Journal of Genetics, Vol. 97, No. 5, December 2018, pp. 1433–1444
https://doi.org/10.1007/s12041-018-1042-5

RESEARCH ARTICLE

Selection and validation of reference genes for normalization of qRT-PCR gene expression in wheat (*Triticum durum* L.) under drought and salt stresses

JAMSHIDI GOHARRIZI KIARASH[1]*, HENRY DAYTON WILDE[2], FARZANE AMIRMAHANI[3], MOHAMMAD MEHDI MOEMENI[1], MARYAM ZABOLI[4], MARYAM NAZARI[5], SAYYED SAEED MOOSAVI[5] and MINA JAMALVANDI[6]

[1] *Department of Plant Breeding, Yazd Branch, Islamic Azad University, Yazd 8616871967, Iran*
[2] *Institute of Plant Breeding, Genetics, and Genomics, University of Georgia, Athens 30602, USA*
[3] *Faculty of Sciences, Genetic Division, Department of Biology, University of Isfahan, Isfahan 8174673441, Iran*
[4] *Faculty of Science, Department of Chemistry, University of Birjand, Birjand 7761676334, Iran*
[5] *Department of Agronomy and Plant Breeding, Faculty of Agriculture, Bu-Ali Sina University, Hamedan 6517838695, Iran*
[6] *Department of Genetic, Science and Research Branch, Islamic Azad University, Tehran 1477893855, Iran*
*For correspondence. E-mail: jamshidi_kiarash@iauyazd.ac.ir.

Received 6 March 2018; revised 10 August 2018; accepted 16 August 2018; published online 29 November 2018

Abstract. Eight candidate housekeeping genes were examined as internal controls for normalizing expression analysis of durum wheat (*Triticum durum L.*) under drought and salinity stress conditions. Quantitative real-time PCR was used to analyse gene expression of multiple stress levels, plant ages (24 and 50 days old), and plant tissues (leaf and root). The algorithms BestKeeper, NormFinder, GeNorm, the delta Ct method and the RefFinder were applied to determine the stability of candidate genes. Under drought stress, the most stable reference genes were *glyceraldehyde-3 phosphate*, *ubiquitin* and *β-tubulin2*, whereas under salinity stress conditions, *eukaryotic elongation factor 1-α*, *glyceraldehyde-3 phosphate* and *actin* were identified as the most stable reference genes. Validation with stress-responsive genes *NAC29* and *NAC6* demonstrated that the expression level of target genes could be determined reliably with combinations of up to three of the reference genes. This is the first report on reference genes appropriate for quantification of target gene expression in *T. durum* under drought and salt stresses. Results of this investigation may be applicable to other *Triticum* species.

Keywords. drought stress; housekeeping genes; quantitative real-time PCR; salt stress; transcription factors; *Triticum durum*.

Introduction

Durum wheat (*Triticum durum*; $2n = 4x = 28$ AABB) is one of the most significant agricultural products in the Mediterranean, mostly in central and west Asia and north Africa (Brennan *et al.* 2002). It is a small part of the global wheat industry, accounting for about 5% of agricultural land and 10% of total wheat production (Mohammadi *et al.* 2015). Drought and salt stress conditions have been found to reduce yield and yield components of wheat (Araus *et al.* 2002). It has been shown that a high level of salinity can decrease important agronomic traits such as leaf area, plant height, crop growth, dry matter, net assimilation rate and seed yield (Joshi and Nimbalkar 1983). Drought stress is another critical factor that limits agricultural production, and the improvement of wheat yield under drought stress is an important target of plant breeding (Cattivelli *et al.* 2008; Mir *et al.* 2012;

Electronic supplementary material: The online version of this article (https://doi.org/10.1007/s12041-018-1042-5) contains supplementary material, which is available to authorized users.

Tuberosa 2012). In Iran, a significant decrease in wheat production has resulted from the shortage of rainfall in recent years (Abdolshahi *et al.* 2013).

Plants react to environmental stress through physiological, morphological, and metabolic changes in all of their organs (Dudley and Shani 2003). At the genetic level, stress tolerance involves multiple mechanisms for regulating gene expression (Knight and Knight 2001). Plant engineering approaches to abiotic stress tolerance often take advantage of regulatory genes that control biochemical and physiological responses. Stress-tolerant genotypes can be valuable for evaluating resistance genes in functional studies to understand the molecular basis of adaptation to abiotic stresses (Zhang *et al.* 2016).

Methods for investigating gene expression, such as microarrays and quantitative real-time PCR (qRT-PCR), have played a critical role in biology research (Kavousi *et al.* 2009). qRT-PCR is a technique used to analyse gene expression quantitatively (Gachon *et al.* 2004; Bustin *et al.* 2009). Factors that play an important role in qRT-PCR include the initial sample size, RNA concentration, cDNA synthesis and the differences in the overall transcription activity in the analysed tissues and cells (Chen *et al.* 2006). To obtain the precise results, the target gene expression levels must be normalized by internal reference genes. Housekeeping genes are often applied as reference genes because of their stable expression (Vandesompele *et al.* 2002). These genes are commonly involved in vital processes of cell metabolism and architecture, such as the formation of the cytoskeleton, protein folding and ribosomal subunit synthesis (Huggett *et al.* 2005; Gutierrez *et al.* 2008). Precise normalization is essential for obtaining biologically significant expression data, therefore qRT-PCR analysis greatly depends upon accurate selection of the reliable reference genes that are expressed stably across various tissue samples, experimental conditions and developmental stages (Bustin *et al.* 2009). The use of unsuitable or unstable reference genes can affect the transcript quantification results and lead to erroneous conclusions (Gutierrez *et al.* 2008; Guénin *et al.* 2009). In addition, the stability of reference gene expression has been found to vary with environmental conditions, such as abiotic stress (Wei *et al.* 2013; Galli *et al.* 2015) and biotic stress (Scholtz and Visser 2012).

Over the past decades, housekeeping genes such as actin (*ACT*), glyceraldehyde-3 phosphate (*GAPDH*), *18SrRNA*, β-tubulin 2 (*β-TUB2*) and eukaryotic elongation factor 1α (*eEF-1α*) have been evaluated as suitable reference genes for the qRT-PCR (Mittler 2006). The expression of housekeeping genes has been reported to change under the various conditions, indicating that the optimal reference genes may vary (Volkov *et al.* 2003). To perform qRT-PCR precisely, it is essential to optimize internal control genes in various experimental conditions for a plant species. The factors influencing the variation in the expression of housekeeping genes are the plant life stage, the sample tissue and the environmental stress applied to the plant (Fischer *et al.* 2005; Goossens *et al.* 2005; Sinha *et al.* 2015). A set of multiple reference genes will be more accurate, with the geometric mean of multiple control genes minimizing variation in expression (Vandesompele *et al.* 2002). Stable reference genes have been validated for some economically important cereal crops, including bread wheat (Jain *et al.* 2006; Paolacci *et al.* 2009; Manoli *et al.* 2012), but not durum wheat. Therefore, this study was performed to assess the stability of the expression of the reference genes *GAPDH*, *ACT*, *18SrRNA*, *25SrRNA*, *eEF1α*, *β-TUB2*, eukaryotic initiation factor 4a (*eIF-4a*), and ubiquitin (*UBQ*) in durum wheat. Two stress-responsive transcription factors, *TaNAC29* and *TaNAC6* were used in the validation of reference genes under conditions of drought and salt stress. To our knowledge, this is the first investigation of reference gene stability in durum wheat under abiotic stress.

Materials and methods

Plant materials, growth conditions and treatments

Seeds of *T. durum* cv. 'Shabrang' were sterilized with 2.5% sodium hypochlorite for 15 min and washed thrice with deionized water. Seeds were then exposed to 70% ethanol for 1 min, washed thrice with deionized water, and placed on 0.8% agar medium in a 90 mm Petri dish for two days in an incubator at 28°C and 55% relative humidity. Germinated seeds were transferred to the new Petri dishes containing sand supplemented with half-strength Hoagland solution for two days, followed by full-strength Hoagland solution for 16 days. The seedlings were then transferred to 200-mL Hoagland solution containing different concentrations of osmoticum (0%, 10% and 20% PEG6000) or salt (0, 75 and 150 mM NaCl), with aeration provided by an aquarium pump. After four days of osmotic or salt stress treatment, leaves and roots of the seedlings were harvested, frozen in liquid nitrogen, and stored at –80°C until RNA isolation. To examine mature plants, plants were maintained in 200-mL Hoagland solution for 46 days and then were exposed to the six stress treatments as described above. After four days of stress treatment, leaf and root samples were harvested, frozen in liquid nitrogen, and stored at –80°C. The tissue samples used for qRT-PCR analysis are presented in table 1 in electronic supplementary material in http://www.ias.ac.in/jgenet/.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using RNX-Plus solution (CinnaGen, Tehran, Iran) and treated with DNase (Thermo Fisher Scientific, USA) according to the manufacturers' instructions. The integrity of isolated RNA was

Table 1. Details on primers used for qRT-PCR analysis.

Gene name	Primer sequence (5′–3′)	Amplicon length (bp)	Amplicon T_m (°C)	PCR efficiency (%)	Regression coefficient (r^2)	GenBank
β-Tubulin	Forward:TGTGGCAACCAGATCGGTGC Reverse: CATAAGGCCCAGTGCGGACAC	211	63	98.84	0.995	XM_020323159.1 U76745.1
EF-1α	Forward:TTTCACTCTTGGAGTGAAGCAGAT Reverse: GACCTCCTTGACAATTTCTTCATAA	103	58	97.23	0.999	XM_020317327.1
eIF-4a	Forward:AGGTTCCCAGTTTGACGCTA Reverse: CGATTCCCCTTTGCTGGATG	197	59	109.17	0.997	KX533925.1
25SrRNA	Forward:GGGTTTAGACCGTCGTGAGA Reverse: TTCAGTCATAATCCGGCACA	163	60	95.29	0.998	HQ825319.1 KY129794.1
UBQ	Forward:CATCGACAATGTGAAGGCGA Reverse: CTTACCAGCGAAGATCAGGC	79	59	92.34	0.999	AY297059.2
GAPDH	Forward:GGTGCCAAGAAGGTCATCAT Reverse: TGGTCATCAAACCCTCAACA	187	60	108.19	0.996	EU022331.1
Actin	Forward:CGTGTTGGATTCTGGTGATG Reverse: AGCCACATATGCGAGCTTCT	208	60	103.53	0.998	GQ339780.1
18SrRNA	Forward:TTGATGTATACTCGCAATGC Reverse: AAATTGCAGATAGCACATTTG	173	60	95.67	0.998	K01229.1 AF475127.1
TaNAC29	Forward:GTCCACCCTTCCCTGAACA Reverse: CACGACCCGTTAAGCCTCT	151	60	92.34	0.999	KT783450.1
TaNAC6	Forward:TACGGCGAGAAGGAGTGGTA Reverse: ACCCAGTCATCCAACCTGAG	278	60	94.23	0.997	HM027571.1

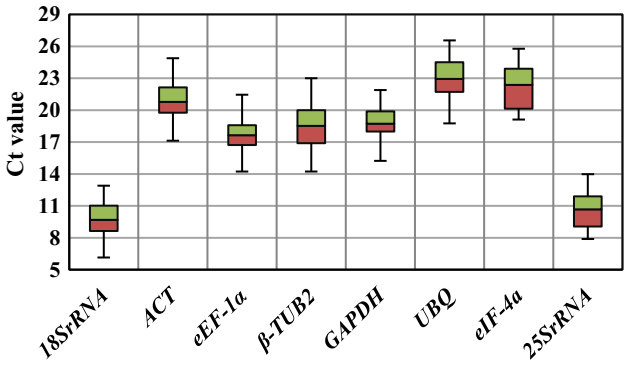

Figure 1. Ct values of candidate reference genes tested under drought stress conditions. The box demonstrates the 25th and 75th percentiles and the whiskers caps show the maximum and minimum values. A centre line across the boxes represents the median.

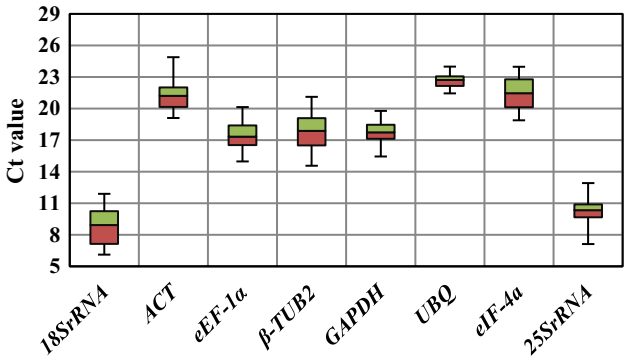

Figure 2. Ct values of candidate reference genes tested under salt stress conditions. The box shows the 25th and 75th percentiles and the whiskers caps demonstrate the maximum and minimum values. A centre line across the boxes represents the median.

tested on 1% agarose gel electrophoresis. The concentration and purification of each sample was checked using NanoDrop-1000 spectrophotometer. High-quality RNA with OD 260/280 and OD 260/230 > 2 was utilized for the subsequent steps. RNA, 2 μg was used for first-strand cDNA synthesis using the Prime-Script RT reagent kit (Takara, Japan) following the manufacturer's guidelines.

Selection of housekeeping genes, target genes, PCR primer design and amplification efficiency test

Sequence information for eight housekeeping genes (*β-TUB2*, *eEF1α*, *eIF-4a*, *25SrRNA*, *UBQ*, *GAPDH*, *ACT* and *18SrRNA*) and two stress-responsive genes (*TaNAC29* and *TaNAC6*) was obtained from the National Center for Biotechnology Information (NCBI) database (table 1). Gene-specific primers were designed using Gene Runner software v. 6.0.28 and Primer3. Dilutions of cDNA (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were made to develop standard

Table 2. Stability values and ranking order of housekeeping genes tested for drought stress conditions, based on results from BestKeeper, GeNorm, NormFinder, Delta Ct and RefFinder.

	BestKeeper		GeNorm		NormFinder		Delta Ct		RefFinder	
Ranking	Gene	Std. dev. [±CP]	Gene	M-value	Gene	Stability value (ρ_{ig} / σ_i)	Gene	Average of st dev	Gene	Geomean of ranking values (GM)
1	*GAPDH*	0.36	*GAPDH*	0.682	*β-TUB2*	0.240	*GAPDH*	0.55	*GAPDH*	1.00
2	*eEF-1α*	0.56	*β-TUB2*	0.684	*UBQ*	0.243	*UBQ*	0.58	*eEF-1α*	1.68
3	*18SrRNA*	0.66	*UBQ*	0.724	*GAPDH*	0.308	*eEF-1α*	0.66	*UBQ*	3.46
4	*25SrRNA*	0.72	*eEF-1α*	0.769	*eEF-1α*	0.314	*18SrRNA*	0.70	*18SrRNA*	3.66
5	*UBQ*	0.82	*25SrRNA*	0.795	*25SrRNA*	0.322	*ACT*	0.79	*ACT*	5.23
6	*ACT*	0.89	*18SrRNA*	0.827	*18SrRNA*	0.385	*β-TUB2*	0.83	*25SrRNA*	6.09
7	*β-TUB2*	1.03	*eIF-4a*	0.948	*eIF-4a*	0.468	*25SrRNA*	0.91	*β-TUB2*	6.45
8	*eIF-4a*	1.04	*ACT*	1.046	*ACT*	0.584	*eIF-4a*	0.97	*eIF-4a*	7.74

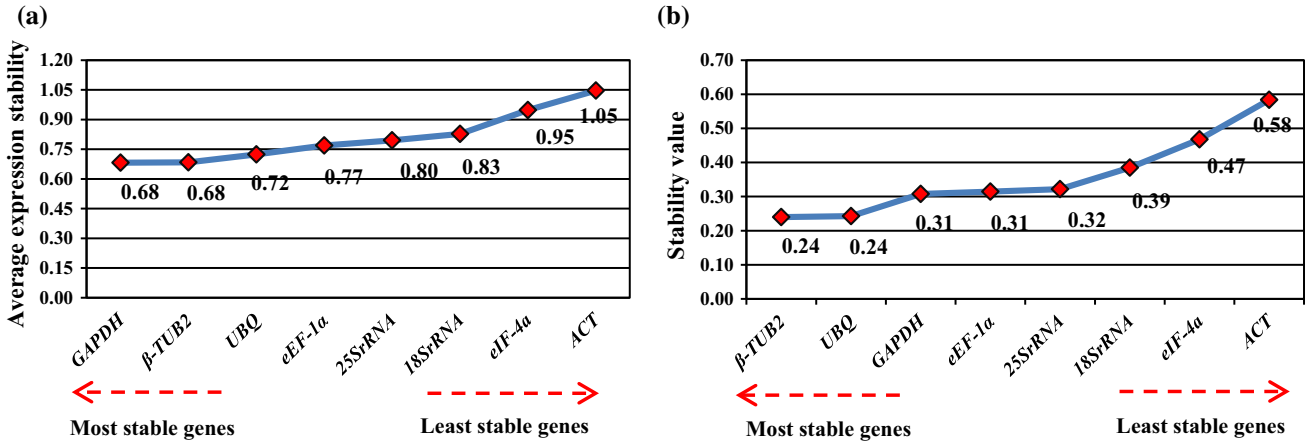

Figure 3. Reference gene ranking for drought stress conditions. (a) Gene expression stability of housekeeping genes using GeNorm program based on an average expression stability value. (b) Gene expression stability using NormFinder algorithm based on stability value.

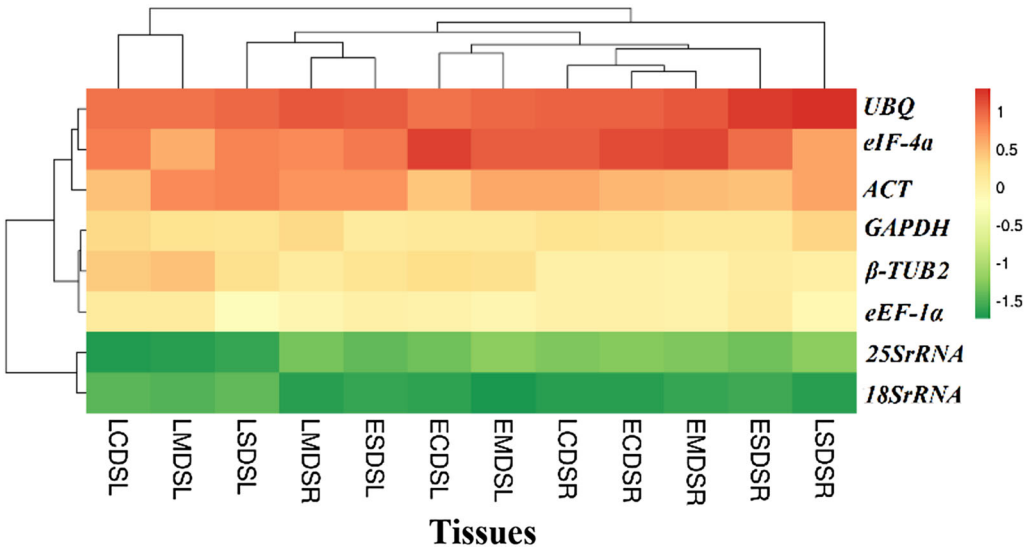

Figure 4. Heat map of candidate genes for drought stress samples. The heat map was derived from normalized Ct mean values of the reference genes in different tissues (root and leaf), stress levels (0%, 10% and 20% PEG 6000) and harvesting time (24 and 50 days old).

curves for the targeted genes. PCR amplification of the cDNA was conducted in a Rotor-Gene 3000 Real time Fast Thermocycler (Sydney, Australia) using 95°C for 30 s, then 40 cycles of 95°C for 5 s and 30 s of annealing at the amplicon T_m (table 1), followed by a melting curve analysis. No-template controls were used to ensure that no reagent or genomic DNA contamination existed. The presence of a single peak in qRT-PCR melting curve products and a single band of the expected size in the 2% agarose gel after electrophoresis verified the specificity of amplicons (figures 1 and 2 in electronic supplementary material). The correlation coefficient (R^2) and amplification efficiency (E) of the primers were calculated from the slope of the standard curve based on the following equation (Radonić *et al.* 2004):

$$\mathrm{E}\,(\%) = \left(10^{\left(\frac{-1}{\mathrm{slop}}\right)} - 1\right) \times 100$$

Gene expression analysis

Reference gene expression was analysed by the programs, BestKeeper, GeNorm, NormFinder, delta Ct method, and RefFinder, a web-tool (https://omictools.com/reffinder-tool) that integrates these programs. The transcription

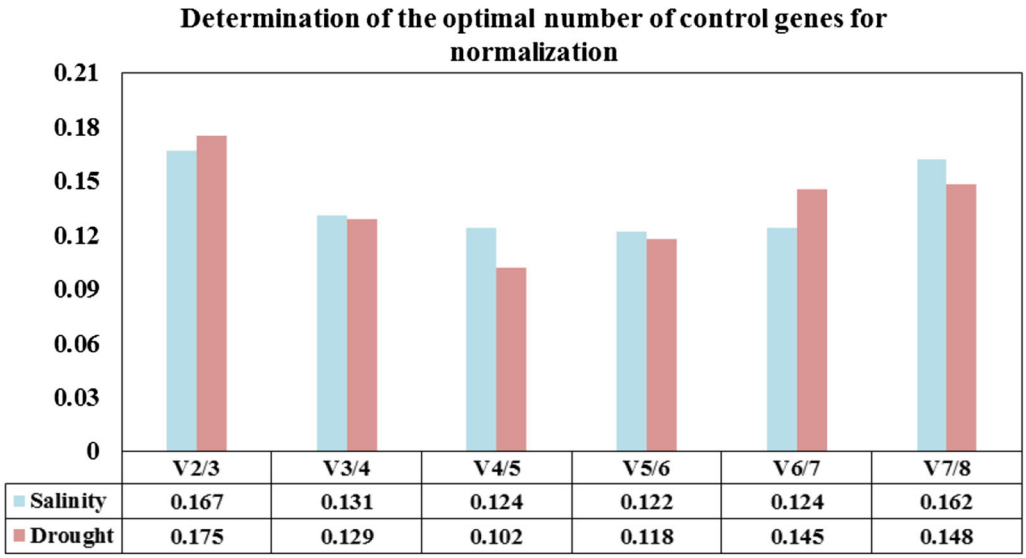

Figure 5. The optimum number of control genes for precise normalization counted by GeNorm algorithm.

factors *TaNAC29* and *TaNAC6* were used as stress-responsive genes to examine reference gene reliability. Through BestKeeper software (Pfaffl *et al.* 2004), reference genes were compared by repeated pairwise correlation and regression analysis of each gene with the other remaining candidate reference genes. The statistical algorithms GeNorm (https://genorm.cmgg.be/) and NormFinder (https://moma.dk/normfinder-software) were applied to identify and rank the most stable housekeeping genes. Reference genes were also examined using the delta Ct method to compare the relative expression of 'pairs of genes' in each sample (Silver *et al.* 2006). Correspondence between the programs GeNorm, NormFinder, BestKeeper, delta Ct method, and RefFinder was evaluated by Pearson's correlations ($P \leq 0.01$) and calculated using SPSS software v. 24.0. A heat map was created from normalized Ct means using the web server ClustVis (http://biit.cs.ut.ee/clustvis/).

Results

Housekeeping genes expression profile

qRT-PCR analysis of the cDNA dilution series determined that amplification efficiencies of the gene targets ranged from 92.34% to 109.17% (table 1). All PCR reactions had efficiencies within the acceptable level of 80–120% (Bustin *et al.* 2009) and they produced a single product (figures 1 and 2 in electronic supplementary material). The mean Ct values of the candidate genes ranged from 6.1 to 26.6 under drought stress conditions and 6.1 to 24.9 for salinity stress conditions (figures 1 and 2). Under both stress conditions, *18SrRNA* is the most expressed gene (lowest mean Ct) and *UBQ* is the least expressed gene.

Determination of the most stable housekeeping genes for drought stress conditions

To identify reference genes under drought stress analysis, the expression of eight reference genes was examined in leaves and roots from plants of two ages (24 and 50 days old) exposed to different levels of osmotica (0%, 10% and 20% PEG 6000). The programs BestKeeper, GeNorm, NormFinder, delta-Ct, and RefFinder were used to rank the reference genes by expression stability (table 2). Through analysis of standard deviation (SD), BestKeeper identified *GAPDH*, *EF-1α*, and *18SrRNA* as the most stable genes and *eIF-4a* and *β-TUB2* as the least stable genes.

The GeNorm and Normfinder results for drought conditions are shown in figure 3. The GeNorm algorithm ranked the reference genes by measuring the average expression stability value (M value; Vandesompele *et al.* 2002). This analysis determined that the most stable genes were *GAPDH*, *β-TUB2* and *UBQ*, based on M-values of 0.682, 0.684 and 0.724, respectively). The least stable genes identified by GeNorm were *ACT* (M 1.046), *eIF-4a* (M 0.948) and *18SrRNA* (M 0.827). NormFinder calculated the stability values (SV) for candidate reference genes using linear mixed-effects modelling. Based on this algorithm, *β-TUB2*, *UBQ*, and *GAPDH* were determined to be the most stable housekeeping genes. Similar to the results of GeNorm analysis, the least stable genes for drought stress conditions were identified to be *ACT*, *eIF-4a*, and *18SrRNA*.

The delta Ct and BestKeeper analyses both ranked *GAPDH* as the most stable reference gene and *eIF-4a* as the least stable gene (table 2). This is consistent with results from the RefFinder analysis, which integrated the

four computational programs. The geomean values determined by RefFinder were 1.00 and 7.74 for *GAPDH* and *eIF-4α*, respectively.

A heat map was produced from normalized Ct mean values for all candidate genes in all samples (figure 4). The heat map analysis demonstrated stable levels of expression of *eEF-1α* and *GAPDH* across the tissues and drought conditions, whereas other genes showed variable levels of expression across samples. The heat map results correlated with the stability ranking of the reference genes (table 2).

Determination of the optimal number of control genes in abiotic stress conditions

To estimate the optimal number of reference genes for qRT-PCR data normalization, a pairwise variation (Vn/Vn+1) was calculated by the GeNorm algorithm. The V2/3 values for drought stress conditions (0.167) and salinity stress conditions (0.175) were above the cut-off value (0.15). This indicates that three reference genes are sufficient for gene expression data normalization in these samples (figure 5). The addition of more reference genes had no significant effect on the normalization of gene expression.

Determination of the most stable reference genes for salt stress conditions

Reference genes under salinity stress were analysed by the same approach used above for drought stress conditions (table 3; figure 6). The GeNorm, NormFinder, delta-Ct, and RefFinder programs all ranked *eIF-1a*, *GAPDH* and *ACT* as the most reliable reference genes and *eIF-4a* to be least reliable. The BestKeeper algorithm identified the most stable genes for salinity stress to be *UBQ*, *GAPDH* and *elF-1a* and the least stable genes to be *18SrRNA*, *elF-4a* and *β-TUB*2. Heat map analysis (figure 7) showed that *eEF-1α* and *GAPDH* had the most stable expression across tissues and salinity stress levels.

Correlation of the candidate reference gene analyses

A comparison of the reference gene analysis programs was conducted using Pearson's correlations. As shown in table 4, Pearson's correlations were positive and significant for all pairwise comparisons of BestKeeper, GeNorm, NormFinder, the deltaCt method and RefFinder. The most significant correlations for drought stress were between RefFinder vs GeNorm ($r = 0.992$), RefFinder vs Delta Ct ($r = 0.991$), and NormFinder vs GeNorm ($r = 0.991$). For salinity stress, the most significant correlations were with BestKeeper vs RefFinder ($r = 0.977$) and delta Ct vs GeNorm ($r = 0.975$).

Table 3. Stability values and ranking order of candidate reference genes obtained from all the analyzed samples from salinity stress conditions.

	BestKeeper		geNorm		NormFinder		delta-Ct		RefFinder	
Ranking	Gene	Std. dev. (±CP)	Gene	M-value	Gene	Stability value (ρ_{ig} / σ_i)	Gene	Average of std. dev.	Gene	Geomean of ranking values (GM)
1	*UBQ*	0.24	*eEF-1α*	0.565	*eEF-1α*	0.223	*eEF-1α*	0.42	*eEF-1α*	1.19
2	*GAPDH*	0.55	*GAPDH*	0.569	*GAPDH*	0.265	*GAPDH*	0.46	*GAPDH*	2.59
3	*eEF-1α*	0.63	*ACT*	0.580	*ACT*	0.309	*ACT*	0.47	*ACT*	2.63
4	*25SrRNA*	0.73	*25SrRNA*	0.593	*18SrRNA*	0.317	*25SrRNA*	0.55	*25SrRNA*	3.72
5	*ACT*	0.77	*β-TUB2*	0.641	*25SrRNA*	0.336	*β-TUB2*	0.61	*UBQ*	4.30
6	*β-TUB2*	0.96	*18SrRNA*	0.705	*β-TUB2*	0.347	*18SrRNA*	0.69	*β-TUB2*	5.44
7	*eIF-4a*	0.98	*UBQ*	0.790	*UBQ*	0.42	*UBQ*	0.71	*18SrRNA*	6.45
8	*18SrRNA*	1.23	*eIF-4a*	0.933	*eIF-4a*	0.429	*eIF-4a*	1.03	*eIF-4a*	7.44

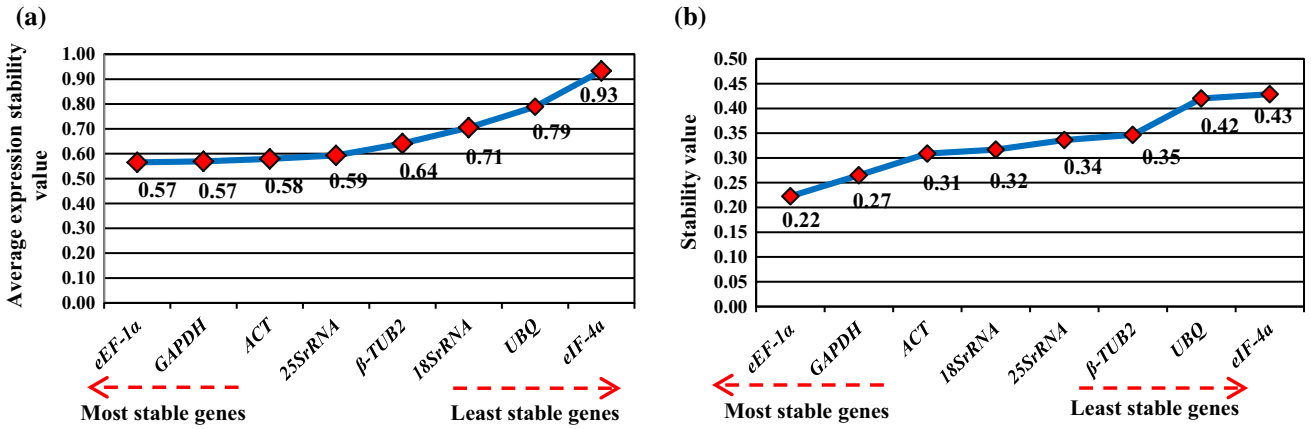

Figure 6. Housekeeping genes ranking for salinity stress conditions. Gene expression studies for determination of most stable housekeeping genes under salinity stress condition using two programs. The direction of arrow shows the most and least stable housekeeping genes in graphs. (a) Gene expression stability graph of housekeeping gene using GeNorm algorithm based on an average expression stability value (M). (b) Gene expression stability graph using NormFinder algorithm based on stability value.

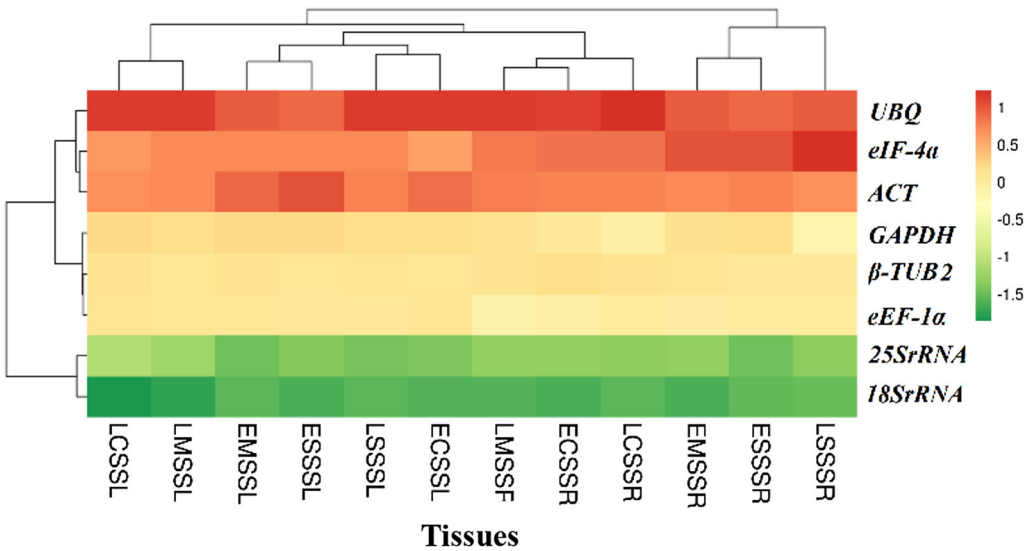

Figure 7. Heat map of candidate genes for salt stress samples. This figure shows a heat map based on normalized Ct mean values of candidate genes. Genes were clustered based on the Ct mean values of single candidate genes among tissues (root and leaf), stress levels (0, 75 and 150 mM NaCl) and harvesting times (Twenty-fourth day and Fiftieth day).

Validation of reference genes for abiotic stress conditions

Reference genes for drought stress conditions were validated with two stress-responsive transcription factors, *TaNAC29* and *TaNAC6*, in roots and leaves (figure 8). Housekeeping genes with the most stable expression (*β-TUB2*, *GAPDH*, *UBQ*), combinations of these genes (*β-TUB2+ GAPDH*, *β-TUB2+ UBQ*, *GAPDH + UBQ*, *β-TUB2+ GAPDH+UBQ*), and the least stable gene (*eIF-4a*) were examined. The relative expression of *TaNAC29* and *TaNAC6* was similar when the three most stable genes and their combinations were used as internal controls, in contrast to *eIF-4a.* For example, for drought stress in root, relative expression of the target gene *TaNAC29* showed induction of 2.0–2.5 fold using *β-TUB2*, *GAPDH*, *UBQ*, and their combinations as controls (figure 8a). With *eIF-4a* as a reference gene, *TaNAC29* expression was calculated to increase 5.5-fold. Similar patterns of expression using *β-TUB2*, *GAPDH*, *UBQ*, and their combinations as controls were also found for *TaNAC29* expression in leaves (figure 8c) and *TaNAC6* in roots (figure 8b) and leaves (figure 8d), in contrast variable results with *eEF-4a.*

For salinity stress conditions, the stable reference genes *eEF-1α*, *GAPDH*, *ACT* and their combinations (*eEF-1α + GAPDH*, *GAPDH + ACT*, *eEF-1α + ACT*, *eEF-1α + ACT + GAPDH*) were validated using *TaNAC29* and *TaNAC6* (figure 9). *EIF-4a* was used for comparison as

an unstable reference gene. The levels of induction of the stress-responsive genes with NaCl treatments were consistent with stable reference genes and their combinations, but the unstable reference gene *eEF-4a* gave different results. For example, for the late moderate salinity stress treatment in root (LMSSR), *TaNAC29* expression was found to increase 2.5–3.0 fold when using stable reference genes, but 4.2 fold when using *eEF-4a* (figure 9a).

Table 4. Correlation matrix of the obtained values of the five different mathematic algorithms (GeNorm, NormFinder, BestKeeper the delta Ct method and the RefFinder web-based tool) used for reference gene evaluation. *R*, Pearson's correlation coefficient; $^{**}P \leq 0.01$ (for drought and salinity stress conditions).

	Correlation	
	Drought	Salinity
NormFinder vs GeNorm	0.991**	0.897**
Delta Ct vs GeNorm	0.958**	0.975**
RefFinder vs GeNorm	0.992**	0.933**
BestKeeper vs GeNorm	0.903**	0.885**
Delta Ct vs NormFinder	0.957**	0.881**
RefFinder vs NormFinder	0.932**	0.970**
BestKeeper vs NormFinder	0.911**	0.956**
RefFinder vs Delta Ct	0.991**	0.935**
BestKeeper vs Delta Ct	0.975**	0.919**
BestKeeper vs RefFinder	0.975**	0.977**

Discussion

qRT-PCR is a useful method for studying the change in gene expression profiles in plants subjected to abiotic stress (Kumar *et al.* 2013). The optimal type and number of reference genes for accurately normalizing target gene expression need to be determined for different experimental conditions (Vandesompele *et al.* 2002; Bustin *et al.* 2009). To select the appropriate reference genes for durum wheat under abiotic stress, we analysed eight housekeeping genes in different tissues (leaves and roots) of plants of different ages (24 and 50 days-old) that had been exposed to different drought and salinity levels.

The five programs used to compare reference gene expression, BestKeeper, geNorm, NormFinder, delta Ct and RefFinder, generally produced the same results, with some exceptions. Under drought stress conditions, all algorithms identified *GAPDH* as the best housekeeping gene

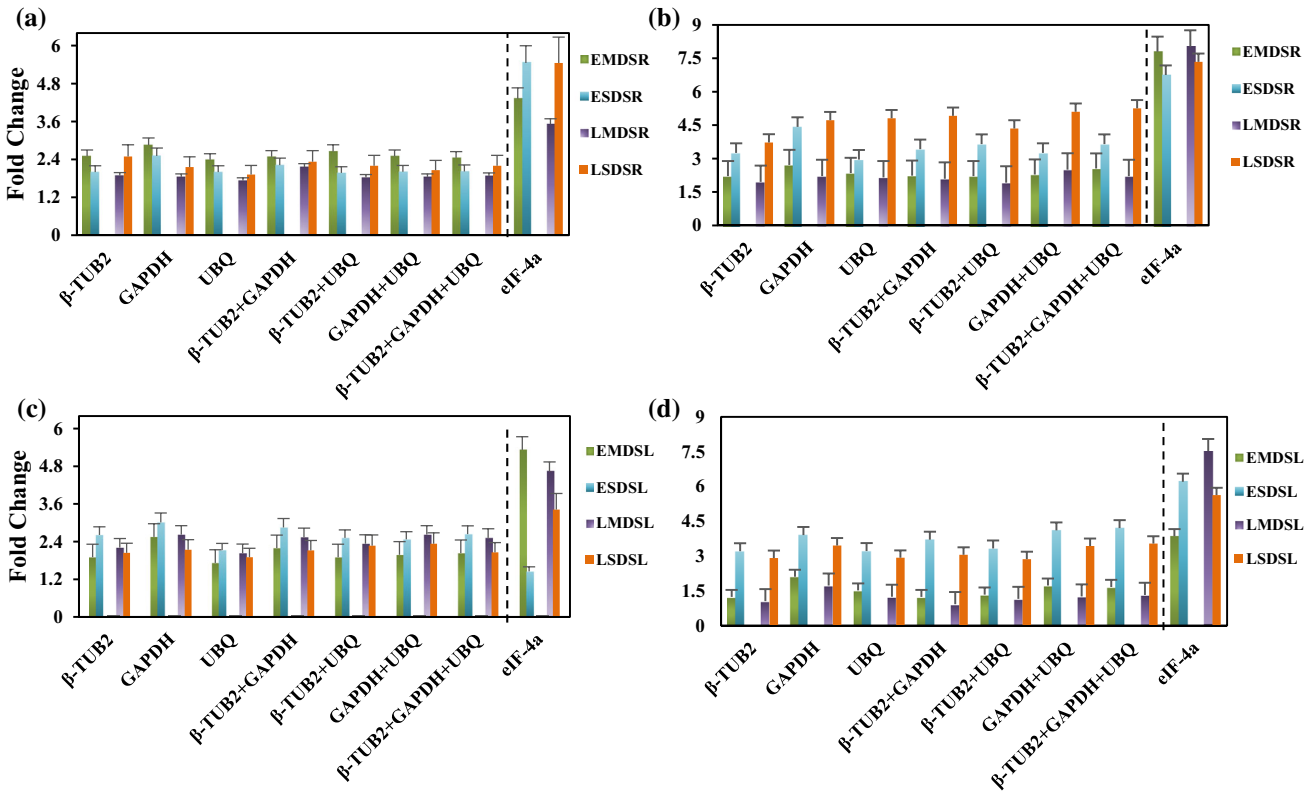

Figure 8. Validation of housekeeping genes under drought stress conditions. Expression profiling of target genes (a): *TaNAC29* in root, (b): *TaNAC6* in root, (c): *TaNAC29* in leaf and (d): *TaNAC6* in leaf) droughty imposed tissues and normalized with (i) *β-TUB* (ii) *GAPDH* (iii) *UBC* (iv) *β-TUB+GAPDH* (v) *GAPDH+UBC* (vi) *β-TUB+UBC* and (vii) *β-TUB+GAPDH+UBC* (left of the vertical dash line) and (viii) *eIF-4a* as the least stable gene (right of the vertical dash line). The analysis was completed in two different stages and tissues with three various drought levels.

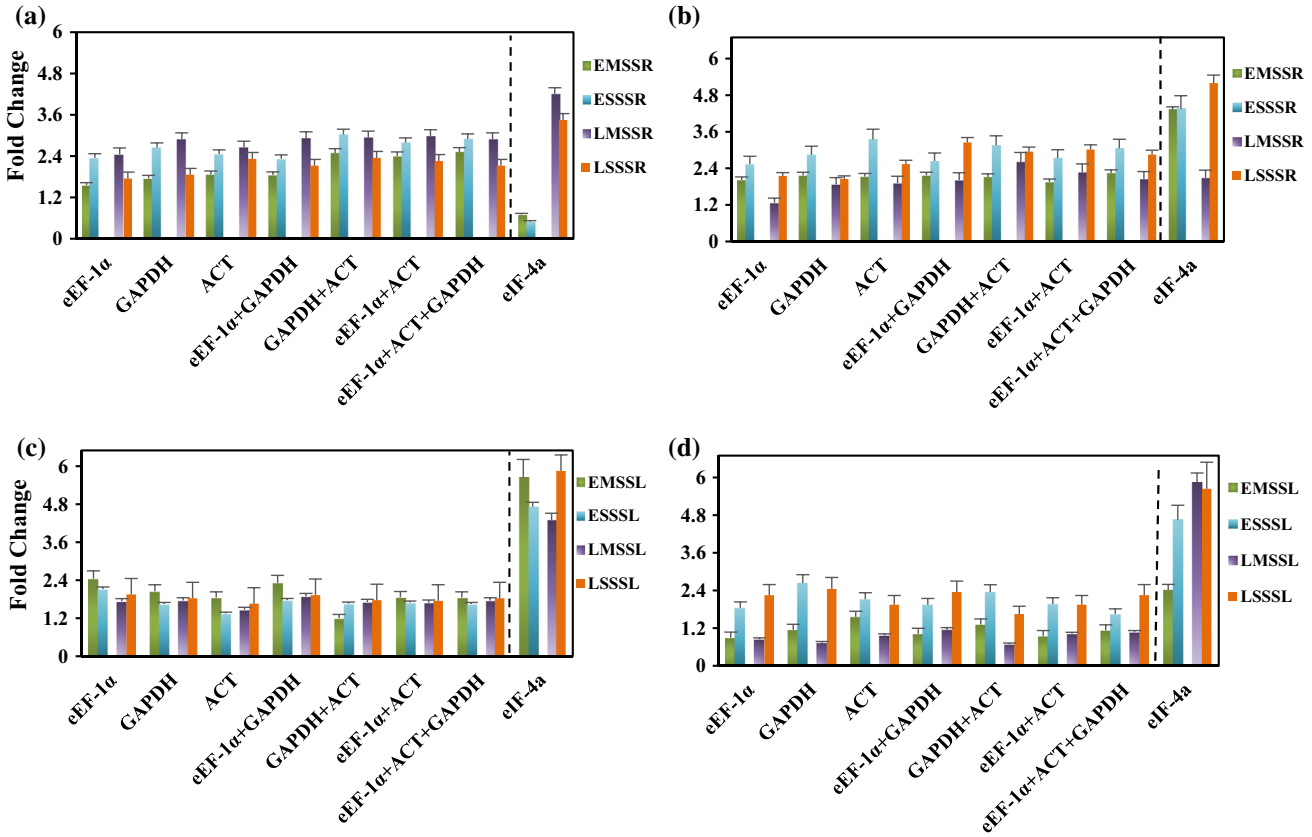

Figure 9. Validation of reference genes under salinity stress conditions. Expression profiling of candidate genes (a): *TaNAC29* in root, (b): *TaNAC6* in root, (c): *TaNAC29* in leaf and (d): *TaNAC6* in leaf) salinity imposed tissues and normalized with (i) *eEF-1α* (ii) *GAPDH* (iii) ACT (iv) *eEF-1α* + *ACT* (v) *eEF-1α* + *GAPDH* (vi) *GAPDH* + *ACT* and (vii) *eEF-1α* + *ACT* + *GAPDH* (left of the vertical dash line) and (viii) *eIF-4a* as the least stable gene (right of the vertical dash line). The analysis was completed in two different stages and tissues with three various salinity levels.

except NormFinder, which ranked *GAPDH* as the third most stably expressed gene. For salinity stress, *eEF-1α* and *GAPDH* were identified as the most stable reference genes by all programs except BestKeeper. Conversely, the least stable reference gene for salinity stress was identified as *eIF-4a* by all programs but BestKeeper. *EIF-4a* was also ranked as the least stable reference gene for drought by BestKeeper, delta Ct and RefFinder, but not GeNorm or NormFinder. The discrepancies observed between the results of these programs are likely due to differences between algorithms (Mallona *et al.* 2010; Mafra *et al.* 2012). BestKeeper specifies the optimum number of housekeeping gene by analysing the pair-wise correlation of all pairs of candidate genes (Pfaffl *et al.* 2004). GeNorm ranks reference genes by using a normalization factor based of the geometric mean of their expression level (Vandesompele *et al.* 2002). The NormFinder algorithm can identify candidate genes in large datasets because it can differentiate intragroup variation from intergroup variation (De Spiegelaere *et al.* 2015). The delta Ct method compares the relative expression of 'pairs of genes' in each sample to determine the ideal reference genes (Silver *et al.* 2006). RefFinder is a comprehensive tool that ranks reference genes according to the geometric mean of the individual gene weights calculated by the other four algorithms. Overall, we found significant, positive correlations ($r > 0.88$) between the results of the five programs. A comparison of GeNorm, Norm Finder and Bestkeeper found that the most and least stable genes identified by these programs were similar to human cell lines (De Spiegelaere *et al.* 2015).

Our results are in accordance with recent findings that identified *eEF-1α* as an accurate reference gene for sugarcane (Guo *et al.* 2014), soybean (Ma *et al.* 2013) under drought and salinity stress and Bermuda grass under drought stress (Chen *et al.* 2015). *GAPDH* was identified as a stable gene in different tissues and genotypes in sugarcane (Iskandar *et al.* 2004). Our results also found *β-TUB* and *UBQ* to rank highly as drought reference genes and *ACT* to be highly ranked under salinity stress conditions. *β-TUB* and *UBC* are widely applied as reference genes and demonstrated high stability under different environmental stresses in several species (Shivhare and Lata 2016). *ACT* is one of the housekeeping genes most commonly used

as an internal control (Li *et al.* 2010; Sun *et al.* 2016). GeNorm analysis found that, for durum wheat, a combination of three reference genes was sufficient accurately normalize gene expression during drought (*GAPDH*, *β-TUB2*, *UBQ*) and salinity (*eEF-1α*, *GAPDH*, *ACT*) stress conditions. Combinations of reference genes gave the most accurate normalization for qRT-PCR of tall fescue (*Festuca arundinacea*) under abiotic stress (Vandesompele *et al.* 2002; Huggett *et al.* 2005) and in human tissues (Yang *et al.* 2015).

Validation of the most stably expressed housekeeping genes and their combinations was conducted using the stress-responsive genes *TaNAC6* and *TaNAC29*. *NAC* genes are transcription factors that play a significant role in the response to stress by plants, including wheat (Xia *et al.* 2010; Baloglu *et al.* 2012). Our results demonstrated that combinations of *GAPDH*, *β-TUB2* and *UBQ* (for drought stress) or *eEF-1α*, *GAPDH* and *ACT* (for salt stress) are appropriate for transcript normalization in durum wheat.

In conclusion, this study identified *GAPDH*, *β-TUB2*, *UBQ* and *eEF-1α*, *GAPDH*, *ACT* as the most stable housekeeping genes for durum wheat under drought and salt stress conditions, respectively. The rankings of reference genes by programs BestKeeper, NormFinder, GeNorm, delta-Ct method and RefFinder were highly correlated. This is the first investigation of appropriate reference genes for durum wheat under abiotic stress.

Acknowledgements

We are grateful to Dr Ali Hadipour, Dr Arman Salehi and Dr Negar Salehi for editing this paper and especially Mrs Sheryl Nikpoor and Navid Jamshidi for their valuable comments. Also, we are thankful to anonymous referees who helped us to improve our paper.

References

Abdolshahi R., Safarian A., Nazari M., Pourseyedi S. and Mohamadi-Nejad G. 2013 Screening drought-tolerant genotypes in bread wheat (*Triticum aestivum* L.) using different multivariate methods. *Arch. Agron. Soil Sci.* **59**, 685–704.

Araus J., Slafer G., Reynolds M. and Royo C. 2002 Plant breeding and drought in C3 cereals: what should we breed for? *Ann. Bot.* **89**, 925–940.

Baloglu M. C., Oz M., Oktem H. A. and Yucel M. 2012 Expression analysis of TaNAC69-1 and TtNAMB-2, wheat NAC family transcription factor genes under abiotic stress conditions in durum wheat (*Triticum turgidum*). *Plant Mol. Biol. Rep.* **30**, 1246–1252.

Brennan J. P., Aw-Hassan A., Quade K. J. and Nordblom T. L. 2002 Impact of ICARDA research on Australian agriculture. *Econ. Res. Rep.* **11**, NSW Agriculture, Wagga Wagga.

Bustin S. A., Benes V., Garson J. A., Hellemans J., Huggett J., Kubista M. *et al.* 2009 The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611–622.

Cattivelli L., Rizza F., Badeck F.-W., Mazzucotelli E., Mastrangelo A. M., Francia E. *et al.* 2008 Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. *Field Crops Res.* **105**, 1–14.

Chen J., Rider D. A. and Ruan R. 2006 Identification of valid housekeeping genes and antioxidant enzyme gene expression change in the aging rat liver. *J. Gerontol. A Biol. Sci. Med. Sci.* **61**, 20–27.

Chen Y., Hu B., Tan Z., Liu J., Yang Z., Li Z. *et al.* 2015 Selection of reference genes for quantitative real-time PCR normalization in creeping bentgrass involved in four abiotic stresses. *Plant Cell Rep.* **34**, 1825–1834.

De Spiegelaere W., Dern-Wieloch J., Weigel R., Schumacher V., Schorle H., Nettersheim D. *et al.* 2015 Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One.* **10**, e0122515.

Dudley L. and Shani U. 2003 Modeling plant response to drought and salt stress. *Vadose Zone J.* **2**, 751–758.

Fischer M., Skowron M. and Berthold F. 2005 Reliable transcript quantification by real-time reverse transcriptase-polymerase chain reaction in primary neuroblastoma using normalization to averaged expression levels of the control genes HPRT1 and SDHA. *J. Mol. Diagn.* **7**, 89–96.

Gachon C., Mingam A. and Charrier B. 2004 Real-time PCR: what relevance to plant studies? *J. Exp. Bot.* **55**, 1445–1454.

Galli V., Borowski J. M., Perin E. C., da Silva Messias R., Labonde J., dos Santos Pereira I. *et al.* 2015 Validation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. *Gene* **554**, 205–214.

Goossens K., Van Poucke M., Van Soom A., Vandesompele J., Van Zeveren A. and Peelman L. J. 2005 Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Dev. Biol.* **5**, 27.

Guénin S., Mauriat M., Pelloux J., Van Wuytswinkel O., Bellini C. and Gutierrez L. 2009 Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *J. Exp. Bot.* **60**, 487–493.

Guo J., Ling H., Wu Q., Xu L. and Que Y. 2014 The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses. *Sci. Rep.* **4**, 7042.

Gutierrez L., Mauriat M., Guénin S., Pelloux J., Lefebvre J. F., Louvet R. *et al.* 2008 The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. *Plant Biotechnol. J.* **6**, 609–618.

Huggett J., Dheda K., Bustin S. and Zumla A. 2005 Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* **6**, 279–284.

Iskandar H. M., Simpson R. S., Casu R. E., Bonnett G. D., Maclean D. J. and Manners J. M. 2004 Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. *Plant Mol. Biol. Rep.* **22**, 325–337.

Jain M., Nijhawan A., Tyagi A. K. and Khurana J. P. 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.

Joshi S. and Nimbalkar J. 1983 Effect of salt stress on growth and yield in *Cajanus cajan* L. *Plant Soil.* **74**, 291–294.

Kavousi H. R., Marashi H. and Bagheri A. 2009 Expression of phenylpropanoid pathway genes in chickpea defense against race 3 of *Ascochyta rabiei*. *Plant Pathol. J.* **8**, 127–132.

Knight H. and Knight M. R. 2001 Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.* **6**, 262–267.

Kumar K., Muthamilarasan M. and Prasad M. 2013 Reference genes for quantitative real-time PCR analysis in the model plant foxtail millet (Setariaitalica L.) subjected to abiotic stress conditions. *Plant Cell Tissue Organ Cult.* **115**, 13–22.

Li Q. F., Sun S. S., Yuan D. Y., Yu H. X., Gu M. H. and Liu Q. Q. 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.

Ma S., Niu H., Liu C., Zhang J., Hou C. and Wang D. 2013 Expression stabilities of candidate reference genes for RT-qPCR under different stress conditions in soybean. *PLoS One*. **8**, e75271.

Mafra V., Kubo K. S., Alves-Ferreira M., Ribeiro-Alves M., Stuart R. M., Boava L. P. *et al.* 2012 Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PLoS One.* **7**, e31263.

Mallona I., Lischewski S., Weiss J., Hause B. and Egea-Cortines M. 2010 Validation of reference genes for quantitative real-time PCR during leaf and flower development in Petunia hybrida. *BMC Plant Biol*. **10**, 4.

Manoli A., Sturaro A., Trevisan S., Quaggiotti S. and Nonis A. 2012 Evaluation of candidate reference genes for qPCR in maize. *J. Plant Physiol*. **169**, 807–815.

Mir R. R., Zaman-Allah M., Sreenivasulu N., Trethowan R. and Varshney R. K. 2012 Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. *Theor. Appl. Genet.* **125**, 625–645.

Mittler R. 2006 Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* **11**, 15–19.

Mohammadi R., Farshadfar E. and Amri A. 2015 Interpreting genotype × environment interactions for grain yield of rainfed durum wheat in Iran. *Crop J.* **3**, 526–535.

Paolacci A. R., Tanzarella O. A., Porceddu E. and Ciaffi M. 2009 Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol. Biol.* **10**, 11.

Pfaffl M. W., Tichopad A., Prgomet C. and Neuvians T. P. 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.

Radonić A., Thulke S., Mackay I. M., Landt O., Siegert W. and Nitsche A. 2004 Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **313**, 856–862.

Shivhare R. and 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.

Scholtz J. J. and Visser B. 2012 Reference gene selection for qPCR gene expression analysis of rust-infected wheat. *Physiol. Mol. Plant Pathol.* **81**, 22–25.

Silver N., Best S., Jiang J. and Thein S. L. 2006 Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol*. **7**, 33.

Sinha P., Singh V. K., Suryanarayana V., Krishnamurthy L., Saxena R. K. and Varshney R. K. 2015 Evaluation and validation of housekeeping genes as reference for gene expression studies in pigeonpea (Cajanus cajan) under drought stress conditions. *PLoS One*. **10**, e0122847.

Sun H. P., Li F., Ruan Q.-m. and Zhong X.-h. 2016 Identification and validation of reference genes for quantitative real-time PCR studies in *Hedera helix* L. *Plant Physiol. Biochem*. **108**, 286–294.

Tuberosa R. 2012 Phenotyping for drought tolerance of crops in the genomics era. *Front. Physiol.* **3**, 347.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. *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).

Volkov R. A., Panchuk I. I. and Schöffl F. 2003 Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *J. Exp. Bot.* **54**, 2343–2349.

Wei L., Miao H., Zhao R., Han X., Zhang T. and Zhang H. 2013 Identification and testing of reference genes for Sesame gene expression analysis by quantitative real-time PCR. *Planta* **237**, 873–889.

Xia N., Zhang G., Sun Y.-F., Zhu L., Xu L.-S., Chen X.-M. *et al.* 2010 TaNAC8, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. *Physiol. Mol. Plant Pathol.* **74**, 394–402.

Yang Z., Chen Y., Hu B., Tan Z. and Huang B. 2015 Identification and validation of reference genes for quantification of target gene expression with quantitative real-time PCR for tall fescue under four abiotic stresses. *PLoS One*. **10**, e0119569.

Zhang S., Zeng Y., Yi X. and Zhang Y. 2016 Selection of suitable reference genes for quantitative RT-PCR normalization in the halophyte *Halostachys caspica* under salt and drought stress. *Sci. Rep*. **6**, 30363.

Corresponding editor: R. S. Sangwan