Selection and validation of reference genes of *Paeonia lactiflora* in growth development and light stress

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Abstract The stem of *Paeonia lactiflora* will bend when it grows in greenhouse at a low light intensity. It is important to explore causes of morphological changes of peony to improve its quality. Gene expression can be evaluated by quantitative real-time PCR, based on reference gene. However, systematic selection of reference genes under weak lighting for herbaceous peony is lacking. To address this problem, we first selected 10 candidate reference genes based on a coefficient of variation of gene expression from peony stem transcriptome data. Then, geNorm, NormFinder and BestKeeper were applied to assess the stability of the genes, and RankAggreg was used to give a comprehensive ranking. The results show that there are some differences in optimal reference genes among samples from different organs and under the two lighting conditions, and the optimal number of suitable reference genes is distinct. Two selected suitable reference genes were then used to normalize target genes, and the results were compared with transcriptome data. Consistent gene expression trends were obtained, indicating the reliability of the method. To the best of our knowledge, this is the first time reference genes

for herbaceous peony were selected in different organs, developmental stages and under two kinds of lighting conditions. The findings can provide a practical method for selecting reference genes for peony under these conditions and demonstrate a useful combination of reference genes.

Keywords Herbaceous peony · Reference gene · Light stress · F-box · GAPDH

Abbreviations

DFG	<i>Paeonia lactiflora</i> ‘Da Fugui’
CTH	<i>Paeonia lactiflora</i> ‘Chui Touhong’
qPCR	Quantitative real-time PCR
CV	Coefficient of variation
F-box	F-box protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
UBQ10	Polyubiquitin
CYP	Cyclophilin
ACT	Actin/actin-like conserved site-containing protein
EF- α	Elongation factor-1 alpha 3
AQU	Probable aquaporin PIP1-2
ETI	Eukaryotic translation initiation factor 5A-2
E3	E3 ubiquitin protein ligase RIE1
pp2A	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform-like
Tm	The melting temperature
Cq	Quantification cycle

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Introduction

By applying high-throughput sequencing technology, large amounts of transcriptome data were produced. To form a reliable conclusion, it is necessary to experimentally verify

the data. qPCR is more sensitive, more precise, faster, requires less of RNA template, and can detect poorly expressed genes, compared with Northern blot analysis. qPCR is also less expensive than Microarray (Bustin et al. 2009; Czechowski et al. 2004; Derveaux et al. 2010; Iskandar et al. 2004). With these advantages, qPCR became the routine method to detect gene expression.

The relative expression of a target gene is normalized by reference genes. Traditionally, there are several commonly used reference genes, such as 18S rRNA, actin and GAPDH (Czechowski et al. 2005). Increasingly more researchers have found that reference gene expression can be influenced by biological and abiotic stresses (Martins et al. 2017; Nicot et al. 2005). Moreover, most suitable reference genes of a plant can be distinguished in different growth development stages, organs and cultivars (Chao et al. 2019; Li et al. 2016, 2017; Sudhakar Reddy et al. 2016; Tong et al. 2009). Improper use of reference genes can lead to biased results or errors (Dheda et al. 2005; Radonić et al. 2004). Thus, selecting and validating reference genes of a particular plant in the specific environment is vital.

Researchers developed three kinds of software to judge the stability of candidate reference genes. In geNorm, the M value is used to represent the arithmetic mean value of the pairwise value of genes (Vandesompele et al. 2002). In NormFinder, the stability value is calculated according to intragroup and intergroup variation (Andersen et al. 2004). In BestKeeper, the standard deviation, Pearson correlation coefficient and the p value of candidate genes are comprehensively analyzed (Pfaffl et al. 2004). However, the order computed from the three methods may be not consistent with each other. A comprehensive rank needs to be performed. Researchers determined the overall rank according to the sum of the ranks (Delparte et al. 2015). Researchers used the online tool, refFinder, to get the comprehensive order (Klumb et al. 2019; Shivhare and Lata 2016; Zhu et al. 2019). RankAggreg (Pihur et al. 2009) was used to calculate the comprehensive rank, which is an R-based package, using cross-entropy algorithms (Amil-Ruiz et al. 2013; Klie and Debener 2011; Mallona et al. 2010; Ponton et al. 2011). RankAggreg assesses the sorted results, calculated by different algorithms, and supports the comprehensive conclusion of the researchers.

Paeonia lactiflora is an excellent cut flower. A short flowering period inhibits its popularity. Greenhouse cultivation can extend the supply period. Previous studies have found that morphological characteristics of some peony cultivars grown in a greenhouse are altered, such as color fading, stem bending and leaf shrinkage, due to the low light intensity (Han et al. 2014). To illuminate the mechanism of morphological change at the molecular level, it is necessary to select the appropriate reference genes.

However, research into reference gene selection in herbaceous peony is lacking at present. On one hand, there are only few studies on selecting reference genes in herbaceous peony (Li 2017). On the other hand, published studies about *Paeonia lactiflora* have only used the actin-encoding gene (JN105299) as a reference gene (Tang et al. 2018; Wu et al. 2018), which was cloned from ‘Hong yan zheng hui’. However, reference genes may be distinct in different cultivars, and there are no sequences in the transcriptome data of ‘Da Fugui’ (DFG) and ‘Chui Touhong’ (CTH) (SRA: PRJNA528693), which were obtained from the stem samples of two peony cultivars in five development stages (from germination stage to the flowering stage), with three biological replicates.

To select the reference genes with stable expression in different peony growth development stages and in a low light intensity environment, experiments were carried out from two aspects. On the one hand, ten candidate reference genes were selected based on the previously published transcriptome data of peony stem (SRA: PRJNA528693). It should be noted that these peonies were grown under normal light conditions. On the other hand, the leaves, flowers and stems were taken from plants in both the field and the single-sided solar greenhouse (light intensity of the greenhouse was $200\text{--}564\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ and it was approximately 50% less in the field at the same time during the experiment period). Quantitative analysis was performed by qPCR. The stability of the candidate reference genes was determined by geNorm, NormFinder and BestKeeper. RankAggreg was used to determine the comprehensive ranking. In addition, the expression of PAL and HCT was normalized against the best and the worst selected candidate reference genes. This study determined the most suitable reference genes for different organs, growth development stages and light stress, which laid the foundation for the future of molecular research of herbaceous peony.

Materials and methods

Plant materials

Paeonia lactiflora ‘Da Fugui’ (DFG) and ‘Chui Touhong’ (CTH) were chosen as experimental material. They were planted in Caozhou Peony Garden, Heze, China, covered with plastic film, and given normal water and fertilizer amounts to create native growth conditions. Leaves, flowers and stems were taken once every week until the flowers bloomed (26th, April, 2018). In addition, DFG was cultured in the single-sided solar greenhouse of the National Engineering Research Center for Floriculture, Changping District, Beijing, without extra artificial lighting to create

light stress condition. The cultivation method used was as described previously (Han et al. 2014). Leaves were collected at 1 week, 3 weeks, 6 weeks and 8 weeks after planting; flowers and stems were collected at the latter three stages. Forty samples in total were collected; every sample was taken from 3 to 6 individual plants and mixed. All the fresh samples were frozen in liquid nitrogen and then stored at -80°C .

Candidate reference gene selection and primer design

After filtering the raw data, Trinity 3.0 was used to assemble the unigene collection (Guo et al. 2018). Reads Per kb per Million reads (RPKM) were calculated according to the previous methods (Mortazavi et al. 2008), as shown in Table S1. In the formula A, RPKM refers to the expression of unigene A, C refers to the number of reads of unigene A, N refers to the total number of reads of all unigenes, and L refers to the number of bases of unigene A.

$$\text{RPKM} = (1000000 * C) / (N * L / 1000) \quad (\text{A})$$

Based on the RPKM, we calculated the coefficient of variation (CV) as a variance/mean. The lower the CV was, the higher the stability of the gene (Czechowski et al. 2005). Six candidate reference genes were selected; the CV of all of which was below 0.15. Another four candidate genes were selected with a CV between 0.19 and 0.38. To more vividly display the stability of candidate reference genes in the transcriptome data, we combined seven other commonly used reference genes to draw heat map, as shown in Fig. 1. Both the unigene expressions of these two groups had some variation, and the expression level of PR II in ‘Da Fugui’ was extremely low. No obvious stability differences were shown between the two groups. The information of unigenes in the two group was shown in Table S1. Primers were designed according to primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and the position of these primers were labeled on the unigenes obtained from the transcriptome, as shown in Figure S1.

cDNA preparation and quantitative real-time PCR

Total RNAs were extracted according to kit manual (RN38, Aidlab Biotechnologies Co., Ltd, Beijing). A NanoDrop was used to examine the concentration and purity. A 1% gel was used to examine the integrity of the RNA. The high quality RNA was collected and stored at -80°C . cDNA was obtained according to the kit manual (FSQ-301, TaKaRa, PrimeScriptTM RT reagent Kit with gDNA Eraser, Japan) using the prepared RNA. Using SYBR[®]Premix Ex TaqTM (TaKaRa, Japan), quantitative real-time PCR were conducted using the CFX96 Real-Time PCR (Bio-

Fig. 1 Heatmap of selected unigenes expression. Each row represents the expression of a particular unigene in five stages of two cultivars. Among them, the unigenes in Group I are some other common used reference genes, and the unigenes in Group II are the candidate reference genes in this experiment. CS1 refers to the samples on stage one of ‘Chui Touhong’, DS1 refers to the samples on stage one of ‘Da Fugui’. Both rows and columns are not clustered by Pheatmap

Rad, USA) instrument using the following procedure: preheat at 95°C for 30 s; run 40 cycles as 95°C for 10 s, T_m ($^{\circ}\text{C}$) for 15 s and 72°C for 15 s; 65°C for 5 s and 95°C for 5 s. The reaction consisted of 2 μL of F primer, 2 μL of R primer, 1 μL of the cDNA template, 5 μL of ddH₂O and 10 μL of the SYBR Green Mix for. Three technical replicates were performed.

Stability and validation of candidate reference genes

GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) were used to determine the stability of candidate reference genes. Based on the RankAggerg package, the results of the three programs was input into R (<http://www.R-project.org>).

PAL and HCT gene sequences were obtained from the transcriptome data (PRJNA528693). The methods of acquisition of RNA and cDNA as well as the qPCR procedure were as previous described. The best and least suitable candidate reference genes as selected by the RankAggerg package were used to validate the stability of reference genes.

Results

Specificity of primer and expression of candidate reference genes

It can be seen from the agarose gel and melting curve (Figure S2 and Figure S3) that the PCR products were

Table 1 List of Primers

Gene	Sequence	Mean C _q	T _m (°C)	Product size	E (%)	R ²
EF- α	CATTGGCCATGTTGACTCTG TGCTGCTTCCTTCTCAAACC	22.61	52	106	101.4	0.996
UBQ10	CGGAGGACAAGATGGAGTGT TCCAAGACAAGGAAGGCATC	22.71	53.9	127	93.2	0.995
GAPDH	GGGCTGAATTCGTTGTTGAG CTTGGGGCTGAGATGATGAC	23.84	58.3	103	97.4	0.997
ACT	TGATCACCTGTCCATCAGGA CGATCTCACAGACTCGCTCA	28.42	59.4	197	94.9	0.982
F-box	GACCTCGACTGACTCCTCCA TCGTCAACCGTGAAATGTGT	30.53	59.4	145	104.3	0.990
CYP	CTGGACCTGGAATTCTGTCTG AGCCACTCAGTCTTGGCAGT	22.73	59.4	88	101.5	0.992
AQU	GCCCTTGATTACACCAGCAC AATCCAGCTGTGACCTTTGG	25.11	59.4	117	92.8	0.997
ETI	ATGTTCCCATGTCACTCGT TCAGTTGGGAGCCTCAAATC	22.19	59.4	115	91.0	0.994
E3	ACGTCATCATCCTCGGTCTC TGTGGAGTTCATGTGGTGCT	30.70	60	119	103.5	0.996
pp2A	ACTGGCTGCAAATTCATTC	29.40	60	82	100.4	0.992

unique, and the melting curves had a single peak, which showed that the primers used in this study were specific. Primer sequences, the melting temperature (T_m), product size, efficiency (E) and R^2 can be obtained from Table 1. The efficiency of primers was 91.0–104.3%, and the R^2 was 0.982–0.997, indicating that the qPCR procedures have been optimized. The C_q values of 40 samples are shown in Fig. 2. The C_q values of all the samples were between 17.30 and 35.54. ETI had the minimum mean C_q values, 22.19, and E3 had the maximum mean C_q values, 30.7. Both in the natural lighting and light stress condition, the C_q of samples showed similar regulation. C_q values of CYP, ETI and UBQ10 were similar, and C_q values of EF- α were the most scattered.

Ranks of candidate reference gene

Three algorithms were used to judge the stability of the candidate reference genes, and their stability ranking was also obtained separately. Then, the R package, RankAggreg, was used to produce the aforementioned three ranked results, and the comprehensive ranking based on cross-entropy algorithm was enumerated.

In geNorm, M values less than 1.5 represented the candidate reference genes of relatively good stability, and the most stable reference gene should be the minimum M value (Vandesompele et al. 2002). M values are shown in Table 2. For all the organs of the two cultivars grown under normal light conditions, the M values were less than or

equal to 1.5. However, different organs did have an influence on the stability of the candidate reference genes. For example, EF- α is the most stable reference gene in flower samples (0.42), and the stability ranking of it was in the middle in both the leaf (0.80) and stem samples (0.48), leading to the lowest ranking in total samples (1.50). For samples under light stress conditions, GAPDH was the most stable (0.69, 0.82, 0.53), ranking in the top three throughout the organs, and UBQ10 was one of the bottom three in all the organs (1.11, 1.67, 0.95). The rankings of AQU and ACT fluctuated greatly throughout the organs.

In general, it is regarded that the best number of reference genes is where the pairwise variation value is below 0.15 (Vandesompele et al. 2002). Pairwise variations among organs and conditions are shown in Fig. 3. It was indicated that only two reference genes were needed in stem samples in normal conditions, while under light stress, four were needed. Most samples needed four to seven reference genes combined to guarantee the relative stability. However, for leaves and total samples under light stress, the ten candidates reference genes above did not seem to be desirable due to the relatively high pairwise variation values.

As seen in Table 3, the ranks of ten candidate reference genes were obtained by NormFinder according to the stability values. By using this kind of algorithm, the ranking among groups varied greatly. ETI ranked first among the three groups, while it was at the bottom in stem samples under light stress. GAPDH ranked in the top three of the

Fig. 2 Box-plot of Cq values. **a** Cq values of DFG and CTH in the field, **b** Cq values of DFG under light stress and in the field. Candidate reference genes are differentiated by colors according to the legend. For each gene, the Cq values among samples are arranged by order,

with the upper and lower edges being the upper and lower edges, respectively. The upper quartile and the lower quartile form two sides of the rectangle, and the horizontal line in the rectangle represents the median. In addition, the outlying points are enclosed by a triangle

Table 2 Average expression stability values (M) for the ten candidate reference genes based on geNorm algorithm

Organs	Condition	Candidate reference genes									
		ACT	AQU	CYP	E3	EF-α	ETI	F-box	GAPDH	pp2A	UBQ10
Flower	Normal	0.90	0.73	0.83	0.86	0.42	0.79	0.94	0.42	0.61	1.03
	Light stress	0.69	1.20	0.93	1.26	1.03	0.76	0.83	0.69	0.88	1.11
Leaves	Normal	0.85	0.70	0.74	1.03	0.80	0.65	0.56	0.89	0.97	0.56
	Light stress	1.22	1.53	1.42	0.61	0.98	1.31	1.10	0.82	0.61	1.67
Stems	Normal	0.28	0.72	0.83	0.57	0.48	0.39	0.28	0.42	0.44	0.29
	Light stress	0.53	0.66	0.70	1.05	0.88	1.16	0.75	0.53	0.84	0.95
Total	Normal	1.14	1.08	1.35	0.88	1.50	1.29	1.01	0.70	0.70	1.24
	Light stress	1.38	1.42	1.27	1.14	1.58	1.34	0.84	0.84	1.03	1.48

Fig. 3 Pairwise variation values for ten the candidate reference genes based on geNorm. The position indicated by the small triangle represents the optimal number of reference genes among organs and conditions

Table 3 Ranks of the ten candidate reference genes based on the NormFinder algorithm

Organs	Condition	Rank									
		1	2	3	4	5	6	7	8	9	10
Flower	Normal	ETI	CYP	E3	F-box	AQU	pp2A	ACT	GAPDH	EF- α	UBQ10
	Light stress	ETI	pp2A	ACT	CYP	GAPDH	F-box	UBQ10	AQU	E3	EF- α
Leaves	Normal	ETI	AQU	CYP	GAPDH	EF- α	UBQ10	F-box	pp2A	ACT	E3
	Light stress	ACT	ETI	GAPDH	E3	F-box	pp2A	CYP	AQU	EF- α	UBQ10
Stems	Normal	F-box	ACT	UBQ10	GAPDH	EF- α	pp2A	ETI	E3	AQU	CYP
	Light stress	GAPDH	F-box	AQU	CYP	ACT	EF- α	pp2A	UBQ10	E3	ETI
Total	Normal	F-box	GAPDH	AQU	E3	ACT	pp2A	UBQ10	ETI	CYP	EF- α
	Light stress	GAPDH	pp2A	ACT	F-box	CYP	ETI	AQU	E3	UBQ10	EF- α

Table 4 Correlation coefficient for the ten candidate reference genes based on the BestKeeper algorithm

Organs	Condition	Coeff. of corr. [r]									
		ACT	AQU	CYP	E3	EF- α	ETI	F-box	GAPDH	pp2A	UBQ10
Flower	Normal	0.961	0.947	0.974	0.979	0.945	0.981	0.947	0.960	0.967	0.946
	Light stress	0.923	0.771	0.928	0.78	0.88	0.972	0.964	0.924	0.948	0.944
Leaves	Normal	0.757	0.91	0.902	0.615	0.873	0.929	0.959	0.831	0.649	0.971
	Light stress	0.853	0.507	0.032	0.83	0.888	0.661	0.958	0.866	0.681	– 0.111
Stems	Normal	0.846	0.732	0.212	0.573	0.843	0.699	0.858	0.724	0.85	0.621
	Light stress	0.934	0.918	0.921	0.949	0.944	0.290	0.968	0.969	0.972	0.788
Total	Normal	0.939	0.918	0.746	0.932	0.900	0.829	0.958	0.950	0.919	0.865
	Light stress	0.843	0.768	0.751	0.821	0.856	0.701	0.892	0.914	0.874	0.694

four groups, while it was the third to last in flower samples in normal conditions. In addition, EF- α was in the bottom five in all groups.

As shown in Table 4, the correlation coefficient (r) of all the samples was obtained by BestKeeper. In all the organs and conditions, F-box (0.858–0.968) was ranked in top three 7 times, which showed the relatively stability. However, the r of CYP and UBQ10 varied among conditions. For leaf samples in normal conditions, the r of CYP was 0.902, while r was only 0.032 under light stress. r was 0.971 in UBQ10 in normal conditions, while under light stress, it was – 0.111.

Based on the cross-entropy algorithm RankAggreg, the comprehensive ranking of the ten candidate reference gene stability was obtained, which is shown in Fig. 4. The most stable reference gene was on the left in each graph, and the least stable reference gene was on the right. It can be concluded that ETI was the most stable reference gene in flower, leaf, and total samples in normal condition, while it became relatively unstable under light stress. GAPDH,

F-box, ACT and pp2A were the most stable genes under light stress.

Validation of the selected reference genes

Using the two most stable and least stable reference genes for stems in normal conditions, the expression of PAL and HCT was measured, which are two vital genes in the phenylpropanoid metabolic pathway. Figure 5 shows the PAL and HCT expression revealed by the transcriptome data, and the qPCR results, which used a combination of ACT and F-box or CYP. In the transcriptome data, the expression of PAL increased from the first to third stages, decreased to the least at fourth stage, and peaked at fifth stage. Using a combination of ACT and F-box as reference genes, the trend was consistent with the expression pattern revealed by the transcriptome data. HCT expression showed consistency between these two methods. However, using CYP as reference gene led to the wrong output; the peaks were shown in the totally different phases.

Fig. 4 Ranks for the ten candidate reference genes based on RankAggreg. **a–c, g** Samples in normal condition; **d–f, h** samples under light stress; **a, d** flowers; **b, f** leaves; **c, e** stems; **g, h** total samples. The gray lines represent the ranked results of geNorm,

NormFinder and BestKeeper, black lines refer to the means of the rankings, and the red lines are the comprehensive rankings for the ten candidate reference genes based on RankAggreg

Discussion

For each organ and environmental condition, the ranking of the stability of the candidate reference genes were obtained using three kinds of software. Due to algorithm differences, these sorting results have similarities, yet they were not identical. To objectively show the comprehensive ranking, the cross-entropy algorithm was used to integrate the three lists to obtain a comprehensive ranking. The results showed that F-box was almost always stable in stem and leaf samples under normal lighting condition and in flower, stem and leaf samples under weak light stress. Though F-box was not a traditional reference gene, it has been used in candidate reference gene screening in recent years, but the stability of F-box expression among different species varied greatly. The F-box family was not the most stable candidate reference gene in different tissues and stress conditions of *Artemisia sphaerocephala* (Hu et al. 2018). It was the most unstable candidate reference gene in *Fagopyrum esculentum* (Demidenko et al. 2011). GAPDH is a metabolically related gene and was one of the commonly used internal controls (Radonić et al. 2004). In this paper, under light stress condition, GAPDH was relatively stable. It has been indicated that GAPDH expression was stable in petals of three tree peony (*Paeonia suffruticosa*) cultivars (Li et al. 2016) and in different tissues of four

sugarcane cultivars (Iskandar et al. 2004), which was consistent with the results of this paper.

As mentioned previously, light stress can weaken the flower color and affect erectness of stem and leaf characteristics of *Paeonia lactiflora*. In this study, some candidate reference genes were instable under weak light stress. Light influenced the expression of UBQ10 in leaves and of E3 in flowers, making UBQ10 and E3 fall from the second most stable genes under normal conditions to last under light stress. However, ETI expression in flowers, F-box expression in leaves and stems, and pp2A expression in stems was not influenced by light. Thus, it can be concluded that the stability of the candidate reference genes differs among individuals, and it needs to be analyzed specifically. A previous study has indicated that gene stability may be different in other kinds of stress conditions. GAPDH and PGK, were the two most stable genes under light stress, but were the least two stable reference genes under nitrogen deficiency and low temperature conditions. Furthermore, the most unstable reference gene under a low intensity of light, EF1, was the most stable under two other kinds of stress (Løvdaal and Lillo 2009).

It is worth noting that there were obvious differences in rankings of reference gene stabilities among flower and other organ samples in this study. For example, ACT was the most unstable reference genes in a normal light

Fig. 5 Expression of PAL (a) and HCT (b) from the stem samples in normal light conditions according to reference genes F-box and ACT. The blue line represents the expression revealed by transcriptome data, the green line represents the expression revealed by CYP, and orange line represents the expression revealed by a combination of ACT and F-box

environment, but it performed well under light stress. GAPDH is a moderately stable gene in both environments. In the study of different flower tissues of *Taihangia rupestris*, it was found that when the flower organs were separated into sepals, petals, stamens and carpels, the order of the stability of reference genes was different from that of the whole flower organs (Li et al. 2017). Therefore, we speculated that because the flower sample used in this study was a mixture of the whole flower tissue, including four parts described previously, there was uneven mixing of various RNA results. In the future, it is vital to distinguish different parts of flower organs.

Conclusions

To the best of our knowledge, this was the first time reference genes in *Paeonia lactiflora* have been selected and validated under light stress conditions. Based on the four algorithms, 40 samples in total from three organs and two cultivars were used to analyze reference gene stability. F-box and GAPDH are two candidate reference genes that are stable in all the samples. In the two light conditions, ETI is the most stable in the flower samples, and ACT and pp2A are the most stable in stem samples. It is necessary to combine multiple reference genes to analyze qPCR data with pairwise variation analysis to get more reliable results.

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