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**Terpene produced by co-expression of *TPS* and *P450* genes from *Lavandula angustifolia* protect
plant from herbivores attacks during budding stages**

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Gene ID:

BankIt2292418 LaTPS7 MN822897

BankIt2292495 LaTPS8 MN822898

BankIt2292498 LaCYP71D582 MN822899

Main conclusion

Abstract

To defend themselves against herbivore attacks, plants produce a variety of volatiles, especially during the early stages of development. During the budding phases of *Lavandula angustifolia*, three genes (*LaTPS7*, *LaTPS8*, and *LaCYP71D582*) were discovered to be highly expressed. The *LaTPS7* catalyzes total eight different compounds *in vitro*, including camphene, myrcene, and limonene, among others. The *LaTPS8* converted ten volatiles using geranyl diphosphate and nerolidyl diphosphate as substrates. The plastid-located *LaTPS7* gene produced limonene when it was overexpressed in tobacco. The endoplasmic reticulum enzyme *LaCYP71D582* may further convert limonene to carveol. The *LaTPS8* present in plastids creates α -pinene and sylvestrene, according to *in vitro* tests. The limonene and carveol were discovered to repel *Myzus persicae* while attracting *Harmonia axyridis* confirmed by standards test. In *Arabidopsis thaliana*, a GUS staining test was used to evaluate the promoter activity of *LaTPS7* and *LaCYP71D582*, and it was discovered that wounding may boost *LaTPS7* expression levels. The volatile compounds generated by *LaTPS7*, *LaTPS8*, and *LaCYP71D582* were found to be important in plant protection. In practice, biological control can be applied to promote human and environmental health via plant approaches.

Key words: lavender, monoterpenes, terpene synthase, cytochrome P450, tritrophic interaction

Introduction

Plants interact with their surroundings by emitting a variety of volatiles that serve physiological and ecological purposes (O'Connor 2015). Ehrlich and Raven (1964) proposed the hypothesis of herbivore-induced plant volatiles (HIPVs), which argued that plants employ volatiles to communicate and defend against herbivore attack. A recent study found that cottons producing higher amounts of volatile compounds have less *Helicoverpa armigera* eggs (Danfeng et al. 2018), implying that volatiles play an important role in pest protection. Many plant-derived volatile chemicals, particularly terpenes/terpenoids, have been shown to protect plants. Farnesene, for example, repels aphids (*Myzus persicae*) while attracting predatory ladybugs (*Harmonia axyridis*) (Francis et al. 2004; Zhu et al. 2005). Linalool, another ubiquitous chemical found in flowers and fruits, has been shown to attract thrips in *Arabidopsis thaliana*, while oxides of linalool (catalyzed by *CYP71D*) repel them (Benoît Boachon et al. 2015). Meanwhile, HIPVs, which include volatiles generated from mint (such as limonene, 1,8-cineole, and carvone), attract herbivore-preying animals (Togashi et al. 2019). As a result, volatile compounds could be part of a defense system used by plants to protect themselves via tritrophic interactions, either directly or indirectly (Meena et al. 2017). Furthermore, the volatiles are likely expressed at higher concentrations during early developmental stages/tissues than mature

stages/tissues (Meena et al. 2017); after that, expression is maintained at a consistent level and reduced over the mature or senescence phase (Niederbacher et al. 2015). This shows that plants dynamically regulate volatile chemical expression in order to adapt to their surroundings and reproduce.

The majority of HIPVs are terpenes/terpenoids that are produced in one of two ways. One is the mevalonate (MVA) pathway, which occurs in the cytosol/endoplasmic reticulum and results in farnesyl diphosphate (FPP), a sesquiterpene precursor. The other is the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, which occurs in plastids and produces geranyl diphosphate (GPP) as a monoterpene precursor. TPSs are a mid-sized gene family in plants, consisting of members of TPS-a, -b, -c, -d, -e/f, and -g. The MEP pathway is involved in numerous terpene synthases (TPSs) (McGarvey & Croteau, 1995; Feng et al. 2011).

Furthermore, in peppermint (*Mentha×piperita*) (Ringer et al. 2005), *Artemisia annua* L. (Teoh et al. 2006), and *A. thaliana*, the phenomena of terpene structure and function that is post-processed by co-expressed genes has been widely explored (Benoît et al. 2015). However, it is yet unknown whether this phenomena occurs in the terpene-rich plant *Lavandula angustifolia*, and which genes are co-expressed to regulate volatile terpene/terpenoid production in *L. angustifolia*.

Lavender (*L. angustifolia*) is a flowering plant that produces up to 70 volatile chemicals in its blossoms, including limonene, α -pinene, and linalool, etc. Lavender has been offered as a model plant for studying terpene biosynthesis control (Yann et al. 2010a). *In vitro* tests were used to identify the enzymes involved in terpene production in lavenders, specifically *LaLIMS*, *LaLINS*, τ -cadinol synthase, and β -phellandrene synthase (Christian Landmann et al. 2007; Demissie ZA et al. 2011; Frédéric J et al. 2014). Despite the fact that TPSs and cytochrome P450 (CYP) are required for terpene and derivative biosynthesis (Karunanithi and Zerbe 2019), and CYP family members account for 1% of the genome in most plant species (Nelson and Werck-Reichhart 2011), no CYP gene has been functionally characterized in lavender.

Volatiles' physiological activities and the genes involved in tritrophic interactions in lavenders are still unknown. Previous research has examined the volatile organic compound (VOC) content of inflorescence ontogeny, revealing that the terpenes involved fell into three distinct groups during the course of three flowering developmental phases (Yann et al. 2010b). Among these, limonene belonged to the first group, and it served as a natural insect repellent. In a recent study, we discovered that aphids and ladybugs were the most common insects in lavender fields in the early spring, implying a tritrophic interaction between volatile predators and preys (Hui et al. 2019). Meanwhile, in Y-tube olfactometer trials, it was discovered that β -trans-ocimene and (+)-R-limonene were able to eliminate 74.71% and 78.41% of aphids, respectively (Hui et al. 2019). As a result, it would be worthwhile to look into how the chemicals react to herbivores and how external stimuli are controlled at the gene level, as this could offer some light on the plant-insect connection.

TPS and CYP genes expressed in immature lavender flowers were cloned and identified using transcriptome data in this work. We then put their gene products to the test *in vitro* and *in vivo*. Simultaneously, the response of genes to environmental stimuli, as well as their regulatory elements, are investigated. In addition, standards were utilized to assess their impacts on aphids and ladybugs in order to demonstrate their ecological potential. As a result of the current study, the spectrum of lavenders' uses may be expanded depending on their distinct development processes, and this strategy can be used for biological control.

MATERIALS AND METHODS

Plants and insects

L. angustifolia 'Jingxun 2' were gathered at various stages of development from an experimental field at the Institute of Botany, Chinese Academy of Sciences. The following are the three distinct stages: B, bud stage: the petals were totally closed and green; I, blossom stage: 60 percent of the flowers had opened and the petals had turned purple; II, fade stage: the petals had shrunk and were about to fall; III, fade stage: the petals had shrunk and were about to fall. Four-week-old tobacco (*Nicotiana benthamiana*) and *A. thaliana* plants were grown in a greenhouse at 25°C with a 16/8h light/dark photoperiod (200 mol m⁻²s⁻¹).

Prof. Su Wang generously provided aphids (*Myzus persicae*) and ladybugs (*Harmonia axyridis*) (Institute of Plant and Environment Protection, Beijing Academy of Agricultural and Forestry Science). The aphids were kept in a growth chamber (30×30×25 cm) at 25°C and fed on a tobacco plant with an L16:D8 photoperiod. The ladybugs emerging from pupae were housed at 25°C, whereas the other pupae were kept at 10-15°C.

Cloning and transcript analyses of the *LaTPS7*, *LaTPS8* and *LaCYP71D582* genes

The glandular trichomes of flowers and leaves were used to extract total RNA. Flowers were made by scraping glandular trichomes down the ditch using a needle. Trichomes were removed from leaves using a brush at various developmental phases, then passed through meshes ranging from 1.6 mm to 0.15 mm, and eventually gathered on a mesh of 0.125 mm. Liquid nitrogen was used throughout the collection process. Following the manufacturer's instructions, reverse transcription was performed with oligo d(T) primers once the RNAs were collected (TSINGKE, China). Target genes were amplified using gene-specific primers and Phanta Max Polymerase after acquiring cDNA (Vazyme, China). The expression level of 18S rRNA was used as a reference gene to calculate normalized expression levels ($2^{-\Delta\Delta CT}$). Finally, SPSS 17.0 was used to examine the significance of gene expression levels among different developmental stages of flowers using LSD (Least Significant Difference). 'abc' was used to denote difference significance. Table S1 lists the primers used in this study.

Sequence analysis of the target genes

Multiple sequence alignment was performed on an ESPript (<http://esprict.ibcp.fr/ESPript/ESPript/>) as reported before using the entire amino acid sequences translated from ORFs (primers are provided in Table S1) (Robert et al. 2014). MEGA7 was used to create phylogenetic trees using the Neighbor-Joining method, and Interactive Tree of Life (<http://itol2.embl.de/>) was utilized for visual modification (Letunic et al. 2007). Tables S2 and S3 contain further information on the genes used to construct the tree.

Subcellular localization of the *LaTPS7*, *LaTPS8*, and *LaCYP71D582* in *N. benthamiana*

The mannopine synthase promoter (MAS)-controlled expression vector super-1300-eGFP was digested with Hind III before ligating target genes with a TreliefTM SoSoo Cloning Kit (TSINGKE, China). As described by Jingjing Jin et al.(2015), the plasmids were then transferred to *E. coli* for amplification before being transferred to *A. tumefaciens* GV3101 for transient transformation in 4-week-old *N. benthamiana* plants. *N. benthamiana* leaves were removed, mounted on slides, and studied under a confocal laser-scanning microscope with a standard filter set after 3 days of infiltration (Leica TCS SP5). Image J (<https://imagej.nih.gov/ij>) was used to process the images.

Heterologous expression of TPSs in *E. coli* and in vitro enzymatic assay

Using a pDE2 Directional Expression Kit Ver.2, shortened TPS sequences without the expected signal peptide were placed into a pDE2 vector with a recombinant C-terminal and poly-histidine tag (TSINGKE, China). The resulting constructions were transformed into *E. coli* BL21 (DE3), and the recombinant proteins were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a

concentration of 1.0 mM for around 8 hours until the OD₆₀₀ reached 0.5, and then purified using a His-Tagged Gravity Column (Merck Millipore). To ensure effective extraction, the purified proteins were visualized using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

In a final volume of 500 mL buffer (25 mM HEPES, pH 7.3; 10 mM MgCl₂; 10% glycerol; 10 mM DTT), about 20 µL pure protein, and 10 µg of either FPP, NPP, or GPP, the *in-vitro* enzymatic assay for TPS activity was done according to Feng et al. (2011). (Sigma-Aldrich). The mixtures were vortexed and incubated at 30°C for 2 hours before receiving 250 mL hexane and vortexed for 1 minute. The upper layers were centrifuged for 30 minutes at 1200 g and 4°C before being transferred to 2 mL glass vials for GC-MS analysis. As a negative control, heat-inactivated recombinant proteins were utilized in the procedure.

Transient expression of *LaTPS7*, *LaTPS8*, and *LaCYP71D582* in *N. benthamiana*

When the OD₆₀₀ reached 1.0, leaves of *N. benthamiana* taken from 4-week-old plants were infiltrated with GV3101 containing specific target TPSs genes. Meanwhile, when the OD₆₀₀ reached 1.0, GV3101 strains carrying TPS or CYP were combined in 1:1 ratios for co-expression. After 5 days of infiltration, leaf tissues were removed, evaluated under UV light for effective agroinfiltration and gene expression, and then crushed in liquid nitrogen. The volatiles were extracted by vortexing in hexane after the leaf powder was kept at -80°C. Before analysis, the extracts were kept at -20°C and oscillated. GC-MS was then used to examine volatile chemicals.

GC-MS Analysis

An Agilent 7890A GC system and an Agilent Technologies 5975C Inert XL Mass Selective Detector fitted with an HP-5MS UI column (30 m 0.25 mm 0.25 µm; Agilent Technologies) were used to perform splitless injection GC-MS analysis. The temperature of the injector was 250°C. Temperature was held at 40°C for 3 minutes, then increased to 130°C at a rate of 10°C·min⁻¹, then to 250°C at a rate of 50°C·min⁻¹, and held for 10 minutes; helium gas was utilized as the vector at a flow rate of 1 mL·min⁻¹.

Ionization energy was 70 eV, electronic impact ion source temperature was 200°C, quadrupole temperature was 150°C, and mass range was 35-550u. Using mass spectra from the Mass Spectral Library of Agilent, products were identified based on their retention time and electron ionization mass spectra.

Determination of promoter activity in *A. thaliana* and gene expression under methyl jasmonate (MeJA) treatment on *L. angustifolia*

FPNI-PCR (Fusion primer and nested integrated PCR) was used to extract a 1.5-kb area of each gene's promoter from genomic DNA using gene-specific and arbitrary primers, with PCR conditions described by Wang et al. (2011). PlantCARE (bioinformatics.psb.ugent.be/webtools/plantcare/html) (Rombauts et al. 1999) was used to analyze the sequences obtained, and they were subsequently inserted into the pCXGUS-P vector, which was digested with BamHI. A cloning cassette is located upstream of GUS in the pCXGUS-P vector (*uidA*). The resultant constructs were transferred into GV3101 *Agrobacterium* and employed for transformation in *A. thaliana* via a floral dip approach after being validated via sequencing (Clough et al. 1998). Transformed lines were chosen using MS plates containing 50 µg·mL⁻¹ hygromycin and validated using PCR. The Jefferson et al. (1987) technique for GUS staining (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) was used.

Meanwhile, at 8:00 AM, lavenders in the flower stage were treated with 8 mM MeJA (MeJA was dissolved in water with 1% ethanol and 0.1% Tween 20) to mimic insect assault. The plants that were given a MeJA-deficient buffer served as the control group. Each plant received a 10 mL buffer until leaves got the drips, and the materials were extracted after 12 hours and stored in liquid nitrogen. Using

the above-mentioned approach, the relative gene expression level in the glandular trichomes of flowers was examined using qRT-PCR. The significance of *LaTPS7* expression treated by MeJA using SPSS17.0 was determined using the Student's t-test.

Insect behavior

A Y-Tube olfactorometer test was used on aphids (*Myzus persicae*) and ladybugs (*Harmonia axyridis*) that had been fasted for 3 hours to see how volatiles altered their behavior. By dissolving the limonene and carveol standards in paraffin oil, different concentrations were produced. The limonene was tested at 0.1%, 4.5% and 8.5%; while carveol was tested at 0.0025%, 0.005%, 0.01% and 0.03%. The flow rate of the olfactometer was 300 mL·min⁻¹, and the insects were classed as responders if they entered more than half-length of the olfactory arms and lingered for at least 10 seconds, and no-choice if they did not pick for either side after 5 minutes. Fresh disks were replaced after 10 aphids or ladybugs were tested. The importance of choices were investigated using the Chi-square test in SPSS17.0.

RESULTS

Candidate genes from *L. angustifolia* and construction of phylogenetic tree

The transcriptomes were constructed using flowers at various stages of development. TPS (TRINITY DN51614 c1 g5) and CYP (TRINITY DN22690 c0 g1) were chosen for further study after reviewing the transcriptome data because their expression levels were high in the bud and indicated a co-expression pattern (Figure S1). Using the same primer pairs for TPS, two TPS genes with sizes of 1803 and 1800 bp were obtained and designated *LaTPS7* and *LaTPS8*, respectively, after being validated by sequencing. In the initial 33 bp at the N-terminal and the last 23 bp at the C-terminal, *LaTPS7* and *LaTPS8* have the same nucleotide sequence (Figure S2).

To examine the relationship with other terpene synthases, neighbor-joining phylogenetic trees were created based on deduced amino-acid sequences. The findings revealed that these two genes were members of the TPS-b subfamily of monoterpene synthases (Feng et al. 2011). (Figure 1a). *LaTPS7* had the most similarity to limonene synthase from *L. angustifolia*, whereas *LaTPS8* had the most similarity to pinene synthase from *L. pedunculata*. Both proteins had the conserved arginine-tryptophan (RRX₈W) motif in the N-terminal, as well as an aspartate-rich (DDXXD) and NSE/DTE motif in the C-terminal (which chelates divalent metal ions like Mg²⁺ or Mn²⁺). These motifs are required for the cyclization of terpene precursors such as GPP, NPP, and/or FPP (Figure S3, S4).

The CYP450 nomenclature committee accepted the CYP sequence and named it *LaCYP71D582*. With conserved motifs (A/G)GX(D/E)T(T/S), EXXR, and FXXGXRXCXG (Pateraki et al. 2017), *LaCYP71D582* showed the highest identity with *PbCYP71D378* from *Plectranthus barbatus* (Figure 1b) (Figure S5). The CYP clans in the phylogenetic tree were linked to terpene metabolism (Nelson and Werck-Reichhart 2011), and *LaCYP71D582* was placed in the CYP71 clan, which is the largest of the CYPs (Figure 1b). As a result of this finding, *LaCYP71D582* is most likely involved in terpene biosynthesis (Nelson et al. 2004).

Quantitative RT-PCR analysis of gene expression during budding stage

Using quantitative real-time PCR, the expression profiles of *LaTPS7*, *LaTPS8*, and *LaCYP71D582* were re-evaluated (qRT-PCR). On leaves and flowers, samples were taken at the budding, blooming, and fading stages of glandular trichomes (Figure 2). During the budding stage, *LaTPS7*, *LaTPS8*, and *LaCYP71D582* were strongly expressed in the glandular trichomes of the flower, but not at other phases of development. During the blossoming and fading stages, the expression levels of *LaTPS7*, *LaTPS8*, and *LaCYP71D582* all reduced, following a similar expression pattern. These findings suggested that these genes could interact throughout plant growth (Figure 2), implying that TPS

products could be accelerated further by CYP.

Subcellular localization of the *LaTPS7*, *LaTPS8*, and *LaCYP71D582* via transient expression in tobacco

LaTPS7 and *LaTPS8* were predicted to have signal peptides by the Phyre2 software, while *LaCYP71D582* was predicted to localize on membranes. For each potential gene, ORF fusion vectors containing enhanced green fluorescent protein (eGFP) were created and transfected into *N. benthamiana* leaves using *A. tumefaciens*. *LaTPS7* and *LaTPS8* were found to be connected with chloroplasts in entire leaves using confocal microscopy (Figure 3), whereas *LaCYP71D582* was found in the endoplasmic reticulum (ER) (Figure 3).

Isolation and *in-vitro* enzymatic characteristic of TPSs

To establish the function of each protein, shortened TPS proteins lacking signal peptides were coupled with six His-tags to create recombinant proteins, which were then expressed and purified in *E. coli* BL21 (DE3). After that, the recombinant proteins were evaluated using GPP, NPP, or FPP as a substrate. *LaTPS7* catalyzed GPP to make seven monoterpenes, including α -pinene, camphene, myrcene, limonene, terpinolene, linalool, and terpineol, and catalyzed NPP to produce α -pinene, camphene, limonene, terpinolene, terpineol, and nerol (Figure. 4a).

α -Pinene, myrcene, sylvestrene, linalool, fenchol, and geraniol were created by *LaTPS8* from GPP, whereas α -pinene, limonene, terpinolene, terpineol, and nerol were made by *LaTPS8* from NPP (Figure. 4b). When FPP was utilized as a substrate for both proteins, however, no products were found, and no novel compounds were examined in the control group (data not shown). These findings revealed that *LaTPS7* and *LaTPS8* are both monoterpene synthases that create distinct volatiles from the identical substrates.

Functional characterization of TPSs and CYP *in vivo*

LaTPS7 and *LaTPS8* were transiently transfected into tobacco leaves to confirm the function of these three genes in plants. Meanwhile, in order to explore *in vivo* functions, *LaCYP71D582* was co-expressed with either *LaTPS7* or *LaTPS8*. In tobacco, *LaTPS7* created only limonene, whereas *LaTPS8* produced α -pinene and sylvestrene, according to the findings (Figure 5a). New product carveol was only detected in the assay with tobacco plants co-expressing *LaTPS7-LaCYP71D582*, but not in the assay with *LaTPS8-LaCYP71D582* (Figure 5b). This result showed that *LaCYP71D582* may further hydroxylate limonene at C6 to produce carveol (Figure 5c).

The spatial-temporal expression pattern of *LaTPS7* and *LaCYP71D582*

We analyzed the specific expression pattern of these two genes as *LaCYP71D582* and *LaTPS7* catalyzed sequentially. FPNI-PCR was used to obtain a 1299bp *LaTPS7* promoter and a 1434bp *LaCYP71D582* promoter, which were dubbed Pro-*LaTPS7* and Pro-*LaCYP71D582*. Both promoters featured regulatory components predicted by PlantCARE in addition to the basic promoter elements (Figure S6, S7). We discovered that *LaTPS7* was expressed in flowers, siliques, trichomes, and leaves after transforming promoter-GUS fusion vectors into *A. thaliana*. Wounding, in particular, induced its manifestation (Figure 6a). In other words, after being harmed, *LaTPS7* was strongly expressed in plants, comparable to the response when plants are attacked by herbivores. In flowers, siliques, trichomes, and leaves, *LaCYP71D582* revealed a constitutive expression pattern (Figure 6b), but it was not stimulated by injury.

Furthermore, methyl jasmonate (MeJA), a phytohormone that responds to herbivore attacks, was utilized to treat lavenders at the blossom stage to mimic insect attacks, which is when the expression level of *LaTPS7* is lowest (Figure 2). The relative expression level of *LaTPS7* from glandular trichomes

of the blossom stage was consistently almost 10 times greater than that of the contrast glandular trichomes (Figure 6c). This phenomenon suggested that *LaTPS7* might be able to respond to herbivore attacks in order to protect plants.

Limonene and carveol repel aphids and attract ladybugs to protect plants

Most herbivores, including aphids, are repellent to limonene, according to previous study (Hui et al. 2019). However, how limonene and its derivatives effect aphid-ladybug interactions is yet unknown. Researchers used a Y-Tube olfactometer to analyze aphid and ladybug responses to limonene and carveol to see if there was a link between volatile chemicals, aphids, and ladybugs. Although several different concentration of limonene and carveol were tested, 47 aphids preferred paraffin oil to the 27 aphids who preferred limonene at a concentration of 4.5%. In the meantime, 48 aphids eluded the 0.05% carveol, while 22 aphids chose it. The repellent effectiveness is estimated to be around 65%. Surprisingly, ladybugs were drawn to limonene at a concentration of 0.1%, with 55 choosing the standards and 19 choosing the paraffin oil. They were also attracted by 0.0025% carveol, with 45 ladybugs choosing the standards vs 25 choosing the paraffin oil. The attraction rates are approximately 60% and 70%, respectively (Figure 7). These findings revealed that limonene and carveol had a potent anti-aphid impact in different concentration, inhibiting aphid attack on the plant.

Discussion

Plants produce as many volatiles as possible during early developmental stages rather than mature levels because juvenile tissues are fragile. In order to grow and reproduce properly, they must protect juvenile tissues such as young leaves and blossoms against herbivore attacks (Meena et al. 2017). This is confirmed by gene expression profiles acquired during the flower development phase (Figure S1), which show that a variety of volatile compounds are critical in plant defense in the early stages. This phenomenon was similarly reported in *A. thaliana* and *Zanthoxylum piperitum* (Yoshiyuki et al. 2017; Boachon et al. 2015). Plants must give energy for both growth and defensive methods at the same time during this period, which is tough because both need a lot of energy (Meena et al. 2017). As a result, plants have had to develop a number of ways to keep themselves alive while growing in a confined space.

Plants are protected by volatile chemicals, which repel herbivores and attract herbivores' natural enemies, producing a tritrophic interplay between plants, herbivores, and carnivores. To establish a sustainable agricultural system, this provides an optimal method of natural biological control and restraint of biospheric sources of pollution — reducing the usage of insecticides (Loreto et al. 2014).

Limonene and carveol repel aphids while attracting ladybugs with different concentration, according to our findings. This indicate that the concentration of volatiles in lavender may be regulated in a dynamic process. In soybeans (*Glycine max*) and mint, the similar phenomena have been observed (Zhu et al. 2005; Togashi et al. 2019). Furthermore, limonene attracts predatory mites *Phytoseiulus persimilis* and *Neoseiulus californicus* (Togashi et al. 2019) and repels western flower thrips (*Frankliniella occidentalis*) (Picard et al. 2012), demonstrating that it can be utilized to resist a wide range of herbivores (Smith et al. 2018). Meanwhile, carveol, pinene, and limonene could play a role in the development of plant-based insect repellents (Picard et al. 2012; Smith et al. 2018). Insects, on the other hand, have acquired resistance to such defenses as well as flexibility. *Musca domestica*, for example, uses endogenous P450 to convert limonene to carveol and carvone, which reduces flies' toxicity (Rossi et al. 2013). This demonstrates that the same terpene performs different functions in different species, which may explain why insects and chemically protected plants are in a

co-evolutionary arms race.

Terpene synthases are enzymes that catalyze terpene/terpenoids formation in plants (TPSs). Even in species with entire genome sequences, such as *Gossypium*, TPS sequences are almost identical, and their actions are repetitive in plants, making annotation and assignment difficult (Xin-Zheng et al. 2017). As a result, function identification requires experimental validation. *LaTPS7* and *LaTPS8* have a high degree of similarity, with 77.61% of their genes being identical; as a result, their products are highly repetitive *in vitro*. Both *LaTPS7* and *LaTPS8* have the ability to catalyze GPP and NPP into distinct products, demonstrating TPS promiscuity and multisubstrate properties (Major 2012). Although the majority of monoterpene synthases, including *LaTPS7* and *LaTPS8*, are found in plastids, TPSs can also be found in mitochondria and the cytosol (Yamaga et al. 1993; Aharoni et al. 2004; Lee & Chappell 2008; Magnard et al. 2015). In addition, some noncanonical metabolic pathways have recently been discovered. In roses, for example, a new enzyme termed *RhNUDX1* (a nudix hydrolase) has been found that produces geraniol and represents a catalysis model that differs from known monoterpene synthases (Magnard et al. 2019). This suggests that our knowledge of terpene metabolism on a network scale, encompassing substrate mediation and environmental cues, is still lacking.

The two TPS genes' promoter activity in terpene synthesis regulation was also investigated. PlantCARE's prediction of the *LaTPS7* promoter led to the identification of MYC transcription factors (TFs), which helped to explain the wounding-related pattern of expression seen with GUS staining. MeJA could specifically promote *LaTPS7* expression in the lavender bloom stage, which was similar to a pattern reported in *Capsicum annuum* (Wu et al. 2019; Wang et al. 2016). Meanwhile, Yuechen et al. (2022) discovered that jasmonate (JA)-mediated signaling led to the development of a plant chemical that was effective against *Empoasca leafhoppers*. Furthermore, research on poplar leaves revealed that juvenile leaves responded to simulated caterpillar feeding with a stronger and faster transcriptome response than adult leaves (Philippe et al. 2010). This could be explained by the fact that juvenile tissue is more vulnerable to herbivore attacks, and TFs such NAC, ethylene-insensitive3-like TF, and R2R3-MYB may activate fast signal transduction (Nieuwenhuizen et al. 2015; Reddy et al. 2017). Although multiple transcription factors (TFs) have been identified in *L. × intermedia* (Sarker et al. 2019), lavenders are likely to uncover new regulatory elements that modulate terpene production and responsiveness to the environment. Nonetheless, the studies described above mostly focused on the vegetative growth stage, but because lavender is a shrub and its blooms are valuable resources, we must also consider the reproductive growth stage.

CYPs, on the other hand, have a role in plant defense, including *CYP51* in *Avena* spp., *CYP99A2* and *CYP99A3* in *Oryza sativa*, and *CYP82G1* in *Arabidopsis* (Qi et al. 2006; Shimura et al. 2007; Lee et al. 2010). These CYPs act on terpenoids (mono-, sesqui-, and di-) generated and kept in the plastid, which are later processed by CYPs in the cytosol/ER-membrane. In the early phases of flowering, *LaCYP71D582*, the first CYP gene cloned from lavenders, protects the plants by converting limonene to carveol. Despite the fact that CYPs have been proven to target plastidial membranes (Kim et al. 2010; Quinlan et al. 2012), *LaCYP71D582* was discovered in the endoplasmic reticulum (ER) (Figure 3), which is near to plastids within plant cells. This spatial relationship allowed for possible communication between the two organelles, and Jean-François et al. (2013) reported a similar result. *LaCYP71D582* shared 56.40% and 58.72% similarity with limonene-6-hydroxylase (*M. canadensis*) (Accession number: QDF63370.1) and limonene-6-hydroxylase (*M. gracilis*) (Accession number: AAQ18706.1), respectively, indicating that determining the function of a CYP solely by its sequence may be unreliable or that CYPs from, which has been demonstrated in details by F. Peter Guengerich

(2018).

The GT1-motif responds to light conditions, and the LTR sequence responds to low temperature, so the *LaCYP71D582*-promoter has elements for responding to environmental stimuli. This suggests that environmental influences may influence CYPs. However, research on the effects of environmental variables on CYPs and terpenoid regulation is scarce. In plants, CYPs are involved in a variety of catalytic and metabolic processes, including fatty acid, alkane, and phytoalexin metabolism (Nelson and Werck-Reichhart, 2011). As a result, it's important to look at not only CYP-related catalysis but also how biotic and abiotic variables affect CYP activity.

So far, according to Li et al. (2021), out of 428 CYP450s, 207 CYP71 clan (associated to mono- and sesquiterpenes hydroxylation) were discovered, which is a valuable resource for further functional characterization of CYP genes in terpene biosynthesis and a better understanding of terpene metabolism in *L. angustifolia*.

In conclusion, we looked at the roles of three lavender genes: *LaTPS7*, *LaTPS8*, and *LaCYP71D582*. During the budding stage, these genes were linked to terpene production for direct and indirect plant defense. We also looked at how plants, aphids, and ladybugs interacted. This tritrophic relationship is necessary for lavender propagation to be successful. Our findings demonstrated that *LaTPS7* and *LaCYP71D582* are most prevalent in lavenders during the budding stage, when they respond to herbivore attack by generating limonene and carveol to defend lavenders. Finally, the creation of a tritrophic connection is critical for plant reproduction and flowering. As a result of our research, the variety of lavenders that can be used will be expanded due to the diverse development processes.

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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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Data availability statements:

Data will be made available on reasonable request.

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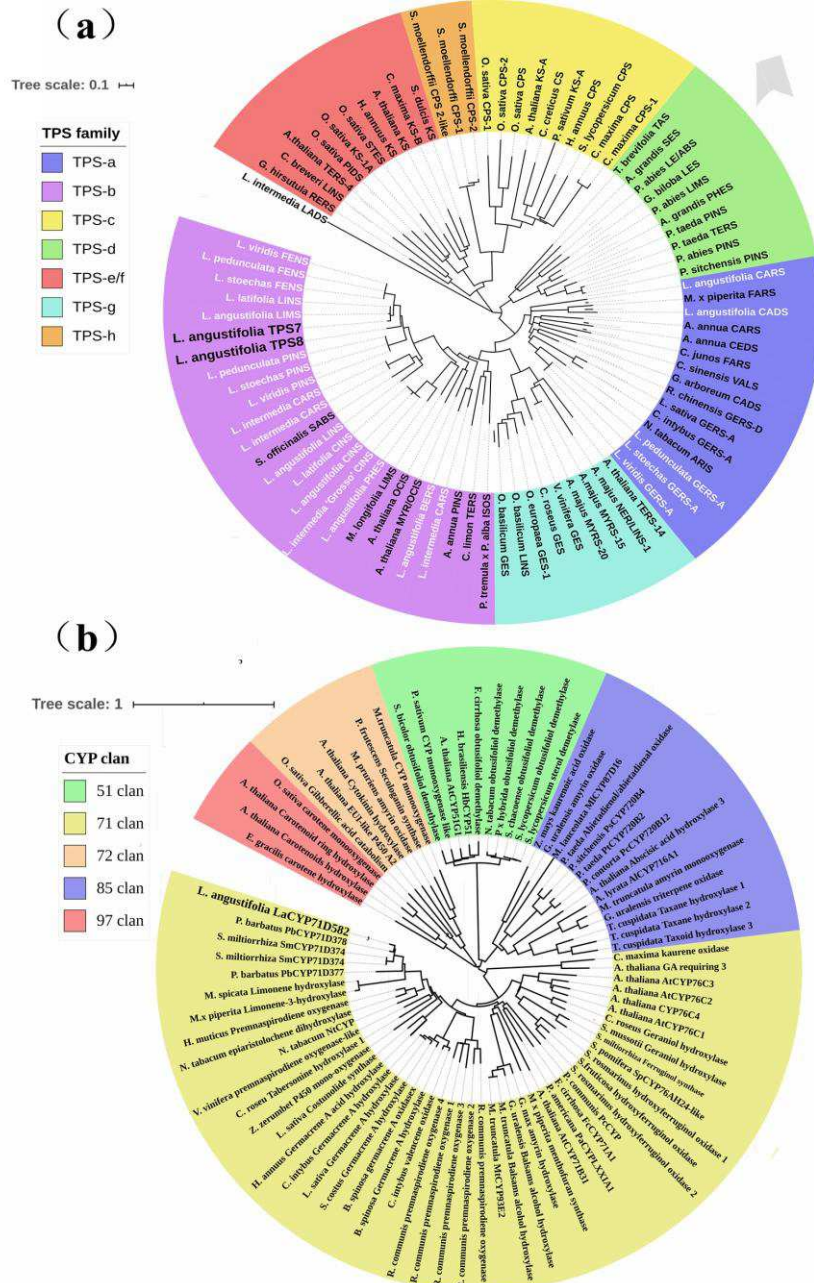


Figure 1. Phylogenetic tree of candidate TPSs and P450 genes. The tree was drawn in MEGA 7 using the Neighbor-Joining method, and then plotted using the web-based iTOL (<https://itol.embl.de>). (a) The candidate TPSs genes were grouped into TPS-b, which is denoted by bold fonts, and the genes in white font are all lavender genes. (b) According to Nelson and Werck-Reichhart (2011), the target gene *LaCYP71D582* was clustered into the CYP 71 clan, which was denoted by bold fonts; CYPs are selected from five clans connected to terpene metabolism. Table S2 for TPSs and Table S3 for CYPs contain the sequences utilized to build the tree.

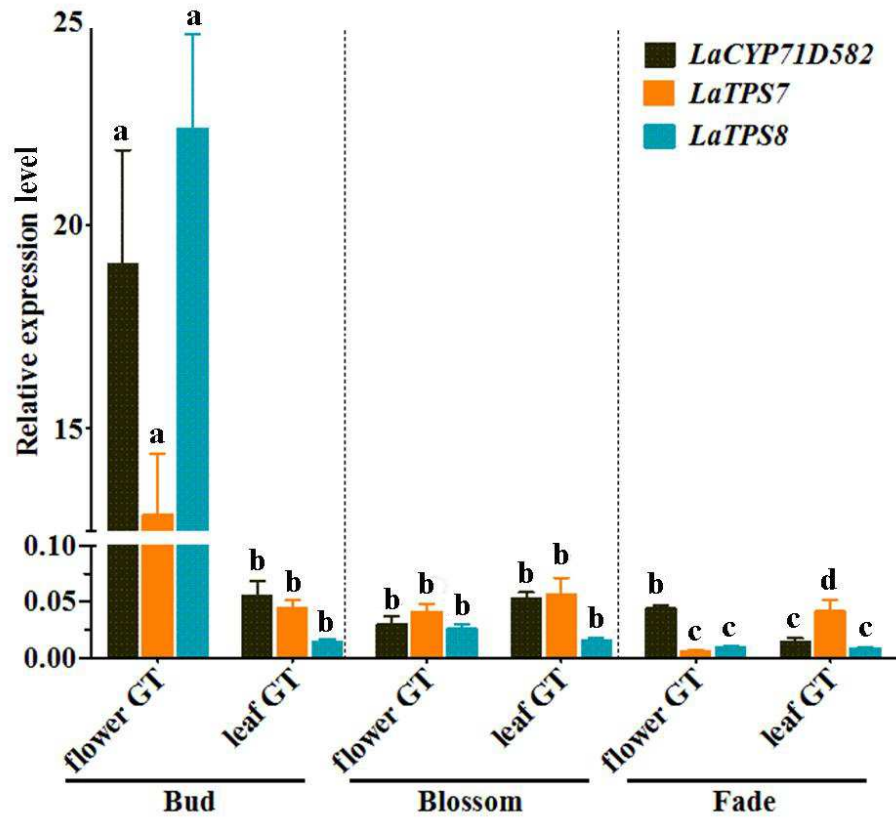


Figure 2. The expression levels of *LaTPS7*, *LaTPS8*, and *LaCYP71D582* in glandular trichomes from various developmental stages were examined using quantitative real-time PCR (qRT-PCR). On the flower, there are glandular trichomes, and on the leaves, there are glandular trichomes. Three genes had the highest expression in glandular trichomes of the flower during the bud phase. The 18S rRNA genes were used to standardize the transcript levels. The results are the average SE of three replicates.

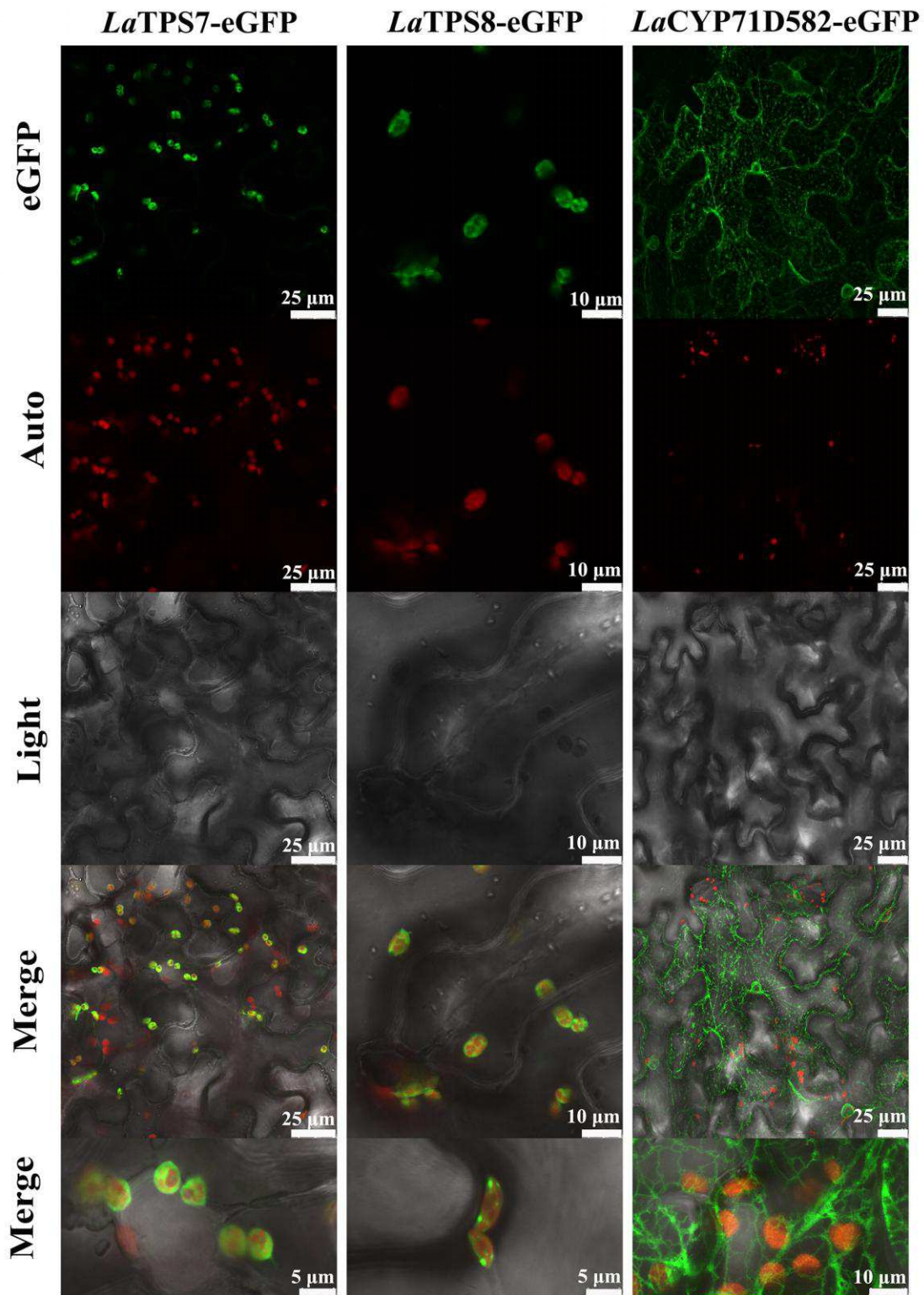


Figure 3. Subcellular localization of *LaTPS7*, *LaTPS8*, and *LaCYP71D582* in *N. benthamiana*. Tobacco leaves after three days of inoculation were observed under confocal fluorescence. *LaTPS7* and *LaTPS8* were discovered in chloroplasts, while *LaCYP71D582* was discovered in the endoplasmic reticulum (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>).

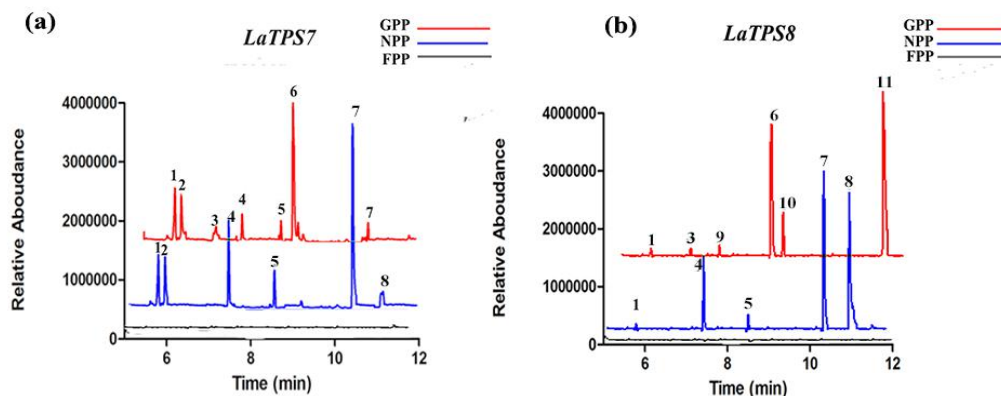


Figure 4. *In vitro* enzymatic assays of *LaTPS7* and *LaTPS8*. Products catalyzed by *LaTPS7* (a) *LaTPS8* (b) with GPP, NPP and FPP. Compounds are marked with number: 1. α -Pinene, 2. Camphene, 3. Myrcene, 4. Limonene, 5. Terpinolene, 6. Linalool, 7. Terpineol, 8. Nerol, 9. Sylvestrene, 10. Geraniol, 11. Fenchol.

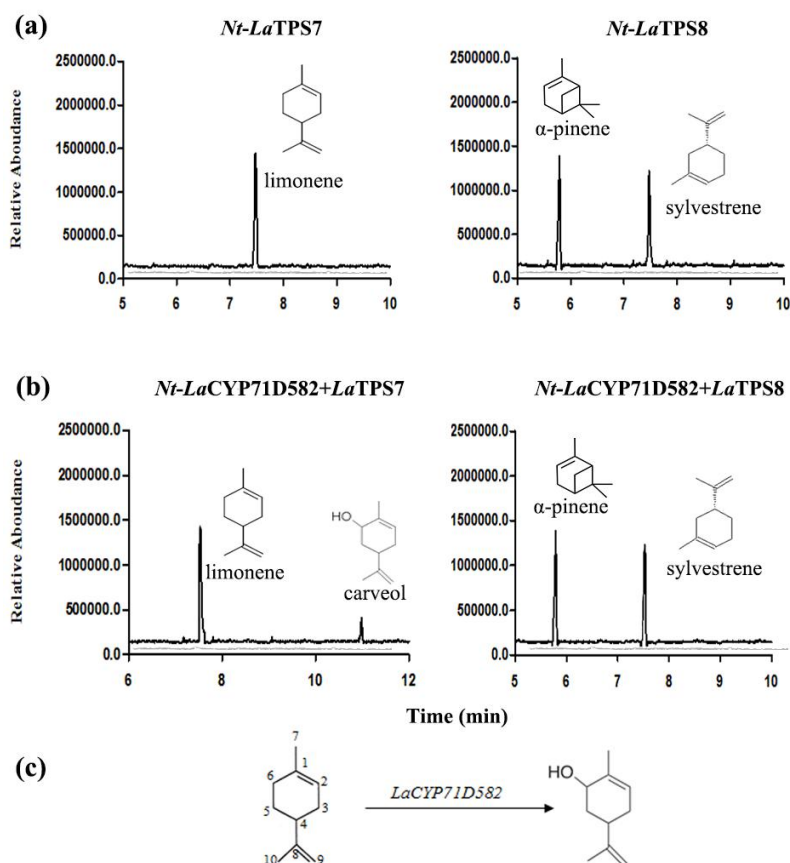


Figure 5. *In vivo* enzymatic activity in tobacco. (a) Volatiles produced in tobacco leaves over-expressing *LaTPS7* or *LaTPS8* respectively, showing that *LaTPS7* only produced limonene in tobacco and *LaTPS8* produced α -pinene and sylvestrene. (b) Volatiles produced in tobacco leaves co-expressing *LaCYP71D582-LaTPS7* or co-expressing *LaCYP71D582-LaTPS8*. It was indicated that limonene can be converted into carveol by *LaCYP71D582*. (c) Limonene was hydroxylated at C6 by

LaCYP71D582 to form carveol. The gray trace in (a) and (b) indicate the control extract of tobacco leaves transformed with empty vector.

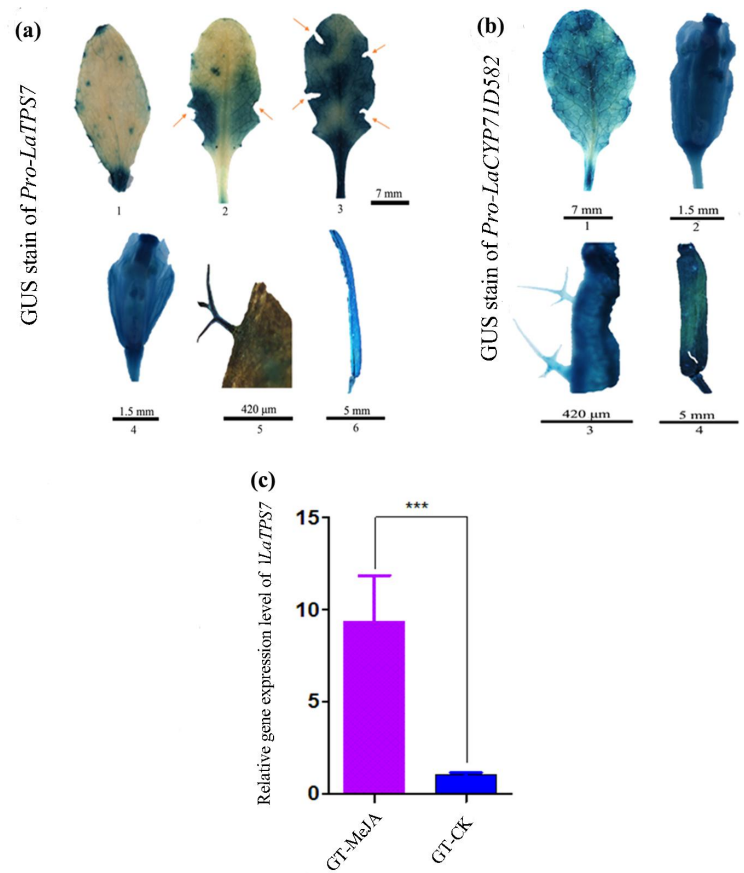


Figure 6. GUS staining and MeJA treatment showing the expression pattern of *LaTPS7* and *LaCYP71D582*. (a) Histochemical stain of Pro-*LaTPS7*. 1-3, leaves; 4, flower; 5, glandular trichome (GT); 6, silique. The red arrows shows the wounding sites. (b) Histochemical stain of Pro-*LaCYP71D582*. 1, leaf; 2, flower; 3, glandular trichome (GT); 4, silique. (c) The expression level of *LaTPS7* was induced by MeJA treatment at the blossom stage, *** $P < 0.001$.

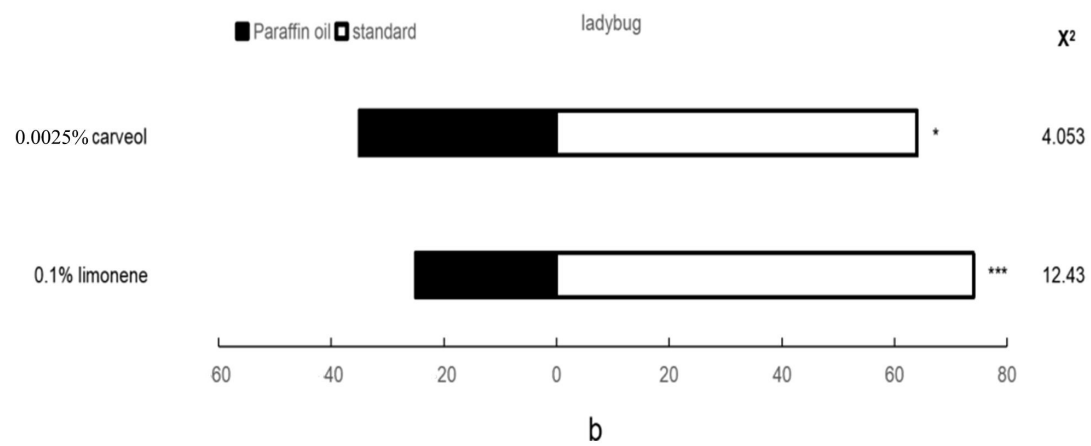
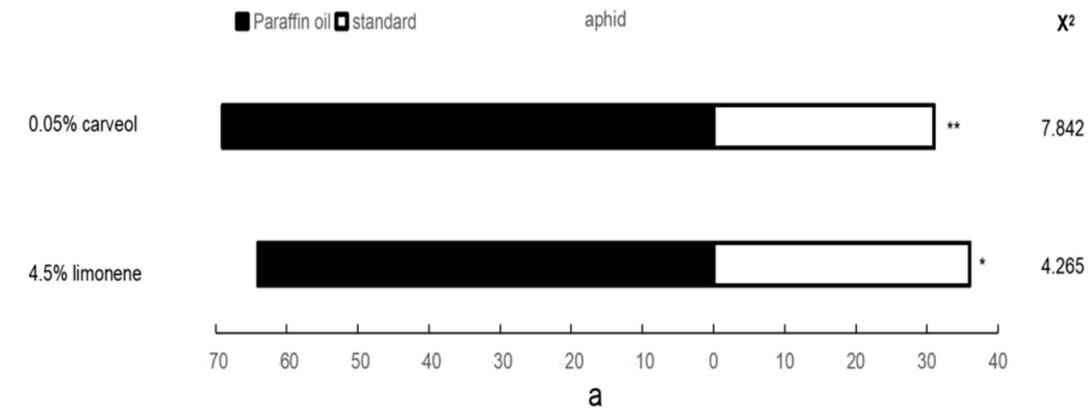


Figure 7. Behavioral responses of aphids and ladybugs toward limonene and carveol stanards. The percentage of insects choosing paraffin oil (black) versus standards (white) are shown, which indicates that limonene and carveol are able to repel aphids and attract ladybugs. Over 70 aphids and ladybugs were tested in total for each odor. Asterisks indicate a significant difference of choice towards different odor source using Chi-Squared Test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Supplementary Files

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