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Prokaryotic Expression of FPPS Gene from Pogostemon cablin and Effect of Methyl Jasmonate on FPPS Expression Level Postprint

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Abstract

Farnesyl diphosphate synthase (FPPS) is a key enzyme in terpenoid biosynthesis via the mevalonate pathway in patchouli, catalyzing the synthesis of the terpenoid precursor farnesyl pyrophosphate from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). To further investigate the molecular mechanisms of terpenoid synthesis in patchouli, this study obtained the cDNA sequence of the FPPS gene through reverse transcription polymerase chain reaction and predicted the physicochemical properties, structure, and function of the FPPS-encoded protein using bioinformatics software. The results indicated that the open reading frame of this sequence was 1,050 bp in length, encoding 349 amino acids, with a predicted molecular weight of 40 kDa and an isoelectric point of 5.43. The protein contained a single domain involved in isoprenoid compound synthesis, lacked a signal peptide, and was subcellularly localized in the cytoplasm. Phylogenetic analysis revealed that the FPPS amino acid sequence from patchouli exhibited the closest phylogenetic relationship with those from Salvia miltiorrhiza Bunge and Salvia officinalis Linn. Furthermore, to investigate protein expression, this study constructed a pET-32b-FPPS prokaryotic expression vector using seamless cloning technology and transformed it into the BL21 (DE3) strain to examine the effects of different concentrations of isopropyl -D-1-thiogalactopyranoside (IPTG) on the expression of the fusion protein. The results demonstrated that the fusion protein was present as inclusion bodies in the pellet, with no significant differences in protein expression observed among the four IPTG concentrations. Finally, to investigate the effect of methyl jasmonate (MeJA) on FPPS expression, this study employed quantitative real-time PCR to analyze the effects of 0.1 and 0.25 mmol · L ^{1} MeJA on FPPS gene expression levels. Following induction with 0.1 mmol · L ^{1} MeJA, the expression level of the FPPS gene exhibited a trend of first increasing, then decreasing, then increasing again, and finally

decreasing again; whereas after induction with 0.25 mmol · L [1] MeJA, the expression trend was first decreasing, then increasing, and then decreasing. It is hypothesized that changes in MeJA concentration in plant tissues can influence FPPS gene expression, with high concentrations exerting an inhibitory effect and low concentrations exerting a promotional effect. This study lays a foundation for research on the terpenoid synthesis pathway in patchouli and provides a theoretical reference for subsequent gene function validation.

Full Text

Preamble

Prokaryotic Expression of FPPS Gene from *Pogostemon cablin* and the Effect of Methyl Jasmonate on FPPS Expression

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Abstract: Farnesyl diphosphate synthase (FPPS) is a key enzyme for terpene biosynthesis in the patchouli mevalonate pathway, catalyzing the conversion of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) into the terpenoid precursor farnesyl pyrophosphate. To investigate the molecular mechanisms of terpenoid synthesis in patchouli, we obtained the FPPS cDNA sequence via reverse transcription PCR and predicted the physicochemical properties, structure, and function of the encoded protein using bioinformatics tools. The results revealed an open reading frame of 1,050 bp encoding 349 amino acids, with a predicted molecular weight of 40 kDa and isoelectric point of 5.43. The protein contains a single domain involved in isoprenoid compound synthesis, lacks a signal peptide, and is localized to the cytoplasm. Phylogenetic analysis showed that patchouli FPPS shares the closest relationship with *Salvia miltiorrhiza* and *Salvia officinalis*. To examine protein expression, we constructed a pET-32b-FPPS prokaryotic expression vector using seamless cloning and transformed it into *E. coli* BL21(DE3), investigating the effects of different IPTG concentrations on fusion protein expression. The results indicated that the fusion protein accumulated as inclusion bodies in the precipitate, with no significant differences observed among the four IPTG concentrations tested. Finally, to assess the effect of methyl jasmonate (MeJA) on FPPS expression, we used quantitative real-time PCR to analyze the impact of 0.1 and 0.25 mmol · L [1] MeJA on FPPS transcript levels. The 0.1 mmol · L [1] MeJA treatment in-

duced a dynamic expression pattern of initial increase, followed by decrease, then increase, then decrease again. In contrast, $0.25\ mmol \cdot L^{1}$ MeJA treatment produced a pattern of initial decrease, then increase, then decrease. These findings suggest that MeJA concentration fluctuations affect FPPS expression, with high concentrations exerting inhibitory effects and low concentrations showing promotive effects. This study establishes a foundation for investigating terpenoid synthesis pathways in patchouli and provides theoretical reference for subsequent gene function validation.

Keywords: *Pogostemon cablin*, farnesyl pyrophosphate, seamless cloning, methyl jasmonate, quantitative real-time PCR

Patchouli, derived from the dried aerial parts of *Pogostemon cablin* (Lamiaceae), is cultivated in Hainan and Lingnan regions of China and is commonly used to treat various exterior syndromes caused by summer dampness and gastrointestinal discomfort resulting from damp turbidity. Currently, patchouli serves both medicinal and aromatic purposes. Patchouli oil contains over 140 bioactive compounds, including terpenoids and flavonoids, and is used to alleviate depression and stress, calm nerves, control appetite, and improve libido, while also exhibiting insecticidal, antibacterial, and antifungal properties. Patchouli alcohol, a major component of patchouli oil, is a tricyclic sesquiterpene widely used in perfumes and cosmetics and can serve as a starting compound for paclitaxel synthesis. With increasing demand from pharmaceutical and fragrance industries, investigating the molecular mechanisms of key compound synthesis and enhancing volatile oil content, particularly patchouli alcohol, has become critically important.

Isoprenyl diphosphate synthases exhibit two stereochemical configurations belonging to the E- and Z-families. In the E-family, carbon atoms in the isoprenoid chain are on opposite sides of the double bond (trans), primarily synthesizing short-chain isoprenoids, whereas the Z-family features carbon atoms on the same side (cis), mainly producing long-chain isoprenoids. Farnesyl pyrophosphate synthase is the backbone elongation enzyme of the isoprenoid pathway and belongs to the E-family, catalyzing the consecutive condensation of dimethylallyl diphosphate (C5) and geranyl diphosphate (C10) with isopentenyl diphosphate (C5) to generate the E-isomer of farnesyl pyrophosphate (C15). FPPS represents a branch point in isoprenoid compound synthesis, and alterations in its activity or structure can significantly affect downstream isoprenoid production by changing carbon flux distribution, making it crucial for metabolic regulation. Beyond its role in isoprenoid synthesis, FPPS participates in fibroblast growth factor-mediated signal transduction, with overexpression enhancing Ras protein farnesylation and activating the Ras/ERK signaling cascade. In pharmaceutical research, FPPS serves as a molecular target for drug development, with studies demonstrating it as the intracellular target of nitrogen-containing bisphosphonates using C14-labeled mevalonate, IPP, and DMAPP in bovine brain.

Methyl jasmonate, derived from jasmonic acid methylation, functions as an endogenous signaling molecule in plants, playing vital roles in metabolism, secondary metabolite synthesis, disease resistance, and stress tolerance. Due to its volatility and molecular properties, MeJA can enter plants through stomata, enabling long-distance signal transduction and inter-plant communication. Upon entry, MeJA is hydrolyzed to jasmonic acid, which stimulates biosynthesis of various secondary metabolites including nicotine alkaloids, isoquinolines, glucosinolates, anthocyanins, sesquiterpenoid artemisinin, and terpenoid indole alkaloids. When herbivores attack plants, specific elicitors trigger volatile compound release for defense, with jasmonic acid as the central signaling component. Studies have shown MeJA-treated tobacco plants exhibit increased volatile production, enhancing parasitism rates of tobacco pests by parasitoid wasps, while lima bean plants release terpenoid volatiles after MeJA treatment, attracting predatory mites to control harmful pests.

Since MeJA signaling increases plant volatiles and sesquiterpenoids constitute important volatile components, with farnesyl pyrophosphate as their key precursor, investigating MeJA regulation of FPPS expression is essential for understanding inter-plant signaling and plant defense mechanisms against pests and diseases.

This study cloned the FPPS gene from patchouli, constructed a pET-32b-FPPS prokaryotic expression vector via seamless cloning for small-scale protein expression, performed bioinformatics analysis of FPPS, and examined MeJA effects on FPPS expression using quantitative real-time PCR to establish a foundation for investigating sesquiterpenoid biosynthesis and MeJA regulatory mechanisms in patchouli.

1.1 Plant Material

Patchouli plants were obtained from Danzhou, Hainan Province, and transplanted to Guangdong Pharmaceutical University, where they were identified as *Pogostemon cablin* by Associate Professor Liu Jizhu.

1.2 Bacterial Strains

E. coli DH5 competent cells and BL21(DE3) expression strains were purchased from Beijing Zoman Biotechnology Co., Ltd.

1.3 Instruments and Reagents

Instruments: Electronic balance CP214 (Ohaus), UV spectrophotometer K5500Plus (Beijing Kaiao Technology), PCR thermal cycler T100 (Bio-Rad), electrophoresis apparatus DYCP-31CN (Beijing Liuyi Biotechnology), gel imaging system Tocan320 (Shanghai Lingcheng Biotechnology), blue light gel cutter BD-BGC1 (Wuxi Bofu Ruide Biotechnology), refrigerated centrifuge 3K15 (Sigma), 恒温摇床 QYC-200 (Shanghai Fuma Experimental Equipment), real-time PCR system (Bio-Rad CFX96), cell disruptor (SONICS VCX750).

Reagents: TRIpure Reagent (Beijing Aidlab Biotechnology), FastKing one-step RT-PCR kit, agarose (Biowest), DNA purification and recovery kit, plasmid mini-prep kit, pGM-T Fast ligation kit (Tiangen Biotech), SYBR® Green Pro Taq HS qPCR kit, Evo M-MLV RT kit (Accurate Biology), 6×protein loading buffer, Blue Plus Protein Marker (Beijing TransGen Biotech), pET-32b plasmid, SE seamless cloning and assembly kit, DL2000 Marker, ExRed nucleic acid dye (Beijing Zoman Biotechnology).

2.1 Total RNA Extraction from Patchouli

Total RNA was extracted using TRIpure Reagent according to the manufacturer' s protocol. cDNA was synthesized using the FastKing one-step RT-PCR kit, and products were identified by 1% agarose gel electrophoresis. Target fragments were recovered by gel extraction, and cDNA concentration and purity were measured and stored at -20°C.

2.2 T-A Cloning of FPPS Gene

T-A cloning was performed using the pGM-T Fast ligation kit. After transformation into DH5 competent cells, positive clones were identified by colony PCR using primers FPPS-F: ATGGCGAATCCGAACGGAGC and FPPS-R: TTATTTCTGTCTCTTGTAAATCTTGCC. Positive clones were cultured overnight, plasmids were extracted using a mini-prep kit, and concentrations were measured. Appropriate amounts were sent to Tsingke Biotechnology for sequencing, with remaining plasmids stored at -20°C. Sequencing results were assembled using ContigExpress software and aligned using BLASTn.

2.3.1 Introduction of Homology Arms for FPPS Gene and pET-32b Vector

FPPS gene PCR reaction system: pGM-T-FPPS recombinant plasmid (100 ng· L ^{1}) 1 L, FPPS-pET-F primer (10 mol·L ^{1}) 1.25 L, FPPS-pET-R primer (10 mol·L ^{1}) 1.25 L, PrimeSTAR® Max DNA Polymerase 25 L, ddH O to 50 L. (Primers: FPPS-pET-F: ACGACGACGACAAGGCGAATCCGAACGGAG, FPPS-pET-R: AGGGGTTATGCTAGTTATTTCTGTCTCTTG)

pET-32b vector PCR reaction system: pET-32b vector (60 ng· L ^{1}) 1 L, pET-F primer (10 mol·L ^{1}) 1.25 L, pET-R primer (10 mol·L ^{1}) 1.25 L, PrimeSTAR® Max DNA Polymerase 25 L, ddH O to 50 L. (Primers: pET-F: GAGACAGAAATAACTAGCATAACCCCTTGG, pET-R: CGTTCGGATTCGCCTTGTCGTCGTCGTCGGTAC)

PCR conditions: 95°C pre-denaturation 3 min; 35 cycles of 98°C denaturation 10 s, 55°C annealing 5 s, 72°C extension 6 s; final extension 72°C 5 min. Products were identified by 1% agarose gel electrophoresis, recovered by gel extraction, and stored at -20°C after concentration and purity measurement.

2.3.2 Circularization of FPPS Gene and Linearized pET-32b Vector

FPPS gene and linearized pET-32b vector were circularized using the seamless cloning kit, then transformed into DH5 competent cells. Single colonies were selected for small-scale culture and verified by colony PCR. Positive clones were cultured, plasmids were extracted and sequenced. Sequencing results were assembled and compared with T-A cloning sequences to confirm correct insertion sites.

2.4 Small-Scale Expression of Patchouli FPPS Protein

Protein expression was examined under different temperatures (20, 25, 30, 37°C) and IPTG concentrations (1, 0.75, 0.5, 0.25 mmol·L 1). For each IPTG concentration, 50 L of induced supernatant, precipitate, and uninduced control were mixed with 10 L 6×protein loading buffer, heated at 100°C for 10 min, and 10 L samples were loaded for electrophoresis at 80 V for 20 min followed by 120 V for 80 min. Gels were stained with Coomassie Brilliant Blue overnight, destained, and photographed.

2.5 Bioinformatics Analysis of FPPS

The primary structure and physicochemical properties of FPPS-encoded protein were analyzed using the ExPASy online platform. Transmembrane structure was predicted using TMHMM 2.0, signal peptides using SignalP 4.1, subcellular localization using ProtComp 9, domain structure using SOPMA, and tertiary structure using SWISS-MODEL based on homology modeling. A phylogenetic tree was constructed using MEGA7.0 by aligning the FPPS amino acid sequence with 61 other plant FPPS sequences from GenBank.

2.6.1 Plant Material Selection and Treatment

Thirty healthy six-month-old patchouli plants were transferred to a constant temperature chamber for acclimation. Solutions of 0.1 and 0.25 mmol·L 1 MeJA were prepared and evenly sprayed on leaves, which were then covered with plastic film for 1 h. Sampling began at 9:00 on August 26, 2019, with mature leaves collected at 0, 2, 6, 12, 24, 48, and 72 h, immediately frozen in liquid nitrogen, and stored at -80°C.

2.6.2 RNA Extraction and First-Strand cDNA Synthesis

RNA extraction followed the method described in Section 2.1. First-strand cDNA synthesis was performed using the Evo M-MLV RT kit according to the manufacturer' s instructions.

2.6.3 Primer Design and Validation

Primers were designed using CmSuite8 software based on patchouli FPPS and internal reference gene 18S rRNA mRNA sequences and synthesized by Ts-

ingke Biotechnology. QFPPS-F: AGGTCCCTAAGGTTGGTATG; QFPPS-R: GGAACTCCACCTCATTGAAC; 18S-F: TCAACCATAAACGATGCCGACC; 18S-R: TTTCAGCCTTGCGACCATACTCC. Primers were validated using a 10 L RT-PCR system: 98°C pre-denaturation 30 s; 40 cycles of 98°C denaturation 10 s, 53°C annealing 30 s, 72°C extension 1 min; final extension 72°C 7 min. Products were detected by 1% agarose electrophoresis.

2.6.4 Quantitative Real-Time PCR

qPCR was performed using the SYBR® Green Pro Taq HS kit with three technical replicates per sample in a 96-well plate. Control plants were sprayed with ethanol diluted in the same manner.

2.6.5 Statistical Analysis

Each sample had three biological replicates. Data were analyzed using the 2^(-ΔΔCT) method, where ΔCT = CT(target gene) - CT(reference gene) and ΔΔCT = ΔCT(treatment) - ΔCT(control). Statistical significance was analyzed using SPSS software.

3.1 Total RNA Extraction and FPPS cDNA Synthesis

Total RNA from three parallel groups showed good integrity with OD260/280 ratios of 1.8. Agarose gel electrophoresis revealed clear 18S and 28S bands, meeting experimental requirements [Figure 1: see original paper]. cDNA synthesized using the one-step RT-PCR kit showed a bright band between 1,000-1,500 bp [Figure 2: see original paper].

3.2 T-A Cloning of Patchouli FPPS Gene

After T-vector construction, colony PCR verified positive clones. Plasmids extracted from positive clones were sequenced by Tsingke Biotechnology. BLAST analysis showed high similarity with FPPS genes from other plants in NCBI. The sequence was deposited in GenBank under accession number MN326318.

3.3 Construction of Prokaryotic Expression Vector via Seamless Cloning

After introducing homology arms, 1% agarose electrophoresis showed a clear FPPS gene band between 1,000-1,500 bp and a bright pET-32b vector fragment at 5,500 bp [Figure 3: see original paper]. Following seamless cloning and transformation into DH5 , eight colonies were selected for colony PCR verification, all showing successful transformation [Figure 4: see original paper].

3.4 FPPS Protein Expression

At 20°C and 130 rpm, induction with 0, 0.25, 0.5, 0.75, and 1.0 mmol·L ¹ IPTG for 6 h revealed that pET-32b-FPPS expressed a thioredoxin-FPPS fusion

protein of approximately 50 kDa. The fusion protein was not expressed in the supernatant but accumulated as inclusion bodies in the precipitate. No significant differences in expression levels were observed among the different IPTG concentrations at 20°C [Figure 5: see original paper].

3.5 Bioinformatics Analysis

The FPPS open reading frame was 1,050 bp, encoding 349 amino acids. Phylogenetic analysis with 61 plant FPPS sequences showed patchouli FPPS clustered most closely with *Salvia miltiorrhiza* and *Salvia officinalis* (both Lamiaceae) with 84% bootstrap support. These formed a major clade with Eucommiaceae, Ranunculaceae, Gentianaceae, and Orchidaceae, while other plants generally clustered by family [Figure 6: see original paper].

The FPPS-encoded protein had a molecular weight of 40 kDa and isoelectric point of 5.43. Amino acid composition included 14.1% acidic residues (Asp, Glu), 11.7% basic residues (Lys, Arg), 26% polar residues (Asn, Cys, Gln, Ser, Thr, Tyr), and 35.2% hydrophobic residues. The aliphatic index was 91.89, instability index 34.47, with predicted half-lives >20 h in yeast and >10 h in *E. coli*, suggesting high stability. TMHMM 2.0 predicted no transmembrane regions, while PSORT II predicted cytoplasmic localization.

SOPMA analysis revealed four secondary structure elements: 214 amino acids (61.32%) in -helices, 25 residues (7.16%) in extended strands, 9 residues (2.58%) in -turns, and 101 residues (28.94%) in random coils [Figure 7: see original paper]. SMART analysis identified a single domain spanning amino acids 39-304 (266 residues) potentially involved in isoprenoid compound synthesis. SignalP 5.0 predicted no signal peptides.

Homology modeling using SWISS-MODEL with monoterpene synthase FDS-5 and chloroplast farnesyl pyrophosphate synthase 1 chimera (SMTL ID: 4kk2.1, 75.66% similarity) as template yielded a reliable 3D structure (GMQE = 0.86, QMEAN = -0.35) [Figure 8: see original paper].

3.6 Effect of Methyl Jasmonate on FPPS Gene Expression

Relative to the control (expression level = 1), 0.1 mmol·L ¹ MeJA induced highly significant differences at 48 and 72 h, while 0.25 mmol·L ¹ MeJA showed highly significant differences at 48 h. Both concentrations peaked at 48 h. The 0.1 mmol·L ¹ MeJA treatment produced a pattern of increase-decrease-increase-decrease, whereas 0.25 mmol·L ¹ MeJA showed decrease-increase-decrease [Figure 9: see original paper].

4.1 Structure and Function Prediction of FPPS Protein

The predicted isoelectric point of 5.43 suggests that refolding buffers should be maintained above this pH to avoid precipitation during inclusion body renaturation. Subcellular localization and transmembrane predictions confirmed

cytoplasmic localization, consistent with previous reports and facilitating future studies on terpenoid synthesis sites and targeted genetic transformation, such as using chloroplast transit peptides to direct FPPS and downstream genes into chloroplasts for enhanced terpenoid production. The SMART-predicted domain likely participates in carbon chain elongation, catalyzing the sequential formation of C10 geranyl pyrophosphate and C15 farnesyl pyrophosphate from C5 substrates.

4.2 Regulatory Effect of Methyl Jasmonate on FPPS Expression

Key enzyme gene expression is influenced by temporal, spatial, and temperature factors, with variation among plant tissues. Previous studies showed diurnal variation in patchouli alcohol synthase expression, peaking at 3:00, and differential squalene synthase expression in *Panax notoginseng* (root > rhizome > stem). This study focused on mature leaves, mixing multiple RNA extractions from the same time point to reduce individual variation. Future studies will examine leaves of different maturity stages to further elucidate MeJA' s role in terpenoid synthesis.

Exogenous MeJA application significantly affected FPPS expression. Under 0.25 $\text{mmol} \cdot \text{L}^{1}$ MeJA, high initial absorption caused inhibition at 0-6 h, followed by increased expression at 12-48 h as MeJA degraded, then decreased expression as levels normalized. Under 0.1 $\text{mmol} \cdot \text{L}^{1}$ MeJA, lower absorption delayed inhibition, causing initial increase at 2 h, gradual decrease at 6-24 h, promotion at 48 h upon reaching optimal concentration, and final decrease at 72 h. Higher MeJA concentrations led to faster accumulation and earlier inhibition. These results suggest high MeJA concentrations inhibit FPPS expression while low concentrations promote it.

In terpenoid biosynthesis, transcription factors regulate synthesis by binding to promoter elements upstream of enzyme genes, simultaneously modulating multiple targets to coordinate metabolic flux toward specific products. As demand for terpenoids increases and chemical synthesis remains challenging due to structural complexity, molecular regulation of plant terpenoid metabolism has become a research focus, with transcriptional regulation of key enzyme genes representing an essential trend for enhancing production.

Note: Figure translations are in progress. See original paper for figures.

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