Gene co-expression modulating terpene metabolism is associated with plant anti-herbivore defence during initial flowering stages

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Abstract

To protect themselves from herbivore attacks, especially during early developmental stages, plants produce volatiles possessing various ecological and physiological functions. Here, we identified three genes cloned from Lavandula angustifolia. These genes, designated as LaTPS7, LaTPS8, and LaCYP71D582, were hypothesized to be active in plant defense during early developmental stages. The in-vitro assays showed LaTPS7 produced eight compounds including camphene, myrcene, limonene etc. and LaTPS8 catalyzed nine volatiles from α-pinene, sylvestrene to fenchol etc. using geranyl diphosphate (GPP) and nerolidyl diphosphate (NPP) as substrate separately. However, LaTPS7 present in plastids only, synthesized limonene within Nicotiana benthamiana. Limonene was then converted into carveol by LaCYP71D582 present in the endoplasmic reticulum. LaTPS8, also located in plastids, synthesized α-pinene and sylvestrene. Odour response of aphids (Myzus persicae) and ladybugs (Harmonia axyridis) showed that volatiles from transgenic tobacco leaves repelled aphids and attracted ladybugs. LaTPS7 promoter GUS stain assay in Arabidopsis thaliana showed that LaTPS7 displayed a wound-induced expression in leaves. Together, our findings show that these compounds and gene-expression pattern played important roles in protecting plants during vulnerable stages. More practically, these plant tactics can be exploited in agriculture to decrease the use of insecticides, thereby contributing to improved human and environmental health.

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Abstract

To protect themselves from herbivore attacks, especially during early developmental stages, plants produce volatiles possessing various ecological and physiological functions. Here, we identified three genes cloned from *Lavandula angustifolia* . These genes, designated as*LaTPS7* , *LaTPS8* , and *LaCYP71D582* , were hypothesized to be active in plant defense during early developmental stages. The*in-vitro* assays showed*La* TPS7 produced eight compounds including camphene, myrcene, limonene etc. and *La* TPS8 catalyzed nine volatiles from*α* -pinene, sylvestrene to fenchol etc. using geranyl diphosphate (GPP) and nerolidyl diphosphate (NPP) as substrate separately. However,*La* TPS7 present in plastids only, synthesized limonene within*Nicotiana benthamiana* . Limonene was then converted into carveol by*La* CYP71D582 present in the endoplasmic reticulum. *La* TPS8, also located in plastids, synthesized *α* -pinene and sylvestrene. Odour response of aphids (*Myzus persicae*) and ladybugs (*Harmonia axyridis*) showed that volatiles from transgenic tobacco leaves repelled aphids and attracted ladybugs. *LaTPS7* promoter GUS stain assay in*Arabidopsis thaliana* showed that *LaTPS7* displayed a wound-induced expression in leaves. Together, our findings show that these compounds and gene-expression pattern played important roles in protecting plants during vulnerable stages. More practically, these plant tactics can be exploited in agriculture to decrease the use of insecticides, thereby contributing to improved human and environmental health.

Key words : lavender, monoterpenes, terpene synthase, cytochrome P450, insects plant-herbivore-natural enemy tritrophic interaction

Introduction

Plants interact with the environment by releasing various volatiles that possess physiological and ecological functions (O'Connor, 2015). The theory of herbivore-induced plant volatiles (HIPVs), proposed by Ehrlich and Raven (1964), states that plants release various volatiles to communicate and defend against herbivore attack. Thereafter, studies have been increasingly focused on relationships between plant compounds and insects. A recent report showed that the cottons emitting increased levels of volatile compounds have fewer eggs of *Helicoverpa armigera* (Liu *et al.* 2018), suggesting that volatiles play important roles in protecting plants from pests and enhancing plant survival. Indeed, numerous plant-derived volatile compounds, particularly terpenes and terpenoids, are documented to protect plants. For example, farnesene protects plants from aphids (*Myzus persicae*) because it acts as an alarm pheromone and attracts the predatory ladybugs (*Harmonia axyridis*) (Francis *et al.*2004; Zhu *et al.* 2005). Another common compound in flowers and fruits is linalool, which has studied in *Arabidopsis thaliana* , indicating it attracts thrips; however, oxides of linalool, catalysed by CYP71D, can repel these insects (Boachon *et al* . 2015). Meanwhile, HIPVs, including mint-derived volatiles such as limonene, 1,8-cineole, and carvone, can also attract species that predate on herbivores, such as predatory mites that feed on two-spotted spider mites (Togashi *et al.* 2019). Therefore,

volatile compounds may be part of a defence system used directly or indirectly by plants to protect themselves via tritrophic interactions (Meena *et al* . 2017). Furthermore, as they deter herbivores, these plant volatiles are likely expressed in higher concentrations during early stages of development than in mature stages and tissues (Meena *et al.* 2017); thereafter, expression of these compounds is maintained at a constant level or reduced(Niederbacher *et al* . 2015). This shows that plants dynamically regulate expression of metabolites in order to adapt to the environment and reproduce.

Most HIPVs are terpenes/terpenoids and are biosynthesized via two pathways. The first is the mevalonate (MVA) pathway, which occurs in the cytosol/endoplasmic reticulum, and leads to the formation of farnesyl diphosphate (FPP) as a precursor to sesquiterpenoids. The other is 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, occurring in plastids; this pathway generates geranyl diphosphate (GPP) as a precursor to monoterpenoids, catalysed by the associated terpene synthases (TPSs) (McGarvey and Croteau, 1995). These TPSs are a mid-sized gene family in plants, consisting of TPS-a, -b, -c, -d, -e/f, and -g (Chen *et al* . 2011).

Recently, neryl diphosphate (NPP), which is an isomer of GPP, and (Z,Z)-FPP, were shown to be precursors to several terpenes in the tomato plant (Schilmiller*et al.* 2009; Akhtar *et al* . 2013). This indicates that terpene/terpenoid biosynthesis needs to be further investigated in various plant species. Moreover, the structure and function of terpenes that are post-processed by co-expressed genes have been extensively studied in the peppermint (*Mentha* x *piperita*) (Rodney*et al* . 2005), *Artemisia annua* L. (Teoh *et al* . 2006), and *A. thaliana* (Boachon *et al* , 2019). However, few studies have examined these compounds in the lavender.

Lavender (*Lavandula angustifolia*) is an important aromatic plant generating as many as 70 volatile metabolites, including limonene,α- pinene, linalool. Lavender has also been proposed as a model to study the regulation of terpene biosynthesis (Guitton *et al.*2010a). Although lavender metabolism has been investigated for several years, enzymes, such as 3-carene synthase, fenchol synthase, α -pinene synthase, andβ -phellandrene synthase are mainly identified using*in-vitro* assays (Demissie ZA*et al* . 2011; Benabdelkader*et al* . 2014; Adal AM *et al* . 2017). In addition, terpenes synthesized by TPSs are potential to be transported into endoplasmic reticulum where these compounds are converted by cytochrome P450 (CYP) into their respective derivatives (Karunanithi and Zerbe 2019). This enables plants to increase the expression of metabolites that can perform multiple functions. Although the CYP enzyme family, which accounts for 1% of the genome in most plants (Nelson and Werck-Reichhart 2011), has not been functionally identified in the lavender, 30 unique CYPs genes were predicted using sequence tags (ESTs) (Lane et al. 2010).

Physiological functions of volatiles and their genes within tritrophic interactions in lavenders remain elusive. Previous studies have described volatile organic compound (VOC) content during inflorescence ontogeny, and revealed that the terpenes involved fell into three groups identifiable via three developmental phases of flowering. 3-carene, limonene, myrcene, bornyl acetate, borneol, camphor, 1,8-cineol, and *trans* -ocimene belonged to the first group and may play a protective role in repelling damaging insects (Guitton*et al* . 2010b). Previously, we found that aphids and ladybugs were dominant insects in lavender fields during early spring in Beijing. This suggests probable tritrophic interaction among predators, preys and volatiles (Li *et al.*2019*)* . Y-tube olfactometer experiments showedβ -trans-ocimene and (+)-R-limonene get rid of 74.71% and 78.41% aphids (Li *et al.* 2019).

In this study, we investigated the tactics used by the lavender plant to defend against herbivore attack during immature developmental stages and in young plant tissues.

MATERIALS AND METHODS

Plants and insects

Lavender (*L. angustifolia*) plants were harvested at different developmental stages from a field belonging to the Institute of Botany, Chinese Academy of Sciences, China. The three distinct stages were as follows: B, bud stage: the petals were completely closed and green; I, blossom stage: the 60% of flowers had opened and

petals turned purple; II, fade stage: the petals had shrunk and were ready to fall. Four-week-old tobacco (*N icotiana benthamiana*) and*A. thaliana* were grown in a green-house at 25°C under a 16/8h light and dark photoperiod (200 μmol $m^{-2}s^{-1}$).

Aphids (*Myzus persicae*) and ladybugs (*Harmonia axyridis*) were kindly provided by Prof. Su Wang (Beijing Academy of Agricultural and Forestry Sciences) and maintained in a growth chamber (30×30×25 cm) at 25°C with a L16:D8 photoperiod feeding on tobacco plant.

Clone and quantification expression analysis of *LaTPS7*,*LaTPS8* and *LaCYP71D582*

Glandular trichomes are tissues located in metabolism-specific sites (Tissier*et al* . 2017). Total RNAs were extracted from glandular trichomes of flowers and leaves in different developmental stages and reverse transcribed using oligo d(T) primers following the manufacturer's directions (TSINGKE, China). After obtaining cDNA, target genes were amplified using cloning primer and Phanta Max Polymerase (Vazyme, China). To clone full-length open reading frames (ORFs), we used a PCR program at the following settings: 95°C for 5 min; 35 cycles at 95°C for 2 min; 58°C for 30 s and 72°C for 2 min; and final extension at 72°C for 5 min. The qRT-PCR was conducted using 2 × T5 Fast qPCR Mix (SYBR Green II) (TSINGKE, China) on an Mx3000P system (Agilent Stratagene), according to the manufacturer's instructions and the following settings: 95°C for 1 min, followed by 40 cycles of 10 s at 95°C, then 5 s at 55°C, and 15 s at 72°C. Leaf trichomes at the blossoming stage were used as controls. Normalised expression values (2^{-T}) were calculated according to the expression of 18S rRNA used as reference gene. The primers used in this part of our study are shown in table S1.

Sequence analysis of target genes

The full amino acid sequences translated from ORFs (primers are provided in table S1) were used for multiple sequence alignent performing on an ESPript (http://espript.ibcp.fr/ESPript/ESPript/) as described previously (Robert *et al.* 2014). Phylogenetic trees were constructed by MEGA7 using the Neighbor-Joining method, and Interactive Tree of Life (i*TOL*) was used for visual editing (http://itol2.embl.de/) (Letunic*et al.* 2007). Analysis of signal peptides in TPSs, and prediction localization of candidate proteins were performed on Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) and signal P (http://www.cbs.dtu.dk/services/SignalP-4.1/) as described previously (Kelley *et al.*2015). Protein secondary structure was predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER).

Determination oftissue-specific gene expression in *A. thaliana*

Approximately 1.5 kb of a promoter for each gene was isolated by FPNI-PCR from genomic DNA using three specific gene primers and three arbitrary primers and PCR settings described by Wang *et al.* (2011). Resulting sequences were analysed on PlantCARE (bioinformatics.psb.ugent.be/webtools/plantcare/html) (Rombauts *et al.* 1999) and then integrated into pCXGUS-P vector harbouring a cloning cassette upstream of *GUS*(*uidA*). After the constructs were confirmed via sequencing, they were introduced into GV3101*Agrobacterium* , which was used to transform *A. thaliana* using a floral dip method described previously (Clough *et al.* 1998). Transformed lines were selected on MS plates containing 50 μg·mL^{-1}hygromycin, and were then confirmed via PCR. Enzymatic assay using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (GUS) was performed according to the method of Jefferson *et al.* (1987) to assess candidate genes tissue-specific expression.

Subcellular localization of the *La*TPS7, *La*TPS8, and *La*CYP71D582 in *N. benthamiana*

GV3101 with eGFP expression vectors controlled by a mannopine synthase promoter (MAS), constructed using a Trelief SoSoo Cloning Kit (TSINGKE, China), were used for transient transformation of 4-week-old*N. benthamiana* plant as described by Jin *et al.* (2015). After 3 days of infiltration, *N. benthamiana* leaves were excised, mounted on slides, and analyzed using a confocal laser-scanning microscope equipped with a standard filter set (Leica TCS SP5). Images were processed by Image J (https://imagej.nih.gov/ij).

Heterologous expression of TPSs in *Escherichia coli* and enzymatic assay*in vitro*

TPS sequences of truncated predicted signal peptide were inserted into a pDE2 vector with a recombinant C-terminal and poly-histidine tag using primers shown in table S1 and a pDE2 Directional Expression Kit Ver.2 (TSINGKE, China). The final constructs was transformed into *E. coli* BL21 (DE3); recombinant proteins were subjected to isopropyl-β-D-thiogalactopyranoside (IPTG) induction at the concentration of 1.0 mM until OD_{600} reached 0.6˜0.8, and then to purification using a His-Tagged Gravity Column (Merck Millipore). The purified proteins were assayed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to ensure that the proteins had been extracted successfully.

The *in-vitro* enzymatic assay for TPS activity was performed according to the method of Chen *et al.* (2011) in a final volume of 500 µL buffer [25mM HEPES, pH 7.3; 10 mM $MgCl_2$; 10 mM $MgCl_2$; 10% glycerol; 10 mM DTT], approximately 20 µL pure protein, and 10 µg of either FPP, NPP, or GPP (Sigma-Aldrich). The mixtures were vortexed, incubated at 30°C for 2 h, after which 250 µL hexane was added to each mixture and vortexed for 1 min. The upper layers were centrifuged at 1200 g and 4°C for 30 min, and then transferred into 2-mL glass vials for analysis via GC-MS. Heat-inactivated recombinant protein was used as negative control.

Transient expression of *La*TPS7, *La*TPS8, and*La*CYP71D582 in *N. benthamiana*

Leaves of *N. benthamiana* collected from 4-week-old plants were infiltrated with *Agrobacterium tumefaciens* GV3101 harboring target genes. Meanwhile, GV3101 was mixed with TPS and CYP at respective ratios of 1:1 when OD_{600} of each strain corresponded to 1.0. Leaf tissues were collected after 5 days of infiltration, examined under UV light to ensure successful agroinfiltration and gene expression, and then ground in liquid nitrogen. Leaf powder was stored at -80°C, and volatiles were extracted by vortexing in 70% alcohol (1:4 m/v). Extracts were maintained at -20°C and oscillated before analysis via solid-phase microextraction (SPME, Agilent). The samples were heated in a water bath at 40°C for 30 min, and then a fibre coated with polydimethylsiloxane and divinylbenzene was used to collect volatiles under the same conditions for 30 min according to a previously published method (Ilc *et al.* 2017). Volatiles were then analysed using GC-MS.

GC-MS Analysis

GC-MS analysis was performed via splitless injection using an Agilent 7890A GC system and Agilent Technologies 5975C Inert XL Mass Selective Detector equipped with an HP-5MS UI column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies). Injector temperature was 250°C. Temperature was initially held for 3 min at 40°C, then increased to 130°C at the rate of $10°C \cdot min^{-1}$, increased to 250°C at the rate of 50°C/min, and maintained for 10 min; helium gas at a flow of 1 mL/min was used as vector.

MS conditions were as follows: ionization energy, 70 eV; electronic impact ion source temperature, 200°C; quadrupole temperature, 150°C; and mass range, 35-550u. Products were identified based on their retention time and electron ionization mass spectra using mass spectra from the National Institute of Standards and Technology (NIST) Mass Spectral Library (NIST-14.0).

Insect behaviour

For aphids (*Myzus persicae*), a leaf disk bioassay was performed as described by Picard *et al* . (2012). Transformed *N. benthamiana* leaves were punched into disks of 1 cm in diameter. These disks were placed onto filter paper that was suitable for a Petri dish. Aphids, which had been starved for 3 h, were placed in these Petri dishes, and the number of insects on either control disks or treatment disks was recorded for 1 hour. Ninety aphids were tested in total. We then used a Y-Tube olfactometer to assess the influence of volatiles on ladybugs (*Harmonia axyridis*) that had been starved for 3 h. Sixty ladybugs were tested in total. Ladybugs entering into more than half-length of the arms of the olphactometer and lingering for at least 10 s were recorded as responders. Disks were exchanged for fresh disks after 10 aphids or ladybugs were tested.

Statistical analysis

The statistics of aphids was assessed by variance (ANOVA). Means were separated using Tukey's HSD test

at a significance level $p = 0.05$. As for ladybugs (*Harmonia axyridis*), the statistics was assessed by usingχ^2 test.

RESULTS

TPS and CYP clustered in phylogenetic tree

Two TPS genes having the sizes of 1803 and 1800 bp were obtained from the same primer, and were named *LaTPS7* and *LaTPS8* , respectively; *LaTPS7* and *LaTPS8* displayed an identical nucleotide sequence in the first 33 bp of N' and in the last 23 bp of C' (Figure S2). Phylogenetic trees based on deduced amino-acid sequences of each gene showed that these two genes belonged to the TPS-b subfamily, which consists of monoterpene synthases (Chen *et al.* 2011) (Figure 1a).*LaTPS7*showed the highest identity with*L. angustifolia* limonene synthase when searched via BLAST in NCBI, and*LaTPS8* showed highest identity with *L. peduncuiata*pinene synthase in a phylogenetic tree. Both of these proteins contained the conserved arginine-tryptophan (RRX_8W) motif in the N terminal, and an aspartate-rich (DDXXD) and (NSE/DTE) motif (which chelates divalent metal ions such as Mg^{2+} or Mn^{2+}) in the C terminal. Synthases need these motifs to cyclise terpene precursors such as GPP, NPP, or/and FPP (Figure S3, S4).

The CYP sequence was submitted to CYP450 nomenclature committee and designated as *LaCYP71D582* . The protein showing highest identity with *La* CYP71D582 was *P. barbatus Pb* CYP71D378 (Figure 1b), which functions in forskolin (Pateraki *et al.* 2017) with conserved motifs (A/G)GX(D/E)T(T/S), EXXR, and FXXGXRXCXG (Figure S5). Secondary protein structure with four membrane-spanning domains and active site is shown in supplementary figure S5. CYP clans, used to build the phylogenetic tree, were related to terpene metabolism (Nelson and Werck-Reichhart, 2011), and the candidate CYP was clustered into the CYP71 clan, which is the largest clan among the CYPs (Figure 1b). This result indicates that it is highly possible to catalyze terpenes (Nelson *et al.* 2004). These results indicate that *La* CYP71D582 may participate in terpene metabolism

Quantitative RT-PCR analysis of candidate-gene expression during budding stage

The expression level of *LaTPS7* , *LaTPS8* and*LaCYP71D582* were accessed via quantitative real-time PCR (qRT-PCR) during different periods of budding, blossoming and fading stages of glandular trichomes in leaves and flowers (Figure 2). *LaTPS7* , *LaTPS8* , and*LaCYP71D582* were highly expressed in the glandular trichomes of flower during budding stage, but showed low expression in other organs. Meanwhile, the expression levels of *LaTPS7* , *LaTPS8* , and*LaCYP71D582* decreased during blossoming and fading stage, showing a similar expression pattern, and indicating that the three genes perform similar functions during plant development (Figure 2). This expression pattern may provide a temporal basis for consecutive catalysis: products from TPSs may be further catalyzed by CYP.

Subcellular localization of the *La*TPS7, *La*TPS8, and *La*CYP71D582 via transient expression in tobacco

When *La* TPS7 and *La* TPS8 were predicted by Signal P, the signal peptide was not detected in the plastid. However, the signal was detected when we used Phyre2, and *La* CYP71D582 was predicted to localize on the membranes by both Signal P and Phyre2. Hence, experimental verification is particularly important when prediction of TPSs subcellular localization is conflicted. Thus, full-ORF fusion vectors enhanced with green fluorescent protein (eGFP) were constructed for each candidate gene, and injected into *N. benthaiana* leaves via *A. tumefaciens* -mediated transfection. Analysis of whole leaves via confocal microscopy showed that both *La* TPS7 and*La* TPS8 were associated with chloroplasts (Figure 3), whereas*La* CYP71D582 was located in the endoplasmic reticulum (ER) (Figure 3).

Isolation and*in-vitro* enzymatic characteristic of TPSs

To determine the function of each protein, six His-tagged TPS recombinant proteins, with truncated putative signal peptides in their amino acid sequences, were expressed and extracted from *E. coli* BL21 (DE3). These recombinant proteins were then tested with either GPP, NPP, or FPP (10 μg for each) used as

substrate. Our results show that *La* TPS7 catalysed GPP to produce seven monoterpenes corresponding to α -pinene, camphene, myrcene, limonene, terpinolene, linalool, and terpineol, and catalysed NPP toα -pinene, camphene, limonene, terpinolene, terpineol, and nerolidol. *La* TPS8 produced α -pinene, β -pinene, sylvestrene, linalool, fenchol, and geraniol from GPP, andα -pinene, limonene, terpinolene, terpineol, and nerolidol from NPP. However, no products were detected when FPP was used as substrate for both genes (Figure 4). These results indicate that these enzymes differ in their substrate recognition and product output.

Functional characterization of TPSs and CYP *in vivo*

To further identify the products of target proteins in plants,*LaTPS7*and *LaTPS8* were separately transiently transfected into tobacco leaves. Similarly,*LaCYP71D582*was co-expressed with either *LaTPS7* or *LaTPS8* to investigate the function of *LaCYP71D582* . Our results showed that*La* TPS7 produced only limonene within tobacco, whereas *La* TPS8 producedα- pinene and sylvestrene; these results are consistent with those of the *in-vitro* enzymatic assay (Figure 5a). Notably, carveol levels were assessed in tobacco plants co-expressing *LaTPS7* and*LaCYP71D582* , but no changes in caveol levels were observed in plants co-expressing *LaTPS8* -*LaCYP71D582* (Figure 5b). In other words, limonene could be hydroxylated at C6 by*La* CYP71D582 to form carveol (Figure 5c).

Promoter clone and tissue-specific expression by GUS strain in*A. thaliana*

Because *LaCYP71D582* and *LaTPS7* showed a relationship of successive catalysation, we further investigated the precise expression pattern of these genes. Thus, a 1299-bp promoter of *LaTPS7* and a 1434-bp promoter of*LaCYP71D582*were obtained via FPNI-PCR and designated as*Pro -LaTPS7*and *Pro -LaCYP71D582* . Both of these promoters not only contained the basic promoter elements, but also harboured several regulatory elements predicted by PlantCARE (Figure S6, S7). We then constructed *A. thaliana* lines transformed with each gene promoter-GUS fusion vectors. Strain assay of*LaTPS7*showed that it was expressed in flowers, siliques, trichomes and leaves. Its expression in leaves, in particular, exhibited a wound-induced pattern (Figure 6a). In other words,*LaTPS7* was only highly expressed in leaves after a leaf had been injured, similar to the response observed in leaves being attacked by herbivores. *LaCYP71D582* showed a constitutive expression pattern in flowers, siliques, trichomes, and leaves (Figure 6b). This response was similar to that of *LaTPS7* , but expression of *LaCYP71D582* did not show a wound-induced pattern.

Products of *La*TPS7 and *La*CYP71D582 protect plants against insects

To the best our knowledge, limonene repels most herbivores including aphids, as shown in our previous work (Li *et al.* 2019). However, how limonene and its derivatives affect interactions between aphids and ladybugs remains undetermined. Therefore, to determine how limonene and its derivatives affect the relationship between aphids and ladybugs, we used a leaf disk assay and a Y-Tube olfactometer to assess responses from aphids and ladybugs, respectively, to tobacco leaves transiently transfected with*LaTPS7*and co-transfected with*LaTPS7*and *LaCYP71D582* .

Our result show that tobacco leaves transfected with *La* TPS7, and leaves co-transfected with *La* TPS7 and *La* CYP71D582, repelled aphids at a rate of approximately 70% (Figure 7b). In contrast, *La* TPS7-expressing tobacco leaves attracted ladybugs at a rate of 48.33%, whereas leaves co-expressing *La* TPS7 and*La* CYP71D582 attracted ladybugs at a rate of 58.33% (Figure 7a). These results suggest that limonene and carveol were more attractive to ladybugs than limonene alone.

Discussion

Plants emit more volatiles during early stages of development than during mature stages because in order to grow and proliferate successfully, they must protect juvenile tissues, such as young leaves and buds, against herbivore attack (Meenaetal*et al* . 2017). This is consistent with our heat map of flower development (Figure S1), showing that various volatile compounds play significant roles in plant defence during early stages. Identification of gene function in the Japanese pepper (*Zanthoxylum piperitum*) (Fujita *et al.* 2017) and in*A. thaliana*(Boachon*et al.* 2019) also showed that genes participating in defence are mainly active during early stages of development and in young tissues of plants. This is a time period when plants need to

supply energy for growth and defensive strategies concurrently, which is difficult because each of these two pathways requires high energy expenditure (Meena et al *et al* . 2017). Collectively, these results indicate that genes and their products in the lavender bud stages likely function in plant defence.

Volatile compounds protect plants not only by repelling herbivores, but also by attracting the natural enemies of herbivores, forming a tritrophic interaction among plant-herbivores-carnivores. This provides means of natural biological control and may serve as a new approach to pest eradication, decreasing the use of insecticides, which are used excessively in modern agriculture and other industrial applications. Our results show that limonene and carveol, expressed in tobacco leaves, repel aphids and attract ladybugs. This has also been shown in soybeans (*Glycine max*) and mint (ZHU *et al* . 2005; Togashi *et al.* 2019). Additionally, limonene attracts predatory mites,*Phytoseiulus persimilis* and *Neoseiulus californicus*(Togashi *et al.* 2019) and repels western flower thrips (*Frankliniella occidentalis*) (Picard*et al* . 2012), indicating that limonene can defend a wide variety of plants (Smith *et al* . 2018). Combining carveol, pinene, and limonene is important in developing plant-derived insect repellents (Picard *et al.* 2012; Smith*et al.* 2018). However, other studies indicate that limonene promotes herbivore and pathogenic attack in the orange fruit (Rodrıguez*et al* . 2011). Moreover, *Musca domestica* L. catalyses limonene into carveol and carvone via endogenous P450, resulting in lower toxicity to flies (Rossi*et al* . 2013). This suggests that the same terpene may function differently among different species; this needs to be investigated in future studies.

Terpenes or terpenoids in plants are mainly catalysed by terpene synthases (TPSs). The sequences and functions of TPSs are redundant, causing difficulty in annotation and assignment even in *Gossypium*genomes (Huang *et al* . 2018). The similarity between*LaTPS7*and *LaTPS8* is quite high, showing 77.61% identity; correspondingly, the products of these genes are highly redundant *in vitro.*However, both of *LaTPS7* and *LaTPS8* can catalyze GPP and NPP into various products, which shows the promiscuous and multi-substrate characteristics of TPSs (Major, 2012). Although most monoterpene synthases, including *La* TPS7 and*La* TPS8, are located in the plastids, exceptions exist and can be located in the mitochondria and cytosol *(* Yamaga *et al* . 1993; Aharoni *et al* . 2004; Lee & Chappell, 2008; Magnard*et al.* 2015). This suggests that there is a complex metabolic network of terpenes. Additionally, *La* TPS7 and*La* TPS8 show different localization patterns in plastids. *La* TPS7 wrapped around chloroplasts, while *La* TPS8 distribution was punctate. Although reasons for these differences remain undetermined, these different patterns of localization may stem from different substrates in organelles. Moreover, several noncanonical metabolic pathways have been recently identified. For example, a novel enzyme called *Rh* NUDX1 (a nudix hydrolase) producing geraniol has been discovered in roses and constitutes a catalysis model that differs from known monoterpene synthases (Magnard *et al* . 2019). This indicates that our understanding of net-regulatory metabolism of terpenes, including substrate mediation and stimuli by environmental factors, are presently incomplete.

We used two promoters to examine regulation of terpene synthesis. Using the *LaTPS7* promoter predicted by PlantCARE resulted in our discovery of MYC transcription factors (TFs), which helped explain the wound-associated pattern of expression observed via the GUS stain bioassay. An analogous pattern was discovered in *Capsicum annuum*L. (Wu et al. 2019; Wang et al. 2016). Studies on relationships between TFs and TPSs, such as those among NAC, ethylene-insensitive3-like TF, and R2R3-MYB, can be conducted in lavenders (Nieuwenhuizen *et al.*2015; Reddy *et al.* 2017). Although some of TFs have been demonstrated in *L.* × *intermedia* (Sarker *et al* . 2019), other regulatory factors modulating terpene release will likely be discovered in lavenders.

Conversely, CYPs participate in defence of a wide variety of plants. Plant CYPS include CYP51 in oats (*Avena* spp.), CYP99A2 and CYP99A3 in rice (*Oryza sativa*), and CYP82G1 in *Arabidopsis*(Qi *et al* . 2006; Shimura *et al* . 2007; Lee *et al* . 2010). *LaCYP71D582* , the first CYP gene cloned from lavenders, protects the plants by converting limonene into carveol to enrich terpenoid metabolites in early stages of flowering. Although some reports show that CYPs target to plastidial membranes (Kim *et al.*2010; Quinlan *et al.* 2012), we found that *La* CYP71D582 was localized to the endoplasmic reticulum (ER) (Figure 3), close to plastids within plant cells. This enabled putative communication between these two organelles, which was also shown by Ginglinger *et al.*(2013). *LaCYP71D582* shared 57.68 and 7.98% similarity with limonene-6-

hydroxylase (*M. canadensis*) (Accession number: QDF63370.1) and limonene-6-hydroxylase (*M* . x *gracilis*) (Accession number: AAQ18706.1), respectively, indicating that determining the function of a CYP only by its sequence may be unreliable.

The*LaCYP71D582* promoter possesses elements for response to environmental stimuli, such as the GT1-motif responsible for responding to light conditions, and the LTR sequence responsible for responding to low temperature. This indicates that CYPs are modulated by environmental factors. Unfortunately, studies on how environmental factors influence CYPs and terpenoid regulation are rare. CYPs participate in various catalytic and metabolic activities in plants, including metabolism of fatty acids and alkane compounds mediated by CYP72, 701, and 90, flavonoid metabolism mediated by CYP73, 98, and 84, and phytoalexin metabolism mediated by CYP79, 80, and 93 (Nelson and Werck-Reichhart, 2011). Therefore, it is important to study not only CYP-mediated catalysis but also biotic and environmental factors that influence CYP activity.

In summary, herein we examined the functions of three genes in lavenders. These genes were associated with direct and indirect plant protection during the budding stage. We also examined the interaction among the plants, aphids, and ladybugs. This tritrophic interaction is vital for the successful propagation of lavenders (Figure 8). Collectively, our results show that metabolism in lavenders during the budding stage involves different regulatory mechanism compared with that occurring during the blossoming stage. Our results also indicate that plants used multiple tactics to protect themselves against herbivores and offer a new perspective on biological pest control.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Authors'

contributions

Z.L., H.L., and L.S. designed the study; Z.L. performed all experiments; J.L. performed bioinformatic analysis; Y.D., W.Z., and H.B. analyzed the chemical data; S.W., and S. L. performed insect rearing. Z.L., H.L., and L.S. analyzed the data and wrote the manuscript. All of the authors read and approved the final manuscript.

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Figure 1 . Phylogenetic tree of candidate gene. The tree was drawn using the Neighbor-Joining method in the MEGA 7 program and were further plotted by web based i*TOL* (https://itol.embl.de). (a) The candidate TPSs genes were clustered into TPS-b which highlighted by an arrow in red, and genes in white font are all from lavenders (b) The target gene *LaCYP71D582* was clustered into CYP 71 clan which was indicated by an arrow in red; CYPs are selected from 5 clans related to terpene metabolism according to Nelson and Werck-Reichhart (2011). The sequences used to construct the tree can be found in table S2 for TPSs and table S3 for CYPs.

Figure 2. Quantitative real-time PCR (qRT-PCR) analysis of *LaTPS7, LaTPS8* and*LaCYP71D582* expression in glandular trichome from different developmental stage. Each organ containing glandular trichome on flower and glandular trichome on leaf. The highest expression of three genes was at period of bud in glandular trichome on flower. Transcript levels were normalized to 18S rRNA genes. Results represent the mean ± SE of three repetitions.

Figure 3. Subcellular localization of *LaTPS7* , *LaTPS8* , and *LaCYP71D582* in*N. benthamiana.* Observation under Confocal Fluorescence of cells of tobacco after injection for three days. Both *La* TPS7 and*La* TPS8 were associated with chloroplasts, and *La* CYP71D582 was located in the endoplasmic reticulum (ER). Auto, chlorophyll autofluorescence; eGFP , enhanced Green Fluorescent Protein channel image; Light, light microscopy image; Merged, merged image between Auto and eGFP . Post processing of pictures were completed by image J (https://imagej.nih.gov/ij).

Figure 4. *La* TPS7 and *La* TPS8 enzymatic activity *in vitro.* Products catalyzed by *La* TPS7 and *La* TPS8 were shown. *La* TPS7-GPP: 1.*α*- Pinene, 2. Camphene, 3. Myrcene, 4. Limonene, 5. Terpinolene, 6. Linalool, 7. Terpineol; *La* TPS7-NPP: 1. *α*- Pinene, 2. Camphene, 3. Limonene, 4. Terpinolene, 5. Terpineol, 6. Nerolidol;*La* TPS8-GPP: 1. *α* -Pinene, 2. *β* -Pinene, 3. Sylvestrene, 4. Linalool, 5. Fenchol, 6. Geraniol; *La* TPS8-NPP: 1.*α*- Pinene, 2. Limonene, 3. Terpinolene, 4. Terpineol, 5. Nerolidol.

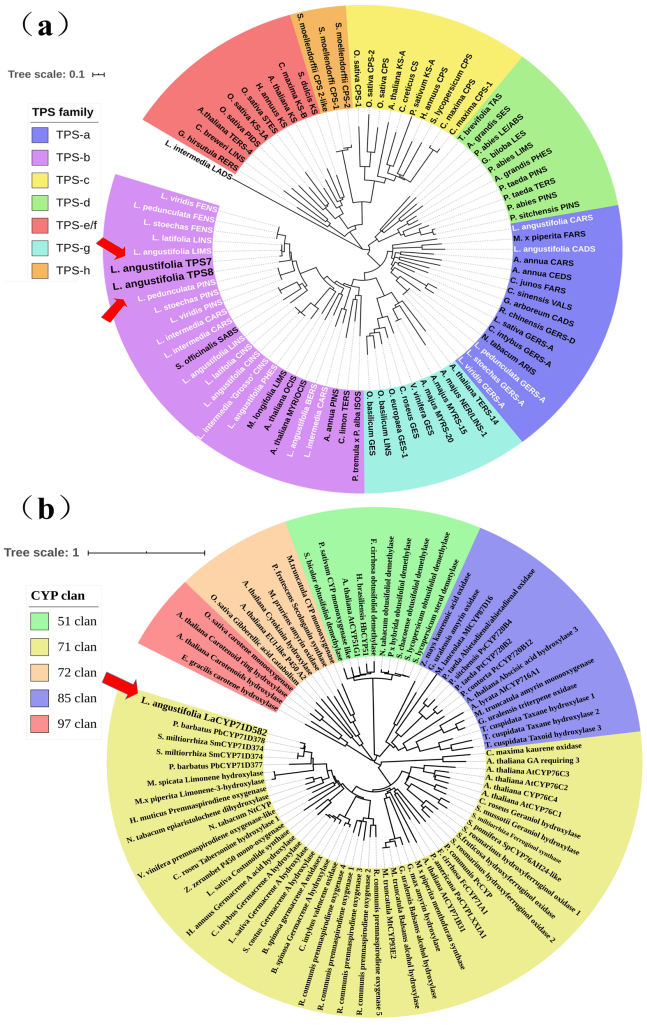
Figure 5. Enzyme activity *in vivo* . (a) Volatiles tested on tobacco leaves transfected with *LaTPS7* or *LaTPS8* respectively showing that *La* TPS7 only produced limonene from tobacco and *La* TPS8 produced *α*- pinene and sylvestrene. (b) Volatiles test on tobacco leaves co-expressed with*LaCYP71D582-LaTPS7*or with *LaCYP71D582-LaTPS8.* It indicated that limonene can be converted into carveol by *La* CYP71D582. (c) Limonene was hydroxylated at C6 by *La* CYP71D582 to form carveol.

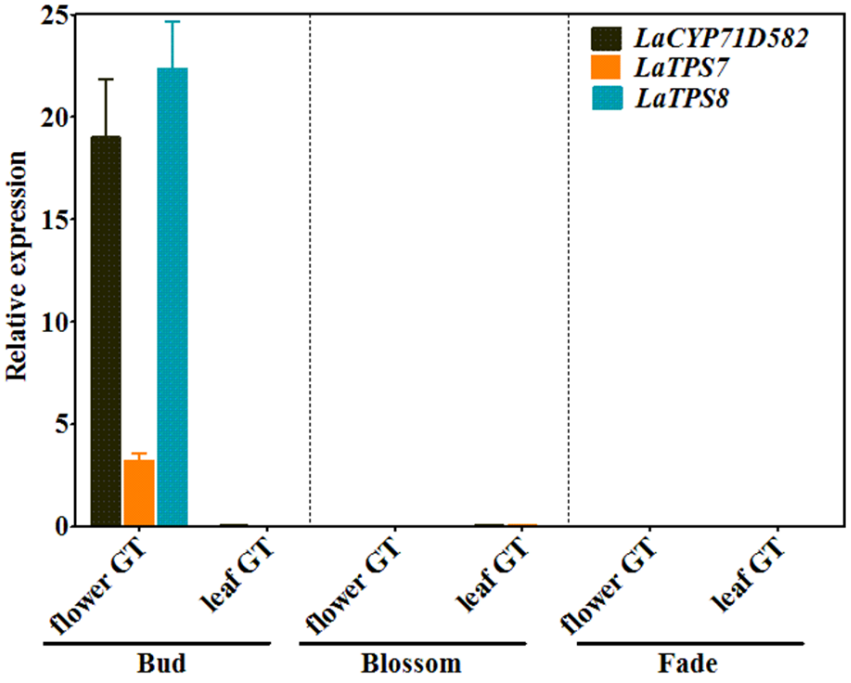
Figure 6. GUS staining showing the expression pattern of *LaTPS7* and *LaTPS8* . (a) Histochemical stain of *Pro -LaTPS7.* 1-3, leaves; 4, flower; 5, trichome; 6, silique. The red arrows shows the wound on leaves

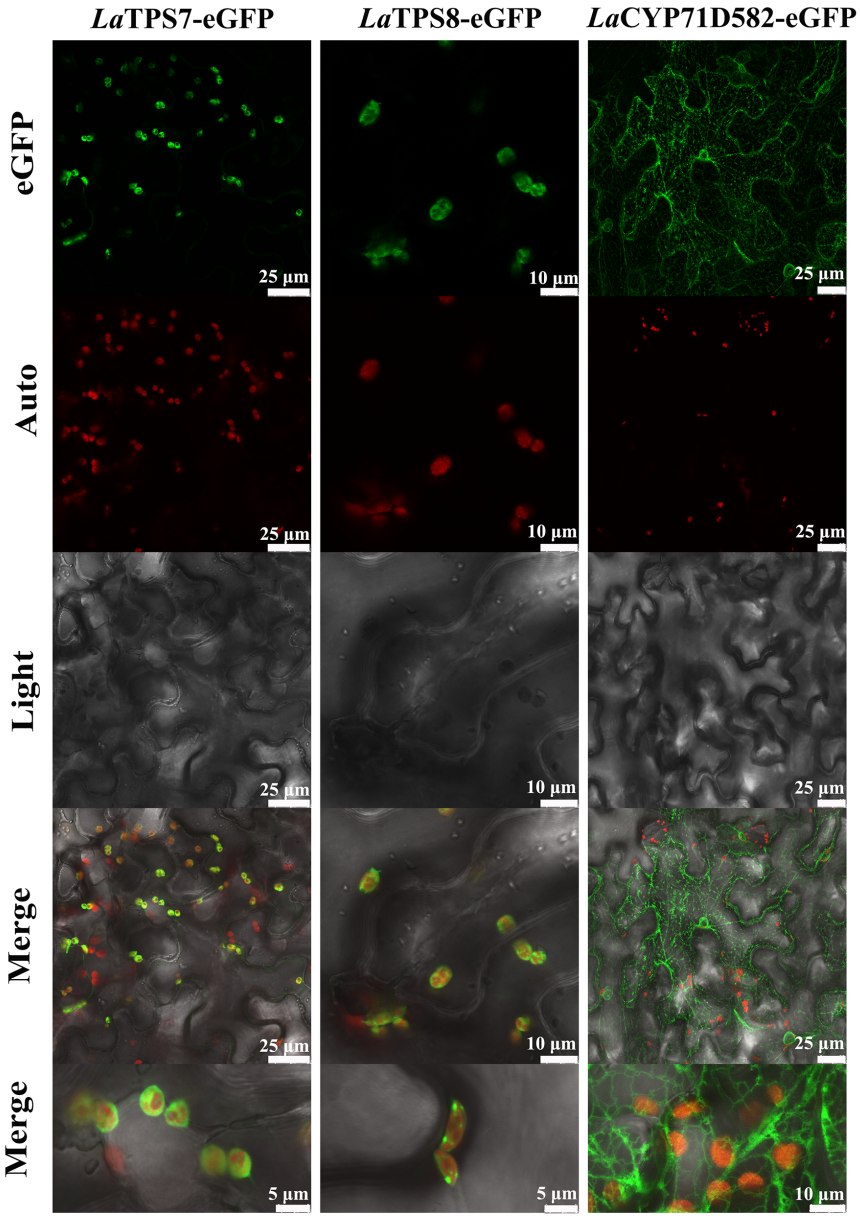
indicating a wound-induced expression pattern of *LaTPS7* . (b) Tissue stain of *Pro -LaCYP71D582* . 1, leaf; 2, flower; 3, trichome; 4, silique.

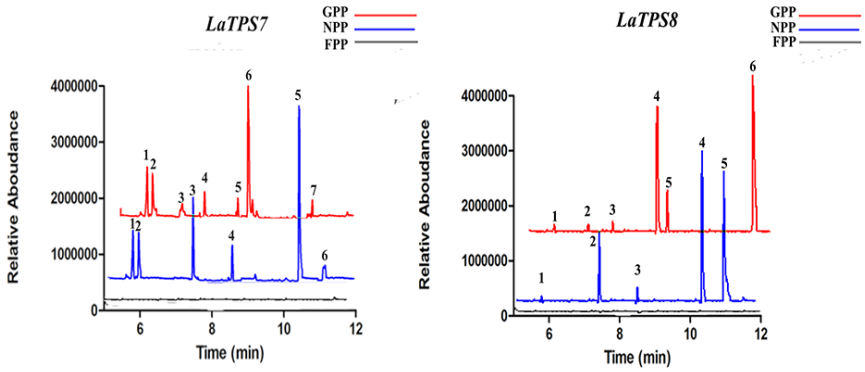
Figure 7 . Behavioral responses of aphids and ladybugs toward tobacco leaves expressing *LaTPS7* and co-expressed *LaTPS7* and *LaCYP71D582* . The percentage of insects choosing wild type leaves (blue bars) versus transfected leaves (green bars) are shown, which indicates that limonene and carveol are able to repel aphids and attract ladybugs. 90 aphids and 60 ladybugs were texted in total. Asterisks indicate a significant difference of choice towards different odor source using χ^2 for ladubugs and variance (ANOVA) for aphids ($^{***}P < 0.05$).

Figure 8 . A summary of the relationship of tritrophic interaction among lavender-aphid-ladybug. Volatiles including limonene, α -pinene catalyzed by terpene synthases in bud stage are able to defend plants against attack from aphids. Sequentially, CYP converts the limonene to carveol with stronger attraction to ladybugs. These regulatory networks work together to protect plants against herbivores.









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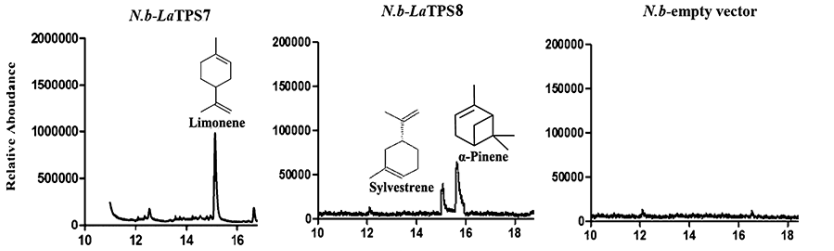

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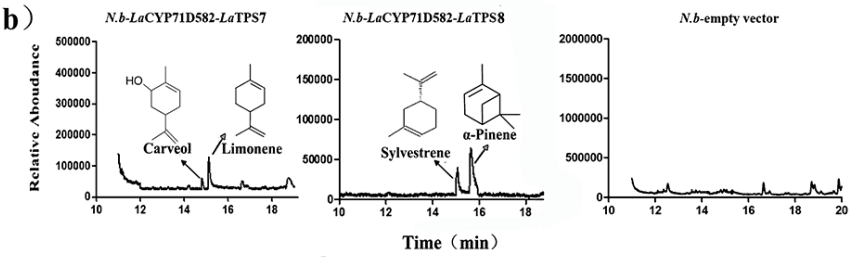

（c）