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Calcium Elevation- and Attenuated Resting Calcium-dependent Abscisic Acid Induction of Stomatal Closure and Abscisic Acid-Induced Enhancement of Calcium Sensitivities of S-type Anion and K^+in Channels *in Arabidopsis* guard cells

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Abstract

Stomatal closure in response to abscisic acid depends on mechanisms that are mediated by intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), and also on mechanisms independent of $[Ca^{2+}]_i$ in guard cells. In this study we address three important questions in reference to these two predicted pathways in *Arabidopsis thaliana*. 1. How large is the relative abscisic acid (ABA)-induced stomatal closing response from a $[Ca^{2+}]_i$-elevation-independent pathway? 2. How do ABA-insensitive mutants affect a $[Ca^{2+}]_i$-elevation-independent pathway? 3. Does ABA enhance (prime) the Ca^{2+}-sensitivity of anion and inward-rectifying K^+ channel regulation? We monitored stomatal responses to ABA while experimentally inhibiting $[Ca^{2+}]_i$ elevations and clamping $[Ca^{2+}]_i$ to resting levels. The absence of $[Ca^{2+}]_i$-elevations was confirmed in ratiometric $[Ca^{2+}]_i$ imaging experiments. ABA-induced stomatal closing in the absence of $[Ca^{2+}]_i$-elevations above the physiological resting $[Ca^{2+}]_i$ showed only ≈30% of the stomatal closure response and was greatly slowed compared to the presence of $[Ca^{2+}]_i$-elevations. The ABA-insensitive mutants *ost1-2, abi2-1, gca2* showed partial stomatal closing responses that correlate with $[Ca^{2+}]_i$-dependent ABA signaling. Interestingly, patch clamp experiments show that exposure of guard cells to ABA greatly enhances the ability of cytosolic Ca^{2+} to both activate S-type anion channels and down-regulate inward K^+ channels, providing strong evidence for the Ca^{2+} sensitivity priming hypothesis. The present study shows and quantifies an attenuated and slowed ABA response, while directly inhibiting $[Ca^{2+}]_i$-elevations in guard cells. A minimal model is discussed, in which ABA increases (primes) the $[Ca^{2+}]_i$ sensitivity of stomatal closing mechanisms.

Introduction

The relationship between guard cell $[Ca^{2+}]_i$ elevations and stomatal closure has been elaborated for the past two decades since pioneering work using Ca^{2+} chelators and Ca^{2+} channel blockers (DeSilva *et al.*, 1985; Schwartz, 1985). Elevation in the cytoplasmic Ca^{2+} concentration of guard cells led to the identification of slow anion channels and $[Ca^{2+}]_i$ down-regulation of inward (-rectifying) K^+ channels, providing important mechanisms that can mediate $[Ca^{2+}]_i$–

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induced stomatal closing and Ca^{2+} inhibition of stomatal opening (Schroeder and Hagiwara, 1989). Experimentally-induced $[Ca^{2+}]_i$ increases in guard cells generated by injection and release of caged Ca^{2+} or IP3 resulted in stomatal closure and inward K^+ channel down-regulation (Blatt *et al.*, 1990; Gilroy *et al.*, 1990). Experimentally imposing $[Ca^{2+}]_i$ transients in guard cells, showed that $[Ca^{2+}]_i$ elevations, independent of their pattern, but with an above threshold amplitude, can cause "Ca^{2+} reactive" stomatal closing (Allen *et al.*, 2001; Mori *et al.*, 2006; Vahisalu *et al.*, 2008). Whereas, "Ca^{2+} programmed" long-term inhibition of re-opening of stomatal pores depends on the imposed pattern of $[Ca^{2+}]_i$ elevations (Allen *et al.*, 2001; Cho *et al.*, 2009; Li *et al.*, 2004; Yang *et al.*, 2003). Externally applied stimuli known to induce stomatal closure have been shown to induce $[Ca^{2+}]_i$ increases in guard cells, including abscisic acid (Allen *et al.*, 2000; Grabov and Blatt, 1998; Marten *et al.*, 2007; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990; Webb *et al.*, 2001), pathogenic elicitors (Klüsener *et al.*, 2002), CO_2 (Schwartz, 1985; Webb *et al.*, 1996), ozone (Clayton *et al.*, 1999; Evans *et al.*, 2005; Vahisalu *et al.*, 2008), reactive oxygen species (McAinsh *et al.*, 1996; Pei *et al.*, 2000; Zhang *et al.*, 2001), external Ca^{2+} (Allen *et al.*, 2001; Gilroy *et al.*, 1991; McAinsh *et al.*, 1995), nitric oxide (Desikan *et al.*, 2002; Garcia-Mata *et al.*, 2003) and other Ca^{2+}-elevating second messengers (Coursol *et al.*, 2003; Leckie *et al.*, 1998; Lemtiri-Chlieh *et al.*, 2003; Ng *et al.*, 2001; Staxen *et al.*, 1999).

The cytosolic Ca^{2+} activation of plasma membrane anion channels (Hedrich *et al.*, 1990; Schroeder and Hagiwara, 1989), vacuolar K^+ (VK) channels (Gobert *et al.*, 2007; Ward and Schroeder, 1994) and slow vacuolar (SV) channels (Hedrich and Neher, 1987; Ward and Schroeder, 1994) and the $[Ca^{2+}]_i$ down regulation of proton pumps (Kinoshita *et al.*, 1995) and inward K^+ channels (Grabov and Blatt, 1999; Kelly *et al.*, 1995; Schroeder and Hagiwara, 1989) together can provide mechanisms for Ca^{2+}-induced guard cell turgor down-regulation which results in stomatal closing (Ward *et al.*, 1995). ABA activates Ca^{2+} permeable channels in the plasma membrane of guard cells (Hamilton *et al.*, 2000; Kwak *et al.*, 2003; MacRobbie, 2000; Pei *et al.*, 2000; Schroeder and Hagiwara, 1990). ABA causes elevations in $[Ca^{2+}]_i$ in several plant species (Allan *et al.*, 1994; Allen *et al.*, 1999a; Gilroy *et al.*, 1991; Grabov and Blatt, 1998; Knight *et al.*, 1997; Marten *et al.*, 2007; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990). These regulatory mechanisms correlate with a central role of Ca^{2+} in stomatal closing. Recently calcium dependent protein kinases (CDPKs) that function in guard cell Ca^{2+} and anion channel activation and stomatal closure were identified (Mori *et al.*, 2006; Zhu *et al.*, 2007). These CDPKs provide Ca^{2+} sensors that link $[Ca^{2+}]_i$ to stomatal closing and anion channel activation, and interestingly also are required for ABA activation of plasma membrane Ca^{2+} channels (Mori *et al.*, 2006).

Studies from many laboratories have also reported experiments that failed to show $[Ca^{2+}]_i$ increases in significant subsets of ABA-treated guard cells (Allan *et al.*, 1994; Allen *et al.*, 1999a; Allen *et al.*, 1999b; Gilroy *et al.*, 1991; Hugouvieux *et al.*, 2001; Kwak *et al.*, 2003; Levchenko *et al.*, 2005; McAinsh *et al.*, 1992; Romano *et al.*, 2000; Schroeder and Hagiwara, 1990). The natural fluctuation of guard cell membrane potential between depolarized and hyperpolarized states (Gradmann *et al.*, 1993; Thiel *et al.*, 1992), coupled with the hyperpolarization dependence of inward Ca^{2+}channels may contribute to different cellular $[Ca^{2+}]_i$ responses to ABA (Grabov and Blatt, 1998; Klüsener *et al.*, 2002). It was shown that ABA shifts the activation of inward Ca^{2+} channels to more positive voltages (Hamilton *et al.*, 2000). However, imaging of $[Ca^{2+}]_i$ in guard cells showed cells in which ABA did not elicit measurable $[Ca^{2+}]_i$ increases. Interpretation of these results has led to hypotheses, which are not strictly mutually exclusive. One posits that $[Ca^{2+}]_i$ is necessary for stomatal closure in response to ABA, but that $[Ca^{2+}]_i$ elevations are not always observed due to technical limitations of the measurements. A second hypothesis is that both $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent pathways function in ABA-induced stomatal closure (Allan *et al.*, 1994), a model that is presently of wide and renewed interest. However, research has not yet analyzed

the relative contributions of the proposed $[Ca^{2+}]_i$- dependent and $[Ca^{2+}]_i$-independent pathways to ABA-induced stomatal closing. A third hypothesis derived from long-term Ca^{2+} imaging studies during guard cell CO_2 signaling, the "Ca^{2+} sensitivity priming hypothesis", posits that physiological stimuli of stomatal closing enhance the Ca^{2+} sensitivity of the appropriate stomatal closing mechanisms, thus allowing specific downstream Ca^{2+} responses to proceed, possibly even when $[Ca^{2+}]_i$ elevations do not occur (Young *et al.*, 2006).

Microinjection of the Ca^{2+} chelator BAPTA at elevated levels into guard cells of *Commelina communis* and *Vicia faba* abolished ABA responses in guard cells providing evidence for a Ca^{2+} requirement in ABA signaling (Levchenko *et al.*, 2005; Webb *et al.*, 2001). Previous studies demonstrated that removal of external Ca^{2+} by chelation with EGTA or BAPTA inhibited spontaneous $[Ca^{2+}]_i$ elevations, as well as $[Ca^{2+}]_i$ elevations induced by ABA or pathogenic elicitors (Klüsener *et al.*, 2002). In parsley, extracellular Ca^{2+} did not need to be completely removed to prevent $[Ca^{2+}]_i$ elevations caused by pathogen elicitors (Blume *et al.*, 2000). Buffering extracellular Ca^{2+} to 200 nM was sufficient to abolish pathogen-induced $[Ca^{2+}]_i$ elevations (Blume *et al.*, 2000). Elevated extracellular K^+ concentrations depolarize guard cells and also dampen $[Ca^{2+}]_i$ elevations (Allen *et al.*, 2000; Grabov and Blatt, 1998; Klüsener *et al.*, 2002). Non-invasive loading of guard cells with the membrane permeable chelator BAPTA-AM together with extracellular Ca^{2+} removal inhibited spontaneous $[Ca^{2+}]_i$ elevations and inhibited long-term stomatal movements induced by $[CO_2]$ (Young *et al.*, 2006). A transient Ca^{2+}-independent CO_2 response was also found under these conditions (Young *et al.*, 2006).

The present study reveals an attenuated partial ABA-induced stomatal closing response that occurs at resting $[Ca^{2+}]_i$. Furthermore, interestingly patch clamp experiments show that ABA enhances (primes) the cytosolic Ca^{2+} sensitivity of both anion and K^+ channels, providing mechanistic support for the Ca^{2+} sensitivity priming hypothesis (Young *et al.*, 2006).

Results

Ca^{2+} transients in guard cells

Yellow cameleon YC2.1 is a protein Ca^{2+} sensor based on a fluorescence resonance energy transfer (FRET) signal between two GFP moieties separated by a Ca^{2+} binding calmodulin (Miyawaki *et al.*, 1999; Miyawaki *et al.*, 1997). Yellow cameleon allows direct monitoring of $[Ca^{2+}]_i$ signals in *Arabidopsis thaliana* guard cells (Allen *et al.*, 1999b). In the present study, we used the yellow cameleon construct 3.6 (YC3.6) with an improved dynamic range (Mori *et al.*, 2006; Nagai *et al.*, 2004) to examine the relative role of guard cell $[Ca^{2+}]_i$ elevations in ABA-induced stomatal closure.

Imposition of transient $[Ca^{2+}]_i$ increases by switching the perfusion buffer between a depolarizing solution with low nanomolar Ca^{2+} (Ca^{2+} buffered by BAPTA) and a hyperpolarizing buffer containing 1 mM Ca^{2+} (Allen *et al.*, 2001) resulted in greater than 3-fold changes in the ratiometric fluorescence change reporting the $[Ca^{2+}]_i$, confirming the strong FRET response of this sensor (Figures 1A and 1B). An accurate *in vivo* calibration of a fluorescent $[Ca^{2+}]_i$ sensor is difficult due to several technical and biological considerations, and therefore imaging studies usually report fluorescence ratio changes, rather than calibrated $[Ca^{2+}]_i$ values. However, we have explored estimating $[Ca^{2+}]_i$ values from our fluorescence ratio data using *in vitro* calibration parameters for YC 3.6 (dynamic range in the fluorescence ratio of 560%, apparent K_D of 250 nM, and Hill coefficient of 1.7 (Nagai *et al.*, 2004)). The large and relatively uniform peak heights in Figure 1A suggest near saturation of the sensor, corresponding to ≥1.4 μM Ca^{2+} (95% of the maximal ratio). A calibration using the above coefficients, with a recorded R_{max} of 6.2 (Figure 1A) and the observed basal R of 2.0 (Figures 1 and 2) would correspond to a resting $[Ca^{2+}]_i$ of approximately 120 to 150 nM. This

approximated basal $[Ca^{2+}]_i$ is in agreement with resting $[Ca^{2+}]_i$ ranges reported by using fura-2, indo-1and YC 2.1 cameleon (Allen *et al.*, 2000; Allen *et al.*, 1999a; Grabov and Blatt, 1998; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990).

Guard cell $[Ca^{2+}]_i$ in freshly prepared intact *Arabidopsis* leaf epidermes continuously perfused with extracellular buffer containing 50 μM added Ca^{2+} (Ca^{2+} buffer C, see Experimental Procedures) displayed frequent spontaneous $[Ca^{2+}]_i$ transients (Figure 1C), similar to previous reports of spontaneous $[Ca^{2+}]_i$ transients in *Arabidopsis* guard cells (Allen *et al.*, 2001; Allen *et al.*, 1999b; Klüsener *et al.*, 2002; Yang *et al.*, 2008; Young *et al.*, 2006). The form and period of these transients were sometimes regular (Figure 1D, gray trace). However, there was no apparent consistent coordination of the spontaneous transient rises even in a guard cell pair from the same stomate (Figure 1D), which correlates with findings from previous research with yellow cameleon 2.1 (Allen *et al.*, 1999b). The baseline ratios from which these transient $[Ca^{2+}]_i$ elevations rise (Figure 1C & 1D) was relatively stable among experiments in the range of approximately 1.7 to 2.0 (n > 15 experiments).

Spontaneous and induced $[Ca^{2}]_i$ transients are abolished by buffering external free $[Ca^{2+}]$ with BAPTA

Previous reports have observed that $[Ca^{2+}]_i$ transients in guard cells could be inhibited by removal of extracellular Ca^{2+} with EGTA or BAPTA (Klüsener *et al.*, 2002) and by incubating the cells with the membrane permeable chelator BAPTA-AM in the absence of added Ca^{2+} in the external buffer (Young *et al.*, 2006). In this study, spontaneous $[Ca^{2+}]_i$ transients were observed during perfusion with 50 μM Ca^{2+} added to the extracellular buffer (Ca^{2+} buffers B and C, see Experimental Procedures). Subsequent perfusion with a buffer that reduced the extracellular free $[Ca^{2+}]$ to 200 nM abolished these spontaneous $[Ca^{2+}]_i$ transients experiments (Figure 2A; n>20 experiments; (BAPTA buffer, see Experimental Procedures). The baseline fluorescence ratio remained between 1.7 and 2, when $[Ca^{2+}]_i$ transients were abolished, which was equivalent to the $[Ca^{2+}]_i$ baseline in guard cells showing repetitive $[Ca^{2+}]_i$ transients (Figure 1).

Extracellular perfusion with hyperpolarizing buffer containing 1 mM Ca^{2+} rapidly restarted the $[Ca^{2+}]_i$ transients (Figure 2A). After leaf epidermes were held in BAPTA buffer with 200 nM free Ca^{2+} for 3 hours or more, perfusion with 50 μM Ca^{2+} was sufficient to cause transient elevations in $[Ca^{2+}]_i$ (Figure 2B). When guard cells were loaded with BAPTA, using the membrane permeable fura-2 analog BAPTA-AM, spontaneous Ca^{2+} elevations were inhibited (Figure 2C,D). An extracellular pulse of 50 μM Ca^{2+} following BAPTA-AM incubation caused increases in guard cell $[Ca^{2+}]_i$ (Figure 2C; n=10 treatments). When guard cells were loaded using BAPTA-AM, the baseline $[Ca^{2+}]_i$ ratio of guard cells was similar to controls (Figures 1 and 2C,D; n > 10 experiments).

Further experiments were pursued to reduce $[Ca^{2+}]_i$ in guard cells to below typical resting levels by extracellular perfusion with millimolar [EGTA] or [BAPTA] without added Ca^{2+}. However, in *Arabidopsis* guard cells EGTA did not reduce $[Ca^{2+}]_i$ ratios in guard cells and extracellular BAPTA (2 mM) only caused intracellular $[Ca^{2+}]_i$ reductions in 12 of 43 guard cells. These findings confirmed that YC3.6 can report lower $[Ca^{2+}]_i$ levels (Nagai *et al.*, 2004), and also suggest relatively robust physiological mechanisms that maintain resting $[Ca^{2+}]_i$ in *Arabidopsis* guard cells.

As incubation in BAPTA buffer with 200 nM free Ca^{2+} and loading guard cells with BAPTA-AM abolished spontaneous transient increases in $[Ca^{2+}]_i$, we examined the effect of BAPTA buffer on ABA-induced $[Ca^{2+}]_i$ increases. Leaf epidermes were incubated in the light in BAPTA buffer for 3 hours to open stomata, and loaded with BAPTA-AM for 10 minutes. The epidermes were then transferred to a microscope stage to monitor $[Ca^{2+}]_i$. Guard cells in these

preparations showed no clear increases in $[Ca^{2+}]_i$ during 20 minutes following application of 2 μM ABA (Figure 2D) (n=12 experiments). As a control to test whether guard cells could report $[Ca^{2+}]_i$ elevations, after this treatment cells were exposed to extracellular buffer containing 50 μM Ca^{2+}. Guard cell $[Ca^{2+}]_i$ transient elevations were triggered in response to the extracellular 50 μM Ca^{2+} buffer (Figure 2C,D). These Ca^{2+}- imaging experiments showed that buffering extracellular Ca^{2+} to a low 200 nM level with 54 mM extracellular K^+ in conjunction with BAPTA-AM loading maintained typical baseline resting $[Ca^{2+}]_i$ ratios, while providing a robust experimental approach to abolish spontaneous $[Ca^{2+}]_i$ transient elevations and ABA-induced $[Ca^{2+}]_i$ increases in guard cells, thus enabling controlled analyses of the Ca^{2+} dependences of ABA responses.

Inhibition of guard cell $[Ca^{2+}]_i$ increases allows a moderate ABA-induced stomatal closure

Using BAPTA buffer (200 nM free Ca^{2+}) in conjunction with BAPTA-AM loading, we examined the effect of blocking ABA-regulated $[Ca^{2+}]_i$ increases on ABA-induced stomatal closure using the same solutions used for $[Ca^{2+}]_i$ imaging. Intact epidermes from wild type plants were placed in a high extracellular Ca^{2+} solution buffered to 0.2 mM free Ca^{2+} or a low extracellular Ca^{2+} solution buffered to 200 nM free Ca^{2+} ("BAPTA buffer") and incubated in the light for three hours to open stomatal pores. Epidermes treated with BAPTA buffer (200 nM Ca^{2+}) were also incubated with 10 μM BAPTA-AM for ten minutes in the light prior to ABA application to further prevent $[Ca^{2+}]_i$ increases (Figure 2). After incubation in the light, photomicrographs of the epidermes were captured for measurements of stomatal apertures. The epidermes were then exposed to ABA and incubated in the light for two hours. Photomicrographs of same leaf epidermis regions were taken for later measurement of the identical stomatal pore apertures after ABA exposures. Pretreatment and post-treatment apertures of the identical stomata were compared pair-wise to determine the degree of stomatal closing.

Guard cell viability remained intact after five hours in the 200 nM free Ca^{2+} external BAPTA buffer solution. The viability dye FDA was added after completion of imaging experiments to monitor whether the analyzed guard cells had remained viable (Young *et al.*, 2006) (see Experimental Procedures). Only few guard cells failed to show fluorescence following FDA staining after several hours of experiments. In addition, the fact that stomata remained open also demonstrates viability, as maintenance of turgor requires an intact plasma membrane and active guard cell signaling and metabolism.

Stomatal apertures were individually mapped and tracked to determine the response of each stomatal aperture (Allen *et al.*, 2001). In high 0.2 mM free $[Ca^{2+}]$ buffer, incubation in the light for 2 hours with 0 ABA following the initial 3 hour opening treatment caused the stomatal apertures to increase by an average additional 5% (Figure 3); this small change was not significant at a 95% confidence level. In the high 0.2 mM free $[Ca^{2+}]$ buffer, ABA-induced stomatal closure showed a sigmoidal response as a function of ABA concentration (Figure 3, open triangles).

When BAPTA buffer and BAPTA-AM were used to prevent $[Ca^{2+}]_i$ increases, incubation in the light with 0 ABA following the initial 3 hour stomatal opening treatment caused stomatal apertures to increase an additional 12%; this change was significant ($p<0.01$) (Figure 3). However, in this low Ca^{2+} BAPTA buffer, ABA caused only a partial attenuated stomatal closure response (Figure 3, open squares). At all concentrations of ABA, stomatal closure under conditions that inhibited $[Ca^{2+}]_i$ elevations was reduced. At the highest ABA concentration (20 μM), stomatal closure under conditions that inhibited $[Ca^{2+}]_i$ elevations was ≈ 30% of the stomatal closure under the high $[Ca^{2+}]$ condition (Figure 3). Similar results are observed if final absolute stomatal apertures rather than percent of initial apertures are plotted (Figure 3, inset).

Although we measured stomatal closure after 2 hours to determine a steady state response of individually mapped stomata (Figure 3), much of the stomatal closure response occurs more rapidly after ABA exposure. We examined whether the reduced response to ABA in the BAPTA treatment was detectable in the early ABA response. The early time course of stomatal closure in response to ABA was analyzed in individually mapped and imaged stomata as shown in Figure 4. In both Ca^{2+} and $[Ca^{2+}]_i$ -elevation-inhibited treatments, the first visible and significant reduction in stomatal aperture occurred at the 4 to 10 minute time point (BAPTA treatment: $P < 0.05$ at 10 min.). The response of the stomata in the low Ca^{2+} BAPTA buffer was substantially reduced compared to the physiological Ca^{2+} buffer (Figure 4A).

Note that when all stomatal apertures for each experiment are averaged together (Figure 4B), the error range of the responses was larger than when the relative (%) response was analyzed (Figure 4A). This increase in error (Figure 4B) is attributed to the fact that in these experiments, each stomatal aperture was individually mapped and the time course of each stomatal aperture was determined. When the degree of the response of each individual stomatal aperture was plotted, we found that less variability occurred in the relative response of each individual stomate (Figure 4A). This is further illustrated in Figures 4C and 4D which show the relative responses of each individual stomate within an imaged field (Figure 4C), illustrating that more open stomata show a larger stomatal aperture change (Figure 4D). These data also illustrate the range of stomatal apertures that are found in *Arabidopsis* leading to the use of blind stomatal response assays when multiple stomatal apertures are measured in parallel-treated samples (e.g. (Allen *et al.*, 1999a; Hugouvieux *et al.*, 2001; Murata *et al.*, 2001)), and the use of independent data confirmations by more than on experimenter (Ichida *et al.*, 1997; Pei *et al.*, 1997). Thus when $[Ca^{2+}]_i$ increases were prevented by BAPTA treatment, a greatly attenuated and slowed "$[Ca^{2+}]_i$-elevation-independent" ABA-induced stomatal closure is revealed here, by analyzing individually mapped stomata (Figures 3 and 4).

ABA-induced stomatal closure in ABA insensitive mutants

Responses of ABA insensitive signaling mutants have not yet been analyzed under $[Ca^{2+}]_i$ -elevation-independent conditions. To examine whether the partial ABA-induced stomatal closure under Ca^{2+}-elevation-inhibited conditions by-passes ABA signaling mutants, we examined this ABA response in the following ABA insensitive mutant lines *ost1-2, abi1-1, abi2-1, gca2*, and *pld∝1* (Himmelbach *et al.*, 1998; Koornneef *et al.*, 1984; Mishra *et al.*, 2006; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002). Stomatal closure induced by ABA was examined in these mutants and compared to wild-type responses. ABA-induced stomatal closure responses in the presence of 0.2 mM extracellular $[Ca^{2+}]$ are shown in Figure 5. ABA caused small average stomatal closure responses in the Landsberg *erecta* mutants *ost1-2, abi2-1* and *gca2* in the presence of 0.2 mM extracellular Ca^{2+} (*ost1-2*: $P < 0.05$; *abi2-1:* $P < 0.05$; *gca2:* $P < 0.01$, paired t-Test). The degree of ABA-induced stomatal closure was significantly less in the *Ler* mutants than that in the wild type (e.g. $P < 0.02$ for *gca2*, n=3 experiments, 1 tailed t-Test).

Results of ABA application to the same mutants under conditions that inhibited $[Ca^{2+}]_i$ elevations are shown in Figure 5 (BAPTA). ABA application to the *ost1-2, abi1-1, abi2-1* mutants caused no average reduction in stomatal apertures. ABA application to the *gca2* mutant caused a weak average stomatal closing response; this amount of stomatal closure in the *gca2* mutant was significantly less than the stomatal closure in the presence of 0.2 mM Ca^{2+} ($p < 0.05$, n=3 experiments). Thus the above ABA signaling mutants show ABA insensitivity in the Ca^{2+} treated samples and show a further average attenuation of slight ABA responses in BAPTA-treated samples.

The phospholipase D mutant, *pld∝1*, was selected for analyses here based on a reported abolishment of ABA-induced stomatal closing (Mishra *et al.*, 2006). The pld*∝1* mutant is in

the Columbia wildtype background and was therefore analyzed in separate experiments. Homozygous T-DNA insertions and the lack of full-length *PLDα1* mRNA were confirmed by PCR and RT-PCR (data not shown) (Mishra *et al.*, 2006). The previously described *pldα1* T-DNA knockout allele (Mishra *et al.*, 2006), showed an ABA-induced stomatal closure response under the imposed conditions (Figure 6). We examined whether this response showed a statistical partial ABA insensitivity compared to wildtype (Columbia) as follows: The difference in stomatal apertures between WT and *pldα1* with no added ABA was not significant (Figure 6 Calcium; 1 tailed t-Test: $P > 0.27$, $n = 5$ *pldα1* experiments). The difference in stomatal apertures between WT and *pldα1* in the presence of ABA was also not significant (Figure 6 Calcium+ABA; 1 tailed t-Test: $P > 0.28$). However, when ABA responses in *pldα1* without and with ABA were subtracted from one another and compared to the same change in wildtype, *pldα1* stomata appeared to show a significant partial ABA hyposensitivity in this subtractive analysis (Figure 6 Calcium - Calcium + ABA; 1 tailed t-Test: $P < 0.02$). In BAPTA-treated samples the ABA response was strongly attenuated in both wildtype and *pldα1* (Figure 6 BAPTA vs. BAPTA + ABA).

Abscisic Acid Sensitizes *Arabidopsis* Guard Cell Anion Channels to Cytosolic [Ca^{2+}]

The above analyses show that although guard cells show spontaneous $[Ca^{2+}]_i$ elevations (Figures 1 and 2), ABA-induced stomatal closing exhibits a clear Ca^{2+}-dependence (Figures 3 and 4). To further investigate this apparent paradox, we analyzed $[Ca^{2+}]_i$ activation of S-type anion channels without and with ABA pre-incubation, as this response is an important mechanism in ABA- and Ca^{2+}-induced stomatal closing (Allen *et al.*, 2002; Mori *et al.*, 2006; Negi J *et al.*, 2008; Pei *et al.*, 1997; Schroeder and Hagiwara, 1989; Vahisalu *et al.*, 2008). We found that $[Ca^{2+}]_i$ activation of S-type anion channels in patch-clamped *Arabidopsis* guard cell protoplasts required that protoplasts were pre-exposed to ABA, as illustrated in Figure 7. When guard cell protoplasts were not exposed to ABA, only small whole-cell currents were measured, both when $[Ca^{2+}]_i$ was buffered to 150 nM (Figure 7A,B) and 2 μM (Figure 7C,D) under the imposed conditions (see also: (Allen *et al.*, 2002)). Interestingly, when protoplasts were pre-exposed to ABA, S-type anion currents were activated when $[Ca^{2+}]_i$ was buffered to 2 μM (Figure 7 C,D). No significant ABA activation of S-type anion currents was observed when $[Ca^{2+}]_i$ was buffered to 150 nM (Figure 7 A,B). These data show that ABA enhances the $[Ca^{2+}]_i$ sensitivity of S-type anion channel activation (Figure 7). These findings also can provide a mechanism to explain the apparent paradox between spontaneous $[Ca^{2+}]_i$ elevations and the $[Ca^{2+}]_i$ -dependence of stomatal closing (Figures 1 to 6).

Abscisic Acid Sensitizes *Arabidopsis* Guard Cell K^+in Channels to Cytosolic [Ca^{2+}]

Cytosolic Ca^{2+} is well-known to cause down-regulation of inward-rectifying K^+ (K^+in) channels (Schroeder *et al.*, 1987) in *Vicia faba* guard cells (Grabov and Blatt, 1999; Kelly *et al.*, 1995; Schroeder and Hagiwara, 1989). Interestingly however, this $[Ca^{2+}]_i$ down-regulation of K^+in channels has not yet been reported in *Arabidopsis* guard cells, and earlier preliminary experiments indicated a lack of this response in *Arabidopsis* guard cells (Z.M. Pei, unpublished). Further experiments confirmed that, in contrast to *Vicia faba*, elevation of $[Ca^{2+}]_i$ to 2 μM did not significantly down-regulate K^+in channel currents in *Arabidopsis* guard cell protoplasts, as illustrated in Figure 8 (Figure 8 A,D top). Average K^+in channel current magnitudes were not significantly down-regulated when $[Ca^{2+}]_i$ was buffered to 2 μM using EGTA in the patch clamp pipette solution compared to 150 nM $[Ca^{2+}]_i$ in the absence of ABA (filled circles in Figure 8 B and E; $P = 0.24$ at -189 mV and $P = 0.60$ at -169 mV for 0 ABA data). Previous research in the larger *Vicia faba* guard cells demonstrated that using BAPTA rather than ABA in the pipette solution allows more effective $[Ca^{2+}]_i$ buffering and K^+in channel down-regulation (Kelly *et al.*, 1995). In *Arabidopsis* guard cells buffering $[Ca^{2+}]_i$ to 2 μM using 4 mM BAPTA did not down-regulate K^+in channel currents either, illustrating a

robust $[Ca^{2+}]_i$ insensitivity under the imposed conditions in the absence of ABA (filled circles in Figure 8 C and F; P = 0.38 at -169 mV for 0 ABA data).

We next analyzed whether ABA exposure mediates $[Ca^{2+}]_i$ down-regulation of K^+in channels in *Arabidopsis*. ABA pre-incubation without elevating $[Ca^{2+}]_i$ in guard cells (150 nM $[Ca^{2+}]_i$) caused no detectable ABA regulation of K^+in currents using both EGTA (Figure 8 A,B) and BAPTA (Figure 8C). Note that ABA was also added to the pipette solution, that dialyzes the cytoplasm, in these experiments. These data are consistent with reports that ABA does not always down-regulate K^+in channel currents (Lemtiri-Chlieh and MacRobbie, 1994; Pei *et al.*, 1997; Wolf *et al.*, 2006).

Interestingly, with ABA application, $[Ca^{2+}]_i$ elevation to 2 μM clearly down-regulated K^+in channel currents using both EGTA (Figure 8 D and E) and BAPTA in the pipette solution (Figure 8F). Taken together new findings in figures 7 and 8 provide strong evidence that ABA enhances (primes) the $[Ca^{2+}]_i$ sensitivity of *Arabidopsis* guard cells towards activation of S-type anion channels and $[Ca^{2+}]_i$ down-regulation of K^+in channels.

Discussion

Observations of guard cells that did not exhibit a $[Ca^{2+}]_i$ increase upon application of ABA have been reported in many independent studies (Allan *et al.*, 1994; Allen *et al.*, 1999a; Allen *et al.*, 1999b; Allen *et al.*, 2002; Gilroy *et al.*, 1991; Hugouvieux *et al.*, 2001; Kwak *et al.*, 2003; Levchenko *et al.*, 2005; McAinsh *et al.*, 1992; Romano *et al.*, 2000; Schroeder and Hagiwara, 1990). The existence of ABA-induced $[Ca^{2+}]_i$-independent stomatal closure has been proposed on the basis of these observations. In this work we experimentally clamped $[Ca^{2+}]_i$ and inhibited $[Ca^{2+}]_i$ increases in guard cells and monitored $[Ca^{2+}]_i$ over prolonged periods of time by ratiometric imaging. Robust experimental inhibition of $[Ca^{2+}]_i$ elevations was possible by buffering extracellular free Ca^{2+} with BAPTA to 200 nM while maintaining a high $[K^+]$ in the external buffer (Allen *et al.*, 2000) and loading guard cells using BAPTA-AM. This approach allowed analyses of whether $[Ca^{2+}]_i$ elevation-independent stomatal closure occurs and allowed for the first time quantification of the relative contributions of Ca^{2+}-elevation-independent and Ca^{2+}-elevation-dependent ABA responses.

Studies have demonstrated that removal of external Ca^{2+} by chelation inhibited spontaneous $[Ca^{2+}]_i$ elevations, as well as $[Ca^{2+}]_i$ elevations induced by ABA, CO_2 and pathogenic elicitors (Klüsener *et al.*, 2002; Webb *et al.*, 1996). It has been reported that extracellular Ca^{2+} need not be entirely removed to completely inhibit the $[Ca^{2+}]_i$ response induced by pathogen elicitors in parsley (Blume *et al.*, 2000). This $[Ca^{2+}]_i$ response, which consisted of a rapid peak followed by a lower plateau, was completely blocked at 200 nM free extracellular Ca^{2+}. In a recent study of $[Ca^{2+}]_i$ responses to cold shock in *Arabidopsis* using an aequorin reporter, a similar temporal $[Ca^{2+}]_i$ pattern of a sharp peak followed by a broad plateau was modeled as resulting from an initial $[Ca^{2+}]_i$ rise in many cells, followed by out of phase $[Ca^{2+}]_i$ oscillations over multiple cells (Dodd *et al.*, 2006). We hypothesized that buffering extracellular free $[Ca^{2+}]$ near 200 nM could provide a "Ca^{2+} neutral" condition. Under these "Ca^{2+} neutral" conditions the long-term viability of guard cells was not adversely affected. Time resolved imaging of individual guard cells showed that these conditions inhibited spontaneous $[Ca^{2+}]_i$ elevations, but did not reduce the baseline resting $[Ca^{2+}]_i$.

Blocking guard cell $[Ca^{2+}]_i$ increases causes attenuation of ABA-induced stomatal closure

YC 3.6 is sensitive to changes in the 50 nM to 1.4 μM range of Ca^{2+} concentrations (Nagai *et al.*, 2004). Therefore, it is unlikely that even small cell wide changes in $[Ca^{2+}]_i$ would be missed within this range (Nagai *et al.*, 2004). Our methodology could have missed rapid $[Ca^{2+}]_i$ spikes of < 1 second duration, or localized microdomain $[Ca^{2+}]_i$ elevations; however, the $[Ca^{2+}]_i$

elevations previously reported in guard cells using the rapid BAPTA-based fura-2 and indo-1 reporters have exhibited durations in the range of ≥ many seconds (Allan *et al.*, 1994; Allen *et al.*, 1999a; Grabov and Blatt, 1998; Marten *et al.*, 2007; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990). Therefore, the observed lack of $[Ca^{2+}]_i$ transients in the BAPTA treatment and the observed stable baseline $[Ca^{2+}]_i$ fluorescence ratios indicate that the $[Ca^{2+}]_i$ was effectively clamped close to resting levels.

Here we demonstrate that ABA induces an attenuated and slowed stomatal closure response *in Arabidopsis* epidermes under conditions that prevented $[Ca^{2+}]_i$ increases in guard cells. The substantially larger stomatal closure when $[Ca^{2+}]_i$ increases can occur shows an important function of ABA-induced Ca^{2+} signaling.

ABA primes the $[Ca^{2+}]_i$ sensitivity of S-type anion and K^+ in channel regulation

The occurrence of spontaneous repetitive $[Ca^{2+}]_i$ transients (Allen *et al.*, 1999b; Klüsener *et al.*, 2002; Staxen *et al.*, 1999; Yang *et al.*, 2008; Young *et al.*, 2006) appears to contradict the conclusion that stomatal closing is $[Ca^{2+}]_i$ dependent. Previous research led to the model that the physiological stimulus of stomatal closing, CO_2, enhances the Ca^{2+} sensitivity of stomatal closing mechanisms, thus allowing Ca^{2+} signaling to proceed (Ca^{2+} sensitivity priming hypothesis) (Young *et al.*, 2006). Interestingly, here we find that pre-exposure of *Arabidopsis* guard cell protoplasts to ABA enhances the ability of $[Ca^{2+}]_i$ to activate S-type anion channels (Figure 7). Furthermore, ABA exposure also enabled $[Ca^{2+}]_i$ down-regulation of K^+in channels (Figure 8).

The presented findings provide new independent mechanistic evidence for the Ca^{2+} sensitivity priming hypothesis (Young *et al.*, 2006) and extend this hypothesis to ABA signaling. $[Ca^{2+}]_i$ has not only been shown to mediate ABA- and CO_2-induced stomatal closing, but data also suggest a link of $[Ca^{2+}]_i$ elevation to stomatal opening (Cousson and Vavasseur, 1998; Curvetto *et al.*, 1994; Harada and Shimazaki, 2008; Shimazaki *et al.*, 1992; Young *et al.*, 2006). The Ca^{2+} sensitivity priming hypothesis could provide a mechanism for specificity in $[Ca^{2+}]_i$ signaling such that physiological stimuli (e.g. ABA, CO_2 or light) prime (de-inactivate) the appropriate Ca^{2+} sensors, hence leading to a specific Ca^{2+} response (Young *et al.*, 2006).

Note that no significant ABA activation of S-type anion currents was resolved when $[Ca^{2+}]_i$ was buffered to 150 nM (Figure 5 A,B), indicating that additional components, such as pH changes (Blatt and Armstrong, 1993; Wang *et al.*, 2001), are likely function in this stomatal closing mechanism. In *Vicia faba* guard cells, ion channels appear to be primed for $[Ca^{2+}]_i$ regulation, even without exogenous ABA application (Grabov and Blatt, 1999; Schroeder and Hagiwara, 1989). However, the question whether ABA may further enhance the $[Ca^{2+}]_i$ sensitivity of these ion channels may be of interest for future studies. Furthermore even in *Arabidopsis* guard cells, the ability of $[Ca^{2+}]_i$ to activate S-type anion channels requires high external Ca^{2+}– pretreatment (in the absence of ABA pre-treatment), showing conditional priming/de-priming of $[Ca^{2+}]_i$ sensitive signaling (see Figure 3 in (Allen *et al.*, 2002)).

Residual ABA signaling dependence on resting intracellular Ca^{2+} levels

Previous studies have demonstrated that injecting high concentrations of the rapid Ca^{2+} chelator BAPTA into *Commelina communis* and *Vicia faba* guard cells using microelectrodes effectively abolished ABA responses, including ABA-induced transcriptional responses, and anion channel activation (Levchenko *et al.*, 2005; Webb *et al.*, 2001). Microinjection with BAPTA of the small *Arabidopsis* guard cells is not practical for analyzing many stomatal responses, and in the present study extracellular applications of millimolar BAPTA only reduced the $[Ca^{2+}]_i$ level below resting levels in a minority of *Arabidopsis* guard cells. Therefore, our data cannot distinguish whether the residual ABA response at resting $[Ca^{2+}]_i$

(Figures 3 and 4) is dependent on physiological resting $[Ca^{2+}]_i$ (Figures 1 and 2). The only condition we have found thus far that consistently reduced the $[Ca^{2+}]_i$ level of *Arabidopsis* guard cells is treatment with the ADP ribosyl cyclase inhibitor, nicotinamide (Klüsener *et al.*, 2002). These findings are consistent with a role for cADPR in ABA responses (Leckie *et al.*, 1998; Wu *et al.*, 1997) and also with a recent study showing that nicotinamide represses the baseline circadian Ca^{2+} oscillation in *Arabidopsis* leaves (Dodd *et al.*, 2007). 50 mM nicotinamide, but not 50 mM sorbitol, completely abolished ABA-induced stomatal closing (Stephen Lee and Schroeder, data not shown), but this represents a fairly high concentration of a pharmacological blocker for lowering baseline $[Ca^{2+}]_i$, that may inhibit additional ABA signaling components. The present results do not exclude the presence of an additional Ca^{2+}-independent pathway (Allan *et al.*, 1994), although a model that $[Ca^{2+}]_i$ elevation is not required for the ABA response (Levchenko *et al.*, 2005) is not supported by the present findings. Previous BAPTA injection studies in *Commelina* and *Vicia faba* (Levchenko *et al.*, 2005; Webb *et al.*, 2001) would support a hypothesis in which Ca^{2+} sensitivity priming may enable baseline $[Ca^{2+}]_i$ to mediate the observed attenuated ABA response at resting $[Ca^{2+}]_i$ (Figures 3 and 4).

$[Ca^{2+}]_i$ elevation-independent ABA-induced stomatal closure in ABA insensitive mutants

The genetics of $[Ca^{2+}]_i$ -elevation-independent ABA signaling have not yet been analyzed. Selected ABA insensitive mutants were analyzed to determine whether a $[Ca^{2+}]_i$ elevation-independent response can by-pass these mutations. In the presence of BAPTA, *ost1*, *abi1-1*, *abi2-1* and *gca2* all showed insensitivities to ABA. These data are consistent with data suggesting roles of these loci very early in ABA signal transduction (Allen *et al.*, 1999a; Belin *et al.*, 2006; Moes *et al.*, 2008; Murata *et al.*, 2001; Mustilli *et al.*, 2002; Pei *et al.*, 2000; Yoshida *et al.*, 2006) which may be upstream of proposed parallel signaling branches. When $[Ca^{2+}]_i$ elevations were inhibited, average ABA responses were more strongly attenuated in *ost1*, *abi2-1*, and *gca2*, consistent with a dominant Ca^{2+}-dependent ABA signaling pathway (Figure 5). The *abi2-1* mutation has been shown to affect mechanisms upstream of Ca^{2+} (Allen *et al.*, 1999a; Murata *et al.*, 2001), but also affects downstream Ca^{2+} signaling events such as downstream of cADPR signaling (Wu *et al.*, 2003), indicating that more than one target may exist for these PP2C phosphatases. *abi1-1* and *abi2-1* are dominant mutants and therefore the dominant proteins might exert their effects indirectly (Moes *et al.*, 2008; Murata *et al.*, 2001; Robert *et al.*, 2006; Yoshida *et al.*, 2006). In addition the ABA responses of *abi1-1* and *abi2-1* show differential phenotypes indicating modulation of non-identical downstream target sets in ABA signaling (de Bruxelles *et al.*, 1996; Gilmour and Thomashow, 1991; Gosti *et al.*, 1995; Pei *et al.*, 1997; Soderman *et al.*, 1996; Strizhov *et al.*, 1997; Vartanian *et al.*, 1994). Note that, *abi1-1* and *abi2-1* are dominant mutants and no inhibitors for these type 2C protein phosphatases are available (Murata *et al.*, 2001). A recent study has shown a requirement for nuclear localization of abi1-1 for ABA signaling (Moes *et al.*, 2008). Therefore, the phenotypes of *abi1-1* and *abi2-1* may be due to indirect or transcriptional effects of the abi proteins, as discussed previously (Moes *et al.*, 2008; Murata *et al.*, 2001; Pei *et al.*, 1997; Robert *et al.*, 2006; Yoshida *et al.*, 2006).

Conclusions

In summary, the present study quantifies an attenuated and greatly slowed abscisic acid-induced stomatal closing response by experimentally clamping $[Ca^{2+}]_i$ to resting levels. Interestingly, ABA is shown to enable $[Ca^{2+}]_i$ to activate S-type anion channels and to down-regulate K^+in channels. These ion channel regulation findings provide strong mechanistic support for a hypothesis in which ABA enhances (primes) the $[Ca^{2+}]_i$ sensitivity of stomatal closing mechanisms, thus enabling specificity in Ca^{2+} signaling (Young *et al.*, 2006). The ABA

insensitive mutants, *abi1-1*, *abi2-1*, *ost1* and *gca2* also show a Ca^{2+} dependence, consistent with Ca^{2+}-dependent ABA signaling.

Experimental Procedures

Stomatal movements of mapped stomata

To avoid effects of leaf to leaf variability, intact epidermal preparations were prepared from a single leaf for two treatments by attaching the abaxial surface of leaf halves to 25 mm round glass cover slips with medical adhesive (Hollister Inc., Libertyville, Il), and then gently removing the upper leaf cell layers with a razor blade. Coverslips were pre-marked on the bottom with a colored grid to allow rapid re-examination of previously photographed stomatal locations. The time of each stomatal aperture was individually tracked (Allen *et al.*, 2001), allowing accurate analyses of each individual stomatal aperture response. Intact epidermes were immediately submerged in 3 ml of test buffer in 35 mm covered Petri dishes, and placed under 300 μE red/blue light (230 μE red + 70 μE blue) from an array of LEDs (LUXEON III, Philips Lumileds Lighting Company, San Jose, CA) at room temperature (21°-23°C) for 3 hours to open stomatal apertures. The following test buffers were used: **BAPTA buffer** (with 200 nM free Ca^{2+}): 25 mM dipotassium (K_2) imminodiacetic acid, 2 mM K_2 BAPTA, 0.7 mM $CaCl_2$, 10 mM MES-TRIS KOH pH 6.1; **Ca^{2+} buffer A** (with 0.2 mM free Ca^{2+}): 25 mM K_2-imminodiacetic acid, 2 mM K_2EGTA, 1 mM $CaCl_2$, 10 mM MES-TRIS, pH 5.6; **Ca^{2+} buffer B** (with 50 μM $CaCl_2$ added): 28 mM K_2-imminodiacetic acid, 50 μM $CaCl_2$, 1.3 mM KCl, 10 mM MES-TRIS pH 6.1, **Ca^{2+} buffer C** (with 50 μM $CaCl_2$ added): 5 mM KCl, 50 μM $CaCl_2$, 10 mM MES-TRIS pH 5.6. Nicotinamide was added at a final concentration of 50 mM and 50 mM sorbitol was added in controls (Dodd *et al.*, 2007; Leckie *et al.*, 1998). Epidermes treated with BAPTA buffer were also loaded with BAPTA-AM for 10 minutes when indicated during the third hour of light exposure by incubating in the same BAPTA buffer to which were added 0.3 mM eserine, 0.025% Pluronic F-127, and 10 to 25 μM BAPTA-AM. Methods used were similar to previously reported methods that allow cytoplasmic loading of the BAPTA analogue fura-2 (Kuchitsu *et al.*, 2002) and BAPTA (Young *et al.*, 2006) into guard cells without need for microinjection.

Each intact epidermal preparation was set into a viewing chamber (a 12 mm hole predrilled in a glass slide) by sealing the cover slip edges with silicon vacuum grease (Dow Corning Corp., Midland, MI) and covered with 0.2 ml buffer from the incubation dish. The epidermal strip was examined with a 40× objective on an inverted microscope (Nikon TS 100, Nikon Instruments Inc., Melville, NY) focused on the inner edge of stomata (Ichida *et al.*, 1997), and an image of open stomata containing at least 5 stomata was acquired with a digital color camera (CFW-1310C, Scion Corp, Frederick, MD). After image acquisition, the epidermal chamber was removed from the viewing chamber, exposed to the indicated ABA containing buffers, and returned to the blue/red LED array for 1 hour (Figures 6, 7 and 8) or 2 hours (Figure 3). The epidermal chamber was returned to the viewing chamber, and a second image was stored of the same field of view taken as in the first image. Widths of the stomatal apertures were measured with ImageJ software (NIH), compared on a pair-wise basis as percent of initial aperture of the same stomata, and all pair-wise comparisons for a treatment were averaged. ABA concentrations were blinded to the experimenter during experiments. When different mutants were used in experiments, genotype was blinded to the experimenter. Viability Staining: At the end of experiments, after having acquired stomatal aperture images, fluorescein diacetate (FDA) was added to the buffer, and cells exhibiting green fluorescence within 5 minutes were designated as viable, as previously described (Young *et al.*, 2006). Time course analyses of stomatal closing: Epidermal strips were prepared and opened and imaged as described above. After imaging, buffer was perfused (e.g. ABA added) at time 0. A second image was acquired within 30 seconds, and then images were collected at 2 minute intervals

from time 0. Widths of individual mapped stomatal apertures were measured and analyzed in pair-wise comparisons as described above, and each time point compared to the original image of the same stomate.

Arabidopsis growth

Arabidopsis mutant lines analyzed in this study were *abi1-1, abi2-1* (Koornneef *et al.*, 1984), *gca2* (Himmelbach *et al.*, 1998), *ost1-2* (Mustilli *et al.*, 2002) kindly provided by Drs. J. Leung and J. Giraudat (CNRS, Gif-Sur-Yvette) and *pld∝1* (Salk 053785) kindly provided by Dr. X. Wang (Danforth Center, St. Louis Missouri). Plants were grown as previously described (Mori *et al.*, 2006).

Statistical treatment of data

Each experiment was repeated in at least 3 independent trials. Calculation of standard error for stomatal apertures is based on the number of independent trials as reported in previous studies (Allen *et al.*, 2000; Murata *et al.*, 2001) rather than the larger total number of independent stomata, which would give a much lower estimate of the error of the measurements as previously described (Li *et al.*, 2006).

Calcium imaging

All Ca^{2+} imaging experiments in this study were performed with a TE300 inverted microscope using a TE-FM Epi-Fluorescence attachment (Nikon Inc. Melville, NY). Excitation from a 75W Xenon lamp (Osram, Germany) was attenuated by 97% (3% light transmission) by using both 4× and 8× neutral density filters to reduce exposure of the fluorescent reporters and cells to epifluorescence excitation. Wavelength specificity was obtained with a cameleon filter set (440/20 nm excitation, 485/40 nm emission1, 535/30 nm emission2, 455 nm DCLP dichroic; filter set 71007a (Chroma Technology, Rockingham, VT). Filter wheel, shutter and CCD camera were controlled with Metafluor software (MDS, Inc., Toronto, Canada). For observation of imposed Ca^{2+} oscillations, intact epidermes from leaves of *pGC1*:Col plants were prepared for microscopy as previously described (Mori *et al.*, 2006; Yang *et al.*, 2008). On the microscope, intact epidermes were perfused with **depolarizing buffer** (25 mM K_2 - imminodiacetate, 100 μM BAPTA 10 mM MES-Tris buffer, pH 6.1) for 10 minutes to obtain a background. Subsequently **hyperpolarizing buffer** containing Ca^{2+} (1 mM K_2 - imminodiacetate, 1 mM $CaCl_2$, 10 mM MES-Tris buffer, pH 6.1) was applied for 2 minute intervals, followed by 5 minutes of depolarizing buffer.

Electrophysiology

Arabidopsis guard cell protoplasts were isolated from rosette leaves of 4- to 6-week-old plants with a protoplast isolation solution containing 1.0% Cellulase R10, 0.5% Macerozyme R10, 0.5% bovine serum albumin, 0.1% kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol (pH 5.5 with KOH). Whole-cell patch clamp experiments were performed as described previously (Allen *et al.*, 2002). For analyses of S-type anion currents, the pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5 mM MgATP, 10 mM Hepes-Tris, pH 7.1, and $CaCl_2$ was added to give the desired free Ca^{2+} concentrations. Guard cell protoplasts were pre-exposed to ABA (20 μM) for 15 minutes prior to patch clamping, when indicated. The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.6. Note that gigaohm seal formation was performed in a 1 mM Ca^{2+} bath solution (Allen *et al.*, 2002) and not in a high $CaCl_2$ bath solution.

For analyses of inward-rectifying potassium channels currents, the pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 5 mM MgATP, 5 mM Tris-GTP, 10 mM Hepes-Tris, pH 7.1. In addition the free Ca^{2+} concentration was buffered with 6.7 mM EGTA

and 2.61 mM $CaCl_2$ (150 nM free Ca^{2+}) or 6.03 mM $CaCl_2$ (2 μM free Ca^{2+}) and for experiments in Figure 8 C and F, EGTA and $CaCl_2$ were replaced by 4 mM BAPTA and 2.12 or 3.8 mM $CaCl_2$ to give the indicated free Ca^{2+} concentrations. The bath solution contained 30 mM KCl, 2 mM MgCl2, 1 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.5. When indicated the pipette solution also contained 50 μM ABA. Guard cell protoplasts were pre-exposed to extracellular ABA (50 μM) for 20 or 120 minutes prior to patch clamping for K^+in channel and S-type anion channel analyses, respectively.

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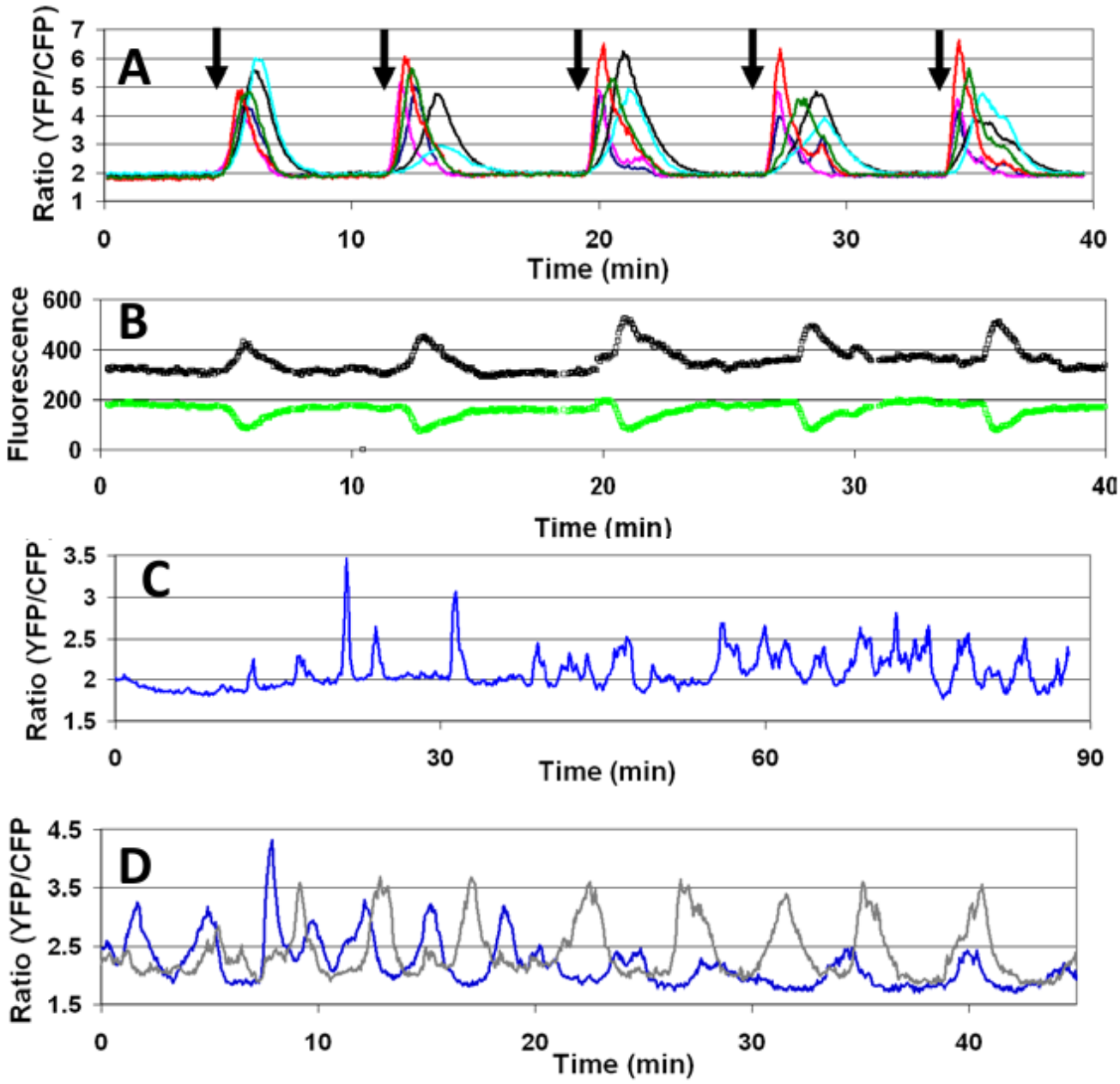

Figure 1.

Characterization of calcium-dependent-ratiometric fluorescence changes in *Arabidopsis thaliana* plants transformed with yellow cameleon 3.6.

A, Fluorescence ratio change of 6 individual guard cells in an intact epidermis in response to 2 minute pulses (at arrows) of hyperpolarizing buffer containing 1 mM Ca^{2+}. **B**, Individual wavelengths from black trace in A. Note fluorescence at 485nm decreases while fluorescence at 530 nm (black) increases confirming fluorescence resonance energy transfer (FRET) of the cameleon reporter. **C**, Spontaneous fluctuations in the Ca^{2+} fluorescence ratio in a guard cell in an intact epidermis during continuous perfusion with Ca^{2+} buffer C (containing 50 μM Ca^{2+}). **D**, Spontaneous repetitive transients in the Ca^{2+} fluorescence ratio in two guard cells

from a single stomate during continuous perfusion with Ca^{2+} buffer A **(**with 0.2 mM free Ca^{2+}).

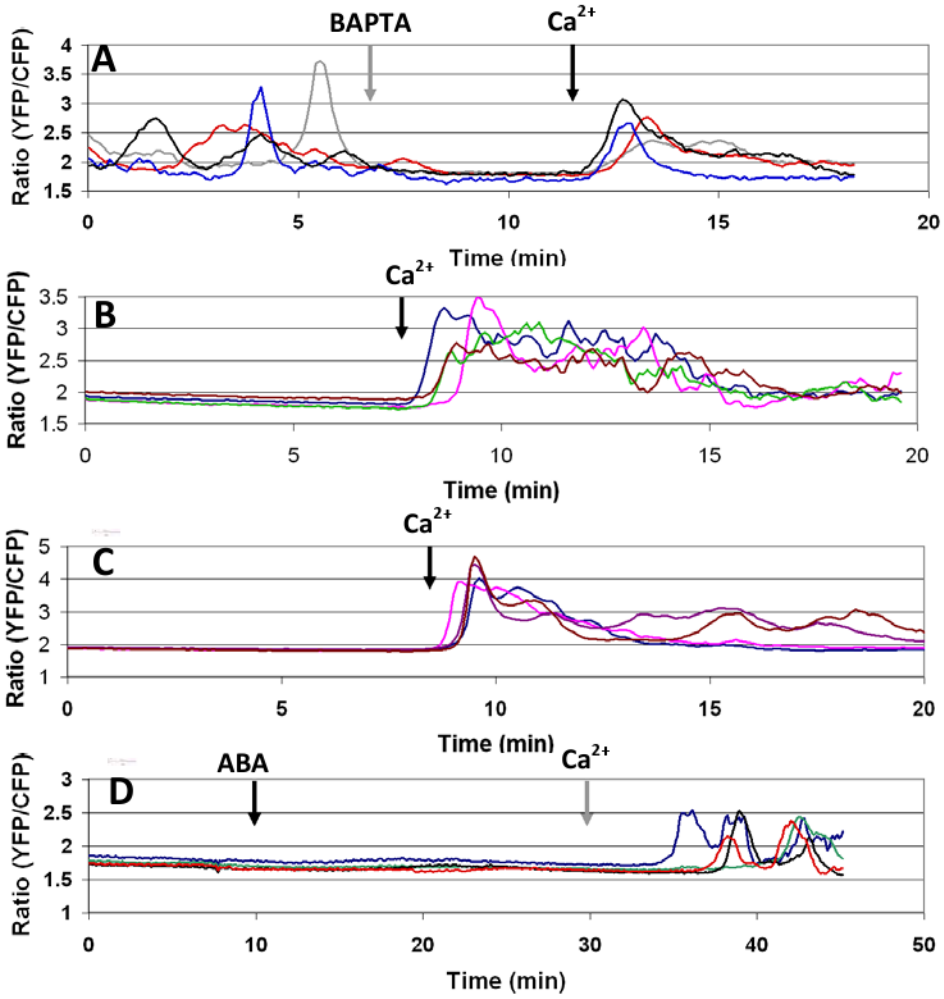

Figure 2.

Inhibition of spontaneous and ABA-induced Ca^{2+} elevations in guard cells.

A, Intracellular Ca^{2+} in four guard cells in an intact epidermis initially perfused with Ca^{2+} buffer A **(**with 0.2 mM free Ca^{2+}). Gray arrow shows switch to BAPTA buffer (200 nM free Ca^{2+}), black arrow shows switch to hyperpolarizing buffer containing 1 mM Ca^{2+}. **B**, Intracellular Ca^{2+} in four guard cells incubated in BAPTA buffer in the light for three hours. Black arrow shows switch to Ca^{2+} buffer B (containing 50 μM Ca^{2+}). **C**, Intracellular Ca^{2+} in four guard cells incubated in BAPTA buffer in light for three hours and also incubated with BAPTA-AM for ten minutes prior to fluorescence imaging. Black arrow shows switch to Ca^{2+} buffer C (containing 50 μM extracellular Ca^{2+} in perfusion buffer). **D**, Intracellular Ca^{2+} in four guard cells incubated in BAPTA buffer in light for three hours and also incubated with BAPTA-AM for ten minutes prior to fluorescence imaging. Black arrow shows switch to the same BAPTA buffer containing 2 μM ABA. Grey arrow shows switch to Ca^{2+} buffer C (containing 50 μM Ca^{2+} in perfusion buffer).

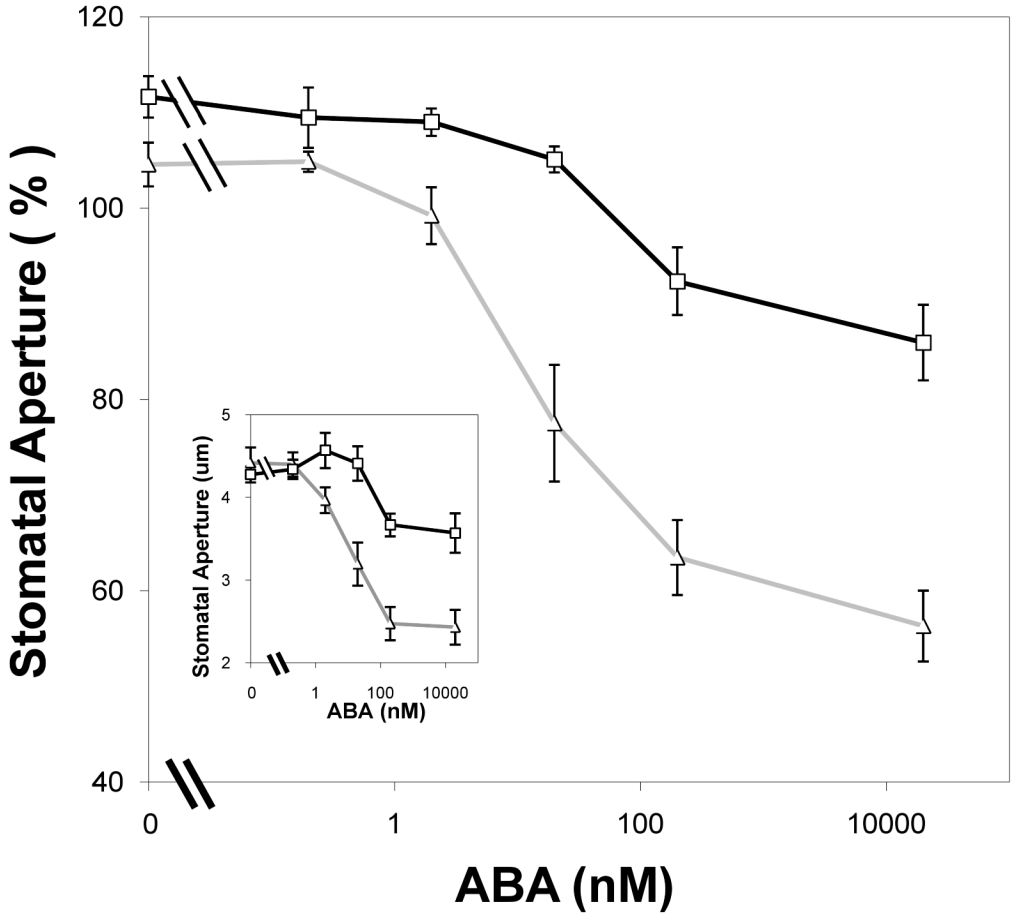

Figure 3.

Quantification of stomatal closure in response to ABA in intact epidermis under conditions that inhibit $[Ca^{2+}]_i$-elevations shows strong inhibition of ABA responses when $[Ca^{2+}]_i$ elevations are inhibited.

Triangles: high 0.2 mM free Ca^{2+} buffer; Squares: 200 nM free Ca^{2+} BAPTA + BAPTA-AM buffer. Y axis shows the percent changes of stomatal apertures relative to the stomatal aperture of each individually mapped stomatal pore prior to ABA incubation. Inset shows the same data of analyzed stomatal apertures in absolute μm. Stomatal apertures were measured after intact epidermes were exposed to light for three hours. Epidermes were placed in buffer containing ABA and incubated in the light for two additional hours before apertures of the identical stomata were measured. Data show averages ± S.E.M. for n=7 experiments with Ca^{2+} buffer A (triangles) and n=8 experiments for BAPTA buffer (squares).

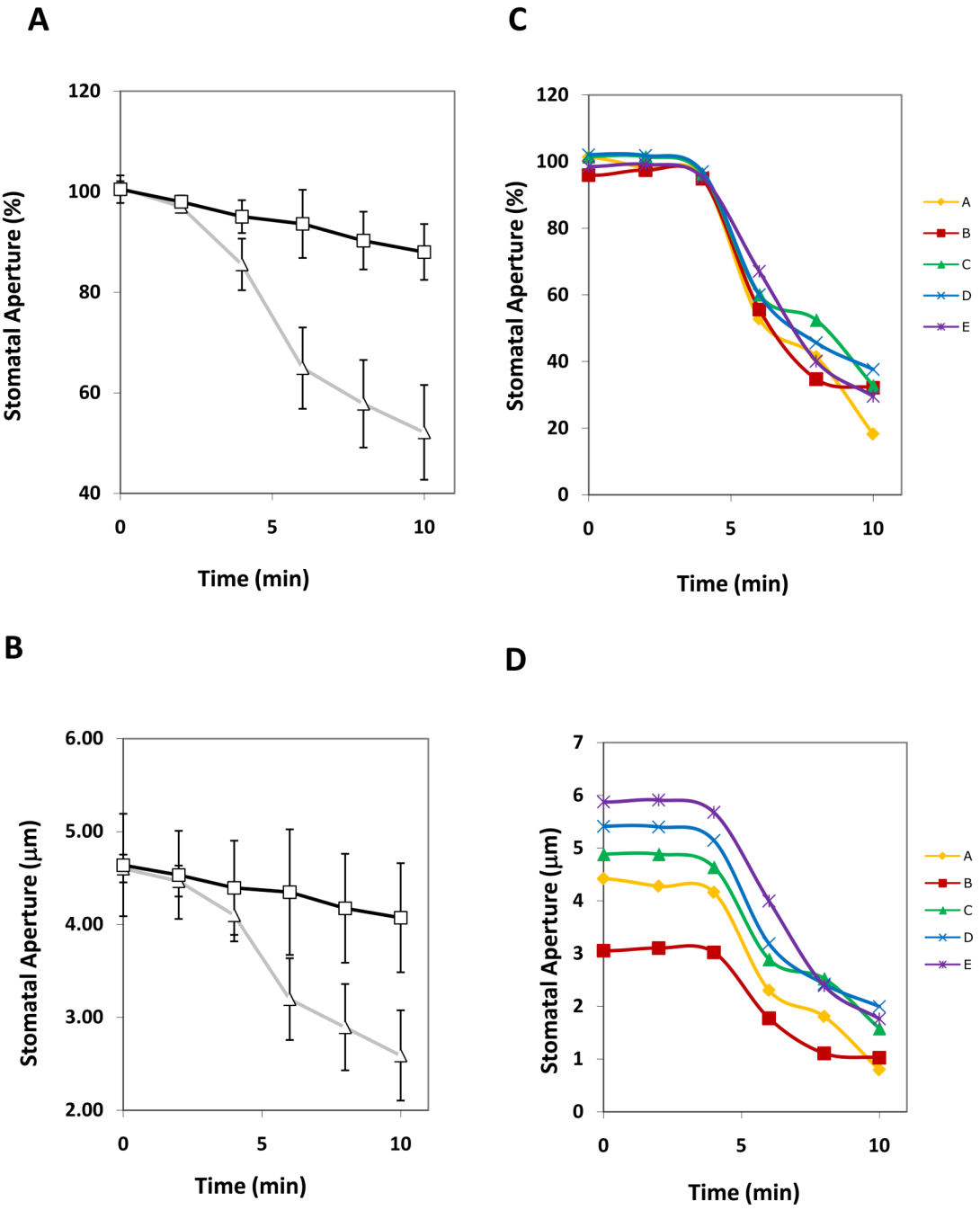

Figure 4.

Time course analysis of response of individually mapped stomatal apertures **(A)** and averages of pooled apertures from the same data **(B)** : Rapid ABA-induced stomatal closing response is strongly attenuated when intracellular Ca^{2+} elevations are inhibited. ABA-induced (2 μM) stomatal closing was analyzed in the presence of 50 μM Ca^{2+} (triangles) and in 200 nM free Ca^{2+} buffered with BAPTA buffer plus BAPTA-AM treatment (squares). Data in (A) and (B) show averages ± S.E.M. of n=3 experiments. **(C)** Individually mapped and tracked stomatal apertures show similar degrees of responses to abscisic acid, although **(D)** the starting stomatal apertures are known to vary in *Arabidopsis* (Ichida *et al.*, 1997; Li *et al.*, 2006; Pei *et al.*, 1997). Data in (C) and (D) are from the same individually tracked stomatal apertures.

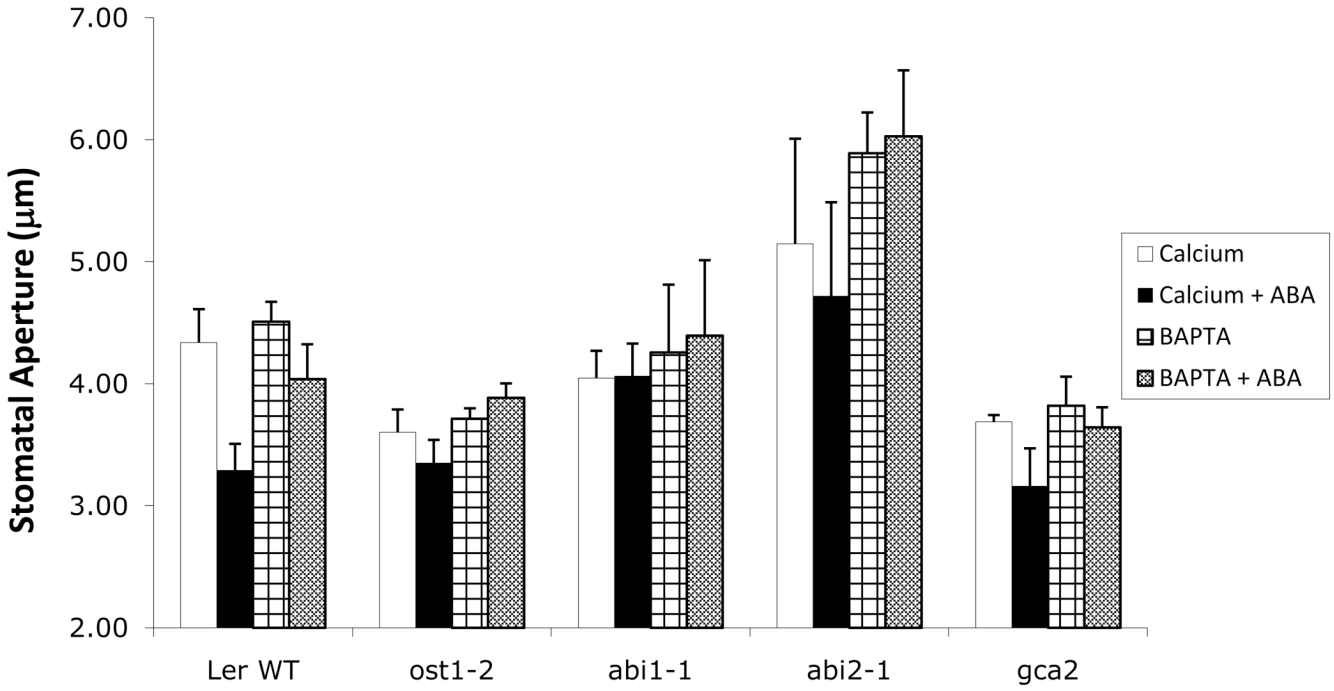

Figure 5.

Analysis of ABA-induced stomatal closure under conditions that inhibit $[Ca^{2+}]_i$ elevations in the ABA insensitive *ost1-2, abi1-1, abi2-1* and *gca2* mutants in the Landsberg erecta (Ler) background.

Calcium (open and black bars) refers to experiments with 0.2 mM free buffered Ca^{2+} in the bath solution (Ca^{2+} buffer A). BAPTA (checkered and grey bars) refers to experiments using 200 nM free Ca^{2+} BAPTA buffer + BAPTA-AM incubation that inhibits $[Ca^{2+}]$i elevations. Stomatal apertures were measured after intact epidermes were exposed to light for one hour. Epidermes were then exposed to fresh buffer containing no ABA or 2 μM ABA (+ABA) and incubated in the light for two additional hours before apertures of the identical mapped stomata were measured. Data show averages ± S.E.M. for n≥3 experiments for each condition.

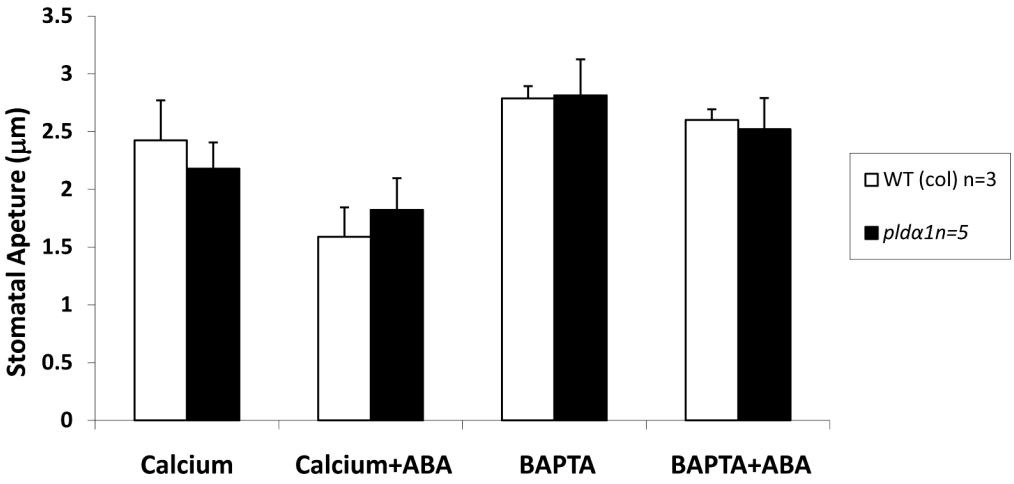

Figure 6.

ABA-induced stomatal closure in the *pldα1 Columbia* mutant.

Calcium (left) refers to experiments with 0.2 mM free extracellular Ca^{2+} and BAPTA (right) refers to conditions with BAPTA buffer containing 200 nM extracellular free Ca^{2+}. Experiments were conducted as described in Figure 5. Data show averages for n≥3 experiments.

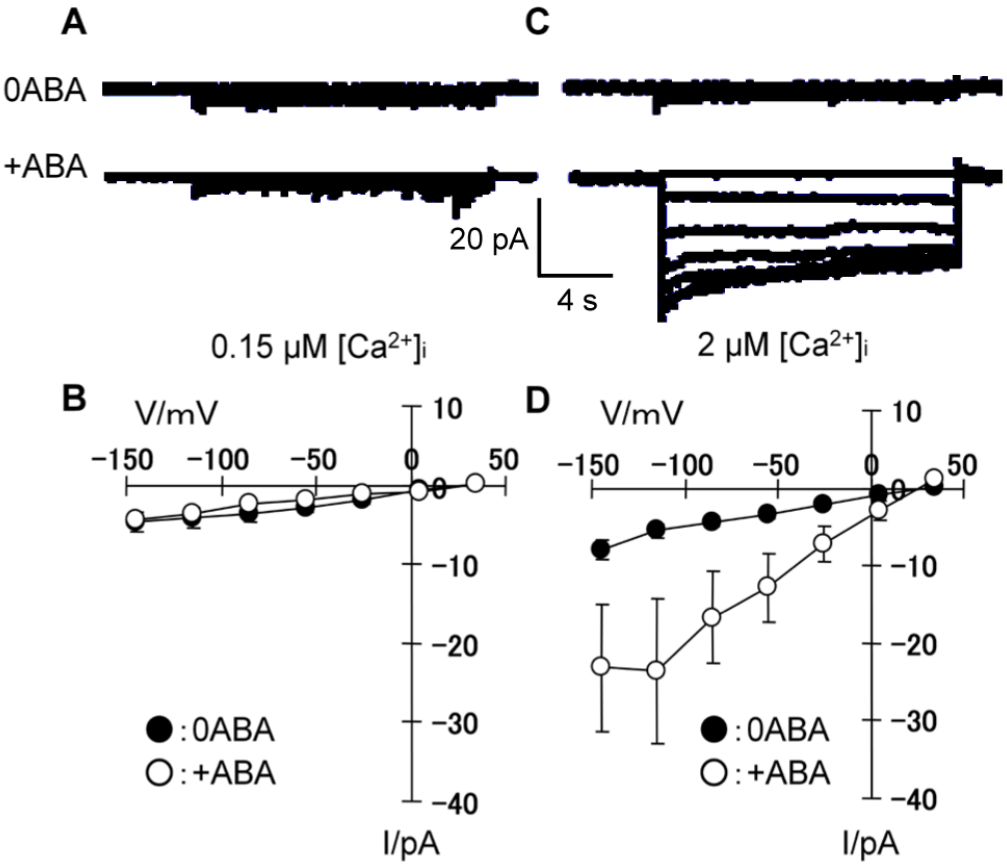

Figure 7.

S-type anion channel activation by $[Ca^{2+}]_i$ in *Arabidopsis* guard cells protoplasts is enhanced by pre-incubation with ABA.

(A) Whole-cell currents in wild-type *Arabidopsis* guard cell protoplasts either not exposed to ABA (top) or pre-exposed to ABA (bottom). $[Ca^{2+}]_i$ was buffered to 150 nM via the pipette solution.

(B) Current-voltage relationships of whole-cell currents recorded in guard cell protoplasts without ABA pre-treatment (filled circles, n = 9) and pre-exposed to ABA (open circles, n = 26) as recorded in (A).

(C) S-type anion channel currents in wild-type *Arabidopsis* guard cell protoplasts either not exposed to ABA (top) or pre-exposed to ABA (bottom). $[Ca^{2+}]_i$ was buffered to 2 μM via the pipette solution.

(D) Current-voltage relationships showing average anion currents recorded from guard cell protoplasts without ABA pretreatment (filled circles, n = 6) and pre-exposed to ABA (open circles, n = 17) as recorded in (C).

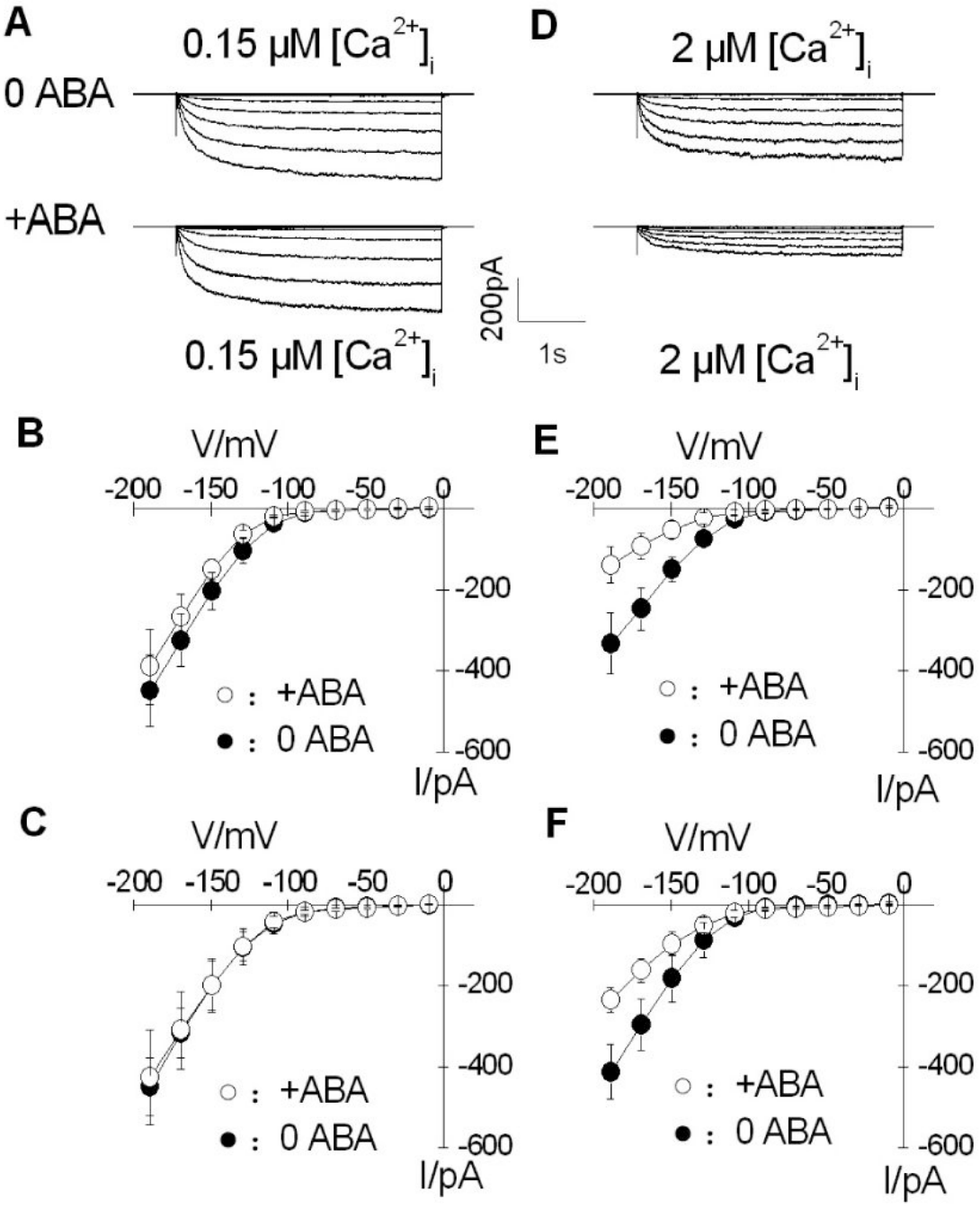

Figure 8.

$[Ca^{2+}]_i$ down-regulation of K^+_{in} channels is primed by pre-exposure to abscisic acid.

(A) Whole-cell currents in wild-type *Arabidopsis* guard cell protoplasts with 0.15 μM $[Ca^{2+}]_i$ either not exposed to ABA (top) or pre-exposed to extracellular ABA (bottom). $[Ca^{2+}]_i$ was buffered to 0.15 μM with 6.7 mM EGTA in the patch clamp pipette solution. In all experiments that included extracellular pre-exposure to ABA in this figure, the patch clamp pipette solution also included 50 μM ABA (see Experimental Procedures).

(B) Current-voltage relationships of whole-cell currents recorded at 0.15 μM $[Ca^{2+}]_i$ in guard cell protoplasts without ABA pre-exposure (0 ABA: filled circles, n = 9) and pre-exposed to ABA (open circles, n = 8) as recorded in (A).

(C) Current-voltage relationships of whole-cell currents recorded in guard cell protoplasts with 0.15 μM $[Ca^{2+}]_i$ without ABA pre-treatment (0 ABA: filled circles, n = 9) and pre-exposed to ABA (open circles, n = 6). $[Ca^{2+}]_i$ was buffered to 0.15 μM with 4 mM BAPTA in the patch clamp pipette solution.
(D) Inward-rectifying potassium currents in wild-type *Arabidopsis* guard cell protoplasts with 2 μM $[Ca^{2+}]_i$ either not exposed to ABA (top) or pre-exposed to ABA (bottom). Cytosolic free $[Ca^{2+}]$ was buffered to 2 μM with 6.7 mM EGTA in the pipette solution. Voltage pulses in (A) and (D) ranged from -9 mV to -189 mV (-20 mV increments), holding potential was -49 mV.
(E) Current-voltage relationships showing average potassium currents recorded from guard cell protoplasts with 2 μM $[Ca^{2+}]_i$ without ABA pretreatment (0 ABA: filled circles, n = 8) and pre-exposed to ABA (open circles, n = 8) as recorded in (D).
(F) Current-voltage relationships showing average potassium currents recorded from guard cell protoplasts with 2 μM $[Ca^{2+}]_i$ without ABA pretreatment (0 ABA: filled circles, n = 8) and pre-exposed to ABA (open circles, n = 6). $[Ca^{2+}]_i$ was buffered to 2 μM with 4 mM BAPTA via the pipette solution.