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RESEARCH ARTICLE

Identification of suitable internal control genes for transcriptional studies in *Eleusine coracana* under different abiotic stress conditions

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Abstract Finger millet [*Eleusine coracana* (L.) Gaertn] is an excellent food and forage crop of arid and semiarid areas in Africa and Asia. It is well adapted to drought, heat, high salinity, poor soil fertility and low pH with an efficient C_4 carbon fixation mechanism for high yield potential. To normalize the target gene expression data, the identification of suitable reference genes is essential. Ten candidate reference genes were selected and their expression stability was analyzed in various samples treated with different abiotic stress conditions. Five different statistical algorithms: geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder were used to determine the stability of these genes. Our results revealed *GAPDH, EEF1a, ACT* and *CYC* as highly stable reference genes and *PP2A* and *eIF4A* as least stable reference genes across all the samples and suggesting that these genes could be used for accurate transcript normalization under abiotic stress. To the best of our knowledge, this is the first report on identification of suitable reference genes for accurate transcript normalization using qRT-PCR in finger millet under abiotic stress.

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Introduction

Quantitative real-time PCR (qRT-PCR) is one of the most powerful and reliable techniques to quantify gene expression. This technique is widely used due to its sensitivity, accuracy and reproducibility in gene expression analysis (Artico et al. 2010; Jatav et al. 2016). Quantification assays are performed to detect differential expression of a gene(s) involved in plant growth, development, signal transduction and metabolism. Development of qRT-PCR has revolutionized the field of gene expression analysis (Barber et al. 2005; Bustin et al. 2009). Quantitative real-time PCR (qRT-PCR) has also been used for other purposes such as clinical diagnosis, gene expression analysis in a specific tissue, or studies involving complex experiments with large numbers of genes (Gachon et al. 2004; Nicot et al. 2005; Paolacci et al. 2009). Although qRT-PCR is a widely used and reliable technique, it is strongly affected by factors such as differences in initial sample amount, quality of RNA (integrity), efficiency of cDNA synthesis, primer performance, and normalization and differences in the overall transcriptional activity of the sample (Bustin 2002, 2010; Bustin et al. 2009; Edmunds et al. 2014). To avoid any such variations and biased results, an appropriate normalization method becomes imperative for obtaining reliable quantitative gene expression data. Normalization of target gene expression requires the selection of a stable internal control gene (ICG) (Bollmann et al. 2012; Guénin et al. 2009). Housekeeping genes are commonly used as ICG as most of them typically have stable expression patterns under specific conditions (Bohle

et al. 2007; Chen et al. 2011a; Yan and Liou 2006) but there are reports which indicate irregular expression of ICG under non-specific conditions (Li et al. 2012; Llanos et al. 2015; Petriccione et al. 2015; Raffaello and Asiegbu 2013; Yin et al. 2013). Large differences between the absolute expression of an ICG and target gene will mislead the actual differences in target gene expression levels between samples (Rieu and Powers 2009). Therefore, it is very necessary to systematically evaluate the stability of potential ICGs for particular experimental system prior to adopting them for use in qRT-PCR normalization strategies. A number of efforts have been made to identify the stably expressed reference genes in different plants including*Arabidopsis* (Czechowski et al. 2005; Remans et al. 2008), rice (Jain et al. 2006; Kim et al. 2003), tobacco (Schmidt and Delaney 2010), wheat (Paolacci et al. 2009), Pearl millet (Saha and Blumwald 2014), soybean (Jian et al. 2008; Libault et al. 2008), coffee (Barsalobres-Cavallari et al. 2009; Cruz et al. 2009), cotton (Artico et al. 2010; Tu et al. 2007), *Brassica napus* (Wang et al. 2014), chicory (Maroufi et al. 2010), cucumber (Wan et al. 2010), pepper (Wan et al. 2011), banana (Chen et al. 2011b), apple (Perini et al. 2014), petunia (Mallona et al. 2010), rose (Klie and Debener 2011; Meng et al. 2013) and tree species (Lin and Lai 2010; Brunner et al. 2004). The frequently used reference genes include elongation factor 1a (EF1a), actin (ACT), ubiquitin-conjugating enzyme (UBC), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tubulin (TUB) and 18S ribosomal RNA (18S rRNA) which are involved in basic cellular functions (Thellin et al. 1999). No single reference gene with stable constant expression across tissues and experimental conditions is available therefore,we performed an investigation to find suitable reference genes in finger millet.

Numerous statistical algorithms such as Stability Index (Brunner et al. 2004), BestKeeper (Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002), ΔCt (Silver et al. 2006), RefFinder (Chen et al. 2011a) and NormFinder (Andersen et al. 2004) have been developed for validation and stability ranking of suitable reference genes for qRT-PCR data normalization.

Finger millet [*Eleusine coracana* (L.) Gaertn] is an excellent food and forage crop of arid and semiarid areas in Africa and Asia. After sorghum, pearl millet, and foxtail millet, it ranks fourth globally as important millets (Gupta et al. 2012; Kothari et al. 2005). Finger millet has a high amount of calcium (0.38%), fiber (18%), phenolic compounds (0.3–3%), and sulphur containing amino acids (Devi et al. 2014; Rurinda et al. 2014; Shobana et al. 2013; Singh and Raghuvanshi 2012). Finger millet is a rich source of tryptophan, cysteine, methionine, and total aromatic amino acids compared to the other cereals (NRC 1996). It is well adapted to drought, heat, high salinity, soil containing Nickel, poor soil fertility and low pH with an efficient C_4 carbon fixation and high yield potential (Dass et al. 2013; Gupta et al. 2017; Hegde and Gowda 1989; Zakeri et al. 2013). So finger millet is an ideal crop for functional genomics studies related to C_4 photosynthesis and abiotic stress tolerance (Zakeri et al. 2013).

To the best of our knowledge, no such study regarding systematic analysis for the identification of suitable reference genes in finger millet have been reported, although functional genomics studies have been reported (Singh 2014). In present study, a panel of candidate reference genes was evaluated under abiotic stress for accurate transcript normalization which is much needed in finger millet.

Materials and methods

Plant materials

Five genotypes of finger millet (*Eleusine coracana* [L.] Gaertn), PR 202, PES 110, GPU 28, GPU 20 and GPU 66 were used in this study and on the basis of germination rate, PR 202 and GPU 28 were selected for stress study. All the genotypes were procured from University Agriculture Sciences, GKVK, Bengaluru. Seeds were thoroughly washed with 20% (v/v) Extran® (Merck, India) for 3 min, followed by rinsing with distilled water three times. Surface sterilization was done with a 0.1% $HgCl_2$ (Merck, India) solution (w/v) for 3 min and then rinsed thrice with sterile distilled water for further use.

Seed germination and stress treatment

Seeds were sown in composite soil (peat:vermiculite:sand mixture, 2:2:1) in controlled environmental condition at 26 ± 1 °C under a 16 h photoperiod with 25 µmol m^{-2} - s^{-1} photon flux and relative humidity oscillating between 40 and 70% at Department of Botany, University of Rajasthan, Jaipur, India. Plants were irrigated with water and 30% strength Hoagland solution (HiMedia, India) on alternate days. After 15 days of germination, finger millet seedlings of each genotype were used for stress treatment. Before stress treatment, they were precultured for 24 h in 30% Hoagland's solution (Lata et al. 2011; Zhang et al. 2007). According to different stress conditions, the seedlings were divided into 13 groups, each containing 10 seedlings, out of which 12 groups were subjected to abiotic stresses treatment (1 group for drought, 3 for salinity, 4 for heat stress and 4 for cold stress). For drought treatment, seedlings were transferred on solution containing 20% PEG 6000. For salinity stress, a range of saline solutions 100, 200, 300 mM NaCl were prepared. Likewise for heat and

cold stress, seedlings were transferred to the solution containing 30% Hoagland medium in plant growth chamber (Thermo, USA) at 38°, 40°, 42° and 45° for heat stress and 4°, 6°, 8°, 10° for cold stress.

Isolation of total RNA, cDNA synthesis and primers

Total RNA was extracted from the different plant samples using Trizol method (Invitrogen). RNA was purified using RNAse free DNase treatment through silica columns (Ribopure^{TM} RNA Purification Kit, Ambion, Thermo, USA). The integrity of the total RNA was checked on 1% agarose gel. Quantity and quality of RNA were evaluated by measuring the optical density at 260 nm and the A260/280 absorption ratio using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and samples were stored at − 80° for further use. cDNA was synthesized using 100 ng RNA with RevertAid RT Reverse transcription Kit (Thermo Fisher Scientific, USA). The mixture of RNA, dNTP mix, random primers and nuclease free water was incubated at 65 °C for 5 min and kept on ice for 3 min. Master mix containing, 5× enzyme buffer, 1 µL Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT) (Fermentas) was added, 6 µL to each sample. The reaction cycle in cycler used the following program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with nuclease free water. To perform the qRT-PCR analysis, primers were either selected from the literature or designed using Primer Express 3.0 software (Applied Biosystem, Foster City, CA, USA). Primer details for all candidate internal control genes are given in Table 1.

PCR and qRT-PCR

Prior to qPCR, specific amplification of all 10 primer pairs was further confirmed in a 20 µL PCR reaction. PCR reaction mixture (20 µL) contained 10× PCR buffer, 10 mM dNTPs mix, 10 µM each of forward and reverse primers, 1 µL of cDNA and 0.2 µL of *Taq* polymerase. PCR amplification protocol followed was: an initial denaturation step of 5 min at 95 °C, and 30 cycles of 30 s at 94 °C, 30 s at annealing temperature of primer and 30 s at 72 °C followed by final extension step of 7 min at 72 °C. Five microliters of the PCR product was evaluated in 2% agarose gel stained with ethidium bromide. qRT-PCR was performed using Step One Plus instrument (Applied Biosystem) in a 96-well optical plate (Applied Biosystem) utilizing SYBR Green detection chemistry. Each reaction mixture contained 4 µL five fold diluted cDNA (equal to 50 ng of initial amount of RNA), with 6 µL of a mixture composed of 5 µL 2× SYBR Green with ROX master mix (Fermentas), 0.4 µL each of 10 pmol gene-specific forward and reverse primers and 0.2 µL DNase/RNase free water. Two biological replicates for each sample were used and two technical replicates were analyzed for each biological sample along with 6-point relative standard curve and negative control for each gene. The real time PCR amplification conditions were as follows: an initial denaturation step of 1 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 60 °C (Annealing + extension) followed by a dissociation protocol with incremental temperatures of 95 °C for 15 s plus 65 °C for 15 s was used to investigate the specificity of qPCR reaction and presence of primer dimers.

Data analysis for expression stability of internal control genes

Five different types of statistical algorithms namely geNorm, NormFinder, BestKeeper, ΔCt and reffinder (Chen et al. 2015) (http://leonxie.esy.es/RefFinder/?type=reference#) were employed to determine the expression stability of the 10 potential internal control genes across all experiment sets. The Ct values of all internal control genes used in NormFinder (http://www.mdl.dk/) and geNorm (http://medgen.ugent.be/~jvdesomp/geNorm/) were converted into relative quantities using the formula $2^{-\Delta Ct}$ where ΔCt is the value obtained after subtracting minimum Ct value from each corresponding Ct value (ΔCt = each corresponding Ct value—minimum Ct value) (Vandesompele et al. 2002). The expression stability is measured as "M" value by geNorm which is based on the overall pairwise variations comparison among the reference genes. "M" value is inversely correlated to gene expression stability and the genes were ranked according to the "M" value. To determine the minimum number of reference genes for optimal normalization, geNorm also calculates Pair-wise variation (V) between the normalization factor (NF) obtained using "n" genes (best references) (NFn) and the NF obtained using n + 1 genes (addition of an extra less stable reference gene) (NFn + 1). For the pair-wise variation (V value) a cut off value 0.15 was considered acceptable.

As an alternative approach we used NormFinder (Andersen et al. 2004) to evaluate the stability among ICGs, in this algorithm CT values were transformed to linear scale expression quantities. Normfinder ranked the candidate ICG by calculating expression stabilities of the candidate reference genes by combining the intra- and inter-group variations in a sample set containing any number of samples organized in any number of groups.

Another statistical algorithm used was Bestkeeper to find out the pair-wise correlations among the candidate ICG (Pfaffl et al. 2004). For Bestkeeper, raw Ct values instead of relative quantities were used as inputs. The

Table 1 Details of primer sequences, accession no., amplicon size and PCR efficiency of candidate reference genes used for qRT-PCR

Gene name	Description	Primer pair 5′–3′	Amplicon size (bp)	Accession no.	PCR efficiency %
ACT	Actin	F-TGCTCAGTGGAGGATCTACTAT	108	HM243500	101.02
		R-CTGGTGGTGCAATCACTTTAAC			
CYC	Cyclophilin, peptidyl-prolyl cis–trans isomerase	F-TTTCGCTCTGACAGACCTTTAG	138	Si014078m.g	102.39
		R-TGGCAGTAGTAGTAGGGAGAAG			
*eEF*1α	Eukaryotic elongation factor 1 alpha	F-GTTACAACCCAGACAAGATTGC	72	EF694165	109.11
		R-TGGACCTCTCAATCGTGTTG			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F-CTGGTATGTCCTTCCGTGTTC	109	GQ398107	122.37
		R-GCAGCCTTGATAGCCTTCTTA			
*TIP*41	Tonoplast intrinsic protein	F-GGTTCTGAACTCAGGCACTAC	96	Si036884m.g	92.08
		R-GAAAGGGCAACAAGGTCAATC			
*UBC*2	Ubiquitin-conjugating enzyme 2	F-CATGGACCCTTCAGTCTGTTT	115	Si018564m.g	98.54
		R-CCTCGGATATCACCCGATCTTA			
*UBQ*5	Ubiquitin 5	F-GTACACCAAGCCCAAGAAGA	79	Si003209m.g	102.91
		R-GTCGTCGACCTTGTAGAACTG			
eIF4A	Eukaryotic initiation factor 4A	F-GCGTGCATGTTGTTGTTGGT	60	EU856535	103.65
		R-GTGACTGCCTACGCAACATGTC			
PP2A	Protein phosphatase 2A	F-TGGTAACATGGCTGCAATTC	95	Si017892m	120.48
		R-AAATTGAGCCGGACACGA			
RPL20	Ribosomal protein L20	F-CCCGTGTTGCTGCGTTTAT	62	KJ490012	132.54
		R-GGGTAGGCAAAAGAGGGATTTT			

program has its assumption that the genes which are stably expressed should be highly correlated to each other. The overall recommended inclusive geomean ranking values of the most suitable reference gene(s) were prevailed using the ranking results of geNorm and NormFinder algorithms in the RefFinder online tool.

Results

Sample size, RNA quality and qRT-PCR conditions

A total of 58 samples from two accessions of finger millet were treated with different kinds of abiotic stresses. RNA extracted from each sample exhibited high quality as determined by then A260/280 ratio. Quality of RNA was checked on agarose gel and NanoDrop 2000 spectrophotometer. To identify suitable ICGs in finger millet, analysis of stressed samples was performed using potential reference genes reported in literature. In the beginning, all the selected reference genes were amplified with a normal thermal cycler and the amplified product was checked on 2% agarose gel for single band and its specific size. Ten reference genes were selected on the basis of primer which did not allow any dimers, non specific bands and poor amplifications across the samples (Table 1). Amplification efficiency observed for each candidate reference gene ranged from 92.08 to 132.54%. Average Ct value for all 10 candidate reference genes ranged from 16.93 (*EEF*1*a*) to 26.86 (*TIP*41) (Table 2). The stability in expression level of each of the candidate reference gene based on their Ct value is shown in box whisker plot (Fig. 1). Box-whisker plot clearly indicate the *EEF*1*a*, *RPL*20 and *ACT* as most abundant genes, while *UBC* 2, *PP2A* and *TIP*41 were least abundant genes in finger millet stressed samples.

Gene expression stability and ranking of internal control genes by the geNorm analysis

geNorm analysis used in the present study to rank 10 internal control genes on the basis of average expression stabilities (M) using pairwise comparison is shown in Fig. 2. "M" value of all the genes was observed to be within the acceptable range (< 1.5).

"M" value is inversely correlated to gene expression stability and *ACT* and *GAPDH* exhibiting the lowest "M" value (0.471) were the most stably expressed amongst all 10 candidate reference genes across all finger millet

Table 2 Expression levels of different housekeeping genes under study in all stress conditions

Genes	Overall Ct ± SD	Heat Ct ± SD	Cold Ct ± SD	Salinity Ct ± SD	Drought Ct ± SD
UBQ5	21.32 ± 0.94	20.62 ± 1.07	21.55 ± 0.72	21.70 ± 0.66	21.49 ± 1.31
UBC2	23.12 ± 1.00	23.06 ± 0.62	22.54 ± 0.80	24.25 ± 1.10	23.06 ± 0.46
ACT	22.52 ± 1.17	23.31 ± 1.09	22.44 ± 0.72	22.17 ± 1.20	20.57 ± 0.34
TIP41	25.49 ± 0.90	25.25 ± 0.57	25.45 ± 0.82	25.75 ± 1.14	26.86 ± 0.21
PP2A	24.43 ± 1.48	23.27 ± 0.65	24.87 ± 1.26	25.51 ± 0.88	26.25 ± 0.32
GAPDH	21.73 ± 1.01	22.14 ± 1.12	21.69 ± 0.69	21.57 ± 1.07	20.16 ± 0.12
EEF1a	18.62 ± 1.10	19.27 ± 1.35	18.72 ± 0.62	18.00 ± 0.55	16.93 ± 0.34
CYC	24.83 ± 1.04	25.06 ± 1.140	24.89 ± 0.60	24.87 ± 1.09	23.44 ± 0.56
RPL20	22.55 ± 0.69	22.82 ± 0.36	22.59 ± 0.63	21.72 ± 0.45	22.83 ± 0.69
eIF4A	24.31 ± 1.44	25.50 ± 1.21	24.36 ± 1.05	23.48 ± 1.07	21.99 ± 0.54

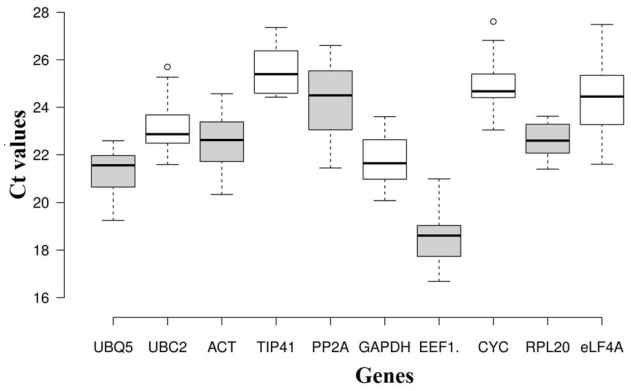

Fig. 1 Gene expression of ten candidate reference genes in all samples. Line across the box depict the median value and inside box show the Ct values. The top and bottom whiskers were determined by the 5th and 95th percentiles, respectively

samples followed by *EEF*1*a* and *CYC* (Table 3). While *PP2A* and *UBC*2 had the highest "M" value indicating that they had least stable expression across all the samples followed by *TIP*41 and *UBQ*5. As shown in Fig. 3, *GAPDH* and *EEF*1*a* were the most stable reference genes in heat stress and cold stress conditions while *GAPDH* and *eIF4A* were best stable in salinity stress and *ACT* and *EEF*1*a* genes were found to be most stable in drought stress treatment. Stability of different reference genes was also evaluated separately in genotype PR202 and GPU 28. In PR202, *ACT* and *GAPDH* gene were most stable across all treatments while *PP2A* and *TIP*41 was found least stable (Fig. 2). Likewise in genotype GPU 28, *EEF*1*a* and *GAPDH* were found to be most stable internal control genes whereas *UBC*2 and *PP2A* were least stable reference genes (Fig. 2). The pairwise variation (Vn/Vn + 1) analysis was performed using geNorm between two sequential normalization factors NFn and NFn + 1 to determine the optimal number of reference genes for accurate normalization of qRT-PCR data. In pairwise variation, a cutoff value of 0.15 is recommended indicating that an additional candidate reference gene will make no significant contribution to the normalization. The $V_{4/5}$ was < 0.15 which suggests that the top 4 reference genes were sufficient for normalization of qRT-PCR data (Fig. 4).

Gene expression stability and ranking of internal control genes by the NormFinder analysis

To identify the most stable reference gene by NormFinder, the Ct values were converted to relative quantities (Table 3). *GAPDH* with stability value 0.119 was found to be most stable gene across all samples followed by *EEF*1*a* (0.153) and *ACT* (0.156) as second and third most stable genes respectively. *PP2A* was found to be least stable gene with stability value 0.320 when used as universal reference gene among all samples. The combination of two genes *GAPDH* and *EEF*1*a* was observed to be most stable with stability value 0.097. The ACT gene which was most stable in geNorm was replaced by *EEF*1*a* gene in Norm Finder. The ranking of most stable expressed gene (*GAPDH*) and least stable expressed gene (*PP2A*) was identical between geNorm and Norm Finder. On the basis of individual stress study, no similar gene was found to be most stable in all four stress conditions, thereby in control, *UBQ*5; in heat stress, *RPL*20; in cold stress, *ACT*; in salinity stress, *EEF*1*a* and in drought stress, *GAPDH* was calculated as most stable reference gene by geNorm (Table 4). Norm Finder analysis was also performed for each accession separately in PR 202 and GPU 28. In PR 202 gene *CYC* with stability value 0.457 was found to be most stable across all treatments and *PP2A* was identified as least stable gene with stability value $M > 1$ (1.111). Likewise in GPU 28,*GAPDH* was identified as most stable gene with stability value 0.479 and the least stable gene was *PP2A* with stability value 1.251. Inter and intra group variations were also evaluated by NormFinder. Intra group variations in PR 202; *UBC2, ACT* and *RPL* 20 genes were more stable in control; *CYC*, *RPL* 20 and *PP2A* genes were more stable in heat stress; *EEF*1*a*, *ACT* and *TIP*41 genes were more stable in cold; *UBQ* 5,

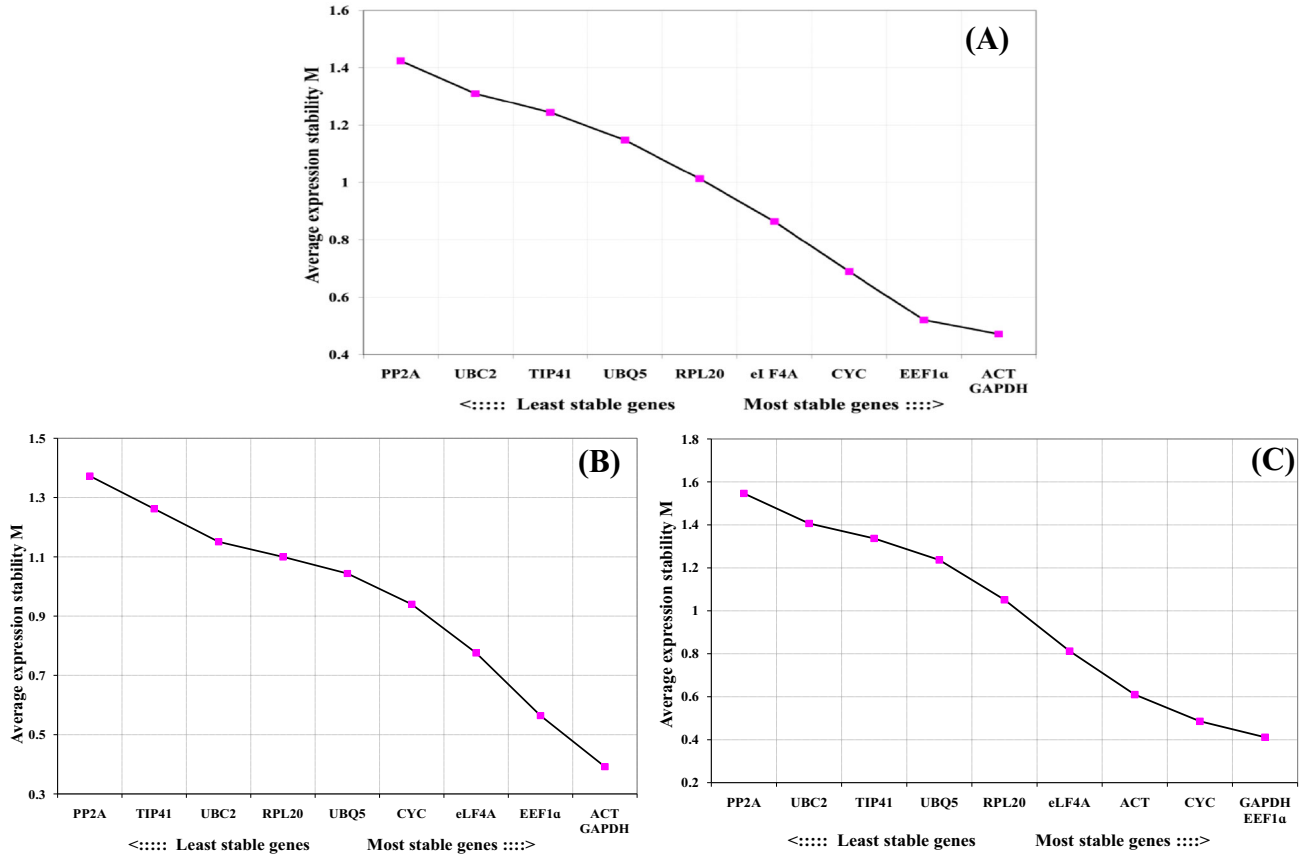

Fig. 2 Expression stability and ranking of 10 candidate reference genes as calculated by geNorm across all stress conditions **a** all samples of finger millet **b** all samples of genotype PR202 **c** all samples of genotype GPU 28

Table 3 Stability of candidate reference genes across all samples using different algorithms in finger millet

Rank	geNorm		NormFinder		BestKeeper		ΔCt		RefFinder	
	Gene	Normalization value	Gene	Stablity value	Gene	SD (± CP)	Gene	Average of STDEV	Gene	Geomean of ranking value
1	ACT | GAPDH	0.471	GAPDH	0.119	RPL20	0.54	GAPDH	1.16	GAPDH	1.57
2			EEF1a	0.153	TIP41	0.75	EEF1a	1.23	ACT	2.91
3	EEF1a	0.52	ACT	0.156	UBQ5	0.76	ACT	1.24	EEF1a	3.03
4	CYC	0.689	CYC	0.162	CYC	0.76	CYC	1.31	RPL20	3.5
5	eIF4A	0.864	RPL20	0.174	UBC2	0.77	RPL20	1.37	CYC	4
6	RPL20	1.013	UBQ5	0.196	GAPDH	0.84	UBQ5	1.45	UBQ5	5.24
7	UBQ5	1.148	UBC2	0.204	EEF1a	0.85	TIP41	1.5	TIP41	5.29
8	TIP41	1.244	TIP41	0.215	ACT	0.97	UBC2	1.55	UBC2	7.33
9	UBC2	1.31	eIF4A	0.247	eIF4A	1.15	eIF4A	1.56	eIF4A	7.77
10	PP2A	1.424	PP2A	0.32	PP2A	1.25	PP2A	1.88	PP2A	10

*EEF*1*a* and *TIP*41 genes were more stable in salinity and similarly *GAPDH*, *CYC* and *UBQ* 5 genes were more stable in drought stress. The inter group variation in all treatments showed *CYC*, *RPL* 20 and *GAPDH* genes to be more stable (Fig. 5). Similarly intra group variations in GPU 28, genes *EIF*4*A*, *RPL* 20 and *PP*2*A* were more stable in control; *ACT*, *RPL* 20 and *GAPDH* genes were more stable in heat stress; *GAPDH*, *EEF*1*a* and *ACT* genes

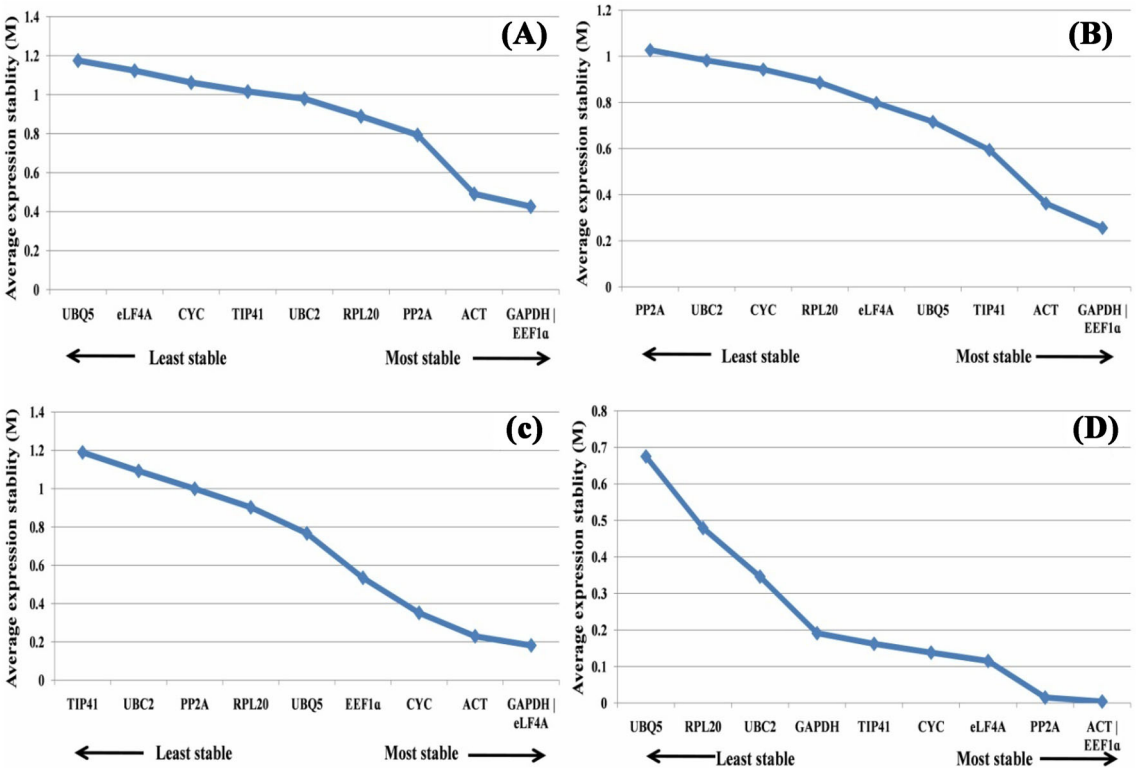

Fig. 3 Expression stability and ranking of 10 candidate reference genes as calculated by geNorm in individual stress condition **a** heat stress treatment **b** cold stress treatment **c** salinity stress treatment **d** drought stress treatment

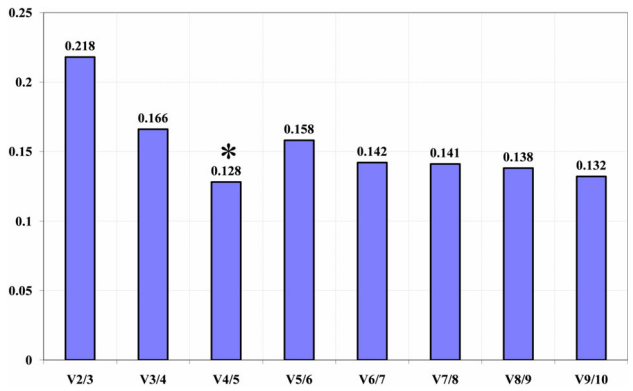

Fig. 4 Determination of the optimal number of reference genes for normalization by pairwise variation (V) across all samples of finger millet using geNorm. The average pairwise variations (Vn/Vn + 1) were analyzed to measure the effect of adding reference gene on the qRT-PCR

were more stable in cold; *UBC*2, *EIF*4*A* and *GAPDH* genes were more stable in salinity and similarly *UBQ* 5, *RPL* 20, *PP*2*A* and *EEF*1*a* genes were more stable in drought stress. The inter group variation in all treatments showed *GAPDH*, *ACT* and *UBQ* 5 genes to be more stable (Fig. 5).

Gene expression stability and ranking of internal control genes by the BestKeeper analysis

To find the optimal candidate reference genes on the basis of pair-wise correlation between the genes, Beestkeeper analysis was performed. A comparative analysis across potential reference genes was performed by estimating correlations of the expression levels between all reference genes. The Bestkeeper analysis suggested that *RPL*20 (0.54) was the most stables reference gene followed by *TIP*41 (0.75), *CYC* (0.76) and *UBQ*5 (0.76) while *PP*2*A* (1.25), *eIF*4*A* (1.15) and ACT (0.97) were least stable genes across all the samples (Table 3). Expression stability of reference genes in individual stress condition as calculated using BestKeeper algorithm and it was found that *TIP*41 in control, *RPL*20 in heat and *EEF*1*a* in cold were most stable reference genes while in salinity and drought stress *RPL*20 and *GAPDH* were most stable reference genes (Table 5). Expression stability of all the genes was also analyzed separately in both the accessions PR202 and GPU28. *UBC*2 was found most stable followed by *RPL*20 and *CYC* whereas *PP*2*A* was found least stable in PR202 among all sample sets. Similarly, *EEF*1*a* was found most stable followed by *CYC* and *GAPDH* while *PP*2*A* was least stable in all sample sets of GPU 28.

Table 4 Expression stability of candidate reference genes as calculated by Normfinder

Rank	Control		Heat		Cold		Salinity		Drought	
	Gene	Stablity	Gene	Stablity	Gene	Stablity	Gene	Stablity	Gene	Stablity
1	UBQ5	0.054	RPL20	0.514	ACT	0.3	EEF1a	0.329	GAPDH	0.043
2	GAPDH	0.054	ACT	0.594	EEF1a	0.337	eIF4A	0.654	TIP41	0.097
3	RPL20	0.37	GAPDH	0.643	GAPDH	0.436	GAPDH	0.682	PP2A	0.288
4	TIP41	0.468	PP2A	0.748	UBQ5	0.661	CYC	0.77	ACT	0.313
5	EEF1a	0.501	EEF1a	0.855	TIP41	0.666	UBQ5	0.777	EEF1a	0.318
6	PP2A	0.636	UBC2	0.858	eIF4A	0.87	ACT	0.839	UBC2	0.467
7	ACT	0.638	TIP41	0.917	RPL20	0.894	RPL20	0.844	eIF4A	0.576
8	eIF4A	0.693	CYC	1	CYC	0.933	PP2A	1.043	CYC	0.59
9	UBC2	0.903	eIF4A	1.112	UBC2	0.962	UBC2	1.23	RPL20	0.741
10	CYC	0.919	UBQ5	1.137	PP2A	1.022	TIP41	1.403	UBQ5	1.452

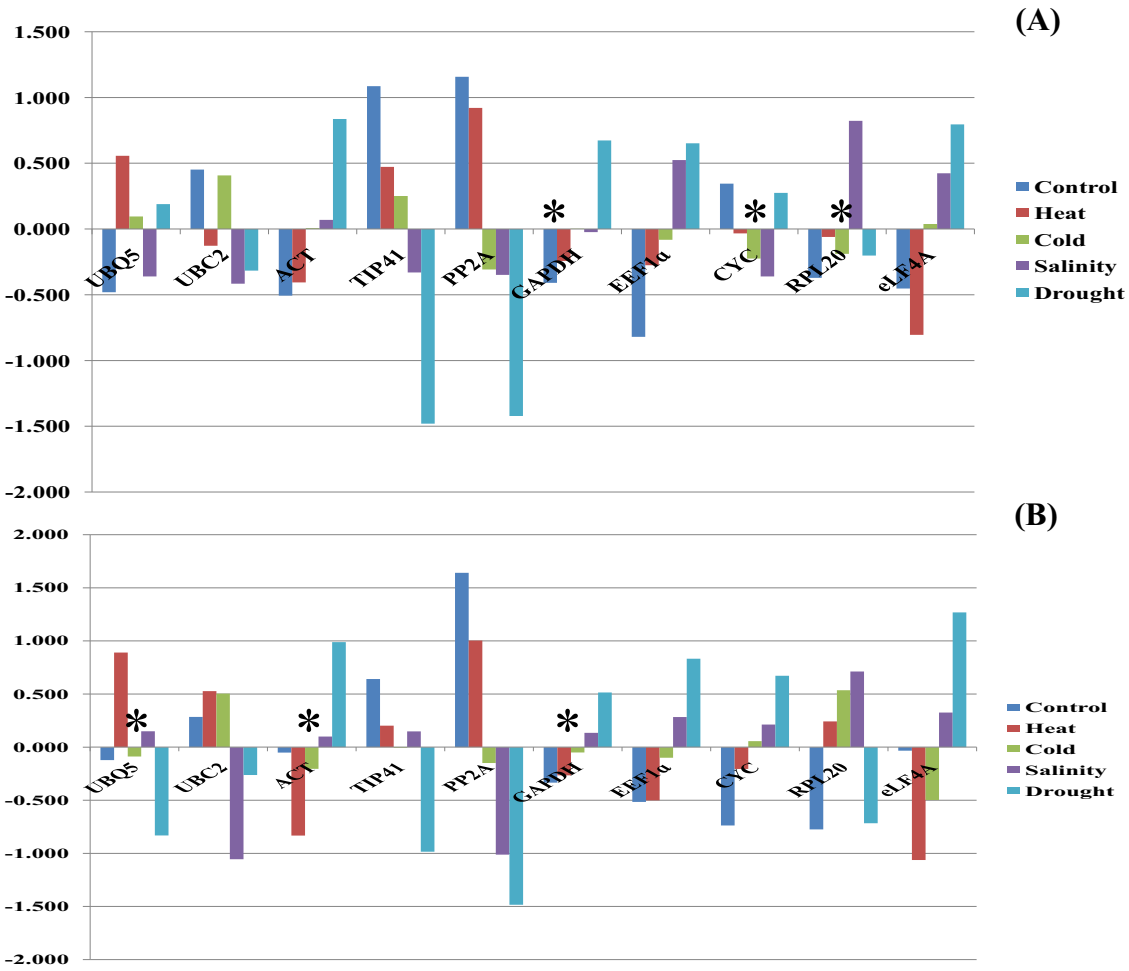

Fig. 5 Normfinder based inter-group variation analysis of candidate reference genes across all stress conditions in both genotypes individually **a** PR202 **b** GPU 28. *Most stable reference genes across all the samples

Table 5 Expression stability of candidate reference genes as calculated by BestKeeper

Rank	Control			Heat			Cold			Salinity			Drought		
	Genes	SD	CV	Gene	SD	CV	Genes	SD	CV	Gene	SD	CV	Genes	SD	CV
1	TIP41	0.05	0.22	RPL20	0.31	1.34	EEF1a	0.34	1.81	RPL20	0.37	1.69	GAPDH	0.09	0.44
2	RPL20	0.11	0.47	TIP41	0.44	1.73	CYC	0.49	1.97	UBQ5	0.48	2.2	TIP41	0.15	0.56
3	CYC	0.22	0.87	UBC2	0.52	2.24	RPL20	0.46	2.03	EEF1a	0.4	2.21	PP2A	0.23	0.87
4	UBC2	0.21	0.92	PP2A	0.56	2.41	GAPDH	0.46	2.11	PP2A	0.68	2.67	ACT	0.24	1.17
5	GAPDH	0.4	1.81	ACT	0.89	3.81	UBQ5	0.54	2.5	CYC	0.73	2.92	UBC2	0.33	1.42
6	UBQ5	0.48	2.2	eIF4A	0.99	3.87	ACT	0.56	2.5	eIF4A	0.82	3.49	EEF1a	0.24	1.44
7	eIF4A	0.84	3.46	UBQ5	0.89	4.31	TIP41	0.64	2.52	TIP41	0.96	3.74	CYC	0.4	1.69
8	ACT	0.8	3.55	GAPDH	0.96	4.35	UBC2	0.58	2.56	UBC2	0.93	3.81	eIF4A	0.39	1.77
9	PP2A	0.8	3.61	CYC	1.11	4.43	eIF4A	0.77	3.16	GAPDH	0.83	3.84	RPL20	0.49	2.15
10	EEF1a	0.72	3.76	EEF1a	1.16	6.02	PP2A	0.95	3.82	ACT	0.94	4.24	UBQ5	0.93	4.33

Gene expression stability and ranking of internal control genes by the ΔCt analysis

ΔCt method was used to compare the relative expression of an internal control gene within every sample, it gives rank on the basis of average of standard deviation (STDEV). Analysis using this method is shown in Table 3. It was observed that *GAPDH* (1.16), *EEF*1*a* (1.23), *ACT* (1.24) and *CYC* (1.31) were more stable reference genes across all the samples (Table 3). Ten candidate reference genes showing highest expression to least expression in individual stress conditions is shown in Table 6. In control, heat, cold, salinity and drought stress: *GAPDH*, *RPL*20*, ACT*, *EEF*1*a* and *PP*2*A* were most stable reference gene.

Gene expression stability and ranking of internal control genes by the RefFinder analysis

Stability ranking of 10 candidate reference genes was further confirmed using comprehensive ranking method by RefFinder across all samples. RefFinder is an online web tool which compares the data obtained from geNorm, NormFinder, ΔCt and BestKeeper and gives comprehensive ranking to authenticate the stability ranking via different softwares. According to the RefFinder tool on the basis of geomean ranking values, *GAPDH* was found to be most stable gene followed by *ACT* and *EEF*1α. The Geomean values were between 1.57 to 10.0 for all samples and *PP*2*A* was least stable gene (Table 3). For reliable identification of most stable internal control genes, 10 candidate reference genes analyzed using four different algorithms were incorporated by RefFinder and the complete results

Table 6 Expression stability of candidate reference genes as calculated by ΔCt

Rank	Control		Heat		Cold		Salinity		Drought	
	Gene	Stablity	Gene	Stablity	Gene	Stablity	Gene	Stablity	Gene	Stablity
1	GAPDH	0.55	RPL20	1.01	ACT	0.8	EEF1a	0.97	PP2A	0.47
2	UBQ5	0.55	ACT	1.02	EEF1a	0.81	eIF4A	0.99	TIP41	0.47
3	EEF1a	0.63	GAPDH	1.03	GAPDH	0.85	GAPDH	1	ACT	0.48
4	RPL20	0.64	PP2A	1.13	UBQ5	1	ACT	1.09	EEF1a	0.48
5	PP2A	0.68	EEF1a	1.14	TIP41	1	CYC	1.09	GAPDH	0.49
6	TIP41	0.68	UBC2	1.18	eIF4A	1.12	UBQ5	1.17	eIF4A	0.62
7	ACT	0.68	TIP41	1.21	RPL20	1.15	RPL20	1.21	CYC	0.63
8	eIF4A	0.72	CYC	1.27	CYC	1.16	PP2A	1.33	UBC2	0.75
9	UBC2	0.92	eIF4A	1.38	UBC2	1.18	UBC2	1.47	RPL20	0.91
10	CYC	0.94	UBQ5	1.39	PP2A	1.21	TIP41	1.58	UBQ5	1.46

are shown in Table 7. The ranking of all 10 reference genes in all samples and individual stress conditions are shown in Fig. 6. *GAPDH* was found to be most stable in overall and control samples while *EEF*1α was most stable reference gene in cold and salinity stress. RPL20 was most stable in heat stress and *PP*2*A* was most stable in drought stress according to RefFinder.

Discussion

Major abiotic stresses like heat, cold, drought and salinity, cause massive losses to agricultural production globally, which is further increased under field conditions due to simultaneous exposure of plants to more than one abiotic stress factor (Mittler 2006; Shivhare and Lata 2016). It has been reported that concurrent exposure of more than one stress may lead to complexity in plant responses due to antagonistic signaling pathways that may interact and impede each other (Mittler and Blumwald 2010; Suzuki et al. 2005). To guarantee sustainable crop productivity and decline the risk of environment, it is necessary to understand the regulation and function of the genes playing important roles under different abiotic stresses. To study variations in gene expression and to evaluate gene regulation patterns, selection of suitable internal control genes is a prerequisite for reliable gene expression analysis of target genes (Guénin et al. 2009; Radonić et al. 2004). Irregular expression of commonly used ICGs was reported and it was found that the genes having high expression level (very low Ct values) are unsuitable for RT-qPCR normalization (Tao et al. 2016). So the accurate

Table 7 Expression stability and ranking of the 10 candidate reference genes as calculated by RefFinder

Method	1	2	3	4	5	6	7	8	9	10
Ranking order without stress treatment (better–good–average)										
Delta CT	GAPDH	UBQ5	EEF1a	RPL20	PP2A	TIP41	ACT	eIF4A	UBC2	CYC
BestKeeper	TIP41	RPL20	UBC2	CYC	GAPDH	UBQ5	EEF1a	PP2A	ACT	eIF4A
Normfinde r	UBQ5	GAPDH	RPL20	TIP41	EEF1a	PP2A	ACT	eIF4A	UBC2	CYC
Genorm	ACT | PP2A		eIF4A	EEF1a	UBQ5	GAPDH	RPL20	TIP41	UBC2	CYC
Comprehensive ranking	GAPDH	UBQ5	RPL20	TIP41	PP2A	EEF1a	ACT	eIF4A	UBC2	CYC
Ranking order under heat stress (better–good–average)										
Delta CT	RPL20	ACT	GAPDH	PP2A	EEF1a	UBC2	TIP41	CYC	eIF4A	UBQ5
BestKeeper	RPL20	TIP41	UBC2	PP2A	ACT	UBQ5	GAPDH	eIF4A	CYC	EEF1a
Normfinder	RPL20	ACT	GAPDH	PP2A	EEF1a	UBC2	TIP41	CYC	eIF4A	UBQ5
Genorm	GAPDH | EEF1a		ACT	PP2A	RPL20	UBC2	TIP41	CYC	eIF4A	UBQ5
Comprehensive ranking	RPL20	ACT	GAPDH	EEF1a	PP2A	UBC2	TIP41	CYC	eIF4A	UBQ5
Ranking order under cold stress (better–good–average)										
Delta CT	ACT	EEF1a	GAPDH	UBQ5	TIP41	eIF4A	RPL20	CYC	UBC2	PP2A
BestKeeper	EEF1a	GAPDH	RPL20	CYC	UBQ5	ACT	UBC2	TIP41	eIF4A	PP2A
Normfinder	ACT	EEF1a	GAPDH	UBQ5	TIP41	eIF4A	RPL20	CYC	UBC2	PP2A
Genorm	GAPDH | EEF1a		ACT	TIP41	UBQ5	eIF4A	RPL20	CYC	UBC2	PP2A
Comprehensive ranking	EEF1a	ACT	GAPDH	UBQ5	TIP41	RPL20	eIF4A	CYC	UBC2	PP2A
Ranking order under salinity stress (better–good–average)										
Delta CT	EEF1a	eIF4A	GAPDH	ACT	CYC	UBQ5	RPL20	PP2A	UBC2	TIP41
BestKeeper	RPL20	EEF1a	UBQ5	PP2A	CYC	eIF4A	GAPDH	UBC2	ACT	TIP41
Normfinder	EEF1a	eIF4A	GAPDH	CYC	UBQ5	ACT	RPL20	PP2A	UBC2	TIP41
Genorm	GAPDH | eIF4A		ACT	CYC	EEF1a	UBQ5	RPL20	PP2A	UBC2	TIP41
Comprehensive ranking	EEF1a	eIF4A	GAPDH	RPL20	CYC	UBQ5	ACT	PP2A	UBC2	TIP41
Ranking order under drought stress (better–good–average)										
Delta CT	PP2A	TIP41	ACT	EEF1a	GAPDH	eIF4A	CYC	UBC2	RPL20	UBQ5
BestKeeper	GAPDH	TIP41	PP2A	ACT	EEF1a	UBC2	eIF4A	CYC	RPL20	UBQ5
Normfinder	GAPDH	TIP41	PP2A	ACT	EEF1a	UBC2	eIF4A	CYC	RPL20	UBQ5
Genorm	ACT | EEF1a		PP2A	eIF4A	CYC	TIP41	GAPDH	UBC2	RPL20	UBQ5
Comprehensive ranking	PP2A	GAPDH	ACT	TIP41	EEF1a	eIF4A	CYC	UBC2	RPL20	UBQ5

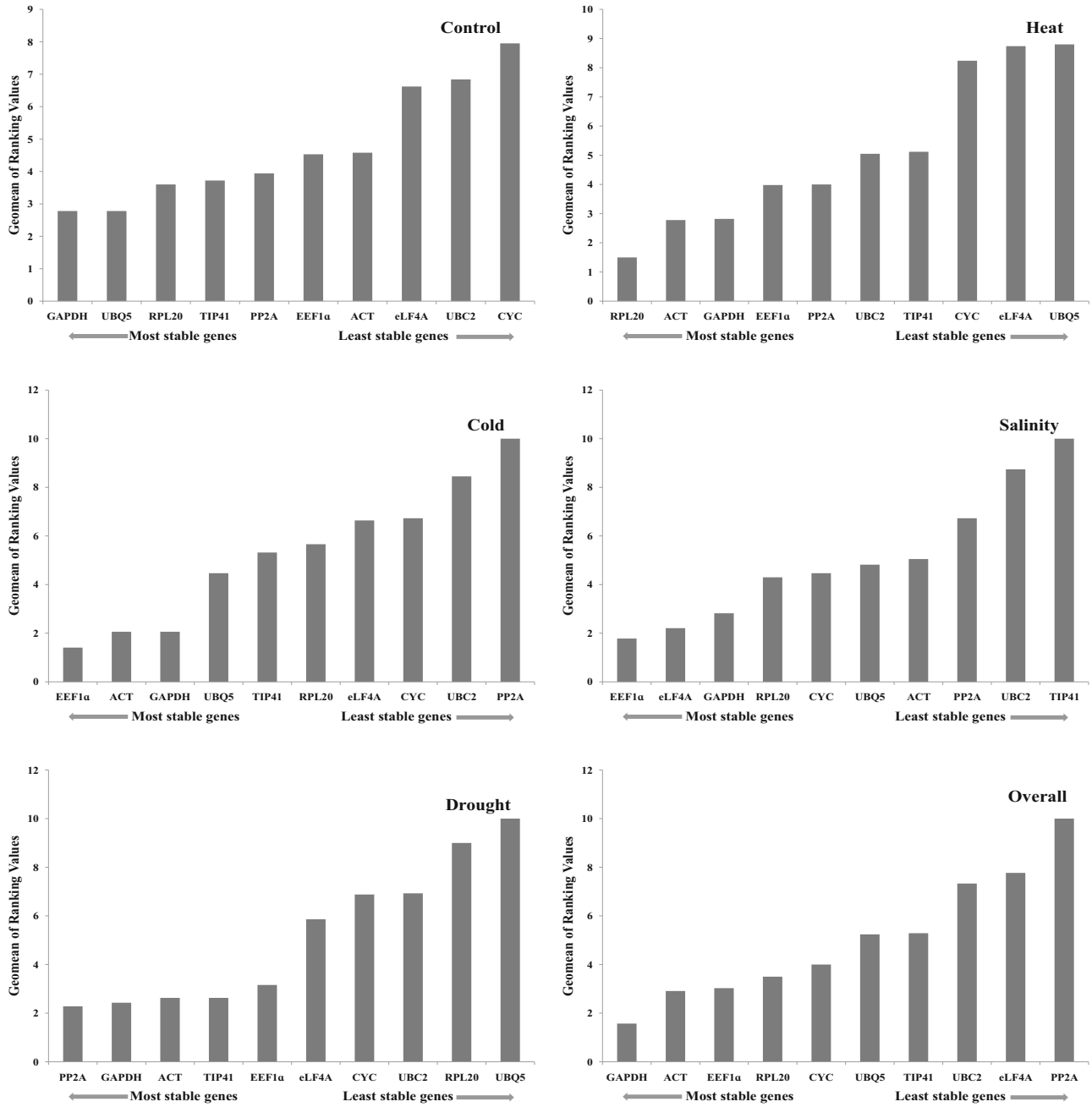

Fig. 6 Expression stability of 10 candidate reference genes as calculated by RefFinder. A lower Geomean value indicates more stable expression

normalization of gene expression remains a most important prerequisite for precise qRT-PCR analysis as normalization helps to minimize variations acquainted at different steps of qRT-PCR arising from sample to sample variations, pipetting variations, variations in RNA integrity and PCR efficiency (Guénin et al. 2009; Huggett et al. 2005). Gene which has constant expression under any experimental condition, cell or tissue types and developmental stages is called an ideal reference gene (Fan et al. 2013; Gutierrez et al. 2008). *ACT*, *GAPDH*, *EF*1*a* and *TUB* are most commonly used internal control genes as these genes are present in all nucleated cell types and involved in primary cellular metabolism which are necessary for cell survival (Migocka and Papierniak 2011). Several reports have documented on variable expression of these reference gens in different plant species in different environmental conditions and in specific cellular functions (Czechowski et al. 2005; Huggett et al. 2005; Lata 2015; Vandesompele et al. 2002; Yang et al. 2015; Zhu et al. 2013). Selection of non suitable internal control genes leads to inappropriate results

and inaccurate gene expression data (Ma et al. 2013). So the identification of suitable internal control genes having stable expression in different crops, biological samples and experimental conditions is very essential for reliable transcript measurements through qPCR (Brunner et al. 2004; Kumar et al. 2013). Several reports are available in which more than one study has been carried out for evaluation of suitable internal control genes for accurate normalization during different experimental conditions e.g. pearl millet (Reddy et al. 2015; Saha and Blumwald 2014), rice (Jain et al. 2006; Kim et al. 2003; Li et al. 2010), soybean (Hu et al. 2009; Libault et al. 2008; Ma et al. 2013), sugarcane (Guo et al. 2014; Ling et al. 2014) and coffee (Barsalobres-Cavallari et al. 2009; Cruz et al. 2009).

Finger millet is an excellent food and forage crop of arid and semiarid areas in Africa and Asia which is known for poor soil fertility, drought and high temperature (Goron and Raizada 2015; McDonough et al. 2000) with these adverse conditions, finger millet is a well adapted crop having excellent nutritional qualities so the researchers are becoming keen to study its cellular and molecular mechanisms of stress tolerance through transcriptomics or functional genomics approaches. This study is focused to understand the molecular mechanisms in finger millet in response to different abiotic stresses which will be helpful for crop improvement and for paving way to transgenic approaches.

Till date, there are no reports available on internal control gene identification in different abiotic stress in finger millet so in this pioneering attempt, study of the expression of ten potential reference genes (*GAPDH*, *EEF*1*a*, *ACT*, *CYC*, *RPL*20, *UBQ*5, *UBC*2, *TIP*41, *eIF*4*A*, *PP*2*A*) were investigated under different abiotic stresses like heat, cold, salinity and drought. Comparison of different statistical approaches could provide a better, unbiased and consistent evaluation of gene stability across different conditions (Migocka and Papierniak 2011). The overall results obtained on the basis of statistical analysis by geNorm, NormFinder, ΔCt and RefFinder showed similarity in determining the most stable candidate reference genes. BestKeeper is a different algorithm from the other algorithms, therefore, the results obtained from BestKeeper showed the least correlation with the others (Guo et al. 2014). Based on reference genes recommended by geNorm, NormFinder, ΔCt, and RefFinder; *GAPDH*, *EEF*1*a* and *ACT* were selected as the top three most stable candidate reference genes across all the samples in different stress conditions. *GAPDH* is one of the most commonly used internal control gene and also referred as "classical" gene and our results confirm work of previous studies where *GAPDH* was identified as most stable reference gene as in *Coffea arabica* (Barsalobres-Cavallari et al. 2009), *Linum usitatissimum* L. (Huis et al. 2010), *Lolium temulentum* (Dombrowski and Martin 2009), sugarcane (Andrade et al. 2017), *Salicornia europaea* (Xiao et al. 2014) and *Petroselinum crispum* (Li et al. 2016). Another gene *EEF*1*a* has also been established as most stable reference gene in pearl millet under different combination of abiotic stresses (Shivhare and Lata 2016), as in soybean under salinity and drought stress (Mittler and Blumwald 2010), wheat under different biotic and abiotic stresses (Long et al. 2010), cucumber under salt and osmotic stress (Migocka and Papierniak 2011), Bermuda grass under drought stress (Chen et al. 2015), sugarcane under drought and salinity stress (Guo et al. 2014) and in *Petroselinum crispum* under abiotic stresses (Li et al. 2016). Several studies have suggested *ACT* as most stable internal control gene for example in pearl millet under different abiotic stress conditions (Saha and Blumwald 2014), in *Salicornia europaea* under drought stress (Xiao et al. 2014), in soybean under drought stress (Stolf-Moreira et al. 2011), in *Oxytropis ochrocephala* under different abiotic stress treatment (Zhuang et al. 2015) and in *Prunus mume* during flowering stress and abiotic stress (Wang et al. 2014). Several reports suggested that use of more than one candidate reference genes would help in more accurate normalization of qRT-PCR data (Le et al. 2012; Vandesompele et al. 2002). There are no reports available regarding internal control gene identification in finger millet. Pairwise variation analysis performed using geNorm suggested that out of 10 candidate genes, 4 genes are suitable for normalization of gene transcript for qRT-PCR analysis in abiotic stress as shown in present study. Earlier studies on identification of suitable internal control genes suggests more than one internal control gene (Chandna et al. 2012; Li et al. 2016; Shivhare and Lata 2016). Study of different types of stresses individually suggested that the comprehensive ranking of most stable reference genes in heat stress were *RPL*20> *ACT* > *GAPDH*; in cold stress *EEF*1*a* > *ACT* > *GAPDH*; in salinity *EEF*1*a* > *eIF*4*A* > *GAPDH* and in drought stress *PP*2*A* > *GAPDH* > *ACT*. *PP*2*A* was reported to be most stable. It shows that at different stress conditions, the stability of internal control genes change (Saha and Blumwald 2014; Shivhare and Lata 2016). So this study indicates that the candidate reference genes should be validated before its use for each study, since the results obtained can rarely be extrapolated to other genotypes or different experimental conditions (Volkov et al. 2003).

Conclusion

To the best of our knowledge, this is the first study of identification of suitable reference genes for accurate transcript normalization using qRT-PCR analyses in finger

millet. In this study, we evaluated ten candidate reference genes in two genotypes of finger millet under abiotic stress conditions of heat, cold, drought and salinity. The study recommends *GAPDH*, *EEF*1*a*, *ACT* and *CYC* as the most stable reference genes and *PP*2*A* and *eIF*4*A* as least stable reference genes. This study will benefit future work on gene expression studies during abiotic stress conditions in finger millet, a crop with limited genomic and transcriptomic information, and will also benefit the researchers for conducting experiments on related crop species.

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