Characterization of Geraniol Synthase from the Peltate Glands of Sweet Basil[1]

Yoko Iijima, David R. Gang, Eyal Fridman, Efraim Lewinsohn, and Eran Pichersky*

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109–1048 (Y.I., E.F., E.P.); Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721–0036 (D.R.G.); and Department of Vegetable Crops, Newe Ya'ar Research Center, Agricultural Research Organization, P.O. Box 1021, Ramat Yishay, 30095, Israel (E.L.)

The monoterpene fraction of the lemon-scented sweet basil (*Ocimum basilicum*) cv Sweet Dani consists mostly of citral (a mixture of geranial and neral), with lower levels of geraniol and nerol. These compounds are stored in the peltate glands found on the leaf epidermis. Younger leaves, which have a higher density of such glands, also have a higher content of monoterpenes than older leaves. Geraniol synthase (GES) activity, generating geraniol from geranyl diphosphate, was shown to be localized exclusively or almost exclusively to glands. GES activity resides in a homodimeric protein that was purified to near homogeneity. Basil GES requires Mn^{2+} as a divalent metal cofactor for activity and produces only geraniol from geranyl diphosphate. K_m values of 21 and 51 μM were obtained for geranyl diphosphate and Mn^{2+}, respectively. In the presence of ^{18}O-labeled water, GES catalyzed the formation of ^{18}O-geraniol from geranyl diphosphate, indicating that the reaction mechanism of GES is similar to that of other monoterpene synthases and is different from the action of phosphatases. A GES cDNA was isolated based on analysis of a glandular trichome expressed sequence tag database, and the sequence of the protein encoded by this cDNA shows some similarity to sequences of other terpene synthases. The expression of the GES cDNA in *Escherichia coli* resulted in a protein with enzymatic activity essentially identical to that of plant-purified GES. RNA gel-blot analysis indicated that GES is expressed in glands but not in leaves of basil cv Sweet Dani, whose glands contain geraniol and citral, and not in glands or leaves of another basil variety that makes other monoterpenes but not geraniol or citral.

Geraniol is an acyclic monoterpene alcohol emitted from the flowers of many species, notably roses (*Rosa hybrida*; Bayrak, 1994; Antonelli et al., 1997; Rao et al., 2000). It is also present in vegetative tissues of many herbs (Charles and Simon, 1992; Mallavarapu et al., 1998; Mockute and Bernotiene, 1999; Vieira et al., 2001) and is often found together with geranial and neral, which are the oxidation products of geraniol (Miyazawa and Kameoka, 1988). The mixture of geranial and neral, also called citral, imparts a "lemon" flavor, and lemongrass (*Cymbopogon citratus* Stapf.; Singh-Sangwan et al., 1993), ginger (*Zingiber officinale* Rosc.; Miyazawa and Kameoka, 1988), and some varieties of sweet basil (*Ocimum basilicum*; Grayer et al., 1996; Simon et al., 1999) such as basil cv Sweet Dani are particularly rich in citral (Morales and Simon, 1997). However, at present, there is no definitive proof of whether citral is synthesized from geraniol by an alcohol dehydrogenase (Sangwan et al., 1993; Singh-Sangwan et al., 1993; Hallahan et al., 1995; Sekiwa-Iijima et al., 2001) or by an oxidase (Potty and Bruemmer, 1970; Banthorpe et al., 1976), nor is it known if geraniol is the only substrate whose oxidation leads to the formation of citral or whether nerol, the cis-isomer of geraniol, can also serve as a precursor (Corbier and Ehret, 1988; Ikeda et al., 1991; Hallahan et al., 1995).

Geraniol itself is likely to be synthesized from geranyl diphosphate, the universal precursor of all monoterpenes (Croteau, 1987; Gershenzon and Croteau, 1993; McGarvey and Croteau, 1995; Wise and Croteau, 1999), although no report to date has identified a specific geraniol synthase (GES). Two types of enzymatic reactions have been hypothesized to lead to geraniol synthesis from geranyl diphosphate, either a phosphatase- or monoterpene synthase-based catalysis. However, in the absence of purified and characterized GES, the question of whether GES employs a similar mechanism to the one used by other monoterpene synthases (and by sesquiterpene and diterpene synthases, acting on farnesyl diphospate and geranylgeranyl diphosphate, respectively) has remained unanswered. The general mechanism of terpene synthases involves the removal of the diphosphate group and the generation of an intermediate with a carbocation as the first step. In the various terpene synthases, such intermediates further rearrange to generate the high number of terpene skele-

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* Corresponding author; e-mail lelx@umich.edu; fax 734–647–0884.

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tons observed in nature (Gershenzon and Croteau, 1993; McGarvey and Croteau, 1995). However, the structure of geraniol, whose carbon skeleton is identical to that of its precursor geranyl diphosphate, hypothetically allows for an alternative mechanism of simply breaking the phosphoester bond by a phosphatase to generate geraniol.

In this paper, we report the purification and characterization of GES from basil cv Sweet Dani and GES cDNA isolation and expression in *Escherichia coli*. Furthermore, the reaction mechanism of GES was investigated using [^{18}O]-labeled water. We show that GES is a member of the terpene synthase family based on sequence analysis, and its reaction mechanism is similar to that of other terpene synthases.

RESULTS

Determination of Terpene Content of Basil Leaves and Glands

There are many basil varieties with distinct terpene compositions (Lawrence, 1988; Grayer et al., 1996; Simon et al., 1999). In the leaves of basil cv Sweet Dani, selected for its lemony flavor, geraniol, nerol, geranial, and neral comprise >99% of the monoterpenes present, and these four compounds also constitute >70% of total essential oil terpenes, with the remainder being sesquiterpenes (Fig. 1A). We have shown previously that basil leaf terpenes are exclusively synthesized in the four-celled peltate glands found on both sides of the leaves and stored in the sac surrounding the gland (Werker et al., 1993; Gang et al., 2001). Direct extraction of material from the sacs showed that geraniol, nerol, geranial, and neral are stored there (Fig. 1B).

The amounts of these four monoterpenes in the leaf depended on age of the leaf, with young leaves having the highest levels per gram fresh weight and the levels getting progressively lower as the leaves mature and expand (Table I). Geraniol and nerol were detected in trace amounts in older, larger leaves. The ratio of geranial to neral was 2:1 at the all stages, with the geraniol to nerol ratio being similar.

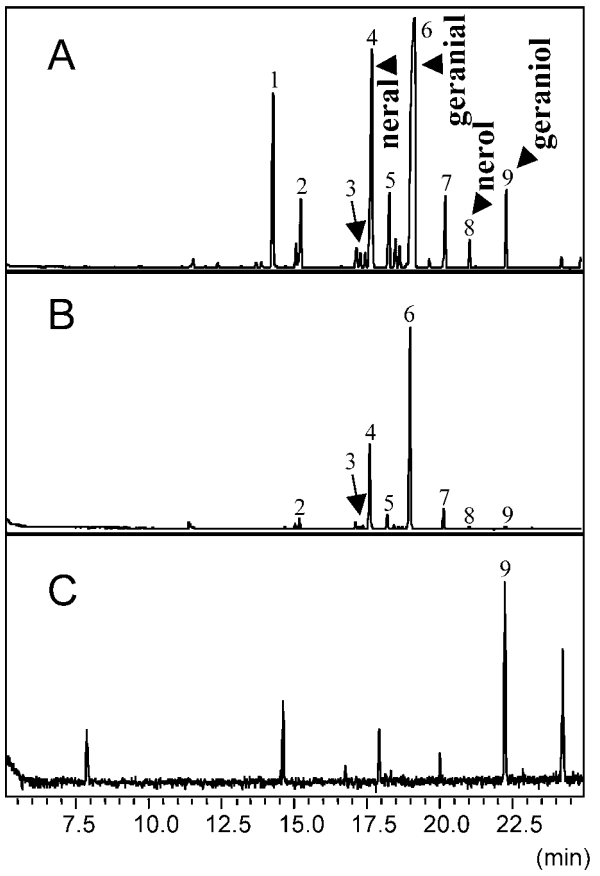

Figure 1. Identification of monoterpenes extracted from leaves and glands of sweet basil cv "Sweet Dani" and produced by a crude protein extract from the glands. A, Extraction from young leaves. B, Extraction from glands. C, Extraction of product after an enzyme assay with crude protein extract incubated with geranyl diphosphate. Samples were analyzed on gas chromatography (GC) fitted with a DB-Wax column, and compounds were identified by mass spectrometry (MS) and, with the exception of Germacrene D, by co-elution with authentic standards. Labeled peaks are: 1, internal standard included for quantification; 2, isocaryophyllene; 3, methyl chavicol; 4, neral; 5, germacrene D; 6, geranial, 7, α-farnesene; 8, nerol; and 9, geraniol. Unlabeled peaks in C are not terpenes and are found in the protein crude extract regardless of whether geranyl diphosphate is included in the assay.

GES Activity in the Glands

To identify and localize GES activity, crude protein extracts were prepared from whole leaves and from isolated glands (prepared according to Gang et al., 2001) and assayed for monoterpene synthase activity with geranyl diphosphate as the substrate. Because small young leaves contained the highest levels of geraniol and related compounds, we used such leaves as the source of the glands. Crude protein extracts from glands produced >99% geraniol upon incubation with geranyl diphosphate (Fig. 1C). In particular, no nerol product was observed. GES-specific activity in the gland extracts was more than 50 times higher than in whole-leaf extracts.

Purification and Characterization of GES from Basil Glands

GES was purified from isolated glands in a protocol employing several chromatographic steps, including DEAE-cellulose anion-exchange column, Mono-Q anion-exchange column, and Superose-12 size exclusion column on an FPLC system (Pharmacia Biotech, Piscataway, NJ; Table II). In general, the enzyme was quite stable during the purification procedure, and freezing, thawing, and dialyzing in buffer resulted in no more than 5% loss of enzyme activity. However, concentrating the enzyme by ultrafiltration centrifugation resulted in complete loss of activity. The addition of octylglucoside as deter-

Table I. *Levels of geranial, neral, geraniol, and nerol in leaves of different age in basil cv Sweet Dani*

Plant Material	Geranial	Neral	Geraniol	Nerol
		mg g^{-1} fresh leaves ± SD		
Small leaves	1.80 ± 0.31	0.92 ± 0.20	0.14 ± 0.08	0.05 ± 0.03
Medium leaves	1.17 ± 0.12	0.57 ± 0.02	0.04 ± 0.02	0.02 ± 0.01
Large leaves	0.83 ± 0.42	0.38 ± 0.13	0.01 ± 0.00	0.01 ± 0.00

gent before ultrafiltration prevented this loss. It was also determined that the presence of KCl, used in eluting the enzyme from the anion-exchange columns, had no effect on reaction rates.

After three successive chromatographic steps, the Superose-12 fraction with highest GES activity had 6,244 pkat mg^{-1} protein, representing 38.3-fold purification with 8.7% yield from crude protein in glands fraction (Table II). SDS-PAGE of this fraction showed one major protein band with an apparent subunit mass of 57.7 kD (Fig. 2). Native, active GES eluted from the size exclusion column as a 140-kD protein. The purified GES catalyzed the exclusive formation of geraniol from geranyl diphosphate, and no other monoterpene product was obtained (Fig. 3B).

Because monoterpene synthases in general are known to require a divalent metal ion such as Mn^{2+} and Mg^{2+} for their activity (Croteau, 1987; Bohlmann et al., 1998; Wise et al., 1999), we tested GES activity at several concentrations of Mn^{2+} and Mg^{2+} ($MnCl_2$, 0–10 mM; and $MgCl_2$, 0–50 mM). GES had maximal activity with Mn^{2+} in the range of 0.1 to 1.0 mM, but activity decreased as the Mn^{2+} concentration was increased further (at 5–10 mM, GES activity was <35% of maximal activity). No GES activity was observed in the absence of Mn^{2+}. On the other hand, Mg^{2+} did not have any effect on GES activity and could not substitute for Mn^{2+}.

K_m values were determined for geranyl diphosphate and Mn^{2+} at the optimum reaction condition. The K_m value of GES for geranyl diphosphate was 21 μM, and the K_m value for Mn^{2+} was 51 μM. The K_{cat} for GES with geranyl diphosphate was determined to be 0.8 s^{-1}.

GES had a pH optimum of 8.5 with more than 70% activity in the pH range of 8.0 to 9.5. However, its activity at the range of pH 6.0 to 7.5 was less than 10% of the maximal activity.

GES was stable at 4°C to 20°C for 30 min and retained 80% activity after incubation for 30 min at 37°C. However, it was completely inactivated after 30 min of incubation at 45°C.

Characterization of the Mechanism of the Reaction Catalyzed by GES

To determine if GES acts as a nonspecific phosphatase, we assayed phosphatase activity using *p*-nitrophenyl phosphate as the substrate. Although whole leaves and glands displayed phosphatase activities of 173.6 ± 18.8 protein and 102.1 ± 21.7 pkat mg^{-1} protein, respectively, purified GES did not exhibit any phosphatase activity.

Next, we assayed the purified GES with geranyl diphosphate in a buffer that contained $H_2{}^{18}O$. The product was extracted, concentrated, and analyzed by GC-MS. A shift in the biosynthetic geraniol molecular mass of +2 was observed for the molecular ion (154:156) and for several fragmentation products (139:141, M^+-CH_3; and 111:113, M^+-C_3H_7) predicted to include the oxygen moiety (Fig. 4).

Isolation of a cDNA Encoding GES and Its Expression in *E. coli*

Because the experiments described above suggested that the reaction mechanism of geraniol formation by GES is similar to the reaction mechanism of other terpene synthases, we examined a large expressed sequence tag (EST) database constructed from the peltate glands of three basil varieties, including basil cv Sweet Dani, for potential GES cDNAs. The two other cultivars, EMX1 and SW, do not produce geraniol (or citral) but they do produce other monoterpenes, such as 1,8-cineole, linalool, and fenchone (Gang et al., 2001). BLAST searches identified five different types of cDNA sequences in Sweet Dani with sequence homology to known terpene synthases, but only one type of sequence was significantly unique to Sweet Dani, encoding a protein that was highly divergent from any terpene synthase-like sequence found in the other cultivars, whereas the other four cDNAs encoded proteins that were >90% identical to proteins from the SW and EMX1 varieties (data not shown). A complete cDNA of this sequence,

Table II. *Purification of GES from gland of basil cv Sweet Dani*

Purification Step	Total Activity	Protein	Specific Activity	Purification	Yield
	pkat	*mg*	*pkat mg^{-1}*		*%*
Crude	3,874.9	23.77	163.0	1	100
DE-53	1,225.7	2.71	153.0	2.8	31.7
Mono Q	265.5	0.43	617.7	3.8	6.9
Superose 12	337.2	0.05	6,244.2	38.3	8.7

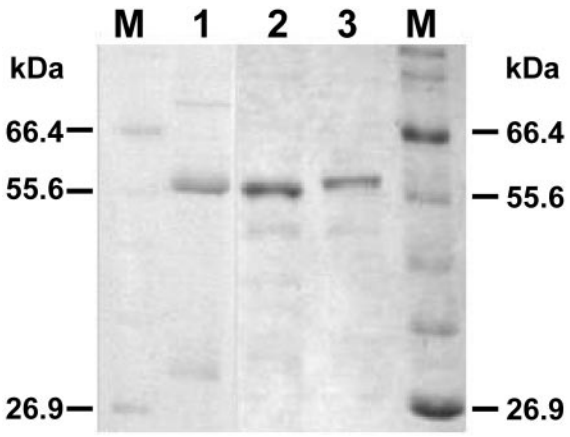

Figure 2. SDS-PAGE analysis of purified basil GES from the leaf glands and from *E. coli* expression system. Lane 1, Purified GES after Superose-12 size exclusion chromatography. Lane 2, MonoQ-purified truncated GES (starting from Met-44) produced in *E. coli*. Lane 3, MonoQ-purified truncated GES (starting from Ser-35) produced in *E. coli*. Lanes marked "M" contain molecular mass markers. Gels were stained with Coomassie Blue.

obtained by 5′-RACE followed by reverse transcriptase-PCR, contains an open reading frame of 1,701 nucleotides that encodes a protein of 567 amino acids (Fig. 5A).

ESI-MS/MS analysis of the trypsin-digested plant-purified GES, together with analysis by the ExPASy peptide mass program of the calculated tryptic peptide masses that could theoretically be generated from the 64,933-kD protein encoded by the full-length cDNA, resulted in the identification of 55 tryptic peptides, totaling 46.7% of its length (Fig. 5A). This result strongly suggests that this cDNA encodes the GES protein purified from the basil glands. Expression in *E. coli* of the full-length cDNA gave a protein that exclusively synthesized geraniol from geranyl diphosphate (Fig. 3C).

Monoterpene synthases are plastidic enzymes, and their N terminus serves as a transit peptide, which is cleaved after the protein is inserted into the organelle (Bohlmann et al., 1998). However, the exact location of the cleavage site has not been determined yet with certainty for any of these enzymes (Williams et al., 1998), although it is believed to be slightly N-terminal to an "RRX_8W" motif (doubly underlined in Fig. 5A) that is present in many, but not all, terpene synthases (Bohlmann et al., 1998; Williams et al., 1998; Dudareva et al., 2003). Examination of the amino acid composition of the N-terminal 50 to 60 residues of GES showed this part of the protein to be rich in hydrophobic, hydroxylated, and positively charged amino acids, which is typical of a transit peptide (Gavel and von Heijne, 1990). In addition, the calculated molecular mass of the GES protein encoded by the complete open reading frame of the cDNA is 64.9 kD, whereas the purified protein from plants migrates on SDS-PAGE as a 57.7-kD protein (Fig. 2). These observations suggested that the GES is initially synthesized as a precursor protein with a transit peptide.

N-terminal sequencing of the plant-purified GES was unsuccessful, indicating that the N terminus might be blocked. However, expression in *E. coli* of GES cDNA of a truncated GES cDNA with an open reading frame that starts with Met-44 (bold and underlined in Fig. 5A), with a calculated molecular mass of 60.2 kD, resulted in a protein that appeared to have nearly identical mobility to plant GES on SDS-PAGE (Fig. 2). Expression in *E. coli* of a truncated open reading frame that starts with Ser-35 (bold and underlined in Fig. 5A), with a Met codon in front of the Ser codon (calcula1ted molecular mass of 61.2 kD), resulted in a protein that migrates on an SDS-PAGE as a protein of 58.6 kD, only slightly slower than plant-purified GES (Fig. 2). Both the Ser-35 and Met-44 truncated GES proteins catalyzed the transformation of geranyl diphosphate exclusively to geraniol with K_m values of 29 and 30 μM, respectively, and K_{cat} values of 0.6 and 1.0 s^{-1}, respectively. These K_m and K_{cat} values are very close to the K_m value of 21 μM and K_{cat} value of 0.8 s^{-1} determined for the plant-purified GES enzyme, suggesting that these truncations resulted in proteins that were very similar to the mature GES enzyme present in the gland. However, the exact position of

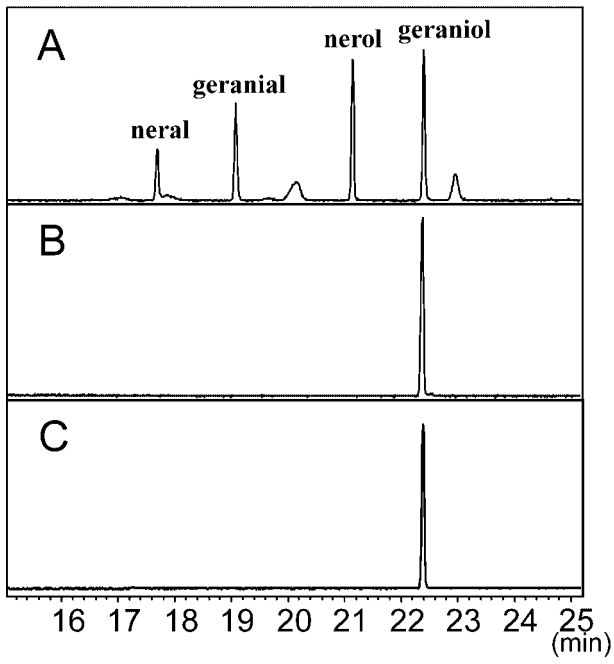

Figure 3. Analysis of the product of the reaction catalyzed purified GES with geranyl diphosphate. A, Gas chromatographic separation of authentic standards of neral, geranial, nerol, and geraniol. B, Solid-phase microextraction (SPME)-gas chromatogram of the reaction solution following catalysis by gland-purified basil GES. Only a single peak was observed, identified as geraniol by MS. C, SPME-gas chromatogram of the reaction solution following catalysis by basil GES purified from the *E. coli* expression system. Only a single peak was observed, identified as geraniol by MS.

the cleavage of the transit peptide remains to be determined, as well as the nature of the modification that blocks the free amino group at the N terminus after processing occurs.

Basil Ser-35-GES produced in *E. coli* eluted from size exclusion chromatography as a 140-kD protein, same as native plant-purified GES. Thus, it appears that active GES is a dimeric enzyme. This conclusion is based on a calculation of a subunit molecular mass of approximately 60 kD (see above).

Similarity of Basil GES to Other Terpene Synthases

BLAST searches with the complete sequence of GES indicate that compared with proteins of known function, GES shares the highest sequence similarity (i.e. percentage identity of amino acid residues) with 1,8-cineole synthase from sage (Wise and Croteau, 1999) and 4*S*-limonene synthase from spearmint (Colby et al., 1993), both monoterpene synthases from Lamiaceae species (Fig. 5A). However, the overall sequence identity of GES to either one is only approximately 30%, whereas the latter two enzymes are approximately 48% identical to each other. A phylogenetic analysis employing the nearest neighbor-joining method suggests that GES occupies a highly divergent branch of the terpene synthase family, but it most likely shares a most recent common origin with the snapdragon ocimene and myrcene synthases and with *C. breweri* linalool synthase (Dudareva et al., 1996, 2003), all terpene synthases catalyzing the formation of acyclic monoterpenes (Fig. 5B).

Analysis of the Expression of the Gene Encoding GES

RNA gel-blot analysis was performed to examine whether GES gene expression is specific to glands producing geraniol. RNA samples from leaves and glands of basil cv Sweet Dani cultivar and RNA samples from leaves and glands of basil cv EMX1, whose monoterpene fraction consists mostly of 1,8-cineole (Gang et al., 2001), were analyzed (Fig. 6). Signal was observed only with the sample containing RNA from Sweet Dani glands but not from Sweet Dani leaves nor from samples of either gland or leaf RNA of basil cv EMX1.

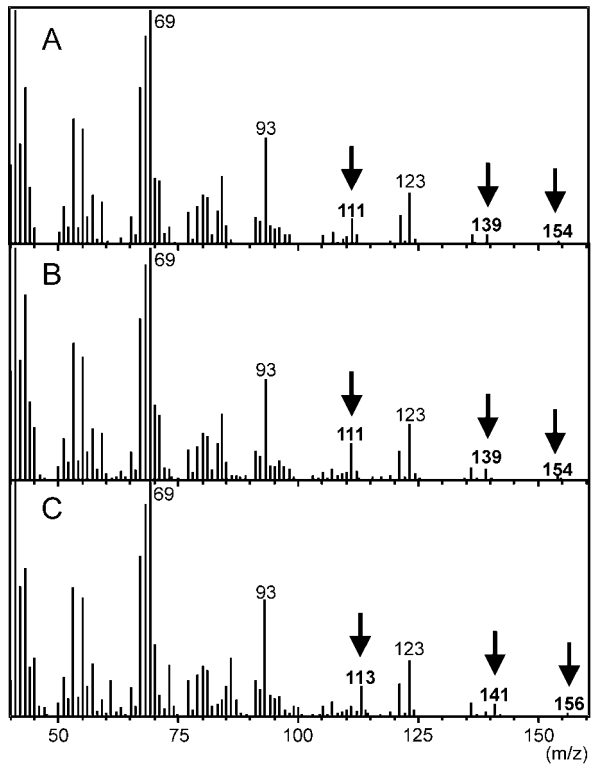

Figure 4. Comparison of the mass spectra of geraniol produced in enzymatic reactions with purified GES in buffers containing either nonisotopic water ($H_2{}^{16}O$) or ^{18}O-labeled water ($H_2{}^{18}O$). A, Mass spectra of a geraniol standard. B, Mass spectra of geraniol from a reaction including nonisotopic water ($H_2{}^{16}O$). C, Mass spectra of geraniol from a reaction containing ^{18}O-labeled water ($H_2{}^{18}O$). Arrows indicate the mass of fragments containing oxygen: 154/156 (M^+), 139/141 (M^+-CH_3), and 111/113 (M^+-C_3H_7).

DISCUSSION

GES from Basil Is a Highly Specific Terpene Synthase

In several Lamiaceae plants (e.g. spearmint), monoterpenoid accumulation and biosynthesis occur in the eight-celled peltate glandular trichomes localized on the both sides of the leaf and attached to the leaf via a stalk cell (Hallahan, 2000). We have shown recently that sweet basil plants, also in the Lamiaceae family, synthesize phenylpropanoids and terpenes in peltate glands and store these compounds in the sac formed by the cuticle that covers peltate glands (Gang et al., 2001).

Here, we show that in Sweet Dani, geraniol, nerol, and citral are stored in the sacs (Fig. 1B) and that GES activity is localized to the peltate gland cells. Further, we show that only geraniol was generated in the enzymatic reaction containing purified GES and geranyl diphosphate (Fig. 1C). Thus, although some terpene synthases have been shown to catalyze the formation of multiple products (Colby et al., 1993; Wise et al., 1999; Lücker et al., 2002), GES is highly specific and produces only geraniol. It is therefore similar to linalool synthase, an enzyme that has now been isolated from several species (although of polyphyletic origin), which also makes a single acyclic monoterpene alcohol, linalool (Pichersky et al., 1995; Crowell et al., 2002; Chen et al., 2003).

We identified the gene encoding GES by the catalytic properties of the protein it encodes, which, when the protein is produced in *E. coli*, are virtually identical to those of plant-purified GES, as well as by the identity of the peptide sequences determined by ESI-MS/MS. The gene encoding GES is specifically expressed in glands of basil cv Sweet Dani but

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                    20                  40                  60                  80                 100
ger syn :           MSCARITVTLPYRSAKTSIQRGITHYPALIRPFSM-CT-----PLASAMPLSSTPLINGDNSQRKNTRQHMEESSSKRREYLEETTR :  83
4S-lim  : MALKVLSVATQMAIPSNLITCLQPGHFKSPKLLSSTNSSSRSRLRVYCS---SSQLTTERFSGNYNFSRWDVNFIQSLLSDYKEDKHVIRASELVTLVK :  97
1,8-cin :     MSSLIMQVVIPKPAKIFHNNLFSVISKRHRFSTTITTRGGRWAHCSLQMGNEIQIGRRTGGYQPTLWDFSTIQLFDSEYKEEKHLMRAAGMIAQVN :  96

                   120                 140                 160                 180                 200
ger syn : KLQRNDTESVEKLKLIDNIQQLGIGYYFEDAINAVLRSPFSTGE----------EDLFTAALRFRLLRHNGIEISPFIFLKFKDERG-KFDE---SDTLG : 169
4S-lim  : MELEKETDQIRQLELIDDLQRMGLSDHFQNEFKEILSSIYLDHHYYKNPFPKEERDLYSTSLAFRLLREHGFQVAQEVFDSFKNEEG-EFKESLSDDTRG : 196
1,8-cin : MLLQEEVDSIQRLELIDDIRRLGISCHFDREIVEILNSKYYTNN------EIDESDLYSTALRFKLLEQYDFSVSQEVFDCFKNDKGTDFKPSLVDDTRG : 190

                   220                 240                 260                 280                 300
ger syn : LLSLYEASNLGVAGEEILEEAMEFAEARIRRSLSEP--AAPLHGEVAQALDVPRHLRMARLEARRFIEQYGKQSDHDGDLLELAILDYNQVQAQHQSELT : 267
4S-lim  : LLQLYEASFLLTEGETTLESARSFATKFLEEKVNEGGVDGDLLTRIAYSLDIPLHWRIKRPNAPVWIEWYRKRPDMNPVVLELAILDLNIVQAQFQEELK : 296
1,8-cin : LLQLYEASFLSAQGEETLHLARDFATKFLHKRVLVD-KDINLLSSIERALELPTHWRVQMPNARSFIDAYKRRPDMNPTVLELAKLDFNMVQAQFQQELK : 289

                   320                 340                 360                 380                 400
ger syn : EIIRWWKELGLVDKLSFGRDRPLECFLWTVGLLPEPKYSSVRIELAKAISILLVIDDIFDTYGEMDDLILFTDAIRRWDLEAMEGLPEYMKICYMALYNT : 367
4S-lim  : ESFRWWRNTGFVEKLPFARDRLVECYFWNTGIIEPRQHASARIMMGKVNALITVIDDIYDVYGTLEELEQFTDLIRRWDINSIDQLPDYMQLCFLALNNF : 396
1,8-cin : EASRWWNSTGLVHELPFVRDRIVECYYWTTGVVERREHGYERIMLTKINALVTTIDDVFDIYGTLEELQLFTTAIQRWDIESMKQLPFYMQICYLALFNF : 389

                   420                 440                 460                 480                 500
ger syn : TNEVCYKVLRDTGRIVLLNLKSTWIDMIEGFMEEAKWFNGGSAFKLEEYIENGVSTAGAYMAFAHIFFLIGEGVTHQNSQLFTQKPYPKVFSAAGRILRL : 467
4S-lim  : VDDTSYDVMKEKGVNVIPYLRQSWVDLADKYMVEARWFYGGHKPSLEEYLENSWQSISGPCMLTHIFFRVTDSFTKET--VDSLYKYHDLVRWSSFVLRL : 494
1,8-cin : VNEMAYDTLRDKGFNSTPYLRKAWVDLVESYLIEAKWYYMGHKPSLEEYMKNSWISIGGIPILSHLFFRLTDSIEED--AESMHKYHDIVRASCTILRL : 487

                   520                 540                 560                 580                 600
ger syn : WDDLGTAKEEQERGDLASCVQLFMKEKSLTEEEARSRILEEIKGLWRDLNGELVYN-KNLPLSIIKVALNMARASQVVYKHDQDTY---FSSVDNYVDAL : 563
4S-lim  : ADDLGTSVEEVSRGDVPKSIQCYMSDYNASEAEARKHVKWLLAEVWKKMNAERVSKDSPFGKDFIGCAVDIGRMAQLMYHN-GDGHGTQHPIIHQQMTRT : 593
1,8-cin : ADDMGTSLDEVERGDVPKSVQCYMNEKNASEEEARERHVRSLIDQTWKMMNKEMMT--SSFSKYFVQVSANLARMAQWIYQHESDGFGMQHSLVNKMLRGL : 585

ger syn : FFTQ   : 567
4S-lim  : LFEPFA : 599
1,8-cin : LFDRYE : 591
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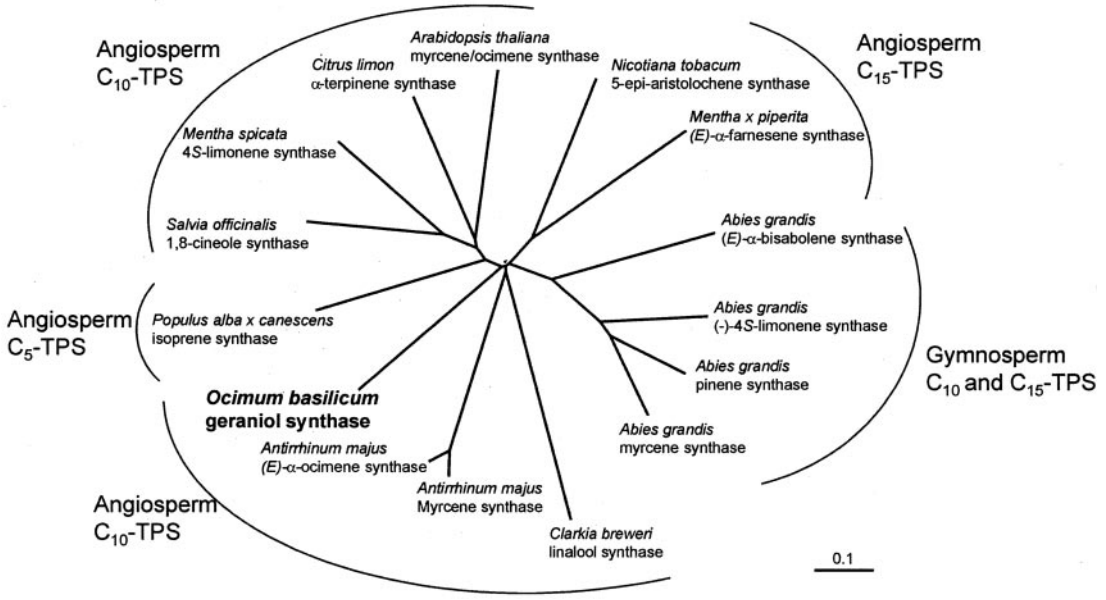

Figure 5. Relatedness of GES to other terpene synthases. A, Amino acid sequence of basil GES, deduced from a full-length cDNA, and compared with the two most similar terpene synthases for which a function is known, 1,8-cineole synthase from sage (*Salvia officinalis*; Wise et al., 1998) and 4*S*-limonene synthase from spearmint (*Mentha spicata*; Colby et al., 1993). Residues identical in at least two of the proteins at a given position are shaded. The tryptic peptides of GES identified by ESI-tandem mass spectrometry (MS/MS) are indicated by lines above the sequence. The RRX_8W motif is double underlined. Ser-35 and Met-44 in GES are underlined and in bold. B, Phylogenetic tree constructed using the nearest neighbor-joining method. The GenBank accession numbers for the sequences mentioned in this figure are as follows: basil GES, AY362553; *Abies grandis* myrcene synthase, U87908; *A. grandis* pinene synthase, U87909; *A. grandis* (−)-4*S*-limonene synthase, AF006193; *A. grandis* (*E*)-α-bisabolene synthase, Af006195; peppermint (*Mentha piperita*) (*E*)-β-farnesene synthase, AF024615; *N. tobacum* 5-epi-aristolochene synthase, L04680; Arabidopsis myrcene/ocimene synthase, At2g24210; *C. limon* γ-terpenene synthase, AF514286; spearmint 4*S*-limonene synthase, L13459; sage 1,8-cineole synthase, AF051899; *Populus alba* × *Populus tremula* isoprene synthase, AJ294819; *Clarkia breweri* linalool synthase, U56314; snapdragon (*Antirrhinum majus*) myrcene synthase, AY195608; and snapdragon ocimene synthase, AY195607.

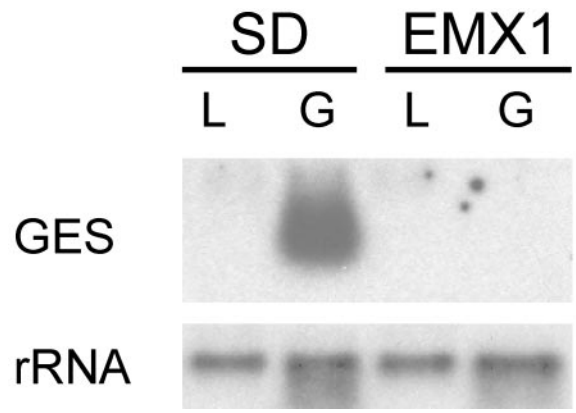

Figure 6. Analysis of the expression of the gene encoding GES. Each lane was loaded with 2 μg of total RNA. After probing with the *GES* cDNA probe, the blot was stripped and reprobed with an 18S rDNA probe to demonstrate equal loading. L, Leaf; G, gland; SD, basil cv Sweet Dani; EMX1, basil cv EMX1.

not in the glands of other basil cultivars that produce other monoterpenes but not geraniol (Fig. 6; data not shown). Furthermore, the gel-blot analysis and comparisons of EST databases of several basil varieties demonstrated that other basil varieties do not express any functional terpene synthase genes whose sequences are >90% identical to GES, nor do Sweet Dani glands express any other functional genes with high similarity to isolated GES cDNA, further demonstrating that this GES cDNA is responsible for encoding GES activity in Sweet Dani glands.

Although the protein sequence of GES clearly indicates it is a member of the terpene synthase family (although a highly divergent one, see Fig. 5B), the molecular mass of active GES was estimated by size exclusion chromatography to be 140 kD, suggesting that it is active as a dimer. Most terpene synthases that have been examined have been shown to be monomeric (Bohlmann et al., 1998). However, bornyl diphosphate synthase (Wise et al., 1998) and possibly pinene synthase (Gambliel and Croteau, 1984), both from sage, have been reported to be dimeric enzymes.

Enzyme Mechanism of GES

The overall structural relatedness of basil GES to other terpene synthases and its requirement of Mn^{2+} for activity suggest that GES catalytical mechanism is similar to that of other terpene synthases. Furthermore, basil GES did not act on *p*-nitrophenol phosphate that can be hydrolyzed by nonspecific phosphatases, suggesting that its mode of action does not involve the hydrolysis of a phosphoester bond. In assays in which $H_2{}^{18}O$ was used for the buffer, ^{18}O was incorporated into the geraniol product in the same proportion as the ratio of $H_2{}^{18}O$ to $H_2{}^{16}O$ in the solution (Fig. 4), directly demonstrating the validity of the model (Croteau, 1987; Gershenzon and Croteau, 1993) positing that terpene biosynthesis involves the removal of the pyrophosphate group and the creation of a carbocation as a reaction intermediate (Fig. 7). Although Croteau et al. (1994) showed, by using ^{18}O-labeled water, that the oxygen in the 1,8-cineole structure was incorporated from water during the enzymatic reaction, the formation of 1,8-cineole from geranyl diphosphate involves a complicated set of steps, and the oxygen present in the final molecule is not bound to the same carbon to which the pyrophosphate group was initially bound. Thus, the enzymatic formation of geraniol, where the oxygen is bound to the same carbon to which the pyrophosphate group was initially bound, allowed us to test the formation of a carbocation more directly, showing that a carbocation is an intermediate. The results also indicate that the reaction mechanism of GES is similar, at least in the initial steps, to that of other monoterpene synthases.

This conclusion is also strengthened by the observation that the protein sequence of basil GES clearly shows it to be a member of the terpene synthase family (Fig. 5), although its overall sequence similarity to other terpene synthases is quite low. Of particular interest may be the relatively high divergence at the N-terminal 100 amino acids, a region that encompasses both the transit peptide and additional sequences from the N terminus of the mature protein. The RRX_8W motif, which is often, but not always, found in the N terminus of mature monoterpene synthases (Dudareva et al., 1996; Williams et al., 1998; Chen et al., 2003; Dudareva et al., 2003), is not found in GES, consistent with the hypothesis that is involved in the synthesis of cyclic terpenes (Williams et al., 1998).

GES Does Not Catalyze the Formation of Nerol

Our results indicate that GES does not synthesize nerol, the cis-isomer of geraniol. Furthermore, we were not able to detect any nerol synthase activity in the glands (or leaves). Nerol, however, is a component of the essential oil of Sweet Dani. An explanation for the presence of nerol in the absence of a

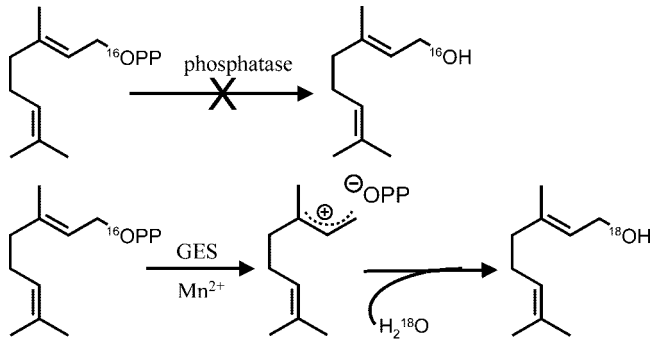

Figure 7. Reaction mechanism of GES. Geraniol is not generated by phosphatase activity from geranyl diphosphate but is formed by the addition of a hydroxyl group to a carbocation intermediate.

specific nerol synthase may lie in the mechanism of generating citral from geraniol. Citral is a mixture of geranial and neral in an approximately 3:2 ratio, and geranial and neral are in fact often found in together in such a ratio in many lemon-scented plants (Singh-Sangwan et al., 1993). This observation is most likely due to the nonenzymatic conversion of geranial to neral via keto-enol tautomerization and additional double-bond migration, with subsequent rotation around the C2-C3 bond (Akhila, 1985). Neral could then be converted to nerol by specific or nonspecific dehydrogeneases, thus explaining the presence of nerol in Sweet Dani glands. Our observations that the geranial to neral ratio is very similar to the geraniol to nerol ratio in Sweet Dani essential oil (Table I) is consistent with this explanation for nerol formation.

MATERIALS AND METHODS

Plant Material

Seeds for basil (*Ocimum basilicum*) cv Sweet Dani were obtained from a local nursery. They were sown in horticultural vermiculite and put in the growth chamber until germination. One week after germination, each seedling was transplanted into a 500-mL pot containing Sunshine Mix No. 1 potting soil (Sun Gro Horticulture Canada Ltd., Seba Beach, AB) and grown in a greenhouse under constant illumination.

Volatile Oil Extraction from Leaves

Basil leaves were categorized into three sizes: small (0.5–1.5 cm), medium (1.5–3 cm), and large (3–4 cm; Gang et al., 2001). Two hundred milligrams of each leaf was added to liquid N_2 and ground by mortar and pestle. The powder was soaked in 2 mL of methyl t-butyl ether containing 0.1 mg of linalool as an internal standard (linalool was used because basil cv Sweet Dani does not contain linalool) and extracted for 2 h at room temperature in 5-mL glass vials with tightly sealed rubber septa caps. The methyl t-butyl ether upper layer, which included the volatile oil, was removed and placed into another vial and concentrated to 200 μL under gentle N_2 gas flow for GC-MS analysis. Data points were obtained in triplicate.

Volatile Extraction from Glands

Volatile oils were extracted from the glands of young leaves with a stretched glass pipette as previously described (Gang et al., 2001).

GC-MS Analysis of Plant Volatiles

A Shimadzu QP-5000 system (Shimadzu, Columbia, MD) equipped with Shimadzu GC-17 gas chromatograph was used for GC-MS analysis of volatile compounds. Separation was performed on DB-WAX (30-m $\times$ 0.32-mm-i.d. $\times$ 0.25-μm film thickness, J&W Scientific, Folsom, CA) capillary column with electron impact mode (1.4 kV). However, some nonpolar compounds eluted with the solvent peak in the DB-WAX column, and their separation was achieved on a CP-5 column (30-m $\times$ 0.32-mm-i.d. $\times$ 1-μm film thickness, Alltech Associates Inc., Deerfield, IL). The oven temperature for DB-WAX methods was held at 60°C for 2 min and raised to 220°C at 4°C min^{-1} with the injector set at 220°C and the interface set at 240°C. The GC condition for the CP-5 method was the same as the previous report (Gang et al., 2001). Ultrapure helium was used as the carrier gas at a rate of 1.3 mL min^{-1}. Samples (2 μL) were injected by the Shimadzu AOC-17 Autoinjector. Eluted compounds were identified by comparing their retention time and mass fragmentation patterns with standard compounds.

GES Enzyme Assays

GES activity was assayed by incubating 5 μL of the enzyme sample in a final volume of 50 μL of buffer containing 50 mM HEPES-KOH (pH 8.0), 1 mM dithioerythritol, 0.5 mM $MnCl_2$, 20 mM $MgCl_2$, 10% (w/v) glycerol, and 0.025 μM [1-^{3}H]-geranyl diphosphate (specific activity 20 Ci mol^{-1}, American Radiolabeled Chemicals, St. Louis). After incubation for 30 min at 32°C, 160 μL of hexane was added to the tube, vortexed briefly, and centrifuged to separated the phases. The hexane layer was directly placed into a scintillation vial containing 2 mL of nonaqueous scintillation fluid (Econo-Safe, Research Products International, Mount Prospect, IL). This extraction procedure was repeated twice, and the total hexane phase was counted by a liquid scintillation counter (LS-6500 model, Beckman Coulter, Fullerton, CA). Boiled enzyme extracts were used as controls.

Identification of Enzymatic Products

GES enzyme assays were also performed by adding 100 μL of enzyme solution with 900 μL of assay solution containing 54 μM nonradioactive geranyl diphosphate (Echelon Research Laboratories, Salt Lake City) and the same buffer described above. The reactions were carried out in an 8-mL DuPont autosampler vial (DuPont-Dow Elastomers L.L.C., Wilmington, DE) with a white solid-top polypropylene cap (Alltech). After letting the reaction proceed for 2 to 4 h at 32°C, the liberated compounds were collected with an SPME device PDMS-100 with a polydimethylsiloxane fiber (Supelco, Bellefonte, PA) by inserting the fiber into the tube and leaving it in for 20 min at 42°C. After this incubation step, the SPME fiber was directly injected into the GC-MS.

Terpene Synthase Assays with ^{18}O-Labeled Water

Assays in buffer containing ^{18}O-labeled water were carried out in 2-mL glass vials with screw cap of PTFE/Silicone Septa (Supelco) by the addition 20 μL of purified enzyme (approximately 1 μg of protein) to a 180-μL assay solution that contained 20 μL of 10$\times$ assay buffer with 150 μL of $H_2{}^{18}O$ (95% atom, Icon Service, Summit, NJ) and 10 μL of 5.4 mM geranyl diphosphate. The final concentration of $H_2{}^{18}O$ in this assay solution was 71.3% (w/v). This solution was incubated for 2 h at 32°C, cooled down on ice, and then extracted with 200 μL of pentane. After concentration to 50 μL, 2 μL of this solution was injected to GC-MS system. To compare the mass spectra pattern, a pentane extract of the product from a reaction in which normal water ($H_2{}^{16}O$) was used was also analyzed.

Phosphatase Activity Assay

Phosphatase activity was measured as described by Hernández and Whitton (1996) with the following modification: Assay samples were prepared by incubating 50 μL of enzyme solution in a final volume of 400 μL of assay buffer containing 2 mM *p*-nitrophenyl phosphate as substrate. The buffer composition was the same as with the GES assay (but without geranyl diphosphate). After incubation for 1 h at room temperature, the reaction was stopped by adding of 700 μL of 0.2 M Na_2CO_3. The yellow color generated from the hydrolysis of *p*-nitrophenyl phosphate was measured at 420 nm in a spectrophotometer (Beckman DU530). Phosphatase activity was calculated using a standard curve for *p*-nitrophenol. For the purified enzyme, this assay was scaled down to 10-fold.

GES Purification

All purification steps were carried out at 4°C unless stated otherwise. Glands were isolated from approximately 300 g of basil cv Sweet Dani, essentially following the procedures previously described by Gang et al. (2001) with a total yield of 4 mL of resuspended glands. The gland preparation was diluted 10:1 (v/v) in ice-cold enzyme extraction buffer (100 mM BisTris-HCl [pH 7.5], 5 mM dithioerythritol, 5 mM $Na_2S_2O_4$, 2% [w/v] polyvinylpolypyrrolidone, and 10% [w/v] glycerol), and sonicated on ice, with rest intervals for cooling down, until gland cells were completely lysed. After centrifugation for 20 min at 10,000*g*, the supernatant (39 mL) was loaded onto a DEAE-cellulose column (10 mL of DE53, Whatman, Fairfield, NJ) installed in a Pharmacia Biotech FPLC apparatus and pre-equilibrated

with a solution containing 50 mM Tris-HCl (pH 7.5), 10% (w/v) glycerol, and 10 mM β-mercaptoethanol (buffer A). After elution of unbound material from the column with 25 mL of buffer A, GES activity was eluted with 200 mL of a linear gradient from 0 to 1 M KCl in buffer A. The flow rate was 1.0 mL min^{-1}, and 3-mL fractions were collected and then assayed for GES activity. The fractions with the highest GES activity were pooled (KCl concentration of 255–435 mM, a total of 38 mL) and dialyzed in buffer A for 4 h to remove KCl. This dialysis step did not result in any decrease in GES activity. The enzyme solution was subsequently loaded onto strong anion-exchange column (Mono Q, 0.5 × 6.0 cm, Pharmacia Biotech) pre-equilibrated with buffer A. After washing off the unbound material with 2 mL of buffer A, GES was eluted with 50 mL of a linear gradient of 0 to 700 mM KCl in buffer A at 0.5 mL min^{-1}, and 1-mL fractions were collected. The highest GES activity was detected in the 2-mL fraction containing 294 mM KCl. Octyl glucoside was added to this fraction (final concentration of 5 mM), and the enzyme was concentrated in an Ultrafree-4 centrifugal device (Millipore, Bedford, MA) to a total volume of 200 μL. The concentrated enzyme solution was loaded onto a size exclusion column (10 × 300 mm) packed with Superose 12 (Pharmacia Biotech), and active fraction was isocratically eluted with 100 mM KCl in buffer A at 0.2 mL min^{-1}. Fractions (0.5 mL each) were collected, and protein purity was examined by SDS-PAGE gel electrophoresis followed by Coomassie Brilliant Blue or silver staining of the gel. The protein concentrations were measured by the Bradford method or by staining intensity on SDS-PAGE compared with bovine serum albumin concentration standards.

Molecular Mass Estimation

Partially purified GES was run on a size exclusion column under the same conditions used during the purification procedure, except that 0.25-mL fractionations were collected instead, and fractions were assayed for GES activity. A standard curve was obtained by plotting the elution volume/void volume of the standard proteins against the log of the molecular mass. The protein standards used included cytochrome C (12.4 kD), carbonic anhydrase (29 kD), ovalbumin (45 kD), bovine serum albumin (66 kD), alcohol dehydrogenase (from equine liver, 80 kD), and alcohol dehydrogenase (from yeast [*Saccharomyces cerevisiae*], 141 kD). The subunit molecular mass was estimated by SDS-PAGE performed on 10% (w/v) polyacrylamide gel and calibrated with molecular mass standard in the range of 14 to 212 kD (New England Biolabs, Beverly, MA).

Characterization of GES Properties

The pH optimum for GES activity was determined using three buffer systems. Reaction was carried out in 50 mM Bis-Tris buffer ranging from pH 6.0 to 7.0, 50 mM Tris-HCl buffer ranging pH 7.0 to 9.0, and 50 mM Gly-NaOH buffer ranging from pH 9.0 to 10.0.

Temperature stability of GES was determined by incubating GES in temperatures ranging from 4°C to 65°C for 30 min and then chilling the samples on ice, followed by enzyme assays at 32°C.

To determine the kinetic parameters of GES, the enzyme was diluted to the appropriate concentration, and incubation time was set for 30 min at 32°C. In determining the K_m value for geranyl diphosphate, Mn^{2+} concentration was set at a saturated level, and geranyl diphosphate concentration was changed from 0.5 to 108 μM with 10 different data points. The K_m value for Mn^{2+} was measured at saturated geranyl diphosphate levels, and Mn^{2+} concentration was changed from 4 to 1,000 μM with nine data points. Lineweaver-Burk plots were made to obtain the K_m value.

ESI-MS/MS Analysis of Purified GES

Mass spectrometric analysis of the purified GES was carried out in the Proteomics Core Facility (Southwest Environmental Health Sciences Center and Arizona Cancer Center, University of Arizona, Tucson). Proteins were first separated by SDS-PAGE, as described above, stained lightly with Coomassie Brilliant Blue R250, excised from the gel, and digested with trypsin (Shevchenko et al., 1996). Extracted peptides were analyzed by liquid chromatography (LC)-MS/MS using a ThermoFinnigan LCQ Classic quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a Michrom MAGIC2002 HPLC (Michrom, Auburn, CA) and a nanospray ion source (University of Washington, Seattle). Peptides were loaded onto 10-cm capillaries (100-μm i.d., packed with 5–6 cm of Vydac C18 material) that were pulled to 3- to 5-μm tips using a Sutter Instruments P2000 capillary puller (Sutter Instruments, Novato, CA). Peptides were eluted at a flow rate of 200 to 300 nL min^{-1} into the mass spectrometer using reversed phase solvent conditions (Shevchenko et al., 1996). Tandem MS spectra of peptides were analyzed with the TurboSequest program to assign peptide sequences to the spectra (Eng et al., 1994). TurboSequest analyses were performed against the sweet basil EST databases housed at the Arizona Genomics Institute (University of Arizona, Tucson). The nonidentified spectra were further analyzed by the ExPASy peptide mass program (http://us.expasy.org/tools/peptide-mass.html) for the calculated tryptic peptide masses from full length GES cDNA.

Isolation of GES cDNAs and Expression in *Escherichia coli*

A basil cv Sweet Dani peltate gland EST database containing 3,200 unique sequences was developed at the Arizona Genomics Institute and the Arizona Genomics Computational Laboratory (University of Arizona, Tucson), using a cDNA library constructed from gland mRNAs as previously described (Gang et al., 2001). BLAST searches revealed numerous ESTs with sequence similarity to terpene synthases. Potential cDNAs encoding GES were examined by reverse transcriptase-PCR cloning of full-length cDNAs (complete sequence were obtained by 5′-RACE when necessary) into the pCRT7/CT-TOPO TA vector (Invitrogen, Carlsbad, CA), expressing these constructs in the *E. coli* expression system, and testing the resulting proteins for activity with geranyl diphosphate, as previously described (Chen et al., 2003). Constructs encoding truncated GES proteins missing the first 34 or 43 amino acids were also constructed, using the method described by Chen et al. (2003) in either the pCRT7/CT-TOPO TA vector or a pET-11a vector. After harvesting recombinant GES proteins from *E. coli* cells (Chen et al., 2003), the proteins were purified using the same method employed in the purification of GES from basil glands.

Sequence Analysis

Alignment of multiple protein sequences was performed using the ClustalX program (Thompson et al., 1997). Sequence relatedness by the neighbor-joining method was determined using the protocol included in the ClustalX package. The phylogenic tree was drawn using the TREEVIEW program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html; Page, 1996).

Analysis of the Expression of the Gene Encoding GES

RNA isolated from glands and leaves and RNA gel-blot analysis were done as previously described (Gang et al., 2001). A fragment consisting of the last one-third of the gene was amplified by PCR with the appropriate oligonucleotides and used as a probe.

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