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## REVIEW ARTICLE

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# GENOMIC HARDWIRING AND PHENOTYPIC PLASTICITY OF TERPENOID-BASED DEFENSES IN CONIFERS

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**Abstract**—Over evolutionary history, conifers have faced a myriad of threats from phloem- and xylem-feeding insects, defoliating insects, and fungal pathogens. Among the trees' defenses, terpenoids appear to play a major role by harming, disabling, deterring, repelling, or otherwise reducing the fitness of potential invaders. Each of the three classes of terpenoids in conifers, monoterpenes, sesquiterpenes, and diterpenes, are composed of a large number of representative compounds. In most cases, the presence of a particular terpenoid compound in the oleoresin or volatile emissions from a specific conifer can be accounted for by the expression of one of many committed terpene synthase (TPS) genes. However, while each TPS may produce one or a few major products, many produce a variety of minor products with relatively constant component ratios in the product blends. TPS genes exist in conifers in large and functionally diverse, yet monophyletic, gene families. Within these gene families, new biochemical functions of TPS appear to have evolved by gene duplication and changes in the amino acid sequence of the enzyme's active site. In addition, TPS genes may be differentially expressed prior to, during, and following attack by insects or pathogens. Thus, while the production of any particular terpenoid is hardwired into a conifer's genome, these trees have the capacity to change the mixture of terpenoids in oleoresin secretions and volatile

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emissions. Anatomical changes may also accompany induced terpenoid production, supplementing the plasticity of the molecular and biochemical events.

**Key Words**—Secondary metabolites, methyl jasmonate, traumatic resin ducts, volatile emissions, chemical defense, terpene synthase, conifer insect defense.

## INTRODUCTION

The class Gymnospermae consists of four divisions: Ginkgophyta (one family, one genus), Gnetophyta (three families, three genera), Cycadophyta (three families, 11 genera), and Coniferophyta (seven families, ~61 genera) (van Gelderen and van Hoey Smith, 1996). Within the diverse Coniferophyta, four genera in the family Pinaceae stand out in terms of economic importance and dominance in the landscape. They are the true firs, *Abies* spp. (~50 species), the pines, *Pinus* spp. (70–100 species), the spruces, *Picea* spp. (~50 species), and Douglas-fir and its allies, *Pseudotsuga* spp. (5–20 species) (Vidaković, 1991; van Gelderen and van Hoey Smith, 1996). Since their origins in the Carboniferous Period (~300 m.y.a.) (Schmidt and Schneider-Poetsch, 2002) and throughout their evolutionary history, conifers have been in contact with insects, which arose at least as early as the Silurian Period (~400 m.y.a.) (Engel and Grimaldi, 2004), and fungi. As a consequence, conifers have evolved sophisticated mechanisms of defense against these antagonists. One of the major defenses mounted against invading pests and pathogens is the secretion of copious oleoresin, comprised mainly of numerous 10-carbon monoterpenes, 15-carbon sesquiterpenes, and 20-carbon diterpenes, and their derivatives (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001a; Langenheim, 2003) (Figure 1).

Terpenoid defenses provide both a physical and a chemical barrier against antagonists. Various oleoresin components in their fluid form are known to have toxic effects on insects and fungi when ingested or contacted by the antagonists (Smith, 1963; Raffa et al., 1985; Himejima et al., 1992; Werner, 1995). The volatile monoterpene and sesquiterpene components of the oleoresin can also act to alter insects' behavior before they feed (Reed et al., 1986; Byers, 1995; Seybold et al., 2000; Wallin and Raffa, 2000). The resin flow (Nebeker et al., 1993) may act to flush out invaders (Raffa and Berryman, 1982), and the non-volatile diterpene component that is left behind following the volatilization of the monoterpenes and sesquiterpenes acts to trap invading insects and pathogens in a crystalline matrix (Hodges et al., 1979).

The efficacy of terpenoid-based defense against a particular antagonist, like other chemical defenses (e.g., polyphenolics, proteinase inhibitors, etc.), depends on multiple factors. These include: (a) the speed at which the defense is mounted, (b) the timing of the defense in comparison to antagonist phenology, (c) the physical characteristics of the defensive secretion (oleoresin), (d) the precise compounds contained in the defensive secretion, (e) the bouquet of volatiles released

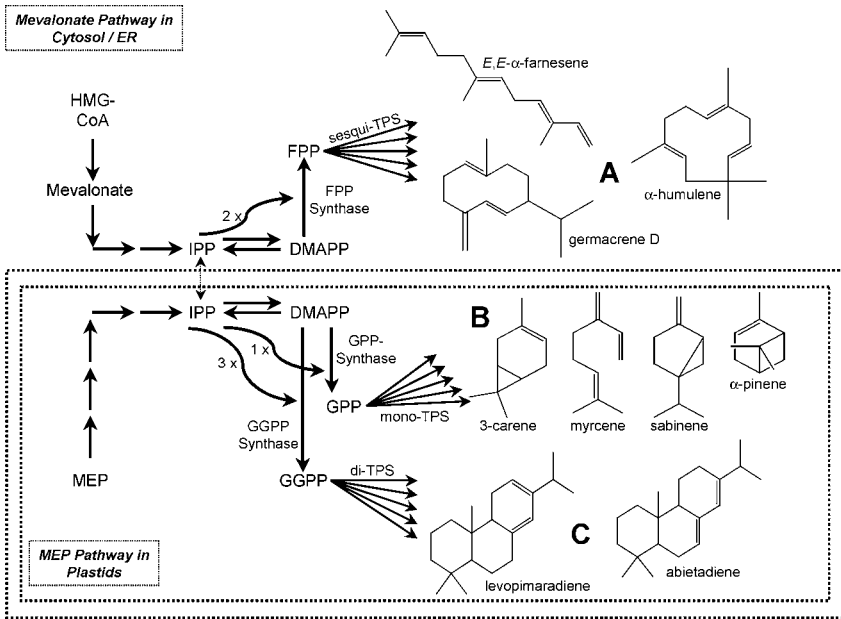


FIG. 1. Schematic diagram of the mevalonate (MVA) pathway in the cytosol/ER and the MEP pathway in the plastids of conifers. Fifteen-carbon sesquiterpenoids (A) (three representatives of which are shown) are produced by numerous sesquiterpene synthases in the late reactions of cytosolic MVA pathway. Ten-carbon monoterpenoids (B) and twenty-carbon diterpenoids (C) (four and two representatives of which are shown, respectively) are produced by numerous monoterpene synthases and diterpene synthases in the late reactions from intermediates of the plastidic MEP pathway. IPP and possibly other intermediates may be able to cross plastid membranes, leading to some interaction (crosstalk) between the two pathways. Abbreviations are as follows: MEP is 2C-methyl-D-erythritol 4-phosphate, HMG-CoA is 3-hydroxy-3-methylglutaryl Coenzyme A, IPP is isopentenyl diphosphate, DMAPP is dimethylallyl diphosphate, GPP is geranyl diphosphate, FPP is farnesyl diphosphate, GGPP is geranylgeranyl diphosphate, mono-TPS refers to the monoterpene synthases, sesqui-TPS refers to the sesquiterpene synthases, and di-TPS refers to the diterpene synthases.

from a wound or from adjacent areas, and (f) the ability of the plant to deliver the defensive secretions to the appropriate location. In the case of long-lived conifer trees, qualitative and quantitative variation, or phenotypic plasticity, and genetic adaptation of the multi-component terpenoid chemical defense also seems to be an important factor in long-term protection against potential insect herbivores or pathogens of much shorter generation times. While the production of each individual terpenoid is hardwired into a conifer's genome, the plasticity

required to mount effective defenses against a plethora of different antagonists is a result of a number of factors: (a) the evolution of a large number of functionally diverse terpene synthases (TPS) organized in multi-gene families, (b) the partitioning of terpenoid biosynthesis at the subcellular level, (c) the complex product profiles of many of the TPS that form terpenoids from simple precursors, (d) differential TPS gene expression in different tissues, and (e) induced TPS gene expression with differential spatial and temporal TPS expression profiles in the challenged tree. Other factors that increase the plasticity of the response against insects or pathogens are the signaling and regulation of the pathways leading to TPS gene expression and an astonishing capacity of conifers to differentiate new anatomical structures for terpenoid formation and resin secretion into traumatic resin ducts that allow accumulation of the products of the TPS enzymes. Our intention with this review is to outline the molecular, biochemical, and related histological and physiological components of the plasticity of conifer terpenoid defenses in order to allow the reader to further investigate this topic.

#### EVOLUTION AND BIOCHEMICAL FUNCTIONS OF CONIFER TERPENE SYNTHASES

Phylogenetic analyses allow the inference that conifer TPS are derived from a common ancestor because, based upon amino acid similarities, all conifer TPS characterized to date cluster together in one subfamily (i.e., subfamily TPS-d) of known plant TPS (Bohlmann et al., 1998b; Aubourg et al., 2002; Martin et al., 2004). Within the TPS-d subfamily, monoterpene synthase (mono-TPS), sesquiterpene synthase (sesqui-TPS), and diterpene synthase (di-TPS) genes mostly cluster separately, indicating separate evolutionary trajectories for each of these three biochemical classes of enzymes (Figure 2). Conifer sesqui-TPS of the TPS-d group apparently evolved independently several times from mono- and di-TPS genes in ancestors (Martin et al., 2004).

Conifers typically contain a large number of functional TPS that together produce a large number of products. For example, grand fir, *Abies grandis*, contains at least seven mono-TPS genes, three sesqui-TPS genes, and one di-TPS gene (Stofer Vogel et al., 1996; Bohlmann et al., 1997, 1998a,b, 1999; Steele et al., 1998a,b). Similarly, Norway spruce, *Picea abies*, contains a large family of diverse TPS of which five mono-TPS genes, three sesqui-TPS genes, and two di-TPS genes have been functionally characterized to date (Fäldt et al., 2003; Martin et al., 2004). Three different mono-TPS genes and one sesqui-TPS gene have been characterized from loblolly pine, *Pinus taeda* (Phillips et al., 2003). These numbers are a gross underestimate of the true number of TPS genes in the large genomes typical of conifer species (estimated 20–40 Gb), considering that more than 30 TPS genes were found in the sequenced *Arabidopsis thaliana* genome

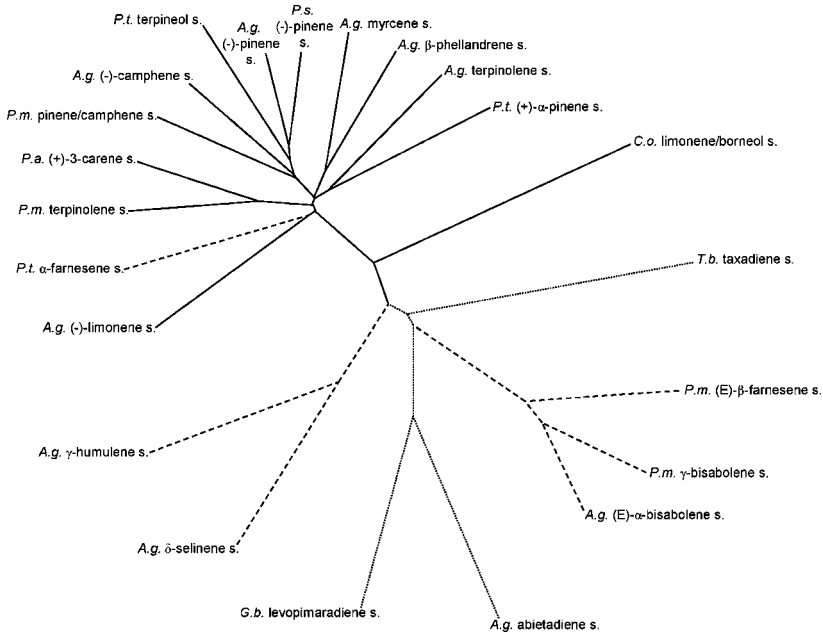


FIG. 2. Phylogenetic tree of representative terpenoid synthases (TPS) in the TPS-d subfamily. Solid lines (—) show branches containing monoterpene synthases, dashed lines (---) show branches containing sesquiterpene synthases, and dotted lines (. . .) show branches containing diterpene synthases. The three different groups mainly cluster separately, though there are exceptions. Taxon abbreviations are as follows: *A.g.* is *A. grandis*; *C.o.* is *Chamaecyparis obtusa*; *G.b.* is *Ginkgo biloba*; *P.a.* is *P. abies*; *P.s.* is *P. sitchensis*; *P.m.* is *P. menziesii*; *P.t.* is *P. taeda*; and *T.b.* is *Taxus brevifolia*. Most amino acid sequences were obtained from GenBank except for those for *P. menziesii*, which were from unpublished data from our lab. The alignment was completed with ClustalX (Thompson et al., 1997) and the tree was visualized with TreeView (Page, 1996).

of ca. 125 Mb (Aubourg et al., 2002). A survey of more than 90,000 expressed sequence tags (ESTs) developed as part of a Sitka spruce, *Picea sitchensis*, and white spruce, *Picea glauca*, genomics project (<http://treenomix.com>) suggests that the number of TPS genes in a spruce genome is much larger than the 30 TPS genes identified in the *Arabidopsis* genome.

The common origin of all conifer TPS genes (Bohlmann et al., 1998b, 2000; Trapp and Croteau, 2001b; Martin et al., 2004), in conjunction with the large number of TPS genes in conifer genomes, suggest that families of functionally diverse TPS genes arose from events of gene duplication, gene mutations, and functional diversification during the evolution of conifers. This pattern of TPS evolution is also becoming apparent from genome-wide analysis of TPS in

*A. thaliana* (Aubourg et al., 2002), and is supported by results from functional characterization and comparative analysis of TPS in *P. abies* (Martin et al., 2004). The fact that the three classes of conifer TPS genes (mono-TPS, sesqui-TPS, and di-TPS) each cluster within the TPS-d group further indicates that a hypothetical ancestral conifer had at least one of each class of genes before the conifer group radiated into the genera and species that we see today. Analysis of TPS genes in more species in the principal genera will test this evolutionary hypothesis. Considering the role of terpenoids in defense against insects and pathogens, it is also likely that co-evolutionary processes (Thompson, 1994) have shaped the array of TPS genes that we find in conifers.

The TPS-mediated reactions, as well as earlier steps of terpenoid biosynthesis, are partitioned in subcellular compartments (Croteau et al., 2000). Specifically, monoterpenoids and diterpenoids are produced in the plastids from five-carbon products of the methylerythritol phosphate (MEP) pathway, whereas sesquiterpenoids are produced in the cytoplasm from products of the mevalonate (MVA) pathway (Figure 1) (Croteau et al., 2000). Our knowledge of the MEP and MVA pathways in plants is largely based on studies with angiosperms, although mining of the rapidly growing spruce cDNA EST databases (<http://treenomix.com>) has identified almost all the genes of both pathways as expressed transcripts in conifers (Bohlmann et al., unpublished data). The subcellular partitioning of the two terpenoid biosynthesis pathways (Figure 1) could allow for the regulation of some terpenoid biosynthesis independently of others, although some crosstalk between the two pathways occurs (Laule et al., 2003).

Following the formation of the five-carbon intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), via the MEP and MVA pathways, terpenoid formation proceeds in the plastids and the cytosol with the activity of prenyldiphosphate synthases that generate the substrates for TPS enzymes (Figure 1). In the plastids, the formation of geranyl diphosphate (GPP) from one molecule of DMAPP and one molecule of IPP by GPP synthase is followed by the activity of mono-TPS that convert GPP to monoterpenoids. Diterpene synthesis, in the plastids as well, proceeds via the activity of geranylgeranyl diphosphate (GGPP) synthase, which makes GGPP from one molecule of DMAPP and three molecules of IPP, followed by the activity of di-TPS that convert GGPP to diterpenoids. To date, GPP synthase and GGPP synthase genes, their functions, and enzyme subunit organizations as homodimers have been described in the literature only for one member of the Pinaceae, grand fir, *A. grandis* (Burke and Croteau, 2002a,b). In the cytoplasm, farnesyl diphosphate (FPP) synthase converts one molecule of DMAPP and two molecules of IPP into FPP. Sesqui-TPS then catalyze the reaction from FPP to 15-carbon sesquiterpenes. Sequences for transcripts with high homology to *A. grandis* GPP synthase, GGPP synthase, and angiosperm FPP synthases are abundantly present and available for functional characterization in the spruce EST databases (<http://treenomix.com>).

The activities of the various TPS enzymes are also localized to the plastids or the cytoplasm (Bohlmann et al., 1998b; Croteau et al., 2000). Following the synthesis of each class of terpenoid, other enzymes may oxygenate them or add other functional groups, increasing the structural complexity of the final terpenoid products. Although secondary modifications of some terpenoids, such as the step-wise oxidations of the diterpene hydrocarbon abietadiene to abietic acid, are important for the physical, chemical, and biological properties of oleoresin defense secretions, little is known about the genes and enzymes of secondary modification of the primary terpenoid products in conifers (Funk and Croteau, 1994). Only recently has it been possible to clone and functionally identify the first cytochrome P450-dependent monooxygenase enzymes of diterpene resin acid formation from *P. taeda* (Ro and Bohlmann, unpublished data).

In contrast, a great deal of detailed information is available on the biochemical functions of known conifer TPS genes (Stofer Vogel et al., 1996; Bohlmann et al., 1997, 1998a, 1999; Steele et al., 1998a; Peters et al., 2000, 2001, 2003; Ravn et al., 2000, 2002; Peters and Croteau, 2002a,b; Fäldt et al., 2003; Phillips et al., 2003; Martin et al., 2004). The similarity in amino acid sequence among TPS enzymes (Figures 3 and 4) suggests similarity in catalytic function within each of the three classes of conifer TPS. While members of each class use the same substrate (GPP, FPP, or GGPP for mono-, sesqui- and di-TPS, respectively), the large number of TPS enzymes in any given conifer and the specific differences in the catalytic activities of each help to produce the enormous array of different terpenoids in conifer oleoresin. In addition, although a single TPS may produce just one terpenoid in substantial quantity (e.g., Bohlmann et al., 1997, 1998a; Martin et al., 2004), many TPS produce significant amounts of numerous products in the same class in fairly consistent ratios (e.g., Steele et al., 1998a; Bohlmann et al., 1999; Peters et al., 2000; Fäldt et al., 2003; Martin et al., 2004). Reaction schemes have been proposed to explain the rich diversity of products from single conifer TPS enzymes acting on single substrates for mono-, sesqui-, and di-TPS (e.g., Steele et al., 1998a; Bohlmann et al., 1999; Fäldt et al., 2003; Martin et al., 2004). Specific examples of multiple product conifer TPS include two multiple product mono-TPS that were cloned and functionally characterized from Douglas-fir, *Pseudotsuga menziesii* (Huber et al., unpublished data). The first mono-TPS, a terpinolene synthase, also produces  $\alpha$ - and  $\gamma$ -terpinene,  $\alpha$ - and  $\beta$ -pinene, limonene, sabinene, myrcene, 3-carene, and trace amounts of camphene and  $\alpha$ -thujene. The second, an  $\alpha$ -pinene/camphene synthase also produces limonene, 3-carene, and  $\beta$ -pinene. Sesquiterpene synthases have been characterized in *A. grandis* and in *P. abies* that produce more than 10 different products each (Steele et al., 1998a; Martin et al., 2004). Characterization of TPS genes in *P. abies* identified a pair of di-TPS of 90% amino acid identity, one of which forms a single diterpene product, isopimaradiene, and the other that produces four different diterpenes, levopimaradiene, abietadiene, neoabietadiene, and palustradiene (Martin et al., 2004).



AAB70707 O24475	MALLSITPLVSRCL----SSSHEIKALRRTIPTLIGICRPGKSVASHIN 45 MALVSTAPLASKSCLHKSLISSTHELKALSRTIPALGMRRRGKSIPTSIS 50 ***:* :*:*:*:*:* *:*:*:*:* *:*:*:*:* *:*:*:*:* *:*:*:*:*
AAB70707 O24475	<sup>RRxxkxxsxxxd</sup> MCLTSVASTDSVQRVRVGNVHSNLWDDDFIQSLISTPYGAPDYRERADRLI 95 MSSTTVVTDGVR <sup>RRMCDFFHSNLW</sup> DDVDVIQSLP-TAYEKSXYLERAEKLI 99 * :*:*:* :*:*:*:*:*:*:*:*:*:*:*:*:*:*:* * :* :* :* :* :* :*
AAB70707 O24475	GEVKDIMFNPKSLEDG----GNDLLQRLLLVDDVERLGIDRHPKKEIKT 140 GEVKN-MFNMSMLEGELMSPLNLDLQRLWIVDSLRLGIHRHPKDEIKS 148 ****: ** * **** *:*:*:* :*:*:*:*:*:*:*:*:*:*:
AAB70707 O24475	ALDYVNSYWNKGGIGCGRESVVTDLNSTALGLRLTLRLHGVTWSSDVLNVF 190 ALDYVYSYWGENGIGCGRESVVTDLNSTALGLRLTLRLHGYPVSSDVPKAF 198 ***** *:*:*:*:*:*:*:*:*:*:*:*:*:*:*:* *:*:*:*:*:*:
AAB70707 O24475	KDKNQFSSSTANIQIEGEIRGVNLNFRASLVAFPGEKVMDEAETFSKYL 240 KGQNGQFSSCSENIQTDEEIRGVNLNFRASLIAFPGEKIMDEAEIFSKYL 248 *:* *:*:*:*:*:*:
AAB70707 O24475	REALQKIPASSILSLEIRDVLEYGWHTNLPRLRLEARNYMDVFGQHTKNKN- 289 KEALQKIPVSS-LSREIGDVLEYGWHTYLPRLRLEARNYIQVFGQDTENTKS 297 :*:*:*:*:* *
AAB70707 O24475	--AAEKLELAKLEFNIFHSLQERELKHVSRWWDKSGSPEMTFCRHRHVE 337 YVSKKLELAKLEFNIFQSLQKRELSLVRWVKESGPEMTCRHRHVE 347 :*:*:*:*:*:*:*:*:*:*:*:*:*:*:* * * * * * * * * * * * * * * * * *
AAB70707 O24475	<sup>DDxxD</sup> YYALASCIAPFPQHSFRLGPTKMSHLITVLDDMYDVFQTVDELELFTAT 387 YTLASCIAPFPQHSFRLGPAKTCHLITVLDDMYDTFQTVDELELFTAT 397 *:*:*:*:*:*:*:*:*:*:*:*:*:*:* * . * * * * * * * * * * * * * * * * *
AAB70707 O24475	IKRWDPsameclPEYMGVYMMVYHTVNEMARVAEAKAQRDRLNARQAW 437 MKRWDPSSIDCLPEYMGVYIAVYDTVNEMARAEAEAKQRDRLTYAREAW 447 :*:*:*:*:*:*:*:*:*:*:* *
AAB70707 O24475	EACFDSYMQEAKWIATGYLPTFEEYLENGKVVSAHRPCALQPIILTLDIPF 487 EAYLDSYMQEARWIATGYLPSFDEYYENGVKSCGHRISALQPIITMDIPF 497 * * : *
AAB70707 O24475	PDHILKEVDFPSKLNLDLICIILRLRGDTRCYKADRARGEAEASSISCYMKD 537 PDHILKEVDFPSKLNLDLACILRLRGDTRCYKADRARGEAEASSISCYMKD 547 *
AAB70707 O24475	NPGLTEEDALNHINFMIRDAIRELNWELLKPDNSVPIITSKKHAFDISRVW 587 NPGVSEEDALDHINAMISDVIKCLNWELLKPDINVPISAKKHAFDIARAF 597 * * * * * : *
AAB70707 O24475	HGgyrYrDgYsFANVETKSLVMRTVIEPVPL 618 HYGYKYrDgYsVANVETKSLVTRTLLESVPL 628 *:*:*:*:*:*:*:*:*:*:* *:*:*:*:* *:*:*:*:* *:*:*:*:* *:*:*:*:*

FIG. 4. Amino acid sequence alignment of two monoterpene synthases [(–)-camphene synthase, GenBank accession AAB70707; and (–)-pinene synthase, GenBank accession O24475] from grand fir, *A. grandis*. Identical residues in the two sequences are marked by “\*”, conserved substitutions by “:” and semi-conserved substitutions by “.”. Marked in the alignment are the DDxxD motif and the RRX8W motif. The sequence similarity is high, making it impossible within a class of terpene synthases to ascertain the catalytic function of an enzyme from only its amino acid sequence. It is necessary to express newly discovered terpene synthases and perform assays with the appropriate substrate to fully characterize the enzyme. The alignment was completed with ClustalW (Thompson et al., 1994) and JalView (Clamp et al., 2004).

This brief review of the molecular and biochemical literature on terpenoid formation in conifers reveals that the many products of conifer terpenoid pathways can be seen as the expression of a genomic blueprint of the various portions of the terpenoid biosynthesis pathways, with multigene families of functionally diverse TPS as the core generators of the chemical diversity and plasticity of conifer terpenoid defense. Comparatively little is known about the molecular genetics and biochemistry of earlier and later steps in the terpenoid pathways in conifers (MEP, MVA pathways, prenyltransferases, and cytochrome P450 enzymes), and their contribution to genetic and biochemical control of terpenoid defense phenotypes. The many genes now available from large-scale conifer genome projects will accelerate functional characterization of these early and late pathway steps in the near future. The expression of terpenoid defense genes in conifers is differentially controlled both by endogenous and exogenous factors (see below), which shape the phenotypic plasticity of terpenoid profiles in conifer defense.

#### PRODUCTION AND DELIVERY OF TERPENOID DURING DEFENSE IN CONIFER STEM TISSUES

The molecular genetics and biochemistry of conifer terpenoid biosynthesis play an overriding role in the variable quantity and quality of terpenoids that a tree can produce. The plasticity of multigene-based terpenoid defense can be considered an important adaptive trait of long-lived conifers that interact on several trophic levels with faster evolving insects and microorganisms. However, regulation at the physiological, cellular, and anatomical levels in response to an insect infestation or a pathogen inoculation, and the associated ability of the tree to allocate terpenoids with appropriate spatial and temporal patterns for effective defense are also important in the flexibility of a conifer's terpenoid defense (Martin et al., 2002, 2003; Hudgins and Franceschi, 2004; Hudgins et al., 2004). Because conifers are typically long-lived and large organisms, an understanding of physiological aspects related to defense against insects and pathogens is not always easy to develop. Vincent Franceschi, Erik Christiansen and co-workers, and our research group, however, have developed systems to study aspects of conifer defenses, including terpenoid and phenolic-based defenses, at several levels, from anatomy and histology to physiology, biochemistry, and genomics in *Picea* spp.

One particularly well-studied system is the response of *Picea* spp. to the white pine weevil, *Pissodes strobi* (Alfaro et al., 2002). After overwintering in the duff, adult weevils climb the stem of small (~1.5–10 m) trees (Turnquist and Alfaro, 1996). When they reach the terminal shoot, they puncture the bark to feed and, after mating, to lay eggs. The larvae hatch and feed within the phloem, cambium, and the youngest outer xylem by burrowing downward from the site of oviposition until they complete their larval and pupal stages and then emerge as adults. The larval feeding takes a large toll on the tree by destroying the terminal

leader and forcing a nearby lateral branch to assume apical dominance in the following year. Repeated infestations by the weevil over a number of years reduce height growth, create infection courts for decay fungi, dramatically reduce wood quality, and increase the risk for a tree to be overgrown by competing vegetation. In response to weevil feeding or oviposition, extensive changes occur in the resin duct anatomy of the affected spruce leader, which normally contains large resin ducts in the bark, but only few, if any, constitutive axial resin ducts in the xylem. Axial traumatic resin ducts, induced by weevil feeding or oviposition, form rapidly in the newly developing xylem where they persist for several months or years (Alfaro, 1995; Byun McKay et al., 2003). The ducts begin to form quickly following early contact with the feeding or ovipositing insect, and it is thought that the rapid formation of ducts near the developing larvae allows the tree to deliver a payload of terpenoids directly to the site of insect residence and feeding. Simulation of insect attack on spruce or inoculation with fungi, often associated with insects, using drill wounding or treatment with an octadecanoid-derived plant defense elicitor, methyl jasmonate (MeJA) (Figure 5), causes similar traumatic resin duct formation in the developing xylem in *P. glauca*, *P. sitchensis*, and *P. abies* (Tomlin et al., 1998; Nagy et al., 2000; Franceschi et al., 2002; Martin et al., 2002, Byun McKay et al., 2003; Miller et al., 2005). Traumatic resin duct formation is also induced by MeJA or ethylene treatment in several other members of the pine family (Hudgins et al., 2003, 2004; Hudgins and Franceschi, 2004; Huber et al., 2005).

Mimicked insect attack of young spruce trees by using MeJA treatment has proven to be a powerful tool in experiments to decipher, in detail, the complex physiological and anatomical changes related to induced terpenoid accumulation, increased biochemical activities of terpenoid pathways, and differential TPS gene expression associated with traumatic resin duct formation in spruce stem tissues.

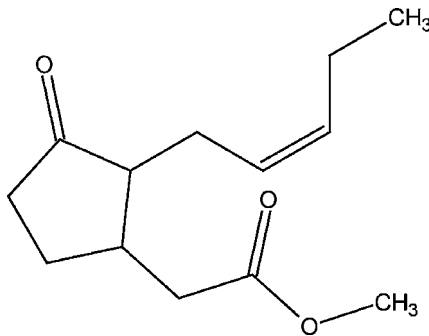


FIG. 5. General structure of methyl jasmonate (MeJA) (Baldwin, 1999). The enantiomeric composition of the biologically active form and endogenous octadecanoids in conifers have not been determined.

Similarly, treatment of spruce trees with MeJA also allows a detailed analysis of induced terpenoid volatiles emitted from spruce needles and of the biochemical processes involved in foliage (Martin et al., 2003). Topical applications of MeJA induce traumatic resin canal formation in species of spruce (Franceschi et al., 2002; Martin et al., 2002; Byun McKay et al., 2003) and other conifers (Hudgins et al., 2003, 2004). Some conifers, however, do not produce traumatic resin ducts upon application of MeJA, but instead either exhibit other histological modifications or no observable changes at all (Hudgins et al., 2003, 2004). MeJA treatment of *P. abies* saplings leads to rapid cell differentiation of traumatic resin ducts *in lieu* of regular xylem tracheids in the youngest developing xylem layers. The new cell structures appear next to the active cambium within a few days after treatment of trees with MeJA (Martin et al., 2002). This process of resin canal differentiation involves a re-direction of the normal developmental program of cambium cells in response to the octadecanoid signal. It is possible that other plant signal molecules are involved in the reprogramming of insect-induced resin canal differentiation downstream or upstream of the octadecanoid pathway. In addition to MeJA, a role for ethylene in the induction of traumatic resin ducts, polyphenolic parenchyma cells, and sclereid lignification in *P. menziesii* has recently been shown (Hudgins and Franceschi, 2004).

MeJA also has profound effects on TPS gene expression and enzyme activities associated with traumatic resinosis in *P. abies*. Transcripts associated with mono- and di-TPS genes increase rapidly in sapling stems following the topical application of 0.01% MeJA (Fäldt et al., 2003). For both classes of TPS genes, the peak in transcript levels occurs about 2 days after MeJA application and declines at day 4. Mono-TPS transcript levels remain slightly elevated even 16 days after MeJA treatment, whereas di-TPS transcript levels decline to levels similar to those prior to MeJA treatment (Fäldt et al., 2003). The increase in TPS mRNA following MeJA application is followed by increased TPS enzyme activities and changes in terpenoid content in *P. abies* stem tissues (Martin et al., 2002).

Monoterpenoids and diterpenoids are not constitutively present in large amounts in the xylem of *P. abies* stems, but they build-up rapidly in xylem of MeJA-treated saplings, and there is also a substantial increase in terpenoids in bark tissues, which are already constitutively rich in resin terpenoids (Martin et al., 2002). The same study showed that the overall enzyme activities of mono- and di-TPS mirrored the terpenoid build-up. Thus, MeJA induces rapid and long-lasting effects in spruce stem tissues including the induction of mono- and di-TPS gene expression, an increase in related enzyme activities (e.g., prenyl diphosphate synthases), an accumulation of induced resin terpenoids, and concurrent *de novo* formation of traumatic resin ducts (Martin et al., 2002; Fäldt et al., 2003). This is consistent with a rapid defense response to a real insect infestation followed by maintenance of terpenoid defense during a growing season. Similar to the induced expression of TPS genes in *P. abies* saplings in response to MeJA, TPS

gene transcripts also accumulate after real insect feeding by white pine weevil and after drill wounding of *P. sitchensis* (Byun McKay et al., 2003). Mono-TPS gene transcripts increase within one day of initial contact with weevils in the terminal growth of lateral branches of *P. sitchensis*, and peak at about 2–4 days. At 7 days, transcript levels are still somewhat elevated in lateral branches. Drill wounding of lateral branches and leaders give similar results. Probing of TPS gene expression on single trees of two genotypes showed that transcript levels of two mono-TPS genes, including a *P. sitchensis* ( $-$ )- $\alpha$ -pinene/( $-$ )- $\beta$ -pinene synthase, increase within 12 hr of drill wounding (Byun McKay et al., 2003). Recent studies with *P. sitchensis* saplings showed some differential, MeJA- and weevil-induced gene expression among 11 different mono-, sesqui- and di-TPS (Miller et al., 2005), suggesting some independent regulation of members of the TPS gene family in response to insect feeding.

Fungal inoculation of conifers can have effects on terpenoid content of resin similar to MeJA-treatment, suggesting the involvement of similar or identical genes. However, to our knowledge, no investigations into the molecular genetic foundation of this phenomenon have been published. Furthermore, microarray gene expression analysis of several thousand *P. sitchensis* genes in response to feeding by *P. strobi*, by the western spruce budworm, *Choristoneura occidentalis*, mechanical wounding, or MeJA treatment, have revealed large-scale changes of gene expression in *P. sitchensis*, with partially overlapping gene expression profiles common to all treatments (Ralph and Bohlmann, unpublished results; <http://treenomix.com>). Such microarray analysis of the transcriptome of conifers in response to real and simulated insect attack is not only leading to the discovery of metabolic pathways, such as terpenoid and phenolic pathways associated with defense in *P. sitchensis*, but also allowing the discovery of signaling cascades and transcription factors that can orchestrate complex, multi-gene defense responses.

#### METHYL JASMONATE- AND INSECT-INDUCED VOLATILE EMISSIONS

Dramatic changes in terpenoid biochemistry are also induced in the foliage of *P. abies* and *P. sitchensis*, in response to treatment with MeJA (Martin et al., 2003; Miller et al., 2005). While the build-up of terpenoids in foliage following MeJA application is not nearly as strong as in bark and wood, MeJA causes a large increase in the emission of volatile monoterpenoids and sesquiterpenoids. Measurements of the headspace around MeJA-treated *P. abies* and *P. sitchensis* saplings reveals not only an increase in terpenoid release from foliage, but also a major shift from emission of monoterpene hydrocarbons towards emission of sesquiterpenoids and oxygenated monoterpenoids, specifically farnesene, bisabolene, and linalool (Martin et al., 2003; Miller et al., 2005). Many angiosperms are known to release volatiles (in many cases terpenoids) in response to herbivory by

insects. These volatiles often function in the attraction of insect natural enemies of the herbivores (Takabayashi and Dicke, 1996; Paré and Tumlinson, 1997a,b, 1999; Dicke and Vet, 1999; Kessler and Baldwin, 2001, 2002; Arimura et al., 2004). Recently, it has also been shown that volatiles are induced from conifers upon herbivory and egg deposition in needles (Litvak and Monson, 1998; Hilker et al., 2002), and it is possible that many of these volatiles are emitted constitutively from insect-damaged resin storage sites in needles. However, simulated insect attack by MeJA treatment of *P. abies* and *P. sitchensis* (Martin et al., 2003; Miller et al., 2005) demonstrated that the induced emission of linalool and sesquiterpenoids from foliage is the result of rapidly induced up-regulation of foliar TPS gene expression and increased enzyme activities of a few monoterpene synthases and sesquiterpene synthases. Moreover, detailed qualitative analysis of temporal patterns of volatile profiles of *P. sitchensis* infested with *P. strobi* showed new emissions of linalool that cannot be explained simply by release from preformed resin terpenoids, but are likely derived from insect-induced *de novo* formation and induced release into the headspace (Miller et al., 2005).

The specific TPS gene expression and enzyme activities that are up-regulated in foliage of *P. abies* and *P. sitchensis* upon treatment with MeJA differ from those that are up-regulated after MeJA-treatment and insect attack in stems, supporting the concept of differential organ-specific regulation of terpenoid defenses in *Picea* spp.

#### SIGNALS INVOLVED IN INDUCED RESIN TERPENOID DEFENSES

The similarity in events of terpenoid responses and related anatomical defenses following insect feeding, simulated wounding, and MeJA application suggests that octadecanoids may play some role in terpenoid-based defense in conifers, specifically as signals leading to traumatic resinosis and terpenoid emissions. While MeJA or other octadecanoid compounds still remain to be detected as endogenous defense signals in *Picea* spp., we have recently identified many of the known octadecanoid pathway genes as transcripts in our *Picea* EST database (<http://treenomix.com>). Increased mRNA levels of key steps of the octadecanoid pathway were found in response to both insect attack by *P. strobi* and in response to MeJA-treatment when the transcriptome of *P. sitchensis* was analyzed using microarrays (Ralph and Bohlmann, unpublished) and verified by Northern hybridizations (Miller et al., 2005). Future work will target additional signaling events and transcription factors that control changes in gene expression, terpenoid accumulation and emission, and anatomical changes in xylem resin duct development. Further, there are possible interactions of these factors with signaling networks, in which ethylene (Hudgins and Franceschi, 2004), auxins, or other signal compounds likely play a role.

## GENOMIC APPROACHES TO CONIFER DEFENSE

Further work is needed in conifers to understand the orchestration of complex insect- or pathogen-induced defense genes and protein expression with the resulting chemical and anatomical defenses. Current genomics approaches in our laboratory use microarray transcriptome analyses and 2DE/LC-MS-MS-proteome analyses of trees challenged by insects, pathogens, or elicitors, as well as metabolite profiling of these trees (<http://treenomix.com>). These approaches allow for the simultaneous profiling of hundreds or thousands of different gene transcript, protein, and metabolite species, requiring careful statistical and bioinformatics analysis of large data-sets, which is now possible due to recent developments in conifer genomics. Genomic resources are also being developed in parallel for conifer-insect pests, specifically for bark beetles (Eigenheer et al., 2003; Tittiger, 2004), western spruce budworm, *C. occidentalis* (Qili Feng and Basil Arif, personal communication), and for conifer pathogens, specifically bark-beetle associated blue-stain fungi (ongoing research in our laboratory). Analysis of large data sets obtained by genomics approaches to conifer defense can result in the *in silico* reconstruction of signaling and metabolic pathway networks under constitutive and insect-induced conditions. Selected components of such signaling and pathway maps of insect-induced processes can then be tested functionally by using a combination of biochemical and genetic methods, that include the modification of target gene expression. Selected candidate genes can be further explored as genetic markers for association with insect-defense or resistance in natural or breeding populations. The simultaneous profiling of gene expression in conifers and their pests or pathogens may also lead to identification of key elements in the interacting genomes of these organisms.

## SUMMARY AND FUTURE DIRECTIONS

The genomic, molecular, and biochemical underpinning of conifer terpenoid-based and other defenses against pests and pathogens is complex and multifaceted. Preceding the TPS-mediated reactions that produce the terpenoids in conifer resin are a number of other reactions contained in two pathways and compartmentalized on a subcellular level. Terpenoids are formed by the action of multiple TPS enzymes, many of which also produce multiple products. TPS genes appear to be differentially transcribed in different tissues following insect or other damage or application of MeJA. Following production of terpenoids via the action of TPS enzymes, the terpenoid products may be modified by the action of other enzymes such as cytochrome P450-dependent monooxygenases. The enzymes involved in these transformations and their regulation are not well understood in conifers. The interplay of the above factors that are influenced by the biotic

and abiotic environment of conifers results in complex blends of terpenoids in the oleoresin defenses and volatile emissions. It is possible that conifers defend themselves differently against different antagonists, and that insects and pathogens, or different species of each, may induce different blends of terpenoids that are, at least somewhat, optimized for defense against the specific threat. For example, foliage-feeding budworms, ovipositing sawflies, or stem-boring weevils, and bark beetles appear to induce different terpenoid responses in conifers. Additional work is needed to better understand the different responses and their specific fine-tuning.

Herbivore-, pathogen- or MeJA-induced biosynthesis of resin terpenoids is often accompanied by anatomical changes that allow the tree to deliver the terpenoid-laced resin to the site of the damage. Similarly phenolic defenses appear to be associated with specialized, inducible cell types in conifers. These anatomical and histological changes, mainly observed in the developing xylem of stems for terpenoids and in the bark for phenolics, will also have complex genetic foundations that are yet to be unraveled.

Natural selection should act on conifers to strengthen their ability to distinguish between antagonists, mount a rapid defense, include the terpenoids in their defensive secretions that will best counter the threat, and deliver their payload to the most appropriate location. Research has just begun to scratch the surface of the molecular genetic and biochemical foundations of these aspects of conifer defense systems. The recently developed genomics tools for conifers will unravel new aspects of defense against herbivores and pathogens in long-lived trees. Sustainability of conifer forests is facing new challenges under scenarios of climate-change-related increases in biotic and abiotic stress. The questions that remain outnumber, by far, the questions that have been answered to date. Research in this field will prove to be useful not only in a basic sense, but also in an applied manner. A more complete understanding of conifer natural defenses at the molecular and genomic levels will provide new strategies to complement existing and proven approaches for sustainable forestry for the future.

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