Validation of reference genes for qRT-PCR data normalisation in lentil (*Lens culinaris*) under leaf developmental stages and abiotic stresses

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Received: 2 August 2018 / Revised: 4 September 2018 / Accepted: 24 September 2018 / Published online: 28 September 2018
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Abstract Lentil (*Lens culinaris*) is one of the most important staple food crops of developing countries. Transcriptome based global gene expression profiling followed by validation of expression of important genes through quantitative real time-PCR (qRT-PCR) has achieved significance in recent years. However, there is a severe scarcity of information regarding stable reference genes in lentil, which is mandatory for qRT-PCR data normalisation. Hence, the present study was under-taken to identify the most stable reference gene(s) in lentil. Expression stability of eight candidate genes viz. ribulose 1,5-bisphosphate carboxylase large subunit (Rbcl), ribosomal protein L2 (RPL2), 18S rRNA, tubulin (Tub), elongation factor 1 α (EF1 α), glyceraldehydes-3-phosphate dehydrogenase (GAPDH), heat shock protein (HSP70), and Maturase (mat K) was evaluated in five varieties of lentil at three different stages of leaf development and abiotic stress conditions using qRT-PCR. The results were analysed using four types of statistical software viz., geNorm, BestKeeper, NormFinder and RefFinder; all softwares identified RPL2 as most stable under abiotic stress conditions and developmental stages followed by Tub and Rbcl; while, HSP70 was identified as least stable. Relative expression of the target genes, defensin and PR4, was evaluated under abiotic stress conditions and data normalisation was done using two stable reference genes,

RPL2 and Tub, either alone or in combination and with two least stable genes, HSP70 and 18S. The present work provides a list of potential reference genes in lentil, which will help in selection of appropriate reference gene for qRT-PCR data normalization depending upon the experiment.

Keywords Abiotic stress · Gene expression · Growth stages · Lentil (*Lens culinaris*) · Reference gene · qRT-PCR

Introduction

Lentil (*Lens culinaris*) is a self-pollinating diploid ($2n = 14$ chromosomes), annual, cool season legume crop. It is a staple food in developing countries and contributes significantly to food and nutritional security. It is a rich source of protein and other micronutrients, like iron and zinc (Grusak and Coyne 2009; Thavarajah et al. 2011). India is the second main lentil producing country in the world, however, its yield is largely compromised due to various biotic and abiotic stresses (Erskine et al. 2011; Abraham 2015). In recent years, transcriptome and gene expression analyses have gained significance for studying fundamental mechanisms of plant development and responses to biotic and abiotic stresses at molecular level (Ford et al. 2011; Musculo et al. 2015; Idrissi et al. 2016). The first gene expression profiling of lentil through microarray was performed to study its response under *Aschochyta lentis* infection (Mustafa et al. 2009). Transcriptional profiling of genes like ADP-glucose pyrophosphorylase (AGPase) and pathogenesis related protein 4 (PR4) in lentil under different physiological and biotic stress conditions (Seferoglu et al. 2013; Vaghefi et al. 2013), have also been performed through quantitative real-time PCR (qRT-PCR).

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12298-018-0609-1>) contains supplementary material, which is available to authorized users.

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The qRT-PCR is most potential technique for studying gene expression owing to its sensitivity, specificity, dynamic range and high throughput capacity (Bustin 2002; Walker 2002; Gachon et al. 2004; Bustin et al. 2005). However, for accurate determination of gene expression, availability of stably expressed reference gene in each experimental condition is a must (Gutierrez et al. 2008). Thus, the qRT-PCR results are screened through MIQE (Minimum information for Publication of Quantitative Real Time PCR Experiment) guidelines to ensure the reproducibility and relevance of data (Udvardi et al. 2008; Bustin et al. 2009). The efficiency of the qRT-PCR experiment depends largely on the amount and quality of template (Bustin et al. 2009), therefore, normalisation step is a pre-requisite to overcome these limitations (Guenin et al. 2009). The most common and widely accepted method for data normalisation is including reference gene(s), whose expression is constitutive under all given experimental conditions (Thellin et al. 1999; Radonic et al. 2004; Czechowski et al. 2005; Huggett et al. 2005; Suzuki et al. 2009). General housekeeping genes, like 18S rRNA (ribosomal RNA), actin, Tubulin, EF1 α (Elongation Factor 1 α), polyubiquitin, GAPDH (glyceraldehydes 3 phosphate dehydrogenase) are commonly used as reference genes (Czechowski et al. 2005; Gutierrez et al. 2008). Owing to the importance of data normalisation, there are substantial reports on stable reference genes in legumes and pulses under various experimental conditions such as Medicago (Kakar et al. 2008), peanut (He et al. 2017), chickpea (Garg et al. 2010; Reddy et al. 2016), soybean (Jian et al. 2008; Libault et al. 2008; Hu et al. 2009; Stolf-Moreira et al. 2011; Le et al. 2012; Ma et al. 2013; Yim et al. 2015; Gao et al. 2017), pea (Die et al. 2010), fava bean (Gutierrez et al. 2011), common bean (Borges et al. 2012), pigeon pea (Sinha et al. 2015a, b) and Vigna (Chi et al. 2016) etc.

Although, identification of most reliable reference gene is a requirement for qRT-PCR studies, only a single study has reported reference gene validation in lentil for different varieties under cold stress and biotic stresses like, *Sclerotinia sclerotiorum* and *Aphanomyces euteiches* infection (Saha and Vandemark 2013). Most of the previous studies related to qRT-PCR analysis in lentil have used either reference gene which has been validated in other species (Seferoglu et al. 2013), or have screened stability of single reference gene as per their experimental conditions (Bhadauria et al. 2013; Vaghefi et al. 2013). However, selection of stably expressed reference gene is a challenge as there is no universally applicable reference gene(s) (Nolan et al. 2006; Chandna et al. 2012). There are substantial evidences which demonstrate that expression of housekeeping genes may vary significantly under different experimental conditions in many plant species (Selvey et al. 2001; Ruan and Lai 2007; Thorrez et al.

2008; Greer et al. 2010; Liu et al. 2012; Duhoux and Délye 2013). It has also been reported that qRT-PCR data, normalized only with one reference gene may generate significant errors (Tricarico et al. 2002). Therefore, in order to facilitate accurate normalisation of qRT-PCR data, the present work was performed to identify the best possible reference gene(s) in lentil. We have evaluated the expression stability of eight reference genes viz. Rbcl (ribulose 1,5 bisphosphate carboxylase large subunit), RPL2 (Ribosomal protein L2), 18S rRNA, Tub (Tubulin), EF1 α , HSP70 (Heat Shock Protein), mat K (Maturase) and GAPDH, in five lentil varieties, under three leaf developmental stages and four abiotic stress conditions viz. salinity, drought, cold and heat, and treatment with abscisic acid and methyl viologen with respect to their corresponding control samples.

Materials and methods

Plant materials, growth conditions and stress treatment

For the gene expression studies, five varieties of lentil: KLS 218, Noori, L4076, HUL 57 and JL3 were procured from Bihar Agricultural University, Sabour, India. Seeds were surface sterilised with 1% sodium hypochlorite (Sigma Aldrich), thoroughly washed with water and soaked overnight. The next day, 4 seeds per pot were inoculated in cocopeat: vermiculite (1:2, v:v) mixture in plastic pots (4 inch diameter). Leaf samples were harvested at different developmental stages. The vegetative growth stages of lentil were marked on the basis of visible nodes and characterised accordingly as VE (seedling emergence), V1 (first simple leaf), V2 (second simple leaf), V3 (first bifoliate leaf), V4 (second bifoliate leaf), V5 (first multifoliate leaf) and Vn (nth multifoliate leaf) (Erskine et al. 1990). In the present work, leaves were harvested at three development stages; V1, V3 and V5 from all five varieties (15 independent samples for each replications), in three biological replicates (total 45 samples) (Online Resource 1) and immediately frozen in liquid nitrogen for further analysis.

For stress treatments, seedlings of KLS 218 were grown for 15d in above mentioned conditions and stress treatments namely, salinity (NaCl 200 mM), drought (18% PEG 6000), cold (4 °C), heat shock (42 °C), abscisic acid (ABA, 100 μ M) and methyl viologen (MV, 5 μ M) were applied in independent pots in three biological replicates. Leaf samples along with control counterparts were collected at different time intervals (1 h, 6 h and 24 h for all stress treatments and for heat 1 h stress, 6 h stress and 24 h recovery) and stored in liquid nitrogen, till further analysis.

Altogether, there were 20 independent samples for each replicate and 60 samples in total for stress (Online Resource 1).

Total RNA extraction and first strand cDNA synthesis

Total RNA was isolated from 100 mg leaf tissue using *iRIS* solution (Ghawana et al. 2011). The RNA concentration and integrity were measured using Biospectrometer (Eppendorf) and RNA quality was assessed by formaldehyde-agarose gel (2.2 M-1.5%) electrophoresis. 1 µg of total RNA was treated with DNase (DNase I RNase-free, Thermo Scientific) to remove genomic DNA contamination. First strand cDNA was synthesised from 1 µg total RNA in 20 µl reaction volume using Verso cDNA synthesis kit (Thermo Scientific). Each cDNA sample was diluted tenfold in nuclease free water, prior to use in qRT-PCR reaction.

Selection of candidate genes and primer designing

Eleven candidate reference genes namely, actin, 18S rRNA, GAPDH, EF1 α , HSP70, Mat K (Maturase K), Rbcl, Tub, ubiquitin, PP2A (protein phosphatase 2A) and RPL2 were selected to identify the stably expressed gene(s) for qRT-PCR studies in lentil. Nucleotide sequence of these genes were retrieved from the genome database of *Glycine max* and *Cicer arietinum* and corresponding homologous nucleotide sequences were BLAST searched against lentil nucleotide sequence using Knowpulse database (<http://knowpulse.usask.ca/>). Primers were designed using Primer Express software version 3.0.1 (Applied Biosystems) with default parameters, except amplicon length was set to 80–120 bp. Out of resulting primers pairs, the one with least penalty was selected. The primer specificity was screened by PCR amplification of target genes from lentil cDNA, followed by gel electrophoresis in 2% agarose gel.

qRT-PCR conditions and PCR efficiency

The qRT-PCR reactions were performed using Step One Plus Real-Time PCR system (Applied Biosystems) with default cycling conditions (initial denaturation at 95 °C, 10 min; 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s) followed by dissociation curve analysis to determine the specificity of amplification. Each reaction was performed in 20 µl reaction mixture containing diluted cDNA (1:10) as template and DyNAmo Flash SYBR Green qPCR mix (Thermo Scientific), and 200 nM of each forward and reverse gene specific primers. Each experiment was conducted with three biological and three technical replicates. In order to

determine the PCR amplification efficiency of each gene in qRT-PCR experiments, tenfold dilution series (10–10,000-fold dilution) of cDNA was performed (Bustin et al. 2009). The slope of standard curve was used to calculate the PCR efficiency of each gene.

Analysis of reference gene expression stability

To estimate the homogeneity of variation among the cycle threshold (Ct) value of different genes, residual analysis and interquartile range calculations were performed. The stability of gene expression among different experimental conditions was determined using four different statistical algorithms viz. BestKeeper (Pfaffl et al. 2004; <https://www.gene-quantification.de/bestkeeper.html>), geNorm (Vandesompele et al. 2002; <https://genorm.cmgg.be/>), Normfinder (Andersen et al. 2004; <https://moma.dk/normfinder-software>) and Reffinder (Xie et al. 2012; <http://leonxie.esy.es/RefFinder/>).

Reference gene validation

The genes identified as most stably expressed were further validated using target genes. In the present study, $\Delta\Delta C_t$ method was used to analyse the expression of general stress inducible genes like defensin and PR4 (pathogenesis related) in abiotic stress samples, with respect to the identified stable housekeeping genes as reference, either alone or in combination. The differential gene expression of target genes in abiotic stress samples was compared to their control counterparts with respect to different reference genes using Relative Expression Software Tool (REST©) (Pfaffl et al. 2002).

Results

Verification of primer specificity and PCR efficiency determination

To identify the most stable housekeeping genes, eleven most commonly used housekeeping genes were selected (Online Resource 2). Specific amplification by designed primers was determined by PCR using lentil cDNA as template followed by agarose gel electrophoresis (2%) and melt curve analysis following qRT-PCR reactions. The presence of a single band of correct size in agarose gel and a single peak in melt curve analysis indicated that a single product of expected length was formed with no primer-dimer formation. However, desired results were obtained for only eight genes-18S rRNA, GAPDH, EF1 α , HSP70, mat K, Rbcl, Tub and RPL2 (Online Resource 3). Thus all the analysis was further continued on these eight genes.

The PCR efficiency of these genes was determined using tenfold dilution series of lentil cDNA. The PCR efficiencies and correlation coefficient (R^2) are listed in Table 1. According to the MIQE guidelines, the acceptable range for PCR efficiency is 80–120%, the ideal being 100% (Bustin et al. 2009). The PCR efficiencies of the genes were found to vary within the acceptable range of 86.3% and 112.3% and the R^2 value ranged within 0.957–0.999.

Expression stability analysis for candidate reference genes

Stability of expression was performed for eight candidate reference genes, across 35 samples of lentil under different experimental conditions viz. abiotic stress conditions (salt, drought, cold and heat), ABA and MV treatments and three leaf development stages in five different varieties (KLS218, Noori, L4076, HUL57 and JL3). Using qRT-PCR, a wide range of Ct values (20.5–28.2) was observed across all the samples, suggesting various levels of transcript abundance of these genes (Online Resource 4). A heat map of individual genes based on their mean Ct values is also plotted (Online Resource 5). The most highly expressed gene was HSP70, with average Ct value of 20.5, while the least expressed was RPL2 with average Ct value of 28.2. Interquartile range was calculated across all the samples, to identify the gene with least dispersal Ct value. The lowest Ct dispersal order was GAPDH < Mat K < EF1 α with dispersal range 1.4–1.9 cycles, which indicates less variation in expression whereas, the higher variation in expression (highest Ct dispersal value) was observed for HSP70 and Tub with dispersal range between 4.9 to 5.0 cycles (Fig. 1), respectively. Dispersal range of Ct value provided an indication of gene expression stability, however to gain a deeper insight and get affirmed results, four types of widely accepted statistical software, like BestKeeper, NormFinder, geNorm and RefFinder were employed for further analyses.

Fig. 1 Ct value of each candidate reference genes in all samples. The box represents 25 and 75 percentile range. The line across the box indicates the median. Asterisk represents maximum and minimum value

BestKeeper analysis

The BestKeeper analysis determines the expression stability on the basis of coefficient of variation (CV) and standard deviation (SD) ($CV \pm SD$). Gene having lowest CV and SD is ranked as most stable reference gene. For this analysis, the raw Ct values (S3 Table) were used directly. Among all samples, the most stable reference gene with lowest CV (6.31) was RPL2, followed by GAPDH and Rbcl with CV values 6.41 and 6.43, respectively. The most unstable gene was HSP70 with highest CV as 13.45. However, when compared individually under abiotic stress samples and developmental stage samples, the results were slightly different. Among abiotic stress samples, RPL2 was identified as most stable gene followed by 18S rRNA and GAPDH with CV values 2.55, 2.72 and 2.9, respectively. While, mat K was found to be least stable with highest CV value (6.72), preceded by HSP70 (CV 5.43). Among developmental stage samples, Rbcl was identified as the most stable gene followed by RPL2 and 18S rRNA with CV values 6.79, 7.25 and 7.68, respectively, while the least stable gene was GAPDH, with CV 10.94, preceded by mat

Table 1 qRT-PCR parameters of selected candidate reference genes and target genes

Sl. no.	Gene name	Correlation coefficient (R^2)	qRT-PCR efficiency (%)
1	RPL2	0.959	86.3
2	EF1 α	0.999	98.5
3	HSP70	0.999	101.3
4	Rbcl	0.986	101.7
5	Tub	0.995	99.3
6	Mat K	0.99	96.9
7	18S rRNA	0.998	98.8
8	GAPDH	0.957	112.3
9	Defensin	0.987	113.4
10	PR4	0.979	98.7

K with CV 10.55. The combined results of BestKeeper analysis are presented in Table 2.

geNorm analysis

The geNorm calculates average expression stability (M value) on the basis of average pair-wise variation between all the genes and the lower M value indicates higher expression stability. The recommended threshold level for M-value is 1.5, gene with M-value less than 1.5 is considered stably expressed, and the one having lowest M-value is considered as most stably expressed, and vice versa. In the present analysis, all the genes were found to have M value below 1.5 (Fig. 2). However, RPL2 was found to be the most stable gene in all three sample types, viz. all the samples together, leaf developmental stages and abiotic stresses, with M-value 0.154, 0.107 and 0.061, respectively. The least stable gene among all sample types and abiotic stress conditions was HSP70 with M-value 0.243 and 0.114, respectively; whereas for the leaf developmental stages, the least stable gene was GAPDH with M value 0.197. The overall results of geNorm are summarised in Table 3.

The geNorm also performs pairwise variations (V_n/V_{n+1}) between sequential normalisation factors (NF_n and NF_{n+1}), to identify the optimal number of reference gene(s) for normalisation among all or in individual sample. For this, the threshold value of 0.15 is accepted (Vandesompele et al. 2002). Our results suggested that among all the three sample categories viz. all sample types, abiotic stress conditions and developmental stages, using two genes is sufficient for data normalisation as $V_{2/3}$ is < 0.15 , and including third gene ($V_{3/4}$) does not makes any significant difference in the threshold values as shown in Fig. 3. Therefore, pairwise variation suggested that a

combination of genes RPL2 and Tub is sufficient for qRT-PCR data normalization in all sets of samples.

NormFinder analysis

The candidate genes were then analysed by NormFinder, which is based on variance estimation approach. It ranks the genes as per their stability under a particular experimental condition. The NormFinder generates average expression stability value (S-value), lower S-value indicates, more stable gene. Among all samples types, the most stable gene was found to be RPL2 followed by mat K with S-value 0.016 and 0.021, respectively (Table 4), while least stable gene was found to be HSP70 with S-value 0.033. Among abiotic stress samples, RPL2 was the most stable gene with minimum S-value (0.005) followed by Tub (0.006), while, the least stable gene was HSP70 (0.015). Similarly, among developmental stages, RPL2 was again observed as the most stable gene with minimum S-value (0.010) followed by Tub (S-value 0.012) and the least stable gene was EF1 α , with highest S value (0.028). NormFinder also identified most stable combination of genes (Tub and RPL2 with S values 0.009 and 0.005, respectively) for all samples in combination as well as abiotic stress samples, while, for developmental stage samples, a combination of 18S rRNA and RPL2, was found to be the best with minimum S value (0.009).

RefFinder analysis

RefFinder provides a comprehensive ranking for gene expression stability, by integrating the commonly used analytical programs like geNorm, NormFinder, BestKeeper and Δ CT method. Ranking of the genes using independent softwares provided varied results however, RefFinder is advantageous as it provides integrated results. The rankings of candidate reference genes as determined using RefFinder (Table 5) were consistent with those obtained by BestKeeper, geNorm and NormFinder, however in some cases slight variations were also observed; which is very likely as it uses raw Ct values, without considering the PCR efficiency of genes. It revealed that, among all sample types the ranking order of the genes were Rbcl, mat K, RPL2, EF1 α ; while for abiotic stress samples: RPL2, Rbcl, Tub and GAPDH were found to be stably expressed. In leaf developmental stage samples, the ranking order was HSP70, RPL2, Rbcl and 18S rRNA. However, according to the comprehensive ranking, HSP70 was the least stable gene for all sample types as well as abiotic stress samples; however, for developmental stages, GAPDH was the least stable followed by EF1 α . The overall compiled ranking order of genes as obtained by independent analysis and comprehensive ranking is shown in Table 6.

Table 2 Ranking and coefficient of variation (CV) of eight candidate reference genes in lentil as calculated by BestKeeper

Rank	All samples		Abiotic stress		Development stages	
	Genes	CV	Genes	CV	Genes	CV
1	RPL2	6.31	RPL2	2.55	Rbcl	6.79
2	GAPDH	6.41	18S rRNA	2.72	RPL2	7.25
3	Rbcl	6.43	GAPDH	2.90	18S rRNA	7.68
4	EF1 α	6.48	Rbcl	3.53	Tub	8.07
5	Mat K	8.89	Tub	3.77	EF1 α	8.1
6	18S rRNA	9.19	EF1 α	4.91	HSP70	9.6
7	Tub	9.9	HSP70	5.43	Mat K	10.55
8	HSP70	13.45	Mat K	6.72	GAPDH	10.94

Fig. 2 Gene expression stability values (M) and ranking of eight reference genes as assayed by geNorm in all sample types (a), abiotic stress samples (b) and different developmental stages (c). The least stable genes are on the left and the most stable genes on the right

Table 3 Ranking and expression stability value (M) of eight candidate reference genes in lentil as calculated by geNorm

Rank	All samples		Abiotic stress		Development stages	
	Genes	M value	Genes	M value	Genes	M value
1	RPL2	0.154	RPL2	0.061	RPL2	0.107
2	RbcL	0.1612	Tub	0.064	Tub	0.116
3	EF1 α	0.1633	GAPDH	0.069	18S rRNA	0.129
4	Mat K	0.1654	RbcL	0.074	RbcL	0.130
5	GAPDH	0.1765	EF1 α	0.081	Mat K	0.132
6	18S rRNA	0.1846	18S rRNA	0.081	HSP70	0.138
7	Tub	0.1937	Mat K	0.089	EF1 α	0.167
8	HSP70	0.2438	HSP70	0.114	GAPDH	0.197

Fig. 3 Pairwise variation (V) of the candidate gene as calculated by geNorm in all sample types, abiotic stress conditions and developmental stages. V_n/V_{n+1} were used to ascertain the optimal number of reference genes

Reference gene validation

The candidate reference genes identified as most stably and least stably expressed from the above results were further validated against target genes. For this, relative fold change of two genes, defensin and PR4 under abiotic stress conditions was determined by $\Delta\Delta C_t$ method and data normalisation was done with respect to two stable reference genes viz. RPL2 and Tub, either alone or in combination and two least stable genes viz. HSP70 and 18S rRNA (Fig. 4). The results showed that expression of both the target genes, defensin and PR4 were relatively comparable in most of the abiotic stress conditions, when normalised with stable reference genes RPL2 and Tub alone and in combination. However, the expression pattern of the genes showed a marked difference when normalised with least stable genes, HSP70 and 18S rRNA, while in some cases even reverse gene expression pattern was observed.

Discussion

The lentil is an important pulse crops for human consumption. Recently high throughput sequencing technology has been employed to generate reference transcriptome of lentil (Sudheesh et al. 2016), and to understand

Table 4 Ranking and expression stability value (S) of eight candidate reference genes in lentil as calculated by NormFinder

Rank	All samples		Abiotic stress		Development stages	
	Genes	S value	Genes	S value	Genes	S value
1	RPL2	0.016	RPL2	0.005	RPL2	0.010
2	Mat K	0.021	Tub	0.006	Tub	0.012
3	Rbcl	0.022	GAPDH	0.007	18S rRNA	0.017
4	18S rRNA	0.025	Rbcl	0.009	Mat K	0.018
5	Tub	0.025	EF1 α	0.009	Rbcl	0.018
6	GAPDH	0.026	18S rRNA	0.010	Hsp70	0.024
7	EF1 α	0.028	Mat K	0.014	GAPDH	0.025
8	HSP70	0.033	HSP70	0.015	EF1 α	0.028

Table 5 Ranking order of the genes as determined by RefFinder analysis

Method	1	2	3	4	5	6	7	8
<i>Ranking order (better-good-average)</i>								
<i>All samples</i>								
Δ CT	Rbcl	Mat K	RPL2	EF1 α	GAPDH	18S rRNA	Tub	HSP70
BestKeeper	Rbcl	Mat K	GAPDH	EF1 α	RPL2	18S rRNA	Tub	HSP70
Normfinder	Rbcl	Mat K	EF1 α	RPL2	GAPDH	18S rRNA	Tub	HSP70
Genorm	Tub/RPL2	Mat K	GAPDH	EF1 α	Rbcl	18S rRNA	HSP70	
Recommended comprehensive ranking	Rbcl	Mat K	RPL2	EF1 α	GAPDH	Tub	18S rRNA	HSP70
<i>Abiotic stress</i>								
Δ CT	RPL2	Tub	GAPDH	Rbcl	18S rRNA	Mat K	EF1 α	HSP70
BestKeeper	18S rRNA	RPL2	GAPDH	Rbcl	Mat K	Tub	EF1 α	HSP70
Normfinder	RPL2	Tub	GAPDH	Rbcl	18S rRNA	EF1 α	Mat K	HSP70
Genorm	Mat K/Rbcl	RPL2	Tub	GAPDH	18S rRNA	EF1 α	HSP70	
Recommended comprehensive ranking	RPL2	Rbcl	Tub	GAPDH	18S rRNA	Mat K	EF1 α	HSP70
<i>Developmental stages</i>								
Δ CT	HSP70	RPL2	18S rRNA	Rbcl	Mat K	Tub	EF1 α	GAPDH
BestKeeper	Rbcl	18S rRNA	HSP70	EF1 α	Mat K	RPL2	Tub	GAPDH
Normfinder	HSP70	RPL2	Rbcl	18S rRNA	Mat K	Tub	EF1 α	GAPDH
Genorm	Tub/RPL2	Mat K	HSP70	18S rRNA	Rbcl	EF1 α	GAPDH	
Recommended comprehensive ranking	HSP70	RPL2	Rbcl	18S rRNA	Tub	Mat K	EF1 α	GAPDH

molecular response of lentil under drought stress (Singh et al. 2017) and *Colletotrichum lentis* infection (Bhadauria et al. 2017). Besides transcriptomic analysis, various kinds of DNA markers, like RAPD, ISSRs, AFLPs, SSRs and SNPs have also been developed and mapped in lentil (Eujayl et al. 1998; Rubeen et al. 2003; Hamwieh et al. 2005; Saha et al. 2010; Sharpe et al. 2013). A rich resource of transcriptome and genome data will further give more opportunities to investigate genetic basis of lentil response to various biotic and abiotic stress conditions. Therefore, determination of stably expressed reference gene for qRT-PCR data normalization in lentil is important. According to MIQE guidelines, using reference genes is the most appropriate method for qRT-PCR data normalisation. Unfortunately, the accurate selection of reference genes to

a robust normalisation of expression data in lentil is very limited. The maiden paper preceding this work has reported gene expression stability of six genes, namely 18SrRNA, actin, GAPDH, β -Tubulin-2, β -Tubulin-3 and translation initiation factor (TIF) in lentil subjected to an abiotic (cold) and biotic stress (inoculation with *Scelrotinia sclerotiorum* and *Aphanomyces euteiches*) employing single statistical software, geNorm (Saha and Vandemark 2013). Therefore, the present study has analysed eight candidate reference genes in five varieties of lentil at three leaf developmental stages and six abiotic stress conditions at three stress regimes. Also, the data was analysed by four independent statistical softwares to generate more accurate and precise results. BestKeeper determines the optimal reference genes on the basis of pair-wise correlation analysis of all pairs of

Table 6 Compiled results of gene expression on stability as per all statistical analysis

Rank	All sample type				Abiotic stress conditions				Developmental stages			
	Best keeper	geNorm	Norm-finder	Ref-finder	Best keeper	geNorm	Norm-finder	Ref-finder	Best keeper	geNorm	Norm-finder	Ref-finder
1	RPL2	RPL2	RPL2	Rbcl	RPL2	RPL2	RPL2	RPL2	Rbcl	RPL2	RPL2	HSP70
2	GAPDH	Rbcl	mat K	Mat K	18S rRNA	Tub	Tub	Rbcl	RPL2	Mat K	Tub	RPL2
3	Rbcl	EF1 α	Rbcl	RPL2	GAPDH	GAPDH	GAPDH	Tub	18S rRNA	EF1 α	18S rRNA	Rbcl
4	EF1 α	Mat K	18S rRNA	EF1 α	Rbcl	Rbcl	Rbcl	GAPDH	Tub	Tub	Mat K	18S rRNA
5	Mat K	GAPDH	Tub	GAPDH	Tub	EF1 α	EF1 α	18S rRNA	EF1 α	Rbcl	Rbcl	Tub
6	18S rRNA	18S rRNA	GAPDH	Tub	EF1 α	18S rRNA	18S rRNA	Mat K	HSP70	GAPDH	HSP70	Mat K
7	Tub	Tub	EF1 α	18S rRNA	HSP70	Mat K	Mat K	EF1 α	Mat K	Hsp70	GAPDH	EF1 α
8	HSP70	HSP70	HSP70	HSP70	Mat K	HSP70	HSP70	HSP70	GAPDH	18S rRNA	EF1 α	GAPDH

candidate reference genes and calculates the coefficient of variance (CV) and standard deviation (SD) to determine expression stability of candidate reference genes (Pfaffl et al. 2004). The geNorm identifies the most stable reference gene based on the average pairwise variation among various candidate genes, and ranks the genes by their expression stability values (M) (Vandesompele et al. 2002). NormFinder calculates the overall variation in the expression of candidate reference genes in all the samples and also the variation of intra- and inter-groups (Andersen et al. 2004). According to NormFinder analysis, gene with the lowest SD and CV is considered as most stable reference gene. Whereas, RefFinder generates a comprehensive ranking by calculating the geometric mean of each reference gene in the above four methods, in which the smaller the ranking, the more stable is the reference gene (Liu et al. 2015). The most dependable reference gene should exhibit least possible variability in transcript level under all experimental conditions. Several reports related to validation of ideal reference genes in various crops have reported that expression of reference genes vary significantly among different tissues, developmental stages and experimental conditions in rice (Kim et al. 2003; Jain et al. 2006) brassica (Wang et al. 2014), wheat (Tenea et al. 2011), *Arabidopsis* (Czechowski et al. 2005; Remans et al. 2008), maize (Lin et al. 2014), banana (Chen et al. 2011a, b) papaya (Zhu et al. 2012) and apple (Perini et al. 2014; Bowen et al. 2014; Kumar and Singh 2015).

The results obtained from this study again showed that there cannot be just one ideal reference gene for all experiments and the selection of reference gene should be made appropriately as per the experimental conditions as

postulated earlier. The gene identified as most stable in the present study, under experimental conditions was RPL2 (Table 6) as also supported by heat map clustering (Online Resource 5). RPL2 is ubiquitously expressed in plants and plays role in ribosome biogenesis and widely accepted as standard reference gene (Hsiao et al. 2001). There are previous reports, which have screened stability of RPL2 under stress conditions in plants, and found to be stably expressed under defined conditions (Kumar and Singh 2015; Nicot et al. 2005). It is also reported that majority of ribosomal protein genes exhibit a consistent stable expression over a wide range of experimental conditions as against conventional housekeeping genes, like GAPDH and actin (de Jonge et al. 2007). Other stably expressed genes were Tub and Rbcl as observed in Table 6. Tubulin (Tub) has also been identified as stably expressed during abiotic stress conditions in pigeon pea (Sinha et al. 2015a). Rbcl gene was found to be stably expressed among all tissue types and in abiotic stress conditions, but not in developmental stages, thus indicating that it has a stage specific expression and should be used very carefully as per the experimental conditions. The HSP70 gene was identified as least stably expressed, especially among all tissue types and under abiotic stresses, which was also in accordance with previous reports (Reddy et al. 2016; Sinha et al. 2015a). HSP gene encodes a group of highly conserved chaperone proteins that maintain protein in their functional native conformations which is important for cell survival under stress as well as normal cellular processes (Mayer and Bukau 2005). Thus, a stable expression of HSP70 was observed during developmental stages, however, various biotic and abiotic stress conditions disrupt protein

Fig. 4 Validation of reference genes under abiotic stress conditions. Relative expression of defensin (a) and PR4 (b) genes as determined by $\Delta\Delta C_t$ method under six abiotic stress conditions, and data

normalisation was done using stable candidate reference genes: RPL2, Tub, RPL2+ Tub and least stable candidate reference genes: HSP70 and 18S rRNA. Error bars represent standard error

homeostasis, involving role of HSP for protein folding, assembly and degradation (Park and Seo 2015). Thus, expression of HSP70 is very liable to vary under stress conditions and hence, it cannot serve as an internal reference gene, which is also in accordance with the present observation. Likewise, 18S rRNA, GAPDH and EF1 α , were also identified as less stably expressed in developmental stages, which is in accordance with previous reports on different plant species (Chi et al. 2016; Tong et al. 2009). The 18S rRNA gene also had low ranking, indicating that ribosomal protein genes cannot be stable in all the conditions, and their expression may vary as per experimental conditions (Thorrez et al. 2008).

To validate the selected candidate reference genes, expression profile of two genes defensin and PR4 (Sels et al. 2008) was determined during different abiotic stress conditions in lentil leaf tissue. It was observed that when $\Delta\Delta C_t$ experiment was normalised using RPL2 or Tub,

either alone or together then both the genes had nearly equal fold change expression pattern. However, with respect to least stable genes, HSP 70 and 18S rRNA, significant difference in expression pattern was observed, and in some cases, even contradictory expression patterns were observed. Therefore, it is again emphasised that using an incorrect and unsuitable reference gene could misinterpret the whole gene expression profiling experiments, leading to incorrect results.

Conclusions

The present study was aimed to identify the most relevant reference gene for qRT-PCR data normalization in lentil under different leaf developmental stages and abiotic stress conditions. Eight candidate genes were selected and the resulting Ct values were analysed by different statistical

software. The comprehensive analysis of the result by RefFinder showed that the most stably expressing genes were RPL2, Tub and Rbcl among all samples and under abiotic stress samples, whereas, for leaf developmental stages, HSP70 was the found to be the most stably expressed gene; in contrast, it was the least stably expressed gene for all and abiotic stress samples. Likewise, the least stable gene in developmental stage samples was GAPDH. The most stable genes as identified by other three software (BestKeeper, geNorm and RefFinder) were also RPL2, Tub and Rbcl, however the results vary for least stable genes as HSP70, matK, GAPDH, EF1 α , and 18S rRNA, were identified as least stable by these software, under different experimental conditions. The present study provides a list of suitable reference genes for normalisation of qRT-PCR data in various lentil cultivars under different leaf development stages and abiotic stress conditions. Taken together, the results suggested that most commonly used housekeeping genes (GAPDH, EF1 α , 18S rRNA) also show variable expression under different developmental stages and abiotic stress conditions. Thus, it can be inferred that a reference gene expressing stably in one organism under particular conditions may not behave similarly in different species, thus choice of reference genes should be made appropriately and must be standardised and validated as per the experimental conditions.

Acknowledgements RS acknowledges Science and Engineering Research Board, Department of Science and Technology, Government of India for the National-Postdoctoral Fellowship (PDF/2016/000924). AKS acknowledges Institute projects IXX12585 and IXX12644 funded by ICAR-Indian Institute of Agricultural Biotechnology, Ranchi. We thank Dr. Madhuparna Banerjee, Associate Professor, College of Biotechnology, Birsa Agricultural University, Ranchi for granting access to her lab facilities during the course of this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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