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A domain swapping approach to elucidate differential regiospecific hydroxylation by geraniol and linalool synthases from perilla

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ABSTRACT

Geraniol and linalool are acyclic monoterpenes found in plant essential oils that have attracted much attention for their commercial use and in pharmaceutical studies. They are synthesized from geranyl diphosphate (GDP) by geraniol and linalool synthases, respectively. Both synthases are very similar at the amino acid level and share the same substrate; however, the position of the GDP to which they introduce hydroxyl groups is different. In this study, the mechanisms underlying the regiospecific hydroxylation of geraniol and linalool synthases were investigated using a domain swapping approach and site-directed mutagenesis in perilla. Sequences of the synthases were divided into ten domains (domains I to IV-4), and each corresponding domain was exchanged between both enzymes. It was shown that different regions were important for the formation of geraniol and linalool, namely, domains IV-1 and -4 for geraniol, and domains III-b, III-d, and IV-4 for linalool. These results suggested that the conformation of carbocation intermediates and their electron localization were seemingly to be different between geraniol and linalool synthases. Further, five amino acids in domain IV-4 were apparently indispensable for the formation of geraniol and linalool. According to three-dimensional structural models of the synthases, these five residues seemed to be responsible for the different spatial arrangement of the amino acid at H524 in the case of geraniol synthase, while N526 is the corresponding residue in linalool synthase. These results suggested that the side-chains of these five amino acids, in combination with several relevant domains, localized the positive charge in the carbocation intermediate to determine the position of the introduced hydroxyl group.

Introduction

Monoterpenes comprise a chemically diverse group of C10 compounds composed of two isoprene units; they are generally volatile and fragrant and are often found in plant essential oils. In the plant body, monoterpenes play important roles in defense against microbes and insect pathogens and in the attraction of pollinators (Chappell, 1995; Chen et al., 2003; Yamasaki et al., 2007). Many monoterpene compounds are used for perfumery and flavoring of either food or medicines, and quite a few have antibacterial or antitumor properties (Chung et al., 2006; Honda et al., 1984); thus, they are attractive compounds in the field of pharmaceutical sciences.

Perilla, an annual Asian herb, contains an essential oil that is composed of unique monoterpene compounds and is designated as a medicine in the Japanese Pharmacopoeia. The oil types of perilla are classified into more than 10 groups according to their principal constituent (Ito et al., 1999). Most of the oil types are monoterpene types and are presumed to be biosynthesized from geranyl diphosphate (GDP, **1**) as a starting compound (Fig. 1). This biosynthetic pathway of perilla oil is genetically controlled (Honda, 1996), and the functions of each gene can be determined by cloning the enzymes that catalyze the relevant reaction steps in the biosynthetic pathway.

The first step in the putative biosynthetic pathway of monoterpene type oil components is dephosphorylation of GDP by either geraniol or limonene synthase (Fig. 1). Linalool (**2**), which is found in all perilla plants regardless of oil type (Pichersky et al., 1995), is also converted from GDP by linalool synthase. The geraniol and linalool synthases of *Perilla frutescens*, *Perilla citriodora*, *Perilla hirtella*, and *Perilla setoyensis* and the limonene synthases of *P. frutescens* and *P. citriodora* were cloned and shown to be highly similar at the sequence level (Ito and Honda, 2007; Masumoto et al., 2010; Yuba et al., 1996). Among these three monoterpene synthases, geraniol and linalool synthases have highly homologous sequences that are more than 70% identical at the amino acid level (Masumoto et al., 2010) and are considered to convert GDP in a similar

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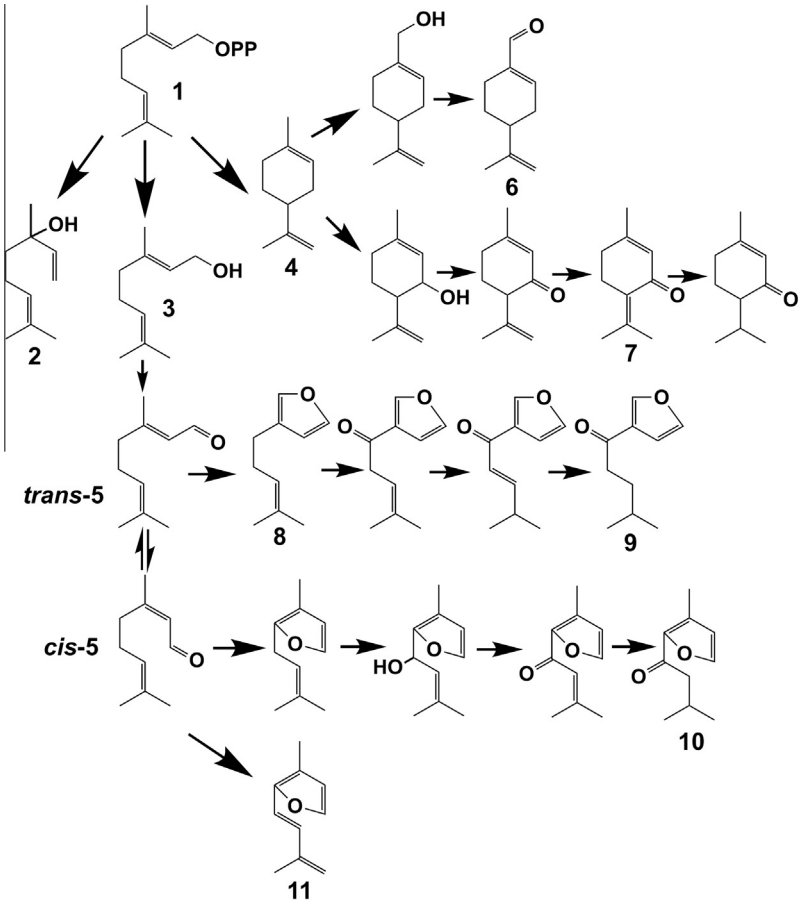

Fig. 1. Putative biosynthetic pathways of the oil constituents of perilla. (**1**) Geranyl diphosphate; (**2**) linalool; (**3**) geraniol; (**4**) limonene; (**5**) citral; (**6**) perillaldehyde; (**7**) piperitenone; (**8**) perillene; (**9**) perillaketone; (**10**) elsholtzia ketone; and (**11**) shisofuran.

manner; GDP is converted to a geranyl cation, and a hydroxyl group derived from water molecules near the active site is added to the intermediate carbocation (Iijima et al., 2004; Jia et al., 1999). As for linalool synthase, it is also conceivable that GDP was changed into linalool **2** via linalyl diphosphate (LDP) and a linalyl cation (Degenhardt et al., 2009). However, a detailed reaction intermediate progression for linalool synthase was unclear. The position of the additional hydroxyl group differs between geraniol (**3**) and linalool; the former is hydroxylated at C-1, while the latter is hydroxylated at C-3 (Fig. 2). It is of great interest to elucidate the mechanism and identify specific domains or amino acids relevant to the regiospecific hydroxylation of GDP. Since linalool (**2**) and the other compounds formed via geraniol (**3**) are found in perilla essential oil, the corresponding synthases for these compounds, namely, linalool and geraniol synthases, are supposed to coexist in perilla, indicating that these two synthases share the same substrate species.

To date, the three-dimensional structures of three plant monoterpene synthases have been determined by X-ray crystallography, namely, bornyl diphosphate synthase derived from *Salvia officinalis* (Whittington et al., 2002), limonene synthase from *Mentha spicata* (Hyatt et al., 2007), and 1,8-cineol synthase from *Salvia fruticosa* (Kampranis et al., 2007). Analysis of their structure provided us with information on amino acid residues and related mechanisms of their synthetic reactions; however, the details are still unknown. In order to identify the functions of the domains of geraniol and linalool synthases, chimeric enzymes were generated using a domain swapping approach between these enzymes.

Results

Identification of the regions responsible for the formation of geraniol (C-1 hydroxylation of geranyl cations)

The amino acid sequences and restriction enzyme recognition sites of geraniol synthase from *P. citriodora* (PcGS) and linalool synthase from *P. hirtella* (PhLS) used in this study are shown in Fig. 3. The transit peptide, which consists of a few dozen residues in the N-terminus that target the initial translation product toward the plastids, was removed from these two monoterpene synthases, because there was no significant difference in catalytic activity between full-length and truncated clones of monoterpene synthases from perilla (Bohlmann et al., 1998; Ito and Honda, 2007). The truncated geraniol and linalool synthases share 76% identity at the amino acid level.

To localize the regions of the enzymes responsible for regiospecific hydroxylation, chimeric enzymes (CHs), named CH1, 2, and 3, were constructed (Table 1). These chimeras were made by exchanging corresponding fragments between mutated PCGS (m-PcGS) and mutated PhLS (m-PhLS) restricted by either *Avr*II, *Ssp*I, or *Mfe*I. The sequence from the N-terminus to the *Avr*II restriction site was named domain I, from *Avr*II to *Ssp*I was named domain II, from *Ssp*I to *Mfe*I was named domain III, and from *Mfe*I to the C-terminus was named domain IV (Fig. 4). Domain III includes the DDXXD motif, which is highly conservative among monoterpene synthases. CH1, 2, and 3, which have the N-terminus of PhLS and the C-terminus of PcGS and had different proportions of PhLS