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Defense Mechanisms of Conifers[1]

Differences in Constitutive and Wound-Induced Monoterpene Biosynthesis Among Species

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ABSTRACT

Levels of monoterpene cyclase activity were determined in extracts from wounded and unwounded saplings of 10 conifer species to assess whether oleoresin biosynthesis is induced by stem wounding. Species of *Abies* and *Picea*, with low to moderate levels of constitutive monoterpene cyclase activity, exhibited a five- to 15-fold increase in cyclase activity 7 days after wounding relative to unwounded controls. In contrast, species of genera such as *Pinus*, with high levels of constitutive cyclase activity, did not significantly respond to wounding by alteration in the level of cyclase activity. The highest fold increase in monoterpene cyclase activity was consistently observed in *Abies grandis*, and the time-course of induction of activity following stem wounding in this species demonstrated a threefold increase at 2 days relative to unwounded controls, rising to a maximum increase in the response at 9 days (greater than 10-fold) followed by an apparent decline. The wound response was localized, and both bark (phloem) and wood (xylem) tissues displayed increased cyclase activity at the wound site. The magnitude of the increase in cyclase activity was dependent on the severity of the wound.

The production of oleoresin (a mixture of roughly equal amounts of cyclic monoterpenes and diterpene resin acids [11, 24]) is an important component of the defense response of conifers against herbivore and pathogen attack (4, 7, 16, 34). Oleoresin is produced by specialized secretory tissues of the tree stem, root and leaves (13), and the anatomical organization of these secretory structures is an important criterion for the systematic classification of conifers (26). Species such as *Thuja plicata* (Cupressaceae) produce individual resin cells scattered throughout the stem, but lack any more organized resin-producing structures (13, 26). The true firs (*Abies*, Pinaceae) produce resin blisters (cysts) in addition to resin cells. Resin blisters are multicellular sac-like structures, filled with oleoresin, and are lined by a layer of epithelial cells. The walls of these cells thicken and lignify with development, and these cells, like the resin cells, are presumed to die during the year of their origin (13, 26). Other members of the Pinaceae (such as *Pinus*) display a much higher level of anatomical development in producing unconstricted resin ducts (6, 13, 26). These interconnected passages are tube-like structures found throughout the stem, including both bark and wood (1, 13, 26). Oleoresin is accumulated within the lumen of these tubes that are fully lined by a layer of thin-walled, long-lived secretory epithelial cells (1, 6, 36, 37). We recently demonstrated that the levels of oleoresin monoterpenes and of monoterpene cyclase activity in different conifer species are generally correlated with the degree of organization of the resin-producing structures (21).

In conifers with well-developed resin duct systems, such as *Pinus* spp., wounding results in the localized accumulation of copious oleoresin. Due to disruption of the resin ducts, preformed resin flows into the wound site, forming a barrier against desiccation and repelling pests and pathogens (29, 31, 33, 34). A secondary response to wounding is believed to be the accelerated *de novo* biosynthesis of oleoresin at the wound (infection) site (4, 22, 23, 28). Conifer types such as *Abies*, which lack high levels of constitutive oleoresin or well-organized secretory structures, also respond to wounding by the rapid accumulation of resin, which in this instance is thought to result primarily by accelerated *de novo* biosynthesis (4, 27, 30). Conifers of the latter type also respond to wounding by forming 'traumatic resin ducts' that are normally absent in unwounded tissue (1, 20, 32). These traumatic resin ducts are cyst-like and accumulate resin; yet, they lack epithelial cells and, therefore, appear limited in oleoresin biosynthetic capacity. By contrast, pines respond to wounding by forming more resin ducts that are anatomically indistinguishable from normal ducts (1, 7, 14), although these structures are sometimes improperly called traumatic resin ducts. In either case, the formation of ancillary duct systems occurs too slowly to account for immediate oleoresinosis at wound sites.

The relative importance of constitutive (primary) oleoresin production compared with induced (secondary) oleoresin formation in conifer defense reactions has been difficult to assess by resin analysis, since it is often not possible to distinguish oleoresin formed before wounding and transported to the wound site, from resin newly synthesized in response to wounding (18). Such analytical evaluations are particularly difficult in species containing large amounts of constitutive

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resin, and the results can be additionally compromised by differential evaporative losses of monoterpenoids *in situ* or during handling (10). Attempts to assess differences in oleoresin biosynthetic capacity by *in vivo* methods are complicated by difficulties in administering labeled precursors at resin-soaked wound sites (10). We recently developed a method for the efficient extraction and *in vitro* assay of monoterpene synthase (cyclase) activity from conifer stem (wood and bark) tissue, and also demonstrated that saplings are a suitable model for evaluating oleoresin-based defense mechanisms (21). Using this system to directly assess monoterpene biosynthetic capability at the cell-free enzyme level (8, 9), we now present evidence that some, but not all, conifer species appear to respond to wounding by a rapid increase in oleoresin biosynthesis. In addition, we describe the conditions and time course of induction of monoterpene cyclase activity in *Abies grandis*.

MATERIALS AND METHODS

Plant Materials, Substrates, and Reagents

Two-year-old saplings of the species listed in Table I were obtained as previously described (21). Additional *Abies grandis* saplings (2 year; average height 20 cm) were purchased from Palouse Seed Co. (Tekoa, WA). All saplings were grown in standard potting mix (Sals Inc., Puyallup, WA) with a 16 h photoperiod (200–300 μmol/m^2·s at 400–700 nm) and a 26°C day/15°C night temperature cycle, and were fertilized (15:30:15 (N:P:K)) weekly and watered daily for 6 weeks prior to use in order to ensure complete breakage of dormancy and active growth. Plants were handled as little as possible prior to experiments.

[1-^{3}H]Geranyl pyrophosphate (150 Ci/mol) was synthesized as previously described (9) and the sources of PVP, polyvinylpolypyrrolidone, and all other reagents and biochemicals have been indicated elsewhere (21).

Stem Wounding and Tissue Extraction

For most experiments, wounding was accomplished by making horizontal incisions of about 2 mm deep with a sharp razor blade at 5 mm intervals along the entire stem on opposite sides. When specified, vertical incisions were similarly made with 5 mm spacings around the circumference of the stem or, alternatively, a 26-gauge needle was used to puncture the stem to a depth of 3 mm. Additional methods of wounding included bending of the stem at ±90° from vertical and continuous pressure applied with a three-eighths inch binder clip.

At periods up to 12 d after wounding, stem sections (~2 cm in length) were cut from the wounded saplings and from the corresponding areas of unwounded controls, and any needles were removed. Either the wood and bark were separated, weighed (the amounts of each tissue are roughly comparable in 2-year saplings), and frozen in liquid N_2, or the entire stem section was weighed and frozen immediately prior to tissue pulverization and extraction as described previously (21), using a buffer system containing 1% (w/v) PVP and 1% (w/v) polyvinylpolypyrrolidone.

The assay for monoterpene cyclase activity has been previously described (9, 21) and is based on the divalent metal ion-dependent conversion of [1-^{3}H]geranyl pyrophosphate to labeled monoterpenes which are separated by solvent extraction and adsorption on silica. Radio-GLC (9) was employed to verify the identity of the labeled products as before (21). Protein was measured by the method of Bradford (5) using BSA as standard.

RESULTS

Survey of Conifer Species for Wound-Inducible Monoterpene Cyclase Activity

In a previous investigation (21), 10 conifer species, differing in the degree of organization of the specialized oleoresin-secreting structures, and in monoterpene content, were surveyed for their level of monoterpene synthase (cyclase) activity using a newly developed method for extracting catalytically active enzymes. Significant differences in constitutive cyclase activity directly responsible for monoterpene formation (8, 9) were observed between these species, and the level of activity in stem extracts of a given species showed good correlation with anatomical complexity of the secretory structures and with oleoresin monoterpene content (21). In the present investigation, the same series of conifer species was examined to evaluate the importance of constitutive monoterpene production compared to monoterpene biosynthesis induced in response to stem wounding. Because of the difficulties in distinguishing preformed oleoresin from newly synthesized oleoresin by *in vivo* labeling or by analysis of resin content (10), comparison of biosynthetic capacity was made at the cell-free enzyme level as before (21). In general, higher levels of monoterpene cyclase activity in stem extracts were found 7 d after wounding relative to control (unwounded) trees

Table I. *Constitutive and Wound-Inducible Monoterpene Cyclase Activity in a Range of Conifer Species*

Saplings were wounded along the entire stem by horizontal slicing and, after 7 d, the stems were extracted and assayed for monoterpene cyclase activity as described in "Materials and Methods." Control saplings were untreated and maintained under identical conditions. The mean and standard error from five determinations are reported, and an asterisk indicates that the difference between wounded and control samples is significant by the Duncan *t* test with $\alpha = 0.05$.

Species	Monoterpene Cyclase Activity	
	Control (constitutive)	Wounded (induced)
	pmol (h·mg fresh wt)$^{-1}$	
Thuja plicata	0.01 ± 0.005	0.06 ± 0.01*
Sequoia sempervirens	0.07 ± 0.02	0.11 ± 0.02
Abies grandis	0.24 ± 0.09	3.30 ± 0.54*
Abies concolor	1.73 ± 0.18	3.34 ± 0.71
Abies lasiocarpa	2.70 ± 0.58	2.52 ± 0.63
Pseudotsuga menziesii	2.11 ± 0.54	2.61 ± 0.55
Larix occidentalis	6.41 ± 1.14	8.56 ± 0.75
Picea pungens	3.78 ± 0.83	13.5 ± 0.77*
Pinus contorta	8.43 ± 1.34	10.2 ± 1.00
Pinus ponderosa	14.5 ± 1.27	16.1 ± 2.11

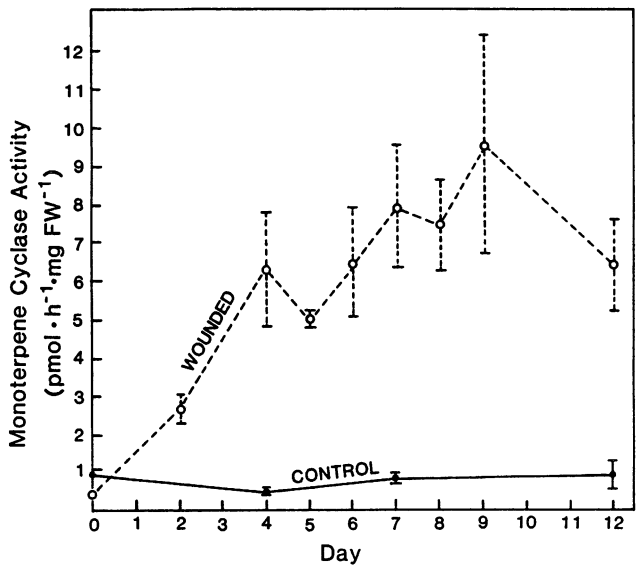

Figure 1. Time-course of the increase in monoterpene cyclase activity following wounding. *A. grandis* saplings were wounded by multiple horizontal slicing along the length of the stem. Samples consisting of three replicates, each containing three stem pieces from the same tree, were extracted as described in "Materials and Methods." Error bars indicate the standard error of the means.

(Table I), and the results of comparing constitutive and wound-induced activity were essentially identical when expressed on a per mg fresh tissue weight or per μg protein basis.

Low constitutive levels of monoterpene cyclase activity were observed in the conifer species that lack organized resin-containing structures (*e.g. Thuja plicata*). A fivefold increase in cyclase activity was found in *T. plicata* extracts 7 d after midstem wounding; however, despite this rise, the absolute level of induced activity was low relative to that of the other conifer species. By contrast, *A. grandis* and *Picea pungens* showed much larger absolute increases in extractable cyclase activity following stem wounding. In *A. grandis*, a 14-fold increase in cyclase activity was observed 7 d after wounding, and in *P. pungens*, a profuse oleoresin producer, a fivefold increase in monoterpene cyclase activity was noted (Table I). All other species tested did not display statistically significant changes in monoterpene cyclase activity relative to controls when stem extracts were assayed 7 d after wounding.

Attempts to confirm the above results by *in vivo* feeding experiments and resin analysis were unconclusive for the reasons previously outlined. Thus, in *Pinus contorta* saplings, the bulk of the [^{14}C]mevalonate applied to a wound was flushed from the wound site by resin, whereas monoterpene analyses of *A. grandis* sapling resin exhibited great variation due, presumably, to differential evaporative losses (*e.g.* 0.56 ± 0.18 mg monoterpene/g fresh weight [constitutive] *versus* 1.00 ± 0.23 mg monoterpene/g fresh weight [7 d after wounding]).

Wound Response in *A. grandis*

Since the highest fold increase in monoterpene cyclase activity was consistently recorded in *A. grandis*, more detailed studies were carried out with this species. The time-course of induction of cyclase activity following wounding demonstrated a threefold increase at 2 d relative to unwounded controls, rising to a maximum increase in the response at 9 d followed by an apparent decline (Fig. 1). After 6 weeks, there was no difference in the level of cyclase activity between wounded and unwounded saplings. Analysis by radio-GC of the monoterpenes generated by the crude cyclase preparation isolated from wounded stems (at the time of maximum increase) demonstrated the presence of all of the monoterpene olefins characteristic of *A. grandis* oleoresin (21). However, the proportion of α-pinene and β-pinene produced in this mixture (these olefins are major components of the oleoresin [21]) was significantly higher than that produced by the constitutive cyclase activity isolated from unwounded controls. Wounded *A. grandis* stems also accumulated brown pigments, presumably phenolic in nature, which represent another component of the conifer defense response (17).

Tissue localization of the response in *A. grandis* was determined by examining monoterpene cyclase activity separately in wood (xylem) and bark (phloem) extracts (21). Constitutive activity was localized primarily in the bark (Table II) as expected, since the resin blisters are restricted to this tissue (1,

Table II. *Tissue Localization of the Wound Response in A. grandis Stem Bark and Wood*

A. grandis saplings were wounded along the entire stem by horizontal slicing and, after 7 d, the entire stem (2 g total tissue) or the separated wood and bark (~1 g total tissue each) were extracted and assayed for monoterpene cyclase activity as described in "Materials and Methods." Control saplings were untreated and maintained under identical conditions. The mean and range from duplicate determinations are reported.

Tissue	Treatment	Monoterpene Cyclase Activity			
		pmol (h·μg protein)$^{-1}$	-fold increase	pmol (h·mg fresh wt.)$^{-1}$	-fold increase
Bark	Control	0.96 ± 0.30		0.64 ± 0.37	
	Wounded	9.84 ± 1.45	10	3.98 ± 0.10	6
Wood	Control	0.23 ± 0.07		0.09 ± 0.04	
	Wounded	29.9 ± 8.10	130	16.9 ± 1.70	187
Combined	Control	0.60 ± 0.22		0.50 ± 0.15	
	Wounded	11.7 ± 0.58	19	7.15 ± 1.76	14

6, 26). Both wood (containing live cambium and xylem mother cells) and bark displayed wound-induced monoterpene cyclase activity, with the most dramatic increase observed in the wood which contains the lower constitutive cyclase activity. The combination of responses of the two tissues gives the roughly 16-fold increase typical of a whole stem extract.

To determine if the induced response was localized or systemic, stem sections above and below a midstem wound site were examined. The response was shown to be localized, since outside 5 cm of the wound site the levels of cyclase activity (after 7 d) were indistinguishable from control levels (Table III). The response in stem sections immediately adjacent to the wound site (within 5 cm above and below) was quite variable, but generally gave activity levels 10 to 40% of the 2 cm wound region after 7 d. The entire stem was capable of the wound response, as wounding along the length of the stem gave substantial increases in cyclase activity in all segments (Table III).

The influence of the type and extent of wounding on the cyclase activity of *A. grandis* stems was next examined. The maximum response was observed either by slicing 2 mm into the stem (vertically or horizontally) with a razor blade or by numerous clustered punctures with a needle (Table IV). A single puncture to a depth of 3 mm was sufficient to elicit a readily detected response, and the cyclase activity of the stem increased with the severity of wounding by increasing the number of punctures locally or dispersed along the stem. Mechanical pressure for 1 week (with a three-eighths inch binder clip) without breaching the outer tissue was sufficient to provoke a significant increase in cyclase activity, and even simple bending of the stem gave an easily measured response (Table IV). Repeated wounding, by any method, over several days of a 1-week time-course did not increase monoterpene cyclase activity levels beyond that which could be obtained by heavy wounding (slice or puncture) on the first day.

Table IV. *Effect of Wound Type and Intensity on Monoterpene Cyclase Activity in A. grandis Stems*

A. grandis saplings were wounded at the midstem or throughout the stem length as indicated and, after 7 d, the entire stem was extracted and assayed for monoterpene cyclase activity as described in "Materials and Methods." The mean and standard error from either three or six determinations is reported.

Treatment	Monoterpene Cyclase Activity	
	pmol $(h \cdot \mu g\ protein)^{-1}$	*-fold increase*
Control (untreated)	0.37 ± 0.09	
Bending[a]	1.45 ± 0.30	3.9
Single puncture[a]	1.46 ± 0.13	3.9
Mechanical pressure[a]	3.26 ± 0.65	8.8
Ten punctures (localized)[a]	3.46 ± 0.80	9.3
Thirty punctures (scattered)[b]	9.98 ± 0.28	27
Thirty punctures (localized)[a]	30.8 ± 4.42	83
Vertical slices[b]	28.2 ± 9.80	76
Horizontal slices[b]	33.4 ± 7.42	90

[a] Midstem. [b] Stem length.

DISCUSSION

Conifers respond to wounding by accumulating high levels of oleoresin and other defensive substances at the wound site (4, 7, 16, 34). However, whether the accumulation of these metabolites is due either to transport of preformed resin from remote sites or to *de novo* synthesis at the wound site has received little attention and has not been unequivocally determined (18). By measuring monoterpene synthase activity in cell-free extracts derived from wounded and nonwounded tissues, we have overcome the difficulties in measuring the rate of monoterpene biosynthesis associated with *in vivo* labeling studies and analytical determinations of changes in resin content. We have shown that, in at least some conifer species, wounding promotes a significant increase in monoterpene synthase activity at the wound site, an observation

Table III. *Localization of the Wound Response in Proximal and in Distal Stem Sections of A. grandis*

Wounding at the midstem or along the length of the stem was performed. After 7 d, monoterpene cyclase activity was assayed from 2 cm sections centered at fixed intervals along the length of the stem (average length 20 cm). The -fold increase in cyclase activity is reported relative to the activity level of equivalent sections of unwounded (control) stems. Extraction and assay procedures are described in "Materials and Methods." The values reported are simple averages of three independent determinations.

Tissue Location	Treatment	-Fold Increase in Cyclase Activity	Treatment	-Fold Increase in Cyclase Activity
% stem length from root				
Upper stem to crown (80%)	Unwounded	0	Wounded	4.4
Upper stem (65%)	Unwounded	4.1	Wounded	7.2
Mid stem (50%)	Wounded	11	Wounded	6.9
Lower stem (35%)	Unwounded	2.2	Wounded	5.9
Lower stem to root (20%)	Unwounded	0	Wounded	7.2

that, based on several lines of evidence (15), suggests a corresponding increase in the rate of monoterpene synthesis.

In *A. grandis*, there was a 3-fold increase in monoterpene cyclase activity at the wound site within 2 d of wounding, rising to a 10-fold increase in a week. This time-course is similar to those of diverse defensive responses in other plants, such as the accumulation of proteinase inhibitors in wounded tomato plants (25) and the increase of chalcone synthase activity in fungus-infected *Phaseolus* seedlings (2), but slightly slower than the observed induction of casbene synthease in fungus-infected *Ricinus communis* seedlings (12). Another characteristic of the defense response in *A. grandis* is its localization to a region close to the wound site. However, the potential to respond to wounding by producing elevated levels of monoterpene cyclase activity is a general feature present throughout the stem. The response is dose dependent and occurs in both wood and bark; constitutive monoterpene cyclase activity is highest in the latter. Inducible monoterpene cyclase activity was observed regardless of the type of wound, and even simple stem bending produced a readily detectable response 7 d after the event. A maximum, absolute level of induced activity of about 30 pmol(h·μg protein)$^{-1}$ was observed, but the -fold increase, relative to controls, varied considerably (on occasion, increases exceeding 200-fold were noted). -Fold increases are highly dependent upon the 'constitutive' levels of activity measured in controls and, since even slight physical manipulation promoted an increase in cyclase activity, the 'basal' levels of activity observed may have depended on the prior handling history of the saplings.

Increases in monoterpene and diterpene cyclase activities have been previously described in *Pinus contorta* saplings after wounding and inoculation with pathogenic fungus or elicitors, and a minor increase in activity (about twofold) was observed on wounding alone (10). However, since PVP was not used in enzyme isolation in this early work, variable inhibition of cyclase activity by bark extractives was likely (21) and these results may have been compromised. Nevertheless, fungus and elicitor-induced sesquiterpene and diterpene cyclases have been documented in tobacco cells in culture (35) and in *R. communis* seedlings (12), respectively. By contrast, the monoterpene cyclase activities described here for *A. grandis* are induced to a significant level by wounding alone, which suggests a different mechanism of regulation of monoterpene biosynthesis in conifers. Additionally, although induced oleoresinosis resembles phytoalexin production, the process represents the greatly enhanced synthesis of prominent, constitutive metabolites, which is quite unlike the typical phytoalexic response (19).

Berryman (3) has shown, by light microscopic evaluation of sections of bark beetle damaged and control *A. grandis* trunks, that constitutive resin accumulation is essentially restricted to the resin blisters of the bark. After wounding, however, oleoresin is also found in tracheids and phloem sieve cells (often within a day) and traumatic cavities are formed after several weeks (3). Using similar methods, Lieutier and Berryman (22) also have demonstrated increased oleoresin accumulation in wounded and infected tissues of pines. However, as pines possess high levels of constitutive resin (21) and an extensive, interconnected system of resin ducts (26), the increased levels of oleoresin observed at the wound site might have been due to translocation of preformed material rather than *de novo* synthesis. By contrast, true firs (*Abies*) have relatively low levels of constitutive resin (21) and the resin blisters, found primarily in bark, are not interconnected (26). Thus, the possibility of oleoresin translocation to wound sites in *Abies* is most unlikely (3). Rather, the present results demonstrate a significant increase in monoterpene cyclase activity after wounding and suggest that wounding induces the synthesis of oleoresin (secondary resin) in cells that normally do not produce this material. These results thus corroborate the earlier microscopic studies by Berryman (3), and later work (27, 30) in which quantitative and qualitative changes in oleoresin monoterpenes of adult *A. grandis* were observed after wounding and fungal inoculation.

Based on the limited survey described here, conifers may be classified into two basic groups according to defense strategy. Species such as *Pinus*, with high endogenous oleoresin levels and a well-developed secretory system, do not appreciably respond to wounding by rapid induction of terpene biosynthesis (constitutive activity is already quite high), but rather seem to rely primarily on the mobilization of preformed resin to the wound site via the extensive network of resin ducts (at least during the first week after wounding). Species such as *Abies* with low to moderate oleoresin levels and limited constitutive biosynthetic capacity, and possessing isolated resin cells or cysts, would appear to rely more heavily on a rapid, localized induction of biosynthetic capability at the wound site. Although this model presents the two extremes of wound response, in reality various conifer species likely exhibit gradations in reliance on constitutive (primary resin) and induced (secondary resin) defense.

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