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Article

Reference Gene Selection for qRT-PCR Normalization in *Iris germanica* L.

Yinjie Wang, Yongxia Zhang, Qingquan Liu, Liangqin Liu, Suzhen Huang and Haiyan Yuan*

Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, 210014, China
*Corresponding Author: Haiyan Yuan. Email: yuanhaiyan416@163.com
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Abstract: Quantitative real-time PCR (qPCR) is an effective and widely used method to analyze expression patterns of target genes. Selection of stable reference genes is a prerequisite for accurate normalization of target gene expression by qRT-PCR. In *Iris germanica* L., no studies have yet been published regarding the evaluation of potential reference genes. In this study, nine candidate reference genes were assessed at different flower developmental stages and in different tissues by four different algorithms (GeNorm, NormFinder, BestKeeper, and RefFinder). The results revealed that *ACT11* (Actin 11) and *EF1α* (Elongation factor 1 alpha) were the most stable reference genes in different tissues, whereas *TUA* (Tubulin alpha) and *UBC9* (Ubiquitin-protein ligase 9) were the most stable ones in different flower developmental stages. *UBC9* and *ACT11* were the most stable reference genes in all of the tested samples, while the *SAMDC* (S-Adenosylmethionine decarboxylase) showed the least stability. Finally, to validate the suitability of the selected reference genes, the relative expression level of *IgTPS* (beta-caryophyllene synthase) was assessed and highlighted the importance of suitable reference gene selection. This work constitutes the first systematic evaluation of potential reference genes in *I. germanica* and provides guidelines for future research on gene function and molecular mechanisms on *I. germanica* and related species.

Keywords: *Iris germanica* L; reference genes; floral scent; qRT-PCR

1 Introduction

Quantitative real time PCR (qRT-PCR) is a reliable technique for quantifying target gene expression. It is the most commonly used technique for studying the expression of genes because of its increased sensitivity, specificity, and accuracy and high throughput compared with reverse transcription PCR (RT-PCR) and Northern blot [1]. However, the accuracy of qRT-PCR is dependent on the stability of the reference genes employed, the RNA integrity and purity, the enzymatic efficiency in cDNA synthesis, and the efficacy of PCR amplification [2]. Therefore, it is necessary to select reliable reference genes to normalize the relative expression of target genes.

A number of common reference genes, such as actin (*ACT*), ubiquitin (*UBQ*), beta-tubulin-4 (*TUB4*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and elongation factor 1 alpha (*EF1α*), involved in primary metabolism or other basic cellular processes have been widely adopted for gene expression

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analyses in different tissues or organs [3–7]. However, recent studies have found that some of these genes are not consistently expressed in different plant species, tissues and developmental stages and various experimental conditions [8,9]. Therefore, an increasing number of studies have focused on the identification and evaluation of potential reference genes under various experimental conditions in different plant species, including cineraria (*Senecio cruentus* Masson ex L' Herit) [10], rose (*Rosa hybrida*) [11], *Chrysanthemum lavandulifolium* [12], tree peony (*Paeonia suffruticosa* Andr.) [13], *Rhododendron molle* [14], lettuce (*Lactuca sativa*) [15], seashore paspalum (*Paspalum vaginatum*) [2] and poplar (*Populus alba* × *Populus glandulosa*) [9].

Iris, a kind of perennial herbaceous plants, consists of approximately 300 species distributed in North America, North Africa and Eurasia [16,17]. Many of them are popular ornamental plants due to their diversity of flower color, and range of volatile organic compounds (VOCs) [18,19]. *Iris germanica* L., belonging to the family Iridaceae, is the best known and most horticulturally important type of bearded iris, and its distinctive floral fragrance makes this species a good model for floral scent research in the *Iris* genus. Recently, chemical components of the floral scent in *I. germanica* have been reported [20], although the molecular mechanisms of floral scent have not yet been described. Revealing the molecular mechanisms of floral scent production requires clarifying the expression pattern of crucial genes involved in floral development. To obtain accurate expression data, a set of reference genes are essential as normalization factors. However, no systematic analysis of suitable reference genes in *I. germanica* has been conducted. Therefore, evaluation of the stability of candidate reference genes in *I. germanica* flower developmental stages and different tissues is necessary.

In this study, based on homolog comparisons between *Iris lactea var. chinensis* transcriptome data and *Arabidopsis* microarray data, 9 candidate reference genes, including *EF1α*, *GAPDH*, *ACT11*, *UBQ*, Ubiquitin-protein ligase 9 (*UBC9*), S-Adenosylmethionine decarboxylase (*SAMDC*), Phosphoglycerate kinase (*PGK*), Tubulin alpha (*TUA*), Protein phosphatase 2A *(PP2A*) were identified. Four different algorithms, GeNorm [21], BestKeeper [22], NormFinder [23], and Ref-Finder (http://www.leonxie.com/referencegene.php) were used to evaluate the most suitable reference genes for normalization. To verify the suitability of the selected reference genes in *I. germanica*, we assayed the relative expression levels of *IgTPS* in different floral development stages and tissues; this gene encodes the beta-caryophyllene synthase, an important enzyme in the terpenoid biosynthesis pathway [24]. This work provides a valuable resource for reference gene selection for qRT-PCR normalization for future gene expression studies in *I. germanica*.

2 Materials and Methods

2.1 Plant Materials

Iris germanica L. plants were obtained from the *Iris* Resource Collection Garden of Institute of Botany, Nanjing Sun Yat-Sen Memorial Botanical Garden. Self-selected cultivars 'Huangjinjia', derived from the crossbreeding of the female parent '93E41076-8' and the male parent 'Jinwuwa', were used as the plant materials. Three different tissues, including roots, stems, and leaves were collected, frozen immediately in liquid nitrogen and stored at −80°C freezer prior to use. Samples of flowers at four developmental stages were collected: Stage 1 (S1), flower bud and bracts are not open. Stage 2 (S2), flower bud has a cleft, and bracts are opening. Stage 3 (S3), flower bud is open, and the tips of florets are visible. Stage 4 (S4), flower is fully open (Fig. 1).

2.2 RNA Extraction and cDNA Synthesis

Total RNA from all samples was extracted using the RNA simple Total kit (TaKaRa Dalian, China) according to the manufacturer's instructions, and then treated with RNase-free DNase I (TaKaRa, Dalian, China) at 37°C for 30 min to eliminate potential DNA contamination. The concentration and integrity of

Figure 1: Four stages of *I. germanica* flower development. Scale bars = 1 cm

the total RNA were evaluated by the procedures described by Gu et al. [25]. The cDNA first strand was synthesized using 1 μg total RNA in a volume of 20 μl with the PrimeScript RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

2.3 PCR Primer Design

Nine candidate reference genes, including *EF1α*, *GAPDH*, *ACT11*, *UBQ*, *UBC9*, *SAMDC*, *PGK, TUA* and *PP2A* were selected from the transcriptome data sequences of *Iris lactea var. chinensis* [26]. All candidate reference genes were cloned and confirmed through sequencing. The gene sequences are stored in GenBank (Tab. 1). Primers were designed using Primer 5.0 software (Premier Biosoft International) to have melting temperatures between 55 and 65°C, primer lengths between 19 and 23 bp, and amplicon lengths between 81 and 280 bp (Tab. 1).

2.4 qRT-PCR Analysis

qPCR reactions were performed using a Mastercycler ep realplex 2 S device (Eppendorf, Germany) with S YBR® Premix Ex Taq™ II (TaKaRa, Dalian, China). Reactions with 20 μL mixture containing 5 μL of diluted cDNA, 0.6 μL of each amplification primer (10 μM), 10 μL of 2 × SYBR Premix and 3.8 μL of ddH_2O. The amplification program comprised an initial denaturation step (95°C for 2 min), followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and 72°C for 30 s, and a melting curve protocol (60–95°C with a temperature increment of 0.5°C s^{-1}). Each qRT-PCR reaction was performed in triplicate.

2.5 Statistical Analysis

PCR efficiency based on the raw fluorescence data was evaluated for each target with the software LinRegPCR program [27]. The stability of the nine potential reference genes were assessed with four programs, including GeNorm [21], NormFinder [23], BestKeeper [22], and Ref-finder (http://www.leonxie.com/referencegene.php). GeNorm, NormFinder and BestKeeper were used to analyze the stability of nine potential reference genes. Finally, Ref-Finder was used to sequence the reference genes and select the highest stability gene in different tissues and during four flower developmental stages. The top two ranked genes and the lowest ranked gene were used to calculate *IgTPS* gene expression to assess the effectiveness of the reference genes. Primers used to amplify *IgTPS* are presented in Tab. 1. The fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [28].

3 Results

3.1 Performance of Primers for Nine Reference Genes

A total of nine genes, including *EF1α*, *GAPDH*, *ACT11*, *UBQ*, *UBC9*, *SAMDC*, *PGK, TUA* and *PP2A*, were selected as candidate reference genes. The specificities of primers were confirmed by analyzing melting

Table 1: Primer sequences and characteristics of PCR amplifications in *I. germanica*

Name	Accession Number	Arabidopsis Homolog Locus	Primer Sequence (5′-3′) Forward/Reverse	Amplicon length (bp)	Primers Tm (°C)	Amplification Efficiency	R^2	Reference
Elongation factor 1 alpha (*EF1α*)	MT302549	At1G07940	5′- CCCTTGCTGGGTCATCTTTAG-3′ 5′-CCGACTGGTTTGACAACTGAA-3	163	59.1/ 58.4	100.31%	0.9996	[50]
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	MT302550	At2G24270	5′-TCCGCATAGCCCAAGATTC-3′ 5′-GCCAGCCACTTATGACGAGA-3′	81	58.4/ 58.5	102.17%	0.9992	[31]
Actin11 (*ACT11*)	MT302548	At2G31200	5′-AAGATTGATGAGAAGCGAAAGG-3′ 5′-GTTTTCAGGAAGAGCAGCCA-3′	93	58.2/ 58.6	98.87%	0.9984	[37]
Ubiquitin (*UBQ*)	MT302551	At4G05320	5′-CATCTTGTTCTCAGGCTCCG-3′ 5′-TTGTCCTGAATCTTTGCCTTAAC-3′	125	59.9/ 59.1	99.82%	0.9996	[48]
Ubiquitin-protein ligase 9 (*UBC9*)	MT302552	At5G53300	5′-CCGACAATCTCCCACAAACA-3′ 5′-GATGATCCGTTGGTTCCAGAG-3′	143	59.5/ 59.5	100.21%	0.9994	[38]
S-Adenosylmethionine decarboxylase (*SAMDC*)	MT302553	At4G01850	5′-CTGGTCGAGAGGGTTCTTAGC-3′ 5′-AACGAACCCATCCACATCAA-3′	120	58.3/ 58.2	99.39%	0.9995	[49]
Phosphoglycerate kinase (*PGK*)	MT302554	At1G56190	5′-ACGGTGCCAAAGTCATCCTC-3′ 5′-GCCTTCAGGAAGTGCAGCA-3′	176	58.4/ 58.0	100.19%	0.9994	[12]
Tubulin alpha (*TUA*)	MT302555	At4G14960	5′-GCCATCTTTGTGGACCTTGA-3′ 5′-AACGGCACTGAACACCAAGA-3′	231	58.1/ 58.6	98.77%	0.9989	[39]
Protein phosphatase 2A (*PP2A*)	MT302556	At1G10430	5′-AGCAGGTTTTTGGGTGAAGTTT-3′ 5′-ATTCCAGTACAGCTACGGGGTT-3′	212	59.9/ 59.7	104.71%	0.9992	[45]
Beta-caryophyllene synthase (*TPS*)	MT302557	At4G16740	5′-CCTCGGTACTCTACTGAAAGGTTAT-3′ 5′-TAGTTTGTATCCAGTGTTTGTCCAT-3′	188	58.2/ 58.0	99.87%	0.9989	[24]

curve assays. The presence of a single peak indicated that the expected amplicons were amplified. A single DNA band in each gel electrophoresis demonstrated that all nine primer pairs amplified a specific product with the expected size and no primer dimers were found. The correlation coefficients (R^2) values were ranged between 0.9984 and 0.9996, and PCR amplification efficiencies ranged between 0.9877 and 1.0471 (Tab. 1).

The quantification cycle (Cq) values of nine reference genes were assayed by qRT-PCR analysis (Fig. 2). Mean Cq values of all reference genes were between 19.84 and 24.87, which represented the different expression levels in *Iris germanica* L. *UBC9* showed the highest expression level and the lowest Cq value (19.84). *GAPDH* presented the lowest expression level and the highest Cq value.

3.2 Stability of Reference Genes

In our study, four software programs included GeNorm, NormFinder, BestKeeper and Ref-Finder were used to assess and rank the expression stability of each reference genes.

3.2.1 GeNorm Analysis

GeNorm software was used to rank the expression stability of the 9 candidate reference genes by calculating the average expression stability (*M*) values. Lower value of *M* indicates higher stability of the reference gene. Stably expressed genes have values below 1.5 [21]. Fig. 2 shows that the *M* values of all tested reference genes were less than 1.5, indicating that they all conformed to the basic requirements for the reference gene. *ACT11* and *UBQ* were the most stable reference genes in different tissues, while the least stable gene was *PGK*. In the different developmental stages of flowers, *UBC9* and *TUA* were the most stable reference genes, while *UBQ* was the least stable. In all of the samples, *ACT11* and *UBC9* were found to be the most stable genes, while *SAMDC* was found to be the least stable (Fig. 3).

The pairwise variation (*V*) between sequential ranked genes (V_n/V_{n+1}) based on the GeNorm algorithm was calculated to determine the optimal number of reference genes for normalization. A low level of

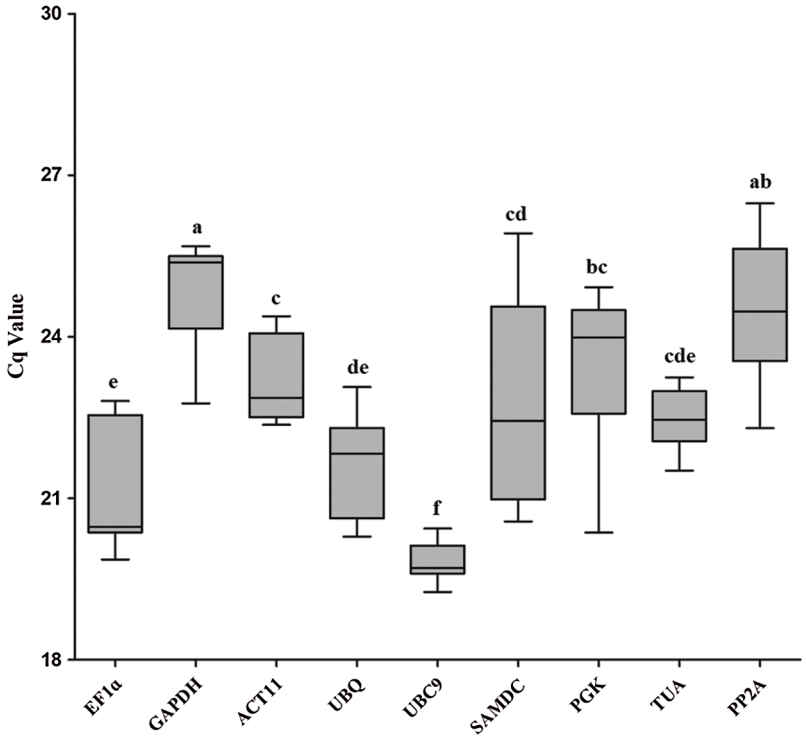

Figure 2: Cq values for 9 candidate reference genes in all samples of *I. germanica*. A line across the box-plot of Cq value indicates the median values. The outside boxes indicate the 25th and 75th percentiles. Whiskers represent the maximum and minimum values. Values are means ± s.d. (n = 7), $p < 0.05$ (student's t-test)

variation between $V_n/_{n+1}$ and V_{n+1}/V_{n+2} indicates no significant effects of the addition of another gene on the normalization, and a V value of 0.15 was used as the threshold to determine whether additional reference genes are necessary [21,29]. In our study, the $V_2/_3$ value in different tissues was below 0.15 (Fig. 4), which indicated that two reference genes are sufficient for accurate normalization. The $V_3/_4$ value of 0.103 for the four developmental stages of flowers indicated that three genes are necessary for normalization (Fig. 4). However, the value of 0.15 should not hold as an inflexible threshold, and higher cutoff values of V_n/n+1 have been used in several reports [30–32]. The low variation between $V_2/_3$ and $V_3/_4$ in all of the samples (Fig. 4) indicated that two reference genes are sufficient for normalization.

3.2.2 NormFinder Analysis

Evaluation values detected by the NormFinder algorithm are shown in Tab. 2, with lower values indicating higher stability [34]. *ACT11* and *UBQ* were ranked as the most stable genes in different tissues. *TUA* and *UBC9* were identified as the most stable reference genes in the four developmental stages of flower. According to NormFinder analysis, *UBC9* and *ACT11* performed as the most stable genes in all of the samples. The ranking order generated by this method was somewhat similar to that determined by GeNorm.

3.2.3 BestKeeper Analysis

The stability of 9 reference genes was also evaluated by BestKeeper algorithm based on SD and CV of the Cq values, with lower SD and CV representing higher stability [22]. Tab. 3 shows the results of the BestKeeper analysis. In different tissues, *TUA* and *ACT11* were identified as the most stable genes by

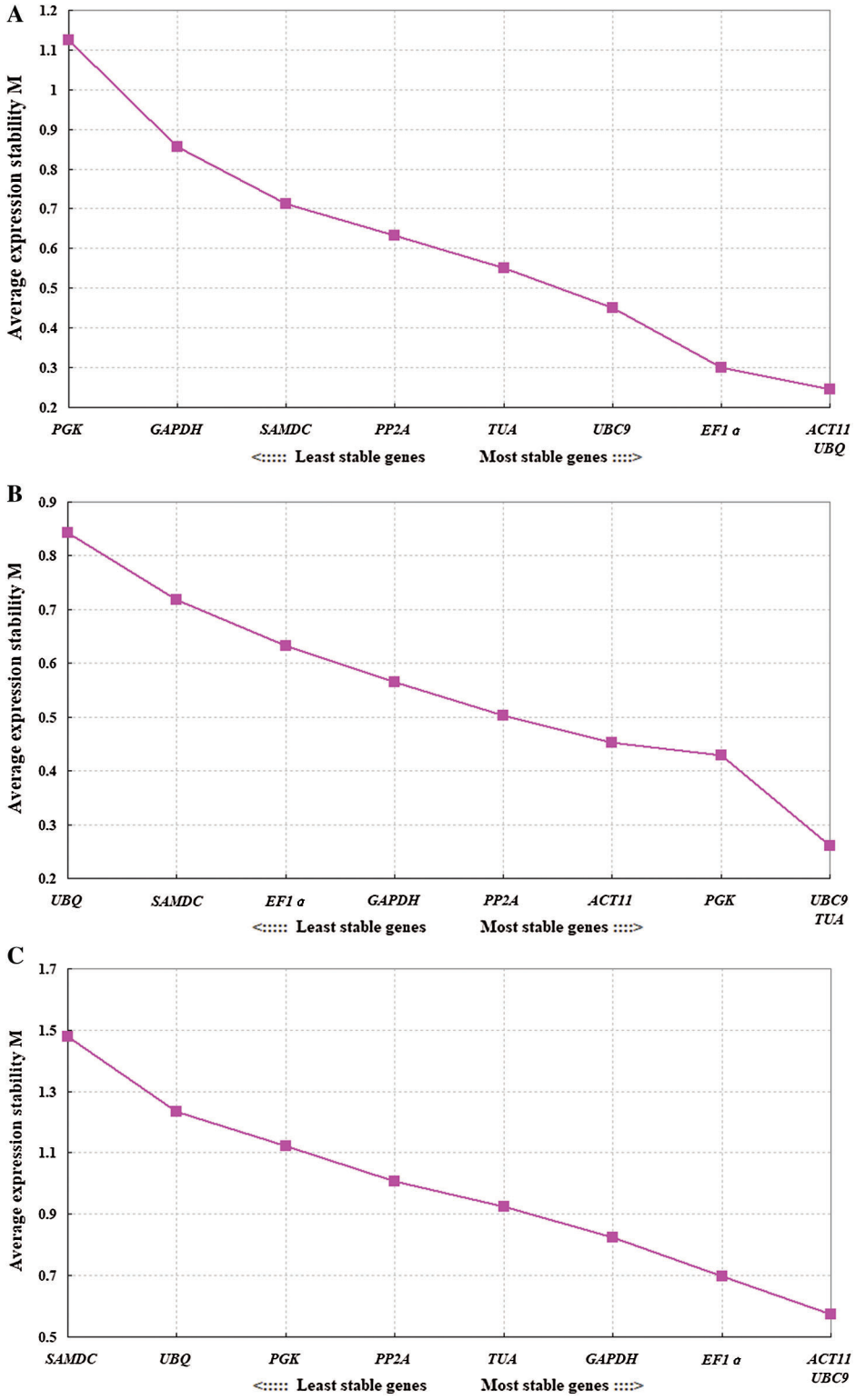

Figure 3: Average expression stability values (M) of 9 candidate reference genes as calculated by GeNorm software. The most stable genes are listed on the right, while the least stable genes are listed on the left. A: Tissues, B: Different developing flowers, C: Total

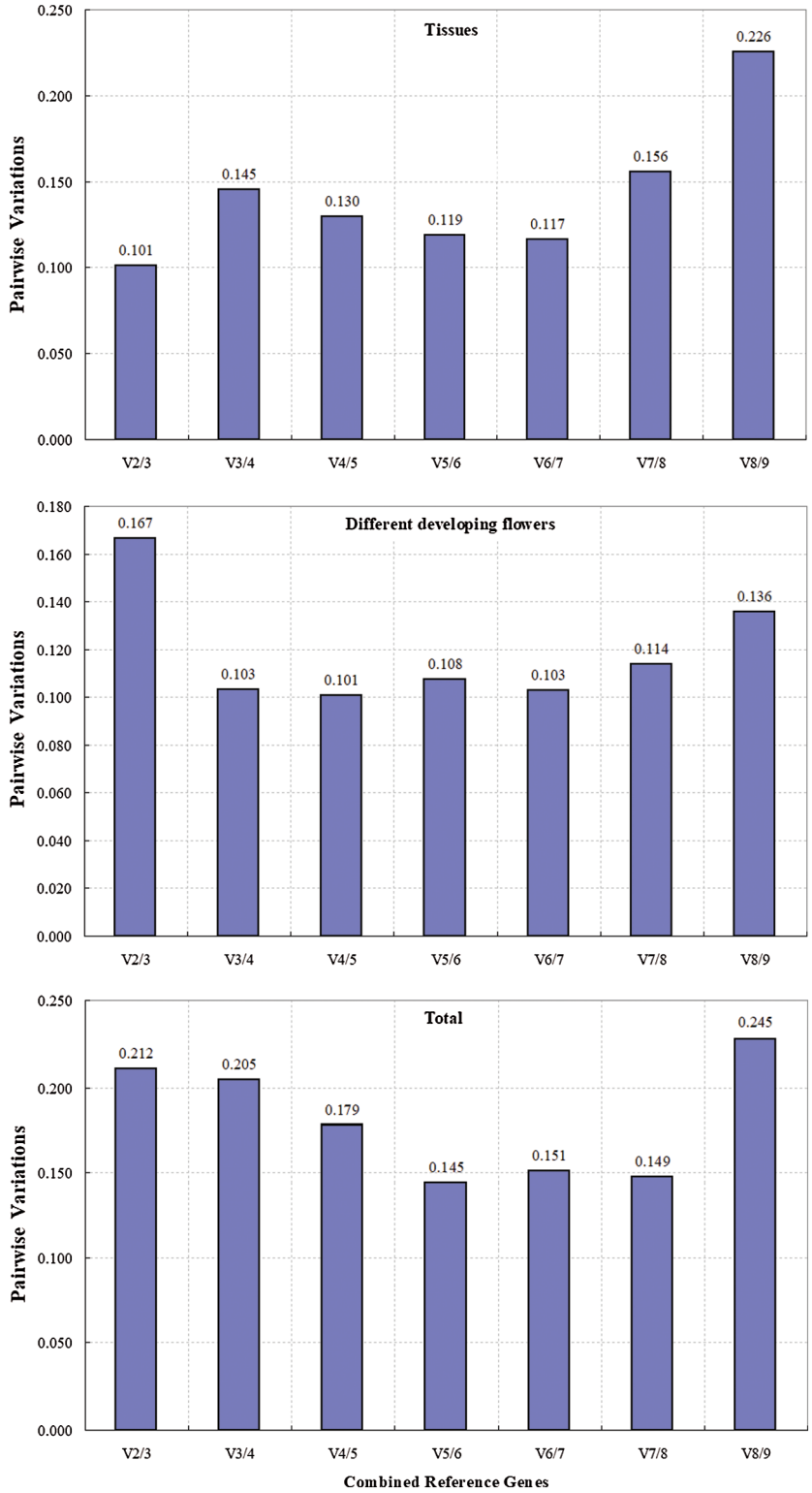

Figure 4: Pairwise variation (V) of 9 candidate reference genes were calculated using GeNorm. V_n/V_{n+1} value were used to determine the optimal number of reference genes

Table 2: Expression stability analysis of 9 candidate reference genes calculated using NormFinder software

Rank	Tissues		Different developing flowers		Total	
	Gene	Stability	Gene	Stability	Gene	Stability
1	*ACT11*	0.094	*TUA*	0.096	*UBC9*	0.206
2	*UBQ*	0.151	*UBC9*	0.129	*ACT11*	0.238
3	*EF1α*	0.161	*PP2A*	0.229	*TUA*	0.399
4	UBC9	0.205	*GAPDH*	0.322	*GAPDH*	0.637
5	*PP2A*	0.522	*PGK*	0.401	*EF1α*	0.720
6	*TUA*	0.574	*ACT11*	0.423	*UBQ*	0.808
7	*GAPDH*	0.756	*SAMDC*	0.577	*PP2A*	0.903
8	*SAMDC*	0.903	*EF1α*	0.700	*PGK*	0.969
9	*PGK*	1.623	*UBQ*	0.926	*SAMDC*	1.619

Table 3: Expression stability analysis of 9 candidate reference genes calculated using BestKeeper software

Rank	Tissues		Different developing flowers		Total	
	Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD
1	*TUA*	1.16 ± 0.26f	*TUA*	1.46 ± 0.33e	*TUA*	1.58 ± 0.36f
2	*ACT11*	1.65 ± 0.37f	*GAPDH*	2.18 ± 0.56d	*UBC9*	2.27 ± 0.46e
3	*PP2A*	1.74 ± 0.41f	*UBC9*	2.24 ± 0.45d	*ACT11*	2.39 ± 0.55e
4	*UBC9*	2.75 ± 0.56e	*ACT11*	2.63 ± 0.60cd	*GAPDH*	2.83 ± 0.72de
5	*UBQ*	3.63 ± 0.82d	*PP2A*	3.17 ± 0.78bc	*PP2A*	3.47 ± 0.84d
6	*GAPDH*	4.07 ± 1.02cd	*PGK*	3.53 ± 0.84b	*UBQ*	4.29 ± 0.94c
7	*SAMDC*	4.65 ± 1.12c	*UBQ*	5.15 ± 1.13a	*PGK*	4.45 ± 1.05c
8	*PGK*	5.39 ± 1.28b	*SAMDC*	5.43 ± 1.22a	*EF1α*	5.75 ± 1.24b
9	*EF1α*	6.38 ± 1.36a	*EF1α*	5.62 ± 1.24a	*SAMDC*	6.86 ± 1.59a

BestKeeper analysis, whereas *TUA* was ranked fifth by GeNorm and sixth by NormFinder. In the four developmental stages of flowers, *TUA* was identified as the most stable reference gene according to BestKeeper analysis, which was similar to the result by GeNorm and NormFinder analysis. In all of the samples, *TUA* and *UBC9* were identified as the most stable reference genes by BestKeeper, whereas *TUA* was ranked fifth by GeNorm and third by NormFinder.

3.2.4 RefFinder Analysis

Finally, Ref-Finder was used to calculate the integrated ranking of the most stable reference genes by GeNorm, NormFinder, and BestKeeper (Tab. 4). Through Ref-Finder analysis, *ACT11* and *EF1α* were the most stable reference genes in different tissues. *TUA* and *UBC9* showed the two highest rankings for the four developmental stages of flowers. *UBC9*, *ACT11*, and *TUA* were found to be three most stable reference genes in all of the samples, whereas *SAMDC* was the least stable gene in all of the samples.

Table 4: Expression stability ranking of 9 candidate reference genes

Tissues		Different developing flowers		Total	
Most	Least	Most	Least	Most	Least
ACT11	*PGK*	*TUA*	*UBQ*	*UBC9*	*SAMDC*
EF1α		*UBC9*		*ACT11*	
				TUA	

PGK and *UBQ* was the least stable gene in three different tissues and the four developmental stages of flowers, respectively.

3.3 Validation of Reference Genes

To confirm the reliability of reference genes, the expression patterns of *IgTPS* were evaluated using different reference genes in different tissues and four developmental stages of flowers. The two most stable genes (*ACT11* and *EF1*a for different tissues, *TUA* and *UBC9* for the different l stages) and the least stable reference gene (*PGK* for different tissues, *UBQ* for the different flower stages) were used in the validation test. When *ACT11* or *EF1a* was used as the reference gene, *IgTPS* expression was highest in leaves among the different tissues, whereas the highest expression level was detected in stems when *PGK* was used as a reference (Fig. 5A). For the four developmental stages of flowers, *IgTPS* expression normalized by *TUA* or *UBC9* was highest in S3 and second highest in S4, but that normalized by *UBQ* was highest in S4 (Fig. 5B). These results showed that the unstable genes *PGK* and *UBQ* failed to effectively standardize the expression data. These results indicate that the accuracy of qRT-PCR analysis could be altered by using different reference genes.

4 Discussion

Although powerful modern technologies, including microarrays and high-throughput sequencing have been used to test gene expression levels, qRT-PCR is still widely used for this purpose owing to its sensitivity, accuracy, and efficiency [34]. Selecting the appropriate reference genes is a fundamental prerequisite for reliable qRT-PCR analysis. The ideal reference genes should have stable expression in different tissues, organs, developmental stages, and treatments [35]. The selection of reference genes that are stably expressed in different samples can improve the accuracy and reliability of qRT-PCR results [14].

GeNorm, NormFinder, BestKeeper, Ref-Finder and delta Ct have been developed to estimate the expression stability of reference genes, but the rankings of most reference genes differ among different programs [36]. In this study, we used four different statistical algorithms, GeNorm, NormFinder, BestKeeper and Ref-Finder, to analyze the stability of candidate reference genes. The gene stability rankings created by GeNorm were similar to those of NormFinder, but they showed quite differences from the ranking obtained with BestKeeper. *ACT11* and *UBC9* were identified as the most stable reference genes in all of the samples by GeNorm and NormFinder. *TUA* ranked in the top two stable genes by BestKeeper, but was ranked fifth by GeNorm and third by NormFinder. The results are similar to many previous studies of different species such as were identified as *Cynodon dactylon* [31], *Chrysanthemum morifolium* [12], *Lilium regale* [37], and *Tolypocladium guangdongense* [38]. Finally, Ref-Finder was used to combine and validate the results. There are some differences among algorithms, but they are not obvious.

In this study, the stability of 9 reference genes in different tissues and different developmental stages of flowers were analyzed. Our study demonstrated that *UBC9*, *ACT11* and *TUA* were good genes for normalization when considering all of the samples. Similar results have been obtained in *Eucommia*

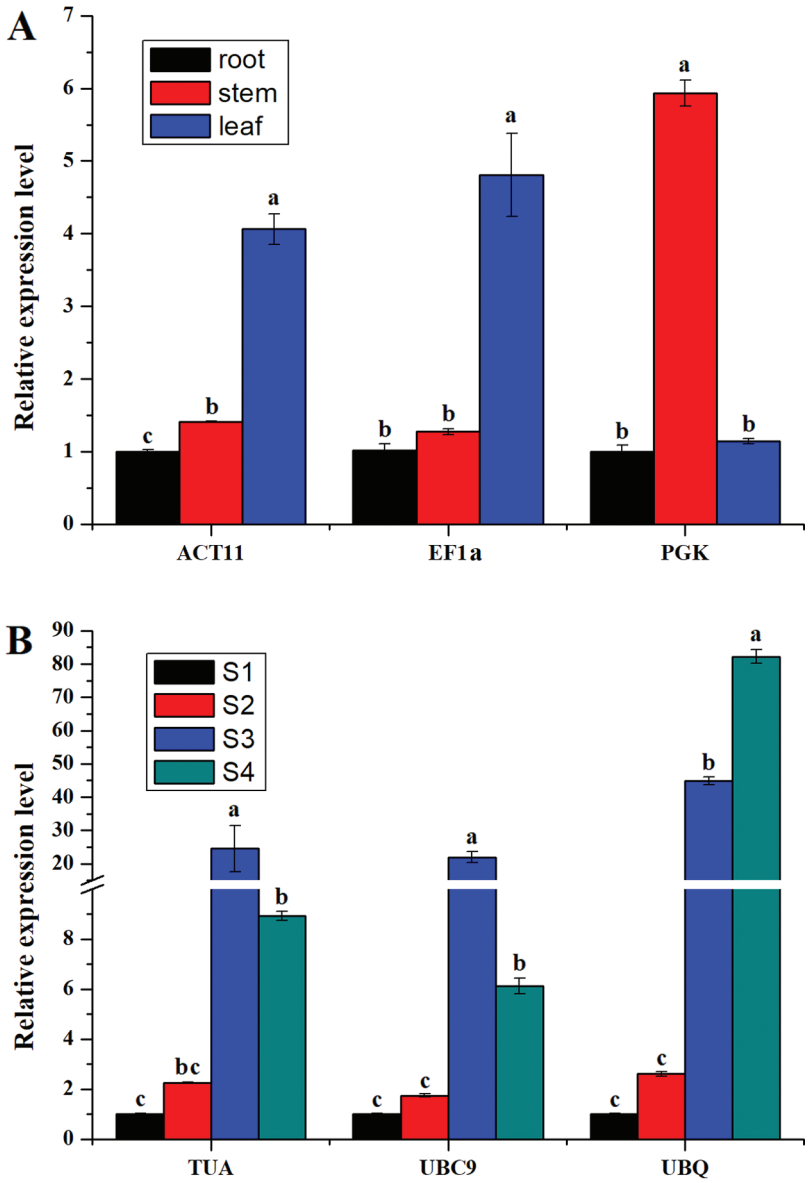

Figure 5: Relative quantification of *IgTPS* gene expression for flower development samples (A) and different organs samples (B) using validated reference genes, including the most and the least stable reference genes for normalization

ulmoides Oliver [39], *Malus domestica* [40], *Fragaria vesca* [41], and *Annona muricata* L. [42]. *GAPDH* and *PP2A* were identified as the most stable reference genes for normalization in seedlings of *Robinia pseudoacacia* L. [43], in tissues of different developmental stages in *Gentiana macrophylla* [44], and in flower development and different organs of *Primula forbesii* [45]. However, these two genes did not perform well in our study. *PGK* plays an important role in glycolytic pathway [46], and it has been widely used as a reliable reference gene by qRT-PCR in *Iris. lacteal var. chinensis* [25] and chrysanthemum [12], while this gene was the least stable reference gene in different tissues in our study. Furthermore, *UBQ* was reported to be one of the most stable reference genes in *Vitis vinifera* L. [47]; however, *UBQ* exhibited the least stable expression in different developing flowers in this study, and

similar studies were found in *Apium graveolens* [48]. In our study, *SAMDC* was ranked least in all of the samples. Consistent with the result in *Hordeum* [49], however, *SAMDC* was appropriate for gene normalization in *Neolamarckia cadamba* [50]. In our study, *EF1α* only performed well in different tissues, which was similar to finding in *Betula platyphylla* [51]. Therefore, these results suggested that different reference genes are required depending on the plant species, tissue types and developmental stages.

Stability of reference genes was further validated by checking the expression patterns analysis of a target gene, *IgTPS*, in different tissues and different flower samples. The expression patterns of *IgTPS* gene showed similar trends when the two most stable reference genes *ACT11*/*EF1a* or *TUA*/*UBC9* were used as internal controls. However, severe disparities occurred when the least stable gene *PGK* or *UBQ* was used for normalization. These results suggest that selecting suitable internal control genes is critically important for the reliable normalization of target gene expression data by qRT-PCR.

5 Conclusions

To our knowledge, this is the first systematic research to validate candidate reference genes for the normalization of gene expression data using qRT-PCR in different tissues or developmental stages of flowers in *I. germanica*. Nine candidate reference genes were tested and their stability was assessed by GeNorm, NormFinder, BestKeeper, and Ref-Finder. Results revealed that *UBC9*, *ACT11*, and *TUA* were identified as the most suitable reference genes in all of the samples, while *SAMDC* showed the least stability. This work will provide useful information for reliable qRT-PCR data normalization in *I. germanica* gene expression studies.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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