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Terpenoid-based defenses in conifers: cDNA cloning, characterization, and functional expression of wound-inducible (*E*)-α-bisabolene synthase from grand fir (*Abies grandis*)

[sesquiterpene synthase/juvenile hormone analogue/plant defense gene/(*E*)-4-(1,5-dimethyl-1,4-hexadienyl)-1-methylcyclohexene/todomatuic acid]

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ABSTRACT **(*E*)-α-Bisabolene synthase is one of two wound-inducible sesquiterpene synthases of grand fir (*Abies grandis*), and the olefin product of this cyclization reaction is considered to be the precursor in *Abies* species of todomatuic acid, juvabione, and related insect juvenile hormone mimics. A cDNA encoding (*E*)-α-bisabolene synthase was isolated from a wound-induced grand fir stem library by a PCR-based strategy and was functionally expressed in *Escherichia coli* and shown to produce (*E*)-α-bisabolene as the sole product from farnesyl diphosphate. The expressed synthase has a deduced size of 93.8 kDa and a pI of 5.03, exhibits other properties typical of sesquiterpene synthases, and resembles in sequence other terpenoid synthases with the exception of a large amino-terminal insertion corresponding to Pro^{81}–Val^{296}. Biosynthetically prepared (*E*)-α-[^{3}H]bisabolene was converted to todomatuic acid in induced grand fir cells, and the time course of appearance of bisabolene synthase mRNA was shown by Northern hybridization to lag behind that of mRNAs responsible for production of induced oleoresin monoterpenes. These results suggest that induced (*E*)-α-bisabolene biosynthesis constitutes part of a defense response targeted to insect herbivores, and possibly fungal pathogens, that is distinct from induced oleoresin monoterpene production.**

The oleoresin of conifers is a complex mixture of volatile monoterpenes and sesquiterpenes (turpentine) and non-volatile diterpene resin acids (rosin) that has arisen during 300 million years of conifer evolution (1–3) as a multifunctional defense against insect herbivores and fungal pathogens. The turpentine fraction of conifer oleoresin may contain up to 30 different monoterpenes (4) and an even larger number of sesquiterpenes (5). These volatiles are toxic to bark beetles (Scolytidae) and their pathogenic fungal symbionts and also furnish the solvent for diterpene resin acids that harden to form a mechanical barrier to seal wound sites upon evaporation of resin volatiles (6).

In grand fir (*Abies grandis*), increased formation of oleoresin monoterpenes, sesquiterpenes and diterpenes is induced by insect attack (7–10), and this inducible defense response is mimicked by mechanically wounding sapling stems (8, 9, 11). Therefore, grand fir has been developed as a model system for the study of the regulation of defensive terpene biosynthesis in conifers (12–15). The induced production of oleoresin monoterpenes and diterpene resin acids has been well defined (11, 16–18). Recent cDNA cloning of abietadiene synthase (19), the major wound-inducible diterpene synthase of grand fir, and of three wound-inducible monoterpene synthases (13), led to the structural analysis of the corresponding catalysts and to the molecular dissection of induced monoterpene and diterpene biosynthesis (14, 15).

Comparatively little is known about the kinetics and functional significance of sesquiterpene biosynthesis in conifers. Sesquiterpenes of the juvabione family, based on the bisabolane skeleton (Fig. 1), mimic insect juvenile hormones and, thus, can disrupt insect development and reproduction (20, 21). Juvabione [the methyl ester of todomatuic acid (Fig. 1)] is a component of "paper factor," a mixture of oxygenated bisabolane sesquiterpenes that is responsible for the failure of certain insects to molt into adults when reared in contact with paper made from fir species (genus *Abies*) (22–25). (*E*)-α-Bisabolene synthase is one of the two inducible sesquiterpene synthases of grand fir that, after stem wounding, is responsible for a dramatic shift in product composition as determined by *in vitro* assay (5). Induced sesquiterpenes could form a second line of chemical defense against insect herbivores and fungal pathogens that is distinct from the initial defense provided by inducible monoterpene production. Todomatuic acid (Fig. 1) has been reported in grand fir, accumulates in response to aphid feeding (10), and has both juvenile hormone activity against *Tenebrio* larvae (26) and antifungal activity (27, 28). Given the obvious structural relationship between bisabolene and todomatuic acid (Fig. 1), these observations, together with the reported wound induction of bisabolene synthase (5), suggest that *de novo* biosynthesis of todomatuic acid may represent an inducible defense against insect herbivores and fungal pathogens.

In this paper, we describe the cDNA cloning and characterization of grand fir *ag1*, a member of the *Tpsd* subfamily of gymnosperm terpene synthases (13, 14), and we report on the bacterial expression of the encoded sesquiterpene synthase, which yields (*E*)-α-bisabolene as the sole product from farnesyl diphosphate. Northern hybridization experiments revealed a wound-induced, transient increase of bisabolene synthase mRNA with a time course of accumulation different from that of the transcriptional activation of monoterpene synthase genes.

MATERIALS AND METHODS

Substrates and Reagents. [1-^{3}H]Geranyl diphosphate (GDP; 250 Ci/mol; 1 Ci = 37 GBq) (29), [1-^{3}H]farnesyl diphosphate

Abbreviations: FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; RACE, rapid amplification of cDNA ends.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF006194 and AF006195).

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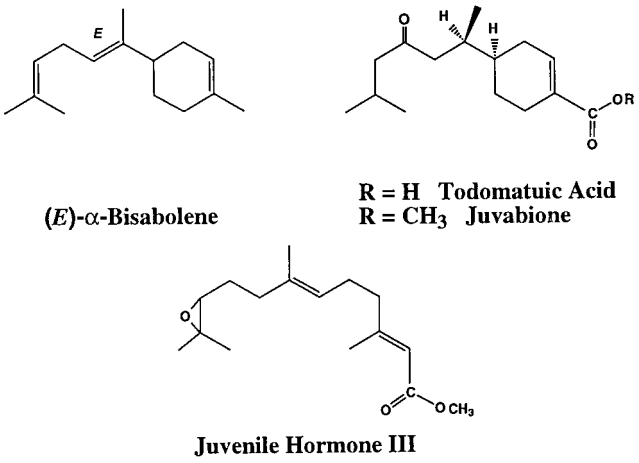

FIG. 1. Structures of (*E*)-α-bisabolene, todomatuic acid, juvabione, and juvenile hormone III.

(FDP; 125 Ci/mol) (30), and [1-^{3}H]geranylgeranyl diphosphate (GGDP; 120 Ci/mol) (31) were prepared as described previously. Authentic (*E*)-α-bisabolene was a gift from Larry Cool (University of California, Berkeley) and was used to confirm the presence of both (*E*)- and (*Z*)-isomers as components of opoponax oil (32). Todomatuic acid and juvabione were gifts from John Manville (Western Forest Products Laboratory, Vancouver). All other biochemicals and reagents were purchased from Sigma or Aldrich, unless otherwise noted.

cDNA Cloning of *ag1*. The homology-based PCR strategy for isolation of the 2424-bp, truncated cDNA clone *ag1.28* from a λ ZAP II grand fir wound-induced stem cDNA library has been described previously (13). To acquire the 5′ terminus of this clone, 5′-rapid amplification of cDNA ends (RACE) was carried out using the Marathon cDNA amplification system (CLONTECH) and the manufacturer's protocol. The reverse RACE primer specific for *ag1.28*, RJ1 (5′-AGA CGG TCG GAC AGC AGA AAG TGG G-3′), was used in combination with primer AP1 (CLONTECH), and the resulting amplicon was ligated into pT7/Blue-vector (Novagen). An internal 404-bp cDNA fragment designated *RJ8* was amplified using primers RJ1 and RJ2 (5′-CTT GGA TCC ATG GCT GGC GTT TCT GCT G-3′) that was designed to introduce a *Bam*HI restriction site for subcloning of *RJ8* into the pBluescript SK(+)-derived plasmid pAG1.28 (13). A *Hin*cII site at nucleotide position 2382 of the cDNA insert in pAG1.28, 32 nucleotides downstream of the stop codon of the 2350-bp ORF, was eliminated by site-directed mutagenesis with the QuikChange kit (Stratagene) following the manufacturer's instructions and using mutagenesis primer F (5′-GTT GCA ATA ATA ATT GAA ATA ATC TCA ACT ATG TTT CAC-3′) and primer R (5′-GTG AAA CAT AGT TGA GAT TAT TTC AAT TAT TAT TGC AAC-3′). cDNA fragment *RJ8* was then digested with *Bam*HI and *Hin*cII and ligated into *Bam*HI–*Hin*cII-digested pAG1.28 resulting in plasmid pAG1 bearing the full-length clone. For functional expression, the 2528-bp *Bam*HI–*Xho*I cDNA insert of pAG1 was subcloned into *Bam*HI–*Xho*I-digested pGEX-4T-2 (Pharmacia) resulting in plasmid pGAG1. Inserts of all recombinant plasmids were completely sequenced on both strands using the DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystems). Sequence analysis was done using the WISCONSIN PACKAGE Version 9.0 (Genetics Computer Group, Madison, WI).

cDNA Expression in *Escherichia coli*, Enzyme Assays, and Product Identification. Plasmid pGAG1 was transformed into *E. coli* XL1-Blue (Stratagene) to yield bacterial strain XL1-Blue/pGAG1, and the putative terpenoid synthase encoded by *ag1* was expressed under conditions described previously (13). The cells were harvested and lysed, and the resulting cell-free extract was assayed for monoterpene, sesquiterpene, and diterpene synthase activity in the presence of the required divalent metal ion using radiolabeled GDP, FDP, or GGDP, respectively, as individual substrates as previously reported (13). A 100-ml culture of *E. coli* XL1-Blue/pGAG1 was also grown for 16 h at 37°C to assess product formation *in situ* from endogenous substrate. The entire culture was codistilled with pentane using the J&W Scientific steam distillation apparatus (33). GLC-MS analysis of the distillate and of products from enzyme assays was performed on a Hewlett–Packard 6890 GC-MSD system (70 eV) using a 5MS (phenyl methyl siloxane, Hewlett–Packard), AT-1000 (polyethylene glycol; Alltech), or Cyclodex B (chiral β-cyclodextrin; J&W Scientific) capillary column (0.25 mm i.d. × 30 m with 0.25-μm film). Split injections were made at an injector temperature of 250°C, and the oven was programmed from 40°C (5-min hold) to 250°C at 10°C/min with constant flow of 0.7 ml of He/min. For monoterpene analysis using the Cyclodex B chiral capillary column, split injections were made at an injector temperature of 230°C, and the oven was programmed from 70°C (15-min hold) to 210°C at 10°C/min with flow rate as above. Mass spectra were analyzed using Hewlett–Packard Chemstation software and were compared with those of authentic standards. Stereochemistry was assigned based on matching of retention times with enantiomerically pure standards.

***In Vivo* Conversion of [^{3}H]Bisabolene in Cultured Grand Fir Cells.** (*E*)-α-[^{3}H]Bisabolene (2.3 μCi), prepared biosynthetically from [1-^{3}H]farnesyl diphosphate using the recombinant enzyme, was added neat to a 40-ml suspension culture of ~2 g of grand fir cells derived from embryos, followed by the addition of 200 μM methyl jasmonate, which has been shown to induce (*E*)-α-bisabolene synthesis (R. E. B. Ketchum, J.C., and R.C, unpublished results). The culture was agitated for 8 d at 25°C before separation of the medium and cells by centrifugation at 1000 × *g* for 15 min. The medium was acidified by addition of HCl to 50 mM and then was extracted with diethyl ether. The ether extract was passed over a 0.5-ml column of $MgSO_4$ and then concentrated for analysis. The cells were frozen in liquid N_2, ground to a fine powder, suspended in 10 ml of 50 mM HCl, and then extracted with ether; the extract was then prepared as before. Samples were injected onto a Spectra Physics 8800 HPLC equipped with an Alltech Econosil C18 RU column (250 mm) and eluted isocratically with 95% acetonitrile/5% water at 1 ml/min. The eluate was monitored at 210 nM, and radioactivity was measured with an in-line Radiomatic A100 liquid scintillation counter. Data were analyzed with Perkin-Elmer Turbochrome software. Tritiated toluene was used for calibration of the detectors.

Northern Blotting. Total RNA was isolated over a 28-d time course from wounded and control grand fir sapling stem tissue as previously described (15) and was electrophoretically separated (20 μg of RNA per gel lane) under denaturing conditions and transferred to nitrocellulose membranes (34). For use as a hybridization probe, a 622-bp fragment of *ag1.28* was amplified and labeled with [α-^{32}P]dATP by PCR with primer 1.28F (5′-TGA CAT AGT TCA CGA GGT GGC-3′) and primer 1.28R (5′-CAG CGG TTC AAT GAG ACA CTC-3′). Blots were hybridized for 24 h at 60°C in 3× SSPE (34) and 0.1% SDS, washed at 60°C in 1× SSPE and 0.1% SDS, and exposed to Kodak XAR film at −80°C for 20 h.

RESULTS AND DISCUSSION

cDNA Cloning and Characterization of *ag1*. A homology-based PCR strategy was recently developed that yielded five cDNA probes for grand fir terpene synthases (probes 1–5) (13). Three different full-length clones were isolated from a grand fir wound-induced stem cDNA library using probes 2 and 3 that were subsequently shown to encode three monoterpene synthases (13). Hybridization using probes 1, 4, and 5

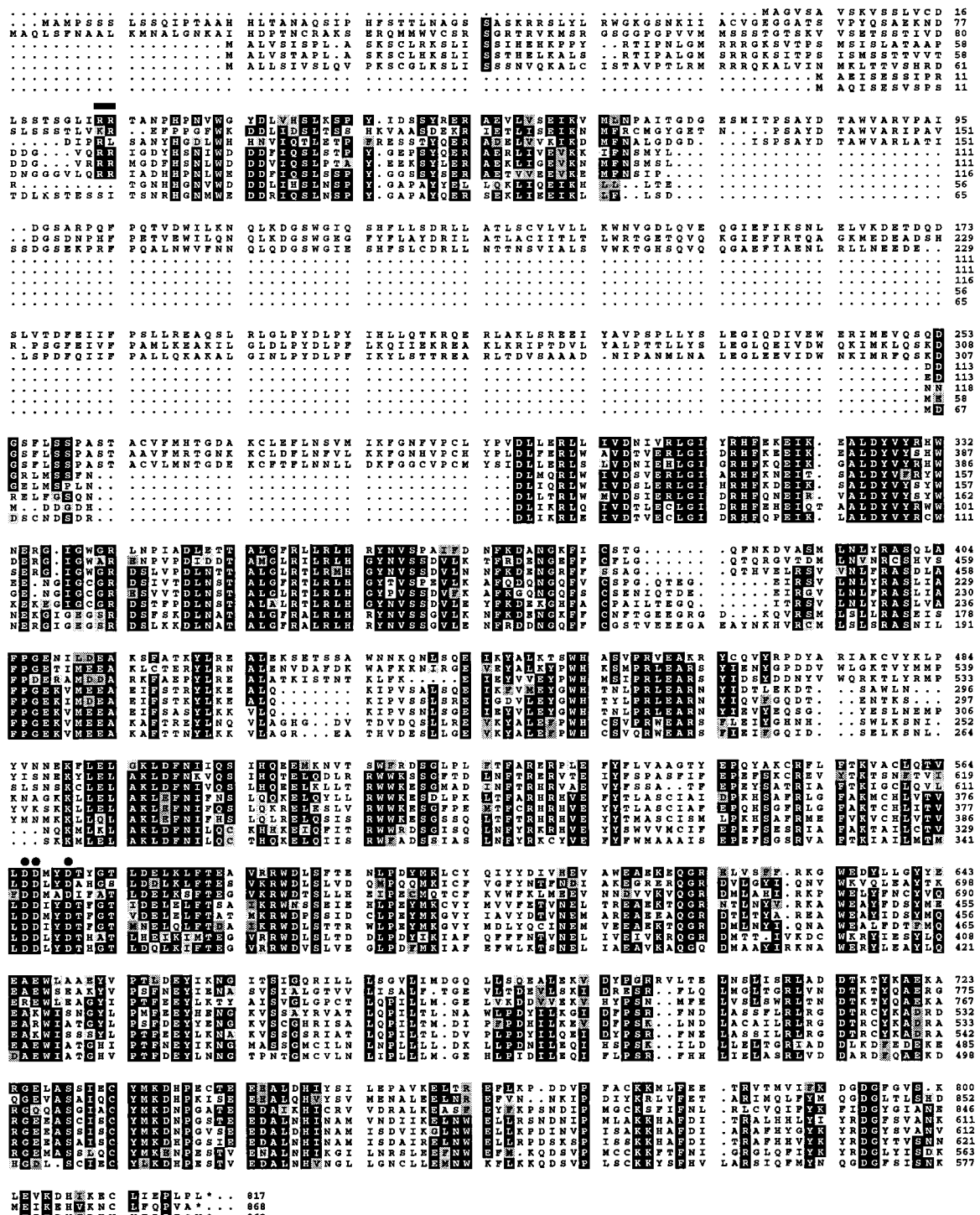

FIG. 2. Alignment of deduced amino acid sequences of gymnosperm monoterpene, sesquiterpene, and diterpene synthases. Sequences are as follows: *1*, (*E*)-α-bisabolene synthase; *2*, abietadiene synthase (19); *3*, taxadiene synthase (35); *4*, myrcene synthase (13); *5*, (−)-pinene synthase (13); *6*, (−)-limonene synthase (13); *7*, δ-selinene synthase (5); *8*, γ-humulene synthase (5). Residues boxed in black are identical for at least five of the eight compared sequences. A horizontal bar marks the tandem arginine motif. Dots mark the aspartate residues of the DDXXD element. The alignment was created with the PILEUP program (Genetics Computer Group).

revealed only 5′-truncated clones. Subsequently, full-length clones corresponding to probes 4 and 5 were generated by 5′-RACE, and these were functionally expressed in *E. coli* and shown to encode multiple-product sesquiterpene synthases (5). The longest clone isolated with probe 1, a 2424-bp cDNA designated as *ag1.28*, is a member of the *Tpsd* subfamily of plant terpenoid synthases (13). RACE was carried out to generate the 5′-upstream region of *ag1.28*. Cloning and sequencing of the amplicon identified a translation initiation site 104 nucleotides upstream of the 5′ terminus of *ag1.28*, thereby extending the deduced amino acid sequence of *ag1.28* by 35 amino acids. Fusion of *ag1.28* with the amplicon, using an

internal *Hin*cII site and the vector *Bam*HI site of pAG1.28, resulted in a 2528-bp full-length cDNA clone designated *ag1*. The 2451-nucleotide ORF of *ag1* encodes a protein of 817 amino acids with molecular weight of 93,776 and calculated pI of 5.03 (Fig. 2).

The deduced amino acid sequence of clone *ag1* was compared with other plant terpene synthases and shown to resemble most closely the two known gymnosperm diterpene synthases, grand fir abietadiene synthase (19) and *Taxus brevifolia* taxadiene synthase (35) (both 71% similarity, 49% identity), and the grand fir sesquiterpene synthases δ-selinene synthase (70% similarity, 48% identity) and γ-humulene synthase (68% similarity, 47% identity) (5) (Fig. 3). The amino acid sequence of ag1 shows 63–65% similarity and 41–42% identity when compared with grand fir monoterpene synthases (13), but only 53–59% similarity and 28–34% identity when compared with monoterpene, sesquiterpene, and diterpene synthases of angiosperm origin (14). The ag1 protein shares several highly conserved residues with other cloned plant terpene synthases (14), including the DDXXD element ($Asp^{566}Asp^{567}$ and Asp^{570} of ag1) (Fig. 2) involved in binding the divalent metal ion-chelated substrate (36, 37). In total length, the ag1 protein (817 residues) is closest to abietadiene synthase (868 residues) (19) and taxadiene synthase (862 residues) (35). It is larger than the grand fir monoterpene synthases (13) by 180–190 residues and larger than the constitutive grand fir sesquiterpene synthases, δ-selinene and γ-humulene synthase (5), by 220–230 residues (Fig. 2). The additional length of ag1, abietadiene synthase and taxadiene synthase, as compared with the grand fir monoterpene and sesquiterpene synthases, is accounted for by a 216-amino acid region corresponding to Pro^{81}–Val^{296} of ag1 (14). This large insert is highly conserved among ag1 and the two diterpene synthases [abietadiene synthase (19) and taxadiene synthase (35); 72–76% similarity, 52–58% identity] but is absent in all previously cloned grand fir monoterpene synthases (13) and sesquiterpene synthases (5).

Monoterpene and diterpene biosyntheses are compartmentalized in plastids, whereas sesquiterpene biosynthesis is cytosolic (38). Thus, all cloned monoterpene and diterpene synthases are encoded as preproteins bearing N-terminal transit peptides for import into plastids (14). As cytosolic enzymes, all cloned sesquiterpene synthases are translated without transit peptides (14). Despite the high level of similarity with gymnosperm diterpene synthases, ag1 does not contain an N-terminal domain bearing features characteristic of plastidial targeting sequences (39, 40). The lack of an apparent transit peptide in ag1 explains the smaller size (by 45–50 amino acids) relative to abietadiene synthase and taxadiene synthase (Fig. 2). The relative location of the starting methionine of ag1 resembles that of the cytosolic sesquiterpene synthases from grand fir (5) and is only 25 residues upstream of a conserved RR motif (Arg^{25}–Arg^{26} of ag1) (Fig. 2); this element may play a role in the isomerization step of the terpenoid cyclization

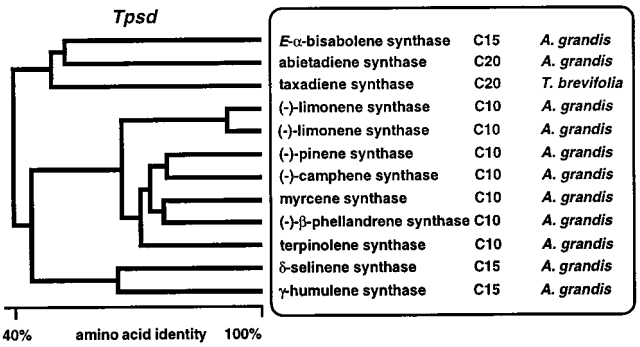

FIG. 3. Amino acid sequence comparison of the gymnosperm *Tpsd* group of the plant terpene synthase (*Tps*) gene family.

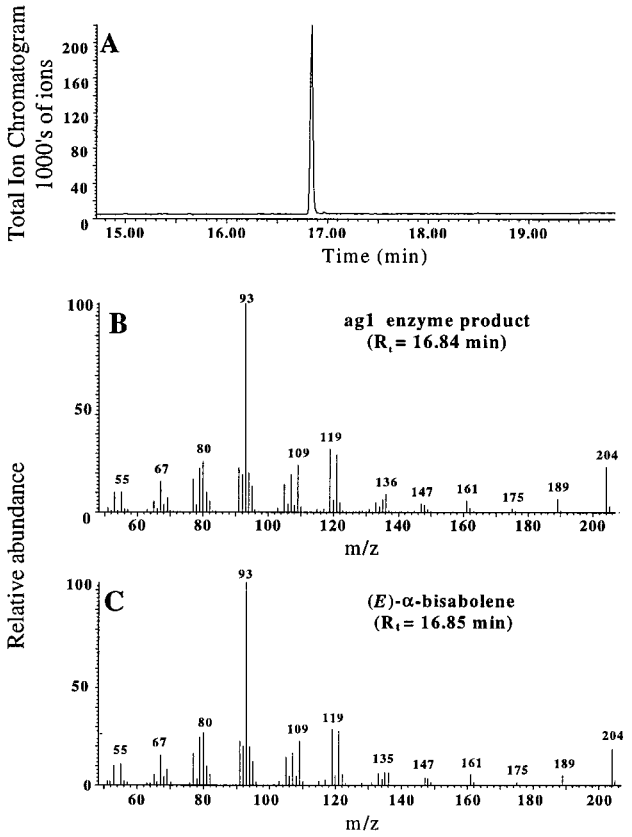

FIG. 4. GLC-MS analysis of the sesquiterpene product of the recombinant enzyme encoded by *ag1*. (*A*) The total ion current profile of the olefinic products generated from FDP by the recombinant enzyme. (*B*) Mass spectrum of the biosynthetic product with R_t = 16.84 min. (*C*) Mass spectrum of authentic (*E*)-α-bisabolene with R_t = 16.85 min.

reaction sequence (D. C. Williams, D. J. McGarvey, E. J. Katahira, and R.C., unpublished results) and represents the presumptive N terminus of mature, proteolytically processed, monoterpene and diterpene synthases (14). In summary, comparative sequence analysis revealed features of the *ag1* gene product that corresponded to both sesquiterpene and diterpene synthases of the gymnosperm *Tpsd* subfamily. Thus, identification of *ag1* required functional expression of the encoded terpenoid synthase.

Functional Expression of *ag1* cDNA and Identification as (*E*)-α-Bisabolene Synthase. For functional characterization, the recombinant ag1 enzyme was expressed from plasmid pGAG1 in *E. coli* strain XL1-Blue, and the derived cell-free extract was assayed for monoterpene, sesquiterpene, and diterpene synthase activity at saturating levels (20 μM) of

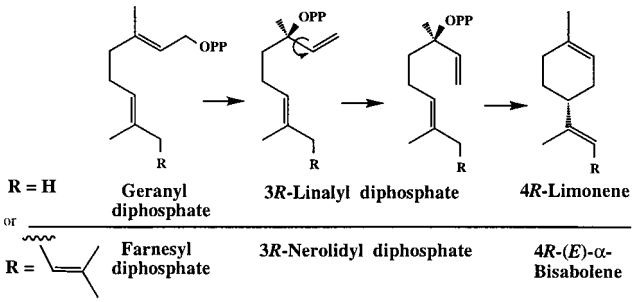

FIG. 5. Proposed mechanism for the coupled isomerization and cyclization of GDP to 4*R*-limonene and of FDP to 4*R*-(*E*)-α-bisabolene via the corresponding tertiary allylic intermediates.

[1-^{3}H]GDP, FDP, and GGDP as the respective substrate in the presence of Mg^{2+} and Mn^{2+}. The enzymatic production of a terpene olefin was observed only with GDP (V_{rel} = 50) and FDP (V_{rel} = 100) as substrates, demonstrating that the recombinant ag1 protein possesses both monoterpene synthase and sesquiterpene synthase activities but is not capable of utilizing GGPP as substrate. Control experiments, with extracts from the bacteria transformed with the vector lacking insert evidenced no terpene synthase activity with any of the three substrates.

As with other terpene synthases, the activity of recombinant ag1 requires a divalent cation cofactor, Mg^{2+} or Mn^{2+}, which is employed to neutralize the negative charge of the diphosphate leaving group in the substrate ionization step of the reaction sequence (41, 42). Similar to previously characterized sesquiterpene synthases utilizing FDP as substrate (5, 43, 44), Mg^{2+} (V_{rel} = 100 at 5 mM) is more efficient in catalysis than is Mn^{2+} (V_{rel} = 70 at 0.5 mM). With GDP as substrate, however, Mn^{2+} at 0.5 mM yielded a 4-fold higher rate of monoterpene synthase activity compared with Mg^{2+} at concentrations up to 50 mM. With both prenyl diphosphate substrates, saturation with Mg^{2+} is reached at ~5 mM, and no apparent inhibition of catalysis occurs up to 100 mM. However, Mn^{2+} at concentrations higher than 1 mM results in a decline of activity with either substrate (V_{rel} = 10 at 50 mM compared with V_{rel} = 100 at 0.5 mM). In contrast to the KCl-dependent monoterpene synthases from grand fir (13), which do not accept FDP as substrate, KCl only weakly influences GDP conversion with the ag1 enzyme (2-fold activation at 100 mM KCl), and the monovalent cation has no effect with FDP as substrate.

The sole sesquiterpene product (>99%) of the recombinant ag1 enzyme was identified as (*E*)-α-bisabolene by combined GLC-MS (Fig. 4). GDP was converted by the recombinant enzyme principally to the monoterpene (+)-4*R*-limonene (Fig. 5) as shown by GLC-MS analysis and GC separation of enantiomers on a chiral capillary column. Conversion of both GDP and FDP, but not GGDP, has been shown recently for other cloned sesquiterpene synthases (5, 43, 44). The lack of an apparent plastidial transit peptide, the higher level of sequence similarity, and the properties in common with the grand fir sesquiterpene synthases, δ-selinene synthase and γ-humulene synthase, compared with the monoterpene synthases, indicate that *ag1* encodes a sesquiterpene synthase now designated as (*E*)-α-bisabolene synthase. The presence of the Arg^{25}–Arg^{26} tandem motif in bisabolene synthase (Fig. 2), an element thus far found only in monoterpene synthases that require an isomerization step in the reaction sequence (D. C. Williams, D. J. McGarvey, E. J. Katahira, and R.C., unpublished results), is consistent with the reaction mechanism of bisabolene synthase, one of few sesquiterpene cyclizations that require a preliminary isomerization to the enzyme-bound, tertiary allylic intermediate, i.e. nerolidyl diphosphate (5) (Fig. 5).

The coupled isomerization and cyclization of FDP and GDP to bisabolene and limonene, respectively, represent analogous reactions that are consistent with established stereochemical considerations (41, 42) (Fig. 5). The cytosolic bisabolene synthase is unlikely, however, to encounter GDP *in vivo*, as the latter arises in plastids (38). Thus, the production of limonene by bisabolene synthase is probably of no physiological significance and may simply represent the lack of evolutionary pressure for the enzyme to discriminate against the adventitious geranyl substrate. Interestingly, (*E*)-α-bisabolene is produced by *E. coli* XL1-Blue/pGAG1 *in vivo* (15 ng/100 ml of culture) from host-derived FDP. However, no limonene is formed *in vivo*, probably due to the lack of endogenous, free GDP (45).

Bisabolene synthase is an unusual terpene synthase, as it forms a single product. Most terpene synthases generate multiple products (5, 13, 43–45) from the prenyl diphosphate substrate, perhaps as a consequence of the electrophilic reaction mechanism in which a series of highly unstable carbocationic intermediates are generated (41, 42). In grand fir, two classes of sesquiterpene synthases have been identified. One class seems to be constitutive, multiproduct enzymes, represented by δ-selinene synthase and γ-humulene synthase, which form, respectively, 34 and 52 different sesquiterpenes (5). These enzymes are responsible for production of many components of the constitutive oleoresin but are not induced by stem wounding. A second class of sesquiterpene synthases is characterized by the generation of a single dominant product, such as δ-cadinene synthase or α-bisabolene synthase, and these enzymes are induced by stem wounding but are otherwise absent (5). The absolute fidelity of bisabolene synthase implies very tight control of the reaction sequence and suggests the evolution of a specialized function for the olefin product, perhaps as the precursor of an important defensive metabolite.

***In Vivo* Conversion of Bisabolene in Grand Fir.** Methyl jasmonate-induced suspension cell cultures of grand fir converted (*E*)-α-[^{3}H]bisabolene into at least two labeled, polar products that were not fully resolved by HPLC (Fig. 6). The principal product recovered from the cells and medium was chromatographically coincident with authentic todomatuic acid, whereas the other eluted before authentic juvabione. Radiolabeled juvabione may have been present as a small shoulder on the trailing edge of the main radio peak; however, juvabione has not been detected previously in grand fir (J. F.

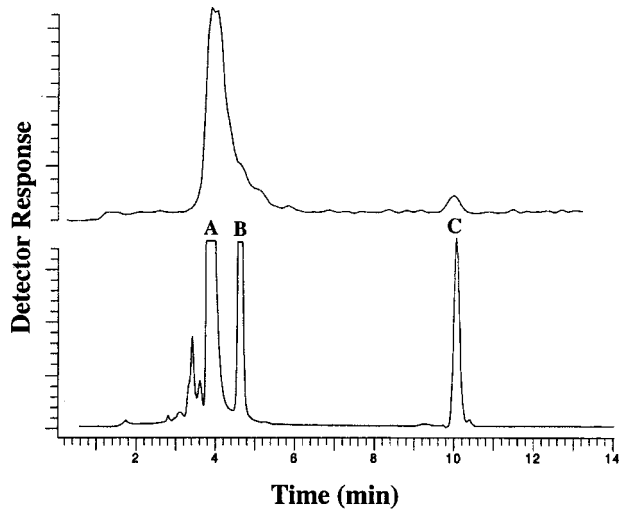

FIG. 6. Radio-HPLC analysis of the ether extract of the media from grand fir suspension cultured cells that had been incubated with (*E*)-α-[^{3}H]bisabolene. The lower trace illustrates the elution pattern of the authentic standards todomatuic acid (*A*), juvabione (*B*), and (*E*)-α-bisabolene (*C*). The *upper trace* illustrates the response of the radio-detector. Note that the first radio peak is a doublet.

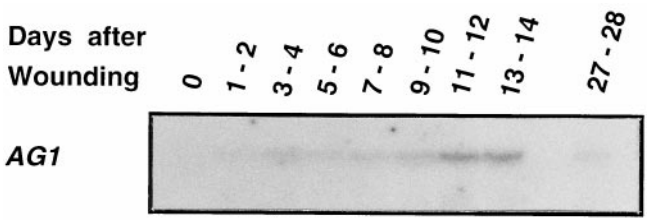

FIG. 7. Northern blot analysis of total RNA isolated from control (day 0) and wounded stem tissue of grand fir saplings as a function of the time (day 1–28) after wounding. The blots (20 μg of RNA per gel lane) were probed with a ^{32}P-labeled cDNA fragment of *ag1*. Equal loading was verified by probing with a fragment of constitutively expressed ubiquitin (data not shown) (15).

Manville, personal communication). Todomatuic acid and the other unidentified polar metabolite together represented at least 7% incorporation of the bisabolene substrate. These results, along with the wound-inducible accumulation of todomatuic acid in intact trees (10), suggest that the inducible bisabolene synthase supplies the precursor for todomatuic acid biosynthesis. The conversion of biosynthetically derived bisabolene to todomatuic acid also establishes the 4*R* stereochemistry of (*E*)-α-bisabolene (as illustrated in Fig. 5), as there is no reason to believe that configuration at this chiral center of the olefin is altered in the transformation.

Wound-Induced Gene Expression of (*E*)-α-Bisabolene Synthase. To test the transcriptional regulation of bisabolene synthase, Northern blots of total RNA extracted from sapling stems prior to wounding and at several time periods after wounding were probed with a cDNA fragment for *ag1*, which does not hybridize to any of the previously isolated grand fir terpene synthase cDNAs (5, 13, 19). mRNA for bisabolene synthase was not detected prior to stem wounding (Fig. 7). Following wounding, the accumulation of *ag1* mRNA increased transiently to a maximum at 11–14 d, demonstrating that bisabolene synthase mRNA induction is responsible for this presumed sesquiterpene-based defense response in grand fir. The increase of *ag1* mRNA accumulation is relatively slow compared with the rapid transcriptional activation of monoterpene synthases, for which maximum steady state mRNA levels are reached 1–2 d after wounding (13, 15). This differential gene activation suggests that both early and late terpenoid-based defense responses are triggered by wounding, possibly involving independent signaling pathways. Whereas monoterpene and diterpene defense compounds are directed against insects during the early attack phase, the delayed, *de novo* biosynthesis of bisabolene (and subsequently todomatuic acid) could be designed for production of fungicidal metabolites or phyto-juvenile hormones as a secondary defense should infestation of the host succeed.

The constitutive and inducible terpenoid-based chemical defenses of grand fir appear to be multi-layered and targeted to both insects and their vectored fungi, with the correct timing of each defense component controlled by differential activation of the large *Tpsd* gene family. The complexity of this multi-component, multi-target chemical defense strategy may represent a mechanism to respond to hundreds of generations of co-evolving insects and fungi that an individual tree may encounter during its lifetime. Cloning of a grand fir bisabolene synthase cDNA furnishes the first molecular probe to evaluate transcriptional regulation of inducible sesquiterpene formation in conifers and provides an avenue for the genetic manipulation of anti-fungal defense and juvenile hormone analogue biosynthesis.

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