RESEARCH ARTICLE Legume Research- An International Journal, Volume 44 Issue 6: 646-651 (June 2021)

Reference Genes for Quantitative Real-Time PCR Analysis of Gene Expression in Mung Bean under Abiotic Stress and *Cercospora canescens* Infection

X.W. Ke[#], L.H. Yin[#], J. Xu, W.N. Sun, X.D. Xu, Y.X. Guo, Y.H. Zuo

10.18805/LR-507

ABSTRACT

The objective of the study was to identify suitable reference genes that can be used for quantitative real-time PCR (qPCR) analysis in mung bean (*Vigna radiata*). Therefore, 10 potential reference genes were selected and the results showed that *ubiquitin-conjugating enzyme* was suitable as reference under drought and pathogen infection stress; *elongation factor 1-α* was the most stable gene under waterlogging; and *actin* performed the best under saline stress. These selected reference genes were further confirmed by analysis of the expression profiles of *catalase* and *peroxidase* under waterlogging. Our results will contribute to the improvement of the accuracy of gene expression evaluation in mung bean.

Key words: Drought, Fungal infection, qPCR, Saline, *Vigna radiata,* Waterlogging.

INTRODUCTION

Mung bean [*Vigna radiata* (L.) R. Wilczek] is one of the most important pulse crops in South, East and Southeast Asia (Gokulakrishnan *et al*., 2012). Similar to other legumes, mung bean fixes atmospheric nitrogen *via* its roots in symbiosis with *Rhizobium* bacteria to not only meet its own nitrogen needs but to benefit subsequent crops (Zang *et al*., 2015; Mondal *et al*., 2011). However, mung bean is often cultivated on marginal soils with low inputs, where it is subjected to the action of numerous abiotic and biotic stress, including high temperature, waterlogging, drought, salinity and pathogen infection and therefore severely hinders the yield (Hanumantharao *et al*., 2016; Manjunath *et al*., 2013; Duraimurugan and Tyagi, 2014; Dutta and Bera, 2014).

Determination of gene expression alterations during stress induction and recovery can provide important insight into how plants cope with stress factors. Thus, mung bean gene expression studies have profound implications on strategies to improve mung bean production. Gene expression analysis using qPCR is a widely used method due to its sensitivity, specificity, dynamic range and high throughput capacity (Wong and Medrano, 2005). However, qPCR analysis requires a suitable reference gene whose expression level remains stable under both control and experimental conditions (Guénin *et al*., 2009). Commonly used reference genes are housekeeping genes, such as actin (*ACT*), beta-tubulin (*β-TUB*), polyubiquitin (*UBQ*), glyceraldehyde-3-phoshate dehydrogenase (*GAPDH*), tonoplast intrinsic protein 41 (*TIP41*), alpha-tubulin (*α-TUB*), ubiquitin-conjugating enzyme (*UBC*), adenine phosphoribosyl transferase (*APT*), 60S ribosomal protein (*60S*) and the elongation factor (*EF*) (Chen *et al*., 2017; Li *et al*., 2017). However, no reference genes are universally applicable across different species and all experimental conditions. It is, therefore, crucial to verify the expression stability of putative normalizers in each set of experimental conditions, as the selection of a stably expressed reference gene is critically important for the accuracy of the results. To date, appropriate reference genes have not been reported for mung bean. Therefore, in order to identify suitable reference genes that could be used for qPCR analysis in mung bean, 10 potential reference genes (*EF1α*, *ACT*, *UBQ*, *GAPDH*, *TIP41*, *α-TUB*, *β-TUB*, *UBC*, *APT* and *60S*) were selected and analyzed in a set of 24 samples. The results of this study should provide useful guidelines for further gene expression analyses in mung bean.

Heilongjiang Bayi Agricultural University, National Coarse Cereals Engineering Research Center, Heilongjiang Provincial Key Laboratory of Crop-Pest Interaction Biology and Ecological Control, Daqing 163319, China.

[#] These two authors contributed equally to this work.

Corresponding Author: Y.H. Zuo, Heilongjiang Bayi Agricultural University, National Coarse Cereals Engineering Research Center, Heilongjiang Provincial Key Laboratory of Crop-Pest Interaction Biology and Ecological Control, Daqing 163319, China. Email: zuoyhu@163.com

How to cite this article: Ke, X.W. , Yin, L.H., Xu, J., Sun, W.N., Xu, X.D., Guo, Y.X. and Zuo, Y.H. (2021). Reference Genes for Quantitative Real-Time PCR Analysis of Gene Expression in Mung Bean under Abiotic Stress and *Cercospora canescens* Infection. Legume Research. 44(6): 646-651. DOI: 10.18805/LR-507.

Submitted: 13-06-2019 **Accepted:** 29-12-2019 **Online:** 15-05-2020

MATERIALS AND METHODS

The experiment was conducted in 2017-05 to 2018-03 at Heilongjiang Bayi Agricultural University, China. Mung bean

cultivar LvFeng 5 (LF5) used in this study were obtained from National Coarse Cereals Engineering Research Center, Daqing, China. The plants were grown in a growth chamber at 24°C - 27°C under short-day conditions (8 h light/16 h dark, 150 $\mu molm^{-2}s^{-1}$). Samples for drought stress was performed on 10-day old seedlings by withholding water (Nasrollahi *et al*., 2014) and euphylla were sampled at 0, 1, 3, 6, 9 and 12 d after treatment. For waterlogging, the potted 10-day old seedlings were placed into larger tanks and the soil surface was submerged approximately 2 cm below the surface of the water (Parelle *et al*., 2006) and samples were collected at 0, 1, 2, 5, 10 and 15 d after treatment. For salinity stress, 10-day old seedlings potted with river sand were treated with 100 mM neutral and alkali salts (NaCl: Na_2SO_4: $NaHCO_3$: Na_2CO_3 = 3:3:5:1) (Zhang *et al*., 2010) and samples were collected at 0 h, 12 h, 24 h, 48 h, 72 h and 144 h after treatment. For biotic stress, the euphylla were inoculated with conidia suspension of *Cercospora canescens* (Booker and Umaharan, 2007). Leaves were harvested at 0, 1, 3, 6, 10 and 15 d post-inoculation (dpi). Each sample with three biological replicates were used for RNA extraction.

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA) following the manufacturer instructions and 1 µg of total RNA was used for cDNA synthesis using a PrimeScript™ RT Reagent Kit following the instructions (TaKaRa, China). The final cDNA products were diluted five folds prior to use in qPCR. Primers of the ten candidate genes were designed based on the sequences obtained from mung bean genome (Kang *et al*., 2014) using a web based PrimerQuest Tool (https://sg.idtdna.com/Primerquest/Home/Index) and listed in Table 1.

qPCR was performed in a Bio-Rad CFX96 PCR system using SYBR Premix EX Taq (TaKaRa, China) following the manufacturer instructions. The PCR conditions were as follows: 95°C for 1 min, 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s; then, the temperature was increased by 0.3°C every 10 s to obtain the product melt curve. All qPCR assays were performed in three technical replicates. Amplification efficiency (E) and correlation coefficient (R^2) were tested by a standard curve based on the diluted (4×) cDNA series. The value of E was estimated by using the slope of the standard curve and the equation $E=(10^{-1/slope}-1)\times100\%$ (Li *et al*., 2012).

Three programs geNorm (Vandesompele *et al*., 2002), NormFinder (Andersen *et al*., 2004) and BestKeeper (Pfaffl *et al*., 2004) were used to evaluate the expression stability of the 10 candidate genes. And the data generated by the three algorithms were further compared using the web-based comprehensive tool RefFinder (Xie *et al*., 2012).

To validate reliability of the reference genes obtained in this study, the expression level of catalase (*CAT*) and peroxidase (*POD*) genes of cv. LF5 under waterlogging were normalized against the most stable, the least stable and the most stable combination of genes. The amount of transcripts accumulated for the two genes normalized using these different reference genes were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three technical and biological replicates were performed for all analyses in this study.

RESULTS AND DISCUSSION

Primer specificity and efficiency

The amplification specificity of the primers was confirmed by a single peak in the melting curve (Fig 1). According to the slope of the standard curve, the amplification efficiency of all primers ranged between 85% (*UBQ*) and 110% (*APT*) with regression coefficient (R^2) varied from 0.991 to 0.999 (Table 1). Auler et al. (2017) mentioned that the amplification efficiency between 80% and 120% were considered acceptable, indicative of a reasonable amplification efficiency of the primers used in current study.

Ct value distribution

To investigate the expression stability of the 10 candidate reference genes, the expression levels of these genes in all samples tested in this study were confirmed by their cycle threshold (Ct) values. The Ct values of the 10 genes varied from 19 to 36 in all samples and most of them were between 20 and 25 (Fig 2). *EF1α* was the most highly expressed gene with lowest Ct values (from 19 to 22), while *APT* showed

Table 1: Reference genes used for gene expression normalization in mung bean.

Gene name	Accession number	Primer sequence (5′-3′) forward / reverse	Amplicon size (bp)	E (%)	R^2
EF1α	XM_014656474.1	CTAACTTCACCTCCCAGGTTATC / CAGCGAACTTGACAGCAATG	108	96	0.999
ACT	XM_014638363.1	AGGCTGTTCTGTCCTTGTATG / GAAGAGCGTAGCCCTCATAAAT	105	93	0.991
UBQ	XM_014636394.1	GAAGGAGTCAACTCTCCACTTG / ACTCTCCACCTCAAGGGTAATA	100	85	0.999
GAPDH	XM_014634520.1	CACTTCACCACCTCACTCATAC / CACCAAACGGCCAATTCTTC	137	107	0.996
TIP41	XM_014655703.1	GCATCCAAAGAGCCCATACT / CCAGAATCGCAACAGAAGAAAC	135	92	0.996
α-TUB	XM_014646147.1	CTGGTATGTGGGTGAAGGTATG / GCCAACCTCCTCGTAATCTTT	91	94	0.997
β-TUB	XM_014634642.1	CTGGTGAGGGAATGGATGAAA / TCATCAGCAGTAGCATCTTGG	100	97	0.986
UBC	XM_014634994.1	ATCACATTGCCAGAGGGATAC / CTTTCAAGATGTCCAGGCAAATAG	115	86	0.996
APT	XM_014644257.1	TGGTGCTCAAGTAGTGGAATGGCG / GCTCAACAAGAACATAAAG	101	110	0.992
60S	XM_014634336.1	GAAGAAGCGTAAGAGGGAAGAG / CGTACTGTTGGGCTCTGTTATAG	116	88	0.997

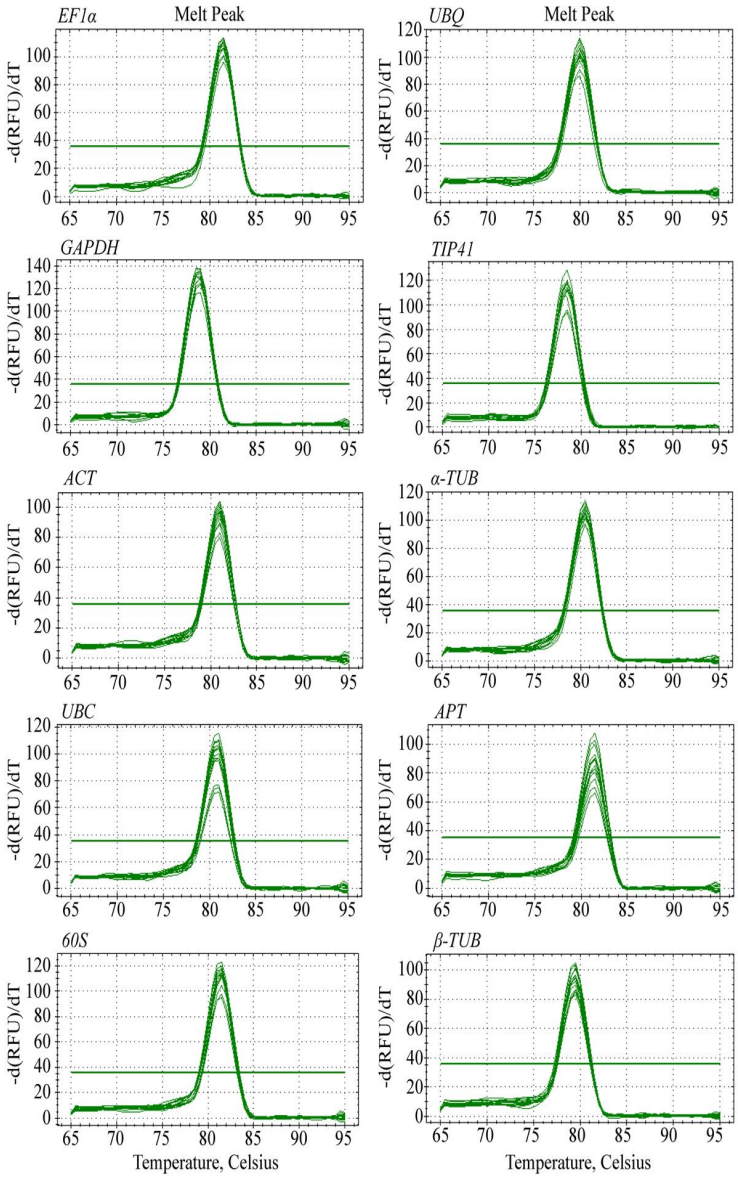

Fig 1: Melting curves of the candidate reference genes. A single peak indicated the specificity of primers.

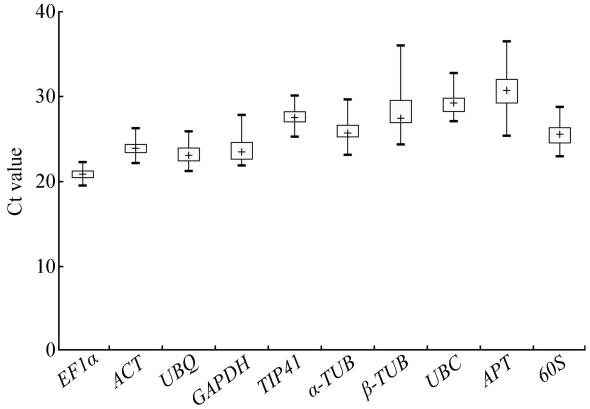

Fig 2: Expression levels of 10 candidate reference genes across all experimental samples. The box graph indicates the interquartile range, the median and maximum/minimum values.

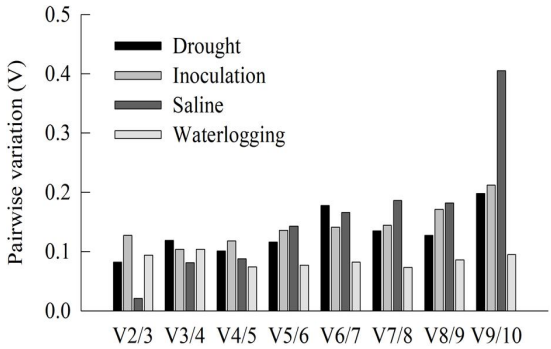

Fig 3: Pairwise variation analyses of the candidate reference genes. The pairwise variation (Vn/Vn+1) was analyzed between the normalization factors NF_n and NF_{n+1} by the geNorm program to determine the optimal number of reference genes required for qPCR data normalization.

Table 2: Gene expression stability ranked by geNorm, NormFinder, BestKeeper and RefFinder.

Group	Rank	geNorm		NormFinder		BestKeeper			RefFinder	
		Gene	Stability	Gene	Stability	Gene	SD(±Ct)*	CV(%Ct)**	Gene	Stability
Drought	1	*UBQ*	0.09	*60S*	0.18	*ACT*	0.37	1.59	*UBC*	2.21
	2	*UBC*	0.09	*EF1α*	0.23	*TIP41*	0.50	1.83	*EF1α*	2.34
	3	*EF1α*	0.20	*TIP41*	0.23	*UBQ*	0.68	2.40	*TIP41*	2.91
	4	*TIP41*	0.34	*UBC*	0.29	*UBC*	0.68	2.77	*UBQ*	3.13
	5	*60S*	0.42	*ACT*	0.34	*EF1α*	0.70	3.36	*60S*	3.64
	6	*ACT*	0.53	*UBQ*	0.35	*β-TUB*	0.82	3.18	*ACT*	3.66
	7	*α-TUB*	0.75	*APT*	0.72	*60S*	0.86	3.58	*α-TUB*	7.48
	8	*APT*	0.85	*α-TUB*	0.74	*α-TUB*	0.97	3.93	*APT*	7.97
	9	*β-TUB*	0.93	*β-TUB*	0.90	*APT*	1.10	3.71	*β-TUB*	8.13
	10	*GAPDH*	1.15	*GAPDH*	1.36	*GAPDH*	1.80	7.07	*GAPDH*	10.00
Waterlogging	1	*α-TUB*	0.19	*UBC*	0.08	*EF1α*	0.36	1.72	*EF1α*	1.57
	2	*UBC*	0.19	*EF1α*	0.08	*ACT*	0.37	1.55	*UBC*	1.68
	3	*EF1α*	0.26	*α-TUB*	0.20	*GAPDH*	0.44	1.94	*α-TUB*	2.82
	4	*GAPDH*	0.34	*GAPDH*	0.21	*UBC*	0.49	1.64	*GAPDH*	3.94
	5	*TIP41*	0.38	*TIP41*	0.24	*β-TUB*	0.53	1.82	*TIP41*	5.32
	6	*UBQ*	0.43	*UBQ*	0.25	*UBQ*	0.56	2.41	*UBQ*	6.00
	7	*60S*	0.48	*60S*	0.43	*α-TUB*	0.56	2.16	*ACT*	6.69
	8	*APT*	0.52	*β-TUB*	0.48	*TIP41*	0.65	2.26	*60S*	7.45
	9	*β-TUB*	0.59	*APT*	0.49	*60S*	0.77	2.84	*β-TUB*	7.54
	10	*ACT*	0.67	*ACT*	0.65	*APT*	0.82	2.57	*APT*	8.71
Inoculation	1	*UBQ*	0.37	*UBC*	0.13	*EF1α*	0.62	2.93	*UBC*	1.57
	2	*UBC*	0.37	*UBQ*	0.13	*UBQ*	0.69	3.05	*UBQ*	1.68
	3	*TIP41*	0.40	*ACT*	0.19	*60S*	0.79	3.17	*TIP41*	3.66
	4	*60S*	0.44	*TIP41*	0.30	*β-TUB*	0.79	2.92	*EF1α*	3.98
	5	*ACT*	0.51	*60S*	0.41	*TIP41*	0.87	3.23	*60S*	4.16
	6	*GAPDH*	0.63	*EF1α*	0.60	*UBC*	0.93	3.26	*ACT*	4.53
	7	*EF1α*	0.75	*GAPDH*	0.74	*ACT*	1.11	4.54	*GAPDH*	7.17
	8	*APT*	0.87	*APT*	0.90	*APT*	1.41	5.00	*β-TUB*	7.35
	9	*β-TUB*	1.03	*β-TUB*	0.93	*GAPDH*	1.42	5.82	*APT*	8.00
	10	*α-TUB*	1.27	*α-TUB*	1.46	*α-TUB*	1.95	7.07	*α-TUB*	10.00
Saline	1	*ACT*	0.01	*TIP41*	0.00	*β-TUB*	0.32	0.89	*ACT*	1.41
	2	*TIP41*	0.01	*ACT*	0.00	*EF1α*	0.38	1.89	*TIP41*	1.86
	3	*UBQ*	0.05	UBQ	0.02	*TIP41*	0.42	1.59	*UBQ*	3.41
	4	*60S*	0.19	60S	0.10	*ACT*	0.43	1.89	*EF1α*	3.76
	5	*EF1α*	0.29	*EF1α*	0.15	*UBQ*	0.48	2.19	*β-TUB*	3.83
	6	*β-TUB*	0.49	*β-TUB*	0.54	*α-TUB*	0.52	1.92	*60S*	4.86
	7	*α-TUB*	0.69	*UBC*	0.83	*60S*	0.62	2.39	*α-TUB*	6.96
	8	*UBC*	0.90	*α-TUB*	0.99	*GAPDH*	1.13	4.69	*UBC*	7.97
	9	*GAPDH*	1.08	*GAPDH*	1.39	*UBC*	1.32	4.30	*GAPDH*	8.74
	10	*APT*	1.68	*APT*	2.80	*APT*	2.86	8.82	*APT*	10.00

* SD[±Ct]: standard deviation of the Ct; ** CV[%Ct]: coefficient of variance expressed as a percentage of the Ct level.

greatest difference range with Ct values from 25 to 36 indicating an unstable expression in different conditions.

Gene expression stability

Under drought stress, *UBQ*, *60S* and *ACT* were the most stable genes according to geNorm, NormFinder and BestKeeper, respectively (Table 2). However comprehensive ranking of genes by RefFinder showed that *UBC* was the most stable gene.

Under waterlogging (Table 2), the stability ranking established by geNorm, NormFinder and BestKeeper showed that *α-TUB*, *UBC* and *EF1α* were the most stable genes, respectively. However, comprehensive ranking of genes by RefFinder indicated that the best reference gene was *EF1α*.

For *C. canescens* infection (Table 2), *UBQ*, *UBC* and *EF1α* was ranked highest by geNorm, NormFinder and BestKeeper, respectively. And further comprehensive

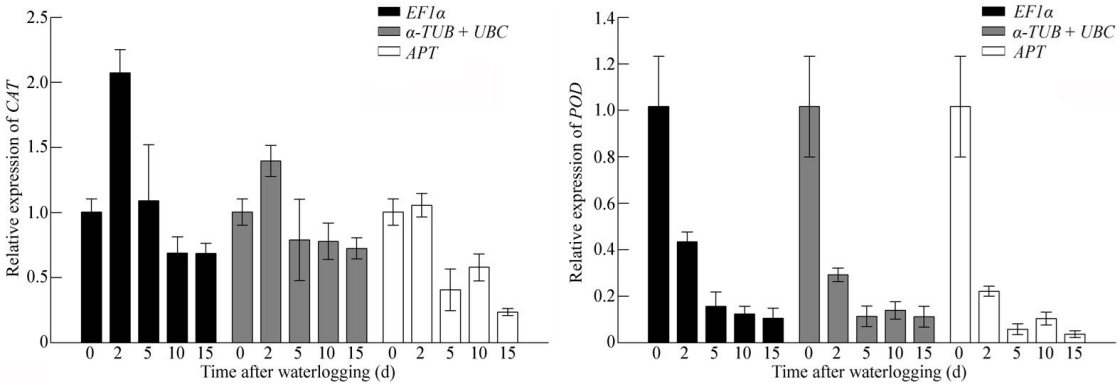

Fig 4: Relative quantification of *CAT* and *POD* expression after waterlogging by using the validated reference gene(s).

comparison by RefFinder demonstrated that *UBC* was the most stable gene.

According to the geNorm, NormFinder and BestKeeper, *ACT*, *TIP41* and *β-TUB* were the most stable genes under saline treatment, respectively, whereas the comprehensive ranking obtained from RefFinder revealed that *ACT* was the optimal reference gene (Table 2).

The discrepancies in these results from different programs were most likely due to the use of different algorithms. NormFinder and geNorm both select optimal internal reference genes based on gene expression stability (M value), while BestKeeper assesses stability based on correlation coefficient, standard deviation and coefficient of variation (Pfaffl *et al*., 2004). Therefore, a web tool RefFinder was used for overall final rankings by measuring the geometric mean of the attributed weights, which has been widely used to evaluate suitable reference genes (Duan *et al*., 2017; Walling *et al*., 2018).

The best pair of reference genes

Pairwise variation (V_n/V_{n+1}) with a threshold 0.15 was used to determine the optimal pair of reference genes by using geNorm (Fig 3). A $V_{2/3}$ = 0.082 was obtained for the pair of *UBQ* and *UBC* under drought stress and the pair of *α-TUB* and *UBC* were determined to be the optimal normalization factors for waterlogging. Yet in fungal infection, the combination of *UBQ* and *UBC* yielded a $V_{2/3}$ = 0.127. According to the $V_{2/3}$ value (0.021), *ACT* combined with *TIP41* was the best pair of genes for salinity stress.

Reference gene validation

To validate the selected reference genes, the expression levels of catalase (*CAT*) and peroxidase (*POD*) under waterlogging were analyzed by qPCR (Fig 3). The relative expression levels of *CAT* and *POD* were not significantly different when normalized by *EF1α* or the combination of*α-TUB* and *UBC*, while the transcript levels were underestimated by using the least stable gene *APT*. These results further confirmed the importance of the appropriate choice of reference gene for normalization of the target gene expression levels.

CONCLUSION

In summary, we identified stable reference genes in mung bean using a set of different experimental stress conditions. *UBC* is an optimal reference gene under drought and inoculation conditions. In the waterlogging treatment, *EF1α* was the most stable reference gene and under saline stress, *ACT* is the best reference gene. These results will provide a basis for accurate gene expression evaluation in mung bean in future research.

ACKNOWLEDGEMENT

The authors would like to acknowledge Dr Yichao Huang (Jinan University, Guangzhou) for assisting in manuscript preparation. This work was supported by University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2016201 and UNPYSCT-2017113), Heilongjiang Bayi Agricultural University Support Program for San Heng San Zong (ZRCQC201901), Research and Development Plan of Applied Technology in Heilongjiang Province (GA19B104) and the Coarse Cereals Specialty Discipline Construction Project.

REFERENCES

Andersen, C.L., Jensen, J.L., Orntoft, T.F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research. 64: 5245-5250.

Auler, P.A., Benitez, L.C., do Amaral, M.N., Vighi, I.L., Dos Santos Rodrigues, G, da Maia, L.C., Braga, E.J. (2017). Evaluation of stability and validation of reference genes for RT-qPCR expression studies in rice plants under water deficit. Journal of Applied Genetics. 58(2): 163-177.

Booker, H.M. and Umaharan, P. (2007). Identification of resistance to Cercospora leaf spot of cowpea. European Journal of Plant Pathology. 118: 401-410.

Chen, X., Mao, Y., Huang, S., Ni, J., Lu, W., Hou, J. (2017). Selection of suitable reference genes for quantitative real-time PCR in *Sapium sebiferum*. Frontiers in Plant Science. 8: 637.

Duan, M., Wang, J., Zhang, X., Yang, H., Wang, H., Qiu, Y., Song, J., Guo, Y., Li, X. (2017). Identification of optimal reference genes for expression analysis in radish (*Raphanus sativus* L.) and its relatives based on expression stability. Frontiers in Plant Science. 8: 1605.

Duraimurugan, P. and Tyagi, K. (2014). Pest spectra, succession and its yield losses in mungbean and urdbean underchanging climatic scenario. Legume Research. 37(2): 212-222.

Dutta, P. and Bera, A. K. (2014). Effect of NaCl salinity on seed germination and seedling growth of mungbean cultivars. Legume Research. 37(2): 161-164.

Gokulakrishnan, J., Kumar, B. S., Prakash, M. (2012). Studies on genetic diversity in mung bean (*Vigna Radiata* L.). Legume Research. 35(1): 50-52.

Guénin, S., Mauriat, M., Pelloux, J., Wuytswinkel, O.V., Bellini, C., Gutierrez, L. (2009). Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions -specific, validation of references. Journal of Experimental Botany. 60: 48.

Hanumantharao, B., Nair, R.M., Nayyar, H. (2016). Salinity and high temperature tolerance in mungbean [*Vigna radiata* (L.) Wilczek] from a physiological perspective. Frontiers in Plant Science. 7: 957.

Kang, Y.J., Kim, S.K., Kim, M.Y., Lestari, P., Kim, K.H., Ha, B.,Jun, T.H., *et al.* (2014). Genome sequence of mungbean and insights into evolution within *Vigna* species. Nature Communications. 5: 5443.

Li, J., Han, X., Wang, C., Qi, W., Zhang, W., Tang, L. Zhao, X. (2017). Validation of suitable reference genes for RT-qPCR data in *Achyranthes bidentata* blume under different experimental conditions. Frontiers in Plant Science. 8: 776.

Li, Q., Jeff, S. and John, E.B. (2012). Evaluation of Reference Genes for Real-Time Quantitative PCR Studies in Candida Glabrata Following Azole Treatment. BMC Molecular Biology. 13 (1): 22.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25: 402-408.

Manjunath, B., Jayaram, N., Muniyappa, V., Prameela, H. (2013). Status of yellow mosaic virus and whitefly *Bemisia tabaci* biotypes on mungbean in southern Karnataka. Legume Research. 36(1): 62-66.

Mondal, M.M.A., Rahman, M.A., Akter, M.B., Fakir, M.S.A. (2011). Effect of foliar application of nitrogen and micronutrients on growth and yield in mungbean. Legume Research. 34(3): 166-171.

Nasrollahi, V., Asghar, M., Khosro, P., Sonbol, N., Rahim, M. (2014). The effect of drought stress on the expression of key genes involved in the biosynthesis of triterpenoid in liquorice (*Glycyrrhiza glabra*). Phytochemistry. 103: 32-37.

Parelle, J., Brendel, O., Catherine B., Berveiller, D., Dizengremel, P., Jolivet, Y. (2006). Differences in morphological and physiological responses to water-logging between two sympatric oak species [*Quercus petraea* (Matt.) Liebl. *Quercus robur* L.). Annals of Forest Science. 63(8): 849-859.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotech--nology Letters. 26: 509-515.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology. 3(7): research0034.1.

Walling J.G., Zalapa L.A., Vinje M.A. (2018). Evaluation and selection of internal reference genes from two- and six-row U.S. malting barley varieties throughout micromalt ing for use in RT-qPCR. PloS ONE. 13(5): e0196966.

Wong, M.L. and Medrano, J.F. (2005). Real-time PCR for mRNA quantitation. BioTechniques. 39: 75-85.

Xie, F., Xiao, P., Chen, D., Xu, L., Zhang, B. (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Molecular Biology. 80: 75-84.

Zang, H., Yang, X., Feng, X., Qian, X., Hu, Y., Ren, C., Zeng, Z. (2015). Rhizodeposition of nitrogen and carbon by mungbean (*Vigna radiata* L.) and its contribution to intercropped oats (*Avena nuda* L.). PLoS ONE. 10(3): e0121132.

Zhang, J., Chen L., Qiao L., Huang L., Li M. (2010). Characteristics and evaluation of salinization of different soil types in daqing city. Journal of Northeast Forestry University. 38(7): 119-122.