Topical Review

The Membrane Transport System of the Guard Cell and Its Integration for Stomatal Dynamics[1][CC-BY]

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Stomatal guard cells are widely recognized as the premier plant cell model for membrane transport, signaling, and homeostasis. This recognition is rooted in half a century of research into ion transport across the plasma and vacuolar membranes of guard cells that drive stomatal movements and the signaling mechanisms that regulate them. Stomatal guard cells surround pores in the epidermis of plant leaves, controlling the aperture of the pore to balance CO_2 entry into the leaf for photosynthesis with water loss via transpiration. The position of guard cells in the epidermis is ideally suited for cellular and subcellular research, and their sensitivity to endogenous signals and environmental stimuli makes them a primary target for physiological studies. Stomata underpin the challenges of water availability and crop production that are expected to unfold over the next 20 to 30 years. A quantitative understanding of how ion transport is integrated and controlled is key to meeting these challenges and to engineering guard cells for improved water use efficiency and agricultural yields.

Stomata are pores that form across the epidermal cell layer of plant leaves and stems. They connect the inner air space of these organs with the atmosphere, thereby serving as the major route for gaseous exchange, bypassing the otherwise impermeable cuticle that forms on the outer epidermal surface. Stomata respond to environmental and endogenous (chemical and hydraulic) signals, opening and closing the pore in order to satisfy the needs of the mesophyll cells for CO_2 in photosynthesis while limiting water loss via transpiration to the atmosphere. In the light, stomata may reduce photosynthetic rates by 50% and more when water supply is limiting (Lawson and Blatt, 2014; Vialet-Chabrand et al., 2017). They have a major impact on global water and carbon cycles. Transpiration by crops has been a key factor in global atmospheric modeling and weather prediction for over a quarter of a century (Beljaars et al., 1996; Berry et al., 2010). Today, stomatal transpiration is widely recognized to lie at the center of the crisis in water availability and crop production now expected over the next 20 to 30 years. Water use around the world has increased 6-fold in the past 100 years,

ADVANCES

- Stomatal guard cells are the premier plant cell model for ion transport and cell signaling, and they are key targets for biological engineering to improve crop production and reduce water demand.
- Although key elements of the signaling pathways and their downstream response elements are well defined, new stimuli and pathway intermediates continue to surface that challenge our understanding of guard cells.
- Phenotypic analysis of mutants must be supported with quantitative kinetics, binding, and related functional data if we are to understand the physiological roles of transporters and the signaling pathways that regulate them.
- The ion transport processes that drive stomatal movements are highly coordinated, both for stomatal opening and closing, with much of this coordination arising from the intrinsic features and biophysical properties of the individual solute transporters at the plasma membrane and tonoplast. Additionally, ion transport must be integrated with membrane vesicle traffic to ensure changes in cell volume and turgor are coregulated with surface area.
- This complexity in the transport interactions of guard cells often defies an intuitive understanding. Emergent behaviors arising from this complexity demand a quantitative systems approach for any meaningful interpretation.

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twice as fast as the human population, and is expected to double again before 2030, driven mainly by agriculture and irrigation (UNESCO, 2015). Indeed, there are some very basic reasons for pursuing an understanding of how stomata work.

Stomata attracted the attention of early microscopists, including Grew (1682), who described stomata as breathing holes on the surface of plant leaves. de Candolle (1827) first confirmed that stomatal apertures are variable, but it was only later that von Mohl (1856) would appreciate the importance of turgor in driving these changes. A number of observations recognizable today followed the advent of the diffusion porometer that enabled measurement of the resistance of the leaf to gaseous flow (Darwin and Pertz, 1911). These included transient movements (Darwin, 1916; Knight, 1916), midday closure (Loftfield, 1921), and the effects of drought (Laidlaw and Knight, 1916). Freudenberger (1940) and Heath (1948) showed that CO_2 within the leaf air space was important in regulating aperture, and Wilson (1948) established the importance for stomatal movements of the vapor pressure difference between inside and outside the leaf.

Stomata were inextricably bound up with the plant hormone abscisic acid (ABA) when Wright and Hiron (1969) at Wye College in the United Kingdom and Mittelheuser and van Steveninck (1969) in the United States discovered ABA to be highly effective in closing stomata and in the subsequent resistance of the leaf to wilting. This same period, during the 1960s and 1970s, marked a recognition of ion transport, especially of K^+ salts, and of solute content contributing to the cell turgor as a driver behind stomatal movements (Fischer and Hsiao, 1968; Humble and Hsiao, 1969). Ironically, research on stomatal movements at the time was motivated by interest in the mechanism of opening and by the new concepts of chemiosmosis (Mitchell, 1969). Opening, but not closing, was thought to be active, requiring coordination and energy for transport. Research came to focus on stomatal closure only following MacRobbie's pioneering radiotracer flux analysis in the 1980s. Her studies showed that ion efflux during closure is a highly coordinated process (MacRobbie, 1981, 1983a).

The last three decades have seen an explosion in research directed to the mechanics of solute transport and its regulation. The majority of this new knowledge comes from electrophysiological studies, both voltage clamp on intact stomatal guard cells and patch clamp on guard cell protoplasts, that allow separate transport activities to be identified and characterized. These efforts have provided an unprecedented depth of quantitative information about the kinetics of individual ion transporters, including those of the H^+-ATPases, K^+, Cl^-, and Ca^{2+} channels at the plasma membrane and several cation- and anion-selective channels at the tonoplast, and about the dynamics of their regulation (Pandey et al., 2007; Sokolovski and Blatt, 2007; Kim et al., 2010; Roelfsema and Hedrich, 2010; Lawson and Blatt, 2014). With the cloning of many of these transporters, it has been possible to connect gene to function through heterologous expression and analysis in isolation. This same strategy has been used to dissect macromolecular protein complexes regulating several K^+ channels (Honsbein et al., 2009; Grefen et al., 2015) and to reconstruct speculative phosphorylation cascades (Geiger et al., 2009, 2011). Imaging techniques combined with voltage clamp studies have shown how individual transporters are regulated in vivo by cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and pH (pH_i; Thiel et al., 1993; Grabov and Blatt, 1998; Hamilton et al., 2000; Loro et al., 2012). Site-directed mutation, complementation studies, and structural analysis have uncovered the molecular mechanics of channel gating (Riedelsberger et al., 2010; Lefoulon et al., 2014) and early events of ABA perception and signaling (Garcia-Mata et al., 2003; Melcher et al., 2009; Cutler et al., 2010; Wang et al., 2013; Eisenach and Di Angeli, 2017; Inoue and Kinoshita, 2017).

The actions of other hormones such as auxin (Blatt and Thiel, 1994; Lohse and Hedrich, 1995), of light and CO_2 (Negi et al., 2008; Kim et al., 2010; Xue et al., 2011; Kinoshita, 2017), and of plant pathogens (Melotto et al., 2008) have not been neglected (Melotto et al., 2017). Resolving the interface between transport and carbohydrate metabolism remains a major challenge (Wang and Blatt, 2011; Horrer et al., 2016; Griffiths and Males, 2017; Santelia and Lunn, 2017). Considerable attention, too, has been drawn in recent years to the unusual pattern of stomatal development within the epidermis and to its evolution (McElwain et al., 2005; Bergmann and Sack, 2007; Chen et al., 2017). Thus, present interest in stomata extends well beyond ion transport and gas exchange. Some of these topics are explored in depth in this Focus Issue, and we direct the reader to the several Updates accompanying this article (Brodribb and McAdam, 2017; Chater et al., 2017; Vialet-Chabrand et al., 2017). Nonetheless, in many respects, the focus has come full circle, returning to issues of membrane transport and its control. If we are to use our knowledge of stomata to improve crop resilience and agricultural capacity in marginal areas, then stomatal gas exchange (Buckley, 2017; Franks et al., 2017) must be linked to an understanding of the mechanics of stomatal ion transport and its regulation as a priority for the future. Here, we review the current knowledge of ion transport in stomatal guard cells. We emphasize its dynamics and coordination, the origins of which often defy intuitive understanding yet are critical to any rational efforts toward stomatal engineering, and we stress the importance of quantitative functional data that are essential to realize such efforts.

STOMATAL OPENING

Stomatal pores form between specialized pairs of epidermal cells, the guard cells (Fig. 1). Guard cells of dicotyledonous plants bow apart as they expand, thereby opening the stomatal pore (Table I). The

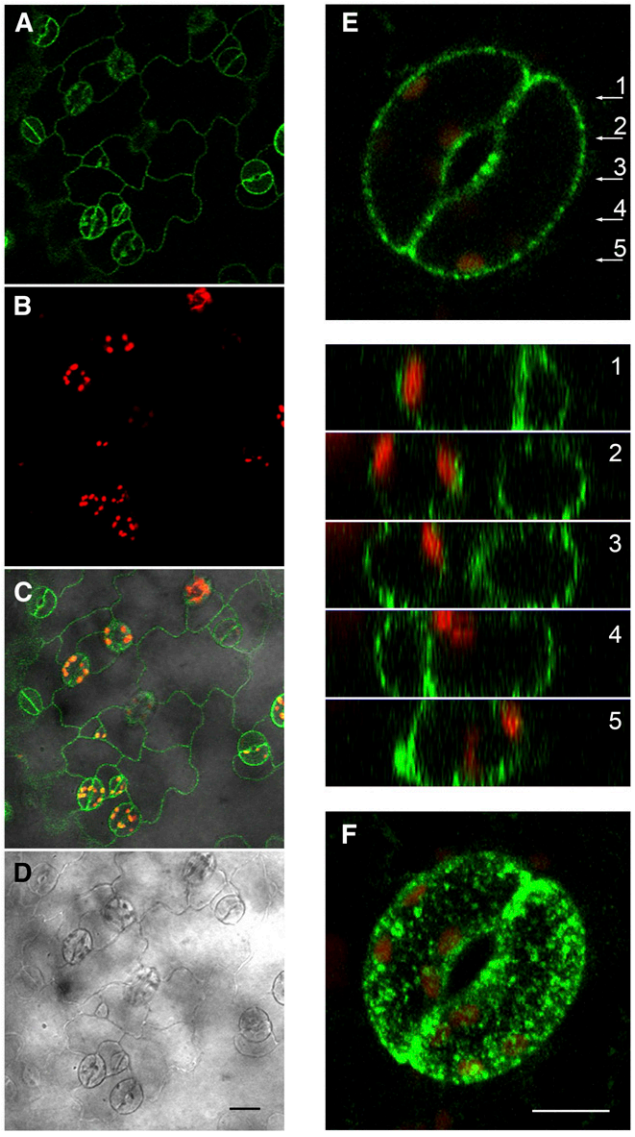


Figure 1. Guard cells expressing the GORK K^+ channel in the Arabidopsis leaf epidermis. A to D, Confocal images of the leaf epidermal surface of Arabidopsis stably transformed with GORK-GFP under the control of the Ubiquitin-10 promoter (Grefen et al., 2010b), showing the distribution of GORK-GFP (A), chloroplast autofluorescence (B), and the overlay of these images (C) with the corresponding bright-field image (D). Bar = 20 μm. E, Single optical section from a Z-stack through two kidney-shaped guard cells surrounding one stoma (center), showing the punctate distribution of GORK-GFP around the periphery of the two guard cells. Z-plane transects taken along the *x* axis at positions 1 to 5 are shown below. F, The full three-dimensional projection of the Z-stack clearly shows the punctate character of GORK localization and the prevalence of the channel at the junctions between the two guard cells. Bar = 5 μm. Data are from C. Eisenach, Ph.D. thesis. See Eisenach et al. (2014) for further details.

anatomy of stomata in many monocotyledonous plants differs, but the principle of their movement is much the same (Chen et al., 2017). In opening the pore, guard cells accumulate solute, mainly K^+ salts with Cl^- and malate (Mal), as well as sugars. In closing the pore, guard cells reverse this process by metabolizing these solutes or releasing them to the apoplast. The changes in solute content between the open and closed states are substantial, often exceeding 300 to 400 mosmol L^{-1} (= 300–400 mosM) on a cell volume basis (Table II), and draw corresponding water fluxes, thereby driving the volume and turgor changes that open and close the pore. Mature guard cells lack plasmodesmata (Wille and Lucas, 1984), so all of the inorganic ions, and during closure much of the organic solute that is not metabolized, must be transported across the plasma membrane. As in other plant cells, the vacuole in guard cells of open stomata comprises some 80% to 90% of the cell volume and remains isotonic with the cytosol. Thus, the bulk of solute transported across the plasma membrane also must pass across the tonoplast surrounding the vacuole.

Guard cells coordinate solute flux actively through a number of major transport pathways at the plasma membrane and the tonoplast during stomatal movements (Box 1; Allen et al., 1999; Chen et al., 2016; Desikan et al., 2002; Grabov et al., 1997; Takemiya et al., 2016; Takemiya and Shimazaki, 2016; Suhita et al., 2004; Yamauchi et al., 2016; Yin et al., 2009; Zhang et al., 2001; Zhao et al., 2016). Like all plant cells, guard cells use ATP to drive H^+ out of the cell via H^+-ATPases, thereby generating a membrane voltage, negative inside, and an electrochemical potential difference ($\Delta\mu_H$) for the H^+. Stomatal opening is promoted by light and by the breakdown of starch (Horrer et al., 2016) and lipid (McLachlan et al., 2016) to organic osmotica. Membrane voltage facilitates K^+ uptake through K^+ channels, in Arabidopsis (*Arabidopsis thaliana*) primarily KAT1 (Nakamura et al., 1995; Pilot et al., 2001, 2003; Szyroki et al., 2001; Lebaudy et al., 2008). The $\Delta\mu_H$ also drives high-affinity K^+ transport (Blatt and Clint, 1989; Clint and Blatt, 1989), most likely coupled 1:1 with H^+ influx through HAK-type transporters, as first described in fungi and in other plant cells (Rodriguez-Navarro et al., 1986; Blatt and Slayman, 1987; Maathuis and Sanders, 1994; Véry et al., 2014). Anion uptake must be energized by coupling with $\Delta\mu_H$ to overcome the membrane voltage, which opposes anion influx. In the few instances in which it has been examined in plants, this flux is coupled with at least two H^+, giving a net movement of one or more positive charges inward with each anionic charge (Sanders and Hansen, 1981; Hawkesford and Miller, 2004; Barbier-Brygoo et al., 2011) and, hence, leading to membrane depolarizations (Meharg and Blatt, 1995; Blatt et al., 1997). Transport at the tonoplast is coordinated with ion flux across the plasma membrane, in part because transporters at both membranes share a common pool of solutes and metabolites in the cytosol. For example, as K^+ and Cl^- are taken up by transport across the plasma membrane, their increased content in the cytosol feeds directly into their transport across the tonoplast via mass action. Other connections depend on common solutes that serve as signaling intermediates, including $[Ca^{2+}]_i$ and pH_i (see "Ca^{2+} Control of Osmotic Solute Efflux" below).

Table I. *Basic biophysical parameters of stomatal guard cells in the open and closed state in V. faba and Arabidopsis*

References are as follows: Humble and Raschke (1971); Raschke et al. (1975); Blatt (1987b); Clint and Blatt (1989); Willmer et al. (1995); Willmer and Fricker (1996); Franks et al. (2001); Shope et al. (2003); Shope and Mott (2006); Meckel et al. (2007); Vialet-Chabrand et al. (2016); Xie et al. (2016). GC, Guard cells.

Parameter	Species			
	V. faba		Arabidopsis	
	Closed	Open	Closed	Open
Aperture (μm)	1.0–6.0	8.0–16.5	1–3	2–6
Pore area (μm^2)	75–124	178–262	3–5	10–15
GC length (μm)	35–45	40–50	9–11	10–20
GC diameter (μm)	10–12	14–16	3–5	5–6
GC surface area ($cm^2 \times 10^{-5}$)	1.2–1.6	1.6–3	0.2–0.3	0.3–0.5
GC volume (pL)	2.7–3.7	4.0–7.5	0.3–0.4	0.5–0.65
GC vacuole surface area ($cm^2 \times 10^{-5}$)	0.96–1.2	1.3–1.6	0.15–0.23	0.25–0.43
GC vacuole volume (pL)	1.8–2.4	3.6–5.8	0.23–0.3	0.43–0.55
GC turgor (atm)	1–4	3.5–6	3–5	6–8
Plasma membrane voltage (mV)	−30 to −70	−100 to −180	−30 to −70	−100 to −180
Tonoplast membrane voltage (mV)	0 to −50	−10 to −40	0 to −50	−10 to −40

STOMATAL CLOSING

The characteristics of ion transport for solute accumulation are generally shared among plant cells, including guard cells. Where guard cells differ from the norm is their ability to coordinate solute release and close the stomatal pore. Voltage clamp studies at the beginning of the 1990s first uncovered concerted alterations in K^+ channel activities associated with plasma membrane depolarization and K^+ and anion efflux in the presence of ABA (Blatt, 1990; Linder and Raschke, 1992). These discoveries were soon tied to changes in $[Ca^{2+}]_i$ and pH_i, each moderating subsets of transporters and their activities (Schroeder and Hagiwara, 1989; Blatt et al., 1990b; Gilroy et al., 1990; McAinsh et al., 1990; Blatt, 1992; Blatt and Armstrong, 1993; Grabov and Blatt, 1997, 1998, 1999). Only later was membrane voltage linked to Ca^{2+} influx, Ca^{2+} release from endomembrane compartments, and their regulation by reactive oxygen species (ROS) and nitric oxide (NO; Hamilton et al., 2000; Pei et al., 2000; Garcia-Mata et al., 2003; Kwak et al., 2003; Sokolovski et al., 2005). The origins of the changes in pH_i observed in the presence of ABA are still to be determined but are most likely an emergent property of interactions between ion transport and metabolism (Chen et al., 2012; Wang et al., 2012). In parallel, these signals are interwoven with phosphorylation cascades, now thought to be triggered by ABA binding with one or more pyrabactin/pyrabactin-like (PYR/PYL) ABA receptors that sequester and inhibit PP2C-type protein phosphatases. The primary effects of ABA are to suppress the activities of the H^+-ATPase and the inward-rectifying K^+ channels (KAT) to prevent K^+ uptake and to activate the SLAC- and ALMT (QUAC)-type anion channels along with outward-rectifying K^+ channels (GORK) to facilitate K^+, Cl^-, and Mal efflux (Box 1).

Table II. *Compartmental ion and sugar concentrations of guard cells of closed and open stomata (in mM)*

Data relate to *V. faba* unless noted otherwise. References are as follows: Fischer (1968); Allaway (1973); Raschke et al. (1975); Raschke and Schnabl (1978); Van Kirk and Raschke (1978a, 1978b); MacRobbie (1983b); Clint and Blatt (1989); Talbott and Zeiger (1993, 1996); Thiel et al. (1993); Marschner (1995); Willmer and Fricker (1996); Guo et al. (2003); Dodd et al. (2005, 2007).

Solute	Apoplast		Cytosol		Vacuole	
	Closed	Open	Closed	Open	Closed	Open
K^+	0.05–114	0.05–24	55–93	150–247	38–92	181–454
H^+ (pH) [a,b]	6.2–7.1	4.8–6.5	7.4–7.9	7.2–7.7	5.2–6.5	4.0–5.3
Ca^{2+} [c]	0.05–1.0	0.5–1.7	0.1–0.9 μM	0.09–0.45 μM	1–5	3–22
Cl^- [d]	0.3–44	0.1–7	3–20	11–50	3–40	40–124
NO_3^- [a,b]	0.1–0.2	1–0.2	2–5	2–5	10–92	10–92
PO_4^{2-} [a]	0.3–0.7	3–0.7	2–6	2–6	4–92	4–92
Mal [d]	0.5–20	0.4–3.5	0.1–6	2–25	5–48	41–464
Suc	0.4–3	1–8	12–20	6–75	1–27	45–200

[a]Values are typical for glycophytic plants. [b]Values incorporate data from *Commelina communis* and Arabidopsis guard cells. [c]Cytosol values are free concentrations. [d]Cl^- will exchange with Mal, subject to availability.

A number of other stimuli promote stomatal closure, including darkness, high CO_2 partial pressures (pCO_2), and several plant pathogens. The mechanics of the changes in transport in each case are thought to follow a pattern similar to that for ABA. Indeed, the characteristics required for closure generally limit transport changes to those evoked by ABA (Chen et al., 2012; Blatt et al., 2014; Wang et al., 2014a). Of course, the signal cascades need not be the same, but what detail is available at present is often fragmentary. For the transition to darkness, there is virtually no quantitative information beyond our knowledge of H^+-ATPase promotion by light for stomatal opening (above) and hints, albeit temporally problematic, of an association with $[Ca^{2+}]_i$ (Marten et al., 2008). The stomatal response to bacterial pathogens probably depends on kinase cascades, NO, and ROS that are common to ABA signaling (Melotto et al., 2006; Deger et al., 2015). The fungal pathogen elicitor of *Cladosporium fulvum* engages phosphorylation cascades that probably overlap with those of ABA to regulate the K^+ and Cl^- channels for net KCl efflux (Blatt et al., 1999). Elevated CO_2 activates the outward-rectifying K^+ channels and SLAC-type Cl^- channels, and it reduces the activity of the KAT-like K^+ channels in *Vicia faba* guard cells (Brearley et al., 1997), actions that are consistent with measurements of apoplastic Cl^- (Hanstein and Felle, 2002). Like ABA, the CO_2 signal cascade is associated with a rise in $[Ca^{2+}]_i$ (Webb et al., 1996), but it is not mediated directly through changes in pH_i (Brearley et al., 1997).

The stomatal response to CO_2 is sensitive to external pH, which suggests that HCO_3^- in solution, rather than CO_2 per se, is the primary ligand sensed within the guard cell (Bown, 1985). More recently, the SLAC1 channel, and its activation, has become a focal point for dissecting the mechanisms of CO_2 regulation. The hydration of dissolved CO_2 is very slow in the absence of carbonic anhydrase (Gutknecht et al., 1977). So it is no surprise that stomata respond sluggishly to pCO_2 changes in the *ca1ca4* double mutant (Hu et al., 2010), which eliminates the two major cytosolic and chloroplastic carbonic anhydrases in Arabidopsis (Fabre et al., 2007). One recent study (Wang et al., 2016) used *Xenopus* oocytes to reconstitute pCO_2 signaling with carbonic anhydrases, the aquaporin PIP2;1 and SLAC1. The relevance of this ex vivo association, like that of several protein kinases (see "Phosphorylation Cascades" below), remains speculative, however, especially given the immense differences in cell volume between guard cells and *Xenopus* oocytes and the implications for CO_2 and HCO_3^- diffusion. The results are also difficult to reconcile with a publication from the same group suggesting that phosphorylation sites on both sides of the membrane are essential for the SLAC1 response to pCO_2 (Yamamoto et al., 2016). Thus, the relationships between aquaporins and carbonic anhydrases in water and CO_2 permeability are a matter of debate (Grondin et al., 2015; Zhao et al., 2016), as is their coupling to activation of the anion channel. For now, the guard cell CO_2 (HCO_3^-) sensors remain unknown, although it is clear that, downstream, the action overlaps with significant elements important also for ABA signaling (Chater et al., 2015; Tian et al., 2015).

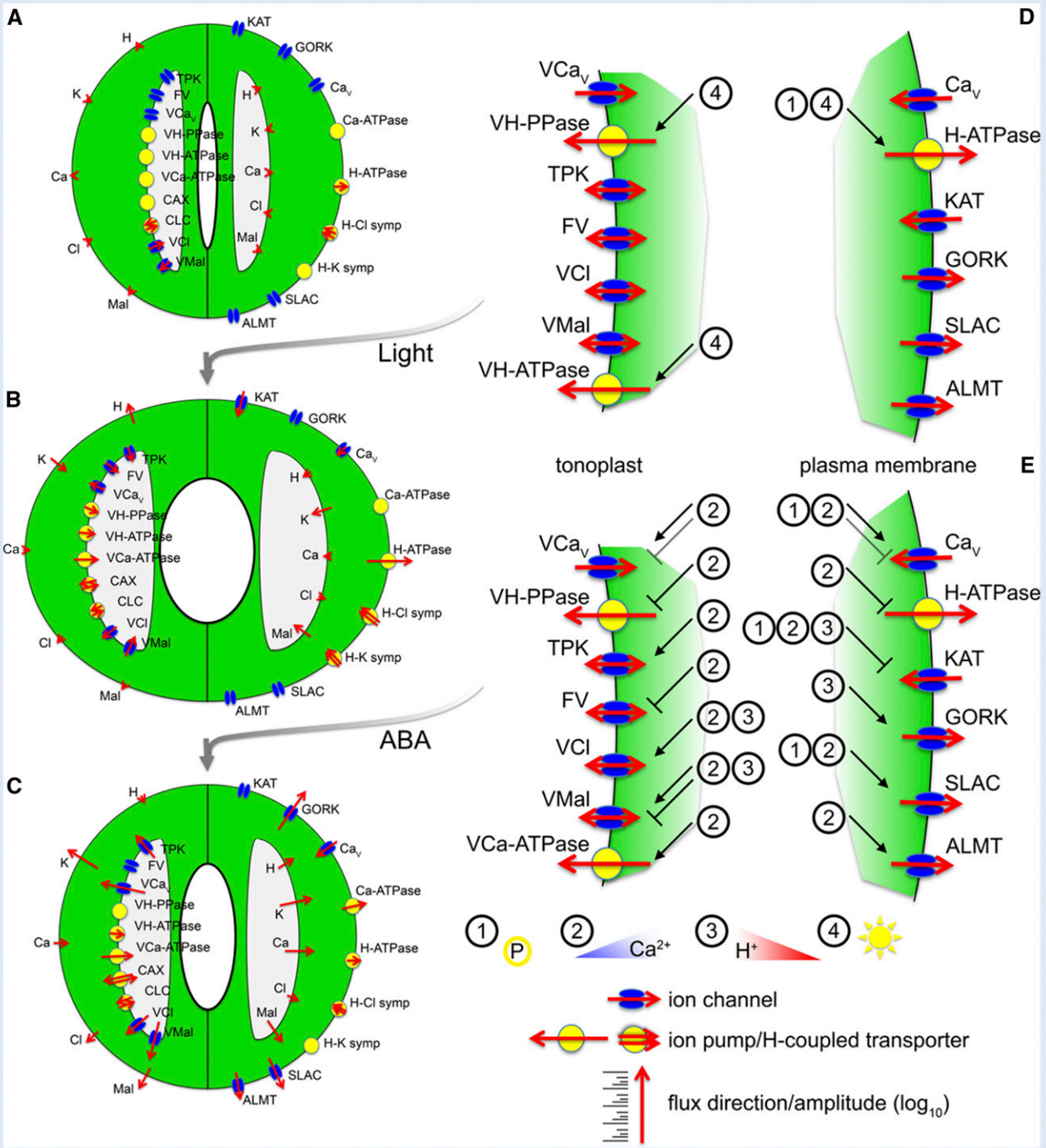
A WEALTH OF MOLECULAR IDENTITIES

The first plant K^+ channel, KAT1, to be cloned and functionally characterized (Anderson et al., 1992; Schachtman et al., 1992) was soon associated with the guard cells of Arabidopsis (Nakamura et al., 1995). These discoveries accompanied a growing knowledge of the principal ion pumps of the plasma membrane and tonoplast, including the H^+-ATPases and their regulation (Kinoshita and Shimazaki, 1999; Moriau et al., 1999; Kinoshita et al., 2001; Ueno et al., 2005; Merlot et al., 2007), Ca^{2+}-ATPases, and the tonoplast H^+-pyrophosphatase (H^+-PPase; Martinoia et al., 2007; McAinsh and Pittman, 2009). Other discoveries followed with signaling proteins, including the OST1 protein kinase associated with ABA and ROS signaling (Merlot et al., 2002; Mustilli et al., 2002), the ABI1 2C-type protein phosphatase (Leung et al., 1994; Armstrong et al., 1995) now known to be part of a set of key ABA receptor complexes (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009), and the first vesicle-trafficking protein affecting stomatal movements (Leyman et al., 1999; Eisenach et al., 2012) now known to interact physically with several K^+ channels (see "Coordinating Ion Transport, Membrane Traffic, and Water Flux" below).

Following publication of the Arabidopsis genome in 1999, the list of guard cell transporters, and even more so that of the regulatory proteins functional in guard cells, has expanded rapidly. These include the full complement of plasma membrane K^+ channels homologous to the mammalian voltage-sensitive (Kv) channels (Dreyer and Blatt, 2009), the tonoplast TPK1 (Gobert et al., 2007) and TPC1 (Peiter et al., 2005; Islam et al., 2010) channels corresponding to the so-called Vacuole K^+ (VK) and Slow Vacuole (SV) currents, and Cl^-- and Mal-permeable channels, including the slow-activating SLAC1 Cl^- channel and several quick-activating, ALMT (QUAC)-type anion channels (Kovermann et al., 2007; Negi et al., 2008; Vahisalu et al., 2008; Meyer et al., 2010, 2011; Sasaki et al., 2010). The molecular identities of several H^+-coupled transporters are known as well, including the endomembrane and vacuolar H^+-coupled alkali cation and Ca^{2+} exchangers (Padmanaban et al., 2007; Bassil et al., 2011; Pittman, 2011; Andrés et al., 2014; Bassil and Blumwald, 2014), the CLC-type Cl^- and NO_3^- transporters observed previously to show channel-like behaviors (De Angeli et al., 2006; Jossier et al., 2010), and plasma membrane transporters for Mal (Lee et al., 2008; Medeiros et al., 2016) and even for ABA itself (Merilo et al., 2015). Less is known for sugar transport (Ritte et al., 1999; Stadler et al., 2003), although it is likely to facilitate organic solute uptake and communication with the photosynthetic mesophyll (Lawson et al., 2008;

BOX 1. Membrane Transport for Stomatal Opening and Closing

Stomatal opening (see fig., A, B, and D) is promoted by red light through photosynthesis (Lawson et al., 2008) and by blue light that activates the plasma membrane H^+-ATPases (H-ATPase) through phosphorylation of the BLUS1 protein kinase and recruitment of 14-3-3 proteins to mask the H^+-ATPase autoinhibitory domain (Kinoshita and Shimazaki, 1999; Takemiya et al., 2016; Takemiya and Shimazaki, 2016; Yamauchi et al., 2016). K^+ uptake utilizes the voltage component of the $\Delta\mu_H$ fostered by the H^+-ATPase (Blatt



and Clint, 1989; Clint and Blatt, 1989). Roughly 50 to 75% of K^+ uptake is thought to be channel-mediated (KAT; Wang et al., 2014a), with the rest carried by high-affinity transport coupled 1:1 with H^+ influx through HAK-type transporters (H-K symp), such as was first described in fungi and other plant cells (Rodriguez-Navarro et al., 1986; Maathuis and Sanders, 1994; Very et al., 2014). Anion uptake is generally opposed by the membrane voltage and must be energized by coupling with $\Delta\mu_H$. The uptake of Cl^-, for example, is thought to depend on coupling with 2 H^+ (H-Cl symp), giving a net movement of one positive charge inward with each anionic charge (Blatt, 2000). Light also entrains a diurnal cycle in vacuolar H^+-ATPase (VH-ATPase) gene expression and activity (Chen et al., 2012) and increases metabolic production of pyrophosphate that feeds the H^+-pyrophosphatase (VH-PPase) to transport H^+ into the vacuole (Rea and Poole, 1993; Martinoia et al., 2007). These pumps generate a $\Delta\mu_H$ to drive into the vacuole the bulk of solutes passing from the apoplast into the cytosol as well as Mal synthesized in the guard cell.

In stomatal closing (see fig., B, C, and E), the earliest event within 2 to 3 s of adding ABA to isolated membrane patches (Hamilton et al., 2000) is a shift in the voltage sensitivity of Ca^{2+} channels at the plasma membrane (Ca_V) that promotes Ca^{2+} influx and its release via endomembrane Ca^{2+} channels (VCa_V) to raise $[Ca^{2+}]_i$ (Grabov and Blatt, 1998). ABA triggers a phosphorylation cascade by binding with one or more PYR/PYL ABA receptors to sequester and inhibit ABI1 PP2C-type protein phosphatases (Melcher et al., 2009; Park et al., 2009; Yin et al., 2009; Cutler et al., 2010). It is unclear whether other ABA receptors occur that trigger one or more signal cascades parallel to those associated with the PYR/PYL receptors. The PYR/PYL-PP2C cascade may be linked to the gasotransmitters H_2S and nitric oxide (NO; Desikan et al., 2002; Scuffi et al., 2014; Chen et al., 2016), and both have immediate actions on K^+ and Cl^- channels and on Ca^{2+} channel activities (Garcia-Mata et al., 2003; Papanatsiou et al., 2015) that include direct *S*-nitrosylation of channels and at least one protein kinase (Sokolovski and Blatt, 2004; Wang et al., 2015). The consequences of PYR/PYL-PP2C binding may well account downstream for the changes in gating of the Ca^{2+} channels (Allen et al., 1999; Koehler and Blatt, 2002; Sokolovski et al., 2005; Wang et al., 2013), but this remains to be explored.

Together, $[Ca^{2+}]_i$ and one or more phosphorylation cascades activate both SLAC (so-called slow or S-type) and ALMT (so-called rapid or R-type) anion channels to potentiate anion efflux (Schmidt et al., 1995; Grabov et al., 1997; Geiger et al., 2009; Meyer et al., 2010), and they suppress H^+-ATPase activity (Merlot et al., 2007), both favoring membrane depolarization. The rise in $[Ca^{2+}]_i$ inactivates the inward-rectifying K^+ channels (KAT) to prevent K^+ uptake (Grabov and Blatt, 1997, 1999). The outward-rectifying K^+ channels (GORK) are engaged to facilitate K^+ efflux as membrane voltage shifts positive of the K^+ equilibrium voltage, E_K; flux through these channels is further enhanced 2- to 3-fold as the pH_i rises during the first 3 to 5 min in ABA (Blatt, 1990; Blatt and Armstrong, 1993; Grabov and Blatt, 1997; Suhita et al., 2004). Thus, the bias on the plasma membrane shifts from hyperpolarization and solute uptake to depolarization and solute loss (Blatt, 2000; Kim et al., 2010; Lawson and Blatt, 2014). Of course, stomatal movements arise as the cumulative sum of the net solute flux over time. So, changes in aperture take place over timescales typically orders of magnitude longer than is needed for changes in transport activities and membrane voltage (Raschke et al., 1975; Blatt and Armstrong, 1993; Roelfsema and Prins, 1995; Zhang et al., 2001). Connecting stomatal movements to changes in membrane transport is inferred only unless flux is measured or transport rates can be integrated over time through quantitative modeling (see "Systems Models for Guard Cell Transport"). Red arrows indicate the directions and, in A to C, the amplitudes in logarithmic scale of the total ion fluxes and the respective fluxes through each transporter at the tonoplast and plasma membrane. In D and E, the regulatory factors acting on each of the several transporters are indicated by the circled numbers as (1) protein (de-)phosphorylation, (2) $[Ca^{2+}]_i$ increase, (3) $[H^+]_i$ decrease, and (4) light. Further details and transporter acronyms are listed in Tables 3 to 6.

Lawson and Blatt, 2014). Finally, the plasma membrane aquaporin PIP2;1 was recently shown to promote ABA-mediated stomatal closure, most likely by enhancing the capacity for water flux (Grondin et al., 2015).

Much attention has been drawn by the recent discovery of the family of PYR and PYL ABA receptor proteins, their regulation of PP2C-type protein phosphatases, including ABI1, and their coordination with Ca^{2+}-mediated control in guard cells (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2010; Wang et al., 2013). These receptors mark the beginning of a key phosphorylation cascade triggered by ABA. Downstream effectors include the SLAC1 Cl^- channel, but lacking still is unequivocal evidence of the relevant phosphorylation targets in vivo (see "Phosphorylation Cascades" below). A similar situation applies to members of the family of small auxin up-regulated RNA (SAUR) proteins. Several SAURs are known to promote stomatal opening and have been associated with the regulation of H^+-ATPase activities through the actions of PP2C-D protein phosphatases (Spartz et al., 2014). The molecular identities of their immediate targets are less well defined, however, nor is it clear whether the H^+-ATPases are the only transporters affected directly by SAUR activity in vivo.

Table III. *Predominant pumps and carriers in the plasma membrane of guard cells and their functional characteristics*

Genetic codes relate to Arabidopsis; functional data relate to *V. faba* unless noted otherwise. For I_{sat}, current saturation is at $V >> 0$ for ATPases or $V << 0$ for coupled transport; I_V are typical currents within the physiological voltage range; currents are converted where necessary assuming 1 μA cm^{-2} = 1 pA pF^{-1}, guard cell surface area of 3×10^{-5} cm^2, and volume of 4 pL. Shorthand identifiers in parentheses cross-reference to Box 1. n.d., not determined. References are as follows: A (Blatt, 1987a; Blatt et al., 1990a; Lohse and Hedrich, 1992; Thiel et al., 1992; Becker et al., 1993; Gaxiola et al., 2007); B (Gräf and Weiler, 1990; Carnelli et al., 1992; Askerlund, 1997; Palmgren and Harper, 1999; Bonza et al., 2000; Geisler et al., 2000; Sze et al., 2000; Bonza and De Michelis, 2011; Pittman, 2011); C (Rodriguez-Navarro et al., 1986; Blatt et al., 1987; Blatt and Slayman, 1987; Clint and Blatt, 1989; Maathuis and Sanders, 1994; Maathuis et al., 1997; Quintero and Blatt, 1997; Kim et al., 1998; Rubio et al., 2000, 2008; Chérel et al., 2002; Gierth et al., 2005; Nieves-Cordones et al., 2008; Remy et al., 2013); D (Beilby and Walker, 1981; Sanders and Hansen, 1981; Sanders et al., 1985, 1989; Meharg and Blatt, 1995; Guo et al., 2003; Hawkesford and Miller, 2004); E (Reddy and Das, 1986; Slone et al., 1991; Buckhout, 1994; Ritte et al., 1999; Stadler et al., 2003; Lee et al., 2008; Bates et al., 2012; Santelia and Lawson, 2016); F (Kang et al., 2010; Kuromori et al., 2010, 2011; Kanno et al., 2012; Zhang et al., 2014).

Transporter	Name	Locus	Function	No.	Stoichiometry	I_{sat}	I_V	E_{rev}	Ion Selectivity	References
				$\times 10^6$ cell^{-1}		*μA cm^{-2}*	*μA cm^{-2}*	*mV*		
H^+-ATPase (H-ATPase)	AHA1 AHA2 AHA5	AT2G18960 AT4G30190 AT2G24520	H^+ extrusion, energization	1–3*	1 H^+:1 ATP	22 ± 5 15 ± 5[a]	2–15 5–9[a]	−359 to −457[3,a,d]	n.d.; assumed selective from pH dependence	A
Ca^{2+}-ATPase (Ca-ATPase)	ACA.L ACA1 ACA3 ACA8 ACA10 ACA11 ACA12	AT1G13210 AT1G27770 AT1G07810 AT5G57110 AT4G29900 AT3G57330 AT3G63380	Ca^{2+} extrusion	0.1–0.3**	1 Ca^{2+}:1 ATP	1–2	n.d.	−200[4]	n.d.	B
H^+/Ca^{2+} antiport	CAX11	AT1G08960	Ca^{2+} extrusion	0.1–0.3**	2 or 3 H^+:1 Ca^{2+}	n.d.	−0.6[1,b]	>+100[5]	Transport of K, Na, Mn, Zn, Li possible	B
H^+-K^+ symport (H-K symp)	KUP/HAK/KT1 KUP/HAK/KT2 KUP3 = KT4 KUP/KT5 HAK5 KUP/HAK/KT6 KUP/HAK/KT7 KUP/HAK/KT8 KUP/HAK/KT9 KUP/HAK/KT10 KUP/HAK/KT11 ZIFL1.3	AT2G30070 AT2G40540 AT3G02050 AT4G33530 AT4G13420 AT1G70300 AT5G09400 AT5G14880 AT4G19960 AT1G31120 AT2G35060 AT5G13750	K^+ uptake	0.1–0.5**	1 H^+:1 K^+	−2 to −12[c] −0.1 to −0.3[b]	−0.08 to −0.3 −1 to −2[c] −0.1[b]	>0[6,c] +22[7,b]	$K^+ \sim Rb^+ >> Cs^+$	C
H^+-Cl^- (NO_3^-) symport (H-Cl symp)	NRT1.1 NRT2.1	AT1G12110 AT1G08090	Inorganic anion uptake	0.1–0.5**	2 H^+:1 Cl^- (NO_3^-)	−3 to −12[b] −1 to 3[a]	−1 to −2[b] −0.4 to −1[b]	Near 0[8,a,b]	n.d.	D

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Transporter	Name	Locus	Function	No.	Stoichiometry	I_{sat}	I_V	E_{rev}	Ion Selectivity	References
H^+-Mal symport	ABCB14	AT1G28010	Malate uptake	n.d.	3 H^+:1 Mal^{2-}***	n.d.	n.d.	>+20[9]	Malate ~ fumarate >> succinate ~ citrate	E
H^+-sugar symport	SUC1 SUC3 STP1 STP4	AT1G71880 AT2G02860 AT1G11260 AT3G19930	Sugar uptake	0.02–0.05**	1 H^+:1 sugar***	n.d.	−0.01 to −0.03[2,e,f]	>+20[10]	Various hexose sugars	E
ABA transport	ABCG22 ABCG40 AIT1 ABCG25 DTX50	AT5G06530 AT1G15520 AT1G69850 AT1G71960 AT5G52050	ABA import ABA import ABA import ABA efflux ABA efflux	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	F

[a]*Chara corallina*. [b]Arabidopsis. [c]*Neurospora crassa*. [d]*Vicia faba*. [e]*Commelina communis*. [f]*Pisum sativum*. [1]Estimated for 3 H^+:1 Ca^{2+}. [2]1 mM sugar, pH_o 5.5. [3]pH_o 4.5 to 7.5. [4]1 mM $[Ca^{2+}]_o$. [5]3 H^+:1 Ca^{2+}, pH_o 5.5, 1 mM $[Ca^{2+}]_o$. [6]200 μM K^+, pH_o 6.1. [7]275 μM K^+, pH_o 4.5. [8]pH_o 7, 100 μM Cl^-/NO_3^-. [9]0.1 mM Mal, pH_o 6.1. [10]0.1 mM Suc, pH_o 6.1. *Calculated assuming a transport rate of 60 H^+ s^{-1} (Sze et al., 1999), a current of 15 to 20 μA cm^{-2} (Blatt, 1987a; Clint and Blatt, 1989), and guard cell surface area of 3×10^{-5} cm^2. **Calculated from I_{sat} or the typical transport current assuming a transport turnover rate of 50 s^{-1} and guard cell surface area of 3×10^{-5} cm^2; estimates for the Ca^{2+}-ATPase are based on assumption of a 30-fold lower density than the plasma membrane H^+-ATPase (Sze et al., 2000) and H^+-coupled transporters scaled accordingly by current densities. ***Stoichiometry determined as the minimum thermodynamic requirement to drive net accumulation.

Table IV. *Predominant ion channels in the plasma membrane of guard cells and their functional characteristics*

Genetic codes relate to Arabidopsis, functional data relate to *V. faba* under typical guard cell ionic contents (see Table II) and external solutions of 10 mM KCl, 1 mM $CaCl_2$, pH 6.1, unless noted otherwise; channel currents are typical values at −200 mV (I_{Cl} S-type, $I_{K,in}$, I_{Ca}), −50 mV (I_{Anion} R-type), and +50 mV ($I_{K,out}$). Shorthand identifiers in parentheses cross-reference to Box 1. n.d., not determined. References are as follows: A (Schroeder et al., 1987; Blatt, 1990, 1992; Fairley and Assmann, 1991; Thiel et al., 1992, 1993; Luan et al., 1993; Obermeyer et al., 1994; Müller-Röber et al., 1995; Grabov and Blatt, 1997; Roelfsema and Prins, 1997; Forestier et al., 1998; Blatt et al., 1999; Sokolovski et al., 2008; Dreyer and Uozumi, 2011; Eisenach et al., 2012); B (Schroeder et al., 1987; Blatt, 1988b, 1999; Hosoi et al., 1988; Thiel and Blatt, 1991, 1994; Fairley-Grenot and Assmann, 1992; Thiel et al., 1992; Armstrong et al., 1995; Lemtiri-Chlieh, 1996; Roelfsema and Prins, 1997; Forestier et al., 1998; Miedema et al., 2000; Sokolovski et al., 2008); C (Keller et al., 1989; Hedrich et al., 1990; Linder and Raschke, 1992; Marten et al., 1992, 1993; Schroeder and Keller, 1992; Schmidt and Schroeder, 1994; Schmidt et al., 1995; Schwartz et al., 1995; Brearley et al., 1997; Grabov and Blatt, 1997; Pei et al., 1997; Dietrich and Hedrich, 1998; Forestier et al., 1998; Raschke et al., 2003; Negi et al., 2008; Vahisalu et al., 2008; Chen et al., 2010; Diatloff et al., 2010; Meyer et al., 2010; Sasaki et al., 2010; Geiger et al., 2011; Wang and Blatt, 2011; Mumm et al., 2013); D (Hamilton et al., 2000, 2001; White, 2000; Köhler and Blatt, 2002); E (Uehlein et al., 2003; Grondin et al., 2015; Byrt et al., 2016; Wang et al., 2016; Zhao et al., 2016).

Type	Name	Locus	Function	No.	γ	G_{max}	I_V	E_{rev}	δ	$v_{1/2}$	Activation voltage	Activation Time	Deactivation Time	Ion Selectivity	References
				cell^{-1}*	*pS*	*mS cm*$^{-2}$	*μA cm*$^{-2}$	*mV*		*mV*	*mV*	*$t_{1/2}$ in s*	*$t_{1/2}$ in ms*		
$I_{K,in}$ (KAT)	KAT1 KAT2 KC1 AKT2	AT5G46240 AT4G18290 AT4G32650 AT4G22200	K^+ uptake	900 −2,000	4–9 4[b]	0.3–0.4[1] 0.3–0.5[d] 0.7–1.1[a]	−50 to 120[f] −400 to −480[a]	−70	1.5–1.9[f]	−176 to −224 −155 to −180[d] −186 ± 2[a]	<−100	0.3–0.5	50–100	K^+>Rb^+>NH_4^+ >>Cs^+>Na^+	A
$I_{K,out}$ (GORK)	GORK	AT5G37500	K^+ release	200 −800	20–30	0.5–0.6[a,d,e]	100–300[a,d]	−70	1.8–2.1	0	>E_K	0.1–0.6	<30	K^+>Rb^+>Na^+ >Li^+>>Cs^+	B
I_{Anion}, R-type (ALMT)	ALMT12/ QUAC1	AT4G17970	Anion release, membrane depolarization	40 −200	38–40 9[a] 36[c]	0.01–0.04[2]	−1 to 2[f]	+20	−2.0–2.2[f]	−58 to −45 −116[a]	>−100	1–2 3.7–20[a]	20–50 8.4[a]	NO_3^-~SO_4^{2-} >I^->Br^- >Cl^-~Mal	C
I_{Cl}, S-type (SLAC)	SLAC1 SLAH2 SLAH3	AT1G12480 AT4G27970 AT5G24030	Cl^- release NO_3^- release	40 −200	34–36	0.01–0.02[3]	−5 to 10[f]	+10	<1[f]	>−200	>−200	10–20	200–500	NO_3^->Br^->F^- >Cl^->I^- >>Mal (SLAH3) Cl^->NO_3^- (SLAC1)	C
I_{Ca} (Ca_V)			Ca^{2+} entry	50 −300	12[f]	0.2–0.4[4]	−0.05 to 2	>+30 +17 (CNGCs)	−0.9 to 1.2 −1.0 to 1.2[a]	−100 to −140 −80 to −140[a] −110 ± 5[d]	<−100	<0.05	<50	Ca^{2+}~Ba^{2+} >>K^+>Cl^- Mg^{2+}~Ba^{2+}>Ca^{2+} (CNGCs)	D
Aquaporins	PIP2;1 PIP1;2	AT3G53420	H_2O transport H_2O_2 transport CO_2 transport	n.d.										can transport Na^+	E

[a]Arabidopsis. [b]*Zea mays*. [c]*Xanthium strumarium*. [d]*Nicotiana tabacum*. [e]*Vicia* faba. [f]Equivalent characteristics available in the literature for *Nicotiana tabacum*, Arabidopsis, and *Zea mays*. [1]pH_o 5.5 to 6.1, $[Ca^{2+}]_i$ < 200 nM. [2]pH_i 7.6 to 7.8, $[Ca^{2+}]_i$ < 200 nM. [3]pH_i 7.6 to 7.8, $[Ca^{2+}]_i$ < 200 nM. [4]$[Ca^{2+}]_i$ < 200 nM. *Calculated from whole-cell and single-channel currents recorded under equivalent conditions.

Table V. *Predominant pumps and carriers in the tonoplast of guard cells and their functional characteristics*

Genetic codes relate to Arabidopsis, functional data relate to *V. faba* unless noted otherwise; for I_{sat} current, saturation is at $V >> 0$ for ATPases or $V << 0$ for coupled transport; I_V are typical currents within the physiological voltage range and E_{rev} range with typical gradients of the relevant ionic species; currents were converted where necessary assuming 1 μA cm^{-2} = 1 pA pF^{-1}, tonoplast surface area of 2×10^{-5} cm^2, and volume of 3 pL. Shorthand identifiers in parentheses cross-reference to Box 1. n.d., not determined. References are as follows: A (Bentrup et al., 1986; Hedrich et al., 1989; Fricker and Willmer, 1990b, 1990a; Davies et al., 1991, 1992, 1994, 1996; Kasamo et al., 1991; Rea and Poole, 1993; Gambale et al., 1994; Ros et al., 1995; Willmer et al., 1995; Obermeyer et al., 1996; Darley et al., 1998; Facanha and de Meis, 1998; Gaxiola et al., 2007); B (Schumaker and Sze, 1986, 1990; Blackford et al., 1990; DuPont et al., 1990; Askerlund and Evans, 1992; Gavin et al., 1993; Askerlund, 1997; Harper et al., 1998; Hirschi, 1999; Palmgren and Harper, 1999; Geisler et al., 2000; Pardo et al., 2006; Pittman, 2011); C (Blumwald and Poole, 1985, 1987; Yamaguchi et al., 2003; Pardo et al., 2006; Rodríguez-Rosales et al., 2009; Chanroj et al., 2011; Barragán et al., 2012; Andrés et al., 2014; Han et al., 2015); D (Accardi and Miller, 2004; De Angeli et al., 2006, 2009a; von der Fecht-Bartenbach et al., 2010; Jossier et al., 2010); E (Burla et al., 2013).

Transporter	Name	Locus	Function	No.	Stoichiometry	I_{sat}	I_V	E_{rev}	Ion Selectivity	References
				$\times 10^6$ cell^{-1}		*μA cm^{-2}*	*μA cm^{-2}*	*mV*		
VH^+-ATPase (VH-ATPase)	VHA-A	AT1G78900	H^+ uptake, energization	3–8*	2 H^+:1 ATP	2[e]	1–2[e]	−94 to +23	Assumed high H^+ selective	A
	VHA-B	AT1G76030					3–3.5[a]			
	VHA-C	AT1G12840					0.6–1[d]			
	VHA-D	AT3G58730								
	VHA-E	AT4G11150								
	VHA-F	AT4G02620								
	VHA-G	AT3G01390								
	VHA-H	AT3G42050								
	VHA-a	AT2G28520								
	VHA-c	AT4G34720								
	VHA-c''	AT4G32530								
	VHA-d	AT3G28710								
	VHA-e	AT5G55290								
VH^+-PPase (VH-PPase)	AVP1	AT1G15690	H^+ uptake, energization	6–20**	1 H^+:1 PP_i	n.d.	0.6–2.1	−60 to −20		A
	AVP2	AT1G78920								
VCa^{2+}-ATPase (VCa-ATPase)	ACA4	AT2G41560	Ca^{2+} uptake	0.3–1***	1 Ca^{2+}:1 ATP****	See I_V	0.01–0.02[c,***]	−100 to −80	n.d.	B
	ACA11	AT3G57330					0.1[b,***]			
H^+/Ca^{2+} antiport (CAX)	CAX1	AT2G38170	Ca^{2+} exchange	0.1–0.3***	3 H^+:1 Ca^{2+}	See I_V	−0.6[1,b,***]	>+100	Assumed selective for Ca^{2+} and H^+	B
	CAX2	AT3G13320								
	CAX3	AT3G51860								
	CAX5	AT1G55730								
	CAX6	AT1G55720								
	CAX7	AT5G17860								
H^+/cation antiport	NHX1	AT5G27150	Cation exchange, pH_i regulation	0.1–0.3***	1 H^+:1 K^+					C
	NHX2	AT3G05030								
H^+/Cl^- (NO_3^-) antiport (CLC)	CLC-B	AT3G27170	Anion exchange	0.3–1***	1 H^+:2 Cl^-	See I_V	0.05–0.1[2,b]	−60 to 0	$NO_3^- > Cl^- >> SO_4^{2-}$	D
	CLC-E	AT4G35440			1 H^+:2 NO_3^-			−48[b]		
	CLC-A	AT5G40890								
	CLC-C	AT5G49890								
ABA transport	ABCC1	AT1G30400	ABA glycosyl ester uptake	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	E
	ABCC2	AT2G34660	ABA glycosyl ester uptake							

[a]*Chara corallina*. [b]Arabidopsis. [c]*Brassica oleracea*. [d]*Commelina communis*. [e]*Beta vulgaris*. [1]> 50 μM $[Ca^{2+}]_i$. [2]4 mM $[NO_3^-]_i$. *Calculated assuming a transport rate of 50 H^+ s^{-1}, a tonoplast surface area of 2×10^{-5} cm^2, and H^+ flux from *Commelina communis* (Willmer et al., 1995). **Calculated from current densities assuming a transport rate of 50 H^+ s^{-1} (Rea and Poole, 1993) and a tonoplast surface area of 2×10^{-5} cm^2. ***Calculated from I_{sat} or the typical transport current assuming a transport turnover rate of 50 s^{-1} and a guard cell surface area of 3×10^{-5} cm^2; estimates for the Ca^{2+}-ATPase are based on assumption of a 10- to 30-fold lower density than the V-type H^+-ATPase and H^+-coupled transporters scaled accordingly. ****Stoichiometry determined as the minimum thermodynamic requirement to drive net accumulation.

Table VI. *Predominant ion channels in the tonoplast of guard cells and their functional characteristics*

Genetic codes relate to Arabidopsis, functional data relate to *V. faba* under typical guard cell ionic contents (see Table II) unless noted otherwise; channel currents are typical values at 0 mV (I_{VK}, I_{Mal}, I_{VCa}, I_{VCl}) and +50 mV (I_{SV}). Shorthand identifiers in parentheses cross-reference to Box 1. n.d., not determined. References are as follows: A (Ward and Schroeder, 1994; Allen and Sanders, 1996; Allen et al., 1998; Bruggemann et al., 1999b, 1999a; Pei et al., 1999; Bihler et al., 2005; Sinnige et al., 2005; Gobert et al., 2007; Rocchetti et al., 2012); B (Allen and Sanders, 1994, 1995, 1996; Ward and Schroeder, 1994; Pei et al., 1999; Bihler et al., 2005; Pottosin and Schönknecht, 2007); C (Alexandre and Lassalles, 1990; Alexandre et al., 1990; Bezprozvanny et al., 1991; Johannes et al., 1992; Muir and Sanders, 1997; Pottosin and Schönknecht, 2007); D (Pei et al., 1996; Cheffings et al., 1997; Pantoja and Smith, 2002; Hafke et al., 2003; Meyer et al., 2011; De Angeli et al., 2013).

Type	Name	Locus	Function	No.	γ	G_{max}	I_V	E_{rev}	δ	$v_{1/2}$	Activation Voltage	Activation Time	Deactivation Time	Ion Selectivity	References
				cell^{-1}*	*pS*	*mS cm*$^{-2}$	*μA cm*$^{-2}$	*mV*		*mV*	*mV*	*$t_{1/2}$ in s*	*$t_{1/2}$ in ms*		
I_{VK} (TPK)	TPK1 KCO3	AT5G55630 AT5G46360	K^+ exchange	20–100	70–90 22–75[a]	1–1.4	0.1–0.2	E_K	n.d.	n.d.		<0.01	<10	K^+>Rb^+ >NH_4^+ >Na^+~Cs^+	A
I_{FV} (FV)			K^+ exchange	50–300	6 4–7[a]	n.d.	1.4–1.6	E_K	1	−30	≤0	<0.01	<10	NH_4^+>K^+ ~Rb^+ >Cs^+>Na^+	A
I_{SV}	TPC1	AT4G03560	Ca^{2+} homeostasis K^+, Ca^{2+} influx	100–500	14 (K^+) 27 (Ca^{2+})	2–3	n.d.	−10 to +30	2–4	20–50	≥0	1–2	n.d.	Ca^{2+}>K^+ >Cl^- Cations >anions	B
I_{VCa} (VCa_{in})			Ca^{2+} signaling	50–200	12–30	n.d.	n.d.	E_{Ca}	1	−25**	≤0	n.d.	n.d.	Ca^{2+}>>K^+	C
I_{VCl} (VCl)	ALMT9	AT3G18440	Cl^- (NO_3^-) exchange	50–300	34	2–4	n.d.	−40 to 0	−1	−50	≤0 <−60 (ALMT9)	<0.05	n.d.	Mal>Cl^- >>K^+	D
I_{Mal} (VMal)	ALMT6	AT2G17470	Mal exchange	100–500	6[a] 3[b]	n.d.	0.2–1.5[a] 0.2–1[b]	−40 to 0	−2	−50	≤0	0.5–1	n.d.	Can transport fumarate	D

[a]Arabidopsis. [b]*Kalanchoë daigremontiana*. *Channel densities calculated from whole-cell and single-channel currents recorded under equivalent conditions. **Characteristics introduced for the purpose of modeling (Hills et al., 2012).

Other transporters undoubtedly remain to be identified, and intermediates such as the gasotransmitter H_2S (Scuffi et al., 2014; Papanatsiou et al., 2015) and NO-dependent *S*-nitrosylation (Sokolovski and Blatt, 2004; Wang et al., 2015) remain to be placed within the broader framework of guard cell physiology. Most notable, we still do not know the molecular identity of the major Ca^{2+}-permeable channels responsible for Ca^{2+} influx across the plasma membrane, nor for the channels mediating Ca^{2+}-evoked release of Ca^{2+} from the several intracellular compartments that serve as Ca^{2+} stores (see "The Ca^{2+} Signal, Its Origins and Oscillations" below). These channels are nonetheless well documented in vivo, with their kinetic and regulatory characteristics defined with respect to single-channel conductance, voltage and Ca^{2+} sensitivities, their dependence on ROS and NO, and their requirements for phosphorylation (Hamilton et al., 2000, 2001; Pei et al., 2000; Garcia-Mata et al., 2003; Kwak et al., 2003; Wang et al., 2013).

Indeed, even without knowledge of individual genes and the proteins they encode, electrophysiological studies provide quantitative information about the kinetic and regulatory properties that are essential to understand the functions of these transporters and their contributions to stomatal movements. For example, the biophysical and regulatory properties of the outward-rectifying K^+ channels in guard cells were known in detail early on, including their dependence on voltage and external K^+, their regulation by pH, and their insensitivity to $[Ca^{2+}]_i$ (Blatt, 1988b; Hosoi et al., 1988; Blatt and Armstrong, 1993). This knowledge informed on their function in vivo more than a decade before the GORK K^+ channel was identified (Ache et al., 2000; Hosy et al., 2003). Such information is essential to understand their mechanics and physiology in the guard cell context (see "Systems Models for Guard Cell Transport" below). A comprehensive list of these transporters and their functional characteristics at the plasma membrane and tonoplast are included in Tables III to VI, together with the corresponding genes from Arabidopsis where known. The major regulatory links to the transporters are summarized in Tables VII and VIII.

Conversely, for stomatal function, knowledge of a gene product based on mutant analysis alone is often uninformative and, without functional information, can be misleading. The Golgi-localized alkaline ceramidase, TOD1, is a case in point (Chen et al., 2015). Ceramidases are enzymes that cleave phospholipids and, together with sphingosine kinases, are important for sphingosine-1-phosphate (S1P) synthesis. Stomatal closure in ABA is promoted by adding exogenous S1P (Guo et al., 2012), suggesting that its synthesis might be important for ABA signaling. The TOD1 promoter is active in guard cells, and its gene product is able to complement a yeast mutant lacking ceramidase activity. However, TOD1 is also present in other cell types, the *tod1* mutation affects stomatal aperture in both the presence and absence of ABA, and its phenotype is pleiotropic, affecting stomata, pollen growth rate, and fertility. So, is TOD1 part of an ABA signal cascade? Or is it part of an assembly necessary for the general integrity of cellular homeostasis, including ABA signaling? Clearly, what is missing is evidence that TOD1 activity responds to ABA, its kinetics, and, downstream, the identity of the targets for S1P.

PHOSPHORYLATION CASCADES

A number of protein kinases and phosphatases have been identified to affect stomatal movements, both opening and closing. Initially, much information was drawn from inhibitor studies, their actions on aperture, ion flux, and transport current (Macrobbie, 1997; Blatt, 2000). Subsequent work has benefitted from mutational screening and site-directed mutagenesis, and in a handful of cases, we now have knowledge of their phosphorylation targets. Table IX summarizes the major groups of these kinases and phosphatases, and we direct the reader to several excellent reviews (Shimazaki et al., 2007; Cutler et al., 2010; Lee et al., 2016), including those in this Focus Issue, for further information.

Recent work has taken advantage of the *Xenopus* oocyte as a platform to reconstitute plausible regulatory cascades with the SLAC1 anion channel and several protein kinases, including the SnRK2-type kinase OST1 originally identified in thermal screening for reduced drought sensitivity (Merlot et al., 2002; Mustilli et al., 2002), the GHR1 kinase (Hua et al., 2012) associated with ROS-mediated control of SLAC1, the HT1 kinase associated with CO_2 (Tian et al., 2015; Hashimoto-Sugimoto et al., 2016), and the kinase partners of calcineurin B-like (CBL) proteins (Luan et al., 2002; Maierhofer et al., 2014). These studies support the idea of phosphorylation cascades that contribute to Cl^- channel regulation by ABA. What they have largely failed to address to date are the connections between protein phosphorylation, whether of SLAC1 or associated targets, and its integration with other signaling intermediates and transporters in vivo. For example, in analyzing the *V. faba* SLAC-like Cl^- channel, Chen et al. (2010) found two separate components to the channel current. Quantitative analysis showed that ABA increased the amplitude of the minor component independent of $[Ca^{2+}]_i$. However, the primary response was a shift in the $[Ca^{2+}]_i$ sensitivity of the major component, from a K_{Ca} of 720 nM to a value near 500 nM, which sets to rest the ill-defined concept of so-called Ca^{2+}-priming (Siegel et al., 2009). Stange et al. (2010) reported a similar $[Ca^{2+}]_i$ sensitivity, although lacking the effects of phosphorylation. In effect, Chen et al. (2010) show that the primary action of phosphorylation in vivo is to enhance the sensitivity of the Cl^- channels to $[Ca^{2+}]_i$.

Oocyte reconstitution studies with SLAC1 to date have not considered the $[Ca^{2+}]_i$ sensitivity of the channel nor tested the effects of $[Ca^{2+}]_i$ on its activity after

Table VII. *Activators and inhibitors of the predominant plasma membrane transporters and ion channels in stomatal guard cells (data relate to V. faba or Arabidopsis unless noted otherwise)*

References are as follows: A (Blatt, 1988a; Blatt and Clint, 1989; Clint and Blatt, 1989; Lohse and Hedrich, 1992; Goh et al., 1995, 1996; Kinoshita et al., 1995; Baunsgaard et al., 1998; Kinoshita and Shimazaki, 1999; Taylor and Assmann, 2001); B (Palmgren and Harper, 1999; Geisler et al., 2000; Sze et al., 2000); C (Blatt et al., 1990b; Blatt, 1992; Fairley-Grenot and Assmann, 1992; Lemtiri-Chlieh and MacRobbie, 1994; Kelly et al., 1995; Ilan et al., 1996; Grabov and Blatt, 1997, 1999; Romano et al., 2000; Garcia-Mata et al., 2003; Dreyer and Uozumi, 2011); D (Blatt and Armstrong, 1993; Ilan et al., 1994; Lemtiri-Chlieh and MacRobbie, 1994; Miedema and Assmann, 1996; Blatt and Gradmann, 1997; Grabov and Blatt, 1997; Garcia-Mata et al., 2003; Hosy et al., 2003; Eisenach et al., 2012); E (Dietrich and Hedrich, 1998; Garcia-Mata et al., 2003; Chen et al., 2010; Diatloff et al., 2010; Meyer et al., 2010; Wang and Blatt, 2011; Xue et al., 2011; Tian et al., 2015; Wang et al., 2016); F (Hamilton et al., 2000; Pei et al., 2000; Sokolovski et al., 2008); G (Maurel et al., 2008; Verdoucq et al., 2008; Grondin et al., 2015; Byrt et al., 2016).

Transporter/ Channel Type	Activators	Inhibitors	References
H^+-ATPase	Fusicoccin ($K_{1/2}$ = 10 μM) Blue light (20–100 μmol m^{-2} s^{-1} via photophosphorylation) Auxin	$[Ca^{2+}]_i$ (K_i = 300 nM)	A
Ca^{2+}-ATPase	$[Ca^{2+}]_i$ ($K_{1/2}$ = 0.5–1 μM) Calmodulin (approximate molar ratio)		B
$I_{K,in}$	$[H^+]_o$ shifts $v_{1/2}$ positive (+15–24 mV per pH unit) and enhances G_{max} (pK_a = 6.7; n_h = 1) Increasing $[H^+]_i$ enhances G_{max}	$[Ca^{2+}]_i$ (K_i = 330 nM; n_h = 3.8–4.1) shifts $v_{1/2}$ negative and suppresses G_{max} $[Al^{3+}]$ (K_i = 15 μM) $[Cs^+]$ (K_i = 0.1 mM)	C
$I_{K,out}$	Decreasing $[H^+]_i$ Increasing $[Ca^{2+}]_i$ ABA	Increasing $[H^+]_i$ (pK_a = 7.4–7.5; n_h = 2–2.4)	D
I_{Anion} (R-type)	$[ABA]_i$ ($K_{1/2}$ = 2.6 μM) [ATP] ($K_{1/2}$ = 0.4 mM) $[Ca^{2+}]_i$ enhances G_{max} (500–700 nM, n_h = 3–4) $[H^+]_i$ enhances G_{max} (pK_a = 6.9, n_h = 1) ABA (Ca^{2+} independent)	$[H^+]_o$ slows kinetics without affecting steady-state I_{Cl}	E
I_{Cl} (S-type)	$[ABA]_{cyt}$ ($K_{1/2}$ = 2.6 μM; Ca^{2+} dependent or independent) $[Ca^{2+}]_i$ enhances G_{max} ($K_{1/2}$ = 500–700 nM, n_h = 3–4) High $[HCO_3^-]_i$ increases $[Ca^{2+}]_i$ sensitivity High $[HCO_3^-]_i$ (Ca^{2+} independent)	Insensitive for $[H^+]_o$	E
I_{Ca}	ROS (H_2O_2) ABA shifts voltage sensitivity for Ca^{2+} gating	$[K^+]_i << 1$ mM $[Ca^{2+}]_i$ shifts $v_{1/2}$ negative (−25–30 mV/$[Ca^{2+}]_i$ decade) $[H^+]_i$ shifts $v_{1/2}$ negative (possible −55 mV per pH unit)	F
Aquaporins	ABA	High $[Ca^{2+}]_i$ $[Cd^+]_i$, $[Mn^{2+}]_i$, $[Sr^{2+}]_i$, $[Ba^{2+}]_i$, $[Ni^{2+}]_i$ High $[H^+]_i$	G

heterologous expression. Indeed, to our knowledge, no evidence has surfaced to confirm that SLAC1 activation is solely dependent on phosphorylation in oocytes, whether via $[Ca^{2+}]_i$-dependent kinases or otherwise. The findings to date, therefore, beg questions about the target sites, kinase and phosphatase specificities, and their relationships to SLAC1 control in vivo. Nor are the data clear cut or consistent. Initial work suggested that $[Ca^{2+}]_i$ could promote phosphorylation by the CPK21 kinase of a SLAC1 N-terminal peptide in vitro (Geiger et al., 2010) and with a similar $[Ca^{2+}]_i$ dependence to that of the current in vivo (Chen et al., 2010). However, coexpression of SLAC1 with CPK21 yielded little current (Geiger et al., 2010), possibly because $[Ca^{2+}]_i$ was too low in the oocytes.

Subsequent studies focused on other kinases. Analysis of SLAC1 peptides phosphorylated in vitro suggested that OST1, CPK3, and CPK6 kinases can target Ser-59 and that OST1 and CPK6 also can phosphorylate Ser-120, both residues located in the cytosolic N-terminal domain of the channel (Vahisalu et al., 2010; Brandt et al., 2015). The $SLAC1^{S59A}$ mutation, which prevents phosphorylation at this site, reduced SLAC1 activation in oocytes by OST1, CPK6, and the CBL1-CIPK23 calcineurin-kinase pair (Maierhofer et al., 2014). Yet, a separate study found OST1 to fully activate the $SLAC1^{S59A}$ channel (Brandt et al., 2015). Other inconsistencies are evident when comparing the reconstituted systems in oocytes with the in vivo characteristics in the guard cell. Most telling, the *ost1*

Table VIII. *Activators and inhibitors of the predominant tonoplast transporters and ion channels in stomatal guard cells (data relate to V. faba or Arabidopsis unless noted otherwise)*

References are as follows: A (Fricker and Willmer, 1990a; Davies et al., 1991; Willmer et al., 1995; Obermeyer et al., 1996; Darley et al., 1998); B (Evans, 1994); C (De Angeli et al., 2009b); D (Ward and Schroeder, 1994; Allen and Sanders, 1996; Allen et al., 1998; Lemtiri-Chlieh et al., 2003); E (Schulz-Lessdorf and Hedrich, 1995; Pei et al., 1999; Carpaneto et al., 2001; Lemtiri-Chlieh et al., 2003; Pottosin et al., 2004; Bihler et al., 2005; Pottosin and Schönknecht, 2007; Beyhl et al., 2009); F (Pantoja and Smith, 2002; Meyer et al., 2011).

Transporter/Channel Type	Activators	Inhibitors	References
VH^+-ATPase	ABA NaCl $[K^+]_i$ $[Cl^-]_v$	$[NO_3^-]_i$ (K_i = 7 mM) H_2O_2 (K_i = 800 μM) Insensitive to ABA and fusicoccin	A
VH^+-PPase	High $[K^+]_i$ ($K_{1/2}$ = 2–50 mM) Low $[K^+]_v$	$[Ca^{2+}]_i$ (K_i = 80 nM)	A
VCa^{2+}-ATPase	Calmodulin (approximate molar ratio)		B
H^+/Cl^- (NO_3^-) antiport		ATP (up to 60% inhibition of CLCa)	C
I_{VK} (TPK)	$[H^+]_i$ (pK_a = 7.4) $[Ca^{2+}]_i$ ($K_{1/2}$ = 1–30 μM)		D
I_{FV}	IP_6 ($K_{1/2}$ = 100 nM) Low $[H^+]_i$	$[Mg^{2+}]_v$ (K_i = 0.23 mM, n_h = 0.67) $[Ca^{2+}]_i$ (K_i = 200 nM) $[H^+]_i$ ($pK_a \sim 6.4$)	D
I_{SV}	$[Ca^{2+}]_i$ ($K_{1/2}$ = 3–30 μM) $[Mg^{2+}]_i$ shifts $v_{1/2}$ negative IP_6 ($K_{1/2}$ = 100 nM)	$[Ca^{2+}]_v$ shifts $v_{1/2}$ positive (+55 mV/$[Ca^{2+}]_v$ decade) $[Zn^{2+}]$, $[Ni^{2+}]$ $[H^+]_i$ ($pK \sim 6_t8$) $[Zn^{2+}]_i$	E
I_{VCl}	$[Ca^{2+}]_i$ ($K_{1/2}$ = 1 μM) $[Ca^{2+}]_i$ independent (ALMT) $[Mal^{2-}]_i$ (ALMT9) High $[H^+]_i$		F
I_{Mal}	$[Ca^{2+}]_i$ ($K_{1/2}$ = 1 μM; activation of ALMT9 Ca^{2+}-independent)	$[H^+]_i$ (pK_i = 7.1, n_h = 2[a]) $[H^+]_v$ shifts $v_{1/2}$ positive (+60 mV per pH unit)	F

[a] *Kalanchoë daigremontiana.*

null mutant suppressed the ABA activation of SLAC1 current in Arabidopsis guard cells (Acharya et al., 2013), even though several other kinases and their associated cascades were present. The mutant *cipk23* showed enhanced sensitivity to ABA and closed the stomata (Cheong et al., 2007). Finally, complementations of the *slac1* mutant with $SLAC1^{S120A}$ had little effect on ABA-mediated stomatal closure and with deletion of the entire N-terminal domain of SLAC1 suppressed closure only partially (Yamamoto et al., 2016). This latter study does present difficulties for interpretation, notably the dissimilar effects of complementations in two different *slac1* mutant lines and a lack of some key controls, but the work appears to highlight differences in SLAC1 activation by elevated pCO_2 and by ABA.

At least for the discrepancies between oocytes and guard cells, the most plausible explanation is that a subset, possibly all, of these kinases engage different targets in vivo from those available when reconstituted in oocytes and that their regulation of SLAC1 is normally indirect. It may be, too, that the work with SLAC1 in oocytes to date has simply refined our knowledge of the minor, $[Ca^{2+}]_i$-independent component of the current found in vivo (Chen et al., 2010). In assessing the actions of the kinase and phosphatase mutants, we need to keep in mind that the guard cells also harbor other anion channels, including ALMT12, which is also affected by the OST1 kinase, but for which less information is available at present (Meyer et al., 2010; Imes et al., 2013). So, in the absence of supporting data in vivo, the relevance of the studies in oocytes must be interpreted with caution. It is time that experiments move beyond reconstitution studies in oocytes. Further progress now will depend, most importantly, on time-resolved phosphorylation assays carried out in vivo.

COORDINATING ION TRANSPORT, MEMBRANE TRAFFIC, AND WATER FLUX

Guard cells integrate ion transport with secretory traffic that adds new membrane surface as the cells expand; conversely, rates of endocytosis coordinate with solute export as the cell volume decreases. While membrane traffic has generally correlated with changes in external osmolality and cell volume (Homann and

Table IX. *Major groups of kinases involved in regulation of guard cell transporters and channels*

Functions listed relate to the kinase group as a whole, not necessarily to the specific kinase on the same line of text. References are as follows: A (Pei et al., 1996; Mori et al., 2006; Zhu et al., 2007; Geiger et al., 2010; Zou et al., 2010, 2015; Ronzier et al., 2014; Brandt et al., 2015; Li et al., 2016); B (Fujii et al., 2009; Geiger et al., 2009; Lee et al., 2009; Park et al., 2009; Sato et al., 2009; Sirichandra et al., 2009; Umezawa et al., 2009; Imes et al., 2013; Osakabe et al., 2013; Wege et al., 2014; Grondin et al., 2015; Yin et al., 2016); C (Guo et al., 2002; Ohta et al., 2003; Xu et al., 2006; Cheong et al., 2007; Held et al., 2011; Maierhofer et al., 2014); D (Gosti et al., 1999; Merlot et al., 2001; Chérel et al., 2002; Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006; Yoshida et al., 2006; Ma et al., 2009; Park et al., 2009; Rubio et al., 2009; Lan et al., 2011; Brandt et al., 2015; Lefoulon et al., 2016; Xie et al., 2016); E (Leonhardt et al., 2004; Saez et al., 2004, 2006; Park et al., 2009; Nishimura et al., 2010); F (Jammes et al., 2009; Hõrak et al., 2016).

Name	Range of Functions	References
Ca^{2+}-dependent protein kinases (CDPKs)	ABA-induced Ca^{2+}-dependent activation of anion currents	A
CPK3	SLAC1 activation	
CPK4	ABA-mediated regulation of CAT3 activity	
CPK5	Inhibition of KAT2 and KAT1	
CPK6	Activation of vacuolar anion channels	
CPK8		
CPK10		
CPK11		
CPK13		
CPK21		
CPK23		
CPK32		
CPK33		
SnRK2 kinases	ABA-induced Ca^{2+}-independent activation of anion currents	B
SRK2E/SnRK2.6/OST1	SLAC1 activation in an ABI1-dependent manner	
SRK2D/SnRK2.2	ALMT12/QUAC1 activation	
SRK2I/SnRK2.3	ABA-induced phosphorylation of K^+ uptake transporter6 (KUP6)	
SRK2C/SnRK2.8	ABA-induced phosphorylation of aquaporin PIP2;1	
	Involved in methyl jasmonate-induced stomatal closure	
	Inactivation of KAT1	
	Activation of NADPH oxidase subunit AtrbohF (ROS production)	
	Activation of CLCa to increase anion efflux from the vacuole	
SnRK3 kinases (CIPKs)	Ca^{2+}-dependent activation of anion currents	C
CIPK6	SLAC1 and SLAH3 activation in the presence of CALCINEURIN-B-LIKE1 and CALCINEURIN-B-LIKE9	
CIPK15/PKS3		
CIPK23	Increases phosphorylation-independent translocation of AKT2 to the plasma membrane	
CIPK24/SOS2	Stomatal closure in response to ABA by interacting with ABI1 and ABI2	
Protein phosphatase 2Cs	Deactivation of ABA-activated SnRK2 protein kinases by dephosphorylation	D
PP2CA/AHG3	SLAC1 dephosphorylation in the absence of ABA	
ABI1	SnRK2 inhibition (interaction with OST1, SnRK2.2, and SnRK2.3)	
ABI2	Decrease of GORK channel activity	
HAB1	Decrease of AKT2 channel activity	
AIP	Dephosphorylation and down-regulation of SLAC1	
	Suppression of CPK activation of SLAC1	
	Inhibition of anion current activation by CBL-CIPK	
RCAR/PYR/PYL ABA receptors	Inhibition of PP2Cs to enable SnRK2-mediated stomatal closure	E
PYR1	Interaction with and repression of ABI1, ABI2, and HAB1	
PYL1		
PYL2		
PYL3		
PYL4		
PYL9/RCAR1		
Mitogen-activated protein kinases (MPKs)		F
MPK4	Involved in ROS-mediated ABA signaling	
MPK9	Positive regulator of CO_2-induced stomatal regulation by inhibition of HT1 kinase	
MPK12		

Thiel, 2002; Shope et al., 2003; Hurst et al., 2004; Meckel et al., 2005; Shope and Mott, 2006), these studies, and those of other plant cell models (Campanoni and Blatt, 2007; Kroeger et al., 2011), have offered few clues to the mechanisms linking membrane traffic and ion transport. One mechanism that surfaced recently follows on the identification of the plasma membrane protein SYP121, and its tobacco (*Nicotiana tabacum*) homolog NtSYR1, associated with the ABA regulation of guard cell ion channels (Leyman et al., 1999). These proteins belong to the superfamily of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that were first characterized in the 1980s and later recognized as part of the molecular machinery for secretory traffic in yeast, animals, and plants (Söllner et al., 1993a, 1993b; Sanderfoot et al., 2000; Duman and Forte, 2003). The association with ion transport is well illustrated by Eisenach et al. (2012), who reported that the *syp121* mutation impairs stomatal reopening following closure in elevated $[Ca^{2+}]_i$ (Fig. 2). The mutant mimicked the phenomenon of so-called programmed closure, previously ascribed to a memory of stress that leads stomata to reopen only slowly (Allen et al., 2001). The *syp121* mutant showed reduced recycling of the KAT1 K^+ channel from endosomal membranes to the plasma membrane, thereby suppressing channel-mediated K^+ uptake by the guard cells, slowing stomatal reopening, and leading to a strong reduction in vegetative growth through stomatal-limited photosynthesis.

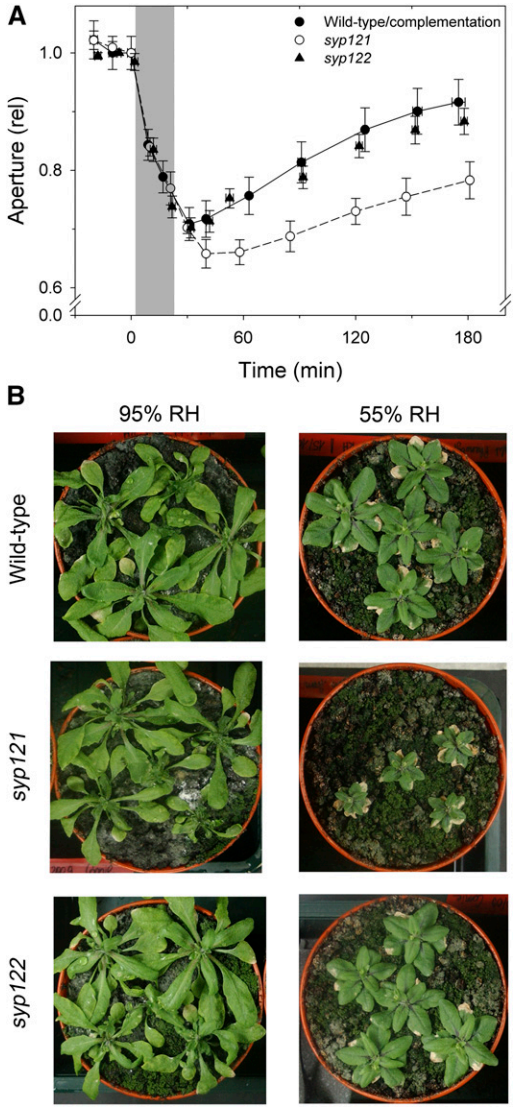


Figure 2. The *syp121* SNARE mutation slows stomatal reopening and shows a strong growth phenotype at moderate relative humidities. A, Stomatal apertures normalized to values at time zero for stomata from the wild type and the SYP121-complemented *syp121* mutant (black circles) and the mutants *syp121* (white circles) and *syp122* (black triangles) before, during, and after the closing stimulus of elevated $CaCl_2$ outside (gray bar). B, Arabidopsis wild-type, *syp121*, and *syp122* plants grown for 3 weeks under 150 μmol m^{-2} s^{-1} light and relative humidities (RH) of 95% and 55%. (This figure was modified from Eisenach et al., 2012.)

The connections of the SNAREs to solute transport go well beyond ion channel traffic, however, as was recognized early on (Leyman et al., 1999; Sutter et al., 2006, 2007). Honsbein et al. (2009, 2011) uncovered direct and selective binding between SYP121 and the K^+ channels KC1 and KAT1. Channel binding occurred at the plasma membrane independent of channel traffic and, in roots as in aerial tissues, binding promoted channel activity and K^+ uptake (Geelen et al., 2002; Sokolovski et al., 2008; Grefen et al., 2010a). Intriguingly, the cognate SNARE VAMP721, which is localized to the vesicle membrane and assembles with SYP121 for vesicle fusion, also binds and modulates the K^+ channels but with opposing effects on channel activity (Zhang et al., 2015). Thus, from the viewpoint of the capacity for K^+ uptake, the ion flux may be temporally coupled with an exchange in channel binding between cognate SNAREs during the process of vesicle fusion (Karnik et al., 2017).

The complementary site for SYP121 binding resides at the base of the K^+ channel S1 α-helix, which forms part of the channel voltage sensor domain (VSD; Grefen et al., 2015). Voltage-sensitive K^+ channels, including KC1, AKT1, and KAT1 in Arabidopsis, belong to the superfamily of so-called Kv channels that are found across all phyla. These channels assemble as tetramers of four subunits, each subunit consisting of six transmembrane α-helices. The first four α-helices (S1–S4) of the channel protein form a semiautonomous VSD structure. These VSDs incorporate a series of fixed positive charges that, with a change in voltage, drive the VSD conformation, moving it partway across the membrane and drawing open the channel pore (Lai et al., 2005; Dreyer and Blatt, 2009; Labro et al., 2012). Grefen et al. (2015) found that the VSD promoted secretory traffic so long as the VSD was locked in the open channel conformation (Lefoulon et al., 2014) or could be driven to this conformation by voltage (Fig. 3). These findings demonstrate that SYP121 commandeers the channel VSD to sense the membrane voltage as a proxy for solute uptake, adjusting the rates of secretory traffic with uptake while promoting K^+ transport.

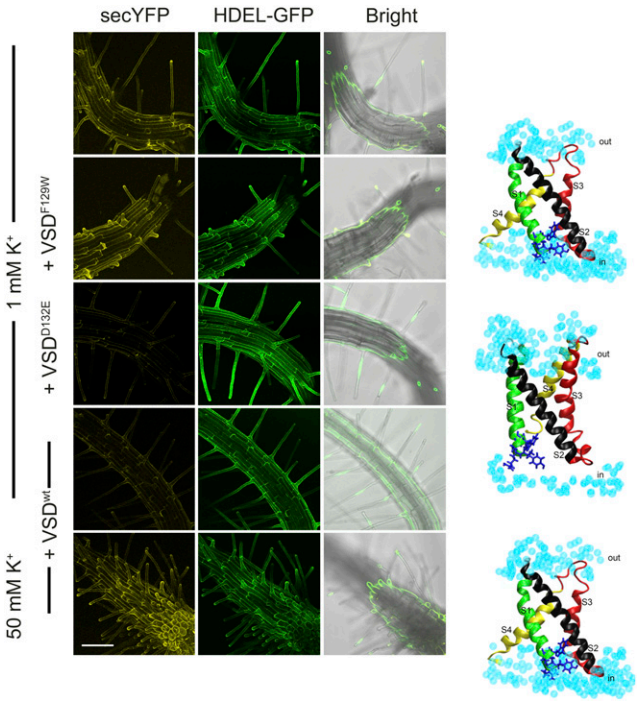


Figure 3. Voltage-dependent conformation of a K^+ channel VSD regulates secretion. Coexpression of the VSD (VSD^{wt}) of the K^+ channel KC1 and its mutant VSD^{D132E} rescues secretory traffic block by the SYP121 soluble domain $SYP121^{\Delta C}$. Coexpression with the mutant VSD^{D132E}, which locks the VSD in the open-channel configuration, also rescues secretory traffic. Traffic is not rescued by coexpression with VSD^{F129W}, which locks the VSD in the closed-channel configuration, nor with VSD^{wt} in 50 mM KCl, which depolarizes the plasma membrane. Images are projections of Arabidopsis roots transiently transformed using the tetracistronic vector pTecG-2in1-CC (Karnik et al., 2013) carrying secretory marker secYFP, GFP-HDEL as a transformation marker and ratiometric reference, $SYP121^{\Delta C}$, and VSD^{wt}, VSD^{F129W}, or VSD^{D132E} in 1 and 50 mM KCl (left). Bright-field images are single medial plane images with fluorescence overlaid. VSD structures are shown in the closed, open, and again closed conformations (right, top to bottom) corresponding to the conditions and VSD constructs used. For clarity, only water molecules (light blue) on either side (in and out) of the membrane are shown. VSD transmembrane α-helices are color coded in green (S1), black (S2), red (S3), and yellow (S4). The RYxxWE motif that forms the binding site for SYP121 is shown with stick representations. Bar = 100 μm. (This figure was modified from Grefen et al. 2015, and Karnik et al. 2017.)

Such coupling between SNAREs and channel VSDs may be common in plants. Both motifs are closely conserved within subsets of plasma membrane SNAREs and K^+ channels in vascular plants (Grefen et al., 2011, 2015), implying their coevolution as the number of SNARE genes expanded when plants colonized land (Sanderfoot, 2007; Karnik et al., 2017). The same SNAREs also interact with several plasma membrane-localized aquaporins (Besserer et al., 2012; Hachez et al., 2014), suggesting functional impacts that extend to plasma membrane water flux. It remains to be seen whether these interactions affect water permeability directly in addition to aquaporin traffic (Chaumont and Tyerman, 2014), possibly to aquaporin function associated with stomatal closure in ABA (Grondin et al., 2015). Certainly, there is reason to suspect that water flux, like that of solute transport, may be coordinated directly with membrane traffic as part of a supermolecular response complex.

THE IMPORTANCE OF VOLTAGE CONTROL

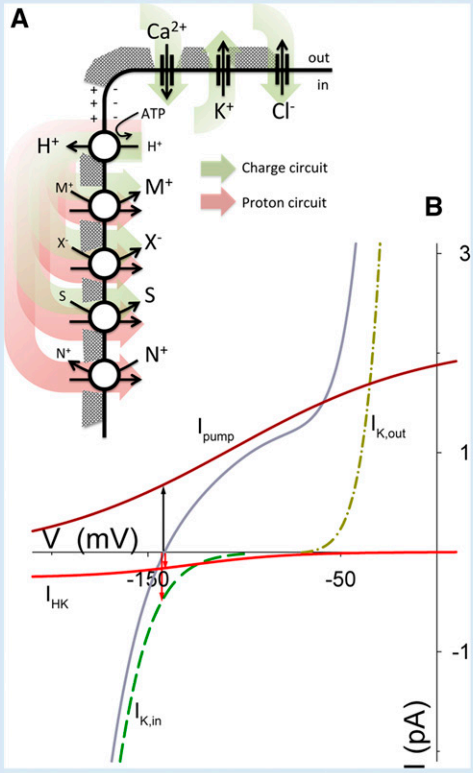
If this wealth of information on guard cell transport is not daunting enough, it is further compounded by the interactions of solute transport across each bounding membrane. Separating the intrinsic characteristics of transport interactions from those of extrinsic regulation, such as by protein phosphorylation, is often challenging. Membrane voltage is a major factor determining osmotic solute flux for stomatal movements (Tables III–VI), and it is central to understanding these transport interactions. From an enzyme kinetic standpoint, voltage serves as a driving force, an electrical substrate, that acts on each charge-carrying transporter in a manner analogous to the mass action effect of adding a chemical substrate to an enzymatic reaction; voltage is also an electrical product of charge-carrying transporters (Blatt, 2004). Following this simple analogy (Box 2; Sanders and Slayman, 1982), plasma membrane voltage is a product of H^+ export by the H^+-ATPases; it is a substrate for K^+ and Cl^- import by H^+-coupled transport; and it is a substrate for K^+ flux through the two major classes of K^+ channels, defined by the currents $I_{K,in}$ and $I_{K,out}$, and for anion efflux. Most important, voltage is a shared intermediate in the charge circuit of each membrane and, therefore, ensures an interdependency between all charge-carrying transporters.

Voltage also determines the activity of several ion channels that contribute to solute flux across both the plasma membrane and the tonoplast. For the K^+ channels that often dominate the plasma membrane (Blatt et al., 2007; Pandey et al., 2007; Lawson and Blatt, 2014), voltage affects channel gating and commonly restricts ion flux. For example, the outward-rectifying K^+ channels of plants, including GORK (Hosy et al., 2003), normally gate open only at voltages positive of the K^+ equilibrium voltage (E_K) when the thermodynamic driving force for K^+ flux is directed out of the cell. When the voltage is situated negative of E_K, the gates close within milliseconds, thereby preventing net K^+ influx through these channels. By contrast, inward-rectifying K^+ channels, including the KAT1 K^+ channel of Arabidopsis, typically gate open at voltages substantially more negative than −120 mV and achieve maximum activity only at voltages negative of −180 mV. These two populations of K^+ channels give rise to two of the most distinctive properties of the guard cell plasma membrane: they effectively delimit the range of physiological voltages normally observed in vivo, and they define the

BOX 2. Electroenzymatics

The "products" of H^+-ATPase transport are the buildup of charge, negative inside relative to the outside, and of [H^+] outside as H^+ ions are pumped across the plasma membrane. Both products, the membrane voltage and the pH gradient, contribute to the $\Delta\mu_H$ and oppose the H^+-ATPase. In turn, voltage and proton concentration are substrates for transporters that use either (or both) driving forces to move other solutes across the membrane (see figure A). The uptake of Cl^- across the plasma membrane, and undoubtedly that of Mal, is coupled to the $\Delta\mu_H$ (Barbier-Brygoo et al., 2011; Chen et al., 2012; Hills et al., 2012). There is evidence for high-affinity, H^+-coupled transport of K^+ in guard cells (Blatt, 1988a), and, of course, voltage drives the movement of ions through all ion channels in the membrane. In short, both voltage and the [H^+] are shared intermediates. Voltage, especially, affects the charge circuit and the kinetics of transport through each transporter interdependently. Here, the membrane circuit (A) for charge is indicated by the green arrows, and the circuit for H^+ is indicated by the red arrows. Carriers that move charged (M^+, N^+, and X^-) and uncharged (S) solutes coupled to the movement of H^+ are indicated along the vertical membrane. Ion channels for Ca^{2+}, K^+, and Cl^- are shown along the horizontal membrane. Physical laws dictate that at the free-running voltage the charge circuit must always sum to zero. The H^+ circuit is not constrained in the same manner; indeed, much of the H^+ substrate for the H^+-ATPase is thought to come from metabolic activity within the cell (Sanders and Slayman, 1982; Chen et al., 2012; Horrer et al., 2016).

An alternative way of viewing this information makes use of current-voltage (IV) curves (B), which also describe the rates of transport, literally the current (=charge per unit time) as a function of voltage. The IV curves shown here were generated using OnGuard and are typical of IV curves of Arabidopsis guard cells during opening (Wang et al., 2012). The total membrane IV curve (grey) is the sum of all current across the membrane at each voltage and the point at which the curve crosses the voltage axis, where sum of all currents is zero, marks the free-running voltage. Away from this point, there is either a net negative (inward) or positive (outward) current. Separating the total membrane IV curve into the component IV curves for each transporter often relies on challenge with (ant-)agonists and, for voltage-gated channels, on the long times needed for channel gating relative to the time needed to clamp voltage (Blatt, 2004). The total membrane IV curve here is broken down to show the major currents relevant to K^+ transport, those of the H^+-ATPase (solid, dark-red line; I_{pump}), the inward-rectifying (dashed green line; $I_{K,in}$) and outward-rectifying (dash-dotted green line; $I_{K,out}$) K^+ channel, and H^+-K^+ cotransport (solid, light-red line; I_{HK}). Current amplitudes and directions are indicated by the arrows at the free-running voltage, the vector sum of which (together with smaller currents of Ca^{2+} and Cl^- transport; data not shown) adds up to zero. Such analysis shows that all four transporters are kinetically limited by voltage (indeed $I_{K,out}$ is not active at this free-running voltage [see Box 3]) and by the balance of currents through each of the other transporters.



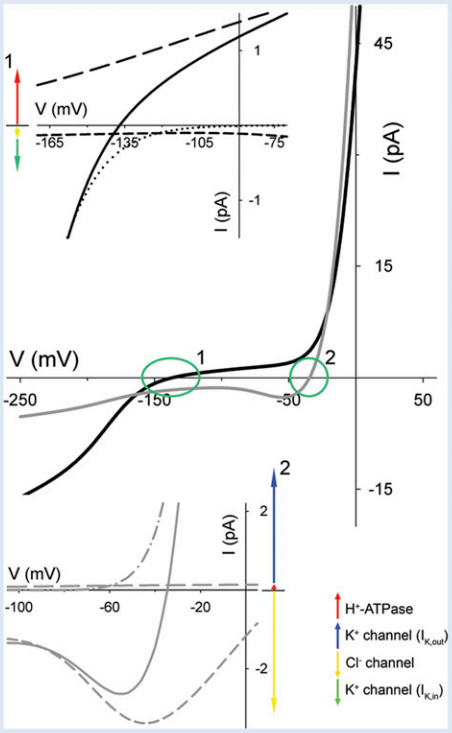
two distinct states of the membrane associated with solute influx and efflux (Box 3).

Ca^{2+} CONTROL OF OSMOTIC SOLUTE EFFLUX

Unquestionably, $[Ca^{2+}]_i$ overlays much of guard cell signal transduction and, especially, of voltage control in membrane transport (Tables III–VIII; Blatt, 2000; Hetherington and Brownlee, 2004; Martinoia et al., 2007; Roelfsema and Hedrich, 2010). Although changes in $[Ca^{2+}]_i$ have not always been associated with stomatal movements (Gilroy et al., 1991; Lemtiri-Chlieh and MacRobbie, 1994; Armstrong et al., 1995; Romano et al., 2000), its elevation is thought to accelerate closure and its reduction is

BOX 3. The Bistable Characteristic of the Plant Plasma Membrane

Like many plant cells, the guard cell membrane shows two quasistable states with two distinct IV characteristics (see figure black and grey). These states are expanded in the two insets with the component currents at the free-running voltages, color-coded beside the plots. One state (voltage region 1, indicated by the green oval and the total membrane IV curve in black) is characterized by voltages negative of the K^+ equilibrium voltage, E_K, and is maintained by H^+-ATPase activity (see Box 2; Lohse and Hedrich, 1992; Thiel et al., 1992; Gradmann et al., 1993), in Arabidopsis primarily AHA1 and AHA2 (Merlot et al., 2007; Blatt et al., 2014). In this state, the H^+-ATPase current (inset, long dashed line) is balanced by K^+ (inset, dotted line) and Cl^- channels (inset, short dashed line) as well as Cl^- and Mal uptake (via H^+-coupled transport; data not shown), and helps drive stomatal opening. The second state is characterized by voltages close to E_K and is associated with solute loss for stomatal closing (voltage region 2, indicated by the green circle and the total membrane IV curve in grey). The membrane in this state is dominated by outward-rectifying K^+ channels (inset, dash-dotted line), including GORK in Arabidopsis (Blatt and Armstrong, 1993; Blatt and Thiel, 1994; Blatt and Gradmann, 1997; Hosy et al., 2003; Eisenach et al., 2014) and by the Cl^- current such as characterized by SLAC1 and its homologs (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2009; Wang et al., 2012). Voltage transitions between these states determine whether the guard cell is actively accumulating or losing solute (Blatt and Armstrong, 1993; Gradmann et al., 1993; Blatt and Thiel, 1994; Minguet-Parramona et al., 2016). Note that the major differences between the black and grey IV curves are the much greater H^+-ATPase and smaller Cl^- channel currents in state 1. Also evident in this state is a larger inward-rectifying K^+ channel current. Thus, the voltage range between the states is dictated primarily by the two K^+ channels and their voltage dependencies.



generally recognized to favor stomatal opening. Raising $[Ca^{2+}]_i$ (Box 1) suppresses H^+-ATPase activity and current through the inward-rectifying K^+ channels, and it promotes SLAC1 and related channel activities (Blatt et al., 1990b, 2007; Kinoshita et al., 1995; Chen et al., 2010; Roelfsema and Hedrich, 2010). Elevating $[Ca^{2+}]_i$ also suppresses H^+ transport via the tonoplast H^+-PPase (Darley et al., 1998) and K^+ flux through FV-type K^+ channels (Tikhonova et al., 1997); it activates the TPK1 K^+ channel (Gobert et al., 2007), the K^+- and Ca^{2+}-permeable TPC1 channel, and vacuolar Cl^-- and Mal-permeable channels (Pei et al., 1996, 1999; Beyhl et al., 2009; Rienmüller et al., 2010; Eisenach and Di Angeli, 2017). Of course, raising $[Ca^{2+}]_i$ also provides substrate for CAX-mediated Ca^{2+}/H^+ exchange (Pittman, 2011) and Ca^{2+} efflux driven by the Ca^{2+}-ATPases, with the net effect at both membranes of removing Ca^{2+} from the cytosol (Geisler et al., 2000; Sze et al., 2000; Bonza and De Michelis, 2011; Pittman, 2011).

A few transporters are either demonstrably $[Ca^{2+}]_i$ insensitive or are likely to be so, including the outward-rectifying K^+ channels such as GORK in Arabidopsis, the H^+-coupled transporters for K^+, Cl^-, Mal, and sugar at the plasma membrane, and the VH^+-ATPase and CLC Cl^-/H^+ antiporters at the tonoplast. Each of these transporters is affected by membrane voltage (Tables III–VIII) and, therefore, will be subject to $[Ca^{2+}]_i$ indirectly through its action on Ca^{2+}-sensitive transport. Thus, resting $[Ca^{2+}]_i$ near 100 to 200 nM favors energization by the plasma membrane H^+-ATPase and tonoplast H^+-PPase, and K^+ and anion flux across both membranes into the vacuole; conversely, raising $[Ca^{2+}]_i$ has the overall effect of reducing metabolically driven H^+ transport that energizes both membranes, and it promotes the net export of K^+, Cl^-, and Mal^{2-} from the vacuole, through the cytosol, and out across the plasma membrane. In short, elevated $[Ca^{2+}]_i$ shifts the balance of solute transport from net solute influx to net solute efflux.

THE Ca^{2+} SIGNAL, ITS ORIGINS AND OSCILLATIONS

Membrane voltage and $[Ca^{2+}]_i$ obviously connect through the gating properties of the plasma membrane Ca^{2+} channels and through the actions of

$[Ca^{2+}]_i$ on one or more endomembrane Ca^{2+} channels (Grabov and Blatt, 1999; Garcia-Mata et al., 2003). As a consequence, voltage and $[Ca^{2+}]_i$ form two interacting control loops that impact directly on guard cell transport. The plasma membrane Ca^{2+} channels (Hamilton et al., 2000, 2001) show a voltage sensitivity near unity and activation sufficient to trigger $[Ca^{2+}]_i$ elevations observed in vivo at voltages more negative than −150 mV (Grabov and Blatt, 1998; Garcia-Mata et al., 2003; Chen et al., 2010). Voltage will also exert control on Ca^{2+} efflux through Ca^{2+}-ATPases at the plasma membrane, in Arabidopsis the ACA8 Ca^{2+}-ATPase (Bonza et al., 2000; Geisler et al., 2000), but antiparallel to its effect on the Ca^{2+} channels. The Ca^{2+}-ATPases almost certainly couple charge flux with ATP hydrolysis in a 2:1 ratio, that is 1 Ca^{2+}:1 ATP (Geisler et al., 2000; Sze et al., 2000), implying a reversal voltage near −200 mV at resting $[Ca^{2+}]_i$ with 1 mM Ca^{2+} outside. These characteristics also imply a strong kinetic enhancement as the membrane depolarizes toward 0 mV. So, between roughly −200 to −50 mV, Ca^{2+} flux across the plasma membrane alternates between net influx and efflux, promoting and suppressing $[Ca^{2+}]_i$ elevation, respectively.

The interactions between voltage and Ca^{2+} channel gating are more subtle, however, and give rise to the phenomenology of $[Ca^{2+}]_i$ oscillations observed during stomatal closing (McAinsh et al., 1995; Grabov and Blatt, 1998; Staxen et al., 1999; Allen et al., 2000; Sokolovski et al., 2008). In general, such oscillations arise only when three prerequisites are met: (1) Ca^{2+} must enter the cytosol from at least two sources or pathways with different temporal kinetics; (2) each pathway must be self-limiting to ensure that $[Ca^{2+}]_i$ elevations are transient and will not overwhelm the cytosol; and (3) each Ca^{2+} source must operate one or more mechanisms for Ca^{2+} recovery following a rise in $[Ca^{2+}]_i$. It is now widely recognized that $[Ca^{2+}]_i$ transients in guard cells and other plant cells depend on Ca^{2+} from outside as well as on its release from endomembrane stores. Similarly, energy-coupled Ca^{2+} transporters, both CAX antiporters mediating Ca^{2+}/H^+ exchange and ACA Ca^{2+}-ATPases, are widely distributed among all of the major membranes within the plant cell (Lopez-Marques et al., 2004; Pardo et al., 2006; Martinoia et al., 2007; Bonza and De Michelis, 2011; Pittman, 2011). These transporters ensure a capacity for Ca^{2+} recovery with affinities suited to scavenging Ca^{2+} across a wide range of free concentrations (Tables III and V). So, both the first and third of the three prerequisites are met.

It is the second prerequisite that has proven more difficult to establish. All evidence points to a set of highly Ca^{2+}-selective channels at the plasma membrane as the major pathway for Ca^{2+} entry into the guard cell (Grabov and Blatt, 1999; Hamilton et al., 2000, 2001), although a number of other channels that are nonselective among cations have been proposed (Véry and Sentenac, 2002), including several of the Glu receptor-like channels (Qi et al., 2006; Cho et al., 2009). The Glu receptor-like channels GLR3.1 and GLR3.5 have been shown to affect resting levels of $[Ca^{2+}]_i$, but their voltage dependence and sensitivity to $[Ca^{2+}]_i$ are insufficient to account for transients in its free concentration and they do not affect stomatal closure in ABA (Kong et al., 2016). By contrast, the Ca^{2+}-selective channels (Hamilton et al., 2000, 2001) show a low (13 pS) single-channel conductance, they activate in a strongly voltage-dependent manner negative of approximately −150 mV, and their gating is affected by ABA. Most important, their gating is suppressed as $[Ca^{2+}]_i$ rises above approximately 1 μM. This $[Ca^{2+}]_i$ sensitivity is crucial and satisfies the essential prerequisite for self-limitation. Indeed, to date, these are the only channels in plants known to exhibit such characteristics.

ABA displaces the gating of the Ca^{2+} channels to more positive voltages (Hamilton et al., 2000), which accounts for the parallel shift with ABA in the voltage threshold for $[Ca^{2+}]_i$ elevations (Grabov and Blatt, 1998). How this displacement in gating arises has yet to be explored in detail, but it may be linked to phosphorylation of the channels (Box 1) or associated proteins (Köhler and Blatt, 2002; Sokolovski et al., 2005; Mori et al., 2006). Additionally, the Ca^{2+} channels are activated by ROS, in Arabidopsis subject to the *atrbohd* and *atrbohf* mutants, which encode plasma membrane-localized NADPH oxidases (Kwak et al., 2003). The quadruple ABA receptor mutant *pyr1/pyl1/pyl2/pyl4*, which is much reduced in its response to ABA (Park et al., 2009; Nishimura et al., 2010), also suppresses $[Ca^{2+}]_i$ elevation in ABA. This impairment has been linked to a loss in ROS production in ABA, and adding the ROS hydrogen peroxide (H_2O_2) recovers both the enhanced activity of the Ca^{2+} channels in vivo and stomatal closure (Wang et al., 2013). Until the molecular identity of the Ca^{2+} channels is determined, however, further processes to understand their regulation will be hampered.

Less obvious is how Ca^{2+}-permeable but nonselective cation channels contribute to the regulation of $[Ca^{2+}]_i$, among these OSCA1 (Yuan et al., 2014) and its close homolog CSC1 (Hou et al., 2014). The *osca1* mutant is impaired in its response to hyperosmotic stress, leading to the proposal that OSCA1 is important for $[Ca^{2+}]_i$ elevation under mechanical or osmotically induced stress. However, stomata of the mutant responded normally to ROS (H_2O_2) and ABA, the channels show no evidence of self-limitation, and their localization to the plasma membrane, based solely on the diffuse distribution of an overexpressed, GFP-tagged construct, is unconvincing. Thus, neither OSCA1 nor CSC1 is likely to trigger the cyclic elevations in $[Ca^{2+}]_i$ that have been observed in vivo, although they may contribute to osmotic equilibrium across the tonoplast or other endomembranes.

If Ca^{2+} entry is important to trigger $[Ca^{2+}]_i$ increases, it is its release from intracellular stores that contributes the bulk of the Ca^{2+} to raise $[Ca^{2+}]_i$. Best estimates (Chen et al., 2012) indicate that over 95% of the total Ca^{2+} entering the cytosol during $[Ca^{2+}]_i$ transients comes from within endomembrane compartments, much as it does in animals (Bezprozvanny et al., 1991; Hille, 2001). These compartments almost certainly include the endoplasmic reticulum (Garcia-Mata et al., 2003; Blatt et al., 2007; Bonza et al., 2013), the vacuole (Allen et al., 1995; Beyhl et al., 2009) and, over a higher $[Ca^{2+}]_i$ range, also mitochondria and chloroplasts (McAinsh and Pittman, 2009; Loro et al., 2012; Loro and Costa, 2013). In guard cells, Ca^{2+} is released as $[Ca^{2+}]_i$ rises, triggered by Ca^{2+} influx across the plasma membrane (Grabov and Blatt, 1999; Garcia-Mata et al., 2003), a process that leads to oscillations and is often identified as Ca^{2+}-induced Ca^{2+} release. Endomembrane Ca^{2+} release is key to producing the $[Ca^{2+}]_i$ oscillations and their interactions with voltage observed in vivo (Grabov and Blatt, 1998; Allen et al., 2001; McAinsh and Pittman, 2009; Minguet-Parramona et al., 2016). Several Ca^{2+}-permeable channels associated with endomembrane stores are activated by cytosolic Ca^{2+} and ligands, including IP_3, cADP-ribose, NO, and inositol hexakisphosphate (IP_6), that are known or have been implicated in promoting Ca^{2+} release (Alexandre et al., 1990; Muir and Sanders, 1996; Wu et al., 1997; Leckie et al., 1998; Grabov and Blatt, 1999; Garcia-Mata et al., 2003; Lemtiri-Chlieh et al., 2003). Yet, although essential for $[Ca^{2+}]_i$ to oscillate (Chen et al., 2012; Minguet-Parramona et al., 2016), missing for all of the channels characterized to date, including the Ca^{2+}- and K^+-permeable channel TPC1 (Peiter et al., 2005; Dadacz-Narloch et al., 2011), is evidence of self-limitation leading to suppressed Ca^{2+} release at elevated $[Ca^{2+}]_i$.

SYSTEMS MODELS FOR GUARD CELL TRANSPORT

Although essential for any rational approach to engineering stomata, relating the transport capacity of guard cells to stomatal movements in quantitative mechanistic terms poses a number of difficulties (Buckley, 2017). As a consequence, relatively few studies have progressed beyond the qualitative analysis of mutant associations. One of the difficulties, as we note above, arises because flux through the predominant transporters for K^+, Cl^-, and Mal, as well as the H^+-ATPases, depends strongly on membrane voltage. The physical requirement for charge to balance means that the transport of each ionic species is necessarily joined to that of all others across the same membrane, unless this connection is bypassed by the circuit of a voltage clamp (Blatt, 2004). So knowledge of the prevailing voltage and of the voltage dependence for each transporter is critical (see "The Importance of Voltage Control" above).

A second difficulty arises from the general finding that the ion fluxes needed for stomatal movements reflect only a small fraction of the maximal capacity of several transporters mediating these fluxes (Thiel et al., 1992; Hamilton et al., 2000; Pottosin and Schönknecht, 2007; De Angeli et al., 2009b). As a case in point, during stomatal opening, the solute content of a typical *V. faba* or Arabidopsis guard cell rises by approximately 200 to 300 mM on a cell volume basis, roughly half of this K^+ (Table II). For Arabidopsis, the changes are equivalent to 0.03 to 0.07 pmol of K^+ per guard cell and, over the period of opening (typically 60 min), translates to a K^+ flux of 6 to 10 amol s^{-1} and a current of 0.5 to 0.9 pA or 1 to 3 μA cm^{-2}. Such currents are typical for the inward-rectifying K^+ channels at voltages near −150 mV in vivo (see Boxes 2 and 3), but they are no more than 2% to 3% of the K^+ current when maximally activated, such as recorded at −200 mV (Wang et al., 2012, 2013). One general conclusion, then, is that the capacity for transport, especially through the individual ion channels that facilitate K^+, Cl^-, and Mal flux, is not inherently limiting. Instead, it is the balance between the sum of all transporters at the membrane that limits solute flux. Again, manipulating solute flux through any one transporter inevitably affects this balance and, thereby, directly affects other transporters at the same membrane.

Systems modeling offers one approach to overcoming these difficulties. It enables the detailed knowledge available for the individual transporters to be reconstructed within the physiological framework of the cell. Effective physiological models are constrained by fundamental physical laws and the known kinetic relationships, ligand binding, and related regulatory properties for each transporter. Such models address the difficulties inherent to understanding how transport and metabolic activities are temporally connected. A growing number of studies employ systems approaches, for example to validate a role for K^+ transport in the phloem loading of sucrose as an energy reserve (Gajdanowicz et al., 2011) and to describe oscillatory characteristics in H^+ and K^+ flux (Gradmann et al., 1993; Shabala et al., 2006). Boolean network models (Li et al., 2006; Sun et al., 2014) also have been applied to guard cell signaling and transport control, although these models operate with nodes and links that can only be on or off, so they omit the most important insights that arise from the quantitative and dynamic interactions between transporters.

Of course, the real test of any model is its capacity not only for reproducing experimental observations but for predicting new and unexpected behaviors. In this regard, the development of the OnGuard platform for modeling stomata (Chen et al., 2012; Hills et al., 2012; freely available at www.psrg.org.uk) has proven the most successful to date, demonstrating true predictive power in uncovering previously unexpected and emergent features of guard cell

physiology, several of which have been verified experimentally. Among these, OnGuard analysis of the Arabidopsis *slac1* mutant predicted a connection between the Cl^- channel and the plasma membrane K^+ channels that was subsequently confirmed experimentally (Wang et al., 2012). The results showed how *slac1* slowed K^+ uptake and stomatal opening, even though the SLAC1 Cl^- channel contributes directly only to solute loss and stomatal closure. OnGuard models have since resolved an unforeseen coordination between the plasma membrane and tonoplast transport in the Arabidopsis *ost2* H^+-ATPase mutant (Blatt et al., 2014); they accurately predicted (Wang et al., 2014a) the consequences of overexpressing the KAT1 K^+ channel and AHA2 H^+-ATPase in Arabidopsis guard cells (Wang et al., 2014b); and they have shed new light on the connection between $[Ca^{2+}]_i$ oscillation frequency and the osmotic solute flux (Minguet-Parramona et al., 2016). The latter study is especially informative, not only in validating the concept of the $[Ca^{2+}]_i$ signal cassette (Blatt, 2000) but in illustrating how quantitative modeling is essential as an approach to physiology that otherwise confounds intuitive understanding and leads to misinterpretations.

CONCLUDING REMARKS

If we are to design crops with improved water use efficiency and able to cope with reduced water availability, then manipulating stomatal conductance is an obvious target. Reducing stomatal density has already proven successful in some contexts (Condon et al., 2002, 2004), but to date, although conceptually the most promising approach there are no examples in which manipulating guard cell behavior per se has resulted in improved water use efficiency without a cost in carbon gain. The challenge, therefore, will be to moderate stomatal conductance without a significant cost in photosynthetic assimilation (Lawson and Blatt, 2014). Ideally, improved stomatal function also needs to be achieved without increasing vulnerabilities to the naturally fluctuating environment or to pathogens. This problem is a highly complex one. It demands a comprehensive and quantitative understanding of the metabolic and signaling pathways that determine the physiological responses of guard cells. It also requires an understanding of how the functions of guard cells are coordinated within the plant, notably with carbon assimilation (Lawson et al., 2012) and with hydraulic water flux (Caldeira et al., 2014; Chaumont and Tyerman, 2014). From research on guard cell ion transport and its regulation over the past three decades, there is now a substantial body of quantitative information, all essential to inform rational efforts in manipulating stomatal responses. Even so, assembling this information to anticipate the consequences of specific genetic manipulations clearly is not straightforward (see Outstanding Questions).

How might we engineer guard cell transport to reduce water use without a cost to carbon gain by the plant? Although separate ion transporters are responsible for driving solute flux during stomatal opening and closing (Li et al., 2006), a primary difficulty remains that all of these transporters interact. We come back to the studies of SLAC1, which amply demonstrate that manipulations of transport to affect stomatal closing have profound effects on opening as well and are predictable outcomes of the intrinsic interactions between transport at the plasma and tonoplast membranes (Wang et al., 2012). Thus, insights from quantitative systems modeling of guard cell transport clearly will be vital in guiding efforts toward rational molecular designs in the future. Wang et al. (2014a) used OnGuard to survey manipulations of each of the major transporters, one at a time, in the guard cell. Their results confirm experimental observations from known mutants, including *gork* (Hosy et al., 2003), *slac1* (Negi et al., 2008; Wang et al., 2012), *ost2* (Merlot et al., 2007), *clca* (De Angeli et al., 2006), and *tpk1* (Gobert et al., 2007). They also show that the transport interactions inherent to guard cells preempt most, if not all, intuitive approaches to

OUTSTANDING QUESTIONS

- Despite the wealth of biophysical and functional data on Ca^{2+} flux across the plasma membrane, tonoplast, and other endomembranes, we still do not know the molecular identities of the major Ca^{2+} channels that mediate these fluxes. Resolving the identity of the 13-pS Ca^{2+} channel at the plasma membrane will be a major step forward to understanding how $[Ca^{2+}]_i$ is regulated and its connections to the known ABA receptors and signal intermediates, including ROS.
- Identifying the mechanisms of perception for several environmental stimuli, notably CO_2, poses a major challenge for the future. Additionally, we may ask if the PYR/PYL family of proteins represents the only receptors by which the ABA stimulus is perceived.
- To advance an understanding of how several protein kinase cascades affect stomatal function, it is now vital that their the phosphorylation kinetics and their targets are resolved in vivo. Further studies may then use mutant complementation approaches to explore the consequences for stomatal physiology.
- Systems analysis of guard cell transport and stomatal physiology needs now to be extended to the whole plant canopy, bridging the gap between the molecular mechanics of guard cells and whole-plant gas exchange.

altering stomatal behavior. In short, the problem of manipulating stomata throws up a surprising degree of complexity that will need more subtle methods if practical solutions are to be found to reverse engineer stomata. Of course, these models are still in their infancy and will need to be extended to the scales of the leaf and the whole plant canopy (Pieruschka et al., 2010) in order to bridge the modeling gap between microscopic functionalities and macroscopic outputs. Once this micro-macro link is made, we expect substantial and rapid progress to be realized through quantitative modeling of guard cell membrane transport.

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The literature from the past two decades yields some 40,000 research articles on stomatal guard cells, over 3,000 published in each of the past two years, with this number rising at a rate of 5% per year. A comprehensive review on such a scale would be virtually impossible to write, or to read, and we apologize to those researchers whose work we have been unable to highlight here. Figure 1 is from the Ph.D. thesis of Cornelia Eisenach (Glasgow, 2011), and we are grateful for her permission to include these data here.

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