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REVIEW

Major intrinsic proteins (MIPs) in plants: a complex gene family with major impacts on plant phenotype

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Abstract The ubiquitous cell membrane proteins called aquaporins are now firmly established as channel proteins that control the specific transport of water molecules across cell membranes in all living organisms. The aquaporins are thus likely to be of fundamental significance to all facets of plant growth and development affected by plant–water relations. A majority of plant aquaporins have been found to share essential structural features with the human aquaporin and exhibit water-transporting ability in various functional assays, and some have been shown experimentally to be of critical importance to plant survival. Furthermore, substantial evidence is now available from a number of plant species that shows differential gene expression of aquaporins in response to abiotic stresses such as salinity, drought, or cold and clearly establishes the aquaporins as major players in the response of plants to conditions that affect water availability. This review summarizes the function and regulation of these genes to develop a greater understanding of the response of plants to water insufficiency, and particularly, to identify tolerant genotypes of major crop species including wheat and rice and plants that are important in agroforestry.

Keywords MIPs · Aquaporins · Water transport · Salinity · Drought

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Introduction

Plant–water relations for the improvement of plant tolerance to abiotic stresses and/or selection of appropriate genotypes for improved agricultural productivity or reforestation provide major challenges for the new genetic technologies. Water uptake and its supply throughout the plant body are required for a large number of key physiological processes, such as cell enlargement, stomatal movement, photosynthesis, phloem loading, and transpiration. Drought- and salt-affected plants suffer from affected root function, slower growth rates, stunted form, metabolic changes, reduced germination, reduced yields, and plant death (Bernstein 1975; Munns 2002). Proteins called aquaporins have emerged in the last 10 years as major factors controlling the *specific* transport of water molecules across cell membranes, not only in human cells where they were discovered, but in cells of all living organisms. The aquaporins are thus likely to be of fundamental significance to all facets of plant growth and development affected by plant–water relations and the behavior of plants under osmotic stress induced by factors such as salinity and drought. This review summarizes the key structural and functional characteristics of aquaporins garnered mainly from studies of human and bacterial proteins and key observations in aquaporin research in plants. A particular focus on cereals brings together the trends observed in behavior of aquaporins under osmotic stress in cereals and presents some of the emerging issues and challenges.

The major intrinsic proteins and aquaporins

The main biochemical factors associated with plant–water relations include the "*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 transcellular 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). Since the discovery of the first aquaporin as a 28-kDa integral membrane protein in human red blood cells and renal tubes (Denker et al. 1988; Preston et al. 1992), MIPs have been identified in a diverse range of other organisms, including plants (Fortin et al. 1987; Kammerloher et al. 1994; Maurel et al. 1993), insects (Beuron et al. 1995), yeast (Carbrey et al. 2001), bacteria (Calamita et al. 1995), protozoa (Mitra et al. 2000), and Archaea (Kozono et al. 2003). This illustrates their ancient evolutionary history and functional significance. The MIPs have been reviewed extensively (Maurel et al. 2002; Tyerman et al. 1999; Zardoya 2005; Zardoya and Villalba 2001). Figure 1 summarizes the key features of aquaporins-mediated transport of water molecules across a cell membrane.

Establishment of the significance of aquaporins in water permeability

In a landmark experiment, the RNA of human AQP1, transcribed in vitro from a plasmid vector, was injected and expressed in a *Xenopus* oocyte, and the cell was then placed into hypertonic medium, resulting in high swelling rate in contrast to the control oocyte, indicating the increased water permeability attributable to the AQP1 protein (Preston et al. 1992). This property was confirmed by other methods including channel blockage with mercurial reagents (Preston et al. 1992, 1993; Zhang et al. 1993) and reconstitution of the AQP1 protein into proteoliposomes, which resulted in increase in water permeability (Zeidel et al. 1992). Osmotic water permeability of aquaporins is inhibited by mercurial sulfhydryl reagents (Macey 1984) and was utilized as a test in the first *Xenopus* oocyte swelling assay (Preston et al. 1992). It has since been found, through site-directed mutagenesis of human AQP1, that the Cys189 near the asparagine–proline–alanine (NPA) motif of loop E of the protein structure (Fig. 2a; discussed below) is sensitive to mercury (Preston et al. 1993). These studies showed that water does not only diffuse across cellular membranes. It is thought that mercury can block the pore aperture via oxidation of cysteine residues (Maurel 1997). Assays similar to these have now been applied to aquaporins from a range of organisms to define them functionally and compare their functions.

Osmotic water permeability (P_{os}) describes the overall water movement in response to hydrostatic or osmotic pressure gradients (and is controlled by aquaporins), while

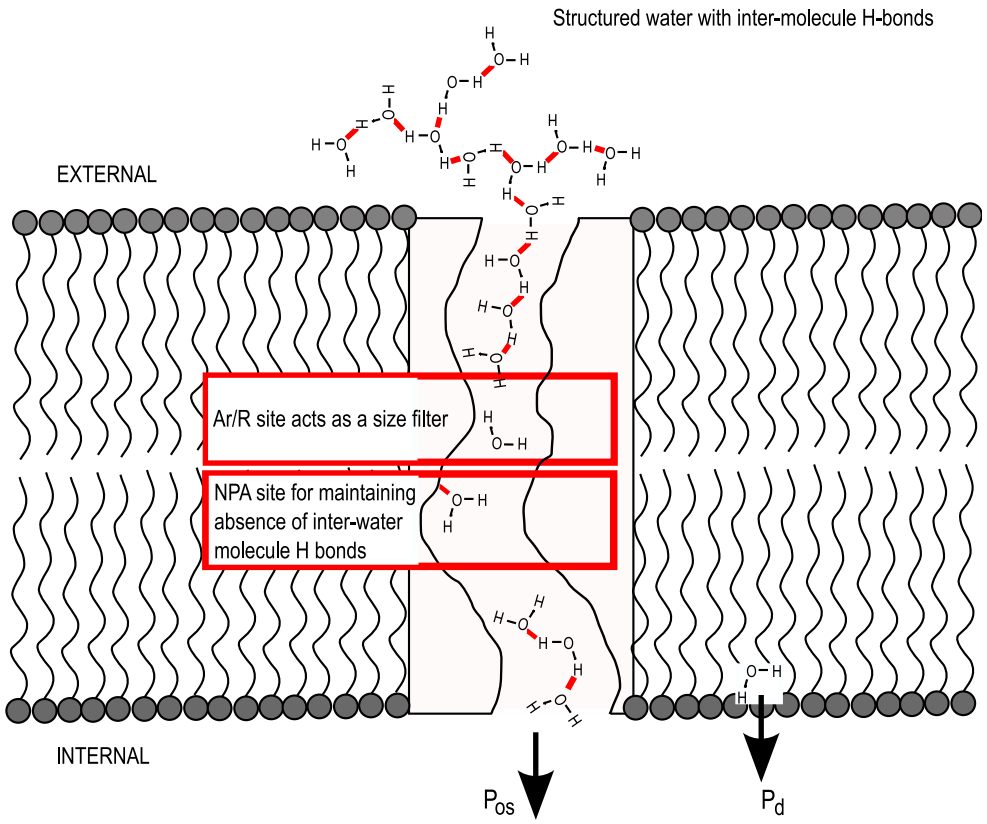

Fig. 1 Water transport as mediated by aquaporins in cellular membranes. Water mostly permeates cellular membranes via aquaporins (P_{os}, osmotic water permeability) in a single file, or to a lesser degree via simple diffusion (P_d, diffusional water permeability). The two NPA (Asn-Pro-Ala) sites serve as a proton exclusion mechanism by reorientation of the water molecules (see Fig. 2). The ar/R site consists of four residues that together act as a substrate selectivity filter at the pore's constriction

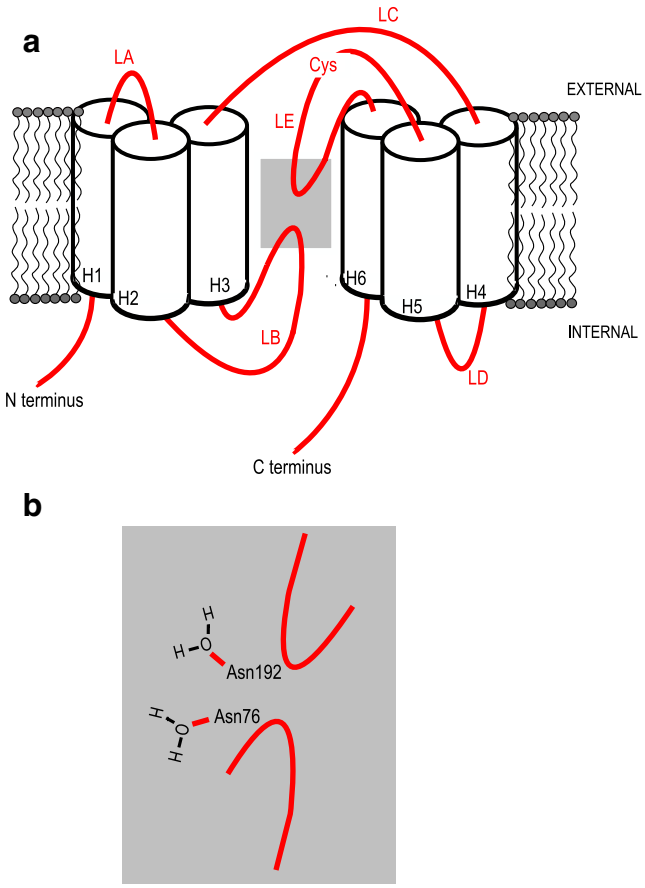

Fig. 2 Membrane topology of aquaporins and importance of NPA motifs. **a** Aquaporins typically consist of six transmembrane helical domains (H1–H6) and five interconnecting loops (LA–LE; not to scale). LB and LE have a partial helical nature and are partly embedded into the membrane, meeting at their respective NPA motifs. The Cys shown corresponds to Cys189 of AQP1, which confers mercury sensitivity. **b** The amide group of each Asn of the two NPA motifs (Asn76 and Asn192, respectively) forms a hydrogen bond with a water molecule, capturing the oxygen atom and causing the water molecule to be reoriented. This prevents formation of a continuously hydrogen-bonded chain of water molecules, and thus, prevents proton transport

the diffusional permeability coefficient (P_d) describes diffusion of water molecules that still occurs across the membrane without any driving force (Finkelstein 1987). Diffusion of individual water molecules across the phospholipid bilayer was shown to be characterized by a relatively low P_{os} (i.e., P_{os}/P_d ratio <1) and high activation energy (E_A) of membranes, whereas the transport of water molecules via aquaporins exhibited high P_{os} (i.e., P_{os}/P_d ratio >1) and low activation energy (Finkelstein 1987; Haines 1994). The water-transporting capacity (and subsequently P_{os}) of individual MIPs or entire cells/protoplasts is now typically evaluated using cell swelling assays, including the *Xenopus* oocyte assay mentioned above (reviewed in Maurel 1997; Verkman 2000).

A number of experimental and modeling studies have now led to a greater understanding of unique structural characteristics and physiological roles of MIPs, and these have been reviewed elsewhere (King et al. 2004; Luu and Maurel 2005; Tyerman et al. 2002). Their significance is highlighted by the fact that human genetic diseases or conditions such as cataracts, reduced urine concentration, and nephrogenic diabetes insipidus appear to be associated with lack of an aquaporin or mutations in its critical amino acid residues (reviewed in Agre and Kozono 2003). Furthermore, animal studies show the aquaporins to be mislocalized under bacterial infections in mice (Guttman et al. 2007) and involved in cell migration (Saadoun et al. 2005; Hu and Verkman 2006) and cell-to-cell adhesion (Harries et al. 2004), suggesting other functions and effects.

Key structural properties and functional specialization of MIPs

Individual MIP proteins range in size from 195 (animals) to >500 amino acids (fungi) (Zardoya 2005). They are known to function as a tetramer, composed of individually functional monomeric units (discussed below). Each MIP monomer has a highly conserved structure and typically exhibits (1) six alpha-helical hydrophobic transmembrane domains (H1–H6); (2) five inter-helical loops (LA–LE), of which loops A, C, and D are hydrophilic in nature and have an extracellular (loops A, C, and E) or cytoplasmic (loops B and D) location, while loops B and E are hydrophobic and partially embedded in the membrane; (3) an often observed AEF (Ala-Glu-Phe) or AEFXXT motif in the N-terminal domain (Zardoya and Villalba 2001); and (4) two highly conserved NPA (asparagine–proline–alanine) motifs (the "NPA box") in the loops B and E (Reizer et al. 1993) (Fig. 2a). The protein appears structured into two halves, each with three transmembrane helices (TMH) (H1–H3 or H4–H6) and a small hydrophobic loop (LB or LE) containing the NPA box. In the case of an aquaporin-type of MIP, the two halves together form a narrow water-filled channel ("the hour glass model") that mediates single-file water molecule movement in *either direction* (Jung et al. 1994) (Fig. 1). The various subfamilies within the MIP superfamily transport substances other than, or in addition to, water; thus, the term "MIPs" is more appropriate for the whole superfamily or its non-water conducting members, as the term "aquaporins" suggests involvement in water transport only, a function not necessarily associated with, or tested for, all MIPs.

NPA motifs play an important role in substrate selectivity of aquaporins

A number of mechanisms are suggested to contribute to the selectivity of aquaporins for water molecules only. The two NPA motifs have long been recognized as potentially

important in this regard due to the high degree of conservation of their sequence and position in numerous MIPs from animals, plants, protozoa, yeast and other fungi, eubacteria, and Archaea (Zardoya 2005). These motifs are predicted to meet in the center of the two halves, forming an hourglass-shaped pore, thought to contribute to the channel transport function (Jung et al. 1994) (Figs. 1 and 2). Their importance was established through site-directed mutagenesis of residues within or near these motifs that resulted in reduced osmotic water permeability and failure of the protein to localize to the plasma membrane (Jung et al. 1994). The amide functional groups on Asn (N) were shown to bond with water molecules (Murata et al. 2000) (Fig. 2b), a fact confirmed through a higher resolution structural analysis of human AQP1 (Sui et al. 2001). Use of molecular modeling simulations showed that replacing the Asn from each NPA with Leu in AQP1 led to the double mutant showing a complete breakdown of the aqueous pathway (Kong and Ma 2001) confirming their significance in water permeability. In contrast, as detailed later, mutations in these boxes in a plant MIP subgroup (NIP), were ineffective, suggesting that the main role of NPA motifs, as a selectivity barrier for transport of water molecules, appears to apply only to aquaporins, and other factors may contribute to selectivity for other substrates, e.g., residues of the ar/R filter (Fig. 1), while the NPA might play a greater role in proton exclusion.

The elucidation of 3.8 Å resolution of the human AQP1 has led to the model wherein the water molecules are proposed to be reoriented as they traverse the pore by disruption of hydrogen (H) bonds between them (Figs. 1 and 2b), which would prevent proton exchange from occurring (Murata et al. 2000). It was predicted that the positive ends of the helix dipoles of the two pore transmembrane helices induce water molecules to align with the water dipole perpendicular to the pore axis, which breaks the H-bonds between neighboring water molecules and allows new ones to form between the oxygen atom of the water molecule and the amide groups of the Asn residues from the two NPA motifs which extend into the pore (Murata et al. 2000). The NPA motifs thus function to capture the oxygen of single water molecules by hydrogen-bonding and prevent the proton transfer that could normally occur. Higher resolution X-ray crystal structure of bovine AQP1 showed a lack of a continuous H-bonded water chain through the pore (Sui et al. 2001), consistent with this theory. Molecular modeling simulations of a MIP from bacteria (GlpF, a glyceroporin) also predicted that the water molecules undergo "orientational tuning" to be oriented in opposite directions in the two halves of the pore (Tajkhorshid et al. 2002), and such global reorientation was also predicted for a water-specific (aquaporin) MIP from bacteria (AQPZ) (Savage et al. 2003). More recently, an alternative proton exclusion model has been proposed wherein protons come up against a strong electrostatic field at the NPA site due to the TMH macrodipoles, and a secondary barrier exists at the ar/R selectivity filter, both barring proton transport in AQP1 (de Groot et al. 2003) and GlpF (Chakrabarti et al. 2004a, b). These studies (reviewed in de Groot and Grubmuller 2005) oppose "global reorientation" as being the main barrier, but as neither water molecule dynamics nor proton transport can be studied experimentally, these simulations are predictive. Nonetheless, the NPA motifs are clearly important, a fact supported by observations of mutations close to these causing reduced water permeability and being involved in human diseases; for example, a human AQP1 with a point mutation in the NPA box, resulting in a KPA box in loop E was found in a Co (Colton blood group antigen)-null homozygous individual and led to medical problems including anemia, cataracts, and uterine sarcoma (Agre and Kozono 2003; Chretien et al. 1999).

The aromatic/arginine selectivity filter further contributes to pore selectivity

The second major player in substrate selectivity of different MIPs for water or other polar molecules is amino acid residues including the ar/R (aromatic/arginine) region. The ar/R region is a tetrad composed of one residue each from the alpha helices H2 and H5 and two from loop E (LE1 and LE2) that meet in the center of the pore, forming its narrowest constriction in a region separate from the NPA motifs (Fig. 1) and thought to constitute the primary selectivity filter for substrate specificity (Fu et al. 2000; Sui et al. 2001; Thomas et al. 2002; Wallace and Roberts 2004). The highly conserved Arg (R) in LE2 is thought to be important for providing hydrogen bonds for transport of water or glycerol molecules and to repel cations from the pore (Sui et al. 2001). It is thought that cations are repelled by hydrophilic residues and the positive dipole of the transmembrane helices lining the pore, and anions are repelled by the carbonyl groups of the amino acids lining it (Murata et al. 2000; Sui et al. 2001).

The properties of the four residues making up the ar/R selectivity filter appear to govern the substrate specificity of the pore and are thought to be useful for predicting the function of the proteins, such as the specific transport of water (aquaporin-type MIPs) vs glycerol (glycerol facilitator-type MIPs) or both water and glycerol (aquaglyceroporin-type MIPs) (Thomas et al. 2002). The selectivity filter residues of aquaporins tend to be smaller and more hydrophilic (Thomas et al. 2002) (Wang et al. 2005), with human and bovine AQP1 containing Phe58, His182, Cys191, and Arg197, resulting in a pore diameter of 2.8 Å, the same as that of a single water molecule (Sui

et al. 2001). In contrast, the bacterial glycerol transporter GlpF contains Trp48, Gly191, Phe200, and Arg206, of which Gly191 is smaller and less hydrophilic than the corresponding His182 of AQP1, and Phe200 is more hydrophobic than Cys191 of AQP1. This results in a larger, more hydrophobic pore for GlpF, with a diameter of 3.5 Å that allows transport of the bulkier, less polar glycerol molecule (Fu et al. 2000; Thomas et al. 2002). Bacterial aquaglyceroporin Gla_{Llac} contains a selectivity filter comprising of two hydrophilic (Pro232 and Arg238) and two hydrophobic (Tyr49 and Val223) residues, which again allows for a wider, more hydrophobic pore compared to AQP1, but allowing the transport of glycerol (Thomas et al. 2002). In addition, for the transport of glycerol, a much larger energy barrier exists for the bacterial aquaporin AQPZ compared to GlpF (Lu et al. 2003; Wang et al. 2005). A recent study on point mutations in rat AQP1 ar/R residues using the *Xenopus* oocyte assay system provided further evidence of the selectivity of this filter, wherein replacement of Phe56 with Ala widened the outer constriction and replacement of H180 with Ala enlarged the pore, but neither transported glycerol or urea, while double mutations at residues Phe56+H180 and H180+Arg195 allowed transport of urea and glycerol and replacement of Arg195 with Val (removal of positive charge) allowed transport of protons (Beitz et al. 2006). The critical role of the ar/R region is further indicated by the observations that mutations at its R (LE2) residue are associated with human disease conditions (reviewed in Agre and Kozono 2003). For example, substitution of Arg with Cys in AQP2 was shown to result in a nonfunctional aquaporin (when the mutant RNA is expressed in the *Xenopus* oocyte assay) and cause severe nephrogenic diabetes insipidus, resulting in extremely large urinary output per day (Deen et al. 1994).

MIPs comprise tetramers, consisting of four functional monomers

The oligomeric nature of MIPs was indicated for a plant MIP (of the TIP class, i.e., tonoplast integral protein; discussed below) from *Arabidopsis thaliana* by studies showing low electrophoretic mobility and a tendency to aggregate (Johnson et al. 1989), and indications that aquaporins function as individually functional monomers came from radiation inactivation of rat renal cortex membranes which resulted in aquaporins of a functional size of 30 kDa (van Hoek et al. 1991). The tetrameric nature of human AQP1 was determined by hydrodynamic studies and affinity chromatography (Smith and Agre 1991), its study in native and artificial membranes through freeze fracture electron microscopy (Verbavatz et al. 1993), and electron microscopy where four-lobed particles were discerned (Walz et al. 1995). Evidence also came from co-expression of wild type (mercury-sensitive) and mutant (mercury-insensitive) AQP1 mRNAs in frog oocytes, where exposure to the mercurial reagent reduced the water permeability to the level of the mutant aquaporin *only* (Preston et al. 1993), suggesting that AQP1 monomers act as functional pores. Mixed oligomers were also reported for AQP1 mutants (Jung et al. 1994). The oligomeric assembly of AQP1 monomers is suggested to be important for their folding, stability, and/or targeting to the plasma membrane (Fetter et al. 2004; Jung et al. 1994).

Plant MIPS comprise larger and more diverse families

The sedentary nature of plants and the absence of a circulatory system, combined with the greater number of intracellular compartments (e.g., water-storage and protein-storage vacuoles), are consistent with the expectation that the MIP superfamilies in plants are much more diverse and complex than in animals, and importantly, their subfamilies appear to have specialized locations and nonredundant functions involving transport of diverse substrates and exhibiting diverse effects on plant physiology. For example, *A. thaliana* is reported to contain 35 different MIPs (Johanson et al. 2001; Quigley et al. 2001) compared to the 13 in humans (Agre et al. 2002; Ishibashi et al. 2000; Takata et al. 2004).

Plant MIPs comprise a superfamily with subfamilies, classes, and subclasses and share key structural properties and principles of function

All plant MIP superfamilies identified over the last 10 years could be classified into four main subfamilies until recently: the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nodulin 26-like membrane intrinsic proteins (NIPs), and small, basic membrane intrinsic proteins (SIPs) (Johanson et al. 2001; Zardoya 2005). The newly reported GlP1;1 from a moss comprises a new, fifth subfamily closely related to bacterial glycerol transporters (Gustavsson et al. 2005). It should be noted that the names of the subfamilies are indicative of their common, *but not the only*, subcellular location (Barkla et al. 1999; Inoue et al. 1995). Each subfamily (e.g., PIP) has further groups (e.g., PIP1), each with various isoforms (e.g., PIP1;1), some of which have specialized locations and/or functions. For example, different types of vacuoles in plant cells, each with specialized functions, are reported to have different TIPs associated with their membranes (Inoue et al. 1995; Jauh et al. 1999; Johnson et al. 1989); the *A. thaliana* PIP1 is specific to plasma membrane invaginations (Robinson et al. 1996), and the α-TIP in the bean is seed-specific

(Johnson et al. 1989), while a tobacco TIP (TobRB7) is root-specific (Yamamoto et al. 1991).

PIPs appear to cluster into two evolutionary groups (PIP1 and PIP2) (Kammerloher et al. 1994), while TIPs cluster into five groups (TIP1, TIP2, TIP3, TIP4, and TIP5). PIPs and TIPs are far more prevalent than NIPs and SIPs, with *Arabidopsis* having 13 PIPs, 10 TIPs, 9 NIPs, and 3 SIPs (Chaumont et al. 2001; Johanson et al. 2001; Quigley et al. 2001). In terms of function, many of the TIPs and PIPs are 'aquaporins', i.e., have water-channel activity, although some isoforms additionally transport other substances (see below). The plant NIPs and SIPs are comparatively less studied. While some NIPs have shown little or no water transport ability (Wallace and Roberts 2005), most isoforms transport alternative substrates, including both glycerol and water (Rivers et al. 1997) or ammonia (Niemietz and Tyerman 2000; Tyerman et al. 2002) (see below). The function of plant SIPs is largely undefined, although in vitro studies indicate water channel activity for some forms, and the first endoplasmic reticulum (ER)-membrane localized MIP, of a SIP-type, has been reported recently in *Arabidopsis* (Ishikawa et al. 2005). The in vivo roles of the new fifth subfamily mentioned above (Gustavsson et al. 2005) need investigation.

The residues of the NPA motifs are highly conserved in plant PIPs and TIPs, with alternative motifs generally found only in the NIP or SIP groups (Ishibashi 2006; Zardoya 2005). NPAs in NIPs appear to have little effect on their substrate selectivity, as found in studies on AtNIP6;1 from *A. thaliana*, a glycerol, formamide and urea transporter with NPA (loop B) and NPV (loop E) motifs and very low water flux rate, where substitution of Val in the NPV with Ala exhibited no apparent water transport and high urea and glycerol transport rates (as in the wild type) (Wallace and Roberts 2005). The pore specificity of the plant MIPs appear to be more attributable to the ar/R selectivity filter residues as indicated by the homology modeling of the pore regions of the four plant MIP subgroups against the crystal structures of bovine AQP1 and bacterial GlpF (Wallace and Roberts 2004). The importance of this filter was demonstrated in a study on an aquaglyceroporin NIP (LIMP2) from a legume where substitution of Trp with His at position H2 abolished glycerol transport when expressed in *Xenopus* oocytes (Wallace et al. 2002). Moreover, substitution of Ala with Trp at position H2 of the urea-transporting AtNIP6;1 resulted in the predicted narrowing of the pore aperture and increased water permeability and exclusion of urea (Wallace and Roberts 2005). Mutagenesis studies of NPAs and ar/R residues in plants have been limited, and considering the great variability in the ar/R filter residues in the TIPs in plants (see below), further research is required for both plant PIPs and TIPs to assess the roles of both the NPA boxes and the various combinations of the ar/R residues on selectivity and permeability for water and other substrates.

The tetrameric assembly also appears to occur in plant aquaporins (Fotiadis et al. 2001), and interactions can also occur between different isoforms, leading to heteromer formation, which can lead to changes in stability and increases in water permeability and expression levels of some isoforms (Fetter et al. 2004). Furthermore, the importance of loop E in the protein interactions was demonstrated when replacement of the loop E residues of ZmPIP1;1 (a PIP1 isoform with no positive interaction with ZmPIP2s) with those of the ZmPIP1;2 resulted in a positive interaction of the mutant ZmPIP1;1 with native ZmPIP1;1 and with another isoform, ZmPIP2;5 (Fetter et al. 2004).

The *MIP* gene superfamilies in cereals are also large and complex

Wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.), belonging to different subfamilies of the grass family Poaceae (Kellogg 2001), make up a very large proportion of the staple grain worldwide. It is thus particularly important to focus our efforts on analyzing key genetic factors involved in water transport such as the MIPs. The following sections focus on the current status of MIP research in cereals, particularly on the PIP and TIP subfamilies due to their significance in water transport.

All cereals studied to date exhibit large MIP superfamilies, consisting of the four major subfamilies mentioned above. The first cereal in which the *MIP* genes were studied was maize, wherein 33 genes were identified through sequencing of full-length cDNAs, grouping into 13 PIPs, 12 TIPs, 5 NIPs, and 3 SIPs (Chaumont et al. 2001). Our analysis of the TIGR rice genome assembly (TIGR Release 10.0, January 2005; http://www.tigr.org/tdb/e2k1/osa1/index.shtml; last accessed November 2006); using tblastx searches in Gramene (http://www.gramene.org/Multi/blastview, accessed June 2005) indicated 38 *MIP* genes ranging in size from 669 to 1,113 bp and spread across all rice chromosomes except chromosome 11. The MIPs could be split into 13 *PIP*s, 10 *TIP*s, 13 *NIP*s, 2 *SIP*s, including at least two possible pseudogenes (Table 1). These results are similar to those of Sakurai et al. (2005), who independently identified 33 expressed genes and some pseudogenes (Sakurai et al. 2005) and Guo et al. (2006) who identified 10 expressed *PIP* genes. Of the five additional genes (OsPIP2;9, OsPIP2;10, OsNIP1;5, OsNIP3;4, and OsNIP3;5) identified (Forrest and Bhave, unpublished), OsNIP3;5 showed a corresponding EST and is thus unlikely to be a pseudogene (unless its protein product is nonfunctional), while the other four loci did not have expression data (EST/TCs), and of these, *OsPIP2;9*

and *OsPIP2;10* have major gaps in their putative protein sequences. Most of the rice PIPs were predicted to be located in the plasma membrane, with the notable exception of OsPIP1;2, which was predicted to exist in the chloroplast or mitochondria but contained a nuclear localization signal (Table 1). Some of the genes appear closely linked, e.g., four *PIP* loci (*OsPIP2;1*, *OsPIP2;4*, *OsPIP2;5*, and *OsPIP2;9*) at ~15.4 Mb on chromosome 7, suggesting gene duplication events, and the highest identity (86–94%) between the putative protein products of these four genes compared to other rice PIPs suggests origin by recent duplications.

Although there has been no whole genome project data or a major report on barley MIPs, a number of *PIP* cDNA and/or genomic sequences (Doering-Saad et al. 2002; Hollenbach and Dietz 1995; Katsuhara et al. 2002) and several *TIP* cDNAs have been reported (Schunmann and Ougham 1996; Shirasu et al. 2000). Our analysis of this data and sequences available in Genbank, translations of coding sections into putative protein sequences and their clustalW alignments (data not shown) show 3 PIP1s, 2 PIP2s and 3 TIPs (Supplemental data Figs. 1 and 2), plus one likely pseudogene (BM817412) that shows only one NPA motif and a stop codon after loop B and another protein (BM816065) with atypical sequence. Forrest and Bhave (unpublished) have shown that wheat has at least 6 PIP and 2 TIP genes and numerous isoforms within these subclasses, some of this complexity being related to the hexaploid nature of wheat (i.e., presence of homeologous genes), in addition to multigenes of a particular subclass *per genome*.

MIP gene function: PIP and TIP genes in cereals exhibit conserved structures within the subclasses, but a higher degree of identity between the PIPs than the TIPs

The overall structures of the *PIP* genes of rice (Table 1; Sakurai et al. 2005), barley (Katsuhara et al. 2002), and wheat (Forrest and Bhave, unpublished) are highly conserved in relation to those of *A. thaliana* (Johanson et al. 2001; Quigley et al. 2001) (DNA alignment data not shown). The genes contain 0 to 3 introns (Table 2), the locations of these (when present) being at the start of the coding region for loop B, the end of that for loop D, and the start of that for transmembrane helix 6 (DNA alignment data not shown). The intron positions are also conserved between the *TIP* genes of *A. thaliana* (Johanson et al. 2001; Quigley et al. 2001), rice (present work; Sakurai et al. 2005), and wheat (Forrest and Bhave, unpublished), with these genes containing up to two introns, which when present, occur in the coding region for loop A and loop C (DNA alignment data not shown). However, significant variations are noticed in the intron lengths of both *PIP* and *TIP* genes, especially introns I and II of *PIP*s (Table 2). While most of the *PIP* and *TIP* genes exhibit the universal GT/AG splice donor and acceptor sites, a GC/AG motif has been identified by us in the rice gene *OsTIP5;1* in its TIGR genomic data (data not shown) and also experimentally in the wheat isoform *TaPIP2;1*, as also a GC/TG in a putative pseudogene *TaPIP1;1* (Forrest and Bhave, unpublished).

The cereal *PIP* and *TIP* genes appear more closely related to one another than to those in *A. thaliana*, both at the level of coding sequences (DNA alignments not shown) and their respective putative proteins, as would be expected (Table 3; supplemental data Figs. 1 and 2). Significant identities are seen in the coding sections of *PIP* genes of all plants (53–92%) and in the putative protein sequences (54–96%) (Table 3, supplemental data Fig. 1); however, the *TIP* sequences show significantly lower identities and a wider range, both between coding sections of genes (10–90% identity) and at the putative protein levels (17–95%) (Table 3, supplemental data Fig. 2).

The TIP gene and protein sequences of cereals are also generally not as highly conserved as the PIPs (Table 3). A high degree of conservation is seen in the amino acid sequences of subclasses of cereal PIPs, PIP1's (64–98 % identity), and PIP2's (58–93% identity) (supplemental data Figs. 1 and 2), excluding products of the OsPIP2;9 and OsPIP2;10 pseudogenes (which have large internal gaps; data not shown). Members of a major PIP subclass show a higher degree of interspecies sequence conservation (e.g., 93% identity between maize ZmPIP2;4 and rice OsPIP2;2, and between ZmPIP2;6 and OsPIP2;9) than intraspecies (87% between all PIP1s of barley and 89% between all PIP1s of wheat). This suggests that at least some of the gene duplication events in the MIP superfamily predate the separation of the subfamilies within Poaceae. As with the PIPs, the highest identities between members of the same TIP subclass are often shared between rather than within species; e.g., the putative proteins of wheat TaTIP2;3 (Forrest and Bhave unpublished; supplemental data Fig. 2) and maize ZmTIP4;2 share 98% identity, while there is 17–95% identity between all cereal TIPs.

The PIP and TIP proteins in cereals show conservation and/or specialization of functionally important areas

The six transmembrane helices and loop B and E regions are more highly conserved than the inter-helical loops in all putative protein members of the PIP and TIP subfamilies of rice, maize, barley, and wheat, as also in *Arabidopsis*. The N and C-terminal sequences appear to be the most divergent between the PIPs compared to the rest of the sequence area, while the TIPs exhibit a much greater extent of diversity throughout the protein, and more so, at the N termini than the C termini (supplemental data Figs. 1 and 2).

Table 1 Rice *MIP* loci identified using *in silico* methods

MIP isoform[a]	Locus[b]	Gene position[c]		*I*[d]	*E*[e]	AA[f]	NPA[g]	TMH[h]	Location[i]
		Start (bp)	Stop (bp)						
OsPIP1;1	LOC_Os02g44630	27,038,450	27,041,579	3	4	289	2	6	PM[o]
OsPIP1;2	LOC_Os04g47220	27,827,487	27,829,439	2	3	282	2	4[l]	Chl/M[o], Chl[p], NLS[q]
OsPIP1;3	LOC_Os02g57720	35,343,804	35,345,571	1	2	288	2	6	PM[o]
OsPIP2;1	LOC_Os07g26690	15,404,748	15,408,054	2	3	290	2	6	PM[o]
OsPIP2;2	LOC_Os02g41860	25,157,007	25,160,748	3	4	288	2	6	PM[o]
OsPIP2;3	LOC_Os04g44060	25,880,249	25,882,687	2	3	290	2	6	PM[o]
OsPIP2;4	LOC_Os07g26630	15,357,266	15,358,697	2	3	286	2	7[m]	PM[o]
OsPIP2;5	LOC_Os07g26660[j]	15,375,222	15,376,595	2	3	283	2	6	PM[o]
OsPIP2;6	LOC_Os04g16450	8,929,872	8,928,144	3	4	282	2	6	PM[o]
OsPIP2;7	LOC_Os09g36930	21,309,651	21,311,060	1	2	290	2	6	V[o]
OsPIP2;8	LOC_Os03g64330	36,299,579	36,300,677	0	1	280	2	6	PM[o]
OsPIP2;9[r]	LOC_Os07g26640	15,367,441	15,368,473	3	4	223	1	5	V/PM[o]
OsPIP2;10[r]	LOC_Os10g34000[j]	17,811,393	17,813,051	3	4	242	1	4[l]PM[o]	
OsTIP1;1	LOC_Os03g05290	25,437,57	25,419,20	1	2	250	2	6	PM/V[o]
OsTIP1;2	LOC_Os01g74450	43,444,069	43,442,595	1	2	252	2	7	PM/V[o]
OsTIP2;1	LOC_Os02g44080	26,603,789	26,602,493	1	3	248	2	6	PM[o], S[p]
OsTIP2;2	LOC_Os06g22960	13,406,320	13,407,829	2	3	248	2	6	PM[o]
OsTIP3;1	LOC_Os10g35050	18,371,554	18,372,931	2	3	264	2	6	PM[o]
OsTIP3;2	LOC_Os04g44570	26,161,182	26,162,454	2	3	265	2	6	PM/V[o]
OsTIP4;1	LOC_Os05g14240	7,980,002	7,982,306	2	3	251	2	6	V[o]
OsTIP4;2	LOC_Os01g13130	7,299,199	7,300,573	1	2	256	2	7	PM/V[o]
OsTIP4;3	LOC_Os01g13120	7,294,484	7,295,547	1	2	251	2	6[l]	V/PM[o]
OsTIP5;1	LOC_Os04g46490	27,357,899	27,356,566	2	3	269	2	6	PM[o], S[p]
OsNIP1;1	LOC_Os02g13870	7,519,336	7,516,220	3	4	284	2	6	PM[o]
OsNIP1;2	LOC_Os01g10600	5,665,705	5,660,125	4	5	303	2	6	PM[o]
OsNIP1;3	LOC_Os05g11560	6,524,124	6,519,140	4	4	286	2	5[l]	PM[o]
OsNIP1;4	LOC_Os06g35930	20,959,709	20,960,822	3	4	273	2	6	PM[o]
OsNIP1;5[r]	LOC_Os01g10530	5,590,435	5,588,074	5	6	303	2	6	V/PM[o]
OsNIP2;1	LOC_Os02g51110	31,260,459	31,260,459	4	5	298	2	6	PM[o]
OsNIP2;2	LOC_Os06g12310	6,652,003	6,647,391	4	5	298	2	6	PM[o]

OsNIP3;1	LOC_Os10g36924[j,k]	19,455,302	19,448,706	2	3	241	0	6	PM[o], S[p]
OsNIP3;2	LOC_Os08g05590	2,981,082	2,984,672	7	7	371	2	6[n]	V[o]
OsNIP3;3	LOC_Os08g05600	2,990,687	2,992,113	3	4	278	2	6	V[o]
OsNIP3;4[r]	LOC_Os08g05580	2,977,397	2,980,418	3	4	276	2	6	V[o], Chl[p]
OsNIP3;5[r]	LOC_Os12g10280	5,434,714	5,438,021	2	3	310	2	6	PM[o]
OsNIP4;1	LOC_Os01g02190	643,524	642,417	1	2	286	2	6[l]	V/PM[o]
OsSIP1;1	LOC_Os01g08660	4,312,614	4,310,471	2	3	246	1	5[l]	PM[o], S[p]
OsSIP2;1	LOC_Os03g20410	11,527,917	11,525,413	2	3	250	1	6	PM[o], M[p]

A tblastx search was performed on each identified *A. thaliana* gene against the rice genomic sequence database in Gramene to find all homologous genes in rice (http://www.gramene.org/Multi/blastview, accessed June 2005; last accessed November 2006). The putative protein sequences were aligned and compared to the 35 from *A. thaliana* (Johanson et al. 2001; Quigley et al. 2001) using ClustalW (http://www.ebi.ac.uk/clustalw/), and a cladogram was produced using TreeView. The corresponding homology scores and cladogram were used to classify each rice gene into a subfamily (e.g., PIP or TIP) and further into isoforms. Gene and protein information was originally obtained from the TIGR Rice Genome Annotation Release 3 (December 2004, now obsolete) and recently updated from TIGR Rice Genome Annotation Release 4 (January 2006, http://www.tigr.org/tdb/e2k1/osa1/index.shtml; last accessed November 2006).

PM Plasma membrane, *C* cytoplasm, *V* vacuole, *Chl* chloroplast, *M* mitochondria, *NLS* nuclear localization signal, *S* secretory

[a] Isoform determined from clustalW alignments with *A. thaliana* gene sequences and a TreeView cladogram. Isoforms in italics are predicted pseudogenes.

[b] Locus name as given by TIGR Rice Genome Annotation Release 4.

[c–f] Taken from the TIGR gene locus reports (http://www.tigr.org/tdb/e2k1/osa1/LocusNameSearch.shtml)

[c] Position of the locus on rice chromosome

[d] Number of introns

[e] Number of exons

[f] Putative protein length (number of amino acids)

[g] Number of NPA motifs, determined from analyses of putative protein sequences

[h] Number of transmembrane helical domains (TMHs) determined using the TMHMM server

[i] Subcellular location and other characteristics of putative proteins

[j] Some sequences appear to be incorrectly annotated in the new TIGR release (by comparison with previous locus information and other published rice data (Sakurai et al. 2005), and, therefore, information obtained from the previous Release 3 has been retained for these.

[k] The gDNA of LOC_Os10g36924 (OsNIP3;1) was annotated as locus LOC_Os10g36910 in Release 3 of the TIGR Rice Genome Annotation database (locus now obsolete). The previous annotation (including cDNA and protein sequences) is confirmed by Sakurai et al. (2005), and therefore, the number of introns, exons, AAs, NPAs, and TMHs is carried over from cDNA and protein sequences from LOC_Os10g36910.

[l] One additional TMH identified using the TMpred server

[m] One less TMH identified using the TMpred server

[n] Two additional TMHs identified using the TMpred server

Programs used for prediction of subcellular locations:

[o] pSort

[p] TargetP

[q] PredictNLS

[r] Not identified by Sakurai et al. (2005) as expressed genes

Table 2 Comparison of intron lengths and numbers in plant *PIP* and *TIP* genes

PIP genes[a]	*A. thaliana*[b]			Rice[c]			Barley[d]			Wheat[e]		
	Intron I	Intron II	Intron III	Intron I	Intron II	Intron III	Intron I	Intron II	Intron III	Intron I	Intron II	Intron III
PIP1;1	264	238	94	992	380	488				105	122	553
PIP1;2	225	80	88	–	104	562				–	88	142
PIP1;3	157	84	89	–	–	234	–	101	110			
PIP1;4	309	128	94	NA	NA	NA						
PIP1;5	273	94	133	NA	NA	NA	–	–	–			
PIP1;6	NA	NA	NA	NA	NA	NA						
PIP2;1	468	127	90	–	1887	125	116	979	739	105		
PIP2;2	121	91	98	107	1742	563				–	90	112
PIP2;3	121	91	89	–	711	510				–	–	156
PIP2;4	199	97[f]	95	–	45	115				–	–	–
PIP2;5	116	555	89	–	96	133						
PIP2;6	505	730	78	89	106	84						
PIP2;7	92	259	93	–	–	102						
PIP2;8	229	385	107	–	–	–						
PIP2;9	NA	NA	NA	180	84	100						
PIP2;10	NA	NA	NA	115	89	358						

TIP genes[a]	*A. thaliana*[b]		Rice[c]		Barley[d]		Wheat[e]	
	Intron I	Intron II	Intron I	Intron II	Intron I	Intron II	Intron I	Intron II
TIP1;1	–	91	755	–			500	–
TIP1;2	–	172	82	–				
TIP1;3	–	–	NA	NA				
TIP2;1	103	399	81	119			101	93
TIP2;2	–	180	82	103				
TIP2;3	–	205	NA	NA				
TIP3;1	91	119	103	86				
TIP3;2	300	130	101	262				
TIP4;1	106	391	101	922				
TIP4;2	NA	NA	153	–				
TIP4;3	NA	NA	84	–				
TIP5;1	79	84	112	66				

The darker grey cells indicate lack of published data in case of barley and incomplete data in case of wheat to conclude whether or not a certain gene isoform and/or intron is present (work in progress; Forrest and Bhave, unpublished).

NA Indicates gene types that have not been reported and/or not found in analysis of the genomic sequence of *A. thaliana* or rice.

Introns for *A. thaliana* and rice were identified by aligning gDNA and cDNA sequences for each gene. These sequences were obtained by searching for loci for:

[a]Length in base pairs (bp)

[b]*A. thaliana* (Johansson et al. 2001) (e.g. At3g61430 for AtPIP1;1) in TIGR *Arabidopsis thaliana* Database (http://www.tigr.org/tdb/e2k1/ath1/LocusNameSearch.shtml, last accessed October 2006) or

[c]Rice in TIGR Rice Database (http://www.tigr.org/tdb/e2k1/osa1/LocusNameSearch.shtml, last accessed November 2006) (present work; Table 1).

[d]Intron sizes for HvPIP1;3, HvPIP1;5 and HvPIP2;1 from barley were obtained from Katsuhara et al. (2002).

[e]Introns in wheat genes were predicted by comparing wheat gDNA sequences (Forrest and Bhave, unpublished) with gDNA and cDNA sequences of rice. All alignments were manually performed in Bioedit v. 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

[f]This intron is in a different position compared to that of intron II in all other PIPs shown in the table.

—Indicates absence of that intron in the gene structure.

All cereal PIP and TIP sequences also contain the two highly conserved NPA motifs.

All PIPs contain the ar/R selectivity filter residues Phe-His-Thr-Arg (FHTR) (Figs. 3, 4 and 5), very similar to the human AQP1 selectivity filter FHCR (Sui et al. 2001). In contrast, a variety of ar/R sequence combinations is observed in the TIP, NIP, and SIP subfamilies, including differences between various subclasses within each family (Table 4; supplemental data Table 1), some of which may be associated with functional specialization. For example, the highly conserved Arg at LE2, shown to be important for transport of water (see above), is found in all cereal PIPs, but a subclass of TIPs, the TIP1s, contain a Val residue here (Table 4, supplemental data Table 1), a change suggested to affect their water transport function (Wallace and Roberts 2004). Up to seven different combinations are seen among the cereal TIPs, some of which (e.g., His-Met-Ala-Arg of OsTIP3;1) are not seen in *A. thaliana*. This diversity in filter residues and reports of alternative transport functions (e.g., for urea or ammonia; see below) suggest that the TIP subfamily members include aquaporins but some may (also) have other roles; these need to be tested thoroughly. None of the plant MIP ar/R filter residues, except for the highly conserved R, is identical to those of the bacterial glycerol transporter GlpF (Fu et al. 2000) or aquaglyceroporin Gla_{Llac} (Thomas et al. 2002), even though some

Table 3 Extent of sequence identity in coding sequences and putative PIP and TIP proteins of cereals

cDNA/proteins	Barley[a]	Maize[b]	Rice[c]	*A. thaliana*[d]
PIPs				
Barley		*59–91*	*60–92*	*57–75*
Maize	61–93		*59–91*	*57–75*
Rice	54–94	54–96		*53–75*
A. thaliana	63–88	60–88	54–87	
TIPs				
Barley		*32–90*	*29–89*	*16–72*
Maize	18–90		*53–89*	*10–72*
Rice	17–89	32–95		*12–74*
A. thaliana	19–74	30–76	30–80	

Numbers in italics refers to percent identity between the cDNA sequences (alignment data not shown; sequence sources as above and Table 1) and numbers in regular text refer to percent identity between their putative protein sequences (supplemental data Figs. 1 and 2). The TIP cDNA sequences were obtained from Genbank and aligned in ClustalW (http://www.ebi.ac.uk/clustalw/) to obtain identity scores. The PIP cDNA sequences were first aligned in Muscle (http://www.ebi.ac.uk/muscle/) to obtain a more accurate DNA alignment, and the corresponding alignment was then imported into ClustalW to calculate identity scores. Protein sequences were obtained from Genbank (where available) or predicted from cDNA sequences or coding sections of genomic sequences by translation in Bioedit v7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using ClustalW. Some barley cDNA sequences appear to be of poor quality as they result in many changes to the reading frame, and thus, poor percent identity scores. Predicted pseudogenes from rice (OsPIP2;9 and OsPIP2;10) were excluded from comparisons.

[a] Barley HvPIP1;3 (AB009308), HvPIP1;5 (AB009309), HvPIP1:6 (CO720031),H HvPIP2;1 (AB009307), HvPIP2;4 (AB219525), HvTIP1;1 (X80266), HvTIP2;1 (AF254799), HvTIP5;1 (AF254799) from Genbank (Katsuhara et al. 2002; Schunmann and Ougham 1996; Shirasu et al. 2000)

[b] (Chaumont et al. 2001)

[c] Analysis of the rice MIP sequences in The TIGR Rice Database (present work; Table 1).

[d] (Johanson et al. 2001).

plant MIPs are reported to transport these substrates (Rivers et al. 1997; Tyerman et al. 2002).

The mechanisms for differential localization of PIPs mostly to the plasma membrane and TIPs mostly to the tonoplast are as yet unclear, as no specific signal peptides or other signature sequences have been reported, and these could not be identified with certainty in our alignments due to the extent of variations seen (supplemental data Figs. 1 and 2). However, presence of the highly conserved NPA motifs and ar/R selectivity filter residues suggests that the PIPs and at least several of the TIPs from cereals are highly likely to be functional aquaporins. It also appears from the high degree of similarity of various PIP and TIP isoforms between different cereal species, particularly rice, wheat and barley, that they may retain orthology between their genomes. This information offers substantial inroads for crop improvement and is discussed later.

Water permeability functions of plant aquaporins (TIP and PIP subclasses of MIPs)

Water uptake and its movement throughout the plant are required for a large number of cellular and physiological processes in plants such as cell enlargement, stomatal movement, photosynthesis, phloem loading, and transpiration. Water is absorbed by the roots into the apoplast, but for water to cross the hydrophobic barrier of the Casparian strip, water molecules are transferred to the symplast via the transcellular pathway, which is proposed to be regulated by the aquaporins (Amodeo et al. 1999; Johansson et al. 2000). Many other roles have been shown for various MIPs, in addition to, or instead of, water transport; therefore, functional assays must be utilized to test water permeability of a MIP and define it as an "aquaporin". The first plant aquaporin to be defined by expression of mRNA in the frog oocytes (the assay that originally identified the human aquaporins; Preston et al. 1992) was AtTIP1;1 (γ-TIP) from *A. thaliana*, its expression resulting in a six- to eightfold increase in osmotic water permeability (Maurel et al. 1993). A number of plant MIPs, particularly PIP and TIP isoforms, have passed such tests and can now be justifiably called aquaporins due to functional assays demonstrating their water permeability.

Functional evidence for water transport functions of plant PIPs

The plasma membrane is the first cellular barrier for the water molecules to cross, and any PIPs within it would be expected to have a more significant effect on cell–water relations than the (generally) tonoplast-located TIPs. Many

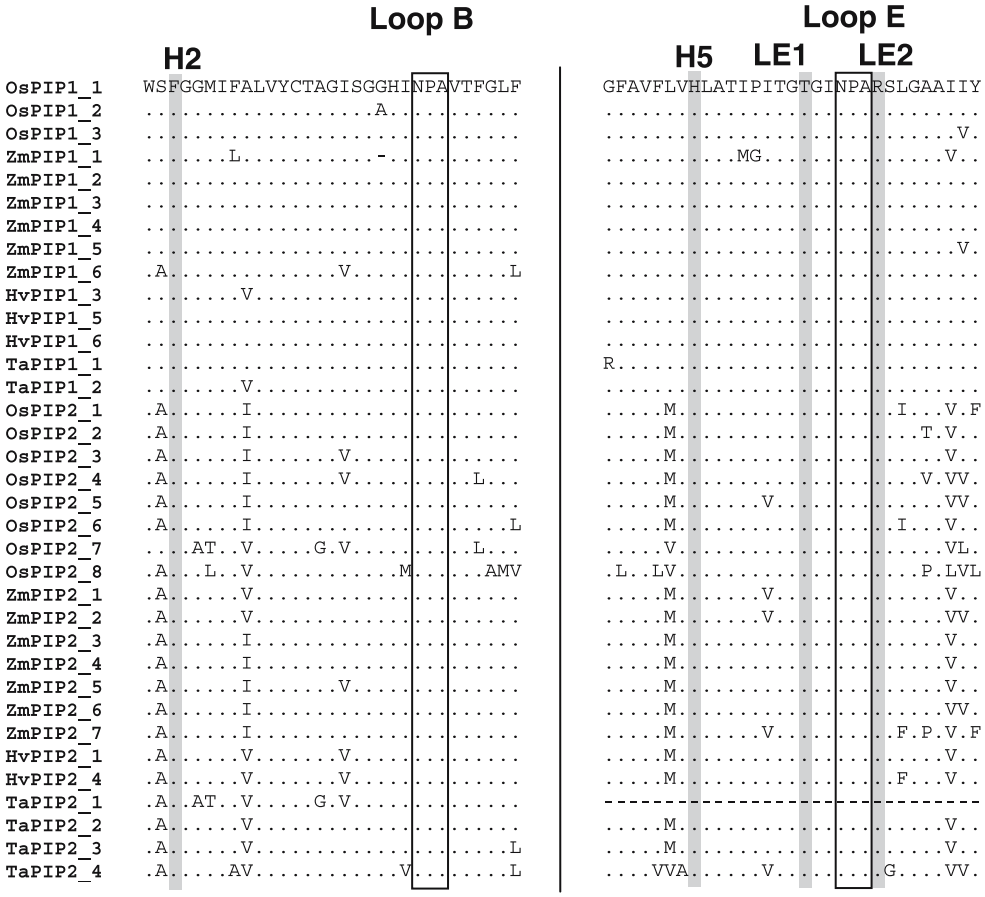

Fig. 3 Alignment of ar/R selectivity filter and NPA motif residues in the putative PIP proteins of cereals. *Dots* indicate conserved residues with OsPIP1;1. *Grey shading* indicates the position of an ar/R residue, and *boxes* indicate the NPA motifs. *Dashes* indicate sequence data not available

PIP isoforms from a diverse range of plants have been tested using the oocyte swelling and mercury blockage assays, and tools such as antisense technology to reduce or knock out the expression of a specific gene. Indeed, high water permeability has been detected in oocytes expressing numerous genes, e.g., two PIPs from *A. thaliana*, AtPIP2;3 (RD28) (Daniels et al. 1994; Johanson et al. 2001) and AtPIP1;1-1;3 (PIP1a-c) (Kammerloher et al. 1994) and rice OsPIP1;3 and OsPIP1;1 (RWC3 and RWC1, respectively) (Li et al. 2000; Lian et al. 2004). HvPIP2;1 from barley also tested positive for water transport in oocyte swelling assays and mercurial blockage (Katsuhara et al. 2002), whereas HvPIP1;3 showed no activity (Katsuhara et al. 2003). However, mercury inhibition does not always apply; for example, a PIP of *A. thaliana* (AtPIP2;3 or RD28) is insensitive to pore blockage by mercury despite containing a number of cysteine residues (Daniels et al. 1994), whereas two of its TIPs (δ-TIP and γ-TIP) are mercury-sensitive, the property attributed to Cys116 and Cys118, respectively, in the transmembrane helix 3 (Daniels et al. 1996).

PIPs seem to play a particularly important role in controlling transcellular water transport, supported by observations of a transgenic *A. thaliana* plant expressing double antisense isoforms of two *PIP* genes (of subclasses *PIP2* and *PIP1*) that resulted in a decrease in osmotic hydraulic conductivity in root and leaf protoplasts (Martre et al. 2002) and a gene knockout of an *A. thaliana PIP* gene (*AtPIP2;2*) showing similar results for root cortex cells and root (Javot et al. 2003). Transgenic *A. thaliana* plants expressing an antisense copy of a *PIP* gene (*PIP1;2)* showed its reduced expression and a corresponding threefold decrease in water permeability of protoplasts (Kaldenhoff et al. 1998), and overexpression of a barley *PIP* (*HvPIP2;1*) in transgenic rice also increased the radial hydraulic conductivity of roots (Katsuhara et al. 2003).

While members of the PIP2 subclass generally show high water transport capabilities, some members of the subclass PIP1 have shown little such ability. For example, a maize *PIP* gene (*ZmPIP2a*) was found to be functional in *Xenopus* oocytes, whereas two other isoforms (*ZmPIP1a* and *ZmPIP1b*) showed no activity (Chaumont et al. 2000), four rice *PIP* genes (*OsPIP1;1, OsPIP1;2, OsPIP2;4* and *OsPIP2;5*) expressed in yeast showed greater water permeability for the PIP2 members than the PIP1s (Sakurai et al. 2005), and expression of PIP2a and PIP2b in oocytes showed faster cell bursting compared to PIP1a, PIP1b, and PIP1c from *A. thaliana* (Kammerloher et al. 1994).

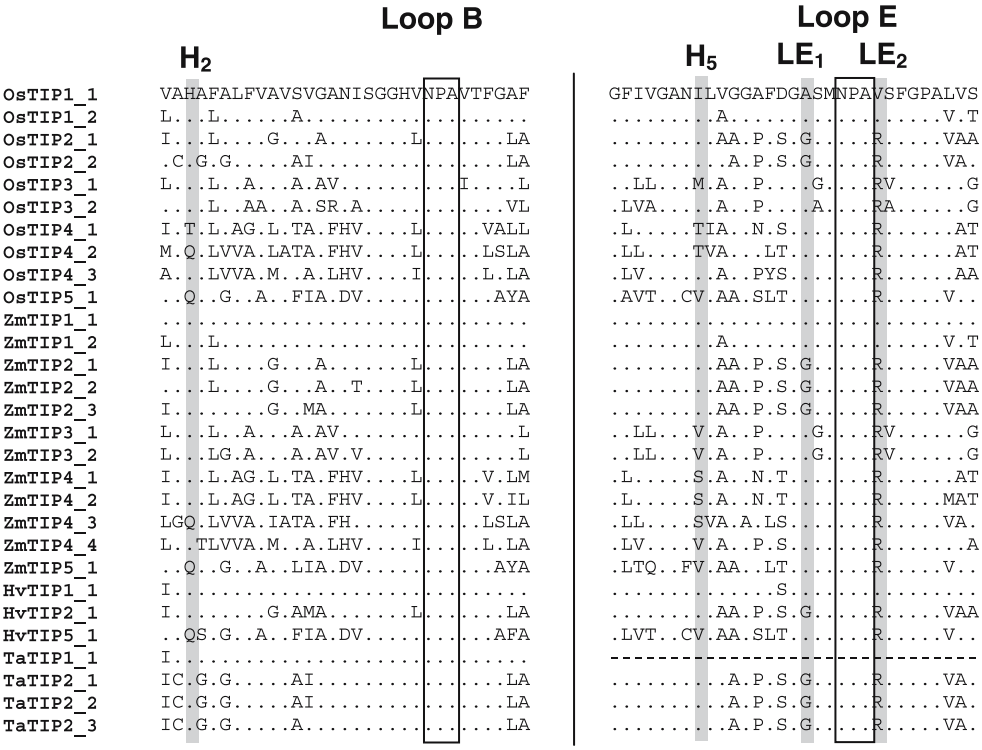

Fig. 4 Alignment of ar/R selectivity filter and NPA motif residues in the putative TIP proteins of cereals. *Dots* indicate conserved residues with OsTIP1;1. *Grey shading* indicates the position of an ar/R residue, and *a black box* indicates an NPA motif. *Dashes* indicates sequence data not available

Functional evidence for water transport functions of plant TIPs

The water permeability function of plant aquaporins was first demonstrated in a TIP from *A. thaliana* via expression in oocytes (Maurel et al. 1993) and has since been confirmed using similar assays for TIP isoforms from diverse species including sunflower (Sarda et al. 1997), ice plant (*Mesembryanthemum crystallinum*) (Vera-Estrella et al. 2004), maize (Chaumont et al. 1998), tobacco (Gerbeau et al. 1999), bean (Maurel et al. 1995), *A. thaliana* (Maurel et al. 1997a), cauliflower (Barrieu et al. 1998), and radish (Higuchi et al. 1998). TIP1;1 of Arabidopsis and a TIP (McTIP1;2) from the ice plant show a high level of aquaporin function (Maurel et al. 1993; Vera-Estrella et al. 2004). This correlates with a higher water permeability for tonoplasts compared to the plasma membranes; for example, tonoplast permeability was 100-fold greater than that of plasma membrane from tobacco suspension cells (Maurel et al. 1997b), and the water permeability in wheat root cells was higher for endomembrane vesicles than plasma membrane vesicles (Niemietz and Tyerman 1997), although in both cases, the aquaporin isoform(s) involved with this function is not known. TIPs seem to control water exchange between cytosolic and vacuolar compartments, and hence, regulate cell turgor. This is indicated by the upregulation of transcripts of a *TIP* gene (*SunTIP7*) in sunflower leaf guard cells during stomatal closure (Sarda et al. 1997) and studies on the ice plant in response to osmotic stress showing an increased distribution of TIP1;2 to tonoplast fractions, possibly as "a homeostatic process to restore and maintain cellular osmolarity" (Vera-Estrella et al. 2004). The strongest clue yet to the potential significance of TIPs to plant survival was provided when RNA(i)-targeting of the gene *TIP1;1* was shown to result in plant death (Ma et al. 2004).

Other assays suggesting involvement of aquaporins in water transport

Although no specific genes were addressed, a number of other assays have demonstrated that water transport in root membranes occurs via aquaporins. For example, a rapid decrease in hydraulic conductance (i.e., the overall water permeability of the membrane; L_p) of single wheat root cortical cells was reported during anoxia (Zhang and Tyerman 1991), while mercury inhibited hydraulic conductivity in intact wheat root cells (Zhang and Tyerman 1999). Water permeability measurements in wheat root membrane vesicles in hyperosmotic medium showed that both endomembrane and plasma membrane vesicles possessed an osmotic to diffusional water permeability ratio (P_{os}/P_d) >1, indicating the presence of aquaporins (Niemietz and Tyerman 1997). Endomembrane vesicles measured greater osmotic permeability (P_{os}) and lower activation energy (E_A) compared to plasma membrane vesicles and showed no

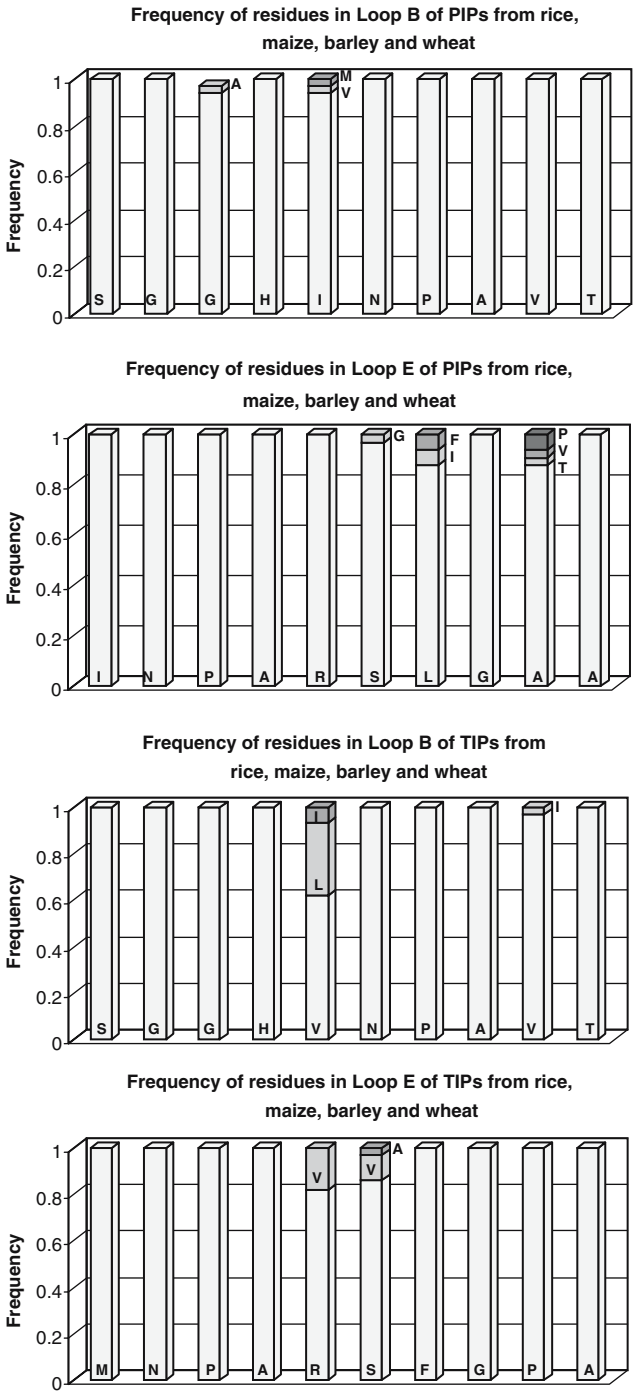

Fig. 5 Frequency of residues in loop B (LB) and loop E (LE) sequences of PIPs and TIPs in wheat, barley, rice, and maize. Alignments of PIP1, PIP2, and TIP putative protein sequences were created in ClustalW. The *height of each letter* reflects the relative frequency of the amino acid at that position in the ClustalW alignment

mercury inhibition, while water transport in plasma membrane vesicles was inhibited (Niemietz and Tyerman 1997). In another study, P_{os} was found to be reduced in protoplasts from a drought-sensitive bread wheat but remained constant for a drought-tolerant durum, suggesting a possible difference in their aquaporins content (Morillon and Lassalles 2002).

Significance of aquaporins to plant–water relations during osmotic stress

Factors creating osmotic stress on plants, such as drought and salt, affect water uptake into and distribution within plants and have numerous negative effects on plant growth, development, and even survival (Bernstein 1975; Munns 2002). For example, studies using a pressure probe showed that maize roots exposed to salt stress showed a decrease in hydraulic conductivity (L_p) of cortical root cells, increased root diameter, and increased the diameter of root cells, suggesting adverse effects on water transport of root cells (Azaizeh et al. 1992), and the hydraulic conductivity of wheat and *A. thaliana* roots was reported to be vastly reduced during salt stress (Boursiac et al. 2005; Ktitorova et al. 2002). Such alteration of water transport in plants provides strong grounds to study aquaporins in this context.

Involvement of MIP subclass PIP aquaporins in abiotic stress response: the comparative evidence

Numerous comparative studies have tested the effects of salt, drought, or cold on the expression of mRNA transcripts and/or proteins of members of two main MIP subfamilies, PIPs and TIPs, in different tissues of cereal plants, and many have shown differential response (summarized in Table 5). For example, transcripts and proteins of the barley PIP gene *HvPIP2;1* were found to be down-regulated in roots but transcripts were up-regulated in shoots of plants under salt stress (Katsuhara et al. 2002), while over-expression of *HvPIP2;1* in transgenic rice actually increased salt sensitivity (Katsuhara et al. 2003). In maize, transcripts of several members of the PIP subgroups *ZmPIP1* and *ZmPIP2* were down-regulated in response to salt, with a few exceptions which were up-regulated (Aroca et al. 2005; Zhu et al. 2005).

Under osmotic stress caused by water insufficiency, the rice *PIP* gene *OsPIP1;3* (*RWC3*) was found to be up-regulated in only the drought tolerant rice cultivar (Lian et al. 2004). During osmotic stress induced by 10% polyethylene glycol (PEG), the rice *OsPIP1;1* and *OsPIP1;2* transcripts were up-regulated, whereas *OsPIP1;3* remained unchanged (Guo et al. 2006). Surprisingly, *OsPIP1;1* was found to be *down-regulated* in response to alternative methods of water stress such as drought (lack of water) (Malz and Sauter 1999) or use of a different chemical (250 mM mannitol) (Li et al. 2000). Several studies have simultaneously monitored

transcript or protein levels of multiple *MIPs;* for example, *OsPIP1;1* and *OsPIP1;2* were found to be down-regulated, while *OsPIP1;3* was up-regulated during chilling (Sakurai et al. 2005). Studies in wheat have been limited, but in one study (Morillon and Lassalles 2002), no changes were seen in PIP1 and PIP2 protein levels during drought stress in durum wheat (*Triticum turgidum* L.) and a soft common wheat (*Triticum aestivum* L.) using immunoblot. However, the antibodies had been designed against the C-terminal region, one of the highly divergent regions of PIPs (Johanson et al. 2001) (Supplemental data Fig. 1); therefore, the results cannot be taken as representative of all PIP isoforms. In addition, changes to protein activity can be affected by abiotic stress (Aroca et al. 2005), which would not be differentiated by this type of approach. Another study identified a *PIP1* (similar to *AtPIP1;4*) and a *PIP2* (similar to *AtPIP2;5*) sequence in wheat ESTs libraries from abiotically stressed plants (Houde et al. 2006), but their expression levels were not investigated, so it cannot be assessed whether these isoforms were in steady state or specifically induced.

Cold (chilling) stress appeared to increase osmotic water permeability (P_{os}) to a much higher degree in maize with a chilling-tolerant phenotype compared to a sensitive one, and transcripts of several members of *ZmPIP1* and *ZmPIP2* were found to be down-regulated (Aroca et al. 2005). Chilling also increased the abundance of total PIP1 proteins of both chilling-tolerant and -sensitive phenotypes, and although the total amount of PIP2 proteins remained unchanged, that of phosphorylated form of PIP2 (active state, discussed later) increased dramatically in both phenotypes (Aroca et al. 2005), suggesting that activation of the protein by phosphorylation plays some role in the response process.

In other plants, the *PIP* gene *AtPIP2;3* (*RD28*) of *Arabidopsis* was one of the earliest studied and found to be induced under water deficit (Yamaguchi-Shinozaki et al. 1992). Several studies utilizing diverse techniques have now led to identification of specific members of the *AtPIP1* and *AtPIP2* subgroups which are down-regulated in roots and/or shoots in response to salt stress but also several that are up-regulated (Jang et al. 2004; Alexandersson et al. 2005; Boursiac et al. 2005; Kobae et al. 2006) (summarized in supplemental data Table 2). Overexpression of a rapeseed (*Brassica napus) PIP* (*BnPIP1)* in transgenic tobacco plants has been shown to result in increased tolerance to water stress, while an antisense plant shows reduction in growth and germination and tolerance to water stress (Yu et al. 2005). Response to dehydration and/or salt has been studied in a succulent, the resurrection plant *Craterostigma plantagineum* (able to recover completely from severe dehydration but sensitive to salt), and shows up-regulation of certain transcripts during dehydration, but their down-regulation by salt (Smith-Espinoza et al. 2003) and the salinity-tolerant ice plant (*M. crystallinum*) also shows down-regulation of a number of PIP transcripts during salt stress but up-regulation of a PIP protein (Yamada et al. 1995). In the grapevine, which typically shows sensitivity to salinity but a degree of tolerance to drought, transcripts of a *PIP* aquaporin (*PIP2;1*) were up-regulated under salinity stress but down-regulated under water deficit (Cramer et al. 2007). The data clearly shows that a number of *PIP* subfamily members play significant roles in stress response. However, due to the great diversity observed, further studies, especially those involving individual gene expression and knock outs, would be required to develop a better understanding of the key players. Possibilities such as heteromeric associations and post-translation regulatory controls such as phosphorylation (discussed later) need to be considered also.

Involvement of TIP aquaporins in abiotic stress response: the comparative evidence

Specific TIP isoforms of rice and maize (Table 5) or *A. thaliana* (supplemental data Table 2) also show differential responses to water stress, salinity, or chilling. For example, transcripts of *OsTIP1;1* were down-regulated during chilling (Sakurai et al. 2005) but up-regulated during water stress (300 mM mannitol) and salinity (150 mM NaCl) (Liu et al. 1994). However, studies focusing on the role of TIPs in abiotic stress response have been somewhat limited, which is surprising, considering extent of the changes noted. In the case of resurrection grass (*Eragrostis nindensis*), TIP3;1 was immuno-localized only to desiccated leaves and in small, drought-induced vacuoles, suggesting it might be important in increased water permeability and mobilization of solutes from these vacuoles upon rehydration (Vander Willigen et al. 2004). The salinity-tolerant ice plant shows a TIP protein (MIPF) to be down-regulated in leaves under salt stress (Kirch et al. 2000).

Involvement of PIPs and TIPs based on whole genome expression studies

Many studies have monitored transcript abundance ('digital expression') of the whole genome of a species in response to abiotic stresses, e.g., by the use of microarrays, and MIPs are noted in many of these reports. For example, among all genes in the *A. thaliana* genome, a number of *PIP* and *TIP* transcripts were significantly up-regulated in response to drought (Rizhsky et al. 2004) and up-regulated or down-regulated in response to rehydration after dehydration (Oono et al. 2003) and cold acclimation and deacclimation (Oono et al. 2006). In rice, using DNA microarray encompassing 1,728 transcripts, PIPs were found to be

Table 4 Aromatic/arginine selectivity filter residues in MIP proteins in plants

Plant	NPA motifs		Ar/R selectivity filter residues[a]				MIP protein(s)
	LB	LE	H_2	H_5	LE_1	LE_2	
PIPs							
A. thaliana[b]	NPA	NPA	F	H	T	R	All
Rice[c]	NPA	NPA	F	H	T	R	All
Maize[d]	NPA	NPA	F	H	T	R	All
Barley[e]	NPA	NPA	F	H	T	R	HvPIP1;3, HvPIP1;5, HvPIP1;6, HvPIP2;1, HvPIP2;4
Wheat[f]	NPA	NPA[g]	F	H[g]	T[g]	R[g]	TaPIP1;1, TaPIP1;2, TaPIP2;1[g], TaPIP2;2, TaPIP2;3, TaPIP2;4
TIPs							
A. thaliana[b]	NPA	NPA	H	I	A	V	AtTIP1;1, AtTIP1;2, AtTIP1;3
			H	I	G	R	AtTIP2;1, AtTIP2;2, AtTIP2;3
			H	I	A	R	AtTIP3;1, AtTIP3;2, AtTIP4;1
			N	V	G	C	AtTIP5;1
Rice[c]	NPA	NPA	H	I	A	V	OsTIP1;1, OsTIP1;2
			H	I	G	R	OsTIP2;1, OsTIP2;2
			H	M	A	R	OsTIP3;1
			H	I	A	R	OsTIP3;2, OsTIP4;3
			T	T	A	R	OsTIP4;1
			Q	T	A	R	OsTIP4;2
			Q	V	A	R	OsTIP5;1
Maize[d]	NPA	NPA	H	I	A	V	ZmTIP1;1, ZmTIP1;2
			H	I	G	R	ZmTIP2;1, ZmTIP2;2, ZmTIP2;3
			H	V	A	R	ZmTIP3;1, ZmTIP3;2
			H	S	A	R	ZmTIP4;1, ZmTIP4;2
			Q	S	A	R	ZmTIP4;3
			H	V	A	R	ZmTIP4;4
			Q	V	A	R	ZmTIP5;1
Barley[e]	NPA	NPA	H	I	A	V	HvTIP1;1
			H	I	G	R	HvTIP2;1
			Q	V	A	R	HvTIP5;1
Wheat[f]	NPA	NPA[g]	H	I[g]	A[g]	V[g]	TaTIP1;1[g]
			H	I	G	R	TaTIP2;1, TaTIP2;2, TaTIP2;3
NIPs							
A. thaliana[b]	NPA	NPG	W	V	A	R	AtNIP1;1
			W	V	A	R	AtNIP1;2
		NPA	W	V	A	R	AtNIP2;1

			W	I	A	R	AtNIP3;1
			W	V	A	R	AtNIP4;1
			W	V	A	R	AtNIP4;2
	NPS	NPV	A	I	G	R	AtNIP5;1
	NPA		A	I	A	R	AtNIP6;1
	NPS	NPA	A	V	G	R	AtNIP7;1
Rice[c]	NPA	NPA	W	V	A	R	OsNIP1;1, OsNIP1;2 OsNIP1;3, OsNIP1;4 OsNIP1;5
			G	S	G	R	OsNIP2;1, OsNIP2;2
	NPS	NPV	A	I	G	R	OsNIP3;1
	NPA						
		NPA	A	A	A	R	OsNIP3;3
			A	I	A	R	OsNIP3;3
			A	A	P	R	OsNIP3;4
			I	V	A	R	OsNIP3;5
			C	G	G	R	OsNIP4;1
Maize[d]	NPA	NPA	W	V	A	R	ZmNIP1;1
			G	S	G	R	ZmNIP2;1, ZmNIP2;2, ZmNIP2;3
	NPS	NPV	A	I	G	R	ZmNIP3;1
SIPs							
A. thaliana[b]	NPT	NPA	T	V	P	I	AtSIP1;1
	NPC	NPA	T	F	P	I	AtSIP1;2
	NPL	NPA	S	H	G	A	AtSIP2;1
Rice[c]	NPT	NPA	V	V	P	N	OsSIP1;1
	NPL	NPA	V	H	G	S	OsSIP2;1
Maize[d]	NPT	NPA	T	I	P	N	ZmSIP1;1
			V	V	P	N	ZmSIP1;2
	NPL		V	H	G	S	ZmSIP2;1

[a] The residue H_2 is located in the trans-membrane helix 2 (TMH2), H_5 is in TMH5, and LE_1 and LE_2 are located in the inter-helical loop E.
[b] *A. thaliana* sequences from Johanson et al. (2001)
[c] Rice (*Oryza sativa*) sequences from TIGR Rice Database (http://www.tigr.org/tdb/e2k1/osa1/LocusNameSearch.shtml, last accessed November 2006) (present work; supplementary data Table 1 and Fig. 1)
[d] Maize (*Zea mays*) sequences from Chaumont et al. (2001)
[e] Barley (*Hordeum vulgare*) sequences HvPIP1;3, HvPIP1;5 and HvPIP2;1 from Katsuhara et al. (2002); HvTIP1;1 (g-TIP) from Schunmann and Ougham (1996), HvTIP2;1(TIP1) and HvTIP5;1 (TIP2) from Shirasu et al. (2000), HvPIP1;6 (CO720031) and HvPIP2;4 (AB219525) from Genbank
[f] Wheat (*Triticum aestivum*) sequences from K. Forrest and M. Bhave (unpublished)
[g] Indicates that the full gene sequences for these isoforms are not available yet to identify whether the marked residues are present (work in progress). Pseudogenes not included in the analysis

Table 5 Changes in expression patterns of *MIP* genes in cereals under abiotic stresses

MIP Gene	Abiotic stress	Effect	Tissue	Method used	Reference
Rice *(O. sativa)*					
OsPIP1;1	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsPIP1;1	Osmotic (10% PEG)	↑ of transcripts	Root	Quantitative RT-PCR	(Guo et al. 2006)
WCP-1 (BE607367) (OsPIP1;1)	Salt stress (150 mM)	↓ of transcripts initially after salt stress, ↑ of transcripts later, in salt tolerant variety only	Root	Microarray	(Kawasaki et al. 2001)
RWC1 (AB009665) (OsPIP1;1)	Osmotic (250 mM mannitol)	↓ of transcripts	Root	Northern blot	(Li et al. 2000)
	Salt stress (150 mM) NaCl	↓ of transcripts, quick (3h) recovery	Leaf		
	Chilling (4°C), mannitol (250 mM)	Chilling, pretreatment with mannitol then chilling: ↓ of transcripts	Leaf		
OsPIP1a (AJ224327) (OsPIP1;1)	Drought (no water)	↓ of transcripts, recovered later	Stem	Northern blot	(Malz and Sauter 1999)
OsPIP1;1	Chilling (7°C)	↑ of transcripts during recovery in tolerant variety	Shoot, root	Real time RT-PCR	(Yu et al. 2006)
OsPIP1;2	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsPIP1;2	Osmotic (10% PEG)	↑ of transcripts	Root	Quantitative RT-PCR	(Guo et al. 2006)
OsPIP1;2	Osmotic (20% PEG)	↑ of transcripts in drought-tolerant rice	Root, leaf	RT-PCR	(Lian et al. 2006)
OsPIP1;3	Chilling (4°C)	↑ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
RWC3 (AB029325) (OsPIP1;3)	Osmotic (20% PEG)	↑ of transcripts and protein in drought tolerant rice, ↓ of protein in drought-sensitive rice	Root, leaf	RT-PCR, Immunoblot	(Lian et al. 2004)
WCP-1 (BE607372) (OsPIP1;3)	Salt stress (150 mM)	↑ of transcripts in salt tolerant variety	Root	Microarray	(Kawasaki et al. 2001)
OsPIP1;3	Osmotic (20% PEG)	↑ of transcripts in drought-tolerant rice	Root, leaf	RT-PCR	(Lian et al. 2006)
OsPIP2;1	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsPIP2;1	Osmotic (10% PEG)	↓ of transcripts ↑ of transcripts	Leaf Root	Quantitative RT-PCR	(Guo et al. 2006)
OsPIP2a (AF062393) (OsPIP2;1)	Drought (no water)	↓ of transcripts, recovered later	Stem	Northern blot	(Malz and Sauter 1999)
OsPIP2;1	Osmotic (20% PEG)	↑ of transcripts in drought-tolerant variety	Root	RT-PCR	(Lian et al. 2006)
OsPIP2;1	Chilling (7°C)	↑ of transcripts during recovery in tolerant variety	Shoot, root	Real time RT-PCR	(Yu et al. 2006)
OsPIP2;2	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
WCP-1 (OsPIP2;2)	Salt stress (150 mM)	↑ of transcripts in salt tolerant variety	Root	Microarray	(Kawasaki et al. 2001)
OsPIP2;3	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsPIP2;2[a]	Osmotic (10% PEG)	↑ of transcripts	Root	Quantitative RT-PCR	(Guo et al. 2006)

Table 5 (continued)

MIP Gene	Abiotic stress	Effect	Tissue	Method used	Reference
OsPIP2;3[a]			Leaf, root		
OsPIP2;4			Root		
OsPIP2;5	Osmotic (20% PEG)	↑ of transcripts in drought-tolerant rice	Root	RT-PCR	(Lian et al. 2006)
OsPIP2;4 OsPIP2;5 OsPIP2;6	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsPIP2;5[a]	Osmotic (10% PEG)	↓ of transcripts	Leaf	Quantitative RT-PCR	(Guo et al. 2006)
OsPIP2;6[a]		↑ of transcripts	Root		
OsPIP2;7[a]		↑ of transcripts	Root		
OsPIP2;7	Chilling (7°C)	↑ of transcripts during recovery in tolerant variety	Shoot	Real time RT-PCR	(Yu et al. 2006)
OsTIP1;1	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
OsTIP1;1 (rTIP) (D25534)	Salt (150 mM NaCl), drought (300 mM mannitol)	↑ of transcripts	Shoot, root	Northern blot	(Liu et al. 1994)
OsTIP2;2	Chilling (4°C)	↓ of transcripts	Root	Semiquantitative RT-PCR	(Sakurai et al. 2005)
Barley *(Hordeum vulgare)*					
HvPIP2;1	Salt (200 mM NaCl)	↓ of transcripts, ↓ of protein ↑ of transcripts	Root Shoot	RT-PCR, western RT-PCR	(Katsuhara et al. 2002)
Maize *(Zea mays)*					
ZmPIP1	Chilling (5°C)	↑ of proteins	Root	immunoblot	(Aroca et al. 2005)
ZmPIP1;1		↓ of transcripts		Northern blot	
ZmPIP1;1	Salt (100 mM NaCl)	↑ of transcripts	Root	DNA array hybridization, real-time RT-PCR	(Zhu et al. 2005)
ZmPIP1;2	Salt (200 mM NaCl)	↓ of transcripts			
ZmPIP1;2 ZmPIP1;3 ZmPIP1;4	Chilling (5°C)	↓ of transcripts	Root	Northern blot	(Aroca et al. 2005)
ZmPIP1;5	Salt (100 mM NaCl) Salt (200 mM NaCl)	↑ of transcripts ↓ of transcripts	Root	DNA array hybridization, real-time RT-PCR	(Zhu et al. 2005)
ZmPIP1;5	Chilling (5°C)	↓ of transcripts	Root	Northern blot	(Aroca et al. 2005)
ZmPIP1;6	Salt (200 mM NaCl)	↓ of transcripts	Root	DNA array hybridization, real-time RT-PCR	(Zhu et al. 2005)
ZmPIP1;6	Chilling (5°C)	↓ of transcripts	Root	Northern blot	(Aroca et al. 2005)
ZmPIP2		↑ of proteins in chilling-tolerant cultivar only		Immunoblot	
ZmPIP2;1 ZmPIP2;2 ZmPIP2;3 ZmPIP2;4 ZmPIP2;5 ZmPIP2;6		↓ of transcripts		Northern blot	
ZmPIP2;4	Salt (100 mM NaCl) Salt (200 mM NaCl)	↑ of transcripts ↓ of transcripts	Root	DNA array hybridization, real-time RT-PCR	(Zhu et al. 2005)
ZmPIP2;5 ZmPIP2;6	Salt (200 mM NaCl)	↓ of transcripts			

Table 5 (continued)

MIP Gene	Abiotic stress	Effect	Tissue	Method used	Reference
ZmTIP1;1 ZmTIP1;2 ZmTIP2;1 ZmTIP2;4 ZmTIP2;2	Salt (200 mM NaCl)	↓ of transcripts	Root	DNA array hybridization, real-time RT-PCR	(Zhu et al. 2005)
ZmTIP2;3	Salt (37 mM NaCl), osmotic (8% PEG)	↑ of transcripts	Root	Northern blot	(Lopez et al. 2004)
Durum wheat *(Triticum turgidum)* and bread wheat *(Triticum aestivum* L.*)*					
PIP1	Osmotic stress (250 mM kg^{-1} sorbitol;	No change	Root	Immunoblot	(Morillon and Lassalles 2002)
PIP2	Salt stress (125 mM NaCl)				

↑ indicates up-regulation, ↓ indicates down-regulation

[a] These sequences are identical to sequences designated as OsPIP2;3; OsPIP2;2; OsPIP2;6, OsPIP2;7 and OsPIP2;8, respectively, by Sakurai et al. (2005).

affected by salinity (Kawasaki et al. 2001), and in wheat, *PIP* genes were found to be more abundant in cDNA libraries constructed from plants exposed to cold, salt, and dehydration stresses compared to those from unstressed ones (Houde et al. 2006). Microarrays of grapevine total RNA expressed under salinity and water deficit conditions also identified a number of genes, which were differentially expressed, including the MIPs (Cramer et al. 2007).

Comparative studies on the *MIP* gene expression such as those outlined above have provided substantial new information towards an understanding of differential expression of different aquaporins in relation to stress conditions. Specific, universal physiological changes brought on by the change in aquaporin expression have not been reported yet. However, it would seem likely that reduced aquaporin expression and/or function in the plasma membrane would result in prevention of loss of water to the hypertonic media outside of the plant (e.g., to saline soil), or reduced aquaporin function in the tonoplast would prevent loss of water from the vacuole and help maintain turgidity. It would also be interesting to see if the variable ar/R selectivity filter residues of certain MIP isoforms would allow transport of osmolyte molecules such as certain sugars or amino acids, considering the range of nonaqueous substrates transported by some MIPs.

It has also been difficult to identify a concerted pattern or specific gene type(s) of most significance, as it appears that the degree of response and the responding gene(s) vary depending on the nature of stress, its degree, and the type of plant and its tissue under investigation. This could be due to a number of factors such as (1) differences in expression of different aquaporin genes under un-induced conditions; (2) variations in functions/efficiency of different isoforms (e.g., due to differences in amino acid sequence including in the ar/R filter or NPA box residues); (3) some isoforms may be serving a "house-keeping" function of water transport, while others might be specialized for stress response; (4) regulation by gating or posttranscriptional factors. Some multigene families are known to have members with specialized functions or tissue or developmental stage specific expression (reviewed in Zhang 2003). It would thus be important to investigate if MIPs corresponding to all *PIP* and *TIP* genes in a species are actually expressed and membrane-integrated, to truly understand the roles of various isoforms and the significance of the up- or down-regulation of their transcripts and/or proteins in response to stress.

Limitations of the comparative evidence and need for concerted approaches

In spite of the significant commonalities seen in the salt or drought stress responses of a number of plants pointing to most likely candidates (Table 5), caution needs to be exercised in extrapolating the results and conclusively identifying the PIP or TIP isoforms that might be most important in a given plant or universally in every plant. One factor could be possible variations in the number and subclasses of *PIP* genes in various species and variations in nomenclature, as studies on MIP gene families are incomplete for a majority of plants except rice and *Arabidopsis*, with the cDNA and some genomic data being available in maize. Furthermore, application of "drought" in the laboratory can mean lack of water or the use of osmotic

stressor such as PEG or mannitol (Table 5), but these treatments could result in different responses, as observed in the case of *OsPIP1;1* transcripts. Use of different concentrations of test treatments must also be considered, as thresholds may exist; for example, different concentrations of NaCl result in up- or down-regulation of *ZmPIP1;5* transcripts, respectively, in maize (Zhu et al. 2005). Different tissue types can also differ in responses; e.g., varying transcript levels of rice *OsPIP2;1* in root vs leaf (see above) (Guo et al. 2006), and many tissues need to be investigated for interpretation of biological roles. Furthermore, the time points (e.g., hours vs days) of observations might be critical, as different isoforms may have different response times, e.g., reduction in TIP1 protein in *A. thaliana* occurs after 24 h in response to salt, while that in PIP1 occurs in 30 min (Boursiac et al. 2005). Another important issue to consider is that biotic stresses in "real life" situations often occur simultaneously, e.g., drought would be often accompanied by high temperatures in natural environments, compared to constant temperatures set in plant growth chambers/greenhouses. One study of *A. thaliana* exposed to simultaneous drought and heat stress shows that levels of certain aquaporins (e.g., AtPIP2;5) were elevated only during the *combination of stresses* but not individual stress (Rizhsky et al. 2004), confirming possible interactions of key players. A few studies have taken such a combinational approach (possibly due to logistical reasons), as seen from the comparative studies discussed earlier, but it appears essential for developing a better picture for field applications. Finally, many studies have focussed on transcript or protein abundance; however, differences in these may not necessarily reflect protein activity (see below); therefore, functional assays are required to fully assess isoform-specific MIP responses to abiotic stress.

The transcriptional and posttranscriptional regulation and membrane distribution of aquaporins

In spite of the aforementioned numerous correlative studies, the precise mechanism whereby aquaporins respond *in planta* to the demands of the abiotic stress remain unclear. Further work is particularly required to address questions such as the physiological mechanisms whereby the abiotic stresses regulate aquaporin expression, whether mRNA levels directly correlate to protein levels, whether the protein levels correlate directly with *functional* protein levels, and whether the aquaporins are the end-game in abiotic stress response and/or are they part of a signaling pathway.

Having established the effects of abiotic stress on *MIP* gene and protein expression, one obvious direction to investigate is *how* the gene expression is altered by variations in the promoter regions. In the few such studies, fusions of the putative promoters of two *PIP* genes from ice plant with the *GUS* (beta-glucuronidase) reporter gene were found to direct individual, tissue-specific expression patterns (Yamada et al. 1997; Yamada and Bohnert 2000), fusions of various lengths of upstream regions of root-specific TIP (*TobRB7*) of tobacco with GUS identified a specific upstream region (−299 to −630) for root-specific expression and a negative regulatory element (Yamamoto et al. 1991), and a similar region (−650 bp) in a carrot TIP (DcRB7) was shown to direct root-specific and drought-inducible expression (Liu et al. 2004). In studies emerging on identification of potential abiotic stress-responsive elements, a *cis*-acting element (ACTCAT) has been found within a 1,000-bp upstream region of many rehydration-inducible *A. thaliana* genes, including the motif TG*ACTCAT*TT, thought to be involved in response to rehydration (Oono et al. 2003). The promoter regions of aquaporins also need to be examined for other abiotic stress-responsive elements reported extensively in plants, e.g., the ABA-responsive element (ABRE) and dehydration-responsive element (DRE) (reviewed in Ingram and Bartels 1996). Such information will provide not only a better understanding of the mechanisms of responses to osmotic stress, but also potentially help to identify the genetically better-suited cultivars.

Factors that regulate aquaporin protein activity post-translationally under osmotic stress need to be considered, and phosphorylation has been identified as such a mechanism (Johansson et al. 1998); reviewed in (Chaumont et al. 2005; Hedfalk et al. 2006). As shown for a PIP2 (PM28) from spinach, although its protein and transcript levels were not affected by drought, increase in the osmolarity caused a decrease in phosphorylation (Johansson et al. 1996), and mutant isoforms of PM28 with the serine phosphorylation sites knocked out showed decreased water permeability (Johansson et al. 1998). This and other regulatory and gating mechanisms of aquaporins and their impact thus need to be considered.

A number of studies provide credence to membrane trafficking also being an important aspect in regulation of plant aquaporins during abiotic stress, possibly through physically moving these proteins to alternative membranes, and hence, preventing water loss. Studies on plant species adapted to osmotic stress, for example, Crassulacean acid metabolism (CAM) plants, which are succulent and drought-tolerant, may provide more information. For example, a low level of a TIP protein was identified in *Kalanchoë daigremontiana* vacuolar membrane isolated from leaf tissue (Maeshima et al. 1994), and expression of a number of PIP and TIP isoforms in *Graptopetalum paraguayense* was found to be low in plasma membrane and tonoplast leaf fractions, and water permeabilities for corresponding vesicles were absent or low, respectively

(Ohshima et al. 2001). The immuno-localization of a TIP only to the desiccated leaves of the resurrection grass (*Eragrostis nindensis*) in small, drought-induced vacuoles in the bundle sheath cells suggests its potential role in increased water permeability and mobilization of solutes from the small vacuoles upon rehydration (Vander Willigen et al. 2004). In another study, a TIP from ice plant was found to be up-regulated during osmotic stress but also redistributed from the tonoplast to other membrane fractions including Golgi, endoplasmic reticulum, prevacuolar compartments, and the vacuolar-sorting receptor BP80 (Vera-Estrella et al. 2004). Thus, exposure to drought or salt conditions appears to regulate the number of aquaporins (or MIPs) in cellular membranes and alter their water permeability to help reduce water loss. Membrane protein studies, therefore, would help fully understand aquaporin functions.

Pleiotropic effects of control of water movement by plant MIPs

As a result of the water uptake and cell turgor functions discussed above, plant MIPs are thought to be involved in specialized functions such as regulation of cell expansion (Ludevid et al. 1992), stomatal opening (Sarda et al. 1997), differentiation (Kaldenhoff et al. 1995), leaf unfolding (Siefritz et al. 2004), and seed germination (Gao et al. 1999). Their potential roles in two important issues for cereal productivity and quality, i.e., seed dormancy and preharvest sprouting, are of particular interest. During germination, the transport of water across plasma membranes after imbibition is essential for initiation of metabolism of the storage nutrients for cell proliferation and growth (Gao et al. 1999), and several studies suggest that aquaporins might have roles in this process. Several MIPs have been localized to the seed only, e.g., α-TIP from *A. thaliana* (Höfte et al. 1992; Johnson et al. 1989), which is progressively replaced by γ-TIP during germination, suggesting that each may have different roles (Maurel et al. 1997a). In other studies, the *BnPIP1* transcripts were only found in canola seeds shortly after water imbibition (Gao et al. 1999). Treatment of aged pea seeds with an aquaporin inhibitor reduced the rate of seed hydration (Veselova et al. 2003), and mercury reduced the speed of germination. Different isoforms were expressed in *Arabidposis* seeds before and after germination (Vander Willigen et al. 2006). Preharvest sprouting (PHS), i.e., seed germination under untimely rainy conditions during seed maturation, results in reduced yield and grain quality and is a serious issue to the wheat grower. It has been suggested that the key to preventing preharvest sprouting lies in the selection of lines that do not express aquaporins rapidly when seeds are exposed to wet conditions (Meristem Land and Sciences 2002). This would thus comprise an important area in study of aquaporins, not just in cultivars but also in exotic germplasm such as synthetic wheats.

Other transport substrates of plant MIPs of interest

Some of the plant MIPs have been shown to be selective for the transport of other hydrophobic molecules (reviewed in Kaldenhoff and Fischer 2006), although the *in planta* significance of some of these substrates is unclear. MIPs involved in CO_2 transport are of interest due to relevance to photosynthetic and plant growth processes. Tobacco NtAP1, a membrane CO_2 pore, has shown significant function in photosynthesis and stomatal opening (Uehlein et al. 2003) and its over-expression increases membrane permeability for CO_2 and results in heightened leaf growth. Barley HvPIP2;1 over-expressed in rice also shows increased internal conductance for CO_2 diffusion inside rice leaves (Hanba et al. 2004). MIPs that can transport both water and glycerol molecules have been discovered in the PIP, TIP, and NIP subfamilies (reviewed in Tyerman et al. 2002). A high degree of urea transport (and little water transport ability) has been detected for ZmPIP1;5b from maize (Gaspar et al. 2003), and AtTIP2;1 of *Arabidopsis* (Liu et al. 2003), and the localization of the former in root tissue may facilitate a urea-transporting function. Numerous NIPs have been reported in legumes and are thought to be involved in regulating the flux of water, ammonia, and other solutes between the plant and the bacterial symbionts (reviewed in Kaldenhoff and Fischer 2006). Ammonia transporters have been reported in nonleguminous plants also, for example, three wheat TIP2 proteins including TaTIP2;1 which show ammonia transport in *Xenopus* oocytes (Jahn et al. 2004) and two *A. thaliana* TIP2 proteins (AtTIP2;1 and AtTIP2;3), which possibly mediate the extracytosolic transport of methyl-NH_2 and NH_3 across the tonoplast (Loque et al. 2005). A NIP from rice (OsNIP2;2) (Table 1) is reported to show silicic acid permeability and localizes to the plasma membrane of the exodermis and endodermis of roots (Ma et al. 2006); the significance of this function is unclear.

Involvement of other genes in response to osmotic stress

Finally, abiotic stress responses in plants are described as complex traits, as they are multigenic in nature (see Bartels and Sunkar 2005 for a review). Other well-known physiological mechanisms for salinity or drought tolerance include, for example, osmoprotectant sugars such as trehalose or mannitol or modified amino acids such as glycine-betaine or mechanisms such as salt exclusion

(reviewed in Bartels and Sunkar 2005), and a number of other QTLs and single genes are potentially involved in drought (Zeng et al. 2006) and salt (Sahi et al. 2006) tolerance in rice. Thus, while the numerous results presented earlier clearly establish that MIPs play a key role in water uptake and distribution in the plant and thus contribute significantly to these traits, they may not be *solely* responsible. It would thus be most beneficial to consider a "barcode" approach, which includes assessment of at least some of the other protective biochemicals (such as the osmoprotectants) or other key genes, in addition to identifying the appropriate genotypes for aquaporins, to select the best possible germplasm for cultivation in saline or drought-affected areas.

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