Identification of Candidate Reference Genes in Perennial Ryegrass for Quantitative RT-PCR under Various Abiotic Stress Conditions

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Abstract

Background: Quantitative real-time reverse-transcriptase PCR (qRT-PCR) is an important technique for analyzing differences in gene expression due to its sensitivity, accuracy and specificity. However, the stability of the expression of reference genes is necessary to ensure accurate qRT-PCR assessment of expression in genes of interest. Perennial ryegrass (Lolium perenne L.) is important forage and turf grass species in temperate regions, but the expression stability of its reference genes under various stresses has not been well-studied.

Methodology/Principal Findings: In this study, 11 candidate reference genes were evaluated for use as controls in qRT-PCR to quantify gene expression in perennial ryegrass under drought, high salinity, heat, waterlogging, and ABA (abscisic acid) treatments. Four approaches – Delta C_T, geNorm, BestKeeper and Normfinder were used to determine the stability of expression in these reference genes. The results are consistent with the idea that the best reference genes depend on the stress treatment under investigation. Eukaryotic initiation factor 4 alpha (eIF4A), Transcription elongation factor 1 (TEF1) and Tat binding protein-1 (TBP-1) were the three most stably expressed genes under drought stress and were also the three best genes for studying salt stress. eIF4A, TBP-1, and Ubiquitin-conjugating enzyme (E2) were the most suitable reference genes to study heat stress, while eIF4A, TEF1, and E2 were the three best reference genes for studying the effects of ABA. Finally, Ubiquitin (UBQ), TEF1, and eIF4A were the three best reference genes for waterlogging treatments.

Conclusions/Significance: These results will be helpful in choosing the best reference genes for use in studies related to various abiotic stresses in perennial ryegrass. The stability of expression in these reference genes will enable better normalization and quantification of the transcript levels for studies of gene expression in such studies.

Introduction

Perennial ryegrass (Lolium perenne L.) is dominant forage and turf grass specie in temperate regions due to its good grazing tolerance, extraordinarily high digestibility and adequate seed production [1,2]. Perennial ryegrass is cultivated in the USA, China, Japan, UK, Australia, New Zealand, South Africa and South America [3,4]. The species may also aid China in mitigating food shortages. For example, the stress tolerance 

stress [6]. Under waterlogging treatment, the emergence and seedling growth of cv. S.24 perennial ryegrass is significantly reduced in a glasshouse environment [7], with dry matter yield reduced by up to 25% [8]. Moreover, as a cool season grass, perennial ryegrass has poor heat resistance, making it more likely to die in high-temperature, and high-humidity regions in summer. The susceptibility of perennial ryegrass to freezing temperature also limits its cultivation [9].

Understanding the genetic responses of different varieties of perennial ryegrass to differential abiotic stresses could lead to better germplasm utilization strategies for successful agronomic production in regions experiencing different climate conditions. For example, the stress tolerance-conferring miR398 could potentially be used as a genetic component or a genetic marker for plant stress tolerance [10–12]. Numerous studies clarifying
genetic mechanisms of abiotic stress responses in plants have been based on gene expression analysis [13–15]. In perennial ryegrass, the expression of C-repeat (CRT) binding factors (CBF) gene can be rapidly induced in response to low temperature [16], while expression of myoinositol 1-phosphate synthase (EC 5.5.1.4) and galactinol synthase (EC 2.4.1.123) genes can be decreased by drought stress [17]. Therefore, the exploration of expression patterns of candidate stress tolerance gene is critical to illustrate the mechanisms underlying abiotic stress tolerances in perennial ryegrass.

Quantitative real-time reverse-transcriptase PCR (qRT-PCR) together with Northern blots, microarrays, and RNA-Seq analysis [18] are useful for inferring the transcription levels of genes, and especially for detecting low-quantity miRNAs. In particular, qRT-PCR is valuable for its accurate quantification of target genes across a relatively broad dynamic range [19,20]. However, numerous factors –RNA stability, quality, or quantity, transcription efficiencies, and PCR reaction conditions – can all affect the reliability of qRT-PCR. In order to ensure that any variation observed in qRT-PCR estimates of transcript levels are due to changes in expression of the target gene(s) rather than overall variations in mRNA among biological samples or replicate, multiple genes should be analyzed simultaneously. To avoid bias, qRT-PCR is also typically referenced to an internal control gene having relatively stable expression for comparison to the target genes [21].

Reference genes maintain stable gene expression based on the need to support cell function or cell structure across various experimental conditions, and can contribute to the identification of subtle differences in expression among genes of interest [22,23]. Numerous reference genes are commonly used in gene expression studies, such as β-actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor (TEF), tubulin (TUB), ubiquitin conjugating enzyme (UBC), 18S ribosomal RNA (18S rRNA), and 25S ribosomal RNA (25S rRNA) [24–26]. Nevertheless, some of these reference genes may differ in expression among plant tissues, species and growth conditions (e.g., abiotic stresses). If this is the case, a study aiming to compare gene across such conditions may have no reference genes with a suitably stable expression profile [27–30], making the unbiased assessment of target genes difficult. Therefore, it is crucial to assess the expression stability of reference gene for various conditions prior to a complete study. Eukaryotic elongation factor 1 alpha (eEF1A (s)) and YT521-B-like family protein (YT521-B) have previously been identified as suitable reference genes for normalizing expression of target genes under different defoliation regimes in perennial ryegrass [31], but few similar studies assessing expression stability under other stresses have been published. Therefore, for further development of qRT-PCR as a tool for studying transcriptional responses in perennial ryegrass, it is important to screen potential candidate reference genes under various abiotic stresses.

In this study, we contrasted the expression stability of 11 candidate reference genes (Zeitlupe [ZTL], 60S ribosomal protein [60S], Histone 3 [H3], TEF1, GAPDH, UBG, eEF1A (s), TBP-1, eIF4A, YT521-B, and E2) in perennial ryegrass under drought, heat, salt, waterlogging and ABA treatments. We used the results to identify the best reference genes for normalization of the expression of target genes in perennial ryegrass gene under each of these abiotic stresses.
### Table 1. List of primer sequences and related information for 13 genes (11 reference gene and 2 object genes).

| Gene    | Gene function     | Amplification efficiency (%) | Primer sequence (5'-3') (Forward) | Primer sequence (5'-3') (Reverse) | Tm (°C) | Amplification length (bp) |
|---------|-------------------|-----------------------------|-----------------------------------|-----------------------------------|---------|--------------------------|
| ZTL     | Zeitlupe          | 98.64                       | AGTTCCAGGGTGATCTGCTG              | TCACAACTTCCTTTGCCACA              | 77.19   | 180                      |
| 60S     | 60S Ribosomal protein L18A | 93.57             | GCAGAATACGAGGGCAATGT              | ACCAAAGACACGGTTTCCAG              | 81.16   | 132                      |
| TEF1    | Transcription elongation factor 1 | 96.09             | CGTGTGATCGAGAGGTTTGA              | CGAATTTCCAGAGGGCAATA              | 79.78   | 133                      |
| GAPDH   | Glyceraldehyde-3-phosphate dehydrogenase | 97.56             | TCTGACCGTTAGACTTGAGAAGG          | CTTGAGCTTACCCTCAGACTCCT           | 77.98   | 83                       |
| UBQ     | Ubiquitin         | 96.04                       | AAAATTCCCCAATCAATCTCCT            | CTTCACAAAGATCTGCATCTTGA           | 80.08   | 89                       |
| eEF1A   | Eukaryotic elongation factor 1alpha | 101.02            | CCGTTTTGTCGAGTTTGGT              | AGCAACTGTAACCGAACATAGC           | 77.64   | 113                      |
| TBP-1   | 26S proteasome regulatory subunit 6A homolog | 98.45             | TGCTTAGTTCCCCTAAGATAGTGA         | CTGAGACCAAACACGATTTCA             | 74.11   | 112                      |
| eIF4A   | Eukaryotic initiation factor 4alpha | 103.02            | AACTCAACTTGAAGTGTTGGAGTG         | AGATCTGGTCCTGGAAAGAATATG          | 78.49   | 168                      |
| YT521-B | YT521-B-like family protein | 93.61             | TGTAGCTTGATCGCATACCC             | ACTCCCTGGTAGCCACCTT              | 79.61   | 122                      |
| H3      | Histone 3         | 99.09                       | CACCAATGTTCTGCCTATCG              | CAGACCAACGAACAC                  | 76.67   | 135                      |
| E2      | Ubiquitin conjugating enzyme | 99.10             | CGGTTCTGTGCCAAAATGT              | CAGCTATCTCCAACGGTTCA             | 77.28   | 111                      |
| POD     | Guaiacol peroxidase | 95.34             | CTCTACAACGAGACCAACATCAA          | GACGCTGCGACAAGGCTA               | 76.61   | 122                      |
| SOD     | Superoxide dismutase | 95.37             | CTCTACAACGAGACCAACATCAA          | GACGCTGCGACAAGGCTA               | 76.61   | 122                      |

### Plant Materials, Growth Conditions and Stress Treatments

Perennial ryegrass cv. Barnauta were germinated and grown in 3-L pots containing 1 kg soil (soil pH of 5.18; organic qualitative content of 1.41%; N, P, K of 100.36 mg kg\(^{-1}\), 4.32 mg kg\(^{-1}\), and 337.24 mg kg\(^{-1}\), respectively) in a growth chamber at 25°C. A light source provided 100 μmol of photons m\(^{-2}\) s\(^{-1}\) on a 16/8-h light/dark regime. Plants at the 6–8 leaf stage were used for all stress treatments. Control plants were watered every other day to maintain 80% soil water content, and each pot was watered and weighed every daily to maintain a total mass of 1 kg (0.97 kg potting medium plus 0.03 kg pot). The amount of water that added to each pot was recorded. Plants were exposed to a drought treatment by ending the above watering regime for 15 days. At the end of drought treatment, the leaf water content was 10% as measured by a method [32]. For heat treatment, plants were moved to a growth chamber set at 37°C for 7 days, and all other environmental conditions were held constant relative to the early growth stage. For salinity treatment, Plants were watered with 250 mmol L\(^{-1}\) NaCl for 12 days. For waterlogging treatment, Plants were flooded with water with 2.5 cm above the soil surface daily for 15 days, with control and treated pots maintained in the same growth chamber. For ABA treatment, Plants were treated with a concentration of 100 μmol L\(^{-1}\) ABA sprayed on the plants for 12 days, while control plants were treated with equal quantity of water. All experimental and control plants (leaves and roots) were separated three times with three biological replicates for expression analysis under different conditions.

### RNA isolation and cDNA Synthesis

For all the experiments, RNA was extracted from plants using the Total RNA Kit II (Genebase Bioscience, China) according to the manufacturer’s instructions. Possible DNA contamination was removed from RNA samples by treatment with RNase-free DNase I provided in the kit. Concentration and purity of RNA samples were assessed spectrophotometrically with a NanoDrop ND-1000 Micro-Volume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), with 260/280 nm ratios in the range of 1.9 to 2.2 and 260/230 nm around 2.0 considered acceptable for use in qRT-PCR. Integrity of the RNA was also confirmed using 1% agarose gel electrophoresis. A total of 0.8 μg RNA was used for reverse transcription with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, California, USA) with Poly (A) and random primers in a 20 μl reaction volume according to the manufacturer’s instructions. The cDNA obtained for each sample was diluted 1:20 with nuclease-free water for qRT-PCR.

### Primer Design and Quantitative RT-PCR Analysis

The 60S, GAPDH, UBQ, ZTL, TEF1, peroxidase (POD), and superoxide dismutase (SOD) orthologous genes of wheat were used as ‘query’ to BLAST against available perennial ryegrass expressed sequence tags in the GenBank database (NCBI). All genes were named based on their similarity to known genes with sequence similarity from 90% to 98%. Primer sequences for an additional six reference genes were identified from a previous study [31].

Primer pairs, length of 18 to 24 nucleotides in optimal length of 20 nucleotides, and GC content from 40% to 60%. In order to check the primers’ specificity, the PCR products were analyzed using the primers' specificity, the PCR products were analyzed using a method [32]. For heat treatment, plants were moved to a growth chamber set at 37°C for 7 days, and all other environmental conditions were held constant relative to the early growth stage. For salinity treatment, Plants were watered with 250 mmol L\(^{-1}\) NaCl for 12 days. For waterlogging treatment, Plants were flooded with water with 2.5 cm above the soil surface daily for 15 days, with control and treated pots maintained in the same growth chamber. For ABA treatment, Plants were treated with a concentration of 100 μmol L\(^{-1}\) ABA sprayed on the plants for 12 days, while control plants were treated with equal quantity of water. All experimental and control plants (leaves and roots) were separated three times with three biological replicates for expression analysis under different conditions.
2.0% agarose gel electrophoresis and stained using Gelred (Biotium, USA).

QRT-PCR reactions were executed in 96-well blocks with a BIO-RAD CFX96 Real-Time PCR system (Bio-Rad, USA) using SYBR Premix Ex Taq™ (TaKaRa, Japan). The PCR reactions were 20 μl in volume and contained 10 μl 2×SYBR Premix Ex Taq™, 2 μl diluted cDNA reaction mixture, 0.4 μl ROX Reference Dye II, and 1 μl 10 μM for each primer. The cycling conditions were as recommended by the manufacturer: 5 min at 95°C, 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. At the end of the cycling process, the temperature was raised from 60°C to 95°C to obtain the dissociation curve. Three technical replicates were performed for each biological replicate – gene combination. The final cycle threshold (CT) values were calculated as the means of all nine values.

Gene Expression Stability Analysis

The stability of reference gene expression was analyzed using four different VBA (Visual Basic Applet) applets: Delta CT method [33], geNorm (ver. 3.5) [34], BestKeeper (ver. 1.0) [35] and Normfinder (ver. 0.953) [36]. Results from CFX manager (Bio-Rad) were exported into Microsoft Excel 2007 and transformed to create input files for each target according to the requirements of each software. The geNorm tool ranked the reference genes by calculating the gene expression stability value (M1) based on the average pairwise expression ratio. The most stable reference gene has the lowest M1 value, while the least stable one presents the highest M1 value. The program considers M1 values below 1.5 to indicate stable expression. Normfinder is a Microsoft Excel application which ranks candidate genes according to stability index M2 based on the average pairwise variation of a given gene compared to all other studied genes [33]. The more stably expressed genes exhibit lower M2 values. BestKeeper is another Excel-based tool that identifies the most stably expressed genes by making comparisons of the coefficient of variance (CV) and the standard deviation (SD) [35]. The most stable genes are those with the lowest coefficient of variance and standard deviation (CV ± SD). Finally, the reference genes' stability values derived from each of the three software tools were then used to create a comprehensive rank each of the 11 candidate reference genes in order from most to least stable expression under each abiotic stress condition using RefFinder [37]. This is a user-friendly, web-based tool developed for comprehensively evaluating and screening reference genes from extensive experimental datasets [37].

Gene-specific PCR efficiency was also calculated based on standard curves using a 10-fold serial dilution of the mixed cDNA template for each primer pair. The correlation coefficients (R²) and slope values were acquired from the standard curve. Calculate the coefficient of variation (CV) according to the equation: 

\[ E = 10^{(1/\text{slope})} - 1) \times 100 \] [38].

To detect the influence of reference genes on the outcome of an experiment, the relative expression patterns of two genes SOD and POD were evaluated using the most stable and unstable genes in Days 0, 3, 6 and 9 under drought treatment.

Figure 2. Median cycle threshold (Ct) values for each reference gene for all samples. The filled diamond symbol indicates median Ct values. The bars indicate standard deviation. doi:10.1371/journal.pone.0093724.g002

Candidate reference genes

- ZTL
- 60S
- TEF1
- UBQ
- GAPDH
- eEF1A (s)
- TBP-1
- eIF4A
- YT521-B
- H3
- E2

Ct values
Figure 3. Average expression stability values (M₁) of 11 candidate reference genes calculated by geNorm. (a) drought stress, (b) salt stress, (c) heat stress, (d) waterlogging stress, (e) ABA treatment. Lower M₁ values indicate more stable expression.

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Table 2. Expression stability values for perennial ryegrass candidate reference genes calculated using Normfinder under five treatments.

| Rank | Drought stress | Salt stress | Heat stress | Waterlogging stress | ABA |
|------|----------------|-------------|-------------|---------------------|-----|
| 1    | TEF1 (0.568)   | eIF4A (0.628) | eIF4A (0.592) | UBQ (0.700) | eIF4A (0.700) |
| 2    | eIF4A (0.573)  | TEF1 (0.748)  | YT521-B (0.757) | TEF1 (0.837) | TEF1 (0.718)  |
| 3    | TBP-1 (0.698)  | YT521-B (0.786) | TBP-1 (0.796) | YT521-B (0.847) | YT521-B (0.737) |
| 4    | UBQ (0.713)    | TBP-1 (0.790) | H3 (0.839) | E2 (0.870) | H3 (0.754)   |
| 5    | YT521-B (0.757) | H3 (0.799) | UBQ (0.920) | eIF4A (0.925) | E2 (0.990) |
| 6    | H3 (0.862)     | UBQ (0.819) | TEF1 (0.931) | H3 (0.983) | UBQ (1.037) |
| 7    | E2 (0.924)     | E2 (0.973) | E2 (0.939) | TBP-1 (1.031) | TBP-1 (1.085) |
| 8    | ZTL (1.224)    | ZTL (1.080) | ZTL (1.340) | ZTL (1.209) | ZTL (1.240) |
| 9    | 60S (1.483)    | eEF1A (s) (1.592) | eEF1A (s) (1.559) | 60S (1.403) | GAPDH (1.581) |
| 10   | GAPDH (1.516)  | GAPDH (1.611) | GAPDH (1.563) | GAPDH (1.577) | eEF1A (s) (1.610) |
| 11   | eEF1A (s) (1.661) | 60S (1.953) | 60S (1.814) | eEF1A (s) (1.581) | 60S (1.841) |

Note: Expression stability and ranking of 11 candidate reference genes calculated with Normfinder under drought, salt, heat, waterlogging stresses and ABA treatment. Lower average expression stability (M₂ value) indicates more stable expression.

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Table 3. Expression stability values for perennial ryegrass candidate reference genes calculated using BestKeeper under five treatments.

| Rank | Drought | Salt stress | Heat stress | Waterlogging stress | ABA |
|------|---------|-------------|-------------|---------------------|-----|
| 1    | ZTL (1.02±0.02) | ZTL (1.32±0.03) | ZTL (0.85±0.05) | ZTL (1.23±0.03) | ZTL (1.32±0.09) |
| 2    | TEF1 (2.35±0.70) | TEF1 (2.04±0.61) | eEF1A (2.94±0.92) | TEF1 (1.71±0.51) | TEF1 (3.17±0.94) |
| 3    | eEF1A (2.55±0.81) | eEF1A (2.17±0.69) | GAPDH (3.79±1.04) | eEF1A (2.19±0.69) | eEF1A (3.11±0.97) |
| 4    | GAPDH (3.75±1.03) | eIF4A (2.83±0.85) | H3 (4.27±1.22) | GAPDH (3.99±3.09) | GAPDH (3.89±1.06) |
| 5    | eIF4A (3.72±1.10) | TBP-1 (3.49±0.96) | TEF1 (5.74±1.53) | E2 (3.96±1.30) | H3 (4.42±1.26) |
| 6    | TBP-1 (5.13±1.38) | ZTL (1.40±1.28) | TEF1 (4.48±1.47) | eEF4A (4.67±1.37) | E2 (4.25±1.40) |
| 7    | H3 (4.83±1.38) | YT521-B (4.35±1.34) | TBP-1 (5.74±1.53) | UBQ (4.94±1.38) | TBP-1 (5.24±1.41) |
| 8    | YT521-B (5.05±1.55) | GAPDH (4.85±1.35) | UBQ (6.03±1.66) | TBP-1 (6.17±1.64) | UBQ (5.47±1.52) |
| 9    | E2 (4.88±1.60) | UBQ (5.01±1.42) | YT521-B (5.47±1.67) | YT521-B (5.39±1.64) | YT521-B (5.10±1.56) |
| 10   | 60S (6.14±1.83) | 60S (7.29±2.21) | 60S (6.35±1.89) | 60S (6.04±1.78) | 60S (6.68±1.99) |

Note: Expression stability and ranking of 11 candidate reference genes calculated with BestKeeper under drought, salt, heat, waterlogging stresses and ABA treatment. Eleven reference genes are identified as the most stable genes, as evaluated by the lowest values of the coefficient of variance (CV) and standard deviation (SD).

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Results

Verification of PCR Amplicons, Primer Specificity, and PCR Amplification Efficiency

The description of 13 genes (11 candidate reference genes and 2 object genes), primer sets, melting temperatures (Tm) values, and amplicon lengths assessed using qRT-PCR in this study are listed in Table 1. The melting temperatures (Tm) values of all PCR products ranged from 74.11°C to 93.57°C for 60S (Table 1). Amplicon sizes were determined by testing each primer pair using electrophoresis on a 2% agarose gel (Figure 1A). This and the single peak melting curves of the qRT-PCR products (Figure 1B) demonstrated that a single PCR amplification product pair using electrophoresis on a 2% agarose gel (Figure 1A). This single peak melting curve of the qRT-PCR products (Figure 1B) demonstrated that a single PCR amplification product of the expected size was obtained for each reference gene. The amplification efficiency of all primers ranged from 103.02% (eIF4A) to 93.57% (60S; Table 1).

Determination of Ct Values and Variation in Reference Gene Expression

Cycle threshold (Ct) values were obtained from nine qRT-PCR reactions (three biological replicates, each with three technical replicates) for each of the 11 candidate reference genes under each of the abiotic stress treatments. To quantify overall differences in transcript levels for the 11 reference genes, we calculated the median Ct range, and CV of Ct for each gene across all samples. As expected, the median Ct values for each of the 11 candidate reference genes ranged from 15 to 30 cycles (Figure 2). Most displayed median Ct values ranging from 20 to 25, indicating a moderately high level of expression. The gene eEF1A was the highest expression level among the tested candidate reference genes (i.e., had the lowest median Ct value), while H3 had the lowest expression level (i.e., the highest median Ct value). To reveal the expression stability of candidate reference genes, it is necessary to assess the standard deviation of Ct values. In our results, some genes with high standard deviations (for instance, ZTL, 60S and YT521-B) had relatively large standard deviations, indicating more variable expression levels, while others such as UBQ, H3, and eIF4A had smaller standard deviations and more stable expression patterns.

Stability Ranking of Candidate Reference Genes

To compare stability of expression among the candidate reference genes, the computational methods, BestKeeper, NormFinder, and geNorm were applied to Ct values for each gene’s expression data. These tools are based on different models and assumptions and each produced different results for the same gene’s expression data. RefFinder was used to calculate a recommended comprehensive ranking based on the results of computational analysis, which in turn allowed us to identify the best reference genes for qRT-PCR data normalization in perennial ryegrass samples.

a) geNorm analysis. We used geNorm to rank reference genes’ expression stability based on average pairwise expression ratios (Mi), with values below 1.5 considered to represent stable expression. In our study, all genes except 60S and GAPDH exhibited Mi values <1.5, indicating stable expression (Figure 3). Similar results were produced across different stress treatments. The TBP-1, E2, and eIF4A were the top three genes under drought, salt, heat, and ABA treatments.

To evaluate the optimal number of the reference genes required for accurate normalization, the pairwise variation between consecutively ranked genes (Vn/Vn+1) was calculated using geNorm. The optimal number of reference genes was identified as the lowest number of genes producing a pairwise variation of no more than 0.15 [34]. When all samples (i.e., across all stress treatments) were considered together to determine the optimal number of reference genes, the pairwise variation of V2/V3 was higher than 0.15 (0.173), the V3/V4 (0.145), indicating that three reference genes should be included for gene expression studies in perennial ryegrass that encompass multiple stress conditions.

b) NormFinder analysis. NormFinder ranks gene expression stability based on average pairwise variation of a gene compared to all other genes (M2). The expression stability calculated by NormFinder for each gene in this study is shown in Table 2. The results showed that TEF1 and UBQ were most stable in expression, and therefore, they were the most appropriate reference genes, under drought and waterlogging stresses, with M2 values of 0.568 and 0.700, respectively. When considering salt, heat stresses, and ABA treatment, eIF4A was the most reliable reference gene, with M2 values of 0.628, 0.592, and 0.700, respectively. In contrast, eEF1A was the least stable reference gene under drought and...
Table 4. Stability ranking of 11 candidate reference genes.

| Methods | A. Ranking Order under drought stress (Better–Good–Average) | B. Ranking Order under salt stress (Better–Good–Average) | C. Ranking Order under heat stress (Better–Good–Average) | D. Ranking Order under waterlogging stress (Better–Good–Average) | E. Ranking Order under ABA treatment (Better–Good–Average) |
|---------|---------------------------------------------------------|------------------------------------------------------|--------------------------------------------------------|---------------------------------------------------------------|----------------------------------------------------------|
|         | 1  2  3  4  5  6  7  8  9  10  11                        | 1  2  3  4  5  6  7  8  9  10  11                    | 1  2  3  4  5  6  7  8  9  10  11                        | 1  2  3  4  5  6  7  8  9  10  11                           | 1  2  3  4  5  6  7  8  9  10  11                          |
| Delta CT | eIF4A  TBP-1  TEF1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | eIF4A  TBP-1  TEF1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | eIF4A  TBP-1  TEF1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | eIF4A  TBP-1  TEF1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | eIF4A  TBP-1  TEF1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) |
| BestKeeper | ZTL  TEF1  eEF1A (s)  GAPDH  eIF4A  UBQ  TBP-1  H3  YT521-B  E2  60S | ZTL  TEF1  eEF1A (s)  GAPDH  eIF4A  UBQ  TBP-1  H3  YT521-B  E2  60S | ZTL  TEF1  eEF1A (s)  GAPDH  eIF4A  UBQ  TBP-1  H3  YT521-B  E2  60S | ZTL  TEF1  eEF1A (s)  GAPDH  eIF4A  UBQ  TBP-1  H3  YT521-B  E2  60S | ZTL  TEF1  eEF1A (s)  GAPDH  eIF4A  UBQ  TBP-1  H3  YT521-B  E2  60S |
| Normfinder | TEF1  eIF4A  TBP-1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | TEF1  eIF4A  TBP-1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | TEF1  eIF4A  TBP-1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | TEF1  eIF4A  TBP-1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) | TEF1  eIF4A  TBP-1  UBQ  YT521-B  H3  E2  ZTL  60S  GAPDH  eEF1A (s) |
| geNorm | TBP-1  | E2  eIF4A  H3  YT521-B  UBQ  TEF1  60S  ZTL  GAPDH  eEF1A (s) | TBP-1  | E2  eIF4A  H3  YT521-B  UBQ  TEF1  60S  ZTL  GAPDH  eEF1A (s) | TBP-1  | E2  eIF4A  H3  YT521-B  UBQ  TEF1  60S  ZTL  GAPDH  eEF1A (s) | TBP-1  | E2  eIF4A  H3  YT521-B  UBQ  TEF1  60S  ZTL  GAPDH  eEF1A (s) | TBP-1  | E2  eIF4A  H3  YT521-B  UBQ  TEF1  60S  ZTL  GAPDH  eEF1A (s) |
| Recommended comprehensive ranking | eIF4A  TEF1  TBP-1  E2  UBQ  ZTL  YT521-B  H3  eEF1A (s)  GAPDH  60S | eIF4A  TEF1  TBP-1  E2  UBQ  ZTL  YT521-B  H3  eEF1A (s)  GAPDH  60S | eIF4A  TEF1  TBP-1  E2  UBQ  ZTL  YT521-B  H3  eEF1A (s)  GAPDH  60S | eIF4A  TEF1  TBP-1  E2  UBQ  ZTL  YT521-B  H3  eEF1A (s)  GAPDH  60S | eIF4A  TEF1  TBP-1  E2  UBQ  ZTL  YT521-B  H3  eEF1A (s)  GAPDH  60S |

Identifying Reference Genes of Perennial Ryegrass

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indicated that few differences occurred across stress treatments making it the least stable. The results obtained from BestKeeper and Normfinder indicated that the most stably expressed reference gene was TBP-1, followed by TEF1 and eIF4A, respectively, under drought, salt, heat, waterlogging, and ABA treatments, respectively, making it the least stable. The results obtained from BestKeeper indicated that few differences occurred across stress treatments (Table 3).

c) BestKeeper analysis. BestKeeper analysis determined which genes exhibited the lowest (CV±standard deviations) in order to judge stability of gene expression (Table 3). Results indicate that ZTL had values of 1.02±0.02, 1.32±0.03, 0.85±0.05, 1.23±0.03, and 1.32±0.09 under drought, salt, heat, waterlogging, and ABA treatments, respectively, making it the most stably expressed reference gene in all cases. However, 60S had CV values of 6.14±1.83, 7.29±2.21, 6.35±1.89, 6.04±1.78 and 6.68±1.99, respectively, under the same abiotic stresses, making it the least stable. The results obtained from BestKeeper indicated that few differences occurred across stress treatments (Table 3).

d) RefFinder analysis. Finally, we used RefFinder to create a comprehensive ranking of the most stably expressed candidate reference genes within each experimental treatment condition (Table 4).

Under drought stress, RefFinder’s component programs identified five genes (eIF4A, TEF1, TBP-1, E2, and UBQ) as potential suitable reference genes. The three best reference genes were eIF4A, TEF1, and TBP-1 (Table 4A). With respect to salt stress, a similar group of five genes (eIF4A, TBP-1, TEF1, E2, and ZTL) were identified as potential reference genes, and the same three genes identified as most stable under drought stress were again the three most stable reference genes in roots and leaves of salt-stressed perennial ryegrass (Table 4B). The five most suitable reference genes identified for use in heat stress experiments were eIF4A, TBP-1, E2, YT321-B, and H3, with the first three of these (eIF4A, TBP-1, and E2) the most stably expressed (Table 4C). Under waterlogging treatment, five genes (UBQ, TEF1, eIF4A, E2, and YT321-B) were identified as potential suitable reference genes based on the component programs of RefFinder. UBQ, TEF1, and eIF4A were the three most stable reference genes in both roots and leaves (Table 4D). Under ABA treatment, the component programs identified different gene pairs as the most stably expressed: eIF4A and TEF1 for Delta C T and Normfinder, ZTL and TEF1 for BestKeeper, and TBP-1 and E2 for geNorm. RefFinder’s comprehensive ranking identified eIF4A, TEF1, and E2 as the overall best three reference genes (Table 4E). Finally, under different abiotic stress treatments overall, 60S and GAPDH were found to be last two least stably expressed reference genes using most of programs (Table 4).

Validation of the Usefulness of the Reference Genes Identified from this Study

To validate the performance of the reference genes identified in this study on known abiotic-stress inducible genes, we quantified the expression of SOD and POD genes which are up-regulated under dehydration drought stress [39–41]. After quantifying, a representative least stable reference gene (60S) and a representative most stable reference gene (eIF4A) were used to normalize their expression. As shown in Figure 4, using 60S for normalization, suggests that both SOD and POD genes are induced ten or six fold, respectively, on both Day 3 and Day 6 after initiation of drought treatment. In contrast, using eIF4A as the reference gene reveals greater overall fold changes in expression of SOD and POD compared to Day 0 and greater expression of both genes on Day 6 than on Day 3. Furthermore, if 60S was used as reference gene, we would have consistently failed to detect drought-induced gene expression changes in perennial ryegrass leaves for SOD (Day 3 to Day 6) and POD (between Days 3, 6, and 9) that were clearly identified when normalization was carried out with respect to eIF4A (Figure 4). Gel electrophoresis of PCR products also showed (Figure 5) that eIF4A was expressed more stable than 60S under drought treatment.

Figure 4. Expression levels of SOD (a) and POD (b) in different time (Days 0, 3, 6, and 9) of perennial ryegrass leaves under drought stress. Genes were normalized to highly stable (eIF4A) and unstable (60S) reference genes. Error bars indicate one standard error of the mean. doi:10.1371/journal.pone.0093724.g004

Figure 5. Agarose gel results of SOD, POD, eIF4A, and 60S PCR amplicons in perennial ryegrass leaves exposed to drought stress treatment. doi:10.1371/journal.pone.0093724.g005
Discussion

Because of its high sensitivity, specificity and broad quantification range, qRT-PCR has significantly improved the quantification and detection of gene expression differences in distinct biological samples [42–44]. Quantification of gene expression is affected by several factors, such as RNA quality, efficiency of reverse transcription, cDNA quantity, expression of reference genes, and statistical methods [35,45]. The selection of reference genes is an important step because they are the basis for comparison of expression changes in target genes and it is necessary to select suitable, stably-expressed reference genes for each experiment [46]. In this study, expression stability was evaluated for 11 perennial ryegrass genes under five stress treatments to determine which are the most suitable for experimental use. While no single gene had perfectly stable expression under all treatment conditions, the candidates showed differing levels of stability allowing preference rankings for different types of experiments. Validating the expression stability of candidate reference genes in the species and treatments of interest before qRT-PCR normalization is preferable to using published reference genes without such initial screening.

Given the differences among rankings from the four computational methods used to determine expression stability, some overall patterns based on different stress treatments could be identified. In particular, eIF4A-I was the most stable reference gene under drought, salt, heat stresses, and ABA treatment, but not under waterlogging stress (Table 4). This gene has previously been identified as a stable reference gene in Arabidopsis, rice (Oryza sativa) and Lolium perenne [47–49]. However, eIF4A-I has performed poorly as a reference gene in oats (Avena sativa) and barley (Hordeum vulgare), so its use as a reference gene which is not recommended for these species [50]. Therefore, we believe that good reference genes should be selected based on different species or different stress treatment involved. Similarly, UBQ witnessed the most stable expression under waterlogging treatment in this study (Table 4E) and has previously performed well in the developing seeds of Tung tree (Vernicia fordii Hemsl.) [22] and Arabidopsis [51]. Nevertheless, recent studies have shown that UBQ reference gene can have unstable expression under other conditions [30,47] and should not be used as an internal control gene in rice or soybean (Glycine max) [48,52]. Although traditional housekeeping function genes are often considered good candidate reference genes due to their functional nature [53–55], some studies have found that these genes are frequently unstable. For example, Hong et al. [56] selected GAPDH as a stably expressed gene under various abiotic stress conditions in Brachypodium distachyon, but in our analyses, the stability of GAPDH ranked much lower than other reference genes under a variety of abiotic stresses (Table 4A). Previous studies have also demonstrated that GAPDH has unstable expression in rice and

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