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Research article

Selection and validation of reference genes for quantitative real-time PCR in different tissues of *Clematis lanuginosa* ☆

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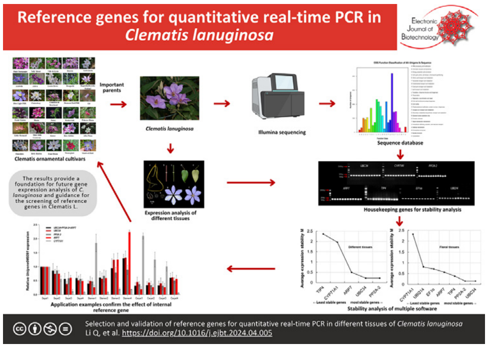
GRAPHICAL ABSTRACT

Reference genes for quantitative real-time PCR in *Clematis lanuginosa*



The results provide a foundation for future gene expression analysis of *C. lanuginosa* and guidance for the screening of reference genes in Clematis L.

Selection and validation of reference genes for quantitative real-time PCR in different tissues of *Clematis lanuginosa*
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ABSTRACT

The lack of reference genes makes it difficult to conduct molecular biology research on the plant genus, *Clematis* L. *Clematis lanuginosa* belongs to Sect. Viticella DC of *Clematis* L. It is also an important ornamental cultivated variety parent of the early and late large-flowered groups. Studying the reference genes of *C. lanuginosa* in different tissues will provide a theoretical basis for the reference selection of early and late large-flowered groups of *Clematis*, which could promote research progress on molecular biology of ornamental *Clematis*.

Results: The roots, stems, leaves, sepals, stamens, and carpels of *C. lanuginosa* were used as research materials, and seven candidate reference genes were used for quantitative real-time PCR analysis. Comprehensive stability analysis using geNorm, NormFinder, BestKeeper, and RefFinder software showed that suitable reference genes in *C. lanuginosa* root, stem, and leaf were *PP2A-2* and *UBC34*; and in floral tissue were *UBC34*, *PP2A-2*, and *ARP7*. These reference genes can be used as internal reference either alone or in combination. The pairwise variation value evaluated with geNorm software showed that two internal reference genes were needed for gene expression correction in the tissues. In the floral organs, three reference genes were required for gene expression correction.

Conclusions: Our results provide a foundation for future gene expression analysis of *C. lanuginosa* and guidance for the screening of reference genes in *Clematis*.

☆ Audio abstract available in Supplementary material.

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1. Introduction

Screening reference genes for *Clematis lanuginosa* will provide scientific evidence for quantitative analysis of the genes of *Clematis* cultivars. Floral tissue development and flower color are important agronomic traits of ornamental plants, and there are few studies on molecular characteristics of related traits in *Clematis* cultivars. Thus, it is necessary to study the expression regulation of genes related to these important traits, and it is meaningful for molecular mechanism research and molecular breeding of *C. lanuginosa*, early- and late-flowering *Clematis* cultivars, and even *Clematis* germplasm.

Quantitative real-time PCR (qRT-PCR) requires reference genes to correct expression differences both between and within samples. Housekeeping genes, frequently described as "essential for cellular existence regardless of their specific function", are often selected because of their high copy number and their expression level is rarely affected by the environment, or experimental factors [1,2]. In different organs of *Narcissus pseudonarcissus*, *ACT* (Actin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were expressed stably [3]; expressions of *SAND* (SAND family protein) and *PGK* (phosphoglycerate kinase) were stable in *Chrysanthemum lavandulifolium* [4]; *EF1α* (elongation factor 1-alpha) and *18S rRNA* were stably expressed in *Lilium brownii* [5]; and *Actin* was expressed stably in *Hibiscus hamabo* under different experimental conditions [6]. However, an increasing number of studies have shown that no gene has absolutely stable expression, and different cell types and experimental conditions may lead to differences in expression [7]. In our pre-experiment, we found that the reported *Actin* reference gene could not be stably expressed in tissues of *C. lanuginosa* and so could not be used as a reference gene. To solve this problem, it is necessary to seek new reference genes or simultaneously use several reference genes.

Based on transcriptome sequence data of *C. lanuginosa* [8] and *C. courtoisii* in previous studies, 25 reported reference genes were selected and 45 pairs of primers were designed and used in the pre-experiment. Finally, seven candidate reference genes selected in this experiment were *ARP7* (actin-related protein 7), *UBC34* (ubiquitin-conjugating enzyme E2 34-like isoform 1 34), *UBQ14* (Polyubiquitin 14), *CYP71A1* (Cytochrome P450 71A1), *EF1α* (Elongation factor 1-alpha), *TIP4* (Aquaporin TIP4-1), and *PP2A-2* (serine/threonine-protein phosphatase PP2A-2 catalytic subunit-like catalytic subunit). These included both traditional housekeeping genes and novel reference genes like *CYP71A1*. Detection and selection were conducted to provide a theoretical basis for qRT-PCR analysis of *C. lanuginosa*.

2. Materials and methods

2.1. Plant materials

The experimental material was *C. lanuginosa*. This was grown in the Germplasm Garden of Clematis, Institute of Botany, Chinese Academy of Sciences in Jiangsu Province. Roots, stems, and leaves were collected from individuals grown under natural conditions. Fleshy roots, green stems, and young leaves were selected. Fruit was collected when green. Flowers were collected at four periods: I, 33% of the buds were about to bloom; II, 66% of the buds were about to bloom; III, buds about to bloom; and IV, early flowering buds. All samples were quickly frozen in liquid nitrogen and then stored at −80°C.

The *C. lanuginosa*. was identified by botanist Dr. Shuan Wang from the Research Center of Ornamental Plants, Institute of Botany, Jiangsu Province and Chinese Academy of Science. The voucher specimen was deposited in the herbarium at the Institute of Botany, Jiangsu Province and Chinese Academy of Science. The certificate number was 000736328.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted with a Quick RNA Isolation Kit 3.0 (Waryong, Nanjing, China). The RNA integrity, concentration, and purity were checked by 1% agarose gel electrophoresis and spectrophotometer (JS-680B, P & Q Science & Technology, Shanghai, China). The 28S and 18S bands were clear, and the criteria of 260/280 nm and 260/230 nm ratios were 2.13–2.25. First-strand cDNA was synthesized based on 1 μg of RNA by HiScript® III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, Nanjing, China).

2.3. Selection of candidate reference genes and qRT-PCR primer design

Based on transcriptome sequence data of *C. lanuginosa* [8] and *C. courtoisii* in previous studies, 25 reported reference genes (**Table S1**) were selected in a pre-experiment. Finally, seven genes with relatively stable expression were selected as candidates: *ARP7*, *CYP71A1*, *EF1α*, *PP2A-2*, *TIP4*, *UBC34*, and *UBQ14*. Primer pairs were designed using Beacon Designer 8.0 software according to the design requirements of primers for qRT-PCR, and synthesized by Tsingke Biotechnology Co., Ltd. (Table 1).

2.4. PCR and qRT-PCR amplification

The PCR was performed using a 2 × Taq PCR Mastermix II PCR Amplification Kit (Tiangen, Nanjing, China). The product's length and the specificity of the primers were then determined using 2% agarose gel electrophoresis. The qRT-PCR was carried out according to the instructions of the fluorescence quantitative kit (TAKARA, Beijing, China), and each reaction was repeated three times to reduce the error caused by sample addition.

2.5. Stability analysis and validation of candidate reference genes

The expression stability of seven candidate reference genes was evaluated using geNorm, NormFinder, and BestKeeper software [7], and the stability values and the numbers of suitable reference genes were compared. Finally, RefFinder software was used to comprehensively evaluate the candidate reference genes [9].

Gene *Unigene046091* (annotated as *AGL8*) was selected and its expression pattern was assessed in different floral tissues. The $2^{-\Delta\Delta Ct}$ method was used to analyze the expression of *Unigene046091*. The *Unigene046091* specific primer pair used was forward 5′-GTTTCCACACCTCTTTGTCCTAT-3′ and reverse 5′-TTATTCTCATCCTCAAGTGCCCT-3′.

Table 1
Seven candidate reference genes and primer sequences.

Gene symbol	Gene ID	Gene name	Primer Sequence (5′-3′)	Amplicon length (bp)
ARP7	CL228.Contig1_Maoye	Actin-related protein 7	F:TGCTCATCTTCTATCCGTCCTT; R:TCATCGTACTCTGCCTTGGT	149
CYP71A1	CL307.Contig4_Maoye	Cytochrome P450 71A1	F:TCCGCCTAAACTGCCGATTA; R:AGCCATCTCTGCCGATTGTA	151
EF1α	Unigene023234	Elongation factor 1-alpha	F:GGCAGCAGACTTGGTGACCTTAG; R:GAGTATCCACCGCTTGGTCGTT	120
PP2A-2	CL393.Contig3_Maoye	Serine/threonine-protein phosphatase PP2A-2 catalytic subunit-like catalytic subunit	F:GGTGAGGTGAAGTCGTTA; R:ATTGGTGTCAGGAGCATT	162
TIP4	Unigene17471_Maoye	Aquaporin TIP4-1	F:ATAAGGGAGACATTGGGGGACT; R:TCGGTCCAGACTCCGCTTATC	145
UBC34	CL3948.Contig1_Maoye	ubiquitin-conjugating enzyme E2 34-like isoform 1 34	F:CTGAGAAGCAGCGGTTAGC; R:AGGTGATGTCTGATGTGGAGAT	179
UBQ14	Unigene11235_Maoye	Polyubiquitin 14	F:TGGTGGTAGTTGGCTTGAGA; R:TGACGCTGAGGATGGTGT	176

3. Results

3.1. Specificity of primer and expression of candidate reference genes

Gel electrophoresis indicated that the primer amplification of candidate reference genes in each sample had only a single band (**Fig. S1**), and the primer specificities of the seven reference genes were revealed using melting curves that only had a single obvious peak, indicating that the primer pairs had good specificity (**Fig. S2**).

The average expression levels of the five candidate reference genes in tissues (roots, stems, leaves, sepals, stamens, carpels, and fruits) ranged between 21.72 and 31.07 (Fig. 1a). If the Ct value of a gene is low, the gene is highly expressed under the condition. Gene *UBC34* had a greater expression quantity with an average of fewer than 23.64 cycles (Fig. 1a). The changes in *ARP7*, *UBC34*, *ARP7*, and *CYP71A1* were all within five cycles (Fig. 1a), meaning that their expression was relatively stable, while the variation range of *TIP4* was greater than five cycles (Fig. 1a), indicating that its expression was not stable.

Among the 12 tested floral tissues, the average expression levels of seven candidate reference genes ranged between 17.17 and 27.47 (Fig. 1b): *EF1α* had the highest expression abundance, while *CYP71A1* had the lowest. The variation range of *CYP71A1* was greater than five cycles (Fig. 1b), indicating that its expression in floral tissues was not stable.

3.2. Ranks of candidate reference genes

Commonly used geNorm, NormFinder, and BestKeeper software were used to analyze the stability of seven candidate reference genes, and RefFinder software was used to comprehensively analyze the stability of each gene.

In geNorm, the expression stability value (*M*) of each candidate reference gene can be calculated, and the calculated pairwise variations (*V*) of reference genes can determine the appropriate number of parameter genes. The *M* values of *PP2A-2* and *UBC34* were the smallest for all tissues (roots, stems, leaves, sepals, stamens, carpels, and fruits) (Fig. 2), indicating that these two genes had the highest expression stability; while the *M* value of *TIP4* was greater than 1.5, indicating unstable expression. Genes *PP2A-2* and *UBC34* were still the most stable genes in floral tissues. Pairwise variation analysis showed that the $V_{2/3}$ value was 0.15 in all tissues, indicating that only two reference genes were required. The $V_{3/4}$ value was less than 0.15 in floral tissues, indicating that three reference genes were required (Fig. 3).

The ranks of the seven candidate reference genes were gained by NormFinder according to stability values. Gene *PP2A-2* ranked first in all tissues (Fig. 4). Gene *CYP71A1* ranked in the bottom two in all groups, suggesting that its expression was not stable in *C. lanuginosa*.

The expression stability value of candidate reference genes was also calculated using BestKeeper. Moreover, the software can compare the values of standard deviation (SD) and the coefficient of variation to determine the stability of reference genes. When SD > 1, the expression of the reference gene is unstable. The analysis results for BestKeeper software (Fig. 5) showed that the SDs of *CYP71A1* and *TIP4* were greater than 1 in all tissues, suggesting that these two genes were unstable. In floral tissues, *UBC34* and *EF1α* had SDs $\geq$ 1, showing that their expression was not stable. The software RefFinder was used to comprehensively evaluate the stability of the seven candidate reference genes. The expression stability values of *UBC34* and *PP2A-2* were the top two in roots, stems, leaves, fruits, and floral tissues Genes *PP2A-2* and *UBC34* were selected as the reference genes required for gene expression correction in all tissues. In floral tissues, three reference genes were required, and *UBC34*, *PP2A-2*, and *ARP7* were chosen (Fig. 6).

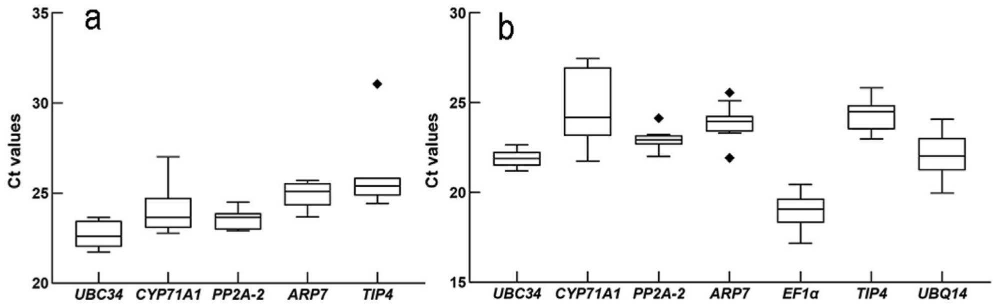

Fig. 1. Box-plot of Ct values: a, roots, stems, leaves, sepals, stamens, carpels and fruits; b, sepals, stamens and carpels at different development stages.

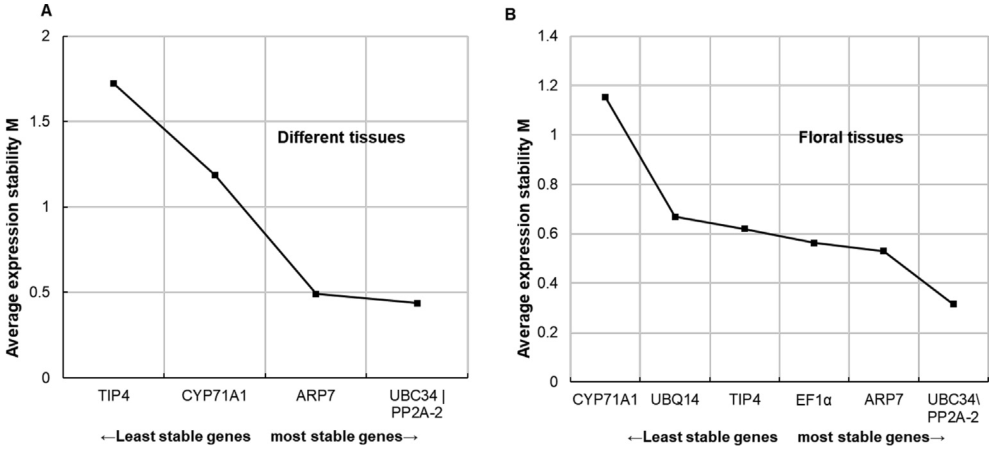

Fig. 2. Average expression stability M evaluated by geNorm.

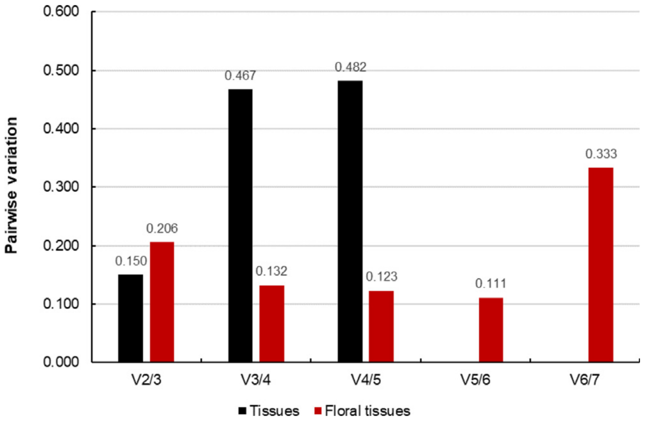

Fig. 3. Pairwise variation evaluated by geNorm. The pairwise variation (Vn/Vn + 1) was analyzed between the normalization factors (NFs, NF_n and NFn + 1) using geNorm software, where n is the number of genes involved in the NF. Once the pairwise variation is below 0.15, no additional genes are required for normalization.

3.3. Validation of the selected reference genes

The expression level of the target gene *Unigene046091* gradually decreased in sepals and gradually increased in stamens and pistils with growth and development, when using *UBC34*, *PP2A-2*, and *ARP7* as reference genes. Moreover, the target gene was more highly expressed in sepals for periods I and II. This was consistent with the mechanism of *Unigene046091* regulating the growth and development of floral tissues. However, the expression levels of the target genes differed when *CYP71A1* was the reference gene (Fig. 7). Therefore, *UBC34*, *PP2A-2*, and *ARP7* could be used as reference genes to study the expression of related functional genes in floral tissues of *C. lanuginosa*.

4. Discussion

The expression level of reference genes will vary greatly due to different materials, tissues, and experimental conditions because they not only participate in cell metabolism but also perform other functions at the same time; and reference genes filtered out in one experiment do not necessarily work in another [2]. For example, Casas et al. [10] found that the expression level of the same gene in-vivo and ex-vivo varies greatly. Therefore, before the expression analysis of functional genes, it is necessary to analyze the stability of the reference genes, which is important for obtaining more accurate experimental results.

The choice of reference gene greatly affects the reliability of RT-qPCR data [11]. Due to the different algorithms of the three software types, the results obtained by each software will slightly differ [12]. For example, in *C. lanuginosa* floral tissues, the most stable gene evaluated by NormFinder software was *PP2A-2*, while the most stable genes evaluated by geNorm software were *PP2A-2*

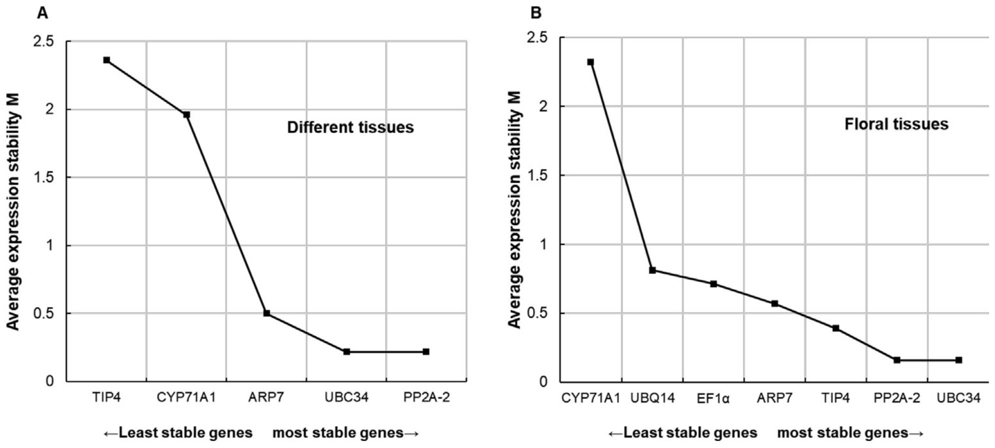

Fig. 4. Average expression stability M evaluated by NormFinder.

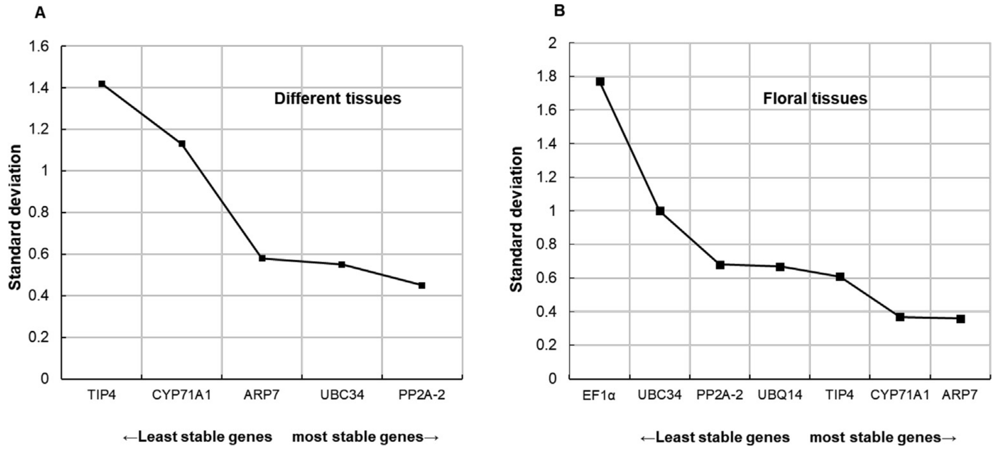

Fig. 5. Standard deviation evaluated by BestKeeper.

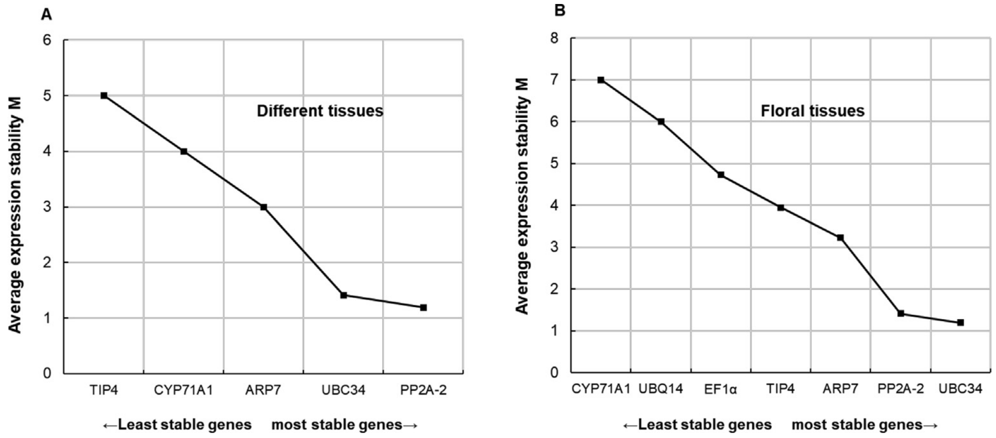

Fig. 6. Average expression stability M evaluated by RefFinder.

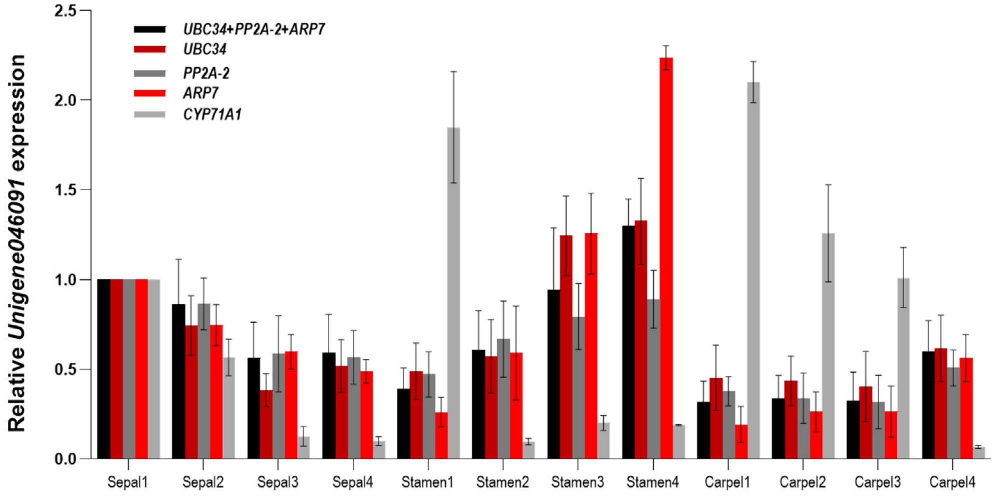

Fig. 7. Expression analysis of *Unigene046091* and *CL715.Contig6_Maoye* ($p \leq 0.05$).

and *UBC34*. Hence, the analysis results of geNorm, NormFinder, and BestKeeper needed to be fully considered. Finally, a comprehensive evaluation was carried out using RefFinder software.

Although no reference gene was expressed most stably under all experimental conditions, *UBC34* and *ARP7*, as non-traditional housekeeping genes, were relatively stable in different tissues of *C. lanuginosa* in this study. Thus, they are good choices for studies of *C. lanuginosa* gene expression.

Gene *ARP7* is actin-related. In previous studies, *Actin* was often used as a reference gene, but reports on the selection of *ARP* genes are rare. Because actin encoded by *Actin* is ubiquitous and very important in eukaryotes, it was widely used as a reference gene in the study of functional genes of many plants such as *Hibiscus hamabo* [6]. However, in this study, *Actin* was not stable, and expression of *ARP7* was relatively stable in the roots, stems, leaves, fruits, and floral tissues of *C. lanuginosa*.

Gene *UBC34* encodes a ubiquitin-conjugating enzyme. According to previous studies, during the fruit development of *Euscaphis konishii*, suitable reference genes were *UBC23*, *CYP38*, and *GAPDH2* [13]. In *Cryptomeria japonica* var. *sinensis*, *CYP*, *Actin*, *UBC*, and *18S* were suitable reference genes [14]. Under different biotic and abiotic stresses, both *UBC17* and *18S* were expressed stably in *Psathyrostachys huashanica* [15]. Expression of *UBC34* was relatively stable in root, stem, leaf, fruit, and floral tissues of *C. lanuginosa*, similar to the results of these previous studies.

Gene *AGL8* (AGAMOUS-LIKE 8), a member of the MADS-box gene family, is homologous to *AP1*, belongs to the *SQUA* (*AP1*) subfamily gene along with *AP1*, and is a class A functional gene [16]. Previous studies found that *AGL8* acts on the inflorescence apical meristem to affect flowering in *Arabidopsis thaliana* [17]. Gene *AP1* can regulate flower meristem characteristics of *A. thaliana* in the early stage of flower development. With the further development of floral tissues, *AP1* regulates the growth and development of the outermost two rounds of tepals [18]. In our study, when *UBC34*, *PP2A-2*, and *ARP7* were used as reference genes, the expression level of *Unigene046091* in sepals at stages I and II of flower buds was higher than that in stamens and carpels; however, when *CYP71A1* was used as a reference gene, the analysis results were opposite and did not match the expression pattern of *Unigene046091*.

Author contributions

Study conception and design: Linfang Li and Ya Li.
Draft manuscript preparation: Qiao Li.
Analysis and interpretation of results: Qiao Li.
Material collection: Shuan Wang, Yongdong Liu.
Data analysis: Fenni Lv, Sumei Li, Lulu Gao and Peng Wang.
Revision of the results and approval of the final version of the manuscript: Linfang Li.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

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