LbCML38 and LbRH52, two reference genes derived from RNA-Seq data suitable for assessing gene expression in Lycium barbarum L.

Lei Gong¹⁺, Yajun Yang²⁺, Yuchao Chen³, Jing Shi², Yuxia Song¹ & Hongxia Zhang³,⁴

For quantitative real-time PCR (qRT-PCR) analysis, the key prerequisite that determines result accuracy is the selection of appropriate reference gene(s). Goji (Lycium barbarum L.) is a multi-branched shrub belonging to the Solanaceae family. To date, no systematic screening or evaluation of reference gene(s) in Goji has been performed. In this work, we identified 18 candidate reference genes from the transcriptomic sequencing data of 14 samples of Goji at different developmental stages and under drought stress condition. The expression stability of these candidate genes was rigorously analyzed using qRT-PCR and four different statistical algorithms: geNorm, BestKeeper, NormFinder and RefFinder. Two novel reference genes LbCML38 and LbRH52 showed the most stable expression, whereas the traditionally used reference genes such as LbGAPDH, LbHSP90 and LbTUB showed unstable expression in the tested samples. Expression of a target gene LbMYB1 was also tested and compared using optimal reference genes LbCML38 and LbRH52, mediocre reference gene LbActin7, and poor reference gene LbHSP90 as normalization standards, respectively. As expected, calculation of the target gene expression by normalization against LbCML38, LbActin7 or LbHSP90 showed significant differences. Our findings suggest that LbCML38 and LbRH52 can be used as reference genes for gene expression analysis in Goji.

Fluorescent quantitative real-time PCR (qRT-PCR) is a fast, accurate method for nucleic acid analysis. Unlike the standard reverse transcription polymerase chain reaction (RT-PCR), which detects the reaction product at the end, qRT-PCR detects and quantifies the amplified target nucleic acid in “real time” by measuring accumulated fluorescent signal during each cycle of polymerization. Therefore, qRT-PCR is more specific, sensitive and reproducible compared with standard RT-PCR¹. However, reference gene is required for qRT-PCR to adjust the initial cDNA levels and transcriptional efficiency to offset the variation in nucleic acid purity and concentration during sample preparation, and to avoid the errors generated during sample treatment². Previous studies demonstrated that very few reference genes were absolutely stable, but were only “relatively” stable under certain conditions in specific types of cells or tissues³-⁴. To date, some reference genes including those encoding actin, α- and β-tubulin, GAPDH, EF1α and ubiquitin have been identified. However, expression of these reference genes varied with different treatments and at different developmental stages of plants, which greatly affected the accuracy of target gene expression evaluation¹,⁵,⁶. Thus, stable reference gene screening and evaluation are essential for functional studies of target genes.

¹Ningxia Key Laboratory for Agrobiotechnology, Agricultural Bio-Technology Center, Ningxia Academy of Agriculture and Forestry Science, 590 Huanghe East Road, Yinchuan, Ningxia Hui Nationality Autonomous Region, 750002 China. ²School of Life Sciences, Ningxia University, 489 Helanshan West Road, Yinchuan, Ningxia Hui Nationality Autonomous Region, 750021 China. ³National Key Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai, 200032 China. ⁴College of Agriculture, Ludong University, 186 Hongqizhong Road, Yantai, 264025 China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Y.X.S. (email: songyx666@163.com) or H.X.Z. (email: hxzhang@sippe.ac.cn)
As an importantly dietary and medicinal plant, Goji (Lycium barbarum L., 2n = 24) is cultivated in the northwest part of China for over 5 millennia due to its strong resistance to abiotic stresses as well as its economic value. Its roots, leaves, and fruits contribute significant medicinal ingredients such as polysaccharide, betaine, carotene and anthocyanin, which function in improving immunity, anti-oxidative stress and anti-tumor ability, scavenging free radicals, as well as promoting sexual function. Current researches on Goji are mainly limited in the isolation, extraction and development of active ingredients. Studies related to pharmacologically active intermediate synthesis and molecular mechanisms underlying plant metabolism, development and stress resistance are still unavailable. Unlike plants from the same Solanaceae family such as tobacco, tomato, pepper and potato, the whole genome data of Goji are still not available. Previously, Liu and co-workers used actin as a reference gene to analyze the expression pattern of genes involved in carotene synthesis in Goji. However, the validity of results is questionable due to the lack of systematic and scientific screening of reference genes.

For plants lacking whole genome information, one of the standard approaches for reference gene identification is to clone gene homologous to the known housekeeping gene identified in other model plants. Alternatively, emerging chip or next-gen sequencing technology provides ample data, which can be used for reliable reference gene screening. In Arabidopsis, a new approach using ATH1 chip was used to screen the super reference genes. They found that a novel reference gene such as PP2A showed stable expression at different developmental stages and under different treatments than the classical reference gene such as actin. Macrae et al. used the spliceosome and proteasome genes from RNA-Seq data to normalize and calibrate target gene expression pattern in human cancer tissues. Similarly, based on the RNA-Seq data of sika deer antlers, Liu et al. evaluated the stability of 16 standard reference genes and 5 expression-stable genes from the sequencing platforms of different tissues and treatments.

In woody plants, screening of stably expressed reference genes using gene expression data from next-generation sequencing platform has been performed in plum and wine grape (Vitis vinifera). But no such kind of research has been done in Goji. Here, the transcriptomic sequencing data from the leaves, flowers and fruits of Goji were systematically assessed, and 8 classical reference genes and 10 stably expressed transcripts with little variation among different tissues and treatments were selected as candidate reference genes. Their expression levels in different organs, at different development stages, and under drought stress condition were investigated by qRT-PCR. The stability of candidate gene expression was evaluated to select the best reference gene, and the newly selected reference gene was tested to normalize and analyze the expression level of a target gene LbMYB1 under different conditions. Our findings provide a foundation for the functional studies of genes in Goji.

### Results

#### Sequencing data analyses.

From the transcriptomic sequencing of 14 sample databases, a total of 8,091,979,192 raw reads were obtained, including 7,514,959,092 clean reads and 67,634,558,280 clean nucleotides after impurity filtration. The average Q20 value was up to 97.6%. We also found 144,250 Unigenes with a total length of 172,036,673 nt after sequence assembly. The average length of Unigene was 1193 nt, and that of N50 was 1885 nt.

#### Candidate reference gene selection.

The mean values of raw fragments, coefficient of variance and annotation of the 18 selected candidate references were listed in Table 1. LbTUB displayed the maximal coefficient of variance (CV) value (1.005) of raw fragments. On the contrary, LbEIF4A showed the minimal CV value (0.079). The CV value variation among different reference genes suggests that these genes have different expression levels in different organs, at developmental stages and under different treatment conditions. Four classical reference genes LbCYC, LbEIF4A, LbEF1β and LbUBQ using our screening criteria (CV < 0.3, mean raw fragments > 500) were identified, suggesting the validity of our approach using raw fragment CV as preliminary screening criteria.

#### qRT-PCR analyses of reference genes.

Ct value reflects the abundance of reference gene expression. The higher the Ct value, the lower the expression level, and vice versa. The Ct values of the 18 reference genes ranged from 17.18 to 25.02 (Table 2). LbHIS3 showed the lowest Ct value, whereas LbSKIP exhibited the highest Ct value. Compared with LbActin7 and the newly identified reference genes LbRH52 and LbCML38, reference genes LbEF1α, LbHSP90 and LbHIS3 manifested a higher expression upon different treatments. In addition, the dispersion level (standard deviation, SD) of Ct values is a schematic indicator of the stability of candidate reference gene expression in all tested samples. Among the 18 candidate reference genes, LbPP2A showed the lowest SD value. LbCML38 and LbRH52 also showed lower SD values (Table 2). In addition, PCR products of these candidate reference genes were checked on 1% agarose gel, and unique amplicons of expected length without distinct dimmers also showed lower SD values (Table 2).

#### Evaluation of reference gene expression stability.

In order to screen the best reference gene or gene combination in different organs, at different developmental stages, and under different treatment conditions, geNorm, NormFinder, BestKeeper and RefFinder were employed to evaluate and rank their expression stability, as shown in Tables S1, S2 and S3.

#### geNorm analysis.

We used geNorm to compare and rank the M value of each candidate reference gene in terms of expression stability. The higher the M value, the lower the stability, and vice versa. The default cutoff value of geNorm software is 1.5. All candidate reference genes showed an M value lower than 1.5 (Fig. 1b). Based on the scores obtained from the 14 samples in different organs, at different developmental stages, and after different treatments, LbCML38 and LbRH52 were chosen as the best reference genes with an M value of 0.374. In leaves, LbCML38 and LbRH52 showed the best expression stability with an M value of 0.273 (Supplementary Table S3). In fruits and flowers, LbTBP demonstrated better stability with an M value of 0.249 and 0.142, respectively.

Under
drought stress condition, LbCYP and LbCML38 were the most stably expressed genes with an M value of 0.32 (Supplementary Table S2). However, commonly used reference genes such as LbCYP, LbActin7 and LbHIS3 showed mediocre expression stability. LbHSP90, LbTUB and LbGAPDH showed poor expression stability with the lowest ranking among these tested candidate reference genes (Supplementary Tables S2 and S3).

We also determined the optimal number of reference genes required under a particular condition by analyzing their paring difference value V_{n\times 1}. Typically, the threshold was set to 0.15 to select the best reference gene. When the paired value is lower than 0.15, additional (n + 1) reference genes are not necessary. After combining the analyses of all samples in different organs, at different developmental stages and after drought stress treatment together, V_{2\times 3} (0.139) was lower than the threshold of 0.15, indicating that the optimal number of reference genes needed was 2 (Fig. 2), and no need to introduce a third reference gene for calibration. The best combination of reference genes was LbCML38 and LbRH52. A combination of LbCYP and LbCML38 is optimal to analyze samples in different organs and at different developmental stages (Supplementary Tables S1, S2 and S3).

**NormFinder analysis.** Similar to geNorm, NormFinder evaluates reference genes by calculating their expression stability. Combined analyses of all samples showed that expression of LbCML38 and LbRH52 was the most stable with values of 0.16 and 0.254, respectively (Fig. 3). In different organs, LbRH52 was the most stably expressed reference gene in leaves (0.079), whereas LbActin7 was appropriate reference gene in fruits and flowers (Supplementary Table S3). Under drought stress condition, LbCML38 showed the highest expression stability with a value of 0.16 (Supplementary Table S2). Among the commonly used reference genes, NormFinder analyses confirmed the validity of LbCYP, LbHIS3 and LbEIF4A, with a value less than 0.5. On the contrary, LbHSP90, LbGAPDH and LbTUB showed poor expression stability (Fig. 3, Supplementary Table S3), and were not suitable to be used as reference genes.

**BestKeeper analysis.** Results from BestKeeper analysis is slightly different from that of geNorm and NormFinder analyses. When combining the analyses of all samples in different organs, at different

### Table 1. Characteristics of candidate reference genes.

| Gene ID | Gene abbreviation | Mean raw fragments | Standard Deviation (SD) | Coefficient of variation (CV) | Swissprot annotation |
|---------|-------------------|--------------------|------------------------|-----------------------------|---------------------|
| CL10201.Contig1_All | LbCYP | 1142.214286 | 718.6318388 | 0.62915676 | Peptidyl-prolyl cis-trans isomerase CYP2-2, chloroplastic OS = Arabidopsis thaliana GN = CYP2-2 PE = 1 SV = 1 |
| CL1082.Contig1_All | LbTBP | 138.571426 | 38.5141618 | 0.27793275 | TATA-box binding protein OS = Solanum tuberosum GN = TBP PE = 2 SV = 1 |
| Unigene36249_All | LbGAPDH | 145.428571 | 68.2852774 | 0.46954513 | Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic OS = Arabidopsis thaliana GN = GAPC1 PE = 1 SV = 2 |
| CL13903.Contig10_All | LbTUB | 57.7142857 | 57.88412069 | 1.00531377 | Tubulin beta-2 chain OS = Solanum tuberosum GN = TUBB2 PE = 2 SV = 1 |
| CL2539.Contig4_All | LbEF1α | 3198.571429 | 823.6067456 | 0.25769347 | Elongation factor 1-alpha OS |
| CL4826.Contig3_All | LbActin7 | 626.571426 | 191.7922411 | 0.30699768 | Actin-7 OS = Arabidopsis thaliana GN = ACT7 PE = 1 SV = 1 |
| Unigene44516_All | LbHSP90 | 126.071426 | 53.97501457 | 0.428130427 | Heat shock protein 90-2 OS = Arabidopsis thaliana GN = HSP90-2 PE = 1 SV = 1 |
| CL13810.Contig1_All | LbHIS3 | 2307.928571 | 612.964365 | 0.26559275 | Histone H3.3 OS = Vitis vinifera PE = 2 SV = 3 |
| CL10058.Contig1_All | LbCUC | 1009 | 92.2297394 | 0.091450371 | Cycin-B1-5 OS = Arabidopsis thaliana GN = CYCB1-5 PE = 2 SV = 3 |
| Unigene55248_All | LbEIF4A | 2087.928571 | 165.8161459 | 0.07941658 | Eukaryotic initiation factor 4A-3 OS = Nicotiana plumbaginifolia PE = 2 SV = 1 |
| Unigene19346_All | LbPP2A | 831.8571429 | 99.63923937 | 0.119779268 | Serine/threonine-protein phosphatase PP2A catalytic subunit OS = Nicotiana tabacum PE = 2 SV = 1 |
| CL11175.Contig21_All | LbUBQ | 1211.428571 | 186.5371542 | 0.153981141 | Polyubiquitin 10 OS = Arabidopsis thaliana GN = UBQ10 PE = 1 SV = 2 |
| Unigene15131_All | LbCML38 | 695 | 106.1066227 | 0.1526714 | Calcium-binding protein CML38 OS = Arabidopsis thaliana GN = CML38 PE = 2 SV = 1 |
| CL7969.Contig1_All | LbLEA | 525.714287 | 113.3995174 | 0.215705604 | Late embryogenesis abundant gene Lea14-A OS = Gossypium hirsutum GN = LEA14-A PE = 2 SV = 1 |
| Unigene31075_All | LbEF1α | 5663 | 113.8141849 | 0.201026285 | Elongation factor 1-beta OS = Oryza sativa subsp. japonica GN = Os07g0662500 PE = 1 SV = 3 |
| Unigene26461_All | LbSKI1 | 612.5 | 120.2361459 | 0.196303912 | F-box protein SKP31 OS = Arabidopsis thaliana GN = SKP31 PE = 1 SV = 1 |
| Unigene55138_All | LbRH52 | 950.4285714 | 113.8166621 | 0.119821379 | DEAD-box ATP-dependent RNA helicase S2 OS = Arabidopsis thaliana GN = RH52 PE = 2 SV = 1 |
| Unigene59903_All | LbRPL7A | 793.642857 | 149.1166849 | 0.187889802 | 60S ribosomal protein L1-1 OS = Arabidopsis thaliana GN = RPL7A PE = 2 SV = 1 |
| Unigene59939_All | LbMYB1 | 2982.571429 | 697.3143085 | 0.233796348 | Transcription factor MYB1 OS = Solanum tuberosum PE = 2 SV = 1 |
developmental stages and under drought stress condition together, LbPP2A (SD = 1.13) was identified as the best reference gene (Fig. 4). LbPP2A was the most stably expressed gene in leaves (SD = 0.85). Whereas LbEF1α (SD = 0.77) and LbHSP90 (SD = 0.11) were respectively selected as the best reference gene in fruits and flowers (Supplementary Table S3). LbGAPDH (SD = 1.8) and LbTUB (SD = 2.03) were the worst candidate genes.

Table 2. Primer sequences and amplicon characteristics of the 18 reference genes.

| Gene abbreviation | Forward primer (5′-3′) | Reverse primer (5′-3′) | TM (°C) | Amplicon size (nt) | PCR efficiency (%) | Correlation coefficient (R²) | Mean CT | Standard Deviation (SD) | Coefficient of variation (CV) |
|-------------------|------------------------|------------------------|--------|-------------------|-------------------|-----------------------------|--------|-----------------------|-----------------------------|
| LbCYP             | TCGTGTGCTGTGGCAATGCGG  | CTGTGTGCTGTGGCAATGCGG  | 61     | 208               | 91.3              | 0.9953                     | 20.19  | 2.16656889            | 0.107307139                |
| LbTFP             | CGACGAAAGCAGATTCAAGG   | CAAGTTCACGAGCAGCAATTT  | 62     | 108               | 101.0             | 0.9937                     | 24.67  | 2.14058409            | 0.08674429                |
| LbGAPDH           | CAGGTCAATGGAGGGAGAT   | GCAGGCACCTTGCTTCTAC    | 60     | 179               | 94.8              | 0.9775                     | 22.46  | 2.267361358           | 0.10092507                |
| LbTUB             | GTCCAGAAAGCAGATCGCTCT | CGCCCTCTCTACATCACCTCTC | 60     | 325               | 96.1              | 0.9747                     | 23.58  | 2.294896689           | 0.097316984               |
| LbEF1α            | TGTTGAGAAGCTGTTGAATAC | TGGCCTGCTCATTGGCTGCAAA| 60     | 352               | 92.2              | 0.9973                     | 17.43  | 2.401203192           | 0.137725635               |
| LbActin7          | GGTCCTCTGAGAGCCATCAT  | TGAGCCACCTGAGCAGCAAA  | 62     | 133               | 90.6              | 0.9962                     | 20.29  | 1.841419014           | 0.090745794               |
| LbHSP90           | TCTCGTGTGCTGTGGGATGGG | TCTCGTGTGCTGTGGGATGGG | 60     | 121               | 97.3              | 0.9502                     | 18.55  | 2.043184756           | 0.110139076               |
| LbHIS3            | CACTACAGGAGTTCTGGAAG  | CAGAAACAGCCTTGAGGAG  | 61     | 128               | 91.9              | 0.9957                     | 17.18  | 1.561123661           | 0.090835934               |
| LbCYP             | TTTCTTGTGCACACCTGCGGT | CGAAGTGCTGTGGCTCTGTGA | 60     | 119               | 98.5              | 0.9977                     | 21.01  | 1.875417615           | 0.089258037               |
| LbEF1α            | ACGAGAATGCTGCCAGAAGAG | AGAGGACAAAGGATTGGAATG | 60     | 182               | 92.2              | 0.9543                     | 20.79  | 1.830072065           | 0.087990277               |
| LbPP2A            | GAGATGCTGTGAGAGTGGTGA | AGAAGATTACAGCAGCTCATGGA| 60     | 246               | 105.0             | 0.9857                     | 21.17  | 1.30967704            | 0.061843202               |
| LbUBQ             | GCGATCTCCCTCACATGCA   | GTTGGCAAGCCTCACCTCA   | 60     | 291               | 106.1             | 0.9993                     | 20.14  | 2.090803559           | 0.103768094               |
| LbML38            | CGGTTGCTCTCTGCCTCAATC | TCTCTCTGCTGCCTCACCTCA | 62     | 192               | 105.7             | 0.9958                     | 21.68  | 1.708817146           | 0.078819113               |
| LbLEA             | TGTCTCGCTCCTGCTGGCA   | TCCCTCCTCCTACATCACCTTCA| 61     | 310               | 92.6              | 0.9851                     | 18.37  | 1.741954661           | 0.094816225               |
| LbEF1β            | GACGATGAGCGAGCGAGCAT  | GCAGCACAGCGGTCCTCAAGT | 60     | 186               | 94.9              | 0.9947                     | 18.88  | 1.674620435           | 0.088683577               |
| LbSKIP            | TGGAGGGAGGAGAGGAGAGAGA | TGAGGATGAGCGACAGCAA   | 60     | 293               | 102.4             | 0.9936                     | 25.02  | 2.10058639            | 0.083924345               |
| LbRHB2            | GCGAGCAATGCTGAGATGCA  | CGCATAAGGCAACCCATCAG  | 62     | 123               | 97.5               | 0.9972                     | 22.02  | 1.636191649           | 0.074287127               |
| LbRFL7A           | CTTTTACCTCTCACAGCAGAA | TCCAACTCTGATAACCCCAAGA| 60     | 162               | 110.1             | 0.9536                     | 20.99  | 1.774507767           | 0.084515702               |
| LbMYB1            | TCCGGCAAGCAACCTCACAC | CATAGGAACAGGCAACCAAGC | 60     | 290               | 108.2             | 0.9809                     | 21.11  | 3.398526812           | 0.16096409                |

Figure 1. Sampling of materials and ranking of candidate reference genes. (a) Samples representing different developmental stages of fruits for sequencing. (b) Comprehensive ranking of candidate reference genes based on their expression stability in all 14 samples calculated by geNorm. Stage 2 represents the flowering stage. Stages 3, 4 and 7 represent samples 18, 31 and 40 days after flowering, respectively. Scale bar = 1 cm. The y-axis represents the expression stability of gene based on normalization M. Mthreshold < 1.5 for solo calculation.
reference genes. Under drought stress condition, \textit{LbHIS3} (SD = 0.98) was the most stably expressed reference gene (Supplementary Table S2).

**Reffinder analysis.** Statistical analyses showed that the stability value ranged from 1.57 to 18 among the 18 selected candidate reference genes in the combined analyses of all samples in different organs, at different developmental stages and under drought stress condition evaluated by Reffinder (Fig. 5). \textit{LbCML38} and \textit{LbRH52} were identified as the most stably expressed two reference genes with an average value less than 2. \textit{LbGAPDH}, \textit{LbHSP90} and \textit{LbTUB} were the worst. In addition, Reffinder ranked \textit{LbCML38}, \textit{LbRH52}, \textit{LbHIS3} and \textit{LbCYC} as the top four reference genes, which was consistent with the rankings obtained with geNorm and NormFinder. Collective evidence suggested that \textit{LbCML38} and \textit{LbRH52} were the best reference genes under the tested conditions. Individual factor analysis indicated that \textit{LbCML32} was the best reference gene under drought stress condition (Supplementary Table S2). \textit{LbRH52} and \textit{LbActin7} were identified as the best reference genes for target gene calibration in leaves, fruits and flowers (Supplementary Table S3).

In summary, these four software algorithms yielded various results in selecting the best reference genes in different organs, at different developmental stages, and under different treatment conditions in Goji. \textit{LbCML38} and \textit{LbRH52} showed relatively stable expression (Fig. 6), whereas \textit{LbTUB}, \textit{LbHSP90} and \textit{LbGAPDH} were not so stable (Fig. 7). Specifically, \textit{LbActin7} should be carefully used for calibrating gene expression in fruits and flowers. \textit{LbCML38}, especially combination of \textit{LbCML38} and \textit{LbRH52} performed the best stability under most conditions.
Target gene expression analyses. To further re-evaluate the validity of selected reference genes, the expression level of \( LbMYB1 \) in 14 samples was normalized with \( LbCML38, LbRH52, LbActin7 \) and \( LbHSP90 \). As illustrated in Fig. 8, no significant difference between \( LbCML38 \) and \( LbRH52 \) was observed in most of the treatments when they were used for normalization of \( LbMYB1 \) (\( P < 0.01 \)). However, compared with \( LbCML38 \), \( LbActin7 \) yielded a higher normalization value of \( LbMYB1 \) in leaves and lower values in fruits and flowers after some specific treatments (\( P < 0.01 \)). \( LbMYB1 \) normalization against \( LbHSP90 \) resulted in higher values in most cases (\( P < 0.01 \)). These results demonstrate that \( LbActin7 \) and \( LbHSP90 \) introduced errors when they were used as reference genes. Therefore, it is crucial to select appropriate reference gene(s) for evaluation of gene expression.

Discussion

The acreage of Goji in Ningxia and Qianghai provinces of China is growing rapidly due to its economical and medicinal value. However, the molecular mechanism of fruit development, pharmacologically active ingredient accumulation, and stress resistance of it is largely unknown. Screening and selection of stable reference genes for gene expression study in Goji will provide a foundation for elucidating the molecular mechanism. In the absence of systematic profiling of reference genes, we screened the reference genes suitable for samples in different organs, at developmental stages and under drought stress condition of Goji using transcriptomic sequence database for the first time.
Several studies on the screening of reference genes in Solanaceae family plants have been reported. In pepper, EF1α and UEP were found to be the most stably expressed genes in roots, stems, leaves and flowers under different treatment conditions (salicylic acid, gibberellic acid, cold, heat, salt, and drought)\(^\text{21}\). EF1α and APRT were the most stably expressed reference genes among 8 commonly used ones when potato plants were exposed to salt and drought stress, respectively\(^\text{22,23}\). In tomato (\textit{Solanum lycopersicum}, cv. Suzanne), RPL2 and PP2Acs exhibited as stable expression as ACT and UBI under nitrogen deficiency, low temperature and different light conditions\(^\text{24}\). Gantasala and co-workers\(^\text{25}\) investigated 6 commonly used reference genes (18sRNA, APRT, GAPDH, CYP, Actin and RuBP) in egg plants (\textit{Solanum melongena}) and found that 18sRNA, CYP and APRT had the best expression stability.

Figure 6. Venn diagram showing the most stable reference genes identified by the geNorm, NormFinder, BestKeeper and RefFinder algorithms. The intersection part shows the most stable genes in common, specifically, \textit{LbCML38}, \textit{LbRH52}, \textit{LbCYC} and \textit{LbHIS3}. Mapping data were derived from Figs 1b, 3, 4 and 5.

Figure 7. Venn diagram showing the most unstable reference genes identified by the geNorm, NormFinder, BestKeeper and RefFinder algorithms. The intersection part shows the most unstable genes in common, specifically, \textit{LbTUB}, \textit{LbEF1α}, \textit{LbGAPDH}, \textit{LbRPL7A} and \textit{LbHSP90}. Mapping data were derived from Figs 1b, 3, 4 and 5.
From the results discussed above, EF1α or Actin showed relative stable expression in some of the Solanaceae family plants. Liu et al. used Actin as a reference gene in fruits of two goji cultivars (Lycium barbarum L. and L. ruthenicum Murr.) for the profiling of genes involved in carotenoid biosynthesis and metabolism. However, our study demonstrated that LbActin7 exhibited moderately stable expression in some of the treatments in flowers and fruits, but introduced significant errors when normalizing the target gene expression level in leaves and fruits at specific developmental stages. These results suggest that special caution should be paid when using LbActin7 as a reference gene. Besides, commonly used LbGAPDH, LbHSP90, and especially LbTUB, were not suitable to be used as reference genes in Goji (Figs 6 and 7). This was further confirmed in the normalization of LbMYB1 gene expression. We also tested the expression stability of established reference genes in Solanaceae family plants including LbRPL, LbPP2A and LbEF1α. Our data suggest that they are not suitable to be used as reference genes in all the tested samples of Goji (Figs 1b, 2, 3, 4, 5 and Supplementary Tables S1, S2 and S3). The expression stability of GAPDH, EF1α, Actin and other reference genes has been questioned in some reports. The discrepancies between these results tested in Solanaceae materials and our data could be due to the different genetic backgrounds of plant species as well as the different treatment conditions. Therefore, selection of appropriate reference genes is critical in the genomic function study of Goji or a comparative study of different plant lines in the Solanaceae family.

DNA chips and next-generation sequencing provide a novel approach for reference gene screening of non-model organisms that lack whole genome information. Czechowski and co-workers firstly proposed to screen reference genes using Arabidopsis whole genome Affymetrix ATH1 chips. González-Agüero et al. further summarized and refined the analytic process for reference gene screening from RNA-Seq data in grape. A total number of 19 candidate reference genes were identified from 242 non-differentially expressed genes (NDE) using CV < 0.4 of the total read as the screening threshold. qRT-PCR results showed that VvAIG1 and VvTCPB were the most stably expressed reference genes in 14 grape lines, at 4 developmental stages, and under gibberellic acid treatment condition. Similarly, studies with oil-tea camellia, Striga hermonthica and plums corroborated the approach of using RNA-Seq database to screen reference genes. In this study, we adopted even stricter screening threshold (CV < 0.3) to select 10 functional genes from 1272 raw fragments according to the analytic protocol proposed previously, and three classical reference genes LbEIF4A, LbUBQ and LbEF1α were included with a CV value less than 0.1 (Table 1). They showed better stability than that of LbTUB, LbHSP90 and LbGAPDH as confirmed by qRT-PCR (Table 2), all of which had higher CV values of raw fragments and Ct values.

Taken together, our studies confirmed the correlation between the transcriptional expression stability and qRT-PCR results, thus it is appropriate to select the transcripts with smaller CV values as candidate reference genes during preliminary screening with sequencing data. Collective evidence suggests that LbCML38 and LbRH52 could be used as the best reference genes for gene expression study in Goji.

Methods
Sample preparation and treatment. Ningxia goji (Lycium barbarum L.) Ningqi I was cultivated in field and collected. Plants grown under normal condition were used as control (control, C). Drought stress treatment was performed as described previously with minor modification: four-year-old adult plants were transplanted...
into pots filled with a mixture of fertilizer, sand and loam (1:1:3 v/v). The maximal water holding capacity of soil in field was measured and determined as 18%. Transplanted plants at vegetative growth stage with similar physiological stage were selected for drought stress treatment. During the drought stress period, water holding capacity of soil in pots was maintained at 40% to 45% of the maximal field level. The water capacity of soil was measured at a fixed time point every day using soil moisture meter TDR300 (Spectrum, USA), and maintained at a specific level by artificial replenishment. Ten plants were individually cultivated in pots for each treatment. Sequencing samples including equal amounts of leaves (L), flowers (F) and fruits (G) from control and drought-treated plants were collected 18 (developmental stage 3), 31 (developmental stage 4) and 40 (developmental stage 7) days after flowering (Fig. 1a). Control leaves were collected from the same plants at developmental stage 3.

**Total RNA isolation and sequencing database establishment.** Total RNA was extracted using plant total RNA extraction kit (Tiangen Biotechnology, PRC). Genomic DNA was eliminated by treating each sample with RNase-free DNase I (TAKARA BIO INC., code 2270A) according to the instruction manual. The purity of total RNA extracted was checked using a NanoDrop 2000 spectrophotometer. Samples with an absorbance ratio at OD260/280 between 2.0 and 2.2 were used for further analyses. The concentration and quality of extracted RNA was determined using Agilent 2000 bioanalyzer (Agilent, USA). First-strand cDNA was synthesized from magnetic beads-enriched poly(A)-mRNA using random hexamers, followed by buffer addition to synthesize the complement strand. Synthesized cDNA was purified using Qiaquick PCR purification kit (Qiagen, USA) and eluted with EB elution buffer. Purified cDNA was mixed with poly(A) tail and sequencing adaptors. Appropriately sized cDNA fragments were selected by agarose electrophoresis and amplified by PCR. The library was sequenced on Illumina HiSeq2000 using de novo PE 100 sequencing strategy.

**Sequence assembly and annotation of basic bioinformatics.** The original imaging data obtained from sequencing were processed into raw reads by base calling, followed by filtration of noises and low quality data to obtain clean reads. The de novo assembler program Trinity was used to assemble the short reads into contigs, scaffolds and Unigene, respectively. Unigene sequences were blasted against that of Nr, SwissProt, GO, KEGG and COG databases (E-value was used to predict the coding region and sequence orientation) to obtain homologous proteins with high sequence similarity to the reference protein. When Unigene sequences failed to match sequences in the databases, ESTScan software was used to predict the coding region and sequence orientation. Unigene expression level was calculated using FPKM method (Fragments Per kb per Million fragments). The RNA-Seq data used in this study is available at the Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) of the National Center for Biotechnology Information (NCBI) with an accession number SRP063577.

**Reference gene screening and primer design.** Two approaches were used to select reference genes: 1) Excavation of classical reference genes: 8 classical reference genes including CYP, TBP, GAPDH, TUB, EF1α, Actin7, HSP90 and HIS3 derived from tomato or Arabidopsis were used as template to screen homologous genes from prepared RNA-Seq database of L. barbarum; 2) Based on the method described by González-Agüero et al., 10 stably expressed Unigenes (LbCYC, LbEIF, LbPP2A, LbUBQ, LbCML38, LbLEA, LbEF1α, LbSKIP, LbRH52 and LbRPL7A) were selected out of 1272 transcripts (raw fragments > 500, and the coefficient of variation < 0.3). Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). Data information about the candidate genes and primers are listed in Tables 1 and 2.

**qRT-PCR and data analysis.** Synthesis of cDNA was performed with 0.5–1μL total RNA (the final content of RNA in the reaction mixture was adjusted to 1μg for all samples) according to the instruction manual of the cDNA synthesis System (TRANSGEN BIOTECH INC., code AU311–02) in a total volume of 20μL. Quantitative RT-PCR analysis of the cDNA of 14 samples was performed using StepOne Real-time PCR Systems (Applied Biosystems, USA). A total amount of 20μl of PCR reaction mix containing 10μl of Power SYBR Green PCR Master Mix, 5μl of diluted cDNA, 0.5μl (10 pmol) each of forward and reverse primers, and ddH2O was prepared. The thermocycling condition was set as follows: initial denaturation at 95 °C for 10 mins, 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60–62 °C for 15 seconds, and extension at 72 °C for 25 seconds. Fluorescent signals were collected after each cycle. Each sample was run in triplicate along with a negative control. Amplified products were checked on 1% agarose gel.

The first-strand cDNA was synthesized after six rounds using a five-fold serial dilution. Each PCR reaction was conducted in triplicate using diluted cDNA as template. The Cv values of samples were generated automatically after qRT-PCR. A standard curve was also generated and the melting curve was analyzed to determine the specificity of PCR products. The amplification efficiency (E) of each candidate reference gene was calculated with the slope of the standard curve according to the equation $E = 10 \left( - \frac{1}{Slope} \right) \times 100\%$. The stability of candidate reference genes was evaluated by geNorm, NormFinder, BestKeeper and RefFinder using obtained Cv values of the samples. The average expression stability value (M) of each candidate reference gene was calculated by geNorm. The higher the M value, the less stable the gene expression was, and vice versa. Meanwhile, paired difference analysis (V_mic,1) of candidate reference gene normalization factor was used to determine the optimal number of required reference genes. NormFinder was used to determine the candidate reference gene stability using the combined variances within and between groups. The lower the stability value, the more stable the reference gene expression was, and vice versa. BestKeeper was used to determine the candidate reference genes’ standard deviation (SD) and coefficient of variation (CV). Reference gene with the smallest CV ± SD value was considered the most stably expressed one. All reference genes with a SD value less than 1 were established as stably expressed genes. The smaller the SD value, the more stable the reference gene expression was, and vice versa. To calculate the geometric mean values and the ranking of each candidate reference gene, results respectively obtained with geNorm, NormFinder, BestKeeper and Delta Ct were integrated.
using RefFinder (http://www.leonxie.com/referencegene.php). The lower the ranking index, the more stable the reference gene expression was, and vice versa.

Reference gene validation. For confirmation of selected reference gene validity, LbMYB1 which encodes a MYB transcription factor involved in drought stress response and flavonoid anabolism was selected as a target gene\(^3\). The expression levels of LbMYB1 in difference samples were normalized against those of the most stable reference genes LbCML38 and LbRH52, the moderate stable reference gene LbActin7, and the less stable reference gene LbHSP90, respectively. Data were compared and analyzed with analysis of variance (ANOVA) and multiple comparisons using the statistical analysis software of SPSS 21. Values of P < 0.01 were considered statistically significant difference.

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Author Contributions
H.X.Z. and L.G. initiated and designed the research. L.G. and Y.J.Y. performed the experiments. L.G., Y.J.Y., Y.C.C., J.S., and Y.X.S. analyzed the data. L.G. and H.X.Z. wrote the paper. All of the other authors reviewed and approved the manuscript.

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