Research Article

Selection and Validation of Appropriate Reference Genes for Quantitative RT-PCR Analysis in *Rubia yunnanensis* Diels Based on Transcriptome Data

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Real-time quantitative polymerase chain reaction (RT-qPCR) has been widely applied in gene expression and transcription abundance analysis because of its high sensitivity, good repeatability, and strong specificity. Selection of relatively stable reference genes is a precondition in order to obtain the reliable analysis results. However, little is known about evaluation of a set of reference genes through scientific experiments in *Rubia* plants. Here, 15 candidate reference genes were selected from *R. yunnanensis* transcriptome database and analyzed under abiotic stresses, hormone treatments, and different tissues. Among these 15 candidate reference genes, heterogeneous nuclear ribonucleoprotein (hnRNP), TATA binding protein (TBP), ribosomal protein L5 (RPL5), malate dehydrogenase (MDH), and elongation factor 1-alpha (EF-1α) were indicated as the five most stable reference genes by four statistical programs (geNorm, NormFinder, BestKeeper, and RefFinder). Ultimately, the validity of reference genes was confirmed by normalizing the expression of o-succinylbenzoate-CoA ligase (OSBL) and isochorismate synthase (ICS) involved in the anthraquinone biosynthesis pathway in different tissues and hormone treatments. Meanwhile, four other putative genes involved in the anthraquinone biosynthesis pathway were also normalized with the selected reference genes, which showed similar expression levels with those given by transcriptome data. This work is the first research that aims at a systematic validation on the stability of reference genes selected from *R. yunnanensis* transcriptome data and will be conducive to analyze gene expression in *R. yunnanensis* or other *Rubia* species.

1. Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a technique for precise quantification of nucleic acids by monitoring the entire PCR process using a real-time fluorescence quantifier. It also plays an extremely important role in gene expression analysis and is the most extensive method compared with the other three methods (northern blot, microarray, and high-throughput sequencing) due to its high sensitivity, good repeatability, strong specificity, high throughput, wide application, and low cost [1].

RT-qPCR is a powerful tool for understanding metabolic pathways, genetics, signaling pathways, and several complex regulatory networks in organisms. The accuracy of results using this method is unavoidably affected by sample amounts, RNA integrity, cDNA quality, efficiency of enzymatic reaction, and gDNA contamination [2, 3]. In order to obtain stable and reliable results in the RT-qPCR assay, a suitable and stable reference gene is a prerequisite to normalize gene expression and avoid errors caused by the factors mentioned above.

Reference genes are usually housekeeping genes, which are expressed in all kinds of cells in organisms, and their products are essential proteins to maintain the basic life activities of cells. There are hundreds of housekeeping genes, among which GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ACT (actin-related protein), TUB (beta-1 tubulin), 18S rRNA (ribosomal RNA), and 28S rRNA are often used as internal reference genes for the standardization of target genes owing to their stable expression levels under different conditions and various tissues in many plant
species [4, 5]. However, these reference genes have also shown some shortcomings that their expression levels vary greatly under certain factors even if they can be stably expressed in some cells or tissues under some experimental conditions in recent studies [6, 7]. Therefore, it is completely necessary to select suitable internal reference genes as the standard to study the expression levels of target genes according to the different sample types and test conditions in order to make the results more reliable. With the development in science and technology, several new statistical algorithms such as geNorm, NormFinder, BestKeeper, and RefFinder have emerged for screening stable internal reference genes in recent years, which have been developed as efficient tools to determine conveniently the most stable reference genes for RT-qPCR normalization [8–10]. Hence, many novel reference genes were validated, and their suitability was evaluated by employing these programs in different plants, such as Eucaphis konishii [11], Sapium sebiferum [12], and Neolamarckia cadamba [13].

Rubi a yunnanensis Diels is a Chinese medicinal herb mainly distributed in Yunnan Province in Southwest China, and its medicinal parts are the roots and rhizomes named “Xiaohongshen,” which are used to treat many diseases in folk medicines such as cancer, vertigo, insomnia, rheumatism, tuberculosis, menstrual disorders, and contusions [14]. It can be used as a substitute in Yunnan Province for R. cordifolia listed in Chinese Pharmacopoeia. Three major types of important constituents, i.e., quinones, Rubiaceae-type cyclopeptides, and terpenoids, have been isolated from R. yunnanensis by our group [15–17]. Several of them have been reported to have important pharmacological activities, such as rubiadi [18], RA-V [19, 20], RA-XII [21, 22], and rubiarbonol G [23]. Among them, anthraquinones are the most abundant and important bioactive compounds [17], and there are still no data reported on gene expression and also about biosynthetic pathways of anthraquinones in R. yunnanensis. Based on previous research progress on anthraquinone biosynthesis in Rubiaceae [24, 25], we hope to increase the knowledge of anthraquinone biosynthesis in R. yunnanensis, particularly in the areas such as gene expression analyses and mechanisms responsible for anthraquinone biosynthesis and regulation at their enzyme and gene levels. Moreover, fewer reference genes of Rubia were identified through scientific and systematic experiments, and randomly selected reference genes or single gene quantification RT-qPCR assays have shown frequently variability and unreliability in gene expression under various experimental conditions [26].

In our study, 15 genes including 10 common reference genes (GAPDH, eIF (eukaryotic translation initiation factor), EF-1α (elongation factor 1-alpha), UBCE (ubiquitin-conjugating enzyme), RPL5 (ribosomal protein L5), TBP (TATA binding protein), TUB, ACT2, MDH (malate dehydrogenase), and PEPC (phosphoenolpyruvate carboxykinase)) and 5 novel reference genes (SAND (SAND family protein), PP2A (serine/threonine-protein phosphatase 2A), PTBP2 (polypyrimidine tract-binding protein homolog 2), hnRNP (heterogeneous nuclear ribonucleoproteins), and F-box (F-box protein)) were selected as candidate genes according to transcriptome data of the root and stem leaf (mixed stem and leaf) of R. yunnanensis from our laboratory (Yi et al., School of Traditional Chinese Pharmacy, China Pharmaceutical University) (unpublished data). Their expression stability in R. yunnanensis was evaluated by RT-qPCR across different experimental conditions including various tissues (root, stem, and leaf), abiotic stresses (osmotic stress, salt stress, oxidative stress, metal stress, and cold and heat stress), and hormone treatments referring to previous study [27]. For hormone treatments, methyl jasmonate (MeJA) and salicylic acid (SA) were used to treat plants because they could act as the important elicitors and signaling molecule and also regulate the activity of various enzymes related to secondary metabolic pathways. Optimal reference genes were confirmed for each experimental condition through four statistical algorithm programs. The results indicated that hnRNP, TBP, RPL5, MDH, and EF-1α were the five most stable reference genes, while GAPDH, F-box, SAND, TUB, and PEPC were the least stable reference genes in all sample sets. In addition, in order to confirm the suitability of selected reference genes from our results, o-sucinalbenzoate-CoA ligase (OSBL) and isochoisimurate synthase (ICS), two critical common enzymes involving shikimate and terpenoid pathways of anthraquinone biosynthesis, were chosen for normalizing their expression levels in different tissues and different time points after MeJA treatment using the least stable genes, the two most stable genes, and their combinations as the reference genes, respectively. Finally, the relative expression levels of other four putative key genes (isopenetynl-diphosphate delta-isomerase (IPPI), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), D-deoxy-D-xylulose 5-phosphate synthase (DXS), and acetyl-CoA acetyltransferase (AACT)) involved in the anthraquinone biosynthesis pathway normalized with hnRNP and TBP as reference genes were compared with those calculated by the FPKM (fragments per Kilobase Million) method [28] of transcriptome data to ensure again the reliability of selected internal reference genes.

2. Materials and Methods

2.1. Plant Material. Seeds of R. yunnanensis were collected legally in the wild in Kunming, Yunnan Province, China, in October 2016. After identification by Professor Heng Li from Kunming Institute of Botany, Chinese Academy of Sciences, the seeds were sown in 25 cm × 20 cm plastic pots containing a mixture of vermiculite, nutrient soil at a ratio of 2:1 under a set photoperiod (16 h light and 8 h dark) at 25°C, 65% relative humidity, and PAR of 30000 lux of light intensity in Kunming Institute of Botany, Chinese Academy of Sciences (25°08’N, 102°44’E). After germination, one-year-old R. yunnanensis seedlings were obtained and then transported to the greenhouse of China Pharmaceutical University in November 2017 (31°54’N, 118°54’E) under routine management of the greenhouse as described above. The seeds samples were deposited at Prof. Tan’s lab.

The experiment was conducted in May 2018. For different tissue samples, fresh roots, stems, and leaves were
| Gene abbreviation | Gene name                          | Primer sequence (5′–3′) | Amplicon length (bp) | Tm (°C) | E (%)  | R² |
|-------------------|------------------------------------|-------------------------|----------------------|---------|--------|----|
| GAPDH             | Glyceraldehyde-3-phosphate dehydrogenase | GCTGAGATTTCCTTGATGGGGAG  
Rev: CTTGTTGGTAGAATTCTACATGGCA | 101 | 58.7/58.5 | 97.90 | 0.998 |
| eIF               | Eukaryotic translation initiation factor | TCAGTTTACTGCTGGGACATCG  
Rev: GGTTCACTACATGGGCAAGCCCTCG | 181 | 60.1/61.3 | 93.58 | 0.992 |
| EF-1α             | Elongation factor 1-alpha           | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 117 | 59.2/59.9 | 102.78 | 0.995 |
| SAND              | SAND family protein                | CTCAGTTTACTGCTGGGACATCG  
Rev: GGTTCACTACATGGGCAAGCCCTCG | 259 | 58.9/59.4 | 94.18 | 0.998 |
| PP2A              | Serine/threonine-protein phosphate 2A | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 208 | 58.8/59.0 | 103.91 | 0.995 |
| hnRNP             | Heterogeneous nuclear ribonucleoprotein | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 187 | 59.2/61.1 | 99.36 | 0.996 |
| UBCE              | Ubiquitin-conjugating enzyme        | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 203 | 60.4/59.0 | 93.36 | 0.996 |
| RPL5              | 60S ribosomal protein L5            | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 150 | 58.2/61.2 | 93.62 | 0.994 |
| PTBP2             | Polyribosyl tract-binding protein homolog 2 | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 115 | 60.4/59.0 | 93.36 | 0.996 |
| TBP               | TATA binding protein                | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 146 | 59.9/58.4 | 101.57 | 0.967 |
| TUB               | Beta-1 tubulin                     | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 116 | 58.3/58.4 | 98.83 | 0.995 |
| ACT2              | Actin-related protein 2             | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 149 | 60.1/61.2 | 102.79 | 0.995 |
| MDH               | Malate dehydrogenase               | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 119 | 58.7/59.4 | 95.76 | 0.998 |
| PEPC              | Phosphoenolpyruvate carboxykinase   | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 106 | 60.2/60.8 | 94.86 | 0.996 |
| F-box             | F-box protein                      | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 129 | 58.9/58.8 | 99.03 | 0.994 |
| OSBL              | o-Succinylbenzoate-CoA ligase       | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 143 | 58.9/60.3 | 95.27 | 0.997 |
| ICS                | Isochorismate synthase              | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 218 | 59.2/58.5 | 98.46 | 0.998 |
| IPP1              | Isopentenyl-diphosphate delta-isomerase | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 152 | 59.3/58.9 | 97.35 | 0.997 |
| HMGR              | 3-Hydroxy-3-methylglutaryl A reductase | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 116 | 59.8/58.6 | 93.79 | 0.997 |
| DXS               | 1-Deyoxy-D-xylulose-5-phosphate synthase | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 119 | 58.2/58.8 | 94.86 | 0.996 |
| AACT              | Acetyl-CoA acetyltransferase        | GAGGTTTTGAGGCTGGGATTGGTGCATGGGACATCG  
Rev: GCAAACGATCCACAGAGCG | 249 | 60.0/59.4 | 96.41 | 0.994 |

*bp, Tm, E, and R² indicate base pair, melting temperature, PCR efficiency, and correlation coefficient, respectively. Standard curves of 15 candidate reference genes and six target genes are shown in Additional Figure S1.
harvested and frozen immediately in liquid nitrogen and stored at −80°C until RNA extraction. For drought treatment, plants were subjected to 200 mL of 25% PEG 6000 once a day for seven days. For salinity stress treatment, plants were irrigated with 200 mL of 600 mM NaCl once a day for a week. For cold and heat stress treatments, plants were placed at 4°C and 42°C for two days in the illumination incubator, respectively. For heavy metal and oxidative stress, 500 mM copper sulfate (CuSO₄) and 50 mM hydrogen peroxide (H₂O₂) were applied to plant leaves once for 24 h, respectively. Exogenous H₂O₂ could enhance stress tolerance of many plants by activating the antioxidative system [29]. For hormone treatments, plant leaves were sprayed once with 25 mM MeJA and 5 mM SA for 6 h, respectively. Leaf samples in all of the treatments were cut with alcohol-sterilized scissors and collected in three biological replicates for further study. The plants without treatments were collected as control, and the harvested samples after treatments were washed with MILLI-Q-filtered water and frozen immediately in liquid nitrogen and stored at −80°C until RNA extraction.

2.2. RNA Isolation, Quality Control, and cDNA Synthesis. Total RNA was isolated from collected samples using the EASYspin Plus Complex Plant RNA kit (catalog number: RN53, Aidlab Biotechnologies Co., Ltd, Beijing, China) according to the manufacturer’s instructions and treated with DNase I (catalog number: RN34, Aidlab, Beijing, China) to eliminate DNA contamination. The RNA concentration and purity were determined with a NanoDrop 2000c Spectrophotometer (Thermo Scientific, US), and RNA integrity was checked on 1% agarose gel electrophoresis. The extracted RNA had a 260/280 ratio between 1.9 and 2.1, which was considered to meet the requirements of following experiments. 0.6 µg of total RNA was prepared for synthesizing first-strand of cDNA by the Goldenstar™ RT6 cDNA Synthesis kit (Tsingke, Nanjing, China) according to the manufacturer’s instructions.

2.3. Selection of Candidate Reference Genes and Primer Design. Fifteen candidate reference genes, including ten traditional reference genes (GAPDH, eIF, EF-1α, UBCE, RPL5, TBP, TUB, ACT2, MDH, and PEPC) and five novel reference genes (SAND, PP2A, PTBP2, hnRNP, and F-box), were selected as candidate genes to normalize RT-qPCR experiments by screening genes with relatively stable expression, which was based on their FPKM and fold change values from transcriptome sequencing data in our lab. All data on sequences and FPKM values of screened genes are shown in Additional file 1 and Additional Table S1. The selected candidate reference genes included GAPDH, eIF, EF-1α, UBCE, RPL5, TBP, TUB, ACT2, MDH, PEPC, SAND, PP2A, PTBP2, hnRNP, and F-box that have been reported to be stably expressed in many plants such as soybean [5], *Eucaphis konishii* [11], poplar [30], potato [31], flax [32], wheat [33], maize [34], foxtail millet [35], sorghum [36], pearl millet [37], and cabbage [38].

2.4. Data Analysis. The three most common Microsoft Excel-based statistical softwares (geNorm, NormFinder, and BestKeeper) were applied for the evaluation of the expression stability of 15 selected candidate reference genes across all the experimental sets. Expression levels of the tested genes were determined by the amplification cycle threshold (Ct) values. The raw Ct values could not be used directly by geNorm and NormFinder, which should be converted into relative quantified data “2^{−ΔΔCt}.” ΔCt was calculated by the Ct value of each sample set subtracted with their minimum Ct (ΔCt = sample Ct − minimum Ct). Gene expression stability value (M) and pairwise variation (V) were calculated by geNorm according to the criterion that the smaller M value had the more stability, and V value was used to determine the optimal numbers of reference genes with a cutoff value of 0.15 [8, 39]. NormFinder was not only used to compare the expression differences of candidate reference
### Table 1: Melt Curve Temperatures

| Gene         | Tm (°C) |
|--------------|---------|
| GAPDH        | 79.14   |
| ACT2         | 77.07   |
| EF-1α        | 81.69   |
| eIF          | 79.01   |
| TBP          | 78.86   |
| TUB          | 78.71   |
| PP2A         | 78.71   |
| UBCE         | 79.46   |
| SAND         | 79.16   |

### Figure 2: Continued.
Figure 2: Specificity of primer pairs for RT-qPCR amplification. Melt curves with single peaks produced for all amplicons.

Figure 3: RT-qPCR raw Ct values for candidate reference genes in different samples. The line across the box depicts the median. The box indicates the 25th and 75th percentiles, and whisker caps show the maximum and minimum values. The lower the boxes and whisker, the smaller the variations.
Figure 4: Average expression stability values $M$ of 15 candidate reference genes calculated by geNorm. Total: the mixed samples of all given conditions; PEG: drought treatment; NaCl: salinity treatment; CuSO$_4$: heavy metal treatment; H$_2$O$_2$: oxidation treatment; cold and hot: temperature treatment; MeJA and SA: hormone treatment; tissue: total tissues samples. The least stable genes are on the left and the most stable genes on the right.
genes based on the calculated stability value but also used to calculate the intra- and intergroup variations. Like geNorm, the smaller the M value, the better the stability of internal reference gene. However, the NormFinder program can only select out the most suitable internal reference gene compared to geNorm. BestKeeper was applied with the raw Ct directly to obtain standard deviations (SDs) and coefficient of variation (CV) between pairs of genes, and the criterion for gene stability was smaller CV and SD values. Ultimately, considering the differences in ranking orders of candidate reference genes given by these three different algorithms, the web-based tool RefFinder (http://leonxie.esy.es/RefFinder/) was used to rerank the candidate genes, and the results indicated that the most stably expressed gene has the lowest value [40].

2.5. Validation of Reference Genes and Expression Analysis of Putative Key Genes of Anthraquinone Biosynthesis Pathway. In order to evaluate the reliability of the selected reference genes, we analyzed the relative expression levels of two key enzyme-coding genes OSBL and ICS involved in the anthraquinone biosynthesis pathway in R. yunnanensis [24]. The expression levels of OSBL and ICS were determined and normalized using the two most and one least stable reference genes and their combinations as reference genes under various tissues (root, stem, and leaf) and MeJA treatments. For MeJA treatments, plants were sprayed once with 200 μM MeJa and leaves were then taken as samples after 1, 6, 12 h, and 24 h.

To further confirm the reliability of the proposed reference genes, we selected six putative key genes all identified as differentially expressed genes (DEGs) involved in the known anthraquinone biosynthesis pathway in root and stem leaf of R. yunnanensis. Expression levels of these six target genes were normalized to those of hnRNP and TBP. Moreover, RT-qPCR results of these genes were compared with their relative expression levels exhibited by FPKM values of R. yunnanensis transcriptome data (Table S1). The RT-qPCR experimental method was the same as described above, and the 2^ΔΔCt method was used to calculate their relative expression levels. RT-qPCR data were obtained with three biological replicates and analyzed with variance (ANOVA) followed by Student’s t-test (P < 0.05).

3. Results

3.1. Verification of Amplicons, Primer Specificity, and PCR Amplification Efficiency. The specific amplification of all primer pairs of candidate reference genes was confirmed with regular PCR and RT-qPCR. A single PCR band was found with expected size by 2.0% agarose gel electrophoresis analysis (Figure 1). A single amplification peak for each candidate reference gene was observed in the melting curve (Figure 2). The PCR amplification efficiencies for these genes ranged from 93.36% for PTBP2 to 108.08% for UBCE, and the correlation coefficients varied from 0.999 to 0.990 (Table 1).

3.2. Expression Profile of Candidate Reference Genes. The raw Ct values of the 15 candidate reference genes for RT-qPCR were collected and are shown in Figure 3. The Ct values varied from 17.13 (EF-1α) to 30.55 (TUB), and the average Ct values ranged from 19.40 (EF-1α) to 27.49 (TUB), which indicate that obvious differences exist in the expression profiles. Low Ct values correspond to high expression levels. Therefore, EF-1α exhibited the highest expression abundance and TUB expressed the lowest level. Moreover, Ct values also have shown the differential expression variability, and RPL5 and hnRNP had a relative narrower Ct range than other genes, showing that these two genes might be expressed more stably.

3.3. Expression Stability of Candidate Reference Genes. In order to further confirm the stability of candidate reference genes, different treatments including osmotic stress, salt stress, heavy metal stress, oxidative stress, hormone, and temperature stress and different tissues were selected for the RT-qPCR experiments of 15 candidate reference genes. Then, 1485 Ct values (three biological and technical replicates, Additional Table S2) were obtained for further analysis by geNorm, NormFinder, BestKeeper, and RefFinder.

3.3.1. geNorm Analysis. In geNorm analysis, measurement (M) values of expression stability of the reference genes were generated as the level of pairwise variation for each reference gene with all other control genes until the most stable reference genes were identified [8]. A cut-off M value of 1.5 was recommended for evaluating all genes stability and a lower M value meant a higher stability. In our study, the M values of the candidate genes were all lower than 1.5 and were ranked by average expression stability values of the reference genes. As shown in Figure 4, all of the most stable reference genes in the 15 candidate genes were not identical under different treatments and tissues. The most stable genes were hnRNP for the groups of tissue, PEG, and cold treatments, RPL5 for the groups of CuSO4, MeJA, and hot treatments, TBP for the groups of total (all the samples were mixed), H2O2 and SA treatments, and EF-1α for the NaCl group according to their lowest M values. Correspondingly, the least stable genes were as follows: F-box for the total, CuSO4, MeJA, and NaCl groups, EF-1α, PP2A, PEPC, GAPDH, TUB, and SAND for the PEG, H2O2, hot, cold, SA, and tissue groups, respectively.

However, an interesting finding was that the same reference genes could have different stabilities under various groups such as EF-1α, which was the most stable reference gene in the NaCl group but was the least stable reference gene for the PEG group. Another important information also observed was that MeJA treatment had a greater impact on plants than other treatments based on its wider M value (0.23 (RPL5) to 0.94 (F-box)), while PEG had the least impact with the low span of M value (0.09 (hnRNP) to 0.29 (EF-1α)), meaning that EF-1α was the least stable reference gene in the PEG group, but it could be selected as a reference gene for other experimental conditions because of its low M value. This phenomenon might also be used to explain why EF-1α
could become the most stable reference gene in the NaCl group. Finally, one of the most important findings was that \( hnrNRP \), \( RPL5 \), and \( TBP \) seemed to be the three most stable reference genes according to their number of occurrences on the first four most stable genes in each group (\( hnrNRP \) appeared 10 times and 6 times for \( TBP \) and \( RPL5 \), respectively).

Moreover, multiple reference genes were taken into consideration for obtaining more reliable normalization results according to the calculated pairwise variation (\( V_{n(n+1)} \)). In order to find the appropriate number of reference genes for normalization, an ideal ratio below 0.15 was chosen for the determination on whether additional reference genes should be required [8]. According to the results of this work, two reference genes were sufficient to meet the requirements for normalization analysis of RT-qPCR due to that a pairwise variation value of V2/3 from each sample group was lower than 0.15 (Figure 5). The best combinations were as follows: \( TBP + PP2A \) for total samples and \( TBP + hnrNRP \) for tissues, \( hnrNRP + eIF \) for PEG treatment and \( EF-1\alpha + UBCE \) for salinity stress, \( RPL5 + MDH \) for MeJA treatment and \( TBP + UBCE \) for SA treatment, \( TBP + RPL5 \) for oxidative stress and \( RPL5 + PEPC \) for heavy metal stress, \( RPL5 + hnrNRP \) and \( hnrNRP + F-box \) for hot and cold stress, and respectively.

### 3.3.2. NormFinder Analysis

In order to further ensure the accuracy of experimental analysis by geNorm, NormFinder was used for analyzing expression stability values of the reference genes. Similar to geNorm, the raw data from RT-qPCR could not be used directly and should be transformed [9]. However, the difference between NormFinder and geNorm was that the former consider intra- and intergroup variations for calculating normalization factors using ANOVA to evaluate expression stability. A variation value of each reference gene was given by NormFinder, and lower values also indicate better stability (Table 2). The most stable genes could be found directly under different experimental conditions. \( hnrNRP \) was the most stable gene with the minimum variation in PEG, \( CuSO_4 \), \( H_2O_2 \), MeJA, cold treatment groups, and tissue samples, while \( RPL5 \), \( EF-1\alpha \), \( eIF \), and \( TBP \) were the most stable genes for hot, NaCl, SA stresses, and total samples, respectively.

### 3.3.3. BestKeeper Analysis

Unlike geNorm and NormFinder, the raw Ct data were used directly without conversion by BestKeeper, which compared the expression differences in samples, rather than pairwise comparisons for analyzing the expression stability of the candidate reference genes. Standard deviations (SDs) and coefficient of variation (CV) between pairs of genes were obtained to evaluate the stability of reference genes in each experimental group by BestKeeper. The most stable reference gene was considered with the lowest CV and SD, and these values should be invalid and abandoned if the value of SD was found to be greater than 1. As shown in Table 3, CV±SD values were calculated and ranked by BestKeeper and the stability of the reference genes was gradually decreasing from the top to the end in the table similar to NormFinder. Besides, ten reference genes in group “Total” and eight genes in group “Tissue” were above the cut-off value of 1.0 and could not be applied, while all SD values of other eight groups were under 1.0, except one gene in the PEG group (\( F-box \)), two in the \( CuSO_4 \) group (\( TUB \) and \( PTBP2 \)), four in the MeJA group (\( TUB, F-box, PEPC, \) and \( PP2A \)), and four in the hot group (\( SAND, ACT2, GAPDH, \) and \( MDH \)). Because of the different analytical principles, the most stable reference gene evaluated by BestKeeper differed somewhat from the results of geNorm and NormFinder. In addition, the top five most stable reference genes in each group from BestKeeper also had some relatively consistent rankings with geNorm and NormFinder, especially \( hnrNRP \), \( RPL5 \), and \( TBP \) appeared with the most frequency. Unexpectedly, the top two most unstable genes analyzed by the three softwares might be unified and confirmed in different sample sets such as \( F-box \) and \( GAPDH \) for the total group, \( PEPC \) and \( GAPDH \) for the
Table 2: Gene expression stability across sample sets calculated by NormFinder.

| Rank | Total | PEG | NaCl | CuSO$_4$ | H$_2$O$_2$ | MeJA | SA | Cold | Hot | Tissue |
|------|-------|-----|------|----------|------------|------|----|------|-----|--------|
| 1    | TBP (0.32) | hnRNP (0.03) | EF-1α (0.02) | hnRNP (0.04) | hnRNP (0.04) | hnRNP (0.07) | eIF (0.08) | hnRNP (0.02) | RPL5 (0.03) | hnRNP (0.17) |
| 2    | EF-1α (0.34) | eIF (0.06) | UBCE (0.02) | RPL5 (0.05) | TBP (0.05) | RPL5 (0.09) | hnRNP (0.09) | PTBP2 (0.03) | hnRNP (0.03) | TBP (0.21) |
| 3    | UBCE (0.42) | RPL5 (0.06) | hnRNP (0.02) | PEPC (0.08) | MDH (0.07) | TBP (0.15) | TBP (0.10) | F-box (0.03) | EF-1α (0.05) | MDH (0.41) |
| 4    | MDH (0.49) | MDH (0.06) | TUB (0.04) | EF-1α (0.11) | RPL5 (0.07) | ACT2 (0.22) | EF-1α (0.11) | RPL5 (0.04) | TBP (0.06) | EF-1α (0.41) |
| 5    | RPL5 (0.51) | UBCE (0.13) | TBP (0.06) | MDH (0.12) | eIF (0.08) | MDH (0.29) | UBCE (0.12) | PP2A (0.09) | UBCE (0.09) | RPL5 (0.44) |
| 6    | hnRNP (0.52) | SAND (0.13) | RPL5 (0.11) | TBP (0.13) | ACT2 (0.11) | PTBP2 (0.30) | ACT2 (0.15) | EF-1α (0.11) | PP2A (0.20) | UBCE (0.44) |
| 7    | PTBP2 (0.58) | ACT2 (0.13) | MDH (0.15) | eIF (0.15) | TUB (0.16) | EF-1α (0.30) | PEPC (0.17) | MDH (0.13) | PTBP2 (0.22) | F-box (0.44) |
| 8    | eIF (0.58) | PTBP2 (0.15) | SAND (0.18) | ACT2 (0.17) | UBCE (0.16) | SAND (0.33) | RPL5 (0.18) | UBCE (0.14) | TUB (0.29) | PEPC (0.47) |
| 9    | PP2A (0.60) | TBP (0.15) | PEPC (0.20) | SAND (0.19) | F-box (0.17) | TUB (0.37) | SAND (0.21) | PEPC (0.16) | eIF (0.32) | ACT2 (0.50) |
| 10   | PEPC (0.63) | PP2A (0.16) | PP2A (0.21) | PP2A (0.24) | PTBP2 (0.19) | eIF (0.44) | PP2A (0.22) | TUB (0.18) | F-box (0.33) | PP2A (0.52) |
| 11   | TUB (0.65) | F-box (0.18) | PTBP2 (0.22) | UBCE (0.24) | SAND (0.20) | UBCE (0.55) | PTBP2 (0.22) | eIF (0.20) | SAND (0.35) | TUB (0.66) |
| 12   | ACT2 (0.67) | GAPDH (0.20) | ACT2 (0.24) | GAPDH (0.24) | EF-1α (0.24) | ACT2 (0.24) | GAPDH (0.30) | TBP (0.20) | ACT2 (0.39) | GAPDH (0.66) |
| 13   | SAND (1.21) | PEPC (0.22) | eIF (0.38) | PTBP2 (0.54) | GAPDH (0.24) | GAPDH (0.80) | F-box (0.31) | ACT2 (0.28) | GAPDH (0.40) | eIF (0.75) |
| 14   | GAPDH (1.23) | TUB (0.23) | GAPDH (0.45) | TUB (0.63) | PEPC (0.26) | PP2A (0.81) | MDH (0.41) | SAND (0.28) | MDH (0.64) | PTBP2 (0.84) |
| 15   | F-box (1.25) | EF-1α (0.23) | F-box (0.47) | TUB (0.64) | PP2A (0.29) | F-box (0.116) | TUB (0.42) | GAPDH (0.31) | PEPC (0.79) | SAND (2.08) |
PEG group, GAPDH and elF for the NaCl group, TUB and PTBP2 for the CuSO4 group, GAPDH and EF-1a for the H2O2 group, F-box and PP2A for the MeJ A group, MDH and GAPDH for the SA group, GAPDH and SAND for the cold group, MDH and ACT2 for the hot group, and SAND and elF for the tissue group.

3.3.4. RefFinder Analysis. Taking into account the discrepancy on stability analysis among different gene, RefFinder program was applied for reanalyzing the 15 reference genes based on the geometric mean of their attributed weight, which used geNorm, Normfinder, BestKeeper, and the comparative ΔCt method to rank and compare candidate reference genes [41] (Table 4). The ranking order of the top five most stable and unstable reference genes obtained by RefFinder was broadly in consistent with the results provided by geNorm and NormFinder and had slight differences with the results from BestKeeper. For instance, under all given experimental conditions, the top two most unstable genes generated by RefFinder appeared in the top five least stable genes yielded by geNorm, Normfinder, and BestKeeper. Ranking orders of the stability of 15 reference genes were summarized in order to better observe the rankings of the analysis results from the four softwares (Additional Table S3).

3.4. Validation of Reference Genes and Expression Analysis of Putative Key Genes of Anthraquinone Biosynthesis Pathway. To confirm the stability of reference genes in this study, two key enzyme-coding genes OSBL and ICS involved in the anthraquinone biosynthesis pathway in Rubiaceae [24] were selected for analyzing the relative expression levels under different tissues and MeJA treatments with two unstable reference genes (SAND and F-box), three stable reference genes (hmRNP, TBP, and PTBP2), and their combinations as reference genes according to the comprehensive rankings of reference genes by four software programs mentioned above. As depicted in Figures 6(a) and 6(b), slight differences were produced in the relative expression levels of both OSBL and ICS when hmRNP, TBP, and hmRNP + TBP were used for the normalization in the group of different tissues. However, when using SAND as the normalization factor, OSBL and ICS exhibited significant differences in their relative expression levels of root and leaf samples compared with those normalized by the other three genes. The expression levels of OSBL and ICS were the highest in root, followed by leaf, and lowest in stem. For MeJA treatments (1 h, 6 h, 12 h, and 24 h), differences also emerged in the relative expression levels of both OSBL and ICS at each corresponding time point when normalized with hmRNP, RPL5, and hmRNP + RPL5. Nevertheless, extremely significant differences were observed when using the unstable gene F-
Table 4: Comprehensive ranking of 15 candidate reference genes integrated by RefFinder.

| Rank | Total | PEG | NaCl | CuSO₄ | H₂O₂ | MelA | SA | Cold | Hot | Tissue |
|------|-------|-----|------|-------|------|------|----|------|-----|--------|
| 1    | TBP (1.19) | hnRNP (2.34) | EF-1α (1.68) | hnRNP (1.97) | PTBP2 (2.11) | RPL5 (1.68) | TBP (1.97) | RPL5 (2.14) | RPL5 (2.06) | PTBP2 (2.34) |
| 2    | hnRNP (3.08) | MDH (3.03) | UBCE (2.45) | RPL5 (2.58) | hnRNP (2.21) | MDH (1.86) | hnRNP (2.45) | PTBP2 (3.56) | hnRNP (2.34) | hnRNP (2.94) |
| 3    | EF-1α (3.56) | eIF (3.83) | hnRNP (3.57) | PEPC (3.08) | TBP (2.82) | hnRNP (2.43) | UBCE (2.99) | hnRNP (3.83) | TBP (3.94) | TBP (3.13) |
| 4    | PTBP2 (3.83) | RPL5 (3.87) | TUB (4.00) | MDH (4.40) | MDH (4.47) | ACT2 (3.46) | eIF (3.13) | EF-1α (3.98) | EF-1α (4.28) | EF-1α (4.12) |
| 5    | RPL5 (4.23) | TBP (4.43) | TBP (4.40) | EF-1α (4.43) | RPL5 (4.95) | TBP (5.42) | SAND (5.05) | PTBP2 (5.05) | TBP (3.63) | UBCE (4.23) |
| 6    | UBCE (4.82) | EF-1α (5.19) | RPL5 (4.74) | TBP (6.00) | F-box (6.45) | PTBP2 (5.89) | EF-1α (5.48) | PP2A (4.90) | F-box (5.61) | MDH (6.19) |
| 7    | MDH (6.45) | ACT2 (6.15) | MDH (7.48) | SAND (6.34) | PEPC (6.84) | EF-1α (7.36) | ACT2 (6.24) | UBCE (6.05) | PP2A (5.73) | F-box (6.21) |
| 8    | eIF (6.96) | SAND (6.16) | F-box (7.62) | eIF (7.24) | eIF (7.14) | SAND (7.52) | PEPC (7.65) | PEPC (6.70) | eIF (5.83) | PP2A (6.67) |
| 9    | TUB (7.88) | UBCE (7.33) | PEPC (7.98) | F-box (7.62) | ACT2 (7.24) | eIF (7.84) | PP2A (8.57) | ACT2 (6.71) | PTBP2 (6.09) | PEPC (6.93) |
| 10   | PTBP2 (9.08) | PTBP2 (8.74) | PTBP2 (8.21) | ACT2 (8.38) | PP2A (9.06) | TUB (9.93) | TUB (9.06) | TUB (6.83) | TUB (8.71) | RPL5 (6.96) |
| 11   | PEPC (10.70) | TUB (10.00) | SAND (9.46) | PP2A (8.57) | TUB (9.19) | GAPDH (10.70) | RPL5 (9.16) | MDH (9.32) | PEPC (10.00) | GAPDH (9.00) |
| 12   | SAND (12.20) | F-box (10.60) | ACT2 (11.00) | GAPDH (10.60) | UBCE (9.74) | UBCE (11.20) | PTBP2 (11.50) | TUB (10.50) | SAND (11.00) | ACT2 (10.20) |
| 13   | ACT2 (12.20) | PTBP2 (12.70) | PTBP2 (12.20) | UBCE (12.00) | SAND (11.70) | PEPC (12.00) | F-box (12.50) | eIF (12.50) | GAPDH (12.70) | TUB (12.50) |
| 14   | GAPDH (14.20) | GAPDH (12.20) | eIF (13.20) | PTBP2 (13.50) | EF-1α (12.50) | PP2A (14.20) | GAPDH (12.70) | SAND (14.00) | ACT2 (12.70) | eIF (14.00) |
| 15   | F-box (14.70) | PEPC (13.50) | GAPDH (14.20) | TUB (14.00) | GAPDH (14.00) | F-box (14.50) | MDH (14.20) | GAPDH (15.00) | MDH (14.20) | SAND (14.70) |

*RefFinder exploits computational programs (such as BestKeeper, geNorm, Normfinder, or the comparative delta-ct method) to rank and compare candidate reference genes. The values following the genes indicate the geometric mean of the attributed weights measured by this software for the overall final ranking.
box as a reference gene for normalizing the relative expression levels of OSBL and ICS in 1h, 6h, 12h, and 24h samples, respectively (Figures 6(c)–6(d)). Therefore, incorrect results would be obtained when using the improper reference gene for the normalization of target genes.

In our study, six putative key genes involved in the anthraquinone biosynthesis pathway were analyzed. The KEGG enrichment analysis (ko00130) showed that anthraquinones could be biosynthesized by two pathways consistent with the previous reports [24]. To better determine the accuracy and applicability of the selected reference genes and understand the expression patterns of key genes in the anthraquinone biosynthesis pathway, hnRNP and TBP were selected as reference genes for RT-qPCR data normalization of relative expression levels of six putative key genes involved in the anthraquinone biosynthesis pathway in the root and stem leaf of R. yunnanensis (Table 5; Additional file 1 and Table S1). The RT-qPCR and FPKM results were compared and presented in Figure 7, revealing that the relative expression levels of six target genes were consistent with differential expression patterns in the root and stem leaf of R. yunnanensis and their expression levels in root were higher than those in stem leaf.

4. Discussion

Our current research on R. yunnanensis has been focused in the fields of the extraction and separation of chemical compositions and pharmacological activities [15–17, 19–23] but still not focused on its molecular function and gene expression. Therefore, de novo transcriptome sequencing of R. yunnanensis had been completed in our group for further studies concerning about the biosynthesis of secondary metabolites and related gene function mining (unpublished). To obtain accurate and reliable results for the realization of these goals, a stable and suitable reference gene would be selected and evaluated for the normalization of gene expression analysis by RT-qPCR in our research.

An ideal reference gene should be stably expressed in all samples under a range of given experimental conditions to ensure the accuracy and reproducibility of measurement of
Table 5: Six putative key genes involved in the biosynthetic pathways of anthraquinones in *R. yunnanensis*.

| Enzyme name                                      | Abbreviation | EC number    | Query ID                                      |
|--------------------------------------------------|--------------|--------------|-----------------------------------------------|
| Shikimate pathway key genes forming rings A and B |               |              |                                               |
| Isochorismate synthase                           | ICS          | 5.4.99.6     | TRINITY_DN125672_c2_g2                       |
| o-Succinylbenzoate-CoA ligase                    | OSBL         | 6.2.1.26     | TRINITY_DN125216_c2_g1                       |
| Isopentenyl-diphosphate isomerase                | IPPI         | 5.3.3.2      | TRINITY_DN131965_c2_g1                       |
| Terpenoid pathway key genes forming ring C       |               |              |                                               |
| Acetyl-CoA acetyltransferase                      | AACT         | 2.3.1.9      | TRINITY_DN130086_c0_g2                       |
| 3-Hydroxy-3-methylglutaryl-coenzyme A reductase   | HMGR         | 1.1.1.34     | TRINITY_DN129754_c4_g1                       |
| 1-Deoxy-D-xylulose 5-phosphate synthase           | DXS          | 2.2.1.7      | TRINITY_DN124410_c3_g1                       |

Figure 7: RT-qPCR validation of relative expression levels of six putative key genes involved in anthraquinone biosynthesis pathways using *hnRNP* and *TBP* as reference genes in the root and stem leaf (SL) of *R. yunnanensis*. Columns indicate relative expression levels of six genes calculated by the FPKM method (left y-axis) (Additional Table S1); lines indicating the relative expression levels were obtained by RT-qPCR (right y-axis) (2\(^{-\Delta \Delta C_{T}}\) method). Error bars indicate the standard deviation of mean values of three repeats.
target genes expression abundance. However, no universally applicable reference genes with an invariant expression are available, which made it particularly important to screen stable reference genes. Indeed, the 15 candidate reference genes showed a relatively wide range of expression profiles in our study, confirming again that no single gene could be used for normalization in all the samples of different tissues and various experimental conditions, similar to the results in Eusaphis konishii [11], Pennisetum glaucum [37], Bixa orellana [42], and Klebsiella pneumoniae [43]. Owning to no reports on reference genes of R. yunnanensis, 15 candidate reference genes including 10 general housekeeping genes (GAPDH, eIF, EF-1α, UBCE, RPL5, TBP, TUB, ACT2, MDH, and PEPC) and 5 novel genes (SAND, PP2A, PTBP2, hnRNP, and F-box) were selected from the R. yunnanensis transcriptome database for evaluation according to some literatures [44–46]. RT-qPCR is considered the most common method for gene expression analyses based on its high sensitivity and specificity. The primer pairs of 15 candidate reference genes were validated and presented high specificity by regular PCR gel electrophoresis and RT-qPCR melting curve. The correlation coefficient ($R^2 > 0.99$) (Additional Figure 1) and PCR efficiency (93.36% to 108.08%) (Table 1) indicated that the curves had a good linear relationship and the amplification conditions were acceptable. Therefore, the standard curve and amplification efficiency of each primer pair meet the requirements of the following experiments, which proved that the designed primers for 15 candidate reference genes were suitable for screening and evaluating the reference genes in R. yunnanensis.

The raw Ct data were obtained by RT-qPCR under the different treatments and tissues and analyzed using four most popular computational programs of gene expression analysis (geNorm, NormFinder, BestKeeper, and RefFinder) for ranking the reference genes. Different rankings of the 15 candidate reference genes were shown according to the four algorithms, indicating the necessity of utilizing more than one program to obtain the best results. Firstly, the expression levels of the candidate genes could be observed directly by the mean Ct values (Figure 3), and the lower values indicated higher expression levels. A prerequisite for accurate normalization of genes was the expression level with a suitable range of Ct values [13], and Ct values ranged from 17.13 (EF-1α) to 30.55 (TUB) in this study were sufficient for experimental needs (Additional Table S2). The stability of genes could be reflected in the ranges of different transcriptional abundances from candidate reference genes. For instance, the expression of RPL5 and hnRNP might be more stable than others according to the relative narrower Ct range. Importantly, these results were somehow consistent with the outcomes calculated by geNorm, NormFinder, BestKeeper, and RefFinder (Figure 4 and Tables 2–4), indicating that the expression level and the stability analysis of reference genes need to be combined with each other. Moreover, these results also revealed that none of 15 reference genes could be expressed constantly in all different conditions and tissues from R. yunnanensis.

For different analysis softwares, geNorm evaluated the stability of reference genes with the average pairwise variation [8], and NormFinder exhibited the expression stability of reference genes by analyzing their intra- and intergroup variation. However, the CV and SD values were the key factors in determining the stability rankings of reference genes obtained by BestKeeper [10]. Thus, the results were also reasonable for some differences in the ranking order shown by the three programs in our research. In geNorm analysis, hnRNP, RPL5, TBP, and EF-1α were the most stable genes and F-box, EF-1A, PP2A, PEPC, GAPDH, TUB, and SAND were the least stable genes under different conditions, which was relatively consistent with the results given by NormFinder, and thus further ensured the accuracy and reliability of the analysis results.

However, F-box, the most unstable reference gene in NaCl and CuSO4 groups from geNorm and NormFinder analyses, was ranked at a top position in BestKeeper analysis. Similar research findings were reported in some literatures on the selection and validation of reference genes in other plant species such as sorghum [36] and pearl millet [37]. Fortunately, BestKeeper still showed some conformance in the top five most stable reference genes with geNorm and NormFinder (Figure 4; Tables 2 and 3). Finally, a widely used web-based tool RefFinder was used to obtain a consensus stability ranking of each candidate gene under the given conditions according to the geometric mean of the attributed stability values of each gene [45, 47]. For RefFinder analysis, hnRNP, RPL5, TBP, and EF-1α were ranked as the top four most stable reference genes similar with the results from geNorm, Normfinder, and BestKeeper. Synthetically, hnRNP, TBP, RPL5, MDH, and EF-1α were the five most frequent and stable genes ranked by the four programs with hnRNP occurring for 37 times, TBP for 26 times, RPL5 for 25 times, MDH for 18 times, and EF-1α for 17 times. Among them, the top three stable genes given by BestKeeper were hnRNP for 8 times, TBP for 5 times, and RPL5 for 4 times. Correspondingly, the top five least stable genes in all groups were also obtained for GAPDH with 36 times, F-box with 20 times, SAND with 19 times, TUB with 17 times, and PEPC with 16 times. Noticeably, the most unstable gene seemed to be determined from the top five least stable genes in each group (Additional Table S3).

Based on the stability value sequence, hnRNP, RPL5, and TBP ranked firstly and were considered the most stable reference genes for analyzing all sample sets. Traditional housekeeping genes have been used as internal reference genes for normalization for a long time because proteins encoded by housekeeping genes were either used to maintain the cell structure or to participate in basic cellular metabolic processes. Theoretically, they could be stably expressed in different types of cells or in different physiological states. However, previous reports have demonstrated that some housekeeping genes have shown poor expression stability in some experimental conditions, which could not be used as internal reference genes for RT-qPCR analysis [48, 49]. In this study, the traditional housekeeping genes RPL5, TBP, MDH, and EF-1α exhibited quite good stability under the given conditions. Unexpectedly, a novel reference gene, hnRNP, vital for the translation of mRNA in the cytoplasm [50] also showed particularly excellent stability in all
sample sets. Furthermore, increasing studies in various species [51–53] tended to apply multiple references rather than a single one considering the reliability and accuracy for the normalization of RT-qPCR data. In our study, the combination of two reference candidate genes has already satisfied the normalization under all the experimental conditions and various tissues (Figure 5).

To confirm the suitability of the reference genes selected from our study, a validation experiment is prerequisite for the evaluation of the reference genes. Three stable reference genes (hnRNP, TBP, and RPL5) and their combinations and two of least stable genes (SAND and F-box) were used for the normalization of OSBL and ICS. The relative expression levels of OSBL and ICS involved in anthraquinone biosynthesis were assessed under different tissues and MeJA treatments. Apparently, only the slight differences were noticed in the relative expression levels of OSBL and ICS between both the groups of different tissues and MeJA treatments when using the pairs of selected stable reference genes alone or their combinations for normalization. However, the opposite results were exhibited when the least stable reference genes were used for the normalization of relative expression levels of OSBL and ICS between different tissues and MeJA treatments, suggesting that the selection and confirmation of suitable and stable reference genes were particularly critical for the proper normalization for the RT-qPCR data in R. yunnanensis. Besides, significant differences were found in which the relative expression levels of OSBL and ICS in root were much higher than those on stem and leaf (Figures 6(a) and 6(b)).

These results might be regarded as a reference to reflect the contents of anthraquinones in R. yunnanensis and used to explain why the root of R. yunnanensis is used as the major medicinal parts. For the MeJA stress, the relative expression levels of OSBL and ICS were markedly upregulated after R. yunnanensis was treated for 1 h and then significantly downregulated in 6 h and 12 h until they tend to rise slowly again between 12 and 24 hours, indicating that some strong responses were produced and enhanced in the relative expression levels of OSBL and ICS of R. yunnanensis at the beginning of the MeJA stimulation for 1 h (Figures 6(c) and 6(d)). This phenomenon might be elucidated by previous literature reports indicating that MeJA plays critical roles in the increase of anthraquinone contents in Rubiaceae plants by upregulating the expression levels of genes related to anthraquinone biosynthetic pathways [24, 54]. It is also known that MeJA could upregulate the expression of defense-related genes [55], so the upregulation of OSBL and ICS in response to MeJA treatment on R. yunnanensis might indicate that anthraquinone biosynthesis is possibly related to its defense response.

Anthraquinones are the most abundant and important bioactive compounds, and their biosynthesis pathway is still unclear in R. yunnanensis. However, major routes of biosynthesis of anthraquinones in Rubiaceae have been reported that its rings A and B are formed via the shikimate pathway and ring C for the terpenoid pathway [24]. In anthraquinone biosynthesis, we selected the six putative key DEGs encoding anthraquinone biosynthetic enzymes based on KEGG enrichment pathway analysis from the transcriptome database of R. yunnanensis and previous literature report [24]. A further comparison was conducted with the RT-qPCR and FPKM results on relative expression levels of six target genes in the root and stem leaf of R. yunnanensis. These two-part results both showed the similar differential expression patterns of six target genes in the root and stem leaf of R. yunnanensis, indicating that hnRNP and TBP could be indeed stable reference genes in the tissue group (Figure 7). The higher expression levels of these target genes in the root than stem leaf might indicate that root was the main organ for anthraquinone synthesis. Consequently, the results of our analysis not only highlighted the importance and necessity of selecting suitable and stable reference genes for the quantitative experiments by RT-qPCR but also established a foundation to explore the biosynthetic pathway of anthraquinones.

5. Conclusions

Our study presented the evaluation of the expression stability of 15 candidate reference genes, including 10 common housekeeping genes and 5 novel genes obtained from the transcriptome data in R. yunnanensis under a wide range of experimental conditions. According to our results, comprehensive rankings order was showed by four widely used programs (geNorm, NormFinder, BestKeeper, and RefFinder), indicating that hnRNP, RPL5, and TBP displayed better stability in all groups. Meanwhile, unstable genes were relatively consistent combining the two most unstable genes by RefFinder with the top four least stable genes by three other softwares under the corresponding conditions. In addition, the expression analysis of OSBL and ICS emphasized the importance of selecting suitable and stable genes for the normalization of gene expression analysis by RT-qPCR. Meanwhile, the expression patterns of putative key genes in anthraquinone biosynthesis pathway of R. yunnanensis were investigated by RT-qPCR for the first time. This study is the first report on the selection and validation of reference genes and will supply an important foundation for the future research on gene expression analyses by RT-qPCR in R. yunnanensis and related species of Rubia.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

The funding bodies were not involved in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.
**Authors’ Contributions**

Shanyong Yi and Qianwen Lin contributed equally to this work. Shanyong Yi, Ninghua Tan, and Yuanyuan Miao designed the experiments. Yuan Yuan Miao, Xuejia Zhang, and Qianwen Lin selected the material. Shanyong Yi, Qianwen Li, and Jing Wang performed the experiments and analyzed the data. Shanyong Yi, Yuanyuan Miao, and Ninghua Tan wrote the paper. All authors read and approved the final manuscript.

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**Supplementary Materials**

Additional Figure S1: standard curves of 15 candidate reference genes and six target genes were directly generated by StepOne™ Real-time PCR system. Additional File 1: sequence information of 15 candidate reference genes and six target genes selected from *R. yunnanensis* transcriptome database. Table S1: information of 15 candidate reference genes and six target genes selected for evaluation and validation based on *R. yunnanensis* transcriptome database. Table S2: raw Ct values in *R. yunnanensis*. Table S3: expression stability ranking of the 15 candidate reference genes estimated by geNorm, NormFinder, BestKeeper, and RefFinder. (Supplementary Materials)

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