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A NEW MONOTERPENE SYNTHASE GENE INVOLVED IN THE MONOTERPENE PRODUCTION FROM *LILIUM* 'SIBERIA'

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ABSTRACT

Floral scent is thought to be an important characteristic to evaluate ornamental flowers, playing a key role in plant ecophysiology. Monoterpenes are an important category of floral scent compounds. In this study, the monoterpene emission from the *Lilium* 'siberia' flowers was investigated using the automated thermal desorption-gas chromatography/mass spectrometry (ATD-GC/MS) technique. It was found that monoterpenes were the dominant components of floral scent, and showed a significantly developmental emission. The release amounts of monoterpenes first increased and then decreased during the developmental stages, peaking at the full opening stage. In the following study, the first monoterpene gene in *Lilium*, named *Li-mTPS*, was cloned. Due to the presence of conserved DDxxD motif and RRx8W motif, the deduced Li-mTPS was classified into TPS-b monoterpene synthase subfamily. The tissue-specific expression of *Li-mTPS* revealed the highest level in the outer petals. *Li-mTPS* expression showed similar pattern with monoterpene emission except that the peaking time was earlier than monoterpene emission. It was concluded that the *Li-mTPS* expression played a key role in monoterpene emission from *Lilium* 'siberia' flowers. But other regulatory mechanisms also may contribute to the production and emission, which needs to be investigated in the future study.

Key words: Floral scent; *Lilium* 'siberia'; Monoterpene emission; Monoterpene synthase gene.

INTRODUCTION

Releasing floral scent is an important feature of flowering plants (Knudsen *et al.* 2006). Floral scent produced with other specific morphology such as color, nectar composition contributes to reproductive success by attracting pollinators and by limiting out-crossing with other species, and plays a key role in floral evolution (Galliot *et al.* 2006). The floral scent is a mixture of volatile compounds, belonging to terpenoids, aromatics, and fatty acid derivatives with low vapour pressure and low molecular weights (Tholl *et al.* 2004). Terpenoid compounds are the largest class of plant secondary metabolites and important components of floral scent in a wide range of species (Knudsen *et al.* 1993; Gershenzon and Kreis 1999).

A large number of TPS belonging to a large family have been found in plants, and many TPS genes and proteins have been isolated and characterized from several plants (Chen *et al.* 2011), and more than 200 TPS genes including 90 monoterpene synthase genes have been cloned in more than 40 plant species (Degenhardt *et al.* 2009). Among the monoterpene synthases, linalool synthase gene is the first gene isolated and characterized from the flowers of *Clarkia breweri* (Pichersky *et al.* 1995; Dudareva *et al.* 1996). In the flowers of *Antirrhinum majus*, (*E*)-β-ocimene and myrcene synthase genes are also isolated, and present tissue-specific, developmental, and rhythmic expression (Dudareva *et al.* 2003). In addition, the *TPS* has been successively isolated from many plants' flowers including *Arabidopsis thaliana* (Tholl *et al.* 2005), *Lavandula angustifolia* (Landmann *et al.* 2007), *Actinidia deliciosa* (Nieuwenhuizen *et al.* 2009), and *Alstroemeria* (Aros *et al.* 2012).

Lilium is a world famous fragrant bulb flower. At present, there are more than one hundred cultivars found in market, and new cultivars are continuously cultivated. A significant difference in floral scent is found among different *Lilium* cultivars, which is though to result from different emission of monoterpenes (Zhang *et al.* 2013). The scented oriental hybrid lilies emit much more monoterpenes than Asiatic hybrid lilies (Zhang *et al.* 2013). However, little is known about the emission mechanism of monoterpenes in *Lilium*.

Lilium 'siberia' belonging to the oriental hybrids, is an artificially cultivated famous breed. The flowers of this cultivar are large and white, and can release rich fragrance, so this cultivar is commercially important and highly appreciated by consumers, and is a hot-selling flower. In this study, the monoterpene emission from

Lilium 'siberia' flowers was investigated, and one monoterpene synthase gene, named *Li-mTPS*, in the flowers was cloned, and its tissue-specific and developmental expression was also examined.

MATERIALS AND METHODS

Plant materials: In this study, *Lilium* 'siberia' plants grown from bulbs were used. The bulbs were cultured in plastic pots (20 cm diameter, 20 cm height) containing medium composed of peat and vermiculite at the ratio of 2 to 1. The seedlings were placed in the greenhouse at the Science Park of Beijing University of Agriculture under 16/8 h light/dark 25/20 cycle. They were irrigated every three days and supplied with a full Hoagland nutrient solution every two weeks. The experiments were carried out in August 2013. The whole flowering phase of *Lilium* 'siberia' was classified into four stages including flower bud stage, middle opening stage, full opening stage, and wilting stage. After collection of floral scent, the different tissues of flowers were collected and quickly thrown into liquid nitrogen for gene cloning and expression analysis.

Floral scent collection and analysis: The dynamic headspace sampling was used to collect the floral scent (Zhao *et al.* 2012; Hu *et al.* 2013). An individual flower was put in a Reynolds oven bag (16 × 17.5 IN) which releases and absorbs few volatiles. A stainless steel tube (0.25 × 3.5 IN, USA) containing Tenax-GR (60-80 mesh, Chrompack) was used as the volatile trap, and avoided touching the flower. A portable air sampler (QC-1; Beijing Municipal Institute of Labour Protection, China) served as the pump, and air filtered through a drying column filled with charcoal was pumped into the bag. The volatiles were collected for 20 min at a flow rate of 300 $mL \cdot min^{-1}$. Afterwards, the stainless steel tubes were sealed and placed in a refrigerator.

Then automated thermal desorption - gas chromatography / mass spectrometry (ATD-GC/MS) technique was used to analyze the floral scent. The floral scent collected in the stainless steel tube was desorbed by heating in an ATD (Auto Thermal Desorber, TurboMatrix 650, PerkinElmer) at 260 °C for 10 min, and then cryofocused in a cold trap whose temperature was maintained at -25 °C for 3 min. The cold trap was then quickly heated to 300 °C maintained for 5 min to transport the volatiles to GC (Clarus 600, Perkin EImer). The GC was equipped with a capillary DB-5MS column (30 m × 0.25 mm i.d., with a 0.25 μm film thickness). Helium was used as the carrier gas. The GC was programmed at 40 °C for 2 min, 4 $°C \cdot min^{-1}$ up to 160 °C, then 20 $°C \cdot min^{-1}$ up to 270 °C, and held at 270 °C for 3 min. The MS (Clarus 600T, Perkin EImer) was operated in EI ionization mode at 70 eV, and a mass scan range of 29-600 amu was monitored. Interface and ion source temperatures were 250 °C and 220 °C, respectively.

Preliminary identification of the compounds was made by searching the NIST08 and WIELY library in the TurboMass Ver5.4.2 software and checked according to its retention index. In order to enable the release amounts of volatile components to be compared, α-pinene (Fluka, USA) was used as an external standard. As described previously (Hu *et al.* 2013) with some modification, the α-pinene was dissolved in ethyl acetate with different solution concentrations. $\mu g \cdot h^{-1}$ was used as unit to describe the release amount.

Isolation of the full-length cDNA: The TRIzol method was used to isolate the total RNA from *Lilium* 'siberia' flowers. First-strand cDNA reverse transcribed from total RNA was used as a template for cloning genes. The monoterpene synthase gene sequences on GeneBank were compared, and according to their conserved sequence a pair of degenerate primers, MidTPS-F and MidTPS-R were designed for PCR amplification. The purpose fragment was recycled with plastic recycling kit, and then connected to the cloning vector pMD18-T. The recombinant plasmid was transformed into *E. coli* JM 109 cells. Some single colonies were picked and identified through PCR. Then the positive clones of bacterial colonies were sequenced by Beijing Liuhe Huada Gene Technology Company.

According to the sequences of middle segment, 3' RACE Outer and Inner primers, and 5' RACE Outer and Inner primers were designed and synthesized respectively. The sequence information of 3' and 5' ends was referred to the illustrations of 3'-full RACE Core Set Ver. 2.0 and 5'-full RACE kit from TAKARA. After electrophoresis validation and gel extraction, PCR amplification product was connected to the cloning vector pMD18–T to form recombinant plasmid transformed into *E. coli* JM 109 cells. Positive clones of bacterial colonies were screened and sent to sequencing analysis.

Bioinformatics analysis: The comparison of nucleotide sequence and deduced amino acid sequence was analysed by BLAST tool on the http://www.NCBI.com website. ORF finder tool was used to analyse open reading frame. Homology and phylogenetic evolution of amino acid sequence and members of the gene family known were analysed by DNAMAN software. Molecular weight and isoelectric point of the protein were predicted by the online software ProtParam (http://web.expasy.org/protparam/) on ExPASy.

Gene expression analysis: Total RNA was extracted from different tissues of *Lilium* 'siberia', including petals, stamens, pistil, and leaves, cDNA reverse transcribed from total RNA was used as reaction template. Primers of RTTPS-F and RTTPS-S were designed for real-time PCR

amplification. Reaction system was 25 μL, the amount of each reagent was referenced SYBR®*Premix Ex Taq*™II instruction. Real-time PCR was proceeded on Bio-Rad apparatus, the program was: 95 degeneration 3 min, 40 cycles of 95 for 30 s, 55 for 30 s, 72 for 30 s, fluorescent signal acquisition in 55 . Three sample replications were run.

Statistical analysis: The data obtained after experimentation was statistically evaluated using ANOVA at significance level of $P < 0.05$

RESULTS

The developmental patterns of monoterpenes: The floral scent emitted from single, living flower at different four stages were collected by headspace method and then examined by ATD-GC/MS technique. The total release amount of floral scent first increased and then decreased along with the flower development, and significant difference in the total release amount was found at different flowering stages (Fig. 1, $P < 0.05$). Compared to at the flower bud stage, a strong increase in total release amount was found at the middle opening stage, and increased to the maximum at the full opening stage followed by a sharp decrease at the wilting stage. The total release amount at the full opening stage was nearly 12-fold and 6-fold higher than that at the flower bud stage and wilting stage respectively. Among the floral scent components, the release amount of monoterpenes exhibited the highest proportion, and showed the similar pattern with the total release amount. At the full opening stage, the release amount of monoterpenes increased to the peak, and was significantly higher than other stages ($P < 0.05$), which almost accounted for 80% of the total release amount. It could be found that the release amount of other volatile compounds except monoterpenes also first increased and then decreased along with the flower development, but the change extent was lower than monoterpenes, and no significant difference was found between the middle opening stage and the full opening stage ($P < 0.05$). The significant increase and decrease in the total release amount was dominantly contributed by the remarkable change in release amount of monoterpenes. So the monoterpenes played a crucial role in change pattern of the total release amount along with flower development, and were the most dominant components in floral scent of *Lilium* 'siberia'.

Seven (7) monoterpenes were detected in the floral scent of *Lilium* 'siberia', and their release amounts also first increased and then decreased along with the flower developing, (Fig. 2). Their maximum release amounts all appeared at the full opening stage, which was significantly higher than those at other three stages ($P < 0.05$). Among these 7 monoterpenes, linalool showed the highest release amount followed by α-ocimene and β-myrcene, and at the full opening stage the release amounts of these three volatile compounds accounted for nearly 50%, 31%, and 9% of the total release amount of mononterpenes. The release amount of α-pinene was the lowest, which was only 1/64 of the release amount of linalool at the full opening stages.

Isolation and characterization of monoterpene synthase: The total RNA was extracted from the flower of *Lilium* 'siberia' at the full opening stage. The total RNA was first reverse transcribed into cDNA as a template, with degenerate primers for PCR amplification. A 772 bp amplified product was obtained after recovering and sequencing. The nucleotide sequence of the 772 bp DNA fragment revealed high homology with monoterpene synthase genes of plants through BLAST comparative analysis. Based on this conserved sequence, 656 bp and 700 bpamplified products were obtained through the nested PCR amplification of 3’ and 5’ ends respectively. After three-fragment assembly, a full-length monoterpene synthase gene of 1507 bp in the flower of *Lilium* 'siberia' was obtained, named *Lilium monoterpene synthase* (*Li-mTPS*).

Li-mTPS was analyzed by DNAMAN software, and it was found that the gene contained an open reading frame (ORF) of 1410 bp, a poly (A) tail, and an untranslated region with 5' UTR of 36 bp and 3'UTR of 49 bp. The deduced protein of the coding region containing 469 amino acid residues, whose theoretical molecular weight was 55.04 kDa and isoelectric point was 4.85 (Fig. 3). The homology analysis of protein sequence revealed 53% homology with myrcene synthase in *A. peruviana* flowers, 52% homology with linalool synthase in *Freesia hybrid* flowers, and 50% homology with linalool synthase in *Hedychium coronarium* flowers (Fig. 4). It was also found that this protein contained the DDxxD motif, a terpene synthase conserved base sequence, and the RRx8W motif, an angiosperm monoterpene synthase.

The evolutionary relationship between Li-mTPS and the related monoterpene synthases in other plants was also analyzed. The phylogenetic tree exhibited Li-mTPS had the closest genetic relationship with myrcene synthase in *A. peruviana* flowers, and had the longest-distanced relationship with the monoterpene synthase of *A. majus* (Fig. 5).

Tissue-specific and developmental expression of Li-mTPS: To determine tissues specificity, the *Li-mTPS* expressions in leaves and floral tissues (floral outer petals, inner petals, stamens and pistil) were examined (Fig. 6 a). It was found that there was no detectable expression of *Li-mTPS* in *Lilium* 'siberia' leaves, so *Li-mTPS* was expressed in floral tissues specifically, and different expression levels were showed among these tissues. *Li-mTPS* was expressed at significant levels in of *Lilium* 'siberia' tepal tissues. The highest expression level

was observed in the outer petals, which was nearly two folds as much as that in the stamens, where the *Li-mTPS*was expressed at the lowest level.

The expression patterns of *Li-mTPS* in the outer petals at different flowering stage were investigated. It was found that during flower developing the *Li-mTPS* expression level also first increased and then decreased (Fig. 6 b). The general pattern was similar to the monoterpene emission, but the highest level of expression occurred at the middle opening stage not at the full opening stage, the time difference was observed between these two peaks. Following the high expression level at the full opening stage, a sudden drop occurred at the wilting stage, when *Li-mTPS* expressed at the lowest level.

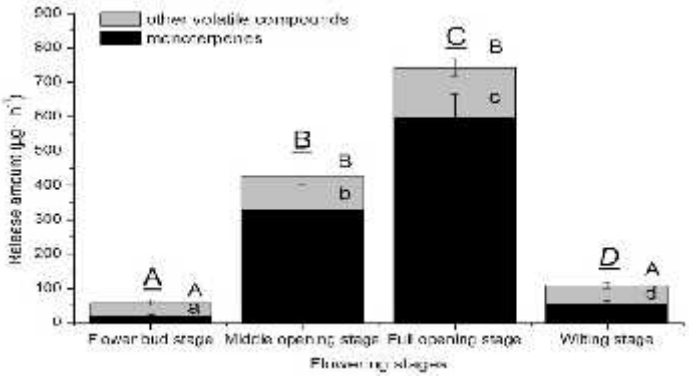

Fig. 1.The release amounts of floral scent emitted from *Lilium* 'siberia' flowers at different flowering stages. Each bar is the average of three independent replications, and standard errors are shown. Statistical significance [least significant difference (LSD) test] of difference in total release amount of floral scent, the release amount of monoterpenes, and the release amount of other volatile compounds except monoterpenes among flowering stages are indicated by different capital letters with an underline, different small letters, and different capital letters respectively ($P < 0.05$).

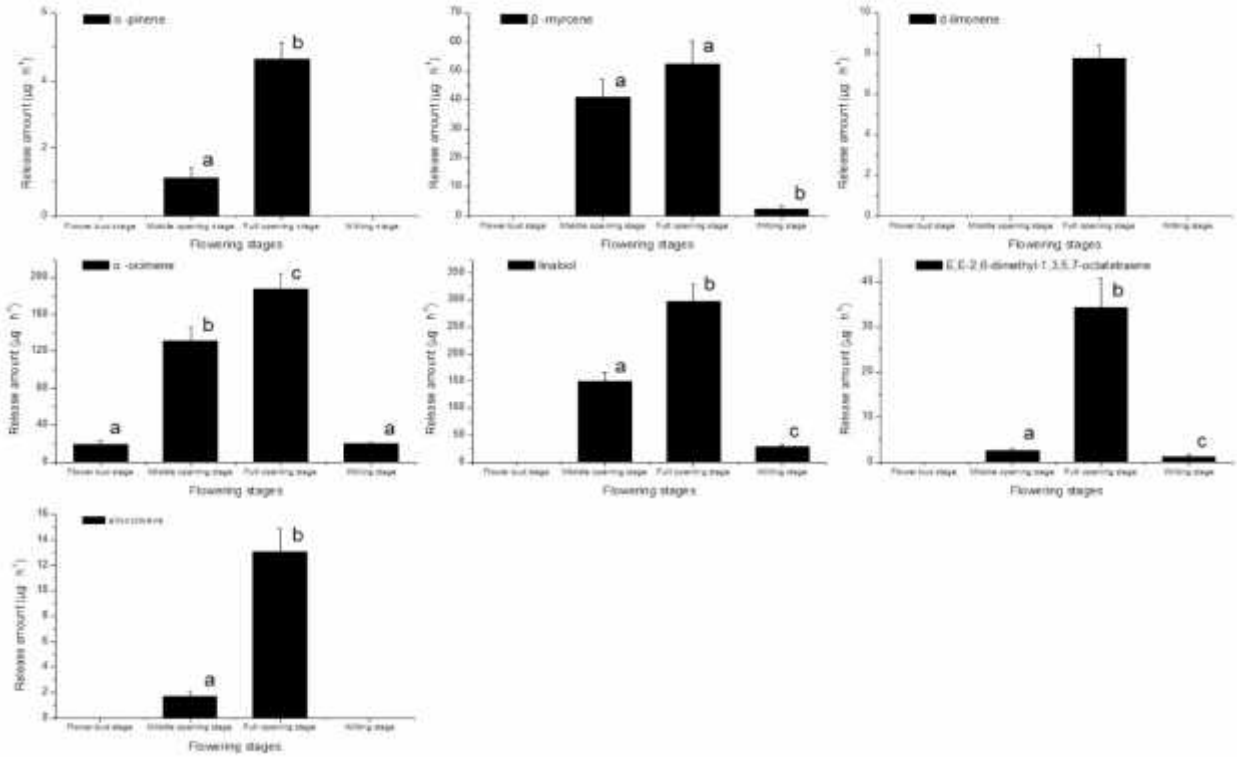

Fig. 2.The release amounts of 7 monoterpenes in the floral scent of *Lilium* 'siberia' at different flowering stages including α-pinene, β-myrcene, d-limonene, α-ocimene, linalool, E,E-2,6-dimethyl-1,3,5,7-octatetraene, and alloocimene. Each point is the average of three independent replications, and standard errors are shown.

Statistical significance [least significant difference (LSD) test] of difference among flowering stages is indicated by different small letters ($P < 0.05$).

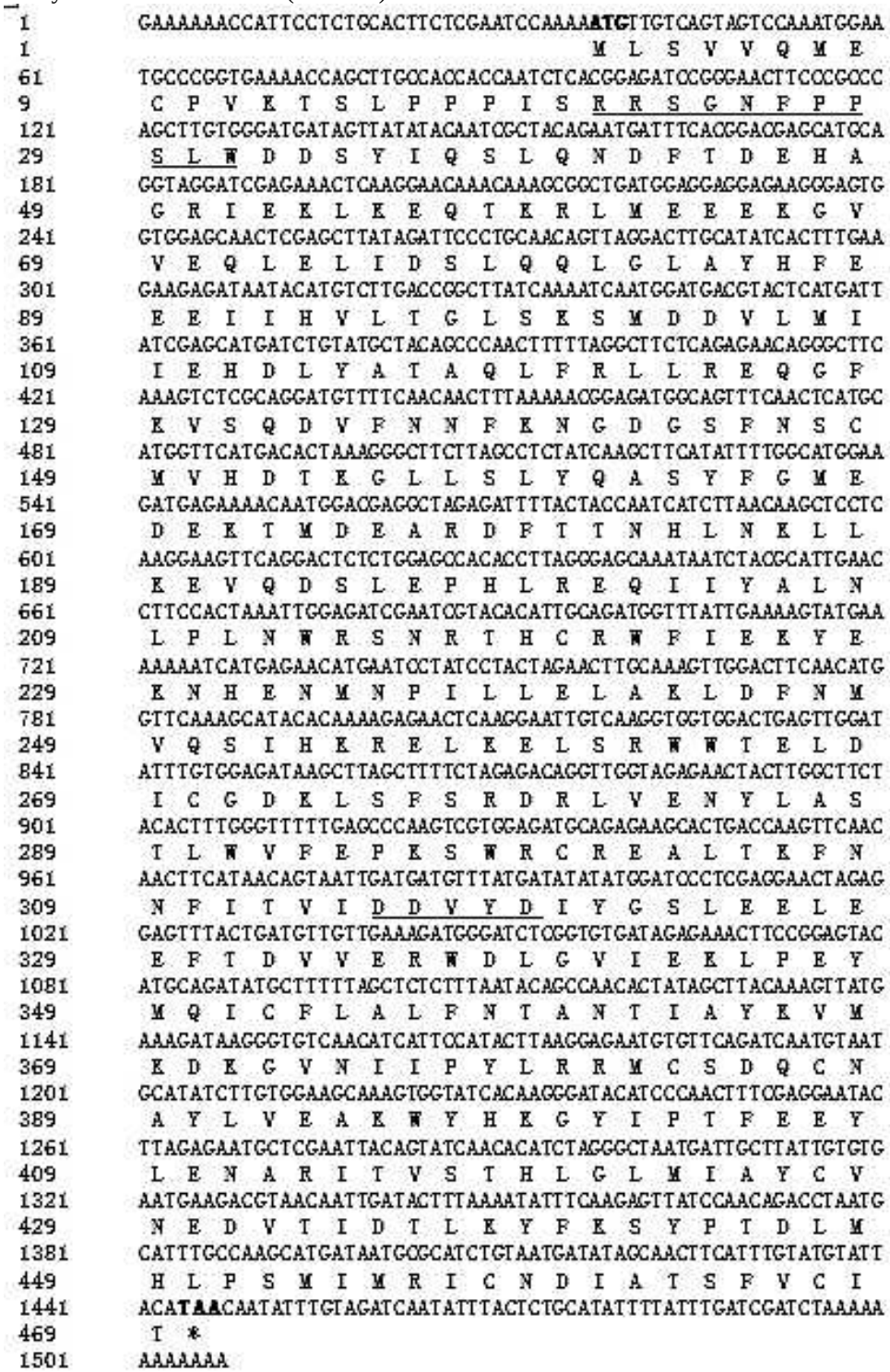

Fig. 3.The nucleotide and deduced amino acid sequences of *Li-mTPS* cNDA. The predicted amino acid sequence was shown directly below the nucleotide sequence. The asterisk indicated the stop codon .Experimentally determined amino acid sequences were underlined.

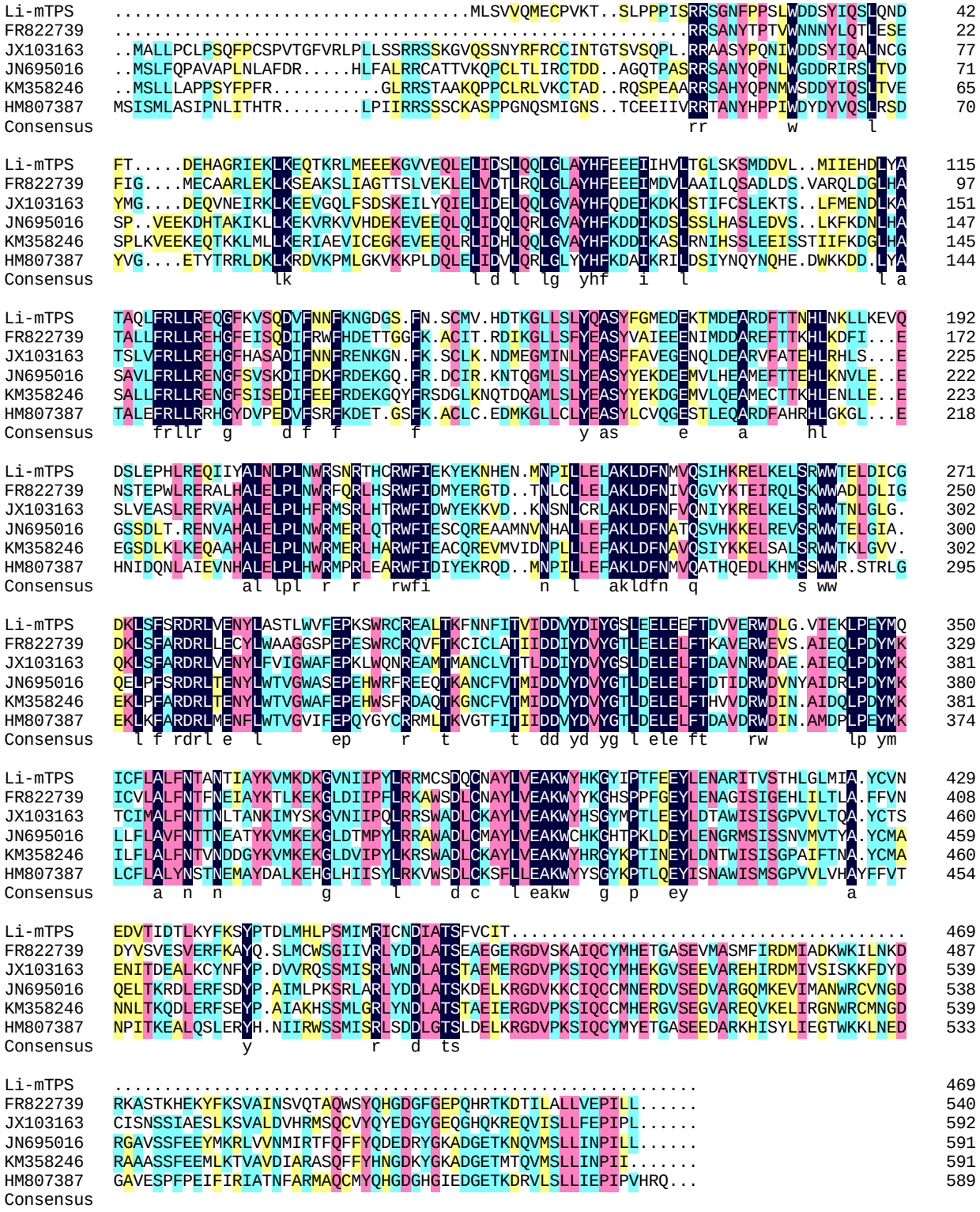

Fig. 4.Comparison of the predicted amino acid sequence of Li-mTPS to related proteins. Black, red, green, yellow shadow represented homology =100%,>=75%,>=50%, and >=33% respectively. The underlined parts indicated conservative region. Li-mTPS: *Lilium* Monoterpene synthase; FR822739: *Alstroemeria* Myrcene synthase; JX103163: *F. hybrid* Linalool synthase; JN695016: *H.coccineum* Linalool synthase; KM358246: *H. coccineum* Terpene synthase; HM807387: *V. vinifera* Myrcene synthase/Ocimene synthase.

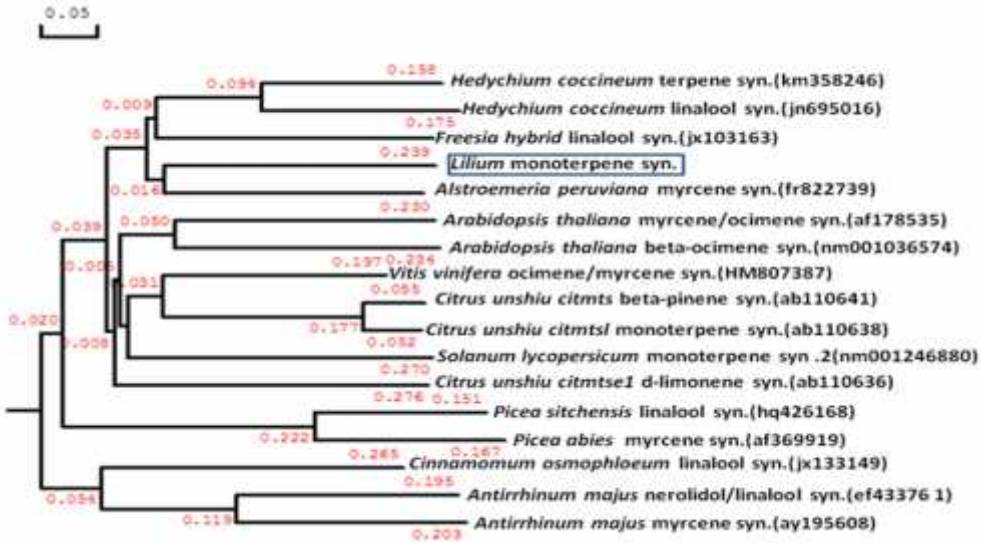

Fig. 5.Phylogenetic tree of the amino acid sequences. Li-mTPS had closest genetic relationship with myrcene synthase in *A. peruviana* flowers. The monoterpene synthase of *A. majus* belonging to TPS-g group presented the longest distance to Li-mTPS.

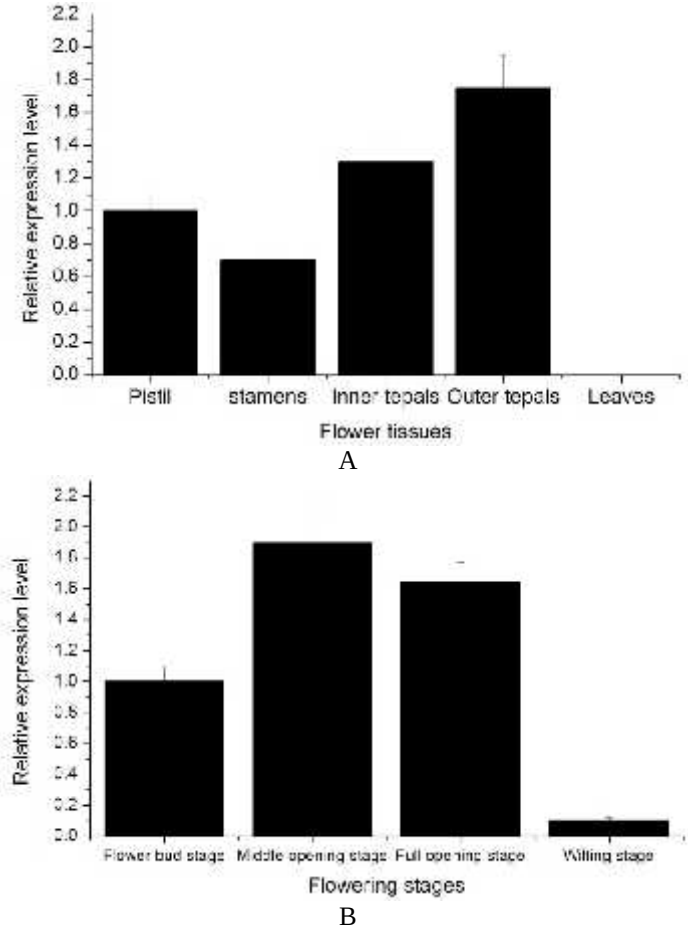

Fig. 6.The tissue-specific(A) and developmental(B) expression of *Li–mTPS* in *Lilium* 'siberia' flowers. The *Li–mTPS* expression level in different tissues was evaluated by RT-PCR, and the expression level in pistil was taken as 1. In the analysis of developmental expression, the expression level at early opening stage was taken as 1. Each point is the average of three independent experiments.

DISCUSSION

As an important part of plant volatile compounds, the floral scent plays a key role in plant ecophysiology, and represents a decisive communication channel between plants and animals (de Vega *et al.* 2014). Moreover, for ornamental flowers, floral scent is thought to be an important characteristic to evaluate flowers. So, more and more researchers focused on floral scent in recent years (Grausgruber-Grögera *et al.* 2012; Demissie *et al.* 2013; Sharkey *et al.* 2013). In this study, the emission of monoterpenes from *Lilium* 'siberia' flowers was detected, and a synthase gene was cloned, followed by the expression of gene under study.

The monoterpenes were found to be emitted from *Lilium* 'siberia' flowers at a high level, and showed a significant developmental pattern during the flower development: first increase and then decrease. Due to the high relative content of monoterpene, especially at the full opening stage when the release amount constituted 80% of the floral scent emitted from *Lilium* 'siberia', monoterpenes were thought to be the most important components playing a dominant role in the floral scent of *Lilium* 'siberia'. Moreover, among the 7 monoterpenes detected, linalool and α-ocimene were emitted at a high level, and the sum of release amounts of these two components accounted for more than 80% of the total monoterpene compounds. Monoterpenes has been also found to contribute significantly to the floral scent of numerous plant species (Chen *et al.* 2003; Yu and Utsumi 2009; Fähnrich *et al.* 2011). It has been reported that gene expression of monoterpene synthase play a key role in the emission pattern of monoterpne (Pichersky *et al.* 1994; Dudareva *et al.* 1996; Dudareva *et al.* 2003).

In previous study, a classification of TPS is divided into seven clades: TPS-a, TPS-b,TPS-c, TPS-d, TPS-e/f, TPS-g, and TPS-h, and the genes encoding monoterpene synthases fall into four clades: TPS-b, TPS-d, TPS-e/f, and TPS-g (Bohlmann *et al.* 1998). In this study, a new monoterpene synthase gene (*Li-mTPS*) was isolated from the flowers of *Lilium* 'siberia'. In the sequence of deduced protein, the RRx_8W motif was found, which was characteristic of other monoterpene synthases of the angiosperm TPS-b group and gymnosperm TPS-d. In addition the Li-mTPS showed relatively high sequence relatedness with other TPS-b monoterpene synthases, so it was a member of TPS-b subfamily. *Li-mTPS* was the first monoterpene synthase gene involved in the biosynthesis and emission of floral scent in the flowers of *Lillium.*

Previously, monoterpene synthase gene has been discovered in some plant floral tissues. In *C. breweri* flowers, the mRNA transcripts of linalool synthase gene accumulate to a high level in pistil and styles, but a lower level is present in petals and stamens (Dudareva *et al.* 1996). In *A. majus* flower tissues the highest-level expression of β-ocimene and myrcene synthase genes are found in the upper and lower lobes of petals, and a very low level of transcripts is detected in the tube and stamens, but no detectable signals are found in pistils, sepals, and leaf tissues (Dudareva *et al.* 2003). Using real-time PCR technique, high levels of *Li-mTPS* expreression were found in petals, especially in outer tepals. It was found that in *Alstroemeira* flowers, a monoterpene synthase gene *AlstroTPS* also exhibited a high-level expression in outer tepals (Aros *et al.* 2012). The tissue-specific expression of volatiles synthase genes was thought to be related to their main production and emission sites.

In addition, the developmental expression of the *Li-mTPS* was found in the petals of *Lilium* 'siberia' flowers. The gene expression level was found to reach the maximum at the middle opening stage, and presented a relative high level at the full opening stage followed by a sudden decrease at the wilting stage. Though the similar pattern was found between *Li-mTPS* expression and the monoterpene emission, the peak of *Li-mTPS* expression was earlier than monoterpene emission. The previous study reveals that the steady state mRNA levels of *monoterpene synthase* in snapdragon petals peaks on day 4 after anthesis, 1 to 2 days ahead of maximum emission (Dudareva *et al.* 2003). On one hand this positive correlation indicated that the changes in transcript level was an important determinant of monoterpene production and emission, while on the other hand, the delayed emission implied that the developmental emission of monoterpenes was regulated by diverse factors besides gene expression. So the possible regulatory mechanisms at the precursor, the post-transcriptional, translational, and post-translational levels could not be excluded, which will become a focus in future research.

Conclusion: In summary, it was found that monoterpnenes were the dominant components in the floral scent emitted from *Lilium* 'siberia', and showed significantly developmental pattern. Then *Li-mTPS*, the first monoterpene synthase gene was cloned from *Lilium*, presenting the highest-level expression in petals. The similar pattern between *Li-mTPS* expression and the monoterpene emission indicated that *Li-mTPS* expression played an important role in the monoterpene emission. But the gene function and the different regulatory mechanisms on monoterpene emission, especially at the post-transcriptional, translational, and post-translational levels should be investigated in the future studies.

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