. Control saplings for the wounding treatment were not handled at all. The normalizer gene used was ubiquitin. Error bars indicate standard deviations

β -pinene, camphene, limonene, and myrcene released from the cultured cells following addition of MeJA to the growth medium (Fig. 6). Based on comparison to the internal standard, all were detected at concentrations corresponding to 25–50 ng/ml culture. These monoterpenes were absent in all controls, including cells not treated with MeJA, MeJA-induced cells grown in the absence of XAD4 resin, and XAD4 resin incubated in culture medium without cells (data not shown).

This inducible cell culture provided an excellent opportunity to learn more about the signaling pathway involved in triggering oleoresin terpene formation in conifers. Given the dramatic effects of MeJA on resin production in Norway spruce (Martin et al. 2002) and the elevated transcript levels of octadecanoid pathway genes observed on insect attack in Sitka spruce (Miller et al. 2005; Ralph et al. 2006), we searched for jasmonic acid (JA) and other oxylipins after elicitation with chitosan. Compared to unelicited controls (0 h), JA levels more than doubled 2 h after application of chitosan (Fig. 7A), consistent with previous reports of induced JA formation in conifer cell cultures (Blechert et al. 1995). In addition to

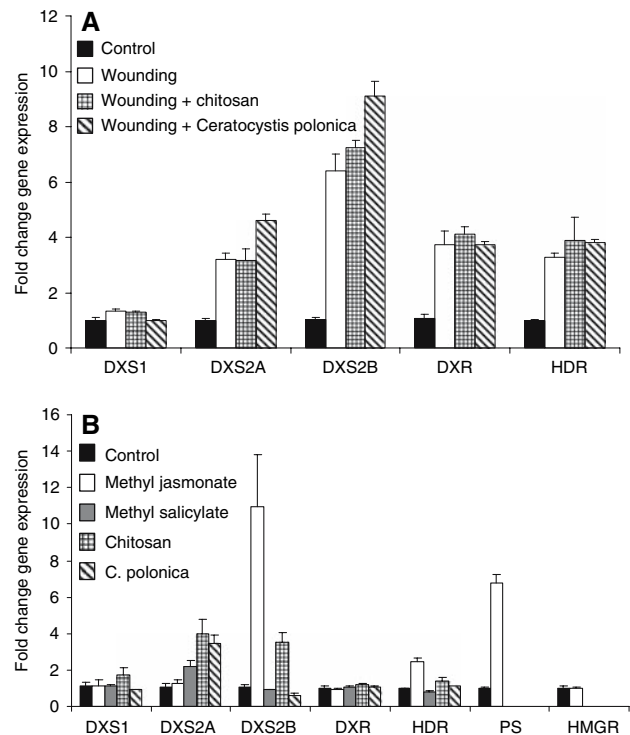


Fig. 5 Steady-state transcript levels of isoprenoid biosynthetic genes in Norway spruce saplings (**A**) and suspension cultured cells (**B**) after various treatments as determined by qRT-PCR ($n = 12$). Saplings were harvested 3 days after treatment, while cultured cells were harvested 18 h after treatment. The normalizer gene used was ubiquitin. Fold changes were calculated according to the efficiency corrected method (Pfaffl 2001). DXR (1-deoxyxylulose 5-phosphate reductoisomerase) and HDR (1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase) catalyze the second and seventh steps, respectively, of the MEP pathway and have previously been shown to be regulated steps. PS ($(-)$ - α/β -pinene synthase) was functionally characterized previously (Martin et al. 2004) and HMGR (3-hydroxy 3-methylglutaryl CoA reductase) was identified from an EST collection (Ralph et al. 2006; <http://www.treenomix.ca>) based on sequence similarity. Error bars indicate standard deviations

the increase in JA levels, the isomeric composition shifted towards a greater proportion of the less stable *cis*-JA (18.4% in controls rising to 70.0% at 2 h after treatment) compared to the *trans*-isomer, suggesting *de novo* JA biosynthesis in induced cells. Another oxylipin detected was 12-oxophytodienoic acid (OPDA), a JA precursor potentially active in defense signaling. Levels of OPDA increased more than ten-fold (4 h) after chitosan elicitation (Fig. 7B).

Elicitor treatment stimulates monoterpene synthase activity in cultured cells

To measure the activation of terpene biosynthesis in cultured Norway spruce cells, we monitored TPS enzyme activity by measuring the conversion of various prenyl

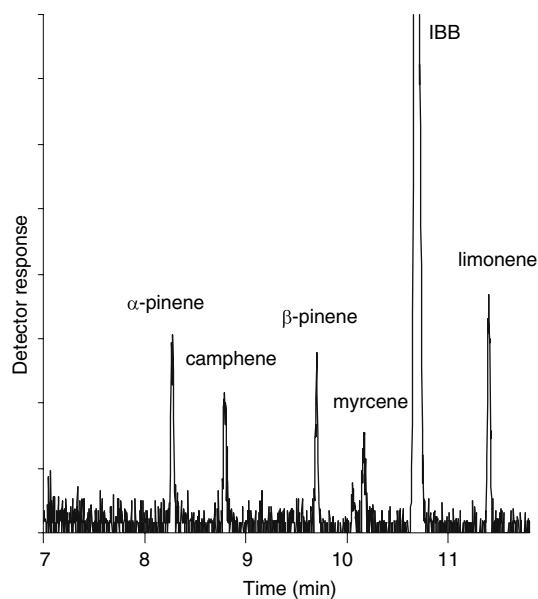


Fig. 6 Profile of monoterpene products accumulated in Norway spruce cell suspension culture after application of MeJA to the growth medium. Depicted is the total ion trace of GC-MS analysis performed on a pentane extract of XAD4 resin beads which had been co-incubated with cultured spruce cells as a monoterpene trap. Compounds were identified by their mass spectra and retention times compared with authentic standards. IBB, isobutylbenzene, was used as an internal standard

diphosphate substrates to terpene products. Mono-TPS activity (conversion of geranyl diphosphate to monoterpenes) was readily detectable under constitutive conditions, and increased substantially after application of chitosan or MeJA (Fig. 8A). A time course of mono-TPS activity after application of MeJA showed a detectable increase in enzyme activity in as little as two hours (Fig. 8B). Mono-TPS activity continued to rise after 2 h and reached levels approximately five times higher than those measured in control cells after 48 h. In contrast to mono-TPS activity, neither sesqui-TPS (farnesyl diphosphate to sesquiterpenes) nor di-TPS (geranylgeranyl diphosphate to diterpenes) activity was found in control or MeJA-induced cell cultures, nor were any sesquiterpenes or diterpenes detected in organic extracts (data not shown). Taken together, the formation of jasmonates, the induction of monoterpene synthase activity and the accumulation of monoterpenes suggest that at least a portion of the signaling and metabolic pathways of induced terpenoid defense are functional in cultured Norway spruce cells.

Type II *DXS* genes are differentially induced in cell cultures by fungal treatment, elicitors, and signaling compounds

The expression of *PaDXS* and other isoprenoid biosynthetic genes was measured in our inducible, terpene-producing

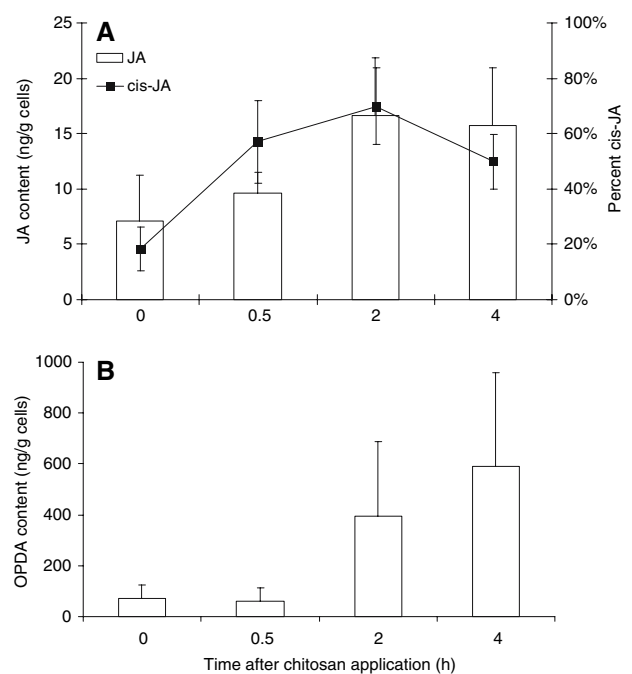


Fig. 7 Content of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA) in Norway spruce cell suspension cultures following treatment with chitosan, a fungal cell wall component. Results are expressed per gram fresh weight of cells. The ratio of *cis*-jasmonic acid (*cis* JA) to *trans*-jasmonic acid is an indicator of *de novo* biosynthesis since JA biosynthesized in the *cis* form tautomerizes to the more stable *trans* isomer

cell cultures to compare and extend the results obtained with intact plants. The use of cell cultures allowed us to test the effect of certain signaling compounds on Norway spruce tissue in a more direct fashion than by application to mechanical wounds of intact saplings. Transcript levels were analyzed 18 h after treatment of cells. In agreement with the results obtained from intact saplings (Fig. 5A), *PaDXS1* transcript levels were not affected by any of the treatments tested on cell cultures (Fig. 5B), suggesting again that *PaDXS1* does not encode a regulated step in the formation of induced terpenoid defenses in Norway spruce. Similarly, transcript levels of *DXR* were also not affected, and a response of *HDR* transcript was detected only in response to MeJA treatment ($n = 12$, $P < 0.01$) (Fig. 5B).

In contrast, the type II *DXS* genes, *PaDXS2A* and *PaDXS2B*, were both activated by some of the signaling compounds and elicitors tested, but not in a similar manner. *PaDXS2A* transcript levels were not affected by MeJA but increased approximately fourfold in response to chitosan and *C. polonica* spores (Fig. 5B). *PaDXS2A* also responded slightly to treatment with methyl salicylate (MeSA) ($n = 12$, $P < 0.01$) consistent with its activation by fungal elicitors. On the other hand, transcript levels of *PaDXS2B* responded strongly (more than 10-fold increase) to MeJA, but were not affected by MeSA or *C. polonica* spores.

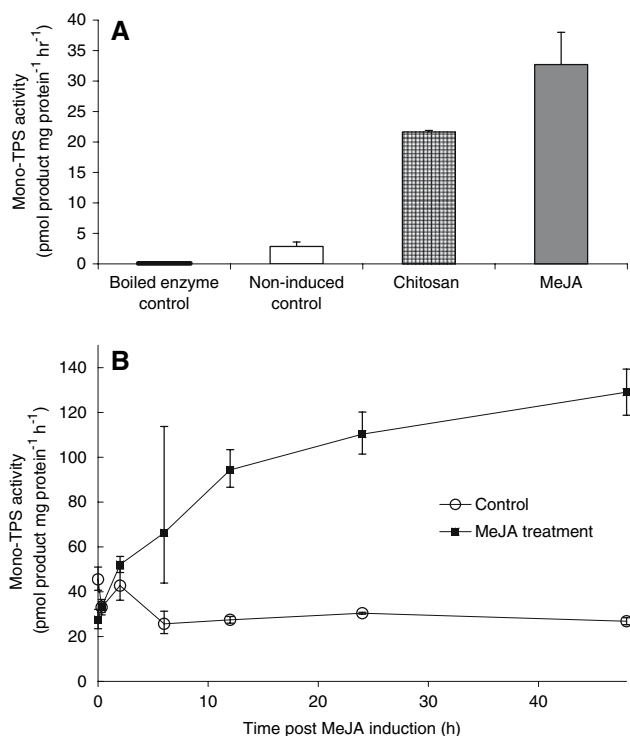


Fig. 8 The effect of elicitors on the induction of monoterpane synthase activity in Norway spruce cell suspension cultures. Chitosan ($50 \mu\text{g ml}^{-1}$) or MeJA ($50 \mu\text{M}$) was applied (A), and activity was measured by the conversion of GPP to monoterpane products 18 h after induction. The product spectrum was similar to that in Fig. 6. A time-course of monoterpane synthase activity in Norway spruce cell cultures after induction with $50 \mu\text{M}$ MeJA (B) was conducted separately. Error bars in A and B indicate standard deviations

Chitosan was the only common elicitor for both type II *DXS* genes.

Among other genes studied, transcripts of the monoterpane synthase, ($-$)- α/β -pinene synthase (PS) (Martin et al. 2004) responded to MeJA like *PaDXS2B* (Fig. 5B), indicating that genes of other stages of terpene biosynthesis may be coordinately regulated with specific *DXS*-type isogenes. The lack of response of the mevalonate pathway gene, HMG-CoA reductase (HMGR; Fig. 5B) upon MeJA treatment supports the dominant role of the MEP rather than the mevalonic acid pathway in supplying substrate for monoterpane formation in Norway spruce.

Discussion

Transcription of MEP pathway genes regulates defensive oleoresin formation in Norway spruce

Despite the intensive study of genes and enzymes participating in oleoresin terpene biosynthesis in conifers, almost no information is available about the role of the MEP

pathway in this process. The MEP pathway, along with the mevalonate pathway, constitutes the first stage of terpene biosynthesis, producing the C_5 intermediates, IPP and DMAPP, from basic precursors, such as glyceraldehyde-3-phosphate and pyruvate. Here we have isolated and functionally characterized three genes from Norway spruce encoding *DXS*, the first step of the MEP pathway, and examined their expression and the expression of other MEP pathway genes after wounding and simulated pathogen attack.

We observed a general increase in transcripts of all three MEP pathway genes studied in response to mechanical wounding, fungal elicitors, and treatment with defensive signaling compounds known to activate terpene oleoresin formation (Martin et al. 2002; Phillips et al. 2006). These results suggest that the MEP pathway plays an important role in regulating the formation of oleoresin components, and that this regulation is at least partly at the level of transcription. Among the steps of the MEP pathway, *DXS* appears to have particular regulatory significance since there is a small family of *DXS* genes, differentially responsive to various wounding treatments and biotic stresses. In addition, *DXS* transcripts collectively increase to a much greater extent than transcripts for the two other MEP pathway genes examined, *DXR* and *HDR*.

Type II but not Type I *DXS* genes are activated by wounding, fungi, and signaling compounds

The two types of *DXS* genes were previously reported in plants based on differences in sequence and expression pattern (Walter et al. 2002). Both were found in Norway spruce. Type I genes have been implicated in primary terpenoid metabolism due to their constitutive expression in photosynthetic tissues, consistent with a role in providing precursors for chlorophyll and carotenoid biosynthesis. Here we found *PaDXS1* to be constitutively expressed under all conditions, and not induced by any wounding, fungal elicitor or defensive signal, indicating it is likely to be involved in primary metabolism (Walter et al. 2002).

In contrast, type II *DXS* genes have been shown to be active in isoprenoid metabolism in specialized tissues, such as in the apocarotenoid-accumulating roots of legumes following mycorrhizal colonization (Walter et al. 2002), in the monoterpane synthesizing gland cells of mint leaf trichomes (Lange et al. 1998), or in tissues forming diterpenes in *Ginkgo biloba* (Kim et al. 2006). Here we demonstrated for the first time the involvement of type II *DXS* in inducible terpene defenses. The two type II *DXS* genes of Norway spruce respond to a variety of stimuli previously associated with the activation of induced resin defenses in conifers, including fungal infection,

mechanical wounding, chitosan and MeJA treatment (Steele et al. 1995; Martin et al. 2002, 2005), and their up-regulation is linked to the activation of other genes involved in the defense response, such as terpene synthases. While some small differences were noted in the timing and extent of the responses of *PaDXS2A* and *PaDXS2B* to various forms of defense induction in saplings, these differences were much more pronounced in cell suspension culture. For example, while *PaDXS2B* was highly activated by MeJA in culture, *PaDXS2A* was completely unresponsive. Methyl salicylate (MeSA) had the opposite effect, producing a slight induction of *PaDXS2A* ($n = 12$, $P < 0.01$) but not of *PaDXS2B*. Treatment with live *C. polonica* spores resembled MeSA with a slight induction of *PaDXS2A* and a slight repression of *PaDXS2B* ($n = 12$, $P = 0.05$). Chitosan was the only treatment that had a similar effect on both genes in cell culture.

The distinct expression patterns of the two type II *DXS* genes in spruce cell culture may be indicative of separate jasmonate- and salicylate-mediated signaling pathways in gymnosperms homologous to those known in angiosperms (Kunkel and Brooks 2002; Glazebrook et al. 2003). Jasmonates have been demonstrated to induce defense signaling and accumulation in a variety of gymnosperm species, especially in cell cultures (Ketchum et al. 1999; Kozlowski et al. 1999; Lapointe et al. 2001; Khosroushahi et al. 2006). However, only rarely has an attempt been made to measure endogenous jasmonate levels in gymnosperms (Blechert et al. 1995). In this investigation, we found measurable levels of jasmonic acid in Norway spruce cell suspension cultures that increased 2 h after treatment with chitosan. The presence of OPDA, an intermediate in jasmonic acid biosynthesis, at levels more than 30 times higher than JA is notable because this octadecanoid has been postulated to serve as a signal independent of JA (Stintzi et al. 2001; Taki et al. 2005; Buseman et al. 2006). To our knowledge, this is the first report of OPDA in a gymnosperm. There is also evidence for salicylic acid (SA) as an endogenous signal in gymnosperms. Increases in bound SA have been observed in Norway spruce seedling roots inoculated with *Pythium* sp. (Kozlowski and Metraux 1998) and increases in both free and bound SA have been detected in seedlings treated with MeJA (Kozlowski et al. 1999).

In angiosperm defense signaling, jasmonate is more commonly associated with responses to herbivores and salicylic acid with responses to pathogens. Our results on *DXS* expression in spruce cell culture show the rudiments of a similar pattern in that one transcript (*PaDXS2A*, but not *PaDXS2B*) is inducible by both MeSA and *C. polonica* spore treatment, while the other transcript (*PaDXS2B*, but not *PaDXS2A*) is inducible by MeJA. However, both genes respond similarly to chitosan, and the response pattern in

saplings is not qualitatively different. This may not be too surprising because herbivore and pathogen attack often occur simultaneously in gymnosperms. Bark beetle attacks, for example, are nearly always associated with fungal invasion (Bohlmann et al. 1997; Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and Bohlmann 2006a, b), so responses to insects, pathogens and wounding may appear to be similar. Studies with mature Norway spruce showed increased formation of traumatic resin ducts after inoculation with *C. polonica*. However, control trees in which a wound was made for insertion of sterile agar plugs also developed more traumatic ducts than non-wounded controls indicating similar response to pathogens and wounding (Nagy et al. 2000).

Inducible accumulation of terpenes in Norway spruce cell suspension cultures provides a convenient experimental system for studying induced oleoresin formation

Despite the enormous differences in tissue organization between spruce cell suspension cultures and intact spruce trees, suspension cultures also exhibit induced terpene formation making them a useful system for a detailed dissection of this process. A wide range of elicitors can be tested on suspension cultures in known concentrations, and experiments may allow separation of specific wound and pathogen-specific pathways. In addition, suspension cultures allow uniform stimulation of a large population of cells which should facilitate isolation of components of the signaling pathway. In our cultures, treatment with MeJA led to the accumulation of a similar profile of monoterpenes as in intact trees. Such accumulation is likely a result of increased biosynthesis since MeJA treatment also caused an increase in monoterpene synthase activity, as measured by in vitro enzyme assays, and an increase in the transcript level of one of the major monoterpene synthases as measured by qRT-PCR. Norway spruce cultures were previously described as incapable of *de novo* monoterpene biosynthesis (Lindmark-Henriksson et al. 2003, 2004), but this conclusion may be blamed on the difficulty of detecting low levels in culture.

Measurements of gene expression in our cultures demonstrated that genes encoding key steps in the MEP pathway were stimulated by MeJA. However, such an increase was not observed for HMGR, a key gene of the mevalonate pathway, suggesting that the MEP pathway is primarily responsible for providing precursors for induced oleoresin biosynthesis. Interestingly, the gene expression pattern measured in suspension culture differed from that observed in intact saplings. For example, *PaDXS2B* responded significantly to MeJA in culture and in saplings,

while *DXR* and *HDR* only responded in saplings. These observations suggest that the lack of differentiated cells and specialized anatomical structures in the suspension cultures may limit transcriptional activation. Incomplete activation of the MEP pathway may be responsible for the low levels of monoterpenes detected in cultures, in comparison to intact plants, and the lack of sesquiterpene and diterpene accumulation in cultures.

In spite of its importance in terpenoid biosynthesis, the MEP pathway was first discovered slightly more than ten years ago (Rohmer et al. 1993). Hence there are still many unanswered questions about the enzymology of the pathway and its regulation, and about which terpenes are formed from MEP pathway products, and how this pathway complements the mevalonate pathway, the other route for producing the C₅ units of terpenes. We have now demonstrated an important function of the MEP pathway in controlling production of terpene metabolites in conifer oleoresin, using Norway spruce as a model. Knowledge of how oleoresin formation is regulated should contribute to a greater understanding of conifer defense and facilitate our ability to manage many of the destructive pests of conifer forests.

Acknowledgements We thank Kerstin Manke (IPB, Halle) for skillful technical assistance, Xue Mei Niu (Hans Knöll Institute, Jena) for the preparation of *C. polonica* spore cultures and Ms. Sharon Jancsik (UBC Michael Smith Laboratories) for maintaining the EST/FLcDNA-collections of the TREENOMIX:Conifer Forest Health Project. We also thank Paal Krokene of the Norwegian Forest Research Institute (Ås, Norway), for providing the *C. polonica* strain. The research reported in this paper was supported with funds from the Max Planck Society (to JG), Genome British Columbia and Genome Canada in support of the TREENOMIX:Conifer Forest Health Project (grant to JB), and the Natural Sciences and Engineering Council of Canada (NSERC, grant to JB). JB is an NSERC E.W.R. Steacie Memorial fellow.

References

- Bishop-Hurley SL, Zabkiewicz RJ, Grace L, Gardner RC, Wagner A, Walter C (2001) Conifer genetic engineering: transgenic *Pinus radiata* (D. Don) and *Picea abies* (Karst) plants are resistant to the herbicide Buster. *Plant Cell Rep* 20:235–243
- Blechert S, Brodschelm W, Holder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proc Natl Acad Sci USA* 92:4099–4105
- Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from Grand fir (*Abies grandis*)—cDNA isolation, characterization, and functional expression of myrcene synthase, (–)(4S)-limonene synthase, and (–)-(1S,5S)-pinene synthase. *J Biol Chem* 272:21784–21792
- Buseman CM, Tamura P, Sparks AA, Baughman EJ, Maatta S, Zhao J, Roth MR, Esch SW, Shah J, Williams TD, Welti R (2006) Wounding stimulates the accumulation of glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoic acid in *Arabidopsis* leaves. *Plant Physiol* 142:28–39
- Byun-McKay SA, Hunter WL, Godard KA, Wang SX, Martin DM, Bohlmann J, Plant AL (2003) Insect attack and wounding induce traumatic resin duct development and gene expression of (–)-pinene synthase in Sitka spruce. *Plant Physiol* 133:368–378
- Campos N, Rodriguez-Concepcion M, Sauret-Gueto S, Gallego F, Lois LM, Boronat A (2001) *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. *Biochemical J* 353:59–67
- Croteau R (1987) Biosynthesis and catabolism of monoterpenoids. *Chem Rev* 87:929–954
- Croteau R, Gurbekov S, Johnson MA, Fisk HJ (1987) Biochemistry of oleoresinosis—Monoterpene and diterpene biosynthesis in Lodgepole pine saplings infected with *Ceratocystis clavigera* or treated with carbohydrate elicitors. *Plant Physiol* 85:1123–1128
- Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J (2006) Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia* 148:426–436
- Estevez JM, Cantero A, Reindl A, Reichler S, Leon P (2001) 1-deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J Biol Chem* 276:22901–22909
- Fäldt J, Martin D, Miller B, Rawat S, Bohlmann J (2003) Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol Biol* 51:119–133
- Franceschi VR, Krekling T, Christiansen E (2002) Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. *Am J Bot* 89:578–586
- Franceschi VR, Krokene P, Christiansen E, Krekling T (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol* 167:353–375
- Glazebrook J, Chen WJ, Estes B, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J* 34:217–228
- Huber DP, Philippe RN, Godard KA, Sturrock RN, Bohlmann J (2005) Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*. *Phytochemistry* 66:1427–1439
- Hudgins JW, Franceschi VR (2004) Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiol* 135:2134–2149
- Hudgins JW, Christiansen E, Franceschi VR (2003) Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the Pinaceae. *Tree Physiol* 23:361–371
- Hudgins JW, Ralph SG, Franceschi VR, Bohlmann J (2006) Ethylene in induced conifer defense: cDNA cloning, protein expression, and cellular and subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in resin duct and phenolic parenchyma cells. *Planta* 224:865–877
- Keeling CI, Bohlmann J (2006a) Diterpene resin acids in conifers. *Phytochemistry* 67:2415–2423
- Keeling CI, Bohlmann J (2006b) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol* 170:657–675
- Ketchum RE, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol Bioeng* 62:97–105
- Khosroushahi AY, Valizadeh M, Ghasempour A, Khosrowshahi M, Naghdibadi H, Dadpour MR, Omid Y (2006) Improved Taxol

- production by combination of inducing factors in suspension cell culture of *Taxus baccata*. Cell Biology Intl 30:262–269
- Kim SM, Kuzuyama T, Chang YJ, Song KS, Kim SU (2006) Identification of class 2 1-deoxy-D-xylulose 5-phosphate synthase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase genes from *Ginkgo biloba* and their transcription in embryo culture with respect to ginkgolide biosynthesis. Planta Medica 72:234–240
- Kozlowski G, Metraux JP (1998) Infection of Norway spruce (*Picea abies* (L.) Karst) seedlings with *Pythium irregulare* Buism and *Pythium ultimum* Trow: histological and biochemical responses. Eur J Plant Path 104:225–234
- Kozlowski G, Buchala A, Metraux JP (1999) Methyl jasmonate protects Norway spruce *Picea abies* (L.) Karst. seedlings against *Pythium ultimum* Trow. Physiol Mol Plant Path 55:53–58
- Krushkal J, Pistilli M, Ferrell KM, Souret FF, Weathers PJ (2003) Computational analysis of the evolution of the structure and function of 1-deoxy-D-xylulose-5-phosphate synthase, a key regulator of the mevalonate-independent pathway in plants. Gene 313:127–138
- Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol 5:325–331
- Lange BM, Ghassemian M (2003) Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. Plant Mol Biol 51:925–948
- Lange BM, Wildung MR, McCaskill D, Croteau R (1998) A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc Natl Acad Sci USA 95:2100–2104
- Lapointe G, Luckevich MD, Seguin A (2001) Investigation on the induction of 14–3–3 in white spruce. Plant Cell Rep 20:79–84
- Lindmark-Henriksson M, Isaksson D, Sjodin K, Hogberg HE, Vanek T, Valterova I (2003) Transformation of alpha-pinene using *Picea abies* suspension culture. J Nat Prod 66:337–343
- Lindmark-Henriksson M, Isaksson D, Vanek T, Valterova I, Hogberg HE, Sjodin K (2004) Transformation of terpenes using a *Picea abies* suspension culture. J Biotechnol 107:173–184
- Lois LM, Campos N, Putra SR, Danielsen K, Rohmer M, Boronat A (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. Proc Natl Acad Sci USA 95:2105–2110
- Lois LM, Rodriguez-Concepcion M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J 22:503–513
- Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol 129:1003–1018
- Martin DM, Gershenzon J, Bohlmann J (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. Plant Physiol 132:1586–1599
- Martin DM, Faldt J, Bohlmann J (2004) Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol 135:1908–1927
- Miller RH, Berryman AA, Ryan CA (1986) Biotic elicitors of defense reactions in Lodgepole pine. Phytochemistry 25:611–612
- Miller B, Madilao LL, Ralph S, Bohlmann J (2005) Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. Plant Physiol 137:369–382
- Mori H, Cline K (2001) Post-translational protein translocation into thylakoids by the Sec and Delta pH-dependent pathways. Biochim Biophys Acta 1541:80–90
- Nagy NE, Franceschi VR, Solheim H, Krekling T, Christiansen E (2000) Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): Anatomy and cytochemical traits. Am J Bot 87:302–313
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007
- Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4:184–190
- Phillips MA, Wildung MR, Williams DC, Hyatt DC, Croteau R (2003) cDNA isolation, functional expression, and characterization of (+)-alpha-pinene synthase and (–)-alpha-pinene synthase from loblolly pine (*Pinus taeda*): stereocontrol in pinene biosynthesis. Arch Biochem Biophys 411:267–276
- Phillips M, Bohlmann J, Gershenzon J (2006) Molecular regulation of induced terpenoid biosynthesis in conifers. Phytochem Rev 5:179–189
- Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, Marra MA, Douglas CJ, Ritland K, Bohlmann J (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. Plant Cell Environ 29:1545–1570
- Ralph SG, Hudgins JW, Jancsik S, Franceschi VR, Bohlmann J (2007) Aminocyclopropane carboxylic acid synthase is a regulated step in ethylene-dependent induced conifer defense. Full-length cDNA cloning of a multigene family, differential constitutive, and wound- and insect-induced expression, and cellular and subcellular localization in spruce and Douglas fir. Plant Physiol 143:410–424
- Ro DK, Bohlmann J (2006) Diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*): Functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienol oxidase (PtAO, CYP720B1). Phytochemistry 67:1572–1578
- Ro DK, Arimura G, Lau SY, Piers E, Bohlmann J (2005) Loblolly pine abietadienol/abietadienol oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase. Proc Natl Acad Sci USA 102:8060–8065
- Rodriguez-Concepcion M, Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130:1079–1089
- Rohmer M, Knani M, Simonin P, Sutter B, Sahn H (1993) Isoprenoid biosynthesis in bacteria—a novel pathway for the early steps leading to isopentenyl diphosphate. Biochemical J 295:517–524
- Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen E, Gershenzon J (2005) Induced chemical defenses in conifers: Biochemical and molecular approaches to studying their function. In: Romeo JT (ed) Recent advances in phytochemistry 39: chemical ecology and phytochemistry of forest ecosystems, Vol 39. Elsevier, Amsterdam, pp 1–28
- Schulze B, Lauchli R, Sonwa MM, Schmidt A, Boland W (2006) Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. Anal Biochem 348:269–283
- Sprenger GA, Schorken U, Wiegert T, Grolle S, deGraaf AA, Taylor SV, Begley TP, Bringer-Meyer S, Sahn H (1997) Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. Proc Natl Acad Sci USA 94:12857–12862

- Steele CL, Lewinsohn E, Croteau R (1995) Induced oleoresin biosynthesis in grand fir as a defense against bark beetles. *Proc Natl Acad Sci USA* 92:4164–4168
- Steele CL, Crock J, Bohlmann J, Croteau R (1998a) Sesquiterpene synthases from grand fir (*Abies grandis*)—Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization and bacterial expression of delta-selinene synthase and gamma-humulene synthase. *J Biol Chem* 273:2078–2089
- Steele CL, Katoh S, Bohlmann J, Croteau R (1998b) Regulation of oleoresinosis in grand fir (*Abies grandis*). Differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding. *Plant Physiol* 116:1497–1504
- Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. *Proc Natl Acad Sci USA* 98:12837–12842
- Taki N, Sasaki-Sekimoto Y, Obayashi T, Kikuta A, Kobayashi K, Ainai T, Yagi K, Sakurai N, Suzuki H, Masuda T, Takamiya K, Shibata D, Kobayashi Y, Ohta H (2005) 12-Oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. *Plant Physiol* 139:1268–1283
- Walter MH, Fester T, Strack D (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the ‘yellow pigment’ and other apocarotenoids. *Plant J* 21:571–578
- Walter MH, Hans J, Strack D (2002) Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. *Plant J* 31:243–254
- Zeneli G, Krokene P, Christiansen E, Krekling T, Gershenzon J (2006) Methyl jasmonate treatment of large Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. *Tree Physiol* 26:977–988
- Zhao J, Zheng SH, Fujita K, Sakai K (2004) Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to beta-thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. *J Exp Bot* 55:1003–1012