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Full Paper

Identification of floral development reference genes in *Prunus triloba* cultivars with different petal types

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Abstract: *Prunus triloba*, a well-known ornamental plant, is native to northern China. There are three kinds of petals of *P. triloba*: simple flower, semi-double flower and double flower. At present, the specific molecular mechanisms of floral development in *P. triloba* is not clear. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) has been widely used to measure gene expression level to further explore gene function, while suitable reference genes must maintain stable expression under different conditions to obtain accurate data. In order to select appropriate reference genes for three cultivars of *P. triloba* with different petal types, 9 candidate reference genes were evaluated, namely *Pt18sRNA*, *PtTEF2*, *PtACTIN*, *PtGAPDH*, *PtEF 1-α1*, *PtEF 1-α2*, *PtEF 1-α3*, *PtRPL13* and *PtRPII*. The geNorm and NormFinder algorithms were used to analyse the data from three kinds of flower buds and organs of different petal-type cultivars (*P. triloba* 'Dahuazi', *P. triloba* 'Fuban Tiaozhi' and *P. triloba* 'Hanyan'). Our results show that the suitable internal reference genes are different among cultivars: *PtRPL13* and *PtRPII* present greater stability in *P. triloba* 'Dahuazi' and *P. triloba* 'Fuban Tiaozhi', whereas *PtGAPDH* is more stable in the study of gene expression of *P. triloba* 'Hanyan' organs by qRT-PCR.

Keywords: *Prunus triloba*, reference genes, qRT-PCR, floral development

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INTRODUCTION

Prunus triloba, a widely distributed deciduous frutex in northern China, blossoms in early spring with large flowers and bright colours. According to the current classification system, the flower can be divided into three groups on the basis of petal types: simple petal, semi-double petal and double petal. In this important octaploid plant (8n=64) within the *Prunus* genus [1], the quantity of its petals increases rapidly during a relatively short period of selection. Therefore a variety of flower shapes are produced and the plant's ornamental value greatly increases. Additionally, the plant provides appropriate material for research on floral development [2, 3].

The genes related to floral development of *P. triloba* have been screened in our previous research [3], but their expression needs to be further studied. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is a widely used tool for analysing the temporal and spatial expression patterns of genes [4]. It is considered to be the most effective way to evaluate the expression of genes related to floral development in *P. triloba* [5]. However, the accuracy is strongly affected by many factors such as the amount of material, recovery of RNA, integrity of RNA extraction, quality of cDNA, tissue activity and many other factors [6]. Therefore, a normalisation step is necessary. The most effective method is the use of suitable reference genes that maintain stable expression across different experimental conditions and various developmental stages [7, 8].

So far, housekeeping genes have been used as reference genes for gene expression study by many researchers. It is unlikely that a suitable universal reference gene will be stable for different species. For *P. mume* and *P. salicina*, *actin 2/7* (*ACT*) is suitable for standardising gene expression quantification [9, 10], while *translation elongation factor 2* (*TEF2*), *ubiquitin 10* (*UBQ10*) and *RNA polymerase II* (*RPII*) are stably expressed in *P. persica* [11]. There are many studies related to the screening of reference genes in *Prunus* spp.; however, research on suitable reference genes in *P. triloba* is still scarce.

The analysis of floral-development-related gene expression in tissues of different petal types of *P. triloba* can provide important clues to gene functions involved in floral development, which would be helpful in elucidating the molecular mechanism of floral development from single petal to double petals. In this study flower buds and organs of three *P. triloba* cultivars with different petal types were chosen. Nine candidate reference genes were selected according to former reports [4, 11-16]; they are *18S ribosomal RNA* (*Pt18sRNA*), *PtTEF2*, *PtACTIN*, *glyceraldehyde-3-phosphate dehydrogenase* (*PtGAPDH*), *elongation factor1-α1* (*PtEF1-α1*), *elongation factor1-α2* (*PtEF1-α2*), *elongation factor1-α3* (*PtEF1-α3*), *ribosomal protein L13* (*PtRPL13*) and *PtRPII*. The purpose is to screen suitable floral-development-related reference genes in the different petal types of *P. triloba*. This may help in understanding floral-development-related gene expression patterns and functions, and at the same time provide guidance for selecting reference genes of related *Prunus* plant species.

MATERIALS AND METHODS

Plant Material

All *P. triloba* samples were obtained from the National Flower Engineering Center, Beijing, China. Flower bud samples at different developmental stages were collected: pre-differentiated stage (P1), differentiated stage of flower primordia (P2), sepal primordia (P3), petal primordia (P4), stamen primordia (P5) and pistil primordia (P6) (Figure 1). The fully open and pollen retaining

flowers were also picked and divided into sepal, petaloid sepal, petal, petaloid stamen, stamen and pistil (Figure 2).

Flower buds and organs were collected from three cultivars of *P. triloba* with varying numbers of petals: *P. triloba* 'Dahua Zi' (simple flower with 4-6 petals), *P. triloba* 'Fuban Tiaozhi' (semi-double flower with 10-20 petals) and *P. triloba* 'Hanyan' (double flower with more than 20 petals) (Figure 3). The samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated using E.Z.N.A.™ Plant RNA kit (Omega Bio-Tck, USA) and the method described therein. Potentially contaminated DNA was removed by RQ1 RNase-free DNase (Promega, USA) according to the manufacturer's instructions. RNA integrity was checked by 1% agarose gel electrophoresis. RNA concentration and purity was examined by NanoDrop 1000 spectrophotometer (Thermo, USA) at 230, 260 and 280 nm. cDNA was synthesised by TransScript II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (Takara, China) following the manufacturer's directions in a total volume of 10μL. cDNA was diluted 1:50 with nuclease-free water for qRT-PCR and stored at -20°C.

Selection of Reference Genes and Primer Design

Nine candidate reference genes were selected according to the existing reports on *P. pseudocerasus* [4], *P. mume* [9], *P. sibirica* [14], *Malus domestica* [15] and *P. persica* [16]. Primer Premier 6.0 software (Premier, Canada) was used to design primers based on common conserved sequence shared between peach, plum, apricot and sweet cherry (Table 1). The specificity of the primers was detected by their melting curves.

PCR and qRT-PCR Analysis

Real-time PCR amplification reactions were carried out with SYBR ® Premix Ex Taq™ II kit (Takara, China) in a qTower 2.2 Real-time PCR System (Jena, Germany). The PCR volume was 10 μL, containing 5 μL of SYBR® Premix Ex Taq™ II solution, 0.8 μL of each primer (10 μM), 2 μL of cDNA template and 2.2 μL of ddH_2O. The amplification programme was at 95°C for 90 sec., followed by 40 cycles of 5 sec. at 95°C, 15 sec. at 60°C and 15 sec. at 72°C. Melting curve analysis ranged from 60°C to 95°C. All qRT-PCRs were carried out with three biological and technical replicates.

Data Analysis

The expression stability of the nine reference genes was analysed by geNorm and Normfinder, which are publicly available Microsoft excel-based software packages. All the software packages were used according to the manufacturer's instructions. The quantification cycle (Cq) was collected from the samples and converted into relative quantities for geNorm and Normfinder.

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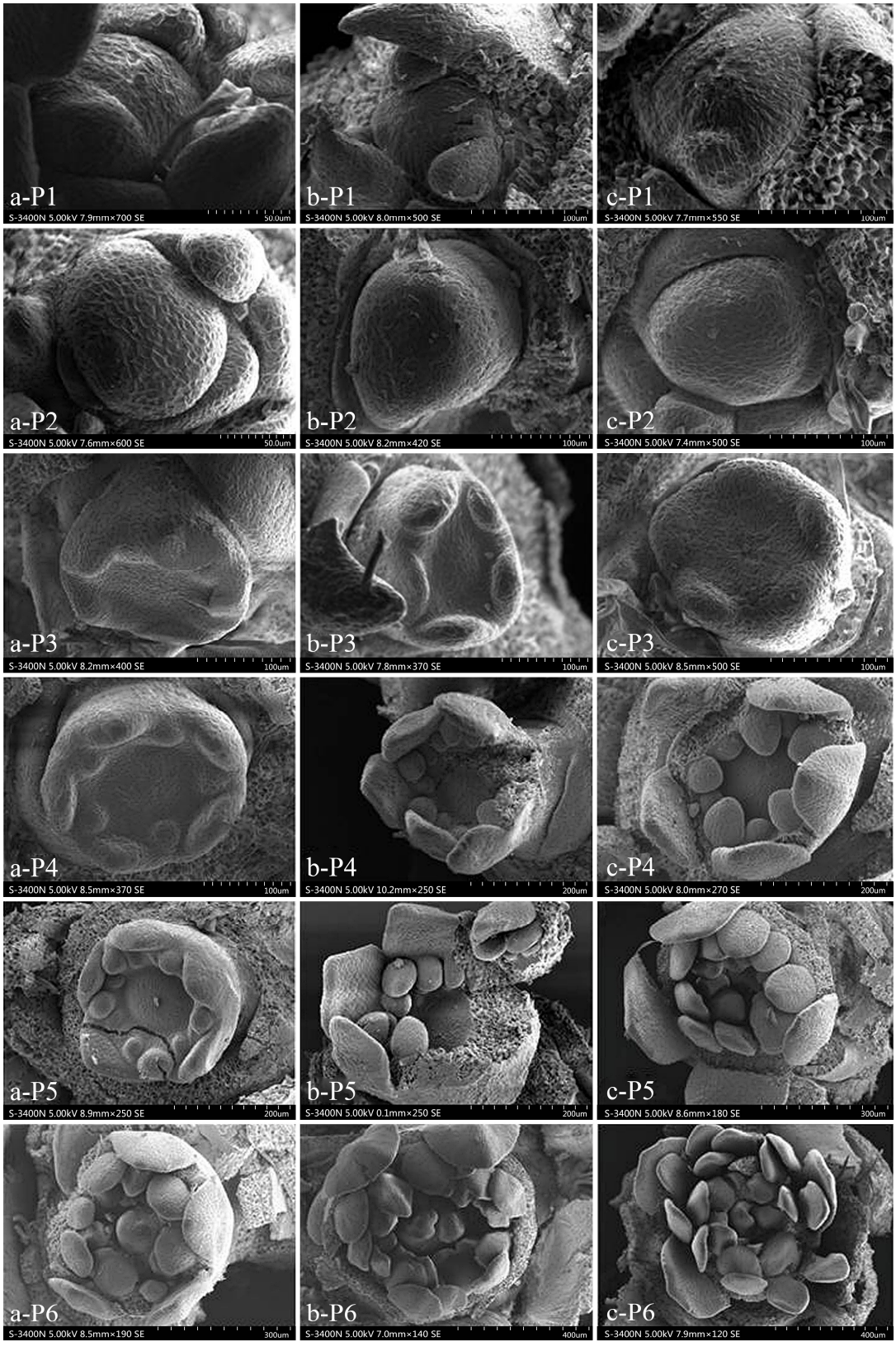

Figure 1. Flower bud differentiation of three *P. triloba* cultivars: a) Flower bud differentiation stage of *P. triloba* 'Dahuazi'; b) Flower bud differentiation stage of *P. triloba* 'Fuban Tiaozhi'; c) Flower bud differentiation stage of *P. triloba* 'Hanyan'

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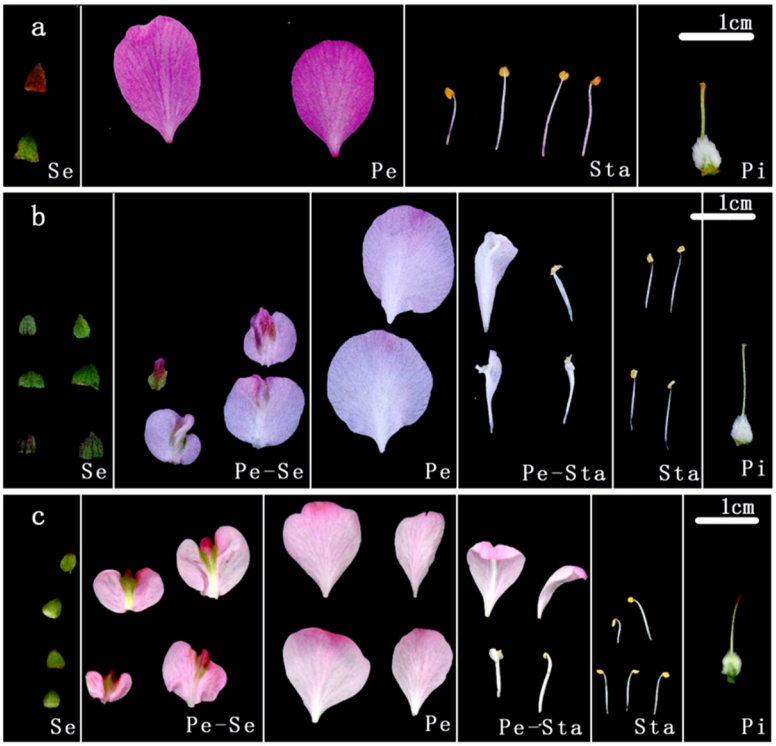

Figure 2. Flower organs of three *P. triloba* cultivars: a) *P. triloba* 'Dahua Zi'; b) *P. triloba* 'Fuban Tiaozhi'; c) *P. triloba* 'Hanyan' (Se = Sepal, Pe-Se = Petaloid sepal, Pe = Petal, Pe-Sta = Petaloid stamen, Sta = Stamen, Pi = Pistil)

Figure 3. Flower anatomy (front and back): a) *P. triloba* 'Dahua Zi'; b) *P. triloba* 'Fuban Tiaozhi'; c) *P. triloba* 'Hanyan'

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Table 1. Primers of candidate reference genes for qRT-PCR

Gene abbreviation	F/R	Primer sequence 5′ —3′	Count of G+C (%)	Annealing temperature (°C)	Product length (bp)
PtACTIN	F	TGCCCTCCCTCATGCCATTCTT	54.5	59.7	110
	R	GCTCAGCAGTGGTGGTGAACAT	54.5		
PtGAPDH	F	TCTGATGACCACCGTTCACTCCAT	50	60.15	86
	R	CAGCTCTTCCACCTCTCCAATCCT	54.2		
PtEF1-α1	F	CTGTGAAGGATCTCAAGCGTGGTT	50	59.6	147
	R	TGTGGCAATCAAGAACTGGAGCAT	45.8		
PtEF1-α2	F	CTGGTGTTGTGAAGCCTGGTATGG	54.2	59.9	165
	R	AACCACGCTTGAGATCCTTGACAG	50		
PtEF1-α3	F	GCTCCTGTTCTCGACTGTCACACCTC	57.7	63.5	91
	R	CCAGTTCCTTACCAGATCGCCTGTCA	53.8		
Pt18sRNA	F	TGTTGGCCTTCGGGATCGGAGTAAT	52	62.4	118
	R	GGCAAATGCTTTCGCAGTTGTTCGT	48		
PtRPII	F	AGCCTGGGCTTGTGGATATCATTGC	52	62.1	138
	R	AAAGGCGCACTGCATCCAGTTTCT	50		
PtRPL13	F	GGAGAAGCCTTCCGTTGAGCTAGTG	56	62.05	109
	R	CCTGGCACCTACATGACGTTGGTTC	56		
PtTEF2	F	CTTGTTGCTGCTGCTGGTATCATTGC	50	62.65	82
	R	GCTCTGCCTCATCTGCACGGGTAT	58.3		

RESULTS AND DISCUSSION

Selection of Primers

To determine the specificity of the designed primers, melting curve analysis was performed. The amplification efficiency of the 9 reference genes varies from 96% for *PtEF1-α1* to 105% for *PtRPII*, the slope is in a range of -3.55 to -3.21, and all primer pairs show $R^2 > 0.98$ (Table 2). Additionally, the melting curves have a single peak (Figure 4), which indicates that the selected primers match the requirements for the qRT-PCR experiment.

Analysis of Reference Genes Expression

The Cq value determines the relative expression level of the candidate reference genes. *Pt18sRNA* has the lowest Cq value (11.89), indicating the highest expression level. The average Cq

values of other genes are between 22 and 25 (Figure 5). Taken together, the expression level varies widely and none of the selected genes has a constant expression level.

Table 2. Screening of candidate reference gene primers for qRT-PCR

Gene	R^2	Slope	Offset	PCR efficiency/%
PtACTIN	0.984	-3.28	21.86	1.02
PtGAPDH	0.999	-3.25	20.85	1.03
PtEF1-α1	0.997	-3.43	19.66	0.96
PtEF1-α2	0.998	-3.55	24.23	1.02
PtEF1-α3	0.994	-3.31	21.57	1.00
Pt18sRNA	0.997	-3.33	10.99	1.00
PtRPII	0.993	-3.21	23.46	1.05
PtRPL13	0.995	-3.24	22.70	1.03
PtTEF2	0.999	-3.22	24.48	1.04

Analysis of Reference Genes Expression Stability

GeNorm analysis

GeNorm program [7] was used to evaluate reference genes by calculating the stability value M: the lower the M value is, the more stable the gene expression becomes. Among 11 samples of *P. triloba* 'Dahua Zi', *PtRPL13* and *PtEF1-α3* are the most stably expressed genes with the lowest M values, whereas the M value of *Pt18sRNA* is higher than 1.5 (Figure 6a), which may not be suitable as a reference gene [7,17]. *PtRPL13* and *PtTEF2* are the most stable genes among the 13 samples of *P. triloba* 'Fuban Tiaozhi' while *Pt18sRNA* is the least stable (Figure 6b). As for the 13 samples of *P. triloba* 'Hanyan', *PtGAPDH* and *PtEF1-α1* are the most stable genes while *PtACTIN* is the least stable (Figure 6c). For all samples we discover that *PtEF1-α2* and *PtRPL13* are two most stable genes while *Pt18sRNA* is the most unstable (Figure 6d).

Genorm program also estimates the optimal number of internal reference genes by calculating the pairwise variation of the normalisation factor. The pairwise variation ($V_{n/n+1}$) parameter 0.15 is considered as the threshold value, below which the inclusion of an additional control gene is not required. If the pairwise variation values are higher than 0.15, (n+1) genes will be needed for accurate normalisation. In our study, for *P. triloba* 'Fuban Tiaozhi' and all samples, the $V_{5/6}$ pairwise variations are less than 0.15, indicating that 5 stable genes are sufficient for gene expression analysis (Figure 7). The $V_{6/7}$ pairwise variations in *P. triloba* 'Hanyan' are less than 0.15, which indicates that 6 stable genes are needed for normalisation (Figure 7). However, the pairwise variations of all $V_{n/n+1}$ in *P. triloba* 'Dahua Zi' are higher than 0.15 (Figure 7). Higher threshold values of V n/n+1 are used in several research reports, which points out that the value of 0.15 should not be held as an inflexible threshold [18,19].

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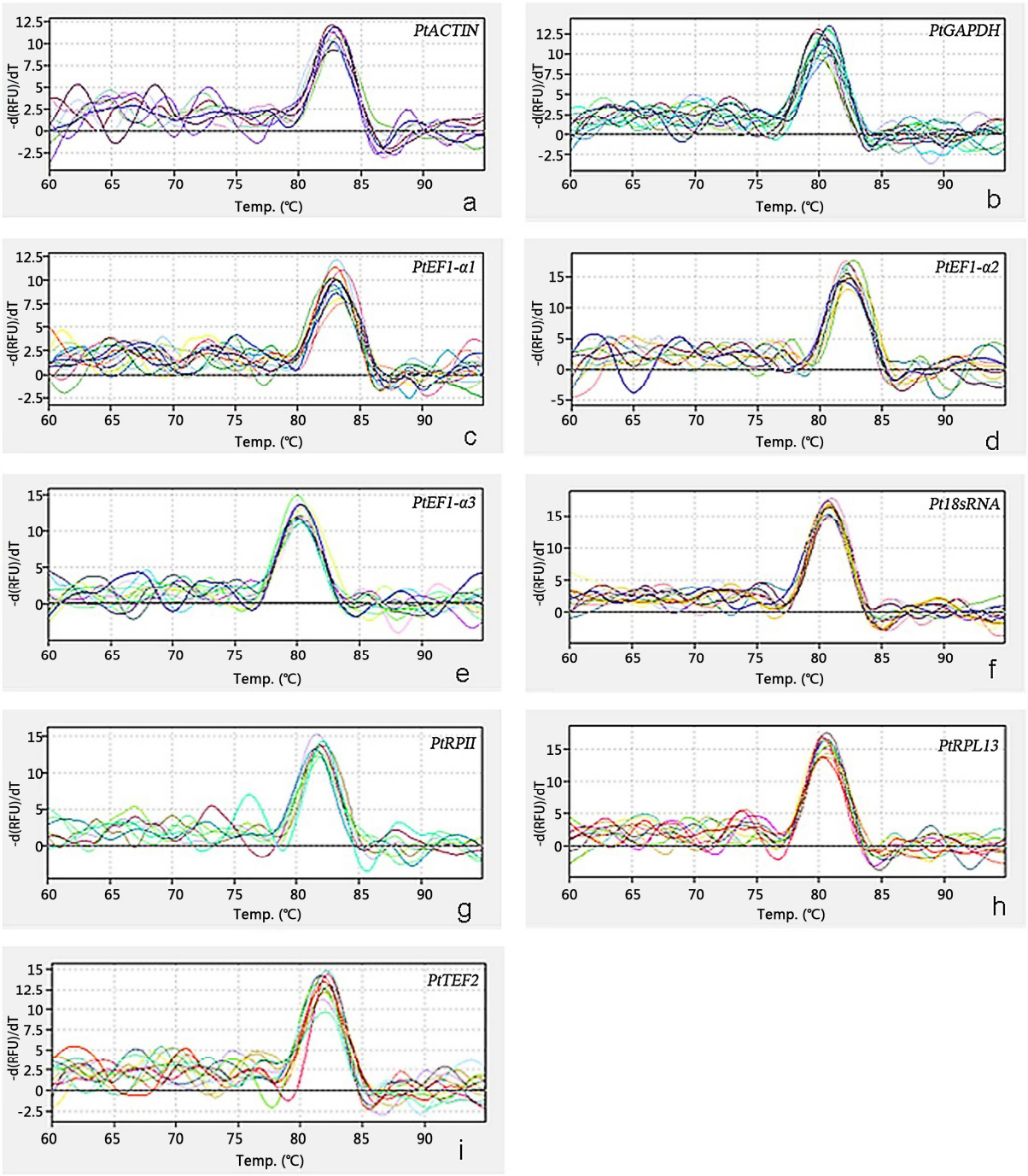

Figure 4. Melting curves of primers of candidate reference genes for qRT-PCR: a) *PtACTIN*; b) *PtGAPDH*; c) *PtEF1-α1*; d) *PtEF1-α2*; e) *PtEF1-α3*; f) *Pt18sRNA*; g) *PtRPII*; h) *PtRPL13*; i) *PtTEF2* (-d(RFU)/dT = rate of fluorescence change)

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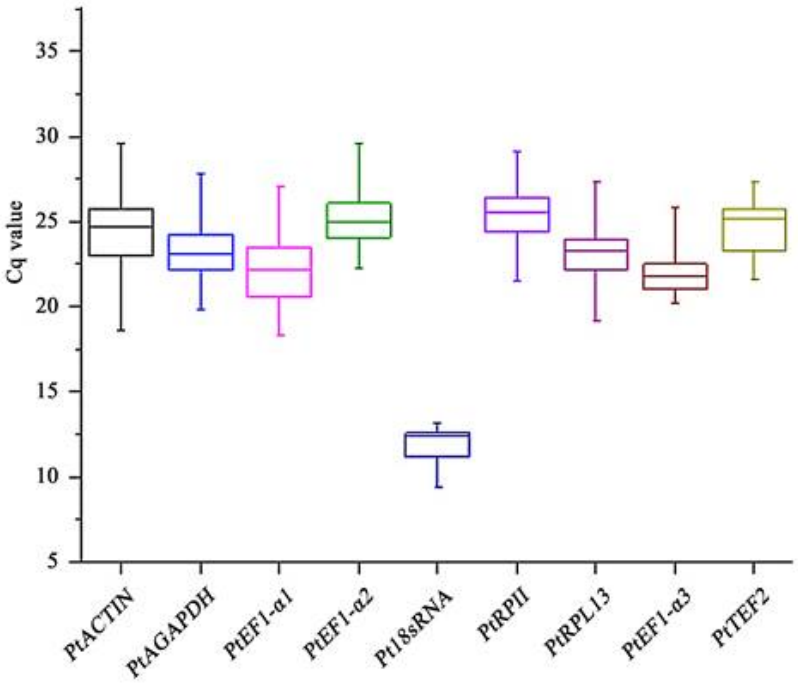

Figure 5. Cq values for 9 candidate reference genes. The line across box signifies the median, the box indicates 25th and 75th percentiles, and whiskers represent maximum and minimum Cq values.

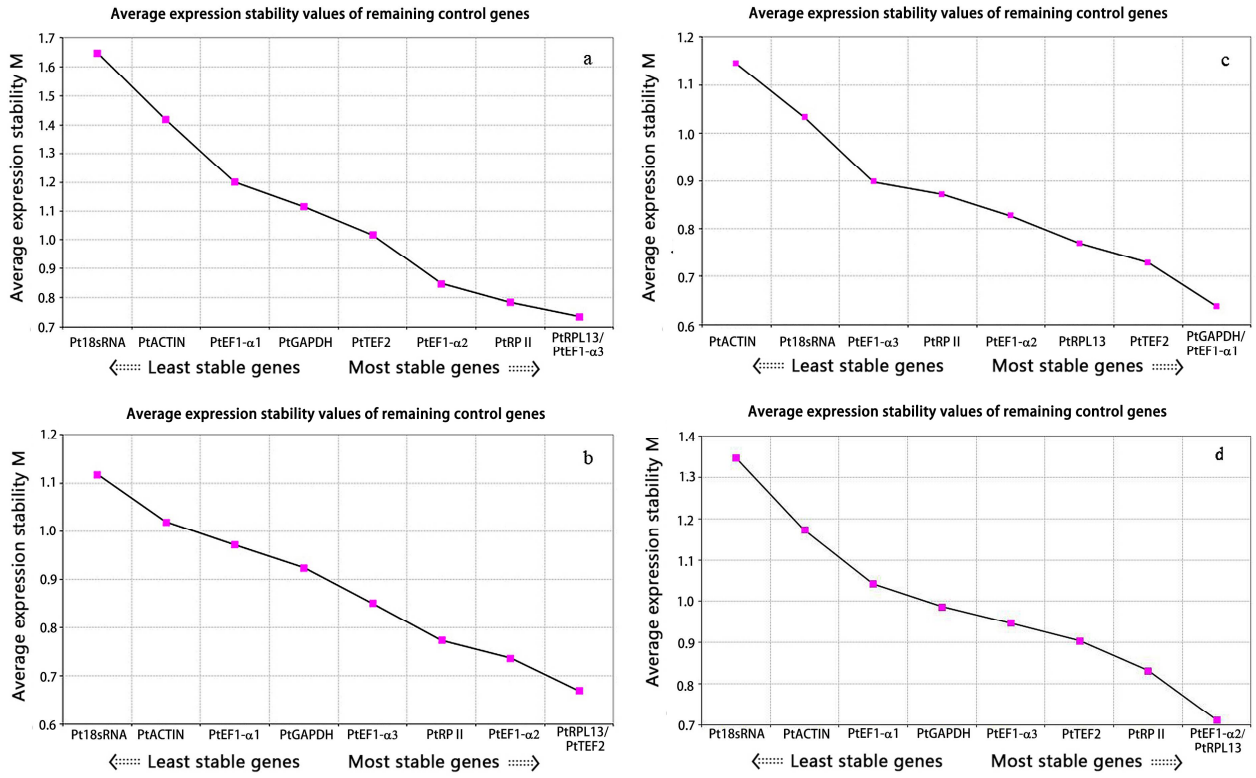

Figure 6. M values of nine candidate reference genes: a) *P. triloba* 'Dahua Zi'; b) *P. triloba* 'Fuban Tiaozhi'; c) *P. triloba* 'Hanyan'; d) all samples

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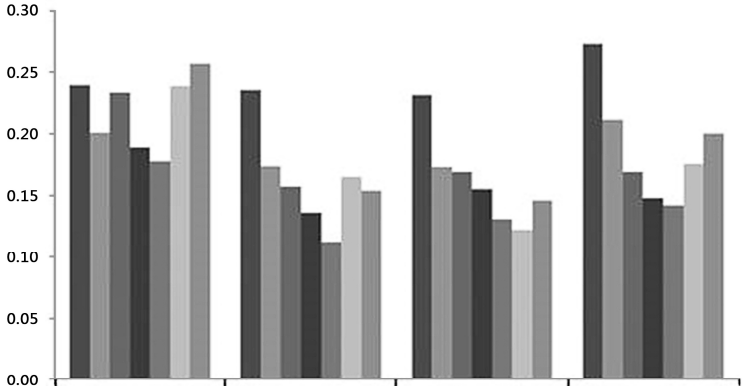

	'Dahua Zi'	'Fuban Tiaozhi'	'Hanyan'	All samples
V2/3	0.240	0.236	0.232	0.273
V3/4	0.201	0.174	0.173	0.212
V4/5	0.234	0.157	0.169	0.169
V5/6	0.189	0.136	0.155	0.148
V6/7	0.178	0.112	0.131	0.142
V7/8	0.239	0.165	0.122	0.176
V8/9	0.257	0.154	0.146	0.200

Figure 7. Pairwise variation analysis of normalisation factors to determine the optimal number of reference genes

NormFinder analysis

Evaluaion values detected by the NormFinder algorithm are shown in Table 3, with lower values indicating higher stability [20]. *PtGAPDH* is ranked as the most stable genes in all samples (Table 3). The stability of *PtRPL13* is similar to *PtGAPDH*, their stability values differing by only 0.001; however, *PtRPL13* is the most stable reference gene by geNorm analysis (Table 3). Combining the two algorithms, *PtRPL13* is the most suitable reference gene in all samples. In *P. triloba* 'Dahua Zi', *PtTEF2* is considered as the most stable one, whose stability value is only 0.001 higher than reference gene *PtRPL13*. However, the *PtRPL13* ranks first in the stability ranking based on geNorm. So we can draw the conclusion from the above analysis that *PtRPL13* is the most stable reference gene. In *P. triloba* 'Fuban Tiaozhi', *PtRPII* performs as the most stable gene while *Pt18sRNA* is not recommended as a reference gene. Combining the stability ranking of the two algorithms, *PtRPII* is considered as the most suitable reference gene. In *P. triloba* 'Hanyan', *PtEF1-α2* is considered the most suitable reference gene followed by *PtGAPDH* while *PtRPII* is unsuitable. However, *PtGAPDH* is one of the most stable reference genes based on geNorm, and the above result suggests that *PtGAPDH* is the most stable reference gene.

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Table 3. Stability values of nine candidate reference genes

	'Da Huazi'		'Fuban Tiaozhi'		'Hanyan'		All samples	
Rank	**Gene**	**Stability value**	**Gene**	**Stability value**	**Gene**	**Stability value**	**Gene**	**Stability value**
1	*PtTEF2*	0.011	*PtRPII*	0.000	*PtEF1-α2*	0.002	*PtGAPDH*	0.063
2	*PtRPL13*	0.012	*PtGAPDH*	0.003	*PtGAPDH*	0.005	*PtRPL13*	0.064
3	*PtRPII*	0.020	*PtEF1-α3*	0.006	*PtRPL13*	0.009	*PtTEF2*	0.070
4	*PtEF1-α2*	0.028	*PtEF1-α2*	0.007	*PtACTIN*	0.013	*PtACTIN*	0.078
5	*PtEF1-α3*	0.035	*PtACTIN*	0.014	*Pt18sRNA*	0.019	*PtEF1-α2*	0.086
6	*Pt18sRNA*	0.040	*PtEF1-α1*	0.020	*PtEF1-α1*	0.021	*PtRPII*	0.092
7	*PtGAPDH*	0.043	*PtRPL13*	0.035	*PtEF1-α3*	0.026	*PtEF1-α1*	0.113
8	*PtACTIN*	0.062	*PtTEF2*	0.053	*PtTEF2*	0.027	*PtEF1-α3*	0.142
9	*PtEF1-α1*	0.073	*Pt18sRNA*	0.076	*PtRPII*	0.030	*Pt18sRNA*	0.234

Comprehensive Analysis of Expression Stability

Statistical algorithms such as geNorm [7], NormFinder [20] and BestKeeper [21] are recommended for gene expression stability evaluation and selection of reference genes. In order to select suitable reference genes for accurate normalisation, two statistical approaches, geNorm and Normfinder, were used to evaluate nine reference genes in *P. triloba*. Results based on the two types of software show that *PtEF1-α2*, *PtEF1-α3* and *PtTEF2* perform poorly while *Pt18sRNA*, *PtACTIN* and *PtEF1-α1* seem to be unsuitable as reference genes. This may be due to the fact that the same reference gene has different stability in different species and different tissues [22-24]. *18s RNA* is the most suitable reference gene for hard branch cuttings of poplar [25]. However, *ACTIN* can be selected as a reference gene for peach [26] and cherry [4] under chilling stress conditions. Moreover, *EF1-α* is stably expressed in soursop fruits stored at 15±1°C [27].

Genes that can be stably expressed under different conditions are generally selected to be reference genes. The frequently used reference genes include *ACT*, *UBQ*, *UBC*, *18sRNA*, *TEF and GAPDH*. Ideal reference genes are not affected by different conditions such as developmental stage and tissue type. However, there is currently no reference gene that can be applied to all species and environments. Selection of reference genes differs from species to species. *GhACT4* and *GhUBQ14* are the most suitable reference genes for different flower development stages in *Citrus sinensis* [28] while *CsUBL5* and *CsGAPDH* are the most stable genes for the development of different flower organs [29]. At different stages of floral organ development in *Lonicera japonica*, *Lonja. ACT2/7* and *Lonja. G6PD* are more stable reference genes [30]. Moreover, it has been found that selection of reference genes is different between cultivars [16], which is consistent with the results of this study.

In our study *PtRPL13* is identified as the most stable reference gene for all sample sets based on geNorm and Normfinder. It is suitable for *P. triloba* 'Dahuazi' and *P. triloba* 'Fuban Tiaozhi' but not for *P. triloba* 'Hanyan'. Similar results can be found in the studies of peach and sweet

cherry plants [31, 32]. Suitable internal reference genes are different among cultivars and it is necessary to select suitable reference genes according to the flower petal type of cultivars.

CONCLUSIONS

This study has shown that *PtRPL13* and *PtRPII* are the most suitable reference genes for gene expression normalisation of floral development in *P. triloba* 'Dahuazi' (simple flower) and *P. triloba* 'Fuban Tiaozhi' (semi-double flower) while *PtGAPDH* presents greater stability for *P. triloba* 'Hanyan' (double flower). This work will provide effective information for the standardisation of qRT-PCR data for gene expression studies related to floral development.

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