Arabidopsis abi1-1 and abi2-1 Phosphatase Mutations Reduce Abscisic Acid-Induced Cytoplasmic Calcium Rises in Guard Cells

Gethyn J. Allen,1 Kazuyuki Kuchitsu,2 Sarah P. Chu, Yoshiyuki Murata, and Julian I. Schroeder

Department of Biology and Center for Molecular Genetics, University of California–San Diego, La Jolla, California 92093-0116

INTRODUCTION

Many physiological stimuli in plant cells induce elevations in cytoplasmic calcium ([Ca\textsuperscript{2+}]\textsubscript{cyt}) which is an essential second messenger in plant signal transduction cascades (Bush, 1995; Trewavas and Malhó, 1997). However, as far as only one report has directly demonstrated a mutation that impairs stimulus-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations (Bush, 1996). The hormone abscisic acid (ABA) controls a wide variety of stress responses and developmental processes in plants (Leung and Giraudat, 1998), and [Ca\textsuperscript{2+}]\textsubscript{cyt} has been proposed to function as a second messenger in several ABA responses (McAinsh et al., 1990; Sheen, 1996, 1998; Wu et al., 1997). ABA is produced in response to drought stress and mediates a reduction in stomatal aperture that prevents excessive evaporation-mediated water loss. Stomatal closure is elicited via a reduction in the Cl\textsuperscript{-}, K\textsuperscript{+}, and organic solute content in the two guard cells that border the stomatal pore (Assmann, 1993; MacRobbie, 1997; Müller-Röber et al., 1998). In guard cells, ABA induces an increase in [Ca\textsuperscript{2+}]\textsubscript{cyt}, which precedes the reduction in stomatal aperture (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992; Allan et al., 1994; Grabov and Blatt, 1998). The [Ca\textsuperscript{2+}]\textsubscript{cyt} rise initiates the processes required for guard cell turgor loss through the modulation of ion channels and pumps in both the plasma and vacuolar membranes (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Fairley-Grenot and Assmann, 1991; Luan et al., 1993; Lemtiri-Chlieh and MacRobbie, 1994; Ward and Schroeder, 1994; Kinoshita et al., 1995; Allen and Sanders, 1996).

Studies have indicated that outwardly rectifying K\textsuperscript{+} currents and slow (S-type) anion currents play an important role in the process of ion efflux that drives stomatal closure (reviewed in Assmann, 1993; MacRobbie, 1997; Müller-Röber et al., 1998). Ca\textsuperscript{2+} activation of S-type anion currents results in anion efflux and depolarization of the guard cell plasma membrane (Schroeder and Hagiwara, 1989). ABA activates S-type anion currents in guard cells of Arabidopsis (Pei et al., 1997), tobacco (Grabov et al., 1997), and fava bean (Schwarz and Schroeder, 1998). However, ABA activation of anion channels is impaired in guard cells of the Arabidopsis ABA-insensitive mutants abi1 and abi2 (Pei et al., 1997, 1998). Because the stomata of abi1 and abi2 do not close in

1To whom correspondence should be addressed. E-mail gallen@biomal.ucsd.edu; fax 858-534-7108.

2Current address: Department of Biotechnology, National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba 305-8602, Japan.

Elevations in cytoplasmic calcium ([Ca\textsuperscript{2+}]\textsubscript{cyt}) are an important component of early abscisic acid (ABA) signal transduction. To determine whether defined mutations in ABA signal transduction affect [Ca\textsuperscript{2+}]\textsubscript{cyt} signaling, the Ca\textsuperscript{2+}-sensitive fluorescent dye fura 2 was loaded into the cytoplasm of Arabidopsis guard cells. Oscillations in [Ca\textsuperscript{2+}]\textsubscript{cyt} could be induced when the external calcium concentration was increased, showing viable Ca\textsuperscript{2+} homeostasis in these dye-loaded cells. ABA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations in wild-type stomata were either transient or sustained, with a mean increase of ~300 nM. Interestingly, ABA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} increases were significantly reduced but not abolished in guard cells of the ABA-insensitive protein phosphatase mutants abi1 and abi2. Plasma membrane slow anion currents were activated in wild-type, abi1, and abi2 guard cell protoplasts by increasing [Ca\textsuperscript{2+}]\textsubscript{cyt}, demonstrating that the impairment in ABA activation of anion currents in the abi1 and abi2 mutants was bypassed by increasing [Ca\textsuperscript{2+}]\textsubscript{cyt}. Furthermore, increases in external calcium alone (which elevate [Ca\textsuperscript{2+}]\textsubscript{cyt}) resulted in stomatal closing to the same extent in the abi1 and abi2 mutants as in the wild type. Conversely, stomatal opening assays indicated different interactions of abi1 and abi2, with Ca\textsuperscript{2+}-dependent signal transduction pathways controlling stomatal closing versus stomatal opening. Together, [Ca\textsuperscript{2+}]\textsubscript{cyt} recordings, anion current activation, and stomatal closing assays demonstrate that the abi1 and abi2 mutations impair early ABA signaling events in guard cells upstream or close to ABA-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations. These results further demonstrate that the mutations can be bypassed during anion channel activation and stomatal closing by experimental elevation of [Ca\textsuperscript{2+}]\textsubscript{cyt}.
response to exogenous ABA or drought stress (Roelfsema and Prins, 1995; Pei et al., 1997), these findings emphasize
that activation of S-type anion currents is one of the essential early events in the ABA signal transduction cascade in
guard cells.

The abi1 and abi2 loci encode (semi)dominant mutations in
two distinct type 2C protein phosphatases (Leung et al.,
1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998). The
mutations have been proposed to inhibit ABA signal transduc-
tion at the biochemical level as dominant negative mutations,
binding to a putative substrate and preventing activity of the
wild-type protein (Merlot and Giraudat, 1997; Sheen, 1998).

S-type anion currents are modulated by phosphorylation
events in fava bean, Arabidopsis, and tobacco (Schmidt et
al., 1995; Grabov et al., 1997; Pei et al., 1997; Schwarz and
Schroeder, 1998). The above-mentioned studies show that
[Ca\(^{2+}\)]\(_{cyt}\) elevations, S-type anion currents, and phosphory-
elation events are integral to ABA signaling in guard cells, al-
though the exact sequence of events has not been fully
resolved.

Biophysical and functional analyses of genetic signal
transduction mutants provide quantitative approaches for
characterizing mechanisms and their relative locations in
early signaling cascades. To determine whether the ABA-
sensitive mutants abi1 and abi2 affect early [Ca\(^{2+}\)]\(_{cyt}\) rises
and to analyze events downstream of [Ca\(^{2+}\)]\(_{cyt}\), we adapted
techniques to monitor [Ca\(^{2+}\)]\(_{cyt}\) levels in Arabidopsis guard
cells by using ratiometric Ca\(^{2+}\)-sensitive fluorescent dyes.
Due to their small size, microinjection of Arabidopsis guard
cells has proven technically difficult. Therefore, we adapted
techniques developed to noninvasively load these dyes into
plant cells (Bush and Jones, 1987; Darjania et al., 1993) to
load intact guard cell pairs. In addition, [Ca\(^{2+}\)]\(_{cyt}\) depend-
ence of guard cell S-type anion currents and of stomatal
movements in Arabidopsis wild type and abi1 and abi2 mu-
tants were investigated to study signaling events down-
stream of [Ca\(^{2+}\)]\(_{cyt}\). Quantitative analyses show that the abi1
and abi2 mutations reduce ABA-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations
that can mediate stomatal closure in Arabidopsis. In addi-
tion, S-type anion currents in Arabidopsis guard cells and
stomatal closing can be induced by elevation of [Ca\(^{2+}\)]\(_{cyt}\),
and Ca\(^{2+}\) activation is intact in the abi1 and abi2 mutants.
These data suggest that in guard cells, the abi1 and abi2
mutations disrupt early signal transduction events upstream
or close to ABA-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations, whereas sig-
ning components downstream of [Ca\(^{2+}\)]\(_{cyt}\) remain intact.

RESULTS

Acid-Loaded Ca\(^{2+}\)-Sensitive Fluorescent Dyes Localize
in the Cytoplasm of Arabidopsis Guard Cells

Ratiometric Ca\(^{2+}\)-sensitive fluorescent dyes were loaded
into the cytoplasm of Arabidopsis guard cells by incubation
of epidermal fragments in buffers containing micromolar
concentrations of the dyes (fura 2 or indo 1) at acidic pH.

Ca\(^{2+}\)-independent fluorescence imaging indicated that both
of these dyes were loaded predominantly into the cytoplasm
with some nuclear localization but were excluded from the
vacuole and chloroplasts (Figures 1A to 1C). In agreement
with the confocal images, a low resting Ca\(^{2+}\) level in unstimu-
lated cells indicated a cytoplasmic location of the dye. Guard
cells of the abi1 and abi2 mutants also loaded in the
same way (Figure 1B). Autofluorescence from unloaded
guard cells was <1% of loaded cells (Figure 1D, enhanced
image). To quantify dye loss from dye-loaded cells, the fluo-
rescence signal at the two excitation wavelengths of fura 2
(f\(_{340}\) and f\(_{380}\)) and the ratio were measured for brief (30 to 50
sec) periods at defined intervals over a 25-min period. This
allows separation of dye loss effects from dye bleaching.

Figure 1G shows that [Ca\(^{2+}\)]\(_{cyt}\) elevations, whereas sig-
nal recordings under these conditions

Fura 2-Loaded Guard Cells Allow [Ca\(^{2+}\)]\(_{cyt}\) Analyses

Fura 2-loaded guard cells maintained low [Ca\(^{2+}\)]\(_{cyt}\). The
mean resting [Ca\(^{2+}\)]\(_{cyt}\) in the wild-type stomata was 188 ±
20 nM (n = 178) and was not significantly different in the
abi1 and abi2 mutants (see below). To test the viability of
Ca\(^{2+}\) homeostatic mechanisms in guard cells loaded using
this technique, we challenged fura 2-loaded guard cell pairs
with increases in external Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{ext}\)) from 50 μM to ei-
ther 750 μM or 10 mM. An increase in [Ca\(^{2+}\)]\(_{ext}\) previously
has been demonstrated to cause oscillations in [Ca\(^{2+}\)]\(_{cyt}\) in
Commelina communis guard cells (McAinsh et al., 1995).

Ratiometric [Ca\(^{2+}\)]\(_{cyt}\) recordings under these conditions
are shown in Figure 2. In addition to these cells, controls
were also performed in which [Ca\(^{2+}\)]\(_{ext}\) was not changed. In
none of these control cells were changes in [Ca\(^{2+}\)]\(_{cyt}\) ob-
served (n = 27; data not shown). In 40% of stomata (n = 18
of 45) subjected to an increase in [Ca\(^{2+}\)]\(_{ext}\), there was no
resolvable effect on the resting [Ca\(^{2+}\)]\(_{cyt}\) (Figure 2A). How-
ever, in the remaining 60% of stomata (n = 27 of 45), in-
creases in [Ca\(^{2+}\)]\(_{ext}\) caused oscillations (n = 21) or increases
(n = 6) in [Ca\(^{2+}\)]\(_{cyt}\). When [Ca\(^{2+}\)]\(_{ext}\) was increased from 50 to
750 μM [Ca\(^{2+}\)]\(_{cyt}\) oscillations were induced with a small am-
plitude of <60 nM and a peak-to-peak period of 5.8 ± 0.1
min (n = 8) (Figure 2B). If [Ca\(^{2+}\)]\(_{ext}\) was increased from 50
μM to 10 mM, oscillations in [Ca\(^{2+}\)]\(_{cyt}\) could be induced that
had an amplitude >120 nM and a peak-to-peak period of
6.7 ± 0.2 min (n = 13) (Figure 2C). These [Ca^{2+}]_{cyt} oscillations in Arabidopsis guard cells are comparable to the oscillations in Commelina communis guard cells in that the greater the increase in [Ca^{2+}]_{ext}, the larger their magnitude and the slower their period (McAinsh et al., 1995). These data show that fura 2–loaded Arabidopsis guard cells are able to maintain [Ca^{2+}]_{cyt} homeostasis and faithfully report changes in [Ca^{2+}]_{cyt}, indicating that cells loaded by this technique are viable.

**ABA Induces [Ca^{2+}]_{cyt} Increases in Arabidopsis Guard Cells**

To determine whether ABA can elicit increases in [Ca^{2+}]_{cyt} in Arabidopsis guard cells, as demonstrated for guard cells of other species (reviewed in McAinsh et al., 1997), wild-type guard cells were loaded with fura 2 in 50 mM KCl and 50 μM [Ca^{2+}]_{ext}. These cells were then challenged by the addition of 10 μM ABA to the external medium. Ratiometric [Ca^{2+}]_{cyt} measurements showed distinguishable types (classes) of responses to this ABA stimulus (Figure 3). In 47% of wild-type stomata tested (n = 38 of 80), no increase in [Ca^{2+}]_{cyt} was resolved when ABA was applied, despite a low resting level of [Ca^{2+}]_{cyt} (Figure 3A). In 53% of the wild-type stomata (n = 42 of 80), ABA induced increases in [Ca^{2+}]_{cyt} (Figures 3B to 3D). In 38% of the stomata tested (n = 30), ABA application led to a transient increase in [Ca^{2+}]_{cyt} levels (Figure 3B), whereas in the remaining 15% (n = 12) of stomata, application of ABA caused an increase in [Ca^{2+}]_{cyt} with different kinetic properties. In some stomata, increases in [Ca^{2+}]_{cyt} were

---

**Figure 1.** Calcium-Independent Fluorescence from Arabidopsis Guard Cells Acid-Loaded with Ca^{2+}-Sensitive Fluorescent Dyes.

(A) Confocal fluorescence image from wild-type stomata loaded with fura 2. Ca^{2+}-independent excitation wavelength is 351 nm. Bar = 20 μm.

(B) Fluorescence from an abi1 guard cell pair loaded with fura 2. Excitation wavelength is 351 nm. Bar = 12 μm.

(C) Confocal fluorescence image from a wild-type guard cell pair loaded with indo 1. Bar = 10 μm.

(D) Autofluorescence from an unloaded wild-type guard cell pair under the same conditions as in (A) to (C) but with the photon multiplier tube voltage increased from 498 to 600 V to amplify background fluorescence. Bar = 12 μm.

(E) Confocal microscope image of wild-type Arabidopsis guard cell pair in an epidermal fragment mounted on an electron microscope grid to prevent movement during photometric measurements. Bar = 20 μm.

(F) Increased magnification of the cells in (E) to show the intact organelle structure.

(G) Change in fluorescence at 380 nm (f380), and 340 nm (f340) excitation wavelengths and their ratio in dye-loaded cells over the time course of a typical experiment. Error bars indicate standard error of the mean.

All cells in (A) to (G) were loaded in 50 mM KCl, 50 μM CaCl₂, and 10 mM Mes-KOH, pH 4.5.
relatively rapid and resulted in a sustained level of \([\text{Ca}^{2+}]_{\text{cyt}}\) over the time of the measurement (Figure 3C), whereas other stomata showed gradual increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) after ABA application (Figure 3D).

The ABA induction of \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations in only a proportion of the stomata (53%) and the variability in the nature of \([\text{Ca}^{2+}]_{\text{cyt}}\) rise are similar to reports of ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in guard cells of other species under similar conditions (Schroeder and Hagiwara, 1990; Gilroy et al., 1991; McAinsh et al., 1992; Allan et al., 1994). The reason for this variability is not known, although limited local resolution or the presence of parallel, calcium-independent, pathways has been suggested (Allan et al., 1994). Recently, plasma membrane hyperpolarization has been demonstrated to have an important influence on ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases (Grabov and Blatt, 1998), suggesting that nonresponsive cells may be cells that do not maintain an adequately hyperpolarized membrane potential. Using Arabidopsis guard cells, we found that lowering external KCl levels did enhance \([\text{Ca}^{2+}]_{\text{ext}}\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) rises in some cells, as reported for Commelina communis (Gilroy et al., 1991) and fava bean (Grabov and Blatt, 1998) guard cells (n = 10; data not shown).

**Figure 2.** Ratiometric Microphotometry Measurements of Cytoplasmic \([\text{Ca}^{2+}]\) Changes in Fura 2-Loaded Wild-Type Guard Cell Pairs Challenged with Increases in Extracellular \([\text{Ca}^{2+}]\):

(A) The addition of 10 mM CaCl\(_2\) to the external medium elicits no increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) (n = 18).

(B) The addition of 750 \(\mu\)M CaCl\(_2\) to the external medium elicits oscillations in \([\text{Ca}^{2+}]_{\text{cyt}}\) with amplitudes <60 nM (n = 8).

(C) The addition of 10 mM CaCl\(_2\) to the external medium elicits oscillations in \([\text{Ca}^{2+}]_{\text{cyt}}\) with amplitudes >120 nM (n = 13). All cells in (A) to (C) were loaded in 50 mM KCl, 50 \(\mu\)M CaCl\(_2\), and 10 mM Mes-KOH, pH 4.5. Measurements were made at pH 5.6.

**ab1 and ab2 Mutations Reduce ABA-Induced \([\text{Ca}^{2+}]_{\text{cyt}}\) Increases in Guard Cells**

The ABA-insensitive mutants ab1 and ab2 (Koornneef et al., 1984) were investigated to evaluate further the role of \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in ABA signal transduction and to analyze at which level in the signaling cascade these mutations impinge upon ABA signaling. Guard cells from these mutants were loaded with fura 2 (Figure 1B). The addition of 10 \(\mu\)M ABA to the external medium elicits no transient small transient increases in \([\text{Ca}^{2+}]_{\text{cyt}}\) in 40% (n = 32 of 80) of ab1 and 34% (n = 17 of 50) of ab2 guard cell pairs tested (Figures 4A and 4B), with the remaining cells showing no \([\text{Ca}^{2+}]_{\text{cyt}}\) change. In neither of the two mutants were sustained or rising \([\text{Ca}^{2+}]_{\text{cyt}}\) increases measured, as were observed in the wild-type guard cells (Figures 3C and 3D).

Quantitative comparison of \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in all stomata that responded to ABA showed that the ab1 and ab2 mutations dramatically reduced ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation (P < 0.001). Figure 4C shows the average maximal ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in responsive guard cells of the wild type and the ab1 and ab2 mutants. The mean \([\text{Ca}^{2+}]_{\text{cyt}}\) increase in the wild type was 278 ± 40 nM (n = 42), whereas this increase was reduced to 97 ± 16 nM (n = 32) in ab1 and 88 ± 20 nM (n = 17) in ab2. The mean timing of the maximal \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in all responsive cells was not significantly different at 16.1 ± 5.4 min in the wild type and 15.5 ± 3.9 min and 12.8 ± 4.5 min in ab1 and ab2, respectively (P > 0.5 for the wild type versus ab1 or ab2). In further analyses, the average ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase from all responsive cells over the time of the recordings was determined by integrating all responses in the wild type and in the two mutants. This analysis yielded mean \([\text{Ca}^{2+}]_{\text{cyt}}\) increases of 3584 nM min\(^{-1}\) in the wild type and 583 and 536 nM min\(^{-1}\) in ab1 and ab2, respectively. Therefore, both the magnitude and (in some cells) the nature of the ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) change are significantly reduced although not entirely abolished in the ab1 and ab2 mutants. These data show that the ab1 and ab2 mutations reduce ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in guard cells.
Calcium Signaling in abi Guard Cells

Results in Figures 3 and 4 indicate that experimental elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ might bypass the abi1 and abi2 mutations and activate ABA signaling mechanisms if components downstream of Ca$^{2+}$ are not directly affected by these mutant protein phosphatases in guard cells. To test this possibility, Arabidopsis guard cell protoplasts were patch clamped to analyze S-type anion currents as a potential downstream, Ca$^{2+}$-dependent activity in the ABA signal transduction cascade, as demonstrated for fava bean guard cells (Schroeder and Hagiwara, 1989).

Patch clamping wild-type Arabidopsis guard cell protoplasts in the whole cell mode (with CsCl solutions to inhibit K$^+$ currents) resulted in small background currents in all cells when $[\text{Ca}^{2+}]_{\text{cyt}}$ was buffered to 100 nM (Figure 5A; $n = 10$). However, if $[\text{Ca}^{2+}]_{\text{cyt}}$ was buffered to 1 or 2 mM, S-type anion currents were activated (Figures 5B to 5E). These currents showed slow, voltage-dependent relaxation kinetics and steady state currents at negative potentials, all of which are characteristic of S-type anion currents (Figures 5B, 5C, and 5E). The steady state current–voltage relationship of these currents reversed at $-125 \pm 6$ mV ($n = 11$), which was close to the equilibrium potential for chloride of $+34$ mV, indicating a high permeability to anions (Figure 5C). Titration of free Ca$^{2+}$ in the pipette between 100 nM and 10 $\mu$M Ca$^{2+}$ yielded a half-maximal activation concentration ($K_{1/2}$) for steady state current activation of 1.2 $\mu$M at $-145$ mV with a steep Ca$^{2+}$ dependence at physiological Ca$^{2+}$ elevations from 0.1 to 1 $\mu$M (Figure 5D).

Activation of S-type anion currents by elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ to 2 $\mu$M (Figure 5E) was abolished in all cells when Mg-ATP was omitted from the pipette solution ($n = 10$) or when ATP was replaced by the nonhydrolyzable analog adenosine 5'-(\beta,\gamma-imino)triphosphate (App[NH]p) ($n = 9$; Figures 5F and 5G). These data indicate that Ca$^{2+}$-dependent activation of S-type anion currents requires hydrolyzable ATP and therefore may involve protein kinase activity (see Discussion). Kinase involvement was further supported by an inhibition of Ca$^{2+}$-dependent anion channel activation after a 2-hr preincubation of the protoplasts with the kinase inhibitors K-252a (2 $\mu$M) or staurosporine (50 $\mu$M) (Figures 5H and 5I).

ABA activation of S-type anion currents previously has been shown to be impaired in guard cells of abi1 and abi2 mutants (Pei et al., 1997, 1998). The absence of ABA-induced anion currents correlates closely with the insensitivity of stomatal closure in these mutants, even at high ABA concentrations of 10 to 50 $\mu$M (Roelfsema and Prins, 1995; Pei et al., 1997). These mutations have now been found to reduce ABA-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increases (Figure 4). To test whether $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation is sufficient to suppress the lack of anion channel activity in abi1 and abi2 mutants, guard cell protoplasts from these mutants were patch clamped at elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ levels. The data in Figure 6 clearly show that S-type

---

**Figure 3.** Ratiometric Microphotometry Measurements of Cytoplasmic Ca$^{2+}$ Changes in Fura 2-Loaded Wild-Type Guard Cell Pairs Challenged with 10 $\mu$M ABA.

(A) The addition of 10 $\mu$M ABA to the external medium elicits no increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ ($n = 38$).
(B) The addition of 10 $\mu$M ABA elicits a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ ($n = 30$).
(C) The addition of 10 $\mu$M ABA elicits a sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ ($n = 8$).
(D) The addition of 10 $\mu$M ABA elicits a gradual rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ ($n = 4$).
The removal of Mg-ATP from the cytoplasm (0.05), although the reason for this observation is unclear. In fact, the average magnitude of the currents was larger in cell pairs with characteristics similar to those in the wild type (Figures 6A to 6C).

The addition of 10 μM ABA to the external medium of ab1 guard cell pairs elicits a small transient increase in \([Ca^{2+}]_{\text{cyt}}\) (see text for details).

Figure 4. Ratiometric Microphotometry Measurements of Cytoplasmic Ca\(^{2+}\) Changes in Fura 2–Loaded ab1 and ab2 Guard Cell Pairs Challenged with 10 μM ABA.

(A) The addition of 10 μM ABA to the external medium of ab1 guard cell pairs elicits a small transient increase in \([Ca^{2+}]_{\text{cyt}}\) (n = 32 from 80 cell pairs), the remaining 48 cells showing no change in \([Ca^{2+}]_{\text{cyt}}\); see text for details).

(B) The addition of 10 μM ABA to the external medium of ab2 guard cell pairs elicits a small transient increase in \([Ca^{2+}]_{\text{cyt}}\) (n = 17 from 50 cell pairs).

(C) Quantification of ABA-induced \([Ca^{2+}]_{\text{cyt}}\) increases in responsive wild-type (WT), ab1, and ab2 guard cell pairs. ABA-induced changes were measured from all responding cells, and values were measured as the peak \([Ca^{2+}]_{\text{cyt}}\) averaged over 1 min minus the resting \([Ca^{2+}]\) (n = 42 for the wild type, 32 for ab1, and 17 for ab2). Bars represent standard error of the mean.

Cells in (A) and (B) were loaded in 50 mM KCl, 50 μM CaCl\(_2\), and 10 mM Mes-KOH, pH 4.5, as described in Methods. Measurements were made at pH 5.6.

ABA-induced stomatal closure is abolished in the ab1 and ab2 mutants (Figure 7A), as previously reported (Roelfsema and Prins, 1995; Pei et al., 1997). The physiological relevance of the reduction in ABA-induced \([Ca^{2+}]_{\text{cyt}}\) elevations in the ab1 and ab2 mutants was investigated by measuring stomatal closure initiated by increasing extracellular Ca\(^{2+}\) alone in the absence of exogenous ABA. Increasing the extracellular Ca\(^{2+}\) concentration leads to increases in \([Ca^{2+}]_{\text{cyt}}\) (Figure 2) and elicits Ca\(^{2+}\)-dependent signal transduction pathways that result in stomatal closure, as demonstrated in other species (De Silva et al., 1985; Schwartz, 1985; Schroeder and Hagiwara, 1989; McAlinsh et al., 1995). Interestingly, Figure 7B clearly demonstrates that when \([Ca^{2+}]_{\text{ext}}\) was increased from 0 to 0.05, 0.75, 2, 5, or 10 mM, a decrease in stomatal aperture was triggered in the wild type as well as the ab1 and ab2 mutants. The degree of closure was similar at all five extracellular calcium concentrations tested for ab1, ab2, and the wild type. These physiological data support the finding that the ab1 and ab2 mutations repress early ABA-induced \([Ca^{2+}]_{\text{cyt}}\) elevations and that these mutations can be bypassed by elevation of \([Ca^{2+}]_{\text{cyt}}\) at the levels of anion channel activation (Figure 6) and stomatal closing (Figure 7).

In addition to analyzing preopened stomata (Figures 7A and 7B), we analyzed Ca\(^{2+}\)-dependent inhibition of stomatal opening (Figure 7C). Studies have shown that stomatal opening may also involve elevations of \([Ca^{2+}]_{\text{cyt}}\) (Irving et al., 1992; Cousson and Vavasseur, 1998), indicating a complex Ca\(^{2+}\) response (see Discussion). When wild-type leaves were transferred from low KCl in the dark to high KCl in the light, stomatal opening was induced (Figure 7C, bars at center). Stomatal opening was inhibited by 5 mM \([Ca^{2+}]_{\text{ext}}\) in the wild type, opening being reduced to only 9% of that measured in the absence of Ca\(^{2+}\) (Figure 7C, left). In ab1 and ab2 plants, stomatal opening was inhibited to a much lesser extent by Ca\(^{2+}\). Stomatal opening in the presence of Ca\(^{2+}\) was 63 and 45% of that induced in the absence of Ca\(^{2+}\) in the ab1 and ab2 mutants, respectively. These data show that whereas preopened stomata could be closed by \([Ca^{2+}]_{\text{ext}}\) to the same extent in the wild type and ab1 and

anion currents were activated by increases in \([Ca^{2+}]_{\text{cyt}}\) in ab1 (n = 17 from 21) and ab2 (n = 11 from 11) with characteristics similar to those in the wild type (Figures 6A to 6C). In fact, the average magnitude of the currents was larger in the ab1 and ab2 mutants compared with the wild type (P < 0.05), although the reason for this observation is unclear. The removal of Mg-ATP from the cytoplasm (n = 7) or replacement of ATP with App(NH)p (n = 15) prevented anion channel activation in these mutants (Figures 6A and 6B, right), as was also found for the wild type (Figures 5F and 5G).

These data are consistent with the observation that early \([Ca^{2+}]_{\text{cyt}}\) signaling is disrupted in the ab1 and ab2 mutants (Figure 4), whereas Ca\(^{2+}\)-dependent signaling components downstream remain unaffected. Furthermore, whereas ABA activation of S-type anion currents is impaired in ab1 and ab2 (Pei et al., 1997, 1998), elevations of \([Ca^{2+}]_{\text{cyt}}\) are able to activate anion currents in ab1 and ab2 guard cells in the absence of ABA.

Elevating External Ca\(^{2+}\) Causes Stomatal Closure in the Wild Type and ab1 and ab2 Mutants

ABA-induced stomatal closure is abolished in the ab1 and ab2 mutants (Figure 7A), as previously reported (Roelfsema and Prins, 1995; Pei et al., 1997). The physiological relevance of the reduction in ABA-induced \([Ca^{2+}]_{\text{cyt}}\) elevations in the ab1 and ab2 mutants was investigated by measuring stomatal closure initiated by increasing extracellular Ca\(^{2+}\) alone in the absence of exogenous ABA. Increasing the extracellular Ca\(^{2+}\) concentration leads to increases in \([Ca^{2+}]_{\text{cyt}}\) (Figure 2) and elicits Ca\(^{2+}\)-dependent signal transduction pathways that result in stomatal closure, as demonstrated in other species (De Silva et al., 1985; Schwartz, 1985; Schroeder and Hagiwara, 1989; McAlinsh et al., 1995). Interestingly, Figure 7B clearly demonstrates that when \([Ca^{2+}]_{\text{ext}}\) was increased from 0 to 0.05, 0.75, 2, 5, or 10 mM, a decrease in stomatal aperture was triggered in the wild type as well as the ab1 and ab2 mutants. The degree of closure was similar at all five extracellular calcium concentrations tested for ab1, ab2, and the wild type. These physiological data support the finding that the ab1 and ab2 mutations repress early ABA-induced \([Ca^{2+}]_{\text{cyt}}\) elevations and that these mutations can be bypassed by elevation of \([Ca^{2+}]_{\text{cyt}}\) at the levels of anion channel activation (Figure 6) and stomatal closing (Figure 7).

In addition to analyzing preopened stomata (Figures 7A and 7B), we analyzed Ca\(^{2+}\)-dependent inhibition of stomatal opening (Figure 7C). Studies have shown that stomatal opening may also involve elevations of \([Ca^{2+}]_{\text{cyt}}\) (Irving et al., 1992; Cousson and Vavasseur, 1998), indicating a complex Ca\(^{2+}\) response (see Discussion). When wild-type leaves were transferred from low KCl in the dark to high KCl in the light, stomatal opening was induced (Figure 7C, bars at center). Stomatal opening was inhibited by 5 mM \([Ca^{2+}]_{\text{ext}}\) in the wild type, opening being reduced to only 9% of that measured in the absence of Ca\(^{2+}\) (Figure 7C, left). In ab1 and ab2 plants, stomatal opening was inhibited to a much lesser extent by Ca\(^{2+}\). Stomatal opening in the presence of Ca\(^{2+}\) was 63 and 45% of that induced in the absence of Ca\(^{2+}\) in the ab1 and ab2 mutants, respectively. These data show that whereas preopened stomata could be closed by \([Ca^{2+}]_{\text{ext}}\) to the same extent in the wild type and ab1 and
abi2 mutants (Figure 7B), Ca^{2+} inhibition of stomatal opening was partially affected by the abi1 and abi2 mutations.

**DISCUSSION**

**Arabidopsis abi1 and abi2 Mutations Reduce [Ca^{2+}]_{cyt} Elevations Involved in ABA Signal Transduction**

[Ca^{2+}]_{cyt} acts as a second messenger system in many signal transduction cascades in plant cells (Bush, 1995; McAinsh et al., 1997). However, few studies have shown disruption of signal-induced [Ca^{2+}]_{cyt} changes by specific mutations in plant cells (Bush, 1996). [Ca^{2+}]_{cyt} has been implicated as a second messenger in ABA responses in a variety of plant cells (McAinsh et al., 1990; Gilroy and Jones, 1992; Sheen, 1996, 1998; Wu et al., 1997). Using a technique to load Ca^{2+}-sensitive ratiometric fluorescent dyes into Arabidopsis guard cells, we were able to monitor quantitatively [Ca^{2+}]_{cyt} changes in response to ABA in large sample numbers to determine the effects of specific mutations on [Ca^{2+}]_{cyt} changes.

Similar effects of ABA on [Ca^{2+}]_{cyt} were observed in wild-type Arabidopsis guard cells, as previously reported for other species. In particular, the nature of the ABA-induced [Ca^{2+}]_{cyt} increase was variable, and in some cells no perceivable increase was observed (Schroeder and Hagiwara, 1990; Gilroy et al., 1991; McAinsh et al., 1992) (Figure 3). These observations have led to the hypotheses that Ca^{2+}-independent ABA signal transduction pathways also occur in guard cells, and their activity may depend on growth, development, or experimental conditions (Allan et al., 1994; Trewavas and Malhó, 1997), and/or that localized changes are difficult to resolve (McAinsh et al., 1992; discussed in MacRobbie, 1997).

The effect of physiological concentrations of ABA on [Ca^{2+}]_{cyt} was measured in the ABA-insensitive mutants abi1 and abi2. Although [Ca^{2+}]_{cyt} transients were induced by ABA in the guard cells of these mutants (Figure 4), quantitative...
the plasma membrane and release from internal stores (Schroeder and Hagiwara, 1990; Lemtiri-Chlieh and MacRobbie, 1994; Wu et al., 1997; Grabov and Blatt, 1998; Leckie et al., 1998). The greatly reduced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations in the \(\text{abi1}\) and \(\text{abi2}\) mutants indicate the possibility that specific components contributing to \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations might remain intact, whereas other mechanisms are impaired in these mutants. Further research is needed to determine which \(\text{Ca}^{2+}\) pools and channels are affected by the \(\text{abi1}\) and \(\text{abi2}\) mutations in guard cells. Only transient increases were observed in the \(\text{abi1}\) and \(\text{abi2}\) mutants; no large or sustained increases were apparent in a total of 130 mutant stomata tested. This demonstrates that in these mutant plants, ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) signaling is disrupted, although not entirely abolished, and that physiological ABA concentrations fail to induce large enough \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in the mutants to elicit stomatal closure (Koornneef et al., 1984; Roelfsema and Prins, 1995; Pei et al., 1997).

The fact that ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases were reduced in \(\text{abi1}\) and \(\text{abi2}\) mutants led us to analyze whether experimental elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) might bypass the points of action of these mutations in the guard cell ABA signaling cascade. This was studied using patch-clamp analysis of Arabidopsis guard cells (Pei et al., 1997, 1998) at elevated pipette (cytoplasmic) calcium concentrations. \(S\)-type anion currents were analyzed because it has been demonstrated previously that \(S\)-type anion channels are activated by increased \([\text{Ca}^{2+}]_{\text{cyt}}\) (Schroeder and Hagiwara, 1989) or by ABA (Grabov et al., 1997; Pei et al., 1997, 1998), and importantly, ABA activation is disrupted in guard cells of the \(\text{abi1}\) and \(\text{abi2}\) mutants (Pei et al., 1997, 1998).

Calcium activation of \(S\)-type currents in guard cells of \(\text{abi1}\) and \(\text{abi2}\) mutants was unaffected (Figure 6), suggesting that the \(\text{abi1}\) and \(\text{abi2}\) mutations impinge upon the signaling cascade upstream or close to ABA-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases in guard cells, and that activation of anion channels lies downstream of \([\text{Ca}^{2+}]_{\text{cyt}}\) increases. Interestingly, support for this hypothesis is lent by the finding that increasing \([\text{Ca}^{2+}]_{\text{cyt}}\) (and thereby increasing \([\text{Ca}^{2+}]_{\text{cyt}}\); Figure 2) leads to stomatal closure in both the wild type and the \(\text{abi1}\) and \(\text{abi2}\) mutants, indicating that activation of processes downstream of \([\text{Ca}^{2+}]_{\text{cyt}}\) still results in stomatal closure in the \(\text{abi1}\) and \(\text{abi2}\) mutants.

Differences in Ca\(^{2+}\) Signaling during Stomatal Opening and Stomatal Closing

Bypassing of \(\text{abi}\) mutants by \(\text{Ca}^{2+}\) initially appears to conflict with a recent study that suggested that the \(\text{abi1}\) and \(\text{abi2}\) mutants were impaired in \([\text{Ca}^{2+}]_{\text{cyt}}\) dependent stomatal responses (Webb and Hetherington, 1997). However, this apparent discrepancy can be explained by an interesting difference in the interactions of \(\text{Ca}^{2+}\) with the \(\text{abi1}\) and \(\text{abi2}\) mutants during stomatal closing compared with stomatal opening. When stomata are preclosed, either by low KCl

![Diagram](image-url)
Calcium Signaling in abi Guard Cells

and darkness (Figure 7C) or by isolating an epidermal peel at low K⁺ concentrations (Webb and Hetherington, 1997), opening induced by high KCl and light is inhibited by the addition of external Ca²⁺ (Figure 7C, left bars; Webb and Hetherington, 1997). In the abi1 and abi2 mutants, this inhibition by Ca²⁺ is partial and incomplete (Figure 7C). However, when stomata are preopened before [Ca²⁺]ᵣₑₙ addition, the Ca²⁺-induced closure is similar for the wild type and the abi1 and abi2 mutants (Figure 7B). Many signal transduction pathways in plants are Ca²⁺ dependent (Bush, 1995); therefore, it is not surprising that the signaling mechanisms involving Ca²⁺ that mediate stomatal closure are different for those that control stomatal opening, as has been proposed previously (Irving et al., 1992; Cousson and Vavasseur, 1998). These data are supported by the data in Figure 7, which clearly demonstrate different effects of abi1 and abi2 mutants on Ca²⁺-dependent signal transduction pathways that control stomatal closing compared with stomatal opening.

Other recent studies also have demonstrated that different signal transduction mechanisms become rate limiting, depending on whether stomatal opening or closing is analyzed. For example, the protein phosphatase inhibitor okadaic acid potentiates ABA-induced stomatal closing in fava bean, Commelina communis (Schmidt et al., 1995), and pea (Hey et al., 1997). However, in the same study on pea, okadaic acid was shown to inhibit the ABA response when stomatal opening rather than stomatal closing was analyzed (Hey et al., 1997). All of these studies support a model in which different signaling pathways are employed in a complex nonlinear network to control stomatal opening versus stomatal closing.

In this study, the combination of [Ca²⁺]ᵣₑₙ measurements, electrophysiology, and physiological analysis of stomatal movements clearly demonstrates that the abi1 and abi2 mutations reduce ABA-induced [Ca²⁺]ᵣₑₙ elevations. Furthermore, the partial impairment of ABA-induced [Ca²⁺]ᵣₑₙ elevations correlates with comparisons of stomatal closing and opening responses and together indicate a complex signaling network in guard cells.

![Figure 7. ABA Does Not Close Stomata of abi1 and abi2.](image)

Positively Regulating Phosphorylation Events Act Downstream of abi1, abi2, and [Ca²⁺]ᵣₑₙ and Control Anion Currents in Arabidopsis Guard Cells

Increases in [Ca²⁺]ᵣₑₙ previously have been shown to activate S-type currents in fava bean guard cells (Schroeder and Hagiwara, 1989), and these currents are activated by phosphorylation events (Schmidt et al., 1995). However, these previous studies had not yet analyzed whether the [Ca²⁺]ᵣₑₙ activation of S-type anion currents requires hydrolyzable ATP and whether some kinase activities lie downstream of [Ca²⁺]ᵣₑₙ in guard cells. In this study using Arabidopsis guard cells, we provide evidence for a link between these activating factors because [Ca²⁺]ᵣₑₙ increases are effective only in activating S-type currents in the presence of

![Diagram](image)
hydrolyzable ATP (Figure 5). This suggests a requirement for kinase activity in the calcium-dependent activation of S-type currents and is further supported by the inhibition of [Ca\(^{2+}\)\(_{cyt}\)]-activated anion currents after a 2-hr preincubation with the kinase inhibitors K-252a (2 μM) and staurosporine (50 μM) (Figures 5H and 5I). These inhibitors would act as positive regulators of ABA-induced stomatal closure mediated by ABA-induced increases in [Ca\(^{2+}\)\(_{cyt}\)]. Calcium- and ATP-dependent activation of S-type anion currents in guard cells of the abi1 and abi2 mutants (Figure 6) indicates that these positively regulating kinases remain functional in the abi1 and abi2 mutant plants. Therefore, these kinases can be positioned downstream of the abi1 and abi2 phosphatases and upstream of anion channels in the signaling cascade.

A recent study in fava bean in which S-type anion currents were preactivated before patch clamping further indicated that the final step in anion channel activation in guard cells is Ca\(^{2+}\) independent and requires ATP (Schwarz and Schroeder, 1998). Together, these data suggest that in ABA signaling, calcium-dependent proteins may turn activate downstream calcium-independent kinases. Biochemical data that correlate with this model come from studies of ABA activation of guard cell kinases (Li and Assmann, 1996; Mori and Muto, 1997) and aleuron cell kinases (Knetsch et al., 1996). In guard cells, ABA has been shown to activate calcium-independent protein kinases (Li and Assmann, 1996; Mori and Muto, 1997), and Ca\(^{2+}\)-dependent steps have been proposed to lie upstream of these (Mori and Muto, 1997). In aleuron cells, calcium-independent MAP kinase activity is activated by ABA (Knetsch et al., 1996).

In maize mesophyll protoplasts, overexpression of a constitutively active calcium-dependent protein kinase can activate ABA signaling pathways, and this activation is partly counteracted by transgenic expression of mutant abi1 protein phosphatases (Sheen 1996, 1998). These data indicate that additional protein kinases may act upstream of abi1 or that different systems do not have identical early signaling cascades (see above discussion on stomatal opening).

**Positively and Negatively Regulating Kinases Are Conditionally Revealed in Arabidopsis Guard Cells**

Interestingly, in guard cells of Arabidopsis abi1 mutants, ABA activation of anion channels can be restored by the simultaneous application of protein kinase inhibitors (Pei et al., 1997). Similarly, in transgenic tobacco expressing abi1-1, ABA-induced stomatal closure and regulation of potassium channels can be restored by the application of the protein kinase inhibitor staurosporine (0.5 μM; Armstrong et al., 1995). These data lead to a model in which additional, negatively regulating kinases also function in the ABA signal transduction cascade close to the abi1 phosphatase. Surprisingly, simultaneous treatment of wild-type Arabidopsis guard cells with ABA and 2 μM K-252a did not inhibit the ABA activation of anion channels (Pei et al., 1997; Figure 7A), whereas in this study, Ca\(^{2+}\)-dependent anion channel activation was inhibited after preincubation with kinase inhibitors (Figures 5H and 5I). To analyze further the apparent counteracting effects of kinase inhibitors, wild-type guard cells were treated with ABA after a 30-min preincubation with 2 μM K-252a. Under these conditions, ABA activation of anion channels was inhibited (n = 10; Y. Murata and J.I. Schroeder, unpublished data). These data indicate that pharmacological modification of both positively and negatively regulating kinases can depend not only on the mutant background but also perhaps on the relative timing of kinase inhibitor and ABA application and on the concentration (staurosporine) of kinase inhibitors. Taken together, these data indicate a complex phosphorylation cascade in ABA signaling with both positively and negatively regulating kinases and phosphatases.

This study shows that the final steps in Ca\(^{2+}\)- and kinase-dependent activation of anion channels are not disrupted by abi1 and abi2 mutations. Further research is necessary to identify the discussed kinases and phosphatases at the biochemical and molecular genetic levels to understand fully their interactions in ABA signaling.

**Conclusions and Working Model**

New data presented here allow us to present a simple model for the positioning of [Ca\(^{2+}\)\(_{cyt}\)], abi1 and abi2 phosphatases, anion channels, and positively regulating kinases in the portion of the ABA signal transduction network in Arabidopsis guard cells that leads to stomatal closure (Figure 8). ABA can lead to an increase in [Ca\(^{2+}\)\(_{cyt}\)] via activation of multiple calcium influx and/or release pathways. [Ca\(^{2+}\)\(_{cyt}\)] elevations, in turn, act via a signaling cascade involving positively regulating kinase(s) to activate S-type anion channels that mediate anion efflux. Anion efflux depolarizes the plasma membrane potential, leading to activation of outwardly rectifying K\(^{+}\) channels, K\(^{+}\) loss, and hence, stomatal closure. The abi1 and abi2 phosphatases act upstream of or at the level of certain ABA-induced [Ca\(^{2+}\)\(_{cyt}\)] elevation mechanisms because the mutant abi1 and abi2 proteins severely reduce [Ca\(^{2+}\)\(_{cyt}\)] increases. Increases in [Ca\(^{2+}\)\(_{cyt}\)] that raise [Ca\(^{2+}\)\(_{cyt}\)] result in stomatal closure in both the wild type and the abi1 and abi2 mutants by the combined study of [Ca\(^{2+}\)\(_{cyt}\)] changes, ion channel regulation, and stomatal movements will lead to further functional dissection of early guard cell signal transduction cascades.
ABA can cause elevations of [Ca^{2+}]_{cyt} that activate S-type anion channels via protein kinase activities. Activation of anion currents will depolarize the plasma membrane, activating K^-release channels, and thus result in turgor loss and stomatal closure. That the dominant abi1 and abi2 mutations are positioned upstream of or close to [Ca^{2+}]_{cyt} increases is confirmed by both the reduction (but not abolition) of ABA-induced [Ca^{2+}]_{cyt} changes in these mutants (Figures 3 and 4) and the ability of [Ca^{2+}]_{cyt} to bypass these mutations by activating anion (An^-) channels (Figure 6) and eliciting stomatal closure (Figure 7). Note that abi1-1 and abi1-2 may repress certain Ca^{2+} influx pathways but leave others unaltered, based on residual [Ca^{2+}]_{cyt} elevations (see text). Evidence for additional mechanisms and complex branch points in the ABA signal transduction network are not shown but are discussed in the text.

**Plant Material**

Seeds (*Arabidopsis thaliana* ecotype Landsberg erecta) and the abscisic acid (ABA)-insensitive abi1-1 (abi1) and abi2-1 (abi2) mutants (Koomen et al., 1984) were grown in soil (Redi-Earth Peat-Lite Mix; Scotts, Marysville, OH) in a controlled environment growth chamber (Conviron model E15; Controlled Environments, Asheville, NC) under a 16-hr-light and 8-hr-dark cycle, a photon fluency rate of 100 μmol m^{-2} sec^{-1}, and a temperature of 20°C. Pots were watered every 2 to 3 days with deionized water, and plants were misted with deionized water daily to keep the humidity close to 70%. Seeds were obtained from the Ohio State University Arabidopsis Resource Center (Columbus).

**Ca^{2+}-Sensitive Fluorescent Dye Loading**

In initial studies, a method was developed to load the Ca^{2+}-sensitive dye calcium green-AM (Molecular Probes, Eugene, OR) into guard cells (K. Kuchitsu, J. Ward, I. Schelle, and J. I. Schroeder, unpublished data; see http://www-biology.ucsd.edu/labs/schroeder/protocols/calcium.html). However, this method did not allow accurate [Ca^{2+}]_{cyt} quantification due to the nonratiometric nature of the dye. Therefore, an alternative method was developed in which ratiometric fluorescent dyes were acid-loaded into guard cells in epidermal fragments (Figures 1A to 1D) (Bush and Jones, 1987; Darjania et al., 1993). One or two rosette leaves were clipped from the plant, blended in 400 mL of deionized water twice for 20 sec each in a Waring blender at 20,000 rpm, and carefully filtered through a cone of 30-μm pore diameter nylon mesh. Epidermal fragments were collected at the tip of the cone. A small sample of the fragments was transferred using a pointed spatula into a microcentrifuge tube (with holes pierced in the lid to facilitate gaseous exchange). The tube contained 50 μL of buffer comprising 50 mM KCl, 50 μM CaCl₂, 10 mM Mes-KOH, pH 4.5, 0.02% (w/v) pluronic F-127 (Sigma), and 100 μM fura 2 pentapotassium salt (Molecular Probes). Fragments were incubated in this buffer in the dark at room temperature for 2 hr and then in the light (photon fluency rate of 100 μmol m^{-2} sec^{-1}) for 2 hr.

After this loading period, the fragments required mounting to prevent movement during photometric measurements. Fragments were mounted on 100/100 mesh (100 lines per inch) folding electron microscope grids (EM grids) (Ted Pella, Inc., Redding, CA) by the following procedure. One lobe of the two-lobe EM grid was held with needle nose tweezers. A 4-μL sample of the loaded fragments was pipetted gently onto the other lobe using a 200-μL pipette tip with the last 5 mm removed. The corner of a tissue was gently touched onto the underside of the lobe containing the fragments. This had the effect of drawing the buffer into the tissue and pulling the fragments onto the grid. Care was taken not to dry the fragments out. The grid was immediately inverted, and the lobe without the fragments was pushed into a blob of vacuum grease that previously had been placed on an Ultra thin (0.083 mm) glass cover slip (MatTek Corp., Ashland, MA). This cover slip formed the bottom of a 200-μL chamber made in a glass microscope slide. The lobe of the EM grid was gently pushed into the vacuum grease until the lobe containing the epidermal fragments just made contact with the cover slip surface, effectively trapping the epidermal fragments between the grid and the cover slip. The chamber was immediately filled with loading buffer (see above), with the pH adjusted to 5.6. The chamber was then mounted on a Nikon (Tokyo, Japan) diaphot inverted microscope and continuously perfused at a rate of 20 μL sec^{-1} with the same buffer for 5 to 15 min before photometric measurements were commenced. To quantify dye loss, in separate experiments, recordings were made for 30 to 50 sec at 1, 5, 15, and 25 min after this initial period. The cells were not exposed to UV excitation between these control experiments to prevent dye bleaching.

In all experiments, [Ca^{2+}]_{cyt} measurements were performed only on those stomata in which both cells had maintained a significant turgor, the turgor of the two cells was equal, and the stomatal aperture was large (see Figures 1E and 1F).
Microphotometry

UV excitation light for fura 2 fluorescence was provided by a 100-W mercury light source passing through a spinning filter wheel rotating at 50 Hz. Excitation light of wavelengths 340 ± 15 nm and 380 ± 15 nm (filters from Omega Optical, Brattleboro, VT) were passed into the back of the microscope via a fiber optic cable and deflected toward the sample with a 430-nm long pass dichroic mirror (Omega Optical). Excitation light was focused on the guard cells through a Nikon Fluor ×40 objective. Excitation light was limited to the area of one stomate by using an iris positioned before the dichroic mirror. Emission light passed through the dichroic mirror and an emission filter (510 ± 20 nm; Omega Optical) before passing to the photomultiplier tube (End-on 9B28B; Electron Tubes, Inc., Runslip, UK). The emission signal area was also limited to the size of one guard cell pair by a pinhole placed before the photomultiplier. The emission signals were synchronized with the excitation wavelength by using a Spectrophotometric demodulator (Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia), and the ratio of the two emission signals was determined on line with an analog ratio circuit. The two emission signals and the ratio were recorded using Axotape (Axon Instruments, Foster City, CA), sampled at 8 Hz, and filtered during later analysis at 1 to 4 Hz, depending on the signal-to-noise ratio. Measurements were made simultaneously on the two guard cells of a stomatal complex to achieve a satisfactory signal-to-noise ratio from these small cells. The system was calibrated in vitro using Ca2+ calibration standards (catalog No. C-3722; Molecular Probes) containing 100 mM KCl, 1 mM MgCl2, and 10 mM MOPS, pH 7.2; 0 to 10 mM CaEGTA (0 to 39.8 μM free calcium) as ionophores failed to consistently elevate [Ca2+]cyt. Autofluorescence correction was conducted on line by applying a suppression voltage to each emission signal. These voltages were calculated as the average from 20 to 30 unloaded guard cell pairs and did not exceed ~1% of the signal from the loaded cells (see Figure 1D).

To confirm the cytoplasmic location of the acid-loaded dyes, confocal images were obtained from fura 2- and indo 1-loaded wild-type and ab1 guard cells on a separate system from that used for photometry. Fura 2 was excited close to its Ca2+ type and loaded cells (see Figure 1D).

Electrophysiology

Arabidopsis guard cell protoplasts were prepared from rosette leaves of 4- to 6-week-old plants, and patch-clamp electrophysiology was performed in the whole-cell mode exactly as described previously (Pei et al., 1997). To measure anion currents, the pipette solution contained 150 mM CsCl, 2 mM MgCl2, 6.7 mM EGTA, 5 mM Mg-ATP, 10 mM Hepes-Tris, pH 7.1, and a concentration of CaCl2 calculated to give the desired free Ca2+ concentration. Free calcium concentrations were calculated with the program CALCIUM (Foehr et al., 1993). The bath solution contained 30 mM CsCl, 2 mM MgCl2, 1 mM CaCl2, and 10 mM Mes-Tris, pH 5.6. This solution was supple-mented with 40 mM CaCl2 to aid giga seal formation. Any further changes to these solutions are indicated in the legends to Figures 5 and 6. The liquid junction potential in these solutions was 0.45 mV. Steady state currents were sampled during the last 3 sec of voltage pulses. The standard voltage protocol stepped the voltage from a holding potential of +30 to −145 mV for 40 sec. Subsequent voltage steps were reduced by 30 mV per pulse. The interpulse period was 12 sec, and no leak subtraction was made. All recordings were made 7 to 10 min after access to the whole-cell configuration. To measure the effects of inhibitors, protoplasts were incubated at 22°C with 2 μM K-252a or 50 μM staurosporine for 2 hr before recordings. The inhibitors also were included in the pipette solution at the same concentration for these experiments.

Stomatal Aperture Bioassays

To measure stomatal closing, rosette leaves from 4- to 6-week-old plants were detached and floated in opening solution consisting of 50 mM KCl and 10 mM Mes-Tris, pH 6.15 or 5.6, for 2 hr in the light (photon fluency rate of 100 μmol m−2 sec−1). After 2 hr, either ABA (10 or 50 μM) or CaCl2 (0.05, 0.75, 2, 5, or 10 mM) was added to the buffer. After an additional 2 hr, the leaves were blotted in 400 mL of deionized water in a Waring Blender for 20 sec. The resulting epidermal fragments were filtered out with a 30-μm nylon mesh, placed on a microscope slide, and covered with a cover slip. Aperture ratios (width to height) were measured as previously described (Pei et al., 1997). To measure stomatal opening, three leaves were floated on 10 mM KOH and 10 mM Mes, pH 6.15 (Webb and Hetherington, 1997), in the dark for 2 hr. After 2 hr, one leaf was blotted and apertures measured. The other two leaves were transferred to 50 mM KCl, 10 mM KOH, 10 mM Mes, pH 6.15, in the light (Webb and Hetherington, 1997) either with or without 5 mM CaCl2. After an additional 2 hr, these leaves were blotted and apertures measured.

ACKNOWLEDGMENTS

We thank Drs. Mark Ellisman and Hisao Fujisaki (National Center for Microscopy and Imaging Research, University of California, San Diego) for assistance in obtaining the confocal images in Figure 1. Thanks also to Norman Graham (Research Instrumentation Shop, University of Pennsylvania, School of Medicine, Philadelphia) for technical advice. This research was supported by National Science Foundation Grant No. MCB-9506191 and U.S. Department of Energy Grant No. DE-FG03-94-ER20148 (to J.I.S.) and by a Human Frontiers Science Program long-term fellowship (to G.J.A.) and a National Science Foundation REU supplement. We dedicate this publication to the memory of Richard C. Crain for his pioneering research on lipid and calcium signaling in plants.

Received April 7, 1999; accepted June 17, 1999.

REFERENCES

Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H., and Trewavas, A.C.


Calcium Rises in Guard Cells

Gethyn J. Allen, Kazuyuki Kuchitsu, Sarah P. Chu, Yoshiyuki Murata and Julian I. Schroeder

*Plant Cell* 1999;11;1785-1798

DOI 10.1105/tpc.11.9.1785

This information is current as of October 20, 2017