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Converting S-limonene synthase to pinene or phellandrene synthases reveals the plasticity of the active site

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ABSTRACT

S-limonene synthase is a model monoterpene synthase that cyclizes geranyl pyrophosphate (GPP) to form S-limonene. It is a relatively specific enzyme as the majority of its products are composed of limonene. In this study, we converted it to pinene or phellandrene synthases after introducing N345A/L423A/S454A or N345I mutations. Further studies on N345 suggest the polarity of this residue plays a critical role in limonene production by stabilizing the terpinyl cation intermediate. If it is mutated to a non-polar residue, further cyclization or hydride shifts occurs so the carbocation migrates towards the pyrophosphate, leading to the production of pinene or phellandrene. On the other hand, mutant enzymes that still possess a polar residue at this position produce limonene as the major product. N345 is not the only polar residue that may stabilize the terpinyl cation because it is not strictly conserved among limonene synthases across species and there are also several other polar residues in this area. These residues could form a “polar pocket” that may collectively play this stabilizing role. Our study provides important insights into the catalytic mechanism of limonene synthases. Furthermore, it also has wider implications on the evolution of terpene synthases.

1. Introduction

Terpenes form one of the largest groups of natural products (Wise and Croteau, 1999; Tholl, 2006; Christianson, 2006, 2007; Degenhardt et al., 2009; Gao et al., 2012). All of them are derived from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). The head-to-tail condensation of DMAPP and IPP forms geranyl pyrophosphate (GPP) that can be condensed with more IPP units to form farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and longer polyprenyl diphosphates (Wise and Croteau, 1999; Tholl, 2006; Christianson, 2006, 2007; Degenhardt et al., 2009; Gao et al., 2012). These linear molecules are then cyclized by terpene synthases, creating a diverse set of carbon skeletons that are further modified to form final terpenoid products (Wise and Croteau, 1999; Tholl, 2006; Christianson, 2006, 2007; Degenhardt et al., 2009; Gao et al., 2012). Terpenes play important roles in biology, as they function as hormones, regulators of membrane fluidity (e.g., cholesterol) and insect repellants (Pare and Tumlinson, 1999; Umehara et al., 2008). They also have a variety of applications in food, medicine and chemical industries (Ajikumar et al., 2008; Kirby and Keasling, 2009).

It has been estimated that there are more than 55,000 different terpene metabolites (Gao et al., 2012). This striking diversity is created by terpene synthases (Wise and Croteau, 1999; Tholl, 2006; Christianson, 2006, 2007; Degenhardt et al., 2009; Gao et al., 2012). The product profile of terpene synthases is determined by the conformation of the substrate or intermediate in the active pocket (Starks et al., 1997; Christianson, 2006, 2007; Gao et al., 2012). Stabilization of carbocations also plays a significant role (Lesburg et al., 1997; Starks et al., 1997; Christianson, 2007; Gao et al., 2012). For example, aromatic or polar side chains may stabilize the initially formed carbocation intermediate long enough for deprotonation to occur, “short-circuiting” its migration that can lead to more complicate products (Christianson, 2007; Zhou and Peters, 2011; Gao et al., 2012).

Terpenes derived from cyclization of GPP are monoterpenes. S-limonene synthase is a typical monoterpene synthase. It produces 4S-limonene, the precursor of oxygenated monoterpene in essential oils such as (-)-menthol, (-)-carvone and (-)-perillaldehyde (Guenther, 1975; Croteau et al., 2005; , Hyatt et al., 2007). Previous studies have revealed its mechanism (Fig. 1; Pyun et al., 1993;

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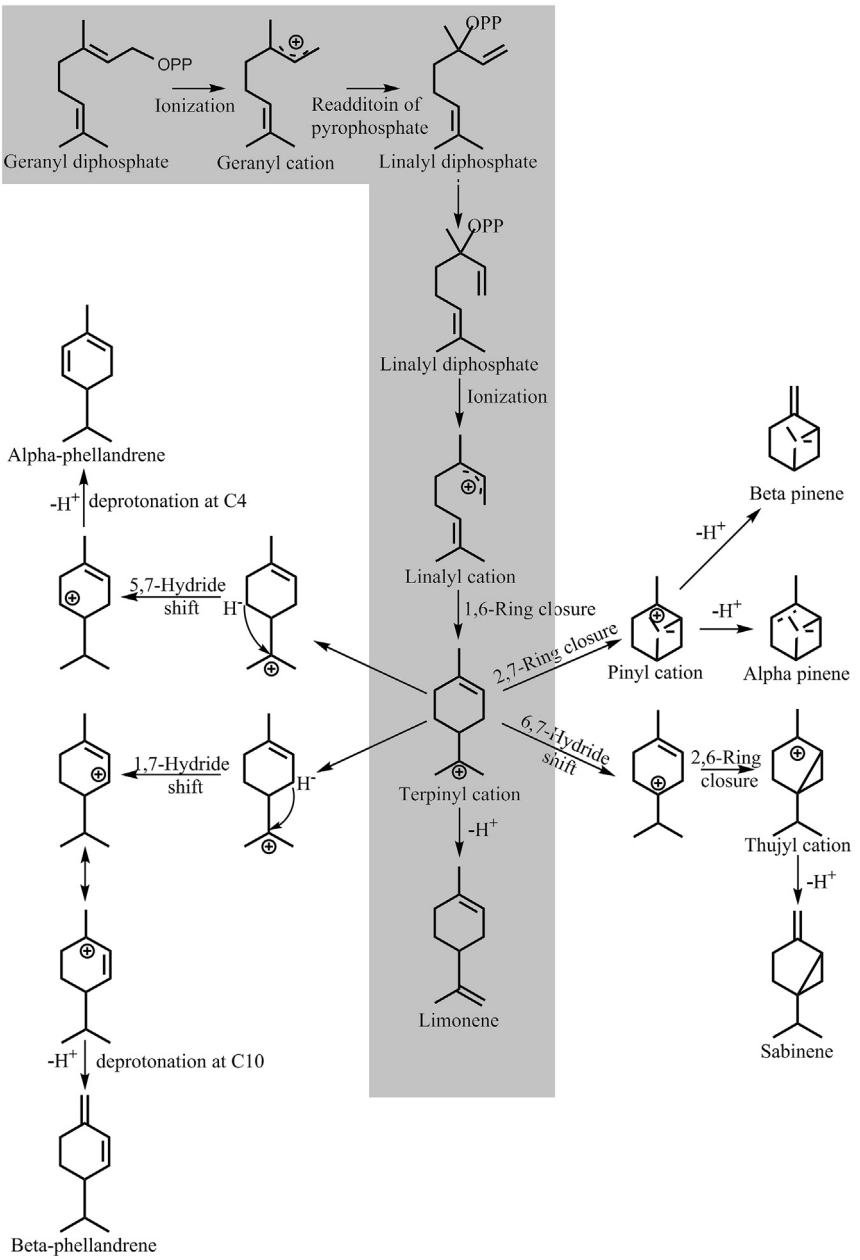

Fig. 1. Proposed reaction mechanism for the synthesis of limonene, pinene, phellandrene and sabinene.

Williams et al., 1998; Schwab et al., 2001; Hyatt et al., 2007). First, the pyrophosphate group migrates to C3 to form 3S-linalyl pyrophosphate (3S-LPP), which is followed by rotation about C2-C3 to create the cisoid conformer. This is followed by the ionization of 3S-LPP and C1-C6 cyclization, creating the (4S)-α-terpinyl cation. Deprotonation of the terpinyl cation forms 4S-limonene. Although the principle product is 4S-limonene (94%), side products, including myrcene (2%), α-pinene (2%) and β-pinene (2%), are also produced (Croteau et al., 2005; Hyatt et al., 2007; Srividya et al., 2015). Despite the availability of its crystal structures (Hyatt et al., 2007), it is still unclear about the mechanism of its product selectivity. A previous study reported that the activity of *A. grandis* limonene synthase can be converted to that of limonene/pinene synthase by domain swapping (Katoh et al., 2004). However, this study has not identified any key residues that determine the product selectivity (Katoh et al., 2004).

In order to delineate the detailed catalytic mechanism, it is critical to identify residues that contribute to discrete steps within the reaction. One strategy is to perform systematic mutagenesis analysis (Yoshikuni et al., 2006; O'Maille et al., 2008; Srividya et al., 2015). For example, by performing a systematic alanine-scanning study on *M. spicata* S-limonene synthase, Srividya and colleagues have identified W423 and H579 as key residues for the stabilization and deprotonation of the terpinyl cation intermediate (Srividya et al., 2015). Other studies are focused on switching the enzyme activity by mutating only a few residues (Wilderman and Peters, 2007; Xu et al., 2007; Keeling et al., 2008; Morrone et al., 2008; Zerbe et al., 2012). Sometime this can be achieved by changing only one amino acid (Wilderman and Peters, 2007; Xu et al., 2007; Morrone et al., 2008).

Here, we demonstrate that altering a few residues is sufficient to convert *M. spicata* S-limonene synthase to pinene synthase or

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