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Selection and Validation of Reference Genes for qRT-PCR Analysis of Gene Expression in *Tropaeolum majus* (Nasturtium)

Qing Tang [1,†], Guang-Can Zhou [2,†], Si-Jie Liu [1], Wen Li [3,4], Yi-Lei Wang [2], Gao-Ying Xu [2], Teng-Fei Li [2], Guo-Qing Meng [2,*] and Jia-Yu Xue [1,*]

[1] College of Horticulture, Academy for Advanced Interdisciplinary Studies, Nanjing Agricultural University, Nanjing 210095, China; tangqing_zcmu@163.com (Q.T.); lsj1550909103@163.com (S.-J.L.)
[2] College of Agricultural and Biological Engineering, Heze University, Heze 274015, China; zgcan2009@163.com (G.-C.Z.); wangyilei001@163.com (Y.-L.W.); xugaoying011104@163.com (G.-Y.X.); litengfei200212@163.com (T.-F.L.)
[3] School of Life Sciences, Jiangsu Normal University, Xuzhou 221131, China; hfdy0706@163.com
[4] Jiangsu Xuzhou Sweetpotato Research Center, Chinese Agricultural Academy of Sciences, Xuzhou 221131, China
* Correspondence: mengguoqing@hezeu.edu.cn (G.-Q.M.); xuejy@njau.edu.cn (J.-Y.X.)
† These authors contributed equally to this work.

Abstract: *Tropaeolum majus* (nasturtium) is an important ornamental and medicinal plant due to its colorful flowers, shield-shaped leaves, and richness in mineral elements and bioactive compounds. However, the key genes related to these important biological traits, as well as their expression patterns and functions, remain obscure. In this study, to choose appropriate reference genes for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis, we screened 14 candidate genes from the transcriptome of *T. majus* and evaluated their expression stability. Through evaluation with four commonly used algorithms (geNorm, NormFinder, BestKeeper, and RefFinder), *EXP1*, *EXP2*, and *TUB6* were found to be the most stably expressed genes among different organs, while *EXP1* combined with *CYP2* was identified as the optimal reference gene combination for seeds at different development stages. For all the tested samples, *EXP1*, *EXP2*, *CYP2*, and *ACT2* were the most suitable reference genes. Moreover, the target gene *KCS11* involved in very-long-chain fatty acid biosynthesis was employed to confirm the most and least stable reference genes in different organs, seeds at different development stages, and all the tested samples. The expression profiles of *KCS11* were similar, with minor differences based on the analysis of different stable reference genes (either alone or in combination), while the expression profiles were diverse and the relative expression level was overestimated when using the least stable ones. These results suggest that the appropriate selection of reference genes is critical for the normalization of gene expression. Furthermore, the reference genes screened in this study will greatly improve the accuracy of the qRT-PCR quantification of candidate genes involved in the many biological characteristics of nasturtium.

Keywords: *Tropaeolum majus*; quantitative real-time PCR; reference genes; expression stability; organs; seeds of developmental stages

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1. Introduction

The quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) technique has been widely used for transcript quantification due to its high accuracy, sensitivity, specificity, low cost, and high-throughput characteristics [1–7]. Gene expression can be significantly influenced by a series of steps in an experimental procedure, such as the initial material amount, the quality of mRNA templates, reverse-transcription efficiency, and cDNA quality [1,8–10]. For accurate qRT-PCR results, it is essential to select one or more stably expressed genes as internal reference genes for the evaluation of gene expression levels [10–12]. Housekeeping genes, such as *GAPDH*, *ACT*, *TUB*, *18S rRNA*, *EF-1α*, *UBC*,

and *PP2AA3*, are regarded as stably expressed genes in all tissues at various developmental stages influencing basic cellular components and maintaining basal cellular functions and have been widely used to normalize the qRT-PCR data of plants and animals [6,13–17].

However, numerous studies have shown that the transcript levels of these housekeeping genes vary considerably in response to variable experimental conditions, different tissue types, and/or across taxa [6,7,14,18–21]; thus, none of the commonly developed genes reported so far can act as a universal reference. Hence, it is necessary to select a set of appropriate reference genes for the specific set of chosen experimental conditions and tissue types [13,22–25]. The use of proper reference genes for normalization in the relative quantification of gene expression can allow one to obtain valid results and correct conclusions from qRT-PCR analysis [1,19,26].

Tropaeolum majus (nasturtium) is an annual garden plant in the genus *Tropaeolum* and the family Tropaeolaceae native to South and Central America with shield-shaped leaves and pungent, edible yellow, orange, or red spurred flowers (www.iplant.cn/foc/ accessed on 10 February 2023). It is also cultivated as a medicinal plant due to its richness in mineral elements and bioactive compounds [27]. Additionally, it is a source of elongase involved in the synthesis of very-long-chain fatty acids, and this plant specifically contains significant amounts of erucic acid (70–75% of its total fatty acid content) in its seeds [28]. Mietkiewska et al. found a fatty acid elongase gene (nasturtium *KCS11*) in nasturtium embryos using the degenerate primer PCR approach, and seed-specific expression resulted in an up to eight-fold increase in erucic acid proportions in transgenic *Arabidopsis* seed oil [29]. The investigation of gene expression profiling related to the above-described important biological characteristics via qRT-PCR can help to advance our understanding of the molecular mechanisms regulating these characteristics in nasturtium. In a previous study, *ACTIN* was used as a positive control in a qRT-PCR analysis of the candidate genes involved in adaxial–abaxial differential growth during spur formation in *T. longifolium* [30]. However, there has not been a systematic selection of reference genes for qRT-PCR analysis in nasturtium so far.

Benefitting from high-throughput sequencing technology, growing plant transcriptome datasets have provided potential resources for the identification of appropriate reference genes [6,7,31–33]. In this study, 14 candidate reference genes (*TUA4*, *TUB6*, *ROC3*, *CYP2*, *GAPC2*, *YLS8*, *PTB1*, *TIP41*, *EXP1*, *EXP2*, *ACT2*, *PP2AA3*, *EF1-α*, and *UBC9*) were assembled from the nasturtium seed transcriptome database (Table 1). qRT-PCR analysis was used to assess the expression profiles of these candidate genes in different organs and seeds across different nasturtium developmental stages. Four statistical algorithms, namely, NormFinder, geNorm, BestKeeper, and RefFinder, were utilized to evaluate the stability of these putative reference genes. Moreover, the nasturtium fatty acid elongase gene *KCS11* was used to validate the stability of the exploited reference genes. The aims of this study were to evaluate the expression stability of 14 candidate reference genes and select the most stable reference genes expressed in different organs (roots, stem, leaves, and flowers) and seeds in different developmental stages in nasturtium. Additionally, we assessed the expression patterns of nasturtium *KCS11* in different organs and seed developmental stages of nasturtium. The results provide a reference for the further expression profiling of functional genes and facilitate studies on the mechanisms regulating erucic acid synthesis in nasturtium.

Table 1. Information on candidate reference genes in nasturtium.

Gene Name	Gene Symbol	*Arabidopsis* Homologue	Primer Sequence (Forward/Reverse)	Size (bp)	Efficiency (%)	R^2
Actin-2	*ACT2*	*AT3G18780*	CAGACCGTATGAGCAAGGAGA ATTGATGGACCAGACTCGTCG	178	98.07	0.9979
Glyceraldehyde-3-phosphate dehydrogenase GAPC2	*GAPC2*	*AT1G13440*	GGCTGCTATCAAGGAGGAGTCTG GATCAACCACCCGGGTACTGT	203	115.20	0.9913
Tubulin alpha-4 chain	*TUA4*	*AT1G04820*	GCATTGGTATGTTGGTGAGGGT ACTCATCCCCATCATCATCTTCC	134	95.79	0.9942
Tubulin beta-6 chain	*TUB6*	*AT5G12250*	ATGTTCAGGAGGGTGAGTGA CTCATCAGCGGTAGCATCTTG	168	104.13	0.9996
Peptidyl-prolyl cis-trans isomerase CYP19-1 ROC3	*ROC3*	*AT2G16600*	CTCAGTTCTTCGTCTGTACCGAG AGAAGCGCAGATCCTCCACT	213	98.84	0.9985
Cyclophilin-like peptidyl-prolyl cis-trans isomerase 2	*CYP2*	*AT4G33060*	CAGCTCAGTTGAAGTTTGTGCC CAGCCTCATCCAGGTTATAGAACTC	235	99.89	0.9964
GTP binding Elongation factor EFTu/EF1A	*EF1-α*	*AT1G07920*	ATCTCCAAGGATGGGCAGAC TCCAACCTTCTTCAGGTAGGAAG	168	108.64	1.0000
Expressed protein 1 (Dimethylallyl, adenosine tRNA methylthiotransferase)	*EXP1*	*AT4G33380*	CTGAGTTTGGACGACGTGAAG CCAGCTTTCTTCTTCCTCATCG	173	105.26	0.9979
Expressed protein 2 (S-adenosyl-L-methionine-dependent methyltransferases)	*EXP2*	*AT2G32170*	CCGAGTATGGATGCTATTCTCCAG GATTGACTGCTCATTGTGAAGTCC	203	105.54	0.9939
Serine/threonine-protein phosphatase PP2AA3	*PP2AA3*	*AT1G13320*	GCCTGAAGATTGTGTTGCTCAC CTGCTATTCGTACTTCAGCCTCA	197	97.97	0.9959
Polypyrimidine tract-binding protein 1	*PTB1*	*AT3G01150*	TGCTGTCACTGTGGATGTGC TGCGTCTCTAGCAACTGCTG	142	106.44	0.9974
Type 2a phosphatase activator tip41	*TIP41*	*AT4G34270*	CGCAAACGCTCCATTCTCACTTC CCTGCTGAGAAGGTTTGCATCTG	241	98.15	0.9944
Ubiquitin-conjugating enzyme 9	*UBC9*	*AT4G27960*	TTGCTCTCAATCTGCTCGCTG TCCTATCGCAACCATCATAGCG	175	119.79	0.9978
Mitosisprotein YLS8	*YLS8*	*AT5G08290*	TGATTGGGATGAAACATGCATGC TTGTGGATGGATCGTACAGCTC	146	110.54	0.9908
3-ketoacyl-CoA synthase 11	*KCS11*	*AT2G26640*	AGGAAGACTCGGACGGGAAGAT GACATGGGTAGAACAAGTGGTCCG	115	100.36	0.9982

2. Materials and Methods

2.1. Plant Material, Candidate Reference Gene Isolation, and Primer Design

The plant materials were grown in a greenhouse under natural light conditions at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (32.06° N, 118.84° E). Four organ samples—taken from the roots, stem, leaves, and flowers (just after blooming)—and seeds at different developmental stages were collected from the same plant. Flowers were hand-pollinated, and the seeds were classified into four developmental stages: 7, 14, 21, and 28 days after pollination (dap). All samples were collected from three individuals in each organ or seed developmental stage, frozen using liquid nitrogen, and then stored at −80 °C prior to use. Homologs for the *ACT2*, *GAPC2*, *YLS8*, *ROC3*, *CYP2*, *TIP41*, *PTB1*, *EXP1*, *EXP2*, *PP2AA3*, *TUA4*, *TUB6*, *EF1-α*, and *UBC9* genes from nasturtium were isolated from a reference transcriptome based on mixed nasturtium seeds at different developmental stages. RNA-seq experiments were conducted using a TruSeq mRNA library construction kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina NextSeq 500 platform, and 150 bp paired-end reads were read using a product developed by the sequencing company Personalbio (Shanghai, China). The transcriptome was assembled de novo using Trinity (r20140717, k-mer 25 bp) [34]. The search for homolog genes was performed using BLASTN on the assembled transcriptome, using *ACT2*, *GAPC2*, *YLS8*, *ROC3*, *CYP2*, *TIP41*, *PTB1*, *EXP1*, *EXP2*, *PP2AA3*, *TUA4*, *TUB6*, *EF1-α*, and *UBC9* gene sequences from *Arabidopsis thaliana* as queries. Raw sequence data were deposited at the NCBI Sequence Read Archive (SRR25909845). Specific primers were designed based on these candidate reference genes sequences (Table S1) using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools accessed on 5 September 2021) with the following parameters: primer lengths of 20–25 bp, amplicon lengths of 110–250 bp, GC content of 40–55%, and melting temperature (Tm) in a range of 55–60 °C.

2.2. Total RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR Analysis

Total RNA was isolated from roots, stems, leaves, flowers (just after blooming), and seeds at different developmental stages using an RNAprep Pure Plant Kit (Tiangen Biotech, Beijing, China) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Isolated RNA was treated with DNase I (Ambion, Foster City, CA, USA). Only the RNA samples with absorption ratios of A260/230 higher than 1.8 and A260/280 = 1.8–2.2 were used for cDNA synthesis. A total of 1 μg per sample was used as a template for cDNA synthesis using the PrimeScriptTM RT Reagent Kit with Oligo dT primers (TaKaRa Bio Inc., Dalian, China). The cDNA was diluted five-fold with nuclease-free water and stored at −20 °C for qRT-PCR analysis.

qRT-PCR was performed in a 96-well plate using a QuantStudio^{TM} 3 Flex System (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR reaction mix amounted to 20 μL in total and contained 10 μL of TB Green Premix (TaKaRa Bio Inc., Dalian, China), 2 μL of diluted cDNA, 0.4 μL of upstream and downstream primers (10 μmol/L), and ddH_2O, adding up to its final volume. The qRT-PCR reaction procedures were as follows: 95 °C for 5 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s.

Melting curve analysis and PCR products run on a 1.5% agarose gel were used to verify the specificity of each primer pair. The cycle threshold (Ct) was measured automatically and used to define the expression level of each reference gene. The correlation coefficients (R^2) and slope were calculated from a standard curve based on a five-fold serial dilution of all sample cDNA templates, and the equation (% Efficiency = $(10^{[-1/\text{slope}]} - 1) \times 100\%$) was used to calculate the mean amplification efficiency (E) for each primer pair [35,36].

2.3. Statistical Analysis of Candidate Reference Genes

GeNorm [18], NormFinder [37], BestKeeper [38], and RefFinder [39] were used to statistically assess the stability of the 14 selected reference genes across all experimental subsets. The qRT-PCR data of all tested samples were collated and summarized using Excel 2010 software. The stability of each reference gene was analyzed in strict adherence to the

algorithm principles stipulated by the software programs. As recommended by GeNorm, a reference gene with an M value below 1.5 was considered stably expressed, and a lower M value represented more stable expression [18]. NormFinder was used to calculate the stability value (SV) of reference genes in evaluating expression variation, and a lower SV indicates higher stability [37]. For BestKeeper, the Ct values of these 14 candidate reference genes were used to calculate the coefficient of variance (CV) and the standard deviation (SD). The lowest CV ± SD value represents the most stable gene, and genes with an SD of less than 1 were considered acceptable [38,40,41]. Finally, a comprehensive analysis integrating M values from GeNorm, SV from NormFinder, CV and SD from BestKeeper, and delta Ct values was performed in RefFinder to generate a comprehensive, stable ranking of the candidate reference genes.

2.4. Validation of Selected Reference Genes

To confirm the utility of the candidate reference genes in the final standardization results, the relative expression levels of the nasturtium *KCS11* gene in different organs and in seeds at different developmental stages were analyzed using the most stable and unstable reference genes or a combination of stable reference genes after normalization via RefFinder across all experimental sets. The *KCS11* sequences were obtained from the transcriptome data (Table S1). The qRT-PCR amplification conditions of the target gene were the same as those described above. The $2^{-\Delta\Delta CT}$ method was used to calculate the fold change in relative gene expression [42].

3. Results

3.1. Selection of Candidate Reference Genes and PCR Amplification

Fourteen genes (*ACT2*, *GAPC2*, *YLS8*, *ROC3*, *CYP2*, *TIP41*, *PTB1*, *EXP1*, *EXP2*, *PP2AA3*, *TUA4*, *TUB6*, *EF1-α*, and *UBC9*) were selected as candidate reference genes. All of these genes were retrieved from the transcriptomic data of nasturtium via BLASTX using *Arabidopsis* orthologous as queries. The primer sequences and PCR amplification characteristics of the 14 candidate reference genes are described in Table 1. Agarose gel electrophoresis and melting curve analysis were used to evaluate the primer specificities, and the results showed that all the target reference genes had single-peak melting curves and a single amplicon with the expected size (Figure 1). The amplification product lengths ranged from 134 bp for *TUA4* to 241 bp for *TIP41*. For each primer pair, the amplification efficiencies ranged from 95.79% for *TUA4* to 119.79% for *UBC9*, while the correlation coefficients (R^2) ranged from 0.9908 for *YLS8* to 1.0000 for *EF1-α* (Table 1).

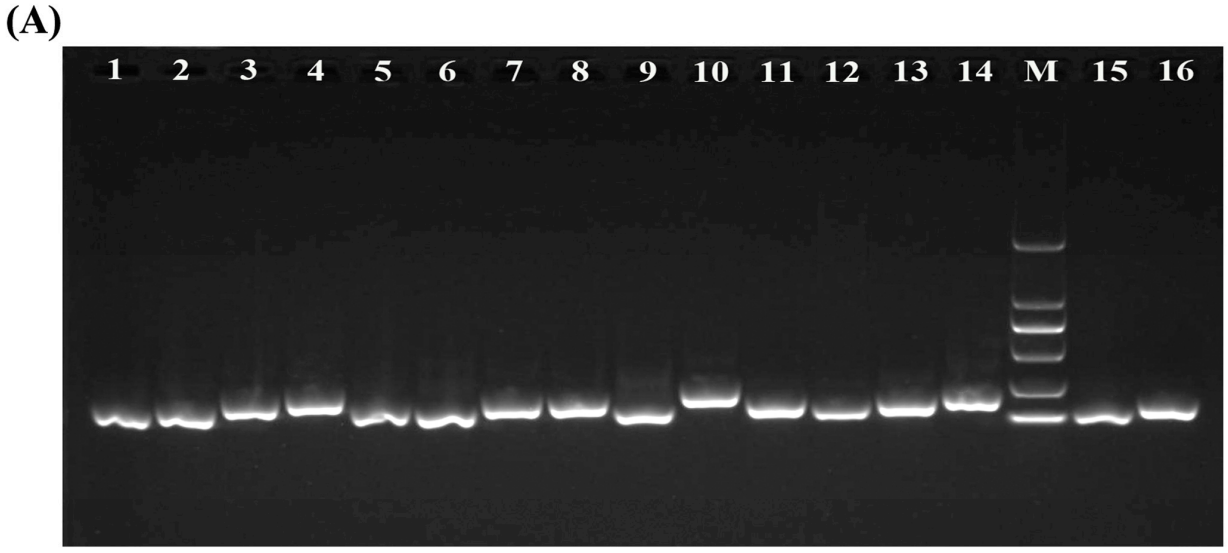


Figure 1. *Cont.*

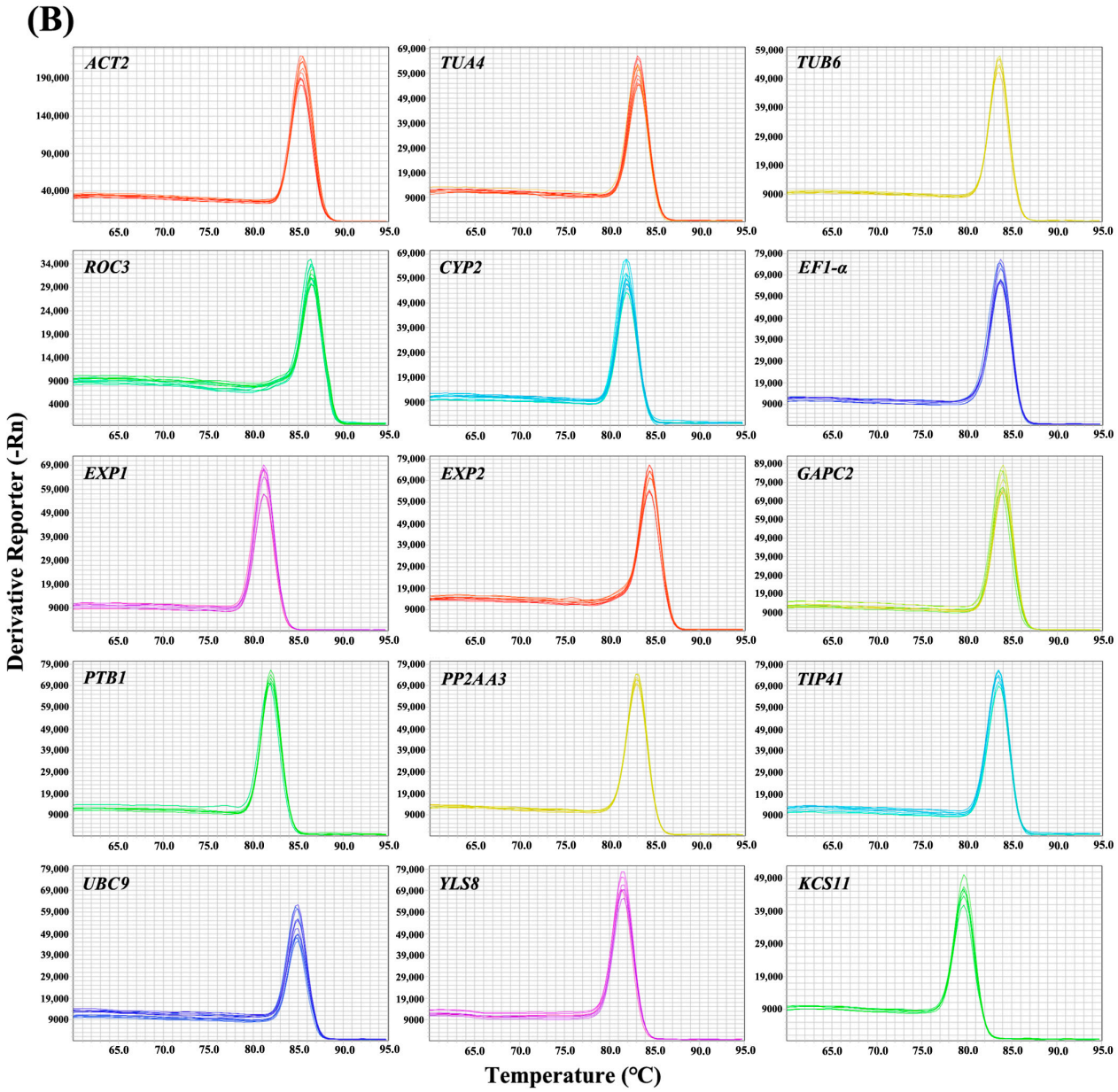


Figure 1. Agarose gel electrophoresis of PCR products and melting curves. (**A**) Agarose gel (1.5%) electrophoresis indicating the amplification of a single PCR product of the expected size for 14 candidate reference genes and the *KCS11* gene in nasturtium (lines 1–15: *TUA4*, *TUB6*, *ROC3*, *CYP2*, *EF1-α*, *EXP1*, *EXP2*, *PP2AA3*, *PTB1*, *TIP41*, *UBC9*, *YLS8*, *ACT2*, *GAPC2*, and *KCS11*). M represents a DL2000 DNA marker. (**B**) Melting curves for the 15 genes showing single peaks.

3.2. Expression Profiles of Candidate Reference Genes

The expression profiles of these 14 reference genes in the roots, stems, leaves, and flowers and in four seed developmental stages were examined using their mean cycle threshold (Ct) values. The mean Ct values of the 14 reference genes considering all the samples varied from 17 to 23 (Figure 2). Among these reference genes, *EF1-α* exhibited the highest expression levels, with the lowest average Ct value, namely, 17.37 ± 1.21 (mean ± SD), followed by *ROC3* (18.02 ± 0.75), *GAPC2* (18.68 ± 0.76), *ACT2* (18.92 ± 1.02), *UBC9* (18.97 ± 1.03), *TUA4* (20.06 ± 2.84), *TUB6* (20.1 ± 1.87), and *YLS8* (20.48 ± 1.3). *EXP1* had the lowest expression level of all the tested genes, with mean Ct values of 23.08 ± 0.81, followed by *TIP41* (22.9 ± 2.05), *PTB1* (22.8 ± 1.75), *PP2AA3* (22.62 ± 1.25), *CYP2* (22.25 ± 0.8), and *EXP2* (21.67 ± 0.78) (Figure 2). Moreover, the standard deviations

(SD) of the Ct values ranged from 0.75 to 2.84. The reference genes with a lower SD of Ct values indicate higher stability. *ROC3* (18.02 ± 0.75) showed the smallest variation in gene expression, while *TUA4* (20.06 ± 2.84) showed the most variable expression levels (Figure 2).

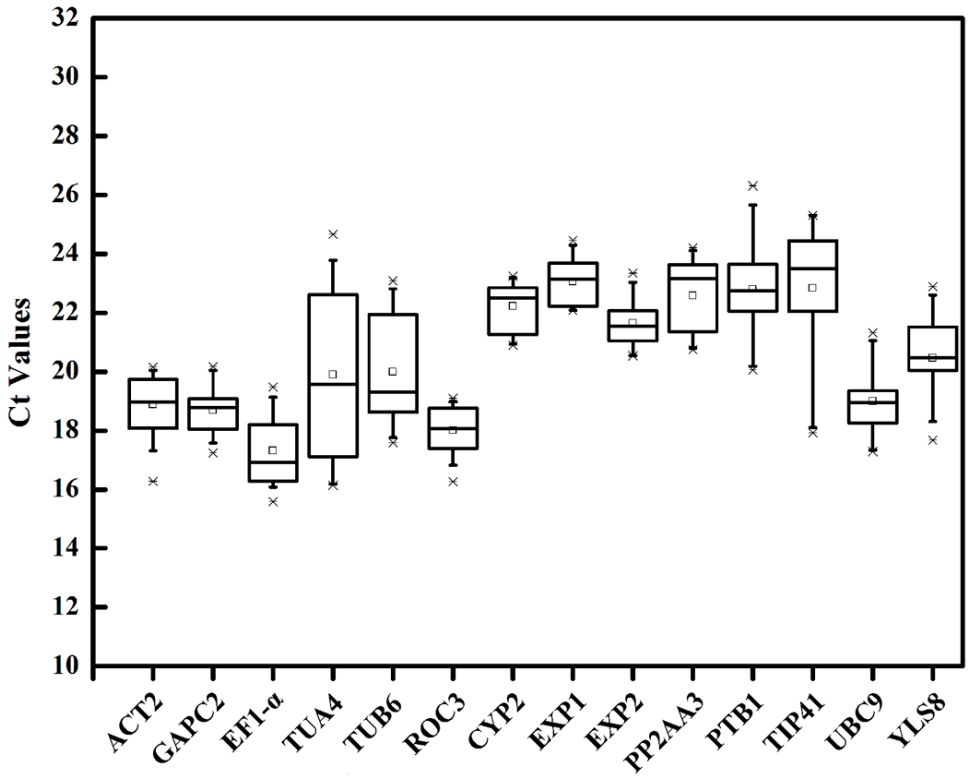


Figure 2. Distribution of Ct values of candidate reference genes across all samples. The Ct distribution boxes indicate the 25th and 75th percentiles. The whiskers represent the maximum and minimum values, while the lines and the squares within the boxes indicate the median and mean, respectively. The asterisk represents the outliers.

3.3. Expression Stability of Candidate Reference Genes

To obtain the optimal reference genes for qRT-PCR analysis in the investigation of different organs and seeds in different development stages, we divided these tested samples into three groups: different organs, seeds at different developmental stages, and all samples. Four statistical algorithms (geNorm, NormFinder, BestKeeper, and RefFinder) were used to further assess the stability of the 14 reference genes. As shown in Figure 3, all these tested candidate reference genes were considered stable genes on the basis of their M values being less than 1.5 when assessed using geNorm. *CYP2* and *EXP2* were recommended as the most stable reference genes in the seeds at different developmental stages and all samples, while *EXP1* and *EXP2* were identified as the most stable reference genes in different organs. In contrast, *TUA4* in the seeds at different developmental stages and all samples, and *PTB1* in different organs with the highest M values, were considered to be the most unstable reference genes (Figure 3). Additionally, the pairwise variation (V_n/V_{n+1}) between the normalization factors was calculated using the geNorm program, and the optimal number of reference genes for accurate normalization was determined (Figure 4). A ratio of V2/3 in the seeds at different development stages less than 0.15 suggests that at least two reference genes should be used in the qRT-PCR analysis. For the different organs and all samples, V3/4 and V4/5 were 0.119 and 0.126, indicating that three and four internal reference genes, respectively, were required to obtain accurate results (Figure 4). The stability values (SVs) of the candidate reference genes were determined using Normfinder (Table 2). A lower SV indicates higher stability. For all three sample groups, *EXP1* was deemed the most stable

reference gene, followed by *EXP2*, *ROC3*, and *ACT2* in different organs, seeds at different developmental stages, and all samples, respectively. Similar to geNorm, *TUA4* in seeds at different developmental stages and all samples and PTB1 in different organs were the least stable genes.

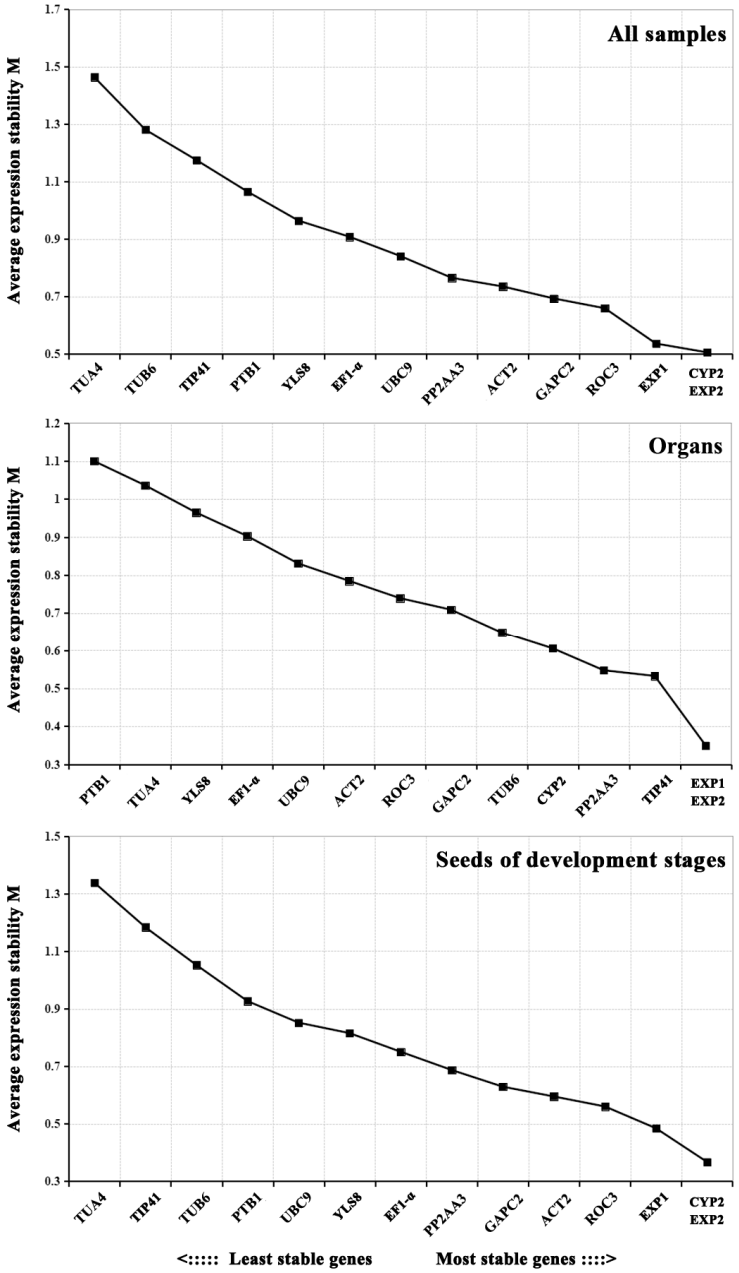
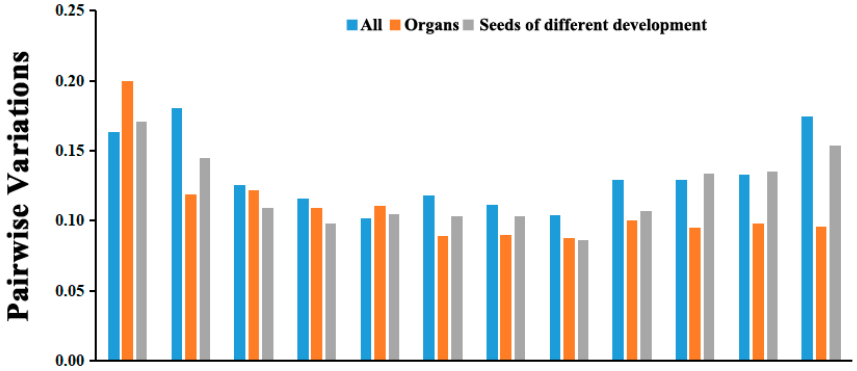


Figure 3. Expression stability (M) values of the 14 candidate reference genes determined using geNorm software v 1.0. The lowest M value represents the most stable gene.

According to the criteria of the BestKeeper program, *TUB6* (2.39 ± 0.44) was identified as the most stable gene for normalization in different organs (Table 3). In the seeds of the different development stages group, *UBC9* (2.23 ± 0.42) had the lowest CV ± SD values, showing remarkably stable expression. In all samples, *EXP2* (2.94 ± 0.64) was considered the most stable reference gene, followed by *CYP2* (3.06 ± 0.68) and *EXP1* (3.11 ± 0.72), while *TUA4* (11.72 ± 2.35) was the most unstable reference gene (Table 3).



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11	V11/12	V12/13	V13/14
All	0.164	0.180	0.126	0.116	0.101	0.118	0.112	0.104	0.129	0.129	0.133	0.175
Organs	0.200	0.119	0.122	0.109	0.111	0.089	0.090	0.088	0.100	0.095	0.098	0.096
Seeds of different development	0.171	0.145	0.109	0.098	0.105	0.103	0.103	0.086	0.107	0.134	0.135	0.154

Figure 4. Determination of optimal number of reference genes for normalization via pairwise variation analysis using GeNorm software. V_n/V_{n+1} values were used to determine the optimal number of reference genes.

Table 2. Stability of candidate reference genes according to NormFinder software v 1.0.

Ranking Order	Organs		Seeds in Different Developmental Stages		All Samples	
	Gene Name	Stability Value	Gene Name	Stability Value	Gene Name	Stability Value
1	*EXP1*	0.175	*EXP1*	0.155	*EXP1*	0.070
2	*EXP2*	0.268	*ROC3*	0.158	*ACT2*	0.262
3	*TUB6*	0.300	*ACT2*	0.257	*PP2AA3*	0.375
4	*ACT2*	0.346	*CYP2*	0.399	*EXP2*	0.376
5	*UBC9*	0.422	*EF1-α*	0.416	*CYP2*	0.443
6	*PP2AA3*	0.504	*PP2AA3*	0.424	*ROC3*	0.499
7	*CYP2*	0.505	*EXP2*	0.434	*GAPC2*	0.553
8	*GAPC2*	0.535	*GAPC2*	0.463	*EF1-α*	0.554
9	*TIP41*	0.576	*YLS8*	0.643	*UBC9*	0.658
10	*ROC3*	0.642	*UBC9*	0.714	*YLS8*	0.722
11	*YLS8*	0.687	*PTB1*	0.941	*TUB6*	1.053
12	*EF1-α*	0.699	*TUB6*	0.987	*PTB1*	1.091
13	*TUA4*	0.859	*TIP41*	1.179	*TIP41*	1.099
14	*PTB1*	0.921	*TUA4*	1.481	*TUA4*	1.686

Due to the different principles of the geNorm, NormFinder, and BestKeeper algorithms, the results for the most stable reference genes were not completely consistent. Therefore, a comprehensive evaluation of gene expression stability for each candidate gene was performed using RefFinder to calculate the geometric mean of the three algorithms' corresponding rankings (Table 4). This comprehensive analysis indicated that *EXP1* was the most stable gene in different organs, in seeds at different developmental stages, and in all the tested samples. Conversely, the traditional housekeeping gene *TUA4* was unstably expressed in different organs and in seeds at different developmental stages in nasturtium.

3.4. Validation of the Selected Reference Genes

To examine the reliability of the reference genes for normalization, the relative expression patterns of nasturtium *KCS11*, which is related to erucic acid biosynthesis [29], were evaluated in different organs, in seeds at different developmental stages, and in all the samples. The most stable reference genes (*EXP1*, *EXP2*, *TUB6*, and *EXP1/EXP2/TUB6* for different organs; *EXP1*, *CYP2*, and *EXP1/CYP2* for seeds at different development stages; and *EXP1*, *EXP2*, *CYP2*, *ACT2*, and *EXP1/EXP2/ CYP2/ACT2* for all samples) were

selected in accordance with the pairwise variation (V_n/V_{n+1}) using geNorm to normalize *KCS11* gene expression in the three group samples. In addition, the least stable reference genes (*PTB1* for different organs, *TIP41* for seeds at different development stages, and *TUA4* for all samples) were used to normalize the target gene expression profiles for a comparative analysis.

Table 3. Stability of candidate reference genes according to Bestkeeper software v 1.0.

Ranking Order	Organs		Seeds in Different Developmental Stages		All Samples	
	Gene Name	Stability Value	Gene Name	Stability Value	Gene Name	Stability Value
1	*TUB6*	2.39 ± 0.44	*UBC9*	2.23 ± 0.42	*EXP2*	2.94 ± 0.64
2	*ROC3*	2.91 ± 0.52	*CYP2*	2.26 ± 0.51	*CYP2*	3.06 ± 0.68
3	*EXP1*	3.20 ± 0.73	*EXP2*	2.37 ± 0.52	*EXP1*	3.11 ± 0.72
4	*EXP2*	3.23 ± 0.69	*GAPC2*	2.40 ± 0.45	*GAPC2*	3.28 ± 0.61
5	*GAPC2*	3.65 ± 0.68	*YLS8*	2.50 ± 0.51	*ROC3*	3.47 ± 0.63
6	*EF1-α*	3.85 ± 0.65	*ACT2*	2.62 ± 0.51	*UBC9*	4.08 ± 0.77
7	*CYP2*	4.00 ± 0.88	*EXP1*	2.89 ± 0.67	*ACT2*	4.26 ± 0.81
8	*UBC9*	4.54 ± 0.88	*PTB1*	3.57 ± 0.79	*YLS8*	4.55 ± 0.93
9	*ACT2*	4.84 ± 0.90	*ROC3*	3.68 ± 0.66	*PP2AA3*	5.15 ± 1.17
10	*TIP41*	4.88 ± 1.13	*PP2AA3*	4.66 ± 1.06	*PTB1*	5.95 ± 1.36
11	*PP2AA3*	5.57 ± 1.23	*EF1-α*	5.71 ± 1.03	*EF1-α*	6.05 ± 1.05
12	*YLS8*	5.96 ± 1.24	*TUB6*	6.05 ± 1.29	*TIP41*	6.80 ± 1.56
13	*PTB1*	6.11 ± 1.44	*TUA4*	7.53 ± 1.67	*TUB6*	8.39 ± 1.69
14	*TUA4*	7.97 ± 1.43	*TIP41*	9.04 ± 2.03	*TUA4*	11.72 ± 2.35

Table 4. Stability of candidate reference genes according to RefFinder.

Ranking Order	Organs		Seeds in Different Developmental Stages		All Samples	
	Gene Name	Stability Value	Gene Name	Stability Value	Gene Name	Stability Value
1	*EXP1*	1.57	*EXP1*	2.21	*EXP1*	1.97
2	*EXP2*	2.11	*CYP2*	2.83	*EXP2*	2.45
3	*TUB6*	2.71	*ROC3*	3.44	*CYP2*	2.99
4	*ACT2*	6.00	*ACT2*	3.66	*ACT2*	3.60
5	*PP2AA3*	6.31	*EXP2*	4.12	*GAPC2*	3.96
6	*ROC3*	6.32	*UBC9*	5.01	*ROC3*	4.12
7	*UBC9*	6.47	*GAPC2*	5.24	*PP2AA3*	5.69
8	*GAPC2*	6.51	*YLS8*	5.38	*UBC9*	7.67
9	*CYP2*	6.65	*PP2AA3*	7.93	*EF1-α*	8.74
10	*TIP41*	7.02	*EF1-α*	8.80	*YLS8*	9.46
11	*EF1-α*	8.30	*PTB1*	10.46	*PTB1*	11.24
12	*YLS8*	11.49	*TUB6*	12.00	*TUB6*	12.22
13	*TUA4*	13.00	*TUA4*	13.00	*TIP41*	12.49
14	*PTB1*	14.00	*TIP41*	14.00	*TUA4*	14.00

KCS11 was expressed in the roots, stems, leaves, flowers, and seeds (7 dap) but at different levels (Figure 5A). According to the analysis of different stable reference genes, either alone (*EXP1*, *EXP2*, or *TUB6*) or in combination (*EXP1/EXP2/TUB6*), the expression patterns of *KCS11* were similar, with minor differences, and the results showed that *KCS11* was relatively highly expressed in leaves and flowers and weakly expressed in stems and seeds (7 dap). However, the expression patterns of *KCS11* were observed to be diverse in different organs, especially in stems, leaves, and flowers, when using the least stable gene, PTB1, for normalization. Although the expression level of *KCS11* was higher in the leaves and flowers, its relative expression was overestimated (Figure 5A). When the different stable reference gene(s), either alone (*EXP1* or *CYP2*) or in combination (*EXP1/CYP2*), were used for normalization in seeds at different development stages, the *KCS11* expression patterns

were similar, with minor differences (Figure 5B). *KCS11* transcripts were significantly increased from 7 to 14 dap and then successively decreased at 21 and 28 dap. When the least stable gene, *TIP41*, was used for normalization, the expression patterns and transcript levels were very different. The expression levels increased, peaked at 21 dap, and then decreased. Based on the suggestions of RefFinder and the geNorm pairwise variation analysis, a single (*EXP1*, *EXP2*, *CYP2*, or *ACT2*) reference gene and a combination (*EXP1/EXP2/CYP2/ACT2*) of four reference genes were selected to normalize *KCS11* gene expression in all samples. For either a single reference gene or a combination of stable reference genes, the expression patterns of *KCS11* were similar in all the tested samples (Figure 5C). The expression levels were remarkably lower in roots, stems, leaves, flowers, and seeds (7 dap), while they increased, peaked at 14 dap, and then decreased at 21 and 28 dap. However, when *TUA4* was regarded as the reference gene, the *KCS11* expression patterns were diverse, and the relative expression level was overestimated (Figure 5C). Overall, our results suggest that these genes' expression patterns are closely correlated with the stability of the reference genes.

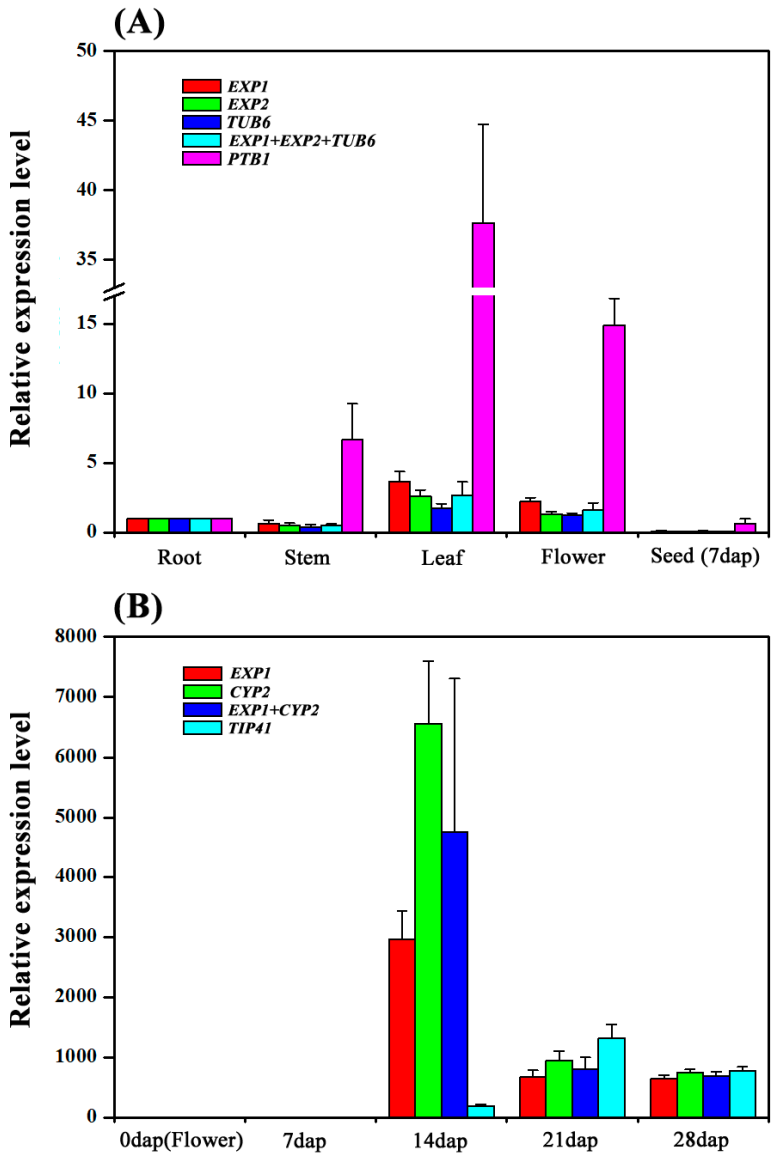


Figure 5. *Cont.*

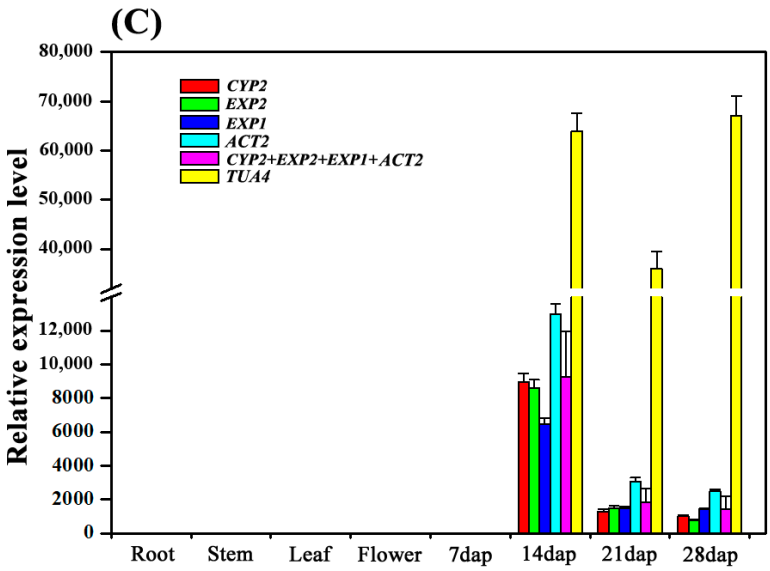


Figure 5. *KCS11* expression patterns normalized using an unstable gene and the most stable genes in different organs (**A**), seeds at different development stages (**B**), and all tested samples (**C**).

4. Discussion

The qRT-PCR technique is widely used in gene expression analysis due to its rapid and high-throughput properties [26]. To obtain reliable quantification results in qRT-PCR analysis, it is essential to select and validate suitable reference genes for data normalization. Ideally, reference genes should be expressed at a constant level regardless of the various organs, tissue types, or developmental stages. In this study, 14 candidate reference genes were selected based on the transcriptome data of mixed individual developing nasturtium seeds, and their expression stability was measured in different nasturtium organs and in nasturtium seeds at different development stages. The PPI network made in the STRING database v11.5 with high confidence (0.700) using housekeeping genes (gene names following *Arabidopsis* gene ID names) was analyzed in the present work. The analysis revealed that in 14 species, some of the genes were used as a reference (https://version-11-5.string-db.org/cgi/network?networkId=bFxkb046DcDI, accessed on 10 October 2023). Due to the different principles of evaluating gene stability of the geNorm, NormFinder, and BestKeeper algorithms, the stability orders of 14 candidate reference genes were not completely identical (Figure 3, Tables 2 and 3). For example, in different organs, *TUB6* was selected as the most stable reference gene by BestKeeper, while it was ranked third and fifth in NormFinder and geNorm, respectively. In all the tested samples, *EXP2* was ranked first in geNorm and BestKeeper, while it was ranked fourth in NormFinder. A similar phenomenon has been reported for many other plants, such as *L. aurea*, *H. salicornia*, *C. capsularis*, and two *Chrysanthemum* species [6,41,43,44]. For determining the stable reference genes for nasturtium, RefFinder, a widely used comprehensive tool for screening appropriate reference genes in plants [6,7,44–51], was employed to determine the final overall ranking of candidate reference genes according to the geometric mean of the weights of each gene calculated using the different algorithms [39]. Based on the RefFinder analysis, *EXP1* was marked as the most stable reference gene for normalization in different nasturtium organs and seeds at different development stages (Table 4). Moreover, we noticed that *ACT2* displayed relative expression stability and ranked fourth in the RefFinder analysis (Table 4), and it was used as a positive control gene in *Tropaeolum longifolium* [30]. Thus, a better accuracy for each candidate gene was acquired by integrating the results of the three algorithms used in this study, and it is necessary to utilize more than two algorithms for evaluating the stability of reference genes.

Increasing evidence has shown that the expression levels of classic housekeeping genes vary greatly among species, organs, tissues, specific conditions, and developmental

stages [6,7,13,45,52–60]. In this study, the expression stability of commonly used housekeeping genes, such as *ACT2*, *GAPC2*, *UBC9*, *TUA4*, *EF-1α*, and *TUB6*, displayed remarkably differential expression patterns in nasturtium organs and seeds at different developmental stages. Of these selected housekeeping genes, *ACT2* ranked fourth among all tested samples. *GAPC2* and *UBC9* were the most stable reference genes for flower development in *Paeonia suffruticosa* and *Jatropha curcas* [52], while they ranked in medium positions in the present study. Moreover, *UBC9* exhibited strong stability in *L. aurea*, *Corchorus olitorius*, and *Platycladus orientalis* [6,40,43]. *EF1-α* showed a less stable expression pattern, with a lower ranking order in RefFinder. *EF1-α* was also identified as unstable across the tissues and organs of tomato at various developmental stages [61], but it was highly stable in rice tissues [62]. *TUA4* was identified as the most unstable in all the tested nasturtium samples. The lower stability of *TUA4* in nasturtium was consistent with the results regarding *L. aurea* and *Taihangia rupestris* flowers [6,32]. Additionally, the qRT-PCR validation results showed that the *KCS11* expression profiles were strongly biased when the least stable gene *TUA4* was used as a reference for all the tested samples (Figure 4). These results indicate that it is necessary to select suitable reference genes to normalize the transcription levels of target genes under a given experimental condition.

Recently, several new reference genes have been developed in qRT-PCR analysis for non-model plants in addition to the classic ones [45,54,63–66]. In this study, eight newly developed reference genes, *ROC3*, *CYP2*, *EXP1*, *YLS8*, *EXP2*, *PP2AA3*, *PTB1*, and *TIP41*, were tested. As shown in Table 4, *EXP1* ranked ahead of *EXP2* and was judged to be the most stable gene across all the sample groups. High expression stability of *EXP1* has been reported in the developing stages of anthers and pollen in rice [67] and in most experimental subsets, including tissue samples and all samples in *L. aurea* [6]. CYPs (Cyclophilins) belong to a large family of proteins with peptidyl-prolyl cis-trans isomerase activity relevant to a variety of biological processes and are widely expressed in bacteria, fungi, animals, and plants [68]. *CYP* is the most stable reference gene for different tissues in *Isatis indigotica* [69]. In *L. aurea*, *ROC3* and *CYP2* have also been selected as the most stable reference genes for methyl jasmonate treatments and cold stress, respectively [6]. In this study, *ROC3* and *CYP2* were more stable in the seeds at different developmental stages than in different organs and all the tested samples. *PP2AA3* and *YLS8* have also been reported to be stable reference genes in *Setaria viridis*, *Corchorus olitorius*, *Oenanthe javanica*, peanut, *Humulus lupulus*, and *Arabidopsis* [36,70–74] while also being less stably expressed in *L. aurea* [6] and our tested samples. The stability results of *PTB1* and *TIP41* were similar to those of *PP2AA3* and *YLS8*, although they displayed a relatively stable expression profile in some plants and experimental conditions [6,41,75–80]. They were the least stable reference genes in the different organs and seeds of nasturtium at different developmental stages. However, *TIP41* was identified as the second most stable gene in organs only via geNorm software (Figure 3; Tables 2–4). This phenomenon was similar to that regarding *PP2AA3* in some *L. aurea* experimental conditions [6] and could be attributed to the fact that *TIP41* plays a role in the regulation of the TOR signaling pathway and indirectly activates PP2A phosphatase via interaction with its suppressor TAP46 [54].

It is widely acknowledged that more accurate expression quantification results can be obtained by using multiple reference genes as opposed to a single one in qRT-PCR analysis [61,81]. Based on the pairwise variation analysis and validation of target gene expression, *EXP1/EXP2/TUB6* for different organs, *EXP1* combined with *CYP2* for seeds at different development stages, and *EXP1/EXP2/CYP2/ACT2* for all samples were chosen as suitable reference genes in the qRT-PCR analysis for nasturtium (Figure 5). The results indicated that the gene expression level of *KCS11* was relatively low in roots, stems, leaves, flowers, and seeds (7 dap), while it increased, peaked at 14 dap, and then decreased at 21 and 28 dap (Figure 5). However, *PTB1*, *TIP41*, and *TUA4* were validated as reference genes for the normalization of *KCS11*, and their expression patterns and levels were misestimated (Figure 5). Therefore, it is crucial to screen the appropriate reference genes for the normalization of target gene expression via qRT-PCR for nasturtium. In addition,

previous studies showed that nasturtium *KCS11* is involved in erucic acid biosynthesis and expressed in seeds [28,29], and the expression level of *KCS11* increasing and peaking at 14 dap might be in line with erucic acid accumulation. A similar expression pattern of *KCS11* in the developing nasturtium seeds was found by analyzing the Jensen's RNA-Seq data [82], where the expression of *KCS11* reached the highest level at 16 DAP (Table S2).

5. Conclusions

Here, we investigated the expression stability of 14 candidate reference genes in different organs and seeds at different developmental stages in nasturtium using four statistical algorithms. Additionally, the expression pattern of the target gene nasturtium *KCS11* was determined in different organs and seeds at different development stages to further confirm the reliability of the identified stable genes. *EXP1*, *EXP2*, and *TUB6* were the most stably expressed across different organs. *EXP1* and *CYP2* expression were the most stable for the seeds at different development stages, and *EXP1*, *EXP2*, *CYP2*, and *ACT2* were the best genes for all the tested samples. The findings presented in this study could facilitate functional genomic studies on many biological characteristics of nasturtium.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9111176/s1, Table S1: Sequences of the 14 candidate reference genes assembled from the nasturtium seed transcriptome; Table S2: Results of nasturtium *KCS11* expression level analysis using Jensen's RNA-Seq data.

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Data Availability Statement: Sequences of the 14 candidate reference genes assembled from the nasturtium seed transcriptome. Results of nasturtium *KCS11* expression level analysis using Jensen's RNA-Seq data.

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