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Mutations in SLAC1 anion channel slow stomatal opening and severely reduce K^+ uptake channel activity via enhanced cytosolic [Ca^{2+}] and increased Ca^{2+} sensitivity of K^+ uptake channels

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Summary

- The *Arabidopsis* guard cell anion channel SLAC1 is essential for stomatal closure in response to various endogenous and environmental stimuli. Interestingly, here we reveal an unexpected impairment of *slac1* alleles on stomatal opening.
- We report that mutations in SLAC1 unexpectedly slow stomatal opening induced by light, low CO_2 and elevated air humidity in intact plants and that this is caused by the severely reduced activity of inward K^+ (K^+_{in}) channels in *slac1* guard cells.
- Expression of channels and transporters involved in stomatal opening showed small, but significant reductions in transcript levels in *slac1* guard cells, however this was deemed insufficient to explain the severely impaired K^+_{in} channel activity in *slac1*. We further examined resting cytosolic $[Ca^{2+}]_{cyt}$ levels and K^+_{in} channel sensitivity to cytosolic $[Ca^{2+}]_{cyt}$ in *slac1*. These experiments showed higher resting $[Ca^{2+}]_{cyt}$ in *slac1* guard cells and that reducing $[Ca^{2+}]_{cyt}$ to <10 nM rapidly restored the activity of K^+_{in} channels in *slac1* closer to wild type levels.
- These findings demonstrate an unanticipated compensatory feedback control in plant stomatal regulation, that counteracts the impaired stomatal closing response of *slac1*, by

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Supporting Information

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down-regulating stomatal opening mechanisms and implicates enhanced $[Ca^{2+}]_{cyt}$ sensitivity priming as a mechanistic basis for the down-regulated K^+_{in} channel activity.

Keywords

abscisic acid (ABA); anion channel; Ca^{2+} signaling; cytosolic Ca^{2+} concentration; guard cells; potassium uptake channel; SLAC1; stomatal opening

Introduction

Each stomatal pore in the epidermis of aerial plant tissues is formed by a pair of guard cells. They enable carbon dioxide (CO_2) uptake into leaves for photosynthesis and control transpirational water loss to the atmosphere. The aperture of stomatal pores is regulated by guard cell turgor pressure adjusted by modification of guard cell ion and organic solute content (Raschke *et al.*, 1988). Stomata respond to various environmental and endogenous factors, such as light, air humidity, CO_2 concentration, abscisic acid (ABA) and cytosolic Ca^{2+}. Elevation of cytosolic Ca^{2+} functions in ABA-, and CO_2-induced stomatal closing (McAinsh *et al.*, 1990; Webb *et al.*, 1996; Grabov & Blatt, 1998; Staxen *et al.*, 1999; MacRobbie, 2000; Allen *et al.*, 2001; Marten *et al.*, 2007a; Young *et al.*, 2006; Siegel *et al.*, 2009; Hubbard *et al.*, 2012).

Stomatal opening is driven by the guard cell plasma membrane H^+-ATPase, which pumps protons from the guard cell cytosol to the cell wall and creates an electrochemical gradient across the plasma membrane (Assmann *et al.*, 1985; Shimazaki *et al.*, 1986; Thiel *et al.*, 1992; for review see Shimazaki *et al.*, 2007). Membrane hyperpolarization triggers the activation of voltage-gated potassium uptake channels (K^+_{in}) that mediate the uptake of K^+ ions (Schroeder *et al.*, 1984). Guard cell K^+_{in} channels function as multimers and single channel gene knockouts do not have apparent stomatal and K^+_{in} channel activity phenotypes (Szyroki *et al.*, 2001; Very & Sentenac 2003). However, over-expression of a dominant negative K^+_{in} channel mutant (Uozumi *et al.*, 1995) resulted in a slowing and decrease in light-induced stomatal opening by *c.* 45% (Kwak *et al.*, 2001). Furthermore, even stronger repression of K^+_{in} channels in *kincless*, which is a knockout of KAT2 transformed with a dominant negative version of KAT2, resulted in substantially reduced light-induced stomatal opening (Lebaudy *et al.*, 2008). The activity of K^+_{in} channels in guard cells is rapidly down-regulated by $[Ca^{2+}]_{cyt}$ elevation in guard cells (Schroeder and Hagiwara, 1989; Kelly *et al.*, 1995; Grabov and Blatt, 1999). Recent research has shown that ABA enhances the $[Ca^{2+}]_{cyt}$ sensitivity of K^+_{in} channel down-regulation in guard cells (Siegel *et al.*, 2009), which is in-line with ABA inhibition of stomatal closing.

When plants are subjected to factors causing stomatal closure, slow (S-type) and rapid (R-type) type anion channels are activated (Keller *et al.*, 1989; Schroeder and Hagiwara, 1989; Pei *et al.*, 1997; for review see Barbier-Brygoo *et al.*, 2011; Kollist *et al.*, 2011). Efflux of anions leads to depolarization of the plasma membrane, which in turn activates K^+ efflux channels (Schroeder *et al.*, 1987; Blatt and Armstrong, 1993; Roelfsema *et al.*, 2001). A screen for *Arabidopsis* ozone sensitive mutants (Overmyer *et al.*, 2000; Overmyer *et al.*, 2008, Vahisalu *et al.*, 2008) and CO_2-insensitive mutants (Negi *et al.*, 2008) led to the identification of SLAC1, a protein essential for guard cell plasma membrane S-type anion channel function (Vahisalu *et al.*, 2008). SLAC1 is required for stomatal closure induced by ozone, CO_2, ABA, calcium, light/dark transitions and reduction in air humidity (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Mutations in *SLAC1* impaired S-type anion channel function, but R-type anion channels and ABA-activated Ca^{2+}-permeable channel activity was not altered (Vahisalu *et al.*, 2008). Furthermore, guard cell protoplasts of *slac1* over-accumulated of osmoregulatory ions, such as malate, fumarate, Cl^- and K^+ (Negi *et al.*,

2008). The plasma membrane localization of SLAC1 and the homology of SLAC1 to anion transporters (Camarasa *et al.*, 2001) suggested that *SLAC1* encodes guard cell S-type anion channel (Vahisalu *et al.*, 2008). Heterologous expression of *SLAC1* in Xenopus oocytes induced S-type anion currents similar to those observed in guard cells confirming this model (Geiger *et al.*, 2009; Lee *et al.*, 2009).

During stomatal opening, SLAC1 must be inactivated (Schroeder *et al.*, 1993; Schwarz *et al.*, 1995; Marten *et al.*, 2007). This implies that stomatal opening should be faster in *slac1* plants. However, in complete contrast to this prediction, we found that *slac1* exhibited a reduced rate of stomatal opening in response to three physiological stimuli of stomatal opening: light, low CO_2 and high humidity in intact plants. Interestingly, although *SLAC1* encodes for S-type anion channels, we demonstrate that *slac1* alleles exhibit strongly impaired K^+_{in} channel activities, which provides a mechanistic basis for slower stomatal opening in *slac1* mutants. Furthermore, the dramatic reduction in K^+_{in} channel currents in *slac1* guard cells is shown to be rapidly reversed upon lowering the cytosolic free Ca^{2+} concentration, showing an enhanced $[Ca^{2+}]_{cyt}$ sensitivity of K^+_{in} channels in *slac1*.

These findings imply that eliminating SLAC1 function (Vahisalu *et al.*, 2008; Negi *et al.*, 2008) unexpectedly affects the activities of other membrane proteins essential for mediation of stomatal opening, and they point to compensatory mechanisms that counteract transpirational water loss.

Materials and Methods

Plant growth and gas exchange

Arabidopsis thaliana (L.) Heynh (Col-0 as wild-type) seeds were planted in soil containing 4:3 (v:v) peat:vermiculite and grown through a hole in a glass plate as described (Kollist *et al.*, 2007). Soil moisture was kept at 60-80% of maximum water capacity. Plants were grown in growth chambers (AR-66LX and AR-22L, Percival Scientific, IA, USA) at 12 h : 12 h photoperiod and 150 μmol m^{-2} s^{-1} light. Temperature was 23°C in the light and 18°C in the dark, relative humidity of the air was 70-80%. For experiments 23-26 d old plants were used.

Stomatal conductance was measured in intact plants using a rapid-response gas exchange measurement device consisting of eight through-flow whole-rosette cuvettes (Kollist *et al.*, 2007). Inlet air composition was monitored and adjusted to desired values. Air humidity and CO_2 concentration were decreased by filtering air through a granular potassium hydroxide. Humidity was increased by a humidifying thermostat system and CO_2 was added to inlet air as necessary. Light conditions were changed manually by covering plants from the light source. Stomatal conductance measurements for air humidity and CO_2 treatments were carried out during 11:00-15:00 h; light treatment was applied at the onset of regular light regime (07:30 h).

Guard cell protoplast extraction and RT-PCR

Guard cells from plants grown as described above were isolated using the protocol by Leonhardt *et al.* (2004). Plant RNA was extracted using the RNeasy Plant Mini Kit and converted to cDNA using QuantiTect Reverse Transcription Kit (both from Qiagen GmbH, Germany). 7900HT Fast Real-Time PCR System was used with MicroAmpTM Optical 384-well Reaction Plate (Applied Biosystems, Singapore) and MicroAmpTM Optical Adhesive Film (Applied Biosystems, USA).

For qPCR reactions, the Maxima™ SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) was used. The TIP41-like family protein (At4g34270; (Czechowski *et al.*, 2005) was used as

normalization standard. Primers used for RT-PCR are listed in Supporting Information Table S1. The following genes were analysed: Guard cell expressed H^+ATPase genes *AHA1* (At2g18960), *AHA2* (At4g30190), and *AHA5* (At2g24520) (Ueno *et al.*, 2005). The inward rectifying K^+ channels *KAT1* (At5g46240) (Nakamura *et al.*, 1995), *KAT2* (At4g18290) (Pilot *et al.*, 2001), *AKT2* (At4g22200) (Cao *et al.*, 1995), *KC1* (At4g32650) (Reintanz *et al.*, 2002) and K^+ outflow channel GORK (At5g37500) (Ache *et al.*, 2000). We also measured expression levels of *AtABCB14* (At1g28010) which mediates malate uptake (Lee *et al.*, 2008), aluminum activated malate transporter, *ALMT12* (Meyer *et al.*, 2010; Sasaki *et al.*, 2010) and *TPC1* (At4g03560), a vacuolar Ca^{2+} channel required for Ca^{2+}-inhibition of stomatal opening (Peiter *et al.*, 2005).

Patch clamping

Arabidopsis thaliana plants (Columbia ecotype) were grown in soil (Sungro Special blend Professional Growing Mix; Seba Beach, Alberta, Canada) in a growth chamber (Conviron, Manitoba, Canada) under a 16-h-light : 8-h-dark cycle at a photon fluence rate of 75 μmol m^{-2} s^{-1} and a temperature of 20-22°C with ≥70% humidity described previously (Mori *et al.*, 2006). *Arabidopsis* guard cell protoplasts were isolated enzymatically as described previously (Vahisalu *et al.*, 2008). To analyze inward potassium channel currents at physiological $[Ca^{2+}]_{cyt}$, the bath solution contained 30 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Mes-Tris, pH 5.5, pipette solution contained 30 mM KCl, 70 mM K-Glu, 2 mM $MgCl_2$, 6.7 mM EGTA, 10 mM HEPES-Tris, pH 7.1, and 3.35 mM $CaCl_2$ was added to result in 250 nM free Ca^{2+} as described previously (Pei *et al.*, 1997). Inward potassium currents were recorded within 2 to 3 min after accessing whole-cell configurations and for continued durations >5 min. Thus the pipette solution that dialyzes the cytoplasm (Pusch & Neher, 1988) enabled the cytoplasmic free Ca^{2+} concentration to reach a stable state. To analyze inward potassium channel currents at low free $[Ca^{2+}]_{cyt}$, no Ca^{2+} was added to the pipette solution, other components in the pipette solution remained unchanged, resulting in a calculated free Ca^{2+} concentration of <10 nM. The same bath solution was used for all conditions. The osmolarities of the bath and pipette solutions for inward potassium current recordings were adjusted with D-sorbitol to 485 mM and 500 mM, respectively (Pei *et al.*, 1997). To analyze outward malate currents, the bath solution contained 30 mmol/L Cs-Malate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Mes-Tris, pH 5.6, and the pipette solution contained 150 mM Cs-Malate 2 mM $MgCl_2$, 10 mM Hepes-Tris, pH 7.05, plus 6.67 mM EGTA and 5.86 mM $CaCl_2$ to result in 2 μM free Ca^{2+}. The osmolarities of bath and pipette solutions were adjusted with D-Sorbitol to 485 mM and 500 mM, respectively. For malate current analyses, guard cell protoplasts were extracellularly pre-incubated for 30 min in the same bath solution with 40 mM $CaCl_2$ added before patch clamping, the $CaCl_2$ concentration in the bath solution was reduced from 40 mM to 1 mM by perfusion before patch clamping, and whole-cell recordings with giga seals (>10 GΩ) were achieved within 30 min after the pre-incubation as described previously (Vahisalu *et al.*, 2008).

Ratiometric quantitation of cytosolic Ca^{2+} with yellow cameleon 3.6

Cytosolic Ca^{2+} concentration changes were monitored using the non-invasive FRET Ca^{2+} reporter yellow cameleon. 3.6 as previously described (Mori *et al.*, 2006). Intact leaf epidermes adhered to a cover slip were incubated 3 h in stomatal opening buffer (50 mM KCl, 10 mM MES-Tris (pH 5.6), 0 mM added $CaCl_2$) for 3 h in 200 μmol $m^{-2}s^{-1}$ white light and baseline cytosolic Ca^{2+} concentrations were monitored for 0.5 h in the same stomatal opening buffer in > 30 wild-type and >30 *slac1* guard cells, as indicated.

ABA-induced stomatal closure

Stomatal measurements were performed by excising *c.* 4 wk old rosette leaves. Intact leaves were glued abaxial side down onto cover slips using Hollister medical adhesive and

mesophyll cells were removed from the intact epidermis using single edge industrial razor blades (Young *et al.*, 2006). The glued epidermes were submerged in stomatal opening buffer (10 mM KCl, 7.5 mM iminodiacetic acid, 10 mM MES and pH 6.2 adjusted with KOH) for 3 h in light (100 μmol m^{-2} s^{-1}) and then incubated for an additional 3 h or 1 h in stomatal opening buffer in which the indicated ABA concentrations had been added. Stomatal apertures (30 stomata per treatment per experiment) were then measured on a light microscope (Nikon Diaphot 300) via scion image. Measurements were performed as double blind in which genotype and ABA concentrations were not known to the experimenter.

Results

Mutations in SLAC1 anion channel slows high humidity-, light- and low CO_2-induced stomatal opening

In initial experiments, we observed an unexpected slowing of stomatal opening when plants kept in darkness for 2 h were exposed to 130 μmol m^{-2} s^{-1} white light (Fig. S1a). As in these experiments there were differences in pre-treatment stomatal conductances between wild-type and *slac1* mutant alleles (Fig. S1b), we sought for conditions that produce similar steady-state starting stomatal conductances for further investigations of stimulus-induced stomatal opening. We found that by adapting plants initially to low air humidity (water vapor concentration difference between leaf and air 23.5 ± 0.5 mmol mol^{-1}, relative air humidity in cuvette 17.9 ± 0.7%) for 2-3 h the *slac1-1* and *slac1-3* and wild-type plants equilibrated at similar stomatal conductances. To analyze humidity-induced stomatal opening air humidity was increased (water vapor concentration difference between leaf and air 14.9 ± 0.3 mmol mol^{-1}, relative air humidity in cuvette 47.3 ± 0.6%) while keeping CO_2 and light conditions constant. Such treatments induced first a sudden decline in stomatal conductance (Fig. 1a,b), which has been proposed to be caused by a rapid decrease of water efflux from epidermal cells and concomitant increase of their pressure on guard cells (Ivanoff, 1928). This was followed by a rapid increase in stomatal opening in both wild type and in *slac1* mutants. However, the initial rates of stomatal conductance changes were unexpectedly faster in wild type than in *slac1* mutants (at 12 min 0.74 ± 0.19, 0.21 ± 0.07 and 0.15 ± 0.05 mmol m^{-2} s^{-1} per min for wild type, *slac1-1* and *slac1-3*, respectively, $P < 0.01$).

To address light–induced stomatal opening, plants were kept in gas-exchange cuvettes over regular night (12 h) period. In the morning *slac1* and wild-type plants had similar stomatal conductance. After onset of light, conductance began to increase both in wild-type and in *slac1* mutants (Fig. 1c,d). However, the initial rates of increases in stomatal conductance were significantly slower in *slac1* mutants than in wild-type (at 12 min 2.9 ± 0.4, 0.9 ± 0.3 and 1.1 ± 0.4 mmol m^{-2} s^{-1} per min, in wild-type, *slac1-1* and *slac1-3*, respectively, $P < 0.01$). After 30 min in white light, the increase in stomatal conductance in wild type plants ceased and even slightly declined until it reached a steady state. In *slac1* only a gradual slower rate of stomatal opening was observed without the subsequent slight closure.

To study the stomatal responsiveness of *slac1* to low CO_2, we first acclimatized plants under reduced humidity for 2-3 h to achieve similar stomatal conductances of *slac1* mutants and wild-type plants. Thereafter CO_2 was reduced from 400 to 40 μmol mol^{-1}. Such treatment caused a rapid, three-fold increase in stomatal conductance in all genotypes (Fig. 1e,f). Still, the initial rate of stomatal opening was clearly slower in *slac1* mutants (at 12 min 1.5 ± 0.1 and 1.5 ± 0.2 mmol m^{-2} s^{-1} per min in *slac1-1* and *slac1-3*, compared to 2.4 ± 0.2 in wild-type, $P < 0.01$).

Earlier experiments have shown that *slac1* plants are largely insensitive to high CO_2-induced stomatal closure (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). To research whether elevation of

CO_2 from 100 μmol mol^{-1} to 400 μmol mol^{-1} will induce stomatal closure in *slac1*, plants were acclimatized under low CO_2 for 3 h followed by an increase of CO_2 to 400 μmol mol^{-1}. This caused clear, though significantly slower, stomatal closure also in *slac1* mutants (Fig. S2). Also stepwise reduction of CO_2 concentration from 400 to 0 μmol mol^{-1} followed by increase to 800 μmol mol^{-1} indicated that *slac1* had a clear, though reduced, stomatal response to CO_2 changes (Fig. S3). These results are in agreement with the findings that SLAC1 is not the only anion channel in guard cells, as R-type anion channels and SLAH3 remain intact in *slac1* (Vahisalu *et al.*, 2008; Geiger *et al.*, 2011).

slac1 guard cells exhibit dramatically reduced K^+_{in} channel currents

Inward K^+ (K^+_{in}) channels provide a pathway contributing to K^+ uptake during stomatal opening driven by H^+-ATPases in guard cells (Schroeder *et al.*, 1984; Schroeder *et al.*, 1987; Thiel *et al.*, 1992; Kwak *et al.*, 2001; Lebaudy *et al.*, 2008). In parallel independent research, we analyzed K^+_{in} channel currents by whole-cell patch clamping. These measurements showed dramatically reduced K^+_{in} channel activity in *slac1-1* and *slac1-3* guard cells compared to wild type (Fig. 2), indicating that the disruption of S-type anion channel function in *slac1* unexpectedly also greatly reduced the activity of K^+_{in} channels. In *slac1-1* and *slac1-3* the reduction (by 84% and 79%, respectively) in K^+_{in} channel current magnitude of *slac1* alleles at -180 mV was similar to low K^+_{in} current magnitudes that caused slow stomatal opening when K^+_{in} channels were blocked or genetically impaired (Kelly *et al.*, 1995; Kwak *et al.*, 2001; Lebaudy *et al.*, 2008). Next we investigated possible mechanisms causing the dramatic reduction in K^+_{in} channel activity in *slac1* alleles.

Mutations in SLAC1 cause partly reduced expression levels of ion channels in guard cells

We investigated whether the reduced K^+_{in} channel activity in *slac1* could be a result of reduced K^+_{in} channel subunit mRNA levels, and/or whether impaired SLAC1 has affected the expression of other known transporters expressed in guard cells (Leonhardt *et al.*, 2004; Yang *et al.*, 2008) involved in stomatal opening. We analyzed mRNA levels of several guard cell ion channels and transporters in *slac1* and wild type isolated guard cells, including *AHA1, AHA2, AHA5, KAT1, KAT2, AKT1, AKT2, AtABCB14, GORK, TPC1, AtALMT12* and *AtKC1* (For details see Table S1). Transcript levels of several guard cell-expressed transporter genes were reduced in *slac1* guard cells in four independent experiments (Fig. 3). The three guard cell-expressed H^+-ATPase transcripts showed slight average reductions in *slac1* mutants guard cells, albeit at a 90% statistical confidence level only for AHA1 and AHA2 in *slac1-3*. Also the transcript levels of guard cell malate importer *AtABCB14* which was reported to limit light and CO_2-induced stomatal responses (Lee *et al.*, 2009) were reduced in *slac1-1*. Moreover, *KAT2* transcript level was significantly reduced in both *slac1* alleles at a 95% statistical confidence level (Fig. 3). However, we concluded that the observed differences in ion channel transcript levels were deemed unlikely to cause the severely reduced K^+_{in} channel activity in *slac1* mutants.

Decreasing the cytosolic Ca^{2+} concentration rapidly restores K^+_{in} channel currents in *slac1* guard cells

Elevation in the cytosolic Ca^{2+} is known to activate S-type anion channels in guard cells (Schroeder & Hagiwara, 1989; Mori *et al.*, 2006; Vahisalu *et al.*, 2008; Siegel *et al.*, 2009; Chen *et al.*, 2010). Furthermore, $[Ca^{2+}]_{cyt}$ elevation is also known to down-regulate K^+_{in} channel activity, as a mechanism contributing to inhibition of stomatal opening (Schroeder & Hagiwara, 1989; Kelly *et al.*, 1995; Grabov & Blatt, 1999; Siegel *et al.*, 2009). To explore whether cytosolic Ca^{2+} plays a role in the substantially reduced K^+_{in} channel activity of *slac1* guard cells, patch clamp experiments were performed in which the free Ca^{2+} concentration in the patch clamp pipette solution was decreased from 250 nM to <10 nM by adding 6.7 mM EGTA and no Ca^{2+} to the pipette solution (see Methods). Interestingly,

recording of K^+_{in} channel currents only 2 to 3 min after reducing $[Ca^{2+}]_{cyt}$ to <10 nM by whole-cell patch clamp access to the cytoplasm of guard cells (Hamill *et al.*, 1981) resulted in markedly increased K^+_{in} channel current magnitudes in *slac1* mutant guard cells. At <10 nM free $[Ca^{2+}]_{cyt}$ in the pipette solution that dialyzes the cytoplasm of guard cells, K^+_{in} channel currents were only *c.* 20% smaller than in wild type guard cells (Fig. 4a,b). This lay in stark contrast to experiments in which $[Ca^{2+}]_{cyt}$ was buffered to higher levels (250 nM) in the pipette solution (Fig. 2). These results showed that K^+_{in} channel currents could be rescued by lowering $[Ca^{2+}]_{cyt}$ to an unphysiologically low concentration of 10 nM, thus revealing an enhanced sensitivity of K^+_{in} channels to $[Ca^{2+}]_{cyt}$ in *slac1* guard cells. To determine whether resting cytosolic Ca^{2+} levels are higher in *slac1* than in wild-type guard cells, we used plants transformed with the non-invasive yellow cameleon construct 3.6 (YC3.6) (Mori *et al.*, 2006; Siegel *et al.*, 2009; Vahisalu *et al.*, 2008). Analysis of YC3.6 transformed wild-type guard cells, showed that average baseline fluorescence ratio in wild-type guard cells was 1.71 ± 0.10 SD (± 0.02 SEM, $n = 35$ guard cells). The average baseline fluorescence ratio of *slac1* guard cells was 1.79 ± 0.1 SD (± 0.01 SEM, $n = 39$ *slac1* guard cells). Note that we compared baseline FRET ratios of the cameleon Ca^{2+} reporter, rather than attempt calibration of free $[Ca^{2+}]_{cyt}$ ratios. Calibrations usually do not calibrate each individual cell and therefore introduce errors with any Ca^{2+} reporter. Therefore comparative $[Ca^{2+}]_{cyt}$ imaging analyses often report unbiased raw $[Ca^{2+}]_{cyt}$ ratios (Siegel *et al.*, 2009), as analyzed here. Thus analyses of a large number of wild-type and *slac1* guard cells indicated that *slac1* guard cells had slightly but statistically significant ($P < 0.001$)higher baseline $[Ca^{2+}]_{cyt}$ ratios (Fig. 4c), which suggests that *slac1* mutants guard cells have an elevated cytosolic Ca^{2+} concentration.

Prolonged treatment with ABA induces partial stomatal closure in *slac1* mutants

We have previously shown that ABA-induced stomatal closure was strongly impaired in *slac1* when up to 10 μM ABA was applied for 40 min (Vahisalu *et al.*, 2008). Since R-type anion channels remained functional in *slac1* guard cells (Vahisalu *et al.*, 2008), we analyzed *slac1* stomatal responses to higher ABA concentrations and longer exposure times. This demonstrated that application of 50 μM ABA for 3 h induced a clear but partial stomatal response in *slac1* (Fig. 5a). To exclude that this result was due to changes in experimental conditions, we repeated earlier experiments with application of a more physiological lower ABA concentration and analyzed short-term responses (Fig. 5b). In line with previous findings (Vahisalu *et al.*, 2008), these conditions did not induce stomatal closure in *slac1* (Fig. 5b). Together, these data reinforce the observation that both S-type and R-type anion channels in guard cells contribute to stomatal closure, and suggest that the S-type anion channel is important for fast responses as also indicated by the lack of rapid ozone/ROS responses in *slac1* (Vahisalu *et al.*, 2010).

Given that, in contrast to wild type, *slac1* guard cells retain high intracellular organic anion concentrations, including malate (Negi *et al.*, 2008), we analyzed whether S-type anion channels can mediate malate efflux from *Arabidopsis* guard cells. Patch clamp studies with malate in the pipette solution that dialyzes the cytoplasm of guard cells did not display clear anion efflux currents (outward currents), suggesting that S-type anion channels in *Arabidopsis* guard cells are not highly permeable to malate anions (Fig. S4). These results correlate with a report that S-type anion currents are suppressed by malate (Wang & Blatt, 2011). Thus the high malate and fumarate content of *slac1* guard cells (Negi *et al.*, 2008) may also result from an influence of impaired SLAC1 function on additional transport processes in guard cells.

Discussion

The guard cell plasma membrane S-type anion channel SLAC1 is required for stomatal closure in responses to multiple environmental and endogenous stimuli (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). *slac1* guard cells exhibit greatly reduced S-type anion channel function, whereas R-type anion channels and ABA-activated Ca^{2+}-permeable channels remained functional (Vahisalu *et al.*, 2008). These data illustrate the rate-limiting role of S-type anion channels for the onset of stomatal closure (Schroeder & Hagiwara, 1989; Schroeder *et al.*, 1993; Kim *et al.*, 2010; Kollist *et al.*, 2011). Stomatal opening, on the contrary, requires accumulation of anions and thus S-type anion channels are inactivated (Schwartz *et al.*, 1995; Marten *et al.*, 2007). This implies that the absence of SLAC1 function might result in faster stomatal opening. However, our experiments show the opposite, the impaired SLAC1 activity in two independent *slac1* mutant alleles clearly reduced the rate of stomatal opening in response to three distinct stimuli in intact plants: light, low CO_2 and an increase in air humidity (Fig. 1). As SLAC1 mediates anion efflux, it is unlikely that impairment in SLAC1 activity would directly be responsible for the slow stomatal opening phenotype observed in *slac1*. Furthermore, humidity, light and CO_2 induce stomatal opening by different signaling pathways (Roelfsema & Hedrich, 2005; Shimazaki *et al.*, 2007), which suggests that a basic property of stomatal opening and not only a single signaling pathway is affected in *slac1*. The basic property, which is the same for light-, CO_2- and humidity-induced stomatal opening, could be the uptake of ions leading to water inflow and swelling of guard cells.

Down-regulation of guard cell potassium uptake channel activity by enhanced $[Ca^{2+}]_{cyt}$ sensitivity in SLAC1 mutants

We found a dramatic reduction of K^+_{in} channel activity in *slac1* (Fig. 2). Based on previous knock-down and pharmacological experiments, blocking of K^+_{in} channel activity by 80% or more in guard cells, causes a slowing in the rate of stomatal opening (Kelly *et al.*, 1995; Kwak *et al.*, 2001; Lebaudy *et al.*, 2008). Therefore, the observed reduction (≈ 80%) in K^+_{in} channel activity in *slac1* alleles (Fig. 2) provides a functional basis that can cause slowed stomatal opening responses to light, low CO_2 and high humidity (Fig. 1).

We also analyzed K^+_{in} channel and other ion transporter transcript levels which revealed decreased expression of *KAT2, AKT2, AtABCB14, AHA1* and *AHA2* (Fig. 3). However, the decreases in transcript levels were modest and are unlikely to fully explain the large reduction of K^+_{in} currents. Further exploration of the reduction in K^+_{in} channel activity in *slac1* guard cells (Fig. 2) revealed that the K^+_{in} current activities were rapidly restored in *slac1* guard cells by lowering $[Ca^{2+}]_{cyt}$ to below physiological resting levels of <10 nM (Fig. 4). A recent study showed that ABA enhances the $[Ca^{2+}]_{cyt}$ sensitivity of K^+_{in} channel down-regulation in *Arabidopsis* guard cells demonstrating that the $[Ca^{2+}]_{cyt}$ sensitivity of K^+_{in} channels is tunable (Siegel *et al.*, 2009). This stimulus-induced enhancement of the $[Ca^{2+}]_{cyt}$ sensitivity of guard cell signaling mechanisms has been termed 'Ca^{2+} sensitivity priming' (Young *et al.*, 2006; Siegel *et al.*, 2009; Xue *et al.*, 2011). The present results suggest that an impaired SLAC1 channel may similarly enhance the Ca^{2+} sensitivity of K^+_{in} channel, such that free $[Ca^{2+}]_{cyt}$ levels of 250 nM dramatically down-regulate K^+_{in} channel activity (Fig. 2). This response could provide an adaptive response of guard cells to counteract the tendency of *slac1* mutant stomata to open too widely.

Ca^{2+} imaging analyses showed that *slac1* guard cells have a slightly elevated cytosolic $[Ca^{2+}]_{cyt}$ concentration (Fig. 4c). The impairment in S-type anion channel activity in *slac1* guard cells (Vahisalu *et al.*, 2008), is predicted to cause more negative membrane potentials. This in turn can increase the activation of hyperpolarization-activated Ca^{2+} permeable I_{Ca} channels in guard cells (Pei *et al.*, 2000; Hamilton *et al.*, 2000), which may provide a basis

for the observed elevation in baseline $[Ca^{2+}]_{cyt}$ in *slac1* guard cells. Although the increase in $[Ca^{2+}]_{cyt}$ was statistically significant, more research would be needed to determine the relative contribution of this elevation to the slowed stomatal opening, as K^+_{in} channels also exhibited an enhanced sensitivity to $[Ca^{2+}]_{cyt}$ *per se*. Previous studies have shown that exposure of guard cells to elevated extracellular Ca^{2+} concentrations primes guard cells to respond to cytosolic Ca^{2+} (Allen *et al.*, 2002; Mori *et al.*, 2006). Thus the elevated baseline $[Ca^{2+}]_{cyt}$ concentrations in *slac1* guard cells (Fig. 4c), may contribute to the constitutively primed (enhanced) Ca^{2+} sensitivity of K^+_{in} channels in *slac1* guard cells (Figs 2, 4).

Intracellular Ca^{2+} elevation regulates several key mechanisms and ion channels that function in stomatal closing (Schroeder & Hagiwara, 1989; McAinsh *et al.*, 1990; Hedrich *et al.*, 1990; Webb *et al.*, 1996; MacRobbie, 2000; Siegel *et al.*, 2009). The rapid "Ca^{2+}-reactive" phase of stomatal closing is mediated by $[Ca^{2+}]_{cyt}$ activation of SLAC1 channels (Vahisalu *et al.*, 2008) and has been shown to be independent of the $[Ca^{2+}]_{cyt}$ oscillation frequency, in contrast to the long-term 'Ca^{2+}-programmed' phase (inhibition of stomatal re-opening after initial closure) (Allen *et al.*, 2001). The rapid initial Ca^{2+}-reactive phase of stomatal closing (Allen *et al.*, 2001) has been shown to include ABA- and CO_2-induced enhancement (priming) of the $[Ca^{2+}]_{cyt}$ sensitivity of S-type anion channel activation (Siegel *et al.*, 2009; Chen *et al.*, 2010; Xue *et al.*, 2011) and enhancement in the $[Ca^{2+}]_{cyt}$ sensitivity of K^+_{in} channel down-regulation (Siegel *et al.*, 2009).

Furthermore, knock-out of *SLAC1* can be predicted to cause more negative guard cell membrane potentials, based on the lack of the major depolarizing SLAC1 anion efflux activity (Schroeder & Hagiwara, 1989), which would drive enhanced K^+ influx and more rapid stomatal opening. To counter these effects, an enhanced Ca^{2+} sensitivity of K^+_{in} channels would provide a mechanism to at least partially counteract the detrimental effects of *slac1* mutation. The unexpected strongly reduced K^+_{in} channel activity previously found in *abi2-1* guard cells also correlates with the much wider stomatal apertures in *abi2-1* guard cells under the imposed conditions (Pei *et al.*, 1997). Thus the present study suggests that guard cells adapt to mutations through compensatory feedback Ca^{2+} signaling mechanisms.

Recent studies indicate a direct link between regulation mechanisms of SLAC1 and the K^+ uptake channel KAT1. Phosphorylation of SLAC1 by OST1 and by Ca^{2+} dependent protein kinases can activate S-type anion currents in guard cells and SLAC1-dependent anion currents in oocytes (Geiger *et al.*, 2009, 2010; Lee *et al.*, 2009; Brandt *et al.*, 2012) add: Mori *et al.*, 2006). At the same time OST1-dependent phosphorylation of the guard cell K^+ uptake channel KAT1 leads to inhibition of K^+_{in} currents in oocytes (Sato *et al.*, 2009). Activation of OST1 is controlled by PYR/PYL/RCAR receptor proteins and ABA (Ma *et al.*, 2009; Park *et al.*, 2009; Nishimura *et al.*, 2010). The concentration of ABA tends to be increased in seeds of ABA insensitive mutants such as *abi2-1* (Koornneef *et al.*, 1984; Ma *et al.*, 2009). Thus it is possible that a feedback mechanism exists which leads to increased ABA biosynthesis in plants when SLAC1 is impaired and which in turn leads to enhanced activation of protein kinases such as OST1 and concomitant inactivation of KAT1 and reduction of K^+_{in} currents. However, we did not observe any statistically significant differences in ABA concentration between *slac1* mutants and wild type plants in whole plant extracts (Fig. S5), which does not exclude that there may be differences at the guard cell level.

CO_2- and ABA-induced stomatal closure in *slac1* mutants

Previously it was shown that *slac1* mutants are insensitive to increases in CO_2 concentration (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Here we observed that stomatal closure induced by an increase in $[CO_2]$ was reduced, but not completely abolished in *slac1* plants (Figs S2, S3). In addition, as shown earlier (Vahisalu *et al.*, 2008), SLAC1 is required for rapid 10

μM [ABA]-induced stomatal closure (Fig. 5b). Interestingly, application of a higher concentration of ABA for a longer period of time induced partial stomatal closure in *slac1* (Fig. 5a). Stomatal closure induced by darkness or reduction of air humidity was also present in *slac1*, albeit delayed and with slower kinetics compared to wild type plants (Vahisalu *et al.*, 2008). Both S- and R-type anion channels are activated by ABA and CO_2 in *Vicia faba* (Raschke *et al.*, 2003). Recently it was shown that AtALMT12 encodes a guard cell R-type anion channel (Meyer *et al.*, 2010) and indeed CO_2-, darkness- and ABA-induced stomatal closure were partially suppressed in plants lacking AtALMT12 (Meyer *et al.*, 2010; Sasaki *et al.*, 2010). On the basis of this information we suggest that, whereas SLAC1 is required for fast stomatal responses, other channels such as R-type anion channels and possibly also SLAH3 (Geiger *et al.*, 2011) can contribute to stomatal closure when the stimuli are sufficiently strong, depending on growth conditions and/or after extended time courses.

S-type and R-type anion channels have different dynamics (Linder & Raschke, 1992; Schroeder & Keller, 1992). R-type anion channel currents are malate permeable (Keller *et al.*, 1989). By contrast, S-type anion currents did not show large malate efflux currents in *Arabidopsis* guard cells (Fig. S4) or in oocytes expressing SLAC1 (Geiger *et al.*, 2009). Note that previous research (Vahisalu *et al.*, 2008) analyzed an upper limit for relative malate permeability through extrapolation of chloride carrying currents with malate on the extracellular membrane side and therefore did not analyze malate transport mediated by S-type anion channels.

Conclusions

The present study demonstrates that *slac1* mutants exhibit an unexpected pronounced slowing of stomatal opening and a dramatic reduction in K^+_{in} channel currents. These findings provide evidence that guard cells have a compensatory machinery that counteracts mutations that would cause enhanced stomatal apertures. Here, mechanisms have been identified that can provide a mechanistic basis for the slowed stomatal opening and the reduced K^+_{in} channel activity in *slac1* alleles. The sensitivity of guard cell K^+_{in} channels to the free cytosolic Ca^{2+} concentration is enhanced ('primed'). Large K^+_{in} channel activity can be rapidly recovered in *slac1* guard cells by lowering $[Ca^{2+}]_{cyt}$. Furthermore, *slac1* guard cells exhibit slightly elevated baseline $[Ca^{2+}]_{cyt}$ levels. In addition, the partially reduced expression of guard cell ion channel and transporter transcripts, may also contribute a small component to the reported compensatory slowing of stomatal closing mechanisms. The compensatory slowing of stomatal opening in response to light, low CO_2 and high humidity may provide an important feedback mechanism for gas exchange regulation in plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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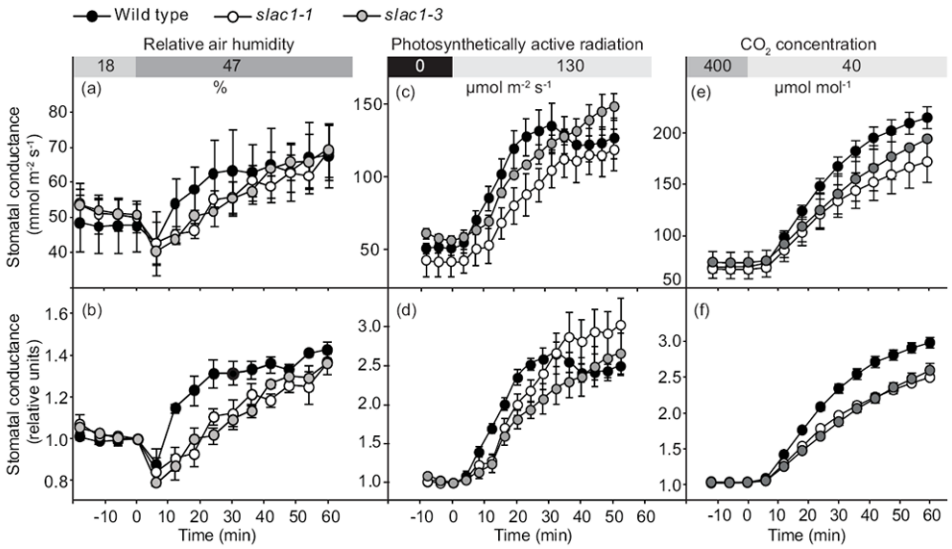

Fig. 1.

Humidity-, light- and low CO_2-induced increase in whole-plant stomatal conductance is reduced in *Arabidopsis slac1* mutants. (Wild type, black circles; *slac1-1*, white circles; *slac1-3*, grey circles.) (a, b) Wild type, *slac1-1*, and *slac1-3* plants were kept in low relative air humidity (17.9 ± 0.7%) for 120–180 min, thereafter humidity was abruptly increased to 47.3 ± 0.6% at time 0 as indicated in the upper panel. Error bars indicate ±SEM, $n = 3$. (c, d) Stomatal conductance patterns of wild type, *slac1-1*, and *slac1-3* plants kept in darkness overnight (12 h) and then exposed to 130 µmol m^{-2} s^{-1} light as indicated in upper panel are shown. Error bars indicate ±SEM, $n = 4$. (e, f) To address low CO_2-induced stomatal opening of wild type, *slac1-1*, and *slac1-3* plants were kept in 400 µmol mol^{-1} of CO_2 and 20-25% relative air humidity until stomatal conductance values had stabilized (2-3 h), thereafter CO_2 concentration was decreased to 40 µmol mol^{-1} at time 0 (±SEM, $n = 4$-8). Patterns of absolute (a, c, e) as well as relative stomatal conductance normalized to value at 0 time point (b, d, f) are shown.

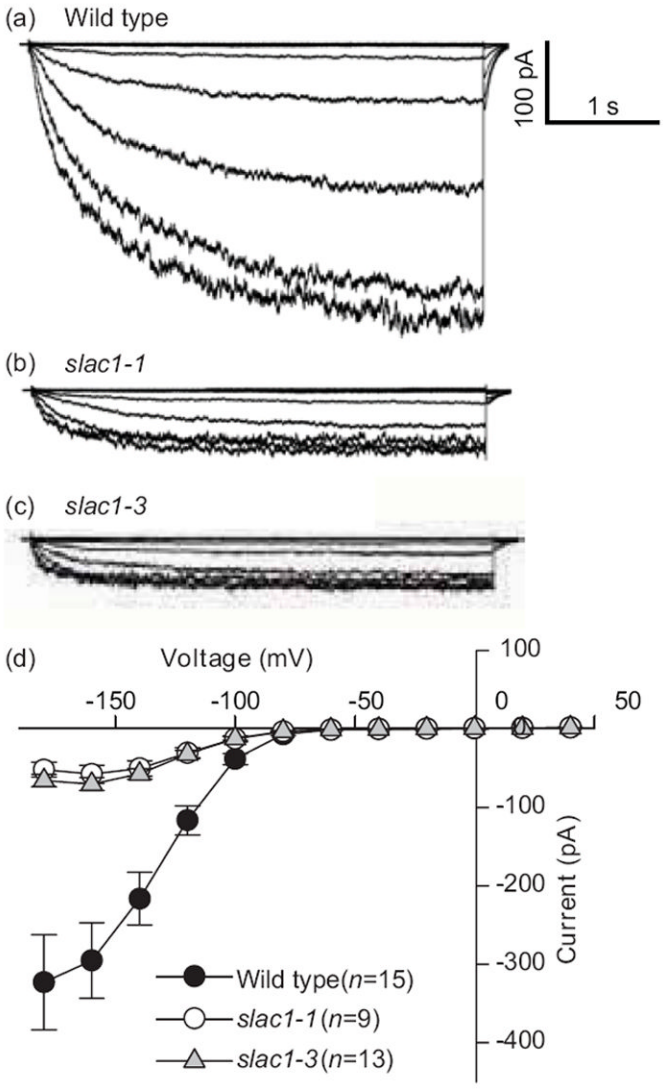

Fig. 2.

Impaired SLAC1 leads to reduced K^+_{in} channel activities in *Arabidopsis* guard cells. Whole-cell recordings of inward K^+ currents in the presence of 30 mM KCl in the bath solution in (a) wild-type, (b) *slac1-1*, and (c) *slac1-3* guard cells. (d) Average current–voltage relationships. K^+_{in} channel currents were activated by voltage pulses with +20 mV increment from -180 mV to +40 mV. Error bars indicate ±SEM.

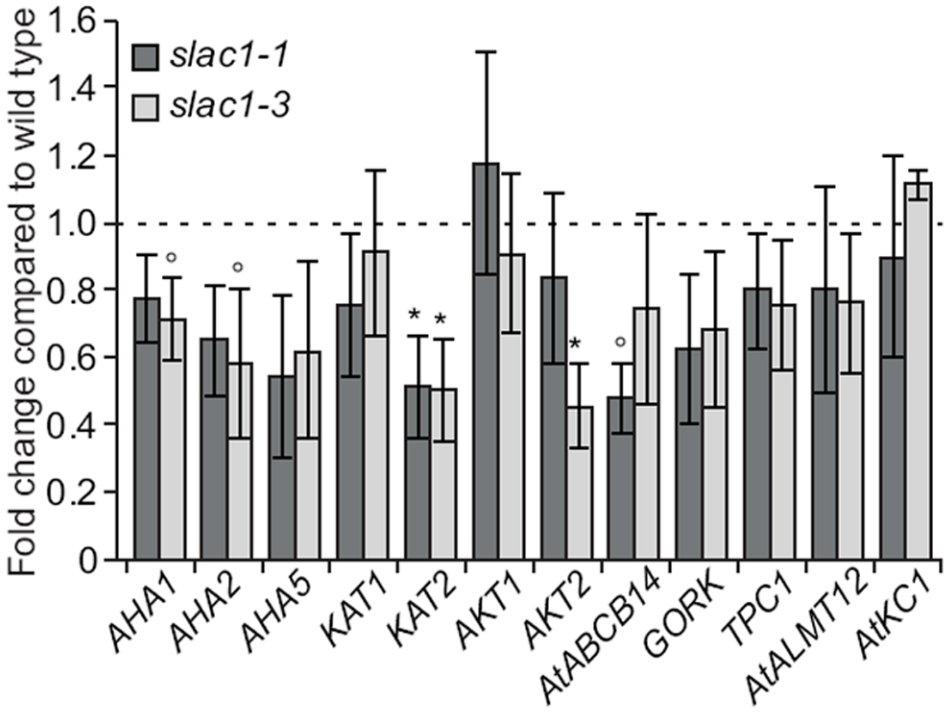

Fig. 3.

Mutations in SLAC1 suppress expression of several guard cell ion channel and transporter genes. Relative expression of *Arabidopsis* plasma membrane H^+-ATPases *AHA1, AHA2, AHA5*, transporter *AtABCB14* and ion channels *KAT1, KAT2, AKT1, AKT2, TPC1, GORK, AtALMT12*, and *AtKC1* was measured in isolated guard cells of wild type, *slac1-1* (dark bars), and *slac1-3* (light bars). Error bars indicate ±SEM, $n = 4$ (*, $P < 0.05$; °, $P < 0.10$; One-way ANOVA and LSD test).

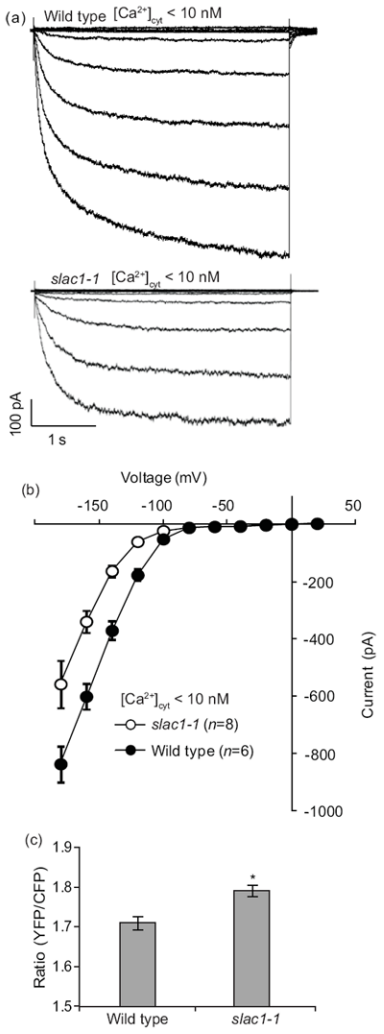

Fig. 4.

Decrease of intracellular Ca^{2+} concentration rapidly restores K^+_{in} channel activity and cameleon Ca^{2+} reporter analyses indicates slightly elevated cytosolic Ca^{2+} in *Arabidopsis slac1* mutant guard cells. (a) Whole-cell recordings of K^+_{in} currents with <10 nM free $[Ca^{2+}]_{cyt}$ in the pipette solution in *slac1-1* and in wild type guard cells. (b) Average steady state current–voltage relationships for guard cells, as recorded in (a). (c) Baseline cytosolic Ca^{2+}-dependent ratiometric fluorescence levels in wild type (n = 35) and *slac1-1* (n = 39) guard cells transformed with yellow cameleon 3.6; *, $P < 0.001$. Error bars indicate ±SEM.

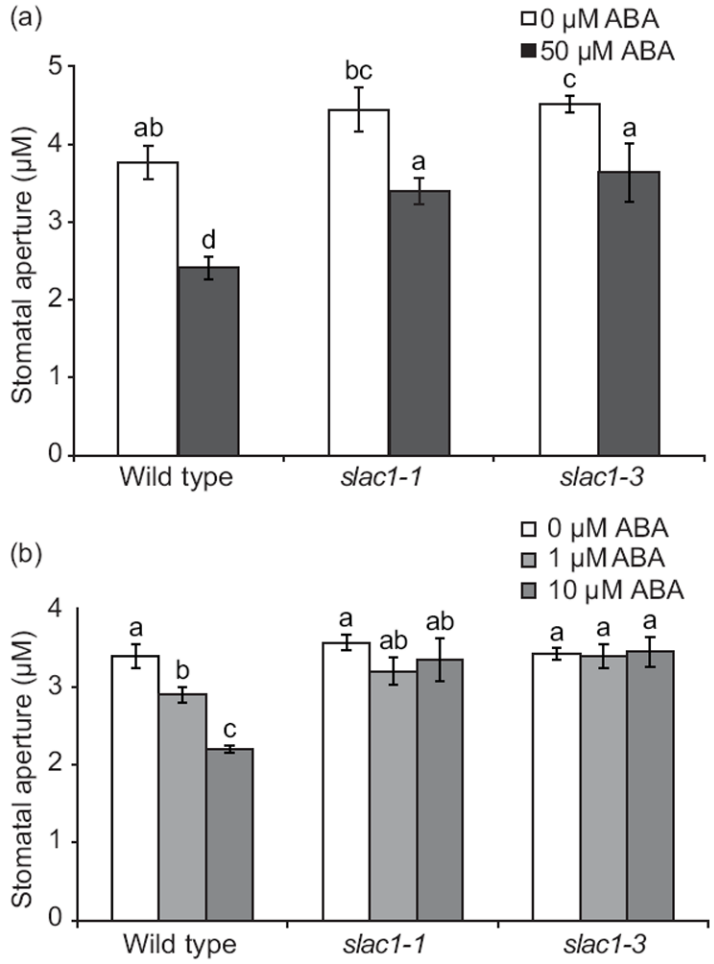

Fig. 5.

Partial abscisic acid (ABA)-induced stomatal closure in *Arabidopsis slac1* mutants depends on ABA concentration and duration of ABA exposure. (a) Leaves of wild type, *slac1-1*, and *slac1-3* were incubated in 0 µM ABA or 50 µM ABA for 3 h (±SEM, n = 20-33). (b) Impaired ABA-induced stomatal response in *slac1-1* and *slac1-3* mutants, at lower ABA concentrations (1 and 10 µM ABA) and reduced exposure time to ABA (1 h). ABA-induced stomatal closure in wild type, *slac1-1*, and *slac1-3*, intact leaf epidermis treated with the indicated ABA concentrations for 1 h (±SEM, n = 3 experiments, 30 stomata per condition per experiment). Experiments were conducted as genotype and [ABA] blind experiments. Letters refer to significant ($P < 0.05$) differences (ANOVA and LSD test).