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Genome-wide *in silico* analysis of dehydrins in *Sorghum bicolor*, *Setaria italica* and *Zea mays* and quantitative analysis of dehydrin gene expressions under abiotic stresses in *Sorghum bicolor*

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ABSTRACT

Dehydrins (*DHNs*) are highly hydrophilic, thermo stable, calcium dependent chaperons involved in plant developmental processes as well as in diverse abiotic stresses. A systematic survey resulted in the identification of 7 dehydrins (*DHNs*) in *Setaria italica* and *Zea mays*, but 6 in *Sorghum bicolor*. They are classified into 5 sub-groups, namely YnSKn, SKn, KnS, S, and YnS. *DHNs* of *Sorghum* exhibit 1 ortholog with *Oryza sativa* and *Z. mays* and 3 with *S. italica*. Unlike other *DHNs*, Sb*DHN*5 has been found as an ordered protein with many phosphorylation sites. Network analyses of novel YnS subgroup showed interaction with HSP70 and FKBP genes. *In silico* promoter analysis revealed the presence of abscisic acid (ABA), drought, salt, low temperature stress-responsive elements. The miRNA target analysis revealed *DHNs* are targeted by 51 miRNAs responsive to abiotic stresses. High transcript expressions of *DHNs* were observed in root, stem and leaf compared to inflorescence in *S. bicolor*. All *DHN* genes exhibited high levels of expression in stem under cold, heat, salt, and drought stresses. In contrast to other *DHNs*, the *SbDHN*2 of YnS subgroup, exhibited the highest expression, under multiple stresses in all the tissues indicating its involvement against a wide array of abiotic stresses.

1. Introduction

Dehydrins (*DHNs*) or group 2 LEA protein family members are expressed under cellular dehydration and play crucial roles in response to abiotic stresses. Due to their hydrophilicity and high glycine content, *DHNs* assist cells to withstand dehydration stress (Anchordoguy and Carpenter, 1996). *DHNs* are unstructured proteins and share many features with other types of intrinsically disordered/unstructured proteins. Due to their disordered state, *DHNs* escape from denaturing under abiotic stress conditions (Livernois et al., 2009; Hincha and Thalhammer, 2012). Under dehydration stress, tissue and developmental specific expressions of *DHNs* have been observed. Some *DHNs* are more responsive to the developmental stages of the plant than to abiotic stresses. They act as chaperons involved in developmental processes like late embryogenesis and stabilize macromolecules, denatured proteins, and membrane structures in stressed plants (Close, 1996; Hinniger et al., 2006). *DHNs* contain a consensus sequence of lysine rich residues (K-segment), representing a highly conserved 15 amino acid (EKKGIMDKIKELLPG) motif, with repeated glycine and polar amino acids forming amphipathic-helices. These helices interact with lipid components and hydrophobic sites of the partially denatured proteins of cell membranes and protect the proteins from denaturation (Koag et al., 2009). *DHNs* have a serine rich segment (S-segment), which can be modified by phosphorylation. The phosphorylated *DHNs* binding activity is generally conserved in the acidic subfamily of *DHNs* (Kovacs et al., 2008). *DHNs* consist of 1–3 tandem copies of the consensus Y-segment (V/T) DEYGNP, near the N terminus. They show similarity to the plant and bacterial chaperonin nucleotide binding site

Abbreviations: ABRE, abscisic acid-responsive elements; CK, casein kinase; *DHNs*, dehydrins; DRE, drought-responsive elements; HSE, heat shock-responsive elements; IDP, intrinsically disordered proteins; ILP, intron length polymorphism; LEA, late embryogenesis abundant proteins; LTR, low temperature-responsive elements; miRNA, micro RNA; MW, molecular weights; NJ, neighbour joining; pI, iso electric point; PKA, protein kinase A; PKC, protein kinase C; qRT-PCR, quantitative real-time PCR; SSR, simple sequence repeats; UTRs, untranslated sequence regions

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motifs (Hanin et al., 2011). *DHNs*, based on the number and order of the Y-, S- and K-segments, are classified into several classes, such as KS, SK_3, YSK_2, Y_2SK_2, Kn, Y_2SK_3, and YSK_3 (Close, 1996). In barley, YSK_2-type *DHN* was up-regulated by drought, but not by cold stress. SK_3-, Kn-, and KS-type *DHNs* are induced by low temperature and drought (Tommasini et al., 2008). Overexpression of *Sorghum DHN1* (YSK_2) in tobacco displayed enhanced tolerance to high temperature and osmotic stress conditions (Halder et al., 2017). YnSKn-type *DHNs* are expressed during drought, salt, frost, ABA, gibberellic acid, methyl jasmonate, and salicylic acid (SA) treatments. KnS-type *DHNs* bind to metal and scavenge hydroxyl radicals and protect the membrane integrity (Hanin et al., 2011). KS-type *DHNs* on the other hand are small proteins expressed in the reproductive tissues like anthers during chilling stress (Wang et al., 2014). The SKn-type acidic *DHNs* consist of compositional and structural features, and membrane binding properties. They protect the membranes from freezing and desiccation by acting as molecular chaperones or ion sequestration agents to prevent the damage of membrane lipids (Alsheikh et al., 2003; Kovacs et al., 2008).

DHNs scavenge reactive oxygen species (ROS), causing enhancement in the antioxidative enzyme activity under dehydration stress (Kumar et al., 2014). *DHNs*/DHN-like proteins with ion (calcium in particular) binding activity might act either as calcium buffers or as calcium-dependent chaperones like calreticulin and calnexin (Alsheikh et al., 2003). Eight *DHNs* have been reported earlier in rice (Wang et al., 2007; Verma et al., 2017), 13 in barley (Tommasini et al., 2008), 10 in *Arabidopsis* (Hundertmark and Hincha, 2008), 11 in poplar (Liu et al., 2012), 9 in *Malus* (Liang et al., 2012), 4 in *Vitis* (Yang et al., 2012), and 23 in *Brassica napus* (Liang et al., 2016). However, the information regarding the number of diverse *DHN*-types in warm grasses like *Setaria italica*, *Sorghum bicolor*, and *Zea mays* is lacking. Hence, the present investigation was carried out with an objective to find out the number, type, distribution, characterization, motif and promoter analysis, phosphorylation sites and structure of *DHNs* in 3 economically important warm grasses; *S. italica*, *S. bicolor*, and *Z. mays* with special focus on *S. bicolor* and their evolutionary relationships with *Oryza* and *Arabidopsis*, besides tissue specific expression profiles.

2. Materials and methods

2.1. Plant material and stress conditions

To investigate the expression levels of *DHNs*, seeds of *Sorghum bicolor,* variety BTx623, were sown in pots containing 4.5 kg of black clay soil under glass house conditions at 28/20 °C day/night temperatures. After the emergence of inflorescence, the plants were subjected to drought stress by withholding water for 5-days, cold stress by keeping the plants at 4 °C for 4 h and heat stress by exposing the plants to 40 °C for 4 h in a growth chamber and salinity stress by treating the plants with 150 mM NaCl solution for 24 h. Respective controls were maintained under similar conditions. Roots, stems, leaves, and inflorescences were collected and snap frozen immediately in liquid nitrogen and stored at −80 °C until further use.

2.2. In silico identification of DHN genes

DHN gene sequences of *Arabidopsis*, *Oryza*, *Hordeum*, *Vitis*, *Lycopersicum*, and *Malus* were retrieved from NCBI database and searched against *Sorghum bicolor*, *Setaria italica,* and *Zea mays* genomes in Gramene database (http://www.gramene.org/) to find out their homologs. Edit plus (http://www.editplus.com/) and Genscan (http://genes.mit.edu/GENSCAN.html) programs were used to retrieve the *DHN* gene cds and protein sequences. Based on the homology, all the identified putative DHN protein sequences were subjected to SMART program (http://smart.embl-heidelberg.de/) to identify their conserved domains (Letunic et al., 2004).

2.3. Sequence analysis of DHNs

The identified *DHN* genes were mapped to their respective chromosomes based on the information provided in the Gramene Database. Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) software was used for obtaining the *DHN* gene structures - exons, introns, and untranslated sequence regions (UTRs) based on the alignments of their coding sequences (Guo et al., 2007). Multiple sequence alignment was performed using ClustalX (Larkin et al., 2007) to explore conserved sequences and regulatory domains including their functional homology. Multiple Em for Motif Elicitation (MEME) software (http://meme-suite.org/) was employed with default parameters: number of motifs (1 − 10), motif width of (5–50) and the number of motif sites (5–10) to analyze sequence patterns and their significance (Bailey et al., 2006). Molecular weight (MW), isoelectric point (pI), and GRAVY (grand average of hydropathy) of *DHNs* were identified using ProtParam of Expasy tools (Gasteiger et al., 2005) (http://web.expasy.org/protparam), while phosphorylation sites were predicted by employing NetPhosK1 software of Expasy tools (Blom et al., 2004). Disorder tendencies of all the identified *DHNs* were analyzed using the IUPred (http://iupred.enzim.hu/) (Dosztanyi et al., 2005). The putative transmembrane helices within *DHNs* were identified using TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) (Moller et al., 2001). Subcellular localization of *DHNs* was identified using CELLO V2.5 (http://cello.life.nctu.edu.tw) (Yu et al., 2006) and WoLFPSORT programs (http://wolfpsort.org/) (Horton et al., 2007). Secondary structures of DHN proteins were predicted using PSIPRED v3.0 program (Jones, 1999). All the *DHNs* were queried against the Protein Data Bank (Berman et al., 2000) to identify the best template with similar amino acid sequences and known 3D structures for developing the homology models. Homology structures of *DHNs* were built by employing the Modeller 9.15 software (http://www.salilab.org/) (Webb and Sali, 2014) and validated by PROCHECK software to identify phi-psi angles of amino acids (Laskowski et al., 1993). Amino acids that were not found in the allowed regions were brought back into the allowed regions by loop building with the help of Swiss Protein Data Bank viewer programme. The protein-protein interaction of Sb YnS-subgroup was generated by employing STRING (http://string-db.org/) software.

2.4. In silico prediction of potential cis-regulatory elements

To predict the putative *cis*-regulatory elements of *DHN* promoter regions PLACE (Higo et al., 1999) and PLANTCARE (Lescot et al., 2002) software programs were used. Genomic sequence of length 2000 bp upstream to start codon was retrieved from *S. bicolor*, *S. italica*, and *Z. mays* and used for analysis.

2.5. Phylogenetic analysis of DHNs

The N-J phylogenetic tree was constructed with the DHN protein sequences of *S. bicolor*, *S. italica*, *Z. mays*, *O. sativa*, and *A. thaliana* using MEGA 6.2 software (Tamura et al., 2013) by employing the Poisson correction, pairwise deletion and bootstrap value (1000 replicates) parameters.

2.6. In silico prediction of gene specific molecular markers (SSRs and ILPs) and miRNAs targeting DHNs

Gene specific molecular markers including SSRs and ILPs were developed in genomic transcripts of identified *DHN* genes using BatchPrimer3v1.0 (http://probes.pw.usda.gov/batchprimer3/) server. Further, putative miRNAs in different plant species targeting the *DHN* genes were identified using psRNATarget server (Dai and Zhao, 2011) with default parameters.

2.7. Classification, signature amino acid analysis and evolutionary relationship of DHNs

DHN sequences belonging to different crops were retrieved from Gramene and Phytozome databases. They are further classified manually using MEME (Bailey et al., 2006) to identify the nature of motifs with default parameters; number of motifs (1–10), motif width (5–50), and the number of motif sites (5–10). The amino acid percentages were calculated by Protparam tool (Gasteiger et al., 2005), to identify the signature amino acids. To know the evolutionary relationship, phylogenetic tree for a set of 451 DHN sequences was constructed using MEGA 6.2 software (Tamura et al., 2013) by employing the Maximum Parsimony (MP) search method Tree-Bisection-Reconnection (TBR) that uses all sites and bootstrap value (1000 replicates) parameters.

2.8. RNA isolation and qRT-PCR analysis

Total RNA was extracted from different tissues of *S. bicolor* exposed to different abiotic stresses along with their respective controls using MACHEREY-NAGEL kit by following the manufacturer's instructions. A total of 2.5 µl RNA (2.5 µg concentration) was converted to cDNA using Superscript III first strand synthesis kit (Invitrogen) and used as template after diluting it with nuclease free water (1:12). The SYBR Green Master Mix (2×) was used according to the manufacturer's recommendations on the RealPlex (Eppendorf) to study the gene expression. Gene expression analysis was performed for 6 *SbDHNs* (*SbDHN1* to *SbDHN6*) with expected product sizes of 80–124 bp (Supplementary Table 1) in 96-well optical PCR plates. Three biological replicates were taken for qRT-PCR analysis with the following thermal cycles: 1 cycle at 95 °C for 10 min, followed by 40 cycles alternatively at 95 °C for 15 s and 62 °C for 1 min. Amplicon dissociation curves were recorded with fluorescence lamp after 40th cycle by heating from 58 to 95 °C within 20 min. Transcript levels of eukaryotic initiation factor4α (*SbEIF4α*) and protein phosphatase2A (*SbPP2A*) genes were used as internal controls (Reddy et al., 2016). Experiments were repeated thrice and average values are represented. Relative gene expressions were calculated by employing Rest software (Pfaffl et al., 2002).

3. Results

3.1. In silico identification of DHN genes

A total of 43 *DHN* nucleotide sequences; 10 from *Arabidopsis*, 4 from *Vitis*, 9 from *Malus*, 7 from *Oryza* and 13 from *Hordeum* were retrieved from NCBI database. Blast search of the DHN sequences against the genomes of *S. italica*, *S. bicolor*, and *Z. mays*, resulted in the identification of 17 putative genes in *S. italica*, 23 in *S. bicolor* and 19 in *Z. mays* (total 59). On testing these sequences by SMART software, a conserved domain search tool, only 20 of the 59 were confirmed to be *DHNs*; 7 each in *S. italica*, and *Z. mays* and 6 in *S. bicolor* (Table 1).

3.2. Chromosomal location and gene structure of DHNs

The 7 and 6 *DHNs* identified in *S. italica* and *S. bicolor* are localized on 3 different chromosomes each, while 7 *DHNs* identified in *Z. mays* are distributed on 6 different chromosomes. Among the 7 *DHNs* in *S. italica*, *SiDHN1* is mapped on chromosome 1, *SiDHN2*, *3*, and *4* on 5 and *SiDHN5*, *6*, and *7* on 8. Of the 6 *DHNs* in *S. bicolor*, *SbDHN1* and *2* are located on chromosome 3, *SbDHN3*, *4* and *5* on 9, and *SbDHN6* on chromosome 10. Out of the seven *DHNs* in *Z. mays*, *ZmDHN1* is tagged on to chromosome 1, *ZmDHN2* and *3* on 4, *ZmDHN4* and *5* on 5, *ZmDHN6* on 8 and *ZmDHN7* on chromosome 9 (Fig. 1 and Table 1). *DHN* gene structures revealed that only 2 of them contain one exon while all other *DHNs* contain 2 to 4 exons. In *S. italica*, 2 exons were identified in *SiDHN1*, *2*, *6* and *7* genes, 3 in *SiDHN4* and 4 in *SiDHN3* and *5*. In the case of *S. bicolor,* one exon was identified in *SbDHN3*; 2 in *SbDHN4* and *6*, 3 in *SbDHN1* and *2*, and 4 exons in *SbDHN5*. In *Z. mays*, only one exon was noticed in *ZmDHN4*; 2 in *ZmDHN1*, *3*, *5*, *6* and *7* genes and 3 exons in *ZmDHN2* (Fig. 2 and Table 1).

3.3. Conserved domains and motif analysis of DHNs

Multiple sequence alignment showed highly conserved domains of K- (lysine), S- (serine) and Y- (tyrosine) rich segments in all the *DHNs* of *S. italica*, *S. bicolor*, and *Z. mays* (Fig. 3A). The motif search by MEME software revealed that the K-rich domain is the most common among all the *DHNs*, while S- and Y-segments varied among these taxa. Motifs 1, 3, and 5 represented the K-segment, motif 2 the S-segment, and motifs 4 and 9 the Y-segment. In all the *DHNs*, the S- and Y-segments are present only once with an exception in *SiDHN4*, *SbDHN1*, and *SbDHN2*. In *Z. mays*, some *DHNs* have been found without Y-segments, while K-segment is repeatedly found 2–3 times similar to that of *Arabidopsis* (Fig. 3B and Supplementary Fig. 1). *DHNs* in these three crops are classified into YnSKn, SKn, KnS, S, and YnS types. The YnSKn group of *DHNs* is common among all the three crops, while in *S. italica* and *S. bicolor*, only the newly identified YnS group is present but absent in *Z. mays*. On the other hand, KnS and S groups appeared only in *Z. mays*. Based on the presence of 3 and 4 motifs, the *SbDHN*5 is grouped into SK-type (Table 1). The YnS sub group of *Sorghum* contained DnaJ domain, whereas it is absent in *Setaria*.

3.4. Analysis of DHN proteins in S. bicolor, S. italica, and Z. mays

*Zm*DHN3 is the smallest confirmed protein with 108 aa while the largest one (*Sb*DHN6) is 388 aa in length. MWs of *DHNs* in *S. italica* ranged between 14,126.34 and 33,741.27 Da and pI values from 4.79 to 10.11, while in *S. bicolor*, they ranged from 15,399.74 to 37,488.09 Da and pI from 5.79 to 9.25. In *Z. mays*, MWs ranged from 12,199.09 to 35,266.67 Da and pI from 5.51 to 9.92. Most of the identified *DHNs* are basic in nature. The *Zm*DHN6 of YnSKn type and all the SKn-type *DHNs* exhibited low isoelectric point, with an exception of SKn-type (*SiDHN*5 and *SbDHN*5) which have high pI compared to YnSkn *DHN*s. The GRAVY values of *S. italica* varied between −1.246 and −0.374, whereas in *S. bicolor DHNs*, they ranged from −1.282 to −0.330 and in *Z. mays DHNs* between −2.158 and −0.306 indicating their hydrophilicity. Both the WoLFPSORT and CELLO software predicted the subcellular localization of *DHNs* in nucleus, mitochondria, chloroplast, and extra cellular matrix, however, these software exhibited varied localization of *SiDHN*4, *SiDHN*5, *SbDHN*2, *ZmDHN*2, and *ZmDHN*6. The protein instability index of the *DHNs*, as explored by Protparam software, indicated that 5 of the 7 (71.4%) *SiDHNs* are stable, whereas 3 out of 6 (50%) *SbDHNs* and 3 out of 7 (42.8%) *ZmDHNs* are stable. The IUPred Server (http://iupred.enzim.hu/) predicted that all the *DHNs* are IDPs with the exception of *Sb*DHN5, which is an ordered or folded protein (Table 1). The NetPhos software predicted that all the *DHNs* contain higher number of PKC than CK1, CK2, and PKA types. The YnSKn-type *DHNs* contained more number of putative PKC sites than protein kinase CK2. In SKn *DHNs*, CK2 sites are more in number than PKC, with an exception of *SiDHN*5 and *SbDHN*5. Besides PKC and CK2 sites, SKn *DHN*s also contained PKA, DNAPK, RSK and CK1 sites, which are absent in YnSKn type. However, *ZmDHN*5 does not contain any PKC but contains more number of CK2 sites (Supplementary Table 2). The Psipred software analysis of secondary structures of all the DHN proteins of *Setaria*, *Sorghum*, and *Zea* exhibited highly disordered regions with less helix or strand motifs, except *SbDHN*5 which contained fewer disordered regions and consisted of high number of strand motifs. The helices are located within K-segments. Generally, YnSKn *DHN*s displayed less number of helices or strands, due to their disordered tendency, but *Si*DHN1 and *Si*DHN3 exhibited the highest amount (80%) of disordered tendency of motifs with high number of helices. Three SKn-type 3 (*SbDHN*3 and *ZmDHN*3, and *ZmDHN*5) proteins showed 90% of motifs with disordered tendency and the highest number of helices.

Table 1
List of identified dehydrins exhibiting chromosomal location, sub group, length, DNA binding domains (DBD), molecular weight (MW), iso-electric point (Pi), GRAVY, no. of exons, localization, instability index and disordered tendency.

ACC number	Common name	Sub-group	No. of amino acids	Chromosome number	DBD	pI/MW	GRAVY	No. of exons	Localization (CELLO)	Localization (PSORT)	Instability index	Disordered tendency
Si02g0669100	SiDHN1	SK3	290	1	156–285	5.76/31644.05	−1.246	2	N	N	57.93[a]	0.85
Si101756646	SiDHN2	YSK2	138	5	1–138	9.19/14126.34	−1.178	2	N	N	27.68	0.88
Si11g0454200	SiDHN3	YSK2	218	5	1–157	9.69/23222.86	−0.684	4	N	N	30.83	0.59
Si01g0702500	SiDHN4	Y2S	307	5	12–153	4.79/31173.12	−0.374	3	EC	C	44.42[a]	0.57
Si101755847	SiDHN5	SK2	150	8	1–150	10.11/15117.94	−0.829	4	NC	C	35.09	0.81
Si10g003700	SiDHN6	YSK3	347	8	13–287	8.99/33741.27	−0.804	2	N	N	11.92	0.76
Si11g0454000	SiDHN7	YSK2	169	8	14–169	8.81/16914.33	−1.050	2	NC	N	14.74	0.82
Sb01g20440	SbDHN1	Y2SK2	277	3	81–277	8.99/29881.14	−0.972	3	N	N	49.39[a]	0.72
Sb03g032255	SbDHN2	Y2S	188	3	12–178	8.37/19842.84	−0.535	3	N	C	52.57[a]	0.54
Sb03g037700	SbDHN3	SK3	283	3	141–279	5.79/31029.23	−1.282	1	N	N	59.66[a]	0.88
Sb09g018420	SbDHN4	YSK2	152	9	2–152	8.81/15399.74	−1.132	2	N	N	23.87	0.85
Sb05g50710	SbDHN5	SK	310	9	127–180	9.25/34491.4	−0.836	4	M	M	20.44	0.37[#]
Sb10g003700	SbDHN6	YSK3	388	10	293–388	8.50/37488.09	−0.330	2	N	N	35.69	0.77
Zm02g98750	ZmDHN1	K2S	326	1	9–264	7.37/31690.09	−0.798	2	N	N	24.17	0.62
Zm09g026210	ZmDHN2	S	325	3	9–266	8.56/31828.43	−0.745	3	N	EC	22.23	0.47
Zm04g032250	ZmDHN3	SK3	108	4	2–108	6.22/12199.09	−2.158	2	N	N	29.71	0.90
Zm01g013000	ZmDHN4	KS	290	5	2–164	6.05/31440.76	−1.250	1	N	N	54.20[a]	0.89
Zm03g037700	ZmDHN5	SK3	289	5	1–165	5.51/31466.47	−1.300	2	N	N	57.06[a]	0.89[b]
Zm01g20440	ZmDHN6	YSK3	143	8	39–123	6.06/15096.02	−0.306	2	EC	N	51.01[a]	0.72
Zm11g0840	ZmDHN7	YSK3	331	9	213–311	9.72/35266.67	−1.112	2	N	N	62.13[a]	0.76

Si:*Setaria italica*; Sb: Sorghum bicolour; Zm:*Zea mays*; N:Nuclear; EC: Extracellular; M: Mitochondrial; C: Chloroplast.

[a] Unstable.

[b] Ordered.

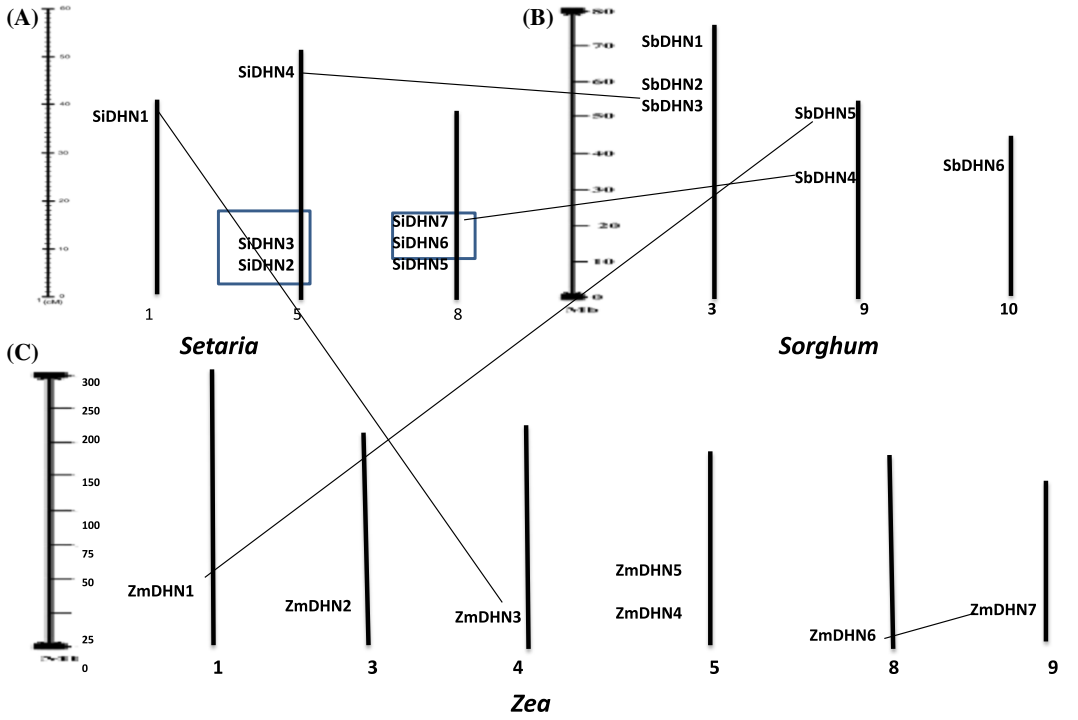

Fig. 1. Locations and duplications of *DHNs* in *Setaria* (A), *Sorghum* (B) and *Zea* (C); scale represents the mega bases. The chromosome numbers are indicated at the bottom of each bar.

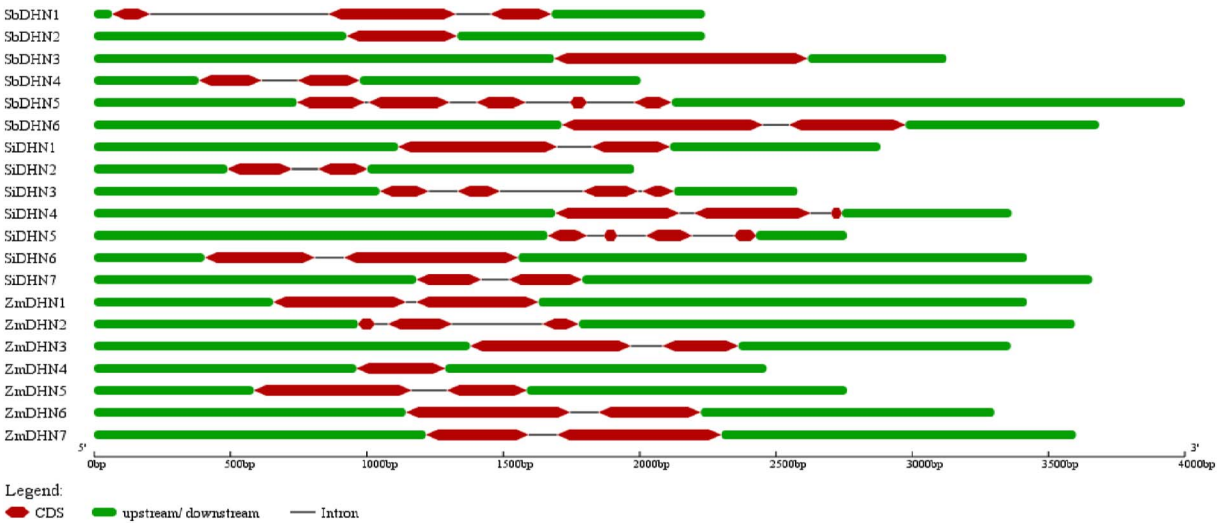

Fig. 2. Distribution of exons, introns, upstream and downstream regions in *DHNs*.

Further, it is observed that a KS-type *ZmDHN*4 and YSK-type *ZmDHN*7 lacked the strand motifs (Fig. 4A). Three-dimensional models of all 20 proteins generated at 80.4–95.2% confidence levels by similarity search software, the BLASTP are shown in Fig. 4B and Supplementary Table 3. Based on the highest homology, their structures have been visualized using Pymol tool (https://www.pymol.org/). The predicted 3-D structures of 20 *DHNs* revealed the presence of conserved DHN domain of nearly 150 amino acids. The β-sheets are absent in *SiDHN*1, 6 and *ZmDHN*1, while *ZmDHN*2, a KS-type *DHN*, lacked α-helices.

3.5. Identification of cis-regulatory elements of DHN promoters

Analysis of *cis*-acting elements revealed the presence of ABRE, DRE, DPBF, MYB and MYC, HSE, salt stress-responsive and LTR elements. *DHNs* are rich in Skn-1 type motifs, with endosperm specific expression elements that play an important role in seed development. They contain KST1 elements, involved in guard cell-specific gene expression, and pollen specific elements associated with pollen and anther development (Table 2). The motif analysis of *DHN* promoters revealed that 1, 3, 11, 14, 23, 25, 27 and 29 motifs have ABRE elements; 12, 13, 14, 21 and 23 have DRE; 2, 5, 6, 7, 9, 13 and 16 have HSE; 8, 25, 26 and 27 have LTRE; 3, 21, 23, 27 and 29 have CGCG (salt-responsive elements); 7, 14 and 24 have TAAG (endosperm-responsive elements); 6 has AGAAA (pollen and anther-responsive elements); 5 and 12 have GTCAT (guard cell-responsive elements); 9, 10, 11, 15, 17, 19, 27 and 30 have MYB; and 1, 6, 14, 21, 28 and 30 have MYC, the water stress-responsive elements (Supplementary Table 4; and Supplementary Figs. 2 and 3).

3.6. Phylogenetic and gene duplication analysis of DHNs

All *DHNs* of *Setaria, Sorghum*, and *Zea* were grouped into YnSKn, YnS, SKn, and KnS-types. While YnSKn has been found to be the largest

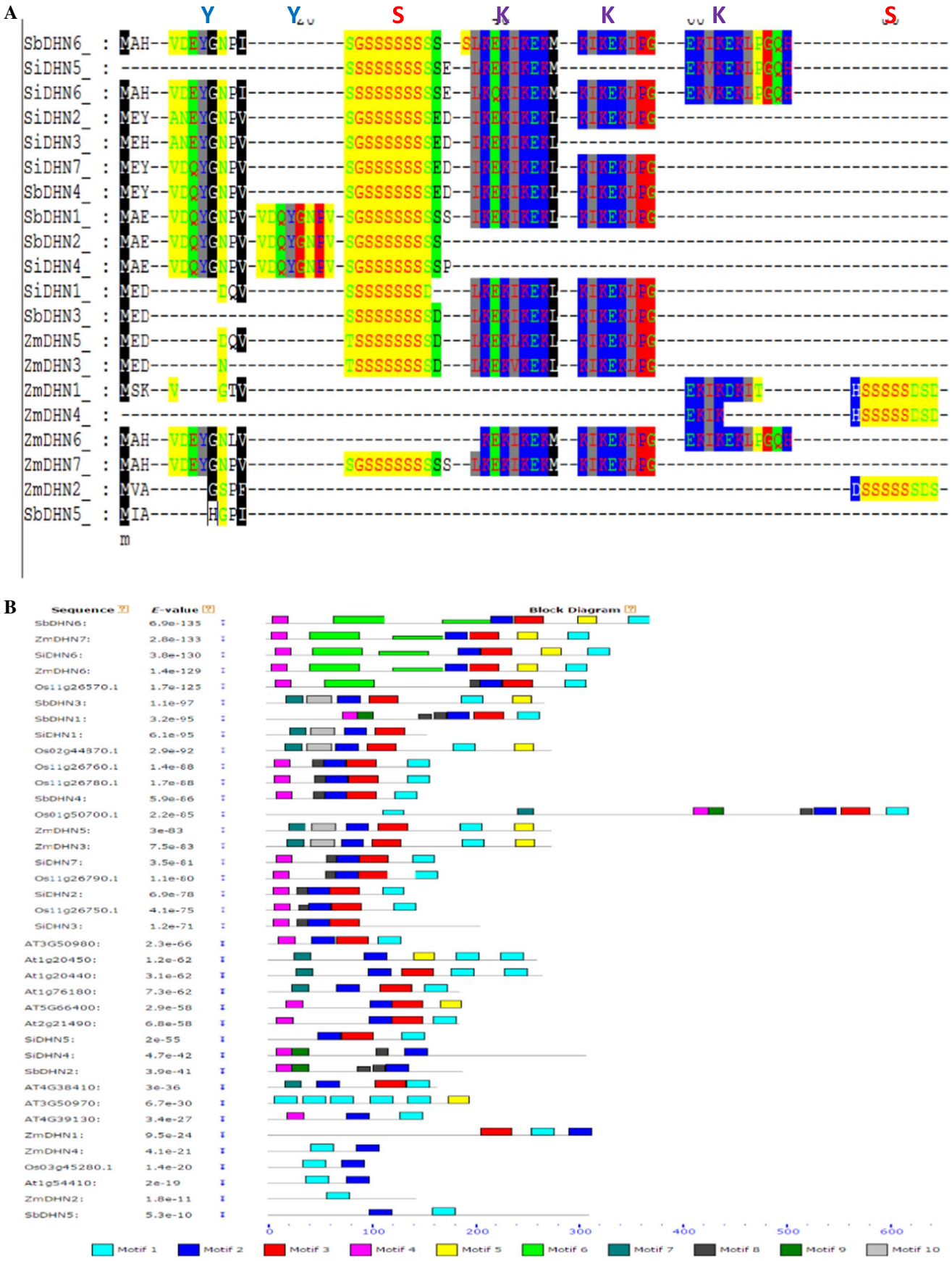

Fig. 3. A. *DHNs* exhibiting multiple sequence alignments and highly conserved Y, S, and K domains in *Setaria, Sorghum* and *Zea.* B. Distribution of 1–10 MEME identified *DHN* conserved motifs in *Setaria*, *Sorghum*, *Zea*, *Oryza*, and *Arabidopsis* are shown in colors. Gene clusters and p values are shown on the left side and motif sizes at the bottom of the figure.

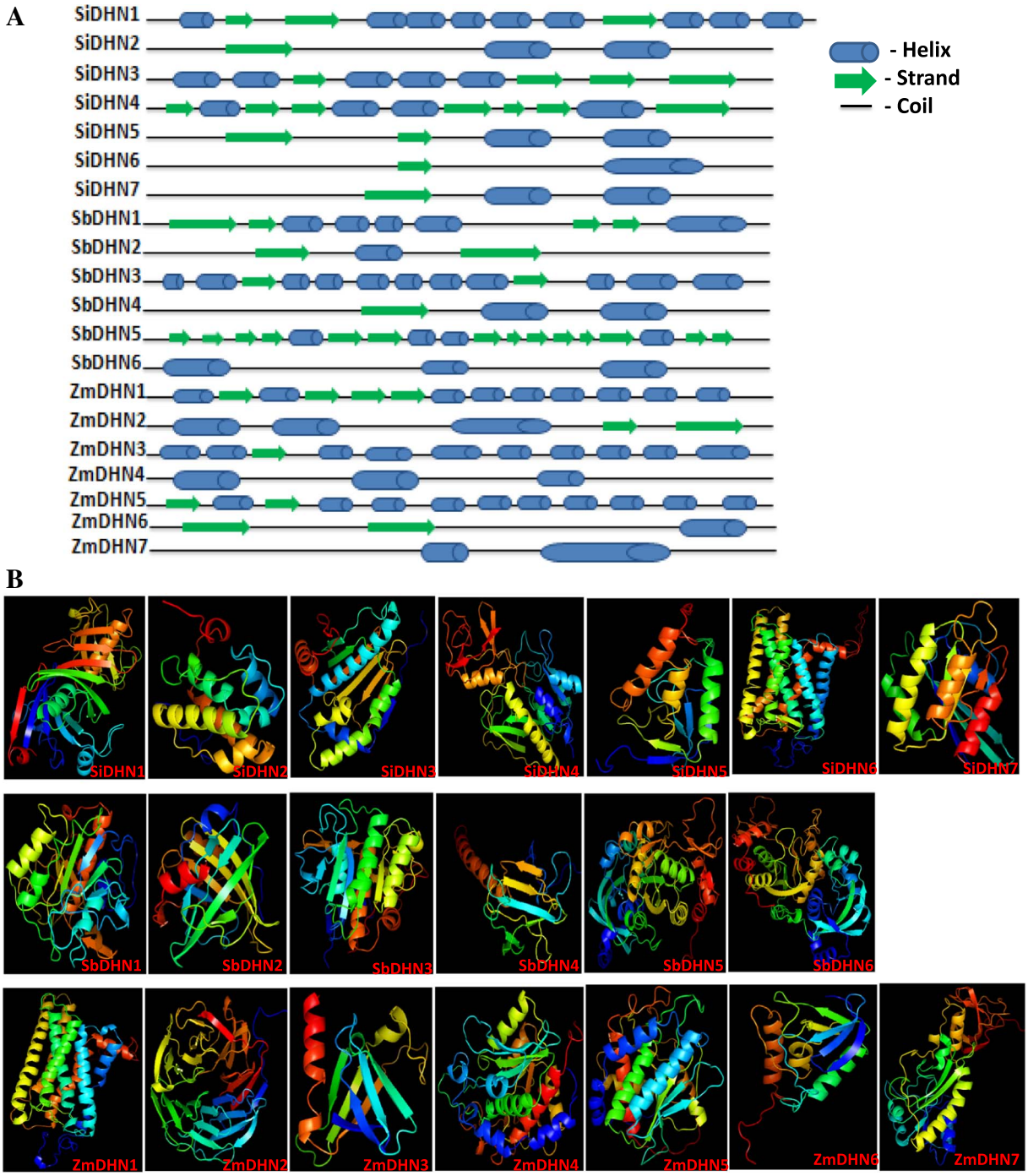

Fig. 4. A. *DHN* protein secondary structures of *Setaria*, *Sorghum* and *Zea*. B. Modelled 3D structures of *DHN* proteins; *Si* = *Setaria italica*, *Sb* = *Sorghum bicolor* and *Zm* = *Zea mays*.

subgroup (4/7 in *Setaria*, 3/6 in *Sorghum*, and 3/7 in *Zea*), followed by SKn subgroup (2/7 in *Setaria*, 2/6 in *Sorghum* and 2/7 in *Zea*), and the smallest has been KnS observed in *Zea* (2/7). But, only one KnS was found each in *Oryza* and *Arabidopsis*. A new subgroup, YnS, an intermediate type between YnSKn and SKn, and an ortholog clustered with YnSKn subgroup, has been noticed only in *Setaria* and *Sorghum* but absent in *Zea*. Two paralogs, the regional duplication events; *SiDHN*2 and *SiDHN3,* and *SiDHN5* and *SiDHN6*, were observed in *Setaria*, which might have resulted due to the gene duplication of their ancestral genes. One paralog *ZmDHN6* and *ZmDHN7* was reported as segmental duplication event in *Zea*, but no such paralogs were noticed in *Sorghum*. Out of the 4 common orthologs of *Setaria, SiDHN1* and *SbDHN3*, *SiDHN4* and *SbDHN2*, and *SiDHN7* and *SbDHN4*, are common to *Sorghum*, while only one *SiDHN1 and ZmDHN3* with *Zea*. Further, only one common ortholog, *SbDHN5* and *ZmDHN1* were found common among *Sorghum* and *Zea* (Figs. 1 and 5).

3.7. In silico prediction of gene specific molecular markers and miRNAs targeting DHNs

In the present study, a total number of 49 SSRs and 2 ILPs were discovered among genomic transcripts of identified *DHNs* (Supplementary Table 5). Tri-nucleotide SSR repeats (25/49) outnumbered the other repeats, while hexanucleotide SSR repeats (8/49) were found less than tri nucleotide SSRs. The dinucleotide and tetranucleotide SSR repeats were found more than pentanucleotide repeats,

Table 2
Conserved *cis*-acting elements in *DHN* promoters of *Setaria italica*, *Sorghum bicolor* and *Zea mays*.

DHNS	Cis elements										
	ABRE (CACGTG)	DRE (ACCGAC)	HSE (AGAAAATTCG)	LTR (CCGAAA)	*CGCGBOX* (*VCGCGB*)	*DPBF* (*ACACNNG*)	*GT1GMSCAM4* (*GAAAAA*)	*KST1* (*TAAAG*)	*MYB* (*WAACCA*/ *YAACKG*/ *CNGTTR*)	Myc (CANTTG)	SKN1(GTCAT)
SiDHN1	5	6	0	7	14	1	0	2	3	8	0
SiDHN2	7	1	2	1	2	2	1	4	24	8	2
SiDHN3	10	1	1	2	2	3	0	0	18	8	1
SiDHN4	14	4	2	3	4	5	6	0	20	6	3
SiDHN5	3	0	0	1	0	3	6	10	15	20	3
SiDHN6	13	5	5	7	10	8	2	12	44	24	2
SiDHN7	13	4	1	5	8	3	2	4	25	10	1
SbDHN1	10	8	0	6	12	4	0	2	3	10	1
SbDHN2	6	4	0	7	10	3	2	1	4	4	3
SbDHN3	17	1	0	5	34	1	2	3	14	21	0
SbDHN4	5	7	0	3	6	3	0	1	5	4	0
SbDHN5	9	5	0	8	32	4	3	1	12	15	0
SbDHN6	12	7	0	6	2	3	5	3	27	10	3
ZmDHN1	6	1	0	4	0	5	5	10	38	50	0
ZmDHN2	11	0	0	1	10	2	0	0	20	2	4
ZmDHN3	4	4	0	9	18	1	1	1	8	14	1
ZmDHN4	3	0	1	3	2	2	3	7	22	18	2
ZmDHN5	5	8	1	10	12	2	1	3	15	12	3
ZmDHN6	14	3	0	3	4	4	2	3	16	20	2
ZmDHN7	11	3	0	3	4	2	2	3	18	20	2

ABRECTAL: Response to ABA, CGCGBOX: Multiple signal transduction, DPBF: ABA, DRE: Dehydration responsive elements, GT1GMSAM4: Salt and pathogenesis related, LTRE: Low temperature and cold responsive, MYB: Response to drought and ABA, MYC: Response to drought, cold and ABA, POLLEN: Pollen and anther development, TKST1: Guard cell-specific gene expression.

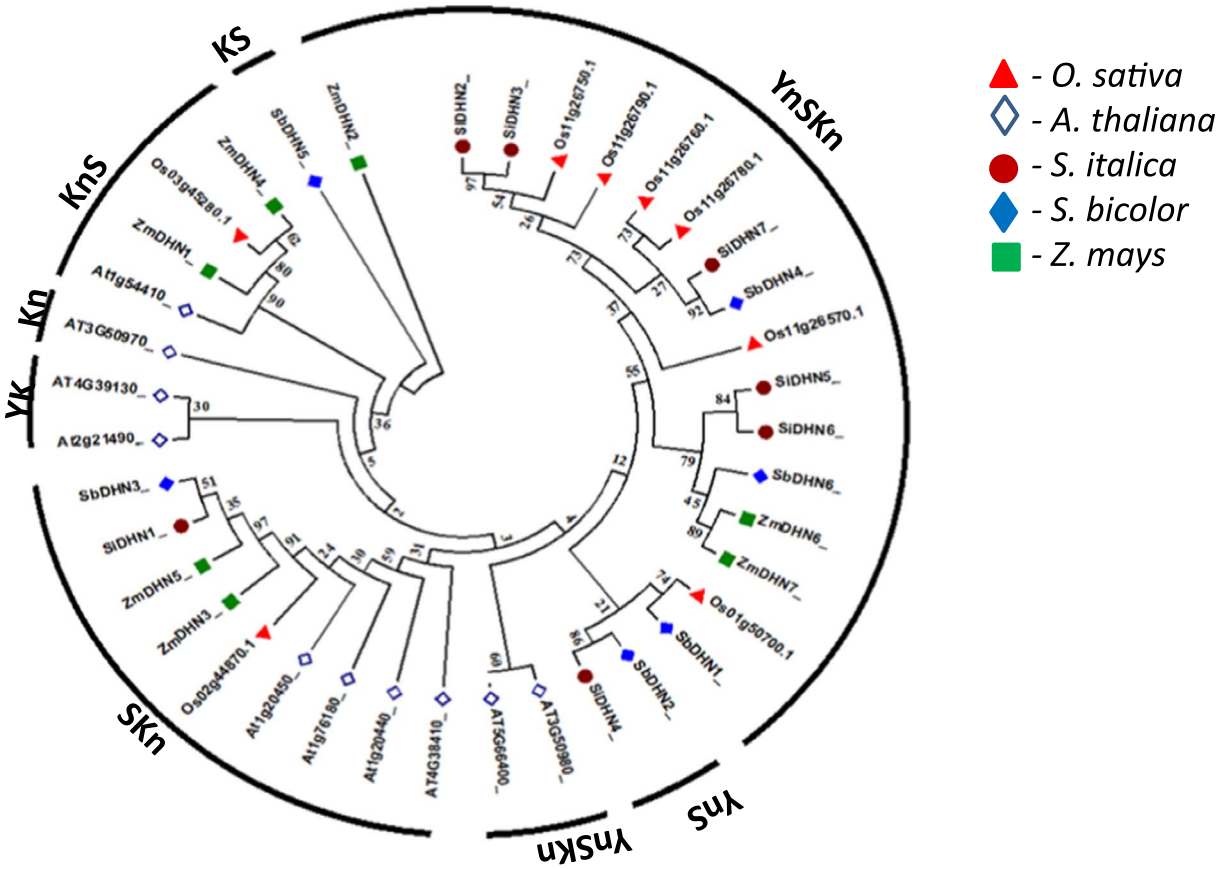

Fig. 5. Neighbor joining phylogenetic tree of YnSKn, YnS, SKn, KnS, YK and Kn *DHN* proteins of *O. sativa*, *A. thaliana*, *S. italica*, *S. bicolor* and *Z. mays*; *Os* = *Oryza sativa*, *At* = *Arabidopsis thaliana*, *Si* = *Setaria italica*, *Sb* = *Sorghum bicolor* and *Zm* = *Zea mays*.

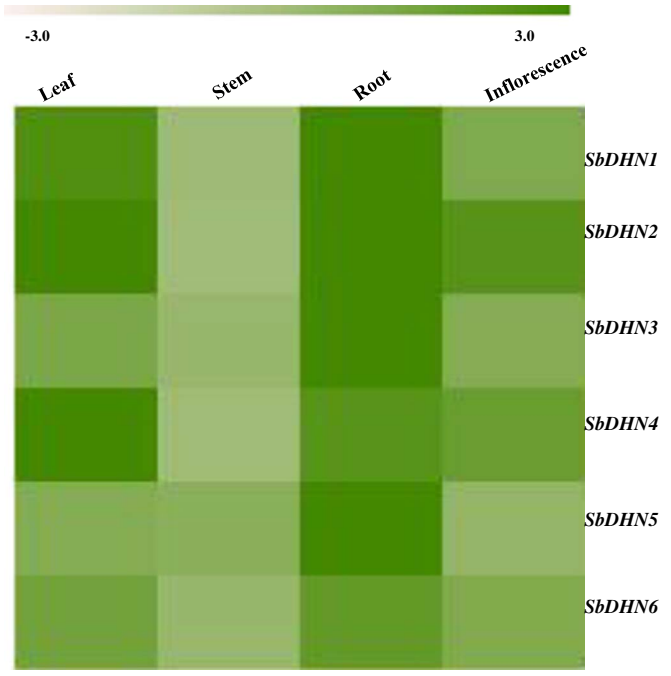

Fig. 6. Transcriptional profiling of *SbDHNs* in leaf, stem, root and inflorescence tissues of *Sorghum bicolor*.

but less than hexanucleotide repeats (Supplementary Fig. 4). Two ILP markers were also mined in *SbDHN2* gene. Besides, putative microRNAs (miRNAs) targeting the *DHNs* genes were identified using psRNATarget server. The analysis revealed 14 *DHN* (*ZmDHN1, ZmDHN3, ZmDHN4, ZmDHN6, SbDHN3, SbDHN5, SbDHN6, SbDHN1, SbDHN4, SiDHN2, SiDHN4, SiDHN6, SiDHN7,* and *SiDHN3*) genes are targeted by 51 miRNAs which belong to diverse classes of miRNA families responsive to various abiotic stresses (Supplementary Table 6).

3.8. Classification, signature amino acid analysis and evolutionary relationship of DHNs

A total of 451 *DHNs* belonging to 17 families and 53 crops were identified and classified into YnSKn, SKn, KnS, Kn, S, YnKn, and YnS based on their conserved characteristic domains. Out of them, 223 were divided into YnSKn, 123 SKn, 23 KnS, 47 Kn, 4 YnS, 23 YnKn, and 8 S sub-types. The YnSKn is the most common sub-group in all the families, while SKn members appeared less in number in monocots when compared to dicots. Both KnS and Kn appeared only in fewer species (Supplementary Fig. 5 and Supplementary Table 7), suggesting that they are evolved in particular genome or they might have lost during the course of evolution. Further evaluation of segments revealed several truncated segments especially with K segment. Out of 451 *DHNs*, it has been observed that they are absent in 24 K segments (Supplementary Table 8 and Supplementary Figs. 6 & 7). The amino acid composition analyses illustrated that *DHNs* are rich with glutamic acid, glycine, histidine, alanine, arginine, aspartic acid, leucine, proline, threonine, and valine along with lysine and serine. But, cysteine and tryptophan are completely absent. *DHNs* exhibited variations in glutamic acid and glycine percentages, and if glutamic acid residues are more, glycine residues are less and *vice versa*. Interestingly, all the *DHNs* exhibited the highest percentage of glycine, except SKn sub-type, which contained more amount of glutamic acid. The KnS and Kn sub-groups are rich with histidine. Further, proline levels also exhibited variations alongside glutamic acid (Supplementary Table 9).

3.9. Transcriptional profiling of SbDHNs

All the 6 identified and confirmed *DHN* genes in *S. bicolor* exhibited better expression in roots in comparison with leaves, inflorescences, and stems. Though *SbDHN2* and *SbDHN4* were constitutively expressed in all the four tissues, their expression levels were high in roots. The

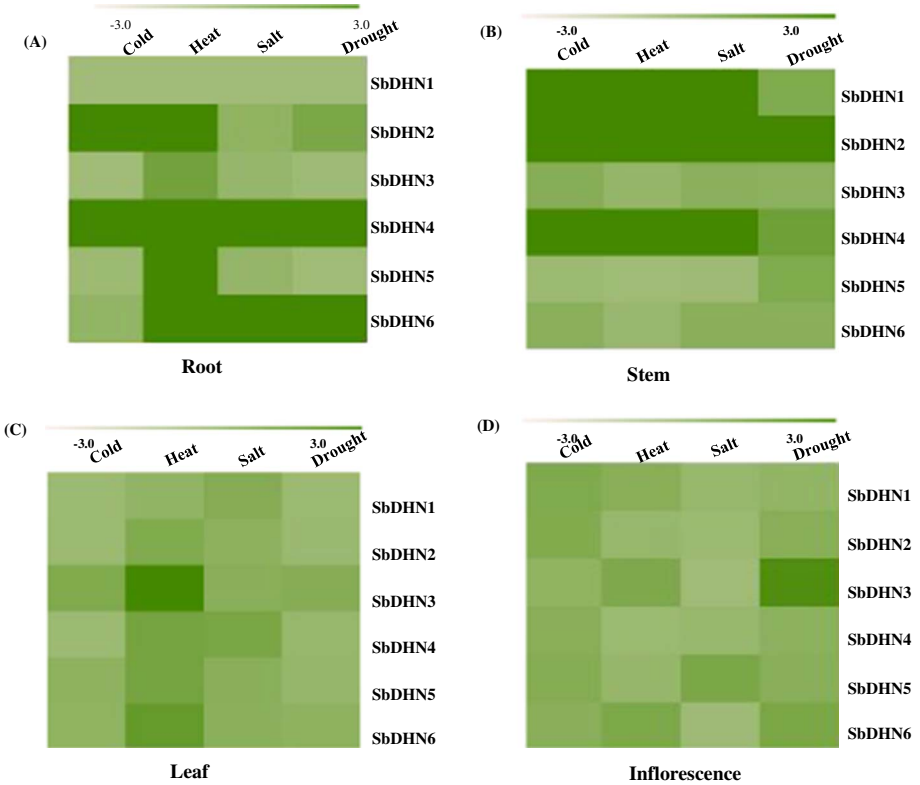

Fig. 7. Relative expression patterns of *SbDHNs* in different tissues under cold, heat, salt and drought stresses in *Sorghum bicolor*, (a) root, (b) stem, (c) leaf and (d) inflorescence.

other 4 *DHNs* exhibited upregulation in leaves and inflorescences but down-regulation in stems (Fig. 6 & Supplementary Table 10). Under drought, salt, heat, and cold stresses, *Sorghum DHNs* displayed differential expressions in roots, stems, leaves, and inflorescences. Among the 6 *DHN* genes, *SbDHN2* exhibited the highest expression levels under stress in roots, stems, leaves, and inflorescences followed by *SbDHN4* and *SbDHN6*. While *SbDHN1* and *3* did not exhibit upregulation in any one of the stress conditions, expression of *SbDHN2* in roots was 114.9-folds higher under high temperature compared to cold, salt, and drought. *SbDHN4* recorded upregulation (10.3-folds) in roots treated with high temperature stress followed by drought (7.6-folds). Fold-wise increase in the expression of *SbDHN5* was 3.5 under elevated temperature. In contrast, *SbDHN6* showed 18.8 and 5.5-folds higher expression levels in roots under salt and high temperature stresses respectively (Fig. 7A & Supplementary Table 10). In leaf, *SbDHN1*, *2, 4, 5*, and *6* did not exhibit any upregulation during stress. Expression level of *SbDHN3* was 3.2-folds better under high temperature stress in leaf (Fig. 7B & Supplementary Table 10). Contrarily, *SbDHN1* expression was 7.2, 3.8, and 3.8-folds higher under cold, high temperature, and drought stresses respectively in stem tissues. *SbDHN2* recorded 160.4-folds increase in its expression levels under cold followed by drought (136.4-folds), high temperature (123.6-folds), and salt stresses (50.8-folds). Thus, upregulation of *SbDHN2* appeared high in the stem compared to other *DHNs*. In contrast, *SbDHN3*, *5* and *6* did not exhibit higher levels of expression under stress conditions (Fig. 7C & Supplementary Table 10). In inflorescence, barring *SbDHN3*, other *DHNs* were not much upregulated. Only *SbDHN3* recorded 2.6-folds higher expression under salt stress (Fig. 7D & Supplementary Table 10).

3.10. Protein – protein interaction of SbYnS DHNs

To explore the functions of novel SbYnS sub-group *DHN*s, protein – protein interaction network map was constructed (Supplementary Fig. 8). The map showed interaction among the proteins Hsp 70, FKBP-type peptidyl-prolyl *cis*-trans isomerise, tankyrase 2, ser/thr protein phosphatase 6, ankyrin repeat, SOCS box protein 3 and ankyrin protein 3 that contained tetratricopeptide and ankyrin repeats with YnS type *DHN*s. Their functions are retrieved based on protein – protein interaction network, and it appeared that they participate in endocytosis, spliceosome and protein processing in the endoplasmic reticulum.

4. Discussion

4.1. Characterization of DHNs

Comparison between *Setaria, Sorghum*, and *Zea DHNs* revealed variation in the number and patterns of exons and introns. Presence of more than one intron in all the 3 cereals have been observed with an exception of *SbDHN3* and *ZmDHN4* which are devoid of any introns, similar to their common ancestor, the *Oryza* (Wang et al., 2007). The number of exons and introns in a gene revealed the divergent relationship between the gene families as pointed out by Cao (2012). More number of introns may be causing the delay of transcription by extending the length of nascent transcript and thus burdening the gene expression (Jeffares et al., 2008). All the *DHNs* exhibited distinct differences between pI and kinases. The positively charged YnSKn-type *DHNs*, with higher pI are bound to the cell membranes during stress, thereby protect the cells and thus confer stress tolerance (Yang et al., 2012). The present investigation revealed that YnSKn type *DHNs* are phosphorylated by PKCs, while SKn *DHNs* by CK2s, and both the types may be promoting the activity of *DHNs* for conferring tolerance against stress. *DHNs* are highly hydrophilic and unstructured, and due to this nature, they escape from stress and protect other proteins too (Hincha and Thalhammer, 2012). However, the *SbDHN5* has been found as an ordered protein with higher pI and phosphorylation sites.

The YnSKn *DHNs* are common in all the three crops (*Setaria* 4, *Sorghum* 3 and *Zea* 3), and are triggered in response to severe drought, salt, frost, ABA, methyl jasmonate, salicylic acid, and high temperature (Close, 1997; Rahman et al., 2010; Halder et al., 2017), compared to one SKn-type *DHN* in *Setaria*, 2 each in *Sorghum* and *Zea*. Two KnS-type chilling stress-responsive *DHNs* are present in *Zea*, but absent in *Setaria* and *Sorghum*. Expression of KnS-type *DHN* was reported in reproductive tissues in response to chilling stress (Wang et al., 2014). In the present investigation, a new group, called YnS-type *DHNs*, endowed with more number of phosphorylation sites and abiotic stress regulatory *cis*-acting elements, 1 each in *Setaria* and *Sorghum*, have been identified for the first time. Kn-type *DHNs* identified in *Arabidopsis* have not been observed in all the 3 crops (Close, 1996). It appears that LEA genes are highly conserved (Liang et al., 2016) among plants though gene losses or gains were noticed. Two regional duplication events were noticed in *Setaria*, while one segmental duplication event in *Zea*, mays. But, no duplication event was noticed in *Sorghum*, indicating less number of *DHNs* in *S. bicolor* compared to *Setaria* and *Zea*. Perhaps, it is lost in the evolution. The distributions of *DHNs* in monocots are crowded on only few specific chromosomes, with an exception of *Zea*, compared to dicots.

4.2. Phylogenetic analysis of DHNs

Analysis of phylogenetic tree revealed presence of 4 divergent subgroups of *DHNs* in *Setaria*, *Sorghum*, and *Zea* and on comparison exhibited similarity with *Oryza* (monocot), but wide variation was noticed with that of *Arabidopsis* (dicot), indicating that the *DHNs* are derived from their common ancestor *Oryza*. Expansion of *DHN* family generally occurs through tandem and genome duplication events. In *Arabidopsis*, 3 tandem duplications and 3 whole genome duplication events resulted into 6 *DHNs*, thus increasing the original 4 *DHNs* to a total of 10 *DHNs* (Hundertmark and Hincha, 2008). Similarly, 3 tandem duplication events in *Oryza* resulted into 3 *DHNs* and thus enhanced the original 5 *DHNs* to a total of 8 *DHNs* (Wang et al., 2007). It appeared that the whole genome duplication event must have occurred at least once in poplar, *Oryza*, and *Arabidopsis*, while such an event is unlikely in *Setaria*, *Sorghum*, and *Zea* (Jaillon et al., 2007), but resulted into a varied number of *DHNs* in these crops. The YnSKn *DHNs* are expressed during drought and salt stress, while SKn, KnS and Kn mostly during cold stress though some of them appeared to be associated with desiccation and salt stresses (Liang et al., 2012; Wang et al., 2014). This indicated that YnSKn *DHNs* are relatively associated with desiccation and salt stress, whereas SKn, KnS, and Kn seemed to be associated with plants like *Triticum* and members of Rosaceae family that grow in low temperatures.

4.3. Promoter analysis of DHNs

In the present study, endosperm specific *cis*-elements SKn-1 were noticed closer to translation start sites in majority of the *DHN* promoters and are upregulated during the late embryogenesis stage (Washida et al., 1999). Baker et al. (1994) demonstrated that *DHN* promoters rich in low temperature-responsive elements confer tolerance against cold, drought, and ABA-induced stresses. Heat shock elements (HSE) have been noticed in *Setaria* and *Zea DHNs*, but surprisingly not in *Sorghum*. The guard cell specific and stomatal conductivity regulating KST1 *cis*-elements noticed in *DHN* promoters may participate in K^+ influx and guard cell movement during stress (Plesch et al., 2001). This study also revealed the presence of AGAAA-rich POLLEN1LELAT52 *cis*-elements inferring the involvement of *DHNs* in anther and pollen development (Flicchkin et al., 2004).

4.4. In silico analysis of gene specific molecular markers and miRNAs

S. bicolor is sensitive to cold, drought and salt stresses. Several molecular markers (simple sequence repeats) have been identified in *S.*

bicolor for cold (Burow et al., 2011) and drought (Zhu et al., 2017). Therefore, the observed gene specific markers in *DHNs* might aid further in the development of drought, salinity, and cold stress tolerant sorghum cultivars using genotyping and marker-assisted selection approaches.

Most of the stress-responsive miRNAs target transcription factors. For example, miR164 targeted the NAC mRNAs in *Arabidopsis thaliana* and rice and altered the plant developmental and abiotic stress responses (Fang et al., 2014). In *Arabidopsis*, miR156-mediated down-regulation of SPL enhanced the abiotic stress tolerance, and heat stress memory (Stief et al., 2014). Since several miRNAs were shown to be upregulated under multiple abiotic stresses (Sunkar and Zhu, 2004; Zhou et al., 2010), Our *in silico* analysis points out that miRNAs target *DHNs* and therefore, they may be validated further for understanding the mechanism of DHN activities and for improving abiotic stress tolerance in *S. bicolor*.

4.5. Network analysis of SbYnS–type DHN

It has been observed that *SbYnS DHN* contained a Dnaj domain. DnaJs are co-chaperones which assist Hsp70 and bring about temperature stress tolerance. Work by Mulaudzi-Masuku et al. (2015) demonstrated that transfer of plant Hsp70 gene brought about thermal tolerance in *E. coli*. The Hsp 70 proteins along with DnaJ prevented aggregation of proteins, participated in protein translocation and mediated assembly or dis-assembly of multimeric proteins and targeted proteosomes for degradation (Hartl, 1996). Tetratrico peptide repeat (TPR) motifs are protein-protein interaction modules that are associated with the regulation of diverse cellular functions. Schapire et al. (2006) have identified TITAN LIKE protein (TTL1) containing TPR motifs. Such motifs have been found to be required for abscisic acid responses and osmotic stress tolerance. Therefore, proteins containing TPRs have emerged as essential determinants for signal transduction mediated by stress-related hormone. Association of *DHNs* with TPR protein indicates that this interacting partner is helping in signal transduction during stress. The SbYnS DHN along with other proteins in the network might maintain the membrane integrity, protect proteins from denaturation, and scavenge ROS under diverse abiotic stress conditions.

4.6. Transcript profiling of DHNs in different tissues under abiotic stress

High expression of *DHNs* was observed at the late embryogenesis stages compared to vegetative tissues and very limited expression at the seedling stages in *Arabidopsis* (Rorat et al., 2004). But upon exposure of plants to stress, higher amounts of *DHN* expressions were noticed in the vegetative tissues (Bray, 1994). Similar to *OsDHN3* in rice, *SbDHN*2 in *Sorghum* exhibited the highest expression levels under stress in roots, stems, leaves, and inflorescences (Verma et al., 2017). Higher expression levels of *SbDHN2*, *4*, and *6* observed in root, stem, leaf, and inflorescence indicated that they play an important role during vegetative as well as reproductive stages by their participation in plant development, pollen germination and seed filling; similar to that of higher expression levels recorded in *Arabidopsis* LEA gene (At5g27980) (Wang et al., 2008). *SbDHN2* and *4* were highly induced under all abiotic stresses in roots and stems in comparison with other *DHNs*. Massarelli et al. (2006) used a functional screening method based on random overexpression of a plant cDNA library in *E. coli* to identify plant genes related to salt tolerance. They found that DHN2 gene is induced by NaCl. This suggests that DHN2 protein is associated with salt stress and is conserved across prokaryotes as well as plants. Expression of three *DHN* genes was noticed in sugarcane under heat stress, but the expression was independent of changes in water relations in leaves (Wahid and Close, 2007). Similarly, grapevine *DHN2* was induced by both heat and cold stress with different expression profiles (Yang et al., 2012). Xu et al. (2008) found that expression of brassica *BjDHN2* and *BjDHN3* resulted in higher tolerance to Cd^{2+} and Zn^{2+} metals by attenuating lipid peroxidation and protecting cellular membranes. Thus, *DHN2* gene stands apart by associating with multiple stresses like salt, heat, cold and metal unlike that of other *DHN* genes. Significantly high expression of *SbDHN3* was observed in the inflorescence under salt and heat stresses, similar to the expression of grapevine *DHN1* during late embryogenesis under drought, cold and heat (Yang et al., 2012). High activity levels in different tissues under varied abiotic stress conditions inferred the involvement of *DHNs* during developmental processes also.

5. Conclusion

In the present study, a novel Y2S subgroup was identified in *Sorghum bicolor*. *SbDHN2* gene, belonging to Y2S subgroup, upregulated especially in stems under different abiotic stress conditions indicated its potential role in stress. *DHNs* expressed abundantly in roots, leaves, and stems, particularly *SbDHN2*, *4*, and *6* under cold, high temperature, salt, and drought stress conditions. The present investigation laid a foundation for further functional validation of *DHNs* and the development of cereals for abiotic stress tolerance.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2018.01.004.

Conflict of interest

The authors declare that no conflict of interest exists.

Author contribution

PBK and DMR conceived and designed the experiments. MN, PSR, SAK, AK, and AA performed the experiments. MN, PBK, SAK, PS, RKS and DMR prepared the manuscript. All authors have read and approved the manuscript.

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