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ORIGINAL PAPER

Physical mapping of wheat aquaporin genes

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Abstract Aquaporins are water channel proteins that control the flow of water across cellular membranes and play vital roles in all aspects of plant–water relations. Our previous identification of 35 wheat *PIP* and *TIP* aquaporin genes showed they formed a large family with many conserved features that are thought to be important in structure and function. The present work focussed on determining the positions of these genes in the wheat genome in order to help investigate their functions in water uptake and transport. Genomic locations of wheat *PIPs* and *TIPs* were predicted using a number of reported rice–wheat comparative maps and additional in silico approaches. Physical mapping of select genes utilising aneuploid stocks and progenitor DNAs placed these on chromosomes 2B, 2D, 6B and 7B and helped to clarify the individual genes and homoeologues. The compilation of all in silico and physical mapping work confirmed many of the orthologous relationships between wheat and rice and/or barley genes, and synteny in the related areas of genome. These results further reinforce that wheat PIP and TIP proteins are most likely to have similar functions to those closely related in rice, including water permeability and abiotic stress response, and provide important tools for future investigations into the involvement of this complex gene family in traits related to plant-water relations and osmotic stress response.

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Introduction

The key mechanisms associated with plant-water relations include 'aquaporins', the 26–30 kDa water channel proteins, belonging to the Major Intrinsic Protein (MIP) superfamily of integral membrane proteins. These proteins specifically facilitate the passive flow of water molecules across cellular membranes, appear to regulate the *trans*-cellular route of water (Agre et al. 1993; Maurel 1997) and fulfil a vital role by transporting a large volume of water with minimal energy expenditure (Tyerman et al. 1999). The hour-glass structure of aquaporin proteins that allows selective, bidirectional flow of water, and the amino residues involved in the transport substrate selectivity and functional regulation have been reviewed extensively (Heymann and Engel 2000; Chaumont et al. 2005; Hedfalk et al. 2006; Törnroth-Horsefield et al. 2006; Forrest and Bhave 2007).

Investigations of aquaporin genes in a variety of plant species including mosses have shown the plant aquaporins fall into seven groups (PIPs, TIPs, NIPs, SIPs, GIPs, HIPs and XIPs) (Chaumont et al. 2001; Danielson and Johanson 2008). Certain key structural features, and most importantly, transport substrate specificities ranging from water to glycerol, CO_2, ammonia and urea, are conserved across numerous plant species (Wallace and Roberts 2004; Kaldenhoff and Fischer 2006). Of the plant aquaporins, the plasma membrane integral proteins (PIPs) and tonoplast

integral proteins (TIPs) have been studied extensively and a number of these genes have been shown to be directly involved in the transport of water across cell membranes (Kaldenhoff et al. 1998; Li et al. 2000; Johanson et al. 2001). Reports demonstrate that genes belonging to these two subgroups are important for many aspects of plant water relations, including plant development and adaptation to environmental stresses such as salinity, drought and cold (reviewed in Luu and Maurel 2005; Hachez et al. 2006; Forrest and Bhave 2007).

The *PIPs* and *TIPs* in the rice genome have been reported earlier (Sakurai et al. 2005; Forrest and Bhave 2007) and many of these have already been shown to be important for water transport through cellular membranes (Li et al. 2000; Lian et al. 2004; Sakurai et al. 2005, 2008). Rice aquaporins have been implicated in various plant functions including response to abiotic stresses, e.g., chilling (Sakurai et al. 2005; Yu et al. 2006), salt (Li et al. 2000; Kawasaki et al. 2001), osmotic (Li et al. 2000; Guo et al. 2006) and drought (Liu et al. 1994; Malz and Sauter 1999) stresses, cell expansion (Malz and Sauter 1999), and seed germination (Takahashi et al. 2004; Liu et al. 2007). However, as yet little information exists on the function of aquaporins in wheat, one of the two largest crops in the world. Our work (Forrest and Bhave 2008) was the first to identify twenty-four *PIP* and eleven *TIP* aquaporin genes in wheat, all of which showed a high degree of identity to those in rice. Conservation of important protein features in both subfamilies suggested some may be water specific (PIPs), while others (TIPs) are likely to transport water and/or other substrates. Residues involved in potential post-translational protein regulation were identified, and many of the genes were highly identical to those of rice.

By considering these orthologous relationships between wheat and rice aquaporin genes, and the reported functions of the proteins in rice (Li et al. 2000; Lian et al. 2004; Sakurai et al. 2005, 2008), it may be possible to predict the functions of wheat aquaporins. Rice and wheat *PIP* and *TIP* gene orthologues were predicted in our earlier work based on sequence homology (Forrest and Bhave 2008). This study aimed to physically map the wheat *PIP* and *TIP* genes. Firstly, the location of each wheat gene was predicted using a number of published rice–wheat comparative maps that established the synteny (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007). Additionally, the GrainGenes database containing mapped wheat ESTs (Qi et al. 2004) was searched to find aquaporin probes and hence gene locations. The barley HarvEST database was also searched to identify likely barley aquaporin orthologues and potential synteny between these three genomes. Experimental physical mapping was also undertaken to help clarify whether some of these wheat genes were individual genes or homoeologues.

While the functions of aquaporins remain relatively untested in wheat, the evidence to date suggests important roles for many of these genes in salt, drought and cold response in other plants, e.g. rice (Li et al. 2000; Lian et al. 2004; Luu and Maurel 2005; Guo et al. 2006; Yu et al. 2006; Sakurai et al. 2008). Knowledge of gene location will be of great significance for functional testing, and exploration and exploitation of the reported data on synteny of many sections of the wheat genome to that of rice and/or barley (Cho et al. 2006; Gale and Devos 1998; La Rota and Sorrells 2004; Singh et al. 2007; Sorrells et al. 2003; Thiel et al. 2009) provide an ideal tool for mapping of the wheat genes. The results of this work provide a foundation for genetic mapping and identifying their relationships to quality traits, including abiotic stress tolerance, potentially enabling cultivar growth in difficult growing conditions. Identification of stress tolerant genotypes of wheat would immensely help agricultural productivity worldwide to meet the increasing demands as well as cope with changing environment.

Materials and methods

Predictive mapping of wheat *PIP* and *TIP* genes

Prediction of wheat chromosomes using rice–wheat comparisons

In order to predict the positions of *PIP* and *TIP* aquaporin genes on wheat chromosomes, the comparative data available for rice and wheat genomes were utilised. Initially the locations of the wheat genes were predicted roughly using the extensive comparative maps of rice and wheat chromosomes (Sorrells et al. 2003; La Rota and Sorrells 2004). Additionally, each rice *PIP* and *TIP* locus ID (Sakurai et al. 2005; Forrest and Bhave 2007) was entered into the TIGR Rice Genome Browser site (http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/, now at http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/, last accessed May 2009), to identify the Genbank accession number of the BAC (bacterial artificial chromosome) or PAC (P1-derived artificial chromosome) clones to identify the section of rice genome containing each rice *PIP* and *TIP* gene. The Genbank numbers were then used to search the supplementary material of Sorrells et al. (2003) to find the corresponding wheat chromosome(s) for each BAC/PAC.

In a different approach, the VanshanuDhan Rice Gene Database (National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, India) was explored for the extensive rice–wheat synteny data of Singh et al. (2007). The ‘search by molecular function’ tool and the keywords ‘aquaporin’, ‘intrinsic’ and ‘channel’ were used

for the chromosome-by-chromosome search of the gene ID numbers of the rice aquaporins (http://www.nrcpb.org/RICE_GENOME/INDEX.htm; last accessed October 2009). Each resulting gene number was typed into the 'search by gene ID' tool to obtain further information and determine which database gene number corresponded to which of the rice *PIP* and *TIP* genes (through analysis of sequence information). Thus the supplementary data of Singh et al. (2007) was searched to locate each rice gene number, to find any *PIP* or *TIP* genes used in the synteny work and hence identify corresponding wheat chromosome bins containing orthologous genes. Additionally, the locus ID of each rice gene was typed into the Rice Genome Annotation site—Release 5 Genome Browser (http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/, now available as Release 6 at http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/, last accessed August 2009) and all wheat bin-mapped markers (expressed sequence tags: ESTs) aligning to these genes were recorded.

Prediction of chromosomal location utilising wheat bin-mapped markers

Wheat *PIP* and *TIP* deduced coding sequences (CDS; exon contigs) or expressed sequences (tentative consensus sequences, TCs; or expressed sequence tags, ESTs) reported in Forrest and Bhave (2008) were each queried in the MPBCRC SOE BLAST (http://www.genica.net.au/mpbcrcsoeblast/; accessed May 2009) site using a blastn search of the 'GrainGenes wEST SQL Binned' database (expect limited to 0.01). Sequences of the top six hits were obtained from the NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide; accessed May 2009). All resulting marker sequences were aligned with the query sequences in ClustalW (http://www.ebi.ac.uk/clustalw/; last accessed May 2009). Each wEST was entered into the 'GrainGenes-SQL query resources' (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi; last accessed May 2009) to obtain relevant probe information including the locus and wheat bin. Cultivar and tissue information was obtained from Genbank (http://www.ncbi.nlm.nih.gov/Entrez/; last accessed May 2009).

Prediction of wheat chromosomes using barley–wheat comparisons

The barley HarvEST database v.1.74 (http://harvest.ucr.edu/HBarley174.exe; accessed October 2009) was used to identify similar genes that have been mapped in barley. Blast searches were performed in the HarvEST Blast Server against the Barley 2943 Mapped SNPs database to identify barley unigenes with associated SNP markers, where the best Blast hit was recorded for each wheat *PIP* and *TIP* CDS (wheat sequences reported in Forrest and Bhave 2008). Barley unigene information (consensus EST sequence, associated barley marker, integrated barley map chromosome and best rice BLASTX hit) was obtained from the HarvEST database. Sequence alignments (*PIP* and *TIP*) were performed in ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/; accessed October 2009) using barley unigene consensus sequences against wheat coding sequences.

Physical mapping of wheat *PIP* and *TIP* genes

Optimisation of allele specific-PCR and cleaved amplified polymorphic sequences

Primers specific for select genes, called 'allele specific (AS) primers', were designed to anneal to variable intron regions of the closely related genes *TaPIP1;2* to *TaPIP1;6* reported earlier (Forrest and Bhave 2008) (Supplementary Table S1). They were designed to contain at least one single nucleotide polymorphism (SNP) at the 3′ end and preferably additional ones along the length. Primers were designed using NetPrimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html; last accessed May 2009) and tested using the 'In silico PCR' function of FastPCR (http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm; last accessed May 2009). Primer pairs that predicted amplification of only the target gene (>80% identity with primer) and a product size of <1000 bp were synthesised commercially. To further confirm the specificity of amplifications, sequences of the gene sections to be amplified by AS primers were entered into the SDSC (San Diego Supercomputer Center) Biology Workbench v3.2 (http://workbench.sdsc.edu/; last accessed May 2009) and restriction maps generated using the 'TACG-Analyze a NS for Restriction Enzyme Sites' tool to identify sites that would produce characteristic cleaved amplified polymorphic sequence (CAPS) patterns for each gene. The genes were amplified from the genomic DNA (gDNA) of *T. aestivum* cv. Cranbrook using reported PCR conditions (Forrest and Bhave 2008) except that gradient PCR was utilised over a 5°C range to optimize the annealing temperature for each AS primer pair so as to obtain a strong and specific band. One μL of the PCR products was then digested with 5 U of the appropriate enzyme based on predicted CAPS pattern, in 10 μL volumes and the results analysed on 2% agarose gels.

Physical mapping using nullisomic–tetrasomic lines and deletion lines and AS-PCR

Genomic DNA (gDNA) was isolated from seedlings of the *T. aestivum* cv. Chinese Spring nullisomic–tetrasomic

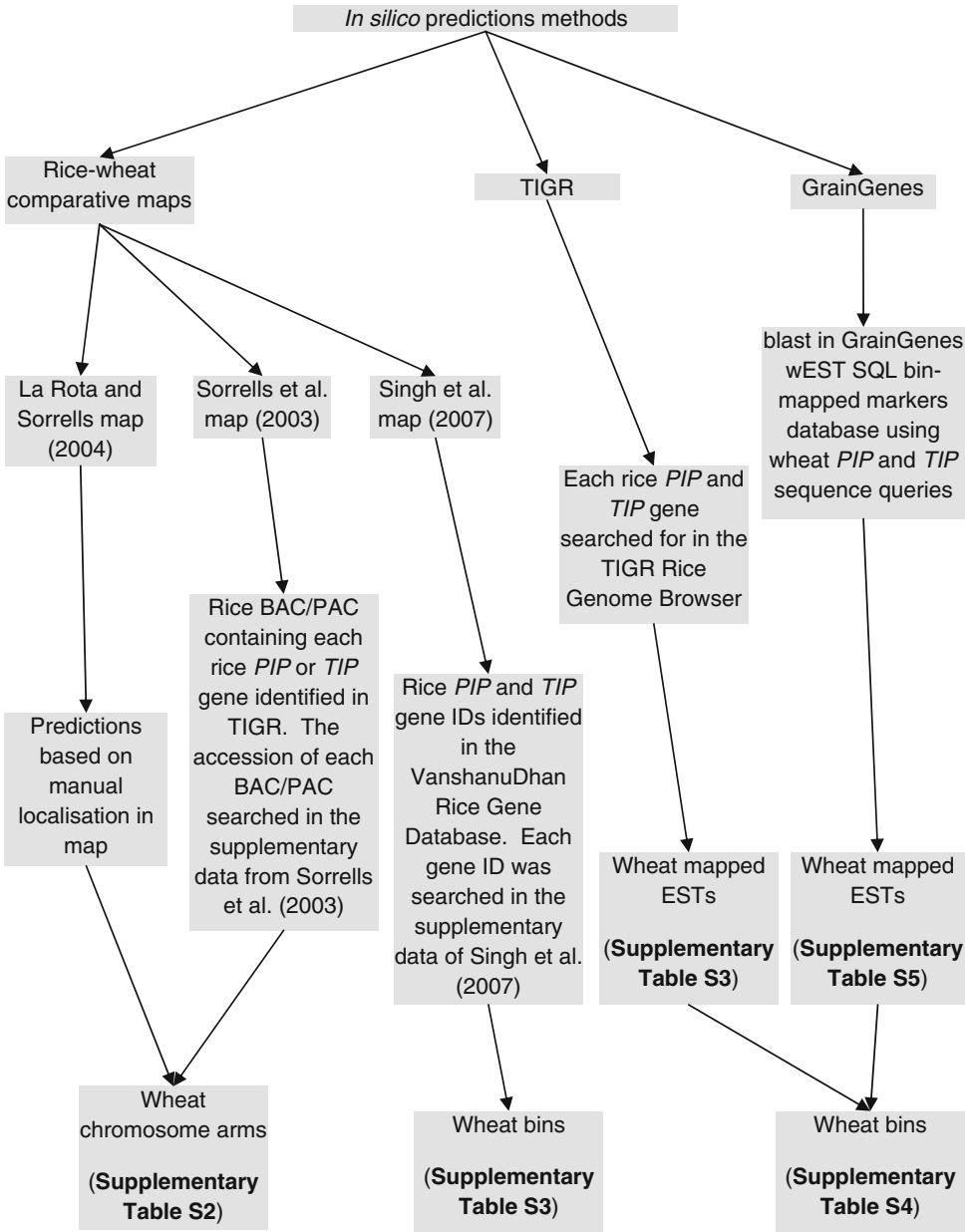

Fig. 1 Summary of in silico methods used to predict wheat *PIP* and *TIP* locations

(N/T) lines using the Wizard Genomic DNA Purification Kit (Promega Australia). The gDNA of some N/T lines was also kindly donated by Prof Rudi Appels (State Agricultural Biotechnology Centre, Murdoch University, Western Australia). The first round of PCR was performed using 100 ng of gDNA and the degenerate primer pair PIPF1 and PIPR1 under the reported conditions (Forrest and Bhave 2008), at an annealing temperature of 52°C, in 25 µL volumes. These PCR products (multiple bands expected; see Forrest and Bhave 2008) were used as templates (0.5–1.0 ng) for second round (nested) PCR in 25 µL volumes using 0.3 µM of each AS-primer pair and the respective optimized annealing temperatures (Supplementary Table S1). The genes *TaPIP1;2* and *TaPIP1;4* were amplified together (multiplex PCR), as were *TaPIP1;3* and *TaPIP1;5,* while the gene *TaPIP1;6* was amplified separately. The results were analysed on 1% (w/v) agarose gels and absence of a band of appropriate size from a particular N/T line was taken as indicative of that gene being located on the missing chromosome. Using these results, gDNAs of appropriate *T. aestivum* cv. Chinese Spring deletion lines (Sourdille et al. 2004) were amplified using PIPF1 and PIPR1, and the products used for separate nested PCRs with the AS primers for *TaPIP1;2*, *TaPIP1;3*, *TaPIP1;4*, *TaPIP1;5* and *TaPIP1;6*. Absence of a band of appropriate size in a particular deletion line was taken as indicative of that gene being located in the missing section of the chromosome.

Additional *PIP* genes (*TaPIP1;6*, *TaPIP2;4*, *TaPIP2;5* and *TaPIP2;7*) were amplified with primers designed to

amplify the small amplicons (for expression analysis; Forrest and Bhave, unpublished) in a multiplex PCR assay (Supplementary Table S1). Genomic DNAs (100 ng) of N/T lines were used as templates, with 0.3 μM of each primer (*TaPIP1;6* and *2;4*, or *TaPIP2;5* and *2;7*) in 25 μL volumes. Altered reaction conditions were utilized to suit the small amplicons, consisting of 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 15 s, and the products were electrophoresed on 2% agarose gels.

Genome assignments using progenitor DNAs and AS-PCR

The gDNAs of the diploid (*T. urartu,* AA; *Ae. speltoides*, SS; *Ae. tauschii,* DD) and tetraploid (*T. turgidum* ssp. *durum* var. Abyssinia 37, AABB) progenitors of wheat were amplified with PIPF1-PIPR1 and the products used as templates in separate nested PCRs with the five AS primer pairs, as above (Supplementary Table S1). Additionally, multiplex PCR of small amplicons (*TaPIP1;6* and *TaPIP2;4*, *TaPIP2;5* and *TaPIP2;7*) was performed on gDNA of these progenitors as well as N/T as described above.

Results

Prediction of wheat *PIP* and *TIP* loci using rice–wheat comparative maps

The chromosome position (in bp) of the wheat gene(s) corresponding to each of the rice *PIP* and *TIP* genes (Sakurai et al. 2005; Forrest and Bhave 2007) were estimated in a rice–wheat comparative map (La Rota and Sorrells 2004) and wheat chromosome arms corresponding to these were recorded. Thus, positions of the potentially orthologous wheat genes could be identified (Supplementary Table S2; Table 1). All wheat chromosomes were predicted to contain *PIPs* and *TIPs* orthologous to those of rice, the majority being on chromosomes 1 and 2. Although this map (La Rota and Sorrells 2004) was more up to date and contained more probe information than an older version (Sorrells et al. 2003), the latter was utilised to look for more detailed information available in its supplementary data. The Genbank accession number of each BAC/PAC containing the rice *PIP* or *TIP* gene was identified in the TIGR rice genome browser, and each resulting accession was used to search the supplementary tables (Sorrells et al. 2003). Only five different wheat probes (ESTs) could be identified which mapped to five BAC/PAC sequences containing eight rice aquaporins (Supplementary Table S2). Some probes were specific to a BAC/PAC containing one aquaporin gene only (BE406840, BG606824 and BF483796, mapping to *OsPIP1;3*, *OsPIP2;3* and *OsPIP2;8*, respectively), while others (BE518349 and BE489244) mapped to sequences containing >1 aquaporin (AP004668 containing *OsPIP2;1*, *2;4*, *2;5* and *2;9*, and AP001550 containing *OsTIP4;2* and *4;3*). The BAC/PACs containing 14 other rice *PIPs* and *TIPs* had no corresponding mapped probes. A potential limitation is that the probes might map to any gene within the BAC/PAC, not necessarily an aquaporin; however, the mapped gene is likely to be closely linked to the aquaporin gene and hence this tool and the maps offer a useful predictive method.

Further information was obtained from a more recent map utilising single-copy rice genes (Singh et al. 2007). Using the unique identification system in this work (gene IDs instead of Genbank accessions), the gene ID of each rice *PIP* and *TIP* was identified, where possible, from the VanshanuDhan Rice Gene Database (http://www.nrcpb.org/RICE_GENOME/INDEX.htm; last accessed October 2009). Keyword searches were performed and protein sequences from each hit were compared to those previously identified (Forrest and Bhave 2007) to determine which gene ID corresponded to which rice *PIP* and *TIP*. Gene IDs were identified for seven *PIPs* (*OsPIP1;1*, *1;3*, *2;1*, *2;4-2;5*, *2;8* and *2;10*) and four *TIPs* (*OsTIP1;1*, *2;1, 2;2, 3;1*) but only the *PIPs* contained mapping information in the supplementary data of Singh et al. (2007) and all seven matched the same wEST contig (#17857) mapping to chromosome 6DL, between breakpoints 0.29–0.47 (Supplementary Table S3). Therefore, it is likely that only one rice gene matches this wheat contig, but that the threshold used for analysis (Singh et al. 2007) was too low for the highly similar *PIP* subfamily. Thus, probably only one wheat *PIP* was included in this comparative map.

Additionally, each rice *PIP* and *TIP* was viewed in the TIGR Genome Annotation (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/; last accessed August 2009) and wheat bin-mapped ESTs mapping to each rice gene were recorded. Four ESTs mapped to rice *PIPs* (BE518349, CD453579, BQ166341, BQ171235) and four to *TIPs* (BE489586, CD452377, BE403397, CD452377, BQ172112) (Supplementary Table S3). Of these, BQ166341 is specific to *OsPIP2;8*, BQ171235 is specific to *OsPIP2;10* and BQ172112 is specific to *OsTIP3s*. The remaining ESTs are specific to a subfamily (*PIP* or *TIP*) but not to a group (e.g. *TIP1* or *TIP2*). The ESTs BE518349 and CD453579 (corresponding to *PIPs*), and BE489586 and CD452377 (corresponding to *TIPs*) were frequently identified and may consist of highly conserved sequences. No ESTs mapped to *OsTIP4* or *OsTIP5* groups.

Predicted location of wheat *PIP* and *TIP* genes utilising the graingenes wEST SQL bin-mapped markers database

Due to a degree of ambiguity of above results (many wheat EST probes from different chromosomes mapping to one

Table 1 Compilation of in silico and physical mapping data

Wheat gene	Location on wheat chromosomes			Rice orthologue	
	Rice–wheat comparative maps[a]	GrainGenes wEST-SQL database	Physical mapping	Rice gene	Chrom
TaPIP1;1	3L, 4L, 5L, 6L, 7L	6A, C-L4-0.55 6B, L3-0.36-0.40 6D, L6-0.29-0.47	NT	OsPIP1;1	2
TaPIP1;2	1L, 1S, 2L, 5L, 5S, 6L, 6S, 7L	–	2B, 0.53S-0.89L	OsPIP1;2	4
TaPIP1;3	1L, 1S, 2L, 5L, 5S, 6L, 6S, 7L	–	6B, 0.76S-0.36L	OsPIP1;2	4
TaPIP1;4	1L, 1S, 2L, 5L, 5S, 6L, 6S, 7L	–	7B, 0.33S-0.63L	OsPIP1;2	4
TaPIP1;5	1L, 1S, 2L, 5L, 5S, 6L, 6S, 7L	–	2D, 0.49-0.76L	OsPIP1;2	4
TaPIP1;6	1L, 1S, 2L, 5L, 5S, 6L, 6S, 7L	–	ND	OsPIP1;2	4
TaPIP1;7-12	2L, 5L, 6S, 6L, 7L	–	NT	OsPIP1;3	2
TaPIP2;1	1S, 2S, 3L, 4L, 5L, 6L, 6S, 7S	7A, C-7AS8-0.45 5B, 5BS6-0.81-1.00 7B, C-7BS1-0.27	NT	OsPIP2;1	7
TaPIP2;2	3L, 6L, 4L, 5L, 7L, 7S	6A, 6AL 6D, 6DL6-0.29-0.47 6B, C-6BL5-0.40	NT	OsPIP2;2	2
TaPIP2;3	2L, 3S, 4L, 4S, 5L	–	NT	OsPIP2;3	4
TaPIP2;4	1S, 2S, 3L, 4L, 5L, 6L, 6S, 7S	–	ND	OsPIP2;1/4/5	7
TaPIP2;5	1S, 2S, 3L, 4L, 5L, 6L, 6S, 7S	–	ND	OsPIP2;4/5	7
TaPIP2;6	1S, 2S, 3L, 4L, 5L, 6L, 6S, 7S	–	NT	OsPIP2;4/5	7
TaPIP2;7	1S, 2S, 3L, 4L, 5L, 6L, 6S, 7S	–	ND	OsPIP2;4/5/9	7
TaPIP2;8	2S, 4L, 4S, 6L, 7L	–	NT	OsPIP2;6	4
TaPIP2;9	2S, 4L, 4S, 7L	–	NT	OsPIP2;6	4
TaPIP2;10	4L, 4S, 5L, 6L	–	NT	OsPIP2;7	9
TaPIP2;11-12	4L, 4S, 5L	–	NT	OsPIP2;7	9
TaTIP1;1	1L, 2L, 3S, 4L, 4S, 5L, 6L	4A, 4AL5-0.66-0.80 3D, 3DS6-0.55-1.00 4B, 4BL5-0.86-1.00	NT	OsTIP1;1	3
TaTIP1;2	1L, 2L, 3S, 4L, 4S, 5L, 6L	4A, C-4AL12-0.43 4B, C-4BS4-0.37 4D, C-4DS1-0.53 3D, 3DS6-0.55-1.00	NT	OsTIP1;1	3
TaTIP1;3	1S, 2L, 2S, 3L, 5S, 7L	–	NT	OsTIP1;2	1
TaTIP2;1	3L, 4L, 5L, 6L, 7L	6B, 6BL5-0.40-1.00 6A, 6AL4-0.55-0.90 2D, 2DL3-0.49-0.76 2A, C-2AL1-0.85	NT	OsTIP2;1	2
TaTIP2;2-4	1L, 3L, 4L, 5L, 5S, 6L, 7S	–	NT	OsTIP2;2	6
TaTIP3;1	1L, 3L, 4L, 5L, 5S, 7L, 7S	–	NT	OsTIP3;1	10
TaTIP3;2	2L, 3S, 4L, 4S, 5L	6A, 6AL8-0.90-1.00 6D, 6DL10-0.80-1.00	NT	OsTIP3;2	4
TaTIP4;1	1L, 1S, 2L, 3L, 4L, 6S	–	NT	OsTIP4;1	5
TaTIP4;2	1L, 2L, 3S, 7L, 7S	–	NT	OsTIP4;2/3	1

Underlined wheat chromosome arms are those that show an overall homology with the rice chromosome containing an orthologous aquaporin gene

NT not tested, *ND* not detected through PCR. *Chrom* location (chromosome) of rice gene

[a] Combination of predictions from the three comparative maps and TIGR Rice Genome Browser

rice gene, or some rice genes having no probe hits, or some probes mapping to >1 gene), the wEST probe information from the GrainGenes wEST-SQL database (http://wheat.pw.usda.gov/wEST/; last accessed May 2009) was analysed to directly determine which probe was specific to which wheat gene(s). The wheat *PIP* and *TIP* cDNA

sequences (exon contigs, TCs, or ESTs) identified earlier (Forrest and Bhave 2008) were used to search (blastn) the GrainGenes wEST-SQL database using the MPBCRC SOE BLAST tool (http://www.genica.net.au/mpbcrcsoeblast/; accessed May 2008; now available at https://ccg.murdoch.edu.au/yabi//) and the resulting hits aligned with the wheat query sequences using ClustalW. This led to the identification of five wESTs corresponding to *PIPs* and 5 to *TIPs*. Of these, BQ171235 and CD452546 were both linked to *TaPIP1;1*, BQ166341 was specific to *TaPIP2;1*, both BE518349 and CD453579 to *TaPIP2;2*, BE489586 and CD452377 to *TaTIP1;1*, BE398525 to *TaTIP1;2*, BE403397 to *TaTIP2;1*, and BQ172112 was specific to *TaTIP3;2* (Supplementary data Tables S4 and S5; Supplementary data Fig. S1; Table 1). Thus most *PIPs* are predicted to be located on chromosome 6, and *TIPs* on chromosomes 4 and 6. Each wEST was analysed in the GrainGenes wEST-SQL database to determine probe information including ESTs for each probe, as well as in Genbank (http://www.ncbi.nlm.nih.gov/Entrez/; last accessed May 2009) to determine the cultivar and tissue used for probe preparation. All probes were prepared from *T. aestivum cv.* Chinese Spring, except one (from Cheyenne), and from a variety of tissue libraries (Supplementary data Table S4). In summary, a combination of in silico approaches (summarised in Fig. 1) were used to predict the location of wheat *PIP* and *TIP* genes putatively orthologous to those in rice (Table 1).

Predicted barley orthologues of wheat *PIP* and *TIP* genes

Six barley unigenes (three *PIP2s*, two *TIP2s* and one *TIP4*) were identified from the barley HarvEST database v. 1.74 (http://harvest.ucr.edu/HBarley174.exe; accessed October 2009) that are associated with mapped markers (Supplementary data Table S6). These showed a high degree similarity to wheat *PIPs* and *TIPs* (≥91% coding DNA sequence identity; data not shown), compared to rice (≥84% coding DNA sequence identity; Forrest and Bhave, 2008), as would be expected between members of Triticeae (Pooideae subfamily) compared to *Oryza* (Ehrhartoideae subfamily). By inference, this also suggests these barley genes are orthologous to *OsPIP2;1*, *OsPIP2;3*, *OsPIP2;6*, *OsTIP2;1*, *OsTIP2;2* and *OsTIP4;1*, which is supported further by the best rice blastx hit reported in HarvEST (Supplementary Table S6). The chromosomal locations of these potentially orthologous genes are mostly in regions syntenic between rice and barley (Thiel et al. 2009), for example *OsTIP2;1* on rice chromosome 2 is syntenic to barley 6H which contains a similar gene. Similarly, the equivalent gene in wheat (*TaTIP2;1*) is likely to be located on wheat chromosome 6 (Table 1), a region syntenic to rice chromosome 2 (Thiel et al. 2009) and Triticeae group 6 (Gale and Devos 1998; Cho et al. 2006).

Identification of genomes of the *PIP* genes using progenitor species of wheat

Analysis of gene sequences were used to predict a number of restriction enzymes that could cut the *TaPIP1;2-1;6* genes in unique positions; thus these could be differentiated through CAPS type of molecular markers: *Sty*I to differentiate *TaPIP1;2*, *1;3* and *1;5*; *Bst*EII to differentiate *TaPIP1;4* from the rest; *Hind*III to differentiate *TaPIP1;6*; and *Alu*I or *Nla*III to differentiate all five genes (Supplementary data Fig. S2, Supplementary data Table S7). The patterns were confirmed by digestions of inserts amplified from clones of these genes in pGEM-T Easy plasmids (Forrest and Bhave 2008) (Supplementary data Fig. S3). Amplification of the inserts directly by AS primer pairs (Supplementary data Table S1) confirmed the predicted results (data not shown) indicating the specificity of the primers.

Amplification of DNAs of the diploid and tetraploid progenitors of wheat with the degenerate primers PIPF1 and PIPR1 and use of these products for nested PCR with AS primers showed that *TaTIP1;2* and *TaPIP1;4* could be amplified from durum wheat (AABB), but not from *T. urartu* (AA), *Ae. speltoides* (SS), and *Ae. tauschii* (DD) (Supplementary data Fig. S4; Supplementary data Table S8). The results suggest these genes are most likely located in the B genome of common wheat, this genome of common and durum wheat being likely to have diverged compared to that of *Ae. speltoides,* with possible variation(s) at primer annealing site(s) in *Ae. speltoides*. *TaPIP1;3* was amplified from common and durum wheat, *Ae. speltoides* and *Ae. tauschii*, but not from *T. urartu*, suggesting it may be located in the B and D genomes. *TaPIP1;5* was amplified from common wheat, *Ae. tauschii* and *Ae. speltoides*, but not durum or *T. urartu*, suggesting it is located on D and B/S genomes, while *TaPIP1;6* was amplified from all species except *Ae. tauschii*, suggesting it is located on the A and B genomes (Supplementary data Table S8). Further analysis of progenitors using small amplicons in a multiplex PCR assay using gDNA template showed *TaPIP1;6* and *TaPIP2;4* were amplified from all species except *Ae. tauschii* (Supplementary data Fig. S5) suggesting they are on A and B genomes but not D. *TaPIP2;5* was only amplified from *Ae. tauschii* and common wheat (but not durum), suggesting it is on the D genome, while amplification of *TaPIP2;7* from all species suggests it may represent closely related homeologs in all three genomes.

Identification of physical positions using aneuploid genetic stocks

In order to confirm some of the predicted locations of aquaporin genes in wheat and further understand their physical relationships, select highly similar *PIP* genes were physically mapped using aneuploid genetic stocks. Forty-one *T. aestivum* cv. Chinese Spring nullisomic–tetrasomic (N/T) lines were used to amplify the *PIP* genes with the degenerate primers PIPF1/PIPR1, and these products were used as templates for nested, multiplex PCR with AS primers (*TaPIP1;2* and *1;4*; *TaPIP1;3* and *1;5*). Products of expected sizes (Supplementary data Fig. S2) were absent from the Null2B-Tetra2A (N2BT2A) and N2B-T2D lines for the *TaPIP1;2*-specific primers, from N6B-T6A and N6B-T6D for *TaPIP1;3*, from N7B-T7A and N7B-T7D for *TaPIP1;4* and from N2D-T2A and N2D-T2B for *TaPIP1;5* (Supplementary data Fig. S6). This implied that these genes were located on chromosomes 2B, 6B, 7B and 2D, respectively, and supported the findings from the progenitors (Supplementary data Table S8). Only one line (N4A-T4B) lacked the product for *TaPIP1;6*, but N4A-T4D did produce it (data not shown), suggesting the gene is unlikely to be on 4A. Work repeated using another source of chromosome 4 NT lines (from SABC, Murdoch University) confirmed PCR products for both null 4A lines (data not shown), suggesting *TaPIP1;6* is not located on this chromosome. PCR of small amplicons of *TaPIP1;6*, *TaPIP2;4*, *TaPIP2;5* and *TaPIP2;7* led to bands in all NT lines (data not shown), suggesting that these genes might have homoeologues in the aneuploid stocks, and the current primers are not allele-specific, thus physical mapping is not possible until all homeologues are characterised. The amplification of *TaPIP1;6*, *TaPIP2;4* and *TaPIP2;7* from multiple genomes supports these results. Depending on the results of N/T lines, relevant *T. aestivum* cv. Chinese Spring deletion lines were then used to amplify genes using the PIPF1/PIPR1 followed by nested AS-PCR (Supplementary data Fig. S7). The chromosomal positions of genes deduced from all experimental mapping results are shown in Supplementary data Fig. S8.

Summary of mapping data

After combining the data from the in silico and experimental methods, many of the wheat gene locations predicted from the reported rice–wheat comparative maps and the orthologies based on sequence similarity to rice genes were confirmed (summarised in Table 1). For example, *TaTIP1;1* and *1;2* were both found on wheat chromosomes 3 and 4, reinforcing their orthology with *OsTIP1;1* located in the reported macrocolinear region of rice chromosome 3 (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007). In contrast, *TaTIP3;2* was predicted to be located on chromosome 6 based on similarity to a GrainGenes mapped wEST, a position not expected from the rice–wheat comparative map. The reason for this difference is unknown; however, the high sequence similarity between *TaTIP3;2* and rice *OsTIP3;2* suggests these genes are orthologous. Interestingly, the highly similar *TaPIP1;2-1;6* genes (which are all orthologous to *OsPIP1;2*) were found to be located over at least three different chromosomes. This suggests gene duplications before chromosomal rearrangements or translocations. The lack of mapping data for a *TIP5* equivalent gene in wheat was not surprising, as this group appears to be lacking in wheat (Forrest and Bhave 2008). Further work is required to confirm the remaining predictions and clarify ambiguities such as *TaTIP3;2*.

Discussion

Physical locations of wheat *PIP* and *TIP* genes

Our previous characterisation of wheat aquaporin genes (Forrest and Bhave 2008) identified their potentially orthologous relationships to rice *PIPs* and *TIPs,* thus enabling in the present work the predictions of the physical locations of wheat genes through searches of the reported rice–wheat comparative maps (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007). However, the predictions often led to more than one possible location for the wheat genes, and not all matched the reported macrocolinearity with rice. Additionally, some wheat aquaporin probes (ESTs) corresponded to more than one rice gene (Supplementary data Tables S2 and S3), even those with only one potential orthologue in wheat (Forrest and Bhave 2008). Thus these predictions are very useful to narrow down the potential sites, but need to be treated as preliminary, as the accuracy of results relies on a number of factors, e.g. (i) estimation of the position of rice aquaporins in the La Rota and Sorrells (2004) map; (ii) the degree of *PIP/TIP* linkage to other genes in a BAC/PAC clone for the Sorrells et al. (2003) map; and (iii) degree of the similarity between probes and wheat *PIP* and *TIP* genes for the Singh et al. (2007) map and TIGR Rice Genome Annotation Project. The high degree of identity within members of the *PIP* and *TIP* subfamilies in rice and wheat is likely to influence our interpretations of data from the TIGR Rice Genome Browser and Singh et al. (2007). Some of these predictions were successfully clarified further through comparison of sequences of the wEST probes from the GrainGenes database with the wheat *PIP* and *TIP* genes (Supplementary data Table S4), e.g., *TaPIP1;1* was localised on chromosome 6 long arm using comparative maps as well as a probe from GrainGenes. Experimental mapping results for four

PIP1 genes matched the predicted genome locations, but did not show complete correspondence of the genes on a wheat chromosome to those on a rice chromosome. The three comparative maps (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007) indicate genes in the vicinity of *OsPIP1;2* (on rice chromosome 4) mostly correspond to wheat probes (ESTs) mapping to wheat 2L. Experimental mapping also localised two orthologues (*TaPIP1;2, 1:5*) to wheat chromosome 2, but another two to wheat chromosome 6 and 7 (Supplementary data Fig. S8), the latter two also deduced so from the La Rota and Sorrells (2004) map (Table 1). The results thus support the reported interruptions to synteny of rice chromosome 4 to wheat chromosome 2 (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007). There is evidence for *TaPIP2;1* mapping to chromosome 4A, as this gene (AF139814) was analysed as a candidate in seed dormancy (Mares et al. 2005). However, results from the GrainGenes wEST SQL database (Supplementary data Tables S3 and S4) suggest it is on 7A, 7B and 5B. Thus the location of this gene currently remains unclear. In summary, the reported rice–wheat comparative maps have been extremely useful for preliminary predictions of physical loci of most wheat *PIP* and *TIP* genes, but due to aquaporins being members of multi-gene families with members of similar sequences, further work would be required to clarify some of the ambiguities, e.g., through genetic mapping using established populations (Kammholz et al. 2001).

The work could also be extended further by utilizing/investigating the synteny of wheat genome with related Triticeae species such as barley. For example, a barley *TIP2* unigene (U35_14113) was identified from the HarvEST database to be located in barley chromosome 6H. The equivalent gene in rice (*OsTIP2;1*, Supplementary data Table S6) is located on rice chromosome 2, to exhibit synteny with barley 6H (Thiel et al. 2009). The wheat equivalent, *TaTIP2;1*, was predicted in this work to be located on chromosome 2A, 2D, 6A or 6D (Table 1), of which chromosome 6 is syntenic with rice chromosome 2 (Sorrells et al. 2003; La Rota and Sorrells 2004; Singh et al. 2007). The reported synteny between barley and wheat was also confirmed; for example, a region containing a seed dormancy QTL was found to be syntenic between wheat chromosome 4 and barley 4H (Kato et al. 2001), and high-affinity K^+ transporter-encoding genes were identified in comparative positions of wheat and barley chromosome groups 7L, 2L, 6S and 2L, which are also syntenic to chromosome regions containing orthologues in rice (Huang et al. 2008). This comparative approach can be applied for optimal benefit to research on both plants, once the wheat and barley aquaporin genes are fully mapped. The functional equivalency of the barley genes will need to be tested also.

Possible evolutionary relationships within the *TaPIP1* genes

The physical mapping also provided clues as to whether the *PIP1* genes are all individual, or homoeologues. Despite the highly identical nature of some of these (up to 98%; (Forrest and Bhave 2008)), the AS-primers designed were successful in specifically amplifying *TaPIP1;2-1;5* and mapping them to chromosomes 2B (*TaPIP1;2*; 0.53S-0.89L), 6B (*TaPIP1;3*; 0.76S-0.36L), 7BL (*TaPIP1;4*; 0.33L-0.63L) and 2DL (*TaPIP1;5*; 0.49L-0.76L) (Table 1; Supplementary data Fig. S8). This distribution suggests *TaPIP1;3* and *TaPIP1;4* may be separate non-homeologous genes, and the homeologues of each (e.g., copies of *TaPIP1;3* on 6A, 6D) are yet to be identified. *TaPIP1;2* and *TaPIP1;5* also appear to be non-homeologous to these two genes. Interestingly, their similar positions on chromosome 2L suggest they may be homoeologous to each other, with potentially another (either unidentified, or very closely related) isoform on chromosome 2A. Although the AS primers and small amplicon primers both were specific for *TaPIP1;6*, it could not be mapped using N/T lines, suggesting it have very closely related homeologues.

The predictions of wheat aquaporin genome positions summarised in Table 1 might be useful for selecting genes for further study. For example, *TaPIP2;2*, *TaTIP2;1* and *TaPIP1;1* appear closely linked on chromosome 6L (Table 1; Supplementary data Figure S1), in a region that is syntenic with rice 2L containing *OsPIP2;2*, *OsTIP2;1* and *OsPIP1;1*. Thus, these genes could be of interest for investigation into their functions or association with QTLs for traits similar to those reported for the rice orthologues, e.g. transport of water and/or other molecules (Li et al. 2000; Sakurai et al. 2005) and abiotic stress response (Malz and Sauter 1999; Li et al. 2000; Kawasaki et al. 2001; Sakurai et al. 2005; Guo et al. 2006; Yu et al. 2006). Interestingly, the TIP5 gene group appears to be lacking in wheat, but is present in *A. thaliana* (Johanson et al. 2001), rice (Sakurai et al. 2005) and maize (Chaumont et al. 2001). A narrow region of rice chromosome 4 contains *OsPIP2;3, OsTIP3;2*, *OsTIP5;1*, and *OsPIP1;2*; orthologues of three of these are found in wheat (*TaPIP2;*3, *TaTIP3;2* and *TaPIP1;2-1;*6), but not that of *TIP5* (Table 1). *TIP5* appears to have evolved early, before the separation of monocots and dicots. Thus an equivalent gene may be highly diverged in wheat and not amplifiable by PCR (Forrest and Bhave 2008), or not expressed and hence not identified from ESTs/TCs. Alternatively, the region containing a *TIP5*-type gene may have been disrupted during wheat evolution, with its abundance of insertions, deletions, duplications, pericentric inversions and translocations (Devos et al. 1995; Qi et al. 2006); this possibility will need further investigations.

There is evidence for a number of *PIP* and *TIP* gene duplications both before and after speciation of rice and wheat. For example, *TaPIP1;7-1;12* (predicted to be on wheat chromosome 6L) may be duplicate genes and inparalogues, orthologous to *OsPIP1;3*; the *TaPIP2;4-2;7* (on wheat 2S) may be outparalogues, orthologous to *OsPIP2;4, 2;5* and *2;9; TaPIP2;8* and *2;9* (wheat 2S) may be inparalogues, orthologous to *OsPIP2;6*; *TaPIP2;10-2;12* (wheat 5L) may be inparalogues, orthologous to *OsPIP2;7*; *TaTIP1;1* and *1;2* (wheat 3 or 4) may be inparalogues, orthologous to *OsTIP1;1*; *TaTIP2;2-2;4* (wheat 7S) may be inparalogues, orthologous to *OsTIP2;2*; and *OsTIP4;2* and *4;3* (rice 1S) might be inparalogues, orthologous to *TaTIP4;2*. The greater number of genes in wheat may be explained by its larger, hexaploid genome. Further physical as well as genetic mapping will clarify these relationships.

Conclusions

This work describes in silico mapping of wheat *PIPs* and *TIPs*, as well as physical mapping of select *PIP*s. Many genes were confirmed to be orthologues of those in rice and hence likely to possess similar properties in water permeability and abiotic stress responses reported in rice. Specific aquaporins have been shown to be directly involved in water homeostasis, hence the present data will be important for marker development, assessing their association with QTLs for relevant traits and breeding for improvement of abiotic stress tolerance.

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