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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(ZH_2) 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

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Abbreviations: Ac-DEVD-CHO - acetyl-Asp-Glu-Val-Asp-aldehyde; ACT - actin; *BLAST* - basic local alignment search tool; Cq - the threshold (or quantification) cycle; CV - coefficient of variation; DMSO - dimethyl sulfoxide; EF-1b - elongation factor 1b; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; M - the average expression stability values; PCD - programmed cell death; RT-qPCR - reverse transcription quantitative polymerase chain reaction; 18S - ribosomal RNA *18S*; TUB - β-tubulin; UBQ - ubiquitin; V - the pairwise variation.
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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 (Patankar *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-phospahate 1-dehydrogenase* (*G6PD*) 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*, *UBI1* 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*, *α-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 hypogea* L.) cultivars Zhonghua 2 (ZH_2; 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 Eastep®* 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_{280} 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^{-3} and checked by 1 % (m/v) agarose gel electrophoresis (Fig. 1. Suppl.). cDNA was synthesized using *Prime 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 mm^3 of reaction mixture containing 10 mm^3 of *SYBR® Primix Ex Taq*™ (*Bio-Rad*), 2.0 mm^3 of cDNA template, 1.0 mm^3 of each primer (10 mM) and ddH_2O; RT-qPCR reactions with no-template controls (NTC, containing ddH_2O 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 every primer pair was carried out *via* a three-fold serial dilution of pooled cDNA. The amplification efficiency (E) was calculated using the formula $E = (2^{-1/slope} - 1) \times 100$. The slope value (regression coefficient) and the correlation coefficient (r^2) 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 V_n/V_{n+1} is also used in *geNorm* between two sequential normalization factors, NF_n and NF_{n+1}, to determine the ideal number of reference genes. Once the V_n/V_{n+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, Zsóri *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 ZH_2 were treated with 100 μM Al for different times (0, 4, 8, 12, and 24 h) after pretreatment at 0.1 mM $CaCl_2$ solution (pH 4.5) for 1 d, and the root tips, stems, and leaves were collected for RT-qPCR. The primers (*AhMC1*-F: 5' TGACGATGATGGAAGTGCGG 3'

and *AhMC1*-R: 5' CATAGCATTCAGCAAGCGGC 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 $2^{-\Delta\Delta Cq}$ formula (Kundu *et al.* 2013).

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.

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.).

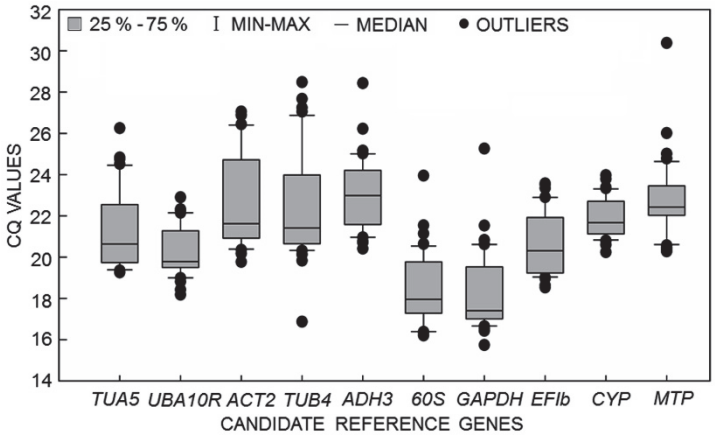


Fig. 1. Box-whisker plot of expression of ten candidate reference genes obtained from all the tested peanut samples. The figure was drawn by *Sigmaplot 10.0* software according to the raw Cq values obtained from RT-qPCR. A lower Cq value represents more stable expression. The *box* represents the first (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.

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. 2*A*), 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 ZH_2, whereas *MTP* (M = 2.08) and *ACT2* (M = 0.60), respectively, ranked the least stable in root tip and stem tissues of ZH_2 (Fig. 2*B*-*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 ZH_2, suggesting that two reference genes were sufficient

for normalization, but that three reference genes were necessary for the root tip and leaf tissues of ZH_2. 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

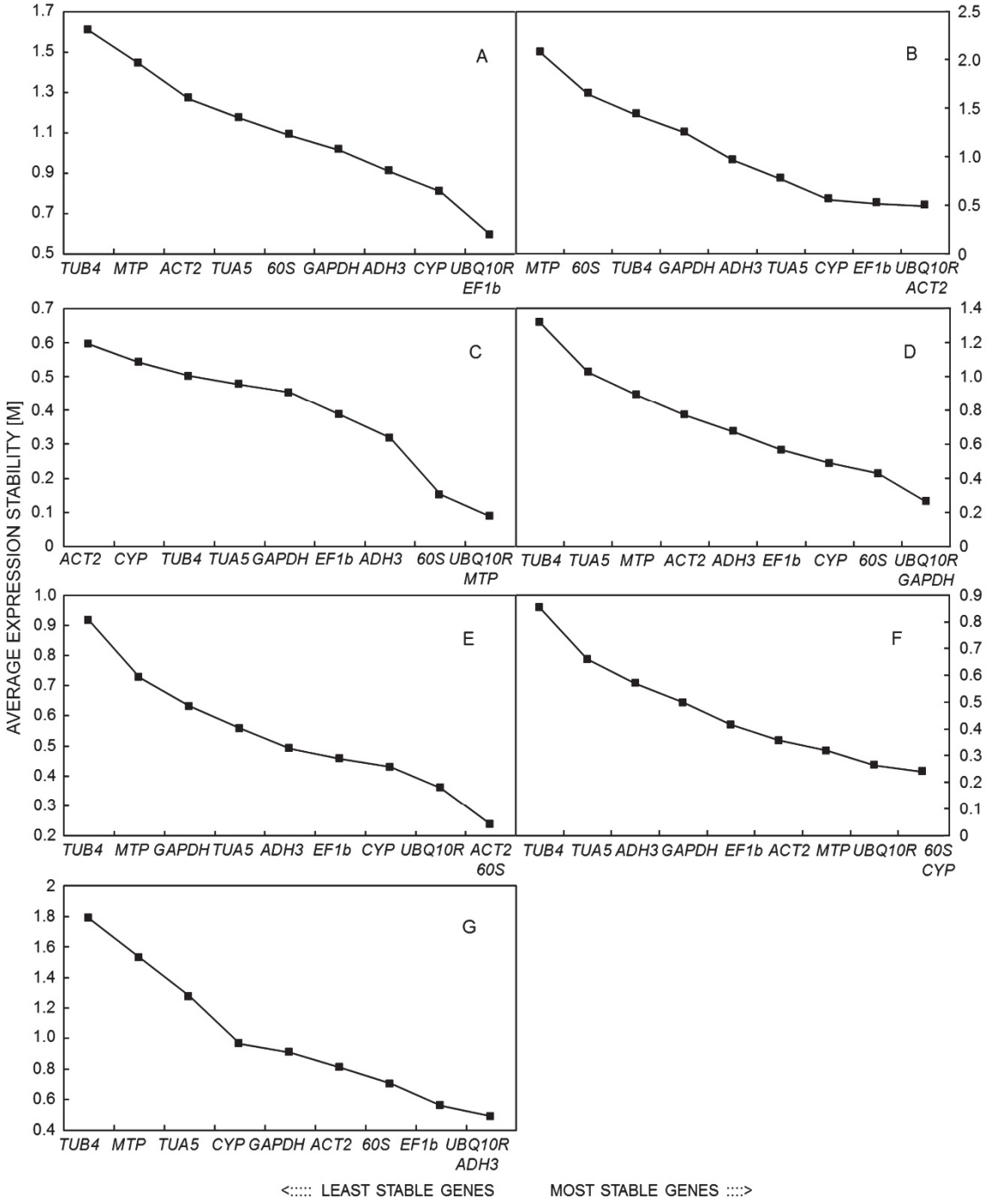
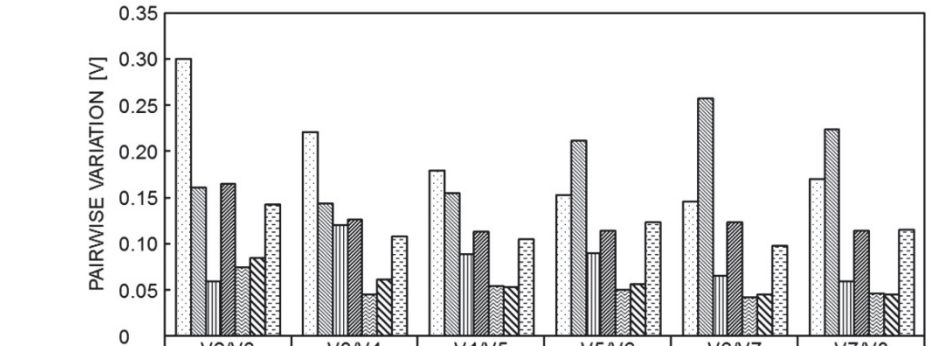


Fig. 2. Gene average expression stability (M) and ranking of potential reference genes in different tissues calculated by *geNorm*. A lower M value represents more stable expression analyzed in different sets, including all samples (*A*), roots of ZH_2 (*B*), stems of ZH_2 (*C*), leaves of ZH_2 (*D*), 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 expect for the root tip tissue of ZH_2, 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 *UBQ10R* were highlighted as the best stable reference genes in the root tissue of ZH_2 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 *UBQ10R* > *EF1b* > *CYP* = *TUA5* > *ACT2* > *ADH3* > *GAPDH* > *60S* > *MTP* > *TUB4*. For the root, stem, and leaf tissues of ZH_2, *UBQ10R* and *TUA5*, *UBQ10R* and *EF1b*, and *UBQ10R* and *EF1b*, respectively, were suggested to be the most



	V2/V3	V3/V4	V4/V5	V5/V6	V6/V7	V7/V8
ENTIRE	0.3	0.221	0.179	0.153	0.146	0.17
ZH2 ROOT	0.161	0.144	0.155	0.212	0.258	0.224
ZH2 STEM	0.06	0.12	0.089	0.09	0.066	0.06
ZH2 LEAF	0.165	0.127	0.113	0.114	0.124	0.114
99-1507 ROOT	0.075	0.045	0.054	0.05	0.042	0.046
99-1507 STEM	0.085	0.062	0.053	0.057	0.045	0.045
99-1507 LEAF	0.143	0.108	0.105	0.124	0.098	0.115

Fig. 3. Pairwise variation of the ten candidate genes was predicted by *geNorm*. The pairwise variation (V_n/V_{n+1}) was calculated between the normalization factors NF_n and NF_{n+1}, with a recommended cut off threshold of 0.15.

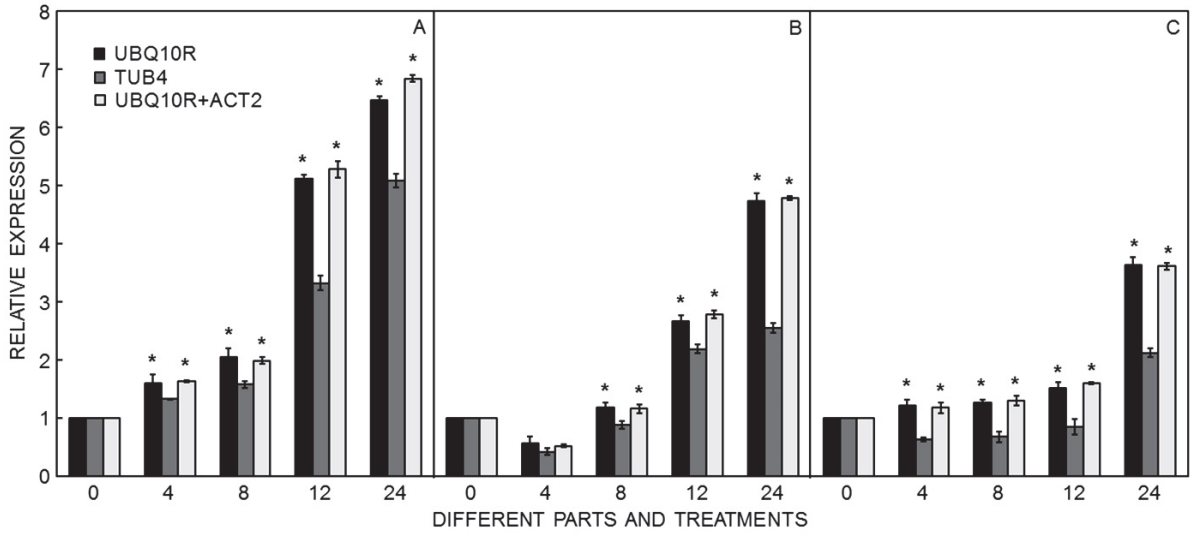


Fig. 4. Relative expression of the *AhMC1* gene during Al-induced PCD determined in roots (*A*), stems (*B*), and leaves (*C*) of cv. ZH_2 treated with 100 μM $AlCl_3$ for 0, 4, 8, 12, and 24 h, respectively. We selected the most stable reference gene *UBQ10R* and the worst one *TUB4* identificated by *RefFinder*, and the best combination [*UBQ10R +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*] identificated 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 ZH_2 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 ZH_2, *ACT2* in the stem of 99-1507, *TUA5* in the root of ZH_2 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 normalization of reference genes. In the present study, the ranking orders of reference genes generated by *geNorm* and *NormFinder* were different, but their selection for the best was nearly uniform, while the result from *BestKeeper* was quite different. *RefFinder* was needed to rank the candidate genes on the basis of outputs from three different algorithms. The results of *RefFinder* algorithms further recommended *UBQ10R* and *EF1b* to be the most suitable reference genes for normalization during gene expression studies in Al-induced PCD. In previous studies, *UBQ* had also been used as the reference gene for the analysis of RT-qPCR in banana fruit under stress (chilling, high temperature, and pathogen) (Chen *et al.* 2011), cotton under salinity and drought (Wang *et al.* 2013), perennial ryegrass under different abiotic stresses (Yan *et al.* 2014), and *Sapium sebiferum* affected by sucrose (Chen *et al.* 2017), indicating that *UBQ* is related to stress response. Elongation factor *EF1* has been reported to be the most stable gene in terms of expression in peanut under cold treatment (Chen *et al.* 2011, Chi *et al.* 2012), and in switch grass (Huang *et al.* 2014a) and African oil palm (Xia *et al.* 2014) under abiotic stresses. The expression stability of genes is related to the function they play in biological metabolic pathways (Wang *et al.* 2017). Ubiquitin is a small protein existing in all eukaryotes with a conserved sequence and structure, which plays important roles in controlling cellular signal transduction (Zhang 2011). *EF1b* is mainly in charge of the initial steps of protein synthesis, and also exhibits conserved sequences. Acevedo *et al.* (2018) reported that *elongation factor 1-alpha + tubulin alpha chain* (*EF1a + α-Tub*) is one of the most suitable genes to compute a normalization factor in *Ilex paraguariensis* leaves during drought. The central and ubiquitous roles of these genes are likely to be the reasons which *UBQ10R* and *EF1b* exhibit stable expression in many species.

On the other hand, *TUB4* was the most unstable reference gene as ranked by all four algorithms, but it is interesting to note that *TUB* was identified to be one of the best reference genes for gene expression in maize under different abiotic stresses (Lin *et al.* 2014) and in

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 expressional 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 ZH_2 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.

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