RESEARCH ARTICLE

Selection of appropriate reference genes for the detection of rhythmic gene expression via quantitative real-time PCR in Tibetan hulless barley

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Abstract

Hulless barley (Hordeum vulgare L. var. nudum hook. f.) has been cultivated as a major crop in the Qinghai-Tibet plateau of China for thousands of years. Compared to other cereal crops, the Tibetan hulless barley has developed stronger endogenous resistances to survive in the severe environment of its habitat. To understand the unique resistant mechanisms of this plant, detailed genetic studies need to be performed. The quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) is the most commonly used method in detecting gene expression. However, the selection of stable reference genes under limited experimental conditions was considered to be an essential step for obtaining accurate results in qRT-PCR. In this study, 10 candidate reference genes—ACT (Actin), E2 (Ubiquitin conjugating enzyme 2), TUBα (Alpha-tubulin), TUBβ6 (Beta-tubulin 6), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), EF-1α (Elongation factor 1-alpha), SAMDC (S-adenosylmethionine decarboxylase), PKABA1 (Gene for protein kinase HvPKABA1), PGK (Phosphoglycerate kinase), and HSP90 (Heat shock protein 90)—were selected from the NCBI gene database of barley. Following qRT-PCR amplifications of all candidate reference genes in Tibetan hulless barley seedlings under various stressed conditions, the stabilities of these candidates were analyzed by three individual software packages including geNorm, NormFinder, and BestKeeper. The results demonstrated that TUBβ6, E2, TUBα, and HSP90 were generally the most suitable sets under all tested conditions; similarly, TUBα and HSP90 showed peak stability under salt stress, TUBα and EF-1α were the most suitable reference genes under cold stress, and ACT and E2 were the most stable under drought stress. Finally, a known circadian gene CCA1 was used to verify the service ability of chosen reference genes. The results confirmed that all recommended reference genes by the three software were suitable for gene expression analysis under tested stress conditions by the qRT-PCR method.
Introduction

The continual growth of populations and excessive use of chemical fertilizers in agricultural productivities have remarkably and negatively influenced the natural environment: arable lands have decreased, air and water have been polluted, severe desertification has increased, global warming is changing the climate, and frequent natural disasters threaten plant growth [1, 2]. According to the characteristics of these stress factors, they can be classified into two categories of environmental stresses to organisms: biotic and abiotic stresses. Among all abiotic stress factors—such as drought, high temperatures, cold, UV radiation, and mechanical injury—salt, cold, and drought stresses are the most regular abiotic stresses, which may be encountered by crops throughout their life spans and which also negatively affect growth, yield, and quality of cereal crops [3–5]. The focus of research has always been on the abiotic stresses of plants [6]. Owing to the rapid development of the new generation of sequencing technology, a variety of biological genome sequencing studies have been completed. The genome sequences of many important crops have also been recently reported, such as rice and barley [7, 8]. These studies have accelerated the development of genomics and comparative genomics research in crops enormously, and provided a shortcut for revealing the constitution and regulations of plant stress-resistance mechanisms in different species via comparative genomics method.

Tibetan hulless barley (Hordeum vulgare L. var. nudum hook. f.) is a type of traditional crop in the Qinghai-Tibetan plateau, which has been cultivated as an irreplaceable cereal by the local inhabitants for hundreds of years. As a true functional food, the hulless barley flour is rich in dietary fiber, which significantly reduces the risk of type II diabetes, cardiovascular disease, and colorectal cancers [7, 9–12]. As a result of its very high altitude, the environment of the Qinghai-Tibetan plateau has been believed to be one of the harshest conditions for agriculture on our planet. Compared with the normal cultivated barley and all other plants within the genus Hordeum, hulless barley has evolved stronger endogenous resistance systems to resist the intensive UV radiation, lower oxygen pressure, and other abiotic stresses, such as salt, cold, and drought stresses, to better adapt to the severe environmental conditions of this area [13]. Still, the mechanism of how hulless barley stress defense systems are organized and the expression profiles of most key stress-resistant genes remained unknown for this plant. Further, as a diploid, hulless barley can also serve as a new model species for the genetic research within the genus Hordeum [7]. Recently, with the rapid development of molecular biology technology, crops have been multiple genome-wide sequenced, providing a solid foundation to investigate the molecular mechanism of plant resistance features that has been thought to be controlled by multiple genes [7, 13, 14].

At present, the detection of gene expression profiles in plants is usually conducted via Northern blotting, competitive RT-PCR, microarray, or qRT-PCR techniques. All these commonly used methods have shown shortcomings, such as Northern blot being an experience-requiring and time-consuming technique [15], while the selection of appropriate internal standard RNAs significantly affects the accuracy of competitive RT-PCR experiments [16]. To ensure the veracity and repeatability of experiments, high-throughput methods, such as microarrays or RNA-seq, are always expensive and not only require high quality, but also a significant amount of RNA samples [17]. Compared to these methods, qRT-PCR has many advantages, such as high sensitivity, high specificity, high accuracy, ease of operation, and lower consumption of supplies. On account of these features of qRT-PCR, it is more convenient for it to be used as a rapid detection protocol for the mRNA titers or gene expression among limited numbers of sample groups [18, 19], either in different species [20, 21], in different developmental stages [22, 23], or in various responding processes to abiotic and biotic stresses [14, 24]. A primary requirement for the detection of the gene expression quantity is
that the total amount of RNAs of individual samples should be equal. Qualified reference genes have often been employed as internal standards for volume calibration of RNA samples, which always played a pivotal role in obtaining reliable quantitative analysis data from genes expression [25, 26]. The use of optimized reference genes remarkably reduces experimental errors that have been generated during the sample preparing procedure. Housekeeping genes of plants, which have usually been constantly expressed in most of the developmental stages during their life spans, are preferred candidates for reference genes in qRT-PCR experiments [23, 27]. However, the expression profiles of most generally-considered housekeeping genes would be influenced by changes in environmental conditions, especially by being induced or suppressed by environmental stresses to various degree [23, 28]. Recent studies have proven that no tested genes could be used as universal reference gene under all circumstances [18, 29]. This means the expression profiles of optimized reference genes can only be chosen to meet one of the following requirements, such as to be constant in a manner of environmental condition-specific, tissue-specific, or developmentally specific, as well as in cultivar-dependent manners [30–32]. Therefore, to accurately detect expression patterns of functional genes in plants under various stress conditions, it is necessary to select optimized reference genes whose expression remains constant under certain stresses [25, 26].

In this present study, 10 candidate reference genes from hulless barley (as shown in Table 1)—ACT (Actin) [33], E2 (Ubiquitin conjugating enzyme 2) [34], TUBa (Alpha-tubulin), TUBβ6 (Beta-tubulin 6) [35], GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) [36], EF-1α (Elongation factor 1-alpha) [37], SAMDC (S-adenosylmethionine decarboxylase) [38], PKABA1 (Gene for protein kinase HvPKABA1) [39], PGK (Phosphoglycerate kinase) [40], and HSP90 (Heat shock protein 90) [41]—were chosen as candidates to evaluate expression stability via qRT-PCR. Three popular software packages, including geNorm, NormFinder, and BestKeeper, were employed to conduct the following data analysis [35, 42–44]. The performance of most suitable reference genes were then tested with a time course expression pattern of the well-studied clock gene, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) (JN603242.1) [45–48]. This study has achieved optimal reference genes for hulless barley under individual cold, salt, and drought stresses. The results provided a solid experimental basis for further research either on subsequent functional gene expression pattern analysis of hulless barley or for the exploration of the mechanism with which this crop has adapted to the harsh environments of its habitats.

**Materials and methods**

**Plant materials and treatments**

The seeds of hulless barley used for this study were purchased from the seed company of Xining City, Qinghai Province in China. After soaking in sterilized water for 16 h, the seeds were first treated with 70% ethanol for 3 min, and surface sterilized with 0.1% HgCl₂ for 10 min. After fine washing with sterile water five times, the pretreated seeds were placed onto plastic dishes, containing filter paper soaked with sterile water for germination, in an incubator under 100 μE•M⁻²•s⁻¹ constant illumination provided via cool white fluorescents at 25°C for 10 days. When the height of seedlings reached 10 cm, the illumination condition was adjusted from constant to a 12/12 h light/dark cycle for 2 days before simulating drought, cold, and salt stress treatments, as an entrainment of the transcriptome-wide gene expression. For the cold treatment, seedlings were treated for 1 day in the 12/12 h light/dark cycle at 5°C, and then 0.1 g leaf tissues were then harvested every 4 h under constant light. For the drought and salt treatments, seedlings were first cultured in 15% (w/v) PEG6000 (Sangon, China) or 300 mM NaCl (Sangon, China) for 24 h under the 12/12 h light/dark cycle, and then
0.1 g leaf tissues were harvested every 4 h under constant light. All plant samples were immediately frozen in liquid nitrogen and stored at -80˚C until RNA extraction.

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted from 0.1 g frozen leaf samples via modified Trizol method (TaKaRa, Dalian, China) [49]. Both the quality and purity of these RNAs were assessed via electrophoresis on a 1.0% agarose gel, and quantified via Biospec-nano (SHIMADZU, Japan). All qualified RNA samples with an $A_{260}/A_{230}$ ratio of approximately 2.0 and $A_{260}/A_{280}$ ratio ranging from 1.9 to 2.1, were first diluted to 1.0 μg/μL and then used as templates for the following reverse transcription experiments. Subsequently, a PrimeScript™ RT reagent Kit with genome DNA Eraser (TaKaRa, Dalian, China) was applied to conduct cDNA synthesizing processes by a recommended procedure, following the instructions of the manufacturer. Following the standard protocol of the manufacturer, cDNAs were purified with a Universal DNA Purification Kit (TIANGEN, China) after which cDNAs were used as templates for following qRT-PCR amplifications.

**Selection and primer design of reference genes**

Ten known housekeeping genes from normal barley (*Hordeum vulgare*), a relative species of hulless barley, were selected as candidate reference genes in this study. These genes were ACT (GenBank: AY145451.1), *E2* (AY220735), *TUBα* (U40042.1), *TUBβ6* (AM502854.1), GAPDH (AK359500.1), *EF-1a* (JN107538.1), SAMDC (AK368996.1), *PKABA1* (AB058924.1), PGK (AK251528.1), and *HSP90* (AY325266.1). The homologous mRNA sequences of the chosen candidate reference genes were downloaded from the NCBI of the National Institutes of Health via the online Blast tool. According to these mRNA sequences, the primers for qRT-PCR amplification were then designed via software Primer Premier (version 5.0) with the

| Gene    | Gene description       | Primer sequence 5'-3'                  | Product size(bp) | TM(˚C) | Efficiency(%) | $R^2$ | Mean Ct | SD | CV(%) |
|---------|------------------------|----------------------------------------|------------------|--------|---------------|------|---------|----|-------|
| EF-1a   | Elongation factor 1-alpha | CCACCTCTTGTGCCGGTCTTA                    | 293              | 56     | 97            | 0.999| 22.34   | 1.78| 7.96  |
| TUBα    | Alpha-tubulin          | CCATCCGAGCAAGGACACTA                    | 239              | 56     | 94.9          | 0.999| 24.19   | 0.78| 3.27  |
| TUBβ6   | Beta-tubulin 6         | ACTGGGCAAGGAGCACTA                      | 217              | 56     | 107.9         | 0.996| 27.39   | 1.07| 4     |
| GAPDH   | Glyceraldehyde 3-phosphate dehydrogenase | GCAGAAGGGAAGGATT               | 250              | 56     | 91.1          | 0.997| 25.99   | 1.61| 6.17  |
| HSP90   | Heat shock protein 90  | AGAGCAAGATGGAGAGGTCG                  | 265              | 56     | 90            | 0.998| 28.39   | 1.52| 5.36  |
| ACT     | Actin                  | GCCGTGCCTCTCCCTCTATG                   | 234              | 56     | 95.4          | 0.997| 26.17   | 0.93| 3.61  |
| E2      | Ubiquitin conjugating enzyme 2 | CCATCCGAGCAAGGACACTA           | 112              | 56     | 95            | 0.997| 23.52   | 1.45| 6.15  |
| SAMDC   | S-adenosylmethionine decarboxylase | GTCCTCTGACCATCTTCTGGC               | 291              | 58     | 102.1         | 0.996| 27.32   | 2.17| 7.96  |
| PGK     | Phosphoglycerate kinase | GGAAGAAGAAGAAGCAAC                | 218              | 60     | 107.1         | 0.996| 24.75   | 2.1 | 8.47  |
| PKABA1  | Gene for protein kinase HvPKABA1 | AACCATCGTGCTACTCACACGC           | 263              | 60     | 101.3         | 0.998| 29.65   | 2.55| 8.59  |

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0.1 g leaf tissues were harvested every 4 h under constant light. All plant samples were immediately frozen in liquid nitrogen and stored at -80˚C until RNA extraction.
following criteria: the primers should be with limited lengths of 18–25 bp, melting temperatures (Tm) in the range of 50–60˚C, GC contents varying from 45 to 55%, and product lengths of 100–300 bp. All primers that have been used in this study were synthesized by Sangon Co. Ltd., in Shanghai, China.

**Semiquantitative RT-PCR and qRT-PCR analysis**

The specification and validity of all primers were firstly tested via semiquantitative RT-PCR and the amplifications were triggered by Premix Taq™ kits (TaKaRa, Dalian, China). The amplified products of each candidate gene were identified via electrophoresis on a 1.5% agarose gel. The validated PCR primers should account for a single specific amplified product with the correct size.

The quantitative real-time PCR reactions were performed with the Bio-Rad CFX96 Real-Time PCR system (Bio-Rad, USA) using a SYBR® Premix Ex Taq™ II qPCR kit (TaKaRa, Dalian, China). The qRT-PCR reactions were conducted in mixtures constituted with 12.5 μl 2×SYBR Premix Ex TaqII (TaKaRa, Dalian, China), 0.5 μl of each amplification primer (20 μM), 10.5 μl ddH2O, and 1 μl cDNA templates. Each group of reactions was accomplished with three repetitions and one negative control, in which 1 μl ddH2O was used instead of cDNA as a template for PCR. After pre-denaturation at 94˚C for 30 s, a 2 step-program of qRT-PCR was set as follows: denaturing at 94˚C for 5 s, subsequently annealing at 56˚C for 30 s, and repeated for 40 cycles. Finally, melting curves of qRT-PCR amplifications were performed to confirm the specificity of the primers again by heating up the products from 56˚C to 95˚C.

**Analysis of reference gene expression stability**

Three public software packages—geNorm, NormFinder, and BestKeeper—were introduced to perform the expression stability analysis of candidate reference genes under various abiotic stresses. When geNorm was used in this analysis, a relative quantity (2^{ΔCt}) of each gene was firstly obtained by calibrating their raw Ct data to the one with the highest Ct value. The normalized Ct values from all tested candidates were then employed to calculate the average M value of test genes [23, 50]. A significant negative correlation was detected between the precise M value of a target gene and its expression stability when assuming 1.5 as critical value for the M value. An M value below 1.5 indicates a confidential candidate for the reference gene; in contrast, an M value above 1.5 has often been used as a rejection criterion for a candidate gene [23]. The smallest M value from geNorm analysis in all examined reference genes always presented the most stably expressed candidate gene. In addition, the pairwise variation (Vn/Vn+1) between two sequential normalization factors can determine the optimal number of reference genes, which needed to be normalized. The recommended cut-off threshold was 0.15, when the pairwise variation was lower than this value, an additional reference gene was not required for the following normalization process. In terms of the software NormFinder, the 2^{ΔCt} value of each individual gene also served as input data for analyzing gene expression stability. For BestKeeper, the raw data of the Ct value from qRT-PCR was used to calculate the coefficient of variation (CV) and the standard deviation (SD), while the best reference gene was chosen via the lowest CV±SD value. Finally, the appropriate reference genes for qRT-PCR were obtained via combinatorial analysis of the results from all three abovementioned algorithms. To verify the reliability of all selected proper reference genes, the relative expression of a known circadian gene, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, was analyzed in a set of time course samples from various stressed hulless barley leaf tissues. Additionally, a standard curve was first generated from a tenfold gradient dilution of cDNA in a qRT-PCR assay by using the software...
Microsoft Excel 2003. The efficiency (E) and the regression coefficient ($R^2$) of the qRT-PCR reactions were then calculated using the slope of the standard curve according to the following equation: $E = [10^{(-1/slope)} - 1] \times 100\%$. The average cycle threshold (Ct) values from three independent biological repetitions were used to carry out the statistical analysis of the target genes’ relative expression, while data from each of the biological repetitions was presented by three technical repeats.

**Results**

**Genetic information of candidate reference genes for qRT-PCR**

A total of the ten most commonly used reference genes in the reports of qRT-PCR in graminaceous crops, including $ACT$, $E2$, $TUB\alpha$, $TUB\beta6$, $GAPDH$, $EF-1\alpha$, $SAMDC$, $PKABA1$, $PGK$, and $HSP90$, were chosen as candidates for gene expression stability assessment under various abiotic stresses (cold stress, salt stress, and drought stress) The specification of primers for all candidate genes was verified via PCR amplification before performing the qRT-PCR reactions. Only those ones that could trigger an amplification of identical products with predicted size were identified as qualified primers (S1 Fig). Subsequently, the performances of these chosen primers were further verified by observing their melting curves during qRT-PCR, and a single sharp peak in the melting curves always presented high quality primers (S2 Fig). The qRT-PCR products of all tested genes in this study ranged from 112 bp to 293 bp. The efficiencies of each qRT-PCR reaction of all candidate reference genes were above 90% and varied from 90% to 107.9% with egression coefficients, denoted as $R^2$, distributed from 0.996 to 0.999.

To analyze the expression level of these 10 candidate reference genes under three different experimental conditions, the Ct values of all analyzed samples were obtained via qRT-PCR, and the average Ct values of each gene under all experimental groups were calculated. The results revealed that the Ct value of 10 candidate reference genes varied from 22.30 to 29.63 (Fig 1), and the most abundant expression gene was $EF-1\alpha$ with the lowest average Ct $\pm$ SD (22.34$\pm$1.78), followed by $E2$, $TUB\alpha$, $PGK$, $ACT$, $GAPDH$, $TUB\beta6$, $SAMDC$, $HSP90$, and $PKABA1$ (Table 1). $PKABA1$ was the gene with the lowest expression level, but the highest average Ct $\pm$ SD (29.65$\pm$2.55). The SD values of $TUB\alpha$ and $ACT$ were minimal (24.19$\pm$0.78 and 26.17$\pm$0.93), indicating that they shared the smallest variation of the ten candidate genes. The coefficient of variation (CV) of the Ct values also represents the stability of gene expression. In other words, a smaller CV value of the reference gene indicated a more stable expres- sion. Among the ten candidate reference genes, the CV values of $TUB\alpha$ (3.27%) and $ACT$ (3.61%) had the lowest variation, while the $PKABA1$ (8.59%) had the highest across all tested samples (Table 1). In summary, these results show that the expression abundance of candidate reference genes under different experimental conditions has played a pivotal role in the screen- ing of appropriate reference genes in Tibetan hulless barley.

**geNorm analysis**

The software geNorm determines the ranking of the ten candidate reference genes by calculating the average pairwise expression ratios (presented as M) of each candidate reference gene. The M value of the gene is negatively related to its stability, while 1.5 is definitively configured as the threshold of the M value. If the M value of a candidate gene is below 1.5, this gene can be used as a confident reference gene, otherwise it should be rejected for use as a reference gene. The results of this present study reveal that the M values of all tested candidate reference genes were below 1.5 either under drought, cold, or salt stressed conditions. However, when combining the data for all the samples from three stressed conditions, the M value of $SAMDC$ and $PKABA1$ were larger than 1.5. In summary, it could be confirmed that the M value of the
selected candidates in this study were relatively smaller, demonstrating that these reference genes are relatively stable under individual stress environments. Although the different performances of individual genes have been detected under various stressed conditions in general, the TUBβ6 and E2 with the lowest M value were found to be the most stable reference genes under all tested situations, while the genes of SAMDC, PKABA1, and PGK were the least stable. Concerning of the situation of various stress treatments, the two best reference genes for samples from the salt stress condition were EF-1α and HSP90. For the drought stress conditions, ACT and TUBα were the most stable reference genes. The most suitable reference genes under cold stress were TUBα and EF-1α (Fig 2).

Subsequently, by analyzing the pairwise variation (Vn/Vn+1) of 10 candidate reference genes, the optimal number of reference genes was determined under different stress treatments. As described in Fig 3, the V2/3 values from the salt stress, drought stress, and cold stress
groups were below 0.15 (0.095, 0.121, and 0.146, respectively), signifying that only two reference genes—EF-1α and HSP90 for salt stress, ACT and TUBα for drought stress, and TUBα and EF-1α for cold stress—were required to calibrate the expression of target genes under each stress condition. When all stresses were considered, the V5/6 value (0.172) was the lowest; however, it was still above the threshold of 0.15. This result indicates that no tested reference genes could be generally used to normalize the expression of target genes under the three tested stress conditions.

**NormFinder analysis**

The rank of expression stability of the candidate reference genes via NormFinder was determined according to the stability values of each reference gene. Lower stability data collected by this software represented higher expression stability of the candidates. Under the salt stress conditions, HSP90 and TUBα displayed most stable genes expression, while GAPDH exhibited the least stability. For cold stress, the most stably expressed reference genes were E2 and
In contrast, the PKABA1 gene, which had been newly used as a reference gene in some reports for barley proved to be the least stable under cold stress. When the general performance of the candidates under all tested stresses was considered, GAPDH and TUBβ6 were the most suitable reference genes for qRT-PCR via NormFinder, although TUBβ6 and GAPDH were ranked ninth and tenth, respectively for the salt stress condition. Under drought stress subset, TUBβ6 and GAPDH were ranked seventh and eighth, respectively. Simultaneously, the data of Table 2 also shows that TUBβ6 and GAPDH are ranked second and fourth, respectively, under cold the stress condition. These results indicate that the stability of individual reference genes exhibits enormous variations under different stresses. As shown in Fig 2 and Table 2, for salt stress, drought stress, and the combined results from three tested stresses, no significant differences were identified between the results from the evaluation of expression stabilities of

![Fig 3. Pairwise variation (V) of 10 candidate reference genes under various stresses calculated via geNorm to determine the optimal number of reference genes for normalization. Pairwise variation (Vn/n+1) was calculated between the normalization factors (NFn and NFn+1) with the geNorm program to determine the optimal number of reference genes for qRT-PCR data normalization of various samples.](https://doi.org/10.1371/journal.pone.0190559.g003)
these candidate reference genes, which were conducted either via the software NormFinder or via geNorm methods. However, under cold stress, the hierarchy of gene expression stability analysis performed by NormFinder was distinctly different from the results produced by geNorm. The best reference genes, TUBα and EF-1α, that were recommended by geNorm were located as the sixth and seventh in all 10 tested candidates sorted by NormFinder.

**BestKeeper analysis**

The rank of the expression stability of reference genes via BestKeeper was determined according to the CV±SD of their average Ct values (Table 3). Synthesizing all the data from various sample subsets, two genes with the lowest CV±SD values, 2.28±0.54 for TUBα and 2.89±0.74 for ACT, were confirmed to be the most stable reference genes. Individually, under the salt stress condition, the TUBβ6 and E2 genes showed lowest CV±SD values with 1.17±0.3 and 1.38±0.3, and were ranked as the top two stable reference genes. Similar to this, the results from the samples of drought stressed subset indicated that the TUBβ6 (with a CV±SD value of 1.91±0.53) and E2 (with a value of 3.15±0.77) genes were the most stable genes. Furthermore, in the cold stress sample subsets, TUBα (with a CV±SD value of 1.6±0.38) and EF-1α (with a CV±SD value of 2±0.42) were chosen to be the most stable reference genes by the software BestKeeper.

Table 2. Expression stability of candidate reference genes calculated by NormFinder.

| Rank | Total Gene | Stability | Salt Gene | Stability | Drought Gene | Stability | Cold Gene | Stability |
|------|------------|-----------|-----------|-----------|--------------|-----------|-----------|-----------|
| 1    | GAPDH      | 0.423     | HSP90     | 0.087     | E2           | 0.053     | E2        | 0.112     |
| 2    | TUBβ6      | 0.470     | TUBα      | 0.123     | ACT          | 0.272     | TUBβ6     | 0.186     |
| 3    | E2         | 0.490     | ACT       | 0.164     | TUBα         | 0.332     | HS90      | 0.311     |
| 4    | HSP90      | 0.552     | EF-1α     | 0.171     | PGK          | 0.338     | GAPDH     | 0.347     |
| 5    | TUBα       | 0.568     | PGK       | 0.234     | SAMDC        | 0.381     | ACT       | 0.517     |
| 6    | EF-1α      | 0.823     | SAMDC     | 0.325     | EF-1α        | 0.416     | EF-1α     | 0.618     |
| 7    | ACT        | 0.851     | PKABA1    | 0.378     | TUBβ6        | 0.450     | TUBα      | 0.710     |
| 8    | PGK        | 1.012     | E2        | 0.455     | GAPDH        | 0.510     | SAMDC     | 0.792     |
| 9    | PKABA1     | 1.260     | TUBβ6     | 0.533     | HSP90        | 0.542     | PGK       | 1.289     |
| 10   | SAMDC      | 1.427     | GAPDH     | 0.607     | PKABA1       | 0.588     | PKABA1    | 1.386     |

Table 3. Expression stability of candidate reference genes calculated via BestKeeper.

| Rank | Total Gene | SD | CV | Salt Gene | SD | CV | Drought Gene | SD | CV | Cold Gene | SD | CV |
|------|------------|----|----|-----------|----|----|--------------|----|----|-----------|----|----|
| 1    | TUBα       | 0.54| 2.28| TUBβ6     | 0.30| 1.17| TUBβ6        | 0.53| 1.91| TUBα      | 0.38| 1.60|
| 2    | ACT        | 0.74| 2.89| E2        | 0.30| 1.38| E2           | 0.77| 3.15| EF-1α     | 0.42| 2.00|
| 3    | TUBβ6      | 0.89| 3.31| PGK       | 0.39| 1.69| TUBα         | 0.77| 3.19| ACT       | 0.47| 1.80|
| 4    | E2         | 1.10| 4.66| TUBα      | 0.41| 1.75| ACT          | 0.79| 3.13| TUBβ6     | 0.63| 2.31|
| 5    | HSP90      | 1.20| 4.20| HSP90     | 0.41| 1.53| HSP90        | 0.88| 3.00| E2        | 0.75| 3.09|
| 6    | GAPDH      | 1.23| 4.70| EF-1α     | 0.41| 1.90| PKABA1       | 0.89| 2.74| GAPDH     | 0.93| 3.59|
| 7    | PKG        | 1.27| 5.15| ACT       | 0.45| 1.79| EF-1α        | 0.98| 4.01| PKG       | 1.10| 4.32|
| 8    | EF-1α      | 1.41| 6.33| SAMDC     | 0.46| 1.65| PGK          | 0.98| 3.82| HSP90     | 1.10| 3.77|
| 9    | SAMDC      | 1.61| 5.90| PKABA1    | 0.76| 2.74| SAMDC        | 0.98| 3.40| SAMDC     | 1.54| 6.19|
| 10   | PKABA1     | 2.20| 7.43| GAPDH     | 0.96| 3.83| GAPDH        | 1.12| 4.09| PKABA1    | 1.84| 6.41|
Comprehensive stability analysis of reference genes

To get more reliable stably expressed reference genes, comprehensive ranking (Table 4) of stability for reference genes was often integrated by the results from three open access software packages [51]. This strategy has also been used in the present study. As shown in Table 5, the most and least stable combinations of reference genes were based on geNorm, NormFinder, and BestKeeper. In terms of total sample subsets, TUB6, E2, HSP90, and TUBα were ranked as the most stable reference genes, while HSP90 and TUBα were confirmed to be the optimal stable reference genes in the salt stress subset. Furthermore, TUBα and EF-1α were the most stable reference genes in the cold stress subset. Moreover, the data of the drought stress subset indicated that the ACT and E2 genes were the most stable genes. Interestingly, although PKABA1 has been used as a novel reference gene by some studies on the detection and quantifying of nucleic acid molecules in wheat and barley [39], the results of this study demonstrated that the Ct values of PKABA1 under various stress conditions were too large to be acceptable. This reflects that the expression of PKABA1 has been down-regulated by environmental stress conditions. Accounting for the lower abundance of its mRNA in cells of Tibetan hulless barley,

Table 4. Expression stability ranking of the 10 candidate reference genes.

| Method               | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   |
|----------------------|------|------|------|------|------|------|------|------|------|------|
| (A) RANKING ORDER UNDER ALL SAMPLES (BETTER - GOOD - AVERAGE) |
| geNorm               | TUBβ6| E2   | HSP90| TUBα | ACT  | GAPDH| PGK  | EF-1α| PKABA1| SAMDC|
| NormFinder           | GAPDH| TUBβ6| E2   | HSP90| TUBα | EF-1α| ACT  | PGK  | PKABA1| SAMDC|
| BestKeeper           | TUBα | ACT  | TUBβ6| E2   | HSP90| GAPDH| PGK  | EF-1α| SAMDC | PKABA1|
| Comprehensive ranking| TUBβ6| E2   | TUBβ6| HSP90| GAPDH| ACT  | PGK  | EF-1α| PKABA1| SAMDC|
| (B) RANKING ORDER UNDER SALT STRESS (BETTER - GOOD - AVERAGE) |
| geNorm               | EF-1α| HSP90| TUBα | ACT  | SAMDC| PGK  | PKABA1| E2   | TUBβ6| GAPDH|
| NormFinder           | HSP90| TUBα | ACT  | EF-1α| PGK  | SAMDC| PKABA1| E2   | TUBβ6| GAPDH|
| BestKeeper           | TUBβ6| E2   | PGK  | TUBα | HSP90| EF-1α| ACT  | SAMDC| PKABA1| GAPDH|
| Comprehensive ranking| HSP90| TUBα | EF-1α| ACT  | PGK  | E2   | TUBβ6| SAMDC| PKABA1| GAPDH|
| (C) RANKING ORDER UNDER DROUGHT STRESS (BETTER - GOOD - AVERAGE) |
| geNorm               | ACT  | TUBα | SAMDC| E2   | TUBβ6| PGK  | EF-1α| GAPDH| HSP90 | PKABA1|
| NormFinder           | E2   | ACT  | TUBα | PGK  | SAMDC| EF-1α| TUBβ6| GAPDH| HSP90 | PKABA1|
| BestKeeper           | TUBβ6| E2   | TUBα | ACT  | HSP90| PKABA1| EF-1α| PGK  | SAMDC | GAPDH|
| Comprehensive ranking| ACT  | E2   | TUBα | TUBβ6| SAMDC| PGK  | EF-1α| HSP90| GAPDH | PKABA1|
| (D) RANKING ORDER UNDER COLD STRESS (BETTER - GOOD - AVERAGE) |
| geNorm               | TUBα | EF-1α| ACT  | TUBβ6| E2   | GAPDH| HSP90| SAMDC| PKABA1| PGK  |
| NormFinder           | E2   | TUBβ6| HSP90| GAPDH| ACT  | EF-1α| TUBα | SAMDC| PGK  | PKABA1|
| BestKeeper           | TUBα | EF-1α| ACT  | TUBβ6| E2   | GAPDH| PGK  | HSP90| SAMDC| PKABA1|
| Comprehensive ranking| TUBβ6| E2   | TUBβ6| ACT  | E2   | GAPDH| HSP90| PGK  | SAMDC| PKABA1|

Table 5. Comprehensive results of selected suitable reference genes based on geNorm, NormFinder, and BestKeeper.

| Total     | Salt        | Drought     | Cold       |
|-----------|-------------|-------------|------------|
| Most      | Least       | Most        | Least      | Most      | Least      | Most      | Least      |
| TUBβ6     | SAMDC       | HSP90       | GAPDH      | ACT       | PKABA1     | TUBα      | PKABA1     |
| E2        | TUBα        | E2          | EF-1α      | PKABA1    | SAMDC      | PKABA1    |            |
| HSP90     |             |             |            |           |            |           |            |

https://doi.org/10.1371/journal.pone.0190559.t005
the ranks of *PKABA1* are on the rear part of the list for the expression stability of candidate reference genes under various stresses from all three software packages.

**Validation test of the chosen reference genes**

To validate the suitability of candidates, the reference genes that have been ranked as the best or the worst were used to calibrate the expression of the target gene CIRCADIAN CLOCK ASSOCIATED 1, under the stress conditions of drought, salt, and cold. Serving as one of the most important morning-phased components in the circadian rhythmic controlling system of higher plants, CCA1 is a MYB-like transcription factor with enormous abundance in the morning phase [52, 53]. As shown in Fig 4, the expression profile of CCA1 was analyzed with the qRT-PCR method under drought stress condition, while the optimized two reference genes *ACT* and *E2* were used as references. The results are consistent with the Alabadı’s report in Arabidopsis, where the expression peak of CCA1 was observed at time point CT0, and the trough was located at CT12 [45]. Although similar results also can be achieved with the

![Fig 4. Normalized expression level of CCA1 using validated reference genes.](https://doi.org/10.1371/journal.pone.0190559.g004)
PKABA1 gene (the least suitable candidate), which was used as a reference gene, the SD value of CCA1 expression data normalized via PKABA1 was too large to be accepted. This result showed that the data calibrated by PKABA1 was not as stable as the data calibrated by ACT and E2. Furthermore, the average cycle threshold (Ct) values of the PKABA1 gene in certain stress samples varied around 30, which was much higher than that of other candidate reference genes. This phenomenon indicated that the abundance of PKABA1 mRNA in the transcriptome of drought stressed hulless barley was too low to meet the primary requirement of a qualified candidate gene. In the salt stress subset, positive results were obtained via software, recommending the optimal genes TUBa and HSP90 as references to analyze the relative expression of CCA1. While negative results were detected for GAPDH (a rejected reference gene judged by software), this was used to calibrate the expression of CCA1 with unacceptable SD values and disturbed qRT-PCR results. The risk of generating unrealistic results during data normalization processes has rendered the GAPDH gene unsuitable as a reference for analyzing the expression of target genes under salt stress condition. Similarly, when PKABA1 was used to calibrate the expression of CCA1 under cold stress, large SD values and two unexpected distinct peaks located at false time points in the relative expression profiles of CCA1 were found in 24 h time-course samples stressed by low temperature. This result is inconsistent with a report by Seo et al. for Arabidopsis [54]. In contrast, when the two optimal reference genes TUBα and EF-1α were used as references under the same cold stressed condition, results consistent with a previous report were obtained [54]. In summary, PKABA1 is not a suitable reference gene to normalize the expression of the target gene under cold and drought stress conditions.

Discussion

The expression pattern of luxury genes under a particular environment is one of the most important features of the target genes during the identification of their functions. Northern blotting, competitive RT-PCR, microarray, and qRT-PCR are the methods generally used for gene expression pattern detection. However, when the accuracy, sensitivity, specificity, and efficiency of these methods are concerned, qRT-PCR was the optimal choice for a small amount of genes in large sample pools. The selection of appropriate reference genes is essential for the performance of qRT-PCR reactions. Ideally, the reference genes should express stably under various environmental conditions, across different developmental stages, or in individual tissues. Before the large-scale sequencing methods were widely applied in the RNA studies, some so-called housekeeping genes were used by researchers as reference genes, such as EF-1α, UBQ, CYP, ACT, and TUB. The translated products of most of these genes were involved in controlling and carrying out some basic metabolic activities in the cell, or maintaining the primary structures of the cell skeleton system. However, an increasing number of studies have shown that the expression of these abovementioned housekeeping genes are not as constant as they were once assumed to be, especially under various environmental stress conditions [55]. These revelations have negatively affected the reliability of the results of many qRT-PCR studies in which housekeeping genes were used as references without optimization. Reliable reference genes have played a pivotal role during the process of gene expression evaluation via qRT-PCR. In recent years, Carvalho et al. have reported that the two optimized stable expressed housekeeping genes EF-1α and ADP and a commonly used reference gene TUB were used to calibrate the expression of a same target luxury gene, P5CS, in a comparative study. The EF-1α and ADP genes were selected and evaluated with the software geNorm, where the TUB gene was not obtained from the software analysis, but from previous reports where it was most commonly used as a reference for qRT-PCR in the studies from Citrus
species. The results of Carvalho’s study demonstrated that the expression profiles of the target gene, were rather similar when the raw expression data from P5CS had been normalized by the EF-1α and ADP as references; however, significant differences were detected between this and the expression data of the target gene from the same set of RNA samples only when TUB was introduced as the reference gene [56]. This indicates that it is necessary to assess and analyze the stability of candidate reference genes before the implementation of qRT-PCR reactions. For this purpose, various software packages have been developed to conduct the tasks of evaluating the stability of reference genes. GeNorm, NormFinder, and BestKeeper were the most widely used programs [42–44, 57, 58].

Abiotic stresses, such as drought stress, salt stress, or extreme temperatures, always remarkably limited the growth and yield of plants. Studies concerning how those abiotic stresses influenced the growth of plants have been one of the predominant issues for plant sciences research. In particular, many researchers have focused on the identification and characteristics of plant resistance genes to abiotic stresses or the breeding of new plant germplasm resources with significantly promoted abiotic stress resistance, either via genetic engineering or cellular engineering methods. The selection of the most stable reference genes under different stressed conditions would provide a reliable basis for the accurate analysis of resistance gene expression.

In terms of the expression stability of reference genes under low temperature stress, diverse results have been obtained in different plant species. Studies from cucumber have shown that the expression of both CYP and ACT2 were the most stable reference genes under cold stress [59]. However, some other reports from S. sibiricum, tomato, and rice indicated that the EF-1α gene was the most stable reference gene under cold stress condition [38, 40, 60]. In the present study, the results showed that TUBα and EF-1α were the most suitable reference genes for hulless barley under low temperature stress when both stability and repeatability were concerned. Generally consistent with the abovementioned reports by Hong et al., Løvdal and Jain, found that the comprehensive rank of EF-1α is located at the second place in the list of the expression stability of all 10 candidate reference genes tested under cold treatment [38, 40, 60]. Therefore, EF-1α can be used as a reference gene for qRT-PCR in hulless barley under low temperature stress. In addition, we also found that TUBα is a more stable reference gene than EF-1α, which was reported as the most unstable reference gene in Lycoris aurea under cold stress subset [61].

In contrast, the results from our present work showed that the TUBα was ranked first place in the list of the expression stability of all 10 candidate reference genes tested under cold treatment. As far as drought stress condition was concerned, the results from our study revealed that ACT and E2 were also the two candidate reference genes with top expression stability in hulless barley. In contrast to studies in maize under drought stress, TUBβ and EF-1α were confirmed as the best reference genes, while GAPDH and ACT2 ranked third and fifth, respectively [62]. Our results from hulless barley have demonstrated that ACT and E2 were the most stable reference genes, while TUBβ6, EF-1α, and GAPDH ranked fourth, seventh, and ninth place on the list, respectively. In summary, it can be concluded that ACT and E2 are competent reference genes for qRT-PCR tests under drought stress.

Under the salt stress condition, distinct qualified reference genes have been used to analyze gene expression in different plant species. The studies in Oxytropis ochronocephala Bunge grown under salt stress indicated that GAPDH2, HIS, and ACT101 were the top three reference genes with highest stability, while GAPDH1 and 18S were the least stable reference genes [63]. A report in Jute (Corchorus capsularis) revealed that RAN, ACT7, and EF-1α were the top three stable reference genes, while 18S rRNA and ACT were the least stable reference genes under salt stress [51]. This is contrary to results by Hua et al. [14], which have reported that ACT2, UPL, and TIP41 were the most stable reference genes in barley under abiotic stress [14].
Results of the present reference gene screening work in hulless barley convinced that HSP90 and TUBα were the two most competent candidates with top stability, followed by EF-1α, ACT, E2, PGK, TUBβ6, SAMDC, PKABA1, and GAPDH with gradient dropped stability.

It is quite difficult to find an identically optimal reference gene that is universally suited to evaluate gene expressions in plants in different environments or diverse growth stages that is verified by a large body of evidence from either different plant species under various abiotic stress, or the same species of plant but in different developmental stages [50, 64, 65]. This might be partially attributable to differences among the categories of reference genes that have been selected for stability detections in separated research reports [66, 67]. However, by employing different types of software (using various weighted stability parameters from qRT-PCR data via different algorithms), the stability of certain groups of candidate reference genes could be evaluated in a combinative manner. The optimized reference genes selected with this method can be used to calibrate the expression of individual target genes under various conditions with significantly promoted accuracy and efficiency [68, 69].

**Conclusion**

Three different statistical algorithms were used to study the stability of 10 candidate reference genes in hulless barley under abiotic stress conditions. The results showed that under the salt stress condition, the most stable expression of the reference genes were HSP90 and TUBα, while GAPDH had the worst stability. Meanwhile, the best stable reference genes were ACT, E2 and TUBα, EF-1α, respectively, at the drought and cold stress conditions. The most stable reference genes screened in this work can efficiently improve the accuracy and standardization of the expression of target genes under abiotic stress conditions by qRT-PCR analysis in Tibetan hulless barley.

**Supporting information**

S1 Fig. Agarose gel (1.5%) electrophoresis shown the amplification of a single PCR product with expected size. M represents 2000 bp DNA marker. (TIF)

S2 Fig. Melting curves of 10 candidate reference genes shown single peaks in qRT-PCR. (TIF)

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References

1. Abdala-Roberts L, Rasmann S, Berny-Mier YTJC, Covelo F, Glauser G, Moreira X. Biotic and abiotic factors associated with altitudinal variation in plant traits and herbivory in a dominant oak species. American journal of botany. 2016; 103(12):2070–8. https://doi.org/10.3732/ajb.1600310 PMID: 27965243.

2. Abdallah NA, Moses V, Prakash C. The impact of possible climate changes on developing countries: the needs for plants tolerant to abiotic stresses. GM crops & food. 2014; 5(2):77–80. https://doi.org/10.4161/gmcr.32208 PMID: 25075960.

3. Mikkelsen MD, Thomashow MF. A role for circadian evening elements in cold-regulated gene expression in Arabidopsis. The Plant journal: for cell and molecular biology. 2009; 60(2):328–39. https://doi.org/10.1111/j.1365-313X.2009.03957.x PMID: 19566593.

4. Zhu JK. Abiotic Stress Signaling and Responses in Plants. Cell. 2016; 167(2):313–24. https://doi.org/10.1016/j.cell.2016.08.029 PMID: 27716505.

5. Mba C, Guimaraes EP, Ghosh K. Re-orienting crop improvement for the changing climatic conditions of the 21st century. Agriculture & Food Security. 2012; 1(1):1–17. https://doi.org/10.1186/2048-7010-1-7.

6. Gehan MA, Greenham K, Mockler TC, McClung CR. Transcriptional networks-crops, clocks, and abiotic stress. Current opinion in plant biology. 2015; 24:39–46. https://doi.org/10.1016/j.pbi.2015.01.004 PMID: 25646608.

7. International Barley Genome Sequencing C, Mayer KF, Waugh R, Brown JW, Schulman A, Langridge P, et al. A physical, genetic and functional sequence assembly of the barley genome. Nature. 2012; 491(7426):711–6. https://doi.org/10.1038/nature11543 PMID: 23075845.

8. Han B, Huang X. Sequencing-based genome-wide association study in rice. Current opinion in plant biology. 2013; 16(2):133–8. https://doi.org/10.1016/j.pbi.2013.03.006 PMID: 23562592.

9. Zeng X, Long H, Wang Z, Zhao S, Tang Y, Huang Z, et al. The draft genome of Tibetan hulless barley reveals adaptive patterns to the high stressful Tibetan Plateau. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112(4):1095–1000. https://doi.org/10.1073/pnas.1423628112 PMID: 25583503.

10. Hua W, Zhu J, Shang Y, Wang J, Jia Q, Yang J. Identification of Suitable Reference Genes for Barley Gene Expression Under Abiotic Stresses and Hormonal Treatments. Plant Molecular Biology Reporter. 2014; 33(10):1002–12. https://doi.org/10.1016/S1110-0148(07)0070-0.

11. Fehr JE, Trotter GW, Oxford JT, Hart DA. Comparison of Northern blot hybridization and a reverse transcriptase-polymerase chain reaction technique for measurement of mRNA expression of metalloproteinases and matrix components in articular cartilage and synovial membrane from horses with osteoarth. American Journal of Veterinary Research. 2000; 61(8):900–5. https://doi.org/10.2460/ajvr.2000.61.900 PMID: 10951979.

12. Pagliarulo V, George B, Beil SJ, Groshen S, Laird PW, Cai J, et al. Sensitivity and reproducibility of standardized-competitive RT-PCR for transcript quantification and its comparison with real time RT-PCR. Molecular Cancer. 2004; 3(1):1–11. https://doi.org/10.1002/1476-4598-3-5 PMID: 14741054.

13. Choi WY, Kim JS, Park SJ, Ma CJ, Lee HY. Microarray-based gene expression profiling to elucidate effectiveness of fermented Codonopsis lanceolata in mice. International journal of molecular sciences. 2014; 15(4):5907–15. https://doi.org/10.3390/ijms15045907 PMID: 24717412.

14. Demidenko NV, Logacheva MD, Penin AA. Selection and validation of reference genes for quantitative real-time PCR in buckwheat (Fagopyrum esculentum) based on transcriptome sequence data. PloS one. 2011; 6(5):e19434. https://doi.org/10.1371/journal.pone.0019434 PMID: 21589908.

15. Derveaux S, Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. Methods. 2010; 50(4):227–30. https://doi.org/10.1016/j.ymeth.2009.11.001 PMID: 19969088.
20. Lu J, Sivamani E, Azhakandam K, Samadder P, Li X, Qu R. Gene expression enhancement mediated by the 5' UTR intron of the rice rubi3 gene varied remarkably among tissues in transgenic rice plants. Molecular genetics and genomics: MGG. 2008; 279(6):563–72. https://doi.org/10.1007/s00438-008-0333-6 PMID: 18320227.

21. Wiesner M, Hanschen FS, Schreiner M, Glatt H, Zrenner R. Induced production of 1-methoxy-indol-3-ylmethyl glucosinolate by jasmonic acid and methyl jasmonate in sprouts and leaves of pak choi (Brassica rapa ssp. chinensis). International journal of molecular sciences. 2013; 14(7):14996–5016. https://doi.org/10.3390/ijms140714996 PMID: 23873294.

22. Koo SC, Bracko O, Park MS, Schwab R, Chun HJ, Park KM, et al. Control of lateral organ development and flowering time by the Arabidopsis thaliana MAD-box Gene AGAMOUS-LIKE6. The Plant journal: for cell and molecular biology. 2010; 62(5):807–16. https://doi.org/10.1111/j.1365-313X.2010.04192.x PMID: 20230491.

23. Vaucheret H, Vazquez F, Crete P, Bartel DP. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes & development. 2004; 18(10):1187–97. https://doi.org/10.1101/gad.1201404 PMID: 15131082.

24. Pan L-j, Jiang L. Identification and expression of the WRKY transcription factors of Carica papaya in response to abiotic and biotic stresses. Molecular biology reports. 2014; 41(3):1215–25. https://doi.org/10.1007/s11303-013-1966-6 PMID: 24390238.

25. Guerin S, Mauriat M, Pelloux J, Van Wytsvinkel O, Bellini C, Gutierrez L. Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. Journal of experimental botany. 2009; 60(2):487–93. https://doi.org/10.1093/jxb/ern305 PMID: 19264760.

26. Kozera B, Rapacz M. Reference genes in real-time PCR. Journal of applied genetics. 2013; 54(4):391–406. https://doi.org/10.1007/s13353-013-0173-x PMID: 24078518.

27. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of Biochemical and Biophysical Methods. 2000; 46(1):69–81. https://doi.org/10.1007/s13353-000-00129-9.

28. Valasek MA, Repa JJ. The power of real-time PCR. Advances in physiology education. 2005; 29(3):151–9. https://doi.org/10.1152/advan.00019.2005 PMID: 16109794.

29. Dekkers BJW, Willems L, Bassel GW, Ligterink W, Hilhorst HWM, Bentsink L. Identification of Reference Genes for RT–qPCR Expression Analysis in Arabidopsis and Tomato Seeds. Plant & cell physiology. 2010; 62(5):807–16. https://doi.org/10.1111/j.1365-313X.2010.04192.x

30. Fan C, Ma J, Guo Q, Li X, Wang H, Lu M. Selection of reference genes for quantitative real-time PCR in bamboo (Phyllostachys edulis). PloS one. 2013; 8(2):396-. https://doi.org/10.1371/journal.pone.0036573 PMID: 23437174.

31. Hu R, Fan C, Li H, Zhang Q, Fu YF. Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC molecular biology. 2009; 10:93. https://doi.org/10.1186/1471-2199-10-93 PMID: 19785741.

32. Park SC, Kim YH, Chang YJ, Park S, Jeong JC, Lee HS, et al. Stable Internal Reference Genes for the Normalization of Real-Time PCR in Different Sweetpotato Cultivars Subjected to Abiotic Stress Conditions. PloS one. 2012; 7(12):-. https://doi.org/10.1371/journal.pone.0051502 PMID: 23251557.

33. Enrico P, Tanzarella OA, Paolacci AR, Mario C. Identification and validation of reference genes for quantitative RT–PCR normalization in wheat. BMC molecular biology. 2009, 10(1):1–27. https://doi.org/10.1186/1471-2199-10-11 PMID: 19232096.

34. Shrivhare R, Lata C. Selection of suitable reference genes for assessing gene expression in pearl millet under different abiotic stresses and their combinations. Scientific reports. 2016; 6:23036. https://doi.org/10.1038/srep23036 PMID: 26972345.

35. Jaroslav J, Kunda JK. Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. BMC plant biology. 2010; 10(1)::. 146. https://doi.org/10.1186/1471-2229-10-146 PMID: 20630112.

36. Zhu YN, Shi DQ, Ruan MB, Zhang LL, Meng ZH, Liu J, et al. Transcriptome analysis reveals crosstalk of responsive genes to multiple abiotic stresses in cotton (Gossypium hirsutum L.). PloS one. 2013; 8(11):158-. https://doi.org/10.1371/journal.pone.0080218 PMID: 24224045.

37. Guo J, Hui L, Wu Q, Xu L, Que Y. The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. Scientific reports. 2014; 4:7042-. https://doi.org/10.1038/srep07042 PMID: 25391499.

38. Hong SY, Seo PJ, Yang MS, Xiang F, Park CM. Exploring valid reference genes for gene expression studies in Brachypodium distachyon by real-time PCR. BMC plant biology. 2008; 8(1)::. 112. https://doi.org/10.1186/1471-2229-8-112 PMID: 18992143.
39. Rønning SB, Berdal KG, Andersen CB, Holst-Jensen A. Novel reference gene, PKABA1, used in a duplex real-time polymerase chain reaction for detection and quantitation of wheat- and barley-derived DNA. Journal of Agricultural & Food Chemistry. 2006; 54(3):682–7. https://doi.org/10.1021/jf052328n PMID: 16484168

40. Levdal T, Lillo C. Reference gene selection for quantitative real-time PCR normalization in tomato subjected to nitrogen, cold, and light stress. Analytical Biochemistry. 2009; 387(2):238–42. https://doi.org/10.1016/j.ab.2009.01.024 PMID: 19454243

41. Ovesná J, Kucera L, Vaculová K, K Š, Svobodová I, Milella L. Validation of the β-amy1 transcription profiling assay and selection of reference genes suited for a RT-qPCR assay in developing barley caryops. PloS one. 2012; 7(7):e41886. https://doi.org/10.1371/journal.pone.0041886 PMID: 22866024

42. Vandesenompele J, Preter KD, Pattyn F, Poppe B, Roy NV, Paepe AD, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002; 3(7). https://doi.org/10.1186/1471-2105-3-7-research0034

43. Andersen CL, Jensen JL, Ørntoft TF. 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 research. 2004; 64(15):5245–50. https://doi.org/10.1158/0008-5472.CAN-04-0496 PMID: 15289330

44. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. Biotechnology Letters. 2004; 26(6):509–15. https://doi.org/10.1023/B:BILE.0000019559.84930.47 PMID: 15127793

45. Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Más P, Kay SA. Reciprocal regulation between TOC1 and LHY/CCA1 within the Arabidopsis circadian clock. Science. 2001; 293(5531):880–3. https://doi.org/10.1126/science.1061320 PMID: 11486091

46. Lu SX, Knowles SM, Andronis C, Ong MS, Tobin EM. CIRCADIAN CLOCK ASSOCIATED1 and LATE HYPOCOTYL function synergetically in the circadian clock of Arabidopsis. Plant physiology. 2009; 150(2):834–43. https://doi.org/10.1104/pp.108.133272 PMID: 19218364

47. Lu SX, Tobin EM. CCA1 and ELF3 Interact in the Control of Hypocotyl Length and Flowering Time in Arabidopsis. Plant physiology. 2012; 158(2):1079–88. https://doi.org/10.1104/pp.111.189670 PMID: 22190341

48. Yakir E, Hilman D, Kron I, Hassidim M, Melamed-Book N, Green RM. Posttranslational regulation of...
58. Zeng S, Liu Y, Wu M, Liu X, Shen X, Liu C, et al. Identification and validation of reference genes for quantitative real-time PCR normalization and its applications in lycium. PloS one. 2014; 9(5):e97039. https://doi.org/10.1371/journal.pone.0097039 PMID: 24810586

59. Wan HJ, Zhao ZG, Qian CT, Sui Y, Malik AA, Chen JF. Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Analytical Biochemistry. 2009; 399(2):257–61. https://doi.org/10.1016/j.ab.2009.12.008 PMID: 20005862

60. Jain M, Nijhawan A, Tyagi AK, Khurana JP. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochemical & Biophysical Research Communications. 2006; 345(2):646–51. https://doi.org/10.1016/j.bbrc.2006.04.146 PMID: 16690022

61. Ma R, Xu S, Zhao Y, Xia B, Wang R. Selection and Validation of Appropriate Reference Genes for Quantitative Real-Time PCR Analysis of Gene Expression in Lycoris aurea. Frontiers in plant science. 2016; 7:536. https://doi.org/10.3389/fpls.2016.00536 PMID: 27200013

62. Lin Y, Zhang C, Lan H, Gao S, Liu H, Liu J, et al. Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. PloS one. 2014; 9(5):e95445. https://doi.org/10.1371/journal.pone.0095445 PMID: 24810581

63. Zhuang H, Fu Y, He W, Wang L, Wei Y. Selection of appropriate reference genes for quantitative real-time PCR in Oxytropis ochrocephala Bunge using transcriptome datasets under abiotic stress treatments. Frontiers in plant science. 2015; 6:475. https://doi.org/10.3389/fpls.2015.00475 PMID: 26175743

64. Li XY, Cheng JY, Zhang J, Silva JATD, Wang CX, Sun HM. Validation of Reference Genes for Accurate Normalization of Gene Expression in Lilium davidii var. unicolor for Real Time Quantitative PCR. PloS one. 2015; 10(10):e0141323. https://doi.org/10.1371/journal.pone.0141323 PMID: 26509446

65. Yang T, Chaudhuri S, Yang L, Du L, Poovaiah BW. A calcium/calmmodulin-regulated member of the receptor-like kinase family confers cold tolerance in plants. Journal of Biological Chemistry. 2009; 285(10):7119–26. https://doi.org/10.1074/jbc.M109.035659 PMID: 20026608

66. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. PloS one. 2010; 5(12):5525–56. https://doi.org/10.1371/journal.pone.0015208 PMID: 21179435

67. Kong Q, Yuan J, Gao L, Zhao S, Wei J, Huang Y, et al. Identification of Suitable Reference Genes for Gene Expression Normalization in qRT-PCR Analysis in Watermelon. PloS one. 2014; 9(2):e90612. https://doi.org/10.1371/journal.pone.0090612 PMID: 24387403

68. Yang Q, Yin J, Li G, Qi L, Yang F, Wang R, et al. Reference gene selection for qRT-PCR in Caragana korshinskii Kom. under different stress conditions. Molecular biology reports. 2014; 41(4):2325–34. https://doi.org/10.1007/s11033-014-3086-9 PMID: 24452712

69. Zhong HY, Chen JW, Li CQ, Chen L, Wu JY, Chen JY, et al. Selection of reliable reference genes for expression studies by reverse transcription quantitative real-time PCR in litchi under different experimental conditions. Plant cell reports. 2011; 30(4):641–53. https://doi.org/10.1007/s00299-010-0992-8 PMID: 21901853