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Hypersensitivity of Abscisic Acid–Induced Cytosolic Calcium Increases in the Arabidopsis Farnesyltransferase Mutant *era1-2*

Gethyn J. Allen,[1] Yoshiyuki Murata, Sarah P. Chu, Majse Nafisi, and Julian I. Schroeder[2]

Cell and Developmental Biology Section, Division of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0116

Cytosolic calcium increases were analyzed in guard cells of the Arabidopsis farnesyltransferase deletion mutant *era1-2* (enhanced response to abscisic acid). At low abscisic acid (ABA) concentrations (0.1 μM), increases of guard cell cytosolic calcium and stomatal closure were activated to a greater extent in the *era1-2* mutant compared with the wild type. Patch clamping of *era1-2* guard cells showed enhanced ABA sensitivity of plasma membrane calcium channel currents. These data indicate that the ERA1 farnesyltransferase targets a negative regulator of ABA signaling that acts between the points of ABA perception and the activation of plasma membrane calcium influx channels. Experimental increases of cytosolic calcium showed that the activation of S-type anion currents downstream of cytosolic calcium and extracellular calcium-induced stomatal closure were unaffected in *era1-2*, further supporting the positioning of *era1-2* upstream of cytosolic calcium in the guard cell ABA signaling cascade. Moreover, the suppression of ABA-induced calcium increases in guard cells by the dominant protein phosphatase 2C mutant *abi2-1* was rescued partially in *era1-2 abi2-1* double mutant guard cells, further reinforcing the notion that ERA1 functions upstream of cytosolic calcium and indicating the genetic interaction of these two mutations upstream of ABA-induced calcium increases.

INTRODUCTION

Farnesyltransferases (FTases) are heterodimeric enzymes in eukaryotes that catalyze the attachment of farnesyl lipids to the C-terminal region of target proteins (Schafer and Rine, 1992; Zhang and Casey, 1996; Nambara and McCourt, 1999). Farnesylation occurs at a conserved CaaX domain, where "C" is a Cys, "aa" are most often alipathic amino acids, and "X" is usually Cys, Met, Ser, Ala, or Gln. Farnesylated proteins play central roles in cell signaling in yeast and animal cells and include Ras, GTP binding proteins, nuclear lamin B, yeast mating factors, and protein kinases (Glomset and Farnsworth, 1994; Schmitt et al., 1996; Yalovsky et al., 1997). Attachment of a farnesyl lipid to these proteins allows targeting to a membrane surface or facilitates protein–protein interactions and therefore is essential in the construction of functional signaling complexes.

In plants, FTases have been identified at the molecular level in tomato, pea, and Arabidopsis (Yang et al., 1993; Cutler et al., 1996; Schmitt et al., 1996), and a range of farnesylated proteins have been identified through biochemical studies and the recognition of conserved CaaX domains in potential signaling proteins (Zhu et al., 1993; Nambara and McCourt, 1999; Rodríguez-Concepción et al., 1999; Yalovsky et al., 2000a, 2000b). However, in most cases, it remains unknown at which point in the various signaling cascades farnesylated signaling proteins have their effects in plants.

An Arabidopsis mutant carrying a fast neutron–induced deletion in the β-subunit of a FTase has been isolated from a screen to identify mutants with an enhanced response to abscisic acid (ABA) (Cutler et al., 1996). The *era1-2* mutant allele carries a 7.5-kb deletion in the FTase β-subunit and increases the dormancy of seeds in response to ABA (Cutler, 1995; Cutler et al., 1996) but does not affect endogenous ABA levels (Ghassemian et al., 2000).

ABA reduces water loss from plants during drought stress via a signal transduction network in guard cells that leads to stomatal closure (MacRobbie, 1998; Schroeder et al., 2001b). The *era1-2* FTase mutation causes ABA-hypersensitive S-type anion current activation and stomatal closure (Pei et al., 1998). Stomatal closure and anion channel activity in wild-type Arabidopsis were rendered hypersensitive to ABA by the application of the FTase inhibitor α-hydroxyfarnesylphosphonic acid, indicating that FTases may function in the guard cell ABA signal transduction pathway (Pei et al., 1998).

[1] In memoriam.

[2] To whom correspondence should be addressed. E-mail julian@biomail.ucsd.edu; fax 858-534-7108.

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Enhanced ABA sensitivity in *era1-2* guard cells leads to reduced rates of water loss from *era1-2* plants compared with wild-type plants under drought stress (Pei et al., 1998). The *era1-2* mutation deletes the only FTase β-subunit gene present in Arabidopsis and therefore affects a variety of signal transduction and developmental processes (Pei et al., 1998; Nambara and McCourt, 1999; Yalovsky et al., 2000a, 2000b; Ziegelhoffer et al., 2000). The ABA hypersensitivity of the *era1-2* mutant in both germination and stomatal responses indicates that the Arabidopsis FTase targets one or more negative regulators of ABA signal transduction to a signal-transducing complex that affects ABA signaling in both Arabidopsis seeds and guard cells.

Increases in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in guard cells have been shown to be early events in the signaling cascade that results in ABA-induced stomatal closure in a number of plant species (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992; Grabov and Blatt, 1998; Allen et al., 1999a, 2001; Staxén et al., 1999). Recently, the kinetics of $[Ca^{2+}]_{cyt}$ oscillations were shown to tightly control stomatal movements in response to stimuli such as ABA (Allen et al., 2001). Furthermore, studies in suspension culture cells have shown that ABA induction of *Rab18* gene expression requires the activation of plasma membrane calcium and anion channels (Ghelis et al., 2000a, 2000b) and that phospholipase C and inositol 1,4,5-trisphosphate contribute to the ABA regulation of seed germination and *KIN2* gene expression (Sanchez and Chua, 2001; Xiong et al., 2001).

These data suggest that $[Ca^{2+}]_{cyt}$ increases function as a second messenger in a wide variety of ABA signal transduction cascades. Recently, signaling mutants in Arabidopsis and other species are being defined and "mapped" with respect to their effects on stimulus-induced $[Ca^{2+}]_{cyt}$ signals with mutations acting either upstream or downstream of $[Ca^{2+}]_{cyt}$ (Allen et al., 1999a, 2001; Baum et al., 1999; Knight et al., 1999; Wais et al., 2000; Walker et al., 2000; Hugouvieux et al., 2001; Schroeder et al., 2001a). To date, only one plant mutation has been shown to enhance the sensitivity of $[Ca^{2+}]_{cyt}$ signal induction (Hugouvieux et al., 2001).

In this study, we used Arabidopsis plants expressing the calcium indicator yellow cameleon 2.1 (YC2.1) (Allen et al., 1999b; Miyawaki et al., 1999) combined with stomatal movement assays and electrophysiological measurements of guard cell S-type anion channels, potassium channels, and calcium influx channels to map the position of the *era1-2* mutation with respect to $[Ca^{2+}]_{cyt}$ in the guard cell ABA signaling cascade. The data presented demonstrate that the *era1-2* mutation acts upstream of ABA-induced $[Ca^{2+}]_{cyt}$ increases and the activation of plasma membrane Ca^{2+} influx channels, resulting in a ABA-hypersensitive $[Ca^{2+}]_{cyt}$ increase and stomatal closure in *era1-2*. In addition, the *era1-2* mutation can partly rescue the insensitivity of the *abi2-1* mutant to ABA at the level of $[Ca^{2+}]_{cyt}$ signaling and stomatal closure. These data suggest that the *era1-2* mutation acts very early in the ABA signaling cascade at a point between the perception of the ABA stimulus and the activation of Ca^{2+} channels.

RESULTS

Hypersensitivity of ABA-Induced $[Ca^{2+}]_{cyt}$ Increases in *era1-2* Guard Cells

ABA elicits repetitive transients, or oscillations, in $[Ca^{2+}]_{cyt}$ in stomatal guard cells maintained in low (5 mM) extracellular KCl buffers (Grabov and Blatt, 1998; Allen et al., 1999b, 2000; Staxén et al., 1999). In 75% ($n = 36$ from 48 cells) of wild-type (Columbia) guard cells expressing the green fluorescent protein–based calcium indicator YC2.1 (Allen et al., 1999b; Miyawaki et al., 1999), application of 10 μM ABA induced repetitive transients in $[Ca^{2+}]_{cyt}$, with an average peak-to-peak period of 7.6 ± 0.3 min and an amplitude increase of 0.42 ± 0.021 ratio units ($n = 36$), as shown in Figure 1A. However, in wild-type guard cells, application of only 0.1 μM ABA elicited no, or only small, $[Ca^{2+}]_{cyt}$ increases (change in ratio units of 0.04 ± 0.01; $n = 46$) (Figure 1B).

As a positive control, 10 mM extracellular calcium ($[Ca^{2+}]_{ext}$) was applied to these cells at the end of the experiment and elicited large $[Ca^{2+}]_{cyt}$ increases, indicating that they were viable to report $[Ca^{2+}]_{cyt}$ changes (Figure 1B). However, when *era1-2* guard cells expressing YC2.1 were treated with 0.1 μM ABA, repetitive $[Ca^{2+}]_{cyt}$ transients were induced in 67% of cells ($n = 29$ from 43 cells; Figure 1C). Increases in $[Ca^{2+}]_{cyt}$ in *era1-2* guard cells induced by 0.1 μM ABA had a mean transient peak-to-peak period of 9.1 ± 0.9 min, an amplitude increase of 0.17 ± 0.033 ratio units, and mean and modal (most common) numbers of transients of 2.5 and 3, respectively ($n = 29$), within a recording period of up to 45 min. No significant differences in resting $[Ca^{2+}]_{cyt}$ were apparent between wild-type and *era1-2* guard cells ($P > 0.61$ for the wild type [$n = 36$] versus *era1-2* [$n = 43$]).

The *era1-2* mutant shows ABA-hypersensitive stomatal closure (Pei et al., 1998). Here, we analyzed ABA responses on stomatal movements using experimental conditions identical to those used in calcium imaging experiments. Hypersensitive induction of $[Ca^{2+}]_{cyt}$ transients in *era1-2* guard cells by ABA correlated with the hypersensitivity of ABA-induced stomatal closure, measured at 0.1 μM ABA, under identical experimental conditions (Figure 1D). Furthermore, the additional T-DNA insertion allele *era1-1* (Cutler et al., 1996) also showed hypersensitive stomatal closure at 0.1 μM ABA (Figure 1E), whereas the *era1-2* mutant complemented with the β-subunit of the FTase from tomato rescued the ABA hypersensitivity phenotype, with stomatal closure at 0.1 μM ABA being similar to that of the wild type (Figure 1E). Together, these data suggest that the *era1* mutation renders stomata hypersensitive to ABA and affects

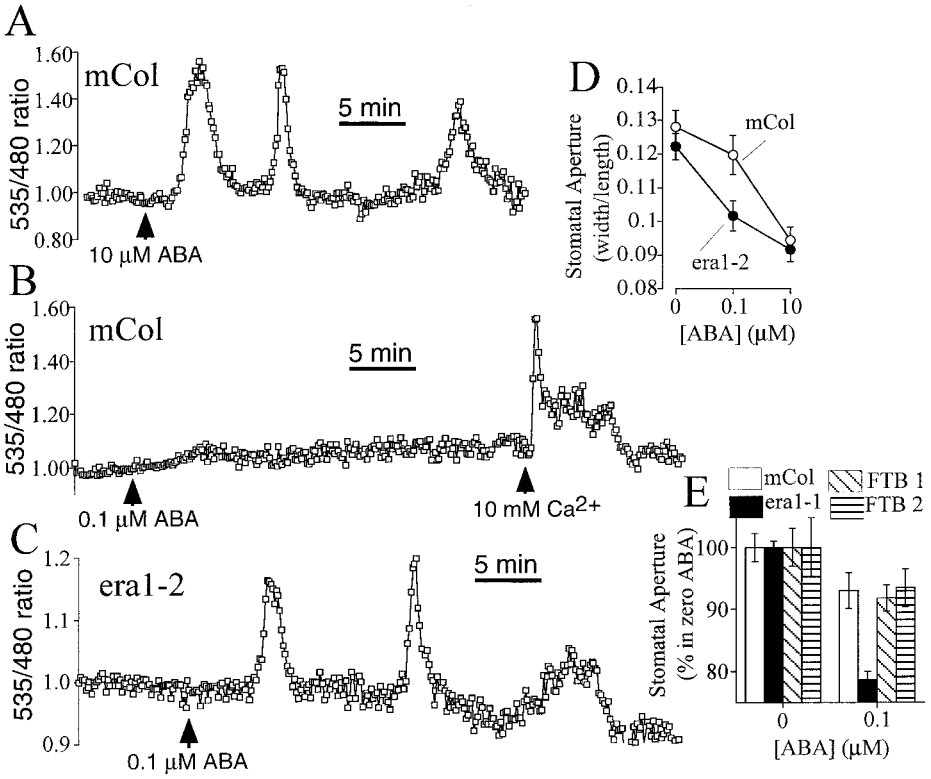

Figure 1. ABA-Hypersensitive Induction of $[Ca^{2+}]_{cyt}$ Increases and Stomatal Closure in *era1-2*.

(A) Repetitive $[Ca^{2+}]_{cyt}$ transients induced by 10 μM ABA in wild-type Arabidopsis guard cells expressing YC2.1.
(B) ABA at 0.1 μM fails to elicit $[Ca^{2+}]_{cyt}$ transients in wild type (Columbia ecotype) Arabidopsis guard cells, although application of extracellular calcium elicits $[Ca^{2+}]_{cyt}$ transients.
(C) ABA at 0.1 μM elicits $[Ca^{2+}]_{cyt}$ transients in *era1-2* guard cells.
(D) Hypersensitive stomatal closure is induced in *era1-2* stomates at 0.1 μM ABA.
(E) Hypersensitive stomatal closure is induced in *era1-1* stomates at 0.1 μM ABA but not in lines FTB1 and FTB2 complemented with the tomato FTase β-subunit.
Data are means ± SEM relative to $n = 6$ replicates comprising 120 stomates per point in **(D)** and relative to $n = 3$ replicates comprising 75 stomates per bar in **(E)**. Fluorescence ratios were normalized relative to baseline ratios before ABA application (see Methods). mCol, Columbia.

guard cell signal transduction at a point upstream of $[Ca^{2+}]_{cyt}$ increases.

Calcium Influx Mediated by Calcium-Permeable Currents Shows ABA-Hypersensitive Activation in *era1-2*

Hyperpolarization-activated calcium-permeable currents (I_{Ca}) in the guard cell plasma membrane are activated by ABA and H_2O_2 and mediate extracellular calcium influx (Hamilton et al., 2000; Pei et al., 2000). Using Ba^{2+} as a charge carrier, we measured I_{Ca} activation by low concentrations of ABA (0.1 μM) in the presence of 1 mM cytosolic NADPH in wild-type and *era1-2* guard cell protoplasts, as shown in Figure 2. I_{Ca} in the presence of 0.1 μM ABA in *era1-2* were significantly greater ($P < 0.01$) than in wild-type guard cells (Figures 2A to 2C). These data reinforce the observation that the *era1* mutation affects ABA signaling in guard cells at a point upstream of ABA-induced $[Ca^{2+}]_{cyt}$ signaling and show that *era1-2* amplifies the ABA activation of I_{Ca} channels.

S-Type Anion Channel Activation by $[Ca^{2+}]_{cyt}$ in *era1-2* Guard Cells

To determine whether the *era1-2* mutation also can affect signal transduction mechanisms downstream of $[Ca^{2+}]_{cyt}$ in guard cells, we measured S-type anion channel activation by $[Ca^{2+}]_{cyt}$. S-type anion currents can be activated by both ABA (Grabov et al., 1997; Pei et al., 1997, 1998; Leonhardt et al., 1999; Li et al., 2000) and $[Ca^{2+}]_{cyt}$ (Schroeder and

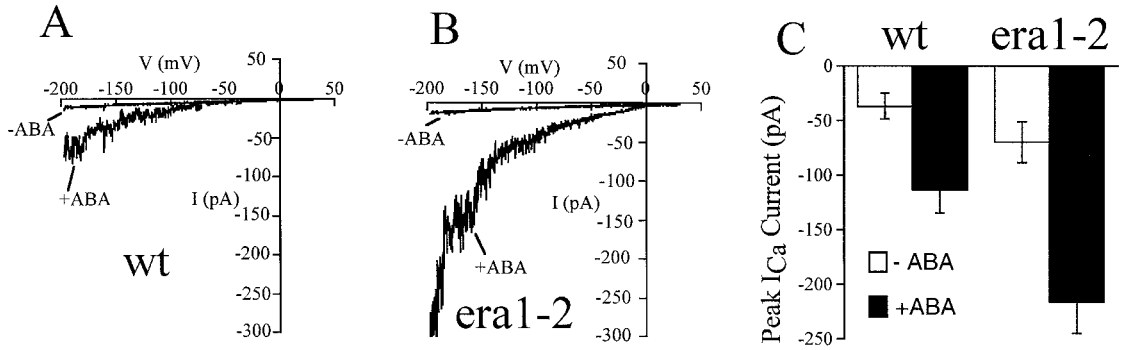

Figure 2. I_{Ca} Channels Show Hypersensitive ABA Activation in *era1-2*.

(A) and **(B)** I_{Ca} measured before and after the addition of 0.1 μM ABA in wild-type **(A)** and *era1-2* **(B)** guard cell protoplasts ($n = 6$ to 8 protoplasts for each).
(C) Average maximal ABA-induced I_{Ca} in wild-type and *era1-2* protoplasts at −198 mV.
wt, wild type.

Hagiwara, 1989; Allen et al., 1999a) and are central components of stomatal closure (Schmidt et al., 1995).

Unexpectedly, we found that $[Ca^{2+}]_{cyt}$ activation of S-type anion channels in patch-clamped Arabidopsis guard cell protoplasts was dependent on the protoplasts being preincubated with high $[Ca^{2+}]_{ext}$ concentrations, as illustrated in Figure 3. When protoplasts were preincubated in a solution containing 1 mM $[Ca^{2+}]_{ext}$, no significant cytosolic calcium activation of S-type anion currents was measured when $[Ca^{2+}]_{cyt}$ was buffered to 280 nM ($n = 9$) (Figures 3A and 3C) or 2 μM ($n = 26$) (Figures 3B and 3C). After 1 h of preincubation with 40 mM $[Ca^{2+}]_{ext}$, only small S-type anion channel currents were recorded when $[Ca^{2+}]_{cyt}$ was buffered to 280 nM ($n = 13$) (Figures 3D and 3F), but they were activated when $[Ca^{2+}]_{cyt}$ was buffered to 2 μM (Figures 3E and 3F) ($n = 8$; $P < 0.001$ for 2 μM versus 280 nM $[Ca^{2+}]_{cyt}$ at −145 mV).

Consistent with these findings, $[Ca^{2+}]_{cyt}$ activation of anion currents measured previously in Arabidopsis guard cell protoplasts followed gigaohm seal formation in a bath solution containing 40 mM $CaCl_2$ (Allen et al., 1999a). In the experiments represented in Figures 3A to 3F, protoplasts were sealed and recorded in bath solution containing 1 mM $CaCl_2$ after preincubation in either 1 or 40 mM $[Ca^{2+}]_{ext}$, indicating that the continued presence of high $[Ca^{2+}]_{ext}$ per se is not essential for $[Ca^{2+}]_{cyt}$ activation of anion currents, although a previous exposure to high $[Ca^{2+}]_{ext}$ is required.

Calcium activation of S-type anion currents was compared in wild-type and *era1-2* guard cell protoplasts at 1 mM $[Ca^{2+}]_{ext}$ after preincubation in 40 mM $[Ca^{2+}]_{ext}$, as shown in Figure 4. S-type anion currents were similar in the wild type and *era1-2* at $[Ca^{2+}]_{cyt}$ levels of 500 nM ($P > 0.79$) or 2 μM ($P > 0.58$) (Figure 4). Because the activation of anion currents occurs downstream of $[Ca^{2+}]_{cyt}$ (Schroeder and Hagiwara, 1989; Allen et al., 1999a), similar $[Ca^{2+}]_{cyt}$ activation of S-type currents is consistent with the positioning of the *era1-2* mutation upstream of $[Ca^{2+}]_{cyt}$ signaling in ABA-induced stomatal closure (Figures 1 and 2).

The *era1-2* Mutation Reduces Inwardly Rectifying K^+ Current Channel Activity in Guard Cells

To determine if the *era1-2* mutation affects other ion channels involved in stomatal movements, inwardly rectifying K^+ (K^+_{in}) currents were measured in guard cell protoplasts. As shown in Figure 5, K^+_{in} currents were reduced significantly (2.8-fold lower at −180 mV; $P < 0.0001$; $n = 26$) in *era1-2* guard cells compared with the wild type. K^+_{in} channels provide an important route for K^+ uptake during stomatal opening (Schroeder et al., 1987; Thiel et al., 1992; Kwak et al., 2001). To determine whether lower K^+_{in} currents affected stomatal movements in *era1-2*, stomatal opening was measured in low and high extracellular K^+ (Figure 5D). In low K^+ (5 mM KCl and 50 μM $CaCl_2$), stomatal opening was similar in the wild type and *era1-2* (Figure 5D, left bars; $P > 0.46$; see also Figure 1D).

However, when extracellular K^+ was increased (50 mM KCl and 50 μM $CaCl_2$), wild-type stomata opened farther than those in *era1-2* (Figure 5D, right bars). Similarly, at 20 mM KCl, stomatal apertures were smaller in *era1-2* compared with the wild type in the absence of ABA (Pei et al., 1998). These data suggest that the *era1-2* mutation can act as a limiting factor on K^+_{in} channel activity in guard cells, reducing stomatal opening.

Epistatic Interaction between *abi2-1* and *era1-2* in $[Ca^{2+}]_{cyt}$ Signaling in Guard Cells

The Arabidopsis mutants *abi1-1* and *abi2-1* are dominant mutations in homologous protein phosphatases of type

2C, which render stomatal closure insensitive to ABA (Koornneef et al., 1984). Using fura-2 dye–loaded Arabidopsis guard cells, it was demonstrated that *abi1-1* and *abi2-1* both act upstream of ABA-induced $[Ca^{2+}]_{cyt}$ increases in guard cells, suppressing ABA-induced $[Ca^{2+}]_{cyt}$ transients (Allen et al., 1999a). Furthermore, experimental induction of $[Ca^{2+}]_{cyt}$ increases in *abi1-1* and *abi2-1* resulted in the recovery of S-type anion channel activation and stomatal closing, indicating that processes downstream of $[Ca^{2+}]_{cyt}$ were unaffected in guard cells by these protein phosphatase type 2C mutants (Allen et al., 1999a).

A recent study showed that the *abi2-1* mutation disrupts calcium signaling downstream of ABA perception but upstream of I_{Ca} activation (Murata et al., 2001), analogous to the positioning of *era1-2* found here. Therefore, we analyzed interactions of the *abi2-1* and *era1-2* mutations and their effects on guard cell signaling. As shown in Figure 6, we confirmed the *abi2-1* impairment of ABA-induced $[Ca^{2+}]_{cyt}$ increases using *abi2-1*–expressing YC2.1. ABA at 10 μM induced repetitive $[Ca^{2+}]_{cyt}$ transients in wild-type (Landsberg *erecta*) guard cells (Figure 6A) ($n = 36$ of 42 cells) but failed to elicit significant changes in $[Ca^{2+}]_{cyt}$ in all *abi2-1* guard cells tested ($n = 26$) (Figure 6B). Positive controls using the addition of 10 mM $[Ca^{2+}]_{ext}$ induced $[Ca^{2+}]_{cyt}$ transients in *abi2-1* (Figure 6B).

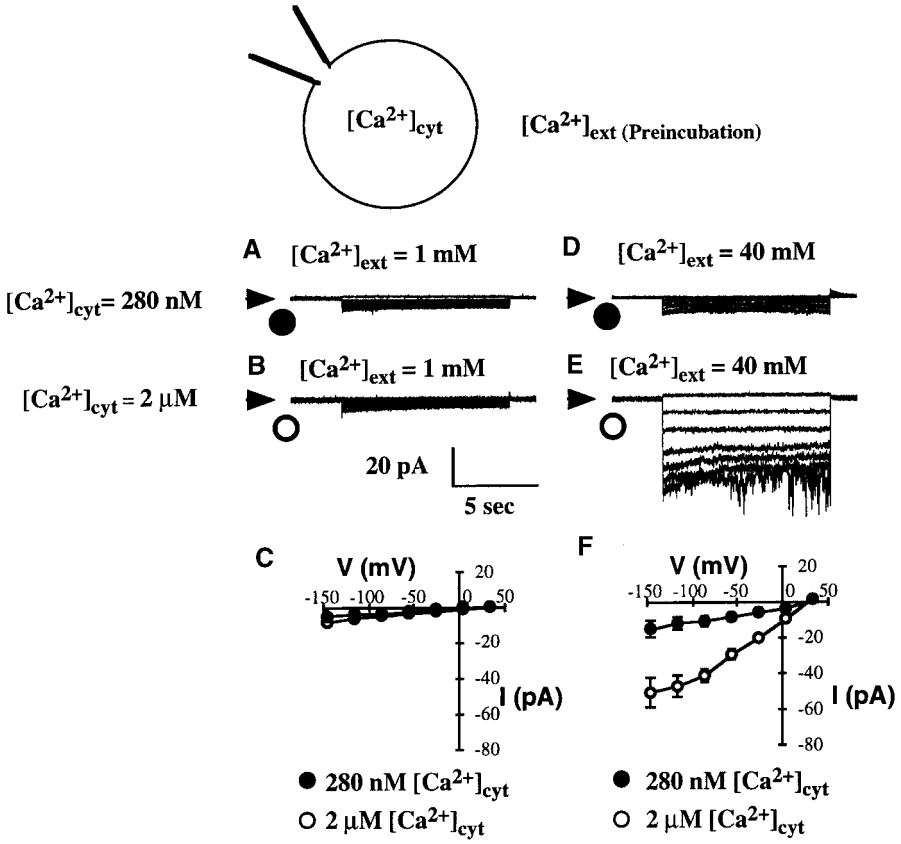

Figure 3. S-Type Anion Channel Activation by $[Ca^{2+}]_{cyt}$ in Arabidopsis Guard Cell Protoplasts Requires Preincubation at High $[Ca^{2+}]_{ext}$ Calcium Concentrations.

(A) S-type anion channel currents in a wild-type Arabidopsis guard cell protoplast preincubated in 1 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered to 280 nM via the patch pipette solution.

(B) S-type anion channel currents in a guard cell protoplast preincubated in 1 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered to 2 μM.

(C) Current-voltage relationships for S-type anion currents recorded from guard cell protoplasts preincubated in 1 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered at 280 nM (closed symbols; $n = 9$) or 2 μM (open symbols; $n = 26$) as in **(A)** and **(B)**.

(D) S-type anion channel currents in a guard cell protoplast preincubated in 40 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered to 280 nM.

(E) S-type anion channel currents in a guard cell protoplast preincubated in 40 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered to 2 μM.

(F) Current-voltage relationships for S-type anion currents recorded from guard cell protoplasts preincubated in 40 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered at 280 nM (closed symbols; $n = 13$) or 2 μM (open symbols; $n = 8$) as in **(D)** and **(E)**.

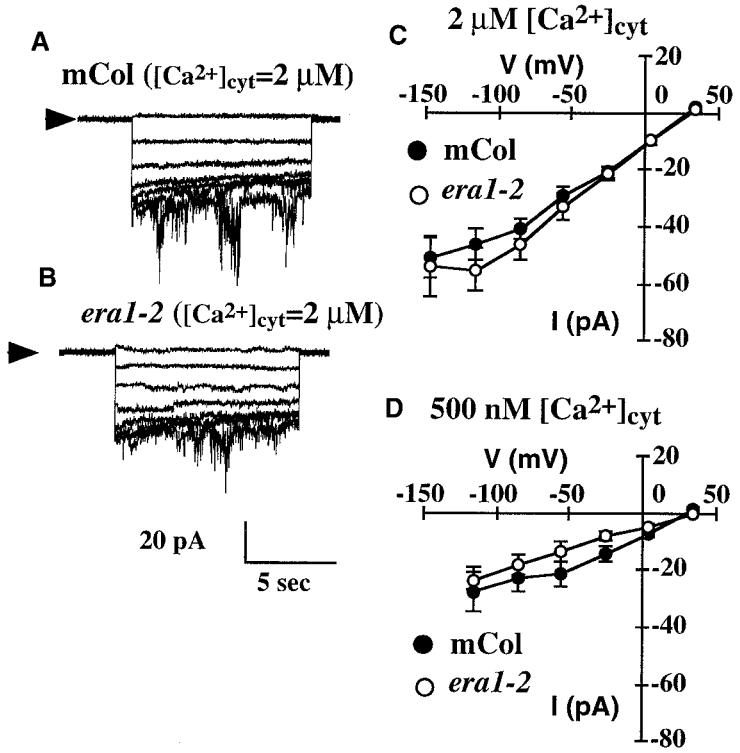

Figure 4. S-Type Anion Channel Activation by $[Ca^{2+}]_{cyt}$ Is Similar in Wild-Type and *era1-2* Guard Cell Protoplasts.

(A) S-type anion channel currents in a wild-type guard cell protoplast with $[Ca^{2+}]_{cyt}$ buffered to 2 μM.
(B) S-type anion channel currents in an *era1-2* guard cell protoplast with $[Ca^{2+}]_{cyt}$ buffered to 2 μM.
(C) Current-voltage relationships for S-type anion currents recorded from wild-type (closed symbols; $n = 8$) or *era1-2* (open symbols; $n = 8$) guard cell protoplasts with $[Ca^{2+}]_{cyt}$ buffered at 2 μM as in **(A)** and **(B)**.
(D) Current-voltage relationships for S-type anion currents recorded from wild-type (closed symbols; $n = 8$) or *era1-2* (open symbols; $n = 7$) guard cell protoplasts with $[Ca^{2+}]_{cyt}$ buffered to 500 nM.
In all experiments, protoplasts were preincubated in 40 mM $[Ca^{2+}]_{ext}$ and then perfused with 1 mM $[Ca^{2+}]_{ext}$ before patch clamping (see Results and Figure 2). mCol, Columbia.

To determine whether the hypersensitivity of ABA-induced $[Ca^{2+}]_{cyt}$ signals in *era1-2* (Figure 1C) or the insensitivity of ABA-induced $[Ca^{2+}]_{cyt}$ increases in *abi2-1* (Figure 6B) was epistatic in relation to ABA-induced $[Ca^{2+}]_{cyt}$ signals, we measured $[Ca^{2+}]_{cyt}$ and stomatal closure in the *era1-2 abi2-1* double mutant in response to low levels of ABA (0.1 μM). (Note that Landsberg *erecta*/Columbia cross-controls did not show any significant variation from either ecotype in ABA sensitivity in seed germination assays [data not shown].) In the majority of double mutant guard cells (69%; $n = 29$ from 42 cells tested), there was no induction of $[Ca^{2+}]_{cyt}$ transients after the application of 0.1 μM ABA (Figure 6C), although subsequent application of 10 mM $[Ca^{2+}]_{ext}$ as a control was able to induce $[Ca^{2+}]_{cyt}$ transients.

However, in some *era1-2 abi2-1* guard cells (31%; $n = 13$ from 42 cells tested), 0.1 μM ABA induced a transient increase in $[Ca^{2+}]_{cyt}$ (Figure 6D). In these responsive guard cells of the double mutant, a single $[Ca^{2+}]_{cyt}$ transient was induced in all cells that responded to 0.1 μM ABA ($n = 13$). Therefore, $[Ca^{2+}]_{cyt}$ signaling in guard cells of the double mutant showed a phenotype that was intermediate between those of the two mutant phenotypes (cf. Figures 1C and 6B).

The partial recovery of the *abi2-1* $[Ca^{2+}]_{cyt}$ signaling phenotype is reflected in ABA-induced stomatal closure in the double mutant. Under the same experimental conditions used for $[Ca^{2+}]_{cyt}$ imaging here, ABA-induced closure in *era1-2 abi2-1* was significantly greater than in *abi2-1* (Figure 6E) but less than in the wild type or in *era1-2* alone (Figures 1D and 6E) (see Pei et al. [1998] for other experimental conditions). These data support the notion that both *era1-2* and *abi2-1* mutations act upstream of $[Ca^{2+}]_{cyt}$ signaling in guard cells but that neither has a clear epistatic effect.

To test this finding further, $[Ca^{2+}]_{cyt}$ activation of S-type anion currents and $[Ca^{2+}]_{ext}$-induced stomatal closure were measured in the *era1-2 abi2-1* double mutant, as illustrated in Figure 7. S-type anion currents were activated (after a 40

mM $[Ca^{2+}]_{ext}$ preincubation, as in Figures 3 and 4) by $[Ca^{2+}]_{cyt}$ at 500 nM and 2 μM (Figure 7A), and activation was not significantly different from that in the wild-type or *era1-2* guard cell protoplasts (Figure 7B) ($n = 13$ *era1-2 abi2-1* guard cells; $P > 0.58$).

The addition of $[Ca^{2+}]_{ext}$ to preopened stomates can cause $[Ca^{2+}]_{cyt}$ increases and oscillations (McAinsh et al., 1995; Allen et al., 1999a, 2000) that cause stomatal closure. Stomatal closure induced by $[Ca^{2+}]_{ext}$ showed no dramatic differences between the wild type, *era1-2*, and the *era1-2 abi2-1* double mutant (Figure 7C), although interestingly, at 0.1 mM $[Ca^{2+}]_{ext}$, stomatal apertures in the *era1-2* mutant were consistently larger than at 0.01 or 1 mM (Figure 7C). The signaling mechanism underlying this phenotype is not known, but this finding suggests additional points of action of the FTase.

Together, the $[Ca^{2+}]_{ext}$-induced stomatal closure in the wild type, *era1-2*, and the *era1-2 abi2-1* double mutant (Figure 7C) and the calcium activation of anion currents (Figures 7A and 7B) reinforce the finding that the *era1-2* and *abi2-1* mutations both affect guard cell signaling upstream of $[Ca^{2+}]_{cyt}$.

DISCUSSION

The role of cytosolic calcium in controlling plant cell signal transduction is becoming more fully understood with the application of new molecular genetic and imaging techniques (Sanders et al., 1999; Rudd and Franklin-Tong, 2001). In particular, the ability to map the positions of various mutants in relation to the calcium signal is revealing interactions and relationships in defined signaling cascades (Allen and Schroeder, 2001). However, the identities of many signaling components remain to be identified, and their interactions with calcium-based signaling pathways remain unknown.

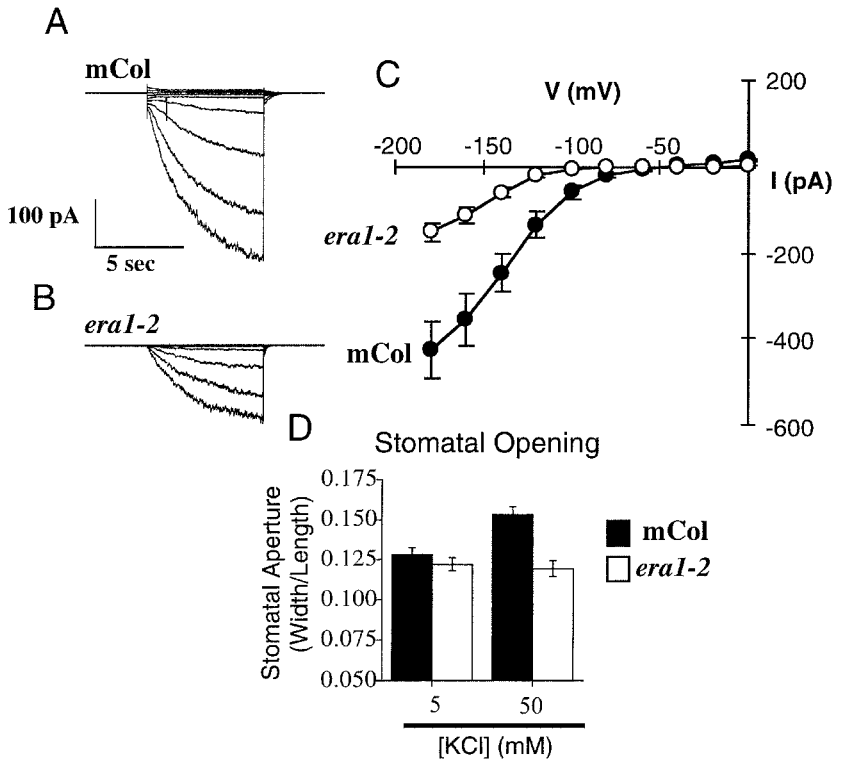

Figure 5. K^+_{in} Channel Current and Extracellular K^+-Dependent Stomatal Opening Are Reduced in *era1-2*.

(A) Representative K^+_{in} currents in a wild-type guard cell protoplast.

(B) Representative K^+_{in} currents in an *era1-2* guard cell protoplast.

(C) Current-voltage relationships for K^+_{in} currents recorded from wild-type (closed symbols; $n = 13$) or *era1-2* (open symbols; $n = 13$) guard cell protoplasts.

(D) Stomatal apertures measured after a 2.5-h opening period in the light in the wild type and *era1-2*. Apertures were measured in 5 mM KCl and 50 μM $CaCl_2$, pH 6.15, opening buffer (left bars) or in 50 mM KCl and 50 μM $CaCl_2$, pH 6.15, opening buffer (right bars). Data are means ± SEM relative to $n = 3$ replicates comprising 120 stomates per bar. mCol, Columbia.

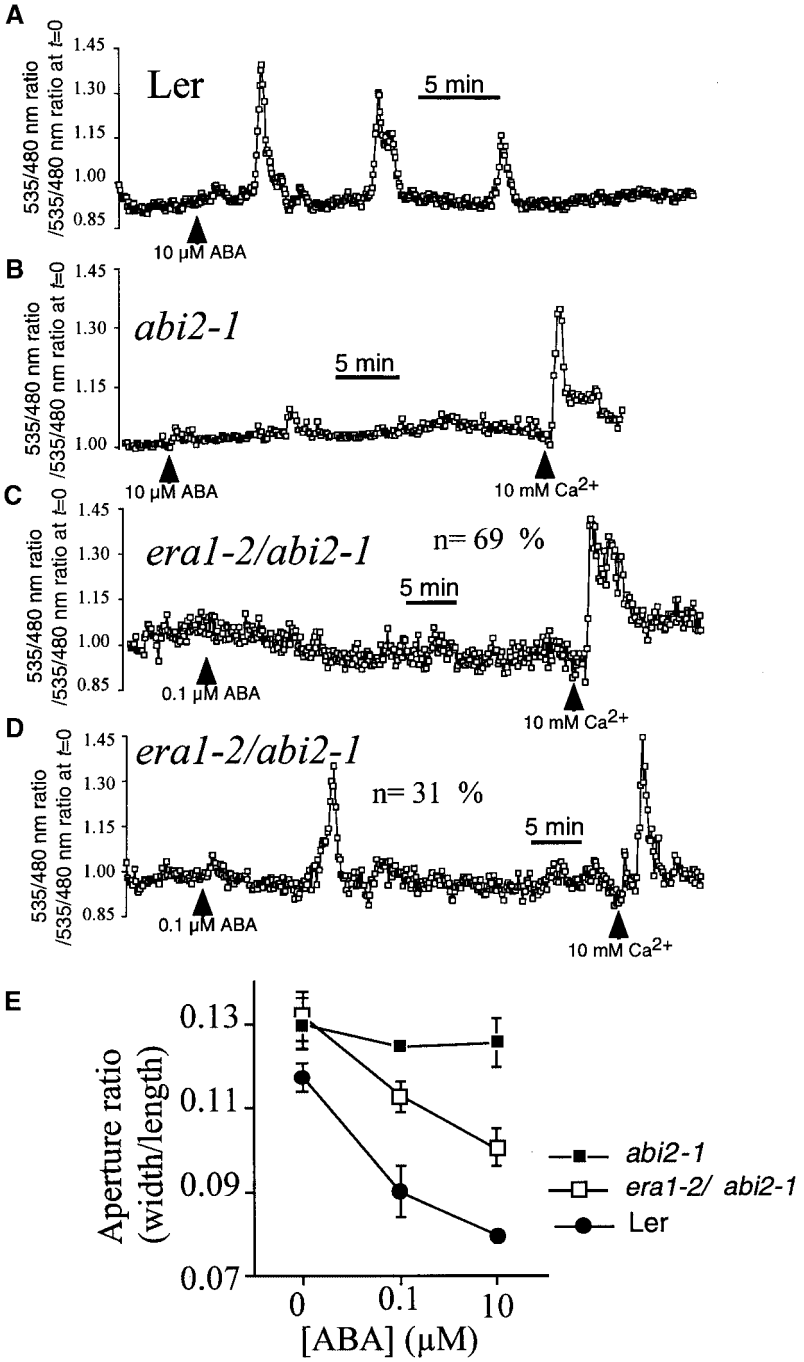

Figure 6. The *era1-2* Mutation Partly Restores ABA-Induced $[Ca^{2+}]_{cyt}$ Increases and Stomatal Closure in the *era1-2 abi2-1* Double Mutant.

(A) Repetitive $[Ca^{2+}]_{cyt}$ transients induced by 10 μM ABA in wild-type (Landsberg *erecta*) Arabidopsis guard cells expressing YC2.1.

(B) $[Ca^{2+}]_{cyt}$ transients are not induced by 10 μM ABA in *abi2-1* guard cells expressing YC2.1, although application of extracellular calcium elicits $[Ca^{2+}]_{cyt}$ transients.

(C) ABA at 0.1 μM fails to elicit $[Ca^{2+}]_{cyt}$ transients in the majority (69%; $n = 29$ from 42 cells) of *era1-2 abi2-1* guard cells, although application of extracellular calcium elicits $[Ca^{2+}]_{cyt}$ transients.

(D) ABA at 0.1 μM elicits single $[Ca^{2+}]_{cyt}$ transients in a fraction (31%; $n = 13$ from 42 cells) of *era1-2 abi2-1* guard cells.

(E) ABA-induced stomatal closure is impaired in the *abi2-1* mutant but is partly restored in the *era1-2 abi2-1* double mutant at 0.1 μM ABA using the same bathing solutions that were used for calcium imaging studies. Data are means ± SEM relative to three replicates comprising 120 stomates per point.

Ler, Landsberg *erecta*.

To date, five Arabidopsis mutants have been shown to affect guard cell calcium signaling. These mutants fall into three categories: the mutants *abi1-1* and *abi2-1* impair ABA-induced calcium oscillations (Allen et al., 1999a); the mutant *abh1* enhances the sensitivity of ABA-induced calcium signals (Hugouvieux et al., 2001); and the mutations *det3* and *gca2* affect the kinetics and dynamics of stimulus-induced calcium signals (Allen et al., 2000, 2001). In these mutants, the changes in the guard cell calcium signaling phenotypes correlate well with equivalent changes in stomatal movement and ion channel regulation phenotypes, underscoring the importance of cytosolic calcium signaling in the control of guard cell turgor.

In this study, we investigated the effect of the FTase deletion mutant *era1-2* on calcium signaling in guard cells. This mutation renders stomatal closure hypersensitive to the hormonal stimulus ABA (Pei et al., 1998) (Figure 1D). Here, we show that cytosolic calcium increases could be induced in this mutant at low ABA concentrations that were ineffective in the wild type (Figure 1).

Recent studies have suggested that calcium influx into guard cells across the plasma membrane plays a critical role in ABA-induced stomatal closure (Hamilton et al., 2000; MacRobbie, 2000; Pei et al., 2000). In particular, at high ABA concentrations (1 to 10 μM) in *Commelina communis*, guard cell Ca^{2+} influx predominated, whereas at low ABA concentrations (0.1 μM), internal calcium release was more prevalent (MacRobbie, 2000). These data correlate with only a small activation of I_{Ca} in wild-type Arabidopsis guard cells at 0.1 μM ABA (Figure 2A) and an increase in this activity in the *era1-2* mutant (Figure 2B).

Hyperpolarization-activated calcium channels that mediate Ca^{2+} influx have been identified in tomato suspension cells (Gelli and Blumwald, 1997), in Arabidopsis root cells (Kiegle et al., 2000; Véry and Davies, 2000), and in Arabidopsis and *Vicia* guard cells (Hamilton et al., 2000; Pei et al., 2000). The I_{Ca} channels in guard cells are activated by reactive oxygen species (ROS) (Pei et al., 2000), and ABA can generate ROS in both Arabidopsis (Pei et al., 2000) and *Vicia* (Zhang et al., 2001) guard cells and in maize embryos (Guan et al., 2000). In *era1-2* guard cells, activation of the I_{Ca} was hypersensitive to ABA, indicating that *era1-2* modulates signaling between ABA perception and the activation of ROS-sensitive I_{Ca} (Figure 2).

Our data do not exclude additional effects of ERA1 in modulating intracellular calcium release mechanisms (reviewed in Schroeder et al., 2001a). The *era1-2* mutant is hypersensitive to ABA, suggesting that the FTase deleted in this mutant acts to target a negative regulator of ABA signaling to the plasma membrane or to an ABA signaling complex in guard cells. Modulation of the calcium channel or a closely related protein is possible. The positioning of a negative regulator between ABA perception and I_{Ca} activation might act to prevent spurious I_{Ca} activation in response to low levels of ABA.

The activation of S-type anion currents by an increase of cytosolic calcium has been shown to be a component of guard cell turgor reduction (Schroeder and Hagiwara, 1989; Grabov and Blatt, 1998; Allen et al., 1999a; Leonhardt et al., 1999). To further test the positioning of *era1-2* in relation to cytosolic calcium in guard cells, we measured the activation of S-type anion channels in guard cells. S-type anion currents activated by calcium were identical in wild-type and *era1-2* guard cells, reinforcing the finding that *era1-2* acts upstream of cytosolic calcium increases and that mechanisms downstream of $[Ca^{2+}]_{cyt}$ remain largely unaffected by *era1-2*.

A previous report has shown that S-type anion current activation in *era1-2* guard cell protoplasts was hypersensitive to ABA (Pei et al., 1998) when protoplasts were preincubated in ABA and subsequently patch clamped in the presence of ABA and cytosolic calcium was buffered at the intermediate level of 280 nM, as described by Pei et al. (1997). In the present study, ABA was applied to guard cells 15 to 30 min after whole-cell configurations had been achieved to measure rapid events in ABA-induced calcium signaling. With these shorter ABA treatments, I_{Ca} were activated to a greater extent in the *era1-2* mutant (Figure 2), showing that ERA1 acts upstream of calcium influx.

These data suggest two possible explanations for the ABA-hypersensitive anion channel phenotypes observed previously (Pei et al., 1998). Cytosolic calcium increases occur in protoplasts preexposed to ABA and result in the ABA-hypersensitive activation of anion channels, even though the calcium is buffered subsequently to 280 nM during the patch clamp experiments (Pei et al., 1997), and/or anion channel activation may occur via a calcium-independent pathway that has been proposed (Allan et al., 1994; Li et al., 2000), and this pathway also may be hypersensitive to ABA in *era1-2* guard cells. To unequivocally characterize parallel Ca^{2+}-independent pathways, Arabidopsis mutants or experimental conditions that show no ABA-induced $[Ca^{2+}]_{cyt}$ increases but that continue to show stomatal closure would be required.

Interestingly, a new component of the regulation of S-type anion currents by calcium was revealed in this study (Figure 3). Cytosolic calcium activated S-type currents most effectively when guard cells were preincubated in high external calcium. High extracellular calcium concentrations are known to cause $[Ca^{2+}]_{cyt}$ oscillations in guard cells (McAinsh et al., 1995; Allen et al., 2000), and $[Ca^{2+}]_{cyt}$ oscillations program long-term stomatal closure (Allen et al., 2001). The external calcium enhancement of S-type anion current activation by $[Ca^{2+}]_{cyt}$ may be linked to the programming of stomatal responses. Importantly, the requirement for $[Ca^{2+}]_{ext}$ incubation found here provides a new and powerful approach for dissecting mechanisms and mutations that affect the $[Ca^{2+}]_{cyt}$ sensitivity and regulation of S-type anion channels.

Another new effect of the *era1-2* mutant on guard cell K^+_{in} channel activity also was characterized here (Figure 5). K^+_{in} currents were significantly lower in *era1-2* compared with

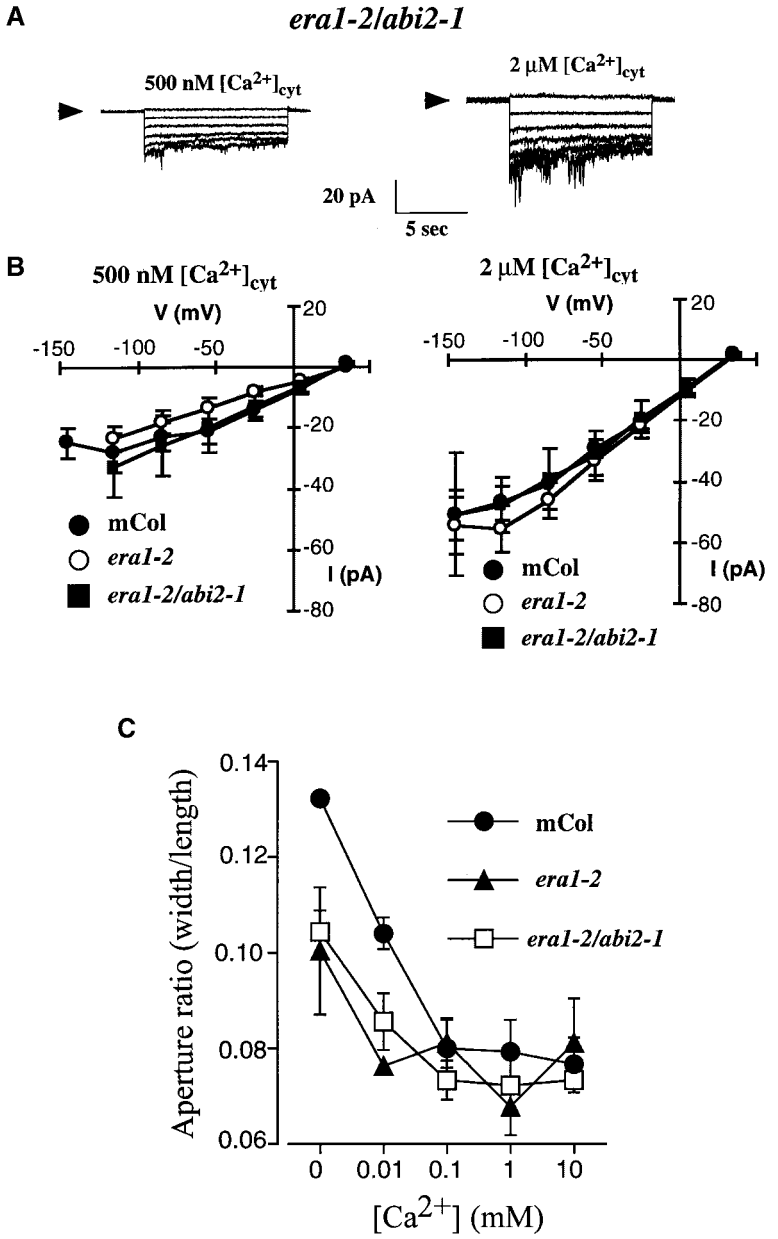

Figure 7. S-Type Anion Channel Activation by $[Ca^{2+}]_{cyt}$- and $[Ca^{2+}]_{ext}$-Induced Stomatal Closure Is Not Impaired in the *era1-2 abi2-1* Double Mutant.

(A) S-type anion channel currents in *era1-2 abi2-1* double mutant guard cell protoplasts preincubated in 40 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered to 500 nM (left) and 2 μM (right).

(B) Current-voltage relationships for S-type anion currents recorded from wild-type (closed circles), *era1-2* (open circles), or *era1-2 abi2-1* double mutant (closed squares) guard cell protoplasts preincubated in 40 mM $[Ca^{2+}]_{ext}$ with $[Ca^{2+}]_{cyt}$ buffered at 2 μM (right) or 500 nM (left) as in **(A)**. The wild-type and *era1-2* data are as presented in Figures 4C and 4D and are shown for direct comparison with *era1-2 abi2-1* data.

(C) $[Ca^{2+}]_{ext}$-induced stomatal closure in the wild type (closed circles), *era1-2* (closed triangles), and *era1-2 abi2-1* double mutant (open squares). Note that stomata were opened in buffer containing 5 mM KCl and no $CaCl_2$, pH 6.15, and that in the absence of $[Ca^{2+}]_{ext}$, *era1-2* and *era1-2 abi2-1* double mutant stomates failed to open to the same aperture as the wild type. Data are means ± SEM relative to three replicates comprising 120 stomates per point.

mCol, Columbia.

wild-type guard cells. This was not observed to have an effect on stomatal aperture under the imposed conditions of low extracellular K^+ and Ca^{2+} concentrations (Figure 1D), but it led to reduced apertures relative to those in the wild type in *era1-2* at higher extracellular K^+ (Figure 5) (Pei et al., 1998) or at low extracellular K^+ in the absence of extracellular calcium (Figure 7C).

The idea that ERA1 acts upstream of $[Ca^{2+}]_{cyt}$ was further strengthened by analyzing *era1-2* and *abi2-1* interactions. The hypersensitivity of ABA-induced cytosolic calcium increases in *era1-2* guard cells could partly rescue the impairment of ABA-induced calcium increases in the *abi2-1* protein phosphatase type 2C mutant (Figure 6). These data correlate with the intermediate ABA-induced stomatal closing phenotype in the *era1 abi2* double mutant (Pei et al., 1998) (Figure 6E). Double mutant analyses presented here show that both of these mutants act upstream of calcium in guard cells. The intermediate phenotype of the double mutant may be explained in part by the genetic dominance of *abi2-1*. This double mutant analysis suggests that the signaling pathway between ABA perception and calcium increases is complex and contains many distinct signaling elements.

Overall, the data presented in this study demonstrate how a combination of genetics and a multifaceted cell biological approach can be a powerful tool for dissecting signal transduction pathways and placing mutations at specific points in defined signaling networks. For the *era1-2* mutant, this analysis has placed the action of this mutant in guard cells at a point downstream of ABA perception and upstream of plasma membrane calcium influx channel activation.

METHODS

Plant Growth

Arabidopsis thaliana plants 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-h-light/8-h-dark cycle at a photon fluence rate of 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of 20°C. Pots were watered every 2 to 3 days with deionized water. The wild-type background was Columbia for the *era1-2* mutant and Landsberg *erecta* for *abi2-1*. The *era1-2* mutant line used in this study had been backcrossed previously into the wild type (Columbia) three times (Cutler, 1995). All mutants and wild types were transformed with yellow cameleon 2.1 (YC2.1) by direct *Agrobacterium tumefaciens* transformation using floral dip (Allen et al., 1999b). At least three independent YC2.1-transformed lines were used for each wild type and mutant, except for the *era1-2 abi2-1* double mutant, for which only two lines were available.

Calcium Imaging

Calcium imaging was performed as described previously using Arabidopsis expressing the green fluorescent protein–based calcium indicator YC2.1 (Allen et al., 1999b, 2001). To open stomata, abaxial epidermal strips were incubated for 2.5 h in the light (125 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in 5 mM KCl, 50 μM $CaCl_2$, and 10 mM Mes-Tris, pH 6.15, before cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) measurements. In all ecotypes and mutants analyzed, ~30% of all guard cells exhibited spontaneous oscillations in $[Ca^{2+}]_{cyt}$. These cells were not included in the analysis; only cells exhibiting a stable resting $[Ca^{2+}]_{cyt}$ level were used to analyze stimulus-induced $[Ca^{2+}]_{cyt}$ increases. (Note that because measurements of $[Ca^{2+}]_{cyt}$ in this study were collected from two different microscopes that used charge-coupled device cameras with different 480-nm sensitivities, the 535:480-nm ratios were normalized by dividing all measured ratios in any given recording by the initial, steady state ratio from that recording that represents prestimulus $[Ca^{2+}]_{cyt}$). The yellow fluorescent protein of YC2.1 bleaches at a faster rate than the cyan fluorescent protein, leading to a slow decline in baseline 535:480-nm ratios. Baseline decline was eliminated by a linear correction factor that was calculated from the rate of decrease in baseline ratio before the application of a stimulus.

Aperture Measurements

Stomatal apertures were measured using whole leaves as described previously (Pei et al., 1997; Allen et al., 2000). To open stomates, leaves were floated for 2.5 h (at light intensity of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 20°C) on buffer containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM Mes-Tris, pH 6.15, for abscisic acid (ABA)–induced stomatal closure experiments, or 5 mM KCl and 10 mM Mes-Tris, pH 6.15, for $[Ca^{2+}]_{ext}$-induced closure experiments. After 2.5 h, ABA or $CaCl_2$ was added as indicated, and incubation was continued for another 3 h. Then, leaves were blended briefly (15 s) in the same opening buffer in a Waring blender, and the epidermal fragments were collected on a 30-μm (pore size) nylon mesh. Fragments were washed gently onto a microscope slide with 500 μL of opening buffer and covered with a cover slip, and aperture size was measured (pore width/length) by focusing on the focal plane of guard cells in epidermal strips, as described previously (Allen et al., 1999a).

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 as described previously (Pei et al., 1997; Allen et al., 1999a).

To measure calcium influx currents (I_{Ca}), the pipette solution contained 10 mM $BaCl_2$, 0.1 mM DTT, 4 mM EGTA, and 10 mM Hepes-Tris, pH 7.1, and the bath solution contained 100 mM $BaCl_2$, 0.1 mM DTT, and 10 mM Mes-Tris, pH 5.6. NAD(P)H (1 mM) was added to the pipette for the measurement of ABA-activated I_{Ca} activity. The addition of 5 mM NAD(P)H to the pipette (Pei et al., 2000; Murata et al., 2001) caused unstable, bursting activity of background I_{Ca} in the absence of ABA in the present study, particularly in the *era1-2* mutant (data not shown). The voltage was ramped from +30 mV to −196 mV and back to +30 mV in 3 s. Sixteen ramps were applied at 40-s intervals before ABA addition and after the activated currents had stabilized. ABA was added 15 to 30 min after whole cell configurations had been achieved.

To measure S-type anion currents, the pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5 mM MgATP, 10 mM Hepes-Tris, pH 7.1, and a concentration of $CaCl_2$ calculated to give

the desired free Ca^{2+} concentration. Free calcium concentrations were calculated with the program CALCIUM (Foehr et al., 1993). The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.6. The liquid junction potential in this solution was 0.45 mV. Preincubation in 1 or 40 mM $[Ca^{2+}]_{ext}$ was for 30 to 60 min in either standard bath solution (1 mM $CaCl_2$) or bath solution supplemented with 39 mM $CaCl_2$. (Note that gigaohm seal formation and current recording were performed in the 1 mM $CaCl_2$ standard bath solution.) Steady state currents were sampled during the last 3 s of voltage pulses.

The standard voltage protocol stepped the voltage from a holding potential of +30 mV to −145 mV for 40 s. Subsequent voltage steps were reduced by 30 mV per pulse. The interpulse period was 12 s. No leak subtraction was made. All recordings were made 7 to 10 min after access to the whole cell configuration. There was no preincubation of protoplasts with ABA in these experiments (in contrast to the procedure reported by Pei et al. [1997, 1998]).

To measure inwardly rectifying K^+ currents, the pipette solution contained 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$ 6.7 mM EGTA, 3.35 mM $CaCl_2$, 5 mM Mg-ATP, and 10 mM Hepes-Tris, pH 7.1. The bath solution contained 30 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Mes-Tris, pH 5.6. From a holding potential of 0 mV, the voltage was stepped to −180 mV in −20-mV increments for 5 s each. Steady state current was sampled in the last 100 ms of these voltage steps.

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