Transcriptome-based screening and the optimal reference genes for real-time quantitative PCR in Rehmannia chingii and R. henryi

X. Zuo, F.-Q. Wang*, X.-R. Li, and M.-M. Li

College of Agronomy, Henan Agricultural University, Zhengzhou 450002, P.R. China

Abstract

Real time quantitative PCR (qPCR) is a powerful tool for studying the expression of specific genes. The accuracy and reliability of qPCR analysis data require the selection of reference genes with stable expression. However, the reference genes that can be used for qPCR of Rehmannia chingii and R. henryi have not yet been identified. In this study, based on the transcriptome data of R. chingii and R. henryi, we initially selected genes with relatively stable expression in different samples. We screened six candidate reference genes in R. chingii and R. henryi and calculated their expression abundance by real time qPCR. Their expression stability was evaluated by three algorithms geNorm, NormFinder, and BestKeeper. Although the results obtained by different algorithms were not completely consistent, R. chingii type 2A phosphatase activator TIP41 and R. chingii 18S ribosomal RNA had the highest expression stability in six different samples of R. chingii, and R. henryi 18S ribosomal RNA and R. henryi actin showed the most stable expression in different samples of R. henryi. In addition, based on transcriptome data, four genes were screened in R. chingii and R. henryi, and the expression stability of the selected reference genes was further verified. This study laid the foundation for further analysis and verification of the functions of important genes in R. chingii and R. henryi.

Additional key words: BestKeeper, gene expression stability, geNorm, NormFinder.

Introduction

Real time quantitative PCR (qPCR) is an important method for studying the transcription and expression patterns of target genes. It has the advantages of accurate quantification, high sensitivity, high throughput, and good reproducibility (Bustin 2000, 2002, Bustin et al. 2005). The premise of qPCR for accurate correction and standardization of target gene expression is to screen out the reference gene (Huggett et al. 2005, Kozera and Rapacz 2013), and then to calculate the relative expression of a target gene by \(2^{\Delta\Delta Cq}\) method. This method is of the great significance for the study of gene expression regulation and biological functions. However, the stability of the reference gene directly affects the quantitative results of the target genes, and the relative stability of the reference gene in different species, tissues at different developmental stages, and under different experimental conditions is not constant and universal (Gutierrez et al. 2008). Therefore, screening and determining the most suitable reference gene with relatively stable expression characteristics according to the research materials are the prerequisites to ensure the accuracy and reliability of qPCR results.

Diploid (n=28) Rehmannia chingii and R. henryi are two wild relatives of tetraploid (n=56) R. glutinosa. Zheng (2014) and Liu (2016) isolated eight new chemical components and nine iridoid compounds from the shoots and the whole plant of R. chingii. In addition, Zhou (2019a,b) isolated thirteen new iridoid compounds and an ionone glycoside from R. henryi. Among them, iridoid compounds have important biological activities in anti-inflammatory, anti-tumour and neuroprotection. R. chingii and R. henryi have obvious differences in appearance, especially the shape of flowers. Among them, the R. chingii flower is large and gorgeous, the corolla is fuchsia, there are yellow oil purple spots in the corolla tube, while the corolla of R. henryi is pale yellow-white, which is often used as one of the important characteristics to distinguish R. henryi. Different flower colours can attract insects to spread pollen, reflect light, and produce certain cell protection effects. Moreover, the material basis for the formation of different flower colours is mainly flavonoid compounds, which have important biological activities in anti-inflammatory, anti-tumour and neuroprotection.
compounds such as anthocyanins and carotenoids, which have great development and application value. However, in R. chingii and R. henryi, the biomass of the leaves is the largest, and the flowers are also one of the important tissues in the aerial part. It can be seen that further research on the flowers and leaves of R. chingii and R. henryi is of great significance.

The analysis of gene expression is the basis of our further study of genes function and metabolic regulation network at the molecular level. Real time qPCR can further verify the accuracy and reliability of transcriptome results by measuring gene expression. However, there are no reports about the selection and determination of reference genes in R. chingii and R. henryi, which will limit further research. The use of transcriptome data has the advantages of high efficiency and ideal results for screening reference genes. Based on the transcriptome results, we tried to select genes with relatively high and stable fragments per kilobase of transcript per million fragments mapped (FPKM) values from different samples of R. chingii and R. henryi, combined with gene annotation and identified common reference genes in plants as candidates. They are Actin, 18S ribosomal RNA (18S), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), elongation factor 1-alpha (EF-1α), ubiquitin (UBQ), and TIP41. Among them, actin supports the cytoskeleton; 18S participates in the translation of amino acids; GAPDH is a key enzyme in the glycolysis process; EF-1α participates in RNA transcription extension; UBQ participates in the plant's response to stress and has a proteolytic effect. Qualitative PCR analysis and statistical software geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004) were used to comprehensively evaluate the expression stability of these candidate genes in the corolla (five different developmental stages) and leaves of R. chingii and R. henryi. It is hoped that the most suitable reference genes will be screened out in R. chingii and R. henryi, respectively, which will provide a reference for further research on genes relative expression and function analysis in R. chingii and R. henryi.

Materials and methods

Collection of plant samples: Corollas of Rehmannia chingii Li. and Rehmannia henryi N.E. Brown in five stages including small flower bud stage (C1, bud length less than 1 cm), middle flower bud (C2, bud length between 2 and 3 cm), big flower bud (C3, bud length between 4 and 5 cm), opening flower (C4), and fully opened flower (C5) and leaves were used as experimental materials (Fig. 1). A single sample was taken by mixed sampling and three biological replicates were performed. Samples were quickly frozen in liquid nitrogen and stored in a refrigerator at -80 °C for RNA extraction.

Candidate reference genes selection and primers design: According to the gene annotation information obtained by transcriptome sequencing, combined with the FPKM values of the genes in different samples, we screened the common reference genes in six samples of R. chingii and R. henryi as candidates, namely 18S, GAPDH, EF-1α, UBQ, Actin, and TIP41. The FPKM values of these candidate genes are relatively high and stable in different samples. (You can find the specific information of these candidate genes through the online website https://pan.baidu.com/s/1Vjc26DUB1DqwvePNe2ryYA, the extraction code is 9y6). The ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), the NCBI online analysis software, was used to make an open reading frame (ORF) prediction of the cDNA sequence of each gene. Quantitative specific primers were then designed using the NCBI-Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) online software based on the gene sequences (Table 1 Suppl.). The parameters are set as follows: primer length is generally around 20 bases, the length of the PCR product is 80 to 200 bp, the range of primer melting temperature (Tm) is 57 to 60 °C, and the optimal temperature is 58 °C. All primer sequences were synthesized by Sangon Biotech Co. (Shanghai, China) and primer PCR products were detected by 1 % (m/v) agarose gel electrophoresis (Table 1 Suppl.).

Total RNA extraction and cDNA synthesis: Total RNA was extracted from each sample according to the instructions of RNA extraction kit (TaKaRa, Dalian, China), and the starting amount of the sample was 50 mg. The total RNA obtained was treated with DNase to remove genomic DNA contamination. The concentration and purity of total RNA were measured using an ultramicro nucleic acid protein analyzer, RNA was quantified by absorbance at 260 nm, and purity was evaluated by absorbance ratio at 260/280 nm. The integrity of total RNA was detected by agarose gel electrophoresis. The obtained RNA bands are clear and free of DNA and other impurities and can be used in the next experiment. The first strand of cDNA was synthesized using reverse transcription kit 6210A (TaKaRa), which requires approximately 1 μg of total RNA, 1 mm3 of oligo dT primer (concentration: 50 μM), 1 mm3 of dNTP, and 0.5 mm3 of RNase. Inhibitor (40 U mm3-1), 1 mm3 of PrimeScript II reverse transcriptase (200 U mm3-1), and deionized water were added to form a total system of 20 mm3. The reaction procedure was: 65 °C for 5 min; 42 °C for 60 min; 95 °C for 5 min; and 4 °C for 2 min to terminate the reaction.

Fig. 1. Characteristics of buds and flowers in different growth periods (C1 to C5). A - Rehmannia chingii; B - R. henryi. L - leaf.
Real time qPCR: Reaction was performed according to the instructions of SYBR® Premix Ex Taq™ II kit (TaKaRa) and performed on a 96-well plate of the Bio-Rad iQ5 real time PCR system (Bio-Rad, Hercules, USA), each reaction was repeated three times. The reaction system is followed: 12.5 mm³ of SYBR® Premix Ex enzyme, 1 mm³ of forward primer (10 μM), 1 mm³ of reverse primer (10 μM), 2.0 mm³ of cDNA template, and deionized water 8.5 mm³ for a total volume of 25 mm³. The qPCR reaction program was: 95 °C for 30 s; 95 °C for 5 s, 60 °C for 30 s, 40 cycles; after the last cycle, the set temperature was continuously increased between 60 °C and 95 °C to obtain a melt curve. In addition to determining the Cq value of each candidate reference gene in different samples, a standard curve for each gene was also drawn to identify parameters such as the amplification efficiency ($E$) and correlation coefficient ($R^2$) of the primers. The cDNA solution obtained by reverse transcription was sequentially diluted 5, 10, 15, 20, and 25 times to draw a standard curve. Quantitative PCR was performed using the diluted cDNA as a template, and the obtained standard curve was analyzed by CFX-Manager™ software. The parameters such as primer amplification efficiency and correlation coefficient were calculated. The amplification efficiency of primers was related to the slope ($\Delta \Delta Cq$) of the primers. The Cq values of each candidate reference gene obtained by the qPCR analysis were transformed according to relevant requirements and then introduced into geNorm, NormFinder, and BestKeeper to analyze the stability of candidate reference genes.

Results

Total RNA extracted from different tissues of *R. chingii* and *R. henryi* was detected by 1% agarose gel, and 28S, 18S and 5S clear bands were seen (Fig. 1 Suppl.). The absorbance ratio $A_{280}/A_{260}$ of all RNA was between 1.8 and 2.0, with an average value of 2.0 (SD = 0.05), indicating that the RNA had good integrity and quality, with no obvious degradation and could be used for cDNA synthesis.

Quantitative PCR analysis was performed using the diluted cDNA of *R. chingii* and *R. henryi*, and the qPCR products were all specific strips after detection by 1% agarose gel electrophoresis (Fig. 2), and the fragment sizes were correct. Quantitative PCR results showed that the melt curve of each candidate reference gene also had a single main peak (Fig. 2 Suppl.). The standard curve of each candidate reference gene was obtained through the CFX-Manager™ software, and the PCR amplification efficiency ($E$) and correlation coefficient $R^2$ (Table 1 Suppl.) of the primers were further obtained according to the standard curve, where the range of $E$ needs to be 90 to 110 %. The above results indicate that qPCR primers of candidate reference genes have high specificity and amplification efficiency, and can be used in subsequent experiments.

Fig. 2. Polymerase chain reaction products of candidate reference genes in *Rehmannia chingii* and *R. henryi*. For the names of the genes see Table 1.
genes in all samples of *R. chingii* and *R. henryi* were shown in Fig. 3 and their Cq values are inversely related to the gene expression abundance. The Cq values of candidate reference genes fluctuated to a certain extent in all samples, indicating that the gene expression in these samples was not completely constant. From the results of qPCR, the Cq values of the six candidate reference genes in all samples were relatively consistent. The Cq values of *GAPDH* were relatively small, the Cq values of *UBQ* were relatively large, and the Cq values of *EF-1α* fluctuated greatly. The Cq values of *TIP41*, *Actin*, and *18S* in all samples were relatively moderate and concentrated. The fluctuation range of Cq values of *TIP41* gene was 23.57 to 26.87, the Cq values of the *Actin* gene were 23.62 to 27.03, and the Cq values of *18S* gene were 24.89 to 26.14.

Before using the geNorm algorithm for analysis, the Cq values of each gene need to be converted into relative expression by $Q = 2^{-\Delta Cq}$, where the $\Delta Cq$ value is equal to the Cq value of each corresponding candidate gene minus the minimum Cq value. The geNorm program calculates the stability measure (M) of each gene expression based on the average paired variation (V) of a specific gene relative to all other reference genes. Generally, M < 1.5 is used as the stability threshold. The smaller the M value, the more stable the gene expression, and the more unstable conversely.

In addition, the $V_{n/n+1}$ value of the gene pair variation analysis calculated by the geNorm program can determine the optimal reference gene number, and the threshold is 0.15. When $V_{n/n+1} < 0.15$, there is no need to introduce the $n+1$th reference gene. The average expression stability value (M) of each candidate reference gene in *R. chingii* and *R. henryi* was calculated by the geNorm program (Fig. 4). The default M threshold of the program is 1.5. According to the smaller M value, the higher the gene stability, the expression stability of each candidate reference gene was ranked. GeNorm analysis results show that the M values of the six candidate reference genes for both *R. chingii* and *R. henryi* were less than 1.5. In *R. chingii*, the highest expression stability had *RcTIP41* and *RcActin* ($M = 0.26$), and the lowest expression stability had *RcGAPDH* ($M = 0.67$). In *R. henryi*, *RhActin*, and *RhGAPDH* ($M = 0.26$) had the highest expression stability, and *RhUBQ* ($M = 0.83$) had the lowest expression stability.
stability.

The paired variation values \((V_{n/n+1})\) derived from the geNorm program were shown in Fig. 5. Taking \(V_{n/n+1} = 0.15\) as the threshold, the \(V_{2/3}\) of *R. chingii* and *R. henryi* are 0.097 and 0.092, respectively, which are both less than 0.15. Therefore, the optimal reference gene number during the quantitative normalization of *R. chingii* and *R. henryi* both are two, and there is no need to introduce a third reference gene.

NormFinder uses a conversion method similar to geNorm to calculate the expression stability of candidate reference genes. Unlike geNorm, the NormFinder program calculates the intra-group and inter-group variation and then combines the two results into the stability of each candidate gene (Schmidt and Delaney 2010).

The stability values (M) of six candidate reference genes were calculated by NormFinder in *R. chingii* and *R. henryi* (Table 1). According to the results of NormFinder analysis, the expression stability of six candidate reference genes in *R. chingii* and *R. henryi* were ranked from high to low. Among them, in *R. chingii*, *RcTIP41*, *RcEF-1α*, and *Rc18S* were relatively high in expression stability. The expression stability of *RhGAPDH*, *Rh18S*, and *RhActin* in *R. henryi* were relatively high. It can be seen that the results of NormFinder and geNorm programs were basically the same in the stability calculation of reference genes of *R. chingii* and *R. henryi*.

The BestKeeper software can directly import the Cq value (geometric average of 3 repetitions) of the gene in the numerical input area. Through the obtained correlation coefficient \((r)\), standard deviation (SD), and coefficient of variation (CV), the expression stability of candidate reference genes was analyzed (Li *et al.* 2019). The larger the correlation coefficient, the smaller the standard deviation, and the coefficient of variation, the better the stability of gene expression. The results are shown in Table 2. According to the combination of CV ± SD and r values, in *R. chingii*, the CV ± SD values of *Rc18S* and *RcTIP41* were relatively small, and the r values were relatively high. In *R. henryi*, *Rh18S* and *RhActin* had lower CV ± SD value and higher r value. Comprehensive analysis based on BestKeeper data showed that the genes with relatively stable expression in *R. chingii* were *Rc18S*, *RcActin*, and *RcTIP41*, and the most unstable gene was *RcGAPDH*. The relatively stable genes in *R. henryi* were *Rh18S* and *RhActin*, and *RhUBQ* had the most unstable expression. This result is basically consistent with the results of geNorm and NormFinder. Because the number of optimal reference genes was two, and combined with the analysis results of geNorm, NormFinder, and BestKeeper,

![Fig. 5. Pairwise variation \((V_{n/n+1})\) analysis for selection of the number of real time quantitative PCR best reference genes with the geNorm algorithm. A - Rehmannia chingii, B - R. henryi.](image)

Table 1. The ranking of expression stability of candidate reference genes in *Rehmannia chingii* and *R. henryi* based on NormFinder analysis. *TIP41* - type 2A phosphatase activator TIP41, *EF-1α* - elongation factor 1-alpha, *18S* - 18S ribosomal RNA, *UBQ* - ubiquitin, *GAPDH* - glyceraldehyde 3-phosphate dehydrogenase.

| Rank | *Rehmannia chingii* | stability value | *Rehmannia henryi* | stability value |
|------|---------------------|----------------|-------------------|----------------|
| 1    | *RcTIP41*           | 0.090          | *Rh18S*           | 0.193          |
| 2    | *RcEF-1α*          | 0.144          | *RhActin*        | 0.264          |
| 3    | *Rc18S*            | 0.183          | *RhGAPDH*        | 0.298          |
| 4    | *RcActin*          | 0.260          | *RhTIP41*        | 0.300          |
| 5    | *RcUBQ*            | 0.429          | *RhEF-1α*        | 0.312          |
| 6    | *RcGAPDH*          | 0.726          | *RhUBQ*          | 0.990          |
the optimal reference genes of *R. chingii* were *Rc18S* and *RcTIP41* and the optimal reference genes of *R. henryi* were *Rh18S* and *RhActin*.

Using *RcTIP41*, *Rc18S*, *RcTIP41 + Rc18S*, and *RcGAPDH* as reference genes we verified the expressions of the *Rc4CL*, *RcERF*, *RcC4H*, and *RcPAL* (Fig. 6). The correlation analysis of the qPCR results of the four verification genes under different reference genes conditions and the FPKM values of the transcriptome in *R. chingii*. The results show that when the stably
expressed RcTIP41, Rc18S, RcTIP41 + Rc18S were used as reference genes for *R. chinigii*, the changes in the relative expression of all the verification genes in all samples were well consistent with the FPKM values (Fig. 6). However, when qPCR was performed with RcGAPDH as the reference gene, the expression trends of RcC4H and RcPAL in different samples were inconsistent with their FPKM values of transcriptome analysis. Correlation analysis showed that relative expressions and FPKM values of the four verification genes were significantly correlated \((P < 0.05)\) when RcTIP41, Rc18S or RcTIP41 + Rc18S were used as reference genes (Table 2 Suppl.). When RcGAPDH was used as a reference gene, the correlation between the relative expressions and FPKM values of RcC4H and RcPAL genes didn’t reach a significant level (Table 2 Suppl.). The results of verification experiments suggested that RcTIP41, Rc18S or RcTIP41 + Rc18S were good reference genes (reference gene combinations) for expression analysis in flower and leaf development of *R. chinigii*.

Similar to the verification results in *R. chinigii*, when Rh18S, RhActin, Rh18S + RhActin were used as reference genes of *R. henryi*, the change trends in gene expression of RhC4H, RhPAL, RhERF and RhEXP A8 were basically consistent with the results of the transcriptome (Fig. 7). When RhUBQ was used as a reference gene, the consistency of the changes in the expression levels of the four verification genes and the transcriptome data was relatively poor (Fig. 7). Correlation analysis results showed that when Rh18S, RhActin and Rh18S + RhActin were used as reference genes, the correlation between the relative expressions of the four verification genes (RhERF, RhEXP A8, RhC4H and RhPAL) and the FPKM values in the RNA-Seq data reached a significant level \((P < 0.05)\) (Table 3 Suppl.). However, when RhUBQ was used as a reference gene, the correlation analysis results of the relative expressions of the four verification genes and FPKM values were not significant (Table 3 Suppl.).
This shows that RhActin, Rh18S, RhActin + Rh18S were suitable as reference genes (reference gene combinations) for gene expression level analysis during flowers and leaves development of R. henryi.

Discussion

Quantitative PCR is an important method for gene expression analysis, and the key to accurate analysis of gene expression by real-time qPCR is to select the suitable and stable reference gene. The ideal reference gene is expressed in all cells and tissues of the organism (Tong et al. 2009), the stability of gene expression is not affected by external environmental conditions (Huggett et al. 2005), and the expression abundance of the reference gene and the target gene is similar (Paolacci et al. 2009).

GeNorm, NormFinder, and BestKeeper are all free statistical algorithms based on Excel, which are specifically used for reference gene stability calculations and are widely used and convenient. GeNorm and NormFinder need to convert the Cq value to the ΔCq value to analyze the stability of the gene, and BestKeeper directly compares the standard deviation and the coefficient of variation of the Cq value to evaluate the stability of the gene. GeNorm analyzes the M values of candidate reference genes by pairwise comparison (Vandesompele et al. 2002), which can precisely control the error caused by the cDNA input because the systematic error can affect the two paired genes at the same time (Expósito-Rodríguez et al. 2008). In addition, geNorm can only determine the most suitable reference genes, but also the number and combination of the most suitable genes. However, geNorm is more sensitive to co-regulatory genes, which makes it more inclined to select genes with higher expression similarity (Andersen et al. 2004). NormFinder can make up for the shortcomings of geNorm, it is not sensitive to co-ordination, and can analyze the differences within and between the sample groups to balance the sources of variation, but NormFinder does not consider the systematic errors generated during the sample preparation process (Expósito-Rodríguez et al. 2008, Paolacci et al. 2009). In addition, to analyze the reference genes, BestKeeper can also compare the expressions of target genes. Therefore, when using three different algorithms to comprehensively evaluate the stability of candidate genes, the results are more stable and reliable.

So far, no related research has been reported on diploid R. chingii and R. henryi reference gene screening, which has limited the application of qPCR in these organisms. In this study, Actin, GAPDH, 18S, UBQ, EF-1a, and TIP41, which are six common endogenous reference genes in plants, were screened based on FPKM data of genes in the transcriptome as candidate genes (Guénin et al. 2009, Manoli et al. 2012). The results of qPCR and gene stability analysis showed that the expression abundance and stability of expression of the six candidate reference genes in different developmental stages of R. chingii and R. henryi were not consistent. This further validates the previous view that the stability of the expression of reference genes in different species and tissues is not completely constant (Suzuki et al. 2000, Paolacci et al. 2009). In addition, the standardization of a single reference gene is prone to large errors (Thellin et al. 1999, Nicot et al. 2005). Based on the comprehensive evaluation of \( V_{\text{area}} \), geNorm, NormFinder, and BestKeeper results, the number of optimal reference genes for R. chingii and R. henryi were determined to be two.

In R. chingii, the results of geNorm and NormFinder programs showed that RcTIP41, RcActin, and Rc18S gene expression stability was relatively high, but BestKeeper results showed that \( r \) (0.79) of RcActin gene was lower than that of RcTIP41 (0.97) and Rc18S (0.95). The expression stability of RcUBQ and RcGAPDH genes were relatively poor, and qPCR showed that the expression abundance of RcUBQ gene was relatively low and the expression abundance of RcGAPDH gene was relatively high. Therefore, RcUBQ and RcGAPDH genes are not suitable as reference genes for R. chingii. In R. henryi, the results of geNorm and NormFinder showed that the gene expression stability of Rh18S and RhActin was the best, and it was the same as in BestKeeper analysis. While the expression stability of RhEF-1a and RhGAPDH was relatively poor, they were not suitable as reference genes. Although the stability of RhGAPDH gene expression in R. henryi was relatively high, it was also not suitable as a reference gene for R. henryi because of the high abundance of RhGAPDH expression. 18S is one of the common reference genes. It has a highly conserved sequence and plays an important role in maintaining basic cellular metabolism and intracellular functions (Weigand et al. 2012, Yeap et al. 2014). It is often controversial that the expression of 18S gene is too high, but in this study, the 18S gene was moderately abundant in different tissues of R. chingii and R. henryi, 25.31 (SD = 0.35) and 25.48 (SD = 0.49), so it can be used as a reference gene.

Conclusions

Combining the expression abundance of candidate reference genes and the gene stability results obtained by geNorm, NormFinder, and BestKeeper, it was finally determined that the suitable reference genes for R. chingii were RcTIP41 and Rc18S, and for R. henryi were Rh18S and RhActin. This study can provide appropriate references for the standardization and normalization of quantitative expressions of related genes in R. chingii and R. henryi, and lay a foundation for further research on the functions of key genes in R. chingii and R. henryi.

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