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ORIGINAL RESEARCH PAPER

Characterization of a monoterpene synthase from *Paeonia lactiflora* producing α-pinene as its single product

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Abstract
Objectives To identify a terpene synthase that catalyzes the conversion of geranyl pyrophosphate (GPP) to α-pinene and is involved in the biosynthesis of paeoniflorin.
Results Two new terpene synthase genes were isolated from the transcriptome data of *Peaonia lactiflora*. Phylogenetic analysis and sequence characterization revealed that one gene, named *PlPIN*, encoded a monoterpene synthase that might be involved in the biosynthesis of paeoniflorin. In vitro enzyme assay showed that, in contrast to most monoterpene synthases, *PlPIN* encoded an α-pinene synthase which converted GPP into α-pinene as a single product.
Conclusions This newly identified α-pinene synthase could be used for improving paeoniflorin accumulation by metabolic engineering or for producing α-pinene via synthetic biology.

Keywords Monoterpene synthase · *Paeonia lactiflora* · Paeoniflorin biosynthesis · α-Pinene · Terpene synthase

Introduction

The roots of *Paeonia* species, including *Baishao* (white peony) from *Paeonia lactiflora* Pall., *Chishao* (red peony) from *P. lactiflora* Pall. and *Paeonia veitchii* Lynch, and *Mudanpi* from *Paeonia suffruticosa* Andr.,

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are traditional Chinese medicines (Chinese Pharmacopoeia Committee 2015). The active compounds in *Paeonia* roots are primarily monoterpene glucosides, including paeoniflorin and its derivatives. Paeoniflorin is the most abundant (>90 %) component among the active compounds (Yuan et al. 2013). It has diverse biological functions and exhibits anti-oxidative, anti-inflammatory and anti-apoptotic activities (Chen et al. 2015). However, the accumulation of paeoniflorin and its derivatives in plants requires about 5–6 years. Metabolic engineering and synthetic biology are effective methods for obtaining increased yields of active components, while, mostly rely on the characterization of its biosynthetic pathway in *P. lactiflora*.

Paeoniflorin is a monoterpene glucoside whose biosynthesis includes the formation of benzoic acid via the cinnamic acid pathway and the formation of α-pinene from the isoprenoid pathways (Fig. 1). Analysis of the *P. lactiflora* transcriptome revealed that 24 genes related to the biosynthesis of paeoniflorin and its derivatives were involved in the formation of benzoic acid and geranyl pyrophosphate (GPP) (Yuan et al. 2013). Pinene synthase, one of the key enzymes involved in the conversion of GPP to the α-pinene skeleton of paeoniflorin, remains to be characterized.

Two new "terpene synthase-like" cDNAs from *P. lactiflora* were isolated in this work. The full-length clones were functionally expressed in *Escherichia coli* and one was identified as α-pinene synthase, that could catalyze GPP to form α-pinene as a single product. The functional characterization of α-pinene synthase in *P. lactiflora* provides a starting point for downstream biosynthetic pathway analysis.

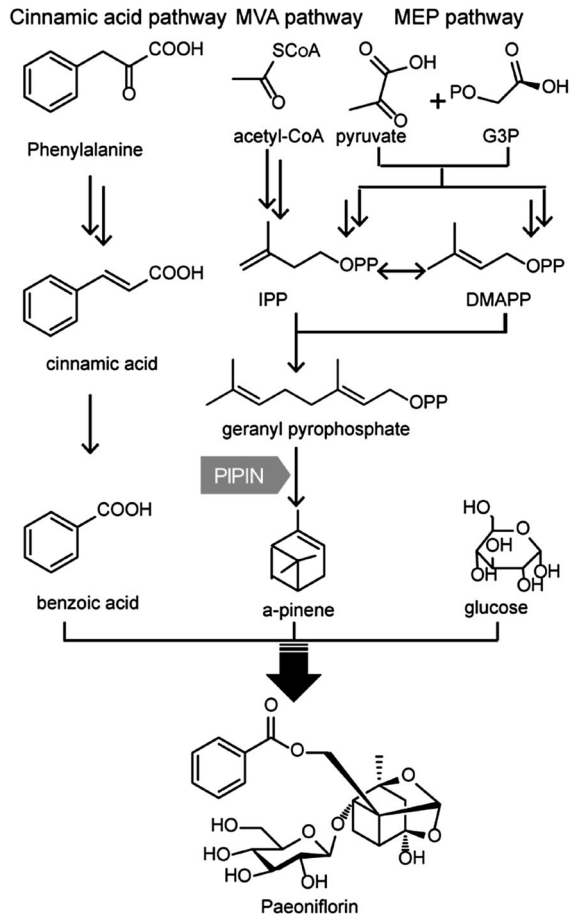


Fig. 1 Hypothetical biosynthetic pathway of paeoniflorin. Multiple *arrows* indicate the multi-step reaction

Materials and methods

Plant materials and chemicals

Paeonia lactiflora Pall. plants were collected locally. α-Pinene was purchased from the National Institutes for Food and Drug Control. Geranyl pyrophosphate ammonium salt (GPP), farnesyl pyrophosphate ammonium salt (FPP), geranylgeranyl pyrophosphate ammonium salt (GGPP) and neryl pyrophosphate lithium salt (NPP) were purchased from Sigma-Aldrich. The purity of these commercial chemicals was ≥95 %.

cDNA expression in *E. coli* and enzyme assays

Total RNA was extracted from *P. lactiflora* roots using the CTAB method (Smart and Roden 2010). The total RNA was reverse-transcribed using PrimeScript RT reagent Kit with gDNA Eraser.

The ORFs of the two genes (*PlPIN* and *PlTPS2*) were amplified using PrimeSTAR HS DNA polymerase with the primers listed in Supplementary Table 1, and subcloned into the pET-32a(+) vector via *Nco*and *Eco*RI.

The plasmids were transformed into *E. coli* Transetta (DE3) (TransGen Biotech, Beijing, China). The expression and purification of recombinant proteins were carried out as previously described (Cui et al. 2015; Matsuba et al. 2013). The recombinant proteins were tested for terpene synthase activities using

50 μM GPP, 50 μM FPP or 50 μM GGPP as substrates in 500 μl assay buffer containing 50 mM HEPES (pH 7.0), 100 mM KCl, 7.5 mM $MgCl_2$, 10 % (v/v) glycerol and 5 mM DTT. After incubation at 30 °C, 150 rpm for 2 h, the reaction mixtures were extracted with an equal volume of n-hexane and submitted to GC–MS analysis. *E. coli* Transetta (DE3)/pET-32a(+) was used as negative control.

GC–MS analysis

GC–MS was carried out using a Trace 1310 series GC and TSQ8000 MS. Chromatographic separation was performed using a TR-5 ms column capillary column (30 m × 0.25 mm ID DF = 0.25 μm). He was used as the carrier gas at 1 ml min^{-1} with a 50:1 split ratio. The injector was at 280 °C. A gradient program was applied: 50 °C (2 min), 50–150 °C linear (5 °C min^{-1}), 150–230 °C linear (20 °C min^{-1}), 230–300 °C linear (30 °C min^{-1}), 300 °C (5 min). One 1 μl sample was injected into the GC system.

Results and discussion

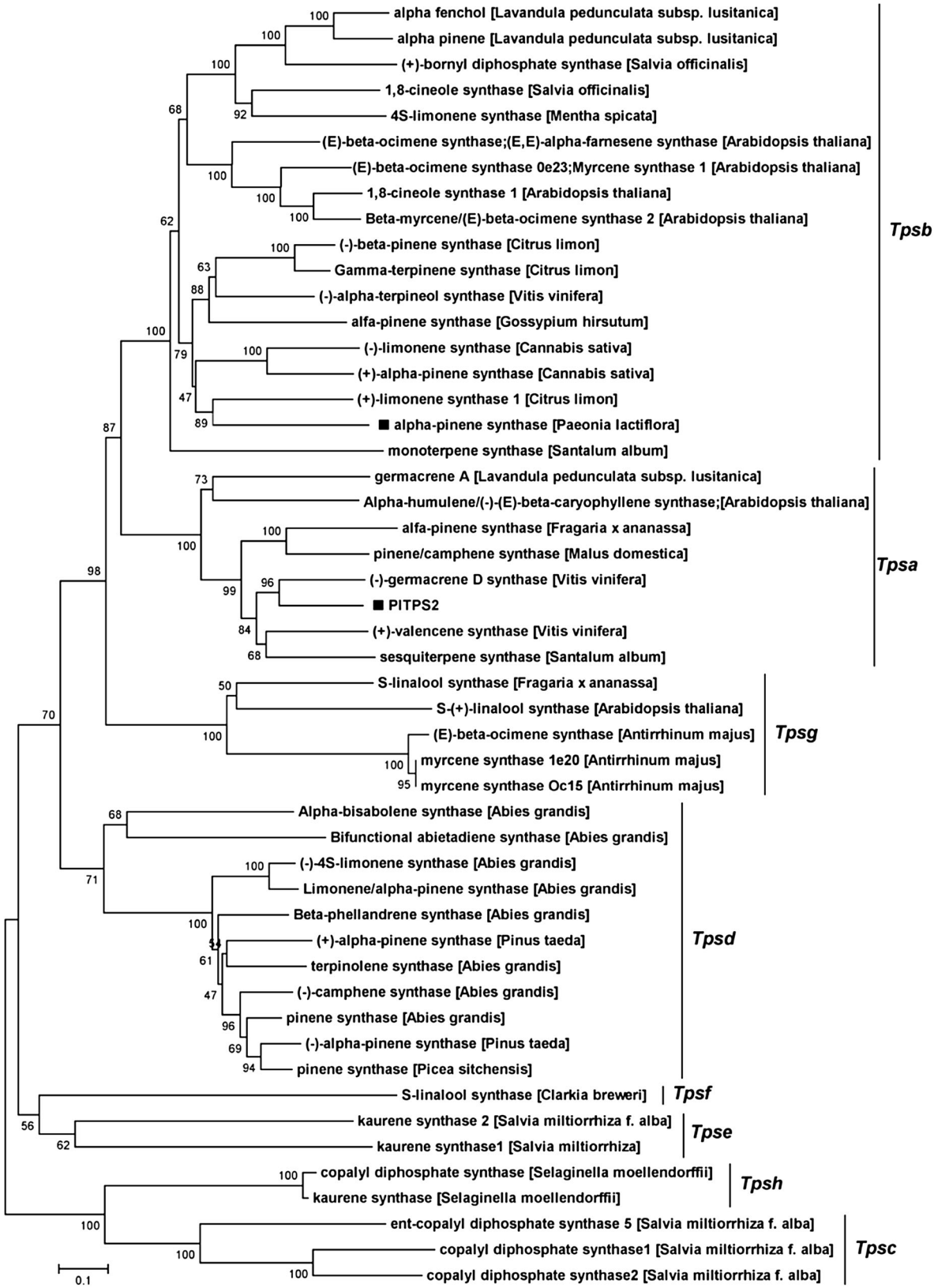
Cloning of *Paeonia lactiflora* terpene synthases and sequence analysis

Two genes (*PlPIN* and *PlTPS2*) annotated as "Terpenoid synthase-like" with full-length cDNA were identified from the transcriptome data of *Paeonia lactiflora*. *PlPIN* (accession No. KU187411), encoding 592 amino acids exhibited 53 % sequence similarity to (+)-limonene synthase 2 from *Citrus limon* (Lücker et al. 2002). *PlTPS2* (accession No. KU187412), encoding 554 amino acids, was similar to two sesquiterpene synthases from *Vitis vinifera* (Lücker et al. 2004). The lengths of the deduced proteins (592 and 554 amino acids) and their predicted molecular weights (69 and 63.7 kDa) are in the range of known terpene synthases. Sequence alignment showed that the protein products of both *PlPIN* and *PlTPS2* contain the highly conserved RRX_8W motif at the *N*-terminal and the DDXXD domain at the *C*-terminal, which are important for substrate binding and catalysis (Supplementary Fig. 1). Compared with sesquiterpene synthase, which is always located in the cytosol, the *PlPIN* protein product with an *N*-terminal transit peptide targets the plastids where it uses the available GPP to make a monoterpene.

Terpene synthases are divided into eight clades, designated *Tpsa* through *Tpsh*, sharing a minimum of 40 % identity among members in each clade (Benabdelkader et al. 2015; Bohlmann et al. 1998a). All plant terpene synthases share a common evolutionary origin. Within the subfamilies, terpene synthases with similar functions or similar reaction mechanisms are subdivided into the same clade (Bohlmann et al. 1998a). Most of monoterpene synthases in angiosperm are clustered in *Tpsb* subfamily, and most of angiosperm sesquiterpene synthases are divided into *Tpsa* subfamily (Bohlmann et al. 1998a). Phylogenetic analysis showed that PlTPS2 is similar to the sesquiterpene synthases in clade *Tpsa*, and is most closely related to sesquiterpene synthase and (−)-germacrene D synthase (Fig. 2). Phylogenetic analysis and sequence characterization indicated that PlTPS2 might be a sesquiterpene synthase. PlPIN was clustered with monoterpene synthases in clade *Tpsb*, indicating that PlPIN might be a monoterpene synthase involved in the biosynthesis of paeoniflorin.

Functional characterization of *PlPIN*

Some terpenoid synthases from gymnosperms and angiosperms convert both GPP and FPP into a monoterpene and sesquiterpene, respectively (Bohlmann et al. 1998a, b; Nieuwenhuizen et al. 2013). Recombinant expressed proteins of *PlPIN* and *PlTPS2* were extracted and assayed for monoterpene, sesquiterpene, and diterpene synthase activities using GPP, FPP and GGPP as the substrates, respectively. A terpene olefin was produced from the reaction of GPP with the fusion protein of *PlPIN* compared with control. This product was identified to be α-pinene by GC–MS by comparison to the authentic standard (Fig. 3). As monoterpene synthases are promiscuous in vitro, when PlPIN was identified as an α-pinene synthase, further analysis using NPP, a general substrate for monoterpene synthases, was carried out (Bohlmann and Gershenzon 2009). However, no products were detected. Moreover, no products were found in the reaction of FPP and GGPP with the fusion protein of *PlPIN* (Supplementary Fig. 2). This indicated that PlPIN employs GPP as a specific substrate to produce α-pinene as a unique product. The activity of PlPIN was in accordance with the phylogenetic


alpha fenchol [Lavandula pedunculata subsp. lusitanica]
alpha pinene [Lavandula pedunculata subsp. lusitanica]
(+)-bornyl diphosphate synthase [Salvia officinalis]
1,8-cineole synthase [Salvia officinalis]
4S-limonene synthase [Mentha spicata]
(E)-beta-ocimene synthase;(E,E)-alpha-farnesene synthase [Arabidopsis thaliana]
(E)-beta-ocimene synthase 0e23;Myrcene synthase 1 [Arabidopsis thaliana]
1,8-cineole synthase 1 [Arabidopsis thaliana]
Beta-myrcene/(E)-beta-ocimene synthase 2 [Arabidopsis thaliana]
(-)-beta-pinene synthase [Citrus limon]
Gamma-terpinene synthase [Citrus limon]
(-)-alpha-terpineol synthase [Vitis vinifera]
alfa-pinene synthase [Gossypium hirsutum]
(-)-limonene synthase [Cannabis sativa]
(+)-alpha-pinene synthase [Cannabis sativa]
(+)-limonene synthase 1 [Citrus limon]
alpha-pinene synthase [Paeonia lactiflora]
monoterpene synthase [Santalum album]
Tpsb
germacrene A [Lavandula pedunculata subsp. lusitanica]
Alpha-humulene/(-)-(E)-beta-caryophyllene synthase;[Arabidopsis thaliana]
alfa-pinene synthase [Fragaria x ananassa]
pinene/camphene synthase [Malus domestica]
(-)-germacrene D synthase [Vitis vinifera]
PITPS2
(+)-valencene synthase [Vitis vinifera]
sesquiterpene synthase [Santalum album]
Tpsa
S-linalool synthase [Fragaria x ananassa]
S-(+)-linalool synthase [Arabidopsis thaliana]
(E)-beta-ocimene synthase [Antirrhinum majus]
myrcene synthase 1e20 [Antirrhinum majus]
myrcene synthase Oc15 [Antirrhinum majus]
Tpsg
Alpha-bisabolene synthase [Abies grandis]
Bifunctional abietadiene synthase [Abies grandis]
(-)-4S-limonene synthase [Abies grandis]
Limonene/alpha-pinene synthase [Abies grandis]
Beta-phellandrene synthase [Abies grandis]
(+)-alpha-pinene synthase [Pinus taeda]
terpinolene synthase [Abies grandis]
(-)-camphene synthase [Abies grandis]
pinene synthase [Abies grandis]
(-)-alpha-pinene synthase [Pinus taeda]
pinene synthase [Picea sitchensis]
Tpsd
S-linalool synthase [Clarkia breweri]
Tpsf
kaurene synthase 2 [Salvia miltiorrhiza f. alba]
kaurene synthase1 [Salvia miltiorrhiza]
Tpse
copalyl diphosphate synthase [Selaginella moellendorffii]
kaurene synthase [Selaginella moellendorffii]
Tpsh
ent-copalyl diphosphate synthase 5 [Salvia miltiorrhiza f. alba]
copalyl diphosphate synthase1 [Salvia miltiorrhiza f. alba]
copalyl diphosphate synthase2 [Salvia miltiorrhiza f. alba]
Tpsc
0.1

◀**Fig. 2** Phylogenetic relationship of the two terpene synthases from *Paeonia lactiflora* and known functional plant terpene synthases (TPSs). TPSs were aligned using BioEdit and were used for neighbor-joining phylogenetic analysis using Mega 5. α-Pinene synthase (*Paeonia lactiflora*) and PlTPS2 from *P. lactiflora* are highlighted with a *black square*. Names of TPS (GenBank accession number) are shown as follows: (−)-β-pinene synthase (AAM53945), γ-terpinene synthase (Q8L5K4) and (+)-limonene synthase 1 (AAM53944) are from *Citrus limon*. (+)-valencene synthase (AAS66358), (−)-germacrene D synthase (AAS66357) and (−)-α-terpineol synthase (NP_001268216) are from *Vitis vinifera*; myrcene synthase Oc15 (AAO41726), myrcene synthase 1e20 (AAO41727) and (E)-β-ocimene synthase (AAO42614) are from *Antirrhinum majus*, β-myrcene/(E)-β-ocimene synthase 2 (Q9LRZ6), α-humulene/(−)-(E)-β-caryophyllene synthase (Q84UU4), *S*-(+)-linalool synthase (Q84UV0), myrcene/(E)-β-ocimene synthase (Q9ZUH4), (E)-β-ocimene synthase; (E,E)-α-farnesene synthase (A4FVP2) and 1,8-cineole synthase (P0DI76) are from *Arabidopsis thaliana*; monoterpene synthase (ACF24767) and sesquiterpene synthase (ACF24768) are from *Santalum album*; (−)-camphene synthase (AAB70707), (−)-4S-β-phellandrene synthase (Q9M7D1), terpinolene synthase (AAF61454), Limonene/α-pinene synthase (Q9M7C9), (−)-4*S*-limonene synthase (AAB70907), pinene synthase (AAB71085), (E)-α-bisabolene synthase (O81086) and abietadiene synthase (Q38710) are from *Abies grandis*; (−)-limonene synthase (ABI21837) and (+)-α-pinene synthase (ABI21838) are from *Cannabis sativa*; 4*S*-limonene synthase (AAC37366) is from *Mentha spicata*; 1,8-cineole synthase (AAC26016) and (+)-bornyl diphosphate synthase (AAC26017) are from *Salvia officinalis*; pinene synthase (AAP72020) is from *Picea sitchensis*; α-pinene synthase (AGX84977) is from *Gossypium hirsutum*; α-pinene synthase (CAD57092) and *S*-linalool synthase (CAD57106) are from *Fragaria x ananassa*; pinene/camphene synthase (AGB14627) are from *Malus domestica*; copalyl diphosphate synthase 1(AHJ59321), copalyl diphosphate synthase 2 (AHJ59322), *ent*-copalyl diphosphate synthase 5 (AHJ59324) and kaurene synthase 2 (AHJ59325) are from *Salvia miltiorrhiza f. alba*; kaurene synthase 1 (ABV08817) is from *Salvia miltiorrhiza*; (+)-α-pinene synthase (AAO61228) and (−)-α-pinene synthase (Q94G53) are from *Pinus taeda*; (−)-β-pinene synthase (Q94G53) is from *Artemisia annua*; copalyl diphosphate synthase (EFJ31965) and kaurene synthase (EFJ37889) are from *Selaginella moellendorffii*; *S*-linalool synthase (AAC49395) is from *Clarkia breweri*; α fenchol (AGN72798), α-pinene (AGN72799) and germacrene A (AGN72800) are from *Lavandula pedunculata* subsp. *lusitanica*

analysis in which PlPIN was clustered with monoterpene synthase from *Cannabis sativa* and *Citrus limon*. These synthases have been functional characterized to catalyze GPP to form (−)-limonene, (+)-limonene, or (+)-α-pinene as the main product (Günnewich et al. 2007; Lücker et al. 2002). Though phylogenetic analysis indicated that PlTPS2 was clustered with sesquiterpene synthases, in vitro analysis of *PlTPS2* with GPP, NPP, FPP and GGPP as the substrates did not yield any products (Supplementary Fig. 3). The function of PlTPS2 still needs further investigation.

α-Pinene is a natural and active monoterpene, which is widely used in flavorings, fragrances, medicines, fine-chemicals and high-density renewable fuels (Yang et al. 2013). As the key enzyme involved in the biosynthesis of α-pinene, many α-pinene synthases catalyze the formation of multiple products in addition to α-pinene. α-Pinene synthase from *Cannabis sativa* produces a small amount of β-pinene, myrcene and limonene in addition to α-pinene (Günnewich et al. 2007). α-Pinene is also produced by other monoterpene synthases, and was considered to be a byproduct of (+)-limonene synthase, (−)-β-pinene synthase and γ-terpinene synthase from *Citrus limon* (Lücker et al. 2002). Although the biosynthesis of α-pinene as the principle product or byproduct was elucidated for many plants, an α-pinene synthase from strawberry was reported to produce α-pinene as a single product in angiosperm (Aharoni et al. 2004). The biosynthetic pathway of α-pinene has been assembled in *E. coli* (Yang et al. 2013). Both the α-pinene synthase from strawberry and PlPIN in this study may be useful in synthetic biology for production of α-pinene.

Conclusion

Biosynthesis of α-pinene, the skeleton of paeoniflorin, remains elusive in *P. lactiflora*. In this study, an α-pinene synthase was identified in *P. lactiflora*, which catalyzed the conversion of GPP to α-pinene as a unique product. The α-pinene synthase from *P. lactiflora* would help improve our understanding of the structure–function relationship of α-pinene synthase and provide material for producing α-pinene by synthetic biology. The α-pinene synthase cloned here allowed further study of the formation of paeoniflorin in *P. lactiflora* and will ultimately permit its metabolic engineering in plant or tissue culture. However, the function of α-pinene synthase in the biosynthesis of paeoniflorin in vivo needs further study by RNA

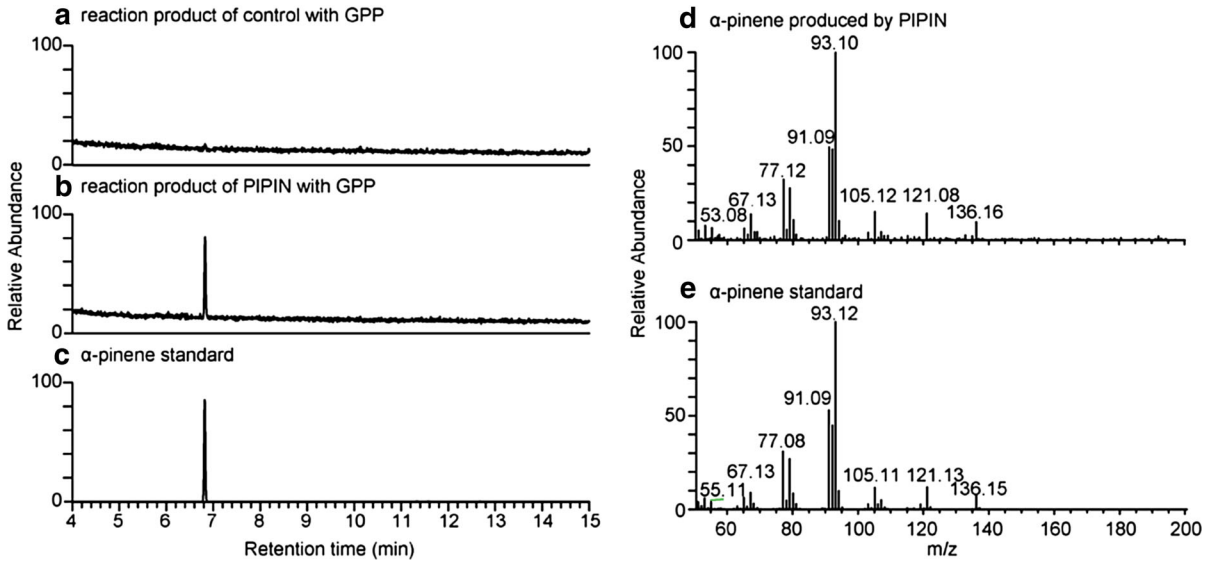


Fig. 3 α-Pinene synthase catalytic activity. GC–MS total ion chromatograms of the reaction products obtained from the control reaction (**a**) and the protein product of *PlPIN* (**b**) using GPP as a substrate, and the authentic α-pinene standard (**c**). Mass spectrum of α-pinene produced by PlPIN (**d**), and the authentic α-pinene standard (**e**)

interference (RNAi), virus-induced gene silencing (VIGS), CRISPR/Cas 9 or overexpression.

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Supporting information Supplementary Table 1 Primers for full-length cloning of *PlPIN* and *PlTPS2*.

Supplementary Figure 1 Alignment of the deduced amino acid sequences of terpene synthases from angiosperm using DNAMAN.

Supplementary Figure 2 GC–MS total ion chromatograms of the reaction products.

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