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Genome-wide identification and expression profiles of flavonoid O-methyltransferase gene family in *Scutellaria baicalensis*

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Author contributions

Fei CH was responsible for conceptualization, methodology, visualization, and writing – review and editing; He YB performed the software development and formal analysis; Chen P, Qian K and Chen B were involved in data curation; Zhou PN oversaw project administration, writing – review and editing, and funding acquisition. All authors have reviewed and approved the final manuscript.

Competing interests

The authors declare no conflicts of interest.

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Abbreviations

COMT, caffeic acid O-methyltransferase; UV, ultraviolet; SbOMTs, *Scutellaria baicalensis* O-methyltransferase; OMTs, O-methyltransferases; CCoAOMT, caffeoyl-CoA O-methyltransferase; ABA, abscisic acid; MeJA, methyl jasmonate; HPLC, high-performance liquid chromatograph.

Citation

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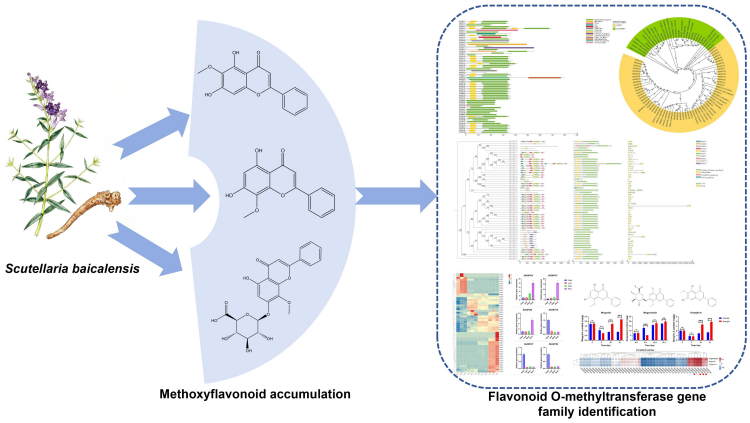
Abstract

Background: *Scutellaria baicalensis* Georgi is a medicinal plant prized for its bioactive flavonoid derivatives. Flavonoid O-methyltransferases (OMTs) in this species play a vital role in enhancing these compounds' pharmacological activities, including their antioxidant, anti-inflammatory, and anticancer effects. However, a comprehensive genomic overview of the OMT gene family in *S. baicalensis* is lacking. **Methods:** This study conducted a genome-wide identification of the OMT gene family in *S. baicalensis* using bioinformatics approaches. The identified genes were characterized through phylogenetic, physicochemical, and structural analyses. Furthermore, the response of methoxylated flavonoids and key *SbOMT* genes to drought stress was investigated. **Results:** A total of 54 *SbOMTs* were identified and classified into 9 CCoAOMT and 45 COMT subfamily members. These proteins, with lengths from 129 to 695 amino acids and molecular weights from 14.42 to 76.94 kDa, were predominantly acidic. Subcellular localization predicted 43% to be cytoplasmic. Structurally, the CCoAOMT subfamily was more conserved than the COMT subfamily. Promoter analysis revealed hormone- and stress-responsive cis-elements. Under drought stress, the root content of methoxylated flavonoids (wogonin, wogonoside, and oroxylin A) decreased initially and then increased. The expression of *SbOMT06*, *SbOMT41*, *SbOMT27*, and *SbOMT29* was positively correlated with this accumulation, suggesting their involvement in biosynthesis. **Conclusion:** This study provides foundational insights into the *SbOMT* gene family, revealing key candidates likely involved in methoxyflavonoid biosynthesis. The findings advance our understanding of the molecular mechanisms in *S. baicalensis* and offer valuable resources for future metabolic engineering and pathway optimization efforts.

Keywords: *Scutellaria baicalensis*; flavonoids; O-methyltransferase; bioinformatics; expression analysis



Highlights

1. Genome-wide analysis identified 54 *SbOMT* genes of *Scutellaria baicalensis*, classifying them into CCoAOMT and COMT subfamilies with distinct structural features.
2. Promoter and expression analyses revealed that key *SbOMT* genes are responsive to drought stress and correlate with methoxylated flavonoid accumulation.
3. The study provides crucial genetic resources and candidate genes for engineering the biosynthesis of bioactive methoxyflavonoids.

Medical history of objective

Scutellaria baicalensis was first documented as a medicinal substance in *Shennong Bencao Jing* (compiled around 200–250 C.E.). Later, Shizhen Li of the Ming Dynasty elaborated on its applications in *Compendium of Materia Medica* (compiled in 1578 C.E.), emphasizing its role in treating "damp-heat disorders" (the body exhibits simultaneous accumulation of water metabolism stagnation and inflammation/feverish reactions), respiratory infections, and bleeding syndromes. Modern pharmacological studies have confirmed that *Scutellaria baicalensis* exhibits anti-inflammatory, antiviral, antioxidant, antiallergic, hepatoprotective, and neuroprotective properties. Its active compounds, such as baicalin and wogonoside, contribute to these therapeutic effects, aligning with its traditional uses.

Introduction

Flavonoids, a structurally diverse and widely distributed class of plant secondary metabolites, have attracted significant scholarly interest due to their notable bioactive properties, which offer substantial health benefits [1]. As critical medicinal components in plants, the biological activities of flavonoids are intricately associated with their distinct chemical structures. Among the various structural modifications, O-methylation is particularly common and plays a crucial role in modulating the physicochemical properties of flavonoids. Specifically, O-methylation enhances the chemical stability and protein-binding affinity of flavonoids and, most importantly, significantly improves their bioavailability [2]. These enhancements collectively augment the therapeutic efficacy of O-methylated flavonoids. Empirical evidence from numerous studies consistently indicates that O-methylated flavonoids exhibit superior antioxidant, anti-inflammatory, and anticancer activities compared to their non-methylated counterparts [1, 3, 4]. Furthermore, the increased bioavailability of O-methylated flavonoids significantly enhances their potential applications within the pharmaceutical industry. This improved bioavailability is essential for the efficient delivery and utilization of these compounds in therapeutic settings, thereby broadening their applicability in medicinal contexts. The prominence of O-methylation in flavonoids highlights the necessity of understanding the biochemical mechanisms and evolutionary adaptations that have contributed to the widespread occurrence of this modification in nature. Future research should aim to elucidate the specific pathways and enzymes involved in O-methylation, as well as investigate the potential for synthetic or genetic engineering approaches to optimize the production and efficacy of O-methylated flavonoids for medicinal use.

The O-methylation of plant flavonoids represents a crucial biochemical modification that profoundly affects their biological activities and metabolic stability. This process is catalyzed by a group of enzymes known as S-adenosylmethionine (SAM)-dependent O-methyltransferases (OMTs) [5]. These enzymes facilitate the transfer of a methyl group from SAM to various hydroxyl groups on flavonoid substrates, thereby modulating the substrates' chemical properties and bioavailability. Plant OMTs are classified into two distinct subfamilies based on differences in protein molecular weight and cation dependence: (1) CCoAOMTs and (2) COMTs [6]. This classification underscores the functional and structural diversity within the OMT family, which is essential for elucidating their roles in plant metabolism and the biosynthesis of secondary metabolites. The COMT subfamily, which contains a larger number of members, comprises of homodimeric proteins (38–43 kDa per subunit) that exhibit cation-independent catalytic activity [7]. In contrast, the CCoAOMT subfamily is characterized by a smaller number of members, each possessing lower-molecular-weight protein subunits (approximately 23–30 kDa). Most enzymes within this subfamily exhibit a strict dependence on cations for their catalytic activity [8]. These variations in protein structure, molecular weight, and catalytic requirements between CCoAOMTs and COMTs highlight the functional specialization within the OMT family. Comprehending these differences is crucial for elucidating the specific roles of O-methylation in plant secondary metabolism, especially in the biosynthesis of flavonoids and other phenolic compounds. The OMT gene family has been identified and characterized across numerous plant species, including *Cucumis melo*, *Chrysanthemum indicum*, *Capsicum annuum*, and *Vitis vinifera* [9–11]. In *C. indicum*, correlation analysis between OMT gene expression and flavonoid accumulation has suggested that four OMTs (CHR00029120, CHR00029783, CHR00077404, and CHR00078333) may be involved in the biosynthesis of the majority of methylated flavonoids in the capitulum. Additionally, CiCCoAOMT1 has been identified and demonstrated to catalyze the O-methylation of quercetin and luteolin at the 3'-OH position [5]. Within the OMT gene family in grapevine, VvCCoAOMT1, VvCCoAOMT4, and VvCOMT1 have been identified as significant contributors to the synthesis of methylated flavonoids in berry skin. Notably, *VvCCoAOMT1* and *VvCCoAOMT4* exhibit high expression levels specifically in the skin and are induced by abscisic acid and elevated temperatures. In contrast, *VvCOMT1* is markedly upregulated during fruit development and in response to ultraviolet (UV) treatment. Furthermore, *VvMYBA1*, a key regulator of anthocyanin biosynthesis in grape berries, has been shown to directly activate *VvCCoAOMT4*, thereby influencing the production of methylated flavonoids [10]. These findings collectively highlight the essential catalytic roles of the OMT gene family in the biosynthesis of O-methylated flavonoids.

S. baicalensis, a medicinally significant species within the Lamiaceae family, has been an integral component of traditional Chinese medicine for centuries. Its dried roots are clinically employed for their properties in "clearing heat" (eliminating the inflammatory or feverish reactions), "drying dampness" (removing the excessive water or sticky substances), "purging fire" (relieving intense, blazing heat), and detoxifying [12]. To date, nearly 100 distinct flavonoids have been identified in *S. baicalensis*, exhibiting notable tissue-specific accumulation patterns: the aerial parts are rich in flavonoids such as scutellarin, scutellarein, apigenin, and luteolin, whereas the roots predominantly accumulate methoxylated flavonoids, including baicalin, wogonoside, baicalein, and wogonin [12–14]. The abundance of mono- and poly-methoxylated flavonoids in *S. baicalensis* makes it an exemplary model system for investigating the biosynthesis of methoxylated flavonoids [15]. This research involved a thorough genome-wide analysis of the OMT gene family in *S. baicalensis* using bioinformatics methods, examining its phylogenetic relationships, chromosomal positions, sequence features, subcellular localization, and gene structures. Additionally, qRT-PCR was employed to outline the tissue-specific expression patterns of certain *SbOMTs*. This comprehensive study laid a strong groundwork for understanding the biosynthetic pathways of pharmacologically active methoxylated flavonoids in *S. baicalensis*.

Materials and methods

Plant materials and treatment

The plant specimens of *S. baicalensis* were procured from the greenhouse at Nanjing Agricultural University, where they were cultivated under controlled conditions of a 16-h light/8-h dark photoperiod and a temperature regime of 25 °C for day and night. The identification of the specimens as *S. baicalensis* (Lamiaceae) was confirmed by Professor Zhaibiao Zhu of Nanjing Agricultural University. During the flowering phase, samples from the flowers, leaves, stems, and roots were collected, thoroughly cleaned, and stored at −80 °C for subsequent analyses. Concurrently, a drought stress treatment was administered during the flowering stage of *S. baicalensis*. The treatment group was subjected to natural drought conditions, receiving only a single irrigation to saturate the soil, with no further watering provided thereafter. In contrast, the control group (CK) was maintained under standard conditions with daily irrigation. The drought stress treatment commenced on Day 0 (CK) and continued for a duration of 15 days. Sampling was conducted on Days 0, 5, 10, and 15 of the drought stress period. At each sampling interval, three plants were randomly selected, and their roots, stems, and leaves were harvested for subsequent experimental analyses.

Identification of *SbOMTs* family members in *S. baicalensis*

The genomic data of *S. baicalensis* were procured from the Genome Warehouse database (https://bigd.big.ac.cn/gwh) under Accession No. GWHAOTO00000000. Subsequently, Hidden Markov Model (HMM) profiles corresponding to the OMT gene family (PF01596, PF00891) were obtained from the Pfam database (http://pfam.xfam.org/) and employed in HMMER searches to identify conserved domain sequences of the OMT family within the protein sequences of the *S. baicalensis* genome [16]. The integrity of these characteristic domains was then validated using the batch CD-search tool available on NCBI (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and the SMART database (https://smart.embl-heidelberg.de/). Sequences of *SbOMTs* that exhibited incomplete or absent domains were excluded from subsequent analyses.

Physicochemical characteristics and subcellular localization prediction of the *SbOMTs* gene family in *S. baicalensis*

The protein sequences encompassing the complete conserved domains of the OMT gene family were analyzed using the ExPASY database (https://web.expasy.org/protparam/) to determine their physicochemical properties. These properties included the isoelectric point (pI), relative molecular weight, protein hydrophobicity, instability index, and aliphatic index [17]. Following this, the sequences were subjected to subcellular localization prediction using the WoLF PSORT tool (https://wolfpsort.hgc.jp/) [18].

Phylogenetic analysis of SbOMT proteins of *S. baicalensis*

For the purpose of phylogenetic analysis, OMT protein sequences from various species (Supplementary Table S1) were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). These sequences were integrated with the SbOMT protein sequences into a consolidated file. Subsequently, multiple sequence alignment was conducted utilizing the MUSCLE program. This was followed by the construction of a maximum likelihood (ML) phylogenetic tree using MEGA X software, with the bootstrap value configured to 1,000 replicates [19]. The optimal evolutionary tree model was JTT + G. Finally, the phylogenetic tree was visualized and refined for enhanced graphical representation using the iTOL online tool (https://itol.embl.de/) [20].

Conserved motif, conserved domains and exon-intron structure and analysis of *SbOMTs*

The analyses of motifs, conserved domains, and gene structures of *SbOMTs* were conducted in accordance with the methodologies outlined in reference [17]. Chromosomal localization of *SbOMTs* was visualized utilizing the Gene Location Visualize from GTF/GFF tool available in TBtools. To examine intra-species synteny and genome visualization, circos plots and collinearity diagrams for *S. baicalensis* were generated using the One Step MCScanX, Advanced Circos, and Dual Synteny Plot for MCScanX features in TBtools (https://github.com/CJ-Chen/TBtools). The upstream 2,000 bp sequences of the *SbOMTs* coding regions were extracted and designated as putative promoter regions. These sequences were submitted to the PlantCare online platform (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for the identification of cis-regulatory elements. The distribution of the analyzed cis-elements was visualized using TBtools.

Expression profiles of *SbOMTs* with RNA-Seq data

The transcriptomic data utilized in this study were obtained from a series of experiments, encompassing tissue-specific transcriptomes of *S. baicalensis* (BioProject ID: PRJNA1079421), drought-stress transcriptomes (BioProject ID: PRJNA1141697), and MeJA-induced transcriptomes (BioProject ID: PRJNA961700). The raw transcriptomic reads were aligned to the reference genome, facilitating the generation of gene expression matrice. Subsequently, gene expression heatmaps were constructed using the read count values for each gene.

RNA extraction, cDNA synthesis and qRT-PCR

For the purpose of qRT-PCR analysis, tissues from *S. baicalensis*, including flowers, leaves, stems, and roots, as well as roots subjected to varying durations of drought stress, were collected. Total RNA extraction and reverse transcription were conducted in accordance with the protocol outlined in reference [21]. Primers specific to the target genes were designed utilizing Primer 5 software, as detailed in Supplementary Table S2. All qRT-PCR reactions were performed on LineGene 9600 Plus (Bioer Technology Co. Ltd., Hangzhou, China). The whole procedure was executed following the manufacturer's guidelines for the ChamQ™ SYBR Color qPCR Master Mix (Without ROX) (Q421) (Vazyme Biotech Co., Ltd., Nanjing, China). The *Sb18S* gene served as an internal control, and the relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ method [22]. Four biological repetitions were performed.

Total flavonoid content analysis

The total flavonoid content was quantified employing the $NaNO_2$-$Al(NO_3)_3$-NaOH method as described in reference [23]. A standard calibration curve was established using a 1 mg/mL rutin solution, resulting in the regression equation $y = 0.7068x + 0.05062$ with a coefficient of determination (R^2) of 0.999. Dried leaf samples (0.2 g) subjected to various light treatments were mixed with 5 mL of 70% ethanol, sonicated in a water bath at 60 °C for 30 min, and subsequently allowed to stand for an additional 30 min. The mixtures were then centrifuged at 120,000 rpm for 10 min, and the supernatants were transferred to 10 mL volumetric flasks. The extraction process was repeated once, and the final volume was adjusted to 10 mL with 70% ethanol. Precisely 1 mL of the test solution was extracted, to which 0.3 mL of a 5% $NaNO_2$ solution was added, followed by thorough mixing and a 6-minute incubation. Subsequently, 0.3 mL of a 10% $Al(NO_3)_3$ solution was introduced, mixed well, and allowed to stand for another 6 min. Finally, 4 mL of a 4% NaOH solution was added, the mixture was shaken, and the volume was brought to 10 mL with 70% ethanol. A 200 μL aliquot of the prepared reaction solution was dispensed onto a 96-well plate, and the absorbance at 510 nm was measured. This absorbance value was then substituted into the rutin standard curve to calculate the total flavonoid content in the sample.

Determination of three compounds by high-performance liquid chromatograph (HPLC)

Initially, the tissues were subjected to lyophilization using a freeze dryer (model: Scientz-100F). Subsequently, the sample was pulverized into a fine powder employing a grinding mill (MM 400, Retsch GmbH, Haan, Germany) operating at a frequency of 30 Hz for a duration of 90 seconds. Following this, an exact mass of 0.1 g of the powdered sample was measured using an electronic balance (MS105DM) and

introduced into 5 mL of 70% methanol for ultrasonic extraction. After 30 min of extraction, the mixture was centrifuged at 12,000 rpm for 10 min. The resulting supernatant was filtered through a 0.22 μm microporous membrane and stored in a vial for subsequent analysis. HPLC analysis was performed on an Agilent 1260 Infinity II High-Performance Liquid Chromatograph system (Agilent Technologies Inc., Santa Clara, CA, USA) with a BDS Hypersil C18 reversed-phase chromatographic column (250 × 4.6 mm, 5 μm) (Thermo Fisher Scientific, Waltham, MA, USA), utilizing solvent A (0.01% formic acid solution) and solvent B (acetonitrile) as the mobile phases. The gradient elution was programmed as follows: from 0 to 5 min, solvent B increased linearly from 30% to 35%; from 5 to 10 min, solvent B increased linearly from 35% to 40%; from 10 to 15 min, solvent B increased linearly from 40% to 50%; from 15 to 22 min, solvent B linearly kept 50%; from 22 to 35 min, solvent B linearly from 50%–30%; from 35 to 40 min, solvent B was maintained at 30%. The entire HPLC separation process was monitored at a wavelength of 280 nm and a temperature of 30 °C, with a flow rate of 1.0 mL/min [24, 25].

The standards for wogonoside (DSTDH002602, CAS 51059-44-0, HPLC ≥ 98%), wogonin (DSTDH002501, CAS 632-85-9, HPLC ≥ 98%) and oroxylin A (DSTDQ004102, CAS 480-11-5, HPLC ≥ 98%) was purchased from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). Analytical curves with coefficients of determination (R^2 > 0.990) were constructed to quantify the contents. The analytical curves for these compounds are presented in Supplementary Table S3.

Results

Identification of *SbOMTs* in *S. baicalensis*

In the *S.baicalensis* genome database, a total of 64 members of the OMT gene family, designated as *SbOMTs*, were identified. Following an analysis of conserved domains, it was determined that *SbOMT11*, *52*, *53*, *56*, *57*, *58*, *59*, *60*, *63*, and *64* did not possess the conserved domain characteristic of the AdoMet_MTases superfamily (Supplementary Figure S1). Consequently, these sequences were excluded from further study, leaving 54 *SbOMTs* for subsequent analyses. Examination of the physicochemical properties of the proteins revealed that the SbOMTs varied in length from 129 to 695 amino acids, with molecular weights ranging from 14.42 to 76.94 kDa. Among these, SbOMT48, SbOMT08, SbOMT12, SbOMT55, SbOMT62, and SbOMT10 exhibited isoelectric points (pI) greater than 7, classifying them as basic proteins, whereas the remaining members had pI values below 7, classifying them as acidic proteins. Among the 36 SbOMTs analyzed, those with protein stability indices below 40 were categorized as stable proteins, whereas the remaining 18 proteins were deemed unstable. The Grand Average of Hydropathicity (GRAVY) analysis indicated that the majority of SbOMTs exhibited hydrophilic characteristics, as evidenced by their negative average hydrophilicity values. Subcellular localization analysis revealed that the predominant proportion (43%) of SbOMTs were localized within the cytoplasm, followed by localization in the cytoskeleton (26%) and chloroplasts (20%) Supplementary Table S4.

Phylogenetic analysis of SbOMTs and sequence analysis

A phylogenetic analysis was conducted utilizing ML methods based on OMT proteins from various plant species, including *Arabidopsis thaliana*, *Mesembryanthemum crystallinum*, *Oryza sativa*, *Eucalyptus globulus*, and *S. baicalensis*. The analysis identified that the CCoAOMT subfamily comprises 9 members, while the remaining 45 members are classified under the COMT subfamily (Figure 1). By integrating phylogenetic tree construction, gene structure analysis, and conserved

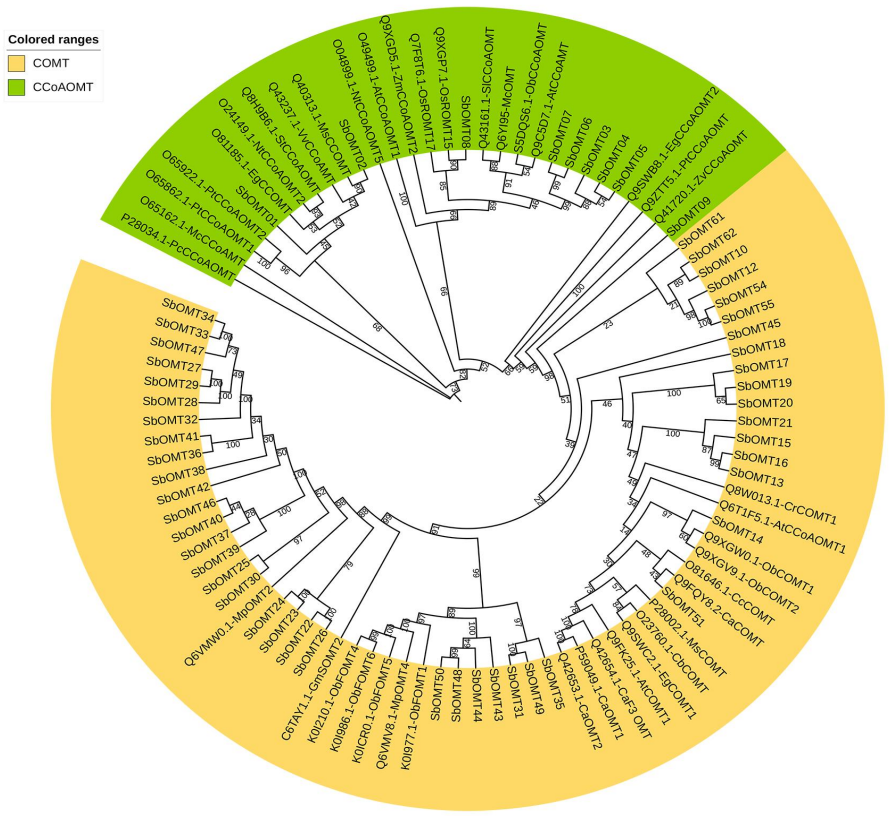


Figure 1 Phylogenetic analysis of SbOMTs reveals the distinct clustering of COMT and CCoAOMT subfamilies.
The yellow clade comprises the COMT subfamily, while the green clade comprises the CCoAOMT subfamily. The evolutionary tree was constructed using full-length protein sequences of OMTs from different species. The results show a clear separation into two major clades, indicating an ancient divergence within the OMT family. OMT, O-methyltransferase; COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase.

motif identification, we obtained enhanced insights into the gene architecture and evolutionary relationships within the OMT gene family of *S. baicalensis*. The phylogenetic classification was found to be consistent with the OMT groupings observed in other plant species [5, 26]. In our phylogenetic and structural analyses of the CCoAOMT and COMT subfamilies, we identified a distinct pattern of motif distribution and domain architecture. Within the CCoAOMT subfamily, most members, with the exception of SbOMT09, contained motifs 9 and 10. This indicates a high degree of conservation in motif composition among these enzymes, potentially reflecting conserved functional or structural roles. Conversely, the COMT subfamily displayed a different motif profile, predominantly featuring motifs 1–8. This variation in motif distribution may suggest functional specialization or evolutionary divergence between the two subfamilies (Figure 2).

Conserved domain analysis further elucidated that the CCoAOMT subfamily generally possesses shorter amino acid sequences in comparison to their COMT counterparts. This disparity in sequence length may be linked to differences in structural complexity or the presence of functional domains within the enzymes. Notably, CCoAOMT proteins were identified to contain solely a single AdoMet_MTases superfamily domain, whereas COMT proteins typically encompass both a dimerization domain and an AdoMet_MTases superfamily domain. Gene structure analysis offered additional insights into the evolutionary dynamics of these subfamilies. Members of the CCoAOMT subfamily exhibited relatively minor variations in gene length, indicative of a high degree of conservation in their genetic architecture. Conversely, COMT members demonstrated significant length variability, suggesting a higher degree of intra-subfamily diversification (Figure 2). These findings imply that the CCoAOMT subfamily is evolutionarily more conserved, whereas COMT subfamily members exhibit greater intra-subfamily diversification.

Chromosome location and cis-acting element analysis of the *SbOMTs*

Genomic analysis demonstrated that *SbOMTs* are distributed across all nine chromosomes of *S. baicalensis*, albeit with significant variations in gene density per chromosome (Figure 3A). The CCoAOMT subfamily exhibited a localized distribution pattern, predominantly concentrated

Figure 2 Analysis of the conserved motifs, domains and structural of characteristics of the SbOMTs in *S. baicalensis*.
(A) The MEME, CDs, and gene structure analysis of SbOMTs. (B) Discovered motifs in the amino sequences of SbOMTs. The arrangement and type of motifs are highly consistent among members belonging to the same subfamily. COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; MEME, multiple expectation maximization for motif elicitation; CDs, coding DNA sequence; SbOMTs, *Scutellaria baicalensis* O-methyltransferase; PPR, pentatricopeptide repeat; UTR, untranslated region.

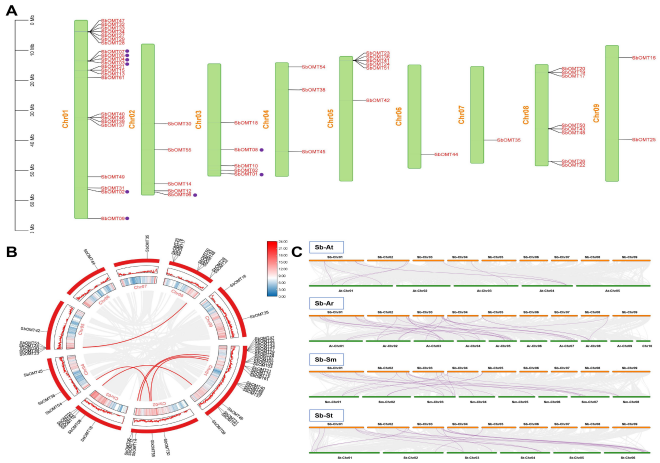


Figure 3 Chromosomal localization of *SbOMTs* in *S.baicalensis*.
(A) Chromosomal distribution and localization of *SbOMTs*. The purple circles represent members of the CCoAOMT subfamily, and the rest are members of the COMT subfamily. (B) Chromosomal distribution and gene replication events of the *SbOMTs*. (C) Collinear relationship between *S. baicalensis* and other species. The Sb, At, Ar, Sm, and St represent *S. baicalensis*, *A. thaliana, A. rugosa, S. miltiorrhiza*, and *S. tenuifolia.* The two subfamilies exhibit distinct distribution patterns on chromosomes, indicating they may have expanded through different evolutionary events. COMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; SbOMTs, *Scutellaria baicalensis* O-methyltransferase.

on chromosomes 01, 02, and 03, whereas members of the COMT subfamily were dispersed across all chromosomes. Notably, gene clustering was observed on chromosomes 01, 05, and 08, with chromosome 01 displaying particularly dense groupings, suggesting the occurrence of potential gene duplication events (Figure 3A). Additionally, intra-genomic synteny analysis identified six segmentally duplicated gene pairs within the SbOMT family: *SbOMT06/SbOMT07*, *SbOMT14/SbOMT21*, *SbOMT02/SbOMT01*, *SbOMT55/SbOMT54*, *SbOMT23/SbOMT26*, and *SbOMT24/SbOMT26* (Figure 3B). These pairs likely originated from duplication events and exhibit high sequence similarity, indicating potential functional conservation or subfunctionalization within flavonoid biosynthesis pathways.

Interspecies synteny analysis is an essential methodology for examining genome evolution. In this study, we conducted comparative genomic analyses between *S. baicalensis* and four representative species: *Arabidopsis thaliana*, *Salvia miltiorrhiza*, *Agastache rugosa*, and *Schizonepeta tenuifolia*. Our analysis identified 7, 17, 17, and 19 syntenic OMT gene pairs, respectively (Figure 3C). Importantly, the number of syntenic gene pairs between *S. baicalensis* and its taxonomically related species within the Lamiaceae family (such as *S. miltiorrhiza*, *S. tenuifolia,* and *A. rugosa*) was significantly greater than that observed with the more distantly related *A. thaliana*, reflecting their phylogenetic divergence. These conserved syntenic blocks likely represent ancestral genomic configurations, offering valuable insights into the evolutionary trajectory of the OMT gene family and its functional diversification across different plant lineages.

Through cis-element analysis of the 2,000 base pair upstream promoter regions of *SbOMTs*, potential transcription factor binding sites were identified, revealing 26 distinct regulatory motifs. The most prevalent elements were linked to light responsiveness, followed by those associated with abscisic acid responsive element (ABA-RE), methyl jasmonate responsive element (MeJA-RE), and anaerobic responsive element (ARE) (Figure 4). These findings suggested that light signaling, abscisic acid, jasmonate, and hypoxic conditions are critical regulatory factors influencing the biological functions of the *SbOMT* gene family members.

Expression pattern of the *SbOMTs* in *S. baicalensis*

A transcriptome analysis of *S. baicalensis* tissues, including roots, stems, leaves, and petals, revealed distinct expression patterns of *SbOMTs*. Notably, petals and roots exhibited higher expression levels compared to stems and leaves. In petals, where anthocyanin accumulation is responsible for blue-to-purple pigmentation, several *SbOMTs* (*SbOMT16*, *SbOMT37*, *SbOMT39*, *SbOMT13*, *SbOMT40*) showed tissue-specific upregulation, indicating potential involvement in anthocyanin biosynthesis. In contrast, roots, which are the primary medicinal organs for the production of methoxylated flavonoids such as wogonoside and wogonin, preferentially expressed *SbOMT04, SbOMT07*, *SbOMT28*, *SbOMT26*, and *SbOMT41*, suggesting a role for these genes in the biosynthesis of bioactive flavones (Figure 5A).

To examine the expression profiles of *SbOMTs* involved in flavonoid biosynthesis across various tissues (root, stem, leaf, and petal), qRT-PCR was conducted on selected genes that exhibit tissue-specific expression patterns: root-preferential *SbOMT04*, *SbOMT07*, and *SbOMT28*, as well as flower-preferential *SbOMT16*, *SbOMT37*, and *SbOMT39*. The findings confirmed that *SbOMT04*, *SbOMT07*, and *SbOMT28* are predominantly expressed in roots, whereas *SbOMT16*, *SbOMT37*, and *SbOMT39* demonstrate elevated expression levels in petals, aligning with the transcriptome data (Figure 5B). This validation substantiates the reliability of the expression trends derived from the transcriptome, thereby supporting their functional association with tissue-specific flavonoid biosynthesis.

Identification of *SbOMTs* involved in flavonoid synthesis and flavonoid content during drought

S. baicalensis is mainly cultivated in dry and semi-dry areas north of the Qinling Mountains. Studies have shown that moderate drought stress can increase flavonoid levels in this medicinal plant [22]. To explore this, experiments were conducted with drought stress lasting 5–15 days, and the total flavonoids and three specific methoxylated flavonoids – wogonin, wogonoside, and oroxylin A – were analyzed in the roots under varying drought conditions (Figure 6A–6B, Supplementary Figure S2).

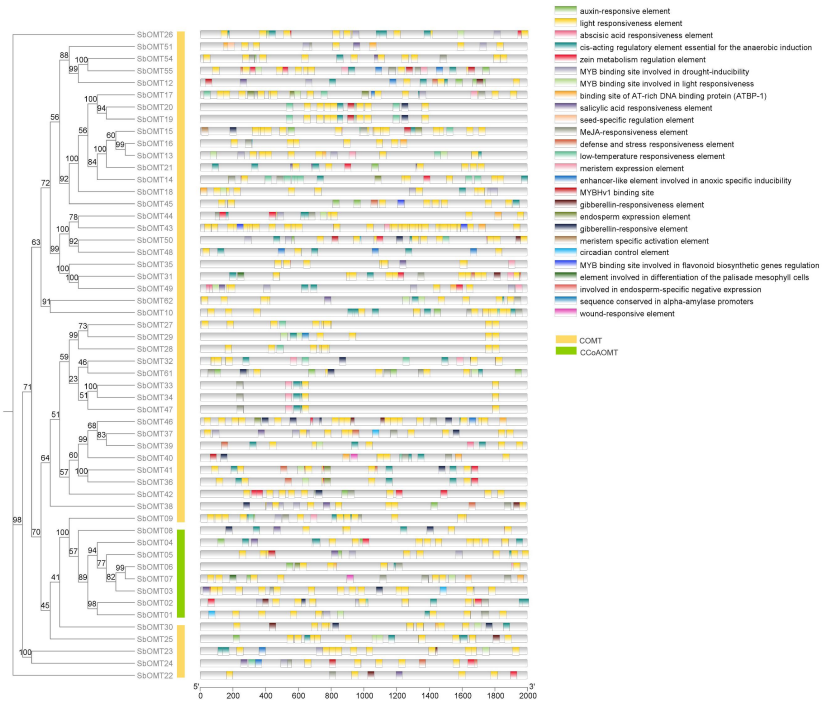


Figure 4 Analysis of cis-acting elements of *SbOMTs* in *S. baicalensis*.

The promoters harbor a diverse set of regulatory elements, particularly those associated with light, abscisic acid, methyl jasmonate and anaerobic stress responses, pointing to a complex regulatory network governing *SbOMT* gene expression. CCoAOMT, caffeoyl-CoA O-methyltransferase; SbOMTs, *Scutellaria baicalensis* O-methyltransferase.

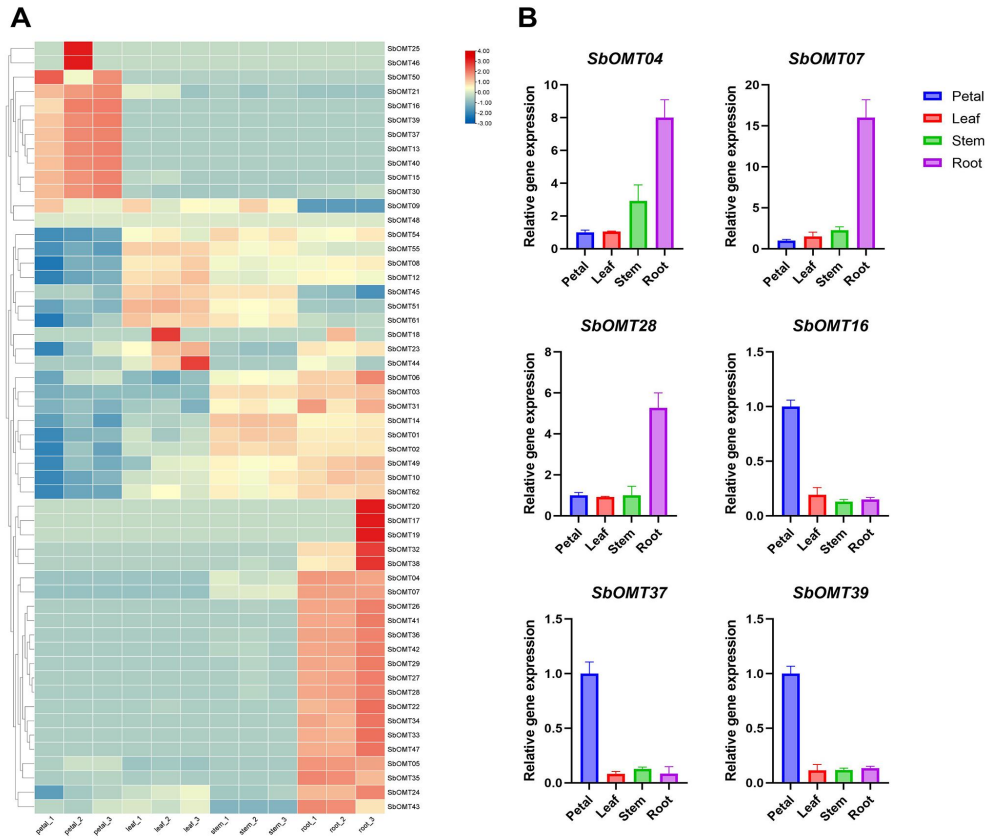


Figure 5 Heat maps of *SbOMTs* gene expression.

(A) The gene expression of *SbOMTs* in different tissues. Blue indicated low gene expression, red indicates high gene expression. (B) The qRT-PCR analysis of *SbOMTs* in different tissues. The error bars represent standard deviations (SD; n = 3). These patterns align with the biosynthesis of root-specific methoxylated flavones and petal-specific anthocyanins. SbOMTs, *Scutellaria baicalensis* O-methyltransferase.

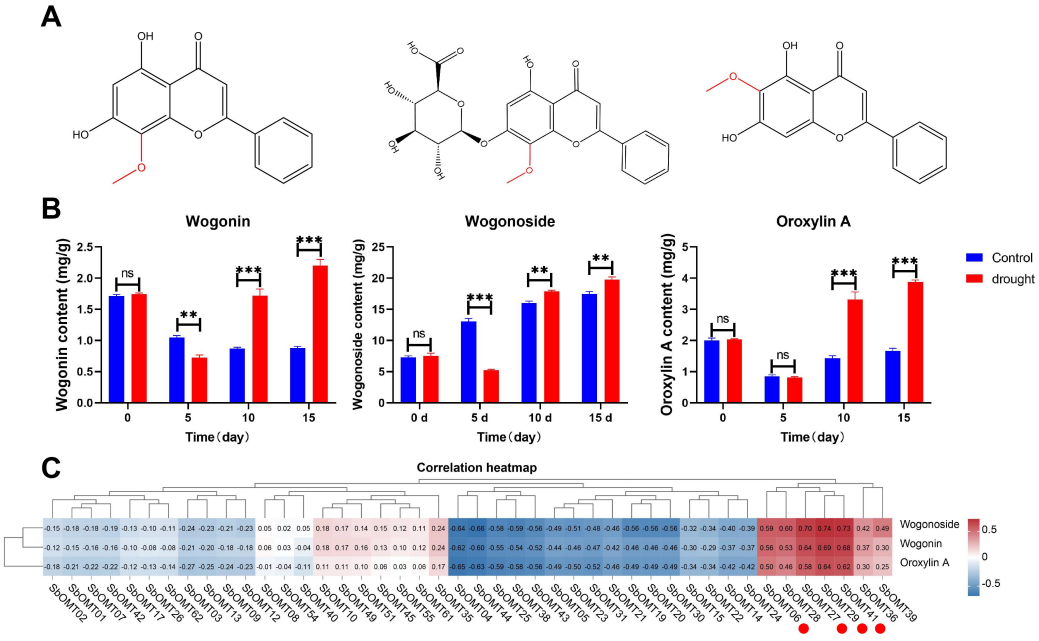


Figure 6 The content of methoxyflavonoids in *S. baicalensis* after drought stress.

(A) The chemical structural formulas of wogonin, wogonoside, and oroxylin A. (B) Contents of wogonin, wogonoside and oroxylin A in the drought stress group. $^{**}P < 0.01$, $^{***}P < 0.001$, and ns indicates no significance, n = 3. (C) Correlation analysis of the expressions of wogonin, wogonoside, oroxylin A and *SbOMTs*. These results demonstrate that drought stress significantly induces the accumulation of bioactive methoxyflavonoids and identifies *SbOMT06*, *SbOMT41*, *SbOMT27*, *and SbOMT29* as key candidates responsible for their biosynthesis. SbOMTs, *Scutellaria baicalensis* O-methyltransferaseS; ns, no significance.

Under drought stress conditions, plants synthesize a crucial stress-signaling hormone known as abscisic acid (ABA) [27]. The production and accumulation of ABA play a pivotal role in coordinating plant responses to arid environments while enhancing water-use efficiency [28]. In this study, we examined the expression of *SbOMTs* utilizing transcriptomic data from *S. baicalensis* subjected to treatments with both polyethylene glycol (PEG) and ABA. Pearson correlation analysis between these genes and the levels of three chemical components following 15 days of drought treatment indicated that *SbOMT06*, *SbOMT41*, *SbOMT27*, and *SbOMT29* demonstrated correlation coefficients (R) greater than 0.5 (Figure 6C). Expression profiling further revealed that these *SbOMTs* are predominantly expressed in roots and exhibit significant inducibility under drought conditions.

The *SbOMTs* exhibited elevated expression levels in roots and were induced by drought conditions. Analysis of expression patterns under drought stress revealed that their transcriptional trends corresponded with variations in the concentration of wogonin, wogonoside, and oroxylin A. Considering that methoxylated flavonoids predominantly accumulate in the roots of *S. baicalensis*, these genes are strongly implicated in the biosynthesis of methylated flavonoids. Importantly, their dynamic expression patterns under increasing drought stress demonstrated significant positive covariation ($P < 0.05$, $R > 0.5$) with the accumulation profiles of three key methoxylated flavonoids: wogonin, wogonoside and oroxylin A. This spatiotemporal correlation, along with the established root-specific accumulation pattern of methoxylated flavonoids in *S. baicalensis*, provides compelling evidence for the functional involvement of these *SbOMTs* in the methylation-mediated flavonoid modification pathway. Furthermore, it highlights the plant's adaptive response to water deficit through the production of specialized metabolites.

Discussion

The flavonoid compounds present in *S. baicalensis* are acknowledged as the principal bioactive constituents responsible for its pharmacological effects, encompassing methoxylated flavonoids such as wogonin, wogonoside, oroxylin A, and oroxylin-A-7-glucuronide [29, 30]. OMTs play essential roles in the biosynthesis of these methoxylated flavonoids. In this study, we performed the inaugural genome-wide identification of the OMT gene family in *S. baicalensis*, identifying 54 *SbOMTs*, which were categorized into 9 members of the CCoAOMT subfamily and 45 members of the COMT subfamily. This distribution pattern is consistent with those observed in *Stephania japonica*, *Populus*, and *Chrysanthemum indicum*, as well as with the established classification of plant OMT families into COMT and CCoAOMT subfamilies [5, 10, 31, 32]. In particular, the CCoAOMT subfamily members in *S. baicalensis* were fewer in number compared to its COMTs, displaying minimal variation in protein molecular weight (ranging from 26.05 to 31.39 kDa). In contrast, COMT members accounted for over 83% of the total and exhibited a significantly broader range of protein weights (14.42 to 76.94 kDa). These observations align closely with the results of the gene structure analysis, suggesting that the CCoAOMT subfamily members are more evolutionarily conserved, with limited variability in both protein characteristics and gene structures. Conversely, the COMT subfamily exhibited considerable intra-subfamily diversity in these attributes.

Cis-acting elements, which serve as essential binding sites for transcription factors, are crucial in regulating gene expression, thereby influencing plant developmental processes and the accumulation of secondary metabolites. An analysis of the OMT gene family in soybean has demonstrated that *GmCOMTs* are implicated in growth, light response, stress adaptation, and hormone signaling [33]. In maize, light-responsive elements are conserved across all OMT members, with *ZmCOMT22* and *ZmCOMT10* exhibiting specific responsiveness to light [34]. The promoter region of *DfCCoAOMT* from *Dendrocalamus farinosus* is enriched with cis-acting elements associated with light, hormones (ABA/MeJA), and drought stress. The expression of *DfCCoAOMT2/5/6/8/9/14/15* is induced by ABA and MeJA. Moreover, the overexpression of *DfCCoAOMT14* significantly enhances lignin content, xylem thickness, and drought tolerance, underscoring its role in the coordinated regulation of lignin biosynthesis and stress adaptation [35]. In this study, a comprehensive genome-wide analysis of *SbOMT* promoters identified a significant enrichment of 705 elements responsive to biotic and abiotic stress, alongside 333 regulatory elements associated with hormonal pathways. Remarkably, light-responsive elements accounted for 70% of all stress-related cis-elements, indicating that photoregulation is a predominant environmental factor influencing the biosynthesis and accumulation of methoxylated flavonoids in *S. baicalensis*.

Studies have reported that combined red-blue light treatment in *S. baicalensis* significantly upregulates genes related to plant hormone signaling, such as CYCD3, while downregulating genes associated with jasmonic acid signaling, such as JAR1. This modulation promotes flavonoid accumulation [36]. These findings suggest that precise light regulation could be a promising strategy for manipulating specific flavonoid biosynthetic pathways, potentially by modulating the interplay between different phytohormone signals. Furthermore, under varying concentrations of 6-benzylaminopurine (6-BA), UV-B radiation significantly increased flavonoid content in *S. baicalensis* adventitious shoots, particularly enhancing the levels of aglycones such as baicalein and wogonin. This indicates that UV-B treatment significantly enhances methoxylated flavonoid production [37]. The results imply a synergistic interaction between cytokinin signaling and the UV-B stress response, which may converge on the activation of key OMTs. This offers a potential strategy for enhancing the production of valuable methoxylated flavonoids in in vitro cultures.

Moreover, the biosynthesis of methoxylated flavonoids in *S. baicalensis* is meticulously regulated by various environmental response elements, such as drought and low temperature. These environmental factors function as critical signaling molecules that initiate complex signal transduction pathways, ultimately influencing methoxylated flavonoid biosynthesis. For example, in *Citrus reticulata* "Chachiensis", there is a strong correlation between methoxylated flavonoid biosynthesis and environmental stress, particularly in plants subjected to saline stress conditions in the Xinhui region. Under these conditions, there is a significantly higher expression of AP2/ERF transcription factors compared to non-authentic producing areas, which in turn activate methoxylated flavonoid biosynthetic genes, leading to enhanced production of methoxylated flavonoids in the fruits [38]. These findings underscore an evolutionarily conserved mechanism by which environmental stresses augment secondary metabolite production through transcriptional activation, offering valuable insights for developing cultivation strategies that leverage controlled stress conditions to enhance the quality of medicinal plants.

The expression patterns of genes in specific tissues are typically closely linked to their biological functions, with genes exhibiting tissue-specific expression often playing a direct role in the biosynthesis of chemical components that accumulate in those tissues. In *S. baicalensis*, the blue-to-purple coloration of petals is attributed to the significant accumulation of delphinidin-type and cyanidin-type anthocyanins. The initial stages of anthocyanin biosynthesis are facilitated by key enzymes such as chalcone isomerase (CHI), chalcone synthase (CHS), flavonoid 3'-hydroxylase, and flavanone 3-hydroxylase (F3H). In contrast, the later stages involve enzymes like anthocyanidin 3-glycosyltransferase, dihydroflavonol 4-reductase, anthocyanidin synthase, and OMTs [39]. Although the biosynthetic pathways for cyanidin and delphinidin anthocyanins are relatively well-characterized, the subsequent methoxylation process catalyzed by OMTs remains inadequately documented. In this study, the genes *SbOMT13*, *SbOMT15*, *SbOMT16*, *SbOMT21*, *SbOMT30*, *SbOMT37*, *SbOMT39*, and *SbOMT40* were found to be highly expressed specifically in the petals of *S. baicalensis*, indicating their potential roles in the methoxylation of anthocyanidins within these tissues. Future investigations should prioritize the functional characterization of these petal-specific SbOMTs to fully elucidate the anthocyanin

modification pathway, which may facilitate the biotechnological production of novel pigments with improved stability and color properties. Concurrently, the underground parts of the plant are known to accumulate significant quantities of methoxylated flavonoids, such as wogonoside and wogonin, whose biosynthetic pathways remain partially understood, with OMTs playing pivotal catalytic roles. Twenty-two SbOMTs were identified as being highly expressed in roots and are considered potential key enzymes in the biosynthesis of these methoxylated flavonoid compounds. Notably, the expression levels of *SbOMT06*, *SbOMT41*, *SbOMT27,* and *SbOMT29* were positively correlated with the accumulation of methoxylated flavonoids. These candidate genes constitute optimal targets for metabolic engineering strategies designed to enhance the biosynthesis of pharmaceutically significant methoxylated flavonoids via genetic overexpression or genome editing methodologies.

Research on methoxylated flavonoid pathways has delineated two functionally distinct classes of OMTs exhibiting catalytic activity in the roots of S. baicalensis roots. Class I OMTs, comprising SbFOMT3, SbFOMT5, and SbFOMT6, predominantly facilitate the C-7 methoxylation of flavonoids, with SbFOMT5 also exhibiting methylation activity at the C-5 and C-6 hydroxyl positions of baicalein [15]. In contrast, Class II OMTs, including SbPFOMT2 and SbPFOMT5, selectively methylate one of two adjacent hydroxyl groups on flavonoids, catalyzing modifications at the C-6, C-8, and C-30 positions to produce oroxylin A, tenaxin II, and chrysoeriol, respectively. Despite these insights, the biosynthetic pathways of numerous other methoxylated flavonoids in *S. baicalensis* remain uncharacterized, underscoring the need for further investigation of *SbOMTs* through extensive gene mining and functional characterization. The present study systematically identified members of the *SbOMT* gene family through whole-genome screening, followed by an extensive bioinformatics analysis and validation of expression patterns. This approach elucidated the genetic diversity and functional complexity of *SbOMTs* in *S. baicalensis*. The research not only establishes foundational knowledge regarding the *SbOMT* gene family but also offers a critical theoretical framework for exploring OMT catalytic mechanisms and their essential roles in the biosynthesis of methoxylated flavonoids.

Conclusion

This study elucidates the identification of 54 *SbOMTs* within the genome of *S. baicalensis*, categorizing them into the CCoAOMT (9 members) and COMT (45 members) subfamilies. Members within the same subfamily exhibit conserved gene structures, motifs, and classifications. Analysis of promoter cis-acting elements revealed numerous elements responsive to hormonal signals and environmental stresses within the promoters of *SbOMTs*. Notably, the expression levels of four specific genes (*SbOMT06, SbOMT41, SbOMT27*, and *SbOMT29*) demonstrated a significant positive correlation with the concentrations of key bioactive methoxylated flavonoids – wogonin, wogonoside, and oroxylin A – under conditions of drought stress. These findings strongly imply that these *SbOMTs* are likely integral to the biosynthetic pathway of these pharmacologically significant compounds. The comprehensive genomic and expression analyses offer critical molecular insights into the biosynthesis of methoxyflavonoids in *S. baicalensis*, thereby establishing a valuable foundation for future research endeavors in metabolic engineering and the optimization of flavonoid production pathways.

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