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Genome-Wide Characterization and Expression Analysis of Annexin Genes under High-Temperature Stress in *Sorghum bicolor* L.

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ABSTRACT

The annexin family is found in animals and plants and responds to a wide range of environmental stressors. In this study, 19 annexin proteins were identified based on their putative annexin repeats in *Sorghum bicolor* and further grouped on a phylogenic tree. These genes were randomly distributed across seven chromosomes. The amino acid length of sorghum annexins (SbAnn) varied from 241 (SbAnn3) to 370 (SbAnn2). Additionally, selective pressure analysis suggests the role of purification selection in conserving the annexin gene family in sorghum. Most SbAnns were located in the nucleus, cytoplasm, chloroplast, and mitochondrion. Also, higher syntenic relationships were observed between *S. bicolor* and monocots (*S. italica, Z. mays,* and *O. sativa)* than in *A. thaliana*. Cis-acting elements in the *SbAnns* gene promoters were grouped into response to light, hormone and environmental stress, and development-related elements. A number of miRNAs related to abiotic stress response targeted eleven *SbAnns*. Furthermore, five (*SbAnn 4, 5, 14, 15,* and *18*) of the seven *SbAnn* genes utilized had relatively higher fold change under high-temperature treatment. This research provides insights into the characterization and functions of annexins under high temperatures.

Keywords: sorghum, annexins, gene family, bioinformatics, high-temperature stress.

INTRODUCTION

The Sorghum (*Sorghum bicolor* L. Moench), which has different cultivars such as grain sorghum as a food crop, sweet sorghum as a source of sugar and bioethanol, and forage sorghum as a feed source, is a C4 crop and ranks fifth in terms of production in the world. It can flourish in semi-arid tropical and subtropical areas with low rainfall and high-temperature patterns (Barcelos et al., 2011; Elkhalifa et al., 2005). However, environmental stress factors such as drought, salinity, and high temperatures, which are increasing globally, negatively impact sorghum production and other crops. Plants exposed to abiotic stress, also increase in reactive oxygen species, leading to membrane lipids, proteins, and nucleic acids damage and dysfunctional metabolism. Plants however possess a diverse range of molecular activities that assist them in adjusting to their surroundings (Nagaraju et al., 2020). Identifying key genes responsible for stress tolerance and adaptation and determining their functions is one of the important approaches in stress studies.

Several studies have proved that plant annexin (*Ann*) genes exhibit dynamic expression to increase stress tolerance as well as plant growth and development (Mortimer et al., 2008). In *Arabidopsis thaliana*, the annexin gene (*AtAnn1)* functions in cold tolerance, wounding caused by herbivores, and heat stress mediation (Wang et al., 2015; Liu et al., 2021; Malabarba et al., 2021). Also, the *AtAnn8* gene is involved in negatively regulating cell death and boosting disease resistance (Zhao et al., 2021). *GhAnn1* in transgenic cotton played a role in fiber elongation and conferred tolerance to drought and salt stresses (Tang et al., 2014). In *Oryza sativa*, *OsAnn1* enhances tolerance to heat stress (Qiao et al., 2015) and *OsAnn3* improves abscisic acid stress tolerance and cold tolerance (Shen et al., 2017; Li et al., 2019). In *Raphanus sativus, RsANN1α* is involved in heat stress tolerance (Shen et al., 2021). Additionally, the *StAnn1* gene in *Solanum tuberosum* also promotes drought tolerance and mitigates light stress (Szalonek et al., 2015). The expression of *SpANN2* in *Solanum pennellii* improved the tolerance to

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drought and salt stress (Ijaz et al., 2017). Alfalfa over-expressing *MsAnn2* are tolerant to drought and ABA (Kovács et al., 1998). *TdAnn12* isolated from *Triticum durum* was up-regulated under salt and osmotic stress in transgenic tobacco and expressed differently in various tissues (Saad et al., 2019).

Annexins have four annexin repetitions in the C-terminal region, each around 75 amino acids long, separated by variable length sequences. The first and fourth repeats of plant annexins usually contain a 'type II' motif (with the endonexin sequence K-G-X-G-T-{38 variable residues}-D/E) that can bind calcium (Ca^{2+}) ions (Moss and Morgan, 2004). Additionally, repeat III and IV contain an actin-binding site and a GTP binding site (DXXG), respectively. The N-terminal region of plant annexins is highly variable and short (approximately 10 amino acids). In the evolutionarily conserved multigene annexin family, the N-terminal region shows the greatest variation in sequence and length among family members. A few post-translational modification sites may also act as regulators of Ca^{2+}-dependent signaling in plant annexins (Konopka-Postupolska et al., 2011).

In this study, we identified and characterized the annexin gene family members in *Sorghum bicolor*. Analyses including phylogenetics, conserved motifs, gene structure, synteny, protein-protein, and miRNA interactions were carried out. The chromosomal positions, physiochemical properties, and putative cis-elements were also predicted. RT-qPCR was also used to understand the response of some *S. bicolor Anns* during high-temperature treatment.

MATERIAL AND METHODS

Bioinformatics analyses of annexin in sorghum

The Arabidopsis genome database (https://www.arabidopsis.org/) was used to retrieve the 8 identified Annexin proteins (AtAnn) and gene sequences. Initially, the AtAnn protein sequences were used in NCBI protein BlastP search against the *S. bicolor* genome (https://www.ncbi.nlm.nih.gov/). The blast results were examined, and redundant sequences were removed. Phytozome 13 database was used to retrieve sequences of the corresponding phytozome identifiers. The putative annexins in sorghum were identified using the annexin domain (PF00191.23) via HMMR online software v3.3.2 and SMART (https://www.ebi.ac.uk/Tools/hmmer/, and http://smart.embl-heidelberg.de/).

To predict the physiochemical properties of sorghum annexins, the Expasy online software (https://web.expasy.org/protparam/) (Gasteiger et al., 2005) was used. Post-translational modification was determined by (https://prosite.expasy.org/scanprosite/) (de Castro et al., 2006). Also, in analyzing conserved motifs, MEME v5.5.2 (https://meme-suite.org/meme/tools/meme) (Bailey et al., 2009) was utilized and set to find 10 motifs. TBtools software v1.120 was used to draw Heatmaps (Chen et al., 2020).

Amino acid sequences of annexins in *A. thaliana*, *S. bicolor*, *Triticum aestivum*, and *Hordeum vulgare* were aligned via the ClustalW and used to construct the Neighbor Joining (NJ) phylogenetic tree with method with 1000 bootstrap replicates using MEGA 11 software (Felsenstein, 1985; Saitou and Nei, 1987; Tamura et al., 2021). The gene structures were carried out on the Gene structure display server v2.0 (http://gsds.gao-lab.org/).

The locations of the sorghum annexin genes were retrieved from NCBI. TBtools was used to draw the positions of the annexin genes on the sorghum chromosome. Utilizing genomic data retrieved from *Setaria italica*, *Zea mays*, *Oryza sativa*, and *A. thaliana*, syntenic relationships were studied. Ka/Ks calculations were also accomplished on the TBtools software v1.120 (Chen et al., 2020).

The 1000-bp upstream of gene sequences were manually retrieved from the Phytozome 13 database https://phytozome-next.jgi.doe.gov/) and queried on the PLANTCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002), to find putative cis-acting elements. The cis-acting elements were grouped into four main groups, and HeatMaps were drawn

using TBtools software (Chengjie Chen, Guangdong, China).

Employing the STRING database (https://string-db.org/) (Szklarczyk et al., 2019), the interaction between annexins and other proteins in sorghum was predicted. The disconnected nodes were hidden, and the color of the links indicates interaction evidence between proteins. Also, psRNATarget (https://www.zhaolab.org/psRNATarget/analysis?function=2) (Dai et al., 2018) was utilized to decipher possible miRNAs that target annexin genes in sorghum.

Planting material and heat stress treatment

Sorghum cv. Theis seeds were planted in pots at room temperature and watered to inhibit water stress. The two-week-old plants were grown in 30ºC, 65% moisture, 370 μmol m^{-2} s^{-1} of light, and a day/night photoperiod of 16/8 h in a Nukleon manufacturer growth chamber. Every 30 minutes, the temperature was raised by 2ºC, bringing it from 30ºC to 42ºC. After four hours of maintaining the temperature at 42ºC, the leaves of 3 plants grown in the same pot were taken together and placed in liquid nitrogen and further moved to a -80ºC refrigerator. Samples from the control plant were collected at room temperature.

RNA isolation, cDNA, and RT-qPCR gene expression analysis

Using the EZ-10 Spin Column Plant RNA Mini-Preps Kit from (Bio Basic Inc.), RNA was extracted from the leaves. cDNA was produced from RNA using the OneScript Plus cDNA Synthesis Kit from (Applied Biological Materials Inc). The 2X qPCR SYBR-Green MasterMix (A.B.T.™) was used for the qPCR analysis. PP2A (Serine/Threonine-Protein, XM-002453490), served as the internal reference gene (Sudhakar Reddy et al., 2016). Real-time qPCR was performed using the Applied Biosystems 7500 Real-time PCR system (USA) and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for gene expression analysis. qPCR conditions include a holding period of 50ºC for 2 min, 40 cycles of 95ºC for 5 m, 95ºC for 15 s, 48ºC for 30 s, and 72ºC for 10 s, and a melting curve of 95ºC for 15 s, 60ºC for 60 s, and 95ºC for 30 s. Gene-specific primers were designed on (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences for *SbAnn*s were as follows:

F: TCACCAACCCACCCCTTCTA, R: TACGGCTCGGTCGATGTTAG for *SbAnn4*;

F: TTACAAGGGTCGTGGTGACG, R: GGAACGCCCGATAGTTTCCT for *SbAnn5*;

F: ACGACACCAAGAACAGTGGT, R: TGGGATGCTCCTCTTGCTGA for *SbAnn7*;

F: TGGCCCATTCTGAAGCCAAA, R: TGCTCCGTGTGGTGAGAATC for *SbAnn12*;

F: TCAACCACGTCAGGGAACAC, R: CCAGCAACACATAGCACACG for *SbAnn14*;

F: TCCCTTCCCGTGTGTTTTTG, R: ATGTTACAGTCCCGCTCGAT for *SbAnn15*;

F: GCATTTGGCCACCGAATCAA, R: CCTGGCGACCTTCTCAAAGT for *SbAnn18*.

The experiment was conducted with three biological replicates.

RESULTS AND DISCUSSION

A total of 19 annexins were identified in sorghum and named based on the chromosome positioning as SbAnn1 to SbAnn19. While most SbAnns contained three or four of the annexin domain repeats (PF00191), SbAnn4 contained only the domain. In understanding the relationship between annexins in monocots and dicots, a phylogenetic tree was constructed using 11 *H. vulgare*, 25 *T. aestivum*, 8 *A. thaliana*, and 19 *S. bicolor* annexin proteins (Figure 1). The phylogenetic tree containing 63 annexins were clustered into 4 main groups. Group I was further sub-grouped into Ia and Ib and contained 11 SbAnns. In Group Ia,

Arabidopsis ANNAT1, ANNAT2, ANNAT6, and ANNAT7 annexins formed a species-specific subgroup, which was consistent with the results of (Cantero et al., 2006). Group II contained 3 SbAnns and also ANNAT4 as its outgroup. Two and three SbAnns were found in groups III and IV, respectively. The fact that Arabidopsis annexins are separated from annexins of other species as outgroups and monocotyledons are also closely clustered in groups.

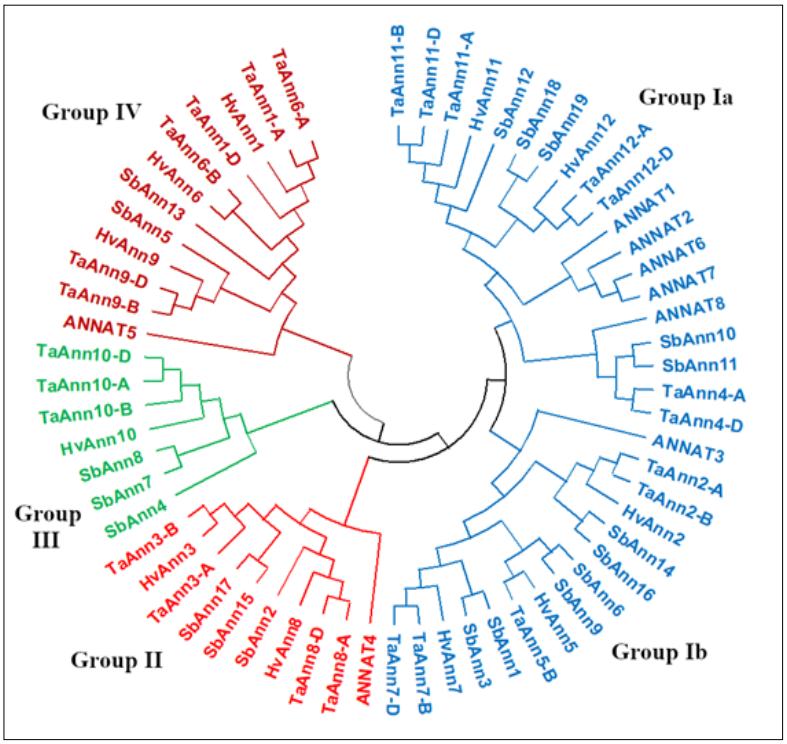

Figure 1. Neighbor-joining phylogenetic tree of annexins from *Arabidopsis thaliana*, *Sorghum bicolor*, *Triticum aestivum*, and *Hordeum vulgare*. The numbers represent bootstrap values based on 1000 replications.

Table 1 shows the protein properties of SbAnns. The amino acid numbers of SbAnns varied from 241 (SbAnn3) to 370 (SbAnn2) with molecular weight in KDa varying from 26.13 (SbAnn11) to 41.01 (SbAnn2). The negative GRAVY index values of SbAnns indicate the hydrophilic nature of these proteins. Additionally, the aliphatic index varied from 78.68 (SbAnn17) to 95.22 (SbAnn12). The isoelectric point ranged from 5.37 (SbAnn6) and 10.12 (SbAnn4). The majority of the proteins had an instability index greater than 40, indicating the unstable nature of these proteins. The annexin repeat number ranged from 1 to 4, with a variety of residues relating to calcium-binding activity similar to plant annexins. Phosphorylation sites such as casein kinase II and protein kinase C varied across SbAnn proteins. The cAMP and cGMP, N-glycosylation site, N-myristoylation site, proline, and cysteine-rich sites, RGD, and amidation sites were also found.

Ten protein motifs in total were identified (Figure 2b). Motifs 1, 2, 3, 6, and 9 were found to be associated with the annexin domain involved in calcium binding. Motif 1 was present in all SbAnn proteins and SbAnn4 had only three motifs. The SbAnns genes mostly ranged from 1 to 4.5 kb, except for SbAnn4 which has a length of approximately 7.5kb (Figure 2c). The exon numbers varied from 1 (SbAnn13) to 6 (SbAnn4 and SbAnn5). Interestingly, with intron numbers up to 5, only SbAnn13 did not have an intron. The longest intron was found in SbAnn4, which possibly contributed to its longer gene length. Remarkably, the divergent motif and intron/exon patterns were identified in subfamilies.

Table 1. Physiochemical properties of SbAnns

Protein ID (Phytozome)	Sorghum Annexins	Length (aa)	Mol. weight (kDa)	pI	GRAVY	Aliphatic index	II Instability index
Sobic.001G367900.2	SbAnn1	246	27.79	5.85	-0.365	86.46	46.65
Sobic.001G368000.1	SbAnn2	370	41.01	8.91	-0.476	79.32	50.74
SbRio.01G391900.1	SbAnn3	241	27.23	5.69	-0.419	82.99	48.51
Sobic.001G489150.1	SbAnn4	323	35.59	10.12	-0.332	80.25	70.73
Sobic.002G194100.1	SbAnn5	316	35.80	8.89	-0.431	82.18	33.57
Sobic.002G404100.2	SbAnn6	251	27.76	5.37	-0.255	90.92	39.82
Sobic.002G223100.2	SbAnn7	323	36.16	8.18	-0.414	82.85	54.93
SbRio.02G233600.1	SbAnn8	359	39.88	8.69	-0.341	81.64	51.13
SbRio.02G424700.1	SbAnn9	268	29.68	5.38	-0.363	86.27	41.5
Sobic.003G058400.1	SbAnn10	327	35.86	6.02	-0.221	86.94	46
SbRio.03G062800.2	SbAnn11	242	26.13	6.32	-0.252	84.01	44.36
Sobic.004G232300.1	SbAnn12	314	35.22	6.77	-0.339	95.22	36.02
Sobic.007G131800.1	SbAnn13	320	35.59	9.38	-0.202	87.28	37.69
Sobic.009G122000.1	SbAnn14	361	39.83	5.98	-0.207	91.69	40.75
Sobic.009G121900.1	SbAnn15	327	36.36	6.53	-0.411	78.87	40.17
SbRio.09G127800.1	SbAnn16	361	39.85	5.98	-0.217	91.41	40.67
SbRio.09G127700.3	SbAnn17	303	33.88	7.02	-0.435	78.68	41.25
Sobic.010G089500.1	SbAnn18	314	35.46	7.16	-0.412	91.78	38.14
SbRio.10G108600.1	SbAnn19	314	35.45	7.16	-0.413	91.46	37.87

Amino acid (aa), Kilodalton (kDa), Isoelectric point (pI).

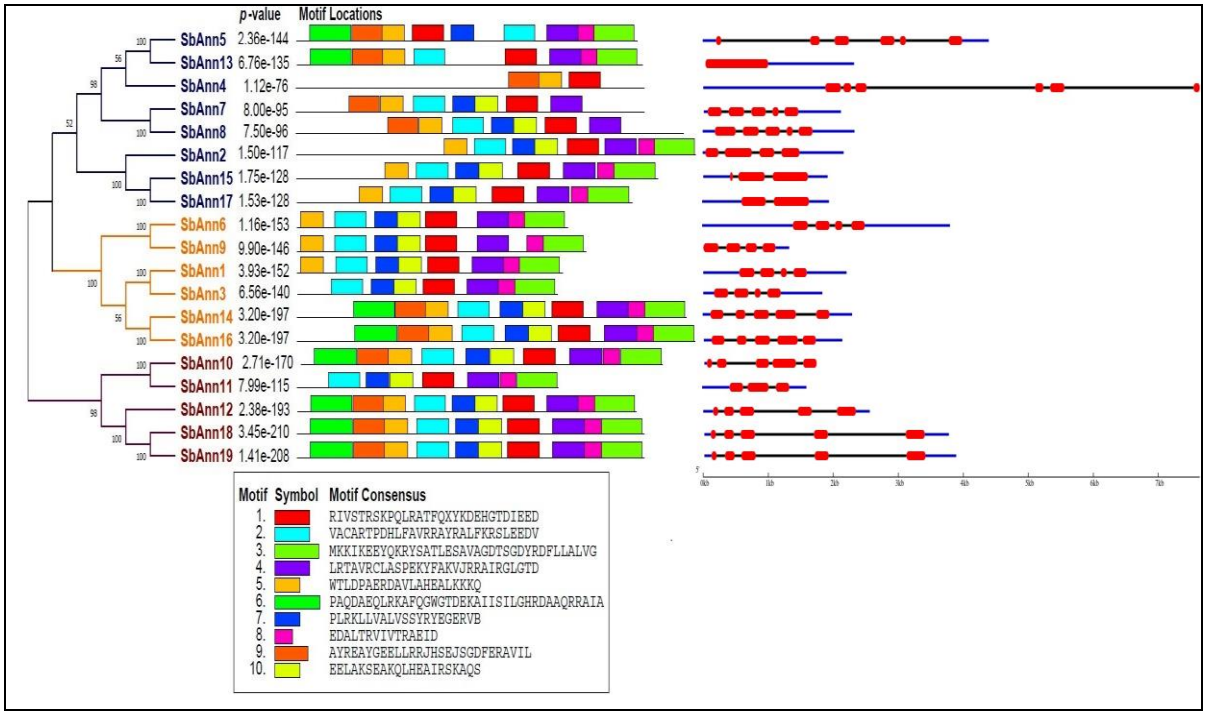

Figure 2. **a** - Neighbor-joining phylogenetic tree showing the relationship among SbAnns, **b** - conserved motifs, and **c** - gene structure of annexins of *S. bicolor*. The motif compositions and distributions were identified using MEME, and different colors indicated divergent motifs. In the gene structures, the solid blue and black lines indicate UTRs and introns, respectively, and red rectangles show exons.

The 19 SbAnn genes were randomly distributed across seven chromosomes, except for chromosomes 5, 6, and 8 (Figure 3). The largest number of SbAnn genes (SbAnn5, -6, -7, -8, -9) is on chromosome 2. Chromosomes 4 and 7 however contained one gene each. Seven duplication events were observed.

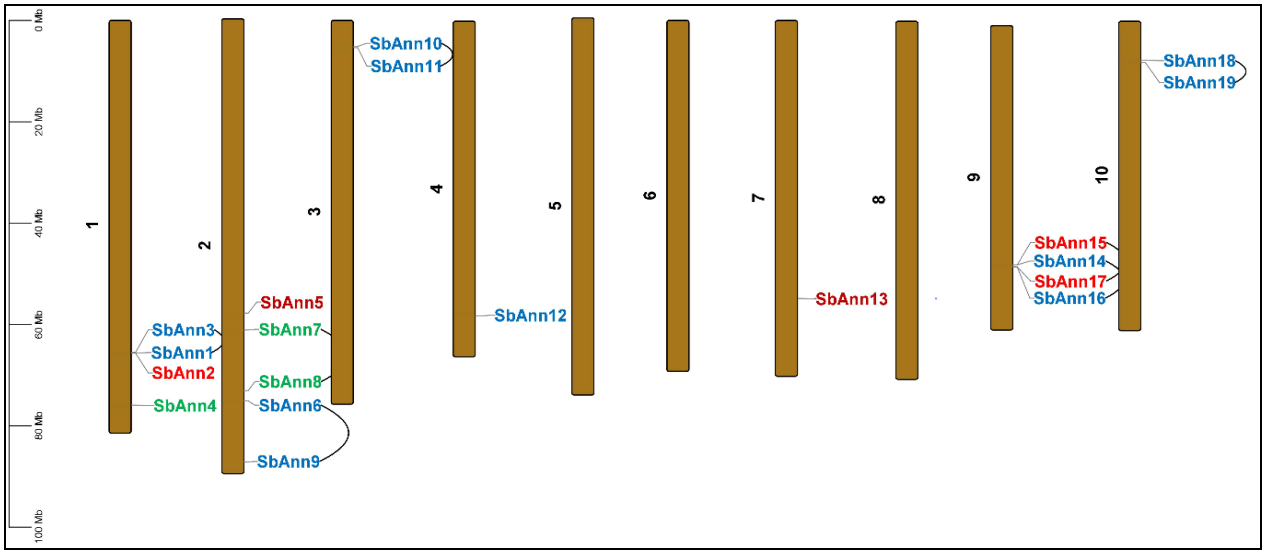

Figure 3. Chromosomal locations of *SbAnn* genes in sorghum chromosomes

The synteny analysis revealed various syntenic relationships between *S. bicolor* and *S. italica*, *Z. mays*, *O. sativa*, and *A. thaliana* (Figure 4a-d). It was observed that the syntenic relationship among monocots was much more than with dicots. Of the *SbAnn* genes, 9 were orthologous with *S. italica* and *Z. mays* and 10 with *O. sativa* (Figure 4a-c). Only one gene (*SbAnn12*) had an orthologous gene in *A. thaliana* (Figure 4d).

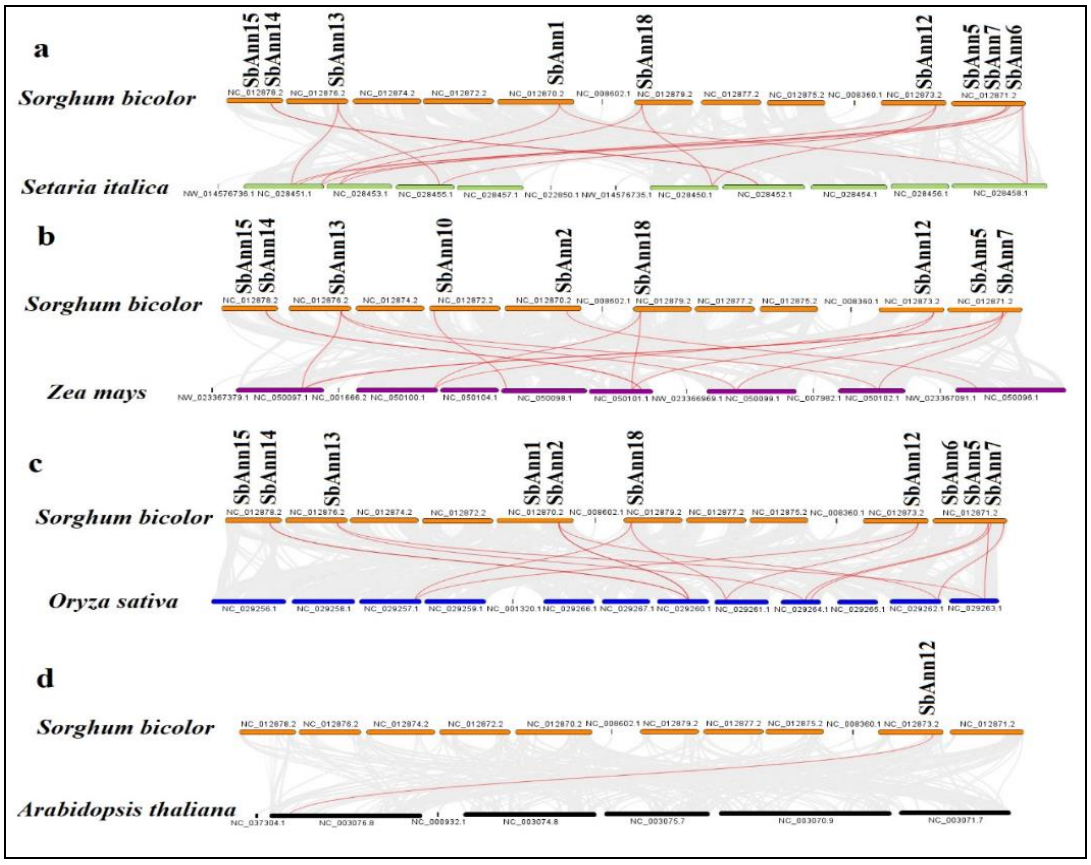

Figure 4. **a** - Syntenic relationship between *S. bicolor* and *S. italica*, **b** - *S. bicolor* and *Z. mays*, **c** - *S. bicolor* and *O. sativa*), and **d** - *S. bicolor* and *A. thaliana*. Red lines represent syntenic lines while the grey color represents shared genomic blocks between the organisms.

The presence of cis-acting elements provides important information needed to understand the functions of genes. The cis-acting elements in the promoter regions of *SbAnn* genes were grouped into four categories: light, hormone, environmental stress, and development-related cis-acting elements (Figure 5a-d). Of the sixteen light response cis-elements identified, the G-box was detected in 12 *SbAnn*. ACE element was found in only *SbAnn12* (Figure 5a). Additionally, twelve hormone response elements were found, the highest being the CGTCA-motif (involved in MeJA response) was present in all but four *SbAnns* (Figure 5b). A review of twelve environmental stress-associated cis-elements reveals that most of the SbAnns gene promoters have MYB, MYC, STRE, and WRE3 (Figure 5c). TC-rich repeats were however found in only *SbAnn9*. Finally, seven development-related elements represent the group with the least number of cis-elements (Figure 5d). The most abundant element was as-1 (involved in root-specific expression), present in all but four *SbAnns*. The endosperm-specific element (GCN4_motif) was observed in only *SbAnn9*. *SbAnn2* and *SbAnn14* on the contrary lacked any of the development-related elements. The presence of these elements in the promoter regions of *SbAnns* suggests variant possible functions.

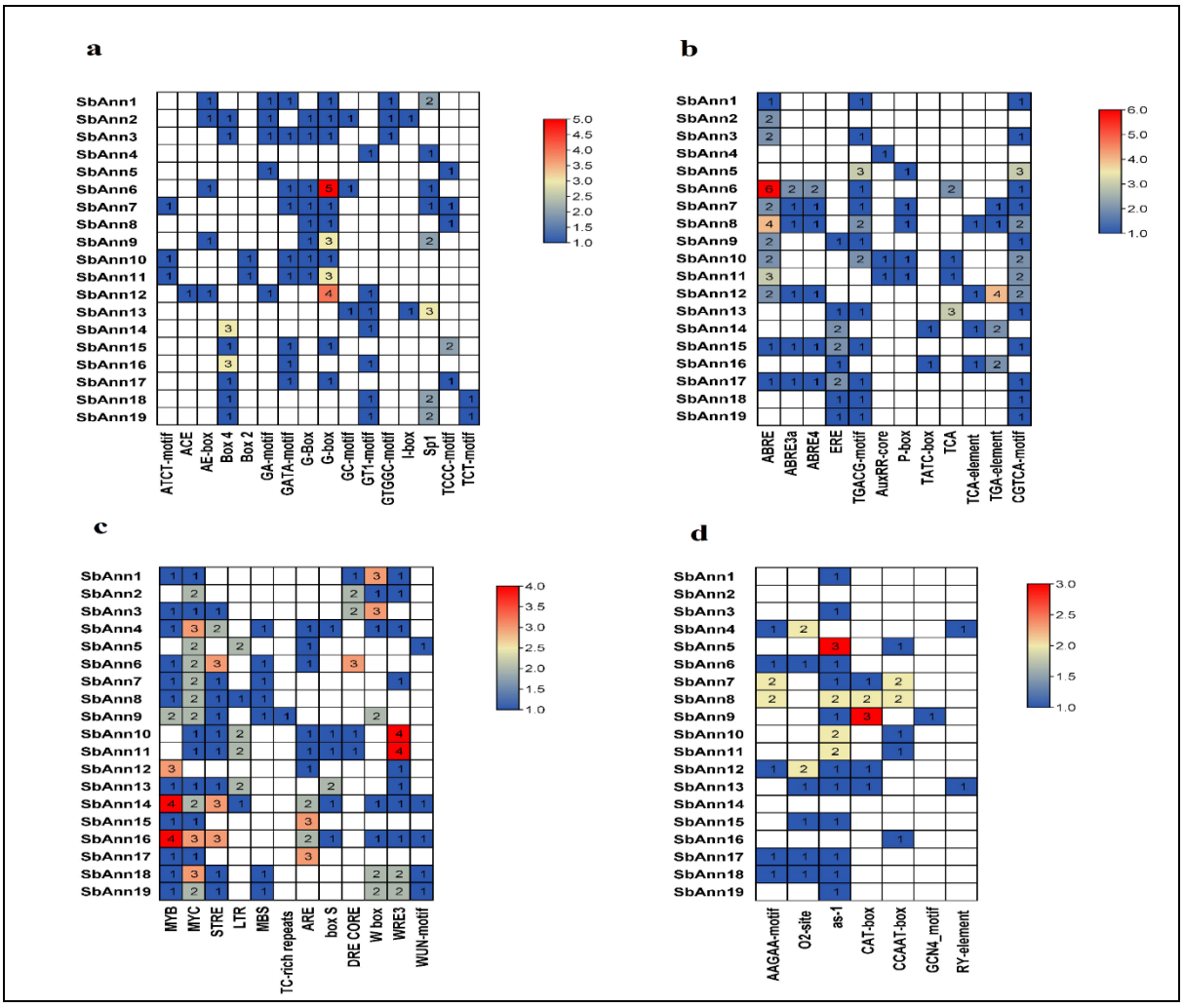

Figure 5. **a** – The identified light response cis-elements, **b** –hormone response, **c** –environmental stress response, and **d**–development-related cis-acting elements in *SbAnn* gene promoters

To elucidate the proper function of a protein, identifying other collaborating proteins and revealing their functions can be beneficial. Therefore, protein interactions of SbAnns were predicted (Figure 6). Eleven SbAnns were seen to interact with 5 proteins

namely, Sb03g045400.1, Sb01g032210.1, Glutaredoxin-C8, Sb03g013110.1, and Sb07g024140.1. The STRING platform provided putative functions of these proteins, except for uncharacterized proteins (Sb03g013110.1 and Glutaredoxin-C8). The Sb03g045400.1 protein has a peptidase domain involved in serine-type peptidase activity and proteolysis. Sb01g032210.1, the most interactive protein, contains AAA and ClpB domains involved in a variety of cellular activities and protein disaggregation. Finally, the vesicle-associated membrane protein (Sb07g024140.1) which consists of the longin and synaptobrevin domains, is involved in the transportation of proteins from the ER to the plasma membrane and an integral component of membranes. The overall interaction network consisted of 17 nodes, 30 edges, and a PPI enrichment value of 7.8e-14. Signifying the partial biological relationships shared between these proteins.

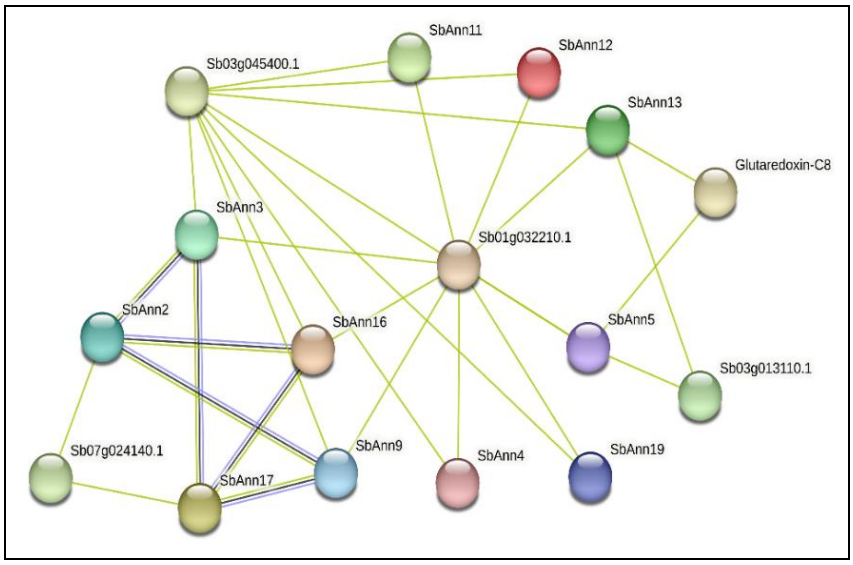

Figure 6. The STRING network illustration of SbAnn proteins with other proteins

Revealing miRNAs with which *SbAnn* genes interact may be an insinuation to the function of the protein. Thus, miRNA target analysis was carried out. 19 miRNAs were targeted at 11 *SbAnns* (Figure 7). It was interesting to note that a family of miRNA (Sbi-miR395) targeted only *SbAnn13*. Also, sbi-miR5566, sbi-miR5568f-5p, sbi-miR5568c-5p, sbi-miR5387b, sbi-miR408 and sbi-miR6229-3p also targeted two *SbAnns* each. Lastly, sbi-miR5568e-5p and sbi-miR172f were the lone targets of *SbAnn5* and *SbAnn12*, respectively. The inhibition modes were largely by cleavages rather than translations.

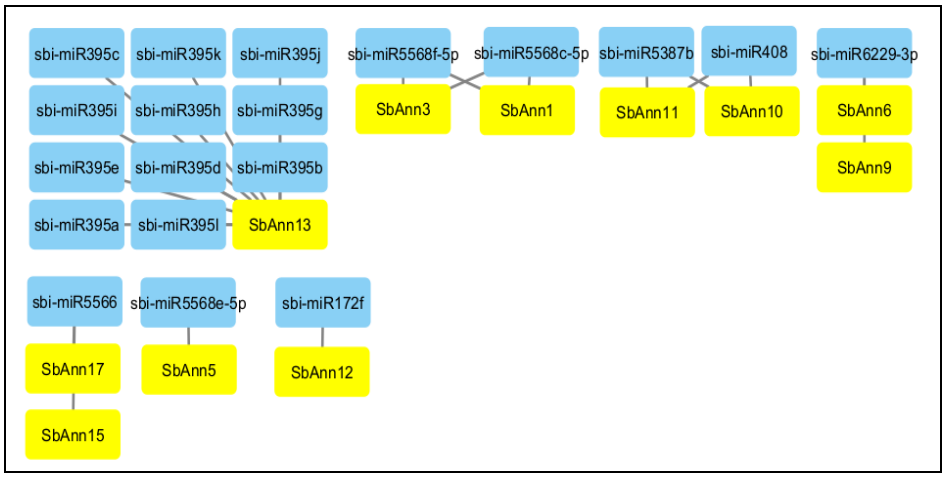

Figure 7. miRNA interaction network of *SbAnn* genes

To validate the potential roles of *SbAnn* genes under high-temperature stress, the expression of seven *SbAnn* genes (*SbAnn4, -5, -7, -12, -14, -15,* and *-18*) that have cis-elements for response to environmental stress were evaluated using qRT-PCR with gene-specific primers (Figure 8). The expression level of *SbAnn4, -5, -14, -15,* and *-18* genes was increased with high-temperature treatment. The higher relative fold change ranged from 1.5 (*SbAnn4*) to 1.2 (*SbAnn5, 14* and *15*).

SbAnn7 and *SbAnn12* with -0.6- and -1.0-fold changes, respectively were down-regulated. There were however no statistically significant differences ($P \geq 0.05$) between the medians of the control and the treatment. These results illustrated that *SbAnn*s have differentiated in response to high-temperature treatment and five of them are possibly involved in the response to high-temperature treatment.

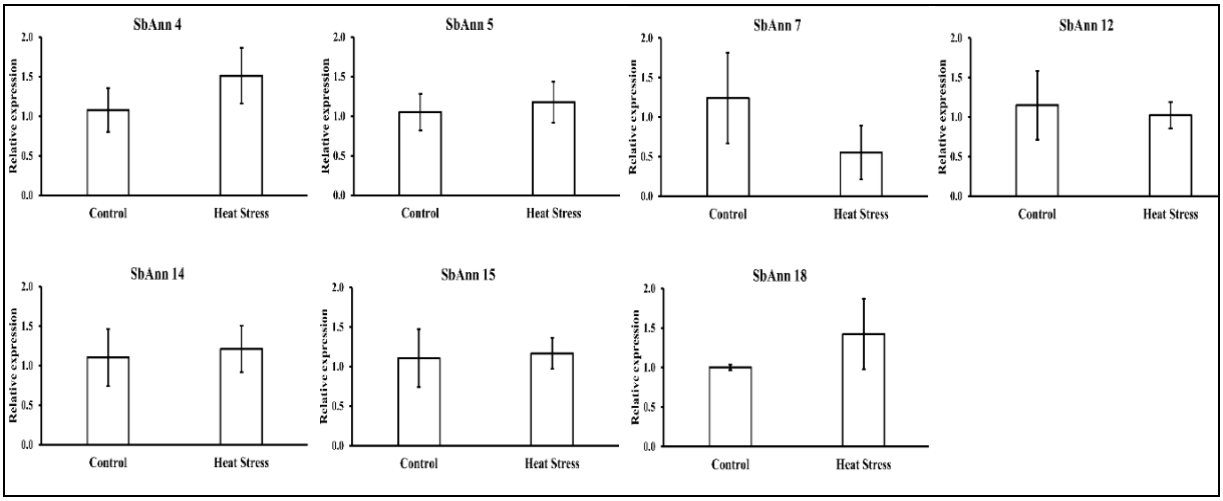

Figure 8. Expression levels of *SbAnn* genes under high-temperature stress. The control and stress treatments are on the x-axis while the fold change is shown on the y-axis. The *PP2A* gene from sorghum was utilized as an internal control. Data is shown as mean ±SE (n=3).

The annexins have activities such as nucleotide phosphodiesterase (Calvert et al., 1996), and peroxidase (Konopka-Postupolska et al., 2009). Also, they have roles in binding to membranes (Hayes et al., 2004; Rescher and Gerke, 2004), complex saccharide organization (Capila et al., 2001), and ion transport (Laohavisit et al., 2009). Thus, the annexins sense Ca regulates the signal and mediates cell elongation, plant growth and development, auxin, and stress responses (Konopka-Postupolska et al., 2011).

Annexins that belong to a family of calcium- and phospholipid-binding proteins are believed to have sprung from a single common ancestor, and gene duplication events may have expanded the number of annexins in existence. This is supported by similarities between amino acid sequences in different annexins and by the positions of these genes in the genome (Clark et al., 2012). As expected, the *S. bicolor* annexin proteins intermingled with other monocots, suggesting their close evolutionary fate (Figure 1). As an exception, SbAnn4 being the outgroup of Group III, could be attributed to its possession of only one annexin domain. In each cluster, dicot species (*A. thaliana*) were relatively distant amongst monocot species (*S. bicolor, T. aestivum, H. vulgare*), which is similar to the phylogenetic divergence of eudicot, dicot, and monocot species reported by (Xu et al., 2016) and (Hyeon Jeong et al., 2022).

Insertions and deletions in some SbAnns resulted in the loss of some regular annexin domains or motifs, like the Ca^{2+} binding site. As with other plant annexins, SbAnns also contained conserved residues. Eight sorghum annexins contained His residues. Recent studies have shown His residues to be involved in maintaining the secondary

structure of the protein (Konopka-Postupolska et al., 2009). The IRI motif enhances the binding of annexins to F-actin which governs several cellular processes (Clark et al., 2001). All but SbAnn7 and SbAnn8 contained an IRI motif or its modified form.

The numerous post-translational sites in SbAnns were identified. The activity and functions of annexins significantly rely on post-translational modification. For example, (Gorecka et al., 2005) studied the peroxidase activity in recombinant AnxAt1 in *E. coli* and observed a low activity compared to higher activity in *N. benthamiana*. They concluded that the increased peroxidase activity was caused by post-translational modification, perhaps through phosphorylation. Additionally, numerous potential phosphorylation sites identified in SbAnns may function as substrates for various kinases, which was consistent with previous reports in *Arabidopsis* and rice (Rohila et al., 2006; Konopka-Postupolska et al., 2011).

Furthermore, the intron number in *SbAnn* genes ranged from 0 to 5, similar in wheat and barley (Xu et al., 2016), though 3 to 5 were observed in *Arabidopsis* (Cantero et al., 2006). Also in barley, two HvAnns did not possess introns like SbAnn13. The similarity in intron numbers and gene structure between these monocots might be a shared evolutionary ancestor. However, a longer intron length has the advantage of gene function preservation by preventing mutation interference (Jo and Choi, 2015). In the subgroups, SbAnns had either similar conserved motifs or different motifs. The motif diversity in the subgroups indicates functional diversity.

Synteny analysis is an indispensable topic in comparative genome analysis because it serves as the foundation for gene and genomic evolutionary investigations (Cheng et al., 2012). Even though there is no comprehensive study of annexins in *S. italica*, we found nine orthologs common in the sorghum genome (Figure 3). Thus, the need for detailed research in that plant. The orthologs of *SbAnn18* and *SbAnn12*, in *Z. mays ZmAnx6.1* and *ZmAnx7*, are involved in pathogen stress tolerance and JA induction, respectively. *ZmAnx1*, ortholog of *SbAnn2* is induced in the presence of Zinc (Zhou et al., 2013). Also, *SbAnn5* and *SbAnn13* orthologs in *O. sativa*, *Os08g32970* is expressed during heat stress (Jami et al., 2012). Lastly in *Arabidopsis*, *AnnAt6* the lone ortholog of *SbAnn12* is upregulated during far red light exposure at the seedling stage (Cantero et al., 2006).

Moreover, cis-acting elements in *SbAnns* varied in quantity and function. The most abundant light response element G-box in SbAnns together with those reported in tobacco annexins by (Baucher et al., 2011) has been shown to control how plant genes are expressed in response to light (Giuliano et al., 1988). ABRE, ABRE3a, and ABRE4 (involved in the abscisic acid response), the CGTCA-motif, and the TGACG-motif (involved in the MejA response) are a few hormone response elements observed and were consistent with the results of in cis-elements of rye annexins (Hyeon Jeong et al., 2022). The environmental stress response cis-elements such as MYB and MYC (involved in stress response), STRE and WR3 (involved in heat stress response), TC-rich repeats (involved in defense and stress responses), and WUN-motif (wound response elements) reported in the promoter regions of *Schrenkiella parvula* and *Eutrema salsugineum* annexin genes (Moinoddini et al., 2023), were almost consistent with the results of the current study. Plant development elements in *SbAnns* including, as-1 (the root-specific element), CAT-box and CCAAT-box (involved in meristem expression), O2-site (involved in zein metabolism), and AACA_motif (involved in endosperm specific negative expression) were also found. The wide range of these predicted cis-elements suggests their importance during growth and stress response.

Interaction between proteins can reveal details of their probable biological functions. From the string interaction (Figure 4), possible cellular functions such as proteolysis and protein transport can be deduced. The Glutaredoxin-C8 protein that interacted with SbAnn5 and SbAnn13 is a reactive oxygen species-scavenging enzyme (Lin et al., 2014) activated during stress response.

In this study, *11 SbAnns* were found to interact with 6 different miRNA types, many of which have been proven by various studies to have roles in controlling biological processes such as adaptive response against abiotic stress. For example, miR395 (-a, b, c, d, e, g, h, j, k, l) which was induced during sulfur starvation (Kawashima et al., 2009) targeted SbAnn3. miR5568 that targeted SbAnn5, 3, and 1 was expressed during drought stress (Katiyar et al., 2015). miR408 which targeted *SbAnn10* and *SbAnn11* was upregulated during drought in *Arabidopsis* (Liu et al., 2008), and barley (Kantar et al., 2011) and downregulated in rice (Zhou et al., 2010), suggesting species-specific expression. Also, it has been shown that miRNA172-SSR markers targeting *SbAnn12* could be used as markers to distinguish resistant and susceptible individuals in rice under salt stress (Mondal and Ganie, 2014). All these examples indicate that potential functions of *SbAnns* may exist under stress conditions.

In this research, differential expression of *SbAnn* genes was observed under high temperature. *SbAnn 4, 5, 14, 15,* and *18* had relatively higher fold changes of 1.5, 1.2, 1.2, 1.2, and 1.4, respectively. These results were consistent with the findings that AnnAt2 in Arabidopsis (Cantero et al., 2006), *OsANN1* in rice (Qiao et al., 2015), most of *RsAnn* genes in radish (Shen et al., 2021), and four *ScANNs* in rye (Hyeon Jeong et al., 2022) were induced significantly their expression levels against high-temperature treatment. On the contrary, *SbAnn7* and *SbAnn12* genes had low fold change levels. Our findings contribute to the fact that some annexin family genes are involved in response to high-temperature stress.

CONCLUSIONS

Bioinformatic analyses of structural features such as domains, motifs, cis-elements in the promoter region, and protein-protein and microRNAs interactions, and detection of gene expression levels by RT-qPCR would be useful to elucidate sorghum annexin proteins features and characteristics and functions. A total of 19 SbAnns were identified based on their conserved domains, characterized, and grouped on a phylogenic tree. The promoter region and miRNA analysis indicate the involvement of SbAnns in biotic and abiotic stress. Additionally, the post-translational modification sites identified require more experimental research for confirmation. Synteny analysis also showed varied relationships between monocots and dicots. Also, some *SbAnns* are induced by heat stress, suggesting roles during high-temperature conditions.

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