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IDENTIFICATION AND VERIFICATION OF THE OPTIMAL REFERENCE GENES FOR THE FLORAL DEVELOPMENT OF *LUCULIA GRATISSIMA* (RUBIACEAE) BY qRT-PCR

HONG MA[1,2], XIONGFANG LIU[1], XIUJIAO ZHANG[1], ZHENGHONG LI[1*] AND YOUMING WAN[1*]

[1]*Research Institute of Resource Insects, Chinese Academy of Forestry, Kunming 650224, China*
[2]*Key Laboratory of Breeding and Utilization of Resource Insects, National Forestry and Grassland Administration, Kunming 650224, China*
[*]*Corresponding author's email: lzh4949@163.com; wanyouming@126.com*

Abstract

Luculia gratissima is a perennial short-day woody plant with important ornamental value, of which flowering is affected by photoperiod. Currently, the studies on the perennial woody plants remains scarce, especially on the short-day woody plants. However, the expression analysis of genes related to flowering is the basis of understanding the molecular mechanism of flowering regulated by photoperiod. Selecting the appropriate reference genes is an essential prerequisite for the qRT-PCR which is extensively applied into gene expression analysis. Therefore, based on the transcriptome data of the floral development of *L. gratissima*, seven housekeeping genes with low RPKM variation coefficient were chosen as candidate genes, and four softwares were applied to evaluate the optimal reference genes. The result of the study indicated that *Actin, EF-1α* and *TUB* had good stability in geNorm and NormFinder analysis. *SKIP, UBQ* and *TUB* had good stability in BestKeeper analysis. In RefFinder analysis, it was found that *Actin, EF-1α* and *TUB* had good stability, totally different with that of BestKeeper. In order to further verify the stability of the selected reference genes, the expressions of *LgPRR7* and *LgFKF1* in different floral development stages were standardized. The result indicated that when three individual genes (*Actin*, *EF-1α* and *TUB*) or the combination of five genes (*Actin*, *EF-1α*, *TUB, UBQ* and *SKIP*) were used as reference genes, the expression trends of *LgPRR7* and *LgFKF1* were basically consistent. According to the analysis of economic cost and correction accuracy of qRT-PCR, 2 or 3 gene combinations in *Actin, EF-1α* and *TUB*, as reference genes, can accurately correct the expression of genes involved in the floral development of *L. grandissima.* The results can provide a correction basis for the gene expression analysis on the floral development of *L. grandissima* in the future.

Key words: *Luculia gratissima*, Flower development, Photoperiod, Housekeeping genes, Reference genes, qRT-PCR analysis.

Introduction

Luculia grandissima is an evergreen shrub or small tree of the family Rubiaceae with flowering period from August to December (Zhou *et al*., 2011), which belongs to a typical perennial short-day woody plant. The critical day length and suitable day length of its flowering are 14 h and 10-12 h, respectively (Wan *et al*., 2018). *L. grandissima* has dense inflorescences, pink, fragrant flowers and long flowering period, so it can be applied to cut flowers, pot culture and garden landscaping (Lin & Lin, 2016; Murray, 1990). In ornamental horticulture, as one of the most important phenotypic characters, flower determines its ornamental value and economic value to a great extent, which has always been the research focus (Yao *et al*., 2019). Floral development, as a complex biological and morphological process, is regulated by many genes (Liu *et al*., 2016). In recent years, great progress has been made in the study on the molecular mechanism of flowering regulated by photoperiod in annual model herbaceous plants (Shrestha *et al*., 2014), but little is known in perennial woody plants. Perennial woody plants do not die immediately after flowering, on the contrary, they produce new vegetative branches and flower buds every year, with the characteristics of long reproductive cycle and seasonal flowering (Albani & Coupland, 2010). Therefore, it is of great reference value to elucidate the flowering mechanism of photoperiod regulation in perennial woody plants.

qRT-PCR has been extensively applied to gene expression reserches in many fields due to its quantitative, sensitive, specific and rapid characteristics (Li *et al*., 2021; Qi *et al*., 2016; Ren *et al*., 2021). However, its accuracy is affected by many factors including the quality of sample RNA, extraction method and reverse transcription efficiency (Zhou *et al*., 2017). Inappropriate reference genes may cause misunderstanding of expression data, thus resulting in incorrect results (Fu *et al*., 2013). Appropriate reference genes can eliminate the variability brought by the differences among samples (Wu *et al*., 2016). In addition, the study also found that the reference gene did not have versatility under different materials and experimental conditions (Artico *et al*., 2010). So it is an important prerequisite for the selection of appropriate reference gene under different materials and experimental conditions before qRT-PCR analysis.

Currently, many studies have been carried out the reference gene screening for floral development of *Chrysanthemum morifolium* (Fu *et al*., 2013) and *Ch. lavandulifolium* (Qi *et al*., 2016), *Paeonia suffruticosa* (Li *et al*., 2016), *Jatropha curcas* (Karuppaiya *et al*., 2017), *Gossypium hirsutum* (Artico *et al*., 2010), *Lagerstroemia indica* and *Lagerstroemia speciosa* (Zheng *et al*., 2018). Nevertheless, the related studies on *L. grandissima* has not been reported, which restricted the exploration on the molecular mechanism of flowering in *L. grandissima*. In this study, based on the transcriptome data of floral transformation in *L. grandissima*, 7 housekeeping genes with low expression variation coefficient were selected as candidate genes. Our research will provide a correction basis for the gene expression study on the floral development of *L. grandissima*.

Materials and Methods

Experimental material: The propagation of the experimental materials was carried out in the central Yunnan Plateau experimental station of Resaerch Institute of Resources Insects, Chinese Academy of Forestry (Yunnan, China; 25° 13′ N, 102° 12′ E, 1826 m a. s. l.). On December 15, 2018, cuttings with apical buds were collected from the plant of *L. gratissima* var. 'Xiangfei' and used for cuttage. All apical meristem of rooting seedlings were removed and transplanted into flowerpots, and cultured in a natural greenhouse with high pressure sodium lamp from 22:00 to 2:00. At the same time, some plants were placed in the natural environment as the control. When the bud differentiation appeared in the control plant, 10 h light/14 h dark photoperiod treatment (short-day) was carried out. After that, according to the anatomical observation of flower bud morphology, the apical tissues of seven development stages (Fig. 1) were selected for reference gene screening, including 0 d (vegetative growth stage), 16 d (floral transition stage), 30 d (small flower primordium differentiation stage), 45 d (budding stage), 55 d (pigmented bud stage), 75 d (bud enlargement stage) and 85 d (fully opened flower stage). Sampling was repeated 3 times in each period, and in each repetition at least 30 apical buds or 10 buds (or flowers) was taken. All collected samples were quickly frozen using liquid nitrogen and stored in the refrigerator of -80°C.

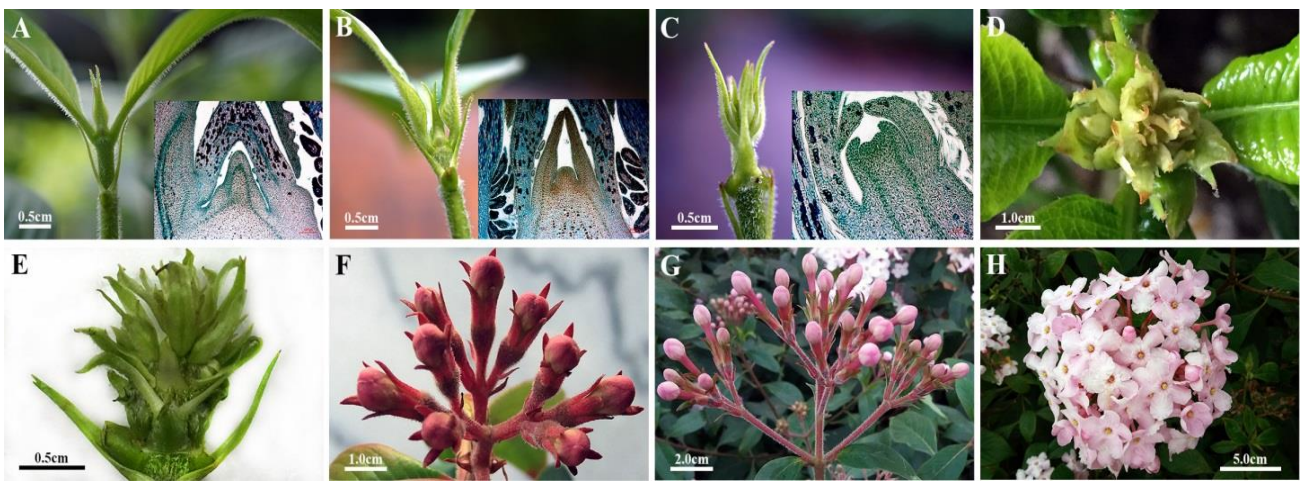

Fig. 1. Floral developmental stages of *L. gratissima*. A: the vegetative growth stage (0 d); B: the floral transition stage (16 d); C: the small flower primordium differentiation stage (30 d); D, E: the budding stage (45 d); F: the pigmented bud stage (5 d); G: the bud enlargement stage (75 d); H: the fully opened flower stage (85 d).

Selection of candidate genes: After assembly and quality control, All-Unigene sequences were compared with four major protein databases (NR, Swiss-Prot, KEGG and COG) and 39090 Unigenes were annotated. The standard deviation (SD), RPKM mean (MV) and coefficient of variation (CV) of these genes in all samples were calculated. Subsequently, 7 housekeeping genes with an average RPKM greater than 30 and a coefficient of variation less than 0.16 were selected as candidate genes (Table 1).

Primer design and amplification efficiency detection: The melting temperature was 57-59°C, the length of primers was 19-25 bp, and the size of amplified fragments was 100-160 bp (Table 1). The PCR reaction system of the reference genes was as follows: 0.5 µl of template cDNA, 0.5 µl of forward and reverse primer, 0.5 µl of dNTP, 2.5 µl of Taq buffer (10 ×), 2 µl of $MgCl_2$ (25 mM), 0.2 µl of Taq enzyme (5 U/µl) and 18.3 µl of H_2O. The reaction conditions were: initial denaturation for 3 min at 95°C, denaturation for 30 s at 94°C, annealing for 30 s at 57°C, extension for 30 s at 72°C; repair extension for 8 min at 72°C, 35 cycles. Finally, the amplified products were detected by 1.5% agarose gel.

qRT-PCR reaction: The reaction was carried out on lightcycle480 II system (Roche, Switzerland). The reaction system: SYBRGREEN qPCR Master Mix (2x) 10 µl, forward primer (10 µm) 0.4 µl and reverse primer (10 µm) 0.4 µl, ddH_2O 5.2 µl, cDNA template 4 µl. The reaction conditions were: initial denaturation for 90s at 95°C,denaturation for 5S at 95°C, annealing for 15 s at 60°C, elongation for 20s at 72°C, 40 cycles.

Statistical analysis: The expression stability of 7 candidate genes in the process of floral formation of *L. gratissima* was analyzed by geNorm (vandesompele *et al*., 2002), NormFinder (Anderson *et al*., 2004; Brunner *et al*., 2004) and BestKeeper (Michael *et al*., 2004). For the geNorm and NormFinder, the raw Cq values of each sample was calculated into Q values before the data analysis using the formula $Q=2^{(minCq\text{-}sampleCq)}$ (minCq is the minimum Cq value of each candidate gene in each sample; sample Cq is the Cq value of each candidate gene in each sample) (Chen *et al*., 2015; Hellemans *et al*., 2007). Then, the obtained Q values of each sample were input into geNorm and NormFinder to calculate the average stable expression value (M or S) of each gene. By the geNorm software, the number of required reference genes was identified by analyzing the pairing difference value (Vn/n+1) of reference genes (Zheng *et al*., 2018). In BestKeeper analysis, the original Cq value was directly analyzed without conversion of the original Cq value (Michael *et al*., 2004). RefFinder was finally applied to calculate the ranking geometric average value of each gene obtained through geNorm, NormFinder and BestKeeper (Hao *et al*., 2014; Li *et al*., 2016; Qi *et al*., 2016; Zhou *et al*., 2017).

Table 1. Primer sequences and amplification characteristics for candidate reference genes.

Gene	Mean -RPKM	CV	Primer sequence (5′ - 3′)	Tm (ºC)	PCR efficiency (%)	Product length (bp)	Regression coefficient (R^2)	Cq value [Mean ± SD]
Actin	365.84	0.09	F:TGACCCTCCAATCCAGACAC	57.5	106.2	143	0.996	24.08 ± 1.29
			R: TTGTGCTCAGTGGTGGTTCA	57.5				
PGK	187.57	0.08	F: TGCCATCTTAACCTCAACGC	58.7	100.7	109	0.993	27.85 ± 1.48
			R: CATCCTTTCTTCCCATCTCG	57.0				
MTP	30.92	0.09	F: TCGAAACTGTTTAGCCCACTC	57.2	109.5	148	0.999	28.90 ± 1.52
			R: GGTTGTAGATTCTGTCTATGATTGC	57.3				
UBQ	1890.94	0.16	F: ATAACATCCAGAAGGAGTCTACCC	57.9	105.1	156	0.999	23.82 ± 1.09
			R: CATTCAAAGCGGCTTAAACAC	58.1				
TUB	65.61	0.11	F: TCGCTAACACGCCTGAACA	58.2	106.9	118	0.999	26.74 ± 1.26
			R: GGATTCCCAACAATGTCAAGTC	58.4				
SKIP	53.90	0.05	F: GACCCTATGGAGCCACCTAAG	58.1	101.4	143	0.998	28.46 ± 0.75
			R: TTTGAAATACAAGGCGGGAT	57.5				
EF-1α	845.26	0.10	F: AACAGCAACAGTTTGACGCAT	58.3	109.4	125	0.999	21.78 ± 1.42
			R: TGAAGAACGGTGATGCTGGT	58.5				

Stability verification: To verify the reliability of the reference genes, the relative expression level of *LgPRR7* and *LgFKF1*, involved in flowering pathway regulated by the photoperiod, were standardized by the selected stable expressed genes and their combinations in the vegetative growth stage (S1), floret primordium differentiation stage (S2), floret coloring stage (S3), floret expansion stage (S4) and floret opening stage (S5). The primer sequence of *LgPRR7* was F:5′-GGGAGTGAAAGTGGGATACGG-3′, R:5′-CACGGTGACATTGGTTGAGG-3′; the primer sequence of *LgFKF1* was F:5′-CCTGCTGAAGAAAAA CCATCCTG-3′, R:5′-TGCCCACCAAGAACCAATAC-3′.

Results

Efficiency of primers: The amplified PCR products showed only a single band (1.5%) by agarose gel electrophoresis (Fig. 2), indicating that the primers could specifically amplify the candidate gene products, and there was no primer dimer.

The melting curve of each gene had single peak. The curve between the duplicate samples had good repeatability (Fig. 3). The primers of each candidate reference gene were designed reasonably, with good specificity, which could be used for the subsequent qRT-PCR analysis. The amplification efficiency was between 100.7-109.5%, and the correlation coefficient (R^2) > 0.990, which met the experimental requirement of qRT-PCR (Table 1).

Cq value analysis of reference gene: The Cq value reflects the gene expression abundance. The smaller the Cq value, the higher the gene expression abundance.The average Cq value of 7 candidate genes in all samples was between 21.78 ± 1.42 and 28.90 ± 1.52, with small variation range (Table 1). The average Cq value of *EF-1α* in each sample was the lowest (21.78), indicating the highest expression abundance in each sample; the value of *MTP* in each sample was the highest (28.90), showing the lowest expression abundance in each sample. *SKIP* had the smallest coefficient of variation, indicating that, it expressed steadily and was preliminarily determined as an appropriate reference gene.

GeNorm: GeNorm determined the most stable reference gene by calculating the average value (M) of expression stability for each candidate reference gene in different samples. The smaller the M value, the better the expression stability of the gene. Generally, the genes with M value less than 1.5 can be used as reference genes. The M values of the seven candidate genes are all less than 1.5 (Table 2), indicating that these genes can be used as candidate genes. According to the order of stability from high to low: *Actin* = *EF-1α* > *TUB* > *MTP* > *UBQ* > *PGK* > *SKIP*. All Vn/n+1 values were greater than threshold value of 0.15 (Fig. 4), indicating that there was no optimal combination number of selected candidate genes. However, many studies revealed that 0.15 was only a relative value, but not an absolute value, which could be adjusted according to the experimental situation (Karuppaiya *et al*., 2017). When the fifth gene was added, V4/5 was relatively low but when the sixth gene was added, V5/6 reached its peak. Therefore, the data can be accurately corrected by selecting 5 combinations of reference genes.

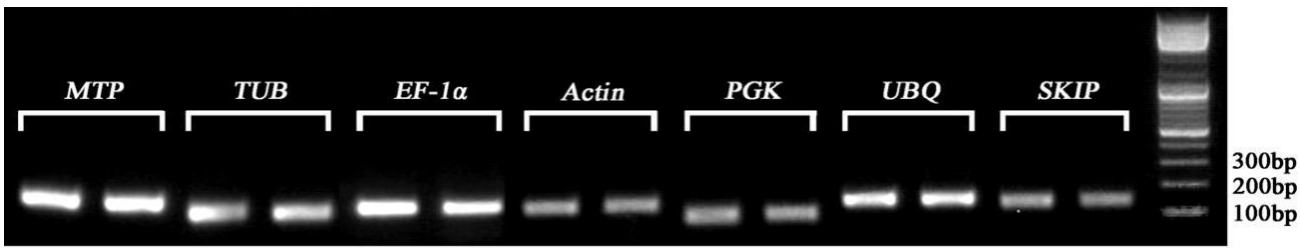

Fig. 2. Agarose gel electrophoresis analysis of 7 candidate reference genes for PCR amplification.

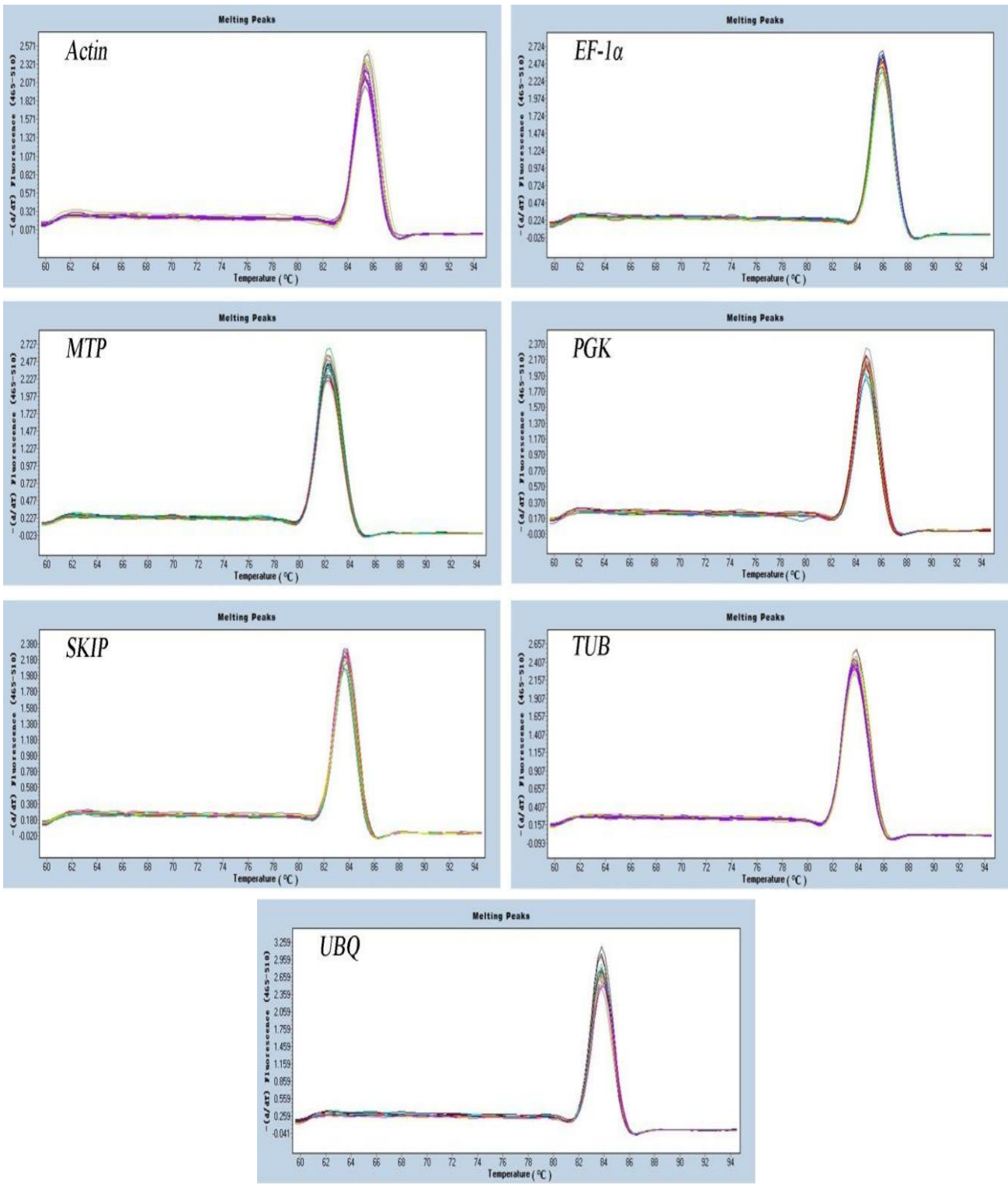

Fig. 3. Melting curves of 7 candidate reference genes.

Table 2. Expression stability of candidate reference genes analyzed by GeNorm, NormFinder and BestKeeper.

Rank	GeNorm		NormFinder		BestKeeper	
	Gene	Stability	Gene	Stability	Gene	CV ± SD
1	*Actin/EF-1α*	0.351	*Actin*	0.121	*SKIP*	2.634 ± 0.749
2	*TUB*	0.480	*EF-1α*	0.265	*UBQ*	4.567 ± 1.088
3	*MTP*	0.599	*TUB*	0.276	*TUB*	4.728 ± 1.264
4	*UBQ*	0.709	*UBQ*	0.449	*MTP*	5.251 ± 1.518
5	*PGK*	0.854	*MTP*	0.553	*PGK*	5.298 ± 1.476
6	*SKIP*	0.972	*PGK*	0.724	*Actin*	5.345 ± 1.287
7			*SKIP*	0.768	*EF-1α*	6.515 ± 1.419

Table 3. Expression stability of the reference gene analyzed by RefFinder.

Methods	1	2	3	4	5	6	7
GeNorm	*Actin/EF-1α*	*TUB*	*MTP*	*UBQ*	*PGK*	*SKIP*	
NormFinder	*Actin*	*EF-1α*	*TUB*	*UBQ*	*MTP*	*PGK*	*SKIP*
Bestkeeper	*SKIP*	*UBQ*	*TUB*	*MTP*	*PGK*	*Actin*	*EF-1α*
RefFinder	*Actin*	*EF-1α/TUB*	*UBQ*	*SKIP*	*MTP*	*PGK*	

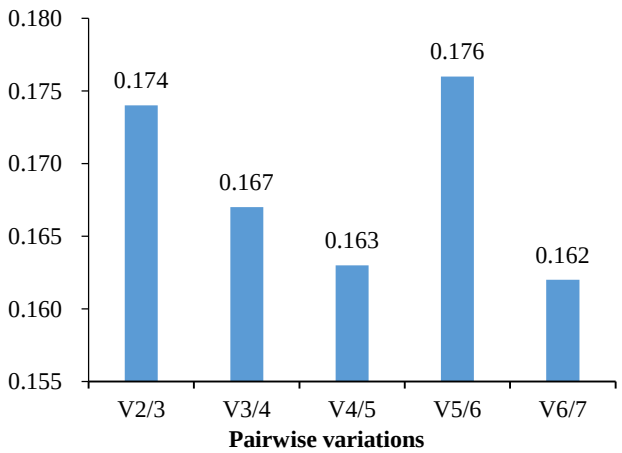

Fig. 4. Pairwise variation (V) of 7 candidate reference genes analyzed by GeNorm.

NormFinder: NormFinder is to evaluate the stability of S-value of candidate reference genes in different samples. The smaller the S-value, the more stable the gene expression. The rank of the expression stability from high to low was: *Actin* > *EF-1α* > *TUB* > *UBQ* > *MTP* > *PGK* > *SKIP* (Table 2). Compared with geNorm, the stability ranking results were basically the same, indicating is, *Actin* and *EF-1α* were still the most stable genes, while *SKIP* was the most unstable gene.

Best Keeper: BestKeeper selects the reference genes with stable expression in different samples by comparing the SD and CV of the Cq value of candidate reference genes. The smaller the SD and CV, the better the stability. The results of BestKeeper analysis (Table 2) indicated that the rank of the expression stability from high to low was: *SKIP* > *UBQ*> *TUB*> *MTP*>*PGK* >*Actin*>*EF-1α*. This was the opposite of the ranking results based on geNorm and NormFinder analysis.

Comprehensive evaluation of candidate reference genes: The smaller the comprehensive index, the more stable the reference gene expression. According to RefFinder's comprehensive ranking results (Table 3), the sequence of expression stability was: *Actin* >*EF-1α*=*TUB* >*UBQ* >*SKIP* >*MTP* >*PGK*. It was evident that the ranking result was basically similar to that of geNorm and NormFinder, that is, *Actin*, *EF-1α* and *TUB* were all ranked as the most stable genes, which was totally different from the results of BestKeeper.

Verification of stability: In order to fully verify the stability of the candidate reference genes, the individual candidate genes and the five gene combinations determined by geNorm differential pairing analysis were used as the reference genes to standardize the expression of *LgPRR7* and *LgFKF1* in different stages of flower development, so as to determine the most suitable reference genes. The five gene combinations were C1 (*Actin* + *EF-1α*), C2 (*Actin* + *EF-1α* + *TUB*), C3 (*Actin* + *EF-1α* + *TUB* + *UBQ*) and C4 (*Actin* + *EF-1α* + *TUB* + *UBQ* + *SKIP*). The expression trends of *LgPRR7* and *LgFKF1* were basically the same when the single gene *Actin*, *EF-1α*, *TUB* and the combination genes C1, C2, C3 and C4 were used as reference genes. The expression patterns of *LgPRR7* and *LgFKF1* were different when *UBQ, SKIP, MTP* and *PGK* were adopted as reference genes (Fig. 5).

Discussion

qRT-PCR is one of the main methods for the detection of genes expression. Howere, it is a prerequisite to improve the detection accuracy of qRT-PCR for the selection of suitable reference genes (Niathalie *et al*., 2005). Three of the best candidate genes, *Actin*, *EF-1α* and *TUB*, were selected from 7 candidate reference genes. Previous studies have confirmed that *Actin* is suitable as an reference gene in the flower development of *Gossypium hirsutum* (Artico *et al*., 2010) and *Lagerstroemia indica* (Zheng *et al*., 2018). However, *Actin* is not suitable as an reference gene in the flower development of *Paeonia suffruticosa* (Li *et al*., 2016) and *Chrysanthemum lavandulifolium* (Qi *et al*., 2016). *EF-1α* and *TUB* are both suitable as reference genes in the male flower development of *J. curcas* (karuppaiya *et al*., 2017). However, *EF-1α* is the appropriate reference gene in the flower development of *P. suffruticosa* (Li *et al*., 2016) and *Lagerstroemia indica* (Zheng *et al*., 2018), while *TUB* is not suitable. In addition, *PGK* is considered to be an reference gene in the flower development of *Ch. morifolium* (Qi *et al*., 2016), but its stability is the worst in our study. Therefore, for the flower development of different plants, the reference genes should be screened and verified specifically.

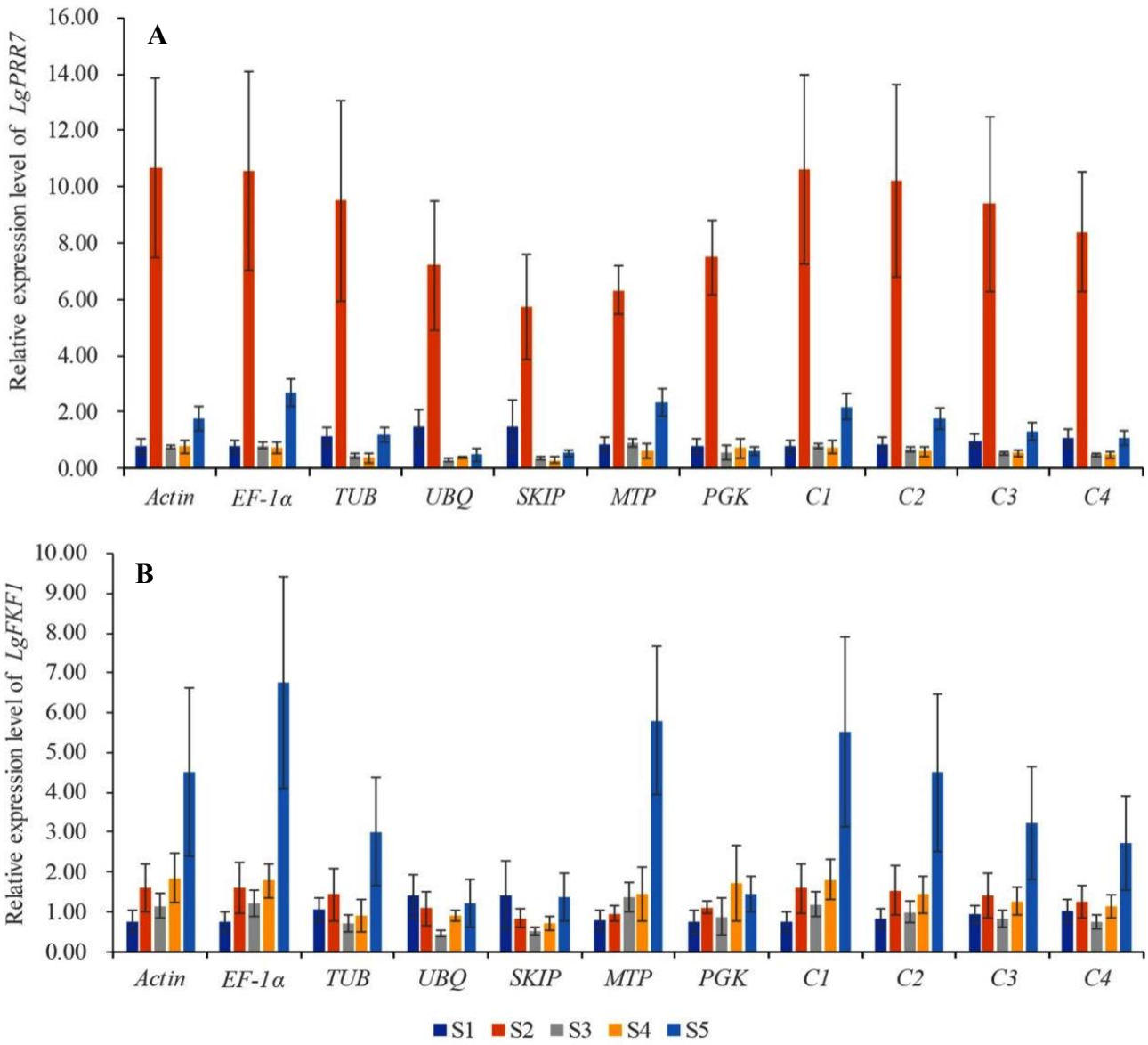

Fig. 5. Relative expression levels of *LgPRR7* and *LgFKF1* at the different floral developmental stages of *L. gratissima* using candidate genes and their combinations for normalization. (A) Relative expression level of *LgPRR7*; (B) Relative expression level of *LgFKF1*. Combination genes: C1 (*Actin* + *EF-1α*), C2 (*Actin* + *EF-1α* + *TUB*), C3 (*Actin* + *EF-1α* + *TUB* + *UBQ*) and C4 (*Actin* + *EF-1α* + *TUB* + *UBQ* + *SKIP*). Flower developmental stages: S1 (the vegetative growth stage), S2 (the budding stage), S3 (the pigmented bud stage), S4 (the bud enlargement stage) and S5 (the fully opened flower stage).

For the same data, different statistical algorithms may produce contradictory results (Wan *et al*., 2010). Therefore, at least three different softwares are required to obtain the best results (Qi *et al*., 2016). The analysis results based on geNorm and NormFinder are basically similar, and *Actin* and *EF-1α* are the most stable genes. However, the results of BestKeeper analysis are different from those of the former two, that is, *Actin* and *EF-1α* are the most unstable genes. Finally, the results of RefFinder comprehensive analysis are basically consistent with those of geNorm and NormFinder. In geNorm analysis, the default value (Vn/n+1) for the program is 0.15. If it is less than 1.5, there is no need to add the N+1 gene (vandesompele *et al*., 2002). All Vn/n+1 values in this study are greater than 0.15, which has been reported in *Lycoris* (Jiang *et al*., 2015) and *Ch. lavandulifolium* (Fu *et al*., 2013), and this result may be due to the large development span of experimental materials. In addition, the geNorm operation manual also emphasizes that the default value of Vn/n+1 is 0.15, which is not a strict limit value, and it can be adjusted according to the test situation. Among all Vn/n+1 values, the value of V4/5 is the smallest and closed to V6/7, but the value of V5/6 is the largest. Therefore, the number of optimal genes is determined as five. Although many reference genes can improve the accuracy of qRT-PCR, it is necessary to balance the accuracy of qRT-PCR with the economic cost. In cases where there are too many reference genes or there is no optimal number of combinations, the 3 most stable genes should be combined as the reference genes (Maroufi *et al*., 2010).

To further verify the reliability of reference genes, the expressions of *LgPRR7* and *LgFKF1* were corrected by using the individual candidate gene and the 5 gene combinations identified by geNorm differential pairing analysis as reference genes. The expression patterns of

LgPRR7 and *LgFKF1* were basically the same when the top 3 individual genes, *Actin*, *EF-1α*, *TUB* and the top 5 gene combinations were used as the reference gene for correction. In consideration of economic cost, standardized correction could also be achived by using one reference gene. But a single reference gene may lead to contradictory results and multiple stable combinations of reference genes can obtain more accurate and reliable results (Zhou *et al*., 2017; Gutierrez *et al*., 2008; Reid *et al*., 2006). Given the economic cost of qRT-PCR and the accuracy of standardized correction, we believe that the expression of genes related to the development of the *L. gratissima* can be accurately corrected by using 2 or 3 gene combinations among *Actin*, *EF-1α* and *TUB*. The results would provide a basis for the gene expression analysis of the development of *L. gratissima* in the future.

Conclusion

The expression stability of 7 candidate genes at 7 flower developmental stages was evaluated using 4 statistical approaches. A combination (*Actin*, *EF-1α* and *TUB*) was validated by the expression of *LgPRR7* and *LgFKF1*. These data drawn from this research will provide useful information and lay a solid foundation on flower development in *L. gratissima*.

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