Calcium-Dependent and -Independent Stomatal Signaling Network and Compensatory Feedback Control of Stomatal Opening via Ca^{2+} Sensitivity Priming[W]

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In the past 15 years or more, many mutants that are impaired in stimulus-induced stomatal closing and opening have been identified and functionally characterized in Arabidopsis (*Arabidopsis thaliana*), leading to a mechanistic understanding of the guard cell signal transduction network. However, evidence has only recently emerged that mutations impairing stomatal closure, in particular those in slow anion channel SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1), unexpectedly also exhibit slowed stomatal opening responses. Results suggest that this compensatory slowing of stomatal opening can be attributed to a calcium-dependent posttranslational down-regulation of stomatal opening mechanisms, including down-regulation of inward K^+ channel activity. Here, we discuss this newly emerging stomatal compensatory feedback control model mediated via constitutive enhancement (priming) of intracellular Ca^{2+} sensitivity of ion channel activity. The CALCIUM-DEPENDENT PROTEIN KINASE6 (CPK6) is strongly activated by physiological Ca^{2+} elevations and a model is discussed and open questions are raised for cross talk among Ca^{2+}-dependent and Ca^{2+}-independent guard cell signal transduction pathways and Ca^{2+} sensitivity priming mechanisms.

Stomatal pores formed by two guard cells enable CO_2 uptake from the atmosphere, but also ensure leaf cooling and provide a pulling force for nutrient uptake from the soil via transpiration. These vitally important processes are inevitably accompanied by water loss through stomata. Stomatal opening and closure is caused by the uptake and release of osmotically active substances and is tightly regulated by signaling pathways that lead to the activation or inactivation of guard cell ion channels and pumps. Potassium ions enter guard cells through the inward-rectifying K^+ channels (K^+_{in}) during stomatal opening and are released via outward-rectifying K^+ channels during stomatal closure (Schroeder et al., 1987; Hosy et al., 2003; Roelfsema and Hedrich 2005). Cytosolic Ca^{2+}, an important second messenger in plants, mediates ion channel regulation, particularly down-regulation of inward-conducting K^+_{in} channels and activation of S-type anion channels, thus mediating stomatal closure and inhibiting stomatal opening (Schroeder and Hagiwara, 1989; Dodd et al., 2010; Kim et al., 2010). Stomatal closure is initiated by anion efflux via the slow S-type anion channel SLAC1 (Negi et al., 2008; Vahisalu et al., 2008; Kollist et al., 2011) and the voltage-dependent rapid R-type anion channel QUICK-ACTIVATING ANION CHANNEL1 (Meyer et al. 2010; Sasaki et al., 2010).

In recent years, advances have been made toward understanding mechanisms mediating abscisic acid (ABA)-induced stomatal closure (Cutler et al., 2010; Kim et al., 2010; Raghavendra et al., 2010). The core ABA signaling module, consisting of PYR/RCAR (for pyrabactin resistance 1/regulatory components of ABA receptors) receptors, clade A protein phosphatases (PP2Cs), SNF-related protein kinase OPEN STOMATA1 (OST1), and downstream targets, is Ca^{2+}-independent (Ma et al., 2009; Park et al., 2009; Hubbard et al., 2010). However, ABA-induced stomatal closure was reduced to only 30% of the normal stomatal closure response under conditions that

[1] This work was supported by the National Science Foundation (grant no. MCB0918220) and National Institutes of Health (grant no. R01GM060396) to J.I.S. K^+ channel analyses were supported in part by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (grant no. DE–FG02–03ER15449). H.K. was supported by an Institutional Research Funding Grant (grant no. IUT2–21) and by the European Regional Fund (Center of Excellence in Environmental Adaptation).

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[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.113.220343

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inhibited intracellular cytosolic free calcium ($[Ca^{2+}]_{cyt}$) elevations in Arabidopsis (Siegel et al., 2009), consistent with previous findings in other plants (De Silva et al., 1985; Schwartz, 1985; McAinsh et al., 1991; MacRobbie, 2000). Together these and other studies show the importance of $[Ca^{2+}]_{cyt}$ for a robust ABA-induced stomatal closure. Here, we discuss Ca^{2+}-dependent and Ca^{2+}-independent signaling pathways in guard cells and open questions on how these may work together.

Plants carrying mutations in the SLAC1 anion channel have innately more open stomata, and exhibit clear impairments in ABA-, elevated CO_2-, Ca^{2+}-, ozone-, air humidity-, darkness-, and hydrogen peroxide-induced stomatal closure (Negi et al., 2008; Vahisalu et al., 2008; Merilo et al., 2013). Recent research, however, unexpectedly revealed that mutations in SLAC1 also down-regulate stomatal opening mechanisms and slow down stomatal opening (Laanemets et al., 2013).

UNEXPECTED SLOWING OF STOMATAL OPENING IN *SLAC1* MUTANT ALLELES

Stomatal opening in plants is mediated by increased light intensity or enhanced air humidity and by decreased CO_2 concentrations inside the leaf (C_i) that occur as a result of photosynthesis. During light-induced stomatal opening, phototropin-related blue-light signaling leads to the activation of H^+-ATPases, resulting in H^+ efflux and plasma membrane hyperpolarization (for review, see Shimazaki et al., 2007), which in turn leads to the uptake of K^+ via K^+ channels (Schroeder et al., 1984). Simultaneously, due to active photosynthesis, C_i is reduced and S-type anion channels are inactivated, which further favors stomatal opening (Roelfsema et al., 2002). Mutations in the *SLAC1* gene result in impaired anion efflux, and would therefore be expected to accelerate stomatal opening in response to opening stimuli. Unexpectedly, the opposite was detected: Stomatal opening of intact whole rosettes induced by three independent biological stimuli (light, low C_i, and high humidity) was slower in *slac1* mutants (Laanemets et al., 2013). Independent research showed that *slac1* mutant guard cells show a greatly reduced activity of K^+_{in} channels (Laanemets et al., 2013), which contribute to stomatal opening (Kwak et al., 2001; Figs. 1 and 2). These independent findings suggest that plants possess a system that counteracts the impaired stomatal closing of S-type anion channels in *slac1* mutants by down-regulating stomatal opening mechanisms to prevent excessive water loss.

IMPAIRED ANION EFFLUX LEADS TO A CHANGED IONOMIC PROFILE IN *SLAC1* GUARD CELLS

Severely reduced S-type anion channel activity and reduced anion efflux in *slac1* guard cells change the entire ionomic profile of guard cells. Elevated accumulation of anions such as chloride, malate, and fumarate, but also potassium was observed (Negi et al., 2008). Hyperaccumulation of chloride and malate can suppress H^+-coupled anion transport (Sanders et al., 1989). Accordingly, the cytosolic pH (pH_{cyt}) of *slac1* guard cells was slightly more alkaline (Wang et al., 2012). Furthermore, the removal of S-type anion channel activity in *slac1* mutant guard cells (Vahisalu et al., 2008) is expected to cause a more negative ("hyperpolarized") membrane potential due to the reduced anion efflux from guard cells. This is predicted to enhance the activity of hyperpolarization-activated Ca^{2+} influx channels resulting in a slightly

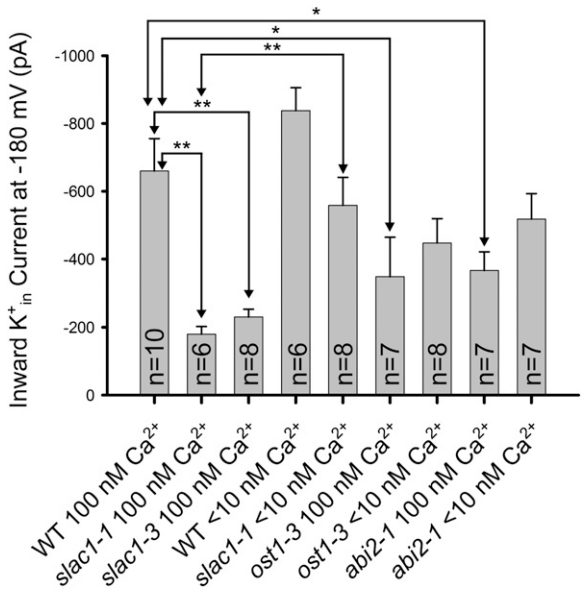

Figure 1. K^+_{in} channel current activity is reduced in the stomatal closing impaired mutants *slac1*, *ost1*, and *abi2-1* and this K^+ channel down-regulation is rapidly reversed in *slac1* guard cells by lowering $[Ca^{2+}]_{cyt}$ to less than 10 nM. Average K^+_{in} channel current magnitudes at −180 mV are shown for wild-type Columbia-0 (WT) and the *slac1*, *ost1*, and *abi2-1* alleles. The concentrations of buffered $[Ca^{2+}]_{cyt}$ concentrations are indicated. Whole-cell patch clamp recordings were performed on guard cell protoplasts at the indicated cytosolic free Ca^{2+} concentrations. Note that an *abi2-1* allele in the Columbia-0 accession was analyzed (Nishimura et al., 2004). Error bars (SEM) for the indicated number of guard cells analyzed are depicted. K^+_{in} channel current magnitudes in *slac1* recovered at less than 10 nM $[Ca^{2+}]_{cyt}$ compared with 100 nM $[Ca^{2+}]_{cyt}$ ($P < 0.005$). Statistical analyses showed significant down-regulation of K^+_{in} channel current magnitudes in the *slac1-1*, *slac1-3* ($P < 0.001$), *ost1* ($P < 0.04$), and *abi2-1* (Columbia-0; $P < 0.02$) mutants compared with wild-type guard cells at 100 nM free $[Ca^{2+}]$ in the cytosol. Small but statistically nonsignificant differences for comparisons of K^+_{in} channel current magnitudes in response to lowering $[Ca^{2+}]$ from 100 nM to less than 10 nM for *ost1-3* (P value = 0.525) and *abi2-1* (P value = 0.109) were found. *$P <$ 0.05; **$P < 0.01$. Unpaired Student's t tests were applied to assess significance. Data from WT < 10 nM and *slac1-1* < 10 nM are from Laanemets et al., 2013. Methods were as described in Laanemets et al., 2013 (see Supplemental Text S1).

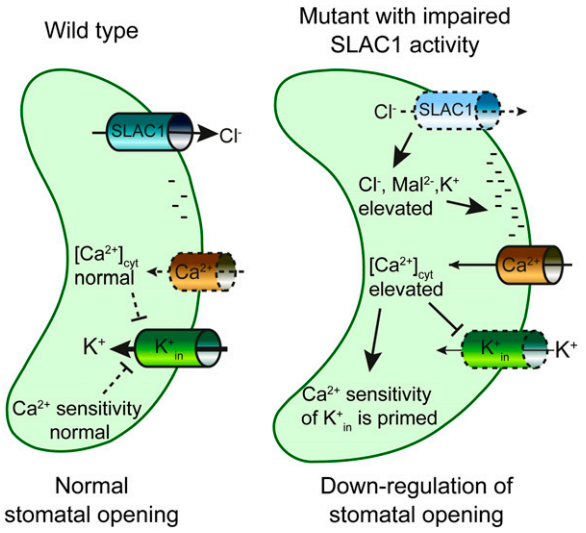

Figure 2. Schematic model for K^+_{in} down-regulation in mutants with impaired SLAC1 activity. Without SLAC1, ions accumulate in guard cells, and the plasma membrane is charged more negatively (hyperpolarization) due to reduced anion efflux, which leads to activation of hyperpolarization-dependent Ca^{2+}-permeable influx channels and elevated levels of $[Ca^{2+}]_{cyt}$. The increased $[Ca^{2+}]_{cyt}$ concentration down-regulates K^+_{in} channel activity. Furthermore, K^+_{in} channels exhibit an enhanced (primed) Ca^{2+} sensitivity, thus enhancing K^+_{in} channel down-regulation. The negative charges shown at the inner side of the plasma membrane (right model) indicate the more negative (hyperpolarized) membrane potential expected for *slac1* mutant alleles.

elevated $[Ca^{2+}]_{cyt}$ in *slac1* guard cells (Grabov and Blatt, 1998; Hamilton et al., 2000; Pei et al., 2000). Slightly elevated $[Ca^{2+}]_{cyt}$ in *slac1* guard cells was experimentally observed in two studies (Wang et al., 2012; Laanemets et al., 2013) and causes down-regulation of K^+_{in} activity (Schroeder and Hagiwara, 1989; Siegel et al., 2009), thereby slowing stomatal opening in *slac1*.

DOWN-REGULATION OF GUARD CELL K^+ UPTAKE CHANNEL ACTIVITY BY ENHANCED $[Ca^{2+}]_{CYT}$ SENSITIVITY IN *SLAC1*

Analyses of guard cell ion channel transcript levels showed only partially reduced expression of K^+_{in} and H^+-ATPase gene transcripts in *slac1* guard cells, suggesting that posttranslational mechanisms may down-regulate K^+_{in} (Laanemets et al., 2013). The patch clamp method enables clamping of defined $[Ca^{2+}]_{cyt}$ and pH_{cyt} conditions by rapidly equilibrating the cytosol with the patch pipette solution. The first experiments showing dramatic down-regulation of K^+_{in} channel activity in plants lacking SLAC1 (*slac1-1* and *slac1-3*) were performed at 250 nM free $[Ca^{2+}]_{cyt}$. Interestingly, the reduction of K^+_{in} activity was rapidly reversed by lowering $[Ca^{2+}]_{cyt}$ to a subphysiological $[Ca^{2+}]_{cyt}$ concentration of less than 10 nM (Laanemets et al., 2013), indicating that Ca^{2+}-induced inhibition of K^+_{in} is more sensitive to $[Ca^{2+}]_{cyt}$ in *slac1* than in wild-type plants (Fig. 1). However, whether K^+_{in} channel activity in *slac1* guard cells is also affected at resting $[Ca^{2+}]_{cyt}$ levels had not yet been investigated. Additional patch clamp experiments show that K^+_{in} activity of *slac1-1* and *slac1-3* is also greatly reduced at physiological resting $[Ca^{2+}]_{cyt}$ of 100 nM (Fig. 1; Supplemental Fig. S1), indicating a down-regulation of K^+_{in} activity in *slac1* plants even at resting $[Ca^{2+}]_{cyt}$. These results demonstrate that the sensitivity of K^+_{in} channels to physiological $[Ca^{2+}]_{cyt}$ is constitutively enhanced (primed) in *slac1* guard cells. These findings support the hypothesis that the sensitivity of Ca^{2+} signaling mechanisms in guard cells can be enhanced such that guard cells respond to resting $[Ca^{2+}]_{cyt}$ levels, thus resulting in possible residual $[Ca^{2+}]_{cyt}$ signaling (Siegel et al., 2009).

The priming of K^+_{in} channel sensitivity to $[Ca^{2+}]_{cyt}$ leads to reduced K^+ influx representing a mechanism to counteract the potential adverse effect of more open stomata in *slac1* plants (Laanemets et al., 2013). Guard cell $[Ca^{2+}]_{cyt}$ elevation alone is not sufficient to explain the slowed stomatal opening of *slac1* mutants. As mentioned above, anion accumulation in the *slac1* mutant resulted in elevated pH_{cyt} (Wang et al., 2012), which might also slow stomatal opening. In sum, the primed Ca^{2+} sensitivity of K^+_{in} channels, together with higher $[Ca^{2+}]_{cyt}$ and more alkaline pH_{cyt}, provide a feedback mechanism helping to prevent excessive water loss in *slac1* mutant plants that are defective in stomatal closure (Fig. 2).

The next question was whether *slac1* plants always show higher Ca^{2+} sensitivity of K^+_{in} channels or whether this is reversible. Patch clamp experiments showed that the reduction of K^+_{in} activity was reversed by lowering $[Ca^{2+}]_{cyt}$ to a subphysiological Ca^{2+} concentration (Laanemets et al., 2013). If stomata of *slac1* plants are in a more closed state, does this provide feedback to $[Ca^{2+}]_{cyt}$, pH_{cyt}, and most importantly to Ca^{2+}-priming of K^+_{in} channels, resulting in a wild-type-like stomatal opening rate? The answer to this question requires further research, but results so far indicate that the Ca^{2+}-priming of K^+_{in} channels is indeed reversible and depends on initial stomatal "openness". When *slac1* and wild-type plants showed nearly similar steady-state starting stomatal conductances (Laanemets et al., 2013), the differences in half-times for stomatal opening between *slac1* and wild-type plants were only moderate (Table I). However, when the starting stomatal apertures of *slac1* plants were considerably larger than those of the wild type, the differences in half-times for stomatal opening were also larger, about 2-fold (Wang et al., 2012), indicating that in the latter experiments the compensatory feedback control of stomatal opening functioned to counteract further stomatal opening in this already open state.

Table I. *Increase in stomatal conductance is slower in slac1-1, slac1-3, and ost1-3 mutants (background Columbia-0) and in abi2-1 mutant (background Ler)*

*$P < 0.1$; **$P < 0.05$ (statistical difference from the wild type, one-way ANOVA, $n = 5–18$).

Genotype	Half-Times for Stomatal Opening[a]	
	Low CO_2	Light
	min	
Wild type (Columbia-0)	18.8 ± 0.9	15.4 ± 0.6
slac1-1	22.8 ± 2.0*	21.1 ± 1.1**
slac1-3	26.3 ± 2.3**	19.7 ± 1.4**
ost1-3	24.3 ± 1.2**	22.1 ± 1.3**
Wild type (L*er*)	19.8 ± 0.8	10.1 ± 0.8
abi2-1	24.9 ± 3.3	16.6 ± 3.9**

[a]In light experiments, plants were kept in the measurement cuvettes (Kollist et al., 2007) overnight and stomatal opening was measured during the onset of the light period in the morning. In CO_2 experiments, plants were kept at ambient CO_2 (400 mmol mol^{-1}) for 2 h, then the CO_2 was decreased to 40 mmol mol^{-1}The stomatal opening response, within first 45 min, was scaled to the range from 0% to 100%, directly yielding the half-times for stomatal opening. Plant growth conditions were as described in Laanemets et al., 2013. See Supplemental Text S1 for further experimental details.

SLOWED STOMATAL OPENING AND DOWN-REGULATION OF GUARD CELL K^+ UPTAKE CHANNEL ACTIVITY IS ALSO OBSERVED IN OTHER MUTANTS WITH MORE OPEN STOMATA

If down-regulation of K^+_{in} channel activity via Ca^{2+} priming is caused by the open stomata phenotype of *slac1* mutants, the question arises whether the same phenotype is also present in other mutants with constitutively more open stomata. To address this point, experiments were performed with *ost1-3* (Mustilli et al., 2002; Yoshida et al., 2002) and the dominant mutant *aba insensitive2* (*abi2-1;* Koornneef et al., 1984). OST1 is a protein kinase that activates SLAC1 anion channels via phosphorylation (Geiger et al., 2009; Lee et al., 2009; Vahisalu et al., 2010) and functional ABA activation of SLAC1 channels via OST1 was reconstituted in oocytes (Brandt et al., 2012). An unexpected reduced K^+_{in} channel activity in *abi2-1* mutant guard cells was shown in an earlier study (Pei et al., 1997). The dominant *abi2-1* mutation generates a mutant ABI2 protein phosphatase that is refractory to ABA-induced inhibition by PYR/RCAR receptors and suppresses OST1 activation (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009). Guard cells lacking functional OST1 or having a dominant active ABI2, are likely to hyperaccumulate ions and exhibit more negative plasma membrane potential, which would lead to an increase in $[Ca^{2+}]_{cyt}$ (Grabov and Blatt, 1998; Hamilton et al., 2000; Pei et al., 2000). Our gas-exchange experiments showed that both light- and low-CO_2-induced stomatal opening responses were slower in *ost1-3* and *abi2-1* plants compared with corresponding wild types (Table I). Additional patch clamp experiments with *abi2-1* and *ost1-3* guard cells were performed and K^+_{in} channel activity was found to be reduced in *ost1-3* guard cells and confirmed to be reduced in *abi2-1* guard cells (Pei et al., 1997; Fig. 1; Supplemental Figs. S2 and S3). However, reducing $[Ca^{2+}]_{cyt}$ to a subphysiological Ca^{2+} concentration (less than 10 nM) only slightly improved K^+_{in} activity in *ost1-3* and in *abi2-1* guard cells compared with *slac1* (Fig. 1; Supplemental Figs. S2 and S3; Laanemets et al., 2013). These recent studies also highlight that K^+_{in} channel activities in guard cells do not always correlate with the predominant phenotype of a given mutant, as illustrated for the *slac1*, *abi2-1*, and *ost1* mutants (Pei et al., 1997; Laanemets et al., 2013; Fig. 1; Supplemental Figs. S1–S3).

Taken together, elevated $[Ca^{2+}]_{cyt}$, combined with an increased sensitivity of Ca^{2+}-mediated K^+_{in} inhibition in *slac1* plants (Wang et al., 2012; Laanemets et al., 2013), leads to the down-regulation of K^+_{in} channel activity, even at physiological resting $[Ca^{2+}]_{cyt}$ concentrations (Fig. 1; Supplemental Fig. S1). This results in slowed stomatal opening of intact *slac1* plants in response to several stimuli such as air humidity, CO_2, and light. Reduced K^+_{in} activity and slowed stomatal opening of *ost1-3* and *abi2-1* mutants further suggests that this may be a general characteristic of plants with more open stomata or of plants with impaired S-type anion channel activation. Further research of mutants with an enhanced open stomata phenotype independent of S-type anion channels is needed to address this point. Importantly, in *slac1* mutants the down-regulation of K^+_{in} channel activity was reversible at low $[Ca^{2+}]_{cyt}$, whereas it was not clearly reversible in *ost1-3* and only partly reversible in *abi2-1* mutants, indicating that either active OST1 is involved in the increase of K^+_{in} at low $[Ca^{2+}]_{cyt}$ or this type of reversible compensatory regulation of ion channel activity is a unique characteristic related to the impaired SLAC1 anion channel.

PHYSIOLOGICAL STIMULI RAPIDLY ENHANCE $[Ca^{2+}]_{CYT}$ SENSITIVITY

Considering that the Arabidopsis genome encodes over 200 calcium binding (EF-hand containing) proteins (Day et al., 2002), understanding the mechanisms that mediate specific responses to Ca^{2+} is a subject of current research interest in plants and in eukaryotes in general (Berridge, 2012). Several mechanisms have been proposed to mediate specificity in Ca^{2+} signaling in plants, all of which may contribute to this phenomenon (Dodd et al., 2010; Kudla et al., 2010). However, strong cellular and biochemical evidence for any given model is missing and needed in plants, as well as in other systems (Berridge, 2012). Research on guard cell signal transduction has led to a new model that can contribute a mechanism for specificity in Ca^{2+} signaling. Studies in different plant species have shown that calcium is required for both ABA- and

CO_2-induced stomatal closing (De Silva et al., 1985; Schwartz, 1985; Webb et al., 1996; Grabov and Blatt, 1998; Staxén et al., 1999; MacRobbie, 2000; Mori et al., 2006; Young et al., 2006; Siegel et al., 2009). Several independent findings support the model that the stomatal closing signals, ABA and elevated CO_2, "prime" specific early Ca^{2+} sensing mechanisms, switching them from a relatively inactivated state to a Ca^{2+}-responsive "primed" state, and therefore tightly controlling Ca^{2+} responsiveness. Here, we briefly review evidence supporting this Ca^{2+} sensitivity priming model (Table II).

Ca^{2+} imaging in guard cells resolved "spontaneous" repetitive $[Ca^{2+}]_{cyt}$ transients that are more likely to occur at increasingly negative membrane potentials (Grabov and Blatt, 1998; Allen et al., 1999; Klüsener et al., 2002; Young et al., 2006; Siegel et al., 2009; Table II). Surprisingly, repetitive $[Ca^{2+}]_{cyt}$ elevations even occurred when the stomatal opening stimulus low CO_2 was applied (Young et al., 2006). The following question arose: How can $[Ca^{2+}]_{cyt}$ be required for stomatal closing if $[Ca^{2+}]_{cyt}$ elevations are also observed while applying stomatal opening stimuli (Young et al., 2006)? Previous research showed that any imposed $[Ca^{2+}]_{cyt}$ elevation above a threshold value can cause a rapid Ca^{2+}-reactive stomatal closure (Allen et al., 2001; Table II). Moreover, the $[Ca^{2+}]_{cyt}$ oscillation frequency and pattern did not affect this rapid "Ca^{2+}-reactive" stomatal closure response (Allen et al., 2001). (Note that the Ca^{2+} elevation pattern does affect the ability of closed stomata to reopen later, a response called "Ca^{2+}-programmed" stomatal response [Allen et al., 2001; Cho et al., 2009; Eisenach et al., 2012].) The above findings together led to the hypothesis that stomatal closing stimuli may modulate and thus enhance the Ca^{2+} sensitivity of specific Ca^{2+}-activated stomatal closing mechanisms (Young et al., 2006).

Further studies are consistent with the stimulus-induced Ca^{2+} sensitivity priming hypothesis (Table II). In brief, an early study showed that raising $[Ca^{2+}]_{cyt}$ alone does not trigger S-type anion channel activation in Arabidopsis guard cells (Allen et al., 2002). However, if the guard cell protoplasts were preexposed to high external Ca^{2+} during isolations prior to recordings, then elevated $[Ca^{2+}]_{cyt}$ rapidly activated S-type anion currents (figure 3 in Allen et al., 2002). A similar and physiologically more relevant effect was found for ABA: when guard cells were preexposed to ABA, elevated $[Ca^{2+}]_{cyt}$ strongly activated S-type anion currents by shifting the $[Ca^{2+}]_{cyt}$ sensitivity to lower $[Ca^{2+}]_{cyt}$ levels (Siegel et al., 2009; Chen et al., 2010). Interestingly, ABA preincubation also primed K^+_{in} down-regulation by $[Ca^{2+}]_{cyt}$ (Siegel et al., 2009). An increase in the Ca^{2+} sensitivity of S-type anion channel activation was also triggered by elevated CO_2 (Xue et al., 2011). Intracellular bicarbonate and CO_2 levels lead to strong S-type anion channel activation in the presence of 2 μM $[Ca^{2+}]_{cyt}$, but not at 0.1 μM $[Ca^{2+}]_{cyt}$, already 3 to 5 min after achieving the patch clamp whole-cell configuration, which allows equilibration of the pipette solution with the cytosol (Xue et al., 2011). This rapid Ca^{2+} sensitivity priming indicates that the underlying processes are less likely mediated by transcriptional changes. Early ABA signaling mechanisms were determined to indirectly or partially affect CO_2 control of stomatal closing (Merilo et al., 2013), which could be explained by the finding that both pathways require S-type anion channels and the OST1 protein kinase (Roelfsema et al., 2004; Hu et al., 2010; Xue et al., 2011; Merilo et al., 2013). Furthermore, basal ABA signaling in guard cells may partially prime guard cells to respond stronger to other stimuli such as CO_2 elevation (Merilo et al., 2013).

In preliminary experiments we have observed that simply continuously increasing the extracellular Ca^{2+} concentration appears to show a weaker Ca^{2+} reactive stomatal closure response than when oscillations in extracellular Ca^{2+} are imposed. As hyperpolarization of guard cells causes Ca^{2+} oscillations (Grabov and Blatt, 1998; Staxén et al., 1999; Klüsener et al., 2002; Siegel et al., 2009), *slac1* mutants may enhance (prime)

Table II. *Evidence for stimulus-induced Ca^{2+} sensitivity enhancement (priming) in guard cells*

Experimental Observations	Reference
Spontaneous calcium transients found in guard cells	Grabov and Blatt, 1998; Allen et al., 1999; Staxén et al., 1999; Klüsener et al., 2002; Young et al., 2006
Spontaneous calcium transients found in guard cells even when stomatal opening stimulus is applied	Young et al., 2006
Rapid Ca^{2+} reactive stomatal closing occurs for any Ca^{2+} elevation pattern above a threshold level	Allen et al., 2001; Supplemental Fig. S4: http://www.nature.com/nature/journal/v411/n6841/extref/4111053a0_S1.htm
Calcium is required for both ABA and CO_2 induced stomatal closing	e.g De Silva et al., 1985; Schwartz, 1985; Webb et al., 1996; Staxén et al., 1999; MacRobbie, 2000; Mori et al., 2006; Young et al., 2006; Zhu et al., 2007; Siegel et al., 2009
Priming (enhancement) of $[Ca^{2+}]_{cyt}$ sensitivity of S-type anion and K^+_{in} channel regulation by ABA, elevated CO_2 and high external Ca^{2+}	Allen et al., 2002; Siegel et al., 2009; Chen et al., 2010; Xue et al., 2011
Constitutive priming of Ca^{2+} sensitivity of K^+_{in} channel down-regulation in *slac1* guard cells	Laanemets et al., 2013

the cytosolic Ca^{2+} sensitivity via this pathway. Thus, prior Ca^{2+} exposure itself can play a role in Ca^{2+} sensitivity priming (see figure 3 in Allen et al., 2002). More work is needed, however, to identify the underlying mechanisms.

The result showing that the compensatory down-regulation of K^+_{in} channels in *slac1* guard cells can be rapidly reversed by lowering $[Ca^{2+}]_{cyt}$ provides additional strong evidence that the $[Ca^{2+}]_{cyt}$ sensitivity of mechanisms leading to stomatal movements can be primed (Laanemets et al., 2013). Interestingly, in *slac1* mutants, $[Ca^{2+}]_{cyt}$ down-regulation of K^+_{in} channels is constitutively primed under these conditions (Laanemets et al., 2013; Fig. 1). Moreover, it was reported that ABA-induced stomatal closure does not require preceding $[Ca^{2+}]_{cyt}$ signaling (Levchenko et al., 2005; but see De Silva et al., 1985; Schwartz, 1985; Grabov and Blatt, 1998; Staxén et al., 1999; MacRobbie, 2000; Mori et al., 2006; Young et al., 2006; Siegel et al., 2009; Chen et al., 2010). Ca^{2+} sensitivity priming, such that physiological resting $[Ca^{2+}]_{cyt}$ levels enable Ca^{2+} signaling, may explain this (Levchenko et al., 2005).

Modulation of the sensitivity of calcium sensors provides a mechanism which could contribute to the specificity in Ca^{2+} signaling in other plant responses and might help to resolve the crucial question of how Ca^{2+} elevations are "translated" into specific responses with numerous Ca^{2+} binding proteins expressed in individual cells. Further research is needed to determine whether this mechanism might also occur in other cell types and represent a more broadly used option to achieve specificity in responses to $[Ca^{2+}]_{cyt}$ in plants.

PUTATIVE BIOCHEMICAL MECHANISMS THAT MAY MEDIATE Ca^{2+} SENSITIVITY PRIMING

In vivo research has shown that CPKs are important mediators of Ca^{2+}-dependent stomatal closing and S-type anion channel activation (Mori et al., 2006; Zhu et al., 2007; Zou et al., 2010). The CPKs that are presently known to function in Ca^{2+}-induced stomatal closing in vivo are CPK6, CPK3, CPK4, CPK10, and CPK11 (Mori et al., 2006; Zhu et al., 2007; Zou et al., 2010; Hubbard et al., 2012). In addition, CPK23 and CPK21 mutants were reported to show enhanced drought resistance (Ma and Wu, 2007; Franz et al., 2011), whereas recent data showed slightly impaired stomatal closing phenotypes in response to environmental stimuli for a CPK23 mutant (Merilo et al., 2013). However, the cellular and molecular signaling mechanisms mediating Ca^{2+} sensitivity priming remain unknown. Research in *Xenopus laevis* oocytes and in vitro biochemistry are providing insights into how CPKs may mediate stomatal closing. Expression of the Ca^{2+}-dependent protein kinases CPK23, CPK21, and CPK6 showed that these CPKs activate SLAC1 anion channels in oocytes (Geiger et al., 2010; Brandt et al., 2012). Furthermore, expression of a truncated and constitutively active CPK3 also resulted in SLAC1 activation (Scherzer et al., 2012).

Although CPK6 functions in Ca^{2+}-, ABA-, and methyl jasmonate-induced activation of S-type anion channels in vivo (Mori et al., 2006; Munemasa et al., 2011), CPK6 was reported to interact with SLAC1 only weakly (Geiger et al., 2010) and not to show physiologically relevant Ca^{2+}-activated protein kinase activity in vitro (Scherzer et al., 2012). However, quantitative phosphorylation analyses showed a strong preference for CPK6 to phosphorylate the N terminus of SLAC1 in a Ca^{2+}-dependent manner (Brandt et al., 2012). A stringent biochemical analysis (modified after Hastie et al., 2006) of CPK6 protein kinase activity reveals that CPK6 is strongly activated by elevation in $[Ca^{2+}]$ in the physiological range of $[Ca^{2+}]_{cyt}$ increases from baseline levels of approximately 100 to 150 nM to concentrations greater than or equal to 300 nM (Fig. 3). Taken together, CPK6 is activated by physiological $[Ca^{2+}]$ increases and interacts with SLAC1 to phosphorylate SLAC1 Ca^{2+} dependently (Fig. 3; Brandt et al., 2012). One hypothesis for a mechanism mediating Ca^{2+} sensitivity priming is that the clade A PP2Cs directly down-regulate CPK activity (see Fig. 4), similar to PP2C-mediated down-regulation of OST1 activity (Belin et al., 2006; Yoshida et al., 2006; Umezawa et al., 2009; Vlad et al., 2009). However, to date no study has shown the down-regulation of CPK activity by PP2Cs,

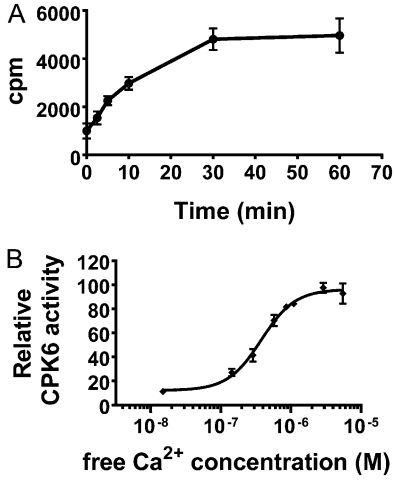

Figure 3. Quantitative phosphorylation assays show a strong Ca^{2+} activation of CPK6 activity for physiological $[Ca^{2+}]$ elevations. A, To determine proper conditions, the time-dependent phosphorylation of Syntide-2 (400 μM) by CPK6 (75 nM) was analyzed by measuring the incorporation of ^{32}P into the substrate (in counts per minute [cpm]). B, A time point in the linear range of product phosphorylation in A (4.5 min) was chosen to determine CPK6 activities at defined free Ca^{2+} concentrations. CPK6 activity is strongly dependent on the free Ca^{2+} concentration (fit parameters: K_A = 508 nM; Hill coefficient = 1.8 ± 0.2 SE; R^2 = 0.98). Error bars represent SD (n = 3 experiments). See Supplemental Text S1 for a detailed description of the method used.

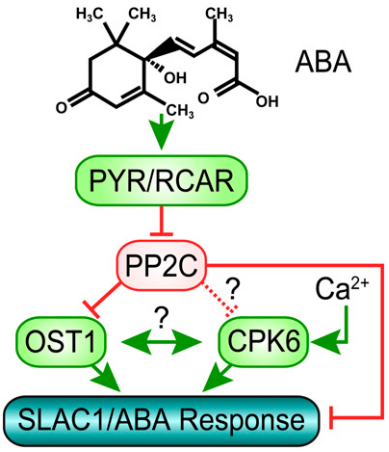

Figure 4. Simplified model of abscisic acid signaling in guard cells. In the presence of ABA, PYR/RCAR proteins inhibit PP2C phosphatases. This enables activation of the protein kinase OST1, which in turn phosphorylates and activates SLAC1, representing the Ca^{2+}-independent branch of SLAC1 activation. Furthermore, the ABI1 PP2C directly dephosphorylates SLAC1 leading to deactivation of SLAC1. Decreased PP2C activity induced by ABA also leads to decreased negative regulation of SLAC1 activation by calcium dependent protein kinases. Whether this regulation solely occurs by dephosphorylation of SLAC1 or whether in addition PP2Cs directly regulate CPKs remains to be determined (see text). A potential mechanism of cross talk between Ca^{2+}-dependent and -independent SLAC1 activation may occur by cross regulation of OST1 and CPKs, which is indicated by ?, but is hypothetical and remains to be investigated.

neither in vivo nor in vitro, and thus more research is needed to address this or other hypotheses.

Studies showed that the Ca^{2+}-independent protein kinase OST1 can activate SLAC1 in *X. laevis* oocytes and that this is inhibited by the presence of clade A PP2C phosphatases (Geiger et al., 2009; Lee et al., 2009). Moreover, a recent study demonstrated that functional ABA-activation of SLAC1 channels can be reconstituted in *X. laevis* oocytes by coexpression of ABA receptors, PP2Cs, protein kinase, and SLAC1 (Brandt et al., 2012). Either the Ca^{2+}-dependent protein kinase CPK6 (Fig. 3) or the Ca^{2+}-independent protein kinase OST1 was sufficient for functional reconstitution of ABA activation of SLAC1 (Brandt et al., 2012). Further research is needed to determine the genetic and cell signaling mechanisms that mediate stimulus-induced enhancement (priming) of $[Ca^{2+}]_{cyt}$ -dependent signal transduction.

COMMUNICATION AMONG Ca^{2+}-DEPENDENT AND Ca^{2+}-INDEPENDENT MECHANISMS

It remains unknown how the above-described Ca^{2+}-dependent and Ca^{2+}-independent pathways communicate with one another in guard cells in vivo. Several nonexclusive models can be envisioned as discussed below, although other mechanisms may also mediate this communication. A hypothesis in which PP2Cs may down-regulate CPKs (Fig. 4) remains to be investigated, as discussed above. Given that Ca^{2+}-dependent and Ca^{2+}-independent stomatal closing appear to depend on one another quantitatively (Mustilli et al., 2002; Siegel et al., 2009), an additional hypothesis is that CPKs and OST1 (cross) regulate each other (Fig. 4). However, no biochemical evidence for such cross regulation or protein-protein interaction has presently been reported, in vivo or in vitro, and this hypothesis would need to be investigated. In addition to these models, recent research demonstrated that the ABI1 PP2C phosphatase directly dephosphorylates the N terminus of SLAC1 (Fig. 4; Brandt et al., 2012). (Note that PP2Cs are Mg^{2+}-requiring protein phosphatases and millimolar Mg^{2+} concentrations are best included at all times, including during all PP2C protein purification steps, to assess their roles in target dephosphorylation.) The dephosphorylation of SLAC1 by ABI1 provides a mechanism for the required tight regulation of S-type anion channel activity in guard cells (Fig. 4; Pei et al., 1997). Direct regulation of ion channels by protein phosphatases has been reported for other plant and animal ion channels (Westphal et al., 1999; Chérel et al., 2002; Lee et al., 2007; Zhou et al., 2010). Furthermore, OST1 may regulate $[Ca^{2+}]_{cyt}$ levels via the NADPH oxidases respiratory burst oxidase homolog D and F and subsequent reactive oxygen species bursts (Sirichandra et al., 2009). Through this pathway, OST1 could control $[Ca^{2+}]_{cyt}$ (Kwak et al., 2003) and regulate CPK activities. A fourth hypothesis, which does not exclude the above models, is that SLAC1 serves as a coincidence detector for phosphorylation and activation by OST1 and CPKs (Fig. 4). OST1 has been shown to phosphorylate residues including Ser 120 (S120) in the N terminus of SLAC1 and S120 phosphorylation is essential for the SLAC1 activation by OST1 in oocytes (Geiger et al., 2009) and for stomatal closing (Vahisalu et al., 2010). However, recent experiments showed that stomatal closure induced by environmental factors were clearly less impaired in *slac1-7* plants that carry S120F mutation than those observed for SLAC1 knockout plants, further suggesting that SLAC1 activation is a process that involves phosphorylation of multiple amino acids by multiple protein kinases (Merilo et al., 2013). In line with this assumption, S120A mutation did not disrupt activation of SLAC1 by CPK23 (Geiger et al., 2010). Moreover, CPK6 phosphorylated Ser 59 (S59) in the SLAC1 N terminus and S59 phosphorylation is essential for SLAC1 activation by CPK6 (Brandt et al., 2012). Data show that S59 can also be phosphorylated by OST1 in vitro (Vahisalu et al., 2010). However, whether this is required for OST1 activation of SLAC1 remains unknown. Thus, a combination of the above options and/or additional mechanisms may mediate Ca^{2+} specificity and sensitivity priming and synergistic effects of Ca^{2+}-dependent and Ca^{2+}-independent signal transduction. These models await investigation and could lead to a detailed mechanistic understanding of

a network that mediates specificity in plant calcium signal transduction.

SUMMARY

In conclusion, recent findings show that stomata compensate for excessively open apertures by mechanisms that include constitutive priming (enhancement) of Ca^{2+} sensitivity, as found in *slac1* guard cells (Laanemets et al., 2013; Figs. 1 and 2; Supplemental Fig. S1). The finding that stomatal regulation can adapt to and compensate for impaired stomatal responses (Laanemets et al., 2013) could be of broader relevance for plant-environment interactions. A precise biochemical and cellular understanding of the mechanisms that ensure compensatory regulation of stomatal movements and detailed mechanisms mediating specificity in Ca^{2+} signaling remain to be elucidated in plants. ABA- and CO_2-induced Ca^{2+} sensitivity priming in guard cells (Young et al., 2006; Siegel et al., 2009; Chen et al., 2010; Xue et al., 2011) provides a system that can explain calcium signaling specificity in guard cells and adds to other (nonexclusive) models for Ca^{2+} signaling specificity in plants (Kudla et al., 2010). An in depth biochemical and cellular understanding of mechanisms mediating specificity in Ca^{2+} signaling is also a present goal in animal cell signaling research (Berridge, 2012). The hypotheses and models proposed here (Fig. 4) could enable the underlying specificity mechanisms to be characterized at an in depth mechanistic level in a plant cell system.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers supplied in Supplemental Text S1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *slac1* shows enhanced Ca^{2+} sensitivity at resting $[Ca^{2+}]_{cyt}$.

Supplemental Figure S2. K^+_{in} channels are down-regulated in *ost1-3* mutant plants.

Supplemental Figure S3. K^+_{in} channels are down-regulated in *abi2-1* mutant plants.

Supplemental Text S1. Supplemental Materials and Methods.

Received April 23, 2013; accepted June 10, 2013; published June 13, 2013.

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