Journal of Oleo Science
Copyright ©2021 by Japan Oil Chemists' Society
J-STAGE Advance Publication date : October 12, 2021
doi : 10.5650/jos.ess21172
J. Oleo Sci.

Functional Characterization of a New Bifunctional Terpene Synthase LpNES1 from a Medicinal Plant *Laggera pterodonta*

Zhan-Ku Shi[1], Xiao-Wei Gong[1,2], Jiang-Yuan Zhao[1], Ming-Gang Li[1], Xiu-Lin Han[1]*, and Meng-Liang Wen[1]*

[1] *National Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education, Yunnan Institute of Microbiology, School of Life Sciences, Yunnan University, Kunming 650091, Yunnan, CHINA*
[2] *R&D Center of China Tobacco Yunnan Industrial Co., Ltd., Kunming 650231, Yunnan, CHINA*

Abstract: *Laggera pterodonta*, known in China as 'Choulingdan' for its stimulous odor, has long been used as traditional herbal medicine. The essential oil of *L. pterodonta*, which exhibits various pharmacological activities, is a rich resource of monoterpenes and sesquiterpenes. To date, however, the terpene synthases responsible for their production remain unknown. In present study, a new terpene synthase gene (*LpNES1*) was identified from *L. pterodonta*, transcript level of which was significantly upregulated in response to methyl jasmonate treatment. Recombinant LpNES1 could synthesize (*E*)-nerolidol and minor *β*-farnesene from farnesyl diphosphate and linalool from geranyl diphosphate *in vitro*. Whereas, only sesquiterpenes including (*E*)-nerolidol and minor *β*-farnesene were released when LpNES1 was reconstituted in yeast, even coexpressed with a geranyl diphosphate synthase (ERG20^{WW}). Combined with subcellular localization experiment, the result indicated that the cytosol-targeted LpNES1 was responsible for (*E*)-nerolidol biosynthesis exclusively in *L. pterodonta*. Additionally, the expression level of *LpNES1* gene was more prominent in floral buds than that in other tissues. LpNES1 characterized in present study not only lays the molecular foundation for sesquiterpene biosynthesis of *L. pterodonta*, but provides a key element for further biosynthesis of bioactive compound in microbes.

Key words: *Laggera pterodonta*, essential oil, nerolidol/linalool synthase, (*E*)-nerolidol

1 Introduction

Laggera pterodonta, belonging to Asteraceae family, is widely distributed in the sub-Saharan Africa and in the southwestern China. As a traditional herbal medicine, *L. pterodonta* has long been used as ethnomedicine to treat bronchitis, pneumonia, and epidemic influenza, etc.[1,2]. Additionally, the essential oils of *L. pterodonta* are reported to exhibit antiviral[3], antioxidant[4], and antifungal properties[5]. Due to these important pharmacological activities and practical applications, up to now, many studies have focused on characterization of the chemical compositions of the essential oils and/or extracts of *L. pterodonta*, which lead to the identification of 42 monoterpenoids, and 151 sesquiterpenoids[1,6].

In higher plants, terpenoids are biosynthesized from two universal building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are produced by two distinct pathways, the plastidic methylerythritol 4-phosphate (MEP) pathway and the cytosolic mevalonate (MVA) pathway. Further condensation of DMAPP and 1-3 IPP(s) gives rise to geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP) by corresponding prenyltransferases. Subsequently, these prenyldiphosphate precursors are converted into a wide range of structurally diverse mono-, sesqui-, and diterpenes by the action of terpene synthases (TPSs)[7,8]. In general, mono- and di-terpenes are produced compartmentally in plastids, whereas sesquiterpenes are produced in the cytosol[9]. A few studies, however, reveal that mono- and diterpenes can be formed in the cytosol and sesquiterpenes can be formed in plastids[10–12], suggesting that there is a 'cross-talk' between subcellular compartments for their substrates formation in plants.

To date, many terpenoids have been identified from *L.*

*Correspondence to: Meng-Liang Wen, Yunnan Institute of Microbiology, School of Life Sciences, Yunnan University, Kunming 650091, Yunnan, CHINA; Xiu-Lin Han, Yunnan Institute of Microbiology, School of Life Sciences, Yunnan University, Kunming 650091, Yunnan, CHINA
E-mail: mlwen@ynu.edu.cn (MLW); xlhan@ynu.edu.cn (XLH)
ORCID ID: https://orcid.org/0000-0002-7621-1563 (MLW)
Accepted July 30, 2021 (received for review May 25, 2021)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

pterodonta. However, the underlying terpene synthases for their production have yet to be characterized. In this study, through transcriptome dataset, a novel terpene synthase (LpNES1) was isolated from *L. pterodonta*. Recombinant LpNES1 could convert GPP and FPP into linalool and (*E*)-nerolidol *in vitro*, respectively. However, when reconstituted in yeast, LpNES1 failed to form the same compounds as those obtained *in vitro*, even coexpressed with a geranyl diphosphate synthase ($ERG20^{WW}$), which is a mutant of farnesyl diphosphate synthase ERG20 from yeast and has high specificity for GPP synthesis[13]. The expression level of *LpNES1* in different tissues was also investigated. LpNES1, to our knowledge, was the first characterized terpene synthase from *L. pterodonta*.

2 Materials and Methods

2.1 Plant materials and agents

After treatment with 0.25% methyl jasmonate (MeJA)[14, 15] or the 1.4% ethanol solvent (as control) for 3 days, leaves from *L. pterodonta* were sampled separately, immediately frozen in liquid nitrogen and then delivered to OE Biotech Co., Ltd. (Shanghai, China) with dry ice for RNA sequencing, as described in the supporting information. The leaves, floral buds, stems, and roots were collected and immediately frozen in liquid nitrogen and stored at −80℃ for subsequent total RNA extraction.

Substrates including geranyl diphosphate ammonium salt, farnesyl diphosphate ammonium salt, geranylgeranyl diphosphate ammonium salt, and standards linalool and (*Z*/*E*)-nerolidol were purchased from Sigma-Aldrich (Shanghai, China). T4-DNA ligase, restriction enzymes, and HRV 3C Protease were purchased from Takara (TaKaRa Bio, Beijing, China). All other chemical agents were of analytical grade and purchased from Tianjin Guangfu Tech Co., Ltd (Tianjing, China), Guangzhou Saiguo Biotech Co., Ltd (Guangzhou China), and Aladdin (Shanghai China). The synthesis of primers and DNA sequencing were done by Tsingke (Kunming, China).

2.2 Identification of terpene synthases and sequence analysis

Transcripts were annotated by using as a query for BLASTX-searches by UniProtKB/Swiss-Prot25 and KEGG (https://www.kegg.jp/kegg/kegg1.html). Phylogenetic analyses were performed using MEGAX to generate a neighbor-joining tree using 1000 bootstrap replicates. Multiple amino acid sequence alignments of LpNES1 and other TPSs belonging to TPS-g subfamily were aligned using ClustalW 2.1[16].

2.3 Plasmids construction

Plasmids used and constructed in this study are listed in Table S1. All the primers are listed in Table S2. Full-length ORF sequence of *LpNES1* gene was amplified using primers LpNES1-F/R and subcloned into the pMD20-T vector to get 20T-LpNES1. For expression in *Escherichia coli*, PCR-amplified *LpNES1* from 20T-LpNES1 was inserted into the *Bam*H I/*Hin*d III site of plasmid pCold–ProS2 to generate expression plasmid ProS2-LpNES1; For overexpression of LpNES1 in yeast, *LpNES1* gene was codon-optimized and synthesized by GenScript Inc (China), designated as *OP-LpNES1* (Genebank Accession NO: MZ190022). The auxotrophic marker gene *LEU2* was amplified from vector pRS605 (stored in our lab), upstream and downstream homologous region of the *GAL80* gene (indicted as *80up* and *80down* respectively), and terminator *tTEF1*, *tCYC1* were amplified from the genomic DNA of *Saccharomyces cerevisiae* BY4742. The *80up-LEU-tCYC1* and *tTEF1-80down* fragments were obtained through overlap extension PCR (OE-PCR) and then sequentially ligated to vector pESC-URA (harboring the *GAL1,10* bidirectional promoter) by *Xho* I/*Bam*H I and *Not* I/*Sac* I, respectively, to get backbone plasmid pZK10. The *OP-LpNES1* was then ligated to plasmid pZK10 to get pZK11 (finished by GenScript). The mutant $ERG20^{WW}$ (*F96W-N127W*) gene was obtained by OE-PCR according to Ignea, C. *et al.*[13], first cloned to pMD-20T and then subcloned to plasmid pZK10/11 at *Bam*H I site using infusion ligase (Biomed Beijing China) to get pZK12/13, respectively. Final fragments *80up-leu-GAL1-*$ERG20^{WW}$*-80down* and *80up-leu-GAL1,10-*$ERG20^{WW}$, *OP-LpNES1-80down*, could be obtained through *Xho* I/*Sac* I digestion from plasmid ZK-12 and ZK-13, respectively (Fig S1); For transient expression of LpNES1 in *Nicotiana benthamiana*, full-length of *LpNES1* (without stop codon), fused with *GFP* gene, driven by 35S promoter and terminator *tNOS1* were subcloned into plasmid pCAMBIA1301 using infusion ligase to obtain plasmid 1301-LpNES1-GFP.

2.4 Subcellular localization analysis of LpNES1

N-terminal signal peptide sequence and subcellular localization was predicted using ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/) and Predotar (http://genoplante-info.infobiogen.fr/predotar/). For subcellular localization analysis, the plasmid 1301-LpNES1-GFP was transformed into *Agrobacterium tumefaciens* LBA4404 competent cell (Transgen Biotech, China). *Agrobacterium*-mediated transient expression was performed using syringe-infiltration on 4 weeks old *N. benthamiana* leaves. The agroinfiltrated plants were maintained in long-day conditions (16 h light / 8 h dark, 25℃) for 3 days, then *N. benthamiana* leaves were viewed under confocal laser scanning microscopy (TCS SP8, Leica, Germany).

2.5 Expression and purification of the recombinant LpNES1

ProS2-LpNES1 was chemically transformed into *Rosetta* (DE3) for expression. The expression and purification procedures for recombinant LpNES1 were performed as previously described[17] with some modifications: 25 μg/mL ampicillin and 17 μg/mL chloramphenicol were used during cultivation and the expression was induced at 15℃, 170 rpm/min for 20 hours with 0.8 mM IPTG. The Ni-NTA resin purified protein was concentrated, desalted, and exchanged with PBS buffer (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 136 mM NaCl, 2.6 mM KCl, pH 7.4). The ProS2 tag was removed by HRV 3C Protease (TaKaRa Bio, Beijing, China), and reaction was performed according to the instructions. Purified LpNES1 was detected by 12% SDS–PAGE and the protein concentration was determined using the BCA Protein Assay Kit (TaKaRa Bio, Beijing, China).

2.6 Functional characterization of LpNES1 *in vitro*

To assay catalytic activity of the purified LpNES1, the reaction was conducted in 20 mL headspace bottle with 25-50 μg purified protein in assay buffer (25 mM HEPES pH 7.4, 100 mM KCl, 7.5 mM $MgCl_2$, 1 mM $MnCl_2$, 5% (v/v) glycerol, 5 mM DTT), and initiated by addition of 6 μg of three prenyldiphosphate precursors, respectively, at 30℃ for 2 h. Terpene products were collected and analyzed by solid-phase micro extraction (SPME) and gas chromatography–mass spectrometry (GC–MS). Heat-inactivated (at 95℃ for 15 min) protein was used as a negative control. Products were identified by comparison of retention time and mass spectra with those of authentic standards.

2.7 Functional characterization of LpNES1 in yeast

Gel-purified *80up-leu-GAL1-ERG20^{WW}-80down* and *80up-leu-GAL1,10-ERG20^{WW}*, *OP-LpNES1-80down* fragments were transformed into *S. cerevisiae* BY4742 to obtain strain 4742-01 (control) and 4742-02, respectively, by the LiAc/PEG/ssDNA method[18]. Transformants were selected on SD-leu solid medium (20 g/L glucose, 15 g/L agar, 6.7 g/L yeast nitrogen base, 2 g/L amino acids without leucine) and further validated by primers Δ80-F and Δ80-R (Table S1). For solid fermentation, verified colonies were inoculated on yeast extract-peptone-dextrose (YPD) plates at 28℃, for 6 days. Pellets were transferred to a 20 mL headspace bottle for SPME-GC-MS analysis. Liquid fermentation was performed by transferring the overnight culture to 20 mL of fresh YPD medium with an initial OD_{600} of 0.2, terpenes were captured by overlaying dodecane 10% (v/v). The mixture was then incubated at 28℃, 200 rpm for 6 days. Then, the dodecane layer was harvested, dehydrated with anhydrous Na_2SO_4, and detected by GC-MS.

2.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted using the plant RNA extraction kit (Omega Guangzhou, China). Reverse transcription was performed using PrimeScript™ RT reagent Kit (TaKaRa Bio, Beijing, China) and the final cDNAs were diluted 4-fold before use. 18S ribosomal RNA was used as the internal reference gene, and the primers were designed using NCBI Primer software (https://www.ncbi. nlm.nih.gov/tools/primer-blast/, Table S1). qRT-PCR was performed by Applied Biosystems (AB) 7500 Real-time PCR system and AB power SYBR green PCR master mix (Life Technologies). The expression levels of LpNES1 in the four various tissues were calculated using the $2^{-\triangle\triangle CT}$ method. All the experiments were performed by three biological replicates and three technical replicates.

2.9 GC-MS analysis

Samples were injected at 1 μL volume in splitless mode on Agilent 7890A GC coupled with 5977B inert XL MS detector (Agilent Technologies). The separation was achieved by using a HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies) following the program: 50℃ for 1 min, ramped at 8℃ min^{-1} to 300℃ for 5 min. MSD Chem-Station Data Analysis program was used for data processing (Agilent Technologies). Details of GC-MS analysis were described in the supporting information.

3 Results

3.1 Identification and phylogenetic analysis of LpNES1

Based on comparative transcriptome analysis of *L. pterodonta*, one transcript named *LpNES1*, which was upregulated with 8-fold increase after MeJA treatment, was screened out. The full-length *LpNES1* (Genebank Accession NO: MW701393) contained an open reading frame (ORF) of 1749 bp encoding a protein of 493 amino acids with theoretical isoelectric point (pI) and molecular weight of 5.69 and 56.9 kDa, respectively (http://web.expasy.org/compute_pi/). According to the multiple alignments (Fig. 1), despite from different origins, these terpene synthases shared the highly conserved motifs, including the absence of the RRx8W motif in the N-terminal domain which is essential for cyclic monoterpenes biosynthesis[19], and two highly conserved aspartate-rich motifs, consistently occurring as DDTFD and DDLGS (C) AKD (N) E among these enzymes. This two conserved motifs were demonstrated to be located oppositely in the entrance of active cave for fixing the phosphate group of prenyldiphosphate precursors via binding Mg^{2+} or Mn^{2+} cofactors[20–22]. To define the evolutionary relationship of LpNES1, the phylogenetic tree was constructed based on the representative TPSs from other species (Table S1), which were downloaded from

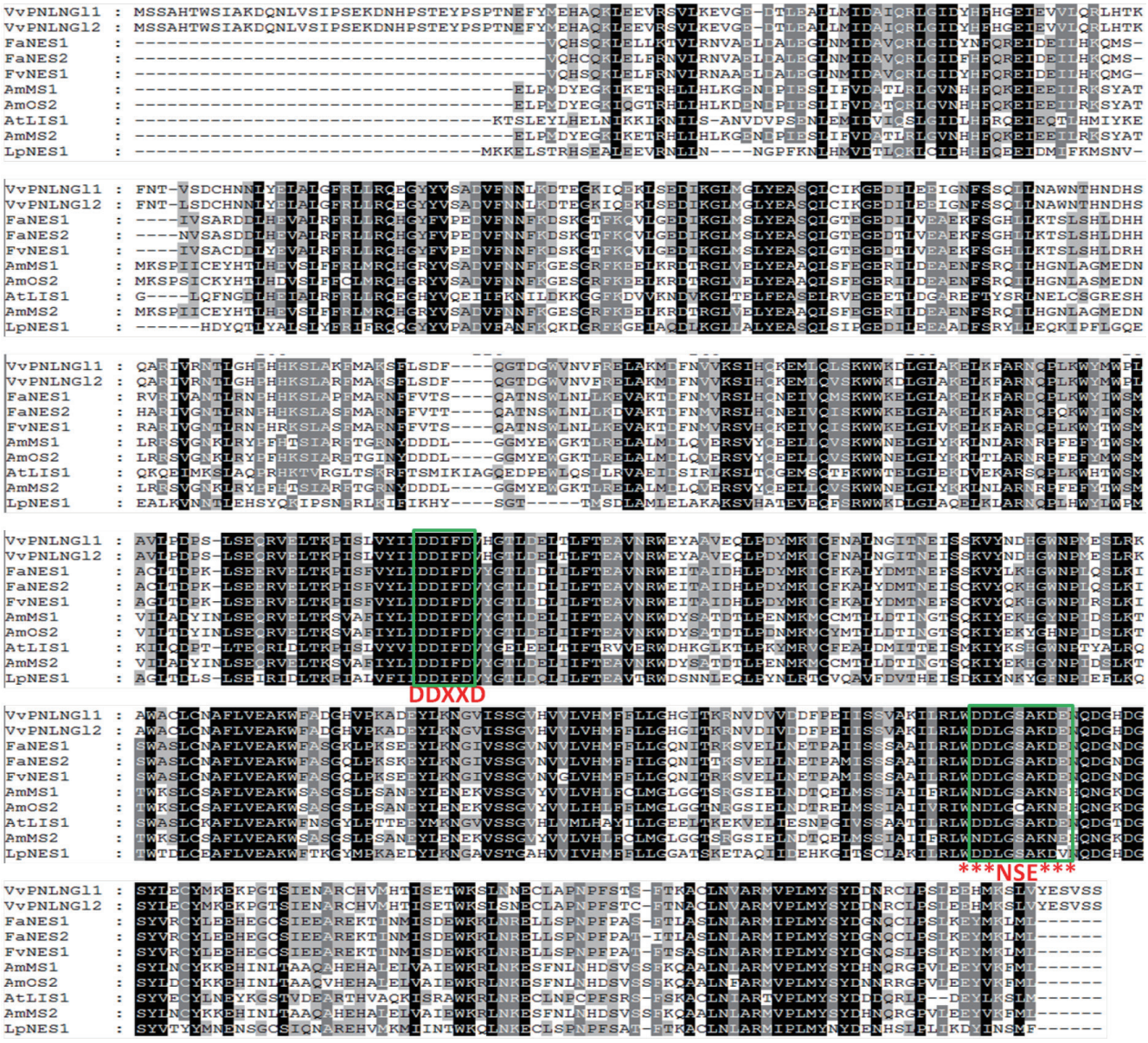

Fig. 1 Amino acid sequence alignment of LpNES1 with other plant linalool/nerolidol synthases, belonging to TPS-g. The Asp-rich domain DDXXD, and the NSE/DTE motif are highlighted by green box, which are highly conserved in plant TPSs. Completely conserved residues are shaded in black and similar residues are shaded in light grey. Dashes indicate gaps introduced to maximize sequence alignment. VvPNLNGl1 (ADR74213): linalool/(*E*)-nerolidol /(*E,E*)-geranyl linalool synthase from *Vitis vinifera*; VvPNLNGl2 (ADR74214): linalool/(*E*)-nerolidol /(*E,E*)-geranyl linalool synthase from *Vitis vinifera*; FaNES1/2 (P0CV94/P0CV95): (3*S*,6*E*)-nerolidol synthase from *Fragaria X ananassa*; FvNES1 (P0CV96): (3*S*,6*E*)-nerolidol synthase from *Fragaria vesca*; AmOS1/2 (AAO42614/Q84NC8): (*E*)-beta-ocimene synthase from *Antirrhinum majus*; AtLIS1 (Q84UV0): *S*-linalool synthase from *Arabidopsis thaliana*; AmMS2 (Q84ND0): Myrcene synthase from *Antirrhinum majus*.

NCBI database. Inspection of the phylogenetic tree clearly indicated the LpNES1 clustered into the terpenoid synthase (TPS) subfamily TPS-g (Fig. 2).

3.2 Cytosol-targeted *LpNES1*

According to the software, *LpNES1* gene was predicted to be localized in the cytosol, as described in the supporting information. To experimentally determine the subcellular localization of *LpNES1* gene, the agroinfiltrated leaves were analyzed for transient GFP expression using confocal laser scanning microscopy. It was obvious that GFP fluorescence was observed in the cytosol of protoplasts ex-

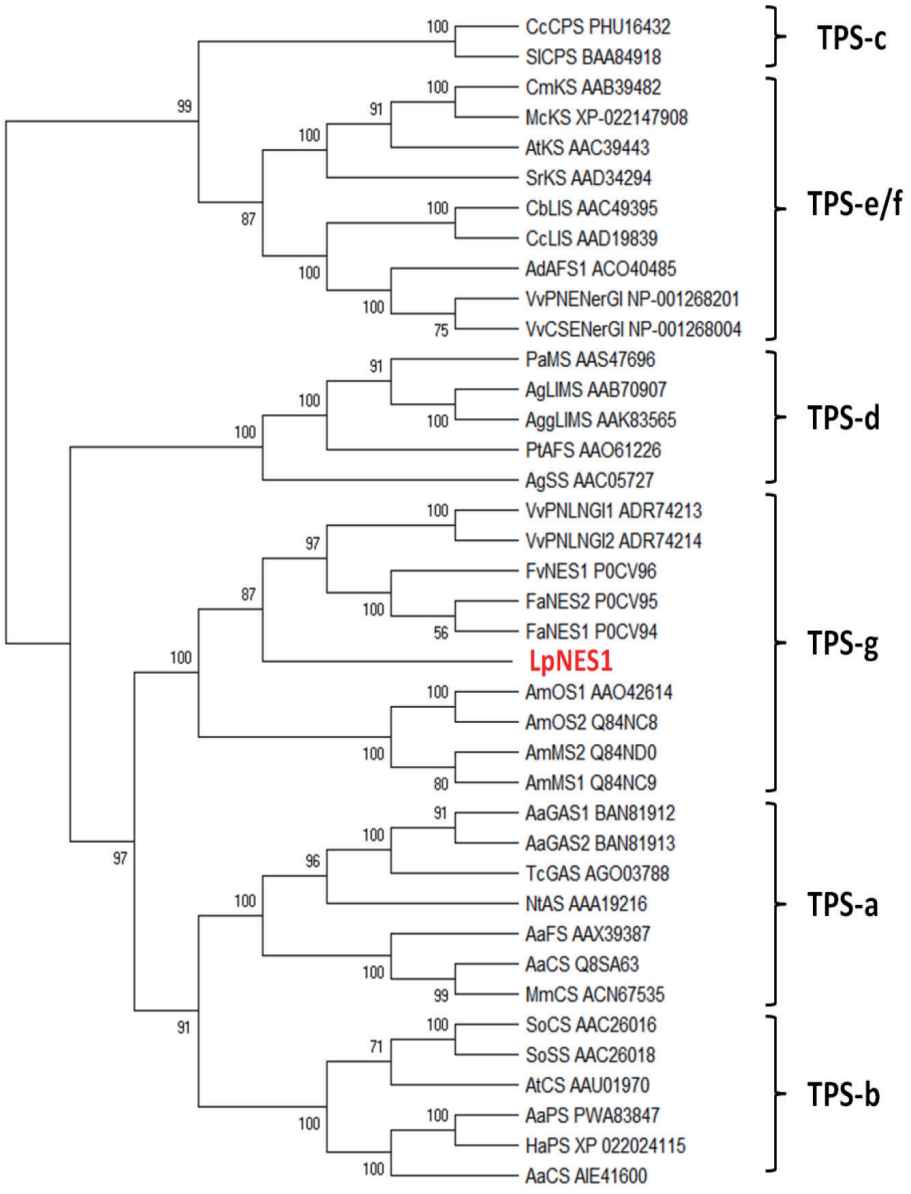

Fig. 2 Phylogenetic analysis of LpNES1 and other plant-identified and/or functionally-annotated TPSs. Information on TPSs was listed in supplement (Table S2).

pressing LpNES1-GFP (Fig. 3), indicating *LpNES1* gene was targeted to the cytosol.

3.3 *In vitro* biochemical characterization of recombinant LpNES1 revealed its bifunction

It is difficult to accurately predict the products profiles of a candidate TPS enzyme only by protein sequence analysis[23]. Therefore, to determine the catalytic function of LpNES1, enzymatic assay experiment was conducted. The purified LpNES1 protein was detected in SDS-PAGE (Fig. S2). GPP, FPP, and GGPP were used as substrates for enzymatic assay *in vitro*, and products were analyzed by SPME-GC-MS. When incubated with GPP, LpNES1 protein could form the linalool as its main compound (Fig. 4A); when incubated with FPP, LpNES1 protein could form the (*E*)-nerolidol as its principal product, accompanied with minor β-farnesene (Fig. 4C). LpNES1 protein did not accept GGPP as a substrate for no terpenoids were generated (Fig. S3), and no terpenoids were detected in control assays. The major products of the recombinant LpNES1 exhibited identical retention time and mass spectrum to the linalool and nerolidol standard (Figs. 4B and 4D).

3.4 Formation of sesquiterpenes exclusively by LpNES1 in yeast

To further investigate whether LpNES1 could produce

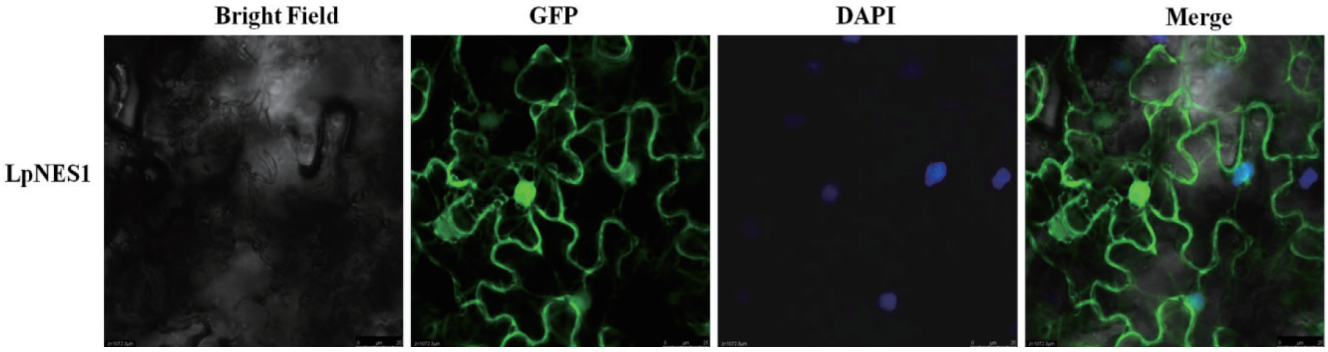

Fig. 3 Subcellular localization of LpNES1 in *N. benthamiana*. Confocal image of *N. benthamiana* leaves infiltrated with *Agrobacterium* harboring the plasmid 1301-LpNES1-GFP. Bright field image, GFP channel image, DAPI image, and Merged image. Scale bar, 25 μm.

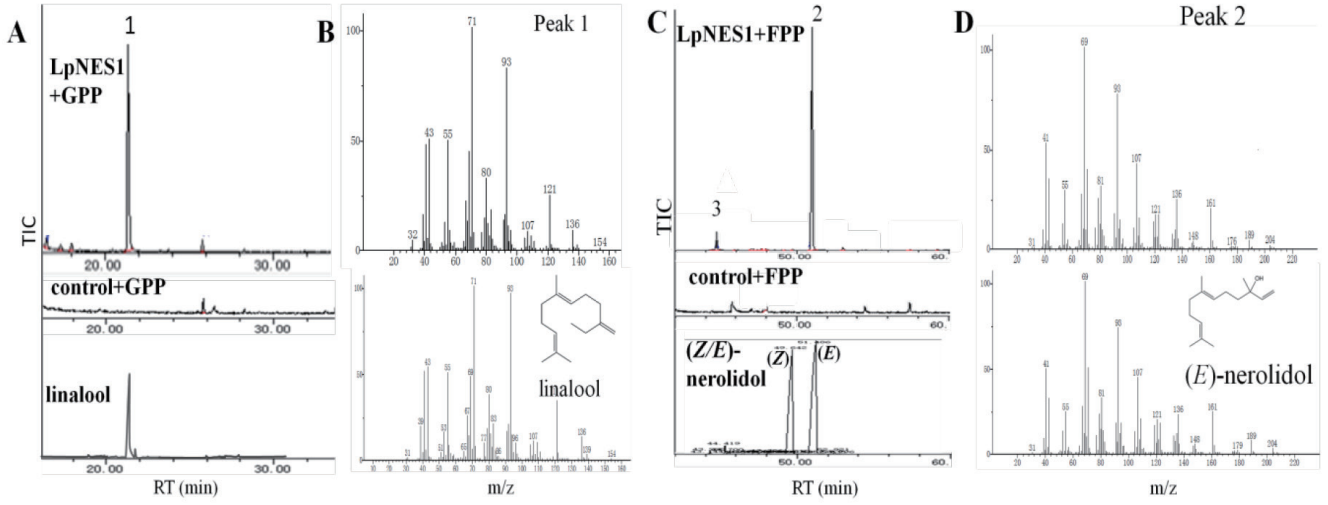

Fig. 4 GC-MS analysis of reaction products catalyzed *in vitro* by purified LpNES1. Reaction products were achieved by incubation of LpNES1 and control (heat-inactive LpNES1) with GPP (A) and FPP (C) as substrate. Peaks were identified and confirmed by the mass spectra reference library and authentic standard including linalool and (*E*)-nerolidol. The mass spectrums of peak 1 and peak 2 were identical to that of the standard linalool (B) and (*E*)-nerolidol (D) respectively; peak 3 was *β*-farnesene which was identified by mass spectra reference library. TIC, total ion chromatogram; RT, retention time; *m/z*, mass-to-charge ratio.

the same terpene products in eukaryote, we introduced the *OP-LpES1* (codon-optimized *LpNES1*) gene into *S. cerevisiae* BY4742. Given the Gal80p was a negative regulatory factor that could repress the *GAL* promoter[24], we disrupted the *GAL80* gene by integrating the expression cassettes of *OP-LpNES1* at this loci. Initially, linalool was not detected by SPME-GC-MS (data not shown). This might be attributed to the fact that the pool of GPP in yeast is too small, for GPP was an intermediate compound for FPP synthesis[25]. To improve the pool of GPP, the mutant *ERG20*^{WW} (*F96W-N127W*) gene, the encoding protein of which showed more efficient in biosynthesis of GPP[26] was coexpressed with *OP-LpNES1* under the control of *Gal1,10* bidirectional promoter in strain 4742-02. Surprisingly, compare to strain 4742-01 (control), only sesquiterpenes including (*E*)-nerolidol, and minor *β*-farnesene were released from strain 4742-02, and still no monoterpenes were detected (Fig. 5A). In order to avoid the errors caused by volatility of linalool, we further detected the products captured by dodecane from liquid fermentation, which got the same results (Fig. S4).

3.5 Expression analysis of *LpNES1* gene

Biosynthesis and emission of volatile terpenoids from specific plant tissues often correlate with the spatial expression of their terpene synthases[27]. In order to investigate the spatial regulation patterns of *LpNES1* expression in *L. pterodonta*, relative gene expression analysis was carried out in various tissues. The results showed that the expression level of *LpNES1* was significantly predominant in floral buds and leaves, approximately 300- and 180-fold higher than that in roots respectively (Fig. 5B).

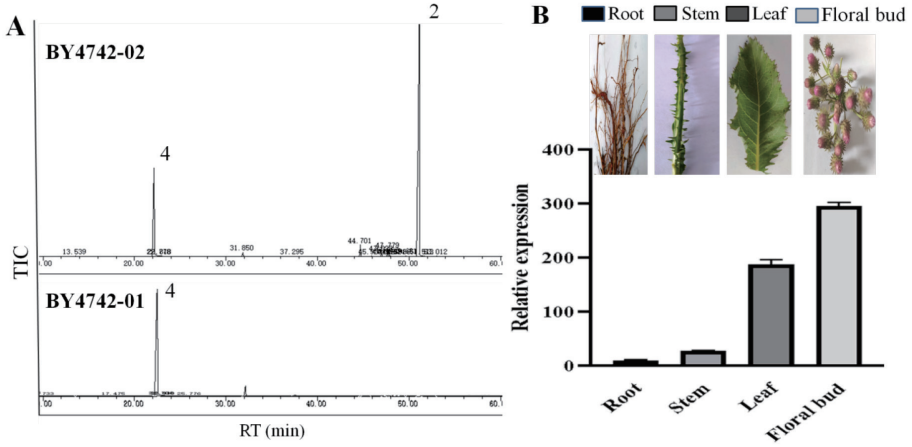

Fig. 5 (A) GC-MS analysis of the terpene products produced by BY4742-02 expressing OP-LpNES1 and ERG20^{WW} and control (BY4742-01). Peak 4 was phenylethyl alcohol; (B) qRT-PCR analysis of LpNES1 in four different tissues including the root, stem, leaf, and floral bud. Amplification of 18S ribosomal RNA gene was applied as an internal control. The value represents the means of the results, which were generated via six samples: three biological replicates and three technical replicates and standard error were shown.

4 Discussion

LpNES1, characterized in this study, belongs to TPS-g clade proteins. One prevalent property of these proteins is their acyclic products such as linalool, (*E*)-nerolidol, and geranyllinalool, which is attributed to lack of RRx8W motif shared by them[28]. Moreover, the members from TPS-g clade are more promiscuous than conventional terpene synthases in regard of substrates, for two or more of prenyldiphosphate precursors including GPP, FPP, and/or GGPP can be converted into linalool, nerolidol, and/or geranyllinalool[29, 30]. This characteristic is similar to that of an aromatic prenyltransferase AtaPT from *Aspergillus terreus*, which could accept three prenyldiphosphate precursors (DMAPP, GPP, FPP). The study also revealed the residue G326, the short hydrophobic side chain of which contributes to enlarge substrate binding pocket, was key for AtaPT to accept prenyl diphosphates with longer chain such as GPP and FPP[31]. We, therefore, speculated that enzymes of TPS-g family might possess a spacious active-site cavity that allows for accommodating substrates of varying size. To shed light on the catalytic mechanism of linalool/nerolidol synthases with different substrates and the determinants of substrate specificity, further work with protein crystal structure will be badly needed.

Generally, FPP biosynthesis through MVA pathway is known to happen in the cytosolic compartment in plants[9]. However, it has also been revealed that a small pool of GPP exists in the cytoplasm of plants[11, 12]. Similar to that of plant cell, in the cytosol of yeast, FPP is the major component synthesized by ERG20 with a minor component of GPP released through the same pathway[32]. Thus, whether a small pool of cytosolic GPP can be encountered by linalool/nerolidol synthases like LpNES1 and converted into linalool *in vivo* is interesting to explore. Several studies demonstrate that cytosolic linalool/nerolidol synthase are only responsible for biosynthesis of (*E*)-nerolidol when expression in recombinant yeast or in plants[33–35]. The same result was obtained in our study that only FPP was applied by LpNES1 for (*E*)-nerolidol generation in yeast. In fact, previous study demonstrated that *S. cerevisiae* cell harboured enough free GPP that could be catalytically converted into monoterpenes through introducing monoterpene synthases[36]. Moreover, ERG20^{WW} was overexpressed under the bidirectional *GAL1,10* promoter to further improve the pool of GPP in yeast. Thus, we can say, LpNES1 exhibited preference to FPP than GPP. Combined with cytosolic location, LpNES1 was suggested to be responsible for (*E*)-nerolidol synthesis exclusively in *L. pterodonta*.

MeJA is often used experimentally as mimic of herbivory or abiotic stress to elicit the expression of many defense genes and the production of defense compounds[37, 38]. The *LpNES1* gene was obviously up-regulated by the MeJA treatment, indicating its significant role in plant defense. (*E*)-nerolidol is ubiquitous compound in floral aroma of flowers, involved in attracting pollinators[39]. Together with 4,8-dimethylnona-1,3,7-triene (DMNT), a degraded derivative from (*E*)-nerolidol, they are emitted from plant leaves in response to stimuli and exert defensive activities in a direct or indirect way[40, 41]. Thus, LpNES1 might be involved in ecological interactions against abiotic stress or biotic factors through regulating the biosynthesis of (*E*)-nerolidol.

5 Conclusion

In our study, a new terpene synthase (LpNES1) was first identified from *L. pterodonta* (Asteraceae). Functional characterization *in vitro* and demonstrated that LpNES1 was a bifunctional linalool/nerolidol synthase. Further investigation in yeast indicated that LpNES1 possessed a robust activity for (*E*)-nerolidol generation whereas no linalool formation. Gene expression patterns showed that *LpNES1* was expressed prominently in floral buds and leaves. The Lp*NES1* gene identified in our study can be applied for further (*E*)-nerolidol production in microbes through metabolic engineering strategy.

Authors' Contribution

MLW and XLH conceived and designed the research, and revised the manuscript. ZKS performed the experiment and wrote the draft manuscript. XWG performed the analysis of samples. JYZ and MGL provided suggestions on the process of the experiment.

Acknowledgement

This work was partially supported by the National Natural Science Foundation of China (Grant No.: 31760189).

Conflict of Interest

The authors declare no conflicts of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21172

References

1) Getahun, T.; Sharma, V.; Gupta, N. The genus *Laggera* (Asteraceae) ethnobotanical and ethnopharmacological information, chemical composition as well as biological activities of its essential oils and extracts: A review. *Chem. Biodivers.* **16**, e190013 (2019).
2) Shang, X.; Liabsuetrakul, T.; Sangsupawanich, P.; Xia, X.; He, P.; Cao, H.; McNeil, E. Efficacy and safety of *Laggera pterodonta* in children 3-24 months with acute bronchiolitis: A randomized controlled trial. *Clin. Respir. J.* **11**, 296-304 (2017).
3) Wang, Y.; Zhou, B.; Lu, J.; Chen, Q.; Ti, H.; Huang, W.; Li, J.; Yang, Z.; Jiang,Z.; Wang, X. Inhibition of influenza virus via a sesquiterpene fraction isolated from *Laggera pterodonta* by targeting the NF-κB and p38 pathways. *BMC Complement. Altern. Med.* **17**, 25-32 (2017).
4) Usman, L.A.; Ismaeel, R.O. Chemical composition and antioxidant potential of fruit essential oil of *Laggera pterodonta* (DC.) Benth grown in north central, Nigeria. *J. Biol. Act. Prod. Nat.* **10**, 405-410 (2020).
5) Zeng, Z.Y.; Li, Q.Q.; Huo, Y.Y.; Chen, C.J.; Duan, S.S.; Xu, F.R.; Cheng, Y.X.; Dong, X. Inhibitory effects of essential oils from Asteraceae plant against pathogenic fungi of *Panax notoginseng*. *J. Appl. Microbiol.* **130**, 592-603 (2021).
6) Gu, J.L.; Njateng Guy, S.S.; Li, Z.J.; Zhang, H.X.; Dai, Q.; Du, Z.Z. Chemical composition and antimicrobial activities of essential oils of *Laggera pterodonta* collected in four different locations in Yunnan, China. *Plant Div.* **36**, 116-122 (2014).
7) McGarvey, D.J.; Croteau, R. Terpenoid Metabolism. *Plant Cell* **7**, 1015-1026 (1995).
8) Bohlmann, J.; Meyer-Gauen, G.; Croteau, R. Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 4126-4133 (1998).
9) Aharoni, A.; Jongsma, M.A.; Bouwmeester, H.J. Volatile science? Metabolic engineering of terpenoids in plants. *Trends Plant Sci.* **10**, 594-602 (2005).
10) Aharoni, A.; Giri, A.P.; Deuerlein, S.; Griepink, F.; de Kogel, W.J.; Verstappen, F.W.; Verhoeven, H.A.; Jongsma, M.A.; Schwab, W.; Bouwmeester, H.J. Terpenoid metabolism in wild-type and transgenic Arabidopsis plants. *Plant Cell* **15**, 2866-2884 (2003).
11) Wu, S.; Schalk, M.; Clark, A.; Miles, R.B.; Coates, R.; Chappell, J. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nat. Biotechnol.* **24**, 1441-1447 (2006).
12) Dong, L.; Miettinen, K.; Goedbloed, M.; Verstappen, F.W.; Voster, A.; Jongsma, M.A.; Memelink, J.; Van Der Krol, S.; Bouwmeester, H.J. Characterization of two geraniol synthases from *Valeriana officinalis* and *Lippia dulcis*: Similar activity but difference in subcellular localization. *Metab. Eng.* **20**, 198-211 (2013).
13) Ignea, C.; Pontini, M.; Maffei, M.E.; Makris, A.M.; Kampranis, S.C. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphosphate synthase. *ACS Synth. Biol.* **3**, 298-306 (2014).
14) Ho, T.T.; Murthy, H.N.; Park, S.Y. Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *Int. J. Mol. Sci.* **21**, 716 (2020).
15) Gao, W.; Meng, Q.; Luo, H.; Chen, F.; Zhou, Y.; He, M. Transcriptional responses for biosynthesis of flavor

volatiles in methyl jasmonate-treated *Chrysanthemum indicum* var. *aromaticum* leaves. *Ind. Crops Prod.* **147**, 112254 (2020).
16) Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882 (1997).
17) Zuo, S.S.; Wang, Y.C.; Zhu, L.; Zhao, J.Y.; Li, M.G.; Han, X.L.; Wen, M.L. Cloning and characterization of a ginsenoside-hydrolyzing *alpha*-L-arabinofuranosidase, CaAraf51, from *Cellulosimicrobium aquatile* Lyp51. *Curr. Microbiol.* **77**, 2783-2791 (2020).
18) Gietz, R.D.; Schiestl, R.H. Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2**, 38-41 (2007).
19) Williams, D.C.; McGarvey, D.J.; Katahira, E.J.; Croteau, R. 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 (1998).
20) Starks, C.M.; Back, K.; Chappell, J.; Noel, J.P. Structural basis for cyclic terpene biosynthesis by tobacco 5-*epi*-aristolochene synthase. *Science* **277**, 1815-1820 (1997).
21) Christianson, D.W. Structural biology and chemistry of the terpenoid cyclases. *Chem. Rev.* **106**, 3412-3442 (2006).
22) Degenhardt, J.; Köllner, T.G.; Gershenzon, J. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry* **70**, 1621-1637 (2009).
23) Wei, G.; Tian, P.; Zhang, F.; Qin, H.; Miao, H.; Chen, Q.; Hu, Z.; Cao, L.; Wang, M.; Gu, X.; Huang, S.; Chen, M.; Wang, G. Integrative analyses of nontargeted volatile profiling and transcriptome data provide molecular insight into VOC diversity in Cucumber plants (*Cucumis sativus*). *Plant Physiol.* **172**, 603-618 (2016).
24) Xie, W.; Liu, M.; Lv, X.; Lu, W.; Gu, J.; Yu, H. Construction of a controllable β-carotene biosynthetic pathway by decentralized assembly strategy in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **111**, 125-133 (2014).
25) Anderson, M.S.; Yarger, J.G.; Burck, C.L.; Poulter, C.D. Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 19176-19184 (1989).
26) Zhao, J.; Bao, X.; Li, C.; Shen, Y.; Hou, J. Improving monoterpene geraniol production through geranyl diphosphate synthesis regulation in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **100**, 4561-4571 (2016).
27) Nagegowda, D.A. Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. *FEBS Lett.* **584**, 2965-2973 (2010).
28) Dudareva, N.; Martin, D.; Kish, C.M.; Kolosova, N.; Gorenstein, N.; Fäldt, J.; Miller, B.; Bohlmann, J. (*E*)-beta-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. *Plant Cell* **15**, 1227-1241 (2003).
29) Martin, D.M.; Aubourg, S.; Schouwey, M.B.; Daviet, L.; Schalk, M.; Toub, O.; Lund, S.T.; Bohlmann, J. Functional annotation, genome organization and phylogeny of the grapevine (*Vitis vinifera*) terpene synthase gene family based on genome assembly, FLcDNA cloning, and enzyme assays. *BMC Plant Biol.* **10**, 226 (2010).
30) Richter, A.; Schaff, C.; Zhang, Z.; Lipka, A.E.; Tian, F.; Köllner, T.G.; Schnee, C.; Preiß, S.; Irmisch, S.; Jander, G.; Boland, W.; Gershenzon, J.; Buckler, E.S.; Degenhardt, J. Characterization of biosynthetic pathways for the production of the volatile homoterpenes DMNT and TMTT in *Zea mays*. *Plant Cell* **28**, 2651-2665 (2016).
31) Chen, R.; Gao, B.; Liu, X.; Ruan, F.; Zhang, Y.; Lou, J.; Feng, K.; Wunsch, C.; Li, S.M.; Dai, J.; Sun, F. Molecular insights into the enzyme promiscuity of an aromatic prenyltransferase. *Nat. Chem. Biol.* **13**, 226-234 (2017).
32) Fischer, M.J.; Meyer, S.; Claudel, P.; Bergdoll, M.; Karst, F. Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol. Bioeng.* **108**, 1883-1892 (2011).
33) Nagegowda, D.A.; Gutensohn, M.; Wilkerson, C.G.; Dudareva, N. Two nearly identical terpene synthases catalyze the formation of nerolidol and linalool in snapdragon flowers. *Plant J.* **55**, 224-239 (2008).
34) Green, S.A.; Chen, X.; Nieuwenhuizen, N.J.; Matich, A.J.; Wang, M.Y.; Bunn, B.J.; Yauk, Y.K.; Atkinson, R.G. Identification, functional characterization, and regulation of the enzyme responsible for floral (*E*)-nerolidol biosynthesis in kiwifruit (*Actinidia chinensis*). *J. Exp. Bot.* **63**, 1951-1967 (2012).
35) Li, W.; Yan, X.; Zhang, Y.; Liang, D.; Caiyin, Q.; Qiao, J. Characterization of *trans*-nerolidol synthase from *Celastrus angulatus* Maxim and production of *trans*-nerolidol in engineered *Saccharomyces cerevisiae*. *J. Agric. Food Chem.* **69**, 2236-2244 (2021).
36) Herrero, O.; Ramon, D.; Orejas, M. Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. *Metab. Eng.* **10**, 78-86 (2008).
37) Ho, T.T.; Murthy, H.N.; Park, S.Y. Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *Int. J.*

Mol. Sci. **21**, 716-733(2020).
38) Zulak, K.G.; Bohlmann, J. Terpenoid biosynthesis and specialized vascular cells of conifer defense. *J. Integr. Plant Biol.* **52**, 86-97(2010).
39) Shields, V.D.; Hildebrand, J.G. Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J. Comp. Physiol. A* **186**, 1135-1151(2000).
40) Kappers, I.F.; Aharoni, A.; Van Herpen, T.W.; Luckerhoff, L.L.; Dicke, M.; Bouwmeester, H.J. Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. *Science* **309**, 2070-2072(2005).
41) Liu, G.; Yang, M.; Fu, J. Identification and characterization of two sesquiterpene synthase genes involved in volatile-mediated defense in tea plant(*Camellia sinensis*). *Plant Physiol. Biochem.* **155**, 650-657(2020).