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Rhizome Diameter-Dependent Regulation of Glycyrrhizin Biosynthetic Genes and Glycyrrhizin Accumulation in Glycyrrhiza glabra

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Abstract

Glycyrrhiza glabra L. (licorice) is a valuable medicinal plant renowned for its triterpenoid saponin glycyrrhizin, which exhibits anti-inflammatory, antiviral, and hepatoprotective properties. Both developmental and environmental factors influence variations in glycyrrhizin accumulation; however, the role of rhizome morphology remains poorly understood. This study investigated the impact of rhizome diameter (0.5 cm, 1–1.5 cm, and 2 cm) on glycyrrhizin content and expression of key biosynthetic genes in wild *G. glabra* from Mansuriyeh, Khuzestan Province, Iran. Medium-diameter rhizomes (1–1.5 cm) accumulated the highest glycyrrhizin (21.46 mg/g DW), followed by large (17.83 mg/g DW) and small (14.59 mg/g DW) rhizomes, with leaves showing the lowest levels (12.75 mg/g DW). qRT-PCR analysis revealed upregulated expression of pathway genes (LUS, BAS, CYP88D6, CYP72A154, UGTs, and SQS) in medium- to large-diameter rhizomes, correlating strongly with glycyrrhizin content (Pearson's r up to 0.79). These findings demonstrate diameter-dependent transcriptional regulation of glycyrrhizin biosynthesis, highlighting rhizome size as a developmental proxy for metabolite optimization. This work provides insights for selective harvesting and cultivation strategies to enhance pharmaceutical-grade licorice production.

Introduction

Glycyrrhiza glabra, commonly known as licorice, is a perennial herb widely cultivated for its subterranean rhizomes and roots, which are rich in bioactive secondary metabolites (Sharifi-Rad et al. 2021). Among these, glycyrrhizin a triterpenoid saponin, stands out as the principal active compound, accounting for the plant's characteristic sweet taste and diverse pharmacological properties (Fanani et al. 2021; Chiyo et al. 2024). Glycyrrhizin exhibits potent anti-inflammatory, antiviral, hepatoprotective, antioxidant, and anticancer activities, making it a valuable ingredient in traditional medicine, pharmaceuticals, and food industries (Semwal et al. 2025). Its therapeutic potential has been demonstrated in various studies, including its role in inducing apoptosis in cancer cells and modulating immune responses (Lu et al. 2017). However, the accumulation of glycyrrhizin in licorice varies significantly depending on environmental, genetic, and developmental factors, which directly impact the medicinal quality and commercial value of harvested material (Li et al. 2025; Chiyo et al. 2024). Environmental factors like climate, soil conditions, water deficit, sunshine, and rainfall significantly influence the content of glycyrrhizin (Fujii et al. 2016). For instance, increased sunshine can be advantageous for glycyrrhizin accumulation (Yu et al. 2015). Genetic factors also play a crucial role, with significant genotypic effects observed on glycyrrhizin content, and specific genes and their expression patterns determining accumulation levels in different licorice species (Chiyo et al. 2024). Developmental factors, such as the age of the plant and the specific part of the root (taproot vs. stolon), also affect glycyrrhizin distribution and content (Liu et al. 2020; Guo et al. 2014). These variations are critical because glycyrrhizin is considered the most important bioactive compound determining the quality of medicinal licorices and is widely used as a natural sweetener and pharmaceutical agent (Li et al. 2025).

The biosynthesis of glycyrrhizin follows the mevalonate (MVA) pathway, initiating from squalene and progressing through a series of enzymatic steps to form glycyrrhetinic acid, the aglycone precursor, followed by glycosylation (Sakanishi et al. 2024). Key genes in this pathway include squalene synthase (SQS), which catalyzes the formation of squalene; lupeol synthase (LUS) and β-amyrin synthase (BAS), responsible for cyclization to lupeol and β-amyrin, respectively; cycloartenol synthase (CAS), involved in sterol branching; CYP88D6 (β-amyrin 11-oxidase), which oxidizes β-amyrin at the C-11 position; CYP72A154 (11-oxo-β-amyrin 30-oxidase), facilitating further oxidation at C-30; and UDP-glucuronosyltransferases (UGTs), which add glucuronic acid moieties to yield glycyrrhizin (Fig. 1) (Majidi et al. 2024). These genes are highly conserved across *Glycyrrhiza* species and are modulated by phytohormones, biotic stresses, and developmental stages (Peres et al. 2019; He et al. 2025). Despite advances in elucidating the molecular basis of glycyrrhizin production through genome editing and transcriptomic approaches, the influence of rhizome morphology—particularly diameter—on gene expression and metabolite accumulation remains underexplored (Wang et al. 2021; Zhong et al. 2022). Rhizome size is a critical agronomic trait, often correlating with plant maturity and metabolite content, yet its regulatory role in biosynthetic pathways is not well understood (Son et al. 2025).

In natural populations, licorice rhizomes exhibit variability in diameter, which may reflect differential resource allocation and metabolic activity. Previous studies have shown that larger rhizomes tend to accumulate higher levels of secondary metabolites due to enhanced vascular development and storage capacity, but the underlying transcriptional and biochemical mechanisms are unclear (Dang et al. 2020). This knowledge gap hinders efforts to optimize cultivation practices for high-glycyrrhizin yields, especially in regions like Iran, where *G. glabra* is endemic and economically important. The present study addresses this by investigating the expression profiles of key glycyrrhizin biosynthetic genes and quantifying glycyrrhizin content in rhizomes of varying diameters (0.5 cm, 1–1.5 cm, and 2 cm) collected from a uniform ecological and temporal context in Mansuriyeh, Khuzestan Province, Iran. By elucidating size-dependent gene regulation and metabolite accumulation, this work provides insights into developmental control of metabolite biosynthesis, with implications for selective harvesting and genetic improvement of licorice for pharmaceutical applications.

Materials and Methods

Plant Material Collection

Rhizomes of *Glycyrrhiza glabra* were collected from wild populations in Mansuriyeh, located in Khuzestan Province, southwestern Iran (coordinates: approximately 31°20'N, 48°30'E) (Fig. 2). Sampling was conducted during the optimal growth season to minimize temporal and environmental variations. Only healthy, mature plants of the same species (*G. glabra*) were selected. Rhizomes were categorized based on diameter measured at the midpoint using a digital caliper: small (0.5 cm), medium (1–1.5 cm), and large (2 cm). For each category, three biological replicates (n = 3) were harvested, each consisting of rhizomes from distinct plants to ensure genetic diversity. Immediately after excavation, the rhizomes

were washed with distilled water, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction and metabolite analysis were performed.

Glycyrrhizin Extraction and Quantification

Glycyrrhizin was extracted from dried rhizome samples following a standard methanolic extraction protocol. Approximately 500 mg of powdered rhizome tissue was macerated in 10 mL of 70% methanol (v/v) at 60°C for 2 hours with occasional shaking. The extract was filtered through Whatman No. 1 filter paper, and the residue was re-extracted twice under the same conditions. The combined filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C and reconstituted in 5 mL of HPLC-grade methanol. Samples were filtered through a 0.22 µm syringe filter before analysis (Nagar et al. 2022; Brovchenko et al. 2020).

Quantification of glycyrrhizin was performed using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) on an Agilent 1260 Infinity system (Agilent Technologies, USA). Separation was achieved on a reversed-phase C18 column (4.6 mm × 250 mm, 5 µm particle size) maintained at 30°C. The mobile phase consisted of acetonitrile (A) and 0.1% phosphoric acid in water (B) in a gradient elution: 0–5 min, 30% A; 5–20 min, 30–50% A; 20–25 min, 50% A; followed by re-equilibration to initial conditions. The flow rate was 1.0 mL/min, the injection volume was 10 µL, and detection was at 254 nm. A standard curve was prepared using glycyrrhizin ammonium salt (Sigma-Aldrich, purity ≥ 95%) at concentrations ranging from 10 to 500 µg/mL. Glycyrrhizin content was expressed as mg/g dry weight (DW). All analyses were conducted in triplicate (Brovchenko et al. 2020).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 25–50 mg of frozen rhizome and leaf tissue powdered in liquid nitrogen using the EZ-10 DNAaway RNA Mini-Preps Kit (Bio Basic Inc., Markham, Canada) according to the manufacturer's protocol. To eliminate genomic DNA contamination, purified RNA was treated on-column with RNase-free DNase I (Takara Bio, Kusatsu, Japan). RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Eppendorf, Hamburg, Germany), and integrity was verified by 1% agarose gel electrophoresis.

First-strand cDNA was synthesized from 1 µg of total RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan) following the manufacturer's instructions. The reaction employed both oligo(dT) and random hexamer primers and included an initial gDNA removal step at 42°C for 2 min, followed by reverse transcription at 37°C for 15 min and enzyme inactivation at 85°C for 5 s.

Primer design

Coding DNA sequences (CDS) of the target genes and the reference gene (ACTIN) were obtained from relevant transcriptomes or public databases. Primer pairs were designed using Primer3Plus with a product size of 80–150 bp, primer length of 18–24 nt, Tm of 58–62°C, and GC content of 40–60%. Specificity was verified through Primer-BLAST against the corresponding transcriptome and by melt-curve analysis. Primer efficiency and specificity were further confirmed using Beacon Designer 8.0 software (PREMIER Biosoft, San Francisco, CA, USA). All primers were synthesized by Metabion International AG (Planegg, Germany), diluted to a 10 μM working concentration, and stored at − 20°C. Primer sequences and characteristics are listed in Table 1.

Quantitative real-time PCR (qRT-PCR)

Relative expression levels of the target genes were determined by qRT-PCR using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) master mix (Takara Bio, Kusatsu, Japan) on an Applied Biosystems StepOne™ Real-Time PCR System. Each 10 μL reaction contained 5 μL 2× TB Green Premix Ex Taq II, 0.4 μL each forward and reverse primer (10 μM), 0.2 μL ROX Reference Dye II (50×), 2 μL of 5-fold diluted cDNA template, and 1.8 μL nuclease-free water.

Amplification conditions were as follows: initial denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 34 s. A melting curve was generated by heating from 60°C to 95°C with a ramp rate of 0.5°C/s to confirm amplicon specificity. All reactions were performed in triplicate, including no-template controls.

Table 1
Primers used for quantitative real-time PCR analysis

Name primers	Accession No.	Nucleotide sequences(5' → 3')	Annealing temperature
LUS-Forward	BAL41371.1	TTCCAGCAGCAGTGAAATTG	56.4
LUS- Reverse		CGTAGCTCAATGCAGATCCA	58.4
CAS-Forward	Q9SXV6.1	TAAAAGGGCTGATTGCTGCT	56.4
CAS- Reverse		CAGCATAGCCCACCCAGTAT	60.5
BAS-Forward	ADE88148.1	TGCGGACATTGTAGTTGAGC	58.4
BAS- Reverse		ACCAAGTGCGAACCAAGAAC	58.4
CYP88D6-Forward	B5BSX1.1	TCGGCGATCTATTGTCCTTC	28.4
CYP88D6- Reverse		GGGTCTACATCGTGCCAACT	60.5
CYP72A154-Forward	H1A988.1	GGACTTCCCCAAACCAAAAT	56.4
CYP72A154- Reverse		CCCCTTCCATTTGCTAATCA	56.4
UGTs-Forward	ADV71366.1	CCCAACGACTTCAACCTCAT	58.4
UGTs- Reverse		ACCCAAGTTTTGTGCTCCAC	58.4
SQS-Forward	D86410.1	GCGAGGTCTAACTGCCAAAG	60.5
SQS- Reverse		CCGTTCCCATTCCTCAATAA	59.4
Actin-Forward	EU190972.1	TCCTTCGTCTGGATCTTGCT	59.4
Actin- Reverse		GCAACGGAATCTCTCAGCTC	60.5

Statistical Analysis

Data for both gene expression and glycyrrhizin content were analyzed using one-way ANOVA followed by Duncan test for comparing means in GraphPad Prism (version 10.0). Differences were considered significant at $p < 0.05$. Expression and metabolite data are presented as mean ± standard error (SE) from three biological replicates.

The qRT-PCR results analysis was conducted using the $2^{-\Delta CT}$ method. The $2^{-\Delta CT}$ equation is used when the primary goal is to assess the relative expression of each gene and the internal control gene, as well as the non-control test (Livak and Schmittgen 2001).

Results

Glycyrrhizin Content in Rhizomes of Varying Diameters and Leaves

To assess the impact of rhizome diameter on glycyrrhizin accumulation, we quantified the metabolite levels in rhizomes categorized by size (0.5 cm, 1–1.5 cm, and 2 cm) and compared them to leaf tissues. Significant differences in glycyrrhizin content were observed across the groups (one-way ANOVA, $p < 0.0001$; Fig. 3). The medium-diameter rhizomes (1–1.5 cm) exhibited the highest glycyrrhizin concentration at 21.46 mg/g dry weight (DW), which was significantly greater than all other groups (Duncan, $p < 0.05$). This was followed by large-diameter rhizomes (2 cm) with 17.83 mg/g DW, small-diameter rhizomes (0.5 cm) with 14.59 mg/g DW, and leaves with the lowest content at 12.75 mg/g DW.

Expression Profiles of Glycyrrhizin Biosynthetic Genes in Rhizomes of Varying Diameters and Leaves

To elucidate the molecular basis underlying the observed variations in glycyrrhizin accumulation, we examined the relative expression levels of seven key genes in the glycyrrhizin biosynthetic pathway—LUS (lupeol synthase), CAS (cycloartenol synthase), BAS (β-amyrin synthase), CYP88D6 (β-amyrin 11-oxidase), CYP72A154 (11-oxo-β-amyrin 30-oxidase), UGTs (UDP-glycosyltransferases), and SQS (squalene synthase)—using qRT-PCR. Expression was analyzed in rhizomes categorized by diameter (0.5 cm, 1–1.5 cm, and 2 cm) and in leaf tissues. Overall, gene expression patterns revealed tissue- and size-specific regulation, with most genes showing upregulated expression in larger rhizomes compared to small-diameter rhizomes and leaves (Figs. 4 and 5). Statistical analysis indicated significant differences across sample groups for LUS ($p < 0.05$), BAS ($p < 0.05$), CYP88D6 ($p < 0.001$), CYP72A154 ($p < 0.01$), UGTs ($p < 0.05$), and SQS ($p < 0.05$), while CAS showed no significant variation ($p > 0.05$; ns).

The early pathway gene SQS exhibited the highest relative expression in medium-diameter rhizomes (1–1.5 cm) at 1.76, representing a ~ 9.5-fold increase over the 0.5 cm baseline. Expression was moderate in leaves (0.91) and lower in large-diameter rhizomes (0.55), suggesting a peak in squalene production during intermediate developmental stages. Similarly, LUS was markedly upregulated in 1–1.5 cm (3.54) and 2 cm (3.03) rhizomes (~ 23- and 20-fold increases, respectively) compared to 0.5 cm (0.15), with intermediate levels in leaves (1.74). BAS showed a distinct pattern, with peak expression in 1–1.5 cm rhizomes (0.63, ~ 13-fold increase), followed by reduced levels in 2 cm (0.32), leaves (0.16), and 0.5 cm (0.05). In contrast, CAS displayed consistently low expression across all samples (0.01–0.08), with no significant differences, indicating minimal regulation by rhizome size.

Downstream oxidation genes CYP88D6 and CYP72A154 followed trends aligned with glycyrrhizin accumulation. CYP88D6 expression was highest in 2 cm rhizomes (2.20, ~ 8.5-fold increase), followed by 1–1.5 cm (1.70, ~ 6.6-fold), with lower levels in leaves (0.42) and baseline (0.26). CYP72A154 exhibited a similar profile, with maximum expression at 1–1.5 cm (0.13, ~ 16-fold increase), moderate expression at 2 cm (0.06) and in leaves (0.04), and minimal at baseline (0.008). The glycosylation gene

UGTs mirrored this trend, peaking at 1–1.5 cm (0.25, ~ 4.7-fold) and 2 cm (0.22, ~ 4.1-fold), with reduced expression in leaves (0.09) and at 0.5 cm (0.05).

Correlation Between Glycyrrhizin Content and Biosynthetic Gene Expression

To further explore the relationship between glycyrrhizin accumulation and transcriptional regulation of its biosynthetic pathway, Pearson's correlation analysis was performed across all samples. The results revealed strong positive correlations between glycyrrhizin content (GLY) and the expression of most pathway genes, supporting coordinated transcriptional control (Fig. 6).

Glycyrrhizin content showed the strongest correlation with CYP72A154 (r = 0.79, $p < 0.01$), followed by BAS (r = 0.77, $p < 0.01$), UGTs (r = 0.70, $p < 0.05$), CYP88D6 (r = 0.69, $p < 0.05$), LUS (r = 0.60, $p < 0.05$), and SQS (r = 0.59, $p < 0.05$). These significant positive correlations indicate that upregulation of these genes is closely associated with higher glycyrrhizin levels. Notably, no significant correlation was observed with CAS (r = − 0.18, ns).

Inter-gene correlations further highlighted tight co-regulation within the pathway. For instance, BAS exhibited very strong correlations with CYP72A154 (r = 0.92, $p < 0.001$), UGTs (r = 0.86, $p < 0.001$), and LUS (r = 0.81, $p < 0.001$). Similarly, CYP88D6 and UGTs were highly correlated (r = 0.93, $p < 0.001$) with and glycosylation steps.

Discussion

The present study reveals a clear diameter-dependent pattern in glycyrrhizin accumulation and biosynthetic gene expression in *G. glabra* rhizomes.

Glycyrrhizin, a major triterpenoid saponin in *G. glabra* roots and stolons (Seki et al. 2011; Sharifi-Rad et al. 2021) exhibited the highest content in 1–1.5 cm diameter rhizomes (21.46 mg/g DW), with a slight decline in larger (2 cm) rhizomes—a pattern consistent with reports that intermediate developmental stages optimize resource allocation for triterpenoid saponin biosynthesis in licorice and related species (Mochida et al. 2017; Seki et al. 2011). Lower accumulation in smaller rhizomes likely reflects immature metabolic pathways, whereas reduced levels in leaves align with tissue-specific partitioning, in which subterranean organs act as primary storage sites for defense-related saponins (Hayashi and Sudo 2009). Although glycyrrhizin biosynthesis involves specific enzymatic pathways and some studies note its accumulation in thickened roots or compare overall content across *Glycyrrhiza* species (Yang et al. 2018), the precise non-linear correlation between rhizome size, developmental maturity, and glycyrrhizin accumulation described here is not explicitly supported by existing literature.

Wei et al. (2012) investigated the effects of cultivation duration (years) and tissue type (leaves, roots, and rhizomes) on glycyrrhizin content in *Glycyrrhiza* species, finding that wild populations exhibited higher glycyrrhizin levels than cultivated ones, with no significant differences between roots and

rhizomes, but negligible amounts in leaf tissue. In cultivars aged 1–4 years, glycyrrhizin content varied significantly, followed by a rapid increase after five years (Wei et al. 2012). The production of secondary metabolites in medicinal plants is typically influenced by genetic and environmental factors, likely including temperature, humidity, developmental stages, and tissue-specific differences that affect enzyme activity and substrate availability along the glycyrrhizin biosynthesis pathway, thereby altering the accumulation of this compound or its precursors. Furthermore, biosynthesis of secondary metabolites can be enhanced by increased precursor availability or induction of catalytic activity in pathway-related enzymes (Nomura et al. 2019).

Transcriptional profiling demonstrated coordinated upregulation of key genes in the glycyrrhizin pathway—particularly LUS, BAS, CYP88D6, CYP72A154, and UGTs—in medium- to large-diameter rhizomes. This pattern mirrors enhanced flux toward β-amyrin-derived triterpenoids, with BAS and CYP72A154 emerging as rate-limiting steps, as previously characterized in *G. uralensis* and *G. glabra* (Seki et al. 2011; Nomura et al. 2019). The strong positive correlations between gene expression and glycyrrhizin content ($r = 0.59–0.79$) support direct transcriptional control, akin to observations under elicitation or stress, where phytohormones like methyl jasmonate modulate pathway genes (Nomura et al. 2019). Notably, CAS remained unchanged, reinforcing its role in diverting precursors toward sterols rather than saponins (Moses et al. 2013).

These findings extend prior evidence on environmental and genetic influences on glycyrrhizin variation in Iranian populations (Esmaeili et al. 2019), highlighting rhizome diameter as an overlooked developmental regulator. In wild *G. glabra* from Khuzestan, uniform sampling minimized ecological confounders, isolating morphological effects.

Practically, selective harvesting of medium-diameter rhizomes could maximize yields, addressing sustainability concerns amid overexploitation (Yamamoto et al. 2025). Future studies should explore upstream regulators and epigenetic mechanisms governing size-dependent expression (Lin et al. 2024; Chang et al. 2020). Integrating rhizosphere microbiomes, which modulate saponin accumulation (Yu et al. 2024), may further enhance cultivation strategies.

In conclusion, rhizome diameter profoundly influences glycyrrhizin biosynthesis through transcriptional reprogramming, offering a novel framework for optimizing medicinal licorice quality.

Conclusions

This study demonstrates that rhizome diameter serves as a critical developmental determinant of glycyrrhizin accumulation in *Glycyrrhiza glabra*, with medium-sized rhizomes (1–1.5 cm) exhibiting maximal metabolite levels and upregulated expression of biosynthetic genes (LUS, BAS, CYP88D6, CYP72A154, UGTs, SQS). Strong correlations between gene expression and glycyrrhizin content highlight coordinated pathway regulation, independent of CAS-mediated sterol branching. These insights reveal morphological maturity as a key regulator of triterpenoid saponin production, providing a basis for

targeted harvesting and breeding to improve pharmaceutical yields while supporting sustainable utilization of this economically vital species.

Declarations

CRediT authorship contribution statement

A.K.: Investigation, Methodology, Data curation, Writing original draft, Software, Formal analysis. **M.Z.:** Conceptualization, Supervision, Review and editing, Validation, Project administration. **K.S.:** Review and editing, Visualization, Methodology, Project administration.

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Data availability

Not applicable.

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Figures

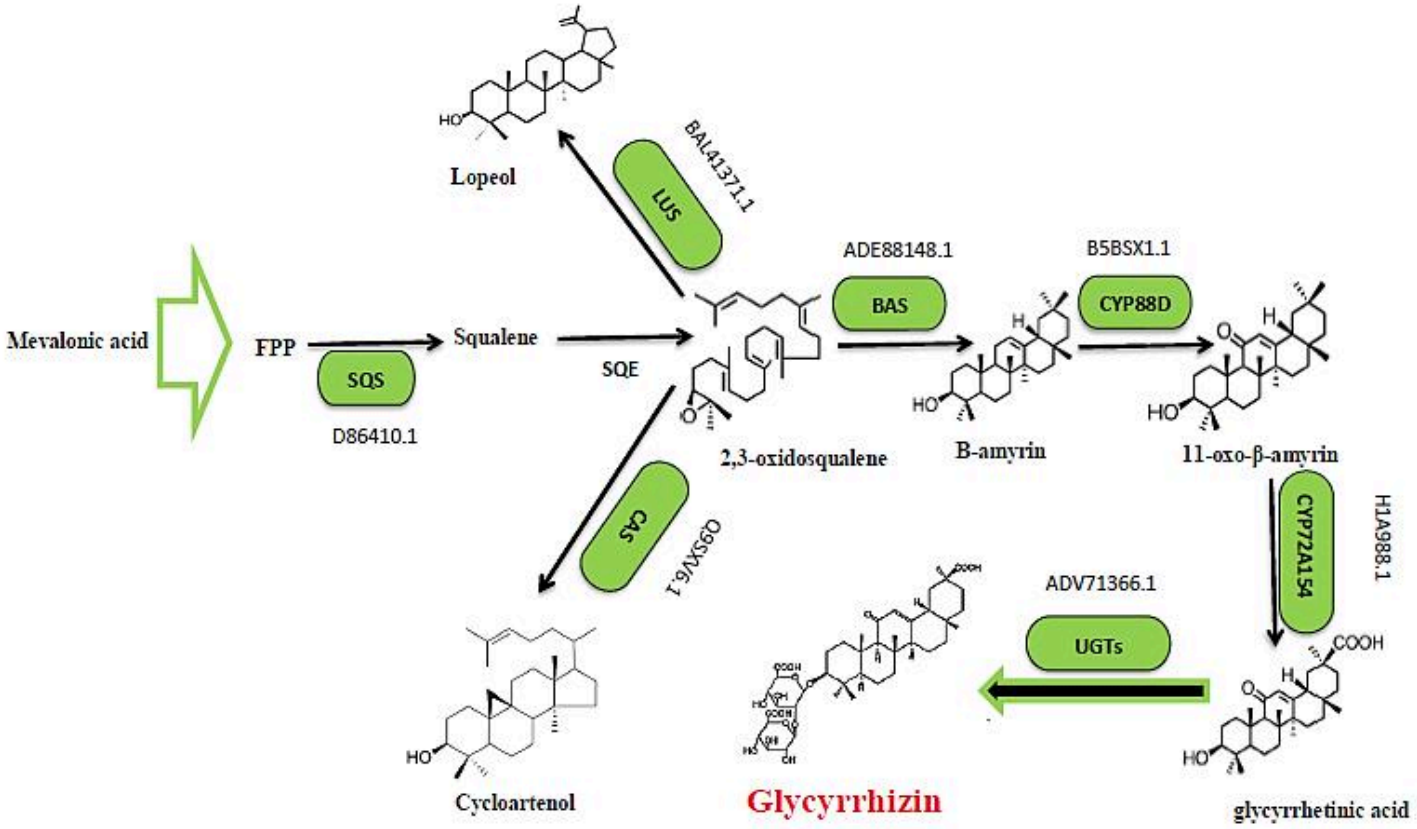


Figure 1

Schematic representation of the glycyrrhizin biosynthetic pathway in Glycyrrhiza glabra. Key enzymes analyzed in this study are highlighted in bold: SQS (squalene synthase), LUS (lupeol synthase), BAS (β-amyrin synthase), CAS (cycloartenol synthase), CYP88D6 (β-amyrin 11-oxidase), CYP72A154 (11-oxo-β-amyrin 30-oxidase), and UGTs (UDP-glycosyltransferases responsible for the final glycosylation steps).

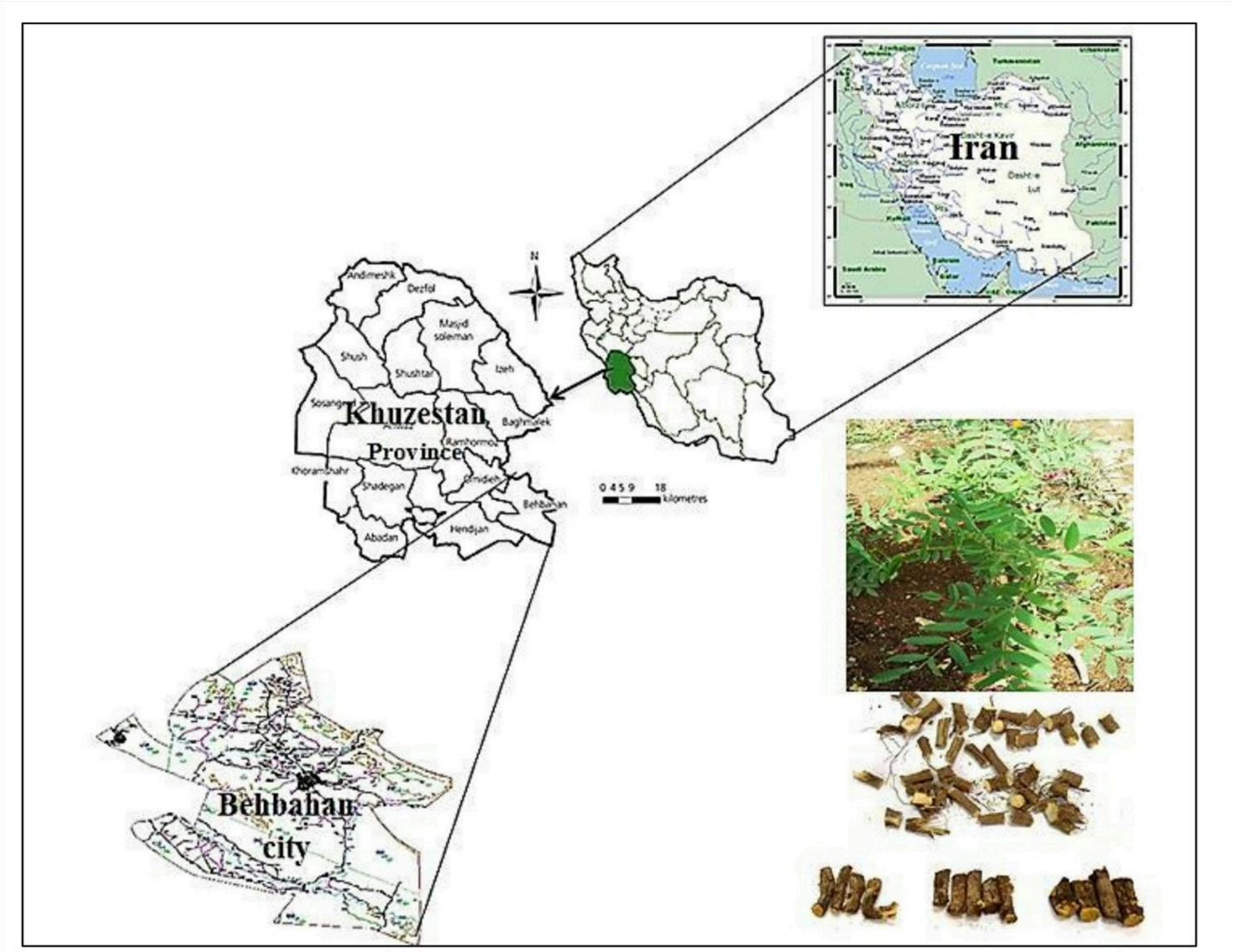


Figure 2

Collection site and rhizome samples of Glycyrrhiza glabra L. The map shows the location of the sampling area in Mansuriyeh, Behbehan, Khuzestan Province, Iran (30°35'37"N, 50°14'28"E; 318 m a.s.l.). Rhizomes were harvested from a single wild population to ensure genetic and environmental uniformity. Insets show the natural habitat and representative rhizomes classified into three diameter classes: 0.5 cm, 1.0–1.5 cm, and 2 cm.

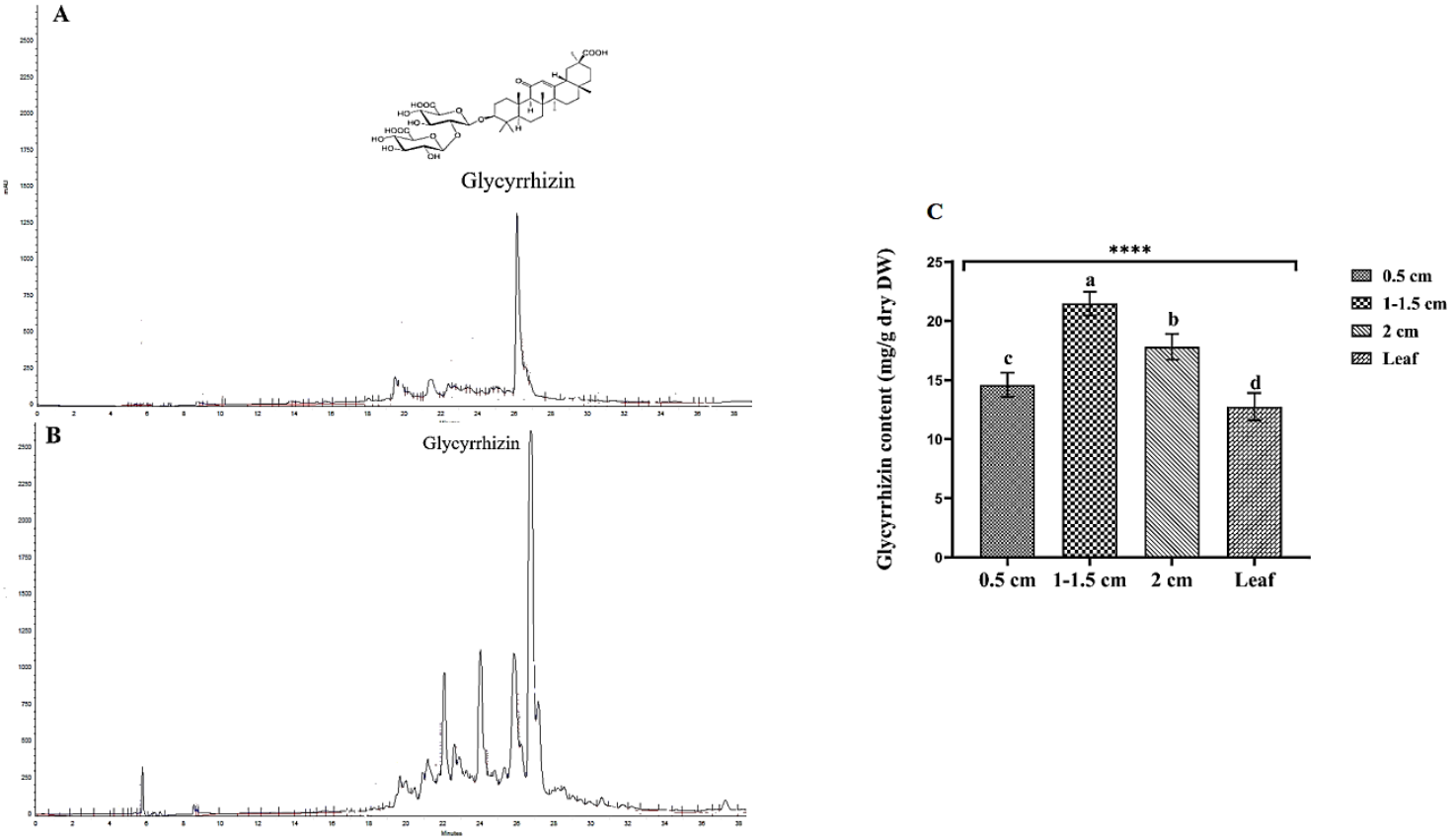


Figure 3

(A) HPLC chromatogram of a standard solution of glycyrrhizin; (B) Representative HPLC chromatogram (observed at λ 254 nm); (C) Glycyrrhizin content in *Glycyrrhiza glabra* rhizomes of different diameters (0.5 cm, 1–1.5 cm, and 2 cm), and in the leaves. Bars represent mean ± standard error (SE) from three biological replicates. Different lowercase letters (a–d) above bars indicate significant differences (Duncan test, $p < 0.05$). **** denotes overall significance ($p < 0.0001$).

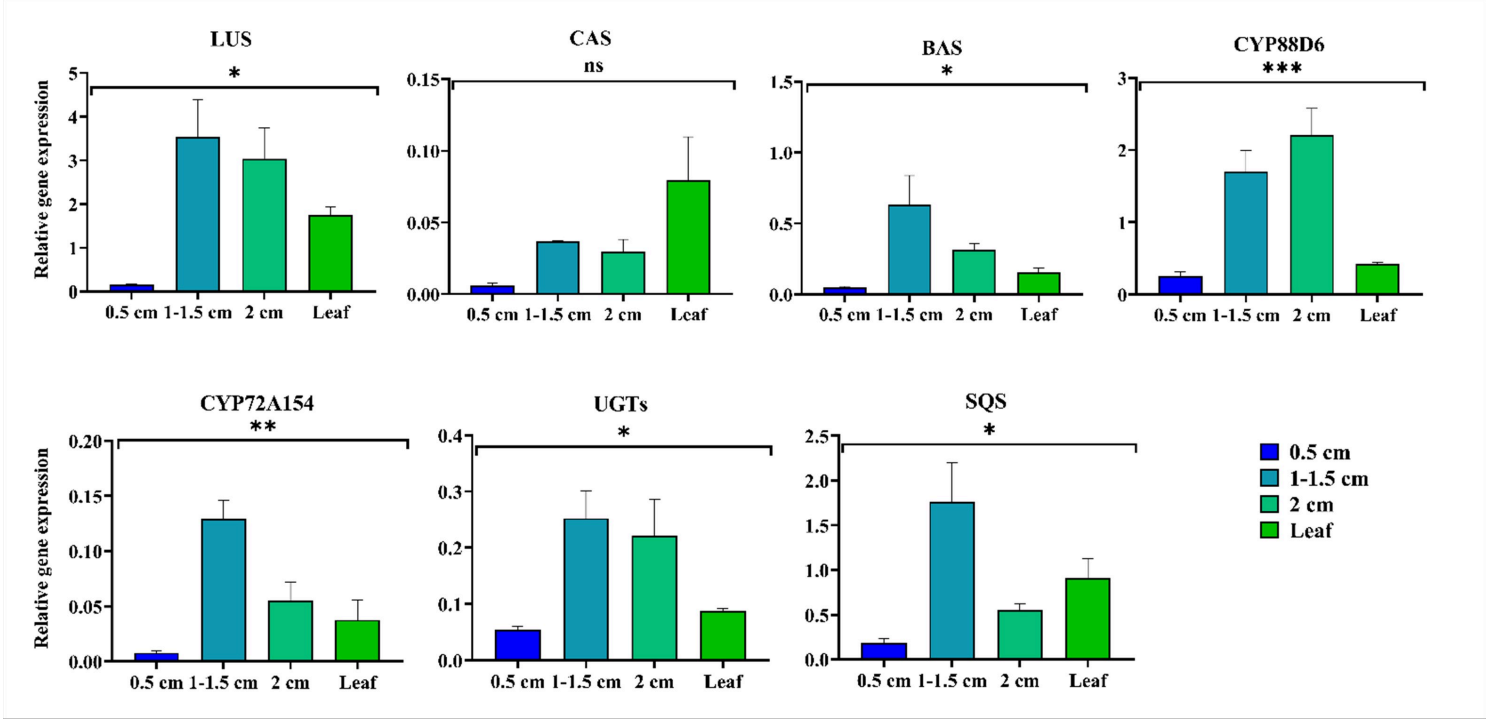


Figure 4

Relative expression levels of key genes involved in glycyrrhizin biosynthesis in rhizomes of *Glycyrrhiza glabra* with different diameters and in leaves. SQS, squalene synthase; LUS, lupeol synthase; BAS, β-amyrin synthase; CAS, cycloartenol synthase; CYP88D6, β-amyrin 11-oxidase; CYP72A154, 11-oxo-β-amyrin 30-oxidase; UGTs, UDP-glycosyltransferases (UGT73F3 and UGT72AY1). Vertical bars represent mean ± SE of three independent biological replicates. *, **, ***, and ns indicate significance at probability levels of $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and non-significant, respectively.

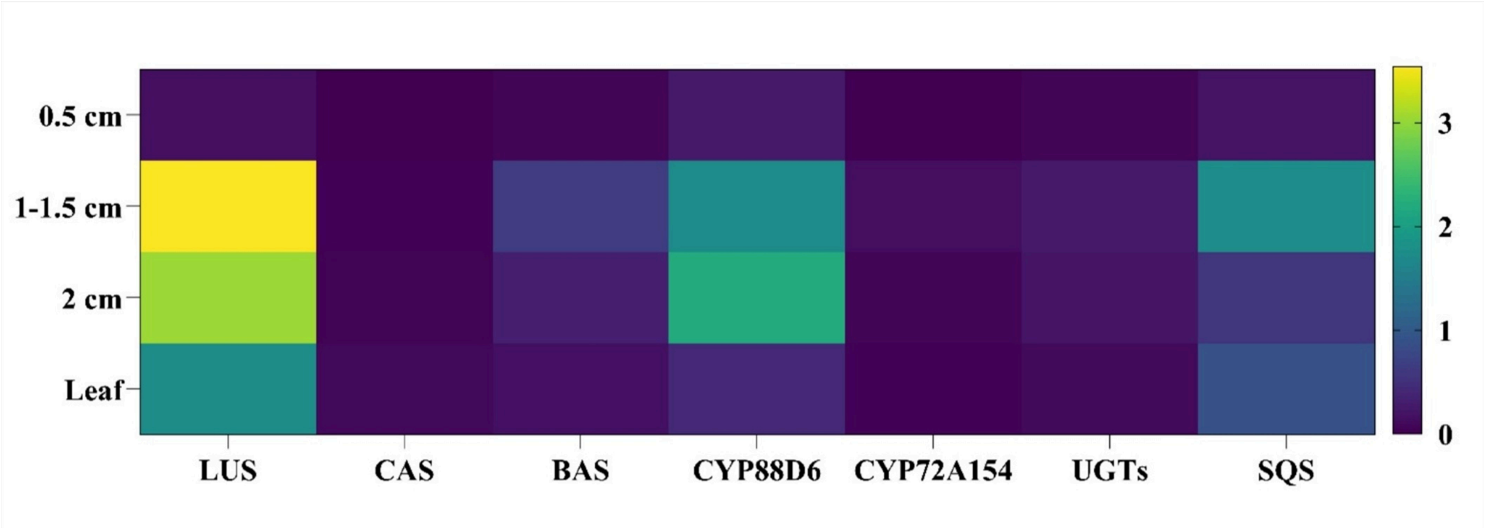


Figure 5

Heatmap summarizing the relative expression profiles of genes involved in glycyrrhizin biosynthesis in Glycyrrhiza glabra. Samples include leaves and rhizomes of three diameter classes (0.5 cm, 1–1.5 cm,

and 2 cm). Genes analyzed: SQS (squalene synthase), LUS (lupeol synthase), BAS (β-amyrin synthase), CAS (cycloartenol synthase), CYP88D6 (β-amyrin 11-oxidase), CYP72A154 (11-oxo-β-amyrin 30-oxidase), UGT73F3, and UGT72AY1 (UDP-glycosyltransferases). Color scale ranges from blue (lowest expression) to yellow (highest expression).

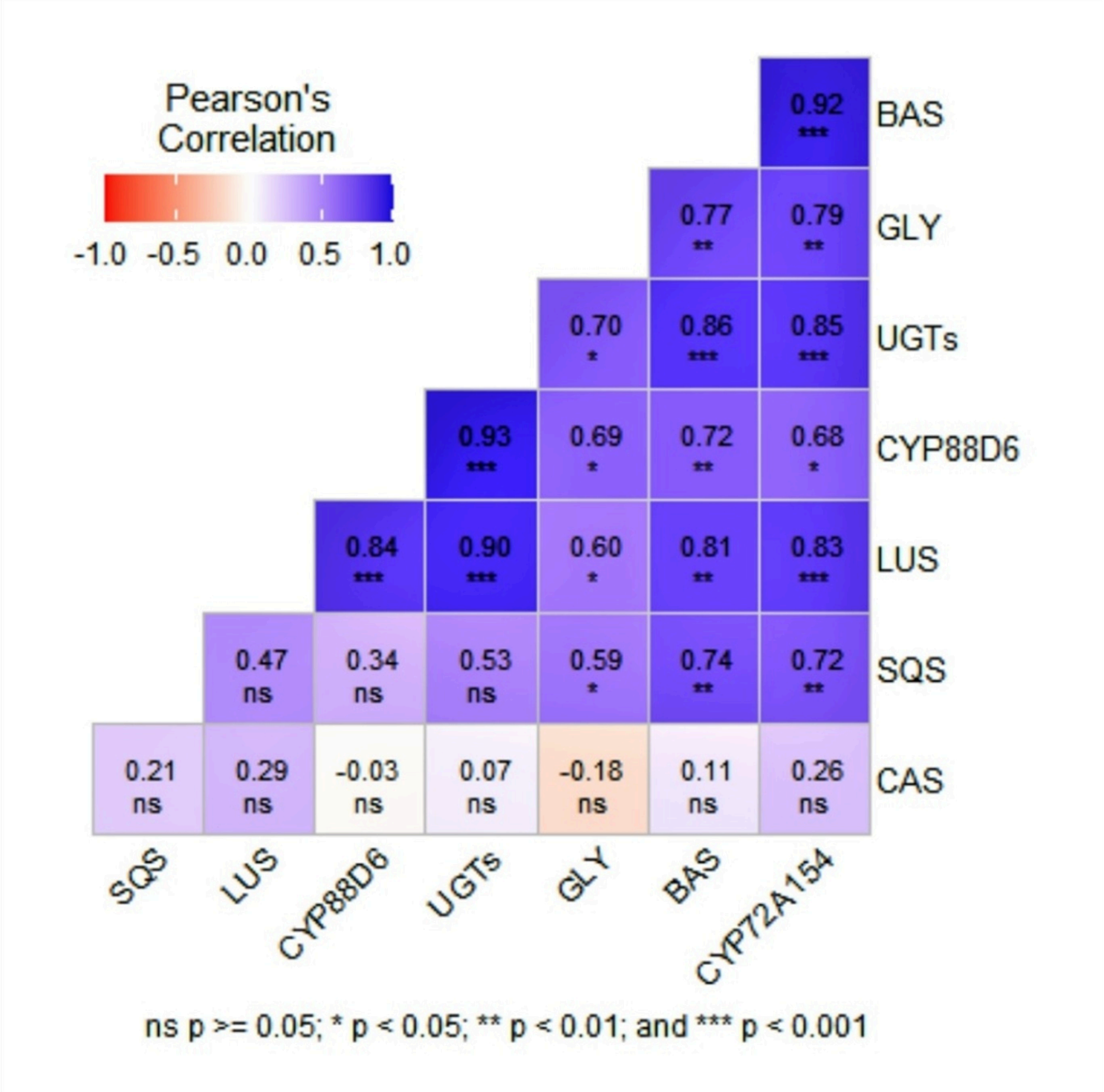


Figure 6

Heatmap of Pearson's correlation coefficients between glycyrrhizin content (GLY) and relative expression levels of biosynthetic genes in *Glycyrrhiza glabra* rhizomes and leaves. Color scale ranges from blue

(strong negative correlation, −1.0) to red (strong positive correlation, +1.0). SQS (squalene synthase), LUS (lupeol synthase), CAS (cycloartenol synthase), BAS (β-amyrin synthase), CYP88D6 (β-amyrin 11-oxidase), CYP72A154 (11-oxo-β-amyrin 30-oxidase), UGTs (UDP-glycosyltransferases).