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AQUAPORINS IN THE PLANT KINGDOM: THE REGULATORY MECHANISMS REVISITED.

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Running Title: ***Regulation in plant aquaporins.***

More than 30 years ago, biophysicist and animal physiologists supported the hypothesis of the existence of pores facilitating water transport through membranes since certain animal structures were unusually permeable to water. Although plant physiologists also discussed the existence of water channels since the early 1960s, the survey remained marginal in the field (reviewed by Chrispeels and Maurel 1994). It was in 1992 that the hypothetical proteinaceous water channel was identified (named CHIP28, now AQP1) by Preston *et. al.* This discovery opened the molecular detection of homologous proteins in all kingdoms. The term "aquaporins" (AQPs) was suggested later, when other two proteins belonging to the MIP26 family (WCH-CD -from mammalian collecting duct- and γ-TIP -from tonoplast of *Arabidopsis thaliana*-) were also characterized as water channels (Agre *et al.,* 1993). The first cloned and functionally expressed aquaporin from plants was therefore γ-TIP (now TIP1;1) (Maurel *et al.,* 1993). Since this event, plant aquaporins captured significant attention.

This article intends to explore the regulatory mechanisms in plant aquaporins and to contrast them with those achievements made on their animal homologues. The aim is to merge the contributions made by both fields of research.

Aquaporins in plants.

In mammals, 13 aquaporins have been identified and named by numbers, from AQP0 to AQP12. In contrast, an unexpectedly large number of these proteins have been found in plants; for instance, 35 aquaporin genes have been detected in the *Arabidopsis thaliana* genome and 36 in *Zea mays* (reviewed by Chaumont 2005). This huge amount of plant water channels has been divided into four subfamilies on the basis of sequence homology: TIPs (tonoplast intrinsic proteins), PIPs (plasma membrane intrinsic proteins), NIPs (nodulin26-like intrinsic proteins) and a small group named SIPs (small and basic intrinsic proteins), a classification which also seems to be coupled to its cellular localization. It is remarkable to observe that animal and plant aquaporins remained interestingly amalgamate if they are analyzed through a phylogenetic point of view. (**Figure 1**).

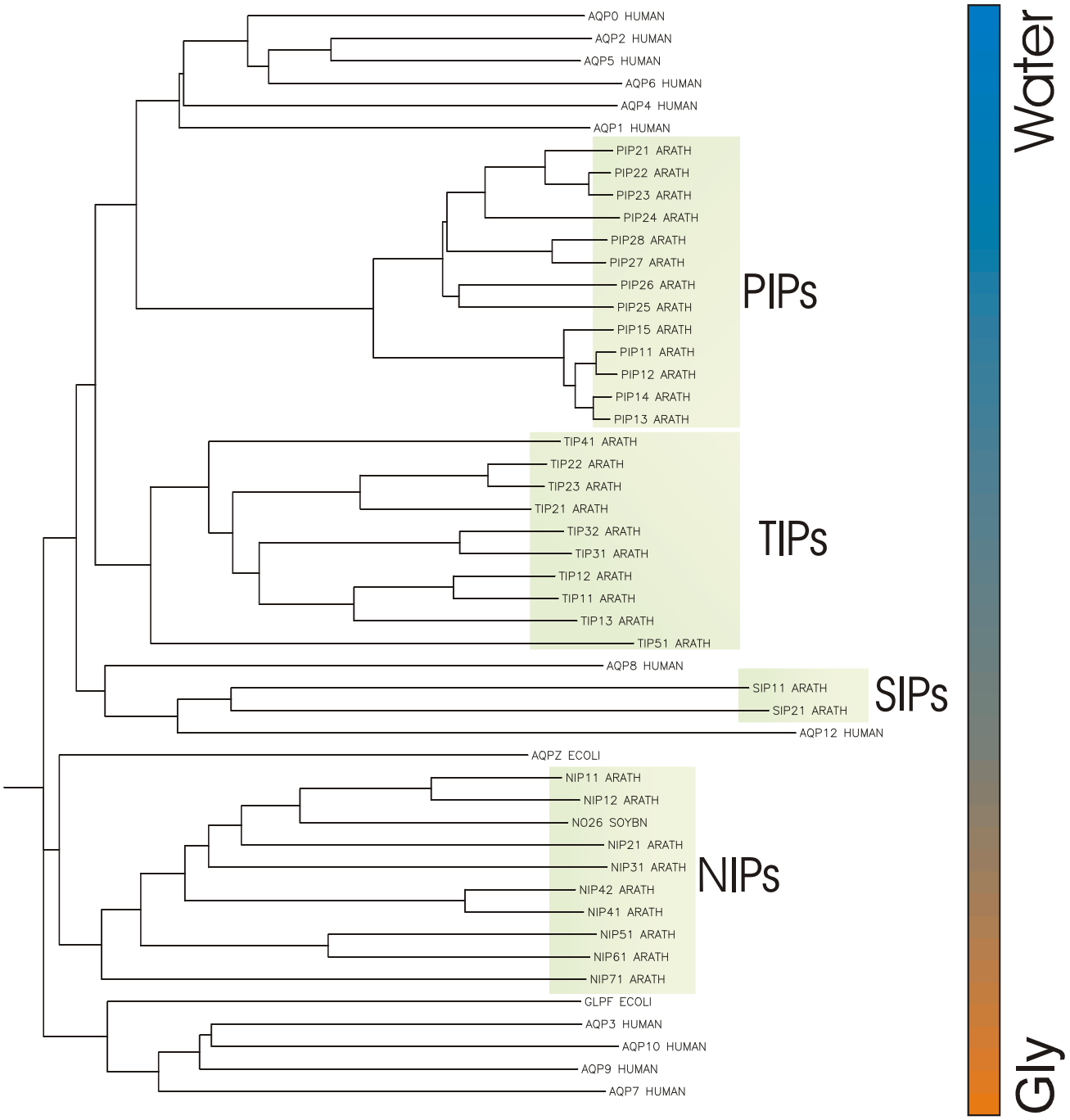

Fig. 1. Dendogram of AQPs *The phylogenetic sequence relationship between aquaporin homologues of human, the plant model Arabidopsis and the bacteria E. coli is shown. The figure indicates the classical subfamilies of plant aquaporins, PIPs, TIPs, NIPs and SIPs (see text) and suggest the degree in water vs. glycerol transport capacities of different subclasses of aquaporins. Amino acid sequences were aligned using ClustalW. The length of each pair of branches represents the distance between sequence pairs.*

The **TIP** subfamily includes plant aquaporins located in the plant vacuolar membrane. Five different subclasses of TIP have been identified in *Arabidopsis thaliana* genome (Quigley *et al.,* 2001) and it was described that TIP specific isoforms could be unambiguously related with distinct vacuole subtypes. For instance, expression of TIP2 is associated with vegetative storage protein vacuoles whereas TIP1 corresponds to lytic

vacuoles. Moreover, TIP3 in conjunction with TIP2 is commonly found in protein storage vacuole membranes in seeds while TIP3 expressed alone is linked with autophagic vacuoles (Jauh *et al.,* 1999). It is possible that this distribution of TIPs in separate plant vacuoles could be pointing to their active role in a functional specialization of vacuolar subtypes.

The other well-studied subfamily is **PIPs**. This is the largest and most conserved plant water channel subfamily and can be further divided into two subgroups, PIP1 and PIP2. The PIP subfamily received its name from their localization in the plasma membrane. Compared with PIP1 proteins, PIP2 have a shorter N-terminal fragment and a longer C-terminal end containing putative phosphorylation sites. PIP1 and PIP2 are known to show different water permeabilities when expressed in Xenopus oocytes -the classical technique to assay aquaporin activity after Preston (1992) (**Figure 2**). While PIP2 show high water transport activity, PIP1 are either inactive or have very low water transport activity when tested by the same technique (review by Chaumont *et al.,* 2005). Recently this fact was disclosed by Fetter *et al.* (2004). The authors showed that PIP1 could increase water permeability only if it is co-expressed with PIP2 due to physical interaction through loops E.

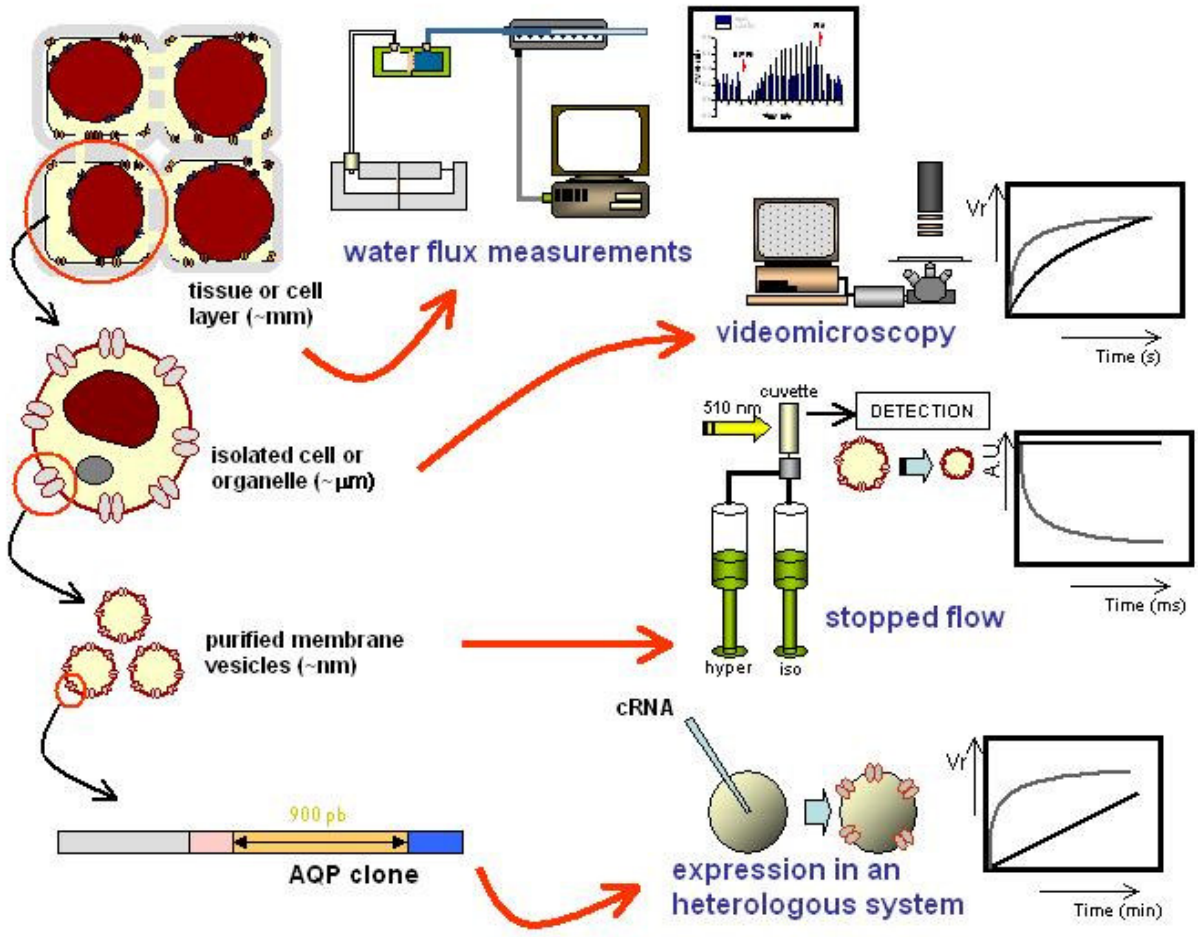

Fig. 2. Methods used to measure water transport. *Different techniques can be used to assess water transport depending on the organization level of the material employed. Water flux measurements at tissue level are usually performed by means of techniques where the hydraulic properties of a membrane or cell barrier are estimated. Water permeability can be obtained through measurement of volume changes by video-microscopy or stopped-flow depending on the size of the structure. For a direct analysis of the*

aquaporin, it is required to express the protein in a heterologous system (Xenopus oocytes) [for a detailed description of biophysical properties of water transport see Finkelstein (1987) and Parisi et al., 1987]

The first **NIP** identified was NOD26 (Weaver *et al.* 1994), a MIP member expressed in the peribacteroid membrane of soybean root nodules. Several NOD26 homologues have been identified but their subcellular localization remains elusive. NIP family members studied so far show similar functional properties, i.e. glycerol transport and low water transport (review by Vander Willigen *et al.,* 2004). Sequence analysis made on *Arabidoposis thaliana* genome indicates that NIP family members maintain higher homology to bacterial GlpF than to TIPs or PIPs (Quigley *et al.,* 2001).

The last group corresponds to SIP subfamily. This is the most diverged subclass of MIPs. Sequence analyses provided most of the information on them. SIPs present the shortest amino terminus and substitutions at loop B (see later), being NPT or NPL the sequences detected instead of the characteristic NPA motif of all other aquaporins. There is no data available on their function but a recent work described their localization at the level of the ER membrane (Ishikawa *et al*., 2005).

Aquaporin structure

Nowadays several aquaporin structures have been solved: mammalian AQP1 (the first one, cristalized by Waltz *et al*., 1994), AQP2 and AQP0, the bacterial ones AqpZ and GlpF -a homologous glycerol facilitator- and concerning plant aquaporins, αTIP and SoPIP2;1 (reviewed by Engel and Stahlberg, 2002; Vander Willigen *et al.,* 2004). The great number of available amino acid sequences along with the information provided by molecular dynamics simulation data, have opened the perspectives regarding function and selectivity of water channels. A very recent work reveals for instance the initial events governing gating in the SoPIP2;1 (Törnroth-Horsefield *et al*., 2005).

Aquaporins are very hydrophobic proteins with a molecular mass fluctuating between 27 and 31 kDa. All these proteins have six membrane-spanning α-helical domains and N- and C-termini located in the cytoplasm (**Figure 3**). Two loops, one cytosolic (Loop B) and other extra-cytosolic (Loop E) contain a characteristic and conserved Asn-Pro-Ala (NPA) motif and form two additional short hydrophobic helices that immerse halfway into membrane (**Figure 3**). A second narrower constriction, the aromatic/Arg (ar/R) region is formed above the NPA motif. Studies held on human AQP1 and GlpF, shows that the ar/R region and the NPA constrictions constitute the selectivity filters (review by Chaumont *et al.* 2005). Aquaporins assemble in membranes as tetrameters in which each monomer consists in a single water pore (**Figure 4**). Three-dimensional structures determined for AQP1 and GlpF shows that the functional unit in both proteins is a homotetramer (review by Engel and Stahlberg 2002; Vander Willigen *et al.,* 2004). On the contrary, plant aquaporin heterotetramers have been described. Heterooligomers of two tonoplast aquaporins from lentil seed have been detected in cross-linking experiments (Harvengt *et al.,* 2000). More recently, Fetter *et al.* (2004) demonstrated that plasma membrane aquaporins from different subfamilies (PIP1 and PIP2) could physically interact to promote water transport activity, opening a new regulating water permeation scheme.

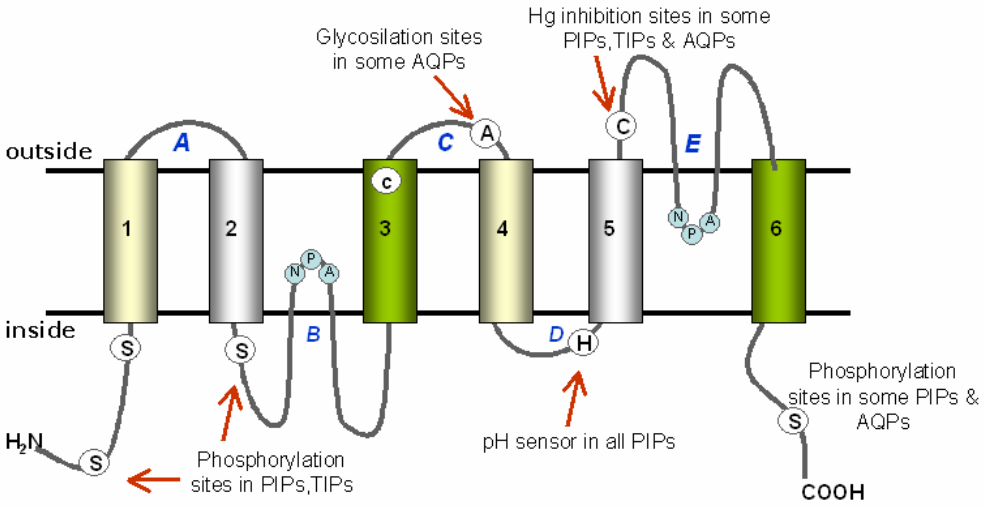

Fig. 3. Topological model of an AQP. *A schematic representation of the structure of an aquaporin based on topological studies from both plant and animal AQP homologues. The six transmembrane spanning domains (helices 1-6) and five connecting loops (A-E) are represented, loop B and E with the conserved NPA (Asn-Pro-Ala) motifs shown. Other residues with different degree of conservation are shown encircled.*

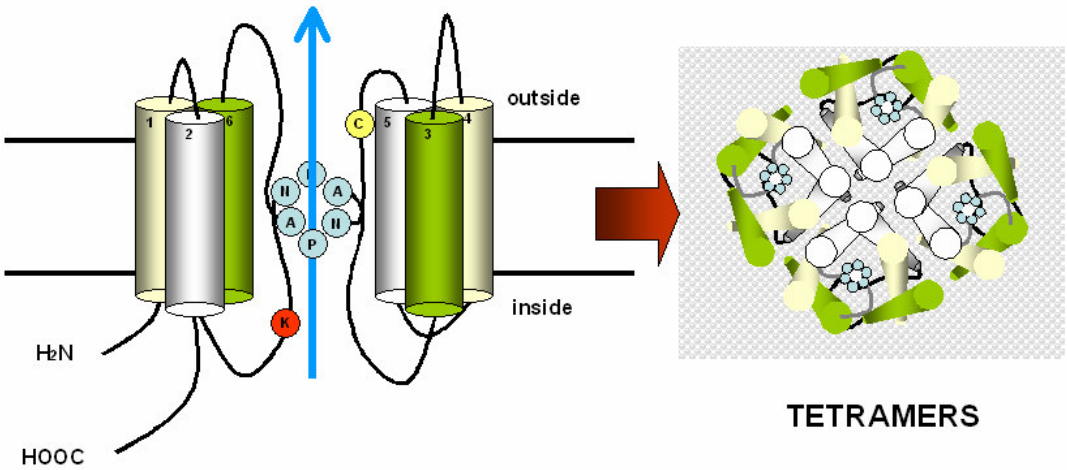

Fig. 4. Three-dimensional schematic organization of AQPs. *Each aquaporin monomer folds with both NPA motif forming the water pore. Tetrameric organization of aquaporin monomers is well established for all water channels being each monomer a unique water pore.*

What are water channels made for?

All aquaporins share the feature of having a pore with relatively high selectivity to water. However, several members of this broad family can transport small uncharged molecules instead of *or* in addition to water. For instance, mammalian AQPs 3, 7, 9 and 10

are known as aquaglyceroporins since they transport not only water but glycerol as well. Moreover, AQP1 seems also to be able to transport CO_2 and ammonia (Verkman, 2005). Interestingly enough, among plant aquaporins the spectra of solutes that can be transported is also wide. For example, members of PIP and NIP subfamilies have been shown to transport glycerol, *Nt*TIPa to transport urea and CO_2 in addition to glycerol and water (Gerbeau *et al.,* 1999). Other TIP homologues have been also identified as permeable to urea (review by Vander Willigen *et al.,* 2004) and NOD26 seems to transport ammonia (Niemietz and Tyerman, 2000). Besides these observations, there are also descriptions about transport of boron, antimonite or hydrogen peroxide by some plant water channels (Tyerman *et al.,* 2002).

All these selectivity attributes are well reflected in the already shown dendogram (**Figure 1**) where most channels that transport solutes are clustered together independently of their phylogenetical source. However, it must be pointed out that besides this selectivity profile can be explained on a structural basis alone, the physiological relevance of most of these transport activities is still an unresolved issue. Knock-out aquaporin studies performed in transgenic mice is an important tool that is helping not only to elucidate some of these aspects but also to find novel and unexpected functional roles (Verkman 2005).

Regulatory mechanisms

Long and short term regulatory mechanisms have been described in certain aquaporins. This water channel activity modulation helps to control the cell water balance. Among the short term regulatory mechanisms are included channel gating and trafficking while changes in aquaporin pattern expression are integrated in the long term ones.

a) *aquaporin trafficking:* In animals there are evidences of aquaporin trafficking between membranes, which allow fast changes in membrane water permeability. It has been confirmed than some mammalian aquaporins shuttle from intracellular vesicles to the plasma membrane in response to different stimuli (Brown, 2003; Marinelli *et al*., 2005). A well-known case are AQPs 1, 2, 5 and 8 whose redistributions depend on hormone levels or in second messenger stimuli. In plants, several evidences point also to aquaporin trafficking: i) changes in water permeability of protoplast membranes (Moshelion *et al.,* 2004), ii) unexpected patterns in the localization of PIPs, e.g. two *Mc*PIP isoforms located in the tonoplast fraction of a discontinous sucrose gradient (Kirch *et al.,* 2000); iii) *At*PIP1 homologues found in invaginations of the plasma membrane known as plasmalemmasomes (Robinson *et al.,* 1996); iv) redistribution of *Mc*TIP1;2 to endosomal compartments under osmotic stress (Vera-Estrella *et al.,* 2004) and v) detection of *Zm*PIP1;2 and *Zm*PIP2;5 fused to GFP, not only in plasma membrane, but also in intracellular membranes and perinuclear compartments (Chaumont *et al.,* 2000).

It is still not clear if plant aquaporin distribution follows a process similar to the one described for some mammalians AQPs which are submitted to recycle between the plasma membrane and internal vesicles according to hormonal or second messengers stimuli (review by Vander Willigen *et al.,* 2004). However, assuming that this scenario applies to plants, regulation of plant aquaporin trafficking seems to be a possible mechanism to modulate membrane water permeability in response to environmental conditions. It is important to stress that plant cells have evolved with a unique endomembrane system, where vacuoles can account for 90% of the total cell volume. This internal compartmentalization among the presence of the cell wall are responsible of water

homeostasis as plant cells are always exposed to wider osmolar fluctuations when compared to animal ones.

b) *aquaporin gating:* In comparison to animal AQPs, great advances have been achieved on the modification of channel gating by pH, phosphorylation and cations.

Acidic pH has been pointed out as responsible for inhibition of water transport since 1980´s, when reduced water permeability for some animal membranes was reported (Parisi *et al.,* 1983, 1984a,b). First signs of plant aquaporin regulation by medium acidification were obtained in organelles, as demonstrated in isolated vacuoles employing a viceomicroscopy technique by Amodeo *et al.* (2002). Other evidences of pH inhibition of water transport were provided using the stopped-flow spectrophotometry technique on isolated tonoplast vesicles (Sutka *et al.,* 2005) and plant plasma membrane vesicles (Gerbeau *et al.,* 2002, and Alleva *et al.,* 2005). The gating mechanism of the inhibitory effect was finally associated to the protonation of a conserve histidine residue (His^{197} of *At*PIP2;2) allocated on intracellular loop D of the protein (Tournaire Roux *et al*., 2003). In accordance with these results, Alleva *et al.* (2005) found that only the cytoplasmic side of *Beta vulgaris* PIPs was able to reduce water transport at low pH. Recently Chaumont *et al.* (2005) via molecular dynamic simulation showed that a protonated histidine model of *At*PIP2;2 was found in a closed state in which loop D was folded over the cytoplasmic vestibule of the water channel. Chaumont *et al*. suggested that His^{197} forms a crucial part of a charge network, including Arg^{194} and Asp^{195}, that stabilizes the closure of the water channel by ionic interaction between loop D and acidic N-terminus of the PIP. Recent evidence of *So*PIP2;1 X-ray structure and its dynamic simulation reveal that in the closed conformation loop D caps the channel from the cytoplasm and thereby occludes the pore (Törnroth-Horsefield *et al*., 2005). In the open conformation, loop D is displaced up to 16 Å and this movement opens a hydrophobic gate blocking the channel entrance from the cytoplasm.

Many studies have shown that **phosphorylation** of water channels is also an important step in the regulation of their water transport activity. *In vivo* and *in vitro* phosphorylation of serine residues within the N- or C-termini of several plant aquaporins has been reported (review by Chaumont *et al.,* 2005). These phosphorylation processes are suggested to occur via a membrane-associated calcium-dependent protein kinase. Additionally to N- or C-terminal serines, there is another residue candidate for phosphorylation of water channels, a well-conserved serine sited in the cytoplasmic loop close to the first NPA motif both in plant PIPs and TIPs. Mutation of this serine by an alanine in *So*PIP2;1 as well as *Pv*TIP3;1, resulted in reduced water transport activity when proteins were expressed in Xenopus oocyte (Maurel *et al.,* 1995, Johansson *et al.,* 1998). Both developmental stages and environmental factors are known to modulate plant aquaporin phosphorylation and it has been proposed that phosphorylation is directly involved in rapid and reversible channel gating (Johansson *et al.* 2000). On the contrary, in animals, phosphorylation of water channels is generally associated with signaling processes and membrane targeting.

Evidence for a direct action of **cations** on water channels has been extensively described. Mercurial compounds are known to block water transport by acting on clue cystein residues and have been widely used as an indicator of aquaporin presence in different systems. However, some instances where low or no mercurial inhibition was observed have also been reported (Daniels *et al.,* 1994; Biela *et al.,* 1999, Alleva *et al.,* 2005). Niemietz and Tyerman (2002) found that silver and gold were more potent inhibitors of aquaporins than the classical mercury. Divalent cation action on aquaporins has also been studied. Fotiadis *et al.* (2002) pointed to the existence of a putative Ca^{2+} binding site at the C-terminus of AQP1 based on sequence analysis. In plants, Gerbeau *et al*. 2002

reported a Ca^{2+} inhibitory effect for *Arabidopsis thaliana*, and Alleva *et al.* (2005) showed that intracellular calcium was responsible for the shut down of water transport in *Beta vulgaris* plasma membrane vesicles. Nevertheless, molecular mechanisms of Ca^{2+} dependent channel closure remain cryptic.

c) *Changes in aquaporin expression*

The participation of aquaporins in plant water management can be deduced not only from their ubiquity but also from the amazing variability of water permeability values among plant species as well as between plant cell types from the same species (Chaumont *et al.,* 2005). Physiological studies showed that drought, low temperature, salinity, light, pathogens, or nutrients might modify plant water transport properties (Steudle 2001, Tyerman *et al.*, 2002). At the present time, studies on transcription levels of MIPs in conjunction with already described physiological approaches contribute to explain how water channels are involved in plant responsiveness to diverse environmental conditions (review by Luu and Maurel 2005). Hormones such as ABA or GA3 and those environmental stimuli studied by physiologists are now revealed to lead modifications on transcriptional levels of aquaporins (Maurel *et al.,* 2002). Other evidences for the role of aquaporins in plants emerged from manipulation of gene expression. Overexpression of water channels, gene silencing by anti-sense suppression and T-DNA insertion (review by Chaumont *et al*., 2005) are all strategies that contributed to unravel the close interrelation between plant aquaporins and water handling by whole plants.

In agreement with different regulatory features described in the literature for both TIPs and PIPs, changes in the expression pattern as well as short-term regulation must take part of a synchronized response to adjust water fluctuations.

Conclusions

The identification of water channels opened a novel chapter in physiology. Since the discovery of aquaporins, plant water flow could be revisited. Research is now flourishing in aquaporin regulatory mechanisms, allowing going further into the comprehension of plant water management. Unraveling these mechanisms will contribute to understand not only the aquaporin *per se* but also the physiological events in which they are involved. The actual picture regarding regulation of plant membrane water permeability can be described as follows: long term regulation leads to strong changes at the level of transcription, while for short term, it is proposed a fine-tuning *via* gating given by the differential sensitivity of PIPs and TIPs to identical stimuli. This fine-tuning may allow cells to best-manage the distribution of water according to the exposed conditions.

A relevant aspect in the aquaporin field that remains to be solved concerns the study of aquaporins as a family of transporters of small and neutral solutes besides water, and not to merely describe them as a family of water channels that can also move solutes. This role is supported for instance by recent evidence of the role of AQP7 as a novel regulator of fat accumulation in aquaporin-7-deficient mice, where it is reported a progressive adipocyte hypertrophy (Hara-Chikuma *et al.*, 2005).

These findings widen the perspective not only for water transport but also for analyzing the membrane permeability as a whole and will contribute to uncover new cellular roles of aquaporins beyond the already proposed (Verkman *et al*., 2005; Hill *et al.*, 2004). As it was stated before, the field is just about emerging.

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XXII CONGRESO LATINOAMERICANO Y 1ER IBERO-AMERICANO DE CIENCIAS FISIOLÓGICAS

Organizado por la Sociedad Argentina de Fisiología, por decisión de la Asociación Latinoamericana de Ciencias Fisiológicas (ALACF) y con el auspicio de la Sociedad Española de Ciencias Fisiológicas
Buenos Aires, 4 al 7 de noviembre de 2006

Este año tendrá lugar en Buenos Aires el XXII Congreso de la Asociación Latinoamericana de Ciencias Fisiológicas (ALACF). Esta reunión congregará a científicos originarios de América Latina trabajando en sus países de origen, en Estados Unidos, en Europa y alrededor del mundo. Fisiólogos no latinoamericanos de primer nivel son también regularmente invitados. Esta vez la Sociedad Española de Ciencias Fisiológicas se asocia al evento, dándole especial interés y relevancia.

El objetivo central del Congreso es dar, a los fisiólogos trabajando y viviendo en Latinoamérica, la posibilidad de entrar en contacto con referentes en su campo de trabajo. Esto será especialmente cierto esta vez para aquellos radicados en el Cono Sur del continente (Bolivia, Brasil, Chile, Paraguay, Uruguay y Argentina).

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