Identification of appropriate reference genes for RT-qPCR analysis in *Juglans regia* L.

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Abstract

Reverse transcription quantitative real-time PCR (RT-qPCR) is a popular adopted technique to detect gene expression, and the selection of appropriate reference genes is crucial for data normalization. In the present study, seven candidate reference genes were screened to evaluate their expression stability in various flower buds, leaf buds, tissues and cultivars of the English walnut (*Juglans regia* L.) based on four algorithms (geNorm, Normfinder, Bestkeeper and Reffinder). The results demonstrated that TUA, EF1 and TUB were appropriate reference genes for flower buds at different stages of female flower buds differentiation; TUB and 18S rRNA were best for leaf buds at different stages of female flower buds differentiation; TUB and TUA were suitable for different cultivars; and ACT2, 18S rRNA and GAPDH were useful for different tissues. Moreover, the expression of ACT was not stable among different flower buds, leaf buds and cultivars. The stability of reference genes were confirmed through the analysis of the expression of SPL18 gene. These results will contribute to a reliable normalization of gene expression in *J. regia*.

Introduction

With high sensitivity, rapidity, specificity and reliability, reverse transcription quantitative real-time PCR (RT-qPCR) is a popular technique applied to detect relative gene expression [1, 2]. Nevertheless, the reliability of the data is influenced by the quantity and quality of the templates, and the efficiency of the reverse transcription reaction and amplification [3, 4]. Consequently, it is very important to assess gene expression levels with a stable reference gene. Numerous genes related to basic metabolism or cellular processes have been applied as reference genes in various fruit trees, including Chinese jujube [5, 6], peach [7, 8], litchi [9], grape [10], citrus [11], apple [12–14], pear [15, 16] and cherry [17]. Only one study has analyzed the reference genes stability for *Juglans sigillata* Dode [18].

The English walnut (*J. regia* L.) has abundant nutrition and commercial value, and is one of the most important nut fruit trees in the world. Flowering is an important stage for nut production. For most walnut trees, there is a long juvenile period of 8 to 10 years before first flowering. However, the *J. regia* cv. Xinxin 2, an early-seeding cultivar, has a short juvenile phase of
2 to 3 years, and few studies have explored the molecular mechanism of floral induction. Recently, we used high-throughput sequencing technology to detect the transcriptome profiles of flower buds and leaf buds before, during and after the critical period of female flower bud differentiation. The transcriptome sequence dataset provided abundant information for the selection of appropriate reference genes.

An ideal reference gene should be stably expressed not only in various tissues but also at all developmental stages. Furthermore, the reference gene should not be affected by experimental treatments [19]. However, an increasing number of studies indicated that expression of many classic reference genes varies among different tissues, genotypes and experimental treatments [4]. Hence, it is necessary to screen a suitable reference gene before RT-qPCR analysis.

In the present study, we screened seven candidate reference genes based on the transcriptome dataset of *J. regia* cv. Xinxin 2. Their expression stability among various flower buds, leaf buds, tissues and cultivars was assessed. Furthermore, the expression of one target gene (*SPL18*) was explored to validate the effectiveness of the selected reference genes.

**Materials and methods**

**Plant materials**

The plant samples in this study were collected from the southern part of Xinjiang Uyghur Autonomous Region, China. Flower buds and leaf buds were collected from *J. regia* cv. Xinxin 2 at different stages of female flower buds differentiation. Flower buds were collected at the critical period of female flower buds differentiation from the following four cultivars: Xinxin 2, Hetian, Wuhuo and Wen 185. Different tissues (leaves, leaf buds, branches and flower buds) were collected at the critical period of female flower buds differentiation from Xinxin 2. All samples were immediately frozen in liquid N and then stored at −70˚C. For flower buds and leaf buds, three buds made a sample. Three samples were used for each period. (For each period, nine buds were used.). For different tissues, three samples were used. Eight periods of flower buds and five periods of leaf buds were considered to analysis the stability of the seven candidate reference genes.

**RNA isolation and cDNA synthesis**

Total RNA was isolated from the flower buds, leaf buds, leaves and branches using a Plant RNA Extract Kit (Aidlab, Beijing, China). The quality of RNA was detected by NanoDrop 2000 (Thermo Scientific) and 1.1% agarose gel electrophoresis. For each sample, one microgram of total RNA was used to synthesize cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

**Selection of candidate reference genes and primer design**

Seven candidate reference genes, including actin (*ACT*), actin-related protein 2 (*ACT2*), elongation factor (*EF1*), α-Tublin (*TUA*), β-Tublin (*TUB*), glyceraldehyde-3-phosphatedehydrogenase (*GAPDH*), and 18S ribosomal RNA (*18S rRNA*) were selected based on their low variance of gene expression in the transcriptome dataset of *J. regia* cv. Xinxin 2. The primers were designed using Primer Premier 6 [20] and the detailed information were listed in S1 Table.

**RT-qPCR and statistical analysis**

The RT-qPCR was conducted using a SYBR Green based PCR assay (Toyobo, Japan) on a Bio-Rad CFX96 real time PCR system. Each 10 μL PCR reaction mixture covered cDNA (1 μL),
forward and reverse primer (0.4 μL), SYBR Green Mix (5 μL) and ddH₂O (3.2 μL). The PCR procedure were 94°C for 30 s, followed by 40 cycles of 94°C for 15 s, 60°C for 15 s and 72°C for 30 s. A melting curve was performed to confirm the specificity of primers. Each reaction was carried out three times. In addition, standard curves were generated using a five-fold cDNA dilution series to calculate the amplification efficiency (E) and correction coefficients (R²) of the candidate reference genes [2].

Quantification cycle (Cq) values were obtained and analyzed using three Microsoft Excel-based softwares, geNorm [21], Norm Finder [22], and BestKeeper [23]. The comprehensive ranking order of the candidate reference genes were obtained through an online-based program: ReFinder (http://150.216.56.64/referencegene.php?type=reference), which integrates the analysis of geNorm, Normfinder and Bestkeeper.

**Validation of candidate reference gene**
To confirm the stability of reference genes, RT-qPCR was performed to detect the expression levels of squamosa promoter-binding-like protein 18 (SPL18) using the 2⁻ΔΔCq method. Three of the most stable genes and the least stable gene were used to normalize the expression levels of SPL18 in different flower buds and leaf buds at different stages of female flower buds differentiation (five periods were analyzed), different tissues and four cultivars. The primers of SPL18 were 5’−AGCAGTGCAGCAGGTTCCATTC (forward) and 5’−GTCGTCGGTTGTGTCCATCAAGG (reverse).

**Results**

**Primer specificity and PCR amplification efficiency**
The specific primers for seven reference candidates were designed for RT-qPCR. The size of amplicons ranged from 98 to 166 bp. All primer pairs amplified a single PCR product of the expected size (S1 Fig). All the primers demonstrated a single peak melting curves (S2 Fig). The R² of seven candidates ranged from 0.947 for ACT2 to 0.997 for EF1 and E varied from 97.8% to 111.1% for TUB and ACT2, respectively (S3 Fig).

**Expression profile of the reference genes**
The Cq values showed an overview of the expression level of seven candidate reference genes among the tested samples. The Cq values of the seven reference genes ranged from 18.63 (EF1) to 28.42 (TUB). The mean Cq values of the seven reference genes ranged from 19.34 to 25.58 (Fig 1, S2 Table). A high Cq value suggested a low gene expression level [24]. Among the seven candidate reference genes, 18S rRNA and ACT2 demonstrated low expression with high Cq values, whereas TUA and EF1 showed high expression levels with low Cq values. The outliers suggested that no candidate reference genes had constant expression levels among all the tested samples. Therefore, it’s necessary to screen appropriate reference genes via statistical methods.

**Expression stability of candidate reference genes**

**geNorm analysis.** GeNorm algorithm determines the ranking of candidate reference genes by calculating the expression stability measure (M). The M value of the gene is negatively related to its stability, and 1.5 is set as the threshold of M value [21]. As showed in Fig 2, M-values of all the seven candidate reference genes were below 1.5. The stability of the seven candidate reference genes varied across the tested samples. Among the various flower buds, TUA and EF1 were the most stable genes with a same M-value of 0.23 (Fig 2A). EF1 and TUB were the most stable genes with a same M-value among the different leaf buds (Fig 2B) and the four
cultivars (Fig 2D). For different tissues, GAPDH and ACT2 were the most stable genes with a same M-value of 0.26 (Fig 2C). When all the samples were analyzed, 18S rRNA and ACT2 were the most stable genes (Fig 2E). ACT was found to be the least stable gene among the various flower buds, leaf buds, different cultivars and all the test samples (Fig 2A, 2B, 2D and 2E).

The geNorm also generated the pairwise variation ($V_{n/n+1}$), which can help to select the optimal number of references. If the value of $V_{n/n+1}$ was less than 0.15, there is no necessary to apply an additional reference gene (Vandesompele et al., 2002). For various flower buds, the value of $V_{2/3}$ was 0.087 (Fig 2F), suggesting that two reference genes, EF1 and TUA, were needed for normalization. For leaf buds at different flowering stages, the variation value was 0.121 at $V_{3/4}$, so three reference genes, TUB, EF1 and GAPDH, were required for normalization. The value of $V_{2/3}$ was 0.075 in the cultivars group, suggesting that EF1 and TUB were needed for normalization.

**NormFinder analysis.** The results of NormFinder analysis were shown in Table 1. For various flower buds, TUA (0.085), TUB (0.145) and EF1 (0.193) ranked the most stable genes, which was similar to the results generated from geNorm. Similarly, ACT was the least stable gene among the various flower buds, leaf buds, cultivars and all the tested samples calculated by NormFinder, which was consistent with the results of geNorm. For various leaf buds, tissues and cultivars, the most stable genes got from NormFinder were different from the results of geNorm. Specifically, 18S rRNA (0.155) and TUB (0.235) for various leaf buds; ACT (0.115) and 18S rRNA (0.199) for different tissues; GAPDH (0.097), TUA (0.167) and TUB (0.206) for different cultivars were ranked the most stable by NormFinder.
BestKeeper analysis. Bestkeeper was applied to evaluate the stability of candidate references according to the standard deviation (SD) and the coefficient of variation (CV) of Cq values. SD values less than 1 were acceptable. A lower SD and CV (CV ± SD) value indicated a more stable gene. For various flower buds, the values of SD for seven candidate reference genes and pairwise variation analysis to select the optimal number of reference genes. A: flower buds, B: leaf buds, C: different tissues, D: four cultivars, E: all samples. F: pairwise variation.

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Fig 2. geNorm ranking of the seven candidate reference genes and pairwise variation analysis to select the optimal number of reference genes. A: flower buds, B: leaf buds, C: different tissues, D: four cultivars, E: all samples. F: pairwise variation.

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Validation of selected reference genes

The expression levels of SPL18 were assessed in various flower buds, leaf buds, tissues and cultivars to confirm the stability of the selected reference genes. Three of the most stable reference genes and the most unstable gene were applied for data normalization. The expression levels of genes were below 1 (Table 2). EF1 (CV ± SD = 1.60% ± 0.31) were ranked the best stability, followed by TUA (CV ± SD = 1.69% ± 0.33) and TUB (CV ± SD = 1.73% ± 0.41). This result was similar with that got from geNorm and NormFinder. However, in other experimental groups, the results of Bestkeeper analysis were different from those got from geNorm and NormFinder. For various leaf buds, 18S rRNA was the most stable (CV ± SD = 2.89% ± 0.76), followed by ACT2 (CV ± SD = 3.10% ± 0.79) and GAPDH (CV ± SD = 3.60% ± 0.82). All other genes, with SD values bigger than 1, were not acceptable. For different tissues, EF1 had the lowest CV ± SD values (1.87%±0.36), followed by ACT2, GAPDH and 18S rRNA, whereas ACT, TUA and TUB had SD values bigger than 1. For different cultivars, all the genes had SD values less than 1, and TUB were ranked the most stable, followed by TUA and EF1. For all samples, EF1 was the most stable gene.

Comprehensive ranking of the candidate reference genes by RefFinder. The comprehensive ranking order of the candidate reference genes were obtained through RefFinder, which integrated the methods of geNorm, NormFinder and Bestkeeper. As shown in Table 3, TUA, EF1 and TUB were the most three stable genes in flower buds. 18S rRNA and TUB expressed most stably in leaf buds. For different tissues, ACT2, 18S rRNA and GAPDH were the most suitable reference genes. For different cultivars, TUB, TUA and GAPDH were identified as the most stable genes. Among all the samples, 18S rRNA, ACT2 and GAPDH were expressed most stably. The expression of ACT was unstable in flower buds, leaf buds and different cultivars. The expression of TUB was unstable across different tissues.

Validation of selected reference genes

The expression levels of SPL18 were assessed in various flower buds, leaf buds, tissues and cultivars to confirm the stability of the selected reference genes. Three of the most stable reference genes and the most unstable gene were applied for data normalization. The expression levels of genes were below 1 (Table 2). EF1 (CV ± SD = 1.60% ± 0.31) were ranked the best stability, followed by TUA (CV ± SD = 1.69% ± 0.33) and TUB (CV ± SD = 1.73% ± 0.41). This result was similar with that got from geNorm and NormFinder. However, in other experimental groups, the results of Bestkeeper analysis were different from those got from geNorm and NormFinder. For various leaf buds, 18S rRNA was the most stable (CV ± SD = 2.89% ± 0.76), followed by ACT2 (CV ± SD = 3.10% ± 0.79) and GAPDH (CV ± SD = 3.60% ± 0.82). All other genes, with SD values bigger than 1, were not acceptable. For different tissues, EF1 had the lowest CV ± SD values (1.87%±0.36), followed by ACT2, GAPDH and 18S rRNA, whereas ACT, TUA and TUB had SD values bigger than 1. For different cultivars, all the genes had SD values less than 1, and TUB were ranked the most stable, followed by TUA and EF1. For all samples, EF1 was the most stable gene.

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Validation of selected reference genes

The expression levels of SPL18 were assessed in various flower buds, leaf buds, tissues and cultivars to confirm the stability of the selected reference genes. Three of the most stable reference genes and the most unstable gene were applied for data normalization. The expression levels of
SPL18 were similar in different flower buds under the normalization of TUA, EF1 and TUB (Fig 3A). Similar results were obtained when 18S rRNA, TUB and EF1 were used as reference genes. However, when the least stable gene (ACT) was used as the reference gene, the expression of SPL18 was considerably biased at Period 5 (Fig 3B). Analysis of four cultivars suggested that the expression levels of SPL18 were consistent when TUB and TUA were used for normalization, while there was a slightly difference when EF1 was used as reference gene. However, there was a significant difference when ACT was applied for normalization (Fig 3C). For different tissues, the expression trends of SPL18 were consistent when ACT2, 18S rRNA and GAPDH were used for normalization. TUB was not suitable for normalization among different tissues (Fig 3D).

Discussion

Different normalization approaches can change the calculation of P-values and fold changes of a large number of genes depending on the normalization method applied [25]. RT-qPCR is considered as the most appropriate method for gene expression due to its high sensitivity, rapidity, specificity and reliability [1, 2]. An ideal reference gene is assumed to have constant expression levels among different samples and under different experimental conditions. However, there is no universal reference gene. The expression of putative reference genes varies across different tissues, genotypes and various experimental conditions [26]. The expression levels of target genes were evaluated according to the expression of reference genes, however,
an unstable reference gene can result in inaccurate evaluation of target gene expression. For example, the expression of FaWRKY1 in roots of tall fescue under salinity and drought stress peaked at 3 h when the most stable reference genes (TUB and SAND) were used. However, the expression of FaWRKY1 exhibited fluctuations and failed to achieve a consistent pattern when the least stable gene (EF1α) was used [27]. As reported in the previous study, the expression patterns of miR159 for leaf, stem and root were consistent when the most stable reference genes, EF-1α, Ubiquitin and GAPDH, were used for data normalization. However, when the least stably expressed reference genes, Actin and 18S rRNA, were used for data normalization, the expression of miR159 was considerably biased. This result indicated that the least stable reference genes, Actin and 18S rRNA, failed to standardize the expression data effectively [28].

In this study, the expression level and stability of seven candidate reference genes were evaluated by RT-qPCR using geNorm, Normfinder and Bestkeeper. As a result, no single reference gene was consistently expressed across all the samples tested due to the different statistical algorithms of the programs [29]. For example, EF1, TUA and TUB were identified as the most stable genes in various flower buds and different cultivars by geNorm, Normfinder and Bestkeeper. Similar to our results, EF1 and TUB were steadily expressed during flower development in other plants [30, 31]. However, EF1 and TUA in various leaf buds; TUA, EF1 and TUB in different tissues were not stable by Normfinder (Table 2). TUB and TUA were also identified to be unstable reference genes in peach [7]. Similarly, EF1 was not stably expressed in grapevine [10]. A previous study indicated that β-ACT2 was not an appropriate reference gene in Juglans sigillata [18]. In the present study, ACT was also identified as the least stable in various

Fig 3. Relative expression of SPL18 based on candidate reference genes. A: flower buds. B: leaf buds. C: different cultivars. D: different tissues. The relative expression levels were depicted as mean ± SD (standard deviation) calculated from three technical replicates.

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flower buds and leaf buds and different cultivars by the three programs (Fig 2, Tables 1 and 2), whereas it was ranked first in different tissues by Normfinder (Table 2). These results may be due to different species and tissues that were assessed as reliable reference genes that were highly specific to an individual experimental condition. Furthermore, we applied RefFinder to get a comprehensive ranking order of the seven candidate reference genes based on the geometric mean of every reference gene evaluated through delta Cq, geNorm, Normfinder and Bestkeeper [19, 32, 33]. To confirm the stability of selected reference genes, the expression levels of SPL18 in different flower buds, leaf buds, tissues and four cultivars were detected. The results revealed that SPL18 were consistently expressed under the normalization of the most stable reference genes (Fig 3).

**Conclusion**

In conclusion, the stability of seven candidate reference genes for RT-qPCR data normalization was evaluated by geNorm, Normfinder and Bestkeeper, and the comprehensive ranking orders were obtained by RefFinder. The results demonstrated that TUA, EF1 and TUB for flower buds at different stages of female flower buds differentiation, 18S rRNA and TUB for leaf buds at different stages of female flower buds differentiation, TUB and TUA for different cultivars, ACT2, 18S rRNA and GAPDH for different tissues were considered to be the suitable reference genes. Moreover, ACT was not a suitable reference gene for different flower buds, leaf buds and cultivars. TUB expression was unstable among different tissues. Additionally, the expression level of SPL18 was analyzed to validate the stability of selected reference genes. Our results will contribute to a reliable normalization of RT-qPCR data for gene expression in *Juglans regia*.

**Supporting information**

S1 Fig. Agarose gel (1.2%) electrophoresis shown amplification of seven candidate reference genes with a single PCR product of expected size. (TIF)

S2 Fig. Melting curves of seven candidate reference genes in RT-qPCR. (TIF)

S3 Fig. Standard curves of seven candidate reference genes in RT-qPCR. (TIF)

S1 Table. Primer sequences and amplification characteristics of seven candidate reference genes for RT-qPCR analysis. (XLSX)

S2 Table. The Cq values of seven candidate reference genes. (DOCX)

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