Identification and Validation of Reference Genes for Quantitative Real-Time PCR Normalization and Its Applications in Lycium

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Abstract

Lycium barbarum and L. ruthenicum are extensively used as traditional Chinese medicinal plants. Next generation sequencing technology provides a powerful tool for analyzing transcriptomic profiles of gene expression in non-model species. Such gene expression can then be confirmed with quantitative real-time polymerase chain reaction (qRT-PCR). Therefore, use of systematically identified suitable reference genes is a prerequisite for obtaining reliable gene expression data. Here, we calculated the expression stability of 18 candidate reference genes across samples from different tissues and grown under salt stress using geNorm and NormFinder procedures. The geNorm-determined rank of reference genes was similar to those defined by NormFinder with some differences. Both procedures confirmed that the single most stable reference gene was ACN1 for L. barbarum fruits, H2B1 for L. barbarum roots, and EF1α for L. ruthenicum fruits. PGK3, H2B2, and PGK3 were identified as the best stable reference genes for salt-treated L. ruthenicum leaves, roots, and stems, respectively. H2B1 and GAPDH1+PGK1 for L. ruthenicum and SAMDC2+H2B1 for L. barbarum were the best single and/or combined reference genes across all samples. Finally, expression of salt-responsive gene NAC, fruit ripening candidate gene LrPG, and anthocyanin genes were investigated to confirm the validity of the selected reference genes. Suitable reference genes identified in this study provide a foundation for accurately assessing gene expression and further better understanding of novel gene function to elucidate molecular mechanisms behind particular biological/physiological processes in Lycium.

Citation: Zeng S, Liu Y, Wu M, Liu X, Shen X, et al. (2014) Identification and Validation of Reference Genes for Quantitative Real-Time PCR Normalization and Its Applications in Lycium. PloS ONE 9(5): e97039. doi:10.1371/journal.pone.0097039

Editor: Ji-Hong Liu, Key Laboratory of Horticultural Plant Biology (MOE), China

Received February 8, 2014; Accepted April 14, 2014; Published May 8, 2014

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Funding: This work was supported by National Natural Science Foundation of China (Grant no.31100223), Scientific Research Equipment Project of Chinese Academy of Sciences (YY2012227), and Key Laboratory of Plant Resources Conservation and Sustainable Utilization. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Lycium belong to the Solanaceae family and include seven Chinese species, L. chinense Miller, L. ruthenicum Murray, L. truncatum Y. C. Wang, L. barbarum L., L. cylindricum Kuang et A. M. Lu, L. truncatum Y. C. Wang, and L. yunnanense Kuang. Of those, L. barbarum and L. ruthenicum have been extensively used as medicinal and functional foods in China for more than 2000 years. Several Chinese medicinal monographs depict their functions in nourishing the liver and kidney, enhancing eyesight, enriching blood, invigorating sex, reducing rheumatism, curing heart disease and correcting abnormal menstruation. These health-promoting phytochemical compounds, including anthocyanins and carotenoids, accumulate in Lycium fruits [1,2]. At this time, anthocyanin biosynthesis is well known [3] and the anthocyanin regulatory model of BMW tricomplex, formed by bHLH, MYB, and WD40 transcription factors, has been established [4]. The BMW tricomplex is responsible for transcription of several anthocyanin structural genes, including flavonoid 3’hydroxylase (F3’H, EC: 1.14.13.21) and flavonoid 3’5’hydroxylase (F3’5’H, EC: 1.14.13.88). All anthocyanin structural genes were recently isolated and characterized in L. ruthenicum [5]. In addition, petunidin derivatives account for 95% of the anthocyanins in L. ruthenicum fruits [1], suggesting that metabolic flux was largely introduced into the delphinidin branch by F3’5’H enzymes while not into the cyanidin branch by the F3’H enzyme. In the anthocyanin pathway, F3’5’H enzymes compete with F3’H enzymes for the same substrate, dihydrokaempferol, and the anthocyanin pathway in L. ruthenicum fruit has been predicted (Fig. S1).

L. barbarum and L. ruthenicum are widely cultivated and distributed in Northwest China because they are drought-, alkaline-, and salt-resistant. These unique characteristics enable Lycium to prevent soil desertification and improve soil salinity/alkalinity, which is necessary for ecosystem protection and agricultural stability in remote areas of Northwest China. Recently, SIACI transcripts were reported to be increased in tomato (Solanum lycopersicum) roots under salt-stress [6]. Thus, SIACI was thought to be a salt stress-responsive gene marker. Lycium NAC, which is homologous to SIACI, is a candidate gene for investigating molecular mechanisms behind Lycium tolerance to salt stress.
Several fruit-specific genes, including polygalacturonase (PG) [7,8] and Elt [9], have been identified in the tomato and the PG gene encodes major cell wall degradation enzymes [8]. Previous studies showed that ripening is thought to have ripening-specific expression in tomato fruits.

Studies thus far in Lycium species have focused on phytochemical extraction [1,2,11] and medical usage of the extracts [12,13]. Fewer studies have attempted to uncover the underlying molecular biosynthetic and regulatory mechanisms of these medicinal, phytochemical components. Thus, understanding gene expression patterns may offer clues of complex regulatory networks and help us identify genes relevant to novel biological processes such as salt-resistance, fruit ripening and anthocyanin biosynthesis in Lycium. To this end, we screened and evaluated candidate reference genes using quantitative reverse-transcription PCR (qRT-PCR) to measure expression across different samples.

An ideal gene reference should have stable expression in all tissues and under various experimental conditions. Housekeeping genes (HKGs) are usually presumed to be stable in this way, and they are often chosen as candidate reference genes. In the past, HKGs have been extensively used to evaluate gene expression by qRT-PCR without systematic experimental verification. Such universally-used HKGs include elongation factor-1-α (EF-1α) [14–17], actin (ACTIN) [14–17], cyclin (CYC) [14,15,17], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [14–16], ubiquitin (UBQ) [18,19], phosphoglycerate kinase (PGK) [20,21], alpha-tubulin (TUA) [14–16], ubiquitin conjugating enzyme (UBCE) [14,16], and s-adenosyl methionine decarboxylase (SAMDC) [14,16]. Unfortunately, ideal reference genes do not exist [22–25], so using an undefined HKG would not be prudent for normalizing gene expression, especially when only one HKG is used as a reference gene [23].

Several procedures, including geNorm [22] and NormFinder [26], were exploited to identify the optimal reference gene(s) stably expressed in a given set of tissues and experimental conditions. geNorm is defect-sensitive to co-regulated genes among candidates [22], which can be surmounted by NormFinder [26]. The principle difference between geNorm and NormFinder also results in discrepancy in ranking candidate genes [26]. Using these two procedures, an increasing number of reference genes have been identified in various species, including Arabidopsis [27], rice [28], potato [17], Brachypodium [16], flax [15], banana [14], and the common bean [29]. At this time, identification of stable reference genes has only been investigated in L. barbarum ripening fruit [18] but not in other Lycium species or tissues. Additionally, many stably expressing reference genes have been identified in fruit [14] and seeds [28], as well as reference genes that are responsible for different plant developmental stages [14,15,28], responses to biotic stress [14,17], responses to abiotic stress such as cold [17,28,29], drought [29], salinity [17,29], and responses to hormone treatment [14,16].

In this study, with the purpose of identifying suitable reference genes for accurate evaluation of gene expression in ripening fruits or plants under salt stress. Thus, the expression stability of 18 candidate reference genes was calculated using geNorm and NormFinder procedures. Data show that candidate reference genes ranked by geNorm algorithm were similar to those defined by the NormFinder algorithm. Also, expression of the salt-responsive gene MG, the fruit-specific gene PG, and several anthocyanin genes were normalized using the selected reference genes. The reference genes identified here will help researchers more precisely assess gene expression and better understand novel gene function to elucidate specific molecular mechanisms of particular biological/physiological processes in Lycium.

Materials and Methods

Plant Materials and Stress Treatments

Both L. barbarum and L. ruthenicum fruits were harvested from Zhongning County, Ningxia Hui autonomous region, P. R. China and Shibezi County, Xining Uygur autonomous region, P. R. China, respectively. No specific permissions were required for these locations/activities. Fruit samples were divided into five specimens corresponding to five developmental stages (S1–S5) to identify fruit-specific candidate reference genes. In L. ruthenicum fruits, phenotypic changes of S1 fruits converting to S2 were from green to light pink. S3 fruits were dark purple while S4 fruits were black. Furthermore, black S5 fruits were fully expanded. In L. barbarum fruits, green S1 fruits turned light yellow S2 fruits. Also, variegated S3 fruits turned red S4 and red S5 fruits were matured and fully expanded. In addition, L. ruthenicum sepal, petal, stamens, pistils, roots, stems, and leaves were harvested to determine the stability of tissue-specific candidate reference genes. Of those tissues, roots, stems, and leaves were derived from forty-day old L. ruthenicum seedlings. For salt treatment, forty-day old seedlings of L. barbarum and L. ruthenicum were treated with 500 mM for 0, 0.5, 1, 2, 4, 8, and 16 h. Seedlings dissected into roots, leaves, and stems were sampled. Under 21–23°C growth conditions, forty-day old L. barbarum and L. ruthenicum seedlings were cultured under a photoperiod of 16/8 h (day/night). Samples were prepared in triplicate.

Data Acquisition and Statistical Analysis

In this study, 17, 18, and 17 candidate reference genes were used to identify reference gene targets suitable for evaluating gene expression in different tissues, salt-treated seedlings, and developmental fruits of L. ruthenicum, respectively (Table S1). Also, 12 and 9 candidate reference genes were used to identify the reference gene targets suitable for evaluating gene expression in fruits and salt-treated roots of L. barbarum, respectively (Table S1). Candidate reference gene expression was defined as the number of cycles needed to reach a threshold fixed in the exponential phase of PCR (Cp) [30]. As suggested in algorithm manual, Cp values generated by LightCycle480 Detection System (Roche, USA) were transformed using the delta-Cp method. Resulting values were put into geNorm [22] and NormFinder [26] to measure gene expression.

The geNorm procedure calculated the expression stability value (M) for each gene and the pairwise variation (V) of a certain gene compared with remains. Finally, all candidate reference genes were ranked according to their stability in the samples, and the optimal number of reference genes benefit for accurate normalization is suggested [22]. In contrast, NormFinder independently ranks the stability of candidate reference genes and it calculates not only the overall candidate reference gene variation but also the variation between sample subgroups of the sample set [26]. Statistical analyses were performed with ANOVA.
Total RNA Extraction and Template Preparation

Total RNA was extracted from all samples using Trizol kit (Invitrogen, USA). The quality and amount was confirmed with 1% gel electrophoresis and Nanodrop, respectively. Only high quality RNA samples were used for subsequent analyses. Total RNA (1 μg) was reverse-transcribed using PrimeScript RT Reagent Kit with gDNA Eraser (DDR047, TaKaRa), which digested residual RNA sample DNA and reverse-transcribed in one step. cDNA templates were diluted and used (20 ng cDNA per reaction) for qRT-PCR.

Primer Design, Verification of PCR Products and qRT-PCR

Several potential reference genes, including ACTIN, EF-1α, GAPDH, UBQ, SAMDC, H2B, PGK, CYC, TUA, and UBCE, were retrieved from our Lycium EST database (Table S2). According to the reference gene sequence, primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) based on these criteria: GC% of 40–80%, Tm of 60–90°C, length of 18–24 bp, and PCR product length of 150–250 bp (See Table 1 for detailed primer sequence information). Gel electrophoresis was performed to confirm PCR product amplification. In addition, PCR products were cloned and sequenced to confirm amplicon correspondence to the reference gene. qRT-PCR was performed in an optical 96-well plate with a LightCycler480 detection system (Roche systems) and universal cycling conditions (30 s 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C) followed by a dissociation curve to assure specific amplification. For the qRT-PCR reaction, 2 μl 10X SYBR Green Master Buffer (RR047A, Takara, Japan), 10 μM of a gene-specific forward and reverse primer, and 20 μg cDNA template were mixed in each 20 μl reaction. To evaluate PCR efficiency, calibration curves of a cDNA five-fold dilution series were constructed to calculate PCR efficiency and the regression coefficient (R²) for each candidate reference gene.

Verification of Fruit-specific and Stress Responsive Gene Expression

To validate the selected reference gene, relative expression of several genes were measured, including LpPG homologous to SbPG involved in fruit ripening [7,8], Lycium XADC homologous to the salt-stress responsive gene SAMDC [6], and anthocyanin genes. Expression of structural genes (F3'5'H and F3'5'H) and BMW regulatory genes involved in anthocyanin biosynthesis were also investigated. Two sets of primers were designed for both F3'5'H and F3'H genes and used for qRT-PCR assay. PCR products amplified by one set of primers corresponded to the conserved functional domain of F3'5'H or F3'H protein. Thus, this primer pair was used to estimate transcripts of all copies of LfF3'5'H or LfF3'H. Another set of gene-specific primers across the coding region and the 3' untranslated region was designed for LfF3'SH1 or LfF3'SH1 to measure expression of the single copy of LfF3'SH1 or LfF3'SH1. These primers were designed using Primer3 (See Table S3). All experiments were performed in triplicate.

Results

Characterization of Potential Reference Genes in Lycium

To identify stable reference genes suitable for particular tissues and/or experimental condition (Table S1), we retrieved 18 candidate reference genes from the Lycium EST library (Zeng et al. unpublished data). Gene function, primer sequence, amplicon size, and PCR efficiency of these potential reference genes are shown in Tables 1 and S2. Amplification specificity for each gene was confirmed by agarose gel electrophoresis (Fig. S2) and single peaks of melting curves (Fig. S3). A calibration curve was created for each gene tested using a serial five-fold dilution. Subsequently, a significant linear relationship between cycle number and dilution-fold was confirmed for calibration curves in both L. ruthenicum and L. barbarum (Table 1). As shown in Table 1, PCR efficiency for these candidate reference genes ranged from 80.1% for TUA1 to 105.1% for PGK1 in L. ruthenicum and from 87.6% for H2B1 to 108.3% for SAMDC1 in L. barbarum.

Candidate Reference Gene Expression

To identify stable reference genes, expression of 18 candidate reference genes across all samples were detected by qRT-PCR. The variations in candidate reference gene mRNA were revealed by the spectrum of Cp values across all samples. Theoretically, the candidate reference gene with the least amount of variation is the most stable. As shown in Fig. 1A, EF1α, GAPDH2, and TUA2 were more stably expressed across L. barbarum samples than remained reference genes. In L. ruthenicum, transcripts of all candidate reference genes except SAMDC1 and UBQ were stable across all samples (Fig. 1B). Interestingly, the Cp variance of a candidate reference gene in L. barbarum was greater than its counterpart in L. ruthenicum (Fig. 1). These results indicate that no candidate reference gene was consistently expressed across different tissues, experimental treatments, or species. Therefore, identifying the best reference gene target is necessary for normalizing gene expression in a particular experimental system.

Normfinder-determined Rank of Candidate Reference Genes

For NormFinder analysis, inter- and intra-group variations were taken into account, and both results were combined and presented as the stability value of each candidate reference gene. Candidate reference genes with lower stability values are more stably expressed. In L. barbarum fruits, the most stable reference gene was ACTIN1 followed by EF1α and H2B1 (Fig. 2A). The reference gene target was H2B1, which was suitable for salt-treated L. barbarum roots (Fig. 2B). When evaluating across L. barbarum samples, the top two ranked stable reference genes were SAMDC1 and ACTIN1 and the best combination of reference genes were SAMDC2+H2B1 (hereafter reference gene A+B corresponding to the best stable reference gene A combined with B; Fig. 2I). The stability value for SAMDC2+H2B1 is 0.099, which is lower than that of SAMDC1 (0.310). In L. ruthenicum fruit, EF1α and CYC were the top two ranked stable reference genes followed by SAMDC2, H2B1, GAPDH1, and PGK2 (Fig. 2C). PGK3, PGK1, EF1α, CYC were the most four stable reference genes in salt-treated L. ruthenicum leaves (Fig. 2D). In salt-treated L. ruthenicum roots, the stability values of H2B2, H2B1, PGK3, and ACTIN1 were lower than that of remaining candidate reference genes (Fig. 2E). PGK3, CYC, ACTIN1, and UBCE were more stably expressed than other candidate reference genes in salt-treated L. ruthenicum stems (Fig. 2F). The top four ranked candidate reference genes with PGK3, UBCE, ACTIN1, and H2B2 in all salt-treated L. ruthenicum samples were imperfectly identical to that in leaves, roots, and stems (Figs. 2D–2G). Furthermore, the best combination of two genes optimal to evaluate the expression of genes in salt-treated L. ruthenicum seedlings were EF1α+GAPDH1, the stability value of which was 0.112 lower than that of the best single reference gene PGK3 (0.185). Correctly estimating the spatial expression profile of genes in L. ruthenicum is essential to elucidate their biological function. The top four ranked most stable reference genes were PGK1, UBCE, GAPDH1, and H2B2 across stems, leaves, roots, sepal, petals, stamens, and pistils (Fig. 2H). When all samples were analyzed together, the best single stable reference gene was H2B1 and the best combination of two genes were GAPDH1+PGK1.
| Genes   | Primer Name | Primer Sequence (5’-3’) | Product Length (bp) | PCR Efficiency (%)± SD | R² |
|---------|-------------|-------------------------|---------------------|-------------------------|----|
| Actin   | ACTIN1-F    | CTGACCACTTCTCAAGCAGAT   | 162                 | 99.8±1.4                | 1  | 0.9996 |
|         | ACTIN1-R    | TAACACTGAAACGCATTTTC    |                     |                         |    |        |
|         | ACTIN2-F    | CACCTCCAACAGATGTGGATT   | 150                 | 98.0±2.5                | 1  | 0.9984 |
|         | ACTIN2-R    | TCCTGCTCAAGACTCCGACT    |                     |                         |    |        |
| EFTα    | EF1α-F      | GAAAGGTTGCTCTAGATCA     | 180                 | 101.2±1.2               | 1  | 0.998  |
|         | EF1α-R      | CGTCCGTCCTGCTGTTT       |                     |                         |    |        |
| GAPDH   | GAPDH1-F    | CAGTGGAAATGGAGCAGAGG    | 191                 | 104.1±4.2               | 1  | 0.9963 |
|         | GAPDH1-R    | AACTGTTCTCAAGGCTACTACCAC |                     |                         |    |        |
|         | GAPDH2-F    | CAGGTGTTGAGTCAGAGG      | 192                 | 104.4±1.1               | 1  | 0.9939 |
|         | GAPDH2-R    | TGGGACTCCCCTGAACTTT     |                     |                         |    |        |
|         | GAPDH3-F    | GTCACTGGAGATGACAGG      | 208                 | 109.6±2.2               | ND | 0.9874 |
|         | GAPDH3-R    | TCCTGTTACATTATTCAGAAAGG |                     |                         |    |        |
| UBQ     | UBQ-F       | TGAATGTGGTGCTGGAACCTT   | 202                 | 105.6±8.7               | 1  | 0.9910 |
|         | UBQ-R       | GCTGGGCAACATACACAAAAAA  |                     |                         |    |        |
| SAMDC   | SAMDC1-F    | TCTGTAGGCAAGGTCATCGA   | 169                 | 102.8±3.4               | 1  | 0.9983 |
|         | SAMDC1-R    | CATTATTCGACTGCCGAT      |                     |                         |    |        |
|         | SAMDC2-F    | ATCTCCCAACTGCTGCTG     | 200                 | 105.0±3.4               | 1  | 0.9982 |
|         | SAMDC2-R    | TCAGAAATCTGCCAACAGAG    |                     |                         |    |        |
| H2B     | H2B1-F      | AGTGCTTCCTGGTGAATTG    | 163                 | 96.3±2.1                | 1  | 0.9944 |
|         | H2B1-R      | TGGGAAATACCCTGCCGATTGTC |                     |                         |    |        |
|         | H2B2-F      | GTGGTGGCTCTGGTGAAAT     | 156                 | 102.9±8.1               | 1  | 0.9678 |
|         | H2B2-R      | TCAAACAGAAACCTGAAAGAG   |                     |                         |    |        |
| PGK     | PGK1-F      | GCGACTGCGGAGGAGATGAG    | 154                 | 105.3±1.4               | ND | 0.9975 |
|         | PGK1-R      | CAGAAGTGCAAAAGAAAGAAA   |                     |                         |    |        |
|         | PGK2-F      | CAGTGGGTAAGCTGAAAGG     | 150                 | 100.8±1.1               | 1  | 0.9943 |
|         | PGK2-R      | ACCTAACATTTCCAACTGGA    |                     |                         |    |        |
|         | PGK3-F      | ATGCTGCGGAGCAGTAA       | 154                 | 96.3±3.9                | ND | 0.9948 |
|         | PGK3-R      | AAAATGATGGCACCACACAGG   |                     |                         |    |        |
| CYC     | CYC-F       | AATTGCGTGAAGGAAAACAT    | 172                 | 89.1±0.4                | ND | 0.9969 |
|         | CYC-R       | TCACAGGCTTCTAAGATCTGACAA |                     |                         |    |        |
| TUA     | TUA1-F      | TGAAAGCAGAGAAGCTGGG     | 161                 | 88.1±2.7                | ND | 0.9971 |
|         | TUA1-R      | TCGCACGACTAGGAAGAAACCC  |                     |                         |    |        |
|         | TUA2-F      | GATGGTGAGGGCGATGAG      | 153                 | 104.7±0.9               | ND | 0.9995 |
|         | TUA2-R      | TCAGCAGCAGAAATCATGG     |                     |                         |    |        |
Table 1. Cont.

| Product Length (bp) | PCR Efficiency (%) ± SD R² | Primer Name | Primer Sequence (5'-3') |
|---------------------|-----------------------------|-------------|-------------------------|
| 157                 | 95.2 ± 7.8                  | UBCE-F      | TTCCCAACTTGGTTGTTGCT    |
|                     |                             | UBCE-R      | ACCAGAGCAGGGATGACAAC    |

Note: ND, not determined. Lr, L. ruthenicum; Lb, L. barbarum.

doi:10.1371/journal.pone.0097039.t001

Validation of Target of Reference Genes

To confirm whether normalization by different candidate reference genes altered qRT-PCR-measured expression of genes of interest, expression of several genes related to salt stress response, anthocyanin biosynthesis, and fruit ripening were analyzed.

For salt stress, a Lycium NAC transcription factor was selected to validate its expression level in salt-treated Lycium roots. Amino acid sequence analysis revealed that Lycium NAC was 85% identical to Solanum lycopersicum NAC (SINAC, NP_001234482), which was previously identified as a salt stress-responsive genes in tomato roots [6]. In L. ruthenicum roots, the top two ranked reference genes PGK3 and H2B1, recommended by geNorm and/or NormFinder (Fig. 2E and 3E), were chosen to evaluate Lycium NAC expression (Fig. 5A). SAMDC1, serving as an unstable reference gene, was also used to comparatively assess NAC expression (Fig. 5A). As shown in Fig. 5A, Lycium NAC expression revealed by qRT-PCR using reference genes PGK3, H2B1, PGK3+H2B1, and SAMDC1 were similar.
However, the fold-change of LrNAC expression at 8 h compared to 16 h was 1.89 for PGK3, 2.24 for H2B1, and 2.02 for PGK3/H2B1, which were lower than that of 5.65 for SAMDC1 (P<0.01; Fig. 5A). These results indicate that exact expression of NAC as revealed by stable reference genes PGK3 and/or H2B1 were more precise than those calculated using the unstable gene SAMDC1. In L. barbarum roots, H2B1 and UBQ, identified by NormFinder and/or geNorm as the best stable reference genes, were utilized as internal controls to estimate LrNAC expression (Fig. 5B). Also, LrNAC expression was estimated with the unstable reference gene SAMDC1 as an internal control. As presented in Fig. 5B, the qRT-PCR-determined expression of LrNAC using stable reference genes H2B1, UBQ or H2B1+UBQ as internal control were in agreement, which is a different finding from those revealed by the unstable reference gene SAMDC1. Summariy, in salt-stress treated Lycium seedling roots, more precise and reliable results were offered by stable reference genes systematically identified by geNorm and NormFinder compared to unstable reference gene (Fig. 5).

To identify candidate fruit-specific expression genes in L. ruthenicum, an EST sequence encoding PG homologous to tomato PG, which was previously reported to be fruit-specific expression [8,9,31], was retrieved from a Lycium EST library. Expression of LrPG in roots, stems, leaves, flowers, and ripening fruits was investigated using several reference genes recommended by NormFinder and/or geNorm (Fig. 6). With regard to the selection of reference genes, H2B1/EF1a and H2B1 and PGK1 were recommended as the most stable top two ranked reference genes across all samples by geNorm and NormFinder, respectively (Fig. 2J and 3J). Additionally, NormFinder predicted that the stability value of the best combination of two genes GAPDH1+ PGK1 was lower than that of the single most stable gene H2B1, suggesting that GAPDH1+PGK1 was more stable than H2B1. Consequently, these candidate reference genes were selected to evaluate the expression of LrPG in roots, stems, leaves, flowers, and stage S1 fruits (Fig. 6A). As shown in Fig. 6A, LrPG transcripts were undetectable in roots and stems and were highly expressed in stage S1 fruits followed by flowers and leaves when using the reference genes H2B1, GAPDH1, H2B1+EF1a, and GAPDH1+PGK1 as normalization factors. In ripening fruits, expression of LrPG was normalized by EF1a, UBCE, and CYC, which were the most stable top two ranked reference genes (Figs. 2C and 3C). Normalized expression of LrPG was identical using the three reference genes. Furthermore, LrPG transcripts were increasingly enhanced and peaked at stage S4 (Fig. 6B). Summarily, LrPG was abundantly expressed in fruits, especially at stage S4 prior to the expansion stage S5 (Fig. 6), suggesting that LrPG was involved in L. ruthenicum fruit ripening.

For anthocyanin biosynthesis in L. ruthenicum fruits, several genes, including structural genes (F3'5'H and F3'H) and regulatory genes homologous to petunia AN2, AN11, JAF13, and AN1, were used as target genes of interest to demonstrate the utility of the validated target reference genes in qRT-PCR (Fig. 7). To better understand underlying molecular mechanism of most metabolites being introduced to the delphinidin branch in L. ruthenicum fruits, we measured expression of LrF3'5'H and LrF3'H during fruit development. Expression level of these genes was

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**Figure 1.** RNA transcription levels of candidate reference genes presented as Cp mean value in different samples of L. barbarum (A) and L. ruthenicum (B). doi:10.1371/journal.pone.0097039.g001
Figure 2. Rank of candidate reference genes by NormFinder procedure based on data generated by qRT-PCR analysis in L. barbarum fruits (A), L. barbarum roots treated by salt (B), L. ruthenicum fruit (C), L. ruthenicum leaves treated by salt (D), L. ruthenicum roots treated by salt (E), L. ruthenicum stems treated by salt (F), L. ruthenicum leaves, roots, and stems treated by salt (G), different L. ruthenicum tissues (H), all L. barbarum samples (I), and all L. ruthenicum samples (J).
doi:10.1371/journal.pone.0097039.g002
Figure 3. Rank of candidate reference genes by geNorm procedure based on data generated by qRT-PCR analysis in *L. barbarum* fruits (A), *L. barbarum* roots treated by salt (B), *L. ruthenicum* fruit (C), *L. ruthenicum* leaves treated by salt (D), *L. ruthenicum* roots treated by salt (E), *L. ruthenicum* stems treated by salt (F), *L. ruthenicum* leaves, roots, and stems treated by salt (G), different *L. ruthenicum* tissues (H), all *L. barbarum* samples (I), and all *L. ruthenicum* samples (J).

doi:10.1371/journal.pone.0097039.g003
normalized using the most stable reference gene, EF1a, a moderately stable reference gene, ACTIN1, and an unstable reference gene, GAPDH3. With EF1a as an internal control, expression profiles of these genes were consistent with those normalized by the moderately stable reference gene ACTIN1 (Fig. 7). However, gene expression normalized with the unstable reference gene (GAPDH3) was significantly different from those estimated with EF1a or ACTIN1 (Fig. 7). Furthermore, gene transcripts were over-estimated at stage S4 and/or S5 when GAPDH3 was used as an internal control. These results suggest that using an unstable reference gene(s) may give rise to less accurate or misleading results.

Discussion

*L. barbarum* and *L. ruthenicum* are widely utilized as traditional Chinese medicine (TCM) because of their purported health-promoting phytochemical compounds [1,2,32,33]. Such phytochemicals include carotenoids and anthocyanins and which are thought to have anti-aging, neuroprotective [12], anti-diabetic, anti-glaucoma, anti-oxidant, immunomodulatory, anti-tumor and cytoprotective effects. To date, the biosynthetic and regulatory mechanisms of these phytochemicals are unclear and how *Lycium* resists abiotic stresses (saline and alkaline conditions) remains uncertain. Both *L. barbarum* and *L. ruthenicum* plants are tolerant to salt and alkalinity, so precise evaluation of gene expression is needed to identify the functional genes involved in physiological and/or biological processes that mediate these stress-resistant properties.

qRT-PCR has been used to quantify gene expression [17], and reliable qRT-PCR results depend on the choice of a stable reference gene(s). However, increasing evidence indicates that HKG expression, which is used as a candidate reference gene, is variable across different tissue samples and/or experimental conditions [24,25]. Thus, systematically identifying stable reference genes prior to their use in qRT-PCR normalization is necessary [27,28]. Because few reference genes have been systematically evaluated in *L. barbarum* ripening fruits [18], we identified suitable reference genes in *Lycium* samples from different tissues and in plants grown under salt stress.

First, we measured expression stability of 18 candidate reference genes across seven experimental sets (Table S1). NormFinder and geNorm procedures were used to calculate and identify the best suitable reference genes for specific investigations (Figs. 2 and 3). Generally, both geNorm and NormFinder identify the same subset of reference genes but rank them differently in certain tissues or under stresses (Figs. 2 and 3). For instance, although geNorm and NormFinder identified ACTIN1, EF1a, and H2B1 in *L. barbarum*
fruits as the most stable reference genes, their ranks were different in each algorithm (Figs. 2A and 3A). Similarly, PGK3, CYC, ACTIN1, and UBCE which differed with respect to rank were identified as the most stable reference genes in salt-treated L. ruthenicum stems with these two procedures (Figs. 2F and 3F). This situation agree with a previous study of L. barbarum fruits [18]. However, in citrus case, remarkable divergence was observed in the most stable reference genes ranked by geNorm and NormFinder [34]. Divergence in the top-ranked stable reference genes predicted by the two procedures could be attributed to differences in statistics used by geNorm and NormFinder. Previous research indicates that GAPDH/EF1a is the best stable reference gene for normalizing gene expression in L. barbarum ripening fruits [18]. Noticeably, GAPDH and EF1a were in a previous L. barbarum study [18] and have high and low sequence identity with L. ruthenicum GAPDH3 and EF1a, respectively. However, GAPDH3 was identified as the unstable reference gene in L. ruthenicum fruits (Figs. 2C and 3C). Differences in expression stability between L. ruthenicum GAPDH3 and L. barbarum GAPDH may be species-specific. Alternatively, the set of candidate reference genes investigated possibly affect the rank of reference genes. In our study, the best stable reference genes in L. barbarum fruits were EF1a and EF1a/ACTIN1 as predicted by NormFinder and geNorm, respectively. The stability value of the best combination genes GAPDH1+PGK1 recommended by NormFinder was lower than that of the best single reference gene H2B1. Double asterisks show statistical significant differences between flowers or S1 fruits and leaves at P<0.01 level. B, the top2-ranked most stable reference genes EF1a and CYCNormFinder-determined and UBCE and EF1a/NormFinder-identified were used as normalization factors to evaluate UrPG expression in ripening fruits. Double asterisks show statistical significant differences between S2–S5 fruits and S1 fruits at P<0.01 level. doi:10.1371/journal.pone.0097039.g006

Figure 5. Relative quantification of Lycium NAC gene using validated reference genes for normalization in salt-treated L. ruthenicum (A) and L. barbarum (B) roots. The validated reference genes used as normalization factor were PGK3 and H2B1 for L. ruthenicum and H2B1 and UBQ for L. barbarum, which were most stable reference genes recommended by geNorm and/or NormFinder procedure(s). The most unstable gene SAMDC1 was also selected as a comparative normalization factor to evaluate NAC gene expression in both Lycium species. Double asterisks show statistical significant differences between samples salt-treated and control sample at P<0.01 level. doi:10.1371/journal.pone.0097039.g005

Figure 6. Relative quantification of a putative fruit-specific expression gene LrPG in different tissues (A) and developmental fruits (B) of L. ruthenicum. A, the validated reference genes were H2B1, PGK1, EF1a, and GAPDH1. Of those, H2B1 and H2B1/EF1a were the most stable reference genes recommended by NormFinder and geNorm, respectively. The stability value of the best combination genes GAPDH1+PGK1 recommended by NormFinder was lower than that of the best single reference gene H2B1. Double asterisks show statistical significant differences between flowers or S1 fruits and leaves at P<0.01 level. B, the top2-ranked most stable reference genes EF1a and CYCNormFinder-determined and UBCE and EF1a/NormFinder-identified were used as normalization factors to evaluate UrPG expression in ripening fruits. Double asterisks show statistical significant differences between S2–S5 fruits and S1 fruits at P<0.01 level. doi:10.1371/journal.pone.0097039.g006

Identification and Application of Reference Genes in Lycium

PLOS ONE | www.plosone.org 10 May 2014 | Volume 9 | Issue 5 | e97039
PGK3, CYC, ACTIN1, UBCE, and GAPDH1 for salt-treated stems (Figs. 2D–2F), which was confirmed by geNorm-determined results (Figs. 3D–3F). Previous studies also document alterations in candidate reference gene rankings in distinct sample sets [14,19,38,39].

To validate the geNorm- and NormFinder-determined results for suitable reference genes in experimental systems, expression of several genes was investigated (Figs. 5–7). Previous study demonstrated that the salt-sensitive gene SlNAC1 is significantly upregulated in tomato roots [6]. As presented in Fig. 5, LrNAC and LbNAC, SlNAC1 homologs, were significantly upregulated by salt stress. Specifically, LrNAC transcripts decreased in the first 1 h followed by a significant increase at 2 h and peaked at 8 h whereas LbNAC transcripts were enhanced at 1 h and almost peaked at 2 h. The divergent expression pattern of Lycium NACs suggested that cis- and/or trans-elements controlling NAC expression responding to salt stress were evolutionarily divergent in Lycium species. In this case, using the unstable reference gene SAMDC1 as an internal control offered less precise expression patterns for LrNAC and incorrect expression profiles for LbNAC (Fig. 5).

In tomato, several genes, including PG [8,9,31] and E8 [40,41], were reported to be relative to fruit ripening. In this study, a unigene encoding LrPG was retrieved from our EST database (Zeng et al., unpublished data) and data show that LrPG was highly
expressed in stage S1 fruit when using the best single reference gene \( H2B1 \), or the best combination of two genes \( GAPDH \)+PGK1 and H2B1+EF1α as an internal control (Fig. 6A). During \( L. \) ruthenicum fruit ripening, \( LiPG \) transcripts were enhanced and peaked at stage S4 (Fig. 6B). These results suggested that \( LiPG \) expression was fruit-specific, and that the \( LiPG \) promoter would be a good choice for investigating the function of genes of interest in \( L. \) ruthenicum fruit.

Petunidins are abundantly accumulated in \( L. \) ruthenicum fruits [1], so expression of structural and regulatory genes involved in anthocyanin biosynthesis was normalized using the most stable, moderately stable, and most unstable reference genes (Fig. 7). As shown in Fig. 7, expression of genes normalized by the unstable reference gene \( GAPDH \) was significantly distinct compared to those normalized by the most stable reference gene EF1α or the moderately stable reference gene ACTIN1, suggesting that normalization using unstable reference gene resulted in misinterpretation of anthocyanin gene expression. These results also indicated that the moderately stable reference gene ACTIN1 was stable enough to precisely calculate the expression of anthocyanin genes in ripening fruit. qRT-PCR data suggested that, except for \( LrF3 \) and \( H2B1 \), all tested gene expressions were enhanced at the first four stages when using \( EF1α \) or \( ACTIN1 \) as a normalization factor (Fig. 7). Furthermore, the fold-change of \( LrF3 \)’ expression at the first four stages was higher than that of \( LrF3 \)’ when using internal gene \( EF1α \) or \( ACTIN1 \) (Figs. 4A–4D), and these data were confirmed with data generated with \( EF1α \) as an internal control (Fig. S4). These results may account for 95% of anthocyanins being petunidin-derivatives in mature fruits of \( L. \) ruthenicum (Figs. 7 and 8, [1]).

**Conclusion**

In conclusion, reference gene targets in different tissues and samples under salt-stress were identified in \( Lycium \). Data show that reference gene ranking as determined by geNorm and NormFinder were similar with minor change. Our results also indicate that \( EF1α \) and \( ACTIN1 \) were the top two most stable reference genes in \( L. \) barbarum fruits, and that \( EF1α \) was the most stable reference gene in \( L. \) ruthenicum fruits. Additionally, \( H2B1 \) and \( H2B2 \) were the best reference genes in salt-treated \( L. \) barbarum and \( L. \) ruthenicum roots, respectively. Expression of \( Lycium \) NAC, \( LiPG \), and genes involved in \( L. \) ruthenicum anthocyanin biosynthesis were analyzed to emphasize the importance of validating reference genes to obtain accurate and reliable qRT-PCR results. Results show that \( LiPG \) expression was fruit-specific, and that \( Lycium \) NACs were upregulated and divergent in expression in response to salt stress. Also, both enhanced anthocyanin gene transcripts and increased ratios of \( LrF3 \)'s transcripts in ripening fruits may have accounted for accumulation of petunidin-derivatives in \( L. \) ruthenicum fruits. Summarily, reference gene targets identified herein will provide a foundation for achieving accurate and reliable qRT-PCR results and help us understand complex molecular mechanisms of \( Lycium \) physiological and biological processes such as salt resistance, fruit ripening, and secondary biosynthesis pathways.

**Supporting Information**

**Figure S1** A predicted anthocyanin biosynthetic pathway in \( L. \) ruthenicum. According to previous result (Zheng et al. 2011), the petunidin-derivatives are the major component of anthocyanins in \( L. \) ruthenicum fruits and the anthocyanin pathway was postulated. The arrow weight indicates the size of metabolic flux. The dashed arrows indicated that BMW tricomplex possibly regulate the transcription of \( F3'H \) and \( F3'5'H \) genes. CHS, chalcone synthase; CHI, chalcone isomerase; \( F3'H \), flavanone 3'-hydroxylase; \( F3'H \), flavonoid 3'-hydroxylase; \( F3'5'H \), flavonoid 3'5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; MT, anthocyanin methyltransferase.

(DOC)

**Figure S2** Electrophoresis analysis of the specificity of primer pairs for RT-PCR amplification. Of 2.0% agarose gel electrophoresis indicated amplification of a specific product of the expected size for each candidate reference genes. 1, \( ACTIN1 \); 2, \( ACTIN2 \); 3, \( EF1α \); 4, \( GAPDH \); 5, \( GAPDH2 \); 6, \( GAPDH3 \); 7, \( UBQ \); 8, \( SAMDC1 \); 9, \( SAMDC2 \); 10, \( H2B1 \); 11, \( H2B2 \); 12, \( PKG1 \); 13, \( PKG2 \); 14, \( PKG3 \); 15, \( CIT \); 16, \( TUA1 \); 17, \( TUA2 \); 18, \( UBCE \); M, DL2000 DNA Marker.

(DOC)

**Figure S3** Dissociation curves for the eighteen reference genes tested in this study.

(DOC)

**Figure S4** The expression level of \( F3'5'H \) relative to \( F3'H \) in \( L. \) ruthenicum fruits. The expression ratio of \( F3'5'H/F3'H \) was quantitatively evaluated by qRT-PCR using primers designed on the basis of functionally conserved domains in \( F3'5'H \) or \( F3'H \) protein while the expression ratio of \( F3'5'H/F3'H \) was quantitatively estimated by qRT-PCR using primers designed across 3’ untranslated region and coding region of \( F3'5'H \) or \( F3'H \).

(DOC)

**Table S1** Experimental design to identify reference genes in \( Lycium \) species. Note: TRG, Target of Reference Genes, *indicated that the number of biological replicates.

(DOC)

**Table S2** Annotation of \( Lycium \) HKGs.

(DOC)

**Table S3** qRT-PCR primer for genes used to validate target of reference genes.

(DOC)

**Author Contributions**

Conceived and designed the experiments: SZ YW. Performed the experiments: SZ YL XL. Analyzed the data: SZ YL. Contributed reagents/materials/analysis tools: MW CL XS. Wrote the paper: SZ YW.
12. Li S, Yang D, Yeung C, Yu W-Y, Chang RC, et al. (2011) Reference gene selection for quantitative real-time PCR in transgenic plants. Plant Mol Biol 11: 651–652.

13. Nicholas F, Smith CS, Schuch W, Bird C, Grierson D (1995) High levels of ripening-specific reporter gene expression directed by tomato fruit polygalacturonase gene-flanking regions. Plant Mol Biol 28: 423–433.

14. Chen L, Zhong HY, Kuang JF, Li JG, Lu WJ, et al. (2011) Reference gene selection for quantitative real-time PCR during Chinese wolfberry fruit development. Plant Physiol 156: 641–653.

15. Lovdal T, Lillo C (2009) Analysis of flavonoids from leaves of cultivated Lycium barbarum. Plant Food Hum Nutr 64: 199–204.

16. Hong SY, Seo PJ, Yang MS, Xiang F, Park CM (2008) Exploring valid reference genes for gene expression studies by reverse transcription quantitative real-time PCR during Chinese wolfberry fruit development. Plant Physiol Biochem 46: 304–310.

17. Nicot N, Hausman J, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. Mol Biotechnol 30: 595.

18. Wang L, Wang Y, Zhou P (2013) Validation of reference genes for quantitative real-time PCR during Chinese wolfberry fruit development. Plant Physiol Biochem 70: 304–310.

19. Zheng H, Chen J, Li C, Chen L, Wu J, et al. (2011) Selection of reliable reference genes for expression studies by reverse transcription quantitative real-time PCR in litchi under different experimental conditions. Plant Cell Rep 30: 641–653.

20. Gu C, Chen S, Liu Z, Shan H, Luo H, et al. (2011) Reference gene selection for quantitative real-time PCR in Glycine max subjected to biotic and abiotic stress. Mol Biotechnol 49: 192–197.

21. Lovdal T, Lillo C (2009) Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Anal Biochem 387: 238–242.

22. Vandenseimple J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: research0034.0031-0034.0011.

23. Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distanti V, et al. (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to 18S rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309: 295–300.

24. Suzuki T, Higgens PJ, Crawford DR (2000) Control selection for RNA quantitation. Biotechniques 29: 332–337.

25. Theelin O, Zorzi W, Lakaye B, De Borman B, Goumans B, et al. (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 73: 291–295.

26. Andersson CL, Jensen JL, Omrøft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64: 5245–5250.

27. Czechowski T, Sutি M, Altmann M, Udvardi MK, Schible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139: 5–17.

28. Naras O, Ivanova A, Nė S, Whelan J (2010) Defining reference genes in Oryza sativa using organ, development, biotic and abiotic transcriptome datasets. BMC Plant Biol 10.

29. Borges A, Tsi SM, Caldas DGG (2012) Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. Plant Cell Rep 31: 827–838.

30. Walker NJ (2002) A technique whose time has come. Science 296: 557–559.

31. Tucker G, Grierson D (1982) Synthesis of polygalacturonase during tomato fruit ripening. Plant 155: 64–67.

32. Yao X, Peng Y, Xu L, Li L, Wu Q, et al. (2011) Phytochemical and biological studies of Lycium medicinal plants. Chem Biodivers 8: 976–1010.

33. Amagase H, Farmsworth NR (2011) A review of botanical characteristics, phytochemistry, clinical relevance in efficacy and safety of Lycium barbarum fruit (Goji). Food Res Int 44: 1702–1717.

34. Liu Z, Ge X, Wu X, Koi S, Chai L, et al. (2013) Selection and validation of suitable reference genes for mRNA qRT-PCR analysis using somatic embryogenic cultures, floral and vegetative tissues in Citrus. Plant Cell Tiss Org 113: 469–481.

35. Reid K, Olson N, Schlosser J, Peng F, Lund S (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6: 27.

36. Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Co 345: 646–651.

37. Dombrowski JE, Martin RC (2009) Evaluation of reference genes for quantitative RT-PCR in Lollum temulentum under abiotic stress. Plant Sci 176: 390–396.

38. Artico S, Nardelli S, Brilliante O, GrossideSa M, Alves-Ferreira M (2010) Identification and evaluation of new reference genes in Gossypium hirsutum for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol 10: 49.

39. Hu RR, Fan CM, Li HY, Zhang QZ, Fu YF (2009) Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 10.

40. Deikman J, Klime R, Fischer RL (1992) Organization of ripening and ethylene regulatory regions in a fruit-specific promoter from tomato (Eysperison coelestis). Plant Physiol 100: 2013–2017.

41. Lincoln J, Fischer R (1988) Diverse mechanisms for the regulation of ethylene-inducible gene expression. Mol Gen Genetics 212: 71–75.