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Phytochemistry 66 (2005) 285–293
PHYTOCHEMISTRY
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A geraniol-synthase gene from *Cinnamomum tenuipilum*

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Received 10 August 2004; received in revised form 27 October 2004

Abstract

Geraniol may accumulate up to 86–98% of the leaf essential oils in geraniol chemotypes of the evergreen camphor tree *Cinnamomum tenuipilum*. A similarity-based cloning strategy yielded a cDNA clone that appeared to encode a terpene synthase and which could be phylogenetically grouped within the angiosperm monoterpene synthase/subfamily. After its expression in *Escherichia coli* and enzyme assay with prenyl diphosphates as substrates, the enzyme encoded by the putative *C. tenuipilum* monoterpene synthase gene was shown to specifically convert geranyl diphosphate to geraniol as a single product by GC–MS analysis. Biochemical characterization of the partially purified recombinant protein revealed a strong dependency for Mg^{2+} and Mn^{2+}, and an apparent Michaelis constant of 55.8 μM for geranyl diphosphate. Thus, a new member of the monoterpene synthase family was identified and designated as CtGES. The genome contains a single copy of CtGES gene. Expression of *CtGES* was exclusively observed in the geraniol chemotype of *C. tenuipilum*. Furthermore, in situ hybridization analysis demonstrated that *CtGES* mRNA was localized in the oil cells of the leaves.

Key word index: Cinnamomum tenuipilum; Lauraceae; Camphor tree; Functional expression; Chemotypes; Essential oil; *CtGES* gene; Geraniol synthase; Monoterpene synthases

1. Introduction

Cinnamomum tenuipilum Kosterm (Lauraceae) is an evergreen camphor tree native to southwestern China, and is subdivided into 12 chemotypes according to the major components of their leaf essential oils. For instance, geraniol may constitute up to 86–98% of leaf essential oils in geraniol chemotypes, linalool up to 84–98% in linalool chemotypes, and farnesol chemotypes may contain up to 54–71% of farnesol in their leaf essential oils (Cheng et al., 1993). Chemotypes of other plant species with such a high percentage of geraniol and farnesol are very uncommon in nature. Moreover, those wild plants are becoming increasingly rare, thus the remaining gene pool urgently needs to be investigated. The presence of genes coding for the enzymes responsible for the production of geraniol, linalool or farnesol, and their phenotypical expression was to be expected.

Monoterpenes are principal compounds in plant-derived essential oils, of which a few members are of acyclic types (e.g. myrcene). Like all regular monoterpenes, acyclic monoterpenes are also produced from geranyl diphosphate (GDP) by related synthases such as linalool synthase and myrcene synthase based on a common ionization-dependent reaction mechanism (Bohlmann et al., 1998). However, the fact that the carbon skeleton of geraniol is identical to that of its precursor GDP could have been interpreted such that a phosphatase-based catalysis is responsible for generating geraniol.

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0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.12.004

While this manuscript was under preparation, a geraniol synthase found in sweet basil was first reported to synthesize geraniol from GDP by a reaction mechanism similar to that of other terpene synthases (Iijima et al., 2004). Here we report the isolation of a cDNA clone from *C. tenuipilum*, which was functionally expressed in *Escherichia coli* and thereby identified as a geraniol synthase.

2. Results and discussion

2.1. Essential oil analysis

The subdivision of three chemotypes of *C. tenuipilum* used in this study was confirmed by essential oil analyses (Table 1). Constituents of their leaf essential oils were comparable with those described before (Cheng et al., 1993). Geraniol was not detected in the leaf essential oils of both linalool and farnesol chemotypes.

2.2. cDNA isolation and sequence analysis

Because the geraniol chemotype of *C. tenuipilum* was shown to contain more than 86% geraniol in its leaf essential oil, this species appeared to be an ideal source for the isolation of the related monoterpene synthase. A homology based PCR cloning strategy, which has previously been proven to be successful for the isolation of terpene synthase genes (Bohlmann et al., 1998; Fischbach et al., 2001), was used in this study. With primers 1F and 1R (Jia et al., 1999), the cDNA from the geraniol chemotype produced a specific fragment, which showed significant sequence similarity to plant terpene synthase genes. Based on the sequence of this fragment, new primers were designed to direct upstream of the 5′ end and downstream of the 3′ end of the coding cDNA. This approach yielded a complete 5′ segment of 724-bp and 3′ segment of 1436-bp. Subsequently, a new primer pair was designed specifically for the 5′ end and 3′ end of the cDNA that allowed amplification of the complete gene from the original full-length cDNA. Sequencing of both strands of the cloned full-length cDNA (pCt5) resulted in a 2005-bp segment (GenBank Accession No. AJ457070) with an open reading frame of 1812 nucleotides encoding 603 amino acids for a protein of 69-kDa and calculated p*I* of 5.67.

Alignment of the deduced amino acid sequences of the *C. tenuipilum* cDNA with their closely related monoterpene synthases (Fig. 1), showed that the *C. tenuipilum* cDNA appeared to encode a polypeptide with a typical N-terminal plastidial targeting sequence, which is anticipated for a monoterpene synthase, and a tandem pair of arginine residues ($Arg^{51}Arg^{52}$) which is highly conserved in most of the monoterpene synthases (Bohlmann et al., 1998; Dudareva et al., 2003). The expected DDXXD motif is located at residues 338–342. This aspartate-rich element is now generally recognized as a binding site for the metal ion-chelated diphosphate ester substrate. In a phylogenetic tree calculated for plant terpenoid synthases (Fig. 2), the deduced peptide sequence of *C. tenuipilum* grouped together with angiosperm monoterpene synthases and belonged to the *Tpsb* subfamily according to Bohlmann et al. (1998) and Martin et al. (2004). The relatedness strongly suggests that the *C. tenuipilum* cDNA encodes a monoterpene synthase rather than a sesquiterpene synthase. However, functional expression was required to confirm its catalytic activity.

2.3. cDNA expression and product analysis

Monoterpene synthase is a plastidial enzyme; its N-terminal plastid targeting sequence is cleaved after the protein is inserted into the organelle (Bohlmann et al., 1998). The location of the cleavage site is believed to be slightly N-terminal to an RRX_8W motif (Fig. 1) that

Table 1
Chemical compositions of leaf essential oils of *C. tenuipilum*

Compound[a]	% Peak area of the essential oils		
	Fc	Gc	Lc
Linalool	0.39	<0.06	**89.11**
Nerol	0.62	Not detected	0.22
Geraniol	Not detected	**87.51**	Not detected
α-Copaene	8.87	0.94	1.32
β-Elemene	5.22	0.71	0.73
β-Caryophyllene	3.82	3.03	0.80
β-Cubebene	9.21	0.82	Not detected
Farnesene	1.49	Not detected	0.50
δ-Cadinene	0.85	0.54	0.92
Farnesol	**57.73**	<0.06	1.94
Farnesal	0.52	Not detected	Not detected

[a] Not all compounds are listed in the table. Relative proportions are shown in average values from three replicates. Fc, Gc and Lc refer to farnesol chemotype, geraniol chemotype and linalool chemotype, respectively.

Fig. 1. Sequence alignment of the deduced amino-acid sequences of monoterpene synthases catalyzing acyclic, monocyclic and bicyclic terpene formation. The alignment of CtGES (the geraniol synthase from *C. tenuipilum*, AJ457070), ObGES (the geraniol synthase from *O. basilicum*), MsLIS (the 4S-limonene synthase from *Mentha spicata*, L13459), SoSAS [the (+)-sabinene synthase from *Salvia officinalis*, AF051901], QiMYS (the myrcene synthase from *Quercus ilex*, AJ304839) and MaLIS (the linalool synthase from *Mentha aquatica*, AY083653) was created with Clustal W (MegAlign 5.01, DNAStar, Inc., Madison, WI). RRX_8W motif located at the N-terminus is underlined. The highly conserved tandem arginine pair, DDXXD element and amino acids are in the bright yellow shade. The residues preceding the RR (yellow shade) encode a typical N-terminal plastidial targeting sequence. Elimination of the sequence generates a truncated form which is usually applied for functional expression. The geraniol synthase from *O. basilicum* lacks RRX_8W motif. Missing the first 34 or 43 amino acids results in the Ser-35 (green shade) or Met-44 (green shade) truncated basil GES proteins.

is present in many, but not all, terpene synthases (Bohlmann et al., 1998; Williams et al., 1998; Dudareva et al., 2003). The truncation of monoterpene synthases (in which the N-terminal transit peptide immediately preceding the arginine pair was eliminated) resulted in 'pseudo-mature' fully active enzymes (Williams et al., 1998). Therefore, truncated versions of the cDNAs are frequently applied for functional expression of monoterpene synthases (Jia et al., 1999; Fischbach et al., 2001; Crowell et al., 2002). As the deduced peptide sequence of the *C. tenuipilum* cDNA preceding the arginine pair ($Arg^{51}Arg^{52}$) was similar to those of plastid transit peptides of other monoterpene synthases (Fig. 1), a 5′-truncated version of the cDNA was cloned without this transit peptide-coding part into an expression vector pET32a+ (Novagen) and expressed as a fusion protein. Crude extracts of induced *E. coli* BL21 (DE3)/pETTR (a truncated version), *E. coli* BL21 (DE3)/pETFL (a full-length version), and *E. coli* BL21 (DE3)/pET32a+ (empty vector) were assayed for monoterpene synthase, sesquiterpene synthase and prenyltransferase activity using the corresponding substrates GDP, FDP, DMADP plus IDP under optional conditions described in Section 3. Enzymatic production of monoterpenes was observed only when GDP was incubated in the presence of the truncated form of the enzyme. Incubation

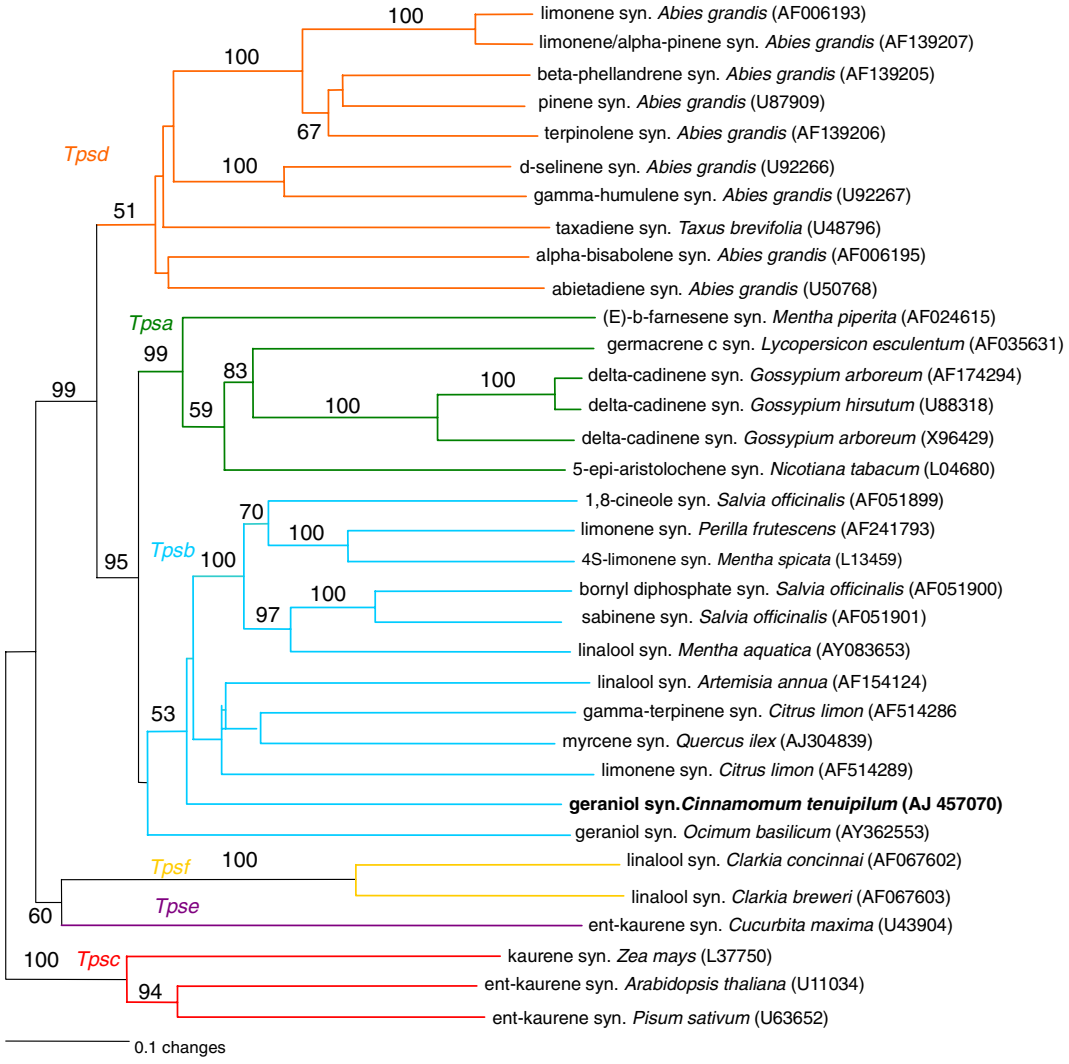

Fig. 2. Phylogenetic tree of plant terpene synthases reconstructed by using Dayhoff's distances between proteins and the neighbor-joining method (program PAUP 4.0b). The percentages in the tree are the bootstrap values for the branches and lower 50 bootstrap values do not show up in the tree. The EMBL or NCBI Database accession numbers of the corresponding genes or the deduced amino-acid sequences are given in parentheses. Scale bar indicates 10% sequence divergence. Each subfamily is distinguished by sharing a minimum of 28.4% identity among members at the amino acid level. The terpenoid synthases involved in secondary metabolism constitute subfamilies *Tpsa* (angiosperm sesquiterpene synthases, green), *Tpsb* (angiosperm monoterpene synthases, blue), *Tpsd* (gymnosperm monoterpene synthases, orange) and *Tpsf* (distant and possibly ancient, gold). The deduced peptide sequence of *C. tenuipilum* (bold) was grouped together with angiosperm monoterpene synthases and belonged to *Tpsb* subfamily. *Tpsc* (red) and *Tpse* (purple) subfamilies, involved in primary metabolism, are distantly related to *Tpsa, Tpsb, and Tpsd.*

with FDP, or DMADP plus IDP as substrates, respectively, neither with the truncated nor with the full-length version of the enzyme led to substantial product formation. Extracts of *E. coli* with empty vector and heat-denatured crude enzymes served as controls, yielding no detectable products.

The purified recombinant protein was used for further characterization. After purification under native condition on Ni-NTA His·Bind Superflow, the analysis of the elute on SDS/PAGE led to the detection of five bands (Fig. 3). One of the more intense bands was the expected size of the recombinant protein. With GDP at apparent saturation (150 μM), the partially purified truncated version of the enzyme generated geraniol as the sole product, identified by GC–MS (Fig. 4); and no product was detected with the partially purified full-length protein. In addition, the absence of geraniol in the full-length enzyme assay preparation and in the empty vector controls indicated that the preparation was free of phosphatase activity under the current assay conditions employed (data not shown).

To determine if the enzyme acts as a nonspecific phosphatase, tests were carried out with *p*-nitrophenyl phosphate as substrate. The purified enzyme did not exhibit any phosphatase activity. Addition of the phosphatase inhibitor molybdate (1 mM) did not prevent the enzyme from conversion of GDP into geraniol. Because monoterpene synthase activities are strongly dependent

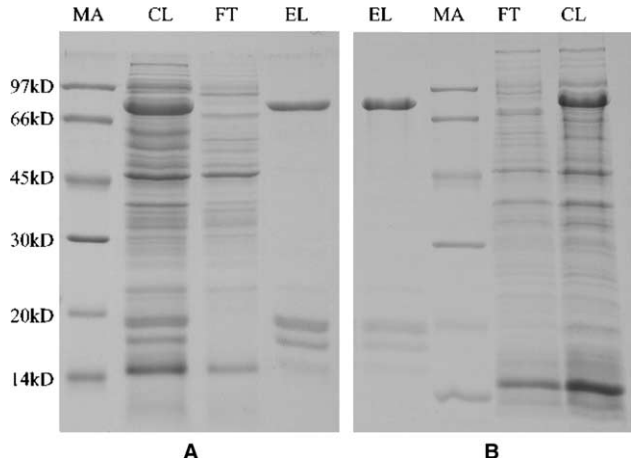

Fig. 3. Purification of the His-tagged proteins analyzed by SDS/PAGE (10% polyacrylamide gel, Coomassie staining). Recombinant proteins of 82.5-kD and 86.7-kD are expected in a fusion expression for the truncated version (A) and the full-length version (B), respectively. Lanes: CL, cleared cell lysate; FT, flow-through of the lysate from a Ni-NTA His·Bind Resin column; EL, eluate; MA, LMW-SDS Marker (Amersham Biosciences: 97 kDa = phosphorylase *b*; 66 kDa = albumin; 45 kDa = ovalbumin; 30 kDa = carbonic anhydrase; 20 kDa = trypsin inhibitor; 14 kDa = α-Lactalbumin).

on bivalent metal ions such as Mg^{2+} or Mn^{2+} (Bohlmann et al., 1998), varying concentrations of Mg^{2+} or Mn^{2+} were tested for enzyme activity. The optimum Mg^{2+} and Mn^{2+} concentration was about 10 and 0.5 mM, respectively. No enzyme activity was observed in the absence of Mg^{2+} or Mn^{2+}. Further addition of K^+ up to 100 mM had no effect on the apparent enzyme activity. The pH dependence was examined and found to be maximal around pH 7.0. At pH 6.2 or 8.2, less than 20% of the maximal activity was obtained. Kinetic properties of the enzyme were determined at the optimum assay conditions. The apparent K_m value of the enzyme was 55.8 μM. Nevertheless, the buffer system has been reported to influence the apparent K_m values. When Hepes buffer was used to assay monoterpene synthase activity responsible for limonene formation in holm oak, the apparent K_m decreased drastically to 5.5 μM instead of 107.6 μM in KPi assay buffer (Fischbach et al., 2000).

The overall structural similarity of the protein encoded by the *C. tenuipilum* sequence to basil GES and other terpene synthases, its requirement of Mg^{2+} or Mn^{2+} for activity and its insensibility to *p*-nitrophenyl phosphate and molybdate, strongly suggest that the enzyme acts as do other terpene synthases. From all this it seems clear that the enzyme encoded by the *C. tenuipilum* sequence specifically converts GDP to geraniol as a single product. Thus, a geraniol synthase has been identified from *C. tenuipilum* and is now designated as CtGES. The reaction mechanism of a geraniol synthase presumably proceeds through ionization of GDP with the assistance of a divalent metal ion, forming a chelate with the diphosphate moiety of the substrate, and the resulting allylic carbocation is captured by water (Iijima et al., 2004) (see Fig. 4).

2.4. DNA and RNA gel blot analyses

Southern analysis at high stringency with a *CtGES*-specific genomic DNA probe detected only one major hybridizing band in genomic DNA of the geraniol chemotype, whereas *Hind*III-digested lambda DNA as a negative control showed no signals. cDNA fragments including the open reading frame of *CtGES* were used as a positive control (Fig. 5A). Results suggest that the genome contains a single copy of *CtGES*. *CtGES* expression was examined at high stringency, hybridizing RNA gel blot with a *CtGES*-specific RNA probe. A hybridization band was detected only in leaf tissues of the geraniol chemotype and not in those of other chemotypes (Fig. 5B), indicating that *CtGES* was specifically expressed in the geraniol chemotype of *C. tenuipilum*. RT-PCR analysis showed a very similar pattern with that of RNA gel blot (Fig. 5B). Results demonstrate that the expression of geraniol synthase gene is chemotype-specific in the leaves of *C. tenuipilum*. Furthermore, in situ hybridization in leaf tissues using digoxigenin (DIG)-labeled antisense RNA probe for CtGES showed the specific accumulation of transcripts in oil cells of the leaf (Fig. 6B). Signals were observed throughout the cytoplasm of developing oil cells, whereas in mature oil cells signals were localized to the parietal region. In situ hybridization was not detected in the section exposed to sense RNA probe as shown in Fig. 6A, supporting the specific hybridization between the antisense RNA probe and the gene transcript. In peppermint peltate glandular trichomes, biochemical studies have revealed that monoterpene biosynthesis occurs only in the secretory cells (Gershenzon et al., 1992). Immunogold labeling further demonstrated that limonene synthase was exclusively located to the leucoplasts of peppermint oil gland secretory cells during the period of essential oil production (Turner et al., 1999). Accordingly, the leaf oil cells of *C. tenuipilum* are probably not only responsible for the essential oil storage, but also serve as the actual site of geraniol biosynthesis.

CtGES and basil GES, the only two members of a geraniol synthase cloned to date, are quite different from each other in their amino acid sequences. Among the members of *Tpsb* subfamily, the CtGES has the highest sequence similarity with γ-terpinene synthase from *Citrus limon* (Accession No. AF514286, 43.3% amino acid identity) and the lowest with basil GES (Accession No. AY362553, 33.2% amino acid identity). Both the CtGES and basil GES occupy a highly divergent branch of *Tpsb* subfamily (Fig. 2). Although its overall similarity to other terpene synthases is rather low, the basil GES catalyzes the formation of geraniol from GDP by

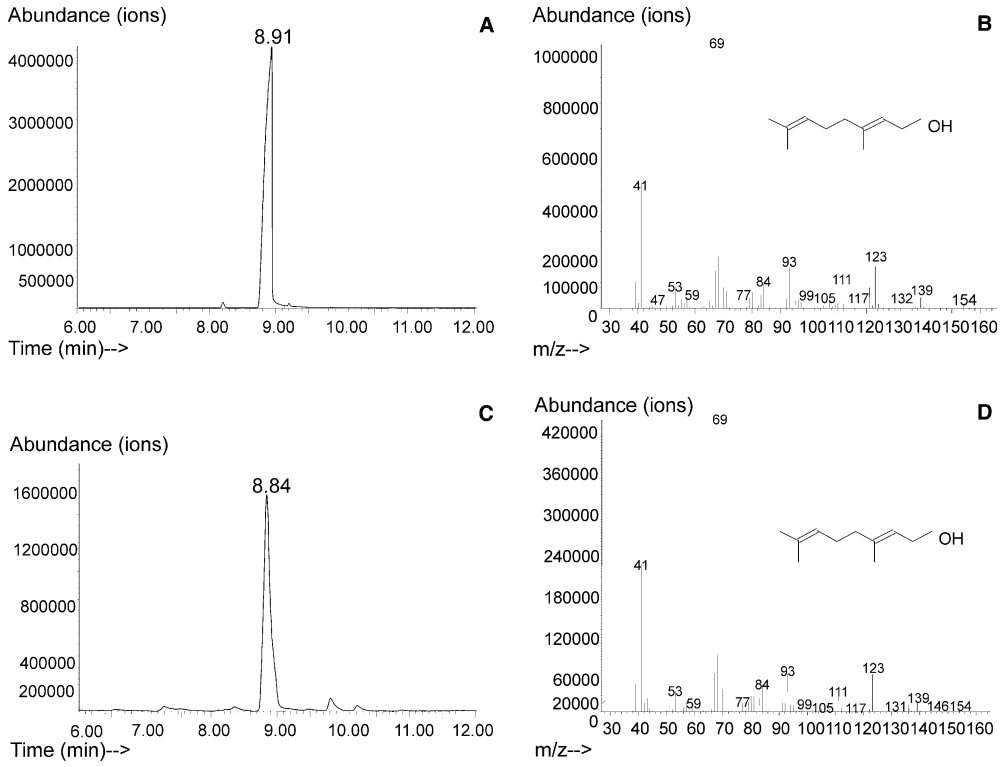

Fig. 4. GC–MS analysis of the product formed by the truncated version of recombinant CtGES with geranyl diphosphate as substrate. The GC profile (A) and the mass fragmentation patterns (B) of authentic geraniol are illustrated. Both the retention time from the GC profile (C) and the mass spectrum (D) of the enzyme product are identical to those of authentic geraniol.

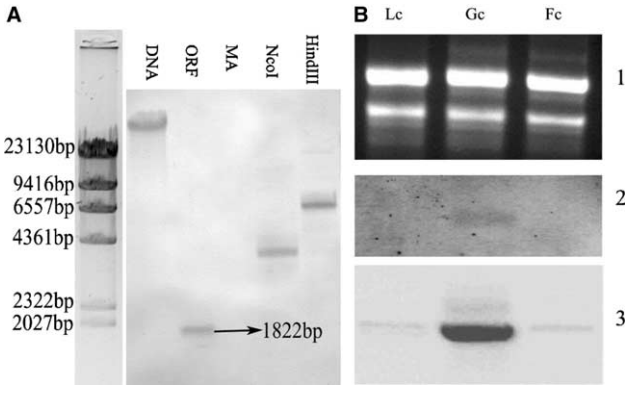

Fig. 5. DNA and RNA gel blot and RT-PCR analysis of the *CtGES* gene. (A) A Southern blot of genomic DNA from geraniol chemotype digested with *Nco*I or *Hind*III; DNA, genomic DNA without digestion. ORF, *CtGES* cDNA with open reading frame as a positive control. MA, 20 μg *Hind*III digested lambda DNA (TaKaRa) as negative control; (B) Northern blot and RT-PCR analysis; (1) Ethidium bromide staining of ribosomal RNA showing the equivalence of total RNA loading between the chemotypes; (2) Northern blot analysis of the *CtGES* transcript in the leaves of three chemotypes (15 μg total RNA per lane). *CtGES* expression was only detected in the geraniol chemotype (Gc). (3) RT-PCR analysis of *CtGES* expression in the leaves of three chemotypes; *CtGES* was much more abundantly expressed in geraniol chemotype (Gc) than in linalool (Lc) or farnesol (Fc) chemotype. Equal amount of total mRNA from three chemotypes was used in the first-strand cDNA synthesis.

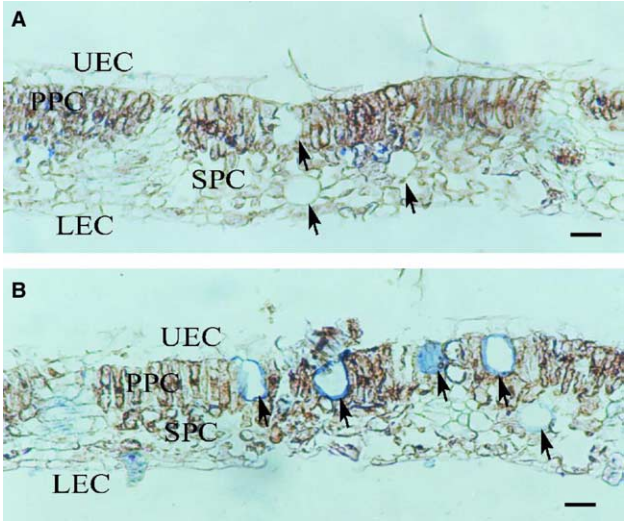

Fig. 6. In situ location of *CtGES* mRNA in leaf tissues of a geraniol chemotype. (A) Cross-section hybridized with sense RNA probe as control. (B) Cross-section hybridized with antisense RNA probe shows *CtGES* expression in the oil cells. UEC: upper epidermis cells; PPC: palisade parenchyma cells; SPC: spongy parenchyma cells; LEC: lower epidermis cells; arrows: oil cells. Bar, 20 μm.

a mechanism similar to that of other monoterpene synthases (Iijima et al., 2004). The CtGES has an N-terminal RRX_8W motif and its truncated protein is enzymatically active like other monoterpene synthases

(Jia et al., 1999; Fischbach et al., 2001; Crowell et al., 2002). Although the RRX_8W motif is not found in basil GES, both the Ser-35 and Met-44 truncated GES proteins exhibit a similar enzyme activity compared to plant-purified basil GES (Iijima et al., 2004). Finally, the basil GES is specifically expressed in glands, not in leaves, of basil cv Sweet Dani, indicating a site- and variety-specific expression pattern. The CtGES gene exhibits a site- and chemotype-specific expression pattern, which presumably implies its critical role in the formation of diverse chemotypes of *C. tenuipilum*.

3. Experimental

3.1. Plant materials, substrates and reagents

Young leaves and shoots from linalool, geraniol and farnesol chemotypes of *C. tenuipilum* were collected from Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences (Yunnan Province), frozen immediately (*liq*. N_2), and stored at −80 °C until use. Isopentenyl diphosphate (IDP), dimethylallyl diphosphate (DMADP), geranyl diphosphate (GDP), farnesyl diphosphate (FDP), geraniol and citronellol standards were purchased from Sigma. Restriction enzymes were from New England Biolabs. T4-DNA ligase and pGEM-T Vector System I was purchased from Promega. All other biochemicals and reagents were purchased from Sigma or Amresco, unless otherwise noted. Primers were synthesized by Shanghai Sangon Co., and automated DNA sequencing was conducted at Shanghai Sangon Co. or at Dalian TaKaRa Co.

3.2. Essential oil analysis

Leaves of *C. tenuipilum* (5 g) were ground to fine powder (liq. N_2) extracted with *n*-hexane (15 mL) (Fisher) and shaken at room temperature for 1 h. The mixture was centrifuged 12000*g* for 30 min at 4 °C. The supernatant oils were dried (anhydr. Na_2SO_4), concentrated under a stream of N_2 and subjected directly to GC [Agilent HP5890, column: 0.32-mm × 30-m (30QC2/AC5)] with temperature programming from 80 to 260 °C at 5 °C/min, or to GC–MS [Agilent HP6890/5973, column: 0.25-mm × 30-m (HP-5MS)] analysis under conditions: electron-impact (EI) mode (70 eV), constant current 1.2 mL/min, temperature programming from 80 to 260 °C at 5 °C/min. The identification of the compounds was achieved by comparing the retention time and the mass spectra with those of the standards included in the library or by co-chromatography with authentic compounds (Fluka, Sigma). The leaf essential oil yield is 1.5–2.0% in fresh leaves of geraniol chemotypes determined by steam-distillation (Cheng et al., 1993)

3.3. cDNA synthesis and RACE

Total RNA was isolated from young leaves of *C. tenuipilum* as described previously (Zeng and Yang, 2002). mRNA was extracted from total RNA (200 μg) using an mRNA purification kit according to the manufacturer's recommendation (Amersham Pharmacia). Single-stranded cDNA was synthesized using Superscript™ First-Strand Synthesis Kit (Gibco-BRL). A set of degenerate primers was designed for conserved regions of known terpene synthase genes from angiosperms and used for PCR with single-stranded cDNA as template by employing a broad range of amplification conditions. Only one pair of primers (designated 1F and 1R) (Jia et al., 1999) generated a specific fragment of approximately 400-bp from the first-strand cDNA of the geraniol chemotype. The plasmid DNA was prepared from 33 individual transformants and the inserts were sequenced. DNA sequences were analyzed by BLASTN and BLASTX programs of the NCBI (Altschul et al., 1997). Three clones with 259-bp overlapping were identified to harbor sequences similar to those of plant monoterpene synthases. Based on the sequence of the longest clone (420-bp), primers for RACE-PCR (Clontech, Smart™ RACE cDNA Amplification Kit) and nested PCR were designed. According to the sequence information of RACE, primers 2F, 5′-CTC TTC TAT ATG TAA TGC CT-3′ and 2R, 5′-CTC ATG GTA AGC TAC CAG AAC-3′ were designed and used for the amplification of full-length cDNA. The amplicon of approximately 2000-bp was cloned, sequenced and designated as pCT5. The complete pCT5 cDNA sequence has been submitted to the GenBank/EMBL database under an Accession No. AJ457070.

RT-PCR was performed with 0.5 μg mRNA using Superscript™ First-Strand Synthesis Kit (Gibco-BRL). About 1.0 μl first-strand cDNA was used for subsequent amplification of the open reading frame of the cDNA. For PCR, 25 cycles of reactions were performed at the condition (95 °C, 30 s; 55 °C, 30 s; 72 °C, 120 s) with final extension at 72 °C, 10 min.

3.4. cDNA expression in E. coli and enzyme purification

For functional characterization of the encoded terpene synthase, a full-length and a truncated version of the open reading frame of the cDNA were prepared from the defined clone pCT5. A unique *Bam*HI restriction site was placed immediately upstream of Arg^{51} Arg^{52} (Fig. 1), resulting in a truncated version of the cDNA. A unique *Xho*I restriction site was placed immediately downstream of the stop codon. Restriction sites of *Bam*HI and *Xho*I for subcloning were introduced by PCR using primer combinations FL-*Bam*HI (5′-CGGATCC ATG GCA TTG CAA ATG ATT G-3′) and FL-*Xho*I (5′-GACCTCGAGT TCA GGC AGA

TCC ACC ATC-3′) for the full-length version (1812-bp); and primer pairs TR-*Bam*HI (5′-CGGATCC AGA AGA TCA GGG AAC TAC-3′) and TR-*Xho*I (5′-GACCTCGAGT TCA GGC AGA TCC ACC ATC-3′) for the truncated version (1662-bp). The amplicons were cloned and sequenced. After verification of the sequence, the *Bam*HI–*Xho*I fragments were subcloned into pET32a+ (Novagen) to yield plasmids pETFL (a full-length version) and pETTR (a truncated version), respectively. Both pETFL and pETTR harbor the gene 5′ fused to the His-encoding region downstream of an inducible *lac* promoter. Considering the fusion head of 17.7-kD, recombinant proteins of 86.7 and 82.5-kD are expected in a fusion expression for the full-length and the truncated version, respectively. Plasmids were transformed into *E. coli* BL21 (DE3) for a fusion expression, using the original pET32a+ as negative control.

Purification of His-tagged proteins was performed according to the Ni-NTA His·Bind Superflow protocol (Novagen). Protein concentration was determined by the method of Bradford (1976) with BSA as a standard. All fractions were analyzed by SDS/PAGE on 10% polyacrylamide gel at 140 V for 1.5 h. Approximately 80% purity of the enzyme was achieved by gel scanning.

3.5. Enzyme assays and properties

Mixtures of crude enzyme (50 μL) with 1 mL assay buffer and 50 μM substrates were overlaid with 1 mL hexane and incubated at 30 °C for 2 h. After extraction with hexane (3 × 1 mL), the hexane phase was collected by centrifugation, dehydrated (anhydr. Na_2SO_4) and concentrated under a stream of N_2 for GC–MS analysis. Crude extracts of induced *E. coli* BL21 (DE3)/pETTR, *E. coli* BL21 (DE3)/pETFL, and *E. coli* BL21 (DE3)/pET32a+ were assayed for monoterpene synthase, sesquiterpene synthase and prenyltransferase activity under optional conditions described by Crowell et al. (2002); Crock et al. (1997) and Burke et al. (1999); respectively.

Further characterization of the enzyme activity was carried out using the partially purified enzyme. The reaction mixture was overlaid with 1 mL hexane and incubated at 30 °C for 30 min. A precise amount (2.5 μg) of citronellol was added as an internal standard into the reaction tube after incubation. The contents of the tubes were thoroughly mixed by vortexing and kept still on ice for 10 min. The tubes were centrifuged at 6000*g* for 10 min, and the supernatant hexane phase was collected. The extraction was repeated with hexane (2 × 1 mL). Then the hexane phase was collected, dehydrated (anhydr. Na_2SO_4) and concentrated under a stream of N_2 for GC–MS analysis. Amounts of geraniol formed in enzyme assays were calculated from the resultant GC integral using the relative response factor with respect to the citronellol internal standard.

The effect of pH on enzyme activity was examined using two buffer systems. The reaction was carried out in 50 mM Mopso buffer ranging from pH 6.2 to 7.4, and in 50 mM Hepes buffer ranging from pH 7.4 to 8.2. To check the specific bivalent metal ion preference, varying concentration of either 0–100 mM $MgCl_2$ or 0–5 mM $MnCl_2$ was used. The affinity for K^+ was examined at different concentrations of KCl ranging from 0 to 100 mM. All assays were performed in duplicate. For determination of the K_m value, the enzyme was incubated in assay buffer for 30 min at 30 °C, with GDP ranging from 5 to 100 μM. Lineweaver–Burk plots were constructed to obtain the K_m value. Phosphatase activity was measured as described (Hernandez and Whitton, 1996). About 2 mM *p*-nitrophenyl phosphate was incubated with increasing quantities of enzyme in a final volume of 3 mL assay buffer without GDP. The reaction was stopped by adding 0.2 M Na_2CO_3 after incubation for 1 h at 25 °C. The product was detected at 420 nm in Beckman DU600 spectrophotometer.

3.6. Southern, northern and in situ hybridization analyses

Genomic DNA from shoots of *C. tenuipilum* was isolated using a modification of the procedure (Li et al., 2001). From a genomic DNA fragment of about 1700-bp (GenBank Accession No. AJ586886), a specific probe of 420-bp was prepared using the PCR DIG Probe Synthesis Kit (Roche), and was applied in genomic DNA hybridization analysis according to a standard procedure (Sambrook et al., 1989). A specific fragment used for RNA probe was prepared using the DIG RNA labeling Kit (Roche), and used in Northern blot or mRNA in situ hybridization analysis. For Northern blot analysis, blots were hybridized for 8 h at 68 °C in DIG Easy Hyb buffer (Roche), washed and subjected to immunological detection (Roche, DIG Nucleic Acid Detection Kit). For in situ hybridization analysis, young leaves of the geraniol chemotype were fixed and dehydrated through conventional ethanol series and embedded in paraffin. Sections were cut to 10 μm and deparaffinized with xylene. In situ hybridization was done essentially according to the procedure described by Cox and Goldberg (1988).

Acknowledgements

We thank Professor Thomas. J. Bach, IBMP-Strasbourg for critical reading of the manuscript, and Professor Chen Xiao-Ya for his helpful suggestion. This work was supported, in part, by grants from the Natural Science Foundation of Yunnan Province; from Chinese Academy of Sciences.

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