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Full Length Article

Identifications and Validations of Reference Genes for Gene Expression Data Normalization of *Chenopodium album*

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Abstract

Quantitative real-time PCR (qRT-PCR) is considered as an important technique for gene expression and validation analysis for several high-throughput proteomics and genomics data. However, reliable qRT-PCR results are dependent on appropriate reference genes used for data normalization under specific experimental condition and such information is limited in case of plants whose genome is not sequenced yet. Therefore, we sequenced and evaluated stability of seven novel internal control genes (*Act*, *Act α*, *β-tub*, *18SrRNA*, *GAPDH*, *EF1α* and *Ubq*) of *Chenopodium album*, an important edible plant with medicinal values and have been recently used in synthesis as well as analysis of several gold and silver nanoparticles. Statistical algorithms like geNorm and NormFinder were used and compared for the samples treated with different types of stresses including, high/low temperatures, drought, heavy metal and salt stress. *β-tubulin* was revealed as the most stable genes under heat, drought and metal stress. While, *18SrRNA* has shown the stable expression only under cold and salt stresses. Our data support the use of the combinations of two stable internal control genes for normalization of the gene expression. We compared the relative expression of a stress induced Chloroplast small Heat Shock Protein (Cp-sHSP) of *C. album* to further support our results. Together with all novel internal controls genes, this study also provides a platform for future gene expression studies of *C.album* using validated housekeeping genes. © 2017 Friends Science Publishers

Keywords: Validation; Internal controls; Abiotic stress; *Chenopodium album*

Introduction

Chenopodium album (commonly known as Bathu) is a dicot C3 edible weed. It has been spread all over the world and recently being used for safer, biocompatible and cost effective gold and silver nanoparticles synthesis and analysis(Roy and Barik, 2010; Dwivedi and Gopal, 2011). *C.album* can tolerate extreme environmental conditions including semi-arid, light-saline and other severe abiotic stresses (Yao *et al.*, 2010; Shakeel *et al.*, 2011; Haq *et al.*, 2013a; Haq *et al.*, 2013b). Limited genomic information of *C.album* is limiting its use for important applications in molecular biology; otherwise it can also be a potential candidate for dissection of several important pathways using expression technologies in future.

Quantitative PCR is powerful technique used for detection and quantification of transcript of any gene (Reid *et al.*, 2006; Paolacci *et al.*, 2009) and is frequently used to understand different biological processes (Bustin, 2002; Ginzinger, 2002) due to its reproducibility, specificity, sensitivity, minimal post-PCR processing and broad dynamic range(Garson *et al.*, 2005). Researchers need to be very careful in interpreting qRT-PCR results as experimental conditions, treatments and tissue types can introduce biasness and errors without utilization of appropriate normalization strategies. Main purpose of data normalization is usually to eliminate the error or variability due to transcript amount or quality. Use of stable internal control genes i.e. reference genes in qRT-PCR with consistent expression are required throughout the experimental conditions (Thellin *et al.*, 1999; Schmittgen and Zakrajsek, 2000). Different internal control genes usually show differential expression in diverse plant parts or different experimental conditions (Maroufi *et al.*, 2010). Researchers have concluded that expression of the internal control gene should not be affected by experimental conditions (Schmittgen and Zakrajsek, 2000). Previous reports showed use of several

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conventional internal control genes e.g. elongation factor-α1, cyclophilin, ribosomal genes, glyceraldehyde-3-phosphate dehydrogenase, tubulin, ubiquitin and actin genes with stable expression and can be used as internal control genes for those specific samples (Sturzenbaum and Kille, 2001; Bezier *et al.*, 2002; Thomas *et al.*, 2003; Dean *et al.*, 2005).

The best internal control genes should have constant expression under given experimental conditions or samples. In past, most commonly used internal control genes were often used for gene expression studies without any verification with the assumption that all internal controls give constant expression under all experimental or environmental conditions. Recently, different internal control genes have been examined with variable transcript levels under different experimental conditions (Thellin *et al.*, 1999; Schmittgen and Zakrajsek, 2000; Warrington *et al.*, 2000; Sturzenbaum and Kille, 2001; Radonic *et al.*, 2004; Huggett *et al.*, 2005; Maroufi *et al.*, 2010). Infect this differential expression of the internal control genes may be due to their involvement in multiple pathways at the same time (Vandesompele *et al.*, 2002).

Recent studies have provided evidences of variable expression of different internal control genes i.e. *Actin*, beta *tubulin* and *ubiquitin* among various plant species under different conditions (Czechowski *et al.*, 2005; Nicot *et al.*, 2005; Jian *et al.*, 2008; Martin *et al.*, 2008; Wan *et al.*, 2009). Therefore, the selection of suitable of internal control genes is recommended for data normalizations. Moreover, two or more internal control genes can also be used as internal controls to normalize to further minimize the error in real-time PCR data (Thellin *et al.*, 1999; Vandesompele *et al.*, 2002).

Recent preference for internal control validations has helped in clarifications of the role of expressed genes in better way in case of many plants e.g. rice (Kim *et al.*, 2003; Jain *et al.*, 2006; Jain and Khurana, 2009; Narsai *et al.*, 2010), potato (Nicot *et al.*, 2005), perennial ryegrass (Martin *et al.*, 2008), *A. thaliana* (Czechowski *et al.*, 2005), soybean (Brechenmacher *et al.*, 2008; Jian *et al.*, 2008), *Brachiaria brizantha* (Silveira *et al.*, 2009), Darnel ryegrass (Dombrowski and Martin, 2009), poplar (Brunner *et al.*, 2004), water lily (Luo *et al.*, 2010), cucumber (Wan *et al.*, 2009), flax (Huis *et al.*, 2010), cotton (Artico *et al.*, 2010), *Salvia miltiorrhiza* (Yang *et al.*, 2010), tobacco (Schmidt and Delaney, 2010), faba bean (Diaz-Ruiz *et al.*, 2010), garden petunia (Mallona *et al.*, 2010), longan tree (Lin and Lai, 2010), chicory (Maroufi *et al.*, 2010), peach (Tong *et al.*, 2009), maize (Scholdberg *et al.*, 2009), coffee (Barsalobres-Cavallari *et al.*, 2009), wheat (Paolacci *et al.*, 2009), *Orobanche ramose* (Gonzalez-Verdejo *et al.*, 2008), tomato (Exposito-Rodriguez *et al.*, 2008; Lovdal and Lillo, 2009), *Litseacubeba* (Lin *et al.*, 2013) and *Atropa belladonna* (Li *et al.*, 2014), *Populus euphratica* (Wang *et al.*, 2015), Chinese medicinal plant i.e. *Gentiana macrophylla* (He *et al.*, 2016).

Different methods have been used to check the stability of internal control genes e.g., NormFinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004) and geNorm (Vandesompele *et al.*, 2002). GeNorm calculates the average expression stability and pairwise changes to determine the stability of housekeeping genes. According to this tool, the most stable gene under specific condition should have the lower average expression stability. BestKeeper performs comparative study based on raw Ct values of the gene to calculate the relation between genes and their rankings. Results are shown with the lowest variations means the most stably expressed gene and vice versa (Pfaffl *et al.*, 2004). NormFinder selects the most suitable normalized gene from a group of housekeeping genes and ranks them according to their stability but here the most stable expressed gene is indicated by the lowest stability. Similalry, researchers have observed that a group of internal control genes can also be used to choose the best suitable internal control gene to use for quantitative PCR data analysis.

In this study, seven novel internal control genes including elongation factor 1α (*EF1α*), 18S ribosomal RNA (*18S rRNA*), actin (*Act*), actin alpha (*Act α*), beta-tubulin (*β-tub*), ubiquitin (*Ubq*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) of *C. album* were identified, sequenced and validated for their use as internal controls to determine relative transcript levels. This will be the first report of identifications and characterizations of internal control genes with their validations of C. *album*. We used two different statistical methods (NormFinder and geNorm) to determine the most appropriate internal control gene for the normalization after heat, drought, cold, metal and salt treatments.

Material and Methods

Plants Growth, Treatment and Sampling

C. album which is a dicot plant commonly and known as Fat hen was selected for internal control gene identification and validation under different environmental stresses. Seeds were grown in pots having mixture of calcined-clay and potting soil (1:1 V:V) under controlled environment in growth chambers at day/night growth temperature regimes of 26°C/20°C with 350 mmol/m^2 per second photosynthetic photon flux density (PPFD). Almost 8–10 weeks old plants were treated independently with five different abiotic stresses (heat, drought, cold, metal, and salt) in three biological replicates.

Heat stress was given by incubating the leaves (Shakeel *et al.*, 2011) of *C. album* at different temperatures (including 30, 35, 37 and 40ºC) for 4 h in sterilized incubation buffer (1% sucrose [W/V], 1 mM KPO_4 pH6 [V/V] and 0.02% Tween-20) while control leaves were incubated at 25ºC for 4 h. Drought stress was imposed by placing the whole plant on dried tissue paper for 1, 3, 5 and

7 h, while control condition plants were kept under normal growth conditions (Haq *et al.*, 2013a). Cold treatment was given by placing the potted plants at 4ºC for 1, 3, 5, 7and 9 h, while control conditions plants were kept at 25°C. Cadmium salt ($CdCl_2.2.5H_2O$) was used for heavy metal stress to *C. album* plants. Whole plants were dipped in the Cd solutions of 10, 15, 20 and 25 mM concentrations for 3 hours with respective controls (Haq *et al.*, 2013b). Sodium chloride was used for salt stress by dipping the roots of the plants in 150, 200, 400 and 600 mM NaCl solution as described (Haq *et al.*, 2013a) while the roots of the control conditions plants were dipped in distilled waterfor5 h. Leaf tissues were collected from individual treatment conditions and kept at -80ºC for RNA isolation.

Total RNA Extraction and cDNA Synthesis

RNA was extracted from *C. album* samples using TRI Reagent (MRC, TR#118), following the instructions provided by manufacturer. RNA quantification was done using Nanodrop spectrophotometer 1000 (Thermoscientific). RNA (5µg) from each sample was made free from any kind of DNA by treatment with DNase I (RNase-free) (Fermentas Cat. # EN0521).Revert Aid first strand cDNA synthesis Kit (Fermentas Cat. # K1621) was used for cDNA synthesis. Samples (cDNA) were checked for the presence of single product peak of their respective transcript.

Sequencing of *C. album* Internal Control Genes

Sequences of the above mentioned candidate reference genes were not present in the database (NCBI). Gene-specific primers were designed for *Ubiquitin, Actin, Actin α, β-tubulin, GAPDH, EF1α* and *18S rRNA* from conserved regions of the sequences of known their homologs. Genomic DNA of *C. album* was extracted and all the above mentioned genes (Table1) were amplified from genomic DNA under PCR conditions: denaturation at 95°C and30 cycles of 94°C (45 s), 54–60°C (45 s) and 72°C (1 min) followed by final extension at 72°C for 5min. Amplified products (12µL) were used for gel electrophoresis at 80 volts for 90 min. The amplified products were sequenced and deposited in database (Accession numbers have been given in Table 1).

Quantitative PCR

Relative quantification of all candidate internal control genes was done using Applied Bio-Systems 7500 Fast Real Time PCR system. Reaction was performed in 96-well reaction plate using a mixture prepared by mixing qPCR master mix (12.5µL), primers (0.3µM each), template transcript (<500ng) and nuclease-free water up to 25µL. PCR was performed using two-step cycling procedure for triplicate technical and biological replicates. PCR master mix was treated with Uracil DNA Glycosylase (UDG) at 50ºC for 2 min. Further reaction was proceeded with initial denaturation at 95ºC (10 min)and50 cycles of initial denaturation at 95ºC for 15sec followed by annealing and extension at 54–58ºC (60sec). Control conditions and negative control samples were also considered for each reaction. Biological and technical triplicates were maintained in the case of each sample. ABI SDS software was used for data analysis.

Normalization of Cp-sHSP Gene for Validation of Reference Gene

The chloroplast small heat shock proteins (Cp-sHSPs) have been reported to protect thylakoid membranes and photosystem II under high temperature and different other abiotic stresses (Haq *et al.*, 2013a; Haq *et al.*, 2013b). Cp-sHSP gene was used as a target gene to evaluate the related gene expression using different internal control genes by qRT-PCR. According to our statistical analysis, a list of different reference genes were selected in terms of the most reliable gene or pairs under different abiotic stress conditions, we picked up only two genes from top most ranking and one least stable gene from each list for further validation using target gene.

Statistical Analysis for Stability of Internal Control Gene

Candidate internal control genes were evaluated independently for stability by two different statistical approaches after analysis of expression relative to the sample with the highest expression. Slope of linear regression model (Pfaffl *et al.*, 2004) was used to determine the efficiency of real-time PCR for each gene under each stress condition. Ct value was determined for each sample by ABI SDS software. GeNormv3.4 software (http://medgen.ugent.be/~jvdesomp/genorm/) was used to analyze the gene expression stabilities. It is an algorithm using geometric averaging of mean pairwise variation (*V*) of a gene and multiple control genes to determine the stability of the gene (*M*) under examination (Vandesompele *et al.*, 2002). Genes under investigation were ranked based on their stability and the appropriate reference gene was selected. Additionally, NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) was used to identify the optimal normalization of selected gene among different candidate genes. It ranks the genes among different internal control genes based on their stability in expression patterns under experimental conditions (Andersen *et al.*, 2004).

Results

Identifications, Sequencing and Characterizations of Potential Internal Control Genes

Due to unavailability of genomic sequences of *C.album*, we identified and sequenced *seven* putative candidate internal

Table 1: Selected reference genes used for gene expression data normalization and validation studies of *C. album* plant treated with different abiotic stresses

Gene name	Gene symbol	GeneBank Accession number	Forward and reverse primer sequence [5'-3']	Primer efficiency %	Approximate product size (bp)	Temp (°C)
Ubiquitin	*Ubq*	KC898958	CCTGACCAGCAGAGGTTGATC TCCGCCAGAGTCCTTCCAT	97.1	500	62
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	KC898962	ATGCTCCCATGTTTGTTGTGGGTG TTAGCCAAAGGTGCAAGGCAGTTC	96.6	100	60
Actin α	*Act α*	KC898960	TTGTTAGCAACTGGGATGATATGG CAGGGTGTTCTTCAGGAGCAA	99.0	100	55
Actin	*Act*	KC898957	TCCATAATGAAGTGTGATGT GGACCTGACTCGTCATACTC	99.0	450	55
Elongation factor 1 α	*EF1α*	KC898961	CCGAGCGTGAACGTGGTAT TAGTACTTGGTGGTTTCGAATTTCC	91.1	90	56
18S ribosomal RNA	*18S rRNA*	HQ827790.1	TCCTGAGTAACGAACGAGACC CACGATGAAATTTCCCAAGAT	99.6	250	58
Beta-tubulin	*β-tub*	KC898959	CGTAAGCTTGCTGTGAATCTCATC CTGCTCGTCAACTTCCTTTGTG	99.7	250	58

control genes including, *Ubiquitin (Ubq), Actin (Act), Actin α (Act α), β-tubulin (β-tub), GAPDH, EF1α* (*EF1α*) and*18S rRNA* using primers from conserved regions of the sequences of known homolog *of* other plants. The gene names, accession numbers, plant names, primer sequences with their melting temperatures, approximate product sizes and average PCR efficiencies are given in Table 1. To select specific transcripts of above mentioned internal control genes, *C.album* leaf samples treated with different abiotic stresses (salt, drought, and heavy metals, low and high temperature) were used for qRT-PCR were used. All the amplified products including *Ubq, GAPDH, Act-α, Act, EF1α, 18S rRNA* and *β-tub* genes of approximate sizes of 450, 300, 220, 350, 100, 250 and 250 bp were first analyzed on agarose gel (Fig. 1a) followed by sequencing and sequences of the candidate internal control genes were submitted to GenBank (Id KC898957-62 and HQ827790.1) after initial analysis using bioinformatics tools. Melting curve analysis were also performed for each gene of interest using Real-time PCR to exclude the possibility of any kind of non-specific amplification or primer dimmers (Fig. 1b).

Expression Levels, Stabilities of Candidate Reference Genes

The average threshold cycle value was determined using triplicate technical of three biological replicates for treated samples. Ct values for all the seven candidate internal control genes were determined and were in the range of 19 to 33 cycles (Fig. 2). Most of these genes have higher expression levels supported by threshold cycle values below than 20. Transcript level of the above mentioned candidate housekeeping genes were determined using quantitative real-time PCR. The amplification curves were generated to determine the cycle threshold (Ct). Ct value of each gene was used to measure the expression stabilities by geNorm and NormFinder algorithms.

geNorm Analysis

Internal control gene expression of all of the above treated and control samples were checked using geNorm (Vandesompele *et al.*, 2002). M and V values of all of the genes were individually computed for each sample in three replicates based on the average pairwise expression ratios. The recommended value for *M* was kept as 1.5 and most stable gene expression has been indicated by lowest *M* value and vice versa. Taking all together, the results of all samples showed that *β-tub* and *18SrRNA* have the lowest *M* values and were considered as the most stable genes and *Ubq* was demonstrated as least stable gene with highest *M* value (Fig. 3).

The effect of different experimental treatments including heat, drought, cold, metal and salt on stability of genes was also checked by their *M* values. Lowest *M* values of β-*tub* and *18SrRNA* indicates that this is good pair of internal controls under heat stress treatment, while *GAPDH* was the least stable gene, while β-*tub* and *EF1α* gave highest gene stability under drought stress (Fig. 3b).Cold stress has shown lowest effect on the expression stability value (M) of *18SrRNA*, which supports its potential use as an internal control gene for cold stress treatment while *Act α* is the least stable gene in *C. album* under cold stress conditions (Fig. 3c). *β-tub* and *EF1α* were the most stable genes based on geNorm analysis and *18SrRNA* was least stable under heavy metal stress (Fig.3d). Salt stress has prominent effect on *Act* stability while *18SrRNA* has shown the highest stability under salt stress conditions (Fig.3e).

Sometimes us of more than one internal control can increase the reliability of normalization data, so we calculated the minimum number of the most suitable internal control genes required for better normalization by checking Pair-wise variations (*Vn/Vn+ 1*) between two highly ranked internal control genes using geNorm.

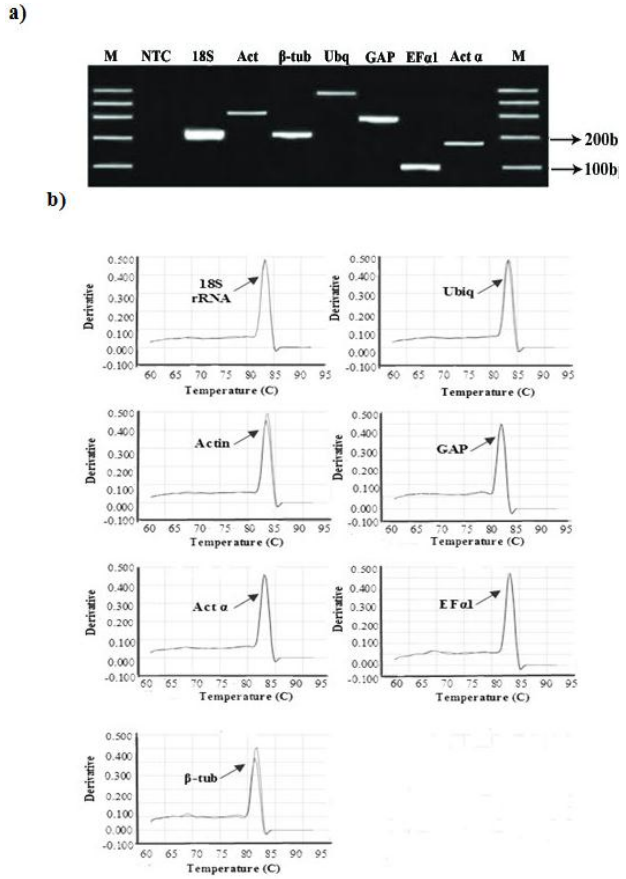

Fig. 1a: Genomic DNA amplification of seven candidate internal control genes under different abiotic conditions. Gene specific primers of *18S ribosomal RNA*(*18S rRNA*), *Actin (Act), Beta tubulin* (*β-tub*), *Ubiquitin* (*Ubq*),*Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *Elongation factor 1 α* (*EF1α*) *and Actin α* (*Act α*) genes were used to amplify genomic DNA. (b) Dissociation curves of putative internal control gene primers used for real time PCR in *C. album*. The gene specific primers of Ubiquitin (*Ubq*), *Actin* (*Act*)*, Actin α* (*Act α*), *Beta tubulin* (*β-tub*), *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *Elongation factor 1 α* (*EF1α*) and *18S ribosomal RNA* (*18S rRNA*) used to amplify the *C. album* transcript by real-time PCR

Cut-off value of pair-wise variations is recommended as 0.15 and value below this shows no need to add additional reference genes for normalizations of particular data. Analysis of the pair-wise variation of all of our data showed that V values of all the selected reference genes were less than 0.15, suggesting that the selected internal control genes were enough to normalize the real-time PCR data in given conditions (Fig. 4). Conclusively, no additional internal controls are required for any of these experimental conditions for *C. album*.

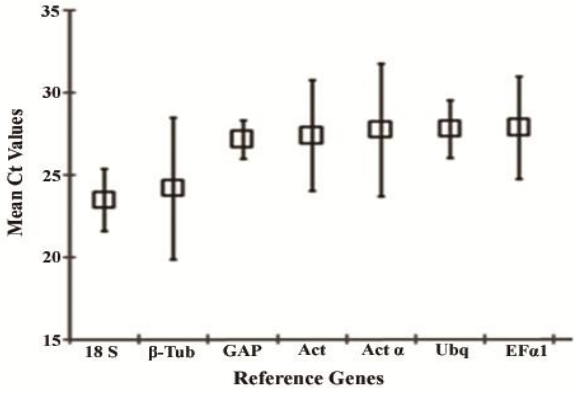

Fig. 2: Expression profile of seven candidate reference genes of *C. album*

The cycle threshold numbers (Ct values) are given as a mean of triplicate technical of three biological replicates. These values represent the points at which a gene shows the maximum expression. The boxes represent the mean *Ct* values of 18S ribosomal RNA (*18S rRNA*), Beta-tubulin (*β-tub*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Actin (*Act*), Actin α (*Act α*), Ubiquitin (*Ubq*) and Elongation factor 1 α (*EF1α*) and the bars represent standard deviations

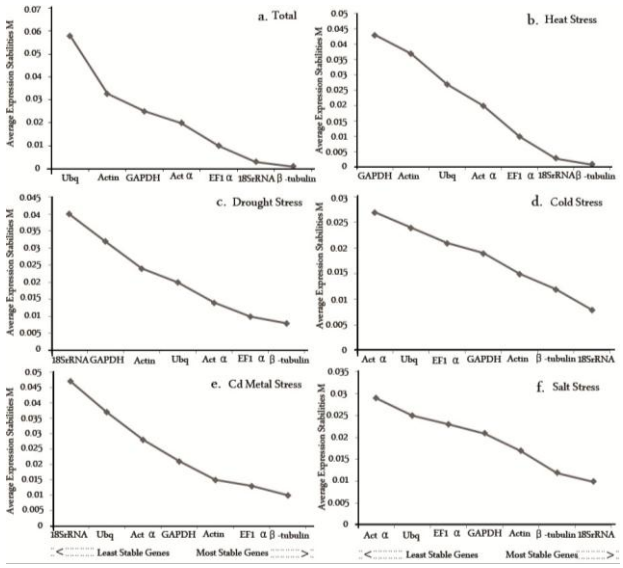

Fig. 3: Gene expression average stabilities (*M values*) of the seven candidate genes of *C. album* determined by geNorm

Mean expression stabilities (M) follows the exclusion of the least stable gene stepwise across all the treated samples of an experiment, (a) total samples, (b) heat stress, (c) drought stress, (d) cold stress, (e) metal stress and (f) salt stress. The least stable genes are on the left of the graph and the most stable pair of the genes are on the right as calculated by the geNorm

Analysis by NormFinder

To get further support for our internal control validations by geNorm, we analyzed the same seven candidate genes of *C. album* with another algorithm: NormFinder, which calculates expression stability value of individual genes and then ranks different genes based on their values.

Table 2: Ranking of 18S ribosomal RNA (*18S rRNA*), Ubiquitin (*Ubq*), Actin (*Act*), Beta-tubulin (*β-tub*), Elongation factor 1 α (*EF1α*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Actin α (*Act α*) internal control genes of *C. album* under different abiotic stresses based on expression stabilities according to NormFinder in order to their expression stabilities

Rank	Internal Control Gene Stability									
	Heat		Drought		Cold		Metal		Salt	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	*18S rRNA*	0.005	*EF1α*	0.213	*β-tub*	0.276	*β-tub*	0.097	*Ubq*	0.002
2	*EF1α*	0.124	*Act α*	0.330	*Act α*	0.325	*Act*	0.187	*β-tub*	0.074
3	*Act*	0.135	*β-tub*	0.340	*EF1α*	0.357	*18S rRNA*	0.269	*Act*	0.123
4	*β-tub*	0.196	*Act*	0.369	*Act*	0.423	*Ubq*	0.321	*18S rRNA*	0.149
5	*GAPDH*	0.234	*Ubq*	0.419	*Ubq*	0.446	*GAPDH*	0.367	*EF1α*	0.218
6	*Ubq*	0.258	*18S rRNA*	0.570	*GAPDH*	0.477	*Act α*	0.380	*GAPDH*	0.276
7	*Act α*	0.515	*GAPDH*	0.580	*18S rRNA*	0.493	*EF1α*	0.509	*Act α*	0.357

Lowest expression stability value represents the most stable gene and vice versa (Ma *et al*., 2013). This algorithm gave almost the same ranking of the above reference genes under different abiotic stresses as geNorm did (Table 2). In the case of heat stress, gene stability pattern was *18SrRNA>EF1α>Actin>β-tub >GAPDH>Ubq>Actα,* while in the case of drought stress, the stability order of the genes was; *EF1α>Act α>β-tub >Actin>Ubq>18SrRNA>GAPDH.* Similarly, the ranking of the gene stability under cold stress has been concluded as; *β-tub >Actα>EF1α>Actin>Ubq>GAPDH>18SrRNA*. Genes stability under metal stress was*β-tub >Actin >18SrRNA >Ubq >GAPDH >Act α>EF1α* while in the case of salt stress, the stability of the genes was*Ubq >β-tub >Actin >18SrRNA>EF1α>GAPDH >Act α.*

Validation of Reference Gene

To experimentally validate the selected candidate internal control genes, we analyzed the expression pattern of Cp-sHSP gene using so, me of the selected reference genes under each experimental condition. In *C. album*, the expression of this Cp-sHSP gene was regulated differentially under different abiotic stresses (Shakeel *et al.*, 2011; Haq *et al.*, 2013a; Haq *et al*., 2013b). In this study, the expression of the particular Cp-sHSP gene was assessed using three different reference genes as internal controls. Cp-sHSP transcript accumulation was determined in relation to the increasing temperature with the help of qRT-PCR while using *β-tub*, *18SrRNA* and *GAPDH* as internal control genes. Using the two most stable reference genes i.e. *β-tub* and *18SrRNA* for normalization resulted the sharp increase in transcript expression level of Cp-sHSP at 30ºC heat treatment after slow decrease in the expression (Fig. 5a).These results were consistent with our previously published results (Shakeel *et al.*, 2011). A noticeable difference was observed in the basal levels of the Cp-sHSP expression profile, when the least stable gene *GAPDH* was used as an internal control.

Similarly, Cp-sHSP transcript level was examined after drought stress using *β-tub, EF1α*and *18SrRNA* genes as internal controls for normalization. Cp-sHSP transcript increased approximately nine folds after 1–3 h of drought stress followed by sudden decrease in expression after prolonged stress (Fig. 5b). Initial higher basal levels of Cp-sHSP expression was observed in the case of *18SrRNA* gene as an internal control. In short, very different expression patterns were observed when less stable genes were used for normalization in the case of cold (Fig. 5c), metal (Fig. 5d) and salt stress (Fig. 5e). The expression of Cp-sHSP gene was almost similar and consistent when two different stable genes were used for normalizations in case of each stress.

Discussion

Plant growth is affected by different environmental stresses. Therefore, the regulation and expression studies at gene/proteins can be helpful to explore the underlying important pathways. Quantitative PCR is widely used to determine the transcript level of the gene due to its large dynamic and high throughput range, accuracy, sensitivity and specificity. But for accuracy and reliability of real-time PCR, it is very important to validate the genes by using as internal controls for data normalization. The most suitable internal control gene should show stable expression level at any growth stage under any specific condition (Ullmannova and Haskovec, 2003). Researchers have used housekeeping genes as internal controls in expression studies because of their constant expression in each cell (Huggett *et al.*, 2005). Experiments have shown the varied transcript level of the housekeeping genes under different environmental conditions (Jian *et al*., 2008; Maroufi *et al.*, 2010). Several studies showed differential stabilities of different internal control genes which can be used for transcript level studies under any experimental condition (Exposito-Rodriguez *et al*., 2008; Cortleven *et al.*, 2009; Artico *et al*., 2010). For more clarity, two or more reference genes can also be used for perfect interpretation of quantitative PCR data. Transcript level of perfect internal control gene should not be affected by any experimental condition (Butte *et al.*, 2001). Therefore, the expression of the internal control gene should be validated before using as internal control for transcript level analysis under different environmental conditions. This kind of analysis can easily be done for

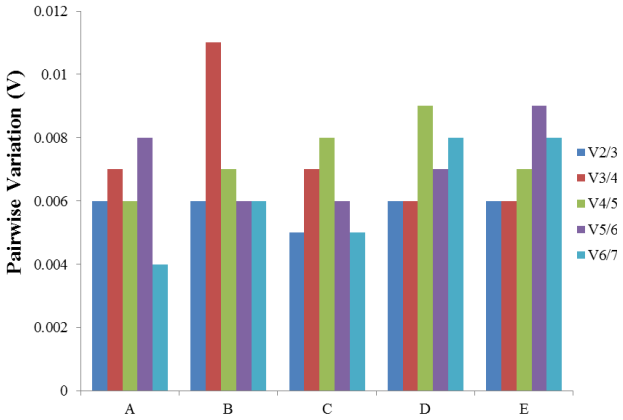

Fig. 4: Pairwise variations (*V values*) to determine the optimum number of reference genes required for qRT-PCR data normalization

geNorm was used to knowpairwise variations for determination the appropriate reference genes for absolute normalization underdifferent stress conditions (a) salt (b) cold (c) heat (d) metal and (e) drought stresses. Each bar shows the variation in normalization accuracy with additionof more reference genes based on the ranking in Fig. 3

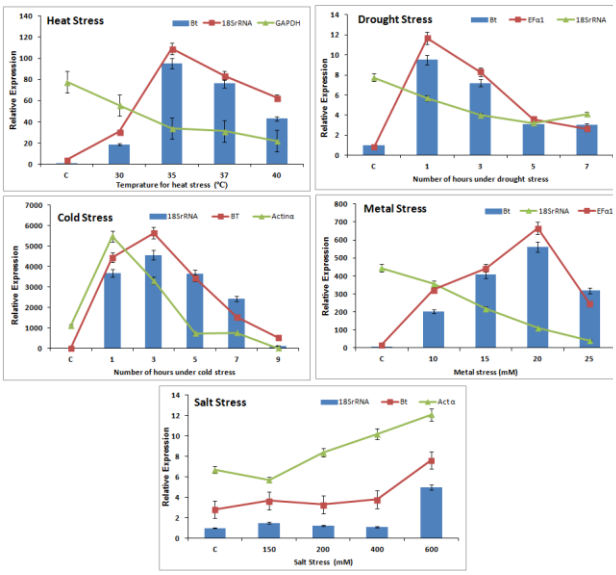

Fig. 5: Validation of selected reference genes of *C. album* for relative expression of Chloroplast small heat shock protein (*Cp-sHSP*) under environmental stress conditions

Chloroplast small heat shock protein(*Cp-sHSP*)gene expression was checked with two most stable genes and one least stable gene for each stress including heat, drought, cold, metal and salt stress. The raw Ct values of Cp-sHSP transcripts were normalized with different internal controls. Standard deviation has been shown by error bars

model plants because of genome sequencing but difficult for non-model plants such as *C. album*. This plant has been recently used in several important studies including biosynthesis of nano-particles used in nano-medicines (Roy and Barik, 2010; Dwivedi and Gopal, 2011). So, it is important to identify and validate appropriate controls for the normalizations of transcript level data.

Here we tested the expression stability of seven candidate reference genes (*Act, Act α, β-tub, GAPDH, EF1α, Ubq* and *18SrRNA*) of *C. album* to use as internal controls for transcript level determination under different abiotic (heat, cold, metal, drought and salt) stresses. This study might be the first survey on validation of the genes to use as internal controls for normalization of qRT-PCR data in medicinally important plant. The most commonly used internal controls are the genes involved in cellular metabolism and are coded by multigene families. There was no genomic information available for the stable internal control genes in *C.album*, so we first downloaded the genomic sequences of orthologs of the candidate internal controls from public database to design the primers.

We used two statistical approaches to evaluate the expression stability of these candidate internal control genes i.e., NormFinder (Andersen *et al.*, 2004) and geNorm (Vandesompele *et al.*, 2002). These methods are Ct based, so the quality, quantity of RNA and initial input of cDNA was critical. Keeping this in mind, we tried to use more than one method to check the quality and quantity of RNA by Nanodrop and gel electrophoresis. The gene expression stabilities of different candidate genes of *C. album* were analyzed and compared for the selection of best internal control gene under specific abiotic stress. Analysis of gene expression stability through geNorm showed that the most stable genes in each case. In the case of high temperature, heavy metal and drought stress, *M* value of *β-tub* was the lowest, supporting the stability and consistent expression of this gene. We also concluded that there is no need for additional internal controls in the case of high temperature, heavy metal and drought stress based on *V* values. Conclusively, *β-tub* and *18SrRNA* were selected as good internal control genes for heat stress treatments of this plant. Similar findings were shown for the stable expression of *β-tub* in soybean (*G.max*)under heat stress conditions (Libault *et al.*, 2008). While no effect of developmental stages and abiotic stresses on Tobacco *β-tub* was observed (Schmidt and Delaney, 2010). Similarly, in case of heat and metal stress *β-tub* was used as an internal control gene. Our data showed that *18S rRNA* can be used as the most stable control gene for salt induced gene expression normalization in *C. album.* While, *18SrRNA* has been used as internal control for the normalization of heat induced gene expression of *A.americana* (Aman *et al.*, 2012). Our data also showed stable expression of *β-tub* along with *EF1 α* in the case of drought and metal stressed *C.album* samples. *EF1 α* is known to be associated with protein translation (Thornton *et al.*, 2003) and has been validated as best internal control gene for rice, potato and ryegrass (Nicot *et al.*, 2005; Lee *et al.*, 2010).

We also used NormFinder to get more support of this algorithm to compare our validated results by geNorm. Interestingly this method also identified almost the same ranking pattern to select the top one ortwo internal control genes under different stress conditions as determined by

geNorm analysis. *β-tub and 18SrRNA* along with *EF1α*were considered as most stable genes underdifferent type of stresses. *EF1α* gene has also been validated as a stable internal control in *Brachypodium* grown under various stressed conditions or treated with different growth hormones (Hong *et al.,* 2010).Recently, *EF1 α4* hasbeen found to be a stable internal control gene for the expression of the abioticstressed in *G. macrophylla* (He *et al.,* 2016). Additionally EF1 α has also been used as an internal control for the rapid detection and quantification of the virusintomato yellow leaf curl disease-OM (TYLCD-OM) (Ammara *et al.,* 2017).

The results of the gene expression stability index determined by the two selected methods were almost similar in our hands and experimental conditions. For plants, geNorm is a good choice because it gives best internal controls for normalization of qRT-PCR data. Our results also supported the use of isoforms of same internal control because their stability and expression may vary underdifferent experimental conditions, e.g. the expression patterns of actin and *Act-α* were quite different in this study. Similarly *UBQ5* and *UBQ10* have been reported to have variable expression patterns in rice (Jain *et al.*, 2006). In another reports, *ACT2/7* and *ACT11* genes have shown variable expression patterns in soybean (Jian *et al.*, 2008). Our data have shown almost consistent expression of *β-tub, 18SrRNA* and *EF1α* so these genes can be used for normalization of *C. album* under abiotic stresses.

Once we confirmed the order of the selected internal control genes using statistical methods, we used a direct approach to compare the expression patterns of Cp-sHSP gene using different types of internal controls genes under same experimental conditions for which they were validated. We already know that the expression of Cp-sHSP gene is regulated differentially under different types of abiotic stresses in *C. album* (Shakeel *et al.*, 2011; Haq *et al.*, 2013a; Haq *et al.*, 2013b). So we tried to switch the internal control genes for Cp-sHSP transcript level abundance using *β-tub, 18SrRNA* and *GAPDH*. If there is no difference on the internal control stability in variable experimental conditions then we should get the same Cp-sHSP patterns as previously reported. The expression of Cp-sHSP gene was almost similar and consistent under any specific stress condition when two different stable internal control genes were used for normalizations but different pattern using least stable gene, was a clear evidence of effect of experimental or environmental conditions on the expression of the internal control gene.

Conclusion

Based on our evaluations and validations, we concluded that *18SrRNA, β-tub and EF1α*are the most stable genes under specific abiotic stress. We also recommend of using more than one reference genes or combinations for the gene expression studies for more reliable gene normalizations. Current study also provides seven novel reference genes of *C. album* for their use in future gene expression or downstream signaling studies after appropriate validations in specific conditions or tissue samples.

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