Functional analysis of *Pogostemon cablin* farnesyl pyrophosphate synthase gene and its binding transcription factor PcWRKY44 in regulating biosynthesis of patchouli alcohol

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Farnesyl pyrophosphate synthase (FPPS) plays an important role in the synthesis of plant secondary metabolites, but its function and molecular regulation mechanism remain unclear in *Pogostemon cablin*. In this study, the full-length cDNA of the FPP synthase gene from *P. cablin* (PcFPPS) was cloned and characterized. The expressions of PcFPPS are different among different tissues (highly in *P. cablin* flowers). Subcellular localization analysis in protoplasts indicated that PcFPPS was located in the cytoplasm. PcFPPS functionally complemented the lethal FPPS deletion mutation in yeast CC25. Transient overexpression of PcFPPS in *P. cablin* leaves accelerated terpene biosynthesis, with an ~47% increase in patchouli alcohol. Heterologous overexpression of PcFPPS in tobacco plants was achieved, and it was found that the FPP enzyme activity was significantly up-regulated in transgenic tobacco by ELISA analysis. In addition, more terpenoid metabolites, including stigmasterol, phytol, and neophytadiene were detected compared with control by GC-MS analysis. Furthermore, with dual-LUC assay and yeast one-hybrid screening, we found 220bp promoter of PcFPPS can be bound by the nuclear-localized transcription factor PcWRKY44. Overexpression of PcWRKY44 in *P. cablin* upregulated the expression levels of PcFPPS and patchouliol synthase gene (PcPTS), and then promote the biosynthesis of patchouli alcohol. Taken together, these results strongly suggest the PcFPPS and its binding transcription factor PcWRKY44 play an essential role in regulating the biosynthesis of patchouli alcohol.

**KEYWORDS**

*Pogostemon cablin, PcFPPS, PcWRKY44, biosynthesis, patchouli alcohol*
Introduction

*Pogostemon cablin* (P. cablin) is a perennial aromatic herb belonging to *Lamiaceae* family. From its Southeast Asia origins, *P. cablin* has been widely cultivated in southern China, including Guangdong, Guangxi and Hainan provinces, for over 1,000 years (Chen et al., 2013). Many parts of *P. cablin* plant, especially leaves and stem, are rich in numerous active components, such as sesquiterpenoids and flavonoids (Li et al., 2014) and alkaloids. Patchouli alcohol, a sesquiterpenoid obtained from the leaves of *P. cablin*, has been reported to relieve depression and stress (Zhao et al., 2020), control appetite, and improve sexual drive (Swamy and Sinniah, 2015). Furthermore, patchouli alcohol shows anti-inflammatory (Lian et al., 2018), antibacterial (Xu et al., 2017), anti-nociceptive (Yu et al., 2019), and antifungal (Kocevski et al., 2013) properties.

Farnesyl pyrophosphate synthase (FPPS) is a key enzyme occupying the branch point of the mevalonate metabolic pathway (MVA). It contains five conserved domains, namely, domain I (GKXXR), domain II (EXXXXXXLLLXDDXXDDXXXXXRRG), domain III (GQXXD), domain IV (KT) and domain V (GXXFXQXXDDXXDDXXXXXGXKXXXXDXXXXXK) and aspartic acid-rich region DDXXD in domains II and V (X represents any amino acid; Srivastava et al., 2008). FPPS catalyzes the condensation of one molecule of dimethylallyl diphosphate (DMAPP) and two molecules of isopentenyl diphosphate (IDP) to form FPP, the precursor of sesquiterpenoids, which is then used for synthesis of sesquiterpenoids with diverse structures in a reaction catalyzed by various sesquiterpene synthases (Szkopinska and Plachochna, 2005). Currently, FPPS genes have been identified in different plant species, such as *Withania somnifera* (Gupta et al., 2011), *Anoectochilus roxburghii* (Yang et al., 2020). These genes regulate plant development, secondary metabolism, and various biological processes, such as terpenoid and sterol biosynthesis. In recent years, an increasing number of studies have investigated the function of FPPS in plants. For example, LeFPPS1, plays an important role in early development of plant organs in tomato (Gaffe et al., 2000). Transferring FPPS of modified signal peptide into *Artemisia annua* significantly increased the content of sesquiterpenoid artemisinin (Chen et al., 2000). In *Gymnostemma pentaphyllum*, binding of transcription factor GpMYB81 to the promoter of GpFPPS1, a key structural gene, activates its expression (Huang et al., 2021). In *Poria cocos*, the expression profile of FPS gene and content of total triterpenoids at different developmental stages indicated that the activity of FPS was positively correlated with the amount of total triterpenes produced by *P. cocos* (Wang et al., 2014). Although FPP synthase gene of *P. cablin* (PcFPPS) has been successfully cloned from *P. cablin* transcriptome library (Tang et al., 2019), its function has not been studied in detail.

It is well known that the expression levels of genes involved in plant metabolic pathways are usually regulated by transcription factors (TFs). Further, the interaction between TFs and gene promoters is considered the most common regulatory mechanism of gene expression. Common TFs include bHLH, MYB, and WRKY. For example, PatSWC4, an MYB-related transcription factor, promotes the biosynthesis of patchouli alcohol by directly binding to and activating the PatPTS promoter (Chen et al., 2020). WRKY transcription factor family is one of the largest TF families in plants that controls expression of genes involved in plant growth and development and secondary metabolic processes (Schluttenhofer and Yuan, 2015). For example, AaGSW2, a GST-specific WRKY transcription factor identified in *Artemisia annua*, is positively regulated by the direct binding of homodomain proteins AaHD1 and AaHD8 to the L1 box of the AaGSW2 promoter. Overexpression of AaGSW2 in *A. annua* significantly increased GST density, whereas AaGST2 knockdown lines showed impaired GST initiation (Xie et al., 2021). Similarly, in *A. annua*, AaWRKY17 directly bound to the W-box motifs in the promoter region of the artemisinin biosynthetic pathway gene amorpha-4,11-diene synthase (ADS) and promoted its expression. Overexpression of AaWRKY17 in *A. annua* decreases its susceptibility to *pseudomonas syringae* (Chen et al., 2021). In addition, 37 putative WRKY transcription factors with intact WRKY domains were identified in *Rhododendron glutinosum*, with overexpression of RgWRKY37 increasing the content of acetoside and total phenylethanols glycosides (PhGs) in hairy roots (Wang et al., 2021). In our previous research, we conducted binding experiment of PcFPPS-Pro with total patchouli protein using DNA pull-down technology. The proteins annotated as TFs were selected from the pulled-down proteins, and the genes with the highest similarity to the candidate protein genes were screened from the patchouli transcriptome database (PRJNA528262) using the Basic Local Alignment Search Tool (BLAST). A total of seven genes were initially screened but four genes, including PcWRKY44 that interacts with PcFPPS-Pro, were further screened using yeast one-hybrid technology (Y1H). Based on their gene expression profiles and previous results, we speculated that PcWRKY44 is very likely involved in the biosynthesis of patchouli alcohol, but the exact mechanism remains unclear.

In this study, a survey and systematic characterization of the FPPS gene in *P. cablin* were carried out. A phylogenetic tree was constructed to test the evolutionary relationships. The expression profiles of PcFPPS were detected using reverse transcription-quantitative PCR (RT-qPCR) in different tissues of *P. cablin*. The subcellular localization of PcFPPS was tested in *Arabidopsis* protoplasts and the biological activity of FPPS enzyme was verified in *Saccharomyces cerevisiae* mutant strain CC25. Additionally, in functional characterization, transient overexpression of PcFPPS significantly increased the content of patchouli alcohol in pCAMBIA1304-PcFPPS leaves. Similarly, overexpression of PcFPPS significantly increased the content of phytol, neophytadiene, and stigmastanol in transgenic tobacco leaves. Furthermore, we identified a 220bp PcFPPS-pro that can be bound by the nuclear-localized transcription factor PcWRKY44 using dual-LUC assay and Y1H assay. Overexpression of PcWRKY44 significantly increased the content of patchouli alcohol in *P. cablin* leaves. Our results suggest that PcFPPS gene and its binding
transcription factor PcWRKY44 play important roles in patchouli alcohol biosynthesis.

**Materials and methods**

**Plant materials**

The *P. cablin* plants used in this study were collected from Yangjiang City, Guangdong Province, China. Cuttage was used to obtain more *P. cablin* seedlings for analysis of expression levels of PcFPPS in leaf, stem, and flower tissues and patchouli alcohol content in leaves. Tobacco cultivar K326 (*Nicotiana tabacum*) was used for the genetic transformation experiments. *P. cablin* plants and transgenic tobacco plants were grown in a growth chamber at 25°C with a day/night cycle of 16/8 h light/dark. *Nicotiana benthamiana* seeds were kept in our laboratory and cultivated in an intelligent incubator with a 16/8 h photoperiod treatment and a 21°C/17°C day/night temperature for use in dual-luciferase reporter system assays.

**RNA extraction and gene isolation**

Total RNA was isolated from tissues using the GeneMark plant RNA purification kit (GeneMarkBio, Taiwan, China). RNA concentration and purity were determined using spectrophotometer (IMPLEN GNBH, Germany). Oligo dT was used for cDNA synthesis. PcFPPS sequence was retrieved from our *P. cablin* PacBio transcriptome project (NCBI Accession no. PRJNA528262). Primer3Plus1 was used to design the cloning primers, when designing primers, multiple repeating bases should be avoided. In addition, the GC% content is preferably between 45 and 55%, the primer length is between 18 and 25 bp, and the Tm value is 40–60°C. The 3′ end is preferably C or G terminated. After primers were designed, Primer-BLAST in NCBI was used for primer validation. All primers used in this study were listed in Supplementary Table S1.

**Sequence feature analysis of PcFPPS**

The molecular weight of PcFPPS was predicted on ExPASy. The localization of deduced protein was predicted on BaCelLo, whereas its transmembrane domains were analyzed on TMHMM. Conserved domains and signal peptide were analyzed using Pfam protein families database. Multiple sequence alignment was performed using DNAMAN. A phylogenetic tree was constructed by the neighbor-joining method using the MEGA7 software.

**Gene expression analysis**

A HiScript®II qRT reagent kit with gDNA wiper (Vazyme, NanJing, China) was used to digest 400 ng of total RNA and residual genomic DNA samples, which were then reverse-transcribed. Reverse transcription–quantitative PCR (RT-qPCR) was performed on a real-time PCR system (Bio-Rad CFX96, CA, United States) with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02/03) and gene-specific primers (Supplementary Table S1). RT-qPCR conditions were as follows: 95°C for 3 min for one cycle, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The 18S rRNA of *P. cablin* served as an internal reference. Relative expression levels of the genes were calculated with the 2^−ΔΔCt method. Each sample was assayed in three independent biological replicates and three technical replicates. ANOVA and Student’s t-test were used to analyze the data.

**Subcellular localization**

Complete ORFs of PcFPPS and PcWRKY44 without a termination codon were inserted into the pAN580 vector driven by 35S promoter. *Arabidopsis thaliana* protoplast cells were isolated and transfected as previously reported (Cao et al., 2016). EGFP fluorescent signals were visualized using ZEISS LSM 800 with Airyscan (ZEISS, Germany). The empty vector pAN580, which has an EGFP-Fusion tag, was used as a negative control.

**Functional complementation of PcFPPS in yeast**

*Saccharomyces cerevisiae* strain CC25, an ergosterol auxotrophic strain, was used to confirm the function of PcFPPS. A fragment containing the coding region of PcFPPS was PCR-amplified using specific primers pair (Supplementary Table S1) and cloned into the expression vector pESC-TRP to yield pESC-TRP-PcFPPS. pESC-TRP and pESC-TRP-PcFPPS plasmids were transformed into CC25 competent cells using PEG/LiAC and cultured with TRP deficient medium at 30°C for 2–3 d. Positive colonies were verified using PCR. CC25, CC25 + pESC-TRP, CC25 + pESC-TRP-PcFPPS transformants were diluted 1x, 100x, and 1,000x respectively, then plated on YPD medium and incubated at 30°C and 42°C, respectively; for 16 h, followed by 37°C for 2 days to observe the growth of yeast.

**Transient overexpression in Pogostemon cablin plants**

The complete ORF fragment without stop codon of PcFPPS was cloned into pCAMBIA1304 vector tagged with GUS to form pCAMBIA1304-PcFPPS construct with two restriction enzyme sites: NcoI and SpeI. *Agrobacterium tumefaciens* strain GV3101 harboring the empty vector pCAMBIA1304 or binary vector

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1 http://www.bioinformatics.nl/cgi-bin/primer3plus/
pCAMBIA1304-PcFPPS was cultured at 28°C in LB liquid media until the OD_{600} of the culture reached ~0.8. Agrobacterium cells were harvested and resuspended in induction buffer and incubated for 3h at 28°C before infiltration. Leaves of *P. cablin* were infiltrated with *Agrobacterium* suspension and maintained for 3 days under normal growth conditions. The untransformed *Agrobacterium* GV3101 was used as the control group. To determine expression efficiency, histochemical staining was performed on the infiltrated leaves to detect the GUS activity. Tissues were incubated using a GUS staining kit (SL7160, Coolaber, China) according to the manufacturer’s instructions. Leaf tissues near the infiltration point were collected and immediately frozen in liquid nitrogen for further analysis.

To evaluate transient overexpression of *PcWRKY44* in *P. cablin* leaves, full-length coding sequences of *PcWRKY44* were cloned into pJLTRBO to construct pJLTRBO-PcWRKY44 with the restriction enzyme sites PacI and NotI. Empty vector pJLTRBO was used as a negative control. Preparation and infiltration of *Agrobacterium* GV3101 (pSoup-p19) was performed, as previously described (Chen et al., 2020).

**Development of PcFPPS transgenic tobacco lines**

Recombinant vector pCAMBIA1304-PcFPPS was transformed into tobacco strain K326 with wild-type K326 as the control. Transformation of tobacco was performed following the infiltration method using *Agrobacterium tumefaciens* GV3101. Seeds were harvested and plated onto the selection medium containing hygromycin to identify transgenic plants. The presence of the appropriate DNA inserts was confirmed in these plants with RT-qPCR.

**FPP enzymatic activity assays of transgenic plants**

Transformed tobacco leaves were fully homogenized in phosphate-buffered saline (PBS) using a homogenizer. Then, the leaf tissue homogenate was centrifuged at 2000–3000 rpm/min for 20 min at 4°C to collect the supernatant. FPPS enzymatic activity was determined using Plant ELISA Kit (MyBioSource, Inc. United States) following the manufacturer’s instructions.

**Analysis of volatile compounds in leaves using GC-MS**

The content of patchouli alcohol in transiently overexpressed *P. cablin* leaves was detected as previously described (Chen et al., 2019).

A total of 0.4g leaves of transgenic tobacco were ground frizzed in liquid nitrogen, extracted with 1.5 ml hexane ultrasonic for 30 min, and then heated in a 56°C water bath for 40 min. The sample was centrifuged at 12,000 rpm/min for 3 min, the supernatant was passed through 0.22µm organic membranes, and the filtrate was transferred into new vials for GC-MS analysis using an Agilent 7890B Gas Chromatograph with 5977A inert Mass Selective Detector (Agilent, United States). The gas chromatograph was equipped with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness). The oven temperature was programmed as follows: from 35°C (5 min hold) to 300°C at a rate of 12°C/min and hold 5 min at 300°C. NIST14/Wiley275 Mass Spectral Library was used for metabolite identification. To analyze transgenic tobacco metabolites, the relative quantification of volatiles was carried out using the external standard method with cyclohexanone as the external standard substance.

**Cloning of the PcFPPS promoter and dual-luciferase reporter assays**

The promoter region of *PcFPPS* was cloned using PCR strategy with primers designed to flank known genomic sequence of *PcFPPS*. A 938 bp DNA fragment was obtained and cloned into pEASY-Blunt Zero vector for sequencing. For the dual-luciferase reporter assay, full-length coding sequence of *PcWRKY44* was cloned into pGreenII 62-SK vector as effectors, whereas *PcFPPS-pro* was recombined with pGreenII 0800-LUC vector as reporters. The Renilla LUC gene in pGreenII 0800-LUC was used as an internal control. Empty pGreenII 62-SK was used as the negative control for the effector. pGreenII 62-SK, effectors, and reporters were then transferred into *Agrobacterium* strain GV3101 (pSoup-p19). The reporter strain harboring *PcFPPS-pro* LUC was mixed with *Agrobacterium* strains containing 62-SK effector or 62-SK: *PcWRKY44* at a ratio of 1:10. The mixture was injected into 5-week-old *N. benthamiana* leaves using a 1 ml syringe. Firefly luciferase and Renilla luciferase were quantified after 4 days of infiltration with the dual-luciferase assay kit (Promega, United States). The transcriptional regulation ability of *PcWRKY44* was assessed based on the LUC to REN ratio. At least six independent experiments were conducted for each combination.

**Yeast one-hybrid assays**

For the Y1H assay, *PcFPPS-pro* was cloned into the bait vector pHIS2 with the restriction sites EcoRI and SacI. pHIS2-PcFPPS-pro was then transformed into yeast strain Y187 competent cells to test for toxicity and autoactivation. The ORF of *PcWRKY44* was cloned and fused to pGADT7 digested with EcoRI and BamHI to construct the prey vector. Then, pHIS2-PcFPPS-pro and pGADT7-PcWRKY44 were cotransformed into Y187 competent cells. In addition, pHIS2-p53 and pGAD53m were cotransformed into Y187 competent cells as the positive control, whereas pHIS2-PcFPPS-pro and the empty
vector pGADT7 were cotransformed into Y187 as the negative control. The transformants were cultured on SD agar medium lacking tryptophan and leucine (SD/−Trp/−Leu) and positive clones were transferred to SD/−Trp/−His/−Leu plates with 3-AT and grown at different concentrations to verify positive interaction between PcFPPS-pro and PwWRKY44.

Results

Isolation of the PcFPPS full-length cDNA and expression analysis

PCR strategies were applied to isolate putative PcFPPS cDNA (Supplementary Figure S4) based on P. cablin PacBio transcriptome (NCBI Accession: PRJNA528262). Gene sequencing and sequence analysis revealed that PcFPPS contained an integrated open reading frame (ORF) with 1,050 bp, encoded 349 amino acid residues, and its estimated molecular weight was 40.11 kDa. Consistent with FPPSs from different plants (Wang et al., 2004), the deduced PcFPPS protein contains five highly conserved domains, two aspartate-rich motifs (DxDD) FARM (First Aspartic Rich Motif) and SARM (Second Aspartic Rich Motif) located in conserved domains II and V, respectively. These motifs are considered the binding sites of IDP and DMAPP (Figure 1A). BaCelLo analysis predicted PcFPPS localized in the cytoplasm. TMHMM and Pfam protein families database analyses showed that PcFPPS lacks transmembrane domain and signal peptide, respectively. A phylogenetic tree illustrated that PcFPPS was highly homologous to Lavandula angustifolia LaFPPS (AGQ04160.1; Figure 1B).

The expression profiles of PcFPPS were investigated using RT-qPCR analysis. As shown in Figure 1C, all PcFPPS transcripts were detected in four different P. cablin tissues, with the highest level observed in flower, followed by young leaf, and lowest levels in stem and old leaf.

Subcellular localization of PcFPPS in Arabidopsis protoplasts

PcFPPS was predicted with a high probability as localized in the cytoplasm using the BaCelLo database. To determine the subcellular localization of PcFPPS, the ORF of PcFPPS without a termination codon was inserted into the C-terminus of the GFP tag in vector pAN580. As shown in Figure 2, robust fluorescence was observed in the cytoplasmic compartments of Arabidopsis protoplasts expressing 35S: GFP-PcFPPS, suggesting that PcFPPS was localized in the cytoplasm.

Functional complementation of PcFPPS in mutant yeast strain CC25

S. cerevisiae mutant strain CC25 (MATa/MATα, Derg20+/+) is a sterol auxotrophic strain with two leaky mutations erg20-2 and erg12-2, which diminish the capacity of FPPS to condense GPP with IDP to produce FPP. In addition, CC25 is heat-sensitive and cannot grow at temperatures higher than 42°C. As shown in Figure 3, different dilutions of CC25, CC25 + pESC-TRP, and CC25 + pESC-TRP-PcFPPS transformants could grow at a suitable temperature (30°C for 16 h and then 37°C for 2 d), but CC25 and CC25 + pESC-TRP died at high temperature (42°C for 16 h and then 37°C for 2 days) after 100-fold dilution. By comparison, CC25 + pESC-TRP-PcFPPS could still grow on the plate, indicating that PcFPPS compensated for the functional defect of CC25 strain, and it encoded a biologically active enzyme that could catalyze the production of FPP.

Transient overexpression of PcFPPS increases the accumulation of patchouli alcohol in Pogostemon cablin leaves

To further analyze the biological role of PcFPPS and its influence on the biosynthesis of patchouli alcohol, an efficient transient overexpression experiment was performed in vivo on P. cablin plants because designing a stable transformation system in this species is a challenge. The coding region without stop codon of PcFPPS was cloned into pCAMBIA1304 vector to generate pCAMBIA1304-PcFPPS construct, which was expressed under the control of the 35S promoter (Figure 4A). Subsequently, homologous transient overexpression was performed in the leaves of P. cablin. Leaf tissues were collected after PcFPPS was overexpressed for 3 days and used for RT-qPCR, GUS histochemical staining, and GC-MS analysis. PcFPPS transcript was significantly expressed (Supplementary Figure S1) in leaves after infiltration with Agrobacterium harboring overexpressed PcFPPS constructs. GUS histochemical staining was observed in leaf tissues infiltrated with empty vector pCAMBIA1304 and recombinant vector pCAMBIA1304-PcFPPS (Supplementary Figure S1). As shown in Figures 4B,C, overexpression of pCAMBIA1304-PcFPPS produced 47% more patchouli alcohol (2.16 mg/g) compared with the control group pCAMBIA1304 (1.47 mg/g). Altogether, these results indicated that PcFPPS could accelerate biosynthesis of patchouli alcohol in P. cablin (Figure 4).

Overexpression of PcFPPS affects terpenoids metabolism in transgenic tobacco plants

To further investigate the roles of PcFPPS in secondary metabolism and its effects on terpenoid production in vivo, a heterologous transgenic tobacco plant overexpressing PcFPPS was developed. PCR analysis using genomic DNA was performed to confirm the integration of PcFPPS gene into the transgenic tobacco lines. A total of 9 positive transgenic PcFPPS gene tobacco plants were obtained (Supplementary Figure S2). Figure 5A shows the phenotypes of wild-type (WT) and transgenic tobaccos used in this study. As shown in Figure 5A,
all five PcFPPS transgenic tobacco lines appeared slightly larger than the WT. Subsequently, RT-qPCR was performed to verify the accumulation of PcFPPS transcript. As shown in Figure 5B, overexpression of PcFPPS upregulated PcFPPS transcripts in leaves of transgenic plants. The expression levels of PcFPPS in the OEL4, OEL12, OEL16, OEL25, and OEL34 lines were 1–10-fold higher than those of WT. ELISA showed that FPPS enzymatic activity was significantly upregulated in most overexpression lines compared with WT. Among them, FPPS activity in OEL4 was 1.7 times higher than that of WT (Figure 5C). Two transgenic plants, OEL4 and OEL12, were chosen for subsequent GC-MS analysis. As shown in Figure 5D, OEL4 and OEL12 transgenic plants exhibited significantly higher levels of phytol (diterpene alcohol) and neophytadiene (diterpene) compared with WT lines. The content of phytol in OEL4 and OEL12 were 1.37-fold and 1.85-fold higher than that of WT, respectively. In addition, the contents of neophytadiene in OEL4 and OEL12 were 1.28-fold and 1.76-fold higher than that of WT. Stigmasterol, one of the triterpenoid sterols, was also detected in transgenic plants. Its content in OEL4 and OEL12 was 1.48-fold and 1.73-fold higher than that of WT, respectively (Figure 5E). The GC-MS results were consistent with the gene expression and FPPS enzymatic activity changes, indicating that PcFPPS played a key role in the formation of farnesyl diphosphate, a precursor of several classes of essential metabolites, including terpenoids and sterols.
**PcFPPS-pro** can be bound by transcription factor **PcWRKY44**

To further analyze the function of **PcFPPS** in *P. cablin*, a 938bp promoter region of **PcFPPS** was successfully cloned and sequenced using PCR method (Supplementary Figure S3). The transcription factor **PcWRKY44** (Supplementary Figure S4) was obtained through DNA-pull down screening in the early stage of our research. To identify whether **PcFPPS-pro** (938bp) can be bound by **PcWRKY44**, a Dual-LUC assay was performed in *N. benthamiana* leaves. **PcFPPS-pro** (938bp) was cloned into vector pGreenII 0800-LUC vector as reporters and the ORF of **PcWRKY44** was

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**FIGURE 2**
Subcellular localization of **PcFPPS** in *Arabidopsis* protoplasts. Control vector (35S:EGFP) and recombinant vector (35S:GFP-**PcFPPS**) are expressed in protoplasts of *Arabidopsis*. GFP, GFP fluorescence; Chlorophyll, Chlorophyll fluorescence; Light field; and Merged; superposition of fluorescence and light field. Bars, 10 μm.

**FIGURE 3**
Functional complementation of **PcFPPS** in mutant yeast strain CC25 (MATa/MATalpha, *Derg20*/+). The growth testing and evaluation of strain CC25(*ΔERG20*/+), empty vector transformed CC25(CC25+pESC-TRP) and pESC-**PcFPPS** transformed CC25(CC25+pESC-TRP-**PcFPPS**) were carried out in parallel at appropriate temperature of 30°C and nonpermissive high temperature of 42°C for 16h. Then the yeast growth was observed after culturing at 37°C for 2 days.
FIGURE 4
Transient overexpression of PcFPPS increases the accumulation of patchouli alcohol in *P. cablin* leaves. (A) The coding region without stop codon of PcFPPS was cloned into the pCAMBIA1304 vector controlled by the 35S promoter to form the pCAMBIA1304-PcFPPS. (B) Patchouli alcohol contents in leaves of CK (wild type), the empty vector pCAMBIA1304, and transformations pCAMBIA1304-PcFPPS. (C) Gas Chromatography–Mass Spectrometer (GC-MS) chromatograms of samples from the pCAMBIA1304-PcFPPS (top panel), pCAMBIA1304 (middle panel), and CK (bottom panel) leaves showing abundance of patchouli alcohol. Data represent the mean ± SEs of three biological replicates. The asterisks represent significant differences, as indicated by t-test (**p<0.01). FW, fresh weight.

FIGURE 5
Overexpression of PcFPPS modulated the relative expression levels of PcFPPS and altered the endogenous terpenoid contents in transgenic tobacco plants. (A) Phenotypes of WT and transgenic tobacco plants. (B) Relative expression levels of PcFPPS in transgenic tobacco plants. (C) Activity of FPPS in WT and transgenic tobacco plants. (D) The content of phytol and neophytadiene detected in WT and transgenic tobacco OEL4 and OEL12. (E) The content of stigmasterol detected in WT, OEL4, and OEL12. Student’s t-test was performed to identify significant differences. One asterisk (*) indicates a significant difference (0.01<p<0.05) and two/three indicate a very significant difference (p<0.01). Scale bars=5cm.
cloned into vector pGreenII 62-SK vector as effectors (Figure 6A). A 5-week-old N. benthamiana was injected by Agrobacterium GV3101-pSoup-p19 cultures containing recombinant constructs (Figure 6B). Agrobacterium injection site containing 62-SK: PcWRKY44 and PcFPPS-pro: LUC recombinant constructs showed a larger red area, revealing a strong interaction between PcWRKY44 and PcFPPS-pro in N. benthamiana vivo. It also suggested that this interaction is likely to exist in P. cablin. Relative firefly Luc activity was quantified after 4 days of infiltration using dual-luciferase assay. As shown in Figure 6C, the activity of PcFPPS-pro (938 bp) was significantly increased by PcWRKY44, suggesting that the expression of PcFPPS gene was activated by PcWRKY44.

Y1H assay was carried out to further investigate whether PcWRKY44 protein could directly bind to PcFPPS-pro in vitro. The PcFPPS-pro (938 bp) was firstly cloned into the bait vector pHIS2 and the full-length coding sequence of PcWRKY44 was cloned into pGADT7. Y1H assays showed that the yeast cells with pHIS2-PcFPPS-pro (938 bp) and pGADT7-PcWRKY44 could not grow on SD/-Trp/-His/-Leu plates, which was consistent with the negative control, suggesting that PcWRKY44 could not bind to the 938 bp PcFPPS-pro (Figure 6E). Subsequently, PcFPPS-pro was truncated to 220 bp (Figure 6D) and Y1H assay was performed again. As shown in Figure 6F, yeast cells with pHIS2-PcFPPS-pro (220 bp) and pGADT7-PcWRKY44 could grow on SD/-Trp/-His/-Leu plates containing different concentrations 3-AT, which was consistent with the positive control, suggesting that 220bp PcFPPS-pro can be bound by transcription factor PcWRKY44 to activate HIS3 expression.

PcWRKY44 located in the cell nucleus and could promote the accumulation of patchouli alcohol

The above results demonstrate that PcWRKY44 can bind to the 220bp PFP promoter and activate the transcriptional activity of genes downstream of the promoter, indicating that PcWRKY44 may play an important role in the biosynthesis of patchouli alcohol. To further analyze the function of PcWRKY44, subcellular localization and transient overexpression experiments were performed.

The ORF of PcWRKY44 without a termination codon was inserted into the N-terminus of the GFP tag in vector pAN580 for subcellular localization analysis. The 35S: PcWRKY44-GFP and 35S: EGFP constructs were separately introduced into Arabidopsis protoplast cells. The GFP fluorescence of the cells transformed with 35S: PcWRKY44-GFP was observed in the
nucleus, indicating that PcWRKY44 is a nuclear protein (Figure 7A).

The full-length coding sequence of PcWRKY44 was cloned into pJLTRBO to construct pJLTRBO-PcWRKY44 (Figure 7B). Subsequently, an Agrobacterium-infiltrated homologous transient overexpression assay in P. cablin leaves, the expression levels of PcWRKY44, PcFPPS, and PcPTS were all higher than that of EV-TO (Figure 7C). Patchouli alcohol content in PcWRKY44-TO and EV-TO was determined using GC-MS. Consistent with the gene expression profiles of PcWRKY44, PcFPPS, and PcPTS, patchouli alcohol accumulation was increased in PcWRKY44-TO. Transient overexpression of PcWRKY44 produced levels of patchouli alcohol (1.19 mg/g FW) 12.3% higher than that of the EV-TO (1.06 mg/g FW; Figures 7D,E). The above results indicate that PcWRKY44 could promote the biosynthesis of patchouli alcohol in P. cablin.

Discussion

Hundreds of different drug products from P. cablin have been registered for use by the State Food and Drug Administration of China (SFDA), including various pills, 245 oral fluid agents, tablets, capsules, and granules (Chen et al., 2013). Therefore, P. cablin represents an effective herbal remedy with great application potential in diverse clinical situations. Patchouli alcohol is sesquiterpene alcohol with antibacterial, antifungal, and antiviral activity, isolated exclusively from patchouli oil. Patchouli alcohol also inhibits lipopolysaccharide-induced inflammation in the cells (Xian et al., 2011). However, there are considerable theoretical and technological lags limiting our understanding of the biosynthesis and regulatory mechanism of patchouli alcohol in P. cablin, which hinders sustainable pharmacological development and utilization of P. cablin. Our study systematically performed functional analysis of PcFPPS encoding farnesyl pyrophosphate synthase and revealed that PcFPPS activates terpenoids and patchouli alcohol biosynthesis in P. cablin. Moreover, PcWRKY44 was characterized and proposed as a critical transcriptional activator of PcFPPS. This study provides a detailed analysis of the roles of PcFPPS and PcWRKY44 in patchouli alcohol biosynthesis, elucidating the functional role and transcription regulatory network of patchouli alcohol production.

FPPS in higher plants has been reported to play an important role in organ development and as a key regulatory enzyme involved in terpenoid biosynthetic pathways (Qian et al., 2017; Qin et al., 2018). To understand and elucidate the functions of PcFPPS in P. cablin terpenoids biosynthesis, we functionally confirmed PcFPPS through a yeast complementation assay and analyzed its function in P. cablin by transient overexpression and stable transformation expression in tobacco. The function of the PcFPPS gene was verified by functional complementation of mutant yeast strains lacking FPPS activity. P. cablin was transiently transformed with a construct harboring PcFPPS under the control of the 35S promoter. GC-MS analysis revealed that PcFPPS accelerated terpene biosynthesis, resulting in an ~47% increase in patchouli...
alcohol compared with the control (Figure 4). The results demonstrate that PcFPPS activated the terpene biosynthesis pathway and accelerated the accumulation of patchouli alcohol. With the overexpression of PcFPPS in tobacco, the transgenic tobacco appeared to grow larger and more vigorous than WT tobacco (Figure 5A). However, Arabidopsis thaliana overexpressing FPS1S showed a senescence-like phenotype (Masferrer et al., 2002), which was contrary to our findings, preliminarily indicating that PcFPPS may have a more unique function in P. cablin growth and development. However, further study is required to verify this function. In addition, the results of FPPS enzyme activity were significantly upregulated, and transgenic plants exhibited significantly higher levels of phytol, neophytadiene, and stigmasterol (Figure 5). Phytol is an acyclic diterpene alcohol molecule and a constituent of chlorophyll with a wide range of biological effects (Islam et al., 2018). Neophytadiene was identified as a natural diterpene herbal component isolated from flue-cured tobacco (Banožić et al., 2021) and has been tested for its analgesic potential. These results indicate that PcFPPS enhanced enzymatic activity and activated terpenoid biosynthesis in plant. Stigmasterol is a phytosterol, one of the most abundant phytosterols in nature, produced through the mevalonate pathway (Aboobucker and Suza, 2019). FPP is a precursor for sterol synthesis, which agrees with our findings that overexpressed PcFPPS significantly increases the accumulation of stigmasterol. Overexpression of PcFPPS in P. cablin and tobacco plants resulted in upregulation of terpene and phytosterol biosynthesis, which is consistent with previous findings (Keim et al., 2012; Yang et al., 2017). Our study shows that in P. cablin plants, PcFPPS plays a key role in the formation of isoprenoid end products such as terpenoids and phytosterols.

In previous studies, it was found that plants contain at least two FPP genes, FPS1 gene that encodes a long isoform FPS1L which is targeted to mitochondria and a short isoform FPS1S, for which the localization has not been reported (Clastre et al., 2011). CrFPPS (which is closely related to FPS2), isolated from the Madagascar periwinkle (Catharanthus roseus), was targeted to peroxisomes in C. roseus cells; however, the CrFPPS protein was retained in the cytoplasm (Thabet et al., 2011). The PcFPPS isolated in this paper may be closely related to FPS2 based on sequence alignment analysis. Therefore, we explored whether PcFPPS is located in peroxisomes. However, we found that 35S: GFP-PcFPPS displayed a diffuse pattern of fluorescence throughout the cytoplasm (Figure 2), which differed from that of the punctate fluorescent signal characteristic of peroxisomal proteins. Our results are consistent with those reported previously with regard to the subcellular distribution of FPP enzymes involved in the early steps of plant isoprenoid biosynthesis. This finding provides a new perspective for characterizing the biosynthesis of terpenes and/or patchouliol. The genome of P. cablin has been reported so far, and there are at least four FPPS genes in the patchouli genome (Shen et al., 2022), which are consistent with our previous transcriptome analysis results (Tang et al., 2019). In this study, FPPS3 in Tang’s paper was selected, and named PcFPPS for study, because it was expressed at a higher level in flowers and leaves than the other three genes. Our results suggest that PcFPPS plays an important role in the biosynthesis of terpenes. As members of the FPPS family, it is reasonable to believe that the other three FPPS genes also have significant effects on the biosynthesis of terpenes in P. cablin, which remains to be further studied.

Currently, the transcriptional regulation of functional synthase genes involved in the biosynthesis of patchouli alcohol is not well understood, and this limits its industrialization exploitation. The growth and development of P. cablin is regulated by transcription factors. For example, PatDREB, a nucleus-localized AP2/ERF TF, was identified as a transcription activator that binds to the promoter of PatPTS to positively regulate jasmonate-induced patchouli alcohol biosynthesis (Chen et al., 2022). In this study, the promoter of PcFPPS was identified for the first time, and the PcWRKY44 transcription factor was identified to bind to 220bp PcFPPS-pro to increase the transcriptional activity of PcFPPS (Figure 6). WRKY transcription factors family, localized in the nucleus, is one of the largest transcription factor families in plants (Rushton et al., 2010). Numerous WRKY transcription factors involved in plant metabolism have been identified in different plant species. For example, subcellular localization analysis of Osmanthus fragrans showed that p35S::GFP OWRYK7/38/95/139 was localized in the nucleus (Ding et al., 2020), which is consistent with our observation that PcWRKY44 was localized in the nucleus (Figure 7A). In Oryza sativa, OsWRKY45 played an important role in the initiation of diterpenoid phytoalexin biosynthesis after inoculation with Magnaporthe oryzae (Akagi et al., 2014). A total of 58 WRKYs were identified in Andrographis paniculata genome, and 7 of them, including ApWRKY01, ApWRKY08, ApWRKY12, ApWRKY14, ApWRKY19, ApWRKY20, and ApWRKY50 may participate in the biosynthesis of andrographolide (Zhang et al., 2020). Overexpression of the WRKY gene may enhance the expression of structural genes and promote accumulation of key secondary metabolites in plants. For example, in Ophiopogon pumila, OpWRKY2 was found to directly bind and activate the OpTDC gene involved in the camptothecin synthesis pathway. Moreover, overexpression of OpWRKY2 resulted in a more than 3-fold increase in camptothecin levels (Hao et al., 2021). In this study, overexpression of PcWRKY44 increased the expression of PcFPPS and PcPTS, thereby enhancing synthesis and accumulation of patchouli alcohol. Taken together, the present findings highlight the transcriptional regulation mechanisms involved in patchouli alcohol biosynthesis. They are expected to promote genetic engineering of patchouli alcohol for industrial production.

### Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.946629/full#supplementary-material

SupPLEMENTARY FIGURE S4
Gene sequencing of PcFPPS and PcWRKY44.

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Publisher contributions

LC, XW, YT, and HH conceived and designed the experiments. LC, YT, HH, XC, JL, HZ, and DW performed the experiments. YT, LC, HH, and DW analyzed the data. LC, YT, and XW wrote the manuscript. All authors contributed to the article and approved the submitted version.

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