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Research article

Selection of suitable reference genes for abiotic stress-responsive gene expression studies in peanut by real-time quantitative PCR

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ABSTRACT

Background: Because of its strong specificity and high accuracy, real-time quantitative PCR (RT-qPCR) has been a widely used method to study the expression of genes responsive to stress. It is crucial to have a suitable set of reference genes to normalize target gene expression in peanut under different conditions using RT-qPCR. In this study, 11 candidate reference genes were selected and examined under abiotic stresses (drought, salt, heavy metal, and low temperature) and hormone (SA and ABA) conditions as well as across different organ types. Three statistical algorithms (geNorm, NormFinder and BestKeeper) were used to evaluate the expression stabilities of reference genes, and the comprehensive rankings of gene stability were generated.
Results: The results indicated that *ELF1B* and *YLS8* were the most stable reference genes under PEG-simulated drought treatment. For high-salt treatment using NaCl, *YLS8* and *GAPDH* were the most stable genes. Under $CdCl_2$ treatment, *UBI1* and *YLS8* were suitable as stable reference genes. *UBI1*, *ADH3*, and *ACTIN11* were sufficient for gene expression normalization in low-temperature experiment. All the 11 candidate reference genes showed relatively high stability under hormone treatments. For organs subset, *UBI1*, *GAPDH*, and *ELF1B* showed the maximum stability. *UBI1* and *ADH3* were the top two genes that could be used reliably in all the stress conditions assessed. Furthermore, the necessity of the reference genes screened was further confirmed by the expression pattern of *AnnAhs*.
Conclusions: The results perfect the selection of stable reference genes for future gene expression studies in peanut and provide a list of reference genes that may be used in the future.

1. Introduction

Peanut is one of the most important legume crops cultivated in the world [1,2]. Its economic benefit is remarkable, and its acreage has gradually expanded in recent years. However, peanut production and quality are adversely affected by various environmental stresses worldwide, such as the emergence of extreme weather, coastal land salinization, and drought and oxidative stress [3,4]. Research on stress tolerance in peanut should be paid extensive close attention. But most of these agronomical traits are difficult to breed by conventional selection techniques because of the multigene nature and the little genetic variation within cultivated peanut [5].

Modern biotechnology approaches such as marker-assisted selection and high-throughput gene expression analyses have been employed in crop improvement programs worldwide. In particular, gene expression analyses have become important for understanding the molecular mechanisms of plant stress responses recently [6,7]. RT-qPCR and semi-quantitative RT-PCR can both be used to detect the expression of target genes [8,9,10,11], while RT-qPCR is the preferred method for this purpose because of its stronger specificity, higher sensitivity, and wider detection range than semi-quantitative RT-PCR. However, there are many variable in RT-qPCR, such as the quantity and quality of the initial sample, the efficiency of reverse transcription, the amplification efficiency, and the analysis method [12]. Selection of an unstable reference gene could add unpredictable errors on the gene expression analysis.

An ideal reference gene should have relatively stable expression in different biological samples, including different developmental stages, distinct cell types, and samples exposed to various experimental conditions. Therefore, house-keeping genes are considered to be the best choices for reference genes for RT-qPCR, e.g., glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [13,14] and *18S rRNA* [11,15,16,17,18,19]. In fact, a number of reports demonstrated that almost no single housekeeping gene or internal control gene is universal and invariable for use as a reference gene for all experiments [20,21,22]. For instance, the expression of *18S rRNA*

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was stable in rice but has different results in papaya [23,24]. *Actin* was expressed stably in the study of tomato virus infection but could not be a reliable reference gene in cucumber under salinity stress or in papaya under numerous experimental conditions [24,25,26]. Therefore, it is necessary to select and optimize reference genes for RT-qPCR according to the experimental material and treatment condition to improve the accuracy of RT-qPCR analysis and interpretation [27,28].

Efforts for identifying suitable reference genes have been reported in a number of plants, including *Glycine max* L. [29,30,31,32,33,34], *Nicotiana tabacum* [35], *Cucumis sativus* L. [26], *Corchorus capsularis* L. [36], *Plukenetia volubilis* [37], *Solanum lycopersicum* [25,38], *Zea mays* L. [39], and *Arabidopsis* [40]. However, very few studies on reference gene validation have been conducted in peanut [41,42,43,44]. These results offered guides for selecting reference genes in different experiment conditions in peanut. Nevertheless, peanut samples treated with $CdCl_2$, ABA, and SA were not studied before. With reference to these experiment results and on a theoretical basis, we systematically investigated the expression of 11 reference genes (namely *ACTIN11*, *ACTIN7*, *ACTIN1*, *ADH3*, *GAPDH*, *UKN2*, *ELF1B*, *YLS8*, *G6PD*, *60S*, and *UBI1*) under conditions of drought, salt, heavy metal, low temperature, and hormone and in different organs (roots, stems, leaves, and flowers). We calculated their stabilities using three statistical algorithms, namely geNorm [45], BestKeeper [46], and NormFinder, which laid a foundation for the expression analysis of genes in peanut.

2. Materials and methods

2.1. Plant material and stress treatments

Peanut material Jihua 2, the control variety of peanut regional test in Hebei province with multi-resistance and wide adaptation, derived from a cross between 7851-24 and 7101-43, was obtained from Hebei Academy of Agriculture and Forestry Sciences. Uniform and full peanut seeds were surface sterilized in 70% (v/v) ethanol for 1 min and 0.1% (v/v) $HgCl_2$ for 10 min successively and washed six times with sterile deionized water subsequently. After peeling off the seed coat, the seeds were plated onto Murashige and Skoog medium containing 3% (w/v) sucrose [47]. The seeds were maintained in the growth chamber (light intensity of 275 mmol m^{-2} s^{-1}, humidity of approximately 80%, and temperature of 27 ± 1°C) under a daily photoperiodic cycle of 14 h light and 10 h dark for 2 weeks before transferring the seedlings to soil in separate pots and water for culture adaptation. For organ-specific expression, samples were collected from roots, stems, leaves, and flowers from a 4-week stage of the same plant. To investigate the expression stability of candidate reference genes under abiotic stress, peanuts were separately passed through solutions containing 250 mM sodium chloride (NaCl), 150 μM cadmium chloride ($CdCl_2$), 10% PEG-6000, 100 μM ABA, and 100 μM SA. We adjusted the incubator to 15°C for low temperature stress condition for leaves. Functional leaves were collected at different times (0, 2, 4, 6, 8, 10, 12, and 24 h) after treatment from uniform growth seedlings, quickly frozen in liquid N_2, and stored at -80°C until further use. Control seedlings were mock treated with water (Water CK). Three independent experiments were performed.

2.2. Total RNA extraction and cDNA synthesis

The total RNA of stress-treated and unstressed leaves, stems, roots, and flowers was extracted using EASYspin Plant RNA mini kit (Aidlab) according to the manufacturer's instructions. RNA integrity was then assessed on 2% agarose gel electrophoresis, and RNA sample quality was determined using a NanoDrop 2000 spectrophotometer (NanoDrop, Thermo Scientific). Finally, RNA samples with an A260/A280 ratio of 1.9–2.1 and an A260/A230 ratio greater than 2.0 were used for further analyses. Subsequently, for real-time PCR, the first-strand cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara). The quality and integrity of the cDNA were checked using a NanoDrop 2000 spectrophotometer and by agarose gel electrophoresis, respectively, and the cDNA was stored at -20°C until further use.

2.3. Primer design

On the basis of previous studies on expression stabilities of reference genes in peanut and soybean, 11 candidate reference genes, namely *ACTIN7*, *GAPDH*, *60S*, *ADH3*, and *YLS8* [41]; *ACTIN1* and *UBI1* [42]; *UKN2* [34,43]; *ACTIN11*, *G6PD*, and *ELF1B* [44], were selected as candidate reference genes. From these previous studies and the cDNA and EST sequences of soybean and peanut published in GenBank, specific amplification primers for each candidate gene were designed. These primers had the following criteria: annealing temperature ranged from 58°C to 62°C, the length was between 17 and 25 bp, and the GC content varied between 45% and 55%. After determining the sequences and screening a series of primers, 11 pairs of primer sequences referred to in the references were selected [34,41,42,43,44].

2.4. Relative expression analysis of reference genes

The specificity of the candidate reference genes was confirmed by the presence of a single peak in RT-qPCR and single amplicon in conventional PCR. cDNA at 10-fold dilution was used as the PCR template for reference gene amplification. Special primers amplification using conventional PCR were performed in a total volume of 20 μL using rTaq DNA polymerase (Takara) at 95°C for 2 min followed by 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 1 min (elongation) for 30 cycles, with a final extension of 15 min at 72°C. The resultant PCR products were detected by 2.0% gel electrophoresis. RT-qPCR analyses were performed in a 96-well plate on an Agilent MX3000P Real-Time PCR system (Agilent Technologies) with gene-specific forward and reverse primers for each reference gene. For RT-qPCR, 2 μL of cDNA (after dilution) was used as template in a reaction volume of 20 μL using SYBR® Premix Ex Taq™ II kit (Takara) with two-step amplification conditions of 95°C for 1 min; 40 cycles of 95°C for 15 s and 60°C for 34 s (data collection); and melting curve analysis at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. The standard curve for each set of primers of reference genes was plotted from RT-qPCR with 10-fold echelon dilution of cDNA as template using Ct value as ordinate and the log of template concentration as abscissa. RT-qPCR analyses were performed with three technical replicates.

2.5. Stability analysis of candidate reference genes

The PCR reaction system and protocol for the candidate reference gene amplification were the same as RT-qPCR analyses. The Ct values were calculated using Agilent MX3000P real-time PCR analysis system (Agilent Technologies) on the basis of the expression of the 11 candidate reference genes in different samples under different treatments. The amplification efficiency of each reference gene was calculated from the slope of each standard curve using the formula, $E = (10^{-1/slope} - 1) \times 100$. The Ct value distribution of the candidate reference genes, which reflected the average expression levels of the candidate reference genes in all peanut samples, was constructed using SigmaPlot 10 software. The expression stabilities of candidate reference genes were analyzed using three statistical algorithms: geNorm, NormFinder, and BestKeeper. The geNorm software could calculate the stability value (M) of each candidate reference gene on the basis of its expression. The lower the value of M, the more stable was the expression of the reference gene, and vice versa. The optimal number of reference genes required for effective RT-qPCR data normalization was determined by analyzing pair-wise