Phyton-
International Journal of
Experimental Botany

Tech Science Press

DOI: 10.32604/phyton.2022.018767

ARTICLE

Selection of Stable Reference Genes for Quantitative Real-Time PCR on *Paeonia ostii* T. Hong et J. X. Zhang Leaves Exposed to Different Drought Stress Conditions

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Received: 16 August 2021 Accepted: 27 October 2021

ABSTRACT

The definition of relatively stable expressed internal reference genes is essential in both traditional blotting quantification and as a modern data quantitative strategy. Appropriate internal reference genes can accurately standardize the expression abundance of target genes to avoid serious experimental errors. In this study, the expression profiles of ten candidate genes, *ACT1*, *ACT2*, *GAPDH*, *eIF1*, *eIF2*, *α-TUB*, *β-TUB*, *TBP*, *RNA Pol II* and *RP II*, were calculated for a suitable reference gene selection in *Paeonia ostii* T. Hong et J. X. Zhang leaves under various drought stress conditions. Data were processed by the four regularly used evaluation software. A comprehensive analysis revealed that *RNA Pol II* was the most stable gene and *eIF2* was the least stable one. In addition, the geNorm program provided the optimal choice of two reference gene combination, *RNA Pol II* and *β-TUB*, for qRT-PCR normalization in *P. ostii* subjected to different drought stress levels. Our research provided convenience for gene expression analysis in *P. ostii* under drought stress and promoted research of effective methods to alleviate *P. ostii* drought stress in the future.

KEYWORDS

Paeonia ostii T. Hong et J. X. Zhang; reference gene; qRT-PCR; drought stress

1 Introduction

The gene expression level analysis has been widely used in different research areas and species [1–6] to screen key genes, confirm specific regulatory mechanisms, as well as to study new gene functions. Quantitative real–time PCR (qRT–PCR) is predominant in different research fields and has been widely applied due to its high accuracy, good specificity, and high throughput at the mRNA level [7,8]. However, many restricting factors influenced the accuracy of results analyzed by qRT-PCR, including quality and integrity of RNA samples, efficiency in complementary DNA (cDNA) synthesis, primer specificity, and overall transcriptional activity of the tissue or cells [9–11]. Thus, with the aim to acquire precise experimental results, the target gene's expression abundance must be corrected with reliable internal reference genes (housekeeping genes) to acquire normalized expression levels [9,12,13].

Some traditional housekeeping genes, such as actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 18S ribosomal RNA (*18S rRNA*), elongation factor 1α (*EF-1α*), α-tubulin (*α-TUB*) and β-tubulin (*β-TUB*), etc., have often been used as expression correction tools due to their stable expressions in previous studies [14,15]. Theoretically, these traditional reference genes maintain the most basic cellular activities and their expression levels are not affected by the external environment. However, many researches revealed that the common housekeeping genes presented different expression modes in different species as well as under different conditions [16,17]. The *RPS2* (ribosomal protein S) and *UBQ2* (ubiquitin) genes were validated as the two most suitable reference genes in various tissues, across different developmental stages and under different stresses in banana (*Musa acuminata* AAA Group, cv. Carven-dish) fruits [18]. In *Eremosparton songoricum* (Litv.) Vass., *EsUBQ* could only be used as a reference gene across multiple adult tissue samples, and *EsEF* and *EsACT* were the most stable reference genes for samples of differing germination stages [19]. The *ACT* and *CACs* (clathrin adaptor complexes medium subunit family protein) genes were identified as the most suitable combination for gene expression correction in several tissues of different collection dates in cork oak (*Quercus suber* L.) [20]. Similarly, common reference genes may display various expression patterns under different abiotic stresses [21]. Also, using unstable internal reference genes will have great impacts on the target gene expression level analysis and cause erroneous experimental results. In *Kosteletzkya virginica* (L.) Presl., *18S rRNA* was identified to be the most stable reference gene under salt stress [22]. In herbaceous peony (*Paeonia lactiflora* Pall.) under light stress, *ETI* (eukaryotic translation initiation factor 5A-2) was recommended to be the most stable gene in the flowers, and *ACT* and *pp2A* (serine/threonine protein phosphatase 2A) showed the least expression variations in stems [23]. Therefore, it is very important to choose relatively stable housekeeping genes according to specific experimental conditions, especially under specific abiotic stresses, before their use in qRT-PCR normalization. Up to now, several housekeeping genes were identified in different species under various abiotic stresses, such as in goosegrass (*Eieusine indica* (L.) Gaertn.) under herbicide stress [24], in cotton (*Gossypium hirsutum* L.) under drought stress [25], in *Moringa oleifera* Lam. under water stress [26] and in *Panax ginseng* C. A. Meyer under heat stress [27].

Paeonia ostii T. Hong et J. X. Zhang is a famous traditional ornamental flower in China, which is internationally popular due to its large, showy, and aromatic flower. Studies have proved that its seed oil enriches a large amount of α-linolenic acid (ALA), which may be beneficial as a nutraceutical/pharmaceutical candidate and is good for human health [28]. Additionally, as an emerging woody oil crop, it has a lot of advantages such as strong adaption and low planting investment for large-scale cultivation promotion in many areas [29]. However, as a common abiotic stress, drought stress has greatly restricted the ornamental characteristics and economic values of *P. ostii*, especially in arid areas [30]. Therefore, it is urgent to find feasible ways to alleviate this damage caused by drought stress. At present, there are few reports in the literature on *P. ostii* internal reference gene selection; some scholars have studied the reference genes selection of *P. ostii* in different tissues and across different developmental stages [31,32]. However, there is no literature report on internal reference genes verification under drought conditions in *P. ostii*. Thus, when clarifying the underlying molecular mechanism of *P. ostii* under drought stress and finding effective mitigation routes, there is a lack of stable internal reference genes for qRT-PCR analysis.

In this study, we selected 10 commonly used candidate reference genes and used the geNorm, NormFinder, BestKeeper, and RefFinder to test their expression stability under different drought stress conditions in *P. ostii*. The identified internal reference genes provide valuable information for further qRT-PCR analysis and drought stress alleviation methods of *P. ostii*.

2 Materials and Methods

2.1 Plant Materials and Treatments

The potted seedlings (top diameter × height × bottom diameter = 20 cm × 25 cm × 12 cm) of *Paeonia ostii* (three-year-old) were used as plant materials. *P. ostii* plants with uniform traits were cultivated in a greenhouse maintained at 25–30°C under natural light in Yangzhou University (Yangzhou, Jiangsu Province, China) by regular agronomic practices. Before the drought treatment, watering was normally conducted for three consecutive days. Then, 12 potted seedlings were subjected to the natural drought treatment. According to our previous study, with the increase of drought treatment days, the damages caused by the drought stresses in *P. ostii* plants were gradually severe [33], and on the 0, 4, 8, and 12 d after the drought stresses, the soil water content was 32.23%, 18.31%, 11.68%, and 4.02%, respectively [34]. In this experiment, leaves were randomly sampled every 4 d on the 0, 4, 8, and 12 d after the drought treatment as described earlier [34], and leaves were collected from 2 to 4 pairs of leaves from top to bottom with three biological repeats. Then, they were quickly frozen in liquid nitrogen and stored at −80°C until further analysis.

2.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted using TRIzol reagent (Takara, Dalian, China). The RNA integrity was analyzed by 1.0% agarose gels, and the RNA purity was assessed using BioPhotometer D30 (Eppendorf AG, Hamburg, Germany). Only qualified RNA samples (260/280 ratio ranging from 1.8 to 2.0; 260/280 ratio > 2.0) were used for subsequent experiments. The first-strand cDNA was synthesized using PrimeScript RT reagent Kit (Takara, Dalian, China).

2.3 Potential Reference Gene Selection and qRT-PCR Primer Design

Based on the RNA-seq database obtained from the transcriptome sequencing using *P. ostii* drought treatment (DT) and control (CK) leaves on the 12th day, a total of 78,392 unigenes' fragments per kilobase million (FPKM) values were obtained. According to the differential expressions of genes in different samples, $\log_2$ (DT/CK) was set as a threshold between −0.1 to 0.1 to find stable expressed internal reference genes. We initially obtained 10 unigenes with the most stable expressions distributed in 8 gene families as candidate internal reference genes for further expression profiles analysis (Table 1). All of these candidate genes were chosen for their essential functions in maintaining the most basic cellular activities.

Table 1: FPKM values of 10 candidate reference genes in *P. ostii* RNA-seq

Gene ID	Gene name	Gene symbol	CK FPKM	DT FPKM	$\log_2$ (DT/CK)
Unigene0040750	Actin	*ACT1*	9.53	9.69	0.023531858
Unigene0029456	Actin	*ACT2*	13.58	14.29	0.07416581
Unigene0043536	Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	19.05	18.33	−0.055462592
Unigene0041281	Eukaryotic translation initiation factor	*eIF1*	56.98	54.97	−0.051803661
Unigene0043455	Eukaryotic translation initiation factor	*eIF2*	9.08	8.90	−0.028507097
Unigene0041583	α-tubulin	*α-TUB*	144.36	136.35	−0.082280223
Unigene0030732	β-tubulin	*β-TUB*	5.34	5.62	0.073786991
Unigene0032356	TATA-box binding protein	*TBP*	16.17	15.81	−0.031465634

(Continued)

Table 1 (continued)

Gene ID	Gene name	Gene symbol	CK FPKM	DT FPKM	Log_2 (DT/CK)
Unigene0006653	RNA polymerase II	*RNA Pol II*	9.99	9.43	−0.084286282
Unigene0043820	RNA polymerase II transcription factor	*RP II*	10.85	10.95	0.013217316

Note: FPKM, fragments per kilobase million; CK, control; DT, drought treatment.

Using the Primer Premier 5 software, we designed qRT-PCR primers for each of the 10 reference genes from *P. ostii*. The specific sequence of each primer was shown in Table 2 and the design standard rules are as follows: the amplification product length: 100 to 200 bp; primer length: 18 to 22 bp; melting temperature (TM): 45 to 55°C; GC content: 40% to 60%. Additionally, we used the mixed cDNA sample of each sample as the cDNA template, and the diluted cDNA template was used to calculate PCR efficiency (E) and determination coefficient (R^2). Finally, the amplified products of each reference gene were visualized on 1.0% agarose gels.

2.4 qRT-PCR Analysis

qRT-PCR reactions were conducted with a Bio-Rad PCR CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA). The 10 candidate genes were amplified by qRT-PCR from different drought stress conditions using SYBR Premix Ex Taq (Takara, Dalian, China). Meanwhile, the reactions without cDNA were generated as the negative controls. The qRT-PCR system was conducted as follows: 95°C for 30 s, followed by 5 s at 95°C, 30 s at 55°C, and 30 s at 72°C (40 cycles). We repeated each reaction in triplicate.

2.5 Data Analysis

The Data were processed by Bio-Rad CFX Manager software. Four different methods based on statistical algorithms including the geNorm [13], NormFinder [10], BestKeeper [35], and RefFinder were used to evaluate gene expression stability and availability in various drought stresses.

The geNorm program calculates the expression stability value (M) of each gene. The final criterion defines the gene with the lowest M value as the most stable one. Moreover, geNorm can also calculate the pair-wise variation (Vn/Vn+1) to determine the optimal numbers of reference genes combination used for qRT-PCR analysis.

The NormFinder's reference gene definition principles are consistent with geNorm which is based on the variance between samples. It uses the M value to rank candidate reference genes for the most suitable reference gene selection.

The BestKeeper program generates the coefficient of variation (CV) and standard deviation (SD) by comparing Ct values. Genes with lower CV and SD values imply more stability; conversely, they imply lower stability.

The RefFinder program (http://www.leonxie.com/referencegene.php) is an online website tool that integrates the ranking results of the Delta Ct, geNorm, NormFinder, and BestKeeper; it obtains an integrated result by combining the results of these four statistical algorithms and recommends the most suitable reference gene.

3 Results

3.1 Validation of PCR Assays

The primers of all candidate genes were designed based on the gene sequences obtained from *P. ostii* RNA-seq data [33]. The fragment length of PCR amplifications ranged from 104 to 199 bp (Fig. 1), and

the PCR amplification results were verified to be consistent with the original RNA-seq. In addition, quality analysis results of each primer showed that the melting curve of each candidate gene revealed a single sharp peak, indicating good specificity (Fig. 2). The PCR efficiencies (E) ranged from 92.1% for *eIF2%* to 108.4% for *RNA Pol II*, and the determination coefficients (R^2) varied from 0.9826 for *GAPDH* to 0.9999 for *β-TUB* (Table 2).

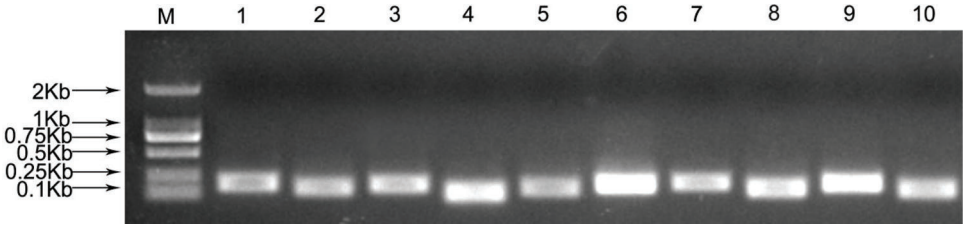

Figure 1: PCR amplifications of 10 candidate reference genes of *P. ostii*

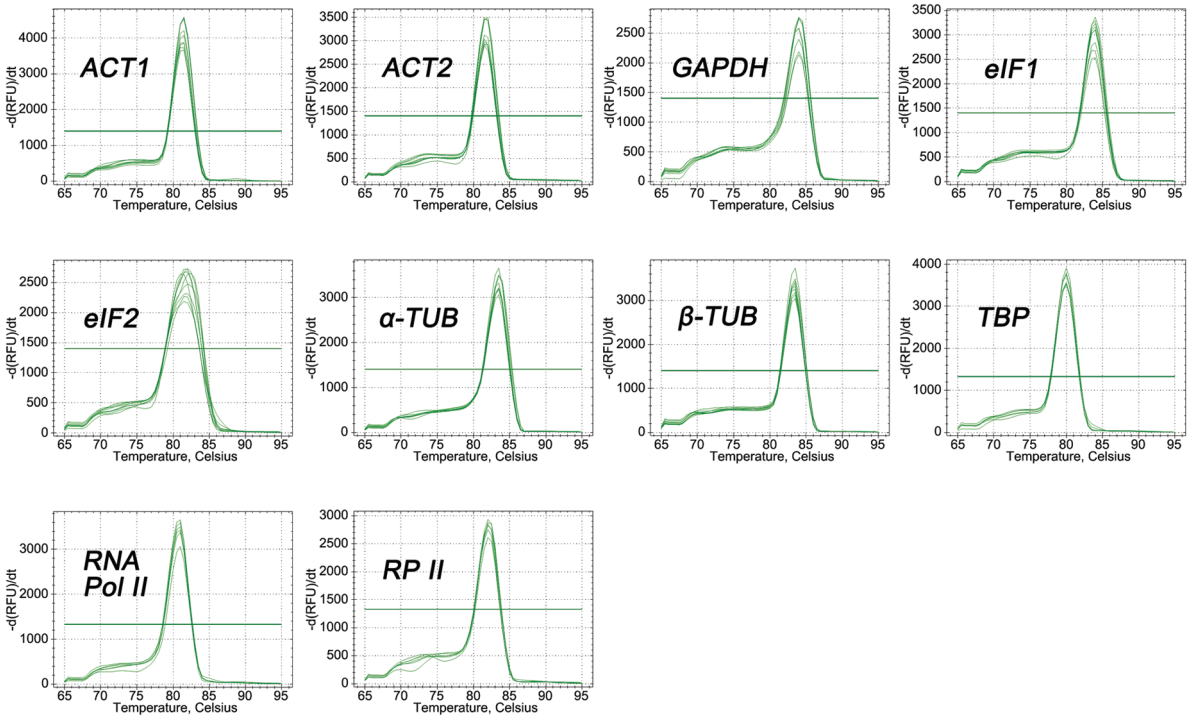

Figure 2: Melting curves of 10 candidate reference genes of *P. ostii*

Table 2: Primers and PCR efficiencies for *P. ostii* reference genes

Gene symbol	Forward primer	Reverse primer	Amplicon length (bp)	Amplicon Temp. (°C)	E (%)	R^2
ACT1	CTTCTTGCTACGACATCC	AGCTAAGGTATTGCCACT	188	51.5	98.7	0.9949
ACT2	GCAGAGGGGTATGTTATT	CTTCACTTTGCGAGCTAC	143	51.5	99.1	0.9874
GAPDH	ATGACCACTGTCCACTCC	TTCCAGTCAACTTCCCAT	168	52.6	108.4	0.9826
eIF1	ACGATTACGAAGACAGACC	CAAAGGACTTGACAGAGTC	104	53.0	99.0	0.9978
eIF2	GGAAGTGGAAAGGAAGAC	ATCCATAATAGCAGCTCC	146	51.5	92.1	0.9942
α-TUB	CTGGGAACTTTACTGTCTC	TCCTGACTTCATCAATGAC	178	51.9	107.7	0.9887

(Continued)

Table 2 (continued)

Gene symbol	Forward primer	Reverse primer	Amplicon length (bp)	Amplicon Temp. (°C)	E (%)	R^2
β-TUB	GTATTACAACGAGGCTTCT	CAGCATCAATCAACTCAG	199	50.6	98.7	0.9999
TBP	GTAATGCTGAATACAATCCC	ATACTTCCGTGCTGCTAA	148	50.8	99.1	0.9891
RNA Pol II	GGTTTGTGACATTTCCTG	ATAGTTTGGTGCATCTCC	190	50.3	108.4	0.9929
RP II	ACGGAGATAGGGTTTGCC	CCCAAAGTTCTTATCGCATT	135	53.1	99.0	0.9921

Note: E, efficiency; R^2, determination coefficient.

M, 2000 marker; 1, *ACT1*; 2, *ACT2*; 3, *GAPDH*; 4, *eIF1*; 5, *eIF2*; 6, *α-TUB*; 7, *β-TUB*; 8, *TBP*; 9, *RNA Pol II*; 10, *RP II*

3.2 Expression Characteristics of the Candidate Reference Genes

The Ct values of 10 candidate genes under different drought conditions were summarized in Fig. 3. It showed that the Ct values of the 10 candidate genes ranged from 24.43 to 31.47. Among them, *RNA Pol II* had the lowest average Ct value (25.30); it indicated that *RNA Pol II* had the highest expression abundance at the mRNA level. Meanwhile, *ACT2* had the highest average Ct value (29.85), indicating it had the lowest expression level. The CV value defined the stability of gene expression under the different drought conditions. Among these ten genes, *ACT1* showed the smallest variation span value of 0.66%, which meant it had the highest expression stability, while *eIF2* showed the highest variation span value of 5.08%, indicating that its expression level varied intensely under the diverse drought stresses. The line across the boxplot reflected the median value. The box showed the percentile range from 25% to 75%. Vertical lines indicate maximum and minimum values.

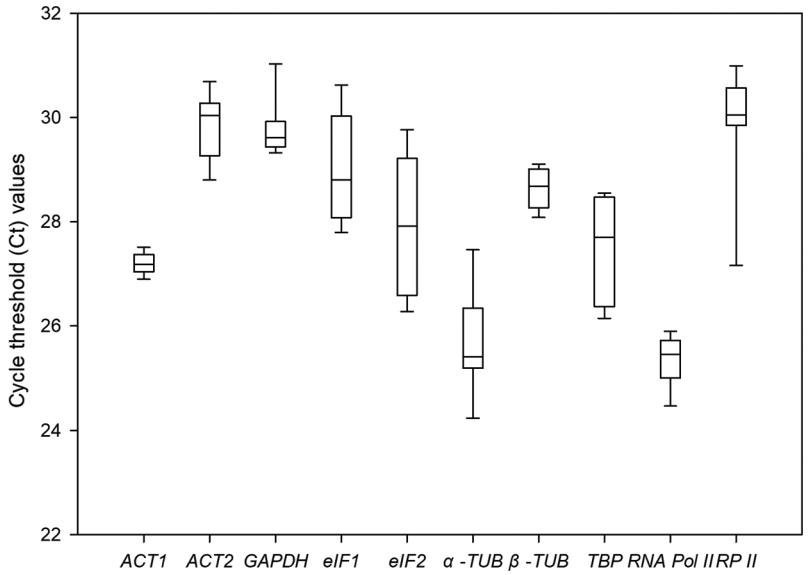

Figure 3: Comparative expression profiles of 10 candidate reference genes in *P. ostii*

3.3 GeNorm Analysis

GeNorm measures the stability of genes based on the average variation degree of the M value calculated by Ct-converted values. The default value of this software is 1.5. It was not recommended to use genes with M values higher than 1.5 as internal reference genes. Moreover, the lower the M value is, the more stable the gene is. In this study, the M values of the 10 candidate genes were all lower than 1.5, ranging from 0.14 to

0.96. Among them, *eIF2* had the least stable expression, while *RNA Pol II* and *β-TUB* ranked first. These latter two genes were stably expressed despite changes in the external environment. The expression stability ranking of other candidate genes is as follows: *TBP* > *ACT1* > *α-TUB* > *eIF1* > *GAPDH* > *RP II* > *ACT2* (Fig. 4).

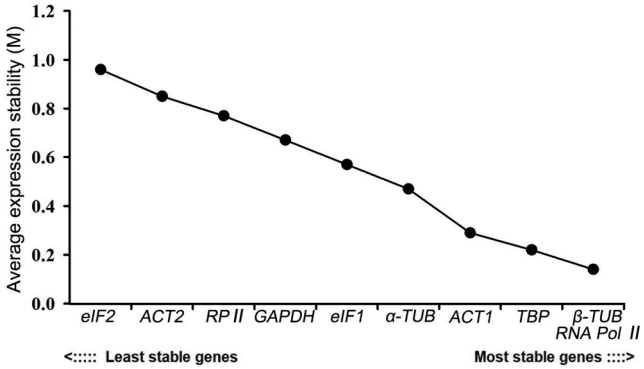

Figure 4: Expression stability analysis of 10 candidate reference genes in *P. ostii* by the geNorm program

Two or more internal reference gene combinations can be used for correcting target gene expressions under certain conditions to obtain more accurate experimental results. The geNorm software can also determine the appropriate number of internal reference genes according to the paired difference value Vn/Vn+1 of the candidate internal reference genes. The 0.15 threshold was proposed to determine optimal reference gene numbers, and the standard was as follows: if Vn/Vn+1 value < 0.15, the optimal number was n; if Vn/Vn+1 value > 0.15, the optimal number was n + 1. In this study, *RNA Pol II* and *β-TUB* were the optimal combinations for accurate qRT-PCR analysis in *P. ostii* with a $V_{2/3}$ value of 0.085 under various drought stress conditions (Fig. 5). Thus, there was no need to introduce more genes for qRT-PCR correction.

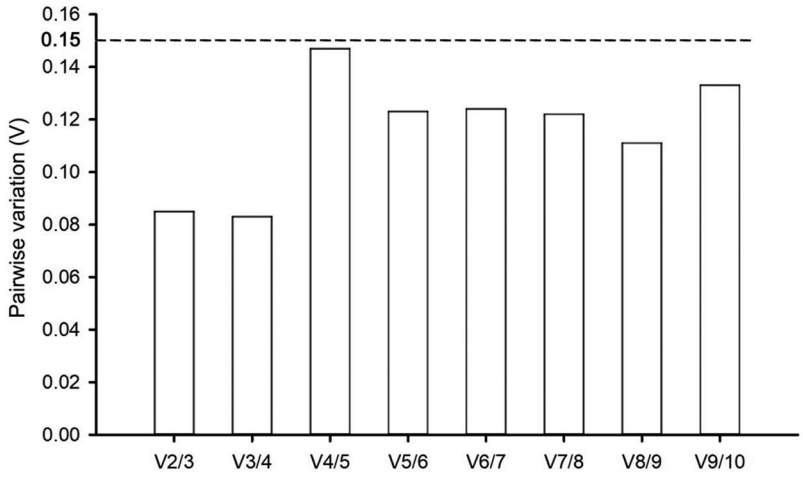

Figure 5: Pairwise variation (V) for the optimal number of candidate genes in *P. ostii* by the geNorm program

3.4 NormFinder Analysis

NormFinder combines the within-group variation and the between-group variation to calculate the stable value of each candidate reference gene. NormFinder considers the gene with the lowest M value as the most

stable one. NormFinder only determines one gene as the most stable reference gene, which is different from geNorm. Among these ten genes, *TBP* had the lowest M value of 0.021, indicating that it was the most stable reference gene. *RNA Pol II* ranked second with an M value of 0.048, followed by *β-TUB* (0.203), *ACT1* (0.322), *α-TUB* (0.381), *eIF1* (0.539), *GAPDH* (0.566), *RP II* (0.626) and *ACT2* (0.734). *eIF2* was the most unstable gene with the largest M value of 0.909, which revealed the same result with geNorm (Fig. 6).

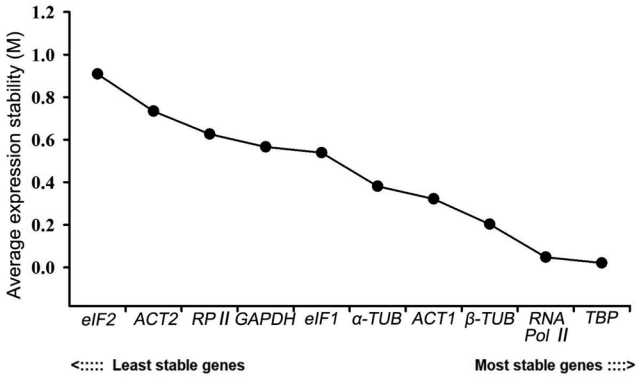

Figure 6: Expression stability analysis of 10 candidate reference genes in *P. ostii* by the NormFinder program

3.5 BestKeeper Analysis

BestKeeper judges the gene expression stability based on the SD and the CV values by directly analyzing the Ct values. The program default threshold value is 1. When the SD value is greater than 1, the gene is considered to be unstable; conversely, a gene with an SD value less than 1 implies high expression stability. In this study, the SD values of all genes were less than 1, except *eIF2*. Thus, they were all suitable as internal reference genes, but *eIF2*. *ACT1* was the most stable with an SD value of 0.07, which was the most suitable choice for an internal reference gene (Table 3).

Table 3: Expression stability analysis of 10 candidate reference genes in *P. ostii* by the BestKeeper program

	ACT1	*ACT2*	*GAPDH*	*eIF1*	*eIF2*	*α-TUB*	*β-TUB*	*TBP*	*RNA Pol II*	*RP II*
std dev [± CP]	0.07	0.76	0.31	0.80	1.34	0.70	0.35	0.29	0.34	0.65
CV [% CP]	0.26	2.53	1.03	2.78	4.83	2.73	1.22	1.07	1.36	2.17

3.6 RefFinder Analysis

RefFinder is an online analysis website that integrates the analysis methods of the Delta Ct, geNorm, NormFinder, and BestKeeper. It calculates the weight of the analysis results of each software to obtain a comprehensive ranking result. The re-rank order analysis result was as follows: *RNA Pol II* > *RP II* > *β-TUB* > *ACT1* > *GAPDH* > *α-TUB* > *ACT2* > *eIF1* > *TBP* > *eIF2*. Thus, *RNA Pol II* was the most stable candidate reference gene which was consistent with the result of geNorm. *eIF2* ranked last, indicating that it had the most unstable expression stability (Fig. 7).

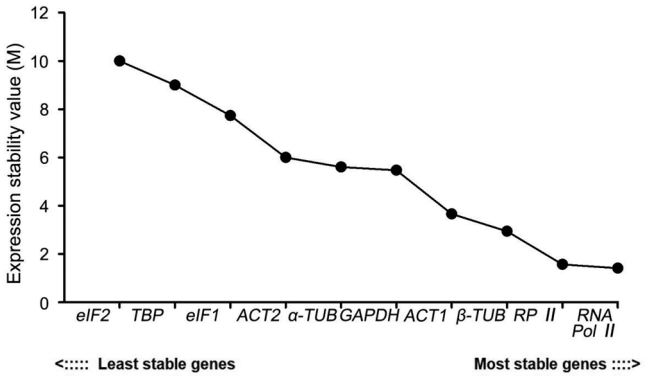

Figure 7: Expression stability analysis of 10 candidate reference genes in *P. ostii* by RefFinder

3.7 Comprehensive Analysis

Based on the ranking results of the geNorm, NormFinder, BestKeeper, and RefFinder, a comprehensive analysis revealed that *RNA Pol II* was better than the other 9 candidate reference genes and ranked first due to its stable expression under various drought stress conditions (Table 4). The final ranking result was as follows: *RNA Pol II* > *β-TUB* > *ACT1* > *TBP* > *GAPDH* > *α-TUB* > *RP II* > *eIF1* > *ACT2* > *eIF2*. Finally, we recommended *RNA Pol II* as the most suitable reference gene; and this result was the same as the geNorm and RefFinder programs.

Table 4: Final Ranking of 10 candidate reference genes of *P. ostii* under drought stress

Method	1	2	3	4	5	6	7	8	9	10
geNorm	*RNA Pol II/β-TUB*		*TBP*	*ACT1*	*α-TUB*	*eIF1*	*GAPDH*	*RP II*	*ACT2*	*eIF2*
NormFinder	*TBP*	*RNA Pol II*	*β-TUB*	*ACT1*	*α-TUB*	*eIF1*	*GAPDH*	*RP II*	*ACT2*	*eIF2*
BestKeeper	*ACT1*	*TBP*	*GAPDH*	*RNA Pol II*	*β-TUB*	*RP II*	*α-TUB*	*ACT2*	*eIF1*	*eIF2*
RefFinder	*RNA Pol II*	*RP II*	*β-TUB*	*ACT1*	*GAPDH*	*α-TUB*	*ACT2*	*eIF1*	*TBP*	*eIF2*
FinalRanking	*RNA Pol II*	*β-TUB*	*ACT1*	*TBP*	*GAPDH*	*α-TUB*	*RP II*	*eIF1*	*ACT2*	*eIF2*

4 Discussion

Compared with conventional quantitative methods, qRT-PCR is superior in terms of its high sensitivity and good specificity contributing to its wide application. However, studies have shown that no gene expresses in the same abundance under any condition, and a single gene may have different expression levels in different tissues, growth stages, and experimental treatments [36–38]. Therefore, it is critically necessary to choose suitable reference genes according to specific experimental treatments before qRT-PCR.

At present, with the wide application of qRT-PCR in many areas [39] such as molecular biology [40], modern medicine [41,42], food detection [43], and environmental monitoring [44], some evaluation methods for judging gene stability have gradually emerged simultaneously. The geNorm, NormFinder, BestKeeper, and RefFinder are among these methods, which improved the efficiency of gene stability analysis and accelerated the pace of suitable reference genes selection. However, the results of each algorithm are different because of the unique algorithm of each program. Therefore, a comprehensive analysis needs to be synthesized according to the analysis results of each statistical algorithm.

P. ostii produces a traditional famous flower in China. Research on *P. ostii* is very rich from multiple viewpoints. It includes research on its antioxidant activity [45], seed dormancy [46], oil resource

promotion [47], ornamental traits regulation [48], and growth resistance and ecological adaptation [49]. In recent years, as the medicinal values and economic benefits of *P. ostii* have become increasingly prominent, the planting scale has continued to expand in many areas. However, in arid and semi-arid areas, due to the lack of water resources, the use of *P. ostii* and the orderly development of a *P. ostii* industry have been greatly restricted. Therefore, many scholars have conducted numerous drought-related studies on *P. ostii*, such as on water physiology [34], cell membrane system [50], osmotic system [51], and photosynthetic mechanisms [34].

In our previous research, some drought stress-relevant indicators were measured, and we have tried to find useful ways to alleviate the drought stress of *P. ostii* [52,53]. At the same time, with the development of transcriptome sequencing, research on drought stress has gradually advanced and deepened from the physiological to the molecular level [54]. Therefore, it is essential to verify the expression of key genes involved in enhancing *P. ostii* drought stress tolerance.

To date, the evaluation of internal reference genes in *P. ostii* was only reported in different tissues across different developmental stages without environmental stresses [31,32]. As a result, different reference gene combinations [*GAPDH/UBC* (ubiquitin conjugating enzyme) for 'Feng Dan' and 'Xi Shi'; *EF-1α/UBC* for 'Que Hao.'] were identified in *P. ostii* flowers [31]. This indicates that it is essential to choose reference genes in different cultivars, even though they pertain to the same species. Some scholars screened the reference genes under different abiotic stresses such as plant hormone stress [55], light stress [23], cold or high-temperature stress [56,57], and salt stress [58], based on the special experimental needs to solve specific scientific problems. Similarly, due to the high industrial values of *P. ostii*, research on *P. ostii* drought stress and its mitigation methods is also very significant. Screening the key genes, and explaining the key mechanisms of drought stress, require suitable reference genes which can correct the expression levels of drought-related genes.

Leaves are the main organ of plants for photosynthesis and transpiration and are also the most sensitive plant part to drought stress [59]. Drought stress can cause leaf wilting and withering, and reduce leaf water content in *P. ostii*. In the drought research of plants, leaves are often used to detect drought-related physical and chemical indicators to measure the overall plant drought degree. Therefore, in this study, we used leaves as the experimental material to screen stable reference genes of *P. ostii* under different drought conditions to provide reference and help for *P. ostii* drought research.

In this study, we selected appropriate internal reference genes based on the unigenes' FPKM values in the transcriptome sequencing database of *P. ostii* leaves under drought stress obtained in the previous stage. Then, we set a strict threshold ($\log_2$ (DT/CK) value ranging from −0.1 to 0.1) to initially obtain 10 stable expressed candidate reference genes. The qRT-PCR results indicated that *α-TUB* had the lowest average Ct value; this was consistent with the transcriptome data, while other candidate genes showed variable expression patterns. Then, a comprehensive evaluation was performed using the geNorm, NormFinder, BestKeeper, and RefFinder programs. Based on the geNorm analysis results, *RNA Pol II* and *β-TUB* were stably expressed under different drought stress conditions, which were identified as the most stable reference genes. *RNA Pol II* and *β-TUB* were also chosen for internal controls in foxtail millet (*Setaria italica* L.) [60] and sugarcane (*Saccharum officinarum* L.) [61] under dehydration stress and drought stress. NormFinder recommended *TBP* as the most stable reference gene, and *RNA Pol II* ranked second. At the same time, the ranking results of NormFinder from the fourth to the seventh-place were the same as those of geNorm. The BestKeeper analysis results revealed that *ACT1* was the most stable gene in *P. ostii*, which ranked fourth in both geNorm and NormFinder; the remaining ranking results were almost different from these two programs. RefFinder determined *RNA Pol II* as the most stable one, which was consistent with the geNorm analysis results. Due to the different ranking results of the four programs, *RNA Pol II* was considered to be the most suitable reference gene because of its persistent stable

expression under various drought stress conditions (Table 4). Additionally, *RNA Pol II* had high expression abundance among these ten candidate genes and could be used to standardize the expressions of highly expressed target genes. MIQE guideline for qPCR methods suggested that there is no perfect gene that is constantly expressed regardless of the external environment under any condition [9]. geNorm program showed that using two genes as internal reference corrections was more reliable than using only one gene. Due to the pairwise variation calculated by geNorm, a combination of two internal reference genes (*RNA Pol II* and *β-TUB*) could be used as internal controls for qRT-PCR analysis in *P. ostii* under drought stress conditions. This study provided convenience for qRT-PCR analysis and provided useful reference data for screening key genes to alleviate drought stress of *P. ostii* in the future.

5 Conclusions

We evaluated 10 candidate reference genes in the leaves of *P. ostii* under different drought stress conditions. These genes were *ACT1*, *ACT2*, *GAPDH*, *eIF1*, *eIF2*, *α-TUB*, *β-TUB*, *TBP*, *RNA Pol II*, and *RP II*. The final comprehensive ranking results analyzed by four programs recommended *RNA Pol II* as the most stable reference gene, and that it could be used to relatively quantify the expression levels of target genes. geNorm program defined *RNA Pol II* and *β-TUB* as the best combination for qRT-PCR normalization in *P. ostii* exposed to drought conditions. Our research facilitated the expression analysis of target genes in *P. ostii* under drought stress, and also promoted the establishment of an optional internal reference gene library in *P. ostii*.

Author Contributions: J. T. and D. Z. designed the experiments. Y. L., C. X., and X. W. performed the experiments. Y. L., C. X., and X. W. analyzed the data and wrote the manuscript. All authors have carefully read and approved the final manuscript.

Acknowledgement: The authors wish to thank three anonymous reviewers for their input and constructive criticisms.

Funding Statement: This work was supported by the National Key R&D Program of China (2018YFD1000405), and Jiangsu Modern Agricultural Industrial Technology System (JATS [2020]436).

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

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Appendix A: Nucleotide sequences of the 10 candidate reference genes in *P. ostii*

Gene symbol	Nucleotide sequences
ACT1	CTTCTTGCTACGACATCCACAGATGGGAAATGCCGGATATTCTCAACTTTAA TCAAAGGTGTGGATGCAAAGGATTCAAAAGCAGGCTCTTCCTCTGATTCAA AATTTGGAGAGCAAATTGTTCAGCTTGATCTCTCACTTTCCTGGGCATTTGG TGTCAAGTGGTCCCCAAGTGGCAATACCTTAGCT
ACT2	GCAGAGGGGTATGTTATTGGGAGCAGCATTAAGTCGATTCCTATTGCAGGGA AAGATGTCACTCTTTTCATCCAGCAGCTCATGCGGGAACGGGGGGAA AATATTCCAACCGAGGACTCCTTCGAAGTAGCTCGCAAAGTGAAG
GAPDH	ATGACCACTGTCCACTCCATCACTGCTACCCAGAAAACTGTTGATGG GCCCTCAATGAAGGACTGGAGAGGTGGAAGGGCTGCTTCCTTCAAC ATCATTCCCAGCAGCACTGGAGCTGCCAAGGCTGTCGGAAAAGTGC TCCCCGCTTTGAATGGGAAGTTGACTGGAA
eIF1	ACGATTACGAAGACAGACCCACTACCTTCGACGATCAAGCCCACCGC AACCCTCAAGATCTCGGCTACGACCCGAACTTCGTCCCCGACTCTGTC AAGTCCTTTG
eIF2	GGAAGTGGAAAGGAAGACAATCCTCCATGTGATGTGCCAGGGTTTGA AAACTGCAGGATGAAATTGTTGAGACATGTATCTTTTGTAGATTGCCC GGGTCACGATATTCTCATGGCTACGATGCTTAATGGAGCTGCTATTATGGAT
α-TUB	CTGGGAACTTTACTGTCTCGAGCATGGCATTCAGCCCGATGGCCAGA TGCCAAGTGACAAAACTGTTGGTGGAGGCGATGATGCTTTCAACACC TTCTTCAGTGAAACTGGTGCTGGGAAGCATGTTCCACGAGCCATTTTT GTAGATCTCGAGCCCACTGTCATTGATGAAGTCAGGA
β-TUB	GTATTACAACGAGGCTTCTGGTGGAAGGTATGTCCCGAGAGCGGTTCT CATGGATCTTGAACCAGGGACCATGGATAGTATCAGATCCGGACCTTAT GGTCAGATCTTTCGGCCGGATAACTTTGTGTTCGGGCAGTCTGGTGCC GGAAATAATTGGGCGAAAGGCCATTATACTGAAGGAGCTGAGTTGAT TGATGCTG
TBP	GTAATGCTGAATACAATCCCAAGCGTTTTGCTGCTGTAATTATGAGGATAAGG GATCCGAAAACCACAGCATTGATATTTGCTTCTGGAAAGATGGTTTGTACTG GAGCTAAGAGTGAACAACAGTCGAAATTAGCAGCACGGAAGTAT
RNA Pol II	GGTTTGTGACATTTCCTGTAAAGTATCAGTGTGTTGTGTTCAGACCATT TAAAGGAGAGATCTTGGAAGCTGTTGTTACCATGGTTAACAAGATGGG TTTCTTTGCCGAAGCTGGGCCAGTTCAAATTTTTGTTTCAAACCATTTG ATACCTGATGATATGGAGTTTCAATCTGGAGATGCACCAAACTAT
RP II	ACGGAGATAGGGTTTGCCGGATGACTGAAATCGAGGATCTTCAGGCA CCTCGCAACCTCCCTTTTGCACCACTTAGCATCAAGGATCCTCGTGAC TATTTTGACTCGCAACAAGCAAATGCGATAAGAACTTTGGG