Overexpression of geranyl diphosphate synthase and farnesyl diphosphate synthase gene from Chrysanthemum indicum var. aromaticum resulted in modified trichome formation and terpenoid biosynthesis in transgenic tobacco

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Abstract

Geranyl diphosphate synthase (GPS) and farnesyl diphosphate synthase (FPS) catalyzes the biosynthesis of monoterpenoids and sesquiterpenoids, two key precursors for terpenoids biosynthesis. Here, the open reading frame (ORF) sequences of GPS (named *CiGPS*) and FPS (named *CiFPS*) with lengths of 1278 bp and 1035 bp were cloned from *Chrysanthemum indicum* var. *aromaticum* (*C. indicum* var. *aromaticum*). Phylogenetic analysis showed that *CiGPS* and *CiFPS* genes were closely related to *Artemisia annua* and *Chrysanthemum × morifolium*, respectively. qRT-PCR results showed that *CiGPS* and *CiFPS* gene had the highest expression level in leaves, while the expression of *CiFPS* was induced to methyl jasmonate. Overexpression of *CiGPS* and *CiFPS* in tobacco resulted in shorter plant height, darker leaf color, higher chlorophyll, and carotenoid content; the number of long handle trichomes increased significantly, while short handle trichomes only increased significantly on the upper epidermis of leaves of *CiGPS* transgenic tobacco lines. The relative content of terpenes of *CiGPS* and *CiFPS* transgenic tobacco lines increased significantly and monoterpenoids and sesquiterpenoids were detected in transgenic tobacco without wild type (WT) and empty vector (EV) lines. The activity of GPS enzyme was increased in *CiGPS* transgenic tobacco lines, but FPS enzyme activity in *CiFPS* transgenic tobacco lines was non-significant. Transcriptional analyses revealed that the expression levels of terpenoid biosynthesis pathway genes were higher in transgenic tobacco lines than in WT and EV lines. All the results demonstrated that *GPS* and *FPS* cDNA were effective to improve growth traits and alter terpenoid metabolism of *C. indicum* var. *aromaticum*.

1 Introduction

Aroma is an important trait of plants, which not only can improve the ornamental value of plants, but also can attract insect pollination, resist biological, and abiotic stress. Aromatic plants can not only volatilize fragrance, but also can be used for the extraction of plant essential oils and the separation of medicinal ingredients, and at the same time, it can also be used as a medicinal plant (Marković et al. 2019). Due to the unique fragrance, aromatic plants are widely used in molecular breeding and landscaping (Gao et al. 2020). At present, the characteristics of chrysanthemum varieties are mostly concentrated in flower color and flower type, and chrysanthemum varieties with strong fragrance are very scarce, only some chrysanthemums are slightly scented. In addition, there are not many fragrant chrysanthemum varieties that can be cultivated in the open field.

Recently, an aromatic *Chrysanthemum* strain, *Chrysanthemum indicum* var. *aromaticum*(*C. indicum* var. *aromaticum*), has been discovered in Shennongjia, Hubei, China by Liu Qihong from Wuhan Institute of Botany, Chinese Academy of Sciences in 1982(Liu et al. 1986). The whole plant has a strong fragrance, and the flowering period is in autumn. In Chinese folk, dried flowers and leaves of *C. indicum* var. *aromaticum* are usually used to make sachets (Zhong et al. 2019). The surface of *C. indicum* var. *aromaticum* has trichomes with secretory function, and the aroma-making mechanism may be directly related to the substances secreted by the trichomes(Gao et al.). Studies found that genetic factors (Mishra et al. 2017), development period (Rodrigues et al. 2013; Liu et al. 2018), culture conditions

(Stojičić et al. 2016; Esmaeili et al. 2019), growing environment (Isah 2019; Hook et al. 2018), and many other factors determine the composition and content of trichomes and the secretions.

The biosynthesis pathways of plant terpenoids mainly include the mevalonic acid (MVA) pathway in the cytoplasm and the methylerythritol phosphate (MEP) pathway in the plastid (Tian et al. 2021). The biosynthetic process of terpenoids mainly consists of three stages. The first stage is the production of intermediate precursor substances of terpenoids; the second stage is the production of direct precursor substances geranyl pyrophosphate (GPP), geranylgeranyl pyrophosphate (GGPP), and farnesyl pyrophosphate (FPP). In the third stage, terpene synthases (TPSs) catalyze the precursor compounds of terpene synthesis to cyclization and rearrangement, and then hydroxylation and methylation under the action of modifying enzyme, finally forming terpenes (Xue et al. 2019; Shang et al. 2020).

The enzymes that catalyze the condensation reaction of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to generate GPP, FPP, and GGPP are collectively called short-chain isoprenyl diphosphate synthases (IDSs), which belong to prenyltransferase (Adal and Mahmoud 2020). IDSs are directly involved in the biosynthesis of different branched terpenes, thereby controlling the distribution of products. Geranyl diphosphate synthase (GPS) is a short-chain IDS, which is an important branching enzyme in the biosynthesis of terpenoids. It can catalyze a molecule of IPP and DMAPP through head-to-tail condensation to form the direct acting precursor GPP for the synthesis of monoterpenoids (Nagegowda and Gupta 2020). Farnesyl diphosphate synthase (FPS) is also a short-chain IDS, which catalyzes the first-to-tail condensation reaction of two molecules of IPP and DMAPP to generate FPP, the direct-acting precursor for the synthesis of sesquiterpenoids (Fei et al. 2019). Changes in *GPS* and *FPS* gene expression will affect the synthesis of a variety of monoterpenoids and sesquiterpenoids. Although monoterpenoids and sesquiterpenoids are very common, *GPS* and *FPS* genes have only been studied in a few plants, such as *Arabidopsis thaliana* (Masferrer et al. 2002), *Artemisia annua* (Lee et al. 2017), *Panax ginseng* (Kim et al. 2014), *Antirrhinum majus* (Tholl et al. 2004), *Eucommia ulmoides* (Wang et al. 2018), and *Picea abies* (Schmidt and Gershenzon 2008), etc. While the research of *GPS* and *FPS* genes in fragrant *chrysanthemum* is rarely reported.

The main components of the leaf volatiles of *C. indicum* var. *aromaticum* are monoterpenes, sesquiterpenes, and their oxygen-containing derivatives (Fan et al. 2018; Gao et al. 2020). Therefore, the cloning and functional study of *C. indicum* var. *aromaticum GPS* and *FPS* genes can provide an effective molecular approach to increase the aromatic content of *C. indicum* var. *aromaticum*. In this study, the terpenoid synthesis pathway genes *GPS* and *FPS* of *C. indicum* var. *aromaticum*, named *CiGPS* and *CiFPS*, were cloned, and their effects on the content of tobacco trichomes and their secretions were studied when overexpression. Besides, the molecular functions of *CiGPS* and *CiFPS* in the synthesis of terpenoids were analyzed. The results will provide theoretical bases for the use of transgenic engineering technology to create high-terpene aromatic materials and to cultivate new aromatic chrysanthemum varieties.

2 Materials And Methods

2.1 Plant materials and bacterial strains

C. indicum var. *aromaticum* was introduced from the Shennongjia area (110°23'57" E, 31°28'7" N), Hubei Province, China, and was cultivated in the nursery of Northeast Forestry University, Harbin, Heilongjiang Province, China. After treatments, fresh leaves were collected and froze in liquid nitrogen for 10 min and then cryopreserved at -80°C for RNA extraction. *E. coli* strain DH5α and pBI121 vector were used for gene cloning and expression vector construction. *Agrobacterium tumefacien* strain EHA105 was used for transformation of *Nicotiana tabacum*.

2.2 Isolation of *CiGPS* and *CiFPS* genes

Total RNA was extracted from *C. indicum* var. *aromaticum* leaves using the Trizol kit (Tiangen Biotech (Beijing), Co., Ltd.). The first strand cDNA was subjected to reverse transcription using the Revert AidTM First Strand kit (Toyobo (Shanghai) Biotech Co., Ltd.). According to the preliminary sequencing results of *C. indicum* var. *aromaticum* transcriptome, the upstream and downstream primers (Table 1) were designed using Primer 5.0 software, and were used to amplify the open reading frame (ORF) of *CiGPS* and *CiFPS* mRNA. The PCR products were assessed with 1% agarose gel electrophoresis, and the recovered fragment were ligated to pMD18-T vector (Takara Biomedical Technology (Beijing) Co., Ltd.), which were introduced into *E. coli* DH5a, with the blue-white screening for *CiGPS* and *CiFPS*. Positive clones were selected and sequenced by Beijing Orcrea Technology Limited.

2.3 Sequence alignment and phylogenetic tree analysis

The amino acid sequence of *CiGPS* and *CiFPS* were compared with other *GPS* and *FPS* sequences using DNAman (V.6.0) software, and the domain of *CiGPS* and *CiFPS* were analyzed by BlastX at NCBI. The phylogenetic tree was created using MEGA4.0 software.

2.4 Assay of *CiGPS* and *CiFPS* expression in plant tissues under MeJA treatment

C. indicum var. *aromaticum* plants with good condition and consistency were selected and sprayed with 0.25% MeJA for 0, 4, 8, 24, and 48 h. Then leaves were collected for RNA extraction using the Trizol method. A reverse transcription kit was used for reverse transcription. The fluorescence quantification kit (SYBR Green, TOYOBO (Shanghai) Biotech Co., Ltd.) was used for qRT-PCR. The reaction system (20 μL) contained 10 μL of SYBR MIX and 0.4 μL of each primer (Table 1) and 0.5 ng of cDNA template. The PCR tubes were placed in a 96-well plate in LightCycler® 96 system (Roche, Switzerland). The PCR reaction program was 94°C for 30 sec, 40 cycles of 94°C for 5 sec, 60°C for 15 sec, and 72°C for 10 sec. Each sample was replicated 3 times. Meanwhile, the roots, stems, leaves, and flowers of the untreated *C. indicum* var. *aromaticum* plants were collected for the same qRT-PCR analysis. *CmEF1α* (KF305681) was used as the internal reference gene. Relative transcript abundances were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen 2001).

2.5 Construction of transformation vector and genetic transformation of tobacco

The gene specific primers of *CiGPS-Sma*I-F/*CiGPS-Spe*I-R, and *CiFPS-Xba*I-F/*CiFPS-Sma*I-R (Table 1) were designed against the *CiGPS* and *CiFPS* full length cDNA sequence. PCR products were cloned into the *Sma*I/*Spe*I or *Xba*I/*Sma*I sites of the binary PBI121 vector, under the control of a constitutive strong promoter, cauliflower mosaic virus (35S2), and containing the neomycin phosphotransferase II (nptII) selectable marker. The 35S::*CiGPS* and 35S::*CiFPS* plasmids were transferred into *Agrobacterium* strain EHA105 by electroporation (Eppendorf, Germany) for tobacco transformaiton.

2.6 Confirmation of transgenic plants

Putative transgenic plantlets of tobacco were rooted in MS solid media supplemented with 0.1 mg/L naphthalene acetic acid (NAA) and 50 mg/L kanamycin (kana). Leaves of 3-week-old kana-resistant seedlings were harvested to isolate genomic DNA using the Plant Genomic DNA Kit (Tiangen Biotech (Beijing), Co., Ltd.). Total RNA was extracted using the Trizol method, and cDNA was synthesized using a reverse transcription kit (Toyobo (Shanghai) Biotech Co., Ltd.). Transgenic events were confirmed using *CiGPS* or *CiFPS*-specific primers. To investigate the transcriptional regulation of *CiGPS* or *CiFPS* in overexpressed lines, its expression level was determined using qRT-PCR. The reference gene, *NtEF1α* (NM001326165), was used as endogenous controls. The transcript levels of target genes were calculated using the $2^{-\triangle\triangle CT}$ method.

2.7 Measurement of plant height and the length and width of leaves

The plant growth features, including height and leaf size, were compared between T1 *CiGPS* transgenic, T1 *CiFPS* transgenic, EV, and WT plants. The plant height was measured from the soil surface to the arch of the uppermost leaf using a ruler. The length of each leaf was measured from the tip to the base of the short petiole. All the measured plants were 3 months old and cultured in a uniform environment.

2.8 Chlorophyll content analysis

WT, EV, and three *CiGPS* and *CiFPS* gene transgenic tobacco lines (5 each) were selected randomly, and the third fully functional leaf were collected. The chlorophyll content was determined according to a previous work (Shu et al. 2010) with some modifications, and the resulting values were used to calculate the contents of carotenoids, chlorophyll a (Chl A), and chlorophyll b (Chl B) (Arnon 1949).

2.9 Measurement of trichome density

WT, EV, and three *CiGPS* and *CiFPS* gene transgenic tobacco lines (5 each) were selected randomly, and the third fully functional leaf were collected and cut in the apical shoot. Then, samples were placed under a microscope for observation (Leica DM2500 fluorescence microscope, Germany). Trichome density was

determined by measuring the span of five different areas (0.4 mm^2) on the upper and lower epidermis of leaves (base, center, and tip portions) and calculated with ImageJ software (Version 1.49).

2.10 Volatile compounds analysis

Tobacco leaves of the T2 generation transgenic lines, the EV lines, and the WT lines in the vigorous period were collected (10 g) and extract with 100 mL dichloromethane for 30 min (repeated twice). All the extracts were combined, filtered with waterman filter paper, dehydrated with 20 g of anhydrous sodium sulfate (dried at 105°C for 2 h before use), and concentrated on a rotary evaporator at 40°C. The final volume was adjusted to a 0.5 mL and stored at 0°C before analysis.

Agilent 7890A-5975C gas chromatography-mass spectrometry (GC-MS) equipped with HP-5ms (30 m × 0.25 mm ×0.25 μm) elastic quartz capillary column (Agilent Technologies, Santa Clara, CA, USA) was used to detect the volatiles. The obtained concentrate with 1 μL was injected into the injection port at 250°C and tested after a solvent delay of 3.5 minutes. The oven temperature started at 50°C and was maintained for 30 min, then raised to 160°C at a rate of 5°C/min, and finally ramped to 270°C at a rate of 10°C/min and was maintained for 10 min. The MS was operated in an electron ionization mode at 70 eV. The temperatures of the ion source, transmission line, and quadrupole were set at 230, 280, and 150°C, respectively, the electron multiplier voltage was 2100 V, the mass scan range was set from 4 to 500 u, and standard mass spectrum library was NIST08L. Turbo Mass 5.4.2 GC/MS software was adopted to analyze the volatiles. After searching the NIST08L database for the different mass spectrum peaks of the analyzed total ion map, the retention index of the component to be tested was compared with the standard substance in the Pherobase database to determine its category. The relative content of each component was calculated using the peak area normalization method.

2.11 GPS and FPS enzyme assay

Aliquot of 2 g of healthy leaves of WT, EV, and 3 selected *CiGPS* and *CiFPS* gene tobaccos were collected, and the GPS and FPS enzyme activities were determined according to the protocols of plant GPS enzyme-linked immunoassay kit and plant FPS enzyme-linked immunoassay kit (Elabscience Biotechnology Co., Ltd., Wuhan, Hubei Province, China).

2.12 Expression analysis of terpenoid biosynthesis pathway genes in transgenic tobacco

In order to detect the expression of genes related to the terpene synthesis pathway after the overexpression of *CiGPS* and *CiFPS* genes in tobacco, Primer 5 software was used to design qRT-PCR specific primers, as shown in Table 1. Tobacco *NtEF1α* (NM001326165) was used an internal reference gene for relative quantitative analysis.

2.13 Statistical analysis

All the experiments were carried out at least in triplicate. Values were analyzed by ANOVA and Duncan's multiple range test (SPSS v19.0). *P*-values less than 0.05 were considered significant.

3 Results

3.1 Cloning and sequence analysis of *CiGPS* and *CiFPS* of *C. indicum* var. *aromaticum*

The *CiGPS* (KX932456) and *CiFPS* (MK425155) genes cloned from *C. indicum* var. *aromaticum* contained an ORF of 1278 bp and 1035 bp, which encoded 426 and 345 amino acids, respectively. DNAman software was used to compare the amino acid sequences of *CiGPS* and *CiFPS* with some known sequences with high homology selected from NCBI, and the results showed that the amino acid sequences of *CiGPS* and *CiFPS* were highly conservative. The deduced amino acid sequence of *CiGPS* contained five conserved domains including PLN02890, prenyl_cyano, IspA, polyprenyl_synt, and Trans_IPPS_HT (Fig. 1A). The deduced amino acid sequence of *CiFPS* also contained five conserved domains including I (GKXXR), II (EXXXXXXLXXDDXXDXXXXRRG), III (GQXXD), IV (KT), and V (GXXFQXXDDXXDXXXXXXXXGKXXXDXXXXK) (Fig. 1B).

MEGA 5.0 software was used to compare *CiGPS* and *CiFPS* genes with GPS and FPS amino acid sequences of other species to construct a phylogenetic tree. Through the comparison of 13 GPS proteins, it was found that *CiGPS* clustered on the same branch with *Artemisia ann*ua, and also clustered on a large branch with *Taraxacum kok-saghyz* and *Atractylodes lancea*, both of the Asteraceae family (Fig. 1C). Through the comparison of 8 FPS proteins, it was found that *CiFPS* was closely related to *Chrysanthemum lavandulifolium* and *Chrysanthemum* × *morifolium* which belong to the genus *Chrysanthemum* (Fig. 1D). These results, to a certain extent, revealed the evolutionary conservatism of near-source species.

3.2 Tissue specific expression profiles of *CiGPS* and *CiFPS* of *C. indicum* var. *aromaticum* in response to MeJA treatment

The qRT-PCR method was used to analyze the differences in the gene expression of *CiGPS* and *CiFPS* in different organs. The results showed that the expression levels of *CiGPS* and *CiFPS* genes in leaves were the highest, which were 6.43 and 2.48 times higher than that in roots, respectively, followed by stems and flowers, while the lowest expression levels in roots (Fig. 2A). After 0.25% MeJA treatment, the expression of *CiGPS* gene was not significantly, and the expression of this gene under 24 h MeJA treatment was the highest but was only 1.48 times higher than that of the control at 0 h. The expression level of *CiFPS* gene

showed a trend of first increase and then decrease with the extension of treatment time, and at 24 h, the expression level was the highest, which was 10.13 times that of the control. However, it decreased significantly after 48 h treatment, but was still 5.28 times higher than the control at 0 h (Fig. 2B). The results indicated that the expression of *CiGPS* gene was not significantly induced by MeJA, while *CiFPS* gene was significant.

3.3 Identification of transgenic tobacco plants overexpressing *CiGPS* and *CiFPS*

After Kana resistance screening, 6 T0 resistant seedlings containing *CiGPS* target gene (Fig. 3A-C) and 8 T0 resistant seedlings containing *CiFPS* target gene were successfully obtained (Fig. 4A-C). PCR verification results showed that the target gene bands were amplified in 6 *CiGPS* transgenic tobaccos (Fig. 3D) and 8 *CiFPS* transgenic tobaccos (Fig. 4D), and the qRT-PCR results showed that 5 *CiGPS* transgenic tobaccos (G1-G5) (Fig. 3E) and 7 *CiFPS* transgenic tobacco plants (F1-F7) were amplified to the target gene band (Fig. 4E). The qRT-PCR analysis also showed that *CiGPS* and *CiFPS* genes were not expressed in WT and unloaded tobacco. Therefore, the gene expression levels of G4 and F2 strains were recorded as 1, and 3 transgenic strains with differential expression G1 (3.16-fold), G3 (2.51-fold), G5 (2.11-fold) (Fig. 3F) and F1 (19.70-fold), F6 (31.12-fold), F7 (21.11-fold) (Fig. 4F) were selected for subsequent gene function analysis.

3.4 Effects of the *CiGPS* and *CiFPS* overexpression on growth

As can be seen from Table 2 and Fig. 5A, the genetically modified tobacco lines (G1, G3, and G5) were lower than that of the WT, while the leaves of G1, G3, and G5 were darker in color, rounder in shape, wider in leaf width, and thicker in stalk. As shown in Table 2 and Fig. 5B, the genetically modified tobacco lines F1, F6, and F7 were lower than that of the WT and EV lines, and the leaves were darker in color, shorter in leaf, and thicker in stalk, while the shape of the leaf did not change much.

3.5 Effects of the *CiGPS* and *CiFPS* overexpression on chlorophyll content

Compared with WT and EV lines, the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in *CiGPS* transgenic tobacco lines G1, G3, G5 and *CiFPS* transgenic tobacco lines F1, F6, F7 were significantly increased. Among them, G5 and F6 strains showed the most significant. G5 line increased 0.95, 0.65, 0.87, 0.92 times and 0.77, 0.54, 0.70, 0.79 times compared with WT and EV, respectively, and F6 increased 0.75, 0.52, 0.68, 0.87 and 0.58, 0.41, 0.53, 0.74 times compared with WT and EV, respectively (Table 3). These data showed that the overexpression of *CiGPS* and *CiFPS* in tobacco increases the content of chlorophyll-related pigments, which was in consistent with the observed darker green leaf color. However, the chlorophyll a/b values were not significant between the transgenic and WT and EV strains, indicating that the ratio of photosynthetic pigments in the transgenic tobacco with *CiGPS* and *CiFPS* genes was not changed.

3.6 Effects of the *CiGPS* and *CiFPS* overexpression on trichome number

The density of the long and short handle trichomes in the tip, center, and base of the leaf was calculated, and the results are shown in Fig. 6. After the *CiGPS* gene overexpressed in tobacco, the number of long and short handle trichomes of tobacco leaves increased significantly. Regardless of the upper and lower epidermis of leaves, the number of long handle trichomes of the transgenic lines G1, G3, and G5 were significantly higher than that of the WT and EV lines, and the long handle trichomes of the transgenic lines had a consistent distribution pattern: leaf base > leaf center > leaf tip. On the upper epidermis of leaves, the number of short handle trichomes of the transgenic lines was significantly higher than that of the WT and EV lines. However, the number of short handle trichomes on the lower epidermis of leaves did not change much, only G5 strains were slightly more than WT and EV lines, and the number of short handle trichomes had no obvious distribution patterns in different leaf positions.

The overexpression of the *CiFPS* gene in tobacco resulted in an increase in the number of long and short handle trichomes of tobacco leaves. The density of the long and short handle trichomes in the tip, center, and base of the leaf was calculated, and the results are shown in Fig. 7. The number of long handle trichomes of the transgenic lines F1, F6, and F7 was significantly higher than that of the WT and EV lines on the upper and lower epidermis of leaves, and F1 and F6 lines showed the most significant. The number of long- handle trichomes of F1 and F6 strains was 3 ~ 5 times that of WT and EV. However, the number of short handle trichomes of tobacco leaves after transgenesis did not change significantly, and only the F6 strain increased nearly twice as much as WT and EV.

3.7 Effects of the *CiGPS* and *CiFPS* overexpression on terpenoid production

GC-MS analysis of the leaf secretions of wild WT, EV, and *CiGPS* transgenic lines G1, G3, G5 and *CiFPS* transgenic lines F1, F6, F7 revealed that tobacco leaf surface secretions were mainly alkaloids, terpenes, alkanes, esters, ketones, and the percentages of these types of compounds in different strains were different. As shown in Fig. 8A, compared with WT and EV strains, the relative content of alkaloids, esters, ketones, and organic acids compounds in the leaf secretions of the *CiGPS* transgenic strains was significantly reduced, while the terpenes and alkanes compounds were significantly increased. Among them, the relative content of terpene compounds in G1 and G5 increased by 13.50%, 14.15%, 18.07%, and 18.71% compared with WT and EV, respectively. From Fig. 8B, it can be seen that the relative contents of alkaloids, esters, and ketones compounds in the leaf secretions of the *CiFPS* gene transgenic strains were lower than those of the WT and EV strains, while the terpenes and organic acids compounds were significantly higher than that of the WT and EV strains. The relative content of terpenes compounds in F6 and F7 changed most, which increased by 33.51%, 34.15%, 29.98%, and 30.62% compared with WT and EV, respectively.

A total of 96 compounds were detected from the surface secretions of WT, EV, and *CiGPS* and *CiFPS* gene transgenic tobacco lines. Among them, there were 39 compounds shared by all the strains; 43 compounds were newly generated after transgenic, and 20 compounds disappeared after transgenic. It can be seen from Table 4 that the relative content of (S)-(-)-nicotine, o-nicotine, and triethyl citrate decreased in the transgenic lines G1, G3, and G5, while the relative content of cembrene, β-ionone, and 1,3,5,6,7-tetramethyl-adamantane increased. Among them, cembrene and β-ionone in the G1 strain increased most, which was 5.49, 6.38 times and 2.40, 1.72 times than that of WT and EV, respectively. In addition, the *CiGPS* gene overexpression in tobacco lead to the production of some new terpenes. In G1 strain, 6.62% monoterpene trans-5-methyl-3- (methylethenyl)-cyclohexene was detected; in G3 strain, thunbergol, (-)-myrtenal, l-picein, 9-(1-methylethylidene)-1,5- cycloundecadiene, 2,7-dimethyl-5-(1-methyl ethenyl)-1,8-nonadiene, and β-iso- methyl-ionone were newly detected. In G5 strain, (1R)-(+)-trans-isolimonene (8.31%), (+)-calarene (0.27%), 4-methylene-6-(1-propenylidene)cyclooctene (1.78%), and D-(+)-camphoric acid (0.14%) were newly detected. As can be seen from Table 5, the relative contents of (S)-(-)-nicotine, o-nicotine), and triethyl citrate in F1, F6, and F7 strains decreased, while the relative content of cembrene and β-ionone increased. The relative content of cembrene in the F6 strain was 3.25%, and the relative content of β-ionone in F1 was 9.59%. In addition, terpene compounds not found in WT and EV lines with *CiFPS* gene, but 12-isopropyl-1,5,9-trimethyl-4,8,13-cyclotetradecatriene − 1,3-diol, a diterpenoid compound. The unique sesquiterpenoids (Z)-caryophyllene and γ-neoclovene were only determined in F1, and sesquiterpenoids α-bulnesene, (+)-calarene, phellandral, thunbergol, and (1R)-(+)-trans-isolimonene were solely found in F7. However, some terpenes, such as sclareol, β-elemene, farnesol, (+/-)-δ-elemene, and valencene, disappeared in tobacco leaves secretions with *CiGPS* and *CiFPS* genes.

3.8 Effects of the *CiGPS* and *CiFPS* overexpression on GPS and FPS enzyme activities

The GPS enzyme activity of WT, EV, and *CiGPS* transgenic tobacco strains and the FPS enzyme activity of *CiFPS* transgenic tobacco strains were measured. As shown in Fig. 9A, the GPS enzyme activities in G1, G3, and G5 lines were significantly higher than that of WT and EV strains, among which G3 has the highest enzyme activity with 551.59 U/L, followed by G1 (538.79 U/L) and G5 (537.84 U/L). GPS enzyme activities in G1, G3, and G5 lines were 1.39, 1.42, 1.39 times and 1.23, 1.25, 1.22 times that of WT and EV, respectively. As shown in Fig. 9B, the FPS enzyme activities in F1, F6, and F7 were all higher than those of WT and EV strains, but only the F6 strain (190.11 U/L) showed significant difference, which was 1.27 and 1.26 times higher than that of WT and EV lines.

3.9 The effect of *CiGPS* and *CiFPS* overexpression on the expression of MVA and MEP pathway genes

In WT, EV, and *CiGPS* and *CiFPS* transgenic tobacco lines, the expression of MVA and MEP metabolic pathways related enzyme genes were determined, which maily included the main rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*) gene in the upstream of the MVA metabolic

pathway, the rate-limiting enzymes 1-deoxy-D-xylulose 5-phosphate synthase (*DXS*) gene and deoxyxylulose 5-phosphate reductoisomerase (*DXR*) gene in the upstream of the MEP metabolic pathway, and the direct precursor synthetase geranyl pyrophosphate synthase gene (*GPS*) gene, sesquiterpene synthesis direct precursor synthetase farnesyl diphosphate synthase (*FPS*) gene, diterpene synthesis direct precursor synthetase geranylgeranyl diphosphate synthase (*GGPPS*) gene, monoterpene synthase (*MTS*) gene, sesquiterpene synthase 5-epi-aristolochene synthase (*EAS*) gene, and carotenoid lyase carotenoid cleavage dioxygenase (*CCD*) gene.

It can be seen from Fig. 10 that the expression levels of *HMGR*, *DXR*, *GGPPS* and *MTS* genes in the *CiGPS* gene overexpression tobacco strains were higher than those of WT and EV, only the expression of sesquiterpene synthase *EAS* gene decreased, while the expression of *DXS*, *FPS*, and *CCD* genes did not change significantly, which indicated that the overexpression of *CiGPS* genes in tobacco could positively increased the expression of genes in the metabolic pathways of MVA and MEP, as well as the expression of *GGPPS* and *MTS* genes, which in turn promoted the synthesis of monoterpene and diterpene substances.

As can be seen from Fig. 11, the expression of *HMGR*, *DXS*, *DXR*, *GGPPS*, *EAS*, and *CCD* genes in the *CiFPS* gene overexpression tobacco strains were all increased, the expression of *MTS* gene decreased, while the expression level of *GPS* gene showed not significant than that of WT and EV lines. Hence, the overexpression of *CiFPS* gene in tobacco could positively regulate the expression of MVA and MEP metabolic pathway genes, as well as *GGPPS*, *EAS*, and *CCD* genes, which in turn up-regulated the synthesis of terpenoids.

4 Discussion

The head-shaped trichomes on the leaves of *C. indicum* var. *aromaticum* can naturally produce colloidal secretions, and the composition and content of the secretions are the main factors that determine its aroma type and intensity. The main compounds detected from the leaf secretions of *C. indicum* var. *aromaticum* were mainly monoterpenes, sesquiterpenes, and their oxygen-containing derivatives. The synthesis of monoterpenes and sesquiterpenes depends on the supply of GPP and FPP precursors, respectively. GPP is synthesized under the catalysis of *GPS* enzyme, while FPP is synthesized by *FPS* enzyme. As the initial key enzymes for the synthesis of plant terpene active substances, the catalytic activities and contents of *FPS* and *GPS* can affect the anabolism of subsequent products (Daviet and Schalk 2010). At present, *GPS* genes (Rai et al. 2013; Bouvier et al. 2000; Tholl et al. 2004) and *FPS* genes (Xiang et al. 2010; Matsushita et al. 1996) have already been cloned from model plants and non-model plants. In the present study, the ORF sequences of *GPS* and *FPS* genes were cloned and identified from the *C. indicum* var. *aromaticum* transcriptome database, which were named *CiGPS* and *CiFPS*, respectively. The sequence homology comparison and phylogenetic tree analysis showed that the CiGPS protein had higher homology with GPS proteins of *Artemisia annua*, *Atractylodes lancea* and *Taraxacum kok-saghyz*; CiFPS protein was closely related to *Chrysanthemum* x *morifolium*, *Chrysanthemum lavandulifolium*, and *Artemisia annua*, inferring that they have similar functions.

The differential expression of terpenoid synthesis pathway genes in different environments or in different tissues under the same conditions may be the result of the co-regulation of gene expression patterns by its own genotype and external factors (Olofsson et al. 2011). The qRT-PCR results in present study showed that both C*iGPS* and *CiFPS* genes were expressed in leaves at the highest level, followed by stems and flowers. This may be related to the distribution of the head-shaped trichomes of *C. indicum* var. *aromaticum* at the leaf site (Guo et al. 2020). Secondly, it was also found that the *CiFPS* gene was significantly induced under the MeJA treatment, and the expression of the gene was 10.13 times at 24 h than that of the control. In *Panax ginseng*, the expression of *PgFPS* gene was also significantly increased under the induction of MeJA (Kim et al. 2010a). In addition, *Am-FaPS-1* of *Aquilaria microcarpa* (Kenmotsu et al. 2011), *MrFPS* of *Matricaria recutita* (Su et al. 2015), and *TwFPSs* genes of *Tripterygium wilfordii* (Zhao et al. 2015) were all up-regulated after MeJA treatment.

The *CiGPS* and *CiFPS* genes were transferred to the model plant WT tobacco and found that the plant height of the transgenic line was significantly lower than that of the WT, which may be caused by the regulation of the diterpene plant growth hormone gibberellin. Gibberellin, which belongs to diterpenoid acids, is one of the final products of the diterpene synthesis branch in the isoprenoid pathway. This type of plant hormones exists widely and plays a regulatory role in all stages of plant growth and development(Olszewski et al. 2002). The overexpression of *GPS* gene in *Camelina sativa*(Xi et al. 2016) lead to larger leaves, taller plants, and earlier bloom than the WT, which was contrary to the results in present study. In *Artemisia annua* (Banyai et al. 2010), the *FPS* gene overexpression made the plant height short than the WT, and the result was consistent with our results. However, whether the content of gibberellin in tobacco lines with *CiGPS* and *CiFPS* genes is reduced requires further investigation.

Plant photosynthetic pigments chlorophyll and carotenoids are very important in the growth and development of plants, and their synthesis mainly depend on the MEP pathway in plastids (Joyard et al. 2009). The results of the study showed that the content of chlorophyll and carotenoids in the tobacco lines with *CiGPS* and *CiFPS* genes were higher than that in WT and EV lines. This may be due to the overexpression of the inserted gene in the chloroplast, causing the excessive consumption of the IPP precursor in this compartment, which bringing about the flow of IPP in the cytoplasm to the plastid in large quantities, resulting in sufficient IPP precursors in the plastid to synthesize chlorophyll and carotenoids. The overexpression of *GPS* gene in tobacco lead to an increase in carotenoid content in plastids(Li et al. 2018), which were consistent with the results of this study.

Trichome, as an important secretory tissue in plants, can specifically synthesize a variety of secondary metabolites(Tian et al. 2018). In recent years, there have been more and more studies on the use of genetically modified technology to regulate the formation of trichomes, thereby increasing the content of plant's main secondary metabolites (Chang et al. 2018; Alahakoon et al. 2016; Gao et al. 2017). The density of leaf trichomes in *Solanum lycopersicum* RNAi10 and RNAi13 lines with *DXR1* and *DXR2* genes knocked out significantly increased, which were twice than that of the WT and EV lines (Paetzold et al. 2010). The transfer of β-glucosidase gene could make the trichome density of *Artemisia annua* leaves and flowers 20% and 66% higher than that of control plants, respectively (Singh et al. 2016). In this study,

it was discovered for the first time that the overexpression of *GPS* and *FPS* genes in tobacco resulted in a significant increase in the density of long and short handle trichomes on the leaves of the transgenic lines. This may be due to the regulation of genes that control the formation of trichomes when the *GPS* and *FPS* genes were inserted, resulting in an increase in the number of trichomes.

Recently, more and more scholars have focused on cloning or regulating the expression of *GPS* and *FPS* genes through molecular biology experiments in order to control the subsequent synthesis or transformation of terpenoids. In *Camelina sativa*, the combination of *Antirrhinum majus GPPS* (SSU) and cytosolic terpene synthase (TS) could catalyze the formation of sesquiterpenoids and monoterpenoids. Especially, the coupled expression of *GPPS* (SSU) and *TS* lead to an increase in the content of monoterpenoids in the cytosol(Xi et al. 2016). The transiently overexpression of *Antirrhinum majus AmGPPS.SSU* on *Catharanthus roseus* leaves resulted in the increment of vindoline (Rai et al. 2013). Also, the overexpression of the *Panax ginseng FPPS* gene in *Centella asiatica* increased the content of sterols and triterpenoids (Kim et al. 2010b). When the *FPS* gene of *Artemisia annua* was overexpressed in itself, the content of artemisinin could be 2.5 times higher than that in the WT plant (Banyai et al. 2010). Similar results were obtained in this study. The overexpression of *C. indicum* var. *aromaticum CiGPS* and *CiFPS* genes in tobacco resulted in the significant increase of terpenes in tobacco leaf secretions including cembrene and β-ionone. In addition, the monoterpenoid (1R)-(+)-trans-isolimonene was detected in the *CiGPS* gene transgenic line, and (Z)-caryophyllene, γ-neoclovene, α-bulnesene, (+)-calarene, phellandral, thunbergol, and (1R)-(+)-trans-isolimonene were were newly detected in the *CiFPS* gene transgenic line. Therefore, the use of molecular biology technology to regulate the expression of key genes in the terpenoid metabolism pathway is an effective molecular means to increase the content of terpenoids.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Dr. Wenjie Gao carried out the experiment, analyzed the data, and drafted the manuscript. Dr. Qingran Meng and Xiang Wang helped carried out the experiment and analyzed part of the data. Prof. Feng Chen reviewed and revised the manuscript. Prof.

Yunwei Zhou and Prof. Miao He designed the experiments. All authors approved the final manuscript version.

Compliance with Ethical Standards

All authors have read and approved this version of the article, and due care has been taken to ensure the integrity of the work. The article is original and unpublished and is not being considered for publication elsewhere. No conflict of interest exits in the submission of this manuscript. No human participants or animal studies are involved in the present study.

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Tables

Table 1

Primer names and sequences used in this study

Primer name	Primer sequences (5'-3')	Primer usage
GPS-F	ATGTCCAATATTTATCGAGGAATTG	ORF gene cloning
GPS-R	TCACTTTGTTCTATTGATAACTCTGTG	ORF gene cloning
FPS-F	ATGAGTAGCAGCATCGATCTGAA	ORF gene cloning
FPS-R	CTACTTTTGCCTCTTGTAGATTTTACC	ORF gene cloning
*CiGPS-Sma*I-F	CCCGGGATGTCCAATATTTATCGAGGAATTG	Vector construction
*CiGPS-Spe*I-R	ACTAGTCTTTGTTCTATTGATAACTCTGTGTGT	Vector construction
*CiFPS-Xba*I-F	TCTAGAATGAGTAGCAGCATCGATCTGA	Vector construction
*CiFPS-Sma*I-R	CCCGGGCTTTTGCCTCTTGTAGATTTTACC	Vector construction
CmEF1α-F	TTTTGGTATCTGGTCCTGGAG	ACTIN
CmEF1α-R	CCATTCAAGCGACAGACTCA	ACTIN
CiGPS-F	AGCAATCCATCCTTGGCTTTCA	qRT-PCR
CiGPS-R	CTTCCCTTCAACGCCCATCTT	qRT-PCR
CiFPS-F	TTTTGGTGCTCCCGAGGTGA	qRT-PCR
CiFPS-R	AGCCGCAGGGTCTTTTTTCC	qRT-PCR
NtEF1α-F	*AAGCCCATGGTTGTTGAGAC*	ACTIN
NtEF1α-R	*GTCAACGTTCTTGATAACAC*	ACTIN
NtHMGR-F	GGTGTCCAAAGGTGTGCAAAAC	qRT-PCR
NtHMGR-R	CAAAAGTTCCCAGATATGCCG	qRT-PCR
NtDXS-F	GGTGTTGTTGAGCTTACAGTTGC	qRT-PCR
NtDXS-R	GTGTTGACATCTTCCCTCTTCTACC	qRT-PCR
NtDXR-F	TTGGGTCCACTGGCTCTA	qRT-PCR
NtDXR-R	AATGTCCTTTCCTGCTTCTAT	qRT-PCR
NtGPS-F	GCTTTAGATGAGGCTGTTAT	qRT-PCR
NtGPS-R	TTATGGTTCGTCGGCTTT	qRT-PCR
NtFPS-F	ATTATTCATTCTACCTCCCA	qRT-PCR
NtFPS-R	ATCTTACCCAGCACCTCT	qRT-PCR

Primer name	Primer sequences (5'-3')	Primer usage
NtGGPPS-F	TGTCGATTCATGGGCTGCT	qRT-PCR
NtGGPPS-R	GTGGATCACAGGTGGGTCTTT	qRT-PCR
NtCCD-F	CACCTGAAACTCCTCCTTTTAAGAAC	qRT-PCR
NtCCD-R	TGTCCTCACAAAACGTGAGACGTAT	qRT-PCR
NtMTS-F	GTCAGCCTTGATTTTGCGGC	qRT-PCR
NtMTS-R	TCTTAGGGAAGGGGTGTGCT	qRT-PCR
NtEAS-F	TGCCGAGTTATTGATGATACAGC	qRT-PCR
NtEAS-R	GCCATTCCTTGAAATTTAGCC	qRT-PCR

Table 2 Comparison of the morphology of WT, EV, and different transgenic tobacco lines

Plants	Leaf color	Leaf length (cm)	Leaf width (cm)	Leaf width (cm)
WT	Yellow green	13.52 ± 0.82 ^{a}	6.28 ± 0.65 ^{c}	55.80 ± 3.47 ^{a}
EV	Yellow green	12.95 ± 0.66 ^{a}	6.42 ± 0.61 ^{c}	54.20 ± 3.08 ^{a}
G1	Dark green	11.62 ± 0.32 ^{bc}	7.62 ± 0.41 ^{a}	42.20 ± 1.36 ^{bc}
G3	Dark green	12.98 ± 0.30 ^{a}	7.33 ± 0.36 ^{ab}	44.60 ± 1.94 ^{b}
G5	Dark green	12.52 ± 0.64 ^{ab}	7.61 ± 0.25 ^{a}	39.10 ± 1.55 ^{cd}
F1	Dark green	11.19 ± 0.53 ^{c}	6.40 ± 0.49 ^{c}	37.40 ± 1.90 ^{d}
F6	Dark green	11.82 ± 0.53 ^{bc}	6.58 ± 0.44 ^{bc}	37.60 ± 0.67 ^{d}
F7	Dark green	11.43 ± 0.46 ^{c}	6.98 ± 0.44 ^{abc}	38.20 ± 2.70 ^{cd}

Note: [i] WT: wild type tobacco; EV: empty vector tobacco.

[ii] G1: *CiGPS* transgenic tobacco strain 1; G3: *CiGPS* transgenic tobacco strain 3; G5: *CiGPS* transgenic tobacco strain 5; F1: *CiFPS* transgenic tobacco strain 1; F6: *CiFPS* transgenic tobacco strain 6; F7: *CiFPS* transgenic tobacco strain 7.

[iii] Different lower-case letters within the same column mean significant difference at 0.05 level.

Table 3 Comparison of chlorophyll contents of WT, EV, and different transgenic tobacco lines

Plants	Chl a (mg/L)	Chl b (mg/L)	Total Chl (mg/dm)	Chl a/Chl b	$C_{x\cdot c}$ (mg/L)
WT	7.27 ± 1.42 [e]	2.93 ± 0.58 [c]	5.10 ± 0.99 [c]	2.49 ± 0.14 [b]	1.07 ± 0.28 [d]
EV	8.04 ± 1.47 [de]	3.15 ± 0.54 [c]	5.59 ± 0.99 [c]	2.55 ± 0.17 [b]	1.14 ± 0.27 [cd]
G1	9.26 ± 1.64 [cde]	3.35 ± 0.59 [bc]	6.30 ± 1.11 [bc]	2.77 ± 0.06 [a]	1.43 ± 0.28 [bcd]
G3	11.82 ± 0.87 [ab]	4.11 ± 0.24 [ab]	7.96 ± 0.55 [ab]	2.87 ± 0.06 [a]	1.80 ± 0.11 [ab]
G5	14.19 ± 2.47 [a]	4.84 ± 0.76 [a]	9.52 ± 1.61 [a]	2.93 ± 0.05 [a]	2.04 ± 0.51 [a]
F1	11.67 ± 0.53 [abc]	4.15 ± 0.42 [ab]	7.91 ± 0.47 [ab]	2.83 ± 0.17 [a]	1.71 ± 0.03 [ab]
F6	12.69 ± 0.57 [a]	4.44 ± 0.23 [a]	8.57 ± 0.38 [a]	2.86 ± 0.10 [a]	1.99 ± 0.16 [a]
F7	9.92 ± 0.77 [bcd]	3.39 ± 0.24 [bc]	6.66 ± 0.50 [bc]	2.92 ± 0.03 [a]	1.57 ± 0.11 [abc]

Note: [i] WT: wild type tobacco; EV: empty vector tobacco.

[ii] G1: *CiGPS* transgenic tobacco strain 1; G3: *CiGPS* transgenic tobacco strain 3; G5: *CiGPS* transgenic tobacco strain 5; F1: *CiFPS* transgenic tobacco strain 1; F6: *CiFPS* transgenic tobacco strain 6; F7: *CiFPS* transgenic tobacco strain 7.

[iii] Different lower-case letters within the same column mean significant difference at 0.05 level.

Table 4 Major compounds and relative contents of secretions in leaves of WT, EV, and *CiGPS* transgenic tobacco lines

Compounds	Relative content (%)				
	WT	EV	G1	G3	G5
(S)-(-)-nicotine	11.16	16.76	6.05	2.37	4.33
o-nicotine	nd	2.88	4.14	0.94	nd
cembrene	0.48	0.42	2.65	1.67	1.51
triethyl citrate	0.91	0.51	0.10	0.44	0.25
β-ionone	4.09	5.71	9.80	2.45	6.37
4-methylene-1-methyl-2- (2-methyl-1-propen-1-yl)-1-vinyl-cycloheptane	2.46	6.17	4.13	0.85	5.68
1,3,5,7-tetramethyl-adamantane	nd	8.33	24.28	9.57	7.18
1,3,5,6-ttetramethyl-adamantane	11.59	nd	7.72	14.86	6.95
trans-5-methyl-3-(methylethenyl)-cyclohexene	nd	nd	6.62	nd	nd
thunbergol	nd	nd	nd	1.13	0.25
(-)-myrtenal	nd	nd	nd	0.75	nd
l-picein	nd	nd	nd	0.12	nd
9-(1-methylethylidene)-1,5-cycloundecadiene	nd	nd	nd	0.07	nd
2,7-dimethyl-5-(1-methylethenyl)-1,8-nonadiene	nd	nd	nd	2.18	nd
β-iso-methyl-ionone	nd	nd	nd	0.68	0.44
(1R)-(+)-trans-isolimonene	nd	nd	nd	nd	8.31
(+)-calarene	nd	nd	nd	nd	0.27
4-methylene-6-(1-propenylidene) cyclooctene	nd	nd	nd	nd	1.78
D-(+)-camphoric acid	nd	nd	nd	nd	0.14
sclareol	1.67	nd	nd	nd	nd
β-elemene	0.07	nd	nd	nd	nd
farnesol	0.30	nd	nd	nd	nd
(+/-)-δ-elemene	1.43	nd	nd	nd	Nd
valencene	nd	0.17	nd	nd	nd

Note: [i] WT: wild type tobacco; EV: empty vector tobacco.

[ii] G1: *CiGPS* transgenic tobacco strain 1; G3: *CiGPS* transgenic tobacco strain 3; G5: *CiGPS* transgenic tobacco strain 5.

[iii] nd, not detected.

Table 5 Major compounds and relative contents of secretions in leaves of WT, EV, and *CiFPS* transgenic tobacco lines

Compounds	Relative content (%)				
	WT	EV	F1	F6	F7
(S)-(-)-nicotine	11.16	16.76	1.8	3.27	2.76
o-nicotine	nd	2.88	5.27	nd	1.45
cembrene	0.48	0.42	1.9	3.25	2.75
triethyl citrate	0.91	0.51	0.82	0.39	0.27
β-ionone	4.09	5.71	9.59	6.67	5.66
4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-cycloheptane	2.46	6.17	0.96	5.18	13.07
1,3,5,7-tetramethyl-adamantane	nd	8.33	4.92	nd	11.81
1,3,5,6-tetramethyl-adamantane	11.59	nd	4.77	nd	nd
12-isopropyl-1,5,9-trimethyl-4,8,13-cyclotetradecatriene-1,3-diol	nd	nd	5.34	3.42	3.9
(Z)-caryophyllene	nd	nd	0.47	nd	nd
phellandral	nd	nd	0.21	nd	nd
thunbergol	nd	nd	0.53	nd	nd
2,7-dimethyl-5-(1-methylethenyl)-1,8-nonadiene	nd	nd	0.69	nd	nd
(E)-3-eicosene	nd	nd	0.26	nd	nd
γ-neoclovene	nd	nd	0.04	nd	nd
13-epitorulosol	nd	nd	nd	0.77	nd
3-(1-methyl-2-propenyl)-1,5-cyclooctadiene	nd	nd	nd	0.82	nd
α-bulnesene	nd	nd	nd	nd	0.64
(1R)-(+)-trans-isolimonene	nd	nd	nd	nd	2.67
(+)-calarene	nd	nd	nd	nd	0.4
2,7-dimethyl-4-phenylthio-octa-2,6-diene	nd	nd	nd	nd	1.92
(E,E,E)-3,7,11,15-tetramethylhexadeca-1,3,6,10,14-pentaene	nd	nd	nd	nd	6.21
(3R)-1-methylidene-3-prop-1-en-2-yl-cyclohexane	nd	nd	nd	nd	2.1
sclareol	1.67	nd	nd	nd	nd
β-elemene	0.07	nd	nd	nd	nd
farnesol	0.3	nd	nd	nd	nd

(+/-)-δ-elemene	1.43	nd	nd	nd	nd
valencene	nd	0.17	nd	nd	nd

Note: [i] WT: wild type tobacco; EV: empty vector tobacco.

[ii] F1: *CiFPS* transgenic tobacco strain 1; F6: *CiFPS* transgenic tobacco strain 6; F7: *CiFPS* transgenic tobacco strain 7.

[iii] nd, not detected.

Figures

Figure 1

The deduced peptide sequence of *CiGPS and CiFPS* to their nearest homologues. A: Alignment of *CiGPS* with other plant protein. B: Alignment of *CiFPS* with other plant protein. C: Phylogenetic tree analysis of *CiGPS* and other plant proteins. D: Phylogenetic tree analysis of *CiFPS* and other plant proteins. The sequence details are as follows: *AaGPS* (PWA67664.1), A*lGPS* (AYA62519.1), *TkGPS* (AMB19718.1), *HhGPS* (APY22349.1), *TaGPS* (AUZ98416.1), *CaGPS* (ATZ76916.1), *WsGPS* (AOX15286.1), *PcGPS* (AHK06506.1), *SlGPS* (NP_001234089.1), *SoGPS* (AQY54372.1), *SmGPS* (AEZ55677.1), *CrGPS* (ACC77966.1), *CmFPS* (ALF46698.1) , *ClFPS* (AFW98433.1) , *AaFPS* (AAC49452.1) , *McFPS* (AHN96159.1) , *LvFPS* (AFW98434.1) , *CnFPS* (ASJ80971.1) , *HaFPS* (XP_021978159.1) , *TkFPS* (AMB19716.1).

Figure 2

Quantitative real-time PCR analysis of expression patterns of *CiGPS* and *CiFPS* in different tissues in response to salt and MeJA treatments in *C. indicum* var. *aromaticum*. A: Expression patterns of *CiGPS* and *CiFPS* in roots, stems, leaves and flowers. B: MeJA treated conditions.

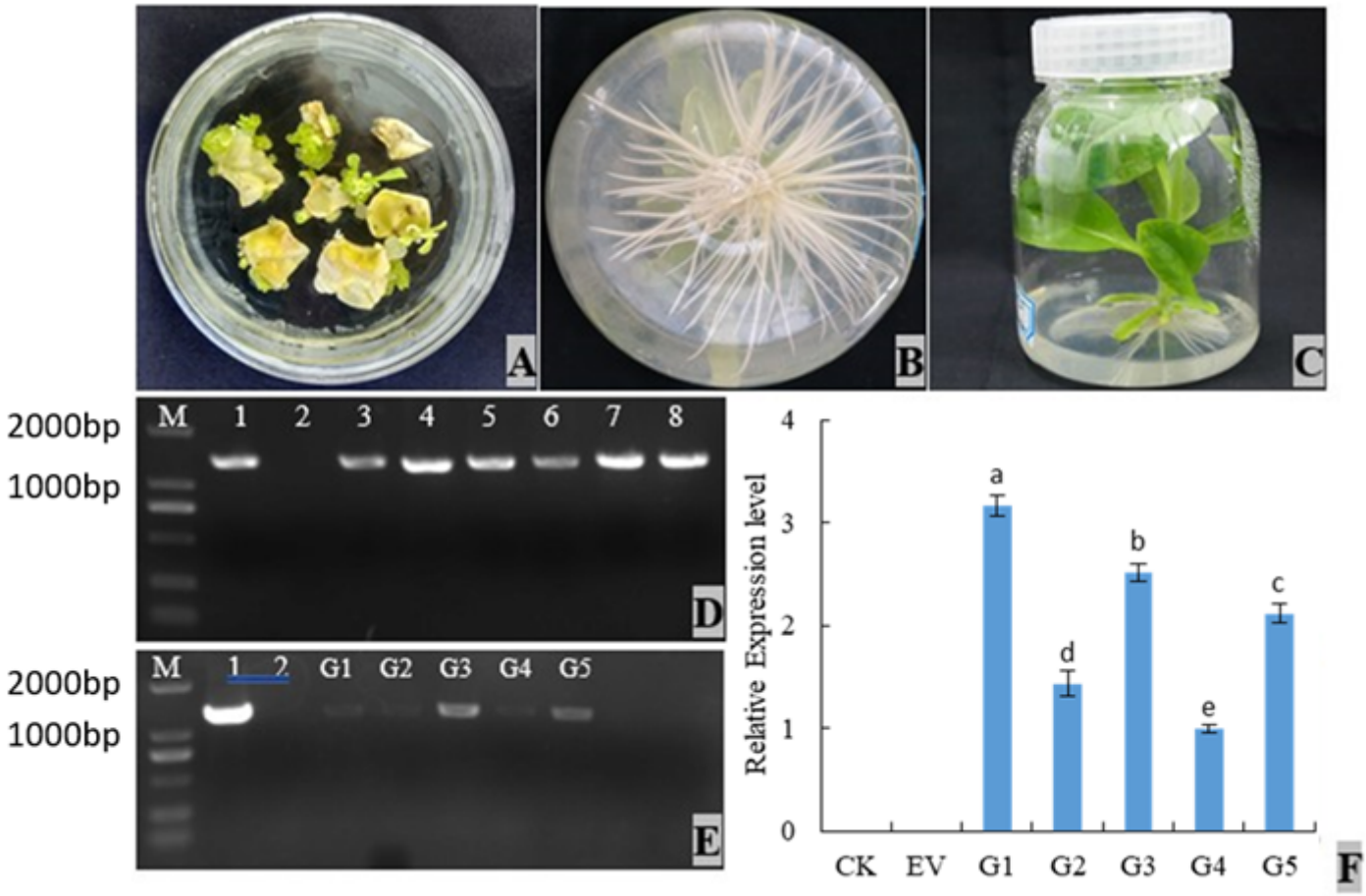

Figure 3

Identification of *CiGPS* transgenic tobacco. A: Resistant buds. B: Seedings root resistance screening. C: Transgenic tobacco. D: PCR identification of transgenic tobacco：1：Positive control；2：Negative control；3-8：Different lines of transgenic tobacco；；E: RT-PCR identification of transgenic tobacco：1：Positive control；2：Negative control；G1-5：Different lines of *CiGPS* transgenic tobacco；；F: Epression patterns of *CiGPS* gene in different transgenic tobaccos；WT：Wild-type tobacco plant；EV：Empty vector tobacco plant；G1-5：Different lines of *CiGPS* transgenic tobacco；.

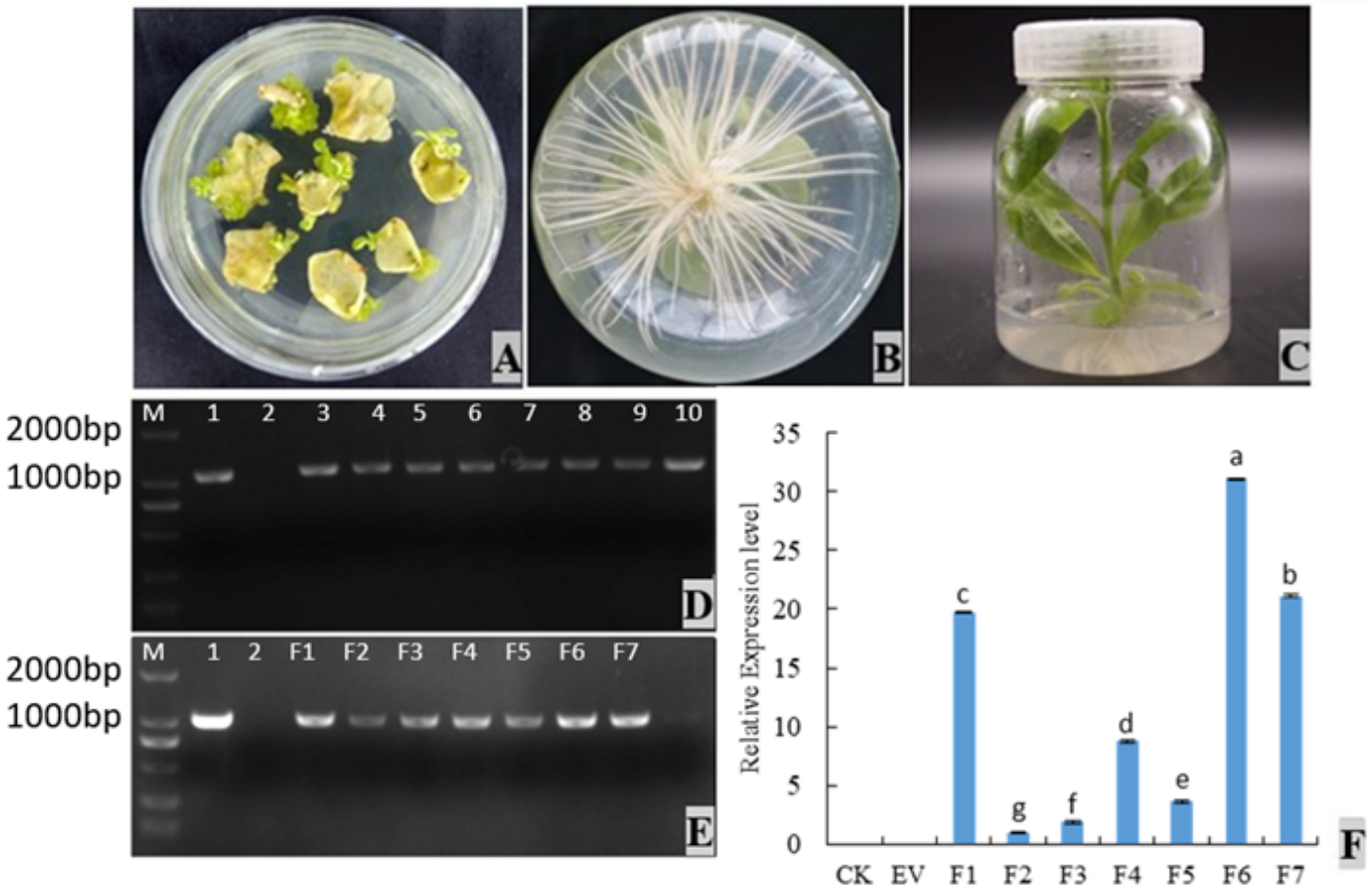

Figure 4

Identification of *CiFPS* transgenic tobacco A: Resistant buds. B: Seedings root resistance screening. C: Transgenic tobacco. D: PCR identification of transgenic tobacco：1：Positive control；2：Negative control；3-10：Different lines of transgenic tobacco；. E: RT-PCR identification of transgenic tobacco：1：Positive control；2：Negative control；F1-7：Different lines of *CiFPS* transgenic tobacco；. F: Epression patterns of *CiFPS* gene in different transgenic tobaccos；WT：Wild-type tobacco plant；EV：Empty vector tobacco plant；F1-7：Different lines of *CiFPS* transgenic tobacco；.

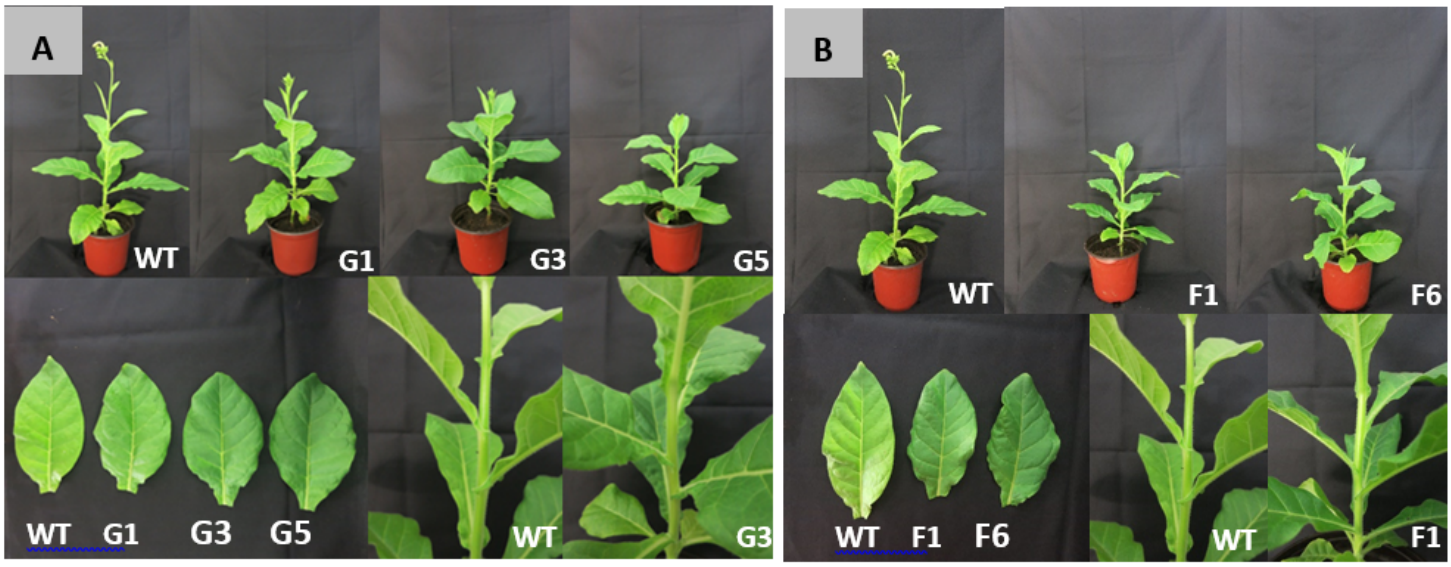

Figure 5

Comparison of the morphology of WT and different transgenic tobacco lines A: Lines of *CiGPS* transgenic tobacco. B: Lines of *CiFPS* transgenic tobacco.

Figure 6

Comparison of trichomes density of WT, EV and *CiGPS* transgenic tobacco lines.

Note: Different lower-case letters within the same leaf position mean significant difference at 0.05 level

Figure 7

Comparison of trichomes density of WT, EV and *CiFPS* transgenic tobacco lines.

Note: Different lower-case letters within the same leaf position mean significant difference at 0.05 level

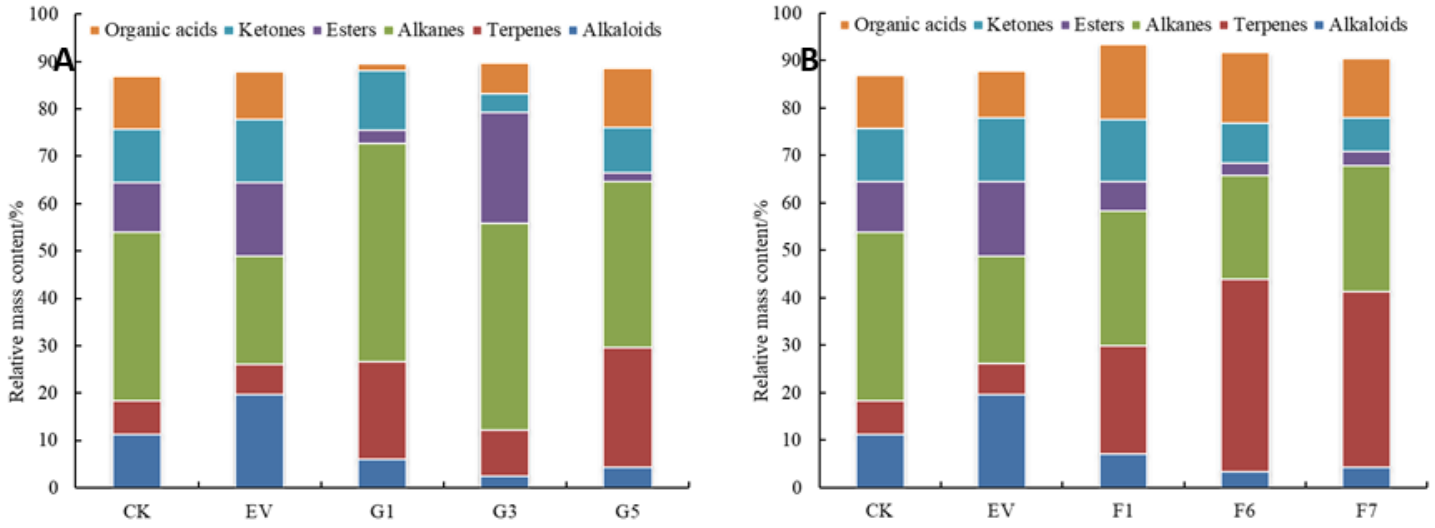

Figure 8

Classification of secretions in leaves of WT, EV and different transgenic tobacco lines. A: Lines of *CiGPS* transgenic tobacco. B: Lines of *CiFPS* transgenic tobacco

Figure 9

Analysis of enzyme activity of WT, EV and different transgenic tobacco lines. A: GPS enzyme activity. B: FPS enzyme activity

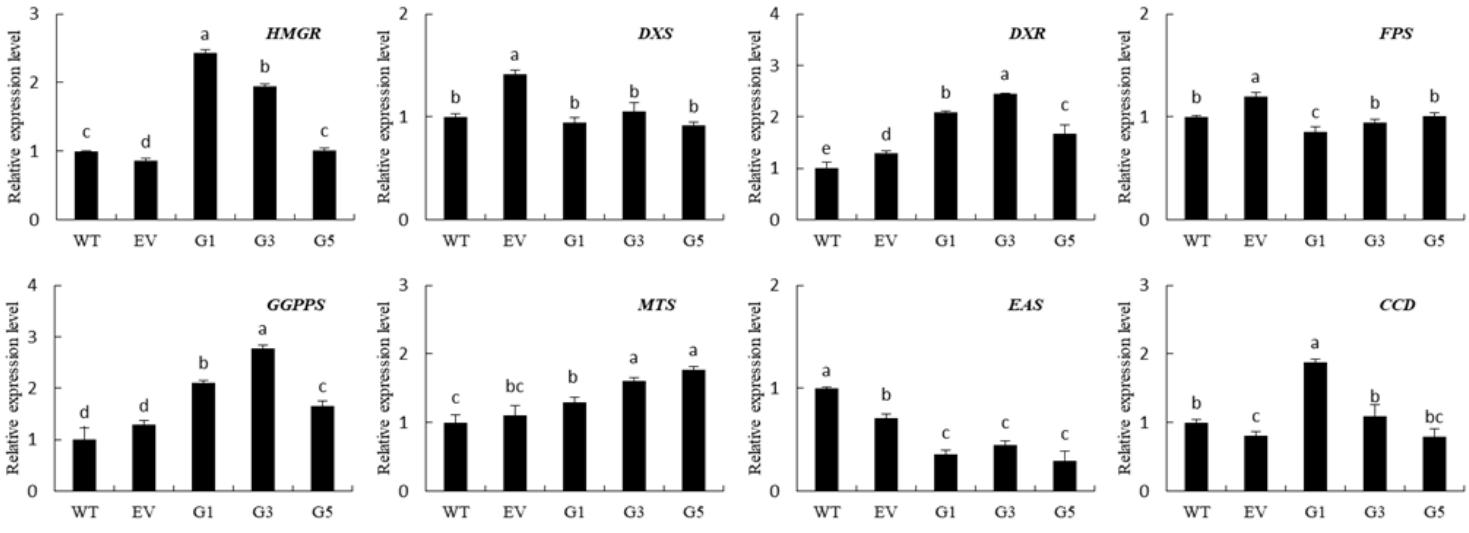

Figure 10

The related gene expression of terpenoids biosynthetsis pathway in *CiGPS* transgenic tobacco lines

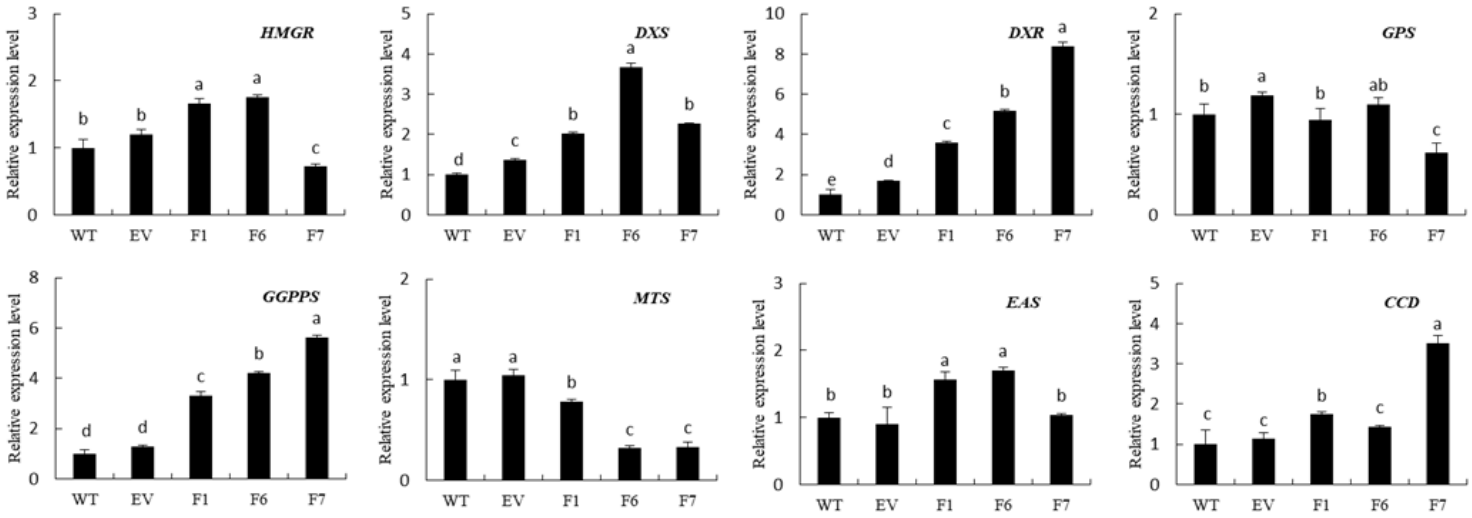

Figure 11

The related gene expression of terpenoids biosynthetsis pathway in *CiFPS* transgenic tobacco lines