Identification and validation of reference genes for real-time qPCR normalization during Al-induced programmed cell death in peanut

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Abstract

The reverse transcription quantitative real-time PCR (RT-qPCR) is becoming increasingly important for gene expression studies. However, the accuracy and reliability of RT-qPCR depend on normalizing expression to reference genes. In this study, ten candidate reference genes, including cyclophilin (CYP), elongation factor 1b (EF1b), α-tubulin (TUA5), β-tubulin (TUB4), ubiquitin10R (UBQ10R), 60S ribosomal RNA (60S), alcohol dehydrogenase (ADH3), metalloprotease (MTP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and actin (ACT2) were evaluated for the stability of expression in three tissues of two peanut cultivars [Zhonghua 2 (ZH2) and 99-1507] under Al stress by four statistical algorithms (geNorm, NormFinder, BestKeeper, and RefFinder). The results suggested that the top-ranked reference genes under Al-induced programmed cell death (PCD) in peanut were UBQ10R, EF1b and CYP, with the most suitable combination of reference genes being [UBQ10R+ACT2]. The UBQ10R exhibited the most stable expression in all samples, while TUB4 was the least stable gene. The relative expression of AhMC1 (the caspase-like protease family gene, which played a significant role in Al-induced PCD) showed that there was no significant difference with the best reference gene and the best gene combination in RT-qPCR normalization, but there was significant difference with the least stable gene TUB4 as reference gene. This is the first study to evaluate the stability of reference genes in peanut under Al-induced PCD, and the results will provide guidance to identify appropriate reference genes for further RT-qPCR analyses under Al stress in peanut.

Additional key words: actin, Arachis hypogea, elongation factor 1b, glyceraldehyde-3-phosphate dehydrogenase, β-tubulin, ubiquitin.

Introduction

Gene expression profiles play an important role in molecular biology (VanGuilder et al. 2008). Several techniques have been used to reveal profiles of gene expression, such as Northern blotting, DNA microarray, in situ hybridization (ISH), gene chip, semi-quantitative (sq) and quantitative (q) PCR (Stephenson 2010). With the advantage of higher sensitivity, specificity, speed, accuracy, throughput, and lower cost, reverse transcription (RT)-qPCR has become the most popular method for the relative quantification of gene expression in recent years (Sugden and Winter 2008, Kulcheski et al. 2010, Li et al. 2012). However, the accuracy and reliability of RT-qPCR...
is restricted by the amount of sample, quality of RNA, and normalization, which used reference genes (Van Guilder et al. 2008). Reference genes are usually recruited from the category of housekeeping genes, including actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin (UBQ), β-tubulin (TUB), elongation factor 1b (EF1b), and 18S or 60S ribosomal RNA (18S or 60S) (Kim et al. 2003). Appropriate reference genes in RT-qPCR should ideally exhibit constitutive expression in various tissues, at different developmental stages, or under different experimental conditions (Wan and Wilkins 1994, Nolan et al. 2006, Xiang et al. 2008, Zhong et al. 2011). However, recent studies have shown that no single reference gene exhibited constant expression under all experimental conditions (Mehdi and Van 2012, Galeano et al. 2014, Ling et al. 2014). Therefore, re-evaluation of a suitable reference gene or multiple stably-expressed reference genes can improve the accuracy of RT-qPCR system, reduce inaccurate data interpretation, and reflect better the actual expressions of target genes.

The expression stability of candidate reference genes was evaluated by different algorithms, estimating the stability parameter in each, in order to identify the most appropriate reference gene from different candidates. Several widely-used statistical algorithm packages (BestKeeper, geNorm, Normfinder, and RefFinder) have been recommended for reference gene evaluation in recent years (Putatunda et al. 2016, Freitas et al. 2017, Hou et al. 2017). Peanut (Arachis hypogaea L.) is an important crop throughout the world, which is generally cultivated in acid soil in southern China. Aluminum is uptaken from the soil as Al\(^{3+}\) once the pH falls below 5.0 (Pan et al. 2001). Our previous studies have shown that Al rapidly induced mitochondria-dependent programmed cell death (PCD) in peanut, and there was a negative relationship between Al-induced PCD and Al tolerance (Zhan et al. 2013, 2014, Huang et al. 2014b). However, the mechanism of Al-induced PCD is still unclear. With the development of high-throughput sequencing technology, more and more genomes have been successfully sequenced, and the expression of genes related to Al tolerance have been studied. This requires an efficient tool, such as RT-qPCR, to analyze the expression profile of Al-responsive genes, to dissect the mechanism of Al-induced PCD.

In recent years, some progresses have been made with respect to reference gene normalization for RT-qPCR in peanut, demonstrating that expression of reference genes changed considerably in different developmental stages and in different experimental conditions. Yael and Ran (2010) suggested that the most stable reference gene is alcohol dehydrogenase (ADH3), followed by 60S and yls8 in various developmental stages of peanut. Reddy et al. (2013) demonstrated that ADH3 and glucose-6-phosphate 1-dehydrogenase (6GPD) were stably expressed across experiments, while EF1b might potentially be better over a diverse set of tissue samples of peanut when subjected to various biotic (virus infection) and abiotic (salinity and drought) stresses. Morgante et al. (2011) found that ACT1, UBII and GAPDH are the most stable genes for different species, organs, and treatments. To date, appropriate reference genes have not yet been validated for Al-induced PCD in peanut.

The aim of present study was to evaluate the expressions of ten candidate housekeeping genes including cyclophilin (CYP), EF1b, a-tubulin (TUA5), TUB4, UBQ10R, 60S, ADH3, metalloprotease (MTP), GAPDH and ACT2 in two peanut cultivars subjected to abiotic stresses (Al and caspase-3-like inhibitor Ac-DEVD-CHO) and in three different tissues and to compare expression stability by GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and RefFinder (Xie et al. 2011) programs.

**Materials and methods**

**Plants and treatments:** Peanut (Arachis hypogaea L.) cultivars Zhonghua 2 (ZH2; Al-sensitive) and 99-1507 (Al-tolerant) were chosen to carry out all the experiments. Plants were cultivated as described by Yao et al. (2016). Some seedlings at 3-leaf stage were treated with 100 µM AlCl\(_3\) in Hoagland nutrient solution (pH 4.2) for five different times (0, 4, 8, 12, and 24 h) after pretreatment with 0.1 mM CaCl\(_2\) solution (pH 4.5) for 1 d. The other seedlings were treated with the caspase-3-like inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) [50 µM dissolved in dimethyl sulfoxide (DMSO), pH 4.5] which can inhibit Al-induced PCD (Jiao et al. 2013). Four treatments of Ac-DEVD-CHO were performed: 50 µM DMSO alone (control), 50 µM Ac-DEVD-CHO alone (DEVD), 100 µM Al alone (Al), and 100 µM Al + 50 µM Ac-DEVD-CHO (Al+DEVD). To achieve the maximum inhibitory effect, Ac-DEVD-CHO was applied 2 h prior to adding 100 µM Al for 8 h. Ten seedlings per replicate and three replicates of each treatment were done. The different tissue samples including root tips, stems, and leaves were collected, snap-frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

**RNA isolation and cDNA synthesis:** Total RNA was extracted from all samples with the Promega Easypure super RNA extraction kit (Promega, Shanghai, China) according to the manufacturer’s instructions. After extraction, the concentration of RNA was quantified with NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Only those RNA preparations, which satisfied the requirement for an A\(_{260}/A_{230}\) ratio of 1.9 to 2.1 and an A\(_{260}/A_{230}\) ratio greater than 2.0, were used.
for further analysis. The total RNA was diluted to 500 μg cm⁻² and checked by 1 % (m/v) agarose gel electrophoresis (Fig. 1. Suppl.). cDNA was synthesized using Primer Script® RT reagent kit with gDNA eraser (Perfect Real Time) kit (Takara, Dalian, China) according to the manufacturer’s instructions. Genomic DNA was cleaned-up using the gDNA eraser procedure before the reverse transcription. The cDNA mixture was stored at -20°C.

Selection of reference genes and primer design: Ten candidate reference genes (CYP, EF1b, TUA5, TUB4, UBQ10R, 60S, GAPDH, ACT2, ADH3, MTP) involved in different functional classes in the cell were selected based on the previous description (Morgante et al. 2011, Chi et al. 2012, Reddy et al. 2013). They were used to BLAST (http://ncbi.nlm.nih.gov/blast) the peanut EST to obtain the corresponding homologous gene sequences in the genome. The primers were designed using Primer Premier 5.0 software (Premier, Toronto, Canada) and referred to previous reports in peanut (Yael and Ran 2010, Chen et al. 2011, Morgante et al. 2011, Chi et al. 2012, Reddy et al. 2013), with melting temperatures of 58 - 60 °C, primer lengths of 18 - 25 bp and amplicon lengths of 64 - 190 bp (Table 1 Suppl.). Synthesis of all the primers was performed by Takara. The amplicons were analyzed by 1.0 % agarose gel electrophoresis before RT-qPCR.

Real-time qPCR analysis for amplification specificity and efficiency: Real-time reactions were performed using the CFX 96TM real-time system (Bio-Rad, Hercules, CA, USA) in 20 mm² of reaction mixture containing 10 mm² of SYBR® Green Ex Taq™ (Bio-Rad), 2.0 mm² of cDNA template, 1.0 mm² of each primer (10 mM) and dDH₂O. RT-qPCR reactions with no-template controls (NTC, containing dDH₂O instead of cDNA) were also carried out for each primer pair to check for template contamination. All PCR reactions were performed under following conditions: an initial denaturation step of at 95 °C for 3 min and 40 cycles (each consisting of 10 s at 95 °C, 30 s at 60 °C, and 10 s at 95 °C), followed by melting curve analysis by heating from 65 to 95 °C with a speed of 0.5 °C per 5 s. A standard curve for each primer pair was carried out via a three-fold serial dilution of pooled cDNA. The amplification efficiency (E) was calculated using the formula E = (2⁻¹/slope - 1) x 100. The slope value (regression coefficient) and the correlation coefficient (r²) were directly obtained from the equation of the line-of-best-fit of the standard curve.

Data handling and analysis: Expressions were usually estimated according to the number of amplification cycles taken to reach a fixed threshold, which is named the threshold (or quantification) cycle (Cq) value. In the relative quantities method, Cq values are converted to ΔCq values and then compared. The smallest Cq is first defined as 1 and other Cq values subtracted from 1 to gain the ΔCq values. The smaller the ΔCq, the more stable a candidate reference gene is.

The geNorm is based on the Excel-VBA applet platform which evaluates the stability of reference genes and recommends the number of reference genes. The principle of geNorm is that the expression ratio of two ideal reference genes should be the same in all the samples despite changes in experimental conditions or types of tissue used (Vandesompele et al. 2002). GeNorm calculates the average expression stability values (M) and the pairwise variation (V) values for that gene, compared to the other genes. To execute, a ΔCq value was obtained and then imported into geNorm. There is a negative relationship between M value and gene stability. The pairwise variation Vₙ/Vₙ+1 is also used in geNorm between two sequential normalization factors, NFₙ and NFₙ₊₁, to determine the ideal number of reference genes. Once the Vₙ/Vₙ+1 ratio is below 0.15, n is considered the suitable number of reference genes.

The NormFinder is also widely used to determine the expression stability of candidate reference genes (Andersen et al. 2004). The calculation of NormFinder is similar to geNorm, and ΔCq values are used. Intra- and inter-group variation among the sample subgroups are also estimated by this software, and the most stable one is identified.

The BestKeeper program calculates the correlation coefficient (r), standard deviation (SD,) and coefficient of variation (CV) by pairwise comparison of all the candidate genes. The genes with smaller SD values, possess greater stability. Reference genes with SD values >1 are considered to be the most unstable ones (Pfaffl et al. 2004).

For more reliable and consistent results, the RefFinder was applied to calculate the geometric mean of each candidate gene and to re-rank the stability (Castro-Quezada et al. 2013, Zsöri et al. 2013). To execute, the ranking of each gene by geNorm, NormFinder and BestKeeper was multiplied by different weighting coefficients and calculated the geometric mean to produce an overall final ranking (Freitas et al. 2017).

Reference gene validation under Al-stress treatments: To determine how the most and the least stable reference genes affected gene expression level, a caspase-like protease gene AhMC1 was chosen to be the target gene. AhMC1 plays an important role in Al-induced PCD in peanut and its ORF sequence has been successfully cloned from peanut (Yao et al., unpublished results). In addition, the most stable reference gene UBQ10R, and the most stable gene combination [UBQ10+ACT2], as well as the least stable reference gene TUB4 were chosen as reference genes. The seedlings of ZH2 were treated with 100 µM Al for different times (0, 4, 8, 12, and 24 h) after pretreatment at 0.1 mM CaCl₂ solution (pH 4.5) for 1 d, and the root tips, stems, and leaves were collected for RT-qPCR. The primers (AhMC1-F: 5' TGACGATGATGGAATGCCGG 3' and AhMC1-R: 5' CAGATGTGATGCAATGATC 3').
and AhMC1-R: 5'CATAGCATTCAAGCGGC 3') of AhMC1 were designed by Primer 5.0 software. The amplified length was sequenced, and specificity was confirmed by melt curve analysis. The relative expression of AhMC1 was calculated by the $2^{-\Delta\Delta C_q}$ formula (Kundu et al. 2013).

**Results**

A set of 42 pooled samples including three different tissues (roots, stems and leaves) of two peanut cultivars exposed to Al stress, was used to evaluate the expression stability of ten candidate genes for normalization of RT-qPCR. A single amplification of expected length was verified by 1 % agarose gel electrophoresis, and a single peak was observed in the dissociation curve by RT-qPCR analysis. Moreover, no signal was observed in the no-template control in qPCR reactions for each primer pair, which suggested that there were no genomic DNA contaminants and primer dimers in the reaction mixture (Fig. 2. Suppl.). Additionally, standard curves were plotted using three-fold serially diluted cDNA for the pooled samples. For all the primer pairs, the linear correlation coefficient ($r^2$) was greater than 0.986, while the specific PCR amplification efficiency (E) ranged from 92.7 to 108.6 %, respectively (Table 1 Suppl.).

**Statistical analysis:** Three repetitions were performed to determine each value and standard deviations were calculated. Data were presented as means ± SDs, and the statistical significance was determined by Student’s t test.

The Cq values of all the candidate reference genes varied from 15.74 to 31.38, with most ranging from 18.63 to 25.01 (Fig. 1). The wide range of expressions reflected the lack of consistency and the necessity for normalization under stress responses. Among the ten tested genes, UBQ10R, 60S, and GAPDH showed relatively low Cq values, indicating that they exhibited high stability. The ADH3 showed the lowest stability (the highest mean Cq value of 22.86), followed by MTP with a mean Cq of 22.43. Using the geNorm, the M values of the candidate genes were calculated and ranked from the lowest to the highest (Fig. 2). The lowest M value belongs to the most stable gene. Taking into account all the samples, UBQ10R and EF1b had the lowest M values (0.59), while TUB4 had the highest M value (1.61) (Fig. 2A), indicating that UBQ10R and EF1b were both the most stable reference genes and TUB4 the least stable reference gene. Except for root tip and stem tissue of 99-1507, UBQ10R was the most stably expressed gene in all the tissues. ACT2/60S (M = 0.24) was the most suitable gene pair for root tip and stem tissues of 99-1507, whereas CYP/60S ranked the best individual reference genes in the stem tissue of 99-1507. However, the gene with the least stable expression was TUB4 in all tissues of 99-1507 and the leaf of ZH2, whereas MTP (M = 2.08) and ACT2 (M = 0.60), respectively, ranked the least stable in root tip and stem tissues of ZH2 (Fig. 2B-G). The results also showed that the most stable genes had an M value below the threshold of 1.5, suggesting that they were suitable for consideration as reference genes for peanut under Al stress. Another function of geNorm was the determination of the optimal number of reference genes using the $V_n/n+1$ value (Fig. 3). The $V_2/V_3$ value was below 0.15 in all tissues of 99-1507 and the stem tissue of ZH2, suggesting that two reference genes were sufficient

![Fig. 1. Box-whisker plot of expression of ten candidate reference genes obtained from all the tested peanut samples. The figure was drawn by Sigmaplot 10.0 software according to the raw Cq values obtained from RT-qPCR. A lower Cq value represents more stable expression. The box represents the first (25 %) and the third (75 %) quartiles, whiskers depict the maximum and minimum values, while the line across the box and the black dots outside the box signifies the median and outlier values, respectively.](image-url)
for normalization, but that three reference genes were necessary for the root tip and leaf tissues of ZH2. For the entire sample dataset, geNorm recommended six reference genes for normalization, because the six most stable genes had no marked effect on the normalization factor ($V_6/V_7 = 0.146$).

The evaluation of reference genes for RT-qPCR normalization was further analyzed by Normfinder (Table 2 Suppl.). Intra- and inter-group variation was used to analyze all the samples and to subsequently calculate the M value to estimate stability. Similar to the geNorm method, the lower the M value, the more stable the gene is. UBQ10R exhibited the most stable expression, with the lowest M value in all the samples, ranking from 0.101 to

Fig. 2. Gene average expression stability (M) and ranking of potential reference genes in different tissues calculated by geNorm. A lower M value represents more stable expression analyzed in different sets, including all samples (A), roots of ZH2 (B), stems of ZH2 (C), leaves of ZH2 (D), roots of 99-1507 (E), stems of 99-1507 (F), and leaves of 99-1507 (G).
0.278. UBAQ10R and ACT2 were recommended to be the best reference combination for all the samples, having an M value of 0.216. TUB4 showed the lowest stability in all the samples except for the root tip tissue of ZH2, where MTP, 60S and TUB4 showed the most variation. The results from the Normfinder were largely consistent with those from the geNorm with regard to the normalization of a single candidate gene. However, the results from the BestKeeper, which mainly determined stability in terms of SD, were slightly different. CYP and UBAQ10R were highlighted as the best stable reference genes in the root tissue of ZH2 and the leaf tissue of 99-1507, respectively, while TUA5 was identified as the most suitable one for other samples (Table 3 Suppl.). The TUB4 had unstable expression in all the samples, as identified by the various software programs.

The geometric mean of each candidate gene from the three different program algorithms was calculated by the RefFinder in order to obtain more reliable and consistent results (Table 4 Suppl.). Based on the geometric mean, the ranking (in the orders of decreasing stability) of the ten candidate genes for the entire sample was UBAQ10R > EF1b > CYP = TUA5 > ACT2 > ADH3 > GAPDH > 60S > MTP > TUB4. For the root, stem, and leaf tissues of ZH2, UBAQ10R and TUA5, UBAQ10R and EF1b, and UBAQ10R and EF1b, respectively, were suggested to be the most

Fig. 3. Pairwise variation of the ten candidate genes was predicted by geNorm. The pairwise variation (Vn/Vn+1) was calculated between the normalization factors NFn and NFn+1, with a recommended cut off threshold of 0.15.

Fig. 4. Relative expression of the AhMC1 gene during Al-induced PCD determined in roots (A), stems (B), and leaves (C) of cv. ZH2 treated with 100 µM AlCl3 for 0, 4, 8, 12, and 24 h, respectively. We selected the most stable reference gene UBAQ10R and the worst one TUB4 identified by RefFinder, and the best combination [UBAQ10R + ACT2] identified by Normfinder. Means ± SDs, n = 3, * - significant difference at P < 0.05 (Student’s t-test).
stable reference genes. Meanwhile, for the root, stem, and leaf tissues of 99-1507, UBQ10R and CYP, UBQ10R and ACT2, and UBQ10R and ADH3, respectively, were highlighted as the reference genes exhibiting the least variation in expression.

To reinforce the results obtained from the software, the relative expression of AhMC1 gene was analyzed, using the most stable reference gene UBQ10R and the least stable gene TUB4 determined by RefFinder algorithms, and the best combination [UBQ10R+ACT2] identified by Normfinder algorithms. The AhMC1 gene from peanut, belonging to the caspase-like protease family, have a positive role in Al-induced PCD (Yao et al. unpublished data). The AhMC1 expression pattern and transcript abundance were similar with the candidate reference gene UBQ10R individually and in the [UBQ10R+ACT2] combination, having no significant difference. On the contrary, normalization with the least stable one (TUB4) demonstrated underestimation of AhMC1 expression in Al-treated samples, which was significantly lower than after normalization with UBQ10R and [UBQ10R+ACT2] (P < 0.05) (Fig. 4).

Discussion

Al toxicity is one of the major limiting factors for peanut production due to induction of root tip PCD. In our previous studies, Al induced PCD rapidly in the Al-sensitive cultivar ZH2 and more slowly in the Al-tolerant cultivar 99-1507. However, the mechanism of Al-induced PCD is still unclear (Huang et al. 2014b). The transcriptomics data from these two cultivars during Al-induced PCD showed that there were many up- or down-regulated genes after Al treatment (Yao et al. unpublished data). Understanding the temporal and spatial expression patterns of these genes would be invaluable in clarifying the mechanism of Al-induced PCD.

Several researchers (Yael and Ran 2010, Morgante et al. 2011, Reddy et al. 2013) have reported different patterns of reference genes at different developmental stages, or various experimental conditions in peanut. Differential stability of reference genes in different tissues has also been reported in other plants (Lin et al. 2013, Imai et al. 2014, Hashemi et al. 2016, Hou et al. 2017). Some reference genes, like ADH3 and TUA5, have previously been highlighted as the most stable genes in peanut, but they are not suitable to all experimental sets. Here, the expression analysis of ten candidate reference genes in a pool of peanut samples containing three tissues, two cultivars, and Al-stress, showed that UBQ10R was the most suitable reference gene for normalization. Except for UBQ10R, the top-ranked stable reference genes in individual samples were quite different, such as EF1b in the stem and leaf of ZH2, ACT2 in the stem of 99-1507, TUA5 in the root of ZH2 and so on (Table 4 Suppl.). This illustrated necessity for validating reference genes in peanut for specific experimental conditions. Prior to conducting a study on gene expression associated with Al-induced PCD in peanut, it was vital to carry out detailed research on the stability of candidate reference genes. RefFinder ranked the candidate genes on the basis of output from the different algorithms, including geNorm, NormFinder and BestKeeper, when the results of algorithms were different (Freitas et al. 2017). Volland et al. (2016) suggested the use of the RefFinder tool as an alternative to a global ranking tool to improve normali-
Amorphophallus under waterlogging (Wang et al. 2017). Nevertheless, it was not surprising that very different results were obtained in our study, reinforcing the necessity of reference gene normalization for expression studies prior to RT-qPCR analysis.

For a long time, GADPH was frequently selected to be the appropriate reference gene in many studies without validation (Gutierrez et al. 2008a,b). However, GADPH expression was found to be generally less stable under abiotic stress in our study, similar to the results from Chrysanthemum (Gu et al. 2011), maize (Galli et al. 2013), strawberry (Galli et al. 2015), lettuce (Borowski et al. 2014) and Setaria viridis (Martins et al. 2016). Hence, we do not recommend GADPH as a reference gene in peanut under Al-induced stress.

A single reference gene can be used if it shows expression consistency in different experimental treatments, but in several instances the use of more than one reference gene as normalizers rather than only one proved to be more reliable (Vandesompele et al. 2002). According to the results obtained by geNorm, more than six genes for entire samples and at least two genes for different tissues should be hypothetically used to normalize the RT-qPCR experiments (Fig. 3). The NormFinder program suggested the combination of [UBQ10R + ACT2] as the most suitable gene combination (Table 2 Suppl). To test this proposal, we analyzed the expression of the AhMC1 gene by using UBQ10R (the most stable gene), TUB4 (the least stable gene) and [UBQ10R + ACT2] (the most suitable combination) in the ZH2 cultivar under Al stress. The expression profiles of the AhMC1 gene were similar when normalized by UBQ10R individually and in the combination of [UBQ10R + ACT2] (Fig. 4), suggesting that almost no significant difference was found between the best reference gene and the best gene combination in RT-qPCR normalization. However, there was a significant difference in target gene expression with the least stable gene TUB4 as reference gene. Therefore, the most stable normalizers selected by RefFinder and the most stable combination opted by NormFinder yielded similar expression profiles of the AhMC1 gene, confirming the significance of validating the stability of reference gene prior to its application in RT-qPCR experiments.

This is the first study to validate a set of ten potential reference genes for RT-qPCR in peanut under Al-induced PCD. Our findings suggested that reference genes should be pre-evaluated for their expression stability in different experimental conditions. The selection of the reference genes recommended here would ensure accuracy in RT-qPCR results, which would also provide precious data for research stress tolerance mechanisms in peanut.

References


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