Plant Cell Rep (2009) 28:291–300
DOI 10.1007/s00299-008-0626-6

BIOTIC AND ABIOTIC STRESS

Expressions of three cotton genes encoding the PIP proteins are regulated in root development and in response to stresses

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Received: 4 August 2008 / Revised: 22 September 2008 / Accepted: 8 October 2008 / Published online: 28 October 2008

Abstract Cotton (*Gossypium hirsutum*), the most important textile crop worldwide, often encounters water stress such as drought or waterlog during its growth season (Summer). To investigate molecular mechanism of water regulation in cotton plants, three cDNAs encoding the plasma membrane intrinsic protein (PIP) were isolated from cotton root cDNA library, and designated *GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2*, respectively. All of the three PIP proteins displayed water channel activity in *Xenopus laevis* oocytes. GhPIP2;1 and GhPIP2;2 proteins, however, showed much higher water transport activity than that of the GhPIP1;1 protein. Northern blot analysis revealed that all of the three genes were preferentially expressed in young roots. Further analysis by Real-time quantitative RT-PCR revealed that the transcripts of all the three genes were accumulated at high levels in 3-day-old young roots, but dramatically declined to much lower levels in 6–14 days old roots during seedling development, suggesting that expressions of the isolated *GhPIP* genes are developmentally regulated in roots. Additionally, expressions of the three genes were remarkably up-regulated or down-regulated under different stresses such as NaCl, cold, PEG (polyethylene glycol) treatments. Collectively, the results suggest that these genes may be involved in root development and in response to stresses.

D.-D. Li and Y.-J. Wu have contributed equally to this work.

Communicated by W.-H. Wu.

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Keywords Cotton (*Gossypium hirsutum*) · Aquaporin · Regulation of gene expression · Root development · Stress

Introduction

Water movement in cells is a precisely regulated process during plant development. Besides diffusion, water pass through cellular membrane rapidly by water channels consisting of aquaporins (AQPs), which belong to the major intrinsic protein (MIP) family. AQPs are small (23–34 kDa) transmembrane proteins, consisting of six transmembrane α-helices (H1–H6) connected by five short loops (loop A–loop E) with both N and C termini facing to the cytosol. Two highly conserved asparagine-proline-alanine (NPA) motifs locate at loop B and loop E which both form hemi-helix (HB and HE) dipping into the membrane from opposite sides. Through the interaction of six transmembrane α-helices and the two hemi-helices, AQPs form a membrane water-selective channel (Chaumont et al. 2001). AQPs generally exist as tetramers which are important for the protein folding and stability in the plasma membrane (Murata et al. 2000; Sui et al. 2001; Chaumont et al. 2005).

In plants, AQP proteins encoded by multigenes form a large family. There are 36 AQP members in maize (Chaumont et al. 2001), and 35 AQPs in *Arabidopsis* (Johanson et al. 2001). Based on the amino-acid sequence similarity, plant AQPs can be classified into four subfamilies, plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs) (Johanson and Gustavsson 2002; Johanson et al. 2001; Chaumont et al. 2001). The PIPs are mainly targeted to the plasma membrane and TIPs are generally localized to the vacuolar

membrane (Chaumont et al. 2005). The NIPs which are close homologues of GmNOD26 are abundantly expressed in the peribacteriod membrane of symbiotic nitrogen-fixing nodules of soybean roots (Wallace et al. 2006), while in non-leguminous plants, they are often localized in plasma and intracellular membranes (Mizutani et al. 2006; Takano et al. 2006; Maurel 2007). SIPs have been mostly found to be present in the endoplasmic reticulum (ER) membrane (Ishikawa et al. 2005). The PIP subfamily can be further divided into PIP1 and PIP2 isoforms based on sequence homology (Fetter et al. 2004). Previous studies indicated that most plant PIP2 proteins show high water channel activity, but PIP1 members are often inactive or have low activity (Chaumont et al. 2000; Marin-Olivier et al. 2000; Moshelion et al. 2002; Fetter et al. 2004). However, coexpression of some plant PIP1s and PIP2s in *Xenopus* oocytes resulted in a greater increase in permeability coefficient (P_f) than did the expression of PIP2s alone (Fetter et al. 2004; Temmei et al. 2005). A number of water-channel activities of AQPs are involved in regulation of water flux and homeostasis at the subcellular, transcellular, tissue and whole-plant levels (Chaumont et al. 2001). The activities of plant AQPs may be regulated by gating mechanisms, and the factors related to the gating action likely contain phosphorylation, heteromerization, pH, Ca^{2+}, pressure, solute gradients, temperature and so on (Chaumont et al. 2005). For instance, the water transport activity of PM28A is regulated by phosphorylation (Johansson et al. 1998). Although most plant AQPs have been found to be water channels (Johansson et al. 2000; Tyerman et al. 2002), a few plant AQPs which do not exhibit a strict specificity for water channel can also transport some small neutral molecules such as glycerol, urea, CO_2 (Gerbeau et al. 1999; Guenther and Roberts 2000; Weig and Jakob 2000; Ciavatta et al. 2001), NH_3 and NH_4^+ (Niemietz and Tyerman 2000; Jahn et al. 2004), boron (Dordas et al. 2000; Takano et al. 2006) and H_2O_2 (Henzler and Steudle 2000; Bienert et al. 2007).

The large numbers of PIP and TIP genes in the plants are expressed in the tissue-specific and developmental-regulated manner. Both PIP and TIP gene products have been localized in the vascular tissue and its surrounding cells, and also in the zones of the elongated cells that are undergoing vacuolization, but seldom in meristematic tissues (Schäffner 1998). A study revealed that many *Arabidopsis* AQPs are predominantly expressed in either root or flower organs, but no AQP members seem to be leaf-specific (Alexandersson et al. 2005). Both *NtPIP2;1* and *NtPIP1;1* genes in tobacco were detected in most tissues of anther, but only *NtPIP1;1* RNA accumulated in the stigma (Bots et al. 2005). Previous study indicated that the water permeability of the cells was declined by repressing *NtAQP1* expression in tobacco (Siefritz et al. 2002). Overexpression of *Arabidopsis AtPIP1b* in tobacco remarkably increased plant growth, transpiration and photosynthesis rate, whereas transgenic tobacco plants wilted more quickly under drought stress (Aharon et al. 2003). In *Arabidopsis*, the expression of all 13 *PIP* genes was up- or down-regulated under abiotic stresses (Jang et al. 2004).

Roots usually provide the input of water in higher plants and establish a critical link in the soil–plant–air continuum which has to be maintained in the most adverse environmental or physiological conditions. Roots, in particular, show a remarkable capacity to alter their water permeability in response to day/night cycles, nutrient deficiency and other stresses. Rapid and significant (>30%) increases or decreases in water permeability (hydraulic conductivity, L_p) of roots may be observed upon short-term exposure to stresses. These rapid changes are mostly accounted for by changes in cell membrane permeability, and are mediated by aquaporins (reviewed in Javot and Maurel 2002). Hence, aquaporins may play significant roles in cotton roots which are usually exposed to irregular cycles of wetting and drying during summer. However, none of cotton aquaporin genes was well characterized in roots, and particularly, the activities and functions of these genes in root development remain to be elucidated so far. Here, we report identification of three new *PIP* genes which were predominantly expressed in developmentally-regulated manner in cotton roots. Overexpression of the cotton *PIP* genes in *X. laevis* oocytes dramatically increased water permeability of the cells, indicating that these PIP proteins possess water transport activities.

Materials and methods

Plant materials and growth

Cotton (*Gossypium hirsutum* cvs. Xuzhou 142, Emian No. 9 and No. 10) seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and 10% (v/v) H_2O_2 for 2 h, then washed with sterile water. The sterilized seeds were germinated on 1/2MS medium under a 16 h light/8 h dark cycle at 25°C for 5–6 days. In the stress experiments, the sterilized seeds were germinated on 1/2MS medium under a 16 h light/8 h dark cycle at 25°C for 4–5 days, and then seedlings were transferred in 4°C for cold treatment, or transferred onto 1/2MS medium containing NaCl or PEG (polyethylene glycol 4,000) for treatment.

Roots, cotyledons and hypocotyls were cut from sterile seedlings, and other tissues for RNA extraction were derived from cotton plants grown in the field. All tissues were collected 2 h after light onset since some plant aquaporins display a diurnal expression pattern, with a peak of

expression at 2–4 h after the beginning of the light period (Hachez et al. 2006).

Construction of cotton root cDNA library and isolation of *GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2* cDNAs

Total RNA was extracted from young roots (3–10 days old) as described previously (Li et al. 2002). Poly(A)$^+$ mRNA was prepared from a pool of root total RNA by using an mRNA purification kit (Qiagen). Complementary DNA was synthesized and cloned into the *EcoR* I–*Xho* I sites of the ZAP express vector and packaged using a ZAP-cDNA Gigapack Gold III cloning kit (Stratagene) according to the manufacturer's instruction.

More than 4,000 cDNA clones were randomly selected from the cotton root cDNA library for sequencing. Some *GhPIP* clones with partial sequences were identified. Then a 500 bp fragment of *GhPIP* including partial coding region and 3′-untranslated region was labeled with α-^{32}P-dCTP and used as probe to screen cotton root cDNA libraries. 2×10^5 cDNA clones were screened and over 100 clones were identified. Among them, the three unique cDNA clones (*GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2*) were obtained.

Northern blot analysis

Total RNA was extracted from young fibers, ovules, anthers, petals, leaves, cotyledons, hypocotyls and roots of cotton. The isolated total RNA was purified by RNA purification kit (Qiagen USA, Valencia, CA). RNA samples (16 μg per lane) from different cotton tissues were separated on 1.2% agarose–formaldehyde gel and transferred onto Hybond-N nylon membranes by capillary blotting. 0.2 kb 3′-UTR (3′-untranslated region) sequences of *GhPIP1*, *GhPIP2;1* and *GhPIP2;2* cDNAs were amplified as gene-specific probes by PCR, respectively. The α-^{32}P-dCTP labeled gene-specific probes were prepared through random primer method (Random Primer DNA Labeling Kit Ver.2, TAKARA). Northern blot hybridization was performed as described previously (Li et al. 2002). The membrane was exposed to X-film (Eastman Kodak, Rochester, NY) with two intensifying screens at −70°C for 1–3 days.

Quantitative RT-PCR analysis

The expression of *GhPIP* genes in cotton roots was analyzed by real-time quantitative reverse transcriptase (RT)-PCR using the fluorescent intercalating dye SYBR-Green in a detection system (MJ Research, Opticon 2). A cotton polyubiquitin gene (*GhUBI1*, access number in GenBank: EU604080) was used as a standard control in the RT-PCR reactions. Two-step RT-PCR procedure was performed in all experiments using a method described earlier (Li et al. 2005). In brief, total RNA was reversely transcribed into cDNAs which were used as templates in real-time PCR reactions with gene-specific primers. The primer sequences were as follows: *GhPIP1;1*: up-chain primer, 5′>TGGGCGCCATATGCGGGGCG<3′, down-chain primer, 5′>AAGCTCATAATCATGGAATCATG<3′; *GhPIP2;1*: up-chain primer, 5′>GCTGATGGATACAGCACTGGC<3′, down-chain primer, 5′>AGCAAAGTAAAAGGCGTAGATTC<3′; *GhPIP2;2*: up-chain primer, 5′>GTCTCTAACTTTAACCTTATCAC<3′, down-chain primer, 5′>AAGGAAAAGGAACTGAACTCCAT<3′. Real-time PCR reaction was performed using Real-time PCR Master Mix (TOYOBO, Japan) according to the manufacturer's instruction. The *Ct* (cycle threshold), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene. Relative quantity of the target *GhPIP* expression level was performed using the comparative *Ct* method. The relative value for the expression level of each *GhPIP* gene was calculated by the equation $Y = 10^{\Delta Ct/3.5} \times 100\%$ (ΔCt is the differences of *Ct* between the control *GhUBI1* products and the target *GhPIP* products, i.e. $\Delta Ct = Ct_{\mathrm{GhUBI1}} - Ct_{\mathrm{GhPIP}}$). For the accurate amplification of each specific target gene among the highly homologous PIP family, we carefully designed the primer sets for each *PIP* gene based on the sequences corresponding to 3′-untranslational region. To achieve optimal amplification, PCR conditions for every primer combination were optimized for annealing temperature, and PCR products were verified by melting curve analysis and confirmed on an agarose gel. The efficiency of each primer pair was detected using *GhPIP* cDNAs as standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair. The data of the real-time RT-PCR are mean values and standard errors (bar) of three independent experiments with three biological replicates of root materials.

DNA and protein sequence analysis

Nucleotide and amino acid sequences were analyzed using DNAstar (DNAstar Inc). The GhPIPs peptide sequences were aligned with the ClustalW program (http://www.ebi.ac.uk), and phylogenetic analysis was employed to investigate the evolutionary relationships among the aquaporins. A minimum evolution tree was generated in MEG3.1. A bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology.

In vitro complementary RNA synthesis

The cDNA of *GhPIPs* were subcloned into pGEM-7Z vector using the flanking restriction sites *EcoR*I and *Xba*I of the ZAP vector. Capped cRNA transcripts were synthesized in vitro with mMAHINE SP6 Kit (Ambion) with *Xba*I linearized vector.

Oocyte preparation, cRNA injection, and osmotic water permeability assay

Xenopus laevis oocytes of stages V and VI were isolated and defolliculated by digestion at room temperature for 1 h with 2 mg/ml collagenase A (Sigma) in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM Hepes-NaOH, pH 7.4, 220 mOsm/kg). 50 nl volume of in vitro transcripts (50 ng) of the target genes, using the same volume of distilled water as negative control, were injected into the oocytes, and then the oocytes were incubated at 19°C for 48 h in ND96 solution supplemented with 10 μg/ml penicillin and 10 μg/ml streptomycin. To measure the osmotic water P_f, a single oocyte was transferred to five-fold diluted ND96 solution. Changes in oocyte volume were monitored at room temperature with a microscope video system by taking digital images at 30 s intervals. Oocytes volumes (V) were calculated from the measured area of each oocyte. The osmotic P_f was calculated for the first 5 min using the formula $P_f = V_0[\mathrm{d}(V_0/V)/\mathrm{d}t]/[S_0 \times V_W(\mathrm{Osm_{in}} - \mathrm{Osm_{out}})]$, with an initial volume (V_0) of 9×10^{-4} cm^3, an initial oocytes surface area (S_0) of 0.045 cm^2, and a molar volume of water (V_W) of 18 cm^3/mol (Zhang and Verkman 1991).

Results

Isolation and characterization of three *GhPIP* genes

By screening cotton root cDNA libraries with a 500 bp *GhPIP* probe, we isolated three unique PIP cDNAs. Sequence analysis predicted that one gene (designated *GhPIP1;1*, accession number in GenBank: EF079900) encodes a protein with 289 amino acids belonging to PIP1 isoform, and the other two (designated *GhPIP2;1*, *GhPIP2;2*, accession number in GenBank: EF079901 and EF079902) encode polypeptides consisting of 285 and 286 amino acids, respectively, belonging to PIP2 members.

The *GhPIP1;1* cDNA consists of 935 bp, including 870 bp open reading frame (ORF). It shares high sequence homology at nucleotide level (approximately 77% identities) in the coding region and at amino acid level (approximately 87% identities) with *GhAQP1* (ABD63904) and *GhPIP1-2* (ABR68794), the other two cotton *PIP1* genes reported previously. There is 13% substitution rate at amino acid level compared with each other (Fig. 1). In addition, the deduced amino acid sequence of *GhP1P1;1* shows 91% identity with *Medicago truncatula* MtPIP1;1 (AAK66766) and 87%–88% identity with *Arabidopsis* AtPIP1s. The *GhPIP2;1* cDNA consisting of 1,116 bp, shares 86% homology at nucleotide level, and 91% identity at amino acid level with *GhPIP2;2* which is 1,105 bp in length. They have 74–83% identity with *Arabidopsis* PIP2 proteins, and shows 81% identity with maize ZmPIP2;1 (AAK26758). Additionally, there is approximately 67% identity between GhPIP1;1 and GhPIP2s.

Although the predicted proteins encoding by these three genes belong to different PIP subgroups, all of them contain six putative transmembrane α-helices, highly conserved amino acid sequence 'HINPAVTFG' and two 'NPA' motifs (Fig. 1). By contrary, the terminal domains of GhPIPs display relatively high variability. GhPIP1s contain large N-terminal domain, while GhPIP2s contain large C-terminal domain (Fig. 1).

Phylogenetic relationship of GhPIP proteins

To investigate the evolutionary relationships of three GhPIP proteins, all 13 PIP protein sequences of *Arabidopsis* as well as cotton AQPs reported previously were selected from GenBank for phylogenetic analysis. GlpF, a member of the MIP family from *Escherichia coli*, was employed as an outgroup. As shown in the phylogenetic tree (Fig. 2), 18 PIP proteins split into two groups obviously. GhPIP1-2 and GhAQP1 form a distinct clade and locate at the clade based to AtPIP1;4 and AtPIP1;5. Although GhPIP1;1 shares high sequence homology with GhAQP1 and GhPIP1-2, it inhabits a single clade which is basal to the branch containing all other PIP1 proteins. The result suggest that GhPIP1;1 may diverge relatively earlier from other GhPIP1s during evolution. In the branch of PIP2s, GhPIP2;1 is a sister group to the AtPIP2;2/AtPIP2;3 clade, while GhPIP2;2 is basal to the clade containing AtPIP2;2, AtPIP2;3 and GhPIP2;1.

Overexpression of GhPIPs in *Xenopus laevis* oocytes enhance water permeability of the cells

To test the water transport activity of GhPIPs, their capped sense cRNA were injected into *Xenopus laevis* oocytes. After a 2-days incubation for cRNA translation and the target proteins to the oocyte membrane, the oocytes were transferred to a hypoosmotic solution and the increase in area was measured in time and used to calculate the increase in volume. This change in volume was used to calculate the relative water permeability (P_f) of the

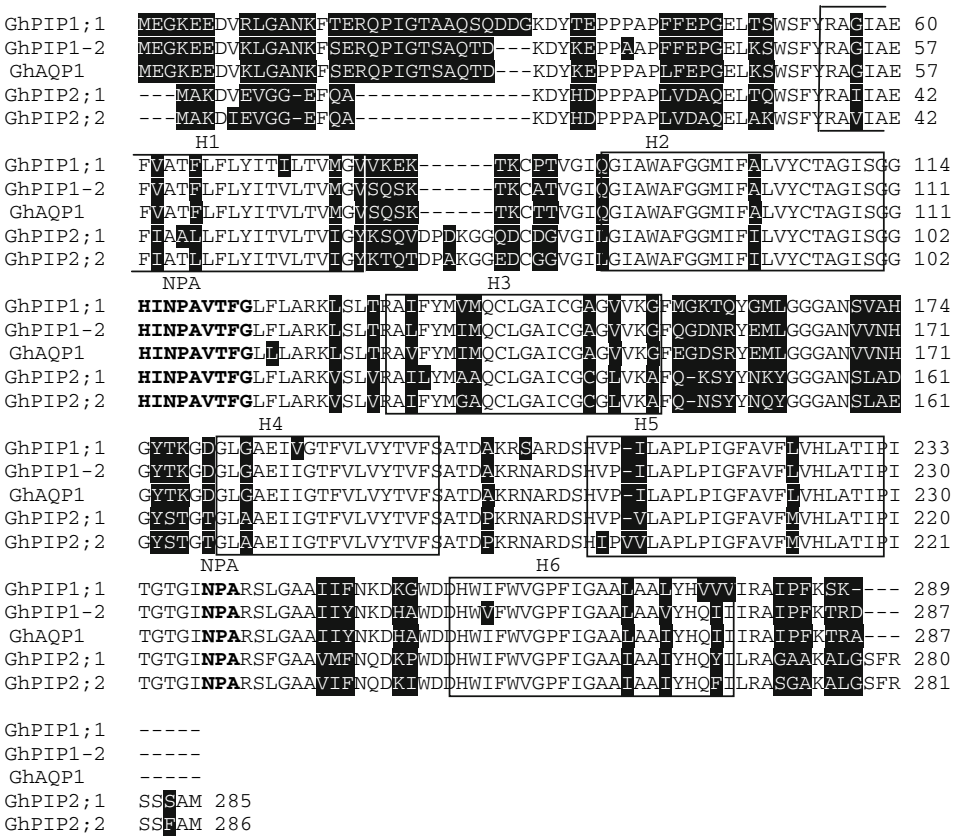

Fig. 1 Comparison of the three predicted amino acid sequences of cotton PIPs with the other known cotton PIP proteins. Amino acid sequences are aligned by ClusterW software. Amino acid substitutions are highlighted in *black* and six transmembrane-helix (H1–H6) are shown in the *box*. *Overstriking letters* refer to the most highly conserved amino acid sequences of MIP. The accession numbers of these known proteins in GenBank are as follows: GhAQP1(ABD63904) and GhPIP1-2 (ABR68794)

oocytes. The results of the experiments are presented in Fig. 3. The oocytes expressed the GhPIP1;1 proteins showed a P_f of 18.79 ± 4.02 μm/s ($n = 30$) which was about two-fold higher than that of the controls injected with water ($P_f = 9.47 \pm 5.30$ μm/s, $n = 30$). Notably, the P_f values of the oocytes expressed GhPIP2;1 and GhPIP2;2, respectively, were 30.58 ± 7.34 μm/s and 37.26 ± 10.01 μm/s ($n = 30$), which were much higher than that of the oocytes injected with GhPIP1;1 cRNA. In addition, We also coexpressed GhPIP1;1 and GhPIP2s in the oocytes. However, statistical analysis revealed that the P_f values of the cells coexpressed GhPIP1;1 and GhPIP2s did not show significant differences from those of the oocytes expressed GhPIP2s alone (data not shown). The above data demonstrated that all of the three GhPIP proteins possess water-transport capacity, and the GhPIP2 activity was higher than that of GhPIP1.

Expressions of the three *GhPIP* genes are organ-specific/preferential and are root-developmentally regulated

Northern blot results showed that the expressions of all the three genes are organ-preferential (Fig. 4). Strong expression of *GhPIP1;1* gene was found in roots, and its moderate expression was detected in petals, but only weak or no signals were observed in other tissues (Fig. 4a). The expression of *GhPIP1;1* in cotton organs and tissues was also analyzed by real-time quantitative RT-PCR. The results revealed that the transcripts of *GhPIP1;1* were predominantly accumulated in 3-day-old roots which is four-fold higher than in petals (data not shown). Similarly, the *GhPIP2;2* was also mainly expressed in young roots. However, its transcripts were relatively largely accumulated in hypocotyls, but not in petals, unlike *GhPIP1;1* (Fig. 4b). On the other hand, the *GhPIP2;1* products were predominantly accumulated in young hypocotyls, and relative high levels of its transcripts were detected in young roots and petals, but no or weak expression was observed in other tissues (Fig. 4b).

Furthermore, as shown in Figs. 4b and 5, both *GhPIP2;1* and *GhPIP2;2* expression are developmentally-regulated in cotton roots. At early stage of development (3-day-old), high levels of the gene products were detected in roots. As roots further developed, the expression activities of both genes were dramatically declined to much lower level (6-day-old roots), and till undetectable level (9–14 days old roots). Similarly, the *GhPIP1;1* gene was also expressed at the highest level in 3-day-old roots, while dramatically declined with further growth of the roots (Fig. 5). Interestingly, the expression levels of all the three genes in the tip regions (20 mm) were much higher than

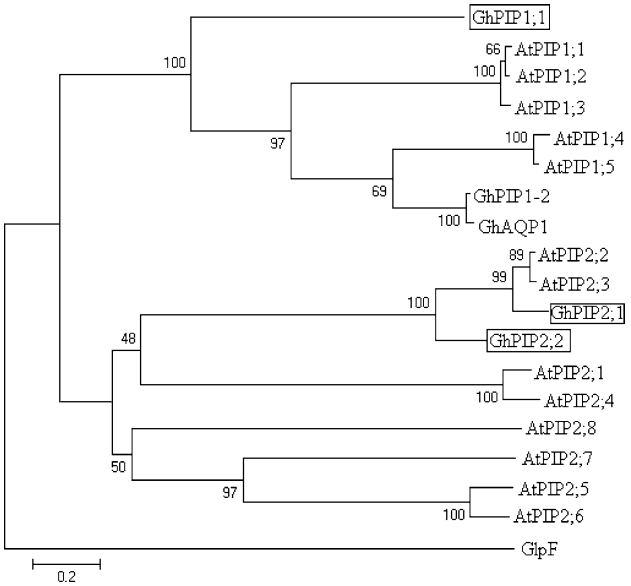

Fig. 2 Phylogenetic relationships of cotton PIP proteins with *Arabidopsis* PIP proteins. The Minimum Evolution tree was constructed in MEGA3.1 from 1,000 bootstrap replicates. The accession numbers of the known proteins of *Arabidopsis* in GenBank are as follows: AtPIP1;1 (AAM19914.1), AtPIP1;2 (AAM14193), AtPIP1;3 (AAK15545.1), AtPIP1;4 (AAN15649.1), AtPIP1;5 (AAL24430.1), AtPIP2;1 (AAK73268.1), AtPIP2;2 (AAD18142), AtPIP2;3 (AAM20335.1), AtPIP2;4 (AAM64801.1), AtPIP2;5 (AAM61408.1), AtPIP2;6 (AAK74048.1), AtPIP2;7 (AAM10142.1), AtPIP2;8 (AAM15086.1). The accession numbers of the known cotton aquaporins are as follows: GhAQP1 (ABD63904), GhPIP1-2 (ABR68794). GlpF is a glycerol facilitator from *Escherichia coli* (Genbank accession number CAH19327) which belongs to MIP family and was used as an outgroup

the upper regions of 6-day-old roots (Fig. 5). The results suggest that all the *GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2* genes are mainly expressed in young cells which are actively undergoing cell division or elongation during early root development.

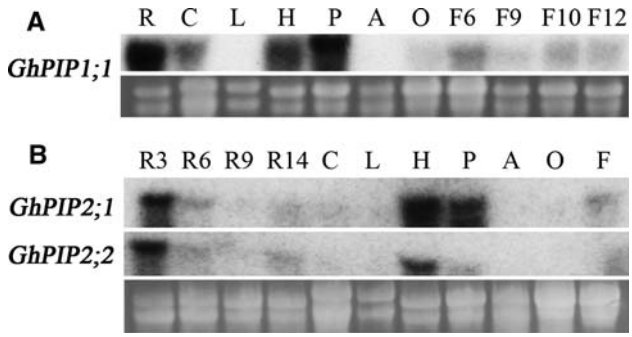

Fig. 4 RNA gel blot analysis of *GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2* genes expression in cotton tissues. *Upper panel* of **a** and the *upper*, *middle panels* of **b**: autoradiograph of RNA hybridization; *bottom panel*: RNA gel before being transfered to membrane showing equal loading of RNAs. **a** *GhPIP1;1* expression patterns: *R* roots; *C* cotyledons; *L* leaves; *H* hypocotyls; *P* petals; *A* anthers; *O* 10 DPA (days post anthesis) ovules; *F6–F12* fiber of 6, 9, 10, 12 DPA, respectively; **b** *GhPIP2;1* and *GhPIP2;2* expression patterns: *R1–R4* Roots of 3, 6, 9, 14-day-old, respectively; *C* Cotyledons; *L* Leaves; *H* Hypocotyls; *P* Petals; *A* Anthers; *O* 10 DPA ovules; *F 10* DPA fiber

Expression levels of the three *GhPIP* genes are regulated in roots upon different stresses

To investigate whether the expression of the *GhPIP* genes are stress-induced, total RNA were exacted from roots under different stresses for 12 h. The expression profiles of the three genes are shown in Fig. 6a. After salt (1% NaCl) treatment, the expressions of all three genes were remarkably up-regulated in roots. A significant increase was also observed in the transcription levels of *GhPIP2;1* after 16% PEG treatment. In contrast, the expression levels of both *GhPIP2;1* and *GhPIP2;2* genes decreased after cold treatment. On the other hand, the expression of *GhPIP1;1* were up-regulated slightly as cold and PEG treatments, and so did *GhPIP2;2* expression with PEG treatment, compared with controls.

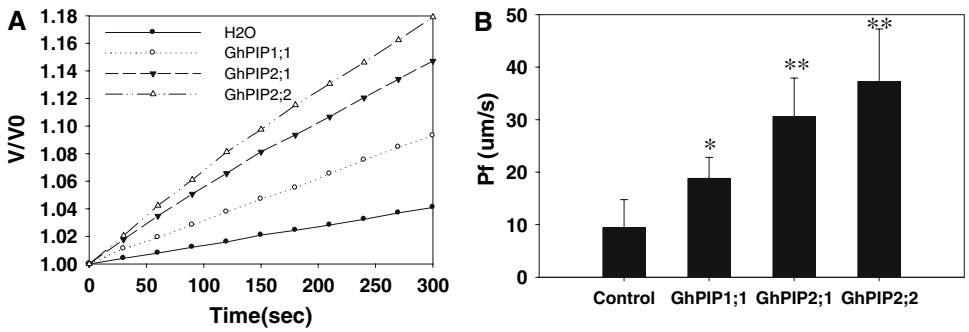

Fig. 3 Functional expression assay of GhPIP proteins in *Xenopus* oocytes. **a** Increase in relative volume of oocytes injected with *GhPIPs cRNA* after transfer to hypoosmotic medium (see "Methods"). **b** P_f-values of oocytes injected with *GhPIPs cRNA*, using water as control. *Independent *t* tests demonstrated that there was significant difference between the P_f-values of oocytes injected with water and *GhPIP1;1* cRNA (*t* test for equality of means, *P* value < 0.05). **Very significant difference between the P_f-values of oocytes injected with water and GhPIP2s cRNA (*P* value < 0.01)

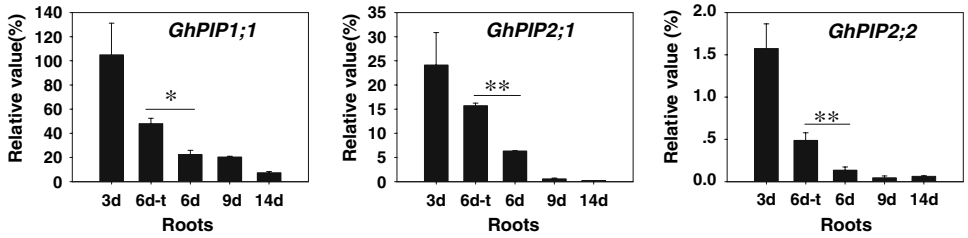

Fig. 5 Expression of *GhPIP* genes during root development. Relative values of the *GhPIP* gene expressions in the development of cotton roots are shown as percentage of *GhUBI1* expression activity (see "Methods"). Error *bars* represent standard deviation. *Independent *t* tests demonstrated that there was significant difference between 6-day-old roots and the tip regions of 6-day-old roots (P value < 0.05). **Very significant difference between 6-day-old roots and the tip regions of 6-day-old roots (P value < 0.01). *3*, *6*, *9* and *14d* refer to 3, 6, 9, 14-day-old roots respectively. And *6d-t* refers to tip regions (20 mm from root tip) of 6-day-old roots

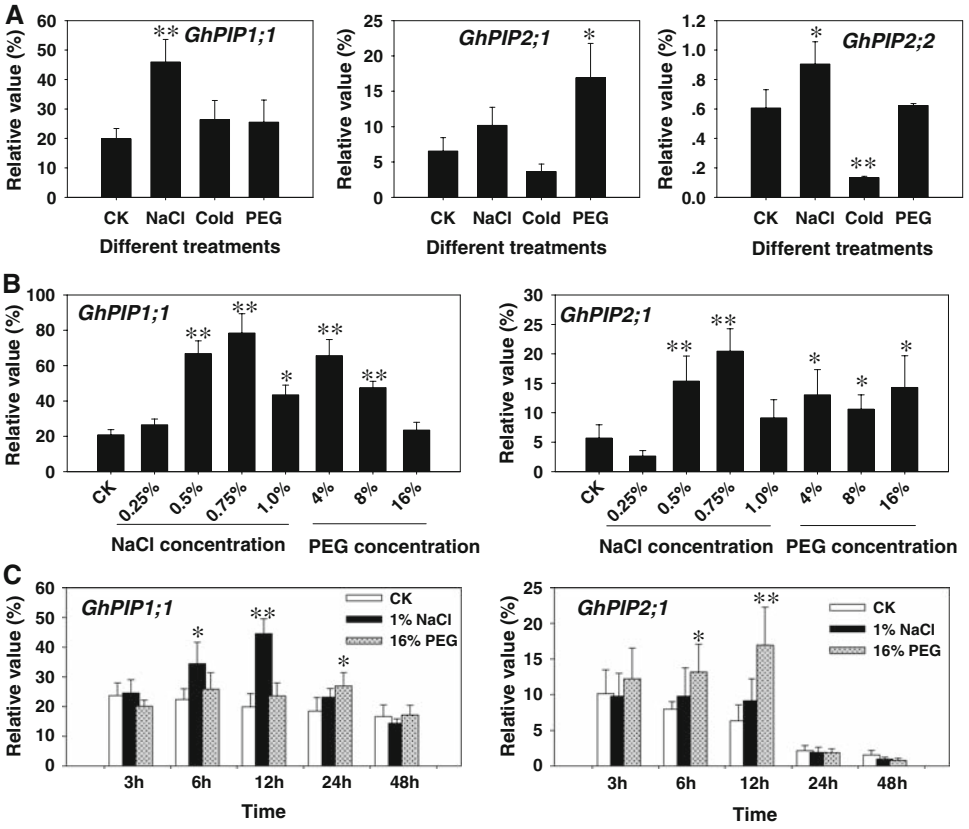

Fig. 6 Expression of the *GhPIP1;1*, *GhPIP2;1* and *GhPIP2;2* genes in roots under salt, cold and PEG stresses. Relative value of *GhPIP* gene expression in cotton roots was shown as percentage of *GhUBI1* expression activity (see "Methods"). Error *bars* represent standard deviation. *Independent *t* tests demonstrated that there was significant difference between (P value < 0.05). **Very significant difference between treated and un-treated roots (P value < 0.01). **a** Different treatments: cotton roots of 5-day-old were dealt with 1% NaCl, 4°C or 16% PEG for 12 h. CK un-treated roots (control); NaCl roots dealt with 1% NaCl; Cold roots dealt with 4°C; PEG roots dealt with 16% PEG. **b** Concentration gradient treatments: 5-day-old roots dealt with different concentrations of NaCl or PEG for 12 h. The concentration of NaCl is 0.25, 0.5, 0.75, 1.0% in turn and that of PEG is 4, 8 and 16% in turn. **c** Time gradient treatments: 5-day-old roots dealt with 1% NaCl or 16% PEG for 3, 6, 12, 24, 48 h, respectively

More details were obtained by investigating the expression patterns of *GhPIP1;1* and *GhPIP2;1* in 5-day-old roots dealt with different concentrations of NaCl or PEG for 12 h as well as in the roots dealt with 1% NaCl or 16% PEG for different time. The same experiments had not been done to *GhPIP2;2* because of its low abundance in the root and the similar expression profiles like *GhPIP2;1* under different stresses.

After a low concentration of NaCl treatment, the expression level of *GhPIP1;1* was slightly up-regulated,

while *GhPIP2;1* was down-regulated obviously in roots. Transcription levels of both genes achieved the peak value in roots dealt with 0.75% NaCl, and then decreased remarkably after a higher concentration of NaCl (1.0%) treatment (Fig. 6b). Unlike the salt stress, the transcripts of *GhPIP1;1* in roots dealt with a low concentration of PEG (4%) were much higher than with the higher concentration of PEG treatment. With different concentrations of PEG treatments, the expression levels of *GhPIP2;1* were all up-regulated significantly in roots, compared to control (Fig. 6b).

Time-course analysis of the expression patterns of *GhPIP1;1* and *GhPIP2;1* under stresses were shown in Fig. 6c. Within 6 h treatment with 1% NaCl, the transcripts of *GhPIP1;1* increased obviously and reached its maximum at 12 h, and then decreased slightly after 48 h treatment. When seedlings were treated by 16% PEG, the expression levels of *GhPIP1;1* in roots were up-regulated slightly from 6 to 24 h and restored to normal level after 48 h treatment. The expression patterns of *GhPIP2;1* response to 16% PEG were similar to that of *GhPIP1;1* response to 1% NaCl. *GhPIP2;1* seems to be less sensitive to 1% NaCl and its expression levels were slightly up-regulated till 12 h treatment. Collectively, the data revealed that the expression of all three *GhPIP* genes in cotton roots was up- or down-regulated in response to different stresses.

Discussion

The data presented in this study revealed that the isolated cotton *PIP1;1*, *PIP2;1* and *PIP2;2* genes encode the proteins which are homologues of the known plant PIP-type aquaporins. Comparing with the *PIP* genes from different species, they share high sequence homology not only at nucleotide level (58–80%) but also at amino acid level (60–91%). In the deduced amino acid sequences, they all display the most conserved amino acid sequence 'HINPATFVG' of MIP family and two 'NPA' motifs (Fig. 1). Furthermore, all of the three PIP proteins also contain the conserved sequence (R/K)DYX(E/D)PP(P/R)X_{3-4}(E/D)XXELXXWSF(Y/W)R which is found in all PIP members (Schäffner 1998), although GhPIP1;1 possesses a longer N-termini than GhPIP2;1 and GhPIP2;2. GhPIP1;1 has the typical C-termini sequence [YHX(I/V)(V/I)IRA(M/I)XF(H/K)XXX] of PIP1, while both GhPIP2;1 and GhPIP2;2 contain the PIP2-conserved sequence [YHQ(F/Y)XLRA(G/S)(A/G)XK(A/X)LGSFRSX(PXX/A)] at the C-termini. Phylogenetic analysis (Fig. 2) showed that GhPIP1;1 had a more close evolutional relationship with AtPIP1s (87–88% identity) than AtPIP2s (63–68% identity), while the situations of GhPIP2;1 and GhPIP2;2 were reverse. Given the data together, our results strongly indicated that the three genes can be classified into two *PIP* subfamilies, i.e. *GhPIP1;1* belongs to plant *PIP1* subfamily and the other two belong to plant *PIP2* subfamily.

It is widely accepted that the plant plasma membrane PIP1 and PIP2 subgroups differ not only in the length of their N and C termini and in several single amino acid residues but also in terms of their water channel activity (Chaumont et al. 2000). When expressed alone in *Xenopus* oocytes, PIP1s are inactive or have low activity, whereas PIP2 s cause a marked increase in the osmotic water permeability coefficient (Fetter et al. 2004; Moshelion et al. 2002). When coexpressed, PIP1 and PIP2 interact to regulate their activity or their subcellular localization (Fetter et al. 2004; Zelazny et al. 2007). In this study, our data revealed that the activities of both GhPIP2;1 ($P_f = 30.58$) and GhPIP2;2 ($P_f = 37.26$) are two- to three-fold higher than that of GhPIP1;1 (Fig. 3). By contrast, the activities of SsAQP2 ($P_f \geq 130$ μm/s) are much higher (about seven-fold higher) than those of SsAQP1 (Moshelion et al. 2002), and the activities of ZmPIP2s ($P_f \geq 70$ μm/s) are as five-fold as those of ZmPIP1s (Fetter et al. 2004). In addition, the coexpression of GhPIP1;1 with GhPIP2;1 or GhPIP2;2 did not lead to an increase in membrane P_f, unlike that of ZmPIP1s with ZmPIP2s (Fetter et al. 2004). These results suggest that regulation mechanism of cotton PIP activities may show some difference from that of the known plant PIPs.

The discovery of aquaporins changed our views about how water moves rapidly across membrane and how plants might regulate water flow in different physiological conditions. Although the water transport across membrane mediated by aquaporins is not the unique approach of water transport in plants, it plays significant role in some special organs and in some certain conditions. Two *GhPIP1* members (*GhAQP1* and *GhPIP1-2*) had been reported previously in our lab (Li et al. 2006) and the other lab (Liu et al. 2008). Although both genes show high sequence identity with *GhPIP1;1* at the amino acid level, the expression patterns of them exhibit completely different features. GhAQP1 is expressed specifically in 6–15 DPA ovules which might be involved in osmoregulation during cotton ovules development (Li et al. 2006), whereas GhPIP1-2 transcripts were accumulated abundantly during rapid fiber elongation (Liu et al. 2008). In this study, Northern blot analysis (Fig. 4) showed that the mRNAs of the three genes all are abundant in young roots. This confirms the general view that cotton AQPs play an important role in facilitating water movement across cell membranes in roots. Additionally, all three genes were preferentially expressed in young roots or young parts of roots, but remarkably declined with further root development, suggesting that the gene activities are

developmental-regulated in cotton roots (Fig. 5). It had been reported that almost all *ZmPIPs* mRNAs were strongly dependant on the developmental stage of the root, with a general increase in expression towards the elongation and mature zones (Hachez et al. 2006). The increase in *ZmPIP* transcript abundance along the longitudinal root axis can be attributed to a decrease in symplastic continuity along the growing and maturing zones and therefore to a need for facilitated water transport through the cellular plasma membrane. *GhPIP* mRNAs are also abundant in the tip of the root, suggesting that these genes might play similar physiological roles in facilitating water uptake and transport during root development of cotton.

In the stress experiments, the expression of three cotton PIP genes was up- or down-regulated in response to different stresses (Fig. 6). *GhPIP1;1* and *GhPIP2;1* are sensitive to different concentrations of salt or PEG treatment. The expression levels of *GhPIP1;1* and *GhPIP2;1* are also related with the duration of treatment. Just within 6 h after salt or drought treatments, both genes were up-regulated (Fig. 6). The expression patterns of *AtPIPs* in response to abiotic stresses had been reported by Jang et al. (2004). The expression levels of most *AtPIPs* were markedly down-regulated in the roots by cold and drought treatment, while were highly up-regulated in the salt-treated roots (Jang et al. 2004). Similarly, the expression of all three genes was up-regulated in roots by higher concentrations (0.5–1%) of salt stress treatment, and down-regulated by cold treatment except *GhPIP1;1*. Under drought stress, however, the expression of *GhPIP2;1* and *GhPIP1;1* was significantly up-regulated in roots, unlike *AtPIPs*. In brief, our results revealed that the isolated cotton PIPs may play important roles in modulating osmotic balance of root cells when plants encountered environmental stresses. Thus, the data presented in this study may contribute to an understanding for water transport and osmotic regulation of cells in root development of cotton.

Acknowledgments This work was supported by National Natural Sciences Foundation of China (grant No. 30871317), and Natural Sciences Foundation of Hubei Province, China (grant No. 2006ABA181).

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