Planta
DOI 10.1007/s00425-013-1937-6

SHORT COMMUNICATION

Cloning of a sesquiterpene synthase from *Lavandula x intermedia* glandular trichomes

Lukman S. Sarker · Zerihun A. Demissie · Soheil S. Mahmoud

Received: 6 June 2013 / Accepted: 18 July 2013

Abstract The essential oil (EO) of *Lavandula* is dominated by monoterpenes, but can also contain small amounts of sesquiterpenes, depending on species and environmental conditions. For example, the sesquiterpene 9-epi-caryophyllene can make up to 8 % of the EO in a few species, including those commercially propagated for EO production. Here, we report the cloning and functional characterization of 9-epi-caryophyllene synthase (*LiCPS*) from the glandular trichomes of *Lavandula x intermedia*, cv. Grosso. The 1,617 bp open reading frame of *LiCPS*, which did not encode a transit peptide, was expressed in *Escherichia coli* and the recombinant protein purified by Ni–NTA agarose affinity chromatography. The ca. 60 kDa recombinant protein specifically converted farnesyl diphosphate to 9-epi-caryophyllene. LiCPS also produced a few monoterpenes when assayed with the monoterpene precursor geranyl diphosphate (GPP), but—unlike most monoterpene synthases—was not able to derive detectable amounts of any products from the *cis* isomer of GPP, neryl diphosphate. The *LiCPS* transcripts accumulated in developing *L. x intermedia* flowers and were highly enriched in glandular trichomes, but were not detected in leaves suggesting that the transcriptional expression of this gene is spatially and developmentally regulated.

Keywords Isoprenoid · Terpenoid · Sesquiterpene · Caryophyllene · Essential oil · Glandular trichome

L. S. Sarker · Z. A. Demissie · S. S. Mahmoud (✉)
University of British Columbia, Kelowna, BC, Canada
e-mail: soheil.mahmoud@ubc.ca

Introduction

Lavenders, *Lamiaceae*, are valued for their essential oils (EOs) which are used in numerous personal care products, cosmetics, foods, and alternative medicines (Upson et al. 2004). Although a few monoterpenes dominate these EOs, sesquiterpenes can also comprise a small amount (<10 %) of the oil depending on species (Lis-Balchin 2002). Although the exact role of *Lavandula* sesquiterpenes is not known, it is anticipated that,as in other plants,they play important roles in plant defense and plant–environment interaction (Cheng et al. 2007). Sesquiterpenes are well-known for their insecticidal, antimicrobial, anti-cancer and anti-inflammatory properties, and are thus industrially significant (Li-Weber et al. 2002; Majdi et al. 2011; Trusheva et al. 2010).

The biosynthesis of mono- and sesquiterpenes is now well established (Okada 2011; Phillips et al. 2008). In plants, two independent but interactive pathways—the mevalonate (MVA) or cytosolic, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) or plastidial pathway—produce the general terpene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Arigoni et al. 1997; Bick and Lange 2003; Laule et al. 2003). The head to tail condensation of IPP and DMAPP yields prenyl diphosphates including geranyl diphosphate and farnesyl diphosphate, the immediate precursors for the synthesis of mono- and sesquiterpenes, respectively (Mahmoud and Croteau 2002; Okada 2011). The MVA pathway is found in animals and fungi as well as in the cytoplasm of phototropic organisms. Precursors produced through this pathway are mainly converted to FPP, used mainly for sesquiterpene and triterpenes biosynthesis (Chappell et al. 1995; McGarvey and Croteau 1995). The MEP pathway, present in most bacteria and in plant chloroplasts, provides precursors

Published online: 06 August 2013

for the biosynthesis of GPP and GGPP that are primarily used to produce monoterpenes and diterpenes, respectively (Mahmoud and Croteau 2002; Okada 2011). Although the two pathways operate independently there is evidence that they interact in the biosynthesis of certain metabolites (Hemmerlin et al. 2003; Laule et al. 2003). For example, FPP molecules containing isoprene units derived from both pathways have been reported, particularly in stressed plants (Adam et al. 1999).

Caryophyllene is one of the most abundant sesquiterpenes (approximately 3–8 %) in *Lavandula* EOs (Lis-Balchin 2002). It is also widely present in other plants and is well-known for its role in allelopathy and plant defense (Ulubelen et al. 1994); for example, this metabolite was produced abundantly in response to herbivore damage in maize (Koellner et al. 2008). It is also pharmaceutically important as it exhibits anti-inflammatory and anti-carcinogenic activities (Kubo et al. 1996; Zheng et al. 1992). Here, we report the cloning and functional analysis of 9-epi-(E)-caryophyllene synthase from *Lavandula x intermedia*.

Materials and methods

Selection and cloning of LiCPS

The construction of a cDNA library and the corresponding expressed sequenced tag (EST) database from the floral glandular trichomes of mature (30 % bloomed) *L. x intermedia* flowers, and the transcript profiling experiment in various tissues of *L. angustifolia*, *L. x intermedia* and *L. latifolia* using microarray technique were recently reported (Demissie et al. 2012; Lane et al. 2010; Sarker 2013). Homology based searches in this library highlighted a unique highly abundant terpene synthase (TPS)-like cDNA (the putative LiCPS) that was strongly expressed in *L. x intermedia* glandular trichomes (51 ESTs). The open reading frame (ORF) for this sequence was analyzed for the presence of a transit peptide using ChloroP1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and signal 3L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/) software. The coding region of the putative *LiCPS* was amplified by PCR using the forward primer 5′-GATAACATATGAGGAGGTCAGC GAATTATAG-3′ (*Nde*I) and reverse primer 5′-AATAT GAATTCTTAATATGGAAGGGGTGAAGG-3′ (*Eco*RI), and iProof high-fidelity taq DNA polymerase (Bio-Rad, USA). The PCR program used was, initial denaturation at 95 °C for 5 min, followed by 95 °C for 1 min, 58 °C for 30 s and 72 °C for 1:30 min for 35 cycles with a final elongation at 72 °C for 5 min. PCR products were purified using a Gel extraction/PCR purification kit from Omega (Omega Bio-Tek, USA). The purified PCR products were digested with *Nde*I, and *Eco*RI restriction enzymes and ligated into pET41b(+) bacterial expression vector, where it was fused to sequences encoding eight C-terminus Histidine residues to facilitate purification by Ni–NTA agarose affinity chromatography (EMD Chemicals, Darmstadt, Germany). The recombinant LiCPS construct was transformed into *Escherichia coli* Rosetta™(DE3)plysS cells (EMD Chemicals, Darmstadt, Germany) and induced at 20 °C with isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM for 12–14 h in Luria–Bertani (LB) media supplemented with 30 mg/L kanamycin and 34 mg/L chloramphenicol. The induced cells were chilled on ice for 15–20 min, collected by centrifugation at 3,220*g* and 4 °C for 20 min, and stored at −80 °C overnight.

Recombinant protein purification and SDS-PAGE

The frozen cells were resuspended in Novagen bind buffer (0.3 M NaCl, 50 mM Na_2HPO_4, 10 mM imidazole, pH 8.0; EMD Chemicals, Germany) containing 1 mM protease inhibitor phenylmethanesulfonyl fluoride, and sonicated on ice using a Sonic Dismembrator Model 100 (Fisher Scientific, Ottawa, ON, Canada) to complete bacterial membrane disruption. The cell debris were removed by centrifugation at 15,000*g* and 4 °C for 15 min (Sorvall, USA), and the recombinant proteins were harvested from the soluble fraction by Ni–NTA agarose affinity chromatography (EMD Chemicals, Germany) following the manufacturer's procedure. Protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with Coomassie Brilliant Blue. Protein concentration was determined by Bradford Assay (Bio-Rad).

Enzymatic assay reaction and GC–MS analysis

In vitro enzyme activity assays were performed in 500 µL reactions containing the assay buffer (50 mM Tris/HCl, 5 % glycerol, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mg/mL bovine serum albumin, pH 7.0), 1 mM DTT, 25 µM substrate (GPP, NPP, or FPP; Echelon, Salt Lake City, UT, USA), and 50 µg of the purified recombinant protein. The mixture was overlaid by 400 µL of pentane and incubated at 30 °C for 60 min. The purified protein extracts from *E. coli* Rosetta™(DE3)plysS cells transformed with the empty expression vector were assayed under the same conditions as controls. Reactions were stopped by vigorous vortexing followed by flash freezing in liquid nitrogen, and stored at −80 °C until analyzed. To concentrate the assay products, ca. 90 % of the pentane was evaporated using a gentle stream of highly purified helium gas. Assay products were identified by gas chromatography–mass spectrometry (GC–MS) using a Varian GC 3800 Gas Chromatographer coupled to a Saturn 2200 Ion Trap mass detector equipped with a ECTM 1000 (30 m × 0.25 mm) capillary

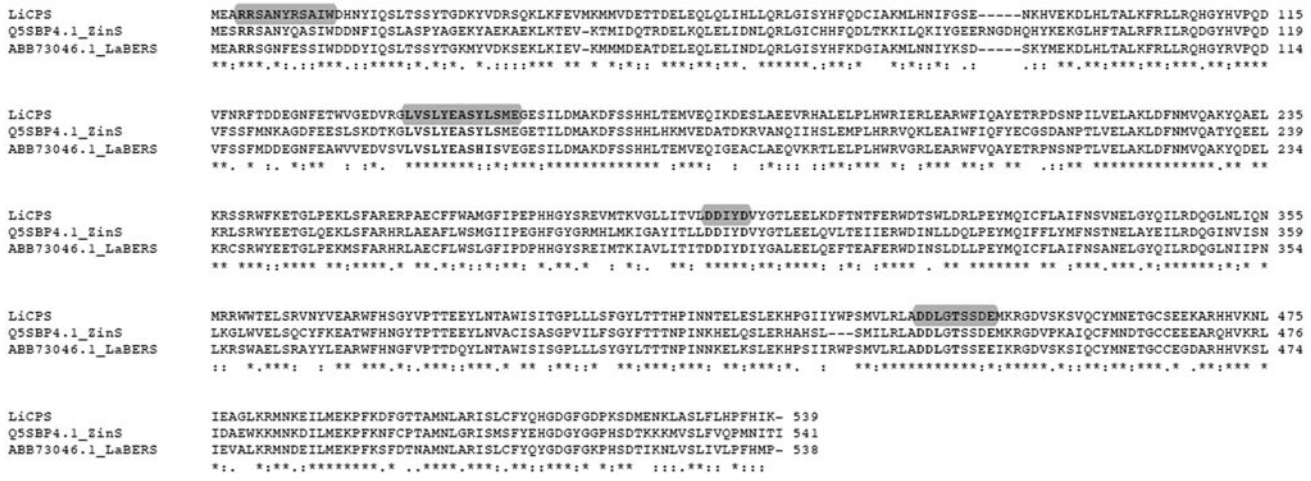

Fig. 1 Multiple alignment of the deduced amino acid sequences of LiCPS (KF470962) with LaBERS from *L. angustifolia* (AAB73046.1), and *Ocimum basilicum* (Q5SBP4.1). *Asterisks* identical; *colon* conserved substitutions; and *dot* semi-conserved substitution of amino acids. *Shaded sequences* are conserved motifs of TPS

column (Altech, Deerfield, IL, USA), and a CO_2 cooled programmable temperature vaporizing injector (Varian Inc., USA). The flow rate of the carrier gas (helium) was set to 1 mL/min. Samples were injected at 40 °C. The oven temperature was maintained at this temperature for 3 min, raised to 130 °C at a rate of 10 °C/min, then to 230 °C at a rate of 50 °C/min, and finally held at 230 °C for 10 min. The identities of products were confirmed by comparing their retention times and mass spectra to those of authentic standards (Sigma, Canada), or to the mass spectra obtained from the NIST library.

Transcript analysis

Total RNA was extracted and treated with DNase I enzyme to remove genomic DNA using Omega Bio-Tek kit, USA. Treated total RNA was reverse transcribed with Oligo (dT) (80 μM) and random hexamers (40 μM) (Custom oligos, IDT, Canada) using M-MuLV Reverse Transcriptase enzyme (New England Biolabs, Ipswich, MA, USA) following the manufacturer protocol. The abundances of *LiCPS* transcripts in young leaf, Bud-I, anthesis, 30 % flowering stage, and glandular trichome from 30 % flowering stages of *L. x intermedia* were analyzed by standard PCR using taq DNA polymerase (New England Biolabs, USA) and the cloning primers used previously with unchanged PCR program.

Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignment was performed using the ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and the phylogenetic tree was constructed using the default parameters of PhyML software available at http://www.phylogeny.fr (Dereeper et al. 2008). PhyML employs MUSCLE software to generate multiple alignments and the maximum likelihood computational method to construct the phylogenetic tree.

Results and discussion

Candidate selection and functional analysis of recombinant LiCPS

We have recently reported the construction of a cDNA library from the secretory cells of *L. x intermedia* floral glandular trichomes, the sequencing of approximately 10,000 ESTs, and the cloning and functional characterization of a number of monoterpene synthases from this library (Demissie et al. 2012, 2013; Lane et al. 2010; Sarker 2013). Our EST database also includes several sequences encoding proteins homologous to known sesquiterpene synthases of higher plants. In particular, the protein encoded by one of the most abundant ESTs (51 ESTs) exhibited over 77 % identity to a bergamotene synthase recently reported from *L. angustifolia* (Landmann et al. 2007), and over 63 % to α-zingiberene synthase (ZIS) of *Ocimum basilicum* (63 %). The protein also exhibited significant sequence identity to monoterpene synthases including α-terpineol synthase of *Vitis vinifera* (52 %), β-ocimene/myrcene synthase of *V. vinifera* (52 %), β-ocimene/myrcene synthase of *V. vinifera* (50 %) (Fig. 1).

The 1,617 bp long ORF of this EST (LiCPS) encoded a 539 amino acid protein with a predicted molecular mass of ca. 60 kDa. The deduced amino acid sequence of LiCPS

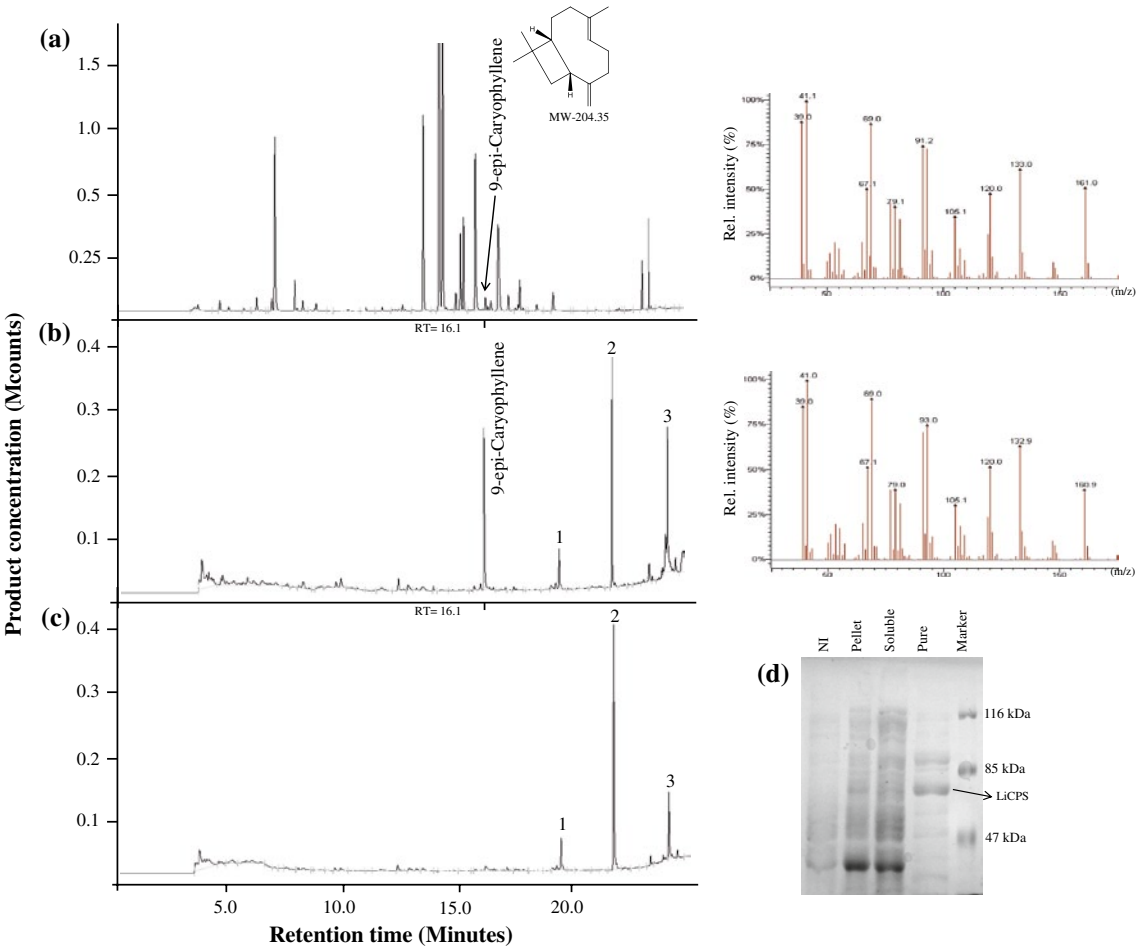

Fig. 2 **a–c** GC–MS analysis of EO of *L. x intermedia* flowers (at 30 % bloom stage), products of LiCPS assay with FPP as a substrate, and products present in the negative control assay, respectively. *Peaks 1–3* correspond to nonspecific unidentified products. Mass spectra correspond to 9-epi-caryophyllene present in the EO of *L. x intermedia* and in the LiCPS assay. *Inset* shows the chemical structure of 9-epi-caryophyllene. **d** SDS gel for LiCPS where NI, non induced cells pellet; pellet, induced cell pellet; soluble, soluble fraction from the induced cell pellets; pure, eluted rLiCPS protein from the Ni–NTA agarose column

contained all the conserve motifs present in typical plant terpene synthases including the RR(x8)W, DDxxD, and (N,D)D(L,I,V)x(S,T)xxxE motifs, which are responsible for the enzymatic activity including coordination of divalent cations, and for substrate binding and ionization (Fig. 1). LiCPS lacked a plastid targeting sequence typically found in monoterpene synthases, suggesting that this TPS was likely a sesquiterpene synthase. The purified bacterially produced recombinant LiCPS (rLICPS) generated 9-epi-caryophyllene as the only product when assayed with FPP (Fig. 2b). As shown in the GC chromatogram detectable quantities of a few other unidentified products were also present in assay reactions. However, these products were also present in the negative control assays (which were devoid of rLiCPS), indicating that they most likely resulted from the non-enzymatic breakdown of FPP (Fig. 2c). Alternatively, they could have resulted from the activity of non-specific contaminating bacterial proteins acting on the FPP substrate (Fig. 2d). The identity of the assay product (9-epi-caryophyllene) was confirmed by comparing it's spectrum to that found in the NIST library, as well as comparison of its retention time and mass spectrum to those of 9-epi-caryophyllene present in *L. x intermedia* essential oil (Fig. 2a). When assayed with monoterpene substrates, rLiCPS produced limonene, thujane, myrcene and ocimene from GPP; however, detectable amount of products was not formed when the enzyme was assayed with NPP as a substrate (data not shown). Our results confirm previous findings indicating that sesquiterpene synthases (e.g. trans-α bergamotene synthase from *L. angustifolia*, ZIS from *O. basilicum*, and β-caryophyllene synthase from *Artemisia annua* produced monoterpenes) can produce monoterpenes when assayed with GPP as a substrate (Cai et al. 2002; Iijima et al. 2004a, b; Kollner et al. 2004; Landmann et al. 2007).

Table 1 Microarray data for LiCPS expression (Sarker 2013)

A vs B	C vs D	E vs F	E vs G	F vs G	F vs H
Do_29	Up_14.76	Up_14	Do_2.28	Do_9.7	Up_8

In this table, A: *L. x intermedia* (Grosso)-gland from bud; B: *L. x intermedia* (Grosso)-gland from anthesis; C: *L. x intermedia* (Grosso)-gland from flower 30 %; D: *L. angustifolia* (Lady)-gland from flower 30 %; E: *L. x intermedia* (Grosso)-total flower; F: *L. angustifolia* (Lady)-total flower; G: *L. latifolia*-total flower; H: *L. angustifolia* (Lady)-total leaf

Up up regulation, *Do* down regulation

Tissue specific regulation of *LiCPS*

Our microarray data (Table 1; Sarker 2013) indicated that *LiCPS* transcripts were 29-fold more abundant in anthesis stage flowers than unopened buds (early flowering stage), indicating that the expression of this gene is developmentally regulated in *L. x intermedia*s flowers. Our data also suggested that *LiCPS* is differentially expressed in various *Lavandula* species. In this context, *LiCPS* transcripts were substantially more abundant (~15-fold) in *L. x intermedia* flowers (both in whole floral tissue and in isolated glandular trichomes) than those of *L. angustifolia* plants. Conversely, *LiCPS* mRNA was 2.28- and 9.7-fold less abundant in *L. x intermedia* and *L. angustifolia* floral tissue, respectively, than those of *L. latifolia* plants. Finally, the microarray data indicated that *LiCPS* transcripts were more abundant in flower than leaf tissue, indicating that this gene is expressed in a tissue-specific manner.

The microarray analysis data were confirmed by standard PCR. The results of this experiment revealed that the abundance of *LiCPS* mRNA was developmentally regulated, peaking in fully mature flowers (Fig. 3). Furthermore, this experiment demonstrated that *LiCPS* transcripts are highly enriched in glandular trichomes in *L. x intermedia* flowers. *LiCPS* mRNA was not detected in young leaf tissue (Fig. 3). These findings parallel previous reports demonstrating that in some instances terpenoid synthesis

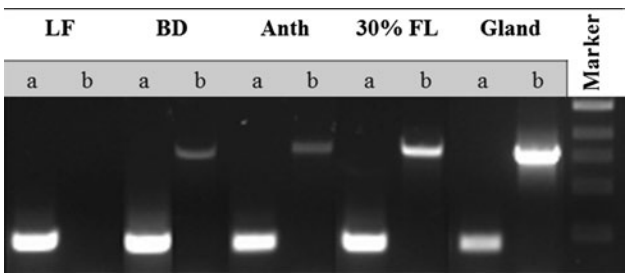

Fig. 3 *LiCPS* transcript abundance in different tissues of *L. x intermedia*. LF, young leaf; BD, bud stage of flower; Anth, anthesis stage of flower; 30 % FL, mature flower at 30 % stage; gland, glandular trichome from 30 % flowering stage. **a** *β Actin*, **b** *LiCPS*

in plants is regulated through the transcriptional control of the corresponding genes. For example, menthofuran in peppermint, and linalool and 1,8-cineole in lavender are directly correlated with the abundance of menthofuran synthase, β-phellandrene synthase, linalool synthase and 1,8-cineole synthase, respectively (Demissie et al. 2011, 2012; Lane et al. 2010; Mahmoud and Croteau 2003). Lane et al. (2010) also demonstrated that the genes encoding for 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and HMG-CoA reductase (HMGR), which represent regulatory steps of the DXP and MVA pathways, respectively, are transcriptionally regulated in glandular trichomes (oil glands). *DXS* transcript was heavily expressed in the glandular trichomes of *L. angustifolia* while *HMGR* mRNA was barely detectable, indicating that essential oil constituents are predominantly produced through the DXP pathway in lavender glandular trichomes.

Sequence comparison and phylogenetic tree analysis

Based on protein sequence and function, plant TPSs were recently organized into seven subfamilies/clades. These include three angiosperm-specific clades (TPS-a, TPS-b and TPS-g), a gymnosperm-specific subfamily (TPS-d), a subfamily most conserved among land plants (TPS-c), a subfamily most conserved among vascular plants (TPS-e/f), and a subfamily specific to *Selaginella moellendorffii* (TPS-h) (Chen et al. 2011). Among these TPS-a clade includes sesquiterpene synthases, while TPS-b includes both mono- and sesquiterpene synthases from angiosperms. The sesqui- and mono-TPS of gymnosperm plants form a specific clade (TPS-d). LiCPS along with LiBERS and ZIS from lavender and sweet basil, belong to the TPS-b group of terpene synthases (Demissie et al. 2012; Landmann et al. 2007). Furthermore, LiCPS shares more structural identity with mono- and sesquiterpene synthases from lavenders, than with other sesquiterpene synthases with similar function including ZIS from *O. basilicum* and caryophyllene synthases from *Zea mays* (Fig. 4). This is as expected, as it has been reported that mono- and sesquiterpene synthases of a given species often share more structural identity with one another, than with TPSs of similar function from a different plant species.

In summary, we have cloned and functionally characterized LiCPS, a sesquiterpene synthase, from the glandular trichomes of *L. x intermedia* plants. The expression of this gene is regulated in a species-specific and tissue-specific manner, and *LiCPS* transcripts are highly enriched in floral glandular trichomes. Given the relatively strong expression of this gene in glandular trichomes, a stronger contribution of this gene would initially be anticipated, and hence as would a higher concentration of caryophyllene in *L. x intermedia* EO. However, given that LiCPS lacks a plastidial

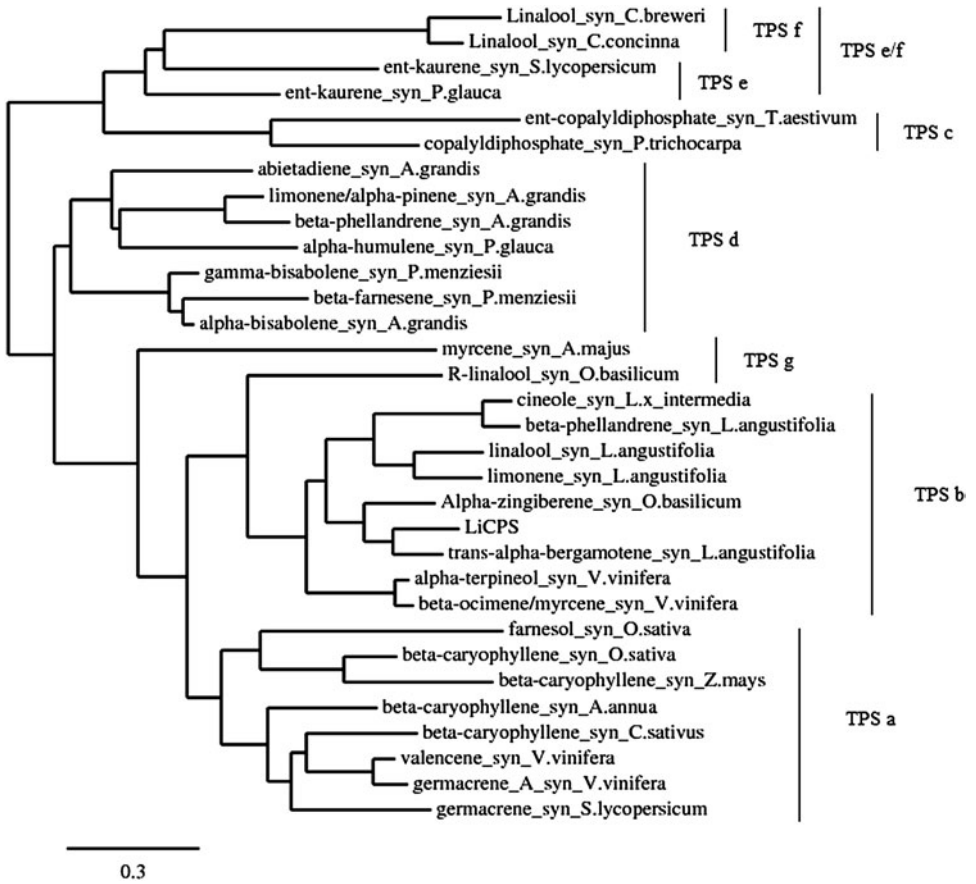

Fig. 4 Phylogenetic tree analysis of LiCPS with other terpene synthases from different plants. The *scale bar* represents 0.3 amino acid substitutions per site. TPSs within the same class share minimum of 50 % amino acid identity. Like trans-alpha-bergamotene synthase of *L. angustifolia*, LiCPS (KF470962) was grouped under TPS-b with other mTPS from angiosperms. Accession numbers of terpene synthases used to generate the phylogenetic tree are beta-phellandrene_syn_*L. angustifolia*: HQ404305; valencene_syn_*V. vinifera*: AAS66358.1; beta-phellandrene_syn_*A. grandis*: AAF61453.1|AF139205_1; limonene-alpha-pinene_syn_*A. grandis*: AAF61455.1|AF139207_1; germacrene_A_syn_*V. vinifera*: ADR66821.1; germacrene_syn_*S. lycopersicum*: AEM05858.1; *trans*-alpha-bergamotene_syn_*L. angustifolia*: ABB73046.1; linalool_syn_*L. angustifolia*: ABB73045.1; limonene_syn_*L. angustifolia*: ABB73044.1; cineole_syn_*L. x intermedia*: JN701459; copalyldiphosphate_syn_*P. trichocarpa*: XP_002306777.1; ent-Copalyldiphosphate_syn_*T. aestivum*: BAH56558.1; abietadiene_syn_*A. grandis*: AAK83563.1; beta-farnesene_syn_*P. menziesii*: AAX07265.1; alpha-bisabolene_syn_*A. grandis*: AAC24192.1; linalool_syn_*C. breweri*: AAD19840.1; linalool_syn_*C. concinna*: AAD19839.1; ent-kaurene_syn_*P. glauca*: ACY25275.1; ent-kaurene_syn_*S. lycopersicum*: AEP82778.1; alpha-terpineol_syn_*V. vinifera*: AAS79352.1 and beta-ocimene/myrcene_syn_*V. vinifera*: ADR74206.1; gamma-bisabolene_syn_*P. menziesii*: AAX07266.1; alpha-humulene_syn_*P. glauca*: ADZ45513.1; R-linalool_syn_*O. basilicum*: AAV63789.1; myrcene_syn_*A. majus*: AAO41726.1; alpha-zingiberene_syn_*O. basilicum*: Q5SBP4.1; beta-caryophyllene_syn_*A. annua*: AAL79181.1; beta-caryophyllene_syn_*Z. mays*: ABY79207.1; farnesol_syn_*O. sativa*: ABJ16554.1; beta-caryophyllene_syn_*O. sativa*: ABJ16553.1; beta-caryophyllene_syn_*C. sativus*: AAU05952.1

targeting sequence, it is most likely located in the cytosol where very little FPP is present due to the inactivity of the MVA pathway in *Lavandula* oil glands (Lane et al. 2010). To confirm this postulate, the subcellular compartmentation of LiCPS must be studied by in situ hybridization or other appropriate methods.

Acknowledgments This work was supported through grants or in-kind contributions to Soheil Mahmoud by UBC Okanagan, Natural Sciences and Engineering Research Council of Canada, Canada Foundation for Innovation, Investment Agriculture Foundation of British Columbia, NRC Plant Biotechnology Institute through the NAPGEN program, and Genome British Columbia. We would also like to thank Lauren Erland for her help with manuscript editing.

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