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IDENTIFICATION AND VALIDATION OF STABLE INTERNAL CONTROL FOR HEAT INDUCED GENE EXPRESSION OF *AGAVE AMERICANA*

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Abstract

18S ribosomal RNA (*18S rRNA*) has been used as housekeeping gene for normalization in gene expression studies of plants. Recently, the effect of experimental conditions and nature of samples have been shown on the stability of internal control gene. *Agave americana* is a monocot heat tolerant plant adapted to arid conditions with Crassulacean acid metabolism (CAM). Few reports have shown the gene expression studies of this or other CAM plants due to lack of suitable reference gene. Here, we partially sequenced *18S rRNA* gene of agave and evaluated its potential use as reference gene under heat stress conditions.

Gene specific primers were designed from highly conserved regions of known *18S rRNA* genes and amplified by using genomic DNA and transcript of *Agave* followed by sequencing (submitted to gene bank with accession # HM991824). To validate the potential use of Agave *18S rRNA* gene for real-time PCR data normalization, we evaluated the expression stabilities of this gene in different tissues and various heat stress conditions. The plants were treated with different temperatures viz., 35ºC, 40ºC, 45ºC, 50ºC and 60ºC. The relative abundance of a heat regulated gene, *Cp-sHSP* (chloroplast small heat shock protein) was examined by real-time PCR. Varied levels of *Cp-sHSP* gene expression under different heat treatments showed the heat regulated expression. Maximum *Cp-sHSP* gene expression was observed in the leaves of Agave after heat stress for four hours at 45ºC. No significant difference in *18S rRNA* expression was observed among control and heat treated samples. Conclusively, this *18S rRNA* gene could be used as a stable internal control for normalization of real-time PCR data of *A. americana*. This work will help to explore many key players in the heat stress related pathways of CAM plants.

Introduction

Gene expression analysis is becoming an important step in analyzing the biological processes in any living organism. Study of the underneath mechanisms and gene expression of complex mechanisms and their networking can lead to the identification of genes necessary for that particular biological process (Teixeira *et al.*, 2009; Zeller *et al.*, 2009; Zuniga *et al.*, 2009; Chou & Huang, 2010; Di Matteo *et al.*, 2010; Miyazaki *et al.*, 2010; Wang & Xu, 2010; Wang *et al.*, 2010). Reverse transcription real-time quantitative polymerase chain reaction (RTqPCR) is supposed to be an accurate and sensitive method of quantifying mRNA transcripts (Bustin *et al.*, 2005; Duquenne *et al.*, 2010; Regier & Frey, 2010; Vaudano *et al.*, 2010). The detection of amplicon accumulation can be observed from beginnings in this method by use of fluorogenic probes or the dyes which can intercalate such as SYBR Green-I, rather. QPCR is a highly sensitive and specific method with excellent reproducibility and less post amplification procedures (Bustin *et al.*, 2005; Valasek & Repa, 2005; Francino *et al.*, 2006; Imène *et al.*, 2011). That's why qPCR has become one of the favorite method for validation of microarray data or a smaller set of genes, transgenic gene expression, molecular diagnostics and biotic or abiotic stress (Arikawa *et al.*, 2008; Kant *et al.*, 2008; Cortleven *et al.*, 2009; Chang *et al.*, 2010; Di Matteo *et al.*, 2010). It is extremely powerful technique to study the expression patterns of any gene in given conditions but a careful normalization of the data is required. Several experimental strategies have been proposed for normalization of transcript data of different organisms (Bonefeld *et al.*, 2008; Olbrich *et al.*, 2008; Axtner & Sommer, 2009; Artico *et al.*, 2010; Borges *et al.*, 2010; De Santis *et al.*, 2010).

Ideally a good reference gene used for normalization of real-time PCR data should express in a stable way in selected tissues and given experimental conditions. In short the selection of a stable reference gene is very important for accurate gene expression quantification and data analysis (Axtner & Sommer, 2009; Gubern *et al.*, 2009; Bagnall & Kotze, 2010; Boava *et al.*, 2010; Chervoneva *et al.*, 2010). Due to the importance of selection of a stable reference gene in normalization of expression data, different housekeeping genes have been identified and evaluated for stable expression in a given set of conditions in various organisms. In case of plants, many housekeeping genes such as ubiquitin, 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase and actin, b-tubulin were proved to be as good internal controls for expression studies because of their uniform expression in various samples and experimental conditions (Exposito-Rodriguez *et al.*, 2008; Barsalobres-Cavallari *et al.*, 2009; Cortleven *et al.*, 2009; Hu *et al.*, 2009; Artico *et al.*, 2010; Boava *et al.*, 2010). It has been shown that most of the well-known and frequently used reference genes cannot be used as a good internal control for normalization in qPCR because the levels transcript vary considerably under given experimental conditions (Thorrez *et al.*, 2008; Teste *et al.*, 2009). The choice of an appropriate internal control is very important for an accurate quantitative data analysis (Teste *et al.*, 2009; Bentsink *et al.*, 2010; Boava *et al.*, 2010; De Santis *et al.*, 2010).

So far only few reports have shown the identification and validations of appropriate housekeeping gene in plants (Wan *et al.*, 2010; Exposito-Rodriguez *et al.*, 2008; Cortleven *et al.*, 2009; Tong *et al.*, 2009; Artico *et al.*, 2010; Jarosova & Kundu, 2010). Therefore quantitative transcript analysis of many important genes involved in novel pathways of non-model and wild plants are limited due to lack of suitable reference genes. Comparative sequence analysis is a powerful approach toward understanding the structure and translational mechanisms. Different internal control genes of cross species plants have been employed in several studies (Artico *et al.*, 2010; Huis *et al.*, 2010).

18S rRNA gene can be used as an internal control for relative quantification (Tong *et al*., 2009; Bagnall & Kotze, 2010; Jarosova & Kundu, 2010) due to highly conserved regions at the 3′ region and greater tendency to form hair pin loops. It is highly conserved throughout many plant species and several recent studies have led to the conclusion that the ribosomal RNA was the best choice of internal control in a variety of cell systems (Goidin *et al*., 2001; Nicot *et al*., 2005). One of the reasons, why some researchers avoid using ribosomal RNA for normalization of real-time PCR data is its high abundance as compared to target gene expression (Schmittgen & Zakrajsek, 2000), although this ratio varies based on the samples and treatments. There are reports of use of 18S and 25S rRNA as good internal control for highly expressed transcript quantifications (Kim *et al*., 2003). 18S ribosomal RNA show less fluctuation under the conditions that affect the expression of mRNAs, due to high abundance i.e., more than 80% and different polymerases are involved for transcription of mRNAs and rRNAs (Goidin *et al*., 2001). Due to their constant basal level expression, no dependence on cell cycle, and non-responsiveness to external treatments or developing stages, these genes can be used as internal controls.

Production of a specific set of proteins, heat shock proteins (HSPs) has been reported in almost all organisms in response to elevated temperatures and many other types of stresses (Vierling, 1991). This is a universal stress response and the mechanism of plant protection under elevated temperatures or other types of stresses is well conserved. Heat shock proteins production is the key for plant survival under heat stress (Wang *et al*., 2004). HSPs range from 15 to 110 KD. Small heat shock proteins (sHSPs), range in size from approximately 15 to 30kD, are more abundant and diverse in plants than other organisms (Vierling, 1991). Five classes of sHSPs in plants have been reported with different subcellular localizations. Chloroplast small heat shock proteins (CP-sHSPs) are produced in the cytoplasm and then targeted to the chloroplast with the help of a transit peptide that can be cleaved off later on (Chen & and Vierling, 1991; Wang & Luthe, 2003) where they can localized to thylakoids or stroma (Osteryoung & and Vierling, 1994; Heckathorn *et al*., 2002).

In the present work, 18S rRNA gene was identified and sequenced from *Agave americana* (*A. americana*) as a house keeping gene, which can be used as an internal control to check the relative transcript expression under particular treatments of this plant. As this gene is highly conserved, we can also use it in cross-species plants like, *A. americana*, a heat tolerant CAM plant. Chloroplast specific small heat shock proteins have been chosen for study the difference in the gene expression in this plant due to its heat regulated differential expression in many plants under stress.

Materials and Methods

Plant material and treatments: Maguey or *Agave americana* is originally adapted to Mexico, although it is now cultivated in many parts of the world. This is very important plant with many uses like, produces fibers for clothing, ropes, bags and to make many tools. In past its thorns were used as an important tool for perforators in bloodletting rituals. A mildly beverage (alcoholic) called pulque, can also be obtained by the fermentation of aguamiel, the sweet, milky juice extracted from the leaves of the plant.

Experiments were performed using *A. americana*, collected from surroundings of Islamabad and were grown at 30°C/20°C (day/night) in growth chamber. Approximately 100mg plant tissues was used for heat stress in sterilized incubation buffer (1% sucrose, 1mM Potassium phosphate pH 6 and Tween-20) at particular temperature starting from 28°C to 55°C and samples were collected each at 0 (control), 1-4hrs respectively in three replicates. Similarly different tissues of agave plant including leaf tips, leaf base and roots were also treated with elevated temperatures at 45°C for four hours. All the samples were frozen immediately in liquid nitrogen and stored at -70°C until used.

Genomic DNA extraction and PCR: Genomic DNA of *A. americana* was isolated by using a modified CTAB method (Zhang & Stewart., 2000). The samples were resuspended in 30µl 10mM Tris HCl. 5µl sample was loaded on 1% agarose gel and run at 80volts for 90mins to check the quality and quantity of DNA.

Sequences of known *18S rRNA* of different plant species were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) database and were aligned by using Bioedit program. The conserved regions were selected for primer design with Primer3 software. Similarly, Chloroplast small heat shock protein (Cp-sHSP) gene (unpublished data) has been used for Cp-sHSP primers.

Sequencing of 18S rRNA: Amplified *18S rRNA* gene of Agave was sequenced as follows and submitted to gene bank with accession number HM991824. PCR products were purified using the rapid PCR Kit (Marligen, USA) as recommended by manufacture, by adding 100µl conversion buffer and 900ul of binding solution (H1= Conc. Guanidine HCl, EDTA, Tris HCl and Isopropanol). The samples were incubated in water bath at 55°C for 5min after adding 8µl of resuspended silica powder. Samples were vortexed and centrifuged at 13000g for 10sec followed by three times washings with 500µl ice cold sequencing wash buffer. The pellet was dissolved in 30µl of preheated TE at 65°C. The samples were incubated at room temperature for 3min followed by spin at 13000g for 3min. Purified products were confirmed by running on 1.8% agarose gel. Sequencing PCR was done at 95°C for 1min, followed by 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 4min, a total of 30 cycles were repeated followed by a final elongation at 72°C for 10min. Finally PCR products were purified and resuspended in 20µl Hi Dye Formamide (HDF) for sequencing in ABI-310 sequencer by Sanger Dideoxy Chain termination method.

Sequence comparisons and phylogenetic tree: BLASTn was used to find out the similarity of agave specific 18S rRNA gene with other known 18S rRNA sequences downloaded from gene bank. Similarly multiple sequence

alignment was also done by using ClustalW software followed by construction of Phylogenetic tree for further comparisons.

RNA extraction and Reverse transcriptase PCR: Total RNA was isolated by using TRI Reagent (MRC, TR# 118) according to the manufacturer`s instructions from 100mg tissue of control and heat stressed plant samples in three replicates. Samples were homogenized in TRI reagent and 200µl chloroform was added, vortexed vigorously for 30 sec, followed by spin at 11000g for 15min at 4°C. Supernatant (0.5ml) was transferred into a fresh tube. Precipitation was done by Isopropanol at room temperature and precipitated at 5000g for 8min at 4°C. Pellet was washed twice with 75% ethanol with centrifugation at 4000g for 5min at 4°C. Pellet was air dried and dissolved in DEPC water. To check the quality and quantity of RNA, 7µl of each sample was used for 1% agarose gel electrophoresis. RNA was quantified both by gel electrophoresis and Nanodrop spectrophotometer (ND/-1000 V3.7.1) at 230, 260 and 280nm. A total of 5µg of RNA of each sample was treated with DNase I RNase free (Fermentas Cat. # EN0521).

Synthesis of cDNA was done by using Revert Aid first strand cDNA synthesis Kit (Fermentas Cat. # K1621) as described by manufacturer by using oligo dT primers. After cDNA synthesis, PCR was performed to confirm the quality and integrity of samples. A total of 5µg RNA was used to make cDNA. Then cDNA of control and treated samples were amplified with candidate internal control (gene) primers: (forward primer 5´-TCCTGAGTAACGAACGAGACC-3`, reverse primer 5`-CACGATGAAATTTCCCAAGAT-3`) and target gene (Cp-HSPs) specific primers (forward primer, 5`-CAGGATGTTTGAGGATGCGATG-3` and reverse primer, 5`-ATGACCAGCACATTGTCCTCCA-3`) by using following conditions: denaturation at 95°C, then at 94°C for 45sec, annealing at 54°C for 45sec, extension at 72°C for 1min for 30 cycles followed by final extension at 72°C for 10min. 10µl of each amplified transcript was checked on 1.2% agarose gel.

Real-time PCR: Relative quantification of the target gene was done by using *18S rRNA* as reference gene and Cp-sHSP as target gene in ABI 7500 real time PCR system (Applied Biosystems). Real-time PCR was carried out in a 96-well reaction plate using a reaction mixture 12.5µl of the containing Maxima SYBR Green qPCR Master Mix (2X), forward primer 0.3µM, reverse primer 0.3µM, template DNA (<50ng) and nuclease-free water to make up the final volume of 25µl. cDNA of control and treated samples were amplified with candidate internal control (gene) primers and target gene (Cp-HSPs) specific primers as described above. DNA or RNA contamination was ruled out by addition of negative controls. Two types of controls were used including non-DNA control and non-treated controls for each run. Thermal cycling was performed by using a two-step cycling procedure in the three replicates. Uracil DNA Glycosylase (UDG) pre-treatment at 50ºC for 2min and 1 cycle, initial denaturation at 95ºC for 10 min followed by denaturation at 95ºC for 15sec, annealing and extension at 54ºC for 60sec for a total of 40 cycles.

Relative quantification of transcript was done with ABI 7500 systems SDS software *V*1.4 (Applied BioSystems, USA). All analysis was based on Ct values of PCR products. Three biological replicates of each sample were used for real-time PCR. Average Ct values were obtained for each sample and data was normalized with this newly sequenced 18S rRNA of Agave as reference gene. Non treated (control) sample was used as a calibrator. Relative quantities were determined for each sample. Error bars indicate the standard deviations of individual replicates.

Results and Discussion

RNA quality/quantity assessment: Quality of RNA is one of the important factors which can cause the variability in the results of real time PCR. We accessed the quality of RNA initially by A_{260}/A_{280} ratio and A_{260}/A_{230} ratio checked by NanoDrop, because presence of contaminants can decrease these ratios. Quality of RNA isolated was found to be good and pure based on these ratios (1.8 and 1.9 respectfully) in each sample. To remove the traces of genomic DNA in isolated RNA, we used RNase-free-DNase I treatment as described in methods. The second important factor which can increase the variability of real-time PCR results is the initial amount of RNA used for cDNA synthesis. We used NanoDrop to find out the concentration of RNA and equal amounts were used for further accuracy. These results demonstrated that RNA used for this study was good enough for relative quantification by real-time PCR.

Identification and characterization of 18S rRNA gene of agave: 3´ region of 18S rRNA gene is highly conserved in eukaryotes (Hagenbüchle *et al*., 1978; Azad & Deacon, 1980). Therefore, to identify and sequence of 18S rRNA gene of *A. americana,* we designed primers from the conserved regions of known 18S rRNA sequences of different plant species, downloaded from NCBI database. Genomic DNA and cDNA was used to amplify approximately 190bp partial sequence of 18S rRNA gene of agave as shown in Fig. 1a. These products were sequenced individually and submitted to gene bank after confirmation by its homology with other known 18S RNA genes downloaded from gene bank. The highly conserved nature of the Agave 18S rRNA genes was confirmed by alignment with other known 18S rRNA sequences of *Typha angustifolia, Dioscorea sylvatica, Burmannia congesta, Chimonanthus campanulatus, Hortonia floribunda, Doryphora sassafras, Restio tetraphyllus, Puya raimondii, Peumus boldus, Laurelia novae-zelandiae, Juncus effuses, Gomortega keule, Galbulimima belgraveana, Daphnandra micrantha, Atherosperma moschatum, Glomero pitcairnia penduliflora, Calycanthus floridus, Acorus gramineus, Calamus caesius, Thalictrum simplex, Aconitum carmichaelii, Clematis gratopsis, Clematis gratopsis, Aquilegia vulgaris, Actaea cimicifuga, Berberis bealei, Fibraurea tinctoria, Coscinium fenestratum, Arcangelisia flava, Nelumbo nucifera, Gymnosiphon bekensis, Gladiolus buckerveldii, Triticum aestivum, Zea mays and Solanum tuberosum.* The *18S rRNA* sequence of agave

has 98-99% similarity with other known *18S rRNA* genes of diverse plants as shown in Fig. 1b. Similarly, phylogenetic tree was constructed to check the evolutionary relationship among *18S rRNA* of *A. americana* and that of from other plants as shown in the Fig. 1c. Analysis of this tree has revealed the conserved nature of *18s RNA* of CAM plants and interestingly it grouped together with another monocot, *Zea mays* which is C4 grass and has different metabolism. These similarities with other monocots have shown the evidence of some changes in the past during their evolution from a common ancestor.

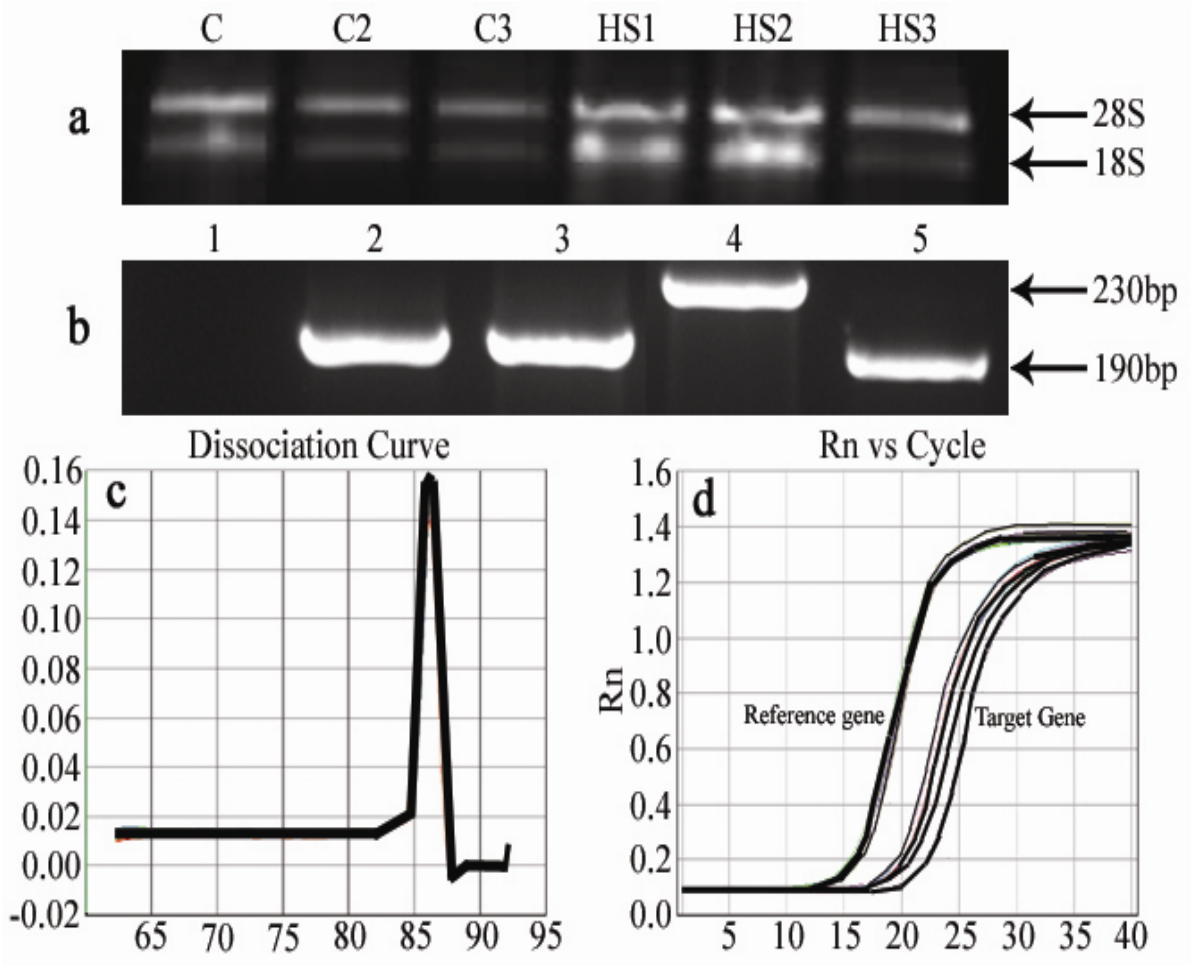

Fig. 1. A) RNA isolated from three individual replicates of control plant samples (C) and heat stressed samples (HS). B) Amplification of partial sequence of *18S rRNA* and Cp-sHSP gene of *A. americana.* Gene specific primers of *18S rRNA* were used to amplify genomic DNA and mRNA extracted from leaves of agave after heat stress (Lane 2 and 3 respectively), and Cp-sHSP primers to amplify, genomic DNA and mRNA extracted from leaves of agave after heat stress (Lane 4 and 5 respectively). PCR products were run on 1.5 % agarose gel along with 100bp DNA ladder. No amplification was seen in the non-template controls (Lanes 1). C) Melting curve analysis of all primers used for real-time PCR. D) Amplification plot of target and reference gene generated by SDS software of real-time PCR.

Expression patterns of candidate reference gene and target gene by relative quantification: There are many reports on the stable and constant expression of different reference genes to use for gene expression studies (Wan *et al.*, 2010; Exposito-Rodriguez *et al.*, 2008; Cortleven *et al.*, 2009; Tong *et al.*, 2009; Artico *et al.*, 2010; Jarosova & Kundu, 2010). For an ideal reference gene, the expression should remain nearly constant under any experimental conditions (Butte *et al*., 2001). In order to check the effect of experimental conditions on the expression of agave *18S rRNA*, we used agave specific Cp-sHSP gene as a target gene for relative quantification by real-time PCR under varied levels of heat treatments. We used agave specific Cp-sHSP primers to amplify one of the heat induced chloroplast small heat shock protein by using *18S rRNA* gene a reference gene for relative quantification in real time PCR. All primers were first tested with standard RT-PCR and melting curve analysis by using real time PCR to optimize the conditions and to exclude the possibility of presence of primer di-mers. The single products of each gene were verified by gel electrophoresis (Fig. 1a). Melting curve analysis of the primers used has revealed the presence of unique melting peak (Tm) showing specific nature of primers with no mismatch or false priming to the selected genes.

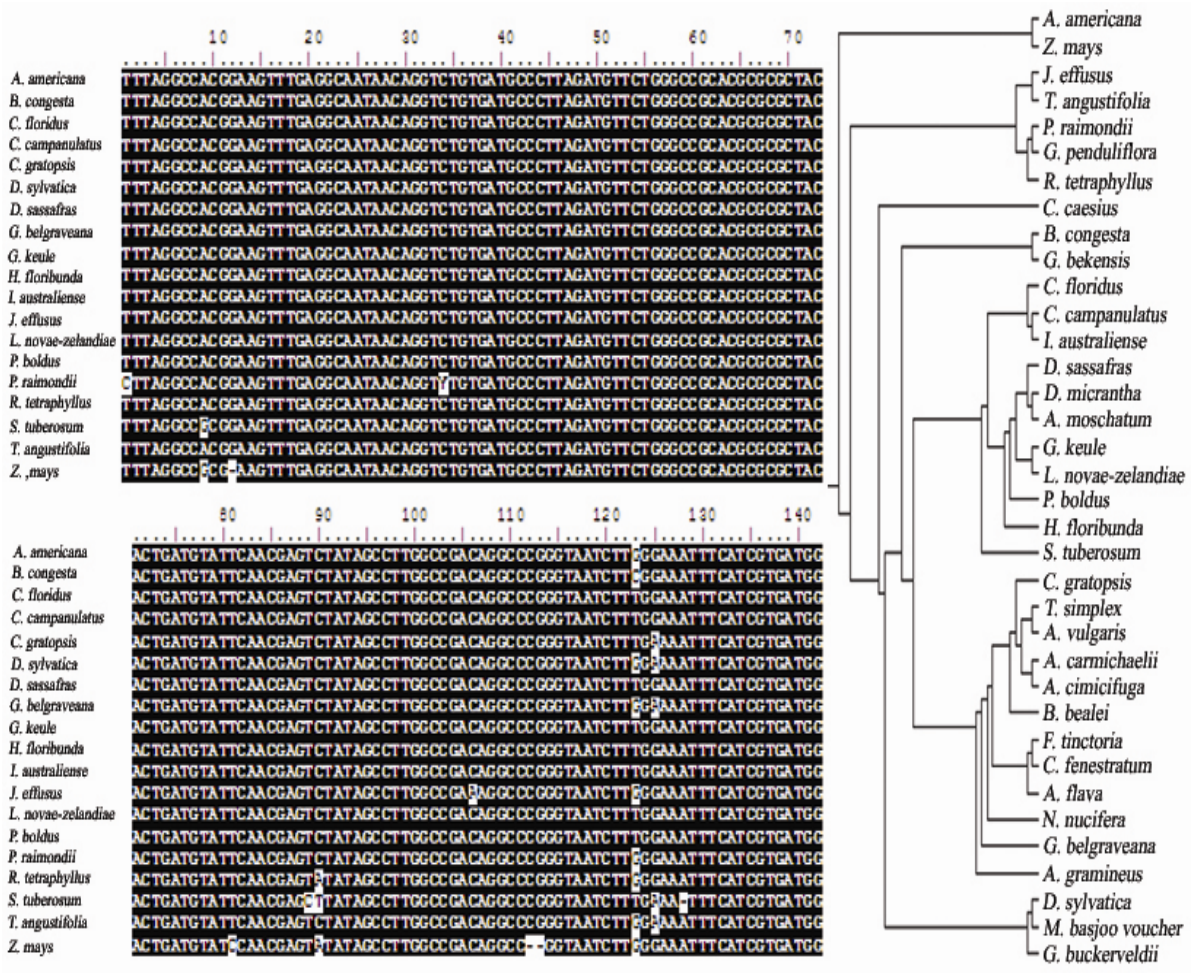

Fig. 2. Sequence alignment and phylogenetic relationship of *18S r RNA* gene of *A. americana* with other known genes downloaded from public database.

Time course expression studies of target gene (Cp-sHSP) and reference gene (*18S rRNA*) was done by giving heat stress to agave plant at 45°C for 1-4hrs. Control plants were kept at 28°C for the given period of time. Total RNA was isolated from all samples. Maximum expression of Cp-sHSP was seen after heat treatment of 4hrs as compared to controls by using 18S rRNA as reference gene (Fig. 2a). While no significant difference in the expression of 18S rRNA gene was seen under heat stress.

Similarly the effect of dose response was seen after heat stress of four hrs at 30°C, 35°C, 40°C, 42°C, 45°C, 50°C and 55°C. Whereas all the control plants were kept at 28°C room temperature and expression levels of target and candidate reference gene was compared. The expression of agave chloroplast small heat shock protein was induced by increasing the heat stress, and maximum expression was seen at 45°C (Fig. 2b). This was followed by a decrease in transcript levels as temperature increases to 55°C, showing the degradation of transcript at higher temperatures. While the expression of *18S rRNA* gene remained constant throughout the heat treatments as shown in Fig. 2. Almost no variability in absolute Ct values of *18S rRNA* of each sample generated with SDS software were examined individually and plotted as shown in the Fig. 2.

To check the expression levels of 18S rRNA in different tissues or parts of agave plant, we examined the relative expression of target gene and reference gene in Leaf tip, leaf base and roots of the agave plant with and without heat stress. Leaf samples accumulated maximum Cp-sHSP as compared to other tissues after heat stress at 45°C for four hours, while no apparent effect of heat stress was seen on 18S rRNA gene expression Fig. 3. Similarly amplification plot of target and reference gene generated by SDS software of real-time PCR has shown less difference in the amplification verses cycles of PCR, which is indicative of variations in the initial levels of transcripts in the given samples (Fig. 3b). Melting curve analysis of primers used for real-time PCR was also done as given in the Fig. 3c; presence of single peak excludes the possibility of presence of primer di-mers. Agave *18S rRNA* can be used as a good internal control for heat stress related studies of high expression genes like sHSPs, because in our hands it had stable expression and no effect of experimental conditions and tissue used was observed. These results are consistent with use of 18S and 25S rRNA as good internal control for quantification of genes with high expression levels in rice (Kim *et al.*, 2003).

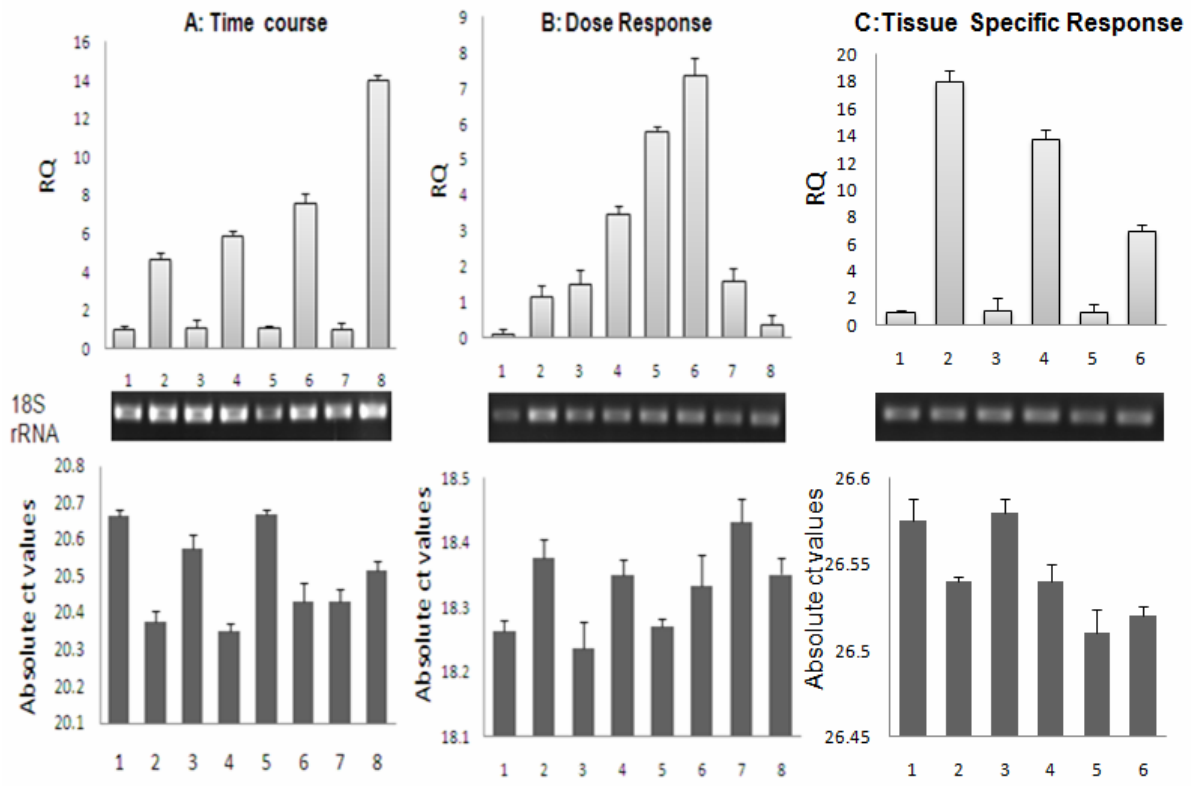

Fig. 3. Time, dose dependence and tissue specific response of target gene and candidate reference gene under heat stress. To study the time course (A), plant were grown 45 ºC for 1-4 hours, for dose response (B) the plants were treated at 28ºC, 30ºC, 35ºC, 40ºC, 42ºC, 45ºC, 50ºC and 55ºC for 4 hours and to check the tissue specific response (C) different tissues (leaf tip, leaf base and roots) with and without heat stress (45ºC for 1-4 hours) were used. Total RNA was extracted from three biological replicates of each treatment and respective controls (without heat treatment). Message levels for *Cp-sHSP* (*mRNA*) were determined by real-time PCR by using gene specific primers of Cp-sHSP genes and *18S rRNA* gene as a reference gene to normalize the data. 6µl of amplified product of *18S rRNA* was run at 1.5% agarose gel for visualizations. Similarly graphical representation of absolute ct values of *18S rRNA* gene of *A. americana* generated by SDS software is given which was used for normalization of target gene.

Conclusion

A lot of transcript information's and data is available now days for the analysis of major model plant species to facilitate the identification of appropriate reference genes for gene expression normalization. However, very less is known for most of the other plant species, especially wild plants. In this study, we have identified a suitable reference gene for studying heat regulated gene expression in *A. americana.* The potential use of *18S rRNA* as reference gene has been shown by studying the expression profiles of chloroplast specific small HSP gene of Agave. As less is known about the CAM plants and CAM metabolism, other researchers can use this gene as an internal control for several gene expression studies of agave and related plants.

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