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Review

The role of aquaporins in cellular and whole plant water balance

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Abstract

Aquaporins are water channel proteins belonging to the major intrinsic protein (MIP) superfamily of membrane proteins. More than 150 MIPs have been identified in organisms ranging from bacteria to animals and plants. In plants, aquaporins are present in the plasma membrane and in the vacuolar membrane where they are abundant constituents. Functional studies of aquaporins have hitherto mainly been performed by heterologous expression in *Xenopus* oocytes. A main issue is now to understand their role in the plant, where they are likely to be important both at the cellular and at the whole plant level. Plants contain a large number of aquaporin isoforms with distinct cell type- and tissue-specific expression patterns. Some of these are constitutively expressed, whereas the expression of others is regulated in response to environmental factors, such as drought and salinity. At the protein level, regulation of water transport activity by phosphorylation has been reported for some aquaporins. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Water is the universal solvent and the most abundant molecule in all living tissue. Growing plants constantly absorb and lose water. Water is absorbed by the roots and evaporates through the stomatal pores in the leaves in the transpiration stream. On a warm, dry day, the leaf may exchange the equivalent of all of its water content in 1 h. Long-distance water transport is carried out in the vascular tissues, by the xylem and the phloem, where water is transported by bulk flow and membrane barriers are in most cases non-existent. In contrast, short-distance transport and transport in non-vascular tissues fre-

quently involve transport across membranes. Transmembrane water transport occurs by diffusion through the lipid bilayer and by transport through proteinaceous water channels, aquaporins. Many events require relatively rapid translocation of large volumes of water across membranes, for example, cell elongation and stomatal movements. Water transport across the lipid bilayer of cellular membranes is not efficient enough to account for these fluxes. Rapid transmembrane water flow is possible due to the presence of aquaporins. Aquaporins are channel-forming membrane proteins with the extraordinary ability to combine a high flux with a high specificity for water. Their physiological importance is revealed by their widespread occurrence in plants, animals, fungi, and bacteria.

Two processes where transmembrane water flow in plants occurs may be distinguished; the fluxes neces-

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sary to fulfill the needs of the individual cell in order to change its size, turgor and/or osmolarity, and the transcellular flow, where water is moving through non-vascular tissues.

Most mature plant cells are characterized by a single vacuole, occupying most of the intracellular space, and a thin layer of cytosol between the plasma membrane and the vacuolar membrane. The composition of the cytosol has to be tightly controlled in order to maintain optimal conditions for the various metabolic activities. Aquaporins in the plasma membrane and the vacuolar membrane, together with transporters of ions and other osmolytes, are likely to be crucial in the maintaining of the proper cytosolic osmolarity. Aquaporins have also been suggested to play an essential role in extension growth. Plants continue to grow throughout their lifetime, and to sustain growth, an influx of water into the expanding cells is necessary. Guard cells and cells within the pulvinus present other examples of sites where efficient transmembrane water transport is essential. These cells exert their function by swelling and shrinking. The guard cells control the stomatal aperture, while the motor cells of the pulvinus control leaf movements. Ion fluxes followed by massive water fluxes across the plasma membrane of these cells produce their swelling and shrinking behavior.

The movement of water through the plant tissues to or from the xylem and phloem may occur by one of, or a combination of, the following pathways: (1) the apoplastic pathway; (2) the symplastic pathway; and (3) the transcellular pathway. In the symplastic pathway, water is transported in the cytoplasmic continuum of adjacent cells connected via plasmodesmata, while the transcellular pathway involves transport across the plasma membrane and the vacuolar membrane. Experiments where the water flow in the apoplast has been compared to the cell-to-cell flow indicate that the preferred pathway differs between species, organs, developmental stage, and environmental parameters. In the root, the Casparian strip of the endodermal cells severely restricts the radial water movement in the apoplast. A Casparian strip may also be formed by the exodermal cells, located just inside the epidermis in the root. Thus, at these sites, water transported between the soil and the xylem vessels is forced to transverse plasma membranes.

Aquaporins are channels, i.e. passive transporters where water always moves down its water potential gradient. Still, the plant has mechanisms to control the cellular water status. The rate of transmembrane water flux may be controlled by changing the abundance or the activity of the aquaporins. In addition, the plant cell may alter the water potential gradient by accumulating or extruding osmolytes in order to favor an influx or efflux of water.

2. Identification and characterization of aquaporins

2.1. Background

Transmembrane water flow in plants occurs primarily in response to a water potential gradient, i.e. differences in osmotic and hydrostatic pressure across the membrane. The buildup of a positive internal hydrostatic pressure, turgor, is possible because of the rigid cell wall. The rate of water flow is determined by the magnitude of the water potential gradient and the permeability of the membrane. The osmotic permeability coefficient, P_f , describes the total water permeability of the membrane. The diffusional permeability coefficient, P_d , on the other hand, describes the permeability due to diffusion, that is, the movement of water molecules across the membrane which occurs even in the absence of a gradient. P_f and P_d are intrinsic properties of each membrane. In order to distinguish between lipid- and channel-mediated water flow, the values of P_f and P_d can be used. In a membrane where the water transport is channel-mediated, P_f exceeds P_d , whereas these values are equal if water transport occurs via diffusion through the lipid bilayer only. Another useful parameter is the Arrhenius activation energy (E_a). E_a reflects the temperature dependence of the flow rate. In lipid-mediated water transport, E_a is high. Water transport mediated by channels, on the other hand, exhibits a low E_a .

Until recently, it was generally assumed that in plants, water moved solely by diffusion across the lipid bilayer and since the water molecule is small and uncharged, this was not considered extraordinary. A few studies, however, indicated the presence of water-transporting pores or channels in plant membranes [1]. In animals, the situation was slightly

different. It had been observed that the plasma membrane of certain cells, such as erythrocytes and the cells of renal epithelia, transported water at rates much too high to be explained by diffusion only [2]. The existence of a protein acting as a water channel was suspected, but could not be proven until 1992 when Preston et al. demonstrated the specific water transport activity of CHIP28 (channel-forming integral protein of 28 kDa; now renamed aquaporin-1, AQP1), an integral, erythrocyte plasma membrane protein [3]. Sequencing of AQP1 showed its homology to the major intrinsic protein (MIP) in the plasma membrane of eye lens fiber cells. The identification of AQP1 was a breakthrough that rapidly led to the identification of several other aquaporins and aquaporin homologs in animals, plants, yeast, and bacteria.

Aquaporins are sequence-related water channel proteins and belong to the MIP superfamily of integral membrane proteins. Hitherto, more than 150 partial or full-length clones encoding MIP family members have been isolated. Functionally, MIPs may be divided into two groups: aquaporins, and transporters of glycerol and other small neutral molecules. It has been suggested that all MIP family members have evolved from two bacterial paralogs, an aquaporin and a glycerol facilitator [4].

The MIP family members generally have a similar molecular weight of 23–31 kDa, six putative membrane spanning regions, and cytosolic N- and C-termini (Fig. 1). One exception to this is the glycerol facilitator Fps1 of *Saccharomyces cerevisiae* which has a higher molecular weight due to extended cytosolic N- and C-termini.

In mammals, the water permeability of the plasma membrane differs widely between cells in different tissues. This is especially true within the kidney, an organ which has been extensively studied with respect to water transport. In addition to cell types with either very high or very low water permeability, the kidney also contains cell types in which the water permeability is strikingly increased within minutes after treatment with the antidiuretic hormone vasopressin. An explanation to these characteristics was offered by the discovery of the aquaporins. Hitherto, ten mammalian aquaporins have been identified, many of them by homology cloning. Demonstration of their water transport capacity was performed by

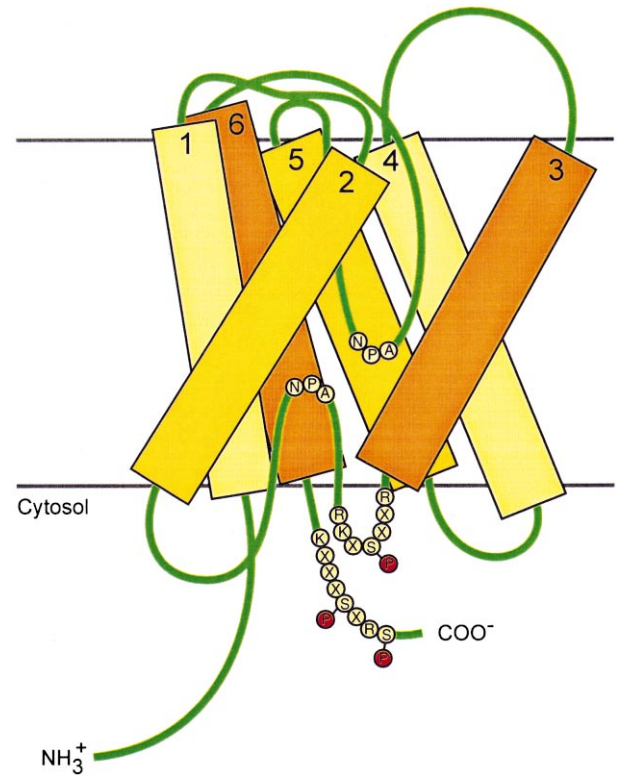


Fig. 1. Putative aquaporin structure. The N- and C-terminal regions are located at the cytosolic side of the membrane both for plasma membrane intrinsic proteins (PIPs) and for tonoplast intrinsic proteins (TIPs), while the other side is either exposed to the apoplast (PIPs) or to the vacuolar compartment (TIPs). The second and fifth loops contain the conserved NPA-boxes. According to the hourglass model [26], these relatively hydrophobic loops penetrate the membrane from each side creating a seventh transmembrane structure. Indicated are also the consensus phosphorylation motifs and the serine residues that in certain aquaporins are known to be phosphorylated either in vitro using membrane vesicles, in the oocyte system, or in planta. The colouring of the membrane helices reflects the internal homology. The N-terminal half of the protein is homologous to the C-terminal half, i.e. helix 1 corresponds to helix 4, etc. The model is based on the structure of AQP1 [106].

expression in oocytes (see below). Their expression patterns correlate well with the observed values of membrane water permeabilities. The mammalian aquaporins are named AQP1–AQP9 (reviewed in [5,6]). In addition, MIP was recently shown to transport water and is therefore sometimes referred to as AQP0 [7]. AQP1 is abundant in many tissues and cell types, e.g. erythrocytes, kidney, lung, and eye. AQP2 is specifically expressed in the collecting duct of the kidney and is responsible for the increased water

permeability of the plasma membrane in response to vasopressin.

2.2. Identification of plant aquaporins

The identification of aquaporins in plants was not mainly a consequence of an active search for a protein with water transport activity. Rather, integral membrane proteins belonging to the MIP family had been identified due to their abundance. A clue to their function came when AQP1 was shown to specifically transport water. The first demonstration of a plant aquaporin was in 1993 when a tonoplast intrinsic protein, γ -TIP in *Arabidopsis*, was shown to exhibit water transport activity when expressed in *Xenopus* oocytes [8]. γ -TIP was isolated by homology cloning using a cDNA corresponding to α -TIP, a seed-specific tonoplast integral protein, which constitutes about 2% of the total extractable protein of bean cotyledons and was identified due to its abundance [9]. The first plasma membrane aquaporins in plants were identified using a polyclonal antiserum raised against total integral plasma membrane proteins of *Arabidopsis* roots. Immunoscreening of an *Arabidopsis* root cDNA library resulted in several positive clones. Many of these were found to encode proteins of about 30 kDa, which were shown to be aquaporins [10].

So far, aquaporins have been found in the plasma membrane and in the tonoplast (vacuolar membrane). The plasma membrane aquaporins and aquaporin homologs are called PIPs for plasma membrane intrinsic proteins. The tonoplast aquaporins and aquaporin homologs are called tonoplast intrinsic proteins (TIPs). PIPs and TIPs form two distinct phylogenetic groups (Fig. 2) and for these two groups, the membrane localization may be determined from the sequence. A third phylogenetic group consists of NOD26, found in the peribacteroid membrane of soybean nodules, NLM1 of *Arabidopsis*, and a few other homologs (Fig. 2). Except for NOD26, the subcellular location for members of this third group is not known.

Water transport activity has been demonstrated for some PIPs and TIPs, e.g. in *Arabidopsis* (seven PIPs and three TIPs [8,10–13]), tobacco (one PIP and two TIPs [14–16]), ice plant (two PIPs [17]), kidney bean (one TIP [18]), sunflower (two TIPs [19]), spi-

nach (one PIP [20]) and maize (one TIP [21]). NOD26 and NLM1 have also been shown to be aquaporins, although NOD26 is not water-specific [13,22,23]. The aquaporin function has been demonstrated by expressing the proteins in *Xenopus* oocytes. In this method, cDNA encoding the putative aquaporin is in vitro transcribed into RNA which is injected into oocytes. After 2–3 days of incubation to allow the protein to be synthesized and inserted into the plasma membrane, the oocytes are transferred to a hypotonic solution, placed under a microscope connected to a videocamera. If the oocyte membrane contains an aquaporin, water will rapidly enter the oocyte, resulting in swelling and eventually bursting. The rate of swelling of the oocytes injected with the putative aquaporin is compared to that of control oocytes, i.e. water-injected or uninjected oocytes. Aquaporins induce a 5–20-fold increase in swelling rate. The osmotic water permeability may be directly calculated from the swelling rate.

In addition, a multitude of aquaporin homologs, whose function has not been determined, have been identified. Most of these will probably turn out to function as aquaporins, but so far not enough is known about water transport specificity to determine function based on sequence data alone.

2.3. Aquaporin structure

Aquaporins have six transmembrane α -helices connected by five short loops with the N- and C-termini located in the cytosol (Fig. 1). This topology, originally proposed based on hydropathy analysis, was confirmed for AQP1 by vectorial proteolysis [24] and for AQP2 by insertion of glycosylation sites [25]. The primary sequence of aquaporins exhibits an internal homology, so that the N-terminal half is homologous to the C-terminal half of the protein. The two halves are inversely located in the membrane. Two amino acid motifs, the NPA boxes, so called after the single letter code for the central amino acids in the motif, are highly conserved. The NPA boxes and adjacent residues are thought to be essential for water transport activity [26]. The first NPA box is located in the first cytosolic loop and the second NPA box is located in the third extracellular (or vacuolar for TIPs) loop. According to a widely accepted model, the hourglass model, these two rela-

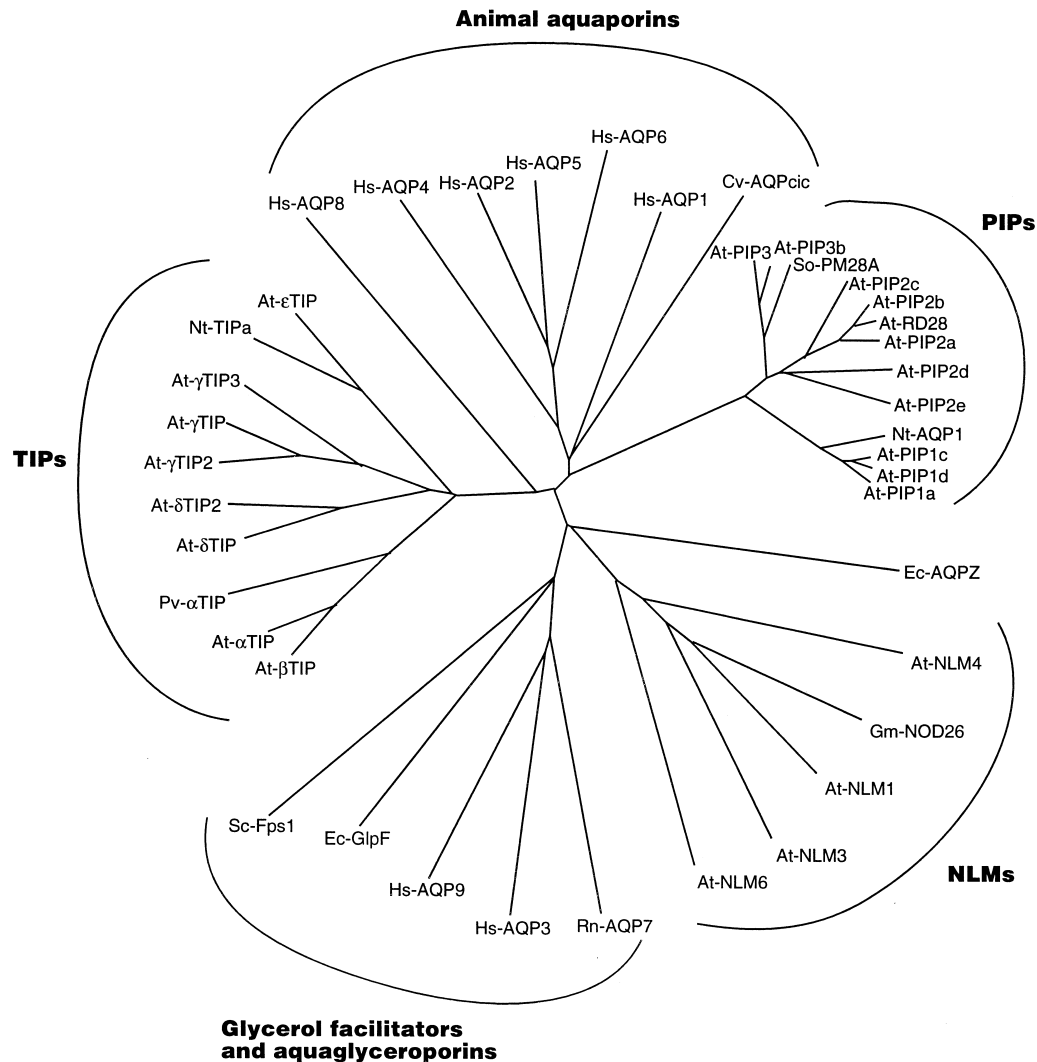


Fig. 2. Phylogenetic analysis of members of the major intrinsic protein (MIP) family from plants, mammals, insects, yeast and bacteria. The glycerol facilitators of *E. coli* (Ec-GlpF) and yeast (Sc-Fps1) transport glycerol and the mammalian aquaglyceroporins AQP3, AQP7 and AQP9 transport glycerol as well as water. NOD26 of the soybean nodule peribacteroid membrane, belonging to the NLM (NOD26-like MIP) subfamily, as well as Nt-AQP1 and Nt-TIPa, have been reported to be permeable to glycerol or to other small uncharged solutes as well as to water. The other MIP family members are either aquaporins or their specificity is unknown. Ec-AQPZ is the *E. coli* aquaporin. Four subfamilies ($\alpha+\beta$, γ , δ , ϵ) can be discerned for the plant tonoplast intrinsic proteins (TIPs), whereas the plant plasma membrane intrinsic proteins (PIPs) fall into two groups, the PIP1 and the PIP2 subfamilies. The PIP3 group is a subgroup within the PIP2 subfamily or may be regarded as a separate subfamily. Several unpublished MIPs were found by Blast searches in sequences generated in the *Arabidopsis* genome sequencing project. These proteins have been named according to their position in the phylogenetic tree. The protein accession numbers are given in parentheses for unpublished protein sequences; At-PIP1d (CAA20461); At-PIP2d (CAB41102), At-PIP2e (AAC79629), At-PIP3b (AAC64216), At- δ TIP2 (CAB10515), At- γ TIP3 (AAC62778), At- ϵ TIP (AAC42249), At-NLM3 (AAC27424), At-NLM6 (CAB39791). NLM4 was found in non-annotated genomic sequence, nucleotide accession number AB016873. At, *Arabidopsis thaliana*; Cv, *Cicadella viridis*; Ec, *Escherichia coli*; Gm, *Glycine max*; Hs, *Homo sapiens*; Nt, *Nicotiana tabacum*; Pv, *Phaseolus vulgaris*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; So, *Spinacia oleracea*. The sequences were aligned using Clustal W [104] and the tree was constructed using PAUP [105].

tively hydrophobic loops dip into the membrane and line the aqueous pore [26]. Recently, the three-dimensional structure of AQP1 has been determined at 6–7 Å resolution by electron crystallographic methods [27–29]. The structural data reveal six tilted transmembrane α -helices surrounding a central density which is thought to be composed of the two NPA box-containing loops, thereby supporting the hour-glass model (Fig. 1). Considering the high homology between aquaporins, especially in the hydrophobic regions, it is reasonable to assume that all aquaporins have a similar structure.

In the membrane, AQP1 forms tetramers which are thought to be comprised of one glycosylated and three non-glycosylated monomers. The tetrameric structure was first suggested based on studies using gel filtration and analytical ultracentrifugation, which resulted in a molecular weight of the multisubunit complex consistent with a tetramer [30]. This was confirmed by various analyses using electron microscopy where four-lobed particles could be discerned [31,32]. MIP and AQPcic, an insect aquaporin, likewise form tetramers, as determined by hydrodynamic and crystallization analysis [33–36]. This tetrameric organization may be a common feature of aquaporins and their well-documented tendency to aggregate *in vitro* may reflect their oligomerization *in vivo*. The tetrameric assembly of AQP1 monomers is likely to be important for the folding, stability, and/or targeting to the plasma membrane [26]. Nevertheless, the monomer is the functional unit, transporting water independently of the other subunits in the complex. When mercury-sensitive and -insensitive forms of AQP1 (see below) were co-expressed in oocytes, no signs of cooperativity were detected [37,38]. In another report, the functional independence of the monomers was concluded from the absence of dominant effects when inactive and active forms of AQP1 were co-expressed in oocytes [26]. In agreement with this, radiation inactivation of aquaporins in kidney membranes predicted a functional size of approximately 30 kDa [39].

Many of the aquaporins are inhibited by mercurial reagents, which act by binding to cysteine residues and thereby presumably blocking the aqueous pathway physically. The mercury-sensitive cysteine has been determined for some aquaporins and its position near one of the NPA boxes in AQP1 and AQP2

is close to the aqueous pore predicted by the hour-glass model [25,37]. In *Arabidopsis* γ -TIP and δ -TIP, the mercury sensitive cysteine is located within the third transmembrane α -helix [12]. In a helical wheel plot of this region, an amphipathic helix with the cysteine residue in the hydrophilic part was observed for both γ -TIP and δ -TIP, suggesting that this part of the helix faces the aqueous pore.

2.4. Aquaporin specificity

The most thoroughly characterized glycerol transporter, GlpF in *Escherichia coli*, transports glycerol, but not water [8,40]. Most of the investigated aquaporins are highly specific for water, excluding ions and small neutral solutes, such as urea and glycerol [3,8]. The basis for this specificity is thought to be a narrow aqueous pore, excluding molecules larger than water, combined with the presence of charged residues at the entrances of the pore, repelling ions. The exclusion of ions is essential since a flow of protons through the aquaporins would make the establishment of a proton gradient impossible, especially considering the abundance of aquaporins. The specificity of plant aquaporins has so far only been investigated in a few reports. High specificity for water has been demonstrated for kidney bean α -TIP, and the *Arabidopsis* aquaporins γ -TIP, PIP1a, and PIP2b [8,41,42]. The first plant aquaporin reported to have a broader specificity was NOD26, which, in addition to water, also permits the passage of glycerol and formamide, but not urea [22]. In tobacco, two aquaporins not specific for water were recently identified. Nt-AQP1, residing in the plasma membrane, transports water, glycerol and urea [15,43], while the tonoplast aquaporin Nt-TIPa transports urea, and, to a lesser extent, water and glycerol [16]. The endogenous substrates for these MIP homologs have not been identified. The specificity has been studied in much more detail for mammalian aquaporins. Although some contradictory results have been presented, most reports indicate that AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8 are specific for water. The remaining aquaporins, AQP3, AQP7, and AQP9 are termed aquaglyceroporins and also transport glycerol and/or other small uncharged molecules [6]. These aquaglyceroporins cluster into one group in the phylogenetic

Cv-AQPcic	HWV YW AGPIV	228
Ec-GlpF	FLV PL FGPIV	242
Sc-Fps1	FWV PM VGPF1	316
So-PM28A	QWI FW VGPF1	251
At-PIP1a	HWV FW VGPF1	264
Nt-AQP1	HWI FW VGPF1	265
Nt-TIPa	HWV YW VGPLI	220
So- δ TIP	HWV YW VGPLV	222
Gm-Nod26	IWI YLL APVV	235
At-NLM1	IWI YL VAPTL	242
At-NLM2	LWI YI VSP1V	256
At-NLM3	IWI YLL APT1	251
At-NLM4	IWV YI VGPFV	240
At-NLM5	IWV YI VGPFV	239
At-NLM6	LWV YL VAPTL	274

Fig. 3. Amino acid residues in the sixth transmembrane helix possibly contributing to aquaporin substrate specificity [46]. The accession number for At-NLM2 is CAA16748 and At-NLM5 was found in non-annotated genomic sequence, nucleotide accession number AB016873.

tree and they are more closely related to the glycerol facilitators of *E. coli* (Ec-GlpF) and yeast (Sc-Fps1) than to the water-specific aquaporins (Fig. 2).

Several attempts have been made to determine the amino acids responsible for the differences in specificity. The second and third apoplastic loops are significantly longer (approximately 15 amino acids) in most glycerol facilitators and aquaglyceroporins as compared to water-specific aquaporins. In [44], the importance of these two loops was investigated in AQP-CE1, a nematode aquaporin permeable to urea, but not to glycerol. The second or third or both apoplastic loops were replaced with the corresponding loops of AQP3, permeable to both urea and glycerol, or GlpF, permeable to glycerol, but not to urea. However, all of the mutants had similar specificity as wild-type AQP-CE1, indicating that these loops are not directly involved in selectivity. In [45], five residues predicted to be important for specificity were identified. Two of these residues (Tyr-222 and Trp-223) in AQPcic, an insect aquaporin, were mutated to the corresponding residues of GlpF, proline and leucine, respectively (Fig. 3; [46]). This mutant lost the ability to transport water, but acquired glycerol transport activity. The mutated residues are located in the sixth transmembrane α -helix and consist of two aromatic amino acid residues in aquaporins while a proline followed by a non-aromatic residue occupy this position in glycerol transporters. However, this rule does not seem to be

true for the recently identified plant aquaglyceroporins. Nt-AQP1 and Nt-TIPa contain two aromatic residues in this position and NOD26 contain one aromatic residue followed by a leucine. This site is clearly not the only determinant for specificity and more aquaporins have to be investigated in order to identify other regions with importance for specificity. It has also been suggested that glycerol facilitators are monomers while aquaporins are tetramers in their native membrane [47].

Whether water and glycerol share the same pathway or if separate pathways exist, is controversial [22,48,49].

2.5. Aquaporin isoforms

Currently, about 30 members of the MIP family have been identified in *Arabidopsis*. Twelve of the MIPs are PIP subfamily members, 12 are TIP subfamily members and six are NLMs (Fig. 2; U. Johansson et al., unpublished data; [13]).

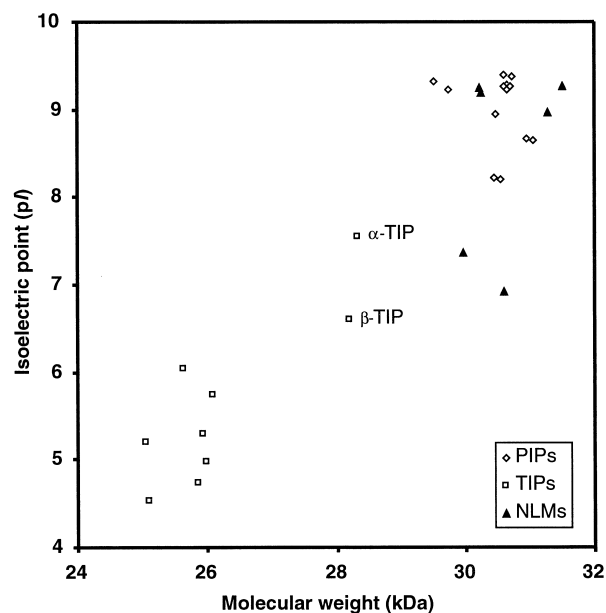


Fig. 4. Distribution in isoelectric point (pI) and molecular weight for members of the plasma membrane intrinsic protein (PIP), the tonoplast intrinsic protein (TIP) and the NOD26-like MIP (NLM) subfamilies of *Arabidopsis* major intrinsic protein (MIP) homologs. α - and β -TIP are both seed specific and have intermediate pIs and also intermediate molecular weights. The differences in pIs are mainly due to the presence or absence of basic residues in the C-terminal domains.

As mentioned above, MIPs are in general fairly small proteins. When the predicted molecular weights and the isoelectric points of all known full length MIPs in *Arabidopsis* are analyzed, an interesting pattern emerges (Fig. 4). TIPs are not only smaller than PIPs and NLMs, but most of them are also much more acidic than both PIPs and NLMs. In phylogenetic analyses, TIPs form a monophyletic group and it is possible that the observed difference is only a relic from the past without a functional basis. A more interesting possibility is if the *pI* is a reflection of a functional constraint imposed on MIPs. A detailed sequence analysis of the *Arabidopsis* MIPs reveals that the cause of the large difference in *pI* is that the C-terminal regions of PIPs and NLMs are more basic compared to the C-terminals of TIPs. The decisive part of the C-terminal region starts at the last third of the sixth transmembrane helix and is thus mainly cytosolic. It is possible that putative phosphorylation sites or sorting signals in the C-terminal regions form part of the hypothesized functional constraint on the sequences.

NLMs are similar to PIPs when molecular weights and isoelectric points are compared. It is not known in what membranes the *Arabidopsis* NLMs are expressed. NOD26, the first described protein in this subfamily, is expressed in the peribacteroid membrane surrounding the symbiotic bacteria in soybean root nodules. As far as is known, *Arabidopsis* lacks bacterial symbionts.

The sequence similarity is higher within one group of aquaporin homologs independent of species as compared to all aquaporin homologs in a single species, indicating that these groups evolved before the divergence of higher plants. The PIPs may be divided into two subgroups, PIP1 and PIP2 based on sequence homology. PIPs have extended N-termini as compared to TIPs (Fig. 5). The PIP1 and PIP2 members differ mainly in the N-terminal domain, which is more extended within the PIP1 subgroup. In addition, the PIP1 members have a shorter C-terminus and certain amino acids in other parts of the sequence are specific for each subgroup [42]. Thus, while the sequence is very well conserved in the transmembrane domains and in the two NPA-containing loops, the N- and C-termini are relatively variable. This suggests that these termini do not directly participate in water transport, but may have

other functions, e.g. in regulation. In agreement with this, a mutated form of the spinach PIP PM28A, lacking eight amino acids in the C-terminus was fully active (I. Johansson et al., unpublished data). Likewise, AQP1, devoid of a 4 kDa C-terminal domain exhibited full activity [50].

In the oocyte expression system, PIPs and TIPs induce a similar increase in water permeability when compared in the same experiment ([11]; I. Johansson et al., unpublished data). In these experiments, the same amount of cRNA was injected, but it is not known if the same amount of protein was inserted into the oocyte plasma membrane or if all aquaporins are open. The water permeabilities of the plasma membrane and the tonoplast has been determined in two reports using light scattering of membrane vesicles. The tonoplast was 100 times [51] or 7 times [52] more permeable to water than the plasma membrane. In [51], it was concluded that water transport across the plasma membrane occurred mainly by diffusion through the lipid bilayer. In contrast, Niemietz and Tyerman [52] found the osmotic water permeability coefficient of the plasma membrane to be three times higher than the diffusional water permeability coefficient, indicating some involvement of aquaporins. Nevertheless, the water permeability of the plasma membrane was surprisingly low in both studies, indicating that plasma membrane aquaporins were either closed or not expressed under the experimental conditions used.

From the phylogenetic tree in Fig. 2, it is evident that PIPs constitute a more homogenous group than any of the other major groups. The relatively conserved sequence among PIPs could be caused by a functional constraint on the sequence. An alternative explanation is that the many PIPs have arisen by a more recent selective advantage for plants with a multitude of different genes encoding PIPs. The latter interpretation is favored if the glycerol facilitators are used to root the tree. The selective advantage in this case could be a regulated tissue-specific expression of aquaporins. The branching of TIPs might represent an older duplication caused by evolution of different types of vacuoles.

It has been suggested that the TIP isoforms can be used as markers for different types of vacuoles. Thus, the presence of α -TIP defines protein storage vacuoles (PSVs) containing seed-type storage proteins,

At-PIP3	MSKEVSEE---GK-----THHGKDYVDPPPAPLLDMGEL	31
At-PIP1a	MEGKEEDVRVGGANKFPERQPIGTSAQSDKDYKEPPPAPFFEPGEL	45
At-NLM1	MVNLKDEVEH-----QEMEDIHNPRLPKQDSLSS	31
At- α -TIP	MATSARRAYGFGRADLA	17
At- δ -TIP	MAGVAFGSFDDS	12
At- γ -TIP	MPIRNIAIGRPDLA	14
At-PIP3	K[SW]S[FY]R[AL]I[A]E[F]I[AT]L[L]F[L]I[V]T[V]A[T]V[I]G[H]K[K]Q[T]G[P]-----C[D]G[V]G	72
At-PIP1a	SSWSFW[RA]GIAE[F]I[AT]F[L]F[L]Y[I]T[V]L[T]V[M]G[V]K[R]S[P]N[M]-----C[A]S[V]G	86
At-NLM1	V[S]V[P]F[L]Q[L]I[A]E[F]L[G]T[Y]F[L]V[F]T[G]C[A]S[V]V[V]N[M]O-----N[D]N[V]V	68
At- α -TIP	T[H]P[D]S[I]R[A]T[L]A[E]F[L]S[T]F[V]F[V]F[A]A[E]G[S]I[L]S[L]D[K]L[Y]W[E]H[A]A[H]A[G]T[N]T[P]G	64
At- δ -TIP	F[S]L[A]S[L]R[A]Y[L]A[E]F[I]S[T]L[L]F[V]F[A]G[V]G[S]A[I]A[Y]A[K]L[T]S[D]-----A[A]L[D]T[P]	54
At- γ -TIP	T[R]P[D]A[L]K[A]A[L]A[E]F[I]S[T]L[L]I[F]V[V]A[G]S[G]S[G]M[A]F[N]K[L]T[E]N-----G[A]T[T]P[S]	56
At-PIP3	-[L]L[G]I[A]W[A]F[G]G[M]I[F]V[L]V[Y]C[T]A[G]I[S]G[G]H[I]N[P]A[V]T[F]G[L]F[L]A[G]K[V]S[L]V[R]A	118
At-PIP1a	-I[Q]G[I]A[W]A[F]G[G]M[I]F[A]L[V]Y[C]T[A]G[I]S[G]G[H]I[N]P[A]V[T]F[G]L[F]L[A]R[K]L[S]L[T]R[A]	132
At-NLM1	T[L]P[G]I[A]I[V]W[G]L[T]I[M]V[L]I[Y]S[L]G[H]I[S]G[A]H[I]N[P]A[V]T[L]A[F]A[S]C[G]R[F]P[L]K[Q]V	115
At- α -TIP	G[L]I[L]V[A]L[A]H[A]F[A]L[F]A[A]V[S]A[A]I[N]V[S]G[G]H[V]N[P]A[V]T[F]G[A]L[V]G[G]R[V]T[A]I[R]A	111
At- δ -TIP	G[L]V[A]I[A]V[C]H[G]F[A]L[F]V[A]V[A]I[G]A[N]I[S]G[G]H[V]N[P]A[V]T[F]G[L]A[V]G[G]Q[I]T[V]I[T]G	101
At- γ -TIP	G[L]V[A]A[V]A[H]A[F]G[L]F[V]A[V]S[V]G[A]N[I]S[G]G[H]V[N]P[A]V[T]F[G]A[F]I[G]N[I]T[L]L[R]G	103
At-PIP3	L[G]Y[M]I[A]Q[C]L[G]A[I]C[G]V[G]F[V]K[A]F[M]K[T]P[Y]N[T]L[G]G[G]A[N]T[V]A[D]G[Y]S[K]G----	161
At-PIP1a	L[Y]Y[I]V[M]Q[C]L[G]A[I]C[G]A[G]V[V]K[G]F[Q]P[K]Q[Y]Q[A]L[G]G[G]A[N]T[V]A[H]G[Y]T[K]G----	175
At-NLM1	P[A]Y[V]I[S]O[V]I[G]S[T]L[A]A[A]T[L]R[L]L[F]G-L[D]H[D]V[C]S[G]K[H]D[V]F[I]G[S]S[P]V[G]S[D]L	161
At- α -TIP	I[Y]Y[W]I[A]Q[L]L[G]A[I]L[A]C[L]L[L]R[L]T[N]----G[M]R[P]V[G]F[R]L[A]S[G]V[G]A[V]----	150
At- δ -TIP	V[F]Y[W]I[A]Q[L]L[G]S[T]A[C]F[L]L[K]Y[V]T[G]----G[L]A[V]P[T]H[S]V[A]A[G]L[G]S[I]----	140
At- γ -TIP	I[L]Y[W]I[A]Q[L]L[G]S[V]V[A]C[L]I[L]K[F]A[T]G----G[L]A[V]P[A]F[G]L[S]A[G]V[G]V[L]----	142
At-PIP3	T[A]L[G]A[E]I[I]G[T]F[V]L[V]Y[T]V[F]S-[A]T[D]P[K]R[S]A[R]D[S]H[I]P[V]L[A]P[L]P[I]G[F]A[V]F[M]	207
At-PIP1a	S[G]L[G]A[E]I[I]G[T]F[V]L[V]Y[T]V[F]S-[A]T[D]A[K]R[N]A[R]D[S]H[V]P[I]L[A]P[L]P[I]G[F]A[V]F[L]	221
At-NLM1	Q[A]F[T]M[E]I[V]T[F]Y[L]M[F]I[S]G[V]A[T]D[N]R[A]-----I[G]E[L]A[G]L[A]I[G]S[T]V[L]L	202
At- α -TIP	N[G]L[V]L[E]I[L]T[F]G[L]V[Y]V[Y]S[T]L[I]D[P]K[R]----G[S]L[G]I[A]P[L]A[I]G[L]I[V]G[A]	193
At- δ -TIP	E[G]V[V]M[E]I[L]T[F]A[L]V[Y]T[V]Y[A]T[A]D[P]K[K]----G[S]L[G]T[I]A[P]L[A]I[G]L[I]V[G]A	183
At- γ -TIP	N[A]F[V]E[I]V[M]T[F]G[L]V[Y]T[V]Y[A]T[A]I[D]P[K]N----G[S]L[G]T[I]A[P]L[A]I[G]F[I]V[G]A	185
At-PIP3	V[H]L[A]T[I]P[I]T[G]T[G]I[N]P[A]R[S]F[G]A[A]V[I]Y[N]N[E]K[A]W[D]D[D]W[I]F[W]V[G]P[F]L[G]A[L]A	254
At-PIP1a	V[H]L[A]T[I]P[I]T[A]T[G]I[N]P[A]R[S]L[G]A[A]I[I]Y[N]K[D]H[S]W[D]D[H]W[V]F[W]V[G]P[F]I[G]A[A]L	268
At-NLM1	N[V]L[I]A[A]P[V]S[S]A[S]M[N]P[G]R[S]L[G]P[A]L[V]Y[G]C---Y[K]G[I]W[I]Y[L]V[A]P[T]L[G]A[I]A	246
At- α -TIP	N[I]L[V]G[G]P[F]S[G]A[S]M[N]P[A]R[A]F[G]P[A]L[V]G[W]R---W[H]D[H]W[I]Y[W]V[G]P[F]I[G]S[A]L	237
At- δ -TIP	N[I]L[A]G[G]P[F]S[G]G[S]M[N]P[A]R[S]F[G]P[A]V[A]A[G]D---F[S]G[H]W[Y]W[V]G[P]L[I]G[G]G[L]	227
At- γ -TIP	N[I]L[A]G[G]A[F]S[G]A[S]M[N]P[A]V[A]F[G]P[A]V[V]S[W]T---W[T]N[H]W[Y]W[A]G[P]L[V]G[G]G[I]	229
At-PIP3	A[A]Y[H]Q[Y]I[L]R[A]S[A]-----I[K]A[L]G[S]F[R]S[N]A[T]N	280
At-PIP1a	A[A]L[Y]H[V]V[V]I[R]A[I]P-----F[K]S[R]S	286
At-NLM1	G[A]W[V]Y[N]T[V]R[Y]T[D]K[P]L[R]E[I]T[K]S-G[S]F[L]K[T]V[R]I[G]S[T]	279
At- α -TIP	A[A]L[Y]E[Y]M[V]I[P]T[E]P[P]T[H]H[A]G[V]H[Q]P[L]A[P]E[D]Y	268
At- δ -TIP	A[G]L[I]Y[G]N[V]F[M]G[S]S[E]-----H[V]P[L]A[S]A[D]F	250
At- γ -TIP	A[G]L[Y]E[V]F[F]I[N]T[T]-----H[E]Q[L]P[T]T[D]Y	251

Fig. 5. Sequence comparison. Primary amino acid sequences of *Arabidopsis* major intrinsic protein (MIP) homologs representing the different MIP subfamilies. Among the plasma membrane intrinsic proteins (PIPs), PIP3 represents the PIP2 subfamily members and PIP1a the PIP1 subfamily members. NLM1 represents the NLM (NOD26-like MIPs) subfamily members, and α -, δ -, and γ -TIP the three major tonoplast intrinsic protein (TIP) subfamilies. PIP1 subfamily members have longer N-terminal regions and PIP2 subfamily members have longer C-terminal regions than the PIP1 members. Both of the PIP subfamilies as well as the NLM subfamily have extended N-terminal regions as compared to the TIPs.

while γ -TIP is present in the tonoplast of vegetative or lytic vacuoles (LVs; reviewed in [53,54]). Recently defined delta vacuoles (Δ Vs) characterized by δ -TIP containing tonoplasts, act as specialized storage organelles for pigments and vegetative storage proteins (VSPs; [55]). Δ Vs can be found in cells together with either PSVs or LVs, but tend to be smaller and lo-

calized in the cell periphery, in some cases just beneath the plasma membrane [54].

By using the C-terminal cytosolic tail of α -TIP or γ -TIP as targeting signals attached to a non-vacuolar reporter protein, two separate pathways for targeting of vacuolar membrane proteins could be followed; directly from the ER to PSVs, as for α -TIP, or via

the Golgi and prevacuolar compartments to the vegetative lytic vacuole for γ -TIP [56].

2.6. Physiological role of aquaporins

Evidence for the involvement of aquaporins at the whole plant level is scarce, but convincing. In tomato roots, the addition of HgCl_2 rapidly resulted in a decrease in water flux by approximately 70%, as well as a decrease in hydraulic conductivity by about 60% [57]. HgCl_2 inhibits the activity of many aquaporins, but not all, hence, the contribution of aquaporins to the water fluxes may be even larger than indicated by these values. Mercurials may affect many sulfhydryl-containing proteins; however, the concentration of K^+ in the xylem sap was not affected by HgCl_2 , indicating that the reduction of water flux was rather due to inhibition of aquaporins than changes in ion permeability of the plasma membrane. Analysis of barley roots treated with HgCl_2 showed an even higher degree of inhibition of the hydraulic conductivity [58]. In *Arabidopsis*, the significance of PIPs in water transport was demonstrated using antisense technology [59]. *Arabidopsis* plants were transformed with a *pip1b* antisense construct, resulting in reduced levels of PIP1b and PIP1a in both roots and leaves. Analysis of water permeabilities of protoplasts prepared from leaves showed a 3-fold reduction of P_f in protoplasts from transformed plants as compared to those from control plants. The phenotype of the transformed plants was similar to that of control plants except for the root system, which was five times larger in the transformed plants. The rate of water uptake by the roots was similar in transformed and control plants, indicating that the plant compensates its reduced level of aquaporins by increasing the size of the root system. Taken together, these results demonstrate the importance of aquaporins in water uptake in vivo.

3. Regulation of aquaporins

The presence of aquaporins in cellular membranes is not only a prerequisite for rapid transmembrane water transport, but also provides the organism with an opportunity to regulate the flux of water between cells and within the cell. Regulation of aquaporins

has been observed at the transcriptional level and in response to post-translational modifications. A third mechanism involving the targeting of aquaporin-containing periplasmic vesicles to the plasma membrane in response to a hormonal signal has been shown for mammalian AQP2 and AQP1 [60,61]. Some aquaporins, are constitutively expressed [62] while the expression of others is regulated by environmental factors such as drought, salinity, hormones, and blue light [17,63–66]. Post-translational modifications reported include phosphorylation, glycosylation, and proteolytic processing [20,67–71].

3.1. Phosphorylation of aquaporins

3.1.1. Identification and activity of phosphorylated aquaporins

A few aquaporins have been shown to be phosphorylated in vivo. In plants, these are NOD26, kidney bean α -TIP, and PM28A [20,67,68]. In planta, NOD26 and PM28A are phosphorylated in the C-terminal domain, at serine-262 and serine-274, respectively (Fig. 1), while α -TIP is phosphorylated at serine-7, in the N-terminus. The effects of phosphorylation on water channel activity was investigated for α -TIP and PM28A transiently expressed in oocytes. An increase in activity following phosphorylation was demonstrated for both aquaporins [18,20]. Phosphorylation of NOD26 has so far not been correlated with a change in transport activity in an in vivo system or in its native membrane [72].

Phosphorylation of PM28A at serine-274 increases at increasing apoplastic water potential, as accomplished by incubation of pieces of spinach leaves in buffers with different osmolarities [20,62]. At high apoplastic water potential, more than 50% of PM28A was estimated to be phosphorylated at this site. These results implicate that, at water deficit, PM28A is less phosphorylated and thus less active. This should be advantageous since the decrease in water efflux from the cell due to closing of aquaporins during drought may provide the plant with additional time to adjust to the stress situation, e.g. by synthesis of osmolytes. When the water potential is high, on the other hand, PM28A becomes phosphorylated and the channel is opened.

A serine corresponding to serine-274, i.e. in a sim-

A Second loop phosphorylation site

	RKXSXX ^R _K	
So-PM28A	ARKV S LLRA	119
At-PIP3	AGKVSLVRA	118
At-PIP1a	ARKLSLTRA	132
Pv- α TIP	GGRIS V IRA	103
At- α TIP	GGRVTAIRA	111
At- β TIP	GGRISVIRA	111
At- δ TIP	GGQITVITG	101
At- γ TIP	GGNITLLRG	103
Gm-Nod26	TRRFPLIQV	115
At-NLM1	CGRFPLKQV	115
Hs-AQP2	GCHVSVLRA	86

B C-terminal phosphorylation site

	K ^K XXSX ^R _K S	
So-PM28A	AAIKALG S FR S NPTN	281
At-PIP3	SAIKALG S FR S NATN	280
At-PIP1a	IPFK S R S	286
Pv- α TIP	HHHQPLATEDY	256
At- α TIP	GVHQPLAPEDY	268
At- β TIP	STHQPLAPEDY	267
At- δ TIP	SEHVPLASADF	250
At- γ TIP	TTHEQLPTTDY	251
Gm-Nod26	SETTK S A S FLKGRAASK	271
At-NLM1	REITK S GSFLKTVRIGST	279
Hs-AQP2	REVRRR S VELHSPQSLPRGTKA	271

Fig. 6. Consensus phosphorylation sites. (A) The consensus phosphorylation site located in the first cytosolic loop. The motif, RKXSXXR/K or only KXSXXR/K, is conserved in all plasma membrane intrinsic proteins (PIPs), i.e. in all PIP1 and PIP2 subfamily members. A similar motif is present in most tonoplast intrinsic proteins (TIPs), although usually with a threonine replacing the serine residue, and in human AQP-2, while this region is less conserved in members of the NLM (NOD26-like MIPs) subfamily. Serines depicted in bold represent serines shown to be phosphorylated in the oocyte system thereby regulating the water transport activity. (B) The consensus phosphorylation site located in the C-terminal region. The motif, SXR/KS, is present in all PIP2 subfamily members. PM28A has been shown to be phosphorylated at Ser-274 in planta, in the oocyte system, and in vitro using plasma membrane vesicles. Both Ser-274 and Ser-277 have been shown to be phosphorylated in planta. A similar motif, KXXSXXK, is present in NOD26 which in planta has been shown to be phosphorylated at Ser-262. *Arabidopsis* NLM subfamily members have a similar motif, K/XX/RXSXXK/R, while all PIP1 and TIP subfamily members lack this phosphorylation motif in the C-terminal region.

ilar position and amino acid environment, is only present in the PIP2 subfamily (Fig. 6B). Within this subfamily, activity may be regulated by phosphorylation of this C-terminal serine in response to an osmotic signal and this mode of regulation may be specific for the PIP2 aquaporins. The amino acid environment of the phosphorylated serine in NOD26 is partly different from that of PM28A.

However, one or two non-polar and a basic residue are present C-terminally of the phosphoserine in both cases. In addition to serine-262, there are three more serines in putative phosphorylation consensus sites in the C-terminus of NOD26. Interestingly, none of these are phosphorylated [72]. Mammalian AQP2 is likewise phosphorylated in the C-terminus, but this does not seem to be directly involved in gating of the aquaporin [73]. Surprisingly, serine-7 of kidney bean α -TIP is not conserved even in the putative orthologs, *Arabidopsis* α -TIP and pumpkin MP23 and MP28 [71,74]. *Arabidopsis* α -TIP has been reported not to be phosphorylated [41].

In contrast, a highly conserved serine is present in the first cytoplasmic loop (Fig. 1; Fig. 6A). This residue is located in the sequence Arg/X-Lys-X-Ser-X-X-Arg/Lys, which is a motif recognized by several protein kinases. This motif is present in the same position in all plant PIPs and a similar motif, Arg-X-Ser-X-X-Arg, is present in the corresponding position in most α -TIPs, including α -TIP of kidney bean. In γ -TIPs, the corresponding sequence is Thr-X-X-Arg and in mammalian aquaporins Ser-X-X-Arg/Lys (except for AQP8). The high degree of conservation indicates that this residue is of major structural or functional importance. Combined with the fact that it is located in a consensus phosphorylation motif, it may be suspected that phosphorylation of this serine regulates activity. Studies of α -TIP and PM28A expressed in oocytes showed that phosphorylation at this conserved serine may occur and results in an increase in water channel activity [18,20]. Thus, although phosphorylation of this residue has not been demonstrated in planta, these experiments in oocytes strongly indicate that regulation of aquaporin activity by phosphorylation/dephosphorylation occurs at this site.

3.1.2. Protein kinases and the role of Ca²⁺

Phosphorylation of PM28A, kidney bean α -TIP, and NOD26 is Ca²⁺-dependent [62,67,68]. Ca²⁺ is well established as an intracellular messenger in plants. In the search for Ca²⁺-dependent protein kinases in plants, a new type of protein kinase, CDPK (calcium-dependent protein kinase or calmodulin-like domain protein kinase) emerged (reviewed in [75,76]). CDPKs have been identified in higher plants, algae, and protists and seem to replace or

complement protein kinase C and Ca^{2+} /calmodulin-dependent protein kinase, which are Ca^{2+} -dependent protein kinases in animals. CDPKs are dependent on micromolar or submicromolar concentrations of Ca^{2+} and bind Ca^{2+} directly without the involvement of effector molecules, such as phosphatidylserine and diacylglycerol (activators of protein kinase C) or calmodulin (activator of Ca^{2+} /calmodulin protein kinase). NOD26 is one of the few endogenous substrates identified for a purified CDPK [67]. The CDPK phosphorylating NOD26 was found in the peribacteroid membrane in root nodules, but also in other parts of the plant, both in membrane and soluble fractions. Since NOD26 is exclusively found in the peribacteroid membrane, this indicates that this CDPK has multiple substrates. PM28A is phosphorylated on serine-274 *in vitro* in purified plasma membrane vesicles showing that the protein kinase is plasma membrane associated [62]. Similarly, α -TIP is phosphorylated by a tonoplast-bound protein kinase [68].

The substrate specificity differs substantially for different CDPKs. In NOD26, the phosphorylated serine is in the sequence basic-X-X-Ser, which is a motif recognized by several CDPKs [75]. Serine-274 of PM28A occurs in the sequence Ser-Phe-Arg which resembles the motif Ser-Phe-Lys recognized by some CDPKs [77,78]. The substrate specificities of CDPK and animal protein kinase C partly overlap and PM28A is phosphorylated *in vitro* by vertebrate protein kinase C (I. Johansson et al., unpublished data).

In vivo phosphorylation of PM28A showed phosphorylation at serine-277 in a minor fraction (approximately 5%) of the protein (Fig. 1; [20]). Phosphorylation at this site only occurred when serine-274 was simultaneously phosphorylated. The reason for this phenomenon may be the substrate specificity of the protein kinase, since the recognition site for some protein kinases involves phosphorylated amino acids. Casein kinase-1 (CK-1) has been shown to preferentially phosphorylate serines (or threonines) in sequences containing phosphoserine (or phosphothreonine) at position -3 relative to the target amino acid (reviewed in [79]). Hence, serine-277 resides in an ideal sequence for recognition by CK1, provided serine-274 is phosphorylated. Interestingly, serine-277 is conserved among PIP2 subgroup members, as is serine-274.

3.1.3. Osmosensors

The response of plant cells to water deficit starts with the perception of osmotic stress, followed by transduction of this information and adaptive responses in order to withstand the stress situation. Changes in activity in response to water deficit have been documented for several proteins, as well as changes in gene expression; however, the mechanism by which the plant cell detects water deficit is not known. In yeast, two independent and unrelated osmosensors have been identified. Both are transmembrane proteins, which, via phosphorylation pathways, regulate a phosphorylation cascade called the high osmolarity glycerol (HOG) pathway. A homolog to one of these osmosensors has been identified in *Arabidopsis* and its function as an osmosensor was demonstrated by functional complementation of a yeast mutant [80]. The mechanism by which the osmosensors sense changes in osmolarity is unknown.

We have proposed a model where, at high apoplastic water potential, a Ca^{2+} channel in the plasma membrane opens in response to a signal, leading to an influx of Ca^{2+} which activates a protein kinase phosphorylating aquaporins [20]. The Ca^{2+} channel may act as an osmosensor in itself. Mechanosensitive (e.g. stretch-activated) ion channels are ubiquitous and have been found in plants, animals, fungi and bacteria. These channels have been suggested to function as osmo-receptors and their activation in response to increased apoplastic water potential has been demonstrated in several reports (reviewed in [81]). Ca^{2+} -specific, stretch-activated channels have been identified in guard cells of *Vicia faba* [82]. Alternatively, the signal could be mediated by an osmosensor similar to the yeast osmosensors. The protein kinase could also be activated by Ca^{2+} originating from intracellular stores.

In another proposed mechanism for perception of water deficit, the roots sense the osmotic stress and transfer this information to the shoot in the form of an as yet unidentified signal. The hypothesis of root-to-shoot signals is supported by several reports demonstrating stomatal closure and inhibition of shoot growth at drought before any changes in total water potential could be detected in the shoot. It has been shown that the synthesis of abscisic acid (ABA) in roots is increased at drought and elevated levels of

ABA have been detected in the xylem sap. Although it seems likely that ABA from the root is responsible for early responses to water deficit in the shoot in many plants, this is probably not the only substance functioning as a root-to-shoot signal. In kidney bean, stomatal closure was observed before any increased levels of ABA could be detected [83]. The delivery of cytokinins from roots is depressed in response to drought and this may act as a negative message to the shoot (reviewed in [84]).

3.2. Translocation of aquaporins

AQP2 is expressed exclusively in the collecting duct of kidney and its location is regulated by the antidiuretic hormone vasopressin. Stimulation by vasopressin leads to an increased water permeability of the plasma membrane of AQP2-expressing cells and the mechanism for this has been investigated in several studies [85,86]. Vasopressin binds to an adenylyl cyclase-coupled vasopressin receptor (V2), resulting in an increase in cAMP and subsequent activation of protein kinase A (PKA). PKA phosphorylates AQP2 located in intracellular vesicles, an event that triggers fusion of these vesicles to the plasma membrane. The translocation of AQP2-containing vesicles in response to vasopressin was elegantly demonstrated by immunoelectron microscopy [60]. Phosphorylation of serine-256, in the C-terminus of AQP2, is required for fusion of the vesicles to the plasma membrane [87,88]. A similar mechanism exists for AQP1, but only in certain cell types. In these cells, the hormone secretin stimulates phosphorylation of AQP1 by PKA followed by translocation of AQP1-containing vesicles to the plasma membrane [61].

In plants, regulation of aquaporins by vesicle shuttling has not yet been demonstrated. Periplasmic vesicles have been speculated to be transient structures by which translocation of PIP1 aquaporins may be mediated [89]. In [90], the membrane localization of three ice plant PIPs was investigated by immunological analysis of sucrose density gradient fractions. All three PIPs (two PIP1 and one PIP2) were detected in intracellular membrane fractions. Phosphorylation of PIP1 aquaporins has so far not been demonstrated but if translocation of PIP1-containing vesicles occurs, fusion of the vesicles to the plasma

membrane does not necessarily have to be triggered by phosphorylation of the aquaporins as in the case of AQP2.

3.3. Transcriptional regulation

3.3.1. Water deficit

Water deficit evokes a multitude of cellular responses. These responses are complex and depend on several parameters, such as extent and rate of water loss, species, age, stage of development, organ, and cell type. Alterations in gene expression have been the focus of several investigations, since the products of these genes are thought to be involved in plant adaptation to drought stress [91]. A few aquaporins and aquaporin homologs were identified by differential hybridization performed in order to isolate drought-induced genes. These include clone 7a in pea which, based on the sequence, belongs to the PIP1 subgroup [63], and RD28, an *Arabidopsis* aquaporin belonging to the PIP2 subgroup [64]. The gene corresponding to TRAMP, a PIP1 homolog in tomato, was likewise upregulated by drought [92]. There are several independent signal transduction pathways resulting in activation of gene expression during water deficit. Some of these are mediated via abscisic acid (ABA), which is accumulated under drought conditions [80]. Experiments with exogenously applied ABA or ABA-deficient mutants indicate that the induction of genes encoding RD28 and TRAMP is ABA-independent. The gene encoding clone 7a was slightly activated following ABA-treatment. Changes in mRNA levels are not automatically reflected in changes in protein levels. Parameters such as mRNA stability and protein turnover may change in a stress situation and have to be considered. In addition, for a protein to perform its task, it has to be properly located in its cellular compartment and post-translational modifications or cofactors may be needed. The levels of RD28 were investigated using a monospecific antiserum [11]. RD28 was abundant in non-stressed plants and no change in abundance could be detected when plants were desiccated.

Aquaporins expressed mainly or only during stress may exist in all plants, especially considering the large number of aquaporin and aquaporin homologs present in *Arabidopsis* and presumably in all plants.

In sunflower, two tonoplast aquaporins, SunTIP7 and SunTIP20 have been identified. These are highly homologous and both are specifically expressed in guard cells. However, their genes are differently regulated. The level of the transcript corresponding to SunTIP7 shows diurnal fluctuations, being low at dawn and dusk and high at noon. This pattern correlates well with stomatal movements. Thus, when the stomata start closing, the level of the *suntip7* transcript is high and at night when the stomata are closed, the transcript level is low, indicating a role for SunTIP7 in the rapid water efflux necessary during stomatal movement. In agreement with this, the *suntip7* transcript accumulated when the plants were subjected to drought. The transcript level of *suntip20*, on the contrary, did not change during the day or in response to water deficit [19].

Several regulatory promoter elements functioning during drought stress, e.g. dehydration-responsive element (DRE) have been identified [80] and analysis of aquaporin gene promoters may provide further insight into the regulation of these genes.

3.3.2. Hormones, blue light, and pathogen attack

Plant hormones may activate aquaporin genes. Gibberellins increase the expression of the gene corresponding to γ -TIP in an *Arabidopsis* mutant with low levels of endogenous gibberellins [65]. Addition of gibberellins to this mutant induces stem elongation by stimulation of cell division and cell elongation. The activation of the γ -*tip* gene in this system may reflect its importance in rapidly elongating cells, a theory supported by the expression pattern of γ -TIP in wild-type *Arabidopsis* where high expression is observed in the elongation zone [93].

The *Arabidopsis pip1b* gene is activated by blue light, ABA, and gibberellins [66,94]. Since ABA and gibberellins often show antagonistic effects, activation by both may seem contradictory. The authors suggest that the balance between the two hormones is crucial. Promoter analysis revealed sequence elements with homology to other ABA and gibberellin induced genes.

The expression of the gene encoding the root-specific tobacco tonoplast aquaporin, TobRB7, was induced by infection by root-knot nematodes. These parasites induce the formation of a feeding site within the plant root and alter the expression of plant

genes at this site. The *tobrb7* promoter sequence responsible for nematode infection-induced expression was determined and found to be different from the sequence responsible for root-specific expression [14].

4. Distribution of aquaporins in the plant

Aquaporins are expressed in organ-, tissue-, and cell type-specific manners. Knowledge of the expression patterns of different aquaporins is essential for several reasons. Firstly, it will provide clues to the function of aquaporins at the whole plant level. Although it is clear that aquaporins play a fundamental role in plants, detailed information about their physiological function is lacking. The expression patterns may suggest in which cells rapid transmembrane water transport is especially important. Secondly, different aquaporins are differently expressed. It is not known whether members from different aquaporin subgroups are present in the same cell. For example, do members from the PIP1 subgroup and the PIP2 subgroup coexist, and if so, do they perform different tasks? Aquaporins of different subfamilies may differ in activity and specificity and are differently regulated. Information about these characteristics combined with information about their cell type specificity will clarify the distinct role of individual aquaporins and how they collaborate.

The expression of aquaporins in different organs has been investigated for several aquaporins and aquaporin homologs. Expression restricted to one organ could be demonstrated for α -TIP, which is seed-specific, and TobRB7, a root-specific TIP in tobacco [9,95,96]. Most PIPs and TIPs are expressed in more than one organ; however, within a specific organ, their expression is often limited to certain cell types.

Several aquaporins have been shown to be expressed in zones of cell enlargement and cell elongation, e.g. γ -TIP of *Arabidopsis* [93] and maize [21,97] and δ -TIP of spinach [98]. Cells in expanding tissues are usually vacuolated, e.g. have one large vacuole occupying most of the cell volume. Cell expansion is believed to be accomplished by loosening of the pectin network of the primary cell wall in combination with the vacuole driving a sustained water influx because of its high osmotic potential. In order for the

vacuolar and cytosolic cell compartments to play this role in cell enlargement, and in order for mature cells to maintain turgor, a tight osmoregulation of the cytosol must be exerted at all times. It is likely that aquaporins of the vacuolar and plasma membrane are involved in buffering osmotic fluctuations of the cytosol caused by ionic flow or lowered apoplastic water potential as during drought stress.

Strikingly, many aquaporins of both the tonoplast and plasma membrane are expressed around vascular bundles throughout plants, e.g. the *Arabidopsis* δ -TIP [12], the maize γ -TIP ortholog ZmTIP1 [97], the spinach δ -TIP ortholog So- δ TIP [98], the *Arabidopsis* PIP1b [99], and the ice plant PIP1 ortholog MipA [17]. Inhibition studies using mercury chloride, known to inhibit aquaporins by binding to cysteine residues near the aqueous pore, suggest that aquaporins of xylem parenchyma cells are involved in maintaining the transpiration stream by refilling gas filled (embolized) xylem vessels thereby increasing the hydraulic conductivity of the tissue [100,101].

5. Conclusions and future perspectives

The recent discovery of aquaporins in plants, animals, fungi, and bacteria has provided new insights into the mechanisms for controlling cellular water balance. In plants, aquaporins are likely to be important both at the whole plant level, for transport of water to and from the vascular tissues, and at the cellular level, for buffering osmotic fluctuations in the cytosol.

The multitude of MIP genes in *Arabidopsis* raises the question of their specific roles. One of the reasons for the apparent redundancy may be different expression patterns. An investigation of the expression pattern of all *Arabidopsis* aquaporin homologs would provide a better understanding of their specificities as well as the overall contribution of aquaporins to water movement in the plant. Furthermore, reporter genes, e.g. the gene coding for the green fluorescent protein (GFP), fused to MIP promoters would be useful for following, e.g. drought stress-induced gene activation in living plants.

Sequence comparisons divide plant aquaporins into three groups: PIPs, TIPs, and NLMs. PIPs and TIPs have been characterized to some extent in

several species; however, close to nothing is known about NLMs. A study of this group of plant aquaporins, primarily their subcellular location, could provide some interesting clues. Knock-out mutants and antisense plants are probably necessary in order to generate phenotypes for understanding the function of individual MIPs and groups of MIPs in maintaining water homeostasis. However, the large number of MIPs, currently more than 30 in *Arabidopsis*, will likely complicate such an approach since compensatory gene expression may occur which might disguise mutant phenotypes. Clues to which knock-out mutants are likely to cause phenotypes may be gained from studying the expression patterns. The fewer MIPs expressed in a certain cell type, e.g. guard cells, the more likely it is to get a mutant phenotype.

The abundance and activity of aquaporins in the plasma membrane and tonoplast may be regulated, hence enabling the plant to tightly control water fluxes into and out of its cells, as well as within the cells. Some aquaporins are regulated by phosphorylation and this is triggered by changes in osmolarity, at least for one aquaporin. The osmosensor and signal transduction pathways leading to phosphorylation/dephosphorylation of aquaporins remain to be identified. A highly conserved putative phosphorylation site is present in the first cytosolic loop in aquaporins of plants and mammals. Although its significance has been demonstrated in plant aquaporins expressed in oocytes, phosphorylation of this site has not yet been shown in planta.

Recently, aquaporins with ability to transport small, uncharged solutes in addition to water have been identified in plants. A comparison of their amino acid sequences to those of water-specific aquaporins do not immediately reveal the reason for this difference in specificity. AQP1 of human erythrocytes has been reported to cause a 4-fold increase in both water and CO₂ permeability when inserted into proteoliposomes [102]. Furthermore, this increase in CO₂ and water permeability was inhibited by HgCl₂ and β -mercaptoethanol reversed the inhibition. Thus, in vivo, AQP1 may facilitate the transport of CO₂ in addition to water, although evidence against physiologically significant CO₂ transport by AQP1 has also been reported [103]. In accordance with these results, the plant MIPs that in the oocyte system have been

shown to be permeable to small uncharged solutes, such as urea and formamide which are unlikely to be important in planta, could be candidate membrane channels for transporting endogenous plant metabolites, such as CO₂ and ethanol, in vivo.

The specificity of more plant aquaporins needs to be determined, and plausible plant metabolites need to be tested, in order to identify regions important to specificity.

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