Postprint: Alkaloid Content and Synthesis-Related Gene Expression in Fritillaria thunbergii from Different Origins

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Abstract

To investigate the relationship between the accumulation of medicinal components and the expression levels of alkaloid synthesis-related genes in Fritillaria thunbergii, this study employed UPLC-MS and qPCR techniques to determine the total alkaloid content (sum of peimine and peiminine) and the expression levels of three genes involved in the alkaloid synthesis pathway (HMGR, FPS, and DXR) in 11 samples from different producing areas, while simultaneously using biostatistical methods to analyze the correlation between alkaloid content in mature bulbs and the expression levels of each gene. The results showed that the total alkaloid content in mature bulbs of Fritillaria thunbergii from different producing areas exhibited significant differences ($P<0.05$), ranging from 0.2105% to 0.4612%. The expression change trends of HMGR and FPS genes in full-bloom stage tissues and in bulbs from full-bloom stage to mature stage were basically consistent with the change trend of alkaloid content. The DXR gene exhibited the highest expression level in mature bulbs, and the expression change trends in full-bloom stage tissues and in bulbs from full-bloom stage to mature stage were largely inconsistent with the change trend of alkaloid content. The expression levels of HMGR and FPS genes showed significant or extremely significant positive correlations with the contents of peimine, peiminine, and total alkaloids ($P<0.05$ or $P<0.01$), with the correlation coefficients between FPS gene expression level and alkaloid content being the highest, at 0.672, 0.631, and 0.664, respectively. The DXR gene showed low correlation with alkaloid content. It can thus be inferred that alkaloid accumulation is significantly affected by the synergistic regulation or modification of HMGR and FPS genes in the MVA pathway, while the regulatory effect of the DXR gene in the MEP pathway is not significant.

Full Text

Preamble

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Alkaloid Content and Synthesis-Related Gene Expression of *Fritillaria thunbergii* from Different Producing Areas

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Abstract

This study investigated the relationship between the accumulation of medicinal components and the expression levels of alkaloid synthesis-related genes in *Fritillaria thunbergii*. Using ultra-high performance liquid chromatography-mass spectrometry (UPLC-MS) and quantitative real-time PCR (qPCR), we determined the total alkaloid content (sum of peimine and peiminine) and the expression levels of three genes involved in alkaloid biosynthesis—HMGR, FPS, and DXR—in 11 samples from different producing areas. Biostatistical methods were employed to analyze correlations between alkaloid content in mature bulbs and the expression of each gene. The results revealed significant differences in total alkaloid content among bulbs at the maturity stage ($P < 0.05$), ranging from 0.2105% to 0.4612%. The expression patterns of HMGR and FPS genes in tissues at the flowering stage and in bulbs from flowering to maturity stage were largely consistent with alkaloid content variation. DXR gene expression was highest in mature bulbs, but its expression trend in flowering-stage tissues and in bulbs from flowering to maturity stage was generally inconsistent with alkaloid content changes. Expression levels of HMGR and FPS genes showed significant or extremely significant positive correlations with peimine, peiminine, and total alkaloid content ($P < 0.05$ or $P < 0.01$), with FPS showing the highest correlation coefficients of 0.672, 0.631, and 0.664, respectively. In contrast, DXR gene expression exhibited only weak correlations with alkaloid content. These findings suggest that alkaloid accumulation is prominently regulated or modified by HMGR and FPS genes in the MVA pathway, while regulation by DXR gene in the MEP pathway appears minimal.

Keywords: *Fritillaria thunbergii*, alkaloid content, alkaloid synthesis-related genes, gene expression, correlation

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Introduction

Fritillaria thunbergii, a perennial herbaceous plant belonging to the Liliaceae family and *Fritillaria* genus, is harvested for its dried mature bulbs, which possess cold properties and are used in traditional medicine to clear heat, moisten the lungs, detoxify, and dissipate nodules. Originally native to Xiangshan, Ningbo, Zhejiang Province, it is primarily cultivated in Zhejiang, which accounts for 90% of the total national cultivation area, hence its common names "Xiangbei" or "Zhebei" (He et al., 2018). Currently, commercial *F. thunbergii* is mainly artificially cultivated, with major production areas in Zhejiang Province concentrated in Yinzhou, Kaihua, and surrounding regions, including neighboring provinces such as Jiangsu, Anhui, and Fujian (Cui et al., 2018). Steroidal alkaloids, represented by peimine and peiminine, are characteristic secondary metabolites in *Fritillaria* plants and constitute the primary medicinal components of *F. thunbergii*. Research has demonstrated that steroidal alkaloids possess significant pharmacological value in expectorant and antitussive effects, blood pressure reduction, blood circulation promotion, analgesia, anti-ulcer activity, anti-inflammatory and antioxidant properties, and antitumor activity (Lee et al., 2015; Tang et al., 2018; Chen et al., 2018; Ruan et al., 2016; Zha et al., 2010).

The precursors of steroidal alkaloids are primarily formed through enzymatic catalysis of isopentenyl pyrophosphate (IPP), an important intermediate in plant terpenoid biosynthesis (Laule et al., 2003). IPP serves as an intermediate in both the mevalonate (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways, which constitute the main routes for steroidal skeleton synthesis and participate in steroidal alkaloid biosynthesis in *F. thunbergii* (Cardenas et al., 2016; Zhao et al., 2018). In the MVA pathway, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) acts as the first rate-limiting enzyme, catalyzing the irreversible conversion of HMG-CoA to mevalonate (Kim et al., 2014). Farnesyl pyrophosphate synthase (FPS) is a key enzyme that catalyzes the formation of farnesyl pyrophosphate (FPP) from geranyl pyrophosphate (GPP), with FPP serving as an important intermediate metabolite in plants (Dhar et al., 2013). 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) represents a crucial rate-limiting enzyme in the MEP pathway. Currently, domestic and international research on steroidal alkaloids in *F. thunbergii* has primarily focused on determination of medicinal component content, extraction and purification processes, and pharmacological activity analysis, with limited reports on the biosynthetic pathways and key enzymes involved. Consequently, further development and utilization of functional genes related to alkaloid synthesis in *F. thunbergii* have been severely constrained.

This study measured alkaloid content (peimine and peiminine) in three tissues (stem, leaf, and bulb) at the flowering stage and in bulbs at the maturity stage from 11 different producing areas of *F. thunbergii* cultivar "Zhebei No. 1." We analyzed differences in alkaloid content among different producing areas and examined correlations between the expression of HMGR, FPS, and DXR genes and

alkaloid accumulation. These findings provide foundational data for elucidating the alkaloid biosynthetic pathway in *F. thunbergii* and establish a basis for revealing the molecular mechanisms underlying quality variation in *F. thunbergii* medicinal materials.

Materials and Methods

1.1 Plant Materials

Plant materials consisted of three tissue types (stem, leaf, and bulb) at the flowering stage and bulbs at the maturity stage of *Fritillaria thunbergii* cultivar "Zhebei No. 1" (a narrow-leaf variety) collected from 11 different producing areas. Samples were harvested in mid-March and mid-May 2018 from Zhangshui (Ningbo, eastern Zhejiang), Ouhai (Wenzhou, southern Zhejiang), Kaihua (Quzhou, western Zhejiang) and Liandu (Lishui, western Zhejiang), Chun' an, Lin' an Changhua, and Yuhang (Hangzhou, northern Zhejiang), Wucheng and Dongyang (Jinhua, central Zhejiang), Nantong (Jiangsu Province), and Liu' an (Anhui Province). Basic meteorological and soil type information for the different producing areas is presented in Table 1 . Freshly collected materials were divided into two portions: one portion was cleaned, sliced, low-temperature dried to constant weight in an oven, ground into powder, and passed through a 65-mesh sieve for alkaloid content determination; the other portion was washed with DEPC-treated water, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent gene expression analysis.

Instruments and Reagents: The ACQUITY UPLC system and SYNAPT G2-Si mass spectrometer (Waters, USA), CFX96 real-time PCR system (Bio-Rad, USA), and SQP electronic analytical balance (Sartorius, Germany) were used. Ammonia and acetonitrile were chromatographic grade. Standard peimine and peiminine were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. PCR primers were synthesized by Shanghai Sangon Biotech Co., Ltd.

1.2.1 UPLC-MS Determination of Alkaloid Content in Different Tissues

1.2.1.1 Chromatographic and Mass Spectrometric Conditions **Chromatographic conditions:** An ACQUITY UPLC® BEH Amide column (2.1 mm × 100 mm, 1.7 m) was employed with a mobile phase of 0.1% ammonia water (A) and 0.1% acetonitrile (B). Gradient elution was performed as follows: 0–1.0 min, 30% A → 30% A; 1.0–5.5 min, 30% A → 5% A; 5.5–5.8 min, 5% A → 30% A. Flow rate was 0.2 mL · min ^{1}, column temperature was 30°C, and injection volume was 2 L.

Mass spectrometric conditions: Electrospray ionization in positive mode (ESI+) was used. The ion mass-to-charge ratios (m/z) were 432.35 for peimine and 430.33 for peiminine. Capillary voltage was 3.0 kV, cone voltage was 4.0 V,

source temperature was 105°C, desolvation temperature was 300°C, desolvation gas flow was 800 L · h ^{1}, and cone gas flow was 50 L · h ^{1}.

1.2.1.2 Standard Curve Preparation Reference standards of peimine (0.0048 g) and peiminine (0.0050 g) were accurately weighed and diluted with methanol to 25 mL volumetric flasks, then further diluted with methanol to prepare mixed reference solutions at concentrations of 96, 48, 38.4, 19.2, and 9.6 mg · L ^{1} for peimine and 100, 50, 40, 20, and 10 mg · L ^{1} for peiminine. After mixing, solutions were filtered through 0.22 m microporous filters. Linear regression equations were established using analyte concentration as the abscissa and peak area as the ordinate under the chromatographic and mass spectrometric conditions described in section 1.2.1.1.

1.2.1.3 Sample Extraction and Determination Alkaloid extraction was performed using a modified method from Luo et al. (2018), with reflux extraction replaced by shaking incubation (conditions: 32°C, 12 h, 120 rpm). Alkaloid content (peimine and peiminine) was determined in three tissues at the flowering stage and in bulbs at the maturity stage from 11 producing areas under the conditions specified in section 1.2.1.1, with three replicates per sample.

1.2.2 Total RNA Extraction and Reverse Transcription

Total RNA was extracted from stems, leaves, and bulbs of *Fritillaria thunbergii* using the Aidlab EASYspin Plus Complex Plant RNA Kit. RNA concentration and purity ratios (A / and A /) were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher, USA), and integrity was assessed via 1.2% agarose gel electrophoresis. Total RNA was reverse-transcribed to cDNA using the Vazyme HiScript® II Q RT SuperMix for qPCR kit and stored at -20°C.

1.2.3 Real-Time Quantitative PCR

Specific primers for *Fritillaria thunbergii* HMGR, FPS, DXR, and -Actin genes were designed and selected using Primer Express 3.0 software based on sequences cloned by our research group. The selected primers are listed in Table 2 (Feng et al., 2017a; Feng et al., 2016; Feng et al., 2017b). The qPCR reaction system (20 L) contained 10 L of 2×ChamQ SYBR Color qPCR Master Mix, 0.4 L each of forward and reverse primers, 0.4 L of template cDNA, and 8.8 L of ddH O. The qPCR program consisted of initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, with a melting curve generated by gradual heating from 60°C to 95°C after PCR amplification. The housekeeping gene -Actin served as an internal reference, and gene expression levels were calculated using the 2 ΔCT method (Wu et al., 2015).

1.2.4 Data Analysis

Statistical analysis was performed using SPSS Statistics 21.0 software, and histograms were generated using Excel 2010.

Results

2.1 Effects of Producing Area on Alkaloid Accumulation in *Fritillaria thunbergii* at Flowering and Maturity Stages

Alkaloid content (peimine and peiminine) in three tissues (stem, leaf, and bulb) at the flowering stage and in bulbs at the maturity stage of "Zhebei No. 1" from 11 producing areas was determined by UPLC-MS, with results shown in Figure 1 [Figure 1: see original paper]. Alkaloids were detected in all tissues and developmental stages across the 11 producing areas. Significant differences in total alkaloid content (sum of peimine and peiminine) were observed in the same tissue type at the flowering stage among different producing areas ($P < 0.05$). The highest total alkaloid content in stems was found in Zhangshui, in leaves from Liu' an, and in bulbs from Kaihua, while the lowest content in leaves occurred in Chun' an, and in both stems and bulbs from Liandu. A consistent trend emerged across tissues, with bulbs containing the highest total alkaloid content, followed by stems, and leaves showing the lowest levels. In stems, leaves, and new bulbs, peiminine content exceeded peimine in most samples, except for stems from Ouhai, Kaihua, and Chun' an, with this trend being more pronounced in leaves than in stems and bulbs.

Among mature bulbs from the 11 producing areas, total alkaloid content varied significantly ($P < 0.05$). Higher alkaloid levels were observed in Ouhai and Liu' an, moderate levels in Lishui, Kaihua, Changhua, Nantong, Wucheng, and Zhangshui, and the lowest levels in Chun' an, Yuhang, and Dongyang. Peimine content exceeded peiminine in all mature bulbs. Notably, alkaloid content in mature bulbs from Ouhai and Liandu was higher than in bulbs at the flowering stage, whereas the opposite pattern was observed in the remaining nine producing areas.

2.2 Effects of Producing Area on Expression of Alkaloid Synthesis-Related Genes

Expression levels of HMGR, FPS, and DXR genes were examined in stems, leaves, and bulbs at the flowering stage and in bulbs at the maturity stage from the 11 producing areas using real-time quantitative PCR, with results presented in Figure 2 [Figure 2: see original paper]. All three genes were expressed across all tissues and developmental stages. HMGR and FPS genes exhibited similar expression patterns, with highest expression in bulbs at the flowering stage, followed by mature bulbs, and lowest expression in leaves and stems. In contrast, DXR gene expression was highest in mature bulbs, but its expression pattern

in the three tissues at the flowering stage differed from that of HMGR and FPS. Significant differences were observed in expression changes from flowering to maturity stage among the three genes ($P < 0.05$). Specifically, HMGR and FPS expression simultaneously increased in Ouhai, while HMGR increased and FPS decreased in Liandu, and both genes decreased in the remaining nine producing areas. DXR expression increased in all producing areas except Dongyang.

Pearson bivariate correlation analysis revealed a significant positive correlation between HMGR and FPS gene expression ($P < 0.05$), while DXR showed only weak positive correlations with HMGR and FPS ($P > 0.05$).

2.3 Relationship Between Alkaloid Synthesis-Related Gene Expression and Alkaloid Accumulation

To further investigate the relationship between gene expression and alkaloid accumulation, Pearson bivariate correlation analysis was performed between expression levels of HMGR, FPS, and DXR genes and alkaloid content in medicinal bulbs at the maturity stage from different producing areas (Table 3). Significant or extremely significant correlations were observed between peimine, peiminine, and total alkaloid content and expression of HMGR and FPS genes, with FPS showing stronger correlations than HMGR. DXR gene expression exhibited weak negative correlation with peimine and weak positive correlations with peiminine and total alkaloid content ($P > 0.05$).

Discussion and Conclusion

Environmental conditions of the producing area represent crucial external factors in the formation of genuine medicinal materials, directly affecting plant growth and development as well as the formation and accumulation of active components (Zhang et al., 2016). Active constituent content has become a key indicator for quality evaluation of genuine medicinal materials (Xie et al., 2016). This study revealed that alkaloid content in different tissues of *Fritillaria thunbergii* at the flowering stage followed a consistent pattern across producing areas, with bulbs containing the highest levels, followed by stems, and leaves showing the lowest content. This distinct tissue-specific distribution of alkaloids aligns with findings for other medicinal plants such as *Polygonum cuspidatum*, *Panax ginseng*, and *Cinnamomum cassia* (Yu et al., 2006; Li and Wu, 2018). Total alkaloid content in mature bulbs from all producing areas met the standard specified in the 2015 edition of the Chinese Pharmacopoeia (0.080%). However, the alkaloid content in bulbs from the genuine producing area of Zhangshui, Yinzhou, ranked at a medium-to-low level among the 11 producing areas, contrasting with studies on *Angelica pubescens* and *Salvia miltiorrhiza* (Hu et al., 2015; Li et al., 2011). This discrepancy may be attributed to several factors: excessively high alkaloid content may negatively impact *F. thunbergii* quality by reducing primary metabolites available for conversion to alkaloids, and the complex

chemical composition of *F. thunbergii*—including amino acids, polysaccharides, nucleotides, and minerals—means alkaloids are not the sole active components for quality assessment. Similar to *Lonicera japonica* and *Notopterygium incisum*, quality grading of *F. thunbergii* may depend on multiple substances (Liu et al., 2016; Jiang et al., 2016). Only Ouhai and Liandu showed higher alkaloid content in mature bulbs compared to flowering-stage bulbs, possibly because their significantly higher average temperatures during the rapid growth period (mid-March to mid-May) were unfavorable for bulb enlargement (Paek and Murthy, 2002; Li et al., 2008).

Advances in molecular biology have shifted research focus toward biosynthetic pathways of active components and their associated enzyme genes. Li et al. (2018) reported that FPS gene expression levels in *Fritillaria cirrhosa* correlated with total alkaloid content variation. Similarly, this study found that expression trends of HMGR and FPS genes in tissues at the flowering stage and in bulbs from flowering to maturity stage were largely consistent with alkaloid content changes, suggesting that up- or down-regulation of these genes may lead to increased or decreased alkaloid synthesis. The significant positive correlation between HMGR and FPS expression ($P < 0.05$) indicates these genes may coordinately regulate or modify alkaloid synthesis in *F. thunbergii*. The opposite expression trends of HMGR and FPS in Liandu from flowering to maturity stage may be related to coordinated regulation by other enzyme genes in the alkaloid metabolic pathway and environmental factors (Zhao et al., 2018). While DXR expression in mature bulbs followed the same trend as HMGR and FPS, its expression pattern in the three tissues at flowering stage differed, and its expression increased from flowering to maturity stage in all producing areas except Dongyang, contrasting with alkaloid content variation. This suggests DXR may not play a critical role in the alkaloid synthesis pathway. Among the three genes examined, FPS expression in medicinal bulbs at the maturity stage showed stronger positive correlations with alkaloid content than HMGR, while DXR showed weak correlations. This may be because the MVA pathway serves as the primary route for steroidal alkaloid synthesis, with the downstream FPS gene exerting stronger regulatory control over alkaloid synthesis than the upstream HMGR gene, whereas the MEP pathway contributes minimally to alkaloid synthesis through the intermediate IPP entering the MVA pathway (Laule et al., 2003; Eva et al., 2013; Zhao et al., 2018). In summary, this study preliminarily demonstrates that alkaloid accumulation in *F. thunbergii* grown in different environments is significantly regulated or modified by HMGR and FPS genes, but not by DXR gene. These findings lay a foundation for future research on key enzyme genes in alkaloid synthesis and the molecular mechanisms underlying quality variation in *F. thunbergii*.

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