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Molecules of Interest

Diterpene resin acids in conifers

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Abstract

Diterpene resin acids are a significant component of conifer oleoresin, which is a viscous mixture of terpenoids present constitutively or inducibly upon herbivore or pathogen attack and comprises one form of chemical resistance to such attacks. This review focuses on the recent discoveries in the chemistry, biosynthesis, molecular biology, regulation, and biology of these compounds in conifers.

Keywords: Diterpene synthase; Cytochrome P450; Resin duct; Conifer genomics; Bark beetle; Defence

1. Introduction

Conifers have several mechanisms to repel, kill, inhibit, or otherwise reduce the success of invading herbivores and pathogens. When wounded by herbivory, such as by an insect, one such mechanism is the secretion of a viscous oleoresin containing approximately equal quantities of monoterpenoids and diterpene acids, and smaller quantities of sesquiterpenoids (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001a; Langenheim, 2003; Huber et al., 2004; Martin and Bohlmann, 2005; Keeling and Bohlmann, 2006). Oleoresin can physically "pitch out" (push the insect out of the site of entry with oleoresin flow) or entomb attacking insects as well as clean and seal the wound from microorganisms. After the more volatile mono- and sesquiterpenoid oleoresin components have evaporated, the non-volatile diterpene resin acids harden to seal the wound. Because of their greater importance in the semiochemistry of plant–insect interactions, the monoterpenes and sesquiterpenes have been studied in more detail than the diterpene resin acids. However, the diterpene resin acids also have interesting biochemistry and are thought to play a role in conifer defence. The chemistry, evolution, ecology, and ethnobotany of plant resins have recently been extensively reviewed by Langenheim (2003). Here we review the recent research in the genomics, molecular biology, biochemistry, and biological significance of conifer diterpene resin acids.

2. Chemistry

Diterpene resin acids are 20-carbon bi- or tricyclic carboxylic acids of several skeletal types with diversity introduced by double-bond isomers, diastereoisomers, and additional functionalization (Fig. 1). Many conifers produce the tricyclic diterpene acids such as abietic, dehydroabietic, isopimaric, levopimaric, neoabietic, palustric, pimaric, and sandaracopimaric acids. Others can produce bicyclic diterpene acids such as agathic, isocupressic, and *trans*-communic acids.

Many examples exist for the analysis of resin acids in conifers. Extraction and analysis of the oleoresin from conifer tissues use the method of Lewinsohn et al. (1993) or a variation thereof, permitting the GC–MS analysis of mono- and sesquiterpenoids at the same time as methylated diterpene acids.

When necessary for enzyme studies and confirmation of product identity, chemical syntheses of the diterpene, diterpene alcohol, and diterpene aldehyde precursors have used the corresponding diterpene acid as starting material

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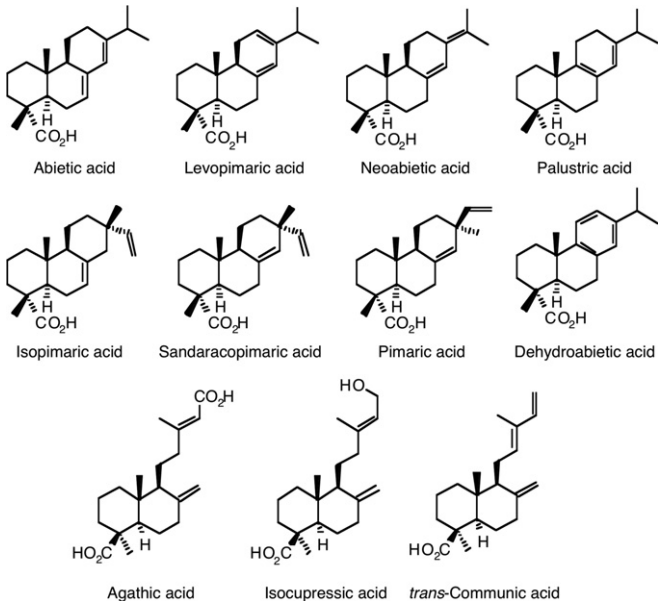

Fig. 1. Examples of conifer diterpene resin acids.

available commercially or from natural sources (Funk and Croteau, 1994; Lee et al., 2001; Ro et al., 2005).

3. Biosynthesis of diterpenes

The cyclic diterpene acids in conifers originate from a common acyclic biosynthetic precursor, geranylgeranyl diphosphate (GGPP), derived from the methylerythritol phosphate (MEP) pathway (Lichtenthaler, 1999). Diterpene synthase enzymes act on this 20-carbon substrate to form diterpenes, which are subsequently hydroxylated and then oxidized by other enzymes to the diterpene acids. These two independent transformations will be discussed separately below.

The first biochemical study of the formation of diterpenes in conifer resin acid biosynthesis used the soluble enzyme fraction of lodgepole pine (*Pinus contorta*) and grand fir (*Abies grandis*) stems in enzyme assays with radio-labelled GGPP (LaFever et al., 1994). The partially purified 80 kDa monomeric enzymes were functionally characterized and found to produce abietadiene with dependence on a divalent metal ion (Mg^{2+} preferred). The purified enzyme from grand fir was partially sequenced to permit the subsequent isolation of the cDNA (AgTPS-AS) and functional characterization of recombinant enzyme (Stofer Vogel et al., 1996), and the comparison of the genomic structure of this gene to other terpene synthases (Trapp and Croteau, 2001b).

Croteau and co-workers have extensively studied the biochemistry of the abietadiene synthase from grand fir. After the initial studies, it was later found that this enzyme in fact produces almost equal amounts of levopimaradiene, abietadiene, neoabietadiene and small amounts of palustradiene, sandaracopimaradiene, and pimara-8(14),15-diene (Peters et al., 2000). The discrepancy apparently resulted from the earlier studies passing the reaction products over silica gel and $MgSO_4$, which resulted in isomerization of levopimaradiene and neoabietadiene into abietadiene. This enzyme has two catalytically independent active sites. The first active site in the N-terminal domain catalyzes the protonation-initiated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate ((+)-CPP) (Fig. 2). The (+)-CPP then freely diffuses to a second active site in the C-terminal domain, which catalyzes the diphosphate ionization-initiated cyclization of (+)-CPP to produce a sandaracopimarenyl carbocation intermediate (Ravn et al., 2000; Peters et al., 2001; Ravn et al., 2002). Following intramolecular proton transfer and a 1,2-methyl migration, a deprotonation from one of four sites produces the four major products levopimaradiene, abietadiene, neoabietadiene, and palustradiene. The biosynthesis of other tricyclic diterpenes can be rationalized by deprotonation of the carbocation without rearrangements, or deprotonation products from a pimarenyl carbocation intermediate. Abietadiene synthase contains two aspartate-rich motifs consistent with the two active sites (Fig. 3), a DXDD motif in the N-terminal domain indicative of a class II terpene synthase fold and a DDXXD motif in the C-terminal domain indicative of a class I terpene synthase fold (Davis and Croteau, 2000; Christianson, 2006).

In a series of elegant experiments, Croteau, Peters, and co-workers have examined the effect of pH on enzyme activity and product profile (Peters and Croteau, 2002b), identified the length of an N-terminal plastidal targeting sequence (Peters et al., 2000), and identified amino acids involved in catalysis of both active sites (Peters et al., 2001; Peters and Croteau, 2002a; Peters and Croteau, 2002b). Kinetically, the second step is the rate-limiting step, with considerable substrate inhibition, specifically non-productive binding of GGPP to the second active site (Peters et al., 2000; Peters et al., 2001). The production and functional characterization of a series of enzyme fragments has indicated that, although catalytically independent, the two active sites cannot be structurally dissected into catalytically distinct domains (Peters et al., 2003). The biochemistry of this enzyme has also been studied with alternate substrates and inhibitors (Peters et al., 2001; Ravn et al., 2002). For further information, a detailed review of terpene synthase biochemistry, including diterpene synthases, has recently been published (Christianson, 2006).

A handful of other conifer diterpene synthases of resin acid biosynthesis have also recently been cloned and functionally characterized (Table 1). A levopimaradiene synthase (GbTPS-LS) has been identified and functionally characterized in *Gingko biloba* (Schepmann et al., 2001). In Norway spruce (*Picea abies*), two diterpene synthases have been identified, a levopimaradiene/abietadiene synthase (PaTPS-LAS) that produces similar products as the grand fir abietadiene synthase, and an isopimaradiene synthase (PaTPS-Iso) that uniquely produces exclusively isopimaradiene (Martin et al., 2004). EST mining and

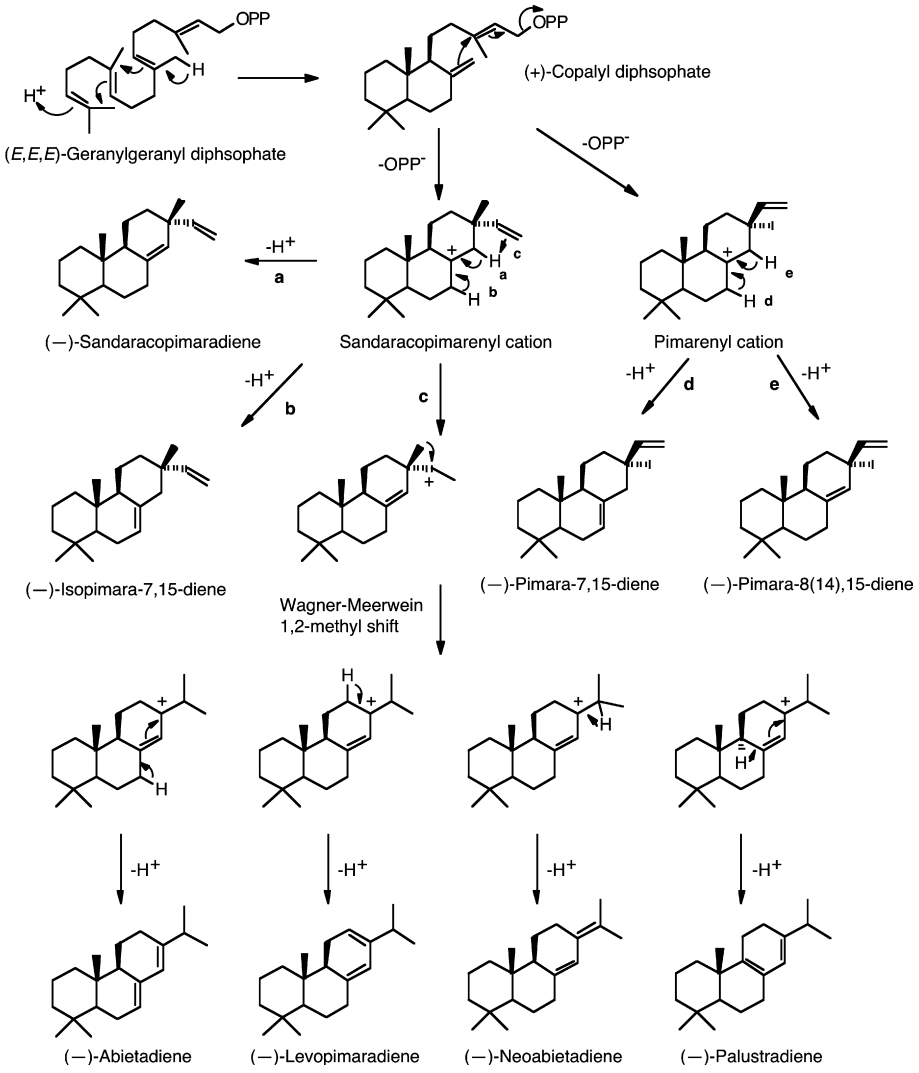

Fig. 2. Proposed reaction mechanism for the biosynthesis of some diterpenes from geranylgeranyl diphosphate. OPP represents a diphosphate group.

full-length cDNA sequencing have recently identified a levopimaradiene/abietadiene synthase (PtTPS-LAS) from loblolly pine (*Pinus taeda*) (Ro and Bohlmann, 2006) and both a levopimaradiene/abietadiene synthase (PsTPS-LAS) and an isopimaradiene synthase (PsTPS-Iso) gene from Sitka spruce (*Picea sitchensis*) (Keeling et al., unpublished). All conifer diterpene synthases have in common a size of approximately 860 amino acids, the presence of an apparent 50–70 amino acid plastid transit peptide, and a 210 amino acid motif that is also found in some conifer sesquiterpene synthases and angiosperm (−)-CPP-synthase and *ent*-kaurene synthases (Fig. 3; Bohlmann et al., 1998; Martin et al., 2004). The conifer diterpene synthases are members of a distinct TPSd-3 group within the larger conifer TPSd group which itself is a distinct subfamily of the plant terpene synthase gene family (Bohlmann et al., 1998; Martin et al., 2004).

Complementing the biochemical studies on the abietadiene synthase in grand fir, we are currently examining the biochemistry of the isopimaradiene synthase and the levopimaradiene/abietadiene synthase from Norway spruce. Although these two enzymes are 92% identical at the amino acid level, they produce non-overlapping product profiles (Martin et al., 2004). We are using domain swapping and reciprocal site-directed mutagenesis strategies to identify those amino acids that differ between the enzymes that result in product profile differences (Keeling et al., unpublished).

Although attempts have been made (Croteau, personal communication) and are currently being initiated (Keeling et al., unpublished) to determine the 3-dimensional structure of a conifer diterpene synthase, no X-ray crystal structure of any diterpene synthase has been published. Thus, our knowledge of conifer diterpene synthase biochemistries has been rationalized by modelling them against the crystal structure of *epi*-aristolochene synthase, a sesquiterpene synthase from tobacco (*Nicotiana tabacum*) (Starks et al., 1997). Although this has been fruitful (Peters and Croteau, 2002a; Peters and Croteau, 2002b; Peters et al., 2003; Martin et al., 2004), having an accurate 3-dimensional structure

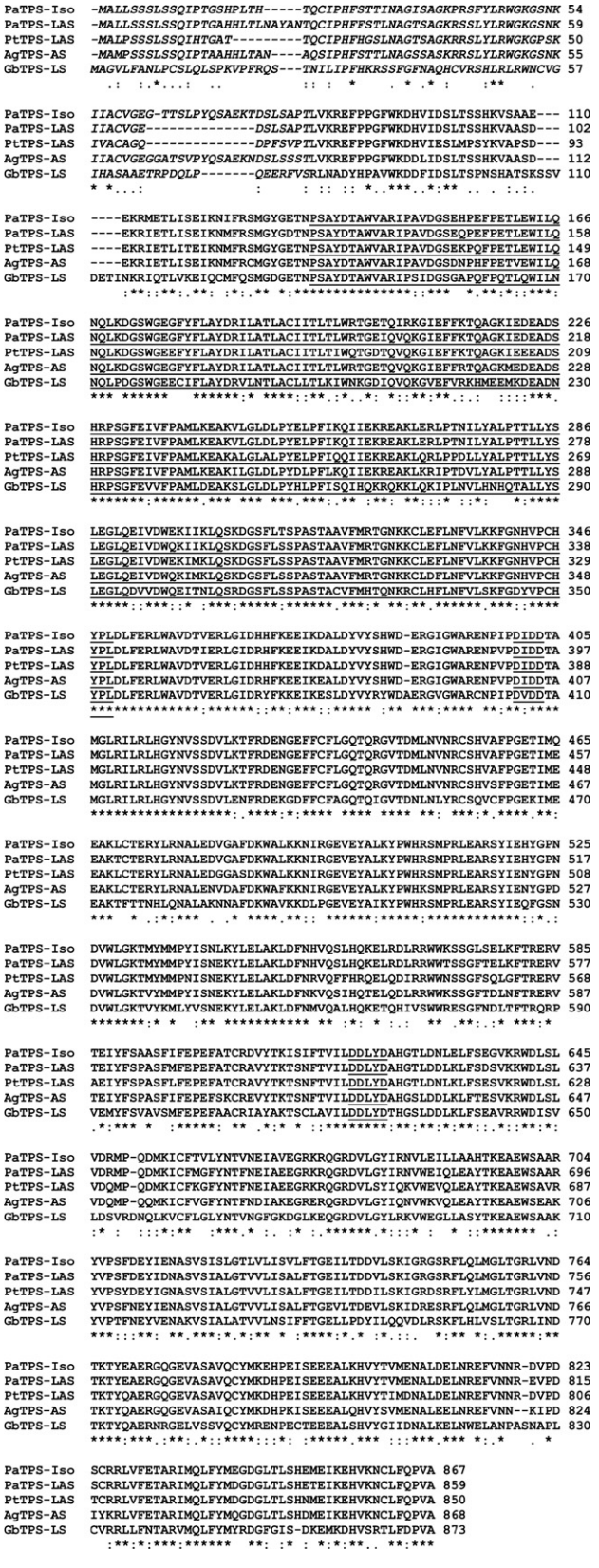

Fig. 3. CLUSTAL W alignment of the deduced amino acid sequences of diterpene synthases from Norway spruce (PaTPS-LAS and PaTPS-Iso), loblolly pine (PtTPS-LAS), grand fir (AgTPS-AS), and *Ginko biloba* (GbTPS-LS). Approximate plastid transit peptide shown in italics, and the conserved 210 amino acid, DXDD, and DDXXD motifs are shown underlined.

of a bifunctional diterpene synthase would lead to much greater insight into diterpene synthase biochemistry.

4. Biosynthesis of diterpene resin acids from diterpenes

Unlike most of the monoterpenes and sesquiterpenes, diterpenoids are not normally found in substantial amounts as hydrocarbons in conifer oleoresin. Instead, diterpenes are modified by hydroxylation and subsequent oxidation to their corresponding alcohols, aldehydes and acids, of which predominantly only the acids are found in oleoresin (Fig. 4). Cytochrome P450 monooxygenases (CYP450) are involved in these modifications. These heme-containing proteins are a very large class of enzymes that utilize NADPH or NADH to cleave atmospheric oxygen reductively while oxidatively functionalizing the substrate (Schuler and Werck-Reichhart, 2003).

The first biochemical study of the transformation of diterpenes into diterpene resin acids used cell free extracts of grand fir and lodgepole pine stem tissue with radiolabelled acetate (Funk and Croteau, 1994). The incorporation of the radio-label could be followed with time into abietadiene, abietadienol, abietadienal, and abietic acid. By fractionating the extracts into soluble and microsomal fractions, the first two oxidation steps were shown to involve two apparently distinct membrane-bound O_2- and NADPH-dependent CYP450 monooxygenase activities and the last step involved a soluble NAD^+-dependent aldehyde dehydrogenase enzyme activity. Unfortunately, no further work has been done in this system. However, a CYP450 cDNA (CYP720B1) from loblolly pine involved in diterpene acid biosynthesis has recently been cloned, heterologously expressed in yeast co-expressing the redox partner cytochrome P450 reductase (CPR), and functionally characterized (Ro et al., 2005). This enzyme can oxidize abietadienol and abietadienal to abietic acid *in vitro* and *in vivo*. It is also able to oxidize the alcohol and aldehyde forms of the related diterpenes dehydroabietadiene, isopimaradiene, and levopimaradiene, but not neoabietadiene, into their respective diterpene acids. Although this enzyme could not hydroxylate abietadiene itself in these *in vitro* assays, an engineered yeast strain that expressed a geranylgeranyl diphosphate synthase, the Norway spruce diterpene synthase PaTPS-LAS, CYP720B1, and CPR produced a small amount of abietic acid, suggesting this CYP450 might be able to catalyze all transformations from abietadiene to abietic acid. Thus, this one multifunctional CYP450 enzyme catalyzes multiple oxidation steps and uses multiple substrates to form many of the diterpene resin acids. However, this CYP450 does not accept all diterpenoid precursors present in conifers and thus is only one of several CYP450s predicted to be involved in diterpene resin acid biosynthesis. Our lab is currently characterizing other conifer CYP450s involved in diterpene acid formation (Hamberger and Bohlmann, in press).

Table 1
Functionally characterized enzymes in conifer diterpene acid biosynthesis

Enzyme	Species	Accession no.	Ref.
Geranylgeranyl diphosphate synthase (Ag-GGPPS)	*Abies grandis*	AAL17614	Burke and Croteau (2002)
Abietadiene synthase (AgTPS-AS)	*Abies grandis*	AAB05407	Stofer Vogel et al. (1996)
Levopimaradiene synthase (GbTPS-LS)	*Ginkgo biloba*	AAL09965	Schepmann et al. (2001)
Isopimara-7,15-diene synthase (PaTPS-Iso)	*Picea abies*	AAS47690	Martin et al. (2004)
Levopimaradiene/abietadiene synthase (PaTPS-LAS)	*Picea abies*	AAS47691	Martin et al. (2004)
Levopimaradiene/abietadiene synthase (PtTPS-LAS)	*Pinus taeda*	AAX07435	Ro and Bohlmann (2006)
Levopimaradiene/abietadiene synthase (PsTPS-LAS)	*Picea sitchensis*	–	Keeling et al. (unpublished)
Isopimara-7,15-diene synthase (PsTPS-Iso)	*Picea sitchensis*	–	Keeling et al. (unpublished)
Abietadienol/abietadienal oxidase (CYP720B1)	*Pinus taeda*	AAX07431	Ro et al. (2005)

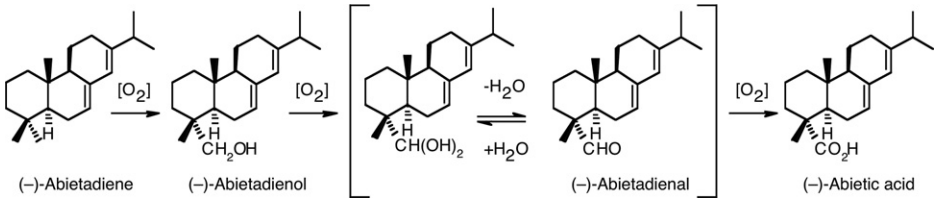

Fig. 4. Proposed reaction mechanism for the biosynthesis of abietic acid from abietadiene.

5. Regulation of biosynthesis

Oleoresin collects in resin ducts as a constitutive chemical defence in conifers. In addition, mechanical damage, herbivory, or fungal inoculation can trigger a local terpenoid response. This results in the activation of epithelial cells lining the axial and radial resin ducts, formation of specialized traumatic resin ducts in the stem xylem, and the induction of gene transcripts for diterpene biosynthesis (Franceschi et al., 2002; Martin et al., 2002; Byun McKay et al., 2003; Miller et al., 2005). Specific mono- and sesquiterpenoids may also be induced and emitted systemically as volatiles (Martin et al., 2003; Miller et al., 2005). Wounding has been shown to induce the enzyme activities of both diterpene synthases and CYP450s in grand fir (Funk et al., 1994).

As part of a larger genomics study of conifer defences against insects and pathogens, a number of microarray transcriptome profiling experiments to better understand induced conifer defences have recently been completed in our laboratory. When challenged by mechanical wounding or herbivory by spruce budworm (*Choristoneura occidentalis*) or white pine weevil (*Pissodes strobi*), the transcripts for diterpenoid biosynthesis increase in Sitka spruce, including the genes in the early steps in the formation of geranylgeranyl diphosphate, as well as the genes for diterpene synthases and CYP450s (Ralph et al., 2006). A genome-wide study of the induction of genes in lodgepole pine upon inoculation of a mountain pine beetle-associated fungus (*Ophiostoma clavigerum*) is currently in progress (N. Kolosova and J. Bohlmann, unpublished).

Methyl jasmonate (MeJA) represents a class of octadecanoid or oxylipin phytohormones involved in signalling mechanical wounding, herbivory, oviposition, or fungal inoculation (Halitschke and Baldwin, 2005; Howe, 2005). The topical application of MeJA as a non-destructive mimic of insect herbivory elicits anatomical changes in several conifer species (Hudgins et al., 2003; Hudgins et al., 2004) and induces the formation of traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in the xylem of Norway spruce (Martin et al., 2002; Fäldt et al., 2003; Hudgins et al., 2003; Hudgins et al., 2004). In foliage however, mono- and sesquiterpenoids, but not diterpene acids, are induced by such treatment (Martin et al., 2003). Similarly in Sitka spruce stems, MeJA induces the transcript levels of diterpene synthases as well as induces the accumulation of diterpene acids in bark and xylem (Miller et al., 2005). Watering a dilute solution of MeJA into the soil of Douglas-fir (*Pseudotsuga menziesii*) seedlings results in traumatic resin formation in stems and roots, and significant increases of abietic, dehydroabietic, levopimaric, palustric, and sandaracopimaric acids in the stems, and abietic and levopimaric acids in the roots (Huber et al., 2005). The defensive responses signalled by MeJA appear at least in part mediated by another phytohormone, ethylene (Hudgins and Franceschi, 2004; Hudgins et al., 2006). In Douglas-fir, inducible 1-aminocyclopropane-1-carboxylate (ACC)-synthase and ACC-oxidase activities leading to the formation of ethylene were detected by immuno-localization in epithelial cells of cortical resin ducts in wound-induced stems (Hudgins et al., 2006; Ralph et al., unpublished).

The sub-cellular localization of the enzymes for conifer diterpene resin biosynthesis predicted based upon N-terminal targeting sequences has recently been confirmed (Ro and Bohlmann, 2006). Fusion proteins containing green fluorescent protein fused to the N-terminal portion of PtTPS-LAS and CYP720B1 expressed in tobacco cells localize to the plastids and the endoplasmic reticulum, respectively. These experiments and the absence of significant diterpene hydrocarbon accumulation in resin-forming tissues suggest

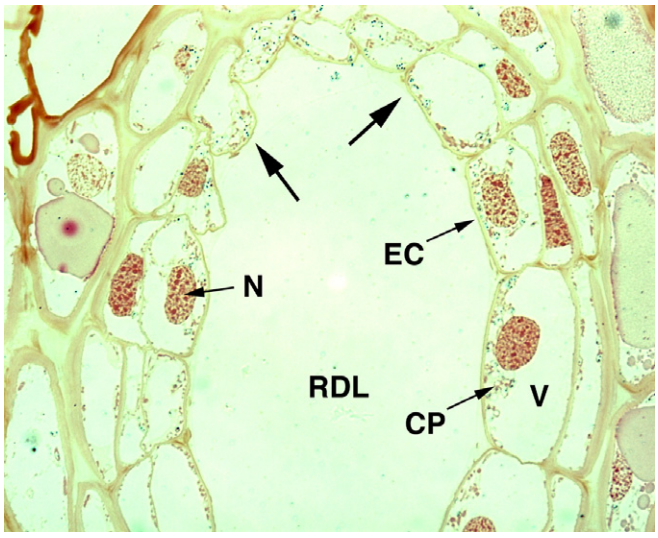

Fig. 5. Localization of PaTPS-LAS to the layer of epithelial cells that surround the resin duct lumen on a cross-section of a Norway spruce cortical resin duct. Large arrows point to example areas of black dots, which are the silver enhanced secondary antibody attached to an anti-PaTPS-LAS antibody. CP, cytoplasm; EC, epithelial cells; N, nucleus; RDL, resin duct lumen; V, vacuole. Image by J.W. Hudgins.

that an efficient transport of intermediates within the cell and the active secretion of diterpene resin acids into the extracellular space are necessary components to this system that require further investigation. In recent experiments, we were able to localize the Norway spruce diterpene synthase PaTPS-LAS to the epithelial cells of cortical resin ducts using protein-specific PaTPS-LAS antibody developed against synthetic peptides (J. W. Hudgins and J. Bohlmann, unpublished). Much of the diterpene synthase protein was detected in the cytoplasm facing the extracellular resin duct lumen (Fig. 5). Future localization of the CYP450 protein involved in diterpene resin acid formation and the identification of the systems for intracellular diterpene resin acid transport and secretion against a steep concentration gradient into the resin duct extracellular lumen are needed to better understand the cellular mechanisms that control the formation, transport, secretion, and extracellular accumulation of the lipophilic and potentially cell toxic diterpene resin acids in conifer defence.

6. Biological activity

Many examples exist that show a relationship between conifer diterpene acid content and tree resistance or an effect on herbivores. For example, the resistance of Sitka and white spruce (*Picea glauca*) to the white pine weevil are positively correlated with conifer diterpene resin acid concentrations (Tomlin et al., 1996; Tomlin et al., 1998; Tomlin et al., 2000; Byun-McKay et al., 2006). Applying MeJA onto Norway spruce to induce terpenoids including diterpene resin acids results in an inhibition of colonization by the bark beetle *Ips typographus* and its associated fungus, *Ceratocystis polonica* (Erbilgin et al., 2006; Zeneli et al., 2006).

In Scots pine (*Pinus sylvestris*), diterpene resin acids have interesting effects on insect herbivores. Pine sawfly larvae (*Neodiprion sertifer*) appear negatively affected physiologically by resin acids but are better protected from predators on trees containing high resin levels (Björkman and Gref, 1993; Saikkonen et al., 1995; Björkman et al., 1997; Larsson et al., 2000). The old house borer (*Hylotrupes bajulus*) actually feeds more intensively on wood containing higher concentrations of levopimaric acid and palustric acid while the brown-rot fungus (*Coniophora puteana*) is unaffected by differences in diterpene resin acid quantities (Nerg et al., 2004).

Surprisingly little research has addressed the biological activity of specific diterpene resin acids in their natural context. Gypsy moth (*Lymantria dispar*) larvae are tolerant to isopimaric and neoabietic acids in their diets and are apparently able to metabolize these compounds as they could not be recovered in the frass (Powell and Raffa, 2003). However, diterpene acids do exhibit antifeedant effects in this insect (Powell and Raffa, 1999). Recently, Kopper et al. (2005) have examined the effects of individual resin acids on the pine engraver beetle (*Ips pini*) and its associated fungus (*Ophiostoma ips*). Abietic acid and isopimaric acid strongly inhibit spore germination, and abietic acid strongly inhibits mycelial growth. However, abietic, isopimaric, and dehydroabietic acids have no effect on *Ips pini* larval survival or adult host acceptance behaviour.

The biological activities of conifer diterpene resin acids have also been studied outside their natural contexts. Isocupressic acid (Fig. 1) has been identified as a potent agent in pine needles that causes abortions of beef cattle (Gardner et al., 1994). The release of diterpene acids into pulp and paper mill effluent may impact downstream aquatic organisms (Kamaya et al., 2005). In humans, dermal and pulmonary sensitization from the use of resin acids in industrial processes (e.g. in rosin for solder) or their release in sawmills and carpentry workshops has been studied (Smith et al., 1998; Eriksson et al., 2004). Diterpene resin acids may also have beneficial properties to human health. For example, compounds such as isocupressic and sandaracopimaric acids from Japanese Thuja (*Thuja standishii*) stems (Iwamoto et al., 2003) and Ryukyu Island pine (*Pinus luchuensis*) cones (Minami et al., 2002) have potential antitumor activity. Several other diterpene acids and diterpenoids from Chinese Arborvitae (*Platycladus orientalis*) have been identified with weak antiplasmodial activity against *Plasmodium falciparum* (Asili et al., 2004). In addition, isopimaric acid shows activity against *Staphylococcus aureus* (Smith et al., 2005).

7. Evolution of diterpene resin acid formation relative to gibberellic acid formation

Diterpene resin acid secondary metabolites resemble both in structure and biosynthesis the *ent*-kaurenoic acid

precursor of the ubiquitous diterpenoid plant hormone gibberellic acid (Fig. 6) (Keeling and Bohlmann, 2006). Albeit quite distant, these compounds and their respective biosynthetic pathways may thus share a common phylogenetic origin, despite their very different roles in plant primary and secondary metabolism. A detailed comparison of the genes involved in both pathways in a gymnosperm system is likely to provide new insights into the evolution of conifer diterpene resin acid secondary metabolism relative to gibberellic acid phytohormone metabolism. At present, none of the diterpene synthases or CYP450s of gibberellic acid formation have been cloned and functionally characterized in a gymnosperm. However, likely candidate genes have recently been identified in our laboratory from the available spruce and loblolly pine EST and FLcDNA resources (Hamberger and Bohlmann, in press).

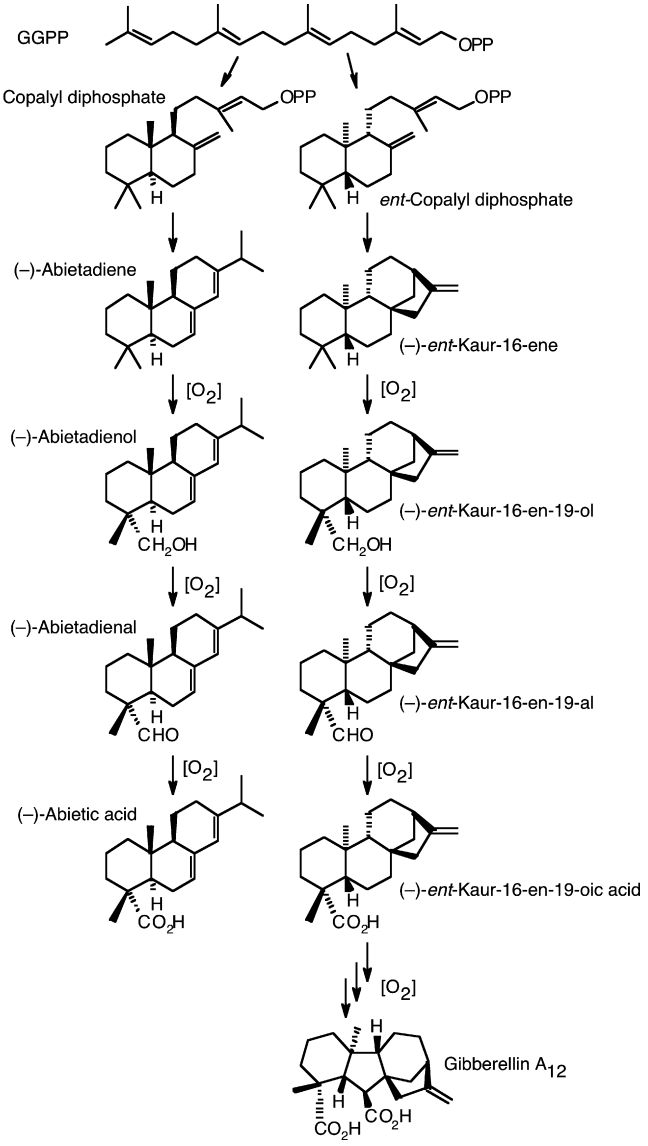

Fig. 6. Parallel biosyntheses of abietic acid and gibberellin from the common GGPP precursor.

Similar to the formation of diterpene precursors for resin acids by conifer diterpene synthases, diterpene synthases have been characterized in angiosperms for the cyclization of GGPP to *ent*-copalyl diphosphate ((−)-CPP) and for the subsequent cyclization of (−)-CPP to *ent*-kaurene, the precursor for gibberellic acid phytohormones (Yamaguchi, 2006). However, unlike the bifunctional diterpene synthases of conifer resin acid biosynthesis, the two active sites for the cyclization of (−)-CPP to *ent*-kaurene reside on two separate diterpene synthase proteins encoded by independent genes, an (−)-CPP synthase and an *ent*-kaurene synthase. Since diterpene synthases for (−)-CPP or *ent*-kaurene formation have not yet been cloned and characterized from a gymnosperm species, it is as of yet unknown if conifers employ a single bifunctional diterpene synthase in gibberellic acid formation as is the case with diterpene resin acid formation, or if they employ two monofunctional diterpene synthases as in the angiosperms.

Formation of the plant hormone gibberellic acid also involves a multifunctional CYP450 in the three-step oxidation of the diterpene *ent*-kaurene to *ent*-kaurenoic acid (Fig. 6; Yamaguchi, 2006). A CYP450 of gibberellic acid formation has not yet been reported for any gymnosperm species. Cloning of a conifer CYP450 involved in the formation of *ent*-kaurenoic acid *en route* to gibberellic acid phytohormones may provide insights into the evolution of CYP450s in diterpene resin acid biosynthesis relative to gibberellin phytohormones.

8. Outlook

Research in recent years has made significant progress in understanding how diterpene resin acids are biosynthesized, the genes involved in their biosynthesis, and the regulation of their biosynthesis. Additional research is still needed to better understand the many aspects of diterpene resin acids production in conifers, the events of cell specialization and transport associated with diterpene resin acid biosynthesis and accumulation, and their role in the chemical defences of conifers. In addition, as we identify new diterpene synthase and CYP450 genes in both primary and secondary metabolism from angiosperms and gymnosperms, a more thorough understanding of the evolution of the genes in conifer resin acid biosynthesis can be achieved.

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References

Asili, J., Lambert, M., Ziegler, H.L., Staerk, D., Sairafianpour, M., Witt, M., Asghari, G., Ibrahimi, I.S., Jaroszewski, J.W., 2004. Labdanes and isopimaranes from *Platycladus orientalis* and their effects on erythrocyte membrane and on *Plasmodium falciparum* growth in the erythrocyte host cells. J. Nat. Prod. 67, 631–637.

Björkman, C., Gref, R., 1993. Survival of pine sawflies in cocoon stage in relation to resin acid content of larval food. J. Chem. Ecol. 19, 2881–2890.

Björkman, C., Larsson, S., Bommarco, R., 1997. Oviposition preferences in pine sawflies: A trade-off between larval growth and defence against natural enemies. Oikos 79, 45–52.

Bohlmann, J., Meyer-Gauen, G., Croteau, R., 1998. Plant terpenoid synthases: Molecular biology and phylogenetic analysis. Proc. Natl. Acad. Sci. USA 95, 4126–4133.

Bohlmann, J., Croteau, R., 1999. Diversity and variability of terpenoid defences in conifers: molecular genetics, biochemistry and evolution of the terpene synthase gene family in grand fir (*Abies grandis*). Novartis Foundation Symposium 223, 132–145, discussion 146–139.

Burke, C., Croteau, R., 2002. Interaction with the small subunit of geranyl diphosphate synthase modifies the chain length specificity of geranylgeranyl diphosphate synthase to produce geranyl diphosphate. J. Biol. Chem. 277, 3141–3149.

Byun McKay, S.A., Hunter, W.L., Godard, K.A., Wang, S.X., Martin, D.M., Bohlmann, J., Plant, A.L., 2003. Insect attack and wounding induce traumatic resin duct development and gene expression of (−)-pinene synthase in Sitka spruce. Plant Physiol. 133, 368–378.

Byun-McKay, A., Godard, K.-A., Toudefallah, M., Martin, D., Alfaro, R., King, J., Bohlmann, J., Plant, A.L., 2006. Wound-induced terpene synthase gene expression in Sitka spruce that exhibit resistance or susceptibility to attack by the white pine weevil. Plant Physiol. 140, 1009–1021.

Christianson, D.W., 2006. Structural biology and chemistry of the terpenoid cyclases. Chem. Rev. 106, 3412–3442.

Davis, E.M., Croteau, R., 2000. Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. In: Leeper, F.J., Vederas, J.C. (Eds.), Biosynthesis: Aromatic Polyketides, Isoprenoids, Alkaloids, vol. 209. Springer-Verlag, Heidelberg, pp. 53–95.

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.

Eriksson, K., Wiklund, L., Larsson, C., 2004. Dermal exposure to terpenic resin acids in Swedish carpentry workshops and sawmills. Ann. Occup. Hyg. 48, 267–275.

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, V.R., 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.

Funk, C., Croteau, R., 1994. Diterpenoid resin acid biosynthesis in conifers: characterization of two cytochrome P450-dependent monooxygenases and an aldehyde dehydrogenase involved in abietic acid biosynthesis. Arch. Biochem. Biophys. 308, 258–266.

Funk, C., Lewinsohn, E., Vogel, B.S., Steele, C.L., Croteau, R., 1994. Regulation of oleoresinosis in grand fir (*Abies grandis*): coordinate induction of monoterpene and diterpene cyclases and two cytochrome P450-dependent diterpenoid hydroxylases by stem wounding. Plant Physiol. 106, 999–1005.

Gardner, D.R., Molyneux, R.J., James, L.F., Panter, K.E., Stegelmeier, B.L., 1994. Ponderosa pine needle-induced abortion in beef cattle: identification of isocupressic acid as the principal active compound. J. Agr. Food Chem. 42, 756–761.

Halitschke, R., Baldwin, I.T., 2005. Jasmonates and related compounds in plant-insect interactions. J. Plant Growth Reg. 23, 238–245.

Hamberger, B., Bohlmann, J., in press. Cytochrome P450 monooxygenases in conifer genomes: Discovery of members of the terpenoid oxygenase superfamily in spruce and pine. Biochem. Soc. Trans.

Howe, G.A., 2005. Jasmonates as signals in the wound response. J. Plant Growth Reg. 23, 223–237.

Huber, D.P.W., Ralph, S., Bohlmann, J., 2004. Genomic hardwiring and phenotypic plasticity of terpenoid-based defenses in conifers. J. Chem. Ecol. 30, 2399–2418.

Huber, D.P.W., Philippe, R.N., Madilao, L.L., Sturrock, R.N., Bohlmann, J., 2005. Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate. Tree Physiol. 25, 1075–1083.

Hudgins, J.W., Christiansen, E., Franceschi, V.R., 2003. Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the Pinaceae. Tree Physiol. 23, 361–371.

Hudgins, J.W., Christiansen, E., Franceschi, V.R., 2004. Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. Tree Physiol. 24, 251–264.

Hudgins, J.W., Franceschi, V.R., 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, J.W., Ralph, S.G., Franceschi, V.R., 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.

Iwamoto, M., Minami, T., Tokuda, H., Ohtsu, H., Tanaka, R., 2003. Potential antitumor promoting diterpenoids from the stem bark of *Thuja standishii*. Planta Med. 69, 69–72.

Kamaya, Y., Tokita, N., Suzuki, K., 2005. Effects of dehydroabietic acid and abietic acid on survival, reproduction, and growth of the crustacean *Daphnia magna*. Ecotoxicol. Environ. Saf. 61, 83–88.

Keeling, C.I., Bohlmann, J., 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol. 170, 657–675.

Kopper, B.J., Illman, B.L., Kersten, P.J., Klepzig, K.D., Raffa, K.F., 2005. Effects of diterpene acids on components of a conifer bark beetle-fungal interaction: tolerance by *Ips pini* and sensitivity by its associate *Ophiostoma ips*. Environ. Entomol. 34, 486–493.

LaFever, R.E., Vogel, B.S., Croteau, R., 1994. Diterpenoid resin acid biosynthesis in conifers: enzymatic cyclization of geranylgeranyl pyrophosphate to abietadiene, the precursor of abietic acid. Arch. Biochem. Biophys. 313, 139–149.

Langenheim, J.H., 2003. Plant resins: chemistry, evolution, ecology and ethnobotany. Timber Press, Portland, OR, U. S. A.

Larsson, S., Ekbom, B., Björkman, C., 2000. Influence of plant quality on pine sawfly population dynamics. Oikos 89, 440–450.

Lee, H.J., Ravn, M.M., Coates, R.M., 2001. Synthesis and characterization of abietadiene, levopimaradiene, palustradiene, and neoabietadiene: hydrocarbon precursors of the abietane diterpene resin acids. Tetrahedron 57, 6155–6167.

Lewinsohn, E., Savage, T.J., Gijzen, M., Croteau, R., 1993. Simultaneous analysis of monoterpenes and diterpenoids of conifer oleoresin. Phytochem. Anal. 4, 220–225.

Lichtenthaler, H.K., 1999. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 50, 47–65.

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, D., Bohlmann, J., 2005. Molecular biochemistry and genomics of terpenoid defenses in conifers. In: Romeo, J.T. (Ed.), Chemical ecology and phytochemistry of forest ecosystems, Recent Advances in Phytochemistry, vol. 39. Elsevier, Toronto, pp. 29–56.

Martin, D.M., 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, D.M., Fäldt, 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, B., Madilao, L.L., 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.

Minami, T., Wada, S., Tokuda, H., Tanabe, G., Muraoka, O., Tanaka, R., 2002. Potential antitumor-promoting diterpenes from the cones of *Pinus luchuensis*. J. Nat. Prod. 65, 1921–1923.

Nerg, A.-M., Heijari, J., Noldt, U., Viitanen, H., Vourinen, M., Kainulainen, P., Holopainen, J.K., 2004. Significance of wood terpenoids in the resistance of Scots pine provenances against the old house borer, *Hylotrupes bajulus*, and brown-rot fungus, *Coniophora puteana*. J. Chem. Ecol. 30, 125–141.

Peters, R.J., Flory, J.E., Jetter, R., Ravn, M.M., Lee, H.J., Coates, R.M., Croteau, R.B., 2000. Abietadiene synthase from grand fir (*Abies grandis*): characterization and mechanism of action of the ''pseudo-mature'' recombinant enzyme. Biochemistry 39, 15592–15602.

Peters, R.J., Ravn, M.M., Coates, R.M., Croteau, R.B., 2001. Bifunctional abietadiene synthase: free diffusive transfer of the (+)-copalyl diphosphate intermediate between two distinct active sites. J. Am. Chem. Soc. 123, 8974–8978.

Peters, R.J., Croteau, R.B., 2002a. Abietadiene synthase catalysis: conserved residues involved in protonation-initiated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate. Biochemistry 41, 1836–1842.

Peters, R.J., Croteau, R.B., 2002b. Abietadiene synthase catalysis: mutational analysis of a prenyl diphosphate ionization-initiated cyclization and rearrangement. Proc. Natl. Acad. Sci. USA 99, 580–584.

Peters, R.J., Carter, O.A., Zhang, Y., Matthews, B.W., Croteau, R.B., 2003. Bifunctional abietadiene synthase: mutual structural dependence of the active sites for protonation-initiated and ionization-initiated cyclizations. Biochemistry 42, 2700–2707.

Phillips, M.A., Croteau, R., 1999. Resin-based defenses in conifers. TRENDS Plant Sci. 4, 184–190.

Powell, J.S., Raffa, K.F., 1999. Effects of selected *Larix laricina* terpenoids on *Lymantria dispar* (Lepidoptera: Lymantriidae) development and behavior. Environ. Entomol. 28, 148–154.

Powell, J.S., Raffa, K.F., 2003. Fate of conifer terpenes in a polyphagous folivore: evidence for metabolism by gypsy moth (Lepidoptera: Lymantriidae). J. Entomol. Sci. 38, 583–601.

Ralph, S.G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J.A., Nelson, C.C., Butterfield, Y.S.N., Kirkpatrick, R., Liu, J., Jones, S.J.M., Marra, M.A., Douglas, C.J., Ritland, K., Bohlmann, J., 2006. Conifer defense 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.

Ravn, M.M., Coates, R.M., Flory, J.E., Peters, R.J., Croteau, R., 2000. Stereochemistry of the cyclization-rearrangement of (+)-copalyl diphosphate to (−)-abietadiene catalyzed by recombinant abietadiene synthase from *Abies grandis*. Org. Lett. 2, 573–576.

Ravn, M.M., Peters, R.J., Coates, R.M., Croteau, R., 2002. Mechanism of abietadiene synthase catalysis: stereochemistry and stabilization of the cryptic pimarenyl carbocation intermediates. J. Am. Chem. Soc. 124, 6998–7006.

Ro, D.-K., 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/abietadienal oxidase (PtAO, CYP720B1). Phytochemistry 67, 1572–1578.

Ro, D.-K., Arimura, G., Lau, S.Y., Piers, E., Bohlmann, J., 2005. Loblolly pine abietadienol/abietadienal oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase. Proc. Natl. Acad. Sci. USA 102, 8060–8065.

Saikkonen, K., Neuvonen, S., Kainulainen, P., 1995. Oviposition and larval performance of European pine sawfly in relation to irrigation, simulated acid rain and resin acid concentration in Scots pine. Oikos 74, 273–282.

Schepmann, H.G., Pang, J., Matsuda, S.P., 2001. Cloning and characterization of *Ginkgo biloba* levopimaradiene synthase which catalyzes the first committed step in ginkgolide biosynthesis. Arch. Biochem. Biophys. 392, 263–269.

Schuler, M.A., Werck-Reichhart, D., 2003. Functional genomics of P450s. Ann. Rev. Plant Biol. 54, 629–667.

Smith, E., Williamson, E., Zloh, M., Gibbons, S., 2005. Isopimaric acid from *Pinus nigra* shows activity against multidrug-resistant and EMRSA strains of *Staphylococcus aureus*. Phytother. Res. 19, 538–542.

Smith, P.A., Gardner, D.R., Drown, D.B., Jederberg, W.W., Still, K., 1998. Oxidized resin acids in aerosol derived from rosin core solder. Am. Ind. Hyg. Assoc. J. 59, 889–894.

Starks, C.M., Back, K.W., Chappell, J., Noel, J.P., 1997. Structural basis for cyclic terpene biosynthesis by tobacco 5-*epi*-aristolochene synthase. Science 277, 1815–1820.

Stofer Vogel, B., Wildung, M.R., Vogel, G., Croteau, R., 1996. Abietadiene synthase from grand fir (*Abies grandis*). cDNA isolation, characterization, and bacterial expression of a bifunctional diterpene cyclase involved in resin acid biosynthesis. J. Biol. Chem. 271, 23262–23268.

Tomlin, E.S., Borden, J.H., Pierce Jr., H.D., 1996. Relationship between cortical resin acids and resistance of Sitka spruce to white pine weevil. Can. J. Bot. 74, 599–606.

Tomlin, E.S., Alfaro, R.I., Borden, J.H., He, F., 1998. Histological response of resistant and susceptible white spruce to simulated white pine weevil damage. Tree Physiol. 18, 21–28.

Tomlin, E.S., Antonejevic, E., Alfaro, R.I., Borden, J.H., 2000. Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. Tree Physiol. 20, 1087–1095.

Trapp, S., Croteau, R., 2001a. Defensive resin biosynthesis in conifers. Ann. Rev. Plant Physiol. Plant Mol. Biol. 52, 689–724.

Trapp, S.C., Croteau, R.B., 2001b. Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 158, 811–832.

Yamaguchi, S., 2006. Gibberellin biosynthesis in Arabidopsis. Phytochem. Rev. 5, 39–47.

Zeneli, G., Krokene, P., Christiansen, E., Krekling, T., Gershenzon, J., 2006. Methyl jasmonate treatment of mature 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.