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Transcriptome-based validation of proper reference genes for reverse trascription quantitative PCR analysis of *Sinocalycanthus chinensis*

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Abstract

Reverse transcription quantitative PCR is a widely used method to detect gene expressions. To obtain accurate expression results, the selection of proper reference genes is important and necessary. However, related works concerning reference gene selection have not been carried on many plant species, especially endangered ones. The aim of the present study was to select dependable reference genes for expression normalization of an endangered plant species with medicinal and ornamental value: *Sinocalycanthus chinensis* (*Calycanthaceae*). Nine reference genes with stable expressions were chosen for further analysis according to transcriptomic sequencing data from *S. chinensis*. The expression stability of these candidate genes in inner and outer petals at different floral developmental stages and in many different tissues was then analyzed with the *geNorm* and *NormFinder* software. The reference genes were evaluated by normalizing the expression of the *anthocyanidin synthase* gene in the outer and inner petals at different floral developmental stages to further verify the expression stability of these genes. *Elongation factor 1-alpha* (*ScEF*) and *50S ribosomal protein L27* were found to be the two most stable genes in the overall ranking of all the samples and different tissues. Furthermore, *ScEF* and *protein phosphatase 2A* were stably expressed in all petal samples. Moreover, among the nine candidate reference genes, *phosphoglycerate kinase* performed poorly in all sample sets. Our results will help to obtain reliable expression data in molecular studies of *S. chinensis*.

Additional key words: anthocyanidin synthase, floral developmental stages, *geNorm*, *NormFinder*, petals.

Introduction

Sinocalycanthus chinensis is an endangered plant species in China that has high medicinal and ornamental value (Cheng and Chang 1964). *S. chinensis* bears large creamy white flowers with a whorl of yellow inner petals, sometimes with pink to purplish around the edges of the outer petals.

Plant petal coloration is caused by the accumulation of pigments such as flavonoids, carotenoids, or betacyanins (Tanaka *et al.* 2008). Biosynthesis of related pigments is likely responsible for the difference in petal coloration between the outer petals and inner petals. However, at present, the factors that regulate the petal coloration of *S. chinensis* are still unclear. Therefore, investigating the expression of genes involved in the biosynthesis of related pigments will help elucidate the underlying mechanisms of petal coloration in *S. chinensis*.

Reverse transcription quantitative PCR (RT-qPCR) is the most reliable technology to detect gene expression because of its high accuracy and sensitivity (Gachon *et al.* 2004, Bustin *et al.* 2005). With respect to RT-qPCR, a suitable reference gene is crucial for gene expression analysis. Actin (*ACT*), beta-tubulin (*TUB*), 18S ribosomal RNA (*18S*), and other traditional genes are often employed as reference genes. However, the expression performance of reference genes is quite different in different plant species or under different experimental conditions. For

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Abbreviations: ACT - actin; ANS - anthocyanidin synthase; Cp - crossing point; EF - elongation factor 1-alpha; EIF - eukaryotic initiation factor; M value - gene expression stability measure; PGK - phosphoglycerate kinase; PP2A - protein phosphatase 2a; PV - pairwise variation; RPL - 50S ribosomal protein L27; RT-qPCR - reverse transcription quantitative PCR; TUB - beta-tubulin; UBC - ubiquitin-conjugating enzyme .

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instance, *18S* is considered a dependable reference gene in developing *Rhododendron molle* flowers (Xiao *et al.* 2016), but this gene is not recommended as a suitable reference gene in *Osmanthus fragrans* (Zhang *et al.* 2015).

To obtain accurate expression results, the selection of proper reference genes under specific research conditions is essential for the normalization of RT-qPCR data. Therefore, in this study, based on the expression data of four transcriptomes of *S. chinensis*, nine reference genes whose expression changed little were chosen: *S. sinensis actin 1* (*ScACT1*), *ScACT2*, *elongation factor 1-alpha* (*ScEF*), *eukaryotic initiation factor* (*ScEIF*), *phosphoglycerate kinase* (*ScPGK*), *protein phosphatase 2a* (*ScPP2A*), *50S ribosomal protein L27* (*ScRPL*), *ScTUB,* and *ubiquitin-conjugating enzyme* (*ScUBC*). The expression performance of these nine genes was then analyzed in different developmental stages of petals and different tissues. The *geNorm* (Vandesompele *et al.* 2002) and *NormFinder* (Andersen *et al.* 2004) were employed to evaluate expression stability in different experimental sample sets of *S. chinensis*. After stability assessment, the reference genes with good or poor performance were further used to normalize the expression of an *S. chinensis* anthocyanin synthase gene, *ScANS,* in the outer and inner petals at different floral developmental stages.

Materials and methods

Plants: *Sinocalycanthus chinensis* W.C. Cheng & S.Y. Chang (*Calycanthaceae*) plants that were grown in the Wintersweet Garden of Zhejiang Agriculture and Forestry University were used for sample collection. The flower opening of *S. chinensis* could be divided into six developmental stages: stage 1 (S1; tight buds, 5 - 7 mm in diameter), S2 (tight buds, 12 - 15 mm in diameter), S3 (soft bud stage), S4 (initial flowering stage), S5 (half flowering stage), and S6 (full flowering stage). The outer and inner petals of flowers at six developmental stages were collected and constituted the samples of all petal sample sets. Additionally, ten different tissue types were collected: pedicels, sepals, outer petals, inner petals, both the androecia and gynoecia from flowers at S6, young leaves, mature leaves, young stems, and mature stems. In total, 22 experimental samples comprised 12 petal samples and ten different tissue samples.

Extraction of RNA and first-strand cDNA synthesis: Total RNA was extracted from all samples using a *MiniBEST* universal RNA extraction kit (*TaKaRa*, Dalian, China). The concentration and quality of RNA samples were measured using a *NanoDrop ND-1000* spectrophotometer (*NanoDrop Technologies*, Rockland, DE, USA). Samples of RNA with absorbance ratios A_{260}/A_{280} of 1.8 - 2.2 and A_{260}/A_{230} >1.8 were used for cDNA synthesis. One microgram of total RNA was used to synthesize the first-strand cDNA with the reverse transcriptase *M-MLV* (*TaKaRa*).

Selection of candidate reference genes: Four cDNA libraries were constructed from the outer and inner petals of *S. chinensis* at S2 and S4 stages and sequenced *via* the *Illumina HiSeq 2000* platform. In total, 125 014 unigenes were assembled and identified. Additionally, the FPKM (fragments per kilobase per million reads) method was used to calculate the unigene expression. The differences in gene expressions among the samples were determined using the FPKM value. Nine reference genes (*ScACT1*, *ScACT2*, *ScEF*, *ScEIF*, *ScPGK*, *ScPP2A*, *ScRPL*, *ScTUB*, and *ScUBC*) were chosen as candidate reference genes in *S. chinensis* because their expression was stable (1/1.5 < fold change <1.5) between any two samples (Table 1 Suppl.).

Amplification efficiency test of primers: The primers for the nine candidate reference genes with amplicon lengths of 87 - 180 bp are shown in Table 2 Suppl. The amplification efficiency of each primer pair was tested as follows: the slope of the standard curve obtained by serial 5-fold dilutions of the cDNA samples was used to calculate the amplification efficiency as:
$E\ [\%] = [-1 + 10\ (-1/\text{slope})] \times 100$.

RT-qPCR was performed using a *LightCycler 480 II* (*Roche*, town?, Switzerland) with *SYBR Premix Ex Taq* (*TaKaRa*). The reaction mixture (20 mm^3 total volume) consisted of 10 mm^3 of *SYBR Premix Ex Taq*, 0.8 mm^3 of each primer (10 μM), 2 mm^3 of cDNA (~50 ng), and 6.4 mm^3 of ddH_2O. The amplification was performed under the following conditions: an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Afterward, a melting curve was obtained. Crossing point (Cp) values of the triplicate reactions were then obtained.

Expression stability analysis: The expressions of the nine genes were determined according to Cp values, and the expression stability was analyzed by *geNorm* (*v. 3.5*) and *NormFinder* (v. 0.953) algorithms. Before their input into this two software programs, the raw Cp values were transformed into relative quantities (Q values) using the following formula: $Q = 2^{-\Delta Cp}$ (ΔCp = Cp value of each sample - the minimum Cp value).

The *geNorm* software determines gene expression stability based on the gene expression stability measure (M value). Additionally, pairwise variation (PV) values were used to choose the optimal number of reference genes (Vandesompele *et al.* 2002). The *NormFinder* software ranks the reference genes by the expression stability of the candidate genes in a certain sample set (Andersen *et al.* 2004).

Expression normalization of *ScANS*: As a target gene, *ScANS* was employed to indicate the usefulness of the selected candidate reference genes. The expression of *ScANS* in the outer and inner petals at different developmental stages was quantified with the three most stable reference genes (*ScEF*, *ScPP2A*, *ScRPL*), the least stable reference gene (*ScPGK*) and a combination of reference genes (*ScEF*+*ScPP2A*) based on the

overall ranking calculated by *geNorm* and *NormFinder*. Primers (5′-GTGTTCGGGCTGTCGGTAAG-3′ and 5′-ACTGGCATTGTTGGCGAGCT-3′) were used to amplify *ScANS.*

Results

Based on the melting curve, only a single peak was observed in the template, while there was no amplicon in the negative control for each gene pair (Fig. 1 Suppl.). In addition, the PCR efficiencies varied from 95.4 % for *ScPP2A* to 101.0 % for *ScTUB*, and the R^2 values ranged from 0.9975 for *ScEIF* to 0.9997 for *ScEF* (Table 2 Suppl.), which indicates that the primers could be used for further gene expression stability with RT-qPCR.

A box plot (Fig. 1) was constructed that gives an overview of the raw Cp values of the nine candidate reference genes in the tested samples. The raw Cp values ranged from 12.51 to 25.85 (Fig. 1). The *ScPP2A* showed the highest average Cp value (21.25), followed by *ScRPL*, and *ScEF* exhibited the lowest mean Cp value (15.23). Furthermore, the Cp value variation was quite different for each gene (Fig. 1). For example, the Cp value variation of *ScPP2A* was below 6 cycles with stable gene expression, while that of *ScPGK* was above 9 cycles with obvious expression variation.

The *geNorm* ranked the candidate genes according to their M values because the genes showed the most stable expression with the lowest M values (Table 3 Suppl.). The M value of 1.5 is usually recommended as a threshold to identify stable reference genes (Vandesompele *et al.* 2002). All M values of the nine reference genes were lower than 1.5 in the four sample sets (Table 3 Suppl.), revealing that all nine genes met the basic reference gene requirements.

The *ScEF* and *ScRPL* were the most stable genes with the lowest M value (0.534; Table 3 Suppl.) for all 22 samples, followed by *ScPP2A*, with the M value of 0.619. Similarly, *ScEF* and *ScRPL* were also the most stable genes in the different tissue samples, followed by *ScPP2A* (Table 3 Suppl.). In the petal sample set, *ScRPL* and *ScPP2A* were the most stable reference genes, followed by *ScEF*. Generally, across all sample sets, *ScEF*, *ScRPL*, and *ScPP2A* were the three most stable reference genes. In addition, *ScPGK*, with the highest M value in all sample sets, was considered the least stable gene (Table 3 Suppl.).

A pairwise variation (V_n/V_{n+1}) of 0.15 is recommended as a threshold to determine the optimal number of reference genes. The V2/3 values of all petals were less than 0.15 (Fig. 2), indicating that the top two reference genes were sufficient for expression normalization. For all samples and different tissues, the V2/3 and V3/4 values were higher than 0.15, but the V4/5 values were lower than 0.15, suggesting that a fifth reference gene was not required to normalize the gene expression and that the top four reference genes were needed for accurate normalization.

The *ScEF* was the most stable gene ranked by *NormFinder* for all samples, including all petal samples and different tissue samples (Table 4 Suppl.). The ranking order of gene stability calculated by *geNorm* and *NormFinder* was the same in the sample set of different tissues and was similar in all petal samples. Regarding the least stable gene, *ScPGK* was ranked last out of the nine candidate reference genes in all sample sets, which is consistent with the results determined by *geNorm*.

The overall ranking with the aggregate results calculated by *geNorm* and *NormFinder* (Table 5 Suppl.) showed that *ScEF* and *ScRPL* were the most stable genes in the overall ranking of all the samples and different tissues. In addition, *ScEF* and *ScRPL* were the most stably expressed genes based on the overall ranking calculated by *geNorm* and *NormFinder* in all petal samples. On the other hand, *ScPGK* was the least stable gene out of all reference genes in all the sample sets.

The three most stable reference genes (*ScEF*, *ScPP2A*, and *ScRPL*), the least stable reference gene (*ScPGK*) and a combination of reference genes (*ScEF*+*ScPP2A*) were used as internal controls for normalization of *ScANS* according to the overall ranking (Table 5 Suppl.) calculated by *geNorm* and *NormFinder*. The expression

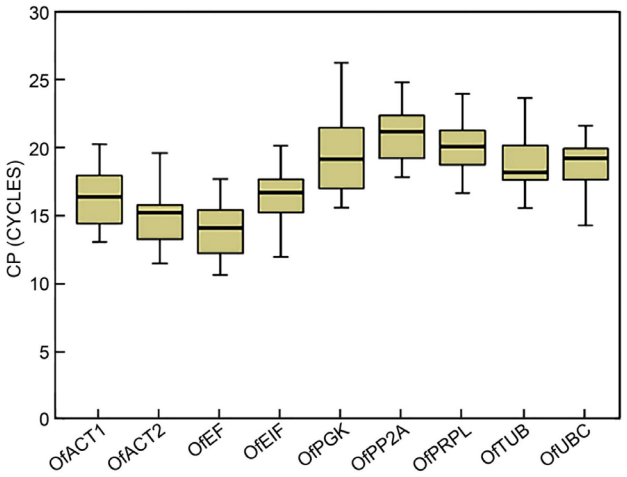

Fig. 1. Expression profiles of nine candidate reference genes from 22 samples. The expression data are displayed as Cp (crossing point) values for each reference gene in all samples. The line across the box is the median, n = 22. The *boxes* indicate the 25/75 percentiles. The *whisker caps* indicate the minimum and maximum values.

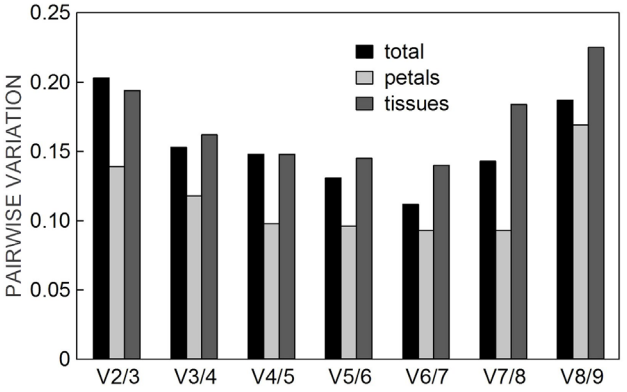

Fig. 2. Pairwise variation (PV, $V_{n/n+1}$) analysis of nine candidate genes in three sample sets by *geNorm*.

patterns of *ScANS* in the outer petals and inner petals at different developmental stages were similar when *ScEF*, *ScPP2A*, *ScRPL*, and a combination of reference genes (*ScEF*+*ScPP2A*) were used for normalization (Fig. 3). However, the expression of *ScANS* with *ScPGK* as an internal control was greatly different, revealing that *ScPGK* performed poorly as a reference gene in all sample sets.

Discussion

Transcriptome sequencing is an excellent technology that can help obtain additional gene information and reveal the complexity of the transcriptomes of many species (Ma *et al.* 2016, Ning *et al.* 2016, Zhang *et al.* 2016). Nine reference genes whose expression changed little in *S. chinensis* transcriptomes were chosen as candidate reference genes in the present study. The expression of these candidate reference genes was tested in outer petals, inner petals, and all petals at different floral developmental stages and in different tissues by RT-qPCR and evaluated using statistical algorithms.

Among the different statistical algorithms, *geNorm* (Vandesompele *et al.* 2002) was the first choice to be used for the evaluation of the stability of candidate reference genes, followed by *NormFinder* (Andersen *et al.* 2004). In our study, the gene stability rankings generated by *geNorm* and *NormFinder* were the same in the sample set of different tissues; however, they were inconsistent in terms of the total samples and all petals (Table 3 Suppl. and Table 4 Suppl.), which can be explained by the fact that the two algorithms are based on different principles (Vandesompele *et al.* 2002, Andersen *et al.* 2004). However, the last ranking order by the two methods was identical in all sample sets. Similarly, based on the rank, the most unstable gene was also the same in many other plant species when different statistical algorithms were used (Fu *et al.* 2013, Zhang *et al.* 2015, Xiao *et al.* 2016). In addition, the two most stable genes generated by the two methods were identical in most sample sets.

With *geNorm* software, a cutoff PV value of 0.15 is recommended (Vandesompele *et al.* 2002). The top four reference genes were required for expression normalization in all samples and different tissues (Fig. 2). In fact, expression normalization with additional reference genes could generate better results, and a PV value of 0.15 is not a necessary criterion (Vandesompele *et al.* 2002). In previous studies, although *geNorm* recommended that more than two genes were needed for optimal results, the top two reference genes were sufficient to obtain accurate expression of interesting genes in *Salix matsudana* (Chen *et al.* 2017) and *Sorghum bicolor* (Sudhakar Reddy *et al.* 2016). Similarly, in our study of *S. chinensis*, the top two reference genes were sufficient for accurate normalization in all samples and different tissues. In other words, *ScEF* and *ScRPL* were proper reference genes used for accurate normalization in all samples and different tissues.

ScEF was an optimal reference gene for all samples, all petals, and different tissues based on the overall rankings calculated by *geNorm* and *NormFinder* (Table 5 Suppl.). *ScEF* is a homolog of the *EF* gene. The *EF* is the most stably expressed gene in different tissues of Chinese cabbage (Qi *et al.* 2010) and tobacco (Schmidt and Delaney 2010). In addition, *EF* is considered the most stable gene not only in flower development but also in the leaf development of 'Mitchell' petunia (Mallona *et al.* 2010) and during the different fruit developmental stages of *Litsea cubeba* (Lin *et al.* 2013).

The *PP2A* was another optimal reference gene for all petals. In cineraria, the expression of *PP2A* was the least variable in developing flowers (Jin *et al.* 2013). In addition, *PP2A* was the third most suitable reference gene for different tissues in *S. chinensis*, and *PP2A* was the most stable gene in all the sample sets of *Sorghum bicolor*, including in different tissues (Sudhakar Reddy *et al.* 2016).

In our study, *ScRPL* was recommended as one of the optimal reference genes for all samples, inner petals and different tissues. Ribosomal proteins are integral to ribosome biogenesis and function in protein synthesis (Szakonyi and Byrne 2011). Thus, homologs of the *RPL*

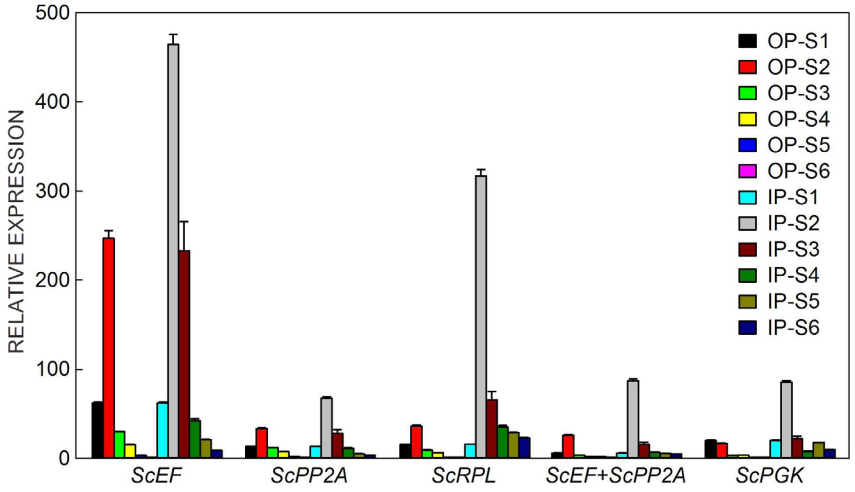

Fig. 3. Relative quantification of *ScANS* expression in outer and inner petals at different developmental stages using different reference genes (*ScEF*, *ScPP2A*, *ScRPL*, and *ScPGK*) and a combination of reference genes (*ScEF*+*ScPP2A*). Means ± SDs, $n = 3$.

gene are often found to serve as suitable reference genes in other plant species, such as wheat (Liu *et al.* 2014), tomato (Løvdal and Lillo 2009), *Theobroma cacao* (Pinheiro *et al.* 2011), and *Rhododendron molle* (Xiao *et al.* 2016). In developing flowers and different tissues of *R. molle*, the *RPL* gene was recommended for the normalization of RT-qPCR data (Xiao *et al.* 2016).

In conclusion, our research reports on the transcriptome-based validation of proper reference genes for RT-qPCR expression normalization in petals at different floral developmental stages and in different tissues of *S. chinensis*. *geNorm* and *NormFinder* were employed to determine the most suitable reference genes. Overall, *ScEF* and *ScRPL* were the two most stable genes in the overall ranking of total samples and different tissues, and *ScEF* and *ScPP2A* were the most stably expressed genes in all petal samples. *ScPGK* performed poorly in all sample sets.

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