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RESEARCH ARTICLE

Transcriptional profiling in pearl millet (*Pennisetum glaucum L.R. Br.*) for identification of differentially expressed drought responsive genes

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Abstract Pearl millet (*Pennisetum glaucum*) is an important cereal of traditional farming systems that has the natural ability to withstand various abiotic stresses. The present study aims at the identification and validation of major differentially expressed genes in response to drought stress in *P. glaucum* by Suppression Subtractive Hybridization (SSH) analysis. Twenty-two days old seedlings of *P. glaucum* cultivar PPMI741 were subjected to drought stress by treatment of 30 % Polyethylene glycol for different time periods 30 min (T1), 2 h (T2), 4 h (T3), 8 h (T4), 16 h (T5), 24 h (T6) and 48 h (T7) respectively, monitored by examining the RWC of seedlings. Total RNA was isolated to construct drought responsive subtractive cDNA library through SSH, sequenced to identify the differentially expressed genes in response to drought stress and validated by qRT-PCR.745 ESTs were assembled into a collection of 299 unigenes having 52 contigs and 247 singletons. All 745 ESTs were submitted to ENA-EMBL databases (Accession no. HG516611- HG517355). After analysis, 10 differentially expressed genes were validated namely Abscisic stress ripening protein, Ascorbate peroxidase, Inosine-5′-monophosphate dehydrogenase, Putative beta-1, 3-glucanase, Glyoxalase, Rab7, Aspartic proteinase Oryzasin, DnaJ—like protein and Calmodulin—like protein by qRT-PCR. The identified ESTs reveal a major portion of the stress responsive transcriptome that may prove to be a vent to unravel molecular basis underlying tolerance of pearl millet (*Pennisetum glaucum*) to drought stress. These genes could be utilized for transgenic breeding or transferred to crop plants through marker assisted selection for the development of better drought resistant cultivars having enhanced adaptability to survive harsh environmental conditions.

Keywords Drought stress · ESTs · Pearl millet · qRT-PCR · Stress responsive genes

Introduction

Water deficiency is an important abiotic factor which restricts the crop productivity in the semi-arid tropics. Along with this, the altering climatic conditions are also expected to contribute towards drought stresses with enhanced severity in the near future. Consequently, sustainable and equitable worldwide food security is mainly dependent on the development of crop plants with better adaptation to water-limited environments (Kholova et al. 2014).

The decrease in rainfall due to world-wide climatic shifts has been predicted to reduce crop yield in semi-arid areas. The reduction of crop yield currently affects approximately 3.6 billion ha (25 % of upland in the world) in semi-arid and arid areas (UNEP report 1991). In these areas, desertification and population growth will exacerbate food shortage. Pearl millet [*Penniseteum glaucum* (L.R.Br)] belongs to the family *Poaceae* and is the most widely grown type of millet, which has strong development of underground organs and tends to have efficient adaptive mechanisms to cope with drought (Bezançon et al. 2009). India is the largest producer of pearl millet in Asia, both in terms of area (about 9 million ha) and production (8.3 million tons) with an average productivity of 930 kg/ha during the past 3 years (ICAR-AICPMIP report

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2014). Drought is one of the main environmental constraints to agricultural productivity worldwide. Many efforts have been made to elucidate the mechanisms of drought tolerance in plants through molecular and genomics approaches, and a number of genes that respond to drought stress at the transcriptional level have been reported (Guo et al. 2009). Drought limits the agricultural production by preventing the crop plants from expressing their full genetic potential (Mitra 2001). Drought is actually a meteorological event which implies the absence of rainfall for a period of time, long enough to cause moisture depletion in soil and plant tissue (Fatima et al. 2014) but it is hard to exactly define drought because it has different meaning in different areas. In a very arid region there has to be really long period of no precipitation to be considered as drought. On the other hand in tropical areas a period of six days with no rain at all can be considered as a drought event.

Several techniques have been utilized for identifying differentially expressed genes (Liang and Pardee 1992; Vos et al. 1995; Hubank and Schatz 1994). Among the various methods for differential transcriptome analysis, Suppression Subtractive Hybridization (SSH) proves to be an efficient approach to isolate and identify cDNAs of differentially expressed genes in the absence of sequence information (Mishra et al. 2007; Almeida et al. 2013; Ding et al. 2014; Khan et al. 2014). In this technique differentially expressed genes can be normalized and enriched over 1000-fold in a single round of hybridization (Diatchenko et al. 1996). This would substantially increase the chances for identification of rare transcripts involved in drought stress. The concept of using cDNAs as a route to expedite gene discovery was first demonstrated in the early 1980s (Putney et al. 1983). Later gene discovery in most plants was done primarily by sample sequencing of expressed sequence tags (ESTs) (Lim et al. 1996; Covitz et al. 1998).

SSH has successfully been used to identify genes responsive to various biotic and abiotic stresses in various plant species such as *Saccharum* (Watt 2003); *Festuca* (Zhang et al. 2005); *Pennisetum* (Mishra et al. 2007); *Agrostis* (Xu et al. 2009); *Triticum* (Chauhan et al. 2011). Thus in the current study we focus on identifying, validating and establishing the putative functions of the major differentially expressed genes in *P. glaucum* during drought stress response using SSH, real-time quantitative qRT-PCR and homology searches.

Materials and methods

Seed material and stress treatment

Seeds of *Penniseteum glaucum* cultivar PPMI741 (a drought tolerant parental line) were procured from the Pearl Millet Breeding Unit, Indian Agricultural Research Institute, New Delhi, India. Seeds, washed thoroughly with distilled water and germinated in duplicates in autoclaved pots (15 cm diameter and 8 in. depth) containing autoclaved Soilrite™ at 33 °C with 16 h light/8 h dark photoperiod at the National Phytotron Facility, IARI, New Delhi.

Drought stress was induced by the treatment of 30 % Polyethylene glycol. One of the two sets of 22 days old seedlings were exposed to 30 % PEG 6000 (Polyethylene glycol) and 1 mM MES for different time periods 30 min (T1), 2 h (T2), 4 h (T3), 8 h (T4), 16 h (T5), 24 h (T6) and 48 h (T7) respectively to stimulate drought stress at −1.25 Mpa (osmotic potential). The other set of seedlings was maintained as control.

Relative water content (RWC)

Another set of drought stressed seedlings were used for determination of relative water content. Relative water content test of seedling subjected to drought stress was measured as per the procedure of Barrs and Weatherley (1962). The seedling samples were weighed to obtain fresh weight (FW) and were immediately hydrated by soaking in double distilled water in a closed Petri dish for 4 h under normal room light and temperature for turgidity. Thereafter the samples were taken out and the surface was wiped using tissue paper and immediately weighed to obtain turgid weight (TW). Samples were then packed in butter paper and dried at 80 °C for 24 h in and dry weight was measured (DW). Relative water content of the samples was calculated as per the following formula:

$$\mathrm{RWC}(\%) = [(\mathrm{FW}-\mathrm{DW})/(\mathrm{TW}-\mathrm{DW})] \times 100$$

The seedlings were then cleansed 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 transcript.

Isolation of total RNA

For each sample including control and corresponding stress exposed Pearl millet cultivar PPMI741 seedlings 100 mg of leaf tissues were ground in pre-chilled mortar and pestle into a fine powder in liquid nitrogen, and total RNA was isolated using the method proposed by Chomczynski and Sacchi (1987). The isolated RNA was dissolved in RNase-free water and quantified by spectrophotometry using Nanodrop (Thermo Scientific, USA) and gel electrophoresis with formaldehyde agarose gel. It was stored at −80 °C till further processing.

Preparation of double-stranded cDNA driver from control plant

Double-stranded cDNA was prepared from 1.5 μg of poly (A) + control RNA (driver population) and stressed RNA (tester population) using the PCR-Select ™ cDNA Subtraction kit (Clonetech, USA). The cDNA from various durations of drought stress (30 min, 2, 4, 8, 12, 24 and 48 h) were pooled so as to include various stress responsive genes expressed at various durations. Control cDNA from corresponding control plants were also pooled and forward subtracted library was constructed using PCR select cDNA subtraction kit.

Construction of drought responsive subtractive cDNA library through SSH

The cDNAs enriched for differentially expressed genes, obtained after the secondary PCR following RsaI restriction digestion, adapter ligation and hybridization, were ligated into a pGEM®-T Easy vector (Promega, USA). Electrocompetent *E. coli* cells (NEB 10 beta, New England Biolabs, MA, USA) were transformed with the ligated product using an electroporator (Eppendorf Multiorator 30672, USA) using standard electroporation protocol. The transformed cells were spread on LA-Ampicillin (100 μg/ml) plates supplemented with of 100 μl of IPTG (0.1 M) and 10 μl X-Gal (100 mg/ml) and incubated at 37 °C for overnight. White colonies were randomly picked and colony PCR was carried out. False positives and colony mixture were also removed via the colony PCR analysis. The confirmed recombinant colonies were stored at −80 ° C, in 96-well format flat bottom cryo-storage plates containing LB medium supplemented with 20 % glycerol.

Sequencing and data analysis

Positive clones from the subtracted cDNA library having insert size of more than 500 bp were sequenced by single pass sequencing using the vector specific sequencing primer T7 by an automated DNA sequencer (sequencing were performed at Macrogen Inc., South Korea, through Sequencher Tech Pvt. Ltd, Ahmedabad, India). The obtained raw sequence reads were screened, edited manually to trim vector/adaptor sequences using VecScreen tool (www.ncbi.nlm.nih.gov/Vecscreen) and EditSeq of DNASTAR™ Navigator Suite. The edited ESTs which had more than 100 bp in length were assembled and clustered into contigs and singletons using SeqMan Pro program of the DNASTAR™ Navigator Suite.

Database searches

BLASTALL (NCBI) is used for batch execution of BLASTX (Altschul et al. 1990) to search for sequence similarity between processed DNA-sequence outputs and public non-redundant amino acid databases. The unigenes obtained were functionally categorized under biological process, cellular component and molecular function by Rice Genome Annotation Project (RGAP).

Validation of differentially expressed genes by qRT PCR

Validation of genes was performed by qRT-PCR using Light Cycler® 480 system (Roche, Germany) equipped with a 96 well plates system and KAPA SYBR® FAST Master Mix reagent (KAPA Biosystems, USA). Tissues from three biological samples of each treatment were pooled. The qRT-PCR experiments were performed with three technical replicates with 10 μl containing 4 μl of cDNA (diluted according to initial concentration so as to contain 150 ng cDNA), 1.0 μl of forward and reverse primers, and 5 μl of KAPA SYBR® FAST qPCR Master Mix according to the manufacturer's instructions.

The following thermal cycling profile was used for all qRT-PCR (Table 1). All quantifications were normalized to the *Pg* -Actin gene (used as housekeeping gene and amplified in the same conditions) as per $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen 2001).

Result and discussion

Relative water content

The relative water content (RWC) of drought treated seedlings decreased with the increased duration of drought stress. RWC was calculated as 79.63 % in 30 min, 75.89 % in 2 h, 74.53 % in 4 h. However it increased to 85.86 % in 8 h, which decreased further to 65.74, 62.37 and 56.20 % respectively in 16, 24 and 48 h (Figs. 1 and 2).

Table 1 Programme for qRT-PCR validation of differentially expressed genes of *p. glaucum*

PCR stages	Sub stage	Temperature	Time	Cycles
Pre incubation		95 °C	3 min	1
Amplification	Denaturation	95 °C	10 s	30
	Annealing	60 °C	20 s	
	Extension	72 °C	10 s	
Mating curve		95 °C	5 s	1
		60 °C	1 min	
		97 °C	Continuous	
		40 °C	Hold	

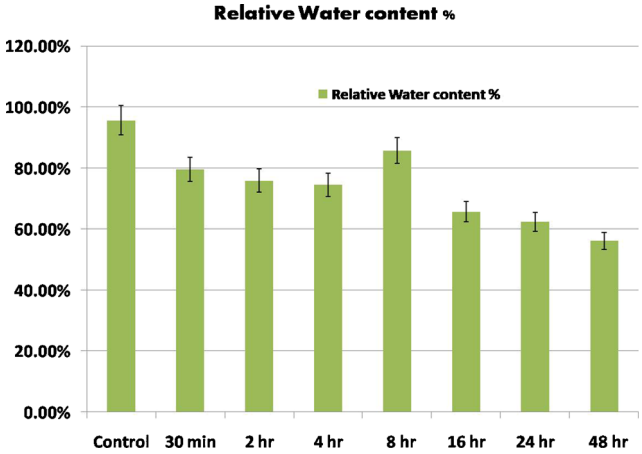

Fig. 1 Variation in Relative Water Content (*RWC*) of drought stressed plants by PEG 6000 from different time duration (A-Control, B-30 min, C-2 h, D- 4 h, E- 8 h, F- 16 h, G- 24 h and H-48 h)

Isolation of total RNA from leave tissues of *P. glaucum* seedling

The integrity of total RNA was examined by electrophoresis. RNA samples were run on denaturing 1.2 % formaldehyde agarose gel stained with ethidium bromide (10 mg/ml) (Fig. 3). The presence of two bright bands 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 2.06 to 2.13 and the concentrations measured by the Nano Drop ND-1000 spectrophotometer quantifications ranged from 765.0 to 2638.0 (ng/μl) (Table 2).

Construction of subtractive cDNA libraries to identify drought stress responsive genes

RNA of 2.0 μg concentration was used to synthesize cDNA in a long distance polymerase chain reaction (LD-PCR) and observed on 1.2 % agarose EtBr gel in 1X TAE buffer. The bands with variable size were visible with absolute background smear showing very high concentration of amplified product (Fig. 4). The amplified cDNA was digested using *Rsa*I restriction enzyme and amplified using primary and secondary PCR to obtain the secondary hybridized product which was then ligated into pGEMT easy vector and transformed into *E. coli*. A total of 2400 clones were obtained based on screening by

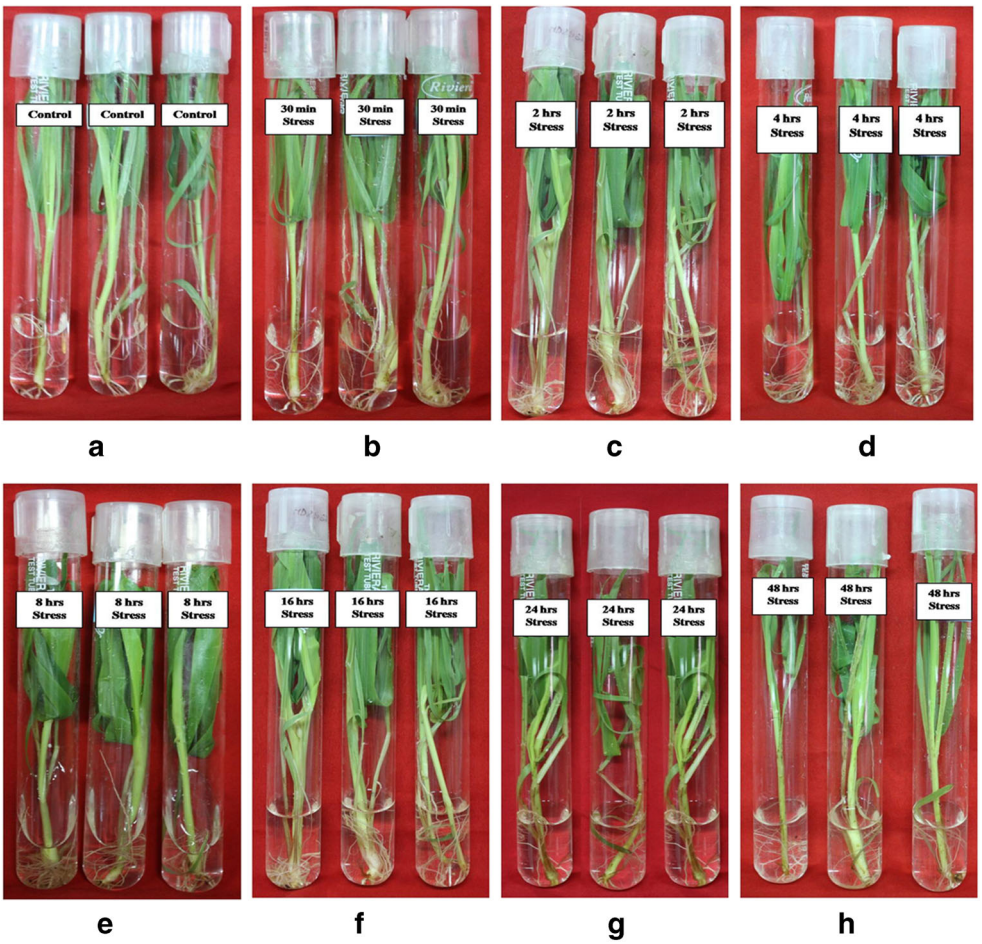

Fig. 2 Expression analysis of whole seedling of *P. glaucum* under drought stress condition. The relative water content (*RWC*) of drought stressed plants by PEG 6000 from different time duration (**a** Control, **b** 30 min, **c** 2 h, **d** 4 h, **e** 8 h, **f** 16 h, **g** 24 h and **h** 48 h)

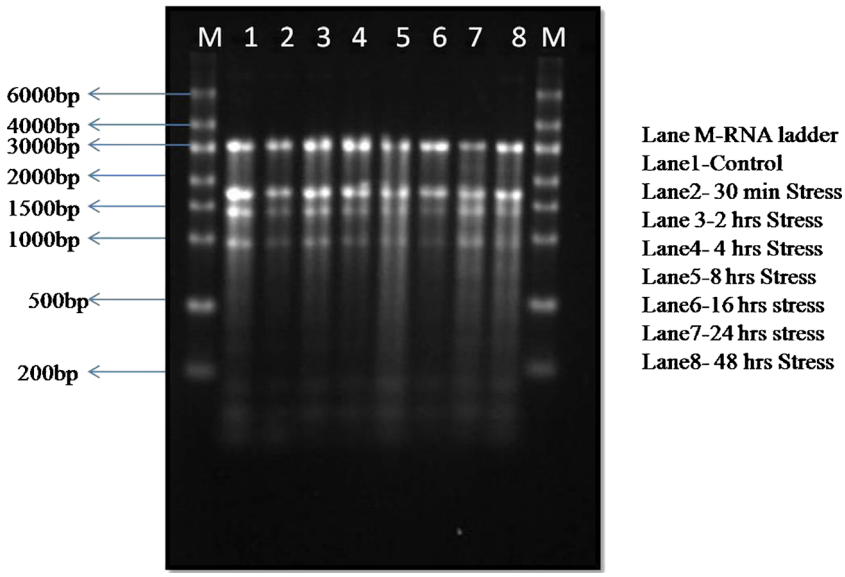

Fig. 3 Agarose gel electrophoresis showing RNA extraction by Qiazol method of *P. glaucum* (whole seedling) treated by drought stress

selection of recombinant clones by alpha complementation and visualized as blue-white colonies on the LA plate with (amp+Xgal+IPTG). Out of these 2400 colonies, 1344 clones (four 96 well-format plates) showing the insert sizes more than 250 bp were identified using colony PCR using the primer Sp6: 5′TATTTAGGTGACACTATA G and T7 primer: 5′TAATACGACTCACTATAGG primer. A total of 745 good ESTs were obtained from single pass sequencing and analysis of the obtained sequences. The EST library thus, constructed contained transcripts responsive to early heat stress (15 min) to transcripts responsive to late heat stress (48 h). Using the DNA STAR version 8 software, the 745 ESTs yielded 299 unigenes which were assembled into 52 contigs and 247 singletons. (Supplementary Table 1). All 745 ESTs were submitted to ENA-EMBL databases (Accession no. HG516611-HG517355).

Table 2 Quantification of total RNA isolated from control and experiment seedlings at different time periods of drought stress

S.N.	Treatment	OD ratio 260/280 nm	OD ratio 260/230 nm	Concentration (ng/μl)
1	Control	2.09	1.89	2638.0 ng/μl
2	30 min	2.10	2.10	1483.4 ng/μl
3	2 h	2.12	2.32	1456.9 ng/μl
4	4 h	2.07	2.21	2820.8 ng/μl
5	8 h	2.07	2.11	1501.8 ng/μl
6	16 h	2.10	2.21	1709.9 ng/μl
7	24 h	2.13	2.09	1655.3 ng/μl
8	48 h	2.06	1.97	765.9 ng/μl

Identification and *in silico* characterization of drought stress responsive ESTs in *P. glaucum*

A total of 745 ESTs were identified in which 16 % were classified under the "Response to Abiotic/Biotic stimulus (GO: 0009628)/ (GO: 0009607). BLASTX was performed against non-redundant protein databases NCBI (www.ncbi.nlm.nih.gov) and Rice Genome Annotation Project (RGAP) to determine putative functions of these unigenes. The products of many of the unigenes predicted by BLASTX were homologous to proteins involved in protection against stress damage. A total of 16 % drought stress modulated genes were categorized using the GO (Gene Ontology) IDs available through RGAP BLASTx Locus IDs. These locus IDs were used to assign GO terms fewer than three main categories *viz.* biological process, cellular component and molecular function (Fig. 5). On the basis of molecular and biological functions, the gene products were categorized into transcription factors, kinase activity, cellular homeostasis during environmental stresses, cell-cell signaling, signal transduction. The result suggests presence of complex network of stress signaling processes (Table 3).

Validation of differentially expressed genes by qRT PCR

The quantitative up-regulation of the selected genes for their expression in response to drought stress clearly showed that the subtractive cDNA libraries constructed in this study were substantially enriched for stress responsive genes. Differential expression analysis of genes encoding Inosine-5′-monophosphate dehydrogenase, Putative beta-1, 3-glucanase, DnaJ-like protein, Calmodulin-like protein and *Rab*7 were up-

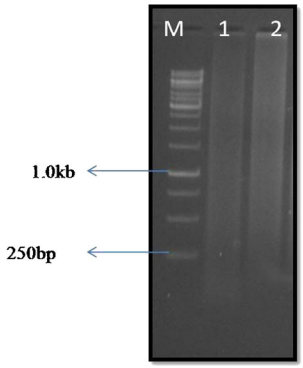

Fig. 4 Agarose gel electrophoresis showing LD- PCR cycle optimization. *Lane M*: 1.0 kb DNA ladder; *Lane 1*: control; *Lane 2*: Final optimization at 24th cycle (Plant sample: *P. glaucum*, drought stress by PEG 6000: 30 min to 48 h)

regulated from early time showing the maximum expression of 6.82, 4.69, 3.43, 3.38 and 4.62 fold respectively, after 2 h of stress treatment. On the other hand *ASR*, *APX* and Aspartic proteinase oryzasin gene exhibited over expression to the level of 10.7, 5.42 and 5.65 fold respectively after 24 h of continuous dehydration. The expression profiling of Glyoxalase and Ubiquitin-Conjugating enzyme E2-7 genes suggested higher early expression level of 2.34 fold after 30 min drought stress. The expression remained stable up to 16 h that again reached to the peak value of 3.36 fold afterwards displaying late expression (Fig. 6).

Discussion

Subtractive suppression hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other (Almeida et al. 2013; Ding et al. 2014; Khan et al. 2014). In the present study, drought stress responsive subtractive cDNA library was constructed from 22 days old pearl millet seedlings [*P. glaucum* cv. PPMI741] subjected to drought stress at room temperature

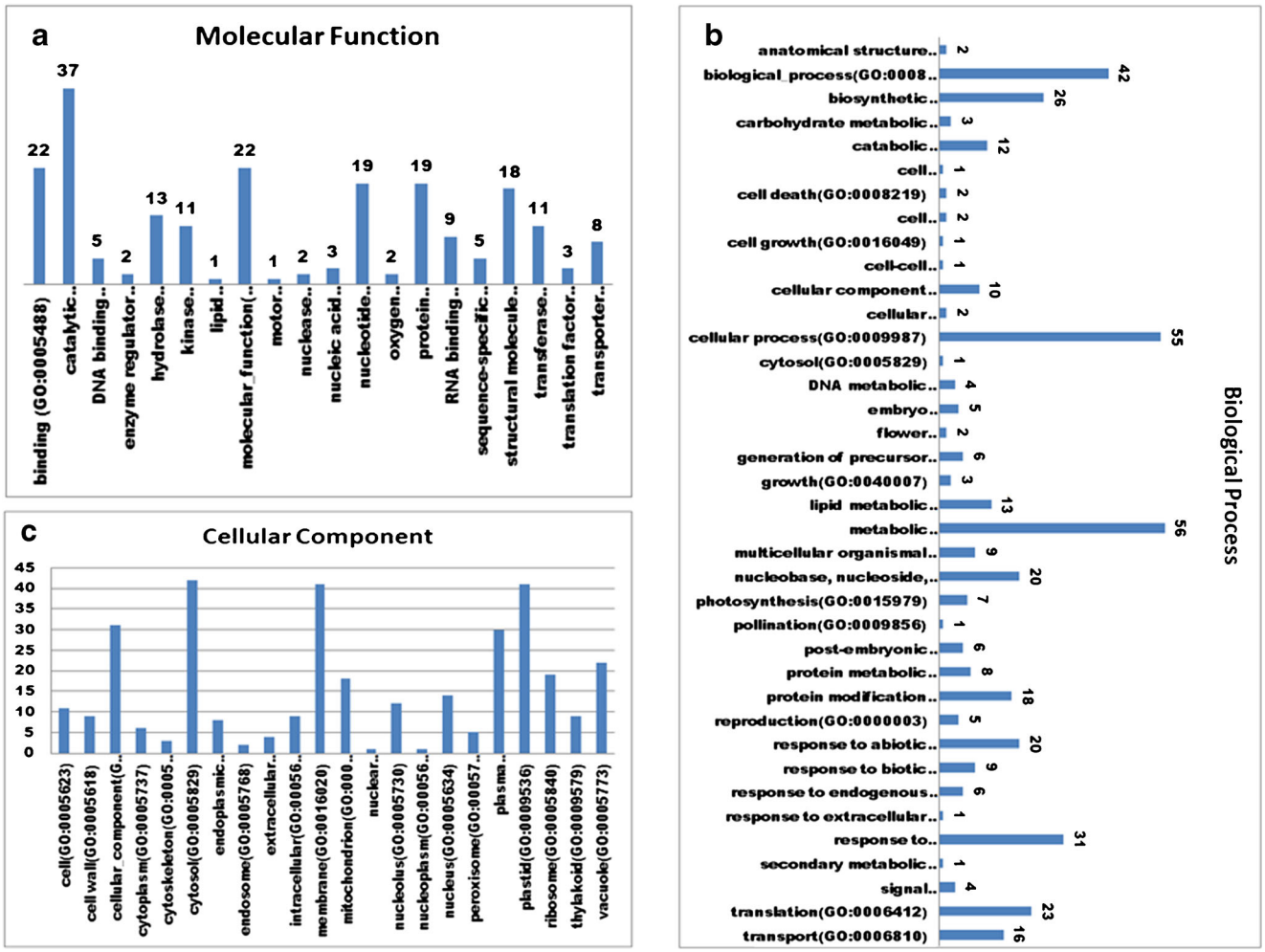

Fig. 5 Functional categorization of annotated unigenes of *P. glaucum* SSH library. **a** Molecular function **b** Biological process and **c** Cellular component

Table 3 Details of some important ESTs obtained in response to drought in the forward SSH library and reverse library their BLASTX analysis

EST Id	Locus ID	Gene product
Forward SSH library		
>Contig_6	LOC_Os01g57962.1	Photosystem I P700 chlorophyll a apoprotein A2,
>Contig_12	LOC_Os11g06720.1	Abscisic stress ripening protein
>Contig_23	LOC_Os08g43560.1	Ascorbate Peroxidase
>Contig_11	LOC_Os11g48090.2	Inosine-5′-monophosphate dehydrogenase
>Contig_53	LOC_Os09g33500.2	Putative beta-1, 3-glucanase
>Contig_44	LOC_Os02g13640.1	Glyoxalase
>Contig_15	LOC_Os03g17870.1	Metallothionein, putative, expressed
>Contig_26	LOC_Os10g38206.1	NADPH-dependent oxidoreductase, putative,
>Contig_33	LOC_Os11g48040.1	Mitochondrial carrier protein, putative, expressed
>Contig_47	LOC_Os12g05860.1	Cupin domain containing protein, expressed
>Contig_60	LOC_Os02g13840.1	Citrate synthase, putative, expressed
>Contig_50	LOC_Os05g47980.1	ATP synthase, putative, expressed
>Contig_35	LOC_Os04g16856.1	Chloroplast 30S ribosomal protein S7, putative,
>Contig_36	LOC_Os09g33500.2	Transketolase, putative, expressed
>Contig_51	LOC_Os01g55470.1	Transposon protein, putative
>Contig_45	LOC_Os05g23740.1	DnaK family protein, putative, expressed
>Contig_58	LOC_Os11g11390.1	Ribosomal protein, putative, expressed
>Contig_43	LOC_Os01g17170.1	Magnesium-protoporphyrin IX monomethylestercyclase, chloroplast precursor, putative, expressed
>Contig_45	LOC_Os08g39140.2	Hsp70 proteins
>Contig_37	LOC_Os05g49200.1	Aspartic proteinase oryzasin-1 precursor, putative, expressed
>Contig_28	LOC_Os06g46770.1	Ubiquitin family protein, putative, expressed
>Contig_35	LOC_Os04g16856.1	Chloroplast 30S ribosomal protein
>Contig_18	LOC_Os06g46149.2	Serine/arginine rich (SR)
Reverse SSH library		
>Contig_11	LOC_Os07g34589.3	Translation initiation factor SUI1, putative, expressed
>Contig_16	LOC_Os05g23610.1	Protein phosphatase inhibitor 2 containing protein, expressed
>Contig_20	LOC_Os07g38110.1	tic20, putative, expressed
>Contig_21	LOC_Os01g16240.1	OsCam1-3—Calmodulin, expressed
>Contig_19	LOC_Os05g33380.1	Fructose-bisphospatealdolaseisozyme, putative, expressed
>Contig_13	LOC_Os01g57966.1	Photosystem I assembly protein ycf3, putative, expressed
>Contig_14	LOC_Os03g17000.2	NAD dependent epimerase/dehydratase family domain containing protein, expressed

by 30 % PEG 6000 for different time period (30 min, 2, 4, 8, 16, 24 and 48 h). Plants with high capacity for water retention can better survive drought stress. During 0–48 h of 30 % PEG600 treatment, *P. glaucum* PPMI741 samples were observed to have high relative water content (RWC) which indicates its drought tolerance ability.

Based on the above observation, *P. glaucum* PPMI741 seedlings were selected to be used to create a subtractive cDNA library. Further, a total of 745 ESTs, found in the drought responsive subtracted cDNA library, were submitted to ENA-EMBL databases accession no. HG516611-HG517355. These EST sequences from the subtracted cDNA library are rich sources of drought stress-related genes that can help in understanding the molecular basis of drought tolerance in pearl millet by revealing a major part of the stress-responsive transcriptome (Mishra et al. 2007). More than 25 % of the ESTs generated were classified into response to abiotic stimulus category. Five percent of the ESTs in the subtracted cDNA library showed no homology to any protein in the database. These uncharacterized ESTs provide new candidate genes for investigation to elucidate their role in drought stress.

Validation of differential expression of ten selected genes in *P. glaucum* at various durations (30 min, 2, 4, 8, 16, 24 and 24 h) *viz*. Abscisic stress ripening protein (EST ID: Contig_12_ Pg _FSSH), Ascorbate peroxidase (EST ID: Contig_23_Pg_FSSH), Glyoxalase (EST ID: Contig_24_Pg_FSSH), Rab7(EST ID: Singlet_223_Pg_FSSH), Aspartic proteinase Oryzasin (EST

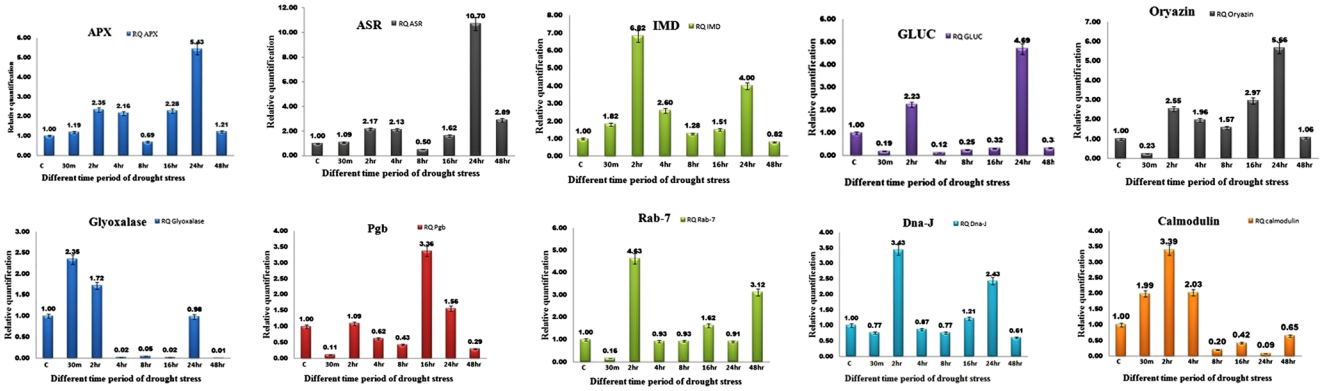

Fig. 6 RT-qPCR analysis of ten drought responsive genes identified in a SSH library of *P. glaucum* cultivar PPMI741. Relative levels of expression of *Abscisic stress ripening protein* (*Asr*), *Ascorbate peroxidase* (*Apx*), *Inosine-5′-monophosphate dehydrogenase*(*IMD*), *Putative beta-1, 3-glucanase* (*GLUC*), *Glyoxalase*, *Rab7*, *Aspartic proteinase Oryzasin*, *DnaJ-like protein* (*Dna-J*), *Calmodulin-like protein*, *Ubiquitin-Conjugating enzyme E2-7* (*Pgb*) in RNA from 22 days old seedlings were determined after exposing seedlings to 30 % PEG 6000 for 30 min to 48 h

ID: Contig_37_Pg_FSSH-P), Ubiquitin-Conjugating enzyme E2-7 (EST ID: Contig_27_ Pg_FSSH), DnaJ-like protein (EST ID:4F_E10_Pg_FSSH), Calmodulin-like protein (EST ID: Singlet_204_Pg_FSSH), Putative beta-1,3-glucanase (EST ID: Contig_53_Pg_FSSH) and Inosine-5′-monophosphate dehydrogenase (EST ID: Contig_11_Pg_FSSH) (Fig. 6) by qRT-PCR analysis corroborated their significant role in drought stress management. Genes like Inosine-5′-monophosphate dehydrogenase, Putative beta-1, 3-glucanase, DnaJ-like protein, Calmodulin-like protein and Rab7 were up regulated from early time having a maximum expression of 6.82, 4.69, 3.43, 3.38 and 4.62 fold respectively after 2 h of stress treatment.

Proteins containing Inosine-5′-monophosphate dehydrogenase (IMPDH) domain (PF00478) along with Cystathionine binding synthase (CBS) domain has been classified in this subgroup. The upregulation of these genes in response to drought stress indicates that CBS domain containing proteins may have an important role to play in plants tolerance to drought, salt and osmotic stress conditions by trying to maintain a balance in the generation and removal of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (Finkel and Holbrook 2000; Allen and Tresini 2000). In present research, Inosine-5′-monophosphate dehydrogenase (ESTS ID: Contig_11-FSSH-Pg) showed higher expression at 2 and 24 h of drought stress (30 % PEG6000) in whole seedling of *P. glaucum* which in coherence with the previous findings.

Plant beta-1,3-glucanase (ESTS ID: Contig_53-FSSH-Pg) one of the typical PR proteins, can catalyze the hydrolysis of β-1,3-glucans, which are a major component of the cell wall of most fungi while little has been found in higher plants so far (Stone and Clarke 1992; Leubner-Metzger and Meins 1999).

Moreover, DNAJ-like protein are mostly used in model plants and crops and do not always maintain stable expression levels among different tissues, experimental conditions and species (Zhong et al. 2011 and Zhu et al. 2013). Systematic validations of reference genes have mainly focused on models and important crop species such as *Arabidopsis* (Czechowski et al. 2005), rice (Jain et al. 2006), wheat (Paolacci et al. 2009), barley (Janska et al. 2013). DNAJ were the most stable genes in PEG treatment. It is known to be up regulated in heat stress but down regulate in salt stress at different time duration in *Ammopipathus mangolicus* (Yan et al. 2014). In the present study, DNAJ-like protein was up regulated gradually at 4, 8, 16 and 24 h but it was observed to be highly expressed at 2 h in drought stress (30 % PEG treatment).

Expression profiling of Calmodulin-like protein gene (ESTS ID: Singlet_204-FSSH-Pg) suggests the up-regulation up to 3.38 fold and down-regulation up to level of 0.087 fold at 2 and 24 h respectively of treatment times. Previous studies have shown that Calmodulin-like protein plays positive role in plant stress tolerance (Liu et al. 1998) and shown to have enhanced salt and drought tolerance in transgenic rice plants (Mallikarjuna et al. 2011). However, its down regulation at 2 h shows that calmodulin-like protein gene might be involved in late drought stress response in *P. glaucum*.

The role of *Rab7* in abiotic stress tolerance, such as salinity and drought has been validated and earlier studies have shown over expression of *AtRab7*, *PgRab7* and *PjRab7* in *A. thaliana* and tobacco under conditions of NaCl stress (Mazel and Levine 2002). The *Rab*7 proteins are important component of the vesicle trafficking system in all eukaryotes (Zerial and McBride 2001). The role of At*Rab*7 protein in eukaryotes seems to be associated with the late endocytosis, where it functions in the fusion of late endosomes to lysosomes or vacuoles.

On the other hand *ASR*, *Apx* and Aspartic proteinase Oryzasin genes were up-regulated at later hours showing 10.7, 5.42 and 5.65 fold up regulation respectively at 24 h. Glyoxalase and Ubiquitin-Conjugating enzyme E2-7 early and late expressed 2.34 and 3.36 fold respectively 30 min, 16 h compared to the control *Pg*- actin of drought stress. Similar results were obtained in previous studies.

The *Asr* gene family (Abscisic acid, Stress and Ripening), classified as a new group of Late Embryogenesis Abundant (LEA) (Caramelo and Iusem 2009; Battaglia et al. 2008), has been reported to be induced under water stress (Chang et al. 1998) and involved in adaptation to dry climates (Frankel et al. 2003). It is also reported to be involved in abscisic acid signaling and has been used to develop transgenic *Arabidopsis* with enhance drought and salt tolerance gene was up-regulated during drought and salt stress in transgenic Arabidopsis (Yang et al. 2005). Our investigation also showed up-regulation of *Asr* gene after 24 h drought stress.

Some genes were found to be up-regulated at later hours of stress induction which were down regulated at initial or extreme late phases of investigation. For example, ascorbate peroxidase (ESTS ID: Contig_23-FSSH-Pg) gene was found to be up regulated in *P. glaucum* up to 5.42 fold at 24 h after drought treatment but after 4 h and 48 h decreased indicating that the gene has transient expression. In stressful environmental conditions, there is an enhanced production of reactive oxygen species (ROS) in plants that causes significant damage to cells. Antioxidant defenses which can detoxify ROS are present in plants. A major hydrogen peroxide detoxifying enzyme is ascorbate peroxidase that catalyses conversion of H_2O_2 into water, using ascorbate as a specific electron donor (Caverzan et al. 2012). Modulated expression of ascorbate peroxidase in our library indicated the *Pennisetum* response to drought stress.

Aspartic Proteinase Oryzasin gene (ESTS ID: Contig_37-FSSH-Pg) classified in aspartic proteinases and metalloproteinase's are present at later stages expressed in salt, heat drought stress in rice, barley and *Coffea arabica* (Dominguez and Cejudo 1996). The said gene is expressed under drought stress in our investigation with up regulated expression at 2 to 24 h of drought stress (30 % PEG6000) and down regulated at 30 min and 48 h indicating that the gene is not responding during early stress conditions and late stress conditions.

Another unigene with its expression profile validated by qRT-PCR is Glyoxalasegene (ESTS ID: Contig_24-FSSH-Pg) that showed higher expression level 30 min and 2 h. After 2 h this gene down regulated in drought stress. Analysis of expression patterns using Gene investigator (Zimmermann et al. 2004) showed that GLX2-1 transcript levels are elevated during several abiotic stresses including anoxia, hypoxia, drought, light, osmotic and salt. In earlier studies exposure of the plants to salt stress had led to increase in GLX2-1 transcript levels of 15 fold increases in 24 h (Devanathan et al. 2014) which confirms that GLX2-1 is induced during abiotic stress and suggests that it may have a role in stress tolerance.

Conclusion

Differentially expressed genes in important crop plants provide a sturdy foundation for the development of drought resistant varieties of economically important crops that can impart sustainable and equitable global food security. The current study focuses on identifying the major differentially expressed genes in *P. glaucum* during drought stress response using SSH and validating them by qRT-PCR analysis. The putative functions of these *P. glaucum* ESTs were established based on the homology searches and the presumed biological implications of the products of these differentially expressed genes in relation to the complex networks of stress-adaptive processes in *Pennisetum* are also discerned. The study will be a valuable resource in the investigations of drought tolerance, as well as other characteristics, of *P. glaucum*.

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