***Pak. J. Bot.*, 58(9) September 2026.**

ARTICLE **DOI: http://dx.doi.org/10.30848/PJB2026-9(5)**

IDENTIFICATION AND VALIDATION OF RELIABLE REFERENCE GENES IN *SAPOSHNIKOVIA DIVARICATA* UNDER LOW-TEMPERATURE STRESS

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Abstract

Based on transcriptomic data, this study screened and validated reference genes in *Saposhnikovia divaricate* under low-temperature stress. Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were conducted, combined with computer software tools such as geNorm, NormFinder, Best Keeper, Delta Ct, and Ref Finder, to identify stably expressed reference genes in the leaves, stems, and roots of *S. divaricata*. The reliability of the selected reference genes was verified by analyzing the expression changes of the sucrose phosphate synthase (SPS) gene under low-temperature conditions. A total of 8 candidate reference genes were screened in this study. The results showed that 18SrRNA-2 exhibited the most stable expression in roots and stems, while 18SrRNA-1 showed the most stable expression in leaves. These findings are of great significance for subsequent studies on changes in the content of active substances in *S. divaricate* under abiotic stress, as they can improve the standardization of qRT-PCR experiments and provide support for research related to gene expression in *S. divaricata*.

Key words: *S. divaricate*; Low-temperature stress; qRT-PCR; Reference gene validation; Cold stress

Introduction

The perennial herbaceous plant *Saposhnikovia divaricate* (Turcz.) Schischk (*S. divaricata*) belongs to the *Apiaceae* family and grows in temperate regions of China, Korea, Japan, and Russia. The roots of this plant contains its principal medicinal ingredients and known for relieving symptoms such as colds, wind, and pain. According to traditional Chinese medicine utilization model, it can dispel wind, alleviate symptoms, and prevent spasms by affecting the channels of the bladder, liver, and spleen (Wang *et al*., 2024). The main active compounds of *S. divaricata* were recognized as flavonoids, coumarins, volatile oils, and polysaccharides. Particularly, the flavonoids such as rutin and quercetin showed powerful antioxidant and anti-inflammatory functions (Gao *et al*., 2023). As the main active ingredients in *S. divaricata*, coumarin compounds exert anti-inflammatory, antiviral, and antibacterial effects (Li *et al*., 2023). Several important volatile oils, such as limonene and pinene, contributed to the antibacterial and anti-inflammatory properties (Yang *et al*., 2020). Moreover, the sterols and polysaccharides in *S. divaricata* were immunomodulatory, demonstrating potential anticarcinogenic properties (Jiang *et al*., 2010). Polysaccharides can improve immune responses by activating macrophages and lymphocytes, indicating their potential utilization in treating immune-related diseases (Randeni & Xu, 2024). Additionally, its antioxidant properties enhance its neuroprotective effects, reducing oxidative stress damage to neurons and offering protection against neurodegenerative diseases. The crucial compounds may moderate the symptoms of illnesses for instance Alzheimer's disease by dampening neuroinflammation and oxidative stress (Meng *et al*., 2021). Both traditional medicine and modern pharmacology benefit from medicinal plants because their bioactive ingredients have significant therapeutic effects. In recent years, the exploitation of molecular biology techniques has made it possible to discover how medicinal plants produce their medicinal components and their biosynthetic mechanisms (Su *et al*., 2023). In order to make these studies more refined and comparable, reference genes must be selected and validated(Bunde *et al*., 2024).

The reference genes need to be used for gene expression studies in order to standardize and correct experimental data, especially with techniques such as quantitative PCR (qPCR). In recent studies, ACTIN, EFIA, GAPDH, and UBQ, which have been customarily utilized as reference genes, may become unstable under certain circumstances, prompting researchers to confirm new reference genes through various screening and validation strategies (Tang *et al*., 2023; Zhang *et al*., 2024). Zhang employed transcriptome sequencing technology to investigate genes linked to bolting and flowering in *S. divaricata*, utilizing the stable TRINITY_DN21702_c2_g1 gene as a reference. This study highlighted the major critical in selecting applicable reference genes for gene expression investigation, particularly given that the steadiness of reference genes could be induced by variable environmental conditions (M. Zhang *et al*., 2023). The selection of reference genes indicates the importance of understanding metabolic mechanisms in *Codonopsis pilosula*, where both traditional and novel reference genes have been assessed (Liang *et al*., 2020). Additionally, Qu selected and validated *Isatis indigotica* reference genes under different stress by using the IiCCR gene (Qu *et al*., 2019). In recent literatures, meaningful advances have been discovered in the study of reference genes of GAPDH, EFIA, 18SrRNA, and UBQ in plants. GAPDH is a commonly used reference gene on account of its committed expression across different plant tissues. Studies indicate that its expression can be exaggerated

Received: 04-09-2025 Revised: 17-03-2026 Accepted: 25-03-2026 Online: 24-04-2026

by certain experimental conditions, requiring careful selection in specific experiments (Tong *et al*., 2009). Elongation factor 1-alpha (EFIA) is another widely used reference gene whose expression levels are generally stable across plant species and tissues, although environmental factors can sometimes affect its expression (Chao *et al*., 2016). 18S ribosomal RNA is one of the most reliable reference genes in cells owing to its abundance and stability. However, its abundance may obfuscate variations in the expression of low-abundance genes (Kuchipudi *et al*., 2012). Ubiquitin (UBQ) family members are also taken as reference genes in plants. While UBQ gene expression is usually stable under various experimental conditions, different UBQ family members may display distinct expression patterns, making it critical to choose the appropriate UBQ gene(Hu *et al*., 2009).

In addition, transcriptomic methods can help elucidate the secondary metabolic pathways of medicinal plants, particularly the biosynthesis of coumarins and flavonoids. Researchers have identified key enzyme coding genes in these pathways by studying gene expression differences under different treatment conditions (Chen *et al*., 2020; Gao *et al*., 2023). The functional validation of these genes can aid in understanding the biosynthetic mechanisms of medicinal components in plants, such as sucrose phosphate synthase (*SPS*), which is an important enzyme involved in carbohydrate metabolism and acts as an vital function in sucrose synthesis, the main transport form of sugar in plants (Tanvir *et al*., 2024). Sucrose synthesis and accumulation are fundamental for plant growth, development, energy storage, and response to environmental stresses (Winter & Huber, 2000; Lao *et al*., 2025). Despite significant improvements in reference gene research in medicinal plants, many areas remain unexplored. Future research could focus on developing cross-species reference genes, identifying stress-specific reference genes, and studying the dynamic regulation of reference genes (Jin *et al*., 2024; Quintieri *et al*., 2024). These explorations will further unlock the medicinal potential of plants, providing important scientific grounds for the modernization of traditional Chinese medicine and new drug development.

This study utilizes existing *S. divaricata* transcriptomic data to select candidate genes such as GAPDH, EFIA, 18S rRNA, and UBQ. It employs RT-qPCR experiments and software packages such as geNorm, NormFinder, Best Keeper, Delta Ct, and RefFinder to screen for stably expressed reference genes in different tissues under cold temperature. The dependability of the selected reference genes was validated based on analyzing changes in the expression of the SPS gene under low temperature, offering reliable reference genes for future investigations on changes in the active substance content of *S. divaricata* under cold stress.

Materials and Methods

Plant materials and treatment: The wild seeds of *Saposhnikovia divaricata* used in this experiment were collected from the Great Khingan Mountains on August 5, 2023 (specific collection coordinates: 121°12′–127°00′ E, 50°10′–53°33′ N). The collection and use of this plant complied with all relevant regulations, and the plant samples were deposited in the Traditional Chinese Medicine Herbarium of Qiqihar Medical University (voucher number: QMU172024935).

The seeds were cultivated at the Astragalus Industry Research Institute of Qiqihar Medical University. Forty-eight days after germination, the plants were transferred to growth chambers for low-temperature stress treatment (maintained at 4°C for 72 consecutive hours). The photoperiod was set to 14 hours of light/10 hours of dark, with an illumination intensity of 80% and a humidity of 55%. Except for temperature, all other environmental conditions were consistent between the low-temperature stress group and the control group.

For the low-temperature stress group, leaves, stems, and roots were collected after 72 hours of treatment at 4°C. For the control group, plants were cultured under standard conditions (25°C), and untreated leaves, stems, and roots were collected. Each treatment included 20 plants, and three biological replicates were set for each tissue sample.

RNA extraction and candidate reference gene screening: The RNA was isolated from samples using TRIzol reagent (Beijing Tiangen Biotechnological Co., Ltd.) following the manufacturer's guidelines. Quantification of RNA absorbance and purity was performed using a Shimadzu BioSpec-Nano spectrophotometer, ensuring that the A260/A280 absorbance ratio ranged from 1.9 to 2.1 and the A260/A230 value was equal to or greater than 2.0. The first strand of cDNA was synthesized using FastKing One-Step Genomic DNA Dispelling and cDNA Synthesis Premix Reagent (TIANGEN KR118) according to the manufacturer's instructions. cDNA samples were diluted to a concentration of 100 ng/μL with RNase-free water, used as qRT-PCR templates, and stored at -80°C for further research. Eight candidate reference genes were identified based on transcriptomic data of Saposhnikovia divaricata (sequence data confirming the findings of this study has been submitted to NCBI with the primary accession number GSE276339). Primers for these genes were designed using Primer Premier 5.0 (Table 1). The primers were synthesized by Sangon Biotech Co., Ltd. and subsequently diluted with purified water to a concentration of 10 μmol/L. The total volume of the qRT-PCR reaction system was 20 μL, with the following amounts of each component: 10 μL of 2×FastKing SYBR Green qPCR Mix, 0.8 μL of forward primer (10 μmol/L), 0.8 μL of reverse primer (10 μmol/L), 2 μL of cDNA template (100 ng/μL), and 6.4 μL of RNase-free water. The reaction program was set as follows: pre-denaturation at 95°C for 3 minutes; followed by 40 cycles (denaturation at 95°C for 5 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds); for the melting curve analysis stage, the temperature was increased from 65°C to 90°C with a 1°C increase every 5 seconds. After amplification, the software automatically analyzed the amplification, exported Ct values, and calculated the amplification efficiency (E), correlation coefficient (R^2), and average Ct value for each candidate reference gene.

Real-time PCR analysis and amplification efficiency verification: The synthesized cDNA products were diluted in a fivefold gradient, and each qRT-PCR was performed across five such gradients, with each reaction set in triplicate. Real-time fluorescence quantitative analysis was carried out utilizing the TIANGEN FastKing One-Step qRT-PCR Kit (SYBR Green, FP313) based on the protocol. The cycle threshold (Ct) values for the candidate genes were measured across five different template gradients. The reaction protocol was outlined as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s. The melting curve analysis was carried out ranging from 65°C to 90°C at 5-second intervals. Each reaction was repeated three times. After amplification, the software automatically analysed the amplification conditions, exported Ct values, and calculated the amplification efficiency (E), correlation (R^2), and average Ct for each candidate gene.

Stability analysis and verification of candidate reference genes: To recognize the most stable reference genes in leaves, stems, and roots of *S. divaricata* under low-temperature condition, we used the geNorm, NormFinder, BestKeeper, and Delta Ct tools based on Ct values from different tissues. A comprehensive analysis was conducted via the online RefFinder tool (http://blooge.cn/RefFinder/) to decide the best reference genes for each tissue under cold stress. The stability of these chosen reference genes was checked by analysing the expression of *SPS*, an important enzyme that plays a regulatory function in the response of *S. divaricata* to cold stress. The expression behaviors of *SPS* in various tissues under low-temperature stress were normalized via both the most stable and least stable reference genes, as recommended by RefFinder. Relative expression value of the target genes were calculated via the $2^{-\Delta\Delta CT}$ approach, with each biological sample examined three times.

Table 1. Candidate reference genes and primer information

Gene abbreviation	Primer sequence(5'→3')	Amplicon size (bp)	Tm °C	%GC
GAPDH-1	GGGAGCAAGGCAGTTGGTAGTG	122	61.2	59.1
	GAAGGGAGGTGCGAAGAAGGTTATC		59.7	52.0
GAPDH-2	CGGTGAGATCAACCACTGAGACATC	132	59.6	52.0
	TTCAACATCATCCCTAAGCAGCACTG		59.2	48.0
EF1A-1	GTCATCAAGAGCGTGGAGAAGAAGG	94	59.8	52.0
	ACACCGATACCCTCCAACACCTC		61.3	56.5
EF1A-2	GCTCACGAGTTTGCCCATCCTTA	95	59.8	52.2
	ACTGGAACTTCTCAGGCTGATTGTG		59.1	48.0
18SrRNA-1	TACTACGGTGGTGACGGGTGAC	90	61.4	59.1
	CCTGCTGCCTTCCTTGGATGTG		61.5	59.1
18SrRNA-2	TCGTTCGTCTGACTTGGGTATAGGG	88	60.2	52.0
	GTACTTCCATTGGTGCCAGCTATCC		60.0	52.0
18SrRNA-3	CCGTGCCGTCCTACCTATTTGAAG	138	60.1	54.2
	GCTGGTTCCCTCCGAAGTTTCC		61.2	59.1
UBQ	CACCACGGAGACGAAGCACAAG	83	61.3	59.1
	GCAGTTGGAAGATGGCAGGACTC		60.9	56.5

Table 2. Primer correlation and amplification efficiency table for the 8 candidate genes.

Gene abbreviation	*E* (%)	R^2	k	C_t span
GAPDH-1	106.76	0.9911	-3.17	24.15~25.55
GAPDH-2	103.09	0.9940	-3.25	23.19~24.93
EF1A-1	92.71	0.9907	-3.51	21.77~24.08
EF1A-2	104.44	0.9973	-3.22	22.89~25.22
18SrRNA-1	90.60	0.9982	-3.57	17.89~22.18
18SrRNA-2	107.23	0.9925	-3.16	22.70~26.33
18SrRNA-3	96.45	0.9939	-3.41	18.46~24.46
UBQ	103.54	0.9970	-3.24	21.30~21.95

Results

Determination of primer accuracy and amplification efficiency: We evaluated the primer specificity of eight candidate reference genes to assure the reliability of our data analysis using qRT-PCR. On the basis of the melting curve results, each primer pair showed one single peak, pointing high specificity and exclusive target gene amplification, which are key requirements for qRT-PCR primers (Fig. 1). Furthermore, all reference genes presented correlation coefficients (R^2) higher than 0.99, indicating good linearity. The amplification efficiency ranged from 92.71% to 107.23% in the roots with a mean Ct ranging from 19.84 to 27.68. In the stems, the efficiency ranged from 100.50% to 103.98% with a mean Ct ranging from 19.38 to 26.08. And the efficiency ranged from 93.07% to 112.23%, with Ct ranging from 17.44 to 21.69 in the leaves. These findings confirmed that all primer pairs met the requirements for qRT-PCR (Table 2).

Expression abundance analysis of candidate reference genes: The cycle threshold (Ct) values from qRT-PCR reflect gene expression levels, where lower Ct values indicate higher expression. We analyzed the Ct values of the eight candidate genes across the roots, stems, and leaves of *S. divaricata*. The average Ct values ranged from 18.83 to 25.12, with 18SrRNA-1 exhibiting the highest expression and 18SrRNA-2 showed the lowest. The box plot analysis illustrates the stability of these candidate genes, and the narrower box widths indicate less variability, indicating greater stability (Fig. 2). For example, 18SrRNA-1 presented the shortest box plot range, suggesting that it is a stable reference gene across these tissues, whereas EF1A-1 presented the greatest variability. To further standardize the experimental data, the Ct values were also investigated via geNorm, NormFinder and BestKeeper, with comprehensive evaluations conducted via RefFinder. The asterisks represent outliers.

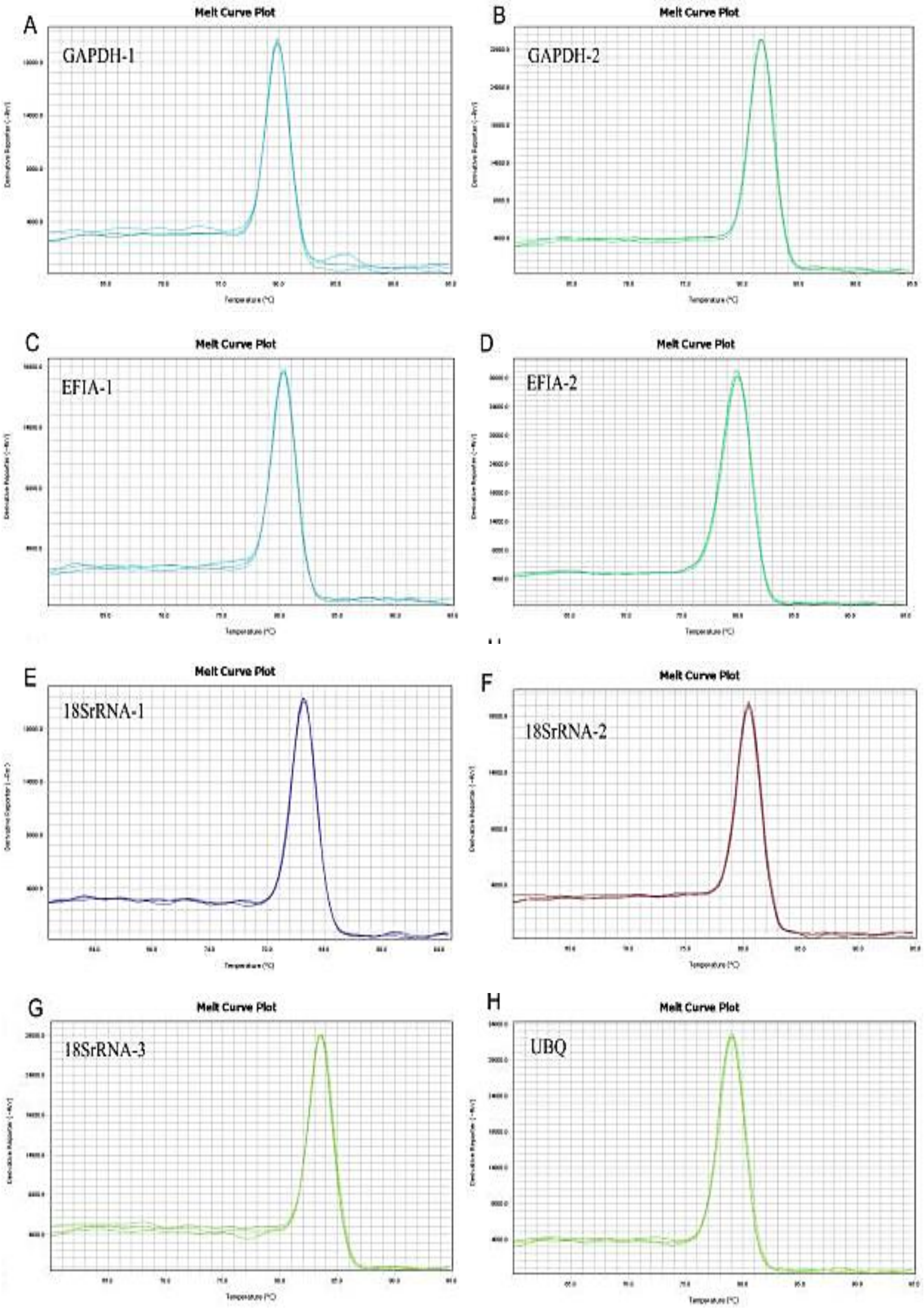

Fig. 1. Melting curves of eight candidate reference genes in *S. divaricata* under cold stress: (A=GAPDH-1; B=GAPDH-2; C=EFIA-1; D=EFIA-2; E=18SrRNA-1; F=18SrRNA-2; G=18SrRNA-3; H=UBQ).

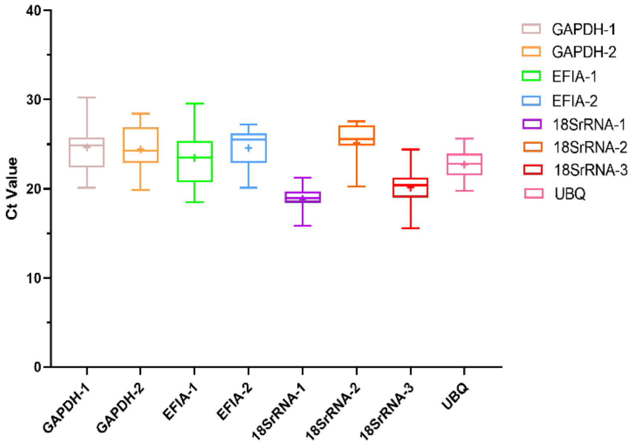

Fig. 2. Cycle threshold value (Ct) of eight candidate reference genes across all samples. The box chart indicates the interquartile range. The outer box represents the 25th to 75th percentiles, and the inner box represents the mean values. The lower and upper dashes depict the minimum and maximum values, respectively. The line across the box is the median.

Stability analysis of candidate reference genes: To classify the optimal reference genes under cold stress in *S. divaricata* leaves, stems, and roots, we utilized tools such as geNorm, NormFinder, BestKeeper, and Delta Ct, integrating all these values with an online RefFinder analysis.

The geNorm calculates an expression maintenance M value for each candidate gene, where a lower M value indicates greater stability. During cold stress, the gene stability ranked as follows in roots: 18SrRNA-2 > EF1A-2 > UBQ > GAPDH-2 > GAPDH-1 > EF1A-1 > 18SrRNA-1 > 18SrRNA-3. In stems, the ranking result was 18SrRNA-2 > EF1A-1 > 18SrRNA-1 > 18SrRNA-3 > GAPDH-1 > EF1A-2 > GAPDH-2 > UBQ. For leaves, the order was 18SrRNA-2 > UBQ > EF1A-2 > 18SrRNA-1 > GAPDH-2 > GAPDH-1 > 18SrRNA-3 > EF1A-1 (Table 3).

NormFinder utilizes intra-group and inter-group variance to calculate stability values, where lower values indicate greater stability. The genes in the roots were ranked as follows: 18SrRNA-2 > EF1A-2 > UBQ > GAPDH-2 > GAPDH-1 > EF1A-1 > 18SrRNA-1 > 18SrRNA-3. In stems, the order was 18SrRNA-3 > 18SrRNA-1 > 18SrRNA-2 > EF1A-1 > GAPDH-1 > EF1A-2 > GAPDH-2 > UBQ. For the leaves, the ranking was GAPDH-2 > 18SrRNA-1 > GAPDH-1 > EF1A-2 > 18SrRNA-2 > UBQ > 18SrRNA-3 > EF1A-1 (Table 4).

BestKeeper assesses stability through the Ct standard deviation (SD) and coefficient of variation (CV); smaller values denote greater stability. The rankings were as follows in the roots, EF1A-2 > 18SrRNA-2 > UBQ > 18SrRNA-1 > GAPDH-2 > 18SrRNA-3 > GAPDH-1 > EF1A-1. In stems, the order was 18SrRNA-3 > 18SrRNA-1 > 18SrRNA-2 > EF1A-1 > GAPDH-2 > GAPDH-1 > UBQ > EF1A-2. For the leaves, the ranking was 18SrRNA-1 > GAPDH-2 > EF1A-2 > 18SrRNA-2 > UBQ > GAPDH-1 > 18SrRNA-3 > EF1A-1 (Table 5).

The Delta Ct method evaluates stability through ΔCt calculations, and smaller ΔCt values indicate greater stability. For the roots, the ranking was UBQ > 18SrRNA-2 > EF1A-2 > GAPDH-2 > GAPDH-1 > EF1A-1 > 18SrRNA-1 > 18SrRNA-3. For the stems was 18SrRNA-2 > EF1A-1 > 18SrRNA-3 > 18SrRNA-1 > GAPDH-1 > EF1A-2 > GAPDH-2 > UBQ. For the leaves, it showed as 18SrRNA-1 > GAPDH-2 > EF1A-2 > GAPDH-1 > 18SrRNA-2 > UBQ > 18SrRNA-3 > EF1A-1 (Table 6).

Using RefFinder, we calculated the geometric mean of the stability rankings of the geNorm, NormFinder, BestKeeper, and Delta Ct analyses to derive the comprehensive stability index. A lower index indicates more stable gene expression. For the roots under cold stress, the most stable genes were 18SrRNA-2 > EF1A-2 > UBQ > GAPDH-2 > GAPDH-1 > 18SrRNA-1 > EF1A-1 > 18SrRNA-3; in the stems, 18SrRNA-2 > 18SrRNA-3 > 18SrRNA-1 > EF1A-1 > GAPDH-1 > GAPDH-2 > EF1A-2 > UBQ; in the leaves, 18SrRNA-1 > GAPDH-2 > 18SrRNA-2 > EF1A-2 > UBQ > GAPDH-1 > 18SrRNA-3 > EF1A-1. These results indicate that 18SrRNA-2 was the most stable reference gene in the roots and stems, whereas 18SrRNA-1 was the best in leaves under cold stress (Table 7)..

Table 3. Expression stability values (M) of the eight candidate reference genes as evaluated by geNorm.

Ranking	Root		Stem		Leaf	
	Gene ID	**values (M)**	**Gene ID**	**values (M)**	**Gene ID**	**Values (M)**
1	*18SrRNA-2*	0.245	*18SrRNA-2*	0.162	*18SrRNA-2*	0.161
	EF1A-2	0.245	*EF1A-1*	0.162	*UBQ*	0.161
2	*UBQ*	0.564	*18SrRNA-1*	0.657	*EF1A-2*	0.219
3	*GAPDH-2*	0.711	*18SrRNA-3*	0.737	*18SrRNA-1*	0.431
4	*GAPDH-1*	0.982	*GAPDH-1*	0.92	*GAPDH-2*	0.634
5	*EF1A-1*	1.157	*EF1A-2*	1.105	*GAPDH-1*	0.86
6	*18SrRNA-1*	1.721	*GAPDH-2*	1.626	*18SrRNA-3*	1.169
7	*18SrRNA-3*	2.176	*UBQ*	1.896	*EF1A-1*	1.457

Table 4. Expression stability of the eight candidate reference genes as calculated by NormFinder.

Ranking	Root		Stem		Leaf	
	Gene ID	**values (M)**	**Gene ID**	**values (M)**	**Gene ID**	**Values (M)**
1	*18SrRNA-2*	0.122	*18SrRNA-3*	0.286	*GAPDH-2*	0.237
2	*EF1A-2*	0.122	*18SrRNA-1*	0.286	*18SrRNA-1*	0.257
3	*UBQ*	0.252	*18SrRNA-2*	0.368	*GAPDH-1*	0.408
4	*GAPDH-2*	1.045	*EF1A-1*	0.562	*EF1A-2*	0.983
5	*GAPDH-1*	1.786	*GAPDH-1*	1.53	*18SrRNA-2*	1.2
6	*EF1A-1*	2.338	*EF1A-2*	2.173	*UBQ*	1.37
7	*18SrRNA-1*	2.474	*GAPDH-2*	2.417	*18SrRNA-3*	1.528
8	*18SrRNA-3*	3.498	*UBQ*	2.629	*EF1A-1*	2.256

Table 5. Expression stability of the eight candidate reference genes as calculated by BestKeeper.

Ranking	Root			Stem			Leaf		
	Gene ID	SD	CV	Gene ID	SD	CV	Gene ID	SD	CV
1	*EF1A-2*	0.527	2.05	*18SrRNA-3*	0.246	1.18	*18SrRNA-1*	0.095	0.51
2	*18SrRNA-2*	0.631	2.36	*18SrRNA-1*	0.432	2.23	*GAPDH-2*	0.401	1.94
3	*UBQ*	1.228	5.41	*18SrRNA-2*	0.931	3.57	*EF1A-2*	0.45	2.08
4	*18SrRNA-1*	1.382	6.96	*EF1A-1*	1.011	4.65	*18SrRNA-2*	0.605	3.21
5	*GAPDH-2*	1.677	6.31	*GAPDH-2*	1.623	6.44	*UBQ*	0.735	3.69
6	*18SrRNA-3*	2.166	9.75	*GAPDH-1*	1.662	6.92	*GAPDH-1*	0.914	4.24
7	*GAPDH-1*	2.184	7.89	*UBQ*	1.775	7.47	*18SrRNA-3*	1.575	9.03
8	*EF1A-1*	2.599	9.76	*EF1A-2*	2.143	8.56	*EF1A-1*	2.235	9.72

Table 6. Expression stability of the eight candidate reference genes as calculated by Delta Ct.

Ranking	Root		Stem		Leaf	
	Gene ID	SD	Gene ID	SD	Gene ID	SD
1	*UBQ*	1.587	*18SrRNA-2*	1.403	*18SrRNA-1*	1.107
2	*18SrRNA-2*	1.608	*EF1A-1*	1.437	*GAPDH-2*	1.11
3	*EF1A-2*	1.632	*18SrRNA-3*	1.486	*EF1A-2*	1.238
4	*GAPDH-2*	1.728	*18SrRNA-1*	1.513	*GAPDH-1*	1.283
5	*GAPDH-1*	2.061	*GAPDH-1*	1.812	*18SrRNA-2*	1.337
6	*EF1A-1*	2.444	*EF1A-2*	2.257	*UBQ*	1.454
7	*18SrRNA-1*	2.807	*GAPDH-2*	2.554	*18SrRNA-3*	1.806
8	*18SrRNA-3*	3.544	*UBQ*	2.708	*EF1A-1*	2.32

Table 7. Expression stability of the eight candidate reference genes based on the RefFinder analysis.

Ranking	Root		Stem		Leaf	
	Gene ID	Geometric average	Gene ID	Geometric average	Gene ID	Geometric average
1	*18SrRNA-2*	1.414	*18SrRNA-2*	1.732	*18SrRNA-1*	1.682
2	*EF1A-2*	1.565	*18SrRNA-3*	2.213	*GAPDH-2*	2.115
3	*UBQ*	2.28	*18SrRNA-1*	2.213	*18SrRNA-2*	3.162
4	*GAPDH-2*	4.229	*EF1A-1*	2.378	*EF1A-2*	3.224
5	*GAPDH-1*	5.439	*GAPDH-1*	5.233	*UBQ*	3.663
6	*18SrRNA-1*	6.086	*GAPDH-2*	6.435	*GAPDH-1*	4.559
7	*EF1A-1*	6.447	*EF1A-2*	6.447	*18SrRNA-3*	7
8	*18SrRNA-3*	7.445	*UBQ*	7.737	*EF1A-1*	8

Validation of reference gene reliability: As an essential enzyme in sucrose synthesis, sucrose phosphate synthase may play a significant regulatory role in the response of *S. divaricata* under cold stress. Studying the role of *SPS* in cold adaptation may provide insights into the stress adaptation mechanisms of plants, supporting their rational use and cultivation. The validity of these selected reference genes was established based on assessing *SPS* expression under different conditions via qRT-PCR.

The primers for the *SPS* gene were as follows:
Forward: 5'-GGAGTTAGCCCTTGATGCCTCTG-3'
Reverse: 5'-TTCCGCCTGATTCTTGCTCGTAG-3'

The *SPS* fragments per kilobase of transcript per million mapped reads (FPKM) values obtained from the *S. divaricata* samples under cold stress at 0, 6, and 48 hours were shown in Fig. 3A. The expression results of the most stable gene 18SrRNA-2, the least stable gene 18SrRNA-3 and *SPS* were analyzed under cold stress (Fig. 3B and C). The results confirmed the consistency between the 18SrRNA-2-normalized expression and transcriptomic data, whereas the 18SrRNA-3-normalized data showed large discrepancies. In the stems, *SPS* gene expression analysis based on the most stable reference gene 18SrRNA-2 was consistent with the sequencing results, whereas the results for UBQ, the least stable reference gene, did not match under cold stress (Fig. 3D, E). With regard to the leaves, SPS expression analysis was conducted using the most stable reference gene 18SrRNA-1 in conjunction with sequencing data. In contrast, the utilization of EF1A-1 led to significantly inconsistent results (Fig. 3F, G). Overall, the most stable reference genes were confirmed to be 18SrRNA-1 for leaves and 18SrRNA-2 for both roots and stems under cold stress.

Discussion

S. divaricata is a significant medicinal plant generally used to treat ailments such as the common cold and rheumatism. In recent years, technological advancements have significantly improved *S. divaricata* research. To better understand its pharmacological effects and how its active ingredients are synthesized, researchers have investigated its molecular biology and biochemistry (Batsukh *et al.*, 2021). Cold temperature showed as one key environmental factors significantly affecting medicinal plant growth, development, and secondary metabolism. In addition to damaging their physiological processes, cold can also modify the content and composition of their medicinal compounds. Therefore, improving the healing value of medicinal plants such as *S. divaricata* requires study of the consequences of cold (Strzemski & Dresler, 2024).

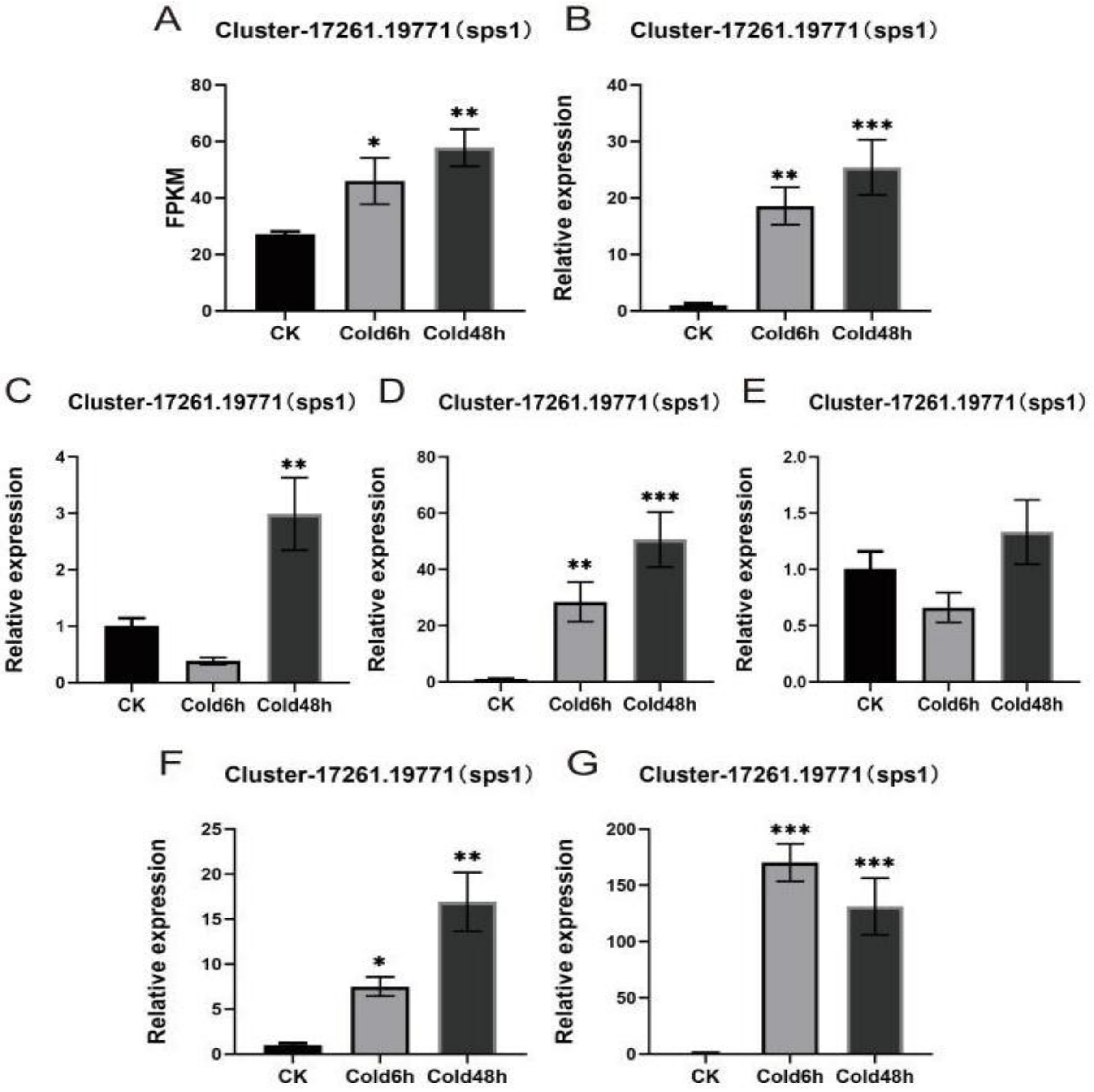

Fig. 3. Relative expression of sps1 using the selected reference genes. The results were normalized to the selected stable reference genes. The data were analysed via one-way ANOVA followed by Dunnett's multiple comparison test using Graphpad 9.0 software. The values are expressed as the means ± SEMs. *<0.05, **< 0.01 and ***<0.001 were considered statistically significant compared with the 0h control group.

Transcriptome has provided new perspectives for the study of medicinal plants, allowing researchers to explore the complicated biochemical pathways that govern the production of particular metabolites. These metabolites are vital for the therapeutic properties of many plants, and understanding their biosynthesis can lead to the development of new drugs and improved medicinal products. Recent progress in transcriptomic technologies, such as high-throughput sequencing, has allowed for a more inclusive research of gene expression in different plant, including those traditionally ignored in metabolic studies (De Luca *et al*., 2012). Moreover, the combination of transcriptomics with other omics methods, such as metabolomics and proteomics, has transformed our understanding of plant biochemistry. For example, studies have shown that systems biology approaches can clarify unknown steps in specialized metabolite pathways, revealing the complicated relationships between genes and the metabolites they are involved in creating (Schilmiller *et al*., 2012) This interdisciplinary method not only improves our knowledge of metabolic pathways but also simplifies the engineering of these pathways in both plant and microbial systems, eventually enhancing the creation of existing medicines and providing lots of biologically functional compounds for screening (D'Amelia *et al*., 2021).

Notably, the relative analysis of wild and cultivated medicinal plants through transcriptomics approaches has emphasized noteworthy differences in metabolite accumulation and gene expression. For example, research on *Ophiocordyceps sinensis* has revealed separate metabolic profiles between wild and cultivated varieties, with awareness of the mechanisms that regulate over these differences (Zhang *et al*., 2023). These findings emphasize the potential of transcriptomics to inform cultivation practices and improve the quality of medicinal plants. It is necessary to choose and validate reference genes to confirm the accuracy and comparability of gene expression data in these studies. Reference genes are conclusive for normalizing and correcting experimental values, notably in techniques such as quantitative PCR (qPCR). In some situations, traditional reference genes such as ACTIN, EF1A, GAPDH, and UBQ may not be stable. For example, in a study of *Kobresia littledalei*, while ACTIN and GAPDH were found to be normally stable, their expression levels varied significantly across different tissues and under various abiotic stresses (Sun *et al*., 2024). Moreover, in *Oenanthe javanica*, the assessment of ten candidate reference genes revealed that TBP and UBQ were the most stable genes at different developmental stages, whereas GAPDH was recognized as the most unstable gene. This finding highlights the necessity of validating reference genes for specific experimental frameworks rather than defaulting to traditional selections (Feng *et al*., 2023). Furthermore, in *Aegilops tauschii* (Coss.), the analysis of transcriptome data resulted in the classification of several stable reference genes, suggesting that confidence in traditional reference genes without validation can lead to imprecise results (Abbas *et al*., 2021).

Therefore, confirming the accuracy of qRT-PCR analyses by validating reference genes under particular investigational conditions is vital. In our research, we assessed eight reference genes, including GAPDH, EF1A, 18SrRNA, UBQ, and their variants, which display fluctuating stability in different tissues under low temperature. In addition to their high abundance and stability in cells, 18SrRNA plays an important role in normalizing gene expression. However, this high abundance can sometimes mask modifications in low-abundance genes (Zhu *et al*., 2021). UBQ gene expression usually remains stable under different experimental conditions, but each UBQ member might display a different form of expression, thus making it vital to identify an appropriate UBQ gene (Wu *et al*., 2021). There are several remaining challenges in reference gene research despite considerable development, including a lack of systematic screening and validation of empirical selections. Additionally, the constancy of reference genes can be considerably altered under different investigational conditions, requiring context-specific screening and validation (Wang *et al*., 2023). The qRT-PCR results revealed high amplification efficiency of the eight reference genes in different *S. divaricata* tissues under cold stress. These findings confirm the reliability of these primers for further stability analysis of these chosen reference genes.

The tools normally utilized for reference gene selection are geNorm, NormFinder, BestKeeper, Delta Ct, and RefFinder. GeNorm estimates M values for each candidate gene; smaller values indicate better stability. NormFinder analyses variance, classifying the most stable reference genes. In BestKeeper, stability is investigated via standard deviations and coefficients of variation, whereas in Delta Ct, stability is determined by the difference between Ct values between the target gene and the candidate gene. RefFinder combines the results of these methods to present a complete resource for choosing stable reference genes (Shukla *et al*., 2019; Zhao *et al*., 2020). Nonetheless, each tool has its limitations. For example, geNorm and NormFinder may be prejudiced by high-abundance genes, and BestKeeper is sensitive to Ct value fluctuation. Therefore, specific experimental conditions should be considered when selecting and using these tools (Wang *et al*., 2025).

SPS is an important enzyme in plant carbohydrate metabolism that probably adjusts the tolerance of *S. divaricata* to cold stress. Calculating *SPS* gene expression levels can validate the stability and reliability of reference genes under cold conditions. The choose of applicable reference genes is vital for precise normalization in quantitative PCR studies, especially when investigating gene expression under different environmental stresses including cold. Recently, the stability of reference genes has been shown to change considerably depending on particular conditions and stresses. In soybean, the steadfastness of expression model of ten recommended reference genes was assessed for different biotic stresses, and the best reference genes were revealed to be stress dependent and expressed similarly under different conditions (Bansal *et al*., 2015). Similarly, in eggplant, spiking is used to select stable housekeeping genes under different biotic stresses, which explains the importance of estimating reference gene stability in various experimental settings (Barbierato *et al*., 2017). Moreover, the validation of reference genes is not solely applicable to biotic stresses, it is similarly important under abiotic stress conditions. Sugarcane key reference genes have been investigated for their stability in different tissues and photoperiodic treatments, emphasizing the significant function of using validated reference genes for reliable gene expression research (da Silva Santos *et al*., 2021). Therefore, by measuring *SPS* gene expression levels in combination with assessing the stability of reference genes under cold temperature, researchers can guarantee the reliability of their qRT-PCR data, ultimately leading to more precise findings on gene expression responses to environmental changes.

Conclusion

The precise assessment of gene expression via qRT-PCR demands the selection of applicable reference genes. The purpose of this study was to scientifically validate a series of candidate reference genes for normalizing gene expression in *S. divaricata* under cold temperature. The perpetuation of expression for 8 candidate genes was evaluated via four extensively used algorithms: geNorm, NormFinder, Delta Ct, and BestKeeper. RefFinder provided a full ranking, classifying 18SrRNA-1 as the most stable reference gene in leaves and 18SrRNA-2 as the most stable in roots and stems under cold stress. These findings were validated through qRT-PCR analysis of the *SPS* gene, which indicated that the chosen reference genes significantly minimized quantification errors. The outcomes of this work will improve the accuracy of qRT-PCR normalization and enable further research on gene expression in *S. divaricata*.

Authors' Contributions: Hui Li and Baoguo He were responsible for experimental design, data analysis, and manuscript composition. Zun Lyu, Jiajia Chen, and Yue Pan undertook tasks related to plant sample cultivation, sample collection, RNA extraction, and qRT-PCR experiments. Ming Jiang and Keyong Zhang were in charge of project topic selection planning and guidance on manuscript writing.

Declaration of Competing Interest: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Ethical Approval: This article does not include any studies with human participants or animals performed by any of the authors.All authors have read and agreed to the journal's publication ethics guidelines.

Acknowledgements

This work was supported by the Academy Project of Qiqihar Medical University (no. QMSI2021M-13).

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