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Reference Gene Selection for Quantitative Real-Time PCR Analyses of *Acer palmatum* under Abiotic Stress

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Abstract: Quantitative real-time reverse transcriptase PCR (qRT-PCR) technology has been extensively used to estimate gene expression levels, and the selection of appropriate reference genes for qRT-PCR analysis is critically important for obtaining authentic normalized data. *Acer palmatum* is an important colorful leaf ornamental tree species, and reference genes suitable for normalization of the qRT-PCR data obtained from this species have not been investigated. In this study, the expression stability of ten candidate reference genes, namely, *Actin3*, *Actin6*, *Actin9*, *EF1α*, *PP2A*, *SAMDC*, *TIP41*, *TUBα*, *TUBβ* and *UBQ10*, in two distinct tissues (leaves and roots) of *A. palmatum* under four different abiotic stress conditions (cold, heat, salt and drought) were investigated and assessed using three statistical methods (GeNorm, NormFinder and BestKeeper). The combinations of reference genes that showed stability in the different stressed samples differed. Specifically, *Actin6*, *UBQ10* and *Actin9* were the most stable reference genes in all the samples, and *Actin3* and *Actin6* wexre stably expressed in cold-stressed leaves and roots. *Actin3*, *Actin6* and *UBQ10* were identified as an appropriate combination of reference genes for the analysis of heat-stressed leaves and roots, whereas the combination of *Actin9*, *UBQ10* and *Actin6* was deemed the most suitable for the analysis of salt-stressed leaves and roots. Similarly, *Actin6* and *UBQ10* exhibited stable expression in drought-stressed leaves and roots. Furthermore, the expression levels of *CBF*, *Cu/Zn-SOD* and *HsfA1* were estimated to determine the reliability of the reference genes assessed in this study. This study revealed stable reference genes in *A. palmatum* that might be used for the normalization of qRT-PCR data obtained under various abiotic stresses.

Keywords: *Acer palmatum*; qRT-PCR; abiotic stresses; reference gene; normalization.

1 Introduction

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR), semi-RT-PCR, Northern blotting and microarray methods are commonly used to reveal gene transcript expression patterns [1]. Compared with other traditional methods, qRT-PCR, a sensitive and accurate method with good specificity and reproducibility and a rapid detection speed, has been extensively used to investigate changes in gene expression under biotic and abiotic stress conditions and spatiotemporal expression patterns [1–3]. The accuracy of qRT-PCR is affected by many factors, such as the template purity and

concentration, primer stability, reaction conditions, PCR amplification cycles, and transcription efficiencies [3–5]. To avoid deviations and obtain normalized data from qRT-PCR analyses, it is crucial to validate the expression stability of several internal reference genes and select reliable reference genes for various conditions and tissues.

The ideal internal reference genes for qRT-PCR analysis should exhibit a constant expression level under various experimental conditions and could be used as a reference when detecting changes in target gene expression levels [1,6]. Internal reference genes are usually a variety of housekeeping genes that are stably expressed in cells and aid the maintenance of cellular function. In plants, the reference genes that are most commonly used are actin (*ACTIN*), elongation factor 1-a (*EF1-a*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tubulin (*TUB*) and polyubiquitin (*UBQ*) [7,8]. Some new reference genes for plants, such as protein phosphatase 2A (*PP2A*), S-adenosylmethionine decarboxylase (*SAMDC*) and TIP41-like family protein (*TIP41*), were recently identified [9]. *PP2A* appears to be stably expressed in hybrid roses (*Rosa hybrida*) and tomato (*Solanum lycopersicum*) [10,11]. *SAMDC* exhibits stable expression in Kentucky bluegrass (*Poa pratensis* L.) [12], and *TIP41* displays expression stability in tea (*Camellia sinensis*) and *Caragana intermedia* [3,13]. However, the expression of these reference genes shows variations among different species and under different experimental conditions. *GAPDH* is stably expressed in *Haloxylon ammodendron* under salt stress [14] but is the least suitable reference gene in tea plants (*C. sinensis*) [15]. *Actin* has been found to be a good reference gene for orchardgrass (*Dactylis glomerata* L.) under drought, salt and heat treatments [16] but is unstable in creeping bentgrass (*Agrostis stolonifera*) under cold and heat treatments [17]. In annual ryegrass (*Lolium multiflorum*), *SAMDC* is the most stable gene under acidic aluminum stress but is the least stable gene under heavy metal stress [18]. In *Bixa orellana*, *18S rRNA* exhibits stable expression in flower buds, in seeds during the first and fourth stages of development and in leaves but unstable expression in flowers and in seeds at the second and third stages of development [19]. In tomato (*S. lycopersicum*), *EF1a* exhibits the highest stability under abiotic stress but the least stability under light stress [11]. Therefore, the selection of an appropriate reference gene according to the species and experimental conditions being tested is important. Many stable reference genes have been identified in different types of plants, including soybean (*Glycine max* L.) [20], peanut (*Arachis hypogaea L.*) [21], switchgrass (*Panicum virgatum*) [22], *Salix matsudana* [23], sugarcane (*Saccharum* spp.) [24], tung tree (*Vernicia fordii* Hemsl.) [25], *Haloxylon ammodendron* [14], black locust (*Robinia pseudoacacia* L.) [26] and annual ryegrass (*Lolium multiflorum*) [18] under different conditions.

A. palmatum is a deciduous arboreous tree belonging to the *Aceraceae* family that has beautiful leaves and is widely popular around the world. As a colorful leaf ornamental tree, *A. palmatum* can have yellow, green or red leaves depending on the season [27,28]. However, changes in the natural environment, including cold, heat, salinity-alkalinity and drought, affect the growth of *A. palmatum*. Detecting changes in gene expression during exposure to various abiotic stresses could aid elucidation of the underlying mechanism. Therefore, the identification of stable internal reference genes is essential for the analysis of gene expression under different abiotic stresses. In this study, the stability of ten potential reference genes (*Actin3*, *Actin6*, *Actin9*, *EF1α*, *PP2A*, *SAMDC*, *TIP41*, *TUBα*, *TUBβ* and *UBQ10*) in two distinct tissues (leaves and roots) under four different abiotic (cold, heat, salt and drought) stress conditions was determined and evaluated using three statistical methods (GeNorm, NormFinder and BestKeeper). The expression levels of C-repeat binding factor (*CBF*), Cu-Zn superoxide dismutase (*Cu/Zn-SOD*) and heat shock transcription factor (*HsfA1*) in *A. palmatum* during different abiotic treatments were evaluated using different reference genes. This study preliminarily describes and selects internal reference genes for *A. palmatum* under different conditions and lays the foundation for future research aiming to elucidate the mechanism underlying target gene expression.

2 Materials and Methods

2.1 Plant Materials and Treatments

A. palmatum "Jinlinghuangfeng" plants were preserved in Acer Research Centre, Jiangsu Academy of Agricultural Sciences, China. Seedlings were cultured in Hoagland's nutrient solution and maintained in a plant growth incubator (RDN-1000D, Dongnan, Ningbo, China) with a day/night temperature of 23/18°C, a 16-h light (200 μmol/m^2/s)/8-h dark photoperiod and 70% relative humidity. Sterilized seedlings were obtained and propagated from sterilized explants through *A. palmatum* "Jinlinghuangfeng" transformation. Thirty-day-old seedlings were transferred under different stress conditions. Cold stress treatment was performed by exposure to 4°C, and heat stress treatment was imposed at 40°C. For the salinity and drought stress treatments, the seedlings were transferred to nutrient solution containing 200 mM NaCl and 20% PEG6000, respectively. Leaves and roots were collected 0, 1, 4, 8, 12, and 24 h after each of the four stress treatments. The samples were frozen immediately in liquid nitrogen and stored at −80°C for RNA extraction. Each treatment and collection time were repeated three times with three plants per replicate.

2.2 Total RNA Isolation and cDNA Synthesis

Total RNA from leaves and roots was isolated using the RNAiso reagent (TaKaRa, Japan) according to the manufacturer's instructions and treated with RNase-free DNase I (TaKaRa, Japan) to remove genomic DNA contamination. The total RNA concentration and purity were measured using a spectrophotometer (K5800, Kaiao, Beijing, China). RNA samples with a 260/280-nm ratio within the range of 1.80 to 2.10 and a 260/230-nm ratio of approximately 2.0 were used for further analysis. First-stand cDNA was obtained using M-MLV reverse transcriptase (TaKaRa, Japan) from 0.8 μg of total RNA, and cDNA samples diluted 30-fold were used for qRT-PCR analyses.

2.3 Selection of Combination of Reference Genes and Primer Design

Ten reference gene (*Actin3, Actin6, Actin9, EF1α, PP2A, SAMDC, TIP41, TUBα, TUBβ* and *UBQ10*) sequences were determined from the transcriptome data sequences of *A. palmatum* leaves (submission number: SRX982234 and SRX833686; ULRs: http://www.ncbi.nlm.nih.gov/sra/ and http://www.ncbi.nlm.nih.gov/sra/) The GenBank accession numbers of the genes are shown in Tab. 1. The specific primers used in the qRT-PCR analysis were obtained using Primer Premier 5.0 software with melting temperatures (Tm) of 55–60°C, a nucleotide length of 18–23 bp, a GC content of 40–60% and an 80–200-bp amplification product (Tab. 1, Appendix A and C).

2.4 Quantitative Real-Time PCR Analysis

Quantitative real-time reactions were performed using the Applied Biosystems 7500 real-time PCR system (Applied Biosystems 7500, Carlsbad, CA, USA). Each reaction was performed in a final volume of 20 μl containing 10 μl of SYBR® Premix Ex Taq™ (TaKaRa, Japan), 5 μl of diluted cDNA, 0.4 μl of 10 mM each primer and 0.4 μl of ROX reference dye in a 96-well reaction plate (Applied Biosystems, Carlsbad, CA, USA). The reaction conditions consisted of an initial denaturing step of 95°C for 30 s followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. A melting dissociation curve was created at 60–95°C, and each qRT-PCR reaction was performed in triplicate.

2.5 Gene Stability Analysis

The specificity of each gene was determined through its standard curve (Appendix B). The correlation coefficients (R^2) and slope values for each primer pair were calculated from a standard curve obtained from a serial dilution of cDNA. The amplification efficiencies (E) of the genes were determined based on the slope of a linear regression model with the following equation: $E = [10^{(1/\text{-slope})}-1] \times 100\%$ (Radonić et al. [6]).

Table 1: Candidate reference gene comparison with *Arabidopsis* orthologs, primer sequences and amplification efficiency of qRT-PCR

Gene symbol	EST GenBank accession	*Arabidopsis* homolog locus	Primer sequences (forward/reverse)	Amplicon length (bp)	Amplification efficiency (%)	R^2
Actin3	MN026863	AT1G13180	CTTGCCATACAAAAGCAGAGT ATTTCAGGGAACAGCATCGG	140	100.42	0.998
Actin6	MN026864	AT3G33520	CACTTTGGATGATGGATCTCTAC TCTCTTCCTGTCCTTACGCTC	94	96.45	0.999
Actin9	MN026865	AT5G43500	AGTCGGTGAGGTCTCTTGTTCTA GCATTCTGCCCTTTGTTTTAT	113	97.23	0.992
EF1α	MN026866	AT5G60390	GCCAACTTCACATCTCAGGTC ACTTGACAGCAATGTGGGAGG	103	98.43	0.997
PP2A	MN026867	AT3G25800	TTCTCTACTTGCCCCAGTTATG GGATTGTCTTCTCCACTACGG	167	98.84	0.995
SAMDC	MN026868	AT5G15950	GGGCTCAGTCATTCCCACAT ACCCATCAAATAGGCTGTGCT	101	97.63	0.977
TIP41	MN026869	AT4G34270	GTGCTTATGAGATTGAGGGACA ACCCTTTTGTAGATAATGCTTGA	121	95.87	0.979
TUBα	MN026870	AT1G50010	CCGTGGTGATGTTGTCCCTA ACTTGAATCCAGTGGGGCA	101	93.40	0.982
TUBβ	MN026871	AT5G62690	CGAGCATTGACTGTTCCTGAG ACGGAACATAGCAGAGGCA	111	99.66	0.969
UBQ10	MN026872	AT4G05320	CTTAGTGCTGAGGCTGCGTG ACTCTCCACTTCCAGGGTTA	82	100.00	0.995
CBF	MN026873	AT5G51990	AACTTTGCGGATTCTGCTTG AAAACCCACCTCCACATTCTC	129	94.92	0.992
Cu/Zn-SOD	MN912733	AT2G28190	TGAGGATGACCTTGGAAAA CAAAAGCAAGCAGCCAATAC	154	95.76	0.982
HsfA1	MN912734	AT5G16820	ACAGCAGAGTCAAGATTTCCT TAACCGAGTCCGTCACCA	88	97.63	0.986

Three programs, GeNorm [29], NormFinder [30] and BestKeeper [31], were used to assess the expression stability of the potential reference genes across all the treatments. Relative quantification cycle (Cq) values were detected using the formula $2^{-\Delta Cq}$, where ΔCq is the corresponding Cq value subtracted by the minimum Cq value. The relative Cq values of each gene were used for GeNorm and NormFinder analysis. The expression stability values (M) for each gene were calculated using GeNorm based on pairwise variations. The typical feature of the stably expressed genes had lower M values. The gene stabilities obtained using BestKeeper are based on the coefficients of variance (CVs) and standard deviations (SDs) in the Cq values, and the genes with the lowest values are ranked as the most stable genes.

3 Results

3.1 The Expression Characteristics of the Candidate Reference Genes

The primers of the 10 candidate reference genes (*Actin3*, *Actin6*, *Actin9*, *EF1α*, *PP2A*, *SAMDC*, *TIP41*, *TUBα*, *TUBβ* and *UBQ10*) and the validation gene (*CBF*) were certified through PCR (Appendix A) and qRT-PCR assays (Appendix B). The specificity of the primers was determined by the presence of a single

PCR product band after agarose gel electrophoresis, and the specificity of each primer pair was assessed by the presence of a single peak in the melting curve obtained by qRT-PCR. The melting temperatures (Tm) of the qRT-PCR products varied from 78.6°C for *TIP41* to 88.8°C for *CBF*. The amplification efficiency of the qRT-PCRs ranged from 94.40% for *TUBα* to 100.42% for *Actin3*, and the correlation coefficients (R^2) varied from 0.969 for *TUBβ* to 0.999 for *Actin6* (Tab. 1).

The quantification cycle (Cq) values reflected the mRNA transcript levels of the ten reference genes in all samples. The Cq values of the ten potential reference genes ranged from 19.98 for *SAMDC* to 31.20 for *TIP41*. Moreover, among the ten candidate reference genes, the lowest average Cq of 22.17 was found for *UBQ10*, which indicated that this gene exhibited the highest expression level, and the highest average Cq of 28.01 was obtained for *TUBβ*, which indicated that this gene exhibited the lowest expression level (Fig. 1). The *Actin6* Cq values exhibited a narrow variation range, whereas the *PP2A* Cq values had the widest variation range. The CVs could reply to the stability of reference genes under different stress conditions. The lower the CV value displayed, higher the stability. *TUBβ* conferred the least variability with a CV value of 2.71%, while *SAMDC* conferred the most variability with a CV value of 5.52%.

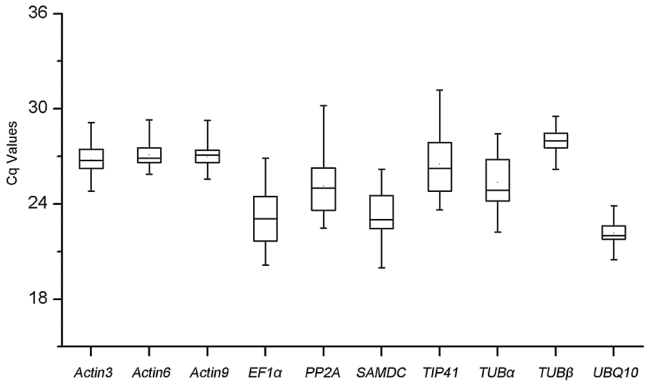


Figure 1: Variations in the quantification cycle (Cq) values of the candidate reference genes in all *A. palmatum* samples under various abiotic stress conditions. The lines across the box-plot graph reflect the median values. The boxes depict the range from the 25% to 75% percentile. The whisker caps indicate the maximal and minimal values

3.2 GeNorm Analysis

The expression stability of the ten potential reference genes was analyzed using the GeNorm program. The average expression stability (M value) of the reference genes in the samples was calculated to filter out the most stable reference genes under different stress treatments. Specifically, a smaller M value indicated improved stability, and a higher M value indicated worse stability [29]. The criterion of an M value below the threshold of 1.5 was used in the analysis in accordance with the GeNorm software guidelines. The rankings of the reference genes after the different treatments according to their M values are shown in Fig. 2. After all the treatments, *Actin6*, *Actin9* and *UBQ10* were the most stable genes in the leaves and roots, as demonstrated by the finding that these genes exhibited the lowest M value (0.70), and *PP2A* was the least stable gene, with the highest M value (1.22) (Fig. 2a). In the cold-treated leaves and roots, *Actin3* and *Actin6* showed the highest expression stability, whereas *EF1α* and *TUBα* showed the lowest expression stability, respectively (Figs. 2b and 2c). In the heat-treated leaves and roots, *Actin3* combined with *Actin6* and *UBQ10* were the most stable genes, whereas *TIP41* and *PP2A* were the least stable genes, respectively (Figs. 2d and 2e). In the salt-treated leaves and roots, *Actin9* in combination with *Actin6* and *UBQ10* exhibited the most stable expression, and *TIP41* and *TUBα* showed the least stable expression, respectively (Figs. 2f and 2g). In the drought-treated leaves and roots, *Actin6* combined with

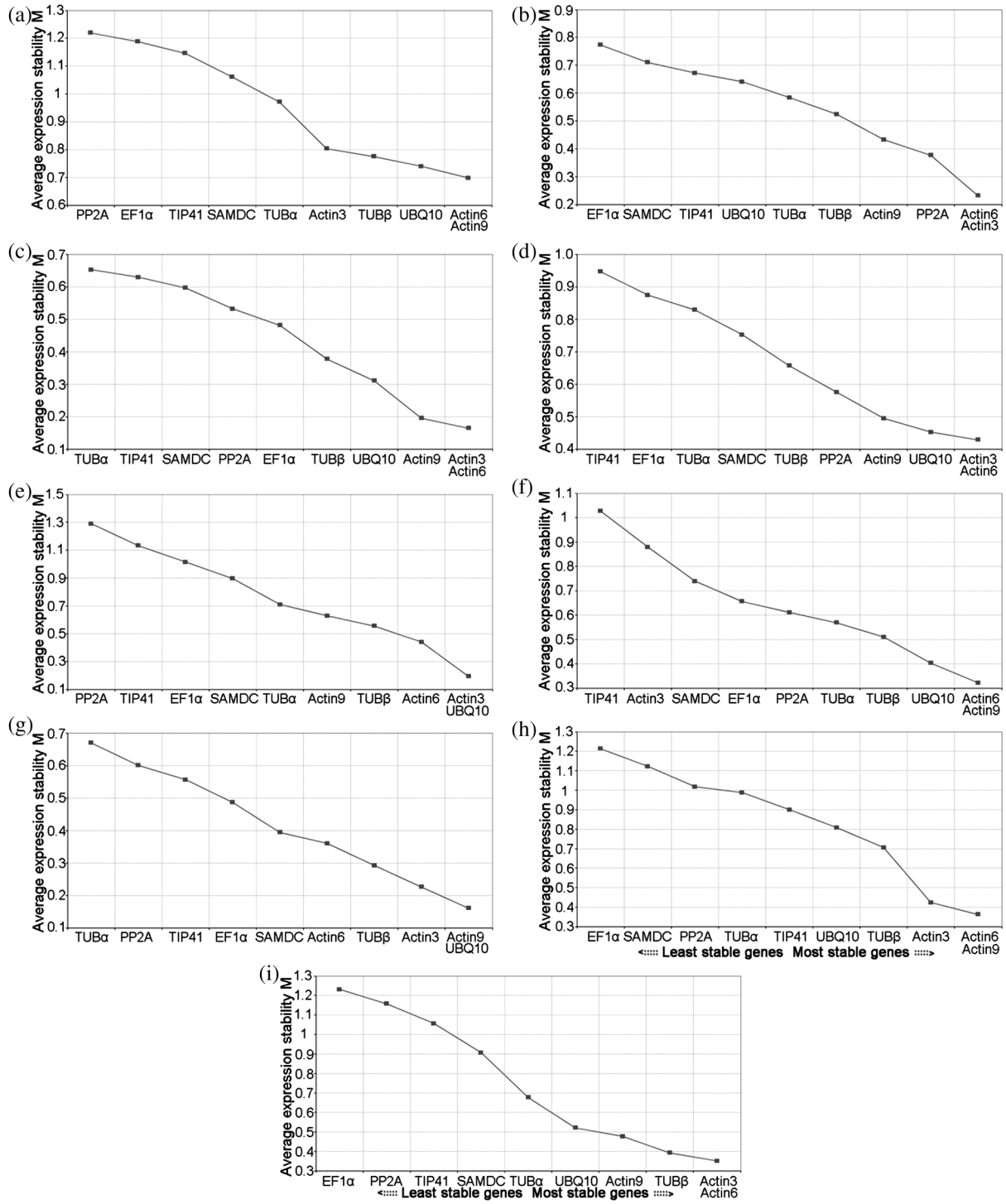
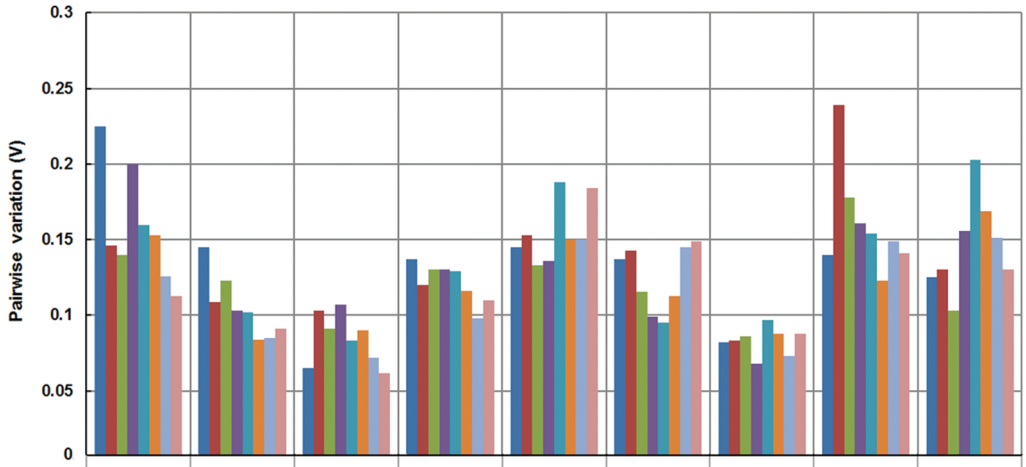


Figure 2: Expression stability values (M) of ten candidate reference genes ranked according using GeNorm software. The genes were ranked in terms of their M values obtained for all the leaf and root samples (a), cold-treated leaves (b), cold-treated roots (c), heat-treated leaves (d), heat-treated roots (e), salt-treated leaves (f), salt-treated roots (g), drought-treated leaves (h) and drought-treated roots (i). The least stable gene is shown on the left, and the most stable gene is shown on the right

Actin3 and *Actin9* were the most stable genes, whereas *EF1α* expression was more variable (Figs. 2h and 2i). Overall, the results showed that *Actin3*, *Actin6*, *Actin9* and *UBQ10* showed the highest stability with lower M values.

To obtain more precise expression values, target gene expression could be corrected using a higher number of reference genes. Pairwise variations (V_n/V_{n+1}) according to the GeNorm algorithm were used to determine the optional number of reference genes. If the pairwise variation value was less than 0.15, multiple reference genes were not needed for correction [22,29]. For the analysis of individual stress treatments, including treated leaves and roots, two reference genes ($V_{2/3} < 0.15$) were found to be sufficient for the normalization of expression data (Fig. 3). The testing of all stress treatments yielded a pairwise variation value ($V_{2/3}$) higher than the threshold (0.15), but the value of $V_{3/4}$ was below the threshold (0.15), which indicated that three reference genes are sufficient for the normalization of gene expression.



	All samples	Cold stress leaves	Cold stress roots	Heat stress leaves	Heat stress roots	Salt stress leaves	Salt stress roots	Drought stress leaves	Drought stress roots
V2/3	0.225	0.145	0.065	0.137	0.145	0.137	0.082	0.14	0.125
V3/4	0.146	0.109	0.103	0.12	0.153	0.143	0.083	0.239	0.13
V4/5	0.14	0.123	0.091	0.13	0.133	0.1154	0.086	0.178	0.103
V5/6	0.2	0.103	0.107	0.13	0.136	0.099	0.068	0.161	0.156
V6/7	0.16	0.102	0.083	0.129	0.188	0.095	0.097	0.154	0.203
V7/8	0.153	0.084	0.09	0.116	0.15	0.113	0.088	0.123	0.169
V8/9	0.126	0.085	0.072	0.098	0.15	0.145	0.073	0.149	0.151
V9/10	0.113	0.091	0.062	0.11	0.184	0.149	0.088	0.141	0.13

Figure 3: Pairwise variation analysis (performed using GeNorm software) showing the optimal number of reference genes for the normalization of qPCR data from various abiotic stress treatments

3.3 NormFinder Analysis

Values corresponding to the expression stability of the candidate genes were calculated using NormFinder software, and the genes were then ranked based on the calculated values. A lower expression stability value indicated higher stability [30]. The results obtained with NormFinder software for the total samples, heat-treated leaves, salt-treated leaves and roots, and drought-treated leaves were consistent with those obtained in the GeNorm analysis, whereas those obtained for cold-treated leaves and roots, heat-treated roots, and drought-treated roots were slightly different from those obtained in the GeNorm analysis (Tab. 2). For all the samples, *Actin6*, *UBQ10* and *Actin9* were the most stable genes, whereas *PP2A* showed expression variation. In the cold-treated leaves and roots, *Actin3* and *EF1α* were ranked as the most stable genes, and *TIP41* and *SAMDC* were ranked as the least stable genes, respectively. In the heat-treated leaves and roots, *Actin6* and *TUBβ* were the most stable genes, and *TIP41* and *PP2A* were the least stable genes, respectively. In the salt-treated leaves, the most and least stable genes were *Actin9*

Table 2: Expression stability of ten candidate reference genes calculated and ranked in *A. palmatum* according to the NormFinder algorithm

Rank	All samples	Cold stress leaves	Cold stress roots	Heat stress leaves	Heat stress roots	Salt stress leaves	Salt stress roots	Drought stress leaves	Drought stress roots
1	*Actin6*	*Actin3*	*EF1α*	*Actin6*	*TUBβ*	*Actin9*	*UBQ10*	*Actin6*	*UBQ10*
Stability	0.494	0.088	0.259	0.127	0.095	0.051	0.109	0.384	0.377
2	*UBQ10*	*Actin6*	*Actin6*	*Actin9*	*UBQ10*	*Actin6*	*Actin6*	*UBQ10*	*Actin6*
Stability	0.514	0.208	0.265	0.265	0.242	0.112	0.232	0.385	0.387
3	*Actin9*	*Actin9*	*UBQ10*	*UBQ10*	*Actin6*	*UBQ10*	*Actin3*	*TIP41*	*TUBα*
Stability	0.562	0.307	0.266	0.364	0.309	0.114	0.236	0.395	0.418
4	*SAMDC*	*UBQ10*	*PP2A*	*Actin3*	*SAMDC*	*TUBβ*	*SAMDC*	*Actin9*	*TUBβ*
Stability	0.569	0.316	0.270	0.441	0.493	0.394	0.275	0.490	0.548
5	*Actin3*	*TUBβ*	*Actin3*	*PP2A*	*EF1α*	*TUBβ*	*EF1α*	*Actin3*	*Actin9*
Stability	0.596	0.371	0.292	0.465	0.592	0.477	0.291	0.545	0.575
6	*TUBα*	*TUBα*	*Actin9*	*SAMDC*	*Actin3*	*SAMDC*	*Actin9*	*TUBβ*	*Actin3*
Stability	0.610	0.380	0.346	0.529	0.712	0.524	0.326	0.579	0.616
7	*EF1α*	*PP2A*	*TUBβ*	*EF1α*	*TUBα*	*EF1α*	*PP2A*	*TUBα*	*SAMDC*
Stability	0.680	0.439	0.372	0.534	0.783	0.527	0.392	0.723	0.720
8	*TIP41*	*SAMDC*	*TIP41*	*TUBβ*	*TIP41*	*PP2A*	*TIP41*	*PP2A*	*TIP41*
Stability	0.682	0.495	0.406	0.558	0.858	0.528	0.395	0.767	0.804
9	*TUBβ*	*EF1α*	*TUBα*	*TUBα*	*Actin9*	*Actin3*	*TUBβ*	*SAMDC*	*PP2A*
Stability	0.691	0.511	0.417	0.559	0.564	0.856	0.414	0.814	0.874
10	*PP2A*	*TIP41*	*SAMDC*	*TIP41*	*PP2A*	*TIP41*	*TUBα*	*EF1α*	*EF1α*
Stability	0.759	0.622	0.419	0.745	1.264	1.026	0.602	0.955	0.882

and *UBQ10*, respectively, and *TIP41* and *TUBα* were the most and least stable genes in the salt-treated roots, respectively. In the drought-treated leaves and roots, *Actin6* and *UBQ10* exhibited the most stable values, whereas EF1α displayed the greatest instability.

3.4 BestKeeper Analysis

The SDs and CVs of the Cq values were determined using the BestKeeper program to assess the stability of the reference genes. The most stable gene among the candidate reference genes was characterized as the gene with the lowest CV and SD values [31]. The results from the BestKeeper analysis for the total samples and the salt-treated leaves were consistent with those obtained in the GeNorm and NormFinder analyses, and those obtained for the other stress treatments showed slight differences between the BestKeeper analysis and the GeNorm and NormFinder analyses (Tab. 3). In all the samples, *Actin6* remained the most stable gene, and *PP2A* was the least stable gene. *Actin6* was also ranked as the most stable gene in the cold-, heat- and drought-treated leaves and the drought-treated roots. *Actin9* was the most stably expressed gene in the salt-treated leaves and roots. *UBQ10* and *Actin3* were the most stable in the cold- and heat-treated roots, respectively. The least stable gene in all the samples, the heat- and salt-treated roots, and the drought-treated leaves was *PP2A*, and *TIP41* showed the least stable expression in the salt-treated leaves

Table 3: Expression stability of ten candidate reference genes calculated and ranked in *A. palmatum* using the BestKeeper algorithm

Rank	All samples	Cold stress leaves	Cold stress roots	Heat stress leaves	Heat stress roots	Salt stress leaves	Salt stress roots	Drought stress leaves	Drought stress roots
1	*Actin6*	*Actin6*	*UBQ10*	*Actin6*	*Actin3*	*Actin9*	*Actin9*	*Actin6*	*Actin6*
CV ± SD	2.01 ± 0.54	0.70 ± 0.17	0.43 ± 0.12	0.96 ± 0.21	0.97 ± 0.27	1.10 ± 0.25	0.34 ± 0.09	2.33 ± 0.64	1.19 ± 0.34
2	*TUBβ*	*PP2A*	*Actin6*	*Actin3*	*Actin6*	*Actin6*	*Actin6*	*TUBβ*	*Actin9*
CV ± SD	2.07 ± 0.58	0.83 ± 0.23	1.08 ± 0.24	1.21 ± 0.33	1.02 ± 0.27	1.44 ± 0.38	1.28 ± 0.45	2.84 ± 0.80	1.26 ± 0.34
3	*Actin9*	*Actin9*	*TUBβ*	*UBQ10*	*Actin9*	*UBQ10*	*TUBβ*	*Actin3*	*Actin3*
CV ± SD	2.11 ± 0.57	1.23 ± 0.34	1.31 ± 0.36	1.38 ± 0.37	1.33 ± 0.36	1.60 ± 0.43	1.36 ± 0.37	3.28 ± 0.86	2.54 ± 0.29
4	*Actin3*	*EF1α*	*Actin9*	*Actin9*	*TUBβ*	*TUBβ*	*Actin3*	*Actin9*	*UBQ10*
CV ± SD	2.41 ± 0.65	1.50 ± 0.33	1.72 ± 0.46	1.60 ± 0.43	1.82 ± 0.49	1.80 ± 0.40	1.36 ± 0.37	3.49 ± 0.94	2.57 ± 0.58
5	*UBQ10*	*Actin3*	*Actin3*	*SAMDC*	*UBQ10*	*SAMDC*	*UBQ10*	*SAMDC*	*TUBβ*
CV ± SD	2.56 ± 0.57	1.82 ± 0.49	1.87 ± 0.51	2.03 ± 0.48	2.05 ± 0.46	2.20 ± 0.56	1.53 ± 0.34	3.85 ± 0.85	2.80 ± 0.78
6	*SAMDC*	*TIP41*	*TIP41*	*TUBβ*	*TUBα*	*Actin3*	*SAMDC*	*UBQ10*	*TUBα*
CV ± SD	5.10 ± 1.19	1.85 ± 0.48	2.52 ± 0.69	2.57 ± 0.73	3.17 ± 0.83	2.57 ± 0.68	2.22 ± 0.54	4.01 ± 0.89	2.92 ± 0.78
7	*TUBα*	*TUBα*	*PP2A*	*SAMDC*	*SAMDC*	*TUBα*	*TIP41*	*TIP41*	*SAMDC*
CV ± SD	5.38 ± 1.37	2.11 ± 0.52	2.54 ± 0.66	3.21 ± 0.71	5.41 ± 1.34	3.00 ± 0.73	2.83 ± 0.78	4.97 ± 1.26	3.09 ± 0.77
8	*TIP41*	*TUBβ*	*EF1α*	*TUBα*	*EF1α*	*EF1α*	*EF1α*	*TUBα*	*PP2A*
CV ± SD	5.57 ± 1.48	2.60 ± 0.74	2.67 ± 0.66	4.03 ± 0.96	5.41 ± 1.35	3.20 ± 0.71	2.85 ± 0.70	5.38 ± 1.32	3.26 ± 0.87
9	*EF1α*	*UBQ10*	*SAMDC*	*TIP41*	*TIP41*	*PP2A*	*TUBα*	*EF1α*	*EF1α*
CV ± SD	6.09 ± 1.53	2.75 ± 0.59	2.98 ± 0.79	4.13 ± 1.05	5.54 ± 1.36	3.57 ± 0.84	3.01 ± 0.81	5.59 ± 1.23	3.38 ± 0.85
10	*PP2A*	*SAMDC*	*TUBα*	*EF1α*	*PP2A*	*TIP41*	*PP2A*	*PP2A*	*TIP41*
CV ± SD	6.70 ± 1.56	3.94 ± 0.87	3.27 ± 0.79	4.72 ± 1.02	7.53 ± 2.08	4.21 ± 1.09	3.07 ± 0.81	6.00 ± 1.45	4.08 ± 1.16

and the drought-treated roots. *SAMDC*, *TUBα* and *EF1α* exhibited the most instability in the cold-treated leaves and roots and the heat-treated leaves, respectively.

3.5 RefFinder Analysis

The RefFinder program, which integrates geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method [32], was used to generate comprehensive rankings of the candidate reference genes (Tab. 4). *Actin3* (cold- and heat-treated leaves and heat- and drought-treated roots), *Actin6* (heat-, salt- and drought-treated leaves and cold- and drought-treated roots), *UBQ10* (drought-treated leaves and cold-, heat- and salt-treated roots), *Actin9* (salt-treated leaves and roots) and *PP2A* (cold-treated leaves) were found to be the top stable genes under the different treatments. Across all samples, *Actin6*, *UBQ10* and *Actin9* were found to be the most stable reference genes.

3.6 Validation of Reference Genes for Determination of Target Gene Expression

To validate the stability of the candidate reference genes and assess their influence on qRT-PCR results, the expression patterns of *CBF*, *Cu/Zn-SOD* and *HsfA1* under various abiotic stresses were analyzed (Fig. 4). The combination of the most stable reference gene and the least stable reference gene was used to quantify the expression of the target genes *CBF*, *Cu/Zn-SOD* and *HsfA1*. After 4 h of cold treatment, the use of *Actin3* and *Actin6* as the reference genes showed that *CBF* was induced 23- to 22-fold, respectively. However, the use of *SAMDC* and *EF1α* for normalization revealed that the expression of *CBF* was induced 10- and 14-fold,

Table 4: Stabilities of candidate reference genes ranked by RefFinder

	Experimental treatments								
	All samples	Cold stress leaves	Cold stress roots	Heat stress leaves	Heat stress roots	Salt stress leaves	Salt stress roots	Drought stress leaves	Drought stress roots
Most	*Actin6* *UBQ10* *Actin9*	*Actin3* *PP2A*	*Actin6* *UBQ10*	*Actin6* *Actin3*	*Actin3* *UBQ10*	*Actin9* *Actin6*	*UBQ10* *Actin9*	*Actin6* *UBQ10*	*Actin6* *Actin3*
Least	*PP2A*	*SAMDC*	*TUBα*	*TIP41*	*PP2A*	*TIP41*	*TUBα*	*EF1α*	*TIP41*

respectively (Fig. 4a). Under salt treatment, the use of the two most stable representative genes (*Actin9* and *UBQ10*) showed that the transcript levels of *CBF* increased rapidly, peaked at 1 h and then decreased during this treatment, whereas normalization using the two least stable reference genes (*PP2A* and *TUBα*) showed that the expression levels of *CBF* fluctuated and showed maximal expression after 4 h of salt stress (Fig. 4b). Under salt and drought treatment, the expression of *Cu/Zn-SOD* was induced with 10-fold and 9-fold increases at 24 h using *Actin9* + *UBQ10* and *Actin3* + *Actin6*, respectively (Figs. 4c and 4d). Under heat treatment, *HsfA1* had the same expression patterns using *Actin6*, *Actin3* and *Actin6* + *Actin3*, for normalization. The expression of *HsfA1* was induced with a peak at 8 h (Fig. 4e).

4 Discussion

qRT-PCR technology is widely used for the detection of gene expression levels because of its simplicity, rapidity and high accuracy. The use of qRT-PCR for relative quantitative analysis requires the use of appropriate internal reference genes for data correction to obtain accurate results. However, no internal reference gene can be completely stable in different plant tissues under different experimental conditions [33]. Therefore, the selection of an appropriate reference gene based on the experimental conditions is important because it would affect the reliability of the results.

A. palmatum is a tree used for its ornamental foliage, and some varieties can display different colors in spring, summer and autumn [27,28]. Recent studies have shown that various environmental changes, such as drought, salt damage and other abiotic stresses, seriously affect the productivity of *A. palmatum*. To ensure sustainable productivity, it is necessary to study the mechanism underlying the regulation of key genes under different abiotic stresses. The selection of an appropriate reference gene is a prerequisite for analyzing the expression level of a target gene. However, internal reference genes have not been systematically screened under different stress conditions. In this study, ten candidate reference genes, including seven housekeeping genes (*Actin3*, *Actin6*, *Actin9*, *EF1α*, *TUBα*, *TUBβ*, and *UBQ10*) and three new genes (*PP2A*, *SAMDC* and *TIP41*), were analyzed to assess their utility for the relative quantification of qRT-PCR data. The expression stability of the ten potential reference genes in leaves and roots under different abiotic stress conditions (cold, heat, salt and drought) was evaluated using three algorithms (GeNorm, NormFinder and BestKeeper). To our knowledge, this study provides the first identification of stable reference genes in the leaves and roots of *A. palmatum* under different abiotic stress conditions.

The Cq values of ten candidate reference genes were determined by qRT-PCR. In all tested samples, the Cq values ranged from 19.98 for *SAMDC* to 31.20 for *TIP41*, and the mean Cq values ranged from 22.17 for *UBQ10* and 28.01 for *TUBβ* (Fig. 1). According to the CVs, the top three genes were *TUBβ* (2.71%), *Actin6* (3.14%), and *Actin9* (3.15%), which implies that these three genes might be stable, whereas the Cq values of *SAMDC* showed the highest variation (5.52%). The Cq values and CVs provided only preliminary results with respect to the stability of the internal reference genes, but these data are obviously insufficient

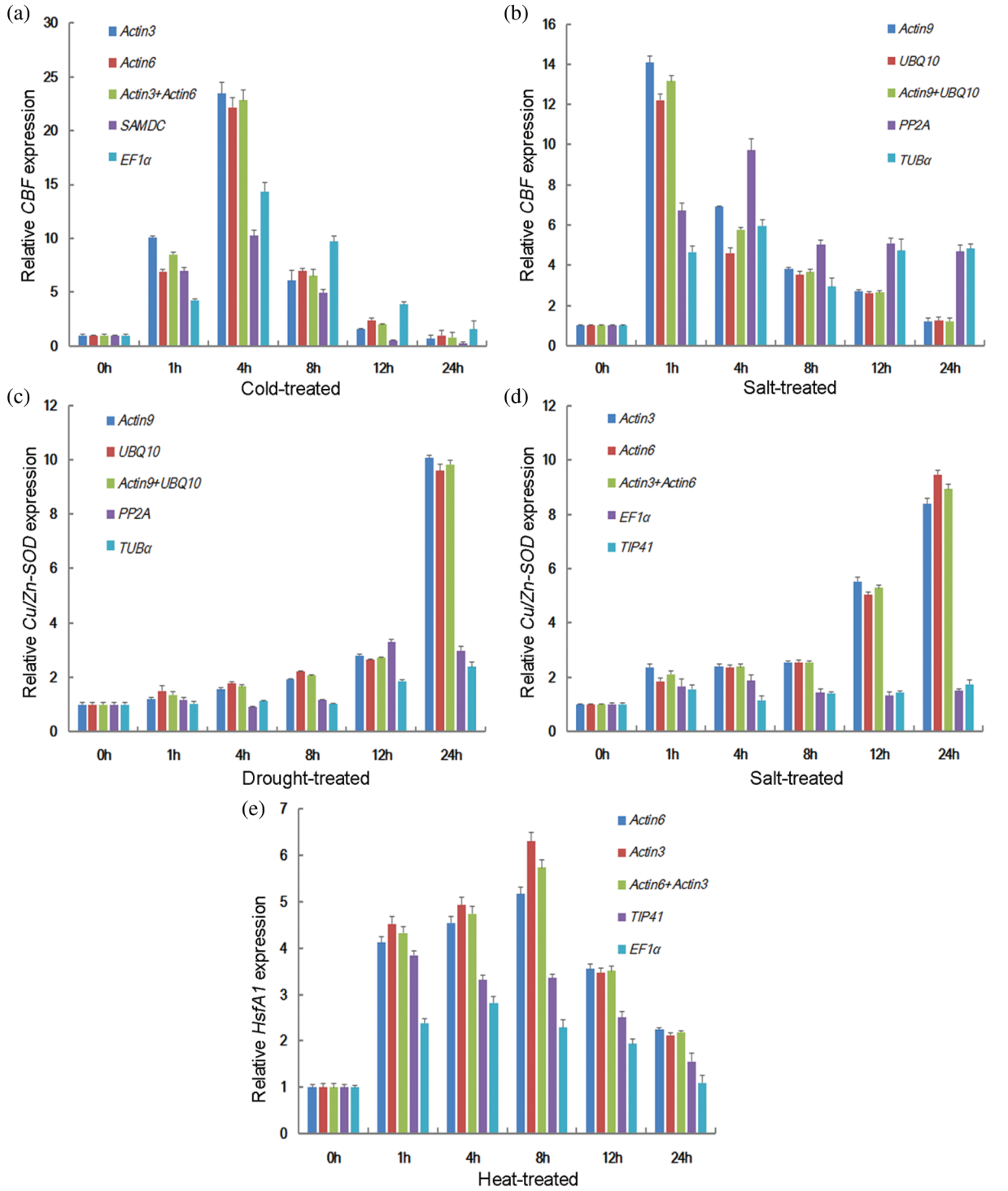


Figure 4: Relative quantification of *CBF*, *Cu/Zn-SOD and HsfA1* expression in *A. palmatum* 0, 1, 4, 8, 12 and 24 h after exposure to different stresses obtained by normalization using different reference genes. (a) Expression levels of *CBF* in cold-treated plants. (b) Expression levels of *CBF* in salt-treated plants. (c) Expression levels of *Cu/Zn-SOD* in drought-treated plants. (d) Expression levels of *Cu/Zn-SOD* in salt-treated plants. (e) Expression levels of *HsfA1* in heat-treated plants. The error bars indicate the standard errors from three biological replicates

[8,12,19]. Three statistical algorithms (GeNorm, NormFinder, and BestKeeper) were used to further analyze the stability of the candidate reference genes. GeNorm could screen out suitable reference genes and determine the optimal number of reference genes. NormFinder could not only compare the expression difference of internal reference genes but also calculate the variation between sample groups; however, it could only select a suitable internal reference gene. The BestKeeper algorithm is different from the GeNorm and NormFinder algorithms, so the result of BestKeeper has some difference from the others. Thus, a comprehensive comparison is needed when we find the appropriate reference genes.

Seven traditional housekeeping genes (*Actin3*, *Actin6*, *Actin9*, *EF1α*, *TUBα*, *TUBβ*, and *UBQ10*) are stably expressed in some plant species and show variable expression in other species [7,8,34]. *Actin* shows stability in cold-, salt-, and heat-treated roots and drought-treated leaves of Kentucky bluegrass (*Poa pratensis* L.) [12]. In addition, *Actin7* is stable under salt stress [14], and *Actin8* is the least stable gene in virus-infected *Arabidopsis thaliana* [35]. In contrast, *Actin* exhibits unstable expression in *Caragana intermedia* under abiotic stress conditions [3]. According to our GeNorm results, *Actin3*, *Actin6*, and *Actin9* displayed stability in leaves under four abiotic stress conditions with the exception of salt stress. Actin protein is commonly expressed in eukaryotic cells and is involved in many important physiological activities, such as cell division, morphological maintenance, signal transduction, and organelle movement [36,37]. The *Actin* gene family has many members and they have different functions. This may be the reason for their different stabilities under different abiotic stresses. *Actin6* was also ranked as one of the three most stable genes in the samples exposed to the four abiotic stresses by NormFinder and BestKeeper. *Actin3* was the most stable gene identified in cold-stressed leaves by NormFinder and in heat-stressed roots by BestKeeper. *Actin3* combined with *TIP41* showed variable expression in the salt-treated leaves, as demonstrated in the GeNorm and NormFinder analyses, whereas *Actin3* was ranked as the sixth most stable gene according to BestKeeper. *Actin9* was ranked as the most stable gene in salt-stressed leaves by NormFinder and in salt-stressed leaves and roots by BestKeeper. In 442 different perennial ryegrasses (*Lolium perenne* L.), *eEF1α(s)* was identified as the most stable gene that could be used for accurate normalization [38]. *EF1α* is one of the four most stable genes that can be used for the assessment of gene expression in *Aeluropus littoralis* under salt stress and recovery conditions [39]. *EF1α* has been determined to not be a suitable stable reference gene in Kentucky bluegrass (*Poa pratensis* L.), Arabidopsis and Moso bamboo (*Phyllostachys edulis*) [9,12,40]. Some *EF1α* genes are induced by abiotic stress [41]. In this study, *EF1α* was identified as the least stable gene in cold-stressed leaves and drought-stressed leaves and roots according to GeNorm, and this result was generally consistent with the NormFinder and BestKeeper results. *TUB* is considered the least reliable gene for the analysis of gene expression in different tissues of rhododendron (*Rhododendron molle* G. Don) [42], and *α-TUB2* and *α-TUB1* have been identified as the most and least stable reference genes in *Salix matsudana* under heavy metal treatment [23]. Different *TUB* genes show different stabilities. TUBα and TUBβ are all tubulin proteins. In our study, *TUBα* was found to be less stable than *TUBβ*. NormFinder analysis showed that *TUBβ* was a stable reference gene only in heat-stressed roots, and *TUBα* was found to be the least stably expressed reference gene in cold- and salt-stressed roots. The results showed that *TUBβ* may be induced by heat stress and that *TUBα* may be induced by cold and salt stress. *UBQ14* has been suggested to be the most stable gene across all tissues and flower stages in cotton (*Gossypium hirsutum*) [43], and *UBQ* is the most stable gene in different tissues and fruit developmental stages in Chinese prickly ash (*Zanthoxylum bungeanum* maxim) [44]. The results obtained with the three software packages in this study showed that *UBQ10* exhibited the highest stability. Specifically, *UBQ10* was ranked as the most stable gene in the salt-stress roots according to GeNorm and NormFinder and in the heat- and cold-stressed roots according to GeNorm and BestKeeper, respectively.

In recent years, three new reference genes (*PP2A*, *SAMDC* and *TIP41*) have been frequently used in the screening of plant internal reference genes [9]. The genes *PP2A* and *UBI9* have been identified as the most

stable reference genes in drought-stressed roots and display high variability among different organs [45]. *PP2A* shows extreme instability among different sample subsets and under cold and heat stress conditions [46]. The *PP2A* gene is involved in eliminating signal transduction of hormones, such as acid, auxin and ethylene [47]. This may be the reason why it is unstable under various abiotic stresses. Based on our results, *PP2A* was the least stable reference gene in all the samples and in heat-stressed roots according to the three programs and in salt-stressed roots and drought-stressed leaves according to BestKeeper. *TIP41* was ranked as the most stable gene in different tissues and at various developmental stages in bamboo (*Phyllostachys edulis*) [40]. In H_2O_2 and cold environments, *TIP41* is one of the least stable reference genes [48]. We previously found that *TIP41* tended to be the least stable reference gene in Chinese tallow (*Sapium sebiferum*) under cold treatment [49]. *TIP41* presented high variability under the four abiotic stresses and was the least stable reference gene in heat- and salt-treated leaves. *SAMDC* was ranked as the least stable gene in chia (*Salvia hispanica*) [50], switchgrass (*Panicum virgatum*) [22], sugarcane (*Saccharum* spp.) [51] and *Litsea cubeba* [52]. In this study, *SAMDC* was also identified as one of the least stable reference genes under the four abiotic stresses and the least stable reference gene in cold-stressed roots and leaves by GeNorm and BestKeeper, respectively.

CBF is a main gene involved in responses to cold, drought and salt stress [53–56]. *Cu/Zn-SOD* is a known salt and drought stress-inducible gene [57,58]. *HsfA1* is the main regulator of the heat shock response [59,60]. To validate the reliability of the tested reference genes, the expression patterns of *CBF*, *Cu/Zn-SOD* and *HsfA1* in various abiotic stresses were determined using the most or least stable reference genes. The results clearly showed that an unstable reference gene affects the expression level of target genes. In addition, using a single gene could lead to a relatively large error, and reference gene combinations are more accurate for normalization [29]. We also used the reference gene combination to determine of target gene expression and obtained more accurate results. The results indicate that the selection of an appropriate reference gene is essential for normalization of qRT-PCR analysis data.

To our knowledge, this study constitutes the first systematic exploration aiming to identify suitable internal reference genes for the normalization of qRT-PCR data from *A. palmatum* under four different abiotic (cold, heat, salt and drought) stress conditions. Ten candidate genes were included in the analysis. *Actin6*, *UBQ10* and *Actin9* were identified as suitable reference genes for the normalization of target gene expression in different tissues under different abiotic stress conditions. Furthermore, the expression profiles of *CBF*, *Cu/Zn-SOD* and *HsfA1* were determined to estimate the suitability of the reference genes investigated in this study. The results showed that *CBF*, *Cu/Zn-SOD* and *HsfA1* expression was induced by various abiotic stresses, and their expression profiles were obtained after normalization using stable reference genes. The identification of stable reference genes in this study can improve our understanding of the molecular mechanism underlying the expression of stress-responsive genes in *A. palmatum* under abiotic stress conditions.

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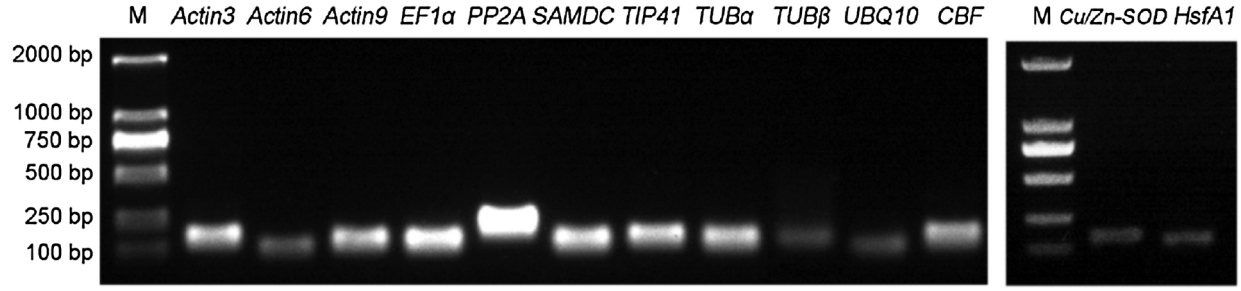
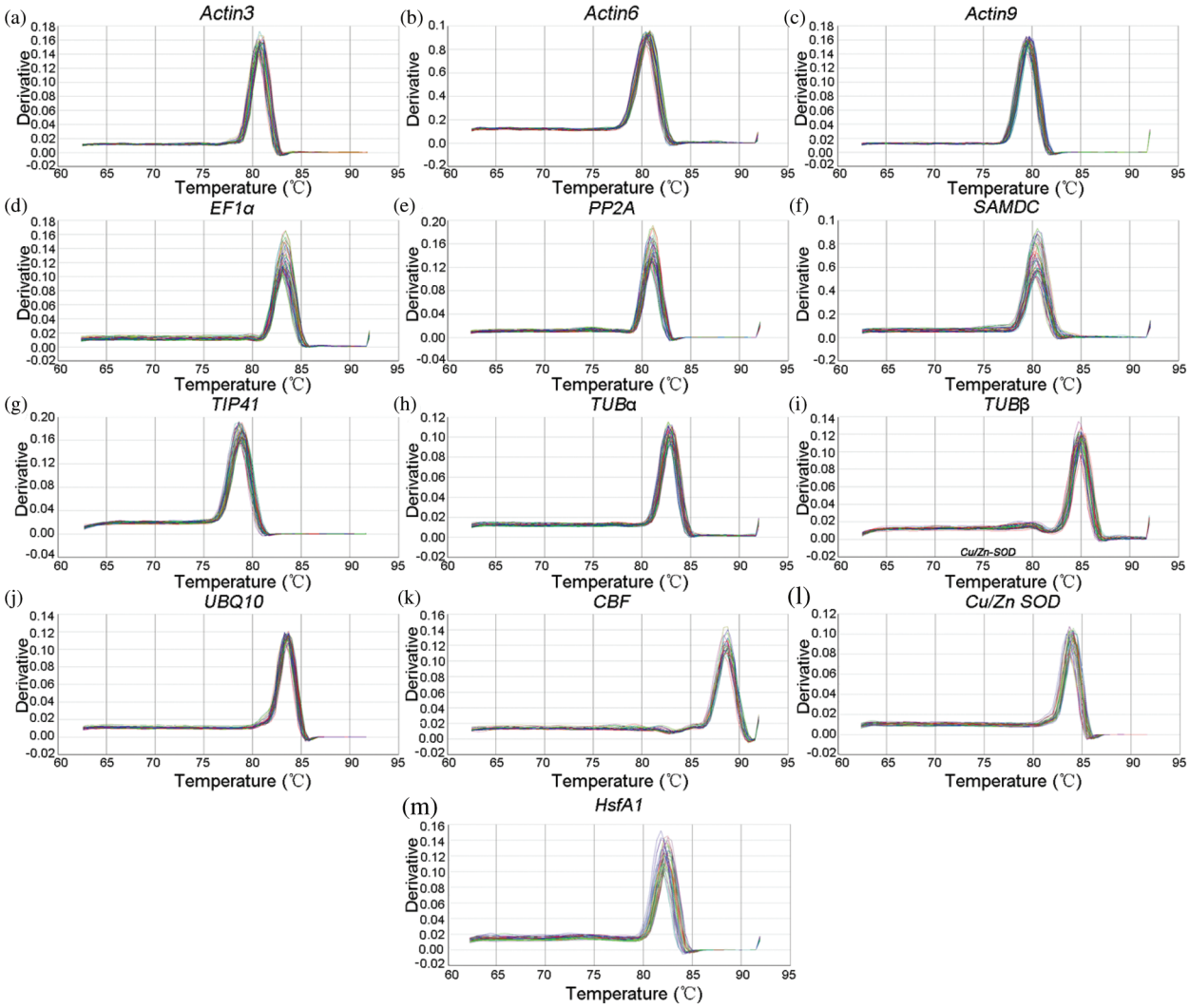
Appendix



Appendix A: PCR products of ten reference genes, *CBF*, *Cu/Zn-SOD* and *HsfA1*. The amplification fragments were separated by agarose gel electrophoresis. M: DNA maker



Appendix B: Melting curves of the ten reference genes, *CBF*, *Cu/Zn-SOD* and *HsfA1*

Appendix C: List of amplified sequences of the 10 reference genes, *CBF*, *Cu/Zn-SOD* and *HsfA1*

Gene abbreviation	Amplified sequences
Actin3	CTTGCCATACAAAAGCAGAGTACGAGGAATATGGAGCGAGCATATGCCGTACAAA TCCTGTTTTCAAGGGGATGTATTGATTTAAAGACTTGTCTTGGCTGATCAAATGGA AGGTATTTCCCGATGCTGTTCCCTGAAAT
Actin6	CACTTTGGATGATGGATCTCTACCTCAACCATCAGAGACAGAGAAGGACATGGTT CAGATAGACGATACAGAGGAGCGTAAGGACAGGAAGAGA
Actin9	AGTCGGTGAGGTCTCTTGTTCTACTAGAGGTTTTGGGATAAGTCGCACTCACATCA ATGTTTTGATGGTGTTACATTTCAGTTTTGCAGAAAATAAAACAAAGGGCAGAATGC
EF1α	GCCAACTTCACATCTCAGGTCATCATCATGAACCACCCTGGGCAAATCGGAAACGG ATATGCTCCAGTGCTTGATTGCCACACCTCCCACATTGCTGTCAAGT
PP2A	TTCTCTACTTGCCCCAGTTATGGGCCCAGAAATTACTTGTTCAAAACTTCTACCTGTG GTCATTAATGCATCAAAAGACAGGGTACCAAACATCAAATTCAATGTGGCTAAGGTG TTGCAGTCACTTATTCCCATAGTGGATCAATCCGTAGTGGAGAAGACAATCC
SAMDC	GGGCTCAGTCATTCCCACATCGTTGCTTCTCTGAGGAAGTAACTGCCCTTGATAATCA CTTCAGTAAGTTTGATATGGACAGCACAGCCTATTTGATGGGT
TIP41	GTGCTTATGAGATTGAGGGACACCCGGATGCATTGTGCTTTTGATGACAGTGCAAGC CCAGTCATTCTTCGAGAGAGCTGCTGGAGAGAAACCACATTTCAAGCATTATCTACA AAAGGGT
TUBα	CCGTGGTGATGTTGTCCCTAAGGACGTGAATGCTGCTGTTGCCACTATCAAGACCAA GCGCACCATTCAGTTTGTTGACTGGTGCCCCACTGGATTCAAGT
TUBβ	CGAGCATTGACTGTTCCTGAGCTTACCCAGCAAATGTGGGATTCAAAGAACATGATG TGTGCAGCTGACCCACGCCATGGTCGTTACCTGACTGCCTCTGCTATGTTCCGT
UBQ10	CTTAGTGCTGAGGCTGCGTGGAGGGATGCAGATTTTTGTCAAGACCTTGACTGGAA AAACCATAACCCTGGAAGTGGAGAGT
CBF	AACTTTGCGGATTCTGCTTGGCGGCTGCCGGTGCCGGCTTCCGCTGACGCTAAGGA TATACAGAAGGCTGCAGCCGAGGCGGCGGAGGCGTTCAGGCCGGCAGGGACGGA GAATGTGGAGGTGGGTTTT
Cu/Zn-SOD	TGAGGATGACCTTGGAAAAGGTGGGCATGAACTTAGTCTATCCACTGGCAATGCAG GTGGAAGATTGGCATGTGGCGTGGTTGGCTTGACTCCAGTGTAAGGTTAGCTCATG CTTGGTACCAGTGTGGTTTGCAGTATTGGCTGCTTGCTTTTG
HsfA1	ACAGCAGAGTCAAGATTTCCTGTCAGTTGCCCATCCACTGGCATTTCGGAAAATCAG TGCTCCCCTTGTGTGGTGACGGACTCGGTTA