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Identification of Suitable Reference Genes for qRT-PCR Normalization in Kiwifruit

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Abstract: Reference genes are used for the correction of qRT-PCR data, and it is necessary to investigate the optimum reference gene under certain conditions. The expression levels of seven traditional reference genes *ACT1*, *ACT2*, *GAPDH*, *18S rRNA*, *UBQ*, *TUB* and *CYP* were analyzed using qRT-PCR in different varieties, tissues, developmental stages and hormone (or pollen polysaccharide) treatments in kiwifruit. Gene expression stability was assessed with the help of three common software (geNorm, NormFinder, BestKeeper), and the minimum number of reference genes necessary for normalization was also determined. *GAPDH*, *ACT1* and *ACT2* were selected as reference genes for different genotypes of kiwifruit. *GAPDH* and *UBQ* were the best combinations of reference genes for root, stem, leaf, flower and fruit. *GAPDH* and *ACT1* could be the preferred reference genes for normalization of qRT-PCR data during fruit development. The pairing of *ACT1* and *UBQ* constituted the optimal combination of reference genes in kiwifruit treated with different hormones (or pollen polysaccharide). This study provides a new and reliable option for the use of reference genes in the analysis of gene expression patterns of interest in kiwifruit.

Keywords: reference genes; kiwifruit; qRT-PCR; stability analysis

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1. Introduction

Quantitative real-time PCR (qRT-PCR) has become a mainstream method for gene expression analysis because of its high sensitivity, high specificity, good reproducibility, ease of operation and short time consumption [1–3]. When performing gene expression analysis on multiple samples, it is often necessary to ensure that the samples have the same RNA quality, cDNA yield and gene amplification efficiency, but in practice, it is often difficult to meet these conditions [4,5]. To eliminate differences in RNA quality, cDNA yield and gene amplification efficiency between samples, it is often necessary to introduce reference genes to normalize the qRT-PCR data [6].

Ideally, the reference gene should be independent of experimental factors and maintain stable transcript levels in all types of tissues and cells [7,8]. However, the study has shown that there is no absolute constant expression of a reference gene that is suitable for all experimental conditions, and that any one reference gene is only relatively consistently expressed under limited conditions [9]. The use of inappropriate reference genes for normalization can lead to bias in the quantitative data [10]. In order to obtain more reliable results, one or more reference genes need to be selected for calibration in the experiment [11,12].

As research into the molecular biology of kiwifruit has progressed, the study of kiwifruit gene regulation function has become a hot topic of research, and the analysis of gene expression patterns is an essential part of the study of gene function, so the selection of suitable reference genes is crucial [13]. Unfortunately, there are few reports on the systematic identification of kiwifruit reference genes [14], let alone literature on the screening of kiwifruit reference genes in different varieties and under hormone treatments. Therefore, in this study, qRT-PCR was used to analyze the expression of seven commonly used plant reference genes, including actin 1 (*ACT1*), actin 2 (*ACT2*), 3-phosphoglyceraldehyde dehydrogenase (*GAPDH*), *18S rRNA*, polyubiquitin gene (*UBQ*), β-microtubulin gene (*TUB*) and procyclin gene (*CYP*), in different genotypes, tissues, fruit developmental stages and hormone (or pollen polysaccharide) treatments of kiwifruit. Meanwhile, the expression stability of reference genes was assessed using three software programs, geNorm [15], NormFinder [16] and BestKeeper [17], to select the appropriate reference genes for gene expression studies in kiwifruit.

2. Materials and Methods

2.1. Plant Materials and Treatments

The tested genotypes included *Actinidia latifolia*, *Actinidia deliciosa* 'Qinmei', 'Hayward' and *Actinidia chinensis* 'Hort16A', 'Jinshi 1', 'Hongyang' and 'Hongshi 2', and their fruits were sampled at 30 days after 75% flower drop. The roots, stems, leaves and flowers of 'Jinshi 1' were collected on 25 April, 2020, and fruits of 'Jinshi 1' were collected at 30, 55, 70, 95, 130 and 145 days after 75% flower drop. All samples were harvested in 2020 from the kiwifruit research base of the Sichuan Academy of Natural Resources Science (104°2′ E, 31°23′ N). 'Jinshi 1' potted live annual seedlings were treated with two hormones (melatonin (MT): M8600 and 14-hydroxybrassinosterol (HBR): B29511), and one pollen polysaccharide (PP), which are some of the substances that are beneficial in improving plant stress resistance, and the latter two substances are receiving a lot of attention. A total of eight processes were set up: CK (water), MT (50 μmol·L^{-1}), PP1 (0.004 mg·L^{-1}), PP2 (0.008 mg·L^{-1}), HBR1 (0.01 mg·L^{-1}), HBR2(0.12 mg·L^{-1}), MT (50 μmol·L^{-1}) + PP (0.008 mg·L^{-1}), MT (50 μmol·L^{-1}) + HBR (0.12 mg·L^{-1}), with root irrigation every 20 days (1 L/time), normal water and fertilizer management for the rest of the year [18]. A total of nine pots per treatment, replicated three times. Leaves were collected from the fourth to eighth positions up the root after three treatments. All samples were then frozen in liquid nitrogen and stored at −80 °C for the following analysis.

2.2. RNA Isolation and cDNA Synthesis

Each sample was extracted for total RNA according to the Plant RNA Extraction Kit-V1.5 (Biofit, Chengdu, China). RNA quality was assessed in a spectrophotometer (Thermo Scientific, Waltham, MA, USA), with only RNA samples showing both an A260/280 ratio between 1.8 and 2.0 and A260/230 ratio more than 2.0 used for subsequent analysis. The integrity of RNA samples was assessed by electrophoresis in 1.0% agarose gels. The first strand of cDNA was synthesized according to the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) using 1000 ng of total RNA in a 20 μL reaction system.

2.3. qRT-PCR Primer Design of Candidate Reference Genes

Seven candidate reference genes were selected based on literature, including *ACT1*, *ACT2*, *GAPDH*, *18S rRNA*, *UBQ*, *TUB* and *CYP*. Five of seven tested primer pairs (*ACT1*, *ACT2*, *GAPDH*, *18S rRNA*, *UBQ*) were obtained from existing reports [16]. The remaining two primer pairs were derived from the kiwifruit genome database (http://kiwifruitgenome, accessed on 7 December 2021). The sequences of the tested primer pairs are shown in Table 1.

Table 1. Primer sequences of candidate reference genes used for qRT-PCR analysis.

Gene	Primer Sequence (5′→3′)	Tm (°C)
ACT1	GCAGGAATCCATGAGACTACC GTCTGCGATACCAGGGAACAT	58
ACT2	TGCATGAGCGATCAAGTTTCAAG TGTCCCATGTCTGGTTGATGACT	57
18S rRNA	CTGTGAAACTGCGAATGGCTC TTCCAGAAGTCGGGGTTTGT	56.5
UBQ	CCACCACGGAGACGGAGCAC TGCAGATCTTCGTGAAAACC	58
GAPDH	ACACTCCATCACTGCGACA CACCTTGCCAACAGCCTTA	56.5
TUB	TGAGCACTAAAGAGGTGGATGA TGGGATGTCACACACACTGG	56.5
CYP	TGATGGCACTGGAGGAGAATC ACTGAGACCCGTTTGTGTTAGG	58

2.4. Quantitative Real-Time PCR Analysis

The qRT-PCR assay was performed using the CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). qRT-PCR assays were carried out in 20 µL reaction volumes, which contained 1.5 µL diluted cDNA (1:100), 1.6 µL primers (0.32 µM forward and reverse primers), 10 µL TB GreenTM Premix Ex TaqTM IIsolution and 6.9 µL sterile water. The amplification program was at 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C for 31 s and a final step for dissociation at 95 °C for 10 s, 65 °C for 30 s and 95 °C for 15 s. Each reaction included three biological replicates with three technical duplicates.

2.5. Standard Curve of Candidate Reference Genes

The standard curves referred to the method of Khanlou et al. [19]. After mixing equal amounts of cDNA from all kiwifruit samples and diluting them into six concentration gradients in sequence with a 5-fold gradient, the expression abundance of the seven reference genes was analyzed using the diluted mixed samples as templates, and the corresponding standard curve for each gene was plotted with the Ct value and the dilution number. The amplification efficiency (E) of the reference genes can be calculated according to the slope of the standard curve, which is calculated as $E = [5^{(-1/\text{slope})} - 1] \times 100\%$. Only reference genes with an amplification efficiency in the range of 90% to 110% are eligible for subsequent analysis [20].

2.6. Data Analysis

The Ct values for each reaction were obtained directly using Bio-Rad CFX Manager v2.0 software (Bio-Rad, Hercules, CA, USA). The arithmetic mean of the Ct values was calculated by Microsoft Excel 2010, and expression stability of seven reference genes was evaluated using the following three software programs: geNorm, NormFinder and BestKeeper (https://seqyuan.shinyapps.io/seqyuan_prosper/, accessed on 23 December 2021).

3. Results

3.1. Amplification Efficiency of Candidate Reference Genes

The amplification efficiency of the genes was calculated by performing qRT-PCR using a 5-fold gradient dilution of an equal volume of mixed cDNA of all kiwifruit samples for testing as the template, followed by plotting the standard curve. The correlation coefficients (Table 2) for all seven reference genes were above 0.98, with good linearity. The amplification efficiencies (Table 2) of all the reference genes except *18S rRNA* reached 91~107%, indicating good primer specificity and reliable quantification results, which met the requirements of the subsequent experiments.

Table 2. Amplification efficiency of candidate reference genes.

Gene	R^2	Amplification Efficiency (%)
ACT1	0.999	94.57
ACT2	0.999	91.01
GAPDH	0.999	92.21
18S rRNA	0.998	87.38
UBQ	0.989	104.07
TUB	0.989	106.91
CYP	0.981	103.66

3.2. Expression Analysis of Candidate Reference Genes

The Ct value reflects the transcriptional level of the gene; the higher the Ct value, the lower the transcriptional level of the gene. The expression abundance of the seven reference genes varied from sample to sample (Figure 1). *18S rRNA* had a low Ct value, suggesting that expression abundance was highest; *CYP* had about twice the Ct value of *18S rRNA* and therefore had the lowest expression abundance, while the expression of the remaining five genes was at an intermediate level.

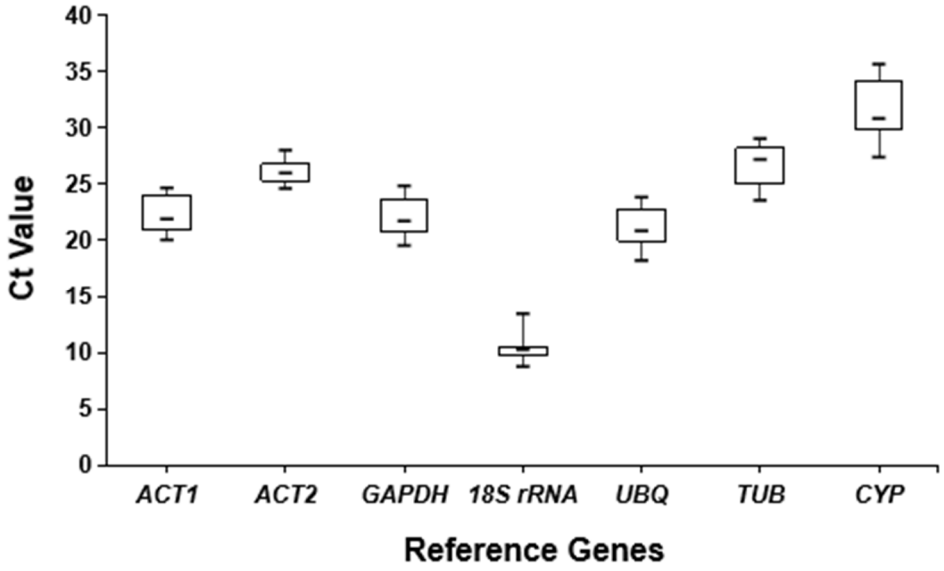

Figure 1. Ct values for seven candidate reference genes in all test samples: fruits of *Actinidia latifolia*, 'Qinmei', 'Hayward', 'Hort16A', 'Jinshi 1', 'Hongyang' and 'Hongshi 2' at 30 days after 75% flower drop; roots, stems, leaves and flowers of 'Jinshi 1', and fruits of 'Jinshi 1' at 30, 55, 70, 95, 130 and 145 days after 75% flower drop; MT, HBR or PP treated leaves of 'Jinshi 1' live seedlings.

3.3. Expression Stability Analysis of Candidate Reference Genes

The geNorm software determines gene stability by calculating the gene expression stability value (M), which is generally considered to be more stable if the M value is less than 0.5, and the lower the M value, the more stable the gene is. As shown in Table 3, *GAPDH* was the most stable gene in nearly all kiwifruit samples. In addition, the M values of *18S rRNA* and *CYP* were less than 0.5 in the fruit of seven different genotypes, and they were also stable, while in the 'Jinshi 1' roots, stems, leaves, flowers and fruits, *18S rRNA* and *CYP* were less stable, and *ACT1* and *UBQ* were the most stable genes. In the 'Jinshi 1' fruit development, the M values of *ACT1* and *ACT2* were about 0.3, which also had good stability. Considering the result from all the hormone (or pollen polysaccharide)-treated samples, the M values of all seven genes were less than 0.5, indicating that the transcript levels of the seven genes were stable.

Table 3. Average expression stability values of seven candidate reference genes as calculated by geNorm. M/H/P treatments means MT, HBR or PP treatments.

Ranking	Varieties		Tissues		Developmental Stages		M/H/P Treatments	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*GAPDH*	0.377	*ACT1*	0.407	*ACT2*	0.322	*GAPDH*	0.242
2	*18S rRNA*	0.377	*GAPDH*	0.407	*GAPDH*	0.322	*UBQ*	0.242
3	*CYP*	0.498	*UBQ*	0.424	*ACT1*	0.372	*ACT1*	0.294
4	*ACT1*	0.693	*ACT2*	0.638	*UBQ*	0.503	*TUB*	0.318
5	*ACT2*	0.810	*18S rRNA*	0.804	*18S rRNA*	0.752	*ACT2*	0.341
6	*TUB*	0.987	*TUB*	0.951	*TUB*	0.966	*CYP*	0.390
7	*UBQ*	1.141	*CYP*	1.221	*CYP*	1.366	*18S rRNA*	0.432

geNorm also calculated the pairwise variation ($V_{n/n+1}$) to determine the minimum number of reference genes. When the pairwise variation $V_{n/n+1}$ is below 0.15, n reference genes are sufficient to correct the data, and conversely, n + 1 reference genes are required. The values of V2/3 for all samples except for the different genotypes were between 0.09 and 0.13 (Figure 2), indicating that two reference genes were sufficient for qRT-PCR data normalization under these conditions. In contrast, the V2/3 values for the different varieties were greater than 0.15 (Figure 2), indicating that the combination of the two reference genes was less stable and that a third reference gene needed to be introduced.

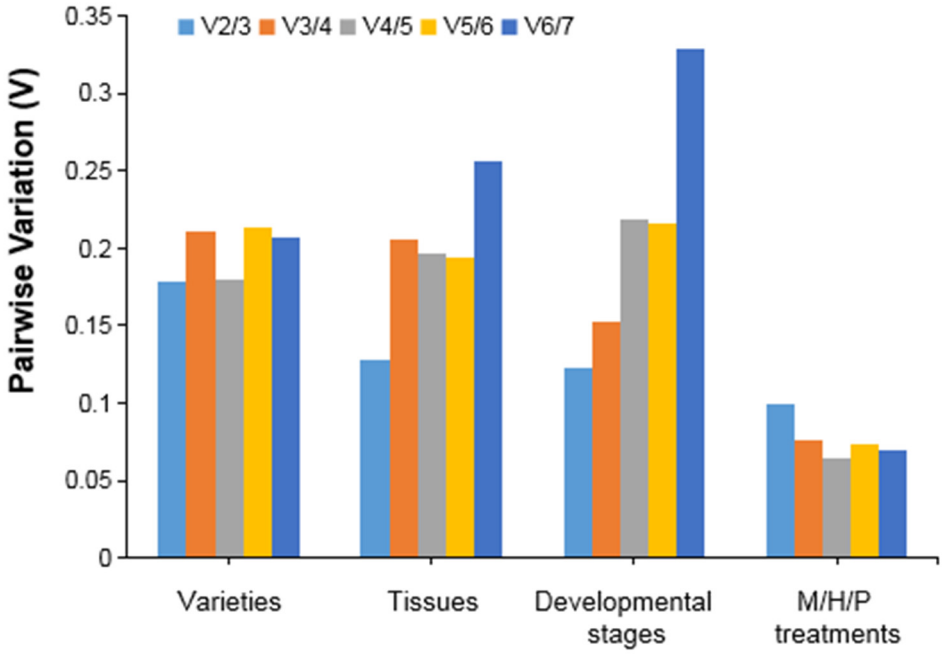

Figure 2. Pairwise variation (V) analysis of the seven candidate reference genes in all tested samples. M/H/P treatments means MT, HBR or PP treatments.

The NormFinder software differs from the geNorm algorithm but also judges gene expression stability based on the M value of the gene; the smaller the M value, the better the stability. The best reference gene screened by NormFinder was similar to the results of the geNorm analysis. *GAPDH* was the most consistently expressed gene in the non-hormone (or pollen polysaccharide)-treated kiwifruit samples, while *ACT1* showed the best stability in the eight different hormone (or pollen polysaccharide)-treated kiwifruit samples (Table 4).

Table 4. Average expression stability values of seven candidate reference genes as calculated by NormFinder. M/H/P treatments means MT, HBR or PP treatments.

Ranking	Varieties		Tissues		Developmental Stages		M/H/P Treatments	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*GAPDH*	0.110	*GAPDH*	0.240	*GAPDH*	0.490	*ACT1*	0.120
2	*18S rRNA*	0.360	*UBQ*	0.270	*ACT1*	0.650	*TUB*	0.210
3	*CYP*	0.480	*ACT1*	0.340	*UBQ*	0.660	*UBQ*	0.230
4	*ACT1*	0.530	*ACT2*	0.640	*ACT2*	0.800	*GAPDH*	0.230
5	*ACT2*	0.810	*18S rRNA*	0.950	*TUB*	0.830	*ACT2*	0.320
6	*UBQ*	1.410	*TUB*	1.240	*18S rRNA*	0.930	*CYP*	0.460
7	*TUB*	1.440	*CYP*	1.770	*CYP*	2.280	*18S rRNA*	0.470

BestKeeper software assesses the expression stability of genes on the basis of the following variables: the standard deviation (SD) and coefficient of variation (CV). SD values of genes less than 1 are considered to be good expression stability, and the smaller the SD and CV values, the better the gene stability. Slightly different from the two previous algorithms for ranking, the results obtained with the BestKeeper algorithm (Table 5) revealed that all five genes except for *UBQ* and *TUB* were stable, as their SD values were less than 0.5 among the different varieties. The transcript levels of *TUB* and *CYP* were unstable in 'Jinshi 1' different tissues and fruits at various developmental stages, whereas the remaining five genes were all relatively stable. The SD values of all seven genes in kiwifruit leaves treated with different hormones (or pollen polysaccharide) were less than 1, indicating that these seven genes had stable expression under this condition.

Table 5. Average expression stability values of seven candidate reference genes as calculated by BestKeeper. M/H/P treatments means MT, HBR or PP treatments.

Ranking	Varieties			Tissues			Developmental Stages			M/H/P Treatments		
	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV	Gene	SD	CV
1	*CYP*	0.427	1.414	*ACT2*	0.422	1.645	*ACT2*	0.253	0.996	*ACT1*	0.184	0.759
2	*GAPDH*	0.535	2.503	*UBQ*	0.614	3.025	*ACT1*	0.259	1.260	*18S rRNA*	0.202	1.962
3	*18S rRNA*	0.683	6.871	*GAPDH*	0.719	3.426	*GAPDH*	0.459	2.248	*UBQ*	0.259	1.128
4	*ACT1*	0.758	3.541	*18S rRNA*	0.867	8.504	*UBQ*	0.647	3.223	*GAPDH*	0.323	1.349
5	*ACT2*	0.773	2.990	*ACT1*	0.955	4.482	*18S rRNA*	0.899	8.536	*TUB*	0.335	1.173
6	*UBQ*	1.470	7.057	*TUB*	1.296	4.970	*TUB*	1.168	4.494	*ACT2*	0.411	1.522
7	*TUB*	1.171	4.520	*CYP*	2.002	6.496	*CYP*	2.375	7.603	*CYP*	0.458	1.328

3.4. Validation of Selected Reference Genes by AcPSY Expression Analysis

PSY, a primary rate-limiting enzyme gene in the carotenoid metabolic pathway, was selected as a target gene to further validate the reliability of the reference genes [21]. As shown in Figure 3, we normalized the expression levels of *PSY* during kiwifruit fruit development using the seven reference genes, respectively. Similar expression patterns and abundances were observed when the data normalization was performed with the most stably expressed reference genes (*ACT*, *GAPDH* and *UBQ*). When the least stably expressed reference genes (*CYP*) were used for data normalization, a consistent pattern of expression was also observed; however, the transcript levels of *PSY* increased dramatically during late fruit development. The expression pattern of *PSY* was considerably biased when the other two reference genes (*18s rRN* and *TUB*) with poor expression stability were used to standard the data. These results were consistent with the stability evaluation of the

seven reference genes and also indicated that less stable reference genes did not effectively calibrate the data from the qRT-PCR assay.

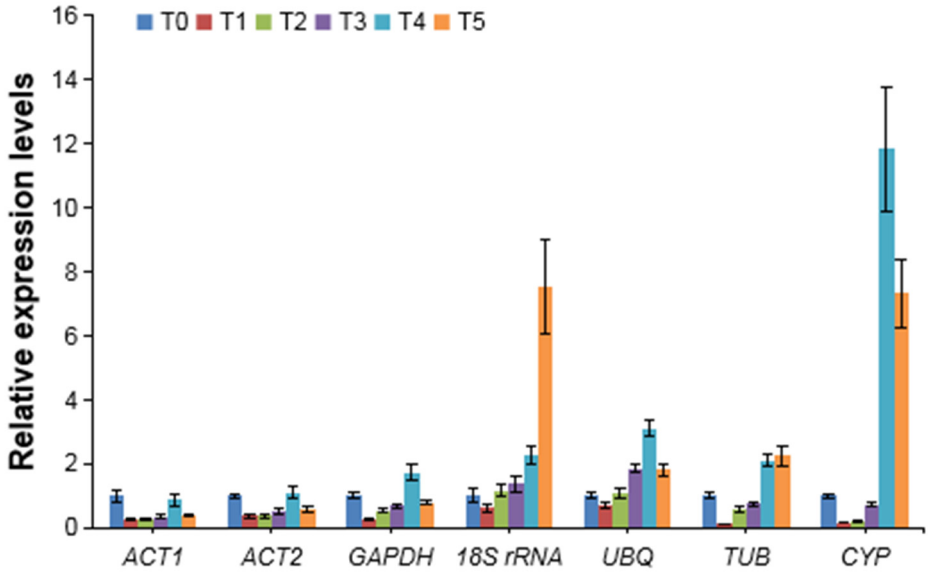

Figure 3. Relative expression levels of *AcPSY* normalized by the seven reference genes during fruit development. T0–T5 represent 30, 55, 70, 95, 130 and 145 days after 75% flower drop.

4. Discussion

qRT-PCR is one of the most common methods used to analyze gene expression patterns, and obtaining reliable results depends on the correct selection of reference genes. There have been many studies involving the screening of fruit tree reference genes. For instance, it has been shown that *TEF2*, *CYP2* and *ACT* are the most suitable reference genes in cherry fruit and flower development [22]. Screening of apple reference genes revealed that *UBQ* had the most stable expression in different genotypes of apples, in different developmental stages of fruit and in different tissues [23]. Another study analyzing the stability of reference genes in different tissues of apple found that *RPL2* and *GAPDH* showed good stability, while *UBQ* showed poor stability [24]. It was also found that *GAPDH* maintained stable expression in different organs and at different stages of fruit development in strawberry [25]. Both *GAPDH* and *CYP* maintained good stability in pomegranate when subjected to biotic and abiotic stresses [26]. The most consistently expressed reference genes in blueberry were *UBC9* and *GAPDH* when attacked by *Monilinia vaccinii-corymbosi* [27]. In summary, no completely universal reference gene existed, and the expression stability of genes varied considerably under different experimental conditions. Therefore, it is necessary to systematically select the most stable reference genes prior to their use in qRT-PCR normalization rather than directly using other published reference genes.

Our data suggested that the widely used kiwifruit reference genes *ACT* and *18s RNA* were not fully applicable under the conditions of this experiment, while *GAPDH* expression was much more stable. *GAPDH* was commonly used as a reference gene due to its stable expression in plants such as Arabidopsis [28], cucumber [29], tree peony [30] and sugarcane [31]. In this study, *GAPDH* was also considered suitable for data normalization of the qRT-PCR assay in kiwifruit, as it obtained high stability assessment values in most of the test samples based on different algorithms. Further analysis suggested that *ACT1* maintained a more stable transcript level during fruit development, a result similar to that of previous screening of reference genes in pitaya [32]. *UBQ* was reported to be the most stable reference gene when identified in different tissues of pineapple [33]. In this study, *UBQ* was similarly considered to have good stability in roots, leaves, flowers and fruits based on its high scores obtained through three software programs. The above results were quite different from those of Ferradá et al. [14] for the identification of the

'Hayward' kiwifruit reference genes, which identified *ACT* and *18s rRNA* as suitable for use as reference genes in different tissues. Our results provided further evidence that the expression of reference genes varied between varieties and that there was a need to conduct reference gene screening for different trials. Meanwhile, we also found that in addition to *GAPDH*, *ACT1* and *ACT2* also presented good stability between different varieties of kiwifruit, which was in agreement with recent studies that *ACT* was the most stable gene between different varieties of peach [34] and jujuba [35]. Both *ACT1* and *UBQ* maintained stable transcript levels in kiwifruit stimulated by different hormones (or pollen polysaccharide), which was similar to the results of a previous study about bananas [36]. The above information indicated that the appropriate reference gene was restricted to a particular experimental setting.

The ideal Ct value for the reference gene should be in the range of 15–30 [37], with either too high or too low a Ct value being highly likely to result in less accurate quantitative results. Therefore, when selecting reference genes, in addition to considering gene expression stability, it is important to ensure that gene expression abundance is maintained at a moderate level. In this study, *18S rRNA* and *CYP* were stably expressed in seven different varieties of kiwifruit, but the expression of *18S rRNA* was high and *CYP* low (Figure 1), both of which were outside the range of Ct values for ideal reference genes and unsuitable for use as reference genes. Further research is needed on the applicability of low (high) level expression of the reference genes.

PSY, a key gene controlling the flow of total carotenoid metabolism in plants [21], has been observed in our previous studies to be expressed at a certain abundance throughout kiwifruit fruit development [38]. To verify the reliability of the selected reference genes, the relative expression of *PSY* was calibrated with different reference genes. The relative expression of *PSY* diverged significantly after normalization with less stable reference genes. These results highlighted the need for the appropriate choice of reference genes in qRT-PCR assays.

In recent years, genome-wide searches have identified new reference genes from fruit crops such as strawberry [39], peach [40] and plum [41], providing new insights into the mining of kiwifruit reference genes. With the improvement of genome-wide information in kiwifruit, it will also be possible in the future to explore new types of reference genes with greater stability and wider applicability by a number of biological means, rather than being limited to traditional reference genes.

5. Conclusions

The result of the stable analysis of the reference genes in the three software programs (geNorm, NormFinder, BestKeeper) revealed that *GAPDH*, *ACT* and *UBQ* were stably expressed in the test kiwifruit samples. Combined with the results of the pairwise variation analysis, *GAPDH*, *ACT1* and *ACT2* were recommended as the best reference genes for different genotypes, *GAPDH* and *UBQ* were the optimal combinations of reference genes for gene expression analysis in different tissues, *GAPDH* and *ACT1* were suitable for use as reference genes during fruit development and *ACT1* and *UBQ* were the optimal choice for correcting qRT-PCR data under hormone (or pollen polysaccharide) stimulation. Our results will provide additional options for future gene expression analysis in kiwifruit.

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