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Evaluation of Reference Genes for Quantitative PCR in *Eustoma grandiflorum* under Different Experimental Conditions

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Abstract: *Eustoma grandiflorum*, commonly known as prairie gentian or Texas bluebells, is among the most popular agriculturally propagated species of cut flowers. Due to its widespread appeal, there is increasing interest in understanding the molecular genetic factors underlying floral development and resistance to abiotic stresses. We analyzed 18 potential reference genes in different organs, at different floral developmental stages and under drought- and salt-stress treatments, for use in RT-qPCR analysis. A total of four analytical tool packages, including geNorm, NormFinder, BestKeeper, and RefFinder were employed to determine the most appropriate reference genes under each treatment condition. The results demonstrate that different reference genes should be used for normalization under different experimental treatments. EgPP and EgPP2A2 were the most stable internal control genes across different organ types, EgPP and Eg18S were the most stable under salt-stress, EgPP and EgACT1 were the most stable across different floral development stages, and EgEF1A and EgTUA were the most stable reference genes under drought-stress. Additional gene expression analyses of EgMIXTA1, EgTOE1, and EgP5CS1 further confirmed the applicability of these reference genes. The results represent a significant contribution to future studies of reference gene selection for the normalization of gene expression in *Eustoma grandiflorum*.

Keywords: drought-stress; *Eustoma grandiflorum*; floral development; salt-stress; reference genes; RT-qPCR

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1. Introduction

Eustoma grandiflorum, commonly known as prairie gentian or Texas bluebells, are widely distributed across North America and bloom year-round [1]. This species has become a popular, high-grade cut flower variety in the international flower market due to its long vase life, diverse colors, and multitudes of flower patterns. In recent years, research on *E. grandiflorum* has gradually shifted from morphological descriptions and flowering physiology to molecular genetic analyses of floral organ development, as well as tolerance to drought-, salt-, and other abiotic-stressors [2,3].

Gene expression analysis, the basis of molecular genetic research, is important for understanding gene function. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is one of the most reliable and widely used techniques to quantify mRNA levels because its high efficiency, sensitivity, and specificity [4,5]. However, protocols vary substantially and are far from standardized, both within and between species [6,7]. The accuracy of results is easily affected by the quality and integrity of RNA [8], the quality

and quantity of template cDNA, the specificity of primers, and PCR amplification [9,10]. To avoid the influence of these factors, expression of the target gene is normalized against that of one or more internal reference genes (RGs) [11]. The ideal RG should be stably expressed across different tissues and experimental treatments. Currently, the most commonly used RGs in plants that are used for RT-qPCR assays are ubiquitin (UBQ), actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin (CYP), eukaryotic translation initiation factor 4A1 (eIF4A1), tubulin (TUB), and 18S ribosomal RNA (18S rRNA) [12–14]. However, the expression levels of some RGs have been shown to vary widely in certain biological samples. Kim et al. (2003) validated the expression levels of 18S rRNA, GAPDH, ACT, and TUB in different rice cultivars and at across different growth stages [15], while Jain et al. (2006) directly compared levels of frequently used RGs from different tissue samples in rice [16]. They found none to be suitable under every experimental condition that was examined. Therefore, for RT-qPCR, it is vital to select internal RGs and experimentally validate that they are stably expressed in different organs and under the experimental conditions that are being employed. In this regard, several statistical algorithms such as geNorm [17], NormFinder [18], BestKeeper [19], and RefFinder [20] have been proposed for assessing reference gene stability and selecting the most appropriate RGs for a given experiment.

Many species-specific RGs have been screened and validated as being stably expressed in different plant organs, including those from *Arabidopsis thaliana* [21], *Oryza sativa* [22], *Petunia hybrid* [23], *Glycine max* [24], *Amygdalus persica* [25], and *Phyllostachys edulis* [26]. A wiki-driven knowledge-base (ICG: http://icg.big.ac.cn) (accessed on 20 December 2021) has integrated experimentally validated internal controls along with their associated experimental conditions [27]. But ACT has been used as an internal control for *E. grandiflorum* studies without any preliminary tests or prior validation efforts to confirm the stability of gene expression [28–30]. Therefore, for future gene expression analyses in *E. grandiflorum*, highly reliable reference genes need to be identified for RT-qPCR result normalization

In the present report, we evaluated the expression stability of these 18 candidate RGs (EgCYP63, EgCYP20, EgACT1, EgACT2, EgUBQ, EgTUA, EgTUB1, Eg18S, EgeIF4A3, EgEF1A, EgGAPC, EgPP2A2, EgTIP4, Eg50SRP1, Eg50SRP2, EgNRPB2, EgNRPB3, and EgPP) in different organs and at different floral developmental stages of *E. grandiflorum*, and under drought- and salt-stresses using several standard algorithms, including geNorm, NormFinder, BestKeeper, and RefFinder. Finally, gene expression analyses that were performed using EgMIXTA1, EgTOE1, and EgP5CS1, were used to validate the best candidate RGs that were identified.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experimental samples, 'Double Flower Development', were collected from *Eustoma grandiflorum* cv. '620' and others were all collected from *E. grandiflorum* cv. '2003-2-2'. *E. grandiflorum* was obtained from the Northeast Forestry University (Harbin, China). They were grown in pots in a greenhouse at 22 °C and under 16 h light/8 h dark cycle.

For the analysis of reference gene expression in organ-specific tissues, *E. grandiflorum* cv. '2003-2-2' were planted in soil in pots to produce mature plant tissues for RNA extraction. The roots, stems, sepals, petals, stamens, carpels, F-leaves, and S-leaves samples were collected from flowering plants. The F-leaves and S-leaves are, respectively, the first pair of leaves and the second pair of leaves below the shoot apical meristem (Figure 1a).

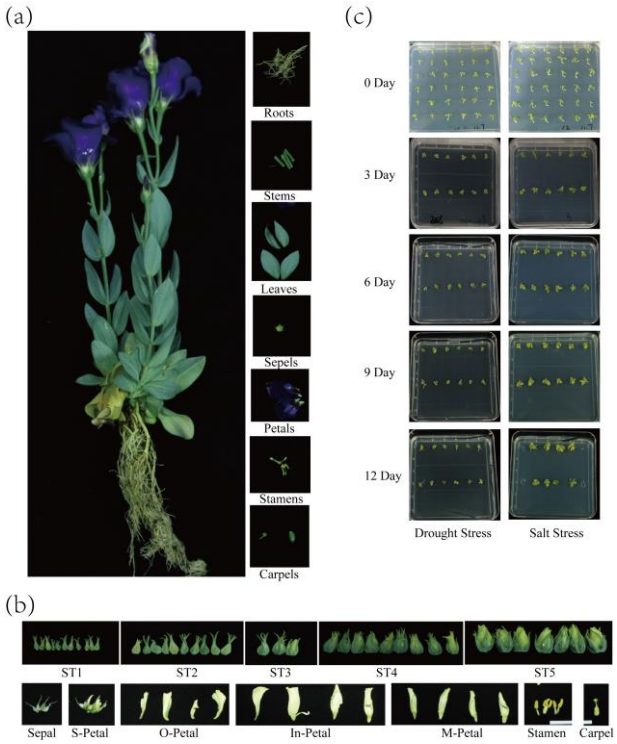

Figure 1. Plant material in *E. grandiflorum*. (**a**) Organ-specific tissues, of *E. grandiflorum* cv. '2003-2-2'. (**b**) The different floral developmental stages of *E. grandiflorum* cv. '620' and flower organ samples of ST6 developmental stage. (**c**) Seedings under two different environmental stress conditions in *E. grandiflorum* cv. '2003-2-2'.

The floral development of *E. grandiflorum* cv. '620' was divided into six stages depending on the size of the bud: ST1 = 0.3 cm, ST2 = 0.5 cm, ST3 = 0.7 cm, ST4 = 0.9 cm, ST5 = 1.2 cm, and ST6 = 2.2 cm. The floral organs, sepals, sepaloid-petals (S-petals), outer wheel petals (O-petals), middle wheel petals (M-petals), inner wheel petals (In-petals), stamens, and carpels were collected from '620' at ST6 developmental stage. In addition, buds from ST1, ST2, ST3, ST4, and ST5 developmental stages were also collected (Figure 1b). Each sample was a mixture of three biological replicates for RNA extraction.

2.2. Drought-Stress Treatment and Salinity-Stress Treatment

For two different environmental stress conditions, the seeds of *E. grandiflorum* cv. '2003-2-2' were disinfected and planted in Petri dishes with 1/2 MS medium. The seeded plates were kept at 4 °C for 2 d and then incubated at 24 °C with a photoperiod of 16 h white light for 4 weeks to grow aseptic seedlings. These 4-week-old seedlings that reached the 4–6 leaf stage, with consensus growth status were selected and treated with drought- and salt-stress. For drought-stress, the seedlings were transplanted into 1/2 MS medium with 30% PEG6000 (*w*/*v*, Shenggong, China). For salt-stress, the seedlings were transplanted into 1/2 MS medium with 300 mM NaCl (Shenggong, China). Entire seedlings were collected at 0, 3, 6, 9, and 12 days from the onset of treatment (Figure 1c). Each sample was a mixture of eight biological replicates.

2.3. Total RNA Extraction and cDNA Synthesis

The total RNA was extracted from all the plant samples using TRIzol reagent (Thermo Fisher, Beijing, China) and using Phasemaker Tubes (Thermo Fisher) to increase the RNA yield and purity. The RNA integrity was assessed by gel electrophoresis. Then, the concentration and purity of the RNA samples were determined using a Nanodrop 2000C Spectrophotometer (Thermo Fisher). Then, we added 1 μg aliquot of total RNA to reverse-

transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biomedical Technology, Beijing, China). First strand cDNA was synthesized using oligo d(T) primer.

2.4. Primer Designing and Primer Efficiency Analysis

The gene sequences of these 18 candidate RGs that were obtained from the transcriptome data were used to design primers for qPCR analysis. (unigene sequences are listed in Figure S1). The primers for each gene were designed using the RT-qPCR primer design website (https://sg.idtdna.com/pages) (accessed on 25 May 2021). To detect the specificity and amplification efficiency of each pair of primers that we designed, cDNA was reverse-transcribed from the 1 μg RNA of leaves was used as a template. Then, a 10-fold concentration dilution series (1, $1/10^1$, $1/10^2$, $1/10^3$) of each cDNA sample was made and used as template for RT-qPCR to generate standard and dissolution curves. A total of three technical replicates were used to ensure accuracy. The PCR primer efficiency (E) was calculated according to the formula: $E = [10^{(-1/S)} - 1] \times 100\%$, where S is the slope of the standard curve [31]. The RT-qPCR experimental method is as follows.

2.5. RT-qPCR

Equal amounts of cDNA that were derived from each sample were used as template, and RT-qPCR for each of the 18 RGs was performed in triplicate on a LightCycler 480 II system (Roche Diagnostics, West Sussex, United Kingdom). The 10 μL reaction mixture consisted of 5 μL SYBR *Premix Ex Taq* (TaKaRa) (2×), 0.8 μL primer mix (each primer 10 μM), and 4.2 μL cDNA as a template (The cDNA was diluted 20-fold). For each gene, a no template control was included using water instead of cDNA as template for evaluation of contaminations. The following thermocycling program was used: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing at 58 °C for 30 s. A gradient dissolution curve analysis consisting of 65–95 °C with a 0.5 °C increase per second was run at the completion of the last cycle. The melting curve was analyzed to determine the primer specificity. All the reactions were performed three times. The means of three technical replicates were used as the final quantification value.

2.6. Reference Gene Stability Analysis

To select the appropriate internal controls, the stabilities of candidate RGs were statistically analyzed using geNorm, NormFinder, and BestKeeper, and then ranked by RefFinder according to the following parameters. The gene expression data (Ct value) was converted to a relative level (Q) for input into the geNorm and NormFinder programs using the following equations, respectively: $Q = 2^{-\Delta Ct}$ and $\Delta Ct = Ct_{min} - Ct$, where Ct_{min} is the smallest Ct value for all the genes in all samples, and Ct is the Ct value of the gene in each sample. Accordingly, the Q values were calculated using Excel for later analysis by the geNorm and NormFinder programs, and the ΔCt values were used in the BestKeeper program for stability analysis. Finally, the RefFinder program was used for comprehensive evaluation of the genes using the three algorithms.

2.7. Validation of Reference Gene Stability

To validate the stability of the recommended candidate RGs, the expression levels of the EgMIXTA1, EgTOE1, and EgP5CS1 genes were quantified using two of the most stable and least stable candidates as RGs across the four experimental sets. These genes are important for horticultural trait research of ornamental plants. First, we verified the expression of the EgMIXTA1 gene, a transcription factor that is required for the development of petal trichomes, in different organ types. EgTOE1, which is involved in the formation and development of flower organs, was chosen as the positive test gene in developmental stages a of double flowers, while EgP5CS1, which is associated with proline accumulation following abiotic stress, was chosen as the positive test gene in the drought- and salt-stress experimental samples. The relative transcript level was calculated with the $2^{-\Delta\Delta Ct}$ method. The primer sequences of EgMIXTA1, EgTOE1, and EgP5CS1 used for RT-qPCR are listed in Table S1.

3. Results

3.1. Selection of Candidate Reference Genes and Primer Specificity and Efficiency Analysis

Some candidate RGs have been reported as top internal control genes ranked in ICG and, as such, were considered for this study. Sequences for internal RG candidates from *Arabidopsis thaliana* were used as queries to search the local transcriptome database of *E. grandiflorum* using the TBLASTN algorithm [32]. Homologous sequences were then used to query the NCBI protein database with BLASTX. Ultimately, we selected 18 RGs (EgCYP63, EgCYP20, EgACT1, EgACT2, EgUBQ, EgTUA, EgTUB1, Eg18S, EgeIF4A3, EgEF1A, EgGAPC, EgPP2A2, EgTIP4, Eg50SRP1, Eg50SRP2, EgNRPB2, EgNRPB3, and EgPP) as candidate RGs (Table 1).

Table 1. Characteristics of candidate reference genes of *E. grandiflorum*.

Gene	Homologous Gene Name and Accession Number	Definition	Gene ID	Primer Sequence (5′→3′)	Product Size (bp)	Amplification Efficiency (%)	R^2
EgCYP63	*NtCYP63* XM_009602865.3	*Nicotiana tomentosiformis* peptidyl-prolyl cis-trans	c88166.graph	F: GCACTGGTGGAGAGAGTATTT R: TGGTGTTTGGACCGCTATT	108	96.9	1
EgCYP20	*VvCYP20* XM_002277727.4	*Vitis vinifera* peptidyl-prolyl cis-trans isomerase	c71397.graph	F: GGGAAGCCTCTTCACTACAAA R: GATTCTCCACCTCTGCCATC	104	96.1	1
EgACT1	*NtACT7* XM_016658880.1	*Nicotiana tabacum* actin-7	c90884.graph_c1	F: AGGCCGTGCTATCTCTCTAT R: GAGCATAACCCTCGTAGATTGG	102	98.1	0.999
EgACT2	*NtACT7* XM_016658880.1	*Nicotiana tabacum* actin-7	c90884.graph_c2	F: CTCGTCCCTTGAGAAGAACTATG R: CGATCATGCTAGGCTGGAAA	104	99.1	0.996
EgUBQ	*CeUBQ* XM_027328677	*Coffea eugenioides* ubiquitin-NEDD8-like protein RUB2	c67028.graph	F: GGTGAAGACTCTTACTGGAAAGG R: CTCTTCCACCCGCTCTTTAAT	85	95.3	0.997
EgTUA	*CaTUA* XM_027217029.1	*Coffea arabica* tubulin alpha-3 chain	c90759.graph	F: CTGCAGGAGATCTCTGGATATTG R: CCATCAAACCGCAAAGAAGTG	102	96.4	0.999
EgTUB1	*SiTUB* XM_011077861.2	*Sesamum indicum* tubulin beta-1 chain	c76703.graph	F: CGCTTCCCTGGTCAACTAAA R: GTGGAGCAAATCCAACCATAAAG	129	95.0	0.999
Eg18S	*Ca18S* XM_027251242.1	*Coffea arabica* 18S rRNA	c62153.graph	F: TCTCTGCTGTTCAGTGGTTATG R: GCTCCTCTTGCCAAACTTCTA	106	95.6	0.998
EgeIF4A3	*CeEIF4A3* XM_027292256.1	*Coffea eugenioides* eukaryotic initiation factor 4A-3	c85413.graph	F: AGTTCGATACACTCTGCGATTT R: GTCAGCCAGTCAACCTTTCT	91	94.2	0.999
EgEF1A	*CaEF1A* XM_027252439.1	*Coffea arabica* elongation factor 1-alpha	c85993.graph	F: GGATATGCTCCAGTCCTTGATT R: GGTTCCTTCTCAAGCTCCTTAC	107	98.6	0.995
EgGAPC	*CeGAPC* XM_027295750.1	*Coffea eugenioides* glyceraldehyde-3-phosphate dehydrogenase 2	c88087.graph	F: TAAGGAGGAGTCAGAGGGTAAG R: GATCTGCTATCACCGACAAAGT	90	96	0.999
EgPP2A2	*SiPP2A* XM_011095100.2	*Sesamum indicum* serine/threonine-protein phosphatase PP2A-2	c88536.graph	F: GTGGCAACATGGCATCTATTT R: GTAACATCAGGCTCTCCTCTTC	97	93.7	1
EgTIP4	*CeTIP4* XM_027304349.1	*Coffea eugenioides* TIP41-like protein	c77390.graph	F: CGGAGCAAGTGATTCTGGATTA R: CTGAACAGTCCTCCCAACATAC	138	94.5	1
Eg50SRP1	*Ha50SRP* XM_022173943.2	*Helianthus annuus* 50S ribosomal protein L3	c82916.graph	F: ATCCAGCAAGGGTTTCCTTTA R: CTTGGCCATCCTTTCCTAACT	99	98.4	0.999
Eg50SRP2	*Ca50SRP* XM_027265126.1	*Coffea arabica* 50S ribosomal protein L3-2	c85685.graph	F: CGGTTCAAGTTGGGTATAGGAG R: GAAGATGCCGAAGAGGGATTAT	97	93.4	1
EgNRPB2	*CaNRPB* XM_027311445.1	*Coffea arabica* DNA-directed RNA polymerase II subunit RPB2	c85245.graph	F: TGGGAAGACTACGCCAATTAC R: CTGGTGCTGTGATCCTTCTT	90	98.05	0.997
EgNRPB3	*PaNRPB* XM_035072035.1	*Populus alba* DNA-directed RNA polymerases II, IV and V	c92646.graph	F: GACTTGGCTTGATTCCTCTCTT R: AATACTCGCACTGTCCATCAC	112	97.49	0.995
EgPP	*CePP* XM_027312588.1	*Coffea eugenioides* threonine-protein phosphatase	c86598.graph	F: ATGGTGGTCTCTCTCCAGAT R: TCCACATAAGGTCACAGAAAGG	102	95.6	0.998

The primers for each gene were designed using the RT-qPCR primer design website (https://sg.idtdna.com/pages) (accessed on 25 May 2021). They produced RT-qPCR standard curves, R^2 was 0.995~1, and the amplification efficiency ranged from 93.4% to 99.1% (Table 1). In addition, the RT-qPCR results using these primer pairs showed that the melting curves of all the genes yielded a single peak (Figure 2), indicating that each pair can be used to specifically amplify the desired gene in a reproducible manner. Therefore, all the primer sets were highly specific and efficient for use in RT-qPCR analysis.

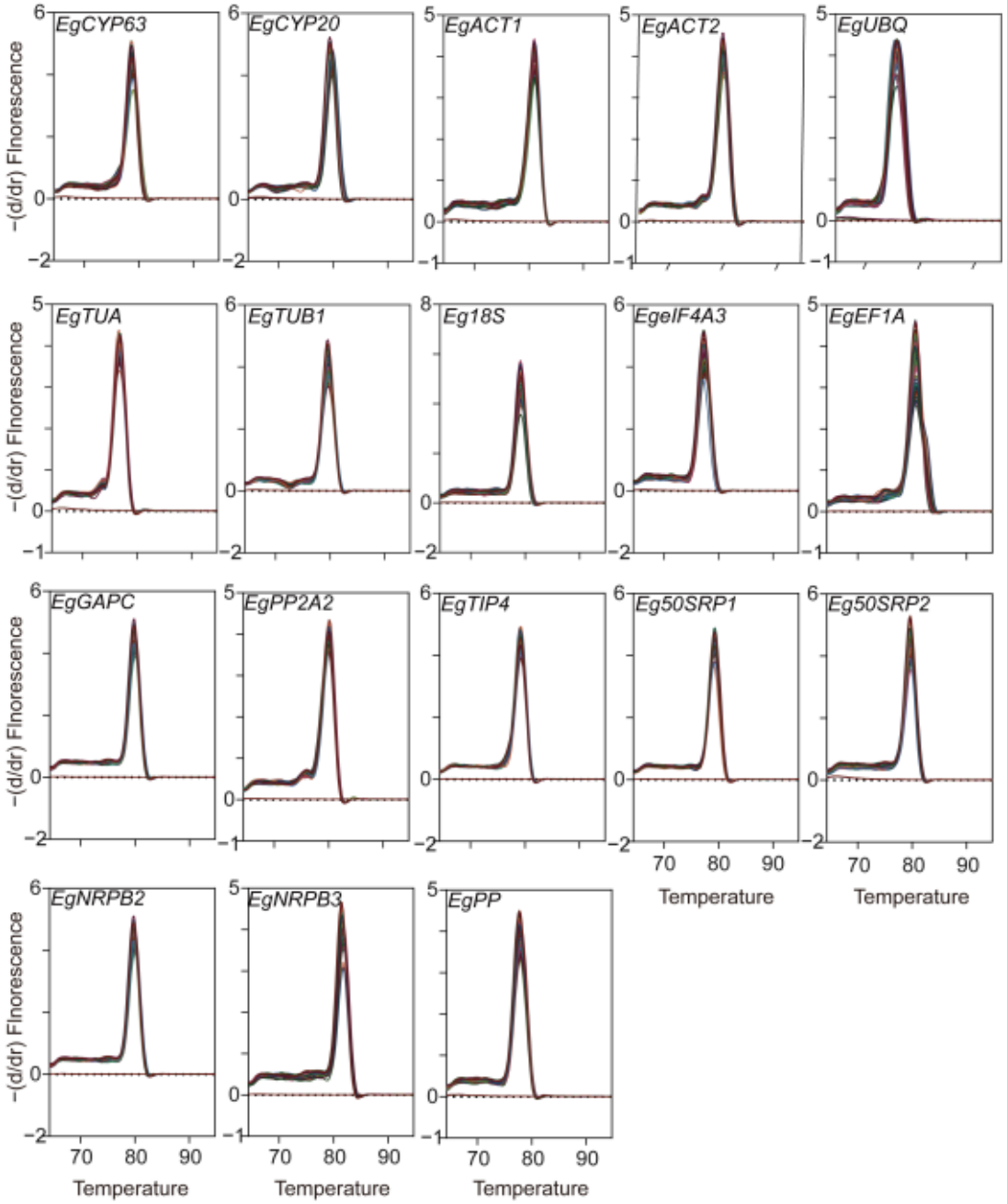

Figure 2. Melting curves of candidate reference genes and negative control. This analysis of melting curves was using LightCycler 480 software 1.5.1.62.

3.2. Analysis of Candidate Reference Genes Ct (Cycle Threshold) Values

The Ct values that were obtained by RT-qPCR indicate the number of cycles that are required for the signal to cross the detection threshold, exceeding the indicated background. Thus, the lower the Ct value, the more abundant the gene target is in a given sample. The variation in Ct values of all the samples indicated that the expression levels of the tested RGs differed very little among the different samples and showed that all the candidate RGs were transcribed at a moderate level (Figure 3).

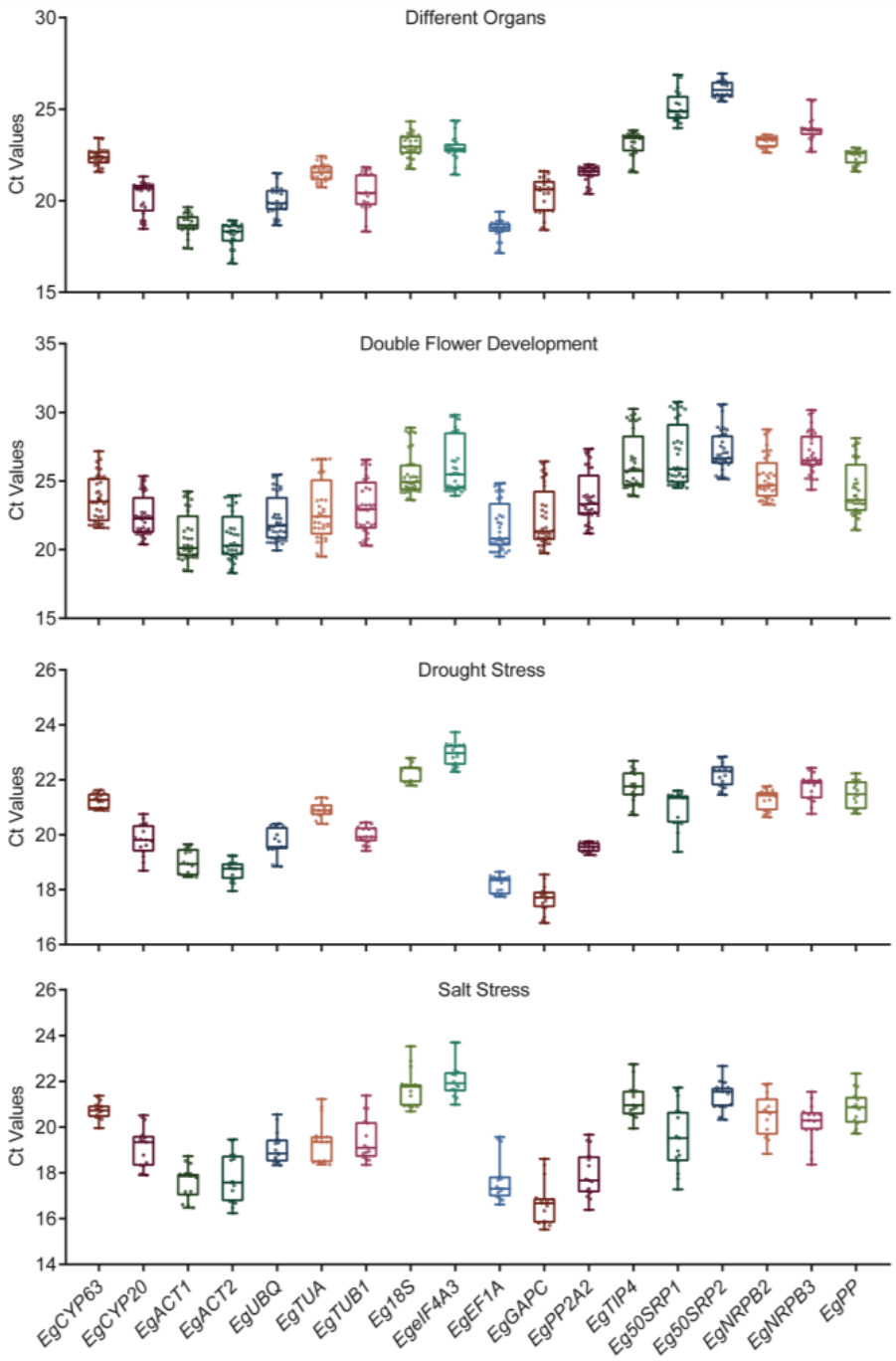

Figure 3. The cycle threshold (Ct) values of 18 candidate RGs across all samples. The final Ct value for each sample is the average of three technical replicates.

As shown in Figure 3, the Ct values of the 18 candidate RGs ranged from 16.48 (*EgACT1*) to 30.59 (*Eg50SRP2*) in the samples that were derived from four different treatments. None of these candidate RGs had a completely stable expression pattern across all the sample types. The fact that the expression level of the candidate genes varies greatly in different samples types indicates that these genes may be not suitable as standard RGs for RT-qPCR normalization under different experimental conditions. Therefore, it was necessary to pre-validate a set of potential RGs in each particular experiment.

3.3. Stability Ranking of Candidate Reference Genes

3.3.1. GeNorm Analysis

The geNorm program is used to determine the most stable RGs by calculating the average expression stability value (*M*) [17]. Lower *M* values indicate higher RG stability, i.e., the most stable RGs have the lowest *M* values, while the least stable have the highest. The ranking order of the *M* values of the RGs in Figure 4 shows that the *M* values for all the tested genes were less than 0.9. EgPP2A2 (*M* = 0.257) and EgNRPB2 (*M* = 0.272) had the highest expression stability with the lowest *M* values, while EgTUB1 had a least stable expression with highest *M* values (0.705) in different organs. EgPP (*M* = 0.28) and EgACT1 (*M* = 0.274) were identified as the most stable genes in double flower development, while EgCYP63 (*M* = 0.834) was the least stable. For drought- and salt-stress, Eg50SRP1 (*M* = 0.403; *M* = 0.513) was identified as the most unstable gene; EgEF1A (*M* = 0.146) and EgeIF4A3 (*M* = 0.149) had the lowest *M* values under drought stress, while Eg18S (*M* = 0.150) and EgPP (*M* = 0.135) had the lowest under salt-stress.

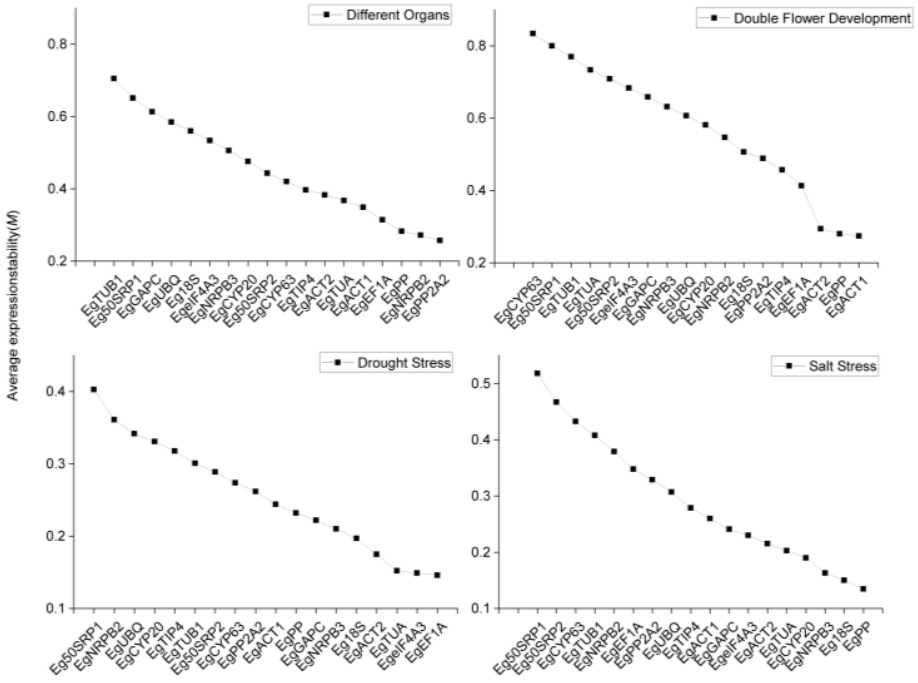

Figure 4. The mean expression stability (*M*) of 18 candidate RGs of *E. grandiflorum* using geNorm software.

geNorm also provides pair–pair comparisons of candidate RGs (Vn/n+1) to determine the optimal number of RGs that are required to ensure the accurate and reliable normalization of RT-qPCR data. Where V (Vn/n+1) is less than 0.15 indicates that n RGs are sufficient to serve as an internal control for RT-qPCR normalization under a particular experimental condition. The optimal number of reference targets in these four experimental situations is two (geNorm V < 0.15 when comparing a normalization factor that is based on the two or three most stable targets) (Figure 5).

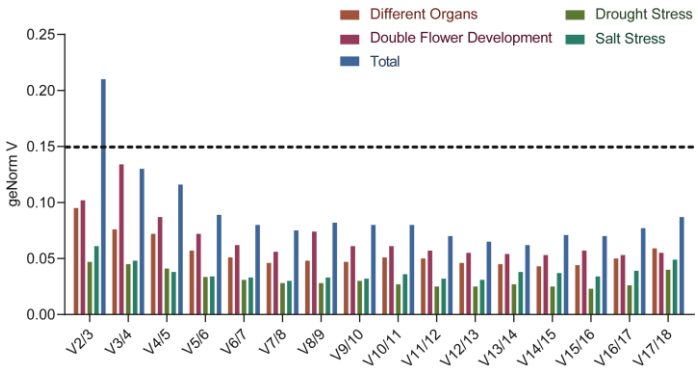

Figure 5. Pairwise variation (Vn/Vn+1) analysis of the candidate reference genes. V-values were calculated by normalization factors NFn and NFn+1, suggesting the optimum number of reference genes for normalization.

3.3.2. NormFinder Analysis

NormFinder is a Visual Basic Application (VBA) for Microsoft Excel that is based on a calculation that is similar to that of geNorm [18]. NormFinder calculates *M* values using the relative expression level (Q) to determine the expression stability of candidate RGs. However, the program can only determine the best RG within a given set and that with the lowest *M* value is considered the most stable. Expression analysis by different organs showed higher stability of the EgEF1A gene. In contrast, EgACT1 was ranked as the best reference gene for double flower development, EgEF1A for drought-stress, and Eg18S for salt-stress, when analyzed separately (Figure 6).

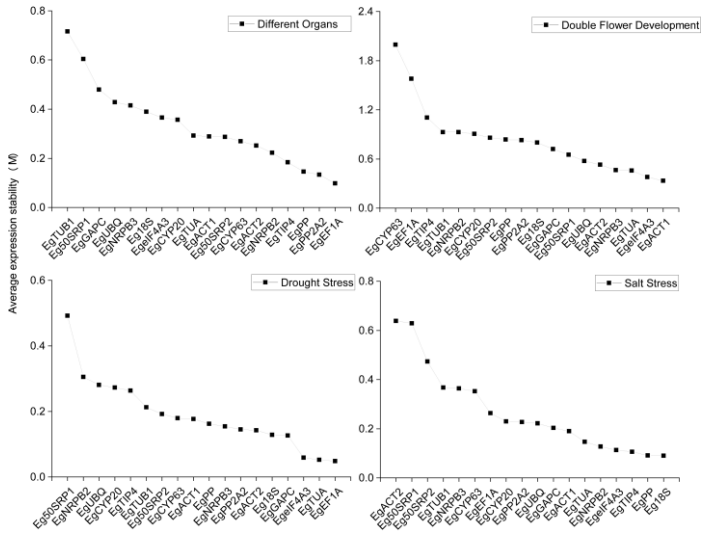

Figure 6. Average expression stability (*M*) of the candidate RGs of *E. grandiflorum* that were determined by NormFinder.

3.3.3. BestKeeper Analysis

The Excel-based BestKeeper program automatically calculates the standard deviation (SD) of Ct values and the coefficient of variation (CV) between each reference gene using the geometric mean of all the candidate RGs Ct values. The most stable RG will have the lowest coefficient of variance and standard deviation (CV ± SD). The expression of the candidate gene is unstable when SD > 1, indicating that the given gene is not suitable as a reference gene. EgNRPB3 and EgPP showed the most stable expression in different organs. EgNRPB2 and EgPP had the most stable expressions in double flower development sets. For drought- and salt-stress, the BestKeeper algorithm showed that EgCYP63 had

the most stable expression. These results were somewhat different from the geNorm and NormFinder results (Table 2).

Table 2. BestKeeper stability analysis of the candidate RGs of *E. grandiflorum*.

Rank	Different Organs		Double Flower Development		Drought Stress		Salt Stress	
	Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD	Gene	CV ± SD
18	*EgTUB1*	4.13 ± 0.85	*EgCYP63*	5.81 ± 1.37	*Eg50SRP1*	2.80 ± 0.91	*Eg50SRP1*	6.20 ± 1.21
17	*EgGAPC*	4.03 ± 0.82	*Eg18S*	7.14 ± 1.01	*EgCYP20*	2.25 ± 0.99	*EgACT2*	5.29 ± 0.94
16	*EgCYP20*	3.89 ± 0.79	*EgTUB1*	7.25 ± 0.89	*EgGAPC*	2.25 ± 0.96	*EgPP2A2*	4.83 ± 0.87
15	*EgUBQ*	3.24 ± 0.65	*EgeIF4A3*	5.79 ± 0.81	*EgACT1*	2.17 ± 0.97	*EgGAPC*	4.22 ± 0.71
14	*EgACT2*	3.14 ± 0.57	*Eg50SRP1*	6.86 ± 0.72	*EgUBQ*	2.14 ± 0.85	*EgTUB1*	4.22 ± 0.82
13	*Eg50SRP1*	2.68 ± 0.67	*EgGAPC*	7.10 ± 0.62	*EgTIP4*	2.03 ± 0.96	*EgEF1A*	4.20 ± 0.74
12	*EgACT1*	2.50 ± 0.47	*EgACT2*	8.04 ± 0.55	*EgNRPB3*	1.92 ± 0.79	*EgCYP20*	4.16 ± 0.79
11	*Eg18S*	2.42 ± 0.56	*Eg50SRP2*	7.11±0.57	*EgACT2*	1.79 ± 0.78	*EgTUA*	3.76 ± 0.73
10	*EgTIP4*	2.38 ± 0.55	*EgTUA*	6.08 ± 0.72	*EgPP*	1.74 ± 0.86	*EgNRPB3*	3.54 ± 0.73
9	*EgeIF4A3*	2.04 ± 0.47	*EgUBQ*	5.41 ±0.56	*EgEF1A*	1.54 ± 0.76	*EgACT1*	3.51 ± 0.62
8	*EgEF1A*	2.02 ± 0.37	*EgTIP4*	6.08 ± 0.34	*Eg50SRP2*	1.53 ± 0.80	*EgNRPB2*	3.51 ± 0.71
7	*EgTUA*	2.00 ± 0.43	*EgEF1A*	5.91 ± 0.43	*EgeIF4A3*	1.47 ± 0.77	*EgTIP4*	3.24 ± 0.69
6	*EgNRPB2*	1.96 ± 0.47	*EgCYP20*	5.81 ± 0.44	*EgNRPB2*	1.33 ± 0.82	*EgPP*	2.96 ± 0.62
5	*EgCYP63*	1.81 ± 0.41	*EgNRPB3*	5.72 ± 0.45	*EgTUB1*	1.32 ± 0.79	*EgUBQ*	2.90 ± 0.55
4	*EgPP2A2*	1.73 ± 0.37	*EgACT1*	5.20 ± 0.46	*Eg18S*	1.27 ± 0.68	*Eg18S*	2.85 ± 0.62
3	*Eg50SRP2*	1.69 ± 0.44	*EgPP2A2*	5.20 ± 0.37	*EgTUA*	1.13 ± 0.48	*EgeIF4A3*	2.78 ± 0.61
2	*EgPP*	1.53 ± 0.34	*EgPP*	3.91 ± 0.55	*EgPP2A2*	0.76 ± 0.33	*Eg50SRP2*	2.44 ± 0.52
1	*EgNRPB3*	1.21 ± 0.28	*EgNRPB2*	3.91 ± 0.44	*EgCYP63*	0.74 ± 0.26	*EgCYP63*	1.39 ± 0.29

3.3.4. RefFinder Analysis

As expected, the use of geNorm, Normfinder, and Bestkeeper resulted in different gene stability rankings given that they are based on distinct statistical algorithms. The RGs were reordered by RefFinder (Figure 7). Combining the ranking results above, the final ranking confirms EgPP and EgPP2A2 as the most stable genes in different organs. EgPP and EgACT1 were the most stable genes in double flower development. For drought stress, EgEF1A and EgTUA were the most stable genes. EgEF1A and EgTUA were the most suitable RGs under salt-stress.

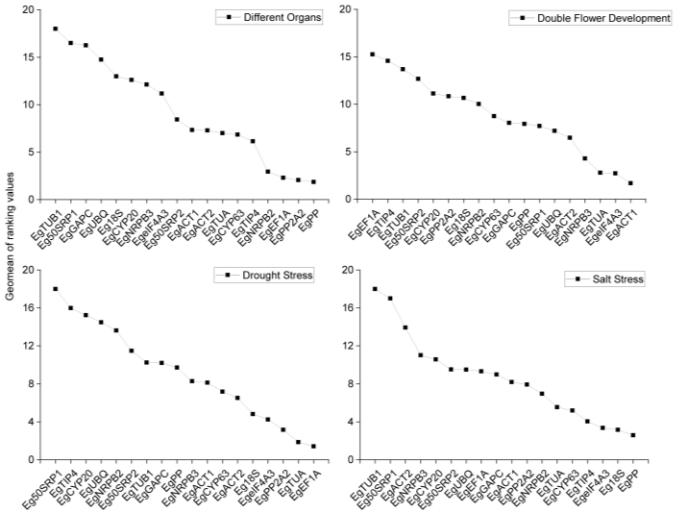

Figure 7. Average expression stability (*M*) of the candidate RGs of *E. grandiflorum* determined by RefFinder.

3.4. Validation of Candidate Reference Gene Stability

To validate the reliability of the best RGs determined in this study, the most stable and unstable RGs were selected to validate the expression of the EgMIXTA1, EgTOE1, and EgP5CS1 genes (Figure 8).

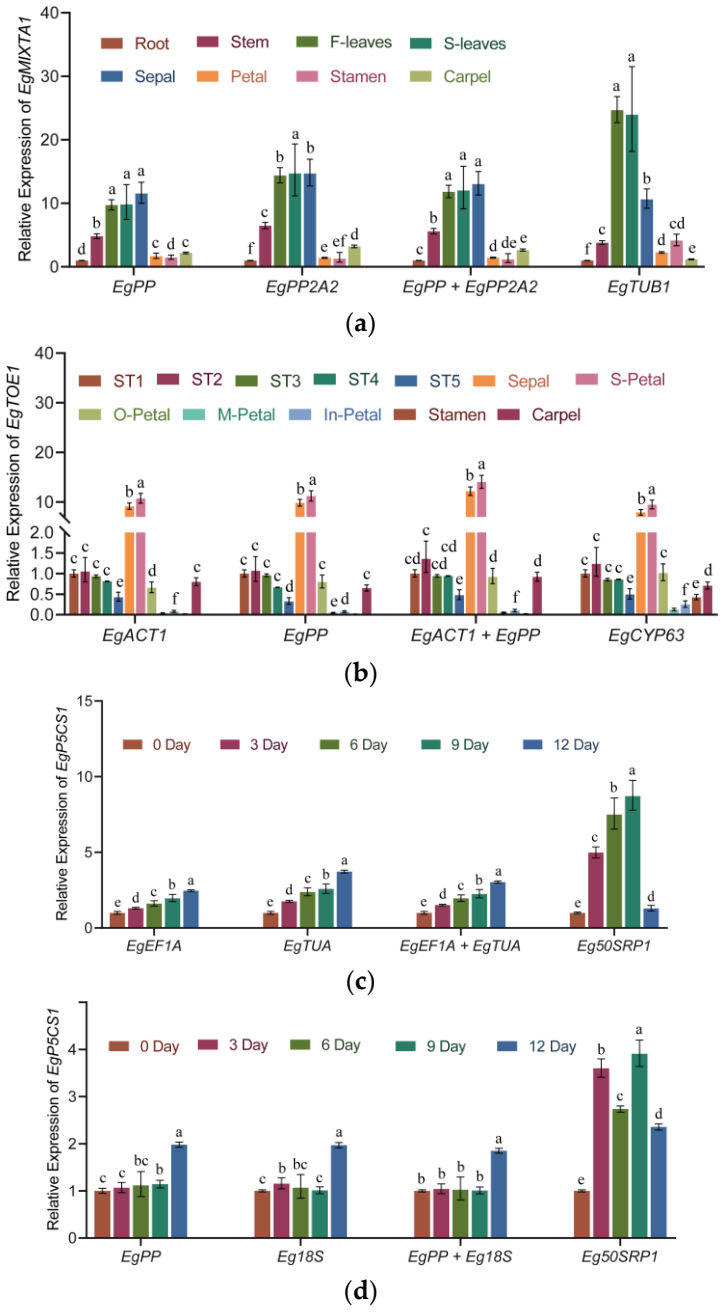

Figure 8. The relative expression of EgMIXTA1, EgTOE1, and EgP5CS1 in *E. grandiflorum*. The results were normalized in sample sets across treatments with (**a**) different organs, (**b**) different floral developmental stages, six stages depending on the size of the bud: ST1 = 0.3 cm, ST2 = 0.5 cm, ST3 = 0.7 cm, ST4 = 0.9 cm, ST5 = 1.2 cm, and ST6 = 2.2 cm. The floral organs, sepals, sepaloid-petals (S-petals), outer wheel petals (O-petals), middle wheel petals (M-petals), inner wheel petals (In-petals), stamens, and carpels were collected from '620' at ST6 developmental stage. (**c**) The drought-stress treatment, and (**d**) the salt-stress treatment. The gene expression levels were calculated individually using different genes as reference, as indicated using the $Q_t = 2^{-\Delta\Delta Ct}$ method. The relative expression levels of EgMIXTA1 of the roots were considered as controls; The relative expression levels of EgTOE1 at ST1 developmental stage were considered as controls; The relative expression levels of EgP5CS1 in 0 days after the stress treatment were considered as the controls. RT-qPCR analysis for each sample was done in triplicate. Different lowercase letters indicate significant differences between treatments, and the same means that there were no such differences ($p < 0.05$, ANOVA).

EgMIXTA1 was demonstrated to function quite similarly to the MIXTA-like MYB TFs that are involved in determining cuticular wax formation in *E. grandiflorum* leaves [33]. In different organs of *E. grandiflorum*, the relative expression levels of EgMIXTA1 standardized by EgPP were similar when EgPP2A2 was used as an internal reference. In contrast, the relative expression of EgMIXTA1 was higher in the leaves and significantly increased relative to the sepals when EgTUB1 was used as the internal control (Figure 8a).

EgTOE1 is related to the euAP2 family. It was expressed in all flower organs but its levels decreased with the number of flower organs from the outside to the inside of double-petaled flowers. Its expression level also decreased with the developmental stage. When EgACT1, EgPP, and EgCYP63 were used for normalization in the double flower development samples, only EgACT1 and EgPP were found to be suitable for normalization of the EgTOE1 transcript expression trends. Using EgCYP63 as the reference gene resulted in EgTOE1 having abnormally high expression in the stamens (Figure 8b).

It was demonstrated that salt- and drought-stress induced EgP5CS1 expression, as well as proline accumulation [34]. Under drought- and salt-stress, when the most stable RGs (EgEF1A and EgTUA for drought; EgPP and Eg18S for salt) were used as RGs, the expression level of the EgP5CS1 gene increased gradually with stress treatment exposure and reached its peak at day 12. Conversely, the expression level was overestimated and showed a significant downward trend at day 12 when calculated using the least stable genes (Eg50SPR1) (Figure 8c,d).

These data show that the results of relative gene expression analysis of a target gene are strongly affected by the choice of reference gene(s) that are used.

4. Discussion

Currently, transcriptome sequencing has been widely used for gene analysis in different samples from various species. Based on transcriptome data, we can obtain gene sequences and gene expression levels, but in some cases, there will be some false positive results, so further validation is required. RT-qPCR, considered the gold standard, is commonly used to determine gene expression levels and verify transcriptome data. In most studies, RGs are differentially expressed in different tissues, organs, stages of development, and under different experimental conditions. Therefore, a set of potential RGs must be pre-validated in each particular experiment. Up to now, there have been no reports on the comparison and selection of RGs for *E. grandiflorum*, which limits further study of gene expression in this species. Reference genes are generally conserved housekeeping genes that are necessary for the survival of living organisms. According to the stable ranking of RGs integrated by the ICG website, 18 genes were selected as candidate RGs under drought- and salt-stress treatment based on RNA-seq data. In addition, we also evaluated the expression stability of these candidate RGs in different organs and at different developmental stages of *E. grandiflorum*. Our results show that the ranking that was calculated by the three evaluation programs, which are based on different algorithms, are incongruent. Therefore, we should combine the rankings that were obtained from each analysis to determine the suitable RGs. The optimal number of reference genes that are required can be determined using paired variants of geNorm. A total of two RGs are sufficient for standardization in each sample group above, but more RGs are needed for standardization in all treatments.

As we expected, many candidate RGs are not suitable for standardized gene expression in certain experiments. Surprisingly, ACT, although the most commonly used reference gene, was not the best in the current analysis. Our laboratory previously used ACT as an internal reference for gene expression analysis of EgMIXTA1 and found inconsistencies in the experimental results. MIXTA is a TF that is required for the development of petal trichomes, the formation of the conical shape of petal epidermal cells and is involved in regulating epidermal cell morphology and cuticle biosynthesis [35]. In contrast, ACTIN is a major constituent of the cytoskeleton and involved in a wide range of biological processes and structures in cells. For instance, ACT7 is involved in root growth and architecture, epidermal cell specification, and cell division, while ACT2 and ACT8 are essential for

root hair tip growth [36]. Therefore, we speculate that ACT affects the expression of the EgMIXTA1 gene and is, therefore, not a suitable reference gene in this instance.

These results validate the need for determining the best reference gene before starting gene expression studies in a particular species, rather than blindly selecting a commonly used reference gene. To verify the accuracy of the reference genes that were selected in this study, we evaluated the expression profiles of EgMIXTA1, EgTOE1, and EgP5CS1 in different samples. The present results for target gene expression, using the two most stable reference genes for standardization separately, were consistent. When we repeated this analysis with the most unstable reference gene as an internal control, the expression pattern of the target genes was different. These results further confirm prior observations that using unstable reference genes result in the generation of misleading target gene expression profiles.

5. Conclusions

Validation of the candidate reference genes that were obtained from RNA-seq data for RT-qPCR normalization under different treatment conditions of *E. grandiflorum* was carried out. A total of 18 RGs were both evaluated and ranked using geNorm, BestKeeper, NormFinder, and RefFinder. Our data showed the differential expression of all RGs in different organs, at different floral developmental stages, and under drought- and salt-stresses. EgPP and EgPP2A2 were the best internal control genes in different organs; EgPP and EgACT1 were the best regardless of developmental stage; EgPP and Eg18S were the best under salt-stress; and EgEF1A and EgTUA were identified to be the most stable internal control genes under drought-stress. The expression patterns of EgMIXTA1, EgTOE1, and EgP5CS1 also confirmed this finding. This essential information for RT-qPCR data normalization can now be used with confidence for the study of gene expression in *E. grandiflorum*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8020164/s1, Figure S1: Unigene sequences of 18 reference genes; Table S1: Primer sequences of EgMIXTA1, EgTOE1, and EgP5CS1 for RT-qPCR.

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Data Availability Statement: The transcriptome datasets that were analyzed during the current study are available in the SRA (SRA data: PRJNA750819, https://www.ncbi.nlm.nih.gov/sra/PRJNA750819, accessed on 10 January 2022) repository. The public access to the databases is open. The datasets supporting the conclusions of this study are available by contacting the corresponding author (summerzhang@126.com). The sequence data of reference genes that were used and analyzed during this study are available in the NCBI (https://www.ncbi.nlm.nih.gov, accessed on 20 January 2022) and UniProt (https://www.uniprot.org, accessed on 15 January 2022) database.

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