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Stereochemical mechanism of two sabinene hydrate synthases forming antipodal monoterpenes in thyme (*Thymus vulgaris*)

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ABSTRACT

The essential oil of *Thymus vulgaris* consists of a complex blend of mono- and sesquiterpenes that provides the plant with its characteristic aromatic odor. Several chemotypes have been described for thyme. In this study, we identified two enzymes of the sabinene hydrate chemotype which are responsible for the biosynthesis of its major monoterpene alcohols, (1*S*,2*R*,4*S*)-(*Z*)-sabinene hydrate and (1*S*,2*S*,4*R*)-(*E*)-sabinene hydrate. Both TPS6 and TPS7 are multiproduct enzymes that formed 16 monoterpenes and thus cover almost the whole monoterpene spectrum of the chemotype. Although the product spectra of both enzymes are similar, they form opposing enantiomers of their chiral products. Incubation of the enzymes with the potential reaction intermediates revealed that the stereospecificity of TPS6 and TPS7 is determined by the formation of the first intermediate, linalyl diphosphate. Since TPS6 and TPS7 shared an amino acid sequence identity of 85%, a mutagenesis study was employed to identify the amino acids that determine the stereoselectivity. One amino acid position had a major influence on the stereochemistry of the formed products. Based on comparative models of TPS6 and TPS7 protein structures with the GPP substrate docked in the active site pocket, the influence of this amino acid residue on the reaction mechanism is discussed.

Introduction

The essential oil of thyme (*Thymus vulgaris* L.) is a natural resource for a wide range of monoterpenes. Due to their antibacterial and spasmolytic activities, these monoterpenes are used in many pharmaceuticals. In addition, thyme is widely used as a spice and as a preservative in the food industry [1]. The composition of the essential oils varies strongly between thyme plants. Natural populations of thyme often consist of several chemotypes which are morphologically identical sub-populations with a distinct composition of their essential oils. These chemotypes are characterized by their major monoterpene alcohols. In Southern France, six chemotypes of *T. vulgaris* are distinguished: α-terpineol (A-type), carvacrol (C-type), geraniol (G-type), linalool (L-type), thymol (T-type) and sabinene hydrate (U-type) [2].

The structural diversity of monoterpenes is formed by the enzyme class of monoterpene synthases which convert geranyl diphosphate (GPP)[2] to the basic monoterpene olefins or alcohols [3]. The terpene synthase reaction mechanism that realizes this diversity of structures has been in the focus of intensive research in the last years [4]. The reactions start with the ionization of the 2,3-(*E*)-GPP substrate in the presence of a divalent cation. The resulting carbocationic intermediate is highly reactive and undergoes a series of cyclizations, hydride shifts, and rearrangements to form the basic carbon skeletons of the monoterpenes. The reactions are terminated by deprotonation, additional endocylizations, or water capture which leads to the formation of monoterpene alcohols [3]. A feature unique to terpene synthases is the formation of multiple products. One terpene synthase can generate complex terpene blends with over 50 compounds [5]. Most terpene synthases have a stereoselective reaction mechanism. The resulting monoterpene enantiomers can differ from each other in their function in plant insect-interactions [6] and are sometimes distinguished by their smell [7].

Many monoterpene synthases convert the achiral GPP substrate to compounds that contain one or more stereocenters. Studies on a fenchol synthase from fennel [8], bornyl diphosphate synthases

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[2] *Abbreviations used:* GPP, geranyl diphosphate; LPP, linalyl diphosphate; TPS, terpene synthase; RACE, rapid amplification of cDNA ends; GC–MS, gas chromatography–mass spectrometry; SPME, solid phase micro extraction; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluorid.

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from tansy and sage [9], or α-pinene synthases of loblolly pine [6] suggested that the stereospecificity of the enzyme reaction is defined in the early steps of the pathway, by the initial folding of GPP in the active-site pocket. The right-handed folding of GPP in the active site leads to the formation of (3*S*)-linalyl diphosphate ((3*S*)-LPP) and the left handed folding leads to (3*R*)-linalyl diphosphate ((3*R*)-LPP), the first chiral intermediates in the pathway (Fig. 1).

Although a large number of plant terpene synthases has been identified to date, little is known about the structure–function relationships in the active center of these enzymes and their impact on product specificity and stereoselectivity [4]. Some conserved sequence motifs and their catalytic function have been identified, like the the magnesium-binding DDxxD and NSE/DTE motifs which are highly conserved among terpene synthases [4,10]. Together, these motifs bind three magnesium ions which interact with the diphosphate moiety of the substrate and cause the initial ionization and rearrangement of the diphosphate group [11]. Another motif typical for monoterpene synthases is the RRx_8W-motif which is located 60 amino acids from the N-terminus. This motif is important for the isomerization of the GPP substrate to the linalyl cation intermediate [12,13]. In monoterpene synthases, site-directed mutagenesis and domain swapping have been utilized to identify structural elements that determine product specificity [14–18]. However, none of these studies reported on structural elements of monoterpene synthases which influence the stereochemical configuration of terpenes.

Here, we describe the isolation and biochemical characterization of two sabinene hydrate synthases from the sabinene hydrate chemotype of *T. vulgaris*. The opposite stereoselectivity of the enzymes provided a chance to investigate the structural base for their stereospecificity. We demonstrated that the stereospecificity is determined by the initial conformation of the GPP substrate in the active site. Mutagenesis experiments revealed a single amino acid residue that determined the stereochemical configuration of terpene products.

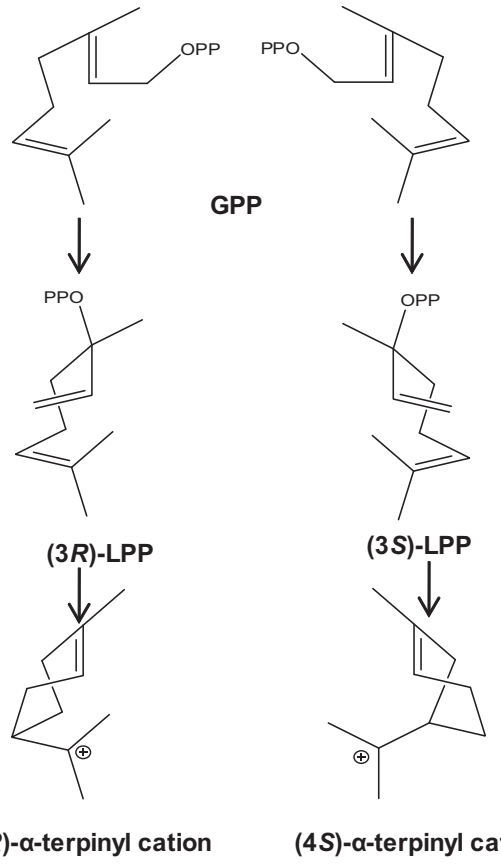

Fig. 1. The binding conformation of GPP determines the stereochemical conformation of LPP which is converted to the respective enantiomers of the α-terpinyl cation intermediate.

Materials and methods

Plant material

The chemotypes of thyme (*T. vulgaris*) were collected and characterized for their terpene content in Southern France at CNRS, Montpellier, France [19]. Plants were grown in the greenhouse under following conditions: temperature day (13 h light) 20–22 °C, temperature night 18–20 °C, humidity 55%, luminosity approximately 320 μmol photosynthetically active radiation.

Sequence isolation and phylogenetic analysis

For the isolation of terpene synthase genes, 5′and 3′-RACE-libraries of the sabinene hydrate chemotype were generated. The libraries were constructed with the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). For the first PCR, degenerate primers based on sequences of terpene synthase genes from other Lamiaceae (Table S1) were used to obtain partial 3′-sequences. The components of the PCR reaction were: 0.8 μl Advantage Taq DNA Polymerase Mix (5 U/μl), 4 μl Advantage Taq PCR buffer, 1 μl dNTPs (10 mM each), 1 μl universal primer mix and 1 μl gene-specific fwd-primer (10 pmol/μl) (Table S1), 3 μl 3′-RACE cDNA and PCR grade water added to a final volume of 40 μl. The PCR was conducted with an initial denaturation at 95 °C for 2 min, 30–35 cycles of denaturation at 95 °C for 30 s, annealing ranging from 48 to 60 °C for 30 s, extension at 68 °C for 2 min, and a final step at 68 °C for 5 min. The PCR fragments were cloned into the pCR4-TOPO vector (TOPO TA cloning kit for sequencing, Invitrogen, Carlsbad, CA, USA) and subsequently sequenced. The obtained sequences were compared by BLAST searches via the NCBI sequence database [20] and showed similarity to monoterpene synthases from other plants. The sequence fragments were used to design primers for the isolation of the 5′ends of the full length gene. This time, the components of the PCR reaction were: 1 μl Advantage Taq DNA Polymerase Mix (5 U/μl), 5 μl Advantage Taq PCR buffer, 1 μl dNTPs (10 mM each), 5 μl universal primer mix and 1 μl gene-specific primer (10 pmol/μl) (Table S1), 2.5 μl 5′-RACE cDNA and PCR grade water added to a final volume of 50 μl. PCR thermocycles were run as follows: initial denaturation at 95 °C for 2 min, 30–35 cycles of denaturation at 94 °C for 30 s, annealing ranging from 64 to 68 °C for 30 s, extension at 72 °C for 1.5 min, and a final step at 72 °C for 5 min. PCR fragments were cloned into pCR4-TOPO vector and sequenced. All 5′end and 3′end sequences were assembled with the SeqMan program (Lasergene DNAStar V5.05, Madison, WI, USA). This assembly revealed two open-reading frames called *tps*6 and *tps*7.

Amino acid sequence alignments and the neighbor-joining tree were constructed using MegAlign software (Lasergene DNAStar V5.05, Madison, WI, USA) and the ClustalW method (protein weight matrix: Gonnet series, gap penalty: 10.00, gap length penalty: 0.20, delay divergent sequences: 30%).

RNA extraction from leaf material and cDNA synthesis

Total RNA was extracted from 100 mg homogenized *T. vulgaris* young leaves pooled from five plants. The RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). To remove residual genomic DNA, RNA was treated with RQ1 RNAse-free