Genome-Wide Identification and Characterization of the Aquaporin Gene Family and Transcriptional Responses to Boron Deficiency in *Brassica napus*

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Aquaporins (AQPs) are an abundant protein family and play important roles to facilitate small neutral molecule transport across membranes. Oilseed rape (*Brassica napus* L.) is an important oil crop in China and elsewhere in the world, and is very sensitive to low boron (B) stress. Several AQP family genes have been reported to be involved in B transport across plasma membranes in plants. In this study, a total of 121 full-length AQPs were identified and characterized in *B. napus* (AC genome), and could be classified into four sub-families, including 43 PIPs (plasma membrane intrinsic proteins), 35 TIPs (tonoplast intrinsic proteins), 32 NIPs (NOD26-like intrinsic proteins), and 11 SIPs (small basic intrinsic proteins). The gene characteristics of *BnaAQP* were similar to those of *BraAQP* (A genome) and *BolAQP* (C genome) including the composition of each sub-family, gene structure, and substrate selectivity filters. The *BnaNIP* was the most complex AQP sub-family, reflecting the composition of substrate selectivity filter structures which affect the permeation of solution molecules. In this study, the seedlings of both B-efficient (QY10) and B-inefficient (W10) cultivars were treated with two boron (B) levels: deficient (0.25 µM B) and sufficient (25 µM B). The transcription of *AQP* genes in root (R), juvenile leaf (JL), and old leaf (OL) tissues of both cultivars was investigated under B deficient and sufficient conditions. Transcription of most *BnaPIP* and *BnaTIP* genes was significantly increased compared with other *BnaAQP* genes in all the three tissues, especially in the roots, of both B-efficient and B-inefficient cultivars under both B conditions. With B deprivation, the expression of the majority of the *BnaPIP* and *BnaTIP* genes was down-regulated in the roots. However, the *BnaNIP* genes were up-regulated. In addition, the *BnaCnn_random.PIP1;4b, BnaPIP2;4s, BnaC04.TIP4;1a, BnaAnn_random.TIP1;1b, and BnaNIP5;1s* (except for *BnaA07.NIP5;1c* and *BnaC06.NIP5;1c*) exhibited obvious differences at low B between B-efficient and B-inefficient cultivars. These results will help us to understand boron homeostasis in *B. napus*.

Keywords: aquaporins (AQPs), *Brassica napus*, boron (B), transcriptional profile, boron homeostasis
INTRODUCTION

Aquaporins (AQPs) are a superfamly of major intrinsic proteins (MIP) that selectively facilitate water and small neutral molecules across biological membranes (Maul and Chrispeels, 2001). Recently, the AQP genes in the genomes of some plants have been identified, with 35 in Arabidopsis thaliana (A. thaliana), 60 in Brassica rapa (B. rapa), 67 in B. oleracea, 31 in maize, 33 in rice, 41 in common bean, and 30 in barley (Chaumont and Jung, 2001; Johanson et al., 2001; Sakurai et al., 2005; Tao et al., 2014; Ariani and Gepts, 2015; Diehn et al., 2015; Tombuloglu et al., 2015). Based on sequence similarity and subcellular localization, the AQPs are divided into five distinct sub-families in higher plants which consists of plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), and uncategorized X intrinsic proteins (XIPs) (Ishikawa et al., 2005; Maurel et al., 2008; Wudick et al., 2009). AQP proteins of different sub-families share a conserved three-dimensional (3D) structure in cell membranes, although they vary in sequence, subcellular localization, and in planta physiological function, especially in substrate selectivity. Each AQP protein contains a six α-helical transmembrane structure (TM1-TM6), five loops (LA-LE), and two additional half-helices (HB, HE) (Daniels et al., 1999; Wallace et al., 2006). There are two selectivity filters within the protein structure that determine substrate specificity. One is composed of two highly conserved NPA motifs located in HB and HE, where HB and HE form a narrow central aqueous pore together, and the other is an ar/R (aromatic/arginine) selectivity filter consisting of four residues, one in transmembrane 2 (TM2), one in transmembrane 5 (TM5), and two in loop E (LE1 and LE2) (Wallace and Roberts, 2006; Hub and Groot, 2008; Mitaniuueno et al., 2011).

Boron (B) is an essential micronutrient (Warington, 1923) for plant growth and development. Boron deficiency is a widespread problem for field crop production, causing large losses of crop yield annually both quantitatively (Wei et al., 1998) and qualitatively (Bell and Dell, 2008). AtNIP5;1 is involved in efficient B uptake in the roots of A. thaliana (Brassicaceae) under B deficiency (Takano et al., 2006) and further studies have indicated that the polar localization of NIP5;1 plays an important role in B absorption, which depends on threonine phosphorylation in the conserved three amino acid (ThrProGly, TPG) repeat of the N-terminal region, and is maintained by clathrin-mediated endocytosis (Wang S. et al., 2017). Moreover, the minimum open reading frame (AUG-stop, “AUGTAA”) in 5′UTR of AtNIP5;1 has been identified as the crucial element in response to cytoplasmic B concentration that regulates gene expression and induces ribosome stalling and mRNA degradation under high B conditions (Tanaka et al., 2016).

The Brassica genus diverged from a common ancestor with Arabidopsis around 18 MYA (Yang et al., 2006). B. napus (AACC), 2n = 4x = 38) is an important oil crop species in the world, which most likely originated in domestication from the natural spontaneous hybridization between the diploid ancestors B. oleracea (CC, 2n = 2x = 18) and B. rapa (AA, 2n = 2x = 20) ~7,500 years ago, resulting in chromosome doubling (Chalhoub et al., 2014). In B. napus, we have previously identified six orthologous genes of NIP5;1, with BnaA03.NIP5;1b, and BnaC02.NIP5;1a proposed as candidate genes underlying the major-effect B efficiency QTLs qBEC-A3a and qBEC-C2a, respectively (Hua et al., 2016a,b). AtNIP6;1 is essential for preferential distribution of B into growing tissues under low B (Tanaka et al., 2008). Yeast complementation assays have confirmed that HvPIP1;3 and HvPIP1;4 can facilitate B transport across the membranes into yeast cells (Fitzpatrick and Reid, 2009). However, the overexpression of AtTIP5;1 and rice OsPIP2;4, OsPIP2;7, OsPIP1;3, and OsPIP2;6 in Arabidopsis notably increased its tolerance to high levels of B, and the latter two are involved the influx and efflux transport of B (Pang et al., 2010; Kumar et al., 2014; Mosa et al., 2015). In addition, AQP proteins can also facilitate transport of many other small molecule nutrients, such as Lsi1 (OsNIP2;1) for silicon transport (Ma et al., 2006), AtTIP2;1 and AtTIP2;3 for NH3 transport (Loqué et al., 2005), AtNIP3;1 for arsenic uptake (Xu et al., 2015), and AtNIP1;2 facilitating Al-malate transport (Wang Y. et al., 2017), respectively.

The ancestral sub-genomes of Brassica species may be classified based on gene density and divergence, and have been nominated as LF (the least fractionized sub-genome), MF1 (the moderate gene fractionized sub-genome), and MF2 (the most gene fractionized sub-genome) (Cheng et al., 2012). A reference genome sequence of B. napus (cultivar Darmor-bzh) has been anchored to all 19 chromosomes (Chalhoub et al., 2014). In this study, we have identified and characterized a complete set of 121 full-length AQPs in the B. napus genome, which can be divided into four sub-families based on a phylogenetic tree that also incorporated A. thaliana orthologues. Phylogenetic relationships were established, and used as a context for investigating variation in gene structure, chromosomal and sub-genome distribution, and protein structural features of AQPs. The transcription of AQP genes in root (R), juvenile leaf (JL), and old leaf (OL) tissues of a B-efficient and a B-inefficient cultivar was also investigated under B deficient and sufficient conditions.

MATERIALS AND METHODS

Identification of BnaAQPs Genes in B. napus

AQP genes were identified in B. napus based on their homology similarity to Arabidopsis. The genome sequences and gene IDs of the 35 AQP genes were obtained from the TAIR10 database (http://www.arabidopsis.org/index.jsp) and used to perform a BLAST analysis and search for the AQP homologous genes in the CNS-Genoscope database (http://www.genoscope.cns.fr/brassicancanus/) (Chalhoub et al., 2014) and Brassica database (BRAD; http://brassicadb.org/brad/index.php), respectively. The genomic, cDNA, CDS, as well as protein sequences, of BnaAQPs were obtained from CNS-Genoscope database with inclusion of each AQP protein determined by checking the candidate genes using the Hidden Markov Model of the Pfam database (http://pfam.sanger.ac.uk/search), SMART database (http://smart.embl-heidelberg.de/), and NCBI Conserved Domain Search.
Multiple Alignment and Phylogenetic Analysis of BnaAQP Family Genes

Multiple sequence alignment of protein sequences of B. napus and Arabidopsis was performed using ClustalW tool of MEGA 5.1 (Tamura et al., 2011). An unrooted phylogenetic tree of the 121 full-length AQP protein sequences was then constructed using MEGA 5.1 with the Neighbor Joining (NJ) method, and the analysis of bootstrap values was conducted using 1,000 replicates.

Chromosome Locations and Gene Structure Analysis of BnaAQP Genes

The chromosome location information of the BnaAQP genes was obtained from BRAD. The MapInspect software was used to draw the gene chromosome location diagrams (Figure 2). The exon-intron structures of the BnaAQP family genes were determined based on alignments of their coding sequences with the corresponding genomic sequences, and the diagram was drawn using GSDS (Gene structure display server: GSDS; http://gsds.cbi.pku.edu.cn/).

Conserved Motifs and Physicochemical Parameters Analysis of BnaAQP Proteins

Conserved motifs were identified by the MEME tool (Version 4.11.2) (http://meme-suite.org/tools/meme). The parameter settings were default values apart from: range of optimum width of each motif from 6 to 50 and maximum number of motifs to search: 15. The physicochemical parameters, including molecular weight (MW) and isoelectric points (pl), of each BnaAQP protein were calculated using the compute pI/MW tool of ExPASy (http://www.expasy.org/tools/). GRAVY (grand average of hydropathy) values were calculated using the PROTPARAM tool (http://web.expasy.org/protparam/). Subcellular localization prediction was conducted using the WoLF PSORT (https://wolfpsort.hgc.jp/) server.

Syntenic Gene Analysis between A. thaliana and B. napus

Syntenic genes between A. thaliana and B. napus were searched by the online tool "syntenic gene analysis" (http://brassicadb.org/brad/searchSyntenyPCK.php) and each syntenic At-Bn ortholog in B. napus of AQP were obtained.

Transcriptional Profile of BnAQP Family Genes

The B. napus seedlings of the B-efficient cultivar Qingyou10 (QY10) and the B-inefficient cultivar Westar10 (W10) were planted in Hoagland solution with 0.25 µM B (low B) and 25 µM B (high B) in an illuminated growth room (300–320 µmol m⁻² s⁻¹; 24°C daytime/22°C night; 16 h light/8 h dark) for 30 d. The roots (R), old leaves (OL, without petiole), and juvenile leaves (JL, without petiole) of QY10 and W10 were individually harvested, and each sample included three independent biological replicates. The total RNA of each sample was extracted using RNA extraction kit (BioTeke, Beijing, China) according to the manufacturer’s recommendations. The RNA-seq sequencing libraries were subsequently sequenced using Illumina HiSeq™ 2000 (Illumina Inc., San Diego, CA, USA). All raw data were deposited in the NCBI Sequence Read Archive (Bioproject accession number, PRJNA393069). High-quality clean reads were mapped to the reference Darmor-bzh genome co-ordinates, and the RPKM (reads per kilo bases per million reads) measure was used to obtain the gene annotation and expression data. The RPKM values of genes were transformed to logarithmic values, which were used to produce a heat map using the program Multiviewer (MeV) (Saeed et al., 2003).

RESULTS

Genome-Wide Identification and Phylogenetic Analysis of BnaAQP Gene Family in B. napus

The sequences and IDs of 35 AtAQP genes were used to carry out BLAST alignment against the B. napus reference genome, with annotation search used to identify 121 full-length BnaAQP genes as well as 15 non-full-length BnaAQP genes (Supplementary Table 1). The latter set lacked the transmembrane domains, resulting in the loss of the NPA motif and/or ar/R selectivity filter (Supplementary Table 1).

A phylogenetic tree was constructed based on the protein sequences of the 121 BnaAQP genes together with the 35 AtAQP genes (Figure 1). Four subgroups of BnaAQPs were readily identified, and coincided with the distribution of AtAQPs. All AtAQP genes had 1–7 corresponding orthologous genes in B. napus except from AtPIP2;8 and AtNIP1;1. Moreover, there were no XIP sub-family genes in either B. napus or A. thaliana. The B. napus sub-families included 43 PIPs, 35 TIPs, 32 NIPs, and 11 SIP members. The PIP sub-family had two sub-groups, 19 members in PIP1 and 24 in PIP2. The TIP sub-family was divided into five sub-groups (TIP1 to TIP5), nine members in TIP1, 13 in TIP2, 10 in TIP3, one in TIP4 and two in TIP5, respectively. SIPs were divided into the SIP1 (6 members) and SIP2 (5 members) sub-groups. The NIPs could be divided into seven sub-groups (NIP1 to NIP7), four members in NIP1, 2 and 6 sub-groups, six in NIP3, 4 and 5 sub-groups and two in NIP7 sub-group. Most (118 members) of the BnaAQPs, were clearly distinguished from each other and within the different sub-families and even
FIGURE 1 | Phylogenetic tree of AQPs in Brassica crops. The AQPs phylogenetic tree was generated by MEGA 5.1 with Neighbor Joining (NJ) method and 1,000 replicates bootstraps, based on the amino acid sequences of 121 BnaAQP genes and 35 AtAQPs genes. The gene name with purple, blue, yellow and cyan represented PIPs, TIPs, NIPs, and SIPs, respectively. AtAQPs, AtPIPs, AtTIPs, AtNIPs, and AtSIPs were marked with circle, square, diamond and triangle, respectively. Different sub-groups of each sub-family. However, the BnaNIP4;1 and one BnaNIP4;2 appeared to have conserved amino acid sequences, distinguished not well each other. The orthologous genes of AtPIPs, BnaA04.PIP1;1a, and BnaAnn_random.PIP1;1c appear to have undergone greater divergence from PIP1;2, although BnaA09.PIP1;1b and BnaC08.PIP1;1a, annotated as PIP1;2, were very close to PIP1;1 in the neighbor joining tree (Figure 1).

The Physicochemical Parameters of BnaAQPs

The length of BnaAQPs ranged from 206 aa (BnaAnn_random.PIP1;1c) to 323 aa (BnaC08.NIP3;1c), with BnaNIPs longer than the other BnaAQPs. The molecular weight (MW) ranged from 21.31 kDa (BnaA02.TIP2;3a) to 34.59 kDa (BnaA02_random.NIP6;1c), which was related to the protein length. All the BnaTIP proteins had a low pI.
(isoelectric point, pI < 7), except for BnaTIP5;1s. The majority of BnaPIPs and BnaNIPs, and all BnaSIPs proteins had pI > 7, while BnaPIP2;1s, BnaPIP2;2/2;3s, BnaPIP2;4s BnaC08.NIP3;1c, BnaC05.NIP7;1a, and BnaA05.NIP7;1a had a relatively low pI (pI < 7). The hydropathy value of all the amino acids was divided by the protein length, which was defined as grand average of hydropathicity (GRAVY). The GRAVY of all BnaAQPs were positive (ranging from 0.21 to 1.07; Table 1), indicating that all they were hydrophobic. Most proteins of each sub-family of BnaAQPs shared similar parameters, while different sub-families of BnaAQPs were distinguished from each other in the values of MW and pI (Table 1 and Supplementary Figure 1).

The main types of subcellular localization for all 121 BnaAQPs were predicted to be plasma membrane (plas) and tonoplast membrane (vacu) (Table 1). 43% of TIPs were localized in the tonoplast membrane and almost all the BnaPIPs were localized in the plasma membrane. BnaNIPs were localized in plasma membrane except for BnaNIP2;1s and BnaNIP3;1s (tonoplast membrane). The members of BnaSIPs were equally localized in the plasma membrane and the tonoplast membrane.

Chromosomal Locations of BnaAQP Genes and Syntenic Block Analysis between A. thaliana and B. napus

Of the genes annotated in this paper as BnaAQPs, 95 full-length and 13 non-full-length had previously been incorporated in the genome scaffolds corresponding to 18 of the 19 pseudochromosomes of B. napus (Figure 2), with the remainder located on scaffolds not yet anchored to a chromosome (Supplementary Table 2). The number of BnaAQPs located on each chromosome varied dramatically, with 12 on C04, two on A08 and none on C09. Genes within each BnaAQP subfamily were distributed unevenly on the chromosomes. Some clustering was observed at the top of chromosomes A05 and C04, the middle of A03 and C03, and the bottom of chromosome A07 and A09. There was an even distribution on each of the A (55) and C (53) genomes (Figure 2), with 76 full length BnaAQPs mapped to 17 syntenic blocks and three non-full-length BnaAQPs to two syntenic blocks (Table 2). Thirty three genes were located within the LF sub-genome with the relative proportion of 76 and 55% in Table 2 syntenic blocks (Table 2). The number of paralogues retained in these sub-genomes of both the LF sub-genome with the relative proportion of 76 and 55% in Table 2 syntenic blocks (Table 2). The number of paralogues retained in these sub-genomes of both diploid ancestors (Liu et al., 2014).

Gene Structure and Conserved Domains of BnaAQPs

The availability of the B. napus gene sequence enabled us to analyze and compare the gene structures of all 121 BnaAQPs (Figure 3). As a whole, there was considerable variation in intron number between the different BnaAQP sub-families. The majority of BnaPIPs had three introns, although BnaA10.PIP1;3a and BnaPIP1;5s had two, and BnaPIP2;4s, BnaA04.PIP1;1a and BnaC07.PIP2;7c had four. Within the BnaTIP sub-family 17 genes had five introns, while two BnaTIP1;3a lacked an intron. Within the BnaNIP sub-family, 17 had four introns and 13, mostly BnaNIP2;1s and BnaNIP3;1s, had three introns. All SIP genes contained two introns. The gene structures of most BnaAQPs were conserved within a sub-family, and although there was some variation among homologous genes, they did not always show similarity amongst paralogous genes within the same sub-family. Additionally, the intron length changed frequently within the homologous genes of the isoforms of BnaPIP2;5, BnaPIP2;6, BnaNIP4;1, and BnaNIP5;1.

In addition, the conserved domains of all BnaAQPs were analyzed and fifteen motifs identified as conserved (Figure 4). Generally, the BnaAQPs within each sub-group shared similar motif compositions, while among different sub-groups their motif compositions varied. Motif 4 was common among all BnaAQPs, and was determined as a conserved region of BnaAQPs (Supplementary Figure 2C). Motif 5 was conserved in BnaPIPs, BnaTIPs and BnaNIPs (Supplementary Figure 2D). Two NPA motifs of all BnaAQPs were included in motif 1 and motif 2 (Supplementary Figures 2A,B). The BnaSIP family had the lowest number of motifs, and shared the lowest similarities with the proteins of other BnaAQP sub-families. The number and type of motifs of BnaNIPs changed frequently, which indicated that the protein diversity of this sub-family was high in B. napus.

The Comparison of NPA motifs and ar/R Selectivity Filters in BnaAQPs

NPA motifs and ar/R selectivity filters in BnaAQPs were identified since they are important for determining the permeation of solution molecules (Table 3). Two NPA motifs were identical in both BnaTIPs and BnaPIPs and all the NPA motifs in each sub-family were conserved. Additionally, the ar/R selectivity filters of BnaPIPs were also identical within different sub-groups, but that of BnaTIPs changed frequently. The second NPA motifs in BnaSIPs were more conserved than the first. The first NPA motifs of BnaSIP2;1s were present in two styles (NPV and NPL). The NPA motif and ar/R selectivity filters of BnaNIPs showed the greatest variation, particularly the ar/R selectivity filters of BnaNIP5;1s (Table 3). Moreover, the amino acids of H5 (BnaC06.random.NIP5;1c and BnaA07.NIP5;1c: A, N, A, R) and LE1 (BnaA03.NIP5;1b and BnaC03.NIP5;1b: A, I, A, R) differed from that of AtNIP5;1 (A, I, G, R) (Wallace and Roberts, 2004). In addition, we found that all the novel differences in the selectivity filters of BnaAQPs were paired in the corresponding A and C genomes (Table 3).

Gene Expression of BnaAQPs in Response to Low B Stress

RNA-seq was used to investigate the transcriptional profile of all the BnaAQPs in contrasting B-efficient (“Qingyou10”) and B-inefficient (“Westar10”) cultivar under B sufficient and deficient conditions. A total of 99 BnaAQPs (81%) were up-regulated or down-regulated in roots and/or leaves of both cultivars. The heat map displayed the transcript abundance pattern of BnaAQPs in roots (R), old leaves (OL), and juvenile leaves (JL) (Figure 5). Most of the BnaPIPs showed relatively higher expression levels in all the three tissues of B-efficient and B-inefficient cultivars compared with other sub-families of BnaAQPs.
TABLE 1 | Gene sequence characteristics of 121 BnaAQP s and their protein physicochemical parameters and putative subcellular localization.

| At AQP | Gene ID | B. napus ID | AQP name | Location | Gene length (bp) | Protein length (aa) | Molecular weight (kDa) | GRAVY | pi | Subcellular localization (Wolf Psort) |
|--------|---------|-------------|----------|----------|-----------------|---------------------|------------------------|-------|----|------------------------------------|
| AtPIP1;1 | At3g61430 | BnaA09g39170D | BnaA09.PIP1;1b | 27787216..27788864 | 1.649 | 286 | 30.64 | 0.36 | 8.66 | vacu |
| AtPIP1;2 | At2g45980 | BnaC07g23630D | BnaC07.TIP1;2b | 28244087..28247766 | 3.680 | 286 | 21.75 | 0.63 | 9.54 | vacu |

(Continued)
| At isoform | Gene ID | B. napus ID | AQP name | Location | Gene length (bp) | Protein length (aa) | Molecular weight (kDa) | GRAVY | pl | Subcellular localization (Wolf Psort) |
|-----------|---------|-------------|----------|----------|-----------------|---------------------|------------------------|-------|----|-------------------------------------|
| AtTIP1;3  | At4g01470 | BnaC07_random.TIP1;3a | 1673039..1673797 | 759 | 252 | 25.86 | 0.81 | 5.35 | pias |
| AtTIP2;1  | At3g16240 | BnaC01g44580 | 4074152..4075768 | 1,617 | 249 | 24.98 | 0.99 | 5.32 | pias |
| AtTIP2;2  | At4g17340 | BnaC01g11690 | 867957..869098 | 1,142 | 250 | 25.05 | 0.10 | 5.10 | vacu |
| AtTIP2;3  | At5g47450 | BnaC02g46870 | 2737513..2738954 | 1,442 | 250 | 25.20 | 1.00 | 6.48 | vacu |
| AtTIP3;1  | At1g73190 | BnaCnn_random.TIP3;1c | 4983652..49837529 | 998 | 266 | 28.03 | 0.56 | 6.70 | vacu |
| AtTIP3;2  | At1g17810 | BnaC08g37510 | 3426321..34264931 | 1,410 | 267 | 28.50 | 0.57 | 6.54 | vacu |
| AtTIP4;1  | At2g25810 | BnaC04g38040 | 39308971..39382403 | 1,433 | 249 | 26.12 | 0.72 | 5.31 | vacu |
| AtTIP5;1  | At3g47440 | BnaCnn_random.TIP5;1a | 14194669..14195620 | 952 | 258 | 26.76 | 0.74 | 8.46 | chlo |
| AtSIP1;1  | At3g04090 | BnaC05g37440 | 2999291..3000207 | 2,797 | 239 | 25.58 | 0.70 | 9.52 | vacu |
| AtSIP1;2  | At5g18290 | BnaC09g53230 | 4140963..4142787 | 1,825 | 243 | 26.21 | 0.74 | 10.13 | vacu |
| AtSIP2;1  | At3g56650 | BnaCnn_random.TIP2;1c | 19195494..19196978 | 1,485 | 238 | 26.01 | 0.66 | 9.61 | vacu |
| AtSIP2;2  | At3g20470 | BnaC04g24760 | 2568147..25683276 | 1,805 | 237 | 25.92 | 0.67 | 9.52 | vacu |
| AtNIP1;1  | At4g19030 | – | – | – | – | – | – | – | – |
| AtNIP1;2  | At4g18910 | BnaC07g35550 | 3794850..37961356 | 2,847 | 298 | 31.66 | 0.42 | 8.83 | vacu |
| AtNIP2;1  | At2g34390 | BnaC04g10830 | 8417418..8418594 | 1,177 | 286 | 30.32 | 0.59 | 8.95 | vacu |

(Continued)
Moreover, approximately half of the BnaPIPs, e.g., BnaPIP1;1s, BnaPIP1;2s, and BnaPIP2;2/2;3s, had high expression levels in roots under the B sufficient condition; and they were dramatically down-regulated under low B stress in both cultivars (Figure 5 and Supplementary Table 3). A majority of BnaPIPs were expressed in all three tissues in both cultivars and both B levels, including BnaPIP1;1s, BnaPIP1;2s, BnaPIP1;4s (expect for BnaCnn_random.PIP1;4b), BnaPIP2;1s, BnaPIP2;2/2;3s, and BnaPIP2;7s (expect for BnaC07.PIP2;7c) (Figure 5 and Supplementary Table 3). However, several other BnaPIPs had tissue-preferential expression patterns in both cultivars, e.g., BnaPIP1;3s, BnaPIP2;4s, BnaPIP2;5s, and BnaPIP2;6s. Under B deprivation, the expression of BnaCnn_random.PIP1;4b was significantly up-regulated in all the three tissues of Qingyou10, but not in Westar10. However, the BnaPIP2;4 members were strikingly down-regulated in the roots of the two cultivars, particularly in Qingyou10. In addition, BnaC08.PIP2;5b and BnaA09.PIP2;5b showed up-regulation, while BnaC06.PIP2;5a and BnaA07.PIP2;5a were down-regulated with B deprivation.

BnaTIP1;1s and BnaTIP1;2s showed high expression levels in all tissues, whereas BnaTIP1;3s, BnaTIP1;3s, and BnaTIP5;1s were rarely expressed in any tissues. BnaTIP2;2s and BnaTIP2;3s showed an obvious root-preferential expression pattern in both B-efficient and B-inefficient cultivars at both B levels (Figure 5 and Supplementary Table 3). Under B deprivation, BnaC06.TIP2;1c, BnaC03.TIP2;1a, and BnaA03.TIP2;1b were distinctly down-regulated in roots and leaves of the two cultivars, while BnaC04.TIP4;1a was only down-regulated in roots. The degree of down-regulation for BnaC04.TIP4;1a in Qingyou10 was much greater than that for Westar10. BnaAnn_random.TIP1;1b only showed significantly up-regulated at low B in Qingyou10.

The expression levels of all the BnaSIPs in all tissues had slightly changed under B deprivation, and there was no significant difference between the B-efficient and B-inefficient cultivars. Moreover, transcription of all BnaSIPs varied in all tissues, apart from BnaA09.SIP2;1b which had no transcripts detected in any tissue, and BnaSIP1;2s which was not transcribed in leaves.

In comparison with other BnaAQPs, the expression levels of BnaNIPs were generally relatively lower in all the three tissues of B-efficient and B-inefficient cultivars under both B conditions. BnaA08.NIP3;1c, BnaC08.NIP3;1c, BnaA02_random.NIP6;1c, BnaA02.NIP6;1a as well as BnaNIP2;1s showed a root-specific...
expression pattern in both cultivars at low and high B levels (Figure 5 and Supplementary Table 3). The transcription of BnaC05.NIP3;1b, BnaA05.NIP3;1b, BnaNIP4;1s, BnaNIP4;2s, and BnaNIP7;1s was not detected in any tissue in both cultivars. In contrast, the transcription of BnaC03.NIP5;1b, BnaC02.NIP5;1a, BnaA03.NIP5;1b, and BnaA02.NIP5;1a was induced at low B in all three tissues of both cultivars, especially in the roots. Interestingly, the level of transcription for all the BnaNIP5;1s mentioned above in the B-inefficient cultivar (Westar10) was significantly higher than that in the B-efficient cultivar (Qingyou10).

**DISCUSSION**

**AQP Characterization in Brassica napus**

Aquaporin is an abundant and high diversity protein family in plants, which facilitates transport of diverse small neutral molecules such as H₂O, H₂O₂, boric acid, arsenic acid, silicic acid, urea and glycerol across membrane (Wallace et al., 2002; Ma et al., 2006, 2008; Takano et al., 2006; Hove and Bhave, 2011). Previously, AQP genes have been identified within the Brassicaceae, with 60 in the diploid A genome of B. rapa (Tao et al., 2014), 67 in the diploid C genome of B. oleracea (Diehn et al., 2015), and 35 in A. thaliana (Johanson et al., 2001). B. napus is a recent allopolyploid that originated by combining the intact genomes of B. oleracea and B. rapa. Gene replication and chromosome rearrangement in B. rapa and B. oleracea is therefore expected to result in a conserved gene distribution of AQP in the respective A and C chromosomes of B. napus. In this study, 121 full-length AQP family genes were identified in B. napus (Table 1), only slightly fewer than the sum of those reported in B. oleracea (Diehn et al., 2015) and B. rapa (Tao et al., 2014). Due to the origin and evolutionary independence of the two diploids (B. rapa and B. oleracea) over the past 4.6 MYA (Liu et al., 2014), the chromosomal location of BnaAQPs in the A genome are not
TABLE 2 | Synteny block and distribution of aquaporin genes in the three sub-genomes of *Brassica napus*.

| At isoform | Gene ID     | Synteny block | Subgenomes        |
|------------|-------------|---------------|-------------------|
|            |             | LF            | MF1               | MF2               |
| AtPIP1;1   | At3g61430   | N             | BnaA09.PIP1;1b    | BnaA04.PIP1;1a    | –                |
|            |             |               |                   |                   |                  |
| AtPIP1;2   | At2g45960   | J             | BnaC04.PIP1;2b    | BnaA03.PIP1;2c    | –                |
|            |             |               |                   |                   |                  |
| AtPIP1;3   | At1g01620   | A             | BnaA10.PIP1;3a    | BnaC05.PIP1;3a    | –                |
|            |             |               |                   |                   |                  |
| AtPIP1;4   | At4g00430   | O             | –                 | BnaC03.PIP1;4a    | –                |
| AtPIP1;5   | At4g23400   | U             | –                 | BnaA07.PIP1;5a    | –                |
|            |             |               |                   |                   |                  |
| AtPIP2;1   | At3g53420   | N             | BnaA09.PIP2;1a    |                   | BnaC06.PIP2;1a   |
| AtPIP2;2   | At2g37170   | J             | –                 |                   | –                |
| AtPIP2;3   | At2g37180   | J             | –                 |                   | –                |
| AtPIP2;4   | At3g60660   | Wb            | BnaA10.PIP2;4c    | BnaC03.PIP2;4a    | BnaA02.PIP2;4a   |
| AtPIP2;5   | At3g54820   | N             | BnaC08.PIP2;5b    |                   | BnaA07.PIP2;5a   |
| AtPIP2;6   | At2g39010   | J             | –                 |                   | –                |
| AtPIP2;7   | At4g35100   | U             | BnaC01.PIP2;7a    | BnaC07.PIP2;7c    | BnaC03.PIP2;7b   |
| AtPIP2;8   | At2g16850   | H             | –                 |                   | –                |
| AtTIP1;1   | At2g36830   | J             | –                 |                   | –                |
| AtTIP1;2   | At3g26520   | L             | BnaC07.TIP1;2b    |                   | –                |
| AtTIP1;3   | At4g01470   | O             | –                 |                   | –                |
| AtTIP2;1   | At3g16240   | F             | BnaC05.TIP2;1b    | BnaA01.TIP2;1a    | BnaC03.TIP2;1a   |
| AtTIP2;2   | At4g17340   | U             | –                 |                   | BnaA03.TIP2;1b   |
| AtTIP2;3   | At5g47450   | V             | BnaC07.TIP2;3a    |                   | –                |
| AtTIP3;1   | At1g73190   | E             | BnaC06.TIP3;1b    | BnaA02.TIP3;1a    | –                |
| AtTIP3;2   | At1g17810   | A             | BnaC05.TIP3;2a    |                   | –                |
| AtTIP4;1   | At2g25810   | I             | BnaC04.TIP4;1a    |                   | –                |
| AtTIP5;1   | At3g47440   | M             | BnaA06.TIP5;1a    |                   | –                |
| AtSIP1;1   | At3g04090   | F             | BnaC05.SIP1;1b    |                   | –                |
| AtSIP1;2   | At5g18290   | R             | BnaA10.SIP1;2a    |                   | –                |
| AtSIP2;1   | At3g56960   | N             | BnaA09.SIP2;1b    | BnaC04.SIP2;1b    | BnaA07.SIP2;1a   |
| AtNIP1;1   | At4g19030   | U             | –                 |                   | –                |
| AtNIP1;2   | At4g18910   | U             | BnaC01.NIP1;2a    | BnaC07.NIP1;2b    | –                |
| AtNIP2;1   | At2g34390   | J             | BnaC04.NIP2;1b    |                   | –                |
| AtNIP3;1   | At1g31885   | B             | –                 | BnaC08.NIP3;1c    | BnaC05.NIP3;1b   |

(Continued)
completely conserved in homeologous regions of the C genome (Figure 2).

In this study, we were able to allocate BnaAQPs according to the four well-established sub-families (43 PIPs, 35 TIPs, 32 NIPs, and 11 SIPs). As expected, no XIP sub-family was detected consistent with their absence in Arabidopsis, B. oleracea and B. rapa (Johanson et al., 2001; Tao et al., 2014; Diehn et al., 2015). There was also evidence of strong conservation within each BnaAQP sub-family since the allopolyploidisation of B. napus ∼7,500 years ago, with membership closely matching the sum of B. rapa (23 PIPs, 16 TIPs, 15 NIPs, and 6 SIPs) and B. oleracea (25 PIPs, 19 TIPs, 17 NIPs, and 6 SIPs).

Compared to formation of diploid Brassica species or subsequent to the evolution of B. napus (Nicolas et al., 2007; Jeonghwan et al., 2009; Liu et al., 2014). Although 121 AQP genes were identified as full-length BnaAQPs (Table 1), 15 had an incomplete protein structure (Supplementary Table 1), mostly lacking fragments containing the conserved motifs corresponding to the NPA and ar/R selectivity filters.

The NPA motifs and ar/R selectivity filters of BnaAQPs showed variation among sub-families (Table 3). Within the PIP sub-families these were highly conserved in A. thaliana, B. rapa, B. oleracea, and B. napus. In addition, compared with those in barley (Tombuloglu et al., 2015), common bean (Ariani and Gepts, 2015) and rubber tree (Zhi et al., 2015), the NPA motifs and ar/R selectivity filters of TIPs were also highly conserved, which suggested that the PIPs have been subject to strong selection in different plant taxonomic lineages. The Val (V) and Ile (I) residues near to P3 site have been considered as the conserved amino acids for water channel activity (Suga and Maeshima, 2004), and their differentiation can be used to distinguish PIP1s and PIP2s. In this study, all BnaPIP2s had the Val (V) and Ile (I) residues near P3 site, except for BnaPIP2s. However, all PIP1s had the Ile (I) and Ile (I) residues near P3 site, except for BnaPIP2s. It was observed that all BnaPIP1s and BnaPIP2s by the two amino acids.

All TIP sub-families of B. napus, B. rapa, B. oleracea, and A. thaliana shared two identical NPA motifs in HB and HE, although there still existed some differences in the ar/R selectivity filters. In Arabidopsis, the ar/R selectivity filters of TIPs were divided into 3 large subgroups (group I, group II and group III), with group II consisting of group IIA and group IIB (Wallace and Roberts, 2004). AtTIP3.2s belongs to group IIB, although all BnaTIP3.2s had a novel compositions of ar/R selectivity filters (H, M, A, R) (Table 3), which were the same as those of B. rapa and B. oleracea, but different from A. thaliana (H, I, A, R) (Tao

### Table 2: Continued

| At isoform | Gene ID | Synteny block | Subgenomes |
|------------|---------|---------------|------------|
|            |         |               | LF         | MF1             | MF2         |
| AtNIP1;1   | At5g37750 | S             | –          | BnaA01.NIP1;1a  | –            |
| AtNIP3;1   | At4g10380 | P             | –          | –               | BnaA05.NIP3;1a |
| AtNIP5;1   | At1g80760 | E             | BnaA06.NIP6;1a | –                | –            |
| AtNIP7;1   | At3g06100 | F             | BnaA05.NIP7;1a | –                | –            |

There were 76 full-length AQP genes in 17 syntenic blocks and 3 incomplete genes in 2 syntenic blocks. LF, Least fractionated subgenome; MF1, medium fractionated subgenome; MF2, more fractionated genome. The genes marked with “*” represented the incomplete genes. “–” indicated that no synthetic ortholog of AtAQP was found in the corresponding sub-genome in Brassica napus.
FIGURE 3 | Gene structure of BnaAQP. The exon-intron structures of the BnAQP s were determined by the alignments of coding sequences with corresponding genomic sequences, and the diagram was obtained using GSDS (Gene Structure Display Server 2.0) Web server. The purple, blue, yellow, and cyan rectangles represented the exons of BnaPIP s, BnaTIP s, BnaNIP s, and BnaSIP s, the gray rectangles represented the UTR and the black lines represented the introns, respectively. BnaC06_random.NIP5;1c was listed alone because it had the longest gene length.
The ar/R selectivity filters in TIP5s of the four species were considerably diverged from other TIPS (Table 3) (Tao et al., 2014; Diehn et al., 2015), indicating that TIP5s probably have a special function for solute permeation. Additionally, BnaTIP5;1s had a high pI and a distinct subcellular localization from the other BnaTIP members (Table 1). TIPs usually facilitate H$_2$O, NH$_3$ and urea transport (Wudick et al., 2009). Overexpression of AtTIP5;1 in A. thaliana significantly increases the tolerance to B toxicity (Pang et al., 2010), and the vacuolar compartmentation could explain the high B tolerance of Arabidopsis overexpression lines.

BnaNIPs and BnaSIPs showed larger variation in the third alanine of the NPA motifs, and a greater diversity in ar/R selectivity filters than BnaPIP and BnaTIPs (Table 3), suggesting that they may have acquired a more specialized function for substrate absorption than the latter. In A. thaliana, the AtNIPs showed a large-scale permeation for substrates such as H$_2$O, H$_2$O$_2$, As, B, Si, urea, and glycerol, which was consistent with their abundant amino acid constitutions of NPA and ar/R selectivity filters of NIP sub-family (Mitaniueno et al., 2011).

The NPAs and ar/R selectivity filters of BnaAQP were similar to those of the combination of BraAQP and BolAQP, but the specific ar/R selectivity filters in BraA.NIP4.c and BolC.NIP4.d (W, S, A, R) (Diehn et al., 2015) had been lost during the allopolyploidisation or subsequent domestication of B. napus (Table 3). The ar/R selectivity filters of BnaTIP3;2s and BnaNIP5;1s differed from those of their homologous genes in A. thaliana, which is consistent with the finding in B. oleracea (Tao et al., 2014). Moreover, all the novel differences in the selectivity filters of BnaAQP were paired in the corresponding A and C genomes (Table 3), which suggested that the differentiation may have occurred before the species divergence of B. rapa and B. oleracea.

| TABLE 3 | Amino acid composition of the NPA motifs and ar/R selectivity filters of AQP in Brassica napus. |
| BnaAQP subgroup | NPA1 | NPA2 | H2 | H5 | LE1 | LE2 |
|------------------|------|------|-----|-----|-----|-----|
| PIP1             | NPA  | NPA  | F    | H   | T   | R   |
| PIP2             | NPA  | NPA  | F    | H   | T   | R   |
| TIP1             | NPA  | NPA  | H    | I   | A   | V   |
| TIP2             | NPA  | NPA  | H    | I   | G   | R   |
| TIP3;1           | NPA  | NPA  | H    | I   | A   | R   |
| TIP3;2           | NPA  | NPA  | H    | M   | A   | R   |
| TIP4             | NPA  | NPA  | H    | I   | A   | R   |
| TIP5             | NPA  | NPA  | N    | V   | G   | C   |
| SIP1;1           | NPT  | NPA  | I    | V   | P   | I   |
| SIP1;2           | NPT  | NPA  | S    | F   | P   | I   |
| BnaA09.SIP2;1b   | NPT  | NPA  | S    | H   | G   | A   |
| BnaCnn_random.SIP2;1c BnaC04.SIP2;1b | NPL  | NPA  | S    | H   | G   | A   |
| BnaA07.SIP2;1a BnaC03.SIP2;1a | NPL  | NPA  | S    | H   | G   | A   |
| NIP1             | NPA  | NPG  | W    | V   | A   | R   |
| NIP2             | NPA  | NPA  | W    | V   | A   | R   |
| NIP3             | NPA  | NPA  | W    | I   | A   | R   |
| NIP4             | NPA  | NPA  | W    | A   | R   |
| BnaA07.NIP5;1c BnaC06_random.NIP5;1c | NPS  | NPA  | A    | N   | A   | R   |
| BnaC03.NIP5;1b BnaA03.NIP5;1b | NPS  | NPA  | A    | I   | A   | R   |
| BnaA02.NIP5;1a BnaC02.NIP5;1a | NPS  | NPA  | A    | I   | G   | R   |
| NIP6             | NPA  | NPA  | A    | I   | A   | R   |
| NIP7             | NPS  | NPA  | A    | V   | G   | R   |

et al., 2014; Diehn et al., 2015).
In this study, the gene expression levels of most AQPs in *B. napus* were relatively higher than other *BnaAQP*s in all the tissues, especially in the roots under both B conditions (Figure 5 and Supplementary Table 3), indicating that they might be involved in the response to the B deprivation in *B. napus*. Additionally, *BnaPIP2;4s* had high expression levels in the roots of both cultivars under the normal B condition, which was similar to their orthologues in *Arabidopsis* (Alexandersson et al., 2010), however, the gene expression of *PIP2;4s* in *B. rapa* and *B. oleracea* was not detected.

*BnaTIP1;1s* (except for *BnaAnn_random.TIP1;1b*) and *BnaTIP1;2s* displayed high expression levels in all tissues of both cultivars at two B levels and their expression was repressed in the roots with low B stress (Figure 5 and Supplementary Table 3), although recent studies report that the losses of *AtTIP1;1* and *AtTIP1;2* do not influence the growth of *A. thaliana* significantly (Schüssler et al., 2008; Beebo et al., 2009), thus suggesting that new functions for these genes in plants still need to be discovered. *BnaTIP2;2s* and *BnaTIP2;3s* clearly showed root-specific expression patterns, which was similar to that of *B. oleracea* (Diehn et al., 2015), *B. rapa* (Tao et al., 2014), and *Arabidopsis* (Alexandersson et al., 2010). Moreover, the *BnaTIP2;2s* and *BnaTIP2;3s* was strongly down-regulated at low B in both cultivars (Figure 5).

The gene expression levels of all the *BnaSIPs* were relatively low in all the tissues of both B-efficient and B-inefficient cultivars under low B condition (Figure 5). Hence, the function of *BnaSIP* genes was independent of the B supplement in this study. As a whole, at both B levels, the expression levels of *BnaNIPs* of both cultivars were lower than that of the other three sub-families in all tissues (Figure 5). Four members of *BnaNIP5;1s* (*BnaA02.NIP5;1a, BnaC02.NIP5;1a, BnaA03.NIP5;1b, and BnaC03.NIP5;1b*) exhibited significantly
up-regulation whether in the root or in the leaves at low B stress, suggesting that they were not only involved in the B absorption in the root, but also in the response to low B in the shoot of *B. napus*.

Some AQPs genes have tissue-specific expression characteristics. For instance, *AtTIP1;3*, *AtTIP5;1s*, *AtNIP4;1s*, and *AtNIP4;2s* have been assigned as pollen-specific genes (Soto et al., 2008; Giorgio et al., 2016) and *AtNIP7;1s* showed an anther-specific expression pattern (Li et al., 2011). Thus, it is not surprising that transcription of *BnaTIP1;3s*, *BnaTIP5;1s*, *BnaNIP4;1s*, *BnaNIP4;2s*, and *BnaNIP7;1s* was not detected in roots, old leaves and juvenile leaves of 30 d seedlings of QT10 and W10 under both B conditions in this study.

Significant differences in the transcription of several AQPs genes between B-efficient cultivar QT10 and B-inefficient cultivar W10 were observed. This included *BnaCnn_random.PIP1;4b*, *BnaC04.TIP4;1a*, *BnaAnn_random.TIP1;1b*, *BnaC03.NIP5;1b*, *BnaC02.NIP5;1a*, *BnaA03.NIP5;1b*, and *BnaA02.NIP5;1a* (Figure 5 and Supplementary Table 3). Of these, we have previously mapped *BnaA03.NIP5;1b* as a B-efficient QTL qBEC-A3a detected in *B. napus* (Hua et al., 2016a). Moreover, the other member *BnaC02.NIP5;1a* was also identified as a B-efficient QTL qBEC-C2a, and possibly facilitates efficient boron absorption under low B condition in *B. napus* (Hua et al., 2016b).

In summary, we identified and characterized 121 full-length AQPs genes which belong to the PIP, TIP, SIP and NIP subfamilies. The characteristics of gene structure, selectivity filters and transcriptional patterns for most AQPs genes of *B. napus* were similar to those of *B. rapa*, *B. oleracea*, and *Arabidopsis*. We confirmed the identity and relationship of two candidate genes (*BnaA03.NIP5;1b* and *BnaC02.NIP5;1a*) underlying B-efficient QTL regions in *B. napus*. We found that most *BnaPIPs* and *BnaTIPS* had high expression levels in all the tissues of B-efficient and B-inefficient cultivars under both B conditions, especially in roots. Moreover, under low B condition, the transcription of these genes was down-regulated in roots although their responses to low B stress in leaves were only slight or with no change.

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**AUTHOR CONTRIBUTIONS**

Conceptual and experiment designs by LS and DY; Experiments were conducted by DY; RNA-seq data analysis performed by WL; Reagents/materials/analysis tools were contributed by LS, and the report was written by DY, YH, GK, FX, and LS. All the authors have commented, read and approved the final manuscript.

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**SUPPLEMENTAL MATERIAL**

The Supplemental Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01336/full#supplementary-material

**Supplementary Figure 1** | Putative MW and PI of BnaPIPs, BnaSIPs, BnaNIPs, and BnaTIPS in *Brassica napus*. The genes in the four sub-families were distinguished by different legends.

**Supplementary Figure 2** | WebLogo plot of the consensus motifs in BnaAQPs. The amino-acid residues of the consensus motifs in FASTA format were obtained from the MEME Web and WebLogo graph plots were generated using the amino acid sequences. (A) Motif 1; (B) motif 2; (C) motif 4; (D) motif 5.

**Supplementary Table 1** | Gene location and description of incomplete BnaAQPs.

**Supplementary Table 2** | BnaAQP genes without chromosome localization information.

**Supplementary Table 3** | The RPKM values of the 121 BnaAQPs.

**Supplementary Data 1** | The multiple sequence alignment of 121 BnaAQPs. The yellow highlighted amino acids represent the residues near P3 site. The letters in a red font below P1 to P5 indicate the amino acid residues in the Froger’s position.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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