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ORIGINAL ARTICLE

Selection of reliable reference genes for gene expression analysis in seeds at different developmental stages and across various tissues in *Paeonia ostii*

Chaoqiong Li[1,2] · Lizong Hu[1] · Xueqin Wang[1] · Hongzhan Liu[1] · Huihui Tian[3] · Junsheng Wang[1]

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Abstract

Paeonia ostii seeds have recently been identified as a new source of α-linolenic acid in China. Studying the gene expression patterns of unsaturated fatty acid-related genes would be helpful for understanding the mechanism of α-linolenic acid accumulation. Quantitative real-time polymerase chain reaction (qRT-PCR) is a useful method for reliably evaluating gene expression, and it is necessary to select reliable reference genes for data normalization in qRT-PCR analysis. In this study, we evaluated the expression stability of 12 candidate reference genes using four mathematical algorithms (ΔC_t, BestKeeper, NormFinder, and geNorm). The web-based tool RefFinder was used to integrate the results and to provide a comprehensive ranking order. The expression stability ranking orders of reference genes were different caculated by these four algorithms, and the ranking order analyzed by the RefFinder was *UBQ* > *Tip41* > *UCE* > *EF-1α* > *α-TUB* > *PP2A* > *ACT* > *GAPDH* > *SAM* > *CYP* > *β-TUB* > *18S* at the different seed development stages, and *UBQ* > *Tip41* > *EF-1α* > *α-TUB* > *PP2A* > *UCE* > *GAPDH* > *SAM* > *ACT* > *CYP* > *18S* > *β-TUB* in *P. ostii* tissues. *UBQ* and *Tip41* are the two most stable whereas *18S* and *β-TUB* are the two least stable reference genes for gene expression in various tissues and seeds at different developmental stages in *P. ostii*.

Keywords *Paeonia ostii* · Reference gene · Normalization · qRT-PCR · Seed development

Introduction

Tree peony (*Paeonia* section *Moutan* DC.), which is a perennial deciduous shrub native to China, is a well-known horticultural and medicinal plant [1]. In 2011, seeds of *Paeonia ostii* (*P. ostii*), which belong to a member of tree peony, were identified as novel sources of edible plant oil in China. The seed oil of this species is characterized by abundant unsaturated fatty acids (UFAs) and a high proportion of α-linolenic acid (ALA), which is a type of n-3 fatty acids with many health benefits [2, 3]. The UFA content in *P. ostii* seed oil exceeds 90% and contains approximately 42.78% ALA, 26.50% linoleic acid (LA) and 22.97% oleic acid (OA) [3, 4]. Consequently, *P. ostii* seed oil is recognized as an excellent health-promoting edible oil that has great potential economic value. To understand the molecular mechanism of UFA biosynthesis (especially ALA biosynthesis) in *P. ostii* seeds, transcriptomic analysis was performed during seed development, and numerous key candidate genes associated with UFA biosynthesis were identified [2]. Investigating the expression profiles of these candidate genes will greatly facilitate the understanding of gene function in seed oil biosynthesis.

Quantitative real-time polymerase chain reaction (qRT-PCR) has become the preferred method to quantify transcript abundance because of its high sensitivity, accuracy,

Chaoqiong Li and Lizong Hu have contributed equally to this work.

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✉ Chaoqiong Li
lcq2013@aliyun.com

[1] College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou 466001, Henan, China

[2] Key Laboratory of Plant Genetics and Molecular Breeding, Zhoukou Normal University, Zhoukou 466001, Henan, China

[3] College of Journalism and Media, Zhoukou Normal University, Zhoukou 466001, Henan, China

specificity and reliability [5, 6]. However, its accuracy is strongly dependent on the constant transcriptional performance of reference genes (RGs), which are often referred to as housekeeping genes and are required for stable expression regardless of experimental conditions [7, 8]. A number of housekeeping genes, which typically encode proteins responsible for the maintenance of basic cellular structures and metabolism, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), 18S ribosomal RNA (*18S*), elongation factor 1 alpha (*EF-1α*), ubiquitin-conjugating enzyme (*UCE*) and ubiquitin (*UBQ*), are common RGs used for gene expression analysis in many plant species [2, 8–10]. However, recent studies have found that there are expression variabilities of these RGs in different tissues, development stages and environmental conditions [7, 8, 11–14]. There are no universal RGs suitable for all experimental conditions. For example, *ACT6*, *ACT8*, and *ACT7* were the best RGs for kumquat under salt stress, drought stress and heavy metal stress conditions, respectively [11]. Protein phosphatase 2A subunit A3 (*PP2AA3*) and *18S* were the most stably expressed genes in tea plant (*Camellia sinensis*) exposed to metal stress [13]. *ACT* and *UCE* were the most suitable RGs for normalization in Sacha inchi during the entire growth cycle [12]. Thus, it is important to select reliable RGs as internal controls under individual experimental condition to ensure reproducible and precise gene expression quantification.

In the last two decades, several prevalent mathematical algorithms have been used to identify appropriate RGs for qRT-PCR under different experimental conditions, such as the delta cycle threshold (ΔC_t) method [15], BestKeeper [16], geNorm [17], NormFinder [18], and RefFinder [19]. These methods have been used to evaluate candidate RGs in different plant species across different developmental stages, tissues and organs, biotic and abiotic stresses, including *Arabidopsis*, rice, potato, papaya, tea plant, and Sacha inchi, among others [8, 12–14, 20–22]. A previous study found that *GAPDH* and *UCE* were the two most stable RGs during flower development in 'Fengdan', which was the cultivated type originated from *P. ostii* [23]; however, suitable RGs during seed development and in various vegetative and reproductive tissues have not been identified for normalization of the expression levels of the genes specially involved in seed oil biosynthesis. In this study, we selected 12 candidate RGs (*UBQ*, *18S*, *ACT*, *α-tubulin* (*α-TUB*), *β-tubulin* (*β-TUB*), *UCE*, *GAPDH*, *Protein phosphatase 2A* (*PP2A*), *Tip41-like protein* (*Tip41*), *S-adenosylmethionine synthetase* (*SAM*), *EF-1α*, *Cyclophilin* (*CYP*) and evaluated the stability of these 12 genes to identify the most suitable RGs for qRT-PCR data normalization in different tissues and seeds at different developmental stages. The reliability of the identified RGs was further validated by analyzing the expression pattern of *fatty acid desaturase 2* (*FAD2*) gene during seed development and in different tissues in *P. ostii* using the stable and unstable genes for normalization. The results may be useful for selecting reliable RGs suitable for the accurate quantification of gene expression and gene function research in *P. ostii*.

Materials and methods

Plant materials

Five-year-old *P. ostii* plants were grown in a field at Zhoukou Normal University, Zhoukou City, Henan Province, China. The roots, stems, leaves, flowers, fruits (5 days after pollination) and seeds of three biological replicates of *P. ostii* plants were collected for experiments. The seeds at different developmental stages were collected at intervals of 10 days after pollination (DAP) until 120 DAP (containing mature seeds). All tissues prepared for qRT-PCR were immediately frozen in liquid N_2 and stored at − 80 °C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from different *P. ostii* tissues using a TIANGEN RNA Prep Pure Plant kit (Tiangen Biotech Co. Ltd, Beijing, China) following the manufacturer's instructions. The RNA integrity, concentration and purity were examined using 1.2% agarose gel electrophoresis and a NanoDrop-2000C spectrophotometer (Thermo Fisher Scientific, USA). Isolated RNA samples, of which the A260/A280 ratio was between 1.9 and 2.1, indicating high purity and no protein contamination, were used for further analysis. First-strand cDNA was synthesized with 0.7 μg total RNA in a 20 μL reaction volume using the PrimeScript® RT Reagent Kit with gDNA Eraser (TAKARA, Dalian, China) according to the manufacturer's instructions. All cDNA samples were diluted (1:5) with RNase-free water prior to use in qRT-PCR.

Selection of candidate reference genes and qRT-PCR primer design

A total of 12 *P. ostii* housekeeping genes were selected as candidate RGs for analysis in this study (Table 1). The cDNA sequences of these RGs were obtained from the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide) from *Paeonia suffruticosa* (*P. suffruticosa*) and a transcriptome data (SRA accession number: SRP051810) from *P. ostii* submitted by Li et al. [2], and the gene information is shown in Table 1. The primer pairs used for qRT-PCR were designed based on selected sequences of the 12 candidate RGs using Primer Premier 5.0 and are listed in Table 1. To

Table 1 Primer sequences and amplification characteristics for 12 candidate reference genes and 1gene of interest

Gene/gene accession number	Gene name	Primer sequences (5′–3′) forward/reverse	Amplicon length (bp)	Slope	Amplification efficiency (%)	Correlation coefficient	T_m (°C)
UBQ/JN699053	*Ubiquitin-like*	GACCTATACCAAGCC GAAG/CGTTCCAGC ACCACAATC	122	−3.323	100.1	0.996	84
18S/U42792	*18S ribosomal RNA*	CCATAAACGATGCCG ACCAG/AGCCTTGCG ACCATACTCCC	109	−3.357	98.6	0.997	82
ACT/KU853024	*Actin*	TTTACTCACAGACCC GCCTC/GTGACACCA TCGCCTGAATC	170	−3.352	98.8	0.995	80
α-TUB/KU853025	*α-tubulin*	GCATTTGAGCCTTCT TCCAT/GTAGCCACA GCAGCATTGAC	119	−3.306	100.7	0.994	82
β-TUB/KU853026	*β-tubulin*	AATGCGGATGAGTGT ATGGT/ GAAGAAGTGAAGTCG TGGAA	210	−3.319	100.1	0.996	85
UCE/KU853027	*Ubiquitin conjugating enzyme*	ATTGGACCTCCCGAA ACTCT/TAGCCATTT GGGTCTTCACC	185	−3.232	103.9	0.998	82
GAPDH/KU853028	*Glyceraldehyde-3-phosphate dehydrogenase*	AGGTGCTCCCATCCC TGAAT/ATCGGTTGA CACCACATCATCTTC	200	−3.331	99.6	0.996	82
PP2A/KU853029	*Protein phosphatase 2A*	TGATTACTTGCCCCT CACAGCC/CATAAC AGGTCGCACATT GGTC	150	−3.301	100.9	0.995	83
TIP41/KU853030	*Tip41-like protein*	AGTTGGTTTCTTCTC TTGCG/GACACTTGG ATCATTATATGCTG	200	−3.288	101.4	0.993	82
SAM/KU853031	*S-adenosylmethionine synthetase*	ATCTTCCACCTCAAC CCATCT/GCACCA CTCCTGTCCACCTT	164	−3.341	99.2	0.996	84
EF-1α/KU853032	*Elongation factor 1α*	ACTTCGTCTCCCACT TCAGG/AGCTTCATG GTGCATCTCAA	160	−3.333	99.5	0.999	82
CYP/KU853033	*Cyclophilin*	GTTCTTCATTACCTT GGCACCT/TGACCG TTGACCGTAGTA TCTT	161	−3.285	101.6	0.994	80
PoFAD2/KT153989	*Fatty acid desaturase 2*	CCATCTACTGGGCAC TACAA/GCCAACGGT GTCATCAAC	110	−3.328	101.1	0.993	82

verify the sequences of the RGs, all the amplicons of the 12 candidate genes were analyzed on 1.5% agarose gels and sequenced.

qRT-PCR assay and data analysis

qRT-PCR was conducted using SYBR Premix Ex Taq (TaKaRa, Japan) on a CFX96 Real-Time System (Bio-Rad, USA). The reaction mixture (20 µL) contained 2.0 µL diluted cDNA, 10 µL SYBR Premix Ex Taq, 0.5 µL of each of the forward and reverse primers (10 µM), and 7 µL PCR-grade water. The PCR program was as follows: 95 °C for 2 min; 40 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s. The final melting curve was obtained between 65 and 95 °C to verify primer specificity. Each assay was carried out with three biological replicates and three technical replicates. To determine the amplification efficiency of each pair of primers, standard curves were generated from five-fold serial dilutions of cDNA samples from the seeds at 10 DAP. The values of PCR amplification efficiencies, slopes

and correlation coefficients were obtained from the standard curves and were calculated using CFX Manager™ software. The quantification cycle (Cq) values were also generated by this software and were used for the gene expression levels.

Four statistical algorithm programs, i.e. ΔC_t [15], BestKeeper [16], geNorm [17], and NormFinder [18] were used to evaluate the stability of the candidate genes during seed development. A comparative web-based method, RefFinder (http://150.216.56.64/referencegene), was used to compare and integrate the comprehensive ranking order of the tested candidate RGs.

To verify the expression stability of the recommended candidate RGs, a *fatty acid desaturase 2* gene in *P. ostii* (*PoFAD2*), which is considered to be one of the key genes involved in UFA biosynthesis, was used as a target gene in the seeds at different stages and diverse tissue samples [2, 24]. The nucleotide sequence of *PoFAD2* was obtained from the NCBI database and the primers used in qRT-PCR were designed with the software Primer Premier 5.0 and are listed in Table 1. The qRT-PCR amplification conditions were the same as described above. The two most and least stable RGs, as determined by RefFinder, either alone or in combination (calculated by geometric mean) were used to normalize the expression level of *PoFAD2*, and the relative expression level of *PoFAD2* was calculated using the $2^{-\Delta\Delta Ct}$ method [25].

Results

PCR amplification efficiency and specificity

Twelve *P. ostii* housekeeping genes, which were obtained from a transcriptome data from *P. ostii* and the NCBI nucleotide database from *P. suffruticosa*, were selected as candidate RGs. Alignment results showed that all the RGs shared the identical sequences between *P. ostii* and *P. suffruticosa*. All the amplicons of the 12 candidate RGs were also sequenced, and we received the same results between sequences obtained from the test, the sequences from transcriptome data and the sequences in *P. suffruticosa*. These results indicated that the selected RGs among *Paeonia* species might very conservative. The gene names, abbreviations, accession numbers, primer sequences and amplicon length, amplification efficiency, correlation coefficient and melting temperature (Tm) values of the 12 candidate RGs are listed in Table 1. The amplicon size ranged from 110 to 210 bp, and the amplification efficiency of each primer pair varied between 98.6% for the *18S* gene and 103.9% for *UCE*. In addition, all correlation coefficient values exceeded 0.99, and the Tm values ranged from 80 °C for *CYP* to 85 °C for *β-TUB*. Moreover, the melting curves for the amplified products of all 12 candidate RGs showed a single peak without peaks originated from the formation of primer dimers and amplification of nonspecific PCR products, corresponding to a specific melting temperature (Fig. 1).

Candidate reference gene expression profiles

The expression levels of candidate RGs during seed development and in various tissues in *P. ostii* were determined according to the Cq values obtained by qRT-PCR, and the mean Cq values of these RGs ranged from 10.02 to 33.98 (Table S1, Fig. 2). A low Cq value indicated a high gene expression level, and a high Cq value meaned a low gene expression level. There were wide differences in the transcript levels among these RGs. *18S* and *ACT* were the most and least abundant transcripts in the tested *P. ostii* tissues, with Cq values ranging from 10.02 to 15.76, and 26.12 to 33.98, respectively (Table S1). The coefficients of variation (CV) of the 12 RGs in all samples were 2.68% (*UBQ*), 10.13% (*18S*), 5.73% (*ACT*), 5.86% (*α-TUB*), 7.12% (*β-TUB*), 5.33% (*UCE*), 7.10% (*GAPDH*), 5.57% (*PP2A*), 3.06% (*Tip41*), 6.64% (*SAM*), 4.31% (*EF-1α*) and 6.20% (*CYP*).

Stability analysis and ranking of reference genes

Four statistical algorithms were used to select the most suitable RGs during seed development and in different tissues of *P. ostii*, and the stability values of 12 RGs in seeds at different developmental stages and across various tissues were shown in Supplementary Tables S2 and S3, respectively. The two most stable RGs recommended by the four programs were consistent, whereas differences were observed for the least stable RGs (Table 2, Supplementary Tables S2 and S3). The ΔC_t method evaluates the expression stability of candidate RGs based on the mean of standard deviation (SD), and the RG with the lowest SD is assumed to be the most stable gene. The BestKeeper program evaluates gene expression stability based on the CV and SD of the Cq values, and the most stable genes have the lowest CV and SD. NormFinder generates the stability values of RGs based on the inter- and intra-group variation in expression level, and lower values indicate higher stability. GeNorm software is a visual basic application tool to select an ideal pair of RGs and creates a stability ranking via the stepwise exclusion of the least stable genes. The stability value (M) of an RG is calculated based on the average pairwise variation between all RGs tested, and is inversely correlated with their exssion stability. Based on ΔC_t, NormFinder, GeNorm analysis, *UBQ* and *Tip41* were the two most stable RGs during *P. ostii* seed development and in all tissues, while *18S* and *β-TUB* were identified as the least most stable RGs (Table 2, Supplementary Tables S2 and S3). Based on BestKeeper analysis, as shown in Table 2, Supplementary Tables S2 and S3, *UBQ*

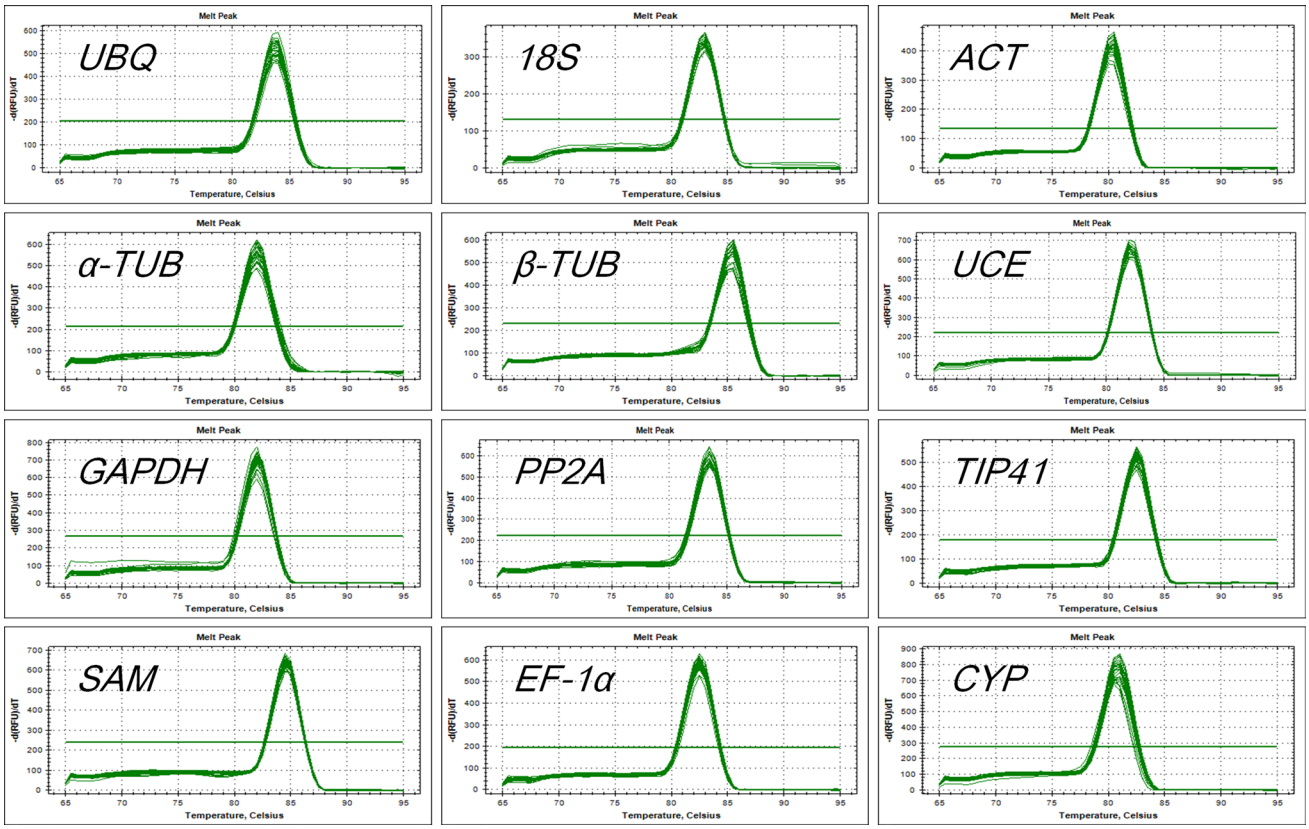

Fig. 1 Melting curves with a single peak for the 12 candidate reference genes

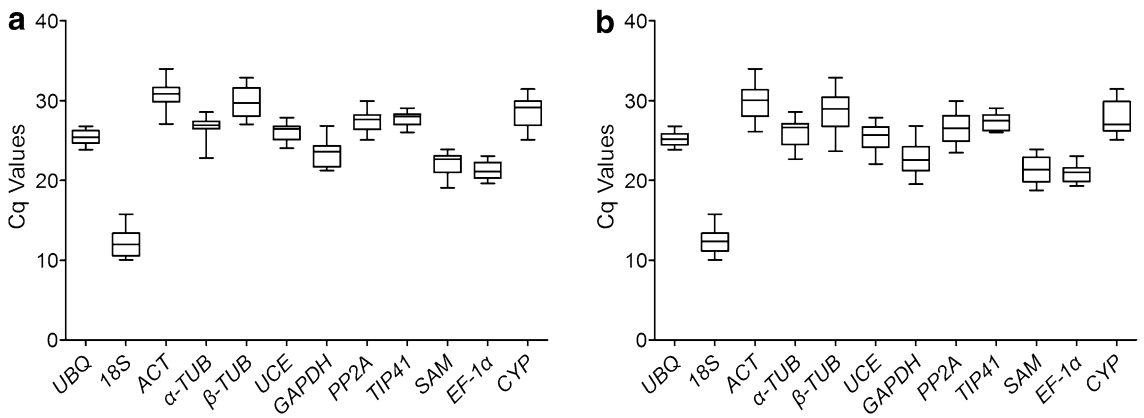

Fig. 2 The quantification cycle (Cq) values of the 12 candidate reference genes in seed samples (**a**) and all tissue samples (**b**). Lines across the box-plot graph of the Cq values represent the median value. The boxes indicate the 25th and 75th percentiles, and the whisker caps represent the maximum and minimum values

and *Tip 41* were ranked as the two most stable RGs in *P. ostii* seed and in all tissues, which was consistent with the results from the three other software analyses. However, *CYP* and *β-TUB* exhibited low stability and were ranked as the least stable RGs, which was different from the results analyzed by the three other algorithms. GeNorm program was also used to analyze pairwise variation (Vn/Vn + 1) for the assessment of the optimal number of RGs required for reliable normalization. A threshold Vn/Vn + 1 value of 0.15 was adopted to determine whether it is necessary to introduce n + 1 RGs as the internal control. As shown in Supplementary Fig. S1, values (V2/3) lower than the recommended threshold of 0.15

Table 2 Stability ranking of candidate reference genes in seeds at different developmental stages and across diverse tissue samples

Method	Ranking order (better–good–average)											
	1	2	3	4	5	6	7	8	9	10	11	12
Seeds at different stages												
Delta CT	*UBQ*	*Tip41*	*UCE*	*EF-1α*	*α-TUB*	*ACT*	*PP2A*	*GAPDH*	*SAM*	*CYP*	*β-TUB*	*18S*
BestKeeper	*UBQ*	*Tip41*	*EF-1α*	*UCE*	*α-TUB*	*PP2A*	*SAM*	*ACT*	*GAPDH*	*18S*	*CYP*	*β-TUB*
NormFinder	*UBQ*	*Tip41*	*UCE*	*EF-1α*	*α-TUB*	*ACT*	*PP2A*	*GAPDH*	*SAM*	*CYP*	*β-TUB*	*18S*
GeNorm	*UBQ/Tip41*		*PP2A*	*GAPDH*	*α-TUB*	*ACT*	*UCE*	*EF-1α*	*CYP*	*SAM*	*β-TUB*	*18S*
RefFinder	*UBQ*	*Tip41*	*UCE*	*EF-1α*	*α-TUB*	*PP2A*	*ACT*	*GAPDH*	*SAM*	*CYP*	*β-TUB*	*18S*
All tissues												
Delta CT	*UBQ*	*Tip41*	*EF-1α*	*α-TUB*	*UCE*	*PP2A*	*GAPDH*	*ACT*	*SAM*	*CYP*	*β-TUB*	*18S*
BestKeeper	*UBQ*	*Tip41*	*EF-1α*	*18S*	*UCE*	*SAM*	*PP2A*	*α-TUB*	*GAPDH*	*ACT*	*CYP*	*β-TUB*
NormFinder	*UBQ*	*Tip41*	*EF-1α*	*α-TUB*	*UCE*	*SAM*	*PP2A*	*GAPDH*	*ACT*	*CYP*	*β-TUB*	*18S*
GeNorm	*UBQ/Tip41*		*PP2A*	*GAPDH*	*α-TUB*	*CYP*	*ACT*	*UCE*	*EF-1α*	*SAM*	*β-TUB*	*18S*
RefFinder	*UBQ*	*Tip41*	*EF-1α*	*α-TUB*	*PP2A*	*UCE*	*GAPDH*	*SAM*	*ACT*	*CYP*	*18S*	*β-TUB*

indicated that two RGs were sufficient for the normalization of gene expression during seed development and across diverse tissues in *P. ostii*.

Finally, the RefFinder tool was used to integrate the above mentioned statistical algorithms and calculate the recommended comprehensive ranking order. The comprehensive ranking order is shown in Table 2, and the ranking order of expression stability was *UBQ* > *Tip41* > *UCE* > *EF-1α* > *α-TUB* > *PP2A* > *ACT* > *GAPDH* > *SAM* > *CYP* > *β-TUB* > *18S* at the different seed development stages, and *UBQ* > *Tip41* > *EF-1α* > *α-TUB* > *PP2A* > *UCE* > *GAPDH* > *SAM* > *ACT* > *CYP* > *18S* > *β-TUB* in *Paeonia ostii* tissues. The result analyzed by RefFinder was similar to those of the ΔC_t method, geNorm and NormFinder algorithm, but there were some differences in the orders of the middle ranking RGs.

Validation of selected reference genes

To assess the reliability of the selected RGs, the relative expression pattern of the target gene, *PoFAD2*, which encodes the enzyme that is essential for UFA biosynthesis in *P. ostii*, was calculated in seeds at different developmental stages and various tissue samples by qRT-PCR. The primer specificity of *PoFAD2* was verified as described for RGs (Table 1). The two most stable RGs (*UBQ* and *Tip41*) and the two least stable RGs (*18S* and *β-TUB*) identified by RefFinder were used either independently or in combination to normalize the expression levels of *PoFAD2* (Fig. 3). Irrespective of normalization by *UBQ* or *Tip41,* alone or in combination, approximately the same expression patterns of *PoFAD2* were obtained (Fig. 3a). *PoFAD2* was predominantly expressed in seeds at the 10–100 DAP stages with the highest expression level at the 70 DAP stage. For other different tissues samples except seed samples, *PoFAD2* was found to be mainly expressed in flowers. However, the expression patterns of *PoFAD2* were different when using *18S* or *β-TUB* alone or in combination as RGs for normalization (Fig. 3b). During seed development in *P. ostti*, the highest expression level of *PoFAD2* appeared at the 80 DAP stage using *β-TUB* as the RG for normalization, and at the 90 DAP stage using either *18S* alone or in combination with *β-TUB* for normalization, respectively (Fig. 3b). In addition, the expression pattern of *PoFAD2* in various tissues was different when using these two least stable RGs alone or in combination for normalization (Fig. 3b). In addition, when stable RGs were used for normalization, significant differences in the expression levels of *PoFAD2* were detected in seeds at 40–90 DAP in comparison with the expression levels in roots, which was used as a calibrator (Fig. 3a). On the other hand, significant differences ($P < 0.001$, $P < 0.01$) and no significant difference ($P > 0.05$) in *PoFAD2* gene expression were found between roots and seeds at 40 DAP when 18S, 18S + *β-TUB* and *β-TUB* used for normalization, respectively. These results revealed that significant differences in the *PoFAD2* expression profiles depending on the RGs used for normalization of the qPCR data, whether stable or unstable. Taken together, these results demonstrate that choosing reliable RGs is important for accurate normalization in qRT-PCR experiments.

Discussion

The accuracy and reliability of gene expression patterns using qRT-PCR technology strongly depend on the use of stable RGs for normalization, which can remove non-biological variations [11, 26]. Previous studies revealed that there were no universal RGs among different plant species, and common RGs, such as *ACT*, *GAPDH* and *18S*, exhibited

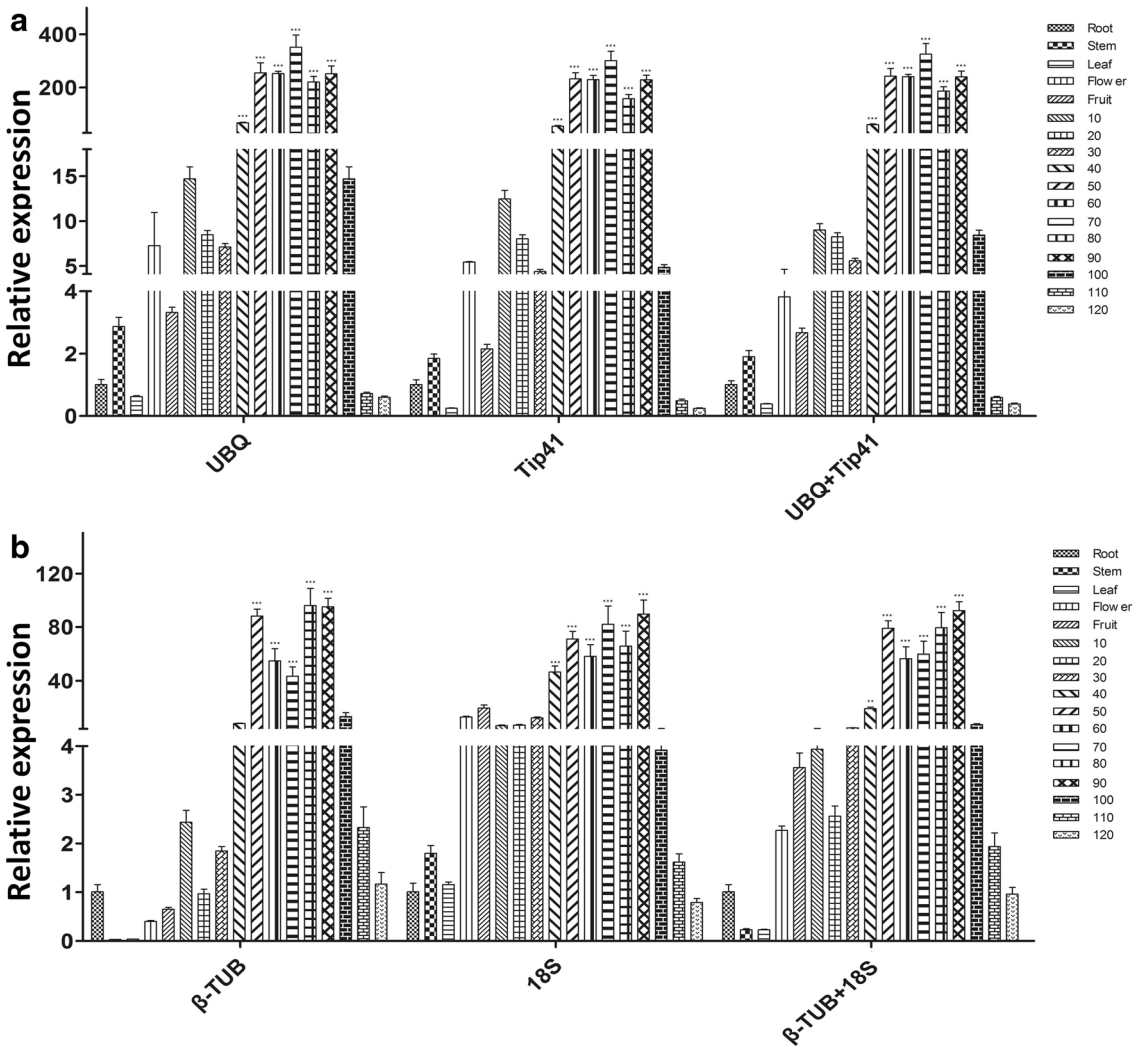

Fig. 3 Relative expression of the *fatty acid desaturase 2* gene in diverse tissue samples using selected reference genes, including the most (**a**) and least (**b**) stable reference genes, for normalization in *Paeonia ostii*. The seed samples were collected at different developmental stages (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 days after pollination). Each data point represents the mean ± SD of three replicates. Student's *t* test was used to determine significant differences from root (calibrator). Significance level: $^{**}P < 0.01$, $^{***}P < 0.001$

expression variation in different plants under different developmental or experimental conditions [12, 27]. Therefore, the selection of reliable RGs for specific developmental or experimental conditions is important in qRT-PCR analysis.

The seed of *P. ostii* is considered as a new source of ALA in oil production, and studying the mechanism of lipid biosynthesis and the accumulation of ALA during *P. ostii* seed development has recently received considerable attention. However, suitable RGs in *P. ostii* seed development have not been identified. In this study, we selected 12 candidate RGs to evaluate their expression stability during seed development and also calculate its stability in different tissues in *P. ostii*. Four statistical methods (ΔC_t, BestKeeper, NormFinder, and geNorm) were used to calculate the stability of gene expression levels. In addition, a web-based tool (RefFinder) was used to integrate the results of these four algorithms and to provide a comprehensive ranking order of all 12 RGs. All four algorithms revealed that *UBQ* and *Tip41* were the two most stable genes among these 12 candidate RGs, but the least stable genes analyzed by these four methods differed (Table 2). The most stable RG ranked by RefFinder was also *UBQ* (Table 2), which is involved in protein degradation and was also identified as the most stable RG in *Platycladus orientalis* under NaCl and ABA treatment [28], across different tissues of *Rhododendron micranthum* Turcz [29], and across all organs and treatments of white clover [26]. However, *UBQ* is also considered to be inappropriate RG in some plant species.

For example, *UBQ* was unsuitable for normalization in *Suaeda aralocaspica* under different salt treatments [27]. The *18S* gene, which forms part of the eukaryotic ribosome and has been exploited as a reference gene for a long time, was calculated to be the least stable RG by ΔC_t, NormFinder, geNorm and RefFinder (Table 2). Previous studies also found that the *18S* gene was the worst RG for normalizing gene expression levels in many plant organs and under different conditions, such as *Lilium davidii* [30], Sacha inchi [12], Chinese tallow [31], *Oxytropis ochrocephala* Bunge [32] and rubber trees [33]. In contrast, *18S* has been demonstrated to be a stable RG in strawberry [34] and rice [21]. In previous study, *Tip41*, one of the most stable RGs in our study, also showed high stability in the different cultivars of tree peony during flower development, and *β-TUB* was identified as one of the least stable RG [23], which was similar to the results in our study. It indicated the stability of *Tip41* and *β-TUB* might display consistent trends among *Paeonia* section Moutan DC cultivars and tissues. These results prove that the most suitable RGs differ among different plant cultivars, plant tissues and under different conditions and it is very important to select reliable RGs according to the specific experiment.

To test the reliability of the selected RGs, the expression pattern of *PoFAD2*, which encodes a key enzyme in UFA synthesis, was evaluated in this study. The *PoFAD2* expression patterns were similar when the gene expression data were normalized using the most stably expressed RGs (*UBQ* and *Tip41*) either alone or in combination, with the highest expression level of *PoFAD2* appearing at the 70 DAP stage (Fig. 3a). However, the expression patterns of *PoFAD2* differed using the least stable RGs (Fig. 3b). For example, the highest expression level of *PoFAD2* was observed at the 80 DAP stage using *β-TUB* as the RG for normalization, and at the 90 DAP stage using either *18S* alone or in combination with *β-TUB* for normalization, respectively. Interestingly, our results showed significant differences in the *PoFAD2* expression profiles depending on the RGs used for normalization, whether stable or unstable. The main divergences were observed in the sample of seeds at 40 DAP in comparison with the expression levels in roots (Fig. 3). Therefore, the preliminary requirement for obtaining accurate relative gene expression levels is the use of suitable RGs.

In conclusion, this study evaluated the expression stability and determined the comprehensive rankings of 12 candidate RGs during *P. ostii* seed development and is diverse tissue samples using four statistical algorithms (ΔC_t, BestKeeper, NormFinder, and geNorm) and one web-based method (RefFinder). *UBQ* and *Tip41* were identified as the two most reliable RGs for qRT-PCR, whereas the least stable RGs were *18S* and *β-TUB*. The results obtained in this study provide useful information for accurate qRT-PCR data normalization in *P. ostii* gene expression studies.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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