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Review

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Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens*

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Contents

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Summary

Insects select their hosts, but trees cannot select which herbivores will feed upon them. Thus, as long-lived stationary organisms, conifers must resist the onslaught of varying and multiple attackers over their lifetime. Arguably, the greatest threats to conifers are herbivorous insects and their associated pathogens. Insects such as bark beetles, stem- and wood-boring insects, shoot-feeding weevils, and foliage-feeding budworms and sawflies are among the most devastating pests of conifer forests. Conifer trees produce a great diversity of compounds, such as an enormous array of terpenoids and phenolics, that may impart resistance to a variety of herbivores and microorganisms. Insects have evolved to specialize in resistance to these chemicals – choosing, feeding upon, and colonizing hosts they perceive to be best suited to reproduction. This review focuses on the plant–insect interactions mediated by conifer-produced terpenoids. To understand the role of terpenoids in conifer–insect interactions, we must understand how conifers produce the wide diversity of terpenoids, as well as understand how these specific compounds affect insect behaviour and physiology. This review examines what chemicals are produced, the genes and proteins involved in their biosynthesis, how they work, and how they are regulated. It also examines how insects and their associated pathogens interact with, elicit, and are affected by conifer-produced terpenoids.

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I. Introduction

Conifers (orders Coniferales and Taxales) are long-lived stationary organisms that must confront a multitude of biotic and abiotic stresses that vary with the season and over their lifetime. The nutrients in these trees are sought after by organisms of many taxa. Of these taxa, insects, and the associated pathogens they can vector, present the major challenge to conifer survival and are currently the greatest threat to the sustainability of conifer forests as ecosystems and for human use. These relationships are ancient and complex. Insects have existed for at least 400 Myr (Engel & Grimaldi, 2004), and the fossil record shows that they have used conifers as their host for over 220 Myr, and also reveals signs of host specificity (Scott *et al.*, 2004).

Conifers have several resistance mechanisms that repel, kill, inhibit, or otherwise reduce the success of invading herbivores and pathogens. These include both mechanical and chemical mechanisms that can be present constitutively or that are induced upon challenge. Although these defences can comprise many classes of chemicals, for example, the phenolics produced in phloem parenchyma cells (Franceschi *et al.*, 1998, 2000, 2005), this review is focused on terpenoids in particular. Oleoresin is comprised of a diverse array of terpenoid compounds mobilized to the site of wounding (Bohlmann & Croteau, 1999; Phillips & Croteau, 1999; Trapp & Croteau, 2001a; Langenheim, 2003; Huber *et al.*, 2004; Martin & Bohlmann, 2005). Oleoresin is a viscous and odoriferous liquid that consists of volatile C_{10} (monoterpenoid) and C_{15} (sesquiterpenoid) components and nonvolatile C_{20} (diterpenoid) components (see Fig. 2 below). The oleoresin of most conifers contains approximately equal amounts of monoterpenes and diterpenes and smaller amounts of sesquiterpenes. However, the ratio of these terpenoid classes and thus the physical and chemical properties of oleoresin can vary with various stresses such as air pollution (Kainulainen *et al.*, 1993), fertilizer application (Turtola *et al.*, 2002), drought stress (Turtola *et al.*, 2003), herbivory (Tomlin *et al.*, 2000; Miller *et al.*, 2005a), and fungal inoculation (Raffa & Smalley, 1995). Oleoresin can physically 'pitch out' (oleoresin flow pushing the insect out of the site of entry) or entomb attacking insects as well as clean and seal the wound from microorganisms. The volatile monoterpenes and sesquiterpenes in oleoresin evaporate over time to leave nonvolatile diterpenoid acids which form a hardened mass upon polymerization (Langenheim, 2003). This seals the wound, can trap the invader, and acts as a further barrier to insects and associated microorganisms. Induction of a local terpenoid response can be triggered by mechanical damage or herbivory which results in the formation of specialized traumatic resin ducts in the stem xylem (Franceschi *et al.*, 2002; Martin *et al.*, 2002; Byun McKay *et al.*, 2003; Miller *et al.*, 2005a). Specific terpenoids may also be induced and emitted systemically (Martin *et al.*, 2003b; Miller *et al.*, 2005a).

Both conifers and insects exploit terpenoids in this system. Conifers produce terpenoids that are toxic to insects or that negatively affect the physiology of the invading insect or offspring. Herbivorous insects use conifer-produced terpenoids to assist in host selection, to choose their species of host, and to identify weakened trees that they can colonize more easily. They can also detoxify some terpenoids and, in some cases, have even adopted a few of these metabolized terpenoids as pheromones to attract mates or to orchestrate mass attacks which rapidly overcome the defences of a tree (Seybold *et al.*, 2000). Insect predators and parasitoids can use terpenoids, both those produced by the tree and those modified or produced by the attacking insect, as semiochemicals in finding their insect host.

This review focuses on the plant–insect interactions mediated by conifer-produced terpenoids. To understand the various roles of terpenoids in conifer plant–insect interactions better, it is important to establish how conifers produce such a wide diversity of terpenoids as well as to elucidate how insects are affected by these specific compounds at the behavioural and physiological levels. In the following discussion, an exhaustive review of the recent literature will not be presented; rather, selected examples will be used to highlight the current state of research in this area. The first section examines the types of terpenoids produced, the genes and proteins involved in their biosynthesis, how they are regulated, how they function, and how they differ between and within species. The second section examines how insects interact with, elicit, and are affected by, conifer-produced terpenoids.

II. Identification and functional characterization of terpenoid pathway genes

1. Overview

Most of the approximately 30 000 known terpenes are derived from structures synthesized by terpene synthases from one of three common prenyl diphosphate precursors (Chappell, 1995; Dewick, 2002). Monoterpenes (C_{10}) originate from geranyl diphosphate (GPP) (Wise & Croteau, 1999), sesquiterpenes (C_{15}) from farnesyl diphosphate (FPP) (Cane, 1999), and diterpenes (C_{20}) from geranylgeranyl diphosphate (GGPP) (Davis & Croteau, 2000; Martin *et al.*, 2004) (Fig. 1). These three prenyl diphosphate precursors are formed by the condensation of dimethylallyl diphosphate (DMAPP) with one or more isopentenyl diphosphates (IPP), catalysed by specific prenyltransferases (Koyama & Ogura, 1999). Isopentenyl diphosphate originates from one of two pathways, the mevalonate (MEV) pathway in the cytosol (Chappell, 1995; Bochar *et al.*, 1999) or the methyl-erythritol 4-phosphate (MEP) pathway in the plastid (Bochar *et al.*, 1999; Lichtenthaler,

*Dedicated to the memory of Dr Vince R. Franceschi, pioneer researcher in conifer plant defence.

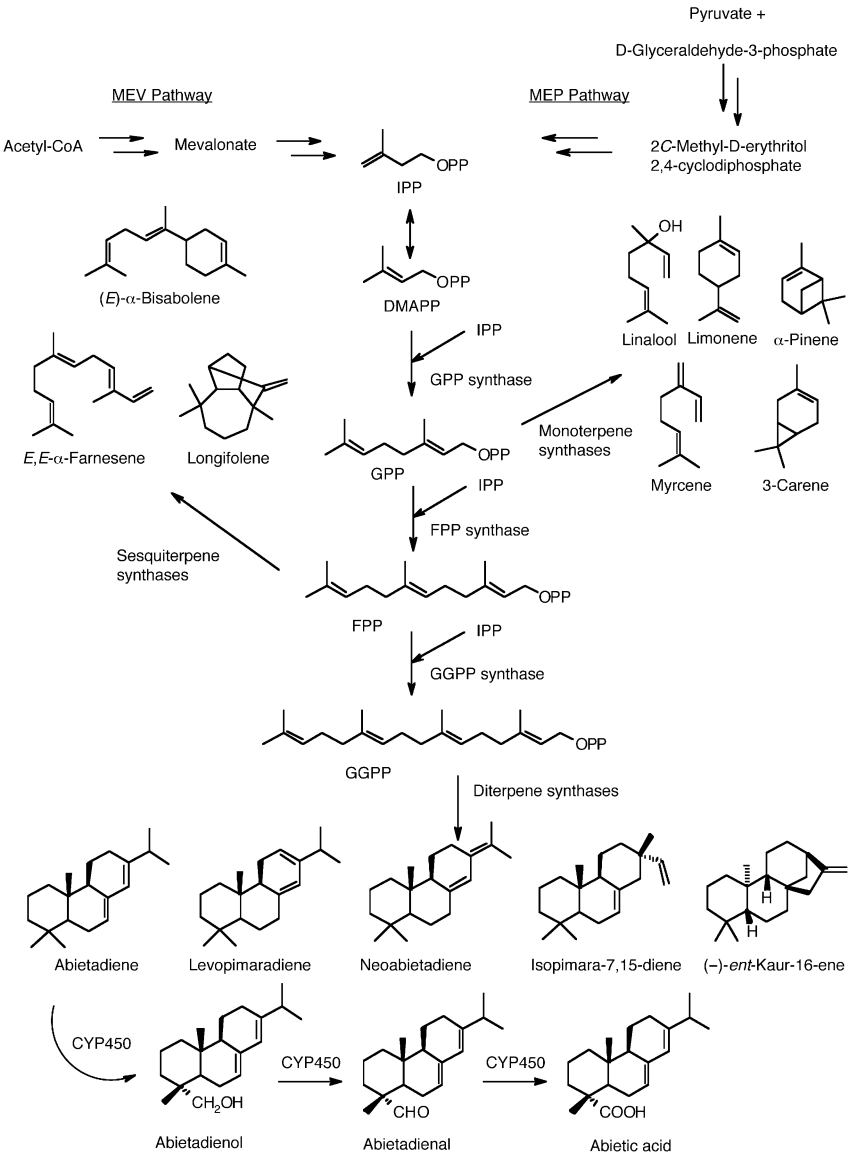

Fig. 1 Biosynthesis of terpenes. Prenyl transferases condense one or more isopentenyl diphosphates (IPPs) with dimethylallyl diphosphate (DMAPP) from the mevalonate (MEV) or methyl-erythritol 4-phosphate (MEP) pathways to produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP), or geranylgeranyl diphosphate (GGPP). Terpene synthases then use these diphosphates as substrates to form the various terpenes. Additional enzymes, such as CYP450s, can further functionalize these terpenes.

1999). Most terpenes are cyclic and many have stereogenic centres. Conifer terpenes, particularly diterpenes, can be further diversified by hydroxylation and further oxidization through the action of cytochrome P450 (CYP450) enzymes (Funk & Croteau, 1994; Jennewein *et al.*, 2004; Ro *et al.*, 2005). The multitude of terpene synthases and modifying CYP450 enzymes, and their varied expression across and within conifer species, make for a very complex area of phytochemistry.

2. Genes involved in the early steps of terpenoid biosynthesis

The genes and enzymes of the early steps in terpene biosynthesis have received relatively little attention in conifers. To our knowledge, no genes of the MEV or MEP pathway from conifers have been functionally characterized, although some work has been completed on prenyltransferases. The geranylgeranyl diphosphate synthase gene from Canada yew (*Taxus canadensis*: Taxaceae) has been isolated and functionally characterized (Hefner *et al.*, 1998) and genes encoding geranyl diphosphate synthase and geranylgeranyl diphosphate synthase have been cloned and characterized in grand fir (*Abies grandis*: Pinaceae) (Tholl *et al.*, 2001; Burke & Croteau, 2002a). Recently, a large-scale sequencing project identified a complete set of full-length cDNAs for the early terpenoid pathway genes in spruce (Picea spp.) (S. Ralph and J. Bohlmann, unpublished results).

3. Terpene synthases

A. Evolution and phylogeny of terpene synthases Terpene synthases (TPSs) comprise a large family of mechanistically

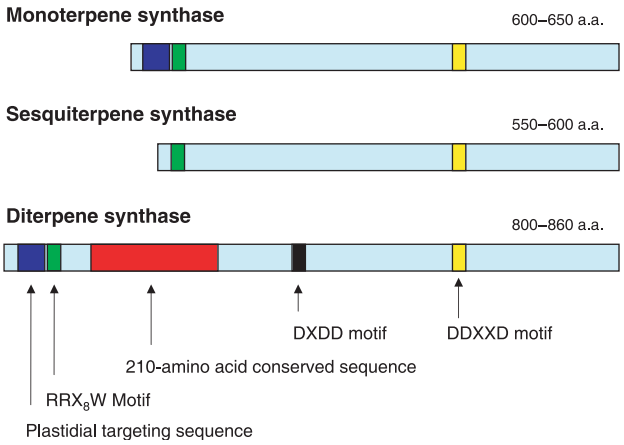

Fig. 2 Schematic of terpene synthase enzymes. a.a., amino acids.

related enzymes involved in both primary and secondary metabolism (Bohlmann *et al.*, 1998b; Martin *et al.*, 2004). Their diversity appears to originate from repeated duplication and subsequent divergence of an ancestral terpene synthase gene of primary metabolism (Bohlmann *et al.*, 1998b; Trapp & Croteau, 2001b). Terpene synthases can be divided into three functional classes, namely monoterpene synthases, sesquiterpene synthases, and diterpene synthases, and are approximately 550–860 amino acids long and 50–100 kDa in size (Fig. 2; Bohlmann *et al.*, 1998b). Sesquiterpene synthases are usually the smallest of these enzymes. Conifer monoterpene synthases are typically 600–650 amino acids long, 50–70 amino acids longer than sesquiterpene synthases, because of their N-terminal plastid-targeting sequence. Conifer diterpene synthases are *c.* 210 amino acids longer than monoterpene synthases because of an additional conserved sequence motif in the N-terminal half of these proteins (Bohlmann *et al.*, 1998b). Interestingly, although not normally found in sesquiterpene synthases, this 210-amino acid feature is found in the sesquiterpene synthases for (*E*)-α-bisabolene in Norway spruce (*Picea abies*: Pinaceae) (Martin *et al.*, 2004) and grand fir (Bohlmann *et al.*, 1998a) and the sesquiterpene synthase for (*E*)-γ-bisabolene in Douglas-fir (*Pseudotsuga menziesii*: Pinaceae) (Huber *et al.*, 2005a), and also in an angiosperm monoterpene synthase (Dudareva *et al.*, 1996). It is also found in all known angiosperm diterpene synthases for the formation of gibberellins. Many terpene synthases have a highly conserved RRX_8W motif near the N-terminus and a DDXXD motif involved in metal cofactor binding. Phylogenetic analysis based on amino acid sequences has grouped the plant terpene synthases into seven subfamilies, named TPS-a to TPS-g (Bohlmann *et al.*, 1998b). Interestingly, the TPS-d subfamily is exclusively comprised of gymnosperm terpene synthases. Recent updates of the TPS-d subfamily to include newly characterized conifer enzymes reinforce the findings of earlier studies and elaborate on the phylogenetic divisions of the TPS-d subfamily (Fig. 3; Martin *et al.*, 2004). The TPS-d subfamily can now be further separated into TPS-d1, TPS-d2, and TPS-d3 groups encompassing primarily monoterpene synthases, sesquiterpene synthases, and primarily diterpene synthases, respectively (Fig. 4; Martin *et al.*, 2004). This analysis also suggests that conifer sesquiterpene synthases evolved independently several times as members of all three divisions, TPS-d1, TPS-d2 and TPS-d3 (Martin *et al.*, 2004).

Comparative genetic mapping of loblolly pine (*Pinus taeda*: Pinaceae) and Douglas-fir suggests conserved chromosomal evolution in conifers (Krutovsky *et al.*, 2004). Although few genomic sequences of terpene synthases have been recovered to date, there are six genomic sequences from grand fir and Pacific yew (*Taxus brevifolia*: Taxaceae) and several from angiosperms. These have provided a framework for an extensive evolutionary analysis resulting in three classes of terpene synthase genes (Trapp & Croteau, 2001b). Trapp & Croteau (2001b) divided genomic sequences of conifer terpene synthases into three classes: classes I, II and III. However, it is important to note that these classes do not refer to classes of terpene synthase gene functions. An original duplication of the ancestral terpene synthase gene resulted in class I genes. This class includes all known angiosperm genes involved in gibberellin metabolism as well as conifer diterpene synthases (some with loss of two introns at the 5′ end) and a few sesquiterpene synthases that retain the 210-amino acid domain but have lost an intron at the 3′ end of the gene. Loss of the 210-amino acid domain differentiates class II terpene synthases, which include the gymnosperm monoterpene and sesquiterpene synthases, from class I. Further loss of several introns differentiates class III genes from class II genes. Class III genes are so far only represented by mono-, sesqui-, and diterpene synthases from angiosperms.

The grouping of conifer terpene synthases as shown in Figs 3 and 4 supports the evolutionary model in which the specialization of these synthases occurred before conifer speciation and functional specialization of terpene synthases occurred independently in angiosperms and conifers (Bohlmann *et al.*, 1998b; Martin *et al.*, 2004). As more terpene synthases are identified in conifers, especially those of primary metabolism (none has been identified to date), their amino acid sequence and genomic structure can be examined within this framework to elucidate the evolution of this highly diverse gene family further. By examining the sequences of homologous enzymes from several conifer species, we can also delineate mutations that lead to functional diversification and place these mutations within the evolutionary context of conifers.

B. Identification and functional characterization of terpene synthases Genomic sequence analysis of *Arabidopsis thaliana* (Brassicales: Brassicaceae) has identified at least 30 putative terpene synthase genes (Aubourg *et al.*, 2002). We would expect at least as many terpene synthases in conifers, if not more. So far, however, only about one-third of this number have been functionally characterized in any one conifer species (Table 1). Several techniques have been used to

Table 1 Functionally characterized enzymes in conifer terpenoid biosynthesis

Enzyme	Species	Accession no.	Reference
Prenyltransferases			
Geranyl diphosphate synthase	*Abies grandis*	AAN01133-5	Burke & Croteau (2002b)
Geranylgeranyl diphosphate synthase	*Abies grandis*	AAL17614	Burke & Croteau (2002a)
Geranylgeranyl diphosphate synthase	*Taxus canadensis*	AAD16018	Hefner *et al*. (1998)
Monoterpene synthases			
(–)-Camphene synthase	*Abies grandis*	AAB70707	Bohlmann *et al*. (1999)
(–)-(4*S*)-Limonene synthase	*Abies grandis*	AAB70907	Bohlmann *et al*. (1997)
(–)-Limonene/(–)-α-pinene synthase	*Abies grandis*	AAF61455	Bohlmann *et al*. (1999)
Myrcene synthase	*Abies grandis*	AAB71084	Bohlmann *et al*. (1997)
β-Phellandrene synthase	*Abies grandis*	AAF61453	Bohlmann *et al*. (1999)
(–)-Pinene synthase	*Abies grandis*	AAB71085	Bohlmann *et al*. (1997)
Terpinolene synthase	*Abies grandis*	AAF61454	Bohlmann *et al*. (1999)
Limonene/borneol synthase	*Chamaecyparis obtusa*	BAC92722	–
(+)-3-Carene synthase	*Picea abies*	AAO73863	Fäldt *et al*. (2003)
(–)-Limonene synthase	*Picea abies*	AAS47694	Martin *et al*. (2004)
(–)-Linalool synthase	*Picea abies*	AAS47693	Martin *et al*. (2004)
Myrcene synthase	*Picea abies*	AAS47696	Martin *et al*. (2004)
(–)-α/β-Pinene synthase	*Picea abies*	AAS47692	Martin *et al*. (2004)
(–)-Limonene synthase	*Picea sitchensis*	–	Byun-McKay *et al*. (2006)
(–)-Pinene synthase	*Picea sitchensis*	AAP72020	Byun McKay *et al*. (2003)
(–)-α-Pinene synthase	*Pinus taeda*	AAO61225	Phillips *et al*. (2003)
(+)-α-Pinene synthase	*Pinus taeda*	AAO61228	Phillips *et al*. (2003)
α-Terpineol synthase	*Pinus taeda*	AAO61227	Phillips *et al*. (2003)
(–)-α-Pinene/(–)-camphene synthase	*Pseudotsuga menziesii*	AAX07267	Huber *et al*. (2005a)
Terpinolene synthase	*Pseudotsuga menziesii*	AAX07264	Huber *et al*. (2005a)
Sesquiterpene synthases			
(*E*)-α-Bisabolene synthase	*Abies grandis*	AAC24192	Bohlmann *et al*. (1998a)
γ-Humulene synthase	*Abies grandis*	AAC05728	Steele *et al*. (1998)
δ-Selinene synthase	*Abies grandis*	AAC05727	Steele *et al*. (1998)
(*E*)-α-Bisabolene synthase	*Picea abies*	AAS47689	Martin *et al*. (2004)
(*E*,*E*)-α-Farnesene synthase	*Picea abies*	AAS47697	Martin *et al*. (2004)
Longifolene synthase	*Picea abies*	AAS47695	Martin *et al*. (2004)
α-Farnesene synthase	*Pinus taeda*	AAO61226	Phillips *et al*. (2003)
(*E*)-γ-Bisabolene synthase	*Pseudotsuga menziesii*	AAX07266	Huber *et al*. (2005a)
(*E*)-β-Farnesene synthase	*Pseudotsuga menziesii*	AAX07265	Huber *et al*. (2005a)
Diterpene synthases			
Abietadiene synthase	*Abies grandis*	AAB05407	Stofer Vogel *et al*. (1996)
Levopimaradiene synthase	*Ginkgo biloba*	AAL09965	Schepmann *et al*. (2001)
Isopimara-7,15-diene synthase	*Picea abies*	AAS47690	Martin *et al*. (2004)
Levopimaradiene/abietadiene synthase	*Picea abies*	AAS47691	Martin *et al*. (2004)
Levopimaradiene/abietadiene synthase	*Pinus taeda*	AAX07435	Ro *et al*. (2005); Ro & Bohlmann (2006)
Taxadiene synthase	*Taxus baccata*	CAC42773	Goerhardt (2001)
Taxadiene synthase	*Taxus brevifolia*	AAC49310	Wildung & Croteau (1996)
Taxa-4(5),11(12)-diene synthase	*Taxus canadensis*	AAR13860	–
Taxadiene synthase	*Taxus chinensis*	AAG02257	Wang *et al*. (2002)
Taxadiene synthase	*Taxus chinensis* var. *mairei*	AAR15329	–
Taxadiene synthase	*Taxus* × *media*	AAS18603	–
P450s			
Abietadienol/abietadienal oxidase (CYP720B1)	*Pinus taeda*	AAX07431	Ro *et al*. (2005)
Taxoid 2-α-hydroxylase	*Taxus canadensis*	AAS89065	Chau and Croteau (2004)
Taxane 13-α-hydroxylase	*Taxus chinensis*	AAX59903	–
5-α-Taxadienol-10-β-hydroxylase	*Taxus chinensis*	AAN52360	Tu *et al*. (2004)
Taxoid 2-α-hydroxylase	*Taxus chinensis*	AAV54171	–
Taxadiene 5-α-hydroxylase	*Taxus cuspidata*	AAQ56240	Jennewein *et al*. (2004)
Taxane 13-α-hydroxylase (CYP725A2)	*Taxus cuspidata*	AAL23619	Jennewein *et al*. (2001)
Taxane 14-β-hydroxylase (CYP725A3)	*Taxus cuspidata*	AAO66199	Jennewein *et al*. (2003)
Taxoid 7-β-hydroxylase	*Taxus cuspidata*	AAQ75553	Chau *et al*. (2004)
5-α-Taxadienol-10-β-hydroxylase (CYP725A1)	*Taxus cuspidata*	AAK00946	Schoendorf *et al*. (2001)
Taxane 13-α-hydroxylase	*Taxus* × *media*	AAX20147	–

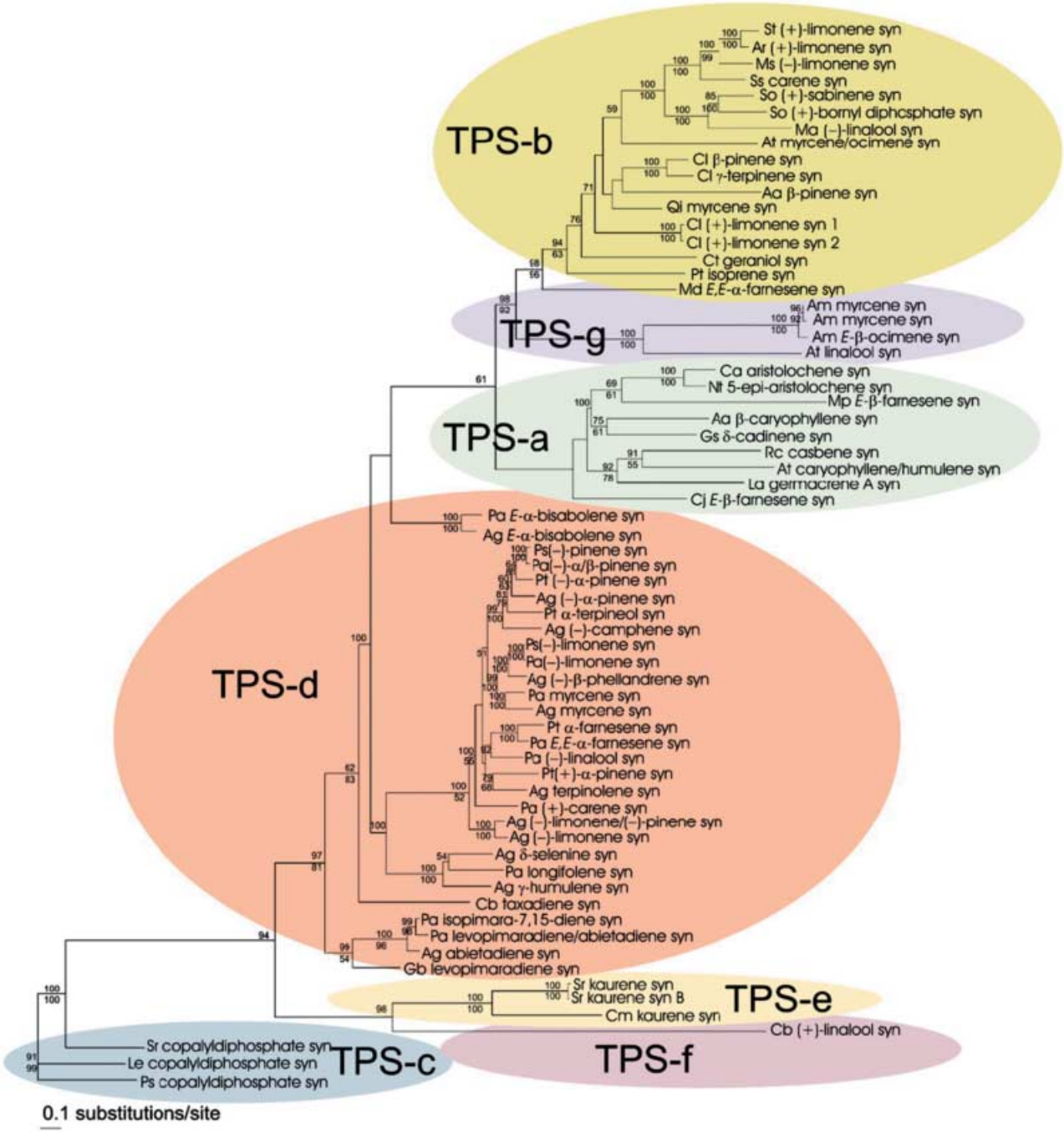

Fig. 3 Phylogenetic tree of terpene synthases involved in primary and secondary metabolism in angiosperms and gymnosperms. (Reproduced with permission from Martin *et al*., 2004; copyright of the American Society of Plant Biologists.) Aa, *Artemisia annua*; Ag, *Abies grandis*; Am, *Antirrhinum majus*; Ar, *Agastache rugosa*; At, *Arabidopsis thaliana*; Ca, *Capsicum annuum*; Cb, *Clarkia breweri*; Cb, *Taxus brevifolia* (for taxadiene syn only); Cj, *Citrus junos*; Cl, *Citrus limon*; Cm, *Cucurbita maxima*; Ct, *Cinnamomum tenuipilum*; Gb, *Ginkgo biloba*; Gs, *Gossypium arboretum*; La, *Lactuca sativa*; Le, *Lycopersicon esculentum*; Ma, *Mentha aquatica*; Md, *Malus x domestica*; Mp, *Mentha x piperita*; Ms, *Mentha spicata*; Nt, *Nicotiana attenuata*; Pa, *Picea abies*; Ps, *Pisum sativum* (for copalyldiphosphate syn only); Ps, *Picea sitchensis*; Pt, *Pinus taeda*; Pt, *Populus alba x Populus tremula* (for isoprene syn only); Qi, *Quercus ilex L*.; Rc, *Ricinus communis*; So, *Salvia officinalis*; Sr, *Stevia rebaudiana*; Ss, *Salvia stenophylla*; St, *Schizonepeta tenuifoli*

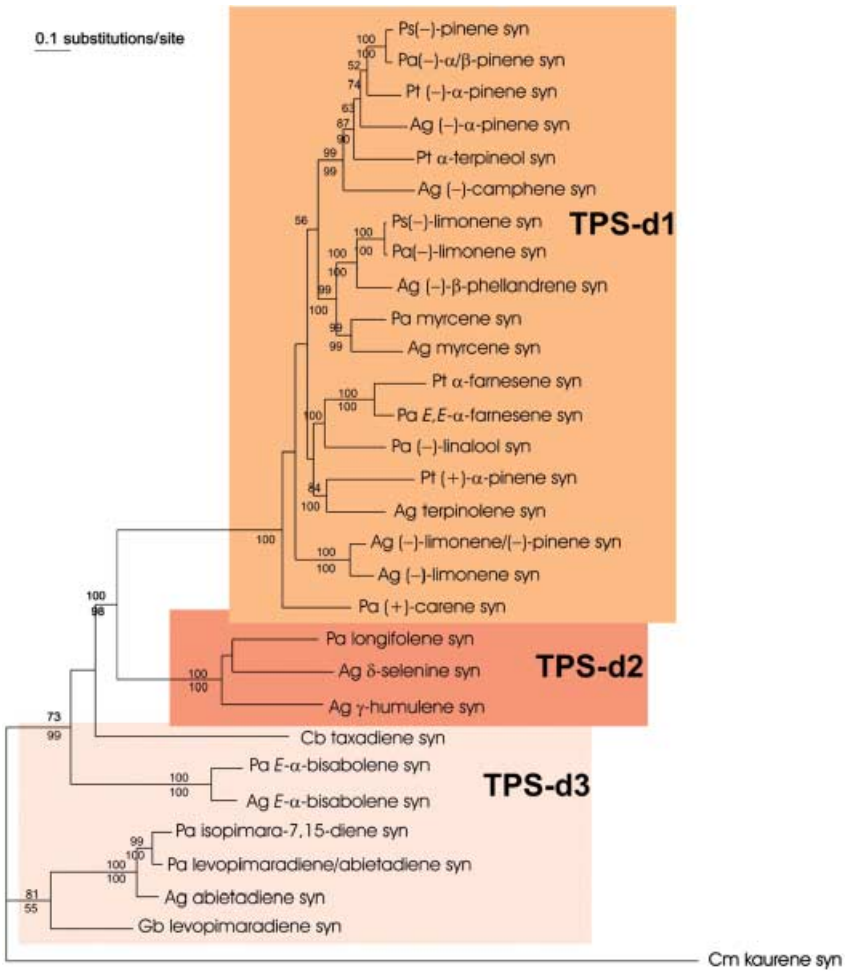

Fig. 4 Phylogenetic tree of gymnosperm terpene synthases. (Reproduced with permission from Martin *et al*., 2004; copyright of the American Society of Plant Biologists.) Aa, *Artemisia annua*; Ag, *Abies grandis*; Am, *Antirrhinum majus*; Ar, *Agastache rugosa*; At, *Arabidopsis thaliana*; Ca, *Capsicum annuum*; Cb, *Clarkia breweri*; Cb, *Taxus brevifolia* (for taxadiene syn only); Cj, *Citrus junos*; Cl, *Citrus limon*; Cm, *Cucurbita maxima*; Ct, *Cinnamomum tenuipilum*; Gb, *Ginkgo biloba*; Gs, *Gossypium arboretum*; La, *Lactuca sativa*; Le, *Lycopersicon esculentum*; Ma, *Mentha aquatica*; Md, *Malus x domestica*; Mp, *Mentha x piperita*; Ms, *Mentha spicata*; Nt, *Nicotiana attenuata*; Pa, *Picea abies*; Ps, *Pisum sativum* (for copalyldiphosphate syn only); Ps, *Picea sitchensis*; Pt, *Pinus taeda*; Pt, *Populus alba x Populus tremula* (for isoprene syn only); Qi, *Quercus ilex L*.; Rc, *Ricinus communis*; So, *Salvia officinalis*; Sr, *Stevia rebaudiana*; Ss, *Salvia stenophylla*; St, *Schizonepeta tenuifoli*

identify new terpene synthases, including reverse genetics, cDNA library screening, and similarity-based polymerase chain reaction (PCR) cloning approaches (reviewed by Bohlmann *et al*., 1998b). More recently, expressed sequence tag (EST) resources have become available for loblolly pine (Allona *et al*., 1998; Dong *et al*., 2004), Japanese cedar (*Cryptomeria japonica*: Cupressaceae) (Ujino-Ihara *et al*., 2000), and white (*Picea glauca*: Pinaceae), interior (*P. glauca* × *engelmanni*), and Sitka (*Picea sitchensis*) spruce species (S. Ralph and J. Bohlmann, unpublished results). In addition, efforts are currently underway to identify and characterize all the terpene synthases in these spruce species using a combination of targeted cDNA cloning and large-scale EST and full-length cDNA mining (Byun McKay *et al*., 2003; Fäldt *et al*., 2003; Martin *et al*., 2004; Miller *et al*., 2005a; C. I. Keeling, S. Ralph and J. Bohlmann, unpublished results).

Functional characterization of terpene synthases is usually achieved by heterologous expression in *Escherichia coli*, incubation of the soluble recombinant enzyme with GPP, FPP, or GGPP in the presence of Mg^{2+} and/or Mn^{2+}, and analysis of the nonpolar products by gas chromatography–mass spectrometry (e.g. Martin *et al*., 2004; O'Maille *et al*., 2004). Two difficulties have been encountered with expression in *E. coli*. First, monoterpene and diterpene synthases are translated as preproteins with an N-terminal transit peptide for targeting to the plastid. With bacterial expression, this transit peptide can sometimes result in inclusion bodies, and thus truncated constructs to remove the transit peptide are sometimes necessary. Secondly, conifer genes use codons that require tRNAs that are rare in the prokaryotic host. Often, cotransformation of a plasmid encoding the rare tRNAs is necessary for sufficiently high expression of recombinant enzymes (Hohn, 1999).

C. Biochemistry and mechanisms of terpene synthases

Conifers produce a large variety of terpenoids that vary both across and within species. This great diversity arises from the expression of multiple terpene synthases and from the multiple products formed by certain individual terpene synthases. Although all terpene synthases use DMAPP, GPP, FPP or GGPP as a substrate, predicting the product profile is almost impossible from the sequence alone. Even if the enzymes have high sequence similarity, some synthases produce multiple products while others produce only one compound. For example, the γ-humulene synthase of grand fir produces 52

unique sesquiterpene products (Steele *et al.*, 1998). Elucidation of the mechanisms of terpene biosynthesis may illuminate the possible origins of conifer resistance, as the mutation of a single amino acid can dramatically change the product profile of an enzyme and thus may affect the behaviour or physiology of a herbivorous insect. Many studies have examined the mechanistic requirements for the activity and product specificity of terpene synthases. These studies have been reviewed for monoterpene synthases (Wise & Croteau, 1999), sesquiterpene synthases (Cane, 1999), and diterpene synthases (Hohn, 1999). Common to most terpene synthases is the ionization of the diphosphate group of the substrate to yield a carbocation that may undergo various cyclizations or rearrangements before being terminated by deprotonation or nucleophile capture (e.g. by water). A divalent metal ion (Mg^{2+} or Mn^{2+}) is also a requirement for catalysis. Monoterpene and diterpene synthases show substrate specificity for GPP and GGPP, respectively. Sesquiterpene synthases appear to accept substrates with less specificity. Some recombinant sesquiterpene synthases may inefficiently use GPP as a substrate, but, under physiological conditions, cytosolic sesquiterpene synthases are not likely to encounter plastid-formed GPP. However, recent studies have revealed a sesquiterpene synthase in the plastid (Aharoni *et al.*, 2004), and also plastid-derived IPP crossing over to the cytosol (Laule *et al.*, 2003; Dudareva *et al.*, 2005). The unique products of a specific terpene synthase are determined by how the enzyme imposes specific conformations on substrate and intermediates while protecting the carbocation intermediate from early termination (Cane, 1999; Wise & Croteau, 1999; Segura *et al.*, 2003). The three-dimensional structure has not been reported for any conifer terpene synthase. However, X-ray structures of the monoterpene synthase bornyl diphosphate synthase from sage (*Salvia officinalis*: Lamiales: Lamiaceae) (Whittington *et al.*, 2002) and the sesquiterpene synthase 5-epi-aristolochene synthase from tobacco (*Nicotiana tabacum*: Solanales: Solanaceae) (Starks *et al.*, 1997) provide structural models for mechanistic studies in conifer terpene synthases.

In grand fir, a pair of monoterpene synthases with high sequence identity, (–)-(4*S*)-limonene synthase and (–)-(4*S*)-limonene/(–)-(1*S*,5*S*)-α-pinene synthase, have been studied through domain-swapping and reciprocal site-directed mutagenesis (Katoh *et al.*, 2004). Two sesquiterpene synthases from grand fir, δ-selinene synthase and γ-humulene synthase, have also been examined using site-directed mutagenesis and truncated constructs (Little & Croteau, 2002). These studies could identify specific amino acids that are critical in determining the product profile. Another pair of synthases, (–)-pinene synthase and (–)-camphene synthase, have also been the subject of a similar study (Hyatt & Croteau, 2005). In this case, the synergistic action of several amino acids, located both within and distant from the putative active site, are important for determining product profiles. These studies highlight the complexity of predicting product profiles based on sequence similarity and show that specific mutations within the active site, or other possible mutations throughout the enzyme, can affect the diversity of products. Such flexibility is an important consideration when comparing sequences between species and studying the evolution of terpene synthase diversity.

The mechanistic aspects of the multiproduct abietadiene synthase in grand fir, a diterpene synthase, have been extensively studied (Peters *et al.*, 2000, 2001, 2003; Ravn *et al.*, 2000, 2002; Peters & Croteau, 2002a,b). This enzyme has two active sites, an N-terminal domain containing a D*X*DD motif that mediates a protonation-initiated cyclization of GGPP to form (+)-copalyl diphosphate (CPP), and a C-terminal domain containing the DD*XX*D motif, conserved in terpene synthases, that catalyses the ionization of CPP and subsequent cyclization to produce the diterpene hydrocarbons via a carbocation intermediate (Peters *et al.*, 2001). Site-directed mutagenesis and investigations with truncated recombinant enzymes have delineated the important residues for catalysis (Peters *et al.*, 2000, 2001, 2003; Peters & Croteau, 2002b). Related to this abietadiene synthase is a pair of paralogous diterpene synthases from Norway spruce with high sequence identity to each other but nonoverlapping product profiles (Martin *et al.*, 2004). One enzyme, isopimaradiene synthase, produces exclusively isopimara-7,15-diene, whereas levopimaradiene/abietadiene synthase produces the same mixture of abietadiene, levopimaradiene, neoabietadiene, and palustradiene as does the abietadiene synthase from grand fir. The important amino acids for product specificity are being determined through domain-swapping and reciprocal site-directed mutagenesis, and single amino acid mutations can completely alter product profiles (C. I. Keeling and J. Bohlmann, unpublished results). Orthologous levopimaradiene/abietadiene-like synthases from loblolly pine (Ro & Bohlmann, 2006) and ginkgo (*Ginkgo biloba*: Ginkgoaceae) (Schepmann *et al.*, 2001) provide comparisons for studying the biochemical origins of these different product profiles across taxa.

4. Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (CYP450) are important in the diversification of terpenoids through oxidative functionalization. These haeme-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 & Werck-Reichhart, 2003). The total number of CYP450 genes in conifers is unknown, but there are 272 and 458 in the *A. thaliana* and rice (*Oryza sativa*: Poales: Poaceae) genomes, respectively, and 99 have so far been identified in loblolly pine (http://drnelson.utmem.edu/CytochromeP450.html).

Diterpene resin acids such as abietic acid (Fig. 5) are important components of conifer oleoresin biosynthesized through the action of CYP450s. Studies with grand fir and lodgepole pine (*Pinus contorta*: Pinaceae) tissue extracts showed that

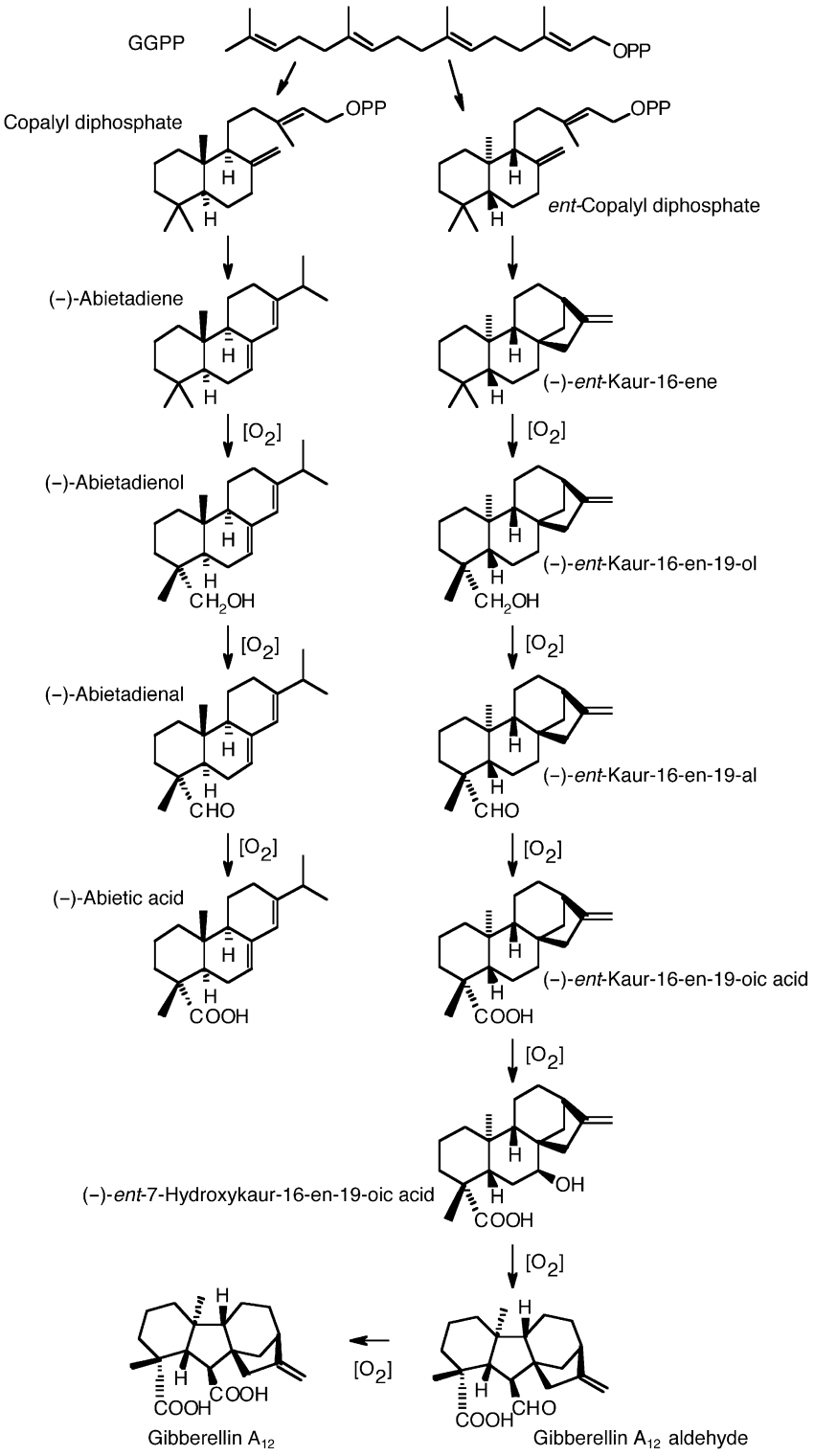

Fig. 5 Parallel biosyntheses of abietic acid and gibberellin. [O_2] indicates an oxidation step thought to be mediated by an NADPH-dependent CYP450.

stepwise oxidation of the diterpene abietadiene to abietic acid can be achieved by membrane-bound CYP450 and soluble aldehyde dehydrogenase enzyme activities (Funk & Croteau, 1994; Funk *et al.*, 1994). The general pathway scheme of oxidation of abietadiene to abietic acid in conifer secondary metabolism parallels that of oxidation of *ent*-kaurene to *ent*-kaurenoic acid in the biosynthesis of gibberellin phytohormones (Fig. 5). A multifunctional CYP450 (CYP701A3) responsible for the three-step oxidation of *ent*-kaurene to kaurenoic acid has previously been identified by using genetic

approaches in *A. thaliana* (Helliwell *et al.*, 1998, 1999). A multifunctional CYP450 (CYP88A) also catalyses the subsequent three-step oxidation from kaurenoic acid to gibberellin A12 in *A. thaliana* (Helliwell *et al.*, 2001).

Recently, a CYP450 cDNA from loblolly pine involved in resin acid biosynthesis was cloned, heterologously expressed in yeast, and functionally characterized (Ro *et al.*, 2005). This enzyme (CYP720B1) is responsible for oxidizing abietadienol and abietadienal to abietic acid and may also hydroxylate abietadiene to abietadienol (Fig. 5). It is also able to oxidize the alcohol and aldehyde forms of the related diterpenes dehydroabietadiene, isopimaradiene, and levopimaradiene but not neoabietadiene (Fig. 1). Thus, this one multifunctional, multisubstrate CYP450 enzyme forms an array of resin acid components. However, this is only one of several CYP450s predicted to act on terpenoids to diversify the chemical nature of the oleoresin.

Several CYP450s acting on diterpenoids have been also characterized in several yew species (*Taxus* spp.: Taxaceae) (Table 1). However, the intense interest in these enzymes is a consequence not of their importance in plant–insect interactions, but rather of their involvement in the biosynthesis of taxol, an important anticancer agent first isolated from the bark of the Pacific yew tree.

5. Regulation of terpenoid biosynthesis

Terpenoid defences exist constitutively in many conifers but they are also inducible upon insect herbivory, oviposition, or fungal inoculation. Conifer terpenoid defences are not without costs to the tree. An evaluation of the metabolic costs of terpenoids suggests that, by weight, they are some of the most expensive secondary or primary metabolites to produce (Gershenzon, 1994). Both constitutive and induced defensive responses in plants require substantial redirection of resources away from growth and reproduction. An excellent demonstration of this exists for an angiosperm (Baldwin, 1998). Induced responses are thought to be less costly as the resources are only used in response to a clear attack detected by the plant. However, induced responses may not act fast enough in some situations to be effective, such as when responding to a rapid, pheromone-mediated, mass attack by bark beetles carrying symbiotic pathogenic fungi. Less severe local wounding may stimulate systemic effects that prepare the whole tree for subsequent herbivory. Inter-tree signalling may also induce a defensive response in adjacent trees. Elucidation of the mechanism of induction of the terpenoid defence response is currently an area of active research in our and a few other laboratories.

Insects are known to induce terpenoid production in several conifer systems. For example, monoterpene synthase activities in needles of Pacific ponderosa pine (*Pinus ponderosa*: Pinaceae), lodgepole pine, and white fir (*Abies concolor*: Pinaceae) increase upon tiger moth (*Halisdota ingens*: Lepidoptera) larva feeding (Litvak & Monson, 1998). Feeding by the white pine weevil (*Pissodes strobi*: Coleoptera) on Sitka spruce induces traumatic resin accumulation in stems and also induces expression of several terpene synthase transcripts, such as the (–)-pinene synthase that catalyses the synthesis of (–)-α-pinene and (–)-β-pinene (Byun McKay *et al.*, 2003; Miller *et al.*, 2005a). One terpenoid that was uniquely up-regulated as an induced volatile emission by weevil feeding was (–)-linalool (Miller *et al.*, 2005a). Although physiochemical effects may be involved (Niinemets *et al.*, 2002, 2004), the unusual diurnal variation of (–)-linalool emission suggests that this compound in particular is controlled by active mechanisms upon herbivory (Miller *et al.*, 2005a). Induced gene expression of (–)-linalool synthase in needles of Sitka spruce further supports a model of active volatile emission as an induced stress response (Miller *et al.*, 2005a). Fungal inoculation can also induce changes in the terpenoid profile (Raffa & Berryman, 1982; Raffa & Smalley, 1995).

The past few years have seen significant progress in the anatomical, biochemical and molecular characterization of the induced terpenoid response in conifers. Much of this progress has been a result of research focused on spruce species as well as comparative analysis in other conifer species (Huber *et al.*, 2004; Martin & Bohlmann, 2005). In addition to using actual insect herbivory to study the conifer terpenoid response, researchers have used mechanical wounding or treatment with chemical elicitors such as methyl jasmonate and ethylene to mimic insect herbivory or oviposition. Jasmonic acid and methyl jasmonate (MeJA) are important signalling molecules for plant response upon mechanical wounding, herbivory, oviposition or fungal inoculation (Halitschke & Baldwin, 2005; Howe, 2005). Topical application of methyl jasmonate has been used as a noninvasive method to mimic herbivory and induce the biochemical and molecular terpenoid response in conifers (e.g. Martin *et al.*, 2002, 2003b; Miller *et al.*, 2005a). However, such treatment does not simulate the physical damage of foraging, nor does it account for insect-produced elicitors that may be present in saliva or on the egg coat. The mechanism by which MeJA elicits the terpenoid response has not been fully explained, but recent evidence suggests that it is mediated by ethylene (Hudgins & Franceschi, 2004).

Norway spruce has been treated with MeJA in several studies of the terpenoid defence response. In the xylem, MeJA treatment induces traumatic resin duct formation, terpenoid accumulation, and the induction of prenyltransferase and terpene synthase activities (Martin *et al.*, 2002). In the foliage, MeJA treatment results in significant but less dramatic increases in terpenoid accumulation and specific changes in volatile terpenoid emission (Martin *et al.*, 2003b). In particular, the emission of linalool and (*E*)-β-farnesene increased dramatically, following a diurnal rhythm with maximum emissions during the light period. In stem tissue, transcript levels of both monoterpene and diterpene synthases increased transiently after MeJA treatment (Fäldt *et al.*, 2003).

Recently, the volatile emissions, oleoresin components, and terpene synthase transcript levels in Sitka spruce were analysed upon treatment with MeJA (Miller *et al.*, 2005a). This treatment induced traumatic resin accumulation in stems and the up-regulation of several terpene synthase transcripts. However, weevil feeding resulted in higher accumulations of resin and more complex emission profiles.

Watering the roots of Douglas-fir seedlings with a dilute solution of MeJA induces traumatic resin duct formation in roots and stems as well as specific changes in the terpenoid profile of roots, stem and foliage (Huber *et al.*, 2005b). Several monoterpenoids, sesquiterpenes, and diterpene acids increased significantly in roots and stems upon treatment, while (*E*)-β-ocimene decreased and linalool and α-humulene increased significantly in foliage.

Relatively little research has been carried out on the regulation of the early terpenoid pathway genes in conifers. Mechanical wounding does not affect the activity of the early pathway enzymes geranyl and farnesyl diphosphate synthases in grand fir (Tholl *et al.*, 2001), but MeJA treatment does induce prenyltransferase activity in Norway spruce (Martin *et al.*, 2002). MeJA also appears to affect CYP450 enzymes in resin acid biosynthesis. It up-regulates both the loblolly pine levopimaradiene/abietadiene synthase and the CYP450 abietadienol/abietadienal oxidase (Ro *et al.*, 2005), suggesting that diterpine synthesis and subsequent diterpine oxidation are coordinately regulated in the formation of diterpine resin acids.

III. Insect interactions with conifers

1. Overview

The existence in conifers of a large terpene synthase gene family, the physical structures to store and release oleoresin, and the constitutive and inducible responses to herbivory suggest that insects exert a strong selective pressure on conifers to maintain and diversify their terpenoid defences. However, there is little evidence to indicate that insects are in fact exerting this selective pressure or that defence is the *raison d'etre* of conifer-produced terpenoids. Although a thorough evolutionary examination of terpenoid-mediated conifer–insect interactions is beyond the scope of this review, one current theory suggests a sequential evolution whereby insect speciation may result from changes in conifers, but insects do not put sufficient selective pressure on conifers to elicit speciation (Rausher, 1992; Schoonhoven *et al.*, 1998). In this framework, genetic changes to the host recognition mechanism of the insect results in an insect identifying a different species of tree as a host tree. Selection then occurs based upon the availability of this new host, the presence of competitors and predators, and the adaptation of the insect to the physiological constraints of the host (including terpenoids). Thus, conifer–insect interactions mediated by terpenoids involve both behavioural and physiological aspects.

2. Behavioural and physiological effects of terpenoids

Conifer terpenoids have a variety of influences on forest insects. Physically, the copious amounts of oleoresin mobilized to the site of wounding can often trap, drown, or pitch out the invading insect. Upon evaporation of the volatile mono- and sesquiterpenes, the semicrystallized diterpene resin acids can polymerize to protect the site of attack further. Physiologically, the terpenoids can be toxic to insects or can negatively impact the development or survival of insect offspring. Terpenoids are involved in a dizzying and sometimes contradictory array of semiochemical interactions between a host and its associates, including host selection and tri–trophic interactions. Host specialization may be one strategy for overcoming or exploiting a highly diverse array of conifer defences. For example, insects that can distinguish suitable hosts from nonhosts by host volatiles may have an adaptive advantage, and, in fact, many coniferophagus insects are highly specialized to only one or a few host species. The ability of insects to differentiate host from nonhost or to identify weakened hosts by making use of conifer volatiles minimizes the time, energy and risks associated with attempting to colonize unsuitable hosts. In addition, adaptations such as pheromone-mediated mass attack and the vectoring of symbiotic fungi by bark beetles can deplete the defensive capacity of conifers before an induced response is sufficient to prevent a successful attack.

There are many important considerations in insect host selection. In addition to the variation seen across tree species, genotypes and physiological states in the terpenoids that insects may use in host selection, insects vary, among individuals and within the same individual over time and with population density, in their response to these terpenoids. The heritability of host selection behaviour has been shown in pine engraver beetles (*Ips pini*: Coleoptera) (Wallin *et al.*, 2002) and has been inferred in the spruce beetle (*Dendroctonus rufipennis*: Coleoptera) (Wallin & Raffa, 2004). In laboratory host choice bioassays, pine engraver beetles increase their acceptance of host material after they have been previously exposed to monoterpenes (Wallin & Raffa, 2002b). They are also more accepting of host material containing monoterpenes such as limonene and α-pinene when beetle densities are greater (Wallin & Raffa, 2002a). Changes in host selection behaviour with population density appear partially heritable in the spruce beetle. Compared with endemic populations, beetles from eruptive populations are more tolerant of high α-pinene levels with increasing population density (Wallin & Raffa, 2004). Mountain pine beetles (*Dendroctonus ponderosae*: Coleoptera) also show differences in responses to the pheromones *cis*- and *trans*- verbenol and exo-brevicomin with population density (Miller *et al.*, 2005b). These studies help to explain the complex dynamics of bark beetle infestations and why eruptive populations are more likely to attack healthy trees that endemic populations would normally avoid.

Much research is still required to understand the mechanisms of host resistance to insect attack as well as to understand the effects of terpenoids on insects and their associated microorganisms. Some terpenoids may act as feeding deterrents (Lindgren *et al.*, 1996). Others, such as limonene, act by inhibiting the attraction of pine weevils (*Hylobius abietis* and *Hylobius pinastrito*: Coleoptera) to α-pinene (Nordlander, 1990). Several monoterpenes have been identified as directly toxic to bark beetles (Cook & Hain, 1988; Raffa & Smalley, 1995; Werner, 1995). One study suggested that resistant Sitka spruce trees prevent the induction of vitellogenin gene expression in feeding white pine weevils, resulting in incomplete ovary development and no egg development (Leal *et al.*, 1997). The underlying molecular mechanism behind this resistance is not yet known. However, conifers do produce compounds that mimic juvenile hormone, an important hormone in insects for development and reproduction. Several sesquiterpenes structurally related to (*E*)-α-bisabolene (Fig. 1), such as juvabione and todomatuic acid, are produced in fir species (Rogers & Manville, 1972; Bohlmann *et al.*, 1998a; Fowler *et al.*, 2001), and have juvenile hormone-like activity in insects (reviewed in Fowler *et al.*, 2001). In testing any of these terpenoids for toxicity or the modification of insect physiology or behaviour, it should be borne in mind that they must be active at biologically relevant concentrations to be important in conifer defence in a natural situation.

Despite the large amounts and chemical diversity of constitutive and induced diterpenoids in conifer resins, the effects of diterpenoids on insects have not been extensively studied. High concentrations of diterpenes in young needles may deter pine sawfly (*Neodiprion* spp.: Hymenoptera) larva feeding and cause increased feeding on mature needles (Ikeda *et al.*, 1977). In Sitka spruce, trees resistant to attack by the white pine weevil produce more diterpene resin acids, and the production of resin acids in these trees is more strongly induced by wounding compared with susceptible trees (Tomlin *et al.*, 1996, 2000). There is also evidence that certain diterpene resin acids do not directly affect certain bark beetles but instead could affect a beetle-associated fungus. For example, abietic acid, dehydroabietic acid and isopimaric acid have no effect on host acceptance behaviour or larval survival for the bark beetle *I. pini* but do influence associated fungi (Kopper *et al.*, 2005).

3. Tree resistance

The resistance or susceptibility of a tree to herbivory by a particular insect is a result of many factors, of which the terpenoid profile is just one. The volatile terpenoid emissions may not be an attractive blend for the insect (Pureswaran & Borden, 2003); the emission may also fail to synergize with insect pheromone components and thus fail to attract the opposite sex or elicit a mass attack (Miller & Borden, 2000; Pureswaran & Borden, 2005), or it may effectively attract insect parasitoids or predators that prey on the insect herbivore. The constitutive and induced oleoresin response in the host may be sufficient and rapid enough to prevent extensive damage. In addition, the oleoresin may contain specific components that are toxic or deterrent to insect herbivores or may physiologically affect the adult or brood and thus prevent successful colonization or reproduction. The tree may also be able to sense the herbivory of nearby trees (through either underground signals or volatiles in the air) and activate its defences before it is itself attacked. Although this has not been demonstrated thus far in conifers, plant–plant signalling has been identified in angiosperms and there are examples of signalling between plants mediated by herbivory-induced terpenoids in angiosperms (reviewed by Dicke *et al.*, 2003; Paschold *et al.*, 2006).

Conifer terpenoid profiles vary geographically (e.g. Manninen *et al.*, 2002), across closely related taxa (Fäldt *et al.*, 2001) and among genotypes of the same species (Martin *et al.*, 2003b). Within a species, several examples exist of differences in tree resistance to specific insects, and some of these correlate with terpenoid profiles. For example, limonene and other terpenes correlate with resistance of Italian stone pine (*Pinus pinea*: Pinaceae) and Aleppo pine (*Pinus halepensis*) to attack by the scale insect (*Marchalina hellenica*: Homoptera) (Mita *et al.*, 2002). Bornyl acetate (Cates *et al.*, 1987) and other terpenes (Chen *et al.*, 2002) are important in the interaction between the western spruce budworm (*Choristoneura occidentalis*: Lepidoptera) and its Douglas-fir host. Resistance of Sitka and white spruce to the white pine weevil appears to be related to the content of diterpene acids or their inducibility (Tomlin *et al.*, 1996, 1998, 2000) and also appears to be correlated with the abundance of specific monoterpenes (Harris *et al.*, 1983). At the molecular level, genotypic differences in resistance of Sitka spruce to white pine weevil correlate with induction of TPS gene expression (Byun-McKay *et al.*, 2006). Resistance of lodgepole pine to attack by the Douglas-fir pitch moth (*Synanthedon novaroensis*: Lepidoptera) varies among clones, and this variation has been correlated with the quantity of the specific monoterpene 3-carene (Rocchini *et al.*, 2000).

Even within the same tree, local differences in terpenoid content result in localization of herbivores to specific areas of the tree. For example, species of spruce aphid (*Elatobium abietinum*, *Cinara pilicornis*, *Cinara costata* and *Cinara pruinosa*: Hemiptera) have differing tolerances to myrcene which parallel the abundance of myrcene in the specific microhabitat each species inhabits on the tree (Jackson *et al.*, 1996). Local terpenoid content may also affect feeding behaviour. Tiger moth larvae start feeding on ponderosa pine needles at the tip and then switch to another needle after consuming about half of the length of the needle. This feeding pattern is consistent with the monoterpene content of the needle, as the base of the needle has a higher total monoterpene concentration, additional monoterpene components, and higher monoterpene synthase activities than the needle tip (Litvak & Monson, 1998). In addition, the anatomy of the resin canal system

varies with species, influencing the success of the white pine weevil feeding upon different spruce and pine species (Boucher *et al.*, 2001).

4. Bark beetles

A. Bark beetle pheromone synergists and precursors Conifer terpenoids play an important role in insect semiochemistry. The interactions between conifer monoterpenes and bark beetles have been particularly well studied (reviewed by Seybold *et al.*, 2006). Some host terpenes are known to synergize with bark beetle pheromones in eliciting the mass attack of a host tree, while other, nonhost volatiles may be inhibitory (Seybold *et al.*, 2000). Many bark beetle pheromones are terpenoid. They once were thought to be derived from simple oxidation of host terpenes for detoxification and to have evolved to act as pheromones. However, there is now clear evidence that at least some monoterpenoid bark beetle pheromone components are made *de novo* via the mevalonate pathway (reviewed by Seybold & Tittiger, 2003). Both the first metazoan GPP synthase (Gilg *et al.*, 2005) and the activity of a monoterpene synthase, myrcene synthase (Martin *et al.*, 2003a), have been identified in the pine engraver beetle. In addition, feeding on conifer phloem induces the mevalonate pathway of this insect (Keeling *et al.*, 2004). Thus, conifers are not the only producers of terpenes in this plant–insect interaction. Other pheromone components, such as *trans*-verbenol, have not yet been shown to be made *de novo* by beetles and may result from insect-oxidized host terpenes such as α-pinene. Insect P450 monooxygenases are likely to play important roles in these detoxification or pheromone biosynthesis steps (Brattsten, 1983; Tittiger *et al.*, 2005; Keeling *et al.*, 2006; Seybold *et al.*, 2006). Thus, the combination of oxidized and unmetabolized plant chemicals may signal that the pioneering beetles have already successfully entered a suitable host tree, encouraging other beetles to aggregate and defeat the defences of this host.

B. Beetle-associated pathogens Integral to the plant–insect interactions of most bark beetles are beetle-associated fungi (Paine *et al.*, 1997). The combined force of beetle and fungi can launch a more formidable attack on the host tree, and the evolution of mycangia on some beetle species to carry fungi between hosts suggests a benefit from this association. In addition, mycangial fungi may be involved beyond the disruption of host tree defences. Some wood-boring beetles feed on ambrosia fungi, and the southern pine beetle (*Dendroctonus frontalis*: Coleoptera) and the western pine beetle (*Dendroctonus brevicomis*) larvae feed on either the mycangial fungus itself or the fungal-modified host tissue (Paine *et al.*, 1997).

Several conifer-produced terpenoids affect beetle-associated fungi, and so the host choice of a beetle may be important not only for its own direct survival, but also for the survival of its symbiotic mycangial fungi. The monoterpenes γ-terpinene and α-pinene inhibit growth and germination of *Leptographium* spp. (Ophiostomatales) (Klepzig *et al.*, 1996), and myrcene inhibits growth of the fir engraver beetle (*Scolytus ventralis*: Coleoptera)-associated fungus *Trichosporium symbioticum* (Deuteromycetes) (Raffa *et al.*, 1985). Diterpene acids affect the pine engraver beetle-associated fungus *Ophiostoma ips* (Ophiostomatales); abietic and isopimaric acids strongly inhibit spore germination and abietic acid strongly inhibits mycelial growth in this fungus (Kopper *et al.*, 2005). Inoculation of bark beetle-associated fungi induces terpenoid production in conifers (Raffa & Berryman, 1982) and may also induce the attraction of bark beetle parasitoids (Sullivan & Berisford, 2004).

Beetle-associated nematodes add further complexity to the beetle–fungi interaction. For example, the pine sawyer beetle (*Monochamus* spp.: Coleoptera) vectors the pinewood nematode (*Bursaphelenchus xylophilus*: Tylenchida: Aphelenchoididae), which causes pine wilt (Wingfield, 1987). This nematode has a phytophagous stage and a mycophagous stage. In the later stage, they feed upon fungi (e.g. *Ceratocystis* spp.: Microascales) vectored by the attacking beetle. Thus, terpenoids could act directly on the nematode or they may inhibit the fungi upon which the nematode feeds. Although the importance of terpenoids in conifer defence against nematodes has not been examined extensively, the heartwood of Chinese red pine (*Pinus massoniana*: Pinaceae) contains the sesquiterpene α-humulene, which repels the pinewood nematode (Suga *et al.*, 1993). In addition, several terpenoids from angiosperms are known to have nematicidal or behaviour-modifying properties (Chitwood, 2002).

An understanding of how conifer-produced terpenoids affect these pathogens and how fungal inoculation may induce terpenoids in conifers is required to explain this complex plant–insect interaction. To this end, the induction of defensive genes such as the terpene synthases upon challenge by a mountain pine beetle-associated fungus (*Ophiostoma clavigerum*: Ophiostomatales) is currently being investigated in lodgepole pine (N. Kolosova & J. Bohlmann, unpublished results).

5. Tri–trophic interactions

Conifer-produced terpenoids do not only influence herbivorous insects. Predatory and parasitic insects use the terpenoids induced or constitutively produced by the tree, often in synergy with the insect host semiochemicals, to locate their herbivorous host (e.g. Pettersson, 2001). For example, linalool and other terpenoid volatiles can be induced in conifer foliage (e.g. Martin *et al.*, 2003b; Miller *et al.*, 2005a) and may be perceived by predators or parasites of insects feeding on conifers. Parasitoids of several Lepidopteran species are attracted to linalool emitted from angiosperm host plants (Turlings *et al.*, 1991). Another example that highlights

the complexity of these interactions involves pine trees, pine sawflies, and their egg parasitoid. Egg deposition by the pine sawfly (*Diprion pini*: Hymenoptera) induces volatiles in Scots pine (*Pinus sylvestris*: Pinaceae) which attract the egg parasitoid *Chrysonotomyia ruforum* (Hymenoptera) (Hilker *et al.*, 2002). However, mechanically wounded controls did not attract the parasitoid. Comparison of the volatile emissions revealed no qualitative differences and, although there were some quantitative differences, only one terpenoid, (*E*)-β-farnesene, had significantly increased concentrations (Mumm *et al.*, 2003). In a related study, oviposition by the pine sawfly on black pine (*Pinus nigra*: Pinaceae), a poorer host for sawfly reproduction, changed the host volatile emission compared with mechanically wounded controls but did not elicit attraction by the parasitoid. Interestingly, egg deposition actually reduced (*E*)-β-farnesene emissions in this pine species (Mumm *et al.*, 2004). Additional studies showed that the parasitoid does not respond to (*E*)-β-farnesene alone, only in combination with other, noninduced, host volatiles (Mumm & Hilker, 2005). This system emphasizes the diversity and specificity of these terpenoid-based interactions. Within this system alone, there is much more to learn about (1) the chemical nature of the egg deposition elicitor of volatile emissions, (2) the regulation of volatile emissions (locally/systemically; the pathways involved), (3) the differences in induced emissions among pine species, (4) whether the egg parasitoid uses semiochemicals from the insect host in addition to the induced and constitutive conifer volatiles to locate its host eggs, and (5) what factors make Scots pine a better host than black pine for the pine sawfly at the expense of the higher parasitism.

IV. Conclusions and outlook

Insects select their hosts, but trees cannot select which herbivores will feed upon them. Thus, a tree must resist many attackers, while a particular insect species has generally specialized to feed on one or a few hosts to whose defences it has at least some resistance. Interactions between a conifer and a specific insect species may depend on a very small subset of the compounds produced by the tree. For example, the different host choices between the very closely related polyphagous mountain pine beetle and monophagous Jeffrey pine beetle (*Dendroctonus jeffreyi*: Coleoptera) may simply be attributable to the presence of heptane in Jeffrey pine (*Pinus jeffreyi*: Pinaceae), creating a niche for the Jeffrey pine beetle where the mountain pine beetle does not feed (Savage *et al.*, 1996).

Much of the chemical diversity of terpenoids resides with the terpene synthase gene family and the *CYP450* genes of terpenoid diversification. The biochemistry of these enzymes is finely tuned to their amino acid sequences. If a certain terpene synthase produces a terpenoid that is important for defence, a single amino acid mutation that alters its efficiency or product profile could decrease the resistance of a conifer to herbivory by certain insects and/or increase resistance to herbivory by others. It is thus important to study how subtle changes to the amino acid sequences generated in the laboratory, or through evolution in nature, can change substrate specificities, product profiles, and the kinetic efficiency of these enzymes. Such studies can provide a framework for understanding the diversity of the terpene synthase gene family and how these enzymes and their products contribute to the natural variation in the resistance of conifers.

Several examples illustrate the correlation of terpenoid profiles with insect resistance. To date, however, few studies have shown a causal relationship of specific terpenoids with resistance in conifers. Identification of the origins of different terpenoid profiles is needed for susceptible and resistant conifers. A change of profiles could occur as a result of gene expression, post-translational regulation, absence or mutation of the gene itself, or a change in a component of the signalling pathways. More importantly, linking the terpene profile changes to insect resistance and uncovering the molecular basis of resistance are crucial to understanding conifer–insect interactions.

Larger scale studies are needed to understand how terpenoids are involved in the evolution of species and geographical differences in both conifers and the insects that feed upon them (e.g. Raffa *et al.*, 2005). More research is also needed on other herbivores, such as deer, which also use terpenoids in host choice (Vourc'h *et al.*, 2002a,b).

Many questions remain to be addressed in future work on the conifer–insect interactions mediated by terpenoids. The identification and functional characterization of a much larger number of conifer terpene synthases are needed to understand the biochemical source of terpenoid diversity and to understand the evolution of these genes and their functions. The regulation of these genes and their products must also be explained. Little is known about the regulation of terpene synthases and other terpenoid-related enzymes, the signalling pathways and transcription factors involved in constitutive and inducible defences, the promoter elements involved in regulation, possible post-translational regulation of the enzymes, and the differences in these aspects of regulation among conifer species. The regulation of terpenoid biosynthesis, terpenoid transport and accumulation, and terpenoid volatile emission at the cellular and subcellular levels are not at all understood. It seems plausible that terpenoid biosynthesis is associated with specialized epithelial cells that line the surfaces of resin ducts or resin blisters. However, direct localization of the mRNAs and proteins involved in terpenoid formation and transport, and imaging of their spatial and temporal patterns of expression in constitutive and induced resin blisters and resin ducts are needed to understand this critical level of cellular regulation of terpenoid chemical defences. In addition, little is known about the impact of seasonal variation or conifer longevity on regulation of terpenoid biosynthesis. The large-scale functional and

quantitative genomics projects on conifer resistance to insect pests (Huber *et al.*, 2004; Rungis *et al.*, 2004; Ralph *et al.*, 2006a; Ralph *et al.*, 2006b; Ritland *et al.*, 2006) and recent development of large-scale proteomic tools (Lippert *et al.*, 2005) in conifers bode well for future studies in these areas of regulation.

Some other areas of research have so far received little attention in conifers. For example, plant communication, such as plant–plant, plant–microbe or plant–insect interactions, may be mediated by terpenoids in the rhizosphere of conifers (Bais *et al.*, 2004). Sesquiterpenoids in the rhizosphere of an angiosperm (*Lotus japonicus*: Fabales:Fabaceae) have recently been shown to induce hyphal branching in arbuscular mycorrhizal fungi (Akiyama *et al.*, 2005). Aboveground tree–tree communication may also exist, such as has been identified in angiosperms (reviewed by Dicke *et al.*, 2003). However, signalling mechanisms such as these await discovery in conifers.

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