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Michiho Ito
Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshida-Shimoadachi, Sakyo-ku, Kyoto 606-8501, Japan.
E: michihoi@pharm.kyoto-u.ac.jp

Lixing Wang
Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshida-Shimoadachi, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail: oureikou@gmail.com

Correspondence:
Michiho Ito
Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshida-Shimoadachi, Sakyo-ku, Kyoto 606-8501, Japan

A Linalool Synthase from Taiwanese *Perilla citriodora*

Lixing Wang, Michiho Ito

ABSTRACT

A linalool synthase gene was isolated from a pure strain of Taiwanese *Perilla citriodora*, which has a different essential oil composition from that of other wild species of perilla and only trace amount of linalool was detected from the plant. The amino acid sequence of the linalool synthase gene was very similar to that of previously reported Japanese wild species. The isolated linalool synthase had a K_m value of about 33 μM for geranyl diphosphate (GDP), which is considerably larger than that of limonene synthase from the same plant and of other known linalool synthases. Semi-quantitative PCR analysis of the linalool and limonene synthases also indicated the gene expression of linalool synthase was lower than that of limonene synthase. It was proposed that the low gene expression level and the large K_m value resulted in the trace amount of linalool in the plants.

Keywords: *Perilla citriodora*, Molecular cloning, Linalool synthase, Kinetic assays, Biosynthetic pathway

1. Introduction

Plant essential oils often contain monoterpenes, which are C10 compounds formed by a combination of two isoprene units. A large number of monoterpene compounds are used for food, perfumery, and flavoring because they are generally volatile and fragrant. Many monoterpenes have antibacterial and antitumor activities [1, 2], and they are important in pharmaceutical science. Perilla, which is a common annual herb in Asia, contains characteristic monoterpene compounds, and is designated as a medicinal in the Japanese Pharmacopoeia [3].

The oils that have been observed in perilla are genetically controlled. The regulatory steps for the oils in their putative biosynthetic pathways have been investigated by crossing experiments, using pure strains developed by repeated self-pollination [4, 5].

The oils produced by perilla can be divided into two groups: the monoterpene (MT) group, which is composed mainly of monoterpene compounds; and the phenylpropene (PP) group, which is composed mainly of phenylpropene derivatives (Fig. 1). Both types of oil contain dozens of aliphatic and terpene compounds, and can be further classified according to their principal constituents. The MT group contains: perillaldehyde (PA, 2), piperitenone (PT, 3), citral (C, 5), perillene (PL, 6), perillaketone (PK, 7), elsholtziaketone (EK, 9), and shisofuran (SF,10). The PP group contains: myristicin (PP-m, 11), dillapiol (PP-dm, 12 + 11), elemicin (PP-em, 14 + 12), PP-dem (12 + 14 + 13), and PP-dmn (12 + 11 + nothoapiol, 13) [6]. The PA oil is used in Japanese medicine. In plant taxonomy, Perilla can be classified into four species: *Perilla frutescens*, which is the cultivated species; and *P. citriodora*, *P. hirtella*, and *P. setoyensis*, which are wild species. The chromosome numbers of the cultivated and wild species are 2n = 40 and 2n = 20, respectively [7]. Previous crossing experiments and DNA polymorphisms suggest that *P. frutescens* is an amphidiploid of two wild species [8, 9]. During the formation of an amphidiploid, the alteration of morphological characteristics and the modification of the secondary metabolite synthetic pathways, such as those for the constituents of essential oils, were thought to occur simultaneously with crossing and natural selection. The generation of various types of oil in perilla can elucidate how the cultivated species was formed from the wild species [10]. Taiwanese *P. citriodora* (strain No. 5601) is a unique species, in which limonene is one of the main compounds. Limonene synthase has previously been cloned from this species, although linalool, which was thought to be present in the essential oils of all strains of perilla, was not detected from the steam distillation of strain No. 5601 by gas chromatography-mass spectrometry (GC-MS) analysis [11]. Thus, the biosynthetic genes of Taiwanese *P. citriodora* are particularly interesting. In this report, the linalool synthase was investigated to clarify the reason for the trace amount of linalool in the plant.

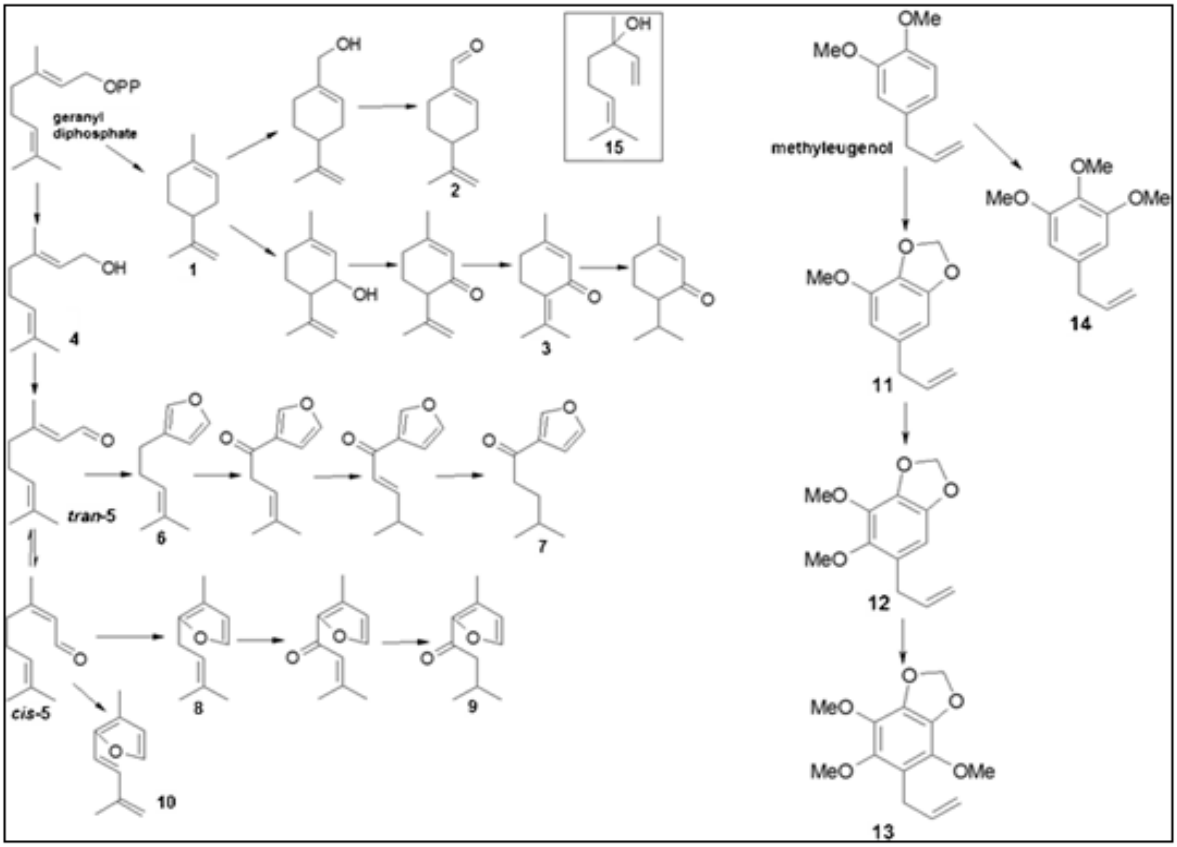

Fig 1: Putative biosynthetic pathways of oil constituents in perilla
(1) limonene; (2) perillaldehyde (PA); (3) piperitenone (PT); (4) geraniol; (5) citral (C); (6) perillene (PL); (7) perillaketone (PK); (8) α-naginatene; (9) elsholziaketone (EK); (10) shisofuran (SF); (11) myristicin; (12) dillapiol; (13) nothoapiol; (14) elemicine; (15) linalool

2. Materials and Methods

2.1 General experimental procedures

Authentic linalool, chemical reagents, and solvents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) or Wako Pure Chemical Industries Co. (Osaka, Japan). The gel and plasmid extraction kits were from NucleoSpin, the His-tagged protein purification kit was from Qiagen (Hilden, Germany), SPME fiber (100 μm polydimethylsiloxane) was purchased from Supelco (Bellefonte, IL, USA), and the vectors and *E. coli* competent cells were purchased from Invitrogen (CA, USA). The polymerase chain reactions were performed on a PCR thermal cycler PERSONAL (TaKaRa, Shiga, Japan). The GC measurements were carried out on a G-5000 gas chromatograph (Hitachi, Tokyo, Japan), and the GC-MS measurements were performed on a G-7000 gas chromatograph (Hitachi, Tokyo, Japan) connected to an M9000 mass spectrometer (Hitachi, Tokyo, Japan). The DNA sequencing was performed by Bio Matrix Research Inc (Chiba, Japan).

2.2 Plant materials

The Taiwanese *P. citriodora* [Strain No. 5601] used in this study was grown in the Experimental Station for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University. It has been bred and maintained as a pure line as previously reported [9].

2.3 *Terpene compounds from strain No. 5601leaves*

Volatile compounds in the leaves of strain No. 5601 were analyzed by SPME-GC/MS. Fresh leaves were pounded with a glass rod in a 4 mL glass vial. The vial was immediately capped and the volatiles in vial headspace were extracted with SPME fiber at 25 °C for 30 s. The essential oils in the glandular trichome were analyzed by cleaving a single glandular trichome, and collecting the secretions with SPME fiber under an optical microscope. The compounds were desorbed from the SPME fiber by placing it for 10 min in the GC injector port of a G-7000 gas chromatograph at 200 °C. The compounds were separated on a TC-WAX-fused silica capillary column (60 m × e0.25 mm, 0.25 μm film thickness; GL Sciences Inc., Tokyo, Japan). The temperature program began at 100 °C for 5 min, increased by 5 °C min^{-1} to 200 °C, and remained at 200 °C for 5 min. Helium was used as the carrier gas, and the column flow was 1.0 mL min^{-1}. An M9000 mass spectrometer was connected to the G-7000 gas chromatograph. The flame ionization detector (FID) temperature was 250 °C. The MS mode was EI and the ionization voltage for MS was 70 eV. The mass range was 40-350 U. The terpene products were identified by comparing the ion spectra and the relative retention times with those of authentic compounds. The GC-MS total ion chromatograms are shown in Fig. 2 and 5.

2.4 Determination of cDNA sequence of putative linalool synthase from strain No. 5601

The total RNA was isolated from fresh young perilla leaves, and was reverse transcribed to synthesize cDNA. The complete sequence of linalool synthase was determined by the RACE method. The details of this procedure have been published elsewhere [9, 12]. The primer used in reverse transcription was add2 (5'-CCACGCGTCGACTACTTTT TTTTTTTTTTT-3'). Primers amm2 (5'-CCACGCGTCGACTAC-

3') and ddmot4 (5'-GATGATGTTTACGATATCTATGGTAC-3') were used in 3'-RACE. Primers 5ann (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIGGGIIG-3'), 5073-3R-7 (5'-GAGCTCCGAAACAAAGTTGTAGAGTA-3'), and 5031-5R-linal-1 (5'-GCGTCTGTAAATAGTTGTAGTTCGTCT-3') were used in 5'-RACE, and were based on the sequence determined by 3'-RACE.

2.5. Expression of genes in E. coli and His-tagged protein purification

There was no significant difference in catalytic activities between the expressions of full-length and targeting-peptide-truncated clones of monoterpene synthases from perilla [9]; therefore, targeting-peptide-truncated clones were used in this study. Truncated Tps-5601L was amplified by PCR, and was ligated to Champion pET101D (Invitrogen, CA, USA). After the sequence was confirmed, the plasmids were introduced into *E. coli* BL21 Star (DE3), and the transformed cells were cultured in LB medium containing 1 mM IPTG for 20-24 h at 16 °C to induce protein expression. Cultivated *E. coli* cells were harvested by centrifuge, and resuspended in buffer containing 100 mM Tris (pH 8.0), 20% (v/w) glycerol, 0.5 mM EDTA, and 1 mM dithiothreitol. The cells were sonicated and purified using a Ni-NTA Spin Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

2.6. Enzyme assays and GC-MS analysis

Enzymatic reactions were performed in glass vials (4 mL) sealed with a polytetrafluoroethylene-coated silicone rubber septa in a solution (250 µL) containing 50 mM Tris (pH 7.0), 10% (v/w) glycerol, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 20 mM $MgCl_2$, 28 µM GDP, and His-tag purified recombinant protein (25 µL). A preparation which was free from Mn^{2+} and Mg^{2+} cofactors and contained a heat-inactivated enzyme preparation was used as a control for the assays. The enzymatic reaction mixture was incubated at 30 °C for 16 h. The headspace of the vial was extracted with SPME fiber at 25 °C for 30 min and was analyzed by GC-MS. The analysis conditions were the same as those described in Section 2.3.

2.7 Kinetic analyses

The kinetic analysis of the enzymatic reactions was performed in screw-capped glass tubes in a solution (1 mL) containing 50 mM Tris (pH 7.0), 10% (v/w) glycerol, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 20 mM $MgCl_2$, 2.8–28 µM GDP, and His-tag purified recombinant protein (100 µL) overlaid with pentane (1 mL). The reaction mixture was incubated at 30 °C for 60 min, and then extracted 3 times with pentane. Limonene (6 nmol) in hexane (5 µL) was added to each tube as an internal standard just before extraction. The pentane fractions were combined, concentrated under an N_2 gas stream, and analyzed on a G-5000 gas chromatograph equipped with a TC-WAX-fused silica capillary column (60 m × 0.25 mm ID, film thickness 0.25 µm; GL Sciences Inc., Tokyo, Japan). The oven program started at 70 °C for 5 min, then increased at 5 °C min^{-1} to 200 °C, and finished at 200 °C for 5 min. Injection Temperature was 180 °C and injection volume was 1.0 µL. Helium was used as the carrier gas and the flow rate was 0.8 mL min^{-1}. Injection spilt was 10:1. The GC-FID detector was 210 °C. The H_2 flow was 40 ml/min. The terpene products were identified by comparison with the relative retention times of authentic standards and quantitated by comparison with an internal standard. The kinetic parameters were calculated with Lineweaver-Burk plots.

2.8 Semi-quantitative RT-PCR

The cDNA was prepared from leaves using the method described in Section 2.4. The gene specific primers for linalool synthase were 5601lina-f (5'-ATGTCTAGCATTAGAATATATGTAGCGATC-3'), and 87LSend (5'-TGCATATTGCTCGAACAGCAGGCTCGC-3'). The primers for limonene synthase were nakano-f1 (5'-GCGATTCCTATTAAGCCAGC-3'), and LSend5601 (5'-CAACCATTGCTCGAACAAGATGTCTG-3'). The histone coding region was amplified as the internal control, using primers 5042histone-f (5'-TAAGAAGAAGCCGGTTACCCGCT-3'), and 5042histone-r (5'-CTTGGTTCTGAATCAAAAGCAC-3').

2.9 Leaf extract assays

Extraction buffer (1 mL) which contained 50 mM Tris-HCl pH 7, 10% (v/v) glycerol, 100 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1% (w/v) polyvinylpyrolidone, 1% (w/v) polyvinylpolypyrolidon, was added to fresh leaves (50 mg). The leaves were then ground on ice with a pestle and mortar. The supernatant was obtained by centrifuging the mixture at 4 °C 11,000 × g for 30 min. Linalool diluted in dimethyl sulfoxide (DMSO) was added to the supernatant and the linalool concentration was adjusted to 0.1 mM. The reaction was incubated at 30 °C for 4 h and the products were analyzed by SPME-GC/MS under the conditions described in Section 2.3.

3. Results and discussion

3.1 Limonene and linalool from fresh leaves of strain No. 5601

In the previous GC-MS analysis, the essential oils were extracted by steam distillation and no linalool was detected in strain No. 5601. It was thought that linalool was oxygenated or degraded by the high temperature or the water [11]. Thus, solid phase microextraction (SPME)-GC/MS, which was carried out at room temperature without the oils coming into contact with water or solvent, was used in this study. Various monoterpenes, phenylpropenes, and sesquiterpenes were detected when the volatile compounds from ground fresh leaves were analyzed by SPME-GC/MS. The detection of limonene and linalool was confirmed by comparing their retention times (Rt) and MS-patterns with those of authentic compounds. The peak areas of GC-MS total ion chromatograms do not accurately correspond to the amount of compound, although they can be used for approximate comparison. The peak area of limonene was about 128-fold larger that of linalool, which showed that the amount of linalool was very small (Fig. 2). However, the detection of linalool was consistent with the previous observation that linalool is present in the essential oils of all strains of perilla.

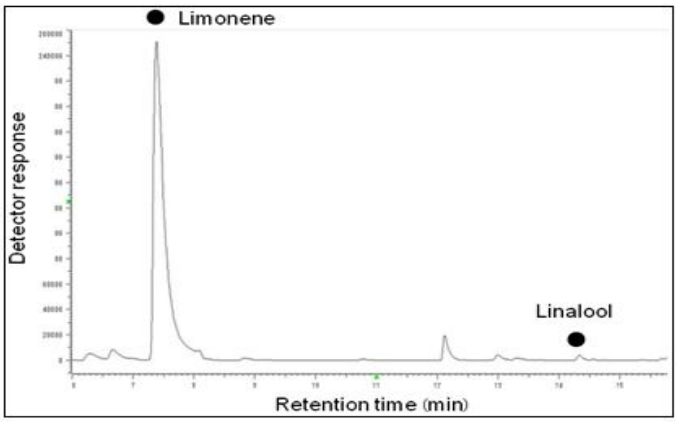

Fig 2: Total ion chromatograms of limonene and linalool from the fresh leaves of *P. citriodora* No. 5601

3.2 Isolation of linalool synthase gene from strain No. 5601 and its expression in *E. coli*

The linalool synthase gene was isolated from the fresh young leaves of strain No. 5601. The full sequence of the clone was determined by the rapid amplification of cDNA ends (RACE) method and was called Tps-5601L. The sequence was composed of 1806 nucleotides encoding 602 amino acids and showed the highest similarity to linalool synthases derived from the Japanese wild species, *P. hirtella* [Strain No. 5042, type PP] (GenBank Accession No. FJ644548) and *P. citriodora* [Strain No. 87, type C] (GenBank Accession No. AY917193), with 98% and 97% identity at the amino acid level, respectively. The DDXXD and RRX_8W motifs, which are highly conserved among monoterpene synthases [13], were also found in Tps-5601L (Fig. 3).

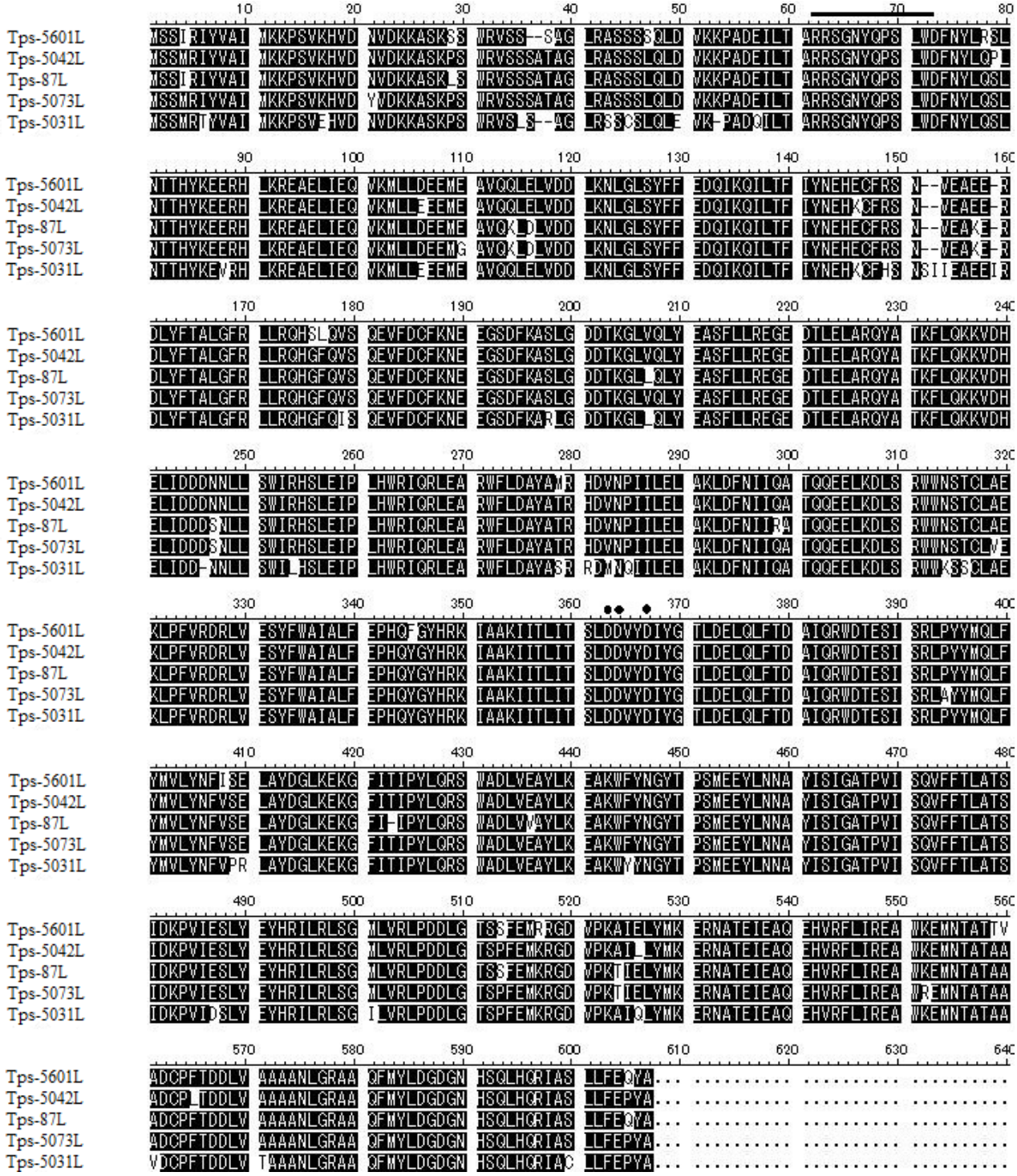

Fig 3: Alignment of the amino acid sequences of the monoterpene synthases described in this study

Tps-5601L: linalool synthase from *P. citriodora* [Strain No. 5601]; Tps-5042L: linalool synthase from *P. hirtella* [Strain No. 5042, PP type] (GenBank Accession No. FJ644548); Tps-87L: linalool synthase from *P. citriodora* [Strain No. 87, C type] (GenBank Accession No. AY917193); Tps-5073L: linalool synthase from *P. hirtella* [Strain No. 5073, PK type] (GenBank Accession No. FJ644546); Tps-5031L: linalool synthase from *P. setoyensis* [Strain No. 5031, SF type] (GenBank Accession No.FJ644544). Filled circles indicate the RRX8W motif and the thick bar indicates the DDXXD motif.

A DDXXD motif, which is a putative binding site for a GDP-divalent metal ion complex [13], is one of the most characteristic features of the amino acid sequences of terpenoid synthases. There are two types of amino acid sequence of this region in perilla monoterpene synthases: DDIYD, which belongs to the cyclic monoterpene synthases such as limonene synthases; and DDVYD, which belongs to the acyclic monoterpene synthases, such as linalool and geraniol synthases [9]. The sequence of this motif in Tps-5601L was DDVYD, which is consistent with an acyclic monoterpene synthase. A transit peptide was also observed directly upstream of the RRX_8W motif in the N-terminal presequence of Tps-5601L. The transit peptide, which is required for the transportation of the immature enzyme across membranes from their original site in the cytoplasm to the operative site in the plastids, is not involved in the enzymatic expression and activities [9, 14]. Therefore, this region was truncated from the sequence and a new methionine was added artificially to the rest of the sequence to produce the expression sequence, called Tps-5601L-RR. The heterologous expression of Tps-5601L-RR was performed using *E. coli*, and the enzymatic assays were conducted using a His-tag-purified protein. The enzymatic reaction with Tps-5601L-RR transformed GDP into linalool (Fig. 4A, 4E). The enzyme solutions controls, which contained no Mn^{2+} or Mg^{2+} cofactors, produced no product (Fig. 4B).

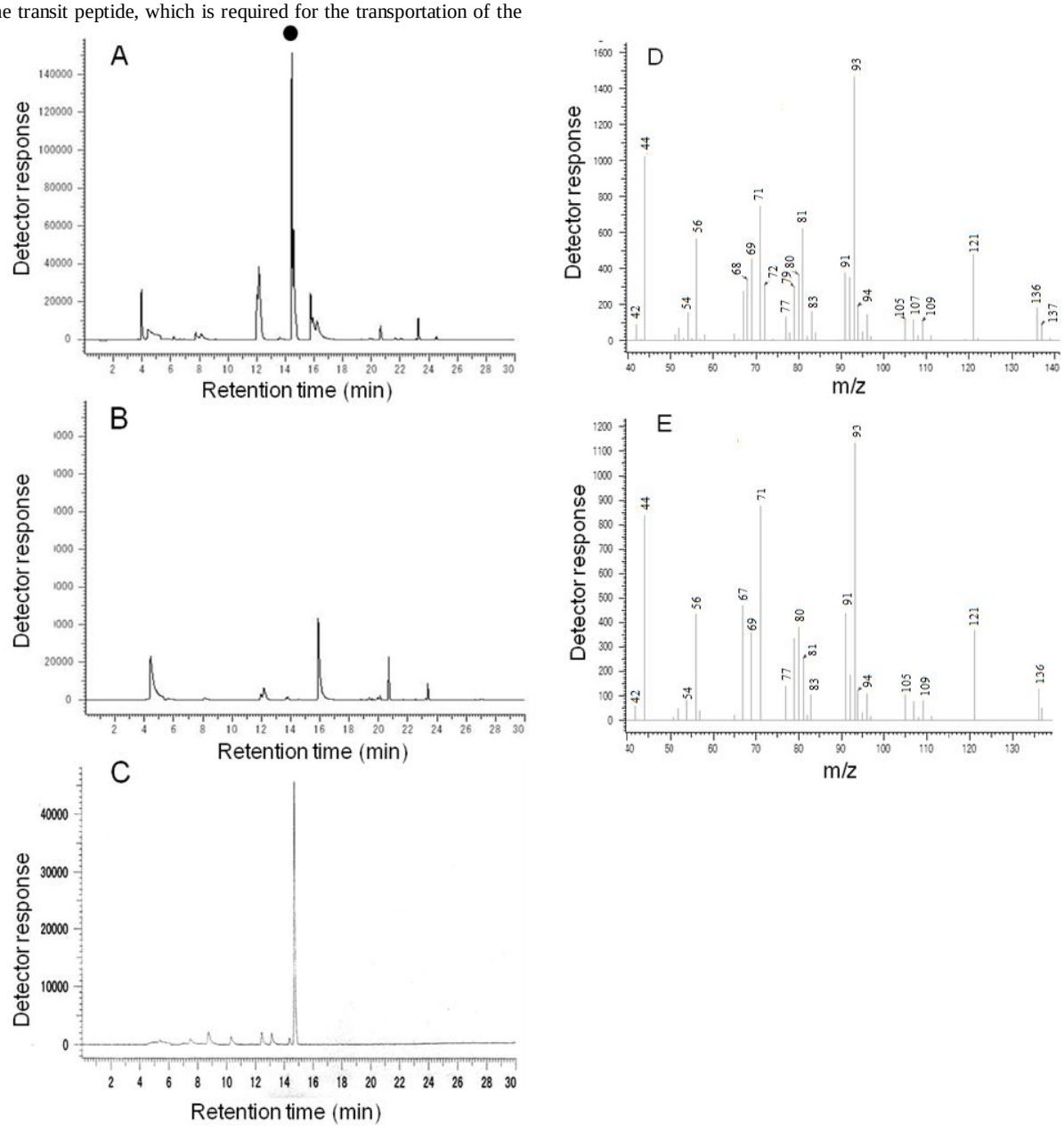

Fig 4: GC total ion chromatograms (A, B, C) and MS patterns (D, E) of the reaction product of linalool synthases from Tps-5601L
(A) Reaction product of Tps-5601L-RR. The peak marked with a filled circle corresponds to linalool. (B) Control Tps-5601L assay with no cofactors. (C) Authentic linalool. (D) MS pattern of authentic linalool. (E) MS pattern of Tps-5601L-RR reaction product with a Rt = 14.7 min, which corresponds to the peak marked with a filled circle in (A).

3.3 Kinetic analysis of the linalool and limonene synthases of strain No. 5601

We hypothesized that linalool and limonene synthases compete for the GDP substrate, because both enzymes are cloned from strain No. 5601. However, if these enzymes are localized in different trichomes, there may be little competition. A single glandular trichome on a fully expanded fresh leaf was analyzed by SPME-GC/MS, which confirmed that both limonene and linalool were produced in the same glandular trichome (Fig. 5).

The kinetic analysis of Tps-5601L-RR showed that the K_m value of Tps-5601L-RR was about 33 μM, which is much larger than that of the truncated limonene synthase from strain No. 5601 (5.3 μM) and also comparatively larger than that of the linalool synthases previously isolated from *Clarkia breweri*, *Mentha* × *piperita* subsp. *citrata*. (0.9–25 μM) [15]. The K_m value expresses the affinity of the enzyme for the substrate; therefore, in strain No. 5601 the affinity of linalool synthase for GDP may have been much lower than that of limonene synthase. A linalool synthase with a K_m value of about 64 μM, which is significantly larger than that of any other known monoterpene synthases was isolated from a plant that does not normally produce linalool [16]. Besides, although linalool was detectable only in trace amount in the oil, a monoterpene synthase producing linalool was isolated from *Melaleuca alternifolia* [17]. They suggested that the K_m value was too large for the linalool synthase and the linalool biosynthesis was physiologically unlikely. Therefore, even if a linalool synthase was isolated, none or trace amount of linalool was detected in the plant. This may be the case with the linalool synthase in strain No. 5601. Although the linalool synthase gene was isolated from strain No. 5601, the much weaker competition for GDP, the substrate than the limonene synthase was thought to be related to the trace amount of linalool in the plant.

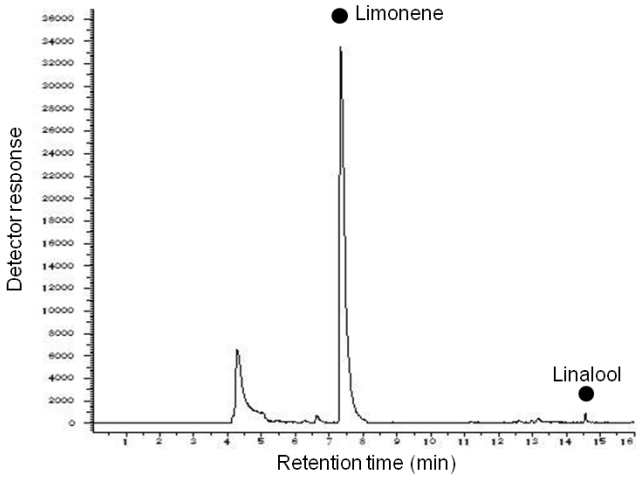

Fig 5: Total ion chromatograms of limonene and linalool from a single glandular trichome on a full-expanded fresh leaf of No. 5601.

However, the K_{cat} value, which is recognized as a measurement of the turnover rate, was larger for Tps-5601L-RR (6.2×10^{-3} s^{-1}) than for limonene synthase (0.8×10^{-3} s^{-1}), which is a cyclic monoterpene synthase in strain No. 5601. This is probably because the linalool synthase catalyzed the initial ionization and rearrangement of GDP, which produced linalool as a principal product [18]. In contrast, limonene synthase catalyzed the synthesis of more structurally complex cyclic products, which resulted in a smaller K_{cat} value. However, the K_{cat} value of Tps-5601L-RR was about 1/40 that of the linalool synthase from *Mentha* × *piperita* subsp. *citrata* (0.24 s^{-1}) [19]. In addition, there was no difference in the K_{cat}/K_m catalytic rates of strain No. 5601 for the limonene synthase (0.15 s^{-1}/mM) and linalool synthase (0.19 s^{-1}/mM). The K_{cat}/K_m rate of linalool synthase from *Mentha* × *piperita* subsp. *citrata* (9.6 s^{-1}/mM) was about 56-fold that of the linalool synthase from strain No. 5601. These results indicate that the activity of the linalool synthase from strain No. 5601 is quite weak.

3.4 Analysis of Tps-5601L and limonene synthase gene expression by semi-quantitative RT-PCR

In addition to the enzymatic kinetics, the gene expression was thought to be another factor that results in the difference in the amount of limonene and linalool. Therefore, the gene expression of the limonene and linalool synthases was also investigated by semi-quantitative RT-PCR. The expression level of linalool synthase was much lower than that of limonene synthase in the fresh leaves of strain No. 5601 (Fig. 6). Interestingly, this suggests that the regulatory systems for these two enzymes are different, which merits further investigation in future studies.

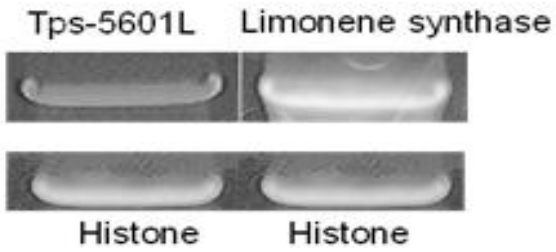

Fig 6: Tps-5601L and limonene synthase cDNA expression in No. 5601 fresh leaves

The expression of histone gene, a housekeeping gene was used as control

3.5 Analysis of volatile compounds emitted by strain No. 5601 and the metabolites of the reaction between the crude enzymes and linalool

It was shown that the low levels of linalool were not caused by its emission from the leaves or its metabolism. The emitted volatile compounds were collected by sealing strain No. 5601 plants in a plastic bag, and then analyzed by SPME-GC/MS. Crude enzyme derived from fresh leaves of strain No. 5601 was prepared and linalool was added to the preparation. Linalool was not found in the emitted gas, and no linalool metabolites were detected in the products of the enzymatic reactions. This suggests that the low levels of linalool were detected because low levels of linalool were produced in the plant.

4. Conclusion

A linalool synthase gene was isolated from a wild species of perilla, Taiwanese *P. citriodora* strain No. 5601. Its sequence showed high homogeneity with those previously isolated from Japanese *P. citriodora*, *P. hirtella*, and *P. setoyensis*.

Both linalool and limonene synthases cloned from strain No. 5601 catalyzed the transformation of GDP into corresponding monoterpenes. Both linalool and limonene were detected in the same glandular trichome, which indirectly indicated that the two enzymes are not separately localized. Linalool was neither emitted nor metabolized, and the amount of linalool accumulated in the fresh leaves was low compared with that of limonene. This difference arose from the large K_m value, which indicated a weak affinity for substrate, and the low expression level of the linalool synthase.

Linalool synthase was not thought to be involved in the biosynthetic network of essential oil compounds [20]; however, its product, linalool, often appears with many other oil compounds. Further elucidation of the regulatory systems of the expression and activities of synthases in the plant body is essential for a comprehensive understanding of the unique biosynthesis of oil constituents.

5. Acknowledgement

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Pharmaceutical Sciences

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