Ion Transport at the Vacuole during Stomatal Movements

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Plant gas exchange with the environment is facilitated by stomata, small pores found on most aerial surfaces of land plants. Stomatal pores are formed between a pair of specialized guard cells. In C3 plants, open stomata allow the uptake of CO₂ for photosynthesis during the day and at the same time the loss of water vapor, maintaining the transpiration stream. At night and during drought, plants close their stomata to conserve water, while they open them in response to low CO₂ and at high temperatures to allow for evaporative cooling. The opening and closure of stomata depends on guard cell turgor, which, in turn, relies on fluxes of osmotically active solutes in and out of the guard cell. During stomatal opening, osmotically active solutes enter guard cells via the plasma membrane or are produced inside the cell and ultimately are stored in the vacuole (Roelfsema and Hedrich, 2005; Kollist et al., 2014; Santelia and Lawson, 2016). This causes water influx, a concomitant increase in turgor and swelling of the guard cell pair, resulting in the opening of the stomatal pore. K⁺ and its charge-balancing anions Cl⁻, NO₃⁻, and malate (Mal₂⁻), as well as sugars, are the osmotica accumulating in guard cell vacuoles for stomatal opening. The ions are transported across the vacuolar membrane (also, the tonoplast) by ion channels and secondary active transporters, while vacuolar proton pumps generate the necessary proton motive force and acidify the vacuolar lumen.

With the dawn of the patch-clamp technique, many ion channels were characterized in the 1980s and 1990s. A lot of these early studies were conducted with the broad bean *Vicia faba* and the monocotyledon dayflower plant *Commelina communis* because of the large size of their guard cells. It was only with the era of molecular genetics that the proteins responsible for the characterized transport were identified in Arabidopsis (*Arabidopsis thaliana*), and this review focuses almost exclusively on vacuolar transport proteins of this species with a role in stomatal physiology (Table I; Fig. 1).

Nevertheless, many discoveries about guard cell function were made using *V. faba*, *C. communis*, and other species but were carried over into Arabidopsis guard cell research and now shape our reasoning. For example, Mal₂⁻ is often listed as an important osmotica. This is true for some species such as *V. faba* (Outlaw and Lowry, 1977; Outlaw and Kennedy, 1978; Van Kirk and Raschke, 1978a, 1978b) and in certain growth conditions (Raschke and Schnabl, 1978; Van Kirk and Raschke, 1978a). But in Arabidopsis guard cells, on average, K⁺ is charge balanced to 50% by Cl⁻ and only to 5% by Mal₂⁻, with Mal₂⁻ concentrations in the range of 1 to 2 mM (Negi et al., 2008; Monda et al., 2011, 2016; Takahashi et al., 2015). Nevertheless,
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<td>AtALMT6</td>
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<td>Mal(^2+), Fum(^2+)</td>
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<td>AtCLCa</td>
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<td>Probably antiporter anion/H(^+)</td>
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<td>AtCAX1 and AtCAX3</td>
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<td>Ca(^2+)</td>
<td>(K_m^{Ca^{2+}} = 13.1 \mu\text{M} ; V_{\text{max}} = 12.4 \text{ nMol mg}^{-1} \text{ protein min}^{-1} (3))</td>
<td>Reduced stomatal opening in light (1, 2) and impaired auxin inhibition of ABA-induced closure (1)</td>
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<td>AtVHA</td>
<td>Vacuolar proton ATPase</td>
<td>H(^+)</td>
<td>H(^+)-ATP coupling ratio = 1.75–3.28 (1); (K_m^{\text{ATP}} = 0.6 \text{ mM} (2))</td>
<td>Reduced ABA-induced stomatal closure (3)</td>
<td>(1) Davies et al. (1994); (2) Hedrich et al. (1989); (3) Bak et al. (2013)</td>
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<td>AtAVP1</td>
<td>Vacuolar proton PP(_2)ase</td>
<td>H(^+)</td>
<td>H(^+)-PP(_2) coupling ratio = 1 (1); (K_m^{\text{PP}_2} = 15–20 \mu\text{M} (2))</td>
<td>Reduced ABA-induced stomatal closure (3)</td>
<td>(1) Schmidt and Briskin (1993); (2) Hedrich et al. (1989); (3) Bak et al. (2013)</td>
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<td>AtMRP5</td>
<td>ABCC transporter</td>
<td>IP(_6) (1)</td>
<td>(K_m^{\text{IP}<em>6} = 263–310 \text{ nM} ; V</em>{\text{max}} = 1.6–2.5 \text{ μMol min}^{-1} \text{ mg}^{-1} \text{ protein} (1))</td>
<td>Impaired stomatal opening and closure; reduced whole-plant transpiration and drought sensitivity (2)</td>
<td>(1) Nagy et al. (2009); (2) Klein et al. (2003)</td>
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Arabidopsis guard cell $\text{Mal}^{2-}$ concentration increases during stomatal opening 2- to 3-fold and generally correlates with stomatal aperture (Monda et al., 2011; Ding et al., 2014; Takahashi et al., 2015; Medeiros et al., 2016).

We have gained good understanding of the importance of individual transport processes at the vacuole. But, by contrast with events at the plasma membrane, the succession of events at the vacuole leading to stomatal opening and closure in response to environmental signals is hardly established. Stomata close in response to drought, which is signaled by the phytohormone abscisic acid (ABA). ABA triggers signaling cascades that regulate plasma membrane ion channels by phosphorylation, increases in cytosolic free Ca$^{2+}$ concentration, and other second messengers (Roelfsema et al., 2012; Munemasa et al., 2015; Jezek and Blatt, 2017). The changed activities of these ion channels result in large changes in the electric potential gradient across the plasma membrane (i.e. the plasma membrane potential), which depolarizes from about $-110$ to $-50$ mV during stomatal closure (Roelfsema and Hedrich, 2005; Roelfsema et al., 2012). By contrast, there are no in vivo measurements of the vacuolar membrane potential in guard cells and no information regarding how it changes during stomatal opening and closure.

Because our knowledge of the events at the plasma membrane is so much more advanced, these events are commonly thought of as initiating and changes in vacuolar membrane potential and vacuolar transport processes are thought of as reactionary, as it were. But there is no actual evidence to assume this is the case. In fact, it is more likely that fluxes at the vacuolar and plasma membranes are coordinated. For example, we know that kinases such as Ca$^{2+}$-dependent kinases (CDPKs) and the SNF1-related kinase2.6, AtOST1, regulate ion channels at the plasma membrane (Kollist et al., 2014; Munemasa et al., 2015; Jezek and Blatt, 2017), but it recently became clear that such kinases also have targets at the tonoplast (Latz et al., 2013; Wege et al., 2014). Furthermore, changes in ATP-dependent transport, at both the tonoplast and the plasma membrane, together seem to be required to generate intracellular Ca$^{2+}$ signals that initiate stomatal closure (Minguet-Parramon et al., 2016).

During stomatal closure, the guard cell volume decreases and, accordingly, so does the vacuolar volume, in Arabidopsis by about 20% (Gao et al., 2011).
VACUOLAR K+ TRANSPORT DURING STOMATAL MOVEMENT

Only recently, it was established that guard cell-expressed K+ and Na+ transporters of the NHX (Na+/H+ exchangers) family are essential for K+ accumulation in Arabidopsis guard cell vacuoles during stomatal opening (Barragán et al., 2012; Andrés et al., 2014). Over a decade ago, NHX-type transporters were shown to be responsible for vacuolar pH homeostasis in morning glory (Ipomoea nil or Pharbitis nil) petals, where the vacuolar pH determines flower color (Yamaguchi et al., 2001). In fact, a double mutant of AtNHX1 and AtNHX2 shows impaired vacuolar pH homeostasis and reduced K+ accumulation in Arabidopsis roots (Bassil et al., 2011; Barragán et al., 2012). This double mutant also displays impaired stomatal opening and reduced whole-plant transpiration in the light, and its vacuolar pH in guard cells was more acidic (by ~0.3 pH units) compared with a wild-type pH of about 5.8/5.9 (Andrés et al., 2014). Thus, K+ is accumulated in the vacuole during stomatal movement via NHX-type H+ antiporters and relies on the proton motive force established by vacuolar proton pumps.

Interestingly, the nhx1 × nhx2 double mutant also shows impaired stomatal closure in response to darkness, ABA, and during drought stress (Andrés et al., 2014). While a role for AtNHX in vacuolar K+ uptake during opening is expected, the reason for the effect observed during closure is unclear. It could be that the altered vacuolar pH in nhx1 × nhx2 double mutants interferes with other vacuolar transport during closure or that the altered K+ concentrations in vacuole and cytosol affect tonoplast voltage.

The transport process involved in K+ loss from the vacuole during closure probably involves ion channels of the TPK (Two-Pore K+) family. AtTPK1 was discovered in 2007 (Gobert et al., 2007) and mediates an ion current formerly known as VK (Vacuolar Potassium; Ward and Schroeder, 1994). This channel presents a marked selectivity for K+ and is voltage independent. Instead, AtTPK1 is activated by cytosolic Ca2+ and is a target of CDPKs and 14-3-3 proteins (Latz et al., 2007, 2013). Interestingly, TPK1 channels from Arabidopsis, rice (Oryza sativa), and barley (Hordeum vulgare) are activated by changes in cross-tonoplast osmotic gradients (Maathuis, 2011). During stomatal closure, as the cytosol loses solutes, hypoosmotic situations might occur, activating AtTPK1 to induce K+ release from the vacuole. Even though AtTPK1 is expressed in guard cells and mediates K+ efflux, analysis of leaf water conductance indicates that the knockout mutant tpk1 shows only a moderately delayed ABA-induced stomatal closure, and no whole-plant drought stress phenotype was reported (Gobert et al., 2007). Other AtTPKs might make up for the loss of AtTPK1 function in the tpk1 mutant, but the role of K+ loss through AtTPKs in stomatal closure and whole-plant water balance needs more detailed investigation.

An important K+ conductance in the vacuole is due to the vacuolar cation channel TPC1 (Peiter et al., 2005). AtTPC1 is the only tandem-pore Ca2+ channel gene present in Arabidopsis. The gene product mediates the so-called Slow Vacuolar (SV) current. This ion channel presents a complex ion selectivity mediating monovalent as well as divalent cation currents, such as K+ and Ca2+ currents (Hedrich and Marten, 2011). This dual permeability fueled a controversy on the intracellular function of AtTPC1 in controlling K+ and Ca2+ fluxes (Ranf et al., 2008; Islam et al., 2010; Riemmüller et al., 2010; Hedrich and Marten, 2011; see below). Despite the importance of AtTPC1 currents, from an electro-physiological point of view, their role in guard cells is still uncertain. The voltage dependence of AtTPC1-mediated currents is puzzling; it activates at positive tonoplast potentials that are commonly thought not to exist in plants (see below). The point mutation D454/fou2 in a vacuolar loop of AtTPC1 disrupts an intravacuolar Ca2+-binding site. This point mutation shifts the activation threshold toward negative, physiological, transtonoplastonic potentials, making the channels constitutively conductive. This induces an unbalance in the K+ and Ca2+ cytosolic/vacuolar distribution (Beyhl et al., 2009), indicating that the control of AtTPC1 opening is tightly regulated in plant cells. However, no stomatal opening analysis has been reported for fou2.

VACUOLAR ANION TRANSPORT DURING STOMATAL MOVEMENT

One of the major advances of the last decade lies in the molecular identification of anion transport proteins at the vacuolar and plasma membranes and their role in stomatal movement (Roelfsema et al., 2012). In the vacuolar membrane, two different families of anion transporters have been found to function during opening and closure: the AtCLC and the AtALMT families. CLC stands for chloride channel and ALMT stands for aluminum-activated malate transporter. But the ALMT channels investigated in Arabidopsis guard cells are not aluminum activated. The plasma membrane ALMTs also are referred to as AtQUAC, for quick-activating channel, which references their function in mediating the so-called rapid-type current. AtALMTs form a family of membrane proteins that is present only in land plants. In Arabidopsis, there are
13 members of the ALMT family, and five of them form a clade that seems to be specific to the vacuolar membrane (Kovermann et al., 2007; clade 2 in Dreyer et al., 2012). So far, two of the vacuolar AtALMTs have been shown to be expressed in stomata. AtALMT6 is expressed specifically in guard cells, and its properties were investigated by patch clamp (Meyer et al., 2011). It was found that AtALMT6 mediates Mal2\(^{-}\) and Fum2\(^{-}\) (fumarate) currents but not NO3\(^{-}\) and Cl\(^{-}\) currents. Interestingly, AtALMT6 is activated by cytosolic Ca\(^{2+}\) in the micromolar range, and an acidic vacuolar pH shifted the threshold of activation of this channel. Because of this shift in activation, at a vacuolar pH around 5, AtALMT6 can mediate Mal2\(^{-}\) efflux from the vacuole to the cytosol. However, so far, it has not been possible to detect a stomatal aperture phenotype in \textit{alm}6 knockout plants. Therefore, the exact role of AtALMT6 in guard cells is still unknown.

More recently, AtALMT9 was found to be important for light-induced stomatal opening (De Angeli et al., 2013). AtALMT9 is localized at the vacuolar membrane and is expressed in both shoots and roots. In the shoots, AtALMT9 is expressed in several cell types, including guard cells. Electrophysiological analysis of AtALMT9 shows that this channel is a voltage-gated inward rectifier, opening at negative membrane potentials. AtALMT9 can mediate fluxes of both Mal2\(^{-}\) and Cl\(^{-}\) from the cytosol to the vacuole. Cytosolic Mal2\(^{-}\) concentrations are estimated to range from 0.5 to 2 mM (De Angeli et al., 2013), and at such concentrations, no AtALMT9-mediated Mal2\(^{-}\) inward current can be detected in patch-clamp experiments (\(K_m\) \text{malate} = 27 \text{mM}; Table I). However, Mal2\(^{-}\) in the 0.5 to 2 mM range acts on the opening probability of AtALMT9 and, consequently, stimulates AtALMT9-mediated Cl\(^{-}\) currents. The fact that Mal2\(^{-}\) regulates the activity of ion channels suggests that, in vivo, cytosolic Mal2\(^{-}\) might serve as a signaling molecule. In guard cells, AtALMT9 participates in stomatal opening in response to light, as \textit{alm}9 knockout mutants display reduced stomatal opening. Moreover, the function of AtALMT9 in stomatal opening is dependent on the presence of Cl\(^{-}\) in the medium. This suggests that, in guard cells, AtALMT9 mediates the accumulation of vacuolar Cl\(^{-}\) rather than Mal2\(^{-}\) during stomatal opening. Recent data show that, also in other tissues, such as root vasculature, AtALMT9 is involved in Cl\(^{-}\) rather than Mal2\(^{-}\) transport (Baetz et al., 2016).

Another family of anion transport systems identified in guard cells is the CLC family. CLCs are a family of membrane proteins that is present in all eukaryotic and prokaryotic organisms. In eukaryotes, CLC proteins can be localized in intracellular membranes and at the plasma membrane (Jentsch, 2015). So far, all intracellular CLCs were found to be anion/H\(^{+}\) exchangers, while the plasma membrane CLCs are anion channels. In Arabidopsis, there are seven CLCs (AtCLCa–AtCLCg), all localized in intracellular membranes, and AtCLCa and AtCLCc have identified roles in guard cell physiology (Jossier et al., 2010; Wege et al., 2014). AtCLCc was the first putative vacuolar anion transporter shown to be involved in regulating stomatal movements (Jossier et al., 2010). The results obtained from stripped epidermis show that AtCLCc has a dual function in light-induced stomatal opening and ABA-induced stomatal closure. However, no direct data on the transport properties of this channel are available to date, but based on the amino acid sequence, it is possible that AtCLCc is an anion/H\(^{+}\) exchanger with a preference for Cl\(^{-}\). The only Arabidopsis CLC expressed in guard cells for which functional data are available is AtCLCa. AtCLCa was the first NO3\(^{-}\) transport system identified in the tonoplast (De Angeli et al., 2006). It was shown that AtCLCa is an anion/H\(^{+}\) exchanger with a marked selectivity for NO3\(^{-}\) over Cl\(^{-}\) and an exchange stoichiometry of 2 NO3\(^{-}\):1 H\(^{+}\). The exchange reaction catalyzed by AtCLCa allows the accumulation of NO3\(^{-}\) into the vacuole up to concentrations 30 to 50 times the ones found in the cytosol.

In guard cells, the capacity of AtCLCa to mediate anion accumulation in the vacuole made it a likely actor during stomatal opening, and indeed, \textit{cla} knockout mutant plants show reduced stomatal opening (Wege et al., 2014). Unexpectedly, AtCLCa also is involved in mediating stomatal closure. Stomata of \textit{cla} plants show impaired ABA-induced closure, and rosettes dehydrate faster than wild-type rosettes during drought stress. Notably, AtCLCa is phosphorylated by the AtOST1 kinase. The phosphorylation of AtCLCa was found to stimulate its activity at tonoplast membrane potentials below 0 mV. These are thought to be physiological, and at these potentials, AtCLCa is able to mediate the efflux of anions from the vacuolar lumen into the cytosol. AtOST1 is a central player of ABA signaling in guard cells (Munemasa et al., 2015); thus, AtCLCa is part of this signaling pathway controlling the stomatal aperture.

The regulation of AtCLCs by AtOST1 and AtIPK1 regulation by CDPK are probably only the beginning of our understanding of how vacuolar anion transporters are regulated by posttranslational modification. A further regulator of vacuolar anion transport was found in cytosolic nucleotides such as ATP. ATPs are voltage-dependent inhibitors of AtALMT9 (Zhang et al., 2014), and also, AtCLCa is inhibited by ATP (De Angeli et al., 2009).

The finding that AtCLCa and AtCLCc are involved in stomatal closure is intriguing. It has been suggested that AtCLCa also mediates the efflux of anions from the vacuole during stomatal movements (Wege et al., 2014), and so could AtCLCc. However, vacuolar solutes move passively downhill their chemical gradient to the apoplastic, making it questionable why secondary active transporters might be required during closure. A more likely explanation could be that, as in the \textit{nhx1} × \textit{nhx2} mutant, \textit{cla} and \textit{clcc} mutants are disturbed in vacuolar pH homeostasis or the maintenance of tonoplast potential, and this, in turn, affects other transport systems required for stomatal closure.

**VACUOLAR CA\(^{2+}\) TRANSPORTERS DURING STOMATAL MOVEMENT**

Ca\(^{2+}\) is an important second messenger in guard cell signaling. The cytosolic concentration of free Ca\(^{2+}\) in a
resting state is below 1 μM, but it increases and oscillates in response to environmental cues leading to stomatal closure. Details of these intracellular Ca2+ signals are reviewed elsewhere (Pottosin and Schönknecht, 2007; Dodd et al., 2010; Roelfsema and Hedrich, 2010; Jezek and Blatt). Suffice it to say that cytosolic free Ca2+ oscillations rely on Ca2+ release from and rapid reuptake into the vacuole and/or other endomembrane stores, where the concentration of free Ca2+ is about 1,000 times higher than in the cytosol (see refs. above). Quantitative modeling suggests that more than 95% of the Ca2+ required for cytosolic signals is of vacuolar/endomembrane origin (Minguet-Parramon et al., 2016). As is the case for the plasma membrane, our knowledge of the molecular identity of guard cell Ca2+ transporters in the tonoplast is poor. There are two Ca2+ uptake systems in plant vacuoles: P-type Ca2+ pumps of the ACA (Autoinhibited Ca2+ ATPases) family, with a high affinity, and H+/Ca2+ antiporters of the CAX (Cation Exchanger) family, with a lower Ca2+ affinity (Pottosin and Schönknecht, 2007). However, to date, a clear role for these transporters in guard cell Ca2+ signaling has not been established. AtACA4 and AtACA11 are known to localize to the vacuole, but no data regarding stomatal behavior are available for the aca4 × aca11 double knockout mutant (Boursiak et al., 2010). AtCAX1 and AtCAX3 are highly expressed in guard cells and are localized to the Arabidopsis vacuole (Cheng et al., 2003, 2005). The double knockout mutant cax1 × cax3 was analyzed by two research groups that found impaired light-induced stomatal opening (Conn et al., 2011; Cho et al., 2012). The double mutant sequestered less Ca2+ into mesophyll vacuoles, and its apoplastic Ca2+ activity was higher compared with the wild type (Conn et al., 2011). Conn et al. (2011) found that the increased apoplastic Ca2+ is responsible for the reduced stomatal aperture. Thus, the stomatal phenotype in the cax1 × cax3 mutant is likely to be indirect, as a consequence of reduced Ca2+ accumulation into mesophyll vacuoles, rather than a direct result of impaired guard cell Ca2+ signaling. Cho et al. (2012) also posited an indirect effect, as they found AtCAX1 and AtCAX3 to modulate apoplastic pH, excluding a direct function of these proteins in the generation of guard cell Ca2+ signals.

To date, we do not know if and/or how Ca2+ is released from the vacuole during stomatal closure. Because the AtTPC1/SV channel is permeable to Ca2+ and activated by the cation, it was suggested that the channel might be involved in intracellular Ca2+ signaling (Hedrich and Marten, 2011). However, the tpc1 mutant is not affected in cytosolic Ca2+ oscillations and signaling (Ranf et al., 2008; Islam et al., 2010). It is not affected in the inhibition of opening induced by ABA, methyl jasmonate, and CO2 (Islam et al., 2010), does not show impaired stomatal closure in ABA (Ranf et al., 2008), and even the impairment of stomatal closure in 10 mM external Ca2+ (Peiter et al., 2005) is not associated with any severe whole-plant mutant phenotype with regard to plant water balance. In brief, the analysis of stomatal responses in tpc1 knockout mutants failed to clarify the functions of AtTPC1 in guard cells, but it is unlikely that guard cell Ca2+ signaling relies on AtTPC1-mediated Ca2+ release from the vacuole. Ligand-gated Ca2+ channels that are sensitive to inositol triphosphate, inositol hexakisphosphate (IP6), and cADP ribose are thought to exist at the guard cell vacuolar membrane. However, while Ca2+ currents sensitive to these ligands have been described for vacuoles, no channel proteins have been identified to date (Roelfsema and Hedrich, 2005).

OTHER VACUOLAR TRANSPORT DURING STOMATAL MOVEMENT

It was shown recently that starch breakdown in guard cells is necessary for stomatal opening in Arabidopsis (Horrer et al., 2016). It is assumed that starch conversion to sugars, but also the import of sugars via the plasma membrane, is required to achieve or maintain open stomata, replacing or adding to K salts as osmotica (Daloso et al., 2016; Santelia and Lawson, 2016; Santelia, 2017). How might sugars be imported into and released from the guard cell vacuole? No data exist addressing exactly these questions; however, AtTMT1 and AtTMT2 are two transporters that have been shown to accumulate Glc and Suc into the vacuole in other cell types (Wormit et al., 2006; Schulz et al., 2011), but their role in stomatal guard cells is not known.

The accumulation of solutes in the vacuole causes massive fluxes of water into the lumen. Although water can permeate membranes by diffusion, aquaporins are probably necessary to mediate the large and fast water fluxes that we have to assume across the vacuolar membrane during stomatal movement. Vacuolar aquaporins are referred to as tonoplast intrinsic proteins (TIPs), and few data on their role in guard cell movement exist. Pou et al. (2013) studied TIP expression in grapevine (Vitis vinifera) and found a strong positive correlation between VvTIP2;1 expression and stomatal conductance, suggesting that TIPs are necessary to achieve maximal stomatal aperture. This view is strengthened by the observation that constitutive overexpression of SITIP2;2 in tomato (Solanum lycopersicum) increases whole-plant transpiration (Sade et al., 2009). Interestingly, under drought stress, overexpressing plants transpired more and had a lower relative water content compared with the wild type. This indicates that SITIP2;2 is important for stomatal opening but not for stomatal closure, suggesting that water flux through SITIP2;2 might be gated or regulated to allow flux in only one direction. Regulation by posttranslational modification was described recently for the plasma membrane-localized aquaporin AtPIP2;1, the activity of which is required for stomatal closure in response to ABA but not for
stomatal opening (Grondin et al., 2015). This raises interesting new aspects of how we commonly think of guard cells: ion flux is regulated and water follows passively. Could there be situations in which water fluxes are regulated and are the driver of guard cell movements and the ions follow? It was suggested recently that this happens in response to changes in air humidity (Peak and Mott, 2011; Mott and Peak, 2013). Water-driven changes in cytosolic water potential could alter the tension of the vacuolar membrane, inducing ion release or uptake through osmosensitive or mechanosensitive channels (Peyronnet et al., 2014). MacRobbie (2006) observed osmotic effects on vacuolar ion release of guard cells and hypothesized the osmosensor to be an aquaporin. In addition, as mentioned above, TPK channels respond to osmotic effects (Maathuis, 2011), and a recent advance identified OSCA1, a Ca^{2+}-permeable channel at the plasma membrane that is gated by osmotic pressure (Yuan et al., 2014).

A vacuolar transport protein with an intriguing role during stomatal movement is AtMRP5/AtABCC5. It is a guard cell-expressed ABC-type transporter that mediates high-affinity uptake of IP_{6} into vacuoles (Nagy et al., 2009). Although AtMRP5/AtABCC5 also is expressed in other cell types, mrp5 knockout mutants show strong guard cell mutant phenotypes (Klein et al., 2003). The mrp5 mutant is impaired in light- and auxin-induced stomatal opening as well as in ABA- and Ca^{2+}-induced stomatal closure. Whole plants of mrp5 knockout mutants show reduced transpiration and, hence, increased drought tolerance (Klein et al., 2003). In patch-clamp experiments, it was found that mrp5 mutant guard cells displayed reduced ABA activation of plasma membrane Ca^{2+} and S-type anion currents as well as reduced activation of anion currents by cytoplasmic Ca^{2+} (Suh et al., 2007). While it was initially thought that the transporter localizes to the plasma membrane, it was later reasoned that it is vacuole localized (Nagy et al., 2009). To explain the observed mutant phenotypes, the authors hypothesized that excess cytosolic IP_{6} in the mrp5 mutant might complex cytosolic Ca^{2+} or increase its release from the vacuole through the stimulation of putative IP_{6}-sensitive vacuolar Ca^{2+} channels.

**VACUOLAR pH DURING STOMATAL MOVEMENT**

The transport of protons across the vacuole is energized by two proton pumps, a vacuolar H^{+}-ATPase (AtVHA) and an Arabidopsis V-PP_{i}-ase (AtAVP). A functional knockout in two transmembrane domain isoforms of the V-type ATPase, vha2 vha3 vha-a3, showed increased root vacuolar pH by 0.5 units with respect to the wild-type pH of 5.9 (Krebs et al., 2010). In fugu5-1, a knockout of the PP_{i}-ase AtAVP1, the vacuolar pH was increased by 0.25 units compared with the wild-type pH of 5.8 (Ferjani et al., 2011). The triple mutant vha2 vha3 vha-a3 shows that these two kinds of proton pumps present an additive function only in some cell types (Kriegl et al., 2015). In petunia (Petunia hybrid) petals, the vacuolar membrane also contains ATPases of the P-type, the type found at the plasma membrane (Verweij et al., 2008; Faraco et al., 2014), but there is no report for such a function in Arabidopsis guard cells.

The nhx1 nhx2 double knockout mutants display more acidic guard cell vacuoles and reduced opening (Andrés et al., 2014). The same study showed that light-induced stomatal opening was accompanied by an alcalinization of guard cell vacuolar pH, from pH 5.8 to 5.9, when epidermal strips were incubated in 10 mM KCl. The fact that mutants of the H^{+-}coupled antiporters AtNHX1, AtNHX2, AtCLCa, and AtCLCc all show impaired stomatal opening phenotypes demonstrates that the existence of a proton gradient is necessary for opening. Therefore, it is surprising that AtVHA and AtAVP1 knockout mutants do not show impaired stomatal opening (Bak et al., 2013), and triple mutants defective in both proton pumps show vacuolar pH values that are still slightly acidic (Schumacher, 2014; Kriegl et al., 2015). Hence, part of the proton gradient necessary for stomatal opening must be generated elsewhere. It has been suggested that vacuolar acidity originates during vacuolar biogenesis and, thus, relies on other acidic compartments, such as the trans-Golgi network/early endosome (Schumacher, 2014; Kriegl et al., 2015). The det3 mutant, a knockout of the trans-Golgi network/early endosome-localized V-type ATPase, however, shows impaired stomatal closure (Allen et al., 2000; Brüx et al., 2008), but stomatal opening was not reported to be affected in this mutant. Therefore, it is still unclear how exactly the cross-ankoplast proton gradient necessary for stomatal opening is established.

With regard to stomatal closure, it now seems clear that, in response to ABA, guard cell vacuoles acidify in Arabidopsis and in V. faba (Bak et al., 2013; Andrés et al., 2014). The respective AtVHA and AtAVP1 mutants are slightly impaired in ABA-induced closure, and AtAVP1 mutants show a reduced acidification during closure (Bak et al., 2013), suggesting that this acidification results, at least in part, from active pumping. This notion is supported by the observation that overexpression of ThVHAc, a tamarisk (Tamarix hispida) VHA subunit, in Arabidopsis led to reduced water loss under cadmium stress (Yang et al., 2016). The above studies indicate that active proton pumping aids stomatal closure, but pumping might not be required only for vacuolar acidification but also to maintain a steady tonoplast potential (Hirata et al., 2000). The fact that the antiporters mentioned above show stomatal closing defects supports the notion that guard cell vacuolar pH also may be regulated through these proton antiport systems (Pittman, 2012).

In summary, recent evidence suggests that, in Arabidopsis, guard cell vacuolar pH becomes less acidic during stomatal opening and more acidic during closure. It is not entirely clear how the proton gradient...
necessary for opening is established, but it seems evident that, in contrast to the plasma membrane, vacuolar proton pumping is required for stomatal closure.

**THE VACUOLAR MEMBRANE POTENTIAL DURING STOMATAL MOVEMENT**

To date, we do not know the resting value of the vacuolar membrane potential in guard cells. Measurements on isolated vacuoles are problematic, as the transtonoplast potential would be artificially determined by the measuring solutions. In intact guard cells, it is technically difficult to access the tonoplast for potential measurements, because of the small size and the dynamic nature of the guard cell vacuole. Thus, measurements were performed on cell types amenable to microelectrodes, like root cells, mesophyll cells, or unicellular green algae, and using fluorescent dyes on isolated vacuoles. The reported transtonoplast potential values varied between 0 and −66 mV (Thom and Komor, 1985; Bethmann et al., 1995; Walker et al., 1996; Miller et al., 2001; Wang et al., 2015). In Arabidopsis, measurements of tonoplast potentials in mesophyll and root hair cells yielded a value of about −30 mV (Miller et al., 2001; Wang et al., 2015). Based on these data, similar resting state values are commonly assumed in guard cell vacuoles. However, it is unknown if and how much the vacuolar membrane potential undergoes variations in response to extracellular and intracellular stimuli such as ABA or Ca$^{2+}$. Like any other membrane potential, the vacuolar potential is theoretically defined by the combination of the permeabilities and the gradients of the different ionic species able to permeate the membrane. The Goldman-Hodgkin-Katz equation provides a theoretical framework to understand the behavior of membrane potentials (Hille, 2001). It highlights that the membrane potential is governed by the membrane permeability for one or more ions. Since the activity of vacuolar ion channels and transporters (i.e. membrane permeability) is strictly regulated, this will translate into changes in membrane potential. In theory, the vacuolar membrane potential could be calculated, and based on the theoretical framework mentioned above, the guard cell modeling platform OnGuard calculates vacuolar transport parameters and retrieves tonoplast potential values between −15 and −35 mV (Chen et al., 2012; Hills et al., 2012). The OnGuard model predicts that the vacuolar membrane potential depolarizes during stomatal opening and hyperpolarizes during closure (Chen et al., 2012). By contrast, in the literature, the vacuolar membrane potential often is hypothesized to depolarize during closure: an activation of the voltage-independent TPK1 by cytosolic Ca$^{2+}$ would lead to K$^+$ efflux, which, being the dominant conductance at this moment, would lead to a depolarization of the membrane potential toward less negative potentials below 0 (Roelfsema and Hedrich, 2005; Hedrich and Marten, 2011; Kollist et al., 2014). Because our knowledge of the activity gradients and permeabilities of all ions, as well as the regulation of tonoplast transport, is limited, it is difficult to make any valid statement on the behavior of the vacuolar membrane potential during stomatal movement. In the future, the use of tonoplast-targeted, voltage-sensitive genetic reporters might help to shed light on this important aspect of guard cell biology.

**CONCLUSION**

In the last decade, our knowledge of vacuolar ion transport in guard cells has increased significantly. Several lines of evidence now indicate that the transport of ions across the vacuolar membrane is crucial for the regulation of the stomatal aperture. In guard cells, the tonoplast does not appear to be a simple follower of the plasma membrane but is likely to be a primary actor of stomatal movements. The transport of ions across the tonoplast emerges to be tightly controlled by posttranslational modifications and intracellular molecules such as phosphorylation, dicarboxylates, and nucleotides. These controls may be required for the coordination of ion fluxes across both the plasma and vacuolar membranes and for efficient stomatal opening and closure. Several open questions exist, and our knowledge of the molecular actors operating at the tonoplast is still incomplete. It will be a challenge for the next years to address issues such as the behavior of the guard cell vacuolar membrane potential, the source of luminal acidification, the release of anions during stomatal closure, and the role of vacuolar transport in guard cell Ca$^{2+}$ signals.
BOX I: An Aid to Interpret Electrophysiological Measurements from Vacuoles

In 1992, a convention was introduced by a group of researchers, by which all measurements on endomembranes are to be presented using the luminal compartment as reference (Bertl et al., 1992). The apoplastic and endomembrane compartments, like the vacuolar lumen, are considered to be “outside” of the cytosol. Consequently, negative tonoplast voltages mean that the electrical potential at the cytosolic side is more negative than the one at the vacuolar side. In older literature one might find similar, but positive, values. For measurements across both membranes, the apoplastic side is the ultimate reference point.

By convention, positive currents are generated by the movement of either cations into the vacuole or anions out of the vacuole and vice versa for negative current. Therefore, to deduce the direction of the ion fluxes associated with a current flowing across a membrane, one needs to know the ion species carrying the charge in the experiment. Positive currents are often referred to as outward currents, whereas negative currents are inward currents.

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