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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

variation (Vn/n + 1) between the normalization factors NFn and NFn + 1. The NormFinder program combined the variance within groups with between groups to calculate the stability value, which was used to evaluate the expression stabilities of reference genes. Lower stability value, the more stable was the expression of the reference gene, and vice versa. For geNorm and NormFinder program, the raw Ct values had to be converted into relative quantities data using the formula $2^{-\Delta Ct}$ [$\Delta Ct = Ct(min) - Ct(Sample)$], and then the relative quantities data was imported into geNorm and NormFinder to analyze the expression stability of each reference gene. The Ct(Sample) was the Ct value of each sample, and the Ct(min) was the minimum value among the entire Ct(Sample). The BestKeeper program analyzed the pairing correlation under a given set of experimental conditions using the Ct values of reference genes of the sample in each group. According to the standard deviation (SD), percentage covariance (CV), and coefficient of correlation of the candidate genes (r), the expression stability of each reference gene was evaluated in the BestKeeper program. The reference gene with lower SD value, lower CV value, and higher r value would be the one with more expression stability. RT-qPCR analyses were conducted using three independent total RNA samples.

2.6. Validation of reference genes

To validate the selected reference genes, the relative expression levels of the target gene *AnnAhs* were evaluated using the most stable and least stable reference genes after normalization across drought and salt stresses. Control samples and samples treated with 10% PEG-6000 were analyzed using *ELF1B* and *ACTIN7* as endogenous reference genes for mRNA normalization. *YLS8* and *ACTIN7* were used as endogenous reference genes in the analyzed samples treated with salt stress. For RT-qPCR, the reaction was same as that of relative expression analysis of reference genes. RT-qPCR analyses were conducted with three independent total RNA samples, and $2^{-\Delta\Delta ct}$ was applied to analyze the expression of *AnnAhs* [48].

3. Results

3.1. Specificity and amplification efficiency of RT-PCR primers

The specificity of primers can be judged using the melting curve (Fig. S1). The melting curves of 11 reference genes in peanut samples represented an obvious single peak, and the amplification curves of the three repeats had good repeatability. Agarose gel electrophoresis of the PCR products of the reference genes showed a single band of the expected size (Fig. S2). These results indicated the high specificities of the primers. As described in Table 1, the amplification efficiency of the primers was 0.91–1.01, which met the requirements of the RT-qPCR experiment. From the above results, these reference genes can be used for the analysis of expression stability.

3.2. Analysis of expression level of candidate reference genes

The Ct value could be used to measure the expression level of each candidate reference gene. The lower the Ct value, the higher is the expression level. The Ct value distribution of candidate reference genes was imbalanced in different treatments and organs of peanut (Fig. 1). To evaluate the stabilities of the reference genes across all experimental samples, the transcript abundances of the 11 candidate reference genes were determined from their mean Ct values. The average Ct value of 11 candidate reference genes in all peanut samples varied from 17.45 to 36.49 (Fig. 1). Among the 11 candidate reference genes, in all samples, the expression level of *UBI1* was the highest, with the lowest average Ct ± SD of 19.88 ± 0.67, followed by *ACTIN7* (20.49 ± 1.60), *GAPDH* (20.79 ± 0.85), *ELF1B* (22.23 ± 1.08), *ACTIN11* (22.42 ± 1.34), *ACTIN1* (23.06 ± 1.02), *ADH3* (23.42 ± 1.02), and *60S* (24.54 ± 0.80). *UKN2* had the highest Ct value (31.42 ± 1.87), which indicated the lowest expression level, followed by *G6PD* (27.13 ± 1.64) (Table 1). A small CV of the Ct value indicates that a given gene is more stably expressed [36]. Among the 11 candidate reference genes, *60S* and *UBI1* showed the least variations in the CV value (3.27% and 3.35%, respectively), and *ACTIN7* (7.83%), *G6PD* (6.03%), *UKN2* (5.96%), and *ACTIN11* (5.97%) showed a greater variation in their expression levels across all tested samples. The ranking of gene stability by CV was as follow (from the most stable to the least): *60S*, *UBI1*, *GAPDH*, *ADH3*, *ACTIN1*, *YLS8*, *ELF1B*, *UKN2*, *ACTIN11*, *G6PD*, and *ACTIN7* (Table 1). Briefly, the results indicated that the expression levels of the candidate reference genes varied across different experiments.

3.3. Stability evaluation of reference genes by geNorm

The stability values (M) of 11 candidate reference genes in each of the tested sample sets was evaluated using geNorm software (Fig. 2). The acquiescent threshold of M value was 1.5. A reference gene with

Table 1
Primer sequences and amplification parameters of candidate reference genes.

Gene name	Tentative annotation	Primer sequence	Amplicon length/bp	RT-qPCR efficiency	Mean Ct	SD	CV (%)
YLS8	Yellow leaf specific 8 gene	F:AACTGCTTAGCTGCTATTACC R:TCGCCAAATAACACGTTGCAT	122	0.91	23.78	1.09	4.59
60S	60S ribosomal protein L7	F:ACAGTTGGTCCTCACTTCAG R:GCTCATTTATGTAAGCTTCCCT	146	0.93	24.54	0.80	3.27
ACTIN7	Actin 7	F:GATTGGAATGGAAGCTGCTG CGGTCAGCAATACCAGGGAA	140	0.99	20.49	1.60	7.82
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F:CCAGCTGCAAAGTCTCTGAA R:GCCGAGCAAAGAGTACATTG	157	0.99	20.79	0.85	4.10
ADH3	Alcohol dehydrogenase class III	F:GACGCTTGGCGAGATCAACA R:AACCGGACAACCACCACATG	140	0.95	23.42	1.02	4.36
ACTIN11	Actin 11	F:ATGCTAGTGGTCGTACAACTGG R:CTAGACGAAGGATAGCATGTGG	108	0.96	22.42	1.34	5.97
ELF1B	Elongation factor1-beta	F:AAGCTTCCCTGGCAAAGCTCAA R:TTCCTCAGCTGCCTTCTTATCC	153	0.95	22.23	1.08	4.87
G6PD	Glucose 6-phosphatedehydrogenase	F:ACCATTCCAGAGGCTTATGAGC R:AAGGGAGTGACTTGAACTCTCC	151	1.01	27.14	1.64	6.03
ACTIN1	Actin1	F:TGGTCTCGGTTTCCTGAGTT R:AATACCACTCCAAAGCAAACG	114	0.99	23.06	1.02	4.44
UBI1	Polyubiquitin	F:TCTTGTCCTCCGTCTTAGGG R:AGCAAGGGTCCTTCCATCTT	196	0.98	19.88	0.67	3.35
UKN2	Hypothetical protein	F:TGTGCTCTGTGAAGAGATTG R:TCATAATCTGTGTGCAGTTC	156	0.93	31.42	1.87	5.96

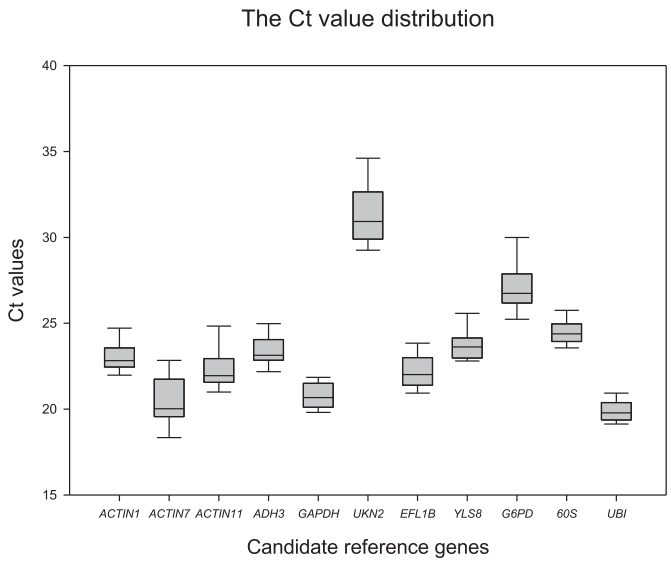


Fig. 1. Ct value distribution of candidate reference genes under different treatments and in different organs of peanut. Global Ct values of the different genes tested are shown as the 25th and 75th quartiles (boxes), median (central horizontal line), and minimal/maximal value (vertical bar).

M value below 1.5 is considered a stable gene. As shown in Fig. 2, the M values of 11 candidate reference genes were lower than 1.5 in all the samples either in synthetic analysis or under different treatments, which indicated that all the candidate reference genes had certain expression stabilities that were different from each other. In total samples, inculding simples under treatment of drought, salt, heavy metal, low temperature, and hormone, and simples in different organs (roots, stems, leaves, and flowers), *GAPDH* and *60S* had the highest stabilities, followed by *UBI1*. For each subset of treatment, the expression stabilities of reference genes were variable. *ELF1B* and *YLS8* were the most stable genes with the lowest M values in PEG-simulated drought stress and water control treatment. Under NaCl stress, the most preferred genes for normalization were *ADH3* and *YLS8*. Under heavy metal stress ($CdCl_2$), *GAPDH* and *UBI1* were the most stable reference genes. Under low temperature treatment, *ACTIN11* and *ADH3* were the most stable reference genes. Under hormone treatments with ABA and SA, all the reference genes had relative smaller M values, and *ACTIN7* and *YLS8* were the most stable genes under ABA treatment, while *ACTIN7* and *UBI1* were suitable genes under SA treatment. In different organs, *GAPDH* and *ELF1B* had the most expression stabilities. In general, *G6PD* and *UKN2* were the least stable reference genes in most conditions (Fig. 2).

It is now a consensus that the expression levels of a single certain reference gene may be unstable under some specific experimental conditions and that multiple reference genes should be evaluated and used to quantify gene expression to improve the accuracy of RT-qPCR analysis. In geNorm, the threshold value for choosing the optimal reference gene number is $Vn/n+1 = 0.15$. When $Vn/n+1$ is below 0.15, the number of reference genes for RT-qPCR should be n, and it is not necessary to introduce the $n+1$ reference gene. As shown in Fig. 3, V5/6 was less than 0.15 in the analysis of all samples, which indicated that the number of reference genes should be at least five in total sample analysis. According to the results shown in Fig. 2, *GAPDH*, *60S*, *UBI1*, *ELF1B*, and *YLS8* should be chosen for total samples analysis. In the analysis of samples under different treatments and in different organs, V2/3 was less than 0.15, which revealed that the optimal number of reference genes was 2.

3.4. Stability evaluation of reference genes by NormFinder

NormFinder software could evaluate the stability of gene expression through the calculation of the stable value (M) of candidate reference genes. The candidate reference genes were ranked according to the numeric value of M (Table 2). The lower the stable value, the higher is the stability of the reference gene expression. Under PEG-simulated drought treatment, reference genes with the highest stability were *ELF1B* and *60S*. Under NaCl treatment, the expressions of *GAPDH* and *YLS8* had the highest stabilities. *YLS8* had the highest stability under $CdCl_2$ treatment. *YLS8* and *ADH3* revealed the highest expression stabilities across samples of water CK. Under low temperature treatment, the expression stabilities of *UBI1* and *GAPDH* were the highest. Under hormone treatment with ABA, most of the candidate reference genes had relative higher stability, and the expression stabilities of *YLS8* and *ACTIN7* were the highest among them. The expression stability of *ACTIN7* was also the highest under hormone treatment with SA. In the organ-specific expression analysis, *UBI1* had the lowest stable value, which indicated the highest expression stability. Moreover, similar to the results obtained by geNorm, in most conditions, *G6PD* and *UKN2* had the lowest gene expression stabilities. Comparison of Fig. 2 and Table 2 demonstrates that the analysis results of gene expression stability by geNorm were consistent with those of NormFinder, especially the results of reference genes with the lowest stability across different treatments and different organs.

3.5. Stability evaluation of reference genes by BestKeeper

In BestKeeper, the number of reference genes cannot exceed 10. Therefore, the gene with lowest expression stability under each single treatment and different organs was rejected according to the results given by geNorm and NormFinder. The stability of gene expression was evaluated in BestKeeper on the basis of three variables: SD (Standard Deviation), correlation coefficient (r), and CV. Ten reference genes from each single analysis are ranked in Table 3 according to the SD value of each reference gene. The smaller the SD value, the higher is the stability of the reference gene. It was obvious that the SD values of the 10 genes under each treatment were all less than 1, which indicated that these genes could be used for the selection of reference genes (Table 3). The ranking results were consistent with the ones given by geNorm and NormFinder under different treatments. Under simulated drought treatment, *ELF1B* and *ACTIN11* had smaller SD and CV values, but also had relatively smaller r values, which showed a low consistency with the tendency of the expression of all the candidate reference genes. By comparison, *YLS8*, with a relatively smaller SD and CV values and a relative larger r value (r =

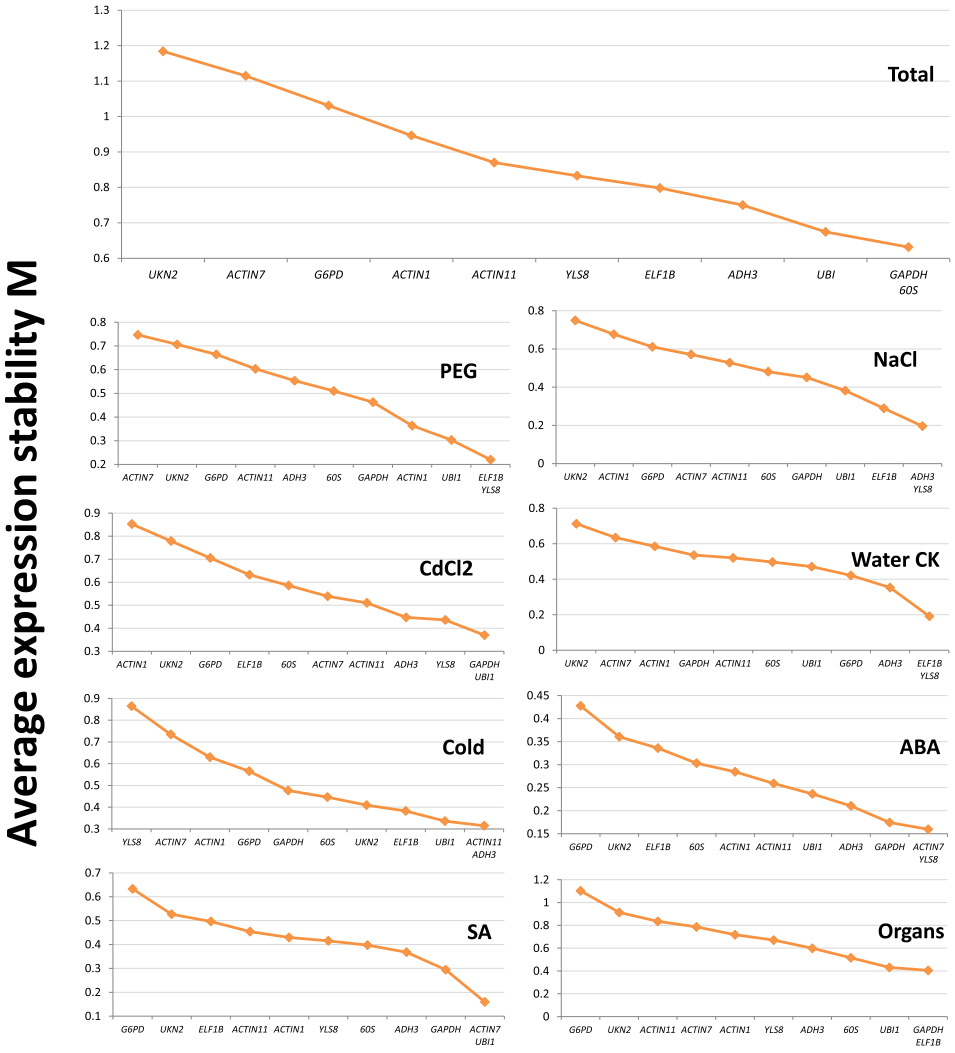
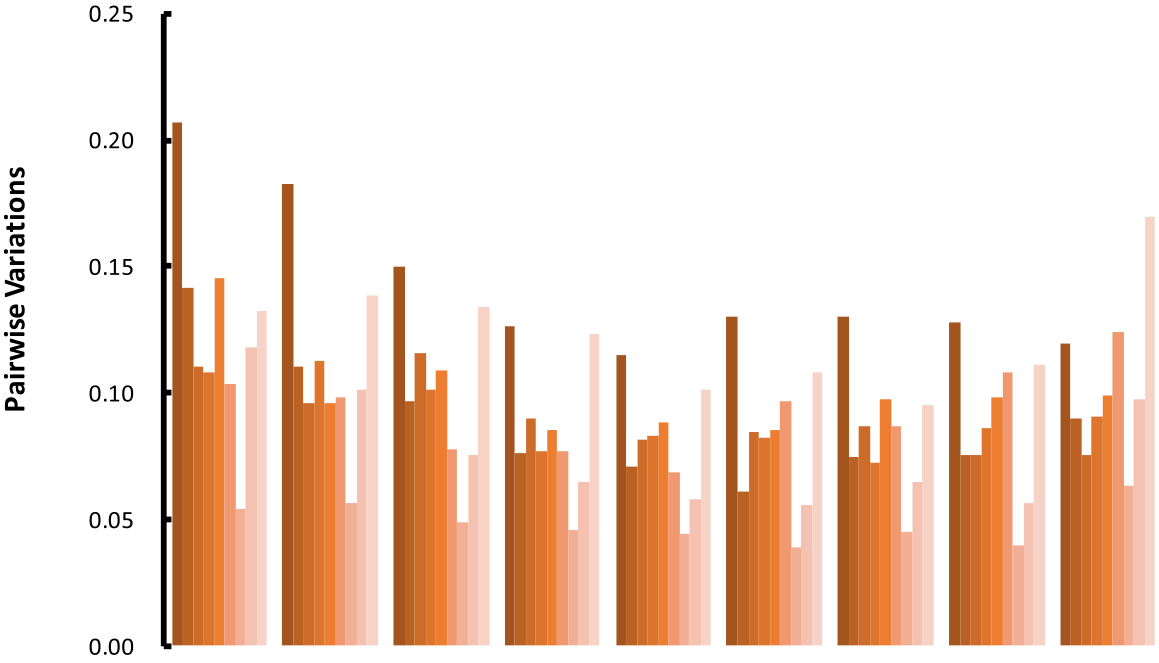


Fig. 2. Expression stability and ranking of the candidate reference genes under different treatments and in different organs of peanut analyzed by geNorm. Average expression stability values (M). A lower M value indicates more stable gene expression. The least stable genes are on the left and the most stable on the right.

0.842), would be the best choice. The expression of *YLS8* showed the highest stability (SD = 0.266, CV = 1.164, r = 0.820) under NaCl treatment. In heavy metal treatment using $CdCl_2$, the expression stabilities of *YLS8* and *UBI1* were the highest. The expressions of *ELF1B* and *ADH3* had the best stabilities in water CK. Consistent with the results of NormFinder, *UBI1* (SD = 0.149, CV = 0.757, r = 0.778) was the most stable gene under low temperature treatment. Under ABA treatment, most of the 10 genes had relative larger r, and especially *ACTIN7* and *ADH3* should be considered to have the best stabilities. Under SA treatment, the expression of *ACTIN7* was the most stable, which was consistent with the result of NormFinder. In organ-specific analysis, *UBI1* showed the highest stability, which was in accordance with the result of NormFinder.

3.6. Validation of reference genes

To validate the selected reference genes, the relative expression levels of the target genes *AnnAh1*, *AnnAh2*, and *AnnAh3* (GenBank accession numbers are KM267643, KM276779 and KM276780, respectively) were evaluated using the most stable and least stable reference genes after normalization across drought stress and salt stress. Annexin is known to be an important gene in response to abiotic stress such as water shortage, osmotic stress, and anaerobic environment [49,50]. Peanut annexin was homologous to other higher plant annexins, including *Glycine max*, *Medicago truncatula*, *Arabidopsis*, and *Brassica juncea* L., which indicated that *AnnAhs* might have a similar function of response to abiotic stress. According to the stability ranking given by the three statistics algorithms (Fig. 2, Table 2, and Table 3), a conjoint analysis was made in Table 4. A higher ranking value indicated lower gene expression stability. Therefore, the most stable reference genes identified for samples treated with 10% PEG-6000 (*ELF1B*) and samples treated with NaCl (*YLS8*) were used as internal controls for data normalization. For comparison, one of the least stable reference genes, *ACTIN7*, identified in two experimental sets, was also considered. The results demonstrated that the expression patterns of *AnnAhs* differed when using the most and least stable reference genes for normalization under different treatments (Fig. 4). The expression of *AnnAhs* showed different levels when *ACTIN7* and *ELF1B* were used as reference genes for untreated samples. Compared to untreated samples, in samples treated with NaCl (250 mM), the level of induction for *AnnAh1* increased from 2–12 h and declined thereafter when *ACTIN7* was used as the reference gene; however, when *YLS8* was used for normalization, the level of *AnnAh1* reached the highest level of transcript at 10 h. Under drought stress with 10% PEG, *AnnAh1* showed a lower expression, particularly when using the most stable reference gene *ELF1B* for normalization (Fig. $4A_1$, Fig. $4A_2$). When *ACTIN7* was used in treatment with NaCl (250 mM) at 8 h and 10 h, the expression level of *AnnAh2*



	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8	V8/9	V9/10	V10/11
Total	0.207	0.182	0.150	0.126	0.115	0.130	0.130	0.128	0.119
Water CK	0.141	0.110	0.097	0.076	0.071	0.061	0.075	0.075	0.090
PEG	0.110	0.096	0.115	0.090	0.082	0.084	0.087	0.075	0.075
NaCl	0.108	0.112	0.101	0.077	0.083	0.082	0.072	0.086	0.091
$CdCl_2$	0.145	0.096	0.109	0.085	0.088	0.085	0.098	0.098	0.099
Cold	0.103	0.098	0.078	0.077	0.069	0.097	0.087	0.108	0.124
ABA	0.054	0.056	0.048	0.046	0.044	0.039	0.045	0.040	0.063
SA	0.118	0.101	0.075	0.065	0.058	0.056	0.065	0.057	0.098
Organs	0.132	0.138	0.134	0.123	0.101	0.108	0.095	0.111	0.169

Fig. 3. Determination of the optimal number of reference genes. The optimal number of reference genes required for effective RT-qPCR data normalization. Pairwise variation (Vn/n + 1) analysis between the normalization factors NFn and NFn + 1 was analyzed.

was higher than that in the water control, but contrasting results were obtained when the most stable reference gene *YLS8* was used. The expression trend and level of *AnnAh2* were also different under treatment with PEG when *ACTIN7* and *ELF1B* were applied for normalization (Fig. $4B_1$, Fig. $4B_2$). Treatment with NaCl (250 mM) caused the downregulation of *AnnAh3* expression at 2–4 h when *ACTIN7* was used as the reference gene, whereas the expression levels increased at 2–4 h when *YLS8* was used for normalization. Under treatment with PEG, the expression levels of *AnnAh3* decreased at all time-points when *ACTIN7* was used as the reference gene, but the expression level increased to the maximum at 2 h when *ELF1B* was used as the reference gene (Fig. $4C_1$, Fig. $4C_2$). These findings revealed that the selection of candidate reference genes in the normalization of target gene expression level was important.

Table 2
Expression stability values (M value) of candidate reference genes under different treatments of peanut calculated by NormFinder.

Ranking	PEG		NaCl		$CdCl_2$		Water CK		Low temperature		ABA		SA		Organs	
	Gene	M	Gene	M	Gene	M	Gene	M	Gene	M	Gene	M	Gene	M	Gene	M
1	*ELF1B*	0.0506	*GAPDH*	0.1188	*YLS8*	0.0762	*YLS8*	0.1291	*UBI1*	0.1184	*YLS8*	0.0592	*ACTIN7*	0.1172	*UBI1*	0.0791
2	*60S*	0.0794	*YLS8*	0.1244	*ADH3*	0.1511	*ADH3*	0.1307	*GAPDH*	0.1220	*ACTIN7*	0.0610	*60S*	0.1365	*GAPDH*	0.1280
3	*GAPDH*	0.0917	*ADH3*	0.1361	*UBI1*	0.1620	*ELE1B*	0.1335	*ADH3*	0.1434	*GAPDH*	0.0656	*ADH3*	0.1373	*ELF1B*	0.1282
4	*YLS8*	0.1099	*60S*	0.1458	*ACTIN11*	0.1638	*G6PD*	0.1476	*ACTIN11*	0.1603	*ADH3*	0.0794	*YLS8*	0.1441	*ACTIN1*	0.1518
5	*ADH3*	0.1648	*ACTIN11*	0.1712	*60S*	0.2015	*60S*	0.1524	*60S*	0.1677	*ACTIN11*	0.0858	*ACTIN1*	0.1453	*60S*	0.1580
6	*ACTIN7*	0.1656	*UBI1*	0.1813	*GAPDH*	0.2047	*ACTIN11*	0.1581	*ELF1B*	0.1776	*ELF1B*	0.0968	*GAPDH*	0.1574	*YLS8*	0.1660
7	*ACTIN11*	0.1681	*ELF1B*	0.1986	*ACTIN7*	0.2275	*ACTIN7*	0.2050	*G6PD*	0.1836	*UBI1*	0.1066	*ACTIN11*	0.1660	*ADH3*	0.2427
8	*G6PD*	0.1920	*UKN2*	0.2265	*ELF1B*	0.2403	*UBI1*	0.2250	*UKN2*	0.2124	*ACTIN1*	0.1092	*UBI1*	0.1775	*G6PD*	0.2649
9	*UBI1*	0.1932	*G6PD*	0.2319	*G6PD*	0.2762	*GAPDH*	0.2336	*ACTIN1*	0.2375	*60S*	0.1694	*ELF1B*	0.2047	*ACTIN11*	0.3153
10	*UKN2*	0.2430	*ACTIN1*	0.2541	*ACTIN1*	0.2951	*ACTIN1*	0.2785	*YLS8*	0.2406	*G6PD*	0.1749	*UKN2*	0.2453	*ACTIN7*	0.3325
11	*ACTIN1*	0.2511	*ACTIN7*	0.2572	*UKN2*	0.3215	*UKN2*	0.2862	*ACTIN7*	0.2663	*UKN2*	0.1859	*G6PD*	0.2728	*UKN2*	0.3521

Table 3
Rankings of the candidate reference genes according to their stability values as determined by BestKeeper.

Ranking	PEG				NaCl				$CdCl_2$				Water CK			
	Gene	SD	CV	r Value	Gene	SD	CV	r Value	Gene	SD	CV	r value	Gene	SD	CV	r value
1	*ELF1B*	0.253	1.080	0.378	*YLS8*	0.266	1.164	0.820	*UBI1*	0.248	1.229	0.418	*ELF1B*	0.318	1.478	0.590
2	*ACTIN1*	0.326	1.413	0.329	*GAPDH*	0.201	0.970	0.542	*YLS8*	0.289	1.248	0.889	*ADH3*	0.355	1.539	0.799
3	*YLS8*	0.364	1.504	0.842	*UBI*	0.371	1.941	0.624	*ACTIN7*	0.392	1.969	0.425	*G6PD*	0.387	1.450	0.741
4	*UBI1*	0.411	2.045	0.656	*60S*	0.383	1.588	0.679	*ADH3*	0.434	1.864	0.672	*UBI1*	0.388	1.907	0.516
5	*ADH3*	0.483	1.936	0.789	*ADH3*	0.390	1.777	0.828	*GAPDH*	0.474	2.261	0.540	*YLS8*	0.419	1.800	0.749
6	*GAPDH*	0.589	2.687	0.913	*ELF1B*	0.453	2.121	0.741	*60S*	0.488	1.990	0.621	*ACTIN7*	0.506	2.736	0.511
7	*60S*	0.634	2.486	0.963	*ACTIN11*	0.483	2.233	0.672	*G6PD*	0.511	1.889	0.287	*60S*	0.513	2.104	0.822
8	*G6PD*	0.651	2.344	0.702	*ACTIN7*	0.586	2.729	0.470	*ACTIN11*	0.520	2.352	0.790	*ACTIN1*	0.520	2.230	0.275
9	*ACTIN11*	0.708	2.891	0.863	*G6PD*	0.591	2.320	0.584	*ELF1B*	0.604	2.800	0.636	*ACTIN11*	0.549	2.512	0.881
10	*ACTIN7*	0.903	4.053	0.895	*UKN2*	0.809	2.684	0.748	*UKN2*	0.790	2.644	0.437	*GAPDH*	0.613	2.885	0.792

Table 3 (*continued*)

Ranking	Low temperature				ABA				SA				Organs			
	Gene	SD	CV	r value	Gene	SD	CV	r value	Gene	SD	CV	r Value	Gene	SD	CV	r Value
1	*UBI1*	0.149	0.757	0.778	*ACTIN7*	0.214	1.075	0.910	*ACTIN7*	0.138	0.697	0.569	*UBI1*	0.137	0.657	0.801
2	*ACTIN11*	0.210	0.963	0.440	*ADH3*	0.218	0.949	0.860	*UBI1*	0.136	0.706	0.137	*ADH3*	0.411	1.676	0.337
3	*ADH3*	0.283	1.216	0.558	*UBI1*	0.242	1.240	0.710	*GAPDH*	0.243	1.196	0.308	*EFL1B*	0.434	1.796	0.724
4	*EFL1B*	0.335	1.516	0.438	*GAPDH*	0.251	1.272	0.918	*60S*	0.280	1.173	0.495	*GAPDH*	0.468	2.207	0.785
5	*60S*	0.370	1.499	0.812	*60S*	0.272	1.138	0.627	*UKN2*	0.316	1.026	0.161	*ACTIN11*	0.581	2.382	0.352
6	*UKN2*	0.390	1.235	0.614	*YLS8*	0.281	1.194	0.934	*ACTIN1*	0.374	1.710	0.719	*60S*	0.594	2.345	0.818
7	*GAPDH*	0.468	2.313	0.914	*UKN2*	0.308	0.998	0.490	*ACTIN11*	0.384	1.797	0.802	*ACTIN7*	0.626	2.762	0.337
8	*G6PD*	0.843	3.142	0.921	*ACTIN1*	0.311	1.395	0.794	*ADH3*	0.400	1.704	0.760	*YLS8*	0.669	2.603	0.927
9	*ACTIN1*	0.871	3.744	0.826	*ACTIN11*	0.344	1.579	0.909	*YLS8*	0.409	1.753	0.681	*ACTIN1*	0.871	3.698	0.918
10	*ACTIN7*	0.938	4.851	0.884	*EFL1B*	0.434	1.975	0.855	*EFL1B*	0.546	2.509	0.826	*G6PD*	1.473	4.920	0.665

Table 4
Comprehensive rankings of gene stability of reference genes by three algorithms.

Gene	PEG				NaCl				$CdCl_2$				Water CK				Low temperature				ABA				SA				Organs			
	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value	gR	NR	BR	Ranking Value
ELF1B	1	1	1	3	2	7	6	15	7	8	9	24	1	3	1	5	3	6	4	13	8	6	10	24	8	9	10	27	4	3	3	10
YLS8	1	4	3	8	1	2	1	4	2	1	2	5	1	1	5	7	10	10	—	—	1	1	6	8	5	4	9	18	5	6	8	19
UBI1	2	9	4	15	3	6	3	12	1	3	1	5	4	8	4	16	2	1	1	4	4	7	3	14	1	8	2	11	2	1	1	4
ACTIN1	3	11	2	16	9	10	—	—	10	10	—	—	8	10	8	26	8	9	9	26	6	8	8	22	6	5	6	17	7	4	9	20
GAPDH	4	3	6	13	4	1	2	7	1	6	5	12	7	9	10	26	6	2	7	15	2	3	4	9	2	6	3	11	1	2	4	7
60S	5	2	7	14	5	4	4	13	6	5	6	17	5	5	7	17	5	5	5	15	7	9	5	21	4	2	4	10	1	5	6	12
ADH3	6	5	5	16	1	3	5	9	3	2	4	9	2	2	2	6	1	3	3	7	3	4	2	9	3	3	8	14	3	7	2	12
ACTIN11	7	7	9	23	6	5	7	18	4	4	8	16	6	6	9	21	1	4	2	7	5	5	9	19	7	7	7	21	6	9	5	20
G6PD	8	8	8	24	8	9	9	26	8	9	7	24	3	4	3	10	7	7	8	22	10	10	—	—	10	11	—	—	8	8	10	26
UKN2	9	10	—	—	10	8	10	28	9	11	10	30	10	11	—	—	4	8	6	18	9	11	7	27	9	10	5	24	10	11	—	—
ACTIN7	10	6	10	26	7	11	8	26	5	7	3	15	9	7	6	22	9	11	10	30	1	2	1	4	1	1	1	3	9	10	7	26

gR: gene expression stability ranking in geNorm.
NR: gene expression stability ranking in NormFinder.
BR: gene expression stability ranking in BestKeeper.
Ranking Value: the sum of gene stability ranking in three algorithms.

4. Discussion

With the continuous development of molecular biology and the accomplishment of diploid genome sequencing of peanut, analysis of gene expression has been widely applied in the research of metabolic regulation mechanism for peanut stress-related genes. A precise expression analysis of stress-related genes can provide useful information for understanding complex regulatory networks in the stress response process in more detail. RT-qPCR is broadly accepted as a suitable method to analyze the expression levels of target genes in biological samples because of its strong specificity, high sensitivity, simplicity, and broad quantification range [51]. However, appropriate reference genes are required for valid RT-qPCR analyses. Selection of suitable reference genes to avoid the influence of random factors and improve the accuracy of RT-qPCR analysis and interpretation has become a key problem that needs to be considered emphatically in gene expression [27,28]. It is considered that different reference genes should be selected according to different experimental conditions and different species. For instance, *UBQ5* and *Eflα* are the most stable reference genes across all rice tissue samples examined, and *18S rRNA* works well in wheat under salinity stress, fungal stress, and drought [11,16,18,52]. *Eflα* is also the most stable reference gene among the seven genes tested under biotic and abiotic stresses in potato [53]. *TUB-B*, *TUB-A*, and *UBC* are the most stable reference genes among all tested samples in celery [54]. Accordingly, it is unanimously held by many researchers that the selection of suitable reference genes expressed stably to normalize is crucial for ensuring the accuracy of the analysis of gene expression.

To date, only a few studies have undertaken the comparison and selection of reference genes in peanut [41,42,43,44]; such a comparison could be of guidance for the selection of peanut reference genes for RT-qPCR analysis under different situations. However, there are barely any studies that report using samples under heavy metal stress and hormone treatments (ABA and SA). According to the studies on peanut, the expression stabilities of 11 candidate genes were analyzed by RT-qPCR method in peanut samples from different organs and under different treatments in this study. Optimal reference genes were evaluated using three statistics algorithms: geNorm, NormFinder and BestKeeper. As shown in Fig. 1, the expression levels of the 11 candidate reference genes in different samples varied, and the Ct values of 11 genes ranged from 17.54 to 36.94. Among them, *UKN2* represented the largest average Ct value, which indicated the lowest gene expression level, followed by *G6PD* with a relative larger Ct value. *UBI1* was considered to display the highest expression level because of the smallest average Ct value. *ACTIN7* and *GAPDH* had a relatively higher expression level. The Ct values of the other reference genes ranged from 22 to 25, without significant difference. The analysis results indicated that the rankings of expression stabilities of reference genes were inconsistent among geNorm, NormFinder, and BestKeeper. Similar results had been reported in the selection of reference genes in peanut, *Brachypodium distachyon*, and citrus [42,55,56]. The main reason for the difference is the difference in the statistical theories used by the three statistics algorithms. Therefore, a conjoint analysis using geNorm, NormFinder, and BestKeeper was necessary. Ranking value, as a new variable, is the sum of gene stability ranking in three statistics algorithms. Therefore, a smaller ranking value indicated higher gene expression stability. The expression stabilities of most of the candidate reference genes were consistent in the three statistics algorithms (Table 4). The conjoint analysis result could provide a reliable basis for the selection of reference genes for RT-qPCR analysis under different treatments.

Our results indicated that *ELF1B* and *YLS8* were the most stable reference genes under PEG-simulated drought treatment, while under high-salt stress, *YLS8* and *GAPDH* were the most stable genes. Under $CdCl_2$ treatment, *UBI1* and *YLS8* showed the maximum stability. *UBI1*, *ADH3*, and *ACTIN11* showed the most stable expression levels under

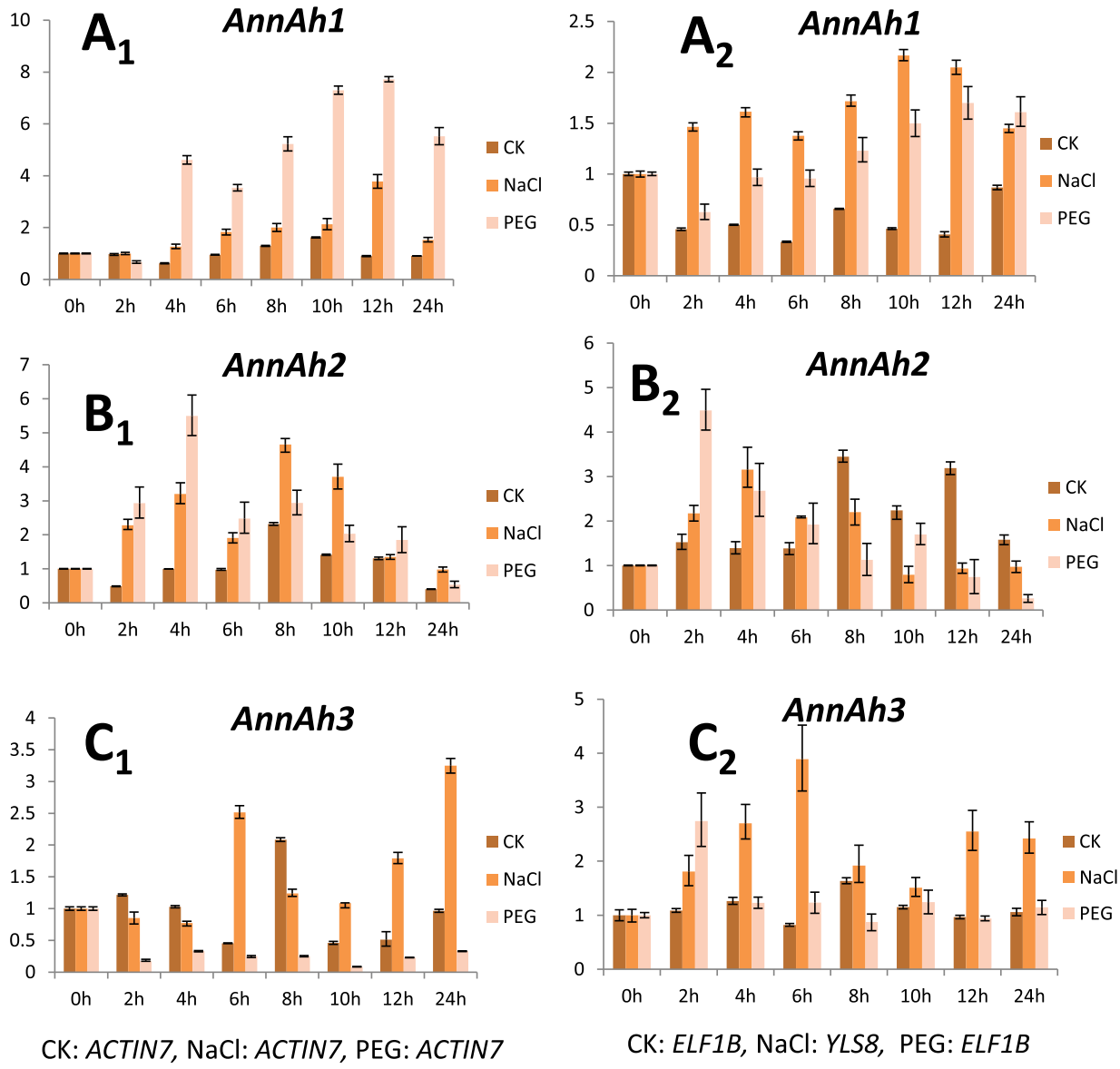


Fig. 4. Relative quantification of targeted genes' (*AnnAhs*) expression in peanut using validated reference genes. Samples collected at 0, 2, 4, 6, 8, 12, and 24 h under drought and salt stress conditions. A_1–C_1: Samples treated with PEG and NaCl used *ACTIN7* as the endogenous reference gene for mRNA normalization. A_2–C_2: The expression levels of *AnnAhs* normalized by different reference genes *ELF1B* for control samples and samples treated with 10% PEG-6000, and *YLS8* for samples treated with NaCl. Data is presented as the fold change in expression levels as normalized to control (value of 1). Data at 0 h is omitted for visual simplicity. Error bars are the standard deviations of three replicates.

low temperature treatment. All the 11 candidate reference genes showed relatively high stability under hormone treatments with ABA and SA, but *ACTIN7* was absolutely the best choice in hormone treatment experiments. In organ-specific analysis, *UBI1*, *GAPDH*, and *ELF1B* showed the maximum stability. Analysis results revealed that *G6PD*, *UKN2*, and *ACTIN7* were relatively unstable reference genes in almost all experimental conditions in this study. The *G6PD* gene, which encodes glucose-6-phosphate dehydrogenase, has been the most commonly used reference gene in plants [42,44,57]. It was also recommended in peanut [44]. However, *G6PD* has been reported to be the least stable reference gene in soybean [29,33]. Similarly, *G6PD* had relative poorer stability in most samples in the present study. *UKN2* was also widely used as a reliable reference gene for RT-qPCR normalization in many previous studies [30,43,58,59]. In our study, *UKN2* was identified as a poor reference gene, which is similar to the results in cambium of poplar [59]. The reason for this result might be because the specificity of primer in soybean was lower than that in peanut [34,43]. Actin, as a main component of cytoskeleton, contributes to a number of essential biological processes in all eukaryotic cells and is the most frequently used reference gene [60, 61]. *ACT7* was found to be one of the best candidate reference genes under biotic stresses and NaCl stress subsets in jute [36]. *ACT7* was also widely used as an appropriate reference gene in a series of developmental stage of seed of *Firmiana simplex* [62] and during the embryo development of *Brassica campestris* [63]. However, *ACT7* did not perform well in studies of *Cichorium intybus* [12] and peanut in our study, which suggested the importance of validating the reference genes for each experimental design.

It is important to note that expression stability of the same reference gene was a little different across different organs, species, or cultivars and under different stresses [34,41,42,43,44]. Our results also proved that there was no single reference gene that is stably expressed in all the samples in this study. For example, *AnnAh1* was more remarkably expressed when the least stable gene *ACTIN7* was used for normalization than that when the most stable gene *ELF1B* was used in samples treated with PEG (Fig. 4A_1, Fig. 4A_2). The same presentation

existed in samples treated with NaCl. When the least stable gene *ACTIN7* was applied, the expression level of *AnnAh2* reduced after 4 h; however, when *ELF1B* was used as reference gene, *AnnAh2* reached the highest expression value at 2 h under PEG stress condition (Fig. $4B_1$, Fig. $4B_2$). The expression of *AnnAh3* also showed different trends and levels when *ACTIN7* and *ELF1B* were used as reference genes under different treatments (Fig. $4C_1$, Fig. $4C_2$). These results further proved the necessity of selection of reliable reference genes for normalization in gene expression studies. The reference gene *ACTIN7* was one of the least stable genes in our study. From the comprehensive comparison of these results, the expression level and pattern of *AnnAhs* showed large differences under PEG treatment when different reference genes were used and less differences under NaCl treatment and in untreated sample, which was consistent with the ranking value of the stability of the reference genes in certain experiments. Therefore, the selection of reference gene for RT-qPCR analysis was important and should be conducted according to the material type and different experimental situations.

To date, a series of studies have reported that the accuracy of RT-qPCR analysis could be improved when multiple reference genes are utilized [36,59,64]. A normalization factor analysis was conducted by geNorm software to calculate the optimal reference gene number in this study (Fig. 3). At least five reference genes, namely *GAPDH*, *60S*, *UBI1*, *ELF1B*, and *YLS8*, were needed for total sample analysis. However, in all samples under different treatments and from different organs, the V2/3 values were less than 0.15, which indicated that the optimal number of reference genes was 2. In general, the number of reference genes should be selected on the basis of the objective of the experiment. If the objective of the research is to simply study the gene expression pattern, a single reference gene with relatively high expression stability is adequate. However, if the purpose of the research is to obtain accurate expression levels or to compare the gene expression in different samples, more number of reference genes should be considered and normalization factor analysis is necessary.

With the rapid development of sequencing technologies, RT-qPCR, microarray, and RNA sequencing (RNA-Seq) approaches have been widely applied in the analysis of gene expression profile [11,17,40]. All these approaches need a reference or standard for comparison to eliminate artificial errors and obtain more accurate results [65]. RT-qPCR based on RNA-seq and microarray data is an effective new strategy to identify and validate reference genes and differentially expressed genes in recent years [22,66,67,68,69]. More new and stable reference genes, which are superior to traditional reference genes, can be screened from RNA-seq and microarray databases and determined through RT-qPCR. Although a rapid increase in research on microarray and RNA-seq in many plants to study gene expression has been witnessed, related research is less in peanut because of the limited availability of sequence information. The completion of diploid genome sequencing of peanut could supply the reference genome sequence for the study on expression patterns and function analysis of many genes [70]. However, a high-quality genome sequence of a tetraploid (cultivated peanut) is extremely essential for achieving the development of molecular breeding approaches to enhance peanut-yielding ability and tolerance to environmental stresses and for offering more accurate reference genes for RNA-seq and microarray approaches. A set of new reference genes with more expression stabilities and wider applications can be developed from large expression microarrays and RNA-seq datasets or searched from whole genome sequences and validated experimentally; this can perfect the utilization of candidate reference genes for gene expression studies in peanut.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ejbt.2017.05.004.

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