Tree Genetics & Genomes (2017) 13: 81
DOI 10.1007/s11295-017-1163-7

ORIGINAL ARTICLE

Identification, phylogeny, and transcript profiling of aquaporin genes in response to abiotic stress in *Tamarix hispida*

Chao Wang[1] · Liuqiang Wang[2] · Chuanping Yang[1] · Yucheng Wang[1]

Received: 23 April 2016 / Revised: 29 May 2017 / Accepted: 26 June 2017 / Published online: 8 July 2017

Abstract Aquaporins belong to the highly conserved major intrinsic protein family and are involved in the transcellular membrane transport of water and other small solutes. However, there has been little work on cloning aquaporin (AQP) family genes and characterizing their functions in plants under various environmental stimuli. In this study, a total of 18 full-length *AQP* genes were identified in *Tamarix hispida*, a woody halophyte. Sequence analysis showed that most of these AQP proteins have six transmembrane domains connected by five loops. Phylogenetic analysis revealed that the members of the AQP family can be divided into four groups based on their structural characteristics, including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). Furthermore, the expression profiles of *AQP* genes were analyzed in the roots, stems, and leaves under salinity, drought, heavy metal, and abscisic acid (ABA) treatments using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. The results demonstrated that the AQP were involved in abiotic stress responses, indicating that they play important roles in response to abiotic stress and are involved in ABA-dependent stress-signaling pathways. These data will be useful to elucidate the complexity of the *AQP* gene family and the molecular mechanisms of abiotic stress tolerance.

Keywords Abiotic stress · Aquaporin · Expression pattern · Phylogenetic analysis · *Tamarix hispida*

Abbreviations

AQP	Aquaporin
ABA	Abscisic acid
NJ	Neighbor-Joining
ORF	Open reading frame
RPKM	Reads per kilobase exon model per million mapped reads
QRT-PCR	Quantitative reverse transcription polymerase chain reaction
PIPs	Plasma membrane intrinsic proteins
TIPs	Tonoplast membrane intrinsic proteins
NIPs	NOD26-like intrinsic proteins
SIPs	Small basic intrinsic proteins

Introduction

Plants constantly experience complex and variable stresses during their growth and development, including salinity, drought, heavy metals, and extreme temperatures. Therefore, plants have evolved a series of protective mechanisms for adequate growth and reproduction. The effectiveness of water transporters, such as the aquaporins (AQPs), is an important component of the plant response to stress (Kaldenhoff and

Chao Wang and Liuqiang Wang contributed equally to this work.

Communicated by C. Dardick

Electronic supplementary material The online version of this article (doi:10.1007/s11295-017-1163-7) contains supplementary material, which is available to authorized users.

✉ Yucheng Wang
wangyucheng@ms.xjb.ac.cn

[1] State Key Laboratory of Tree Genetics and Breeding, Northeast Forestry University, 26 Hexing Road, Harbin 150040, China

[2] State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and Cultivation of the State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, China

Fischer 2006). The AQPs belong to the highly conserved major intrinsic protein family, also called water channels, and interact with the cell membrane system to facilitate the transport of water and a variety of low-molecular weight solutes, such as glycerol and volatile substances (Maurel et al. 2008; Mitani-Ueno et al. 2011). Currently, large numbers of *AQP* genes have been identified in different plant species, including 35 homologs in *Arabidopsis thaliana* (Johanson et al. 2001), 31 in *Zea mays* (Chaumont et al. 2001), 33 in *Oryza sativa* (Sakurai et al. 2005), and 71 in *Gossypium hirsutum* (Park et al. 2010). On the basis of subcellular localization and sequence homology, plant AQPs can be largely divided into at least four different subfamilies, including plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26 intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs) (Gupta and Sankararamakrishnan 2009).

All members of the AQP family are conserved in plants and characterized by six transmembrane domains consisting of α-helices (TM1–TM6) that are connected by five interhelical loops (A–E). The cytosolic loop (loop B) between the second and third transmembrane domain and the extra-cytosolic loop (loop E) between the fifth and sixth transmembrane domain form the aqueous pore. Loops B and E also contain the highly conserved NPA (Asn-Pro-Ala) motifs, which overlap the middle of the lipid bilayer of the membrane forming a 3-D "hourglass" structure where the water flows through (Tajkhorshid et al. 2002). The second constriction is known as the aromatic/arginine (ar/R) selectivity filter and is formed on the extracellular side of the pore by four residues from transmembrane helices (H2 and H5) and loop E (LE1 and LE2). The ar/R filter is able to selectively bind water molecules and prevent other molecules from entering the aqueous pore. Based on the specific structural features, plant AQPs may confer a broad spectrum of selectivity for water and/or solutes (Törnroth-Horsefield et al. 2006).

Although the PIP, TIP, NIP, and SIP subfamilies are conserved in plants, plant AQPs have divergent functions. For example, it is generally known that AQPs play a major role in maintaining water balance by regulating the water transport system in the root to protect against a variety of environmental stimuli and facilitate water transport through inner leaf tissues during transpiration (Maurel et al. 2008; Nguyen et al. 2013). Recently, increasing evidence has demonstrated that plant AQPs play several important roles in numerous physiological processes, including seed germination (Liu et al. 2013), plant reproduction (Bots et al. 2005; Kaldenhoff and Fischer 2006), cell division and elongation (Okubo-Kurihara et al. 2009), fruit ripening (Mut et al. 2008), and leaf movements (Uehlein and Kaldenhoff 2008; Heinen et al. 2009). Furthermore, AQPs promote carbon and nitrogen fixation through their involvement in the passage of many substrates, such as H_2O_2, urea, silicic acid, methylated arsenic species, and ammonia (Bienert et al. 2006; Maurel et al. 2008; Li et al. 2009). However, there have been a limited number of studies on the expression of *AQP*s during the growth and development of native plants or in plant organs over their lifespan, especially in woody halophyte plant species.

Tamarix (Tamaricaceae) is a family of woody halophyte species, which is widely distributed in the saline soils of drought-stricken areas of central Asia and China. *Tamarix hispida*, a species of the genus *Tamarix*, is highly tolerant to salinity, drought, and extreme temperatures. These characteristics make the species suitable for investigation of stress tolerance mechanisms. In this study, we screened the functional annotation of non-redundant unigenes in seven transcriptomes and identified 18 *AQP* genes representing four subfamilies. Then, a comprehensive analysis of their structure and phylogeny was performed. The expression of 18 *AQP*s in the roots, stems, and leaves of *T. hispida* under abiotic stresses (salt, drought, and heavy metal) and with exogenous application of ABA was investigated using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). These systemic overviews are the first comprehensive study of the *AQP* gene family in *T. hispida* and may provide valuable information for further exploring the functions of genes mediated by the water stress-responsive *AQP* genes.

Materials and methods

Identification of AQP family genes from transcriptomes of *T. hispida*

In a previous study, seven transcriptomes were built from *T. hispida* roots treated with $NaHCO_3$ for 0, 12, 24, and 48 h and leaves treated with $NaHCO_3$ for 0, 12, and 24 h by RNA-seq using high-throughput Solexa sequencing technology. The raw reads from each library were respectively assembled into contigs using the SOAPdenovo software; the contigs were further assembled into scaffolds using paired-end joining and gap filling, and tentative unigenes (TUGs) were generated by assembling these scaffolds from each library. Sequence overlaps in the sorted results files are filtered using user-defined criteria: the minimum length of the overlap (default 40 bp), the minimum percent of repeat stringency (default 95%), and the maximum unmatched overhangs (the default starts at 30 nucleotides). The general characteristics of the seven transcriptomes and the length distributions of the unigenes are described in Wang et al. (2014a). A total of 94,359 non-redundant unigenes were generated using the TGI clustering tools (Pertea et al. 2003), and these unigenes were subjected to BLASTX (http://blast.ncbi.nlm.nih.gov/) analysis against the NR and Swiss-Prot protein databases to search for functional annotation. The unigenes with a threshold *E* value of more than 1×10^{-5} were discarded. The key word "aquaporin" was used as a query to search against the

functional annotation of non-redundant unigenes for identifying the *AQP* genes. The open reading frame (ORF) of each *AQP* gene was resolved using ORF Finder from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Then, the primers for amplifying the ORF of *AQP* genes were designed according to these sequences. The PCR products were ligated into the pMD18-T Easy vector (TaKaRa) and subsequently sequencing were used to confirm the *AQP* sequences. Primer sequences are shown in Table S1.

Bioinformatics analysis of the AQP family genes

The isoelectronic point (p*I*) and theoretical molecular weight (M_W) predictions of deduced AQPs were calculated with the ExPASy compute p*I*/M_W tool (http://www.expasy.org/tools/protparam.html). Sequence identity among the AQPs was calculated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). The transmembrane helical domain was predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al. 2001). Prediction of the subcellular localization of putative AQPs was performed using the WoLF PSORT algorithm (http://www.genscript.com/wolf-psort.html) (Horton et al. 2007). Sequence alignments of related proteins were performed with ClustalW using BioEdit software and adjusted manually. The unrooted phylogenetic tree was constructed with MEGA 5.05 using the neighbor-joining (NJ) method, and the internal branch support was estimated with 1000 bootstrap replicates (Tamura et al. 2011). Next, the conserved motifs in the full-length protein sequences were identified using the MEME online program (http://meme-suite.org) with the following parameters: number of repetitions = any, maximum number of motifs = 20, and optimum motif width = 30 to 70 residues (Bailey et al. 2009).

Plant growth and stress treatments

Seeds of *T. hispida* were grown in pots containing a mixture of turf peat and sand (2:1 *v*/*v*) under controlled greenhouse conditions of 70–75% relative humidity, 14 h light, 10 h dark, and an average temperature of 24 °C. Two-month-old seedlings were exposed to 300 mM $NaHCO_3$ for 12, 24, and 48 h. Following the treatment, roots, and leaves from at least 24 seedlings of each group were harvested and stored at −80 °C prior to RNA extraction for building transcriptomes. In addition, the seedlings were exposed to the following solutions: 400 mM NaCl (salt stress), 25% (*w*/*v*) PEG6000 (PEG-simulated drought stress), 150 μM $CdCl_2$ (heavy metal stress), or 150 μM ABA for 0, 3, 6, 9, 12, or 24 h. A fresh water-only control was conducted in parallel. Following these treatments, the leaves, stems, and roots of seedlings from each sample (at least 10 seedlings) were harvested at the indicated times and stored at −80 °C until required.

RNA isolation and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from each sample by the CTAB method with minor modifications and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) to remove genomic DNA contamination. The first-strand cDNA was synthesized with approximately 0.5 μg of purified total RNA using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The synthesized cDNAs were diluted to 100 μL with sterile water and were used as the template for real-time qRT-PCR. qRT-PCR was performed on the Opticon 2 System (Bio-Rad, Hercules, CA) using SYBR Green Real-time PCR Master Mix (Toyobo) and specific primers (Table S1). The reaction mixture (20 μL) contained 10 μL 2 × SYBR Green Real-time PCR Master Mix, 0.5 μM of each of the forward and reverse primers, and 2 μL of cDNA template. The amplification was completed with the following cycling parameters: 94 °C for 30 s; followed by 45 cycles at 94 °C for 12 s, 58 °C for 30 s, 72 °C for 40 s, and 1 s at 79 °C for plate reading. A melting curve analysis of the product was performed immediately after the final PCR cycle by increasing the temperature from 55 to 90 °C at 0.5 °C s^{-1}. To ensure the reproducibility of the results, at least three biological replicates, each with three technical replicates, were performed for each sample. The relative expression ratios were calculated from the threshold cycle according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The relative expression (ΔΔCT) of each gene was normalized to the geometric mean of *α-tubulin*, *β-tubulin*, and *β-actin* as internal control genes (Vandesompele et al. 2002). We used $2^{-\Delta\Delta Ct} > 2$ and *p*-values <0.05 as a threshold to significance of gene expression differences. All of the relative expression levels were $\log_2$ transformed.

Results and discussion

Identification and classification of the *AQP* genes in *T. hispida*

Previously, we generated seven transcriptomes from the roots and leaves of *T. hispida* treated with $NaHCO_3$ at various time intervals. The general characteristics of the seven transcriptomes and the length distributions of the unigenes are described in Wang et al. (2014a). After functional annotation, a total of 34 non-redundant unigenes were identified as AQP family genes. However, because genome sequencing of *T. hispida* is incomplete, we cannot determine the exact number of *AQP* genes in *T. hispida*. To further confirm the reliability of the identified genes, a BLASTP and BLASTX program search of the NR protein and Swiss-Prot databases and an online domain batch search with an E-value of more than

1×10^{-5} were performed. Among them, 18 non-redundant unigenes with full open reading frames (ORFs) were identified in the NR protein database. The genes with 6 homologs classified as plasma membrane intrinsic proteins (PIPs), 5 homologs classified as tonoplast intrinsic proteins (TIPs), 5 homologs classified as NOD26-like intrinsic proteins (NIPs) and 2 homologs classified as small basic intrinsic proteins (SIPs), respectively. The GenBank accession numbers of *ThAQP*s are shown in Table S1. A comparison of 18 deduced AQP protein sequences and those of other plants revealed a high sequence identity at the protein level (67–91%) and at the nucleotide level (55–82%). The amino acid number varied from a minimum of 240 to a maximum of 307, with predicted sizes ranging from a minimum of 25.47 to 31.59 kDa and p*I* values ranging from 4.8 to 9.76 (Table 1).

Structure and phylogenetic analysis of the AQP proteins in *T. hispida*

Sequence homology analysis of the predicted peptide sequences of these AQPs showed that high sequence diversity exists among the four subfamilies of AQPs in *T. hispida*. The highest sequence identity of 27.46–36.73% was found between PIPs and TIPs, and the lowest sequence identity of 16.36–21.76% was found between NIPs and SIPs (Table S2). To examine the phylogenetic relationships between the AQP proteins in *T. hispida* and *Populus trichocarpa*, the AQP protein sequences from the *T. hispida* transcriptomes were subjected to BLAST against the *Populus trichocarpa* proteome (v3.0), which was downloaded from the MIPModDB database (http://bioinfo.iitk.ac.in/MIPModDB/) (Gupta and Sankararamakrishnan 2009; Gupta et al. 2012). Then, the 53 putative *Populus* AQP protein sequences were collected for phylogenetic analysis (Fig. 1). Based on the predicted amino acid sequences, the ThAQPs could be classified into four groups of PIPs, TIPs, NIPs, and SIPs, with different cellular localizations and functions (Zardoya 2005), and designated as ThPIP1;1, ThPIP2;1 to ThPIP2;5, ThTIP1;1 to ThTIP1;3, ThTIP2;1, ThTIP4;1, ThNIP1;1 to ThNIP1;2, ThNIP3;1 to ThNIP3;3, ThSIP1; and 1 to ThSIP1;2. This observation is consistent with previous phylogenetic analyses of plant AQPs (Gupta and Sankararamakrishnan 2009; Rasheed-Depardieu et al. 2012). In addition, we further analyzed the motif distributions among these 18 ThAQP proteins using the MEME program, and a total of 14 conserved motifs were identified and named 1–14 (Fig. 2a). The consensus sequences of these motifs are given in Fig. 2b. Of these, motif 4 was found to be in all the ThAQPs. With the exception of the ThSIPs, the remaining ThAQPs possessed motifs 1, 2, and 3. The four motifs

Table 1 Properties of aquaporin genes and their deduced proteins

Name	ORF (bp)	Deduced peptides			Highest similarity in other plants (%)
		Length (aa)	*M*w (KDa)	p*I*	
ThPIP1;1	864	287	30.924	8.84	*Sesamum indicum*, XP_011084932 (91)
ThPIP2;1	846	281	30.089	8.92	*Theobroma cacao*, XP_007041804 (90)
ThPIP2;2	852	283	30.048	8.51	*Vitis vinifera*, AAV69744 (84)
ThPIP2;3	825	274	29.34	8.30	*Sesamum indicum*, XP_011084932 (83)
ThPIP2;4	861	286	30.567	7.63	*Nicotiana sylvestris*, XP_009772697 (88)
ThPIP2;5	810	269	28.76	9.41	*Malus prunifolia*, AEQ29857 (78)
ThTIP1;1	759	252	25.882	5.38	*Nelumbo nucifera*, XP_010267262 (90)
ThTIP1;2	759	252	26.099	4.80	*Eucalyptus grandis*, XP_010037134 (83)
ThTIP1;3	762	253	26.256	5.49	*Vitis cinerea var.*, AF271660_1 (87)
ThTIP2;1	753	250	25.47	5.67	*Glycine max*, XP_006582836 (83)
ThTIP4;1	747	248	26.001	6.02	*Nicotiana tomentosiformis*, XP_009612974 (81)
ThNIP1;1	852	283	30.063	7.65	*Vitis vinifera*, XP_002264957 (75)
ThNIP1;2	861	286	30.538	6.90	*Tarenaya hassleriana*, XP_010528527 (76)
ThNIP3;1	924	307	31.552	8.21	*Populus euphratica*, XP_011020626 (77)
ThNIP3;2	906	301	31.191	6.22	*Sesamum indicum*, XP_011083233 (77)
ThNIP3;3	909	302	31.598	8.87	*Sesamum indicum*, XP_011083233 (80)
ThSIP1;1	729	242	25.819	8.44	*Ricinus communis*, XP_002525471 (67)
ThSIP2;2	723	240	26.347	9.76	*Populus trichocarpa*, XP_002322583 (69)

The open reading frame and protein length are detailed for all genes identified in this study. The highest sequence identity between the predicted amino acid sequences of aquaporins and those of other plants was determined using BLASTP program. The parentheses indicate the percentage of sequence identity at the amino acid level

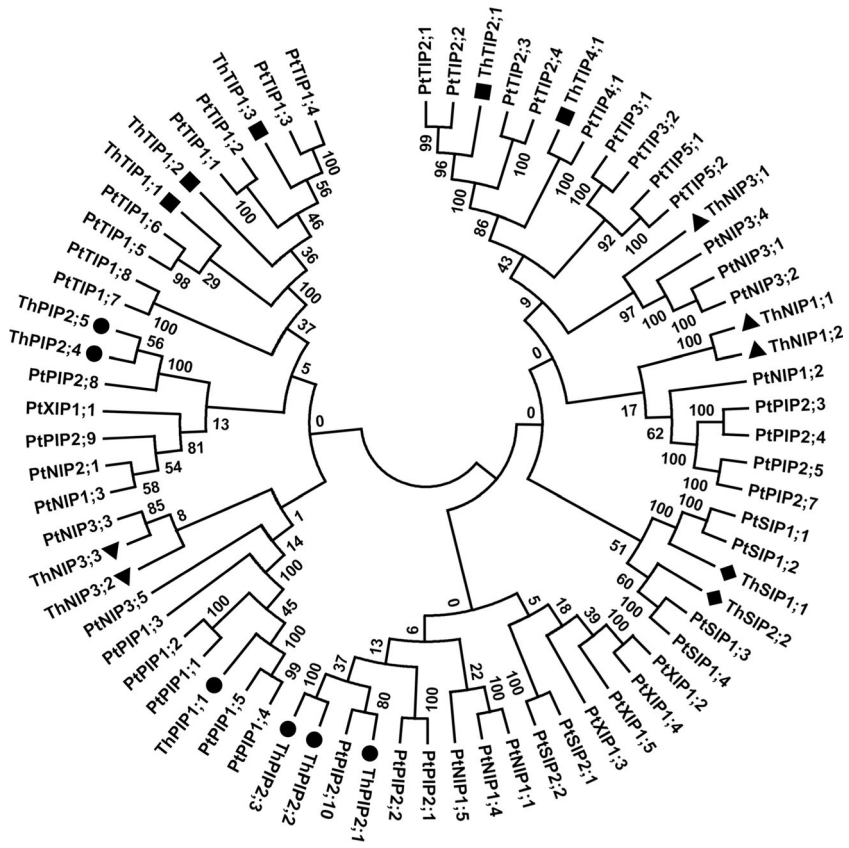
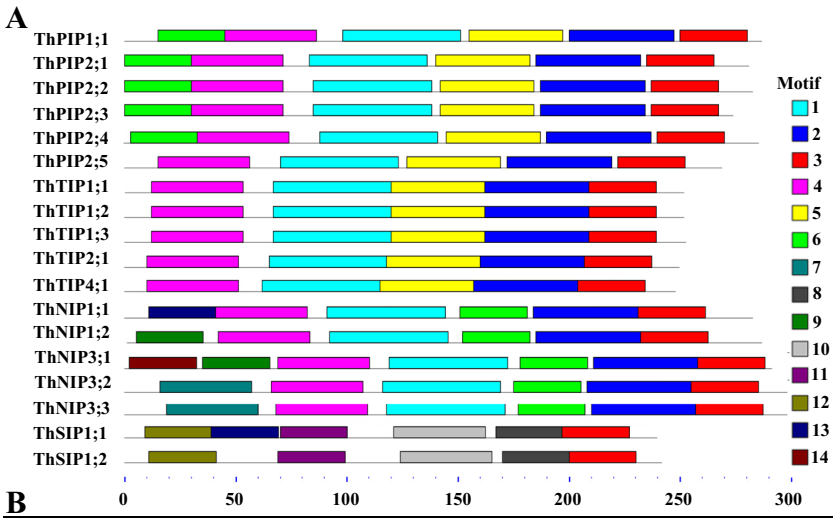


Fig. 1 Phylogenetic analysis of aquaporin proteins from *T. hispida* and *Populus*. Predicted amino acid sequences were aligned using ClustalW2, and the unrooted neighbor-joining tree was constructed using MEGA 5.05 with 1000 bootstrap replicates. Bootstrap support values are indicated on each node. The sequences of the poplar aquaporin proteins were downloaded from the MIPModDB database (http://bioinfo.iitk.ac.in/MIPModDB/) (Gupta and Sankararamakrishnan 2009; Gupta et al. 2012)

correspond to the main transmembrane domain regions. Motif 5 was mainly found in the ThPIPs and ThTIPs, and motif 6 was only present in the ThPIPs and ThNIPs. The remaining 8 motifs were specifically found in the ThNIPs and ThSIPs. Prediction of TMDs (transmembrane domains) by TMHMM Server v. 2.0 showed that most identified putative AQPs contained a typical topology of six TMDs with the exception of ThTIP1;1, ThTIP1;2, ThTIP2;1, ThNIP3;1, ThNIP3;3, and ThSIP1;2. Of these, ThTIP1;1, ThTIP1;2, and ThTIP2;1 had seven TMDs, whereas ThNIP3;1 and ThNIP3;3 had five TMDs, and ThSIP1;2 had four TMDs (Fig. 3, Table 2). The cellular localization prediction analysis showed that the six PIP subfamily members were localized to plasma membranes, which is in agreement with the current literature (Table 2). The TIP members were diverse and predicted localizations were in the cytosol and vacuolar membrane. NIP members were generally predicted to be localized to the plasma membrane. The two members of the SIP subfamily were predicted to be localized to the cytosol and vacuolar membrane.

We further analyzed several features, including the highly conserved dual NPA motifs (NPAI and NPAII), the nature of the ar/R selective filter (H2, H5, LE1, and LE2), and 5 Froger's positions (P1–P5). Multiple sequence alignments of the predicted amino acid sequences of the 18 *Tamarix* AQPs and their dual NPA motifs, ar/R selective filter and Froger's positions are illustrated in Fig. 3, Table 2. In PIPs, the dual NPA motifs and ar/R selective filter (F-H-T-R) are highly conserved across all plant species, the Froger's positions P2 to P5 are strictly conserved and consist of S-A-F-W, and only the P1 position is more variable and can be Met (M), Gln (Q), Gly (G), or Tyr (Y) (Venkatesh et al. 2013). Members of the PIP subfamily in other plant species have been shown to be positively regulated in their water transport activity via phosphorylation (Azad et al. 2008; Whiteman et al. 2008). All of the ThPIP members in *T. hispida* contained the highly conserved dual NPA motifs, exhibited identical amino acids in the ar/R selectivity filter (F-H-T-R) with the exception of ThPIP2;2, in which Thr (T) was replaced with Cys (C) in the LE1 position, and had either Met (M) or Gln (Q) in the P1 position. In the TIPs, dual NPA motifs and the P3, P4, and P5 positions are highly conserved, but the selectivity filter (ar/R) is highly variable across plant species (Venkatesh et al. 2013). However, among the five ThTIPs, dual NPA motifs, ar/R and Froger's positions exhibited no variation as the expected amino acids were identified, such as His (H), Thr (T), Ser (S), and Ala (A) in the H2, P1, P2, and P3 positions, respectively, and the TIPs are characterized by Ala (A) in the LE1 and an unusual Val (V) instead of Arg (R) in the LE2. However, ThTIP2;1 deviated from this pattern and had G (LE1) and R (LE2) residues. ThNIP1;1 and



B

Motif	Width	Best possible match
1	53	MIMVMVYCTAHISGGHINPAVTFGLFLCRKFPWKRAPMYWIAQCLGSICACGL
2	47	KRDPRDRHIGELAPLPIGFTVMMNHLATGPIDGTSMNPARSFGPAVI
3	30	HWNWDDHWIYWVGPFIGALIAAWYYQLVQR
4	41	EMTKWDFYRKCIAEFICTFIFLYAGCLTVMVYKKQDDNEEC
5	42	QKYYYDKQGTGANALSHGYSKWNGLVMEIVMTFVLVYTVYST
6	30	PEKQMSETEPSQHEEQDYHDEPPEPFNDMF
7	41	TPGAPLVSSFRVDCLSYDRKSMPRCVCFSVGNPGYGPSHSC
8	30	KLTMHILGSDYTGPCMNPVNVFGWAYVRDW
9	30	PKEGRRDKECECFRIEEGQNEEKSDPSVSW
10	41	PKLKVDIHHGAITEGFLTFVIVFIQLGIGRKIPGGFMRKTW
11	30	SYNPLGVILFYLSGNFDDFLFSMGLRFPAQ
12	30	DFILSFMWMWCSPSIKIGVSKHLGLGYHDH
13	30	HEEIIRCSLDIDRNFLFSIMAKGNEDCGWR
14	30	CEDPNSLPSDFILPFMWGWPFFGRKILVGK

Fig. 2 Distribution of conserved motifs in the aquaporins proteins. **a** The motifs were identified by MEME. Different motifs are indicated by *different colored numbers* (*1–14*). **b** The detailed motif sequences are shown

ThNIP1;2 showed typical dual NPA motifs, while in ThNIP3;1, ThNIP3;2, and ThNIP3;3, Ala (A) is replaced with Ser (S) in the first NPA motif and Val (V) in second NPA motif. Similar features were observed in the *Arabidopsis* NIP5;1 and NIP6;1 (Miwa et al. 2010). With the exception of the LE2 position, plant NIPs are highly variable in the ar/R selectivity filter. The five ThNIPs showed the expected Trp (W)/Gly (G)/Ala (A)/Thr (T) residues in the H2 position with the exception of ThNIP3;1, which had Ser (S). The H5 position is conserved and was either Ile (I) or Val (V). ThNIP3;2 and ThNIP3;3 had Gly (G) instead of Ala (A) in LE1 position, similar to the *Arabidopsis* NIP7 and potato NIP7;1 (Venkatesh et al. 2013). In Froger's positions, all the ThNIPs showed the expected amino acids. For example, Ala (A) and Tyr (Y) were in the P3 and P4 positions and Leu (L) or Ile (I) in the P5. Plant SIPs show a highly variable first NPA motif with the Ala (A) residue replaced by Leu (L), Ala (A), Thr (T), or Cys (C), but the second NPA motif is conserved. However, ThSIP1;1 showed variation in the first NPA motif, in which Ala (A) is replaced by Thr (T), and in the second NPA motif, in which Ala (A) is replaced by Thr Val (V). All ar/R and Froger's positions (P1 and P2) are highly variable. In contrast, highly conserved Ala (A), Tyr (Y) and Trp (W) residues were observed in the P3, P4, and P5 positions.

Relative abundance of the 18 *AQP* genes in the roots, stems, and leaves of *T. hispida*

The expression profiling of AQPs in the roots, stems, and leaves of *T. hispida* under normal growth conditions was determined by the real-time RT-PCR analysis. The geometric transcript levels of the three internal control genes were used as a calibrator (its expression was set as 1) to normalize the expression levels of *AQP* genes. The results showed that the level of expression varied greatly from gene to gene in each tissue (Fig. 4). *ThPIP1;1* and *ThPIP2;1* were highly abundant in root, stem, and leaf tissues. *ThPIP2;3*, *ThPIP2;4*, *ThTIP1;1*, *ThTIP1;2*, *ThTIP2;1*, and *ThTIP4;1* were relatively highly expressed in leaves compared with root and stem tissues, especially *ThTIP1;1*. The remaining genes were expressed at lower levels in root, stem, and leaf tissues.

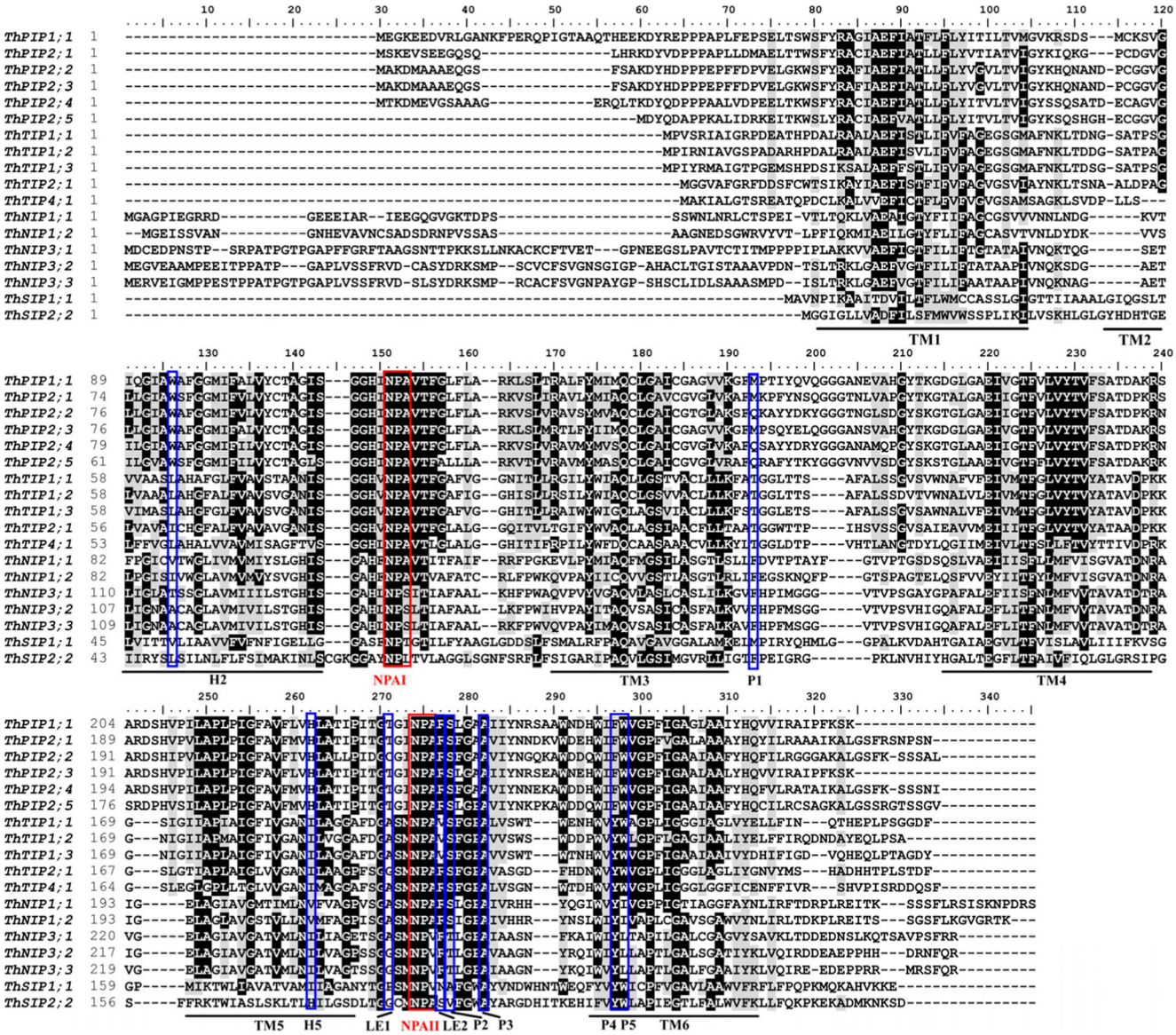


Fig. 3 Multiple sequence alignment of *T. hispida* aquaporins. Predicted amino acid sequences were aligned using ClustalW with BioEdit software. The two conserved NPA motifs (NPAI and NPAII), ar/R selectivity filters (H2, H5, LE1, and LE2) and Froger's positions (P1–P5) are *boxed* and *marked in bold* below the alignment, and the transmembrane domains (TM1–TM6) are *underlined*

Expression patterns of the *AQP* genes under different abiotic stresses

First, we analyzed the expression of these *AQP* genes in response to $NaHCO_3$ by comparing RPKM (reads per kilobase of exon model per million mapped reads) values obtained from transcriptomes (Mortazavi et al. 2008). The results showed that most of these *AQP* genes were differentially regulated by $NaHCO_3$ stress and displayed different expression patterns in response to $NaHCO_3$ (Table S3). We further studied the expression profile of each *ThAQP* in the roots, stems, and leaves of *T. hispida* under different abiotic stresses, including high salinity, PEG-simulated drought and heavy metal stresses. Two-month-old uniform seedlings were subjected to the indicated treatments, and the phenotype was observed, as described previously (Wang et al. 2014a). The expression patterns are shown in the following figures.

Under salt stress, the expression of *ThPIP2;4*, *ThPIP1;1*, *ThPIP2;2*, *ThPIP2;5*, *ThTIP1;2*, *ThTIP2;1*, *ThTIP4;1*, *ThNIP1;2*, *ThNIP3;1*, *ThNIP3;3*, and *ThSIP1;1* displayed similar expression patterns in the roots of the plants (Fig. 5a). They were all rapidly downregulated after 3 h of stress, gradually increased to their peak expression level at 9 h, and then decreased during the subsequent time points. The remaining genes were downregulated at 3 h, *ThPIP2;3*, *ThTIP1;2*, and *ThTIP1;3* were upregulated during the 6 to 24 h time period, but *ThTIP1;1* was downregulated, and the others did not markedly change during salt stress. In the stems and

Table 2 Conserved specificity-determining residues, transmembrane domains (TMDs) in aquaporins of *T. hispida* and their predicated subcellular localization

Name	NPAI	NPAII	H2	H5	LE1	LE2	P1	P2	P3	P4	P5	TMDs	BPCL[a]
ThPIP1;1	NPA	NPA	F	H	T	R	M	S	A	F	W	6	Plas
ThPIP2;1	NPA	NPA	F	H	T	R	M	S	A	F	W	6	Plas
ThPIP2;2	NPA	NPA	F	H	C	R	Q	S	A	F	W	6	Plas
ThPIP2;3	NPA	NPA	F	H	T	R	M	S	A	F	W	6	Plas
ThPIP2;4	NPA	NPA	F	H	T	R	Q	S	A	F	W	6	Plas
ThPIP2;5	NPA	NPA	F	H	T	R	Q	S	A	F	W	6	Plas
ThTIP1;1	NPA	NPA	H	I	A	V	T	S	A	Y	W	7	Cyto
ThTIP1;2	NPA	NPA	H	I	A	V	T	S	A	Y	W	7	Vacu
ThTIP1;3	NPA	NPA	H	I	A	V	T	S	A	Y	W	6	Cyto
ThTIP2;1	NPA	NPA	H	I	G	R	T	S	A	Y	W	7	Vacu
ThTIP4;1	NPA	NPA	H	I	A	R	T	S	A	Y	W	6	Cyto
ThNIP1;1	NPA	NPA	W	V	A	R	F	S	A	Y	I	6	Plas
ThNIP1;2	NPA	NPA	W	V	A	R	F	S	A	Y	I	6	Plas
ThNIP3;1	NPS	NPV	S	I	A	R	F	T	A	Y	L	5	Plas
ThNIP3;2	NPS	NPV	A	I	G	R	F	T	A	Y	L	6	Plas
ThNIP3;3	NPS	NPV	A	I	G	R	F	T	A	Y	L	5	Plas
ThSIP1;1	NPT	NPV	I	I	P	N	M	A	A	Y	W	6	Vacu
ThSIP2;2	NPL	NPA	I	H	G	S	F	V	A	Y	W	4	Cyto

Cyto cytosol, *Plas* plasma membrane, *Vacu* vacuolar membrane

[a] Best possible cell localization prediction by the WoLF PSORT tool

leaves, the members of the ThPIP, ThTIP, ThNIP, and ThSIP subfamilies displayed approximately similar expression patterns (Fig. 5b, c). ThPIP members were upregulated with the exception of several individual time points, especially the *ThPIP2;5* gene. The expression of *ThTIP1;2* was markedly upregulated, and *ThTIP1;1* was noticeably downregulated during salt stress. The remaining genes did not significantly change. However, the members of the ThNIP subfamily were upregulated under salt stress. The *ThSIP* genes were up- or downregulated in response to salt stress but did not markedly change.

Under PEG-simulated drought stress conditions, all of the *ThAQP*s, with the exception of *ThPIP2;5*, *ThTIP1;2* and *ThTIP1;3*, were rapidly downregulated in the roots of plants at 3 h, gradually increased to their peak expression levels at a later time point but were then downregulated (Fig. 6a). The expression of *ThPIP2;5* was upregulated (nearly sixfold induced) after 6 and 9 h of stress, and *ThTIP1;2* and *ThTIP1;3* were upregulated in the roots during the 6 to 12 h time points. In the stems, most of *ThAQP*s were up- or downregulated but did not markedly change during the stress (Fig. 6b). However, *ThPIP2;5* was rapidly upregulated and increased sixfold at 3 h and recovered its relatively low expression at subsequent time points. The expressions of *ThTIP1;2* and *ThTIP1;3* were markedly upregulated during the drought stress. In contrast, *ThPIP2;2*, *ThTIP2;1*, and *ThSIP1;2* expressions were downregulated by approximately twofold. In the leaves, *ThPIP2;2*, *ThPIP2;3*, *ThPIP2;4*, *ThTIP2;1*, *ThNIP1;1*, and *ThNIP1;2*

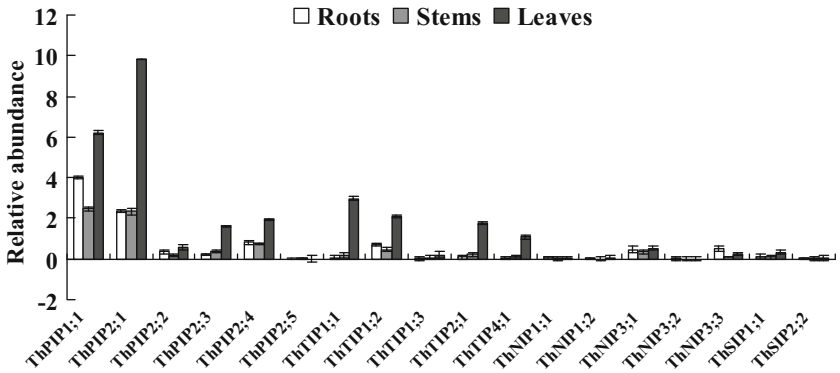


Fig. 4 Relative abundance of 18 aquaporin genes in the roots, stems, and leaves of 2-month-old *T. hispida* seedlings under normal conditions. The *error bars* indicate standard deviations, which were calculated from multiple replicates of real-time PCR

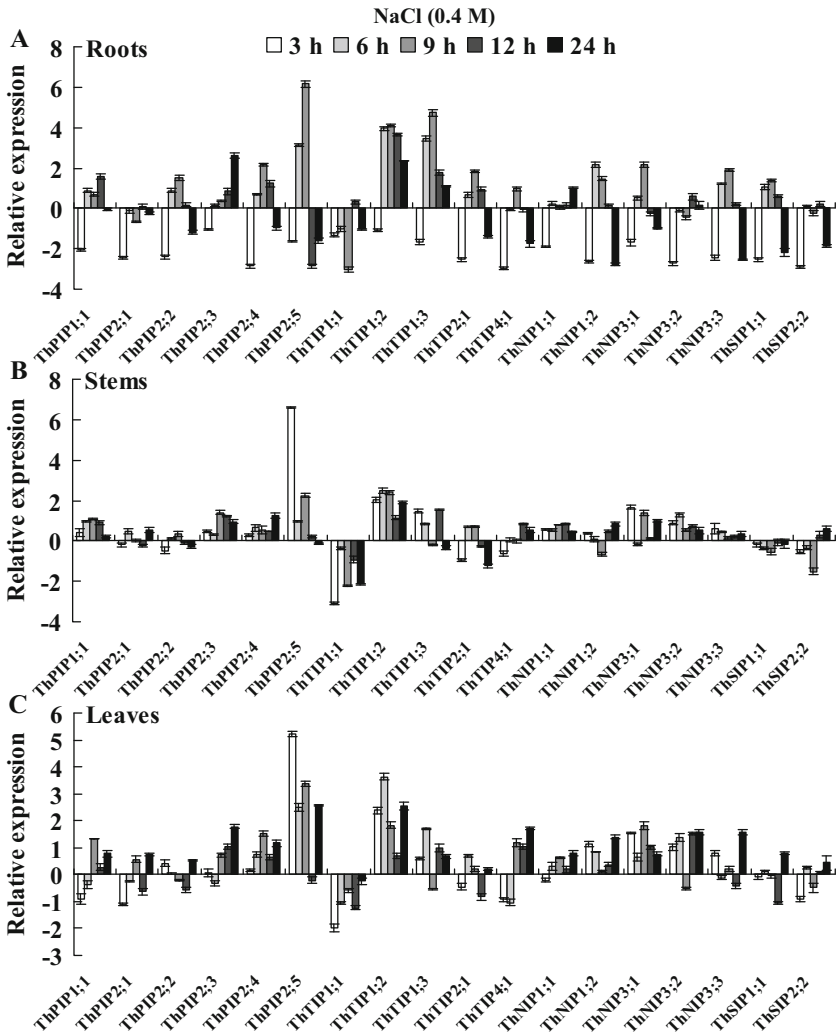


Fig. 5 Expression profiles of the aquaporin genes in the roots, stems, and leaves of 2-month-old *T. hispida* seedlings subjected to salt stress (400 mM NaCl). Relative expression level = $\log_2$ (transcription level under stress treatment/transcription level under control conditions). The *error bars* indicate standard deviations, which were calculated from multiple replicates of real-time PCR

displayed similar expression patterns (Fig. 6c). They were all initially downregulated after 3 h of stress, gradually decreased to the lowest expression levels at a later time point (6, 9, or 12 h), but they were all upregulated at 24 h of stress. *ThPIP2;1*, *ThTIP1;1*, *ThNIP3;1*, *ThNIP3;3*, and *ThSIP1;2* were downregulated during the drought stress, while *ThPIP2;5*, *ThTIP1;2*, and *ThTIP1;3* were upregulated under drought stress, and respectively reached their peak levels at 3 or 24 h (nearly fivefold induced).

Heavy metals, such as cadmium, copper, lead, chromium, and mercury are major environmental pollutants and affect various physiological and biochemical processes in plants (Nagajyoti et al. 2010; Villiers et al. 2011; Datta et al. 2011; Shen et al. 2011; Choudhary et al. 2012). In this study, the expression of *ThAQP*s in response to heavy metal (cadmium) stress was monitored. The results showed that these *ThAQP* genes can roughly be divided into two groups according to their expression patterns (Fig. 7a). The first group included the six members of *ThPIP* subfamily, *ThTIP1;2*, *ThNIP1;3*, and *ThNIP3;3*, whose expression was initially downregulated after 3 h of stress, gradually increased to their peak expression level at a later stress time point (6, 9 or 12 h), and then rapidly decreased at 24 h of stress. The second group included the *ThTIP1;2*, *ThTIP2;1*, *ThTIP4;1*, *ThNIP3;1*, *ThSIP1;1*, and *ThSIP1;2* genes that were downregulated during the heavy metal stress. In addition, *ThNIP3;2* and *ThNIP1;1* expressions were initially downregulated at 3 h and upregulated at subsequent time points. In the stems, with the exception of *ThPIP2;5*, the members of *ThPIP*s did not noticeably change during the heavy metal stress. *ThPIP2;5* expression rapidly increased to the peak level after 3 h of stress and recovered relatively low expression levels at subsequent time points (Fig. 7b). The *ThPIP*s were all downregulated during the heavy metal stress, especially *ThTIP1;2*. The expressions of *ThNIP1;1*, *ThNIP1;2*, and *ThNIP3;2* were upregulated, but the *ThSIP*s were downregulated under the stress. In the leaves, with the exception of *ThPIP2;1*, the members of *ThPIP*s were upregulated during salt stress (Fig. 7c). However, *ThPIP2;4* was downregulated at 24 h of stress and decreased more than twofold. *ThPIP2;1* expression was downregulated after 3 h of

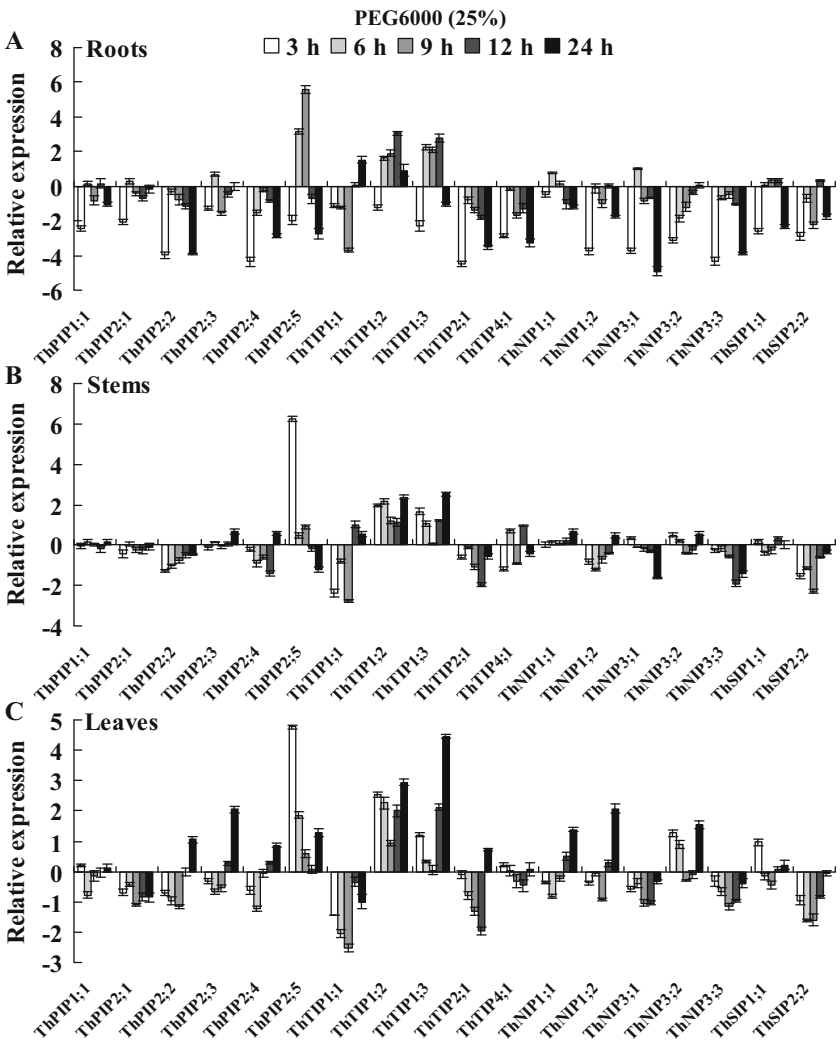


Fig. 6 Expression profiles of the aquaporin genes in the roots, stems, and leaves of 2-month-old *T. hispida* seedlings subjected to PEG-simulated drought stress (25% PEG6000) treatment. Relative expression level = $\log_2$ (transcription level under stress treatment/transcription level under control conditions). The *error bars* indicate standard deviations, which were calculated from multiple replicates of real-time PCR

stress and gradually increased at the subsequent time points. The expressions of *ThTIP1;2*, *ThTIP1;3*, and *ThTIP2;1* were downregulated in response to the heavy metal stress; notably, *ThTIP1;2* was markedly decreased fourfold to sixfold in the leaves. The *ThNIP*s were significantly up- or downregulated (> twofold) at individual time points and did not significantly change during the stress. *ThSIP1;1* was noticeably downregulated during 3 to 24 h.

Plants sense various signals from the surrounding environment and adjust their water transport properties accordingly (Li et al. 2014). Recent studies have shown that a large number of *AQP* genes were induced by different abiotic stresses, such as salt, drought, and cold. Furthermore, overexpression of some *AQP* genes significantly increased the abiotic stress tolerance in transgenic plants (Matsumoto et al. 2009; Hu et al. 2012; Zhou et al. 2012; Ahamed et al. 2012; Reuscher et al. 2013; Rodrigues et al. 2013; Sreedharan et al. 2013; Wang et al. 2014b). In this study, most of the *AQP* genes are differentially regulated by salt, drought, or heavy metal stress, indicating that they may play important roles in response to abiotic stress. For example, the *ThPIP2;2*, *ThPIP2;5*, *ThTIP1;1*, *ThTIP1;2*, and *ThTIP1;3* genes are significantly induced by NaCl and PEG stresses, indicating that they are involved in salt and drought stress tolerance. BLASTX analysis revealed that ThPIP2;2 and ThPIP2;5 had high sequence identity with *TaTIP2;2* from *Triticum aestivum* and PIP2-5 from *Prunus mum*, and *ThTIP1;1*, *ThTIP1;2*, and *ThTIP1;3* had high sequence identity with TIP1–1 from *Nelumbo nucifera* and TIP1–3 from *Eucalyptus grandis*. The wheat *TaAQP7* was very close to the PIP2 subfamily and was induced by PEG and ABA stresses. Overexpression of *TaAQP7* in tobacco increased drought tolerance by enhancing the antioxidant system, increasing the ability to retain water, and reducing ROS accumulation and membrane damage (Zhou et al. 2012). The *TaTIP2;2* gene is downregulated by salinity and drought stress. Heterologous expression of the wheat *AQP* gene, *TaTIP2;2*, compromises the drought and salinity tolerance of transgenic *Arabidopsis* via an ABA-independent pathway (Xu et al. 2013). The *Thellungiella salsuginea TsTIP1;2* gene is expressed highly in the leaves and is induced by several

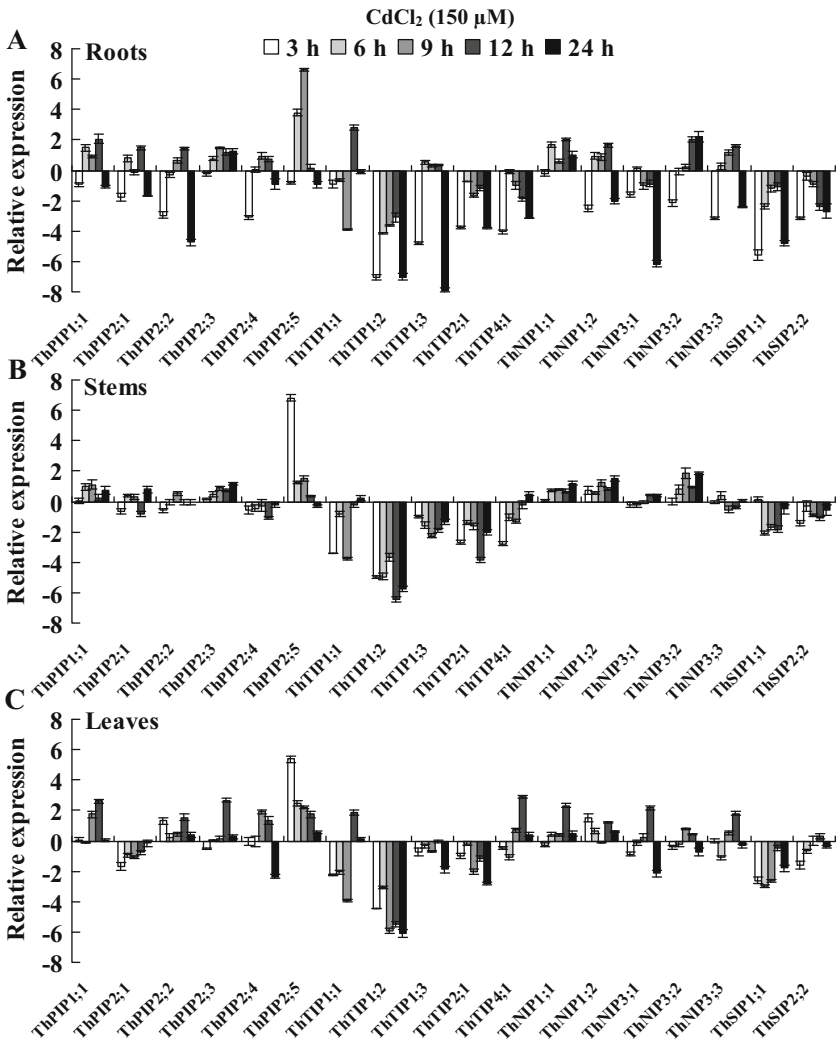


Fig. 7 Expression profiles of the aquaporin genes in the roots, stems, and leaves of 2-month-old *T. hispida* seedlings subjected to heavy metal stress (150 μM $CdCl_2$). Relative expression level = $\log_2$ (transcription level under stress treatment/ transcription level under control conditions). The *error bars* indicate standard deviations, which were calculated from multiple replicates of real-time PCR

abiotic and hormonal stresses. Ectopic overexpression of *TsTIP1;2* significantly increases tolerance to drought, salt, and oxidative stresses in *Arabidopsis* (Wang et al. 2014b). These results will be useful for elucidating the functions of same *ThAQP* genes under different environment stresses.

Effect of abscisic acid on the expression of the *AQP* genes in *T. hispida*

Abscisic acid (ABA) is an important plant hormone that has crucial roles in root and seed development, seed germination, and biotic and abiotic stress responses. ABA interacts with other phytohormones to mediate plant response to stress conditions and modulates expression of many genes at the transcriptional and post-transcriptional levels (Finkelstein et al. 2002; Cutler et al. 2010). Therefore, we measured the expression level of each *ThAQP* gene in *T. hispida* seedlings treated with ABA. In the roots, the expression patterns of *ThAQP*s can be roughly divided into three groups (Fig. 8a). The first group, including the *ThPIP2;2*, *ThPIP2;3*, *ThTIP2;1*, *ThTIP4;1*, *ThNIP1;1*, *ThNIP1;2*, *ThNIP3;3*, *ThSIP1;1*, and *ThSIP1;2* genes, was downregulated during the ABA treatment, especially after 3 or 24 h of stress. The second group included *ThPIP2;4*, *ThPIP2;5*, *ThTIP1;2*, *ThTIP1;3*, and *ThNIP3;1*, whose expression was initially downregulated at 3 h and then gradually increased to their peak expression level at 6 or 9 h and finally decreased again at 24 h. The third group included the *ThPIP1;1*, *ThPIP2;1*, and *ThNIP3;2* genes that were rapidly decreased after 3 h of ABA treatment and then recovered normal levels. In the stems, the *ThPIP*s, *ThTIP2;1*, *ThNIP1;2*, *ThNIP3;1*, *ThNIP3;3*, and *ThSIP1;2* were initially downregulated at 3 h and gradually increased to their peak expression level at 9 or 12 h and then rapidly increased (> twofold) at 24 h (Fig. 8b). *ThPIP2;3*, *ThTIP1;3*, and *ThSIP1;2* were initially upregulated and decreased to their lowest levels at 12 h, then rapidly increased to peak levels at 24 h. However, *ThPIP2;5* and *ThTIP1;2* were upregulated during the ABA treatment. In the leaves, all of the *ThAQP*s, with the exception of *ThPIP2;5*, *ThTIP1;1*, *ThNIP3;3*, and *ThSIP1;2*, did not significantly change during the ABA treatment (Fig. 8c). *ThPIP2;5* and *ThTIP1;2* were rapidly

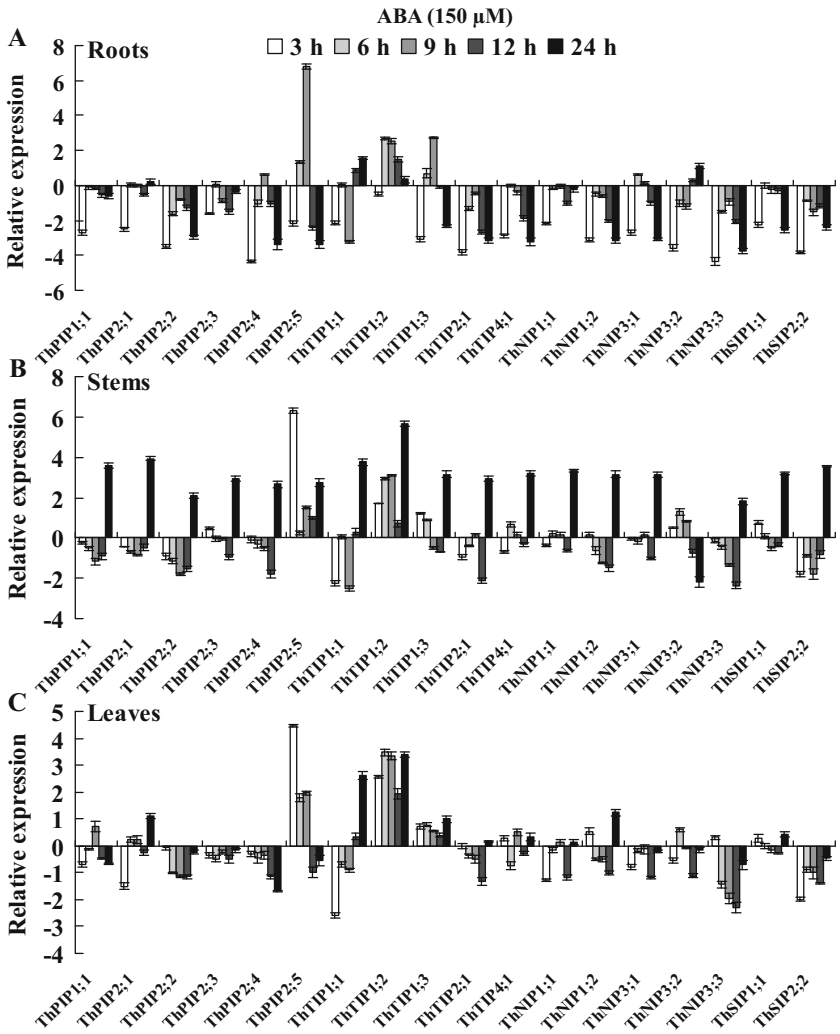


Fig. 8 Effect of ABA on the expression of the aquaporin genes in the roots, stems, and leaves of the 2-month-old *T. hispida* seedlings. Relative expression level = $\log_2$ (transcription level under stress treatment/ transcription level under control conditions). The *error bars* indicate standard deviations, which were calculated from multiple replicates of real-time PCR

increased and had high expression levels after 3 h of treatment; *ThPIP2;5* gradually decreased at subsequent time points, but *ThTIP1;2* maintained high levels. In contrast, *ThPIP2;5* and *ThTIP1;1* showed the opposite pattern. *ThNIP3;3* and *ThSIP1;2* were downregulated under ABA treatment. Overall, some *AQP* genes, such as *ThPIP2;5* and *ThNIP3;3*, were significantly induced and suppressed in different tissues by ABA treatment, indicating that these *AQP* genes participate or might play roles in the ABA-dependent pathways. Previous studies described the effects of exogenous ABA on *AQP* gene expression in different plant species. Following ABA application, *TsTIP1;2* expression was induced, with the highest levels occurring at 1–3 h (Wang et al. 2014b). The *TaNIP* expression was induced by NaCl, drought, and ABA treatments, overexpression of *TaNIP* in transgenic *Arabidopsis* enhanced higher salt tolerance than wild-type (WT) plants (Gao et al. 2010). Lian et al. reported that the expression of *OsPIP1;2*, *OsPIP2;5*, and *OsPIP2;6* in roots and *OsPIP1;2*, *OsPIP2;4*, and *OsPIP2;6* in leaves of upland rice was enhanced under ABA treatment. Jang et al. also investigated the expressions of 13 *Arabidopsis PIP*s under ABA treatment. However, the responsiveness of *PIP* genes to ABA was different, implying that the regulation of *PIP* genes involves both ABA-dependent and ABA-independent signaling pathways during water deficit (Jang et al. 2004; Lian et al. 2006).

Conclusion

This study presents a comprehensive analysis of *AQP* genes in *T. hispida*, including their sequence, structural properties, phylogeny, and expression patterns. Figure 9 illustrates the involvement of the *ThAQP* genes in different stress-related physiological processes. All the *ThAQP* genes can be differentially regulated by NaCl, PEG, $CdCl_2$, or ABA stress in the leaves, stems, or roots of *T. hispida*, especially in the roots. Roots are the primary uptake organ for different salt ions. It is conceivable that AQPs play an important role in response to abiotic stress. *ThPIP2;2*, *ThPIP2;5*, *ThTIP1;1*, *ThTIP1;2*, *ThTIP1;3*, and *ThSIP1;1* were highly induced in response to high salinity, drought, heavy metal stresses, and ABA

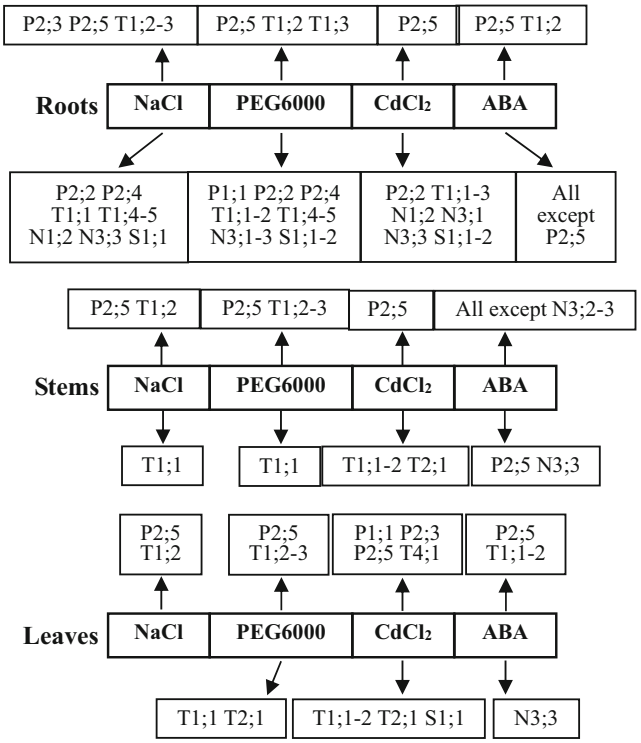


Fig. 9 Involvement of the aquaporin genes in *T. hispida* response to abiotic stresses and ABA treatment. The aquaporin genes are grouped based on their stress responses. *Arrows* indicate the genes that are upregulated or downregulated by at least twofold with the indicated treatments in the roots, stems, and leaves of the *T. hispida* plants. *P* ThPIP, *T* ThTIP, *N* ThNIP, *S* ThSIP

treatment, indicating that they may play roles in response to abiotic stress and were closely regulated by the ABA-dependent stress-signaling pathway. These results will provide further insight into the roles of *AQP* genes in abiotic stress tolerance in plants.

Acknowledgments The work was financially supported by National Natural Science Foundation of China (No. 31300571) and the Fundamental Research Funds for the Central Universities (2572014CA12). We also thank the editor and reviewers for their critical comments and thoughtful suggestions.

Data archiving statement All the identified *AQP* genes were deposited with GenBank with the GenBank accession numbers: *ThPIP1;1* (KT951251); *ThPIP2;1* (ACB71281); *ThPIP2;2* (KT951253); *ThPIP2;3* (KT951254); *ThPIP2;4* (KT951250); *ThTIP1;1* (KT951258); *ThTIP1;2* (ACB71282); *ThTIP1;3* (KT951255); *ThTIP2;1* (KT951256); *ThTIP4;1* (KT951257); *ThNIP3;1* (KT951260); *ThNIP3;2* (KT951262); *ThNIP3;3* (KT951261); *ThPIP2;5* (KT951252); *ThNIP1;1* (KT951263); *ThNIP1;2* (KT951259); *ThSIP1;1* (KT951264); and *ThSIP2;2* (KT951265).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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