Cloning, functional characterization and evaluating potential in metabolic engineering for lavender (+)-bornyl diphosphate synthase

Ayelign M. Adal
UBC: The University of British Columbia
Elaheh Najafianashrafi
UBC: The University of British Columbia
Lukman S. Sarker
UBC: The University of British Columbia
Soheil S Mahmoud (soheil.mahmoud@ubc.ca)
University of British Columbia, Okanagan campus https://orcid.org/0000-0001-6709-1398

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Abstract

The monoterpene (+)-borneol contributes scent and medicinal properties to some plants. It also is the immediate precursor to camphor, another important determinant of aroma and medicinal properties in many plants. (+)-borneol is generated through the dephosphorylation of bornyl diphosphate (BPP), which is itself derived from geranyl diphosphate (GPP) by the enzyme (+)-bornyl diphosphate synthase ((+)-BPPS). In this study we isolated and functionally characterized a novel (+)-BPPS cDNA from *Lavandula x intermedia*. The cDNA excluding its transit peptide was expressed in *E. coli*, and the corresponding recombinant protein was purified with Ni–NTA agarose affinity chromatography. The recombinant (+)-LiBPPS catalyzed conversion of GPP to BPP as the major product, and a few minor products. We also investigated the *in planta* role of (+)-LiBPPS in terpenoid metabolism through its overexpression in sense and antisense orientations in stably transformed *Lavandula latifolia* plants. The overexpression of *(+)-LiBPPS* in antisense resulted in reduced production of (+)-borneol and camphor without compromising plant growth and development. As anticipated, the overexpression of the gene led to enhanced production of borneol and camphor, although growth and development were severely impaired in most transgenic lines strongly and ectopically expressing the *(+)-LiBPPS* transgene in sense. Our results demonstrate that *LiBPPS* would be useful in studies aimed at the production of recombinant borneol and camphor *in vitro*, and in metabolic engineering efforts aimed at lowering borneol and camphor production in plants. However, overexpression in sense may require a targeted expression of the gene in glandular trichomes using a trichome-specific promoter.

Introduction

Several species of the genus *Lavandula* (Lamiaceae) are cultivated worldwide for the production of essential oils (EOs), cut flowers and other horticultural purposes. Of these, *L. angustifolia*, *L. latifolia* and their natural hybrid *L. x intermedia* are widely grown for EO production (Wells et al. 2018; Erland and Mahmoud 2015; Upson and Andrews 2004).

The quality of lavender EO is mainly determined by its monoterpene constituents, which mainly include linalool, linalyl acetate, (+)-borneol, camphor and 1,8-cineole (Upson and Andrews 2004). Linalool and linalyl acetate contribute pleasant scents and are desirable in EOs used in perfumery and aromatherapy. Borneol and camphor are odorous, undesired constituents of EOs used in perfumery and aromatherapy; however, they both have medicinal properties and are abundant in oils obtained from *L. latifolia* plants. These oils have extensive applications in traditional and alternative medicinal preparations (Wells et al. 2018). The EO derived from *L. x intermedia* species has moderate levels of all monoterpenes found in both parents. However, *L. x intermedia* plants have a much higher oil yield (up to 10 X) than either parent. Thus, there is substantial interest in developing *L. x intermeida* plants that produce EOs comparable in composition to those found in *L. angustifolia* or *L. latifolia* species. These objectives may be achieved through plant biotechnology once all related genes are cloned, and necessary molecular tools are developed.

Most *Lavandula* monoterpenes are derived from the head-to-tail condensation of the universal isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) generated through the MEP pathway in plastids. IPP and DMAPP are initially condensed to geranyl diphosphate (GPP) – the linear precursor to most monoterpenes – by geranyl diphosphate synthase. GPP is then converted to other prenyl diphosphate and monoterpenes by specific short-chain isoprenyl diphosphate synthase (IDS), and terpene synthase (TPS) enzymes, respectively (Fig. 1). Several *Lavandula* TPS and IDS genes have been identified and functionally characterized *in vitro* (Landmann et al. 2007; Woronuk et al. 2011; Demissie et al. 2011; Demissie et al. 2012; Sarker et al. 2012; Benabdelkader et al. 2015; Adal et al. 2017; Despinasse et al. 2017; Adal et al. 2019; Adal and Mahmoud 2020).

The biosynthesis of borneol and camphor in plants begins with the formation of bornyl diphosphate (BPP) from GPP through the reaction catalyzed by BPP synthase (BPPS) (Fig. 1). BPP is then used as a substrate for the synthesis of borneol, which is subsequently oxidized into camphor by borneol dehydrogenase (Sarker et al. 2012). To date, five genes encoding BPPS have been reported from *Salvia officinalis* (Wise et al. 1998), L. *angustifolia* (Despinasse et al. 2017), *Lippia dulcis* (Hurd et al. 2017), *Cinnamomum burmanni* (Ma et al. 2021) and *Amomum villosum* (Wang et al. 2018). From these, Wise et al (1998) and Ma et al. (2021) specifically identified the BPPSs as (+)-BPPS type in *S. officinalis* and *C. burmanni*, respectively. Most reported plant BPPSs are multi-product enzymes catalyzing GPP to BPP (major) and multiple monoterpenes (minor) (Wise et al. 1998; Despinasse et al. 2017; Wang et al. 2018; Ma et al. 2021). For example, *S. officinalis* BPPS produces 25% monoterpenes and 75% BPP, while that of *L. angustifolia* generates 70% monoterpenes and 30% BPP. The only exception so far is the *P. dulcis* BPPS, which converts GPP to a single product BPP (Hurd et al. 2017). In this study, we identified a unique *(+)-BPPS* gene in a previously reported *Lavandula* transcriptome database (Adal et al. 2019), cloned the gene from *L. x intermedia*, and functionally characterized the recombinant form of the encoded protein. We also evaluated the effects of overexpressing the gene in sense and antisense on borneol and camphor production in stably transformed *L. latifolia* plants.

Materials And Methods

Plant materials and reagents

Sample flower tissues were harvested from plants grown in an outdoor lavender experimental site at the University of British Columbia Okanagan (Kelowna, BC, Canada). *L. latifolia* seeds purchased from Seed Needs LLC (United States) were used to grow plants for transformation studies. Transformed and wild-type *L. latifolia* plants were maintained in a growth room at 25°C, with a photoperiod of 16 hours light and 8 hours darkness. All sample tissues collected from both *L. x intermedia* and *L. latifolia* plants for RNA extraction were immediately flash-frozen in liquid nitrogen and stored at -80 ^{o}C until used.

Authentic standards of (+)-borneol, (-)-borneol and other monoterpenes for assay product identification were purchased from Sigma (http://www.sigmaaldrich.com/). Geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) substrates

were purchased from Echelon Biosciences (http://www.echelon-inc.com/). Calf intestinal alkaline phosphatase (CIP) used to dephosphorylate the initial product (BPP) to borneol, and restriction enzymes were obtained from New England Biolabs (NEB). Other chemical reagents used in this study were obtained from Sigma unless otherwise noted.

Cloning of (+)-LiBPPS and LaBPPS

We screened a previously reported *L. x intermedia* transcriptome database (Adal et al. 2019) for potential BPPS synthase, and identified a candidate with significant homology to previously reported plant BPPS genes. Using gene-specific primers, the full-length LiBPPS cDNA (GenBank: MW846855) was cloned into pGEM-T (Promega) and, its sequence was confirmed by Sanger sequencing. The full-length genomic DNA for (+)-LiBPPS was amplified from *L. x intermedia* cv. Grosso, cloned into pGEM-T (Promega), and sequenced by Sanger sequencing.

For comparative studies, we also cloned the *L. angustifolia* BPPS (LaBPPS) cDNA from our *L. angustifolia* EST database (Lane et al. 2010). Briefly, the cDNA encoding LaBPPS was amplified by PCR and cloned into various vectors for confirmation of DNA sequence, and for heterologous expression in bacteria (see below). All primers used in this study are listed in Table S1.

Genome organization analysis

Genomic organization of *(+)-LiBPPS* was predicted using the NCBI Spidey genomic DNA-mRNA aligner (Wheelan et al. 2001), targeting intron-exon number and size, as well as intron position in the sequences. The intron-exon structure of *(+)-LiBPPS* was then compared with those previously reported TPSs from *Lavandula*.

Phylogenetic analysis and molecular modeling

Multiple sequence alignments of *(+)-LiBPPS* along with previously reported *BPPS* genes from other plants were made using T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular). The BOXSHADE 3.21 software (www.ch.embnet.org/software/BOX_form.html) was used for shading amino acid sequence alignments. To infer the evolutionary history of LiBPPS, the full-length amino acid sequences encoded functionally characterized BPPS and lavender TPS genes were aligned using ClustalW, and a maximum likelihood phylogenetic tree was constructed using MEGA7 (Kumar et al. 2016) with default settings. All TPSs clustered into previously defined distinct TPS subfamilies (Bohlmann et al. 1998; Chen et al. 2011).

A homology-based model of LiBPPS protein was produced using the SWISS-MODEL automated mode server (Bordoli et al. 2009; Guex et al. 2009). The crystal structure of *S. officinalis* bornyl diphosphate synthase (SoBPPS) (PDB code 1N1Z) containing the substrate analog diphosphate (POP) (Whittington et al. 2002) was detected as the top structural homologous template. A docking study with the model LiBPPS protein and GPP was conducted using autodock4 in a molecular docking server (Bikadi and Hazai 2009). The position of substrate GPP from SoBPPS PDB 1N20 was used as a docking positional

template, and the result was visualized and analyzed using PyMOL v1.1 (The PyMOL Molecular Graphics System, Schrödinger, LLC). The active site cavities were detected from the model using Swiss-pdbviewer (Guex et al. 2009), in which the binding residues at the active site pockets were predicted.

Bacterial expression and recombinant protein purification

For heterologous expression of the gene in *E. coli*, the ORF excluding the transit peptide for *(+)-LiBPPS* or *LaBPPS* was amplified by PCR and cloned into the *Nde*I/*EcoR*I sites of pET41b(+) by sticky-end cloning (Zeng 1998). The respective construct was then expressed in *E. coli* Rosetta™ (DE3)plysS cells (EMD Chemicals, Darmstadt, Germany). *E. coli* cells were grown in LB medium supplemented with 30 mg/L kanamycin and 34 mg/L chloramphenicol at 37°C to an OD_{600} of 0.5–0.6, followed by induction with 0.4 mM IPTG at 20°C overnight. After harvesting, the pelleted cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0) and disrupted by sonication. The soluble (+)-LiBPPS/LaBPPS protein was then purified using PerfectPro Ni-NTA Agarose chromatography (5Prime GmbH, Germany), according to the manufacturer's instructional manual. The purified recombinant proteins were then quantified through Bradford assays.

Enzymatic assays and product identification

The activity of the purified recombinant (+)-LiBPPS (or LaBPPS) protein was assayed with each of GPP, FPP, DMAPP and a combination of IPP and DMAPP as substrates. The assay reactions were prepared in a final volume of 500 µl, containing an enzyme reaction buffer (2 mM dithiothreitol, 12.5% (v/v) glycerol, 1 mM $MgCl_2$ and 1 mM $MnCl_2$), MOPS (pH 6.5) buffer, purified enzyme and substrate(s), and were overlaid with 400 µl of pentane and incubated at 30 ^{o}C for 2 h. Calf intestinal alkaline phosphatase (CIP, 30 U) (NEB Biolabs) was added to the assay, and incubated at 30 ^{o}C overnight to hydrolyze the BPP product to borneol. The assays were frozen at -80 ^{o}C, and the pentane fraction was removed for product analysis according to previous reports (Demissie et al. 2011; Demissie et al. 2012; Adal et al. 2017; Adal and Mahmoud 2020). As a control, heat-denatured (+)-LiBPPS enzyme and purified proteins from *E. coli* lysate cells harboring the pET41b(+) lacking (+)-LiBPPS were assayed with substrates under similar conditions. Assay products were identified using gas chromatography-mass spectrometry (GC-MS), following methods described in Adal et al. (2020). Further, borneol enantiomers ((+) and (-)) were analyzed by GC-flame ionization detector (GC-FID) using a 30 m × 0.25 mm fused silica capillary chiral column coated with Chirasil-DEX (cyclodextrin directly bonded to dimethylpolysiloxane) (Agilent Technologies Canada Inc, Mississauga, ON, CAN) according to Adal et al. (2019). Authentic standards of borneol isomers and thymol were also run on GC-FID under similar conditions to help identify the assay products.

Generation of binary vectors and plant transformation

For expression in *L. latifolia*, the full-length ORF of (+)-*LiBPPS* was cloned in the pCAMBIA1390 vector (which bears a hygromycin resistance gene) between the CaMV 35S promoter and the NOS

transcriptional terminator in either sense or antisense orientation. The resulting constructs (35S::LiBPPS-sense and 35S::LiBPPS-antisense) were separately transformed into *Agrobacterium tumefaciens* strain GV3101. Young leaves (ca. 1 cm in diameter) from tissue culture grown plantlets generated from seeds were transformed by *A. tumefaciens* carrying 35S::LiBPPS-sense or 35S::LiBPPS-antisense, according to Nebauer et al. (2000), with the following modifications. Infected leaves were cultured on Murashige and Skoog (MS) medium supplemented with plant growth hormones (0.6 µM IAA, 8.8 µM BA) and selection antibiotic (7.5 mg/L hygromycin) for shoot initiation. The newly emerged shoots were then excised and elongated on MS media containing 0.06 µM IAA, 8.9 µM BA and 7.5 mg/L hygromycin. Elongated shoots were then transferred to rooting media (half-strength MS salts supplemented with 2.9 µM IAA as previously reported (Erland and Mahmoud 2014)). Wild-type *L. latifolia* plants were used as negative controls for antibiotic selection and further analyses. Rooted plants were transferred to pots and placed in a growth room Until analyzed.

Relative expression

The spatial expression of *LiBPPS* was examined between leaf and flower tissues of *L. x intermedia* cv. grosso and *L. angustifolia cv. Maillette*. The expression of *(+)-LiBPPS* and previously reported *L. angustifolia BPPS* (Despinasse et al., 2017) were also compared in leaf tissues of *L. x intermedia, L. angustifolia* and *L. latifolia*. For transgenic plants, the transcript levels of *LiBPPS* were quantified in transgenic and wild-type *L. latifolia* leaf tissues. All relative expression studies were performed by qPCR assays using the StepOne Plus Real-Time PCR system (Applied Bioscience). In brief, total RNA was extracted from sample tissues of the aforementioned plants using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. RNA from each sample was then converted to cDNA using iScript cDNA synthesis kit (Bio-Rad). *β-actin* was used as a reference gene to normalize the expression levels. qPCR was performed with a final reaction volume of 10 µl containing 5 µl SYBR premix, 0.6 µM of each primer and 150 ng of cDNA. PCR amplifications were then carried out using the following program; (1) holding stage at 50 ^{o}C for 2 min, and 95 ^{o}C for 2 min, followed by (2) amplification stage of 50 cycles at 95°C for 3 s and 60°C for 30 s, as well as (3) a final melting curve stage at 95°C for 15 s and 60°C for 1 min. The generated data were analyzed using the Livak method (Livak and Schmittgen 2001), expressed as $2^{-\Delta\Delta CT}$. The primers used in this study are listed in Table S1.

EO extraction and GC-MS analysis

Young leaves (~ 0.5 g) of transgenic or control *L. latifolia* plants were harvested and immediately placed in separate 15-mL glass test tubes each containing 5 mL pentane, and 50 µg thymol as an internal standard. This was followed by sonication on ice for 30 min. Pentane, containing EO constituents, from each sample was then treated with activated charcoal and run on a Varian CP 3800 GC coupled to a Saturn 2200 MS/MS according to previously described methods (Adal et al. 2017). Briefly, a 1 µl sample was injected with the following instrument setting. Column flow rate: 1 mL min^{-1}, injector temperature: 40 ^{o}C for 10 s, and increased to 100 ^{o}C (20 ^{o}C min^{-1}) for the remaining of the run; column oven

temperature: 40 ^{o}C for 5 min, rise to 170 ^{o}C at a rate of 6 ^{o}C min^{-1}, hold for 4 min, then ramp to 230 ^{o}C at a rate of 30 ^{o}C min^{-1}, and hold for 5 min. The relative percent composition of EO constituents in transgenic and control samples was calculated based on an internal standard (thymol) as previously described (Erland and Mahmoud 2015). Statistical analysis (Student's *t*-test) was performed using SigmaPlot v 12.5 (Systat Software, Germany).

Results

Cloning of (+)-*LiBPPS*

In this study, we identified and cloned the cDNA for (+)-LiBPPS, a terpene synthase enzyme that can convert GPP to BPP *in vitro*. The full-length cDNA was amplified by PCR from reverse-transcribed RNA obtained from *L. x intermedia*, cv. Grosso, flower tissue. The ORF of the cDNA (GenBank: MW846855) encoded a protein of 593 amino acids, bearing a 50 amino acid plastid targeting sequence. The encoded protein was predicted to have a molecular mass of 69.6 kDa and a pI of 5.08. The deduced amino acid sequence was further characterized for the conserved motifs against known orthologous TPS proteins. As with other plant TPSs, the key motifs that are crucial for prenyl pyrophosphate ionization (RRx8W), and for divalent metal ion binding ((DDxxD) and (N,D)D(L,I,V)x(S,T)x3E) were well-conserved in this enzyme (Fig. S1). (+)-LiBPPS exhibited a relatively high degree of amino acid identity with *S. officinalis* BPPS (65.5%), while it was less homologous to other known BPPSs from *L. angustifolia* (LaBPPS; 51.6%), *P. dulcis* (PdBPPS; 47.5%), *C. burmanni* (CbBPPS; 43%) and *A. villosum* (AvBPPS; 37.5%).

Genomic DNA organization and phylogenetic analysis

The genomic sequence for the (+)-LiBPPS was amplified from *L. x intermedia* leaf genomic DNA. The cloned (+)-LiBPPS genomic DNA is 2487 bp long and contains an intron-exon structure typical of *Lavandula* and other angiosperm sesquiterpene (TPS-a) and monoterpene (TPS-b) synthase genes; it bears 7 exons ranging in size from 138 bp in exon-5 to 384 bp in exon-3, and 6 introns ranging in size from 68 bp in intron-1 to 226 bp in intron-3 (Fig. 2). Four of the exons (exon-1, exon-4, exon-6 and exon-7) contained the common TPS conserved motifs at the expected positions. All introns and exons, but exons 4–7, were different in length between the two *Lavandula* BPPSs.

Using encoded amino acid sequences, we constructed neighbor-joining phylogenetic trees for (+)-LiBPPS along with TPS subfamilies from *Lavandula* and BPPSs from other angiosperms (Fig. 3). The tree showed that (+)-LiBPPS was clustered into the TPS-b subfamily, and had a very close evolutionary relationship to that of *S. officinalis* BPPS. Within the genus *Lavandula*, (+)-*LiBPPS* is highly homologous to the *L. angustifolia* limonene synthase (*LaLimS*) (77.6%) and 3-carene synthase (*Li3CarS*) (73.5%), and to *L. viridis* alpha-pinene synthase (LvPinS) (75.5%) and fenchol synthase (LvFencS) (78%). It has a relatively low sequence identity with the recently reported LaBPPS (51.5%).

Homology modeling of (+)-*LiBPPS*

We determined the 3D structure of (+)-LiBPPS based on homology to the previously reported structural model for the *S. officinalis* BPPS (Whittington et al. 2002). Using the information, we predicted the 3D structure of the active site pocket before and after substrate (GPP) docking (Fig. 4). We also identified key amino acid residues positioned within and in close proximity of the active site pocket. Several amino acid residues within the active site are predicted to play important roles in substrate binding and selectivity. These include W323, I344, V452 and F578, as reported for SoBPPS (Whittington et al. 2002). Three of these residues are present in the LiBPPS active site, while the fourth, V452, is replaced by I449. As well, residues S458, A459 and H582, found in LaBPPS (Despinasse et al. 2017), are replaced by T450, C451and F574, respectively, in LiBPPS.

Recombinant (+)-*LiBPPS expression and product identification*

We expressed the (+)-*LiBPPS* ORF, excluding the N-terminus transit peptide, in *E. coli* Rosetta™ (DE3) pLysS strain using pET41b(+) expression vector, and partially purified the protein using Ni-NTA-agarose affinity chromatography. The partially purified recombinant protein was assayed for activity with GPP, FPP, DMAPP as well as a combined IPP and DMAPP as substrate, and the resulting product was treated with calf intestine alkaline phosphatase (CIP) (New England Biolabs) to produce the nearest alcohol. The products of the reactions (before and after alkaline phosphatase treatment) were analyzed along with the authentic standards by GC-MS. Borneol and other monoterpenes (multiple products) were detected from assays of (+)-LiBPPS with GPP followed by CIP treatment (Fig. 5), whereas only monoterpenes were detected from assays of (+)-LiBPPS with GPP. (+)-LiBPPS produced a product mixture that contained ~ 60% BPP, and ~ 40% other minor products (Fig. 5). However, the recombinant (+)-LiBPPS protein was catalytically inactive when assayed with FPP, DMAPP, or IPP and DMAPP (together). The borneol produced in the assay was also analyzed by gas chromatography using a chiral column and identified as (+)-borneol (Fig. S3). As a control, affinity-purified extracts from *E. coli* transformed with the empty vector, or heat-denatured purified recombinant (+)-LiBPPS proteins were assayed. The results revealed that none of the control reactions produced detectable products.

To compare the *in vitro* activity of (+)-LiBPPS with the previously cloned LaBPPS (Despinasse et al. 2017), we cloned the cDNA for LaBPPS from our *L. angustifolia* cv. Lady cDNA library (Lane et al. 2010). The cloned LaBPPS shared over 98% nucleotide identity with the previously reported LaBPPS (from cv. Diva). However, the full-length LaBPPS from cv. Lady contains two nucleotide insertions at the C-terminus that cause frameshift mutations and the introduction of premature termination codons. As a result, we are unable to produce recombinant proteins for LaBPPS in our study.

Using qPCR, we detected transcripts for (+)-*LiBPPS* and *LaBPPS* in *L. x intermedia* leaf and flower tissues, although both transcripts were more abundant in leaves (Fig. 6).

Expression of (+)-*LiBPPS in transgenic L. latifolia*

To evaluate the function of *LiBPPS in planta*, we generated transgenic *L. latifolia* plants expressing the antisense and sense versions of *LiBPPS* cDNA placed under the control of CaMV 35S promoter (Fig. 7a). Most plants transformed with the sense construct exhibited stunted growth and did not survive; only nine independent lines expressing (+)-LiBPPS in sense survived, four of which died soon after early analysis. On the other hand, expression in antisense did not affect plant growth, and over 35 lines expressing the gene in antisense were produced (Fig. 7b). To examine the transcriptional expression of *LiBPPS* in these transgenic lines, qPCR assays were performed for nine transgenic lines in each sense and antisense construct (Fig. 7c-d). The results revealed that, as anticipated, the (+)-*LiBPPS* mRNA was much more abundant in transgenic lines that expressed *(+)-LiBPPS* in sense compared to wild-type controls. Similarly, as expected (+)*LiBPPS* transcript levels were much less abundant in plants expressing (+)-*LiBPPS* gene in antisense compare to wild-type plants.

We examined the transgenic lines for the accumulation of key monoterpenes and total oil yield. Borneol (0.87–2.63%) and camphor (11.6 – 15.9%) levels were substantially lower in the EO of antisense transgenic plants compared to those in wild-type plants, which contained 4.18 ± 0.23% borneol and 23.5 ± 1.45% camphor (Table 2). Additionally, plants expressing *LiBPPS* in antisense accumulated substantially more 1, 8-cineole than WT plants. However, limonene levels were not affected in antisense plants compared to WT controls (Table 2).

Table 1
Percent composition of monoterpenes and borneol from *in vitro* enzymatic assay of LiBPPS with GPP (50 µM) and after treated with calf intestine alkaline phosphatase (CIP) (30 U). Values presented are the means ± standard errors from six independent reactions. Borneol is shown in boldfaced.

Monoterpenes	RT (min)	Proportion (%)	SE
α-Pinene	5.96	12.73	0.06
Camphene	6.22	4.54	0.15
β-Pinene	6.38	1.85	1.83
Limonene	7.52	14.51	0.16
Terpinolene	8.85	5.47	0.35
Borneol	**11.53**	**60.9**	**1.8**

Table 2. Expression of *LiBPPS* impacts the levels of borneol and camphor in *L. latifolia* leaf tissues.

	Lines	Camphor (mg/g FW)	Borneol (mg/g FW)	1,8-Cineole (mg/g FW)	Limonene (mg/g FW)	Total Oil Yield (mg/g FW)
Wild-type	Wt	23.5 ± 1.45	4.18 ± 0.23	23.74 ± 0.76	1.22 ± 0.19	37.30 ± 2.81
Antisense transgenic lines	As4	13.4 ± 0.52	0.87 ± 0.12	35.31 ± 1.46	2.41 ± 0.24	36.80 ± 2.78
	As9	14.6 ± 1.42	1.85 ± 0.09	35.86 ± 2.34	1.96 ± 0.14	37.90 ± 1.21
	As11	12.8 ± 0.39	1.32 ± 0.11	34.89 ± 1.90	2.30 ± 0.05	36.50 ± 2.10
	As14	11.6 ± 1.24	1.87 ± 0.10	35.18 ± 1.72	3.74 ± 0.18	37.80 ± 1.50
	As18	13.5 ± 1.95	2.31 ± 0.17	32.46 ± 2.64	2.39 ± 0.13	36.50 ± 0.58
	As19	12.9 ± 2.17	1.89 ± 0.20	36.33 ± 0.97	2.42 ± 0.25	38.70 ± 2.69
	As20	16.0 ± 3.13	2.14 ± 0.17	28.15 ± 2.66	1.53 ± 0.09	35.80 ± 1.33
	As30	10.8 ± 2.90	1.39 ± 0.16	33.6 ± 3.39	2.04 ± 0.19	36.10 ± 2.61
	As33	15.9 ± 1.23	2.63 ± 0.34	25.24 ± 1.49	1.49 ± 0.15	32.21 ± 2.86
Sense transgenic lines	S1	30.1 ± 2.24	6.88 ± 0.67	19.2 ± 0.56	0.56 ± 0.14	39.89 ± 3.58
	S2	32.8 ± 1.24	7.38 ± 0.41	17.19 ± 1.01	1.01 ± 0.09	40.33 ± 3.58
	S3	18.0 ± 3.43	1.87 ± 0.27	20.15 ± 2.06	1.83 ± 0.30	33.81 ± 1.33
	S4	20.8 ± 2.39	2.32 ± 0.62	19.89 ± 3.80	2.30 ± 0.05	34.50 ± 2.10
	S5	36.38 ± 3.39	9.24 ± 1.01	16.79 ± 2.11	1.01 ± 0.08	41.12 ± 2.34

Altered borneol and camphor levels in leaves of transgenic and control plants. The percent composition of these monoterpenes was calculated from the total major terpenes of fresh weight of leaves. Values presented are the means ± standard errors of five to nine analyses for each transgenic and control line. Asterisks indicate statistically significant differences (Student's *t*-test; *$P \leq 0.05$ and **$P \leq 0.01$).

As noted, most of the regenerated plants overexpressing (+)-*LiBPPS* in sense did not survive for EO analysis. Of the five that did, three lines (S1, S2 and S5) produced substantially and significantly more borneol and camphor, but accumulated less 1,8-cineole than WT plants. In the other two sense lines (S3 and S4), the concentrations of all tested monoterpenes were more or less the same as those in WT plants. Notably, as seen for antisense plants, limonene levels were not affected in any of the sense plants.

Discussion

L. x intermedia, a high EO yielding natural hybrid of *L. angustifolia* and *L. latifolia*, produces an EO constituted of monoterpenes, mainly linalool, linalyl acetate, borneol, camphor and 1,8-cineole. Due to the relatively high levels of borneol and camphor, *L. x intermedia* EOs typically are used in low-value products such as air fresheners and soaps. The cloning of genes responsible for borneol and camphor production opens several avenues for reducing the production of these compounds, and hence increasing the commercial value of the EO in *L. x intermedia* plants. In this context, the cloning of BPPS, an enzyme that catalyzes the committed step (conversion of GPP to BPP) in borneol (and camphor) production is of key importance.

To date, five BPPS genes have been reported from different plants (Wise et al. 1998; Despinasse et al. 2017; Hurd et al. 2017; Wang et al 2018; Ma et al 2021). Most produce BPP and multiple monoterpenes from GPP, with BPP (measured as a derivative alcohol borneol) proportion of 30% (*L. angustifoila)*, 65.9% (*A. villosum)*, 57–75% (*S. officinialis)*, 88.7% (*C. burmanni*) and 90% (*P. dulicis*).

Phylogenetic analysis and genome organization of (+)-LiBPPS

In this study, we identified (+)-*LiBPPS*, which encoded 593 amino acids consisting of all a single copy of conserved motifs present in typical plant TPSs, such as RR(x8)W, DDxxD and (N,D)D(L,I,V)x(S,T)x3E (Wise et al. 1998). The first motif, RR(x8)W, is located at the very beginning of the N-terminus of the protein and is required in mono-TPSs for cyclization (Williams et al. 1998), or protein stabilization (Hyatt et al. 2007). The DDxxD and ((N,D)D(L,I,V)x(S,T)x3E) motifs presented at C-terminus are responsible for substrate binding and coordination of divalent metal ion cofactors (Degenhardt et al. 2009). Uniquely, a double copy of RR(x8)W motif was detected from *P. dulicis* BPPS, in which the first copy inhibited the *in vitro* protein activity (Hurd et al. 2017). However, alterations of the common residues in these conserved motifs are often observed through deletion and/or substitution without compromising the functional activities. For example, a deletion of an arginine (R) at RR(x8)W motif of cadinol and germacrene D synthases, and substitution of an aspartate (D) by glutamate (E) at DDxxD motif of germacrene D synthases were identified in *L. angustifolia* (Jullien et al. 2014).

Based on analysis of deduced amino acid sequences, (+)-LiBPPS is highly homologous to the *L. angustifolia* limonene synthase (LaLimS), *L. x intermedia* 3-carene synthase (Li3CarS), and *Lavandula viridis* α-pinene synthase (LvPinS) and fenchol synthase (LvFencS) (Landmann et al. 2007; Benabdelkader et al. 2015; Adal et al. 2017). All these TPSs are clustered together under the angiosperm

TPS-b subfamily, a monoterpene synthase subfamily (Bohlmann et al. 1998). Intriguingly, (+)-LiBPPS is distantly related to most of the known BPPSs from different plants, including that from *L. angustifolia*, and closes to SoBPPS, with which it shares 65.48% identity at the amino acid level. This indicates that plant mono-TPSs originated from the same ancestor or one has evolved from the other through gene duplication, leading to divergent functional evolution through subfunctionalization and / or neofunctionalization of TPSs. It is not also surprising that (+)-LiBPPS is more homologous to other *Lavandula* TPSs, as most TPS genes with different functions have more similarity to each other within a given species than TPSs with the same function in a different species. For example, Li3CarS shares 75% amino acid identity with LaLimS (from half-parent species) compared to 29–50% sequence homology with 3-carene synthases of *P. abies* and *S. stenophylla* (Adal et al. 2017). While, in Sitka spruce, 3-carene synthase gene had 89–92% nucleotides identity with another mTPS, (-)-sabinene synthase (Roach et al. 2014).

The genomic structure of (+)-*LiBPPS* has typical features of the Class III TPSs, which are known by seven exons and six introns (Chen et al. 2011). The common conserved motifs are also located at the same exon positions of most TPSs in several angiosperms.

In vitro **activity of the recombinant** (+)-**LiBPPS**

The genes and encoding enzymes for most major *Lavandula* monoterpene synthases have been identified and functionally characterized *in vitro* (Landmann et al. 2007; Demissie et al. 2011; Demissie et al. 2012; Adal et al. 2017; Adal et al. 2019). The (+)-*LiBPPS* cloned in this study was found to encode a monoterpene synthase that catalyzes the conversion of GPP to (+)-bornyl diphosphate (BPP), and a few other monoterpenes. The generation of BPP and multiple monoterpenes by (+)-LiBPPS *in vitro* was not surprising, as most of the known BPPSs and some other mono-TPSs are multiproduct monoterpenes (Despinasse et al. 2017; Hurd et al. 2017; Wang et al 2018; Ma et al 2021, Wise et al. 1998; Fäldt et al. 2003; Demissie et al. 2012, Adal et al 2017). The product profile of LiBPPS (which includes 61% BPP, measured as nearest prenyl alcohol, (+)-borneol) is closer to *A. villosum* BPPS (65.9% BPP) (Wang et al. 2018)d *officinalis* BPPS (57–75% BPP) (Wise et al. 1998; Despinasse et al. 2017), than *L. angustifolia* BPPS (30% BPP) (Despinasse et al. 2017), C. *burmanni* BPPS (88.7%) (Ma et al. 2021)d *dulicus* BPPS (90%) (Hurd et al. 2017). A few minor monoterpenes with a total proportion of 39% were also detected from *L. x intermedia* BPPS activity. Production of a few minor monoterpene products by the (+)-LiBPPS was not surprising, as most other reported BPPS proteins are multiproduct enzymes.

(+)-LiBPPS **and** LaBPPS **are differentially expressed in lavenders**

Both (+)-*LiBPPS* and *LaBPPS* are constitutively expressed in *L. x intermedia* leaf and flower tissues, with a strong transcriptional expression observed in leaves. Given that high levels of monoterpenes derived from BPP (i.e., borneol and camphor) are produced in leaves, compared to flowers, in *L. x intermedia* (Boeckelmann 2008; Hassiotis et al. 2014; Seidler- Lozykowska et al. 2014), this finding was not surprising.

Expression of (+)-LiBPPS **impacts the biosynthesis of monoterpenes in the transgenic** L. latifolia

Metabolic engineering of monoterpenes has been reported in Lamiaceaces, particularly in lavender and mint, (Mahmoud and Croteau 2001; Muñoz-Bertomeu et al. 2006; Muñoz-Bertomeu et al. 2008; Lange et al. 2011; Mendoza-Poudereux et al. 2014; Wang et al. 2016; Tsuro et al. 2019; Li et al. 2020). These studies targeted mainly the DXP pathway genes or terpene synthase genes. For example, expression of the *Arabidopsis DXS* gene increases the concentration of monoterpenes and essential oil yield without impacting primary metabolites, such as chlorophyll and carotenoids in *L. latifolia* transgenic plant leaves and inflorescences (Muñoz-Bertomeu et al. 2006). A high level of linalool was also produced through the expression of a full-length clarkia linalool synthase in *L. latifolia* leaves (Mendoza-Poudereux et al. 2014).

In this study, we expressed the full-length (+)-*LiBPPS* cDNA in sense or antisense orientation under the control of the CaMV 35S promoter in *L. latifolia* plants. Plants that most strongly expressed the transgene in sense did not survive to full maturity. The exact nature of this effect is unclear and requires further investigation. However, most likely the unregulated production of large amounts of BPP and monoterpenes derived from BPP (in particular camphor (Fig. 1), a known agent of allelopathy) is lethal to the plant. Given that normally BPPS and other monoterpene synthase genes are expressed in the secretory cells of glandular trichomes, which are specialized to produce and store large quantities of monoterpenes, the above scenario makes sense. To avoid this issue, BPPS should be expressed under the control of a trichome-specific promoter.

Reduced *BPPS* transcript levels in transgenic plants expressing *(+)*-LiBPPS in antisense correlate well with the accumulation of borneol/camphor in these plants. This suggests that (+)-*LiBPPS* controls the synthesis of these monoterpenes through the supply of the primary precursor bornyl diphosphate. This outcome was not unexpected as the production of numerous monoterpenes is controlled through the transcriptional regulation of the corresponding terpene synthase gene. For example, the production of 1,8 cineole was recently shown to be controlled via the regulation of the cineol synthase gene in lavender (Demissie et al., 2012; Tsuro et al. 2019).

Conclusions

We have isolated and functionally characterized a new (+)-bornyl diphosphate synthase ((+)-LiBPPS) cDNA from *L. x intermedia*. (+)-LiBPPS, which is clustered under the TPS-b subfamily, is a typical Class III TPS, and has unique active site residues. The recombinant LiBPPS protein catalyzes the conversion of GPP into BPP and a few other monoterpenes. Ectopic expression of (+)-*LiBPPS* impacted the accumulation of borneol and camphor in *L. latifolia* leaves, prompting decreased levels of these terpenes in antisense transgenic plants. Although overexpression of (+)-*LiBPPS* in sense resulted in increased production of BPP-derived terpenes in transgenic plants, most overexpressors exhibited severely stunted growth. Therefore, the cloned (+)-*LiBPPS* would be useful in metabolic engineering studies, targeting the production of recombinant borneol and camphor in microbial systems and plants. However,

overexpression of this gene to enhance borneol or camphor production in plants must be done using trichome-specific promoters.

Declarations

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Author contributions

AMA and SSM conceived and designed the research. AMA and EN conducted the cloning, functional characterization and metabolic engineering experiments. LSS contributed to the *in vitro* characterization of LiBPPS. ©AMA and SSM wrote the manuscript, and all authors read, edited and approved the manuscript.

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Figures

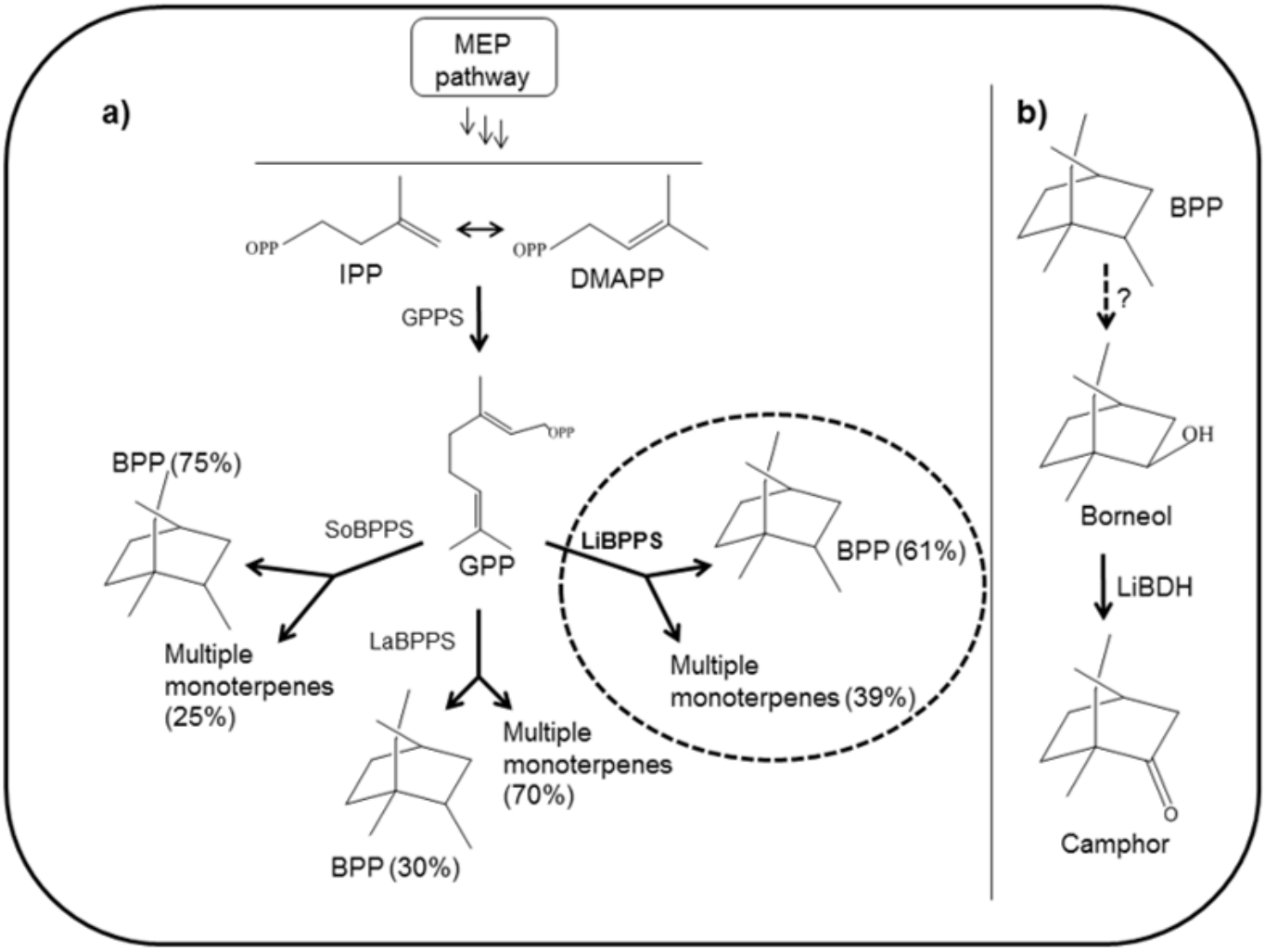


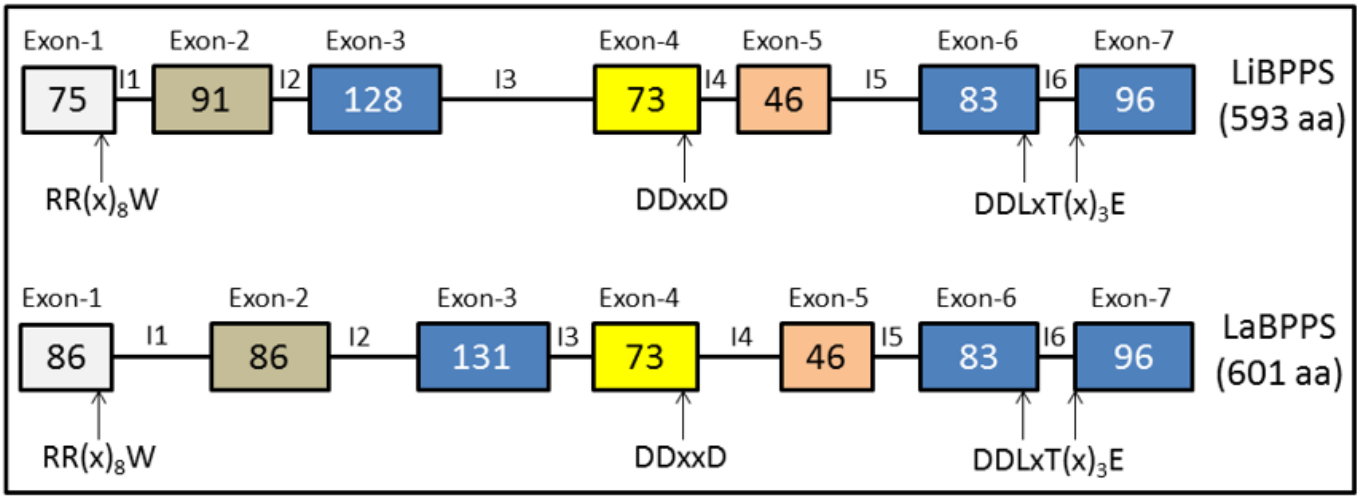
Figure 1

Biosynthesis pathway of borneol and camphor.

a) Methylerythritol phosphate (MEP) pathway leading to the formation of bornyl diphosphate (BPP). Isopentenyl diphosphate (IPP) dimethylallyl diphosphate (DMAPP) are the substrates for the biosynthesis of geranyl diphosphate (GPP). Monoterpene synthases designated as bornyl diphosphate synthases (BPPS) catalyze GPP into BPP, a precursor for borneol, and multiple monoterpenes with different levels in Lamiaceae. These functionally studied monoterpene synthases were previously identified from *S. officinalis* (SoBPPS) (Wise et al. 1998) and *L. angustifolia* cv. Diva (LaBPPS) (Despinasse et al. 2017). Products derived by (+)-LiBPPS shown in a broken circle are from this study.

b) The subsequent biosynthesis of borneol and camphor from BPP as a substrate. The broken arrow with "?" shows unknown catalyzing enzymes from plants.

a)



b)

↓Gene/Introns →	I1	I2	I3	I4	I5	I6	Total gDNA (bp)*
LiBPPS	68	78	226	68	175	92	2489
LaBPPS	217	125	72	178	81	411	2893

*Total gDNA for each gene includes introns and exons (coding regions)

Figure 2

Genome organization and patterns of introns and exons of lavender *BPPS* genes.

a) Genome structure patterns between (+)-*LiBPPS* (upper panel) and *LaBPPS* (bottom panel). The three key conserved motifs known in most monoterpenes are shown in both genes.

b) Intron sizes of *LiBPPS* compared to previously reported BPPS (LaBPPS) from *L. angustifolia* (Despinasse et al. 2017).

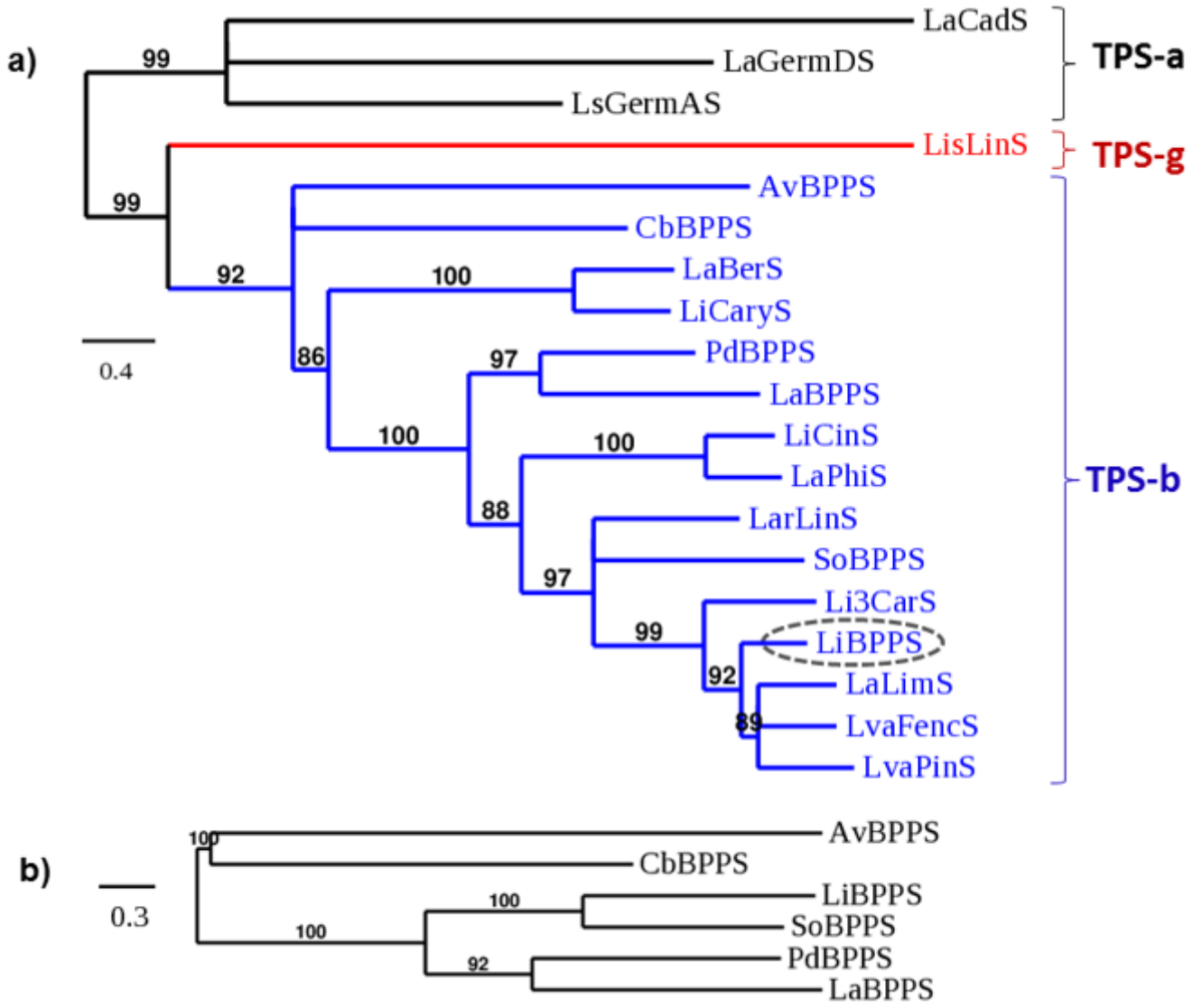


Figure 3

Phylogenetic analysis of (+)-LiBPPS with other selected TPSs based on protein sequences.

a) Relationship of LiBPPS and all known lavender TPSs and BPPSs from other plant species. The taxon in a broken circle indicates the *L. x intermedia BPPS* (LiBPPS) sequence identified in this study. The clades of TPS subfamilies that are designated TPS-a, TPS-b and TPS-g are according to Bohlmann et al. (1998) and Chen et al. (2011).

b) Comparison of LiBPPS with all known BPPS genes. The trees were generated using the Phylogeny Analysis MEGA7.0 program (Kumar et al., 2016). The resulting trees were bootstrap analyzed with 1,000 replicates, and bootstrap values > 70% are mentioned on the trees. The scale bar shows the number of amino acid substitutions per site. Accession numbers for all TPSs used in this study are listed in Table S2.

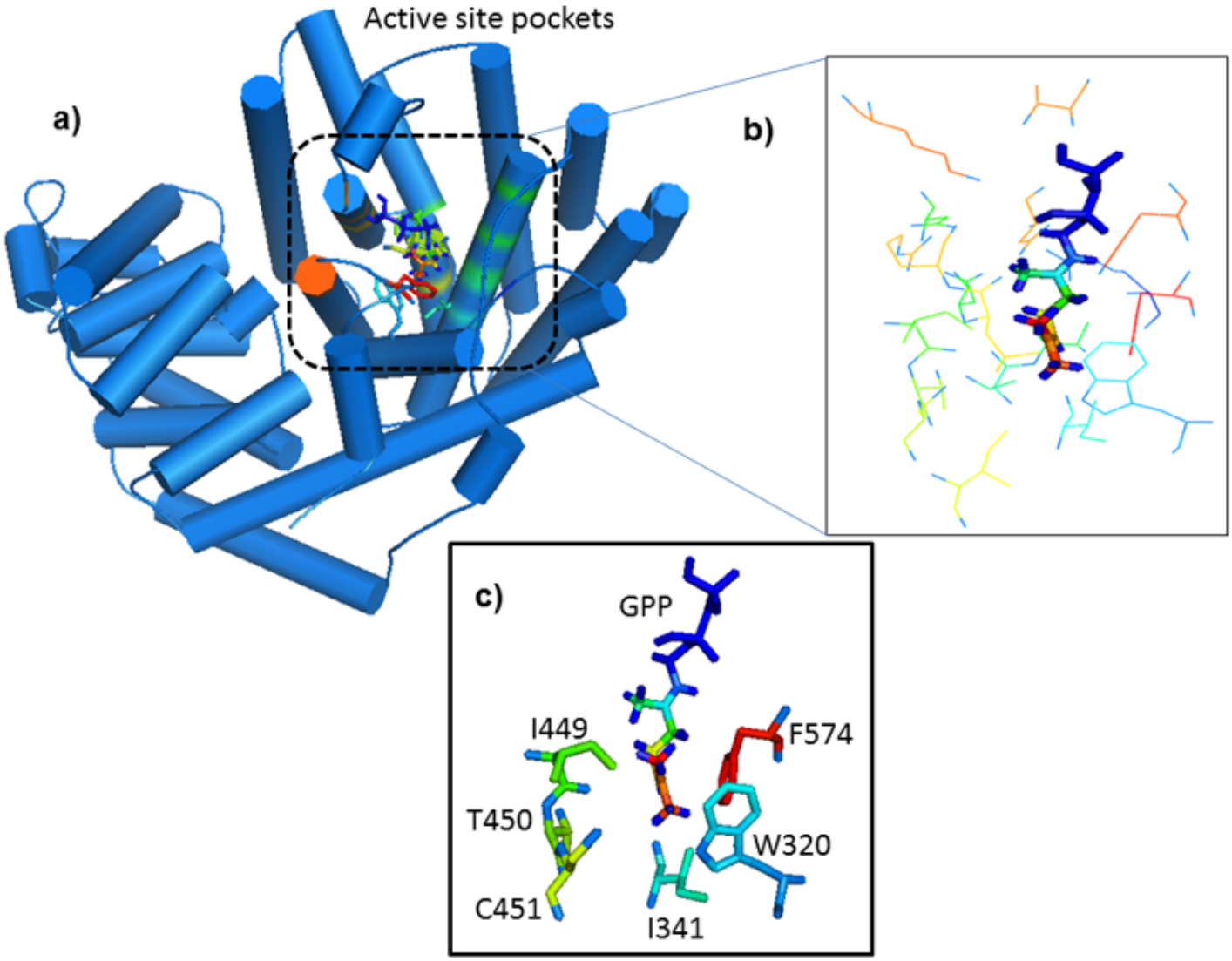


Figure 4

Homology-based modeling of (+)-LiBPPS, displaying active site pocket and binding residues.

a) Three-dimensional protein structure with an active site pocket.

b) The active site pocket with docked GPP and predicted binding residues.

c) Key active site residues involved in carbocation stabilization. These amino acid residues within the active site were compared with previously reported BPPS proteins from *S. officinalis* (Wise et al. 1998) and *L. angustifolia* (Despinasse et al. 2017).

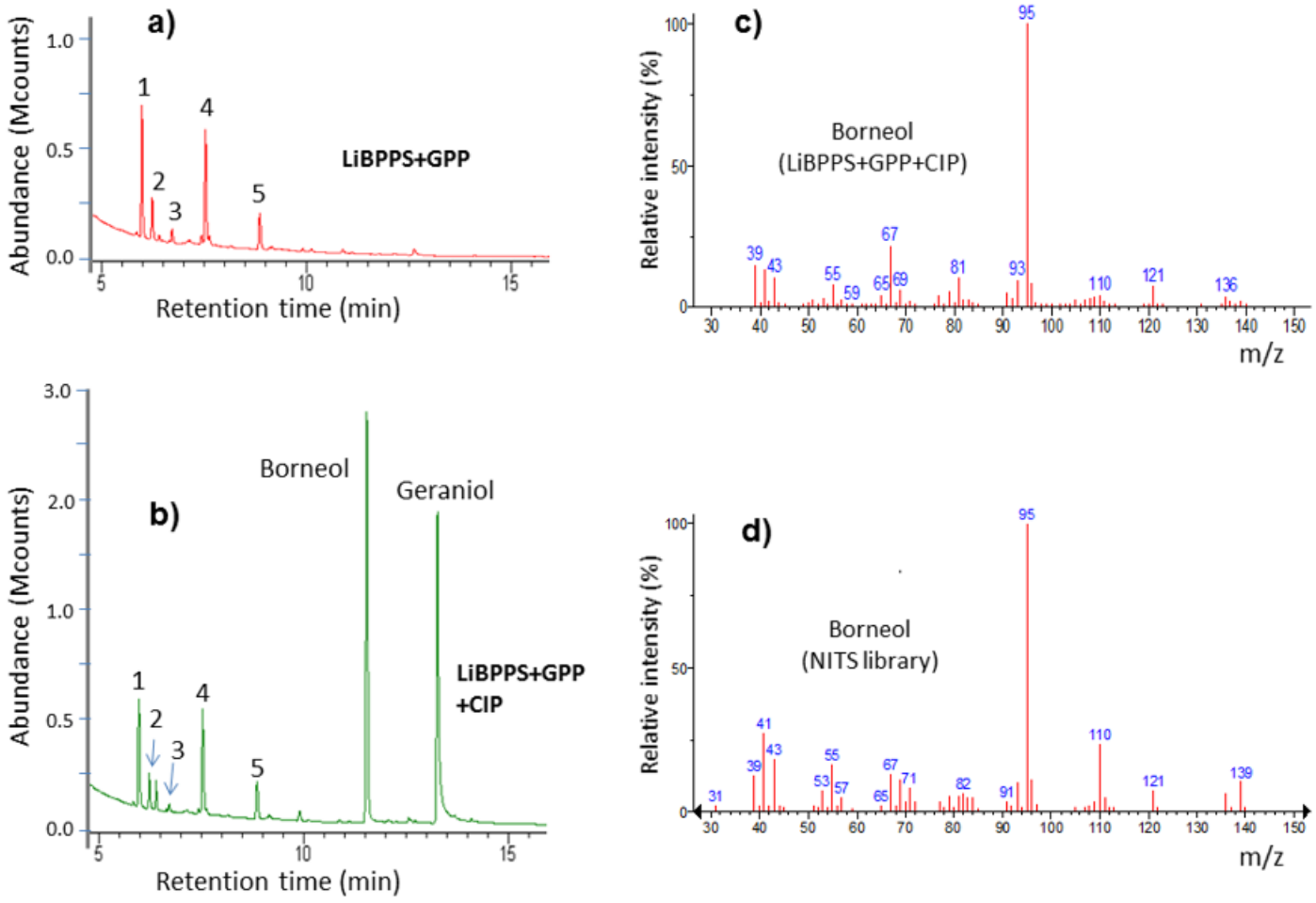


Figure 5

GC-MS analysis of assay products generated by recombinant *L. x intermedia* BPPS (LiBPPS) from geranyl diphosphate (GPP) as a substrate. Reaction products from (+)-LiBPPS assay were analyzed by GCMS before (a) and after (b) treatments of assay products by calf intestinal alkaline phosphatase (CIP). Note that BPP does not show in GCMS analysis, as it is not volatile.

a) Chromatography of monoterpene produced from GPP. Peaks 1 – 5 represent monoterpenes directly formed from GPP by the recombinant (+)-LiBPPS.

b) GCMS analysis of borneol (produced from BPP upon CIP treatment) and other monoterpenes derived from GPP. Assays of recombinant (+)-LiBPPS were run with GPP (50 uM), followed by overnight hydrolysis using calf intestine alkaline phosphatase (CIP) to generate borneol.

c) Mass spectra of (+)-borneol from assay product, and (d) mass spectrum of the borneol authentic standard from the NIST library.

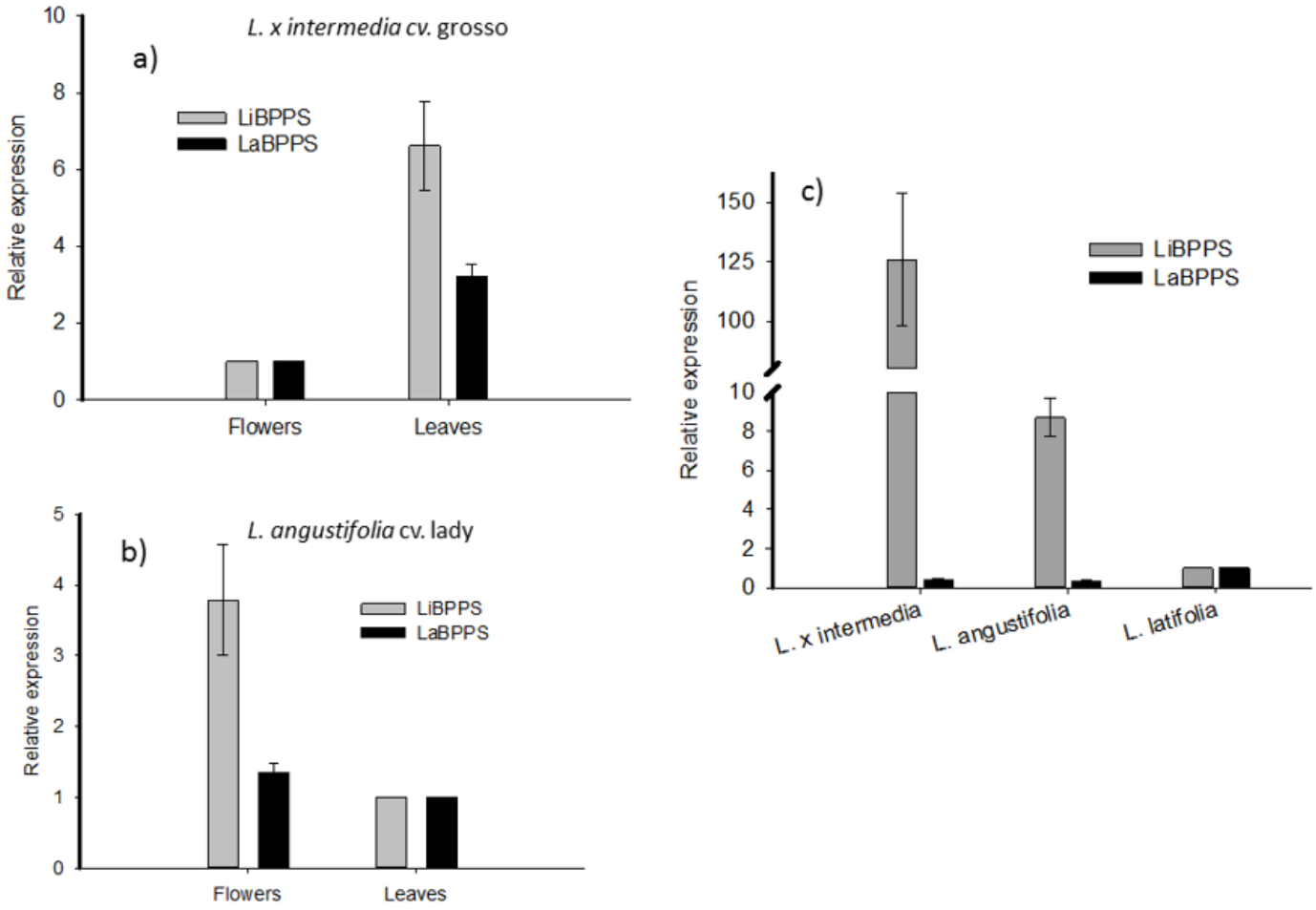


Figure 6

Spatial expression of *L. x intermedia BPPS ((+)-LiBPPS)* and *L. angustifolia BPPS (LaBPPS)* in different tissues of the three species using qPCR assays.

a) The transcript levels of *LiBPPS* and *LaBPPS* in *L. x intermedia* and *L. angustifolia* leaf and flower tissues;

b) *LiBPPS* and *LaBPPS* expressions in leaf tissues across the three species. The transcripts were normalized to *β-actin* gene. The control (in this case leaf tissue) was assigned with the arbitrary value of 1.0. Values presented are the means ± standard errors of three analyses for two biological replications. Asterisks indicate statistically significant differences (Student's *t*-test; $*P \leq 0.05$ and $**P \leq 0.01$).

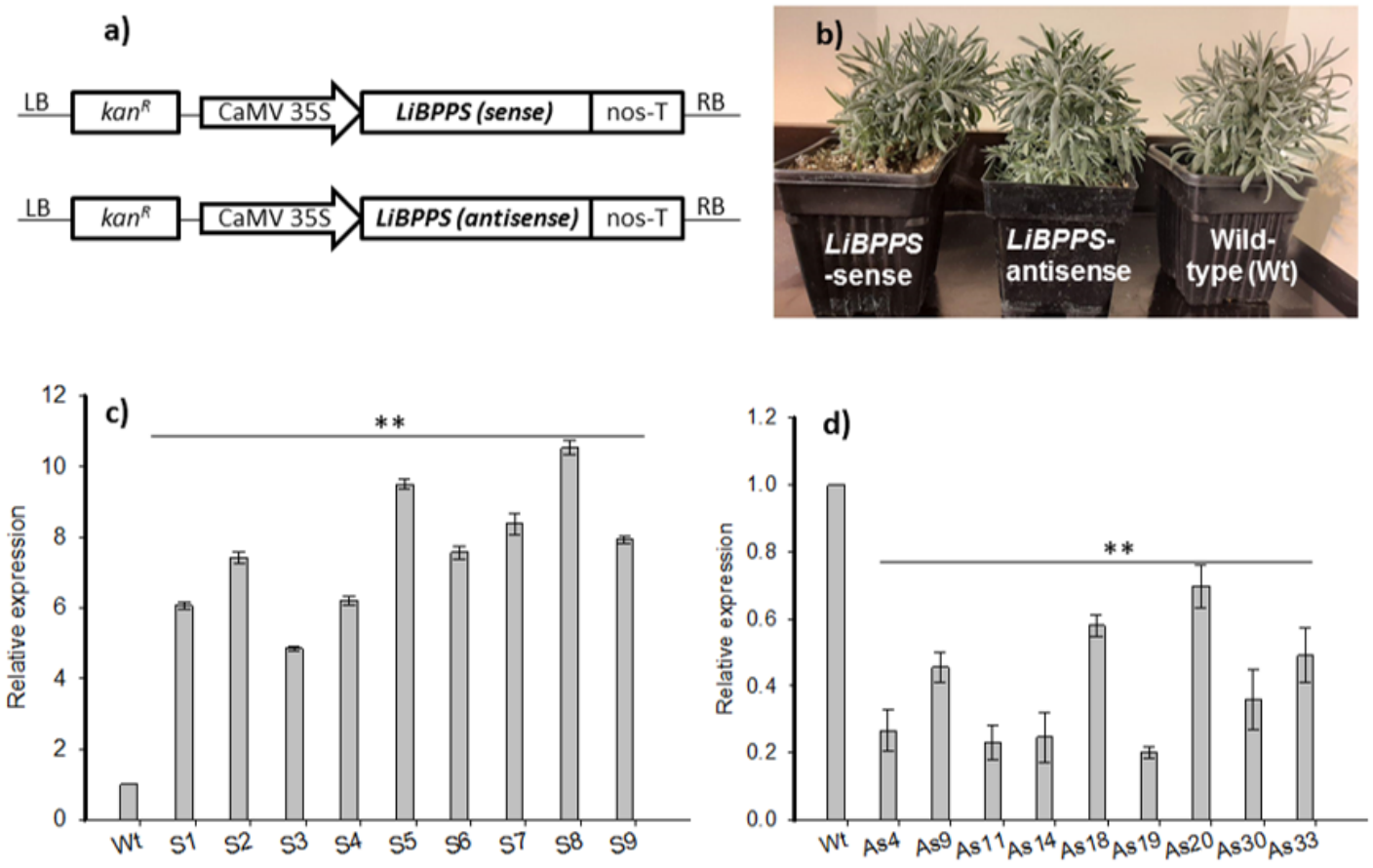


Figure 7

Expression of *(+)-LiBPPS* in transgenic *L. latifolia* leaf tissues.

a) Sense and antisense LiBPPS constructs fused with 35S CaMV promoter and cloned into pCAMBIA 1390;

b) Representative healthy transgenic and wild type *L. latifolia* plants,

c) Transcript levels of *LiBPPS* exhibiting more expression in sense and low in antisense plants compared to that in control plant samples. Values presented are the means ± standard errors of five to nine analyses for each transgenic and control line. Asterisks indicate statistically significant differences (Student's *t*-test; $*P \leq 0.05$ and $**P \leq 0.01$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig.S1.rtf
- Fig.S2.docx

- Fig.S3.docx
- Fig.S4.docx
- TableS1.docx
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