Induced oleoresin biosynthesis in grand fir as a defense against bark beetles
(monoterpenes/turpentine/diterpenoid resin acids/resin/stress effects)

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ABSTRACT

Grand fir (Abies grandis) saplings and derived cell cultures are useful systems for studying the regulation of defensive oleoresinosis in conifers, a process involving both the constitutive accumulation of resin (pitch) in specialized secretory structures and the induced production of monoterpenes (turpentine) and diterpene resin acids (rosin) by nonspecialized cells at the site of injury. The pathways and enzymes involved in monoterpene and diterpene resin acid biosynthesis are described, as are the coinduction kinetics following stem injury as determined by resin analysis, enzyme activity measurements, and immunoblotting. The effects of seasonal development, light deprivation, and water stress on constitutive and wound-induced oleoresinosis are reported. Future efforts, including a PCR-based cloning strategy, to define signal transduction in the wound response and the resulting gene activation processes are delineated.

Many conifer species respond to bark beetle attack by secreting oleoresin (pitch) at the wound site (1, 2). This oleoresin, composed of roughly equal quantities of monoterpenes (turpentine) and diterpenoid resin acids (rosin) (3), is toxic to both beetles and their pathogenic fungal associates (4), and, after evaporation of the volatile turpentine, it forms a hardened resin barrier to seal the injury (5). Interestingly, many species of bark beetles are specifically attracted to the monoterpene compounds emitted from their conifer hosts and can utilize components of the host oleoresin in the synthesis of pheromones for promoting aggregation (6). These same volatile products may also serve as attractants for beetle predators and parasites. Thus, the chemical ecological relationships between conifer host, beetle pest, and beetle predator are exceedingly complex and present several possible avenues for manipulating oleoresin composition to improve tree resistance, for example, by disguising the host, or altering the levels of pheromone precursors or predator attractants (6).

Constitutive oleoresin is synthesized in the epithelial cells of specialized secretory structures such as stem resin blisters or ducts in which the oleoresin is accumulated, whereas induced oleoresin appears to originate in nonspecialized cells, adjacent to the site of injury, that are not normally associated with such large-scale production of terpenoids (7–9). That both constitutive and localized, inducible resin-based defense mechanisms seem to have been selected for in this group of plants is most unusual. However, the major conifer types [pines (Pinus), spruces (Picea), larches (Larix), and true firs (Abies)] do differ considerably in their apparent reliance on constitutive or induced resin defenses (10). Oleoresinosis in species of pines, which accumulate large quantities of stored oleoresin in extensive duct systems, is not highly elevated in response to injury, whereas in true firs, which accumulate relatively little constitutive material in resin blisters, the rate of oleoresin production increases manifolds upon challenge (10, 11). In both constitutive and induced defenses, the analytical and microscopic evidence (12, 13) suggests that the monoterpenes and diterpenoids are formed coincidentally in the same locale, consistent with the requirement for an organic solvent (turpentine) in which the water-insoluble diterpene resin acids can be dissolved and translocated (5).

OLEORESIN BIOCHEMISTRY AND ENZYMOLGY

Grand fir (Abies grandis Lindl.) has proved to be a very useful model for the study of induced oleoresin production (6, 14). Formation of oleoresin monoterpenes in this, and related, conifer species is catalyzed by a series of synthases (cyclases) that transform the common C10 isoprenoid precursor geranyl pyrophosphate to olefins of the various skeletal types (Fig. 1) (11, 15). The increase in monoterpene biosynthesis after wounding of grand fir stem is the result of the apparent enhancement of constitutive activities (principally limonene synthase) and the appearance of distinct, inducible activities (including 3-carene, β-phellandrene, and α- and β-pinene synthases) (15). The enzymes responsible for constitutive turpentine production are very similar in general properties to the inducible forms; however, these gymnosperm monoterpen cyclases are distinguishable in several characteristics (pH optimum, metal ion requirement) from their angiosperm counterparts (16), which they nevertheless resemble in mechanism of action (17).

The principal wound-inducible monoterpen cyclase of grand fir stem produces both (−)-α-pinene and (−)-β-pinene, in a fixed 2:3 ratio, from geranyl pyrophosphate via a common cationic intermediate (18). This most unusual cataytic feature of multiple product formation is, in fact, fairly common among the monoterpen cyclases (19, 20), and it was confirmed in the case of the (−)-pinene synthase by exploiting the phenomenon of isotopically sensitive branching with substrates bearing strategically placed deuterium atoms (21). This 62-kDa monomeric enzyme has been purified to apparent homogeneity, characterized in some detail, and used to raise polyclonal antibodies in rabbits (18, 22). Western immunoblot analysis with the anti-pinene synthase granuloma antisierum indicated strong cross-reactivity with all of the monoterpen cyclases from grand fir but no detectable recognition of any cyclase from pine, spruce, or other conifer genera (22). Thus, this pinene synthase is more closely related to other cyclases of fir

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(that synthesize different monoterpenoid skeletal types) than it is to the pinene synthases from related conifer species.

The principal resin acid of grand fir, (−)-abiatic acid (3), has been shown to originate by cyclization of the corresponding C_{20} isoprenoid precursor, geranylgeranyl pyrophosphate, to (−)-abiet-7(8),13(14)-diene (23), followed by sequential oxidation of the A-ring α-methyl of the olefin to a carboxyl function involving two distinct cytochrome P450-dependent hydroxylases and an aldehyde dehydrogenase (24) (Fig. 1).

Most other common resin acids represent double-bond positional isomers of abiatic acid and are thought to be formed by variations on the same biogenetic theme involving formation of different parent olefins followed by similar oxidation sequences (23). After stem wounding in grand fir, the rate of diterpenoid resin biosynthesis increases significantly, but the composition of the resin products generated differs little from that produced constitutively (25).

The first dedicated step of resin acid biosynthesis is catalyzed by abietadiene synthase. This inducible diterpene cyclase, like the monoterpenoid cyclases of fir, is an operationally soluble enzyme (∼80 kDa), and it has been purified and characterized (23). In general properties, including pH optimum, pl value, cation requirements, inhibitors, and kinetic constants, abietadiene synthase resembles other terpenoid cyclases of angiosperm and gymnosperm origin. Although the enzymatic cyclization sequence from geranylgeranyl pyrophosphate to (−)-abiet-7(8),13(14)-diene (Fig. 1) almost certainly involves the formation of copalyl pyrophosphate and a pimaradiene as stable intermediates, no evidence for separation of the corresponding partial activities has been obtained (23).

**Signaling and Induction of the Wound Response**

The presence of oleoresin, particularly in wounded stem tissue, complicates attempts to examine signaling processes in the induced response and can even compromise the uptake of labeled basic precursors such as sucrose, acetate, and mevalonate (24–26). Thus, resin-soaked tissue forms an effective barrier to the movement of applied water-soluble materials, which may in fact be efficiently expelled from the wound site if resin flow is sufficiently vigorous. In spite of this limitation of in vivo experiments, preliminary studies with lodgepole pine (*Pinus contorta*) saplings and mature trees have indicated that localized, defensive oleoresin production can be stimulated by the application of pectic fragments and chitosan to a wound site (26, 27). These results, while preliminary, suggest that the Pinaceae possess a mechanism for elicitor recognition and induced terpenoid synthesis similar to that of other higher plants, including sesquiterpenoid phytoalexin production in tobacco (28) and diterpene phytoalexin production in castor bean (29–31). A number of investigations of induced isoprenoid formation have focused on 3-hydroxy-3-methylglutaryl CoA reductase, an enzyme thought to catalyze the rate-limiting step of terpenoid biosynthesis (32–35). Differential induction of specific hydroxymethylglutaryl CoA reductase genes by wounding and elicitor treatment of potato indicates that these responses are independent, at least in some species (34, 35).

Elicitation experiments with intact grand fir have not been very successful; however, a cell culture system obtained from sapling stems recently has been developed that is responsive to applied carbohydrate elicitors (36). A major advantage of cell cultures is the virtual absence of endogenous resin; the principal disadvantage is that the spectrum of enzymes induced upon challenge, and thus resin composition, differs from that induced upon wounding sapling stems (36). Although the basis of this difference is not yet clear, cell cultures do offer an experimentally more tractable and well-defined system for examining signaling and signal transduction in this localized defense response.

Monoterpene and diterpene biosynthesis are coordinately induced in wounded fir stems as defined by monitoring the activity of monoterpene and diterpene cyclases, as well as the two cytochrome P450-dependent diterpene hydroxylases involved in the formation of (−)-abiatic acid (25). The activity of these enzymes reaches maximum levels that are 25- to 500-fold higher than those of nonwounded control stems 10 days after wounding, and it is followed by a synchronous decline (25) (Fig. 2).

Western immunoblotting of wounded stem extracts over the time course of induction, using the previously described polyclonal antibodies directed against pinene synthase, demonstrated a close correlation between enzyme activity and enzyme protein, thus providing strong evidence that monoterpene cyclases are synthesized de novo in response to injury (22). The increase in oleoresin biosynthetic activity is consequently followed by the accumulation of a viscous mass of resin acids, with the loss of the volatile monoterpene, at the site of injury. The observed coordinate induction of monoterpene olefin and abiatic acid biosynthesis, and the results of oleoresin analysis (25), are entirely consistent with the role of the volatile monoterpenes as a solvent for mobilization and deposition of resin acids at the wound site to seal the injury with a resin barrier after evaporation of the turpentine. The last step of resin acid biosynthesis (Fig. 1) is catalyzed by an operationally soluble aldehyde dehydrogenase that is not inducible by wounding but seemingly is expressed constitutively at a high level (25).

An interesting feature of wound-induced oleoresinosis is the slowness of the reaction relative to other inducible plant defense responses, which often occur over the course of a few days (37) rather than a few weeks. Nevertheless, this delayed host response, which is typical of conifers (38), is appropriate to the time scale of bark beetle attack initiation which may involve several weeks in boring through the bark to the cambium–sapwood interface, excavation of a nuptial chamber, and preliminary mining of egg galleries (39). It is over this 1-
to 2-week period, when the induced production of the toxic and viscous oleoresin is most intense, that most attacks are abandoned or otherwise terminated (39).

Influences of Development and Stresses on Oleoresinosis

Based on preliminary observations in pines, Lorio (40) has suggested that conifers become more susceptible to bark beetle infestation during flushing. When grand fir saplings were examined for the ability to respond to stem wounding, as measured by alteration in the level of monoterpene cyclase activity, it was shown that this defense response was attenuated during early flush, less impaired at midflush, and then returned to normal (postflush) levels; interestingly, even stems of dormant saplings mounted a greater response than those of saplings at early flush (Fig. 3). Flushing would be expected to represent a strong sink for carbon allocation (41), which in this instance seemingly outcompetes the wound site as a sink for photosynthetic. As a consequence, the capacity for induced oleoresinosis is diminished and greater susceptibility to bark beetle infestation could be expected, providing a clear rationale for Lorio’s proposal (40). It should be noted that, although flushing negatively impacts the ability of the stem to respond to wounding by induction of monoterpene cyclases, flushing promotes an increase in constitutive cyclization activities, suggesting that trees at this developmental stage place a greater priority on establishing their preformed defenses (i.e., the formation and filling of resin blisters).

Conifers in the forest setting often encounter multiple concurrent stresses, particularly drought, that increase susceptibility to bark beetle attack, at least in part by the impairment of oleoresin production (42–45). The influence of stresses can also be interpreted in the context of carbon allocation in response to changes in source (needles)–sink (stems, roots) relationships (46, 47). A simple, direct test of source effects on the wound response was provided by light-deprivation experiments in which it was shown that light deprivation abolished both constitutive and wound-inducible monoterpene biosynthesis (48). With return to normal light conditions, monoterpene cyclase activity returned to normal constitutive levels and to near normal wound-inducible levels. Thus, reduction in the supply of carbohydrate from source tissues attenuated both constitutive oleoresinosis and the response to stem wounding. The effects of light on the accumulation of oleoresin have been observed previously (49, 50); however, the influence of light deprivation on terpene biosynthetic enzymes per se had not (48).

Water deficit can also alter source production and sink strength in conifers (51). When wounded and nonwounded grand fir saplings were subjected to moderate water deficit (−2.1 mPa; 18% soil moisture), the level of constitutive terpene cyclization activity was unaltered. However, wound-inducible cyclase activity was reduced to half of the well-water controls. Under severe water deficit (−3.0 mPa; 8% soil moisture), a significant reduction in both constitutive and wound-inducible cyclase activity was observed (48). Immunoblotting of the corresponding extracts revealed that the levels of cyclase protein were directly correlated with the levels of cyclase activity, indicating that the changes in the rate of monoterpene biosynthesis were due to changes at the enzyme level. The effects of both moderate and severe water deficits are completely reversible after a 2-week recuperation period (48). These results suggest that grand fir under moderate water stress is able to adjust source–sink balance to permit normal rates of constitutive turpentine biosynthesis in nonwounded tissue and near normal induced rates in wounded tissue. Under severe water stress, reduction in source activity seemingly results in the significant decrease in constitutive cyclase production and essentially eliminates the wound response, thus overcoming signaling of sink demand involved in normal wound healing.

A similar hierarchy of stress relationships has been observed in other plants. Thus, the response to heat shock in parsley cells overrides the response to elicitors, which in turn overrides the induced response to UV light (52). In peas, heat shock induction overrides the induced response to pathogen challenge (53). The molecular interactions underlying such hierarchies, the mechanisms involved in detecting source–sink imbalances and in communicating between source tissue and sink sites, and even the wound signaling cascade for localized oleoresinosis are presently unknown.

Future Directions and a Cloning Strategy

Future approaches to examining the control of defensive oleoresinosis will most profitably focus on the wound signaling

![Graph](image-url)
cascade, for which an experimentally tractable system is now available (36), and on regulation of the genes that encode the cyclization enzymes catalyzing the first committed steps of monoterpene and diterpenoid resin acid biosynthesis. In pursuit of the latter goal, an antibody-based screen (anti-pinene synthase) of an induced grand fir stem cDNA library was initiated, but the approach was plagued by false positives and was ultimately abandoned. Amino acid sequence information from the purified, inducible (−)-pinene cyclase from grand fir was then sought for use in designing oligonucleotide probes to enable cDNA isolation by library screening. The protein is N-terminally blocked and has proven difficult to isolate in sufficient amounts for cleavage, peptide separation, and microsequencing; thus far, several such strategies have failed to provide useful sequence information. As an alternative approach, a PCR-based strategy was developed that was based on a set of degenerate primers for PCR amplification designed to recognize highly conserved regions of the three higher plant terpene cyclases whose nucleotide sequences are known (54).

These three cyclases, (−)-limonene synthase [a monoterpene cyclase from Mentha spicata (Lamiaceae)] (54), epi-aristolochene synthase [a sesquiterpene cyclase from Nicotiana tabacum (Solanaceae)] (55), and casbane synthase [a diterpene cyclase from Ricinus communis (Euphorbiaceae)] (56), are remarkably similar (31–42% identity and 53–64% similarity by pairwise comparison at the amino acid level) considering the different substrates and products and the phylogenetic distances involved. The similarity profile (Fig. 4) of the deduced amino acid sequences indicates that these enzymes contain many islands of identity/similarity that could be conserved between angiosperms and gymnosperms and thus offer sites for consensus degenerate oligonucleotide primer construction. Three conserved regions (designated A, B, and C in Fig. 4) were chosen for primer synthesis based on a combination of placement, length, similarity, and codon degeneracy considerations. Primers A and C (Fig. 5), when used to amplify template cDNA sequences synthesized from mRNA isolated from wounded grand fir saplings (58), would be expected to yield a DNA fragment that could be subsequently verified by amplification using primers B and C. Cloning and partial sequencing could confirm the insert(s) as cyclase-like and provide a probe(s) for full-length cDNA isolation. Probes derived by this general strategy might reasonably be expected to recognize monoterpene, sesquiterpene, and diterpene cyclase sequences.

When primer A or B was used individually to amplify cDNA, no DNA fragments were produced, but primer C alone did generate a minor 280-bp fragment. The combination of primers A and C produced a broad distribution of DNA fragments, as evidenced by agarose/TBE gel electrophoresis, indicating nonspecific primer binding. The combination of primers B and C, however, amplified a discrete 580-bp fragment (representing 95% of the DNA synthesized) that was consistent with the predicted length based on the primary structure of the monoterpene cyclase (−)-limonene synthase (54) (Fig. 4). Northern blot hybridization with equivalent amounts of poly(A)+ RNA isolated from wounded and control grand fir stems, with the labeled PCR product mixture as probe, revealed the presence

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**Fig. 4.** Graphic comparison of deduced amino acid sequences of three terpenoid cyclase cDNA species (limonene synthase, epi-aristolochene synthase, and casbane synthase) illustrating the islands of conserved sequences. The data (54) were compared by the plot similarity program of the Genetics Computer Group packet (37). Locations of the PCR primers labeled A, B, and C are indicated, as are the lengths of the corresponding DNA fragments and peptides separating the primers.
of transcripts that were far more abundant in the message pool from wounded tissue, as would be expected for inducible cyclases (Fig. 6). Moreover, the transcript size of ~2300 nt is consistent with information encoding a cyclase protein of 62 kDa plus a plastid targeting sequence, as observed with the plastidial-located limonene synthase (54). These results were sufficiently encouraging to pursue the isolation of terpene cyclase cDNA clones using the PCR product mixture as probe.

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