BIOLOGIA PLANTARUM 58 (2): 274-282, 2014 DOI: 10.1007/s10535-013-0386-1

Characterization of *AQP* gene expressions in *Brassica napus* during seed germination and in response to abiotic stresses

F.W. GE, P. TAO, Y. ZHANG, and J.B. WANG*

State Key Laboratory of Hybrid Rice, College of Life Sciences, Wuhan University, Wuhan 430072, P.R. China

Abstract

Water transport across the cell membranes is regulated largely by a family of proteins known as aquaporins (AQPs). Plasma membrane intrinsic protein (PIP) is an important subfamily of plant AQPs localized on the plasma membrane. To investigate the molecular mechanism of water regulation in seed germination, seven genes encoding PIP were initially cloned and sequenced from the germinating seed cDNA pool of *Brassica napus*. They belong to the PIP1 and PIP2 subfamilies. The transcription of the seven cloned genes plus three previously identified *AQP* genes from *B. napus* were analyzed in different organs and different stages of seed germination by quantitative real-time PCR (qRT-PCR). The results show that the expressions of the ten *AQP* genes were lower or scarcely detected in dry seeds, but were up-regulated during germination as well as in young seedlings. In addition, the expression of these ten *AQP* genes in response to an abiotic stress during seed germination was investigated and the results also show differential responses to abiotic stress treatments. Our findings suggest that these ten genes play different roles during plant development and response to abiotic stresses in *B. napus*.

Additional key words: aquaporin, cold stress, oilseed rape, plasma membrane intrinsic protein, qRT-PCR, salinity, water stress.

Introduction

AQPs belong to a large superfamily known as major intrinsic proteins (MIPs) which selectively allow the flow of water and other small molecules through biological membranes (Baiges *et al.* 2002). Plant AQPs play important roles in seed germination, cell elongation, stoma movement, response to stresses, and other cellular processes (Chaumont *et al.* 1998, Jang *et al.* 2004, Bouton *et al.* 2005, Alleva *et al.* 2010, Hussain *et al.* 2011, Katsuhara *et al.* 2011, Ben Bâaziz *et al.* 2012, Kudoyarova *et al.* 2013). Genome projects have revealed that more than 30 *AQP* genes exist in higher plants (Chaumont *et al.* 2001, Johanson *et al.* 2001), *e.g.*, 35 members in *Arabidopsis* and at least 32 in maize. The plant AQPs are classified into four groups: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (SIPs). The PIPs are further divided into subfamilies PIP1 and PIP2 (Schaeffner 1998, Chaumont *et al.* 2000). Recently, the fifth subfamily, known as X intrinsic proteins (XIPs), has been identified. *XIP* genes were found in *Populus trichocarpa* (Gupta and Sankararamakrishnan 2009).

Many processes of plant growth and development depend on massive water movement into and out of cells. Previous studies have proved that AQPs controll the water transport across cell plasma membranes during seed imbibition and early growth of embryo. For example, the expression of *AQP*s in seeds of *Mesembryanthemum crystallinum* is related to the growth of embryo and is inhibited in dormant seeds (Fukuhara *et al.* 1999). Besides, Gao *et al.* (1999) have demonstrated that two *AQP* genes, *BnPIP1* and *BnTIP2*, are involved in seed germination in *Brassica napus*. In recent years, to detect genes involved in *Medicago truncatula* germination, *AQP1* and *γ-TIP* genes were cloned by suppressive subtractive hybridization (Bouton *et al.* 2005). Liu *et al.* (2007) investigated the role of PIPs throughout germination in rice embryos and confirmed that PIPs are involved in this process.

Several studies have showed the important functions of AQPs in plant-water relations (Weig *et al.* 1997, Kirch *et al.* 2000, Jang *et al.* 2004, Alexandersson *et al.* 2005). Recently, environmental stimuli have been shown to

Submitted 15 December 2012, *last revision* 6 August 2013, *accepted* 21 August 2013.
Abbreviations: AQP - aquaporin; DAP - days after pollination; MIP - major intrinsic protein; PCR - polymerase chain reaction; PEG - polyethylene glycol; PIP - plasma membrane intrinsic protein; qRT-PCR - quantitative real-time PCR; RT-PCR - reverse transcription PCR; TIP - tonoplast intrinsic protein.
Acknowledgments: This work was supported by the National Natural Science Foundation of China (31070204).
* Corresponding author; fax: (+86) 27 68752213, e-mail: jbwang@whu.edu.cn

regulate the expression of both PIPs and TIPs at different levels (Hachez *et al.* 2006). On the transcriptional level, previous studies have pointed out that plant *AQP* genes respond to diverse abiotic stresses. Recently, researchers have reported that the gene expressions of *AQP* are up- or down-regulated by drought, salinity, and cold in *Arabidopsis*, rice, sunflower, cauliflower, wheat, barley, and maize (Kawasaki *et al.* 2001, Seki *et al.* 2002, Maathuis *et al.* 2003, Martinez-Ballesta *et al.* 2003, Wang *et al.* 2003, Jang *et al.* 2004).

Brassica napus is a dicotyledonous plant of the *Brassicaceae* family and it is an important oil crop worldwide. Although *AQP* genes have been extensively studied in *Arabidopsis*, maize, rice and other species, our knowledge is still limited in regulation of water transport during *B. napus* seed germination. In this study, seven new *B. napus PIP* genes from two different groups (*PIP1* and *PIP2*) were identified and the expression patterns of these seven cloned genes plus three previously identified *AQP* genes (*BnPIP1*, *BnPIP2*, and *BnTIP2*) during seed germination and in very young seedlings are reported. In addition, the expressions of these ten *AQP* genes in response to abiotic stresses (drought, cold, and salt) during seed germination was also investigated.

Materials and methods

Oilseed rape (*Brassica napus* L. cv. Zhongshuang 4) seeds were surface sterilized by 10 % (m/v) sodium hypochlorite for 6 min and washed 3 times with sterile water. Then, they were placed in Petri dishes (50 seeds per dish, in triplicate) containing two layers of filter paper moistened with 4 cm^3 of deionized water. The Petri dishes were wrapped in foil and incubated in the dark at 21 °C. Germination percentage was calculated to the total seeds sown in each dish. Samples of the whole seed or seedling were harvested at 0, 6, 12, 24, 48, 72, 96, and 120 h and were immediately frozen in liquid nitrogen.

To investigate the effects of different stresses (drought, cold, and salt), the seeds were germinated in 20 % (m/v) polyethylene glycol (PEG) 6000 solution, at a temperature of 12 °C, or in a 150 mM NaCl solution. The other germination conditions remained the same as mentioned above. The seeds or seedlings were harvested at 6, 12, 24, 48, and 72 h after treatments, frozen in liquid nitrogen, and prepared for a total RNA extraction. Each material was collected twice.

To understand the expression pattern of ten *AQP* genes, their transcription was also analyzed in different organs of adult plants.

Total RNA was extracted using a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). The residual genomic DNA in the extract was removed by several treatments with RNase-free *DNase I* (*Fermentas*, Canada). First strand cDNA was synthesized using *oligo-(dT)*$_{18}$ and *M-MLV* reverse transcriptase according to the manufacturer's instructions. To clone cDNA fragments encoding *B. napus PIP* genes, the reverse transcriptase PCR (RT-PCR) was performed with degenerated primers and first strand cDNA. Due to the high expression of *PIP* genes in germinating seeds, we selected 2 d germinating seeds for first strand cDNA synthesis. The PCR degenerated primers were designed on the conserved regions of different species *PIP* gene sequences derived from GenBank (Suppl. file 1). Five pairs of primers were designed and synthesized for RT-PCR (Table 1). The amplified PCR products were purified and cloned with a *Dual Promoter TA* cloning ® kit (*Invitrogen*). Cloned fragments were sequenced with *M13*, and consequently seven different *PIP* genes were cloned firstly in *B. napus* in our study. The nucleotide sequences of the seven *PIP* genes were submitted to GenBank [accession Nos. KF277205 (*BnPIP1;1*), KF277206 (*BnPIP1;2*), KF277207

Table 1. Degenerated primers for RT-PCR and gene-specific primers for RT-qPCR used in this study.

Name	Forward primers (5' - 3')	Reverse primers (5'–3')
P1	ACAAGTTYCCRGAGAGRC	RAADGGRATNGCTCTGAT
P2	GCHSTYATYGCYGAGTTC	VGTRATBGGRATDGTRGC
P3	BGCDGCTCCRARACTYCT	GCHSTYATYGCYGAGTTC
P4	GAGCNSTYATHGCYGAGT	DGCTCCRARACTYCTDGC
P5	GAGCNSTYATHGCYGAGT	VGTRATBGGRATDGTRGC
BnPIP1;1	GCTAAGAACAGACCAAAAGTG	GGCATTCGGTGGTATGATA
BnPIP1;2	GAATGGTCCAACCCAAAAG	TGTTCCCATTCTTGCACCT
BnPIP1;3	CTGGAAGCCTTTGACAACG	CATAAACCCAGCGGTGACA
BnPIP1;4	TGGCTGGAAACCCTTGACT	GCATCGCTTGGGCTTTT
BnPIP2;2	GGAGTCTCGGGCATTTCTT	CGATGGATACAGCACAGGG
BnPIP2;5	GTGTAGTAAGAAGACTGGAAGGA	GAAAGTGACGTTGGTGAGG
BnPIP2;7	TGGGATGGTAGCCAAATGC	TTCTCCGCGACTGACCCT
BnPIP1	CCAATGATCTCTGCGGCT	GCAGTGACATTCGGGCTA
BnPIP2	ATGCCGCCGAAAGCCCAC	GCAGTCATCGCCGAGTTC
Bnγ-TIP2	CGATTGGTGCGATTGTTC	TCTCTCTGCTGGAGTTGA

(*BnPIP1;3*), KF277208 (*BnPIP1;4*), KF277209 (*BnPIP2;2*), KF277210 (*BnPIP2;5*), and KF277211 (*BnPIP2;7*)]. The sequencing results were analyzed using *BLASTX* algorithms available on the *NCBI* website (http://www.ncbi.nlm.nih.gov/ BLAST/).

Sequence homology analyses were performed using the programs *BLASTN* and *BLASTX* (National Centre for Biotechnology Information, Bethesda, USA), respectively. The BnPIPs peptide sequences were aligned with those of 13 PIPs from *A. thaliana*, using *ClustalX* (Thompson *et al.* 1997). A phylogenetic neighbour-joining analysis was conducted on deduced amino acid sequences using the program *MEGA v. 3.1* (Kumar *et al.* 2004). Distance matrices were based on the Jones-Taylor-Thornton substitution matrix for the amino acid data. A bootstrap analysis with 1000 replicates was performed to assess the relative support for branches of the inferred phylogenetic tree.

The specific primer sets for closely related sequences were designed to carry out the RT-qPCR analysis. The gene-specific primers were designed avoiding conserved regions by the software *Primer5* (Table 1). In our primer pairs, at least one of the primer sets was highly specific.The qRT-PCR was carried out using an *ABI StepOneTM* real-time PCR system (*Applied Biosystems*, USA). *B. napus* β-actin (GenBank accession No. AF111812) was used as standard control in the RT-PCR reactions. Each 20 mm^3 reaction mixture contained 7.4 mm^3 of ddH_2O, 1 mm^3 of a diluted cDNA sample, 0.4 mm^3 of ROX, and 10 mm^3 of a PCR master mix (*Thunderbird SYBR-Green* qPCR mix, *Toyobo*, Japan), and the final concentration of the gene-specific forward and reverse primers was 300 μM. The PCR program was following: 95 ºC for 10 min, 40 cycles at 95 ºC for 15 s, and at 60 ºC for 1 min (48 samples were run in parallel). The specificity of the PCR amplification was verified by the melting curve analysis and confirmed on an agarose gel. Standard curves using five serial dilution points of the bulked cDNA samples, respectively, were built for each primer pair. The relative quantification analysis was investigated by the relative standard curve according to threshold values (CT) generated from the real-time PCR (Zhao *et al.* 2013). The data of the real-time PCR were mean values and standard errors (SD) of two independent experiments with two biological replicates.

Results

We analyzed each cloned gene using the *BLASTX* software and searched homology between *A. thaliana* and *B. napus* (Table 2). *BnPIP2;2* were homologous to *BnPIP2* of *B. napus*, and the other six genes were homologous to the *PIP* genes of *A. thaliana*. Also the amino acid sequences of the seven proteins showed a high similarity with those of known plant AQPs in the GenBank database. In addition, the multiple alignment of the deduced amino acid sequences for the genes cloned in our study together with those previously reported *PIP* genes from *B. napus* as well as five *PIP* genes from *Arabidopsis* was carried out using the *ClustalW* program (Supp. file 2). The deduced amino acid sequences of the seven genes contain two highly conserved asparagine-proline-alanine (NPA) motifs which are known to be important ones for the pore structure of the water channels and are conserved among AQPs. Based on homology analysis and multiple alignment, the seven different genes were thus named as *BnPIP1;1* (GenBank acc. No. KF277205), *BnPIP1;2* (GenBank acc. No. KF277206), *BnPIP1;3* (KF277207), *BnPIP1;4* (KF277208), *BnPIP2;2* (KF277209), *BnPIP2;5* (KF277210), and *BnPIP2;7* (KF277211), respectively.

To investigate the evolutionary relationships of the seven BnPIP proteins identified in our study, all thirteen PIP protein sequences of *Arabidopsis* as well as oilseed rape PIPs reported previously were selected from GenBank for conducting a phylogenetic analysis. As shown in the phylogenetic tree (Fig. 1), 22 PIP proteins were clustered into two separate groups. Four of the PIPs (BnPIP1;1, BnPIP1;2, BnPIP1;3, and BnPIP1;4) clustered with PIP1 subfamily, BnPIP2;2, BnPIP2;5, and BnPIP2;7 with the PIP2 subfamily. In the branch of PIP1s, BnPIP1;1 was a sister group to the AtPIP1;1/AtPIP1;2 clade, whereas BnPIP1;2 was basal to the clade containing AtPIP1;1, AtPIP1;2, and BnPIP1;1. Although BnPIP1;4 shared high sequence homology with BnPIP1;3, it belonged to a single clade which was basal to the branch containing all other PIP1 proteins. The result suggests that BnPIP1;4 might diverge relatively earlier from other *B. napus* PIP1s during evolution. In the branch of PIP2s, BnPIP2;2 and BnPIP2 formed a distinct clade and located at the clade based to AtPIP2;2 and AtPIP2;3. Besides, BnPIP2;5 was a sister group to the AtPIP2;5 clade and BnPIP2;7 was a sister group to the AtPIP2;7 clade.

Table 2. Analysis of sequence homology using *BLASTX* against the *NCBI* database.

Name	Homology	Identity[%]	Protein ID	e-value
BnPIP1;1	AtPIP1;2	96	NP_182120.1	0.0
BnPIP1;2	AtPIP1;2	99	NP_182120.1	0.0
BnPIP1;3	AtPIP1;3	98	NP_171668.1	2e-116
BnPIP1;4	AtPIP1;4	97	NP_567178.1	0.0
BnPIP2;2	BnPIP2	98	AAD39374.1	2e-119
BnPIP2;5	AtPIP2;5	94	NP_191042.1	2e-127
BnPIP2;7	AtPIP2;7	98	NP_195236.1	4e-118

To further understand the expression pattern of ten *AQP* genes, their transcription was analyzed in different organs of adult plants by qRT-PCR (Table 3). The results reveal that most *AQP* transcripts were abundantly accumulated in flowers as compared with the other organs. *BnPIP1;4*, *BnPIP2;2*, *BnPIP2;7*, and two previously reported genes (*BnPIP1*, *BnPIP2*) were highly expressed

in stems or flower organs. However, during seed germination, except for *BnPIP1;4*, other four genes showed a moderate expression. *BnPIP1;1* and three previously reported *AQPs* genes were expressed in roots, stems, leaves, and flowers, but were hardly detected in seeds (21 d after pollination). More *BnPIP1;1* transcripts were detected in roots and leaves than in stems and flowers. Expression patterns of *BnPIP1, BnPIP2*, and *BnTIP2* in various organs were similar. The expression of these three genes was 10- to 19-fold higher in flowers when compared to other organs.

No seeds germinated within 6 h, 56 % of seeds germinated after 12 h, and all seeds germinated after 24 h. Therefore, the stages after 24 h was designated as young

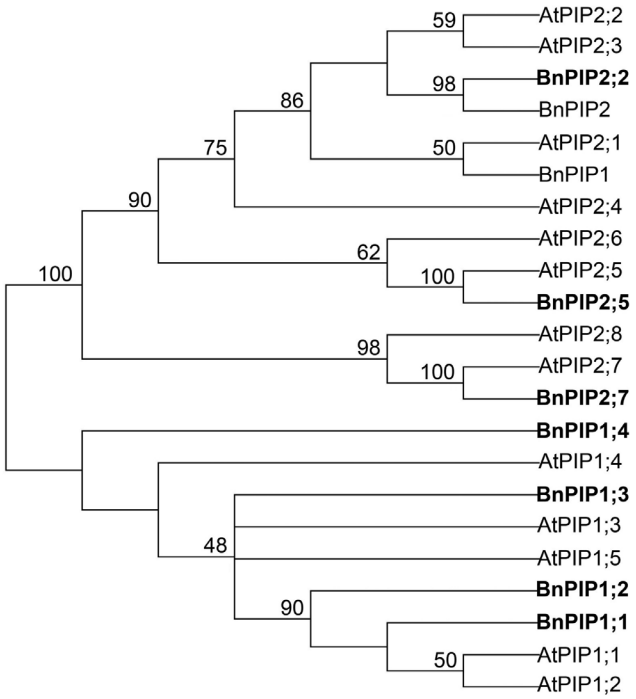

Fig. 1. A phylogenetic tree for the predicated amino acid sequences encoded by PIPs in *B. napus* and *Arabidopsis*. This tree was created by the neighbor-joining method using predicted amino acid sequences from the database and a multiple sequence alignment with *ClustalX*. The Evolution tree was constructed in *MEGA3.1* from 1 000 bootstrap replicates. Numbers above the major branches indicate bootstrap values ≥ 48 %. The sequence sources were as follows: *A. thaliana*, AtPIP1;1 (NP_001078323.1), AtPIP1;2 (NP_001078067.1), AtPIP1;3 (NP_171668.1), AtPIP1;4 (NP_974489.1), AtPIP1;5 (AAM10155.1), AtPIP2;1 (NP_190910.1), AtPIP2;2 (NP_181254.1), AtPIP2;3 (NP_181255.1), AtPIP2;4 (NP_200874.1), AtPIP2;5 (NP_191042.1), AtPIP2;6 (NP_181434.1), AtPIP2;7 (NP_001190920.1), AtPIP2;8 (NP_179277.1); *B. napus*, BnPIP1 (AAD39373.1), BnPIP2 (AAD39374.1), BnPIP1;1 (KF277205), BnPIP1;2 (KF277206), BnPIP1;3 (KF277207), BnPIP1;4 (KF277208), BnPIP2;2 (KF277209), BnPIP2;5 (KF277210), BnPIP2;7 (KF277211). *Bold* indicates the PIPs identified in our study.

Table 3. The relative expression of the *AQP* genes in roots, stems, leaves, flowers, and seeds (21 d post anthesis). Total RNA was extracted from various tissues of 7-month-old plants under non-stressed conditions. Each *AQP* gene was estimated by qRT-PCR. β-actin of *B. napus* was used as internal control. *AQP* expressions were normalized to the *ACTIN* expression (*AQP*/*ACTIN*). Means ± SD of two replicates.

Gene	Roots	Stems	Leaves	Flowers	Seeds
BnPIP1;1	0.0071±0.0013	0.0008±0.0001	0.0046±0.0020	0.0009±0.0002	0.0000±0.0000
BnPIP1;2	0.0076±0.0026	0.0059±0.0020	0.0788±0.0213	0.6965±0.0673	0.0151±0.0021
BnPIP1;3	0.0051±0.0021	0.0020±0.0003	0.0020±0.0010	0.0219±0.0074	0.0158±0.0050
BnPIP1;4	0.0306±0.0162	1.2503±0.1871	0.4528±0.1762	0.4924±0.0615	0.4837±0.1453
BnPIP2;2	0.4657±0.1093	1.3066±0.0958	1.5353±0.4305	3.0087±0.5083	0.0902±0.0204
BnPIP2;5	0.0019±0.0007	0.0009±0.0002	0.0009±0.0002	0.0378±0.0133	0.0117±0.0027
BnPIP2;7	0.0598±0.0274	0.0401±0.0091	0.1914±0.0522	2.1148±0.3481	0.2287±0.0335
BnPIP1	0.4606±0.1477	0.8680±0.2509	1.0226±0.1831	8.3341±1.3831	0.0035±0.0011
BnPIP2	0.0220±0.0085	0.0561±0.0068	0.1897±0.0363	1.7288±0.2977	0.0002±0.0001
BnTIP2	0.0062±0.0017	0.0369±0.0115	0.0335±0.0033	0.2471±0.0428	0.0000±0.0000

Table 4. Expression patterns of *AQP* genes during *B. napus* seed germination and young seedling growth were determined by qRT-PCR. β-actin of *B. napus* was used as internal control. The transcription of each *AQP* gene was compared to *ACTIN* (*AQP/actin*). The values are means ± SD of two replicates.

Gene	0 h	6 h	12 h	24 h	48 h	72 h	96 h	120 h
BnPIP1;1	0	0	0.001±0	0.006±0.001	0.029±0.004	0.031±0.006	0.016±0.002	0.031±0.006
BnPIP1;2	0	0.001±0	0.004±0.001	0.018±0.004	0.057±0.007	0.098±0.015	0.106±0.009	0.085±0.010
BnPIP1;3	0	0.008±0.002	0.003±0.001	0.011±0.001	0.035±0.001	0.029±0.007	0.030±0.003	0.022±0.002
BnPIP1;4	0.010±0	0.986±0.144	0.498±0.108	1.083±0.087	0.987±0.062	0.736±0.094	0.747±0.110	0.453±0.054
BnPIP2;2	0.001±0	0.007±0.002	0.010±0.003	0.047±0.011	0.118±0.007	0.178±0.025	0.203±0.026	0.269±0.032
BnPIP2;5	0.017±0.004	0.014±0.002	0.009±0.002	0.027±0.002	0.035±0.002	0.029±0.004	0.030±0.002	0.031±0.004
BnPIP2;7	0.007±0.003	0.036±0.003	0.024±0.005	0.081±0.011	0.184±0.012	0.233±0.029	0.297±0.017	0.288±0.040
BnPIP1	0.001±0	0	0.001±0	0.010±0.002	0.042±0.003	0.058±0.006	0.087±0.006	0.071±0.009
BnPIP2	0.001±0	0.012±0.002	0.016±0.003	0.103±0.011	0.229±0.022	0.763±0.201	0.430±0.093	0.775±0.152
BnTIP2	0	0	0	0.004±0.001	0.013±0.001	0.021±0.003	0.012±0.002	0.012±0.002

Table 5. The relative expression of the *AQP* genes in germinating seeds subjected to PEG and cold stresses, determined by qRT-PCR. The seeds were treated with 20 % PEG or a temperature of 12 °C for 6, 12, 24, 48, and 72 h, respectively. The expression recorded in the seeds germinated under non-stressed conditions at corresponding time points were used as control. The values are treated/control ratios. Means ± SD of two replicates.

Gene	6 h	12 h	24 h	48 h	72 h	Treatment
BnPIP1;1	0.47±0.10	0.04±0.01	0.06±0.00	0.26±0.02	0.44±0.02	PEG
BnPIP1;2	1.36±0.09	0.20±0.04	0.23±0.03	0.35±0.07	0.23±0.01	PEG
BnPIP1;3	0.36±0.04	0.55±0.12	0.13±0.01	0.14±0.02	0.17±0.03	PEG
BnPIP1;4	0.23±0.03	1.14±0.15	0.31±0.02	0.31±0.04	0.48±0.08	PEG
BnPIP2;2	0.31±0.07	0.15±0.03	0.22±0.03	0.28±0.03	0.22±0.02	PEG
BnPIP2;5	1.26±0.24	0.87±0.34	0.22±0.04	0.28±0.05	0.31±0.10	PEG
BnPIP2;7	0.33±0.06	0.60±0.18	0.19±0.01	0.34±0.04	0.27±0.04	PEG
BnPIP1	0.90±0.14	0.29±0.10	0.55±0.02	0.68±0.15	0.47±0.03	PEG
BnPIP2	0.13±0.02	0.07±0.02	0.03±0.00	0.10±0.01	0.06±0.01	PEG
BnTIP2	8.12±1.68	0.07±0.00	0.19±0.02	0.28±0.03	0.54±0.20	PEG
BnPIP1;1	0.70±0.05	0.06±0.03	0.01±0.00	0.02±0.00	0.11±0.01	cold
BnPIP1;2	1.21±0.18	0.49±0.16	0.08±0.01	0.09±0.02	0.10±0.01	cold
BnPIP1;3	0.67±0.10	1.22±0.28	0.68±0.08	0.10±0.02	0.28±0.05	cold
BnPIP1;4	0.18±0.11	0.66±0.08	1.32±0.17	0.74±0.20	1.02±0.15	cold
BnPIP2;2	1.07±0.19	1.80±0.26	0.41±0.01	0.20±0.06	0.13±0.02	cold
BnPIP2;5	1.91±0.53	2.72±0.40	0.94±0.12	0.19±0.05	0.53±0.04	cold
BnPIP2;7	0.20±0.04	0.60±0.08	0.23±0.03	0.08±0.03	0.11±0.01	cold
BnPIP1	15.38±1.27	1.31±0.27	0.12±0.02	0.20±0.10	0.09±0.03	cold
BnPIP2	0.31±0.02	0.37±0.04	0.08±0.01	0.12±0.03	0.06±0.01	cold
BnTIP2	46.19±9.04	0.77±0.22	0.08±0.01	0.82±0.17	0.78±0.11	cold

seedlings in our study. The expression of ten *AQP* genes was performed by qRT-PCR (Table 4). Except for *BnPIP1;1*, *BnPIP1;2*, *BnPIP1;3*, and *BnTIP2,* other *AQP* genes were expressed in dry seeds at different levels. After imbibition, the expression of almost all *AQP* genes began to increase within 12 h except for *BnPIP2;5*. Among these, the expression of *BnPIP1;3*, *BnPIP1;4*, and *BnPIP2;7* increased gradually within 6 h. However, the expression of *BnPIP2;5* decreased gradually during 12 h of water uptake and then increased rapidly and kept a stable level after germination. The expressions of *BnPIP1;1* and *BnPIP1;2* were enhanced after imbibition for 12 h, then increased rapidly after germination with maximal expression at 72 and 96 h, respectively. The expression patterns of the three previously reported *AQP* genes were similar to *BnPIP1;1* and *BnPIP1;2*. Unlike other *AQP* genes, the expressions of *BnPIP2;2* and *BnPIP2;7* were very low in dry seeds, increased during germination and maintained at a high level during the young seedling phase.

To further understand the effect of abiotic stresses on the expression of ten *AQP* genes, their transcriptions under different stresses were analyzed by RT-qPCR (Tables 5, 6).

Whereas 56 % of seeds germinated after imbibition for 12 h in water at 21 °C, no seeds germinated under the stress treatments at the same time. Similarly, the growth of hypocotyl was also inhibited under all stress treatments (data not shown). The expressions of the *AQP* genes were significantly altered by the dehydration stress induced by 20 % PEG (Table 5). The transcript levels of six genes (*BnPIP1;1*, *BnPIP1;3*, *BnPIP2;2*, *BnPIP2;7*, *BnPIP1*, and *BnPIP2*) were rapidly decreased down to one-tenth of their control mRNA level at the PEG treatment for 6 h, and the expressions of *BnPIP1;2*, *BnPIP2;5*, and *BnTIP2* increased initially and then decreased. The transcription of *BnTIP2* was up-regulated more than 8-fold at 6 h and then decreased rapidly. The expression of *BnPIP2;5* was up-regulated 1.3-fold at the 6-h PEG treatment.

Table 6. The relative expression of the *AQP* genes in germination seeds subjected to the salt stress. Theseeds were dealt with 150 mM NaCl for 6, 12, 24, and 48 h. The relative values are treated/control ratios. Means ± SD of two replicates.

Gene	6 h	12 h	24 h	48 h
BnPIP1;1	1.46±0.21	0.16±0.02	0.04±0.01	0.20±0.01
BnPIP1;2	4.78±0.99	0.99±0.11	1.05±0.18	0.71±0.14
BnPIP1;3	2.35±0.27	3.59±0.34	0.59±0.05	1.23±0.19
BnPIP1;4	3.19±0.24	8.94±1.11	2.03±0.17	1.74±0.43
BnPIP2;2	2.02±0.19	1.49±0.24	1.35±0.18	0.70±0.09
BnPIP2;5	3.17±0.29	2.96±0.30	0.77±0.05	1.16±0.22
BnPIP2;7	1.42±0.11	1.84±0.26	0.80±0.15	0.49±0.05
BnPIP1	6.93±1.08	2.60±0.32	3.18±0.30	1.04±0.12
BnPIP2	0.36±0.05	0.40±0.07	0.20±0.03	0.20±0.03
BnTIP2	22.25±2.29	0.20±0.06	0.25±0.05	0.32±0.06

The expression patterns of the *AQP* genes in response to the cold stress were similar to those in response to the drought stress (Table 5). The expression of *BnPIP1;1*, *BnPIP2;7*, and *BnPIP2* decreased to one-tenth, even one-hundredth of their control mRNA level within 48 h. However, the transcription levels of *BnPIP2;2*, *BnPIP2;5*, *BnPIP1*, and *BnTIP2* increased within 12 h and then decreased. Among these, the expressions of *BnPIP1* and *BnTIP2* were up-regulated 15- to 46-fold at 6 h and then quickly declined. The expression of the genes *BnPIP2;2* and *BnPIP2;5* slightly increased at the 12-h cold treatment, then declined rapidly. In addition, the *BnPIP1;3* and *BnPIP1;4* expressions increased slightly at 12 and 24 h with a subsequent decrease afterward.

Upon the NaCl (150 mM) treatment, the expressions of all the *AQP* genes were rapidly up-regulated and then decreased gradually (Table 6). Among these, the transcriptions of *BnPIP1;1*, *BnPIP1;2*, *BnPIP2;2*, *BnPIP2;5*, and *BnPIP1* were rapidly elevated 1.5- to 7-fold at 6 h, and then the transcripts of *BnPIP1;1*, *BnPIP1;2*, and *BnPIP2;2* decreased to a low level at 12 to 48 h, but *BnPIP2;5* and *BnPIP1* only to their control levels. Interestingly, the expression of *BnTIP2* was up-regulated more than 22-fold after the NaCl treatment for 6 h, and then its expression rapidly decreased to one-tenth of the control. The transcriptions of *BnPIP1;3*, *BnPIP1;4*, and *BnPIP2;7* were up-regulated 1.4- to 9-fold within the 12-h salt treatment and subsequently decreased. The expression of *BnPIP2* was down-regulated after the treatment with NaCl.

Discussion

The *AQP* gene in plants was firstly identified by Maurel *et al.* (1993) and so far a large number of *AQPs* in plants have been reported. For example, in *Arabidopsis*, maize and rice, the whole set of *AQPs* genes have been cloned and classified (Chaumont *et al.* 2001, Johanson *et al.* 2001, Sakurai *et al.* 2005). Nevertheless, for *B. napus, AQPs* genes have been mostly unknown due to lack of detailed genome data. In current study, seven *PIP* genes were cloned from germinating seeds of *B. napus* cv. Zhongshuang 4. The sequence alignment indicates that these seven genes are different from the previously identified *B. napus AQP* genes showing that they are new genes of a *B. napus AQP* family. The phylogenetic analysis (Fig. 1) shows that BnPIP1;1, BnPIP1;2, BnPIP1;3, and BnPIP1;4 had a closer evolutional relationship with AtPIP1s (91 - 99 % identity) than AtPIP2s (70 - 79 % identity), whereas the BnPIP2;2, BnPIP2;5, and BnPIP2;7 were similar to AtPIP2s. Given the data together, our results indicate that the seven PIPs can be classified into two PIP subfamilies, *i.e.*, BnPIP1;1, BnPIP1;2, BnPIP1;3, and BnPIP1;4 belong to the plant PIP1 subfamily and the other three belong to the PIP2 subfamily. Although we could not identify all *PIP* genes in *B. napus*, our results expanded the known *PIP* genes in this species.

AQPs in plants often show a tissue/organ-specific expression (Yamada *et al.* 1995, Takahashi *et al.* 2004). Alexandersson *et al.* (2005) reported that *Arabidopsis AQP* genes are predominantly expressed in roots or flowers, but no *AQP* members are leaf-specifically expressed. In the present study, eight genes were more abundantly expressed in flowers compared to other organs. For example, the *BnPIP2;5* and *BnPIP2;7* transcripts were dominantly expressed in flowers which is consistent with the expression of *AtPIP2;5* and *AtPIP2;7* in *Arabidopsis* flowers (Alexandersson *et al.* 2005). On the other hand, previous study also shows that *PIP* is not seed-specific and may not be involved in the seed maturation (Daniels *et al.* 1994). Similarly, *BnPIP1;1*, *BnPIP1*, *BnPIP2*, and *BnTIP2* were hardly detected in 21 DAP seeds (Table 3). However, all of the four genes were upregulated during seed germination (Table 4). In addition, both *BnPIP2;2* and *BnPIP2* were highly expressed in flowers, and *BnPIP1;4* in stem which is in disagreement with results in *Arabidopsis* (Alexandersson *et al.* 2005). These discrepancies of the gene expressions between

Arabidopsis and *B. napus* might arise from difference in the ploidy level and genetic background of two closely related species.

Seed germination greatly changes the tissue water content, and therefore aquaporins can be expected to play an important function during the process. The expression of ten *AQP* genes were lower or scarcely detected in dry seeds, but were up-regulated during germination as well as in very young seedlings (Table 4). Previous studies have reported that the transcription of certain *PIP*s was low in dry seeds of *Arabidopsis*, oilseed rape, and radish (Daniels *et al.* 1994, Gao *et al.* 1999, Suga *et al.* 2001). Recently, Liu *et al.* (2013) has shown that PIPs play an important role in increasing seed germination rate. In our study, the expressions of *BnPIP1;1*, *BnPIP1;2*, *BnPIP1;3*, and *BnPIP1;4* were enhanced a few hours before radicle emergence, and the *BnPIP1;1* expression was coincident with radicle protrusion. These expression patterns suggested that PIPs might be involved in seed germination. For example, the transcripts of *BnPIP1;4* accumulated at 6 h of imbibition but gradually declined after germination, consistently with the expression patterns of the PIP1 subfamily during rice seed germination (Liu *et al.* 2007). Therefore, the high expression of *BnPIP1;4* in imbibed seeds might contribute to water transport across plasma membranes during early period of seed germination and facilitate water supply to expanding tissues. Other PIPs could also be involved in the movement of water during seed germination because *BnPIP2;2, BnPIP2;5*, and *BnPIP2;7* were also expressed in germinating seeds. Jang (2007) has reported that overexpressing *PIP1;4* or *PIP2;5* enhanced water flow and facilitated germination under cold stress in transgenic *Arabidopsis* plants. Besides, the expressions of *AQP* genes were also up-regulated after 24 h imbibition in water (Table 4), suggesting a possible role of those *AQP*s in the early seedling growth. We measured the hypocotyl growth during germination, and the largest elongation was observed between 24 to 48 h (data not shown). Coincidentally, the expressions of *BnPIP1;1* and *BnPIP1;3* were elevated 3- to 5-fold at 24 h to 48 h and reached almost the maximum at 48 h. Therefore, these *PIP* genes may play a potential role in hypocotyl elongation (Malz and Sauter 1999, Liu *et al.* 2008).

Several studies have reported that a plant *AQP* gene expression is regulated by environmental stimuli, such as drought, cold, and salinity (reviewed by Hachez *et al.* 2006, Gomes *et al.* 2009). A systematic analysis of an *AQP* gene expression in response to abiotic stresses was conducted in *Arabidopsis*, maize, and rice (Alexandersson *et al.* 2005, Zhu *et al.* 2005, Li *et al.* 2008). In the present work, we studied the expression of the *B. napus AQP* genes in response to the salinity stress, cold stress and PEG-induced dehydration. Our results show that the expression of different types of *AQP* genes was up- or down-regulated in response to various stresses (Table 5, 6). The down-regulation of the *AQP* genes expression under drought treatment has been generally observed in various plants, such as in *Arabidopsis* (Jang *et al.* 2004, Alexandersson *et al.* 2005) and *Nicotiana glauca* (Smart *et al.* 2001). Similarly, the expressions of most *BnAQP* genes were significantly down-regulated when the seeds germinated in the PEG solution for 12 h (Table 5). For example, the transcription of *BnPIP2;2* and *BnPIP2* decreased to one-tenth of the control by the 6-h drought stress which is consistent with the expression of *PIP2;2* under a drought stress in *Arabidopsis* (Jang *et al.* 2004). The down- regulation of the *PIP* gene expression indicates the decreased hydraulic conductance in roots when plants experiencing drought stress (Mahdieh *et al.* 2008). However, the expressions of *BnPIP1;4* and *BnPIP2;5* were up-regulated 1.1-fold at 12 h and 1.3-fold at 6 h by the drought treatment. Similarly, *PIP1;4* and *PIP2;5* are up-regulated in *Arabidopsis* by drought treatment (Alexandersson *et al.* 2005, 2010). The up-regulation of the *AQP* genes expression suggests that they play a role in drought tolerance (Almeida-Rodriguez *et al.* 2010).

In contrast to a reduction of some *AQP* gene expressions by the drought stress, the salinity stress caused up-regulation of the most *AQP* gene expressions during the initial treatment stages. The initial up-regulation and the subsequent down- regulation of *PIP* gene expressions have been reported (Jang *et al.* 2004, Du *et al.* 2011). Liu *et al.* (2013) suggested that *PIP* play an important role in increasing root hydraulic conductivity and salt resistance. In our study, the expression of *BnPIP1;3* was up-regulated 3.6-fold at 12 h and then declined gradually, and a similar expression pattern of *PIP1;3* was also observed in root in *Arabidopsis* (Jang *et al.* 2004). After the NaCl treatment for 6 h, the transcription of *BnTIP2* was 22-fold higher than in untreated seeds. Previous study have indicated that over-expression of a *PgTIP1* gene is beneficial for salt-stress tolerance in transgenic *Arabidopsis* (Peng *et al.* 2007).

The expression patterns of the *AQP* genes in response to the cold stress were similar to those in response to the drought stress. In *Arabidopsis*, the expression of most *PIP* genes was markedly down-regulated by cold and drought stresses. However, the transcription of *BnPIP1;4* and *BnPIP2;5* was up-regulated after the 24-h cold treatment, in accordance with the expression of *AtPIP1;4* and *AtPIP2;5* in *Arabidopsis* root under cold stress (Jang *et al.* 2004). Lee *et al.* (2012) indicated that the expressions of *PIP1;4* and *PIP2;5* in *Arabidopsis* seedlings after a low root temperature treatment for 24 h increases. Furthermore, there are no changes in hydraulic conductivity in the *PIP1;4*- and *PIP2;5*-overexpressing transgenic *Arabidopsis* plants after a cold treatment for 1 d. On the other hand, the transcription of *BnPIP1* and *BnTIP2* was 15- and 46-fold up-regulated, respectively, after the cold treatment for 6 h and then declined rapidly (Table 5). *BnPIP1* formed a clade with *AtPIP2;1* in our phylogenetic tree, and the expression of *AtPIP2;1* by a cold treatment in *Arabidopsis* (Jang *et al.* 2004) is up-regulated similarly as *BnPIP1*. In brief, these up- and down-regulations of the *AQP* gene in response to an abiotic stress might contribute to protection from water loss during the early stage of the stress or to acceleration of water uptake to maintain water

homeostasis under adverse conditions (Jang *et al.* 2004).

The *B. napus PIP* gene expression profiles during seed germination and in response to the abiotic stresses were demonstrated. The results show that the expression of ten *AQP* genes were lower in dry seeds, but were up-regulated during germination. These data indicate that the APQs play an important role in seed germination. In addition, the expression of *B. napus PIPs* was significantly up-regulated by salinity and was mostly down-regulated by the cold and dehydration stresses.

References

Alexandersson, E., Danielson, J.A.H., Rade, J., Moparthi, V.K., Fontes, M., Kjellbom, P., Johanson, U.: Transcriptional regulation of aquaporins in accessions of *Arabidopsis* in response to drought stress. - Plant J. **61**: 650-660, 2010.

Alexandersson, E., Fraysse, L., Sjövall-Larsen, S., Gustavsson, S., Fellert, M., Karlsson, M., Johanson, U., Kjellbom, P.: Whole gene family expression and drought stress regulation of aquaporins. - Plant mol. Biol. **59**: 469-484, 2005.

Alleva, K., Marquez, M., Villarreal, N., Mut, P., Bustamante, C., Bellati, J., Martínez, G., Civello, M., Amodeo, G.: Cloning, functional characterization, and co-expression studies of a novel aquaporin (FaPIP2;1) of strawberry fruit. - J. exp. Bot. **61**: 3935-3945, 2010.

Almeida-Rodriguez, A.M., Cooke, J.E.K., Yeh, F., Zwiazek, J.J.: Functional characterization of drought-responsive aquaporins in *Populus balsamifera* and *Populus simonii* × *balsamifera* clones with different drought resistance strategies. - Physiol. Plant. **140**: 321-333, 2010.

Baiges, I., Schäffner, A.R., Affenzeller, M.J., Mas, A.: Plant aquaporins. - Physiol. Plant **115**: 175-182, 2002.

Ben Bâaziz, K., Lopez, D., Bouzid, S., Cochard, H., Venisse, J.S., Sakr, S.: Early gene expression in the walnut tree occurring during stimulation of leaf hydraulic conductance by irradiance. - Biol. Plant. **56**: 657-666, 2012.

Bouton, S., Viau, L., Lelievre, E., Limami, A.M.: A gene encoding a protein with a proline-rich domain (Mtpprd1), revealed by suppressive subtractive hybridization (SSH), is specifically expressed in the *Medicago truncatula* embryo axis during germination. - J. exp. Bot. **413**: 825-832, 2005.

Chaumont, F., Barrieu, F., Herman, E.M., Chrispeels, M.J.: Characterization of a maize tonoplast aquaporin expressed in zones of cell division and elongation. - Plant Physiol. **117**: 1143-1152, 1998.

Chaumont, F., Barrieu, F., Jung, R., Chrispeels, M.J.: Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. - Plant Physiol. **122**: 1025-1034, 2000.

Chaumont, F., Barrieu, F., Wojcik, E., Chrispeels, M.J., Jung, R.: Aquaporins constitute a large and highly divergent protein family in maize. - Plant Physiol. **125**: 1206-1215, 2001.

Daniels, M.J., Mirkov, T.E., Chrispeels, M.J.: The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. - Plant Physiol. **106**: 1325-1333, 1994.

Du, S.Z., Dai, Q.L., Chen, C., Feng, B., Liu, T.T., Tian, X., Gong, Y.Y., Sun, Y.K., Wang, J.: Molecular cloning and characterization of the plasma membrane aquaporin gene (*OvPIP*) from *Orychophragmus violaceus*. - Afr. J. Biotechnol. **10**: 3709-3714, 2011.

Fukuhara, T., Kirch, H.H., Bohnert, H.J.: Expression of *Vp1* and water channel proteins during seed germination. - Plant Cell Environ. **22**: 417-424, 1999.

Gao, Y.P., Young, L., Bonham-Smith, P., Gusta, L.V.: Characterization and expression of plasma and tonoplast membrane aquaporins in primed seed of *Brassica napus* during germination under stress conditions. - Plant mol. Biol. **40**: 635-644, 1999.

Gomes, D., Agasse, A., Thiébaud, P., Delrot, S., Gerós, H., Chaumont, F.: Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. - BBA Biomembranes **1788**: 1213-1228, 2009.

Gupta, A.B., Sankararamakrishnan, R.: Genome-wide analysis of major intrinsic proteins in the tree plant *Populus trichocarpa*: characterization of XIP subfamily of aquaporins from evolutionary perspective. - BMC Plant Biol. **9**: 134, 2009.

Hachez, C., Zelazny, E., Chaumont, F.: Modulating the expression of aquaporin genes *in planta*: a key to understand their physiological functions? - Biochim. biophys. Acta **1758**: 1142-1156, 2006.

Hussain, S.S., Iqbal, M.T., Arif M.A., Amjad, M.: Beyond osmolytes and transcription factors: drought tolerance in plants *via* protective proteins and aquaporins. - Biol. Plant. **55**: 401-413, 2011.

Jang, J.Y., Kim, D.G., Kim, Y.O., Kim, J.S., Kang, H.: An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. - Plant mol. Biol. **54**: 713-725, 2004.

Jang, J.Y., Lee, S.H., Rhee, J.Y., Chung, G.C., Ahn, S.J., Kang, H.: Transgenic *Arabidopsis* and tobacco plants overexpressing an aquaporin respond differently to various abiotic stresses. - Plant mol. Biol. **64**: 621-632, 2007.

Johanson, U., Karlsson, M., Johansson, I., Gustavsson, S., Sjövall, S., Fraysse, L., Weig, A.R., Kjellbom, P.: The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. - Plant Physiol. **126**: 1358-1369, 2001.

Katsuhara, M., Rhee, J.Y., Sugimoto, G., Chung, G.C.: Early response in water relations influenced by NaCl reflects tolerance or sensitivity of barley plants to salinity stress *via* aquaporins. - Soil Sci. Plant Nutr. **57**: 50-60, 2011.

Kawasaki, S., Borchert, C., Deyholos, M., Wang, H., Brazille, S., Kawai, K., Galbraith, D., Bohnert, H.J.: Gene expression profiles during the initial phase of salt stress in rice. - Plant Cell **13**: 889-905, 2001.

Kirch, H-H., Vera-Estrella, R., Golldack, D., Quigley, F., Michalowski, C.B., Barkla, B.J., Bohnert, H.J.: Expression of water channel proteins in *Mesembryanthemum crystallinum*. - Plant Physiol. **123**: 111-24, 2000.

Kudoyarova, G.R., Kholodova, V.P., Veselov D.S.: Current state of the problem of water relations in plants under water deficit. - Russ. J. Plant Physiol. **60**: 165-175, 2013.

Kumar, S., Tamura, K., Nei, M.: *MEGA3*: integrated software for molecular evolutionary genetics analysis and sequence alignment. - Brief Bioinform. **5**: 150-163, 2004.

Lee, S.H., Chung, G.C., Jang, J.Y., Ahn, S.J., J. Zwiazek, J.: Overexpression of PIP2;5 aquaporin alleviates effects of low root temperature on cell hydraulic conductivity and growth

in *Arabidopsis*. - Plant Physiol. **159**: 479-488, 2012.

Li, G.W., Peng, Y.H., Yu, X., Zhang, M.H., Cai, W.M., Sun, WNi., Su, W.A.: Transport functions and expression analysis of vacuolar membrane aquaporins in response to various stresses in rice. - J. Plant Physiol. **165**: 1879-1888, 2008.

Liu, C.W., Fukumoto, T., Matsumoto, T., Gena, P., Frascaria, D., Kaneko, T., Katsuhara, M., Zhong, S., Sun, X.l., Zhu, Y.M., Iwasaki, I., Ding, X.D., Calamita, G., Kitagawa, Y.: Aquaporin OsPIP1;1 promotes rice salt resistance and seed germination. - Plant Physiol. Bioch. **63**: 151-158, 2013.

Liu, D.Q., Tu, L.L., Wang, L., Li, Y.J., Zhu, L.F., Zhang, X.L.: Characterization and expression of plasma and tonoplast membrane aquaporins in elongating cotton fibers. - Plant Cell Rep. **27**: 1385-1394, 2008.

Liu, H.Y., Yu, X., Cui, D.Y., Sun, M.H., Sun, W.N., Tang, Z.C., Kwak, S.S., Su, W.A.: The role of water channel proteins and nitric oxide signaling in rice seed germination. - Cell Res. **17**: 638-649, 2007.

Maathuis, F., Filatov, V., Herzyk, P., Krijger, G., Axelsen, K., Chen, S., Green, B., Li, Y., Madagan, K., Sánchez-Fernández, R., Forde, B., Palmgren, M., Rea, P., Williams, L., Sanders, D., Amtmann, A.: Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. - Plant J. **35**: 675-692, 2003.

Mahdieh M., Mostajeran A., Horie T., Katsuhara, M.: Drought stress alters water relations and expression of PIP-type aquaporin genes in *Nicotiana tabacum* plants. - Plant Cell Physiol. **49**: 801-813, 2008.

Malz, S., Sauter, M.: Expression of two *PIP* genes in rapidly growing internodes of rice is not primarily controlled by meristem activity or cell expansion. - Plant mol. Biol. **40**: 985-995, 1999.

Martinez-Ballesta, M.C., Aparicio, F., Pallas, V., Martinez, V., Carvajal, M.: Influence of saline stress on root hydraulic conductance and *PIP* expression in *Arabidopsis*. - J. Plant Physiol. **160**: 689-697, 2003.

Maurel, C., Reizer, J., Schroeder. J.I., Chrispeels, M.J.: The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus oocytes*. - EMBO J. **12**: 2241-2247, 1993.

Peng, Y.H., Lin, W.L., Cai, W.M., Arora, R.: Overexpression of a *Panax ginseng* tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic *Arabidopsis* plants. - Planta **226**: 729-740, 2007.

Sakurai, J., Ishikawa, F., Yamaguchi, T., Uemura, M., Maeshima, M.: Identification of 33 rice aquaporin genes and analysis of their expression and function. - Plant Cell Physiol. **46**: 1568-1577, 2005.

Schaeffner, AR.: Aquaporin function, structure, and expression: are there more surprises to surface in water relations? - Planta **204**: 131-139, 1998.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., Shinozaki, K.: Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. - Plant J. **31**: 279-292, 2002.

Smart, L.B., Moskal, W.A., Cameron, K.D., Bennett, A.B.: MIP genes are down-regulated under drought stress in *Nicotiana glauca*. - Plant Cell Physiol. **42**: 686-693, 2001.

Suga, S., Imagawa, S., Maeshima, M.: Specificity of the accumulation of mRNAs and proteins of the plasma membrane and tonoplast aquaporins in radish organs. - Planta **212**: 294-304. 2001.

Takahashi, H., Rai, M., Kitagawa, T., Morita, S., Masumura, T., Tanaka, K.: Differential localization of tonoplast intrinsic proteins on the membrane of protein body type II and aleurone grain in rice seeds. - Biosci. Biotechnol. Biochem. **68**: 1728-1736, 2004.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higguns, D.G.: The *CLUSTAL_X* windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. - Nucl. Acids. Res. **24**: 4876-4882, 1997.

Wang, H., Miyazaki, S., Kawai, K., Deyholos, M., Galbraith, D., Bohnert, H.: Temporal progression of gene expression responses to salt shock in maize roots. - Plant Mol. Biol. **52**: 873-891, 2003.

Weig, A., Deswarte, C., Chrispeels, M.J.: The major intrinsic protein family of *Arabidopsis* has 23 members that form three distinct groups with functional aquaporins in each group. - Plant Physiol. **114**: 1347-1357, 1997.

Yamada, S., Katsuhara, M., Kelly, W.B., Michalowski, C.B., Bohnert, H.J.: A family of transcripts encoding water channel proteins: tissue-specific expression in the common ice plant. - Plant Cell **7**: 1129-1142, 1995.

Zhao, P., Zhang, N., Yin, Z.J., Y.D. Liu, Shen, F.F.: Analysis of differentially expressed genes in response to endogenous cytokinins during cotton leaf senescence. - Biol. Plant. **57**: 425-432, 2013.

Zhu, C., Schraut, D., Hartung, W., Schaffner, A.R.: Differential responses of maize MIP genes to salt stress and ABA. - J. exp. Bot. **56**: 2971-2981, 2005.