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Development and characterization of a high temperature stress responsive subtractive cDNA library in Pearl Millet *Pennisetum glaucum* (L.) R.Br.

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Pearl millet (*Pennisetum glaucum* L. R. Br.) is an important cereal crop grown mainly in the arid and semi-arid regions of India known to possess the natural ability to withstand thermal stress. To elucidate the molecular basis of high temperature response in pearl millet, 12 days old seedlings of *P. glaucum* cv. 841A were subjected to heat stress at 46°C for different time durations (30 min, 2, 4, 8, 12 and 24 h) and a forward subtractive cDNA library was constructed from pooled RNA of heat stressed seedlings. A total of 331 high quality Expressed Sequence Tags (ESTs) were obtained from randomly selected 1050 clones. Sequences were assembled into 103 unique sequences consisting of 37 contigs and 66 singletons. Of these, 92 unique sequences were submitted to NCBI dbEST database. Gene Ontology through RGAP data base and BLASTx analysis revealed that about 18% of the ESTs showed homology to genes for "response to abiotic and biotic stimulus". About 2% of the ESTs showed no homology with genes in dbEST, indicating the presence of uncharacterized candidate genes involved in heat stress response in *P. glaucum*. Differential expression of selected genes (*hsp*101 and *CRT*) from the SSH library were validated by qRT-PCR analysis. The ESTs thus generated are a rich source of heat stress responsive genes, which can be utilized in improving thermotolerance of other food crops.

Keywords: Expressed sequence tags, Heat stress, Heat shock proteins, Millets, qPCR validation, SSH library, Stress responsive genes, Thermal stress

With the changes in global climate, environmental stresses such as drought, heat and salinity have gained vital importance because of their adverse impact on agriculture and food security. The most prominent manifestation of climate change is the rising atmospheric temperature which adversely affects plant growth and yield[1]. All climate change models indicate a rise of 1.8 to 4.5°C in the atmospheric temperature by the year 2100[2]. In ecological terms, it is a rapid change that is expected to have a severe effect on countries having agrarian economy such as India. Furthermore, as the world population grows exponentially, there is a need to increase agricultural productivity and also to expand productive areas of the world. Therefore, the ability of the crops to adapt and yield under harsh climate will play a crucial role in determining the sustainability of food production.

Pearl millet (*Pennisetum glaucum* (L.) R.Br.), is the main food source of the poor in India and the African continent. It is generally grown between 40° North and 40° South of the equator, in warm and hot countries characteristic of the semi-arid environment. Pearl millet is highly tolerant to various abiotic stresses and can survive well even at 45°C[3]. Pearl millet which has natural ability to withstand high level of heat stress may house a repository of genes for heat tolerance which may come in use for imparting thermotolerance to other cereals[4]. Among the various techniques utilized, suppression subtractive hybridization (SSH) has proved to be an efficient approach to identify differentially expressed genes in the absence of sequence information. Using SSH, chances for identification of rare transcripts involved in stress is substantially increased. This method has successfully been used to identify genes responsive to various biotic and abiotic stresses in various plants *viz*., *Agrostis*[5]; *Festuca*[6]; *Pennisetum*[7]; *Saccharum*[8] and *Triticum*[9].

Thus, in the current study we focus on identifying the major differentially expressed genes in

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P. glaucum during heat stress response. The identified heat responsive genes, differentially expressed in the subtractive cDNA library were also validated by qPCR.

Materials and Methods

Plant material—Seeds of *P. glaucum* cv. 841A were procured from the Pearl Millet Breeding Unit of the Indian Agricultural Research Institute, New Delhi. Seeds of similar size, visibly free of insect/fungal infection were grown in pots containing autoclaved Soilrite™ at 33°C under a 16 h photoperiod at the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi, India.

Heat stress treatment— Pearl millet, like many cereals, is most susceptible to heat stress in the first 10-15 days of seedling emergence as compared to other vegetative stages[10]. Preliminary physiological and biochemical results helped determine 46°C as adequate thermal stress (Suppl. Fig. 1). Hence, twelve days old seedlings were subjected to heat stress at 46°C for different time periods, i.e., 30 min, 2, 4, 8, 12 and 24 h. The seedlings after being subjected to the stipulated heat stress were cleaned thoroughly with RNAse OUT™ and flash frozen immediately using liquid Nitrogen and stored at −80°C for RNA isolation so as to preserve the stage specific transcripts. Another set of heat stressed plants were used to assess the level of stress by biochemical and physiological studies such as estimation of lipid peroxidation, cell membrane stability (CMS) and relative water content (RWC) of the seedlings (http://www.plantstress.com/methods/index.asp).

The CMS in response to heat stress treatment was measured as per the protocol described by Blum and Ebercon[11] while RWC of the heat stressed samples was estimated under fully turgid and dried conditions adopting the protocol of Barr and Weatherley[12]. Lipid peroxidation for the same samples was estimated followed by the method given by Heath and Packer[13].

Total RNA and mRNA isolation—Total RNA from the leaf sample of seedlings of each treatment and control samples after various durations of heat stress were isolated using QIAzol™ (Qiagen™, Germany). The quantity and quality of total RNA was determined by spectrophotometry using Nanodrop (Thermo Scientific, USA). Poly A^+ mRNA from the total RNA was isolated by Oligotex mRNA Spin-Column kit (Qiagen™, Germany). The presence of two bright bands in agarose gel corresponding to ribosomal 28S and 18S rRNA with a ratio of intensities of ~2:1 confirmed the integrity of RNA. The OD ratio of 260/280 nm of the total RNA isolated from each treatment ranged from 1.98 to 2.10 and the concentrations ranged from 978 to 2567 (ng/µl).

Construction of heat responsive subtractive cDNA library through SSH—Double-stranded cDNA was prepared from 2 µg of poly (A)+ mRNA (tester population) and control RNA (driver population) and the forward Suppression Subtractive Hybridization (FSSH) library was constructed following the manufacturer's instructions using the PCR Select cDNA Subtraction Kit (Clonetech, USA). The cDNA from different heat stressed samples were pooled to include various stress responsive genes expressed at different time periods. *Rsa*I digestion prior to adaptor ligation of the tester cDNAs caused changes in the cDNA size. Amplification of subtracted cDNA was visualized by agarose gel electrophoresis. The primary PCR product was observed as a smear from 0.5 to 2 kb with some distinct bands between 750 bp and 2 kb. The secondary PCR product produced a distinct amplified smear compared to primary PCR product. The cDNAs enriched for differentially expressed genes, obtained after the secondary PCR were ligated[14] into pGEM®-T Easy vector (Promega, USA). Electrocompetent *E. coli* NEB 10 beta cells (New England Biolabs,USA) were transformed with the ligated product using electroporator (Eppendorf Multiorator 30672, USA). The transformed cells were spread on LA-Ampicillin (100 µg/ml) plates supplemented with 100 µl of IPTG (0.1M) and 10 µl X-Gal (100 mg/ml) and incubated at 37°C overnight for blue-white colony screening. White colonies were randomly picked and colony PCR was carried out to confirm the presence of insert. Insert size in recombinant plasmid was analyzed by 1% agarose gel electrophoresis[14].

Sequencing and Data Analysis—The PCR positive recombinant clones with insert size of >500 bp were sequenced by single pass sequencing using the vector specific T7 forward primer by an automated DNA sequencer (Sequence Analyzer Version 2.0, ABI Prism, Chromous Biotech, India). The obtained raw expressed sequence tag (EST) sequences were vector/adaptor screened using DNASTAR™ Navigator Suite. The edited ESTs which were >100 bp in length were assembled and clustered into contigs and singletons using SeqMan Pro program (https://www.dnastar.com). BLASTx was performed

using NCBI databases (http://www.ncbi.nlm.nih.gov/blast) and RGAP (Rice Genome Annotation Project) (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml) to determine similarity of these ESTs with known proteins from other cereal crops or plants. High temperature stress modulated genes were categorized using the GO (Gene Ontology) IDs available with the RGAP database.

Validation of differentially expressed genes by qRT PCR—Selected differentially expressed transcripts identified in heat responsive subtracted cDNA library of *P. glaucum* were validated by qPCR analysis using specific oligonucleotide primers designed using IDT Primer Quest software (Table 1). Indigenous β Tubulin gene of *P. glaucum* was used as an internal control. Expression analysis by qPCR was carried out using Kappa SYBR® FAST qPCR Kit on a Roche Light Cycler 480™ as per manufacturer's instructions. The experiments were repeated at least twice independently, and the fold change data were averaged. Data of the qPCR results was analyzed by 2-ΔΔCt method[15] using the Roche Light Cycler 480™ (qPCR Analysis) Software v.1.5. Furthermore, a heat map of expression profile under different heat stress experiments of genes obtained in the SSH library was generated utilizing publicly available microarray data. Orthologous probes of *Arabidopsis thaliana* were used to hierarchically cluster the genes according to expression levels using the Genevestigator™ database (https://genevestigator.com/gv/)[16].

Table 1—List of primers pairs used for qPCR in this study

Gene Specific Primers	Primer Sequence
Pg β Tubulin	For: 5'-ACGATATACCACCACCACCAC-3' Rev: 5'-CGGACGAAAGGACCTCACC-3'
HSP 101	For:5'-TCACATCCACAAGGACGGTGTCAT-3' Rev:5'-TGCAAGGCCTGTCAAGAGAGTGAT-3'
CRT 1	For: 5'-ACTGTGGTGGTGGTTACGTCAAG-3' Rev: 5'-TGATGAGGTGGTTCTTGCCATCCT-3'

Results and Discussion

Physiological and biochemical analysis—The cell membrane stability (CMS) value of 12 days old seedlings at 30 min heat treatment was 95.2 % as compared to the control sample which showed 98.8% (Fig. 1a). After 8 h of heat treatment, the CMS of the seedling showed significant reduction of 86.25% with 13.75% relative injury. The seedlings after 24 h heat treatment (72.85% of CMS, 27.15% of heat injury) showed necrosis with highly wilted leaves. The relative water content (RWC) of heat stressed seedlings also gradually decreased with the increased duration of stress. The RWC was 95.30 % after 30 min of heat treatment and gradually decreased with increased duration of heat treatment which was 83.23% after 2 h. Though it showed a slight increase (86.93%) after 4 h, it declined sharply thereafter to 76.93% at 8h, and further to 52.77% at 24 h (Fig. 1a). Lipid peroxidation levels indicate the effects of oxidative stress on the cellular membranes of organisms during high temperature stress[17]. Here, the lipid peroxidation during the different time duration of heat treatment, as estimated by TBARS content showed an increasing trend reaching 24.03 nmol/mg after 8h of heat stress followed by a steep increase beyond 8 h of heat stress and reached a maximum of 93.87 nmol/mg after 24 h. Such an increase of more than double from 12 h to 24 h of heat stress, suggests a severe damage to the membrane lipids of the

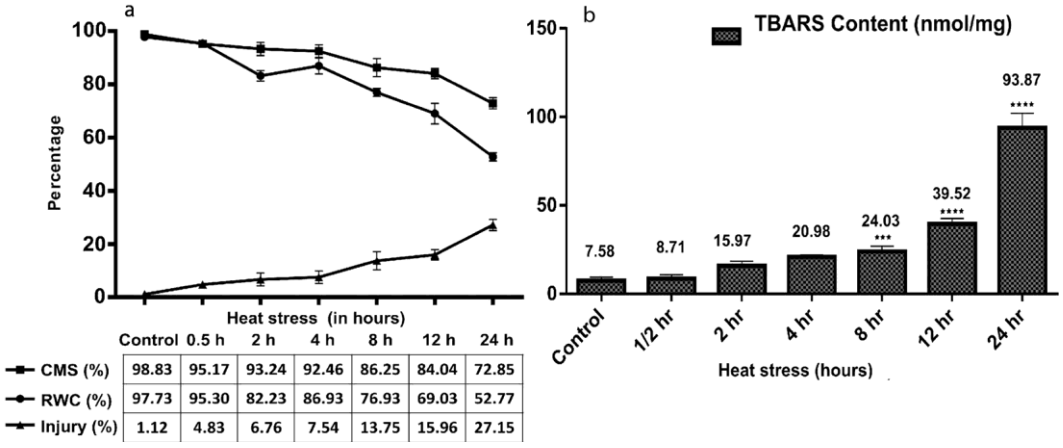

Fig. 1—Estimation of changes in heat stressed seedlings of *Pennisetum glaucum* by physiological and biochemical parameters (in %). (a) Cell membrane stability (CMS), Heat injury (1/CMS) and relative water content; and (b) lipid peroxidation. Data shown are means ± S.D.

seedlings leaf cells due to heat induced by oxidative stress (Fig. 1b). The initial values of low lipid peroxidation up to 8 h stress durations suggest an efficient oxidative damage detoxification system at play in *P. glaucum* under high temperature stress. After 30 min of heat stress, only 4.8 % heat injury was observed and even after 4 h of heat stress the injury percent was only 7.5% indicating that *P. glaucum* cv. 841A is considerably thermotolerant for short periods of heat stress. After 24 h, the heat injury was 27.5%. Similarly, the RWC of the seedlings at 24 h was 52.77% which shows that *P. glaucum* has considerable capability of retaining water even under severe heat stress. Thus, physiological parameters measured during heat stress showed that *P. glaucum* displayed high thermo-tolerance up to 8 h of heat stress. This was a general trend observed in CMS, lipid peroxidation and RWC values.

Construction of SSH library and EST analysis—The Forward subtractive cDNA library (FSSH library) from heat stressed *P. glaucum* cv. 841A was constructed and 1050 white colonies were randomly selected and inserts confirmed by colony PCR. The insert size in the library ranged between 300 bp and 1.5 kb with an average of 750 bp. High throughput sequencing was done for 384 clones and sequences were analyzed. The resulting 331 good ESTs were assembled into 103 unique sequences containing 37 contigs and 66 singletons. Of these, 92 unique sequences were submitted to the dbESTs of NCBI (Accession No. LIBEST_027963: JK999476-JK999567). BLASTx analysis of the EST sequences was done against the non redundant protein database available at RGAP for identifying the putative function of the particular ESTs. The ESTs were assigned with specific GO terms using the GO-IDs available in the RGAP database and all the ESTs were functionally categorized under the biological process. Around 18.0% ESTs of the heat responsive forward subtractive cDNA library were identified as directly or indirectly related to stress. Of these, 12 % of the ESTs were classified under the response to abiotic and biotic stimulus. The maximum number of ESTs, 17 %, was categorized under the class of precursor metabolites and energy, whereas around 15 % of the ESTs were related to the class photosynthesis (Fig. 2).

Stress responsive genes identified in SSH library—A direct result of stress induced cellular changes is the enhanced accumulation of toxic compounds in cells that include reactive oxygen species (ROS). The presence of many genes involved in the ROS detoxification in the subtracted cDNA library was along the expected lines due to the observation of considerable oxidative stress as shown by the lipid peroxidation tests (Table 2). These genes included glutathione peroxidase (GPX), tropinone reductase like (TRL), ATPAP18/PAP18 Purple acid phosphatase. Enzymes such as glutathione peroxidase (GPX) have been shown to be involved in ROS scavenging pathways in plants. It reduces the amount of ROS produced due to oxidative stress linked to the heat stress. Transgenic plants over-expressing GPX were more tolerant than wild type plants to a combination of temperature and high-light stress[18].

Tropinone reductase like (TRL) enzymes[19] and purple acid phosphatases[20] have also been reported in response to oxidative and abiotic stress in many plants. A cytosolic Fe-S cluster assembling factor

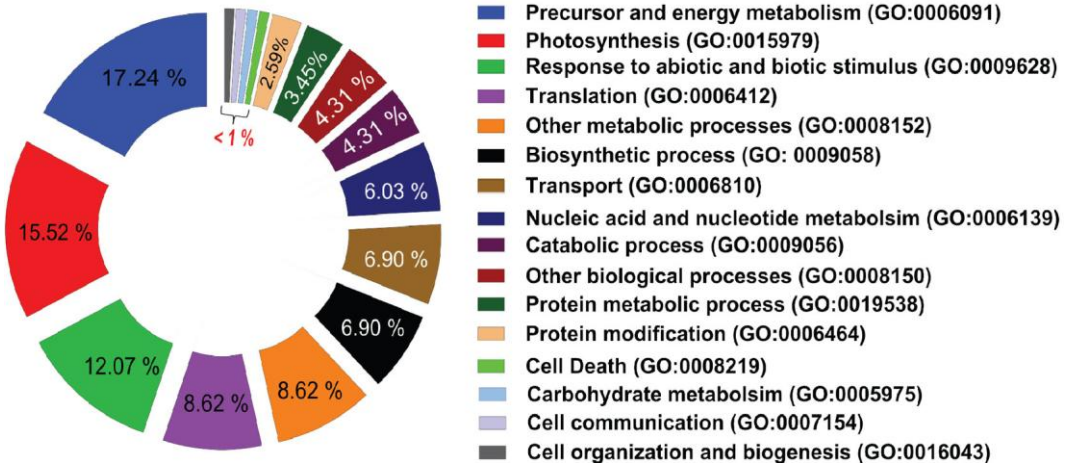

Fig. 2—High temperature stress modulated ESTs identified in forward subtractive cDNA library functionally categorized using the GO (Gene Ontology) IDs available in RGAP database.

Table 2—Some important ESTs obtained in response to heat stress in the forward SSH library and their BLASTx analysis details

EST ID	Locus ID	Gene product	E value	Query coverage (%)	Identity (%)
S6_pg_fssh	Loc_os08g36150.1	Activator of 90 kDa heat shock protein	$9.10e^{-26}$	50.89	83.58
S8_pg_fssh	Loc_os04g16740.1	Calreticulin 1	$3.80e^{-98}$	80.32 s	99.50
C32_pg_fssh	Loc_os08g39140.2	Protein clpb 1, Hsp 101 related cluster	$2.50e^{-49}$	49.76	90.38
S18_pg_fssh	Loc_os06g08670.1	Glutathione peroxidase	$8.70e^{-07}$	38.14	41.33
S27_pg_fssh	Loc_os04g42090.4	Sam decarboxylase (cpu ORF7)	$9.90e^{-52}$	61.95	72.30
S11_pg_fssh	Loc_os01g57966.1	Protein ycf3	$9.40e^{-24}$	22.35	100.00
S42_pg_fssh	Loc_os03g13840.2	Senescence-associated protein	$3.40e^{-58}$	71.70	86.03
S60_pg_fssh	Loc_os02g48780.1	IQ calmodulin-binding and bag domain containing protein	$2.00e^{-06}$	20.67	44.68
S66_pg_fssh	Loc_os03g16210	Tropinone reductase	$3.80e^{-18}$	40.52	74.19
C16_pg_fssh	Loc_os05g23740.1	Dnak family protein	$1.70e^{-118}$	99.60	92.55
C29_pg_fssh	Loc_os05g05270.1	Sucrose-phosphatase	$1.70e^{-53}$	74.07	84.17
C31_pg_fssh	Loc_os04g38870.4	14-3-3 protein	$2.50e^{-117}$	99.58	96.23
C36_pg_fssh	Loc_os04g28420.1	Peptidyl-prolyl isomerase	$6.80e^{-110}$	56.68	88.57
S1_pg_fssh	Loc_os07g43950.1	RNA recognition motif containing protein, SC35-like splicing factor	$2.70e^{-33}$	65.94	69.75

NBP35 was also identified in the library. Yarunin *et al*. have demonstrated the role of such assembly factors in assembling Fe-S cluster containing proteins and ribosome biogenesis under oxidative stress. Thus, an up-regulation of such assembly factors to aid in protein synthesis can be hypothesized as a mechanism to overcome the heat induced oxidative stress[21].

Several ESTs present in the subtracted library had high homology to heat shock proteins (HSPs) of different classes. A few of them are Hsp 101/chaperone protein ClpB1, DnaK family protein, Hsp 90, Hsp70 binding protein (Hsp70 bp) folding regulator, a CS domain containing telomerase-binding protein p23 (hsp90 co-chaperone). The major biological role of these HSPs, known as molecular chaperones, is to maintain and shield the unfolded state of newly synthesized proteins thus preventing them from misfolding or aggregating[22]. The major role of Hsp 90 is to manage protein folding, but they also play a key role in signal transduction networks, cell cycle control, protein degradation, protein trafficking, morphological changes and stress adaptation[23-26]. An activator of 90 kDa Hsp ATPase homolog was also obtained in the subtracted library which is necessary as Hsp 90 requires ATP for its action. The ClpB1 chaperone family generally functions in protein denaturation and degradation[27]. They remove non-functional and harmful polypeptides arising due to misfolding, denaturation or aggregation to maintain cellular homeostasis. A member of the 14-3-3 like protein family was also identified in the library. These proteins have been reported as heat shock related molecular chaperones that dissolve thermally aggregated proteins[28]. The Ycf3 protein is essential for accumulation of photosystem I (PSI) complex and acts at a post-translational level. The thylakoid protein Ycf3 appears to act as a chaperone that interacts directly and specifically with at least two of the PSI subunits during assembly of the PSI complex[29]. Its presence in the differentially expressed library indicates its prime role in maintaining the efficient functioning of photosynthesis during heat stress.

Other proteins which help in maintaining photosynthetic activity were identified in the library. Some of these like the P700 chlorophyll apoprotein A2 photosystem II D2 protein, photosystem II 44 kDa reaction center protein are part of the photosynthetic apparatus, and are likely to be produced at higher rates or in thermotolerant mutein variants to cope with the high temperature stress. A peptidyl-prolyl isomerase gene involved in the biosynthesis of the stress induced amino acid proline was also identified in the FSSH library. This gene is of the FKBP family, reported to be heat induced in wheat[30]. An amidohydrolase type gene involved in the synthesis of polyamines was also identified[31]. Polyamines have been found to be over expressed in response to high temperature stress in many plants and have been implicated as major players in many abiotic stresses[32]. Some genes involved in the complex signaling

pathway associated with heat stress identified in the library are IQ calmodulin-binding protein coding gene and the *CRT* gene. Calreticulin 1 gene is a highly versatile lectin-like chaperone, and it controls intracellular Ca^{2+} homoeostasis by modulation of Ca^{2+} storage and transport. It is involved in the calnexin/calreticulin cycle which is upregulated during stress[33].

Various stresses cause changes in cellular Ca^{2+} level, which acts as a messenger in modulating different physiological processes that are necessary for stress adaptation[34].

A gene homologous to the serine/arginine rich (SR) protein called SC 35 which has a RNA recognition motif has also been identified. Members of the SR protein gene family play a significant role in the regulation of alternative splicing, an important means of generating proteome diversity and regulating gene expression[35]. In addition to these, a zinc finger protein (ZFP) containing a MYB domain has also been identified in the library. However, the protein has not been characterized and its role in abiotic stresses needs to be investigated.

Similarly, around 2% of the ESTs of the subtracted library are novel and uncharacterized, and are putatively expressed proteins with no homology matches to any of the major public databases. Their functional characterization and possible role in heat stress and thermotolerance are yet to be investigated.

Validation of a set of stress responsive genes by qPCR—Quantitative PCR validation was carried out to monitor the relative expression levels of the *CRT* and *hsp* 101 genes that were identified in the heat stress responsive *P. glaucum* EST collection. The qPCR analysis confirmed the differential nature of the subtracted library. The *CRT* gene showed almost 6-fold expression compared to the control after 8 h of heat stress (Fig. 3a). The *CRT* gene expression was observed to be upregulated in the later stages of heat stress. This may answer the extensive increase in calcium dependent signaling pathways in response to increasing duration of heat stress. There was an apparent downregulation of the *CRT* gene after 4 h of heat stress. Such variability in *CRT* gene expression levels has been prominently observed in earlier studies on low temperature stress in wheat[36]. This result was observed in repeated experiments and can be possibly explained by the reasoning that most gene expressions are dynamic in nature and are not expected to be upregulated all the time. In fact, it is quite possible that the gene product might have accumulated in sufficient amounts to warrant the switching off of the gene. A similar observation was made in the case of *hsp*101 after 30 min. of heat stress at 46°C (Fig. 3b). This is along the expected lines as *hsp* 101 is responsible for acquired thermotolerance after some amount of heat stress, and hence, the low amounts initially[33]. Quantitative expression analysis of *hsp 101* gene showed about 20-fold increase in expression after 2 h of heat stress. There was reduced expression of *hsp* 101 during treatments beyond 8 h. The quantitative upregulation in gene expression of the selected genes in their response to heat stress clearly showed that the subtractive cDNA libraries constructed in this study were substantially enriched for stress responsive genes. Relative expression analysis of few genes by qPCR confirmed that the SSH library contained differentially expressed genes such as *hsp101*, *CRT* which are regulated at different stages of the heat stress by varying degrees. The differential nature of the library was further validated

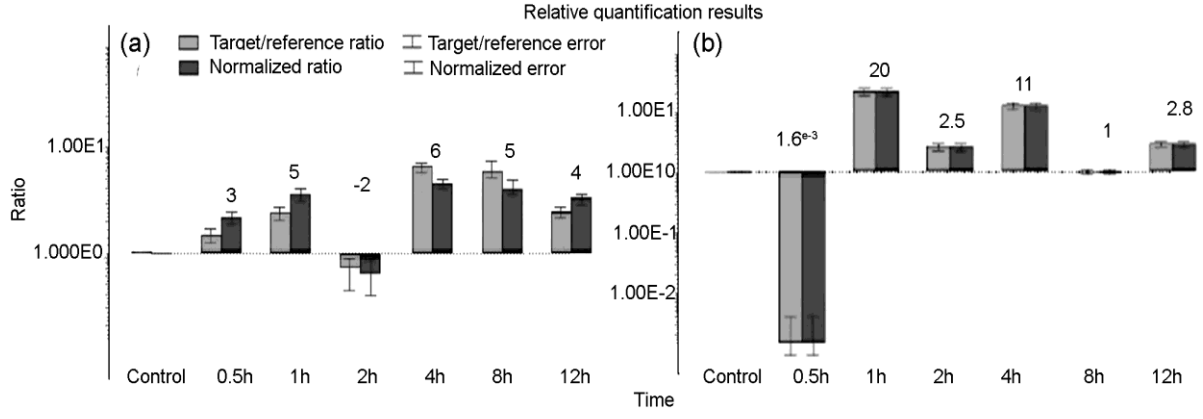

Fig. 3—Validation of differentially expressed gene identified in forward subtractive cDNA library of heat stressed *Pennisetum glaucum* seedlings through qRT PCR analysis. (a) *CRT* and (b) *hsp 101*.

by the high expression levels of orthologous genes in *Arabidopsis* under different heat stress experiments as shown by the hierarchically clustered heat map (Suppl. Fig. 2). The quantitative upregulation of different genes in *P. glaucum* seedlings, in response to heat stress, suggest that *P. glaucum* can effectively reprogram its intricate signaling networks on a global scale to activate regulated expression of several genes. It helps in mitigating the stress-induced cellular damage and grow in regions that are too harsh for other crops to survive.

Generation of differentially expressed ESTs using SSH has been extensively used for identifying genes expressed under heat stress in different crops. Once the differentially expressed genes with higher expression levels have been identified, their function in response to heat stress can be validated by cloning the full length genes by techniques such as Rapid Amplification of cDNA ends (RACE) PCR[38]. The novel and uncharacterized expressed putative proteins in the EST library may be possible candidate genes for thermotolerance. These novel genes can be functionally validated through over-expression and or knockdowns/knockouts in model plant systems via transgenic technology. Allele mining of such full length genes can help us in selection of better variants which will be indispensable tools in crop engineering for thermotolerance. The heat stress responsive EST library generated can be used as a resource for developing genetic markers such as EST SSRs for use in comparative mapping, for tagging/map based cloning of important traits of interest and also in selection of plants with better thermotolerance. By identifying and cloning the upstream promoter elements of such genes, using techniques such as genome walking, it is possible to study regulation of the complex procedure of thermotolerance. Precise genome editing tools based on homologous recombination including nuclease based Zinc Finger Nucleases (ZFNs) and Transcription activator-like effector nucleases (TALENs) are proving to be the next generation tools at the frontiers of plant biology research. With clearer insights into the role of specific sequences in such promoters, we can also think of the possibility of fine-tuning gene expression rates of several genes by subsequent and repeated editing of their regulatory elements using such ground-breaking technologies. This provides a chance to engineer the transcriptome of crops for obtaining thermotolerance at a global scale. Thus, the current study provides essential information to further understand and gain helpful insights into the genetic basis of differential adaptation of plants to adverse thermal stress.

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Disclosure

Authors declare no conflicts of interests.

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