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Identification and functional characterisation of a new terpene synthase gene from *Chrysanthemum indicum* var. *aromaticum*

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Abstract: Terpene synthase (TPS) is a key enzyme in the synthesis of terpenoids in plants and plays an important role in the regulation of the synthesis of aromatic substances in plants. In this study, we treated *Chrysanthemum indicum* var. *aromaticum* as the experimental material to clone the *CiTPS* gene. The open reading frame of *CiTPS* is 1 818 bp, encoding a putative protein of 605 amino acids. There is a monoterpene synthase conserved domain and a highly conserved sequence, DDXXD, (N,D)D(L,I,V)X(S,T)XXXE and RRX8W, respectively, which belong to the monoterpene synthase family. The multiple sequence alignment and phylogenetic analysis showed that the *CiTPS* gene belongs to the TPS-b subfamily. The pBI121-TPS-GFP recombinant plasmid and the pBI121-GFP empty vector plasmid were introduced into *Agrobacterium* by electroporation and transferred into wild-type tobacco by the *Agrobacterium*-mediated method. Three transgenic CiTPS lines were screened, and the secretions of wild-type tobacco and transgenic tobacco T1 leaves in the vigorous growth period were analysed. The volatiles of the transgenic lines were similar to those of wild-type tobacco, but their monoterpenes increased significantly, and the sesquiterpenoids and diterpenoids decreased or remained unchanged, indicating that the *CiTPS* gene is related to the synthesis of the monoterpenes.

Keywords: overexpression; *CiTPS* gene; GC/MS; terpene synthase; monoterpene; gene cloning

Chrysanthemum indicum var. *aromaticum* is a new variety of *Chrysanthemum indicum* (He et al. 2013). With a rich aroma in its flowers, leaves and roots, it is a rare aromatic ornamental flower in the *Chrysanthemum* genus. Studies have found that (–)-α-thujone, (+)-β-thujone, borneol and other terpenoids are the main components of *C. indicum* var. *aromaticum* (Jian et al. 2014). Terpene synthase (TPS) is a key enzyme that is studied in the biosynthesis of steroids. According to the structure and reaction mechanism, TPS in plants can be mainly divided into two categories. Class I refers to the ionisation-dependent TPS caused by the removal of the pyrophosphate groups by metal ions such as Mn^{2+} and Mg^{2+}(Tholl 2015). Class II refers to the protonation-dependent TPS that causes the pyrophosphate group to leave by epoxidation or carbon-carbon double bond protonation (Tholl 2015). The TPS family can be divided into seven subfamilies from TPSa-TPSg according to the correlation of its amino acid

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sequence, and there is no less than a 40% homology between the different amino acid sequences of the same subfamily. Among them, the TPS-a subfamily mainly contains diquat and sesquiterpene synthase of angiosperms (Bohlmann et al. 1998; Dudareva et al. 2003). The monoterpene synthases in the TPS-b subfamily are all present in angiosperms, the amino acid sequences of the enzymes contain one RRX_8W motif (Cheng et al. 2007), the steroidal synthases in the TPS-d subfamily are mainly found in gymnosperms, and their monoterpene synthases also have one RRX_8W motif (Martin et al. 2002). There are only four angiosperms in the TPS-g subfamily, which lack the RRX_8W motif (Yang et al. 2016), and the remaining three subfamilies contain only one or two steroidal synthases (Chen et al. 2011). According to the structural characteristics of TPS genes, terpene synthases in plants are divided into three categories. The first class contains 12–14 introns and 12–15 exons, and this monoterpene synthase is currently only found in a few species, such as *Arabidopsis* (Cai et al. 2002). The second category contains nine introns with 10 exons, which is a unique type of gymnosperm synthase gene. The third type contains only six introns and seven exons. The genes involved in the secondary metabolism of monoterpenes, sesquiterpenes, and catepenoids in plants contain most of the TPS genes of angiosperms, and their introns are conserved among all the plant steroid synthases. However, there are also five introns in some plants, such as *Fragaria* × *ananassa* Duch. Each monoterpene compound has two α-helix regions at the C-terminus and the N-terminus, wherein the C-terminus is the active region, and the R-D carbon-hydrogen bond formed by the N-terminal RRX_8W and DDXXD in the conserved region of arginine not only maintains the structural stability of the terpene, but also has an extremely important heterogeneous function (Xu et al. 2009). Therefore, most monoterpene synthases can catalyse the formation of different products (Peters, Croteau 2003; Landmann et al. 2007).

Terpene synthase genes can effectively regulate the species and content of terpenes in plants, and they vary in species and function. It is a key enzyme gene in the terpene metabolic pathway. A previous study found an essential gene named CiDXR that participates in the regulation of terpenoid synthesis pathways (Gao et al. 2018). To clarify the specific mechanism of terpene synthesis in *C. indicum* var. *aromaticum*, we searched through the transcriptome data of *C. indicum* var. *aromaticum* treated with methyl jasmonate in the early stage and found an upregulated gene in the terpene synthesis pathway. After cloning and identifying the function of this gene, we found it to be a monoterpene synthase (*TPS*) gene. Bioinformatics was used to analyse the main structure and function of the gene and then the *TPS* gene was transferred into the model plant *Nicotiana tabacum* to further discover the features of the *TPS* gene. The objective of this study was to study the aromatic value of *C. indicum* var. *aromaticum,* which could lay a solid foundation for aromatic plant applications.

MATERIAL AND METHODS

Plant material. The *Chrysanthemum indicum* var. *aromaticum* used in this study was introduced from the Shennongjia Region (110'23"57E, 31'28'7"N) in Hubei Province, which was then cultivated in the flower nursery of the Landscape Architecture Department, Northeastern Forestry University. We took cuttings from plants in good condition and planted them in a greenhouse (45°43'15.1"N, 126°37'53.9"E). The plant growth media contained 30 g/L sucrose and 0.6% (w/v) agar and was adjusted to pH 5.8–6.0 and autoclaved at 121 °C for 20 min. The controlled conditions of the greenhouse were as follows: 50–70% relative humidity, 20–30 °C, and 12 h of light alternating with 12 h of darkness, with a G13 fluorescent lamp (Philips, Tianjin, China) as a light source, the light intensity was 75 μmol/m^2/s. The plants were grown in plant culture pots (11 cm high × 7 cm diameter, 300 mL). After rooting, which generally took two weeks, the cuttings were transplanted into pots. Plants with 6–8 leaves were used for the experiments.

RNA extraction and cDNA synthesis. The total RNA was isolated from 100 mg of *C. indicum* var. *aromaticum* leaf tissues using the TRIzol Universal Reagent (TIANGEN) according to the manufacturer's instructions. The quality and concentration were checked by 0.8% agarose gel electrophoresis and nucleic acid protein detection, and the total qualified RNA was stored at –80 °C for further use. The cDNA was synthesised from the total RNA according to the instructions of the ReverTra Ace qPCR RT Master Mix with gDNA Remover, and then the cDNA was stored at –20 °C for further use.

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Cloning of the *CiTPS* gene. The TPS genes (accession number of MH124737) were obtained from the analysis of the transcriptome sequencing data of *C. indicum* var. *aromaticum* in the early stage of the research group (Gao et al. 2020). The primers of the open reading frame (ORF) of the *TPS* genes were designed using Primer 5.0:
TPS-F: 5'-ATGAATGATTCCAGCATCTTAAGATT-3'
TPS-R: 5'-TTATATCCCTTGGATTGGAGTAAACA-3'

Polymerase chain reaction (PCR) was performed using the following system: 1 μL cDNA, 0.4 μL TPS-F/TPS-R, 0.2 μL Taq enzyme, 2 μL 10 × buffer, 2 μL dNTPs, and 14 μL ddH_2O. The extension of the PCR was performed by the following procedures: 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. Then, the PCR was terminated at 72 °C for 5 min and finally stored at 4 °C until further use.

The PCR product was recovered according to the instructions of the agarose gel DNA recovery kit, and the purified DNA fragment was ligated to the pUC-T cloning vector at a molar ratio of 3 : 1 with the following system: 3 μL DNA inserting sequence, 1 μL pUC-T, 5 μL 2 × Quick Ligation Reaction Buffer, and 1 μL Quick T4 DNA Ligase. Then, the connection product was transformed into competent cells of *Escherichia coli* DH5α strains, and the top ten monoclonal colonies that grew in a Luria-Bertani (LB) anti-Amp solid medium were selected. The positive recombinants were identified by PCR screening. The identified bacteria were expanded for culture, and plasmids were extracted from well-cultivated microbes according to the plasmid DNA small extraction kit instructions. The effective plasmids were sequenced by the BOSHI Biology Company.

The cDNA from the total RNA reverse transcription of the *C. indicum* var. *aromaticum* leaves were utilised as the templates, and the target fragment was obtained by PCR amplification (Figure S1 in Electronic Supplementary Material (ESM)). The obtained product was connected to the pUC-T vector and converted into competent *E. coli* DH5α strains. The top three bands of bright clones from ten positive clones filtered out by the PCR amplification were sequenced (Figure S2 in ESM).

Bioinformatics analysis of the gene sequences. The *TPS* gene sequences that were successfully sequenced were translated to amino acid sequences by the DNAMAN software (DNAMAN 6.0; San Ramon, CA, USA), and the molecular characteristics, physical and chemical properties, homology and systematic evolution of the TPS gene sequences were predicted and analysed using the bioinformatics analysis websites ProtParam (https://web.expasy.org/protparam/), ProtScale (https://web.expasy.org/protscale/), SOPMA2.0 (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), SWISS-MODEL (https://swissmodel.expasy.org/) DNAMAN 6.0, MEGA 5.1 (PA, USA), and RasMol_2.7.5.2 (Bellport, NY, USA), while multiple sequence homology alignments were completed using the National Center for Biotechnology Information website (NCBI) (https://www.ncbi.nlm.nih.gov/). Three-dimensional homology modelling was conducted using the SWISS-MODEL Alignment Mode (Figure 1).

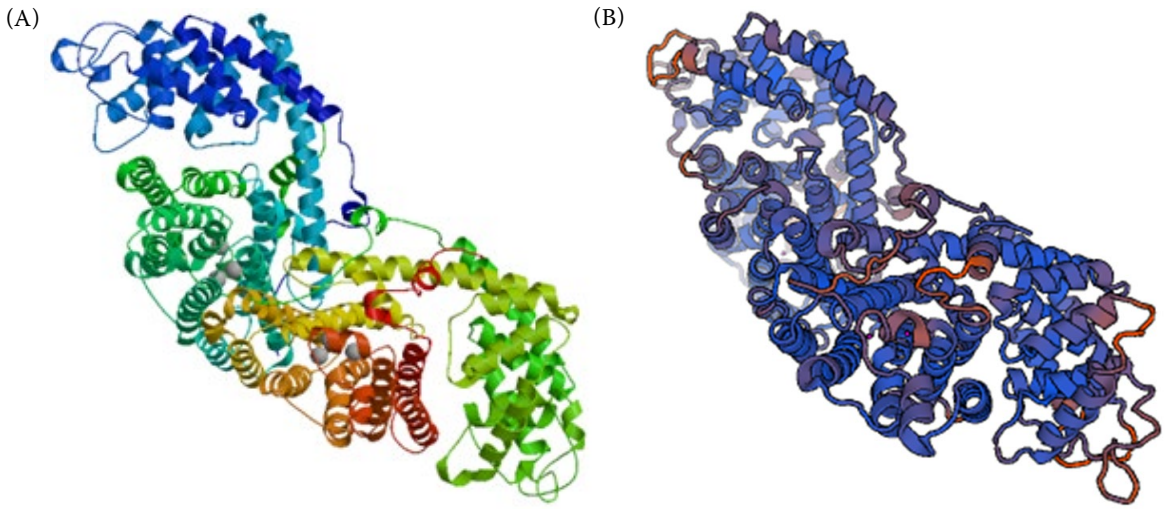

Figure 1. The deduced 3D structure of the CiTPS protein via the SWISS MODEL software: ribbons (A), wireframe (B)

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Construction of the plant expression vector and transformation. The target gene sequences were amplified with two specific primers, *CiTPS*-Sma I and *CiTPS*-Spe I, containing an SmaI and an SpeI restriction spite, and the well-recycled target gene fragments were connected with the pUC-T cloning vector. The intermediate expression vector pUC-T-TPS was produced. According to the proportion of 1 : 100, the positive clonal bacterial solution with the addition of enzyme digestion sites and correct sequencing was further expanded in the LB liquid medium (containing 50 mg/L Amp), and *Escherichia coli* (–80 °C, 35% glycerin) containing the pBI121-GFP empty vector was cultivated on the LB solid medium (containing 50 mg/L Kana) and then added to the LB liquid medium. The positive clone bacterium and the pBI121-GFP plant expression vector plasmid were extracted according to the plasmid DNA kit. After the double digestion of the pUC-T-TPS and pBI121-GFP plasmids with SmaI and SpeI restriction enzymes, respectively, the well-recycled target fragments of *CiTPS* and large fragments of the pBI121-GFP expression vector were connsected with T4-DNA ligase. The recombinant plasmid and the pBI121-GFP empty vector detected by the PCR and restriction enzyme digestion were transferred into *Agrobacterium*.

The genetic transformation of tobacco was infested by the *Agrobacterium*-mediated method according to the method of Gao (2019). Adventitious buds growing to 2-3 cm were transferred to a Murashige and Skoog (MS) rooting screening medium containing 200 mg/L Cef, 50 mg/L kanamycin and 0.1 mg/L NAA to induce rooting, and then 1–2 g of leaves from healthy rooting plants were stored at –80 °C for identification of the transgenic tobacco plants. The remaining plants were grown, and the T_0 seeds were collected.

Gene expression level analysis of the CiTPS using qRT-PCR. Real-time quantitative reverse transcription PCR (qRT-PCR) was used to detect the relative expression of the *TPS* gene in the different transgenic tobacco lines using reverse-transcribed cDNA as a template and three repeats in each group. Using the TransStart® Top Green qPCR SuperMixq kit to establish a 20-μL reaction system, the *CmEF1α* gene (KF305681) was used as the internal reference gene of the amplified fragment, and the *CiTPS* gene primer sequence was designed as follows:

CiTPS-qRT-F: 5'- AGCAAATACATTGAAGGACGCA -3'
CiTPS-qRT-R: 5'- CGATTTCATTCCACTTTTCAGG -3'

The qRT-PCR system was formed using the following components: 10 μL TransStart® Top Green qPCR SuperMix, 0.4 μL for each primer, 1 μL cDNA template, and 8.2 μL ddH_2O.

qRT-PCR was used to analyse the expression of the *CiTPS* gene in the different tobacco strains. We placed the PCR tube in a Light Cycler®96 (Switzerland) 96-well plate for the reaction. The reaction procedure was as follows: first preincubated at 95 °C for 30 s, and then performed a three-step amplification at 95 °C/5 s, 60 °C/15 s, and 72 °C/30 s, then dissolved at 95 °C/10 s, 65 °C/60 s, and 97 °C/1 s, and finally cooling at 37 °C for 30 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the expression richness of the genes.

The PBI121-TPS-GFP recombinant plasmid and the PBI121-GFP empty plasmid were used to transform the tobacco leaves. After two days of coculture, tobacco was induced to differentiate, by inoculation, on the screening medium. The edge of the leaf tray produced a callus, which then differentiated into adventitious buds. The adventitious buds were cut and inoculated on a kanamycin (KANA)-resistant rooting screening medium. After two rooting screenings, the tobacco seedlings with normal rooting and good growth were preliminarily identified as transgenic positive plants.

Detection of the leaf terpenoids by GC-MS. We selected 1 g samples, quickly ground them into powder with liquid nitrogen and transferred them to a 100 mL flask and added 100 mL of dichloride methane, which was extracted by ultrasonication for 20 min, and the dichloromethane was filtered out. The residue was extracted three times in the same way. The collected extract was filtered into a round bottom flask with a funnel containing 20 g of anhydrous sodium sulfate. Then, a rotary evaporator was used to concentrate the solution at 38 °C, and the resulting concentrated solution was transferred to a brown sample bottle, diluted to 0.5 mL and stored in a refrigerator at 0 °C for later use.

An Agilent 7890A-5975C GC/MS GC/MS (dual gas chromatography/mass spectrometry) (USA) was used to analyse and detect the leaf secretions. GC conditions: HP-5 capillary column (60 m × 0.25 mm × 0.25 μm); the injection volume was 1 μl, and the split ratio was 10 : 1; the inlet temperature was 280 °C; the column flow rate was 0.8 mL/min (helium, constant flow); the AUX temperature was 290 °C; the program temperature increase starting from 50 °C, was maintained for 4 min, increased

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at 10 °C/min to 160 °C and held for 5 min, then increased to 220 °C at 3 °C/min, held for 10 min, then increased to 280 °C at 3°C/min, and held for 40 min. MS conditions: EI mode ionisation mode, set quadrupole temperature to 150 °C, ion source temperature of 230 °C, interface temperature of 280 °C, ionisation energy of 70 eV, electron multiplier voltage of 2 100 V, scanning range from 4 to 500 u, Standard Mass Spectrometry Library NIST08L.

Identification method: Turbo Mass5.4.2GC/MS software was used to analyse the volatiles in the tobacco leaves. After searching the NIST08 database for the different mass spectral peaks of the analysed total ion map, the retention index of the component to be tested was compared with the standard substance in the Pherobase database (https://www.pherobase.com) to determine its category. The relative content of each component was calculated using the peak area normalisation method.

RESULTS

Cloning and sequence analysis of CiTPS from *C. indicum* var. *aromaticum*. The cDNA from total RNA reverse transcription of *C. indicum* var. *aromaticum* leaves were utilised as templates, and the target fragment was obtained by PCR amplification (Figure S1 in ESM). The obtained product was connected to the pUC-T vector and converted into competent *E. coli* DH5α strains. The top three bands of bright clones from ten positive clones filtered out by PCR amplification were sequenced (Figure S2 in ESM). The results showed that the gene sequence had a complete open reading frame (ORF), and its sequence length was 1 818 bp, encoding a polypeptide chain of 605 amino acids . This sequence has a typical conserved domain and highly conserved functional sequence of monoterpenoid synthase, namely, DDXXD, (N,D)D(L,I,V)X(S,T) XXXE and RRX_8W, which indicates that the cloned gene belongs to the monoterpenoid synthase family. The gene sequence was named *CiTPS*, and the sequence was registered in GenBank under accession number MH124737.

CiTPS multiple sequence alignment and phylogenetic analysis. The homology alignment with the *CiTPS* sequence by BLASTp indicated that *CiTPS* shared high sequence identity with two trans-linalool synthases, QH1 Q9SPN0.1 and QH5 Q9SPN1.1, from *Artemisia annua*, up to 91% and 89%, respectively, but it had lower than 70% identity with those in other plants, for example: *Arabidopsis thaliana* and *Salvia officinalis*. Figure 2 shows the results of the multiple sequence alignment by DNAMAN between the amino acids encoded by *CiTPS* and the monoterpene synthase amino acid sequences of seven species (*Artemisia annua* Q9SPN0.1, *Populus trichocarpa* AII32477.1, *Cephalotus follicularis* GAV86002.1, *Vitis vinifera* ADR74202.1, *Ricinus communis* B9T536.1, *Actinidia chinensis* AID55337.1 and *Morus notabilis* XP_010088018.1). The typical conserved domains and highly conserved functional sequences of monoterpene synthase, DDXXD, (N,D)D(L,I,V)X(S,T)XXXE and RRX_8W, appeared in each species, and the CiTPS protein had the highest similarity with the amino acid sequence of *Artemisia annua*.

The phylogenetic analysis among the CiTPS protein and TPS proteins of the other 23 species showed consistent results with the outcome of the homology alignment. The protein encoded by CiTPS clustered into the same subgroup as *Artemisia annua* Q9SPN0.1 and *Cynara cardunculus* var. *scolymus* KVG47623.1, which all belong to Asteraceae, but the evolutionary distance of TPS from *Arabidopsis thaliana* TPS10 and TPS of *Salvia officinalis* O81191.1, *Lavandula officinalis* ABD77417.1 and other species increased in turn (Figure 3).

Prediction of the secondary and tertiary structures of CiTPS. The secondary structure of Cats was predicted with SOPMA2.0. The protein was composed of 57.85% α-helices, 13.06% extended strands, 8.26% β-turns and 20.83% random coils, in which random coils and α-helices were the main components (Figure S3 in ESM).

qRT-PCR analysis of the different transgenic tobacco lines. The tobacco genetic transformation and plant regeneration are shown in Figure 4, where Figure 4A shows resistant buds growing from infected tobacco leaves on the screening medium; in Figure 4B, resistant buds were rooted on the rooting screening medium. Figure 4C depicts normal growth of the resistant seedlings.

Since the *CiTPS* gene was not expressed in wild-type lines (WT) and transgenic empty vector lines (EV), the expression of the *CiTPS* gene in transgenic TPS1 line plants was denoted as 1. As shown in Figure 5, in the transgenic *CiTPS* tobacco represented by TPS1-6, the expression of the *CiTPS* gene in the transgenic TPS6 plants was the highest and was 11 times higher than that in the transgenic TPS1

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plants. The expression of the *CiTPS* gene in the transgenic TPS1, TPS4 and TPS5 plants was similar, but relatively low in the transgenic TPS2 and TPS3 plants. Therefore, three transgenic lines, TPS1, TPS5 and TPS6, were screened for further functional identification.

Detection and analysis of transgenic tobacco leaf secretion. Through detection and analysis by GC/MS software, 61 compounds were detected from the surface secretions of wild-type tobacco leaves, and 38 compounds were identified. Sixty compounds were detected from transgenic empty vector tobacco plants, and 35 of these compounds were identified. Sixty-three, 66 and 74 compounds were detected from three transgenic tobacco lines, and 39, 39 and 43 compounds were identified, respectively. There was no significant difference in the total volatile species between the transgenic tobacco and wild type.

The analysis of the terpenoids in the identified tobacco leaf secretions revealed that the contents of monoterpenoids (1R)-(+)-trans-iso-limonene, trans-5-methyl-3-(methyl ethylene)-cyclohexene and d-(+)-camphoric acid in the transgenic tobacco

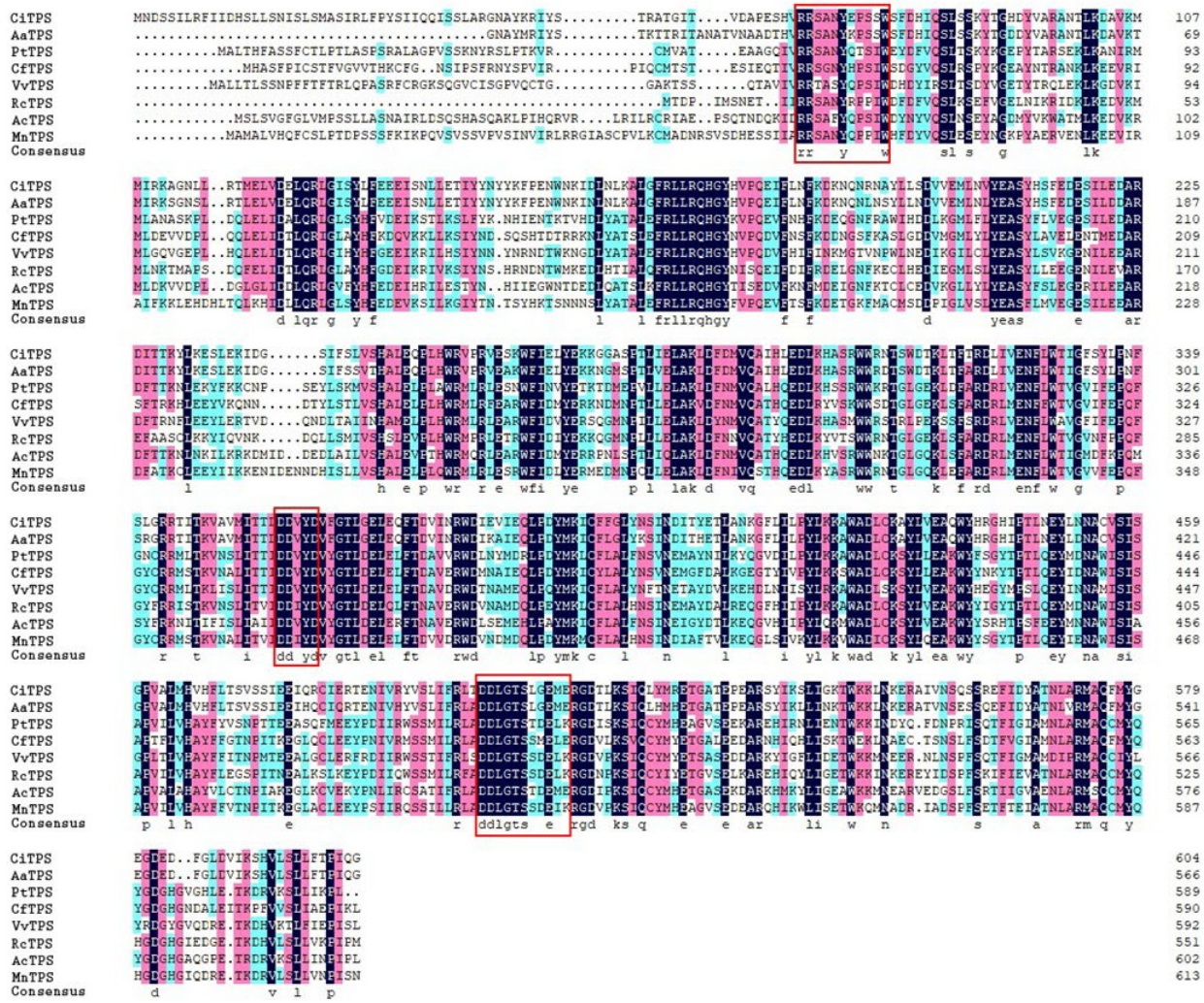

Figure 2. Alignment of CiTPS with terpene synthase (TPS) amino acid sequences of other plants; the highly conserved functional sequences of monoterpenoid synthase are marked by red frames, which are named DDXXD, (N,D)D(L,I,V)X(S,T)XXXE and RRX8W

The sequence details are as follows: CiTPS is from *C. indicum* var. *aromaticum*, AaTPS is from *Artemisia annua*, Q9SPN0.1; PtTPS is from *Populus trichocarpa*, AII32477.1; CfTPS is from *Cephalotus follicularis*, GAV86002.1; VvTPS is from *Vitis vinifera*, ADR74202.1; RcTPS *Ricinus communis*, B9T536.1; AcTPS is from *Actinidia chinensis*, AID55337.1 and MnTPS is from *Morus notabilis*, XP_010088018.1

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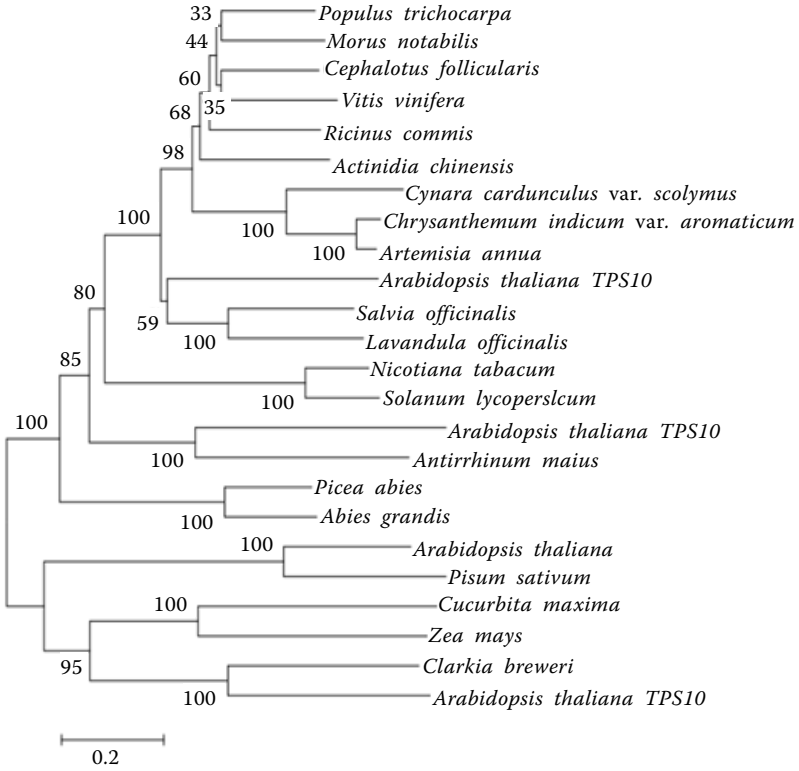

Figure 3. Phylogenetic relationship of the CiTPS amino acid sequences from *C. indicum* var. *aromaticum* and characterised terpene synthase (TPS) sequences from other plant species; the amino acid sequences were aligned using the CLUSTAL W program, and a phylogenetic tree was built using MEGA 5.0 via the maximal parsimony method; bootstrap values obtained after 1 000 replications are shown on the branches

The species and corresponding accession numbers are as follows: AaTPS (*Artemisia annua*, Q9SPN0.1), AcTPS (*Actinidia chinensis*, AID55337.1), AgTPS (*Abies grandis*, O22340.1), AmTPS (*Antirrhinum majus*, Q84NC9.1), AtTPS (*Arabidopsis thaliana*, Q9ZUH4.1, AEE82229.1, AEE33784.1, NP_176361), CbTPS (*Clarkia breweri*, AAC49395.1), CcTPS (*Cynara cardunculus* var. *scolymus*, KVG47623.1), CfTPS (*Cephalotus follicularis*, GAV86002.1), CmTPS (*Cucurbita maxima*, AAB39482.1), LoTPS (*Lavandula officinalis*, ABD77417.1), MnTPS (*Morus notabilis*, XP_010088018.1), NtTPS (*Nicotiana tabacum*, AFJ04408.1), PaTPS (*Picea abies*, AAO73863.1), PsTPS (*Pisum sativum*, O04408.1), PtTPS (*Populus trichocarpa*, AII32477.1), RcTPS (*Ricinus communis*, B9T536.1), SlTPS (*Solanum lycopersicum*, AAG09949.1), SoTPS (*Salvia officinalis*, O81191.1), VvTPS (*Vitis vinifera*, ADR74202.1), ZmTPS (*Zea mays*, Q84ZW8.1)

were significantly higher than those in the wild-type and transgenic empty vector lines. In the transgenic lines, the content of delta-elemene sesquiterpene was significantly lower than that of the wild-type and transgenic empty vector lines. The content of sipinene, a diterpenoid, in the transgenic tobacco also continuously decreased compared with that in the wild type, but the content of diterpene Perilla alcohol was almost unchanged (Figure 6).

DISCUSSION

The tremendous structural variety of terpene synthases (TPSs) has been observed due to their functions of catalysing the formation of the most abundant diverse group of natural metabolites in the plant kingdom. As a primary enzyme in the synthesis of terpenes, it plays an important role in the regulation of the synthesis of aromatic substances in plants. Many monoterpene and sesquiterpene synthase genes have been isolated and characterised from terpene-accumulating cells and tissues, such as leaf glandular trichomes, and from fruits of agriculturally important plants, including the citrus and grape (Sharon-Asa et al. 2003; Iijima et al. 2004; Lucker et al. 2004; Shimada et al. 2004; Picaud et al. 2005). Its functions in plants resistant to abiotic and biological stresses have been studied in a large

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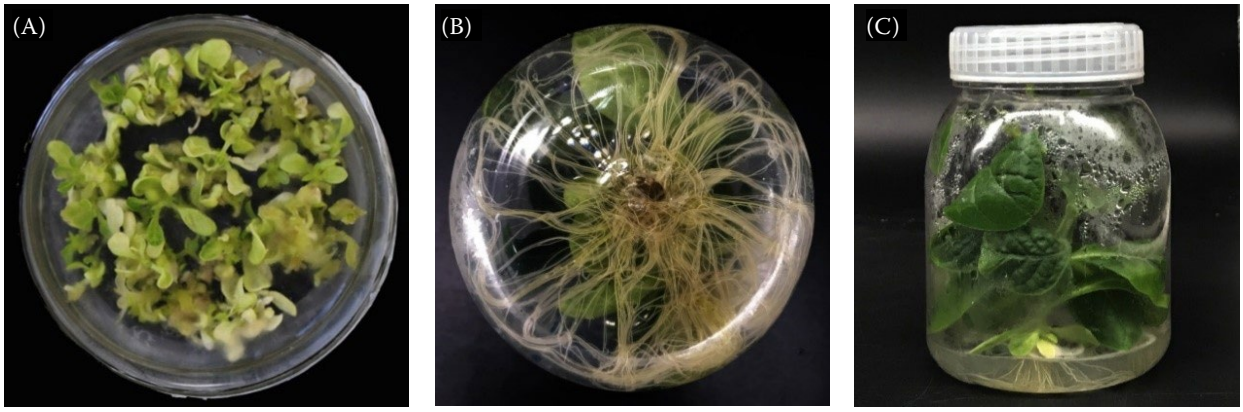

Figure 4. Transgenic tobacco growing *in vitro*: resistant buds growing from infected tobacco leaves on a screening medium (A), resistant buds were rooted on a rooting screening medium (B) and normal growth of resistant seedlings (C)

number of plants. Cotton plants are usually damaged by leaf-chewing caterpillars or piercing-sucking hemipterans. The transcript accumulation of multiple *TPS* genes is related to the increase in herbivore-induced volatile terpenes in cotton, which can defend itself against herbivores (Huang et al. 2015), and the rice terpene synthase gene *OsTPS19* has been reported to enhance resistance to the blast fungus *Magnaporthe oryzae*. (Chen et al. 2018). Ozone and wounding are essential abiotic factors in plants, and after manipulating these two stresses on *Eucalyptus globulus*, the expression profiles of terpene synthase genes were antagonistically altered by combined stress treatments (Kanagenran et al. 2018). It was discovered that *TPS* genes are the pivotal genes determining the spatiotemporal release of volatile scent compounds. Linalool has been identified as a major volatile terpene in the flowers of many other plants, contributing to the aromatic materials that please people. *LoTPS1* and *LoTPS3* have been found to generate (±)-linalool and β-ocimene, which are both monoterpenes in *Lilium* 'Siberia' and are responsible for emitting pleasant aromas (Abbas et al. 2019).

However, much of the research to date has not been related to the *TPS* gene in *C. indicum* var. *aromaticum*. In this study, we cloned and identified the gene encoding a terpene synthase responsible for the monoterpene synthesis from *C. indicum* var. *aromaticum* and denoted it as *CiTPS*. Understanding the genetic and molecular basis of TPS involved in the biosynthesis of volatile terpenes will provide insights for improving breeding strategies aimed at investigating aromatic substances and for developing specific TPS genes possibly useful for fragrance

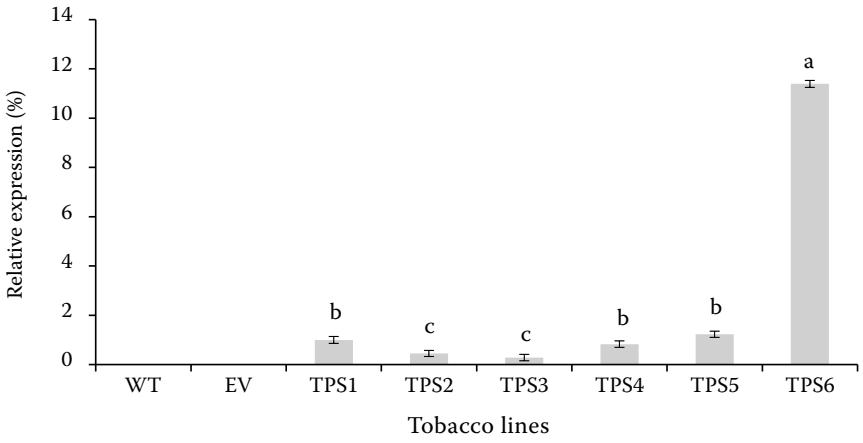

Figure 5. Relative expression patterns (mean + standard error) of the *CiTPS* gene in different transgenic tobacco species
Bars marked with different letters were significantly different ($P < 0.05$) according to Duncan's multiple range test; WT – wild-type lines; EV – transgenic empty vector lines; TPS – transgenic terpene synthase lines

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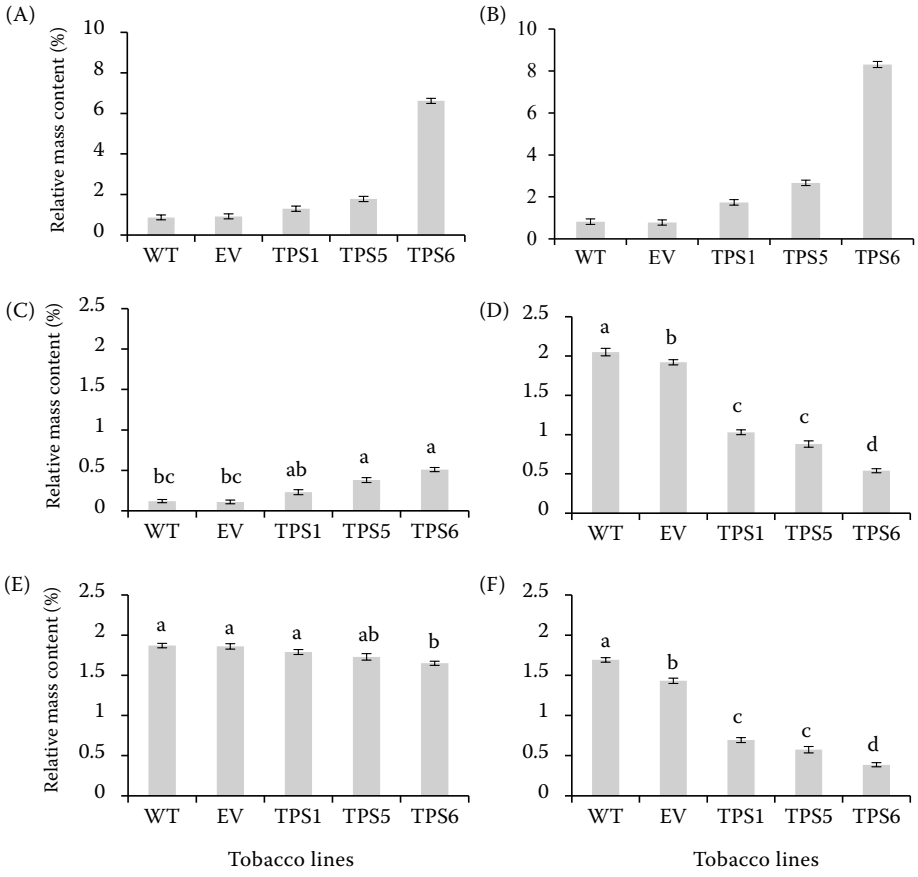

Figure 6. Relative mass contents (mean + standard error) of the terpenoids in the leaves between the different transgenic tobacco lines; six terpenoids were detected here: trans-5-methyl-3-(methyl ethylene)-cyclohexene (A), (1R)-(+)-trans-iso-limonene (B), D-camphoric acid (C), cembranoids (D), sclareol (E), and δ-elemene (F)

Different letters indicate significant differences ($P < 0.05$); WT – wild-type lines; EV – transgenic empty vector lines; TPS – transgenic terpene synthase lines

applications in *C. indicum*. Through the conserved domain prediction of the amino acid sequence encoded by *CiTPS*, we found three conserved domains belonging to terpene synthase (Figure S4 in ESM), which means that *CiTPS* regulates the formation of terpene synthase.

In the past several decades, the terpene synthase gene family has been examined and classified into seven major clades, designated TPS-a, TPS-b, TPS-c, TPS-d, TPS-e/f, TPS-g, and TPS-h. In this study, *CiTPS*, containing the essential DDXXD and RRX_8W motifs, belongs to the TPS-b subfamily, which is recognised as an angiosperm-specific TPS clade (Chen et al. 2011). It has been reported that QH1 and QH5, two cDNAs cloned from *Artemisia annua*, encode monoterpene synthases (Jia et al. 1999). In the phylogenetic tree, *CiTPS* clustered together with QH1 and QH5 in *Artemisia annua*, and the similarity was up to 91% and 89%, respectively, indicating that *CiTPS* encodes monoterpene synthase and is a terpene synthase gene.

After generating the overexpression of *GhTPS12* in transgenic *N. tabacum* plants, there was a relatively improved amount of (3S)-linalool. (Huang et al. 2017). It has been demonstrated that *CitTPS16* in citrus fruits is responsible for the biosynthesis of E-geraniol according to the outcomes of assays of the *in vitro* recombinant protein and the *in vivo* transient expression in sweet oranges (Li et al. 2017). Eight FhTPSs were isolated from *Freesia* x *hybrida*, and six of them were found to be functional. *FhTPS1* and *FhTPS2* are thought catalyse the formation of linalool and α-terpineol, respectively, and *FhTPS6, FhTPS7* and *FhTPS8* have been found to generate sesquiterpenes using FPP as a substrate. *FhTPS4* has been grouped into the TPS-g clade, lacking the essential

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structure of the RRX_8W motif, and can form linalool and nerolidol (Gao et al. 2018). In transgenic rice plants, the overexpression of *OsTPS19* significantly increased the amount of limonene, but the amount of limonene in the RNAi lines declined, indicating that *OsTPS19* produces limonene in plants (Chen et al. 2018). Four terpene synthase genes have been found to be functional in *Ricinus communis*. *RcSeTPS1* is responsible for the formation of two sesquiterpene products, (–)-α-copaene and (+)-δ-cadinene, *RcSeTPS7* produces a single product, (E,E)-α-farnesene, and *RcSeTPS5* and *RcSeTPS10* were found to generate multiple sesquiterpenes (Xie et al. 2012). After isolation from *Acanthopanax sciadophylloides*, *AscTPS1* was acquired, which encodes an enzyme catalysing the formation of β-caryophyllene (BCP) and little α-humulene. *AscTPS2* has been found to encode germacrene D synthase. *AscTPS3*, *AscTPS1*, and *CzTPS1* were examined similarly, and they encode linalool/nerolidol synthase, BCP synthase, and β-eudesmol synthase, respectively. *CzTPS2* was examined and was expected to encode germacrene B synthase (Hattan et al. 2018). In our study, the amounts of three monoterpenes in transgenic tobacco plants expressing *CiTPS* were found to increase significantly: (1R)-(+)-trans-Iso-limonene, trans-5-methyl-3-(methyl ethylene)-cyclohexene and D-(+)-camphoric acid. However, there were decreases in a sesquiterpene, δ-elemene and a diterpene, (±)-cembrene. The amount of sclareol, a diterpene, remained almost unchanged. This result demonstrated that *CiTPS* is a monoterpene synthase gene.

Reports about the function of the terpene synthase gene in *C. indicum* var. *aromaticum* have not been published to date. This study lays the molecular basis for the research on volatile monoterpenes in *C. indicum* var. *aromaticum*. The discovery of the essential TPS gene for the synthesis of terpenes can promote the formation of crucial aromatic substances in *Chrysanthemum* species and can also reveal the horticultural and economic value of other ornamental plants.

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