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Geraniol synthases from perilla and their taxonomical significance

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Abstract

Geraniol synthases were isolated from five pure strains of *Perilla citriodora* and *Perilla frutescens* which vary in essential oil type, the main compounds of which were citral, elsholtziaketone, perillaketone, and perillene, respectively. This result supports the putative biosynthetic pathways of these three furylalkenes which are all produced by way of citral. Nucleotide sequences of geraniol synthases from three oil types of *P. citriodora* were identical, and almost the same as the sequence from *P. frutescens*, a species with twice the chromosome number of *P. citriodora*. This identity in sequence between *P. citriodora* and *P. frutescens*, together with other previous results, indicates that *P. frutescens* was formed as an amphidiploid of *P. citriodora* and an unknown wild species.

Keywords: *Perilla citriodora*; *Perilla frutescens*; Labiatae; *Perilla*; Molecular cloning; Geraniol synthase; Furylalkene; Biosynthetic pathway; Amphidiploid; Essential oil

1. Introduction

Monoterpenes are among the most important and useful compounds in the pharmaceutical sciences and include bioactive compounds such as the insecticidal pyrethrin, the anti-tumoral perillalcohol (**1**), and anti-fungal pinene. Many more monoterpenes are used for food flavoring and perfumery. Plant monoterpenes are formed by a combination of two isoprene units and are often found as constituents of essential oils which are generally volatile at room temperature. Some simple monoterpene compounds can be produced by organic synthesis for commercial use. However, in many cases, natural essential oils obtained by steam-distillation are preferred, since quite a few of the compounds in oil differ in their biological activities as well as in fragrance according to their chirality. Indeed, plants mostly synthesize and accumulate one of the enantiomers (Dewick, 2002). Elucidating the biosynthetic pathways to these essential oil compounds as well as their regulatory systems is important specifically for investigating the theoretical background to the chemotaxonomy of aromatic plants, and to obtain a deeper understanding of phytochemicals in the fields of pharmaceutical sciences and enzymology.

Plant enzymes relating to the pathway for the biosynthesis of oil compounds, particularly mono- and sesquiterpene synthases, have been well investigated in Labiatae plants such as mint (Alonso et al., 1992; Colby et al., 1993; Crowell et al., 2002) and basil (Iijima et al., 2004a). These produce and accumulate a number of terpenoids in a characteristic organ called the grandular trichome, and in Pinaceae as Abies grandis (Bohlmann et al., 1997, 1999; Steele et al., 1998) which develops ducts for the production and secretion of resins comprised of various terpenoids. Basil, mint, and perilla are known to contain various types of oil (Franz, 1993) and are still being bred to create even more varieties for commercial use. This makes studies on the regulatory system and enzymes of biosynthetic pathways of such essential oil compounds even more significant.

Perilla, a common herb in Asia, has several oil types, the most popular of which is type PA (perillaldehyde, **2**) in Japan, Laos and Vietnam and type PK (perillaketone, **3**) in China and Korea. Oil types of perilla can be classified

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into two, type MT (monoterpene) whose oil is mainly composed of monoterpene compounds, and type PP (phenylpropene) whose oil contains mostly phenylpropene derivatives. Each of these can be classified further according to their principal constituent, into types C (citral, **4**), EK (elsholtziaketone, **5**), PA, PK, PL (perillene, **6**), PT (piperitenone, **7**), and SF (shisofuran, **8**) within the MT group, and types PP-m (myristicin, **9**), PP-dm (dillapiol, **10** + **9**), PP-em (elemicin, **11** + **9**), PP-dem (**10** + **11** + **9**), and PP-dmn (**10** + **9** + nothoapiol, **12**), within the PP group (Ito et al., 1999a,b; Fig. 1). The seven types in the MT group are divided into three classes based on the structural features of the main compound; cyclohexene type (PA and PT), furylalkene type (EK, PK, PL and SF), and acyclic type (C). As for the pathways producing these three types of compounds, cyclohexene compounds are synthesized via limonene (**13**) (Turner et al., 1999), and acyclic **4** is likely formed via geraniol (**14**) (Iijima et al., 2004a, 2006). However, while furylalkenes are proposed to be synthesized from **14** via **4** (Hegnauer, 1966), a precise study on this pathway has not been carried out. In the case of perilla, oil types were shown to be determined by genetic control, and regulatory steps in the putative synthetic pathways have been investigated by conventional crossing experiments using pure strains developed by repeated self-pollination (Honda, 1996; Ito et al., 2002). This genetic control of oil types is quite rigid, and the C type *Perilla frutescens* which had not existed was produced artificially by crossing a EK type strain with a PK type strain followed by repeating self pollination to establish a stable strain (Honda et al., 1994b). This approach was based on the employment of genotypes proved by crossing experiments, and was consistent with the putative synthetic pathway of **3** and **5** via **4**. Here we report on the cloning of geraniol synthases from the C type as well as from the furylalkene types of perilla, which strongly supports that monoterpenoid furylalkenes are synthesized from geranyl diphosphate (**15**) via **14** and then **4**.

2. Results and discussion

2.1. Isolation of cDNA encoding monoterpene synthases from a C type strain of Perilla citriodora and its expression in E. coli

The genus *Perilla* includes several species, namely a cultivated species of *P. frutescens* which is known as a common herb, and some wild species whose chromosome numbers are half that of the cultivated species (Ito and Honda, 1996). cDNAs with characteristics of plant monoterpene synthases were cloned from fresh young leaves of one of

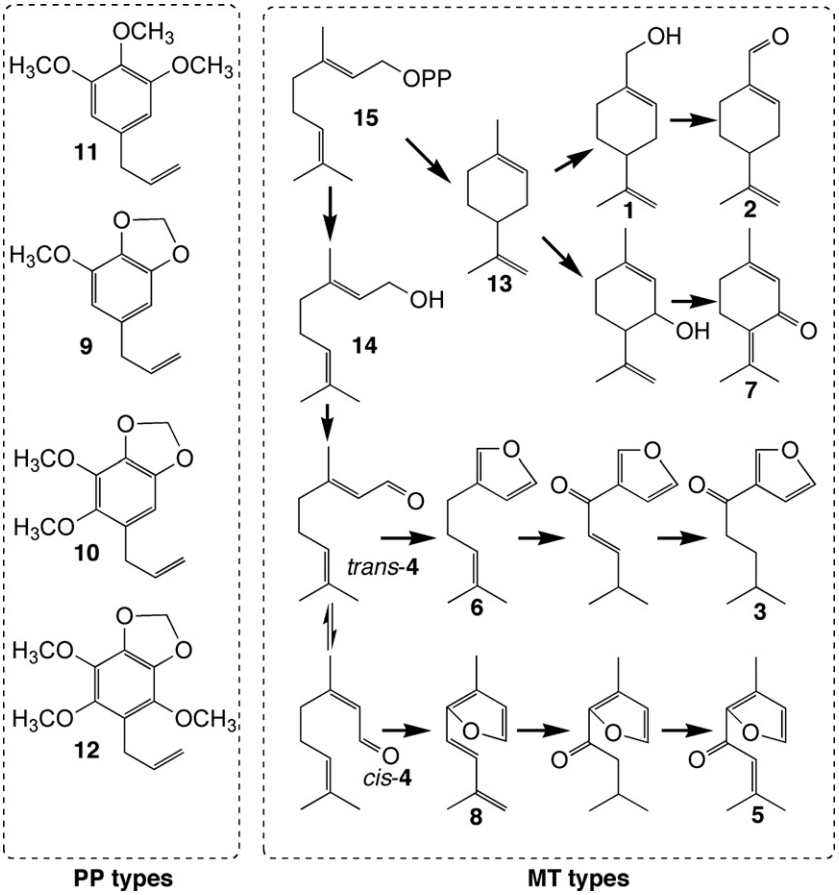

Fig. 1. Essential oil compounds of *Perilla* sp. and their putative synthetic pathways.

the wild species, *P. citriodora* (Strain No. 87), whose essential oil, type C, is the most common oil type among the species. Some distinct sequences of synthases were obtained from Strain No. 87, and the most abundant of these, named PcTps-C (GenBank Accession No. DQ088667) consists of 2057 nucleotides encoding 603 amino acids, whereas the second most abundant turned out to be the sequence of a linalool synthase (GenBank Accession No. AY917193) (Fig. 2). Comparing the sequence of PcTps-C with that of a monoterpene synthase such as linalool synthase (GenBank Accession No. AY917193) or limonene synthase (GenBank Accession No. AF233894, Ito et al., 2000) both derived from the same species of perilla, revealed 72% identity with the former and 50% identity with the latter. One of the most characteristic features of the amino acid sequence of terpenoid synthases is a DDXXD motif which is a putative binding site for a complex of **15** and divalent metal ions (Bohlmann et al., 1998; Fig. 2); however, the deduced amino acid sequence of this region in perilla monoterpene synthases seemed to be of two types, DDIYD of the cyclic monoterpene synthases such as limonene synthases (GenBank Accession Nos. D49368, Yuba et al., 1996; and AF233894, Ito et al., 2000) and DDVYD of the acyclic monoterpene synthases such as linalool synthases (GenBank Accession Nos. AF444798, and AY917193) and myrcene synthase (GenBank Accession No. AF271259, Hosoi et al., 2004). As for PcTps-C, the sequence in this region was DDVYD, and as it showed a higher level of identity to linalool synthase and myrcene synthase (49%) than with limonene synthase, the clone was expected to be an acyclic monoterpene synthase.

The heterologous expression of PcTps-C using *E. coli* and subsequent enzymatic assay with His-tag purified protein revealed that it transforms **15** into **14**, whereas neither a heat-inactivated enzyme solution nor preparations devoid of cofactors which were employed as controls produced no products. Extracts prepared from *E. coli* devoid of a plasmid were also assayed in order to confirm that **15**, in a reaction tube, is not degraded by a phosphatase carried over from *E. coli* (Fig. 3). Enzymatic reaction products were analyzed either by GC–MS for identification or by GC for measurement of their amount. Plant monoterpene synthases have a plastidial targeting sequence at the N-terminus which should be eliminated for better heterologous expression (Williams et al., 1998). Notably, a geraniol synthase from cinnamon was reported to show no catalytic activity when it was expressed with this sequence connected (Yang et al., 2005). However, the geraniol synthase from perilla, PcTps-C, though expressed with the targeting sequence, exhibited adequate activity (78%) yet the truncated PcTps-C without the targeting sequence yielded a k_m value for **15** of about 3.0 μM with a k_{cat} of 0.18 s^{-1}. Further, a RRX_8W motif which shows the cleavage site for this targeting sequence from the active body was found in PcTps-C and is shown with closed circles in Fig. 2. However, it could not be found in the basil geraniol synthase (Iijima et al., 2004a).

Sequence comparison between the geraniol synthase from perilla and those from the species mentioned above revealed 41% identity with cinnamon (GenBank Accession No. AJ457070) and 33% identity with basil (GenBank Accession No. AY362553). Both of the values are much lower than those of monoterpene synthases from *P. citriodora*, which confirms the previously suggested notion that the sequences of functionally different terpenoid synthases derived from either closely related or the same species of plants share greater similarity than the same functional synthases cloned from remotely related species (Bohlmann et al., 1998). A sequence similarity search using Basic Local Alignment Search Tool (BLAST) also showed that monoterpene synthase genes which had great similarity with geraniol synthases from perilla are linalool synthases from perilla (GenBank Accession Nos. AF44798, AY917193), linalool synthase from *Lavandula angustifolia* (GenBank Accession No. DQ263741), and (+)-sabinene synthase from *Salvia officinalis* (GenBank Accession No. AF05901). This result showed the close relatedness between geraniol synthases and linalool synthases, which was previously reported for basil (Iijima et al., 2004b).

The catalytic activity of basil geraniol synthase obtained by heterologous expression in *E. coli* was observed to be greatest at pH 8.0 with less than 1 mM of Mg^{++} (Iijima et al., 2004a); however, very similar conditions, pH 8.0 with 0.5 mM of Mg^{++}, were best for perilla geraniol synthase compared to a pH 7.0, 7.6, or 8.0 HEPES buffer with a Mg^{++} concentration of 0.5 or 20 mM. Additionally, no significant difference was observed in catalytic performance between the use of HEPES or Tris buffers.

2.2. Cloning geraniol synthases from the EK and PK types of *P. citriodora*

There are three types of oil among Japanese *P. citriodora* species other than type C, namely types EK, PK, and PP (Honda et al., 1994a). The EK and PK type oils are considered to partially share a common synthetic pathway with type C (Honda and Ito, 1998). Leaves of *P. citriodora* of these two types were used to isolate cDNAs of monoterpene synthases by the same method used for type C, which resulted in the isolation of sequences PcTps-EK (GenBank Accession No. DQ234299) from type EK (Strain No. 1861) and PcTps-PK (GenBank Accession No. DQ234298) from type PK (Strain No. 4935) respectively. A sequence comparison of PcTps-C from type C, PcTps-EK from type EK, and PcTps-PK from type PK showed that all of them were completely identical at the nucleotide sequence level. Therefore, both PcTps-EK and PcTps-PK code for a geraniol synthase; in other words, geraniol synthase was expressed in the EK and PK types of *P. citriodora*. This finding supports the presumed synthetic pathway of these furylketones that starts with **15** and continues via **14** and then **4** (Fig. 1).

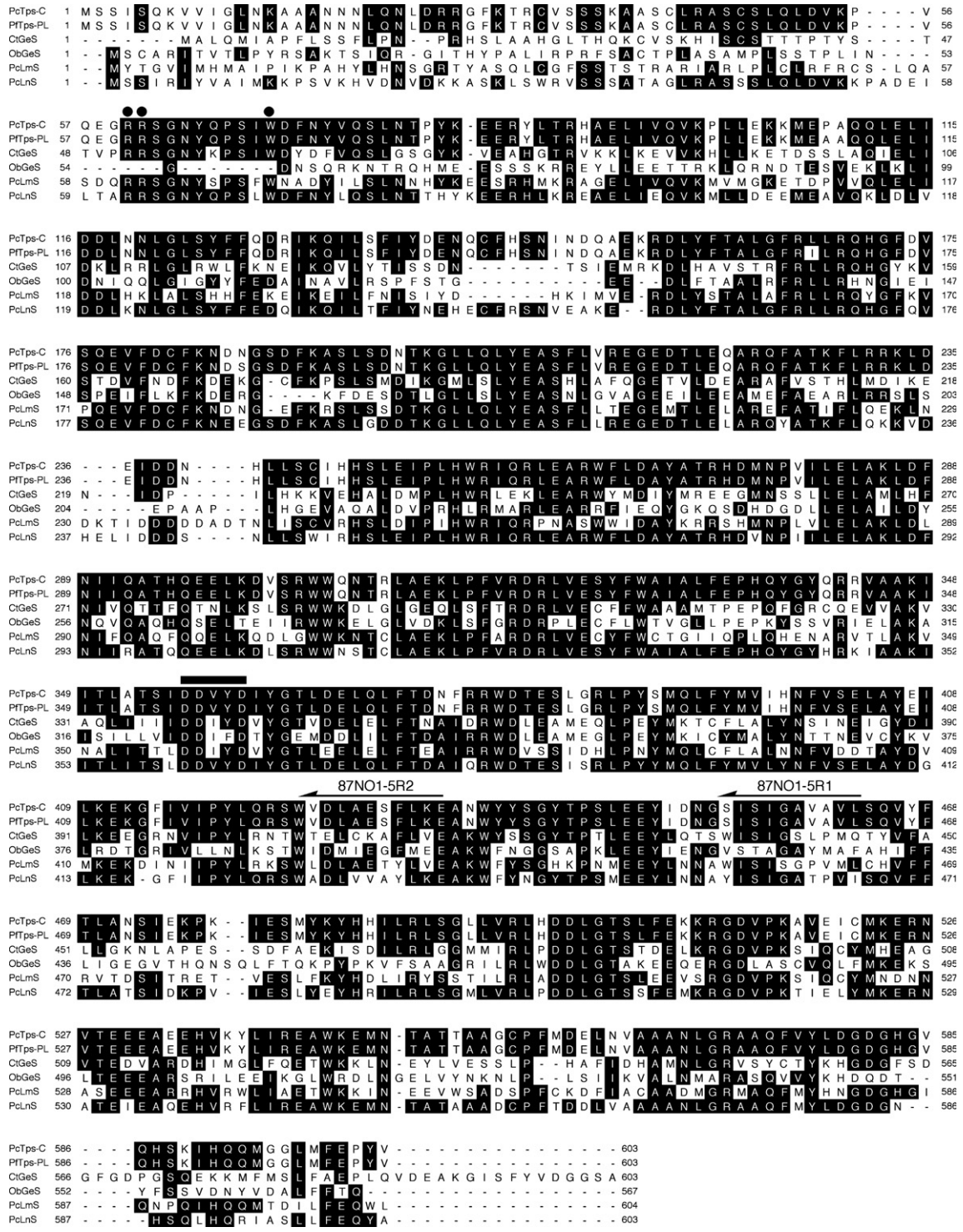

Fig. 2. Alignment of the amino acid sequences of monoterpene synthases. PcTps-C: geraniol synthase from *Perilla citriodora* (Genbank Accession No. DQ088667); PfTps-PL: geraniol synthase from *P. frutescens* (Genbank Accession No. DQ234300); CtGeS: geraniol synthase from *Cinnamomum tenuipilum* (Genbank Accession No. AJ457070); ObGeS: geraniol synthase from *Ocimum basilicum* (Genbank Accession No. AY362553); PcLmS: limonene synthase from *P. citriodora* (Genbank Accession No. AF233894); PcLnS: linalool synthase from *P. citriodora* (Genbank Accession No. AY917193). Closed circles show the RRX_8W motif and a thick bar indicates the DDXXD motif. Arrows indicate the primers 87NO1-5R1 and 87NO1-5R2 used for 5′-RACE.

2.3. Cloning of the geraniol synthase from the PL type of P. frutescens

The same method used for the isolation of monoterpene synthases was applied to the PL type strain of *P. frutescens* (Strain No. 1864) to obtain PfTps-PL consisting of 2083 nucleotides encoding 603 amino acids very similar to PcTps-C (GenBank Accession No. DQ234300). Only three amino acids in the coding region and seven nucleotides within the entire sequence differed between PcTps-C and

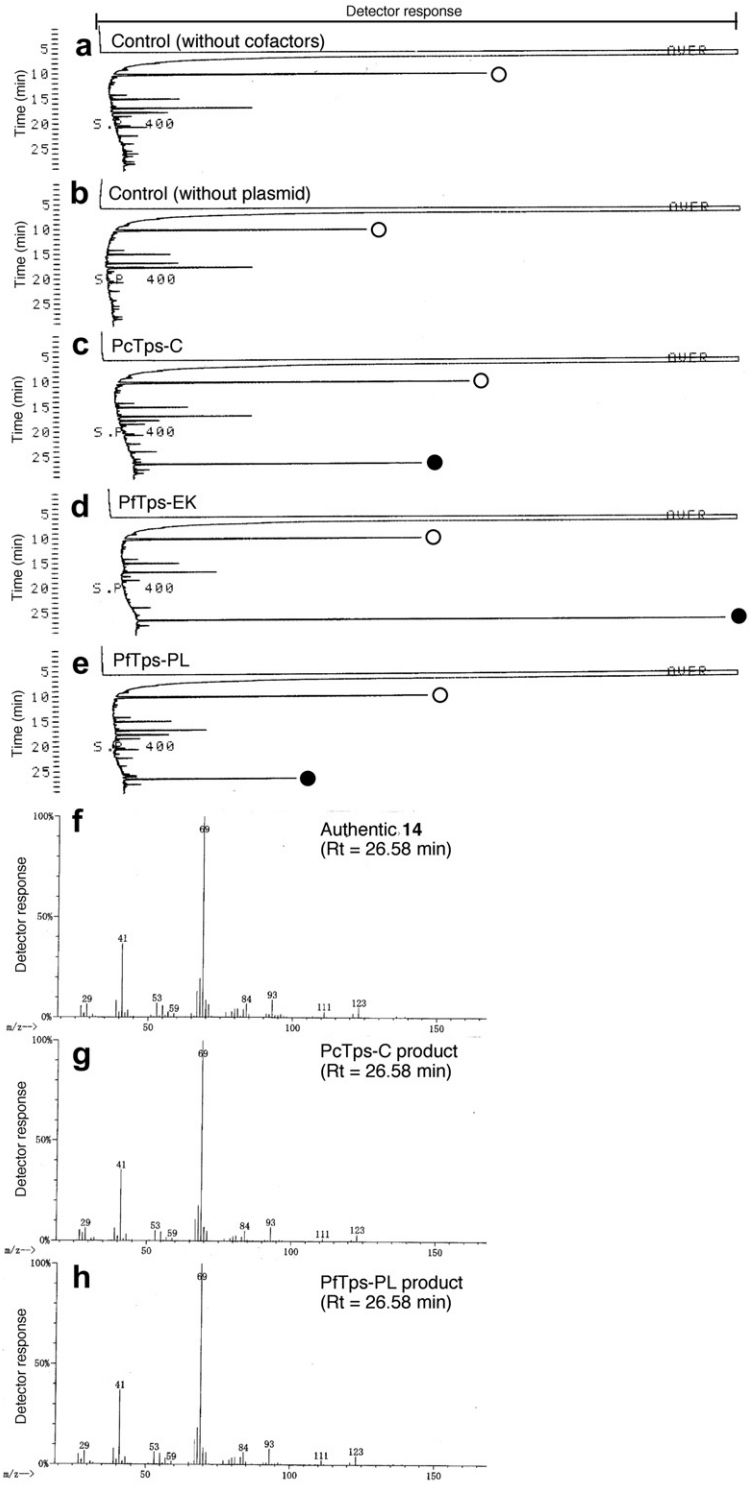

Fig. 3. GC charts (a–e) and MS patterns (f, g, h) of the reaction products of terpene synthases from perilla. (a) Control assay of PcTps-C devoid of cofactors; (b) control assay of extract prepared from *E. coli* devoid of a plasmid; (c) PcTps-C; (d) PfTps-EK; (e) PfTps-PL. Peaks marked with open circles correspond to limonene as an internal standard, and those marked with a closed circle, to **14**. (f) Authentic **14**; (g) reaction product of PcTps-C with Rt = 26.58 min (a peak marked with closed circle in (c)); (h) reaction product of PfTps-PL with Rt = 26.58 min (a peak marked with closed circle in (e)).

PfTps-PL. PfTps-EK was cloned from the EK type strain of *P. frutescens* (Strain No. 79), whose sequence was identical to that of PfTps-PL at the nucleotide level (GenBank Accession No. DQ897973). The heterologous expression of PfTps-PL and subsequent enzymatic reaction of the protein revealed that this enzyme changed **15** into **14** (Fig. 3).

The chromosome number of *P. frutescens* is double that of *P. citriodora*, $2n = 40$ versus $2n = 20$ (Honda and Ito,

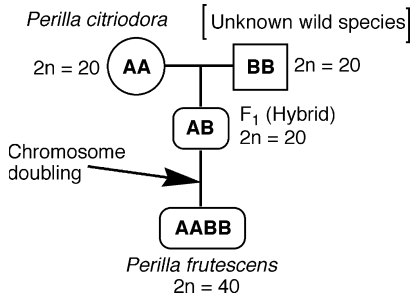

Fig. 4. Hypothesis for the formation of *P. frutescens* as an amphidiploid of two wild species. Each of A and B has a set of chromosomes of the haploid.

1998). The current finding that the sequences of geraniol synthases cloned from *P. citriodora* and *P. frutescens* were mostly the same even in the non-coding regions downstream of the stop codon, supports our assumption that *P. frutescens* is likely to be an amphidiploid of a wild species of perilla (Ito and Honda, 1999). In other words, the cultivated species of perilla (*P. frutescens*) which has $2n = 40$ chromosomes was formed through hybridization between two wild species of perilla, both of which had $2n = 20$ chromosomes and one of which was probably *P. citriodora*. The subsequent chromosome doubling thus resulted from the instability of the chromosome coupling at metaphase in the reproductive cells of the F_1 progeny (Fig. 4). More examples of highly similar sequences derived from *P. frutescens* and *P. citriodora* are found in limonene synthase and in linalool synthase; the limonene synthase from Strain No. 9 (*P. frutescens*, GenBank Accession No. D49368, Yuba et al., 1996) and that from Strain No. 5601 (*P. citriodora*, GenBank Accession No. AF233894, Ito et al., 2000) showed 97% similarity, while the linalool synthase from Strain No. 79 (*P. frutescens*, GenBank Accession No. AF444798) and that from Strain No. 87 (*P. citriodora*, GenBank Accession No. AF917193) were 92% similar (data not shown). The genetic relationship of these very similar enzymes is considered to well reflect the likeliness that *P. citriodora* is an ancestral species of *P. frutescens*.

3. Conclusion

Because the cloning of geraniol synthases from citral (**4**)-containing plants was reported for basil (Iijima et al., 2004a) and perilla, it seems certain that the **4** present in essential oils is formed from geraniol (**14**). In addition, geraniol synthase was cloned from types C, EK, PK, and PL of perilla, which supports the biosynthetic pathway proposed by Hegnauer (1966).

Genetic control patterns of oil types of perilla are mostly the same in both wild species and cultivated species. Here we reported on geraniol synthases in types EK and PK of *P. citriodora*, and type EK of *P. frutescens*. This synthase is supposed to be expressed in the PK type of *P. frutescens*, however, no **14** was detected in our GC–MS analyses of steam-distilled oil from leaves of the types EK or PK of perilla (Ito et al., 1999a). This absence of **14** might be explained by the presence of a highly active **14**-converting enzyme in these plants. Meanwhile, quite a large amount of **14** was found in the C type oil (Ito et al., 1999a,b), which preserves the possibility that the pathway of furylketone formation might have branched from that of **4** formation just after the pathway of **14** formation. We previously isolated myrcene synthase from the PK type of *P. frutescens* (Hosoi et al., 2004) and demonstrated that it converted **15** into myrcene and three other compounds. While suspected that one of these products might be an intermediate of furylketone formation, this turned out to be unlikely.

Since different patterns of furan ring formation are predicted between elsholtziaketone (**5**) and perillaketone (**3**) formation, the substrate of ring formation is likely to be neral (*cis*-**4**) for **5** and geranial (*trans*-**4**) for **3** (Fig. 1). However, nerol was not found among the reaction products of the geraniol synthase. Nor was nerol synthase isolated during the course of cloning, so the *cis*-**4** for **5** synthesis is likely to be formed by a spontaneous tautomeric reaction between *trans*- and *cis*-**4**. A similar observation and calculation were made for the basil geraniol synthase, although neither the presence nor absence of a *trans*-/*cis*-**4** tautomer has been demonstrated (Iijima et al., 2004a). Recently, dehydrogenases capable of oxidizing **14** to produce *trans*- and *cis*-**4** were cloned from a basil expressed sequence tag (EST) library, which confirmed the above deduction (Iijima et al., 2006). Furan ring formation seems to be a rather fast reaction in perilla, which might result in the absence or a trace amount of **4** in the EK or PK type oil (Ito et al., 1999a). In addition, this reaction is expected to be catalyzed by a sort of cytochrome P450 monooxygenase given the menthofuran synthase in mint (Bertea et al., 2001).

Along with the geraniol synthase, many clones of a putative linalool synthase were found in the cDNAs of the C type perilla, which agrees with the finding that linalool was specifically detected in the C type oil as a substantial component (Ito et al., 1999b). On the other hand, a small amount of linalool was present in the oil of almost all strains of perilla. More experiments and discussion are needed to clarify its genetic control.

The almost identical sequences of the geraniol synthases isolated from *P. citriodora* and from *P. frutescens*, along with previously reported morphological comparisons (Ito and Honda, 1996), cytogenetic observations (Honda et al., 1994a), and patterns of RFLP (restriction fragment length polymorphism) and RAPD (randomly amplified polymorphic DNA) (Ito et al., 1998), show the possibility of the formation of *P. frutescens* as an amphidiploid of *P. citriodora* and another unknown wild species. We already demonstrated that two Japanese wild species other than *P. citriodora* were less likely to be involved in the formation of *P. frutescens* by actually making amphidiploids of these wild species and comparing the seed fertility ratio of their progenies for three generations (Ito and Honda,

1999). Field surveys pursuing the ancestral species of perilla have been and will continue to be performed in Eastern Asian countries to check on the results of the experiments carried out in the laboratory.

4. Experimental

4.1. General experimental procedures

Authentic geraniol, and all chemical reagents and solvents were purchased from Nacalai Tesque Inc. or Wako Pure Chemical Industries Co. Gel and plasmid extraction kits were from Viogene, the His-tagged protein purification kit was from Qiagen, and vectors and *E. coli* competent cells were from Invitrogen. Polymerase chain reactions were performed on a PCR Thermal Cycler Personal (Takara). GC measurements were carried out on a G-5000 (Hitachi), whereas GC–MS measurements employed a 5890 series II (Hewlett Packard) connected to an AUTOMASS 50 (JEOL). DNA sequencing was performed by the Genomic Research Center of Shimadzu Co.

4.2. Plant materials

All perilla plants used in this study were grown in the Experimental Station for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University. They have been bred and kept as pure lines through repeated self-pollination, by bagging flowers with paper pollinating bags before they open and collecting seeds when they mature. Strain numbers, oil types and species names of perilla used in this study were as follows; type C {No. 87, *P. citriodora*}, type EK {No. 79, *P. frutescens*} {No. 1861, *P. citriodora*}, type PK {No. 4935, *P. citriodora*}, and type PL {No. 1864, *P. frutescens*}.

4.3. Determination of cDNA sequence of monoterpene synthase from perilla

Total RNA was isolated from fresh young leaves of perilla using an RNeasy Plant Mini Kit, and reverse transcribed with primer add {5′-GGCCACGCGTCGACTA CTTTTTTTTTTTTTTTT-3′} by RevTra Ace (Toyobo). The synthesized cDNA was used as a template for RT-PCR for 3′-RACE (randomly amplified cDNA ends) performed in a reaction mixture containing 0.025 U/μl KOD-Dash, 0.2 mM dNTP, 0.2 μM primer amm {5′-GGCCACGCGTCGACTAC-3′}, 0.4 μM primer ddmot3 {5′-TAGATGATGTTTACGAT-3′}, and 5% DMSO with a temperature program starting at 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 20 s, 74 °C for 30 s, and a final elongation at 74 °C for 30 s. The reaction products were electrophoresed in agarose gel, cut out to be extracted from the gel, ligated to the vector pCR4-TOPO (Invitrogen), and introduced into TOP10F′ *E. coli* (Invitrogen). Amplified plasmids ligated with RT-PCR fragments were subjected to sequencing. Primers 87NO1-5R1 {5′-TAATACTGCAACTGCCCAATTGAAATGC T-3′} and 87NO1-5R2 {5′-TTCTTTTAAAAATGATTCC GCCAGATCTAC-3′} were designed based on the sequence of the RT-PCR fragment. Primer 87NO1-5R1 was used for 5′-RACE reverse transcription. A poly C tail was appended to the cDNA for 5′-RACE by terminal dideoxynucleotidyl transferase (Toyobo), and the cDNA was purified on a PCR-M column (Viogene). This poly C tail-appended cDNA was used as a template for PCR performed in a reaction mixture containing 0.05 U/μl KOD-Plus, 0.2 mM dNTP, 0.75 mM $MgSO_4$, 0.2 μM primer 87NO1-5R1, and 0.2 μM primer 5ann {5′-GGCCACGC GTCGACTAGTACGGGIIGGGIIGGGIIG-3′} with a temperature program starting at 95 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 1 min 30 s, and a final elongation at 68 °C for 1 min 30 s. The reaction products were used as a template for a nested PCR using primer 87NO1-5R2 and primer arm with a temperature program starting at 95 °C for 2 min followed by 39 cycles of 94 °C for 30 s, 50 °C for 20 s, 68 °C for 1 min 45 s, and a final elongation at 68 °C for 1 min 45 s. Cloning and sequencing of the PCR products were the same as for the 3′-RACE to determine the complete sequence of the monoterpene synthase.

4.4. Expression of PcTps-C in E. coli and His-tagged protein purification

Primers for expression clones, primer 87-15ex-f {5′-ATGTCTAGCATTAGCCAGAAGGTGGTAATC-3′} and primer 87-15ex-r {5′-GACATATGGCTCGAACA-TTAGGCCTCCCAT-3′} both for amplification of the full-length clone, and primer 87-15tr-f {5′-ATGCGACG-CAGTGGAAACTACCAACCTTCC-3′} for the targeting-peptide-truncated clone, were designed according to the sequence determined by the RACE method, and the full-length or truncated PcTps-C amplified by PCR using these primers was ligated to pCRT7/CT-TOPO. After confirmation of the sequence, the plasmids were introduced into *E. coli* BL21(DE3)pLysS, and the transformed cells were cultured in LB medium containing 1 mM IPTG for 20 h at 16 °C for protein expression. Cultivated *E. coli* cells were harvested by centrifuge, resuspended in phosphate buffer, and sonicated and purified using a Ni-NTA Spin Kit (Qiagen) following the manufacturer's protocol.

4.5. Enzyme assays and GC–MS analysis

Enzymatic reactions were performed in screw-capped glass tubes with a solution of 1 ml containing 50 mM HEPES or Tris buffer (pH 8.0), 10% (v/w) glycerol, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 20 mM $MgCl_2$, 28 μM GPP, and His-tag purified recombinant protein extracted from a 3-ml culture of *E. coli*, overlaid with 500 μl of pentane. Heat-inactivated protein solution, a preparation devoid of cofactors, and extracts prepared

from *E. coli* devoid of a plasmid were employed as controls. The reaction mixture was kept in an incubator at 30 °C for 10 h (40 min for kinetic analyses), and then extracted 3 times with pentane, while 12 nmol of (−)-limonene in 10 μl of hexane was added to each tube as an internal standard just before extraction. The pentane fractions were combined and concentrated under a N_2 gas stream to be injected into the GC or GC–MS system equipped with a TC-WAX-fused capillary column (60 m × 0.25 mm I.D., film thickness 0.25 μm) conditioned as follows: injector: 180 °C; detector (for GC): FID, 210 °C; ionization voltage (for MS): 70 eV; oven program starting at 100 °C, holding at this temperature for 5 min, then increasing 5 °C per min to 200 °C, and holding at this temperature for 5 min.

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