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Aquaporins in a challenging environment: molecular gears for adjusting plant water status

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ABSTRACT

Plants have to adjust their water balance in response to very challenging environmental conditions such as drought, salinity, and cold but also changes in light, nutrient deficiency or soil acidity. The molecular and functional characterization of aquaporins, a class of membrane proteins that facilitate water diffusion across cell membranes, has revealed the significance of their regulation in response to these environmental stimuli. The aim of this present review is to illustrate the variety of molecular and cellular mechanisms involved. These mechanisms include the control of aquaporin gene transcription and protein abundance, stimulus-induced aquaporin subcellular relocalization, and channel gating by reversible phosphorylation or by intracellular protons. The emergence of novel mechanisms of regulation by hetero-tetramer formation or through control by reactive oxygen species, and osmotic or hydrostatic pressure gradients is also discussed. These various mechanisms do not function individually and a challenge for future research will be to understand how plants respond to stresses by integrating these mechanisms in time and space, to constantly adjust the water transport and solute transport properties of their membranes. Genetic manipulation of aquaporin functions and in particular ectopic expression of deregulated aquaporins in transgenic plants provide promising strategies to address such questions.

Key-words: abiotic stress; gating; membrane channel; water transport.

INTRODUCTION

Plant membranes play a critical role in cell homeostasis and signalling, nutrition and response to stresses. During the 1990s, tremendous insights were gained into the molecular and cellular bases of membrane transport. An unprecedented wealth of membrane transport proteins and of mechanisms for controlling their activity was revealed. Progress has been particularly striking in the field of water relations, the mechanisms of water transport across cell membranes having remained elusive until this period. Thus, the molecular and functional characterization of a new class of membrane proteins, forming the superfamily of Membrane Intrinsic Proteins led most plant biologists to realize that plants are equipped with water channels, namely proteins which facilitate water diffusion across cell membranes (Maurel 1997; Maurel & Chrispeels 2001). The activity of these so-called aquaporins had been largely overlooked in many organisms including plants and microbes. It appears now that these proteins are ubiquitous and fulfil crucial functions in nearly all forms of life.

Aquaporins exhibit a typically conserved structure with six membrane spanning domains linked by three extra- and two intracellular loops, the N- and C-terminal tails of the protein bathing in the cytosol (Fujiyoshi *et al*. 2002) (Fig. 1a). Conserved amino acid motifs, such as two Asn-Pro-Ala boxes also define a characteristic aquaporin sequence signature (Fig. 1a). Aquaporins usually occur as tetramers, each monomer forming a functionally independent pore.

In plants, aquaporins occur as multiple isoforms. For instance, the genome of Arabidopsis encodes 35 aquaporin homologues (Fig. 1b), and a similar diversity is predicted in other plant species (Chaumont *et al*. 2001; Johanson *et al*. 2001; Quigley *et al*. 2001). The plant aquaporin family can be further subdivided into four homology classes, which to some extent reflect distinct subcellular localizations. The most abundant aquaporins in the vacuolar and plasma membranes belong to the tonoplast intrinsic protein (TIP) and the plasma membrane intrinsic protein (PIP) classes, respectively. The PIP class is further subdivided into two homology subgroups, PIP1 and PIP2, which in Arabidopsis comprise five and eight members, respectively (Fig. 1b). Soybean Nodulin-26 is another aquaporin that is expressed in the peribacteroid membrane of symbiotic N_2-fixing root nodules. This aquaporin and its close homologues form the class of Nodulin-26-like intrinsic proteins (NIPs). NIPs also occur in non-leguminous plant species where their subcellular localization is not known. The fourth class of plant aquaporins comprises the small basic intrinsic proteins (SIPs), with three homologues in Arabidopsis (Fig. 1b). Their function and localization are unknown at the moment.

The high isoform multiplicity of plant aquaporins has precluded a thorough analysis of their function. Yet, many early studies have revealed that aquaporins can display exquisite, isoform-specific patterns for subcellular localiza-

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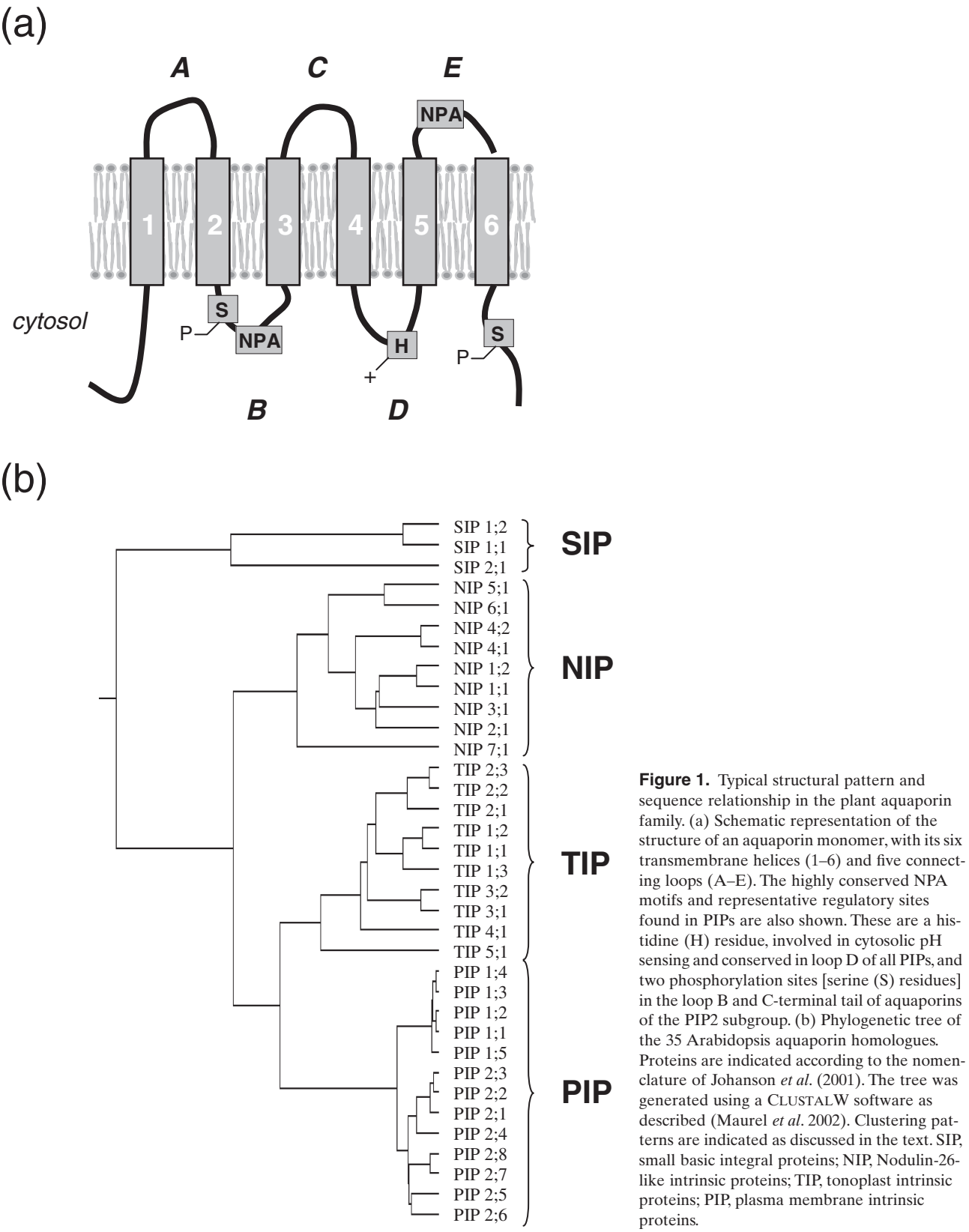

Figure 1. Typical structural pattern and sequence relationship in the plant aquaporin family. (a) Schematic representation of the structure of an aquaporin monomer, with its six transmembrane helices (1–6) and five connecting loops (A–E). The highly conserved NPA motifs and representative regulatory sites found in PIPs are also shown. These are a histidine (H) residue, involved in cytosolic pH sensing and conserved in loop D of all PIPs, and two phosphorylation sites [serine (S) residues] in the loop B and C-terminal tail of aquaporins of the PIP2 subgroup. (b) Phylogenetic tree of the 35 Arabidopsis aquaporin homologues. Proteins are indicated according to the nomenclature of Johanson *et al.* (2001). The tree was generated using a CLUSTALW software as described (Maurel *et al.* 2002). Clustering patterns are indicated as discussed in the text. SIP, small basic integral proteins; NIP, Nodulin-26-like intrinsic proteins; TIP, tonoplast intrinsic proteins; PIP, plasma membrane intrinsic proteins.

tion and cell-specific expression (reviewed in Maurel *et al*. 2002). These observations suggested that the complement of aquaporins expressed in plants contribute to spatially determining the hydraulic properties of cells and tissues, throughout all plant development. Much effort has been concentrated on investigating the function and regulation of PIPs. These aquaporins seem to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots where they mediate most of soil water uptake (Javot & Maurel 2002). In leaves, they may contribute to xylem sap unloading in the mesophyll and determine in part the path followed by the transpiration stream (Morillon & Chrispeels 2001). The TIPs represent another class of well-characterized aquaporins. They mediate water exchange between the cytosolic and vacuolar compartments and may play a central role in cell osmoregulation (Maurel *et al*. 1997; Tyerman *et al*. 1999).

The capacity of aquaporins to transport small neutral solutes and/or gases, in addition to water, has raised the intriguing possibility that aquaporins may work as membrane channels with multiple functions (Tyerman, Niemietz & Bramley 2002). Nodulin-26 transports small uncharged molecules such as glycerol and a role in ammonia gas transport has been hypothesized (Dean *et al*. 1999; Niemietz & Tyerman 2000). Thus, this aquaporin may mediate several facets of exchanges between the infected root cells and the endosymbiot and possibly contributes to the osmoregulation of the peribacteroid space. Recently, evidence for a contribution of PIPs to CO_2 diffusion across leaf tissues has been presented by several groups (Terashima & Ono 2002; Uehlein *et al*. 2003; Hanba *et al*. 2004). These observations establish new important links between aquaporin function and control of photosynthesis and growth.

For the sake of concision, the present review will concentrate on the central function of aquaporins, namely water transport. We will consider an important aspect of this function: the dynamic control of the water permeability of plant cells and organs. Such control occurs during development but also in response to external stimuli. In particular, plants can experience environmental conditions that are very challenging for their water status. A large body of physiological data has revealed that plants alter the water transport properties of their organs and cells in responses to stresses such as drought, salinity, and cold but also changes in light, nutrient deficiency or soil acidity (Steudle 1994; Clarkson *et al*. 2000; Javot & Maurel 2002).

Herein, we will address more specifically the significance in water relations of aquaporin regulation in response to these environmental stimuli. A few examples, selected in the recent literature, will be used to illustrate the variety of molecular and cellular mechanisms involved and show how these contribute to the extended capacity of plants to adjust their water status in response to environmental challenges.

TRANSCRIPTIONAL CONTROL

Because of their abundance in transcripts and their regulation by developmental and environmental cues, aquaporin genes have been the objects of extensive expression studies. Early works have revealed that certain aquaporin isoforms show a strict tissue expression pattern, such as seed-specific α-TIP in bean (*Phaseolus vulgaris*) (Johnson, Höfte & Chrispeels 1990) or root-specific TobRB7 in tobacco (*Nicotiana tabacum*) (Yamamoto *et al*. 1991), whereas other isoforms are expressed throughout the plant. Transcriptional regulation of aquaporin genes by hormones such as ABA or GA_3 or environmental stimuli as diverse as low temperature, drought, salinity, light (daily rhythm), and nutrient deprivation or supply was also revealed. These properties have been assessed in many plant species including Arabidopsis (Liu *et al*. 2003; Maathuis *et al*. 2003; Jang *et al*. 2004), rice (Lian *et al*. 2004), maize (Gaspar *et al*. 2003; Lopez *et al*. 2003, 2004), barley (Katsuhara *et al*. 2002, 2003), radish (Suga *et al*. 2003), and walnut tree (Sakr *et al*. 2003). An extensive review of this topic has been presented elsewhere (Maurel *et al*. 2002). To date, the most striking parallel between aquaporin gene expression levels and regulation of water transport at the tissue and cell level has been observed under conditions of varying light. For instance, diurnal variation of root hydraulic conductivity in *Lotus japonicus* was well correlated to changes in abundance of mRNAs coding PIP1 homologues (Henzler *et al*. 1999). In motor cells of *Samanea saman*, expression of a PIP2 aquaporin gene was under circadian control and was tightly correlated to diurnal leaflet movements and changes in the water permeability of isolated protoplasts (Moshelion *et al*. 2002).

The extensive sequencing of several plant genomes and ESTs libraries has provided the complete or nearly complete set of aquaporins present in several plant species. These resources allowed the development of efficient techniques for thorough transcriptome analysis. Large-scale microarray studies in Arabidopsis, barley and rice have confirmed that transcription of aquaporin genes shows a remarkable responsiveness to multiple stresses such as cold, drought, high salinity, anoxia, or mineral starvation (Kawasaki *et al*. 2001; Bray 2002; Klok *et al*. 2002; Seki *et al*. 2002; Maathuis *et al*. 2003; Bray 2004; Jang *et al*. 2004; Ueda *et al*. 2004). In a study in which expression of all putative membrane transporters was investigated in Arabidopsis roots, Maathuis *et al*. (2003) showed that aquaporins and V-Type ATPases were the only classes that responded to all cation stresses investigated. In particular, a co-ordinated down-regulation of most aquaporin isoforms was observed early after deprivation of plants from calcium or exposure to high salt concentration, suggesting that a global response was required to face these severe stress conditions. Down-regulation of root hydraulic conductivity following salt exposure is observed in many plant species and represents one of best-characterized examples of stress-induced regulation of water transport in plants. In another study, Jang *et al*. (2004) investigated expression of all 13 Arabidopsis PIPs using real-time reverse transcription-PCR. In contrast to microarray studies, this approach allowed a clear identification of the most highly expressed genes in roots and aerial parts and showed differential regulation profiles, thus

pointing to isoform-specific functions for PIPs in the response of plants to abiotic stresses. Whereas extensive expression analyses have been performed in roots, an organ in which aquaporin function has been clearly identified (Javot & Maurel 2002), much needs to be learned about aquaporin gene expression and regulation in other organs. Aquaporins may have a critical role in stomatal movements as suggested by inhibition of stomatal oscillations by treatment with $HgCl_2$, a general inhibitor of aquaporins (Li *et al*. 2004). Yet, there is to date a restricted number of reports on aquaporin expression in guard cells of sunflower (*Helianthus annuus*) (Sarda *et al*. 1997), *Vicia faba* (Sun *et al*. 2001), and *Nicotiana glauca* (Smart *et al*. 2001). Interestingly, Sarda *et al*. (1997) showed that the transcript level of a TIP homologue, *suntip7*, was well correlated with the daily fluctuation of stomatal conductance in sunflower.

REGULATION OF AQUAPORIN ABUNDANCE

Aquaporins mediate mass flows of water within the plant, and the abundance of the proteins is a critical parameter to understand their function at the tissue, cell, or subcellular levels. Most early studies on aquaporin expression have relied on immuno-detection approaches. Yet, cross-reactivity of antibodies with several close aquaporin homologues can be anticipated, in the PIP subfamily in particular where a high sequence identity (> 95%) can be observed between members. In these respects, Kirch *et al*. (2000) pointed out that isoform-specific antibodies should be raised against the most divergent hydrophilic region of these proteins, that is, against the second extracellular loop (loop C). By contrast to immuno-detection techniques, mass spectrometry analysis of aquaporins can provide an accurate distinction of very close aquaporin homologues (Santoni *et al*. 2003). However, as it stands, this approach remains mostly qualitative and does not provide precise information on protein abundance.

There have been several recent reports showing that the respective abundance in aquaporin transcripts and in the encoded proteins are not necessarily correlated. In radish (*Raphanus sativus*) roots for instance, the abundance of mRNAs encoding some PIP2 isoforms was increased transiently in response to an osmotic stress whereas the transcripts for several TIP isoforms were constitutively expressed. Yet, the protein levels of all these isoforms remained constant, suggesting the occurrence of post-transcriptional regulations for PIPs (Suga, Imagawa & Maeshima 2001; Suga, Komatsu & Maeshima 2002; Suga *et al*. 2003). Thus, a note of caution should be taken when interpreting transcriptomic data.

Despite the restrictions above, there are numerous reports providing clear evidence that the abundance of aquaporins can be regulated by developmental and environmental factors (Maurel *et al*. 2002). In the resurrection grass *Eragrostis nindensis* for instance, a TIP3; 1 homologue was detected in the abundant small vacuoles formed in the bundle sheath cells of desiccant-tolerant leaves (Vander Willigen *et al*. 2004). Expression occurred specifically under conditions of leaf desiccation, suggesting that this aquaporin functions during subsequent leaf re-hydration to facilitate both water and solute transport in and out of the small leaf vacuoles (Vander Willigen *et al*. 2004). In walnut trees as in other plants, xylem vessels can undergo embolism due to freeze–thaw cycles during winter. Sakr *et al*. (2003) observed that both PIP transcripts and proteins accumulated during winter in parenchyma cells associated with xylem vessels. It was hypothesized that these proteins facilitate the refilling of embolized vessels by mobilizing water from surrounding cells (Sakr *et al*. 2003).

Although the proteolytic cleavage of a TIP homologue from a 29-kDa precursor to a 23-kDa product has been described in pumpkin seeds (Inoue *et al*. 1995), the cellular pathways that determine aquaporin degradation and/or stability are nearly unknown in plants. Mammalian AQP1 was shown to be ubiquitinated in BALB/c fibroblasts, and a decrease in this modification under hypertonic stress was correlated with an increase in the AQP1 half-life (Leitch, Agre & King 2001). In plants, the ubiquitin/26S proteasome pathway is involved in many facets of growth, development, reproduction, and defence (Vierstra 2003), and may control plant aquaporin stability as well.

DYNAMICS OF AQUAPORIN SUBCELLULAR LOCALIZATION

In animals, the subcellular relocalization of aquaporins is a well-established mechanism for mediating rapid changes in membrane permeability. In particular, immuno-histochemical approaches have revealed how some mammalian aquaporins can shuttle from intracellular vesicles to the plasma membrane in response to various stimuli. For instance, AQP1 is relocated to apical plasma membranes of cholangiocytes upon secretin induction (Marinelli *et al*. 1999) whereas AQP2 is routed to apical membranes of kidney collecting duct epithelia under vasopressin regulation (Brown 2003). Shuttling of AQP5 and AQP8 to apical plasma membranes of parotid acinar cells through nitric oxide/cGMP transduction (Ishikawa, Iida & Ishida 2002) and to canalicular membranes of hepatocytes upon cAMP stimulation (Huebert *et al*. 2002), respectively, has also been reported. In the case of AQP2, it was shown that stimulus-induced subcellular targeting is dependent on phosphorylation of a Ser residue (Ser256) in the C-terminal tail of the protein. The balance between phosphorylated and dephosphorylated AQP2 subunits was found to critically determine the subcellular fate of the aquaporin tetramers (Kamsteeg *et al*. 2000). AQP2 phosphorylation is mediated by a protein kinase A (PKA) following binding of vasopressin to an adenylyl cyclase-V2 coupled receptor, and subsequent increase in cytosolic cAMP levels. Recent studies have shown that transfer of AQP2 through the Golgi apparatus is also controlled by phosphorylation of Ser256, but by another stimulus-independent protein kinase, possibly a Golgi-casein kinase (Procino *et al*. 2003).

In *Mesembryanthemum crystallinum* suspension cells and leaves, immuno-detection of a TIP homologue (McTIP1; 2,

formerly McMIPF) in membrane fractions separated by sucrose density gradient centrifugation revealed that this aquaporin can be redistributed from tonoplast fractions to other endomembrane fractions (Vera-Estrella *et al*. 2004). Redistribution was observed for instance after hyperosmotic treatment of suspension cells with mannitol. Labelling by an anti-McTIP1;2 antibody was then associated with small intracellular vesicles (Vera-Estrella *et al*. 2004). Interestingly, the labelling of similar structures has already been observed in young cotyledon cells of Arabidopsis expressing an AtTIP1;1-GFP protein fusion (Saito *et al*. 2002), but in the absence of any stimulus. Mannitol-induced redistribution of McTIP1;2 may involve complex mechanisms for vesicle sorting. This process was altered by brefeldin A, which inhibits endoplasmic reticulum to Golgi apparatus membrane trafficking; by wortmannin, which blocks endocytosis; and by cytochalasin, which promotes depolymerization of actin filaments. Similar to the observations for vasopressin-induced relocalization of mammalian AQP2, redistribution of McTIP1;2 could directly be induced by cAMP agonists and a PKA-like activation pathway seems to be involved (Vera-Estrella *et al*. 2004). Furthermore, it was observed that treatment of Mesembryanthemum suspension cells by tunicamycin, which inhibits the formation of N-glycosidic protein carbohydrate linkages, blocked mannitol-dependent relocalization of McTIP1;2 (Vera-Estrella *et al*. 2004). Glycosylation of plant aquaporins was first identified in the case of Nodulin 26 (Miao, Hong & Verma 1992) but its functional significance has remained unknown until now. Altogether, the series of elegant experiments reported by Vera-Estrella *et al*. (2004) provides significant insights into a novel mechanism for regulation of aquaporins in response to osmotic stress. It also points to novel roles for aquaporin post-translational modification. By contrast, no re-localization mechanism has so far been identified for plant plasma membrane aquaporins.

GATING OF AQUAPORIN WATER CHANNELS

Reversible phosphorylation

Early studies on protein storage vacuole α-TIP from bean seeds revealed this protein to be phosphorylated at a Ser residue (Ser7) localized on its N-terminal tail (Johnson & Chrispeels 1992). First evidence that phosphorylation regulates the water transport activity of plant aquaporins was provided by heterologous expression of α-TIP in Xenopus oocytes (Maurel *et al*. 1995). The use of cAMP agonists to promote α-TIP phosphorylation by endogenous PKA, together with the analysis of aquaporin mutants carrying substitution of single Ser residues was used to delineate the functional role of Ser7 and of two additional phosphorylation sites, at Ser23 on the N-terminal tail and Ser99 in the first cytosolic loop (loop B). Phosphorylation of each of these sites was found to individually enhance the apparent water transport activity of α-TIP in oocytes. A similar approach was used to show that the activity of PM28A, a PIP2 homologue abundantly expressed in leaves of spinach (*Spinacia oleracea*) is enhanced by phosphorylation of two serine residues, Ser115 in loop B and Ser274 in the C-terminal tail (Johansson *et al*. 1998). By contrast, soybean Nodulin-26 can be activated through phosphorylation at a single site (Ser262) (Guenther *et al*. 2003). Experiments *in planta* using radiolabelled phosphate revealed that phosphorylation of PM28A in spinach leaves is reduced under osmotic stress conditions (Johansson *et al*. 1996). By contrast, phosphorylation of Nodulin-26 as revealed by an antibody specific for phosphorylated Ser262 appeared during nodule development and was enhanced by water deprivation conditions and salinity (Guenther *et al*. 2003). In the latter case, measurements of water transport in purified symbiosome membrane vesicles provided unambiguous evidence that phosphorylation of Nodulin-26 directly gates the water channel and does not exclusively control aquaporin subcellular localization as was demonstrated in the case of mammalian AQP2. In a very recent study, Azad *et al*. (2004a, b) showed that plasma membrane aquaporin homologues present in tulip petals can be phosphorylated by a Ca^{2+}-dependent protein kinase and dephosphorylated by a type-2 A protein phosphatase. Phosphorylation of the aquaporins was closely correlated to temperature-dependent opening and closing of petals, suggesting that aquaporins mediate a large part of water movements accompanying these processes. Altogether, these data establish reversible phosphorylation as a potent mechanism for plant aquaporin regulation, during development and in the response of plants to environmental stimuli.

Protons and calcium

Evidence that water transport across plant membranes can be regulated by pH was obtained in vacuoles isolated from parenchyma of sugar beet (*Beta vulgaris*) storage roots (Amodeo *et al*. 2002). Swelling assays on isolated vacuoles at different pH suggested that protons were blocking water transport by acting on mercury-sensitive channels present in the tonoplast. In a parallel study, Gerbeau *et al*. (2002) used stopped-flow spectrophotometry to show that protons also reversibly inhibit water channels in plasma membrane vesicles purified from Arabidopsis suspension cells. Half inhibition occurred at pH 7.2–7.5, suggesting that protons were acting on the cytosolic side of the membrane.

Tournaire-Roux *et al*. (2003) recently investigated the relevance of this process, for regulation of water uptake in Arabidopsis roots. As in many other plant species, hypoxic stress in Arabidopsis results in a reduction of root hydraulic conductivity. A fall in cytosolic pH is one other early response that typically accompanies hypoxia. To establish a link between these two responses, Tournaire-Roux *et al*. (2003) made parallel measurements in excised roots of water transport using a pressure chamber and of cytosolic pH using *in vivo* ^{31}P-nuclear magnetic resonance spectroscopy. Measurements under hypoxic conditions, after treatment with inhibitors of cytochrome pathway respiration which mimic oxygen deprivation, or after loading of root

tissues with diffusible weak acids provided strong evidence that cytosolic acidosis reversibly inhibits root hydraulic conductivity. This phenomenon was related to the regulation behaviour of several PIP isoforms which upon expression in Xenopus oocytes all showed specific inhibition by acid load-induced cytosolic acidosis (Tournaire-Roux *et al*. 2003). The role in this regulation of cytoplasmically exposed His residues was investigated by site-directed mutagenesis of a representative PIP homologue (PIP2;2). Substitutions of a single His residue (His197) in the second intracellular loop (loop D) of the protein led to pH-insensitive aquaporin mutants, suggesting that this residue plays a critical role in pH sensing. Interestingly, this His residue is conserved in all plant PIPs and is specific for this subclass of aquaporins. Altogether, these data show that regulation of plasma membrane aquaporins by cytosolic protons through a conserved mechanism provides a means for coordinated regulation of water transport in roots under anoxic stress. Whether this mechanism has a broader relevance, for regulation of water transport in other organs or under other stress conditions is under investigation in our laboratory.

Expression of mammalian AQP0, AQP3, and AQP6 in Xenopus oocytes has also revealed a pH-dependent activity for these aquaporins (Zeuthen & Klaerke 1999; Yasui *et al*. 1999; Nemeth-Cahalan & Hall 2000; Nemeth-Cahalan, Kalman & Hall 2004). In these cases, however, protons are sensed extracellularly and the role of a His residue localized in the first extracellular loop (loop A) of AQP0 was established (Nemeth-Cahalan *et al*. 2004). No plant aquaporin has been shown to be regulated by extracellular (cell wall) protons so far.

The His residues involved in pH sensing by plant and animal aquaporins clearly span the inner- and outer-vestibule of the aqueous pore but the precise molecular mechanisms which permit channel gating by intra- or extracellular protons are currently unknown. In the future, the dissection of this regulation process may be instrumental to understanding of what drives the transition of water channels between open and closed states, a key issue in aquaporin research.

Similar to protons, Ca^{2+} and other divalent ions can down-regulate water channels in purified plasma membrane vesicles. This process requires Ca^{2+} concentrations, in the range of 10–100 μM (Gerbeau *et al*. 2002), which is well beyond concentrations commonly observed in the plant cytoplasm and the mechanism of this inhibition, whether direct or indirect, is currently unknown. Physiological measurements in maize and melon (*Cucumis melo*) also showed that treatment with calcium counteracts the negative effects of NaCl on water permeability of whole roots, root cortical cells and protoplasts (Azaizeh, Gunse & Steudle 1992; Carvajal, Cerda & Martinez 2000; Martinez-Ballesta, Martinez & Carvajal 2000). These effects are clearly distinct from those observed in isolated plasma membrane vesicles and the underlying mechanisms, which possibly involve a role for Ca^{2+} in salt stress signalling, remain as yet unknown.

EMERGING MECHANISMS FOR PLANT AQUAPORIN REGULATION

Regulation by hetero-tetramer formation

Structural models of mammalian AQP1 and bacterial GlpF at 2.3 Å resolution have provided exquisite molecular details on how aquaporins spontaneously assemble as homo-tetramers, each monomer delineating a functionally independent water channel (Fujiyoshi *et al*. 2002). Plant aquaporins also exhibit such a conserved homo-tetrameric organization. This was demonstrated by cryo-electron microscopy of two-dimensional crystals formed by bean α-TIP (Daniels, Chrispeels & Yeager 1999) or spinach PM28A (PIP2) and PM28C (PIP1) (Fotiadis *et al*. 2001). An elegant study by Fetter *et al*. (2004) suggests however, that heteromers comprising members of the PIP1 and PIP2 subgroups may occur. When expressed independently in oocytes, aquaporins of the PIP1 subgroup, in contrast to those of the PIP2 subgroup, fail to increase the osmotic water permeability of the oocyte membrane. Surprisingly, co-expression of a seemingly inactive PIP1 (ZmPIP1;2) with reduced amounts of a functional PIP2 (ZmPIP2;5) led to a marked increase in permeability, which was proportional to the amounts of ZmPIP1;2 expressed. The idea that aquaporins of the PIP2 subgroup interact with ZmPIP1;2 to favour targeting of the latter to the oocyte plasma membrane was corroborated by confocal microscopy observations of oocytes expressing a ZmPIP1;2-GFP fusion protein. Physical interaction and heteromerization of ZmPIP1;2 and ZmPIP2;1 were demonstrated by co-purification. Furthermore, an essential role of the third extracellular loop (loop E) in interaction specificity between PIP1 and PIP2 homologues was demonstrated. Although these experiments were exclusively developed in Xenopus oocytes, they suggest the occurrence of a novel mechanism for controlling by hetero-tetramer formation the expression of aquaporins at the plant cell surface. This idea may be related to early observations made in Arabidopsis plants expressing antisense transgenes targeted to members of either the PIP1 or the PIP2 subgroup. In these experiments, it was found that down-regulation of PIP1 or PIP2 aquaporin genes, independently or in combination, resulted in a similar reduction in root hydraulic conductance (Martre *et al*. 2002). This intriguing observation was interpreted to mean that aquaporins of the PIP1 and PIP2 subgroups function in a co-operative manner to determine plant plasma membrane water permeability (Martre *et al*. 2002; Fetter *et al*. 2004).

Regulation by reactive oxygen species

Studies in cucumber (Lee, Singh & Chung 2004a; Lee *et al*. 2004b), tomato (Bloom *et al*. 2004), spinach (Fennell & Markhart 1998), and maize (Aroca *et al*. 2001, 2003; Melkonian, Yu & Setter 2004) have shown that plants respond to chilling by decreasing their root hydraulic conductivity. This effect was recently suggested to be mediated by hydrogen peroxide since exposure of roots to chilling caused a release

of this compound in the vicinity of the plasma membrane and its application decreased the hydraulic conductivity of cortical cells (Lee *et al*. 2004a). Reactive oxygen species were also found to inhibit the activity of water channels in *Chara corallina* internodal cells (Henzler, Ye & Steudle 2004). With an induced decrease in cell hydraulic conductivity by 90%, these compounds proved to be even more efficient than conventional mercury salts. It was proposed that hydroxyl radicals act through a so-called oxidative gating mechanism either by direct oxidation of the aquaporins or indirectly through lipid membrane oxidation and formation of secondary radicals (Henzler *et al*. 2004). Reactive oxygen species have emerged as critical elements in cell signalling (Foreman *et al*. 2003; Kwak *et al*. 2003). They may also trigger a cascade of events ultimately leading to aquaporin down-regulation.

Regulation of aquaporins by osmotic and hydrostatic pressures

Contrary to a preconceived idea, there has been very little evidence until recently that the water permeability of biological membranes is dependent on the forces that drive water transport itself. In animals for instance, there is no clear evidence that aquaporins can be gated by hydrostatic or osmotic gradients. In plants, a few early reports mentioned that the osmotic water permeability of certain plant membranes, such as tonoplast vesicles isolated from wheat root, may be dependent on the strength of the imposed osmotic gradient (Niemietz & Tyerman 1997). Novel insights into these mechanisms were provided by a recent study by Ye, Wiera & Steudle (2004). These authors showed that the water channels of isolated *Chara corallina* internodes can be inhibited in the presence of high concentrations (up to 800 mM) of glycol ethers of different size. Inhibition increased both with increasing concentration and with increasing size of the osmolyte, suggesting a cohesion–tension model whereby exclusion of osmolytes from the water channel pore may cause tensions in the protein. These would in turn result in a reversible deformation and closure of the pore. Although this process is biophysically very intriguing, its significance for regulation of water channels under physiological conditions awaits further evidence.

In another recent study, Moshelion, Moran & Chaumont (2004) developed computational models to account for osmotically induced changes in maize protoplast volume. An initial delay in swelling following imposition of a hypo-osmotic challenge was observed, leading the authors to hypothesize that dynamic changes in water permeability (water channel activity) occurred upon swelling. The molecular and cellular mechanisms that underlie this hypotonicity-induced response are still unknown.

Turgor, namely cell hydrostatic pressure, is still another parameter which may regulate water channel opening. A cell pressure probe consists of an oil-filled microcapillary linked to a mechanical device. The probe is inserted into a living plant cell and can be used to impose hydrostatic pressure pulses. Wan, Steudle & Hartung (2004) recently reported that pressure pulses of varying amplitudes differentially altered the hydraulic conductivity of maize root cortical cells. The extent of inhibition was related to the amplitude of water movements triggered by the pressure changes, suggesting that a high rate of flow in the water channel may impose high tensions at the constriction of a pore, thereby causing a conformational change and its closure. The authors also observed that pressure-dependent inhibition of water channels could be counteracted by application of ABA. This observation does not fit well with a purely mechanical model and suggests that an indirect mechanism involving pressure sensing and downstream cell signalling events may also be involved.

CONCLUSION AND PERSPECTIVES

Prospects in the study of aquaporin regulation

In recent years, a large variety of mechanisms have been unravelled that can account for the regulation of plant aquaporins at the tissue, cellular, subcellular and molecular levels (Fig. 2). Yet, much remains to be discovered and an ever-greater molecular and cellular complexity can be anticipated. Aquaporins probably interact with protein partners, for post-translational modification, subcellular targeting, gating or even degradation and none of these partners has been identified so far. While the functional role of aquaporin glycosylation and phosphorylation is starting to emerge, plant aquaporins, similar to their mammalian counterparts, probably undergo other types of post-translational maturations, such as ubiquitination and/or controlled protein cleavage, or even nitrosylation (Schey *et al*. 1999; Hess *et al*. 2001; Leitch *et al*. 2001). These processes remain to be formally identified in plants.

It also appears that the various mechanisms that determine aquaporin regulation do not function individually. For instance, water stress acts on aquaporin function at the level of transcription, protein relocalization, and gating through reversible phosphorylation or direct effects of osmotic or hydrostatic gradients. Thus, a challenge for future research will be to understand how plants integrate these mechanisms in time and space, to constantly adjust the water transport and solute transport properties of their membranes.

Genetic manipulation of aquaporin functions in plants provides a promising strategy to address such questions. Antisense inhibition of PIP aquaporins in tobacco and Arabidopsis resulted in a marked defect in the plants ability to recover from water stress, suggesting that aquaporin functions, and possibly their adjustment, were of critical importance during this process (Martre *et al*. 2002; Siefritz *et al*. 2002). A novel step forward was made in a recent study by Lian *et al*. (2004). These authors identified a rice PIP aquaporin (RWC3) whose expression is induced by osmotic challenge, specifically in a drought-tolerant rice cultivar (Lian *et al*. 2004). Over-expression of this gene in a drought-sensitive cultivar, under the control of a stress-inducible promoter, was able to improve the growth per-

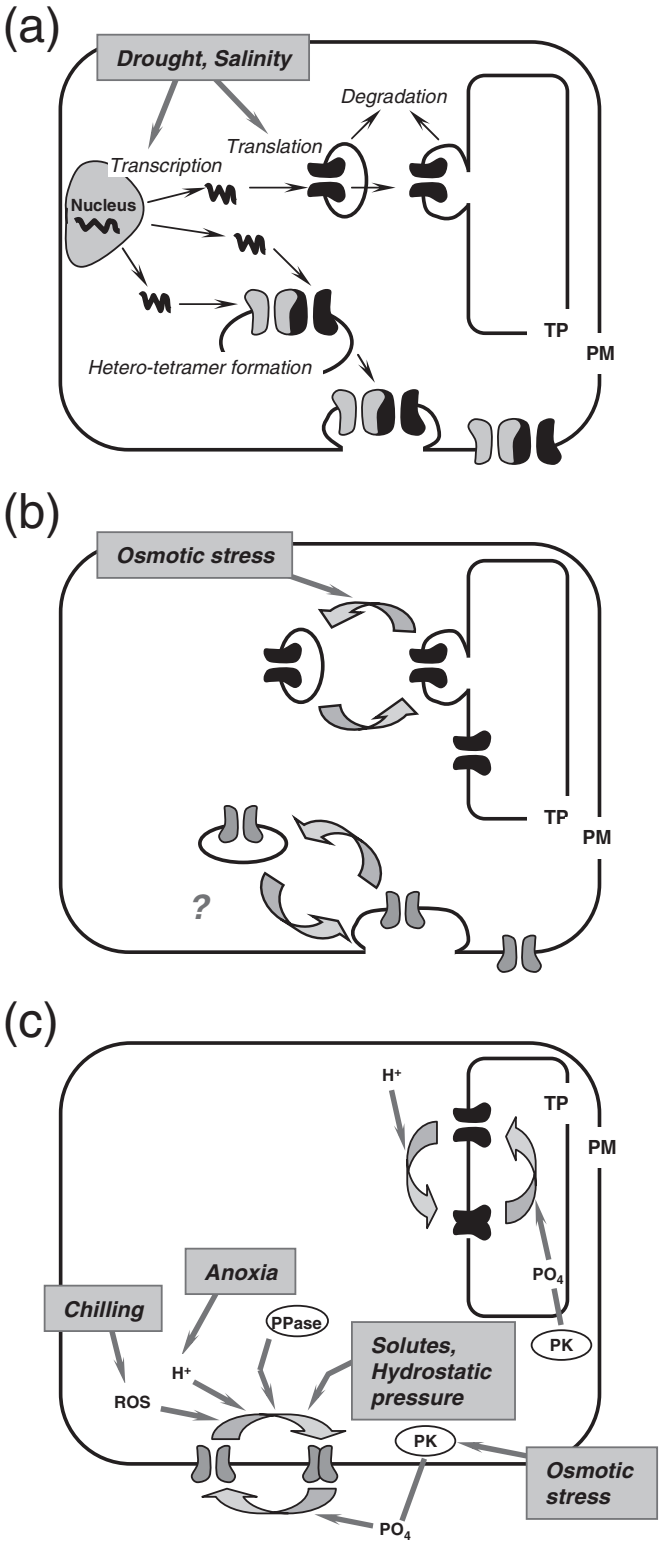

Figure 2. Schematic representation of putative mechanisms involved in plant aquaporin regulation. (a) Control of transcription and protein abundance. Drought and salinity, as other environmental stimuli, are known to act on aquaporin gene transcription and possibly interfere with aquaporin translation and degradation, thereby determining protein abundance. The formation of PIP hetero-tetramers was demonstrated in Xenopus oocytes (Fetter *et al*. 2004) and is still hypothetical in plant cells. This mechanism might favour transfer of PIP1 homologues to the plasma membrane (PM). (b) Sub-cellular relocalization. The redistribution of a TIP aquaporin, from the tonoplast (TP) to small intracellular vesicles, was demonstrated in *Mesembryanthemum crystallinum* suspension cells exposed to a hyperosmotic treatment (Vera-Estrella *et al*. 2004). The occurrence of a similar relocalization mechanism for PIP aquaporins is shown but remains hypothetical. (c) Control of channel gating. Reversible phosphorylation of aquaporins is mediated through the activity of protein kinases (PK) and protein phosphatases (PPase) and regulates their opening and closing in the TP, PM, and peribacteroid membrane of N_2-fixing symbiotic root nodules (not shown). Intracellular protons can induce closure of aquaporins in the TP and PM. Reactive oxygen species (ROS), high solute concentrations, and cell hydrostatic pressure act on aquaporin activity but it remains unclear whether their effects are direct or indirect. The graph also shows the effects of chilling, anoxia, and osmotic stress, on accumulation of ROS, accumulation of intracellular protons, and activity of a PK, respectively.

formance of this cultivar, specifically under stress. In contrast, a study by Katsuhara *et al*. (2003) showed that heterologous over-expression of a barley PIP homologue in transgenic rice had the opposite effects and raised the salt sensitivity of transgenic plants. Altogether, these observations show that isoform-specific functions exist, which may rely in part on their integration in well-defined cell regulation mechanisms

We also anticipate that useful information will be inferred from expression of deregulated aquaporins in

transgenic plants. Following a study on pH regulation of aquaporins in roots under anoxic stress, our laboratory has produced transgenic plants that ectopically express pH-insensitive aquaporin mutants. These plants will hopefully be instrumental for testing the adaptive value of pH-dependent aquaporin regulation.

Towards an integrated view of aquaporin regulation in roots?

A large body of physiological data exists on the regulation of root water transport during development and under stress conditions. This has provided a very valuable ground for the discovery of original mechanisms for aquaporin regulation (Javot & Maurel 2002; Tournaire-Roux *et al*. 2003).

In many plant species, root hydraulic conductivity is under diurnal control due in part to circadian regulation of aquaporin gene expression. During the day, this control would enhance the water uptake capacity of the plant, when the transpiration demand is at its maximum. In contrast to light, many environmental stresses have adverse effects on roots and down-regulate their hydraulic conductivity. Recent studies have revealed that partially distinct mechanisms can account for the response of roots to different stresses. Thus, the effects of salinity, chilling or hypoxia on root aquaporins are mediated in part through transcriptional control, inhibition by reactive oxygen species or cytosolic acidosis, respectively (Maathuis *et al*. 2003; Tournaire-Roux *et al*. 2003; Lee *et al*. 2004a). Root water transport is also down-regulated in response to nutrient deficiency and soil acidity, and the mechanisms involved need to be discovered. Another important objective will be understanding the signalling mechanisms that lead from stimulus perception by root cells to aquaporin inhibition.

In addition, the physiological significance of root aquaporin regulation under stress is still unclear. We speculate that this may reflect a unifying strategy for optimal co-regulation of water and solute transport in varying environmental conditions. Reduced water uptake under salt exposure may prevent a mass flow of salt towards the aerial parts. Anoxia can be induced by abrupt soil flooding and, in these conditions, down-regulation of water uptake may prevent the sudden dilution of xylem sap in conditions where the capacity of cells for solute pumping is reduced because of depleted ATP stocks. We also speculate that these mechanisms may allow plants to adjust their root uptake capacity under conditions of local soil heterogeneity. Reduced water uptake from soil portions contaminated by salt or colonized by microbes (these creating hypoxic conditions for roots) may prevent the drag of toxic salt or microbial toxins to the root surface. In contrast, enhanced water uptake in nutrient rich areas may promote the diffusion of these nutrients to the root vicinity. Here again, we anticipate that plants expressing deregulated aquaporins will provide unique materials to address these crucial physiological questions.

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