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RESEARCH ARTICLE

Functional Exploration of Chaperonin (HSP60/10) Family Genes and their Abiotic Stress-induced Expression Patterns in *Sorghum bicolor*

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Abstract: ***Background*:** *Sorghum*, the C4 dry-land cereal, important for food, fodder, feed and fuel, is a model crop for abiotic stress tolerance with smaller genome size, genetic diversity, and bioenergy traits. The heat shock proteins/chaperonin 60s (HSP60/Cpn60s) assist the plastid proteins, and participate in the folding and aggregation of proteins. However, the functions of HSP60s in abiotic stress tolerance in *Sorghum* remain unclear.

***Methods*:** Genome-wide screening and *in silico* characterization of SbHSP60s were carried out along with tissue and stress-specific expression analysis.

***Results*:** A total of 36 *HSP60* genes were identified in *Sorghum bicolor*. They were subdivided into 2 groups, the *HSP60* and *HSP10* co-chaperonins encoded by 30 and 6 genes, respectively. The genes are distributed on all the chromosomes, chromosome 1 being the hot spot with 9 genes. All the HSP60s were found hydrophilic and highly unstable. The *HSP60* genes showed a large number of introns, the majority of them with more than 10. Among the 12 paralogs, only 1 was tandem and the remaining 11 segmental, indicating their role in the expansion of *SbHSP60s*. Majority of the *SbHSP60* genes expressed uniformly in leaf while a moderate expression was observed in the root tissues, with the highest expression displayed by *SbHSP60-1*. From expression analysis, *SbHSP60-3* for drought, *SbHSP60-9* for salt, *SbHSP60-9* and *24* for heat and *SbHSP60-3, 9* and *SbHSP10-2* have been found implicated for cold stress tolerance and appeared as the key regulatory genes.

***Conclusion*:** This work paves the way for the utilization of chaperonin family genes for achieving abiotic stress tolerance in plants.

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1. INTRODUCTION

Plants face unavoidable environmental challenges like biotic and abiotic stresses, which drastically limit both growth and final productivity [1, 2]. Abiotic stress is the most deterrent and causes 70% yield losses in crop plants [3]. To combat this, plants have developed various mechanisms like morphological (leaf orientation), anatomical (stomatal conductance and increased leaf pubescence), phenological (changes in the developmental stages), physiological (root hydraulic conductance and photosynthesis), metabolic (accumulation of the osmolytes), hormonal balance (ABA, ethylene, and salicylic acid) and secondary metabolites (isopropanoid, flavonoid, anthocyanin, and lignin) [4-7]. Besides these mechanisms, plants should recognize and respond to the stress at cellular and molecular levels, and repress the expression of normal proteins and induce the expression of diverse stress-associated proteins [8]. Abiotic stress generally leads to protein aggregation, which can subsequently cause metabolic dysfunction. To survive under stress conditions, it is imperative for the plants to maintain native conformation of proteins and, at the same time to reduce the accumulation of non-native proteins. To overcome such a problem, plants produce heat shock proteins called molecular chaperones, which are implicated in abiotic and biotic stress tolerance [9, 10]. They act as multi-functional proteins and maintain homeostasis by protein folding, trafficking and disaggregation under stress conditions [9, 11]. Based on their molecular weights, they are categorized into HSP100, HSP90, HSP70, HSP60, HSP40, HSP20, and HSP10 [12, 13].

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HSP60 chaperonins are essential for the viability of cells in all growth conditions because they are required for the efficient folding of numerous proteins that mediate vital cellular functions. They are ubiquitously present in archaea, eubacteria, and eukaryotes. They facilitate protein folding, refolding, aggregation and transportation to chloroplasts and mitochondria [14-16]. The oligomeric protein complexes form double-ring structures, participate in protein functional conformation, folding of denatured and newly synthesized proteins through hydrolysis of ATP [17-20]. So far, two types of chaperonins, type I in bacteria (called GroEL), plastids (HSP60) and mitochondria (Hsp60), and type II in archaea and eukaryotic cytosol (CCT/TriC) [21], with a distinct, conserved crystal structure were identified [18, 22]. In plants, the nuclear genome encodes mitochondrial and plastidial chaperonin homologues [21, 23]. *E. coli* GroEL and mitochondrial HSP60 contain 14 identical subunits, while the plastid HSP60 contains two distinct subunits, α and β [24, 25]. The number of subunits varies in land plants; 3α and 4β subunits in *Physcomitrella patens* [26], 3α and only 1β in *Brassica napus* [27], and 2α and 4β subunits in *Arabidopsis* [21]. In protein folding, while type I chaperonins are assisted by co-chaperonin GroES/HSP10, type II performs by an in-built component "cap" for substrate encapsulation [11, 28, 29]. Bacterial *HSP10* is encoded by a single gene *GroES*, in contrast to the plastid *HSP10* found in algae and plants encoded by several genes [30]. In plants, they are considered as a sub-family of chaperonins [21]. The HSP10, along with HSP60, forms back-to-back double-ring assemblies consisting of closely related and rotationally symmetrical subunits, which help in folding, assembly and sorting of the proteins [17]. The two subunits containing 20-kDa homologue of bacterial HSP10 have been identified in plastids, and the subunits are linked by a TDDVKD-linker in head to tail manner [31].

The HSP60 in plant chloroplasts and *E. coli* [32], protects the rubisco enzyme, a prime protein that mediates CO_2 fixation [12]. It has been observed that folding of plant RbcL subunits is mediated by the chaperonin HSP60, and also its cofactor HSP20 [12, 33]. Chloroplast chaperonin and the auxiliary factors when overexpressed in an *E. coli* strain, functional plant rubisco enzyme was noticed [33]. The results indicate that chaperonins cooperate and play pivotal roles in the functional expression of proteins encoded by transgenes. HSP60 plays an important role in RNA metabolism, RNA protection and processing, too [34]. Hsu *et al*. [35], noticed HSP60-mediated mitochondrial RNA splicing in *Arabidopsis*. Among the *HSP60* gene family members, *AtHSP60α1*, *AtHSP60β1* and *AtHSP60β2* were strongly induced in comparison with others under heat stress conditions in *Arabidopsis* [36]. Prasad and Stewart [37] pointed out that HSP60 plays a vital role in the developmental regulation, mitochondrial biogenesis, heat stress tolerance and in assembly of oligomeric protein structures. The mRNA and protein expression levels of PtHSP60 are altered according to salinity changes and thus appear to play a key role in the regulation of the pathway associated with salinity stress [38]. When HSP60 and HSP10 were overexpressed in *E. coli* and yeast, they exhibited tolerance against osmotic and salt stresses [12]. Thus, the chaperonins are associated with stress tolerance and play crucial roles during the process.

Haq *et al*. [39], noticed a total of 16 *HSP60* genes in pepper, and concluded their defence response against heat and other abiotic stresses. They act as lipo-chaperonins and participate in membrane stabilization, and refolding thus prevent irreversible thermal aggregation. HSP10 homologues were identified in *Oryza sativa* [40], *Hordeum vulgare* [41], *Triticum aestivum*, *Phaseolus vulgaris*, *Pisum sativum* [42], and *Pennisetum glaucum* [43], and these might help in ameliorating abiotic stresses.

Sorghum bicolor, the Great Millet or camel crop is a C_4 crop plant with an extensive root system, waxy bloom on its leaves and culm, can survive to some extent in drought-prone areas [44, 45]. It has the potential to adapt to global warming conditions by maintaining high levels of chlorophyll, known as the stay-green phenotype [46, 47]. Its stay-green character is thus the distinctive feature for its ability to withstand drought stress [48, 49], though the growth is slowed down during drought-like other crops [50]; but it has the potential to adapt to the stress conditions by maintaining high levels of chlorophyll, known as stay-green genotypes. However, the molecular mechanisms of drought and heat stress tolerance and the underlying genes need to be validated. By sharing a double-ring-like structure, chaperonins play a key role in protein functional conformation and transport [20]. Therefore, identification of HSP60s and their expression profiling is highly crucial. They are well studied in prokaryotic systems like *E. coli*, but not in higher plants like sorghum. Hence, the present study aims to find out the number of chaperonins present by screening the whole genome sequence along with their *in silico* characterization and also the expression profiles of chaperonins (*HSP60* and *10*) during abiotic stress tolerance in different tissues of *S. bicolor*.

2. MATERIALS AND METHODS

2.1. Plant Material

Sorghum bicolor BTx623 variety procured from ICRISAT, Hyderabad, India, was used for gene expression analysis. The seedlings of BTx623 were grown in pots under glasshouse conditions. The 40-day-old plants were subjected to abiotic stress treatments as described earlier by Nagaraju *et al*. [51]. Drought stress was imposed by treating the *S. bicolor* plants with 150 mM mannitol for 4 h. Similarly, seedlings were exposed to 150 mM NaCl for 4 h, temperature stress at 40 °C in a growth chamber for 4 h, and cold stress in a refrigerator at 4 °C for 4 h. The treated root, stem, and leaf tissues, along with corresponding controls, were collected separately, frozen immediately in liquid nitrogen and stored at -80 ºC until further use.

2.2. Identification of HSP60 Genes in *Sorghum*

For this study, the HSP60 sequences of *Oryza* and *Arabidopsis* were retrieved from HSPIR (http://pdslab.biochem.

iisc.ernet.in/hspir/) [52], and NCBI (https://www.ncbi.nlm.nih.gov/), and used as query sequences in Gramene (http://www.gramene.org/) [53] database by blasting against *S. bicolor* genome, with default parameters. The non-redundant putative protein sequences were subjected to the MOTIF search tool (http://www.genome.jp/tools/motif/) to identify their conserved domains and other Pfam domains [54] and the reliability was checked by employing the SMART tool (http://smart.emblheidelberg.de/). Proteins that failed to exhibit reliability were eliminated.

2.3. *In silico* Sequence Characterization of *SbHSP60s*

The chromosomal distributions of *HSP60* genes were determined based on the sorghum genome annotation files at Gramene (http://www.gramene.org/) [53]. By blasting the HSP60 amino acid sequences in the ExPASy-ProtParam tool (http://web.expas y.org/ProtParam/) [55], the molecular weight (MW), isoelectric point (pI), grand average of hydropathicity (GRAVY), instability index, and aliphatic indices were calculated. The Gene Structure Display Server (GSDS2.0) (http://gsds.cbi.pku.edu.cn/) [56] was used for determining gene structures (exon/intron and utrs), WOLF PSORT (https://www.genscript.com/wolf-psort.html) [57] for sub-cellular localizations, TMHMM Server V. 2.0 [58] for trans-membrane helices, NetPhosK3 software [59] for putative phosphorylation sites, MEME software (http://meme-suite.org/) [60] for conserved motifs by setting default parameters according to Nagaraju *et al*. [51]. The GeneMANIA platform (https://genemania.org/), STRING database (https://string-db.org/), and STITCH v5.0 (http://stitch.embl.de/) were used to identify the interaction between gene-gene, protein-protein and protein-chemical. Using PLACE [61] and PLANTCARE [62] tools, *cis*-elements were detected in the upstream regions of *SbHSP60s*. The MEGA 6.2 software [63] was used to construct a maximum likelihood (ML) phylogenetic tree with the parameters Poisson correction, complete deletion and bootstrap value of 1,000 replicates for statistical reliability. PAL2NAL (http://www.bork.embl.de/pal2nal/) [64] was used for calculating the substitution rates for non-synonymous to synonymous sites of paralogs and orthologous gene pairs. The putative miRNAs targeting the *SbHSP60s* were predicted by employing the psRNATarget server [65] with default parameters.

2.4. Digital Expression Analysis of *SbHSP60* Genes

In order to perform *in silico* expression profiling of the identified *SbHSP60s*, probe ids for respective genes were manually obtained from the sorghum functional genomics database (SorghumFDB) [66]. Further, these probe ids were utilized to perform expression profiling of *SbHSP60s* using curated whole transcriptome data embedded in the Genevestigator platform [67]. Expression analysis was performed in different anatomical parts and developmental stages under several abiotic stresses, including drought, salt, heat, and cold, with different samples embedded in the platform. Heat map that displays expression profiling was prepared using the clustering tool available on Genevestigator [68] utilizing 30 different transcriptome data sets embedded in the Genevestigator library. Clustering of expressed *SbHSP* genes was performed following the criteria used by Kumar *et al*. [69].

2.5. qRT-PCR Analysis of *SbHSP60s* Under Abiotic Stress Conditions

Due to the availability of limited resources, expression patterns of the identified HSP-60 genes were reduced for this study. The genes were selected based on the homology with other crops, their subcellular localizations and subclasses. From each subclass, at least one representative gene was selected for qRT-PCR analysis. Total RNAs were extracted using MACHEREY-NAGEL RNA isolation kit according to the manufacturer's instructions, and after checking the purity, integrity and quality, 3 µg of RNA was reverse transcribed to cDNA by using Thermo Scientific first-strand synthesis kit. The NCBI PRIMER Blast (www.ncbi. nlm.nih.gov/tools/primer-blast/) [70] was used to design the gene-specific primers with the default parameters: 57-60°C annealing temperature, 18-22 bp primer length, 50-55% GC contents, and 80-140 bp amplicon length (Table **S1**). SYBR Green master mix (2X) (Takara) was used for the qRT-PCR, and the analysis was performed with three biological replicates, and two technical replicates in Agilent Mx3000p with the following thermal cycles: 1 cycle at 95°C for 10 min, followed by 40 cycles alternatively at 95°C for 15 sec and 60°C for 1 min. The amplicon dissociation curves were recorded with a fluorescent lamp after 40^{th} cycle by heating from 58 to 95°C within 20 min and by maintaining triplicates. The sorghum *SbAcp* (*Acyl Carrier Protein 2*) and *SbE-F-P* (*Elongation Factor P*) genes were used as internal controls [71]. The average values ± standard error are represented. The t-test (Tukeys test) was employed to determine the statistical significance for all the values ($*P \leq 0.05$). The relative gene expressions were calculated by employing Rest software [72].

3. RESULTS

3.1. Identification and *In silico* Characterization of HSP60 Genes in *Sorghum*

In the present study, genome-wide scanning of *S. bicolor* resulted in the identification of 36 HSP60 genes which are sub-divided into 2 groups; HSP60 chaperonins with 30 genes and HSP10, the co-chaperonins with 6 genes. The genes were distributed on all the chromosomes, with the highest number of 9 on chromosome 1, while only one each on chromosomes 6 and 8 (Table **1** and Fig. **1**). The number of amino acids varied from 149 (SbHSP60-19) to 2056 (SbHSP60-28), with molecular weights ranging from 16563.89 (SbHSP60-19) to 229911.54 Da (HSP60-28) in HSP60s. In HSP10s, the number of amino acids ranged from 134 (SbHSP10-1) to 1844 (SbHSP10-4) and molecular weights from 14264.54 (SbHSP10-1) to 199040.62 Da (HSP10-4). The iso-electric points varied between 5.14 (SbHSP60-2) to 10.07 (SbHSP60-13). Out of 30 HSP60s, 16 were found acidic, while the remaining basic in nature. Based on the

GRAVY values, SbHSP60s have been found hydrophilic, except SbHSP60-7, 16 and SbHSP10-1. Out of 36 SbHSP60s, 26 proteins displayed instability index ranging between 28.47 (SbHSP60-27) to 62.75 (SbHSP60-28), while the aliphatic index varied from 74.86 (SbHSP60-11) to 62.75 (SbHSP10-6). All the HSP10s revealed the highest index with unstable, basic, and hydrophilic nature and with less molecular weights (Table **1**). Out of 30 SbHSP60s, 6 were localized in the cytosol, 9 in the nucleus, 10 in the chloroplast, 3 in mitochondria, and 2 in the plastid. Of the 6 HSP10s, 3 were localized in chloroplast and 1 each in cytosol, nucleus and vacuole (Table **1**). Among the 36 HSP60s, only 4 exhibited transmembrane helices. Three helices were noticed in SbHSP60-9, one each in HSP60-3, 16 and HSP10-2.

Table 1. Characteristics of identified *SbHSP60* genes in *Sorghum bicolor* number of amino acids, chromosomal location, isoelectric point (pI) and molecular weight (MW), DNA binding domains (DBD), number of exons, localization, GRAVY, instability index, and aliphatic index.

Gene	Common Name	No. of a. a.	Chr. loc.	pI / MW	DBD	No. of Exon	Localization	GRAVITY	Instability Index	Aliphatic Index
Sb01g000380	SbHSP60-1	1217	1	6.22/131849.91	67-547	20	Chl	-0.254	44.29	92.36
Sb01g020010	SbHSP60-2	1077	1	5.14/119175.59	502-1010	26	Chl	-0.410	41.11	90.27
Sb01g033120	SbHSP60-3	1925	1	5.95/215246.07	396-646	15	N	-0.456	48.58	75.67
Sb01g041170	SbHSP60-4	1811	1	9.54/195624.48	1206-1735	24	N	-0.175	44.74	87.33
Sb01g043220	SbHSP60-5	688	1	8.90/74959.68	155-656	16	C	-0.189	38.89*	93.31
Sb01g047360	SbHSP60-6	1180	1	8.74/131338.29	189-554	27	C	-0.433	43.21	84.53
Sb01g049370	SbHSP60-7	628	1	7.20/67781.40	37-592	5	C	0.041	42.98	108.77
Sb02g001450	SbHSP60-8	799	2	8.73/87612.79	40-519	15	C	-0.402	39.63*	84.37
Sb02g011260	SbHSP60-9	1308	2	8.53/141326.78	798-1273	22	P	-0.157	46.24	90.32
Sb02g024300	SbHSP60-10	1285	2	5.41/141642.08	1052-1274	9	N	-0.346	42.05	79.53
Sb02g043440	SbHSP60-11	569	2	7.59/62720.26	63-562	16	M	-0.253	34.15*	96.47
Sb03g009490	SbHSP60-12	775	3	8.80/85157.56	286-676	19	M	-0.385	40.91	82.76
Sb03g005190	SbHSP60-13	871	3	10.07/96258.74	102-557	17	C	-0.259	49.14	90.52
Sb03g041400	SbHSP60-14	1619	3	5.21/182219.68	559-719	17	N	-0.423	45.29	80.15
Sb04g000370	SbHSP60-15	576	4	5.62/61680.65	52-555	14	Chl	-0.143	28.98*	99.51
Sb04g004030	SbHSP60-16	1777	4	5.99/ 190958.85	37-498	17	P	0.033	37.71*	101.93
Sb04g035610	SbHSP60-17	380	4	9.03/40641.08	31-301	12	C	-0.122	38.47*	96.55
Sb05g022470	SbHSP60-18	1706	5	5.68/189340.50	34-522	17	N	-0.445	42.00	76.25
Sb05g008785	SbHSP60-19	149	5	5.61/16563.89	11-51	5	M	-0.125	39.59*	99.53
Sb06g034080	SbHSP60-20	1561	6	5.87/175336.41	197-477	12	N	-0.470	45.02	78.31
Sb07g000590	SbHSP60-21	1814	7	5.64/202460.95	426-566	16	N	-0.503	44.30	75.51
Sb07g020930	SbHSP60-22	1558	7	6.15/173645.21	323-407	15	Chl	-0.425	44.15	79.16
Sb07g022110	SbHSP60-23	1811	7	5.62/199458.67	431-681	13	N	-0.407	51.36	76.21
Sb08g014925	SbHSP60-24	1569	8	8.56/178143.90	126-232	14	Chl	-0.484	50.06	77.23
Sb09g014430	SbHSP60-25	1164	9	7.84/127154.19	150-630	16	Chl	-0.250	38.22*	92.90
Sb09g026970	SbHSP60-26	1061	9	8.92/114446.97	102-588	29	Chl	-0.321	34.99*	88.75
Sb10g001120	SbHSP60-27	665	10	6.92/71398.96	139-648	15	Chl	-0.105	28.47*	100.32
Sb10g009370	SbHSP60-28	2056	10	6.81/229911.54	707-968	16	N	-0.443	49.68	76.31
Sb10g022220	SbHSP60-29	553	10	5.74/61136.68	40-549	9	Chl	-0.156	43.37	98.23
Sb0011s00747	SbHSP60-30	1564	u	9.08/177985.80	192-238	15	Chl	-0.388	46.30	83.08
HSP-10										
Sb01g028650	SbHSP10-1	134	1	7.76/14264.54	47-133	6	Chl	0.110	41.50	105.60
Sb01g034530	SbHSP10-2	319	1	9.65/34727.58	123-199	6	V	-0.082	49.74	105.80
Sb02g040860	SbHSP10-3	169	2	9.26/17502.38	7-87	5	C	-0.470	42.02	90.19
Sb02g025700	SbHSP10-4	1844	2	8.67/199040.62	927-1115	17	N	-0.439	52.49	74.86
Sb04g035040	SbHSP10-5	487	4	9.89/52602.21	63-245	8	Chl	-0.320	48.48	85.91
Sb10g006450	SbHSP10-6	1467	10	8.43/160424.43	1276-1465	4	Chl	-0.482	62.75	78.28

Abbreviations: (a. a.: amino acids, Chrom.: Chromosome, pI: isoelectric point; MW: Molecular weight, Chl.: Chloroplast, C: Cytoplasm, N: Nucleus, P: Plastid, M: Mitochondria, V: Vacuole, GRAVY: Grand average hydropathy, * stable).

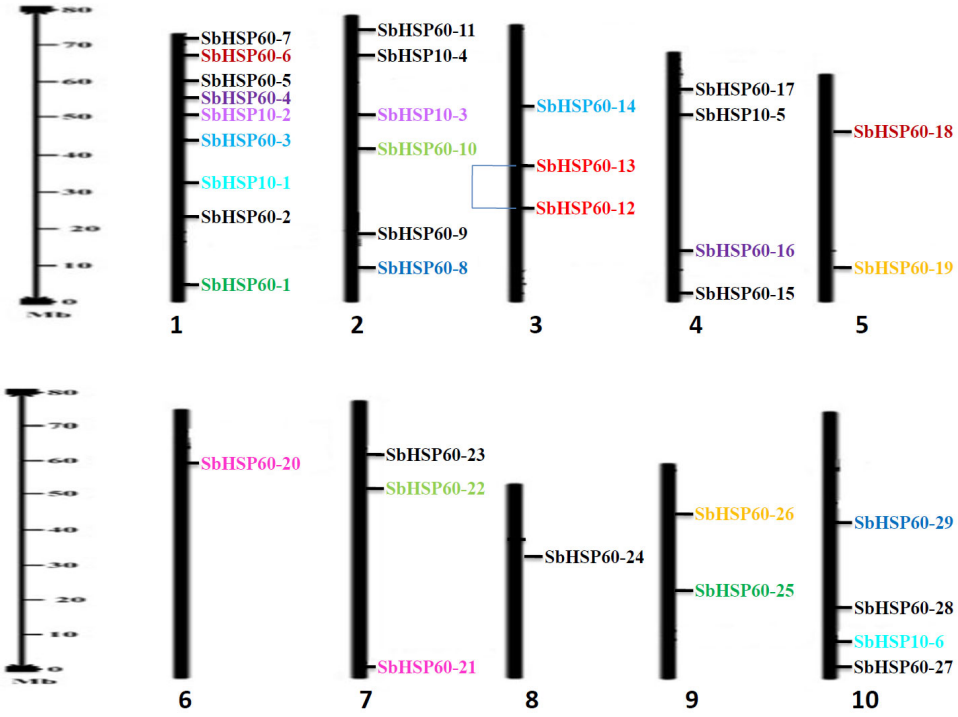

Fig. (1). Distribution of *SbHSP60* genes on 10 chromosomes with duplications. Duplications are illustrated by colours (segmental duplications in same colour) and regional duplications are linked with line. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. Prediction of Phosphorylation Sites in SbHSP60s

The serine and threonine residues, known as the phosphorylation sites, have been identified in the HSP60 family and found dominant. The phosphorylation sites vary in number, 13 (SbHSP60-19) to 172 (SbHSP20-28) for serine, 2 (SbHSP60-19) to 59 (SbHSP20-28) for threonine, and 1 (SbHSP60-19) to 28 (SbHSP20-28) for tyrosine. Among the specific kinases targeting HSP60s, protein kinase C (PKC), casein kinase 2 (CK2), cell division cycle protein 2 (CDC2), protein kinase A (PKA), casein kinase 1 (CK1), P38 MAP kinase and CDK5 were noticed as dominant kinases targeting HSP60s phosphorylation, compared to Ataxia-telangiectasia mutated kinase (ATM), epidermal growth factor receptor (EGFR), insulin receptor tyrosine kinase (INSR) and glycogen synthase kinase 3 (GSK3) (Table **S2**).

3.3. Gene Structure and Motif Analysis of SbHSP60s

The GSDS software analysis of the SbHSP60 family revealed a large number of introns. Twenty-six (86.6%) of the 30 HSP60s have more than 10 introns, the highest number being 28 present in SbHSP60-26. On the other hand, in the HSP10 sub-group, only HSP10-4 exhibited the highest number of 16, while HSP10-6 showed 3 introns (Table **1** and Fig. **2**). MEME identified 10 conserved motifs in each of the SbHSP60 and SbHSP10, with divergent distribution within each of the sub-cellular organelles. Motifs 2, 4, 7, 8, and 9 are the most conserved, commonly found in 66% of the HSP60s. Though motifs 2 and 4 are seen at N-terminus, 6 and 9 have been noticed as highly conserved. SbHSP60-3, 10, 14, 20, 21, 22, 23 and 28 have been found localized in the nucleus. They contain motif 1 at C terminus next to motif 6 repeats, and the conserved motif 2 is completely absent. They were also absent in the rest of the SbHSP60s (Fig. **3A** and Fig. **S1**). The amino acids in motif 1 are aromatic and acidic, responsible for nuclear localization, hence absent in other HSP60s. The stretch EEKK represents the nuclear localization signal. All the HSP10s showed similar motif distribution patterns, with 3 at N terminus and 8 at C terminus (Fig. **3B** and Fig. **S2**).

3.4. Promoter Analysis of SbHSP60 Genes

Biotic and abiotic stress-responsive (drought, salt, heat, cold, light, desiccation), hormone-specific, and developmental-specific *cis*-elements (40-different types) were detected in the promoter regions. MYB, MYC, and HSE were dominant among the abiotic stress-responsive elements, with SbHSP60-2 and 12 exhibiting 20 ABA-responsive elements. Of the 36 HSP60s, 33 (91.6%) were noticed with ABA-responsive elements in their promoter regions and 29 of them exhibit more than 2 ABA elements. The SbHSP60-5, 27 and HSP10-5 were seen devoid of ABA-responsive but rich with heat, low temperature and salt stress-responsive elements. Interestingly, 86% of the SbHSP60s were rich in light-responsive, 41.6% phosphorous starvation-responsive and cytokinin-responsive elements (Table **S3**).

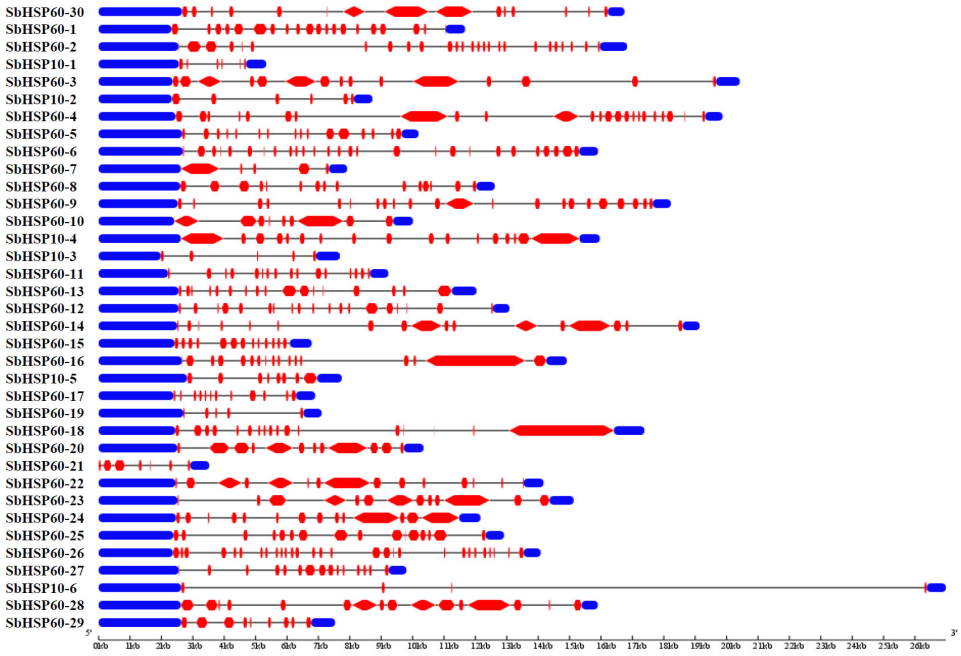

Fig. (2). Structure of the *SbHSP60* and *HSP10s* showing exons, introns and up/drown stream regions. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

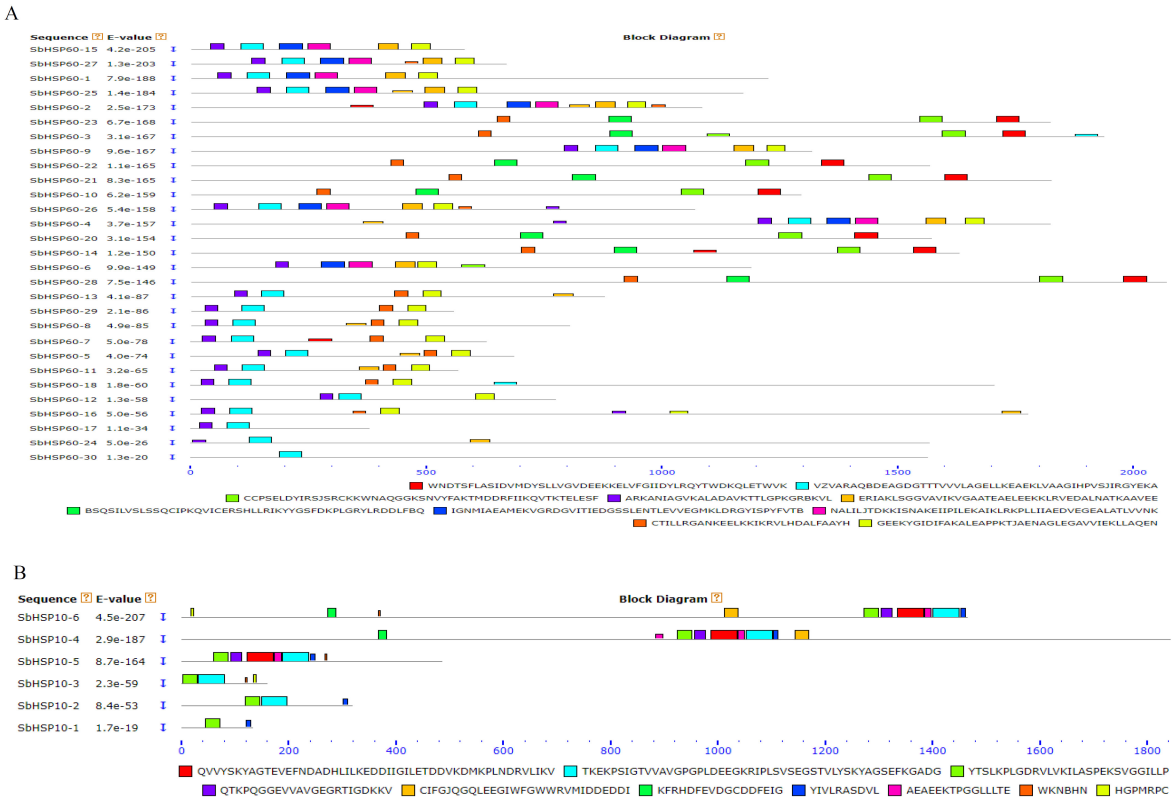

Fig. (3). Conserved motifs in **A**) SbHSP-60s and **B**) SbHSP10s. The scale represents the lengths of the proteins and motifs. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

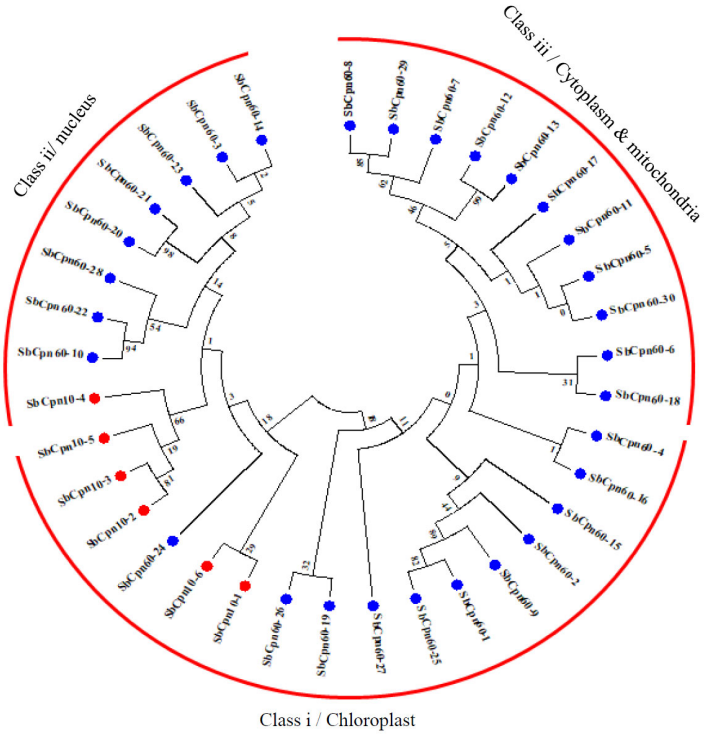

Fig. (4). Phylogenetic tree of S*bHSP60s*. The gene sub groups were classified based on their localization. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

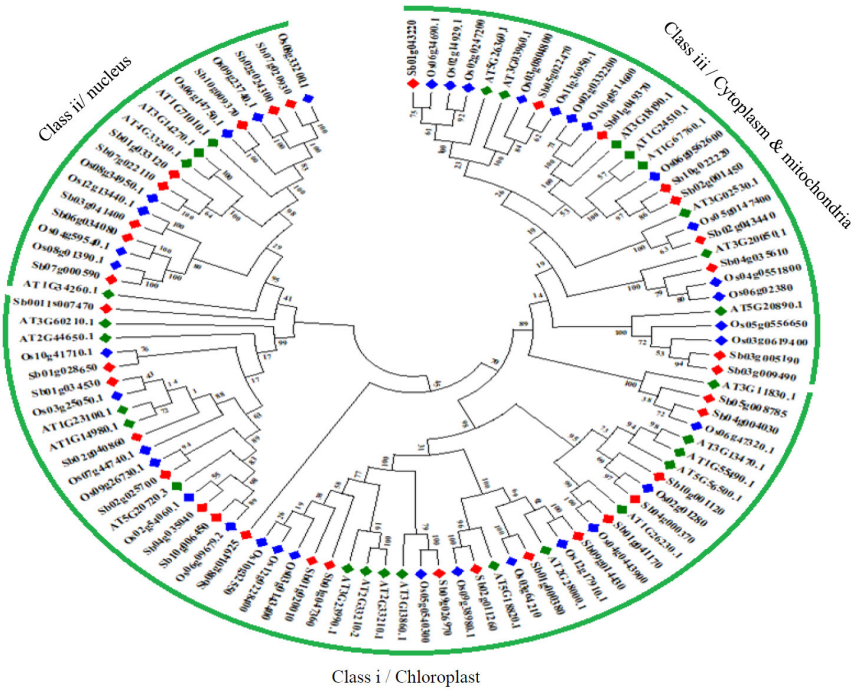

Fig. (5). Phylogenetic tree constructed using protein sequences *Sorghum*, *Oryza*, and *Arabidopsis* to know the evolutionary relationship of HSP60s. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.5. Identification of miRNAs that Target SbHSP60s

A total of 48 different types of miRNAs target and regulate the SbHSP60 family genes, which participate in cleavage and translation inhibition of the RNAs. The sbi-miR6225, sbi-miR5568, miR437, miR6220, miR821, miR166, miR395, and miR5565 are some of the major miRNAs that have been found to target SbHSP60s. The miR167 plays an important role in plant developmental processes, target SbHSP60-11 and 18. The miR166 (drought stress-induced) target SbHSP60-3, 4, 5 and 25. The multiple stress-responsive miR398 targets the SbHSP10-4, SbHSP60-10 and 14. The identified miRNAs (putative) can target more than one HSP60, indicating their nature of interaction with multiple genes. For example, miR6225 interacts with SbHSP10-4, HSP10-6, HSP60-2, HSP60-3, HSP60-4, HSP60-8, HSP60-14, HSP60-18, HSP60-20, HSP60-22, and HSP60-27 (Table **S4**).

3.6. Protein-protein Interaction and Chemical-protein Interaction Network of SbHSP60s

In order to predict the regulatory partners of HSP60s, the automated GeneMANIA server has been utilized. The predicted regulatory network suggests that members of the HSP60 family have various regulatory partners based on the conserved functional domains, physical interactions, and co-expression. As evident from Figure S3, the protein-protein interaction network of HSP60s revealed their complex molecular interaction with (i) 20 functional partners including T- complex protein 1 subunit alpha, (ii) 4 chaperonin proteins, (mitochondrial HSP60-3A, HSP60-2, CPN60, and 10 kDa chaperonin), (iii) 2 putative 1-phosphatidylinositol-3-phosphate 5-kinase (FAB1C, FAB1D), (iv) 4 phosphatidylinositol 4-phosphate 5-kinase (PIP5K3, PIP5K6, PIP5K10, PIP5K11), (v) 4 RING/FYVE/PHD zinc finger superfamily proteins, (vi) phosphoinositide binding protein, (vii) 2 regulators of chromosome condensation (RCC1) family proteins, (viii) GroES-like family protein, and (ix) a protein of unknown function (DUF581). The gene ontology (GO) terms analysis obtained through the GeneMANIA server suggested that predicted regulatory partners of HSP60s play key roles in different biological activity, including phosphatidylinositol phosphate kinase activity 1-phosphatidylinositol-4-phosphate 5-kinase activity, phosphatidylinositol binding, phospholipid binding, lipid binding, apical plasma membrane, and vacuole organization. The interaction between predicted regulatory partners and HSP60s can be validated using different molecular methods, including co-immunoprecipitation (Co-IP) and *in situ* hybridization (Fig. **S3**). The protein-protein interaction analysis predicts that SbHSP60s interact with other chaperones like HSP70s, and 90s along with other proteins like phosphatidylinositol-4-phoshate 5-kinase, Mre/Mbl, ribosomal proteins, elongation factor-1, histidine kinase, glutathione S-transferase, FYVE zinc finger, dynein-related subfamily proteins and Holliday-junction DNA helicase rvuB. By interacting with the above proteins, HSP60s participate in inositol phosphate metabolism, RNA transport and degradation, phagosome, phosphatidylinositol signalling, proteosome, protein processing in endoplasmic reticulum and autophagy (Fig. **S4**). Besides the prediction of the protein interaction network of HSP60s, a chemical-protein interaction network map was generated using the STITCH server. Interestingly, the HSP60s were found to interact with phosphate, ammonia, and lotion (urea or carbamide) (Fig. **S5**). These results support the earlier prediction of HSP60s that interact with phosphorous-starvation-responsive elements. However, since these interactions are not validated, the data need to be taken into consideration with caution.

3.7. Phylogenetic Analysis of HSP60 Proteins

Phylogenetic analysis of HSP60 proteins classified them into three subclasses I to III, with a conserved HSP60_TCP1 domain. All the chloroplast and nuclear-localized proteins are clustered into class I and II, while cytosol and mitochondria localized into class III (Fig. **4**). A total of 12 paralogous pairs have been detected in the SbHSP60 family, of which only one is tandem (SbHSP60-12/13 on chromosome 3) and the remaining segmental duplications (Figs. **1** and **4**, Table **S5**). A phylogenetic tree with 36 *Sorghum*, 35 rice and 28 *Arabidopsis HSP60* genes was constructed to know the evolutionary relationship, exhibiting 3 subclasses according to their conserved motifs and sub-cellular localizations. Sorghum followed the trend like that of rice and *Arabidopsis* HSP60s sub-cellular localizations and also the cluster. It exhibited a total of 23 orthologous events with rice, as shown in Fig. (**5**) and Table **S6**.

3.8. Calculation of Synonymous and Non-synonymous Substitution Rates (d_N/d_S)

The non-synonymous (d_N) to synonymous substitution (d-s) ratios for paralogous and orthologous gene pairs of *HSP60* genes were estimated to know their evolution by Darwinian selection in duplication and divergence. Out of the 12 paralogs, the d_N/d_S ratios ranged from 0.4539 (SbHSP10-2/SbHSP10-3) to 99.0000. The SbHSP10-2/SbHSP10-3 exhibited a d_N/d_S ratio of 0.4539, purifying selection and the rest showed positive/Darwinian selection (Table **S5**). Twenty-three orthologs exhibited ratios between 0.6926-99.0000, of which 2 showed purifying selection (ratio < 1), and the remaining were positive/Darwinian selection with >1 value (Table **S6**).

3.9. Transcriptome-based Gene Expression Profiling of SbHSP60s in Different Tissues and Developmental Stages Under Abiotic Stress Conditions

Of the 36 *SbHSP60* genes, probe ids for 30 were available on the SorghumFDB. These data were utilized for gene expression profiling. Using transcriptome data, expression of these 30 *SbHSP60s* in seven different tissues viz., shoot apex, root, internode, pith, rind, shoot, and leaf were analyzed under normal and abiotic stress conditions such as drought, heat, salt, and cold (Fig. **6A**). In the shoot apex and root tissues, a cluster of 13 *SbHSP60s* (*SbHSP60-26*, *SbHSP10-2*, *SbHSP60-5*, *SbHSP60-12*, *SbHSP60-13*, *SbHSP60-2*, *SbHSP60-17*, *SbHSP60-18*, *SbHSP60-11*, *SbH-*

SP60-16, *SbHSP60-29*, *SbHSP60-8*, and *SbHSP60-7*) portrayed higher expression. Five different stages like booting, dough, flowering, seedling, and stem elongation were taken for studying the expression profiles. *SbHSP60s* were found expressed (either upregulated or downregulated) in all developmental stages. As compared with other *SbHSP60* genes, expression profiling of 11 *SbHSP60* genes such as *SbHSP60-2*, *SbHSP60-13*, *SbHSP60-12*, *SbHSP60-5*, *SbHSP60-7*, *SbHSP60-17*, *SbHSP60-18*, *SbHSP60-11*, *SbHSP60-16*, *SbHSP60-29*, and *SbHSP60-8* have been found higher compared to other *SbHSP60* genes (Fig. **6B**). High expressions of *SbHSP60s* during developmental stages may indicate their involvement in development-related cellular activities followed by metabolic or physiological changes that affect the gene regulation under abiotic stresses. Hierarchical clustering based on expression profiles of *SbHSP60s* under drought, heat, salt, and cold allowed grouping of these 30 genes in two different clusters. One of these clusters contained only one *SbHSP60-25* gene, which depicts upregulation under the experimental conditions. The remaining *SbHSP60s* are distributed among other subclusters of the second and major cluster (Fig. **6C**). The heat map of different *SbHSP60s* following abiotic stresses showed significantly altered expression profiling, either downregulation or upregulation up to 6.60-folds, especially in *SbHSP60-20*. The *SbHSP10-2* and *SbHSP60-20* along with the cluster of eight *SbHSP60s* (*SbHSP60-26*, *SbHSP10-4*, *SbHSP60-2*, *SbHSP10-6*, *SbHSP60-1*, *SbHSP60-15*, *SbHSP60-27*, and *SbHSP60-25*) displayed upregulation under abiotic stress conditions.

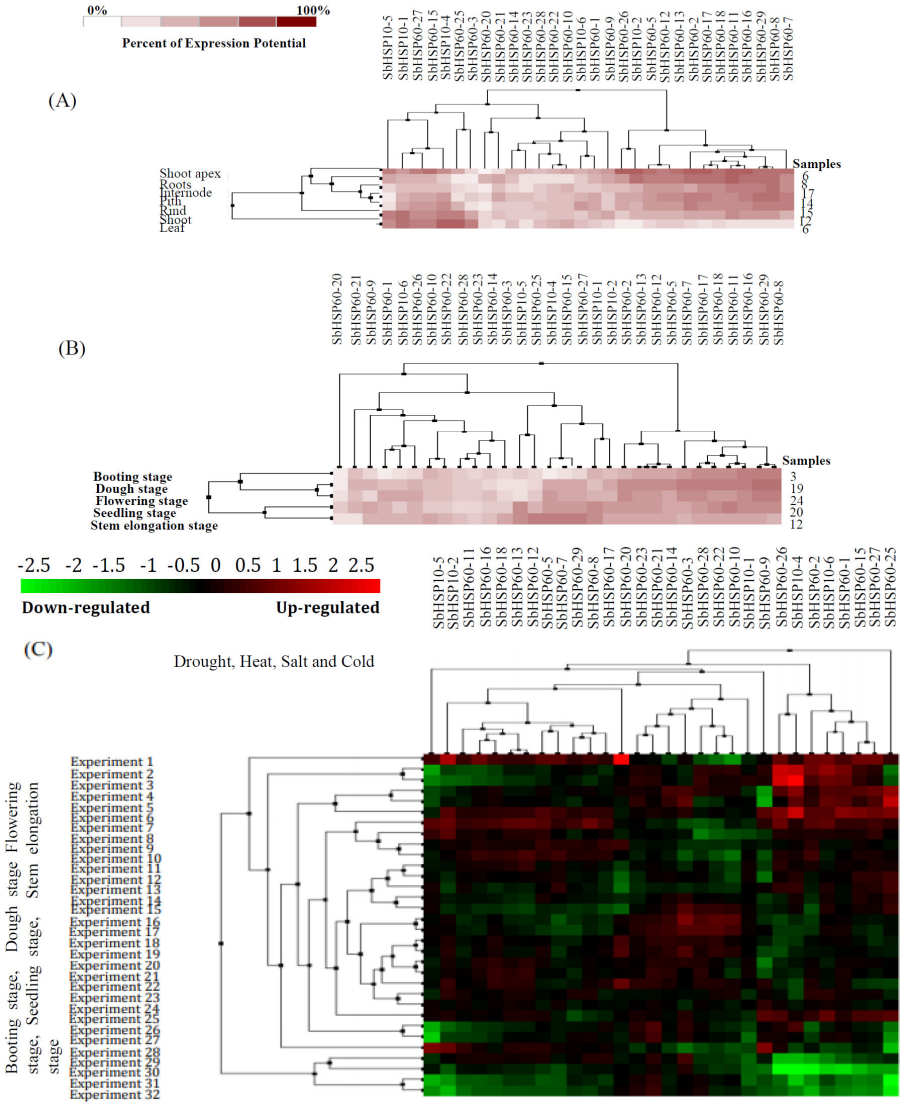

Fig. (6). Hierarchical clustering of *SbHSP60s* based on their expression in (**A**): 6 different tissues; (**B**) 5 different developmental stages and **C**) under different abiotic stress conditions. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.10. Expression Profiling of *SbHSP60* Genes

Further to find out the expression levels of selected *SbHSP60* genes, qRT-PCR was performed under different abiotic stress conditions. Of the 36, only 8 genes; *SbHSP60-1, 2, 3, 6, 9, 24, HSP10-2*, and *10-5* were selected based on the number of introns, homology with other crops, subcellular localizations and their microarray expressions. Expression profiling of 8 selected *SbHSP60* genes was carried out in root, stem and leaf tissues of *S. bicolor*. All the 8 genes depicted uniform expressions only in the leaf tissues. All the 8 selected genes portrayed low expressions in stems (except under cold), moderate profiles in roots (but not under cold), and leaves. *HSP60-1* displayed 2.38-folds higher expression under drought, 2.92- under salt, 6.68- under heat, and 0.11-folds under cold stress in roots. In stem tissues, 0.25-, 7.74-, 0.18-, and 0.43-fold higher expressions were recorded under drought, salt, heat and cold stresses, respectively. Drought exposed leaves of HSP60-1 exhibited high (12.66-folds) expressions in comparison with salt (1.32-folds), heat (0.18-folds) and cold (4.73-folds) stresses (Figs. **7**, **S6** and Table **S7**). Root tissues of *HSP60-2* exhibited 2.22- and 2.01-folds higher expressions under drought and heat stresses, respectively. Stem tissues of *HSP60-2* also showed 2.81-folds higher expressions when exposed to salt stress. Both root and leaf tissues of *HSP60-3* displayed higher expressions in comparison with stem and the expression levels were 4.04-, 14.32-, and 29.24-folds higher in root tissues under drought, salt and heat stresses, respectively. Leaf tissues also displayed 3.79-, 19.07-, 3.13-, and 23.97-folds higher expressions when plants were subjected to drought, salt, heat and cold stresses, respectively. Root tissues of *HSP60-6* exhibited 47.17-folds under salt and 9.44-folds higher expressions in cold stress. Surprisingly, stem tissues displayed 4.16-(drought), 2.11- (salt), and 23.86-folds (heat) higher expressions than the leaf. The gene *HSP60-9* was upregulated under drought (5.92-folds in roots, 17.63-folds in stems, and 3.34-folds in leaf), salt (10.05-folds in roots, 2.87-folds in stem and 16.03-folds in leaf), cold (9.98-folds in root, 224.93-folds in stem, and 5.91-folds in leaf). Stems exposed to heat stress showed 18.33-folds higher expressions, but not the other two tissues (Table **S7**). Stem exhibited 4.16-, 2.11-, 23.6-, and 1.56-folds higher expressions under drought, salt, heat and cold stresses, respectively. In the case of leaf, 1.2-, 1.16-, 0.05- heat and 0.11-folds higher expressions under cold stress were noticed. Expression of root *SbHSP60-24* was significantly high (18.63-folds) under cold stress, but not under other stresses. But the expression of the same gene in the stem was 3.24-, 2.66-, 12.35-, and 82.32-folds higher under drought, salt, heat and cold stresses respectively. Leaf, on the other hand, showed high expression (71.67-folds) only under drought, but not under other abiotic stresses. Expression of *SbHSP10-2* was 5.96-, and 5.81-folds higher in the roots subjected to drought and heat stresses, respectively. Stem tissues displayed 11.65-folds expression under salt, and 100.65-folds in leaf under cold stress but not in other abiotic stresses. In the case of roots, the expression levels of *SbHSP10-5* were 3.96-, 0.59-, 0.04- and 0.01-folds under drought, salt, heat and cold-stresses, respectively. Expression levels in the stem varied from 0.05-(drought), 0.03- (salt), 0.24- (heat) to 30.55-folds under cold stress. The gene showed 0.01-folds higher expression in drought, 0.42- under salt, 0.71- under heat and 3.79-folds under cold stress conditions in the leaf. Surprisingly, any of the *HSP60s*, *HSP10-2* and *HSP10-5* genes did not show any expressions under diverse developmental stages. From the expression analysis, *HSP60-9* and *HSP60-24* for drought, *HSP60-3* and *HSP60-6* for salt, *HSP60-3* and *HSP60-6* for heat, *HSP60-6* and *HSP10-2* for cold stress have been found as candidate genes in *S. bicolor* but need further validation (Figs. **7**, **S6** and Table **S7**).

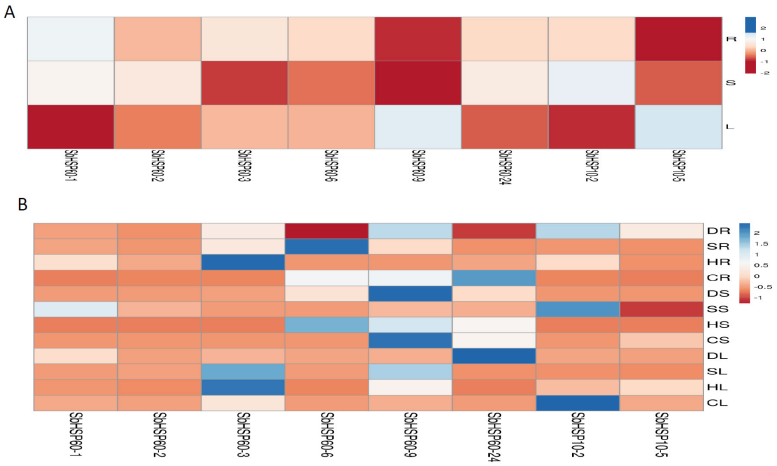

Fig. (7). qRT-PCR expression analysis of selected *SbHSP60* genes **A**) in root, stem, and leaf tissues. **B**) under drought, salt, heat and cold stress in root, stem and leaf.

Abbreviations: (DR: Drought root, DS: Drought stem, DL: Drought Leaf, SR: Salt root, SS: Salt stem, SL: Salt leaf, HR: Heat root, HS: Heat stem, HL: Heat leaf, CR: Cold root, CS: Cold stem, CL: Cold leaf). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

The HSP60 chaperonins play a critical role in folding and aggregation of other proteins present in chloroplasts and mitochondria which are often subjected to stress [14, 26]. The systematic genome-wide analysis of *S. bicolor* resulted in the identification of 36 genes, and subdivided into 2 groups; the HSP60 chaperonins with 30 and HSP10 co-chaperonins with 6. Under stress conditions, *HSP60s* interact with co-chaperones *HSP10* and alleviate the aggregation of denatured proteins and step up the refolding of non-native proteins [73]. The number of chaperonins vary in plants, for example 29 in *A. thaliana* [21, 74, 75], 29 in rice [76], 20 in foxtail millet [77], 49 in *Populus trichocarpa* [78] and 16 in pepper [35]. Based on the earlier reports of their sub-cellular localization, *SbHSP60* genes were subdivided into 3 groups, the cytosol/nucleus, mitochondrial and chloroplast, all of them containing the conserved HSP60_TCP1 domain. But in the present study, 2 additional subgroups were detected based on their localization in plastids (SbHSP60-9 and 16), and vacuole (SbHSP10-2). The *HSP60s* with the highest number of introns, and HSP10s with a smaller number of introns are consistent with the earlier records of Zhang *et al*. [79]. The majority of them were hydrophilic, acidic, unstable with a higher aliphatic index, indicating that these are the characteristic features of stress proteins [80]. The motifs were remarkably conserved within respective subgroups, consistent with the earlier reports [77, 79]. The duplicated genes showed a similar number of introns, conserved motifs, and properties. In the present investigation, 11 of the 12 total duplications were segmental, indicating the expansion of the *SbHSP60* family through segmental duplication events [81, 82], which is in confirmation with the earlier reports in *Populus* and *Setaria* [77, 79].

The HSP60s contain several phosphorylation sites, post-translational modifications and ubiquitination, which are perhaps the reasons behind the multifaceted nature of these proteins. Phosphorylation of the HSPs under dehydration triggers the defense regulatory pathway [83]. By protein-protein interactions, HSP60s mediate the multiple stress-tolerant pathways through phosphorylation. The protein-protein interaction network analysis illustrates that SbHSP60s show interaction with phosphatidylinositol-4-phoshate 5-kinase, which plays a pivotal role in flowering, and plant growth under environmental stress. It phosphorylates phosphatidylinositol-4-phosphate to make it to phosphatidylinositol-4,5-bisphosphate, which is the precursor of inositol-1,4,5-triphosphate and diacylglycerol, and activates cellular proteins that participate in signal transduction and cytoskeletal organization [84]. HSP60s interact with histidine kinase, participate in ethylene, and cytokinin signalling, osmosensing, mega-gametophyte development, cold perception, salt and drought stress resistance [85]. It is known that glutathione S-transferase (GST) contributes to both biotic and abiotic stress tolerance [86]. There are more chances of denaturation of proteins such as GST in stress conditions and HSPs interact with GST and act as molecular chaperones and develop into an HSP-GST complex to restore and maintain its active form. Thus, the chaperonins play very critical roles during stress situations in plants. Many miRNAs are implicated in plant growth, development, metabolism, in abiotic and pathogen stress resistance [87, 88]. In the present investigation, miR171 appears to target several *SbHSP60* genes. miR171 was also found in *Arabidopsis*, which response during all of the abiotic stress conditions [89]. miR393, and miR319 were upregulated under drought stress in rice [90], miR528 and 397 exhibited enhanced expression under arsenic stress [91], miR172c targeted AP2-like transcription factor and played key roles in flowering and abiotic stress, and its over-expression resulted in enhanced water deficit and salt tolerance in *A. thaliana* [92], inferring the prime roles that miRNAs play during plant development.

Promoter analysis of the *SbHSP60* family genes indicated that they may be associated with abiotic and biotic stress tolerance along with other developmental-responsive elements [93-95]. The presence of ABA-responsive elements in the promoter regions indicates that these genes may work in an ABA-dependent manner. This infers that *HSP60s* play vital roles in multiple stresses. Chloroplast *HSP60* in *Arabidopsis* showed enhanced expression levels under high temperature and drought stresses [96], and in pepper, almost all the candidate genes were expressed under multiple abiotic stresses [35], indicating the important roles that these genes play during stress.

In the present investigation, 6 of the *HSP10* subgroup genes identified were found to have similar functions to that of the genes reported earlier in *Arabidopsis* [21]. Chloroplast chaperonins can physically interact with each other, and usually, assist in the refolding of two different target proteins. Also, successful partitioning to the native state-required ATP hydrolysis besides chaperonin 10 [42]. The chloroplast HSP21, the functional homologue of the mitochondrial HSP10 and HSP21 proteins, were identified in *Arabidopsis* and pea [97], but not noticed in *S. bicolor*. *Sorghum* contains 3 chloroplast-localized HSP10s, which exhibit their independent evolution in plants from endocytic events, while only 2 of them were reported in *Arabidopsis* [21]. Kim *et al*. [98], also found that *OsHSP60α1* encodes the plastid chaperonin 60α subunit and is essential for folding of rbcL protein, inferring the importance of chaperonins in protein protection.

Earlier reports indicated the participation of Hsfs and HSPs in several biological processes like in plant development and stress tolerance [4, 76, 99]. In the present investigation, 8 selected *HSP60* genes showed divergent expression levels in roots, stems and leaf tissues, majority of them with high expressions in leaf tissues in comparison with roots and stems implicating their role in leaf developmental processes or protection under stress. Downregulation of *SbHSP60* genes observed in the stem tissues is contrary to the earlier reports in *Populus* [79]. HSPs play critical roles in conferring tolerance against multiple abiotic stresses [99, 100], indicating their overlap response which are crucial in the crosstalk of various abiotic stress conditions [76, 99]. This is akin to the implication of *SbHSP60-3*, *6*, *9*, *24* and SbHSP10-2 in various tissues under diverse stress conditions,

which indicates their crosstalk response. The selected genes exhibited higher levels of upregulation under cold stress (100- and 224-folds) in comparison with drought and salt stresses (Table **S7**). Cross checking of the genes implicated in developmental activities revealed that they are not involved in abiotic stress conditions and *vice versa*. Thus, there is no functional overlap among the genes between the two different events. But, in the case of *PgHSP10*, abundant expression was recorded under salt, heat and drought compared to cold stress [43]. In the present investigation, *SbHSP10-2*, 100-fold enhanced expression was observed under cold stress, especially in the leaf, indicating that it may be involved in the protection of low-temperature stress in the leaves. Turhan *et al*. [101], demonstrated upregulation of *HSP60* in grafted tomato under heat stress, while Taj *et al*. [102], in *AT1G55490* (*Arabidopsis*) and its homologue *PtHSP60-33* by Yer *et al*. [78], under salt stress, indicating the involvement of *HSP60s* in multiple stress tolerance. In *Arabidopsis* and *Zea*, *HSP60* genes showed development-mediated expression during seed germination under heat stress, and in the assembly of macromolecules necessary for mitochondrial biogenesis [33]. Thus, a functional overlap of the genes was noticed in certain plants. In the present investigation, the selected *SbHSP60-1* and *3* genes exhibited high levels of expression in leaf tissues under drought and salt stresses, indicating the prominent roles they play in leaf rather than stem and root. These results are indistinguishable from the results reported in *Setaria italica* [77]. In the present study, *HSP10-2* was found as a candidate gene, which exhibited enhanced expression under drought, salt heat and cold stress conditions. This co-chaperone may interact with *HSP60s* which participate in the aggregation of denatured proteins and refolding the non-native proteins, as has been pointed out by Guo *et al*. [73]. The majority of the selected *SbHSP60* genes showed the highest expression levels under drought, and salt stresses followed by cold stress.

CONCLUSION

Taken together, our findings provide an early insight into the role of *HSP60* genes in plant development and abiotic stress tolerance. The results obtained further pave the path for characterization of *HSP60s* in other crops as well and for understanding the mechanisms associated with stress biology, thereby crop plants can grow better under such harsh environments.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

RESEARCH INVOLVING PLANTS

All experiments on plants used in the present research were in accordance with the international guidelines.

CONSENT FOR PUBLICATION

All authors have read and approved the manuscript for publication.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Fig. S1 MEME identified motif sequences of Sorghum HSP60 proteins.

Fig. S2 MEME identified motif sequences of Sorghum HSP10 proteins.

Fig. S3 Gene-gene interaction and GO annotation analysis of SbHSP60s.

Fig. S4 STRING identified protein-protein interactions of HSP60 in Sorghum.

Fig. S5 Protein - chemical interaction network of SbHSP60s.

Fig. S6 Relative expression values of selected *SbHSP60* genes in the root, stem, and leaves under drought, salt, heat and cold stress obtained through qRT-PCR analysis (DR: Drought root, DS: Drought stem, DL: Drought leaf, SR: Salt root, SS: Salt stem, SL: Salt leaf, HR: Heat root, HS: Heat stem, HL: Heat leaf, CR: Cold root, CS: Cold stem, CL: Cold leaf). Each value represents mean ± SD of three replicates. *indicates significant differences calculated by t-test ($*P \leq 0.05$).

Table S1. Gene-specific primers used in the gene expression analysis of SbHSP60s.

Table S2. Protein kinases that participate in the phosphorylation of SbHSP60s.

Table S3.*Cis*-acting elements present in the promoter regions of *Sorghum* HSP60 family.

Table S4. Identification of miRNAs that target SbHSP60 family of genes.

Table S5. Non-synonymous to synonymous substitution rates (dN/dS) of SbHSP60 paralogs.

Table S6. Non-synonymous to synonymous substitutions of HSP60 orthologs of *Sorghum, Oryza* and *Arabidopsis*.

Table S7. qRT-PCR expression levels of SbHSP60s.

Supplementary material is available on the publisher's website along with the published article.

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