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Genome-wide identification and characterization of the aquaporin gene family in *Sorghum bicolor* (L.)

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ABSTRACT

The aquaporin (*AQP*) gene family constitutes the most conserved class of gene families and plays a key role in water transport and conservation in plants. Although *Sorghum* genome sequencing has been completed, a comprehensive study of *AQP* genes in *Sorghum bicolor* is lacking. In the present study, we identified and characterized *Sorghum AQP* genes using a genome-wide scale, including factors such as their relationship with other species, chromosome distribution, sequence analysis and expression levels. A total of 41 non-redundant *AQP* genes were identified and classified into four subfamilies (PIPs, TIPs, NIPs and SIPs). Analysis of physical distributions revealed that *SbAQP* genes are unevenly dispersed in the *Sorghum* genome. Topological analysis indicated that members of the *SbAQP* gene family have two to seven transmembrane domains, whereas PIPs have four to six transmembrane domains. *SbAQP* genes were disrupted by introns, with intron numbers varying from zero to four. In silico promoter analysis of *SbAQP* genes suggested that it has diverse functions associated with plant development and abiotic stress responsiveness. The transcript analysis of *SbAQP* genes in different tissues and under abiotic stress conditions revealed that AQPs may play an important role in growth and development during abiotic stress conditions. To our knowledge, this is the first systematic study of the *AQP* gene family in *S. bicolor*. This study provides basic insights into the putative role of these genes under different environmental conditions. In summary, our genome-wide analysis of *SbAQP* genes provides a valuable resource for functional analysis aimed towards understanding their role in stress adaptation.

1. Introduction

Water uptake by roots and loss from leaves are driven by hydrostatic and osmotic forces that help plants maintain water homeostasis. Plants achieve this balance by continuously adjusting the hydraulic conductance of their tissues, especially the roots and leaves. The movement of water through the root cylinder occurs through three parallel paths (i.e., apoplastic, symplastic, or transcellular) (Steudle, 2000). The contribution of the different pathways to water transport in all parts of the plant varies from species to species and is dependent on environmental conditions (Steudle, 2000). The symplastic and transcellular pathways cannot be easily distinguished, but both depend on the functioning of aquaporins (AQPs) to transport water through membranes. AQPs are water channel proteins belonging to the membrane intrinsic proteins (MIPs) family that facilitate the rapid and selective transport of water and other small neutral solutes across plant cell membranes (Agre, 2006; Chaumont et al., 2001; Hachez and Chaumont, 2010). AQPs are present in most living organisms and are widely involved in different physiological processes, such as seed germination (Ge et al., 2014), reproduction (Bots et al., 2005), stoma movement (Siefritz et al., 2004; Uehlein and Kaldenhoff, 2008), photosynthesis (Uehlein and Kaldenhoff, 2008; Vera-Estrella et al., 2012), cell elongation (Higuchi et al., 1998), and responses to diverse abiotic stress treatments (Ehlert et al., 2009; Gomes et al., 2009; Luu and Maurel, 2005; Peng et al., 2007). Different AQP isoforms are targeted to distinct subcellular compartments and have emerged as important markers for plant cell membrane differentiation. Based on their subcellular localization and sequence homology, higher plant AQPs can be classified into five major subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and X intrinsic proteins (XIPs) (Bienert et al., 2011; Chaumont et al., 2001; Reuscher et al., 2014; Zhang et al., 2013). However, the XIP subfamily genes identified in a wide variety of non-vascular and vascular plants are absent in *Brassicaceae*, monocots

Abbreviations: AQP, aquaporin; MIPs, membrane intrinsic proteins; PIPs, plasma membrane intrinsic proteins; TIPs, tonoplast intrinsic proteins; NIPs, nodulin 26-like intrinsic proteins; SIPs, small basic intrinsic proteins; XIPs, X intrinsic proteins; ABA, abscisic acid; MEME, Multiple Em for Motif Elicitation; qRT-PCR, quantitative real-time PCR; ACP2, acyl carrier protein; EIF4A, eukaryotic initiation factor-4A; S/T-PP, serine/threonine phosphatases.

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and certain plant species in dicots such as *Arabidopsis* (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009; Lopez et al., 2012; Park et al., 2010; Sade et al., 2009). Although the subfamilies were originally named after the subcellular localization of members, this classification does not always represent actual localization (Wudick et al., 2009). This important aspect of plant AQP multifunctionality has been summarized in some recent studies (Bienert and Chaumont, 2014; Gomes et al., 2009; Hachez and Chaumont, 2010; Kaldenhoff et al., 2008; Maurel et al., 2008; Miwa and Fujiwara, 2010; Tyerman et al., 2002). Due to the boom in genome sequencing projects in several plants, genome-wide analyses have characterized *AQP* genes in several plant species, including rice (Sakurai et al., 2005), maize (Chaumont et al., 2001), *Arabidopsis* (Johanson et al., 2001), *Populus trichocarpa* (Gupta and Sankararamakrishnan, 2009), upland cotton (Park et al., 2010), soybean (Zhang et al., 2013), potato (Venkatesh et al., 2013) and tomato (Reuscher et al., 2014). Although the *AQP* gene family has been analyzed in several model plants, a systematic study is lacking in *Sorghum*, a crop grown in the semi-arid tropics. With the accomplishment of *Sorghum* genome sequencing (Paterson et al., 2009), it has become feasible to identify gene families by in silico cloning.

Sorghum bicolor (L.) Moench is a self-pollinating diploid C4 grass that ranks after maize, wheat, rice, and barley in terms of acreage, with an annual production of approximately 65.5 million tons from an area of 45 million ha (FAO 2010). *Sorghum* is one of the few resilient crops that can adapt well to future climate change conditions, particularly increasing drought and high temperatures. *Sorghum* has a relatively small genome (730 Mbp), extraordinarily diverse germplasm and incremental divergence, which makes it an attractive model for functional genomics and molecular breeding of C4 grasses. *Sorghum* genome sequencing resources (Paterson et al., 2009) allowed us to identify a total of 41 non-redundant *AQP* genes in *Sorghum*. Nomenclature and classification were performed based on phylogenetic tree analysis and existing literature. To provide holistic comprehensive insights into AQP members in *Sorghum*, we analyzed exon–intron junctions as well as putative conserved residues involved in substrate specificity, with a focus on subcellular localization and prediction of transmembrane domains. We also profiled gene expression of *SbAQP* genes in different tissues and in response to abiotic stresses. To the best of our knowledge, this study presents the first report of genome-wide analysis of the *AQP* gene family in *Sorghum* and provides a useful resource for identifying and characterizing *AQP* genes.

2. Materials and methods

2.1. Plant materials and stress treatments

The *Sorghum* cultivar Parbhani Moti was used for gene expression related experiments. Four major tissues representing the seedling, root, panicle and mature seed were collected from the life cycle of *Sorghum*. The plants for all experiments were grown in glasshouse conditions under natural daylight oscillations, with day/night average temperatures of approximately 28/22 °C and relative humidity of 70/90% for the different abiotic stress treatments. For ABA stress, *Sorghum* plants were sprayed with a 100 μM ABA solution and sampled 4 h after treatment. Salinity stress was imposed by drenching the pots containing plants with a solution of 150 mM NaCl followed by leaf sampling after 24 h. For drought stress, the water supply was withheld for 5 days followed by leaf sample collection. For cold and heat shock treatments, the seedlings were kept at 4 and 40 °C, respectively, for 4 h, followed by sample harvesting. Control plants were kept under control condition for

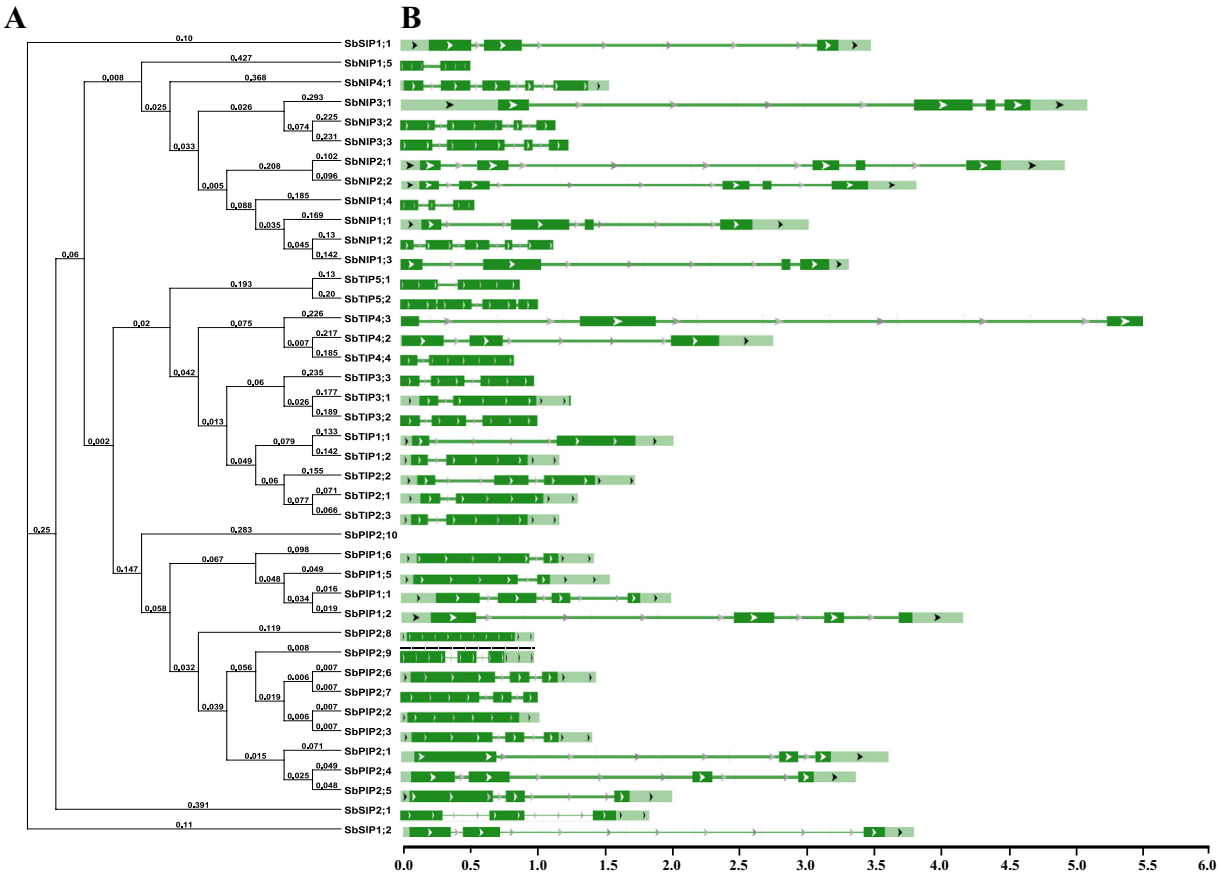

Fig. 1. Phylogenetic relationship and exon–intron structure of *Sorghum* AQP proteins. (A) The unrooted tree was constructed using MacVector software by the NJ method. (B) Exons and introns are indicated by green rectangles and thin lines, respectively. The untranslated regions (UTRs) are indicated by light green rectangles.

Table 1
Details of *Sorghum* AQP genes identified from the genome-wide search analysis. The table shows the following details: Putative AQP gene name, NCBI Accession number ID, open reading frame (ORF) size, amino acids length, predicted molecular mass for the deduced proteins, isoelectricpoint (pI), intron number with size, chromosome location and position), 5' upstream of the translational start site, transmembrane domain helices number and predicted subcellular localization.

S. No	Gene Name	Protein ID	ORF (bp)	Protein (AA)	Molecular weight (kDa)	pI	Chromosome			Intron	5' upstream region (bp)	Transmembrane domain	Subcellular localization
							Location	Start	End				
1	PIP1;1	XP_002446929	867	288	30.66	9.06	6	54149522	54151606	3	-	6	Plastid
2	PIP1;2	XP_002454508	870	289	30.79	9.08	4	62376719	62380912	3	2500	6	Plastid
3	PIP1;5	XP_002453072	873	290	30.79	8.58	4	67357489	67359050	1	1960	6	Plastid
4	PIP1;6	XP_002438067	891	296	30.86	8.31	10	7434249	7435606	1	-	6	Plastid
5	PIP2;1	XP_002461930	873	290	30.27	7.97	2	16901298	16904960	2	2500	6	Plastid
6	PIP2;2	XP_002461932	861	286	29.91	8.43	2	16954792	16955852	2	2500	7	Plastid
7	PIP2;3	XP_002461931	861	286	29.92	8.74	2	16940031	16941473	2	2500	7	Plastid
8	PIP2;4	XP_002452483	870	289	30.38	7.02	4	56555048	56558379	3	2500	6	Plastid
9	PIP2;5	XP_002446796	879	292	30.46	7.97	6	52066384	52068417	2	1795	6	Plastid
10	PIP2;6	XP_002461936	861	286	29.91	8.42	2	17037274	17038686	2	2500	7	Plastid
11	PIP2;7	XP_002461933	861	286	29.9	8.43	2	16963907	16964967	2	2500	7	Plastid
12	PIP2;8	XP_002489214	849	282	29.78	9.28	-	2606099	2607154	0	2500	6	Plastid
13	PIP2;9	XP_002461934	594	197	20.71	10.15	2	16987880	16988880	2	-	5	Vacuolar
14	PIP2;10	Sb02g031390	981	326	35.01	10.28	2	66251124	66252975	3	-	4	Plastid
15	TIP1;1	XP_002465859	753	250	25.77	6.79	1	70246505	70248652	1	2500	6	Plastid
16	TIP1;2	XP_002459183	770	258	26.02	6.5	3	74371021	74372588	1	2500	7	Cytoplasmic
17	TIP2;1	XP_002452808	750	249	25.4	6.94	4	62870930	62872148	1	2500	6	Plastid
18	TIP2;2	XP_002438430	747	248	24.98	6.5	10	41279125	41280840	2	2500	6	Cytoplasmic
19	TIP2;3	XP_002448289	750	249	25.13	6.67	6	53686490	53687675	1	2500	6	Plastid
20	TIP3;1	XP_002467022	801	266	27.64	8.77	1	19171664	19172991	1	2500	6	Mitochondria
21	TIP3;2	XP_002446824	807	268	27.71	9.61	6	52408577	52409875	2	751	6	Cytoplasmic
22	TIP3;3	XP_002468661	816	271	27.63	9.94	1	72866523	72867550	2	2500	5	Plastid
23	TIP4;2	XP_002439483	945	314	32.77	7.7	9	14625543	14628414	2	2500	6	Cytoplasmic
24	TIP4;3	XP_002457068	957	318	32.02	8.76	3	561153	566716	2	2500	6	Vaculoar
25	TIP4;4	XP_002457071	759	252	25.27	7.32	3	614502	615348	1	2029	6	Cytoplasmic
26	TIP5;1	XP_002448288	669	222	22.05	6.91	6	53684983	53685785	1	364	5	Plastid/Vaculoar
27	TIP5;2	XP_002445477	912	303	32.1	10.9	7	522047703	52205718	3	2500	6	Chloroplast
28	NIP1;1	XP_002453573	864	287	30.19	9.04	4	9508112	9511126	3	2500	6	Cytoplasmic
29	NIP1;2	XP_002454982	816	271	28.34	8.21	3	2265494	2266785	4	1449	5	Plastid
30	NIP1;3	XP_002440774	852	283	29.5	8.21	9	9954486	9957794	3	2500	5	Plastid
31	NIP1;4	XP_002437134	423	140	14.73	9.95	10	48255831	48256510	2	149	3	Chloroplast
32	NIP1;5	XP_002437133	441	146	15.48	4.14	10	48255104	48255681	1	2500	2	Cytoplasmic
33	NIP2;1	XP_002454286	894	297	32.04	7.32	4	57971834	57975693	4	2500	6	Plastid
34	NIP2;2	XP_002438105	888	295	31.36	7.93	10	8110238	8115151	4	2500	6	Plastid
35	NIP3;1	XP_002464380	906	301	31.16	8.99	1	17661019	17666112	3	2500	5	Plastid
36	NIP3;2	XP_002445047	870	289	30.01	9.01	7	3939661	3940905	3	2500	6	Vaculoar
37	NIP3;3	XP_002443852	894	297	31.17	7.85	7	3952679	3953866	3	1438	6	Plastid
38	NIP4;1	XP_002455311	870	289	30.15	7.43	3	8731107	8732628	4	1730	6	Plastid
39	SIP1;1	XP_002449310	741	246	25.4	8.47	5	13710573	13714038	2	2500	5	Plastid
40	SIP1;2	XP_002441068	732	243	25.57	9.41	9	48712871	48716641	2	2500	6	Plastid
41	SIP2;1	XP_002465351	750	249	26.76	10.2	1	60518728	60520588	2	1602	7	Chloroplast

the same duration at 28 ± 1 °C. For all conditions, three biological replicates were collected for each sample and immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

2.2. Database search and identification

To identify AQP family genes in *Sorghum*, all known *Arabidopsis*, rice and maize AQP protein sequences were used as queries to perform multiple database searches using BLASTX and BLASTP from the NCBI and Phytozome databases. After filtering *Sorghum* AQPs with at least 50% identity with the query sequence, the candidate *AQP* genes were aligned to ensure that no gene was represented multiple times. All remaining protein sequences were examined using the domain analysis program PROSITE with default cutoff parameters (Sigrist et al., 2013).

2.3. Multiple sequence alignment and phylogenetic analysis

Accurate classification of *AQP* genes into subfamilies was performed using phylogenetic analysis. Amino acid sequences were imported into MacVector software (V13.05) and aligned using ClustalW (Olson, 1994). The alignment file was then used to construct an un-rooted phylogenetic tree based on the neighbor-joining method of MacVector (V13.05) after bootstrap analysis for 1000 replicates. Multiple Em for Motif Elicitation (MEME) software was used to detect conserved motifs of *Sorghum* AQP family genes (http://meme.nbcr.net). The aligned sequences were used to identify conserved regions present in AQP sequences and to analyze the ar/R content and forger positions in the aligned sequences.

2.4. Sequence analysis of AQP genes

The chromosomal distribution of the genes encoding *SbAQPs* was determined by searching *Sorghum* sequences against the Phytozome and NCBI databases using default settings. Prediction of transmembrane regions was performed using http://www.ch.embnet.org/software/TMPRED_form.html. Protein subcellular localization was predicted using the WoLF PSORT algorithm (http://wolfpsort.seq.cbrc.jp). The exon–intron organization of the genes was determined through comparison of the respective full-length cDNA sequence (CDS) with the corresponding genomic sequence. Open Reading Frame (ORF) length and amino acid (AA) translations were obtained from the NCBI database. The molecular weight (kDa) and isoelectric point (pI) of each *AQP* gene were calculated using the MacVector program.

2.5. In silico analysis of promoter sequences

To investigate the putative role of cis-acting elements that are responsible for gene expression under developmental and abiotic stress

Table 2
Primers used in the real time PCR and their product size.

S. No	Name	Sequence	Product Size
1	PIP1;1	TGTTGCTTTCAGATGCCGCC AGCGAACTTGAAAGAGGAACGG	101
2	PIP1;2	CCCACGCCTAGGTCCAAAGT GCTGAGCTCGGTACAGGAAT	110
3	PIP1;5	CCTTCAAGAGCCGCGACTAATT AGTACGTAGCTAGCTCACACGA	159
4	PIP1;6	TAGTGTACCGTACCTTGCTGCT TCCCATGGATGCTAACACGTAG	158
5	PIP2;1	CGTCGTCCAAGGCTTTCAATCT TGAAACAAGAGCGACCAAACCA	134
6	PIP2;2	CTCATGAGTACCCAAAGTCCCA TTTCGTGCTGTCATGGGGTG	106
7	PIP2;3	CTCGTCCTCCTCCGCAGTTT GCACGCTCCACACATCACTT	109
8	PIP2;4	TACGCCGCACAGTACATACATG AAATTAACAGCAGCGTACGTGC	104
9	PIP2;5	TTCAGCCGCTAGATCGACCATC CATTCACGAGTGCAGTGGAGG	109
10	PIP2;6	GCTCCCTCCCGAAGTTTAAGTT CCACAGCACACAAACACACAAA	142
11	PIP2;7	GAGACGCCAAATCAACTAAATGA TGTGTTTTAGGGCTGCCATGG	104
12	PIP2;8	TTGGCATCCTTACGCAACAACT GAATGGACACACGATGCAAACG	141
13	PIP2;9	TGTGCGTTTGTGTGCTGTGAA CGATCGCTTGCATTCCTCCAA	110
14	PIP2;10	TGCCTGAACAAGATTGGCGC GTTCGTGTAATAAGCACCCCGT	116
15	TIP1;1	CTCCCCACCACCGACTACTAAG GAGAGAAACTAAACGGCGAGGG	130
16	TIP1;2	TGCCCACCACTGACTACTGAAT TCGTTTGGACTGGACAATGCAA	143
17	TIP2;1	GTCTTCATCGGCGGCAACTAC GAAGAGCAAAGCAAACGACGAC	129
18	TIP2;2	CTTCTAAGTGCCCTGCCTCTG CCTGAACCAAACAAATGCTGCG	126
19	TIP2;3	CAACCCGTCGTGTTGATTTCAC TAGATCGAACGGTTCTCTCGCT	155
20	TIP3;1	GTGGCGTCGTCTCCTTAGTTG ATTAACCACTCCACACGACACC	107
21	TIP3;2	CCAGCTAACATTTCCGGCACC CTTGACGAGACGATACTGCCTG	103
22	TIP3;3	TGCCCCGCTCTCTGATGAAG TCACTGAAGCAAAGCAACGACA	147
23	TIP4;2	GCCGGGTTCATCTACGAGTCT CTGACTGCCCTGCCCACA	114
24	TIP4;3	CGTCATTCTGTTCTTCCCGGC ATTCAAAATGCTGGGTGGGCC	121
25	TIP4;4	GCTTTCGGATGGATCGTGCAT ATCTTGGCGAGTGAGTGCTGA	104
26	TIP5;1	TGGCGTACACATGAGTCAGTTG CACACACATTCCAAACGCAGC	97
27	TIP5;2	AGGCCTAAATCTCCGGACGAA TGCTTCAAGTGGACAAGGAGGT	125
28	NIP1;1	TGTGGAAGGGCTACTACTGCT CATCCATCCACACACCGCAG	109
29	NIP1;2	GCCGTACCAGCTAACAGACAC GCTTTGGGTACTTGTAGCAGCA	91
30	NIP1;3	AACGCTGCAAGATTGGGTCAC AAACACAGACACGAGCATAGC	129
31	NIP1;4	GCGTACGTGTCTATCGTCCGA ACCATCATCTTCTTGCACGGC	91
32	NIP1;5	AGCAGTGATTCCGTCGTCCTT CTTCTCGCCTTGCCATCGTTC	117
33	NIP2;1	TCTGTGTGTGTCGGTGCTCAT ATTGAGAGAGCGAGCCATCCA	108
34	NIP2;2	AGGTGGTGGGAGAGAGCTAGT CATCACACTTGCTCCGATCCG	130
35	NIP3;1	TCCGTCTCGTACCGTGTCAAG CACACATAGCACTGGACGCC	128
36	NIP3;2	CGTAGTTGCTCCTCCGCTAG TCCGATATCCTGCACTAATCCA	144
37	NIP3;3	TGGAACTGGAGCCTATGTTGCA TCTCGTGTTGCCTGCAGAAGA	145
38	NIP4;1	CCGTTGGATCACTTGCGTCG GTCGCGCCGTTTTGTTGATTG	136
39	SIP1;1	GTTCCTACCACCGGCACCTAA CAAAACCAGCATCCACAACCGA	106
40	SIP1;2	TTCCTAGCACCGCCACCTAAG TGGCGTTCCTCTAATTCTAGCA	112
41	SIP2;1	AGCAGATGAAAACAAGACCAAGA AGACATTTCACCTTGCGTCAT	132

treatments, 2500 bp of genomic DNA sequences of the *SbAQP* genes were extracted from the NCBI database. The sequences were analyzed by different bioinformatics programs, including PlantPAN (Chang et al., 2008), PLACE (Higo et al., 1999), and PlantCARE (Lescot et al., 2002), and the available literature. Whole promoter sequences were searched in both the forward and reverse strands.

2.6. RNA isolation and qRT-PCR

Total RNA was isolated using the Nucleo-Spin RNA plant kit including DNase-I treatment following the manufacturer's recommendations (Macherey-Nagel, Germany). The quantity and quality of total RNA samples were assessed using the NanoVue Plus spectrophotometer (GE Health care, USA) and RNA gel electrophoresis. The DNase-I treated RNA was reverse-transcribed using Superscript III (Invitrogen), and qRT-PCR was performed using a Realplex thermocycler (Eppendorf, Germany). Gene-specific primers were designed using Primer 3.0 software (Untergasser et al., 2007) to amplify 90–160 bp PCR products specific for each *AQP* gene (Table 2). Each reaction contained 5 μl of the 2X Sensi Mix SYBR No-ROX (Bioline, UK) kit mix, 1.0 μl of diluted cDNA sample, and 400 nM gene-specific primers in a final volume of 10 μl. The thermal cycles were as follows: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 62 °C for 1 min. After the qRT-PCR reaction was completed, a melting curve was generated to analyze the specificity of each gene by increasing the temperature from 60 to 95 °C. Three technical replicates were used for each gene. Expression levels of the *SbACP2*, *EIF4A* and *S/T-PP* genes were used as internal controls. These three reference genes are widely used as reference genes in different plant species (Basa et al., 2009; Chandna et al., 2012; Gimeno et al., 2014; Jain et al., 2006; Pinheiro et al., 2011; P.S. Reddy et al. unpublished data). A total of three independent biological repeats of the experiment were performed, and the data were averaged. Relative expression levels of SbAQP transcripts in different tissues and under different abiotic stress treatments were compared to their corresponding control *Sorghum* seedlings using REST software (Pfaffl et al., 2002).

3. Results and discussion

3.1. Identification of AQP family genes in Sorghum

A genome-wide scan was performed using the NCBI and PHYTOZYME databases with rice and Maize *AQP* genes, resulting in the identification of 41 non-redundant *AQP* genes in the *Sorghum* genome (Fig. 1A and Table 1). The number of *AQP* genes in *Sorghum* was slightly greater than that observed for other monocot species, such as rice (34) and maize (36). Most likely, the expansion of AQP members is associated with whole-genome duplication events (Abascal et al., 2014). AQP characteristics including number of amino acids (length), molecular weight (MW) and isoelectric point (pI) of each gene are listed in Table 2. The identified AQP proteins in *Sorghum* ranged from 140 amino acid residues (SbNIP1;4) to 326 amino acid residues (SbPIP2;10). Molecular weights ranged from 14.73 kDa (SbNIP1;4) to 35.01 kDa (SbPIP2;10), and isoelectric points (pI) ranged from 4.14 (SbNIP1;5) to 10.9 (SbTIP5;2). The predicted localization of the SbAQPs subfamily was diverse and included the cytosol, plasma membrane, plastids, vacuoles, mitochondria and chloroplast (Table 1). The majority of SbPIP proteins were localized

to the plastids with the exception of PIP1;6 and PIP 2;9, which were targeted to the cytosol and vacuole, respectively. The TIP subfamily was localized in the cytosol, mitochondria, plastid, and vacuoles. Most of the NIP subfamily members were evenly associated with the vacuoles and plastids, while the SIP subfamily members were located either in the plastids or the chloroplast (Table 1). Based on their localization, we can assume that AQPs are abundant genes that participate in cell metabolism. Identified *AQP* genes were randomly physically distributed on the ten chromosomes of *Sorghum* (Fig. 2), with the highest number of *AQP* genes found on chromosome 1 (8), followed by chromosomes 2, 4, 6, and 10 (5). Three genes were distributed on chromosomes 3, 7, and 9 and two on chromosome 5. Chromosome 8 did not contain any *AQP* genes (Fig. 2). The chromosomal distribution of AQPs in other species differed from that of *Sorghum*, with at least two *AQP* genes on their chromosomes. Complete details of the *AQP* gene distribution are provided in Fig. 2 and Table 2.

3.2. Genomic organization of SbAQP genes

An exon–intron structure analysis was performed to support phylogenetic construction based on the information obtained from the cDNA and genomic DNA sequences. The distribution of introns and exons was analyzed in the 41 SbAQP sequences (Fig. 1B and Table 1). All SbPIPs included 1 to 3 introns with the exception of SbPIP2;2, which contained no introns. One to two introns were found in most SbTIPs. SbNIPs contained variable introns with the majority characterized by 4 introns; all SbSIPs contained 2 introns. The intron insertion positions differed among the four subfamilies and varied within each subfamily. Intron length varied widely, with a range of 30 to 8089 nucleotides (Fig. 1B). While the length of each exon was similar for most members in each subfamily, some deviations were noted. In summary, *Sorghum* AQPs showed a complex gene structure with some differences in the position and length of exons (Fig. 1B).

3.3. Phylogenetic analysis

The evolutionary relationship between SbAQP proteins and closely related species such as rice and maize was investigated for homology by constructing a phylogenetic tree using the NJ method within MacVector (V13.05). Identified AQPs in *Sorghum* were systematically named according to their phylogenetic relationship with *Zea mays* (Chaumont et al., 2001) and rice (Sakurai et al., 2005) AQPs (Fig. 3). Subsequently, the *Sorghum* AQPs were classified into four major subfamilies similar to those observed in rice and maize that consisted of 14 SbPIPs, 13 SbTIPs, 11 NIPs and 3 SIPs (Figs. 1A, 3 and Table 1). The distribution of members of the subfamilies of *Sorghum*, maize and rice were 14, 12, and 11 for PIPs, 13, 12, and 10 for TIPs, 11, 4, and 11 for NIPs, and 3, 3, and 2 for SIPs, respectively (Table 1). The number of PIPs, TIPs, NIPs and SIPs was greater in *Sorghum* when compared with corresponding subfamily members in rice and maize. However, the number of AQPs in *Sorghum* was relatively small compared with other crops such as cotton (71) and soybeans (66). SbPIP family members had a high level of sequence similarity (60–98%) with the maize and rice PIP families, while members of the SbTIP, SbNIP and SbSIP subfamilies had less sequence conservation with their counterparts in maize and rice. The SbPIP subfamily was further divided into two sub-groups including PIP1 and PIP2, while the SbTIP family was further divided into five sub-groups (SbTIP1, SbTIP2, SbTIP3, SbTIP4 and SbTIP5) (Figs. 1A, 3). The very high bootstrap values suggested a common origin for *AQP* genes within each sub-group. While the SbNIP subfamily consisted of four groups (SbNIP1, SbNIP2, SbNIP3, and SbNIP4), SbSIP had only three members with very low sequence similarity (27–79). Among all, the major group of AQPs was SbPIPs, followed by SbTIPs, SbNIPs, and SbSIPs in decreasing order. However, homologous genes corresponding to PIP1;3, PIP1;4 and NIP4;1 were not found in *Sorghum* (Figs. 1A, 3 and Table 2).

3.4. Sorghum AQP protein structure analysis

Sorghum AQP protein sequence analysis revealed a high level of sequence diversity between the four subfamilies. We found 19–23% similarity between PIPs and NIPs, 10–23% similarity between TIPs and SIPs, 14–33% similarity between PIPs and TIPS, 10–21% similarity between SIPs and PIPs, and 11–17% similarity between NIPs and SIPs (Supplementary Fig. 1). In silico transmembrane domain predictions showed that the number of transmembrane helices ranged from 4 to 8. Nearly all AQPs contained 6 helices, except PIP2;9, PIP2;10, TIP3;3, TIP5;1, NIP1;2, NIP1;3, NIP3;1 and SIP1;1 contained 5, PIP2;2, PIP2;3, PIP2;6,

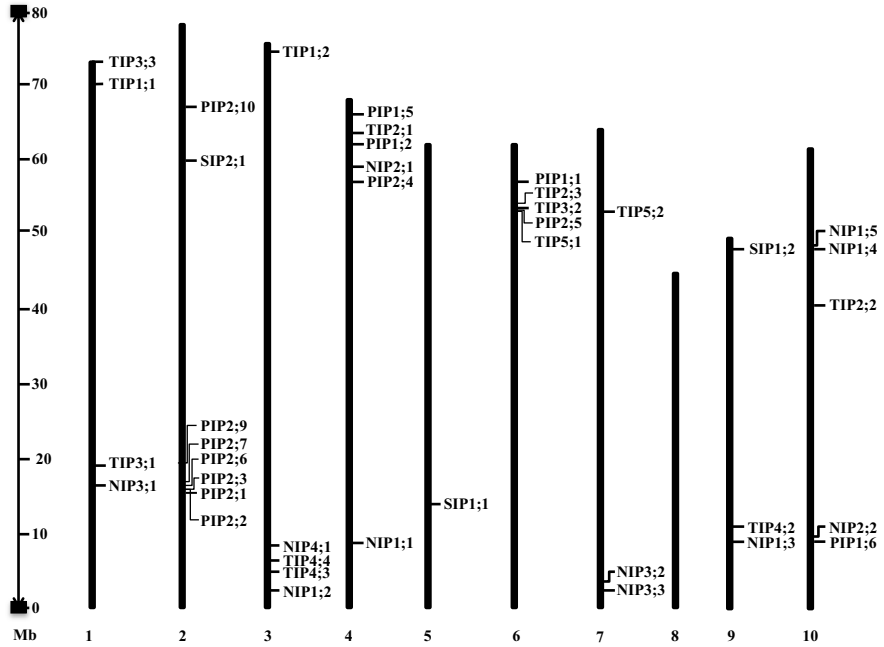

Fig. 2. Chromosomal locations of *Sorghum AQP* genes. The chromosome numbers are indicated on the right side of each chromosome and correspond to the approximate location of each *AQP* gene. Chromosomal distances are given in Mbp.

PIP2;7, TIP1;2 and SIP2;1 contained 7, PIP2;10 contained 4, and NIP1;4 and NIP1;5 contained 3 and 2, respectively (Table 2). MEME motif search analysis revealed that the *Sorghum AQP* gene family contained three major conserved motifs: motif 1 was conserved in all AQP subfamilies except SIP subfamilies NIP1;4 and 1;5; motif 2 was conserved in all subfamilies except PIP2;10, NIP1;5 and TIP5;2; and motif 3 was very specific to the PIP subfamily of *SbAQP* genes (Supplementary Fig. 1). The multiple sequence alignment of *SbAQP* genes showed that sequences for the NPA motif, the ar/R selectivity filter and forger positions were highly conserved (Supplementary Fig. 1). While most *SbAQP* genes possessed the NPA motif, some also contained TPA (TIP5;2), NPS (NIP3;1), NPT (SIP1; 1 and SIP1;2), NPV (SbNIP3;1), and NPI (NIP4;1) motifs. NPA dual motifs, forger positions (P1–P5), and selective filters (H2, H5, LE1, and LE2) are present in all AQPs, although some did not accommodate particular motifs, such as NPA dual motifs in NIP1;4, NIP1;5, SIP2;1, PIP2;10, PIP5;2, and TIP5;1, H2 filters in NIP1;4 and NIP1;5, the H5 motif in NIP4;1 and LE2 and the P2 motif in TIP5;2. Based on this analysis, it was evident that there were structural differences in various *Sorghum* AQP subfamilies. The details of dual NPA motifs, the ar/R selective filter and forger positions are represented in Supplementary Fig. 1.

3.5. Abiotic stress inducible expression

Recent results from transcriptomic studies have provided a better understanding of the molecular mechanisms of the plant stress response, leading to the identification of numerous novel stress-responsive genes. Transcriptomic studies with multiple time points suggest that plants experience different stresses during the initial adjustment period to a particular stress, and plant responses progress from general responses to specific responses. Each of these genes responded differently to abiotic stresses and developmental cues. In *Z. mays* and *Oryza sativa* where the complete genome sequences are available, transcript analysis has shown that different genes of the AQP family are expressed under different abiotic stress conditions in different tissues (Chaumont et al., 2001; Sakurai et al., 2005). In the present study, qRT-PCR analysis of *Sorghum* AQP transcript abundance in different tissues (seedlings, root, panicle and mature seed) and abiotic stress conditions (dehydration, salinity, cold, ABA and heat) aided in the identification of specific expression patterns of individual members of this gene family (Fig. 4A and B).

Transcript analysis demonstrated that all genes displayed differential expression in response to different abiotic stresses during the course of the experiments (Fig. 4A). Among the five treatments, drought and salt stress induced more dramatic changes in SbAQP transcript abundance than cold shock or ABA. Six *SbAQP* genes (*PIP2;7*, *PIP2;8*, *TIP3;1*, *TIP4;4*, *SIP1;1* and *SIP1;2*) were up-regulated and three (*PIP1;6*, *PIP2;1* and *TIP4;2*) were down-regulated in response to the tested abiotic stress conditions (Fig. 4A). AQP transcripts *SbPIP1;6* and *PIP2;1* were down-regulated in all tested abiotic stress conditions but had higher expression in tissues including seedlings, roots and panicles. This type of diverse expression pattern of AQP transcripts in response to abiotic stresses has also been observed in recent studies (Ge et al., 2014; Jang et al., 2004; Mariaux et al., 1998; Wang et al., 2003; Weig et al., 1997; Yamada et al., 1997). These results indicate that AQPs play a major role in maintaining water homeostasis during plant responses to environmental stress (Jang et al., 2004). Plant hormones such as ABA are known to play a crucial role in the regulation of different plant processes, such as signaling and expression during abiotic and biotic stresses. The effect of ABA on *AQP* gene expression has been described for various plant species, such as *Arabidopsis* (Weig et al., 1997), rice (Liu et al., 1994), *Brassica napus* (Gao et al., 1999), and radish (Suga et al., 2002). Expression patterns of *SbAQP* genes under ABA treatment revealed that most SbAQPs were predominantly repressed, with the exception of the up-regulated *SbSIP1;2*, *SbPIP2;10* and *SbTIP3;1* genes, thereby suggesting that *SbAQP* gene expression was controlled in either an ABA-dependent or ABA-independent manner (Suga et al., 2002). This type of gene

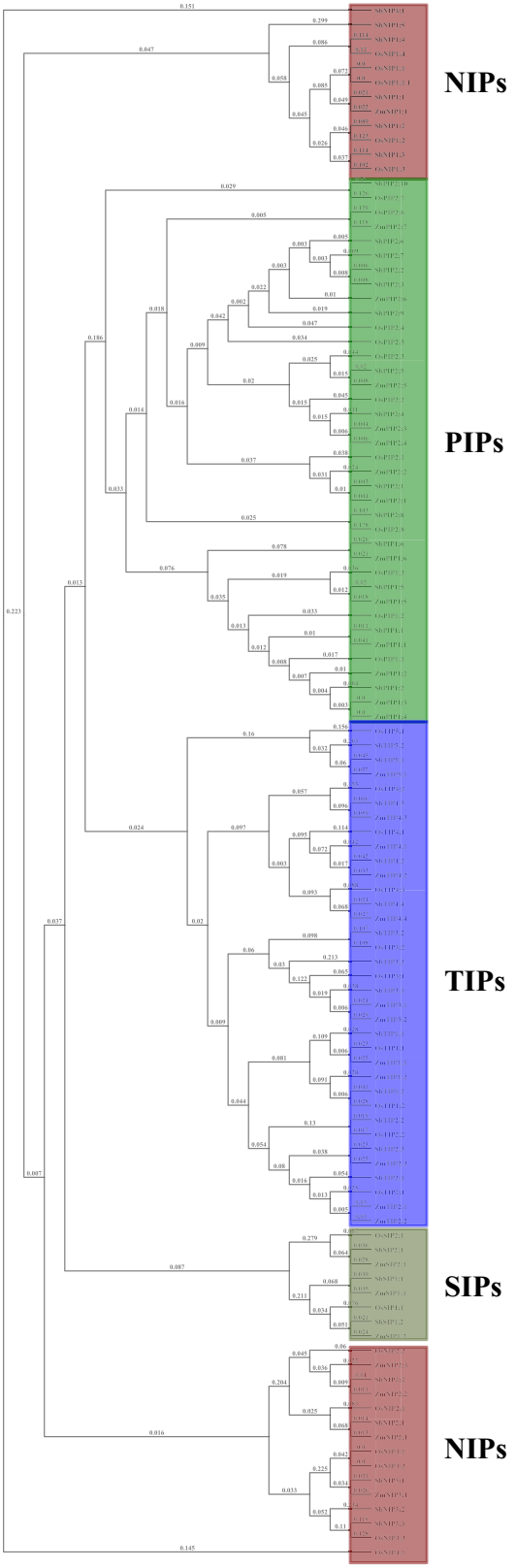

Fig. 3. Phylogenetic analysis showing relative closeness of *Sorghum* AQP proteins with maize and rice AQP proteins. The tree was constructed using the Clustal W program of MacVector by the NJ method from full-length amino acid sequences of maize, rice and *Sorghum* AQP proteins. The numbers above the horizontal lines are proportional to the difference between the sequences.

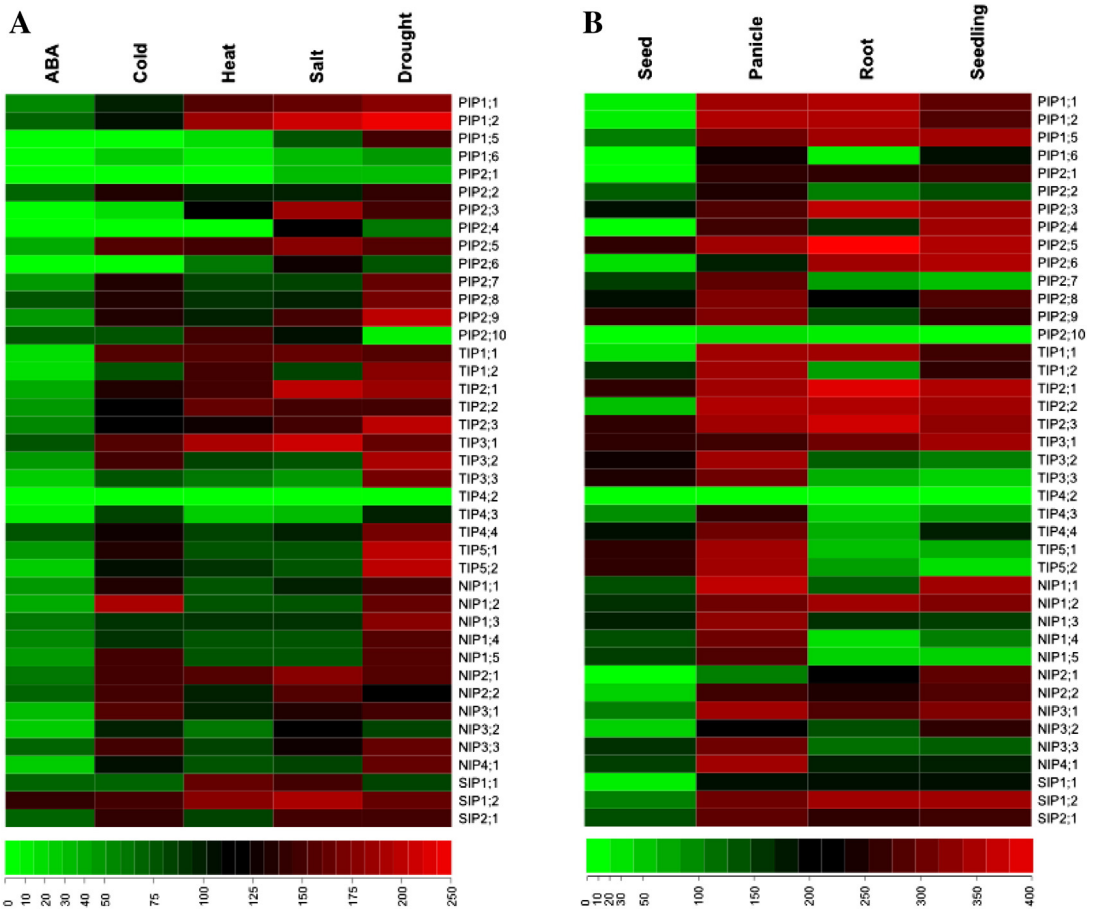

Fig. 4. Heatmap of *SbAQP* gene expression pattern under different abiotic stress treatments (A) and tissues (B). A heatmap displaying the transcript abundance is produced by quantitative real-time PCR analysis. Blocks with colors indicate decreased (green) or increased (red) transcript accumulation relative to the control. All samples were analyzed in triplicate in three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression profile may be due to the presence of ABRE cis-acting elements (involved in abscisic acid responsiveness) in the promoter region of respective *AQP* genes (Supplementary Fig. 2). *SbAQP* genes were significantly up-regulated under cold stress except for *SbPIP1;5*, *PIP1;6*, *PIP2;1*, *PIP2;3*, *PIP2;4*, *PIP2;6* and *TIP4;2* (Fig. 4A). This type of expression pattern has been reported to aid in enhancing the cold stress tolerance of plants, ultimately improving water transport during cold stress (Li et al., 2008). During salt stress, *PIP1;6*, *PIP2;1* and *TIP4;3* were down-regulated, while the rest of the *AQP* genes were significantly up-regulated (Fig. 4A). Increased expression levels may regulate the uptake of water into cells and dilute NaCl accumulation in root cells (Suga et al., 2002). During heat stress, the expression of *SbPIP1;1*, *SbPIP1;2*, *SbTIP1;1*, *SbTIP3;1*, *SbNIP2;1* and *SbSIP1;2* were significantly up-regulated, while *SbPIP1;5*, *PIP1;6*, *PIP2;1*, *PIP2;4*, *PIP2;6*, *SbTIP3;3*, *TIP4;2*, *TIP4;3* and *SbNIP3;2* were down-regulated; other genes were moderately up-regulated during heat stress (Fig. 4A). The expression of SbAQPs under drought stress treatment was significantly up-regulated, with *PIP2.4; PIP2;5* and *PIP2;9* showing the highest expression levels compared to other members; in contrast, *PIP1;6*, *PIP2;1*, *PIP2;4*, *PIP2;10* and *TIP4;2* were predominately repressed under drought stress (Fig. 4A). In summary, SbPIP, TIP and NIP subfamily members were down-regulated in the ABA treatment and up-regulated in the drought, heat, cold and salt stresses with some exceptions like *PIP1;6*, *PIP2;1*, *PIP2;4*, *TIP4;2* and *NIP3;2*. All the *SbSIP* genes were upregulated across the abiotic stress treatments. In the present study, some *SbAQP* genes were constitutively expressed, while others exhibited a distinct expression pattern under different forms of environmental stress, implying that SbAQP family genes reflect their functional diversity. These results increase our knowledge of the molecular mechanisms behind the actions of SbAQPs in plant stress acclimatization. The diverse expression patterns suggest that these genes may perform different physiological functions in order to adapt to complex constraints. This study provides the first step towards future studies of *SbAQP* genes under diverse environmental conditions, such as the creation of a detailed list of SbAQP isoforms and sub-family descriptions, and comparisons to sub-families of rice and maize.

3.6. Tissue specificity of Sorghum AQPs

Sorghum AQP family gene expression in different tissues, including seedling, root, panicle and seed, displayed a complex differential expression pattern (Fig. 4B). This expression pattern reflected their physiological functions in each tissue. Among the four tissues tested, seedling, root and panicle showed a higher level of AQP abundance than the seed, thereby indicating their role in these tissues during intense developmental processes. *SbAQP* genes such as *PIP2;3*, *PIP2;5*, *PIP2;8*, *PIP2;9*, *TIP2;1*, *TIP2;3*, *TIP3;1*, *NIP1;1*, *NIP1;2* and *NIP4;1* were up-regulated in all tested tissues, while *PIP2;10* and *TIP4;2* were repressed (Fig. 4B). SbAQPs such as *TIP3;1* and *PIP2;9* had higher transcript levels in root tissue (Fig. 4B). The SbAQP *PIP2;5*, *PIP2;9*, *TIP2;1 TIP3;1* and *TIP5;2* genes were expressed at lower levels in seeds compared to other tissues, whereas *TIP1;1*, *TIP4;3* and *NIP2;2* were mainly expressed in the panicle and seed (Fig. 4B). Interestingly, some genes were exclusively detected in specific tissues, such as *PIP2;9* in seeds and *PIP1;5* and *NIP1;1* in roots (Fig. 4B). In summary, all SbAQP subfamiliy genes were

Table 3
Putative cis-acting elements identified in the SbAQP promoters and their occurrence and position. The cis-motifs identified 2500 bp upstream of the SbAQP candidate genes in relation to the transcription start site by using in silico search against PlantPan, PlantCARE and PLACE databases.

S. no	Gene	GCN4	HSE	LTR	MBS	ABRE
1	PIP1;2		−1921,+290,−101	−94	+1402,+74,−70	
2	PIP1;5		−629,−560,−430		+1317,+858,+829,	+82
3	PIP2;1		+925		−2214,+1585, −1308,−1216	−1442
4	PIP2;2	+2365		−2218,+740, −571	−2098,−844,+18	−1613,−809, −620
5	PIP2;3	+1758		−506	−777	−2095,−742,
6	PIP2;4		−1650,+1239		+1932,−1619,+803, −789,+444,−119	−1959,−1168, +1092
7	PIP2;5	−328	+594			
8	PIP2;6			+1773	−2266,+2259	−2493,+2310,−249
9	PIP2;8	+939		+524	−1417	−1837,−1615, +2390,+133
10	TIP1;1	−2107	+2391,−2284,−1945, −2286,−733	+2454,+1236		−1618,+791, −1155, +596
11	TIP1;2	−2369, +1883	−195,	+792		+2333,+2316,+1643, +1415,+444
12	TIP2;1				+2412,−2182,+2353, +535,−539,	−209
13	TIP2;2		+2349,+2305, +1529			−349
14	TIP2;3	−2472	+2116,+1398	−1393	−1598,−1365,+893	+2265,−1154, −1149,+230
15	TIP3;1		−2311	−285	−1877,−1430,−484	+589,−529,−213, +71,−43
16	TIP3;2			+526	+717,+710	+186
17	TIP3;3				+2380,+2040, +1726,+407	−825
18	TIP4;2		−496,−495	−2194	+1981,−793,+230	−1490,+1507, +1382,−739
19	TIP4;3				+2270,−1907,+1689, −1593,−900	+1611,+712,+707, −629
20	TIP4;4	+20,	−373,	−179		−1117
21	TIP5;1	+97			−92	−300
22	TIP5;2		+907	+112,−96	+2307,−1837,−1349	+2282,−2250,−1091, −1061,+1059
23	NIP1;1	+2434	−1543,−1294,−484,+596, +1742, +2335,−2233	+1946,+1081, +391	+1976, +1673,−294	
24	NIP1;2				+1089,+722, +715,−330	+1379,+1373, +1036
25	NIP1;3	+2080	+1159		+707,+28	−2426,−1227
26	NIP1;5	−1264, +1026	+2136,+1469, +1179	−738,−1238	+694	+1424,+547
27	NIP2;1	−2169, +1923, −1308		−991	−2187,−2185,−1815, +1639,+1344,+868, +789,+768	+1728,+599
28	NIP2;2	+924	+414,−679		−237	
29	NIP3;1	+1907	−1296,−1208, +468		−2294,+1687,+683, +260,−199	−2356,+2038
30	NIP3;2	+1557,+1603,−1338, +1080,−70	+1165	+161	+2114,−1333,−1084, +649, −515,+61,+50	+1642,+959
31	NIP3;3	−1048			+1178,−1074,+544	−1008,−578,+535
32	NIP4;1		+875	−1241,+878,−869, −862,−545	−1548,−701	−91
33	SIP1;1	−2160	+2409,−1184,+1620,−494,+200	−1029		+2424,−885
34	SIP1;2		−1537,−1196, −486,−173	−2350,+2324, −2076,+82	−2095,+1527,−1114, +1094,−890	+2289,+2285
35	SIP2;1	−199	−1517,−492	−1495,−373		+270

upregulated in the panicle, root and seedling tissues with some exceptions like *PIP2;7*, *TIP3;3*, *TIP4;2*, *TIP4;3*, *TIP5;1*, *TIP5;2*, *NIP1;4* and *NIP1;4*. In case of seeds, all the SbAQP subfamily genes did not follow any unique pattern resulting in their differential expression. Based on these results, it is clear that *SbAQP* genes may play a role during normal plant development in a tissue-specific manner. A detailed study of the expression patterns of *SbAQP* gene family members would facilitate a more comprehensive understanding of the specific functions of these genes. Further studies can aid in selecting candidate genes for functional analysis of their role in specific tissues.

3.7. In silico analysis of SbAQP promoter regions

To identify putative cis-acting elements in the promoter region of *AQP* genes, genomic sequences located approximately 2500 bp from the translational start site were identified and extracted using the PlantCARE, PlantPAN and PLACE databases (Supplementary Fig. 2 and Table 3). In silico sequence analysis showed that the promoter of each gene contained an important putative cis-acting element, such as the ABA-response elements (ABREs) denoting possible ABA dependent regulation (Hobo et al., 1999), dehydration responsive elements (MBS,

RY element	SKN1	Circadian	TGA	W-box
+2086	+2494,−1142	+625	+1889	
	+1458,+1406,−1204,	−1023		
	−1109,+387			
+1854,+2385,	−1888,+1881	−534,−573	+2451,	−579
+874			−2202	
	+1929,+1709,−1466,−1302,	+1686,+1379	+1702	−2099,
	+1150,−694,+285,−99			−17
	−2108,−1802,+1755,−1383,	−1690	−562	+2038
	−1221, +1082,+786,−17			
+1335	−2158,+1613,+1337,−673,	−2427	+2258,	−1741
	+444,−524,+1177,−231		−2112	
	+1709,−1330,−926,−179	+1504		
+2376	+1092,−541	−1529	+2343	
+2138,+1157	−2376,−2204,+2039,+1888,	−2377,−2339,	−1827	−508
	+1342,−1122,−1124,+507	−1213,+656		
	−1314,−1312,−669,−585,		−878,−682	+257
	−418,			
+1433,+640,	+2345,−1730,−1351,−1312,	−1189,−480,	+2091	+1095
+140	+1030,+142	+350		
+1212	+2332,+1781,+1661,−1452,	−291,	−1025	+540
	−815,−1439,−762,+723,			
	−421,+304			
	−1908,+842	+131		
	−1591,+1548,+971,+577			
+413	+2487,−2395,−2397,	−807		
	−2111,−1164,			
+315		−1509,+525,	+2086,+1108,	−2041
			+841,+388	
+535,	−374		+2054,+2038,	
			−1750	
	−780,+156,		+1601,−1618	+2154,
				−817
+570	+1454,−977,−814,+115			−1561
+1034,+1001,	−1993,+1935			+1350,
+250				+2087
+1592,+2370	+1157,−2149,−664,−2480,	−1460,−854,	+1065,	
	+994,+288,+269,−224	281		
	+920,−802	−781		
	−1210,−464	−2288,+494	+1349	
	−1452,+1436,−1263,	+2007	+1783,	−693
	−1450,−967		−987	
	+1328,−678,+435,	+2036		
	−430,−432			
	+562,+712,+1437,+562	−1803,−1233	−1700,+1515,	
			+364	
	−2283,−1638,−1516	−1152,−250		−156,
				+497
	+2238,1537,−1337,	−1981,+613		
	−1339,−251			
	−1156,+635			
+726	+384	+1668	−925,	+1462,+220
+457	+1772,−1759,−1462,	−1385	−7	
	−325,−210			
+1578	−2171,−2123,			−2408
	2090,−2490			
	+1085	−122		

DRE/CRT and G/ACCGCC), low temperature responsive elements (LTRE and CCGAC), heat shock elements (HSEs), cis-elements necessary for induction of many heat shock induced genes (Rieping and Schoffl, 1992), auxin-responsive element (TGA), and hormone responsive elements (CGTCA-motif, TGACG-motif, and TCA). Additionally, several cis-acting motifs related to meristem and seed tissue specific elements (RY and CCGTCC-box), the Skn-1 motif and the GCN4 motif that confers endosperm-specific gene expression were also enriched. The RY motif is a functionally important cis-motif found in many seed-specific gene promoters (Baumlein et al., 1992; Bobb et al., 1997). This seed-specific motif is absent in SbNIPs (except NIP1;1) and SbSIPs. SbTIPs (TIP2;1, TIP2;3, TIP3;1, TIP3;3 and TIP4;4) contained an RY motif and displayed the highest expression in seeds when compared to other plant parts (Fig. 4). Such a tissue-specific expression of *AQP* genes reveals an important role in plant development. The circadian element that is involved in circadian control (Pichersky et al., 1985) is an important factor involved in the regulation of AQPs, particularly during diurnal rhythmicity. Circadian elements were found in most of the SbAQP promoters with some exceptions (TIP2;3,TIP3;2, TIP4;2, TIP4;3, TIP4;4, TIP5;1, TIP5;2, NIP3;3 and SIP1;2). Interestingly, all SbPIP promoters have at least one

circadian motif in their sequences, which may be responsible for diurnal expression regulation patterns (Lopez et al., 2003; Takase et al., 2011; Yamaji and Ma, 2007). Hence, it can be speculated that tissue-specific and stress-related cis-elements in the promoters may be responsible for the multifarious roles of AQPs through complex regulatory mechanisms. Details of this analysis are depicted in Supplementary Fig. 2 and Table 3.

4. Conclusions

In this study, we present a comprehensive assessment of AQP encoding genes in *Sorghum* and their holistic characterization in a genome-wide scale for the first time. Overall, 41 non-redundant *AQP* genes were identified in the *Sorghum bicolor* genome that were phylogenetically clustered into four distinct subfamilies. Phylogenetic comparisons of rice, maize and *Sorghum* AQP proteins showed that tandem repeats and homologous pairs were grouped together into a single class. Analysis of intron/exon length, position, and splicing suggested that introns were highly conserved within the same subfamily. Cis-motif analysis of a 2.5 kb region upstream of *AQP* genes led to the identification of several abiotic stress responsive and developmental specific cis-motifs in *Sorghum*. Expression profiling of *AQP* genes proposed a probable function in abiotic stress responses and during plant development. Further studies are required to ascertain the functions of the individual selected genes identified in this study. The results presented here will help in the design of experiments for functional validation of the precise role of selected AQPs in plant development and abiotic stress responses.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2014.12.002.

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