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Genome-wide Identification and Characterization of *Hsp70* gene family in Pearl millet (*Pennisetumglaucum*)

Kummari Divya[1,2], P. B. Kavi Kishor[2],Nagaraju Maraka[2], Pooja Bhatnagar-Mathur[1], Prashanth Singam[2], Vincent Vadez[1] and Palakolanu Sudhakar Reddy[1]*

[1]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad 502 324, India

[2]Department of Genetics, Osmania University, Hyderabad 500 007, India

*For Correspondence - p.sudhakarreddy@cgiar.org; palakolanusreddy@gmail.com

Abstract

Heat shock proteins (Hsps) are a class of molecular chaperons which are crucial for protein folding, assembly, and translocation in many normal cellular processes. They stabilize proteins and membranes, and can assist in protein refolding under stress conditions in plants. Pearl millet (*Pennisetum glaucum*) is highly abiotic stress tolerant, but its Hsps have not been characterized. In the present study, *PgHsp70* genes were retrieved and gene information analyzed in order to characterize their structure, localization and functions. Genome-wide screening using the tools of bioinformatics identified 18 *PgHsp70* genes in the pearl millet genome which have been categorized into four subfamilies depending on their cellular localization such as endoplasmic reticulum, mitochondria, chloroplast and cytoplasm. Number of introns ranged from 0-11 in *PgHsp70* family genes and the genes are located across 1 to 7 chromosomes. Phylogenetic analysis of *Hsp70s* revealed that they are closely related to *Sorghum Hsp70s*. Promoter analysis showed the presence of *cis*-acting elements such as GCN4, HSE, LTR, MBS, ABRE, MYB, and TC Aassociated with abiotic stress conditions indicating the involvement of these genes in the abiotic stress. Under vapour pressure deficit (VPD) conditions, leaf and root tissues of VPD-sensitive ICMR 1152 line, showed mild expression and in the presence of high VPD, VPD-insensitive ICMR1122 *PgHsp70* genes showed high expression in leaf and root tissues in comparison with VPD-sensitive line. Gene *PgcHsp70-1* displayed high transcript level under high VPD conditions. These results expand our horizon of understanding of the structure and function of *Hsp70s*, especially under abiotic stress conditions which can further be validated and employed in breeding programs and genetic engineering.

Key words: Abiotic stress, Hsps, heat shock proteins, pearl millet, Hsp70 family,*Vapour pressure deficit*

Introduction

Pearl millet (*Pennisetum glaucum*) is the sixth most important cereal crop plant grown in different areas of the world (1). A member of Poaceae family, pearl millet is usually grown well in the arid and semi-arid regions. It is used as food, forage, fuel and construction material (2). The crop accounts for 95% production from the developing countries, India being its largest producer covering an area of 9.8 million hectares (1,3). It is high in carbohydrates, protein and mineral, and hence suitable for animal and human consumption (4,5,6). Plants have developed many stress tolerance mechanisms to cope with adverse conditions. To protect themselves against high temperature stress conditions, plants produce several kinds of heat shock proteins

(Hsps) and heat shock factors (6,7,8,9,10). *Hsp70* class is an important one, among the eukaryotic cells. Hsp70 class proteins have a chaperonic function and prevent the accumulation of unfolded proteins. They also guide proper folding as well as help in the translocation of proteins in an ATP-dependent manner (11, 6, 12). Based on cellular localizations (mitochondria, chloroplasts, endoplasmic reticulum and cytoplasm), 4 subgroups of *Hsp70* have been noticed(6).The number of *Hsp70* genes identified in crop plants vary, for example 18 have been detected in *Arabidopsis* (13), 32 in rice (14), 20 in *Populus* (15), 61 in *Glycine max* (15), 27 in pepper (16), 27 in *Setaria* (17), 29 in *Brachypodium* (18) and 48 in *Sorghum bicolor* (unpublished data).

Pearl millet genomic sequence information (19) aided the present genome-wide screening, identification and characterization of *PgHsp70* gene family. The information about the Hsp gene family number, their cellular and chromosomal localization and also the characterization of the promoter sequences along with tissue specific expressions of the genes under varied abiotic stress conditions are vital for subsequent use of these genes to generate abiotic stress tolerant lines. Multiple sequence alignment of these genes helps in phylogenetic tree construction and their evolutionary tendencies. Present study identifies the number of *Hsp70* class of genes in the whole pearl millet genomes, their cellular and chromosomal localization, promoter sequence analysis and the gene expression data extraction in different tissues under different VPD conditions in the pearl millet crop. This may help ultimately in crop breeding programs aimed at developing stress tolerant lines.

Materials and Methods

Identification and retrieval of *PgHsp70* genes in *Pennisetum glaucum:* In the present study, *Oryza* (14), *Arabidopsis* (13) and *Sorghum* (Nagaraju et al unpublished data) *Hsp70* gene sequences were retrieved from NCBI databaseand searched their homologs in pearl millet genome using TBLASTN. Genscan (http://genes.mit.edu/GENSCAN.html) program was employed in order to identify and retrieve the coding sequences as well as protein sequences.

Nucleotide sequence analysis and characterization: Chromosomal locations of *PgHsp70s* were determined with the information obtained from Gramene data base and NCBI. Gene intron-exon structures were studied using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) (20). Chromosomal localizations were also found out. PAL2NAL software was employed to identify the number of synonymous and non-synonymous sites, their substitutions rates and dN/dS were calculated for the *PgHsp70* orthologs and paralogs (21).

Protein analysis: For *insilico* characterization of proteins, different bioinformatics tools were employed. Total number of amino acids, instability index, protein molecular weight (MW) and isoelectric point (pI) were found out by blasting protein sequences using Expasy ProtoParam. For finding out cellular localization, Wolf PSORT II was used (16). Conserved motifs were identified using MEME suit (22). Multiple alignment of the protein sequences were performed using CLUSTALW. Amino acid sequences of *PgHsp70s* along with their related plant species were taken to construct phylogenetic tree using MEGA 6.0 (23).

Promoter analysis of *PgHsp70* genes: The upstream regions of *PgHsp70* genes were extracted from pearl millet genome and regulatory elements of these genes were retrieved. Putative *cis*-acting regulatory elements of the promoter sequences in both forward and reverse strands were analyzed using Plant CARE database (24). *Hsp70* genes expression pattern in VPD tolerant and susceptible Pearl millet genotypes: To explore the expression of *PgHsp70* family genes under low VPD, high VPD and tissue specific expression in leaf and root tissues was extracted from the our earlier transcriptome data.

Results

***PgHsp70* putative protein identification, classification and cellular localization in**

***Pennisetumglaucum*:** Eighteen putative *PgHsp70* genes were identified in a search by Blast with the homologs of *A. thaliana*, *O.sativa* and *S. bicolor* against pearl millet genome in Gramene database. Based on the presence of these genes in different cellular compartments, PgHsp70s were further sub-divided into 4 groups. In group I *PgcHsp70,* 12 proteins in cytoplasm/nucleus, in group II PgBipHsp70, 3 proteins in endoplasmic reticulum, in group III PgmtHsp70, 2 in mitochondria, and in group IV *PgcpHsp70,* 1 in the chloroplast were localized (Table 1).

Chromosomal localization and gene structure analysis of PgHsp70 genes: *PgHsp70* genes were noticed across all the 7 chromosomes. On chromosomes 1, 2, 3, 4, 5, 6, and 7 (Table 1), 4, 4, 1, 1, 3, 2 and 3 genes were noticed respectively. Structure of *PgHsp70* genes revealed that *PgcHsp70-1* and *PgbipHsp70-15* contain no introns but*PgbipHsp70-14* contains 11. With few exceptions, *PgbipHsp70* contains maximum number of introns.

Characterization and motif distribution of PgHsp70 proteins: In PgHsp70proteins, amino acids ranged from 543 (*PgcHsp70-5*) to 848 (*PgcHsp70-2*). Similarly, pI values ranged from 4.8 (*PgcHsp70-3*) to 8.28 (*PgcHsp70-5*), molecular weights from 59773.3Da (*PgcHsp70-5*) to 93777.37Da (*PgcHsp70-2*) and instability index from 25.64 (*Pgbip Hsp70-15*) to 44.19 (*PgcHsp70-11*) (Table 1). *PgHsp70* proteins werefound localized in the chloroplast, cytoplasm, mitochondria and endoplasmic reticulum with the highest localizationin the cytoplasm(Table 1).Motif 3 at N-terminus and motif 9 and 10 at C-terminus were foundhighly conserved across the whole *PgHsp70* protein family (Fig. 4).The number of amino acids of DBDs in *PgHsp70* proteins ranged from 1 *(PgcHsp70-3)* to 689 *(PgcHsp70-11).*

Phylogenetic analysis and estimation of non-synonymous and synonymous substitution rates in PgHsp70 genes: In the present investigation,out of a total of 7 paralogous events, only one regional duplication (*PgHsp70-3*/ *PgHsp70-16*) was noticed on chromosome 1 and the remaining as segmental duplication events (Fig.2). All the paralogs exhibited non-synonymous substitution (d_N) to synonymous substitution (d_S) ratios above 1, indicating positive/ Darwinian selection pressure (Table 2 and Fig. 2). Out of 12 orthologous events, 9 were observed with S*orghum*, 2 with *Oryza* and 1 with *Arabidopsis* indicating the evolutionary relationship between *Sorghum* and *Pennisetum*. Of the 12 orthologous events,only one (*PgcHsp70-4*(Pgl_GLEAN_ 10006422)/ Sb01g010460) showedd_N/d_S ratio less than 1, suggesting purifying selection whilethe remaining follows the positive/Darwinian selection (Table 3and Fig. 3).

Promoter analysis: Promoter analysis of PgHsp70sshowedvaried*cis*-elements which are categorized into biotic and abiotic stress-responsive elements (DRE, DPBF, MYB, MYC, GT1C, HSE, LTRE, WBOX), light-responsive elements (I Box), hormone-responsive elements (ABA, ERE, ABRE, GARE), tissue-specific elements (CCGTCC-box), and otherelements (Skn, KST1, DOF).

Expression analysis of Hsp70 genes in VPD tolerant and susceptible pearl millet genotypes: To explore the expression of *PgHsp70* family genes under low VPD, high VPD and tissue specific expression in leaf and root tissues was extracted from our earlier transcriptome data. PgcHsp70-4 exhibited the least expression under all conditions in both the leaf and root tissues. Whereas, *PgcHsp70-1* showed maximum expression in all the conditions in comparison to other genes. Rest of the genes *PgcHsp70-2*,*PgcHsp70-3*, *PgcHsp70-5* and*PgcHsp70-16* exhibited mild expressions in both leaf and root tissues with the highest expression in high VPD, followed by low VPD and control conditions.In high VPD stress conditions,VPD insensitive ICMR-1122 cultivar showed higher expression in leaf and root tissues in comparison withVPD sensitive ICMR-1152 cultivar. In low VPD and normal conditions also, VPD insensitiveICMR-1122 showed higher transcript levels in leaf and root tissue when compared to VPD sensitiveICMR-1152 (Fig. 5).

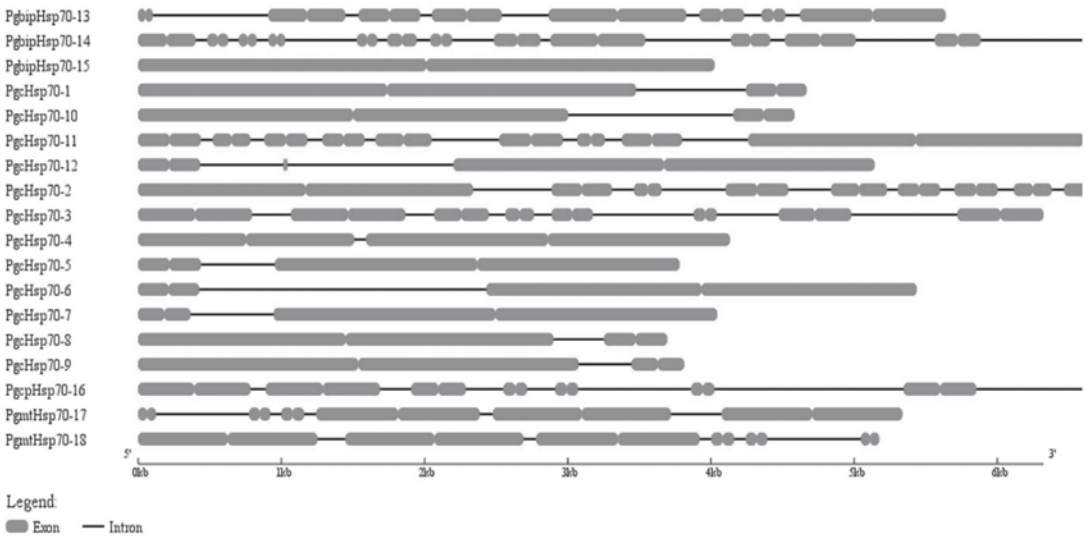

Fig. 1. Intron-exon distribution pattern across *PgHsp70* gene family

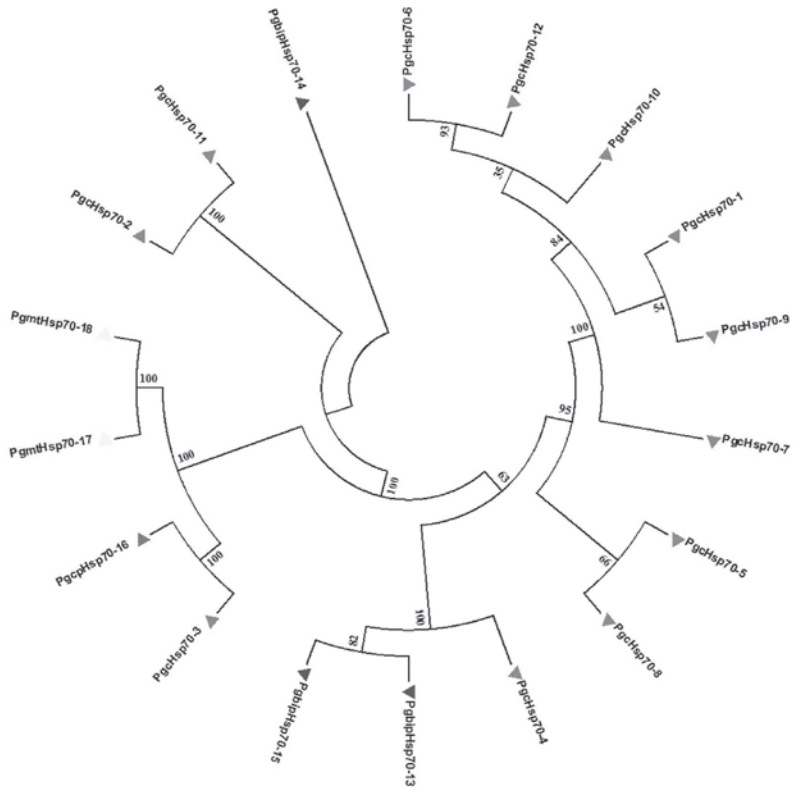

Fig. 2: Phylogenetic tree showing paralogous relation within *PgHsp70* gene family

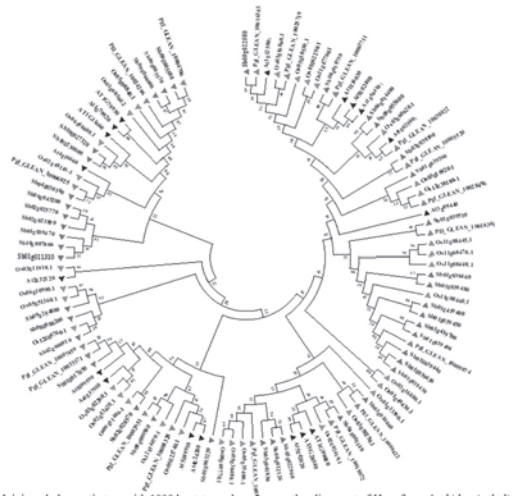

Fig. 3: Neighbor-Joining phylogenetic tree with 1000 bootstraps, based upon the alignment of Hsps from *Arabidopsis thaliana*, *Sorghum bicolor*, *Oryza sativa* and *Pennisetum glaucum.* Numbers on branches represent bootstrap values. At- *Arabidopsis thaliana*, Zm- *Zea mays*, Sb- *Sorghum bicolor*, Os- *Oryza sativa* and Pg-*Pennisetum glaucum*

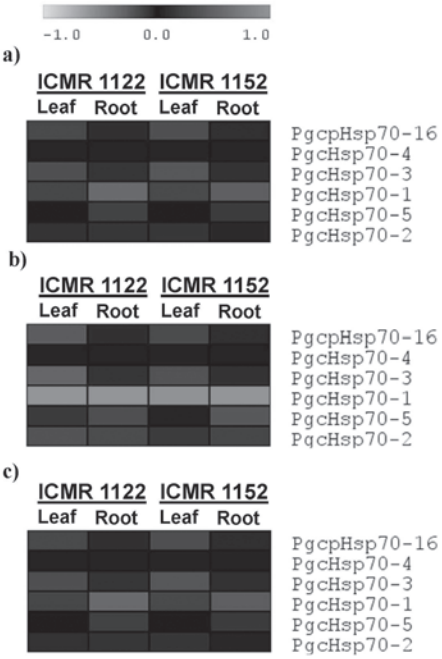

Fig. 5. Expression levels of *PgHsp70* genes in different conditions a) Low VPD, b) High VPD and c) Tissue specific expression

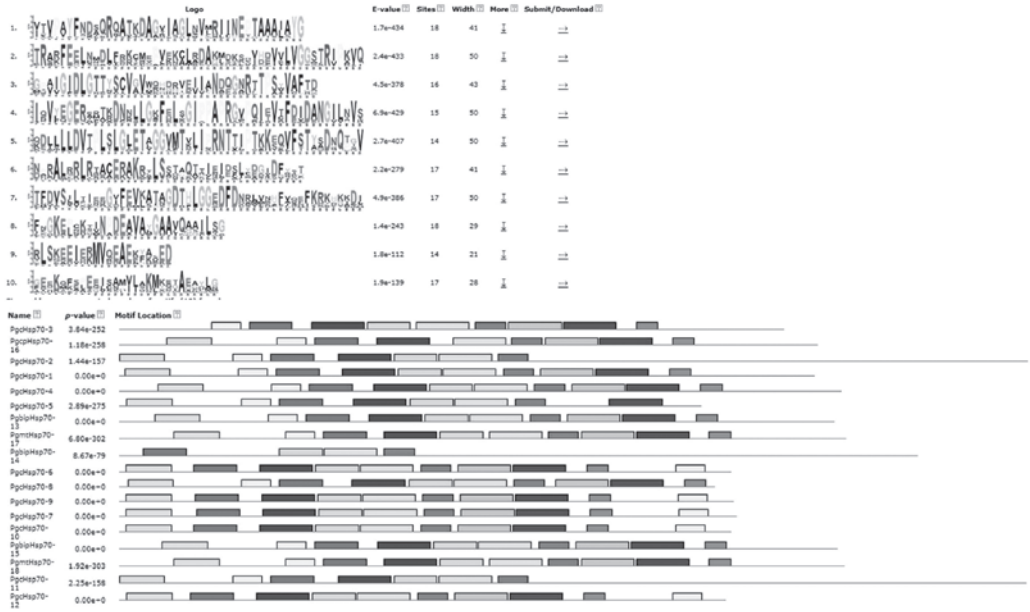

Fig. 4: Conserved protein motifs distribution in 18 PgHsp70s. Conserved motifs were analyzed by MEME Web serve using their respective protein sequences. Ten conserved motifs were identified, and different motifs are assigned different colors

S. No.	Gene name	Acc. No. Genome/ GenBank	Chromosome location	Nucleotide			Protein						Localization	DBD
				ORF (bp)	Introns	Exons	Protein (aa)	M.Wt(daltons)	pI	Instability index	GRAVY	Aliphatic Index	TM helices	WOLF pSORT	
1	PgcHsp70-1	Pgl_GLEAN_10025719	1	1950	0	1	649	71069.5	5.13	33.14	-0.417	81.63	0	Cytoplasm	8-617
2	PgcHsp70-2	Pgl_GLEAN_10004706	1	2547	5	6	848	93777.37	5.17	40.4	-0.467	78.23	0	Cytoplasm	3-688
3	PgcHsp70-3	Pgl_GLEAN_10002651	1	1863	6	7	620	66352.91	4.8	26.59	-0.327	88.37	0	Cytoplasm	1-580
4	PgcHsp70-4	Pgl_GLEAN_10006422	2	2025	1	2	674	73706.53	5.33	30.59	-0.401	85.52	1	Cytoplasm	39-653
5	PgcHsp70-5	Pgl_GLEAN_10009874	2	1632	1	2	543	59773.3	8.28	35.35	-0.298	84.01	0	Cytoplasm	10-540
6	PgcHsp70-6	Pgl_GLEAN_10003711	4	1716	1	2	571	62449.43	4.97	33.82	-0.439	82.54	0	Cytoplasm	9-71 / 71-540
7	PgcHsp70-7	Pgl_GLEAN_10028496	5	1731	1	2	576	62997.08	5.16	33.18	-0.461	82.67	0	Cytoplasm	9-63 / 62-543
8	PgcHsp70-8	Pgl_GLEAN_10016341	5	1671	1	2	556	60963.56	5.39	36.24	-0.194	88.09	0	Cytoplasm	11-554
9	PgcHsp70-9	Pgl_GLEAN_10016343	5	1722	1	2	573	62611.62	5	33.66	-0.447	82.08	0	Cytoplasm	9-62 / 61-542
10	PgcHsp70-10	Pgl_GLEAN_10005520	6	1716	1	2	571	62353.42	4.97	32.47	-0.415	82.35	0	Cytoplasm	9-71 / 71-540
11	PgcHsp70-11	Pgl_GLEAN_10024296	7	2541	8	9	846	93616.12	5.17	44.19	-0.457	78.98	0	Cytoplasm	3-689
12	PgcHsp70-12	Pgl_GLEAN_10030022	7	1701	2	3	566	62112.22	4.98	33.78	-0.444	83.25	0	Cytoplasm	10-75 / 76-537
13	PgbipHsp70-13	Pgl_GLEAN_10018072	2	2004	7	8	667	73439.11	5.07	26.52	-0.474	86.24	1	ER	36-643
14	PgbipHsp70-14	Pgl_GLEAN_10006025	3	2238	11	12	745	82823.38	5.28	41.29	-0.549	82.13	0	ER	1-590
15	PgbipHsp70-15	Pgl_GLEAN_10030426	6	2013	0	1	670	73388.85	5.17	25.64	-0.395	85.73	1	ER	43-651
16	PgcpHsp70-16	Pgl_GLEAN_10004328	1	1959	6	7	652	70026	5.12	29.61	-0.319	84.62	0	Chloroplast	47-275 / 298-614
17	PgmtHsp70-17	Pgl_GLEAN_10033371	2	2037	5	6	571	62449.43	4.97	33.82	-0.439	82.54	0	Mitochondria	54-647
18	PgmtHsp70-18	Pgl_GLEAN_10007459	7	2034	5	6	677	72743.45	5.83	38.03	-0.324	84.93	0	Mitochondria	54-647

Table 1. *In silico* Characterization of pearl millet *Hsp70* genes and proteins

Discussion

When plants are subjected to different biotic and abiotic stress conditions, they display stress tolerance mechanisms by expressing genes like *Hsp70s* that playan importantrole (25,26). Hsps70s have the chaperonic function and play vital roles in many cellularprocesses underboth stress and control conditions. Genome-wide analysis of Hsp70 family revealed 14 *Hsp70* genes in *Arabidopsis*(13), 24 in rice (14), 34 in poplar (27), 61 in soybean (15), 21 in pepper (16), 29 in *Brachypodium* (18), 27 in *Setaria* (17) and 16 in quinoa (28). This indicates that this protein number varies from species to species.In pearl millet, 18 *Hsp70* genes were identified but the number appears less compared to other members of Poaceae family. PgHsp70 proteins are classified into 4 sub-classes, all of them possess the conserved domains at the C and N-termini, similar to *Setaria*Hsp70 (17). The *Hsp70* proteins are located in different sub-compartments of cells (29), probably to protect different cellular proteins. Based upon the sub-cellular localizations, the *PgHsp70* proteinsare categorized to 4 sub-groups; group I with 12 proteins in cytoplasm/nucleus, group II with 4 proteins in mitochondria, group III with 3 proteinsin endoplasmic reticulum and group IV with 1proteinlocalized in chloroplast. Similarly, in rice, *OsHsp70* proteins are localized innucleo/ cytosol (11 proteins), endoplasmic reticulum/Bips (6), mitochondria (3), andchloroplast (2). In*Arabidopsis*, 5 proteins were found in nucleo/ cytoplasm, 2 in plastid, 3 each in mitochondria and endoplasmic reticulum (13, 14). Kose (29) demonstrated that the cytosolic Hsp proteins movedto nucleus underheat stress, and the nuclear *Hsp70s* prevented DNA fragmentation and hence lead to high temperature tolerance. Cytoplasmic Hsp70 genes possess 1 or no introns, whereasorganellar *Hsp70s* have multiple introns with few exceptions (25).

These observations are akin to the present study. In *Sorghum bicolor Hsp70* also,multiple introns were observed, similar to previous reports (13, 16), implying that the exon/intron pattern of *Hsp70* family is not conserved among diverse

plants. Multiple numbers of introns in *PgHsp70s* may play a role in evolutionary process or have a regulatory role for imparting tolerance under diverse abiotic stress conditions (25).

Intandem/regional duplications, two or more genes are noticed on the same chromosome, while in segmental duplication, gene duplications are observed on different chromosomes (30).The gene duplication events play an important role in *Hsp70* family gene expansion in *Pennisetum glaucum*. A total of 7 events were observedin pearl millet, but 5 duplication events in *Sloanum*) (28), 8 in *Quinoa* (28), and 24 in *Glycinemax* (15). Semon et al. (31) pointed out that gene duplications, and chromosomal segments play a crucial role in the evolution of genome structure. In the present investigation, out of the 7 events, 6 were segmental duplications indicating their role in gene family expansion. Similarly, Zhang et al. (15) noticed 19 segmental duplications out of the 24 in *Glycinemax*. *In silico*analysis of *PgHsp70* promoters using the software Plant Care revealed multiple *cis*-acting elements,indicating that these

Table 2. Non-synonymous to synonymous substitution ratios ofPgHsp70 genes

PgHsp70 gene 1	Chromosome number	PgHsp70 Gene 2	Chromosome number	Number of non-synonymous sites (N)	Number of synonymous sites (S)	Non-synonymous substitution rate (d_N)	Synonymous substitution rate (d_S)	d_N/d_S
PgcHsp70-1	1	PgcHsp70-9	5	1430.7	288.3	4.871	0.0492	99
PgcHsp70-2	1	PgcHsp70-11	7	1973.4	564.6	0.4032	0.3335	1.209
PgcHsp70-3	1	PgcpHsp70-16	1	1503.4	356.6	5.9324	0.0599	99
PgcHsp70-5	2	PgcHsp70-8	5	1264.8	364.2	1.2098	1.018	1.1884
PgcHsp70-6	4	PgcHsp70-12	7	1410.5	287.5	6.4921	0.0656	99
PgbipHsp70-13	2	PgbipHsp70-15	6	1530.3	470.7	3.046	2.8728	1.0603
PgmtHsp70-17	2	PgmtHsp70-18	7	1579	452	0.0623	0.0585	1.0637

Table 3. Non-synonymous to synonymous substitution ratios of PgHsporthologs of pearl millet, Sorghum, Oryza, Zea mays and Arabidopsis

Pg Hsp70 Gene	Ortholog Gene	No. non-synonymous sites (N)	No. synonymous sites (S)	Non-synonymous substitution rate (dN)	Synonymous substitution rate (dS)	dN/dS
Pgl_GLEAN_10016343	Sb09g022580	1430.2	288.8	4.9101	0.0496	99
Pgl_GLEAN_10025719	Os03g16860	1615.3	331.7	5.0131	0.0506	99
Pgl_GLEAN_10003711	Sb08g018750	1401.1	311.9	6.0958	0.0616	99
Pgl_GLEAN_10030022	At5g02500	1409.9	288.1	7.6815	0.0776	99
Pgl_GLEAN_10005520	Sb03g039360	1347.5	365.5	2.9632	1.3942	2.1254
Pgl_GLEAN_10028496	Os12g38180	521.8	123.2	7.8399	0.0792	99
Pgl_GLEAN_10016341	Sb01g039510	1290	378	2.8908	1.6094	1.7961
Pgl_GLEAN_10006422	Sb01g010460	1626	396	4.052	8.7376	0.4637
Pgl_GLEAN_10018072	Sb04g001140	1650.2	350.8	6.3938	0.0646	99
Pgl_GLEAN_10002651	Sb08g009580	1492.1	367.9	6.0458	0.0611	99
Pgl_GLEAN_10006025	Sb04g030160	1857.8	377.2	6.5645	0.0663	99
Pgl_GLEAN_10004706	Sb09g005580	1909.3	529.7	0.6553	0.4468	1.4668

promoters couldregulate transcription of the downstream genes under diverse stress conditions, perhaps in a developmental stage-specific and tissue specific manner.

Studies from the past have shown that *Hsp70* genes were expressed variedly in response to different abiotic stress conditions (32,6). This study has been further extended to investigate the expression of *PgHsp70* genes in high VPD, low VPD and normal conditions. The results revealed that *PgcHsp70-1* showed a significantly upregulated expression in response to high VPD stresses which are inconsistent from the previous studies of Devi et al. (32). These findings show the potential roles of *Hsp70s* in the regulation of abiotic stress. However, this has not been validated in the present study.

Conclusions

A genome-wide scanning of *Pennisetum glacum* genome using the tools of bioinformatics resulted in the identification of 18 *Hsp70* genes in pearl millet. These Hsps are categorized into four subfamilies: *PgcHsp70* (12 proteins), *PgcpHsp70* (3 proteins), *PgbipHsp70* (1 protein) and *PgmtHsp70* (2 proteins) based on their subcellular localizations. Phylogenetic relationship revealed that *PgHsp70s* are closely related to *Sorghum Hsp70s.* Motifs at both C- and N-terminiare evolutionarily conserved in all the members. *In silico* promoter analysis showed the presence of several *cis*-elements which indicate that they play a key role under abiotic stress conditions. In high VPD stress,VPD insensitive ICMR-1122 cultivar showed higher expression in leaf and root tissues in comparison with VPD sensitive ICMR-1152 cultivar. In low VPD and normal conditions also, VPD insensitive ICMR-1122 displayed higher expression levels in leaf and root tissues when compared to VPD sensitive ICMR-1152. *PgcHsp70-1* exhibited maximum expression in all the conditions in comparison with other genes with the highest response in high VPD conditions. Our studies provide a point of reference for the functional validation of *Hsp70* family genes in pearl millet crop in the coming times.

Author contributions

PSR and VV designed the experiments, KD, PSR, PBM and PS executed the study, PSR, KD, NM and PBK analyzed data. PSR, PBK, NM and KD wrote the manuscript and critically evaluated.

Conflict of interest

The authors declare that they have no conflict of interest.

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