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Evaluation of Reference Genes for Gene Expression Analysis in *Eichhornia crassipes*

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Abstract: *Eichhornia crassipes* is a notorious invasive aquatic weed, causing enormous ecological and economic losses worldwide. However, it has great potential in agriculture, industry, medical care, and other areas. While being such an important plant, it is poorly understood from the molecular perspective. Aiming to select suitable reference genes for gene expression quantification in *E. crassipes*, this study favors future research at the molecular level. In this work, 12 candidate reference genes were selected. Their expression stability in samples of different tissues, samples treated with various hormones, samples supplied with different levels of phosphorus (P), and pooled samples, were analyzed using GeNorm, NormFinder, BestKeeper, and RefFinder. Meanwhile, the optimal number of reference genes was calculated by GeNorm. The results showed that *eIF* and *ElF1a* were the two most stable reference genes in all samples and in tissue samples. In response to hormone treatments, *Actin* and *eIF* are the best choices of internal controls. In the case of P treatments, *TUA* and *H2A* are recommended to be used as reference genes. Overall, results from this work suggest different reference genes should be applied in qRT-PCR on *E. crassipes*, according to the specific experimental setup.

Keywords: *Eichhornia crassipes*; reference gene; GeNorm; NormFinder; BestKeeper; RefFinder

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1. Introduction

Analysis of transcript abundance is a conventional and important strategy to understand gene function. Commonly applied technologies in determining gene expression include Northern blot, quantitative real-time PCR (qRT-PCR), semi-qRT-PCR, and RNA-seq. Among them, qRT-PCR has become prevalent and indispensable owing to its convenience for operation, relatively low cost, high sensitivity, specificity, and reproducibility [1]. A successful qRT-PCR is shaped by a series of factors, including the RNA quality, the efficiency of the reverse transcription reaction, random errors in experiments, and the reference gene used for normalization [2]. Among these factors, the expression stability of the chosen reference gene is vital in minimizing systematic biases and precisely normalizing the expression of target genes [3]. Therefore, selection of suitable and reliable internal control genes is a critical step in performing qRT-PCR. Reference genes are defined as genes that have sequences different from the target and are used as an internal reaction control, where the expression level is unaffected by the experimental conditions [3]. The term reference gene is often interchangeably used with the term housekeeping gene, while the latter term strengthens its importance in maintaining basic cellular function [3]. Yet, reference genes normally function as housekeeping genes [4]. Mounting research has been devoted to the identification of reference genes in different organisms towards various pathophysiological treatments. In plants, there are some reference genes generally employed, including ribosomal genes such as *18S rRNA* (18S ribosomal RNA), *28S rRNA* (28S ribosomal RNA), *RPL32*

(ribosomal protein L32), cytoskeleton related genes such as *Actin*, *TUA* (tubulin alpha), *TUB* (tubulin belta), genes involved in basic cellular metabolism such as *ElF1a* (elongation factor 1α), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *UBQ* (polyubiquitin), *UBC* (ubiquitin conjugating enzyme), *CYP* (cyclophilin), *PLA2* (phospholipase), genes involved in transcription and translation processes such as *eIF* (eukaryotic translation initiation factor), *H2A* (histone), *TBP* (TATA sequence binding protein), *RPII* (RNA polymerase II), cell division/ cycle related genes such as *CYCC* (cyclophilin C), and so on [4–6]. Ideally, an eligible reference gene should be stably expressed in all tissues and under various treatments. However, it is difficult to have a reference gene that suits all experimental scenarios [7]. For example, the study from Gao et al. (2022) suggested that *PLA2* and *Actin* were the most suitable and statistically reliable reference genes in *Koelreuteria paniculata*, while *RPL13* and *EF1α* were less stable and unsuitable [8]. Therefore, it is necessary to validate and choose specific suitable reference genes for different experiments.

Eichhornia crassipes, commonly known as water hyacinth, is a free-floating perennial aquatic plant [9]. Originating from the Amazon basin in South America, *E. crassipes* is now distributed worldwide. *E. crassipes* is a double-edged sword for human beings. On one hand, it is a notorious invasive weed causing severe ecological problems and great economic losses; however, it has unlimited potential in agriculture, industry, environmental sustainability, medical care, and so on [10–14]. Once it has invaded, *E. crassipes* threatens the survival of other organisms by blocking sunshine, and consuming oxygen and nutrition in water [15]. In addition, massive *E. crassipes* plants may exert potential health risks to nearby communities by providing shelters for pests, such as mosquitos and snakes [16]. Moreover, the occupation of water surfaces by *E. crassipes* hampers irrigation and navigation [16]. It has been estimated that WH's invasion caused economic costs as high as $150 million annually (https://2001--2009.state.gov/g/oes/ocns/inv/cs/2299.htm, accessed on 28 July 2022). On the other hand, owing to its rapid growth, specific physiological structure, and unique chemical composition, it is widely used in feeding animals, remediating diverse pollutants, manufacturing various value-added products, producing a series of clean energies, isolating pharmaceutical compounds, and other applications [12,17,18]. *E. crassipes* related research, therefore, mainly focuses on investigating its ecological and sociological impacts, exploring the control methods, and extending and optimizing its utilization. In contrast, its biology study has stagnated since the last century, and it is poorly understood from the molecular perspective. Up until now, very limited information has been gained on the function of genes in *E. crassipes*, which impedes the investigation of underlying molecular mechanisms of its invasion and utilization. Indeed, there are several publications employing *Actin* as the reference gene to normalize target gene expression [19–21], but no research has been done to evaluate and select suitable reference genes for qRT-PCR in *E. crassipes*. As mentioned above, it is impossible for one reference gene to suit all experiment setups, and neither is *Actin* possible in *E. crassipes*. Therefore, selecting reliable reference genes in *E. crassipes*, a plant that possesses significant ecological and economic importance, yet urgently requires molecular studies, under various experimental conditions, is necessary.

Aiming to ascertain suitable reference genes for *E. crassipes*-related experiments for the first time, and, thereby, to favor future research which wishes to learn gene function or understand molecular mechanisms of its invasion and utilization, this research selected 12 genes, examined their expression in different tissue parts, in plants grown under different phosphorus (P) levels, as well as in plants treated with hormones, and evaluated their expression stability by geNorm, NormFinder, RefFinder and BestKeeper [22–25], four commonly used analyzing tools. Our results indicated that specific reference genes were required for qRT-PCR normalization under various experimental conditions.

2. Materials and Methods

2.1. Plant Materials, Treatments, and Sampling

Plants were routinely grown in plastic trays (40 cm × 32 cm × 12 cm) containing 5 L of half-strength Hoagland solution (0.5 mM KNO_3; 0.125 mM $NH_4H_2PO_4$; 0.05 mM

NH_4Cl; 0.25 mM $MgSO_4$; 0.5 mM $Ca(NO_3)_2$; 0.0125 mM $FeSO_4$; 0.15 μM $CuSO_4$; 0.165 μM $ZnSO_4$; 5.625 μM H_3BO_4; 1.75 μM $MnCl_2$; 0.05 μM $(NH_4)_6Mo_7O_{24}$) which was replaced every week, and kept in the greenhouse (25 ± 5 °C, relative humidity: 60–70%, natural light regimen) [26]. Unless specified, plants were all grown under these conditions. Four-leaf ramets were taken from mother plants, pooled together and precultured for 3 days, and then randomly divided into different trays before subsequent treatments. Plants grown in nutrition solutions containing various levels of P or treated with different hormones were cultured for 3 weeks before sampling. Every treatment has three biological replicates, with three plants per replicate.

For P treatments, plants were supplied with half modified Hoagland solution containing different levels of P: P^-: no $NH_4H_2PO_4$; Ctrl: 0.125 mM $NH_4H_2PO_4$; P^+: 0.75 mM $NH_4H_2PO_4$.

For hormone treatments, every three plants were sprayed every week with 20 mL sterilized ddH_2O, hormone, or biosynthesis inhibitor solutions, including artificial cytokinin 6-Benzylaminopurine (100 μM), gibberellic acid (100 μM), abscisic acid (100 μM), and strigolactones biosynthesis inhibitor Tis108 (5 μM), respectively, on the leaves and peptioles [27,28]. All hormone or biosynthesis inhibitor solutions were prepared by diluting each stock solution at the concentration of 1000× the working concentration with sterilized ddH_2O.

After 3 weeks of culture, plants were surface dried with tissue paper. Afterwards, two of the latest well-developed leaves, the corresponding leaf petioles, stem bases, and roots of each plant were sampled, respectively, and immersed in liquid N_2 immediately, and then stored at −80 °C until RNA isolation.

2.2. RNA Isolation and cDNA Synthesis

Plant samples were ground into a fine powder in liquid N_2 and used for total RNA isolation with an RNA Prep Pure Plant Plus Kit (Polysaccharides and Polyphenolics-rich) (TianGen, China). RNA concentration and quality were determined by NanoDrop, followed by RNA integrity examination by electrophoresis on 1% agarose gel. All RNA samples meeting the criteria, including A260/280 = 1.6–2.1, A260/280 = 1.8–2.0, and showing bright and intact bands on gels were used for the following experiments. Afterwards, genomic DNA contamination was removed, and cDNA was synthesized using FastKing RT Kit (With gDNase) (TianGen, China).

2.3. Candidate Reference Genes, Primer Design

Twelve candidate reference genes belonging to ribosomal RNA genes (*18S*, *28S*), cytoskeleton genes (*Actin*, *TUA*, *TUB)*, basic metabolism genes (*CYP*, *ElF1a*, *GAPDH*, *UBQ*, *UBC*), and transcription- and translation-related genes (*eIF*, *H2A*) were selected to test in this study. The oligonucleotide sequences of these genes are listed in Table S1. Twelve pairs of primers of these genes were designed using Primer 3.0 according to the guidelines suggested by [29]. The detailed information of primers used in this study are listed in Table 1.

2.4. qRT-PCR and Primer Specificity Test

All qRT-PCR were done in 96-well plates with a Quant-StudioTM Real-Time PCR 6 Flex System (Applied Biosystems, Waltham, MA, USA) and SuperReal PreMix Plus (SYBR Green) (TianGen, China). Each 20 μL reaction included: 10.0 μL of 2 × SuperReal PreMix Plus, 0.4 μL of each primer (10 μM), 0.4 μL 50 × ROX dye, 1.6 μL cDNA (10 ng μL^{-1}), and 7.2 μL of RNase-free H_2O. The qRT-PCR program was as follows: 95 °C for 15 min, then 40 cycles of 10 s at 95 °C, 57.5 °C for 20 s, then 72 °C for 30 s. A melting curve analysis was done after 40 cycles of amplification. For each sample, three technical replicates were employed.

Table 1. Oligonucleotide primers for candidate qRT-PCR reference genes in *E. crassipes*.

Gene Name	Gene Product	Primer Sequence 5′–3′	Annealing Temp (°C)	Amplicon Length (bp)	Tm(°C)
18S	18S ribosomal RNA	For: AGTGGCTATGCTATGCTGACAAGTC Rev: TCATCTCCCTCTGGTCCATGTTCTC	82.95	144	58.96 60.20
23S	23S ribosomal RNA	For: CACACCGTCTTGGAGGACATGC Rev: AGGAGCGGAGAAGAACGAGGAC	87.72	81	61.30 61.49
Actin	Actin	For: CTCATTGTGGACTGCGGCTTCTC Rev: CGATACGACGGGCAGCATAGTTG	84.70	85	60.98 60.69
TUA	Tubulin alpha	For: ACACAGCGATGAGAATGAGCAAGG Rev: GGAGCAGAAGGAGCAGATGATGATG	81.68	128	59.72 59.81
TUB	Tubulin beta	For: AGACCACCTCCCAGAACTTGCC Rev: CTTGCCCTTGCCATTCTCTCTCAC	86.55	150	62.23 60.62
CYP	Cyclophilin B	For: TCTCGCTAGGCCAGGTTTCATTATG Rev: ATGCAATCCCAAGACTGTTCCTGAC	80.61	127	58.79 59.41
ElF1a	Elongation factor 1a	For: AGATCCATAACCCAGTCCCTCAGTC Rev: GGTGATCGAGTTGTCGCCTGTC	83.43	150	60.07 60.94
GAPDH	Glycerol-3-phosphate dehydrogenase	For: CCGACCGTTGATGTCTCTGTTGTAG Rev: CGATTCCTCCTTGATAGCAGCCTTG	82.75	93	59.64 59.96
eIF	Eukayotic initiation factor	For: GTGGAGCCGATGCCGATAACTG Rev: TGCTTGAATCCTCTTGCTTCTTCCC	82.25	81	61.15 59.41
H2A	Histone	For: GCT TGGAAGGAGGACATGGATGC Rev: TCTCTGGTGGCTGGATGAATGGG	80.81	94	61.04 61.78
UBC	Ubiquitin conjugating enzym	For: AATCGTCCGCCTCCAACTCCTC Rev: TCCGCTCCCAGATCCGTAAATCC	88.40	115	62.09 61.41
UBQ	Polyubiquitin	For: ACACGAAACACACAACGGGACTC Rev: GCACCTTGTAGACTCGTCATCATC C	81.88	148	60.12 59.60

The primer specificity was determined ahead of primer stability analysis. In detail, qRT-PCR of all primers using the control cDNA and no temperate control (NTC) were performed on one plate. Afterwards, the amplification result and melting curve of each reaction were analyzed.

2.5. Stability Analysis of Candidate Reference Genes and Determination of the Optimal Number of Reference Genes

Ct (cycle threshold) values represent the expression levels of tested reference genes. All Ct values obtained from qRT-PCR were input into GeNorm, NormFinder, BestKeeper, and RefFinder, and the stability of candidate reference genes were analyzed by these softwares. GeNorm ranks stability of reference genes by comparing the so-called M value of each gene, which is calculated by the average degree of change in the expression level of a given gene relative to that of other candidate genes. Similarly, NormFinder calculates intra- and inter-group variation and individual analysis of sample-subgroup expression levels. BestKeeper analyzes multiple pairwise correlations using the raw Ct values of each gene. RefFinder evaluates the stability of candidate reference genes by calculating the geometric average of the analysis results of the other three algorithms.

The minimum optimal number of primer pairs required for qRT-PCR in *E. crassipes* was calculated using GeNorm. When the value of Vn/n + 1 is more than 0.15, the (n + 1)th reference gene is needed.

3. Results

3.1. Evaluation of Primer Specificity

Primer specificity is indicated by the number of peaks in the melt curve. One peak represents one amplification product. As shown in Figure 1, the melting curves of tested primers in qRT-PCR using the control cDNA as a template all had one peak in the corresponding melting curve, while NTCs had no amplification nor any peak in the melting curves except for 23S, suggesting qRT-PCR using these primer pairs had specific amplification and no primer dimers formed under our experimental conditions. In the case of 23S, there was only one peak in the cDNA sample reaction, indicating the primer specificity.

Though another peak was observed in NTC, which is most likely resulting from the primer dimer, its annealing temperature is obviously lower than that in a cDNA reaction, and the amplification was almost below the detection threshold. Therefore, the formation of the primer dimer of 23S under our experimental conditions would have little impact on the final result and is negligible. In conclusion, all the primers tested are suitable for following qRT-PCR experiments.

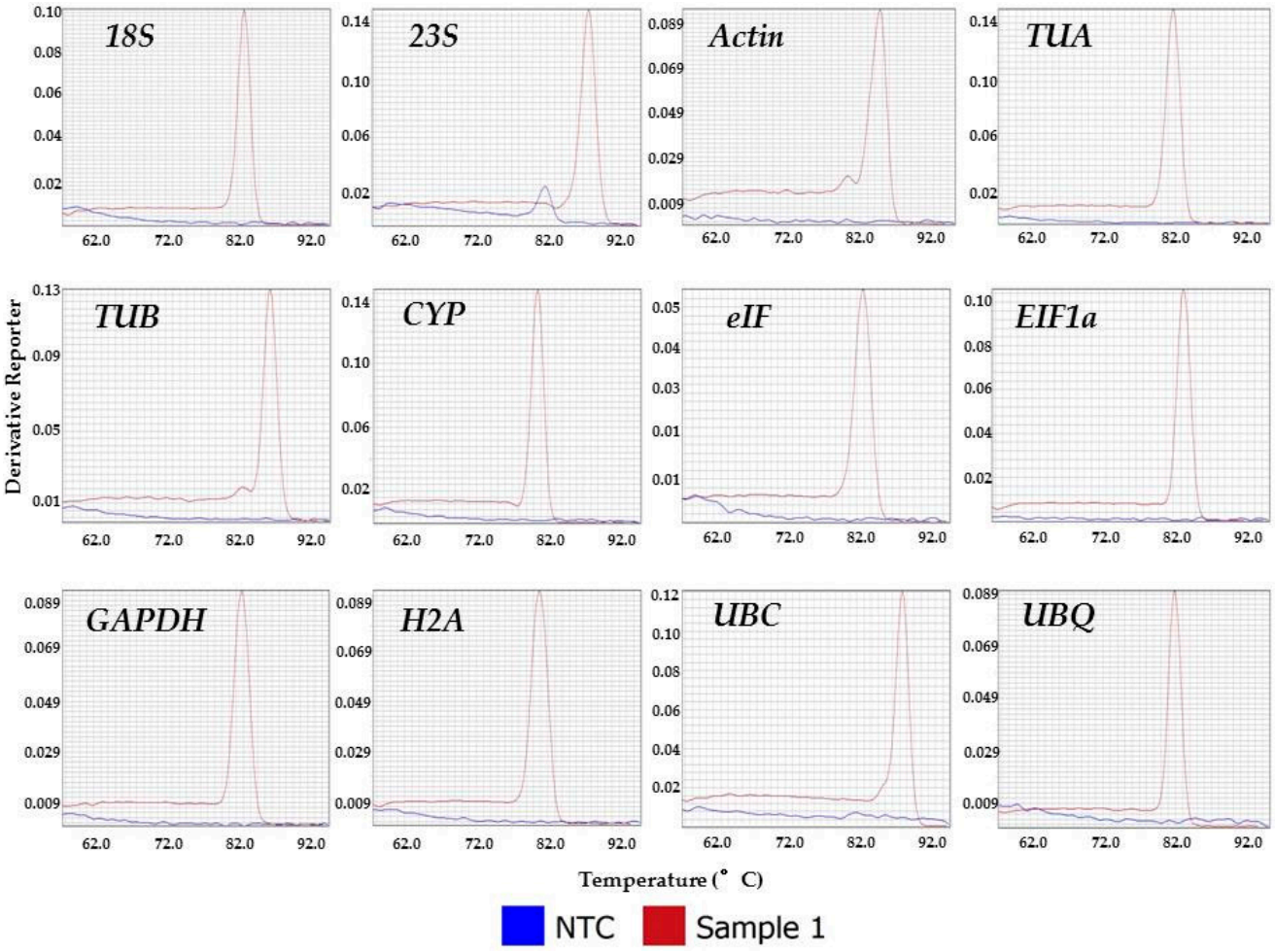

Figure 1. Primer specificity of candidate reference genes. Sample 1 is the cDNA of stem base of Ctrl plants. NTC used sterilized ddH_2O to replace cDNA in the reaction.

3.2. Expression Patterns of Candidate Reference Genes

Ideally, the Ct values of reference genes should be within a range of 15–30 to ensure the accuracy of quantitative results [30]. As shown in Figure 2, the lowest Ct was 15.86 from UBQ-1 whose max value reached 19.77, while the highest Ct was observed on CYP at 31.85, whose minimum value was 22.36. Among all the tested primers, UBQ had a lowest median Ct at 17.71, while ElF1a had the highest median Ct at 24.48. The Ct value of ElF1a ranged from 23.15–25.97, which showed the least variation. Meanwhile, the other tested primers all had variations to a larger extent than that of ElF1a, among which CYP, whose Ct ranged from 22.36–31.85, exhibited the largest variation. These results suggest that the Ct of tested primers varied in different tissues towards various treatments. In other words, none of them presented absolute stable expression in E. crassipes regarding our experimental conditions. Therefore, further evaluation of expression stability of these candidate reference genes by the four algorithms mentioned in Section 2.5 is necessary.

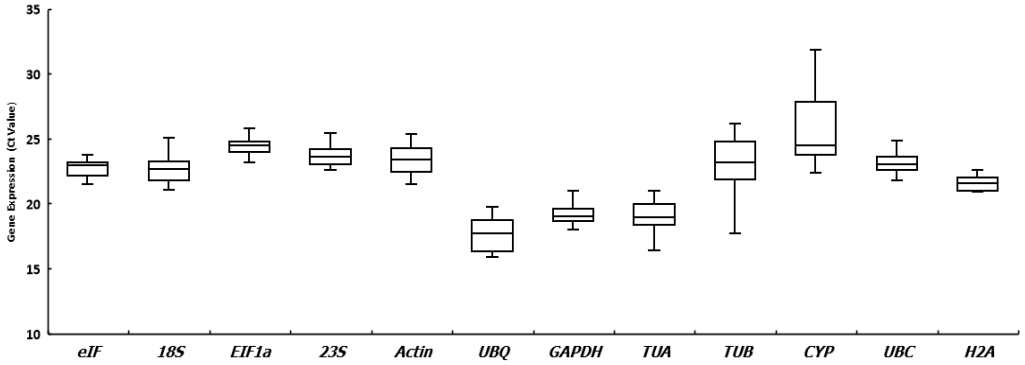

Figure 2. Expression patterns of candidate genes in *E. crassipes*. The expression levels of candidate reference genes are shown as Ct values of all samples. The line in the box represents the median. The upper and lower edges of the interquartile range indicate the 75th and 25th percentiles, respectively. The minimum and maximum values are shown by the whisker caps.

3.3. Expression Stability of Candidate Reference Genes

3.3.1. GeNorm Analysis

In GeNorm analysis, gene expression stability is indicated by the measurement (M) value, which should be less than 1.5, otherwise, the candidate is not suitable to be used as a reference gene. In general, the lower that M is, more stable the gene expression is. As shown in Table 2, *TUA*, *CYP*, *Actin*, and *TUB* should not be used as reference genes in samples that include different tissues, while *TUA* is not suitable for experiments with hormone treatments, as their M values exceed 1.5 in these conditions. According to the calculation of GeNorm, the two most appropriate reference genes are *eIF* and *EIFIa* in different tissues and in total samples, *eIF* and *Actin* in hormones experiment, and *TUA* and *H2A* in P levels experiment, respectively.

Table 2. Rank order of the candidate reference genes in *E. crassipes* under different experimental conditions.

Treatments	Rank	GeNorm		NormFinder		BestKeeper		RefFinder	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Tissues	1	*eIF/ElFIa*	0.688	*eIF*	0.37	*UBQ*	0.83047	*eIF*	0.344
	2	*23S*	0.808	*GAPDH*	0.43	*H2A*	0.948356	*ElFIa*	0.448
	3	*GAPDH*	0.91	*ElFIa*	0.56	*18S*	1.107341	*23S*	0.718
	4	*18S*	0.966	*23S*	0.57	*ElFIa*	1.155646	*GAPDH*	0.734
	5	*UBQ*	1.143	*18S*	0.6	*Actin*	1.260154	*18S*	0.877
	6	*H2A*	1.207	*UBC*	0.62	*GAPDH*	1.500039	*Actin*	1.009
	7	*UBC*	1.262	*TUA*	0.69	*eIF*	1.5658	*UBC*	1.207
	8	*TUA*	1.517	*H2A*	0.84	*UBC*	1.784104	*UBQ*	1.346
	9	*CYP*	1.692	*UBQ*	0.98	*23S*	1.89264	*H2A*	1.439
	10	*Actin*	1.855	*Actin*	1.28	*CYP*	2.461265	*TUA*	1.883
	11	*TUB*	2.03	*CYP*	1.29	*TUA*	3.596017	*TUB*	2.346
	12			*TUB*	2.23	*TUB*	22.69811	*CYP*	2.508
Hormones	1	*eIF/Actin*	0.552	*eIF*	0.27	*UBQ*	0.889151	*Actin*	0.111
	2	*UBC*	0.603	*Actin*	0.35	*CYP*	1.217228	*eIF*	0.276
	3	*GAPDH*	0.65	*UBC*	0.38	*UBC*	1.312575	*UBC*	0.304
	4	*ElFIa*	0.729	*UBQ*	0.4	*18S*	1.399489	*GAPDH*	0.559
	5	*UBQ*	0.79	*ElFIa*	0.41	*eIF*	1.653703	*ElFIa*	0.706
	6	*18S*	0.877	*18S*	0.43	*ElFIa*	1.75747	*UBQ*	0.756
	7	*23S*	0.939	*23S*	0.49	*GAPDH*	1.950305	*18S*	0.828
	8	*TUB*	1.122	*GAPDH*	0.5	*Actin*	2.06208	*23S*	0.909
	9	*CYP*	1.283	*TUB*	0.77	*TUB*	2.49303	*TUB*	1.62
	10	*H2A*	1.402	*CYP*	0.89	*H2A*	2.838247	*CYP*	2.049
	11	*TUA*	1.528	*H2A*	0.94	*23S*	4.371111	*TUA*	2.144
	12			*TUA*	0.95	*TUA*	6.043985	*H2A*	2.19

Table 2. *Cont.*

Treatments	Rank	GeNorm		NormFinder		BestKeeper		RefFinder	
		Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
P levels	1	*TUA/H2A*	0.195	*UBQ*	0.14	*H2A*	1.151808	*TUA*	0.034
	2	*UBQ*	0.256	*H2A*	0.24	*ElFIa*	1.266195	*H2A*	0.097
	3	*23S*	0.293	*TUA*	0.25	*GAPDH*	1.340341	*UBQ*	0.131
	4	*ElFIa*	0.334	*23S*	0.26	*UBQ*	1.965777	*23S*	0.258
	5	*18S*	0.377	*ElFIa*	0.34	*TUA*	2.086377	*ElFIa*	0.331
	6	*eIF*	0.404	*GAPDH*	0.38	*eIF*	2.112718	*GAPDH*	0.461
	7	*GAPDH*	0.442	*CYP*	0.41	*CYP*	2.120346	*eIF*	0.472
	8	*CYP*	0.494	*eIF*	0.41	*TUB*	2.289766	*18S*	0.476
	9	*UBC*	0.686	*18S*	0.49	*Actin*	2.34925	*Actin*	0.505
	10	*TUB*	0.901	*UBC*	0.62	*23S*	2.473408	*CYP*	0.511
	11	*Actin*	1.187	*TUB*	0.84	*18S*	2.53995	*TUB*	0.532
	12			*Actin*	1.1	*UBC*	3.807912	*UBC*	1.622
Total	1	*eIF/ElFIa*	0.738	*ElFIa*	0.26	*UBQ*	0.808993491	*eIF*	1.19
	2	*GAPDH*	0.833	*eIF*	0.27	*18S*	1.24039533	*ElFIa*	1.41
	3	*Actin*	0.924	*GAPDH*	0.27	*UBC*	1.435844982	*GPDH*	3.00
	4	*18S*	0.968	*UBC*	0.32	*ElFIa*	1.468964977	*Actin*	4.86
	5	*23S*	1.045	*23S*	0.41	*Actin*	1.541997044	*18S*	5.18
	6	*UBC*	1.115	*18S*	0.54	*eIF*	1.61899665	*23S*	5.18
	7	*UBQ*	1.244	*Actin*	0.55	*GAPDH*	1.653358981	*UBC*	6.44
	8	*H2A*	1.358	*TUA*	0.60	*CYP*	2.106868104	*UBQ*	8.00
	9	*TUA*	1.494	*UBQ*	0.93	*H2A*	2.639930593	*TUA*	9.24
	10	*TUB*	1.648	*H2A*	0.96	*23S*	3.14792245	*H2A*	9.74
	11	*CYP*	1.844	*TUB*	1.00	*TUA*	4.785600612	*TUB*	11.00
	12			*CYP*	1.48	*TUB*	6.500722183	*CYP*	12.00

In addition to analyzing gene expression stability, GeNorm can be used to calculate the optimal number of reference genes employed in qRT- PCR. When the value of Vn/n + 1 is more than 0.15, the (n + 1)th reference gene is needed. As shown in Figure 3, all the pairwise variation values are less than 0.02, suggesting two reference genes are enough to minimize systemic bias in treatments, including tissues, hormones, and P levels, and in the pooled samples.

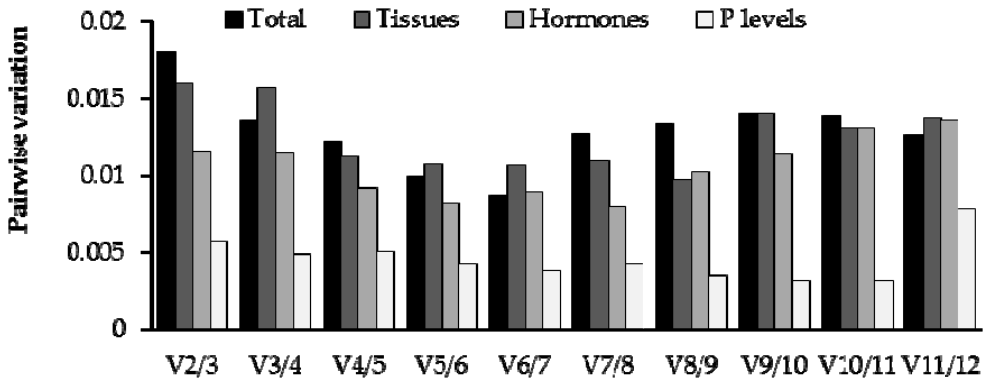

Figure 3. Determination of optimal number of reference genes for different *E. crassipes* samples.

3.3.2. NormFinder Analysis

The NormFinder analysis, as listed in Table 2, suggested that the two most stable reference genes in tissues are *eIF* and *GAPDH*, while the least stable genes are *CYP* and *TUB*. In plants treated with various hormones or supplied with different levels of P, *eIF* and *Actin*, UBQ and H2A, are the two most stably expressed reference genes, respectively, whilst *H2A* and *TUA*, and *TUB* and *Actin*, in that order, are the two least adequate reference

genes and the least stable. In all samples, *eIF* and *UBC* are the top two stable reference genes, whereas *TUB* and *CYP* rank at the bottom of the stability list.

3.3.3. BestKeeper Analysis

According to the analysis of BestKeeper (Table 2), *UBQ* was the most stable reference gene in tissue and hormone treatments and in total samples, while the second most stable genes in these groups were *H2A*, *CYP*, and *18S*, respectively. For the P level treatment, the top two stable genes were *H2A* and *EIF1a*. *TUA* and *TUB* were the least stable expressed genes in tissue samples and total samples. The bottom two genes on the gene stability ranks of hormones and P levels were *23S* and *TUA*, and *18S* and *UBC*, respectively.

3.3.4. RefFinder Analysis

Due to the inconsistency of gene expression stability calculated by GeNorm, NormFinder, and BestKeeper, RefFinder was employed to integrate their results and provide a comprehensive rank (Table 2). As shown in Table 2, *eIF* and *EIFIa* are the two most reliable reference genes in different tissues and all samples of *E. crassipes*, while *TUB* and *CYP* are the least reliable genes. In response to hormone stimuli, *UBQ* and *CYP* show the highest expression stability, whilst *23S* and *TUA* exhibit the highest expression variability. In plants supplied with different levels of P, the two most stably expressed candidate reference genes are *H2A* and *EIFIa*, while *18S* and *UBC* are the least stably expressed genes.

4. Discussion

As a noxious invasive plant, *E. crassipes* also possesses great potential in multi-applications. While being such an important plant, its research from the molecular perspective, for example, in a gene function study, is surprisingly rare in this omics era. Quantification of transcript abundance by qRT-PCR is a prevalent method to study gene function, whereby employment of adequate reference genes is vital in reducing systemic bias. Although reference genes are expected to exhibit stable expression, it is almost impossible for one specific reference gene to suit all experimental setups. Selecting reliable reference genes for a specific organism towards various pathophysiological treatments, hence, should be done prior to performing the expression quantification of target genes. There have been three publications performing qRT-PCR analysis on *E. crassipes* and all of them used *Actin* as the internal control to normalize target gene expression in different tissues [19–21]. As a housekeeping gene functioning in the cytoskeleton, *Actin* is widely used as a reference gene in plants. However, there are cases demonstrating that *Actin* is unreliable in some experiments as well [31,32]. This is verified by the expression profiles of 12 tested candidate reference genes in this study. The expression of all 12 genes, including *Actin*, showed variations to different extents (Figure 1), suggesting that none of them has absolute stable expression and therefore it is necessary to select suitable reference genes to normalize expression of interested genes under various experimental setups.

Evaluation of reference genes can be achieved using several software and online tools. Among them, GeNorm and NormFinder have similar algorithms, whereby both evaluate genes based on the corresponding expression stability. This can explain the similar results obtained from GeNorm and NormFinder in this study. The top five stable genes calculated by GeNorm are also high up the ranks of NormFinder, while the five most unstable genes in the GeNorm rank also appeared on the bottom of the NormFinder list (Table 2). The similarity between the outputs of these two software is often observed in other studies as well [32,33]. BestKeeper calculates gene expression stability by comparing multiple pairwise correlations using the raw Ct values of each gene. Therefore, the rank generated by BestKeeper shared much less similarity with the results of GeNorm and NormFinder. However, they all tended to select the same least suitable reference genes, for example, they all supported that *TUB* was the most unstable expressed gene in tissue samples (Table 2). Finally, RefFinder was employed to comprehensively rank the candidate genes. This is a commonly used strategy in evaluating gene expression stability when

GeNorm, NormFinder, and BestKeeper results are inconsistent [33,34]. Interestingly, the rank generated by RefFinder shares more similarity with the results of GeNorm and NormFinder, rather than BestKeeper (Table 2). This trend has also been reported by other publications [34]. Moreover, applying two or more reference genes in one experiment could further increase the accuracy of results [35,36]. The optimal number of reference genes can be determined by GeNorm and indicated by the $V_{n/n+1}$. Similarly to the situation in *Galium aparine* L. and *Dianthus broteri* [36,37], applying two reference genes is recommended when performing qRT-PCR on *E. crassipes*, based on our results (Figure 3).

All samples in this study can be grouped into three treatments: tissues, hormones, and P levels. In the setup of experiments aiming to select suitable reference genes, tissue is a common variant that needs to be taken into consideration [33,36]. Phytohormones are another typical class of treatments because they are key regulators involved in multi-biological processes of plants. In addition, hormones may play vital roles in the vegetative propagation of *E. crassipes* [38,39]. The reason for choosing P levels as treatments in this work is that P is a crucial factor in controlling clonal reproduction and the invasion speed of *E. crassipes* [40]. Therefore, the selection of reliable reference genes under these experimental conditions could favor a wide range of research on *E. crassipes*, which involves qRT-PCR experiments. According to the results obtained from this study, employing two reference genes in qRT-PCR experiments could ensure the normalization efficiency of target gene expression in *E. crassipes*. The combination of *eIF* and *ElF1a* is the best choice in normalizing expression of target genes in different tissues. In response to hormone treatments, using *Actin* and *eIF* as internal controls could maximize the accuracy of qRT-PCR results. In the gene expression quantification of plants supplied with different levels of P, *TUA* and *H2A* are recommended to function as reference genes.

5. Conclusions

In conclusion, the results obtained from this study support that different reference genes should be chosen when performing qRT-PCR on *E. crassipes* towards various patho-physiological treatments. In addition, to minimize systemic bias and provide accurate analysis of qRT-PCR on *E. crassipes*, two reference genes should be used. Specifically, the combination of *eIF* and *ElF1a*, *Actin* and *eIF*, and *TUA* and *H2A*, are recommended to be used as reliable reference genes in performing qRT-PCR on different tissues of *E. crassipes* and on *E. crassipes* in response to hormone or P stimuli, respectively. To the author's knowledge, this is the first report on the selection of reference genes in *E. crassipes*. The results could favor follow-up research on gene function and the underlying molecular mechanisms of its invasion and utilization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141711071/s1, Table S1: Sequence of 12 candidate reference genes.

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