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Molecular cloning and characterization of a *Perilla frutescens* cytochrome P450 enzyme that catalyzes the later steps of perillaldehyde biosynthesis

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ABSTRACT

Perilla produces the cyclohexanoid monoterpene perillaldehyde as a major constituent of an essential oil that is accumulated in its glandular trichomes. Perillaldehyde is a marker compound for quality control of *soyo* and has biological activities such as antibacterial, sedative, or vasodilatory effects. The predicted perillaldehyde formation involves the cyclization of geranyl diphosphate, hydroxylation, and oxidation, and cytochrome P450 plays a crucial role in perillaldehyde biosynthesis. In this study, a cytochrome P450-type enzyme with perillyl alcohol and perillaldehyde synthase activities was isolated by analyzing an expressed sequence tag library from several oil types of pure lines of perilla. A recombinant protein with a sequence that was highly specific for the type of perillaldehyde was expressed in *Saccharomyces cerevisiae* and evaluated by an *in vitro* enzymatic reaction. The recombinant protein catalyzed the hydroxylation and oxidation of limonene to perillyl alcohol and perillaldehyde. Cytochrome P450 limonene-7-hydroxylase cDNA from *Perilla frutescens* has been previously isolated. The cytochrome P450 isolated in this study shares 37% amino-acid identity with the previously isolated enzyme; however, it may have different characteristics.

1. Introduction

Monoterpenes are often major constituents of essential oils derived from plants. Many have biological activities, such as antibacterial or antitumor activities (Trombetta et al., 2005; Mills et al., 1995), and they are important and useful compounds in pharmaceutical sciences. Monoterpenes are also used in flavors and perfumes because they are fragrant and generally volatile at room temperature. Perilla, a common annual Asian herb, is the source of several oil types. The most popular type in Japan is type perillaldehyde (PA) (**5**), which contains perillaldehyde (**5**) as its major compound (Fig. 1). Perilla leaves are also used as a natural medicine for Kampo prescriptions, and as per the Japanese Pharmacopoeia (JP), such preparations should contain at least 0.08% of PA (**5**), calculated by examination of dried preparations. JP also recommends that *soyo*, the leaves and branch tips of *Perilla frutescens* Britton var. *crispa* W. Deane, should be red on at least one side (The Ministry of Health, Labour and Welfare, Seventeenth ed, 2016). A library of genetically pure lines of perilla has been established and many studies has been conducted showing that the syntheses of oil compounds are genetically controlled (Ito et al., 1999a; Ito et al., 1999b, 2002; Honda, 1996). However, the details of the synthetic pathways await elucidation. The initial reaction step for monoterpene oil constituents is believed to be the dephosphorylation of geranyl diphosphate (GDP) (**1**), catalyzed by either geraniol or limonene synthases. PA (**5**) appears to be synthesized by hydroxylation at the C7 position of limonene (**3**) and subsequent oxidation to the aldehyde (**5**). Almost all monoterpene compounds found in perilla essential oil are considered to be synthesized from acyclic compounds derived from GDP (**1**), and the subsequent reactions of cyclization, oxidation, or reduction are considered to make up the different compound structures (Fig. 1). Oxidation/reduction steps of oil compounds in plants are often catalyzed by cytochrome P450, and previous studies in *Mentha* and *Catharanthus* have shown that limonene (**3**) and geraniol (**2**), both constituents of perilla oil, are employed as substrates for cytochrome P450 (Lupien et al., 1999;

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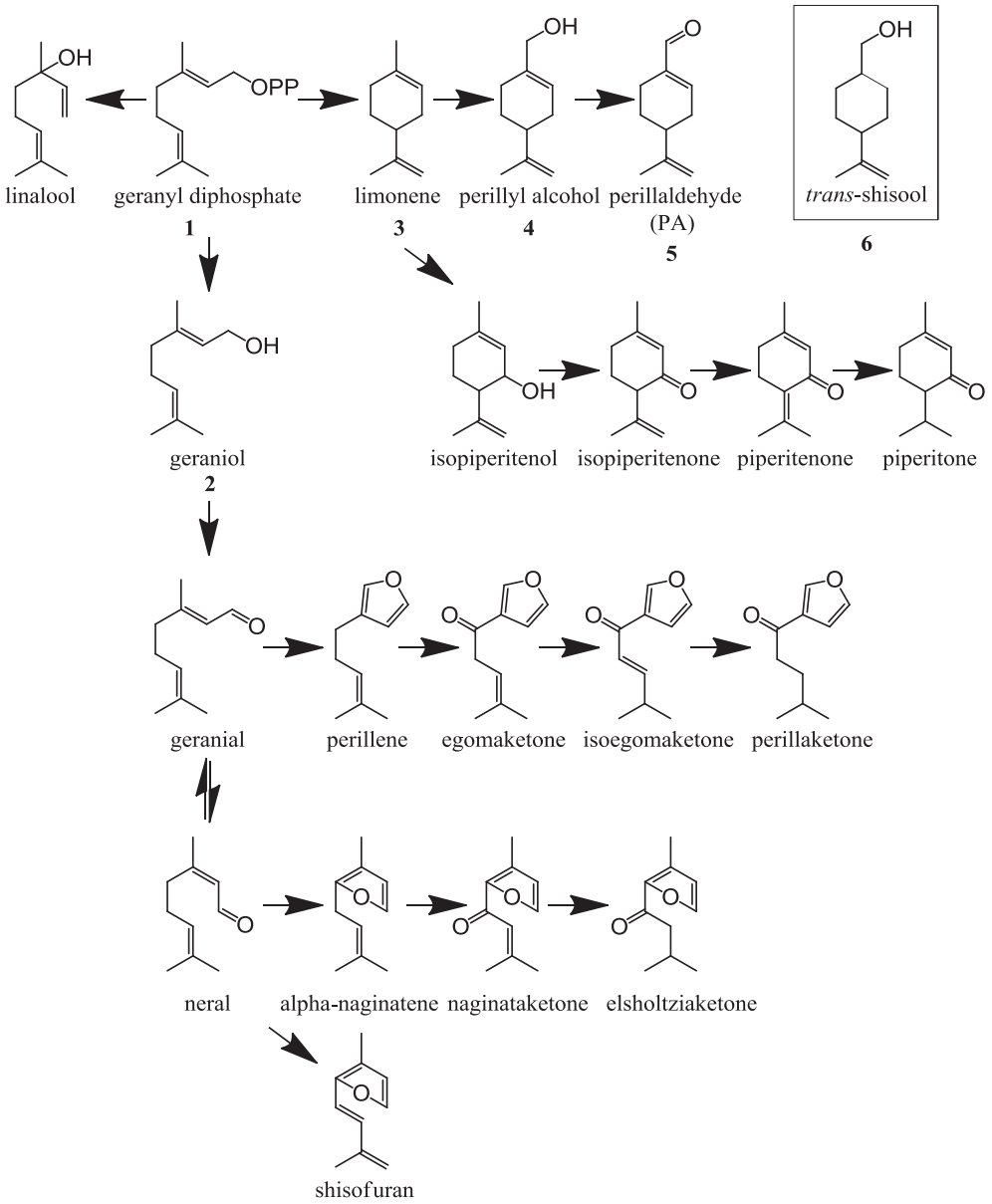

Fig. 1. Putative biosynthetic pathway of oil constituents in MT type perilla. *Trans*-shisool (**6**) may be related to MT biosynthesis. However the step is unclear.

Collu et al., 2001).

Cytochrome P450 monooxygenases are heme-containing enzymes that catalyze a wide range of reactions, including oxidation, hydroxylation, epoxidation, and dealkylation. In plants, many cytochrome P450s mediate the biosynthesis of several secondary metabolites, including plant hormones, fatty acids, and defense compounds. The generation of diverse monoterpenes is usually initiated by cytochrome P450 hydroxylase.

Mau et al. (2010) isolated (−)-limonene-7-hydroxylase from perilla, showing that it catalyzes hydroxylation at the C7 position of limonene (**3**). Their study employed a hybridization strategy using previously cloned mint limonene hydroxylase cDNA as a probe. *P. frutescens*, *M. piperita*, and *M. spicata* are closely related species in the Labiatae family, and their limonene hydroxylases are thought to catalyze (−)-limonene (**3**) hydroxylation with different regiospecificities. A homology-based approach is often employed for cloning enzymes with identical or similar functions from closely related species. However, hydroxylation of limonene (**3**) appears to occur by different mechanisms for 7-hydroxylation and 3- and 6-hydroxylation, and Mau et al. were unsuccessful in isolating the full-length enzyme by a homology-based method. In the present study, cytochrome P450 enzymes relevant to PA synthesis were targeted, and their method of isolation depended on comparison of sequences expressed in pure strains with different oil types. In this context, a library of pure strains of perilla have been obtained and achieved cloning of enzymes catalyzing the synthesis of oil compounds in perilla (Ito and Honda, 2007; Masumoto et al., 2010; Sato-Masumoto and Ito, 2014). The library has been established by collecting various types of perilla and maintaining them for 10–25 years by self-pollination using paper pollination bags.

With this library of pure strains of perilla, it is possible to compare expression levels of specific genes in different oil types and identify sequences relevant to the syntheses of oil compounds specific to the oil type. Herein described as the cloning and characterization of a cytochrome P450 enzyme mediating the synthesis of PA (**5**).

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