

Terpenoid Biosynthesis and Specialized Vascular Cells of Conifer Defense

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Abstract



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Defense-related terpenoid biosynthesis in conifers is a dynamic process closely associated with specialized anatomical structures that allows conifers to cope with attack from many potential pests and pathogens. The constitutive and inducible terpenoid defense of conifers involves several hundred different monoterpenes, sesquiterpenes and diterpenes. Changing arrays of these many compounds are formed from the general isoprenoid pathway by activities of large gene families for two classes of enzymes, the terpene synthases and the cytochrome P450-dependent monooxygenases of the CYP720B group. Extensive studies have been conducted on the genomics, proteomics and molecular biochemical characterization of these enzymes. Many of the conifer terpene synthases are multi-product enzymes, and the P450 enzymes of the CYP720B group are promiscuous in catalyzing multiple oxidations, along homologous series of diterpenoids, from a broad spectrum

of substrates. The terpene synthases and CYP720B genes respond to authentic or simulated insect attack with increased transcript levels, protein abundance and enzyme activity. The constitutive and induced oleoresin terpenoids for conifer defense accumulate in preformed cortical resin ducts and in xylem trauma-associated resin ducts. Formation of these resin ducts *de novo* in the cambium zone and developing xylem, following insect attack or treatment of trees with methyl jasmonate, is a unique feature of the induced defense of long-lived conifer trees.

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Introduction

The conifers (*Coniferales*) include some of the tallest and longest living organisms on earth. Throughout their uncommonly long lifetime, individual conifer trees of the *Pinaceae* family and entire landscapes of conifer forests are exposed to a large number of potentially faster evolving pests and pathogens. Some of the most devastating insect pests of conifers include bark beetles (*Coleopterae*),

which are commonly associated with a symbiotic community of pathogenic and non-pathogenic fungi, and weevils (*Curculionidae*).

Conifers have evolved an array of diverse, mechanical and chemical defenses to cope with herbivore and pathogen attack. One of the main lines of chemical and physical defense is the production of oleoresin, a complex mixture of volatile mono- (C_{10}) and sesquiterpenes (C_{15}), as well as nonvolatile diterpene resin acids (C_{20}) (Figure 1). The composition of

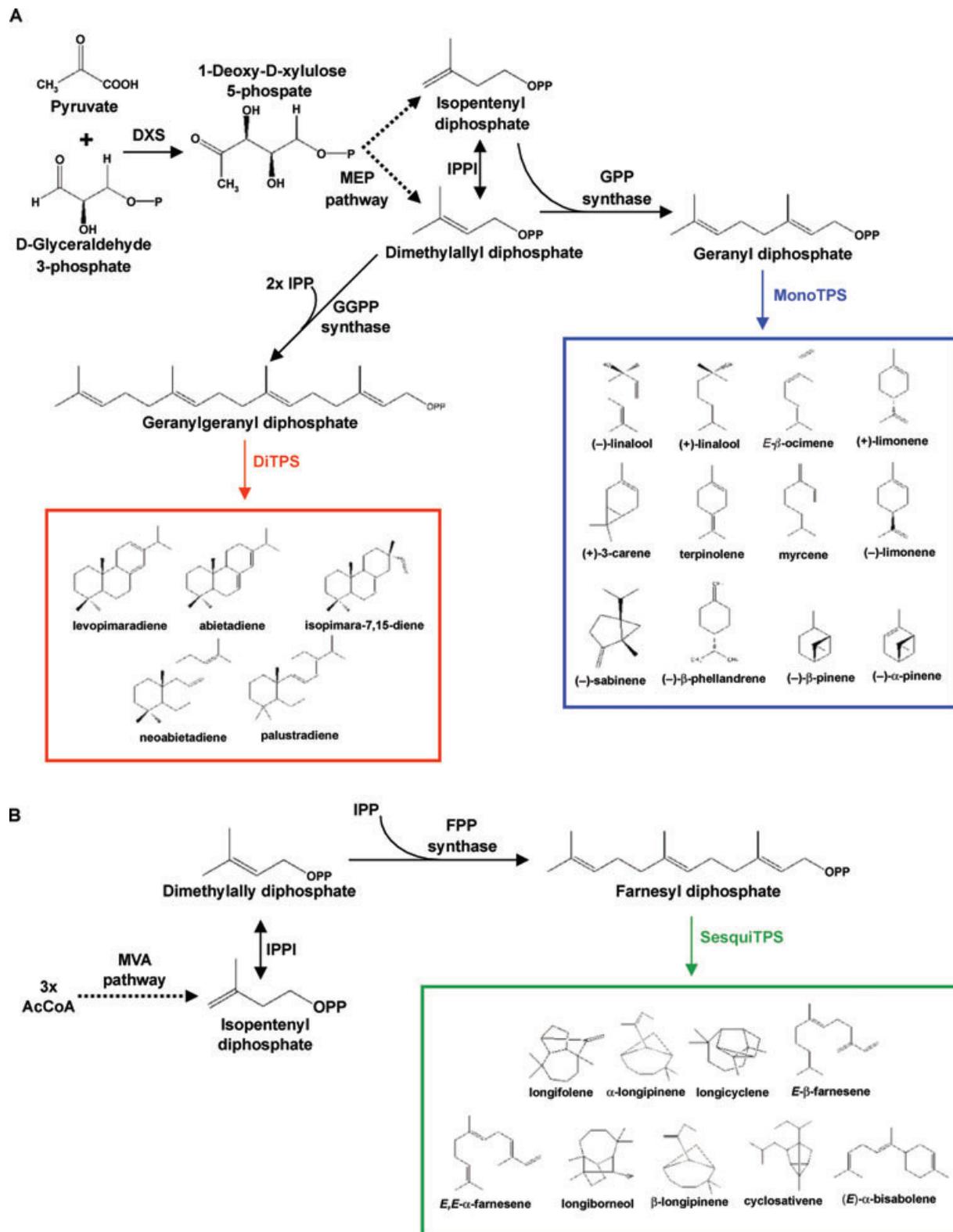


Figure 1. Schematic of terpenoid biosynthesis in conifers.

Representative monoterpenes formed by monoTPS (top right box), sesquiterpenes formed by sesquiTPS (bottom box), and representative diterpenes formed by diTPS (left middle box). TPS, terpene synthases.

oleoresin includes a multitude of structurally different monoterpenes and diterpenoid resin acids in approximately equal proportions, along with a very diverse set of sesquiterpenes as a minor component (Martin et al. 2002; Miller et al. 2005; Zulak et al. 2009).

This review will focus on the dynamics of terpenoid metabolic pathways, and the chemical diversity generated from these pathways, as well as the specialized cell types involved in terpenoid biosynthesis in conifers. Some work in other systems will be referenced for a better understanding of terpenoid biosynthesis, in general. In addition to their role in conifer defense, oleoresin terpenoids are also important as a natural source for industrial chemicals, that is, biomaterials, and can be explored as precursors for biofuels. The aspect of terpenoids as biomaterials and biofuels has recently been reviewed (Bohlmann and Keeling 2008).

Oleoresin Composition Responds to Imposed Environmental Stresses

The chemical composition of oleoresin is dynamic and can change with the type of environmental stress to which the tree is exposed. The biosynthesis of terpenoids in conifer defense is based on the formation of isoprenoid (C_5) units and their elongation to prenyl diphosphates (C_{10} , C_{15} and C_{20}), which are the substrates for the terpene synthases (TPS) (Keeling and Bohlmann 2006b). The formation of diterpenoid resin acids involves the further oxidation of products of diterpene synthase activity by cytochrome P450-dependent monooxygenases (P450s) (Keeling and Bohlmann 2006b). Many of the TPS and P450 enzymes and their transcripts and products are upregulated or increase in abundance in response to insect attack or treatment of trees with the defense hormone, methyl jasmonate (MeJA).

In conifers, oleoresin is present in specialized anatomical structures, such as resin ducts or resin blisters that act as pressurized storage reservoirs for the oleoresin. When a tree is attacked by insect feeding or egg deposition, resin 'pitches out' and entombs the insect. The volatile components of oleoresin can also act as airborne signaling molecules in bark beetle host recognition. The oleoresin terpenoids can act as a deterrent or be directly toxic to insects and pathogens.

Among the conifers, species of the genus *Picea* (spruce) are the best studied in terms of the molecular and biochemical processes and the genomic basis of constitutive and induced terpenoid defenses. Species of spruce also possess a well characterized reticulate system of resin ducts that are both constitutively present in the cortex (Figure 2A) and can be produced *de novo* in the cambium zone and developing xylem (Figure 2B) upon insect attack or treatment with MeJA. Several other anatomical features play an important role in conifer

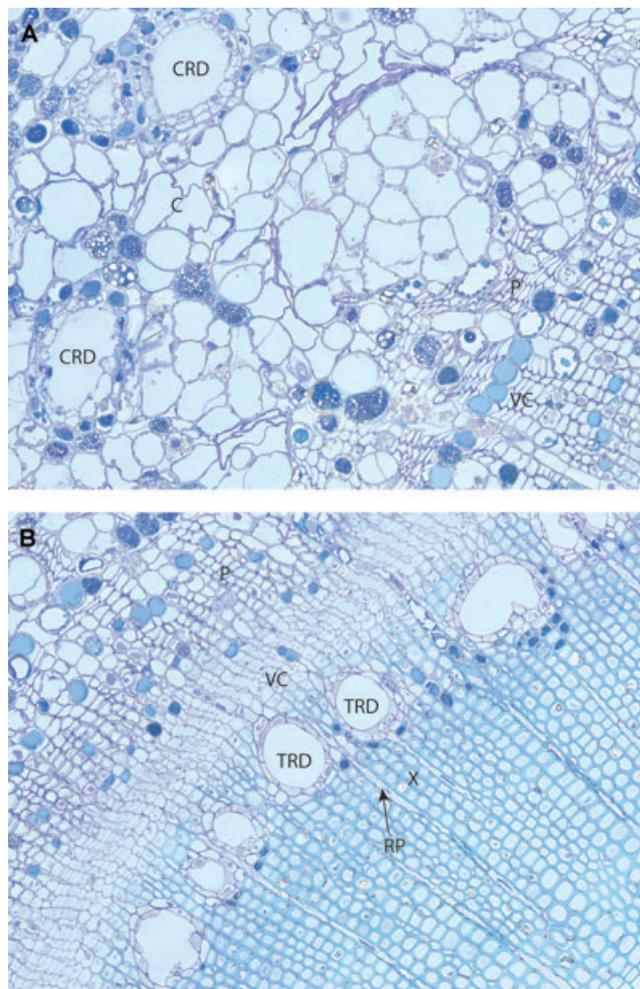


Figure 2. Constitutive resin ducts (A) in untreated Sitka spruce and traumatic resin ducts (B) 24 d after treatment of Sitka spruce with 0.1% methyl jasmonate (MeJA). C, cortex; CRD, constitutive resin duct; P, secondary phloem; RP, ray parenchyma; TRD, traumatic resin duct; VC, vascular cambium; X, secondary xylem.

defense, such as thick periderm, cortex and secondary phloem, which contain a number of specialized cell types such as phenolic parenchyma cells and stone cells (Franceschi et al. 2005).

Formation of Dimethylallyl Diphosphate and Isopentenyl Diphosphate

Dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) are the precursors to all isoprenoid compounds and are biosynthesized via two separate pathways in plants (Figure 1). It was formerly thought that IPP was only formed

from acetyl CoA via the mevalonate (MVA) pathway and isomerized to DMAPP by isopentenyl diphosphate isomerase (IPPI) (Chappell 1995; McGarvey and Croteau 1995). It is now well established that a mevalonate-independent pathway, the methyl-erythritol 4-phosphate (MEP) pathway, exists in bacteria and plants: However, only the MVA pathway is present in fungi and animals (Rodríguez-Concepción 2006).

In plants, the MEP and MVA pathways are responsible for the biosynthesis of different classes of compounds. The MEP pathway biosynthesizes IPP and DMAPP primarily for the formation of carotenoids, abscisic acid, gibberellins, monoterpenes, diterpenes, isoprene and the side chains of photosynthesis-related compounds, such as chlorophyll, tocopherols, phyloquinones and plastoquinone. The MEV pathway produces IPP for the formation of sterols, brassinosteroids, some sesquiterpenes, triterpenes, polyterpenes, polyprenol, dichol and prenyl moieties used in posttranslational modification of proteins. Ubiquinone is also synthesized by MVA-derived IPP, which is transported to the mitochondria (Disch et al. 1998). It is thought that the MEP pathway is prevalent in the biosynthesis of mono- and diterpenoids (Lange and Ghassemian 2003) which are the major components of spruce oleoresin. Therefore, only the MEP pathway will be discussed further.

The first step in the MEP pathway is catalyzed by 1-deoxyxylulose 5-phosphate (DXP) synthase (DXS) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998; Rodríguez-Concepción and Boronat 2002) and can play a rate-limiting role for the production of MEP-derived isoprenoids (Lois et al. 2000; Estevez et al. 2001; Walter et al. 2002). Based on current studies, it appears there are two types of DXS enzymes. Type I is constitutively expressed in photosynthetic tissue and is likely involved in the biosynthesis of isoprenoids of primary or general metabolism, such as carotenoids and phytol, whereas type II DXS enzymes seem to be involved in the biosynthesis of isoprenoids for specialized (i.e., secondary) metabolism (Walter et al. 2002).

In conifers, the genes involved in several steps in the MEP pathway have been cloned and characterized, including DXS, 1-deoxy-xylulose 5-phosphate reductoisomerase (DXR), and hydroxymethylbutenyl 4-phosphate reductase (HDR) (Figure 1). Three DXS isoforms have been identified in Norway spruce (*P. abies*) and are differentially expressed in the outer stem tissue of young saplings in response to various stress treatments (Phillips et al. 2007). No additional DXS transcripts have been identified in the available expressed sequence tag (EST) and full length cDNA collections for spruce species (>500 000 sequences), suggesting there are no additional actively transcribed DXS isoforms in spruce (Ralph et al. 2008). Recently, genes corresponding to two isoforms of DXS (*PdDXS1*, *PdDXS2*), DXR (*PdDXR*) and two isoforms of HDR (*PdHDR1*, *PdHDR2*) have been cloned from *Pinus densiflora* (Kim et al. 2009).

Prenyltransferases

The five carbon building blocks of terpenoid biosynthesis, IPP and DMAPP, undergo sequential condensation reactions to form geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅) and geranylgeranyl diphosphate (GGPP, C₂₀); the precursors to monoterpenes, sesquiterpenes and diterpenes of conifer oleoresin, respectively (Figure 1). The enzymes responsible for catalyzing these condensation reactions are a class of prenyltransferases called isoprenyl diphosphate synthases. GPP, FPP and GGPP are synthesized by specific GPP synthases (GPPS), FPP synthases (FPPS) and GGPP synthases (GGPPS) (Gershenzon and Kreis 1999).

Three types of GPPS have been identified based on sequence comparisons; one contains heterodimeric proteins and two contain homodimeric proteins. Heterodimeric GPPS have been cloned from *Mentha x piperita* (Burke et al. 2004), *Annirrhinum majus* and *Clarkia brewerii* (Tholl et al. 2004). The large subunit of these GPPS has similarity to other plant GPPS and has GPPS activity, but the small subunit has much lower similarity and has low GPPS activity. Of the two homodimeric GPPS classes, only one is found in conifers and is highly similar to known conifer GGPPS sequences from grand fir (*Abies grandis*) and Norway spruce (Hefner et al. 1998; Burke and Croteau 2002a; Burke et al. 2004; Schmidt and Gershenzon 2007). The other class contains only one sequence from *Arabidopsis thaliana* with much lower identity to other isopentenyl diphosphate synthase proteins (Bouvier et al. 2000).

Two different GPPS were cloned and characterized from Norway spruce; both were found to be of a different homodimeric type (Schmidt and Gershenzon 2008). Transcript levels for these genes were measured in control and MeJA treated trees to investigate the potential roles of these GPPS genes in induced oleoresin biosynthesis. One of the Norway spruce GPPS genes (*PaIDS1*) had a high similarity to other known conifer GPPS sequences (Burke and Croteau 2002a). It made GPP as its sole product and the corresponding transcript levels strongly increased in response to MeJA treatment. This suggests that the GPP produced by *PaIDS1* functions as a substrate for induced monoterpene biosynthesis in Norway spruce. However, the second enzyme (*PaIDS2*) was similar to the *A. thaliana* GPPS, and produced substantial amounts of FPP, GGPP as well as GPP, and was not induced by MeJA treatment, suggesting that it is not involved in induced monoterpene production in Norway spruce (Schmidt and Gershenzon 2008).

Several FPPS and GGPPS have been cloned and characterized from conifers, including an FPPS from grand fir (Tholl et al. 2001), GGPPSs from grand fir and *Taxus canadensis* (Hefner et al. 1998; Burke and Croteau 2002b), and one FPPS (*PaIDS4*) and two GGPPS from Norway spruce (Schmidt and

Gershenzon 2007). Phylogenetically, *PaIDS4* clusters separately from the conifer GPPS and GGPPS sequences and with FPPS from other angiosperm species. *PaIDS4* lacks a plastid targeting sequence, thus is likely localized to the cytosol, similar to other known FPPS proteins. The two GGPPS clones from Norway spruce (*PaIDS5* and *PaIDS6*) group differently phylogenetically, with *PaIDS5* grouping with other gymnosperm GGPPS sequences and *PaIDS6* grouping with angiosperm GGPPS genes.

Terpenoid Synthases

The TPS generate much of the enormous chemical diversity found in conifer oleoresin by using GPP, FPP and GGPP in a metal ion cofactor-dependent electrophilic reaction mechanism (Bohlmann et al. 1998b; Davis and Croteau 2000; Keeling and Bohlmann 2006b). The prenyldiphosphate substrates are ionized by cleavage of the diphosphate group, or by protonation, to produce enzyme-bound reactive carbocation intermediates, which are then rearranged within the spatial constraints of the enzyme active site and eventually quenched to form the various cyclic and acyclic terpenoid compounds (Starks et al. 1997; Cane 1999; Wise and Croteau 1999; Christianson 2006). Often, terpenoids exist as pairs of enantiomers. However, TPS typically exert tight stereospecific control over the product profile, with one enantiomer being the dominant product (Davis and Croteau 2000; Phillips et al. 2003). Some TPS enzymes produce a single product, but many are multiple product enzymes (Steele et al. 1998; Fäldt et al. 2003; Martin et al. 2004).

Terpenoid synthase enzymes are grouped into classes based on taxonomy and substrate specificity (Bohlmann et al. 1998a; Martin et al. 2004). Mono-, sesqui- and diTPS are responsible for the conversion of the transoid GPP, FPP and GGPP into a wide array of mono- (C_{10}), sesqui- (C_{15}) and diterpene (C_{20}) compounds, respectively. Recently, it was shown that neryl diphosphate (NPP), the *cis*-isomer of GPP, is also a substrate for monoTPS in tomato glandular trichomes (Schillmiller et al. 2009). Also, a novel pathway for the production of sesquiterpenes has been identified in *Solanum habrochaites*, which uses cisoid *Z,Z*-farnesyl pyrophosphate (Sallaud et al. 2009). Cisoid TPS substrates (Bohlmann and Gershenzon 2009) have not yet been identified in a conifer system.

The biochemical function of TPS cannot be predicted based on sequence similarity alone as changes in only a few amino acids can result in completely different product profiles (Bohlmann et al. 1999; Dudareva et al. 2003; Keeling et al. 2008). Many TPS genes have been identified, cloned and characterized from Norway spruce, Sitka spruce (*P. sitchensis*) and white spruce (*P. glauca*) (Byun McKay et al. 2003; Fäldt et al. 2003; Martin et al. 2004; Byun McKay et al. 2006; Keeling

et al. 2008; Ralph et al. 2006, 2008). Several TPS genes have also been cloned from other conifer species, including grand fir (Bohlmann et al. 1997, 1998a, 1999; Steele et al. 1998), loblolly pine (*Pinus taeda*) (Phillips et al. 2003; Ro et al. 2005) and Douglas fir (*Pseudotsuga menziesii*) (Huber et al. 2005).

Cytochrome P450-Dependent Monooxygenases

The mono- and sesquiterpenes that accumulate in conifer oleoresin are not known to be biochemically modified. However, diterpene compounds formed by conifer diTPS are further oxidized, by multisubstrate, multifunctional cytochrome P450 enzymes, to form diterpenoid resin acids that accumulate in oleoresin (Figure 3) (Keeling and Bohlmann 2006a). To date, only one P450 gene, *CYP720B1* from loblolly pine, involved in diterpenoid resin acid biosynthesis in conifers, has been functionally characterized and described in the literature (Ro et al. 2005; Ro and Bohlmann 2006). The abietadienol/abietadienal oxidase (PtAO or *CYP720B1*) is a member of the newly characterized and apparently conifer-specific *CYP720B* gene family and catalyzes at least two of the three consecutive oxidation steps in diterpene resin acid formation, using various diterpenes, alcohols and aldehydes as substrates. Thus, a relatively small number of diTPS (Martin et al. 2004) and P450 enzymes can account for much of the chemical diversity and plasticity of conifer oleoresin. This feature of *CYP720B1* seems unusual, since most P450s involved in secondary metabolism are highly substrate- and reaction-specific (Schuler and Werck-Reichhart 2003).

Recently, our lab identified additional members of the *CYP720B* gene family in the transcriptomes of loblolly pine and spruce species (Hamberger and Bohlmann 2006). This indicates that *CYP720B* genes of oleoresin biosynthesis exist in conifers with large gene families similar to the *TPS* gene family. A genomic clone of a white spruce (*P. glauca*) *CYP720B4* gene has also been cloned (Hamberger et al. 2009). The corresponding full length cDNA was functionally characterized as an enzyme that can catalyze the oxidation of 24 different diterpenoids in a matrix of homologous series of diterpene olefins, alcohols, aldehydes and resin acids (Hamberger, Ohnishi and Bohlmann, unpubl. data, 2009).

The oxidation of oleoresin diterpenoids resembles closely the formation of *ent*-kaurenoic acid in gibberellin biosynthesis (Ro et al. 2005; Hamberger and Bohlmann 2006), suggesting that the specialized metabolism of conifer diterpene resin acid biosynthesis and the general metabolism of gibberellins share a common origin. Consistent with this notion is the finding that the diTPS of diterpene resin acid formation of conifer oleoresin have many features in common with the diTPS of

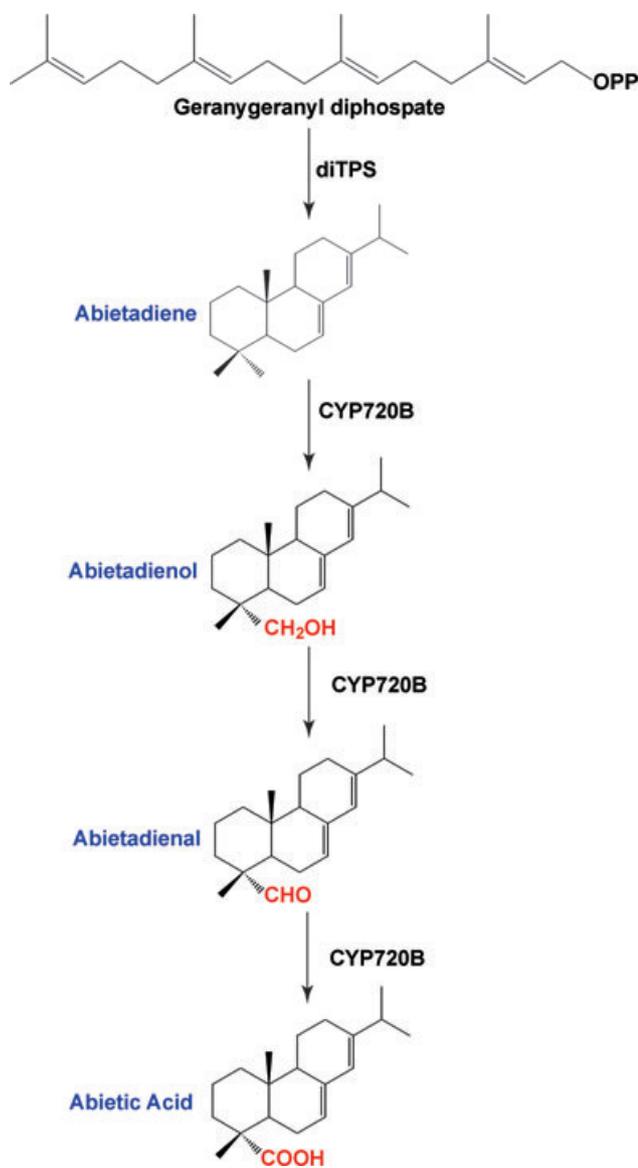


Figure 3. Biosynthesis of diterpene resin acids.

Two classes of enzymes, the diTPSs and P450s of the CYP720B group, generate the diversity of diterpene resin acids. TPS, terpene synthases.

conifer gibberellin formation (Keeling and Bohlmann, unpubl. data, 2009).

Insects Induce a Dynamic Terpenoid Defense Response in Conifers

Much of the research on conifers that has investigated the molecular mechanisms of terpenoid defense in response to

insects has been done using the white pine weevil (*Pissodes strobi*). This weevil is one of the most destructive insect pests of native Sitka and white spruce in Western North America and also destroys plantations of Norway spruce in Eastern North America (Alfaro et al. 2002). Insect attack was found to upregulate monoTPS genes and specifically the gene corresponding to (–)-pinene synthase in Sitka spruce (Byun McKay et al. 2003). The first comprehensive study of the molecular biological response of spruce to insect attack found a massive upregulation of TPS genes and subsequent accumulation of mono-, di-, and sesquiterpenes compounds in response to weevil feeding in both inner (xylem) and outer (bark) tissue in Sitka spruce (Miller et al. 2005).

Compared with MeJA treatment, weevils induced a much stronger increase in TPS transcripts and terpenoid accumulation, which may be due to increased exposure of the tree to the weevil as opposed to a single MeJA treatment, or an additional effect due to the mode of feeding, insect-derived elicitors or fungal symbionts. Also, weevil feeding induced substantial accumulation of longifolene synthase, a sesquiTPS, and accumulation of sesquiterpenes, whereas MeJA treatment did not (Miller et al. 2005), suggesting a special role of sesquiterpenes in the insect-defense response.

A large scale microarray study investigating transcriptome changes in Sitka spruce in response to mechanical wounding, feeding of spruce budworms (*Choristoneura occidentalis*) or white pine weevil, revealed global changes in host gene expression as early as 1–2 d after treatment (Ralph et al. 2006). Major changes in gene expression were induced in a similar manner in response to weevil attack of stems, feeding of foliage by spruce budworm, and wounding treatments. Changes included genes with a general role in plant defense, transport, transcriptional regulation, octadecanoid and ethylene signaling, terpenoid biosynthesis and phenolic secondary metabolism genes (Ralph et al. 2006).

A proteomics study, using 2DE sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis to investigate the time-dependent changes in the Sitka spruce proteome in response to white pine weevil feeding and mechanical wounding, showed significant adaptations occurred as early as 2 h following the onset of insect feeding (Lippert et al. 2007). The major classes of induced proteins included heat shock proteins, stress-response proteins, enzymes involved in secondary metabolism, and oxidoreductases. Several proteins also exhibited characteristics associated with posttranslational modification, suggesting that this type of regulation may play an important role in insect defense in spruce. No TPS proteins were identified in this study, likely due to their low abundance relative to other more ubiquitous proteins within the sample (Lippert et al. 2007).

MeJA Treatment as a Tool to Study Terpenoid Defense Responses in Conifers

Methyl jasmonate is the volatile methylester of the defense signal molecule, jasmonic acid (JA). When exogenously applied on conifers, MeJA is a noninvasive treatment that has been shown to be a good mimic of insect feeding (Martin et al. 2002, 2003). However, terpenoid compounds accumulate to a lesser degree in MeJA treated tissue than in those exposed to weevil feeding (Miller et al. 2005). The fact that MeJA does not inflict physical damage to terpenoid accumulating tissues is important for its use to study resin accumulation in, and volatile emissions from, intact tissue and for studies of the cell types involved in oleoresin biosynthesis. In contrast to MeJA treatment, insect feeding or mechanical wounding cause the release of oleoresin stored in resin ducts and passive emission of volatile mono- and sesquiterpenes, and can also destroy the cells specialized for terpenoid biosynthesis.

In Sitka spruce needle and bark tissue, it has been shown that exposure of trees to weevil attack, or treatment with MeJA, induces transcripts of the octadecanoid or JA pathway (Miller et al. 2005). Treatment of conifer trees with MeJA has enabled biochemical characterization of the defense response, which has led to new insights into the regulation of terpenoid biosynthesis. Until recently, much of the work on the effect of jasmonates on conifers was restricted to cell cultures (Yukimune et al. 1996; Bohlmann et al. 1998a; Ketchum et al. 1999; Lapointe et al. 2001) and only a few conifer tree species (Kaukinen et al. 1996; Regvar et al. 1997; Kozłowski et al. 1999). Work of the last decade is shedding light on the biochemistry and molecular biology of MeJA-inducible terpenoid biosynthesis in differentiated conifer trees.

Enzymes of the MEP pathway are induced upon treatment of spruce trees with MeJA. In Norway spruce, transcript levels of type II DXS enzymes (PaDXS2a and PaDXS2b) increased in abundance relative to the untreated trees; however, transcripts encoding type I DXS enzymes (PaDXS1) do not change (Phillips et al. 2007). Two subsequent steps in the MEP pathway, DXR and HDR, are also upregulated in response to mechanical wounding and fungal treatment (Phillips et al. 2007). Expression levels of genes corresponding to the two isoforms of DXS (PdDXS1, PdDXS2), DXR (PdDXR) and two isoforms of HDR (PdHDR1, PdHDR2) from *P. densiflora* were measured in different tissues and in response to mechanical wounding or MeJA treatment (Kim et al. 2009). Transcripts for all genes were most abundant in wood, but only PdDXS2 and PdHDR2 were upregulated in response to mechanical wounding or MeJA treatment. Of the two isopentenyl diphosphate synthases cloned from Norway spruce, *PaIDS5* transcripts responded strongly to MeJA treatment, whereas *PaIDS6* transcripts did

not. This suggests that *PaIDS5* plays a role in conifer oleoresin biosynthesis, while *PaIDS6* may have a function in general metabolism (Schmidt and Gershenzon 2007).

The dynamics of chemical diversity of defense-related oleoresin production is regulated, in large part, at the level of the TPS enzymes. Proteins, enzyme activity, corresponding transcripts as well as terpenoid products are all strongly induced upon treatment with MeJA in several spruce species. The effect of MeJA on the biochemistry of terpenoid biosynthesis was firmly established in Norway spruce (Martin et al. 2002). Enzyme activity of mono- and diTPS as well as subsequent accumulation of monoterpenes and diterpenoid resin acids was measured in both wood (xylem) and bark tissue (phloem) in response to MeJA treatment. Both TPS activity and metabolite accumulation were induced in wood to a greater extent than in bark, although metabolite levels were generally higher in bark tissue.

The strong induction of TPS and terpenoid accumulation is associated with the MeJA-induced *de novo* formation of traumatic resin ducts (TRD) in the cambium zone and developing xylem of Norway spruce stems (Martin et al. 2002) (Figure 2). Also, activities of GPPS, FPPS and GGPPS were measured and it was found that only GGPPS was induced upon MeJA treatment, indicating that IPP synthases are a possible regulatory control point for diterpenoid resin acid biosynthesis, but perhaps not for mono- and sesquiterpenes (Martin et al. 2002).

Methyl jasmonate induced similar terpenoid responses in both Sitka and Norway spruce (Miller et al. 2005). In young Sitka spruce trees, transcript abundance of TPS was compared between trees treated with MeJA and those exposed to weevil feeding. Along with increases in terpenoid compounds, transcripts corresponding to various mono-, sesqui- and diTPS were also found to increase in abundance in stem tissue response to MeJA over time (Miller et al. 2005). Generally, MeJA was a good mimic of insect feeding on the levels of TPS transcripts, enzyme activity and terpenoid metabolite accumulation, with the exception of sesquiTPS transcripts, which accumulated to a greater degree in weevil-fed tissue than MeJA-treated tissue.

Insect- and MeJA- Induced Emissions of Terpenoid Volatiles

Most of the work characterizing the MeJA-induced conifer defense response has been done with stem tissues, but a few studies also investigated the biochemistry of induced terpenoid biosynthesis and volatile emissions in needles (Martin et al. 2003). Needles release volatile terpenes into the atmosphere, which may act as potential herbivore deterrents, predator attractants or plant-to-plant signals. MeJA treatment elicited

a much less pronounced response (TPS enzyme activity and terpenoid accumulation) in needle tissue than in bark and did not result in as large a change in terpene composition as in stems (Miller et al. 2005).

The composition of terpene compounds released as volatiles changed dramatically upon MeJA treatment. The monoterpene (–)-linalool as well as the sesquiterpenes (*E*)- β -farnescene, (*E,E*)- α -farnescene and (*E*)- α -bisabolene were released in trace amounts without MeJA treatment, but increased dramatically upon treatment. In needles, transcripts corresponding to (–)-linalool synthase were induced upon MeJA treatment with a concomitant increase in volatile emissions and enzyme activities (Martin et al. 2003; Miller et al. 2005). Increases in terpene emissions match increases in TPS transcript abundance and enzyme activity. In addition, the induced terpenes are not found in constitutive oleoresin of needles, suggesting that the emitted volatile compounds are formed *de novo* and are not released from storage reservoirs of resin ducts. Terpene emissions also followed a diurnal cycle, with greater volatilization during the day compared with the night, regardless of treatment.

Proteomic and Transcriptional Analysis of TPS for the Induced Terpenoid Response

Terpene synthase proteins are often highly similar at the amino acid level, yet may have distinct biochemical functions (e.g. Martin et al. 2004; Keeling et al. 2008) and are often of low abundance. Consequently, measuring changes in protein abundance in response to insect attack or MeJA treatment has been difficult using traditional immunological methods. Recently, a targeted proteomics technique called selected reaction monitoring (SRM) was used to analyze changes in protein abundance of five TPS enzymes and three DXS isoforms in Norway spruce bark tissue treated with MeJA (Zulak et al. 2009). In addition, transcript abundance, enzyme activity and terpenoid metabolite accumulation were measured in a target-specific fashion, resulting in a multi-level transcriptomics, proteomics and metabolomics characterization of the terpenoid defense response of Norway spruce bark tissue.

All individual TPS transcripts and proteins measured were differentially induced upon MeJA treatment, and these patterns generally agreed with the other levels of biological data. Mono- and diterpenoid metabolites accumulated with similar patterns, indicating a common multi-product defense response. (+)-3-Carene synthase and (+)-3-carene were induced to a higher degree in all levels of data measured, highlighting its important role in conifer defense.

Cell Specialization of Terpenoid Biosynthesis in Conifer Defense

Conifers have specialized anatomical structures for the accumulation and storage of terpenoid-rich oleoresin such as resin blisters, which are sac-like structures surrounded by lignified short-lived epithelial cells; and resin ducts, which are tube-like structures surrounded by thin-walled long-lived epithelial cells that are thought to secrete terpenoids into the extracellular space of the duct (Figure 2). Resin blisters are found in the stems of fir (*Abies* spp.), Cedar (*Cedrus* spp.), hemlock (*Tsuga* spp.), and golden larch (*Pseudolarix* spp.), whereas resin ducts are found in the wood and bark of spruce (*Picea* spp.), pine (*Pinus* spp.), larch (*Larix* spp.), and Douglas-fir (*Pseudotsuga menziesii*) (Bannan 1936). Resin ducts can be constitutively present, produced *de novo* upon insect attack, or both, depending on the conifer species (Franceschi et al. 2005).

Most anatomical studies of induced terpenoid defense have focused on spruce species, in particular Norway spruce and Sitka spruce (Wu and Hu 1997; Nagy et al. 2000; Franceschi et al. 2002a; Martin et al. 2002; Byun McKay et al. 2003; Hudgins et al. 2003; Hudgins and Franceschi 2004; Krekling et al. 2004). In these species, oleoresin is stored in a three-dimensional system of axial constitutive resin ducts (CRD) in the cortex, radial resin ducts that run from the secondary xylem to the cortex and axial TRD in the secondary xylem, which are induced upon mechanical wounding (Nagy et al. 2000; Byun McKay et al. 2003), insect feeding, fungal elicitation (Christiansen et al. 1999; Krekling et al. 2004) or treatment with MeJA (Franceschi et al. 2002b; Martin et al. 2002; Hudgins et al. 2003). TRDs are formed from xylem mother cells within the vascular cambium that initiate the formation of TRD epithelial cells in lieu of trachyary elements (Krekling et al. 2004) and then return to producing trachyary elements, which embeds the TRD in the secondary xylem tissue (Figure 2).

In Norway spruce, epithelial cells of TRDs are easily identifiable six to nine days after treatment with MeJA (Martin et al. 2002) and 16 d after fungal inoculation (Krekling et al. 2004). The epithelial cells of developing TRDs are first seen as thin-walled, irregularly shaped periclinally and anticlinally dividing cells in the cambial zone and can be distinguished by a dense cytoplasm, an enlarged nucleus and an increased number of plastids. These epithelial cells swell and form a schizogenous gap that will become the extracellular lumen of the mature resin duct. The number of epithelial cells surrounding the lumen increases and the gap enlarges until TRDs are fully developed and become further embedded in the secondary xylem tissue due to activity of the vascular cambium switching back to regular xylem formation.

A row of thin-walled parenchyma cells with several polyphenolic bodies and starch grains often surrounds the developing TRD (Nagy et al. 2000). TRDs can be associated with radial ray

cells, which stretch from the inner secondary xylem tissue to the outer secondary phloem and cortex tissues. Treatment of Norway spruce stems with the root rot fungus, *Heterobasidion annosum*, triggers TRD formation by means of a 'signal' that is axially propagated at a rate of approximately 2.5 cm/d, with TRD size and number becoming attenuated further from the inoculation site in a dose-dependent manner (Krekling et al. 2004).

Work by Franceschi and coworkers elegantly showed that both ethylene and jasmonate signaling is involved in the wound-induced TRD response (Hudgins and Franceschi 2004). These authors suggested that, in spruce, ethylene signaling acts upstream of octadecanoid signaling in TRD formation (Hudgins and Franceschi 2004). In Douglas fir, transcripts and proteins for ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate synthase and 1-aminocyclopropane-1-carboxylate oxidase, are induced and localized to resin duct epithelial cells, polyphenolic phloem parenchyma cells and ray parenchyma (Hudgins and Franceschi 2004; Hudgins et al. 2006; Ralph et al. 2007).

Perspective for Future Work

Defense-related terpenoid biosynthesis in conifers is a dynamic process that allows these trees to adapt to changing environmental conditions, in particular coping with attack from pests and pathogens. Much work has been done on the genomics, proteomics and molecular biochemical characterization of enzymes in conifers that are involved in defense-related terpenoid biosynthesis. However, many genes and enzymes of terpenoid biosynthesis remain to be characterized for their contributions to induced changes of the terpenoid metabolome of conifer defense.

Future structural analyses of conifer TPS and P450s will be essential to understand enzyme active site functions that determine multi-product profiles generated by the TPSs and the substrate promiscuity exhibited by the P450s involved in diterpene resin acid biosynthesis. On the anatomical level, changes of TRD formation in the cambium zone and developing xylem have been described following insect attack or treatment of trees with MeJA. However, the signaling events, transcriptome and proteome changes associated with the reprogramming of the cambium towards formation of TRDs, in lieu of regular tracheid development, remain largely unexplored. Also entirely unknown are the transport mechanisms for the secretion of oleoresin terpenoids from the terpenoid producing cells into the extracellular lumen of the resin duct. Initial work has localized TPS protein to epithelial cells of resin ducts (Keeling and Bohlmann 2006a) but more work is needed to develop an understanding of the spatial and temporal regulation of induced oleoresin defense at cellular and subcellular resolution.

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