

Postprint: Gene Expression Analysis of the MVA Pathway and Its Correlation with Main Component Synthesis in Ketone-type Patchouli

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Abstract

Ketone-type patchouli herb with higher patchouli ketone content is considered to be of the highest quality. Patchouli ketone is a terpenoid component whose biosynthetic pathway remains elusive. The MVA (mevalonic acid) pathway represents an important route for terpenoid biosynthesis. Real-time quantitative PCR was employed to analyze gene expression, and main component content was determined, with the aim of analyzing their correlation to identify potential genes that promote patchouli ketone synthesis. This study utilized two ketone-type patchouli cultivars (Shipai patchouli and Gaoyao patchouli) as materials to investigate the expression of HMGR, MK, and MDD genes related to the mevalonic acid metabolic pathway and chemical components in stems and leaves of test materials at different stages, and analyzed the correlation between gene expression and chemical components. Results showed that HMGR expression was more pronounced in young leaves of Shipai patchouli; MK exhibited similar expression patterns in Shipai and Gaoyao patchouli, with primary expression in old stems; MDD gene expression was higher in leaves of Shipai patchouli than in Gaoyao patchouli, with similar expression patterns in stems of both patchouli types. As both belong to ketone-type patchouli, Shipai and Gaoyao patchouli showed similar chemical components, with the highest patchouli alcohol content in old leaves and higher patchouli ketone content in old stems. Finally, correlation analysis revealed that MDD and MK genes were positively correlated with patchouli ketone synthesis. Based on these results, the gene expression patterns of the MVA pathway were similar between the two cultivars of ketone-type patchouli, and MDD and MK may serve as key genes in the terpenoid metabolic pathway of ketone-type patchouli.

Full Text

Preamble

Gene Expression Analysis of the MVA Pathway in *Pogostemon cablin* and Its Correlation with Main Component Synthesis

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Abstract: Among *Pogostemon cablin* medicinal materials, pogostone-type varieties with high pogostone content are considered the highest quality. Pogostone is a terpenoid component, yet its biosynthetic pathway remains unclear. The MVA (mevalonate) pathway represents an important route for terpenoid biosynthesis. Through real-time quantitative PCR analysis of gene expression and determination of main component contents, this study aimed to analyze the correlation between these factors to identify potential genes that promote pogostone synthesis. Using two pogostone-type *P. cablin* cultivars (Shipai and Gaoyao) as materials, we investigated the expression of HMGR, MK, and MDD genes related to the mevalonate metabolic pathway in stems and leaves at different developmental stages, along with chemical composition, and analyzed the correlation between gene expression and chemical components.

The results showed that HMGR was more prominently expressed in the tender leaves of Shipai cultivar. MK exhibited similar expression patterns in both Shipai and Gaoyao cultivars, with predominant expression in old stems. MDD gene expression was higher in the leaves of Shipai cultivar compared to Gaoyao cultivar, while showing similar expression patterns in the stems of both cultivars. Despite both belonging to the pogostone-type, Shipai and Gaoyao cultivars showed similar chemical profiles, with the highest patchoulol content in old leaves and higher pogostone content in old stems. Finally, correlation analysis revealed that MDD and MK genes were positively correlated with pogostone synthesis. In summary, the gene expression patterns of the MVA pathway were similar between the two pogostone-type cultivars, and MDD and MK may represent key genes in the terpenoid metabolic pathway of pogostone-type *P. cablin*.

Keywords: *Pogostemon cablin*, MVA pathway, pogostone, MDD gene, MK gene

Introduction

Pogostemon cablin (Blanco) Benth., a member of the Lamiaceae family, is a medicinal plant used as a whole herb in traditional medicine. It serves as an

aromatic dampness-resolving drug and constitutes a primary ingredient in numerous Chinese patent medicines such as “Huoxiang Zhengqi Powder,” “Antiviral Oral Liquid,” and “Bao Ji Pills,” while also representing a major raw material for fragrances and cosmetics. During the COVID-19 pandemic, *P. cablin* played a significant therapeutic role, being incorporated into treatment protocols for five related syndromes.

The main components of *P. cablin* essential oil are terpenoid compounds. Terpenoids are primarily synthesized through two pathways: the mevalonate (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway. The MVA pathway, also known as the cytoplasmic pathway, typically produces sesquiterpenes, while the MEP pathway, also called the plastidic pathway, provides precursors for hemiterpenoids, monoterpenoids, and diterpenoids. Multiple enzymes in these terpenoid synthesis pathways represent important regulatory targets for MVA and MEP pathway metabolism.

In the MVA pathway, three acetyl-CoA molecules and one 3-hydroxy-3-methylglutaryl-CoA molecule initially condense, with the intermediate subsequently undergoing reactions catalyzed by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, EC 2.3.3.10) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34). Mevalonate kinase (MVK, EC 2.7.1.36), phosphomevalonate kinase (PMK, EC 2.7.4.2), and mevalonate diphosphate decarboxylase (MDD, EC 4.1.1.33) then produce IPP (isopentenyl pyrophosphate). Subsequently, farnesyl diphosphate synthase (FPPS, EC 2.5.1.10) catalyzes the condensation of cytoplasmic IPP and DMAPP (dimethylallyl pyrophosphate) to form sesquiterpene intermediates (Figure 1 [Figure 1: see original paper]).

With the advent of transcriptome and whole-genome sequencing, research on patchoulol biosynthesis in *P. cablin* has gradually increased. Previous studies reported the identification of 74 terpenoid biosynthesis-related genes from *P. cablin* transcriptome libraries, examining their expression in leaves, stems, and flowers, and measuring enzyme activities related to patchoulol synthesis in the sesquiterpene biosynthetic pathway. Additionally, research has cloned the cDNA of HMGR, a rate-limiting enzyme in the sesquiterpene MEP pathway, conducting bioinformatic analysis and expressing it in *Escherichia coli*.

P. cablin ranks among the ten major characteristic “Guangdong medicinal herbs” and represents one of the “Eight Famous Guangdong Medicinals.” Due to geographical variations, different producing regions yield *P. cablin* with distinct morphological characteristics and volatile oil contents. Commercial *P. cablin* is primarily classified into four types based on origin: Shipai, Gaoyao, Zhanjiang, and Hainan. According to the relative contents of pogostone and patchoulol, *P. cablin* can be divided into alcohol-type (Zhanjiang and Hainan cultivars) and ketone-type (Shipai and Gaoyao cultivars).

Shipai *P. cablin* originally grew in the Shipai area of Guangzhou and is traditionally considered the authentic, highest-quality variety. However, due to

urbanization, Shipai *P. cablin* has become rare in the market, gradually being replaced by Gaoyao *P. cablin*. Pogostone has demonstrated multiple functions, including anti-gastric ulcer activity, anti-proliferative effects, and antimicrobial properties. Consequently, while alcohol-type *P. cablin* is primarily used in cosmetics and fragrances, ketone-type varieties are more commonly applied in traditional Chinese medicine.

Pogostone belongs to terpenoid derivatives, and the MVA pathway represents the upstream biosynthetic route for terpenoids. Stems and leaves constitute the primary medicinal tissues of *P. cablin*. Therefore, clarifying the spatiotemporal expression patterns of metabolic pathway genes, the content of active components in stems and leaves at different growth stages, and their correlation with gene expression represents a crucial prerequisite for elucidating pogostone synthesis pathways and improving medicinal material quality in both Shipai and Gaoyao cultivars. Our previous research analyzed terpenoid metabolic pathway expression in two alcohol-type cultivars (Hainan and Indonesian *P. cablin*), revealing similar expression patterns of terpenoid metabolism-related genes across different tissues and developmental stages, and identifying candidate genes related to patchoulol synthesis, providing a foundation for alcohol-type *P. cablin* applications. Currently, no studies have compared the terpenoid metabolic pathways and expression differences in leaf and stem tissues between Shipai and Gaoyao cultivars. Analyzing these metabolic pathways can provide a molecular basis for understanding differences between ketone-type and alcohol-type *P. cablin*. Meanwhile, correlating gene expression with component content can identify potential genes for improving active component yields. Comparing metabolic pathways and volatile oil content and composition across tissues in the two ketone-type cultivars can also facilitate effective evaluation of *P. cablin* germplasm resources.

Materials and Methods

1.1 Plant Materials

Shipai and Gaoyao *P. cablin* were collected from the Guangzhou Tianhe Longdong Lingnan Traditional Chinese Medicine Garden (113°22' E, 23°12' N, altitude 39 m) and identified by Professor Mo Xiaolu from Guangdong Food and Drug Vocational College. Young leaves from both cultivars were used for full-length gene cloning. For real-time quantitative PCR analysis, leaves and stems were divided into three developmental stages based on size and position on the stem (Figure 2 [Figure 2: see original paper]): tender leaves (first pair of leaves emerging from the shoot apex), mature leaves (fourth to fifth node leaves), and old leaves; young stems (shoot apex), mature stems (non-lignified), and old stems (lignified). Approximately 1 g of each sample was collected, frozen in liquid nitrogen, and stored at -80°C.

1.2 RNA Extraction and RT-qPCR Analysis

RNA extraction was performed according to the method described in Ouyang et al. (2016). After checking RNA quality and integrity, high-quality RNA was used for gene isolation or expression determination. RT-qPCR was conducted as previously described (Ouyang et al., 2016), with each reaction performed in triplicate. RT-qPCR primers are listed in Table 1, and data are presented as mean \pm standard deviation of three biological replicates. Gene expression levels were calculated according to Livak & Schmittgen (2001).

1.3 Chemical Reagents

Reference standards of pogostone (batch No. G-055-180710, Chengdu Ruifensi Biotechnology Co., Ltd.) and patchoulol (batch No. B-037-180122, Chengdu Ruifensi Biotechnology Co., Ltd.), n-octadecane (Sigma), acetonitrile HPLC grade (Merck, Germany), formic acid (Merck, Germany), and analytical grade reagents including anhydrous ethanol, ether, methanol, and anhydrous sodium sulfate were used.

1.4 Equipment

Agilent 1200 HPLC system (including quaternary gradient pump, autosampler, PDA detector, and online degasser, Agilent, USA), Agilent 7892A/5975C GC-MS (Agilent, USA), HP-5 quartz capillary column (0.25 μm \times 30.0 m \times 250 μm), BT 125D analytical balance (Sartorius, Germany), Milli-Q ultrapure water system (Millipore, USA), volatile oil extractor, and moisture analyzer were employed.

1.5.1 Essential Oil Extraction

Mature stems, old stems, mature leaves, and old leaves of Shipai and Gaoyao *P. cablin* were collected (volatile oil content in tender stems and leaves of both cultivars was too low for extraction, so these stages were not analyzed). Samples were air-dried at room temperature in a ventilated area and cut into 1-2 cm pieces. Two hundred grams of dried *P. cablin* material was placed in a 5,000 mL round-bottom flask, soaked in 3,000 mL distilled water for 1 hour, and then extracted according to Method A of General Rule 2204 in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2020).

1.5.2 Patchoulol Content Determination

Preparation of reference and test solutions followed the method described in Chen et al. (2020). GC conditions: initial temperature 180°C held for 2 min, then increased to 230°C at 5°C/min; injector temperature 280°C, auxiliary temperature 280°C, split injection with a 10:1 split ratio. Ion source temperature was 230°C, quadrupole temperature 150°C, with full scan mode and NIST14.1 mass spectral library for patchoulol identification in essential oil.

1.5.3 Pogostone Content Determination

Preparation of reference and test solutions followed Chen et al. (2020). Chromatographic conditions: Phenomenex Gemini 5 μ C18 column (250 mm \times 4.60 mm); mobile phase: acetonitrile-0.2% formic acid aqueous solution (65:35, v/v); isocratic elution at 0.8 mL \cdot min⁻¹; column temperature 30°C; detection wavelength 310 nm; injection volume 10 μ L; run time 15 min (chromatogram shown in Figure 4 [Figure 4: see original paper]).

Linearity was assessed by injecting 10 μ L of pogostone standard solutions at various concentrations under the described chromatographic conditions. The regression equation $Y = 18.35X - 47.19$ ($R^2 = 0.9999$) was obtained with a linear range of 6.25-200 μ g \cdot mL⁻¹ (Figure 5 [Figure 5: see original paper]). Stability test: a prepared test solution was analyzed at 2, 4, 6, 8, 12, and 24 h post-preparation, showing an RSD of 1.05%, indicating stability within 24 h. Precision test: six consecutive injections of 50 μ g \cdot mL⁻¹ standard solution yielded an RSD of 0.63% for pogostone peak area, demonstrating good instrument precision. Repeatability test: six portions (0.15 g each) of the same batch of *P. cablin* oil were prepared and analyzed, with an RSD of 0.86% for pogostone peak area, confirming good method repeatability. Statistical analysis of essential oil content was performed using LSD multiple comparison with R 3.6 software and the agricolae package.

Results

2.1 Expression of HMGR, MK, and MDD Genes in the MVA Pathway of Ketone-Type *P. cablin* at Different Stem and Leaf Stages

To compare the spatiotemporal expression patterns of secondary metabolism-related genes between Shipai and Gaoyao *P. cablin*, we examined the expression of three key enzyme genes in the MVA pathway—HMGR, MK, and MDD—in leaves and stems (Figure 6 [Figure 6: see original paper]). In Shipai cultivar, HMGR, MDD, and MK expression decreased during leaf development but increased again as leaves senesced. During stem development, HMGR, MDD, and MK expression showed an increasing trend, with highest expression in old stems. Similar gene expression patterns were observed in Gaoyao cultivar, except for HMGR in leaves, which showed highest expression in mature leaves, followed by tender leaves, and lowest in old leaves—differing from the pattern in Shipai cultivar.

2.2 Relationship Between Patchoulol and Pogostone Content and Related Gene Expression

To determine the optimal medicinal parts of *P. cablin* for commercial use, we measured patchoulol and pogostone content in mature stems, old stems, mature

leaves, and old leaves of ketone-type cultivars. In Shipai cultivar, pogostone content was highest in old stems at $301 \mu\text{g} \cdot \text{g}^{-1}$, compared to $233 \mu\text{g} \cdot \text{g}^{-1}$ in mature stems. In Gaoyao cultivar, pogostone content showed little difference between old and mature stems ($242 \mu\text{g} \cdot \text{g}^{-1}$ and $239 \mu\text{g} \cdot \text{g}^{-1}$, respectively) (Figure 7 [Figure 7: see original paper]). Volatile oil content in tender stems and leaves of both cultivars was too low for determination. Similarly, patchoulol content in stems was too low for detection. For both ketone-type cultivars, patchoulol content was higher in old leaves than in mature leaves: Shipai cultivar showed $559 \mu\text{g} \cdot \text{g}^{-1}$ in old leaves versus $403 \mu\text{g} \cdot \text{g}^{-1}$ in mature leaves, while Gaoyao cultivar showed $445 \mu\text{g} \cdot \text{g}^{-1}$ in old leaves versus $415 \mu\text{g} \cdot \text{g}^{-1}$ in mature leaves.

To further evaluate the relationship between genes and phytochemicals, Pearson correlation coefficients were estimated using XLSTAT to analyze correlations between patchoulol/pogostone content and corresponding gene expression levels across different tissues and harvest times. The results indicated that expression of MK ($R^2 = 0.758$, $P < 0.05$) and MDD ($R^2 = 0.758$, $P < 0.05$) promoted pogostone accumulation.

Discussion and Conclusion

The authentic medicinal varieties of *P. cablin* belong to the ketone-type, such as Gaoyao cultivar, while Shipai *P. cablin* has become rare in the market due to low yield. Real-time quantitative PCR is considered the most sensitive, efficient, and rapid method for detecting RNA levels and is commonly used for expression analysis of single or multiple genes. Our analysis revealed highly similar expression patterns of the three MVA pathway genes between Shipai and Gaoyao cultivars. In genetics, gene expression serves as the fundamental factor linking genotype and phenotype in an environment-dependent manner, which may molecularly explain why Gaoyao and Shipai *P. cablin* belong to the same chemotype, providing theoretical support for market substitution of Shipai with Gaoyao cultivar. Shipai *P. cablin* was introduced from Indonesia during the Song Dynasty and transplanted to Gaoyao and other regions in Guangdong Province. Current *P. cablin* propagation relies primarily on cutting propagation and tissue culture, resulting in limited genetic variation and speciation that is not conducive to species diversification. This may account for the similar MVA pathway gene expression patterns between Gaoyao and Shipai cultivars.

Terpenoid production and accumulation in plants exhibit tissue-specific characteristics, with their increase being developmentally regulated. Our study found that pogostone content remained very low across all three leaf developmental stages in both Shipai and Gaoyao cultivars, while increasing in senescing stems. Therefore, pogostone biosynthesis is both tissue-specific and growth cycle-dependent, warranting greater attention to stems when cultivating ketone-type *P. cablin*.

From the perspective of *P. cablin* quality evaluation, pogostone content repre-

sents an important quality indicator. Our findings suggest that MK and MDD genes may be crucial for influencing pogostone content in *P. cablin*, providing research directions for improving pogostone yield through metabolic engineering and transgenic approaches. Furthermore, the roles of these two genes in pogostone biosynthesis require further validation through molecular biology and metabolic engineering techniques such as genetic transformation.

It is widely accepted that authentic medicinal materials result from long-term environmental adaptation of medicinal plants, with genetic factors potentially representing the fundamental cause of significant chemical differences between authentic and non-authentic varieties. Our results demonstrate similar MK and MDD gene expression patterns between Gaoyao and Shipai *P. cablin*, with both cultivars showing high pogostone content, indicating that substituting Shipai with Gaoyao cultivar is rational. The MK and MDD genes of the MVA pathway are positively correlated with pogostone synthesis. These findings elucidate the correlation between gene expression and chemical composition in *P. cablin*, contributing to enhanced terpenoid production and providing a foundation for improved utilization of this medicinal plant.

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