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Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha* x *piperita*, L.) that produces the aphid alarm pheromone (*E*)-β-farnesene

(insect pheromone/plant-insect chemical communication/synomone)

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ABSTRACT (*E*)-β-Farnesene is a sesquiterpene semiochemical that is used extensively by both plants and insects for communication. This acyclic olefin is found in the essential oil of peppermint (*Mentha* x *piperita*) and can be synthesized from farnesyl diphosphate by a cell-free extract of peppermint secretory gland cells. A cDNA from peppermint encoding (*E*)-β-farnesene synthase was cloned by random sequencing of an oil gland library and was expressed in *Escherichia coli*. The corresponding synthase has a deduced size of 63.8 kDa and requires a divalent cation for catalysis (K_m for $Mg^{2+} \approx 150$ μM; K_m for $Mn^{2+} \approx 7$ μM). The sesquiterpenoids produced by the recombinant enzyme, as determined by radio-GC and GC-MS, are (*E*)-β-farnesene (85%), (*Z*)-β-farnesene (8%), and δ-cadinene (5%) with the native C15 substrate farnesyl diphosphate ($K_m \approx 0.6$ μM; $V_{rel} = 100$) and Mg^{2+} as cofactor, and (*E*)-β-farnesene (98%) and (*Z*)-β-farnesene (2%) with Mn^{2+} as cofactor ($V_{rel} = 80$). With the C_{10} analog, GDP, as substrate ($K_m = 1.5$ μM; $V_{rel} = 3$ with Mg^{2+} as cofactor), the monoterpenes limonene (48%), terpinolene (15%), and myrcene (15%) are produced.

(*E*)-β-farnesene (Fig. 1) is an acyclic sesquiterpene olefin that occurs in a wide range of both plant and animal taxa. More than 600 papers have been published on the occurrence of this natural product and its deployment as an important courier in chemical communication. The olefin is found in the essential oil of hundreds of species of both gymnosperms, such as *Torreya taxifolia* (Florida torreya) (1) and *Larix leptolepis* (larch) (2), and angiosperms, such as *Robinia pseudoacacia* (black locust) (3), *Medicago sativa* (alfalfa) (4), *Chamomilla recutita* (chamomile) (5), *Vitis vinifera* (grapes) (6), *Cannabis sativa* (hemp) (7), *Zea mays* (corn) (8), *Piper nigrum* (black pepper)†, *Daucus carota* (carrot)†, and *Mentha* x *piperita* (peppermint) (9).

Although socially dominant male mice produce both α-farnesene and (*E*)-β-farnesene in their urine as pheromones (10), it is in the insects and plants that the use of (*E*)-β-farnesene as a semiochemical is most extensive. (*E*)-β-Farnesene is emitted by the Dufour's gland of andrenid bees (11) and by several genera of ants (12–14), where it serves both as a defensive allomone and as a trail pheromone. This sesquiterpene is synthesized *de novo* in the osmetrial glands of larval *Papilio* (Lepidoptera/Papilionidae) as an allomone (15), and it functions as a feeding stimulant to the sand fly *Lutzomyia longipalpis* (Diptera/Psychodidae), an important vector of the blood disease leishmaniasis (16). Several species of predatory carabid beetles use (*E*)-β-farnesene as a prey-finding kairomone (17). When released by corn, this olefin is also a kairomonal oviposition stimulant to the European corn borer (*Ostrinia*) (18). (*E*)-β-farnesene is the major component of pollen odor in *Lupinus* and stimulates pollination behavior in bumblebees (19). Feeding by larval lepidopterans, such as *Heliothis* or *Spodoptera* (Noctuidae), increases the amount of (*E*)-β-farnesene released by corn; the volatile olefin then is detected as a synomone by the parasitic wasp *Cotesia marginiventris* (Hymenoptera/Braconidae) for locating the lepidopteran hosts (8). Circumstantial evidence also suggests the lepidopteran induced production and emission of (*E*)-β-farnesene from corn serves as a synomone for *Cotesia kariyai* (20) and from cotton leaves as a synomone for *C. marginiventris* (21, 22).

Perhaps of greatest significance in plant-insect interactions is the use of (*E*)-β-farnesene by most aphid species as an alarm pheromone (23, 24). Aphids exposed to (*E*)-β-farnesene become agitated and disperse from their host plant (25). Alate aphids are usually more sensitive than are apterae species and often will not colonize a host displaying (*E*)-β-farnesene. Ants that defend aphids are sensitive to host-emitted (*E*)-β-farnesene and, when exposed, will display aggressive behavior (26). (*E*)-β-farnesene also mimics the action of juvenile hormone III in some insects (27), may play a role in control of aphid morphological types, and is acutely toxic to aphids at a dose of 100 ng/aphid (28). (*E*)-β-farnesene vapor is also toxic to whiteflies (29).

Efforts to control aphid behavior by topical application of (*E*)-β-farnesene to crops have met with little success, because of volatility and rapid oxidative inactivation in air (30). Derivatives of (*E*)-β-farnesene with reduced volatility, or increased stability, have shown promise in reducing aphid-transmitted viruses, such as barley mosaic virus (30), potato virus Y (31), and beet mosaic virus (31). The wild potato *Solanum berthaultii*, which produces (*E*)-β-farnesene in type A trichomes, is more repellent to the green peach aphid than are commercial varieties of *S. tuberosum* that produce lower levels of the olefin (32, 33). In alfalfa, repellency to the blue alfalfa aphid and the pea aphid is correlated with the leaf content of (*E*)-β-farnesene, but not with the amount of the co-occurring sesquiterpene caryophyllene (34).

For plants that produce (*E*)-β-farnesene, breeding for increased production has met with some success (34), but has been limited by genetic variation in these species. (*E*)-β-farnesene synthase has been purified from maritime pine (*Pinus pinaster*) and characterized (35), but the gene has not yet been isolated from any source. A cDNA clone for (*E*)-β-

Abbreviations: FDP, farnesyl diphosphate; I, identity; S, similarity.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF024615).

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†Beckstrom-Sternberg, S. M. & Duke, J. A. (1994) The Phytochemical Database, Version 4.3; http://probe.nalusda.gov:8300/cgi-bin/browse/phytochemdb.

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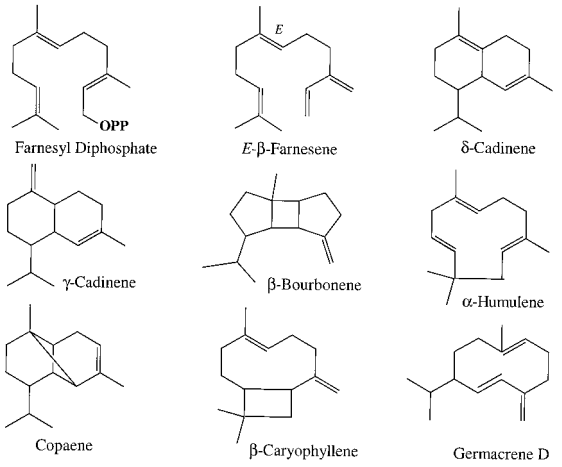

FIG. 1. The sesquiterpene synthase substrate, FDP, and sesquiterpene olefins found in peppermint essential oil.

farnesene synthase would, by transgenic manipulation, provide a valuable addition to the arsenal of natural compounds active in host plant resistance. The substrate for (*E*)-β-farnesene synthase is farnesyl diphosphate (FDP), an ubiquitous isoprenoid intermediate involved in cytoplasmic phytosterol biosynthesis. Sesquiterpene synthases lack plastidial targeting sequences and are localized to the cytoplasm (36). Therefore, even in plants that do not normally produce sesquiterpenes, a recombinant (*E*)-β-farnesene synthase would be directed to the cytoplasm where substrate is supplied by the mevalonate pathway and where production of (*E*)-β-farnesene should result. In this paper, we describe the isolation, sequencing, and functional heterologous expression of a cDNA from peppermint that encodes (*E*)-β-farnesene synthase.

MATERIALS AND METHODS

Plant Material and Reagents. *Mentha* x *piperita* L. cv. "Black Mitcham" was propagated from rhizomes as described (37). The preparations of [1-^{3}H]GDP (250 Ci/mol), [1-^{3}H]FDP (125 Ci/mol), and [1-^{3}H]geranylgeranyl diphosphate (118 Ci/mol) have been reported previously (38–40). Terpenoid standards were from our own collection or were prepared from plant material purchased locally. α-Farnesene was a gift from J. Brown (Washington State University), δ-cadinene was a gift from M. Essenberg (Oklahoma State University), and commercially steam distilled peppermint oil was a gift from I. P. Callison and Sons, Inc., Chehalis, WA. All other biochemicals and reagents were purchased from Sigma or Aldrich unless otherwise noted.

Sesquiterpene Analysis. Young, mature peppermint leaves were harvested and hydrodistilled from NH_4HCO_3-buffered water with simultaneous pentane extraction (41). The organic phase was passed through a column of $MgSO_4$-silica gel (Mallinckrodt SilicAR-60) to provide the olefin fraction for GC-MS analysis. Authentic (*E*)-β-farnesene was prepared by pentane extraction (followed by silica gel fractionation) of macerated ginger (*Zingiber officinale*) root, black pepper oleoresin (*Piper nigrum*), bergamot oil (*Citrus bergamot*), parsley oil (*Petroselinum crispum*), or field-grown (Yakima Valley, WA) commercial peppermint oil (9); all of these sources are reported to contain (*E*)-β-farnesene†.

Cell-Free Assays. Peppermint oil gland secretory cells were isolated from immature leaves as described (37) and sonically disrupted (Braun-Sonic 2000 microprobe at maximum power for three 30-sec bursts with 30-sec chilling period at 0–4°C between bursts) into assay buffer consisting of 25 mM 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid (pH 7.0), 10 mM sodium ascorbate, 25 mM KCl, 10 mM DTT and 10% glycerol, and supplemented with 0.5% (wt/vol) polyvinylpolypyrrolidone and 1% (wt/vol) Amberlite XAD-4 polystyrene resin. The sonicate was centrifuged at 3,700 × *g* for 15 min, and an aliquot of the supernatant then was placed in a 10-ml screw-capped glass test tube containing divalent metal ions (10 mM $MgCl_2$ and 1 mM $MnCl_2$) and substrate (7.3 μM [1-^{3}H]FDP). The aqueous layer was overlaid with 1 ml of pentane, and the sealed tube was incubated at 30°C for 2 hr. The pentane overlay then was collected, and the aqueous layer was extracted twice (1 ml) with pentane. The combined pentane extracts were passed through an anhydrous $MgSO_4$-silica gel column to obtain the labeled hydrocarbon fraction for GC-MS analysis, or for radio-GC analysis by using peppermint oil as an internal standard.

Instrumental Analysis. Radio-GC was performed on a Gow-Mac 550P instrument (He carrier 40 ml/min, injector 220°C, detector 250°C and 150 mA) attached to a Packard 894 gas proportional counter. The column (3.18 mm i.d. by 3.66 m stainless steel with 15% polyethylene glycol ester (AT1000 Alltech) on Gas Chrom Q was programmed from 150°C (5-min hold) to 220°C at 5°C/min. Thermal conductivity and radioactivity outputs were monitored after calibration with an external radiochemical standard, and ≈20,000 disintegrations per minute of tritiated product were injected with data analysis that used Turbochrome Navigator version 4.1 software (Perkin–Elmer). Liquid scintillation counting was performed in toluene/ethanol (70:30, vol/vol) containing 0.4% Omnifluor (DuPont/NEN) by using a Packard 460 CD spectrometer (^{3}H efficiency ≈43%). GC-MS analysis used a Hewlett-Packard 6890–5972 system with a 5MS capillary column (0.25 mm i.d. by 30 m with 0.25 μm coating of 5% phenyl methyl siloxane). Injections were made cool on-column at 40°C with oven programming from 40°C (50°C/min) to 50°C (5-min hold), then 10°C/min to 250°C, then 50°C/min to 300°C. Separations were made under a constant flow of 0.7 ml He/min. Mass spectral data were collected at 70 eV and analyzed by using Hewlett–Packard Chemstation software.

Library Construction and Clone Identification. An enriched cDNA library was constructed from peppermint secretory cell clusters consisting of the eight glandular cells subtending the oil droplet. These cell clusters were harvested by a leaf surface abrasion technique (37), and the RNA contained therein was isolated by using the protocol of Logemann *et al.* (42). mRNA was purified by oligo(dT) cellulose chromatography (Pharmacia), and 5 μg of mRNA was used to construct a λZAPII cDNA library according to the manufacturer's instructions (Stratagene).

Plasmids were excised from the library en masse and used to transform *Escherichia coli* strain XLOLR as per the manufacturer's instructions (Stratagene). Approximately 150 individual plasmid-bearing strains were grown in 5 ml of Luria–Bertani media overnight, and the corresponding plasmids were purified by using a Qiawell 8 Ultraplasmid Kit (Qiagen) before partial 5′-sequencing by the Dye-Deoxy method by using an Applied Biosystems Sequenator at the Laboratory for Biotechnology and Bioanalysis at Washington State University. Putative terpenoid synthase genes were identified by sequence comparison by using the BLAST program of the GCG Wisconsin Package, version 8. Bluescript plasmids harboring unique full-length cDNA inserts with high similarity to known plant terpenoid synthases were tested for functional expression after transformation into *E. coli* XL1-Blue cells. A single extract, from the bacteria containing clone p43, produced a sesquiterpene olefin from [1-^{3}H]FDP, and this clone was selected for further study.

Bacterial Expression and Characterization of (*E*)-β-Farnesene Synthase. *E. coli* XL1-Blue harboring p43 (bearing an apparently full-length insert) or empty pBluescript plasmid as a control were grown overnight at 37°C in Luria–Bertani

medium containing 100 μg of ampicillin/ml. A 50-μl aliquot of the overnight culture was used to inoculate 5 ml of fresh Luria–Bertani medium, and the culture was grown at 37°C with vigorous agitation to A_{600} = 0.5 before induction with 1 mM isopropyl-β-D-thiogalactopyranoside. After an additional 2 hr of growth, the suspension was centrifuged (1,000 × *g*, 15 min, 4°C), the media removed, and the pelleted cells resuspended in 1 ml of cold assay buffer containing 1 mM EDTA. The cells were disrupted by sonication with a microprobe as previously described, except that only two 20-sec bursts were used. The chilled sonicate was cleared by centrifugation, and the supernatant was assayed for sesquiterpene synthase activity as before, or for monoterpene synthase activity (with 4.5 μM [1-^{3}H]GDP) or diterpene synthase activity (with 10 μM [1-^{3}H]geranylgeranyl diphosphate). In all cases, the pentane-soluble reaction products were purified by $MgSO_4$-silica gel chromatography, as above, to prepare the olefin fraction for further analysis. For determination of the pH optimum of (*E*)-β-farnesene synthase, the preparation was adjusted with 50 mM 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid (to a pH of 6.5, 6.75, 7.0, 7.25, 7.5, 8.0, or 8.5) before the assay. Kinetic constants for FDP, GDP, Mg^{2+}, and Mn^{2+} were determined by using a preparation of (*E*)-β-farnesene synthase that was partially purified by anion-exchange chromatography [on a Mono-Q column (Pharmacia) equilibrated with assay buffer and eluted with a linear KCl gradient (0–500 mM) in assay buffer]. The 210–230 mM fraction containing the (*E*)-β-farnesene synthase was used for kinetic evaluation of FDP and GDP as substrates (concentration range 0.31 to 20 μM, with saturating Mg^{2+}). Because of the tenacious binding of divalent cations by the synthase, the partially purified enzyme (prepared in the presence of 10 mM EDTA) was dialyzed overnight against assay buffer containing 50 mM EDTA. The dialysate was buffer-exchanged by ultrafiltration (Amicon Centriprep 30, 450-fold dilution), then desalted (Bio-Rad Econo-Pak 10 DG) into assay buffer. Kinetic constants for Mg^{2+} and Mn^{2+} (assay range 1 μM to 2 mM of the chloride salts) then were determined at 7.3 μM [1-^{3}H]FDP. Triplicate assays were conducted, and control incubations (without enzyme) were included in all cases. A double reciprocal plot (43) was generated for each averaged data set, and the equation of the best-fit line determined (Kaleidagraph version 3.08, Synergy Software, Reading, PA).

RESULTS AND DISCUSSION

Essential Oil Analysis and Cell-Free Assay. To assess the probable abundance of (*E*)-β-farnesene synthase in peppermint gland secretory cells, the exclusive site of essential oil biosynthesis (37), the sesquiterpene olefin fraction of field-distilled peppermint oil was analyzed by GC-MS and shown to contain β-caryophyllene (39%), γ-cadinene (33%), β-bourbonene (11%), (*E*)-β-farnesene (2.9%), δ-cadinene (2.0%), germacrene D (1.3%), copaene (1.3%) and α-humulene (1.2%) (Fig. 1), as well as several other minor components (<1% each). GC-MS analysis of the oil distilled from greenhouse material revealed a similar composition, except that the amount of γ-cadinene was higher (53%), β-bourbonene was conspicuously absent, and the (*E*)-β-farnesene content was 3.4%. Although (*E*)-β-farnesene was not one of the more prominent sesquiterpenes of peppermint, the abundance was sufficient to suggest that cloning of the corresponding synthase by random sequencing of an enriched, oil gland cDNA library might be possible.

To gain a preliminary assessment of the target activity, cell-free extracts of peppermint oil gland secretory cells (37) were assayed for the divalent metal ion-dependent conversion of [1-^{3}H]FDP to sesquiterpene olefins (44). Radio-GC analysis of the derived biosynthetic products (Fig. 2) revealed the presence of two major components identified as caryophyllene

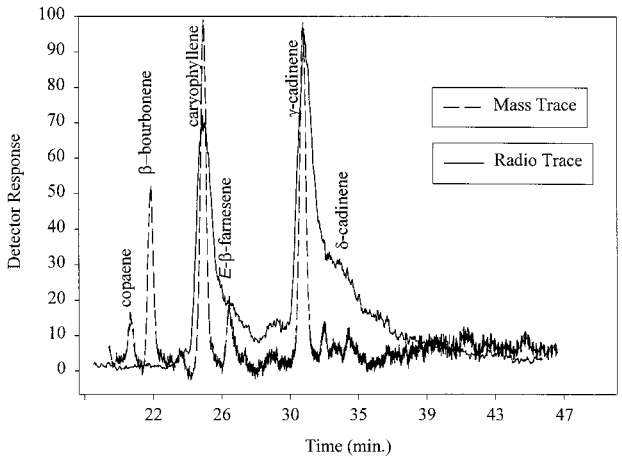

FIG. 2. Radio-GC of the sesquiterpene olefins generated from [1-^{3}H]FDP by an enzyme preparation from peppermint oil gland secretory cells. The olefin fraction of steam-distilled peppermint oil was used as internal standard, and only the portion of the chromatogram containing the sesquiterpene olefins is shown.

and γ-cadinene. However, the separation of the labeled olefins was insufficient to resolve (*E*)-β-farnesene from caryophyllene, or δ-cadinene from γ-cadinene. Both of these minor components appear at the trailing edges of the major peaks but are, nevertheless, coincident with the authentic standards, indicating the corresponding biosynthetic capability. No β-bourbonene was synthesized from FDP by this system.

Isolation and Bacterial Expression of a cDNA Encoding (*E*)-β-Farnesene Synthase. Initial cloning of full-length terpenoid biosynthetic genes from the peppermint oil gland cDNA library was successful and established a very high degree of enrichment for these target sequences. For example, the monoterpene cyclase, limonene synthase (45), represents approximately 4% of the library. This fact, plus the availability of automated sequencing capability, led to the possibility of randomly sequencing the library in search of cDNA species encoding other terpenoid synthases, including the (*E*)-β-farnesene synthase, which was shown to be operational in this plant by both sesquiterpene analysis and cell-free assay.

Of the first 150 random clones, a unique and apparently full-length insert was acquired that was shown by partial sequencing to resemble, in deduced amino acid sequence, other sesquiterpene synthases of plant origin (i.e., a similarity score in excess of 50%, see below). A cell-free extract of *E. coli* XL-1 Blue cells harboring this plasmid (p43) was prepared and shown to be capable of catalyzing the divalent metal ion-dependent conversion of [1-^{3}H]FDP to labeled sesquiterpene olefins. Radio-GC analysis (data not shown) and GC-MS analysis (Fig. 3) of this sesquiterpene olefin fraction demonstrated that the major biosynthetic product (85%) was (*E*)-β-farnesene by matching of both retention time and mass spectrum to those of the authentic standard obtained from several natural sources. Lesser amounts of (*Z*)-β-farnesene (8%) and δ-cadinene (5%), as well as three other minor products (less than 1% each; all seemingly of the cadinene-type based on MS) also were produced. Control reactions, by using extracts of XL1-Blue cells transformed with pBluescript lacking the insert, evidenced no detectable production of sesquiterpene olefins from [1-^{3}H]FDP, thereby demonstrating that a cDNA clone encoding (*E*)-β-farnesene synthase had been acquired.

Multiple product formation is a common feature of the terpenoid synthases and may be a consequence of the electrophilic reaction mechanism catalyzed by these enzymes in which highly reactive carbocationic intermediates are generated (44, 46). (*E*)-β-Farnesene is one of the simplest sesquiterpene olefins that can be derived from FDP, in a reaction

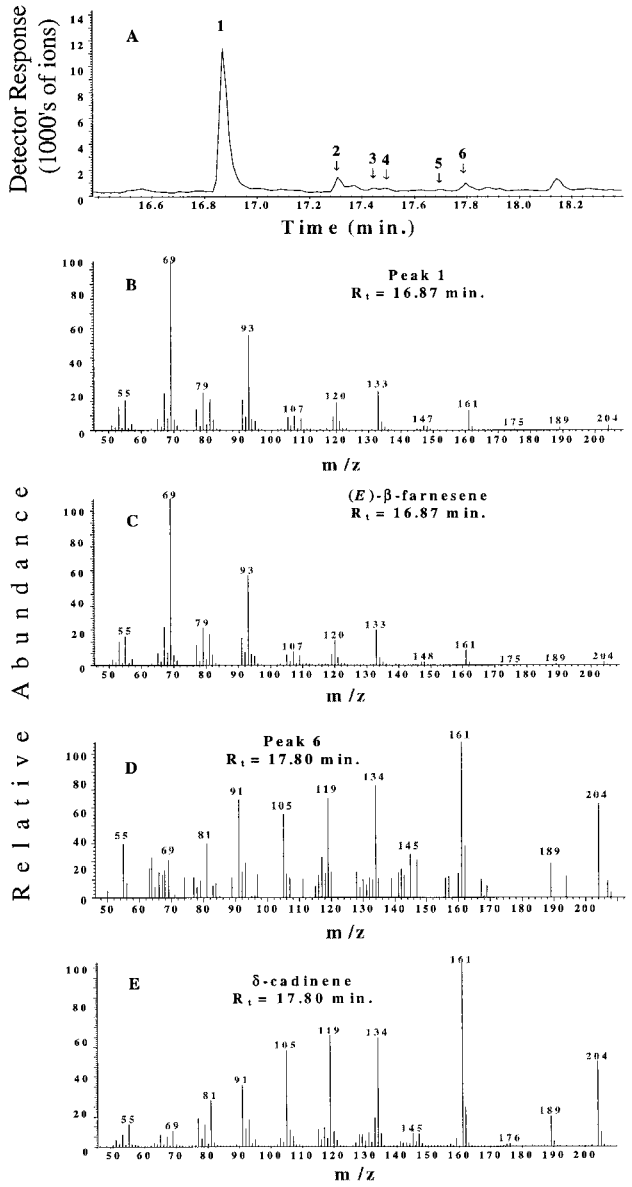

FIG. 3. GC-MS of the products generated from FDP by the recombinant (*E*)-β-farnesene synthase. (*A*) Total ion chromatogram. Numbered peaks are sesquiterpene olefins. (*B*) Mass spectrum and retention time of peak 1 designated in *A*. (*C*) Mass spectrum and retention time of authentic (*E*)-β-farnesene from parley oil. (*D*) Mass spectrum and retention time of peak 6 designated in *A*. The spectrum of this minor product is compromised by the low ion abundance and the corresponding prominence of background ions. (*E*) Mass spectrum and retention time of authentic δ-cadinene.

involving divalent metal ion-assisted ionization of the diphosphate ester and deprotonation from the C-3 methyl of the resulting carbocation (Fig. 4). The formation of δ-cadinene (Fig. 4) involves a considerably more extended reaction sequence, in which a preliminary isomerization step (to nerolidyl diphosphate) is required to permit the ionization-dependent cyclization to the macrocycle, followed by 1,3-hydride shift, closure of the second ring, and deprotonation to the bicyclic product. The small amount of δ-cadinene produced by the recombinant synthase from FDP is interesting in light of the abundance of this bicyclic olefin in the sesquiterpene fraction of peppermint oil and the efficient production of this olefin in oil gland extracts; these observations suggest that an additional and distinct δ-cadinene synthase must operate in peppermint.

The recombinant (*E*)-β-farnesene synthase was inactive with the C_{20} substrate analog [1-^{3}H]geranylgeranyl diphosphate,

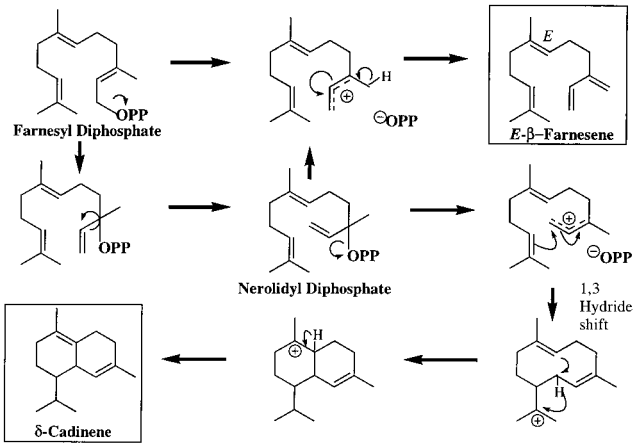

FIG. 4. Proposed mechanism for the formation of (*E*)-β-farnesene and δ-cadinene from FDP. OPP denotes the diphosphate moiety. Ionization of the enzyme-bound nerolidyl diphosphate intermediate and proton elimination also can produce (*E*)-β-farnesene.

but was able to catalyze the divalent cation-dependent conversion of the C_{10} analog [1-^{3}H]GDP to monoterpene olefins. Although the rate of conversion of GDP to these products was less than 3% of the rate of conversion of FDP to sesquiterpene olefins at saturation, a more diverse spectrum of products was formed (see Fig. 5 for structures). The cyclic monoterpenes limonene (48%) and terpinolene (15%) and the acyclic monoterpene analog of β-farnesene, myrcene (15%), were the most abundant products as determined by both radio-GC and GC-MS analysis (data not shown). Lesser amounts of γ-terpinene (7%), (*Z*)-ocimene (6%), (*E*)-ocimene (7%), and sabinene (3%) also were observed as products. Control reactions, by using extracts of XL1-Blue cells transformed with pBluescript lacking the insert, showed no detectable production of monoterpene olefins from [1-^{3}H]GDP, thereby confirming that the monoterpene synthase activity expressed from p43 was a function of the (*E*)-β-farnesene synthase. Because monoterpene biosynthesis is localized to plastids, as is diterpene biosynthesis, whereas sesquiterpene biosynthesis occurs in the cytoplasm (36), the utilization of GDP as a substrate by (*E*)-β-farnesene synthase is unlikely to be of physiological relevance and simply may reflect the lack of evolutionary pressure to discern the chain length of this isoprenoid substrate to which the enzyme is not exposed *in vivo*.

Sequence Analysis. Complete sequencing of the (*E*)-β-farnesene synthase cDNA contained in p43 revealed an insert

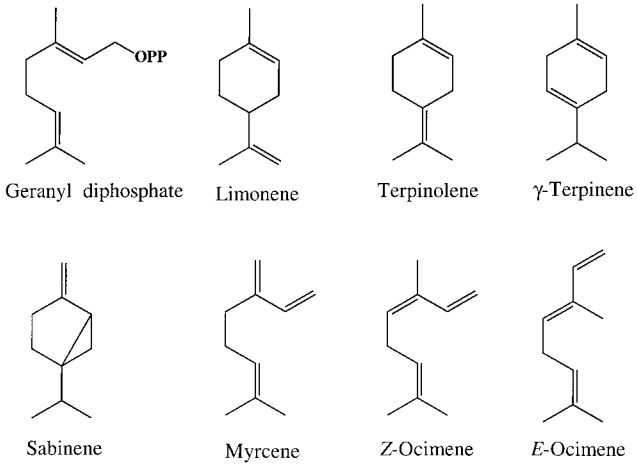

FIG. 5. Monoterpene olefins generated from the alternate substrate GDP by recombinant (*E*)-β-farnesene synthase.

size of 1,959 bp encoding an ORF of 550 amino acids with a deduced molecular mass of 63,829 (Fig. 6). A putative starting methionine codon was identified that was out of frame with the vector β-galactosidase starting methionine; however, a fortuitous stop codon in the 5′-untranslated region, 46 bp upstream of the synthase translation start site and in-frame with the β-galactosidase fusion sequence, allowed polycistronic translation of the cDNA free of vector-derived sequence. The deduced amino acid sequence of the (*E*)-β-farnesene synthase lacks a plastidial targeting peptide (47), typical of monoterpene and diterpene synthases (45, 48, 49), but consistent with all known plant-derived sesquiterpene synthases (50–52) that are directed to the cytoplasm. Like all other known terpenoid synthases, (*E*)-β-farnesene synthase is rich in tryptophan (1.8%) and arginine (5.5%) residues, and bears a DDXXD motif (residues 301–305), which is believed to coordinate the divalent metal ion chelated to the substrate diphosphate group (53); the enzyme has a deduced isoelectric point at pH 5.16.

The deduced amino acid sequence of the farnesene synthase is most similar to that of the sesquiterpene cyclase *epi*-aristolochene synthase from tobacco (50) in exhibiting 62% similarity (S) and 49% identity (I). This peppermint synthase also closely resembles the three other known angiosperm sesquiterpene cyclases (vetispiradiene synthase from *Hyoscyamus muticus* (51) at 63% S and 40% I, δ-cadinene synthase from cotton (52) at 60% S and 37% I, and germacrene C synthase from tomato at 57% S and 34% I (unpublished work), and also the diterpene cyclase, casbene synthase (54), from castor bean (at 61% S and 35% I). Because (*E*)-β-farnesene synthase produces a small amount of δ-cadinene, but cannot be the major source of δ-cadinene in peppermint, it is tempting to speculate that the farnesene synthase represents either a progenitor, or an altered form of cadinene synthase in which the ability to catalyze the more complex bicyclization reaction has been lost.

Surprisingly, (*E*)-β-farnesene synthase is no more closely related to monoterpene synthases from the Lamiaceae [limonene synthase from spearmint (45) with 51% S and 30% I; sabinene synthase and 1,8-cineole synthase from culinary sage with 50% S and 29% I each (unpublished work)] than to the various terpenoid synthases from the gymnosperm *Abies grandis* [monoterpene synthases with 49% S and 28% I (55); sesquiterpene synthases with 53% S and 29% I (unpublished work); diterpene synthases with 51% S and 28% I (48)]. Even a phylogenetically distant diterpene cyclase from *Taxus brevifolia*, taxadiene synthase (49), resembles (*E*)-β-farnesene synthase at the amino acid level (50% S and 24% I) as closely as do the monoterpene synthases of the mint family. These sequence-based relationships may reflect a bifurcation in the evolution of the monoterpene synthases from the higher terpenoid synthases that is as ancient as the separation between the angiosperms and gymnosperms.

Enzyme Characterization. The recombinant, partially purified (*E*)-β-farnesene synthase exhibited a broad pH optimum in the 6.75 to 7.25 range in 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid buffer. This observation is in agreement with the studies of Salin *et al.* (35) in which the purified (*E*)-β-farnesene synthase from maritime pine was shown to possess a pH optimum in the 7.0 to 7.3 range. The K_m value for FDP with the recombinant synthase was calculated to be 0.6 μM, a value typical of other sesquiterpene synthases of plant origin (44) but lower than the value of 5 μM determined for the enzyme from maritime pine (35). Substrate concentrations in excess of 10 μM FDP indicated slight inhibition of activity with the recombinant enzyme. Although the relative velocity at saturating levels of GDP was only 3% of the velocity with FDP for the recombinant synthase, the calculated K_m value for GDP (1.5 μM) was only 3-fold higher than that for FDP, suggesting that the binding of the C_{10} analog was reasonably efficient.

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   1  MATNGVVISC LREVRPPMTK HAPSMWTDTF SNFSLDDKEQ QKCSETIEAL
  51  KQEARGMLMA ATTPLQQMTL IDTLERLGLS FHFETEIEYK IELINAAEDD
 101  GFDLFATALR FRLLRQHQRH VSCDVFDKFI DKDGKFEESL SNNVEGLLSL
 151  YEAAHVGFRE ERILQEAVNF TRHHLEGAEL DQSPLLIREK VKRALEHPLH
 201  RDFPIVYARL FISIYEKDDS RDELLLKLSK VNFKFMQNLY KEELSQLSRW
 251  WNTWNLKSKL PYARDRVVEA YVWGVGYHYE PQYSYVRMGL AKGVLICGIM
 301  DDTYDNYATL NEAQLFTQVL DKWDRDEAER LPEYMKIVYR FILSIYENYE
 351  RDAAKLGKSF AAPYFKETVK QLARAFNEEQ KWVMERQLPS FQDYVKNSEK
 401  TSCIYTMFAS IIPGLKSVTQ ETIDWIKSEP TLATSTAMIG RYWNDTSSQL
 451  RESKGGEMLT ALDFHMKEYG LTKEEAASKF EGLVEETWKD INKEFIATTN
 501  YNVGREIAIT FLNYARICEA SYSKTDGDAY SDPNVAKANV VALFVDAIVF
 551  *
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FIG. 6. Deduced amino acid sequence of (*E*)-β-farnesene synthase encoded by the cDNA insert of p43 (accession no. AF024615). Residues in bold indicate the conserved, aspartate-rich motif involved in binding the divalent cation-chelated substrate.

A K_m value of 150 μM was determined for Mg^{2+} (V_{rel} = 100), and a K_m value of 7.0 μM was determined for Mn^{2+} (V_{rel} = 80). No inhibition of activity was observed at Mg^{2+} concentrations up to 10 mM; however, concentrations of Mn^{2+} exceeding 20 μM resulted in a sharp decline in reaction velocity to a plateau (V_{rel} = 20) in the 0.25 to 2 mM range. Because the product distribution of the recombinant (*E*)-β-farnesene initially had been determined in the presence of excess Mg^{2+}, the conversion of [1-^{3}H]FDP was re-evaluated in the presence of Mn^{2+} alone at apparent saturation (20 μM). The olefin products again were analyzed by GC-MS and found in this case to consist of 98% (*E*)-β-farnesene and 2% (*Z*)-β-farnesene. No δ-cadinene or other sesquiterpenes were synthesized in this instance, indicating that a structural alteration in the binding of Mn^{2+} to the substrate and/or enzyme (relative to Mg^{2+}) improves the fidelity of the reaction.

In operational characteristics (pH optimum, kinetic constants) and physical features (size, pI), the recombinant (*E*)-β-farnesene is a typical sesquiterpene synthase (44, 50–52), suggesting that the enzyme should be highly functional *in planta*. Given that this synthase will be targeted by default to the cytoplasm (36, 47), where the substrate arises from the mevalonate pathway, it should be possible to engineer virtually any plant for the production of (*E*)-β-farnesene to exploit the kairomonal and pheromonal properties of this natural product.

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