the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

*The Plant Journal* (2009) **59**, 207–220 doi: 10.1111/j.1365-313X.2009.03872.x

# Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying $K^+$ channels in Arabidopsis guard cells

**Robert S. Siegel[1,†], Shaowu Xue[1,‡], Yoshiyuki Murata[1,2], Yingzhen Yang[1], Noriyuki Nishimura[1], Angela Wang[1] and Julian I. Schroeder[1,*]**

[1]*Division of Biological Sciences, Cell and Developmental Biology Section, and Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116, USA, and*

[2]*Department of Agriculture, Okayama University, Tsushima, Okayama 700-8530, Japan*

Received 5 December 2008; revised 10 February 2009; accepted 17 February 2009; published online 22 April 2009.

[*]For correspondence (fax +1 858 534 7108; e-mail julian@biomail.ucsd.edu).

[†]Present address: Life Technologies, 5781 Van Allen Way, Carlsbad, CA 92008, USA.

[‡]Permanent address: Institute of Molecular Science, Shanxi University, Taiyuan, 030006, China.

## SUMMARY

**Stomatal closure in response to abscisic acid depends on mechanisms that are mediated by intracellular [$Ca^{2+}$] ([$Ca^{2+}$]$_i$), and also on mechanisms that are independent of [$Ca^{2+}$]$_i$ in guard cells. In this study, we addressed three important questions with respect to these two predicted pathways in *Arabidopsis thaliana*. (i) How large is the relative abscisic acid (ABA)-induced stomatal closure response in the [$Ca^{2+}$]$_i$-elevation-independent pathway? (ii) How do ABA-insensitive mutants affect the [$Ca^{2+}$]$_i$-elevation-independent pathway? (iii) 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 by ratiometric [$Ca^{2+}$]$_i$ imaging experiments. ABA-induced stomatal closure in the absence of [$Ca^{2+}$]$_i$ elevations above the physiological resting [$Ca^{2+}$]$_i$ showed only approximately 30% of the normal stomatal closure response, and was greatly slowed compared to the response in the presence of [$Ca^{2+}$]$_i$ elevations. The ABA-insensitive mutants *ost1-2*, *abi2-1* and *gca2* showed partial stomatal closure responses that correlate with [$Ca^{2+}$]$_i$-dependent ABA signaling. Interestingly, patch-clamp experiments showed that exposure of guard cells to ABA greatly enhances the ability of cytosolic $Ca^{2+}$ to activate S-type anion channels and down-regulate inward-rectifying $K^+$ channels, providing strong evidence for a $Ca^{2+}$ sensitivity priming hypothesis. The present study demonstrates and quantifies an attenuated and slowed ABA response when [$Ca^{2+}$]$_i$ elevations are directly inhibited in guard cells. A minimal model is discussed, in which ABA enhances (primes) the [$Ca^{2+}$]$_i$ sensitivity of stomatal closure mechanisms.**

**Keywords: guard cell, $Ca^{2+}$, intracellular $Ca^{2+}$, abi1, signal transduction.**

## INTRODUCTION

The relationship between elevation of intracellular [$Ca^{2+}$] ([$Ca^{2+}$]$_i$) in guard cells and stomatal closure has been studied in depth for the past two decades since pioneering work using $Ca^{2+}$ chelators and $Ca^{2+}$ channel blockers (DeSilva *et al.*, 1985; Schwartz, 1985). Studies on elevation of 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, thus identifying important mechanisms that can mediate [$Ca^{2+}$]$_i$-induced stomatal closure 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 down-regulation of inward-rectifying $K^+$ channels (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 closure (Allen *et al.*, 2001; Mori *et al.*, 2006; Vahisalu *et al.*, 2008). In contrast, '$Ca^{2+}$ 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-rectifying $K^+$ channels (Grabov and Blatt, 1999; Kelly *et al.*, 1995; Schroeder and Hagiwara, 1989), provide mechanisms for $Ca^{2+}$-induced guard cell turgor reduction that result in stomatal closure (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) and 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 for $Ca^{2+}$ in stomatal closure. Recently, calcium-dependent protein kinases (CDPKs) that function in guard cell $Ca^{2+}$ and anion channel activation and stomatal closure have been identified (Mori *et al.*, 2006; Zhu *et al.*, 2007). These CDPKs contribute $Ca^{2+}$ sensors that link $[Ca^{2+}]_i$ to stomatal closure and anion channel activation, and interestingly are also 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 the various cellular $[Ca^{2+}]_i$ responses to ABA (Grabov and Blatt, 1998; Klüsener *et al.*, 2002). It has been 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 that are not strictly mutually exclusive. One hypothesis postulates 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 considerable interest. However, research has not yet determined the relative contributions of the proposed $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent pathways to ABA-induced stomatal closure. A third hypothesis derived from long-term $Ca^{2+}$ imaging studies during guard cell $CO_2$ signaling, the '$Ca^{2+}$ sensitivity priming hypothesis', postulates that physiological stimuli of stomatal closure enhance the $Ca^{2+}$ sensitivity of the appropriate stomatal closure mechanisms, thus allowing specific downstream $Ca^{2+}$ responses to proceed, possibly even when $[Ca^{2+}]_i$ elevations do not occur (Young *et al.*, 2006).

Micro-injection of the $Ca^{2+}$ chelator BAPTA (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) 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 (*Petroselinum crispum*), 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 (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)), together with extracellular $Ca^{2+}$ removal, inhibited spontaneous $[Ca^{2+}]_i$ elevations and 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 closure response that occurs at resting $[Ca^{2+}]_i$. Furthermore, 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 $Ca^{2+}$-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), which has an improved dynamic range (Mori *et al.*, 2006; Nagai *et al.*, 2004), to examine the 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 threefold changes in the ratiometric fluorescence reporting the $[Ca^{2+}]_i$, confirming the strong FRET response of this sensor (Figure 1a,b). Accurate *in vivo* calibration of a fluorescent $[Ca^{2+}]_i$ sensor is difficult for various technical and biological reasons, and therefore imaging studies usually report fluorescence ratio changes rather than calibrated $[Ca^{2+}]_i$ values. However, we have explored estimation of $[Ca^{2+}]_i$ values from our fluorescence ratio data using *in vitro* calibration parameters for YC3.6 (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 1(a) 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), indicates a resting $[Ca^{2+}]_i$ of approximately 120–150 nM. This approximated basal $[Ca^{2+}]_i$ is in agreement with resting $[Ca^{2+}]_i$ ranges reported using fura-2, indo-1 and the YC2.1 cameleon (Allen *et al.*, 2000; Allen *et al.*, 1999a; Grabov and Blatt, 1998; McAinsh *et al.*, 1990; Schroeder and Hagiwara, 1990).

The guard cell $[Ca^{2+}]_i$ in freshly prepared intact Arabidopsis leaf epidermi continuously perfused with extracellular buffer containing 50 μM added $Ca^{2+}$ ($Ca^{2+}$ buffer C, see Experimental procedures) showed 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 agrees with findings from previous research using YC2.1 (Allen *et al.*, 1999b). The baseline ratios from which these transient $[Ca^{2+}]_i$ elevations increase (Figure 1c,d) was relatively stable among experiments, with a range of approximately 1.7–2.0 ($n > 15$ 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 (BAPTA buffer, see Experimental procedures) abolished these spontaneous $[Ca^{2+}]_i$ transients (Figure 2a; $n > 20$ experiments). 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 had been held in BAPTA buffer with 200 nM free $Ca^{2+}$ for 3 h 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 that of controls (Figures 1 and 2c,d; $n > 10$ experiments).

Further experiments were performed to reduce $[Ca^{2+}]_i$ in guard cells to below typical resting levels by extracellular perfusion with millimolar concentrations of 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 confirm that YC3.6 can report lower $[Ca^{2+}]_i$ levels than resting levels

 

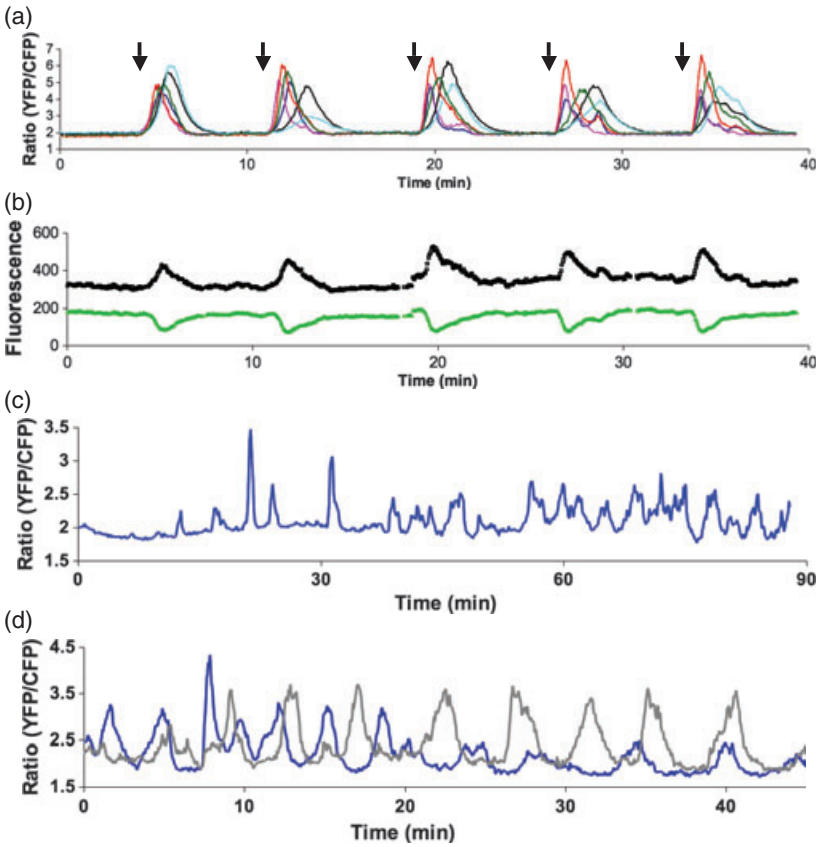


**Figure 1.** Characterization of calcium-dependent ratiometric fluorescence changes in *Arabidopsis thaliana* guard cells transformed with yellow cameleon 3.6.
(a) Fluorescence ratio change of six individual guard cells in an intact epidermis in response to 2 min pulses (at arrows) of hyperpolarizing buffer containing 1 mM $Ca^{2+}$.
(b) Individual wavelengths from the black trace in (a). Note that fluorescence at 485 nm 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 $Ca^{2+}$).

(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 h to open stomata, and loaded with BAPTA-AM for 10 min. 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 min after application of 2 μM ABA (Figure 2d) ($n$ = 12 experiments). As a control to test whether guard cells could report $[Ca^{2+}]_i$ elevations, cells were exposed after this treatment 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 $Ca^{2+}$ dependence of ABA responses.

### Inhibition of guard cell $[Ca^{2+}]_i$ increases allows 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 3 h to open stomatal pores. Epidermes treated with BAPTA buffer (200 nM $Ca^{2+}$) were also incubated with 10 μM BAPTA-AM for 10 min 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 obtained for measurements of stomatal apertures. The epidermes were then exposed to ABA and incubated in the light for 2 h. Photomicrographs of the same leaf epidermis regions were taken for later measurement of the same stomatal pore apertures after ABA exposures. The pre- and post-treatment apertures of the same stomata were compared pairwise to determine the degree of stomatal closure.

Guard cell viability remained intact after 5 h in the 200 nM free $Ca^{2+}$ external BAPTA buffer solution. The viability dye fluorescein diacetate was added after completion of imaging

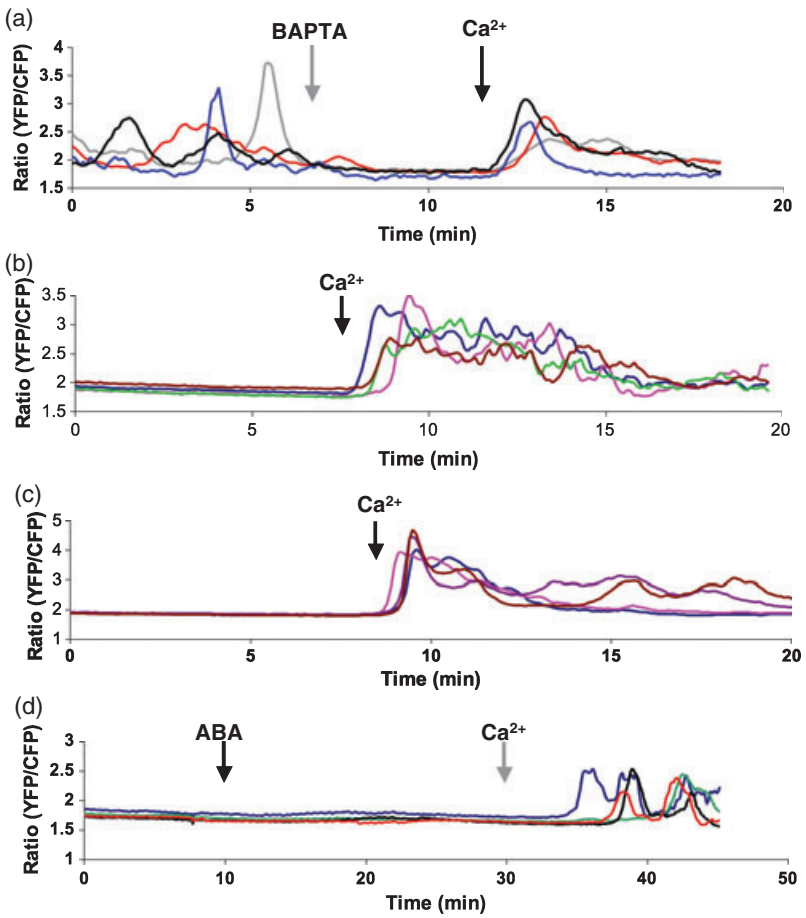


**Figure 2.** Inhibition of spontaneous and ABA-induced $Ca^{2+}$ elevations in guard cells.
(a) Ratiometric recordings of intracellular $Ca^{2+}$ concentrations in four guard cells in an intact epidermis initially perfused with $Ca^{2+}$ buffer A (with 0.2 mM free $Ca^{2+}$). The gray arrow shows the switch to BAPTA buffer (200 nM free $Ca^{2+}$); the black arrow shows the switch to hyperpolarizing buffer containing 1 mM $Ca^{2+}$.
(b) Intracellular $Ca^{2+}$ concentrations in four guard cells incubated in BAPTA buffer in the light for 3 h. The black arrow shows the switch to $Ca^{2+}$ buffer B (containing 50 μM $Ca^{2+}$).
(c) Intracellular $Ca^{2+}$ concentrations in four guard cells incubated in BAPTA buffer in light for 3 h and also incubated with BAPTA-AM for 10 min prior to fluorescence imaging. The black arrow shows the switch to $Ca^{2+}$ buffer C (containing 50 μM extracellular $Ca^{2+}$ in perfusion buffer).
(d) Intracellular $Ca^{2+}$ concentrations in four guard cells incubated in BAPTA buffer in the light for 3 h and also incubated with BAPTA-AM for 10 min prior to fluorescence imaging. The black arrow shows the switch to the same BAPTA buffer containing 2 μM ABA; the gray arrow shows the switch to $Ca^{2+}$ buffer C (containing 50 μM $Ca^{2+}$ in perfusion buffer).

experiments to monitor whether the analyzed guard cells remained viable (Young *et al.*, 2006) (see Experimental procedures). Only a few guard cells failed to show fluorescence following fluorescein diacetate 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 0.2 mM free [$Ca^{2+}$] buffer, incubation in the light for 2 h without ABA following the initial 3 h opening treatment caused the stomatal apertures to increase by an additional 5% (Figure 3); this small change was not significant at the 95% confidence level. In the 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 without ABA following the initial 3 h 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 $[Ca^{2+}]_i$ elevation was reduced. At the highest ABA concentration (20 μM), stomatal closure under conditions that inhibited $[Ca^{2+}]_i$ elevations was approximately 30% of the stomatal closure under high [$Ca^{2+}$] conditions (Figure 3). Similar results were obtained when final absolute stomatal apertures rather than the percentage of initial aperture are plotted (Figure 3, inset).

Although we measured stomatal closure after 2 h to determine the 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 4–10 min time range (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 those in physiological $Ca^{2+}$ buffer (Figure 4a).

 

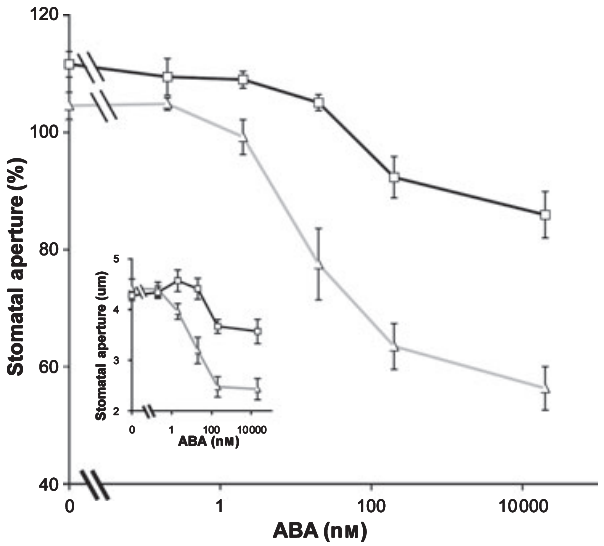


**Figure 3.** Quantification of stomatal closure in response to ABA in intact epidermis under conditions that inhibit $[Ca^{2+}]_i$ elevations shows strong inhibition of ABA responses when $[Ca^{2+}]_i$ elevations are inhibited.
Triangles: 0.2 mM free $Ca^{2+}$ buffer; squares: 200 nM free $Ca^{2+}$ BAPTA buffer + BAPTA-AM-loaded. The *y* axis shows the percentage changes of stomatal apertures relative to the stomatal aperture of each individually mapped stomatal pore prior to ABA incubation. The inset shows the results for the same analyzed stomatal apertures in micrometres. Stomatal apertures were measured after intact epidermes were exposed to light for 3 h. Epidermes were placed in buffer containing ABA and incubated in the light for an additional 2 h before the apertures of the same stomata were measured. Data are means ± SEM for $n = 7$ experiments with $Ca^{2+}$ buffer A (triangles) and $n = 8$ experiments for BAPTA buffer (squares).

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 Figure 4(c,d), 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 favoring 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), or the use of independent data confirmations by more than one 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 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-inhibited conditions. To examine whether the partial ABA-induced stomatal closure under $Ca^{2+}$-elevation-inhibited conditions bypasses ABA signaling mutants, we examined the ABA response in the following ABA-insensitive mutant lines *ost1-2*, *abi1-1*, *abi2-1*, *gca2* and *pldα1* (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 mean 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 L*er* mutants than that in the wild-type (e.g. $P < 0.02$ for *gca2*, $n = 3$ experiments, one-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* and *abi2-1* mutants caused no average reduction in stomatal apertures. ABA application to the *gca2* mutant caused a weak average stomatal closure response; the 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 showed ABA insensitivity in the $Ca^{2+}$-treated samples, and a further average attenuation of slight ABA responses in BAPTA-treated samples.

The phospholipase D mutant, *pldα1*, was selected for analyses here based on reported abolishment of ABA-induced stomatal closure (Mishra *et al.*, 2006). The *pldα1* mutant is in the Columbia wild-type 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 wild-type (Columbia), and found that the difference in stomatal apertures between wild-type and *pldα1* with no added ABA was not significant (Figure 6, 'Calcium'; one-tailed *t* test, $P > 0.27$, $n = 5$ experiments for *pldα1*). The difference in stomatal apertures between wild-type and *pldα1* in the presence of ABA was also not significant (Figure 6, 'Calcium + ABA'; one-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

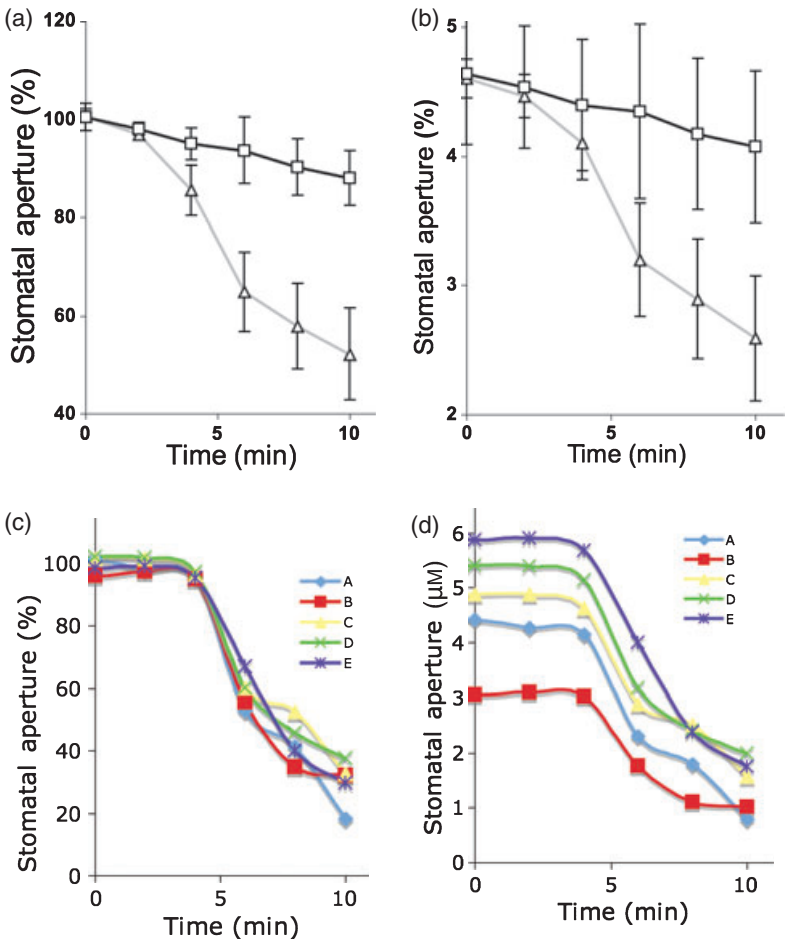


**Figure 4.** Time-course analysis of response of individually mapped stomatal apertures (a) and averages of pooled apertures from the same data (b).
The rapid ABA-induced stomatal closure response is strongly attenuated when intracellular $Ca^{2+}$ elevations are inhibited. ABA-induced (2 μM) stomatal closure 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 are means ± SEM of $n = 3$ experiments.
(c) Individually mapped and tracked stomatal apertures show similar degrees of responses to abscisic acid, although the starting stomatal apertures (d) are known to vary in Arabidopsis (Ichida *et al.*, 1997; Li *et al.*, 2006; Pei *et al.*, 1997). Data in (c) and (d) are for the same individually tracked stomatal apertures.

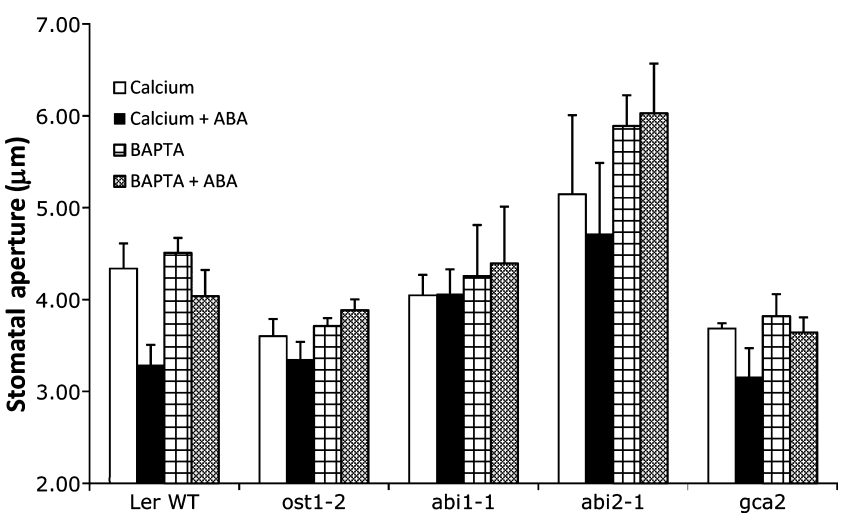


**Figure 5.** Analysis of ABA-induced stomatal closure under conditions that inhibit $[Ca^{2+}]_i$ elevations in the ABA-insensitive *ost1-2*, *abi1-1*, *abi2-1* and *gca2* mutants in the Landsberg *erecta* (L*er*) background.
'Calcium' (open and black bars) refers to experiments with 0.2 mM free buffered $Ca^{2+}$ in the bath solution ($Ca^{2+}$ buffer A). 'BAPTA' (hatched and gray bars) refers to experiments using 200 nM free $Ca^{2+}$ BAPTA buffer+BAPTA-AM incubation, which inhibits $[Ca^{2+}]_i$ elevations. Stomatal apertures were measured after intact epidermes had been exposed to light for 1 h. Epidermes were then exposed to fresh buffer containing no ABA or 2 μM ABA (+ABA), and incubated in the light for an additional 2 h before apertures of the same mapped stomata were measured. Data are means and SEM for $n \geq 3$ experiments for each condition.

same change in wild-type, *pldα1* stomata appeared to show a significant partial ABA hyposensitivity in this subtractive analysis (Figure 6, 'Calcium' – 'Calcium + ABA'; one-tailed *t* test, $P < 0.02$). In BAPTA-treated samples, the ABA response was strongly attenuated in both wild-type and *pldα1* (Figure 6, 'BAPTA' versus 'BAPTA + ABA').

 

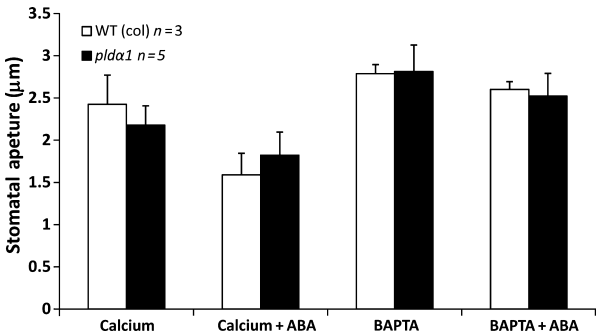


**Figure 6.** ABA-induced stomatal closure in the *pldα1* Columbia mutant. 'Calcium' (left) refers to experiments with 0.2 mM free extracellular $Ca^{2+}$, and 'BAPTA' (right) refers to conditions with BAPTA buffer containing 200 nM extracellular free $Ca^{2+}$. Experiments were performed as described in Figure 5. Data are means and SEM for $n \geq 3$ experiments.

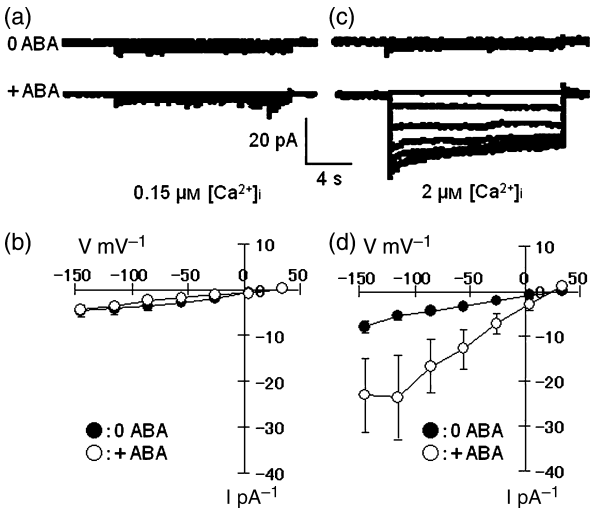


**Figure 7.** S-type anion channel activation by $[Ca^{2+}]_i$ in Arabidopsis guard cells protoplasts is enhanced by pre-incubation with ABA.
(a) Whole-cell currents in wild-type Arabidopsis guard cell protoplasts either not exposed to ABA (top) or pre-exposed to ABA (bottom). $[Ca^{2+}]_i$ was buffered 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 mean anion currents recorded from guard cell protoplasts without ABA pre-treatment (filled circles, $n = 6$) and pre-exposed to ABA (open circles, $n = 17$) as recorded in (c).

### 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 closure 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 with and without ABA pre-incubation, as this response is an important mechanism in ABA- and $Ca^{2+}$-induced stomatal closure (Allen *et al.*, 2002; Mori *et al.*, 2006; Negi *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 when it was buffered to 2 μM (Figure 7c top,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 7c bottom,d). No significant ABA activation of S-type anion currents was observed when $[Ca^{2+}]_i$ was buffered to 150 nM (Figure 7a,b). These data show that ABA enhances the $[Ca^{2+}]_i$ sensitivity of S-type anion channel activation (Figure 7). These findings also suggest a mechanism that explains the apparent paradox between spontaneous $[Ca^{2+}]_i$ elevations and the $[Ca^{2+}]_i$ dependence of stomatal closure (Figures 1–6).

### Abscisic acid sensitizes Arabidopsis guard cell $K^+_{in}$ channels to cytosolic $[Ca^{2+}]$

Cytosolic $Ca^{2+}$ is 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 previous preliminary experiments indicated a lack of this response in Arabidopsis guard cells (Z.-M. Pei, Biology Department, Duke University, unpublished results). 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(a,d top). The mean $K^+_{in}$ channel current magnitudes were not significantly decreased when $[Ca^{2+}]_i$ was buffered to 2 μM using EGTA in the patch-clamp pipette solution compared to when $[Ca^{2+}]_i$ was buffered to 150 nM in the absence of ABA (filled circles in Figure 8b,e; $P = 0.24$ at −189 mV and $P = 0.60$ at −169 mV for experiments without ABA). 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 8c,f; $P = 0.38$ at −169 mV for experiments without ABA).

We next analyzed whether ABA exposure mediates $[Ca^{2+}]_i$ down-regulation of $K^+_{in}$ channels in Arabidopsis.

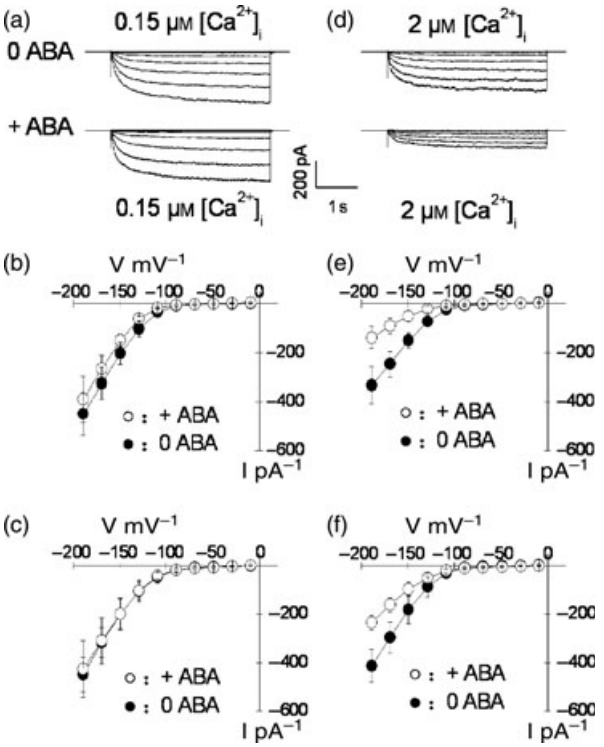


**Figure 8.** $[Ca^{2+}]_i$ down-regulation of $K^+_{in}$ channels is primed by pre-exposure to abscisic acid.

(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 shown here that included extracellular pre-exposure to ABA, 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-treatment (0 ABA: filled circles, $n = 9$) or 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, $n = 9$) or 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 to −189 mV (−20 mV increments); the holding potential was −49 mV.

(e) Current–voltage relationships showing mean potassium currents recorded from guard cell protoplasts with 2 μM $[Ca^{2+}]_i$ without ABA pre-treatment (0 ABA: filled circles, $n = 8$) or pre-exposed to ABA (open circles, $n = 8$) as recorded in (d).

(f) Current–voltage relationships showing mean potassium currents recorded from guard cell protoplasts with 2 μM $[Ca^{2+}]_i$ without ABA pre-treatment (0 ABA: filled circles, $n = 8$) or pre-exposed to ABA (open circles, $n = 6$). $[Ca^{2+}]_i$ was buffered to 2 μM with 4 mM BAPTA via the pipette solution.

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 8a,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 when EGTA (Figure 8d,e) or BAPTA (Figure 8f) were used in the pipette solution. Taken together, the 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

Guard cells that did not exhibit a $[Ca^{2+}]_i$ increase upon application of ABA have been observed 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 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 inhibits 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 modelled as a consequence of 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 $[Ca^{2+}]$ to 200 nM could provide '$Ca^{2+}$-neutral' conditions. 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 inhib-

 

ited 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

YC3.6 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 (Nagai *et al.*, 2004). Our methodology could have missed rapid $[Ca^{2+}]_i$ spikes of <1 sec 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 durations 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 $[Ca^{2+}]_i$ 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 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 closure is $[Ca^{2+}]_i$-dependent. Previous research led to the hypothesis that the physiological stimulus of stomatal closure, $CO_2$, enhances the $Ca^{2+}$ sensitivity of stomatal closure mechanisms, thus allowing $Ca^{2+}$ signaling to proceed (the $Ca^{2+}$ sensitivity priming hypothesis) (Young *et al.*, 2006). Interestingly, here we found 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 findings presented 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 closure, but data also suggest a link between $[Ca^{2+}]_i$ elevation and stomatal opening (Cousson and Vavasseur, 1998; Curvetto *et al.*, 1994; Harada and Shimazaki, 2009; Shimazaki *et al.*, 1992; Young *et al.*, 2006). The $Ca^{2+}$ sensitivity priming hypothesis could suggest 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, 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 5a,b), indicating that additional components, such as pH changes (Blatt and Armstrong, 1993; Wang *et al.*, 2001), probably function in this stomatal closure 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 of 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 $Ca^{2+}$ pre-treatment (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 of the small Arabidopsis guard cells with BAPTA is not practical for analyzing many stomatal responses, and in the present study extracellular application of millimolar concentrations of 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 so 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 cyclic ADP ribose (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). ABA-induced stomatal closure was completely abolished by 50 mM nicotinamide, but not 50 mM sorbitol (Stephen Lee Biology Division, University of California, San Diego, and J.I.S., unpublished results), but this concentration of pharmacological blocker for lowering baseline $[Ca^{2+}]_i$ is fairly high, and 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 suggesting 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) support a hypothesis in which $Ca^{2+}$

 

sensitivity priming may enable $[Ca^{2+}]_i$ 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 bypass these mutations. In the presence of BAPTA, *ost1*, *abi1-1*, *abi2-1* and *gca2* all showed insensitivity to ABA. These data are consistent with data suggesting roles for 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, 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 those downstream of cADPR signaling (Wu *et al.*, 2003), indicating that more than one target may exist for these type 2C protein 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). No inhibitors for these type 2C protein phosphatases are available (Murata *et al.*, 2001). 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). 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 closure 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 closure mechanisms, thus enabling specificity of $Ca^{2+}$ signaling (Young *et al.*, 2006). The ABA insensitive mutants *abi1-1*, *abi2-1*, *ost1* and *gca2* also show $Ca^{2+}$ 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 using medical adhesive (Hollister Inc., http://www.hollister.com), and then gently removing the upper leaf cell layers using a razor blade. Cover slips were pre-marked on the bottom with a colored grid to allow rapid re-examination of previously photographed stomatal locations. The time course of each stomatal aperture was individually tracked (Allen *et al.*, 2001), allowing accurate analyses of the response of each individual stomatal aperture. Intact epidermis 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 light-emitting diodes (LUXEON III; Philips Lumileds Lighting Company, http://www.philipslumileds.com) at room temperature (21–23°C) for 3 h to open stomatal apertures. The following test buffers were used: BAPTA buffer (with 200 nM free $Ca^{2+}$): 25 mM dipotassium ($K_2$) iminodiacetic 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 $K_2$-iminodiacetic acid, 2 mM $K_2$-EGTA, 1 mM $CaCl_2$, 10 mM MES/Tris pH 5.6; $Ca^{2+}$ buffer B (with 50 μM $CaCl_2$ added): 28 mM $K_2$-iminodiacetic 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 to controls (Dodd *et al.*, 2007; Leckie *et al.*, 1998). Epidermes treated with BAPTA buffer were also loaded with BAPTA-AM for 10 min where indicated during the third hour of light exposure by incubation in the same BAPTA buffer to which were added 0.3 mM eserine, 0.025% Pluronic F-127 (CAS No. 11104975) and 10–25 μM BAPTA-AM. The methods used were similar to previously reported methods that allow cytoplasmic loading of the BAPTA analog fura-2 (Kuchitsu *et al.*, 2002) and BAPTA (Young *et al.*, 2006) into guard cells without the need for microinjection.

Each intact epidermal preparation was set into a viewing chamber (a 12 mm hole pre-drilled in a glass slide) by sealing the cover slip edges with silicon vacuum grease (Dow Corning Corp., http://www.dowcorning.com), and covered with 0.2 ml buffer from the incubation dish. The epidermal strip was examined using a 40× objective on an inverted microscope (Nikon TS 100; Nikon Instruments Inc., http://www.nikon.com) focused on the inner edge of stomata (Ichida *et al.*, 1997), and an image of open stomata that contained at least five stomata was acquired using a digital color camera (CFW-1310C, Scion Corp., http://www.scioncorp.com). 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 light-emitting diode array for 1 h (Figures 6–8) or 2 h (Figure 3). The epidermal chamber was returned to the viewing chamber, and a second image was stored of the same field of view as in the first image. Widths of the stomatal apertures were measured using IMAGEJ software (National Institutes of Health, http://rsbweb.nih.gov/ij), compared on a pairwise basis as the percentage of the initial aperture of the same stomata, and all pairwise comparisons for a treatment were averaged. The experimenter was blind with respect to the ABA concentrations used. When mutants were used in experiments, the experimenter was also blind with respect to the genotype.

 

*Viability staining.* At the end of experiments, after having acquired stomatal aperture images, fluorescein diacetate was added to the buffer, and cells exhibiting green fluorescence within 5 min were designated as viable, as previously described (Young *et al.*, 2006).

*Time course analyses of stomatal closure.* Epidermal strips were prepared and imaged as described above. After imaging, buffer was perfused (e.g. ABA added) at time 0. A second image was acquired within 30 sec, and then images were collected at 2 min intervals from time 0. The widths of individual mapped stomatal apertures were measured and analyzed in pairwise comparisons as described above, and the results at each time point were compared to the original image of the same stomate.

### Arabidopsis growth

The 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, France) and *pldα1* (SALK 053785) (kindly provided by Dr X. Wang, Danforth Center, St Louis, MO). Plants were grown as previously described (Mori *et al.*, 2006).

### Statistical treatment of data

Each experiment was repeated in at least three 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 using a TE300 inverted microscope with a TE-FM epi-fluorescence attachment (Nikon Inc.). Excitation using a 75 W xenon lamp (Osram, http://www.osram.com) 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 achieved using a cameleon filter set [440/20 nm excitation, 485/40 nm emission 1, 535/30 nm emission 2, 455 nm DCLP (dichroic long pass) dichroic; filter set 71007a (Chroma Technology, http://www.chroma.com)]. The filter wheel, shutter and CCD camera were controlled using METAFLUOR software (MDS Inc., http://www.mdsinc.com). 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 stage, intact epidermes were perfused with depolarizing buffer (25 mM $K_2$-iminodiacetate, 100 μM BAPTA, 10 mM MES/Tris buffer pH 6.1) for 10 min to obtain a background value. Subsequently, hyperpolarizing buffer containing $Ca^{2+}$ (1 mM $K_2$-iminodiacetate, 1 mM $CaCl_2$, 10 mM MES/Tris buffer pH 6.1) was applied for 2 min, followed by 5 min of depolarizing buffer (Figure 1a).

### Electrophysiology

Arabidopsis guard cell protoplasts were isolated from rosette leaves of 4–6-week-old plants using a protoplast isolation solution containing 1.0% Cellulase R10, 0.5% Macerozyme R10 (Yakutt Horisha Co. Ltd, http://www.yakutt.co.jp/ypi/en/product.html), 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 (buffered to pH 5.5 using 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 Mg-ATP, 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 min 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), not in a high-$CaCl_2$ bath solution.

For analyses of inward-rectifying potassium channel currents, the pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 5 mM Mg-ATP, 5 mM Tris-GTP and 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,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 min prior to patch clamping for $K^+_{in}$ channel and S-type anion channel analyses, respectively.

## ACKNOWLEDGEMENTS

We thank Gethyn Allen for discussions during early planning of this project, Jean Colcombet and Danessa Lerias for pilot experiments using external EGTA solutions, Jean Colcombet for comments on the manuscript, and Stephen Lee for additional stomatal movement analyses. We thank Zhen-Ming Pei for unpublished preliminary data on reduced $[Ca^{2+}]_i$ regulation of $K^+_{in}$ channels in Arabidopsis guard cells. We thank X. Wang for providing *pldα1*, and J. Giraudat and J. Leung for providing *ost1* mutants. This research was supported by National Institutes of Health grant R01GM060396, by National Science Foundation grant MCB0417118, and in part by Department of Energy grant DOE-DE-FG02-03ER15449 to J.I.S and in part by Natural Science Foundation of China 2070/028 to S.X.

## REFERENCES

**Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H. and Trewavas, A.J.** (1994) Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. *Plant Cell*, **6**, 1319–1328.

**Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E. and Schroeder, J.I.** (2001) A defined range of guard cell calcium oscillation parameters encodes inhibition of stomatal re-opening. *Nature*, **411**, 1053–1057.

**Allen, G.J., Chu, S.P., Schumacher, K., *et al.*** (2000) Alteration of stimulus-specific guard cell calcium oscillations and stomatal responses in Arabidopsis *det3* mutant. *Science*, **289**, 2338–2342.

**Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y. and Schroeder, J.I.** (1999a) Arabidopsis *abi1-1* and *abi2-1* phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. *Plant Cell*, **11**, 1780–1798.

**Allen, G.J., Kwak, J.M., Chu, S.P., Llopis, J., Harper, F.J., Tsien, R.Y. and Schroeder, J.I.** (1999b) Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells. *Plant J.* **19**, 735–738.

**Allen, G.J., Murata, Y., Chu, S.P., Nafisi, M. and Schroeder, J.I.** (2002) Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the Arabidopsis farnesyltransferase mutant *era1-2*. *Plant Cell*, **14**, 1649–1662.

**Belin, C., de Franco, P.O., Bourbousse, C., Chaignepain, S., Schmitter, J.M., Vavasseur, A., Giraudat, J., Barbier-Brygoo, H. and Thomine, S.** (2006) Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol.* **141**, 1316–1327.

**Blatt, M.R. and Armstrong, F.** (1993) $K^+$ channels of stomatal guard cells: abscisic-acid-evoked control of the outward-rectifier mediated by cytoplasmic pH. *Planta*, **191**, 330–341.

 

**Blatt, M.R., Thiel, G. and Trentham, D.R.** (1990) Reversible inactivation of $K^+$ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. *Nature*, **346**, 766–769.

**Blume, B., Nurnberger, T., Nass, N. and Scheel, D.** (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell*, **12**, 1425–1440.

**de Bruxelles, G.L., Peacock, W.J., Dennis, E.S. and Dolferus, R.** (1996) Abscisic acid induces the alcohol dehydrogenase gene in Arabidopsis. *Plant Physiol.* **111**, 381–391.

**Cho, D., Kim, S.A., Murata, Y., Lee, S., Jae, S.K., Nam, H.G. and Kwak, J.M.** (2009) Deregulated expression of the plant glutamate receptor homolog AtGLR3.1 impairs long-term Ca-programmed stomatal closure. *Plant J.* doi 10.1111/j.1365-313X.2009.03789.x.

**Clayton, H., Knight, M.R., Knight, H., McAinsh, M.R. and Hetherington, A.M.** (1999) Dissection of the ozone-induced calcium signature. *Plant J.* **17**, 575–579.

**Coursol, S., Fan, L.M., Le Stunff, H., Spiegel, S., Gilroy, S. and Assmann, S.M.** (2003) Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. *Nature*, **423**, 651–654.

**Cousson, A. and Vavasseur, A.** (1998) Putative involvement of cytosolic $Ca^{2+}$ and GMP in stomatal response in *Commelina communis* L. *Planta*, **206**, 308–314.

**Curvetto, N., Darjania, L. and Delmastro, S.** (1994) Effect of two cAMP analogs on stomata in *Vicia faba*: possible relationship with cytosolic calcium concentration. *Plant Physiol. Biochem.* **32**, 365–372.

**Desikan, R., Griffiths, R., Hancock, J. and Neill, S.** (2002) A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **99**, 16314–16318.

**DeSilva, D.L.R., Cox, R.C., Hetherington, A.M. and Mansfield, T.A.** (1985) Synergism between calcium ions and abscisic acid in preventing stomatal opening. *New Phytol.* **101**, 555–563.

**Dodd, A.N., Gardner, M.J., Hotta, C.T., *et al.*** (2007) The Arabidopsis circadian clock incorporates a cADPR-based feedback loop. *Science*, **318**, 1789–1792.

**Dodd, A.N., Jakobsen, M.K., Baker, A.J., Telzerow, A., Hou, S.W., Laplaze, L., Barrot, L., Poethig, R.S., Haseloff, J. and Webb, A.A.** (2006) Time of day modulates low-temperature Ca signals in Arabidopsis. *Plant J.* **48**, 962–973.

**Evans, N.H., McAinsh, M.R., Hetherington, A.M. and Knight, M.R.** (2005) ROS perception in *Arabidopsis thaliana*: the ozone-induced calcium response. *Plant J.* **41**, 615–626.

**Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L. and Blatt, M.** (2003) Nitric oxide regulates $K^+$ and $Cl^-$ channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proc. Natl Acad. Sci. USA*, **100**, 11116–11121.

**Gilmour, S.J. and Thomashow, M.F.** (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. *Plant Mol. Biol.* **17**, 1233–1240.

**Gilroy, S., Fricker, M.D., Read, N.D. and Trewavas, A.J.** (1991) Role of calcium in signal transduction of *Commelina* guard cells. *Plant Cell*, **3**, 333–344.

**Gilroy, S., Read, N.D. and Trewavas, A.J.** (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. *Nature*, **346**, 769–771.

**Gobert, A., Isayenkov, S., Voelker, C., Czempinski, K. and Maathuis, F.J.** (2007) The two-pore channel TPK1 gene encodes the vacuolar $K^+$ conductance and plays a role in $K^+$ homeostasis. *Proc. Natl Acad. Sci. USA*, **104**, 10726–10731.

**Gosti, F., Bertauche, N., Vartanian, N. and Giraudat, J.** (1995) Absicsic acid-dependent and -independent regulation of gene expression by progressive drought in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **246**, 10–18.

**Grabov, A. and Blatt, M.R.** (1998) Membrane voltage initiates $Ca^{2+}$ waves and potentiates $Ca^{2+}$ oscillations with abscisic acid in stomatal guard cells. *Proc. Natl Acad. Sci. USA*, **95**, 4778–4783.

**Grabov, A. and Blatt, M.R.** (1999) A steep dependence of inward-rectifying potassium channels on cytosolic free calcium concentration increase evoked by hyperpolarization in guard cells. *Plant Physiol.* **119**, 277–288.

**Gradmann, D., Blatt, M.R. and Thiel, G.** (1993) Electrocoupling of ion transporters in plants. *J. Membr. Biol.* **136**, 327–332.

**Hamilton, D.W.A., Hills, A., Köhler, B. and Blatt, M.R.** (2000) $Ca^{2+}$ channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proc. Natl Acad. Sci. USA*, **97**, 4967–4972.

**Harada, A. and Shimazaki, K.** (2009) Measurement of changes in cytosolic $Ca^{2+}$ in Arabidopsis guard cells and mesophyll cells in response to blue light. *Plant Cell Physiol.* **50**, 360–373.

**Hedrich, R., Busch, H. and Raschke, K.** (1990) $Ca^{2+}$ and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. *EMBO J.* **9**, 3889–3892.

**Hedrich, R. and Neher, E.** (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature*, **329**, 833–836.

**Himmelbach, A., Iten, M. and Grill, E.** (1998) Signalling of abscisic acid to regulate plant growth. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353**, 1439–1444.

**Hugouvieux, V., Kwak, J.M. and Schroeder, J.I.** (2001) An mRNA cap binding protein ABH1 modulates early abscisic acid signal transduction in Arabidopsis. *Cell*, **106**, 477–487.

**Ichida, A.M., Pei, Z.-M., Baizabal-Aguirre, V., Turner, K.J. and Schroeder, J.I.** (1997) Expression of a $Cs^+$ resistant guard cell $K^+$ channel confers $Cs^+$-resistant light-induced stomatal opening in transgenic Arabidopsis. *Plant Cell*, **9**, 1843–1857.

**Kelly, W.K., Esser, J.E. and Schroeder, J.I.** (1995) Effects of cytosolic calcium and limited, possible dual, effects of G protein modulators on guard cell inward potassium channels. *Plant J.* **8**, 479–489.

**Kinoshita, T., Nishimura, M. and Shimazaki, K.I.** (1995) Cytosolic concentration of $Ca^{2+}$ regulates the plasma membrane $H^+$-ATPase in guard cells of fava bean. *Plant Cell*, **7**, 1333–1342.

**Klüsener, B., Young, J., Murata, Y., Allen, G., Mori, I., Hugouvieux, V. and Schroeder, J.I.** (2002) Convergence of calcium signaling pathways of pathogenic elicitors and ABA in Arabidopsis guard cells. *Plant Physiol.* **130**, 2152–2163.

**Knight, H., Trewavas, A.J. and Knight, M.R.** (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* **12**, 1067–1078.

**Koornneef, M., Reuling, G. and Karssen, C.M.** (1984) The isolation of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **61**, 377–383.

**Kuchitsu, K., Ward, J.M., Allen, G.J., Schelle, I. and Schroeder, J.I.** (2002) Loading acetoxymethyl ester fluorescent dyes into the cytoplasm of Arabidopsis and *Commelina* guard cells. *New Phytol.* **153**, 527–533.

**Kwak, J.M., Mori, I.C., Pei, Z.-M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R., Bodde, S., Jones, J.D.G. and Schroeder, J.I.** (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J.* **22**, 2623–2633.

**Leckie, C.P., McAinsh, M.R., Allen, G.J., Sanders, D. and Hetherington, A.M.** (1998) Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. *Proc. Natl Acad. Sci. USA*, **95**, 15837–15842.

**Lemtiri-Chlieh, F., MacRobbie, E.A., Webb, A.A., Manison, N.F., Brownlee, C., Skepper, J.N., Chen, J., Prestwich, G.D. and Brearley, C.A.** (2003) Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proc. Natl Acad. Sci. USA*, **100**, 10091–10095.

**Lemtiri-Chlieh, F. and MacRobbie, E.A.C.** (1994) Role of calcium in the modulation of Vicia guard cell potassium channels by abscisic acid – a patch-clamp study. *J. Membr. Biol.* **137**, 99–107.

**Levchenko, V., Konrad, K.R., Dietrich, P., Roelfsema, M.R. and Hedrich, R.** (2005) Cytosolic abscisic acid activates guard cell anion channels without preceding $Ca^{2+}$ signals. *Proc. Natl Acad. Sci. USA*, **102**, 4203–4208.

**Li, S., Assmann, S.M. and Albert, R.** (2006) Predicting essential components of signal transduction networks: a dynamic model of guard cell abscisic acid signaling. *PLoS Biol.* **4**, e312.

**Li, Y., Wang, G.-X., Xin, M., Yang, H.-M., Wu, X.-J. and Li, T.** (2004) The parameters of guard cell calcium oscillation encode inhibition of stomatal opening in *Vicia faba*. *Plant Sci.* **166**, 415–421.

**MacRobbie, E.A.C.** (2000) ABA activates multiple $Ca^{2+}$ fluxes in stomatal guard cells, triggering vacuolar $K^+$ ($Rb^+$) release. *Proc. Natl Acad. Sci. USA*, **97**, 12361–12368.

**Marten, H., Konrad, K.R., Dietrich, P., Roelfsema, M.R. and Hedrich, R.** (2007) $Ca^{2+}$-dependent and -independent abscisic acid activation of plasma membrane anion channels in guard cells of *Nicotiana tabacum*. *Plant Physiol.* **143**, 28–37.

**McAinsh, M.R., Brownlee, C. and Hetherington, A.M.** (1990) Abscisic acid-induced elevation of guard cell cytosolic $Ca^{2+}$ precedes stomatal closure. *Nature*, **343**, 186–188.

 

**McAinsh, M.R., Brownlee, C. and Hetherington, A.M.** (1992) Visualizing changes in cytosolic-free $Ca^{2+}$ during the response of stomatal guard cells to abscisic acid. *Plant Cell*, **4**, 1113–1122.

**McAinsh, M.R., Clayton, H., Mansfield, T.A. and Hetherington, A.M.** (1996) Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiol.* **111**, 1031–1042.

**McAinsh, M.R., Webb, A.R., Taylor, J.E. and Hetherington, A.M.** (1995) Stimulus-induced oscillations in guard cell cytosolic free calcium. *Plant Cell*, **7**, 1207–1219.

**Mishra, G., Zhang, W., Deng, F., Zhao, J. and Wang, X.** (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. *Science*, **312**, 264–266.

**Miyawaki, A., Griesbeck, O., Heim, R. and Tsien, R.Y.** (1999) Dynamic and quantitative $Ca^{2+}$ measurements using improved cameleons. *Proc. Natl Acad. Sci. USA*, **96**, 2135–2140.

**Miyawaki, A., Llopis, J., Heim, R., McCaffery, J.M., Adams, J.M., Ikura, J.A. and Tsien, M.** (1997) Fluorescent indicators for $Ca^{2+}$ based on green fluorescent proteins and calmodulin. *Nature*, **388**, 882–887.

**Moes, D., Himmelbach, A., Korte, A., Haberer, G. and Grill, E.** (2008) Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards ABA responses in Arabidopsis. *Plant J.* **54**, 806–819.

**Mori, I.C., Murata, Y., Yang, Y., *et al.*** (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and $Ca(^{2+})$-permeable channels and stomatal closure. *PLoS Biol.* **4**, 10.

**Murata, Y., Pei, Z.-M., Mori, I.C. and Schroeder, J.I.** (2001) Abscisic acid activation of plasma membrane $Ca^{2+}$ channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *Plant Cell*, **13**, 2513–2523.

**Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J.** (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell*, **14**, 3089–3099.

**Nagai, T., Yamada, S., Tominaga, T., Ichikawa, M. and Miyawaki, A.** (2004) Expanded dynamic range of fluorescent indicators for $Ca(^{2+})$ by circularly permuted yellow fluorescent proteins. *Proc. Natl Acad. Sci. USA*, **101**, 10554–10559.

**Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M. and Iba, K.** (2008) $CO_2$ regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature*, **452**, 483–486.

**Ng, C., Carr, K., McAinsh, M., Powell, B. and Hetherington, A.** (2001) Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. *Nature*, **410**, 596–599.

**Pei, Z.-M., Kuchitsu, K., Ward, J.M., Schwarz, M. and Schroeder, J.I.** (1997) Differential abscisic acid regulation of guard cell slow anion channels in Arabidopsis wild-type and *abi1* and *abi2* mutants. *Plant Cell*, **9**, 409–423.

**Pei, Z.-M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E. and Schroeder, J.I.** (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, **406**, 731–734.

**Robert, N., Merlot, S., N'Guyen, V., Boisson-Dernier, A. and Schroeder, J.I.** (2006) A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in Arabidopsis. *FEBS Lett.* **580**, 4691–4696.

**Romano, L.A., Jacob, T., Gilroy, S. and Assmann, S.M.** (2000) Increases in cytosolic $Ca^{2+}$ are not required for abscisic acid-inhibition of inward $K^+$ currents in guard cells of *Vicia faba* L. *Planta*, **211**, 209–217.

**Schroeder, J.I. and Hagiwara, S.** (1989) Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells. *Nature*, **338**, 427–430.

**Schroeder, J.I. and Hagiwara, S.** (1990) Repetitive increases in cytosolic $Ca^{2+}$ of guard cells by abscisic acid activation of non-selective $Ca^{2+}$-permeable channels. *Proc. Natl Acad. Sci. USA*, **87**, 9305–9309.

**Schroeder, J.I., Raschke, K. and Neher, E.** (1987) Voltage dependence of $K^+$ channels in guard cell protoplasts. *Proc. Natl Acad. Sci. USA*, **84**, 4108–4112.

**Schwartz, A.** (1985) Role of calcium and EGTA on stomatal movements in *Commelina communis*. *Plant Physiol.* **79**, 1003–1005.

**Shimazaki, K., Kinoshita, T. and Nishimura, M.** (1992) Involvement of calmodulin and calmodulin-dependent myosin light chain kinase in blue light-dependent $H^+$ pumping in guard cell protoplasts from *Vicia faba* L. *Plant Physiol.* **99**, 1416–1421.

**Soderman, E., Mattsson, J. and Engstrom, P.** (1996) The Arabidopsis homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. *Plant J.* **10**, 375–381.

**Staxen, I., Pical, C., Montgomery, L.T., Gray, J.E., Hetherington, A.M. and McAinsh, M.R.** (1999) Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proc. Natl Acad. Sci. USA*, **96**, 1779–1784.

**Strizhov, N., Abraham, E., Okresz, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, C. and Szabados, L.** (1997) Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. *Plant J.* **12**, 557–569.

**Thiel, G., MacRobbie, E.A.C. and Blatt, M.R.** (1992) Membrane transport in stomatal guard cells: the importance of voltage control. *J. Membr. Biol.* **126**, 1–18.

**Vahisalu, T., Kollist, H., Wang, Y.F., *et al.*** (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature*, **452**, 487–491.

**Vartanian, N., Marcotte, L. and Giraudat, J.** (1994) Drought rhizogenesis in *Arabidopsis thaliana* – differential responses of hormonal mutants. *Plant Physiol.* **104**, 761–767.

**Wang, X.Q., Ullah, H., Jones, A.M. and Assmann, S.M.** (2001) G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. *Science*, **292**, 2070–2072.

**Ward, J.M., Pei, Z.-M. and Schroeder, J.I.** (1995) Roles of ion channels in initiation of signal transduction in higher plants. *Plant Cell*, **7**, 833–844.

**Ward, J.M. and Schroeder, J.I.** (1994) Calcium-activated $K^+$ channels and calcium-induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell*, **6**, 669–683.

**Webb, A.A., Larman, M.G., Montgomery, L.T., Taylor, J.E. and Hetherington, A.M.** (2001) The role of calcium in ABA-induced gene expression and stomatal movements. *Plant J.* **26**, 351–362.

**Webb, A.A.R., McAinsh, M.R., Mansfield, T.A. and Hetherington, A.M.** (1996) Carbon dioxide induces increases in guard cell cytosolic free calcium. *Plant J.* **9**, 297–304.

**Wolf, T., Heidelmann, T. and Marten, I.** (2006) ABA regulation of $K(^+)$-permeable channels in maize subsidiary cells. *Plant Cell Physiol.* **47**, 1372–1380.

**Wu, Y., Kuzma, J., Marechal, E., Graeff, R., Lee, H.C., Foster, R. and Chua, N.-H.** (1997) Abscisic acid signaling through cyclic ADP-ribose in plants. *Science*, **278**, 2126–2130.

**Wu, Y., Sanchez, J.P., Lopez-Molina, L., Himmelbach, A., Grill, E. and Chua, N.H.** (2003) The *abi1-1* mutation blocks ABA signaling downstream of cADPR action. *Plant J.* **34**, 307–315.

**Yang, H., Zhang, X., Wang, G., Li, Y. and Wei, X.** (2003) Cytosolic calcium oscillations may induce stomatal oscillation in *Vicia faba*. *Plant Sci.* **165**, 1117–1122.

**Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S. and Schroeder, J.I.** (2008) Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods*, **4**, 6.

**Yoshida, R., Hobo, T., Ichimura, K., Mizoguchi, T., Takahashi, F., Aronso, J., Ecker, J.R. and Shinozaki, K.** (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. *Plant Cell Physiol.* **43**, 1473–1483.

**Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F. and Shinozaki, K.** (2006) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *J. Biol. Chem.* **281**, 5310–5318.

**Young, J.J., Mehta, S., Israelsson, M., Godoski, J., Grill, E. and Schroeder, J.I.** (2006) $CO_2$ signaling in guard cells: calcium sensitivity response modulation, a $Ca(^{2+})$-independent phase, and $CO_2$ insensitivity of the *gca2* mutant. *Proc. Natl Acad. Sci. USA*, **103**, 7506–7511.

**Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W. and Song, C.-P.** (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol.* **126**, 1438–1448.

**Zhu, S.Y., Yu, X.C., Wang, X.J., *et al.*** (2007) Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. *Plant Cell*, **19**, 3019–3036.

 