Chapter 8
Insect-Induced Terpenoid Defenses in Spruce

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Conifers produce an array of hundreds of different terpenoids as part of their complex chemical, physical, and ecological defenses against insect pests and pathogens. Terpenoid chemicals exist both as constitutive and as massively induced defenses in conifers. It is thought that the diversity of terpenoid chemicals serves as a multilayered chemical shield in long-lived conifer trees that provides a lasting protection against the much faster evolving insect pests and potential pathogens. The formation of terpenoid defenses in conifers involves the activity of two pathways, the methylerythritol phosphate pathway and the mevalonate pathway, which lead to the five-carbon precursors of terpenoid biosynthesis. The many monoterpenoids, sesquiterpenoids, and diterpene resin acids, which are present in oleoresin mixtures, are then formed by families of enzymes belonging to the classes of prenyl transferases, terpenoid synthases, and cytochrome P450 dependent monooxygenases. The genes for almost all the enzymatic steps in terpenoid oleoresin biosynthesis have been identified in species of spruce, which have thus been established as a conifer reference system to study constitutive and induced terpenoid defenses using biochemical, molecular genetic, and genomic and proteomic approaches. At the histological and cellular levels, oleoresin terpenoids are produced and accumulate constitutively in large quantities in specialized anatomical structures that are found in most organs and tissues. In many conifer species, biosynthesis and accumulation of terpenoids is further enhanced as part of the induced defense in response to insect attack or fungal infection. In this chapter, I will discuss selected aspects of terpenoid defenses in conifers against insects and pathogens.

8.1 Introduction

Conifers (i.e., members of the pine family; Pinaceae) include some of the longest-living and tallest organisms on earth. Their tall physical structure and long lifespan

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make them prominent targets in space and time for many potential herbivores and pathogens. The successful defense and resistance of conifers against most herbivores and pathogens can be explained, at least in part, by the formation of a diverse array of monoterpenoid, sesquiterpenoid, and diterpene resin acid defense chemicals. These compounds accumulate in large amounts in form of preformed or induced oleoresin mixtures. Some terpenoids, in particular monoterpenes and sesquiterpenes, can also be actively emitted as volatile organic compounds with functions as semiochemicals from conifer needles. Conifer oleoresin is stored in specialized anatomical structures such as resin canals, resin blisters, or resin cells in stems, roots, or needles. The developmental programs for the constitutive formation of these specialized, resin-accumulating structures are not understood; except, it is now well established that the formation of so-called traumatic resin ducts is induced in the cambium zone of conifer stems in response to insect or fungal attack, by mechanical wounding, or by chemical elicitation with methyl jasmomate (MeJA) or ethylene treatment (Krokene et al. this volume). In recent years, several reviews have been published on the topic of terpenoid oleoresin defenses in conifers (e.g., Bohlmann and Croteau 1999; Phillips and Croteau 1999; Trapp and Croteau 2001a; Keeling and Bohlmann 2006a, b).

The families of terpenoid synthases (TPS) and cytochrome P450 dependent monooxygenases (P450) play a central role in the formation of terpenoid chemical diversity and for phenotypic plasticity in conifer defense (Bohlmann et al. 1998a; Martin et al. 2004; Ro et al. 2005). Both, the TPS and P450 are encoded in multi-gene families in conifers (Martin et al. 2004; Hamberger and Bohlmann 2006). These gene families are thought to contribute, on the genomic, molecular, and biochemical levels, much to the diversity and plasticity of constitutive and induced terpenoid defenses in the long-lived conifers. In contrast to the TPS and P450, we know relatively little about the early steps of terpenoid biosynthesis in conifers with only a few studies published on prenyltransferases (PT; Tholl et al. 2001; Burke and Croteau 2002; Martin et al. 2002; Schmidt and Gershenzon 2007) or earlier steps in conifer isoprenoid biosynthesis (Phillips et al. 2007). However, a recent large-scale spruce genomics project has identified genes (i.e., expressed sequences tags, ESTs; and full-length cDNAs, FL-cDNAs) for almost every single step in conifer monoterpenoid, sesquiterpenoid, and diterpenoid biosynthesis (Ralph et al. 2006; Ralph and Bohlmann, in preparation).

In building on pioneering work by Clarence (Bud) Ryan and coworkers, the use of MeJA as an elicitor to induce plant defenses enabled us and others to develop a detailed characterization of inducible terpenoid defenses in several conifer species such as Norway spruce (*Picea abies*), Sitka spruce (*P. sitchensis*), White spruce (*P. glauca*) and Douglas fir (*Pseudotsuga menziesii*; e.g., Franceschi et al. 2002; Martin et al. 2002, 2003a; Fäldt et al. 2003; Hudgins et al. 2003; Huber et al. 2005a, b; Miller et al. 2005; Ralph et al. 2006; Erbilgin et al. 2006; Zeneli et al. 2006; Phillips et al. 2007). By treating conifer seedlings, mature trees, or even cell cultures with MeJA, it became possible to accurately measure the quantitative and qualitative changes of traumatic resin terpenoids, detect the dynamics of enzyme activities and transcript levels induced in the traumatic terpenoid defense response, and analyze

the induced and active emission of terpenoid volatiles. In addition, the non-invasive treatment with MeJA, as opposed to mechanical wounding or insect attack, allowed for the analysis of temporal and spatial patterns of cell differentiation during traumatic resin duct formation in the cambium zone of spruce stems. Application of MeJA provided a powerful tool to mimic at least some of the effects of insect feeding in spruce, as has been shown in the defense response of Sitka spruce to white pine weevil (*Pissodes strobi;* Miller et al. 2005). Beyond the use of MeJA as an exogenous elicitor, both ethylene and octadecanoid signaling appear to be involved in the endogenous signaling of the induced traumatic resin response in spruce and Douglas fir (Hudgins and Franceschi 2004; Miller et al. 2005; Hudgins et al. 2006; Ralph et al. 2007).

The MeJA- or insect-induced accumulation of terpenoids in traumatic resin ducts in stems and roots, as well as the induced emission of terpenoid volatiles from needles, is controlled at least in part by up-regulation of terpenoid biosynthesis (Martin et al. 2002, 2003a; Huber et al. 2005b; Miller et al. 2005; Keeling and Bohlmann, 2006a). In this process, transcript levels and/or enzyme activities for several steps in terpenoid formation are up-regulated in the methylerythritol phosphate (MEP) pathway, in the subsequent prenyltransferase steps, and in the late steps catalyzed by TPS and P450s. The analysis of more than 200,000 spruce ESTs and 6,464 high-quality finished FLcDNAs along with transcriptome and proteome analyses have identified many other insect- and MeJA-regulated processes in the induced chemical defense and resistance of Sitka spruce against white pine weevil and spruce budworms (*Choristoneura occidentalis;* Ralph et al. 2006, 2007b; Lippert et al. 2007; Ralph and Bohlmann, in preparation).

8.2 Biochemistry of Terpenoid Biosynthesis in Conifer Defense

The progress in research on the biochemistry and molecular biology of terpenoids in conifer defense has recently been reviewed (Martin and Bohlmann 2005; Keeling and Bohlmann 2006a, b; Phillips et al. 2006) and a number of earlier reviews on this topic are available (e.g., Bohlmann and Croteau 1999; Phillips and Croteau 1999; Trapp and Croteau 2001a). Terpenoids represent the largest group of known plant secondary metabolites. The three large classes of monoterpenoids, sesquiterpenoids, and diterpene resin acids are the most abundant terpenoid defense chemicals in conifers. In particular, the monoterpenoids and diterpene resin acids constitute much of the conifer oleoresin volume. In addition, sesquiterpenoids contribute substantially to the structural diversity of chemicals in the oleoresin mixture. The biosynthesis of monoterpenoids, sesquiterpenoids, and diterpene resin acids begins with the formation of the five carbon building blocks, isopentenyl diphosphate (IDP) and its isomer, dimethylallyl diphosphate (DMAPP). In plants, two pathways exist for the formation of these precursors, the mevalonic acid (MEV) pathway and the MEP pathway. Based on large-scale EST and FLcDNA sequencing, all but one gene for the MEV and MEP pathways have been cloned from Sitka spruce and/or white

spruce (Ralph et al. 2006; Ralph and Bohlmann, in preparation). These genomics efforts in Sitka spruce and white spruce, by extension of gene discovery to other conifer species, led to the identification and functional characterization of a small family of differentially MeJA- and fungal-induced genes for the first step of the MEP pathway, deoxyxylulose phosphate synthase (DXPS), in Norway spruce (Phillips et al. 2007). Based on our studies with Norway spruce DXPS in cell suspension cultures, it appears that the different isoforms of DXPS may have specific functions in regulation of substrate flux in primary isoprenoid metabolism and secondary terpenoid defense metabolism. Following the formation of IDP and DMAPP, a group of PTs catalyze 1'-4 condensation reactions coupling IPP with an allylic prenyl diphosphate. Specifically, geranyl diphosphate (GDP) synthase forms the C_{10} precursor of monoterpenoids, farnesyl diphosphate (FDP) synthase forms the C_{15} precursor of sesquiterpenoids, and geranylgeranyl diphosphate (GGDP) synthase produces the C_{20} precursor for diterpenoids. A few conifer PTs have been characterized in grand fir (*Abies grandis;* Tholl et al. 2001; Burke and Croteau 2002) and more recently in Norway spruce (Schmidt and Gershenzon 2007) and a large number of distinct PTs have been identified with ESTs and FLcDNAs in Sitka spruce and white spruce (Ralph and Bohlmann, in preparation). Although it is thought that PTs may control flux of pathway intermediates at branch points of monoterpenoid, sesquiterpenoid, and diterpenoid biosynthesis in the induced defense of conifers, except for a few studies showing up-regulation of some PT activities and transcript levels, this aspect of the regulation of terpenoid biosynthesis is not well understood and will require much further characterization (Martin et al. 2002; Schmidt and Gershenzon 2007; Ralph and Bohlmann, in preparation).

TPS utilize the three prenyl diphosphates GDP, FDP, and GGDP as substrates in the formation of the hundreds of structurally diverse monoterpenoids, sesquiterpenoids, and diterpenoids. The TPS exist in large gene families and are arguably the best characterized genes and enzymes of chemical defenses in conifers (Bohlmann et al. 1998a; Martin et al. 2004; Keeling and Bohlmann 2006a). Much of the work on TPS in induced conifer defense is based on the early and pioneering studies by Rodney Croteau and coworkers at Washington State University. The TPS employ an electrophilic reaction mechanism, assisted by divalent metal ion cofactors (Davis and Croteau 2000). The prenyl diphosphate substrates are ionized or protonated by TPS to produce reactive carbocation intermediates. The carbocation intermediates can then rearrange within the spatial constraints of the TPS active site and are eventually quenched to yield the many different cyclic and acyclic terpenoid products (Starks et al. 1997; Cane 1999; Wise and Croteau 1999; Christianson 2006). While some TPS form only a single product, the majority of TPS characterized to date, including many TPS identified in conifers, produce arrays of multiple products from a single substrate (Steele et al. 1998; Fäldt et al. 2003; Martin et al. 2004). Based on their substrate specificities, the single- and multi-product TPS are grouped into classes of mono-TPS, sesqui-TPS and di-TPS, which are responsible for the formation of the many simple (acyclic or single ring structure) and more intricate (two or more ring structures) terpene skeletons of conifer mono-, sesqui-, and diterpenoids. Many of the known conifer TPS appear to exert tight control over the stereochemistry of

products formed and usually one enantiomer dominates any given TPS product profile. To date, approximately 50 different conifer TPS have been cloned and many of them have been functionally characterized in Norway spruce, Sitka spruce and white spruce (Byun McKay et al. 2003, 2006; Fäldt et al. 2003; Martin et al. 2004; Keeling et al. 2006a; Ralph and Bohlmann, in preparation). In addition, TPS have been cloned and characterized from grand fir (e.g., Stofer Vogel et al. 1996; Bohlmann et al. 1997, 1998a, 1999; Steele et al. 1998), loblolly pine (Phillips et al. 2003; Ro and Bohlmann 2006) and Douglas fir (Huber et al. 2005a).

While the majority of terpenoid products formed by the conifer mono-TPS and sesqui-TPS accumulate in the oleoresin without any further apparent biochemical modifications, the diterpene olefins produced by the di-TPS may be subject to oxidations catalyzed by P450s (Keeling and Bohlmann 2006b). To date only a single P450 in the formation of diterpene resin acids has been cloned and functionally characterized (Ro et al. 2005). The loblolly pine PtAO P450 enzyme, abietadienol/abietadienal oxidase, is a multi-substrate and multi-functional diterpene oxidase in the CYP720 family of plant P450 enzymes. PtAO catalyzes at least two of the three consecutive oxidation steps in the formation of conifer diterpene resin acids and the enzyme efficiently uses several different diterpene alcohols and diterpene aldehydes as substrates. Through the combination of accepting multiple diterpenoid substrates and by catalyzing at least two consecutive oxidations, this P450 enzyme leads to the formation of several different diterpene resin acids found in conifer oleoresin. The ability of PtAO to catalyze consecutive oxidation steps is similar to the activity of angiosperm P450s in the biosynthesis of the diterpene gibberellin phytohormones (Ro et al. 2005; Keeling and Bohlmann 2006b). A further analysis of the nearly half a million conifer ESTs from spruce and loblolly pine revealed a large number of new P450s in the CYP85 clan that may be involved in terpenoid oxidation in conifers (Hamberger and Bohlmann 2006). While conifer P450s putatively involved in terpenoid phytohormone formation, such as gibberellic acid biosynthesis, appear to be expressed as single-copy genes in Sitka spruce and loblolly pine, we found substantial expansion of conifer-specific P450 subfamilies that are likely associated with terpenoid secondary metabolism and defense. Since conifer P450 enzymes are extremely difficult to study as native enzymes in crude or cell-free extracts from bark, needle, or wood tissues, it was necessary to develop an efficient recombinant expression and assay system for functional biochemical characterization of candidate full-length P450 cDNAs. The system developed for the characterization of the PtAO enzyme involved a combination of yeast expression of P450 candidate cDNAs together with expression of cytochrome P450 reductase, in vivo feeding experiments with diterpene substrates, in vitro enzyme assays, and ultimately the development of an engineered yeast strain that produces conifer diterpene resin acids de novo based on the simultaneous expression of GGDP synthase, diterpene synthase, P450, and P450 reductase (Ro et al. 2005).

Based on all current information, the biosynthesis of terpenoids in conifers involves several subcellular compartments (Keeling and Bohlmann 2006a). Enzymes of the MEV pathway are thought to be localized to the cytosol and endoplasmic reticulum, and the MEP pathway is localized in plastids. While GDP and GGDP synthases are

most likely localized to plastids, FDP synthase is thought to reside in the cytosol and possibly in mitochondria. Based on the presence or absence of transit peptides, mono-TPS and di-TPS are also present in plastids, while sesqui-TPS appear to be cytosolic. Finally, the PtAO P450 is associated with the endoplasmic reticulum (Ro et al. 2005; Ro and Bohlmann 2006). It has been speculated that enzymes of terpenoid oleoresin formation are predominantly present in specialized epithelial cells lining the surface of resin ducts or resin blisters (Keeling and Bohlmann 2006b). However, testing this idea requires further validation by immuno-localization or cell-specific transcriptome and proteome analysis. Nevertheless, the involvement of several subcellular compartments in oleoresin terpenoid biosynthesis, the possible association of terpenoid biosynthesis with specialized cells, as well as the massive sequestration and accumulation of oleoresin terpenoids in the extracellular space of resin ducts or resin blister mandates efficient and specialized transport systems for intermediates and end-products of terpenoid biosynthesis. Such transport systems would be essential to deliver the large amounts of lipophilic terpenoids against steep concentration gradients and across cell membranes and the cell wall into the extracellular storage sites of specialized anatomical structures. The combined genomic and proteomic analysis of specialized cells types based on laser-assisted microdissection along with yeast expression systems developed for the de novo formation of conifer terpenoids in vivo (Ro et al. 2005) are now being explored for the discovery of candidate transport systems in conifer oleoresin biosynthesis and terpenoid accumulation.

8.3 Evolution of the Conifer TPS and P450 Gene Families for Terpenoid Defense

The cloning and characterization of several dozen different TPS from several conifer species, including Norway spruce, Sitka spruce, loblolly pine, grand fir, and Douglas fir, enabled phylogenetic reconstruction of the gymnosperm TPS family, thereby shedding some light on the evolution of the larger TPS family in plants in general. Based on overall protein sequence relatedness, gene structure similarities, and catalytic mechanisms, all plant TPS are believed to have arisen from a common ancestor, which may have been closely related to the known conifer di-TPS and to the recently characterized bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens* (Bohlmann et al. 1998a; Trapp et al. 2001b; Martin et al. 2004; Hayashi et al. 2006). It is possible that such an ancestral TPS was involved in the biosynthesis of the precursors of gibberellic acid, copalyl diphosphate and *ent*-kaurene. The evolution of the large family of plant TPS enzymes as we know them today occurred apparently to some large extend independently in the separate angiosperm and gymnosperm lineages involving numerous events of gene duplication and subsequent functional specialization, i.e. neo-functionalization and sub-functionalization (Martin et al. 2004; Keeling and Bohlmann 2006a; Keeling et al. 2008).

Based on amino acid sequence similarity, the TPS family can be divided into seven subfamilies designated TPS-a through TPS-g (Bohlmann et al. 1998a; Martin et al. 2004). The conifer mono-TPS, sesqui-TPS and di-TPS cluster separately from the angiosperm TPS into the TPS-d subfamily, which is further divided into groups TPS-d1 (mostly mono-TPS), TPS-d2 (mostly sesqui-TPS) and TPS-d3 (mostly di-TPS; all containing an ancestral 200-amino acid motif). Both phylogenetic analyses and analysis of gene structure position the conifer di-TPS closest to the putative ancestor of plant TPS (Bohlmann et al. 1998a; Trapp and Croteau 2001b; Martin et al. 2004).

TPS of conifer secondary metabolism are believed to have evolved from TPS involved in primary metabolism. In the evolution of conifer TPS in terpenoid secondary metabolism a massive expansion of gene families and radiation of a diversity of biochemical functions has occurred (Martin et al. 2004). The same may be the case for the P450s (Hamberger and Bohlmann 2006). In contrast to the TPS and P450 of terpenoid secondary metabolism with their adaptive functions in conifer defense and resistance against insects and pathogens, to the best of current knowledge, the TPS and P450s in primary gibberellic acid phytohormone formation seem to be conserved and have undergone very little if any radiation in conifers.

8.4 Biosynthesis and Accumulation of Terpenoid Defenses Requires Specialized Cells

Accumulation of large amounts of hydrophobic terpenoids in constitutive and induced (i.e., traumatic) oleoresin requires specialized anatomical structures in conifers stems, roots, foliage, and cones. Unless sequestered into extracellular spaces of such specialized structures, the hydrophobic terpenoids would interfere with biochemical processes, integrity of membranes and cell structure of the terpenoid producing cells. Sequestration and accumulation of terpenoid oleoresin can be achieved with simple, short-lived resin blisters or with complex, long-lived resin duct systems commonly found in conifer species that produce large quantities of oleoresin. As part of their ability to massively increase the biosynthesis and accumulation of terpenoid defenses, conifers also develop additional traumatic resin ducts (TRD) when challenged by insect attack or fungal inoculation or in response to simulated insect attack induced by treatment with MeJA or ethylene (e.g., Alfaro 1995; Nagy et al. 2000; Franceschi et al. 2002, 2005; Martin et al. 2002; Byun McKay et al. 2003; Hudgins et al. 2003; Krekling et al. 2004; Hudgins and Franceschi 2004; Huber et al. 2005b; Krokene et al. this volume). The de novo formation of TRD occurs within the cambium zone and outermost layers of developing xylem due to a transient change in cambial activity that initiates resin duct epithelial cells in lieu of wood-forming tracheids. Lumenal continuity between TRD, radial ducts, and resin ducts in the bark establishes a three-dimensional resin duct reticulum enabling enhanced biosynthesis, accumulation and flow of resin. The induced formation of TRD is associated with increased biosynthesis and accumulation of terpenoid oleoresin and involves induced gene expression and enzyme activities of several mono-TPS, sesqui-TPS, and di-TPS (Martin et al. 2002; Byun McKay et al. 2003, 2006; Fäldt

et al. 2003; Huber et al. 2005b; Miller et al. 2005) and also involves increased levels of transcripts for octadecanoid and ethylene formation (Miller et al. 2005; Hudgins et al. 2006; Ralph et al. 2007a). Contact of TRD with ray parenchyma cells may enable signaling of induced terpenoid defenses between xylem, cambium, and bark tissues (Nagy et al. 2000; Franceschi et al. 2005; Hudgins et al. 2006; Ralph et al. 2007a). Given the abundance of relevant gene probes available from spruce EST and FLcDNAs projects (Ralph et al. 2006; Ralph and Bohlmann, in preparation) combined with immuno-localization, in-situ hybridization, and cell- or tissue specific micro-dissection techniques, it is now possible to test the cell-specific localization of transcripts and proteins for constitutive and induced terpenoid formation and its defense signaling in conifers (Hudgins et al. 2006; Keeling and Bohlmann 2006b; Ralph et al. 2007a).

8.5 Molecular Biology of Insect-and MeJA-Induced Terpenoid Defenses in Conifers

The use of MeJA to induce chemical and anatomical defenses in conifers has been of critical importance in the characterization of insect-induced terpenoid defense responses in species of spruce (Franceschi et al. 2002; Martin et al. 2002, 2003a; Fäldt et al. 2003; Miller et al. 2005; Erbilgin et al. 2006; Zeneli et al. 2006). Similar to the effect of real insect attack or fungal inoculations, exogenous treatment of conifers with MeJA induces the development of TRD, terpenoid accumulation, as well as terpenoid volatile emissions. Treatment of trees with MeJA provided a means by which to induce conifer defense responses without mechanically injuring the tree. This is especially vital to the quantification of induced terpenoid defenses where any injury may reduce the biological capacity of the bark, cambium, or xylem tissue to respond, but would also enhance the loss of terpenoids through the wound site. Instead, treatment with MeJA allowed for the characterization of the induced enzyme activities and gene expression of terpenoid biosynthesis in stems and foliage of spruces, and enabled the detailed quantitative and qualitative analysis of terpenoids in control and induced trees. This approach also enabled the characterization of active emission of terpenoid volatiles from the needles of Sitka spruce and Norway spruce (Martin et al. 2002, 2003a; Miller et al. 2005).

The cloning and functional characterization of families of TPS and P450 in species of spruce and other conifers provided an initial set of valuable probes for gene expression analysis of MeJA- or insect-induced terpenoid defenses (Fäldt et al. 2003; Byun McKay et al. 2003, 2006; Miller et al. 2005; Ro et al. 2005). TPS of all biochemical classes were induced by weevil-feeding or MeJA-treatment in stems of Sitka spruce, with some of the strongest responses observed for the mono-TPS and di-TPS. In loblolly pine, we also found a strong MeJA-induced increase of transcript levels of the PtAO P450 gene along with increased transcript levels of a diterpene synthase that produces the substrate precursor for PtAO (Ro et al. 2005; Ro and Bohlmann 2006). TPS gene expression and TPS enzyme activities are also elevated in foliage of Norway spruce and Sitka spruce following MeJA

elicitation or weevil attack (Martin et al. 2003a; Miller et al. 2005). Induced gene expression of TPS in Norway spruce and Sitka spruce is associated with increased TPS enzyme activities and with the elevated accumulation of oleoresin terpenoids in stems or the release of terpenoid volatiles from needles (Martin et al. 2002; Miller et al. 2005). In recent work with Sitka spruce, we have established comprehensive maps of weevil-induced gene expression profiles for additional steps of terpenoid biosynthesis, indicating that TPS, P450, PTs, and a few genes in the MEP and MEV pathways are the strongest up-regulated transcripts in the weevil-induced terpenoid defense response in Sitka spruce (Ralph and Bohlmann, in preparation). In addition, microarray analyses on platforms with ~21,800 spotted spruce cDNAs identified thousands of transcript species as differentially expressed in response to insect attack in Sitka spruce (Ralph et al. 2006; Ralph and Bohlmann, in preparation).

8.6 Effects of Conifer Terpenoids on Insects and Insect-Associated Pathogens

Terpenoids in form of oleoresins and volatile emissions are a critical component of conifer interactions with other organisms such as defense and resistance against insects and pathogens (Langenheim 2003; Raffa et al. 2005). In general, terpenoid chemicals can protect conifers by providing mechanical barriers in form of 'pitch' or crystallized resin at wound sites, through toxicity against insects and insect-associated pathogens, or by interrupting essential processes in insect biology. In addition, in indirect defense systems, conifer terpenoids may function as signals for predators and parasites of the attacking herbivore.

Specific functions of individual terpenoid compounds in conifer defense have been difficult to establish experimentally (Keeling and Bohlmann 2006a). While the complexity of hundreds of terpenoid chemicals that form the constitutive and inducible oleoresin blends is likely to provide a major advantage for the sustainability of a conifer defense system that is both stable and flexible, the same chemical complexity also poses a substantial challenge for researchers to pinpoint specific defense activities conclusively to individual terpenoid compounds. It is also possible that the mixture of terpenoids may be more effective than any individual compound alone. The lack of appropriate mutants for most conifers and the slow process of genetic transformation of conifers in general make it extremely difficult to manipulate complete terpenoid profiles or individual terpenoid compounds for in vivo elucidation of their defense functions with intact trees. Therefore, most tests of conifer terpenoids for their effects on insects or insect-associated pathogens have relied on exposure of insects or pathogens to isolated compounds or blends of these compounds. In such experiments, for example, the sesquiterpenoid compounds juvabione, farnesol, and farnesal have been shown to interrupt development and maturation of insects by interfering with insect endocrine systems (Schmialek 1963; Slama et al. 1965).

The tree-killing activity of certain bark beetles involves an association of these insects with a community of fungi and bacteria some of which are known to be

pathogenic and contribute directly to tree mortality. It is therefore important to establish the effect of terpenoid defenses with these pathogens as well. For example, the diterpene resin acids abietic acid and isopimaric acid strongly inhibit spore germination of the blue-stain fungus *Ophiostoma ips*, a conifer pathogen that is symbiotically associated with the pine engraver *Ips pini* (Kopper et al. 2005). The same study also showed that abietic acid inhibits mycelial growth of *Ophiostoma ips*.

Several possible approaches can be considered for future research to test the role of individual terpenoids or groups of terpenoids in conifer defense. Targeted manipulation of terpenoid biosynthesis or genetic association studies are likely to provide the most informative approaches. Both approaches require funding commitments of many years, and genetic association studies also require access to substantial biological resources in form of established breeding programs and provenance trials. Fortunately, relevant biological materials are available to study the role of constitutive and traumatic terpenoids in the resistance of white spruce and Sitka spruce against the white pine weevil (Alfaro et al. 2002, 2004; King et al. 1997, 2004). In Sitka spruce, lines with extremely divergent profiles for individual terpenoids have been identified in metabolite profiling studies and the chemical profiles have been associated with resistance against the white pine weevil (Roberts, Keeling, and Bohlmann, unpublished results). In studies with white spruce, it has been established that insect-induced formation of traumatic resin ducts is positively correlated with resistance to white pine weevil (Alfaro et al. 1996, 2002; Tomlin et al. 1998). As far as targeted manipulation of terpenoids in conifers is concerned, as a proof of principle, we have recently transformed a sesquiterpenoid synthase, *E*-α-bisabolene synthase, under the control of a wound- and insect-inducible promoter in white spruce (Godard et al. 2007). The use of an insect-inducible promoter will allow us to manipulate and test terpenoid defenses in a more realistic fashion than what can be accomplished with constitutive over-expression (Godard et al. 2007).

Although terpenoids have important roles in the protection of conifers against insects, they are also vital for the successful attack against conifers by certain bark beetles and their associated tree-killing fungi. The large topic of conifer terpenoids and terpenoid pheromones as signals in bark beetle biology, as well as the topic of de novo formation of terpenoid pheromones in bark beetles have recently been addressed in several excellent reviews (e.g., Seybold and Tittiger 2003; Seybold et al. 2006; Raffa et al. 2005). It is well established that terpenoid volatiles emitted from conifer host trees function as semiochemicals in the identification of host and non-host conifer species as well as in the identification of individual susceptible trees within a given species. Once a tree has been attacked by a bark beetle, enzymes of the insect and the insect-associated microorganisms are required for detoxification of host terpenoid chemicals. A recent analysis of ESTs of the fungal pathogen *Ophiostoma clavigerum*, a blue-stain fungus associated with the mountain pine beetle (*Dendroctonus ponderosa*), revealed an overrepresentation of transcripts encoding for P450 and transport proteins in fungi exposed to terpenoids present in the conifer host tree (Diguistini et al. 2007). Beyond simple detoxification of terpenoid defenses, oxidation of monoterpenoids by bark beetles or by their associated fungi results in the formation of pheromones for the coordination

of mass attack and mating of hundreds or thousands of insects on individual trees. Recent biochemical and molecular studies showed that bark beetle P450 enzymes play a central role in the conversion of conifer host defense chemicals into insect terpenoid pheromones (Huber et al. 2007; Sandstrom et al. 2006). A mass-attack by bark beetles that is coordinated by terpenoid pheromones allows the insect to overwhelm and exhaust the defense system of the much larger host tree and thus permits the insects and fungal pathogens to kill not only individual conifer trees but in epidemic situations leads to the destructions of conifer forest over landscape areas of millions of hectares. While much research has focused on the role of host monoterpenes as precursors for bark beetle sex and aggregation pheromones, it is now well established, based on precursor feeding experiments, enzyme characterization and gene discovery, that some species of coniferophagus bark beetles are able to produce monoterpenoid pheromones de novo (e.g., Gilg et al. 2005; Huber et al. 2007; Keeling et al. 2004, 2006; Martin et al. 2003b; Sandstrom et al. 2006; Seybold and Tittiger 2003; Seybold et al. 1995, 2000, 2006).

In addition to the prominent role of terpenoids in the direct defense of conifers, indirect defense systems mediated by conifer volatiles have also been established in several conifers. For example, the egg deposition by the sawfly *Diprion pini* elicits emissions of terpenoid and other volatiles from Scots pine (*P. sylvestris*) which can result in the attraction of a parasitic wasp (Mumm and Hilker 2006). Over the millions of years of co-evolution of conifers with insects and insect-associated microorganisms, terpenoids may have played a substantial role in shaping the many interactions of conifers with insects and pathogens.

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