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J. Biol. Chem. 1996, 271:23262-23268.
doi: 10.1074/jbc.271.38.23262

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Abietadiene Synthase from Grand Fir (*Abies grandis*)

cDNA ISOLATION, CHARACTERIZATION, AND BACTERIAL EXPRESSION OF A BIFUNCTIONAL DITERPENE CYCLASE INVOLVED IN RESIN ACID BIOSYNTHESIS*

(Received for publication, May 21, 1996)

Brigitte Stofer Vogel‡, Mark R. Wildung, Guido Vogel§, and Rodney Croteau¶

From the Institute of Biological Chemistry, and Department of Biochemistry and Biophysics,
Washington State University, Pullman, Washington 99164-6340

(–)-Abietic acid, the principal diterpenoid resin acid of the wound-induced oleoresin secreted by grand fir (*Abies grandis*), is synthesized by the cyclization of geranylgeranyl diphosphate to (–)-abieta-7(8),13(14)-diene, followed by sequential three-step oxidation of the C-18 methyl group of the olefin to a carboxyl function. The enzyme catalyzing the cyclization reaction, abietadiene synthase, was purified from stems of wounded grand fir saplings and was digested with trypsin. Amino acid sequence information from the resulting peptides allowed construction of degenerate oligonucleotide primers, which amplified a 551-base pair fragment from a wound-induced stem cDNA library. This hybridization probe was then utilized to screen the wound-induced stem cDNA library, from which three cDNA clones were isolated that were functionally expressed in *Escherichia coli*, thereby confirming that a single protein catalyzes the complex, multistep cyclization of geranylgeranyl diphosphate to abietadiene. cDNA isolate Ac22.1, which yielded the highest expressed level of cyclase activity, was 2861 base pairs in length and encoded an 868-amino acid open reading frame that included a putative plastidial transit peptide. Deduced amino acid sequence comparison to other terpene cyclases revealed an amino-terminal region of the abietadiene synthase, which resembles those of enzymes that employ substrate double bond protonation to initiate the carbocationic reaction cascade, and a carboxyl-terminal region of the synthase, which resembles those of enzymes that employ ionization of the substrate allylic diphosphate ester function to initiate the cyclization reaction. This apparent fusion of segments of the two distinct terpenoid cyclase types is consistent with the novel mechanism of the bifunctional abietadiene synthase in catalyzing both protonation-initiated and ionization-initiated cyclization steps.

Many conifer species secrete oleoresin, composed mainly of monoterpene olefins (turpentine) and diterpenoid resin acids (rosin), in response to wounding and attack by insect pests and pathogens (1, 2). This material is an important defensive secretion that is toxic toward bark beetles and their pathogenic fungal symbionts (3) and that physically seals injuries by solidification of the resin acids following evaporation of the turpentine on the trunk surface (4). (–)-Abietic acid (Scheme I, 5) and its isomers are nearly ubiquitous in the rosin of pines, firs, and spruces (5), and the production of these diterpenoid resin acids may be largely constitutive (as in pines, where oleoresin is synthesized and stored in highly specialized duct-like anatomical structures) or inducible (as in true firs, where new oleoresin originates in nonspecialized parenchyma cells at the site of injury) or both (6–8).

The biosynthesis of (–)-abietic acid (5) in both lodgepole pine (*Pinus contorta*; constitutive resin) and grand fir (*Abies grandis*; wound-inducible resin) was recently demonstrated (9, 10) to proceed from the tricyclic olefin precursor (–)-abieta-7(8),13(14)-diene (4) by sequential oxidation of the A-ring α -methyl group (C-18) to a carboxyl function, via a process similar to that involved in the conversion of *ent*-(–)-kaurene (8) to *ent*-(–)-kaurenoic acid (9) en route to the gibberellin family of plant hormones (11–13) (Scheme I). The cyclization reaction, which leads to the precursor abietane olefin and which is thought to represent the committed, rate-limiting step of the pathway that directs the formation of the various skeletal types of resin acids, has also been examined (9, 14). The reaction sequence involves protonation of the terminal double bond of the ubiquitous C₂₀ isoprenoid precursor geranylgeranyl diphosphate (1) (15) to initiate cyclization to the intermediate (+)-copalyl diphosphate (2), followed by ionization of the diphosphate ester function of (+)-copalyl diphosphate (2) to initiate a second cyclization, via an intermediate pimaradiene (3), to (–)-abietadiene (4) (Scheme I). Soluble enzyme extracts of lodgepole pine stem and of mechanically wounded grand fir stem catalyze the divalent metal ion-dependent cyclization of geranylgeranyl diphosphate (1) to (–)-abieta-7(8),13(14)-diene (4), and the wound-inducible enzyme has been partially purified and identified as a monomeric 84-kDa protein with general characteristics like those of other terpenoid cyclases (14). Although the enzymatic cyclization sequence catalyzed by abietadiene synthase almost certainly involves the formation of (+)-copalyl diphosphate (2) and a pimaradiene (3) as stable intermediates ((–)-sandaracopimaradiene (3) may, in fact, be formed as a minor co-product of the reaction), no evidence for the separation of the corresponding partial cyclization activities was obtained (14). This is unlike the antipodal reaction sequence leading to (–)-kaurene (8) en route to the gibberellins (15–17), in which the cyclization of geranylgeranyl diphosphate (1) to (–)-copalyl diphosphate (6) (kaurene synthase A activity)

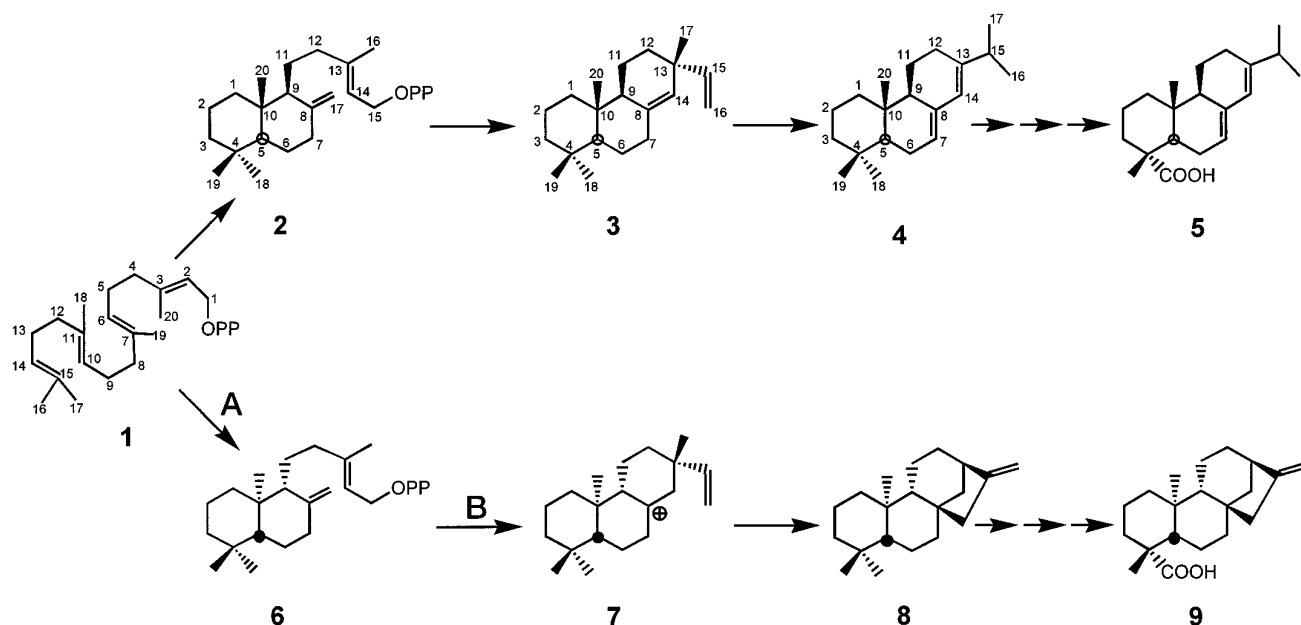
* This investigation was supported in part by United States Department of Agriculture NRI Grant 94-37302-0614, the Tode Foundation, and Project 0967 from the Agricultural Research Center, Washington State University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U50765.

‡ Supported in part by postdoctoral fellowships from the Bernische Krebsliga, Berne, and the Thurgauische Krebsliga, Frauenfeld, Switzerland. Present address: Dept. of Surgery and Research, University of Basel, Hebelstrasse 21, 4031 Basel, Switzerland.

§ Present address: Botanical Institute, University of Basel, Hebelstrasse 1, 4056 Basel, Switzerland.

¶ To whom correspondence should be addressed: Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340. Tel.: 509-335-1790; Fax: 509-335-7643; E-mail: croteau@mail.wsu.edu.



SCHEME I. Pathways for the conversion of geranylgeranyl diphosphate (1) to abietadiene (4), via (+)-copalyl diphosphate (2) and a pimaredienyl intermediate (3), and subsequent oxidation to (-)-abietic acid (5), and for the cyclization of (1) to (-)-copalyl diphosphate (6) by kaurene synthase A, followed by cyclization of (6) to (-)-kaurene (8), via a pimaredienyl intermediate (7), by kaurene synthase B, and subsequent oxidation to (-)-kaurenoic acid (9). OPP denotes the pyrophosphate moiety.

and the cyclization of (-)-copalyl diphosphate, via a pimarenyl intermediate (7), to (-)-kaurene (8) (kaurene synthase B activity) are catalyzed by separate and distinct enzymes (15, 18, 19).

In this paper, we address the question of whether a single enzyme catalyzes the complex, multiple-step cyclization sequence leading to abietadiene by cloning and functional expression of the corresponding cDNA, and we draw structural and functional inferences by deduced sequence comparisons between abietadiene synthase and other terpenoid cyclases.

EXPERIMENTAL PROCEDURES

Plant Materials, Substrates, and Reagents—Two-year-old grand fir (*A. grandis* Lindl.) were grown under conditions previously described (20), and were wounded along the stem length, by standardized protocol, 8–10 days prior to enzyme isolation (21). Methods for the preparation of [$1\text{-}^3\text{H}$]geranylgeranyl diphosphate (90 Ci/mol) and (-)-[$18\text{-}^3\text{H}$]abietadiene (117 Ci/mol), and for synthesis of the unlabeled olefin, have been reported previously (9, 14). (-)-Sandaracopimaradiene was a gift from Robert M. Coates, University of Illinois. All other biochemicals and reagents were purchased from Sigma or Aldrich, unless otherwise noted.

Abietadiene Synthase Isolation, Purification and Assay—Procedures for the isolation and four-step partial purification of abietadiene synthase from grand fir stem have been described (14). These protocols were scaled-up by a factor of 4 to accommodate approximately 1 kg of stem tissue as starting material. The only other modifications were minor; a chilled No. 1 Wiley mill was employed to pulverize the quick-frozen stems to a fine powder; the preparation following the hydroxylapatite chromatography step was diluted 1:1 with water containing 5 mM dithiothreitol and 5% glycerol prior to the Mono-Q HR anion-exchange chromatography step, and only the chromatographic fraction containing the highest level of cyclase activity at each separation step was employed for subsequent purification.

An optimized assay for abietadiene synthase activity has been described that involves the divalent metal ion-dependent cyclization of [$1\text{-}^3\text{H}$]geranylgeranyl diphosphate to the olefin, followed by hexane extraction, purification by column chromatography, LSC¹ of an aliquot for rate determination, and product verification by capillary radio-GC

and capillary GC-MS (9, 14). Protein concentration was determined by the method of Bradford (22) with lysozyme as reference. The purity of the protein was evaluated by SDS-PAGE (23) with silver staining (24).

Protein Sequencing—In preparation for trypsin digestion, approximately 15 μg of purified abietadiene synthase was dialyzed against water, lyophilized in a 1.0-ml vial, and dissolved in 25 μl of 0.4 M NH_4HCO_3 containing 8 M urea (pH 8). The mixture was heated for 5 min at 50 $^\circ\text{C}$ after the addition of 5 μl of 45 mM dithiothreitol, then diluted with 70 μl of water, and a trypsin solution (0.25 $\mu\text{g}/\mu\text{l}$ in 0.1% trifluoroacetic acid; peptide sequencing grade from Boehringer Mannheim) was added in three equal proportions at 8-h intervals over the 24-h incubation period at 37 $^\circ\text{C}$ to give a final trypsin:enzyme ratio of 1:10 (v/v) (25). Proteolysis was stopped by addition of 2 μl of 10% trifluoroacetic acid to the reaction mixture, and the resulting peptides were separated directly by HPLC (2.1 $\times$ 220-mm Aquapore RP-300 column (Applied Biosystems) with a linear gradient from 0.1% aqueous trifluoroacetic acid to 0.08% trifluoroacetic acid in 30% aqueous acetonitrile at 0.5% acetonitrile increase/min) (25). Ten well resolved peaks were collected, the solutions lyophilized, and the corresponding peptides sequenced via Edman degradation on an Applied Biosystems 470 sequencer.

Library Construction, Probe Generation, and Screening—Total RNA was extracted from grand fir sapling stems (6–7 days postwounding; Ref. 21) using a procedure developed for woody gymnosperm tissue (26). Poly(A)⁺ RNA was purified by chromatography on oligo(dT)-cellulose (Pharmacia Biotech Inc.), and 5 μg of this material was employed in library construction using the λ ZAP II-cDNA synthesis kit with Uni-ZAP II vector and packaging via the Gigapack II Gold packaging extract according to the manufacturer's instructions (Stratagene). Using sequence information obtained from the peptides generated by trypsin digestion of the purified abietadiene synthase, a set of degenerate oligonucleotide primers (in both orientations) was synthesized (see Fig. 2). PCR amplification was performed using the primers in all possible combinations to identify a target cDNA fragment for use as a hybridization probe. PCR was performed in a 100- μl reaction mixture containing 20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl_2 , 200 μM of each dNTP, 1–5 μM of each primer, and 5 units of *Taq* polymerase (Life Technologies, Inc.) with 10 μl of induced grand fir stem library cDNA as template (1.5×10^9 plaque-forming units/ml, heated for 5 min at 70 $^\circ\text{C}$) with the following program: denaturation at 95 $^\circ\text{C}$, 1 min; annealing at 55 $^\circ\text{C}$, 1 min; extension at 72 $^\circ\text{C}$, 2 min (35 cycles); then final extension at 72 $^\circ\text{C}$, 5 min. The largest PCR product obtained (~550 bp), which appeared by primer mapping to contain sequences encoding three tryptic fragments (generated between primers tryp 101 forward and tryp 52 reverse, with tryp 127 within), was agarose gel-purified and cloned into pT7Blue (Novagen) resulting in pBS1.1. Sequencing by the silver se-

¹ The abbreviations used are: LSC, liquid scintillation counting; bp, base pair(s); GC, gas chromatography; HPLC, high pressure liquid chromatography; IPTG, isopropyl-1-thio- β -D-galactopyranoside; mAb, monoclonal antibody; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

quencing method (Promega) revealed this 551-bp PCR product to encode the three trypsin sequences (tryp 52, 101, and 127) used for primer construction, plus two additional defined peptide sequences obtained on trypsinization, but not used in primer construction (see Fig. 3). The insert was released from pBS1.1 by *SacI*-*XbaI* digestion and was agarose gel-purified and labeled by random priming in the presence of [α - 32 P]dATP (27). This hybridization probe was used to screen filter lifts of 3×10^5 plaques grown on *E. coli* XL1-Blue (28). Hybridization was performed for 18 h at 42 °C in a solution containing $5 \times$ SSPE, 50% formamide, $5 \times$ Denhardt's, 0.1% SDS, and 100 μ g of denatured sheared salmon sperm DNA/ml. The filters were washed thrice for 10 min at 25 °C with $1 \times$ SSC (0.15 M NaCl in 40 mM sodium citrate, pH 7.0) containing 0.1% SDS, and twice for 30 min at 68 °C with $0.1 \times$ SSC containing 0.1% SDS (28). Of the 75 plaques yielding positive signals, 21 were purified through three additional cycles of hybridization. The purified λ ZAP II clones were *in vivo* excised as Bluescript II SK(-) phagemids and transformed into *E. coli* XL1-Blue according to the manufacturer's instructions (Stratagene), and the size of each cDNA insert was determined by PCR using T3 and T7 promoter primers.

cDNA Expression in *E. coli*—*E. coli* cells harboring the 11 largest clones (bearing inserts >2 kb) were incubated overnight at 37 °C with shaking at 350 rpm in 10 ml of Luria-Bertani medium containing 50 μ g of ampicillin/ml and 1 mM IPTG. Bacteria were harvested by centrifugation ($2,000 \times g$, 15 min, 0–4 °C), resuspended in 1.5 ml assay buffer (9, 14) containing 0.02 μ g of lysozyme (with incubation for 15 min at 0–4 °C), and then disrupted by mild sonication. The lysates were cleared by centrifugation ($18,000 \times g$, 15 min, 0–4 °C), and 1 ml of each resulting supernatant was assayed for diterpene cyclase activity using the standard protocol with saturating levels of the Mg^{2+} cofactor and [1 - 3 H]geranylgeranyl diphosphate as substrate (9, 14). Extracts of three of the bacterial lines (bearing pAC9.1, pAC12.2, and pAC22.1) displayed readily measurable levels of cyclase activity (the pellet preparations from these cells, and bacteria transformed with Bluescript II SK(-) phagemids lacking inserts, contained no activity).

To prepare sufficient biosynthetic product for capillary radio-GC analysis, the hydrocarbon fractions derived by assay of enzyme preparations from multiple cultures were pooled (~ 2 nmol by LSC), diluted with authentic (-)-abietadiene and (-)-sandaracopimaradiene, and analyzed as described previously (9, 14). For capillary GC-MS analysis (Hewlett Packard 6890 GC-MSD system), samples were similarly pooled, the hexane solvent concentrated under argon, and a 0.5- μ l sample (~ 1 pmol) evaluated by cool-on-column injection under conditions previously described (9, 14). Retention times and full spectra (electron impact, 70 eV, electron multiplier at 2800 V) were obtained for comparison to those of the authentic standards.

cDNA clones were sequenced using the DyeDeoxy Terminator Cycle Sequencing method (Applied Biosystems), and both strands of pAC22.1 were completely sequenced via primer walking and nested deletions (28). Sequence comparisons were done using programs from the University of Wisconsin Genetics Computer Group (29), and searches were done at the National Center of Biotechnology Information using the BLAST network service against the SwissProt, Protein Information Resource, and GenPept data bases.

RESULTS AND DISCUSSION

Purification of Abietadiene Synthase—(-)-Abieta-7(8),13(14)-diene synthase has been isolated previously from wounded grand fir sapling stems, partially purified and characterized (14). The isolation and purification protocols for this wound-inducible enzyme were scaled up to allow processing of 1 kg of tissue, and the four chromatographic purification steps (*O*-diethylaminoethyl cellulose, phenyl-Sepharose, hydroxylapatite, and Mono-Q anion-exchange) were monitored for activity (14), total protein (22), and specific protein content by SDS-PAGE (23, 24), such that fractions containing only the highest specific activity were utilized. This procedure afforded the abietadiene synthase at greater than 90% purity (Fig. 1), but the overall recovery was low (<5%). The purified enzyme produced (-)-abietadiene-7(8),13(14)-diene (4) as the major cyclization product of geranylgeranyl diphosphate ($\sim 97\%$) with a small amount of (-)-sandaracopimaradiene (3) as an apparent co-product ($\sim 3\%$). With the unfractionated stem extract, the product mix was 90% abietadiene and 9% sandaracopimaradiene (14), thus suggesting the presence of an additional pimaradiene synthase

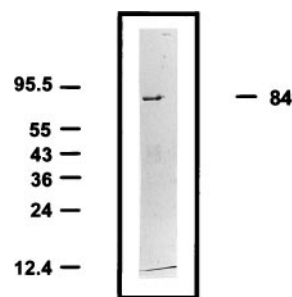


FIG. 1. SDS-PAGE of (-)-abietadiene-7(8),13(14)-diene synthase from grand fir following the four-step purification sequence. The migration of protein standards (in kDa) is indicated. The gel was silver-stained (24).

activity in crude preparations. Only one peak of diterpene cyclase activity was observed throughout the course of purification (*i.e.* abietadiene synthase); however, separation of a low level of a distinct pimaradiene synthase activity could easily have escaped detection.

Because several attempts at amino-terminal sequencing of the abietadiene synthase failed, internal fragments were generated by trypsin digestion for HPLC separation and microsequencing. Ten of the more than 30 peptides separated were partially sequenced to provide information from about 15% of the protein. Only one trypsin fragment, number 127 by elution time, showed significant sequence similarity to proteins in the data bases (Fig. 2). This peptide contains an aspartate-rich segment, ILDDLVD, that corresponds to the consensus sequence motif (I/L/V)XDDXXD found in the mechanistically related prenyltransferases (30–33) and many terpenoid cyclases (34–40). This protein segment is believed to bind the catalytically essential divalent metal ion complexed to the prenyl diphosphate substrates of these enzymes (30, 33, 41–44).

Isolation and Characterization of an Abietadiene Synthase cDNA—Based on size and degeneracy considerations, three amino acid sequences (trypsin peptides 52, 101, and 127 in Fig. 2) were chosen to design degenerate oligonucleotide primers for PCR amplification using library cDNA generated from wounded stem mRNA as template. Electrophoresis of the resulting PCR products, generated using all primer combinations, revealed the longest to be an approximately 550-bp fragment generated between primer trypsin 101 (forward) and primer trypsin 52 (reverse); the combinations of trypsin 101 and 127, and 127 and 52, yielded DNA fragments of about 300 and 250 bp, respectively. This largest DNA fragment was gel-purified and subcloned into pT7Blue, and sequencing of several representative clones revealed the presence of a single 551-bp product encoding the three tryptic fragments employed originally for PCR primer design, as well as the sequences of two other tryptic fragments (see Fig. 3 below). The 32 P-labeled, 551-bp fragment, which was cyclase-like in sequence, was then used as a hybridization probe to screen a λ ZAPII cDNA expression library constructed from mRNA isolated from wounded grand fir sapling stems (26).

From the initial screen of 3×10^5 plaques, 75 clones hybridized to the probe and 21 of these were purified, *in vivo* excised, used to transform *E. coli*, and the inserts sized. Bacteria harboring the largest 11 phagemids (cDNA inserts over 2 kb) were grown in the presence of ampicillin and IPTG, harvested, and homogenized, and the soluble enzyme fraction was assayed for diterpene synthase (cyclase) activity using [1 - 3 H]geranylgeranyl diphosphate as substrate (14). Preparations from three of the 11 transformed bacterial lines (bearing clones pAC9.1 12.2 and 22.1) afforded easily measurable levels of diterpene olefin synthase activity (in the range of 1.0–3.5 nmol/culture). The

Trypsin 52 (699-705)	E A E W S E A
20 nucleotides	GAAGCIGAATGGTCIGAAGC
8x degenerate	G G G
20 nucleotides	GAAGCIGAATGGAGTGAAGC
16x degenerate	G G C G
Trypsin 101 (522-530)	E N Y G P D D V W
27 nucleotides	GAAATATATGGICCGATGATGTITGG
32x degenerate	G C C C C
Trypsin 127 (615-625)	N F T V I L D D L Y D
32 nucleotides	AATTTTACIGTATTCTTIGATGATCTTATGA
384x degenerate	C C CT C CT C
	A

FIG. 2. Amino acid sequences of selected peptides derived by trypsin digestion of abietadiene synthase. The location of the peptides is indicated; numbering of the peptides refers to their HPLC elution times. The corresponding degenerate oligonucleotide primers are also indicated. *I* stands for inosine.

remaining eight transformed lines, and all membranous fractions from cultures of *E. coli* bearing pAC 9.1, 12.2, and 22.1, were devoid of diterpene cyclase activity.

To identify the biosynthetic product(s) generated by the recombinant cyclase(s), preparative scale incubations with each were carried out, and the olefinic products isolated by column chromatography on silica gel were analyzed by radio-GC and capillary GC-MS (14). The olefin fraction generated by the recombinant protein encoded by pAC22.1 (which afforded the highest apparent level of expression) contained principally abieta-7(8),13(14)-diene (~98% by integration of radio or mass response; identification by comparison of GC retention time and mass spectrum to the authentic standard) with a small amount of sandaracopimaradiene along with another unidentified olefin (<2%). The olefin fractions generated by the cyclases encoded by pAC9.1 and pAC12.2 contained only abietadiene, but minor amounts of sandaracopimaradiene, if present, were not possible to quantify due to the lower expression levels observed with these two clones. Comparison of the product distribution generated in crude extracts (abietadiene:pimaradiene ~10:1) to that of the purified cyclase (abietadiene:pimaradiene >33:1) and recombinant enzyme (abietadiene:pimaradiene >49:1), as well as evaluation of stem resin content for diterpene skeletal types (5, 45), indicate that abietadiene synthase is the principal diterpene cyclase of grand fir and that a distinct pimaradiene synthase probably does occur but is present at relatively low levels in stem tissue.

The production of abietadiene by a recombinant cyclase proves that a single enzyme is responsible for the multistep conversion of geranylgeranyl diphosphate to this diterpene olefin (Scheme I). This is unlike the biosynthesis of (–)-kaurene (Scheme I), in which the conversion of geranylgeranyl diphosphate to the product olefin is catalyzed in two discrete steps by two distinct enzymes, the protonation-initiated cyclization of geranylgeranyl diphosphate to (–)-copalyl diphosphate by kaurene synthase A, and the diphosphate ester ionization-dependent cyclization of (–)-copalyl diphosphate to (–)-kaurene by kaurene synthase B (15, 18, 19). The co-production of low levels of sandaracopimaradiene by the abieta-7(8),13(14)-diene synthase, as a side product or intermediate of the catalytic cycle, is entirely consistent with the operation of an integral B-type activity in which this second cyclization event, involving conversion of (+)-copalyl diphosphate to abietadiene, must involve initial C-ring closure to a pimarane intermediate followed by methyl migration. The reaction carried out by the bifunctional abietadiene synthase appears to be among the most complex of any terpenoid synthase, rivaling those catalyzed by squalene synthase (46), phytoene synthase (47), and the squalene/oxidosqualene cyclases (48), in involving two different types of cyclization processes and two stable intermediates, as well as a

rearrangement.

Sequence Analysis—Since the pAC22.1 cDNA gave the highest apparent level of abietadiene synthase expression, this insert was completely sequenced and examined in detail (Fig. 3). The pAC22.1 abietadiene synthase cDNA is 2861 nucleotides in length and contains an open reading frame of 2604 nucleotides, encoding a protein of 868 amino acids at a molecular weight of 99,535, if it is assumed that the first methionine codon represents the start site; there is a recognizable initiator methionine consensus sequence surrounding the designated ATG (49). The locations of all 10 peptide sequence fragments obtained by trypsinization of the native protein were identified within the open reading frame of pAC22.1, confirming the cDNA to represent an abietadiene synthase gene.

As with the biosynthesis of other diterpenoids (50, 51), the abietadiene synthase is likely localized in plastids (in the present instance, the leucoplasts of resin-producing cells; Refs. 20 and 52). Thus, the corresponding cDNA would be expected to encode a plastidial transit peptide. The deduced amino-terminal segment of the abietadiene synthase shows the expected properties of plastid targeting sequences, which are rich in serine and threonine residues and low in acidic amino acids (53), and established criteria (54–57) predict a cleavage site between Ala¹¹⁰ and Ser¹¹¹. Translation of the putative mature abietadiene synthase cDNA (*i.e.*, residues 111–868) yields a protein of 87.9 kDa at a calculated pI of 5.1, values that compare well to a size of ~84 kDa previously established by gel permeation chromatography and SDS-PAGE for the native enzyme (Fig. 1), and to a measured pI of 4.9 (14).

Localization studies with the related diterpene cyclase, kaurene synthase A, encoded by the *GA1* locus of *Arabidopsis thaliana* have demonstrated import of the 86-kDa translation product in isolated pea chloroplasts and processing to a smaller, 76-kDa form (58). Benson,² based on similar plastid import experiments, has indicated the 83-kDa kaurene synthase A preprotein encoded by the *An1* gene of maize (59) to also possess a long transit peptide of about 10 kDa, consistent with the size of the leader sequence predicted for abietadiene synthase from grand fir. However, because the amino terminus of the native abietadiene synthase is blocked and has not yet been identified, the transit peptide/mature protein junction and, thus, the exact lengths of both moieties remain uncertain. The most amino-terminal trypsin peptide (Ile¹⁴⁸–Ile¹⁶⁶) establishes a minimum molecular weight for the mature abietadiene synthase protein of 83,809.

The two other actively expressed abietadiene synthase cDNA clones were partially sequenced. The insert of pAC12.2 appeared to be eight nucleotides (5'-ATCTGAGA) longer at the 5'-end than that of pAC22.1, and two nucleotides (5'-TACTA-CATATTTAAAGT-poly(A)⁺) shorter at the 3'-end. The insert of the third clone, pAC9.1, was 194 nucleotides shorter than that of pAC22.1, with an apparently operational open reading frame of 803 amino acids, and it showed an additional difference at position 75 in pAC22.1, with the substitution of an Asp (AAT) for a Lys (AAG). This small difference likely results from individual variation within the 120 grand fir saplings utilized for cDNA library construction and does not necessarily imply the presence of several cyclase genes in the grand fir genome.

Preliminary RNA blot analysis of total RNA isolated from both wounded and non-wounded (control) grand fir sapling stems (26), using ³²P-labeled insert from pAC22.1 as probe, verified the presence of the homologous sequence in wounded stem at a much higher abundance than in control stem tissue, as might be expected for this induced resinosis response (14,

² R. J. Bensen, personal communication.

1	AGATGGCCATGCTTCCTCTTCAATGTCATCAGAGATGCCACTGCTGCTCATCTAAGCTGCTAAGCACAATCCATTCGCGATTTCTCC	92
2	M A M P S S S L S S Q I P T A A H H L T A N A Q S I P H F S	30
93	ACGAGCGCTGAATCTGGAAGCAGTGTAGCAACCGGAGAGCTTGTACCTACGATGGGGTAAAGGTTCAACACAGATCATTCGCTGTGTT	182
31	T T L N A G S S A S K R R S L Y L R W G K G S N K I I A C V	60
183	GGAGAAGTGGTCAACCTCTGTTCTTATCAGTCTGCTGAAAAGAAATGATTCGCTTTCTTCTTCTACATTTGGTGAACGAGAATTTCTCT	272
61	G E G F A T S V P Y Q S A E K N D S L S S S T L V K R E F P	90
273	CCAGATTTTGAAGGATGATCTTATCGATCTCTAACGTCATCTCACAGGTTGAGCAGTCAGACGAGAGCGTATCGAGACATTAATA	362
91	P G F W K D D L I D S L T S S H K V A A S D E K R I E T L I	120
363	TCCGAGATTAAGAAATGTTTAGATGTATGGGCTATGGGAAACGAATCCCTCTGCAATGACACTGCTTGGGTAGCAAGGATCCAGCA	452
121	S E I K N M F R C M G Y G E T N P S A Y D T A W V A R I P A	150
453	GTTGATGGCTCTGACACCCCTCACTTTCTCTGAGACGGTTGAATGGATTTCTTCAAAATCAGTTGAAAGATGGGCTTGGGGTGAAGGATTC	542
151	V D G S D N P H F P E T V E W I L Q N Q L K D G S W G E G F	180
543	TGCTTCTTGGCATATGACAGAATACCTGGCTACACTTGCATGTATTATACCTTACCTCTGGCGTACTGGGGAGACACAAGTACAGAAA	632
181	Y F L A Y D R I L A T L A C I I T L T L W R T G E T Q V Q K	210
633	GGTATGAAATCTCTCAGGACACAAGCTGGAAGATGGAAGCTGATGATCATTAGGCAAGTGGATTGAAATAGTATTTCTCTGCA	722
211	G I E F F R T Q A G K M E D E A D S H R P S G F E I V F P A	240
723	ATCGTAAGAGACTAAATCTTAGGCTTGGATCTGCTTAGCATTTGCCATCTCTGAAACAAATCATCGAAAGCGGGAGGCTAAGCTT	812
241	M L K E A K L L G L D L P Y D L P F L K Q I I E K R E A K L	270
813	AAAAGATTCCCACTGATGTTCTCTATGCCCTTCCAAACAGCTTATTGTATTCTTTGGAAGGTTTACAAGAAATAGTAGACTGGCAGAAA	902
271	K R I P T D V L Y A L P T T L L Y S L E G L Q E I V D W Q K	300
903	ATAATGAACTCTCAATCAAGGATGGATCAITTTCTCAGCTCTCCGGCATCTACAGCGCTGTATTCTATCGTACAGGGAACAAAAGTGC	992
301	I M K L Q S K D G S F L S S P A S T A A V F M R T G N K K C	330
993	TTGGATTCTTGAACTTTGTCTTGAAGAAATCGGAAACCATTTGCTTCTACTATCCGCTTGATCTATTTTGAAGCTTTGTGGGCGGTT	1082
331	L D F L N F V L K K F G N H V P C H Y P L D L F E R L W A V	360
1083	GATACAGTTGAGCGGCTAGGTATCGATCGTCAATTTCAAGAGGAGATCAAGGAAGCATTGGATTATGTTTACAGCCATTGGGACGAAGA	1172
361	D T V E R L G I D R H F K E E I K E A L D Y V Y S H W D P A	390
1173	GGCATTTGAGTGGGCGAGAGAAATCTGTTCTCTGATATGATGATACAGCCATGGGCTTGAATCTTGAGATTACATGGATACATGTA	1262
391	G I G W A R E N P V P D I D D T A M G L R I L R L H G Y N V	420
1263	TCCTCAGATGTTTTAAAAACATTTAGAGATGAGAATGGGAGTTCTTTTGCTTCTTGGGTCAACACAGAGAGGATTACAGACATGTTA	1352
421	S S D V L K T F R D E N G E F F C F L G Q T Q R G V T D M L	450
1353	AACGTCAATCGTTTTCATCTTTCCTCGGAGAAACGATCATGGAAGAAAGCAAACTCTGTACCGAAAGTATCTGAGGAAATGCT	1442
451	N V N R C S H V S F P G E T I M E E A K L C T E R Y L R N A	480
1443	CTGAAATTTGGATGCTTTGACAAATGGGCTTTTAAAAAGAAATTTCCGGGAGAGGTAGAGTATGCACTCAAAATATCCCTGGCATAAG	1532
481	L E N V D A F D K W A F K K N I R G E V E Y A L K Y P W H K	510
1533	AGTATGCCAAGGTTGGAGGCTAGAAGCTATATGAAACTATGGGCGAGATGATGTGGCTTGGAAAACTGTATATATGATGCCATAC	1622
511	S M P R L E A R S Y R I E N Y G P D D V W L G K T V Y M M P Y	540
1623	ATTTGCAATGAAAGTATTTAGAACTAGCGAAACTGGACTTCAATAGGTGCTATACACCAACAGAGCTTCAAGATCTTGAAGG	1712
541	I S N E K Y L E L A K L D F N K V Q S I H Q T E L Q D L R R	570
1713	TGGTGAATCATCCGCTTTCACGGATCTGAATTTCACTCGTGAGCGGTGTGACGGAATATATTTCTCACCAGCATCTTATCTTTAG	1802
571	W W K S S G F T D L N F T R E R V T E I Y F S P A S F I F E	600
1803	CCCGAGTTTCTAAGTGACAGAGGTTTATACAAAACCTTCAATTTCACTGTTATTTTAGATGATCTTTATGACGCCCATGGATCTTTA	1892
601	P E F S K C R E V Y T K T S N F T V I L D D L Y D A H G S L	630
1893	GACGATCTTAAGTTGTTACAGAAATCAGTCAAAAGATGGGATCTATCACTAGTGGACCAATGCCACAACAAATGAAAATATGTTTGTG	1982
631	D D L K L F T E S V K R W D L S L V D Q M P Q Q M K I C F V	660
1983	GGTTTCTCAATATCTTTTAATGATATAGCAAAAGAGGACGTGAGAGGCAAGGGCGGATGTGCTAGGCTACATCAAAATGTTTGGAAA	2072
661	G F Y N T F N D I A K E G R E R Q G R D V L G Y I Q N V W K	690
2073	GTCCAACTTGAAGCTTACAGAAAGAGCAGATGGTCTGAAGCTAAATATGTGCTCATCTTCAATGAATACATAGAGATGCGAGTGTG	2162
691	V Q L E A Y T K E A E W S E A K Y V P S F N E Y I E N A S V	720
2163	TCAATAGCATTTGGGAACAGTCTCTCATAGTGTCTTTTCACTGGGAGGTTCTTACAGATGAAGTACTCTCCAAAATTTGATCGCGAA	2252
721	S I A L G T V V L I A S L F T G E V L T D E V L S K I D R E	750
2253	TCTGATTTCTTCACTCATGGGCTTAAACAGGCGTTTGGTGAATGACCAAAAATTTATCAGGCAGAGAGGTTCAAGGTGAGGTGGCT	2342
751	S R F L Q L M G L T G R L V N D T K T Y Q A E R G Q G E V A	780
2343	TCTGCCATACAAATGTTATATGAAGGACCATCTAAAATCTCTGAAGAAGAAGCTCTACAAACATGTCTATAGTGTGATGGAAATGCCCTC	2432
781	S A I Q C Y M K D H P K I S E E E A L Q H V Y S V M E N A L	810
2433	GAGAGTTGAATAGGAGTTTGTGAATAACAAAATACCGGATTTTACAAAAGACTGGTTTGTGAAGTCAAGAAATATGCAACTCTTT	2522
811	E A L N R E F V N N K I P D I Y K R L V F E T A R I M Q L F	840
2523	TATATGCAAGGGGATGTTGACATATCAGATGATGGAATTAAGAGCATGCAAAAATTCGCTCTTCCACAGGATTCCTAGATT	2612
841	Y M Q G D G L T L S H D M E I K E H V K N C L F Q P V A	868
2613	AAATATTCAGTTAAAGGCCCTCATGTTATGTTTAAACATTATAACAGATGCTCAAAAGCTTTGAGCGGATTTGTTAAAGGCTATC	2702
2703	TTTGTGTTGTTTGTGTTTACTGCCAACCAAAAGCGTTCTTAAACCTTTGAAGACATTTCCATCCAGAGATGGAGTCTACATTTTATT	2792
2793	TATGAGATTGAATTTTCAAGAGAATATACATATATTTAAAGTAAAAAATAAAAAAAAAA	2861

FIG. 3. Nucleotide and predicted amino acid sequence of grand fir abietadiene synthase clone pAC22.1. The start and stop codons and the sequences of trypsin fragments are underlined. The three trypsin fragments that provided primers for PCR amplification (101, 127, and 52 in order from the amino terminus) are double underlined. The DDDTAM and ILDDLYD motifs are in boldface. The arrowhead (▲) indicates the predicted cleavage site between the transit peptide and mature protein.

60), and provided an estimate of abietadiene synthase mRNA transcript size of about 3500 nucleotides (data not shown). Thus, all lines of evidence, including Northern blotting, indicate that the structural gene for the inducible abietadiene synthase from grand fir has been successfully isolated.

Comparison with Other Terpenoid Cyclases—Comparison of the deduced amino acid sequence of abietadiene synthase with those of the eight other defined terpenoid cyclases of plant origin revealed a significant degree of homology between these enzymes, in spite of significant phylogenetic distances and differences in mechanistic detail. The abietadiene synthase shares a sequence similarity of 51% (28% identity) with a monoterpene cyclase, limonene synthase, from spearmint (38), 55% similarity (30% identity) with a sesquiterpene cyclase, 5-*epi*-aristolochene synthase, from tobacco (37) and 56% similarity (31% identity) with a related sesquiterpene cyclase, vetispiradiene synthase, from *Hyoscyamus muticus* (40), 55% similarity (28% identity) with two isoenzymes of the sesquiterpene cyclase, δ -cadinene synthase, from cotton (61), 54% simi-

larity (28% identity) with a diterpene cyclase, casbene synthase, from castor bean (39), 57% similarity (33% identity) with a diterpene cyclase, kaurene synthase B, from pumpkin (62), and 67% similarity (45% identity) with a diterpene cyclase, taxadiene synthase, from Pacific yew (63). Although these monoterpene, sesquiterpene, and diterpene cyclases employ different prenyl diphosphate substrates (geranyl (C_{10}), farnesyl (C_{15}), and geranylgeranyl or copalyl (C_{20}) diphosphate, respectively), they all operate by a similar mechanism involving ionization of the allylic diphosphate ester, intramolecular attack of the resulting carbocationic center on a remote double bond (often accompanied by hydrogen or methyl migration, or other rearrangement), and termination of the cationic reaction cascade by deprotonation. All of these cyclases, including abietadiene synthase, share the (I/L/V)XDDXXD motif (Fig. 4a) and its approximate placement toward the carboxyl terminus with the mechanistically related prenyltransferases (31–33). This sequence element is considered to function in binding the divalent metal ion-diphosphate complex of the prenyl substrates

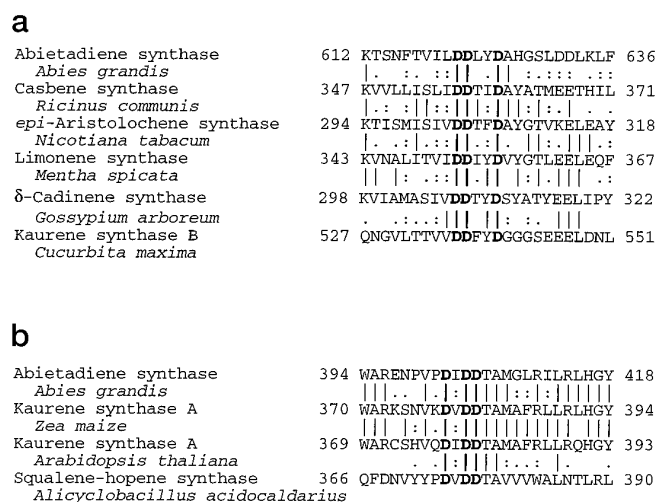


FIG. 4. Amino acid sequence comparison of the aspartate-rich DDXXD (a) and DXDD (b) motifs for a range of terpenoid cyclases. The vertical bar marks identical residues. One and two dots indicate increasing similarity, respectively, as defined using default similarity tables and thresholds within the GAP program of the Genetics Computer Group Packet (29).

(30, 41–43), a proposal supported by directed mutagenesis studies (41–43) and by x-ray structural analysis of farnesyl diphosphate synthase (44). The sequences of the sesquiterpene cyclases of fungal origin (34–36) also contain the aspartate-rich DDXXD motif, but otherwise do not closely resemble those of abietadiene synthase and other plant terpenoid cyclases (43% similarity).

The abietadiene synthase also resembles the two known kaurene synthase A sequences from *A. thaliana* (58) and maize (59), in sharing about 54% similarity (~28% identity) with both. The kaurene synthase A activity catalyzes the protonation-initiated cyclization from the olefinic terminus of geranylgeranyl diphosphate to yield (–)-copalyl diphosphate en route to (–)-kaurene; this reaction is analogous to the first cyclization step catalyzed by abietadiene synthase, but is of opposite stereospecificity (Scheme I). Kaurene synthase A lacks the DDXXD element of the ionization-dependent terpenoid cyclases (58, 59); however, it and the abietadiene synthase share another aspartate-rich D(I/V)DDTAM domain with very similar placement toward the amino terminus (Fig. 4b). A similar sequence motif (DVDDTAV) is also found in the squalene-hopene cyclase of *Alicyclobacillus acidocaldarius* (64), an enzyme that catalyzes a similar protonation-initiated cyclization, in this case of the acyclic triterpene olefin squalene to the pentacyclic olefin hopene; related aspartate/glutamate-rich regions are also present in the eukaryotic oxidosqualene cyclases (65, 66). By contrast, the repetitive electron-rich QW motif of the squalene-hopene cyclase and related oxidosqualene cyclases that is thought to stabilize carbocationic reaction intermediates (67) has no very close analogs in the abietadiene synthase sequence (e.g., Gln¹⁷⁰–Trp¹⁷⁶, Gln²⁹³–Trp²⁹⁸, and Gln⁵⁶⁶–Trp⁵⁷¹). Although the DXDD motif, or related sequence elements, may prove to be as diagnostic for the protonation-initiated terpenoid cyclases, as is the DDXXD substrate-binding motif for the ionization-dependent cyclase and prenyltransferase types (33), a mechanistic role for the former is not yet obvious. Abe and Prestwich (68, 69) have suggested that the D(D/C)TAE motif of the triterpene cyclases corresponds to the DDXXD motif of the prenyltransferases and related monoterpene, sesquiterpene, and diterpene cyclases and, since the triterpene cyclases employ olefinic or epoxide substrates rather than prenyl diphosphates, that this sequence element might

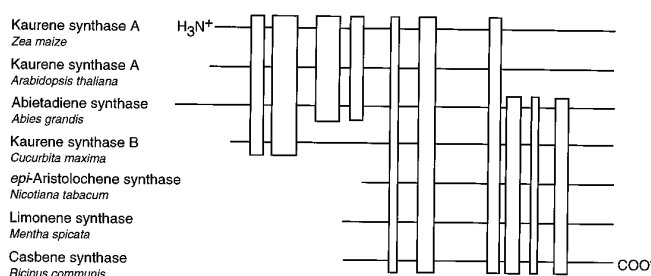


FIG. 5. Similarity comparison between abietadiene synthase and a range of other plant-derived terpenoid cyclases. Cyclases that employ double bond protonation-dependent reactions are listed above abietadiene synthase; those that employ diphosphate ester ionization-dependent reactions are listed below. The plotsimilarity program (29) was employed to locate regions of similarity and, using a minimum 10-residue window at a minimum similarity score of 60%, the results of comparing 10 regions were tabulated; there are a number of less conserved regions below this minimum window setting. The lengths of the horizontal lines are proportional to the sizes of the proteins, which range from about 50 kDa to nearly 100 kDa.

act to stabilize incipient positive charge of intermediates rather than binding the metal ion-diphosphate group. Extension of this argument to the case of abietadiene synthase, which bears both DXDD and DDXXD motifs, suggests that these elements may have separate roles, the ILDDLYD in binding the metal ion-diphosphate complex to assist in ionization, and the DIDDTAM to stabilize cationic intermediates generated in the course of both the protonation-initiated and ionization-initiated cyclization steps.

All of the known plant terpenoid cyclase sequences exhibit significant similarities with abietadiene synthase, with the only other gymnosperm cyclase, taxadiene synthase from the Pacific yew (63), showing the closest similarity (in spite of the difference in mechanism of action). However, inspection of the sequence alignments by several similarity comparison programs (29) revealed that homology with each cyclase type is not evenly distributed throughout the entire sequence. The differences are most apparent when sequences are compared using a minimum 10-residue window at a minimum similarity score of 60% (Fig. 5). Such comparison reveals that the amino-terminal portion of abietadiene synthase, containing the DXDD motif, resembles most closely kaurene synthase A, whereas the carboxyl-terminal region, containing the DDXXD motif, resembles the collection of substrate ionization-dependent terpenoid cyclases, including kaurene synthase B. The central portion of the abietadiene synthase appears to be an overlap region in which elements of both types of cyclases merge. The obvious conclusion is that the abietadiene synthase represents a fusion of elements from both the protonation-dependent cyclases and the ionization-dependent cyclases into a single, relatively large, bifunctional enzyme that catalyzes both of these fundamentally different cyclization reaction types. It is possible, although unlikely, that the more complex abietadiene synthase represents a progenitor of the other two cyclase classes.

The use of amino acid-modifying reagents has implicated histidine and cysteine residues at the active sites of several terpenoid cyclases of plant origin (70, 71). A search of the aligned sequences revealed that the positions of six histidines (residues 371, 416, 457, 486, 509, and 561) and three cysteines (residues 94, 330, and 658) show conservation with other plant terpenoid cyclase genes from spearmint (38), tobacco (37), *H. muticus* (40), castor bean (39), cotton (61), yew (63), maize (59), and *A. thaliana* (58). Only the histidine at position 416 of the abietadiene cyclase (Fig. 4) is conserved in all the plant-derived terpenoid cyclases, including the two kaurene synthase A sequences from *A. thaliana* and maize (58, 59).

The results of this study indicate that a single, bifunctional enzyme, abietadiene synthase, carries out the protonation-initiated cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate and the ionization-initiated cyclization of (+)-copalyl diphosphate to pimaradiene with subsequent methyl migration to afford abieta-7(8),13(14)-diene, and that the responsible protein bears significant primary structural elements from both fundamentally distinct cyclase types. An understanding of the catalytic role of these primary sequence elements of abietadiene synthase will require detailed study of structure-function relationships, an area of investigation that has thus far been precluded by limited availability of the enzyme. The isolation of the abietadiene synthase cDNA permits the development of an efficient functional expression system for this cyclase with which such detailed mechanistic studies can be undertaken and with which molecular tools can be employed to attempt dissection of the complex reaction sequence into its component parts.

Acknowledgments—We thank Gerhard Munske of the Washington State University Laboratory for Bioanalysis and Biotechnology for expert technical assistance in peptide separation and sequencing, cDNA sequencing, and primer synthesis; Reinhard Jetter for sample isolation and the mass spectrometric analysis; Christoph Funk, Christopher Steele, and Jonathan Gershenzon for other technical assistance; Greg Wichelns for raising the plants; and Joyce Tamura-Brown for typing the manuscript.

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