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Geraniol and linalool synthases from wild species of perilla

Naoko Masumoto [1], Miyuki Korin [1], Michiho Ito *

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

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ABSTRACT

Geraniol and linalool synthases were isolated from three pure strains of *Perilla hirtella* and *Perilla setoyensis*, which are wild species of perilla. Their amino acid sequences were very similar to those of *Perilla citriodora* and *Perilla frutescens* that were reported previously. However, comparison of the sequences of the same functional synthases derived from different species of *Perilla* demonstrated that the similarities were high among *P. citriodora*, *P. hirtella* and *P. frutescens*, but low between *P. setoyensis* and any of the others. This result corresponds well with our previous results showing that *P. setoyensis* is remotely related to the other perilla species. Both geraniol and linalool synthases utilize geranyl diphosphate (GDP) as their catalytic substrate and they were expressed simultaneously in perilla. The linalool synthase is considered to be the enzyme whose metabolite seems not to be oxidized nor reduced in the plant body and the geraniol and limonene synthases are the initial-step-catalyzing enzymes for a variety of oil compounds. The regulation of the substrate flow between them would be interesting for further study.

1. Introduction

Monoterpenes, C10 compounds composed of two isoprene units, are generally volatile and fragrant, and often found in plant essential oils. Many monoterpene compounds are used for perfumery and flavoring of either food or medicine, and quite a few have antibacterial or antitumor activities (Chung et al., 2006; Honda et al., 1984). They are thus important compounds in the fields of pharmaceutical sciences. Perilla, an annual oriental herb contains an essential oil composed of unique monoterpene compounds and is designated as a medicinal in Japanese Pharmacopoeia.

Japanese perilla can be classified into four species; one is a cultivated species, *Perilla frutescens*, and the others are wild species, *Perilla citriodora*, *Perilla hirtella* and *Perilla setoyensis*. The cultivated and wild species are different in their chromosome number, which is $2n = 40$ in the former and $2n = 20$ in the latter (Ito and Honda, 1996). *P. frutescens* is hypothesized to be an amphidiploid of two wild species according to the previous results of crossing experiments and DNA polymorphisms (Honda and Ito, 1998; Ito and Honda, 2007). In the process of forming the amphidiploid, crossing and natural selections of the progenies should have occurred and concurrent modification of the synthetic pathways of secondary metabolites such as constituents of essential oils was supposed to be followed along with alteration of morphological characteristics. Investigating the process of generating various oil types that the present perilla harbors would at the same time help elucidate how the cultivated species was formed from wild species (Ito, 2008).

Oil types of perilla can be divided into two different groups; the MT (monoterpene) type whose oil is mainly composed of monoterpene compounds, and the PP (phenylpropene) type whose oil contains mostly phenylpropene derivatives (Fig. 1). Both types contain dozens of aliphatic and terpene compounds in their oils, and can be further classified according to their principal constituent; types C (citral, **1**), EK (elsholtziaketone, **2**), PA (perillaldehyde, **3**), PK (perillaketone, **4**), PL (perillene, **5**), PT (piperitenone, **6**), and SF (shisofuran, **7**) are in the MT group, and types PP-m (myristicin, **8**), PP-dm (dillapiol, **9** + **8**), PP-em (elemicin, **10** + **9**), PP-dem (**9** + **10** + **8**), and PP-dmn (**9** + **8** + nothoapiol, **11**), are in the PP group (Ito et al., 1999). The main oil components of any of the types in the MT group are not found in oils of the PP types, and vice versa, although some compounds like linalool and β-caryophyllene are included in all perilla plants. These oil types of perilla were shown to be genetically controlled, and the regulatory steps in their putative biosynthetic pathways have been investigated by crossing experiments using the pure strains developed by repeated self-pollination (Honda, 1996; Ito et al., 2002).

The function of each gene identified by crossing experiments can be determined by cloning of an enzyme which catalyzes the relevant reaction step in the biosynthetic pathways. The primary reaction step in the putative pathways of the MT type constituents is a dephosphorylation reaction using geranyl diphosphate (GDP,

* Corresponding author. Tel.: +81 75 753 4507.
E-mail address: michihoi@pharm.kyoto-u.ac.jp (M. Ito).
[1] These authors contributed equally to this work.

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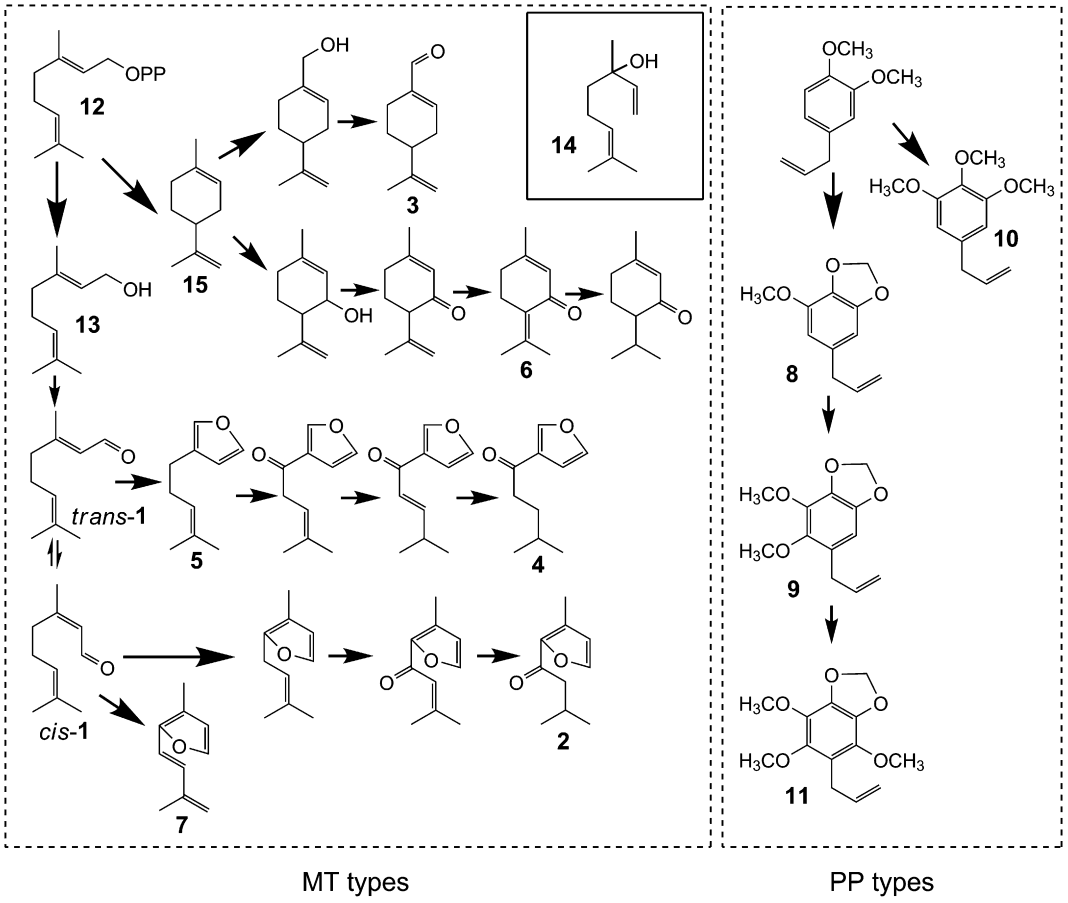

Fig. 1. Putative biosynthetic pathways of oil constituents in perilla. (**1**) citral (C); (**2**) elsholtziaketone (EK); (**3**) perillaldehyde (PA); (**4**) perillaketone (PK); (**5**) perillene (PL); (**6**) piperitenone (PT); (**7**) shisofuran (SF); (**8**) myristicin; (**9**) dillapiol; (**10**) elemicin; (**11**) nothoapiol; (**12**) geranyl diphosphate (GDP); (**13**) geraniol; (**14**) linalool; (**15**) limonene.

12) as a substrate, which is catalyzed by either geraniol or limonene synthase. Synthases derived from both *P. citriodora* and *P. frutescens* were cloned (GenBank Accession Nos. AF323432**,** AF233894**, DQ088667,** DQ234300) and shown to be highly similar (Ito and Honda, 2007; Yuba et al., 1996; Ito et al., 2000). Furthermore, the synthases of linalool (GenBank Accession Nos. AF444798 **,** AY917193), which is known as a "dead end product" in the general monoterpene biosynthetic pathway (Ito, 2008), also have known sequences. Both geraniol (**13**) and linalool (**14**) are acyclic monoterpenes possessing one hydroxyl group in the structure, and their synthetic enzymes demonstrate relatively high homology in the case of perilla. Because linalool is found in essential oils of all perilla, these two synthases should be expressed simultaneously in the MT type plants whose main constituents harbor a furan-ring in their structure, or the compounds which are thought to be formed via geraniol (C, EK, PK, PL and SF types). Studying these two synthases which coexist and share identical substrates in perilla might be interesting both for the fields of botany and enzymology. In this report, geraniol and linalool synthases were isolated from wild species of perilla and were compared with known sequences of the same functionality.

2. Results and discussion

Five clones of cDNA of terpene synthases were isolated from fresh young leaves of the wild species of perilla; one from *P. hirtella* of the PP type [Strain No. 5042], two from *P. hirtella* of the PK type [Strain No. 5073], and two from *P. setoyensis* of the SF type [Strain No. 5031]. The complete sequences of the clones were determined by the RACE method, and named PhTps-5042L (GenBank Accession No. FJ644548) consisting of 1812 nucleotides encoding 604 amino acids, PhTps-5073G (GenBank Accession No. FJ644547) consisting of 1809 nucleotides encoding 603 amino acids, PhTps-5073L (GenBank Accession No. FJ644546) consisting of 1812 nucleotides encoding 604 amino acids, PsTps-5031G (GenBank Accession No. FJ644545) consisting of 1809 nucleotides encoding 603 amino acids, and PsTps-5031L (GenBank Accession No. FJ644544) consisting of 1809 nucleotides encoding 603 amino acids, respectively (Fig. 2). The sequences of PhTps-5073G and PsTps-5031G showed the highest similarity with that of the geraniol synthase derived from *P. frutescens* [Strain No. 79, type EK] (GenBank Accession No. DQ897973), at 96.8% and 97.8% identity in amino acid level, respectively. The sequences of PhTps-5042L and PhTps-5073L showed highest similarity with that of the linalool synthase derived from *P. citriodora* [Strain No. 87, type C] (GenBank Accession No. AY917193), having 97.2% and 98.0% identity. The sequence of PsTps-5031L showed the highest similarity with that of the linalool synthase derived from *P. frutescens* [Strain No. 79, type EK] (GenBank Accession No. AF444798**)** with 95.5% identity. All sequences of these five clones contained DDXXD and RRX_8W motifs which are highly conserved in monoterpene synthases (Bohlmann et al., 1998; Fig. 2). Heterologous expressions of the full-lengths of PhTps-5073G, PsTps-5031G, PhTps-5042L, PhTps-5073L, and PsTps-5031L were performed using *E. coli* ***and enzymatic assays were done using an His-tag-purified protein. As a result, PhTps-5073G and PsTps-5031G transformed GDP (**12**) into geraniol (**13**) (Fig. 3A, C, F–H), and PhTps-5042L, PhTps-5073L, and