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DEFENSIVE RESIN BIOSYNTHESIS IN CONIFERS

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■ **Abstract** Tree killing bark beetles and their vectored fungal pathogens are the most destructive agents of conifer forests worldwide. Conifers defend against attack by the constitutive and inducible production of oleoresin, a complex mixture of mono-, sesqui-, and diterpenoids that accumulates at the wound site to kill invaders and both flush and seal the injury. Although toxic to the bark beetle and fungal pathogen, oleoresin also plays a central role in the chemical ecology of these boring insects, from host selection to pheromone signaling and tritrophic level interactions. The biochemistry of oleoresin terpenoids is reviewed, and the regulation of production of this unusual plant secretion is described in the context of bark beetle infestation dynamics with respect to the function of the turpentine and rosin components. Recent advances in the molecular genetics of terpenoid biosynthesis provide evidence for the evolutionary origins of oleoresin and permit consideration of genetic engineering strategies to improve conifer defenses as a component of modern forest biotechnology.

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INTRODUCTION

Conifers are an ancient group of woody plants that arose at least 200 million years ago (34) and include the oldest living organisms (yew, bristlecone pine, redwood) with life spans of thousands of years (100). Over 600 species of conifers (order Coniferales) of seven families (Pinaceae, Podocarpaceae, Araucariaceae, Taxodiaceae, Cephalotaxaceae, Taxaceae, Cupressaceae) (49) dominate large regions of temperate forest, especially in Western North America, Eastern Asia, and parts of Australia and New Zealand (100). The Pinaceae are the most abundant and widespread of these families, particularly in the Northern Hemisphere where firs (*Abies*), spruces (*Picea*), and pines (*Pinus*) predominate. Like all plants, conifers are subject to predation by a wide range of herbivores and pathogens (but over an uncommonly long life span), with the most serious destruction worldwide resulting from the infestation by tree killing bark beetles and their symbiotic fungal pathogens. The great success of the conifers rests in part on their evolution of complex defense mechanisms to deter such herbivore and pathogen predation. A principal, and unique, chemical and physical defense of conifers is comprised of the constitutive and inducible production of oleoresin (often simply termed resin or pitch). The viscous oleoresin secretion is composed of a complex mixture of terpenoids, consisting of roughly equal parts of volatile turpentine [mostly monoterpene (C_{10}) olefins with some sesquiterpenes (C_{15}), including oxygenated types] and rosin [diterpene (C_{20}) resin acids] (Figure 1) (29, 63, 82, 85). Accumulated resin is released upon tissue injury and/or produced locally at the site of infestation, with the consequence that the beetle and associated fungal pathogen(s) are killed, encased in resin, and expelled from the bore hole point of entry. This process is called pitching out (Figure 2*B*), and it results in not only killing the attackers and flushing the wound site but also moving the oleoresin to the trunk surface where the turpentine evaporates to permit the resin acids to form a formidable physical barrier that seals the wound (29, 52).

With the advent of large-scale commercial lumbering of softwoods at the turn of the century and the replanting of clearcuts with a single conifer species, accompanied by the prevention of forest fires as a natural control agent, predation by bark beetles has become a serious, and occasionally epidemic, problem in monocultural forestry. For these reasons, the biology of bark beetles and their microbial associates as well as their attack strategies and dynamics have become the focus of considerable study. The response of the host conifer, especially the physiological and environmental conditions that result in susceptibility and resistance, and the physical and chemical character of the defensive secretion have also been subjected to substantial investigation. For many such predator-host systems, the players are now well understood, and the chemical bases of the interaction (largely in the context of bark beetle chemical ecology) have been defined. Much recent work has focused on a more refined analysis of conifer resin production, and it is these biochemical, physiological, and molecular advances that

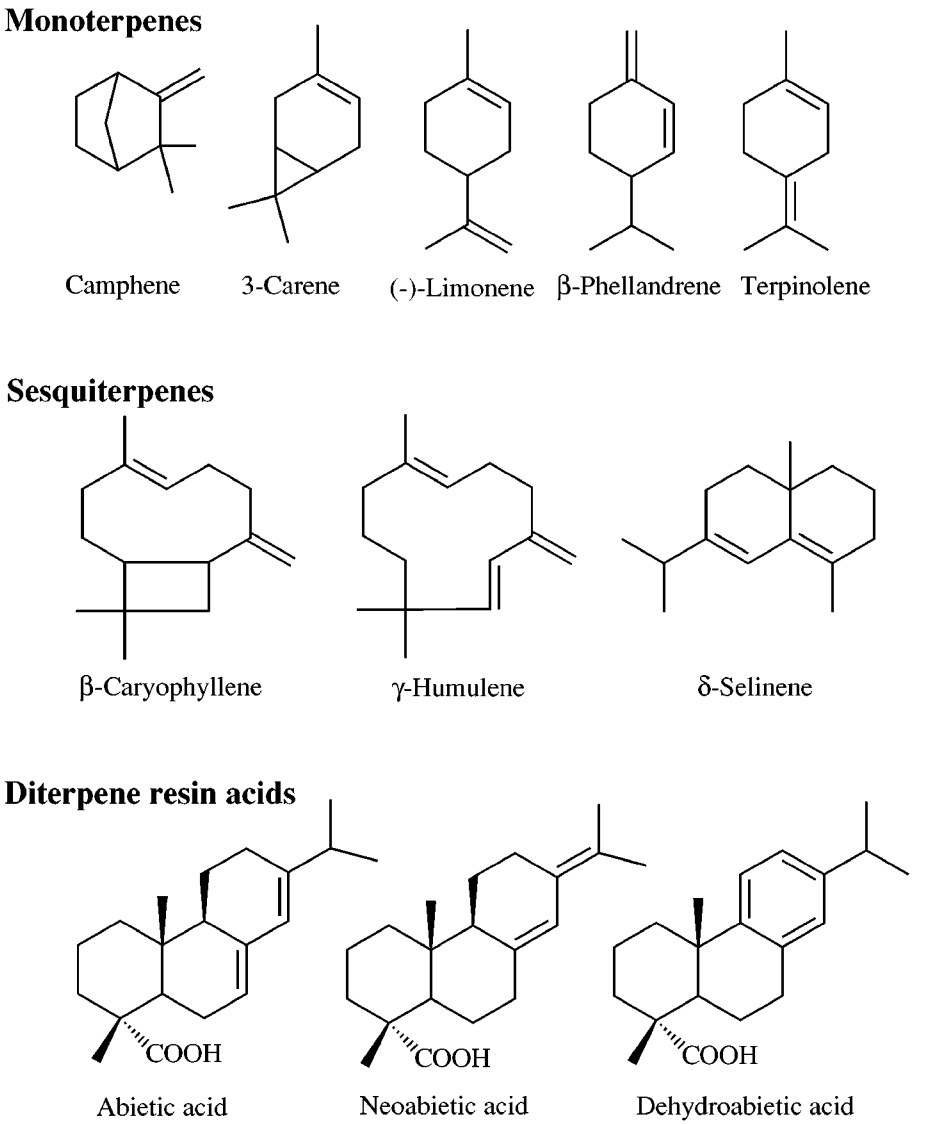


Figure 1 Structures of typical resin components.

are emphasized in this overview of the topic. The evolutionary origins of oleoresin are also briefly described as are the prospects for biotechnological applications using these newer molecular tools. The latter assumes some significance, because there are very few traditional pest control strategies applicable to large-scale forestry.

CONIFER-BARK BEETLE INTERACTIONS

Although conifers encounter a range of pests and pathogens, including insect defoliators (101), root feeders (84), and nematodes (48), bark boring beetles (Coleoptera:Scolytidae) are the most destructive agent of coniferous forests worldwide with annual losses in the United States alone exceeding five million board feet of lumber (8). Most bark beetles live and feed on dead trees; however, aggressive species of *Dendroctonus*, *Ips*, *Scolytus*, and *Dryocoetes* attack living trees exclusively and must kill the host in order to reproduce (8, 118). Bark beetle outbreaks are generally episodic and can rapidly spread over hundreds of square miles of forest before abating. The origins and dynamics of such outbreaks are not yet fully understood, although it is clear that the underlying interactions between the bark beetle and its conifer host involve a very high degree of specialization. This selectivity, wherein a given beetle species will attack only one or two conifer species, reflects the extended period of coevolution between insect and host (112). The biology, behavior, and ecology of bark beetles and their unusual mutualistic relationship with the pathogenic fungi they carry as well as the role of host resin production in resistance against attack have been described for a range of specific beetle-conifer interactions (8, 24, 94, 104, 105), which permit a generalized description for the purpose of this review.

Bark Beetle Life Cycle

The details of the bark beetle life cycle vary with species and geographic location; however, four major phases can be defined as multiplication, development, dispersal, and concentration (8, 105). Adult beetles mass attack the selected host by boring through the bark (Figure 2*C*), where they mate and lay eggs in tunnels (brood galleries) excavated in the phloem, cambium, and sapwood of the dying tree (Figures 2*F*, *G*). In the process of colonization, which has the effect of girdling the tree, the ailing host is also infected with pathogenic fungi carried by the beetles in specialized anatomical structures called mycangia (Figure 2*A*) or on other external, invaginated body parts (5, 43), whereupon the fungi assist in tree killing by invasive growth and toxin production in the conducting elements. The larvae hatch, feed in the tunnels that they construct (Figures 2*F*, *G*), develop, and then pupate and overwinter. In the dispersal phase, young adults emerge from the dead host by boring exit holes and then seek new living hosts by utilizing a variety of visual, tactile, and olfactory cues (122). Once a suitable host is located, often a physically damaged or physiologically compromised tree, pioneer beetles signal for mass attack (Figure 2*C*), via aggregation or sex attracting pheromones (see below), in an attempt to overcome host defenses (7, 97, 122). This concentrated attack often results in the death of the tree in which the annual cycle of multiplication, development, dispersal, and concentration is repeated.

Bark Beetle Microbial Symbiosis

The bark beetle–microbe-tree ecosystem has been described as a supra-organism (6) in which bark beetles associate in a generally mutualistic relationship with a variety of microorganisms (fungi, yeasts, bacteria, and protozoans) borne on and in their bodies (118). As early as the turn of the century, investigators proposed that bark beetles transmit pathogenic fungi to their conifer hosts; however, not until 1929 did experiments show that *Dendroctonus* and *Ips* species vector blue-stain fungi (83). Pathogenic fungi are often designated as blue- or black-staining because they typically discolor the infected wood and reduce its lumber value.

All bark beetle species appear to vector pathogenic fungi, the most common associative types belonging to the genera *Ophiostoma* (formerly *Ceratocystis*), *Leptographium*, *Graphium*, and *Trichosporium*. These vectored fungi, as a consequence of beetle excavation of the host, assist in tree killing; pathogenicity is clearly conditional on entry provided by the wood-boring insect. Not all mutualists are carried externally in the mycangia, as are fungal spores, and the benefits provided to and by these associates are less clear (43, 118). Vectored yeasts may serve as food sources for beetles and their broods (118).

Bark Beetle Chemical Ecology

The volatile monoterpene constituents of conifer oleoresin serve diverse roles in bark beetle chemical ecology, including host recognition and selection; pheromone signaling, which directs beetle aggregation and colonization; and tritrophic level interactions that involve signaling of beetle predators and parasitoids (52, 87, 89, 94, 122). In spite of their toxicity toward bark beetles, resin monoterpenes provide a species-specific chemical signature and thus function as olfactory cues in host location (122); visual cues and gustatory (textural and compositional) features of the bark also serve in final host selection (94). The monoterpene air plume derived from freshly exposed oleoresin resulting from tree damage (lightning strike, wind fall, lumbering activity) is generally attractive to pioneer beetles, as are the emissions from physiologically compromised individuals (e.g. victims of drought stress) (8, 52, 77). Such sites often provide the focus for infestation and subsequent outbreaks by attracting many host-seeking pioneers, which leads to the aggregation and colonization phases of host attack.

The essential advantage of a focused attack, wherein beetles initially aggregate upon a small number of trees, is that the host is unable to respond to the sheer number of invaders. The production of sex attractants and aggregation pheromones (to which both sexes respond) is the critical determinant in the success of a concentrated attack on the selected host (16) and often leads to the colonization of adjacent healthy trees that were not the object of the original pioneers. Many bark beetle species synthesize aggregation pheromones from turpentine components of host oleoresin.

cis-Verbenol, (+)-ipsdienol and (−)-ipsdienol (Figure 3), produced by *Ips paraconfusus*, were the first scolytid aggregation pheromones identified. *I. paraconfusus* converts the acyclic monoterpene myrcene to either enantiomer of ipsdienol, both of which appear to be important in communication in this beetle genus (16). (+)-Ipsdienol acts as an aggregation pheromone for *I. paraconfusus*, whereas (−)-ipsdienol acts as an aggregation inhibitor to regulate attack density and direct colonization to adjacent host trees. In the case of the pine engraver (*Ips pini*), the signaling pattern is reversed (10); the complementary adaptation between these two co-occurring beetle species is thought to regulate interspecific competition (69, 81). The chiral preferences of *I. pini* may also depend upon geographical location because eastern populations of this insect are most strongly attracted by a mixture of both ipsdienol enantiomers (96).

Ips typographus produces (+)-*cis*-verbenol and (+)-*trans*-verbenol (Figure 3) by oxygenation of the monoterpene precursors (+)-α-pinene and (−)-α-pinene, respectively, obtained from the Norway spruce (*Picea abies*) host. *I. typographus*, however, recognizes only (+)-*cis*-verbenol as an aggregation pheromone (69). Host production of both α-pinene enantiomers consequently undermines the effectiveness of pheromonal communication because the beetle transforms both antipodes to the corresponding verbenol isomers with nearly equal efficiency. The verbenols can be further oxidized to the corresponding ketones (+)-verbenone and (−)-verbenone (Figure 3), which act as anti-aggregation (dispersal) pheromones (16). As indicated above, pheromone-based dispersal regulates attack density, such that once a host has been successfully colonized by mass attack, incoming beetles are directed to broaden the offensive to adjacent trees (16, 122). In many *Dendroctonus* species, (+)-*trans*-verbenol is a strong attractant that often acts synergistically with other pheromones or host monoterpenes (58, 92). In the case of the mountain pine beetle (*Dendroctonus ponderosae*), *trans*-verbenol is ineffective as an attractant, unless presented in combination with host monoterpenes (9, 91).

Although the involvement of bark beetles in the production of pheromones is clear, the biosynthetic origin of these signaling compounds is still at issue. Some fungal symbionts and insect gut microorganisms are able to oxidize the host monoterpenes α-pinene and myrcene to the corresponding pheromones (17, 18, 59, 70, 71). However, the production of (+)- and (−)-ipsdienol by *Ips* species, in the absence of the myrcene precursor, has been reported (19, 103), indicating that at least some bark beetle species have the capacity to synthesize pheromones de novo. Francke & Vite (42) have hypothesized that the oxidative modification of host monoterpenes by bark beetles may have arisen as a detoxification mechanism. The ultimate exploitation of these detoxification products in pheromone signaling would thus represent a most unusual and interesting evolutionary adaptation.

Insect predators and parasitoids of bark beetles also respond to bark beetle pheromones and to conifer monoterpenes, but the specificity of these responses is not well understood, in part because these insects, especially predatory beetles, tend to feed on a wide range of prey and may exploit a broad range of chemical signals (87). An illustrative example of these complex interactions is provided by

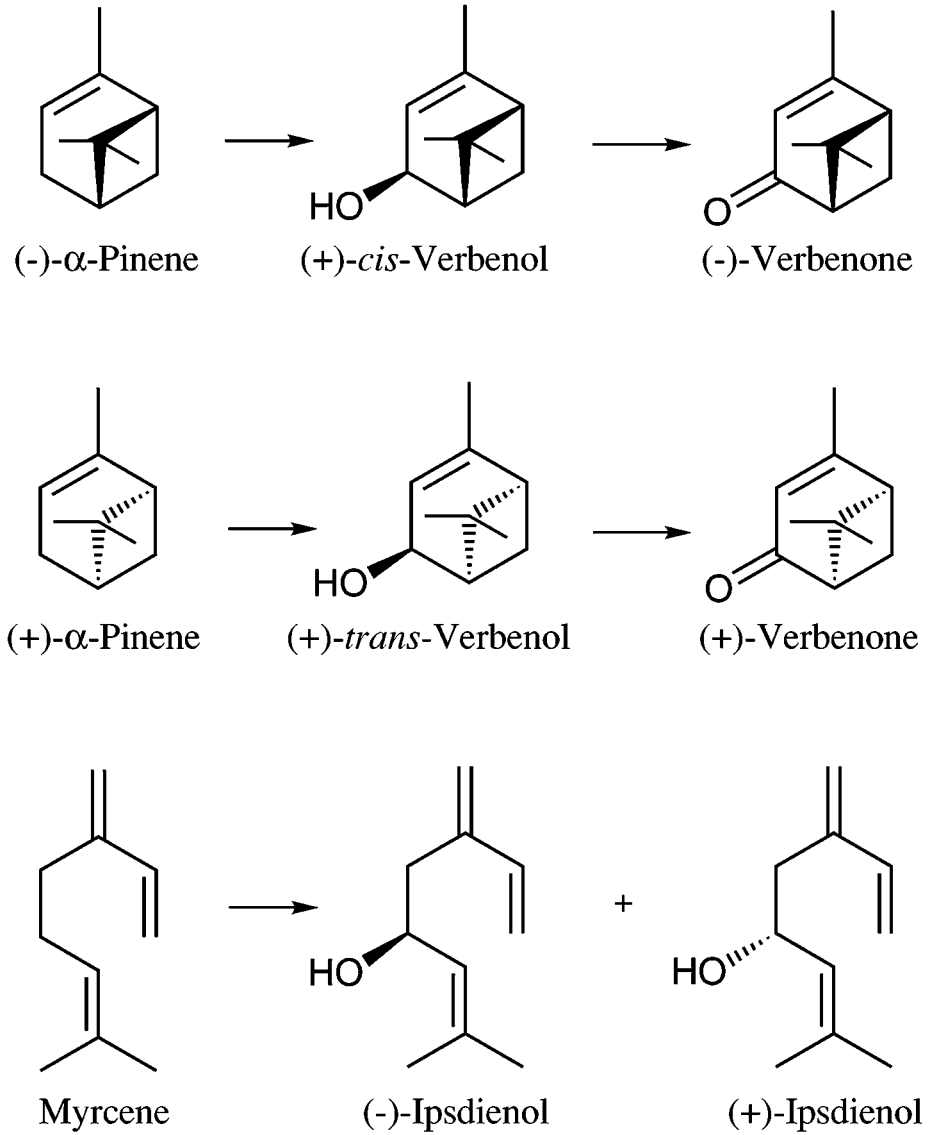


Figure 3 Pheromone biosynthesis. The structures of selected insect pheromones and their corresponding monoterpene olefin precursors are shown. Transformations occur typically in the hindgut of the bark beetle and have been attributed to associated microorganisms. Depending on the species, pheromones may signal aggregation or dispersal, and they may also attract beetle predators and parasitoids (adapted from Gijzen et al; 8).

three trophic levels of Norway spruce (*Picea abies*), the European spruce bark beetle (*Dendroctonus micans*), and the predatory beetle (*Rhizophagus grandis*). Within this interaction, resin monoterpenes attract both bark beetles and predator beetles to mediate host location and bark beetle aggregation, as well as long-range prey location and oviposition stimulation of the *Rhizophagus* predator (54). In this complex ecological context, the selection pressure on the host to alter resin chemistry is clearly multidimensional, and it involves considerations of attraction or repulsion, toxicity, pheromone signaling, and tritrophic level interactions (89, 122).

Raffa & Klepzig (96) have suggested that the chirality of ipsdienol beetle pheromones has evolved as a response to predator recognition. Thus, two species of predatory beetles that feed on *Ips pini* are drawn to the mixture of ipsdienol antipodes produced by the prey; however, the mixture of stereoisomers that is optimally attractive for the predators differs from that which is most attractive to *I. pini*. Such allomonal (signals that benefit the emitter) and kairomonal (signals that benefit the receiver) effects may impose strong selective pressures favoring the use of one or the other enantiomer in chemical signaling (52, 87, 96, 122).

Although it is paradoxical that bark beetles are attracted to and exploit monoterpenes that are both toxic and signal predators, the benefits to the beetle of utilizing specific semiochemical cues to locate a suitable host must outweigh the disadvantages. These interactions illustrate the complexity of the evolutionary relationships between conifer host and insect herbivores, causing selection over many generations to produce bark beetles that are highly adapted to a particular host and conifers that produce highly diverse chemical defenses (36, 52). Most information bearing on resin-based defenses concerns interactions with bark beetles; however, it is important to note that oleoresin also provides the basis of complex interactions with numerous other conifer pests and pathogens, including insect defoliators (101), root feeders (84), nematodes (48), and even mammalian and avian herbivores (31, 56).

CONIFER RESIN PRODUCTION

The term terpene (or terpenoid) derives from the German word for turpentine (Terpentin) from which the first members of this class of natural products were isolated and their structures determined. Historically, the study of conifer resin components is entwined with the development of classical organic chemistry and biogenetic theory (27), and the processing of oleoresin once formed the basis of a substantial “naval stores” industry. With the passing of wooden sailing ships and the growing use of petroleum, the naval stores industry collapsed by the turn of the century, although oleoresin still finds use in the commercial production of turpentine and rosin and as starting chemicals in the preparation of flavorings, adhesives, sealants, and materials for the printing industry (33, 124). As a consequence of this historical legacy, the chemistry of oleoresin terpenes is very well understood. More

recent studies in the context of resin production have focused on those properties most relevant to host defense function (insect toxicity and solvation properties of turpentine components as well as viscosity, crystallinity, and oxidative polymerization properties of rosin components) and on the biological origins of this unique plant secretion.

Copious resin production responsible for the killing and encasing of bark beetles and fungal associates, i.e. the phenomenon of "pitching out" (Figure 2*B*), is the most important chemical defense of the host in repelling initial invasion. Depending on the species, resin production may be largely constitutive (primary resin), composed of material produced and stored in specialized secretory structures, or induced (secondary resin) as the result of injury. Most conifers rely on some combination of preformed and inducible resin defenses, but there is a fairly clear correlation between the anatomical complexity of the specialized resin secretory structures and the reliance on constitutive defenses (76).

Sites of Resin Production and Accumulation

The simplest type of compartmentalized structures are resin cells, isolated cells containing oleoresin that are scattered throughout the stems of certain conifer species, including western red cedar (*Thuja plicata*; Cupressaceae). Other conifer species exhibit a higher degree of anatomical organization in their resin secretory structures. California redwood (*Sequoia sempervirens*; Taxodiaceae) and true firs, such as grand fir (*Abies grandis*; Pinaceae), accumulate oleoresin in resin blisters (often with scattered resin cells also present in the xylem). Resin blisters (or cysts), located in the wood and bark, are oleoresin-containing, multicellular sack-like structures surrounded by a layer of epithelial cells (4). During development, the walls of the epithelial cells thicken and lignify, and presumably die during the year of origin (88). Douglas Fir (*Pseudotsuga menziesii*), western larch (*Larix occidentalis*), and Colorado blue spruce (*Picea pungens*) display an even higher order of organization in which a network of constricted resin passages and ducts reside throughout the trunk. The toxic oleoresin is accumulated within the lumen of these structures, which are surrounded by a layer of thin walled epithelial cells, that presumably remain biologically active for several years (88). Pine species (*Pinus*) contain the most elaborate network of interconnected nonconstricted resin ducts (Figures 2*D*, *E*) located throughout the wood and bark (39, 117). The longevity of the secretory epithelial cells that produce the oleoresin is also loosely correlated with anatomy; thus, the more complex the resin secretory system, as in pines, the more long-lived are the epithelial cells (60).

If a bark beetle severs a resin passage or duct system of a pine or spruce in the process of establishing a gallery, it is often overwhelmed by the rapid and copious exudation of resin, which results in sudden death (Figure 2*B*) (20, 26, 104). The efficacy of the process is primarily dependent upon the composition and amount of oleoresin that may flow under pressure to the site of attack (reaction zone) by emptying connected resin passages along several meters of the trunk

(Figure 2*E*). The response to an individual boring insect depends on attack density and the overall physiological condition of the tree (52). Only conifer species with substantial constitutive reservoir systems (pines and spruces) can depend upon such preformed resistance as a primary defense strategy (8). A secondary response involving the induced production of resin can often be distinguished in these species (28), but the amount is small compared with the initial resin flow.

Bark beetles occasionally penetrate resin cells and blisters in their excavation; however, the scattered nature of these structures in the trunk limits their utility in primary defense. Species lacking extensive resin ducts or passages (*Abies*, *Tsuga*, *Cedrus*, etc) must rely on induced (secondary) oleoresin production as a defense against bark beetle infestation, and this localized response is usually part of a more generalized hypersensitive reaction at the site of injury (8). Induced resin production is not carried out by epithelial cells of secretory structures but rather by parenchyma cells (Figure 2*E*) surrounding the site of injury. Secondary resin can usually be distinguished from primary resin by chemical composition, and studies with grand fir (*Abies grandis*) have indicated that de novo resin biosynthesis is much the same whether induction results from beetle penetration or other physical trauma, with the level of production reflecting the extent of injury (72). Although induced resin production is necessarily a slower process than the immediate flow of stored resin to a wound site, de novo resin production at the site of injury in a healthy response nevertheless can create a formidable barrier to beetle excavation.

Conifers, such as firs, that lack an interconnected duct system often respond to wounding by forming traumatic resin ducts, which are normally absent in uninjured tissue (39, 40, 67). These cyst-like structures accumulate resin but lack epithelial cells. By contrast, pines respond to wounding by forming more resin ducts that are anatomically indistinguishable from normal ducts but are often improperly called traumatic resin ducts (41).

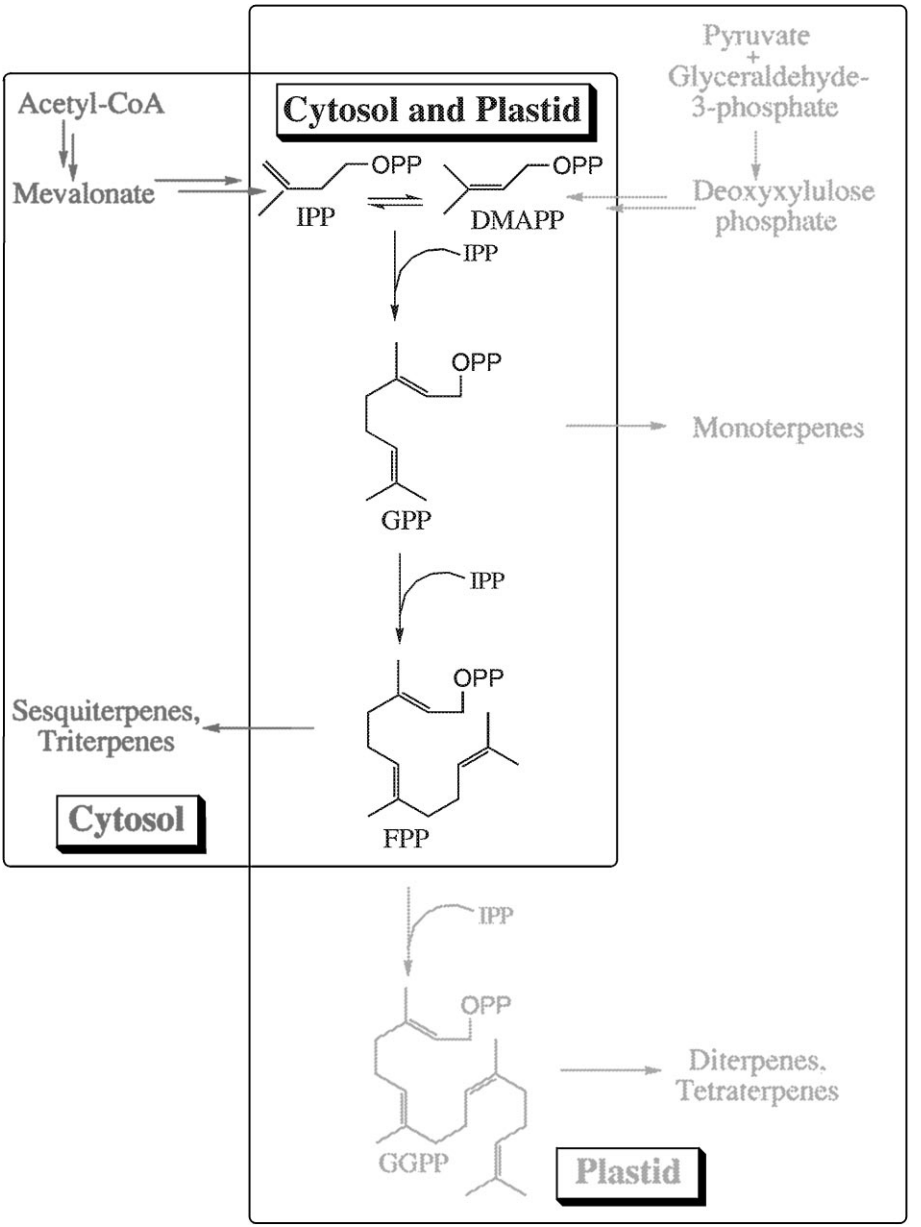
Resin Biosynthesis

Like all terpenoids, oleoresin terpenes arise from the fundamental precursor isopentenyl diphosphate, which in plants arises at one of two subcellular locations. The classic acetate/mevalonate pathway (21) operates in the cytosol compartment in which sesquiterpenes and triterpenes are formed, whereas the mevalonate-independent pathway (initiated from pyruvate and glyceraldehyde 3-phosphate) operates in the plastids to provide the monoterpenes, diterpenes, and tetraterpenes (37, 78). Following production of isopentenyl diphosphate and its isomerization to dimethylallyl diphosphate by isopentenyl diphosphate isomerase, the latter is condensed with one, two, or three units of isopentenyl diphosphate by specific prenyltransferases at the corresponding subcellular locales to give the respective precursors of the monoterpenes (geranyl diphosphate), sesquiterpenes (farnesyl diphosphate), and diterpenes (geranylgeranyl diphosphate) (Figure 4).

The terpenoid synthases next convert the respective acyclic precursors, geranyl, farnesyl, and geranylgeranyl diphosphate, to the various parent structural derivatives of the different terpene families and so represent the committed enzymes of these pathways (32). These enzymes are often called cyclases because most of the products derived from the three central acyclic precursors are cyclic. Terpenoid synthases have been isolated and characterized from several conifer species, but the bulk of this work has been carried out with grand fir (*A. grandis*), a common and widespread species in the Pacific Northwest. The plant is easily raised in the greenhouse, and oleoresin production is wound-inducible, which mimics bark beetle attack (73), thereby offering significant advantage for biochemical and molecular study.

The monoterpene synthases of grand fir are very similar to each other (50), and they differ little in their properties from their counterparts isolated from pine species (99). All are operationally soluble proteins with native (monomeric) sizes in the 50–70 kDa range, pI values around pH 6, and pH optima in the pH 6.8 to 7.8 range (generally higher than angiosperm synthases) (13, 32, 121). They require a divalent metal ion for catalysis (Mg^{2+}, Mn^{2+}, or Fe^{2+}), and activity is stimulated by monovalent cations (preferably K^{+}) (99); this latter property is not shared by angiosperm synthases. Michaelis constants for the prenyl diphosphate substrate rarely exceed 10 μM, and turnover rates typically range from 0.02 to 0.2 s^{-1} (121). The inducible turpentine of grand fir differs in composition from that of the constitutive oleoresin, and these differences are reflected by the differing product outcomes of the inducible monoterpene synthases. In general properties, however, the constitutive and inducible synthases are indistinguishable. All conifer monoterpene synthases appear to arise as preproteins targeted to the plastids (see below), consistent with the origin of the essential geranyl precursor at this locale.

All conifer monoterpene synthases examined thus far produce acyclic or cyclic olefins as products (121), consistent with the composition of turpentine in which oxygenated monoterpenes only make up trace components. As indicated previously, host monoterpene olefins do serve as the precursors of several oxygenated derivatives produced and employed by bark beetles as pheromones (Figure 3). The monoterpene synthase reaction involves the initial, metal ion–assisted ionization of the substrate to the corresponding carbocation-diphosphate anion pair, which may undergo direct deprotonation at various positions to yield the acyclic monoterpenes, such as myrcene (Figure 5). Alternatively, and more commonly, the ionized geranyl precursor may collapse to the corresponding tertiary allylic isomer linalyl diphosphate (Figure 5). The effect of this tightly coupled isomerization step is to convert the original *trans*-C2,C3-double bond of the geranyl substrate (which sterically prohibits direct cyclization) to a single bond of the linalyl intermediate, such that, following transoid to cisoid rotation then ionization, cyclization to the α-terpinyl carbocation is allowed from which all cyclic monoterpenes are subsequently derived (Figure 5). The α-terpinyl cation may suffer direct deprotonation to give, for example, limonene, or it may undergo a range of internal additions to the remaining double bond, hydride shifts, or other rearrangements before the



terminating deprotonation to yield any of several possible bicyclic olefins. The cyclizations to (−)-α-pinene and (−)-β-pinene are illustrative of this reaction type (Figure 5). Many monoterpene synthases essentially produce a single product, but there are several examples of these enzymes that produce multiple products from the geranyl substrate. The inducible pinene synthase from grand fir (74) and the constitutive pinene synthases from several pine species (90, 99), for example, produce substantial amounts of both (−)-α-pinene and (−)-β-pinene by simple variations on the same reaction mechanism. The similarity in properties, and also in primary sequence (see below), of these enzymes indicates that rather subtle differences in structure and mechanism can result in very different product outcomes.

Relatively few sesquiterpene synthases from conifers have been examined thus far. However, it is clear from this work that these cytosolic enzymes resemble the plastidial monoterpene synthases in general properties, although the divalent cation requirement may differ and monovalent cations have no influence on reaction rate. In the case of the sesquiterpene synthases, the longer chain length and additional double bond of the farnesyl substrate permit far greater flexibility in the construction of different carbon skeletons by the same type of electrophilic cyclization mechanisms, involving initial ionization of the diphosphate ester. This great range in mechanistic flexibility is exploited by two constitutive sesquiterpene synthases from grand fir, δ-selinene synthase and γ-humulene synthase, which each produces in excess of 30 different sesquiterpene olefin products from the farnesyl diphosphate substrate (107). These two very prolific enzymes may represent evolutionary adaptations for maximizing chemical diversity with the minimum genetic and enzymatic machinery. A contrasting example is provided by the wound-inducible (*E*)-α-bisabolene synthase from grand fir, which produces this single olefin as product. It is significant to note that (*E*)-α-bisabolene is efficiently converted to todomatuic acid and juvabione by subsequent oxidative metabolism in grand fir and related species (Figure 6) (11). These oxygenated sesquiterpenes are mimics of juvenile hormone III and can disrupt insect reproduction and/or larval development. The production of these modified sesquiterpenes by true firs (*Abies*) might represent a second line of defense directed to insect fecundity, should the initial resinosis response be overcome and successful infestation of the host occur (11, 89).

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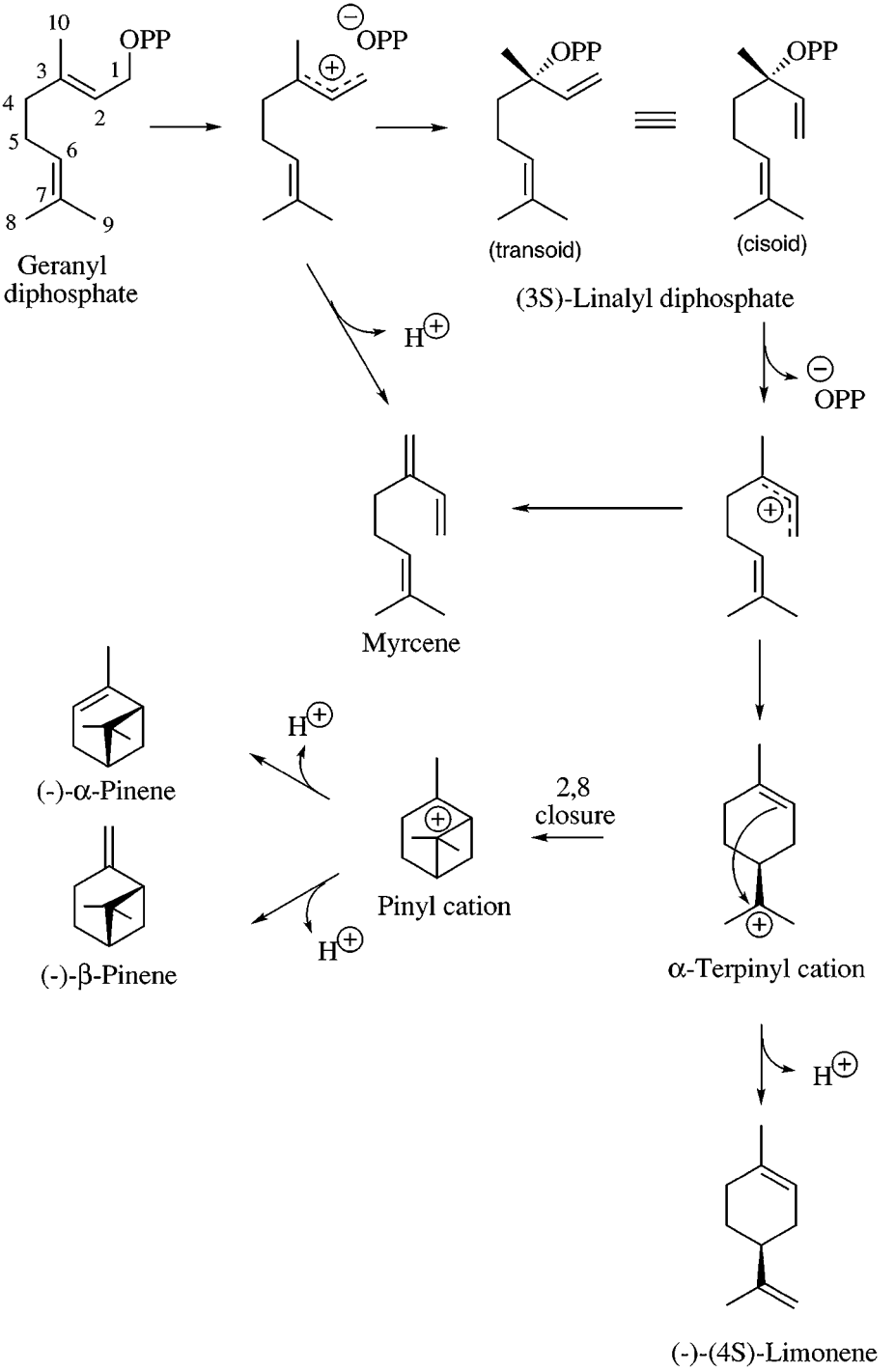
Figure 4 Intracellular compartmentalization of the mevalonate and mevalonate-independent pathways for the production of isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), and associated terpenoids in higher plants. The cytosolic pool of IPP, which serves as a precursor of farnesyl diphosphate (FPP) and, ultimately, the sesquiterpenes and triterpenes, is derived from mevalonic acid. The plastidial pool of IPP is derived from the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate, and it provides the precursor of geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) and, ultimately, the monoterpenes, diterpenes, and tetraterpenes. Reactions common to both pathways are enclosed by both boxes, although separate prenyltransferases are responsible for catalyzing these reactions in each compartment.



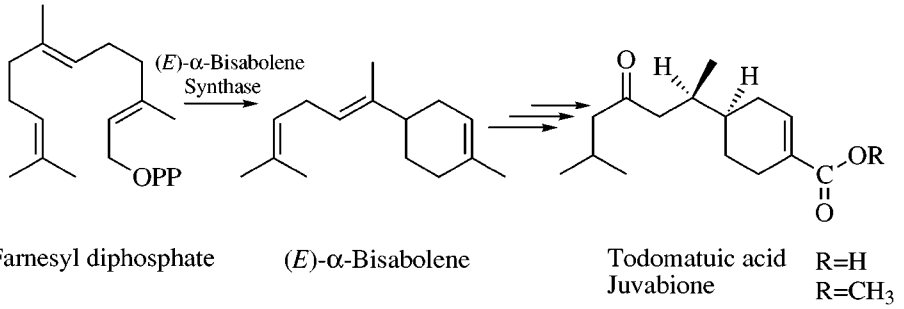


Figure 6 Biosynthesis of the sesquiterpenoid insect juvenile hormone mimics todomatuic acid and juvabione. The sesquiterpene (*E*)-α-bisabolene is derived from farnesyl diphosphate by (*E*)-α-bisabolene synthase of grand fir (*A. grandis*) and is further modified through a series of redox transformations to products that simulate the action of juvenile hormone III, an insect molting hormone.

Although conifer diterpene synthases appear to resemble monoterpene synthases and sesquiterpene synthases in general properties, the cyclization reactions leading to the diterpene olefin precursors of the resin acids are fundamentally different from those of the monoterpene and sesquiterpene series. In these two-step reactions, a proton-initiated cyclization of geranylgeranyl diphosphate leads first to the bicyclic, enzyme-bound intermediate copalyl diphosphate (Figure 7). A second reaction, involving the more typical ionization of the diphosphate ester, allows formation of the third ring that, with or without a methyl migration step and a terminating deprotonation, provides the various pimarane or abietane precursors. These various olefin isomers, differing in the substituents on the third ring and in the positions of the double bonds, undergo oxidative conversion of the C18 methyl to a carboxyl to yield the corresponding resin acid (68). In the case of abietic acid, one of the most common resin acids and the most prone to polymerization, the abietadiene precursor is oxidized in three distinguishable steps to the resin acid by two cytochrome P450 hydroxylases and a dehydrogenase (46). The constitutive and inducible forms of abietadiene synthase from grand fir appear to be indistinguishable and to carry out both of the coupled cyclization steps without the formation of free intermediates (68, 115a). Like the monoterpene synthases, abietadiene synthase arises as a preprotein bearing an

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Figure 5 Mechanism of the monoterpene synthase reaction. The formation of myrcene, (−)-(4*S*)-limonene, (−)-α-pinene, and (−)-β-pinene from the precursor geranyl diphosphate are shown, illustrating the initial isomerization step, which is necessary to overcome the geometric impediment to direct cyclization of the geranyl precursor. Subsequent internal additions in the α-terpinyl cation, methyl migrations, and hydride shifts may also occur, with the ultimate outcome depending on the specific substrate and intermediate folding conformations allowed by the particular synthase.

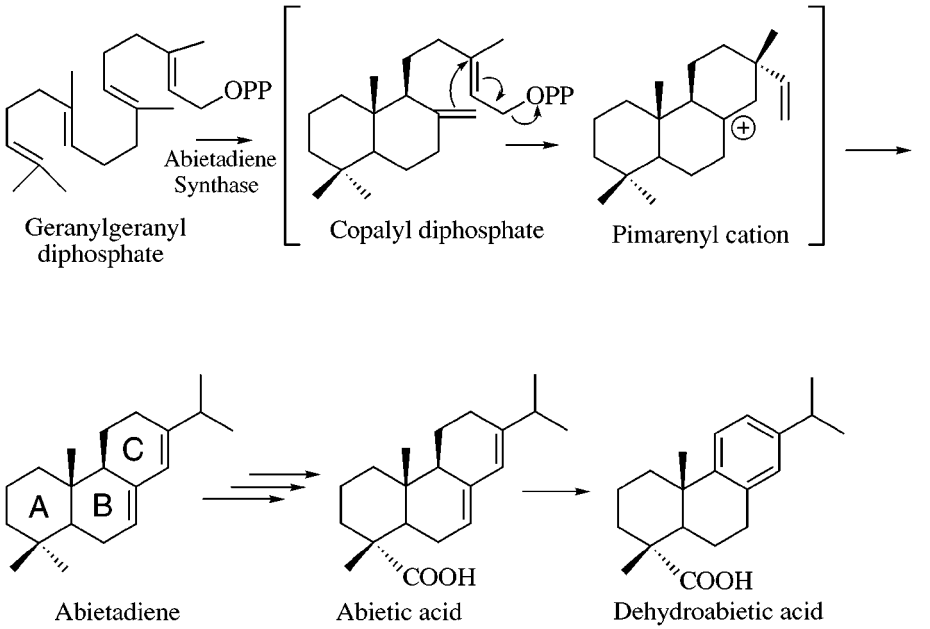


Figure 7 Biosynthesis of the diterpenoid resin acids, abietic acid and dehydroabietic acid. The formation of abietadiene from geranylgeranyl diphosphate is catalyzed by (−)-abietadiene synthase from grand fir (*A. grandis*) via the bound intermediate (+)-copalyl diphosphate. The subsequent oxidations of the olefin are catalyzed by two cytochrome P450-dependent oxygenases and an NAD^+-dependent dehydrogenase.

N-terminal plastidial targeting sequence for localization to the site of diterpene biosynthesis.

Regulation

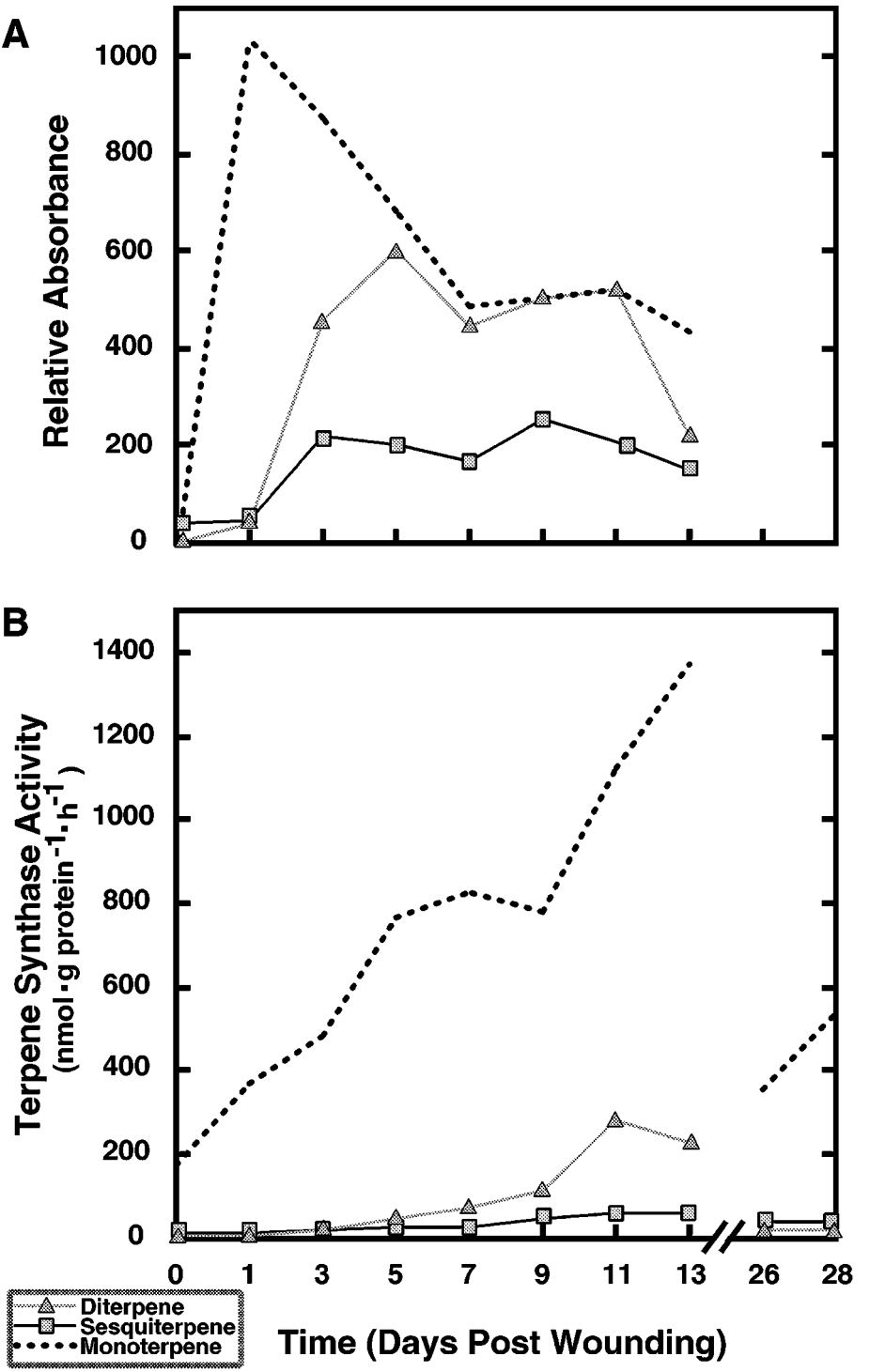
Because of the wound-inducibility of oleoresin production in grand fir saplings under laboratory conditions, this species has been developed as a model for studying the regulation of conifer defense. The time course of the response was initially investigated at the level of resin chemistry by monitoring the levels of the relevant monoterpene, sesquiterpene, and diterpene biosynthetic enzymes (47, 109). The response time is slower than that typical of phytoalexin production in angiosperms but is sustained for several weeks, as is appropriate to the longer time frame involved in beetle colonization and attendant bark boring and tunnel mining activity (Figure 8). The production of induced oleoresin monoterpenes, sesquiterpenes, and diterpenes is coordinated to accomplish a range of defense goals; the first of which is the localized generation of large quantities of sticky, semi-fluid toxic material to physically impede the progress of beetle mining and to poison the invaders. The turpentine components provide the principal toxins (26, 86, 95) and the

solvent for mobilization of the resin acids. Solvation properties of the turpentine components and the composition of the rosin fraction (in terms of crystallinity and susceptibility to oxidative polymerization) are important considerations in producing a sufficiently viscous oleoresin to deter beetle progress. Yet, this material must also be sufficiently mobile to flush the wound and permit flow to the trunk surface, whereupon the turpentine evaporates to deposit a semi-crystalline mass of resin acids that upon exposure to air and UV light polymerizes to form a hardened plastic coating to seal the wound site (29). The cytochrome P450 oxygenases involved in resin acid biosynthesis are upregulated in coordination with the terpene synthases (47); however, timing in the appearance of the enzymatic machinery involved in the oxidative conversion of (*E*)-α-bisabolene to todomatuic acid and juvabione has not yet been evaluated.

Given the complexity of the bark beetle-host interaction, and the many functions of the oleoresin, it is not a trivial exercise (aside from a few broad generalizations, see below) to consider what a chemically and physically optimized resin should contain. Little guidance is provided by an assessment of the induced response among a native population of grand fir in which investigators observed wide variation, especially in the composition of monoterpene production (64). This evidence suggests that there is no single best strategy to deter beetle attack. Studies with water-stressed and light-stressed trees have demonstrated that, regardless of compositional variations in induced oleoresinosis, such stresses delay and/or diminish the response and ultimately eliminate it entirely (75). These results are consistent with field observations indicating that physiologically compromised trees are most readily overwhelmed by massed beetle attacks and often provide the focus for wider spread infestation.

With the availability of cDNA clones that encode several of the monoterpene, sesquiterpene, and diterpene synthases of grand fir (see below) and of polyclonal antibodies directed against some of the corresponding native or recombinant enzymes, researchers are able to examine the defense response in greater detail and demonstrate that induced oleoresinosis arises via the differential transcriptional control of the synthase genes in response to wounding (108). A more precise time course evaluation of the relevant enzyme activities indicates that the monoterpene synthases appear first and proceed to accumulate (Figure 8*B*), correlating closely with the results of immunoblot experiments using broadly crossreacting polyclonal antibodies directed against (−)-pinene synthase (51). RNA-blot hybridization analysis over the same induction time course, using terpene synthase class-specific probes, again indicates that the monoterpene synthases arise first (transcripts detectable in 2 h, reaching a maximum steady-state abundance in 2–4 days) and are followed by a coordinated increase in sesquiterpene and diterpene synthase transcripts (transcription beginning on day 3 or 4 and reaching maximum steady-state abundance at 13–14 days) (Figure 8*A*) (108).

Evaluation of the products of the inducible monoterpene synthases indicate that limonene, a prominent constitutive monoterpene, is the principal monoterpene generated at day 1 after wounding but is replaced by α-pinene and β-pinene



as principal constituents by day 7 after wounding. This dynamic alteration is consistent with the notion that the production of toxic monoterpenes (e.g. limonene) is first upregulated, then followed by the production of monoterpenes that are more notable for their solvent properties [e.g. pinenes (66)] in dissolving resin acids. For all terpene synthase types, enzyme activity levels continue to rise after steady-state mRNA levels are reached, suggesting that the terpene synthases are quite stable in vivo and are not turned over until several weeks later. The time delay in production of diterpene resin acids following the initiation of monoterpene biosynthesis might result from the need to first generate the solvent for rosin mobilization (108), and it fits the time-frame of beetle attack, involving many days, thereby maximizing entrapment of boring beetles and minimizing the possibility of withdrawal to establish a brood elsewhere (89). Although the sesquiterpenes make up only a small portion of conifer oleoresin (about 15% of the turpentine of grand fir), the mixture of constitutive sesquiterpenes is exceedingly complex, and it is now clear that wounding does induce sesquiterpene synthesis as well, suggesting that these resin components may be more important in defense than previously appreciated (11). The wound-inducible sesquiterpene synthases of grand fir, (*E*)-α-bisabolene synthase and δ-cadinene synthase, produce single products with high fidelity, but neither of these olefins accumulates. (*E*)-α-Bisabolene is transformed through a series of steps to the juvenile hormone mimics todomatuic acid and juvabione, whereas δ-cadinene is efficiently converted to oxygenated metabolites of as yet unknown structure. In contrast to diterpene synthase gene expression, (*E*)-α-bisabolene synthase mRNA levels continually increase to a maximum, which is reached at about 12 days after wounding (11), suggesting that the delayed de novo biosynthesis of the juvenile hormone analogs is designed to interfere with insect development as a second line of defense should infestation of the host succeed. Researchers have shown the wound induced accumulation of juvenile hormone analogs in intact fir (93), but they have not evaluated the direct influence of this phenomenon on insect fecundity.

Molecular Genetics

Much progress in understanding the molecular genetics of terpenoid formation has been made with angiosperms as genes encoding a monoterpene synthase (25), a sesquiterpene synthase (38), and a diterpene synthase (79) were first isolated from these species. Each of these initial genes was isolated using tools developed

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Figure 8 Regulation of terpene synthases in grand fir saplings in response to wounding. (*A*). Changes in terpenoid synthase mRNA levels. (*B*). Corresponding changes in terpene synthase enzymatic activities. Developmental Northern blots of total mRNA with respective probes are described in Steele et al 1998 (108). Enzyme activities for monoterpene synthases (dotted line), sesquiterpene synthases (squares), and diterpene synthases (triangles) are plotted as a function of time after wounding (replotted from Steele et al 1998) (108).

from the corresponding protein. The first terpenoid synthase cDNA acquired from a conifer (the diterpene cyclase abietadiene synthase from grand fir) was also obtained by the reverse genetic approach (115a). However, because gymnosperm terpene synthases resemble in primary sequence their angiosperm counterparts, most recent cloning efforts have involved homology-based approaches (13, 109) with which seven monoterpene synthase genes (14, 15), three sesquiterpene synthase genes (11, 107), and two diterpene synthase genes (115a, 119) have been isolated and confirmed by functional heterologous expression. All these genes have been obtained from grand fir with the exception of one, the diterpene cyclase taxadiene synthase from yew (*Taxus*) that catalyzes the first committed step in the biosynthesis of the anti-cancer drug Taxol (119).

Nearly 40 cDNAs encoding gymnosperm and angiosperm terpene synthases have now been acquired, consisting of six phylogenic families. Based on sequence similarity, the conifer synthases are clustered in the *Tpsd* gene family. The monoterpene synthases and diterpene synthases are encoded as preproteins bearing plastidial transit peptides, whereas the sesquiterpene synthases bear no obvious N-terminal targeting information befitting their cytosolic localization. The sequences of all conifer terpenoid synthase types thus far examined contain conserved elements, including the DDXXD motif involved in substrate binding and aromatic residues that may be involved in stabilizing carbocation intermediates of the reaction sequence (13, 121). Tandem arginines near the N terminus of mature monoterpene synthases appear to play a role in the isomerization step required by most enzymes of this class (120). Although no crystal structures of conifer terpene synthases are yet available, modeling studies based upon the structure of *epi*-aristolochene synthase from tobacco (106) suggest that all terpenoid synthases may share a similar overall folding pattern.

A wide range of nomenclatures has been applied to the terpenoid synthases, none of which is systematic. Here we use a unified and specific nomenclature system in which the Latin binomial (two letters), substrate (one to four letter abbreviation), and product (three letters) are specified. Thus, *ag*22, the original cDNA designation for abietadiene synthase from *A. grandis* (a *Tpsd* subfamily member), becomes AgggABI for the protein and Agggabi for the gene; the remaining conifer synthases (and other selected genes) are described accordingly (Table 1).

Protein-Based Phylogenetics

Amino acid sequence comparison of 12 conifer terpene synthases enabled researchers to identify common structural features of this enzyme class (12, 13, 32). Terpene synthase cDNAs encode proteins of 550–850 amino acids (aa) (13), in agreement with observed native molecular masses in the 50–100 kDa range. In general, sesquiterpene synthases are 580–650 aa in length and are 50–70 aa shorter than monoterpene and diterpene synthases due to the absence of the N-terminal plastidial transit peptide (Figure 9) (12). Although conifer monoterpene and diterpene synthases lack significant primary sequence similarity in the transit peptide

region, these targeting sequences characteristically contain a high content of serine and threonine residues and a low content of acidic residues (65). Sequence conservation begins just downstream of an RR (tandem arginine residues) motif of the monoterpene synthases, suggesting that the approximate cleavage site of the transit peptide may be defined by this RR element (Figure 9) (120). Truncation studies with limonene synthase have demonstrated that residues upstream of the tandem arginine element are not required for activity and that the RR motif plays an important role in the isomerization step of the monoterpene cyclization reaction (120). The conifer diterpene synthases AgggABS and TbggTAX (115a, 119) are approximately 210 aa longer than the monoterpene synthases. This difference in length is accounted for by an additional internal sequence element, termed the conifer diterpene internal sequence (CDIS) element (Figure 9), that is conserved in both sequence and position regardless of the type of cyclization catalyzed by these enzymes (13). This CDIS element is also observed in the grand fir sesquiterpene synthase (*E*)-α-bisabolene synthase, AgfEαBIS (11), and in the angiosperm monoterpene synthase linalool synthase from *Clarkia* (30, 35). The CDIS element in these two synthases may be relictual and alludes to their evolutionary origins. No function has been assigned to this element and, based upon the location and distance from the active site, the CDIS element is suspected to serve a purpose other than a catalytic function (13). Based upon modeling from the crystal structure of aristolochene synthase (106), investigators found that conifer terpene synthases, like other terpene synthases, are composed of two distinct structural domains, a C-terminal active site domain and an N-terminal domain that structurally resembles the catalytic cores of glycosyl hydrolases (13) (Figure 9).

The phylogenetic reconstruction based upon amino acid sequence comparison of 33 plant terpene synthases indicates that conifer monoterpene, sesquiterpene, and diterpene synthases are more closely related to each other than they are to their respective, mechanism-based counterparts of angiosperm origin (13), as exemplified by their grouping in the Tpsd family. Additionally, conifer synthases within each class of the same species (producing mono-, sesqui-, or diterpenes) are more closely related to each other than they are to the same cyclase type of other conifers. Prior to phylogenetic analysis, immunochemical studies (51) had indicated that pinene synthase from grand fir is more closely related to other monoterpene synthases from grand fir than to pinene synthases from related conifer species. Consistent with this observation, the six monoterpene synthases (Table 1) from grand fir share 60% or greater sequence identity and form a subgroup within the Tpsd family. The similarity of these six synthases may indicate that they have arisen by multiple gene duplications (13).

The terpene synthases of gymnosperms and angiosperms are divided into six gene families, designated Tpsa through Tpsf, with the groupings based upon a minimum amino acid sequence identity of 40% (13). Terpenoids involved in primary metabolism (e.g. phytosterols, carotenoids, gibberellins, and brassinosteroids) are essential for viability, whereas those derived from secondary metabolism are not. The majority of terpene synthases analyzed to date produce secondary metabolites

TABLE 1 Conifer and other selected terpene synthases

Terpene synthase (products)	Species	Former gene name	Enzyme name	cDNA/gene name	cDNA/ mRNA Acc #	gDNA Acc #
Abietadiene	*A. grandis*	ag22	Ag**gg**ABI	Ag**gg***abi*	U50765 (81)	upub
(*E*)-α-Bisabolene	*A. grandis*	ag1	Ag**f**EαBIS	Ag***f****Eαbis*	AF006195 (78)	upub
(−)-Camphene	*A. grandis*	ag6	Ag**g**-CAM	Ag***g****-cam*	U87910 (96)	-------
γ-Humulene	*A. grandis*	ag5	Ag**f**γHUM	Ag***f****γhum*	U92267 (77)	-------
(−)-4S-Limonene	*A. grandis*	ag10	Ag**g**-LIM1	Ag***g****-lim*	AF006193 (95)	upub
Myrcene	*A. grandis*	ag2	Ag**g**MYR	Ag***g****myr*	U87908 (95)	-------
(−)-(1S,5S)-Pinene	*A. grandis*	ag3	Ag**g**-PIN1	Ag***g****-pin1*	U87909 (95)	upub
(−)-α-Pinene/ (−)-limonene	*A. grandis*	ag11	Ag**g**-PIN2	Ag***g****-pin2*	AF139207 (96)	-------
(−)-β-Phellandrene	*A. grandis*	ag8	Ag**g**-βPHE	Ag***g****-βphe*	AF139205 (96)	-------
δ-Selinene	*A. grandis*	ag4	Ag**f**δSEL1 Ag**f**δSEL2	Ag***f****δsel1* Ag***f****δsel2*	U92266 (77)	upub upub
Taxadiene	*T. brevifolia*	Tb1	Tb**gg**TAX	Tb***gg****tax*	U48796 (97)	upub
Terpinolene	*A. grandis*	ag9	Ag**g**TEO	Ag***g****teo*	AF139206 (96)	-------

5-*epi*-Aristolochene	*N. tabacum*	TEAS3	Nt**f**eARI3	*Nt**f**eari3*	L04680 (93)	L04680 (74)
		TEAS4	Nt**f**eARI4	Nt*f**eari4*	L04680 (93)	L04680 (74)
δ-Cadinene	*G. arboreum*	CAD1-A	Ga**f**δCAD1A	Ga*f*δ*cad1a*	X96429 (121)	Y18484[dcr]
Casbene	*R. communis*	cas	Rc**gg**CAS	Rc*gg**cas*	L32134 (94)	**na**[pc]
(−)-Copalyl diphosphate[b]	*A. thaliana*	GA1	At**gg**-COPP1	At*gg**-copp1*	U11034 (122)	**na** (123) AC004004[p]
ent-Kaurene[b]	*A. thaliana*	GA2	At**gg**-KAU	At*gg**-kau*	443904 (124)	AC007202[dcr]
(4S)-Limonene	*M. spicata*	LMS	Ms**g**-LIM	Ms*g**-lim*	L13459 (92)	-------
(S)-Linalool	*C. concinna*	LIS	Cc**g**LINOH	Cc*g**linoh*	-------	AF067602 (103)
Vetispiradiene	*H. muticus*	CVS1	Hm**f** VET1	Hm*f**vet1*	U20188 (104)	**na**[pc] (104)

[a]Abbreviations are: Acc #, EMBL Accession number; upub, genomic sequences by Trapp & Croteau (in press); na, sequences unavailable in the public databases but discussed in journal reference; pc, sequences obtained by personal communications; dcr, sequences in public database but not published; p, sequences in database with putative function; dotted line (---), no former gene name or accession number. Species names are: *Abies grandis*, *Arabidopsis thaliana*, *Clarkia concinna*, *Gossypium arboreum*, *Hyoscyamus muticus*, *Mentha spicata*, *Nicotiana tabacum*, *Ricinus communis*, *Taxus brevifolia*.

[b]Former names for (−)-copalyl diphosphate and *ent*-kaurene sythase were *ent*-kaurene sythase A and *ent*-kaurene sythase B, respectively.

[c]Nomenclature architecture is specified as follows: The latin binomial two letter abbreviations (abbrev.) are in spaces 1–2. The substrate and product (1–4 letters abbrev.) are in spaces 3–6, consisting of 1–2 letter abbrev. for substrate utilized in bold (e.g. **g**, geranyl diphosphate; **f**, farnesyl diphosphate; **gg**, geranylgeranyl diphosphate; **c**, copalyl diphosphate; **ch**, chrysanthemyl diphosphate in lower case) followed by product stereochemistry and/or isomer definition (e.g. α, β, δ, γ, etc. followed by epi (e), E, Z, −, +, etc.). The three-letter product abbrev. indicates the major product is an olefin, otherwise the quenching nucleophile is indicated (e.g. ABI, abietadiene synthase; BORPP, bornyl diphosphate synthase; CEDOH, cedrol synthase); the upper case specifies protein and the lower case specifies cDNA or gDNA. All letters except species names are in italics for cDNA and gene. Distinction between cDNA and gDNA must be stated or a "g" is added before the abbreviation (e.g.Tb*gg**tax* cDNA and gTb*gg**tax*, or Tb*gg**tax* gene. (Nomenclature system devised by S Trapp, E Davis, J Crock, & R Croteau).

(natural products) and are classified into three families, Tpsa (sesquiterpene and diterpene synthases from angiosperms), Tpsb (monoterpene synthases from angiosperms of the Lamiaceae), and Tpsd (11 gymnosperm mono-, sesqui-, and diterpene synthases from *A. grandis* and a diterpene synthase, TbggTAX, from *T. brevifolia*). The grouping into a single clade of other Tps families (Tpsc, Tpse, Tpsf) involved in primary metabolism (e.g. kaurene synthase and (−)-copalyl diphosphate synthase of gibberellin biosynthesis) suggests that the bifurcation of terpenoid synthases of primary and secondary metabolism occurred before the separation of angiosperms and gymnosperms. Furthermore, the pattern of branching between gymnosperm and angiosperm synthases and among specific synthase classes (monoterpene, sesquiterpene, and diterpene) from a common terpene synthase ancestor implies that independent functional specialization occurred after the separation of angiosperm and gymnosperm lineages. That two limonene synthases, MsgLIM and AggLIM, share only 35% identity reflects this independent specialization and indicates that limonene synthase evolved separately in mint and grand fir (13).

The conifer synthases analyzed thus far primarily represent those from a single species, and, although 33 terpene synthases were used for phylogenetic analysis, the sample size is too small to permit precise analysis. Furthermore, of the sequences analyzed, all but five terpene synthases are involved in secondary metabolism, indicating that synthases of primary metabolism are underrepresented; thus far, no conifer terpene synthases of primary metabolism are available. A variety of additional terpene synthase sequences from different gymnosperm species will be necessary to determine the accuracy of the present Tps phylogenetic scheme and its evolutionary significance.

Genomic Intron/Exon Organization

The examination of terpene synthase genomic (intron/exon) organization (S Trapp & R Croteau, unpublished data) generally supports the protein-based phylogenetic evaluation of this enzyme class (13). In addition, this genomic evaluation provides the first model for the history of the plant terpene synthase gene superfamily, including molecular evolutionary events and ancestral lineage. Until recently, the sequences of only a few angiosperm terpene synthase genes (Nt*fepiari*, Rc*ggcas*, Hm*fvet*) had been described, and these revealed very similar overall structure with six positionally conserved introns (3, 38, 79). The sequences for six conifer terpene synthase genes are now available (S Trapp & R Croteau, unpublished data), including a constitutive and an inducible monoterpene synthase (Ag*glim*, Ag*gpin*), a constitutive and an inducible sesquiterpene synthase (Ag*fαbis*, Ag*fδsel*), and two diterpene synthases (Ag*ggabi*, Tb*ggtax*). The genomic organization of these conifer terpene synthases was analyzed by alignment with seven defined angiosperm terpene synthases and eight putative terpene synthases from *Arabidopsis*. By examining patterns of intron and exon loss, CDIS domain loss, conservation of intron phase and placement, and conservation of

exon size, investigators addressed evolutionary relationships among the plant terpene synthase genes involved in primary and secondary metabolism.

Three classes of terpene synthase genes were established based upon distinct exon/intron patterns (Figure 9), and all conifer synthase genes fell into Class I or Class II. Class I comprises conifer diterpene synthase genes Ag*ggabi* and Tb*ggtax*, a sesquiterpene synthase Ag*fαbis*, and angiosperm synthase genes specifically involved in primary metabolism (At*gg-coppl* and Cc*glin*). Terpene synthase Class I genes contain 11–14 introns and 12–15 exons of characteristic size (Figure 9), including the CDIS domain made up of exons 4, 5, and 6; the first ~20 aa of exon 7; and introns 4, 5, and 6 (based upon A*gggabi* exon structure). Class II terpene synthases comprise only conifer monoterpene synthases and sesquiterpene synthases and contain 9 introns and 10 exons; introns 1 and 2, and the entire CDIS element, have been lost, including introns 4, 5, and 6. Class III terpene synthases contain only angiosperm monoterpene, sesquiterpene, and diterpene synthases involved in secondary metabolism, and these have 6 introns and 7 exons. Introns 1, 2, 7, 9, and 10 and the CDIS domain have been lost in the Class III type. The introns found in Class III terpene synthase genes (introns 3, 8, 11–14) have been conserved among all gymnosperm and angiosperm plant terpene synthase genes, with two exceptions; bisabolene synthase (Ag*fαbis*) has lost intron 14, and linalool synthase (Cc*glin*) has lost intron 3 (as well as introns 1 and 2).

The intron phases of introns 2 through 14 are conserved among gymnosperm and angiosperm terpene synthase genes (Figure 9), and this observation provides a novel means of evaluating the relatedness of genes of this type. Intron phase is defined as the placement of the intron before the first, second, or third nucleotide position of the codon and is referred to as phase 0, 1, and 2, respectively (for example, all terpene synthase genes that contain intron 3 have a phase of 0). The conservation of phases among introns indicates divergent evolutionary events. Conversely, if families of terpene synthase genes evolved by convergent evolution, it is unlikely that the introns would be so precisely placed and intron phase conserved. It is reasonably postulated that the ancestral terpene synthase gene most closely resembles a contemporary gene that contains the largest number of exons and introns because only intron loss would be anticipated (S Trapp & R Croteau, unpublished data). Limited by the sample size of 21 terpene synthase genes, the candidate gene is either At*gg-coppl* or A*gggabi* (because both contain 14 introns and 15 exons), with At*gg-copp* being most likely because it is involved in primary metabolism. Intron loss and CDIS domain loss data, presented in an evolutionary tree model of intron/exon structure (Figure 10), suggest that all angiosperm terpene synthases involved in secondary metabolic processes evolved from a gymnosperm ancestor that contained 9 introns and 10 exons. There are two exceptions to the general genomic organization pattern observed in plant terpene synthases. The sesquiterpene synthase bisabolene synthase (Ag*fαbis*) and the monoterpene synthase linalool synthase (Cc*glin*) gene structures are more similar to conifer diterpene synthase genes, all of which contain the CDIS element. Most likely, both of these are defunct

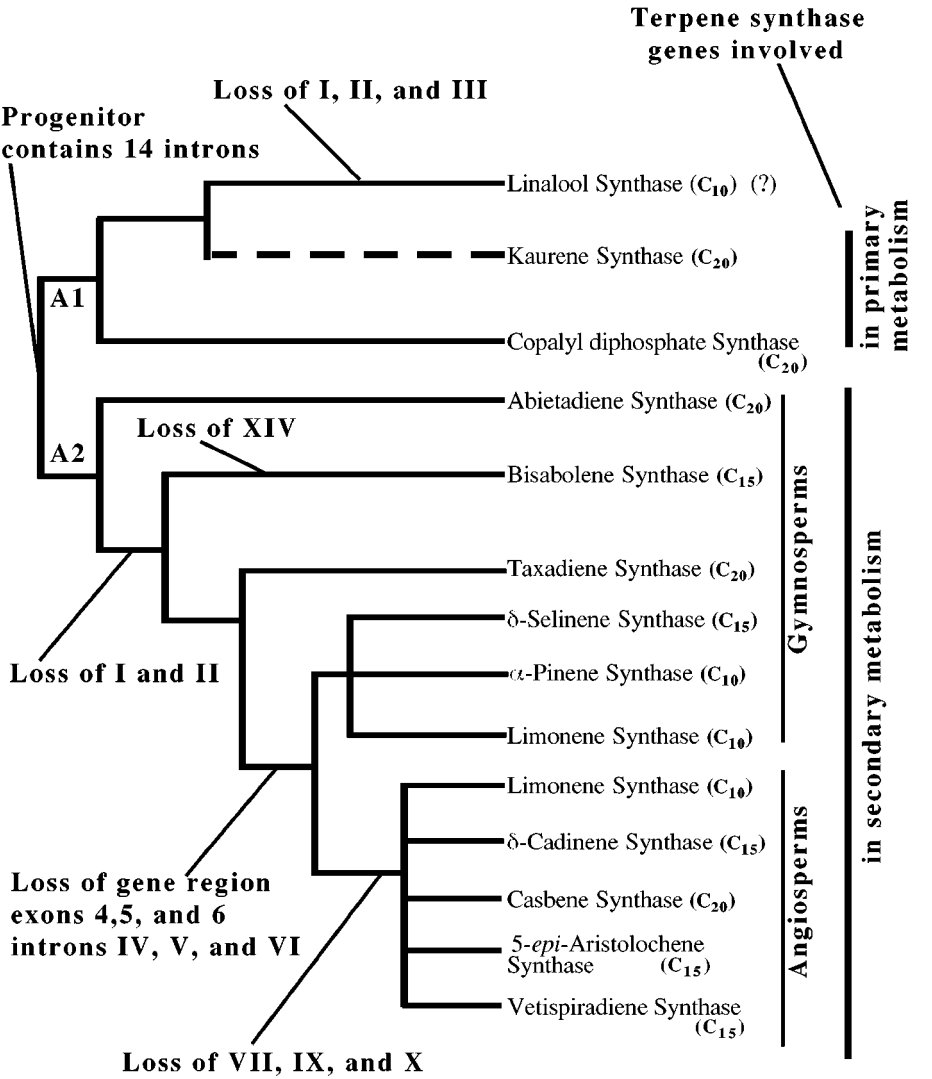

Figure 10 Model for the evolution of Tps gene intron and exon structure. The dendrogram represents an evolutionary tree based on two characters, loss of introns and loss of the CDIS domain (containing 3 exons and 3 introns). Only plant terpene synthase genes of defined function were used for the phylogenetic model (these are listed in Table I). The letters A1 and A2 represent the progeny of the duplicated ancestral gene, both containing a genome organization predicted to be similar to present day gymnosperm diterpene synthase gene-like structures with 14 introns and 15 exons. The question mark (?) symbolizes the uncertain placement of the linalool synthase gene within this scheme. The dotted line indicates prediction of placement in the model for an angiosperm or gymnosperm kaurene synthase gene for which a genomic structure has not yet been described. The symbols C_{10}, C_{15}, and C_{20} represent the enzyme class corresponding to monoterpene, sesquiterpene, and diterpene synthases, respectively.

diterpene synthases whose nondeleterious mutations have evolved to their present day enzymatic function.

Evolutionary Origins

Prior to the availability of any sequence conservation data and based on immunochemical evidence, the suggestion was made that multiple monoterpene synthase genes arose by gene duplication to provide a family of related catalysts for the synthesis of different monoterpene products (51). Limited sequence comparisons between other terpene synthases, based largely on conserved structural features such as the mechanistically relevant DDXXD motif, supported this general notion (3, 25, 38, 79). Ultimately, the protein-based phylogenetic analysis by Bohlmann et al (13) provided substantial evidence that all plant terpene synthases share a common evolutionary origin.

The conservation of genomic organization of plant terpene synthases provides further evidence that the terpene synthases from gymnosperms and angiosperms constitute a superfamily of genes derived from a single ancestor (S Trapp & R Croteau, unpublished results). Prior to the divergence of gymnosperms and angiosperms, during the carboniferous period about 300 million years ago (34), a single initial duplication of an ancestral terpene synthase gene, which most closely resembled a contemporary conifer diterpene synthase, occurred. The terpene synthase multigene family tree arose by subsequent duplication, then functional and structural specialization, by evolutionary processes now considered to be quite common (45, 62). One copy of the duplicated ancestral gene remained conserved in structure and function with little or no intron/exon loss, and this gene may have contemporary descendants in the terpene synthases involved in gibberellin biosynthesis. The second ancestral gene copy diverged in structure and function by adaptive evolutionary processes over millions of years to yield the large multigene superfamily of terpene synthases involved in secondary metabolic pathways. Although entirely speculative, it is plausible that terpene synthase ancestors were functionally less specialized and perhaps able to utilize multiple prenyl diphosphate substrates for the production of multiple terpene types, the specialization into different classes having evolved much later.

The evolution of the extant large number of terpene synthase genes provides an example in which many functionally complementary and nonlethal gene duplication and divergence events were retained by natural selection to provide the great diversity in terpene chemistry as the foundation of conifer defense. Thus, terpene synthase genes evolved to produce new functions that through evolutionary adaptation increased the fitness of the species to defend against predators, pathogens, and herbivores.

Pathway Organization

An important challenge for the immediate future is to identify and isolate all genes of oleoresin terpenoid biosynthetic pathways. The terpene synthases are presumed

to catalyze rate-limiting steps in terpene biosynthesis (e.g. turpentine production) and thus are obvious targets for molecular genetic manipulation to improve tree resistance. Genes encoding enzymes for downstream pathway steps are also important targets. Thus, in the biosynthesis of the common resin acid abietic acid in firs and pines, the cyclization product abietadiene is sequentially oxidized at the C18 methyl group by two cytochrome P450 hydroxylases and a soluble aldehyde dehydrogenase to yield the corresponding carboxyl function (Figure 7) (46). Variations on the cyclization scheme, coupled to the same oxidation sequence, can account for the formation of essentially all of the labdane, pimarane, and abietane resin acids of conifers (68). Similar biosynthetic steps, involving cytochrome P450 and redox enzymes, are likely involved in the conversion of the cyclization product (*E*)-α-bisabolene to todomatuic acid (Figure 6) (11). Genes encoding cytochrome P450 oxygenases and redox enzymes can be isolated based on homology; however, the very large number of these genes makes the sorting of candidates by functional expression of the relevant activities arduous. Furthermore, the extremely large genome size of conifers ($\sim 10^{11}$ for pines versus $\sim 10^{6}$ for *Arabidopsis*) and their exceedingly long development cycles (98) effectively preclude mutagenesis-based approaches for identification of gene function by phenotype.

As an alternative to gene cloning via purification of the target protein, the clustering of secondary metabolic pathway genes may provide rapid access to these sequences. In maize, five genes are clustered on the short arm of chromosome IV that encode the enzymes responsible for the biosynthesis of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (44), an important hydroxamic acid defense compound of cereals (53). Clustering of fungal sesquiterpene biosynthetic genes for simple and macrocyclic trichothecene production exists in several fungal species (57, 115), and three putative sesquiterpene synthase genes, resembling cadinene synthase, vetispiradiene synthase, and *epi*-aristolochene synthase, are located adjacent to each other on chromosome IV of *Arabidopsis* (2). Whether genes encoding enzymes for the oxidative modification of cyclic terpene parent compounds reside in proximity to the corresponding terpene synthases is not presently known.

PROSPECTS FOR FOREST BIOTECHNOLOGY

The prospects for genetically engineering conifer defenses to improve resistance face the same constraints as for forest biotechnology in general. Although forests are internationally recognized as one of the most important of natural resources (102), the agricultural domestication of forest species as "crop plants" is still in its infancy, and the bulk of wood is still harvested from natural forests. Until now, genetic improvement in agroforestry has relied primarily on conventional breeding programs to alter characteristics such as resistance to pests and pathogens, growth rate and form, volume and yield, and quality of end product lumber and paper pulp [see review by Walter et al, (116)]. This approach is clearly limited by the generation time required for selection of improved trees. It is sobering

to realize that even the most advanced forest tree-breeding programs are in their third generation, while maize and wheat have passed through many thousands of generations of cultivation and selective breeding (80).

A decade ago, the application of routine biotechnological techniques (transformation, regeneration, micropropagation) to engineer conifers presented formidable theoretical and practical barriers (80). However, more recently, these approaches, e.g. antisense technologies, are being applied to forest species (102, 116). The current understanding of what constitutes superior traits at the molecular level is still quite rudimentary and lags significantly behind other fields of agrobiotechnology (98). An important challenge is to rapidly acquire genes underpinning desirable traits and to do so without the immediate benefit of a highly revealing conifer genome project. Both poplar and pine expressed sequence tag and genome initiatives are progressing (1, 102, 110), but the large genome size of conifers—up to ten times larger than that of humans—puts these efforts in perspective (80). In addition to the long-term commitment required to make forest biotechnology a commercial reality (98), legitimate environmental concerns will also need to be addressed, and public education will be necessary to overcome the adverse perception of plant genetic engineering, especially when applied on the large-scale in the forest setting (61).

The ecological interactions between conifer hosts, pathogens, and bark beetles and their predators and parasitoids that are mediated by oleoresin terpenoids are exceedingly complex, yet they offer several possible avenues for improving tree resistance by manipulation of oleoresin composition. Understanding the molecular genetics, organization, and regulation of constitutive and inducible oleoresin formation underlies the ability to design protective and management strategies for providing sustainable forest products. Terpene synthases are conceptually attractive and obvious candidates for this purpose, and the recent cloning of a number of terpene synthase cDNAs now offers a biorational approach for improving conifer defenses by altering not only the mix of constitutive and inducible oleoresin but also the yield and composition of oleoresin itself via gene transfer technologies. These approaches to engineered manipulation of oleoresin formation include (*a*) improving the speed and level of the defense response at the critical early attack stages, (*b*) increasing the concentration of resin components that are particularly toxic to invaders by modulating promoter strength and copy number of extant genes, and (*c*) introducing new defense genes. Other more sophisticated strategies, some of which are in development (22, 89), involve (*a*) altering oleoresin chemistry to disguise the host and thereby confuse host selection, (*b*) promoting tritrophic level interactions through improved signaling to foster bark beetle predation or parasitism, (*c*) abolishing production or altering the stereochemistry of pheromone precursors to diminish or mask signaling and thereby disrupt massed attack, (*d*) engineering trees to produce disperal pheromones, and (*e*) improving (or introducing) the production of hormone analogs to disrupt insect reproduction and development.

There are still many aspects of oleoresin-based conifer defense that remain unexplored. No secretory cell-specific constitutive promoters for controlling primary

resin formation, or wound/infection-specific promoters for controlling attack-dependent defense genes, have yet been defined, nor have transcription factors involved in these processes been described. Very little is known about the signaling cues and downstream cascade pathways that mediate the communication between host and pest or pathogen. No crystal structure for a conifer terpene synthase has, to date, been solved to provide information on the determinants of substrate specificity and product outcome that would permit the redesign of these catalysts. Advances in each of these areas can be expected to lead to new strategies for tree protection.

Finally, although this review focuses on specific means of eliminating forest destruction caused by insect pests and their microbial symbionts, we wish to leave the reader with the broader view of sustainable forest management advocated by the International Union of Forestry Research Organizations that is based on the promotion of biological diversity with minimum impact on the functioning equilibrium of the ecosystem. Most insects are not potential pests, and, in a typical forest, insects represent a biomass roughly twice that of vertebrates, with a far greater diversity of species and impact on the structure and function of the complex forest ecosystem (55). For these reasons, biotechnologically based pest management strategies, whether founded on natural defense mechanisms or introduced biopesticides, need to be explored incrementally, in both type and scale, to permit responsible, stepwise consideration of possible, unintended consequences.

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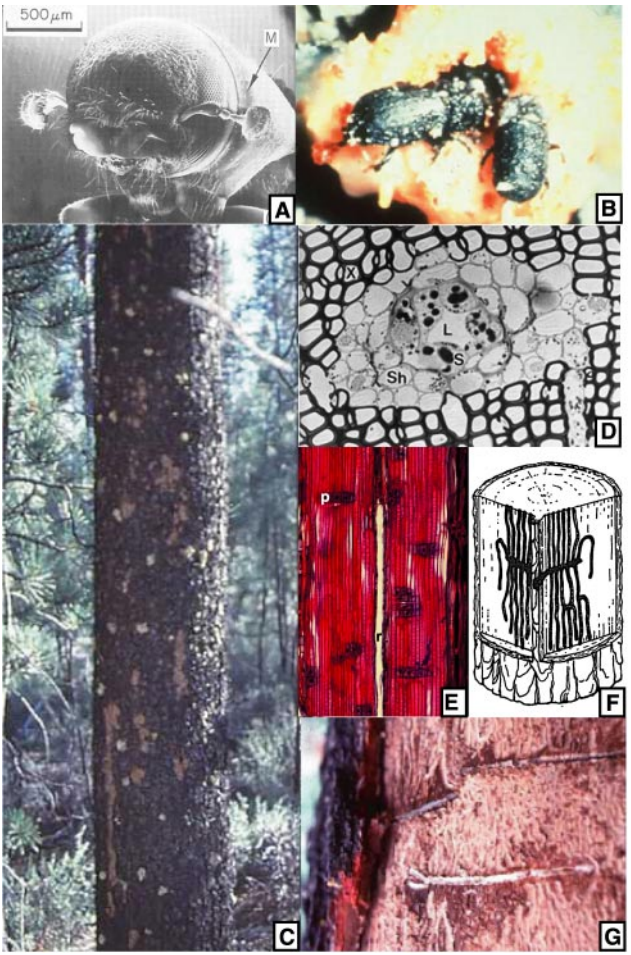

Figure 2 Physical and ecological aspects of bark beetle-conifer interactions. (*A*) Scanning electron micrograph of a Southern pine beetle depicting the mycangia (M), a specialized anatomical structure employed to vector fungal spores (adapted from Barras (5). (*B*) "Pitching out" of a pair of mountain pine beetles (*D. ponderosae*) on ponderosa pine (*P. ponderosa*). (*C*) Mass attack by mountain pine beetles (*D. ponderosae*) on a lodgepole pine (*P. contorta*) bole. Each white spot on the trunk represents a beetle entry point. (*D*) Photomicrograph of a resin duct (transverse section) from Jeffery pine (*P. jeffreyi*) showing lumen (L), secretory cells (S); sheath cells (Sh); and xylem cells (X). (*E*) Photomicrograph of a resin duct (radial section, safranin-fast green stained) from Pinus sylvestris stem showing resin duct (r) length and parenchyma cells (p) (courtesy of Vincent Franceschi). (*F*) Forked tunnel beetle galleries typical of Scolytus spp. [redrawn from Stark (105)]. (*G*) Fir engraver (*Scolytus ventralis*) galleries below the bark of a colonized grand fir (*Abies grandis*). Note that the galleries are filled with mycelia of the symbiotic fungal pathogen *Trichosporium symbioticum*. (Figures 2B, 2C, 2D and 2G are from the authors' laboratory.)

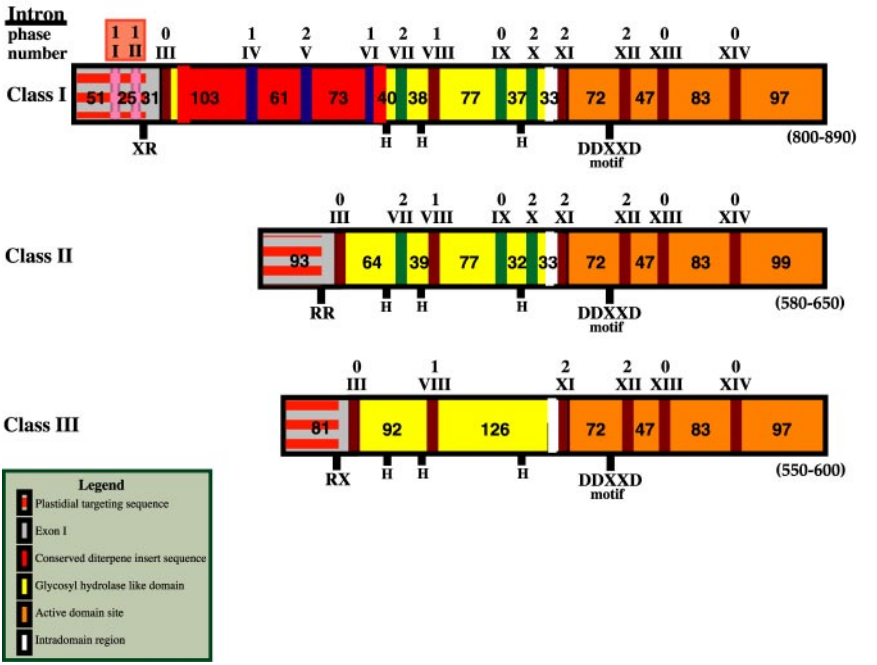

Figure 9 General structural features and organization of plant terpene synthase genes. The generic class I, II, and III terpene synthase (Tps) gene illustrations are based upon defined Tps templates (gA*gggabi*, gA*ggglim*, and Pf*glim*, respectively). General domains pertaining to primary or secondary structure are depicted by colored boxes as described in the legend box. The DDXXD and RR motifs are shown in bold; RX is used where an alternate residue sometimes occurs. Introns I-XIV, represented by colored vertical bars indicating sequential loss during evolution, are indicated as follows: introns I and II (pink bars) are found only in two class I terpene synthase genes gA*gggabi* and gAt*ggcopp1*; introns I and II are also boxed in peach to indicate that their presence is not a class I Tps gene requirement; introns IV,V, and VI (purple bars); introns VII, IX, X (green bars), introns III, VIII, XI, XII, XIII, XIV (brown bars); the later set of introns (brown) are conserved in all plant terpene synthases. The number above the intron number represents the intron phase number (see text). Each block separated by vertical bars represents an exon with the typical amino acid length specified. Class I Tps genes contain 12 to 15 exons; class II and III genes contain 9 and 7 exons, respectively. Exon 12 (last exon) of gCc*gLINOH1* and gA*gfEabis* is 175 aa and 181 aa in length, respectively (gA*gfEabis* has lost intron XIV). The gray box labeled exon I varies in size; monoterpene and diterpene synthases comprise an exon size of 80-107 aa, whereas sesquiterpene synthases comprise an exon I size of 30-50 aa due to the absence of a plastidial targeting sequence. The glycosyl hyrdrolase-like domain (yellow), the catalytic domain (orange), and the intradomain region (white) are predicted (13, 32) based upon the crystal structure of NtfeARI4 (106).

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