Evaluation and Validation of Reference Genes for Normalization of Quantitative Real-Time PCR Based Gene Expression Studies in Peanut

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Abstract

The quantitative real-time PCR (qPCR) based techniques have become essential for gene expression studies and high-throughput molecular characterization of transgenic events. Normalizing to reference gene in relative quantification make results from qPCR more reliable when compared to absolute quantification, but requires robust reference genes. Since, ideal reference gene should be species specific, no single internal control gene is universal for use as a reference gene across various plant developmental stages and diverse growth conditions. Here, we present validation studies of multiple stably expressed reference genes in cultivated peanut with minimal variations in temporal and spatial expression when subjected to various biotic and abiotic stresses. Stability in the expression of eight candidate reference genes including ADH3, ACT11, ATPsyn, CYP2, ELF1B, G6PD, LEC and UBC1 was compared in diverse peanut plant samples. The samples were categorized into distinct experimental sets to check the suitability of candidate genes for accurate and reliable normalization of gene expression using qPCR. Stability in expression of the references genes in eight sets of samples was determined by geNorm and NormFinder methods. While three candidate reference genes including ADH3, G6PD and ELF1B were identified to be stably expressed across experiments, LEC was observed to be the least stable, and hence must be avoided for gene expression studies in peanut. Inclusion of the former two genes gave sufficiently reliable results; nonetheless, the addition of the third reference gene ELF1B may be potentially better in a diverse set of tissue samples of peanut.

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Introduction

Gene expression studies have become increasingly important to understand the molecular mechanisms in animal, human, microorganism, and plant systems [1-4]. Gene expression levels have been determined by techniques including Northern blotting, RNase protection assay, semi-quantitative reverse-transcription PCR, and quantitative real-time PCR (qPCR) [4]. However, qPCR has gained importance over the rest owing to its high sensitivity, accuracy, speed, and high-throughput analysis. The main advantages with the qPCR analysis are ability to detect low-abundance mRNAs [5], quantify mRNA copy number [6], and need for relatively lower amount of test materials and no post-PCR gel analysis, etc. [7,8].

Nevertheless, a substantial technical variability associated with qPCR may exist due to inherent differences in samples, sample collection, quantity and quality of input RNA, reverse transcription and PCR efficiency, and pipetting errors [9]. In order to minimize these, the most common practice is to normalize the gene of interest with the reference gene (an internal control gene), which is also subjected to the similar errors in cDNA preparation, thereby, making results from qPCR more reliable than absolute quantification. The ideal reference gene should notably express stably across the developmental stages and under variable experimental conditions. However, selection of an unstable reference gene can add large unpredictable error to the analysis and result in incorrect evaluations [10]. Several studies have shown that no single internal control gene is universal for use as a reference gene for all experiments [11-13]. Different samples or treatments...
may require the re-evaluation of a suitable reference gene, since changing experimental conditions can sometimes cause a suitable reference gene to become unstable [14]. Hence, the reference genes that do not show variable expression levels in different cells and tissues under different conditions must be the choice so as to reduce measurement errors.

Cellular homeostasis genes, more commonly known as housekeeping genes that are involved in basic and ubiquitous cellular processes such as components of the cytoskeleton, glycolytic pathway, protein folding, protein degradation, and synthesis of ribosome subunits are mostly used as reference genes [9]. The most frequently used housekeeping genes that have been validated as suitable reference genes in many plants include β-actin (ACT), α-tubulin (TUA), ubiquitin (UBQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S or 26S ribosomal RNA and elongation factors (EF) etc. [15-19]. However, transcript levels of housekeeping genes too vary considerably across the developmental stages and under variable conditions [20], thereby, necessitating the selection of other multiple stably expressed reference genes to be considered for accurate normalization of gene expression studies [21]. Reference gene validation have been reported in a number of crop plants such as Oryza sativa L. [22], Triticum aestivum L. [23], Zea mays L. [24], Solanum tuberosum L. [15], Solanum lycopersicum L. [25], Chrysanthemum [19], Vitis vinifera L. [26], Brassica rapa L. [27], Brassica napus L. [28], Brassica juncea L. [9] Arabidopsis [12] Glycine max L. [16,29-33], and Cicer arrietinum L. [17]. However, except for soybean and chickpea, very few studies have been conducted to validate reference genes for qPCR in legume crops, thus necessitating a need to validate experiment-specific reference genes from legumes, including peanut where a major emphasis is on the development of transgenic peanuts for various biotic and abiotic constraints and nutritional enhancement.

Peanut (Arachis hypogaea L.) is the second-most important grain legume crop cultivated in over 100 tropical and subtropical countries of the world [34] and is an important oilseed cash crop containing 48–50% oil and 20–25% protein. The major abiotic factors affecting peanut production include drought, high temperature, low soil fertility, low soil pH and iron chlorosis. Among the biotic factors, diseases caused by fungi, viruses, bacteria, nematodes, and foliar and soil insect pests significantly affect peanut productivity [35]. However, most of these agronomical traits are difficult to breed by conventional selection techniques due to little genetic variation within cultivated peanut. Modern biotechnology approaches including marker-assisted selection, tissue culture, embryo rescue and genetic transformation have been employed in crop improvement programs worldwide including peanut [36]. The use of transgenic technology potentially offers a targeted gene-based approach for the genetic enhancement of field crops. Moreover, with the advent of genetic transformation technology for crop improvement, molecular characterization of transgenic events need to be carried out at various stages from identification of the transgenic event to transgene integration, copy number detection and gene expression.

High-throughput molecular characterization of transgenic events is now possible with the introduction of qPCR based techniques. While, the qPCR based gene expression studies requires reference gene identification, only a limited number of reference gene validation studies have been carried out in peanut [37-39]. Hence, in recognition of the importance of reference genes for the normalization of qPCR data and a need to identify species-specific and experimental conditions-specific reference genes, the present study was conducted to validate suitable reference genes with minimal variations in temporal and spatial expression in cultivated peanut subjected to various biotic (fungal and viral) and abiotic (salt and drought) stresses. Here, we have selected eight candidate reference genes including alcohol dehydrogenase (ADH3), actin (ACT11), ATP synthase (ATPsyn), cyclophilin (CYP2), elongation factor 1B (ELF1B), glucose-6-phosphate 1-dehydrogenase (G6PD), lectin (LEC) and ubiquitin-conjugating enzyme (UBCF1) through bibliographic reviews of studies in crop plants such as peanut, soybean and cotton followed by an in silico analysis. We have compared the expression stability of these candidate reference genes in diverse samples of peanut categorized into distinct experimental sets to check their suitability as stable reference genes for accurate and reliable normalization of gene expression using qPCR.

Materials and Methods

Plant Material

Peanut (Arachis hypogaea L.) varieties including JL24, TTAG24, CS39, ICGV 86699, ICGV 06040, ICGV 91144, ICGV 00350 and ICGV 05155 were obtained from the Groundnut Breeding Unit of ICRISAT. Peanut plants were grown in 6 inch pots containing 3.5 kg of alfisol:sand:compost mixture (3:2:1; 20 % water holding capacity) under greenhouse conditions with 28/20 °C day/night temperature. Leaf samples were collected 3 days after shoot emergence (DAE) from all peanut varieties grown in the greenhouse. Different tissue samples from varieties JL24 and ICGV 86699 including leaf sample of early stage (LES), cotyledons, stems and roots were collected 3 days after shoot emergence (DAE), whereas leaf sample of flowering stage (LFS), immature pods and immature seeds were collected from plants at the pegging stage. The collected tissue samples were immediately frozen in liquid nitrogen and stored in -80 °C until RNA extraction.

Biotic and abiotic stress treatments

For stress treatments, 7 day-old seedlings of cultivars JL24 and ICGV 86699 were used for virus challenging experiments using the Tobacco streak virus (TSV) and Peanut bud necrosis virus (PBNV). While Late Leaf Spot (LLS) caused by Phaeoisariopsis personata, rust caused by Puccinia arachidis and abiotic stress treatments (salinity and drought) were imposed at early flowering stage. For TSV and PBNV infection, the virus inoculum was prepared from the infected plant leaf samples, (TSV from French bean (Phaseolus vulgaris) and PBNV from peanut) and infected to peanut plants by mechanical sap transmission method as described by Kumar and Waliyar [40]. Viral infection symptoms (necrotic lesions) were observed after 3-4 days of inoculation (DOI) on the inoculated mature leaves, and infection was confirmed by
DAC-ELISA as described by Kumar and Waliyar [40]. Young leaf samples were collected in triplicates from the infected peanut plants after 5 DOI.

Spores of LLS and rust were used for fungal infections. Spore collection, inoculum preparation, and inoculation methods for LLS and rust were conducted as described by Subrahmanyam et al. [41]. The spore suspension (30000 spores/mL) was sprayed onto peanut plants at early flowering stage, maintained under controlled conditions at 23 °C with 95 % relative humidity and 12 h photoperiod. Symptoms were evaluated 10 DOI, and leaf samples from control and inoculated peanut plants showing symptoms were collected separately.

Drought and salinity stress was imposed on peanut plants under greenhouse conditions. Plants were given regular irrigation before abiotic stress treatments and irrigated control plants were maintained as such. For drought stress, the water supply was withheld for 5 d followed by leaf sample collection, while the salinity stress was imposed by completely saturating the pots containing plants with 40 mM NaCl, followed by leaf sampling after 24 h.

RNA isolation
Total RNA was extracted from peanut plants using 50 mg of tissue using NucleoSpin RNA plant kit (Macherey-Nagel, Duren, Germany) following the recommended procedures including in-column DNase1 treatment. The isolated total RNA was tested for DNA contamination in PCR using ELF1B and ADH3 primer pairs. The DNA contaminated samples were retreated with DNase1 (Macherey-Nagel, Duren, Germany) in tubes and re-purified using NucleoSpin RNA clean-up kit (Macherey-Nagel, Duren, Germany). The concentration and purity of RNA was determined using NanoVue plus spectrophotometer (GE health care, USA) and the absorbance at 260/280 nm ranging from 1.8 to 2.0 were selected for further analysis. Integrity of the RNA was further checked by electrophoresis through 1.4 % agarose gel. The total RNA isolated was diluted to 100 ng/µl concentration and aliquoted for use in PCR.

Selection of reference genes and primer design
The candidate reference genes were selected through bibliographic reviews of studies in crop plants such as peanut (LEC [42]; ADH3 [37]), soybean (ATPsyn [43]; ACT11, ELF1B, CYP2 and G6PD [29]), and cotton (UBC1) [44]) followed by an in silico analysis using the BLAST tools of the NCBI database [45]. For instance, a previously selected EST in soybean was submitted to the BLASTN tool to obtain EST orthologous in peanut (Table 1). Subsequently, NCBI non-redundant protein sequence database (nr) was used to confirm the sequence function using BLASTX tool (http://blast.ncbi.nlm.nih.gov/Blast). Since the genome sequence of peanut is not known, alignments were made with relevant gene orthologous in Arabidopsis using BLASTN with optimization to ‘somewhat similar sequences’ before primer design to ensure the primer pairs span at least one intron. Primers were designed using primer analysis software PRIMER 3.0 (http://frodo.wi.mit.edu/ primer3/) by considering the following parameters: (a) product size range: 100-160 bp; (b) primer size: 20-22 bp; (3) GC content 50 %. The EST GenBank accession number, primer sequence, amplicon length and primer locations are listed in Table 1.

PCR analysis for specificity and efficiency of primers
In order to test the specificity of the primers and suitable reaction conditions, the primer sets were initially tested by standard PCR reaction with Mastercycler Gradient (Eppendorf, Germany) with temperature gradient (56 °C to 66 °C) using both DNA and cDNA templates. Genomic DNA was isolated from leaf samples of peanut variety JL24 using Nucleospin plant II midi kit (Macherey-Nagel, Duren, Germany) following the manufacturer’s protocol. The cDNA synthesis was carried out using the Thermoscript® RT-PCR system (Invitrogen-life technologies, USA) with total RNA samples according to the manufacturer’s instructions. Primer titrations were carried out to check the effect of primer concentrations in PCR. Template gradient PCR was carried out with different concentrations of cDNA to check the efficiency of the primers by constructing standard curves for each set of primers. All these qPCRs were carried out in Realplex (Eppendorf, Germany), Real Time PCR system using 2X SensiMix™ SYBR No-ROX (Bioline, UK) kit and 400 nM of each primer was used in template gradient PCRs. The reaction conditions were set as 10 min at 95 °C (polymerase activation); 45 cycles of 15 s at 95 °C, 15 s at 62 °C with fluorescent signal recording and 15 s at 72 °C. At the end, a final step of 15 s at 95 °C, 30 s at 58 °C and fluorescence measurement at each 0.5 °C variation from 58 °C to 95 °C in 20 min was included to obtain the melting curve. For each sample, three technical replicates were performed and Cq values were taken for analysis after drift correction. All the PCR amplified products were verified by 2 % agarose gel electrophoresis with SYBR safe DNA gel stain (Invitrogen-life technologies, USA) prior to sequencing the amplified products to check the PCR product specificity.

Real Time qPCR analysis
One step quantitative reverse transcriptase PCR (qRT-PCR) reactions were carried out in Realplex PCR system (Eppendorf, Germany) using 100 ng of total RNA, 2X SensiFAST™ SYBR No-ROX one-Step kit (Bioline, UK), and 400 nM of each primer. The reaction conditions were set as: 10 min at 45 °C (Reverse transcription), 2 min at 95 °C (polymerase activation), 40 cycles of 10 s at 95 °C, and 20 s at 60 °C with fluorescent signal recording for amplification. At the end, a final step of 15 s at 95 °C, 30 s at 58 °C followed by fluorescence measurement at each 0.5 °C variation from 58 °C to 95 °C in 20 min was included to obtain the melting curve. Each sample was tested in three technical replicates.

Data analysis
Expression levels of the eight candidate reference genes in all the sample pools were determined by the number of cycles needed for the amplification-related fluorescence to reach a specific threshold level of detection (quantification cycle Cq). The efficiency (E) of each primer pair was calculated based on
To carry out an in-depth data analysis, 31 diverse samples were categorized under eight experimental sets comprising of condition-specific samplings (Table 2). While the first experimental set included all 31 diverse peanut tissue samples, the second experimental set comprised of tissue samples of varieties JL24 and ICGV 86699 at vegetative stages [cotyledon, early young leaf (LES), stem and root], whereas the third set included tissue samples at reproductive stages (leaf, pods and immature seeds). The fourth set included total samples of both vegetative and reproductive stages. The peanut leaf samples from virus (TSV and PBNV) infected and uninfected controls were included in fifth experimental set, while the LLS and rust infected/non-infected leaf samples at flowering stage were included in sixth experimental set as foliar diseases. The seventh set comprised of leaf samples from salt and drought stressed treatments (abiotic stress), whereas the eighth experimental set included leaf samples from eight diverse peanut cultivars as described in the plant material section (Table 2).

Expression stability of the candidate reference genes was evaluated using two different methods, including geNorm and NormFinder. Firstly, the expression stability of each reference gene and the best combination of normalizer genes for each set of samples were obtained using a pair-wise method by geNorm [20] which is based on the fact that expression ratio of two ideal control genes is identical in all samples and the variation of the expression ratios of two real housekeeping genes reflecting the fact that one (or both) are not constantly expressed, with increasing variation in the ratio corresponding to decreasing expression stability [37].

The raw Cq values were converted into relative quantities after correcting the Cq values according to respective PCR efficiencies for each gene using genEX Professional software (MultiD Analyses AB, Sweden) as the requirement of geNorm analysis to calculate gene expression stability (M). To define the optimal number of genes required for normalization, geNorm platform estimates a normalization factor (NFn) by geometric average of the n best reference genes and performs a stepwise analysis (more stable to less stable genes) to calculate the pair-wise variation (Vn/Vn+1) between two sequential normalization factors, NFn.
and NFn+1, including more genes in each comparison [46]. The pair-wise variation, to define the optimal number of genes required for normalization was carried out using geNorm of qBase plus software (Biogazelle, Belgium).

The expression stability of the eight candidate reference genes was also determined by a model-based variance estimation application called NormFinder [47] to rank the candidate reference genes expression stability for all samples with no subgroup determination according to their stability under given set of experimental conditions. The Cq values of each set of samples were converted to relative quantities after efficiency correction for each gene and expression stabilities were calculated by using the NormFinder tool of genEX Professional software (MultiD Analyses AB, Sweden).

Reference genes validation in transgenic plants

Transgenic peanut plants carrying an antisense PBNV nucleoprotein gene (PBNVnp) were selected for validation of candidate reference genes under biotic stress. Similarly, for abiotic stress, transgenic peanut plants transformed with Arabidopsis thaliana dehydration responsive element binding factor 1A (AtDREB1A) driven by stress inducible promoter rd29A [48] were selected to validate the candidate reference genes under drought stress. Specific primers were designed for PBNVnp and AtDREB1A genes for estimation of relative expression levels in transgenic plants using qPCR assay. Stress treatments were carried out as mentioned in the stress treatment section. Leaf sample were collected from five PBNV infected transgenic peanut (four resistant and one susceptible) plants and one uninoculated (healthy) wild peanut (UT- untransformed) plant after five days of inoculation and different levels of viral gene expression expected in these plants, based on their resistance levels. Similarly, a total of six leaf samples were collected from drought stress experiment which included two transgenics and one wild plant (UT- untransformed), before (WW-well watered) and after treatment (DS-drought stressed), where expression of AtDREB1A gene was expected only in transgenic plants after drought stress. RNA isolation and qPCR analysis were carried out as mentioned in the previous sections. Relative quantification of target genes was estimated by normalizing with different candidate reference genes using qBase plus software. The mean relative expression values of PBNVnp of resistant and susceptible were presented after scaling with values of healthy UT sample. Similarly mean relative expression values for AtDREB1A gene were presented after scaling with values of drought stressed UT sample.

The coefficient of variation was calculated for each reference gene by using the following formula: Coefficient of variation in percentage (CV %) = (standard deviation / mean of Cq)*100

Results

Selection of candidate reference genes and primer design

A real time qPCR assay based on SYBR Green detection was designed for transcript profiling of eight candidate reference genes in 31 diverse samples of peanut. Putative orthologs of six candidate genes including ACT11, ATPsyn, ELF1B, CYP2, G6PD and UBC1 were identified from peanut by BLAST searches in the non-redundant and expressed sequence tag (EST) databases at NCBI. Previously reported two peanut gene sequences ADH3 and LEC were also retrieved from NCBI database. Since the genomic sequence of the candidate genes was not available, additional PCRs were carried out with genomic DNA of peanut using gene-specific primers that were used for real-time PCRs to conform the presence of intron(s) within the amplicon region. Most primer pairs except CYP2 and LEC amplified a specific larger-sized PCR product with DNA (Figure 1A) as compared to that with

| S.No. | Sample        | Description of the tissue sample | Sample set no. |
|------|---------------|----------------------------------|-----------------|
| 1    | RL_JL24       | Root tissue of JL24              | 1,2            |
| 2    | SL_JL24       | Stem tissue of JL24              | 1,2            |
| 3    | C1_JL24       | Cotyledon tissue of JL24         | 1,2            |
| 4    | RL_699        | Root tissue of ICGV 86699        | 1,2            |
| 5    | SL_699        | Stem tissue of ICGV 86699        | 1,2            |
| 6    | C1_699        | Cotyledon tissue of ICGV 86699   | 1,2            |
| 7    | IP_JL24       | Immature pods tissue of JL24     | 1,3            |
| 8    | IS_JL24       | Immature seed tissue of JL24     | 1,3            |
| 9    | LFS_JL24      | Leaf tissue of JL24 variety at Flowering Stage | 1,3 |
| 10   | IP_699        | Immature pods tissue of ICGV 86699 | 1,3 |
| 11   | IS_699        | Immature seed tissue of ICGV 86699 | 1,3 |
| 12   | LFS_699       | Leaf tissue of ICGV 86699 peanut at Flowering Stage | 1,3,4,6,7 |
| 13   | TSV_699       | Leaf tissue of TSV infected ICGV 86699 | 1,5 |
| 14   | TSV_JL24      | Leaf tissue of TSV infected JL24 | 1,5 |
| 15   | PBNV_JL24     | Leaf tissue of PBNV infected JL24 | 1,5 |
| 16   | LS1_699       | Leaf tissue of LLS infected ICGV 86699 pool 1 | 1,6 |
| 17   | LS2_699       | Leaf tissue of LLS infected ICGV 86699 pool 2 | 1,6 |
| 18   | Ru1_699       | Leaf tissue of Rust infected ICGV 86699 pool 1 | 1,6 |
| 19   | Ru2_699       | Leaf tissue of Rust infected ICGV 86699 pool 2 | 1,6 |
| 20   | SS1_699       | Leaf tissue of Salt stressed ICGV 86699 pool 1 | 1,7 |
| 21   | SS2_699       | Leaf tissue of Salt stressed ICGV 86699 pool 2 | 1,7 |
| 22   | DS1_699       | Leaf tissue of Drought stressed ICGV 86699 pool 1 | 1,7 |
| 23   | DS2_699       | Leaf tissue of Drought stressed ICGV 86699 pool 2 | 1,7 |
| 24   | JL24          | Leaf tissue of JL24 peanut variety | 1,2,4,5,8     |
| 25   | TAG24         | Leaf tissue of TAG24 peanut variety | 1,8 |
| 26   | CS39          | Leaf tissue of CS39 peanut variety | 1,8 |
| 27   | ICGV 00350    | Leaf tissue of ICGV 00350 peanut variety | 1,8 |
| 28   | ICGV 05155    | Leaf tissue of ICGV 05155 peanut variety | 1,8 |
| 29   | ICGV 06040    | Leaf tissue of ICGV 06040 peanut variety | 1,8 |
| 30   | ICGV 86699    | Leaf tissue of ICGV 86699 peanut variety | 1,2,4,5,8 |
| 31   | ICGV 91114    | Leaf tissue of ICGV 91114peanut variety | 1,8 |

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Table 2. Details of the peanut tissue sample used for candidate reference genes validation.
cDNA as template (Figure 1B), thereby indicating that primer pairs for these genes span at least one intron. Since we did not find amplification with total RNA as template with ELF1B and ADH3 primer pairs in PCR, the presence of any genomic DNA contamination in the RNA samples was ruled out. Single expected amplicon and no primer dimer formation with all primer combinations with all samples tested in qPCR indicated specificity and efficiency of the primers (Figure 1 and Figure S1).

Expression profiling of reference genes

Expression levels of the eight candidate reference genes were determined and assessed for expression stability in a set of 31 diverse tissue samples including eight samples of different peanut varieties, 12 representing vegetative and reproductive stages, and 11 samples from stressed sets representing various biotic and abiotic stress treatments. The amplification plots for each gene were generated and quantification cycle (Cq) was determined for all the tissue samples (Figure 2). The transcript levels of ELF1B and LEC were higher by several orders of magnitude as indicated by lower average Cq values of 19.09 and 19.25, respectively, than that of other six genes that had average Cq values in the range of 20.15 to 31.82. Among these six genes, CYP2 was expressed at relatively higher level (average Cq value 20.10) followed by ADH3 (average Cq value 25.32), ACT11 (average Cq value 25.74), ATPsyn (average Cq value 25.92), G6PD (average Cq value 26.26), where UBC1 exhibited lowest expression with average Cq value of 31.82. The expression levels of eight genes across all the 31 samples ranked as ELF1B > LEC > CYP2 > ADH3 > ACT11 > ATPsyn > G6PD > UBC1.

Figure 1. Amplification of a specific PCR product with genomic DNA (A) and cDNA (B) as templates on agarose gel (2.0%) using gene-specific primers for each candidate reference gene. Three replicates of the PCR amplicons with each primer set were loaded; M indicates a 100 bp DNA size marker. All primer pairs except CYP2 and LEC amplified a larger size PCR product with DNA template as compared to cDNA template, indicating the position of primer pairs spanning at least one intron.

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Individual candidate reference genes had different expression levels across all the sample pools tested. \textit{ACT11} and \textit{G6PD} showed the smallest gene expression variation (below 4 cycles). \textit{ADH3}, \textit{ATPsyn}, \textit{CYP2}, \textit{ELF1B}, and \textit{UBC1} had the expression variation between 4 to 6 cycles, while \textit{LEC} had highest expression variation (above 9 cycles) as shown in Figure 2. The wide expression range of the eight tested candidate reference genes confirmed that no single gene had a constant expression under tested conditions in peanut. Specifically, in the samples set of vegetative stage, the expression levels of the tested genes are highly variable. These results clearly indicate the necessity to select suitable reference genes to normalize gene expression under a certain experimental condition.

**Gene expression stability analysis**

In the entire set of 31 samples, \textit{G6PD} and \textit{ADH3} had lowest (0.48) average expression stability value (M) followed by \textit{ELF1B} (0.54) and \textit{UBC1} (0.59), and M value of \textit{LEC} was highest (0.94) (Figure 3A), thereby suggesting that \textit{G6PD} and \textit{ADH3} had the most stable expression and that \textit{LEC} was expressed most variably. The results remained very similar in the experimental sets of reproductive stage (Figure 3C) and developmental stages (Figure 3D), with the lowest M value for \textit{G6PD} and \textit{ADH3} and M value of \textit{LEC} was highest. In contrast, \textit{CYP2} and \textit{ACT11} were more stable when the vegetative stage samples were analyzed separately, and \textit{LEC} continued as very unstable (Figure 3B). While \textit{G6PD} and \textit{ADH3} gene were most stable under viral infection (TSV & PBNV), \textit{ACT11} was the least stable (Figure 3E). The data set of foliar diseases (LLS-Rust) suggested that \textit{ELF1B} and \textit{G6PD} were most stable, with \textit{LEC} being the least stable (Figure 3F). Under abiotic stress, \textit{ELF1B} and \textit{CYP2} were the most stable genes, while \textit{ATPsyn} was the most variable one (Figure 3G). In the varietal data set, the M value was least for \textit{G6PD} and \textit{ELF1B} followed by \textit{ACT11}, \textit{ADH3}, \textit{UBC1}, \textit{ATPsyn}, \textit{CYP2}, while \textit{LEC} was again the least stable reference gene (Figure 3H). Notably, the M values for \textit{G6PD} and \textit{ELF1B} in the peanut varietal set were
lower (0.16) than those of all other experimental sets, thereby indicating their high expression stability (Figure 3A-H).

The expression stability rankings of candidate reference gene using NormFinder revealed similar results as of geNorm analysis, where the G6PD was most stable and LEC was least stable genes in the entire sample set, including the reproductive, developmental, and abiotic stress stage sets (Table 3). The stability ranks of the candidate reference genes changed with method (geNorm or NormFinder) used for analysis in the experimental sets of vegetative stage, viral diseases, foliar diseases and in different cultivars sample set. The NormFinder analysis indicated LEC gene to be the least stable in all experimental sets except viral diseases, abiotic stress and peanut cultivars. The NormFinder analysis also indicated ATPsyn to be the least stable in experimental set of abiotic stress and peanut cultivars, while ACT11 to be the one least stable under viral disease set (Table 3).

**Optimal number of internal candidate genes for normalization**

We used geNorm to determine the pairwise variation in eight experimental sets of the samples. When all 31 samples were taken together, the pairwise variation V2/3 was higher than 0.15 (0.176) whereas V3/4 was 0.149 (Figure 4), indicating that ADH3 and G6PD genes together are not sufficient for normalization, and hence need a third gene ELF1B. Similarly the vegetative stage and developmental stages sets too required three genes for normalization viz., CYP2, ACT11 and ADH3 for vegetative stage, ADH3, G6PD and CYP2 for developmental stages, as indicated by their pairwise variation value V2/3 were higher than 0.15 and V3/4 were less than 0.150 (Figure 4).

In the other five experimental sets, addition of the third reference gene for normalization of gene expression showed no significant effect as indicated in pairwise variation (Figure 4). However, different experimental sets required a different pair of genes for normalization of gene expression, as indicated by pairwise variation analysis of G6PD and ADH3 genes for sets of reproductive stage and viral diseases, ELF1B and G6PD genes for foliar diseases (LLS and Rust), and the varietal set, whereas CYP2 and ELF1B genes for abiotic stress (drought and salinity) were sufficient for normalization of gene expression in peanut. When evaluating all the pairwise variations, the least stable reference gene was found to be LEC followed by ATPsyn (Figure 4).

**Reference genes validation**

Transgenic peanut samples from biotic and abiotic stress treatments were used for validation of candidate reference genes. PBNV infected peanut plants carrying antisense nucleoprotein gene of PBNV (PBNVnp) were used for validation under biotic stress. The relative expression levels of the target PBNVnp gene were presented after scaling with uninoculated UT control in each normalization analysis. The relative expression levels of PBNVnp gene in infected peanut samples were similar when normalized with candidate reference genes G6PD or ADH3 individually and in combination, while normalization with CYP2 or ACT11 genes showed a different pattern and a very high relative expression of PBNVnp gene in both the infected samples including resistant and susceptible (Figure 5A). The results were in accordance with the obtained phenotypic data (data not included), when normalized with G6PD and ADH3 reference genes, whereas normalization with CYP2 or ACT11 did not correlate with the phenotypic data.

Similarly, the AtDREB1A gene expression levels in transgenic peanut under drought stress were estimated by normalizing with the candidate reference genes. The expression levels of the target gene AtDREB1A were presented after scaling with drought stressed UT sample in each normalization analysis. The AtDREB1A gene expression levels increased several fold when normalized with ATPsyn and LEC individually or in combination, compared with the values obtained after normalization with CYP2 and ELF1B genes (Figure 5B).

The percentage of coefficient of variation (CV %) calculated for each reference gene in PBNVnp gene expression validation studies indicated high CV with CYP2 (13.8 %) and ACT11 (17.2 %) genes, while G6PD (3.7 %) and ADH3 (5.4 %) showed a lower CV. Similarly, the CV was lower for CYP2 (2.7 %) and ELF1B (4.7 %) reference genes and higher for ATPsyn (11.7 %) and LEC (14.0 %) genes under AtDREB1A gene expression studies. This data is in accordance with stability rankings of the candidate reference gene under biotic and abiotic stress conditions.

**Discussion**

Real-time PCR (qPCR) is a very powerful technique to quantify the expression levels of target genes and stably expressing reference genes required for data normalization to minimize the experimental errors in relative quantification. Nevertheless, no genes are stably expressed universally in any organism, and are regulated to only a certain extent [49]. Several studies on reference gene validation have insisted that multiple internal genes must be evaluated in order to improve the accuracy of a qPCR analysis and interpretation of gene expression [20,50,51]. The present study describes a comprehensive analysis on the validation of eight candidate reference genes in 31 diverse samples of peanut, divided broadly into eight experimental sets. Our analysis based on geNorm and NormFinder algorithms indicate that the choice of reference genes for normalization should be experimental condition-specific. In the present study, we tested the expression stability of commonly used housekeeping genes like ACT11, ELF1B, CYP2, and ADH3 that qualify among the best reference genes [37] and ACT [38] that qualify among the best reference genes under specific experimental sets of peanut were not found to be suitable reference gene across all the eight experimental sets.

The average expression levels of ELF1B and LEC genes were higher by several orders of magnitude than that of other six genes. The expression levels of LEC gene were very high in immature seeds and pods compared to other samples and
Figure 3. Average expression stability and ranking of eight candidate reference genes using geNorm. All 31 tissue samples set (A), vegetative stage (B), reproductive stage (C), developmental stages (D), viral diseases sample set (E), foliar diseases sample set (F), abiotic stress sample set (G), and different peanut cultivars sample set (H). A lower value of average expression stability (M) indicates more stable expression.

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unstable among the tested samples, whereas the ELF1B gene expression is high and stable in all the samples tested. The average expression level of UBC1 gene was lowest among the eight genes tested across experimental sets. This might be due to a possible single copy presence of Ubiquitin-conjugating enzyme (UBC1) gene in the tetraploid genome of peanut as previously reported in cotton [44].

Table 3. Gene expression Stability Ranks of 8 candidate reference genes in different sets of peanut samples calculated using geNorm (GN) and NormFinder (NF) methods.

| Sets                  | All samples | Vegetative stage | Reproductive stage | Developmental stages | Viral diseases | Foliar diseases | Abiotic stress | Peanut cultivars |
|-----------------------|-------------|------------------|-------------------|----------------------|----------------|-----------------|----------------|-----------------|
| Gene                  | GN          | NF               | GN                | NF                   | GN             | NF              | GN             | NF              |
| G6PD                  | 1           | 1                | 5                 | 1                    | 1              | 1               | 1              | 1               |
| ADH3                  | 2           | 5                | 3                 | 2                    | 3              | 2               | 2              | 2               |
| ELF1B                 | 3           | 6                | 4                 | 3                    | 6              | 5               | 3              | 1               |
| UBC1                  | 4           | 4                | 6                 | 5                    | 6              | 5               | 3              | 2               |
| ACT11                 | 5           | 7                | 2                 | 5                    | 4              | 4               | 5              | 4               |
| ATPsyn                | 6           | 2                | 7                 | 6                    | 7              | 7               | 6              | 6               |
| CYP2                  | 7           | 3                | 1                 | 4                    | 3              | 2               | 4              | 7               |
| LEC                   | 8           | 8                | 8                 | 8                    | 8              | 8               | 8              | 8               |

Figure 4. Determination of the optimal number of reference genes for normalization by pair-wise variation using geNorm. All 31 tissue samples set (A), vegetative stage (B), reproductive stage (C), developmental stages (D), viral diseases sample set (E), foliar diseases sample set (F), abiotic stress sample set (G) and different peanut cultivars sample set (H). The pairwise variation (Vn/Vn+1) was analyzed between normalization factors NFn and NFn+1 by geNORM program to determine (V<0.15) the optimal number of reference genes.

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comparison to all other genes being tested and reports the average expression stability (M) of all the genes in a given set of samples [20]. Genes with the lowest M value have the most stable expression, while the highest M value indicates the least stable expression. In the present study, the candidate reference genes displayed high expression stability in all eight experimental data sets indicated by low (<1.0) M values. The G6PD (glucose-6-phosphate 1-dehydrogenase) gene was most stable among the eight genes tested across the six experimental sets, except during the vegetative stage and abiotic stress sets. This is in contrast to the previous report in soybean where G6PD gene expression was least stable under different photoperiodic treatments and developmental stages [29] and under cadmium stress [33]. The G6PD gene has been involved in the glycolytic pathway similar to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) that has been the most commonly reported reference gene [17,38]. Unlike G6PD, GAPDH was recently reported as stably expressing reference gene in two legumes including chickpea [17] and peanut [38]. These results indicate that stability of gene expression is purely based on experimental condition and not only on the species tested.

The second most stably expressing gene in this study was ADH3 that was most stable in the entire sample set including the reproductive stage, developmental stages and in viral diseases experimental sets. The ADH3 encoding for alcohol dehydrogenase class III enzyme that catalyzes the interconversion of alcohols and aldehydes or ketones with the

Figure 5. Relative quantification of PBNVnp and AtDREB1A genes to validate candidate reference genes of peanut under biotic and abiotic stress conditions. (A) Expression of PBNVnp gene in infected transgenic peanut leaf sample relatively quantified with candidate reference genes. (B) AtDREB1A gene expression in leaf sample of transgenic (rd29a:AtDREB1A) peanut relatively quantified with candidate reference genes. The relative quantity values were presented after scaling to control samples in both the (PBNVnp and AtDREB1A) cases.

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The reduction of NAD⁺ to NADH plays an important role in lowering the toxicity of the cell [52]. Although the ADH3 has not been used frequently as a qPCR reference gene, previous reports showed its stable expression in coffee arabica [53] and peanut [37]. While in the previous study with peanut, ADH3 was restricted to kernel developmental stages [37], our study included different experimental sets from developmental stages, biotic stress, abiotic stress and a range of peanut varieties.

The ELF1B (Elongation factor 1-beta) and CYP2 (Cyclophilin) genes were found to be next in ranking in terms of their stable expression after G6PD and ADH3. While the ELF1B was most stable under foliar diseases and across peanut varieties, CYP2 was most stable in vegetative stages, and both ELF1B and CYP2 were most stable under abiotic stress. These observations are in accordance with other studies where ELF1B gene under cold stress in peanut [39] and under abiotic stress [32] and cadmium stress [33] in soybean was reported as most stably expressed. Similarly, CYP2 was reported as most stably expressed gene in different experimental sets of Vicia faba [54]. ELF1B and CYP2 also showed stable expression in different tissues under various developmental stages of soybean [29]. ACT1, a member of Actin gene family has so far been considered to be the most stable across different species of peanut [38], whereas ACT11 has been considered to be the most stable in all the samples tested in peanut [39]. Nevertheless, in the present study ACT11 gene was found to be stable only during the vegetative stages of peanut, indicating that its expression might have been influenced by the experimental conditions in this study.

Our data clearly demonstrates the unsuitability of LEC (lectin) gene as a reference gene for gene expression studies in peanut where it showed the least stable expression across different experimental sets analyzed. Similarly, ATPsyn (ATP synthase) gene that is involved in the synthesis of adenosine triphosphate (ATP) has so far not been reported as stably expressing internal gene, with only one report where it was used as an endogenous gene in soybean [43]. Since ATPsyn was not stable across the experimental conditions, it is not recommended as a reference gene for gene expression studies in peanut.

Although, a single reference gene with high expression stability may be appropriate for normalization of gene expression data in some experimental conditions, in most of the experimental conditions, there may be no single gene suitable as a reliable reference gene and two or more internal reference genes are required for accurate and reliable results [20]. The pairwise variation results in our study indicated that the different pairs of most stable reference genes were found to be optimal for the accurate normalization across the five experimental sets, viz., reproductive stage, viral diseases, foliar diseases, abiotic stresses and different peanut varieties, where pairwise variation values (V2/3) were lower than the cut-off value of 0.15 [20]. Addition of the third candidate reference gene was necessary only to normalize gene expression in sets of all samples, developmental stages and vegetative stage.

The results of these three sets indicated unstable expression of genes in the vegetative stage samples.

Candidate reference genes of peanut were validated with transgenic peanut plants under biotic and abiotic stresses with PBNVnp and AtDREB1A genes, respectively. Normalization of PBNVnp gene expression in infected transgenic peanut plants showed variable expression levels with CYP2 and ACT11 genes as compared to those obtained by normalizing with G6PD and ADH3 reference genes indicating lower stability of CYP2 and ACT11 genes. Similarly, normalization of stress inducible AtDREB1A gene expression in transgenic peanut with CYP2 and ELF1B reference genes indicated its stability under abiotic stress when compared to LEC and ATPsyn genes that had shown extreme expression levels, indicating their instability under the tested experimental conditions. These validation results indicated that stability ranks of the tested candidate reference genes are accurate and more than one stably expressed reference gene should improve the accuracy of normalization.

Conclusion

In the present study, we validated eight candidate reference genes by extending the study to diverse samples including a range of biotic and abiotic stresses, different developmental stages and cultivars. While the ADH3 and G6PD exhibited the most stable expression in all the tissue samples, the ELF1B expression was stable across different varieties, foliar diseases and abiotic stress conditions. Although, two genes like ADH3 and G6PD should be sufficient to give reliable results, the addition of a third gene ELF1B as reference gene may produce even better results in a diverse set of tissue samples of peanut.

Supporting Information

Figure S1. Melting curves of the 8 candidate reference genes of peanut: A-ACT11, B-ADH3, C-ATPsyn, D-CYP2, E-ELF1B, F-G6PD, G-LEC, H-UBC1. (TIF)

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Author Contributions

Conceived and designed the experiments: Dumbala and Pooja and Kiran. Performed the experiments: Dumbala and Katamreddy. Analyzed the data: Dumbala and Pooja. Contributed reagents/materials/analysis tools: Dumbala, Pooja and Kiran. Wrote the manuscript: Dumbala and Pooja.
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