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Identification of Suitable Reference Genes for Studying Expression of Cell Wall-Related and Developmental Associated Genes in Mungbean (Vigna radiata (L.) Wilczek)

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Abstract

Spatio-temporal changes in gene expression are associated with certain biological activities. Therefore, identifying reference genes is paramount to elucidate the gene expression using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Therefore, to identify suitable reference genes, we initially screened 15 putative reference genes by studying their gene expression in different tissues of mungbean. Further, ten candidate reference genes were ranked based on stability using algorithms such as GeNorm, NormFinder, Bestkeeper, and RefFinder. The *EF-1 ALPHA*, *CYP1* and *GAPDH* showed the best stable expression across different tissues as compared to other reference genes. The *EF-1ALPHA* exhibited the lowest Ct value, the highest expression and most stable (using different algorithms) by the comparative analysis among 10 reference genes after analysing its expression in leaf and stem. Further, the suitability of *EF-1 ALPHA* as reference genes was validated by checking the expression of *PHENYLALANINE AMMONIA LYASE (PAL)*, *CINNAMOYL ALCOHOL DEHYDROGENASE (CAD), IRREGULAR XYLEM 14 (IRX14)* and *RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE (RuBisCO*) in three different mungbean genotypes and tissue types. We further tested the expression of *RuBisCO* in seedlings under salt and osmotic stress and found its expression was lower as compared to control plants, which correlated with the phenotype of seedlings. Therefore, we propose *EF1-ALPHA* is the best reference gene to analyse the expression of genes in different tissues and development stages with and without stress conditions.

Introduction

Gene expression profiling is an important tool to understand the complex gene regulatory network functioning at the molecular level. Differential expression of genes in different tissues and developmental stages may determine their function and biological role in the cell. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a sensitive and accurate method which allows the monitoring of gene amplification in real-time and relates it to the amount of gene expression level product present in the sample (Bustin 2000). However, there are several factors that determine the accuracy of gene expression. These includes quality and quantity of tissue used for RNA extraction, presence of inhibitors, primer and retro-transcription efficiencies (Wang et al. 2023). Therefore, the normalization of the gene expression against certain reference or housekeeping genes becomes crucial to adjust sample-to-sample variation and to assess differential expression in different tissue types and genotypes depending on its application (Schmittgen and Zakrajsek 2000). Ideally, a standard reference gene should have a constant level of expression pattern among different cells, different tissues and developmental stages which should be irrespective of experimental parameters (Kozera and Rapacz 2013). There are many reports where different housekeeping genes such as *ELONGATION FACTOR* (*EF-1 ALPHA*), *18S ribosomal RNA (18S)*, *POLYUBIQUITIN (UBQ*), *ACTIN (ACT*), *B-TUBULIN (TUB)*, and *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) and others have been used to determine target gene expression (Kozera and Rapacz 2013; Ling et al. 2014a; Joseph et al. 2018). However, studies suggest several internal control genes for normalization of target gene expression (Hong et al. 2008; Artico et al. 2010; Gu et al. 2011; Manoli et al. 2012a). A specific genes or gene combinations is not found suitable for different experimental conditions or tissue types (Die et al. 2010). Therefore, it is highly imperative to define specific reference genes or gene combinations to normalize gene expression in different experimental conditions. Several reports identified suitable reference genes for specific experimental conditions in cereals and legumes ((Jarošová and Kundu 2010; Ferdous et al. 2015; Shivhare and Lata 2016; Reddy et al. 2016; Dudziak et al. 2020). Specifically, suitable reference genes are reported in Medicago (Kakar et al. 2008; Sun YaLi 2014) chickpea (Garg et al. 2010; Reddy et al. 2016), Pigeon pea (Sinha et al. 2015b, a) and soybean (Libault et al. 2008; Hu et al. 2009). However, less efforts have been made to identify suitable reference genes in *Vigna radiata*. (Kundu et al. 2013; Ke et al. 2020).

Mungbean (*Vigna radiata* (L.) *Wilczek*) is third most important legume in India (Singh et al. 2021). It is cultivated extensively in South-Asia, East-Asia, and South-East Asia from ancient time and native to Central Asia and India ((Ha and Lee 2019). Mungbean is an underutilized legume that is a rich source of dietary protein, vitamin and micronutrients (Manjunatha et al. 2023). It also plays a crucial role in maintaining soil fertility fitting with different crop rotational systems. Considering the huge population growth and hidden hunger, it is highly imperative to improve mungbean production. However, most of the yield-contributing traits, like seed size or seed weight, seed per pod are complex and quantitative in nature. Moreover, several factors including biotic or abiotic stresses can affect mungbean yield ((Nair et al. 2019). These factors may be regulated by the development and re-modulation of cell wall components. The plant cell wall is highly complex structure that is mainly composed of cellulose, hemicellulose, lignin and pectin and varies across plant species and tissue types. The diversity in plant cell shapes and sizes is because of the distinct physicochemical properties of the cell wall. (Farrokhi et al. 2006)Also, in the last couple of decades, tremendous effort has been put forward to understand the genetic regulation of the cell walls which may impact on growth and development, resistance to abiotic or biotic stress and mechanical resistance (Cosgrove 2005; Caffall and Mohnen 2009). Thus, fine-tuning of cell wall composition can lead to improved yield (Ha et al. 2021). The cellulose and lignin content of leaf and stem tissues were significantly different in mungbean. The cellulose content of mungbean leaf and stem was 6.5% and 29%, respectively. Whereas, the lignin content of mung leaf and stem was found to be 8.5% and 8%, respectively (Das et al. 2024). Considering the variation in cell wall composition, gene expression analysis is crucial to infer the gene regulatory function for cell wall biosynthesis and remodelling. In mungbean, less efforts have been made to define reference genes in different experimental conditions and tissue types (Ke et al. 2020; Singh et al. 2021). There is scarce information available for suitable reference gene which can be used for normalization of gene which are differentially expressed under different stress conditions. Moreover, to the best of our knowledge, there is no report to define suitable reference genes to analyse expression of cell wall related genes in mungbean under normal and abiotic stress conditions.

Therefore, the aim of this study was to identify suitable reference genes to normalize the expression of genes regulating cell wall biogenesis. Thus, we employed RT - qPCR to check the expression levels of ten housekeeping genes (*ACTIN, EF-1 ALPHA, UBQ*, *GAPDH, LECTIN, CYP1, TIP41, IF4A, HSP90* and *CYP450*) in leaf and stem tissue of mungbean under normal conditions. Using different algorithm based on GeNorm and RefFinder, the ten candidate reference genes were ranked as per their stability. Three genes - *EF-1 ALPHA*, *CYP1* and *GAPDH* showed stable expression across different tissues and EF1 was most stable among all tested genes. Also, the transcript level was higher for *EF-1 ALPHA* as compared to other reference genes in normal and stress condition in different tissue types. In conclusion, this study has identified several reference genes using robust experimental design which can be used to normalize the expression of gene of interest using RT-qPCR in mungbean.

Material and Method

Plant material

The seeds of mungbean (*Vigna radiata* (L.) *Wilczek*) were procured from the Division of Genetics, Indian Agriculture Research Institute, New Delhi, India. The seeds were thoroughly washed and soaked overnight in reverse osmosis (RO) purified water. The seedlings were grown in autoclaved mixture (1:1) of agropeat and vermiculite in plastic pots at 32°C ± 1°C in a culture room under 16 h light/8 h dark cycle. The leaf and stem of five weeks old mature plant was harvested. At least three independent biological replicates of each tissue sample were harvested and immediately frozen in liquid nitrogen. In this study, three commonly grown genotypes developed by Indian Agricultural Research Institute, New Delhi Pusa i.e., Pusa Vishal, Pusa 1641 and Pusa 1431 were used for analyses. Stress related experiments were performed on above mentioned three genotypes. For stress related experiments, seedlings were initially grown on wet filter paper for 2-day. These seedlings were then subjected to 100 mM NaCl and 20% PEG stress for 3 days. The pictures were recorded, samples were harvested after total 5-day and crushed in liquid nitrogen and stored in -80°C for RNA extraction.

RNA extraction

Total RNA was extracted from seedlings (stress and non-stress conditions), leaf and stem tissues using Ambion Trizol reagent (Sigma Life Science, United States of America) according to the manufacturer's instructions. The DNA contamination was removed using DNase I (Cat no: EN0521, Thermo Fisher Scientific, United States of America). The RNA was quantified using a NanoDrop micro volume spectrophotometer (Thermo Fisher Scientific Inc., United States of America) and all the RNA samples were adjusted to the same concentration. Only the RNA samples with 260/280 ratio from 1.9 to 2.1, 260/230 ratio from 2.0 to 2.5 and RIN (RNA integrity number) more than of 8.0, were used for the analysis. The RIN value was determined using the Bioanalyzer (Agilent, Santa Clara, United States of America). The integrity of RNA samples was also assessed by agarose gel (1.3%) electrophoresis.

cDNA synthesis

Complementary DNA (cDNA) was then synthesized by using the iScript™ cDNA Synthesis Kit (1708891, Bio-Rad, United States of America). In brief, a total of 10 µl reaction mixture was prepared by mixing 1 µl of 500 ng of RNA samples, 2 µl of 5x iScript Reaction mix, 1 µl iScript reverse transcriptase and 6 µl nuclease-free water. Then the reaction mixture was incubated in a thermal cycler following a protocol of priming for 5 min at 25^{o}C, reverse transcription for 20 min at 46^{o}C and RT inactivation for 1 min at 95^{o}C. The PCR product was confirmed using Melt Curve analysis and by running on 1.3% agarose gel.

qRT-PCR Analysis

The sequences of commonly used putative reference genes were retrieved from mungbean genome database; the legume information system (https://www.legumeinfo.org/). These genes are regularly used in different plant species for the selection of suitable reference genes. The details of gene IDs and NCBI IDs are represented in Table 1. The primers for real-time PCR analysis were designed using PrimerQuest™ Tool (Integrated DNA Technologies, Inc., United States of America) under the default parameters; except for minimum primer length was set to 20. The specificity of primer pairs was confirmed by Primer-BLAST with all the nucleotide sequences available for mungbean at National Centre for Biotechnology Information (NCBI). The RT-qPCR was conducted with Quant-Studio 6 Flex Real-Time PCR Systems (Thermo Fisher Scientific Inc., United States of America) using the HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX), 5x. The following reaction set up was done for the preparation of cDNA from all types of tissues under non-stress and stress conditions. The reaction was set up in ten µl reaction containing 2 µl HOT FIREPol® EvaGreen® qPCR Mix Plus, 0.3 µL of each primer (10 µM), 1 µl of cDNA (100 ng/µl) and 6.4 µL ddH_2O. The PCR reaction conditions were as follows – 95°C for 10 min; 95°C for 15 s; 60°C for 1 min; 40 cycles. The PCR amplification specificity was determined from melting curve analysis and semi-quantitative PCR. The melting curve was analysed by heating the amplicon from 60°C to 95°C to confirm primer specificity. Each reaction was performed with three technical and three biological replicates. Relative fold changes in gene expression were calculated using the comparative 2 – $^{\Delta\Delta}$ CT method (Bubner and Baldwin 2004). First the delta Ct value was calculated by subtracting the Ct value of reference gene from the Ct value of the target gene. Then the 2 – $^{\Delta\Delta}$ CT formula is used to obtain the relative fold change.

Table 1
List of all the primers used in this study

Name of primer	Gene ID	Forward Primer	Reverse Primer	Expected band size	Annealing temp	Efficiency (%)	Amp fact
Actin	XM_014638363.1	AGGCTGTTCTGTCCTTGTA	GAAGAGCGTAGCCCTCAT	105	60°C	104.1	2.0
Alpha tubulin	XM_014646147.1	CTGGTATGTGGGTGAAGGT	GCCAACCTCCTCGTAATCT	91	60°C	190.3	2.9
Beta tubulin	XM_014634642.1	CTGGTGAGGGAATGGATG	TCATCAGCAGTAGCATCT	100	58.6°C	168.1	3.6
Elongation factor 1	XM_014656474.1	CTAACTTCACCTCCCAGG	CAGCGAACTTGACAGCAA	108	60°C	106.6	2
Polyubiquitin	XM_022783996	CGTGAAAGCTAAGATCCAGG	CCTCGGAGACGGAGAACTAAG	150	60	131.2	2.3
Ribosomal protein L7-2	XM_014634336	GGATCCAGAGGCTAAGCTC	CAGCATGTTCATGGTGGCC	151	55°C	81.1	1.8
CYP1	AB020612.1	GGGCTCGATCTTCCACCGTG	GTTCCAGGACCGGCGTTCGC	201	60°C	101.3	2.0
Histone H2A	XM_014641486	GGCTGGAAATGCTGCTCGTG	CCTTGGAAGCCTTCTCAGTC	182	60°C	110.6	2.1
Lectin	XM_014639113	CCCGGACACACGAAAATTCTTGA	CTACTCCTTGTTTCATGGGAGC	173	60°C	100.8	2.0
TIP41	XM_014655703.1	CCTTTGGGAAGACTGCAAGG	CCAAGAGCTTGGCATTACTCTC	157	60°C	110.5	2.1
GAPDH	XM_014634520.1	GAGGGTTTGATGACCACAGTTC	CCATTCCAGTCAACTTTCC	181	60°C	98.6	1.9
Initiation factor 4a	XM_014646146	GCTGCGCAGACAGTCACTTAGAC	CAAGTGCCTCAGGAGGCATCG	185	60°C	134.4	2.3
HSP90	XM_014665349	GGTGTTGGATTCTACTCTGCTT	ACCCTGAATCCTGGTTGGCTC	195	60°C	102.2	2.0
CYP450	XM_014637189	GGGGAACCCCGTTAACGTTGGG	CCGAGACAAGGCACAAAGTC	190	60°C	124.4	2.1
18 s RMT	XM_014653246	GGTGACATGGGCCAGGGTTTAG	CTTGAAACACTGCTCTGGCTCC	185	60°C	112.2	2.1
PAL	LOC106778742	GCCACTTAGGGGAACAATC	AGGTTGCAACTCAAAGAAC	195	60°C	110.6	2.0
CAD	LOC106775051	GGTGCGCCACAAGATGAATC	GTCTGCACCAAGCAAGGTC	196	60°C	111.2	2.1
RuBisCO	KU519326.1	GAGGAACAGCTGGGTAAGGAAG	CACATGGTCCAGTACCTTCCAT	188	60°C	121.8	2.0
IRX 14	Vradi11g04510	GCCAAGTCTGGCATCAGAACAATC	CATCAGCAAACACCACAACTCCG	190	60°C	120.3	2.1

Primer efficiency calculations

To estimate the efficiency of the qPCR primers, a cDNA pool was created using equal volumes of leaf and stem cDNA. This pool was then diluted 10 times. From this diluted sample, dilution series of 4X, 16X and 64X were prepared. A RT-qPCR reaction was set up using these dilutions, and a graph of Ct value versus log of cDNA concentration was used to calculate the primer efficiency (in percentage) was then calculated using the following formula-

Efficiency (%) = [10 ^ (-1/The Slope Value) -1] * 100

The primer efficiency for all the candidate reference genes has been determined (Table 1).

Gene expression stability analysis

According to the principle of RT-qPCR analysis, the detection of the amount of fluorescently labelled amplicon is directly proportional to the amount of amplified DNA (Bubner and Baldwin 2004). The threshold cycle (Ct) is adjusted to the cycle number at which the fluorescence generated within a reaction exceeds the fluorescence threshold. The fluorescence threshold is the signal which is above the background fluorescence. The Ct values of each sample were determined using QuantStudio™ Real-Time PCR Software. Several algorithms are recommended for the analysis of stability according to gene expression. In our study, different tools based on different algorithms, including RefFinder (https://blooge.cn/RefFinder/), BestKeeper, NormFinder andgeNorm were used to identify the most stable gene among these selected reference genes. The RefFinder tools provides a comprehensive ranking of all the reference genes according to their stability. However, different algorithm follows different type of calculations (comprehensive gene stability and delta CT method). The geNorm determines the expression stability (M-value) for each candidate genes. The M-value was calculated, and the gene with the lowest M value was recommended as the defined reference gene (Vandesompele et al. 2002). In our study, the M value cut-off of less than 1.5 was set up. The GeNorm algorithm also employed a method to determine the optimal number of reference genes in a given set of samples. The BestKeeper algorithm uses Ct values to estimate the coefficient of variation (CV) and standard deviation (SD) for each reference gene (Pfaffl et al. 2004). The gene with the lowest SD value was considered as the most stable reference gene. The NormFinder algorithm was used to determine the stability value (SV) by measuring the variance in gene expression within and between groups. The genes with the lowest SV were considered the most stable genes.

Validation of reference genes

Phenylalanine ammonia lyase (*PAL*) (LOC106778742), cinnamyl alcohol dehydrogenase (*CAD*) (LOC106775051), *IRREGULAR XYLEM8 (IRX8) (Vradi11g04510), RuBisCO* (gene bank ID KU519326.1) were selected for normalization of their expression using *EF-1 ALPHA* as reference gene. To evaluate the validity of the selection of reference genes, the expression levels of *PAL*, *CAD* and *RuBisCO* were analysed in stem and leaf tissues of mungbean. The expression of these genes was normalized using *EF-1 ALPHA*, *GAPDH* and *HSP90*. The RT-qPCR was performed, and the average Ct value was calculated from three biological and technical replicates. The relative fold change of these genes was subsequently calculated and normalized according to Huis et al. 2010.

Results

Selection and amplification of putative reference genes using RT-qPCR

The PCR was performed on mungbean cDNA for 15 candidate genes to assess the specificity of the primers by semi-quantitative PCR (Table 1). The PCR products amplified from each primer pair was assessed using agarose gel electrophoresis (Fig. 1a, **Fig. S3**). The desired size band was visible for the following ten genes: *EF-1 ALPHA, GAPDH, CYP1, HISTONE H2A, IF-4A, HSP90, LECTIN, TIP41, ACTIN* and *POLYUBQ*. To further validate, we analysed these primer pairs using RT-qPCR. The melting-curve analysis also revealed a single peak corresponding to the expected size of the amplicon (Fig. 1b, **Fig. S2**). These results indicated that primer pairs of these ten genes produce specific amplicons of the target genes. The details of these primers regarding primer sequence, amplicon length, melting temperature, efficiency and correlation coefficient have been provided in Table 1. The RT-qPCR amplification efficiency varied from 98% for *GAPDH* to 134% for *IF-4a*, whereas the correlation coefficients varied from 0.9821 for *IF-4A* to 0.997 for *HSP90*.

EF-1 ALPHA showed the lowest Ct value

Further, we wanted to identify and validate the suitable reference gene to normalise gene expression of cell wall and biomass-related genes in mungbean. Therefore, the Ct value of all the candidate reference genes was analysed independently across stem, leaf and both the tissues (Fig. 2). The Ct value ranged from 19 to 35 across stem and leaf tissues of mungbean (Fig. 2a, 2b). The *EF-1 ALPHA* exhibited the lowest mean Ct value (20) and *LECTIN* had the highest mean Ct value (31), suggesting that *EF-1 ALPHA* has the highest expression and *LECTIN* has the lowest expression level. The *IF4a* (24), *GAPDH* (23), *POLYUBIQUITIN* (22.33), *HSP90* (24.) and *CYP1* (21) showed slightly higher Ct value as compared to *EF-1 ALPHA* suggesting their transcript abundance with moderate expression. The *ACTIN* (27.00), *TIP41* (26) and *HISTONE H2A* (25) exhibited comparatively less transcript abundance as compared to the above genes suggesting moderately lower expression. Interestingly, all the genes followed same Ct value pattern for stem & leaf combined and separately as well. This suggested that most of them with low and moderately high Ct values can be used as reference genes.

Gene expression stability analysis of candidate reference genes

To further understand the gene expression stability of all the candidate genes, and ΔCt was used in GeNorm, NormFinder, BestKeeper and RefFinder statistical tools. GeNorm analysis was performed to define the most suitable reference genes (Vandesompele et al. 2002). The analysis suggested that *GAPDH* and *EF-1 ALPHA* are the most stable genes followed *CYP1*. The *HSP90* was the least stable gene (Fig. 3, **Table S1**). In stem and leaf both tissues, *HSP90* showed the lowest stability (M-value 0.210) and *GAPDH* had the highest stability (M value 0.102) followed by *EF-1 ALPHA* (M value 0.105) and *CYP1* (0.106) (Fig. 3a, **Table S1**). According to the GeNorm principle, lower gene expression corresponds to a high M value, and the lowM value corresponds to stable gene. In our study, a total of ten genes were analysed in stem and leaf tissues using GeNorm. In stem, *HSP90* exhibited the lowest stability with the highest M value (0.185), whereas *EF-1 ALPHA* and *GAPDH* both exhibited the most stability with the lowest M value (0.106) (Fig. 3b, **Table S1**). In the case of leaf tissues, *HSP90* exhibited the highest M value (0.267), with the lowest stability, whereas *EF-1 ALPHA* exhibited the highest stability with the lowest M value (0.080) (Fig. 3c, **Table S1**).

The NormFinder analysis depends on Analysis of Variance (ANOVA), which determines the intra- and inter-group variation of gene expression stability for each reference gene (Anderson et al. 2004). The NormFinder algorithm determines the stability value, which is the lowest for the highest stable gene. *GAPDH* showed the highest stability (SV 0.005) followed by *EF-1 ALPHA* (SV 0.015) and *HSP90* has the lowest stability (SV 0.112) in stem tissue (Fig. 4a, **Table S2**). Whereas, *LECTIN* was most stable (SV 0.006) followed by IF4a (SV 0.008), and *EF-1 ALPHA* (SV 0.013) and *HSP90* (SV 0.183) in leaf tissue (Fig. 4b, **Table S2**). Whereas BestKeeper algorithm determines the standard deviation (SD) across samples to check the expression stability. A gene with SD more than one is not preferred as a reference gene. The gene with the lowest SD value is considered as the most favourable reference gene. In stem, *IF4a* was the most stable (0.57) followed by *H2A* (0.76) and *GAPDH* (1.12) (Fig. 5a). In case of leaf, *IF4a* was the most stable (SD 0.27) followed by *LECTIN* (0.41) (Fig. 5b).

RefFinder that is an integrated tool of all the algorithms, was also used to define the suitable reference gene. The analysis suggests that *GAPDH* followed by *CYP1* and *EF-1 ALPHA* are comprehensively the most favourable reference gene (Fig. 6a). Delta Ct method also suggests that *GAPDH* followed by *CYP1* and *EF-1 ALPHA* are the most suitable reference gene (Fig. 6b). RefFinder also employed BestKeeper, NormFinder and GeNorm algorithm. According to the analysis BestKeeper algorithm suggested *IF4a*, *EF-1 ALPHA* and *GAPDH* were the most stable gene whereas, Polyubiquitin is the least stable gene, which is in line with independent BestKeeper analysis (Fig. 6c). The NormFinder algorithm indicated *GAPDH* as the most stable followed by *EF-1 ALPHA* and *CYP1* (Fig. 6d). The GeNorm algorithm referred *CYP1* and *GAPDH* as the most stable followed by *EF-1 ALPHA* (Fig. 6e). In summary, *EF-1 ALPHA and GAPDH* were identified common reference genes by all the algorithm analysis.

All the reference genes showed optimal pairwise variation value (V)

Generally, a single gene with high expression stability is used for normalization of gene expression data. However, it is suggested to use two or more genes as reference for accurate and reliable results (Die et al. 2010). The GeNorm algorithm determines the optimal number of reference genes in a given set of samples (Vandesompele et al. 2002). The algorithm estimates normalization factor (NF) for the two genes with highest expression stability (NFn and NFn +

1) and then for other genes by stepwise addition of one after the other (Vn/Vn + 1), towards lower expression stability. According to the principle, pairwise variation (V) values for the reference genes in which the value is lower than 0.15 as to be optimized. In our study, no combination exhibited V value of more than 0.15, rather it was very less than 0.15. Therefore, any of two genes can be used for normalization of gene expression. However, based the GeNorm analysis, either *GAPDH* with *EF-1 ALPHA* or *GAPDH* with *CYP1* can be used in combination for normalization of gene expression analysis in mungbean (Fig S4).

EF-1 ALPHA is the best reference gene for expression analysis of RuBisCO, PAL, CAD and IXR14

To validate the reference gene efficacy, we performed gene expression studies in young leaf (2-week-old), old leaf (4-week-old) and stem (4-week-old) in mungbean genotype (Pusa Vishal, Pusa 1641 and Pusa 1451) (Figure S5a). The RNA integrity was tested on agarose gel and 28S or 18S RNA band was intact and this RNA was used for further analysis (Figure S5b).

We first tested expression of *RuBisCO* which is abundant in leaf tissue and its expression will vary depending on development stage and tissue types (SUZUKI et al. 2009). We normalised the expression of *RuBisCO to EF-1 ALPHA* (most stable), *GAPDH* (medium stable) and *HSP90* (less stable) (Fig. 4). Each panel figure relative fold change expression was normalised with one of the genotypes' tissues that is represented by N. The relative fold change expression for N is approximately 1 for all panel figures. The values are calculated based on this formula - ddCt = ((dCtGene of Interest - dCt Reference gene) – (dCt of normalized gene)). Relative fold change = 2^(-ddCt). Based on this formula, we found that relative fold change expression of *RuBisCO* was higher in 2-week - old leaf and 4-week-old leaf when normalized to *EF-1 ALPHA* and *GAPDH* as compared to 4-week-old stem (Fig. 7). However, the gene expression was similar in 4-week-old leaf and stem when normalized to *HSP90*. This further suggested that *HSP90* may not be an appropriate reference gene for normalization.

The expression of lignin and xylan biosynthetic genes can vary in different tissues. Phenylalanine ammonia lyase (PAL) converts phenylalanine to cinnamic acid which is the first step in lignin biosynthesis (Huang et al. 2010). The expression of *PAL* was higher in the stems of all genotypes as compared to 2-week-old leaf and 4-week-old leaf (Fig. 7d-7f). However, average expression of *PAL* when normalized with *EF-1 ALPHA* and *GAPDH* was similar in all the tissues and genotypes. However, relative fold change expression was comparatively higher in Pusa (P) Vishal and variation was also observed after normalizing with *HSP90* (Fig. 2f). Cinnamyl alcohol dehydrogenase converts phenylalanine derived aldehyde to monolignol which is last step in lignin biosynthesis (Wang et al. 2015). Like *PAL* expression pattern, similar pattern of expression was observed for *CAD* gene expression. Expression was higher in stem tissue as compared to leaf tissue (Fig. 7g-i). In P.Vishal stem tissue, relative fold expression of CAD was more than 400 after normalizing with *HSP90* which was very different from other genotypes and as compared to data normalized with *EF-1 ALPHA* and *GAPDH*. Xylan is one of main component of secondary cell wall which abundant generally in stem tissue of angiosperm plants ((Wierzbicki et al. 2019). Irregular Xylem 14 (*IRX14*) belongs glycosyl transferase 43 (GT43) family and is involved in xylan chain elongation. Therefore, we checked the expression of mungbean *IRX14*. We found that expression in stem tissue was approximately 40–50 times higher than leaf tissue (Fig. 7j-l) after normalizing with all the reference genes (*EF-1 ALPHA, GAPDH* and *HSP90*). However, P.Vishal showed approximately 2000 relative fold change expression after normalization with *HSPP90* which was different from other genotypes and compared to after normalization with *EF-1 ALPHA* and *GAPDH*. All these data further confirmed that *EF-1 ALPHA* and *GAPDH* are better reference genes than *HSP90* which again correlated with our previous data.

Further, we wanted to check the expression of *RuBisCO* under salt and osmotic stress after normalisation with different reference genes. RuBisCO activity is generally reduced under different stress. Therefore, we grew mungbean seedling under salt (100 mM, NaCl) and osmotic (20% PEG4000) for 3 days after growing 2 days on wet filter paper and we found reduction in root length after salt and osmotic stress as compared to normal condition grown seedlings (Fig. 8b, Figure S5a). Also, the cotyledonary leaf turned green to greenish white, suggesting a stress phenotype. Further, RNA samples were intact in all seedlings grown under normal and stress conditions (Figure S6b). The Ct values for all references genes were higher as compared to leaf and stem tissue for all reference genes. Single melt curve and PCR product were observed for *EF-1 ALPHA* (Figure S6c). However, multiple size products were observed after amplification with *GAPDH* (Figure S6d). In fact, we did not see expression of *HSP90* in normal as well as stress condition. Therefore, we normalized the expression of *RuBisCO* with *EF-1 ALPHA*. The expression of *RuBisCO* was significantly lower in Pusa Vishal, Pusa 1641 and Pus1431 for salt-treated as compared to control seedlings (Fig. 8b). Also, the expression of *RuBisCo* was lower in osmotic-treated as compared to control seedlings of Pusa 1641 (Fig. 8b). This further confirmed that *EF-1 ALPHA* can be used for normalizing expression of *RuBisCO* under normal and salt or osmotic stress conditions.

Discussion

The reliability and accuracy of RT-qPCR results are primarily dependent on the normalization of the target gene using suitable reference gene. Therefore, the selection of the suitable reference gene is the prime factor for analysis of target gene expression (Die et al. 2010) ; Ling et al. 2014b). An ideal reference gene should have a constant level of expression pattern among cells of different tissues and experimental conditions such as biotic or abiotic stress and irrespective of experimental parameters.

However, very limited number of reference genes have been identified in mungbean which are either in *Vigna mungo* and Chinese mungbean species (Kundu et al. 2013; Ke et al. 2020; Zhou et al. 2023). Zhou et al. 2023 proposed Tubulin (TUA) as the most stable reference gene under biotic stress and hormone treatment in *Vigna radiata* by RT-qPCR only in one genotype. Ke et al. 2021 identified that ubiquitin-conjugating enzyme was suitable as reference under drought and pathogen infection stress; *elongation factor 1* was the most stable gene under waterlogging; and actin performed the best under saline stress. And this study was performed in Chinese LvFeng 5 (LF5) cultivar. Also, *ACT* and *EF-1 ALPHA* was identified as the most suitable reference during MYMIV stress, while *H2A*, *EF-1 ALPHA* and *ACT* were found to be most suitable in salinity stress experiments and *TUB* and *18S* during drought treatments (Kundu et al. 213). The above study was in *Vigna mungo* which has different phenotypic characteristics than *Vigna radiata*. Also, in all these studies, cell wall-related or biomass-related genes were not tested. Therefore, in this study, we systematically tested several reference genes in different tissue types and under stress or non-stress conditions in different Indian genotypes of mungbean (*Vigna radiata*).

Initially, we screened a total of 15 potential reference genes that had been reported in other crops for gene expression analysis in leaf and stem tissues of mungbean. Several algorithm including, GeNorm, NormFinder, BestKeeper and RefFinder has been used to define the most suitable reference gene. The computed result using geNorm and Normfiner algorithm suggested that *GAPDH* and *EF-1 ALPHA* are the most stable genes. These observations were further corroborated with following studies. The GAPDH have been reported as the most suitable reference gene in peanut and chickpea (Garg et al. 2010; Reddy et al. 2016). The *EF-1 ALPHA* was also reported as the suitable reference gene in many legumes (Garg et al. 2010; Gutierrez et al. 2011). BestKeeper analysis revealed that *IF4a* and *EF-1 ALPHA* are the most suitable candidate reference gene. RefFinder analysis defined *GAPDH, CYP1* and *EF-1 ALPHA* as the most stable reference genes. The Cytochromes P450 (P450s or *CYP*s) are heme-cofactor containing enzyme that, function as monooxygenases. There is no report of using *CYP* as reference gene in legume, but in cereal *CYP* have been used as reference gene (Jaiswal et al. 2019). Therefore, in this study, *CYP* is newly identified as a suitable reference gene in mungbean and the homolog of *CYP* can be tested for suitability in other legume species.

Based on the computed algorithms and analyses, *EF-1 ALPHA* exhibited the lowest Ct value across the tissues, hence the highest expression. Therefore, we chose *EF-1 ALPHA* along with *GAPDH* and *HSP90* for comparison to normalize and validate the gene expression of cell wall and biomass-related genes in mungbean using different tissues and developmental stages under stress and non-stress conditions. *PAL*;, *CAD*, *IRX14* and *RuBisCO* were selected for validation. These genes are involved in cell wall biosynthesis and biomass improvement. The gene expression studies were performed using young leaf (2-week-old), old leaf (4-week-old) and stem (4-week-old) in mungbean genotypes (Pusa Vishal, Pusa 1641 and Pusa 1451). The PAL is involved in the first step of the phenylpropanoid pathway. Thus, it regulates the biosynthesis of polyphenol compounds such as flavonoids, phenylpropanoids, and lignin in plants (Wakabayashi et al. 2012). The expression of *PAL* was much higher in the stems of all genotypes as compared to the 2-week-old leaves and 4-week-old leaves. The relative fold change expression was comparatively higher in the stem of Pusa Vishal, suggesting more lignin deposition in the stem secondary cell wall of Pusa Vishal as compared to other genotypes. The *CAD* is the key enzyme involved in the final step of the phenylpropanoid and lignin pathway. Mungbean leaves are abundant with phenolic compounds as compared to other legumes (Bai et al. 2016; Wang et al. 2021). In our previous study, we found that the lignin content was higher in mungbean leaf as compared to *Populus* stem, Arabidopsis stem and rice leaf (Das 2024). However, in this study, we observed that the expression pattern of *CAD* gene was similar to the *PAL* gene expression. The expression of *CAD* gene was higher in stem tissue as compared to leaf tissue (Fig. 7g-i). The *CAD* gene expression was also higher in P. Vishal stem as compared to other genotypes; this may indicate higher lignin deposition in P. Vishal stem as compared to other genotypes. The *PAL* and *CAD* expression was upregulated in stem as compared to leaf. *IRX14* is involved in xylan chain elongation (Qaseem et al., 2024). In this study, *IRX14* expression was much higher in stem tissue as compared to leaf tissue (Fig. 7j-l). Also its expression was higher in stem of Pusa Vishal as compared to other genotypes, suggesting more developed stem of Pusa Vishal as compared to other genotypes at this stage. *RuBisCO* is the carboxylase of the C3 cycle, where it fixes CO_2 onto a ribulose bisphosphate (RuBP) sugar. This is the central carbon cycle reaction which converts around 100 gigatons of carbon from CO_2 into biomass annually (Prywes et al. 2023). It is a major enzyme, abundant in leaf, contributing towards higher plant biomass production. The carbon fixed through RuBisCO is used for cell wall polysaccharide synthesis (Verbančič et al. 2018). The *RuBisCO* is the prime enzyme that plays the most crucial role in C3 cycle of photosynthesis. The level of expression of this enzyme is higher in leaf as compared to stem. We observed much higher expression of *RuBisCO* in leaf as compared to stem of mungbean (Fig. 7a, 7b, 7c). The expression of *RuBisCO* was also checked under salt and osmotic stress after normalizing with *EF1-APLHA*. The expression of *RuBisCO* was significantly lower in Pusa Vishal, Pusa 1641 and Pusa 1431 in salt-treated seedlings as compared to control seedlings Fig. 8b). The expression of *RuBisCo* was also lower in osmotic-treated seedlings as compared to control seedlings of Pusa 1641 (Fig. 8b). Thus, RuBisCO activity may be reduced under different stress.

Conclusion

For the first time, the *CYP1* is reported as unique reference gene for analysis of gene expression in mungbean and its homolog may be tested in other mungbean species. Extensive analysis of selected reference genes in different genotypes of mungbean under stress and non-stress conditions revealed *EF-1 ALPHA* as the best reference gene, followed by *GAPDH*. Also, *HSP90* should be avoided in normalizing expression of genes tested under normal or stress conditions. as well as. Based on this, we propose *EF-1 ALPHA* as the best-suited and robust reference candidate gene to normalize differential gene expression in different tissue types under stress and non-stress conditions.

Declarations

Data availability

No datasets were generated or analysed during the current study.

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Contributions

SD and PM-AP designed and conceptualized the research. AMS performed experiments in revised version. AG performed most of the experiments. VB assisted in some experiments. AG, SD, and PM-AP performed data analysis. SD and PM-AP wrote the manuscript. VB, HKD, and GPM provided mungbean accession and suggestions during manuscript preparation. All authors have read and agreed to publish the manuscript.

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Ethics declarations

Ethics approval and consent to participate

No specific permit was required for the samples analyzed in this study. The authors comply with relevant institutional, national, and international guidelines and legislation for plant studies.

Consent for publication

Not applicable.

Conflict of statement

Authors declare no conflict of interest

References

1. Artico S, Nardeli SM, Brilhante O et al (2010) Identification and evaluation of new reference genes in Gossypium hirsutumfor accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol 10:49. https://doi.org/10.1186/1471-2229-10-49
2. Bai Y, Xu Y, Chang J et al (2016) Bioactives from stems and leaves of mung beans (Vigna radiata L). J Funct Foods 25:314–322. https://doi.org/10.1016/j.jff.2016.06.009
3. Barakat A, Bagniewska-Zadworna A, Choi A et al (2009) The cinnamyl alcohol dehydrogenase gene family in Populus: phylogeny, organization, and expression. BMC Plant Biol 9:26. https://doi.org/10.1186/1471-2229-9-26
4. Bisht A, Bhalla S, Kumar A et al (2021) Gene expression analysis for selection and validation of suitable housekeeping gene(s) in cadmium exposed pigeonpea plants inoculated with arbuscular mycorrhizae. Plant Physiol Biochem 162:592–602. https://doi.org/10.1016/j.plaphy.2021.03.024
5. Bubner B, Baldwin IT (2004) Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep 23:263–271. https://doi.org/10.1007/s00299-004-0859-y
6. Bustin S (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193. https://doi.org/10.1677/jme.0.0250169
7. Caffall KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344:1879–1900. https://doi.org/10.1016/j.carres.2009.05.021
8. Castro P, Román B, Rubio J, Die JV (2012) Selection of reference genes for expression studies in Cicer arietinum L.: analysis of cyp81E3 gene expression against Ascochyta rabiei. Mol Breeding 29:261–274. https://doi.org/10.1007/s11032-010-9544-8
9. Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6:850–861. https://doi.org/10.1038/nrm1746
10. Das S, BV, DBP, GA, MGP, DHK (2024) and PPAM Combining Fourier-transform infrared spectroscopy and multivariate analysis for chemotyping of cell wall composition in Mungbean (Vigna radiata (L.) Wizcek). Res Sq 12–21
11. Die JV, Román B, Nadal S, González-Verdejo CI (2010) Evaluation of candidate reference genes for expression studies in Pisum sativum under different experimental conditions. Planta 232:145–153. https://doi.org/10.1007/s00425-010-1158-1
12. Dudziak K, Sozoniuk M, Szczerba H et al (2020) Identification of stable reference genes for qPCR studies in common wheat (Triticum aestivum L.) seedlings under short-term drought stress. Plant Methods 16:58. https://doi.org/10.1186/s13007-020-00601-9
13. Farrokhi N, Burton RA, Brownfield L et al (2006) Plant cell wall biosynthesis: genetic, biochemical and functional genomics approaches to the identification of key genes. Plant Biotechnol J 4:145–167. https://doi.org/10.1111/j.1467-7652.2005.00169.x
14. Felagari K, Bahramnejad B, Siosemardeh A et al (2023) A Comparison of the Physiological Traits and Gene Expression of Brassinosteroids Signaling under Drought Conditions in Two Chickpea Cultivars. Agronomy 13:2963. https://doi.org/10.3390/agronomy13122963

15. Ferdous J, Li Y, Reid N et al (2015) Identification of Reference Genes for Quantitative Expression Analysis of MicroRNAs and mRNAs in Barley under Various Stress Conditions. PLoS ONE 10:e0118503. https://doi.org/10.1371/journal.pone.0118503
16. Fornalé S, Capellades M, Encina A et al (2012) Altered Lignin Biosynthesis Improves Cellulosic Bioethanol Production in Transgenic Maize Plants Down-Regulated for Cinnamyl Alcohol Dehydrogenase. Mol Plant 5:817–830. https://doi.org/10.1093/mp/ssr097
17. Galli V, da Silva Messias R, dos, Anjos e Silva SD, Rombaldi CV (2013) Selection of reliable reference genes for quantitative real-time polymerase chain reaction studies in maize grains. Plant Cell Rep 32:1869–1877. https://doi.org/10.1007/s00299-013-1499-x
18. Garg R, Sahoo A, Tyagi AK, Jain M (2010) Validation of internal control genes for quantitative gene expression studies in chickpea (Cicer arietinum L). Biochem Biophys Res Commun 396:283–288. https://doi.org/10.1016/j.bbrc.2010.04.079
19. Garrido J, Aguilar M, Prieto P (2020) Identification and validation of reference genes for qRT-PCR normalization in wheat meiosis. Sci Rep 10:2726. https://doi.org/10.1038/s41598-020-59580-5
20. Gho Y-S, Kim S, Jung K-H (2020) Phenylalanine ammonia-lyase family is closely associated with response to phosphate deficiency in rice. Genes Genomics 42:67–76. https://doi.org/10.1007/s13258-019-00879-7
21. Gu C, Chen S, Liu Z et al (2011) Reference Gene Selection for Quantitative Real-Time PCR in Chrysanthemum Subjected to Biotic and Abiotic Stress. Mol Biotechnol 49:192–197. https://doi.org/10.1007/s12033-011-9394-6
22. Gutierrez N, Giménez MJ, Palomino C, Avila CM (2011) Assessment of candidate reference genes for expression studies in Vicia faba L. by real-time quantitative PCR. Mol Breeding 28:13–24. https://doi.org/10.1007/s11032-010-9456-7
23. Ha CM, Rao X, Saxena G, Dixon RA (2021) Growth–defense trade-offs and yield loss in plants with engineered cell walls. New Phytol 231:60–74. https://doi.org/10.1111/nph.17383
24. Ha J, Lee S-H (2019) Mung Bean (Vigna radiata (L.) R. Wilczek) Breeding. Advances in Plant Breeding Strategies: Legumes. Springer International Publishing, Cham, pp 371–407
25. Hong S-Y, Seo PJ, Yang M-S et al (2008) Exploring valid reference genes for gene expression studies in Brachypodium distachyonby real-time PCR. BMC Plant Biol 8:112. https://doi.org/10.1186/1471-2229-8-112
26. Hu R, Fan C, Li H et al (2009) Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 10:93. https://doi.org/10.1186/1471-2199-10-93
27. Huang J, Gu M, Lai Z et al (2010) Functional Analysis of the Arabidopsis *PAL* Gene Family in Plant Growth, Development, and Response to Environmental Stress. Plant Physiol 153:1526–1538. https://doi.org/10.1104/pp.110.157370
28. Huis R, Hawkins S, Neutelings G (2010) Selection of reference genes for quantitative gene expression normalization in flax (Linum usitatissimum L). BMC Plant Biol 10:71. https://doi.org/10.1186/1471-2229-10-71
29. Jain M (2009) Genome-wide identification of novel internal control genes for normalization of gene expression during various stages of development in rice. Plant Sci 176:702–706. https://doi.org/10.1016/j.plantsci.2009.02.001
30. Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345:646–651. https://doi.org/10.1016/j.bbrc.2006.04.140
31. Jain N, Vergish S, Khurana JP (2018) Validation of house-keeping genes for normalization of gene expression data during diurnal/circadian studies in rice by QRT-PCR. Sci Rep 8:3203. https://doi.org/10.1038/s41598-018-21374-1
32. Jarošová J, Kundu JK (2010) Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. BMC Plant Biol 10:146. https://doi.org/10.1186/1471-2229-10-146
33. Joseph JT, Poolakkalody NJ, Shah JM (2018) Plant reference genes for development and stress response studies. J Biosci 43:173–187. https://doi.org/10.1007/s12038-017-9728-z
34. Kakar K, Wandrey M, Czechowski T et al (2008) A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula. Plant Methods 4:18. https://doi.org/10.1186/1746-4811-4-18
35. Ke XW, Yin LH, Xu J et al (2020) Reference Genes for Quantitative Real-Time PCR Analysis of Gene Expression in Mung Bean under Abiotic Stress and Cercospora canescens Infection. https://doi.org/10.18805/LR-507. LEGUME RESEARCH - AN INTERNATIONAL JOURNAL
36. Kiarash JG, Wilde HD, Amirmahani F et al (2018) Selection and validation of reference genes for normalization of qRT-PCR gene expression in wheat (Triticum durum L.) under drought and salt stresses. J Genet 97:1433–1444. https://doi.org/10.1007/s12041-018-1042-5
37. Kozera B, Rapacz M (2013) Reference genes in real-time PCR. J Appl Genet 54:391–406. https://doi.org/10.1007/s13353-013-0173-x
38. Kumar D, Das PK, Sarmah BK (2018) Reference gene validation for normalization of qRT-PCR assay associated with germination and survival of rice under hypoxic condition. J Appl Genet 59:419–430. https://doi.org/10.1007/s13353-018-0466-1
39. Kumar M, Chauhan AS, Kumar M et al (2019) Transcriptome Sequencing of Chickpea (Cicer arietinum L.) Genotypes for Identification of Drought-Responsive Genes Under Drought Stress Condition. Plant Mol Biol Rep 37:186–203. https://doi.org/10.1007/s11105-019-01147-4
40. Kundu A, Patel A, Pal A (2013) Defining reference genes for qPCR normalization to study biotic and abiotic stress responses in Vigna mungo. Plant Cell Rep 32:1647–1658. https://doi.org/10.1007/s00299-013-1478-2
41. Li Q-F, Sun SSM, Yuan D-Y et al (2010) Validation of Candidate Reference Genes for the Accurate Normalization of Real-Time Quantitative RT-PCR Data in Rice During Seed Development. Plant Mol Biol Rep 28:49–57. https://doi.org/10.1007/s11105-009-0124-1
42. Libault M, Thibivilliers S, Bilgin DD et al (2008) Identification of Four Soybean Reference Genes for Gene Expression Normalization. https://doi.org/10.3835/plantgenome2008.02.0091. Plant Genome 1:

43. Lin F, Jiang L, Liu Y et al (2014a) Genome-wide identification of housekeeping genes in maize. Plant Mol Biol 86:543–554. https://doi.org/10.1007/s11103-014-0246-1
44. Lin Y, Zhang C, Lan H et al (2014b) Validation of Potential Reference Genes for qPCR in Maize across Abiotic Stresses, Hormone Treatments, and Tissue Types. PLoS ONE 9:e95445. https://doi.org/10.1371/journal.pone.0095445
45. Ling H, Wu Q, Guo J et al (2014a) Comprehensive Selection of Reference Genes for Gene Expression Normalization in Sugarcane by Real Time Quantitative RT-PCR. PLoS ONE 9:e97469. https://doi.org/10.1371/journal.pone.0097469
46. Ling H, Wu Q, Guo J et al (2014b) Comprehensive Selection of Reference Genes for Gene Expression Normalization in Sugarcane by Real Time Quantitative RT-PCR. PLoS ONE 9:e97469. https://doi.org/10.1371/journal.pone.0097469
47. Manjunatha PB, Aski MS, Mishra GP et al (2023) Genome-wide association studies for phenological and agronomic traits in mungbean (Vigna radiata L. Wilczek). Front Plant Sci 14. https://doi.org/10.3389/fpls.2023.1209288
48. Manoli A, Sturaro A, Trevisan S et al (2012a) Evaluation of candidate reference genes for qPCR in maize. J Plant Physiol 169:807–815. https://doi.org/10.1016/j.jplph.2012.01.019
49. Manoli A, Sturaro A, Trevisan S et al (2012b) Evaluation of candidate reference genes for qPCR in maize. J Plant Physiol 169:807–815. https://doi.org/10.1016/j.jplph.2012.01.019
50. Mu J, Chen L, Gu Y et al (2019) Genome-wide identification of internal reference genes for normalization of gene expression values during endosperm development in wheat. J Appl Genet 60:233–241. https://doi.org/10.1007/s13353-019-00503-0
51. Nair RM, Pandey AK, War AR et al (2019) Biotic and Abiotic Constraints in Mungbean Production—Progress in Genetic Improvement. Front Plant Sci 10. https://doi.org/10.3389/fpls.2019.01340
52. Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10:11. https://doi.org/10.1186/1471-2199-10-11
53. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47
54. Prywes N, Phillips NR, Tuck OT et al (2023) Rubisco Function, Evolution, and Engineering. Annu Rev Biochem 92:385–410. https://doi.org/10.1146/annurev-biochem-040320-101244
55. Quaseem MF, Zhang W, Dupree P, Wu A (2024) Xylan structural diversity, biosynthesis, and functional regulation in plants. Int J Biol Macromol 291:138866. https://doi.org/10.1016/j.ijbiomac.2024.138866
56. Reddy DS, Bhatnagar-Mathur P, Reddy PS et al (2016) Identification and Validation of Reference Genes and Their Impact on Normalized Gene Expression Studies across Cultivated and Wild Cicer Species. PLoS ONE 11:e0148451. https://doi.org/10.1371/journal.pone.0148451
57. Roorkiwal M, Nayak SN, Thudi M et al (2014) Allele diversity for abiotic stress responsive candidate genes in chickpea reference set using gene based SNP markers. Front Plant Sci 5. https://doi.org/10.3389/fpls.2014.00248
58. Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81. https://doi.org/10.1016/S0165-022X(00)00129-9
59. Scholdberg TA, Norden TD, Nelson DD, Jenkins GR (2009) Evaluating Precision and Accuracy When Quantifying Different Endogenous Control Reference Genes in Maize Using Real-Time PCR. J Agric Food Chem 57:2903–2911. https://doi.org/10.1021/jf803599t
60. Scholtz JJ, Visser B (2013) Reference gene selection for qPCR gene expression analysis of rust-infected wheat. Physiol Mol Plant Pathol 81:22–25. https://doi.org/10.1016/j.pmpp.2012.10.006
61. Shivhare R, Lata C (2016) Selection of suitable reference genes for assessing gene expression in pearl millet under different abiotic stresses and their combinations. Sci Rep 6:23036. https://doi.org/10.1038/srep23036
62. Singh CM, Singh P, Tiwari C et al (2021) Improving Drought Tolerance in Mungbean (Vigna radiata L. Wilczek): Morpho-Physiological, Biochemical and Molecular Perspectives. Agronomy 11:1534. https://doi.org/10.3390/agronomy11081534
63. Sinha P, Saxena RK, Singh VK et al (2015a) Selection and Validation of Housekeeping Genes as Reference for Gene Expression Studies in Pigeonpea (Cajanus cajan) under Heat and Salt Stress Conditions. Front Plant Sci 6. https://doi.org/10.3389/fpls.2015.01071
64. Sinha P, Singh VK, Suryanarayana V et al (2015b) Evaluation and Validation of Housekeeping Genes as Reference for Gene Expression Studies in Pigeonpea (Cajanus cajan) Under Drought Stress Conditions. PLoS ONE 10:e0122847. https://doi.org/10.1371/journal.pone.0122847
65. Sudhakar Reddy P, Srinivas Reddy D, Sivasakthi K et al (2016) Evaluation of Sorghum [Sorghum bicolor (L.)] Reference Genes in Various Tissues and under Abiotic Stress Conditions for Quantitative Real-Time PCR Data Normalization. Front Plant Sci 7. https://doi.org/10.3389/fpls.2016.00529
66. Sun YaLi Sun YaLi ZDZDZLZLXCXCCMCM (2014) Reference gene selection for real-time quantitative PCR in black medic (Medicago lupulina L.) root tissue under copper stress. J Agricultural Biotechnol 22:1223–1231
67. SUZUKI Y, MIYAMOTO T, YOSHIZAWA R et al (2009) Rubisco content and photosynthesis of leaves at different positions in transgenic rice with an overexpression of *RBCS*. Plant Cell Environ 32:417–427. https://doi.org/10.1111/j.1365-3040.2009.01937.x
68. Tenea GN, Peres Bota A, Cordeiro Raposo F, Maquet A (2011) Reference genes for gene expression studies in wheat flag leaves grown under different farming conditions. BMC Res Notes 4:373. https://doi.org/10.1186/1756-0500-4-373
69. Tyagi V, Parihar V, Malik G et al (2020) The DEAD-box RNA helicase eIF4A regulates plant development and interacts with the hnRNP LIF2L1 in Physcomitrella patens. Mol Genet Genomics 295:373–389. https://doi.org/10.1007/s00438-019-01628-x
70. Upadhyaya HD, Dwivedi SL, Baum M et al (2008) Genetic structure, diversity, and allelic richness in composite collection and reference set in chickpea (Cicer arietinum L). BMC Plant Biol 8:106. https://doi.org/10.1186/1471-2229-8-106

71. Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3. https://doi.org/10.1186/gb-2002-3-7-research0034. research0034.1
72. Wakabayashi K, Soga K, Hoson T (2012) Phenylalanine ammonia-lyase and cell wall peroxidase are cooperatively involved in the extensive formation of ferulate network in cell walls of developing rice shoots. J Plant Physiol 169:262–267. https://doi.org/10.1016/j.jplph.2011.10.002
73. Wang F, Huang L, Yuan X et al (2021) Nutritional, phytochemical and antioxidant properties of 24 mung bean (Vigna radiate L.) genotypes. Food Production, Processing and Nutrition 3:28. https://doi.org/10.1186/s43014-021-00073-x
74. Wang M, Li Q, Liu L (2023) Factors and Methods for the Detection of Gene Expression Regulation. Biomolecules 13:304. https://doi.org/10.3390/biom13020304
75. Wang P, Dudareva N, Morgan JA, Chapple C (2015) Genetic manipulation of lignocellulosic biomass for bioenergy. Curr Opin Chem Biol 29:32–39. https://doi.org/10.1016/j.cbpa.2015.08.006
76. Wierzbicki MP, Maloney V, Mizrachi E, Myburg AA (2019) Xylan in the Middle: Understanding Xylan Biosynthesis and Its Metabolic Dependencies Toward Improving Wood Fiber for Industrial Processing. Front Plant Sci 10. https://doi.org/10.3389/fpls.2019.00176
77. Xu F, Deng G, Cheng S et al (2012) Molecular Cloning, Characterization and Expression of the Phenylalanine Ammonia-Lyase Gene from Juglans regia. Molecules 17:7810–7823. https://doi.org/10.3390/molecules17077810
78. Zhou Y, Liu H, Wu T et al (2023) Screening of Reference Genes under Biotic Stress and Hormone Treatment of Mung Bean (Vigna radiata) by Quantitative Real-Time PCR. Genes (Basel) 14:1739. https://doi.org/10.3390/genes14091739

Figures

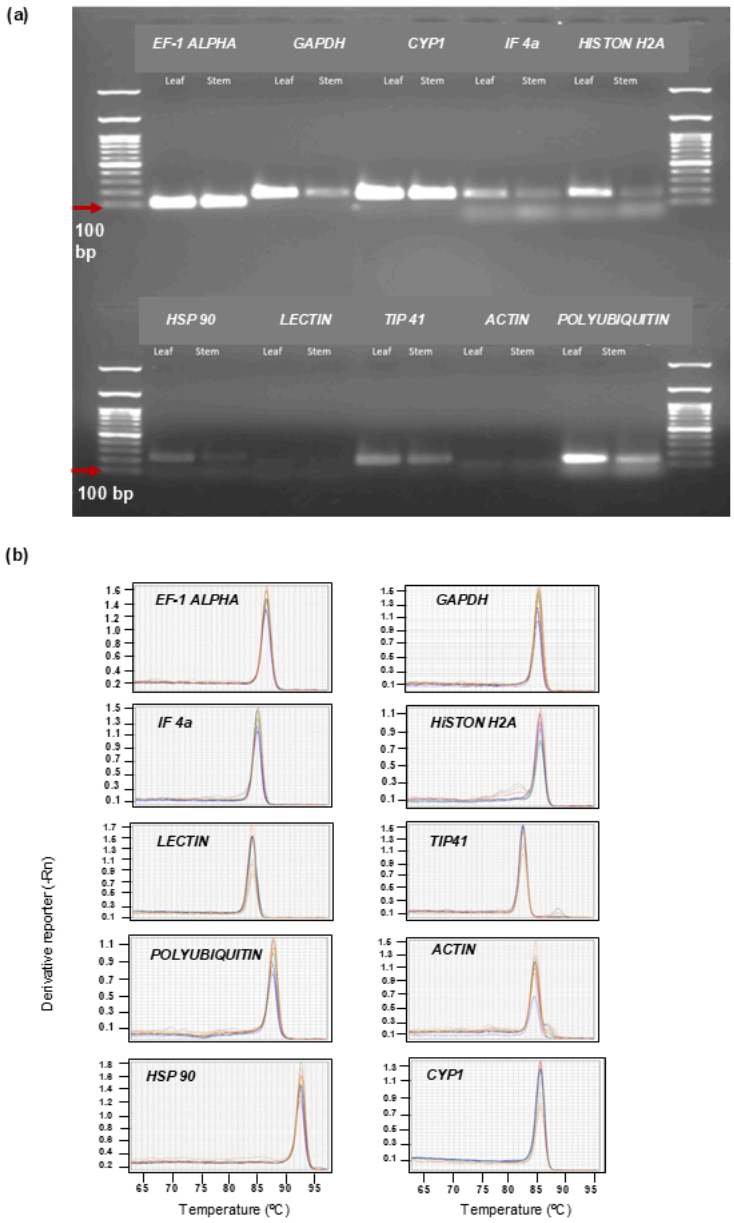


Figure 1

PCR product and melting curve analysis of ten candidate genes (a) PCR product of semi-quantitative PCR for all the ten candidate genes in leaf and stem cDNA (b) melting curve analysis of all the ten candidate genes using RT-qPCR.

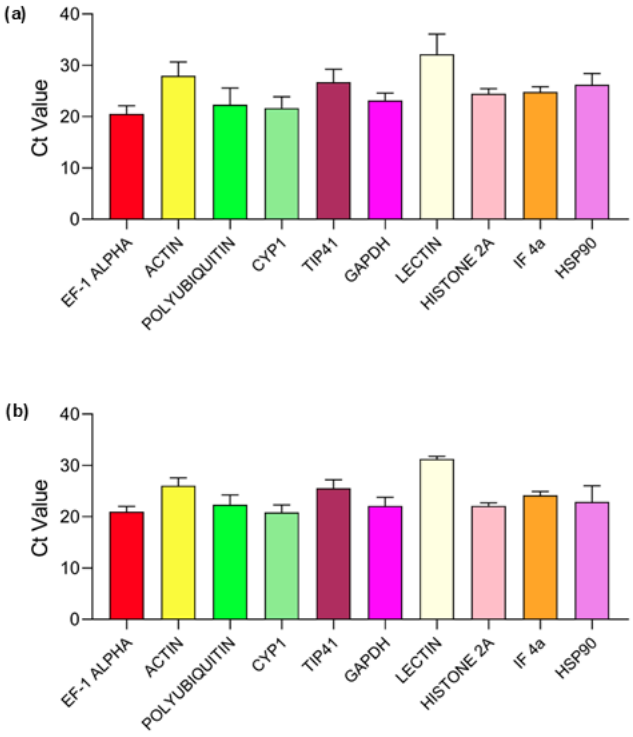


Figure 2. Expression levels of candidate reference genes in mungbean tissue samples. The graphs represent Ct values for each reference gene in **(a)** in stem **(b)** in leaf. The error bar represents mean ± SD from 3 biological replicates.

Figure 2

Expression levels of candidate reference genes in mungbean tissue samples. The Bar graphs represent Ct values for each reference gene in (a) stem and (b) in leaf (b). The average represents Mean ± SD, n = 3 biological replicates.

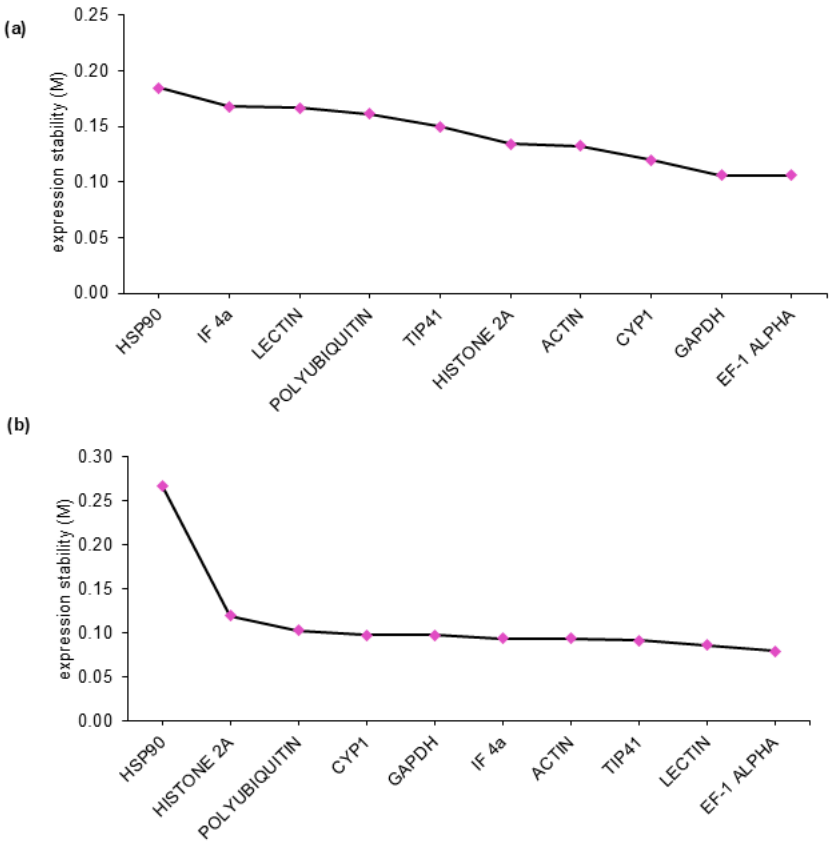


Figure 3

Average expression stability values (M) of Mungbean candidate reference genes. M values of the reference genes were calculated with the geNorm algorithm. Ranking of the stability was performed on (a) stem tissue (b) leaf tissues. The lowest average expression stability value indicates the most stably expressed gene.

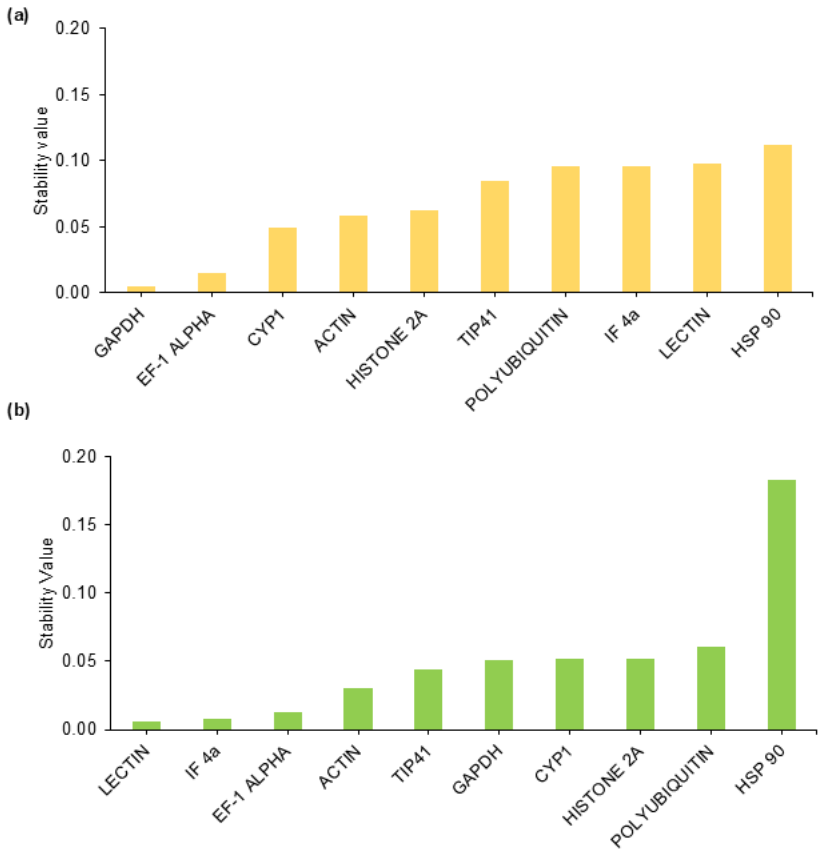


Figure 4

Average expression stability values of Mungbean candidate reference genes. Stability values of the reference genes were calculated with the NormFinder algorithm. Ranking of the stability was performed on (a) stem tissue (b) leaf tissues. The lowest average expression stability value indicates the most stably expressed gene.

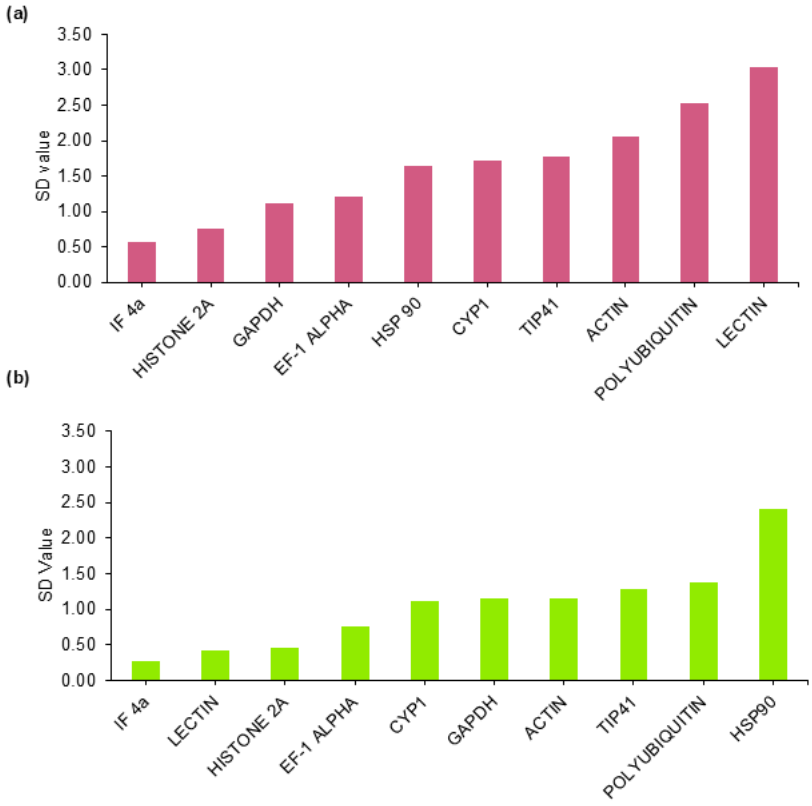


Figure 5

Average expression stability values of Mungbean candidate reference genes. Standard deviation of the reference genes was calculated with the BestKeeper algorithm. Ranking of the stability was performed on (a) stem tissue (b) leaf tissues. The lowest SD value indicates the most stably expressed gene.

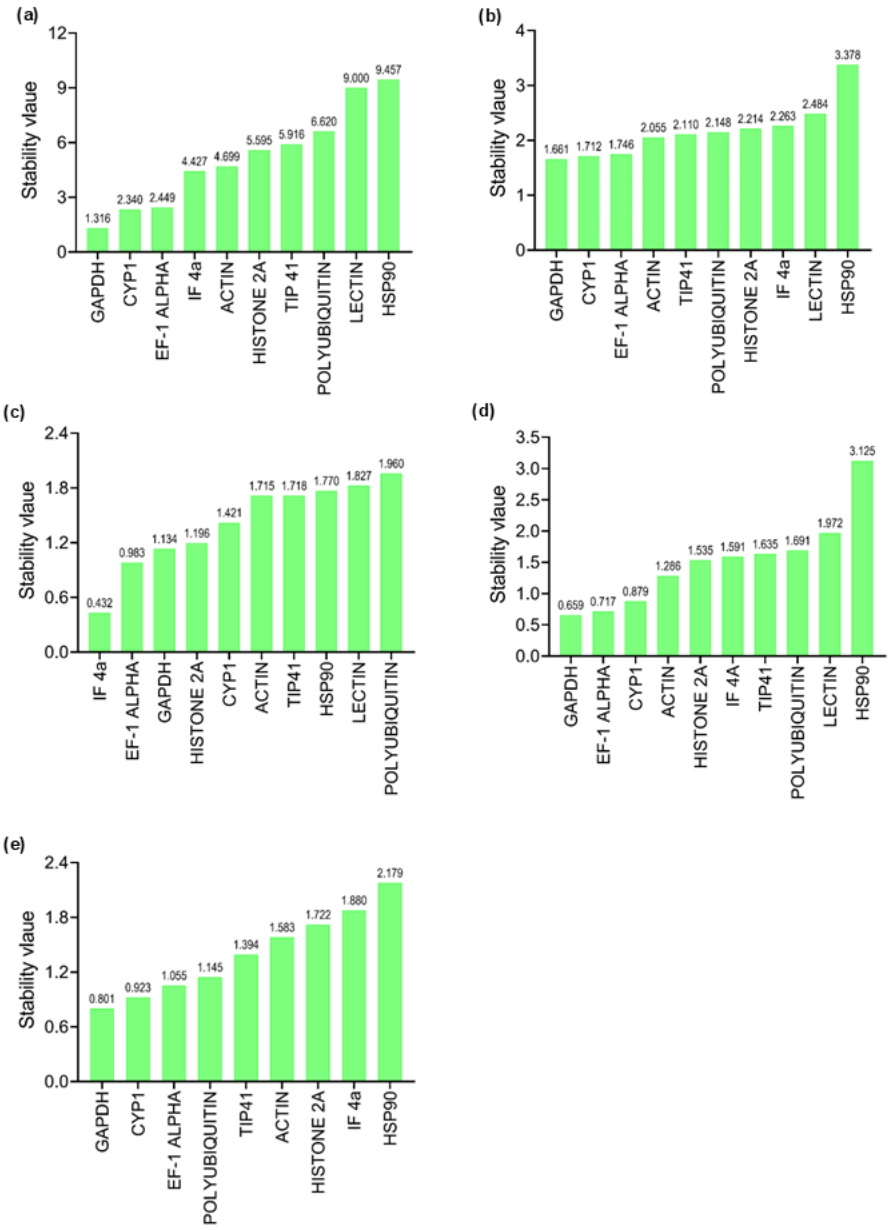


Figure 6

Gene stability analysis of candidate reference genes. The stability value of reference genes was calculated with the RefFinder algorithm. Ranking of the stability was performed on leaf and stem tissues (a) Comprehensive gene stability (b) delta CT method (c) Best Keeper (d) Norm Finder (e) GeNorm. The lowest SD value indicates the most stably expressed gene.

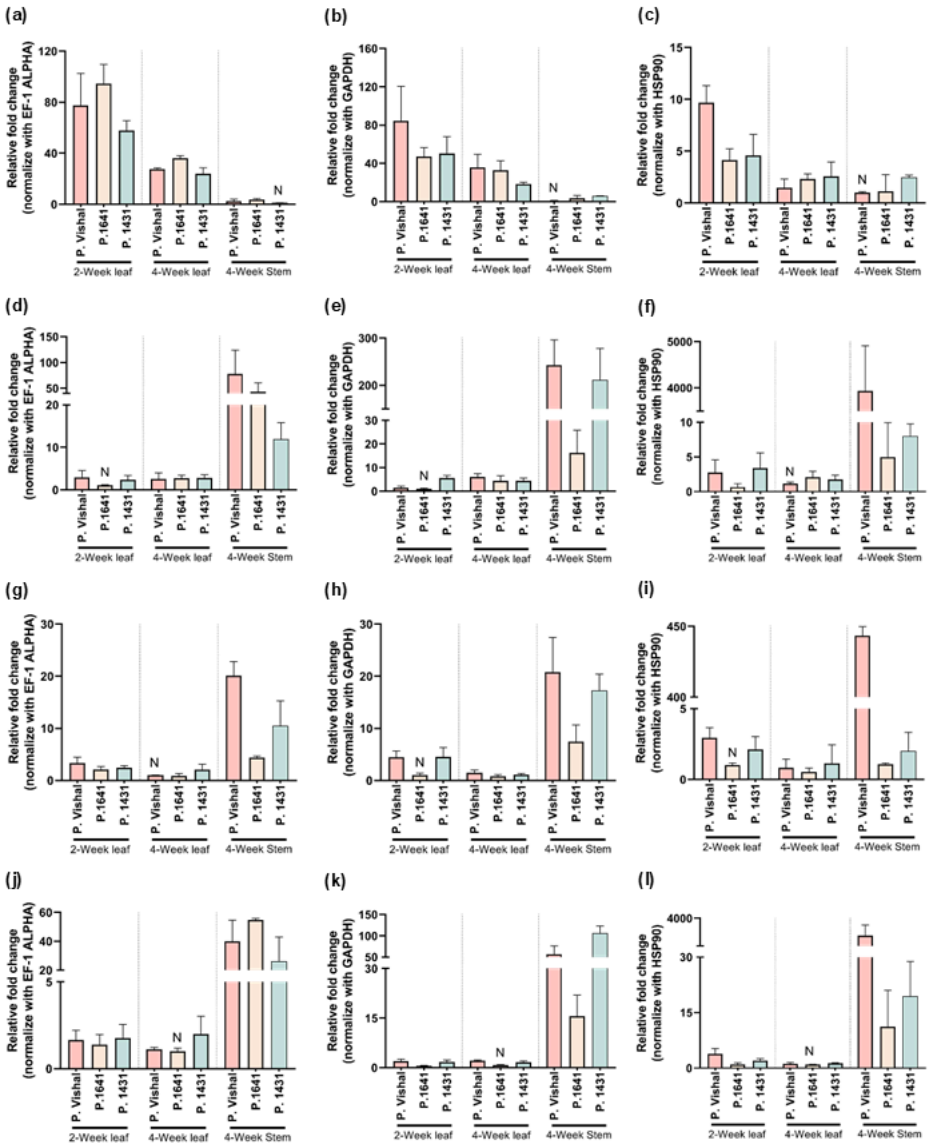


Figure 7

Comparative gene expression analysis (relative fold change) among different mungbean genotypes (Pusa Vishal, Pusa 1641 and Pusa 1431) and tissue types (2-week-old leaf, 4-week-old leaf and 4-week-old stem). P in genotypes corresponds to Pusa. (a), (b) and (c) represent relative gene expression of *Rubisco* (d), (e) and (f) *PAL* (g), (h) and (i) CAD1 (j), (k), (l) IRX8) normalised using *EF-1 ALPHA*, GAPDH and HSP90, respectively as represented on Y-axis. The relative fold change was calculated in each graph by normalising expression using one of genotypes and tissue represented by N. The error bar represents mean ± SD from n =3-4 biological replicates.

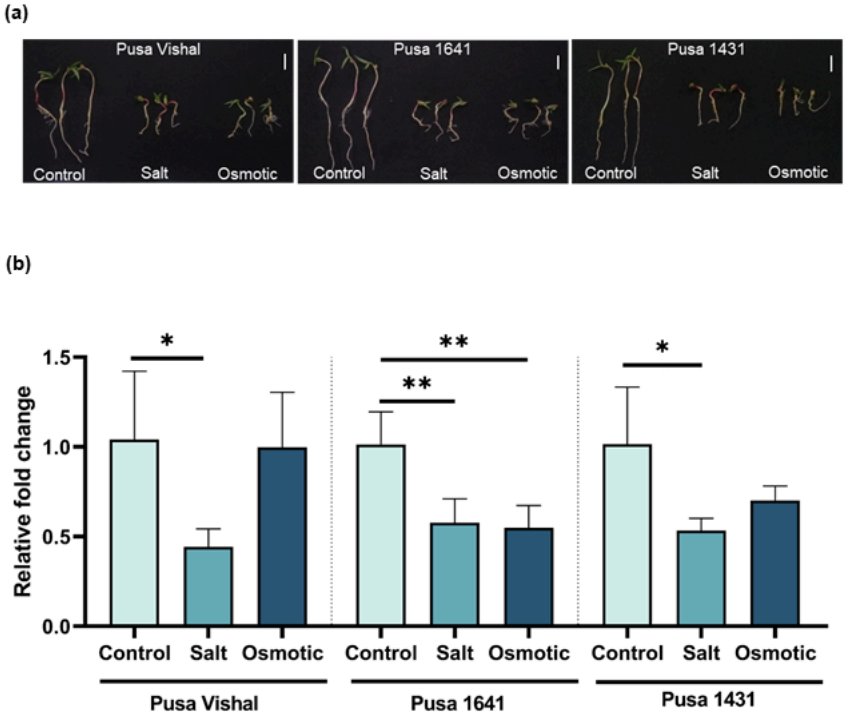


Figure 8

(a) Seedlings picture of 5-day-old mungbean genotypes, i.e., Pusa Vishal, Pusa 1641 and Pusa 1431, grown under control (without treatment), salt (100 mM NaCl) and osmotic (20% PEG4000) stress on germination paper. (b) The relative fold change gene expression of *RuBisCO* using *EF-1 ALPHA* as reference gene and expression normalized to respective control in Pusa Vishal, Pusa 1641 and Pusa 1431 mungbean genotypes. The asterisk represents significant differences as compared to the control using Student's t-test at *p < 0.1, ** p < 0.05, mean ±SD, n =3-4 biological replicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementarytablePMR.xlsx
- SupfigurePMR.pptx