Structure of a Heterotetrameric Geranyl Pyrophosphate Synthase from Mint (Mentha piperita) Reveals Intersubunit Regulation

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Terpenes (isoprenoids), derived from isoprenyl pyrophosphates, are versatile natural compounds that act as metabolism mediators, plant volatiles, and ecological communicators. Divergent evolution of homomeric prenyltransferases (PTSs) has allowed PTSs to optimize their active-site pockets to achieve catalytic fidelity and diversity. Little is known about heteromeric PTSs, particularly the mechanisms regulating formation of specific products. Here, we report the crystal structure of the (LSU·SSU)2-type (LSU/SSU = large/small subunit) heterotetrameric geranyl pyrophosphate synthase (GPPS) from mint (Mentha piperita). The LSU and SSU of mint GPPS are responsible for catalysis and regulation, respectively, and this SSU lacks the essential catalytic amino acid residues found in LSU and other PTSs. Whereas no activity was detected for individually expressed LSU or SSU, the intact (LSU·SSU)2 tetramer produced not only C10-GPP at the beginning of the reaction but also C20-GGPP (geranylgeranyl pyrophosphate) at longer reaction times. The activity for synthesizing C10-GPP and C20-GGPP, but not C15-farnesyl pyrophosphate, reflects a conserved active-site structure of the LSU and the closely related mustard (Sinapis alba) homodimeric GGPPS. Furthermore, using a genetic complementation system, we showed that no C20-GGPP is produced by the mint GGPPS in vivo. Presumably through protein–protein interactions, the SSU remodels the active-site cavity of LSU for synthesizing C10-GPP, the precursor of volatile C10-monoterpenes.

INTRODUCTION

Linear prenyl pyrophosphates (LPPs) are the precursors for the more than 55,000 terpenes (isoprenoids) that have been identified in various organisms. Many are essential for important biological processes, such as protein prenylation (Ras, Rab, and Rho), proper functioning of the electron transport chain (quinone and heme α), glycoprotein biosynthesis (dolichol), and the metabolism of growth hormones (gibberellin, cytokinin, and sterol) (Liang et al., 2002; McTaggart, 2006; Gershenzon and Dudareva, 2007; Christianson, 2008; Kirby and Keasling, 2009). Some terpenes have considerable commercial interest as medicinal agents (taxol for anticancer and Artemisinin for antimalaria), flavors and fragrances (menthol and linalool), and nutritional supplements (carotenoid and vitamin A) (Pichersky et al., 2006; Gershenzon and Dudareva, 2007; Kirby and Keasling, 2009).

In plants, terpenes are derived from the universal five-carbon (C5) precursor isopentenyl pyrophosphate (C5-IPP) (Pichersky et al., 2006). Starting with C5-dimethylallyl pyrophosphate (C5-DMAPP), head-to-tail condensation reactions with one to three molecules of C5-IPP can generate C10-geranyl pyrophosphate (C10-GPP), C15-farnesyl pyrophosphate (C15-FPP), and C20-geranylgeranyl pyrophosphate (C20-GGPP). These products are generated by reactions involving the corresponding enzymes: C10-GPP synthase (GPPS), C15-FPP synthase (FPPS), and C20-GGPP synthase (GGPPS) (Figure 1A). These LPPs are the key intermediates for biosynthesis of diverse terpenes (Pichersky et al., 2006; Kirby and Keasling, 2009). Because the lengths of LPPs determine their distinct physiological roles, the production of LPPs is precisely regulated by their respective prenyltransferases (PTSs), groups of highly conserved enzymes in the cells (Kellogg and Poulter, 1997; Ogura and Koyama, 1998; Wang and Ohnuma, 1999; Liang et al., 2002; Szkołpiska and Plochocka, 2005). PTSs can be further classified into cis and trans types on the basis of the type of double bond formed during C5-IPP condensation (Liang et al., 2002). Here, we focus on the trans-type PTSs.

The function and structure of homomeric PTSs, such as FPPS and GGPPS, have been well studied (Ohnuma et al., 1996; Tarshis et al., 1996; Hemmi et al., 2003; Hosfield et al., 2004; Chang et al., 2006; Gabelli et al., 2006; Kavanagh et al., 2006a, 2006b; Kloer et al., 2006; Rondeau et al., 2006; Guo et al., 2007; Noike et al., 2008). The sequences of different enzymes generally contain <30% conserved amino acids (see Supplemental Figure 1 online). The three-dimensional structure contains a conserved...
Figure 1. Catalytic Reactions and Multiple Sequence Alignment of Plant PTSs.
α-helical bundle that surrounds a cavity that is located adjacent to the active site containing two conserved DD(α)XnD motifs (D indicates Asp, X indicates any residue; n = 2 or 4) facing each other on opposite helices over the central cavity. These motifs are important for substrate and cofactor binding. Previous studies suggest the existence of a molecular ruler mechanism whereby the contour (shape and size) of the catalytic cavity (tunnel) of PTSs determines the product specificity and substrate selectivity (Ohnuma et al., 1996; Tarshis et al., 1996; Hemmi et al., 2003; Guo et al., 2004a, 2004b; Sun et al., 2005; Chang et al., 2006; Kavanagh et al., 2006a; Kloer et al., 2006; Thulasiram et al., 2007; Noike et al., 2008).

By contrast, heteromeric PTSs have so far only been identified in a few prokaryotes and eukaryotes, such as Bacillus subtilis, Homo sapiens, Humulus lupulus, Antirrhinum majus, and Mentha piperita (Fujii et al., 1982, 1983; Koike-Takeshita et al., 1995; Zhang et al., 1997; Burke et al., 1999; Burke and Croteau, 2002; Saiki et al., 2003, 2005; Tholl et al., 2004; Ye et al., 2007; Wang and Dixon, 2009) (see Supplemental Figure 1 online). They are composed of two different types of subunits: one is significantly homologous (50% identity) to homomeric PTSs, while the other is less homologous (15% identity), lacks the DD(α)XnD motif, and is recognized as a noncatalytic subunit (see Supplemental Figures 2 and 3 online). The noncatalytic subunit is not only required for enzymatic activities of the heteromeric PTSs but also serves to modify catalytic fidelity or to promote catalytic activity in plants.

M. piperita GPPS (Mp GPPS), involved in the biosynthesis of essential oil (menthol) in mint glandular trichomes, is such a two-component heteromeric PTS consisting of a large and a small subunit, denoted LSU and SSU, respectively. LSU is a PTS-like protein with ~75% sequence identity to plant GGPPS, and presumably its structure is highly similar to that of Sinapis alba GGPPS (Sa GGPPS) (Kloer et al., 2006). SSU is less similar in sequence to other PTS proteins (Figure 1B). Both LSU and SSU are catalytically inactive by themselves (Burke and Croteau, 1999, 2004; Burke and Croteau, 2002; Croteau et al., 2005). Previous studies have shown that Mp GPPS synthesizes C10-GPP, which is the common precursor of C10-monoterpenes, many of which are plant volatiles involved in important biological activities, such as producing floral or fruit scents that attract pollinators and predators of herbivores, emitting signals that ward off pathogens, and producing floral or fruit scents that attract pollinators and predators of herbivores. Thus far, no structure of a heteromeric PTS is available (see Supplemental Figure 1 online). In an effort to elucidate the molecular mechanism of intersubunit interaction in heteromeric PTSs, we determined the crystal structure of a new (LSU-SSU)2-type heterotetrameric Mp GPPS, in a pseudomature form with the plastid targeting presequence removed. The structure reveals that LSU serves as a catalytic unit, while SSU acts as a regulatory unit. Further kinetic studies and in vivo assay showed that Mp GPPS synthesizes C10-GPP as the major product. No C20-GGPP was produced in vivo, even though LSU possesses a sufficiently large cavity for the accommodation of C20-GGPP. These results provide a significant insight into intersubunit regulation in heteromeric PTS.

**RESULTS**

**Crystal Structure of the Heterotetrameric Mp GPPS**

X-ray crystallographic analysis of the Mp GPPS revealed a novel (LSU-SSU)2 architecture composed of two LSU-SSU heterodimers (Figure 2). The LSU and SSU in each LSU-SSU heterodimer are related by a pseudodyad axis, and two LSU-SSU dimers form a tetramer (LSU-SSU)2 about a third dyad, all of which are parallel to one another. This arrangement differs from that of most other tetrameric assemblies, including those with tetrahedral 222-symmetry (hemoglobin) or square fourfold symmetry (neuraminidase) (Russell et al., 2006). Similar structural assembly has been reported for photosystem II, which contains terpenes (Guskov et al., 2009). Crystallographic symmetry elements are unable to relate the protein subunits in any other way due to the orthorhombic space group of P21·21·21 containing only screw axes. When analyzed by gel filtration, a stable heterotetramer (123 kD) of Mp GPPS formed in solution independent of protein concentration (see Supplemental Figure 4 online). Furthermore, previous studies using immunocytochemical localization have demonstrated that LSU and SSU of Mp GPPS coexist within the leucoplasts of the mint glandular trichomes (Turner and Croteau, 2004).

Our heterotetrameric crystal structures also contain bound ligands, including (1) Mg2+ ions (denoted Mp GPPS-Mg2+), (2) IPP (Mp GPPS-IPP), (3) the nonhydrolyzable DMAPP analog dimethylallyl thiopyrophosphate (DMASPP), IPP, and Mg2+ ions (Mp GPPS-Mg2+/IPP/DMASPP), and (4) GPP and Mg2+ ions (Mp GPPS-Mg2+/GPP; see Supplemental Table 1 and Supplemental Figure 5 online). Taken together, the crystallographically refined models encompass both full-length LSU (residues 1 to 295) and SSU (residues 1 to 266). Excluding regions of ligand-induced conformational change, all structures are virtually identical with an average root mean square deviation of 0.63 Å for 1059 Cα.

![Figure 1](continued).

(A) Schematic diagram of catalytic reactions of PTSs.
(B) SSUs from Mp GPPS (Mp SSU), Am GPPS (Am SSU), and Hi GPPS (Hi SSU), Abies grandis GPPS (Ag GPPS), P. abies GPPS (Pa GPPS), Arabidopsis thaliana GGPPS (At GGPPS), S. alba GGPPS (Sa GGPPS), and LSU from Hi GPPS (Hi LSU), Am GPPS (Am LSU), and Mp GPPS (Mp LSU) are included in the alignment. Regions in the SSU and LSU of Mp GPPS corresponding to their respective structural α-helices are denoted by purple and cyan cylinders. Identical and similar amino acid residues are shaded in black and gray, respectively. The conserved functional motifs, DD(α)XnD, are denoted by yellow boxes. The R loop of SSU is boxed in red. The AC loops 1, 2, and 3 of LSU are boxed in blue, gray, and green, respectively. All sequences presented here have the N-terminal signal peptides omitted.
atoms (see Supplemental Figure 6 online). The LSU and SSU in an LSU-SSU dimer are associated by helices B, F, G, and H of LSU and helices α-2, α-5, α-6, and α-7 of SSU, forming interface A (1980 Å²) (Figure 2). Between two LSU-SSU dimers in an (LSU-SSU)₂ tetramer, helices A and B, and helices α-7, α-8, and α-10 form interface B (380 Å²) (Figure 2). Interface A is similar to the subunit interface of most homodimeric PTSs but is larger in area, particularly compared with the 1570 Å² interface of Sa GGPPS. When LSU or SSU was expressed alone in Escherichia coli, each purified protein did not form a dimer, as analyzed using gel filtration chromatography. These results indicate that the molecular surface for dimer formation is quite different in Mp GPPS and Sa GGPPS.

In fact, interface A has more hydrogen bonds and salt bridges, which contribute to a tighter interaction, relative to the other homodimeric PTSs (see Supplemental Table 5 online). When
analyzing residues in the interface area of mint LSU and that between the two monomers in Sa GGPPS, we found three key differences (Ser-135, Ala-144, Lys-155 of Sa GGPPS versus Ala-126, Glu-134, and Val-145 of LSU, respectively) that may account for the LSU being unable to form a homodimer. In Sa GGPPS, the side chain of Lys-155 forms a direct salt bridge across the dimer interface to Glu-131 of the other subunit, and it also interacts indirectly with the polar residue of Ser-135, mediated by interfacial water molecules. Interestingly, Lys-155 is a conserved residue, also found in other plant GGPPSs and other LSUs possessing GGPPS activity (Tholl et al., 2004; Wang and Dixon, 2009) (Figure 1B). The LSU of Mp GPPS cannot accommodate those interactions because the Lys-155 and Ser-135 of Sa GGPPS are replaced by Val-145 and Ala-126 of LSU, respectively, eliminating the salt bridge interaction. In addition, Ala-144 of Sa GGPPS is replaced by Glu-134 of LSU. If two LSUs are modeled into a dimer such as that of Sa GGPPS, the two twofold related opposing Glu-134 side chains will result in negatively charged repulsion. In yeast GGPPS, mutating only one interface residue of Met-111 into Gly resulted in the disruption of dimer formation, and mutating Leu-8 and Ile-9 in the N-terminal helix, which is also involved in dimer formation, into Gln had a similar disruptive effect (Lo et al., 2009). Consequently, the LSU monomer prefers to associate with SSU rather than to form a homodimer. The monomeric LSU might not have a stable structure to afford an enzyme function.

**Catalytic Activities of Mp GPPS**

Neither LSU nor SSU alone showed detectable PTS activity despite the marked sequence similarity of LSU to those of plant GGPPSs (Burke et al., 1999; Burke and Croteau, 2002; Burke et al., 2004) (Figure 3A). These results are consistent with those using other heteromeric PTSs found in human and yeast DPPS, human and mouse SPPS, and bacterial HPPS and HEPPPS (Fuji et al., 1982, 1983; Koike-Takeshita et al., 1995; Zhang et al., 1997; Saiki et al., 2003, 2005) (see Supplemental Figure 1 online). By contrast, LSU of A. majus GGPPS (Am GPPS) and H. lupulus GPPS (H GPPS) are active under in vitro assay conditions (Tholl et al., 2004; Wang and Dixon, 2009).

When SSU and LSU are coexpressed in E. coli, the purified heterotetrameric Mp GPPS generates not only the anticipated C10-GPP, but also the unexpected C20-GPP in assays using ample amounts of C5-[14C] IPP and C5-DMAUP as substrates (Figure 3A). To further investigate the substrate specificity of Mp GPPS, the enzyme was incubated with other allylic substrates of C10-GPP, C15-FPP, and C20-GPP in the presence of C5-[14C] IPP (Figure 3A). In addition, the well-studied homodimeric Saccharomyces cerevisiae GGPPS (Sc GGPPS) served as a positive control to verify the in vitro assay condition (i.e., whether its functional catalytic site.

To investigate the role that LSU plays in Mp GPPS activity, we constructed the [LSU(D83A/D84A/D89A)·SSU]2 mutant in which the three conserved Asp residues of the DD(X)nD motif, located on loop DE of LSU and important in substrate binding and catalysis, were substituted with Ala. The heterotetrameric [LSU (D83A/D84A/D89A)·SSU]2 mutant was completely inactive (see Supplemental Figure 8 online). These results suggest that the enzymatic activity of Mp GPPS is contributed by LSU but not by SSU. By comparing Mp GPPS and homodimeric PTS structures, we conclude that SSU stabilizes LSU by adopting the position of an identical subunit and creating the proper architecture of a functional catalytic site.

In this regard, three notable regions surrounding the active site cavity (AC) of LSU, denoted as AC loops 1, 2, and 3, were identified by comparing the superposed structures (Figure 3B; see Supplemental Figure 6 online). AC loop 1 between helices D and F contains several critical residues, Asp-83, Asp-84, Asp-89, Asp-91, Arg-94, and Arg-95, that interact with both allylic and homoallylic substrates (see Supplemental Figure 9 online). In Sa GGPPS and Sulfolobus solfataricus HPPS (Ss HPPS) (Sun et al., 2005; Kloer et al., 2006), two homodimeric PTSs having the highest DALI scores, (highest structural similarities) to Mp GPPS, and the regions corresponding to AC loop 1 showed dramatic ligand binding-induced conformational changes necessary for substrate entry or product release (Holm and Sander, 1993) (see Supplemental Figure 10 online). The conformational switch for converting the unliganded (open) to the ligand-bound (closed) structures is located mainly around helices J–N of LSU, including AC loops 2 and 3, which is similar to other homomeric PTSs (Hosfield et al., 2004; Sun et al., 2005; Kloer et al., 2006; Rondéau et al., 2006). AC loop 2 functions as a gate for allylic substrate entry, and AC loop 3 is involved in homoallylic substrate binding. Moreover, it was observed that two conserved DD(X)nD motifs, which are located far apart from each other in the open form, would come closer together by ligand-induced interactions.

**The Active Site of Mp GPPS**

To investigate the role that LSU plays in Mp GPPS activity, we constructed the [LSU(D83A/D84A/D89A)·SSU]2 mutant in which the three conserved Asp residues of the DD(X)nD motif, located on loop DE of LSU and important in substrate binding and catalysis, were substituted with Ala. The heterotetrameric [LSU (D83A/D84A/D89A)·SSU]2 mutant was completely inactive (see Supplemental Figure 8 online). The C5-DMAUP kcat value is significantly higher than C10-GPP and C15-FPP by ~1000-fold and 70-fold, respectively. In sum, C5-DMAUP is the best suitable allylic substrate for Mp GPPS relative to C10-GPP and C15-FPP, as judged by the value of kcat/Km.
Figure 3. In Vitro Product Analysis of Mp GPPS and Conformations of the LSU.

(A) Functional assays (thin layer chromatography) of product synthesis of individual LSU and SSU subunits and complexes of Mp GPPS with three allylic substrates (left column) and C5-[14C] IPP. The products of wild-type Sc GGPPS and a mutant (S71Y), synthesizing C20-GGPP and C15-FPP, were used as markers (Chang et al., 2006).

(B) Surface representations of the open-form (Mp GPPS-Mg2+) and the closed-form [Mp GPPS-Mg2+/IPP/DMASPP (I)] of LSU. The C5-DMASPP (green) and C5-IPP (yellow) ligands are shown on the surface models and the Mg2+ ions as purple balls. AC loops 1, 2, and 3 are highlighted in blue, gray, and green, respectively. Yellow arrows indicate the CP hole for product elongation beyond C10-GPP from the AC (orange dotted circle) into the EC (purple dotted circle).

(C) Superposition of the structures of Sa GGPPS and Mp GPPS. It is likely that C20-GGPP (cyan, from Sa GGPPS) extends through the CP hole into the hydrophobic EC of LSU (green surface). C5-IPP (green, from Mp GPPS-IPP) and C19-GPP (magenta, from Mp GPPS-Mg2+/GPP) are represented as sticks.
(Figure 3B; see Supplemental Figure 9 online). In the catalytic site, obvious conformational changes also cause a shift in the side chain orientation of Lys-44, Arg-95, and Lys-235 for interaction with substrates (see Supplemental Figure 9 online).

By contrast, the SSU of Mp GPPS does not contain either of the catalytically important DD(X)D motifs. The regions in SSU corresponding to AC loops 1, 2, and 3 of LSU are not very conserved in terms of their length and sequence, even among the SSU of different plant GPPS (Figure 1B). Nevertheless, the loop connecting helices α-4 and α-5 of SSU, denoted R loop here, adopts a similar disposition as does its equivalent AC loop 1 of LSU (Figure 2; see Supplemental Figure 11 online). AC loop 2 is completely absent in SSU, and the C-terminal segment shows a conformation quite different from that of the AC loop 3 in LSU. The R loop of SSU may restrict movement of AC loop 1 of LSU, in much the same way as do the two AC loops 1 interact with each other in the homodimeric PTSs (Sun et al., 2005; Kloer et al., 2006). Further studies are needed to elucidate the precise function of this loop.

Roles of the Three AC Loops

The crystal structure of Mp GPPS shows that the active-site cavity itself is limited in size, being just large enough for C10-GPP (Figures 3B and 3C). However, adjacent to it is a second elongation cavity (EC) that can accommodate longer prenyl products (i.e., C15-FPP and C20-GGPP; Figure 3C) and is connected to the AC through a cavity penetration (CP) hole. Overall, this arrangement resembles a flexible hourglass. This two-chamber architecture (Figures 3B and 3C) is probably a result of catalytic site remodeling of LSU by SSU. The strong interactions between LSU and SSU restrict the enzyme’s specificity to products (i.e., C15-FPP and C20-GGPP), the second step (generating C15-FPP) is much slower than the first and third steps (generating C10-GPP and C20-GGPP, respectively; see Figure 1A), allowing time for the open-close movements of AC loop 1 and the release of the first product, C10-GPP. Once C15-FPP is produced, its hydrocarbon moiety immediately penetrates the CP hole, and the reaction proceeds to form C20-GGPP (Figure 3C).

This hypothesis predicts a bottleneck in the C15-FPP production (C10-GPP with C5-IPP) in the second step, which is consistent with our kinetic data in that the value of $k_{cat}/K_m$ in the first step is higher than that of the second step by $\sim$1000-fold (see Supplemental Table 2 online). Our results also suggest that catalytic reaction may be diversified via subunit interactions. This differs from previous studies of homomeric PTSs in which the product chain length was observed to be increased by minimal changes of critical residues to reduce their steric effect and expand the catalytic cavity (Guo et al., 2004a, 2004b; Sun et al., 2005; Chang et al., 2006).

Presumably, the remodeling of a GGPPS-like single-chamber active site for C20-GGPP into a two-chamber, hourglass-like architecture, which prefers C10-GPP over C20-GGPP, can be a result of amino acid substitutions in the vicinity of the active site. However, structural comparison did not reveal any remarkably different residue in the active-site region (see Supplemental Figure 12 online). In fact, all different residues between Sa GGPPS and the LSU of Mp GPPS are found either on the protein surface or at the subunit interface, and all active-site residues are conserved between these two highly homologous proteins (Figure 1B). Further from the active site, near the LSU-SSU interface, occur three residues that are different from those in Sa GGPPS, namely, Cys-161, Val-160, and Ser-107. The former two correspond to Ser-171 and Ile-170 in Sa GGPPS, and they are not likely to change the chamber structure. The smaller Val-160 could make the cavity even larger. Although Ser-107 of LSU, near the C terminus of AC loop 1, makes a hydrogen bond with Arg-89 in the R loop of SSU, the significance of this interaction is uncertain. Consequently, the two-chamber formation of active site in Mp GPPS should be a result of intersubunit interactions.

Based on a structural comparison between the open and closed forms of LSU, we found that AC loop 2 is a highly mobile region, relative to AC loops 1 and 3 (Figure 3B; see Supplemental Figure 13 online). In addition, AC loop 2 has a similar conformation in Mp GPPS-Mg$^{2+}$ and Mp GPPS-IPP and acts like a lid in the structure of Mp GPPS-Mg$^{2+}$/IPP/DMASPP (see Supplemental Figure 13 online). This result indicates that the extensive conformational change on AC loop 2 is induced by allylic substrate rather than by the homoallylic substrate and that AC loop 2 serves to shield the allylic substrate from solvent during the catalytic reaction (see Supplemental Figure 13 online).

Two physically distinct ligand binding sites, for allylic substrate (C5-DMAPP) with Mg$^{2+}$, C15-GPP, and homoallylic substrate (C5-IPP), and one novel misoriented binding site of C5-IPP, are represented in our ligand-bound structures (see Supplemental Figures 5 and 14 online). In a ternary complex, two orientations of the bound C5-IPP in the individual binding pocket are seen, illustrating how the IPP molecule enters in the proper orientation for reacting with the allylic substrate (Figure 4A; see Supplemental Figure 14 online). AC loop 3, especially the three conserved residues (Arg-293, Asp-294, and Asn-295) at the C terminus of LSU, seems to regulate the binding of C5-IPP in the correct position for the ensuing catalytic reaction (Figure 4A). Consistently, the C-terminal deletion mutant of [LSU$\Delta$(293-295)]-SSU$_2$ is essentially inactive; only a minute amount of C10-GPP can be detected when C5-DMAPP reacts with C5-IPP (Figure 4B), attesting to the importance of AC loop 3 in catalysis.

In Vivo Function of Mp GPPS

Because the LSU has EC to accommodate C20-GGPP, and two major products (C10-GPP and C20-GGPP) can be detected by in vitro assay, it is possible that the heterotetrameric Mp GPPS may be a bifunctional enzyme, similar to the heteromeric HI GPPS (Wang and Dixon, 2009). However, there has not been any biological evidence that C20-GGPP is observed as a product of Mp GPPS in nature. Instead, previous studies have shown that the transcription level of Mp GPPS correlates closely with C10-monoterpene biosynthesis in mint (McConkey et al., 2000; Croteau et al., 2005).
In order to investigate whether \( \text{Mp GPPS} \) possesses \( \text{GGPPS} \) activity in nature, we used a genetic complementation method by substituting \( \text{crtE} \)-encoded \( \text{GGPPS} \) in the \( \text{crt} \) gene cluster of \( \text{Pantoea ananatis} \) (formerly \( \text{Erwinia uredovora} \)), which is used for the biosynthesis of carotenoid (a prominent yellow pigment) (Misawa et al., 1990; Zhu et al., 1997; Kainou et al., 1999; Engprasert et al., 2004; Ye et al., 2007). Since \( \text{E. coli} \) does not possess an intrinsic gene encoding \( \text{GGPPS} \), \( \text{E. coli} \) cells harboring \( \text{pACCAR25ΔcrtE} \), which carries \( \text{crtB} \) (phytoene desaturase), \( \text{crtI} \) (phytoene desaturase), \( \text{crtY} \) (lycopene cyclase), \( \text{crtZ} \) (\( \beta \)-carotene hydroxylase), and \( \text{crtX} \) (zeaxanthin \( \beta \)-glucosidase), cannot, without the presence of the \( \text{crtE} \) gene, accumulate carotenoid unless \( \text{C}_{20} \)-GGPP is generated (see Supplemental Figure 15A online). The transformants carrying the \( \text{pACCAR25ΔcrtE} \) and human or yeast \( \text{GGPPS} \) gene (positive control) are expected to show a notable yellow color, indicating the function of \( \text{crtE} \) has been substituted, whereas the empty vectors and the constructs expressing \( \text{GGPPS} \) from orchid (\( \text{Phalaenopsis bellina} \)), \( \text{FPPS} \) from \( \text{E. coli} \), or \( \text{Sc GGPPS (S71Y)} \) mutant are used as negative controls (Kainou et al., 1999; Hosfield et al., 2004; Chang et al., 2006; Kavanagh et al., 2006a; Hsiao et al., 2008) (see Supplemental Figure 15B online). LSU alone again had no \( \text{GGPPS} \) activity by such in vivo assay methods, despite the LSU sequence being \( \sim 70\% \) identical to those of other plant \( \text{GGPPS} \).

Interestingly, neither cotransformation of LSU and SSU nor transformation of a duet vector with LSU and SSU, namely, LSU-SSU/pET-32, in \( \text{E. coli} \) carrying \( \text{pACCAR25ΔcrtE} \), resulted in detectable yellow pigmentation when measured for the optical absorption of the extracted pigments from the transformants (see Supplemental Figures 15B and 15C online). We then considered whether \( \text{Mp GPPS} \) is unable to produce the yellow pigment of carotenoid due to the limited substrate pool of \( \text{C}_{15} \)-FPP in \( \text{E. coli} \) cytosol. According to our kinetic results (Figure 1A; see Supplemental Table 2 online), the second condensation step of \( \text{Mp GPPS} \) from \( \text{C}_{10} \)-GPP to \( \text{C}_{15} \)-FPP is a bottleneck. \( \text{E. coli} \) harboring \( \text{pACCAR25ΔcrtE} \) coexpressing either \( \text{Mp GPPS} \) plus \( \text{Ec FPPS} \), or \( \text{Mp GPPS} \) plus \( \text{Sc GGPPS (S71Y)} \), was employed to supply sufficient \( \text{C}_{15} \)-FPP for \( \text{Mp GPPS} \). However, this construct still failed to produce \( \text{C}_{20} \)-GGPP (see Supplemental Figures 15B and 15C online). Taken together, these data suggest that \( \text{Mp GPPS} \) does not preserve the function of \( \text{GGPPS} \) in vivo even though its LSU is very similar to \( \text{Sa GGPPS} \) in terms of amino acid sequence and the three-dimensional structure (Kloer et al., 2006) (see Supplemental Figure 12 online).

**DISCUSSION**

Our structural and mutagenetic studies offer a new insight into how the product specificity and fidelity of the \( \text{(LSU.SSU)}_2 \)-type \( \text{Mp GPPS} \) is determined via intersubunit regulation through a novel molecular mechanism. In sum, the SSU limits the ability to conduct catalytic reaction beyond the \( \text{C}_{10} \)-GPP by restricting the connection between AC and EC. Based on structure and sequence comparisons with plant \( \text{GGPPSs} \), the two-chamber architecture of LSU in the \( \text{Mp GPPS} \) structure provides additional cavities (CP hole and EC) for accommodating the longer product of \( \text{C}_{20} \)-GGPP (Figure 5; see Supplemental Figure 12 online). Our proposed two-chamber mechanism in which the product chain length is regulated by intersubunit interaction is distinct from the previous single-chamber molecular ruler mechanism for well-studied homomeric PTSs, which use bulky residues to serve as flooring at the bottom of each enzyme active site to block further product chain elongation (Ohnuma et al., 1996; Tarshis et al., 1996; Hemmi et al., 2003; Guo et al., 2004a, 2004b; Sun et al., 2005; Chang et al., 2006; Noike et al., 2008). When the elongation barrier is removed in the active site of these enzymes by substituting the bulky residue with a smaller one, longer chain length products are generated.
In addition, another group of enzymes called terpene synthases, which share a similar \( \alpha \)-helical fold with homomeric PTSs, cyclize the allylic substrates to produce a variety of terpene hydrocarbon scaffolds. Accumulated studies have demonstrated that the contours of the active site of terpene synthases can be changed to generate more diversified products by replacing the critical residues around their individual active-site cavities (Yoshikuni et al., 2006; Thulasiram et al., 2007; Xu et al., 2007; O’Maille et al., 2008). In parallel, our studies of heteromeric Mp GPPS reveal that the shape and size of the active-site cavity can be molded by intersubunit interactions (Figure 5). This new mechanism may shed light on the previous mysteries of heteromeric PTSs regarding their molecular basis for product chain length determination.

As the products generated through PTSs serve as critical precursors for several physiological processes, such as protein prenylation, PTSs need to be regulated precisely (Liang et al., 2002; Szkopinska and Plochocka, 2005; McTaggart, 2006). Heteromeric PTSs possess two-component systems, comprised of a noncatalytic and a PTS-like subunit, though the question concerning the role played by the noncatalytic subunit in vivo remains. In plants, biosynthesis of terpenes is compartmentalized in such a way that \( \text{C}_{10}\text{-GPP} \) and \( \text{C}_{20}\text{-GGPP} \) are generated via the plastidic methylenedioxyphosphate pathway, whereas \( \text{C}_{15}\text{-FPP} \) and its derivatives (\( \text{C}_{15}\text{-sesqui-} \) and \( \text{C}_{20}\text{-triterpenes} \)) are produced through the cytosolic mevalonic acid pathway (Croteau et al., 2005; Pichersky et al., 2006; Kirby and Keasling, 2009). The LSU and SSU of Mp GPPS have also been shown to occur within the leucoplast (a nonpigmented chloroplast) of the glandular trichome, which is the specialized mint tissue responsible for the production and accumulation of essential oil, a derivative of \( \text{C}_{10}\text{-GPP} \) (Turner and Croteau, 2004). Consequently, the unique SSU of Mp GPPS has evolved to interact with LSU to produce \( \text{C}_{10}\text{-GPP} \) from \( \text{C}_{5}\text{-DMAPP} \) and \( \text{C}_{5}\text{-IPP} \), which are generated by the plastidic methylenedioxyphosphate pathway. In addition, as shown in previous studies of \( \text{Am GPPS} \) and \( \text{Hl GPPS} \), expression levels of their SSU mRNA and protein display a tissue specificity, particularly in flowers and glandular trichomes, where \( \text{C}_{10}\text{-monoterpenes} \) are synthesized. However, their LSUs constitutively express in vegetable and \( \text{C}_{10}\text{-monoterpene storage/emission organs} \) (Tholl et al., 2004; Wang and Dixon, 2009). In summary, the expression of SSU, but not LSU, is closely

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**Figure 5.** Schematic Model of the Two-Chamber Architecture for Product Regulation.

The cavities for \( \text{C}_{5}\text{-IPP} \) (misoriented), \( \text{AC}, \text{CP} \), and \( \text{EC} \) are presented in green, gray, cyan, and purple, respectively. The homoallylic substrate of \( \text{C}_{5}\text{-IPP} \), the allylic substrates of \( \text{C}_{5}\text{-DMAPP}, \text{C}_{10}\text{-GPP}, \text{C}_{15}\text{-FPP}, \) and \( \text{C}_{20}\text{-GGPP} \), and \( \text{PPI} \) are shown as sticks. The \( \text{Mg}^{2+} \) ions are presented as purple balls. The \( \text{AC} \) loop 1 of LSU is depicted as a blue line. The size of the gray arrow indicates the level of catalytic efficiency in the three individual steps of the catalytic reactions (see Figure 1A). The red crosses indicate that the catalytic reactions beyond the first step in vivo are blocked via intersubunit interactions.
correlated with the C_{10}-monoterpenoid biosynthesis. It is therefore intriguing to consider that SSUs have evolved to ensure their catalytic specificity and fidelity in a particular specialized tissue by intersubunit regulation. In addition, these findings also encourage investigation of other heteromeric PTSSs in vivo.

Finally, the role played by LSU of Mp GPPS in the production of C_{20}-GGPP in vivo remains to be clarified because no GGPPS activity was detected in LSU of Mp GPPS, in contrast with LSU of Am GPPS and HI GPPS, which were found to be functional GGPPSs by in vitro assay (Thöll et al., 2004; Wang and Dixon, 2009). Whether these two enzymes are active in vivo could be tested by methods such as the genetic complementation assay used here. Judging from the accumulated carotenoid in our in vivo assay, heteromeric Mp GPPS and LSU alone in E. coli cannot produce detectable amounts of C_{20}-GGPP, which is important in protein geranylation (Rac, Rap, and Rho) and the biosynthesis pathway of carotenoid and gibberellin in plant plastid (McTaggart, 2006; Pichersky et al., 2006; Gershenzon and Dudareva, 2007). No GGPPSs has been identified in the ESTs from the mint glandular trichome (Croteau et al., 2005), but GGPPSs and LSU are generally considered to coexist in plastids (Croteau et al., 2005; Pichersky et al., 2006; Kirby and Keasling, 2009). Consequently, it is worthwhile to investigate how C_{20}–GGPP is generated in the leucoplast of mint glandular trichome, if not by the quasifunctional Mp GPPS.

Taken together, our findings provide a new insight into how the catalytic reactions of PTSSs are regulated via intersubunit interactions. Remarkably, only a few heteromeric PTSSs have been discovered in prokaryotes and eukaryotes in previous studies, suggesting that the candidate genes encoding the noncatalytic subunit of heteromeric PTSSs might have been neglected (see Supplemental Figure 1 online). A renewed search of the possible genes of heteromeric PTSSs is warranted.

**METHODS**

**Cloning and Mutagenesis**

The truncated versions of *Mentha piperita* SSU (residues 1 to 266) and LSU (residues 1 to 295) without their plastid targeting sequences in PET-3B (Novagen) with C-terminal His-tag and PET-32a (Novagen) were denoted SSU/pET-37 and LSU/pET-32, respectively, as previously described (Burke and Croteau, 2002). For crystallization, site-directed mutagenesis was performed to yield SSU/pET-32, namely, LSU-SSU/pET-32. The primers are shown in Supplemental Tables 3 and 4 online. A renewed search of the possible genes of heteromeric PTSSs was warranted.

**Protein Expression and Purification**

LSU/pET-32 and SSU/pET-37 D2 were cotransformed to *E. coli* BL21 (DE3) and induced with 0.5 mM isopropyl _β_-thiogalactopyranoside (IPTG) at 10° C for 60 h. Cells were collected, resuspended in extraction buffer [50 mM Tris, pH 8.5, 40 mM imidazole, 0.75 M NaCl, 25% (v/v) glycerol, 0.2 M sorbitol, 10 mM MgCl₂, 2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 2 ng mL⁻¹ of benzonate (Novagen) with added Protease Inhibitor Cocktail (Roche)], and lysed by Cell Disruption Solutions (Constant Systems). After ultracentrifugation at 205,000 g (Beckman Ti45) for 60 min at 4° C, protein complexes were purified by a Ni column (GE Healthcare) and further isolated (LSU-SSU)₂ by gel filtration (Superdex 200; GE Healthcare) in GF buffer (25 mM Tirs, pH 8.5, 0.2 M NaCl, 5% (v/v) glycerol, and 2 mM TCEP). E. coli BL21 (DE3) cells were transformed with SSU/pET-37 D2 to express LSU at 10° C using 0.2 mM IPTG. LSU/pBAD was transformed into E. coli BL21 (DE3) cells and grown in medium (M9 salts, 2% casamino acids, 0.2% glucose, 1 mM MgCl₂) to express LSU by the addition of L-arabinose to 0.002% at 10° C. Purification of Mp GPPS mutants and individual SSU and LSU followed a similar procedure to that described above. LSU and SSU were identified by liquid chromatography–mass electrospray ionization–tandem mass spectrometry search using the program Mascot (Perkins et al., 1999). The LSU-SSU₂ was further determined on a Superdex 200 10/300 GL High Performance column (GE Healthcare) in GF buffer by comparing it with those standards (see Supplemental Figure 5 online).

**Crystalllographic Analyses**

Crystallization was performed by the hanging-drop vapor diffusion method (Hampton Research) at 20° C for 3 to 4 weeks. The Mp GPPS-Mg²⁺ crystals were obtained by mixing protein solution (4 mg mL⁻¹) with an equal volume of reservoir solution (100 mM Bis-Tris, pH 6.5, 200 mM CH₃COONH₄, 13 to 19% [w/v] PEG 3350, and 2 to 5% [w/v] PEG 1000) and soaked in the reservoir solution containing 2.5 mM MgCl₂ and 15% (v/v) ethylene glycol as a cryoprotectant for 12 h at 20° C prior to data collection. For preparing Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals, protein solutions in the presence of 2.5 mM ligands (MgCl₂, C₅-IPP, and C₅-DMASPP or MgCl₂ and C₁₀-GPP) were used, and the crystallization conditions were basically the same as described above. The Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen. For the Mp GPPS-IPP complex, the crystals of Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen. For the Mp GPPS-IPP complex, the crystals of Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen. For the Mp GPPS-IPP complex, the crystals of Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen. For the Mp GPPS-IPP complex, the crystals of Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen. For theMp GPPS-IPP complex, the crystals of Mp GPPS-Mg²⁺/IPP/DMASPP or Mp GPPS-Mg²⁺/GGPP crystals were then soaked for 3 s in the reservoir solution containing 2.5 mM of the appropriate ligands and 15% (v/v) ethylene glycol before flash-cooling in liquid nitrogen.

DIFFERENTIATION CASE STUDY

Structure of Heteromeric GPPS from Mint
In Vitro Enzymatic Analyses and Kinetic Parameters

In vitro assays were performed in reaction solution (100 mM HEPES, pH 7.5, 0.1% Triton X-100, 5 mM MgCl₂, and 50 mM KCl) with 1 μM enzyme and incubated for 6 h at 25°C except for the time course experiment. The in vitro assays and kinetic measurements followed our published procedures (Kuo and Liang, 2002; Guo et al., 2004a, 2004b; Sun et al., 2005; Chang et al., 2006; Lo et al., 2009). The following substrate mixtures (50 μM C₅-DAPP with 200 μM C₁₀-IPP, 50 μM C₁₀-GPP with 150 μM C₁₄-[¹⁴C]IPP, 50 μM C₁₀-FPP with 100 μM C₁₀-[¹⁴C]IPP, and 50 μM C₁₀-GPP with 50 μM C₁₀-[¹⁴C]IPP) were used in assays. The products were identified by thin layer chromatography on silica gel 60 RP-18 F₂₅₄S (Merck) using acetonitrile:water (18:2) as the mobile phase. For Km and kcat measurements of allylic substrates, 0.05 μM purified Mp GPPS was added in reaction solution with 0.5 to 600 μM C₁₀-DAPP, 0.5 to 500 μM C₁₀-GPP, or 0.5 to 400 μM C₁₀-FPP in the saturating concentration of C₁₀-[¹⁴C]IPP (250 μM). The C₅-IPP Km values for C₁₀-DAPP (750 μM), C₁₀-GPP (500 μM), and C₁₀-FPP (170 μM) were determined at C₁₀-[¹⁴C]IPP concentrations from 0.25 to 300 μM. Data were analyzed by nonlinear regression of Michaelis-Menten equation to obtain Km and kcat values. Substrates were purchased from Sigma-Aldrich, Echelon Biosciences, and Amersham Pharmacia Biotech.

In Vivo Genetic Complementation Assay

The pACCAR25acrE, including the crtE gene cluster with the exception of the deleted crtE encoding GGPPS, were developed for identification of GGPPS activity (Misawa et al., 1990; Zhu et al., 1997; Kainou et al., 1999; Engrasert et al., 2004; Ye et al., 2007). The empty vectors of pET-16 and pET-28, and following constructs of Hs GGPPS/pET-46 (human GGPPS), Sc GGPPS/pET-32 (yeast GGPPS), Pb GPPS/pET-15 (orchid GPPS), Ec FPPS/pET-30 (E. coli FPPS), and Sc GGPPS(S71Y)/pET-32 (yeast GGPPS mutant), were used here. The constructs were cotransformed into E. coli BL21 (DE3) carrying pACCAR25acrE and using antibiotics for selection [chloramphenicol for pACCAR25acrE, ampicillin for pET-16, and pET-28, and following constructs of Hs GGPPS/pET-46 (human GGPPS), Sc GGPPS/pET-32 (yeast GGPPS), Pb GPPS/pET-15 (orchid GPPS), Ec FPPS/pET-30 (E. coli FPPS), and Sc GGPPS(S71Y)/pET-32 (yeast GGPPS mutant), were used here. The constructs were cotransformed into E. coli BL21 (DE3) carrying pACCAR25acrE and using antibiotics for selection. The C5-IPP concentrations from 0.25 to 300 μM. Data were analyzed by nonlinear regression of Michaelis-Menten equation to obtain Km and kcat values. Substrates were purchased from Sigma-Aldrich, Echelon Biosciences, and Amersham Pharmacia Biotech.

Accession Numbers

Coordinates and structure factors of Mp GPPS-Mg²⁺, Mp GPPS-IPP, Mp GPPS-Mg²⁺/IPP/DMAPP (I), Mp GPPS-Mg²⁺/IPP/DMASPP (II), and Mp GPPS-Mg²⁺/GPP have been deposited in the Protein Data Bank (http://www.rcsb.org) with the accession codes 3KRA, 3KRC, 3KRF, 3KRO, and 3KR, respectively. Accession codes for other PTSs, when available, are shown in Supplemental Figure 1 online. Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: SSU of Mp GPPS (Mp SSU), ABW86880; SSU of Am GPPS (Am SSU), AAS82659; SSU of Hi GPPS (Hi SSU), ACQ90681; Abies grandis GPPS (Ag GPPS), AAN01133; Picea abies GPPS (Pa GPPS), ACA21458; Arabidopsis thaliana GPPS (At GPPS), NP_195399; Sinapis alba GPPS (Sa GPPS), CAAD73350; LSU of Hi GPPS (Hi LSU), ACQ90682; LSU of Am GPPS (Am LSU), AAS82660; Mp GPPS (MP LSU), ABW86878; human GPPPS, NM_004897; yeast GGPPS, SCA13162; E. coli FPPS, NZ_AAMK02000019; and orchid GPPS, EU023907.

Author Contributions


Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Classification of Prenyltransferases (trans-Type Only).

Supplemental Figure 2. Amino Acid Sequence Alignment of Hetero-meric PTS Subunits That Lack the DD(x)X Motif.

Supplemental Figure 3. Amino Acid Sequence Alignment of Hetero-meric PTS Subunits That Contain the DD(x)X Motif.

Supplemental Figure 4. Analytic Gel Filtration Assays of Heterotetrameric Mp GPPS.

Supplemental Figure 5. Electron Density Maps for the Ligands.

Supplemental Figure 6. Superpositions of Mp GPPS in Complex with a Variety of Ligands.

Supplemental Figure 7. Time-Course Assays of Substrate and Product Specificities of Mp GPPS.

Supplemental Figure 8. Products Synthesized by the [LSU(D83A/D84A/D89A)/SSU] Mutant.

Supplemental Figure 9. Active Sites of Mp GPPS for Ligand Binding.

Supplemental Figure 10. AC Loop 1 Conformational Changes of PTs.

Supplemental Figure 11. Comparison of SSU and Sa GGPPS.

Supplemental Figure 12. Superpositions of LSU of Mp GPPS and Sa GGPPS.

Supplemental Figure 13. Conformational Change of AC Loop 2 Involved in the Allylic Substrate Binding Step.

Supplemental Figure 14. Ligand Binding Locations.

Supplemental Figure 15. In Vivo Activity of GGPPS.

Supplemental Figure 16. Ligand-Induced Conformational Change of Mp GPPS.

Supplemental Table 1. Data Collection and Refinement Statistics.

Supplemental Table 2. Kinetic Parameters of (LSU·SSU)₂-Type Heterotetrameric Mp GPPS.

Supplemental Table 3. The Mutagenic Primers Used to Construct Recombinant Mp GPPS.

Supplemental Table 4. The Constructs Used for in Vitro and in Vivo Assays.

Supplemental Table 5. Parameters of Protein Interface Area.

ACKNOWLEDGMENTS

We thank Rodney B. Croteau (Washington State University) for providing cDNA constructs of Mp GPPS; Makoto Kawamukai (Shimane University, Japan) for the gift of pACCAR25acrE plasmid; Yu-Yun Hsiao (National Cheng Kung University, Taiwan) for supplying the plasmid of Pb GPPS/CGPPS.
pET-15, Rey-Ting Guo (Academia Sinica, Taiwan) for helping with the protein expression, and Kai-Fa Huang (Academia Sinica, Taiwan) for assistance in x-ray data collection. The Protein Crystallography Facility of National Synchrotron Radiation Research Center is supported by the National Research Program for Genomic Medicine. Mass spectrometry analyses were performed by the Core Facilities for Proteomics Research located at the Institute of Biological Chemistry, Academia Sinica. This work was supported by Academia Sinica and Core Facility for Protein Production and X-Ray Structural Analysis (NSC97-3112-B-001-035-B4) to A.H.-J.W.

Received September 29, 2009; revised December 31, 2009; accepted January 20, 2010; published February 5, 2010.

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Structure of a Heterotetrameric Geranyl Pyrophosphate Synthase from Mint (*Mentha piperita*) Reveals Intersubunit Regulation  
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*Plant Cell* 2010;22;454-467; originally published online February 5, 2010;  
DOI 10.1105/tpc.109.071738  
This information is current as of July 9, 2017

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