Aquaporins: Highly Regulated Channels Controlling Plant Water Relations[1]

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Plant growth and development are dependent on tight regulation of water movement. Water diffusion across cell membranes is facilitated by aquaporins that provide plants with the means to rapidly and reversibly modify water permeability. This is done by changing aquaporin density and activity in the membrane, including posttranslational modifications and protein interaction that act on their trafficking and gating. At the whole organ level aquaporins modify water conductance and gradients at key "gatekeeper" cell layers that impact on whole plant water flow and plant water potential. In this way they may act in concert with stomatal regulation to determine the degree of isohydry/anisohydry. Molecular, physiological, and biophysical approaches have demonstrated that variations in root and leaf hydraulic conductivity can be accounted for by aquaporins but this must be integrated with anatomical considerations. This Update integrates these data and emphasizes the central role played by aquaporins in regulating plant water relations.

Water uptake from the soil to the root and its distribution in the plant body is crucial for many physiological processes of vascular plants. Water movement is driven by the gradient of water potential ($\Delta\Psi$), and water moves from a region where Ψ is higher to a region where Ψ is lower. (It should be noted that osmotic gradients as a component of water potential can only generate a flow across a semipermeable membrane. Pressure gradients, however, can generate flows in conduits and across semipermeable membranes.) The most obvious example of water movement in plants is the transpiration stream during which water evaporation through the opened stomata decreases the leaf Ψ and causes water to move from the xylem toward the leaf surface. This process creates a tension in the xylem vessels that draws water from the soil to the root up to the transpirating leaf tissues (Steudle, 2001). In addition to the long-distance water transport during transpiration or sugar transport in the phloem sieve tubes, short-distance water transport is required to maintain and regulate cell water homeostasis, a key element controlling cell turgor involved in essential physiological processes such as cell expansion, opening and closure of stomata, leaf epinasty, etc. While water does not generally meet high hydraulic resistance in the xylem vessels and phloem sieve tubes, water has to flow across different living tissues to reach and exit these conduits or to assure the optimum cell water equilibrium. Three different pathways of water transport through plant tissues have been described: the apoplastic path around the protoplasts, the symplastic path through the plasmodesmata, and the transcellular path across the cell membranes (Steudle and Peterson, 1998). The contribution of the different pathways to the overall water flow in all parts of the plant is dependent on the species, growth conditions, and developmental stages, and the variability in the use of the different paths in roots according to the conditions has been explained by a composite transport model (CTM) based on measurements of the overall root or cell hydraulic conductivities (Steudle, 2000).

The transcellular water movement is tightly controlled by the amount and activity of water channels, known as aquaporins, present in cellular membranes. Aquaporins are found in most living organisms, in which they are involved in many different physiological processes (Gomes et al., 2009). The first water channel activity of a plant aquaporin, Arabidopsis (*Arabidopsis thaliana*) tonoplast *At*TIP1;1, was established in 1993 after its expression in *Xenopus laevis* oocytes and cell-swelling experiments in hypoosmotic medium (Maurel et al., 1993).

Aquaporins are small membrane proteins (21 to 34 kD) consisting of six membrane-spanning α-helices connected by five loops (A to E) and N and C termini facing the cytosol (Fig. 1; Murata et al., 2000). The loops B and E form two short hydrophobic α-helices dipping halfway into the membranes from opposite sides, which, together with the membrane-spanning helices, form a pore with high specificity that mainly results from two filter regions. The first one is formed by the

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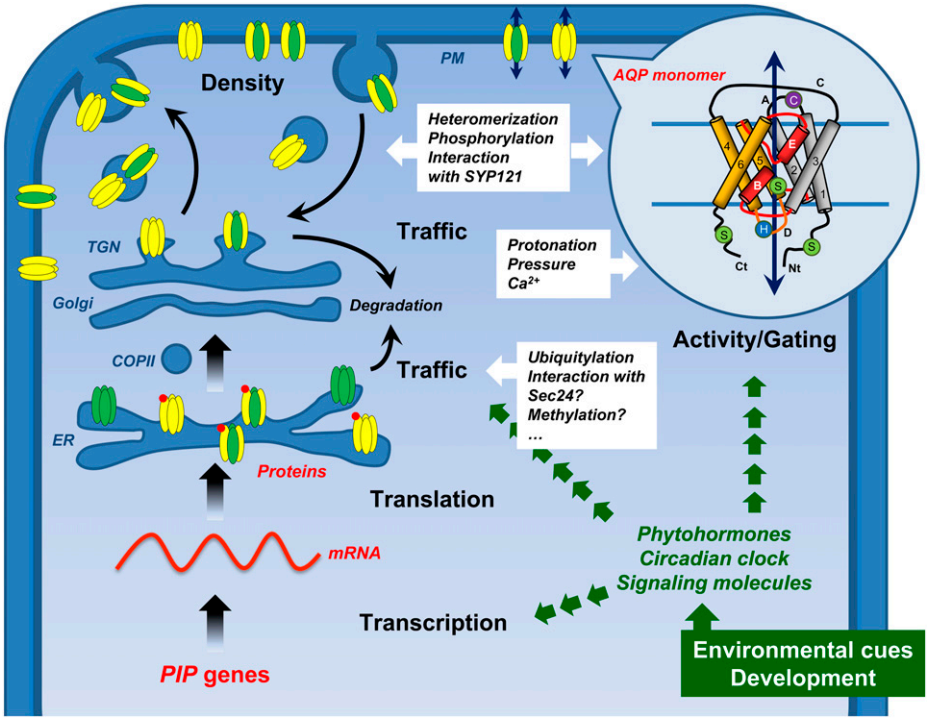

Figure 1. Regulation of PIPs within the cell. *PIP* genes are transcribed, their mRNA translated in the rough ER, and the proteins targeted to the plasma membrane (PM). PIPs belonging to the PIP2 group (in yellow) form homo- or heterooligomers by associating with PIP1 isoforms (in green). Some PIP2s contain a diacidic motif (red circle) in their N terminus that is thought to be recognized by the Sec24 subunit of the COPII coat complex of the vesicles budding at the ER membrane and transiting to the Golgi apparatus. PIP oligomers transit through the Golgi apparatus and trans-Golgi network (TGN) and are then loaded into secretory vesicles and routed to the plasma membrane. Insertion of PIPs into the plasma membrane is mediated by the syntaxin SYP121. Internalization of plasma membrane-localized PIPs occurs as a result of constitutive recycling. Once internalized in vesicles, PIPs are delivered to the trans-Golgi network before being routed back to the plasma membrane or directed into lytic vacuoles for degradation. Salt stress causes dephosphorylation and internalization of PIPs, and drought stress induces ubiquitylation of PIPs, which are then degraded in the proteasome. The water channel activity or gating of PIPs is regulated by different mechanisms (heteromerization, phosphorylation, interaction with SYP121, protonation, pressure gradient, and Ca^{2+} concentration). Question marks indicate possible regulation mechanisms not yet supported by experimental evidence. In the bullet is shown the topological structure of an aquaporin monomer (Murata et al., 2000), which consists of six membrane-spanning α-helices (1–6) connected by five loops (A–E) and N and C termini facing the cytosol. The loops B and E form two short hydrophobic α-helices (in red) dipping halfway into the membranes, which, together with the membrane-spanning helices, create a pore with high specificity. Phosphorylated Ser residues are in green circles (the putative phosphorylated Ser in loop D is not indicated), the protonated His of loop D is in a blue circle, and the Cys residue of loop A involved in disulfide bound formation between monomers is in a purple circle. The transcription, translation, trafficking, and gating of PIPs are regulated by environmental and developmental factors involving signaling molecules, phytohormones, and the circadian clock. See text for more details and references.

Asp-Pro-Ala motifs of the loops B and E that meet at the center of the channel and constitutes a first size exclusion zone, and the second one, the so-called aromatic/Arg is formed by four amino acids and contributes to a size exclusion barrier and the hydrogen bond environment for the substrate transport (Murata et al., 2000). Aquaporins assemble as homo- and/or heterotetramers in the membrane, each monomer acting as independent water channel (Murata et al., 2000; Fetter et al., 2004; Bienert et al., 2012).

Higher plant aquaporins constitute a large and diverse protein family, including 30 to more than 70 homologs found in the monocots rice (*Oryza sativa*) and maize (*Zea mays*) and the eudicots Arabidopsis, tomato (*Solanum lycopersicum*), poplar (*Populus trichocarpa*), upland cotton (*Gossypium hirsutum*), and soybean (*Glycine max*; Chaumont et al., 2001; Johanson et al., 2001; Sakurai et al., 2005; Ishibashi, 2006; Gupta and Sankararamakrishnan, 2009; Sade et al., 2009; Park et al., 2010; Zhang et al., 2013). Based on sequence similarity, they fall into five subfamilies, somehow associated with specific membrane localization: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the Nodulin26-like intrinsic proteins (NIPs) initially identified in the symbiosomes of legumes but also found in the plasma membrane and endoplasmic reticulum (ER), the small basic intrinsic proteins (SIPs) localized in the ER, and the X intrinsic proteins (XIPs) present in the plasma membrane (Kammerloher et al., 1994; Chaumont et al.,

2001; Johanson et al., 2001; Ishikawa et al., 2005; Danielson and Johanson, 2008; Bienert et al., 2011). While the PIP, TIP, NIP, and SIP subfamilies are present in all land plants, including the moss *Physcomitrella patens*, the XIP subfamily, identified in a wide variety of nonvascular and vascular plants, is absent in Brassicaceae and monocots (Borstlap, 2002; Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009; Sade et al., 2009; Shelden et al., 2009; Park et al., 2010; Bienert et al., 2011; Lopez et al., 2012). The large number of plant aquaporins has been explained by their importance in regulating water flow through the plant body and in maintaining cellular water homeostasis at all developmental stages and in all environmental conditions (Hachez et al., 2006b). However, water is not the only molecule diffusing through the aquaporins. Since the discovery of the first water channel, several plant aquaporins have been shown to represent important membrane-selective pathway for small uncharged solutes, including glycerol, urea, ammonia, carbon dioxide, hydrogen peroxide, and the metalloids boric acid, silicic acid, and arsenite, making aquaporins multifunctional channels with important roles not only in water homeostasis, but also in plant metabolism, nutrition, and signaling processes. This important aspect of plant aquaporin multifunctionality has been summarized in recent reviews and will not be addressed in this Update (Tyerman et al., 2002; Maurel et al., 2008; Gomes et al., 2009; Hachez and Chaumont, 2010; Ma, 2010; Miwa and Fujiwara, 2010; Bienert and Chaumont, 2011; Bienert and Chaumont, 2013; Kaldenhoff et al., 2013).

This Update will focus on the latest breakthroughs regarding function and regulation of aquaporins that facilitate water diffusion across membranes (mostly PIPs and TIPs) and on their involvement in plant growth and water relations in roots and shoots. The physiological mechanisms regulating water flow in the plant body will be discussed with special emphasis on the contribution of aquaporins.

AQUAPORIN EXPRESSION

The first clues about aquaporin function in plants come from the study of the level of expression in different organs, tissues, or cell types according to the developmental stages and in response to different environmental conditions (Fig. 1). The mRNA abundance is nowadays measured by reverse transcription-quantitative PCR approaches, a widely used technique that however requires a strict design of the experimental conditions (primer specificity and efficiency, housekeeping genes for normalization, and analysis method; Bustin et al., 2009). The cell type in which a specific aquaporin isoform is expressed can be identified by in situ mRNA hybridization. When available, the use of antibodies raised against specific aquaporin isoforms offers the advantage to be able to quantify the channel amount in the membranes, which does not always correlate to the level of mRNA, especially when changing the growing conditions (i.e. applying water or salt stress or modifying irradiance; Suga et al., 2002; Lopez et al., 2003; Hachez et al., 2012). Many studies comparing PIP and TIP aquaporin expression in different organs and conditions in various plant species have been published and have highlighted their involvement in the control of radial transcellular water transport but also in cell osmoregulation (for review, see Tyerman et al., 1999; Maurel et al., 2002, 2008; Luu and Maurel, 2005; Hachez et al., 2006b; Kaldenhoff and Fischer, 2006; Forrest and Bhave, 2007; Heinen et al., 2009; Bienert and Chaumont, 2011; Prado and Maurel, 2013). It is interesting to mention that, in general, PIP and TIP aquaporin expression seems to be more abundant in roots than in leaves (Alexandersson et al., 2005; Heinen et al., 2009; Besse et al., 2011), but several isoforms are highly or exclusively expressed in leaf tissues (Sakurai et al., 2005; Azad et al., 2008). The cell type localization of aquaporin expression can also provide clues about their physiological roles. For instance, expression of PIP aquaporins in roots and leaves has been correlated to the presence of apoplastic barriers, the exodermis and endodermis in roots or in suberized bundle sheath cells in leaves, suggesting an essential role in the transmembrane water diffusion when its movement is hindered (Schäffner, 1998; Suga et al., 2003; Hachez et al., 2006a, 2008, 2012; Vandeleur et al., 2009; Shatil-Cohen et al., 2011; Prado et al., 2013). Interpretation of global aquaporin mRNA or protein level detected in an organ has however to be done with caution, as an isoform can be expressed, and hence plays an important role, in specific but lowly abundant cell types, such as guard cells or bundle sheath cells. These "gatekeeper" cells are positioned in the flow pathway to have relatively large impacts on plant water relations.

In general, PIP and TIP mRNA and/or protein levels are also higher during the day than the night, an observation that is correlated to the diurnal regulation of the transpiration and the essential root and leaf radial water movement required during this process (Henzler et al., 1999; Moshelion et al., 2002; Lopez et al., 2003; Cochard et al., 2007; Vandeleur et al., 2009; Hachez et al., 2012). Interestingly, a circadian regulation of *PIP* expression in Arabidopsis and maize roots has also been demonstrated with a maximum and minimum mRNA amount near the subjective dawn and dusk, respectively (Lopez et al., 2003; Takase et al., 2011). The expression patterns of specific PIP or TIP aquaporins will be further discussed below in relation to the study of root and leaf hydraulic parameters.

POSTTRANSLATIONAL REGULATION OF AQUAPORINS

While determination of the expression of aquaporins remains essential when studying plant water relations or development, many posttranslational regulation mechanisms have been demonstrated to affect the channel abundance and activity in their target membrane (Fig. 1;

for review, see Chaumont et al., 2005; Maurel et al., 2008; Hachez and Chaumont, 2010). These regulation mechanisms are very often mentioned to explain discrepancies between aquaporin expression data and biophysical measurements aiming at determining organ and/or tissue water permeability (see below). However, these mechanisms have to be always considered, as they are potentially continuously used by plants to regulate membrane permeability. In addition, time scale of the organ/tissue/cell hydraulic responses to environmental cues could involve different regulation mechanisms. While responses to long-term treatment (days) can involve, in addition to regulation of aquaporin expression, anatomical modifications, short-term responses (minutes to hours) are probably mediated by posttranslational modifications, though rapid transcriptional changes have been observed (Horie et al., 2012; Vandeleur et al., 2014). In combination with classical biochemical and cellular biology techniques, the development of mass spectrometry approaches applied to membrane proteins has been essential to determine and quantify posttranslational modifications of aquaporins in different environmental conditions (Johansson et al., 1998; Santoni et al., 2003, 2006; Daniels and Yeager, 2005; Prak et al., 2008; Van Wilder et al., 2008; Kline et al., 2010; di Pietro et al., 2013). These modifications include phosphorylation, methylation, ubiquitylation, deamidation, heteromerization, disulfide bond formation, and protonation and have been demonstrated to affect both their trafficking through the secretory pathway to reach the plasma membrane or the tonoplast and their gating, i.e. the opening and closing of the pore.

Aquaporin Trafficking

Studies of the regulation of plant aquaporin trafficking have mainly focused on PIPs and have recently highlighted the importance and complexity of this process controlling channel density in the membrane and, hence, the water permeability of the cellular membrane (Fig. 1; for review, see Hachez et al., 2013; Luu and Maurel, 2013).

Physical interaction between different aquaporins can change their subcellular localization. Experimental data revealed that plant aquaporins not only form homotetramers (Fotiadis et al., 2001) but also heterotetramers (Harvengt et al., 2000; Fetter et al., 2004; Zelazny et al., 2007). This heteromerization has been shown in maize, where ZmPIP2s, which are predominately expressed in the plasma membrane, affect the localization of ZmPIP1 proteins. When expressed alone in transfected mesophyll protoplasts, ZmPIP1s are retained in the ER, while, when coexpressed with ZmPIP2s, they are relocalized to the plasma membrane (Zelazny et al., 2007). ZmPIP physical interaction has been further confirmed by immunoprecipitation and Förster resonance energy transfer/fluorescence lifetime imaging microscopy (Zelazny et al., 2007). In addition to this trafficking effect, the interaction of ZmPIP1s and ZmPIP2s leads to a synergistic activation effect in *Xenopus* spp. oocytes, resulting in an enhanced membrane water permeability (Fetter et al., 2004), which has subsequently been observed for PIPs from various species (Temmei et al., 2005; Mut et al., 2008; Mahdieh and Mostajeran, 2009; Vandeleur et al., 2009; Alleva et al., 2010; Bellati et al., 2010; Chen et al., 2013; Yaneff et al., 2014). Interestingly, PIP interaction seems to modulate the intrinsic permeability of the channels (Fetter et al., 2004; Yaneff et al., 2014), but the physiological relevance of this mechanism in controlling plant water relations has still to be demonstrated.

Several PIPs contain a diacidic motif in their N-terminal part, which acts as an ER export signal. Diacidic motifs interact with Sec24, which is the main cargo selection protein of the coat protein complexII (COPII) that mediates vesicle formation at ER export sites (Miller et al., 2003). ZmPIP2;4 and ZmPIP2;5, which are targeted to the plasma membrane when expressed in mesophyll protoplasts, are retained in the ER upon mutation of this motif (Zelazny et al., 2009). The functionality of the diacidic motif was confirmed in transgenic Arabidopsis plants. Diacidic motif mutated forms of fluorescent-tagged AtPIP2;1 are retained in the ER and lead to a reduced root hydraulic conductivity (L_{pr}; Sorieul et al., 2011). (Note that L_{pr} is used in this review in a general way and not strictly as a definition of conductivity. In some cases, conductance may be normalized to root area or root weight and flow is measured at one pressure gradient. We assume that conductance normalized to some scaling factor of root surface area will display the same characteristics.) However, the existence of other export or retention signals is supported by the fact that some PIP2s reach the plasma membrane without having a diacidic motif and that fusion of the diacidic motif to the ER-localized ZmPIP1;2 does not redirect the channel to the plasma membrane (Zelazny et al., 2009).

PIP-containing vesicles leaving the post-Golgi network must be correctly inserted in the plasma membrane, a process recently shown to be mediated by the syntaxin of plants (SYP121), a Qa-soluble *N*-ethylmaleimide-sensitive factor protein attachment protein receptor (SNARE) known to regulate vesicular fusion (Besserer et al., 2012; C. Hachez and F. Chaumont, unpublished data). Plasma membrane delivery of ZmPIP2;5 or AtPIP2;7 depends on its physical interaction with SYP121, and this mechanism is partially inhibited by the expression of a truncated form of SYP121 acting as a dominant negative mutant (the so-called Sp2 fragment). Interestingly, this SYP121-Sp2 fragment negatively affects the membrane osmotic water permeability coefficient of ZmPIP2;5- or AtPIP2;7-expressing protoplasts (Besserer et al., 2012; C. Hachez and F. Chaumont, unpublished data), indicating a direct link between the regulation of PIP trafficking and aquaporin-mediated transmembrane water movement (Hachez et al., 2013). In addition, as SYP121 also regulates the delivery and activity of K^+ channels through physical interaction, a central role of this SNARE in controlling the cell water homeostasis in response to the environmental conditions by coordinating membrane transporter traffic and

activity has been hypothesized but needs to be investigated (Sutter et al., 2006; Honsbein et al., 2009, 2011; Grefen et al., 2010; Besserer et al., 2012; Hachez et al., 2013).

Aquaporin relocalization in response to osmotic and salt stress is an important way to rapidly change their content in the target membrane (Vera-Estrella et al., 2004; Boursiac et al., 2005, 2008; Luu et al., 2012). During osmotic stress, the ice plant (*Mesembryanthemum crystallinum*) water channel McTIP1;2 is relocalized from the tonoplast to endosomal compartments, which may contribute to a homeostatic process maintaining cellular osmolarity (Vera-Estrella et al., 2004). In Arabidopsis, salt stress induces the relocalization of TIP1;1 into intravacuolar invaginations (Boursiac et al., 2005). PIPs appear also to be endocytosed from the plasma membrane through the internalization of clathrin-coated vesicles (Dhonukshe et al., 2007) and, in response to salt stress, of raft-associated domains (Li et al., 2011), suggesting the existence of alternative mechanisms regulating PIP abundance in the plasma membrane. Interestingly, salt stress triggers a decrease in the L_{pr}, which is correlated to an internalization of AtPIP2;1-GFP in internal structures and cellular accumulation of H_2O_2 (Boursiac et al., 2005, 2008). In these conditions, the cycling of AtPIP2;1 from and to the plasma membrane (endocytosis and exocytosis) is significantly increased (Luu et al., 2012; Martiniére et al., 2012). In addition, the phosphorylation status of the carboxy-terminal Ser-283 of AtPIP2;1 regulates the salt- or H_2O_2-induced internalization (Prak et al., 2008). In transgenic Arabidopsis plants, salt stress induced a higher internalization of GFP-AtPIP2;1 and GFP-AtPIP2;1S283A compared with GFP-AtPIP2;1S283E, which mimics a constitutive phosphorylated state, suggesting that internalization of AtPIP2;1 under NaCl stress requires the nonphosphorylated form of S283 (Prak et al., 2008). These data suggest that the signaling molecule H_2O_2 induces internalization of PIP proteins in response to the environmental conditions through modifications of their phosphorylation status to regulate the cell membrane water permeability. New developments in imaging and computational techniques to increase the resolution and, hence, to precisely follow the dynamics of single aquaporin complex in the plasma membranes of living samples is underway but remains an important challenge, especially for live plant samples (Li et al., 2011, 2013). Although there are advances in understanding how certain PIPs are regulated by salt stress, it is still not at all clear why the plant should regulate these aquaporins in this way under salt stress.

Aquaporin Gating and Inhibition

A mechanism of aquaporin gating has been proposed using dynamics simulation modeling based on the high-resolution structure of spinach (*Spinacia oleracea*) SoPIP2;1 in a closed and open conformation (Hedfalk et al., 2006; Törnroth-Horsefield et al., 2006; Nyblom et al., 2009). The major difference between the open and closed states of SoPIP2;1 is the position of the cytosolic loop D, which, in the closed conformation, is linked to the N-terminal part through a network of ionic interactions and hydrogen bonds. As a result, the loop D occludes the channel through the insertion of Leu-197 into the cytoplasmic opening (Törnroth-Horsefield et al., 2006). The closed conformation is stabilized by an interaction network around a divalent cation binding site (probably Ca^{2+} in vivo) involving the residue His-193 in the loop D, the Ser-115 in the loop B, and the N-terminal Asp-28 and Glu-31. While protonation of His-193 tightens the closed conformation, phosphorylation of Ser-115 and Ser-188 in the loop D and Ser-274 in the C terminus might lead to the channel opening (Törnroth-Horsefield et al., 2006; Nyblom et al., 2009; Frick et al., 2013b). These molecular dynamics simulations allow unifying of the functional and biochemical experimental data that have highlighted the role of specific amino acid residues or divalent cations in aquaporin gating regulation (Johansson et al., 1998; Tournaire-Roux et al., 2003; Van Wilder et al., 2008; Verdoucq et al., 2008).

From these studies, defined conditions or pharmacological compounds that trigger the closing of aquaporin pores have been largely used to investigate the contribution of aquaporins in the regulation of hydraulic parameters at the plant, organ, and cell levels. Mercury chloride that binds to the thiol group of Cys residues located in the pore region has been historically commonly used to occlude the aquaporin pore, a process that can be partly reversed by treatment with reducing agents (Preston et al., 1993; Kammerloher et al., 1994; Daniels et al., 1996; Chaumont et al., 2000). Interestingly, mercury could also inhibit the channel activity of a heterotetramer composed of ZmPIP1;2 and ZmPIP2;5 through its interaction with a Cys residue located in the loop A of ZmPIP1;2, a residue involved in disulfide bond formation between PIP monomer (Bienert et al., 2012). The structure of the SoPIP2;1/mercury complex has been recently solved and revealed three binding Cys residues for mercury, which could act on the channel gating (Frick et al., 2013a). However, strangely, reconstitution of SoPIP2;1 in liposomes showed that mercury does not inhibit but increases its water channel activity in a Cys-independent way, possibly through changes in the properties of the lipid bilayer. Therefore, due to mercury side effects caused by this compound on the membrane potential, cell respiration, and metabolism, caution has to be taken to correctly interpret the in planta data. Another transition element, silver, has also been used to inhibit plant aquaporins (Niemietz and Tyerman, 2002; Sadok and Sinclair, 2010). The fact that silver inhibition is otherwise rare makes it a potentially more selective tool to test for the aquaporin activity. Silver has been also tested for animal aquaporins and found to be very effective (Yang et al., 2006). Research to uncover better pharmacological agents for modifying (agonists or antagonists) animal aquaporins is well underway because of the role of some isoforms in disease and trauma (Yool et al., 2010). Some that should be examined for effects on plant aquaporins

include AqF026, an aquaporin agonist that is a chemical derivative of the arylsulfonamide compound furosemide, which interacts with loop D in AQP1 (Yool et al., 2013). Interestingly, arylsulfonamide compounds have aquaporin blocking activities (Yool et al., 2010). These include acetazolamide, which is a traditional inhibitor of carbonic anhydrase. One of these compounds, a bumetanide derivative aminopyridine carboxamide analog, AqB013, inhibits AQP1 and AQP4 with 50% inhibitory concentration of 20 μM. Based upon alteration of certain residues in AQP4, the blocking site appears to be on the cytoplasmic side of the water pore (Migliati et al., 2009). Screening a small molecule library for inhibition AQP9 water permeability revealed one compound (HTS13286) that inhibits water permeability in the low micromolar range and that is specific for AQP9. Molecular dynamics simulations and molecular docking have been used to identify other small molecule inhibitors of AQP9 (Wacker et al., 2013). These studies have pioneered the development of AQP-selective pharmacological agents, and plant researchers need to keep abreast of such developments, especially given the close sequence similarity between animal and plant aquaporins and their functional similarities in gating.

Artificial intracellular acidification using propionic acid or anoxia leads to protonation of the His residue located in the loop D and stabilization of the closed conformation of PIP aquaporins (Fig. 1; Tournaire-Roux et al., 2003). This pH inhibition mechanism is reversible and offers a mild method for probing aquaporin activity in living organisms (Alleva et al., 2006; Ehlert et al., 2009; Vandeleur et al., 2014). Finally, H_2O_2 application has also been used in different studies to alter the membrane hydraulic properties (Ehlert et al., 2009; Parent et al., 2009; Pou et al., 2013). The mechanisms by which H_2O_2 regulates the water permeability of the plasma membrane could be direct or indirect, such as direct oxidative gating, the induction of signal transduction pathways leading to the internalization of AQP proteins, or the alteration of their phosphorylation status, a posttranslational modification regulating their gating and subcellular localization (Aroca et al., 2005; Ye and Steudle, 2006; Kim and Steudle, 2007; Boursiac et al., 2008; Prak et al., 2008). However, all these treatments are also potentially not aquaporin specific as they can lead to modification of cell signaling and metabolism. The development of specific plant aquaporin inhibitors would therefore represent an important step to analyze correctly the physiological contribution of aquaporins in plant water relations.

AQUAPORINS IN PLANT GROWTH AND DEVELOPMENT

Plant growth results from cell division and expansion, which requires the continuous uptake of water to maintain turgor pressure. This process is controlled by a gradient in water potential, which itself is generated by the accumulation of solutes. In addition to the regulation of water influx into the expanding cells, the hydraulic properties of the surrounding tissue appear to be important (Volkov et al., 2007). The significance of PIP and TIP aquaporins in tissue elongation has been mainly suggested by a positive correlation between mRNA and/or protein expression and cell expansion in embryos, roots, hypocotyls, leaves, reproductive organs, or fruits, indicating that this process requires a high hydraulic permeability of the plasma membrane and tonoplast (for review, see Maurel et al., 2002; Fricke and Chaumont, 2007; Liu et al., 2008; Ma et al., 2008; Chen et al., 2013). Cell and tissue hydraulic properties during expansion seem to be tightly regulated, as recently illustrated by the involvement of aquaporins in lateral root emergence (Péret et al., 2012). During this developmental process, auxin reduces the expression of most *AtPIP* and *AtTIP* genes, including *AtPIP2;1*, which is excluded from the lateral root primordia but maintained at their base. Interestingly, both suppression and overexpression of *AtPIP2;1* result in delayed lateral root emergence, demonstrating the importance of a tight regulation of the cell water permeability in the growing organ and surrounding tissues (Péret et al., 2012). The specific role of TIPs in this process has still to be elucidated.

The characterization of plants modulated in the expression of aquaporin genes (knockout, knockdown, or overexpression) has been widely used to reveal their physiological function, even if correct interpretation of these data are not always trivial. Using these strategies, some growth defects have been reported, more generally when an aquaporin isoform is overexpressed and exposed in challenging growing environmental conditions (for review, see Hachez et al., 2006b; Maurel et al., 2008). These defects are in general correlated to changes in cell or organ hydraulic permeability parameters (see below). However, it is striking to observe that no obvious growth-related phenotypes were reported for single knockout *PIP* or *TIP* Arabidopsis mutants, probably due to the multigenic diversity of aquaporins and possible compensation mechanisms between close homologs, even if reduction of cell and organ hydraulic conductivities are generally measured (Javot et al., 2003; Beebo et al., 2009; Postaire et al., 2010; Prado and Maurel, 2013).

ROLE OF AQUAPORINS IN ROOT WATER TRANSPORT

The Role of Gatekeeper Cells and Apoplastic Barriers

Symplastic and transcellular pathways and apoplastic barriers occur at specific "gatekeeper" cell layers such as the exodermis and endodermis where large degree of control could be exerted by changes in activity or density of aquaporins. This is where higher expression of specific isoforms of aquaporin proteins is observed (Hachez et al., 2006a; Sakurai et al., 2008; Laur and Hacke, 2013) and also correlated to changes in L_{pr} or cell hydraulic conductivity in some cases (Hachez et al., 2006a; Sakurai-Ishikawa et al., 2011; Laur and

Hacke, 2013). Radial geometric considerations would dictate that flow density per unit surface area will increase toward the center of the root and to xylem vessels, and this is reflected by increased cell hydraulic conductivity (Bramley et al., 2009). Higher expression is generally observed in the inner cortex, endodermis, and stele and around xylem vessels (Hachez et al., 2006a; Vandeleur et al., 2009; Sakurai-Ishikawa et al., 2011; Gambetta et al., 2013; Vandeleur et al., 2014), but the pattern can be different for different transcripts, e.g. in maize, PIP2;5 occurs in the cortex and with increased expression in the exodermis that develops under aeroponic culture (Hachez et al., 2012). The presence of high densities of aquaporins in cells where water flow is concentrated would suggest a primary role for regulating flow across the root.

Recently a study of the PIP expression patterns and development of fine roots of grapevine (*Vitis vinifera*) have shown that the root tip has a high degree of expression, which drops off substantially in the maturation zone (Gambetta et al., 2013). It was expected that, where apoplastic barriers developed, there would be a greater density of aquaporin expression. This was not observed generally, though *VvPIP1;1* remained high in the exodermis and endodermis of the maturation zone. High hydraulic conductivity was associated with the root tip, where aquaporin expression was the highest and apoplastic barriers were not developed. Interestingly and in accordance with the CTM theory, there was over a 100-fold-higher L_{pr} measured with a hydrostatic gradient compared with an osmotic gradient in both the tip zone and the secondary growth zone, but H_2O_2 inhibition was only substantial (45%) for osmotic gradient flow in the meristematic and elongation zones. There was very little inhibition for the secondary growth zone (Gambetta et al., 2013). The authors conclude that aquaporins play a limited role in controlling water uptake in secondary growth zones, contrary to the view that they are more likely to be involved in radial water flow where substantial apoplastic barriers exist according to the CTM theory.

Diurnal and Circadian Regulation

L_{pr} shows diurnal variation with a maximum L_{pr} occurring when transpiration would normally be maximal (Parsons and Kramer, 1974; Henzler et al., 1999; Beaudette et al., 2007; Vandeleur et al., 2009; Sakurai-Ishikawa et al., 2011). Therefore, supply of water by roots can be matched to demand, and this will tend to smooth out diurnal variation in plant water potential (Tsuda and Tyree, 2000). In the case of more isohydric plants (plants with relatively constant water potential), the regulation of L_{pr} in some circumstances might have a greater impact on plant water potential than regulation by stomata (Laur and Hacke, 2013). Over a diurnal cycle, changes in L_{pr} can be 2- to 5-fold from minimum to maximum. These variations generally correlate with PIP transcript and protein abundance. Transcript abundance of root *PIP* genes increases early in the day in roots of maize (Lopez et al., 2003) and rice (Sakurai-Ishikawa et al., 2011), while in grapevine roots, transcripts for *VvPIP1;1* increases while *VvPIP2;2* remains constant (Vandeleur et al., 2009). A diurnal rhythm in expression of a pea (*Pisum sativum*) *PIP2* is correlated with diurnal changes in L_{pr} (Beaudette et al., 2007). Protein levels of various PIPs in maize roots are diurnally regulated, with higher PIP levels generally observed toward the middle and end of the day (Hachez et al., 2012). These changes could be controlled by transpirational demand from the shoot (Laur and Hacke, 2013) and/or via circadian regulation (Takase et al., 2011).

The role of circadian regulation of root aquaporins is clearly evident when it has been examined (Lopez et al., 2003; Sakurai-Ishikawa et al., 2011; Takase et al., 2011). Increased gene expression is still observed for maize root aquaporins during the first subjective day when plants are in continuous darkness, but is reduced by the second subjective day, depending on the particular isoform, e.g. *ZmPIP1;5* does not show a dampened response into the second subjective day (Lopez et al., 2003). The circadian regulation of *PIP* expression in maize roots is correlated with circadian leaf elongation rate observed under adverse environmental conditions, a process associated with hydraulic processes, namely oscillations of leaf water potential and plant hydraulic conductance (C.F. Caldeira, L. Jeanguenin, F. Chaumont, and F. Tardieu, unpublished data). Using ^{1}H-NMR imaging, the water content of Arabidopsis roots shows diurnal oscillation, with continued oscillation under constant light or darkness, a process correlated with the circadian oscillation of *AtPIP1;2* and *AtPIP2;1* transcripts under constant light (Takase et al., 2011). Supporting the role of the circadian clock in modulating water flow and aquaporin expression is the lack of circadian oscillation in water content and aquaporin expression in the early flowering3 (*elf3*) mutant (Takase et al., 2011). ELF3 interacts with ELF4 and LUX ARRHYTHMO (LUX) in regulating the morning clock gene PSEUDO RESPONSE REGULATOR9 (PRR9) and is critical in maintaining circadian rhythms in plants (Herrero et al., 2012). Interestingly, the *elf3* mutant had continuous low expression of *AtPIP2;1* and high expression of *AtPIP1;2* compared with the wild type when maintained in continuous light. Clearly, circadian clock regulation of root aquaporins occurs, and it would be interesting to examine how these genes are entrained with the shoot. Recently, it has been shown that photosynthesis entrains circadian rhythms in Arabidopsis via endogenous oscillations in sugars (Haydon et al., 2013). This opens the possibility that variation in sugar import to the roots via the phloem may be important in entrainment of the clock in the roots and regulation of aquaporins. The question remains as to the role of transpiration.

Control by Transpiration

Both transpiration and circadian controls are evident in regulation of *PIP* transcripts in rice roots (Sakurai-Ishikawa et al., 2011). When darkness was extended after 12 h, most *PIP* genes still show some diurnal regulation with some showing greater dependence on the light signal (increased expression) or higher humidity (decreased expression) around the shoot (*OsPIP2;4* and *OsPIP2;5*). Excising the shoot also results in large decreases in expression of the transpiration regulated isoforms *OsPIP2;4* and *OsPIP2;5* compared with *OsPIP2;1* and *OsPIP2;2* (Sakurai-Ishikawa et al., 2011). It seems, therefore, that both circadian control and transpiration-related signals from the shoot regulate *PIP* gene expression depending on the isoform.

Correlations can be observed between transpiration rate and L_{pr} (Wang et al., 2013) that can account for the large variability between measurements of L_{pr} made on different days (Vandeleur et al., 2014). For wheat (*Triticum aestivum*), there is a strong positive correlation between increasing L_{pr} (with increasing ploidy) and cortex cell hydraulic conductivity and transpiration (Wang et al., 2013), with remarkable correlations between hydraulic conductivity and the expression of both *TaPIP1;2* and *TaPIP2;5*. However, a link between transpiration and L_{pr} has not always been observed. Changes in shoot transpiration are not reflected by changes in L_{pr} in *Lotus japonicus* (Henzler et al., 1999), although these measurement were made on roots that had been excised for some time, and, as outlined further below, excised roots may loose a large component of L_{pr} linked to particular aquaporins (Vandeleur et al., 2014).

Exposure of Arabidopsis shoots to low relative humidity (RH) results in rapid (within 10 min) increase in plant hydraulic conductance by 3-fold (Levin et al., 2007), and roots respond by alterations in gene expression (Levin et al., 2009). Mercury treatment results in reduced response of the hydroponically grown plants, suggesting a role for aquaporins in the humidity response (Levin et al., 2007). Among the many transcripts altered in the roots are several aquaporins, and interestingly, the largest fold changes occur in a TIP aquaporin located close to the xylem in roots (Levin et al., 2009).

Direct responses to increased transpiration were recently investigated for hybrid poplar roots (Laur and Hacke, 2013). Here, transition from shade to higher light or from high to low RH caused an initial reduction in water potential, which subsequently recovered after 28 h (next day). This recovery was not a result of reduced stomatal conductance in the case of the lowered RH but rather an increase in the water flow capacity of the roots. This increased capacity for water flow was correlated with increased expression of *PIP1* and *PIP2* genes, though different responses were observed between isoforms for light and RH transitions. Especially noticeable was the substantial increase in PIP1 protein located in the endodermis and epidermis after only 4 h of reduced RH. This study highlights the importance of regulation of root water flow to maintain plant water potential complementing the regulation exerted by stomata.

Does Driving Force Alone Regulate Root Water Transport?

An interesting observation for root water transport is that often pressure-driven flow gives higher L_{pr} than is measured with osmotically driven flow, but this depends on how pressure gradients are established (Bramley et al., 2007), the species (Bramley et al., 2009), and the part of the root that is measured (Gambetta et al., 2013). Transpiration increases the pressure component of the driving force across the root, so during the day, when transpiration increases, there would be an increase in L_{pr} that would then satisfy the greater demand for water by the shoots. The CTM for water transport across roots (Steudle, 2000) has been used to explain this driving force dependency of L_{pr} in both space and time. In the CTM, it was proposed that the apoplastic pathways in roots becomes more dominant (over transcellular and symplastic pathways = cell to cell) under pressure-driven flow, which would be the situation during transpiration and that aquaporins could account only for finer adjustment or flow through older, suberized parts of the root (Steudle and Peterson, 1998). Pressure gradients do not distinguish between parallel apoplastic or symplastic and transcellular pathways across the root, i.e. an increase in pressure gradient should equally increase the flow through both cell-to-cell and apoplastic pathways. This is not the case for osmotic gradients that can only generate flows across membranes with water-selective membranes, so that at low rates of transpiration (small pressure gradients), more flow would occur via the transcellular pathway relative to the apoplast pathway if osmotic gradients are generated. Higher L_{pr} would be expected for pressure-driven flow when the apoplast contributes significantly to the flow across the root. This test has been used to determine the relative contributions of the apoplast and cell-to-cell pathways (Bramley et al., 2007). However, it assumes that pressure-driven flow does not influence gating of aquaporins in the cell-to-cell pathway, which is a possibility (see below), particularly because different knockouts of *PIP* genes can result in exclusive alteration of osmotic (*AtPIP2;2*; Javot et al., 2003) or pressure-induced flow (*AtPIP1;2*; Postaire et al., 2010).

The CTM in some respects challenges the role of aquaporins in facilitating water transport across the root during transpiration and has been challenged recently based on measurements of the near ideal (membrane-like) semipermeability of the root, implying a small contribution from an apoplastic pathway, at least in barley (*Hordeum vulgare*; Knipfer and Fricke, 2010). (Quantified as a reflection coefficient where ideal semipermeability is indicated by a reflection

coefficient equal to 1 means that an osmotic pressure gradient will generate a pressure gradient of the same magnitude across the root.) Also not in accord with the CTM theory, at least in respect to the role of aquaporins, is gradient-independent diurnal variation in L_{pr} (Henzler et al., 1999; Vandeleur et al., 2009). Diurnal variation in L_{pr} is the same in *L. japonicus*, irrespective of its measurement using osmotic or pressure gradients (Henzler et al., 1999). In grapevine, diurnal variation (2-fold change in L_{pr}) is observed with pressure gradient measurements (Vandeleur et al., 2009). The CTM theory for root water transport clearly needs to be considered more carefully as indicated by Knipfer and Fricke (2010) and particularly in terms of the type of root system anatomies (Bramley et al., 2009) and expression patterns of aquaporins (Gambetta et al., 2013).

Shoot-to-Root Signaling in Aquaporin Regulation

Where transpiration is linked to L_{pr} and changes in root aquaporin gene expression, this would imply that shoot-to-root signaling occurs (McElrone et al., 2007; Levin et al., 2009). Also shoot damage caused by herbivory, biotic stress, weather events, and canopy management techniques in horticulture may impact root water transport via shoot-to-root signaling. Shoot wounding and herbivory are known to activate chemical defenses, changes in carbohydrate storage in the roots (Erb et al., 2009), and relatively rapid changes in root growth (Hummel et al., 2007; Schmidt et al., 2010). The different aspects and mechanisms regarding shoot-to-root communications regulating L_{pr} and aquaporin expression will be discussed below and are summarized in Figure 2.

Transpiration generates xylem tension, which is lost upon excision, and is transmitted to the roots virtually instantaneously depending on capacitative components along the xylem in the stem and root (Bramley et al., 2007). Recently, Vandeleur et al. (2014) showed that shoot wounding reduces L_{pr} of maize, soybean, and grapevine. Even covering a leaf without wounding was enough to reduce L_{pr} in grapevine and soybean by more than 30%, and based on reduced inhibition by weak acid, it was concluded that the response is mediated by changes in aquaporin activity. Closer examination of soybean showed that shoot excision rapidly reduces L_{pr} by over 50% (half time about 5 min), indicating that measurements on excised roots need to be done rapidly and with care to check the impact of shoot excision for each species. Turgor patch-clamp probes (Zimmermann et al., 2008) showed that a pressure signal rapidly propagates down the plant with excision of the top one-third of the shoot in soybean (S.D. Tyerman, unpublished data). Turgor pressure in the root cortex also decreased with shoot wounding, but changes in cortex cell hydraulic conductivity were not observed (Vandeleur et al., 2014). The rapid reduction in L_{pr} was correlated with rapid reduction in transcript of *GmPIP1;6* for both shoot excision and shoot wounding in accordance with a similar observation for a rice *PIP* transcript (Sakurai-Ishikawa et al., 2011). The soybean transcript was present in the cortex but appeared to have higher expression in the stele (Vandeleur et al., 2014).

Possible shoot-to-root signals so far indicated include hydraulic (Boari and Malone, 1993), electric (Davies, 1987; Wegner and Zimmermann, 1998), chemical defense signals, and hormones (Macháčková et al., 1992; Zhang and Baldwin, 1997; Ljung et al., 2002; Shah, 2009). Beveridge et al. (2009) proposed that, in addition to auxin, there is a fast-moving decapitation signal moving basipetally that may be a pressure or electrochemical signal.

Plant hormones may be important in long-distance signaling to control L_{pr}. Abscisic acid (ABA) can alter aquaporin expression in Arabidopsis, depending on the isoform, with six out of 13 *PIP* genes being up-regulated in roots except for *AtPIP1;5*, which is down-regulated (Jang et al., 2004). Both whole root and cortical cell hydraulic conductivity of maize roots are transiently increased by ABA (Hose et al., 2000). Overproduction of ABA in tomato by overexpression of 9-cisepoxycarotenoid dioxygenase (NCED) results in higher L_{pr} (Thompson et al., 2007). Similarly, in transformed maize with high *NCED* expression, the resulting higher ABA translates to high *PIP* gene expression and protein levels giving higher L_{pr} (Parent et al., 2009). Hydraulic conductivity of *Phaseolus vulgaris* roots is also increased by ABA, as is PIP protein abundance (Aroca et al., 2006). In wheat, communication of increased transpirational demand that increased L_{pr} appears to be via ABA translocation to roots in the phloem (Kudoyarova et al., 2011). Redistribution of ABA to roots was proposed to increase L_{pr} sufficiently to allow recovery of shoot turgor and growth without stomatal closure. However, in soybean, shoot-wounding signals that reduced L_{pr} do not appear to be dependent on phloem translocation or root ABA concentration (Vandeleur et al., 2014).

In addition to ABA, other plant hormones have been implicated in regulating root aquaporins (Aroca et al., 2012). These include salicylic acid, which regulates PIPs through a H_2O_2-induced internalization (Boursiac et al., 2008). Auxin inhibits the endocytosis of PIP2 in Arabidopsis (Paciorek et al., 2005), while exogenous auxin has been found to inhibit root and cell hydraulic conductivity in Arabidopsis (Péret et al., 2012). Ethylene can enhance (Kamaluddin and Zwiazek, 2002) or inhibit (Li et al., 2009) L_{pr}. In the case of the latter, inhibition caused by phosphorus deficiency appears to be mediated by ethylene. Vandeleur et al. (2014) found that 10 mM trans-2-amino-4-(2-aminoethoxy)-3-betenoic acid hydrochloride, an inhibitor of ethylene precursor 1-aminocylcopropane-1-carboxylic acid, when sprayed on leaves did not prevent the inhibitory effect of shoot wounding on L_{pr}.

Pressure Signals

Changes in xylem pressure have previously been proposed as a root-to-shoot hydraulic signal of water

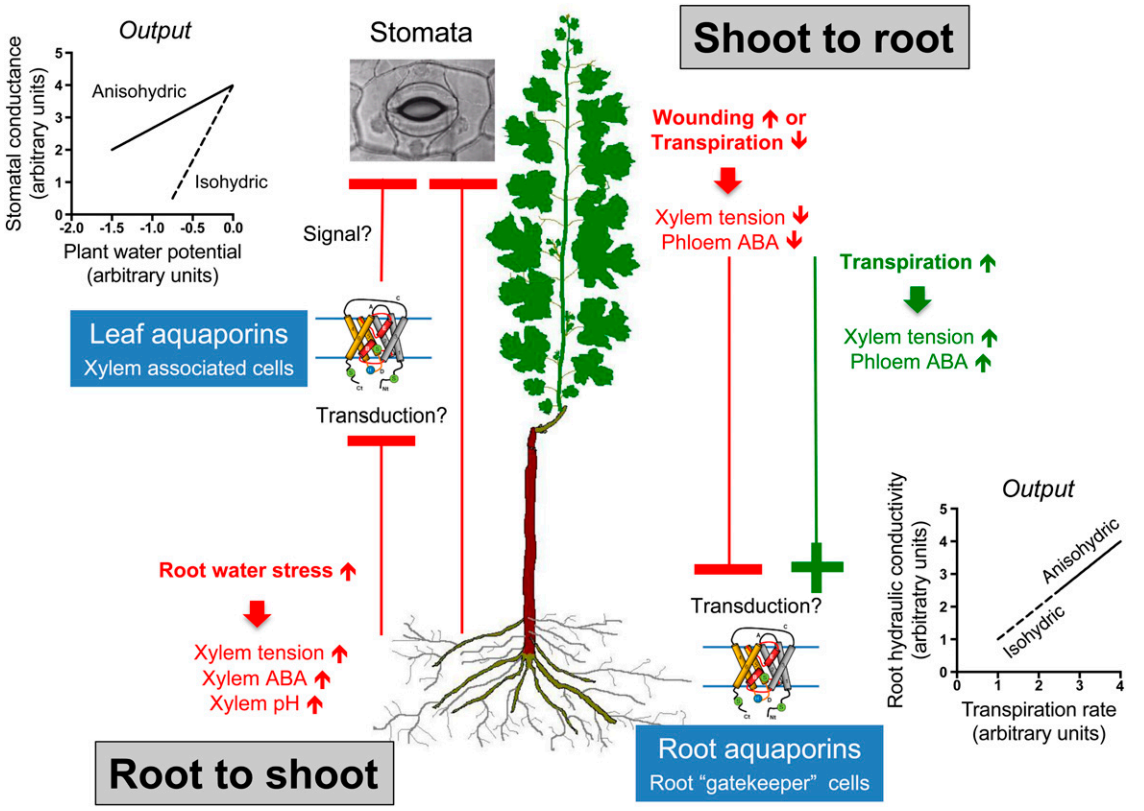

Figure 2. Long-distance signaling within plants involving aquaporins to coordinate water demand by the shoot with supply by the roots. On the left side is shown a summary of root-to-shoot signaling that occurs when roots are subjected to water stress. The classic ABA signaling pathway (including a coordinating effect of xylem sap pH; Wilkinson and Davies, 2002) is shown as a direct link to the stomata or through a hydraulic (pressure) signal that releases ABA in the shoot through an as yet unknown transduction process (Christmann et al., 2007). Also shown is an independent ABA signaling mode wherein bundle sheath cells and/or xylem parenchyma cells respond by reducing the activity of aquaporins (Shatil-Cohen et al., 2011; Pantin et al., 2013). This is proposed to convey a hydraulic signal to the stomata. The degree of isohydry/anisohydry (Output, top left) has been hypothesized to potentially reside in the gain of the ABA transduction process (Pantin et al., 2013). On the right side of the diagram is shoot-to-root signaling that appears to regulate L_{pr} in response to transpiration. The Output graph summarizes observations from various plants (Vandeleur et al., 2014). Increased transpiration increases root aquaporin expression and activity and L_{pr} (Levin et al., 2009; Sakurai-Ishikawa et al., 2011; Laur and Hacke, 2013), and the signal may be an increase in xylem tension that is rapidly transmitted to roots (McElrone et al., 2007); alternatively, phloem ABA may increase and stimulate root aquaporin activity (Kudoyarova et al., 2011). The method of signal transduction is unknown. Lowered transpiration leads to down-regulation of aquaporin activity and reduced L_{pr} possibly via release of xylem tension. Shoot wounding may interfere with this feedback system because similar aquaporin transcripts change in response to shoot decapitation, as in response to reduced transpiration (Sakurai-Ishikawa et al., 2011; Vandeleur et al., 2014). Isohydric and anisohydric plants appear to sit on the same linear response of L_{pr} versus transpiration (Vandeleur et al., 2009), suggesting that the degree of isohydry/anisohydry is more related to the response of the shoot to ABA, though this has not been explicitly tested.

stress (Christmann et al., 2007; Grams et al., 2007). Leaf mesophyll cell turgor declines within 200 s following the imposition of root water stress (Christmann et al., 2007). This pressure signal was proposed to increase shoot ABA, causing stomatal closure in response to root water stress. Considering the possibility that pressure signals are also important from shoots to roots, i.e. in the reverse direction, a change in xylem tension, either due to wounding of xylem by shoot or leaf excision or changes in transpiration rate, propagates rapidly to the roots (Wegner and Zimmermann, 1998) and potentially gates aquaporins (McElrone et al., 2007).

Reduced xylem tension in the roots might be expected to increase turgor pressure in root cells; however, in soybean, turgor declines after 5 min from shoot topping (Vandeleur et al., 2014). These results may be explained in the context of turgor gradients across the root (Rygol et al., 1993). Turgor pressure gradients exist across cortical cells of wheat and maize roots during transpiration, and these gradients respond rapidly to changes in transpiration and leaf area (Rygol et al., 1993). Turgor of inner cortical cells decreased and the normal gradient (low to high) from outer to inner cortex was abolished within 5 to 10 min

following root excision. Contrasting results were observed for the effect of transpiration, through changes in light intensity, on root cortical cell turgor of *Cucurbita ficifolia* seedlings (Lee et al., 2008). Here, turgor decreased within 5 min with increased transpiration, and this corresponded to a decrease in cell hydraulic conductivity. This decrease, when a higher water transport capacity would be in order, seems counter intuitive but was explained in terms of increased water flow through the apoplast (see CTM discussion above). Hachez et al. (2012) reported no significant difference in root cortical cell turgor for plants in the light or dark, and, in this case, hydraulic conductivity of cortical cells was higher for plants taken from the light period that correlated with higher levels of mRNA and protein of various PIP aquaporins. Perhaps these contrasting observations could be explained by different flow pathways across roots between species (Bramley et al., 2009), different cortical cell layers having different responses (Rygol et al., 1993), and circadian regulation imposed on top of transpiration effects. Given the sensitivity of L_{pr} to shoot manipulations, careful experimentation is required to resolve these signals.

One important unresolved issue with regard to intact roots and the gradients that must exist across the radial pathway during transpiration is that osmotic gradients in the apoplast could be significant (Rygol et al., 1993). When the root is excised from the shoot, which is assumed to bring xylem pressure to atmospheric pressure, this does not substantially affect the difference between cell osmotic pressure and turgor pressure measured using the cell pressure probe, suggesting that the apoplastic osmotic pressure is significant. Furthermore, the change in turgor upon excision seems to be associated more so with changes in cell osmotic pressure, indicating potentially rapid fluxes across cell membranes (Rygol et al., 1993).

Is There a Link between Changes in Turgor and Water Transport through Aquaporins?

Turgor pulses in root cortical cells cause large and reversible reductions in cell hydraulic conductivity when the pulses are between 0.1 and 0.2 MPa. The reduction in conductivity is irreversible for larger pressure pulses (Wan et al., 2004). This was explained in terms of mechanosensitive gating of aquaporins that could be reversed by the application of ABA (Wan et al., 2004). Mechanosensitivity of aquaporins was previously proposed to explain the response of *Chara corallina* water transport to osmotic pressure (Ye et al., 2005). Two models of direct gating have been proposed: a pressure-dependent model, where kinetic energy is transferred to the channel to cause a conformational change (Wan et al., 2004), and osmotic pressure dependent (cohesion-tension model), where tension within the pore causes a conformational change (Ye et al., 2005). These models were developed from measurements on intact cells rather than isolated membranes devoid of second messenger systems. However, isolated membrane vesicles can also show aquaporin-mediated water permeability that is highly dependent on osmotic pressure (Vandeleur et al., 2005). Recently, it has been shown that human AQP1 is constitutively open but closes with increases in membrane tension (Ozu et al., 2013).

In respect to pressure-dependent gating of aquaporins, it is now possible to better integrate our knowledge of gating mechanisms via phosphorylation, cytosolic Ca^{2+}, cytosolic pH, and reactive oxygen species (ROS). It is likely that the effects of turgor and ABA on aquaporins observed in intact cells may be via changes in cytosolic Ca^{2+}, pH, and phosphorylation status. Elevated cytosolic Ca^{2+} is commonly observed in mechanostimulation (Monshausen and Gilroy, 2009; Monshausen and Haswell, 2013). Cytosolic pH is reduced in epidermal cells of Arabidopsis roots upon mechanostimulation as well as increased ROS production in the apoplast. This reduction in cytosolic pH together with elevated ROS is likely to close aquaporins or regulate their cycling from the plasma membrane (see above).

The responses discussed so far relate to closing of aquaporins upon mechanostimulation. It is less clear how aquaporins may be opened by mechanostimulation, presumed to occur when xylem tension increases in response to increased transpiration. Also, signal transduction must occur not only for direct aquaporin gating, but also to regulate transcription and/or trafficking. It is possible that different signal transduction occurs for increased aquaporin activity compared with inhibition and also for transcriptional or trafficking regulation. Elevated ABA in roots consistently increases aquaporin transcription and L_{pr}. There is one report of a stimulatory effect of ABA on a barley PIP aquaporin expressed in *Xenopus* spp. oocytes, but this effect was also observed on water-injected oocytes (Wei et al., 2007). Some evidence supports also the direct role of aquaporins as osmo- or pressure sensors (MacRobbie, 2006; Ozu et al., 2013; Shachar-Hill et al., 2013), as was proposed earlier (Hill et al., 2004). However, there is also ample evidence for the presence and functioning of mechanosensitive ion channels in plant membranes that could transduce pressure signals and affect aquaporins via second messenger systems (Monshausen and Haswell, 2013). In Arabidopsis, abolishing mechanosensitive channel activity in root protoplasts by knocking out simultaneously *Mechanosensitive Channel of Small Conductance-Like4* (*AtMSL4*), *AtMSL5*, *AtMSL6*, *AtMSL9*, and *AtMSL10* genes did not result in any other phenotype (Haswell et al., 2008). Perhaps a phenotype is linked to control of water transport, which is difficult to assess in Arabidopsis. These knockouts need to be closely assessed in the light of proposed shoot-to-root hydraulic signals and their transduction via changes in xylem and or cell turgor.

Finally, electrical signals should not be ruled out, because Wegner and Zimmermann (1998) observed

changes in the wheat transroot electrical potential that preceded xylem pressure reduction in response to shoot illumination. Transduction of pressure to electrical signals is clearly evident in their study, indicating that electrogenic ion transport is somehow coupled to xylem pressure in the root. Recently, heat-induced electrical signaling in soybean leaves has been shown to reduce mesophyll conductance to CO_2, which could be due to closure of aquaporins (Gallé et al., 2013).

ROLE OF AQUAPORINS IN SHOOT WATER TRANSPORT

Assessing the Role of Aquaporins in Leaf Hydraulic Conductivity

Leaf (area-specific) hydraulic conductivity (K_{leaf}) can be measured in various ways (Flexas et al., 2013), but compared with roots, it is intrinsically more difficult to determine the relative contributions of the xylem conductance to the postxylem (downstream) conductance, excluding the impact of stomata (Rockwell et al., 2011), let alone to determine relative contributions in the postxylem pathway from apoplastic and cell-to-cell components. Various estimates have been made of the xylem contribution to K_{leaf}, and these average over 50% (Sack and Holbrook, 2006). Recent reviews examine the xylem and hydraulic network in leaves related to coordination between hydraulic and photosynthetic systems of plants (Brodribb, 2009; Nardini et al., 2005; Flexas et al., 2013; Sack and Scoffoni, 2013).

Some measure of the role of aquaporins within the postxylem cell-to-cell pathway can be obtained using inhibitors. Feeding aquaporin inhibitors via the cut petiole yields varying results depending on species or even cultivars (Pou et al., 2013), diurnal timing (Postaire et al., 2010), plus or minus light (Voicu and Zwiazek, 2010), circadian oscillations (Nardini et al., 2005), season (Voicu and Zwiazek, 2010), or water stress and recovery cycles (Pou et al., 2013). For grapevine, a variety of aquaporin inhibitors were examined, with mercury giving the most consistent response for two cultivars (cv Shiraz and Chardonnay), though H_2O_2 was also effective for cv Shiraz. Maximum inhibition of K_{leaf} of between 25% and 50% seems to be an average response from various studies (Pou et al., 2013).

Contributions from apoplast versus symplast flow in leaves can also be made using specific fluorescent tracer dyes (Voicu et al., 2009; Pou et al., 2013). This has indicated no change in apoplastic flow in response to light (see below) in *Quercus macrocarpa* or *Populus tremuloides* (Voicu et al., 2009; Voicu and Zwiazek, 2010), while in grapevine a greater degree of apoplastic flow was observed with drought treatment that corresponded to reduced K_{leaf} and reduced inhibition of K_{leaf} by mercury (Pou et al., 2013). Switching of water pathways involving apoplastic and symplastic transport may allow for some flexibility in responses to water stress (Morillon and Chrispeels, 2001).

Apart from positive correlations between aquaporin expression (*TIP* and *PIP*) and K_{leaf} or stomatal conductance (Baaziz et al., 2012; Pou et al., 2013), there are few studies where alteration in expression of an aquaporin has been linked to changes in K_{leaf} or, more precisely, whole-shoot conductance in Arabidopsis (Postaire et al., 2010; Prado et al., 2013). Knockout of *AtPIP1;2* reduced shoot conductance by about 30%, and this corresponded to reduced osmotic water permeability of mesophyll protoplasts by about 50% (Postaire et al., 2010).

Vein Cells as Chemical/Hydraulic Transducers

Stomatal guard cells as the penultimate water gatekeepers in leaves constitute the major regulator of plant transpiration; however, recent work has indicated that hydraulic regulation upstream of stomata can play a significant role in the dynamics of leaf water potential and stomatal regulation (Shatil-Cohen et al., 2011; Pantin et al., 2013; Prado and Maurel, 2013). Bundle sheath cells around the xylem and the xylem parenchyma cells appear to be the main sensors and regulators of K_{leaf} in Arabidopsis (Shatil-Cohen et al., 2011; Prado et al., 2013; Prado and Maurel, 2013). In contrast to roots (in general), ABA reduces leaf hydraulic conductance when fed via the xylem, an observation correlated with reduced water permeability of isolated bundle sheath protoplasts, which is not observed for leaf mesophyll protoplasts (Shatil-Cohen et al., 2011). Pantin et al. (2013) subsequently showed that feeding ABA to excised leaves of ABA-insensitive mutants of Arabidopsis still caused reduced stomatal conductance. The *OPEN STOMATA2-2* (*ost2-2*) mutant was studied in more detail, where it was found that K_{leaf} was reduced similarly to the wild type, leading the authors to suggest that ABA regulates stomata via an additional indirect mechanism whereby a reduced water permeability within leaf vascular tissues results in local changes in water potential that are sensed by guard cells (Pantin et al., 2013). These results support the hypothesis that bundle sheath cells and xylem parenchyma cells are key gatekeepers within the leaf that function as a hydraulic "control center" (Sack and Holbrook, 2006) transducing ABA signals (Shatil-Cohen et al., 2011).

Response to Light

Dark/light transitions result in variable responses in K_{leaf} between species (Scoffoni et al., 2008; Baaziz et al., 2012). Dark to light can increase K_{leaf} dramatically within 15 min in *Juglans regia* (Cochard et al., 2007) or has only a small positive response in *Salix alba* (Baaziz et al., 2012). In Arabidopsis shoots, the response is an increase conductance from light to dark (Postaire et al., 2010). In *J. regia*, the rapid response in K_{leaf} correlates with increase in transcript of both PIP2 and PIP1

isoforms and is blue light dependent but unrelated to stomatal aperture (Cochard et al., 2007; Baaziz et al., 2012). The effect of light on K_{leaf} and aquaporin expression is not always observed (Scoffoni et al., 2008; Voicu et al., 2009; Rockwell et al., 2011). There is an interaction with dehydration (Guyot et al., 2012), and anoxia can reduce K_{leaf} (Rockwell et al., 2011) as it does in roots. The significance of light effects on K_{leaf} and aquaporins is not yet clear and especially so because there is such variability between species. In one case, modeling suggested that the positive response to light may buffer leaf water relations under variable light (Cochard et al., 2007). There is also an indication that responsive species are those that have heterobaric anatomy (bundle sheath extensions; Scoffoni et al., 2008).

As for ABA signaling, bundle sheath cells and xylem parenchyma cells again appear to be the major controllers of water transport in response to light-to-dark transition in Arabidopsis (Prado et al., 2013). Reverse genetics and complementation showed that water transport in leaves is dependent upon three *PIP* genes expressed in leaf veins (*PIP1;2*, *PIP2;1*, and *PIP2;6*), while *PIP2;1* accounts for dark-elevated hydraulic conductance. Posttranslational modification was indicated by protoplast water permeability increasing for isolated vein protoplasts after dark treatment, while the opposite was observed for mesophyll protoplasts. Phosphorylation of PIP2;1 at both Ser-280 and Ser-283 was necessary for increased conductance in the dark. This study emphasizes the importance of posttranslational modifications of aquaporins in specialized cell layers as gatekeepers of water transport. That just a relatively minor (in size) layer of cells can have such a significant effect in transport is a paradigm for other plant organs.

Integration of the Root and Shoot: Can Control of Aquaporins Determine Isohydric/Anisohydric Responses?

Under various degrees of water stress, plants can show responses in their water potential that range between a relatively constant behavior, i.e. midday values reach a constant minimum value (isohydric), to a more variable response where midday water potential declines (anisohydric). Isohydric plants also tend to maintain more constant water potential with increasing evaporative demand. The difference in response is linked to tighter control by stomata in isohydric plants (Tardieu and Simonneau, 1998). The stomatal response to ABA in the xylem is greater for isohydric plants when under water stress or under high evaporative demand, suggesting that isohydric behavior occurs when there is an interaction between hydraulic and chemical signaling (Tardieu and Simonneau, 1998). There are close links between plant hydraulics and the degree of isohydry/anisohydry. This can be associated with higher xylem conductance through the petiole (Schultz, 2003) and even to grapevine berries, which are less likely to dehydrate in isohydric cv Grenache (J.S. Scharwies and S.D. Tyerman, unpublished data).

The link between isohydry/anisohydry and root hydraulics was investigated by taking advantage of grapevine varieties with contrasting behaviors (Vandeleur et al., 2009). In grapevine, the tighter control of stomatal conductance in the more isohydric variety Grenache compared with cv Chardonnay (anisohydric) under water stress is also reflected in root hydraulic behavior over a diurnal time course. Paralleling the drop in stomatal conductance, there is a greater reduction in L_{pr} in cv Grenache at midday compared with cv Chardonnay. Interestingly, both varieties sit on the same positive linear relationship between L_{pr} and transpiration (Fig. 2); in other words, the lower L_{pr} in cv Grenache under water stress is associated with a lower transpiration rate for a similar water potential. Both varieties show increased root suberization under water stress, but cv Chardonnay seems to partially compensate by increased expression of VvPIP1;1, which correlates with increase cortical cell hydraulic conductivity. cv Grenache does not display this behavior, and an opposite response is observed upon rewatering. This work demonstrates that control of aquaporins is also linked to isohydric/anisohydric behavior. A most impressive dynamic root response involving aquaporins has since been shown for poplar, which is an isohydric species (Laur and Hacke, 2013). Here, increased transpiration in response to light or decreased RH is compensated for by up-regulation of aquaporins and hydraulic conductivity in roots such that water potential is maintained with little change in stomatal conductance. This may at first seem contrary to the results from Vandeleur et al. (2009), but actually it shows the same trend, i.e. roots of isohydric varieties adjust conductivity to maintain a more constant water potential in concert with stomatal regulation.

Changing aquaporin expression also changes isohydric/anisohydric behavior. Isohydric tomato can be converted to anisohydric behavior by overexpression of tomato *TIP2;2* selected for its responses to abiotic stress (Sade et al., 2009). Higher transpiration rates and protoplast water permeabilities are observed for the *TIP2;2* overexpressors, suggesting that removing control on this TIP changes the whole plant isohydric threshold. The work also emphasizes the importance of careful phenotyping when it comes to understanding the role of aquaporins.

Moving now to leaves and attempting to integrate these responses, the bundle sheath hydraulic/chemical transduction (Shatil-Cohen et al., 2011; described above) and apparent dual control of ABA on stomata (Pantin et al., 2013) can be integrated to the formulation of a hypothesis for a biophysical basis of isohydry versus anisohydry. Pantin et al. (2013) have proposed that the vascular ABA-responsive component, putatively aquaporins in bundle sheath cells and K_{leaf}, is more/less sensitive to ABA in isohydric/anisohydric species. This hypothesis is testable especially using varieties of a species such as grapevine that can show contrasting

isohydric/anisohydric behaviors or plants that have altered expression of an aquaporin that leads to change in isohydric to anisohydric behavior. Taking the root responses into account, the question then arises as to whether the signaling is downstream or upstream of the bundle sheath integrators in the leaf, considering that any hydraulic response can rapidly travel through the xylem. We need to find the parallel gatekeepers in roots as the leaf bundle sheath equivalent.

Water and CO_2 Interactions via Aquaporins?

Physiological integration of leaf hydraulic conductance and leaf internal conductance to CO_2 (g_m) has become a major focus, and there are close correlations between the two such that K_{leaf} may be used with other leaf measurements to predict g_m (Flexas et al., 2013). There is recent evidence to suggest that water and CO_2 share diffusion pathways through leaf mesophyll, so that any down-regulation of K_{leaf} in the postxylem pathway may result in reduced g_m and stomatal conductance, both contributing to reduced photosynthesis (Ferrio et al., 2012; Flexas et al., 2012). g_m has been linked to aquaporins, and some aquaporins facilitate CO_2 diffusion across plasma and chloroplast membranes (Kaldenhoff, 2012) and artificial gas-tight membranes (Uehlein et al., 2012). This is not without some controversy, mainly because CO_2 is supposed to permeate very rapidly through lipid membranes and it is very difficult to measure CO_2 permeability accurately (Missner et al., 2008; Missner and Pohl, 2009). There is also a mismatch between modeled CO_2 permeabilities required to account for g_m and the measured values for biological membranes that are much lower (Evans et al., 2009). Recently, water and CO_2 permeability of isolated pea leaf plasma membrane vesicles was examined, and it was found that there was a positive correlation between the two (M. Zhao, J.S. Scharwies, J.R. Evans, and S.D. Tyerman, unpublished data). Although exceedingly high aquaporin-mediated water permeability was recorded (0.06–0.22 cm s^{-1}), the CO_2 permeability (0.001–0.012 cm s^{-1}), deemed to be relatively free of unstirred layer effects and limitation from carbonic anhydrase, was within the range of other low measurements recorded for plant membranes. The pathway for CO_2 diffusion did not appear to occur via the same pathway as for water because blockers showed strong inhibition of water permeability but different effects on CO_2 permeability (M. Zhao, J.S. Scharwies, J.R. Evans, and S.D. Tyerman, unpublished data). Differential response to blockers between water and CO_2 transport has been observed for animal AQP1 (Endeward et al., 2006), and it has been proposed that the homotetramer of NtPIP2;1 is required to facilitate CO_2 diffusion (Otto et al., 2010).

CONCLUSION

Plant water status depends on efficient water supply at the root level and distribution through the plant body. This process involves a tight regulation of the water flow through cells, tissues, and organs and is controlled by internal and external cues. As reviewed here, much progress in the field has highlighted a central role of aquaporins. However, it appears that the mechanisms regulating plant water relations are highly complex and involved various interconnected hormonal and hydraulic signals that lead to regulation of cell and tissue permeability through modifications of aquaporin expression, trafficking, and activity. Here are the outstanding issues that, in our opinion, require more research: (1) What is the role of modification of aquaporin-specific amino acid residues (phosphorylation, deamination, disulfide bond, etc.) and the identity and regulation of the modifying enzymes? (2) How are aquaporins and K^+ channels coregulated to control cell water homeostasis? (3) Identification of highly specific aquaporin inhibitors or agonists. (4) How are CO_2 and water diffusion in leaves coupled, and what is the role of aquaporins? (5) What is the role of TIP aquaporins in both root and leaf hydraulics? (6) Transduction of pressure signals and chemical signals in the control of aquaporins (roots and leaves) and downstream impact on stomatal control.

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