cDNA Isolation and Functional Expression of Myrcene Synthase from *Perilla frutescens*

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cDNA cloning of a monoterpene synthase from *Perilla frutescens* whose steam-distilled oil contains 92.9% perillaketone, was performed by the PCR method using primers designed based on limonene synthase. The full-length nucleotide sequence of this cDNA consisted of 1978 bp including a 1827-bp translational region encoding a deduced protein of 608 amino acids, which was similar to that of limonene synthase from *P. frutescens* (85% identity). Functional expression of this clone in *Escherichia coli* yielded an active monoterpene synthase enzyme, which converted geranyl diphosphate into 53.8% myrcene, 20.9% sabinene, 19.8% linalool and 5.5% limonene. As for the extraction of reaction products, we performed SPME (solid phase micro extraction) as well as conventional solvent extraction, and compared these two extraction methods.

Key words *Perilla frutescens*; myrcene synthase; homology-based cDNA cloning; Labiatae; monoterpene synthase; essential oil

Perilla frutescens (Labiatae) with some wild species,[1] whose leaves and shoots are used as a food, medicinal herb, natural pigment and spice in Japan, China, Korea and Vietnam, and whose mericarps are also used for food and squeezed oil, is grown widely in East Asia. The characteristic odor of its leaves is derived from the essential oil composition and various compounds were detected in steam-distilled essential oils with GC-MS analyses.[2] *Perilla* plants are classified into several chemotypes based on the essential oil constituents, for example a perillaldehyde (PA) type with cyclohexanoid monoterpenes, an elsholztiaketone (EK) type and a perillaketone (PK) type with furanoid monoterpenes, and a phenylpropanoid (PP) type with phenylpropanoids.[2—4] Genetic analyses by cross-breeding in our laboratory have shown that oil composition produced is peculiar to the chemotype, and oils are synthesized under the strict control of several genes[5—13] (Fig. 1). Furthermore, the genetics of these chemotypes are independent of the ones for the color of the leaves or stems, and no link is present between the colors and odors of leaves.[14] Limonene synthase, which produces limonene from universal acyclic precursor geranyl diphosphate, GPP, is thought to be an important enzyme in the biosynthesis of perillaldehyde produced mainly in PA types. The cloning of this synthase was performed previously,[15] but genes relevant to the biosynthesis of essential oil components from different chemotypes have yet to be cloned or expressed functionally. Among the four typical chemotypes mentioned above, in Japan, the chemotype generally used as a food or medicinal herb is the PA type, whereas, for eating mericarps,[16] the PK, EK and PP types are used. Perillaketone and elsholtziaketone with a furan ring which are monoterpenes along with perillaldehyde, are thought not to

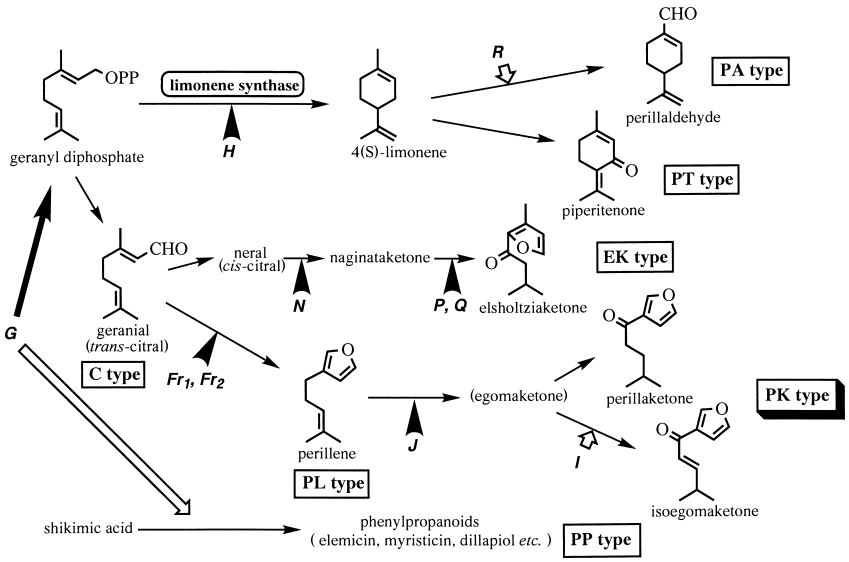

Fig. 1. Hypothetical Biosynthetic Pathways for Essential Oil Constituents of *Perilla frutescens* and Genes Controlling the Reaction Steps[5—13]

Bold alphabets indicate the genes which regulating the biosynthetic steps, black arrows indicate promotion, and white arrows indicate inhibition, of the indicated biosynthetic step.

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be synthesized *via* limonene from GPP,[11] but the pathway of their biosynthesis has not been disclosed. Furanoid monoterpenoid (+)-menthofuran synthase cloned by Bertea *et al*.[17] is a cytochrome P450-relevant synthase which catalyzes a reaction from (+)-pulegone to (+)-menthofuran, thus, it is assumed that perillaketone or elsholtziaketone synthase is a cytochrome P450-relevant hydroxylases which forms a furan ring *via* an intermediate from GPP, but the intermediate is unknown. We planned the cloning of a monoterpene synthase gene related to the biosynthesis of perillaketone from strain No. 5526 containing 92.88% of perillaketone among high perillaketone-producing chemotype PK, in a number of strains of *P. frutescens* which have been preserved by our research group.[2] It was shown previously that the homology of the sequences among terpene synthases from the same family of plants is high regardless of function,[18,19] and linalool synthase from *Mentha citrata* was cloned by primers designed based on this characteristic.[20] In this study, we made use of this feature of terpene synthases and designed primers for consensus sequences of extant limonene synthase from *P. frutescens*,[15] and cloned a new monoterpene synthase gene from a PK type *P. frutescens*.

MATERIALS AND METHODS

Plant Materials and Chemical Reagents Plants for cDNA cloning were grown in the greenhouse at the Botanical Garden for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University. Myrcene was purchased from Tokyo Kasei Chemicals, sabinene from Indofine Chemical Company, Inc and geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) from Sigma-Aldrich Japan. All other biochemical and chemical reagents were purchased from Nacalai Tesque Inc. or Wako Chemical Ind. unless otherwise noted.

RNA Isolation and cDNA Preparation Total RNA of young leaves of *P. frutescens* (strain No.5526) was extracted using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's directions. The RNAs were incubated for 10 min at 65 °C prior to a cDNA synthesis reaction which was performed in a mixture of total RNA, 18 μl of 5× reverse-transcriptase buffer (TOYOBO), 0.1 mM of each dNTP, 100 U of ReverTra Ace (TOYOBO), and 100 nM of oligonucleotide adaptor primer (add: 5′-GGC CAC GCG TCG ACT ACT TTT TTT TTT TTT TTT T -3′) designed to have an adaptor sequence at the 5′-end for the 3′-rapid amplification of cDNA ends (RACE) method or 100 nM antisense primer D designed from the sequence obtained for 5′-RACE (see below). The reaction mixture was incubated at 42 °C for 45 min for reverse transcription followed by 10 min incubation at 70 °C to inactivate the reverse transcriptase. For 5′-RACE, excess primers were removed from the cDNA with a Suprec-2 column (Takara Shuzo), and then an oligo (dC) chain was added at the 3′-end by terminal deoxynucleotidyl transferase (TOYOBO).

Amplification of a Monoterpene Synthase Fragment Primer sets were designed based on the sequence of limonene synthase of *P. frutescens* (GenBank accession No. D49735) within regions highly conserved among plant terpenoid synthases; {A: 5′-GTT GGA GCT TGC CAA ACT CCA C-3′, B: 5′-GCG ATT CCT ATT AAG CCA GC-3′} {A′: 5′-TGA GGG TTT TGT ATG CCA TGT CC-3′, B′: 5′-AAG TCG AGT TTG GCA AGC TCC-3′}. The polymerase chain reactions (PCRs) were carried out with two pairs of primers, sense primer A and antisense primer A′, and sense primer B and antisense primer B′ in volumes of 20 μl containing cDNA of No.5526, 1 U of recombinant Taq DNA polymerase (TOYOBO), 0.2 mM dNTPs, 20 μl of 10× buffer and 0.4 μM of each primer, on a PCR Thermal Cycler Personal (Takara Shuzo) with the following program; 94 °C for 5 min, then 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C, followed by 72 °C for 5 min. PCR products were electrophoresed in 0.7% agarose gel, the product observed at ca. 900 bp was excised, and the DNA fragments were extracted from the gel and sequenced. Primers for 3′- and 5′-RACE were designed based on the sequence of these fragments; a sense primer {C: 5′-TGG AGA TGG ACA TGG CAT GC-3′} (for 3′-RACE) and an antisense primer {D: 5′-CGA AAT GGT GAG AGA GAG CC-3′} (for 5′-RACE).

RACE-PCR and Amplification of Full-Length cDNA For the amplification of 3′-ends, PCR was performed with sense primer C and an antisense adaptor primer {amm: 5′-GGC CAC GCG TCG ACT AC-3′} in the conditions described above. For the amplification of 5′-ends, a RACE-adaptor-specific primer {ann: 5′-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′} and an antisense primer {E: 5′-ATC TTC ACT TGC TCA ACC AG-3′} which was designed upstream of primer D were used for PCR.

Bacterial Expression, Protein Purification and SDS-PAGE The PCR products were ligated into pCRT7/CT-TOPO and the ligates were introduced into *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene) cells. A single colony was cultured overnight in 3 ml of LB medium containing 100 μg/ml of ampicillin and 50 μg/ml of chloramphenicol at 37 °C with shaking at 180 rpm. The overnight culture was inoculated to a 100-ml Erlenmeyer flask containing 20 ml of LB with 100 μg/ml of ampicillin. After 2 h incubation at 37 °C with continuous shaking, IPTG (isopropyl β-D-thiogalactoside) was added to flasks at a final concentration of 1 mM, and then the cultures were incubated at 16 °C for 20 h with shaking at 225 rpm for expression. Cultures were centrifuged at 5500×g for 1 min and the cells were resuspended in IMAC buffer 1 (QuickPick IMAC, Bio-Nobile). Cells were sonicated for 2×20 s with a small probe at an output power of 2.5 (Astrason ultrasonic processor, XL2020, Misonic). After centrifugation of the lysate at 1000×g for 10 min, the resulting supernatant was used for purification of the His_6-tagged proteins with Pick Pen (Bio-Nobile), which was performed according to the QuickPick IMAC protocol (Bio-Nobile). Eluted proteins were separated by SDS/PAGE on a 7.5% polyacrylamide gel at 100 V and the gel was stained with Coomassie Brilliant Blue for visualization.

Enzymatic Assays and GC-MS Analysis Purified enzyme proteins were assayed in screw tight capped glass vials with a total reaction volume of 500 μl consisting of 25 mM K–Pi buffer (pH 7.0), 10% glycerol, 2 mM dithiothreitol, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 2% dimethyl sulfoxide, and 55 μM GPP. Reaction vials containing everything except GPP dissolved in dimethyl sulfoxide were carefully mixed

and the GPP solution was added for initiation of the reaction. Vials were incubated for 3 h at 30 °C with a SPME (solid phase micro extraction) fiber coated with PDMS (polydimethylsiloxane) thrust through the septum of the screw cap for adsorption of the reaction products. PDMS is a sort of polymer which efficiently absorbs mono- and sesquiterpene compounds. The SPME fiber with products of the enzymatic reaction was transferred to the injector of the GC apparatus and the product compounds were desorbed by heating at 50 °C. Products were identified in a GC-MS analysis by comparing retention times and mass spectra with authentic reference compounds. The apparatus and conditions were as follows: apparatus, M9000/3DQMS (HITACHI); column, TC-WAX (GL Sciences, 0.25 mm i.d.×60 m fused silica capillary column, 0.25 μm film thickness); carrier gas, helium (1.0 ml/min); injector temperature, 220 °C; ionization voltage, 15 eV; oven programming, initially 40 °C, increased at 10 °C/min to 130 °C, then at 3 °C/min to 160 °C, and at 15 °C/min to 220 °C, and finally held at 220 °C for 5 min.

For the extraction with solvent, the reaction mixture was overlaid with 1 ml of pentane to trap volatile products and incubated for 3 h at 30 °C. After the incubation, the mixtures were extracted with 500 μl of pentane twice, and the combined extracts were dried on $MgSO_4$ for GC-MS. Reaction products were analyzed on a M9000/3DQMS (HITACHI) or a Hewlett-Packard 5972 MSD directly coupled to a HP5980 gas chromatograph. Analytical conditions for the M9000/3DQMS were the same as for SPME. Those for the Hewlett-Packard 5972 MSD were as follows. EI mass spectra were collected at an ionization voltage of 70 eV over the mass range *m/z* 41—425. Samples of 0.1 μl (5% concentration) were injected. Analytical conditions: column: J&W DB-5, (0.25 mm×30 m, 0.25 μm film thickness); carrier gas: helium at 1 ml/min; injector temperature: 220 °C; split ratio: 10 : 1; oven programming: initial temperature: 60 °C, gradient 3 °C/min, final temperature: 246 °C. The percentages of each compound are TIC (total ion count) values. Identifications were made by searching a volatile oil library,[21] coupled with retention time data for reference compounds.

RESULTS AND DISCUSSION

Isolation and Sequencing of a Putative Terpene Synthase cDNA from *P. frutescens* Two core sequences of 927 bp and 824 bp were obtained by PCRs performed with two primer pairs (A with A′ and B with B′) designed with reference to the limonene synthase cDNA from *P. frutescens* (PFLS) which was cloned previously.[15] Sequences of these two PCR products resembled PFLS in corresponding regions, exhibiting 90% and 86% identity, respectively. Based on sequences of the two fragments, sense primer C for 3′-RACE and antisense primer E for 5′-RACE were designed. Following a common RACE procedure, a full-length putative terpene synthase from strain No.5526 *P. frutescens*, PTS-5526, was obtained by PCR using primer C with the RACE-adaptor-specific primer amm, and E with ann. The complete nucleotide sequence was registered in GenBank (accession No. AF271259).

Sequence Analysis of PTS-5526 Cloned full-length PTS-5526 cDNA consists of 1978 bp with an ATG start codon at position 59—61 and a TGA stop codon at position 1883—1885, including a 1827-bp translational region encoding a deduced protein of 608 amino acids with a mass of 70.8 kDa. This cDNA well resembles PFLS exhibiting 85% sequence identity at the nucleotide level. Figure 2 shows a comparison of the deduced amino acid sequences of PTS-5526, PFLS and other plant terpene synthases. The PTS-5526 sequence has a RR motif which is responsible for the isomerization of GPP to LPP,[24—26] and a DDxxD domain, which is speculated to be the binding site for the substrate (GPP) -divalent metal cation (Mg^{2+}, Mn^{2+} *etc*.) complex, and is absolutely conserved among mono-, sesqui-, and diterpene synthases.[15,26—28] Asterisks in Fig. 2 indicate amino acid residues highly conserved among terpene synthases,[19] which was confirmed in the PTS-5526 sequence. PTS-5526 has a region coding a transit peptide which functions as a signal sequence for the transportation of translated protein to plastids where the enzyme works and is eliminated after the migration to yield mature active enzymes.[24,29,30] Transit peptides are present in mono- and diterpene synthases, but not in sesquiterpene synthases which are active in the cytosol.[31,32]

cDNA Expression and Functional Analysis For the purpose of functional expression, a 5′-truncated cDNA of PTS-5526 was prepared. A sense primer was designed so an ATG was appended to install a new starting methionine immediately upstream of the highly conserved arginine pair to initiate translation, and an antisense primer was designed immediately upstream of the original stop codon in order for the His_6-tagging region to be translated which was encoded on the pCR T7/CT-TOPO vector. DNA fragments amplified by PCR with these primers were ligated downstream of the T7 promoter of pCR T7/CT-TOPO and the plasmids were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells. This *E. coli* strain contains extra copies of the *argU*, *ileY* and *leuW* tRNA genes, and was designed to solve a problem which stems from the poor production of heterologous proteins due to the rarity of these tRNAs in *E. coli.* There are two types in the BL21-CodonPlus (DE3) strain, RIL and RP, suitable for the translation of AT-rich or GC-rich genomes, respectively. The RIL type cells were used in this study because PTS-5526 consists of 57% AT and 43% GC. The expression conditions of the protein were determined as follows; for temperature, the cells harboring the truncated PTS-5526 were incubated at 37 °C after adding IPTG. Expected proteins which were calculated as 64.6 kDa in mass were not observed when the culture was harvested, sonicated, centrifuged and loaded on SDS-PAGE gel. Then the incubation temperature was changed to 30 °C, inclusion bodies were formed and it was difficult to recover the recombinant proteins as active soluble proteins. Finally, an incubation temperature of between 16 and 20 °C was examined, and it turned out that 16 °C was preferable for expression of the recombinant proteins encoded by PTS-5526 in BL21-CodonPlus (DE3)-RIL cells, because of the balance of amounts of expressed soluble protein and inclusion bodies. The period for expression culture was determined by harvesting the cultures 2, 8 and 20 h after adding IPTG, and conducting a SDS-PAGE analysis. The 20 h culture sample yielded much more soluble enzyme protein than the other samples. Cell lysate, recovered soluble proteins and isolated proteins were analyzed by SDS-PAGE (Fig. 3). A control was prepared by expressing the vectors without an insert under the same

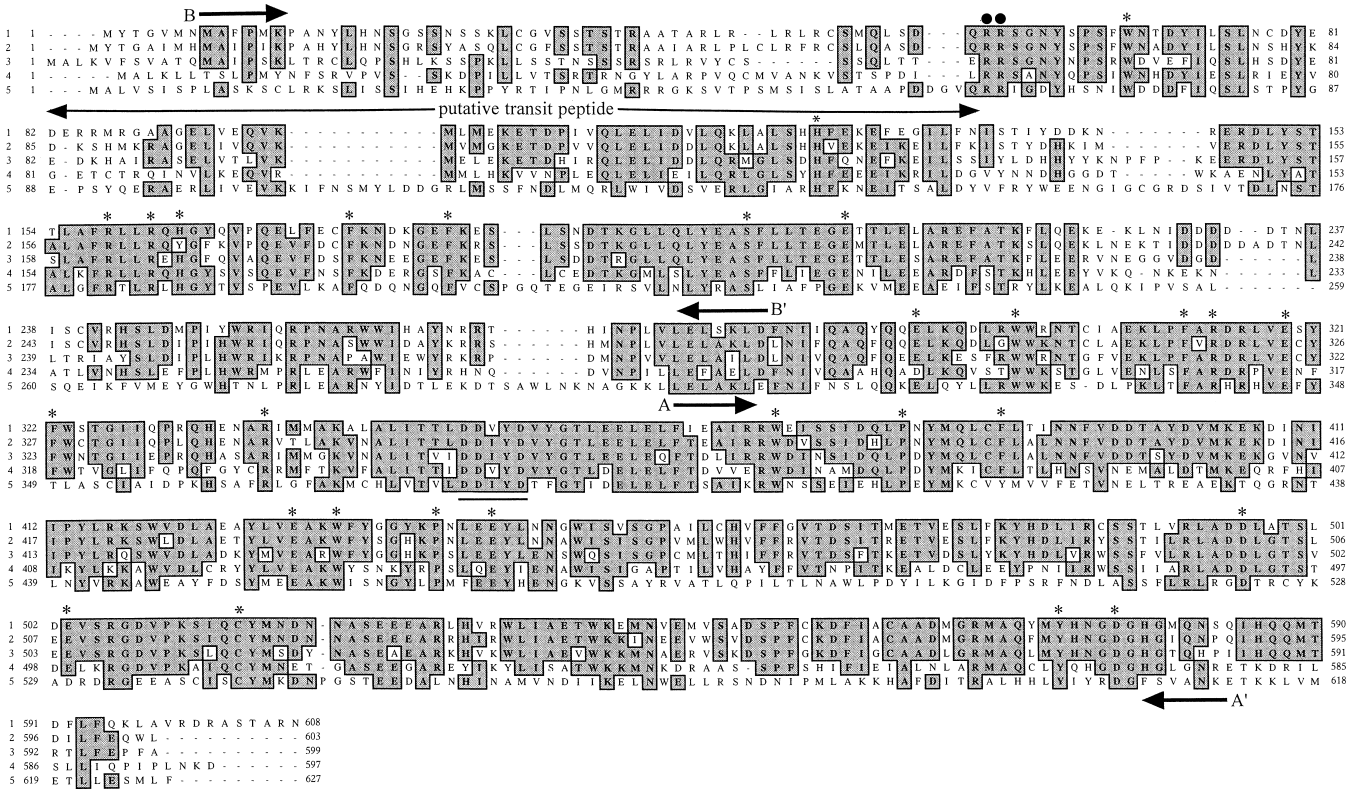

Fig. 2. Alignment of Deduced Amino Acid Sequences of Monoterpene Synthases

The designations correspond to: 1, PTS-5526; 2, limonene synthase from *Perilla frutescens*,[15] 3, limoene synthase from *Mentha longifolia* (GenBank accession No. AF175323); 4, myrcene synthase from *Quercus ilex* (AJ304839); and 5, myrcene synthase from *Abies grandis* (U87908). Asterisks indicate highly or absolutely conserved amino acid residues among many terpene synthases. Gray dots mark the RR motif, and the DDxxD domain is underlined. A and A′, and B and B′ were two primer pairs used for PCR cloning. The alignment was created with the ClustalW program[22] using the MacVector.[23]

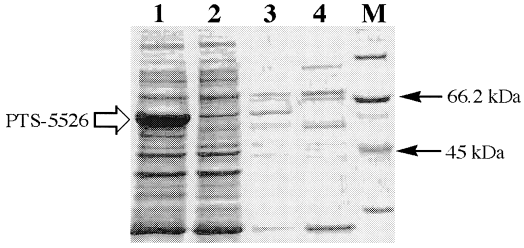

Fig. 3. Purification of His_6-Tagged PTS-5526 Recombinant Proteins Analyzed by SDS-PAGE

Lanes: 1, cell lysate; 2, soluble proteins; 3, isolated proteins; 4, control; and M, marker.

conditions. The PTS-5526 recombinant protein was shown in the lane of PTS-5526 whose size was *ca*. 65 kDa which was close to the expected size of truncated PTS-5526 deduced from its amino-acid sequence. The recombinant protein was incubated with substrates to perform an enzyme assay. The reaction products were extracted using SPME fiber or pentane. The reaction mixture in a 4-ml vial was sealed tightly with a septum and incubated at 30 °C, and the reaction products in the headspace of the vial were adsorbed by the SPME fiber which was thrust through the septum. As for the enzymatic reaction substrate, GPP, FPP, and GGPP were tested, and reaction products were observed only with GPP. The major monoterpene product was identified as myrcene (Fig. 4), and other products as sabinene, linalool and limonene by comparison of their retention times as well as mass fragmentation patterns with those of authentic compounds. The ratio of these monoterpene products was calculated to be 24 : 4 : 1 : 1 from the area of TIC. On the other hand, in the reaction overlaid with 1 ml of pentane and incubated for 3 h at 30 °C, the GC-MS analysis of pentane extracts demonstrated that the products consist of myrcene (53.8%), sabinene (20.9%), linalool (19.8%) and limonene (5.5%) (Fig. 5). Because of the dominance of the myrcene product, the enzyme encoded by PTS-5526 is designated as a myrcene synthase. Comparison of the results of SPME and pentane extracts showed that less linalool was detected by SPME than by pentane extraction. This difference may be derived from the difference in the rate of volatilization of the products since the extraction ratio of SPME was dependent on the volatilization liabilities of compounds, and also from differences in affinity of the fiber towards the different products. However, SPME is considered a valuable method for the detection of monoterpenes in particularly these which trap exclusively volatile compounds and less volatile compounds derived from *E. coli* cells.

The proposed reaction mechanism for many monoterpene synthases is thought to be initiated by the ionization of GPP and subsequent isomerization to form linalyl diphosphate (LPP) as an enzyme-bound intermediate[24,33] (Fig. 5). After ionization of LPP, the resulting linalyl carbocation can undergo deprotonation at the C3 methyl group to form myrcene or at the C4 methylene group to form (*E*)-β-ocimene.[19] Which means that both acyclic products, myrcene and ocimene, are speculated to arise from the same carbocationic intermediate. Actually, terpene synthase which catalyzes the

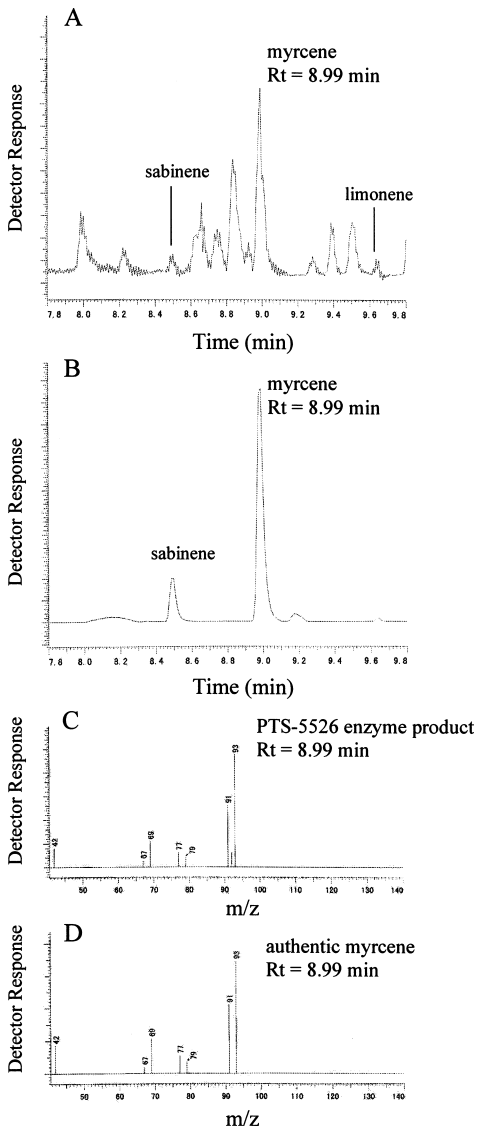

Fig. 4. GC-MS Analysis of the Products of the Recombinant Protein Encoded by PTS-5526

Total ion chromatograms of reaction products extracted with pentane (A), and with SPME fiber (B). The mass fragmentation patterns for the principal monoterpene product with t_R=8.99 min (C), and for authentic myrcene (D). The apparatus used was M9000/3DQMS (HITACHI) with conditions described in Materials and Methods.

coproduction of myrcene and (*E*)-β-ocimene, was cloned from *Arabidopsis thaliana*.[19] However, PTS-5526 discussed in this study encodes an enzyme which produces cyclic monoterpene sabinene and limonene, and acyclic linalool with an OH group as minor products, but does not produce ocimene at all. Thus, this enzyme can be distinguished functionally from a mixed myrcene/ocimene synthase. cDNA of sabinene synthase which produces minor products such as γ-terpinene, terpinolene, limonene and myrcene was cloned from *Salvia officinalis*,[34] and linalool synthase which produces α-terpineol, myrcene and limonene was cloned from *Mentha citrata*.[20] PTS-5526 as well as these enzymes from *Salvia* and *Mentha* is characterized transforming GPP to yield a single main product and some minor products. Here we demonstrated that *Perilla* also has a terpene synthase functionally similar to those of *Mentha* and other plants.

Phylogenetic Analysis A phylogenetic tree was constructed based on the amino acid sequences of PTS-5526, PFLS and terpene synthases from other plants (Fig. 6). In this tree, PFLS is located at the closest branch to PTS-5526, whereas myrcene synthases from other plants belong to different groups from PTS-5526, and terpene synthases other than myrcene synthases were clustered by species of plants. Therefore, it is considered that the homology among synthases of the same plant species, although each of which produces different compounds, is higher than that among synthases of different species but produce identical compounds. As mentioned by Bohlmann *et al.*,[18,19,28] the catalytic function of a terpene synthase cannot be predicted on the basis of the similarity of a phylogenetic relationship.

CONCLUSIONS

The myrcene synthase cloned in this study is the first functionally characterized acyclic monoterpene synthase from *P. frutescens* and also the first myrcene synthase cloned from Labiatae plants. The cloning of PTS-5526 was performed based on the sequence similarity between monoterpene synthases of the same plant species, and this time PFLS was employed as a model. Essential oil analysis shows that steam-distilled oil of strain No.5526 *P. frutescens* contained

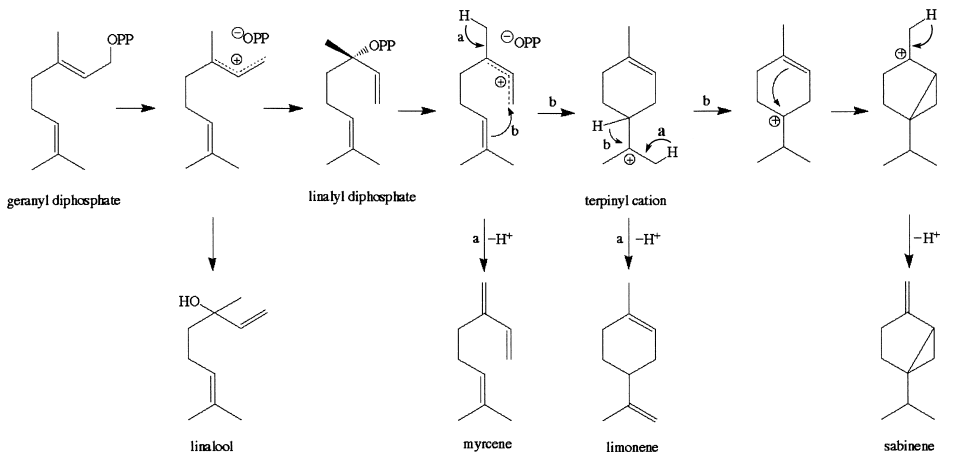

Fig. 5. Proposed Mechanism for the Enzymatic Conversion of Geranyl Diphosphate to Myrcene, Sabinene, Linalool, and Limonene

Formation of the monocyclic and bicyclic products requires preliminary isomerization of geranyl diphosphate to linalyl diphosphate. The acyclic products could be formed from either geranyl diphosphate or linalyl diphosphate *via* carbocations. *OPP* denotes the diphosphate moiety.

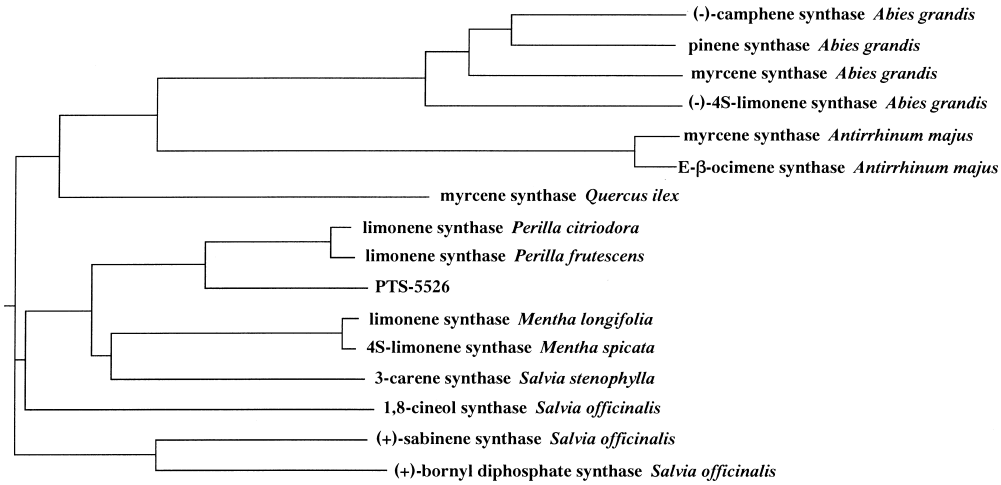

Fig. 6. Phylogenetic Tree of Plant Monoterpene Synthases

The dendrogram was created with the PAUP program[35] using the MacVector.[23] The accession Nos. in the phylogenetic tree are: *Abies grandis* (−)-camphene synthase (U87910); *Abies grandis* pinene synthase (U87909); *Abies grandis* myrcene synthase (U87908); *Abies grandis* (−)-limonene synthase (AF006193); *Antirrhinum majus* myrcene synthase (AY195609); *Antirrhinum majus* E-*β*-ocimene synthase (AY195609); *Quercus ilex* myrcene synthase (AJ304839); *Perilla citriodora* limonene synthase (AF233894); *Perilla frutescens* limonene synthase (D49368); PTS-5526 (AF271259); *Mentha longifolia* limonene synthase (AF175323); *Mentha spicata* 4S-limonene synthase (L13459); *Salvia stenophylla* 3-carene synthase (AF527416); *Salvia officinalis* 1,8-cineol synthase (AF051899); *Salvia officinalis* (+)-sabinene synthase (AF051901); *Salvia officinalis* (+)-bornyl diphosphate synthase (AF051900).

much perillaketone (92.88%), but myrcene and other olefinic monoterpenes at less than detectable amounts.[2] A similar sequence to PTS-5526 was cloned from *P. frutescens* strain No.6 whose main oil component was also perillaketone (data not shown), but it contained neither myrcene nor other olefinic monoterpenes.[2] These results suggest that myrcene is not accumulated in the oil because sensitive myrcene is converted into other compounds by an enzyme such as cytochrome P450 dependent hydroxylases as soon as it is produced by the catalysis of PTS-5526 encoded protein. In the pathway of monoterpene biosynthesis in peppermint (*Mentha*×*piperita*),[36] it was shown that an universal acylic precursor, GPP, is converted to (−)-limonene by plastidial limonene synthase,[36—38] (−)-limonene serves as the common olefinic precursor of the essential oil terpenes of peppermint and spearmint, and menthol and various menthol isomers are formed from (−)-limonene *via* the action of a microsomal cytochrome P450 limonene-3-hydroxylase, (−)-*trans*-isopiperitenol dehydrogenase, (−)-isopiperitenone reductase and so on.[17,36—40] Functional correlations between monoterpene synthases and P450-related hydroxylases seem very close in the biosynthesis of essential oil components, and this might be also true in strain No.5526 *Perilla*. Linalool, which is the only monoterpene alcohol product of this enzyme, is the second principal (1.6%) in the steam-distilled oil of strain No.5526 *P. frutescens*.[2] This indicates that linalool is formed as one of the minor products of PTS-5526 protein and stored without being converted into another compound.

Perillaketone is an oil constituent with a furan ring, but the pathway of its biosynthesis has not yet been elucidated. (+)-Menthofuran is the only furan ring-type monoterpene compound whose ring formation from (+)-pulegone was shown to be accomplished by P450-related enzyme.[17, 41] However, it is suspected that one of the products of PTS-5526 protein might be an intermediate of the perillaketone biosynthetic pathway. Further cloning of enzymes from oil glands of perillaketone type *Perilla* and also other types of monoterpene synthases from various oil types of our perilla collection may help in the elucidation of the pathway of perillaketone biosynthesis as well as hypothetical pathways for the production of essential oil constituents of *P. frutescens*.

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