The Biosynthetic Origin of Irregular Monoterpenes in Lavandula

**ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF A NOVEL cis-PRENYL DIPHOSPHATE SYNTHASE GENE, LAVANDULYL DIPHOSPHATE SYNTHASE**

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**Background:** *Lavandula* accumulate irregular monoterpenes of unknown biosynthetic origin.

**Results:** We cloned a *cis*-prenyl diphosphate synthase (*cis*-PDPS) that produces precursor for irregular monoterpenes in lavenders.

**Conclusion:** Unlike other plants that utilize *trans*-PDPSs, *Lavandula* employ a *cis*-PDPS to initiate the biosynthesis of irregular monoterpenes.

**Significance:** This is the first report of the involvement of a *cis*-PDPS in irregular monoterpenes biosynthesis.

Lavender essential oils are constituted predominantly of regular monoterpenes, for example linalool, 1,8-cineole, and camphor. However, they also contain irregular monoterpenes including lavandulol and lavandulol acetate. Although the majority of genes responsible for the production of regular monoterpenes in lavenders are now known, enzymes (including lavandulyl diphosphate synthase (LPPS)) catalyzing the biosynthesis of irregular monoterpenes in these plants have not been described. Here, we report the isolation and functional characterization of a novel *cis*-prenyl diphosphate synthase cDNA, termed *Lavandula x intermedia* lavandulyl diphosphate synthase (LiLPPS), through a homology-based cloning strategy. The *LiLPPS* ORF, encoding for a 305-amino acid long protein, was expressed in *Escherichia coli*, and the recombinant protein was purified by nickel-nitriotriacetic acid affinity chromatography. The approximately 34.5-kDa bacterially produced protein specifically catalyzed the head-to-middle condensation of two dimethylallyl diphosphate units to LPP *in vitro* with apparent *Kₘ* and *kₘₐₚ* values of 208 ± 12 μM and 0.1 s⁻¹, respectively. LiLPPS is a homodimeric enzyme with a sigmoidal saturation curve and Hill coefficient of 2.7, suggesting a positive co-operative interaction among its catalytic sites. *LiLPPS* could be used to modulate the production of lavandulol and its derivatives in plants through metabolic engineering.

Monoterpenes, the C₁₀ class of the isoprenoids, are derived from the universal terpene building blocks isopentenyl diphosphate (IPP)³ and dimethylallyl diphosphate (DMAPP) predominantly synthesized through the 2-C-methyl-D-erythritol 4-phosphate metabolic pathway in plants (1). *En route* to monoterpene synthesis IPP and DMAPP, or two DMAPP units, can be condensed to form structurally diverse branch point C₁₀ precursor molecules (2). The diversity of these molecules arises from the capability of prenyl diphosphate synthase enzymes (PDPSs) to condense isoprenes in head-to-tail or non-head-to-tail orientations (3, 4) and outcomes with *cis* or *trans* geometric configurations (5). For example, the head-to-tail coupling of two isoprene units to geranyl diphosphate (C₁₀G; GPP) or its cisoid isomer neryl diphosphate (C₁₀N; NPP) is catalyzed by geranyl diphosphate synthase (GPPS) and neryl diphosphate synthase (NPPS), respectively. The non-head-to-tail condensation of isoprene units to lavandulyl diphosphate (C₁₀L; LPP) and chrysanthemyl diphosphate (C₁₀C; CPP), on the other hand, is catalyzed by lavandulyl diphosphate synthase (LPPS) and chrysanthemyl diphosphate synthase (CPPS), respectively (Fig. 1). The final stage of monoterpene synthesis, transforming and elaborating the different precursor molecules to monoterpenes, is catalyzed by another group of enzymes called monoterpene synthases (mTPSs).

The head-to-tail condensation of IPP and DMAPP and the enzymes catalyzing these reactions are the most common in nature. Thus, monoterpenes derived from GPP and NPP such as linalool, 1,8-cineole, limonene, and so forth, are widely distributed and are referred to as “regular monoterpenes.” Subsequently, their biosynthetic route is well established, and cDNAs encoding for GPPS (6–10), NPPS (11), and mTPSs capable of

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³ The abbreviations used are: IPP, isopentenyl diphosphate; CIP, calf intestinal alkaline phosphatase; CPP, chrysanthemyl diphosphate; GPPS, CIP synthase; dDPPS, dehydrodolichol diphosphate synthase; DMAPP, dimethylallyl diphosphate; EO, essential oil; EST, expressed sequence tag; FPP, farnesyl diphosphate; FPPS, FPP synthase; GPPS, geranyl diphosphate synthase; GPPS, GPP synthase; LICINS, *L. x intermedia* 1,8-cineole synthase; LiLPPS, *L. x intermedia* LPP; LPP, lavandulyl diphosphate; LPPS, LPP synthase; mTPS, monoterpene synthase; NPP, neryl diphosphate; NPPS, NPP synthase; PDP, prenyl diphosphate; PDPs, PDP synthase.
transforming GPP and/or NPP into core monoterpenes have been described from diverse plant species (12, 13). GPPS, a trans-PDPS, and NPPS, a cis-PDPS, elongate the linear prenyl chain by coupling DMAPP with IPP through a chain elongation (c1-H1032-4) reaction to generate GPP and NPP, respectively (3). Despite catalyzing the same reaction, however, GPPSs and NPPSs are more distantly related to one another than they are to PDPSs of similar geometric outcomes. GPPSs share high sequence similarity with trans-PDPSs catalyzing the higher order terpene precursors and are distinguished by two aspartate-rich conserved motifs, DDX2–4D and (N/D)DXXD. These motifs serve as a substrate and divalent metal ion cofactor, often Mg2+, binding sites for the carbocation rearrangement-mediated condensation reactions (2, 5). A third conserved motif, the CXXXC motif (where X is any hydrophobic residue), is present in heterodimeric PDPSs (14). Heterodimeric PDPSs, like the GPPSs cloned from *Mentha x piperita* (8), *Salvia miltiorrhiza* (15) etc., are enzymes constituted of two subunits (large and small) which interact through the CXXXC conserved motif to be catalytically active. NPPSs and other cis-PDPSs, on the other hand, do not necessarily retain the above motifs but share five conserved regions designated as Regions I–V. In particular, the aspartate residue in Region IV and the glutamate residue of Region V are catalytically essential (16, 17).

PDPSs catalyzing non–head-to-tail coupling reactions, and monoterpenes derived from LPP and CPP such as lavandulol and pyrethrins, respectively, are encountered less frequently in nature (18–20). Irregular monoterpenes, like pyrethrins, are the major ingredients in leading botanical and EPA-certified insecticides (21, 22). Lavandulol and its ester derivative lavandulyl acetate were identified in pheromones of major insect pests and are subsequently used in artificial pheromone preparations to disrupt the mating behaviors of economically important pests (23, 24). Yet, little is known about the biosynthetic pathways leading to these monoterpenes and their derivatives. Only two cDNAs encoding for CPPSs, both trans-PDPS family members, have been isolated and functionally characterized from *Chrysanthemum cinerariaefolium* (20) and *Artemisia tridentate* ssp. *spiciformis* (25) so far. CPPSs catalyze predominantly the cyclopropanation (c1-H1032-2) reaction in which the non–head-to-tail condensation of two DMAPP molecules generates the pyrethrin branch point intermediate, LPP. In addition to their major product, the A. tridentate CPPS and its chimeric derivatives were found to catalyze the non–head-to-tail coupling of two DMAPP molecules through branching (c1-H1032-2) to generate LPP (3, 4). LPP is the branch point precursor of monoterpenes with head-to-middle condensed PDP backbones, such as lavandulol and lavandulyl acetate (19). It is also the source of the lavandulyl side group of sophoraflavanone G in *Sophora flavescens* Ait (26) that determines their antitumor (27) and phospholipase C-1 inhibition properties (28). To our knowledge a wild type LPPS gene has not been described from plants or other organisms.

Essential oils (EOs) of the genus *Lavandula* (lavenders) are constituted primarily of a few “regular” monoterpenes and their derivatives. For example, the economically important EOs derived from *L. angustifolia* and *L. x intermedia* species, contain large amounts of linalool, linalool acetate, and 1,8-cineole. Consequently, these oils are industrially utilized as ingredients of various cosmetic and antiseptic products (29). The biosynthetic pathway leading to these monoterpenes from IPP and DMAPP has been defined both experimentally (30–32) and...
through in silico analysis of Lavandula EST databases (30, 31, 33). In this regard, four cDNAs encoding for mTPSs that transform GPP and NPP into linalool, 1,8-cineole, limonene, and β-phellandrene in vitro have been cloned from L. angustifolia and L. x intermedia species (30–32). However, lavender EOs, particularly L. x intermedia species, also contain the irregular monoterpane lavandulol and its ester derivative lavandulyl acetate (34) whose biosynthetic origin has not yet been investigated. Here, we report the isolation and functional characterization of a novel cis-PDPS cDNA encoding for LPPS, an enzyme that condenses two DMAPP molecules to generate the lavandulol branch point precursor LPP in vitro, from a L. x intermedia oil gland cDNA library.

EXPERIMENTAL PROCEDURES

LiLPPS Candidate Selection—Our group recently reported the construction of a cDNA library and its corresponding annotated expressed sequence tag (EST) database from L. x intermedia cv. Grosso secretory cells of oil glands, tissues specialized for EO biosynthesis and secretion (31). All PDPS homologs in the database were retrieved by searching the EST database using the strings “diphosphate synthase” and “pyrophosphate synthase.” We then synchronized the search results and manually excluded EST homologs known to be involved in regular terpene biosynthesis including GPPS, trans-farnesyl diphosphate synthase (trans-FPPS), and trans-geranylgeranyl diphosphate synthase (trans-GPPS), and PDPS homologs that are not involved in terpene biosynthesis. This led us to acquire a novel cis-PDPS homolog contig that was later determined to be L. x intermedia lavandulol diphosphate synthase (LiLPPS). The transcriptional expression pattern of this contig and previously described mTPSs throughout L. x intermedia cv. Grosso flower developmental stages was assessed by microarray analysis using the Agilent oligonucleotide-based microarray technology, through services provided by the University Health Network Microarray Centre (Toronto, ON, Canada). The three floral developmental stages were unopened buds, anthesis, and mature flowers in which 30% of the buds were in bloom (for photographic description see Ref. 33). After validating the microarray data using standard PCR, we selected the contig for further detailed analysis.

Cloning, Protein Expression, and Enrichment of LiLPPS—Glandular trichome secretory cells were isolated from mature flowers of L. x intermedia cv. Grosso plants grown at the University of British Columbia, Okanagan campus lavender field following a previously described modified glass bead abrasion method (31). Total RNA was extracted from 100 mg of the tissue using an RNA extraction kit (OMEGA bio-tek) and reverse transcribed in a reaction containing the oligo(dT) primer (Fisher Scientific) and M-MuLV reverse transcriptase enzyme (New England Biolabs) following the manufacturer’s directions. The putative LiLPPS ORF was amplified with the full-length cloning primer sets (Table 1) and iProof™ High-Fidelity DNA Polymerase (Bio-Rad). The PCR program used was 95 °C for 5 min, followed by 37 cycles of 95 °C for 1 min, 58 °C for 30 s, and 72 °C for 1 min, and a 5-min final extension at 72 °C. The amplified fragments were cloned into the Ndel/EcoRI sites of the pET41b(+) expression vector and expressed in Escherichia coli BL21(DE3) strain (EMD Chemicals, Darmstadt, Germany) following previously described procedures (30, 31). Except for the bind buffer that was slightly modified (0.5 M NaCl, 50 mM KH2PO4, pH 8.0) the procedure described in the aforementioned papers was followed to enrich the expressed protein. Sequence information for LiLPPS was deposited in the NCBI public data bank with the accession number JX985358.

**LiLPPS Product Assay and EO Constituent Identification**—Initial in vitro enzyme activity assays were performed in a 500-μl reaction volume containing the assay buffer (50 mM Tris-HCl, 5% glycerol, 1 mM MnCl2, 1 mM MgCl2, pH 8.0), 1 mM DTT, 40 μM IPP, and 40 μM DMAPP (Echelon, Salt Lake City, UT), and 2.5–50 μg of LiLPPS. After a 2-h incubation at 30 °C, the reaction mix was heated at >80 °C for 10 min and kept on ice for 2 min. Then 30 units of calf intestinal alkaline phosphatase (CIP; New England Biolabs) were added to the reaction mix, overlaid by 500 μl of pentane and incubated overnight at 32.5 °C to hydrolyze the prenyl product. GPP and NPP standards (Echelon) were also hydrolyzed in the same reaction mix and conditions, as a control of the hydrolysis reaction. The reaction was stopped by vigorous vortexing followed by flash freezing in liquid nitrogen and stored in a −80 °C freezer until analyzed. Assay product identification and quantification, and EO extraction and quantification were performed by gas chromatography-mass spectrometry (GC-MS) following procedures described previously (32, 35). Purified protein extract of E. coli BL21(DE3) strain transformed with empty pET41b(+) expression vector, i.e. without insert, was also assayed under the same conditions as a negative control.

**Biochemical Assay**—Biochemical characterization of LiLPPS was performed following the procedure described above with slight modifications: the reaction volume was reduced to 125 μl, 2.5 μg of LiLPPS was used, and assays were incubated at 37 °C for 1 h after adding CIP. Six temperature levels (25, 27.5, 30, 32.5, 35, and 37.5 °C) were tested to determine LiLPPS optimum temperature, and the optimum pH was determined using MES and MOPS buffers at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The kinetic properties of LiLPPS were deduced from LPP accumulation data in assays with increasing DMAPP concentrations (6.25, 12.5, 25, 50, 100, 150, 200, 300, 450, and 600 μM) at the optimal conditions. Prism software version 5.0d (GraphPad Software, Inc., La Jolla, CA) was used to fit the data into a sigmoidal substrate concentration-dependent enzyme response.

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**TABLE 1**

Oligonucleotides used in this study

| Primers          | Sequences                  |
|------------------|----------------------------|
| LiLPPS set I     | F: 5′-CCTCTATAGGATCTCCGATGACC-3′ |
|                  | R1: 5′-CTCTATAGGATCTCCGATGACC-3′ |
|                  | R2: 5′-CTCTATAGGATCTCCGATGACC-3′ |
| LiLPPS set II    | F: 5′-CTCTATAGGATCTCCGATGACC-3′ |
|                  | R1: 5′-CTCTATAGGATCTCCGATGACC-3′ |
|                  | R2: 5′-CTCTATAGGATCTCCGATGACC-3′ |
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curve \((y = a + b/(c + x))\), and to calculate the \(V_{\text{max}}, K_m,\) and \(k_{\text{cat}}\) values. Substrate specificity of the enzyme was determined from the type and amount of reactants consumed versus product accumulated in assays containing a constant IPP concentration (150 \(\mu M\)) combined with various DMAPP levels (6.25–200 \(\mu M\)) under the optimized conditions. We also tested LPP as a potential substrate for \(L. x\) intermedia 1,8-cineole synthase (LiCINS), an enzyme that primarily produced 1,8-cineole from both GPP and NPP (31).

Phylogenetic Relationship Analysis—LiLPPS was aligned with cis-PDPSs isolated from tomato using the default parameters of the ClustalW alignment tool available at the EBI platform. The phylogenetic relation of LiLPPS with other PDPSs was constructed using the Jukes-Cantor genetic distance model. The phylogenetic relationship of LiLPPS with other PDPSs involved in the 2-C-methyl-D-erythritol (2-CME) pathway rich plastidial (30 amino acids) and shares 66.4 and 66% sequence similarity with NPPS and cis-FPPS of tomato, respectively. Like many other cis-PDPSs, LiLPPs lacks the two aspartate rich trans-PDPS signature motifs DDX\(_{2,4}\)D and (N/D)DXD but maintains all five semiconserved regions (I–V) that define the cis-PDPSs (Fig. 2). In particular, the catalytically important aspartate and glutamate residues of Regions IV and V were conserved in LiLPPs at 201 and 276 positions from the N-terminal, respectively (Fig. 2). Multiple alignment of LiLPPs and its splice variant, both at the nucleotide and amino acid level, revealed that the splice variant lacks a stretch of 22 amino acids in the middle of its ORF that included a portion of the conserved region III (Fig. 2). Repeated attempts to amplify the splice variant from reverse transcribed total mRNA isolated from \(L. x\) intermedia glandular secretory cells failed possibly due to the low abundance of the corresponding cDNA. It is also possible that the splice variant singleton was an artifact that resulted from sequence assembly errors during database construction. Therefore, only the full-length contig was considered for further investigation.

Cloning, Expression, and Functional Characterization of LiLPPS—The approximately 34.5-kDa recombinant LiLPPs was successfully expressed in bacterial cells and enriched using the nickel-nitritolactric acid-agarose affinity chromatography system. Incubation of the purified recombinant protein with IPP and DMAPP followed by the hydrolysis of the assay mix by CIP resulted in the production of lavandulol as the only product (Fig. 3A). The identity of the product was determined by comparing its mass spectrum and retention time with those of authentic lavandulol standard (Sigma) (Fig. 3B). Given that the alkaline CIP-mediated hydrolysis of LPP is known to yield lavandulol (3, 4, 19), we concluded that LiLPPs catalyzes the synthesis of LPP from isoprene units. In this respect, the hydrolysis of GPP and NPP with CIP under the same conditions produced geraniol and nerol, respectively, as anticipated. Hydrolysis of the control assay mixes contained only the prenyl alcohol derivatives of IPP (buten-1-ol, \(<3\)-methyl-3) and DMAPP (buten-1-ol, \(<3\)-methyl-2) (Fig. S2, A and B).

Substrate Specificity and Kinetic Properties of LiLPPS—When LiLPPs was incubated with IPP alone, detectable amounts of lavandulol or any other terpene prenyl alcohols were not identified after the alkaline hydrolysis step. We also observed that assay reactions containing 5 \(\mu M\) each IPP and DMAPP, or 5 \(\mu M\) DMAPP alone produced equivalent amounts of the preferred substrate for regular monoterpenes (11, 36) and typical sesquiterpenes synthesized from cis-FPP (17) were not detected in the \(L. x\) intermedia EO (supplemental Fig. S1), the identification of NPPS and cis-FPPS homologs was unforeseen. In addition, the results of our transcript-profiling experiment indicated that the transcriptional expression of this EST paralleled those of other lavender mTPSs (31) and was developmentally regulated in \(L. x\) intermedia flowers (data not shown). Therefore, we decided to further investigate this contig which was later determined to be LiLPPS. The ORF of LiLPPS was expressed in \(E. coli\) BL21(DE3) cells for further analysis.

RESULTS

Lavandula x intermedia cv. Grosso EO Composition—GC-MS analysis of EO distilled from \(L. x\) intermedia cv. Grosso floral tissues profiled the following terpenes in their respective abundance order: linalool, linalool acetate, 1,8-cineole, camphor, isoborneol, lavandulyl acetate, \(\alpha\)-terpineol, \(\alpha\)-bisabolol, \(\alpha\)-cadinol, \(\beta\)-ocimene, \(\beta\)-caryophyllene, lavandulon, limonene, \(\delta\)-carene, myrcene, geraniol, nerol, neryl acetate, and a few other minor products (supplemental Fig. S1). Except for the irregular monoterpenes lavandulol and lavandulyl acetate, which are derived from LPP, the remaining EO mono- and sesquiterpene constituents arise from the corresponding linear precursors GPP and trans-FPP, respectively.

PDPS Candidate Selection and Sequence Analysis—Searching our EST library using the string pyrophosphate synthase as a query identified 11 different prenyltransferase homolog unigenes whereas the string diphosphate synthase identified 31. Duplicate search results and prenyltransferases with no known involvement in terpene biosynthesis (e.g. ribose phosphate pyrophosphokinase and cytidine diphosphate diacylglycerol synthase) were disregarded to consolidate the search results. The final synchronized search result identified 25 unigenes that were homologous to known PDPSs involved in terpene biosynthesis (supplemental Table S1). From these sequences 12 were homologous to PDPSs involved in the 2-C-methyl-D-erythritol 4-phosphate pathway, eight were homologous to \(L. x\) intermedia 1,8-cineole synthase, and one was homologous to bornyl diphosphate synthase. The remaining four candidates were homologous to \(cis\)-PDPSs. Two of these homologs corresponded to dehydrodolichol diphosphate synthase (dDPPS) genes, which catalyzes the prenyl chain elongation reaction to produce the poly-prenyl backbone of dolichol. The other unigenes, one of which belonged to a contig with 18 EST members and the other was a singleton, exhibited significant homology to NPPS (FJ797956) and \(cis\)-FPPS (AJ38408) from cultivated \((Solanum lycopersicum)\) (11) and wild tomato \((Solanum habrochaites)\) (17), respectively. The singleton was later determined to be a splice variant of the contig and was disregarded.

The \(trans\)-PDPS and dDPPS homologs were anticipated to be present in a database derived from \(L. x\) intermedia secretory cells because they catalyze reactions that are consistent with EO composition in this tissue. However, given the fact that GPP is the preferred substrate for regular monoterpenes (11, 36) and typical sesquiterpenes synthesized from cis-FPP (17) were not detected in the \(L. x\) intermedia EO (supplemental Fig. S1), the identification of NPPS and \(cis\)-FPPS homologs was unforeseen. In addition, the results of our transcript-profiling experiment indicated that the transcriptional expression of this EST paralleled those of other lavender mTPSs (31) and was developmentally regulated in \(L. x\) intermedia flowers (data not shown). Therefore, we decided to further investigate this contig which was later determined to be LiLPPS. The ORF of LiLPPS was expressed in \(E. coli\) BL21(DE3) cells for further analysis.

The ORF of LiLPPS is 918 nucleotides long encoding a 305-amino acid protein that includes a short N-terminal plastidial transit peptide (30 amino acids) and shares 66.4 and 66% sequence similarity with NPPS and \(cis\)-FPPS of tomato, respectively. Like many other \(cis\)-PDPSs, LiLPPs lacks the two aspartate rich trans-PDPS signature motifs DDX\(_{2,4}\)D and (N/D)DXD but maintains all five semiconserved regions (I–V) that define the cis-PDPSs (Fig. 2). In particular, the catalytically important aspartate and glutamate residues of Regions IV and V were conserved in LiLPPs at 201 and 276 positions from the N-terminal, respectively (Fig. 2). Multiple alignment of LiLPPs and its splice variant, both at the nucleotide and amino acid level, revealed that the splice variant lacks a stretch of 22 amino acids in the middle of its ORF that included a portion of the conserved region III (Fig. 2). Repeated attempts to amplify the splice variant from reverse transcribed total mRNA isolated from \(L. x\) intermedia glandular secretory cells failed possibly due to the low abundance of the corresponding cDNA. It is also possible that the splice variant singleton was an artifact that resulted from sequence assembly errors during database construction. Therefore, only the full-length contig was considered for further investigation.
of LPP. Furthermore, lowering the concentration of DMAPP in the assay resulted in a parallel reduction in LPP production, although reducing the concentration or excluding IPP from the assay had no effect. When LiLPPS was incubated with a constant IPP concentration (150 μM) combined with an increasing DMAPP amount (6.25–200 μM), the amount of LPP accumulated correlated ($R^2 = 0.99; p = 0.0001$) with the amount of DMAPP consumed whereas the IPP consumption remained close to zero at all combinations (Fig. 4).

The optimum temperature and pH of LiLPPS were found to be 30 °C and 8.0 (supplemental Fig. S3, A and B), respectively. Unlike other PDPSs cloned from related plant species, the substrate concentration-dependent saturation curve of LiLPPS did not follow the standard Michaelis-Menten kinetics for single substrate enzymes. Instead, the amount of LPP accumulated in response to increasing DMAPP concentration levels (6.25–600 μM) fitted a sigmoidal saturation curve (Fig. 5), typical of two substrate enzymes. The nonlinear regression equation

$$V = V_{\text{max}} \frac{[S]}{K_M + [S]}$$

was used to calculate the $K_M$ and $V_{\text{max}}$ of LiLPPS which were determined to be 208 ± 12 μM and 448 ± 22 pmol/min, respectively. The Hill coefficient ($H$) value of LiLPPS was 2.7 ± 0.3 whereas the $k_{\text{cat}}$ ($V_{\text{max}}/E$) and catalytic efficiency ($k_{\text{cat}}/K_M$) of the enzyme were calculated as 0.1 s$^{-1}$ and 5 × $10^{-10}$ M$^{-1}$ s$^{-1}$, respectively.

**LPP as a Substrate for Regular mTPSs**—mTPSs involved in the biosynthesis of regular monoterpenes in lavenders accept both GPP and NPP as in vitro substrates. We thus examined the ability of the recombinant LiCINS to utilize LPP as a substrate in standard assay reactions. As anticipated, LiCINS, which produced 1,8-cineole as a major product upon incubation with GPP and NPP (32), did not produce detectable quantities of a product from LPP.
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The cis and trans PDPS family members often share higher sequence similarity with each other irrespective of the genetic relatedness among donor organisms (5, 16). This is contrary to some other enzymes involved in terpene biosynthesis, particularly terpene synthases. Typically, terpene synthases of a given species are more related to one another than to those of distantly related species, even if they catalyze the same reaction. For example β-phellandrene synthase of *L. angustifolia* shares 48 and 47% identity with linalool and limonene synthases of the same species, respectively. However, the gene only shares 31 and 25% similarity with β-phellandrene synthases of grand fir and tomato, respectively (30). In this study, we observed that *L. x intermedia* trans-PDPS homologs (GPPS, FPPS, and GGPPS) share a higher sequence similarity among each other and with other trans-PDPSs cloned from genetically unrelated species than they do with LiLPPS. LiLPPS is closely related to NPPS and cis-FPPS of cultivated (*S. lycopersicum*) (11) and wild (*S. habrochaites*) (17) tomato, respectively. The phylogenetic tree presented in Fig. 6 clearly suggests that LiLPPS together with NPPS and cis-FPPSs diverged very early from trans-PDPSs.

LiLPPS catalyzes the head-to-middle condensation of two DMAPP molecules to synthesize the linear lavandulol branch point precursor, LPP (C10). As demonstrated by Thulasiram *et al.* (3, 4), LPP synthesis proceeds via rearrangement of the double bond position to create the highly reactive carbocation intermediate lavandulyl cation (L^+). L^+ will eventually be transformed to LPP after a mandatory proton loss (Fig. 1). LiLPPS catalyzes these reactions with an apparent *K_m* and *k_cat* of 208 ± 12 μM and 0.1 s⁻¹, respectively. These catalytic properties are within the range of previously reported cis-PDPSs including NPPS, which has a *K_m* and *k_cat* of 177 μM and 0.2 s⁻¹, respectively (11). The *K_m* and *k_cat* values of the aromatic prenyltransferase cloned from fungi were 325 μM and 0.03 s⁻¹, respectively (39). Unlike other PDPSs, LiLPPS displayed a sigmoidal saturation curve (Fig. 5). This is a typical feature of enzymes with multiple substrates, in which the binding of the first substrate affects the affinity of the enzyme for the second substrate through conformational changes or stabilization of the active pocket environment (40). LiLPPS, like any other typical PDPS enzyme, has binding sites for a divalent metal ion.
cofactor and two substrates (5, 16). PDPSs accepting IPP and DMAPP (e.g. GPPS or NPPS) can be saturated with one of the substrates and forced to follow Michaelis-Menten kinetics typical of single substrate enzymes for the other substrate. Because DMAPP was the only substrate for LiLPPS, saturating one site was not an option. Thus, plotting LiLPPS product (LPP) accumulation against increasing substrate (DMAPP) concentrations assumed a sigmoidal saturation curve with a Hill coefficient (H) value of 2.7. The positive Hill coefficient value (2.7) indicates a positive co-operativity among the binding pockets. If LPP was a condensation result of IPP and DMAPP, an equimolar consumption of the two isoprenes would be expected in our assays. However, LPP production was independent of IPP and required only DMAPP. In our assays, a reduction in DMAPP supply resulted in an equivalent reduction in LPP accumulation. However, reducing the concentration or excluding IPP from the reaction did not alter LPP production. This result was also confirmed when IPP was provided at a constant high concentration level (150 μM) while the amount of DMAPP was increased progressively (6.25–200 μM), and the reaction was allowed to run until LPP synthesis ceased or DMAPP molecules were nearly consumed. At all IPP and DMAPP concentration combinations assayed, IPP consumption remained very close to 0, whereas that of DMAPP increased in parallel to the amount of LPP synthesized (Fig. 4). This outcome indicated that, like the CPPS enzymes reported by Thulasiram et al. (4) and Rivera et al. (20), LiLPPS utilizes DMAPP as the only substrate to synthesize LPP. The structural features of LiLPPS underlying this catalytic property or residues involved in DMAPP recognition and c1’-2 coupling reaction catalysis are yet to be determined. Our results, however, suggest that the active sites of LiLPPS selectively bind two DMAPP (and not IPP) units and position them in such a way that the first carbon atom of one unit is in close proximity to the second carbon of the other to facilitate the c1’-2 bond formation. One possibility is that LiLPPS preferentially recognizes DMAPP by identifying its double bond position. It is also possible that the position of the double bond in IPP is not favorable for head-to-middle condensation.

With >55,000 members, isoprenoids are the most structurally and stereochemically diverse biochemical compounds known to mankind (42). Much of this diversity has been attributed to the astounding mechanistic heterogeneity and promiscuity of terpene synthases. PDPSs, cis or trans, also play a major role in the structural diversity of isoprenoids by providing intermediate precursor molecules of varying chain length (C10, C15, C20, C30, etc.) destined for different isoprenoid groups. In addition, PDPSs generate the structurally distinct C10 precursor molecules GPP/NPP, LPP, CPP, maconelliyl diphosphate (C10) or planococcyl diphosphate (C10) by simply changing the position of the carbon-carbon bond (3,4, 20). These linear precursors are then elaborated upon by mTPSs to create various monoterpenes (42, 43). In Lavandula, GPP and LPP are the linear precursors for the biosynthesis of regular and irregular monoterpenes, respectively (30–32).

In conclusion, through the identification and functional characterization of LiLPPS, a novel cis-PDPS, we have elucidated the biosynthetic origin of irregular monoterpene constitu-
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Degenhardt, J., Köllner, T. G., and Gershenzon, J. (2009) Monoterpenes in Lavenders and other plants. The elucidation of this pathway enables researchers to further investigate the biosynthesis of irregular monoterpenes in lavenders and other plants. Further, the cloned gene could be used to modulate the accumulation of lavandulol, lavandulyl acetate, and prenylated metabolites with a lavandulyl group to a desired level through metabolic engineering (44).

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