Phytochemistry xxx (2017) 1–10

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality

Yolande Despinasse [a, b, c], Sébastien Fiorucci [d], Serge Antonczak [d], Sandrine Moja [a, b, c], Aurélie Bony [a, b, c], Florence Nicolè [a, b, c], Sylvie Baudino [a, b, c], Jean-Louis Magnard [a, b, c], Frédéric Jullien [a, b, c, *]

[a] Université de Lyon, F-42023, Saint-Etienne, France
[b] Université de Saint-Etienne, Jean Monnet, F-42000, Saint-Etienne, France
[c] Laboratoire de Biotechnologies Végétales Appliquées aux Plantes Aromatiques et Médicinales, EA 3061, 23 Rue du Dr Michelon, F-42000, Saint-Etienne, France
[d] Institut de Chimie de Nice, UMR-CNRS 7272, Faculté des Sciences, Université de Nice-Sophia Antipolis, 06108 Nice Cedex 2, France

ARTICLE INFO

Article history:
Received 14 November 2016
Received in revised form
19 January 2017
Accepted 21 January 2017
Available online xxx

Keywords:
Lavandula angustifolia (*Lamiaceae*)
Terpene synthase
Transcript regulation
Camphor

ABSTRACT

Lavender essential oils (EOs) of higher quality are produced by a few *Lavandula angustifolia* cultivars and mainly used in the perfume industry. Undesirable compounds such as camphor and borneol are also synthesized by lavender leading to a depreciated EO. Here, we report the cloning of bornyl diphosphate synthase of lavender (*LaBPPS*), an enzyme that catalyzes the production of bornyl diphosphate (BPP) and then by-products such as borneol or camphor, from an EST library. Compared to the BPPS of *Salvia officinalis*, the functional characterization of LaBPPS showed several differences in amino acid sequence, and the distribution of catalyzed products. Molecular modeling of the enzyme's active site suggests that the carbocation intermediates are more stable in LaBPPS than in SoBPPS leading probably to a lower efficiency of LaBPPS to convert GPP into BPP. Quantitative RT-PCR performed from leaves and flowers at different development stages of *L. angustifolia* samples show a clear correlation between transcript level of *LaBPPS* and accumulation of borneol/camphor, suggesting that LaBPPS is mainly responsible of *in vivo* biosynthesis of borneol/camphor in fine lavender. A phylogenetic analysis of terpene synthases (TPS) pointed out the basal position of LaBPPS in the TPSb clade, suggesting that LaBPPS could be an ancestor of others lavender TPSb. Finally, borneol could be one of the first monoterpenes to be synthesized in the *Lavandula* subgenus. Knowledge gained from these experiments will facilitate future studies to improve the lavender oils through metabolic engineering or plant breeding.

Accession numbers: LaBPPS: KM015221.

1. Introduction

In 2015, cultivation of lavenders in the south of France extended over 20,000 ha leading to the production of respectively 1400 and 80 tons of lavandin (*Lavandula x intermedia*) and true lavender (*Lavandula. angustifolia subsp. angustifolia*) essential oils (EOs). Besides the fact that lavender is a touristic emblem of Provence, this agricultural activity has a major economic interest and represents more than half of the world production of lavender EO. The quality of lavender EO depends firstly on the amount of desirable major flavour compounds such as linalool and linalyl acetate that are characteristic terpenes of lavender scent, but also on the specific aromatic touch given by several minor compounds. Lavender EOs of higher quality are produced by a few *L. angustifolia* cultivars and mainly used in the perfume industry. Undesirable compounds such as camphor and borneol can also be produced by lavender leading to a depreciated EO; such terpenes are strongly produced in lavandin, a hybrid between *L. angustifolia* and *L. latifolia*, leading to a restricted use of their EOs in the soap industry. A genetic knowledge of camphor biosynthesis in lavender is therefore an important goal to develop new strategies leading to the production of lavender cultivars with improved EO depleted in camphor.

Monoterpenes and sesquiterpenes are the main components of lavender EO (Charles et al., 2002; Shellie et al., 2002) and are

* Corresponding author. Université de Lyon, F-42023, Saint-Etienne, France.
E-mail address: jullien@univ-st-etienne.fr (F. Jullien).

http://dx.doi.org/10.1016/j.phytochem.2017.01.015
0031-9422/

derived from the condensation of isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP). Condensation of one DMAPP and two IPP molecules catalyzed by farnesyl diphosphate synthase (FPPS) leads to the formation of FPP in the cytosol whereas the condensation of one DMAPP and one IPP catalyzed by geranyl diphosphate synthase (GPPS) leads to the formation of GPP in the plastid. GPP and FPP are substrates of terpene synthases (TPS) for the synthesis of mono- and sesquiterpenes respectively. Terpene metabolism in lavender has already been well depicted (Fig. 1), leading to the characterization of four monoterpene synthases (Demissie et al., 2011, 2012; Landmann et al., 2007) and five sesquiterpene synthases (Jullien et al., 2014; Lukman et al., 2013). Moreover a cis-prenyl diphosphate synthase involved in lavandulyl diphosphate synthesis, a precursor of lavandulol and lavandulyl acetate *in planta*, was also characterized (Demissie et al., 2013). All these enzymes are responsible for the biosynthesis of the major terpenes found in lavender EO, except for camphor and related compounds such as borneol and bornyl acetate.

Bornyl diphosphate synthase (BPPS), the first enzymatic step of camphor biosynthesis, has been cloned only once in *Salvia officinalis* (Wise et al., 1998). This unusual TPS is a multi product enzyme giving both several monoterpenes as minor compounds and bornyldiphosphate (BPP) as a major prenyldiphosphate (Fig. 2). BPP is further dephosphorylated, leading to the monoterpene borneol, then oxidized in camphor by a borneol dehydrogenase (Lukman et al., 2012). The homodimeric BPPS has raised notable attention and was the first monoterpene synthases (mTPS) to be crystallized (Whittington et al., 2002). This study revealed that water molecules firmly anchored in the active site could prematurely quench carbocationic substrates, leading to the synthesis of different monoterpenes. Recently, a computational enzymologic study of BPPS permitted to propose the formation of several possible carbocation intermediates in the active site (Fig. 2) leading to side-product monoterpenes and pointed out electrostatic factors steering favorably the formation of the bornyl cation further stabilized by the diphosphate moiety (Major and Weitman, 2012; Weitman and Major, 2010).

In this study, we characterized the BPPS of lavender as a new TPS involved in major terpene biosynthesis in *Lavandula* species. Molecular modeling of LaBPPS and SoBPPS was carried out to compare their active sites and explain the relationship between their structure and function, especially the differences in their products' distribution. Transcript levels from qRT-PCR and essential oil accumulation in *L. angustifolia* cv Diva were analyzed in leaves and flowers at different developmental stages to examine the regulation of this TPS.

2. Results and discussion

2.1. Sequence analysis

Partial sequences of *LaBPPS* were obtained from 12 sets of ESTs from a 454 cDNA library (Jullien et al., 2014). Amplification of 5′ and 3′ ends was carried out by RACE assays. Primers were then defined in both 5′ and 3′ UTR to amplify the *LaBPPS* in full length (Table S1).

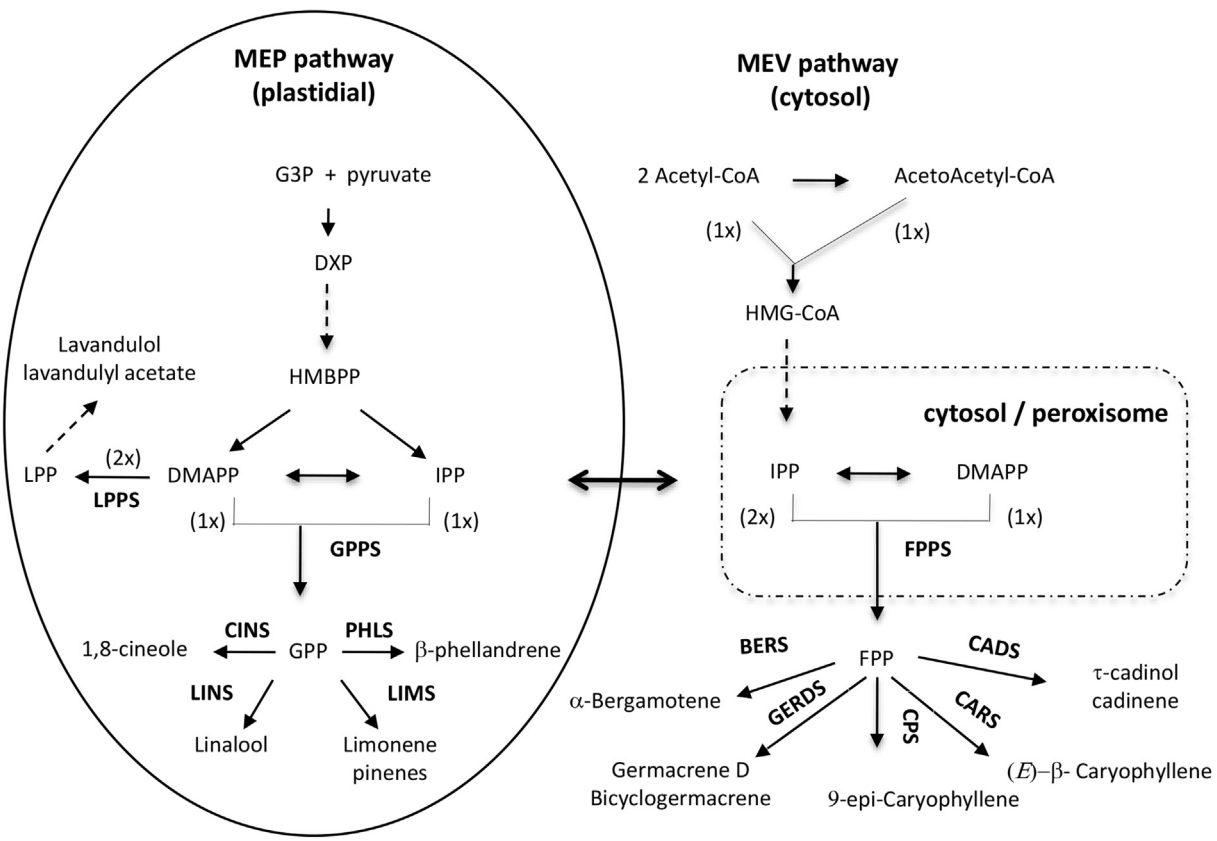

Fig. 1. Pathways of mono and sesquiterpene biosynthesis in *Lavandula angustifolia* and *L.* x *intermedia*. BERS, α-bergamotene synthase; CADS, cadinol synthase; CARS, β-caryophyllene synthase; CDP-ME, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol; CINS, cineole synthase; CPS, 9-epi-caryophyllene synthase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; FPP, farnesyl diphosphate; FPPS, FPP synthase; GERDS, germacrene D synthase; G3P, glyceraldehyde 3-phosphate; GPP, geranyl diphosphate; GPPS, GPP synthase; HMBPP, 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; LIMS, limonene synthase; LINS, linalool synthase; LPP, lavandulyl diphosphate; LPPS, LPP synthase; MEP, 2-C-methyl-D-erythritol 4 phosphate; MEV, mevalonate; PHLS, β-phellandrene synthase.

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015

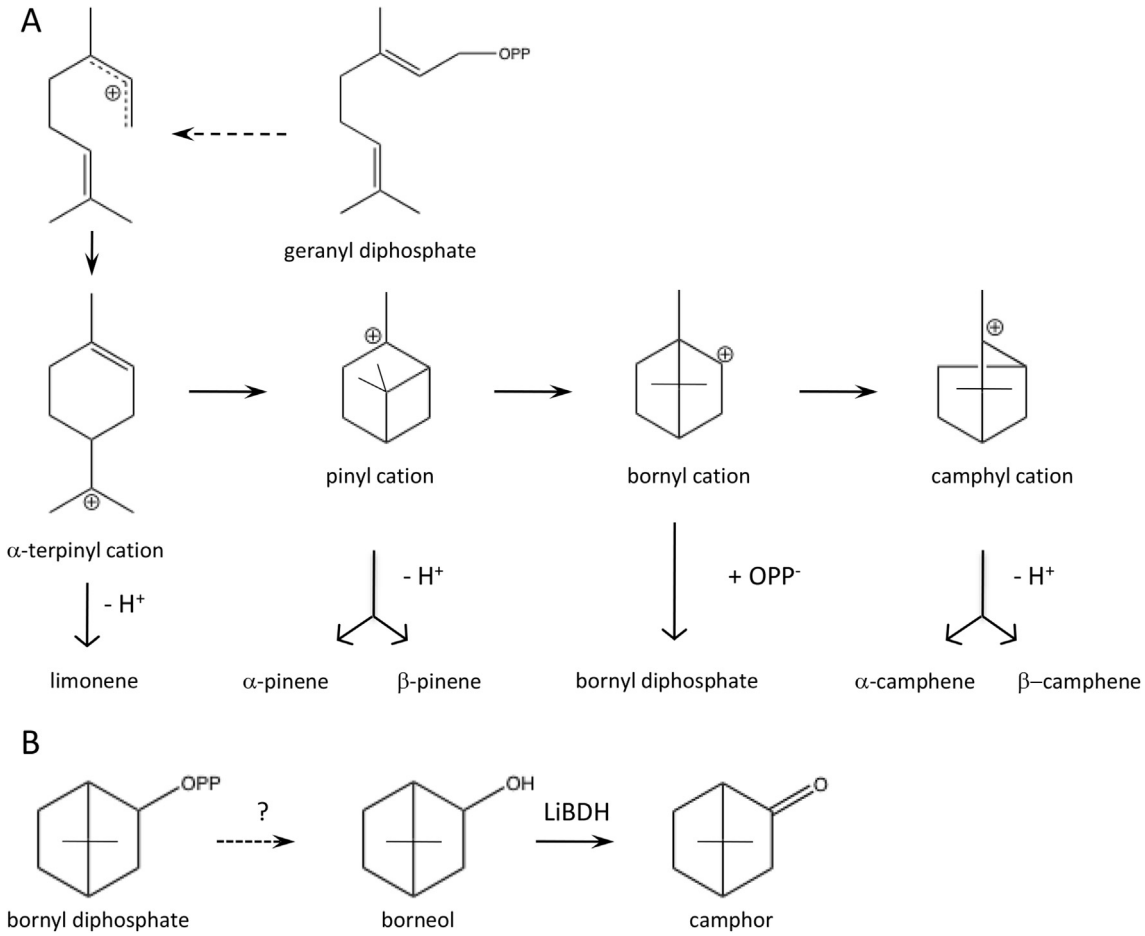

Fig. 2. Biosynthetic pathway of camphor from geranyl diphosphate. A, possible carbocation intermediates in BPPS (adapted from Major and Weitman, 2012). B, Biosynthetic steps from BPP to camphor. LiBDH, borneol dehydrogenase (Lukman et al., 2012).

The open reading frame of the putative *LaBPPS* encoded a 602 amino acids protein with a predicted molecular mass of ca. 70.6 kDa and a pI of 4.92. The deduced amino acid sequence of LaBPPS contained three motifs present in typical plant terpene synthases, namely the RR(x8)W required for cyclisation in mTPSs (Williams et al., 1998) and both the DDxxD and (N,D)D(L,I,V)x(S,T)xxxE motifs (Fig. 3), which are responsible for the enzymatic activity including coordination of divalent Mg^{2+} cations (Degenhardt et al., 2009). On the contrary, the LQLYEASFLL motif that is thought to be part of the active site (McGeady and Croteau, 1995; Wise et al., 1998) was only partially conserved in LaBPPS compared to SoBPPS. These two enzymes shared 50% identity for their amino acids. A sequence of 63 amino acids upstream of the two arginine residues of the RR(x8)W motif suggested the presence of a putative transit peptide, but no clear prediction to address the protein to a specific subcellular compartment could be found from an analysis with chloro-P (http://www.cbs.dtu.dk/services/ChloroP/), target-P (http://www.cbs.dtu.dk/services/TargetP/) and predotar https://urgi.versailles.inra.fr/predotar/predotar.html) softwares.

Finally, the genomic structure of *LaBPPS* was investigated. The gene was amplified from genomic DNA using the same primers as the one employed for cDNA amplification defined in Table S1. Sequencing results showed that *LaBPPS* contained six introns and seven exons placing this gene in the Class III TPS (Fig. 4). The genomic structure of LaBPPS was very similar to that of other lavender genes encoding monoterpene synthases, hence confirming the very conserved length of the exons 4 and 5 (Table S2). Introns 3 and 4 of *LaBPPS* were respectively the smallest (72 versus 155 for *L. angustifolia* cineole synthase (LaCINS)) and the longest (178 versus 58 for LaCINS) of the five lavender TPS genes compared.

2.2. Functional characterization

Recombinant LaBPPS was produced in the *E. coli* Rosetta ™ (DE3) pLysS strain using the pHXGWA expression vector (Busso et al., 2005) and incubated with GPP. The purified LaBPPS produced several monoterpenes (Fig. 5A) and bornyldiphosphate (BPP). Alcaline phosphatase treatment was needed, after incubation with GPP, to dephosphorylate this prenyldiphosphate in borneol (Fig. 5B). A comparison between MS spectra of borneol from standard and enzymatic activities is shown in Fig. 5C–D. A large amount of release GPP was transformed in geraniol by phosphatase treatment, pointing out the very poor activity of the enzyme in our experimental conditions. Regarding the fact that borneol and camphor are main products in some lavenders we should expect that *in vivo* enzymatic environment is more suitable for this enzyme.

The optimum pH for the recombinant LaBPPS was found between 6.5 and 7 with a dramatic loss of enzymatic activity when the pH raised up to 7.5. Variations in pH did not affect the synthesis of all compounds to the same extent, the synthesis of BPP being mostly inhibited with a pH over 8 (data not shown).

In order to compare product distribution of *L. angustifolia* and *S. officinalis* BPPS in the same experimental conditions, SoBPPS was

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015

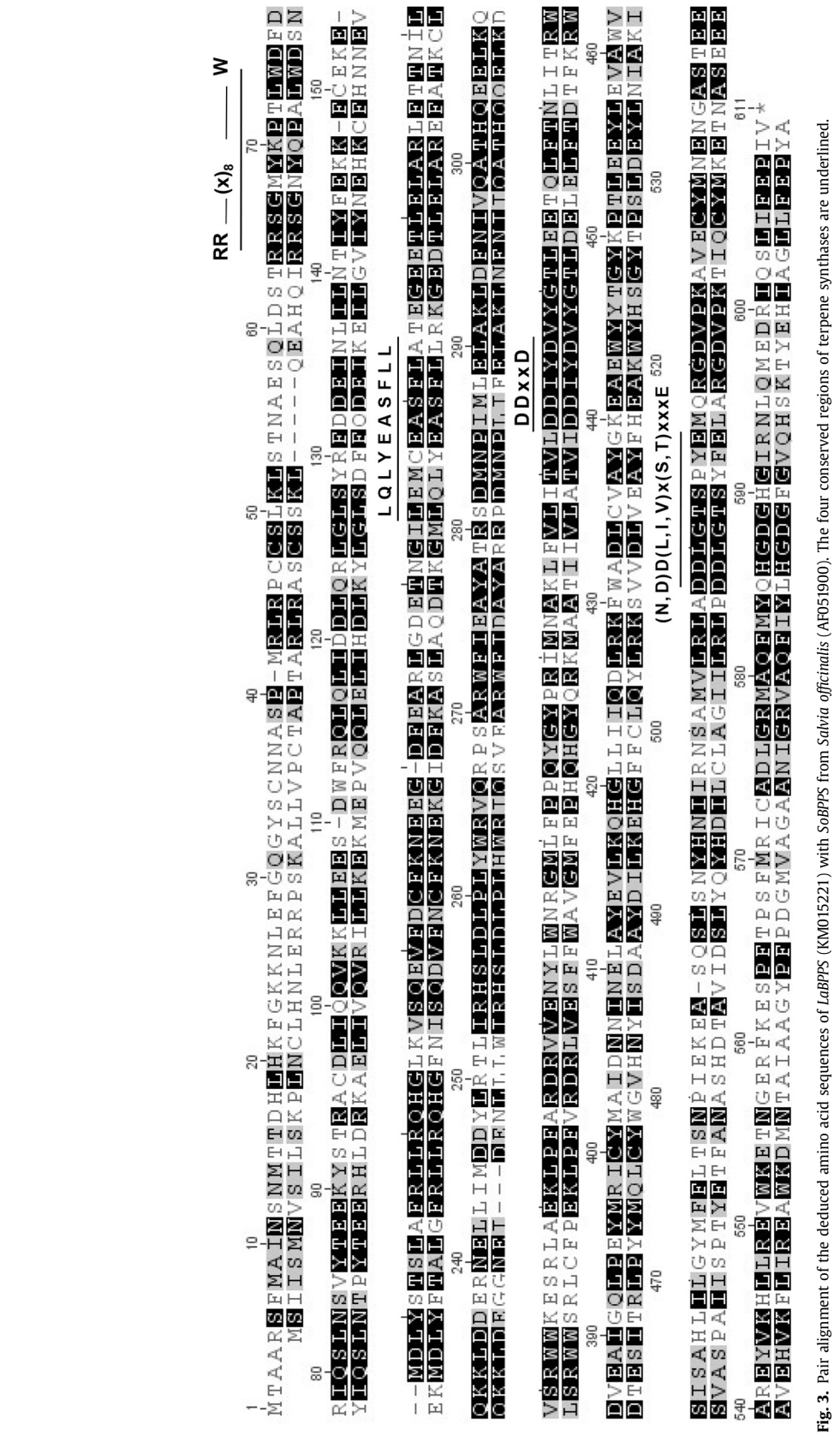

Fig. 3. Pair alignment of the deduced amino acid sequences of *LaBPPS* (KM015221) with *SoBPPS* from *Salvia officinalis* (AF051900). The four conserved regions of terpene synthases are underlined.

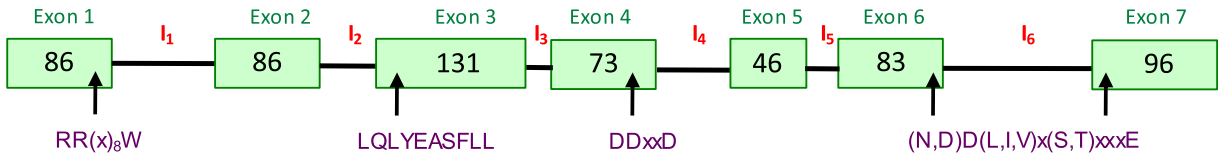

Fig. 4. Schematic representation of *LaBPPS* genomic DNA. Exons are denoted by rectangular boxes (Exon1 through Exon7), and introns are denoted by lines connecting adjacent exons (I_1 through I_6). Numbers inside the box indicate the number of amino acids encoded by that exon. The four conserved motifs are given below the exons, and the black arrows indicate their approximate position. Note: the conserved motif (N,D)D(L,I,V)x(S,T)xxxE amino acids are partly encoded by "Exon 6" and partly by "Exon 7".

cloned using primers defined by Wise et al. (1998). The truncated *SoBPPS* cDNA was inserted in the pHXGWA expression vector, as reported previously for the *LaBPPS*, and *in vitro* enzymatic activity of SoBPPS was performed at pH 6.5 (Fig. S1). SoBPPS catalyzed mainly the synthesis of borneol (75%) and fewer olefins (25%) as already reported by Wise et al. (1998), whereas LaBPPS produced less BPP and more terpenes such as pinenes and camphene (Table 1). LaBPPS catalyzed specifically the synthesis of β-pinene whereas α-terpinolene and α-terpineol were found only in SoBPPS enzymatic assays.

2.3. Molecular modeling of SoBPPS and LaBPPS and structure/function relationship

The high degree of sequence identity between LaBPPS and SoBPPS (roughly 50% of identity and more than 77% of similarity) strengthens the choice of performing homology modeling to produce reliable structures of the enzyme. Subtle structural and physico-chemical differences have been observed between the active sites of LaBPPS homology model and SoBPPS crystal structure. These modifications might affect their catalytic property and

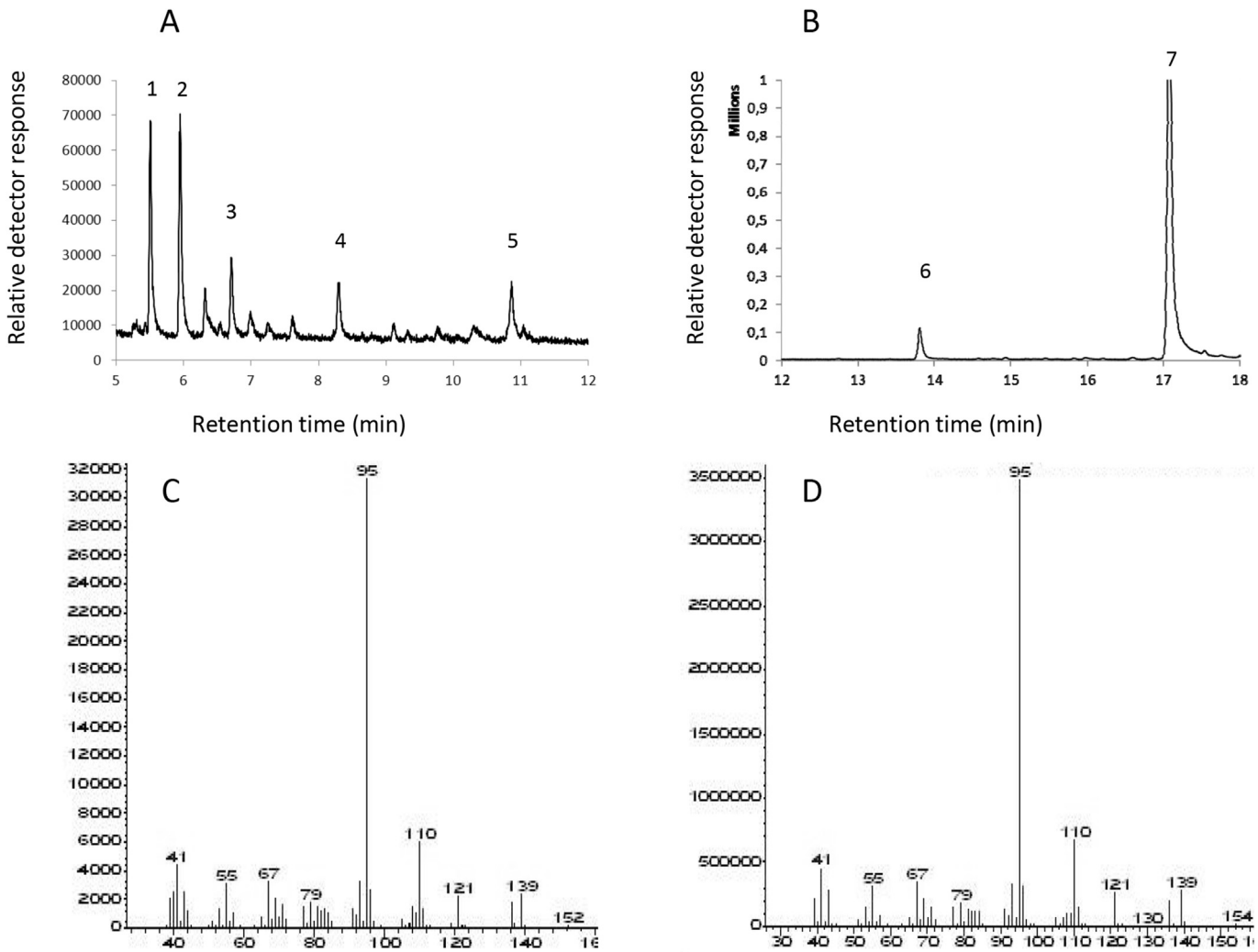

Fig. 5. Monoterpene products of LaBPPS measured *in vitro* and mass spectrum of borneol from NIST library and product of LaBPPS. The enzyme was expressed in *E. coli*, extracted and incubated with 50 μM GPP and 10 mM $MgCl_2$ and 1 mM $MnCl_2$ ions. **A** Products of LaBPPS after incubation, **B** products recovered after alkaline hydrolysis, **C** mass spectrum of compound 6, **D** mass spectrum of borneol from NIST library. Key to the terpene products was: *1* α-pinene, *2* camphene, *3* β-pinene, *4* limonene, *5* linalool, *6* borneol, *7* geraniol.

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015

Table 1
Identification and percentage distribution of compounds from *in vitro* enzymatic assay of LaBPPS and SoBPPS. Average and standard deviation are calculated from six and four independent experiments respectively for LaBPPS and SoBPPS. The three main compounds of both enzymatic assays are in bold letters.

Compounds	AI Adams 2007	AI Calculated	Average ± standard deviation	
			LaBPPS	SoBPPS
α - pinene	**932**	**931**	**23.6 ± 3.8**	**12.5 ± 4.4**
camphene	**946**	**947**	**18.5 ± 2.8**	**9.3 ± 4.7**
β - pinene	974	976	8.0 ± 1.4	0
myrcene	988	986	0	1.6 ± 0.6
limonene	1024	1027	8.9 ± 2.3	8.9 ± 2.1
terpinolene	1086	1084	0	5.6 ± 0.3
linalool	1095	1098	10.7 ± 1.9	1.8 ± 1.8
borneol	**1165**	**1171**	**30.2 ± 7.6**	**57.8 ± 9.6**
α-terpineol	1186	1196	0	2.5 ± 0.1

might explain enzyme selectivity towards terpene and terpenoid compounds. Whittington et al. (2002) deciphered the substrate binding and selectivity of SoBPPS, therefore emphasizing the crucial role played by residues W323, I344, V452 and F578. Among SoBPPS active site residues, V452, A453, S454 and F578 are replaced by I457, S458, A459 and H582 in LaBPPS, respectively (Fig. 6). Interestingly, the active site cavity volume is slightly higher in the LaBPPS model than in the SoBPPS crystal structure (780 *vs* 720 Å^3) and the fraction of polar surface within the binding pocket is clearly in favor of LaBPPS (58 *vs* 44%). Such structural features, in conjunction with modifications of the physico-chemical properties of active site residues would explain the observed differences in substrate selectivity. In SoBPPS, the perfect fit of the substrate within the enzyme binding pocket prevents carbocation stabilization. The tight bonding between carbocations (terpinyl and bornyl cations) and the pyrophosphate co-product favors anti-Markovnikov reactions leading to the production of bornyl cations as the main carbocation (Major and Weitman, 2012; Whittington et al., 2002). In the case of LaBPPS, a higher fraction of polar residues interacting with the carbocation intermediate should increase its lifetime. The size of the binding pocket would enable the classical Wagner-Meerwein rearrangement leading to the formation of more stable tertiary carbocations (pinyl and camphyl cations) and might explain a larger proportion of side products in LaBPPS, for instance (α- and β-) pinene and camphene. The structural analysis of the LaBPPS homology model could guide mutagenesis experiments to validate these hypotheses but such experiments are beyond the scope of the present study and will be examined in a future work. Moreover, it is not excluded that differences in activity and products specificity of LaBPPS and SoBPPS arise from amino acids substitution outside of the active site.

2.4. Bornyl diphosphate synthase expression in fine lavender cultivars and L. latifolia

In order to correlate terpene production and lavender *BPPS* gene expression, leaves and inflorescences were harvested at the same time on the same three fine lavender cv 'Diva' plants. Inflorescences were harvested once a week, from very young spikes closed by green bracts up to fully opened flowers (Fig. 7). GC-MS analyses were performed in duplicate on each plant to quantify the average of borneol and camphor accumulation as an evaluation of the *in vivo* LaBPPS activity. Side products of the BPPS such as pinene or camphene were not taken into account because they could be synthesized by others terpene synthases such as limonene synthase (Landmann et al., 2007). Quantitative RT-PCR was performed from three independent plants. Two reverse transcription reactions were performed for each RNA extraction and the cDNA sample was analyzed in two repetitions leading therefore to 12 results per biological assay. A normalized expression relative to *LaBPPS* gene expression in leaves was applied to compare more clearly gene expression between leaf and the different flower samples.

The amount of borneol/camphor was the lowest in leaves compared to flower samples of different maturity (Fig. 8A). This result could be linked to a very low LaBPPS gene expression in leaf (Fig. 8B). During flower development LaBPPS gene expression raised the highest level in the two first stages of inflorescence development preceding therefore terpene accumulation in the following stages of development as it is usually observed in plants where terpene are accumulated inside glandular trichomes. Lastly, LaBPPS gene expression decreased dramatically whereas borneol/camphor accumulation was lower than volatilization process across gland cuticle. These results therefore bring a clue that LaBPPS is responsible for the *in vivo* synthesis of BBP and then derived terpenes. Moreover, it appeared that the biosynthesis of these compounds is regulated at the transcriptional level even if several

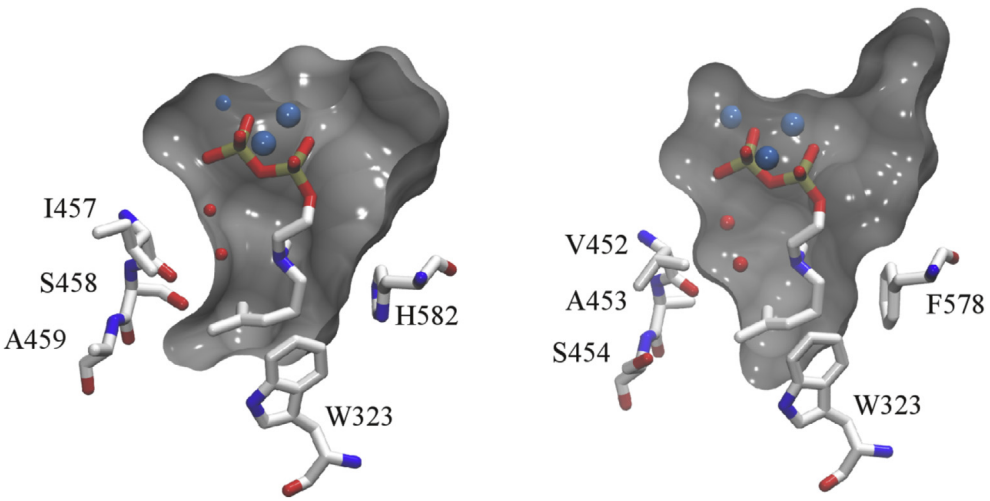

Fig. 6. LaBPPS (left) and SoBPPS (right) active site residues involved in carbocation stabilization. Shape of the active site is delimited in grey. Mg^{2+} ion and water molecules in the active site (#110 and #111 in SoBPPS) have been reported respectively as blue and red dots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015

Fig. 7. Developmental stages of the lavender spike. S1, green spike composed of immature flowers covered by bracts; S2, spike with closed bracts turning purple; S3, spike with opened bracts showing purple calices; S4, Opened calices with closed corolla; S5, Fully opened flowers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

others regulation processes act probably in camphor biosynthetic pathway.

2.5. Phylogenetic analysis

A phylogenetic tree was constructed using amino acid sequences of all the available terpene synthases of *L. angustifolia* and *L. stoechas* species and some sesquiterpene synthases of *Lamiaceae* (Fig. 9). LaBPPS was clustered into the TPSb subfamily, as expected for a monoterpene synthase, and rooted the TPSb clade (Fig. 9). This result should underline that LaBPPS have retained ancestral features and so borneol and perhaps camphor would be among the first monoterpenes to be synthesized in the *Lavandula* species. Polyploidy is common in the genus and several gene duplications have probably occurred further leading to the neofunctionalization of several mTPSs, providing at least two subclade in lavender TPSb. This evolution process reaches probably its paroxysm in species from the subgenus *Lavandula* and the section *Stoechas* (including *L. stoechas, L. viridis and L. pedunculata*) that synthesize the highest diversity of monoterpenes.

3. Conclusions

The culture of lavender in Provence is endangered by both the phytoplasma disease and the production of cheaper lavender EO from emergent countries. The development of lavandin culture, a highly productive hybrid of *L. angustifolia* and *L. latifolia* allowed the enhancement of EO yields but with a lower value due to the presence of borneol and camphor. To date, the improvement of lavandin EO is attained by a costly fractionated distillation to remove camphor. A better knowledge of camphor biosynthesis in lavender could help to develop genetic markers for selective breeding and to produce new lavandin cultivars with an EO depleted in camphor. Moreover, *L. latifolia* is the lavender species that exhibits the highest resistance to phytoplasma disease but is known to produce a camphor-rich EO. A strategy of RNA interference could be applied to this species for which a transformation technology has been developed (Munoz-Bertomeu et al., 2008; Mendoza-Poudereux et al., 2014). Finally, camphor is a major compound in all lavender species of the subgenus *Lavandula*. The characterization of the *BPPS* gene in several lavender species could lead to rebuild the evolutive history of this gene in the *Lavandula* genus.

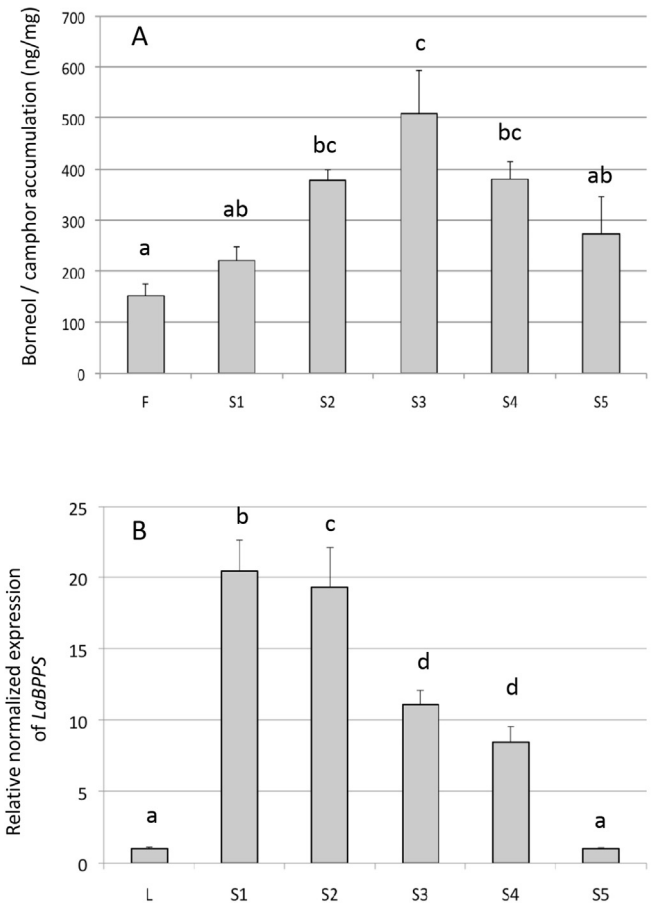

Fig. 8. Borneol/camphor accumulation and transcriptional activity of *LaBPPS* in *L. angustifolia* cv Diva leaf (F) and five inflorescence stages (SI to S5). (A) Borneol and camphor accumulation corresponding to the *in vivo* activity of LaBPPS. (B) Transcript levels of *LaBPPS* was normalized to β-actin and tubulin then *LaBPPS* expression level in leaf was set to 1 in order to express *LaBPPS* expression in inflorescences as a ratio of the expression level in leaf. Error bars indicate standard deviation with n = 6 in A and n = 12 in B. Statistical analyses were performed with the software REST in (B). In (A) for terpene accumulation, an ANOVA was performed with R (R version 3.1.2, 2014) followed by a posthoc mean comparison with Tukey HSD (normality and homosedacsticity were first verified). Same letter indicated no statistical difference with a p value < 0.05.

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015

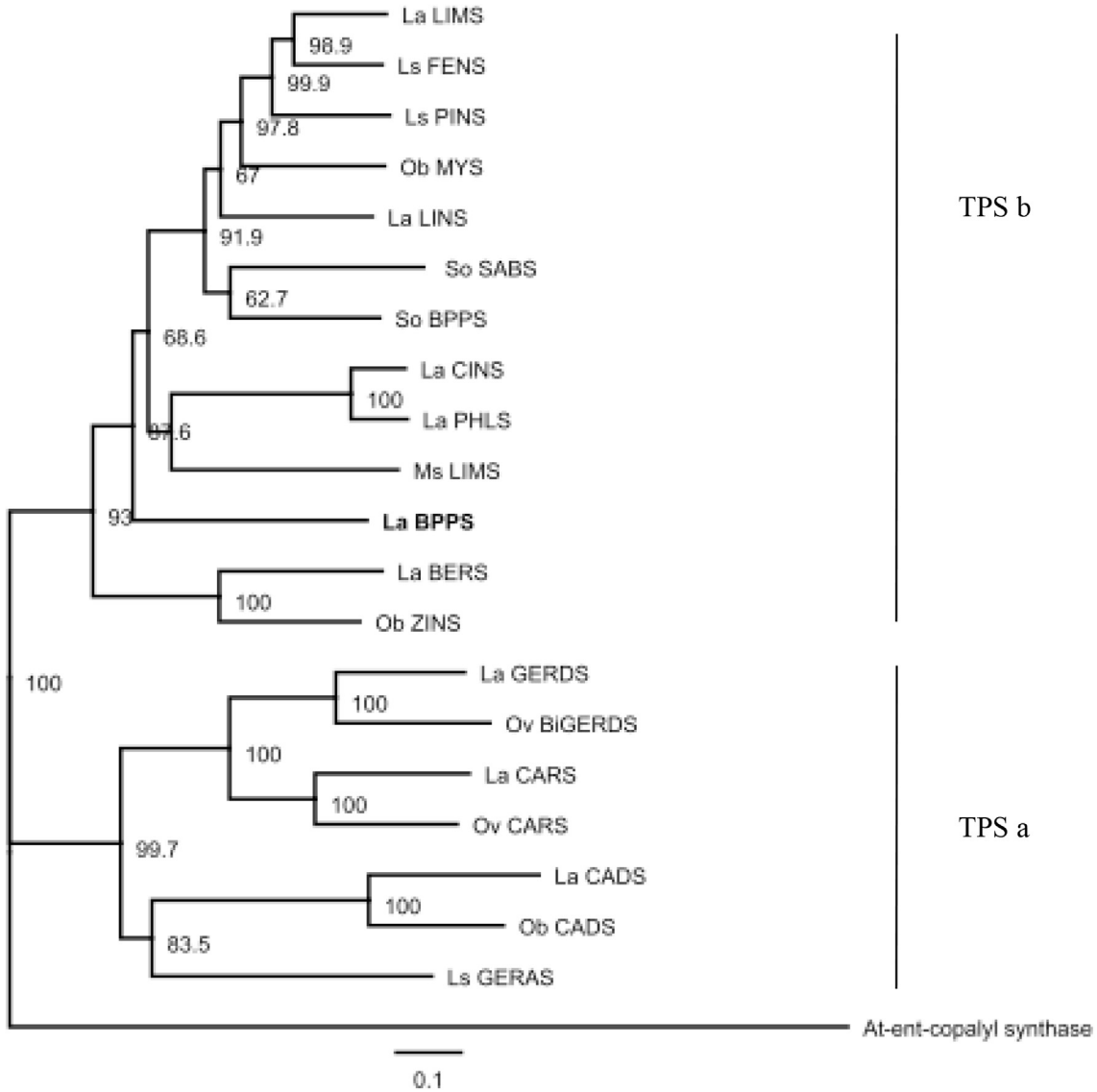

Fig. 9. Dendrogram analysis of monoterpene synthases of *L. angustifolia* and sesquiterpene synthases of *Lamiaceae* using the neighbour-joining method. At *Arabidopsis thaliana* ent-copalyl synthase (NM116512); La, *Lavandula angustifolia* BERGS, α-bergamotene synthase (DQ263741); CADS, t-cadinol synthase (JX401282); CARS, (*E*)-β-caryophyllene synthase (JX401283); CINS, 1,8-cineole synthase (JN701461); GERDS, germacrene D synthase (JX401284); LIMS, limonene synthase (DQ263740); LINS, linalool synthase (DQ263741); PHLS, phellandrene synthase (HQ404305); BPPS bornyl diphosphate synthase (KM015221); Ls, *Lavandula stoechas* FENS, fenchol synthase (JX501514); PINS, a-pinene synthase (JX501515); GERAS, germacrene A synthase (JX501516); Ms, *Mentha spicata* LIMS (L13459); Ob, *Ocimum basilicum* CADS, γ-cadinene synthase (AY693645); ZINS, zingiberene synthase (AY693646); MYS, myrcene synthase (AY693649); Ov, *Origanum vulgare* CARS (GU385970); BiGERDS, bicyclogermacrene D synthase (GU385973); So, *Salvia officinalis* SABS, sabinene synthase, BPPS, (AF051900).

4. Experimental

4.1. Plant material

L. angustifolia plants cv 'Diva' grown in an experimental field in Manosque (Alpes de Haute Provence - France) were used to construct a 454 library previously reported in Jullien et al., 2014. *L. angustifolia* from a population of Calabria were provided by the CNPMAI and *L. latifolia* plants were collected in wild populations. Both plants for quantification of *BPPS* gene expression were grown in greenhouses in Saint-Etienne.

4.2. RNA isolation and cloning

mRNA isolation using the nucleospin® RNA plant kit (Macherey Nagel) was followed by a reverse transcription. RACE assays were carried out to amplify 5′ and 3′ ends of the BPPS using the Marathon® cDNA amplification kit (Clontech, San Diego, CA), following the manufacturer's instructions. Sequences of primers used to clone LaBPPS in pGEMT®-Easy were defined in both UTR (Table S1). DNA inserts cloned in the pGEMT®-Easy vector were sequenced by Eurofins MWG Operon (Germany, Ebersberg). For functional characterization, the BPPS was cloned in the pHXGWA vector (Busso et al., 2005) using the Gateway technology. The 5′ primer used to clone the gene in the entry vector (pENTR/D-TOPO) (Invitrogen, USA) started three amino acids before the RR(x8)W sequence in order to delete the signal peptide and comprised an additional sequence for the hydrolysis of the GST-TPS recombinant protein by thrombin.

4.3. Expression of recombinant LaBPPS in E. coli

The *E. coli* strain Rosetta (DE3) pLysS cells (Novagen, Darmstadt,

Germany) were transformed with pHXGWA carrying the full length *LaBPPS* by heat shock. Production of the heterologous protein was performed during 14–16 h at 17 °C and 150 rpm in terrific broth supplemented with 0.5% glycerol, 0.25 M D-sorbitol, 2.5 mM betaine after induction with 0.2 mM IPTG. The cells recovered by centrifugation were disrupted by incubation in native binding buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, 5% glycerol, 5 mM DTT, pH 8) supplemented with 0.5 mg/ml lysozyme and sonication. After clarification of the lysate by centrifugation, the recombinant protein was purified by binding to the Talon® metal affinity resin (Clontech) according to the manufacturer's instructions. The resin-bound protein was incubated overnight at 4 °C in 200 µl of native binding buffer supplemented with 10 units of thrombin. The next day the TPS was recovered from the mixture by filtration. Protein concentration was measured using the Bio-Rad reagent with bovine serum albumin as standard (Bradford, 1976).

4.4. Recombinant protein purification and electrophoresis

Enzymatic assays were performed in a final volume of 500 µl containing 15–70 µg of purified recombinant protein, buffer (25 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM DTT, 1 mg/ml BSA) and cofactors (10 mM $MgCl_2$, 1 mM $MnCl_2$). The reaction was started by addition of 50 µM of geranyl diphosphate and the mixture was overlaid with 500 µl of hexane supplemented with methyl undecanoate (2 mg/L) as an internal standard. After a 2-h incubation at 30 °C, the mixture was vigorously mixed and the upper hexane phase was collected, concentrated under nitrogen stream and analyzed by GC/MS. Negative controls were performed using the purified product from Rosetta (DE3) pLysS without expression vector.

4.5. GC-MS analysis

GC-MS analyses were performed on an Agilent GC 6850 gas chromatograph coupled with an Agilent 5973 ion trap mass detector. The instrument was equipped with a 30 m × 0.25 mm DB5 apolar capillary column. Temperatures of injector and detector were 250 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. Oven temperature settings were: 4 min at 60 °C after injection followed by a 4 °C/min temperature ramp from 60 °C to 240 °C. Temperature was then kept on hold at 240 °C for 5 min. Injection volume was 2 µl in split 1:2 mode. Molecule identification was performed using Wiley, NIST 05 and Adams mass spectra databases (Adams, 2007). The GC products were in fine confirmed by comparing their retention times and mass spectra to those of authentic α–pinene, β–pinene, camphene myrcene, limonene, linalool and borneol (Payan Bertrand France). Terpenes of *L. angustifolia* cv Diva leaves and flowers were extracted with hexane as previously reported (Guitton et al., 2010) and analyzed as described above.

4.6. Transcript quantification by qPCR

LaBPPS expression levels were determined in leaves of both *L. angustifolia* and *L. latifolia* cultivars. Total RNAs were extracted from the leaves of three plants using the Tri reagent kit (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions and treated with the RQ1 RNAse-free DNase (Promega, Madison, USA). A total of 2 µg of RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, USA) at 50 °C for one hour with an oligo-dT primer. Each RNA sample was reverse-transcribed in two independent reactions.

Quantitative PCR was performed with CFX96™ real-time detection system (Bio-Rad, Hercules, USA) using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Hercules, USA). All reactions were carried out in a 20 µl volume using 2 µl of reverse transcribed cDNA as template and 500 nM of each of the primers according to the manufacturer's protocol. Each cDNA sample was amplified twice in two independent qPCR runs. As the RNA samples were reverse-transcribed in duplicate, this yielded four technical replicates per original sample. Three biological replicates were performed for each cultivar. The following thermal profile was used for all PCR reactions: 95 °C for 30 s, followed by 40 quantification cycles (95 °C for 5 s, Tm (Table S1) for 30s). After 40 cycles, a melting-curve analysis (65 °C–95 °C, one fluorescence read every 0.5 °C) was performed to check the specificity of the amplification.

Normalized expression values were calculated by the CFX96TM data manager (Bio-Rad, Hercules, USA) using *β-ACTIN*, *TUBULIN* as referenced in Jullien et al., 2014. We used REST 2009 (Pfaffl et al., 2002) to compare the expression level of a gene in a 'sample' group using a 'control' group as a reference by implementing a pairwise fixed reallocation randomization test (10000 iterations). Differences in expression between 'sample' and 'control' cDNAs are considered significant for p-values <0.05.

4.7. Model building and refinement

Blast search and sequence alignment were performed using the UniProt webserver and CLUSTAL software to identify sequences similar to LaBPPS. Homology modeling of LaBPPS was carried out with Modeller (Marti-Renom et al., 2000) using Bornyl diphosphate synthase (PDB identifier 1N20) as protein template. The homology model of LaBPPS with the highest DOPE score and no steric clash was then energy minimized using AMBER (Case et al., 2012) and the AMBER ff03 force-field Parameters. The solvation was treated implicitly with a generalized born model. The structural integrity of the homology model of LaBPPS was verified with Procheck (Laskowski et al., 1993). The positions of the magnesium ions, the water molecules and the substrate obtained from SoBPPS crystal structure were kept in order to compare structural features of enzyme active sites. The analysis of structural and physico-chemical properties of the binding pocket was achieved with the fpocket software (Le Guilloux et al., 2009).

Acknowledgements

This work was granted by the Rhône-Alpes country. We thank Dr Busso who kindly provided the pHXGWA vector. We also thank Nicolas Boyer for the culture of lavender and tobacco plants. The CRIEPPAM is also thanked for providing *L. angustifolia* cv'Diva' plants. We are also grateful to the CNPMAI for providing wild fine lavender plants of a population from Calabria. Frédéric Hache is thanked (Department of Foreign Languages, UJM) for English text revision.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2017.01.015.

References

Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/mass Spectrometry, fourth ed. Allured Publ Corporation, Carol Stream.

Busso, D., Busso, B.D., Moras, D., 2005. Construction of a set gateway-based destination vectors for high throughput cloning and expression screening in *Escherichia coli*. Anal. Biochem. 343, 313–321.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Case, D.A., Darden, T.A., Cheatham III, T.E., Simmerling, C.L., Wang, J., Duke, R.E.,

Luo, R., Walker, R.C., Zhang, W., Merz, K.M., Roberts, B., Hayik, S., Roitberg, A., Seabra, G., Swails, J., Goetz, A.W., Kolossváry, I., Wong, K.F., Paesani, F., Vanicek, J., Wolf, R.M., Liu, J., Wu, X., Brozell, S.R., Steinbrecher, T., Gohlke, H., Cai, Q., Ye, X., Wang, J., Hsieh, M.-J., Cui, G., Roe, D.R., Mathews, D.H., Seetin, M.G., Salomon-Ferrer, R., Sagui, C., Babin, V., Luchko, T., Gusarov, S., Kovalenko, A., Kollman, P.A., 2012. AMBER 12. University of California, San Francisco.

Charles, D.J., Renaud, E.N.C., Simon, J.E., 2002. Comparative study of essential oil quantity and composition from ten cultivars of organically grown lavender and lavandin. In: Lis-Balchin, M. (Ed.), Lavender, the Genus Lavandula. Taylor & Francis, London, pp. 232–242.

Degenhardt, J., Köllner, T.G., Gershenzon, J., 2009. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 70, 1621–1637.

Demissie, Z.A., Sarker, L.S., Mahmoud, S.S., 2011. Cloning and functional characterization of β-phellandrene synthase from *Lavandula angustifolia*. Planta 233, 685–696.

Demissie, Z.A., Cella, M.A., Sarker, L.S., Thompson, T.J., Rheault, M.R., Mahmoud, S., 2012. Cloning, functional characterization and genomic organization of 1,8-cineole synthases from *Lavandula*. Plant Mol. Biol. 79, 393–411.

Demissie, Z.A., Erland, L.A.E., Rheault, M.R., Mahmoud, S.S., 2013. The biosynthetic origin of irregular monoterpenes in *Lavandula*. J. Biol. Chem. 288 (9), 6333–6341.

Guitton, Y., Nicolè, F., Moja, S., Valot, N., Legrand, S., Jullien, F., Legendre, L., 2010. Differential accumulation of volatile terpene and terpene synthase mRNAs during lavender (*Lavandula angustifolia* and *L.* x *intermedia*) inflorescence development. Physiol. Plant 138, 150–163.

Jullien, F., Moja, S., Bony, A., Legrand, S., Petit, C., Benabdelkader, T., Poirot, K., Fiorucci, S., Guitton, Y., Nicolè, F., Baudino, S., Magnard, J.-L., 2014. Isolation and functional characterization of a τ-cadinol synthase, a new sesquiterpene synthase from *Lavandula angustifolia*. Plant Mol. Biol. 84, 227–241.

Landmann, C., Fink, B., Festner, M., Dregus, M., Engel, K.H., Schwab, W., 2007. Cloning and functional characterization of three terpene synthases from lavender (*Lavandula angustifolia*). Arch. Biochem. Biophys. 465, 417–429.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK - a program to check the stereochemical quality of protein structures. J. Appl. Cryst. 26, 283–291.

Le Guilloux, V., Schmidtke, P., Tuffery, P., 2009. Fpocket: an open source platform for ligand pocket detection. Bioinformatics 10, 168.

Lukman, S.S., Galata, M., Demissie, Z.A., Mahmoud, S.S., 2012. Molecular cloning and functional characterization of borneol dehydrogenase from glandular trichomes of *Lavandula x intermedia*. Arch. Biochem. Biophys. 528, 163–170.

Lukman, S.S., Demissie, Z.A., Mahmoud, S.S., 2013. Cloning of a sesquiterpene synthase from *Lavandula x intermedia* glandular trichomes. Planta 238, 983–989.

Major, D.T., Weitman, M., 2012. Electrostatically guided dynamics - the root of fidelity in a promiscuous terpene synthase? J. Am. Chem. Soc. 134, 19454–19462.

Marti-Renom, M.A., Stuart, A.C., Fiser, A., Sanchez, R., Melo, F., Sali, A., 2000. Comparative protein structure modeling of genes and genomes. Annu. Rev. Biophys. Biomol. Struct. 29, 291–325.

McGeady, P., Croteau, R., 1995. Isolation and characterization of an active-site peptide from a monoterpene cyclase labeled with a mechanism-based inhibitor. Arch. Biochem. Biophys. 317 (1), 149–155.

Mendoza-Poudereux, I., Munoz-Bertomeu, J., Navarro, A., Arrillaga, I., Segura, J., 2014. Enhanced levels of S-linalool by metabolic engineering of the terpenoid pathway in spike lavender leaves. Metab. Eng. 23, 136–144.

Munoz-Bertomeu, J., Ros, R., Arrillaga, I., Segura, J., 2008. Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. Metab. Eng. 10, 166–177.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in realtime PCR. Nucleic Acids Res. 30, e36.

Shellie, R., Mondello, L., Marriott, P., Dugo, G., 2002. Characterisation of lavender essential oils by using gas chromatography-mass spectrometry with correlation of linear retention indices and comparison with comprehensive two-dimensional gas chromatography. J. Chromatogr. A 970, 225–234.

Weitman, M., Major, D.T., 2010. Challenges posed to bornyl diphosphate synthase: diverging reaction mechanisms in monoterpenes. J. Am. Chem. Soc. 132, 6349–6360.

Whittington, D.A., Wise, M.L., Urbansky, M., Coates, R.M., Croteau, R.B., 2002. Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. Proc. Natl. Acad. Sci. U. S. A. 99 (24), 15375–15380.

Williams, D.C., McGarvey, D.J., Katahira, E.J., Croteau, R., 1998. Truncation of limonene synthase preprotein provides a fully active pseudomature form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry 37, 12213–12220.

Wise, M.L., Savage, T.J., Katahira, E., Croteau, R., 1998. Monoterpene synthases from common sage (*Salvia officinalis*. J. Biol. Chem. 273 (24), 14891–14899.

Please cite this article in press as: Despinasse, Y., et al., Bornyl-diphosphate synthase from *Lavandula angustifolia:* A major monoterpene synthase involved in essential oil quality, Phytochemistry (2017), http://dx.doi.org/10.1016/j.phytochem.2017.01.015