Roles of Aquaporins in Stomata

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Abstract Stomata can be regarded as tightly regulated hydraulically driven valves that control the fluxes of water vapor and carbon dioxide between the plant and the atmosphere. In this chapter, we will focus on the mechanisms and regulation of the movement of fully developed stomata, which requires rapid and controlled fluxes of ions and water. Guard cells are symplastically isolated from their neighboring cells, implying that the regulation of transmembrane water movement is central to the control of their aperture/closure mechanism. Such hydraulic regulation of stomatal movement can be modulated by the activity of aquaporins, acting as water and small uncharged solute facilitators. Despite the existence of a wide range of transcriptomic and proteomic data showing that multiple plasma membrane aquaporins are expressed in these structures, there is currently only a limited number of experimental data supporting a functional involvement of these water channels in stomatal movements. The present review will highlight the main reverse genetics data linking the modulation of aquaporin activity to the control of the aperture of stomata.

1 Introduction

Stomata are microscopic pores in the epidermis of the aerial parts of virtually all extant land plants. They are bordered by two specialized cells, known as guard cells, which control the aperture of the pore (called an ostiole) following endogenous and environmental signals. Understanding the mechanistic aspects of stomatal movements has raised the interest of plant biologists for as early as the eighteenth century. First statements of observations of variable apertures of the *breathing holes* can indeed be traced back to the German botanist Johannes Hedwig at the end of the eighteenth century (Hedwig 1793). In 1812, Moldenhauer noticed three major signals affecting stomatal movement: light, humidity, and time of the day (Moldenhauer 1812). A few decades later, von Mohl put forward the hypothesis that guard cells open the stomatal

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pore thanks to an increase in their turgor pressure and close it by collapsing down (Von Mohl 1856). The concept of turgor-dependent aperture mechanism is therefore nearly as old as the discovery of stomata themselves. However, the molecular mechanisms responsible for such turgor regulation were still totally ignored.

The major role of stomata is to act as a physical checking point for gas exchange, balancing the undesirable loss of water vapor via evapotranspiration with the essential CO_2 uptake, indispensable for photosynthesis. These structures are therefore key players affecting plant development and biomass production. In this respect, the impact of stomata at the global scale is considerable on both plant-mediated water and carbon fluxes. Indeed, the closely coupled controls of stomata on CO_2 and water vapor diffusion processes impact the global distribution of these compounds in the atmosphere (Hetherington and Woodward 2003). Plants optimize CO_2/H_2O fluxes to suit prevailing environmental conditions by (i) regulating the number of developing stomata in the epidermis and (ii) fine-tuning the stomatal movement. The process and genes underlying stomatal development have been intensively investigated for the last decade (reviewed in Dong and Bergmann 2010; Bergmann and Sack 2007; Casson and Hetherington 2010; Dow et al. 2014). Stomatal opening occurs via the activation of plasma membrane and tonoplast transporters, resulting in solute accumulation in guard cells and consequently to water movement into the cells, which increases their turgor. A swelling guard cell changes shape due to the physical properties of its cell walls. This process leads to the opening of the pore. In contrast, an osmotically driven turgor decrease restores the initial closed state. Stomatal aperture and closure are affected by both endogenous and exogenous cues like perceived light intensity, leaf CO_2 concentration, vapor pressure deficit, temperature, and water status of the plant (Kim et al. 2010; Daszkowska-Golec and Szarejko 2013; Andrès et al. 2014). Under constant environmental conditions, the circadian clock was shown to regulate stomatal movements and to influence the sensitivity of the guard cells to extracellular signals (for review, see Webb 2003).

In this chapter, we will focus on the mechanisms regulating the movement of fully developed stomatal complexes that are central players in plant water relations and carbon assimilation. Experimental data highlighting the role of aquaporins in mediating this process will be pinpointed.

2 The Stomatal Complex: Morphological Characteristics

Two major morphological types of stomata exist. Stomatal complexes of grasses exhibit typical dumbbell-shaped guard cells surrounded by two subsidiary cells (Fig. 1a), while kidney-shaped guard cells are found in other species (Fig. 1b). The thin and linear dumbbell-shaped stomata of grasses, with or without subsidiary cells, are considered to be more evolutionary advanced compared to their kidney-shaped relatives (Hetherington and Woodward 2003). Their design translates smaller changes in guard cell volume to larger apertures with probably little energy waste (Raschke 1979; Hetherington and Woodward 2003). The efficient and fast

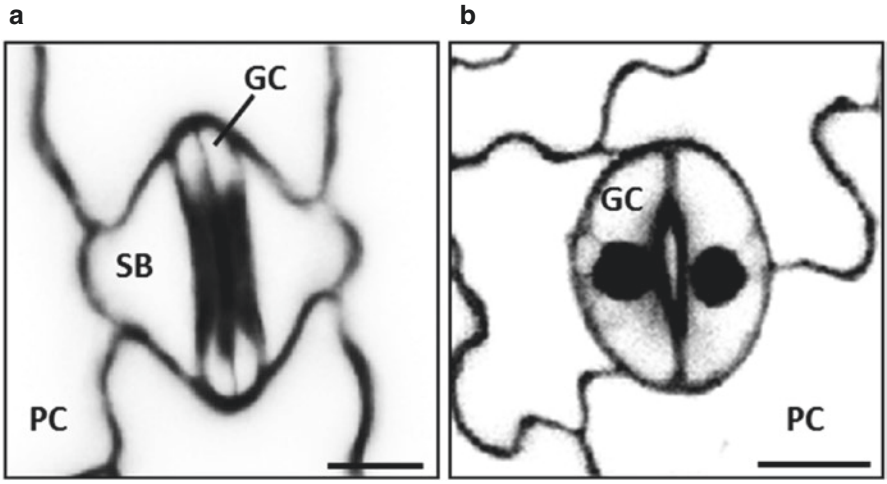

Fig. 1 Morphology of stomata in model mono- or dicotyledonous species as revealed by propidium iodide (*PI*) staining of leaf epidermal cells. (**a**) *Zea mays* stomata complex composed of two dumbbell-shaped guard cells (*GC*) flanked by two subsidiary cells (*SB*). (**b**) *Arabidopsis thaliana* stomata composed of two kidney-shaped guard cells (*GC*). Note that nuclei were stained in GC due to intense PI labeling. In both mono- and dicotyledonous species, stomata are separated from each other in the leaf epidermis by at least one pavement cell (*PC*). Scale bars: 10 μm

stomatal response to fluctuating environmental conditions in grasses is believed to enhance their water use efficiency compared to non-grass species (Franks and Farquhar 2007; Grantz and Assmann 1991; Hetherington and Woodward 2003).

3 The Opening/Closure Mechanism

3.1 The Basic Mechanism

Like most plant movements, stomatal opening and closure mechanisms are based on hydraulic forces (Roelfsema and Hedrich 2005), and such gating mechanism shows a complete reversibility. During stomatal opening, the activation of plasma membrane and tonoplast transporters results in solute accumulation in the guard cell, building up an osmotic gradient. To reestablish the perturbed osmotic equilibrium, water follows the solutes into the guard cells which increases their turgor pressure. Since mature guard cells lack plasmodesmatal connections with their neighboring cells (Erwee et al. 1985; Willmer and Sexton 1979), signaling molecules (including H_2O_2 that permeates aquaporins), ions (K^+, Cl^-), and water most likely reach the guard cell via the surrounding apoplast. As fine-tuned transmembrane water movement is a requisite of such aperture mechanism, water movement might be regulated by channels present in the guard cell membranes (see below).

3.2 Guard Cell Hydromechanics: Water Potential, Turgor Pressure, Cell Wall, Cytoskeleton, and the Mechanical Advantage

During stomatal opening, solute accumulation (n) increases the prevailing osmotic pressure (π) in the guard cells, which is defined by the equation $\pi = nRT/V$, where R, T, and V are the gas constant, the absolute temperature, and the cell volume, respectively. As a consequence, the guard cell water potential (Ψ) decreases as described by the equation $\Psi = P - \pi$ with P being the turgor pressure (reviewed in Roelfsema and Hedrich 2005; Buckley 2005). Water enters the guard cells to re-equilibrate the water potential difference $\Delta\Psi$ with the apoplast. The water inflow causes a rise in guard cell turgor pressure, which induces their swelling, limited by the cell wall elasticity and the increasing backpressure of the adjacent cells. The volume change has been shown to be as much as 40–50 % and goes hand in hand with a cell surface increase (Franks et al. 2001; Raschke and Dickerson 1973; Shope et al. 2003). To accommodate such large changes in surface area, the plasma membrane, whose elasticity is limited to 3–5 % (Wolfe and Steponkus 1983; Morris and Homann 2001), is internalized and remobilized as the cells shrink and swell (Shope et al. 2003; Shope and Mott 2006).

Stomatal aperture (a^-_s) is positively related to the turgor pressure of guard cells (P_g), but negatively related to the pressure of adjacent subsidiary or epidermal cells (P_e) (reviewed in Buckley 2005; Roelfsema and Hedrich 2005). Raschke noticed the collapsing of subsidiary cells during transient pore opening in *Zea mays* leaves, when dipping the leaf in a mannitol solution (Raschke 1970). The decreasing water potential released the backpressure on the guard cells exerted by the subsidiary cells and facilitated stomatal opening.

3.3 Aquaporins Are Involved in the Control of Water Flows During Stomatal Movements

The regulation of stomata aperture is fast (guard cells can adjust their volume by up to 40 % in a few tens of minutes, Franks et al. 2001) and totally reversible. It relies on the controlled shrinking/swelling of guard cells as a consequence of osmotically driven water movement. Such variations in cell volume depend, among other things, on finely tuned efflux/influx of water into guard cells based on a facilitated diffusion mechanism. Given the fact that guard cells are symplastically isolated from their neighboring cells, water movement in or out of the guard cells can only occur via the transmembrane path. By ensuring a higher water permeability to biological membranes, it is tempting to speculate that aquaporins may play a role in rapid changes of turgor/osmotic potentials implicated in stomatal movements. In addition to facilitating water diffusion across the plasma membrane and tonoplast, several plant aquaporins have been involved in the diffusion of small uncharged solutes,

including H_2O_2, or CO_2, solutes playing an important role in stomatal regulation (for reviews, see Bienert and Chaumont 2011; Heinen et al. 2009a; Chaumont and Tyerman 2014). This includes aquaporins belonging to the plasma membrane intrinsic proteins (PIPs).

4 Regulation of Stomatal Movement

Guard cells respond within a few minutes to a broad range of signals so that the plant can rapidly adapt to changing environmental conditions or react to threatening stresses (Franks et al. 2001). Multiple endogenous and external factors such as the circadian rhythm, light, CO_2 concentration, stress hormones and secondary messengers (ABA, Ca^{2+}, H_2O_2), drought, and pathogens attacks affect and/or regulate stomatal movement that involves controlled ion and water effluxes. The reader should however keep in mind that the stomatal response is a result of complex cross talks and interactions between different signaling pathways. The detailed mechanisms of stomatal regulation are beyond the scope of this review, but we chose to briefly emphasize the effects of the circadian clock, CO_2 concentration, and abscisic acid (ABA) signaling on stomatal movements.

4.1 Circadian Regulation

Under constant conditions, the circadian clock regulates stomatal movements and increases guard cell sensitivity to endogenous or environmental stimuli (reviewed in Webb 2003). In general, well-watered C_3 and C_4 plants open their stomata during the day and close them at night following the circadian clock. The movements anticipate light–dark transitions and persist under continuous light or darkness. It is worth noting that the expression pattern of plant PIP aquaporins follows the same diurnal pattern, which could help to finely tune the cell membrane water permeability in stomata (Heinen et al. 2014).

4.2 CO_2 Signaling

In the short term, stomata close in response to elevated CO_2 concentrations. Such closure probably involves, among other things, a transient modification of the cell water permeability of guard cells allowing a fast efflux from the cell. The kinase protein HIGH LEAF TEMPERATURE1 (HT1) is the first identified molecular regulator in the CO_2 pathway that negatively regulates CO_2-induced stomatal closing (Hashimoto et al. 2006) through phosphorylation and inhibition of the OST1 protein kinase (Tian et al. 2015), which is required for further CO_2 signal transduction

in plants (Merilo et al. 2013; Xue et al. 2011). RHC1, a MATE-type transporter protein, acts as a bicarbonate sensor (Tian et al. 2015) and inhibits HT1 thereby preventing the inhibition of OST1 at elevated CO_2 levels.

4.3 ABA Signaling

ABA is a key hormone regulating plant water status and stomatal movement. The cellular and molecular mechanisms underlying ABA-induced stomatal closure have been extensively studied (Popko et al. 2010; Wilkinson and Davies 1997; Assmann 2003; Hubbard et al. 2010; Lim et al. 2015). Many secondary messengers intervene in the ABA signaling network, such as G proteins, cytosolic pH, intracellular Ca^{2+}, nitric oxide (NO), reactive oxygen species (ROS), and lipid-derived signaling intermediates (phosphoinositides). For an extensive review on the role of ABA in stomatal response to (a)biotic stress, the reader can usefully refer to Lim et al. 2015.

Under stress conditions, the ABA concentration in leaves increases rapidly (Cutler et al. 2010; Hubbard et al. 2010). ABA functions there as a chemical messenger inducing stomatal closure through (in)activation of water and ion channels by protein kinases and phosphatases (Cutler et al. 2010; Hubbard et al. 2010; Gowing et al. 1993; Pei et al. 2000; Schroeder and Hagiwara 1989; Grondin et al. 2015; Lee et al. 2009). The common belief that abscisic acid (ABA) is a xylem-transported hormone that is synthesized in the roots, while acting in the shoot to close stomata in response to a decrease in plant water status, has been challenged by several studies showing that foliage-derived ABA is the predominant source of ABA during drought stress (Christmann et al. 2005, 2007; McAdam et al. 2016). A model in which the leaf senses the root dehydration by the drop in hydraulic pressure and consequently synthesizes ABA on-site was proposed (Christmann et al. 2005; Christmann et al. 2007). ABA might also be involved in the stomatal response to changes in relative air humidity (Hartung et al. 1988). After a drop in air humidity, stomata initially open shortly as a consequence of a general turgor loss and a decreased mechanical advantage of the epidermal pavement cells. This short-term (5–15 min) response, also called the wrong-way response, is followed by stomatal closure under prolonged low air humidity to avoid excessive water loss (reviewed in (Buckley 2005). Altogether, ABA accelerates the plasma membrane depolarization and induces a massive ion and water efflux leading to stomatal closure. Plant mutants affected in ABA synthesis and transduction pathway do not close their stomata in response to drought stress and show a wilty phenotype, which could be rescued by application of exogenous ABA (Xie et al. 2006) (reviewed in Belin et al. 2010).

ABA-triggered stomatal closure was recently shown to require an increase in guard cell permeability to water (Grondin et al. 2015). As will be discussed below, this could occur through phosphorylation-mediated activation of PIP aquaporins in the plasma membrane via a specific kinase protein. Among other phosphorylation events, OST1-dependent phosphorylation of PIP2;1 at Ser-121 indeed activates that

water channel. By activating the water channels via such specific phosphorylation events, this model postulates that this posttranslational modification would facilitate greater water efflux from guard cells in response to ABA signaling (Grondin et al. 2015). This could occur via a conformational change impacting the gating of the pore or via an impact on the trafficking or stability of the protein (see chapters "Plant Aquaporin Trafficking" and "Plant Aquaporin Posttranslational Regulation"). Finally, this could also affect the ability of the PIP2 to interact with other PIP isoforms and thereby regulates its activity (see chapter "Heteromerization of Plant Aquaporins").

5 Many Aquaporins Are Expressed in Stomata

Many studies, including guard cell-specific transcriptomic and proteomic approaches, have shown that multiple PIP aquaporins were expressed in guard cells of various plant species (Kaldenhoff et al. 1995; Sarda et al. 1997; Sun et al. 2001; Huang et al. 2002; Leonhardt et al. 2004; Cui et al. 2008; Fraysse et al. 2005; Wei et al. 2007; Hachez et al. 2008; Uehlein et al. 2003, 2008; Flexas et al. 2006; Heinen et al. 2014).

One of the earliest evidence of aquaporin expression in stomata was reported by Kaldenhoff and co-workers, using both GUS fusion and immunodetection to show that AtPIP1;2 was expressed in guard cells of *Arabidopsis thaliana*, although not restricted to this particular cell type (Kaldenhoff et al. 1995). Sarda et al. (1997) reported the mRNA accumulation of two tonoplast aquaporin genes, *SunTIP7* and *SunTIP20,* in sunflower guard cells. Whereas *SunTIP20* expression did not seem related to stomatal movements, the authors observed a *SunTIP7* mRNA accumulation increase at the end of the day suggesting a role in the process of stomatal closure by helping water to exit the vacuole of guard cells. Another *TIP* aquaporin expression was detected by *in situ* hybridization in guard cells of seedlings and mature organs of *Picea abies* (Oliviusson et al. 2001). The expression of the plasma membrane aquaporin SoPIP1;1 was detected in the guard cells of spinach using immunogold labeling (Fraysse et al. 2005). This isoform was also found in phloem and mesophyll cells but showed an interesting localization encircling the guard cells. On the other hand, this study further showed that a PIP isoform could be specifically not expressed in guard cells, like SoPIP1;2. A similar case was observed for AtPIP2;7 whose expression was also specifically null in stomata (Hachez et al. 2014). The expression in guard cells has also been reported for broad bean (*Vicia faba*) VfPIP1 (Cui et al. 2008).

For cereals, HvPIP1;6 was found to be expressed in barley guard cells using *in situ* PCR (Wei et al. 2007). In maize, Hachez et al. (2008) described the expression of ZmPIP1;2 and ZmPIP2;1/2;2 in stomatal complexes using immunocytochemistry. A transcriptomic study of *PIP* gene expression in maize laser-microdissected stomatal complexes (guard cells and subsidiary cells) showed that almost every PIP was expressed, except *ZmPIP2;7* (Heinen et al. 2014). However, the expression of seven of them accounts for more than 98 % of the total *PIP* transcripts. This study was conducted on whole leaf tissue, on peeled epidermis, and on isolated stomata

during night and day, thus allowing to detect isoforms specifically expressed in stomatal complexes. For instance, *ZmPIP1;1* was strongly expressed in the leaf mature zone but less in stomata. Most *PIP* genes followed the same expression pattern, with a basal level at night in both whole leaf tissue and isolated stomata. Interestingly, *ZmPIP1;6* was the only isoform found to be specifically expressed in stomata, only during the day. Along with ZmPIP1;5, ZmPIP1;6 was characterized as a water channel when co-expressed with a PIP2 in *Xenopus* oocytes and also as a CO_2 diffusion facilitator across membranes when expressed alone in yeast cells (Heinen et al. 2014). These two isoforms could either play a role in water fluxes in guard cells or help CO_2 assimilation through stomata as suggested for NtAQP1 (Uehlein et al. 2003, 2008; Flexas et al. 2006). They could also be key players in CO_2 sensing in interaction with carbonic anhydrases, as recently demonstrated for the CO_2-permeable aquaporin AtPIP2;1 (see below) (Wang et al. 2016).

6 Reverse Genetics as a Tool to Probe Aquaporin Role in Stomatal Gating Mechanisms

While expression data may give some hints about the importance of aquaporins in stomata regulation, there is only limited evidence using non-transgenic approaches of the functional involvement of aquaporins in stomatal movement, reverse genetics being the best way to prove such a role. When the expression of a single aquaporin isoform is deregulated, either by overexpression, silencing, or knockout, plants usually use compensation mechanisms to adapt their physiology. Such adaptation may involve an altered density of stomata formed in the leaf epidermis (Aharon et al. 2003; Ding et al. 2004; Li et al. 2015) or altered regulation and/or physical properties of the stomatal apparatus (Bi et al. 2015; Heinen et al. 2009; Cui et al. 2008).

Contrasting examples from the literature, mostly about PIP aquaporins, will be discussed thereafter, highlighting the key findings linking the modulation of aquaporin expression through reverse genetics approaches to stomatal density, morphology, and gating mechanism (summarized in Table 1). For instance, tobacco plants overexpressing the *Arabidopsis AtPIP1;2* grew better than the wild-type (WT) plants under favorable conditions (Aharon et al. 2003). A higher transpiration was observed due to a higher stomatal density at both sides of the leaves, indicating that plants took advantage of the heterologous aquaporin expression to use more water, but had to adapt morphologically. This modification of stomatal density did not confer any advantage under drought stress as transgenic plants wilted faster than WT (Aharon et al. 2003). A positive correlation between stomatal conductance (g_s) and *NtAQP1* expression was demonstrated in tobacco using both overexpressing and silenced lines, although no morphological changes were observed (Uehlein et al. 2003, 2008; Flexas et al. 2006). Mesophyll conductance was enhanced by overexpressing the ice plant aquaporin McMIPB in tobacco, leading to a higher photosynthetic rate under well-watered conditions, but g_s did not differ between

Table 1 Effects of deregulation of plant aquaporin expression

Author	Species	Aquaporin subfamily	Transgene	Type of deregulation	Effect
Martre et al. (2002)	*Arabidopsis thaliana*	PIP1 and PIP2	RNAi against PIP1 and PIP2 (antisense)	Silencing	Lower leaf water potential after re-watering, but no difference in stomatal conductance
Cui et al. (2008)	*Arabidopsis thaliana*	PIP1	*VfPIP1* (heterologous)	Overexpression	Decreased transpiration, stomata close faster after dark or ABA treatment
Sade et al. (2014)	*Arabidopsis thaliana*	PIP1	*NtAQP1* (heterologous)	Overexpression	Increased stomatal conductance, mesophyll conductance, and photosynthesis
				Expression restricted to photosynthetic tissue	Increased stomatal conductance, mesophyll conductance, and photosynthesis
				Expression restricted to stomata	Stomatal conductance, mesophyll conductance, and photosynthesis not different from WT
Sade et al. (2014)	*Arabidopsis thaliana*	PIP1 and PIP2	RNAi against PIP1 (amiRNA)	Silencing	Decreased stomatal conductance, mesophyll conductance and photosynthesis for the most affected line
Li et al. (2015)	*Arabidopsis thaliana*	PIP2	*AcPIP2* (heterologous)	Overexpression	Decreased stomata density
Grondin et al. (2015)	*Arabidopsis thaliana*	PIP2	*AtPIP2;1* (homologous)	Knockout	Stomatal closure and guard cell protoplast permeability lack of response to ABA

(continued)

Table 1 (continued)

Author	Species	Aquaporin subfamily	Transgene	Type of deregulation	Effect
Wang et al. (2016)	*Arabidopsis thaliana*	PIP2	*AtPIP2;1* (homologous)	Knockout	No effect on stomatal closure in response to ABA
Aharon et al. (2003)	*Nicotiana tabacum*	PIP1	*AtPIP1;2* (heterologous)	Overexpression	Increased transpiration and photosynthetic efficiency, higher stomatal density
Uehlein et al. (2003)	*Nicotiana tabacum*	PIP1	*NtAQP1* (homologous)	Overexpression	Increased stomatal conductance and net photosynthesis, especially at high CO_2 concentration
			RNAi against *NtAQP1*	Silencing	Decreased stomatal conductance and net photosynthesis, effect disappear at high CO_2 concentration
Ding et al. (2004)	*Nicotiana tabacum*	PIP1	*AqpL1* (heterologous)	Overexpression	Greater stomatal density in young tissues, stomata more open at light
Flexas et al. (2006)	*Nicotiana tabacum*	PIP1	*NtAQP1* (homologous)	Overexpression	Increased stomatal conductance, mesophyll conductance, and photosynthesis
				Silencing	Decreased stomatal conductance, mesophyll conductance, and photosynthesis
Kawase et al. (2013)	*Nicotiana tabacum*	PIP1	*McMIPB* (heterologous)	Overexpression	Increased photosynthesis rate and mesophyll conductance, no change in stomatal conductance

Table 1 (continued)

Author	Species	Aquaporin subfamily	Transgene	Type of deregulation	Effect
Secchi and Zwieniecki (2013)	*Populus tremula x alba*	PIP1	RNAi against PIP1 (antisense)	Silencing	Decreased mesophyll conductance
Bi et al. (2015)	*Populus* × *canescens*	PIP1 and PIP2	RNAi against PIP1 or PIP2 (antisense)	Silencing	Increased stomatal conductance and photosynthesis, increased stomata size
Hanba et al. (2004)	*Oryza sativa*	PIP2	*HvPIP2;1* (heterologous)	Overexpression	Increased stomatal and mesophyll conductance, decreased stomata size and density
Perrone et al. (2012)	*Vitis vinifera*	PIP2	VvPIP2;4 (homologous)	Overexpression	Increased mesophyll conductance

transgenic and WT plants (Kawase et al. 2013). Interestingly, immunolocalization of the McMIPB protein in WT plants revealed its absence in stomata, contrary to overexpressing plants where McMIPB presented a strong signal in chloroplasts in both mesophyll and guard cells. Ding et al. (2004) reported a higher stomatal density in younger leaves of tobacco plants overexpressing a *Lilium PIP1* (*AqpL1*) and that stomata opened slightly faster with light than WT by studying the stomatal aperture on collodion printings of leaf surface. It was also proved that stomata of transgenic *Arabidopsis* isolated epidermis expressing the broad bean *VfPIP1* closed significantly faster than those of control plants when subjected to ABA or dark treatment (Cui et al. 2008). On the other hand, density and size of stomatal apparatus in rice plants overexpressing the barley *HvPIP2;1* were reduced while g_s, CO_2 internal conductance and CO_2 assimilation were higher than in WT plants (Hanba et al. 2004). This could be explained by the fact that HvPIP2;1 was characterized as a CO_2 transporter (Mori et al. 2014). Stomatal density was also reduced in *Arabidopsis* plants overexpressing *AcPIP2* from *Atriplex canescens* while the growth rate was enhanced (Li et al. 2015). The overexpression of *VvPIP2;4* in grapevine induced an increase in g_s under normal conditions (Perrone et al. 2012). As VvPIP2;4 is not present in guard cells in WT plants, the authors proposed a direct effect of this isoform in guard cell turgor pressure. Constitutive overexpression of aquaporins can result in a wide range of phenotypes, depending on the selected isoform and promoter strength. It shows that modulating aquaporin expression clearly impacts the plant physiology, even though endogenous aquaporin expression pattern might also change upon heterologous expression of specific isoform and should be carefully investigated, but does not provide clear evidence of direct implication in one

physiological process. For that matter, silencing approaches or milder tissue-specific expression were also used to study aquaporins' implication in stomata-related traits.

Silencing both *PIP1* and *PIP2* aquaporin genes in poplar lines resulted in wider and longer guard cells compared to WT and in a higher proportion of open stomata per leaf area (Bi et al. 2015). Furthermore, in contrast with the usually positive correlation between aquaporin expression and g_s, trees with reduced *PIP* expression had a greater g_s and transpiration rate compared to WT, but had growth defects despite showing a better CO_2 net assimilation. Using a proteomic approach, the authors discovered that *PIP* down-expression induced an upregulation of proteins involved in synthesis and signaling of ABA, trafficking or in cell wall synthesis, indicating a drought stress response that explains the reduced growth. This is however not in accordance with results found by Secchi and Zwieniecki (2013) also using poplar *PIP1* RNAi lines in which no morphological or g_s differences were observed, although mesophyll conductance to CO_2 was greatly reduced. Sade et al. (2014) used a very interesting tissue-specific approach to determine the contribution of *NtAQP1* heterologous expression on several gas-exchange parameters in *Arabidopsis* plants. The authors observed an increase in g_s in normal conditions when targeting the expression of *NtAQP1* to photosynthetic tissues, but surprisingly not in the plants in which the expression was restricted to stomata, that behave like WT in both normal and salt stress conditions. Moreover, no difference in photosynthetic rate or g_s were observed between constitutive and mesophyll-specific expression of *NtAQP1* while the expression was much less important in the latter case but still sufficient to induce a similar increase in mesophyll CO_2 conductance (Sade et al. 2014). According to this study, *NtAQP1* overexpression in photosynthetic tissues had an indirect role on stomata opening by raising mesophyll conductance and photosynthetic rate without the need for higher expression of aquaporins in the guard cells. The use of artificial micro-RNAs to reduce *PIP1* gene expression in *Arabidopsis* led to the generation of lines deregulated in *PIP1* but also *PIP2* expression (Sade et al. 2014). Both lines showed a decrease in g_s, whereas only the most affected line in *PIP1* expression exhibited a significant decrease of mesophyll conductance compared to WT. This was not the case for *Arabidopsis* plants silenced for both *PIP1* and *PIP2* genes which showed no significant difference in g_s in normal conditions, during soil drying or after soil re-watering (Martre et al. 2002).

The first functional evidence of direct implication of aquaporins in stomatal movements was provided by a recent study using *Arabidopsis pip2;1* knockout plants (Grondin et al. 2015). The authors showed that the stomata in isolated epidermal peals of knockout plants responded correctly to light but showed a reduced response to ABA, which should have induced a rapid stomatal closure, as seen for the WT plants. The permeability of guard cell protoplasts was measured and showed that ABA triggered a twofold increase in P_f for WT plants, which was abrogated in the *pip2;1* mutants. The authors concluded that AtPIP2;1, was necessary for ABA-dependent closure and dispensable for CO_2- or light-induced stomatal movements. Based on these observations, a model whereby the stomatal closing response to ABA involves an increase in guard cell water permeability mediated by AtPIP2;1 was proposed (Grondin et al. 2015). However, the authors also highlighted the

putative role of AtPIP2;1 in ROS signaling in response to ABA as this isoform is able to facilitate H_2O_2 diffusion across membrane, which is one important messenger in ABA signaling (Dynowski et al. 2008; Bienert et al. 2014).

These results were however contradicted by a recent study (Wang et al. 2016). In this study, genotype-blind stomatal movement imaging analyses of individually mapped stomata of leaf epidermal layers showed that stomata from *pip2;1* single mutant lines (including the mutant line used in the Grondin's study) retained intact responses to ABA and closed to similar levels as the wild type 1 h after ABA treatment. These authors reported that *pip2;1* mutation alone was insufficient to impair the ABA-induced stomatal closing pathway. Such contradictory results could be explained by different experimental growing conditions or measurements methodology (Wang et al. 2016) but raise question regarding the pivotal role of AtPIP2;1 alone in mediating ABA-induced stomatal closing.

A direct interaction between AtPIP2;1 and the carbonic anhydrase βCA4, implicated in stomatal movements in response to CO_2 changes was also described (Wang et al. 2016). This interaction allowed a greater rise in CO_2 permeability of *Xenopus* oocytes compared to each interactor injected separately, proving that AtPIP2;1 is a functional CO_2 channel and could act synergistically with other proteins to increase the CO_2 permeability in plant tissues.

7 Concluding Remarks: Cell-Type-Specific Transgenic Approaches Are Needed to Investigate the Role of Specific Aquaporin Genes in Stomata

Modulating endogenous aquaporin expression levels most often leads to similar observations, although similar phenotypes can have different origins. This is especially true when working with plants as different as *A. thaliana*, *O. sativa*, or *P. trichocarpa* and with aquaporin isoforms facilitating the passage of a wide range of small uncharged molecules. Aquaporins can play a role as water channels or as CO_2 or H_2O_2 facilitators, making the interpretation of physiological observations tricky. As water, H_2O_2, and CO_2 fluxes are strongly impacting leaf physiology, it is difficult to distinguish between an impact on photosynthesis efficiency and an impact on transpiration.

Recent advances in Crispr-CAS9 technology offer the possibility to knock out specific aquaporin genes in virtually any plant species via genome editing and could be an interesting way to probe the role of specific aquaporin isoforms in plant physiology. It is however worth noting that using T-DNA or genome editing approaches will affect gene expression in the whole plant, thereby complicating the interpretation of their role in specific processes. Given the various phenotypes observed when investigating the role of specific aquaporin isoforms in stomata physiology, cell-specific approaches might be required. In the future, it would be interesting to specifically silence/overexpress PIP aquaporins in mature guard cells and to measure the impact of such silencing/overexpression on stomatal gating mechanisms. This

will require the identification of guard cell (and/or subsidiary cell)-specific promoters. Such promoters are already available for some plants such as maize, rice, or *Arabidopsis* (Liu et al. 2009; Yang et al. 2008).

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References

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15:439–447

Andrés Z, Pérez-Hormaeche J, Leidi EO, Schlücking K, Steinhorst L, McLachlan DH, Schumacher K, Hetherington AM, Kudla J, Cubero B, Pardo JM (2014) Control of vacuolar dynamics and regulation of stomatal aperture by tonoplast potassium uptake. Proc Natl Acad Sci 111:1806–1814

Assmann SM (2003) OPEN STOMATA1 opens the door to ABA signaling in Arabidopsis guard cells. Trends Plant Sci 8:151–153

Belin C, Thomine S, Schroeder JI (2010) Water balance and the regulation of stomatal movements. In: Pareek A, Sopory SK, Bohnert HJ, Govindjee (eds) Abiotic stress adaptation in plants. Springer, The Netherlands, pp 283–305

Bergmann DC, Sack FD (2007) Stomatal development. Annu Rev Plant Biol 58:163–181

Bi Z, Merl-Pham J, Uehlein N, Zimmer I, Mühlhans S, Aichler M, Walch AK, Kaldenhoff R, Palme K, Schnitzler JP, Block K (2015) RNAi-mediated downregulation of poplar plasma membrane intrinsic proteins (PIPs) changes plasma membrane proteome composition and affects leaf physiology. J Proteomics 128:321–332

Bienert GP, Chaumont F (2011) Plant aquaporins: roles in water homeostasis, nutrition, and signaling processes. In: Geisler M, Venema K (eds) Transporters and pumps in plant signaling, Signaling and Communication in Plants, vol 7. Springer, Berlin/Heidelberg, pp 3–36

Bienert GP, Heinen RB, Berny MC, Chaumont F (2014) Maize plasma membrane aquaporin ZmPIP2;5, but not ZmPIP1;2, facilitates transmembrane diffusion of hydrogen peroxide. Biochim Biophys Acta 1838:216–222

Buckley TN (2005) The control of stomata by water balance. New Phytol 168(2):275–292

Casson SA, Hetherington AM (2010) Environmental regulation of stomatal development. Curr Opin Plant Biol 13:90–95

Chaumont F, Tyerman SD (2014) Aquaporins: highly regulated channels controlling plant water relations. Plant Physiol 164:1600–1618

Christmann A, Hoffmann T, Teplova I, Grill E, Müller A (2005) Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed arabidopsis. Plant Physiol 137:209–219

Christmann A, Weiler EW, Steudle E, Grill E (2007) A hydraulic signal in root-to-shoot signalling of water shortage. Plant J 52:167–174

Cui XH, Hao FS, Chen H, Chen J, Wang XC (2008) Expression of the *Vicia faba* VfPIP1 gene in Arabidopsis thaliana plants improves their drought resistance. J Plant Res 121:207–214

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol 61:651–679

Daszkowska-Golec A, Szarejko I (2013) Open or close the gate – stomata action under the control of phytohormones in drought stress conditions. Front Plant Sci 4:138

Ding X, Iwasaki I, Kitagawa Y (2004) Overexpression of a lily PIP1 gene in tobacco increased the osmotic water permeability of leaf cells. Plant Cell Environ 27:177–186

Dong J, Bergmann DC (2010) Stomatal patterning and development. Curr Top Dev Biol 91:267–297
Dow GJ, Bergmann DC, Berry JA (2014) An integrated model of stomatal development and leaf physiology. New Phytol 201:1218–1226
Dynowski M, Schaaf G, Loque D, Moran O, Ludewig U (2008) Plant plasma membrane water channels conduct the signalling molecule H_2O_2. Biochem J 414:53–61
Erwee MG, Goodwin PB, Bel AJE (1985) Cell-cell communication in the leaves of *Commelina cyanea* and other plants. Plant Cell Environ 8:173–178
Flexas J, Ribas-Carbo M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R (2006) Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. Plant J 48:427–439
Franks PJ, Farquhar GD (2007) The mechanical diversity of stomata and its significance in gas-exchange control. Plant Physiol 143(1):78–87
Franks PJ, Buckley TN, Shope JC, Mott KA (2001) Guard cell volume and pressure measured concurrently by confocal microscopy and the cell pressure probe. Plant Physiol 125:1577–1584
Fraysse LC, Wells B, Cann MC M, Kjellbom P (2005) Specific plasma membrane aquaporins of the PIP1 subfamily are expressed in sieve elements and guard cells. Biol Cell 97:519–534
Gowing DJG, Davies WJ, Trejo CL, Jones HG (1993) Xylem-transported chemical signals and the regulation of plant growth and physiology. Phil Trans Biol Sci 341:41–47
Grantz DA, Assmann SM (1991) Stomatal response to blue-light – water-use efficiency in sugarcane and soybean. Plant Cell Environ 14:683–690
Grondin A, Rodrigues O, Verdoucq L, Merlot S, Leonhardt N, Maurel C (2015) Aquaporins contribute to ABA-triggered stomatal closure through OST1-mediated phosphorylation. Plant Cell 27:1945–1954
Hachez C, Heinen RB, Draye X, Chaumont F (2008) The expression pattern of plasma membrane aquaporins in maize leaf highlights their role in hydraulic regulation. Plant Mol Biol 6:337–353
Hachez C, Laloux T, Reinhardt H, Cavez D, Degand H, Grefen C, Rycke R, Inze D, Blatt MR, Russinova E, Chaumont F (2014) Arabidopsis SNAREs SYP61 and SYP121 coordinate the trafficking of plasma membrane aquaporin PIP2;7 to modulate the cell membrane water permeability. Plant Cell 26:3132–3147
Hanba YT, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K, Terashima I, Katsuhara M (2004) Overexpression of the barley aquaporin HvPIP2;1 increases internal CO2 conductance and CO2 assimillation in the leaves of transgenic rice plants. Plant Cell Physiol 45:521–529
Hartung W, Radin JW, Hendrix DL (1988) Abscisic acid movement into the apoplastic solution of water-stressed cotton leaves. Plant Physiol 83:908–913
Hashimoto M, Negi J, Young J, Israelsson M, Schroeder JI, Iba K (2006) Arabidopsis HT1 kinase controls stomatal movements in response to CO_2. Nat Cell Biol 8:391–397
Hedwig DJ (1793) D. Johann Hedwig's Sammlung seiner zerstreuten Abhandlungen und Beobachtungen über botanisch-ökonomische Gegenstände. Erstes Bändchen mit fünf illuminierten Kupfertafeln, Leipzig
Heinen RB, Ye Q, Chaumont F (2009) Role of aquaporins in leaf physiology. J Exp Bot 60:2971–2985
Heinen RB, Bienert GP, Cohen D, Chevalier AS, Uehlein N, Hachez C, Kaldenhoff R, Thiec D, Chaumont F (2014) Expression and characterization of plasma membrane aquaporins in stomatal complexes of *Zea mays*. Plant Mol Biol 86:335–350
Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. Nature 424:901–908
Huang RF, Zhu MJ, Kang Y, Chen J, Wang XC (2002) Identification of plasma membrane aquaporin in guard cells of *Vicia faba* and its role in stomatal movement. Acta Bot Sin 44:42–48
Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Genes Dev 24:1695–1708
Kaldenhoff R, Kolling A, Meyers J, Karmann U, Ruppel G, Richter G (1995) The blue light-responsive AthH2 gene of Arabidopsis thaliana is primarily expressed in expanding as well

as in differentiating cells and encodes a putative channel protein of the plasmalemma. Plant J 7:87–95

Kawase M, Hanba YT, Katsuhara M (2013) The photosynthetic response of tobacco plants overexpressing ice plant aquaporin McMIPB to a soil water deficit and high vapor pressure deficit. J Plant Res 126:517–527

Kim T-H, Bohmer M, H H, Nishimura N, Schroeder JI (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu Rev Plant Biol 61:561–591

Lee SC, Lan W, Buchanan BB, Luan S (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. Proc Natl Acad Sci U S A 106:21419–21424

Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI (2004) Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell 16:596–615

Li J, Yu G, Sun X, Liu Y, Liu J, Zhang X, Jia C, Pan H (2015) AcPIP2, a plasma membrane intrinsic protein from halophyte *Atriplex canescens*, enhances plant growth rate and abiotic stress tolerance when overexpressed in Arabidopsis thaliana. Plant Cell Rep 34:1401–1415

Lim CW, Baek W, Jung J, Kim JH, Lee SC (2015) Function of ABA in stomatal defense against biotic and drought stresses. Int J Mol Sci 16:15251–15270

Liu T, Ohashi-Ito K, Bergmann DC (2009) Orthologs of Arabidopsis thaliana stomatal bHLH genes and regulation of stomatal development in grasses. Development 136:2265–2276

Martre P, Morillon R, Barrieu F, North GB, Nobel PS, Chrispeels MJ (2002) Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol 130:2101–2110

McAdam SA, Manzi M, Ross JJ, Brodribb TJ, Gomez-Cadenas A (2016) Uprooting an abscisic acid paradigm: shoots are the primary source. Plant Signal Behav 11(6):e1169359

Merilo E, Laanemets K, Hu H, Xue S, Jakobson L, Tulva I, Gonzalez-Guzman M, Rodriguez PL, Schroeder JI, Brosche M, Kollist H (2013) PYR/RCAR receptors contribute to ozone-, reduced air humidity-, darkness-, and CO2-induced stomatal regulation. Plant Physiol 162:1652–1668

Mohl H (1856) Welche Ursachen bewirken die Erweiterung und Verengung der Spaltöffnungen? Bot Ztg 14:697–704

Moldenhauer JJP (1812) Beiträge zur Anatomie der Pflanzen. Königliche Schulbuchdruckerei, Kiel

Mori IC, Rhee J, Shibasaka M, Sasano S, Kaneko T, Horie T, Katsuhara M (2014) CO2 transport by PIP2 aquaporins of barley. Plant Cell Physiol 55:251–257

Morris CE, Homann U (2001) Cell surface area regulation and membrane tension. J Membr Biol 179:79–102

Oliviusson P, Salaj J, Hakman I (2001) Expression pattern of transcripts encoding water channel-like proteins in Norway spruce (*Picea abies*). Plant Mol Biol 46:289–299

Pei Z-M, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406:731–734

Perrone I, Gambino G, Chitarra W, Vitali M, Pagliarani C, Riccomagno N, Balestrini R, Kaldenhoff R, Uehlein N, Gribaudo I, Schubert A, Lovisolo C (2012) The grapevine root-specific aquaporin VvPIP2;4N controls root hydraulic conductance and leaf gas exchange under well-watered conditions but not under water stress. Plant Physiol 160:965–977

Popko J, Hansch R, Mendel RR, Polle A, Teichmann T (2010) The role of abscisic acid and auxin in the response of poplar to abiotic stress. Plant Biol (Stuttg) 12:242–258

Raschke K (1970) Stomatal response to pressure changes and interruptions in the water supply of detached leaves of *Zea mays* L. Plant Physiol 45:415–423

Raschke K (1979) Movements of stomata. In: Haupt W, Feinleib ME (eds) Encyclopedia of plant physiology, vol 7. Springer, Berlin, pp 383–441

Raschke K, Dickerson M (1973) Changes in shape and volume of guard cells during stomatal movement. J Plant Res 1972:149–153

Roelfsema MRG, Hedrich R (2005) In the light of stomatal opening: new insights into 'the Watergate'. New Phytol 167:665–691
Sade N, Shatil-Cohen A, Attia Z, Maurel C, Boursiac Y, Kelly G, Granot D, Yaaran A, Lerner S, Moshelion M (2014) The role of plasma membrane aquaporins in regulating the bundle sheath-mesophyll continuum and leaf hydraulics. Plant Physiol 166:1609–1620
Sarda X, Tousch D, Ferrare K, Legrand E, Dupuis JM, Casse-Delbart F, Lamaze T (1997) Two TIP-like genes encoding aquaporins are expressed in sunflower guard cells. Plant J 12:1103–1111
Schroeder JI, Hagiwara S (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. Nature 338:427–430
Secchi F, Zwieniecki MA (2013) The physiological response of Populus tremula x alba leaves to the down-regulation of pip1 aquaporin gene expression under no water stress. Front Plant Sci 4:507. doi:10.3389/fpls.2013.00507
Shope JC, Mott KA (2006) Membrane trafficking and osmotically induced volume changes in guard cells. J Exp Bot 57:4123–4131
Shope JC, DeWald DB, Mott KA (2003) Changes in surface area of intact guard cells are correlated with membrane internalization. Plant Physiol 133:1314–1321
Sun MH, Xu W, Zhu YF, Su WA, Tang ZC (2001) A simple method for in situ hybridization to RNA in guard cells of *Vicia faba* L.: the expression of aquaporins in guard cells. Plant Mol Biol Rep 19:129–135
Tian W, Hou C, Ren Z, Pan Y, Jia J, Zhang H, Bai F, Zhang P, Zhu H, He Y, Luo S, Li L, Luan S (2015) A molecular pathway for CO_2 response in Arabidopsis guard cells. Nat Commun 6:6057
Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R (2003) The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 425:734–737
Uehlein N, Otto B, Hanson DT, Fischer M, McDowell N, Kaldenhoff R (2008) Function of Nicotiana tabacum aquaporins as chloroplast gas pores challenges the concept of membrane CO_2 permeability. Plant Cell 20:648–657
Wang C, Hu H, Qin X, Zeise B, Xu D, Rappel WJ, Boron WF, Schroeder JI (2016) Reconstitution of CO_2 regulation of SLAC1 anion channel and function of CO_2-permeable PIP2;1 aquaporin as CARBONIC ANHYDRASE4 interactor. Plant Cell 28:568–582
Webb AAR (2003) The physiology of circadian rhythms in plants. New Phytol 160:281–303
Wei W, Alexandersson E, Golldack D, Miller AJ, Kjellbom PO, Fricke W (2007) HvPIP1;6, a barley (*Hordeum vulgare* L.) plasma membrane water channel particularly expressed in growing compared with non-growing leaf tissues. Plant Cell Physiol 48:1132–1147
Wilkinson S, Davies WJ (1997) Xylem sap pH increase: a drought signal received at the apoplastic face of the guard cell that involves the suppression of saturable abscisic acid uptake by the epidermal symplast. Plant Physiol 113:559–573
Willmer CM, Sexton R (1979) Stomata and plasmodesmata. Protoplasma 100:113–124
Wolfe J, Steponkus PL (1983) Mechanical properties of the plasma membrane of isolated plant protoplasts: mechanism of hyperosmotic and extracellular freezing injury. Plant Physiol 71:276–285
Xie X, Wang Y, Williamson L, Holroyd GH, Tagliavia C, Murchie E, Theobald J, Knight MR, Davies WJ, Leyser HMO, Hetherington AM (2006) The identification of genes involved in the stomatal response to reduced atmospheric relative humidity. Curr Biol 16:882–887
Xue S, Hu H, Ries A, Merilo E, Kollist H, Schroeder JI (2011) Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO2 signal transduction in guard cell. EMBO J 30:1645–1658
Yang Y, Costa A, Leonhardt N, Siegel RS, Schroeder JI (2008) Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. Plant Methods 4:6