

# Aquaporins in Plants: From Molecular Structure to Integrated Functions

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## ABSTRACT

Aquaporins belong to a superfamily of membrane channels with members in all living organisms. In plants, aquaporins mediate a large part of the cell-to-cell and intracellular water movements. The ability of certain plant aquaporins homologues to transport nutrient such as boron or gas such as CO<sub>2</sub> has recently been demonstrated. This present chapter specifically examines how our current understanding of aquaporin structure and function can be integrated into whole plant physiology. Expression studies coupled with physiological and genetic analyses have allowed to delineate a variety of functions for aquaporins in roots, leaves, and during plant reproduction. In addition, a large variety of molecular and cellular mechanisms have been identified that lead to fine regulation of membrane water transport, during plant development, or in response to environmental stimuli. However, central physiological questions remain, such as the role of aquaporins in carbon assimilation, or in a hydraulic control of growth and cell movements.

## ABBREVIATIONS

CAM, crassulacean acid metabolism; GFP, green fluorescent protein;  $g_m$ , conductance of mesophyll to CO<sub>2</sub>;  $G_s$ , stomatal conductance; HPFM, high pressure flow meter;  $K_{leaf}$ , leaf hydraulic conductance;  $Lp_r$ , root hydraulic conductivity; MIP, major intrinsic protein; NIP, nodulin26-like intrinsic protein;  $P_f$ , osmotic water permeability; PIP, plasma membrane intrinsic protein;  $\Psi$ , water potential; SIP, small basic intrinsic protein; TIP, tonoplast intrinsic protein.

## I. INTRODUCTION

Aquaporins belong to a superfamily of membrane channels named after its founding member, the major intrinsic protein (MIP) of lens fibers. MIP homologues have now been identified in all living organisms (Agre *et al.*, 1998). In recent years, tremendous progress has been made in understanding their molecular structure and primary transport properties. CHIP28, an abundant protein in erythrocytes and kidney tubules was the first MIP to be identified as a molecular water channel (Preston *et al.*, 1992) and hence was renamed aquaporin-1 (AQP1). In the following years, several other MIPs including plant  $\gamma$ -tonoplast intrinsic proteins ( $\gamma$ -TIP or TIP1;1) were also shown to transport substantial amounts of water and were also designated as aquaporins (Chrispeels and Agre, 1994). By contrast, other MIPs, such as bacterial glycerol facilitator GlpF, function as solute channels and

have no or a weak water channel activity (Maurel *et al.*, 1995). These MIPs have been named aquaglyceroporins (Agre *et al.*, 1998).

In plants, aquaporins show a typically high isoform multiplicity. Plant genome sequencing has identified 35 and 33 aquaporin homologues in *Arabidopsis thaliana* and rice, respectively (Johansson *et al.*, 2000; Quigley *et al.*, 2001; Sakurai *et al.*, 2005). A similar number of homologues are found in maize (Chaumont *et al.*, 2001). Sequence analysis also showed that plant aquaporins fall into four major homology subgroups that somehow reflect specific subcellular localizations (Fig. 1). For instance, the plasma membrane intrinsic proteins (PIPs; 13 homologues in *Arabidopsis*) and tonoplast

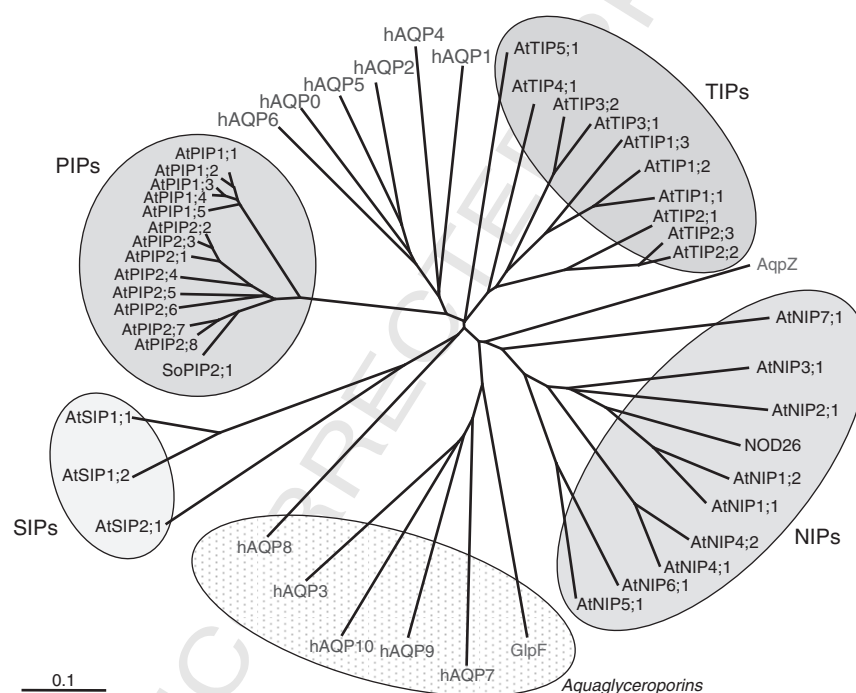


Fig. 1. Sequence relationship between aquaporin homologues of *Arabidopsis thaliana*, human, and *Escherichia coli*. The amino acid sequences were aligned with ClustalW and a phylogenetic tree was constructed with TreeView. The tree illustrates the subdivision of the *Arabidopsis* aquaporin family in four subfamilies: PIPs, TIPs, NIPs, and SIPs. Two other well-characterized plant aquaporins, SoPIP2;1 and NOD26 from spinach and soybean respectively, are also included in the tree for reference. The two *E. coli* aquaporins (GlpF and AqpZ) are represented in blue, while the 11 human aquaporins (hAQP0 to hAQP10) are represented in red. Note that the human aquaporins group into two subfamilies. One of them comprises GlpF and corresponds to the aquaglyceroporin subfamily.

intrinsic proteins (TIPs; 10 homologues in *Arabidopsis*) sit predominantly in the plasma membrane and in the tonoplast, respectively, hence their name (for review, see Maurel *et al.*, 2002). A third subclass of Nodulin26-like Intrinsic Proteins (NIPs) is formed by homologues of Nodulin-26 (NOD26), an aquaporin of the peribacteroid membrane of symbiotic nitrogen-fixing root nodules (Weig *et al.*, 1997). NIPs are also present in non-legume plants (nine homologues in *Arabidopsis*), and some of them have been localized in the plasma membrane of rice and *Arabidopsis* cells (Ma *et al.*, 2006; Takano *et al.*, 2006). The fourth subclass of so-called small basic intrinsic proteins (SIPs) comprises three homologues in *Arabidopsis*, which show preferential localization in the endoplasmic reticulum (Ishikawa *et al.*, 2005).

More than 10 years after their discovery in plants, it now appears that aquaporins mediate a large part of the cell-to-cell and intracellular water movements in these organisms (Maurel *et al.*, 2002; Tyerman and Niemietz, 2002). Their characterization has pointed to a large variety of molecular and cellular mechanisms that lead to fine regulation of membrane water transport, during plant development or in response to environmental stimuli (Chaumont *et al.*, 2005; Luu and Maurel, 2005). More recently, several plant aquaporin homologues have been associated to the transport of small neutral molecules, including gas or micronutrients. Therefore, the function of MIP channels in plants appears to go much beyond plant water relations (Kaldenhoff and Fischer, 2006; Tyerman and Niemietz, 2002).

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In this present chapter, we want to summarize our current knowledge on the integrated function of aquaporin homologues in plants. For this, we will in the first place examine how novel insights were gained from enhanced knowledge of their structure–function relationship. Our aim is to show how this knowledge, combined to genetic and physiological evidences, now defines a large array of functions in roots, leaves, and during plant reproduction.

## II. SIGNIFICANCE OF AQUAPORIN MOLECULAR STRUCTURE FOR TRANSPORT SPECIFICITY AND GATING

### A. AQUAPORINS ARE TETRAMERIC TRANSMEMBRANE CHANNELS

MIPs have emerged as a family of general interest among membrane proteins, as the atomic structures of AQP1 and GlpF were among the first to be solved for polytopic membrane proteins (Murata *et al.*, 2000; Sui *et al.*, 2001).

To date, 18 structures of aquaporin homologues are available. These structural studies have allowed to validate earlier structure–function analyses. Most importantly, they have also provided an exquisite understanding of water and solute permeation mechanisms through the aquaporin channel. Very recently, the first atomic structure of a plant aquaporin was solved (Tornroth-Horsefield *et al.*, 2006). Because, for the first time, an aquaporin structure was determined in both the open and closed states, this work gave unique insights into the gating mechanisms of aquaporins.

Primary sequence analyses indicate that most MIPs consist of small (25–34 kDa), hydrophobic proteins (Chrispeels and Agre 1994; Zardoya and Villalba, 2001), with a typical symmetrical organization due to an early gene duplication. All MIPs comprise six transmembrane domains with the N- and C-terminal ends of the protein being located in the cytoplasm. Early structure–function analyses of AQP1 in oocytes suggested an “hourglass-fold” model for this aquaporin (Jung *et al.*, 1994). In this model, two highly conserved loops (loops *B* and *E*), each of them carrying a conserved Asn-Pro-Ala (NPA) motif, deep into the pore from either side of the membrane (Chrispeels and Agre 1994; Jung *et al.*, 1994). The NPA motif constitutes the most prominent signature sequence of aquaporins. Biochemical and functional studies also suggested that aquaporins assemble as homo-tetramers. Unlike tetrameric potassium channels, the water transporting pore is not localized at the center of the aquaporin tetramer but instead one channel is formed at the center of each monomer (Jung *et al.*, 1994). This typical arrangement was first visualized by freeze-fracture studies (Verbavatz *et al.*, 1993) and then by electron microscopy of two-dimensional crystals. A similar approach showed that plant TIPs and PIPs also exhibit a tetrameric organization (Daniels *et al.*, 1999; Fotiadis *et al.*, 2001).

A major breakthrough in aquaporin research was the resolution by cryoelectron microscopy of a 3.8 Å atomic structure for human AQP1 (Murata *et al.*, 2000), and by X-ray crystallography of 2.2 Å atomic structures for bacterial GlpF (Fu *et al.*, 2000) and bovine AQP1 (Sui *et al.*, 2001). These pioneering studies confirmed the “hourglass-fold” model and showed how each aquaporin monomer is folded according to a conserved structural core of six transmembrane  $\alpha$ -helices tilted along the plane of the membrane. These studies also revealed in great detail how each monomer contributes to form an individual, narrow, aqueous pathway through the membrane. The 2.1 Å atomic structure of spinach *SoPIP2;1* (formerly named PM28A) revealed a very similar “hourglass-fold” that therefore has been conserved between 1.6 billion year distant proteins, with a root to mean square deviation of only 0.8 Å (Tornroth-Horsefield *et al.*, 2006).

## B. METHODS FOR FUNCTIONAL CHARACTERIZATION OF PLANT AQUAPORINS

Accurate measurements of water permeability at the level of isolated membranes, single cells, or whole organs have proved crucial to investigate the various aspects of plant aquaporin function, including their transport selectivity, the molecular and cellular mechanisms of their regulation, and their integrated function. These measurements can be made in plant materials or after expression of an individual aquaporin in a heterologous system. In many cases, the membrane water permeability of the cells or vesicles can be derived from the kinetics of volume adjustment in response to a rapid osmotic challenge. The kinetics of all of these objects are determined by both their intrinsic water permeability and their surface-to-volume ratio. The latter is inversely proportional to the object size and can span over four orders of magnitude from *Xenopus* oocytes (diameter  $\sim 10^{-3}$  m) to membrane vesicles (diameter  $\sim 10^{-7}$  m).

### 1. Stopped-flow techniques

These techniques allow to monitor fast (in the 0.1–1 s range) flow kinetics in small objects like isolated vesicles (Beuron *et al.*, 1995; Verkman, 2000). Stopped-flow techniques have proved useful to characterize the activity of plant aquaporins in their native membranes, after heterologous expression in yeasts, or after purification and reconstitution in proteoliposomes (Karlsson *et al.*, 2003; Laizé *et al.*, 1995). Water permeability of the membrane vesicles is deduced from the kinetics of osmotic volume adjustments and from an independent size determination, usually made by electron microscopy or dynamic light scattering. Stopped-flow techniques have also proved useful to elucidate various aspects of aquaporin transport selectivity. For instance, the membrane permeability to small neutral solutes, such as glycerol or urea, can be determined by iso- or hyper-osmotic challenges in the presence of a concentration gradient for the solute (Gerbeau *et al.*, 1999). Stopped-flow spectrophotometry can also be used to monitor CO<sub>2</sub> transport on vesicles loaded with exogenous carbonic anhydrase and pH-dependent probes (Prasad *et al.*, 1998).

Although these measurements exhibit a great biophysical accuracy, there have been concerns that the water or solute transport properties of aquaporins may be altered during membrane isolation from plant materials. In studies on plasma membranes purified from *Arabidopsis* cell suspensions, Gerbeau *et al.* (2002) suggested that protection of aquaporins from dephosphorylation by protein phosphatases or from inhibition by divalent ions (see Section II.E.4) may be critical to maintain purified plasma membranes with their native water permeability.

## 2. *Swelling and shrinking of plant protoplasts and vacuoles*

Kinetic size measurements on objects such as plant protoplasts or vacuoles are problematic because the rapid imposition of an osmotic gradient can be destructive because of the great fragility of these objects. Video-microscopy has been used to record early changes in protoplast/vacuole volume after a quick transfer of the object in solutions of varying osmolalities (Ramahaleo *et al.*, 1999). This transfer can be achieved by micromanipulation using a pipette or by immobilization in a microscopic observation chamber that support a continuous perfusion. Using the latter technique, Moshelion *et al.* (2002) observed a delay of a few seconds in the swelling behavior of protoplasts subjected to a hypotonic challenge. This delay was tentatively explained as a complex adjustment of membrane water permeability during the course of cell swelling (Moshelion *et al.*, 2004).

## 3. *Cell pressure probe*

Cell pressure probe techniques allow to determine cell water relation parameters in intact plant tissues (Steudle, 1993). The instrument can be assimilated to a miniature syringe with an oil-filled micropipette linked to a pressure transducer. The micropipette is used to impale a living cell. Displacement of the meniscus, formed at the tip of the pipette by the contact between the oil and the cell sap, is used to monitor volume exchanges between the probe and the cell. When the micropipette enters the cell, the turgor pressure shifts the meniscus and this shift can be compensated by applying a pressure in the instrument, which corresponds to the initial cell turgor. Osmotic or hydrostatic perturbations can thereafter be imposed by altering the osmolarity of the bathing solution or by imposing pressure shifts via the syringe. These maneuvers result in cell pressure relaxations, which provide crucial informations on the mechanical properties of the cell (cell wall elastic modulus) and its hydraulic conductivity. The membrane reflection coefficients for different solutes can also be determined. Over the past two decades, this technique has been extensively used to uncover a large variety of physiological regulations of membrane water transport in plants. More recently, the technique has been applied to the phenotypic characterization of aquaporin knock-out mutants and has allowed a precise quantification of the contribution of a single aquaporin (*AtPIP2;2*) to water transport in root cortical cells (Javot *et al.*, 2003).

## 4. *Water measurement in heterologous systems*

*Xenopus laevis* oocytes represent one of the most favorable system for assaying the water and solute transport of cloned individual aquaporins. First, these cells are virtually devoid of endogenous water channels and have thus a

low basal water permeability. Second, oocytes can efficiently express exogenous membrane proteins upon intracellular injection of *in vitro* transcribed complementary RNAs. Third, because of their large size, oocytes exhibit relatively slow swelling kinetics that can easily be followed by video microscopy. Historically, expression in oocytes has provided a central evidence to prove that MIPs cloned from animals, bacteria, or plants encoded functional water channels (Maurel *et al.*, 1993, 1994; Preston *et al.*, 1992). In addition, the capacity of aquaporins to transport solutes such as glycerol or urea can also be monitored in this system by the uptake of radio-labeled molecules (Maurel *et al.*, 1993). Luckily enough, many plant aquaporins, even those such as TIPs which are targeted to intracellular plant membranes, are at least in part routed to the oocyte surface and thus can be functionally characterized in these cells. In all plant species, PIP aquaporins can be subdivided into two typical subclasses, PIP1 and PIP2. Several laboratories have noticed that PIP1 homologues are recalcitrant to oocyte expression (Fetter *et al.*, 2004). One reason is a deficient targeting of PIP1s to the cell surface (see Section II. E). Finally, plant aquaporins are regulated by a variety of cytoplasmic effectors. Some of them can be efficiently altered in oocytes and therefore these cells have been instrumental for dissecting the structural basis of aquaporin regulation by phosphorylation or by cytoplasmic pH (Johansson *et al.*, 1998; Tournaire-Roux *et al.*, 2003).

Aquaporins are among the most highly expressed membrane proteins in animals or plants and can easily be purified from their native organisms (for review, see Maurel *et al.*, 2002). High-expression levels of recombinant plant aquaporins can also be obtained in heterologous systems, such as yeasts (Daniels and Yeager, 2005; Karlsson *et al.*, 2003). The purified proteins can then be inserted in artificial membranes (Daniels and Yeager, 2005; Dean *et al.*, 1999; Karlsson *et al.*, 2003). This so-called proteoliposomes provide a unique system for studying the molecular and structural determinants involved in aquaporin gating. In particular, the specific intrinsic water permeability of a single aquaporin can be determined in an accurately controlled environment. Thus, this approach may be adequate for deciphering the molecular basis of PIP regulation by cytoplasmic calcium since oocytes or other living cells tightly control their cytoplasmic calcium concentration (see Section II.E.4).

### C. PLANT AQUAPORINS ARE NOT JUST WATER CHANNELS

It has been proposed that the great diversity of plant aquaporins may reflect, in addition to distinct subcellular localizations, a broad range of transport specificities (Tyerman and Niemietz, 2002). Functional expression in



*Xenopus* oocytes revealed that *Arabidopsis* TIP1;1 is only permeable to water (Maurel *et al.*, 1993), whereas its tobacco homologue *NtTIPa* transports urea and glycerol in addition to water (Gerbeau *et al.*, 1999). Other TIP homologues have recently been identified as permeable to urea through complementation of a yeast mutant defective in its corresponding endogenous transport system (Klebl *et al.*, 2003; Liu *et al.*, 2003). Solute transport is, however, not restricted to TIPs and other plant aquaporins of the PIP (Biela *et al.*, 1999) and NIP subfamilies (Rivers *et al.*, 1997; Weig and Jakob 2000) transport small neutral solutes in addition to water. In particular, functional reconstitution in proteoliposomes of soybean NOD26 has shown that this protein transports glycerol with a high efficiency and has a low intrinsic water permeability (Dean *et al.*, 1999). Very recently, Lsi1, a *AtNIP7;1* relative, was characterized from a rice mutant defective in silicon uptake and was shown to transport silicon in *Xenopus* oocytes (Ma *et al.*, 2006). In an other study, it was shown that boron uptake in *Arabidopsis* plants is mediated in large part by NIP5;1 (Takano *et al.*, 2006). Other molecules relevant to plant physiology, such as antimonite (Sanders *et al.*, 1997) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Bienert *et al.*, 2007; Henzler and Steudle, 2000), have also been shown to be transported by MIPs. The permeability of plant and animal aquaporins to reactive oxygen species, such as H<sub>2</sub>O<sub>2</sub> (Bienert *et al.*, 2007; Henzler and Steudle, 2000) or NO (Herrera *et al.*, 2006), raises the exciting possibility that aquaporins may participate in cell-signaling cascades, and therefore supports the general idea that aquaporins fulfill multiple functions, besides water and nutrient transport.

In these respects, the capacity of certain aquaporins to transport gaseous compounds has recently raised a great interest. CO<sub>2</sub> transport, in particular, may be one of most significant. It is important to note that CO<sub>2</sub>, similar to water, is a highly diffusive molecule that can freely pass the lipid bilayer of cell membranes. This property has long been considered as sufficient to account for the diffusion of the gas, from the stomatal chamber to mesophyll cell chloroplasts. The transport of CO<sub>2</sub> by mammalian AQP1 has been unambiguously demonstrated after heterologous expression of the protein in oocytes (Cooper and Boron 1998; Nakhoul *et al.*, 1998) and after functional reconstitution in proteoliposomes (Prasad *et al.*, 1998). Recently, functional expression in oocytes of a tobacco PIP aquaporin, *NtAQP1*, has shown that this aquaporin also significantly transports gaseous CO<sub>2</sub> (Uehlein *et al.*, 2003). In addition, ammonia transport has been hypothesized for NOD26 (Niemietz and Tyerman, 2000) and demonstrated for several *Arabidopsis* and wheat TIP homologues (Jahn *et al.*, 2004; Loque *et al.*, 2005). Interestingly, some human aquaporins such as AQP1 and AQP6 can function as ionic channels provided that they are placed under very specific

conditions, that is, acidic extracellular pH (Yasui *et al.*, 1999) or stimulation by cGMP (Anthony *et al.*, 2000; Yu *et al.*, 2006). Similar behavior has not yet been identified in plant aquaporins.

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#### D. MOLECULAR AND STRUCTURAL BASES OF AQUAPORIN SELECTIVITY

The determination of atomic structures for human AQP1 (Murata *et al.*, 2000), bacterial GlpF (Fu *et al.*, 2000), and bovine AQP1 (Sui *et al.*, 2001) has allowed to uncover fundamental mechanisms of transport selectivity in aquaporins. Two conserved structural motifs have been determined as crucial.

One motif is formed by loops *B* and *E* that fold as half-helices and dip into the center of the channel from opposite sides of the membrane, therefore gathering the two NPA motifs in close vicinity (Fig. 2). In this arrangement, the Asn residues of the two NPA motifs play a key role in the formation of

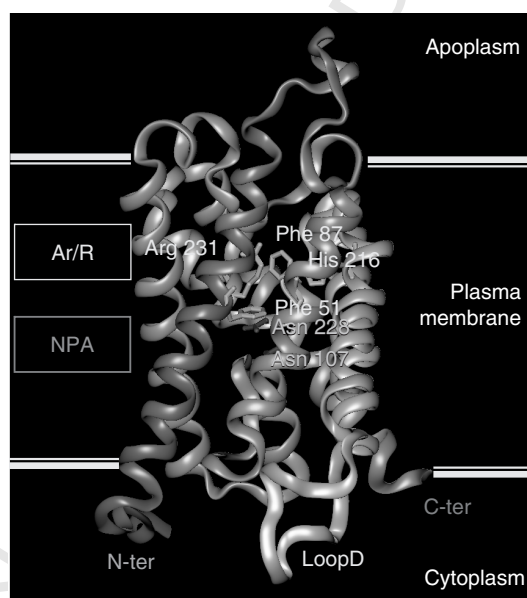


Fig. 2. Structural model of *Arabidopsis* PIP2;1. A predictive structure (amino-acids 28–269) was obtained by homology modeling (using the Swiss-Model server at <http://www.expasy.org/swissmod/swiss-model.html>) based on the X-ray structure of spinach *SoPIP2;1* in its open conformation (PDB ID: 2B5F). The picture represents a single monomer with the six transmembrane domains and the two constrictions Ar/R and NPA shown. The Asn residue of the two NPA motifs and the residues forming the Ar/R motif are shown in red and yellow, respectively (See Color Plate Section in the back of the book.)

the pore selectivity filter. However, exceptions to the NPA aquaporin signature exist in plants, and some NIP and SIP isoforms show a substitution of at least one Ala residue by another residue (for review, see Ishibashi, 2006). In the canonical aquaporin structure, the positive electrostatic potential at the NPA constriction is supposed to prevent proton translocation through the pore (Chakrabarti *et al.*, 2004; De Groot *et al.*, 2003). This model is supported by molecular dynamic simulations on animal or plant aquaporins showing a 180° dipole reorientation of the water molecule upon its passage (De Groot *et al.*, 2001; Tajkhorshid *et al.*, 2002; Tornroth-Horsefield *et al.*, 2006). This reorientation breaks the single file of water molecules and therefore blocks proton transfer through the channel.

A second arrangement named Ar/R, because it is composed of aromatic (Ar) and polar (Arg or R) residues, forms a constriction in the extracellular half of the pore. This constriction is narrow for strictly water selective aquaporins, with a diameter of 2.8 Å for mammalian AQP1, 2.4 Å for bacterial AQPZ, and 2.1 Å for plant *SoPIP2;1* (Murata *et al.*, 2000; Savage *et al.*, 2003; Tornroth-Horsefield *et al.*, 2006). By contrast, glycerol facilitators display a wider constriction, of 3.3 Å in bacterial GlpF for instance (Fu *et al.*, 2000). Such channel widening is predicted to occur in all aquaporin homologues permeable to small solutes such as glycerol or urea. In addition, the positive charge of Arg in the Ar/R arrangement is thought to generate an electrostatic repulsion, which together with size restriction, would also act as an efficient filter for protons and ions (for review, see Fujiyoshi *et al.*, 2002). A model of polyol permeation in GlpF has proposed that the carbon backbone of the solute slides on a hydrophobic side of the pore, whereas its hydroxyl groups form hydrogen bonds with residues on the opposite side of the pore (Fu *et al.*, 2000). This model explains why GlpF is permeable to linear polyols that have their OH groups lined up in the same direction with respect to the carbon backbone (i.e., glycerol, ribitol), whereas the channel protein is poorly permeable to asymmetrical stereoisomers, such as xylitol or D-arabitol.

Although most of the structural information available on aquaporins was obtained on water specific aquaporins, the overall molecular structure of plant aquaporins displaying non-classical solute specificities, such as TIPs or NIPs, can be predicted from the available structures and can help identify the specificity determinants of these proteins. Again, these modeling approaches pointed to the importance of the Ar/R constriction (Wallace and Roberts, 2004). Structure–function analyses with *Lotus japonicus* LIMP2 aquaporin, a close homologue of soybean NOD26, confirmed that a single amino acid substitution in this domain can alter transport selectivity (Wallace *et al.*, 2002). More recently, an elegant work, combining biochemical and structural approaches demonstrated that residues at the Ar/R constriction, and

especially in helix two, play a critical role as a selectivity filter in the *Arabidopsis* aquaporin AtNIP6;1 (Wallace and Roberts, 2005). This study is especially relevant to a subgroup of NIPs (subgroup II). These plant MIPs seem to have evolved from multifunctional aquaglyceroporins and are now specialized in the transport of solutes such as silicon or boron (Ma *et al.*, 2006; Takano *et al.*, 2006; Wallace and Roberts, 2005). One striking example is rice LSI1, which was genetically identified as a silicon channel after characterization of mutant plants defective in silicon uptake. The mutant LSI1 allele carried a single Ala to Thr substitution in helix three. Molecular modeling showed that this substitution causes a severe conformational change of the Asn located in the first NPA motif, and therefore inactivates the channel (Ma *et al.*, 2006).

#### E. MOLECULAR MECHANISMS OF AQUAPORIN GATING

A current challenge in the study of membrane proteins, and especially channel proteins, is to understand the structural aspects of their gating properties. The aim is to describe a sequence of conformational changes that lead to pore opening or closing. While biochemical and physiological studies have indicated that plant aquaporins are regulated by numerous factors related to intracellular metabolic state or signaling, recent structural study has provided critical insights into the molecular events that underlie these regulations (Tornroth-Horsefield *et al.*, 2006).

##### 1. Blockade by mercury

Mercury has long been recognized as a common blocker of aquaporins and, despite its low specificity and cellular toxicity, has been extensively used in physiological studies that addressed the role of aquaporins in plants. Cys residues are known to be preferential targets for oxidation by mercury. In human AQP1, mercury acts at a unique Cys residue, which is located right at the Ar/R constriction, in front of His180 and Arg195. This site of action provides a clear mechanistic explanation to the blocking effects of mercury (Sui *et al.*, 2001). By contrast, the structural basis of inhibition of plant aquaporins is as yet unclear. In particular, there is no conserved cysteine residue in the pore of these proteins. However, introduction of one such residue in the pore-forming loop B of AtPIP2;3, a mercury insensitive aquaporin isoform, was able to confer sensitivity to the blocker (Daniels *et al.*, 1996). The structure of SoPIP2;1 revealed that a conserved Cys residue at the N-terminal end of helix two can make a disulfide bridge between two adjacent monomers (Tornroth-Horsefield *et al.*, 2006) and may stabilize them. Therefore, this residue may be a target for mercury inhibition of PIPs. Silver

and gold ions have also been described as efficient blockers ( $IC_{50} = 1\text{--}10\ \mu\text{M}$ ) of aquaporins in membranes from roots, soybean nodules, and human red blood cells (Niemietz and Tyerman, 2002; Siefritz *et al.*, 2004), but the molecular basis of this inhibition is still unknown.

## 2. Regulation by reactive oxygen species

Direct gating of plant aquaporins by reactive oxygen species has been suggested from experiments made in *Chara corallina* internodal cells (Henzler *et al.*, 2004) and more recently in corn roots (Ye and Steudle, 2006). Henzler *et al.* (2004) proposed that hydroxyl radicals, produced by a Fenton reaction from exogenously supplied  $\text{H}_2\text{O}_2$ , act through on aquaporin gating either by direct oxidation of the aquaporins or by indirectly through lipid membrane oxidation and formation of secondary radicals. In addition, reactive oxygen species are also critical elements in cell signaling and may trigger a cascade of events ultimately leading to aquaporin downregulation in living cells (Boursiac *et al.*, unpublished results).

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## 3. Regulation by osmotic and hydrostatic pressures

Evidence exists that high concentrations of osmolytes act not only as a driving force for water transport but also can interfere with aquaporin gating. Niemietz and Tyerman (1997) reported that the osmotic water permeability of tonoplast vesicles isolated from wheat root was dependent on the strength of the imposed osmotic gradient. In another study, the hydraulic conductivity of *C. corallina* internodal cells was shown to be inhibited by high concentrations of high molecular weight, cell permeable solutes (Steudle and Tyerman, 1983; Ye *et al.*, 2004). A proposed model for this regulation was that the osmolytes, present on either side of the membrane, were excluded from the channel pore due to their size. This resulted in tensions (negative pressures) inside the water-filled pore and a collapse of the pore eventually leading to its closure (Ye *et al.*, 2004). At variance with these ideas, molecular dynamics simulations have shown that the aquaporin structure (in this case human AQP1) was stable up to applied pressures of 200 MPa (Zhu *et al.*, 2002, 2004). This suggested that aquaporins can tolerate extraordinary high hydrostatic and osmotic pressures.

The cell hydrostatic pressure is another parameter that may regulate water channel opening. Pressure pulses of varying amplitudes, generated by a pressure probe device, differentially altered the hydraulic conductivity of young maize root cortical cells. Whereas medium-sized pressure pulses ( $<0.2\ \text{MPa}$ ) caused a reversible inhibition of the hydraulic conductivity, larger pressure pulses induced changes that were not reversible except in

the presence of ABA (Wan *et al.*, 2004). The authors suggested that the high rate of water flow through the aquaporin, which was proportional to the pressure pulse, could cause conformational changes in the channel, *and thereby induce its closure.*

#### 4. Regulation by protons and calcium

The first evidence that plant aquaporins are regulated by pH was initially obtained in purified membrane vesicles or organelles. For instance, Amodeo *et al.* (2002) observed pH sensitivity of tonoplast water channels in *Beta vulgaris* storage roots, using swelling assays on isolated vacuoles. In another study, Gerbeau *et al.* (2002) used stopped flow spectrophotometry on plasma membrane vesicles purified from *Arabidopsis* suspension cells to show that protons can inhibit water channels with an half inhibition at pH 7.2–7.5. Expression of wild-type and site-directed mutant aquaporins in *Xenopus* oocytes provided further functional insights into this regulation. It was first showed that all *Arabidopsis* PIP isoforms investigated were blocked by intracellular protons (Tournaire-Roux *et al.*, 2003). In addition, a pH-sensing His residue, which is specifically conserved in loop *D* on the cytosolic side of all PIPs, was identified as central for aquaporin gating. In particular, point mutations at this site resulted in active aquaporins that were insensitive to cytosolic pH (Tournaire-Roux *et al.*, 2003).

A sensitivity of plasma membrane aquaporins to  $\text{Ca}^{2+}$  inhibition, with an  $\text{IC}_{50}$  of 75  $\mu\text{M}$ , has been described in *Arabidopsis* plasma membrane (Gerbeau *et al.*, 2002). A recent study on plasma membranes from *B. vulgaris* storage roots confirmed these observations (Alleva *et al.*, 2006). In this study, however, both a high apparent affinity component with an  $\text{IC}_{50}$  of 5 nM and a lower apparent affinity component with an  $\text{IC}_{50}$  of 200  $\mu\text{M}$  were identified.

Au6

The high-resolution structures of *SoPIP2;1* in a closed (2.1 Å) and in an open (3.9 Å) conformation has given critical clues to the plant aquaporin gating properties mentioned above (Tornroth-Horsefield *et al.*, 2006). These structures confirmed the key role of loop *D* in pH-dependent channel gating. Moreover, this work showed that the binding of cadmium ( $\text{Cd}^{2+}$ ) (assumed to be replaced by  $\text{Ca}^{2+}$  *in vivo*) in the vicinity of two conserved acidic residues at the N-terminal part of the protein (Asp28, Glu31 in *SoPIP2;1*) can also gate the channel. Here, the divalent cation acts through a chain of ionic interactions and hydrogen bonds involving the loop *B* and the loop *D*, and two acidic residues carried by the N-terminal tail. While the physiological significance of PIP regulation by pH is fairly well understood, the significance of inhibition by divalent cation ( $\text{Ca}^{2+}$ ) remains as yet unknown.

### 5. *Posttranslational modifications: Phosphorylation and methylation*

In contrast to phosphorylation of mammalian aquaporins, which generally mediates membrane targeting of aquaporins (Nejsum *et al.*, 2002; Procino *et al.*, 2003, 2006), the reversible phosphorylation of plant aquaporins is thought to directly gate the pore itself. The first demonstration that phosphorylation regulates the activity of a plant aquaporin was obtained after expression of the seed specific  $\alpha$ TIP isoform from *Phaseolus vulgaris* (homologous to *AtTIP3;1*) in *Xenopus* oocytes (Maurel *et al.*, 1995). Site-directed mutation of serine residues within the protein allowed to show that three phosphorylation sites can regulate the protein water transport activity, with two of these sites being located on the N-terminal tail of the protein and one on loop *B*. A similar approach was used with spinach *SoPIP2;1* and showed that a similar Ser phosphorylation site in loop *B*, together with a major Ser phosphorylation site in the C-terminal tail can regulate this aquaporin in oocytes (Johansson *et al.*, 1998). Molecular dynamics simulations based on the molecular structure of *SoPIP2;1* suggested that the opening of the pore induced by phosphorylation of loop *B* is dominant over the closure induced by cation binding (Tornroth-Horsefield *et al.*, 2006). This work also proposed an original model in which phosphorylation of the C-terminal part of the protein can modulate pore opening by interaction of the phosphorylated residue (Ser274 for *SoPIP2;1*) with the loop *D* of an adjacent monomer. These models point to intricate mechanisms for AQP gating by multiple factors. Of course, these mechanisms will need to be further assessed through functional analysis of aquaporins carrying well-defined point mutations.

The phosphorylation of all plant aquaporins examined thus far seems to be mediated by calcium-dependent protein kinases and can be modulated in response to environmental stimuli. For instance, phosphorylation of *SoPIP2;1* was decreased in response to a hypertonic treatment on excised spinach leaves (Johansson *et al.*, 1996). The biochemical characterization of two distinct kinase activities acting on the loop *B* or the C-terminal tail of *SoPIP2;1* should be instrumental for deciphering the signal transduction pathways that control the gating of this aquaporin (Sjövall-Larsen *et al.*, 2006). In tulip flowers, a decrease in phosphorylation of PIPs was correlated with the cold-induced closure of petals (Azad *et al.*, 2004b). Finally, it was shown that phosphorylation of the C-terminal tail of NOD26 in the peribacteroid membrane of symbiotic root nodules was enhanced by both drought and salinity stress (Guenther *et al.*, 2003).

While aquaporin phosphorylation has attracted much attention, it should be stressed that aquaporins can be posttranslationally modified and possibly regulated by other mechanisms. Although no such evidence is available in plants, ubiquitination of mammalian AQP1 controls the differential stability

of the protein between normal and osmotic stress conditions (Leitch *et al.*, 2001). In addition, N-linked glycosylation of human AQP2 and plant vacuolar *McTIP1;2* aquaporin seems to control the specific subcellular localization of these proteins (Hendriks *et al.*, 2004; Vera-Estrella *et al.*, 2004). In the latter example, the appearance of a glycosylated form of *McTIP1;2* in response to an osmotic stress correlated with the redistribution of the protein from tonoplast to other intracellular membrane fractions. Finally, highly resolution techniques, such as mass spectrometry, have assisted in the discovery of novel posttranslational modifications in aquaporins. In particular, methylation of two residues (Lys and Glu) in the N-terminal tail of *AtPIP2* aquaporins was recently described. While these decorations seem to have no effect on the intrinsic water permeability of *AtPIP2;1* (Santoni *et al.*, 2006), it remains to be tested whether they interfere with the subcellular distribution of the protein.

#### 6. Regulation by heterotetramer formation

Structural models of mammalian AQP1, bacterial GlpF, and plant *SoPIP2;1* 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; Tornroth-Horsefield *et al.*, 2006). However, heteromers comprising members of the PIP1 and PIP2 subgroups may occur in maize or mimosa (Fetter *et al.*, 2004; Temmei *et al.*, 2005). This hypothesis is supported by the observation that, when independently expressed in oocytes, most PIP1 aquaporins, in contrast to PIP2 aquaporins, fail to increase the osmotic water permeability of the oocyte membrane. By contrast, coexpression of a seemingly inactive PIP1 with reduced amounts of a functional PIP2 led to a marked increase in permeability, which was proportional to the amounts of PIP1 expressed (Fetter *et al.*, 2004; Temmei *et al.*, 2005). This suggested that isoforms of the two subgroups interacted, probably as hetero-tetramers. This idea was corroborated by microscopic observations of oocytes expressing PIP1 and/or PIP2 aquaporins fused to the green fluorescent protein (GFP). Physical interaction of *ZmPIP1;2* and *ZmPIP2;1* was also demonstrated by copurification experiments. Yet structural evidence that PIP1 and PIP2 assemble as hetero-tetramers is still lacking.

The idea that hetero-tetramer formation of PIPs controls their expression at the cell surface is also consistent with early observations made in transgenic *Arabidopsis* plants showing that antisense inhibition of PIP1 or PIP2 aquaporin expression, independently or in combination, resulted in a similar reduction in root hydraulic conductance (Martre *et al.*, 2002). All these evidences suggested that aquaporins of the PIP1 and PIP2 subgroups act in



a cooperative manner to form the same active water channels (Fetter *et al.*, 2004; Martre *et al.*, 2002; Temmei *et al.*, 2005). The proposal that the C-terminal tail of an individual PIP monomer can interact with the pore of an adjacent monomer may bring some structural clues to this mechanism (Tornroth-Horsefield *et al.*, 2006).

### III. AQUAPORINS IN ROOT WATER UPTAKE

#### A. PRINCIPLES OF ROOT WATER UPTAKE

Roots are organs specialized in water and nutrient absorption from the soil (Figs. 3–4). The rate of water uptake ( $J_v$ ) is proportional to both the difference in water potential ( $\Delta\Psi$ ) between the soil and the xylem vessels and the hydraulic conductance ( $L$ ) of the root system (Steudle, 1989):

$$J_v = L \cdot \Delta\Psi$$

$L$  is determined by both the intrinsic root water permeability (root hydraulic conductivity,  $L_p$ ) and the exchange surface ( $S$ ).  $\Delta\Psi$  reflects the additive

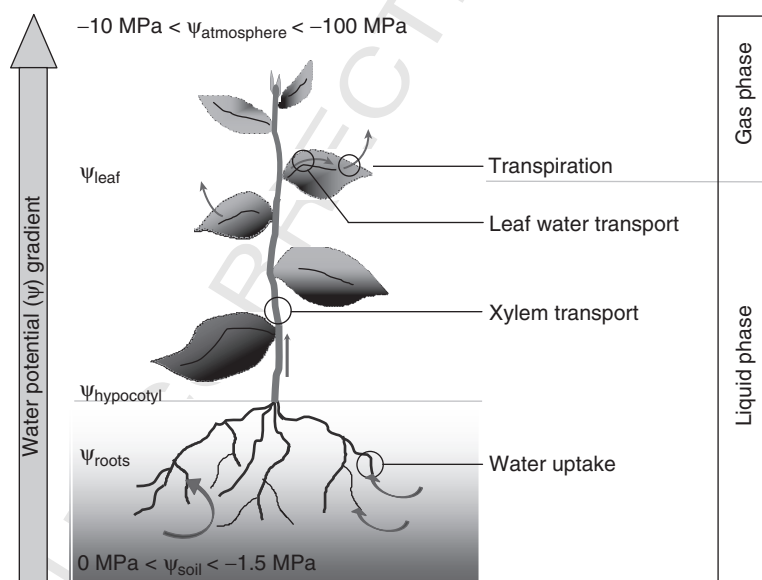


Fig. 3. Water flows in the soil–plant–atmosphere continuum. Water transport is driven by a gradient of decreasing water potentials and occurs in both liquid and gas phase. For all processes indicated, the rate of water flow ( $J_v$ ) is determined by the water potential gradient ( $\Delta\Psi$ ) and the hydraulic conductance of the plant organ considered ( $K$ ):  $J_v = K \Delta\Psi$  (See Color Plate Section in the back of the book.)

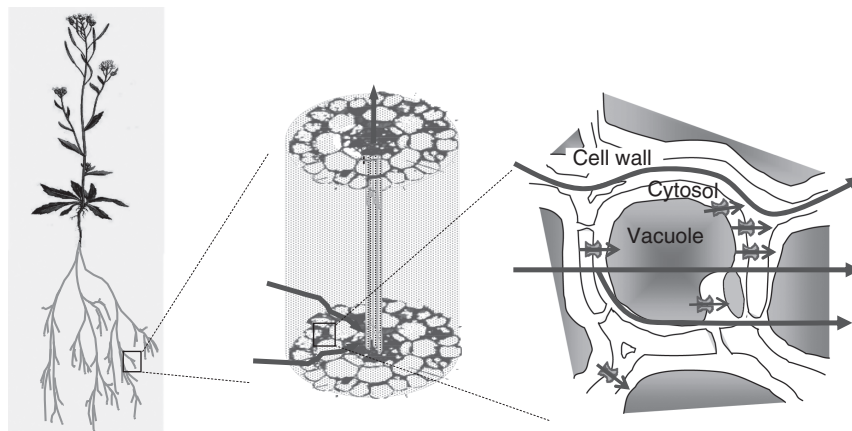


Fig. 4. Water uptake by roots. The graph shows how water passes through concentric cell layers (epidermis, cortex, endodermis, and stele) before being loaded into the xylem vessels where it is pulled up by tensions. According to the composite model, water can flow along both the apoplastic and the cell-to-cell pathways. The latter comprises the symplastic and the transcellular paths. The contribution of plasma membrane and tonoplast aquaporins to the transcellular path is indicated (See Color Plate Section in the back of the book.)

contribution of hydrostatic ( $\Delta P$ ) and osmotic ( $-\sigma\Delta\pi$ ) driving forces. In the latter case, the difference in osmotic potential between the soil and the xylem vessels ( $\Delta\pi$ ) is modulated by the reflection coefficient ( $\sigma$ ), which is equal to unity in hemipermeable barriers and smaller when the barrier is permeable to solutes. Therefore, water uptake can be modeled according the following equation:

$$J_v = L_{p_r} S (\Delta P - \sigma \Delta \pi)$$

Although roots form highly branched tortuous organs, they can be schematically reduced to elemental cylindrical segments, in which water first flows radially from the soil into the xylem vessels (Fig. 4). Water is then transported axially along the vessels. Measurements of the hydraulic resistance of roots and comparison to the axial resistance calculated from vessel diameter using Poiseuille's law have indicated that, in most plant species, axial water transport does not oppose a significant resistance within the root. This point was spectacularly exemplified by Steudle and Peterson (1998), who indicated that a cylindrical vessel with a diameter of  $23 \mu\text{m}$  would require a length of 24 km to exhibit a resistance comparable with that of a single cell membrane.

Radial water transport in roots can be mediated through two parallel pathways (Fig. 4). The composite model of root water transport developed by Steudle and co-workers (for review, see Steudle, 2000; Steudle and Peterson, 1998) has explored all physiological and biophysical significance of this dual configuration. First, the cell wall continuum defines the apoplastic pathway in which water flow is essentially driven by hydrostatic forces (i.e., pressures or tensions) (Steudle, 1994). Second, the cell-to-cell pathway includes water transport through the continuum of cytoplasmic linked by plasmodesmata (symplastic path) and through cell membranes (transcellular path). Because cells and membranes create efficient barriers against solute diffusion, both osmotic and hydrostatic forces are at work in the cell-to-cell pathway (Steudle, 1994; Steudle and Peterson, 1998).

The respective contribution of the different pathways is biophysically difficult to establish, and it has been assumed for a long time that the apoplast offered the pathway of lesser resistance. The realization that aquaporins, by enhancing the water permeability of cells, can significantly decrease the resistance of the cell-to-cell pathway has changed our classical views on root water transport (Maurel and Chrispeels, 2001). Aquaporins have also provided solid molecular bases, with which to explore the amazing properties of roots to adjust their water transport properties ( $L_p$ ) in response to environmental stimuli.

## B. AQUAPORIN EXPRESSION IN ROOTS

Numerous data are available in the literature that describe in great detail the cell specific expression pattern of aquaporins in roots. These data have been reviewed in detail elsewhere (Javot and Maurel, 2002; Maurel *et al.*, 2002) and will not be further commented here. Overall, they show that individual aquaporins can be detected in virtually all root tissues investigated including the root apical meristem and lateral root primordia, the elongation and differentiation zones, the epidermis and root hairs, the cortex and the vascular bundles including a variety of cell types such as tracheary elements, xylem parenchyma cells, phloem, phloem-associated cells and cambium (for review, see Javot and Maurel, 2002). This variety of expression patterns is consistent with putative role of aquaporins in root-specific functions, that is, radial water transport, but also more general functions such as cell expansion or sap transport into and out of vascular tissues. Recently, a few proteomic and transcriptomic studies have provided complementary information and helped address expression of the whole aquaporin family (Alexandersson *et al.*, 2005; Birnbaum *et al.*, 2003; Hachez *et al.*, 2006; Santoni *et al.*, 2003). All these approaches have confirmed that roots express a large number of

aquaporin isoforms, some of them being highly abundant. In particular, mass spectrometry analysis of plasma membranes purified from *Arabidopsis* roots has unambiguously established the expression of at least 6 PIP isoforms (PIP1;1 and/or PIP1;2, PIP1;5, PIP2;1, PIP2;2; PIP2;4, and PIP2;7) (Santoni *et al.*, 2003). A first global view of cell-specific expression of mRNAs (including those encoding aquaporins) within the *Arabidopsis* roots was provided by Birnbaum *et al.* (2003). Protoplasts were prepared by digestion of various root tissues and automatically sorted on the basis of expression of cell-specific reporter genes. Hybridization to Affymetrix chips of cDNAs prepared from various cell fractions was used to generate an expression map comprising 15 root zones. Aquaporin expression was detected in all of these zones, and, for example, PIP1;1 was more expressed in the differentiated zone than in the apex or the elongation zone. Moreover, this isoform was strongly expressed in the stele, the endodermis, and the cortex but was barely expressed in the epidermis. Hachez *et al.* (2006) have developed a thorough analysis of plasma membrane aquaporin expression in maize primary root using quantitative RT-PCR on microdissected root segments, *in situ* RT-PCR and immuno-localization. This study allowed to identify expression of two predominant isoforms (*ZmPIP1;5*, *ZmPIP2;5*) in the differentiated zone of the root. Strong expression of aquaporins, including *ZmPIP2;5*, was observed in the endodermis and exodermis, consistent with a controlling role of these two cell layers in radial water transport. A role of PIP2;5 in primary water uptake was further suggested by polar expression of this aquaporin on the external periclinal side of epidermal cells.

### C. MEASUREMENTS OF ROOT WATER TRANSPORT

The whole set of techniques developed to measure water transport at the cell and subcellular levels, that is, stopped-flow spectrophotometry on isolated membrane vesicles, osmotic swelling of isolated protoplasts, and cell pressure probe techniques has been applied to root materials (see Section II.B). The techniques discussed below allow to measure water transport specifically at the whole root level.

#### 1. Pressure chamber

Pressure chambers have been instrumental to measure water potentials of plant organs. Pressure chambers can also be used to measure the hydrostatic hydraulic conductivity ( $L_p$ ) of excised roots (Boursiac *et al.*, 2005; Markhart *et al.*, 1979). Briefly, an excised root system is inserted in a chamber filled with soil or a bathing solution and the cut stem is threaded through and sealed to the chamber lid. A pneumatic pressure is delivered into the chamber to create

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a hydrostatic pressure gradient across the root and to induce a water flow. The rate of sap flow is determined by collecting the expressed sap into a graduated capillary, whereas the surface area (or the mass) of the root is determined independently. Within certain pressure limits, the flow of sap exuding from the stem section is proportional to the hydrostatic pressure gradient present between the root solution and the xylem solution and the  $L_{p_r}$  value is easily derived from the pressure-to-flow relationship and the root surface area (or mass). The effects of osmotic gradients of varying amplitude on the balancing pressure required for null-flow conditions, together with the difference in osmotic potential between the bathing solution and exuded sap, can be used to calculate the root reflection coefficient (Boursiac *et al.*, 2005; Steudle, 1989).

### 2. High pressure flow meter (HPFM)

Here, a flow of sap is pushed, using a pump, within the excised root, through the stem section (Siefritz *et al.*, 2002). Flow rates are measured for varying pressures, and the slope of the flow versus pressure relationship, divided by the root surface area, provides an  $L_{p_r}$  value. Therefore, this and the pressure chamber technique rely on very similar principles, except that they work with flows of opposite directions. The two techniques have also raised the same types of criticism. First, the forced flow of water leads to tissue (air space) infiltration and therefore may change the respective contributions of the different pathways. In addition, unstirred layers may lead to a biased measurement of  $L_{p_r}$ . In particular, an intense water flow across root tissues may induce an accumulation or sweep away of solutes in the vicinity of membrane barriers and may build up local, artifactual osmotic gradients (Steudle, 1994).

### 3. Root pressure probe

An excised root, maintained at atmospheric pressure, is tightly connected, via silicon seals, to a device that allows to measure and control the pressure within the xylem vessels (Steudle, 1993). In resting conditions, the basal root pressure ( $P_{root}$ ) can be measured, and reflects the hydrostatic pressure developed through spontaneous osmotic transport of water across the root. By analogy to pressure probe measurements at the cell level, the whole root is considered as an elastic barrier that can support both hydrostatic and osmotic pressure gradients. Therefore, water flows can be induced by changes in either of the two forces and the relaxation over time of  $P_{root}$  is indicative of the root osmotic or hydrostatic hydraulic conductivities. In case of a permeable solute, the relaxation curve of  $P_{root}$  is biphasic and can be used to calculate the root permeability to the solute together with the root reflection coefficient (Azaizeh and Steudle, 1991; Steudle, 1993).

#### D. EVIDENCE FOR WATER TRANSPORT BY AQUAPORINS IN ROOTS

##### 1. Cell level

The contribution of aquaporins to water transport in roots was first probed using mercury inhibition. Cell membrane fractionation and stopped-flow measurements on isolated tonoplast vesicles revealed extremely high water permeability values ( $P_f > 500 \mu\text{m s}^{-1}$ ) and strong inhibition by mercurials (>80%) in membranes from wheat roots and radish taproots (Niemietz and Tyerman, 1997; Ohshima *et al.*, 2001). Water transport in intact vacuoles isolated from protoplasts of rape or red beet roots confirmed the idea that the tonoplast of most plant cells exhibits a high  $P_f$  ( $>200 \mu\text{m s}^{-1}$ ) (Morillon and Lassalles 1999). Niemietz and Tyerman (1997) and Ohshima *et al.* (2001) have also measured the  $P_f$  of plasma membrane (PM) vesicles from wheat roots and radish taproots respectively, and have shown this to be several-fold lower than the  $P_f$  of the corresponding tonoplast vesicles. This difference in water permeability between plasma membranes and endomembranes has also been observed in tobacco suspension cells, and its general significance in terms of cell osmoregulation has been discussed elsewhere (Maurel *et al.*, 2002; Tyerman *et al.*, 1999). We note that by contrast to the studies mentioned above, Alleva *et al.* (2006) found a high  $P_f$  ( $542 \pm 40 \mu\text{m s}^{-1}$ ) and active water channels in plasma membranes vesicles prepared from red beet storage roots. In most cases, however, it has been assumed that plasma membranes represent the limiting barrier for transcellular water transport.

The specific contribution of PIP1 or PIP2 aquaporins to water transport in *Arabidopsis* root cells has first been inferred from osmotic swelling assays in protoplasts prepared from wild-type plants or plants expressing a PIP1;2 or a PIP2;3 antisense gene (Martre *et al.*, 2002). Reduced expression of PIP1 or PIP2 aquaporins resulted in significant decreases in root protoplast water permeability. For instance, protoplasts isolated from control and PIP2;3 antisense plants had a mean hydraulic conductivity of  $117 \pm 17 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$  and  $4 \pm 1 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , respectively. Cell pressure probe measurements in *Arabidopsis* roots, either wild-type or carrying a T-DNA insertion in the *PIP2;2* gene, showed that this single aquaporin isoform contributes to about 25% of the cell hydraulic conductivity of cortical cells (Javot *et al.*, 2003).

##### 2. Organ level

*a. Mercury inhibition.* Mercury inhibition has provided the first indication that water channels can represent a major path for root water uptake (Maggio and Joly, 1995). The significance and limitations of this approach have been discussed in detail elsewhere (Javot and Maurel, 2002). Briefly, effects of  $\text{HgCl}_2$  on root water transport have been investigated in more

than 10 plant species. Although differences exist in the dose applied ( $10^{-6}$  to  $10^{-3}$  M) and the duration of treatment (10 min to several hours), all studies pointed to a significant inhibition of  $Lp_r$  by mercury, from 32% in *Opuntia acanthocarpa* to 90% in barley (Javot and Maurel, 2002). Although mercury is now a widely used inhibitor for water transport studies, we insist that this compound can react with exposed cysteine residues of any cellular proteins and therefore can have profound and toxic cellular effects. For instance, mercury reduced the membrane electrical potential of wheat root cortex cells with a dose dependency similar to that for the inhibition of hydraulic conductivity (Zhang and Tyerman, 1999). Wan and Zwiazek (1999) observed that a long (>1 h) mercury treatment reduced both root cell respiration and stomatal conductance in aspen seedlings. These side effects reflect an overall metabolic inhibition in mercury treated plants, with a possibly strong impact on their overall water and solute transport properties. To evaluate a possible role on solute pumping, several authors have checked that mercury treatment did not alter  $K^+$  transport in the root sap (Carvajal *et al.*, 1999; Maggio and Joly, 1995; Wan and Zwiazek, 1999). It is also important to assess that the primary effects of mercury are mediated through an oxidation of cellular components (possibly aquaporins) and can rapidly be reversed by reducing agents such  $\beta$ -mercaptoethanol or dithiothreitol. This mandatory control is missing in certain studies.

*b. Acid loading, a new procedure for aquaporin inhibition.* Because all plant PIPs display the same  $H^+$ -gating properties (Tournaire-Roux *et al.*, 2003), cytosolic acidification can provide a powerful means for blocking aquaporins, specifically at the plasma membrane. This can be experimentally achieved by acid loading, that is, treating plant tissues with a weak acid, which diffuses through the cell membrane preferentially in its neutral (acidic) form and therefore releases protons intracellularly. For instance, Tournaire-Roux *et al.* (2003) observed that exposure of *Arabidopsis* roots to 20 mM of propionic acid/potassium propionate, pH 6.0, induced a rapid ( $t_{1/2} = 3.7 \pm 0.3$  min) and marked ( $71\% \pm 3\%$ ) decrease in  $Lp_r$ . Remarkably, this inhibition was reversible upon removal of the propionic acid/potassium propionate from the bathing solution. Pressure probe measurements revealed that acid load treatments induced an even stronger inhibition of hydraulic conductivity in cortical cells providing evidence that effects were truly exerted on membrane water transport (Tournaire-Roux *et al.*, 2003).

The same study also established that respiratory poisons such as azide or cyanide, which had been previously identified as potent inhibitors of water transport in roots (Zhang and Tyerman, 1991) induced a marked drop in cytosolic pH and therefore blocked  $Lp_r$  (Tournaire-Roux *et al.*, 2003).

Here again, the effects were reversible, upon washing the root with a control bathing solution, suggesting that the poison molecules rapidly diffused out of the cells and/or were efficiently metabolized. Altogether, the data indicated that water transport in the *Arabidopsis* root is largely dominated by the transcellular pathway (Tournaire-Roux *et al.*, 2003) (Fig. 5). In addition, acid load treatments were established as an efficient procedure for inhibiting aquaporins in living plant tissues. This procedure is reversible and possibly less toxic than mercury treatment.

*c. Reverse genetics.* Because of a lack of specific aquaporin inhibitor, reverse genetics remains a central approach to explore the physiological function of aquaporins. In principle, the function of specific isoforms or subgroups of aquaporins can be addressed.

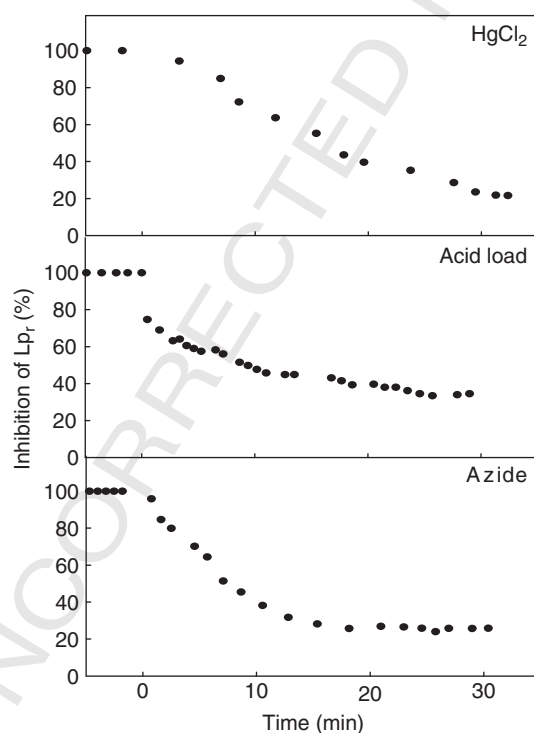


Fig. 5. Inhibition of water transport in the *Arabidopsis* root by aquaporin blockers. Excised roots were inserted into a pressure chamber and the rate of water flow at constant pressure (equivalent to  $L_{p_r}$ ) was measured over time. At time 0, roots were treated with 50  $\mu$ M  $\text{HgCl}_2$ , 20 mM propionic acid, pH = 6.0, or 1 mM azide. The inhibition response, very similar between the three treatments, indicates that, in the *Arabidopsis* root, the cell-to-cell pathway is largely predominant.



Antisense inhibition of PIP aquaporin expression in *Arabidopsis* and tobacco has revealed the importance of this group of aquaporins in root water transport. For instance, expression of a PIP1 (*NtAQP1*) antisense gene in tobacco reduced  $L_{pr}$  by 58%, to an extent which was comparable to the reduction in  $P_f$  observed at the level of isolated root protoplasts (55%) (Siefritz *et al.*, 2002). In *Arabidopsis*, expression of a PIP1;2 or a PIP2;3 antisense gene, individually or in combination, reduced the root hydraulic conductivity by 60, 47, and 68%, respectively. The finding that the simple and double antisense lines induced similar reductions in water permeability, both at the whole root and at the protoplast levels, was interpreted to mean that PIP1 and PIP2 aquaporins cooperate to form the same active water channels in the plasma membrane (Martre *et al.*, 2002). Both Kaldenhoff *et al.* (1998) and Martre *et al.* (2002) also observed that antisense inhibition of PIP aquaporins in *Arabidopsis* lead to an increase in root mass by two- to fivefold, depending on the lines. Therefore, the reduced water permeability in the plasma membrane of root cells was compensated by an increase in the size of the root system. This morphological adjustment allowed the root conductance; in other words, the overall capacity of the root to deliver water to the shoot to be maintained.

Knockout approaches in *Arabidopsis* have also revealed the contribution of a single aquaporin isoform (PIP2;2) to root water uptake (Javot *et al.*, 2003). Whereas this aquaporin contributed to about 25% of cortical cell hydraulic conductivity, no significant contribution to the overall root hydrostatic hydraulic conductivity ( $L_{pr}$ ), as measured with a pressure chamber, could be resolved. By contrast, free exudation by excised roots yielded sap of greater osmolality in mutant than in wild-type plants (Javot *et al.*, 2003). This reflected a reduced osmotic hydraulic conductivity in roots of the mutant plants. The overall data allowed to propose that PIP2;2 is an aquaporin specialized in osmotic fluid transport. This specific function may possibly be related to the preferential expression of this aquaporin in the inner root tissues. Interestingly, PIP2;2 and its close homologue PIP2;3 have likely evolved through a recent gene duplication and share 97% identity in their amino acid sequence. The detection of a phenotype in PIP2;2 knockout plants indicates that despite their high isoform multiplicity, plant aquaporins do not have fully overlapping functions (Javot *et al.*, 2003).

A reverse strategy has been to overexpress a single aquaporin gene in transgenic plants. Katsuhara *et al.* (2003) showed that expression of a barley PIP aquaporin in rice yielded an increase in  $L_{pr}$  by 140%. A note of caution should be taken when interpreting physiological changes due to ectopic expression of an aquaporin in another plant species, since crucial physiological regulations of the aquaporin of interest may not be maintained. A more

consistent strategy was reported by Lian *et al.* (2004) who overexpressed in rice a PIP1 homologue from the same species, the transgene being placed under the control of a stress responsive promoter. Consistent with this,  $L_{p_r}$  was similar between wild-type and transformed plants in standard conditions, whereas, under water stress conditions, it was higher by 25% in the latter plants.

#### E. EFFECTS OF STIMULI ON ROOT WATER TRANSPORT

The ability of plants to adjust their root water transport properties during development or in response to environmental stimuli has received much attention from plant physiologists. Long-term adaptations can in part be accounted for by morphological changes, that is, overall changes in root architecture or differentiation of specialized cell types. For instance, drought can lead to the development of Casparian bands and suberin lamellae in the exodermis and endodermis and to the production of a suberized periderm outside the stele (see Zimmermann and Steudle, 1998, and references therein). It now appears that regulation of aquaporins also significantly contributes to the regulation of root water permeability and the characterization of short-term effects on root water transport of hormonal and environmental stimuli has allowed the effects of aquaporins regulation to be dissociated from slower morphological changes.

##### 1. *Water stress*

In nature, drought, that is, a drop in soil water potential develops continuously over days. Martre *et al.* (2001) have performed a thorough analysis of water transport in the root of *O. acanthocarpa* and observed both deep anatomical changes and a significant decrease in  $L_{p_r}$  after 45 days of soil drying. In addition, the  $L_{p_r}$  of water stressed plants had become fully insensitive to inhibition by 50  $\mu$ M HgCl<sub>2</sub>. North *et al.* (2004) reported that roots of *Agave deserti* that had been drought-stressed for 10 days had a lower hydraulic conductivity than control roots grown in a moist soil. In addition, mercury inhibition of water transport was only found in control roots. These two reports provide some evidence that an inhibition of aquaporins contributed to the adaptation of roots to drought. Although the significance of drought-induced inhibition of  $L_{p_r}$  is not fully clear yet, this response has been interpreted as a strategy to avoid a backflow of water from the plant toward the dry soil. The effects of drought can be mimicked in plants grown in hydroponic conditions and subjected to a hyperosmotic treatment (Boursiac *et al.*, 2005; Shangguan *et al.*, 2005). For example, treatment of sorghum seedlings by a poly ethylene glycol (PEG) solution at  $-0.3$  MPa reduced their  $L_{p_r}$  by up to 70% in 30–60 h (Shangguan *et al.*, 2005).

ABA is synthesized in response to drought and plays a central role in regulating the plant water status. ABA not only induces stomatal closure but also controls root water transport. For instance, treatment of maize roots with 1  $\mu$ M ABA transiently increased the hydraulic conductivity of cortical cells by 27-fold after 1 h and had no effect after 2 h (Hose *et al.*, 2000). An increase in  $L_{pr}$  by a factor of 3–4 was observed after 70–100 min of an ABA treatment. This strategy may allow the plant to take up residual soil water, before severe drought develops.

The regulation of PIPs aquaporin expression in response to water stress has been investigated in several plant species (for review, see Tyerman and Niemietz, 2002). In rice, for instance, water deficit, as induced by treatment with 20% PEG6000 for 10 h, resulted in an accumulation of PIP proteins in roots (Lian *et al.*, 2004). A thorough RT-PCR analysis of expression of all 13 PIP isoforms in the *Arabidopsis* root revealed that a hyperosmotic treatment (250 mM of mannitol, 12 h) induced an upregulation of some isoforms (PIP1;3, PIP1;4, PIP2;1, and PIP2;5), whereas transcripts of other isoforms (PIP1;5, PIP2;2, PIP2;3, and PIP2;4) were markedly downregulated (Jang *et al.*, 2004). However, the relation between changes in aquaporin expression and water transport regulation has not been established in these studies.

Plants with genetically altered expression of aquaporins have provided stronger direct evidences for a role of aquaporins during water stress. For instance, antisense inhibition of PIPs in tobacco and *Arabidopsis* resulted in a marked defect of the plants to adapt to water deprivation (Martre *et al.*, 2002; Siefritz *et al.*, 2002). Upon rewetting after drought, the signs of leaf wilting took longer to dissipate in PIP1 antisense than in control tobacco plants (Siefritz *et al.*, 2002). This suggested that the capacity of the whole plant to take up and transport water was limiting in the antisense lines. Using a different approach, Lian *et al.* (2004) have overexpressed in a lowland, drought sensitive rice cultivar, a PIP aquaporin (RCW3 or OsPIP1;3) that was specifically induced by drought in an upland, drought tolerant cultivar. Interestingly, this manipulation enhanced the ability of the first cultivar to avoid drought. While these observations suggest that an enhanced mobility of water within the plant body and possibly enhanced capacity to take up soil water are beneficial to plants under water stress, more work is needed to draw a clear picture of aquaporin function during water stress.

## 2. Salt stress

Soil salinity exerts noxious effects on plant yield in part by challenging the plant water status. One of the primary responses of plants to salt is inhibition of their root water uptake capacity. In many plant species, including paprika (Carvajal *et al.*, 1999), melon (Carvajal *et al.*, 2000), *Arabidopsis* (Boursiac

*et al.*, 2005), and tomato (Peyrano *et al.*, 1997), salinization with 50–100 mM of NaCl reduced  $L_p$  by  $\geq 60\%$  after 2–4 days. This response can be observed in a large variety of glycophytic or halophytic plant species. However, roots of certain species, such as tobacco, do not respond to a salt treatment (Tyerman *et al.*, 1989). The inhibition of root water transport by salt can be counteracted by external calcium, but the molecular basis of these ameliorative effects is not understood yet (Azaizeh and Steudle, 1991; Carvajal *et al.*, 2000; Martinez-Ballesta *et al.*, 2000, 2006). The idea that the primary inhibition of  $L_p$  by salt could be accounted for by aquaporin inhibition was raised from early experiments showing that the residual  $L_p$  observed after salt treatment of paprika, melon, or *Arabidopsis* roots was insensitive to mercury (Carvajal *et al.*, 1999, 2000; Martinez-Ballesta *et al.*, 2003). Microarrays experiments by Maathuis *et al.* (2003) also revealed that exposure for 6–24 h of *Arabidopsis* roots to salinity stress induced an overall reduction in aquaporin expression, whereas longer-term treatments lead to enhanced aquaporin expression. By combining water transport assays and Northern blot analyses in *Arabidopsis* roots treated for 24 h with various salt concentrations, Martinez-Ballesta *et al.* (2003) further showed a correlation between the extent of  $L_p$  inhibition and the downregulation of PIP1 mRNAs. To investigate further the molecular and cellular mechanisms of aquaporin downregulation under salt stress, Boursiac *et al.* (2005) analyzed the short-term responses of *Arabidopsis* roots to salt. Kinetic analyses revealed that the decrease in  $L_p$  induced by 100 mM of NaCl occurred rapidly with a half-time of about 45 min. All most abundant PIP and TIP transcripts were stably expressed until 2 h of treatment and showed a coordinated and marked decrease in abundance on the long term (6–24 h). ELISA and Western blot analyses using antibodies that specifically recognized aquaporins of the PIP1, PIP2, and TIP1 subclasses revealed a decrease in PIP1 abundance by 30–40% as soon as 30 min after salt exposure, whereas aquaporins of the other subclasses showed a reduced abundance only after 8 h. In addition, transgenic lines expressing various aquaporins fused to GFP revealed that TIP1;1 was partially relocated into circular structures associated to the main vacuole and firstly described by Saito *et al.* (2002) as vacuolar bulbs. The labeling of intracellular structures by PIP–GFP fusions after 2–4 h of salt exposure also suggested that internalization of PIPs contributed to the reduced water permeability of the plasma membrane. Therefore, altered expression at different levels of aquaporins in *Arabidopsis* roots can provide a basis to explain the inhibitory effects of salt on  $L_p$ , mostly in the long term. Other mechanisms such as altered aquaporin phosphorylation may contribute to the early (<2 h) effects of salt.

Findings about the regulation of NOD26 in the peribacteroid membrane of nitrogen fixing symbiotic of soybean roots support these ideas. Guenther *et al.* (2003) showed that phosphorylation of the aquaporin C-terminal tail, as probed with anti-phosphopeptide antibodies was stimulated in response to drought and salt stress. This suggested a role for NOD26 in the osmoregulation of root nodule cells during water stress. The finding that pretreatment of melon protoplasts by okadaic acid, a potent inhibitor of phosphoprotein phosphatases 1 and 2A, counteracted the inhibition of protoplast  $P_i$  by salt stress provides another line of evidence for a role of protein phosphorylation during the regulation of water transport (Martinez-Ballesta *et al.*, 2000).

### 3. Anoxia

Soil flooding or compaction reduces oxygen diffusion and can result in a severe hypoxic stress for plant roots. This physiological context can be mimicked by bubbling  $N_2$  instead of air in a root bathing solution or by treating roots by respiratory poisons such as azide or cyanide. In most plant species, these treatments lead to a marked inhibition of  $Lp_r$ . For instance, oxygen depletion in sunflower roots reduced  $Lp_r$  by 50% in <2 h, whereas treatment of red-osier dogwood roots with 0.5 mM azide led to a decrease by 35% in both  $O_2$  uptake and  $Lp_r$  after 2 h (Everard and Drew, 1989; Kamaluddin and Zwiazek, 2001). In *Arabidopsis* roots, hypoxic stress or treatment with 1 mM azide for 30 min reduced the  $Lp_r$  by 49 and 87%, respectively (Tournaire-Roux *et al.*, 2003). Parallel *in vivo*  $^{31}P$  nuclear magnetic resonance measurements revealed that these treatments also resulted in a rapid drop in cytosolic pH by about 0.5 units. The mechanism of this cell acidosis is primarily due to a depletion in cellular ATP accompanied by an increase in cytosolic inorganic phosphate, which prevents  $H^+$ -ATPases to extrude cytosolic protons (Gout *et al.*, 2001). As discussed above (see Section III.D.2b), the work by Tournaire-Roux *et al.* (2003) has established how the inhibition of  $Lp_r$  under anoxic stress can directly be accounted for the pH-dependent closure of PIP aquaporins.

### 4. Light

More than 30 years ago, Parsons and Kramer (1974) observed that cotton roots exhibited a diurnal cycling of their hydraulic conductivity that was minimal at night and was increased two- to threefold at midday. This kind of observation has now been extended to many other species including wheat (Carvajal *et al.*, 1996), paprika pepper (Carvajal *et al.*, 1999), tomato (Dell'Amico *et al.*, 2001), *L. japonicus* (Henzler *et al.*, 1999), and maize (Lopez *et al.*, 2003). This phenomenon may provide a means for reducing tension in the root xylem and therefore avoiding cavitation during transpiration. A link between diurnal  $Lp_r$

fluctuations and aquaporin functions has been established by several authors. For instance, Henzler *et al.* (1999) found that the diurnal rhythm of the hydraulic and osmotic conductivities of *Lotus* roots over a 24-h period was tightly correlated to the abundance of PIP1 transcripts, as measured by Northern blots (Henzler *et al.*, 1999). Similar evidence has been obtained in maize roots where the abundance of the four most highly expressed *ZmPIPs* transcripts started to raise just before dawn and was maximal after 4 h of light (Lopez *et al.*, 2003). Therefore, the expression of *ZmPIPs* transcripts slightly preceded the increase in osmotic root water transport observed during the day. In addition, the abundance of PIP2 proteins was well correlated to the diurnal variations in root water transport (Lopez *et al.*, 2003).

### 5. Nutrient status

The plant nutrient status is another factor that governs the root hydraulic properties (Clarkson *et al.*, 2000). For example, roots excised from maize or barley plants grown under nitrate or sulphate deprivation for 7 days and 4 days, respectively, exhibited a reduction by 50–80% in exudation rate or  $L_{p_r}$ , respectively, with respect to roots from plants grown in nitrate or sulfate replete conditions (Hoarau *et al.*, 1996; Karmoker *et al.*, 1991). In wheat, a significant part of root water uptake is mediated through aquaporins, as suggested by the marked (66%) inhibition of  $L_{p_r}$  by mercury (Carvajal *et al.*, 1996). Under 5 or 7 days of a complete deprivation in nitrogen (N) or phosphorus (P), respectively, the  $L_{p_r}$  was reduced by 80–85%. Interestingly, the residual root water transport activity had become fully insensitive to mercury and could be reversed to initial control activity in about one day, suggesting that the effects of nutrient starvation were mediated through inhibition of aquaporin expression or activity (Carvajal *et al.*, 1996). This idea is further substantiated by a study in cotton showing that the hydraulic conductivity of root cortical cells, as measured with a cell pressure probe, was decreased by 60 and 80% after 4 days of deprivation in P or N, respectively (Radin and Matthews, 1989).

In a recent study, Shangguan *et al.* (2005) have investigated the interactions between water and P deprivation stresses in sorghum. It was found that the latter stress accentuated the inhibitory effects of drought on  $L_{p_r}$  and slowed the reversal of  $L_{p_r}$  toward initial value that was normally observed in the 12 h following plant rewatering. These experiments support the idea that P-starved plants were more susceptible to drought than P-replete plants.

### 6. Low temperatures

Studies in cucumber (Lee *et al.*, 2004), tomato (Bloom *et al.*, 2004), spinach (Fennell and Markhart, 1998), and maize (Aroca *et al.*, 2001, 2005; Melkonian *et al.*, 2004) have shown that plants respond to chilling by

decreasing their root hydraulic conductivity. In a chilling sensitive maize cultivar, for instance, the  $L_{pr}$  of 11-day-old plants was decreased by up to 80% after 6 h at 5 °C. The osmotic hydraulic conductivity was also decreased and reached a null value after 54 h of chilling treatment (Aroca *et al.*, 2001). In all plant species investigated, chilling-induced inhibition of root water transport resulted in plant water deficit, as shown by a rapid decrease of leaf relative water content (Aroca *et al.*, 2001; Yu *et al.*, 2006) or leaf water potential (Melkonian *et al.*, 2004).

The finding that chilling enhances  $H_2O_2$  production in cucumber roots and that treatment of these roots by 2 mM of  $H_2O_2$  (Lee *et al.*, 2004) leads to a twofold inhibition of hydraulic conductivity of cortical cells has led to the idea that the primary effects of chilling on  $L_{pr}$  may be mediated through an inhibition of root aquaporins by  $H_2O_2$ . However, the mechanisms that underlie this inhibition are not fully understood yet (see Section II.E.2).

In maize, the ability of certain cultivars to resist to chilling is linked to their ability to restore a normal root osmotic conductivity under long-term (3 days) exposure to cold. Aroca *et al.* (2005) observed a parallel increase in PIP proteins abundance and phosphorylation. However, a similar response was also observed in a sensitive cultivar that showed sustained osmotic conductivity inhibition. This suggested that the aquaporin upregulation response was not sufficient in the latter plants and was probably dominated by other mechanisms. In particular, a more important membrane damage was observed in the sensitive cultivar. In rice, the recovery of root osmotic conductivity after a transient chilling treatment (7 °C, 24 h) was total for a resistant cultivar and partial for a sensitive cultivar (Yu *et al.*, 2006). A stronger increase in specific *OsPIP* transcripts was observed during recovery in the resistant cultivar as compared to the sensitive one. However, no difference in protein abundance, as measured by Western blot, was detected between the two cultivars (Yu *et al.*, 2006).

#### F. TRANSPORT OF NUTRIENTS

Whereas the analysis of water transport in plants altered in aquaporin expression has revealed phenotypes of reduced amplitude (Javot *et al.*, 2003; Martre *et al.*, 2002; Siefritz *et al.*, 2002), recent genetic analyses have provided compelling evidences about the role of NIP aquaporins in nutrient transport in plants.

First, molecular characterization of *lsi1*, a rice mutant defective in silicon uptake, revealed that the corresponding locus encoded a plasma membrane localized NIP homologue expressed in root exodermis and endodermis (Ma *et al.*, 2006). The role of LSI1 was confirmed in rice plants that expressed

a corresponding RNAi construct and showed a capacity to take up silicon reduced by 8- to 10-fold. In addition, mRNA expression of LSI1 in wild-type plants was downregulated by continuous silicon supply. Therefore, LSI1 represents the first silicon transporter identified in higher plants. It has close homologues in maize, and its characterization provides a unique opportunity to manipulate silicon transport and possibly stress resistance in crops.

As discussed above (see Section II.C), functional expression in *Xenopus* oocytes has revealed a boron channel activity for AtNIP5;1 (Takano *et al.*, 2006). A first hint at this function was obtained after observing that the abundance in AtNIP5;1 transcripts was reversibly increased by 12-fold in response to boron deficiency. Promoter- $\beta$ -glucuronidase fusions showed NIP5;1 to be preferentially expressed under boron limitation in the root elongation zone and the root hair zone. The characterization of two independent *Arabidopsis* knockout lines allowed to clearly establish the physiological significance of boron transport by NIP5;1 (Takano *et al.*, 2006). First, a reduction in root and shoot growth was observed at limiting boron concentration ( $<3 \mu\text{M}$ ), exclusively in the mutant plants. These phenotypes can be associated with a failure of mutant plants to take up boron. Under low boron supply, mutant plants accumulated less boron than wild-type plants, and the rate of boron uptake, as estimated using a [ $^{10}\text{B}$ ] isotope, was reduced by at least fivefold. Another class of boron transporters, whose founding member is BOR1, has been identified in plants. Whereas NIP5;1 can account for most of the primary uptake of boron from the soil, BOR1 seem to be rather involved in xylem loading and root-to-shoot translocation of boron (see Takano *et al.*, 2006, and references therein).

#### IV. AQUAPORINS IN LEAVES

Water transport in leaves occurs in both liquid and gas phases (Fig. 3). Water is lost by transpiration, that is, mainly by diffusion of water vapor through the stomata. This process results in a drop in leaf water potential that drives a flow of liquid water through the petiole and the inner leaf tissues to the cell surfaces that delimit the stomatal cavity (Fig. 6). This process requires water transport through the veins (vascular compartment) and eventually through the vascular bundles and the mesophyll, which together define the extravascular compartment (Fig. 6).

Although the rate of water transport through transpiring plants is primarily controlled by their stomatal conductance ( $G_s$ ), the hydraulic resistance of inner leaf tissues may be critical in determining the water potential gradients



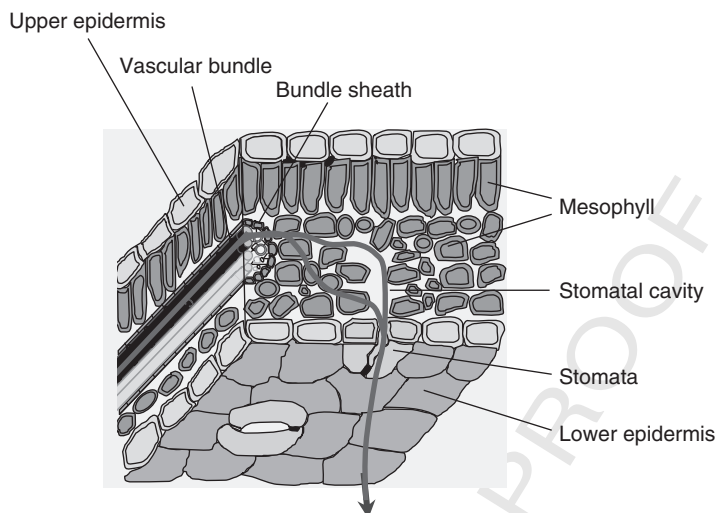


Fig. 6. Water transport within an angiosperm leaf. The contributions of the vascular and the extravascular compartments are indicated. In the latter compartment, water flows through both the apoplastic and cell-to-cell pathways (See Color Plate Section in the back of the book.)

through the leaf. Also, stomatal regulation implies significant changes in guard cell volume. Therefore, a limiting role of transmembrane water transport can be expected in various cell types and physiological contexts in leaves.

#### A. AQUAPORIN EXPRESSION IN LEAVES

The expression pattern of aquaporins in leaves can provide a first glance at their possible role in the leaf water relations. In a recent study, Alexandersson *et al.* (2005) have developed microarrays carrying aquaporin gene specific tags and were the first to monitor the expression profile of all 35 *Arabidopsis* aquaporins in rosette leaves, and compare it to that in other organs such as roots and flowers. Whereas some aquaporin isoforms were found to be specifically expressed in roots or flowers, none of the aquaporin transcripts detected in leaves was specific for this organ. In addition, the overall expression level of aquaporins was lower in leaves than in roots. For instance, with the exception of SIP1;1, expression in leaves of members of the NIP and SIP classes was barely detectable with this approach. By contrast, two TIP isoforms (TIP1;2 and TIP2;1) and three PIP isoforms (PIP1;2, PIP2;1, and PIP2;6) showed strong expression, whereas five additional PIP isoforms (PIP1;1, PIP1;4, PIP1;5, PIP2;2, and PIP2;7) had detectable but less abundant transcripts.

A large number of other studies have provided a more accurate description of cell-specific expression patterns of aquaporin isoforms. For instance, Fraysse *et al.* (2005) have analyzed the expression pattern in spinach leaves of two PIP1s, *SoPIP1;1* and *SoPIP1;2*. Immunolocalization experiments revealed that the two aquaporins were specifically expressed in guard cells and phloem sieve elements, respectively. Expression of PIP and TIP aquaporins has also been reported in guard cells of *Arabidopsis*, *Vicia faba*, and sunflower (Kaldenhoff *et al.*, 1995; Sarda *et al.*, 1997; Sun *et al.*, 2001). In the latter species, expression of a TIP transcript was under diurnal regulation and maximal during stomatal closure (Sarda *et al.*, 1997). In leaves of *Brassica napus*, strong expression of aquaporins recognized by anti-PIP1 and anti-TIP1 antibodies was detected in bundle-sheath cells (Frangne *et al.*, 2001). Expression of two PIP isoforms has also been reported in the xylem parenchyma of walnut twigs (Sakr *et al.*, 2003).

In summary, expression of aquaporins seems to be lower in leaves than in roots. Yet expression has been detected in most leaf cell types of all herbaceous or woody species investigated. In particular, the various expression patterns suggest multiple functions of aquaporins in mediating water transport through the extravascular compartment during transpiration, in loading and unloading xylem and phloem sap, and during stomatal movement. Functional data in favor of some of these hypotheses have recently emerged and are discussed (see Sections IV.D and E).

## B. MEASUREMENTS OF WATER TRANSPORT IN LEAVES

Various techniques have been developed to measure leaf water transport at the cell and organ levels. The accuracy of these techniques is and will be crucial for exploring aquaporin function in leaves.

### 1. Cell level

Most of generic techniques discussed above (see Sections II.B and III.C) and used for characterizing water transport in plant cells or isolated membranes have been applied to leaves. Pressure probe measurements, in particular, have been used to characterize the water transport properties of specific leaf cell types. For instance, Westgate and Steudle (1985) found high cell hydraulic conductivity values ( $0.3\text{--}2.5 \times 10^{-6} \text{ m s}^{-1} \text{ MPa}^{-1}$ ) in the parenchymatous midrib tissues of *Zea mays* leaves. In *Tradescantia virginiana* leaves, epidermal cells had a hydraulic conductivity in the range of  $10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ , which was 10-fold higher than in the corresponding mesophyll cells (Tomos *et al.*, 1981; Zimmermann *et al.*, 1980). Crassulacean acid metabolism (CAM) was developed by certain plant species to improve their water use efficiency.

Au8

Water transport in two CAM species, *Kalanchoe daigremontiana* and *Graptopetalum paraguayens*, has been characterized by pressure probe and stopped-flow techniques, respectively (Ohshima *et al.*, 2001; Steudle *et al.*, 1980). Because these two studies revealed strikingly high- and low-water permeabilities, respectively, it has been difficult to conclude whether CAM metabolism is associated with particular water transport properties. More recently, cell water transport has been characterized by means of swelling assays in protoplasts isolated from *Arabidopsis*, petunia, and tobacco leaves (Ding *et al.*, 2004; Martre *et al.*, 2002; Morillon and Chrispeels 2001; Ramahaleo *et al.*, 1999). This approach, however, has been restricted to mesophyll protoplasts.

## 2. Organ level

Numerous techniques have been developed to measure transpiration and stomatal conductance ( $G_s$ ) in intact leaves or plants. The simplest method consists of measuring the overall loss of weight from a potted plant. More sophisticated techniques consist of measuring water vapor release by porometry or infra-red gas exchange analysis. These techniques that primarily reflect the limiting role of stomata for transpiration will not be further addressed in this chapter.

As explained above, transpiration also drives a flow of liquid water from the petiole to the stomatal chamber. As exemplified for water transport in roots (see Section III.A), this transport depends on the difference in water potential between the petiole and the stomatal cavity, on the one hand, and on the inner leaf hydraulic conductance ( $K_{\text{leaf}}$ ), on the other hand. Therefore,  $K_{\text{leaf}}$  integrates the water conductance in series of the petiole, leaf veins, and extravascular tissues. Three major techniques have been developed to measure  $K_{\text{leaf}}$  in intact plants or excised leaves, the latter system providing the most accurate measurements.

First, in the so-called “vacuum pump method,” water is pulled through an excised leaf using a vacuum pump, while the flow rate is measured through the rate of water uptake into the petiole (Sack *et al.*, 2002). The  $K_{\text{leaf}}$  is derived from flow measurements at varying vacuum pressures. It is noteworthy that, in these experiments, the leaf is carefully maintained at saturating water vapor. Therefore, the drop in atmospheric pressure can fully account for the drop in water potential across the leaf. Also, the possible resistance of stomata to water diffusion in gas phase, which normally dominates the process of transpiration, is close to zero because of the absence of a vapor pressure deficit. Thus, the measured  $K_{\text{leaf}}$  mostly reflects a liquid phase conductance of inner leaf tissues (Sack *et al.*, 2002).

A second method called the evaporative flux method relies on the relationship that exists between the flux of transpiration across the plant or an

excised leaf and the corresponding drop in water potential (Martre *et al.*, 2002; Sack *et al.*, 2002). Briefly, the transpiration flow ( $E$ ) throughout the whole plant is measured under steady-state conditions, using gravimetric methods for instance. A leaf is then excised to measure the leaf water potential ( $\Psi_{\text{leaf}}$ ). In parallel to this, a leaf of the plant that had been previously covered with a bag to locally prevent any transpiration is used to measure the stem water potential ( $\Psi_{\text{stem}}$ ). The  $K_{\text{leaf}}$  can then be calculated as the ratio of  $E$  to  $(\Psi_{\text{stem}} - \Psi_{\text{leaf}})$ .

Finally, the high pressure flow meter (HPFM) method requires a flow of solution to be pushed using a pump, from the petiole throughout the leaf (Sack *et al.*, 2002; Tyree *et al.*, 2005). During the measurements, the leaf is submerged in a liquid solution and leaf airspaces rapidly become infiltrated. As for other methods, the leaf conductance can be deduced from the flow versus pressure relationship. Note that in these conditions, the technique assays the resistance of the inner leaf tissues and the stomata in series and therefore can theoretically underestimate the  $K_{\text{leaf}}$ . The assumption that stomata do not offer any limiting resistance to water flows in a liquid phase is based on simple calculations using Poiseuille's law. This has however been disputed, particularly in contexts where irradiance induces apparent changes in  $K_{\text{leaf}}$  (Sack *et al.*, 2002; Tyree *et al.*, 2005). For instance, Sack *et al.* (2002) have argued that the reduced aperture of stomata in *Quercus rubra* leaves at low irradiance could account for their reduced  $K_{\text{leaf}}$ . By characterizing the effects of irradiance in various plant species, Tyree *et al.* (2005) found however that the extent of light-induced increase in  $K_{\text{leaf}}$  was not always correlated to the accompanying increase in stomatal aperture. In addition, treatment of *Juglans regia* leaves with ABA induced a marked closure of stomata without any alteration in  $K_{\text{leaf}}$  (Tyree *et al.*, 2005).

Finally, the three methods discussed above have been validated through a comparative study showing that similar  $K_{\text{leaf}}$  values could be determined by these methods in each of six woody angiosperm species examined (Sack *et al.*, 2002).

### C. WATER TRANSPORT PATHWAYS IN LEAVES

Sack *et al.* (2003) have shown that among 34 plant species, leaf hydraulic resistance ( $R_{\text{leaf}} = 1/K_{\text{leaf}}$ ) can account for at least one quarter of the whole plant water resistance and, therefore, can significantly contribute to the drop in water potential across the plant. In some species, leaves can contribute up to 80% of the whole plant resistance (Brodribb *et al.*, 2002; Nardini *et al.*, 2005). A recent survey of all data on leaf hydraulic properties existing in the literature (Sack and Holbrook, 2006) also showed a large variability in  $K_{\text{leaf}}$

Au9

Au10

values, from  $0.76 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$  in *Adiantum lunulatum* to  $49 \text{ mmol m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$  in *Macaranga triloba*. In general,  $K_{\text{leaf}}$  tends to be the lowest for conifers and pteridophytes, intermediate for woody angiosperms, and the highest for crop plants.

The total leaf hydraulic resistance sums up the contribution of two compartments that act in series, in dicots especially: the vascular compartment that comprises all the veins and the extravascular compartment that consists of the vascular bundles together with the mesophyll (Sack and Holbrook, 2006) (Fig. 6). Although vein networks are very variable among tracheophyte species in terms of vein density, size, and arrangement, it is assumed that the bulk of transpired water always follows the path of lesser resistance down the vein network, through the midrib, the major vein, and finally the minor veins, and then exits the vessels (Sack and Holbrook, 2006). By analogy to what was previously discussed in roots, water can flow throughout the extravascular compartment via parallel apoplastic and cell-to-cell pathways (Figs. 3 and 6).

Two different approaches have been developed to quantify the partitioning of hydraulic resistance within a lamina, that is, the respective resistances of the vascular and extravascular compartments. One method is to determine how  $R_{\text{leaf}}$  is modified when the extravascular water pathway is progressively bypassed by cutting an increasing number of minor veins. Different studies have applied this technique to woody (*Acer saccharum*, *Q. rubra*) and herbaceous (sunflower) leaves and observed that following up to several hundred cuts, the measured  $R_{\text{leaf}}$  progressively decreased and converged toward a stable value, the supposed vascular resistance (Nardini *et al.*, 2005; Sack *et al.*, 2004). Note that the procedure by which minor veins are cut is still a matter of debate. Alternatively, the disruption of all living structures of the leaf, after freezing or boiling the entire organ, reduces  $R_{\text{leaf}}$  to its vascular component (Cochard *et al.*, 2004). However, this conclusion is only valid, provided that the treatments do not alter the xylem vessel diameter or the extensibility of the walls.

A comparison of numerous plant species, all being grown in standard conditions, that is, with a nonlimiting supply of light and water, showed that the partitioning of hydraulic resistance in leaves is extremely variable between species. For instance, the hydraulic resistance of the extravascular compartment contributed to 26, 36, and 58% of  $R_{\text{leaf}}$  in red oak, sugar maple (Sack *et al.*, 2004), or sunflower (Nardini *et al.*, 2005), respectively. These data indicate that leaves can exhibit a large diversity of hydraulic architectures and, due to their branched network of minor veins, often oppose a significant vascular hydraulic resistance to water. However, leaves can also, similarly to roots, show a significant hydraulic resistance in extravascular tissues, this being possibly under the control of aquaporins.

#### D. FUNCTIONS OF AQUAPORINS IN LEAF WATER TRANSPORT

##### 1. Functional evidence for aquaporins in leaf water transport

*a. Effects of mercury.* Effects of mercury were first investigated in isolated protoplasts and, for instance, Kaldenhoff *et al.* (1998) reported that exposure of *Arabidopsis* leaf protoplasts to 1  $\mu\text{M}$   $\text{HgCl}_2$  resulted in a fivefold reduction in their  $P_f$ . However, the low basal water permeability of protoplasts with respect to that of walled cells and the fact that leaf protoplasts quite exclusively originate from the mesophyll restrict the physiological significance of this and other similar studies (Ding *et al.*, 2004).

More recently, a few reports have addressed the contribution of aquaporins to water transport in whole leaves. Nardini *et al.* (2005) investigated the dose-dependent effects of  $\text{HgCl}_2$  on the  $K_{\text{leaf}}$  of sunflower plants collected during the light period. They found that treatment of leaves for 1 h with 200  $\mu\text{M}$  of  $\text{HgCl}_2$  induced a significant reduction of  $K_{\text{leaf}}$  by 33% (from  $4 \pm 0.4 \times 10^{-4} \text{ kg s}^{-1} \text{ m}^{-2} \text{ MPa}^{-1}$  to  $2.5 \pm 0.3 \times 10^{-4} \text{ kg s}^{-1} \text{ m}^{-2} \text{ MPa}^{-1}$ ) and increased the leaf extravascular resistance by about 100%. In view of the very high  $\text{HgCl}_2$  concentration used and the long time of exposure, it was critical to examine the toxicity of the mercury treatment. The authors found that mercury-induced reduction of  $K_{\text{leaf}}$  could somehow be reversed by subsequent treatment with 30 mM of mercaptoethanol for 1 h (Nardini *et al.*, 2005). Altogether, the data point to a significant contribution of the cell-to-cell (aquaporin) pathway for extravascular transport in illuminated sunflower leaves.

In another study, Aasamaa and Sober (2005) analyzed the effect of a mercury treatment (1 mM of  $\text{HgCl}_2$ ) on leaf water transport in six temperate deciduous trees. More specifically, they measured the hydraulic conductance of the shoot, petiole, stem, and lamina during a seasonal course of growth.  $\text{HgCl}_2$  treatment was found to decrease the hydraulic conductance of leaf lamina ( $K_{\text{lamina}}$ ) in all species, and throughout the whole season. For example,  $K_{\text{lamina}}$  in the growing period was decreased by about 40% for *Populus tremula* and 45% for *Quercus robur*. A conclusion similar to that drawn by Nardini *et al.* (2005) in sunflower was made for the six trees species investigated. However, extremely high concentrations of mercury were used, and their cellular toxicity was not investigated.

*b. Effects of temperature.* Sack *et al.* (2004) took another approach to probe for the contribution of the cell-to-cell pathway and analyzed the effects of temperature on  $K_{\text{leaf}}$ . Measurements were made on intact leaves, or on leaves whose minor veins, tertiary veins, or petiole had been cut to bypass the extravascular compartment. The effects of temperature on water flow through intact leaves were significantly greater than those expected from

the effects of temperature on the viscosity of water. By contrast, there was no such discrepancy in leaves with dissected veins suggesting that the temperature-sensitive component was localized in the extravascular compartment. However, further interpretation was difficult, since the effects of temperature could not be analyzed through a single Arrhenius relationship.

*c. Genetic evidence.* Contribution of aquaporins to liquid water transport in leaves has also been inferred from measurements made in transgenic *Arabidopsis* plants expressing PIP antisense genes (Martre *et al.*, 2002). For instance, antisense genes for *AtPIP1;2* or *AtPIP2;3* or both reduced the  $P_f$  of mesophyll protoplasts by 5- to 20-fold. However, the hydraulic conductivity of the rosette under transpiring conditions, as measured by the evaporative flux method, was similar in wild-type and transgenic plants expressing either one or both antisense genes. These results suggest that mesophyll cells do not oppose any significant hydraulic resistance and that in the mesophyll, the apoplastic pathway is dominant.

#### E. PHYSIOLOGICAL REGULATIONS OF $K_{LEAF}$

The hydraulic resistance of veins can be altered through different mechanisms. For instance, drought or chilling conditions induce cavitations and/or wall collapse in vessels, which create important barriers to leaf water transport. The xylem sap composition, and in particular its potassium concentration, determines hydrogel effects, which interferes with the wall permeability of tracheids (Zwieniecki *et al.*, 2001). Although of great importance for leaf water transport, these processes do not directly involve aquaporins. More recently, evidence has emerged that the extravascular path is also subject to important physiological regulations. The observations described below offer interesting contexts in which to explore the contributing role of aquaporins to leaf hydraulic regulation.

##### 1. Transpiration demand

Morillon and Chrispeels (2001) investigated the  $P_f$  of *Arabidopsis* mesophyll protoplasts from plants grown under various transpiring regimes. In transpiring conditions, most of  $P_f$  values were low ( $<70 \mu\text{m s}^{-1}$ ). By contrast, treatment of plants with  $1 \mu\text{M}$  ABA during 24 h led to a significant increase in mean leaf protoplast  $P_f$  from 69 to  $126 \mu\text{m s}^{-1}$ . This apparent increase in aquaporin activity was however not associated to any increase in PIP1 and PIP2 aquaporin expression, as evaluated by Western blotting. Direct application of ABA to protoplasts did not increase their  $P_f$  suggesting that the drop in transpiration flow due to ABA-induced stomatal closure was

the determining factor for increasing  $P_f$ . This idea was somehow confirmed in ABA-insensitive mutants where a fourfold reduced transpiration due to an increase in air humidity from 45 to 85% significantly increased leaf protoplast  $P_f$  (Morillon and Chrispeels, 2001). The overall data suggested that the water permeability of *Arabidopsis* mesophyll cells would be tightly linked to the leaf transpiration regime. The significance of a high cell water permeability at low transpiration is not clear and may favor water potential equilibration within the leaf. By contrast, under transpiring conditions, mesophyll cells would have a low water permeability and the apoplastic pathway would predominate.

## 2. Light

Changes in  $K_{\text{leaf}}$  due to changes in irradiance have now been reported in several plant species (Nardini *et al.*, 2005; Sack *et al.*, 2002; Tyree *et al.*, 2005). In sunflower, for instance,  $K_{\text{leaf}}$ , as measured with the HPFM method (Nardini *et al.*, 2005), was reduced by 30–40% during the night. Tyree *et al.* (2005) have analyzed the effects of a transition between a very low ( $<10 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) and a very high irradiance ( $>1000 \text{ mmol m}^{-2} \text{ s}^{-1}$ ) onto the  $K_{\text{leaf}}$  of 11 tropical plant species. Whereas 5 of them were insensitive to this treatment, 6 species showed an increase in  $K_{\text{leaf}}$  from 21% (*Cordia alliodora*) to 196% (*Miconia argentea*) in the 30 min following the transition to high light.

Light-induced increases in  $K_{\text{leaf}}$  have been interpreted as a mechanism to avoid cavitations in the xylem. Under high light, that is, under transpiring conditions, the leaf water potential can drop significantly and the xylem sap can be under great tensions. An increase in  $K_{\text{leaf}}$  would favor water supply, reduce the drop in water potential throughout the leaf and would maintain it above a critical vulnerability threshold where cavitation can be avoided (Tsuda and Tyree, 2000).

The mechanisms that underlie the light-dependent regulation of  $K_{\text{leaf}}$  have been addressed in a few recent reports. Nardini *et al.* (2005) observed that when sunflower plants were kept in the dark for several days, the  $K_{\text{leaf}}$  continued to oscillate in phase with their subjective period, meaning that these changes were driven by the circadian clock. The authors also found that the hydraulic conductivity of the veins remained unchanged, whereas the conductivity of the extravascular compartment increased by 62.5% during the transition from dark to light. The hypothesis that the increase in  $K_{\text{leaf}}$  would be mediated through aquaporin activation has found strong support in a study by Cochard *et al.* (2007) in walnut trees. The authors analyzed, using quantitative RT-PCR, the abundance of two major PIP2 aquaporin transcripts during a transition from dark to high light and found a very good kinetic correlation between the increase in  $K_{\text{leaf}}$  and the increase in PIP2 aquaporin expression.



### 3. Water stress

Nardini and Salleo (2005) have analyzed the effects of long-term water stresses on the leaf hydraulic architecture of developing sunflower plants. An increase in the hydraulic resistance of the veins was observed under mild ( $\Psi_{\text{soil}} = -0.58$  MPa) and strong ( $\Psi_{\text{soil}} = -0.94$  MPa) water stresses and could be explained by a reduced diameter of xylem vessels formed under drought. However, the overall  $R_{\text{leaf}}$  was unchanged or decreased under mild and strong water stress, respectively, suggesting that, superimposed on effects on veins, water stress induced a progressive decrease in the hydraulic resistance of the extravascular compartment. The authors hypothesized that the latter effect was due to activation of leaf aquaporins.

Whereas they did not exhibit any change in water status in normal growth conditions, *Arabidopsis* plants expressing both a *AtPIP1;2* or a *AtPIP2;3* antisense transgene revealed interesting phenotypes during water stress (Martre *et al.*, 2002). At the end of an 8-day soil drying period, the leaf water potential of the antisense plants was significantly lower than that of control plants, indicating that the former plants were more affected by water stress. The difference between the two plant genotypes was even stronger during rewatering, since antisense mutants could hardly restore the initial value of leaf water potential even after 4 days. The whole plant water conductance was similarly decreased by drought in the two genotypes but progressively restored on rewatering, exclusively in wild-type plants. The mechanisms that underlie the recovery process are not elucidated yet and may involve proliferation of new roots, embolism repair, and/or aquaporin activation. Nevertheless, the overall data indicates that aquaporin function can be important to withstand a water stress and even more important during plant recovery.

### 4. Cold embolism repair

During winter, freeze-thaw cycles can induce the formation of air bubbles (embolism) within the xylem vessels of woody plants and therefore induce a significant decrease in vascular hydraulic conductance (Ameglio *et al.*, 2001; Cochard and Tyree, 1990). Different plant species may exhibit different vulnerability to winter embolism, depending on the anatomy and diameter of their vessels. Yet plants have developed mechanisms for embolism repair (Holbrook and Zwieniecki, 1999). One strategy is to create a positive pressure in the lumen of the xylem vessels to chase out the air bubbles by an incoming flow of water. For this, parenchyma cells transform starch to sugar and extrude it to the xylem vessels leading to an increase in osmotic potential. This process pulls water from the surrounding cells into the vessels and push the air bubbles. Sakr *et al.* (2003) have proposed that, in walnut twigs,

aquaporins can account for this kind of water transport. They have characterized two PIP2 aquaporins from a xylem cDNA library and followed their expression in xylem parenchyma cells from October to March. The abundance in transcripts of the two isoforms was strongly increased during winter and dramatically decreased in March. The abundance of the corresponding proteins in xylem tissues was consistently increased from January to February. Immunolocalization studies revealed that expression was the highest in parenchyma cells that are in direct contact with xylem vessels, probably to facilitate water influx into embolized vessels. Drought also induces embolism in vessels of woody plants. Therefore, a role of aquaporins in water-stressed plants, for embolism repair during the night or on rewatering, can be expected.

#### F. CO<sub>2</sub> TRANSPORT

Diffusion of CO<sub>2</sub> from the stomatal cavity to the stroma of chloroplasts is important in determining the concentration of CO<sub>2</sub> that is available for fixation by ribulose biphosphate carboxylase in mesophyll cells. Therefore, a limitation in this process may ultimately affect the rate of carbon assimilation and possibly growth. It has also been observed in several plant species that the efficiency of CO<sub>2</sub> transport, that is, the conductance of mesophyll to CO<sub>2</sub> ( $g_m$ ), varies in response to environmental signals (Flexas *et al.*, 2006, and references therein). However, the molecular basis for this phenomenon remains unclear. The finding that certain aquaporins can somehow mediate CO<sub>2</sub> transport *in vitro* or after expression in *Xenopus* oocytes (Uehlein *et al.*, 2003) has opened interesting perspectives into this area of research and has led several authors to directly investigate a role for aquaporins in CO<sub>2</sub> diffusion in inner leaf tissues.

In a first study, Terashima and Ono (2002) have analyzed the effects of mercury on *V. faba* and *P. vulgaris* leaves. A combination of gas exchange and fluorescence measurements revealed that, at moderate mercury concentrations, the stimulation of photosynthesis by intercellular CO<sub>2</sub> was reduced, whereas the dependency of photosynthesis on chloroplastic CO<sub>2</sub> was unchanged. This was interpreted to mean that mercury blocked CO<sub>2</sub> diffusion into the chloroplast without affecting the CO<sub>2</sub> fixation machinery. More specifically, treatment of *V. faba* leaves by 0.3 mM of HgCl<sub>2</sub> decreased  $g_m$  by 56%. However, due to the overall toxicity and lack of selectivity of mercury, this approach has remained insufficient to unambiguously establish a physiological role of plant aquaporins in CO<sub>2</sub> transport.

Uehlein *et al.* (2003) have taken a genetic approach and used transgenic tobacco with altered expression in *NtAQP1* due to expression of an antisense or a tetracycline-inducible sense transgene. In these plants, the rate of [<sup>14</sup>C]

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incorporation in leaf disks fed with  $^{14}\text{CO}_2$  was positively correlated to the level of *NtAQP1* expression. Gas exchange measurements also showed that at ambient, that is, limiting  $\text{CO}_2$  (380 ppm), net photosynthesis was reduced in the antisense line, whereas it was enhanced in the overexpressing line. A thorough biophysical characterization of the same plant lines has recently been reported by Flexas *et al.* (2006). Values of  $g_m$  were evaluated by two different methods, gas exchange, and chlorophyll fluorescence, on the one hand, and online  $^{13}\text{C}$  discrimination on the other hand. Both methods unambiguously showed that leaf conductance to  $\text{CO}_2$  was altered in parallel to *NtAQP1* expression. Overall,  $g_m$  in the overexpressing line was twice as high as in the antisense line. This result was interpreted to mean that *NtAQP1* functions as a  $\text{CO}_2$  channel in the mesophyll. However, two other major leaf parameters, that is, stomatal conductance ( $G_s$ ) and maximal  $\text{CO}_2$  assimilation were also tightly linked to *NtAQP1* expression. It has been argued that all these parameters are known to be coregulated and therefore any change in  $g_m$  will result in an overall change in leaf properties. It may also be argued that, conversely, altered expression in *NtAQP1* primarily alters stomatal behavior ( $G_s$ ), as a result of altered leaf water relations, and therefore induces a change in  $g_m$ .

To a certain extent, similar questions had been raised by a previous study (Hanba *et al.*, 2004) in which a barley aquaporin (*HvPIP2;1*) was overexpressed in transgenic rice. While a good correlation was found between  $g_m$  and *HvPIP2;1* expression, transgenic plants also showed an increase in  $G_s$ . In addition, fine alterations in leaf morphology were uncovered in transgenic plants. In particular, stomata were reduced in density and size, mesophyll cells were smaller and their walls were thicker. It remains unclear whether, in this study, these changes result from heterologous expression of a barley aquaporin in rice. Nevertheless, the notion that all these changes may directly or indirectly contribute to alteration in  $g_m$  makes it difficult to interpret the gas exchange properties of the transgenic plants with regard to  $\text{CO}_2$  transport by aquaporins.

## V. AQUAPORINS IN REPRODUCTIVE ORGANS

During their life cycle and especially during sexual reproduction, higher plants pass through highly desiccated forms that facilitate their dissemination and allow their resistance to adverse environmental conditions. In particular, pollen grains that carry the male gametes and seeds that are produced from fertilized flowers differentiate through well-defined dehydration sequences to finally contain <30% water (Bots *et al.*, 2005b). The subsequent germination of seeds and pollen grains is triggered by a rapid

imbibition that precedes a dramatic growth of the embryo or pollen tube. All these events rely on precise water exchange processes and possibly involve the contribution of membrane water transport and aquaporins.

#### A. AQUAPORINS IN FLOWERS

##### 1. *Aquaporin expression in flowers*

A number of studies have revealed that aquaporins are abundantly expressed in flowers (Alexandersson *et al.*, 2005; Bots *et al.*, 2005b; Dixit *et al.*, 2001; Marin-Olivier *et al.*, 2000; O'Brien *et al.*, 2002). Aquaporin microarrays experiments in *Arabidopsis* have shown that several PIP (PIP1;2, PIP2;1, PIP2;6, and PIP2;7) and TIP (TIP1;1, TIP1;2, and TIP3;1) isoforms are predominantly expressed in flowers (Alexandersson *et al.*, 2005). Expression in wild potato *Solanum chacoense* of *ScPIP2a*, a flower-specific PIP2 homologue, has been described in detail by O'Brien *et al.* (2002). The transcript levels of *ScPIP2a* were ninefold higher in reproductive tissues than in leaves with preferential expression in the anther, pistil, and young developing fruit. In *Brassica oleracea*, two PIP1 aquaporins that had been initially isolated from an anther and stigma cDNA library were shown to have abundant transcripts in petals, sepals, ovary, stigma, and anthers (Marin-Olivier *et al.*, 2000).

##### 2. *Functions of aquaporins in flowers*

A role for aquaporins during specific processes of plant reproduction, such as anther dehiscence, pollen recognition, pollen germination, and petal movement, has been inferred from a combination of gene expression and genetic studies.

The dehydration of anthers results from water losses by evaporation or via osmotically driven water efflux through the vascular bundle (Bots *et al.*, 2005b). Aquaporins may facilitate both of these processes by increasing water permeability of the anther cells. This idea was recently investigated in tobacco by Bots *et al.* (2005b). Western blot analyses have shown that anthers of tobacco flowers express aquaporins of both the PIP1 and PIP2 subclasses (Bots *et al.*, 2005b). In this species, the dehydration of anthers starts between stages 7 and 8. During stage 8, the abundance of PIP2 proteins was increased and the proteins were the most abundant in the vascular bundle and connective tissues. Bots *et al.* (2005a) have introduced into transgenic tobacco an RNAi construct that targeted this aquaporin subclass. The percentage of flowers that contained fully dehiscent anthers was reduced from 76% in wild-type controls to 36–63% in transgenic lines that expressed the RNAi construct. The latter lines also exhibited a reduced rate of anther dehydration, as measured by *in vivo*  $^1\text{H-NMR}$ . The data were interpreted to

mean that PIP2 aquaporins facilitate the dehydration and therefore the dehiscence of tobacco anthers.

The role of aquaporins in the pistil may vary between species. In tobacco flowers, PIP1 aquaporins are expressed in the stigma, mostly at the latest stages of floral development, indicating that these aquaporins may be involved in pollen grain imbibition and/or pollen tube growth. The two functions are supported by *in situ* hybridization experiments showing a strong expression signal in the neck-cells of the stigma and transmitting tissue of the style (Bots *et al.*, 2005b). In *B. oleracea*, *in situ* hybridization revealed a strong expression of PIP1 homologues in the stigmatic cells underneath the papillae cell layer and in the style tissue (Marin-Olivier *et al.*, 2000). By contrast, no expression was detected in the papillar cells, that is, in the cells that are in contact with the pollen grains and directly supply water during their imbibition. Thus, in this species, aquaporins do not seem directly involved in pollen grain germination. In *S. chacoense*, expression of *ScPIP2a* in the pistil was found to be strongly increased after cross-pollination, whereas it remained low after self-incompatible pollination. Precise kinetic analyses suggested that the increase in *ScPIP2a* mRNA expression was actually triggered by fertilization. The abundance of *ScPIP2a* transcripts was also strongly correlated to the rate of style elongation, indicating a possible role for this aquaporin in cell expansion (O'Brien *et al.*, 2002).

Self-incompatibility, a central process in sexual plant reproduction, is based on recognition of self-incompatible pollens that leads to signaling cascades that will ultimately induce a blockade of pollen tube development. A mutation that prevents self-incompatibility in *Brassica* has been associated to a genetic locus encoding MIP-MOD, a PIP1 aquaporin homologue (Ikeda *et al.*, 1997). This gene is expressed in the pistil, essentially in the stigma (papillar cells) and the distal portion of the style (Dixit *et al.*, 2001). By reference to the studies discussed above, it is somewhat difficult to explain how inactivation of a pistil aquaporin may favor pollen tube growth. Ikeda *et al.* (1997) have suggested that in self-incompatible (wild-type) plants, the activation of MIP-MOD would redirect water flow away from the pollen tube. Although not yet established, this mechanism supports the general idea that regulation of water transport from the stigma to the pollen is a checkpoint in early events of pollination in crucifers.

Recent work by Azad *et al.* (2004a) has pointed to an original function of aquaporins during tulip petal movements. In dark conditions, petals open at 20°C and close at 5°C. Petal opening is accompanied by water transport from the stem to the petals. Azad *et al.* (2004a) showed that the amount of titrated water ( $[^3\text{H}_2\text{O}]$ ) taken up by the flower was grossly proportional to flower aperture and that once the flower was opened, the  $[^3\text{H}_2\text{O}]$  content remained

constant. During petal closure, water content decreased rapidly. These authors also found that, during petal opening, a PIP homologue was phosphorylated by a  $\text{Ca}^{2+}$ -dependent protein kinase activity and that dephosphorylation of this aquaporin was associated to petal closure. These results support the idea that reversible phosphorylation of PIPs provides a means for regulating water transport during temperature-dependent tulip petal oscillation.

## B. AQUAPORINS IN SEEDS

Seed germination is triggered by tissue imbibition and, after an apparent lag phase, is followed by the elongation of the radicle and thereafter of the whole embryonic axis. More specifically, rupture of the maternal seed coat (testa) occurring in some species can be taken as the first external sign of initiation of germination, whereas subsequent rupture of the zygotic tissue, the endosperm, and radicle protrusion indicate the completion of the process. The primary imbibition of seeds, the subsequent lag phase, and finally embryo growth can be associated to triphasic kinetics of water uptake, which represent a landmark feature of germinating plant seeds (Bewley, 1997; Bewley and Black, 1994). Water transport in seeds has classically been described using gravimetric methods (Bewley and Black, 1994). More recently, magnetic resonance imaging revealed a precise spatial distribution of water in germinating seeds and strikingly different patterns between species (Krishnan *et al.*, 2004; Manz *et al.*, 2005; McEntyre *et al.*, 1998; Terskikh *et al.*, 2005). In addition, rapid imbibition of seeds can cause mechanical injuries (Veselova *et al.*, 2003). Thus, a tight control of water transport operates within seeds.

The expression of aquaporins in developing seeds and during germination has been investigated in several plant species. Aquaporins of the TIP3 ( $\alpha$ -TIP) subclass are seed-specific and expressed in the membrane of protein storage vacuoles (Höfte *et al.*, 1992; Johnson *et al.*, 1989; Olaviusson and Hakman 1995). Their expression strongly decreases during seedling establishment. Based on these expression properties, Maurel *et al.* (1997) have proposed a role for these aquaporins in cell osmoregulation and maturation of the vacuolar apparatus during late seed development and during the early stages of germination. Expression of the whole aquaporin family in dry and germinating seeds has recently been investigated in *Arabidopsis* by Vander Willigen *et al.* (2006). This study confirmed that TIP3 homologues represent together with TIP5;1 the most highly expressed aquaporins in dry seeds. Surprisingly, the abundance of all PIP mRNAs was very low. This expression profile was changed shortly after germination to a profile dominated by strong expression of aquaporins of the PIP1, PIP2, TIP1, and TIP2 subclasses.

A role for aquaporins in controlling water transport across cell plasma membranes during seed germination has first found some support in a study by Gao *et al.* (1999) in *B. napus*. These authors observed that expression of a lowly abundant PIP2 aquaporin in dry seeds was markedly increased by priming and therefore was tightly linked to the efficiency of seeds to germinate under normal or stress conditions.

There are very few functional evidences for a role of aquaporins in seeds, and all of them are based on the inhibitory effects of mercurials. For instance, Veselova *et al.* (2003) have shown that the aquaporin blocker *p*-chloromercuriphenylsulfonic acid significantly reduces the rate of pea seed imbibition. This idea is in accordance with the work by Schuurmans *et al.* (2003) that uncovered strong expression of a PIP1 homolog in dry and germinating pea seeds. *Arabidopsis* seeds are much smaller in size and show a faster rate of imbibition, with a half time of about 22 min. Treatment of *Arabidopsis* seeds by 5  $\mu$ M HgCl<sub>2</sub> did not delay this process (Vander Willigen *et al.*, 2006).

The idea that hydraulic factors, that is, tissue water transport, could be limiting for embryo growth was initially discarded based on general models of plant tissue expansion (Cosgrove, 1993). However, there have been recent concerns about the significance of growth-induced water potential gradients indicating a possible involvement of aquaporins in cell expansion (Fricke, 2002; Tang and Boyer, 2002). In support of this, Fukuhara *et al.* (1999) observed that aquaporin expression in seeds of *Mesembryanthemum crystallinum* was associated with embryo growth and was retarded in dormant seeds. In addition, Vander Willigen *et al.* (2006) showed that mercury treatment (5  $\mu$ M HgCl<sub>2</sub>) of *Arabidopsis* seeds delayed both testa rupture and radicle emergence and therefore altered very early steps of embryo growth (Fig. 7). These effects could be fully reversed by the reducing molecule DTT

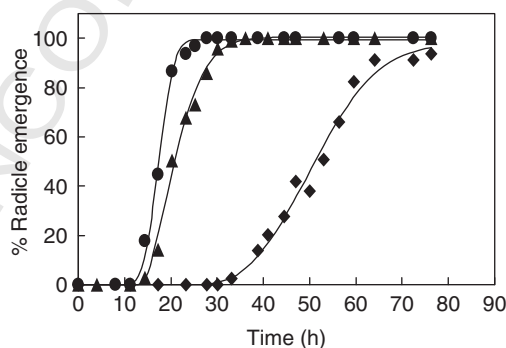


Fig. 7. Effects of mercury on the germination of *Arabidopsis* seeds. Germination curves of seeds grown in the absence of HgCl<sub>2</sub> (circles), or in the presence of 5  $\mu$ M HgCl<sub>2</sub> (diamonds) or 5  $\mu$ M HgCl<sub>2</sub> + 2 mM DTT (triangles).

and were not associated to any morphological abnormality at the time of radicle emergence.

## VI. CONCLUSION

The existence of water channels in plants was largely unsuspected just 15 years ago (Maurel, 1997). Thus, the molecular characterization of aquaporins, in plants and other organisms, is relatively recent and has insuflated novel ideas into the field of water transport. The high resolution atomic structure of spinach *SoPIP2;1* gives a striking illustration of the accuracy of knowledge gained into the molecular mechanisms of transport selectivity and gating of plant aquaporins (Tornroth-Horsefield *et al.*, 2006). Also, biochemical and functional studies have revealed that plant aquaporins can be more than water channels and can be permeated by a large variety of molecules, including gas and solutes of high physiological significance such as CO<sub>2</sub>, NH<sub>3</sub>, or H<sub>2</sub>O<sub>2</sub>.

One aim of this present chapter was to put into perspective the significance of molecular and functional knowledge on plant aquaporins with respect to integrated physiological processes. Here, we showed that, although still rare, examples exist in which a continuum in knowledge between aquaporin molecular structures and whole plants was established. In particular, the conserved gating properties of PIP aquaporins by H<sup>+</sup> have been interpreted in terms of protein conformational changes and their significance with respect to the response of plant roots to anoxia has been established (Tornroth-Horsefield *et al.*, 2006; Tournaire-Roux *et al.*, 2003). Also, the deficiency of mutant rice plants to take up silicon was linked to an inactivating, point mutation in the vicinity of the pore of a NIP aquaporin homologue (Ma *et al.*, 2006). By contrast, the gating of aquaporins by divalent ions including calcium has been established *in vitro* (Gerbeau *et al.*, 2002), can be interpreted by molecular modeling (Tornroth-Horsefield *et al.*, 2006), but its significance in the plant remains largely conjectural.

Understanding the integrated function of aquaporins, whether coupled or not to molecular approaches, remains a central objective. Recent studies have provided strong support to the initial assumption that aquaporins are central to water relations. They have also revealed unsuspected links to other great physiological functions.

It is now fairly well established that aquaporins play a predominant role in root water uptake and in its regulation by environmental factors. A further dissection of aquaporin functions in the differentiated cell layers of the root, by gene expression mapping and reverse genetics, should provide a more precise interpretation of the biophysical and physiological properties of



this organ. Beyond this, and because of the knowledge already gained, roots will remain a unique system to explore general mechanisms of aquaporin regulation.

Recent studies have also pointed to the leaf as another organ in which aquaporins mediate transcellular water transport and therefore confer highly regulated hydraulic properties. We realize that inhibition studies and molecular and cellular analyses of aquaporin regulation in this organ rely on progresses made in roots. Yet aquaporins in leaves raise very original issues, one of them being a role in CO<sub>2</sub> transport. Also, the role of aquaporins in stomatal regulation has been barely examined and a gap in knowledge needs to be filled.

The function of aquaporins in other organs such as seeds and flowers has remained much more conjectural, in particular because a biophysical description of water relations has been much more difficult to establish than in roots or leaves. Nevertheless, molecular and genetic analyses have brought interesting insights and underscored an original role of aquaporins during tissue desiccation and imbibition.

Central physiological questions also remain, beyond the dissection of aquaporin function in each single plant organ. One such question is to understand the hydraulic aspects of growth and cell movements and their links to aquaporins. This question has been punctually addressed in roots (Hukin *et al.*, 2002), leaves (Siefritz *et al.*, 2004), or seeds (Vander Willigen *et al.*, 2006) and will require a more careful examination. A role of aquaporins in the transport of carbon assimilates and carbon metabolism has been suggested (Ma *et al.*, 2004) but not evaluated in detail. Finally, aquaporins transport reactive oxygen species. The significance of this property in terms of cell signaling and response to oxidative stress remains largely unknown.

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