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Differential Abscisic Acid Regulation of Guard Cell Slow Anion Channels in Arabidopsis Wild-Type and *abi1* and *abi2* Mutants

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Abscisic acid (ABA) regulates vital physiological responses, and a number of events in the ABA signaling cascade remain to be identified. To allow quantitative analysis of genetic signaling mutants, patch–clamp experiments were developed and performed with the previously inaccessible Arabidopsis guard cells from the wild type and ABA-insensitive (*abi*) mutants. Slow anion channels have been proposed to play a rate-limiting role in ABA-induced stomatal closing. We now directly demonstrate that ABA strongly activates slow anion channels in wild-type guard cells. Furthermore, ABA-induced anion channel activation and stomatal closing were suppressed by protein phosphatase inhibitors. In *abi1-1* and *abi2-1* mutant guard cells, ABA activation of slow anion channels and ABA-induced stomatal closing were abolished. These impairments in ABA signaling were partially rescued by kinase inhibitors in *abi1* but not in *abi2* guard cells. These data provide cell biological evidence that the *abi2* locus disrupts early ABA signaling, that *abi1* and *abi2* affect ABA signaling at different steps in the cascade, and that protein kinases act as negative regulators of ABA signaling in Arabidopsis. New models for ABA signaling pathways and roles for *abi1*, *abi2*, and protein kinases and phosphatases are discussed.

INTRODUCTION

The plant hormone abscisic acid (ABA) regulates many vital processes, including seed maturation, control of vegetative growth, promotion of dormancy, and the adaptation of plants to environmental stresses such as drought conditions (Zeevaart and Creelman, 1988; Mansfield et al., 1990; Hetherington and Quatrano, 1991). In response to drought stress, ABA causes closing of stomatal pores, which are formed by pairs of guard cells in the leaf epidermis, resulting in a decrease in transpirational water loss (Raschke, 1979; MacRobbie, 1981). Guard cells provide a powerful system to dissect early signal transduction mechanisms because they respond to diverse hormonal, light, and other environmental stimuli. In guard cells, the early events in ABA signal transduction after receptor activation involve ion channel regulation, cytosolic Ca^{2+} changes, and intracellular coupling mechanisms. Patch–clamp studies have led to the identification of a number of ion channel types in the plasma membrane and vacuolar membrane of guard cells that can function in unison to inhibit stomatal opening and mediate stomatal closing (for model and reviews, see Schroeder and Hedrich, 1989; MacRobbie, 1992; Assmann, 1993; Ward et al., 1995). However, many mechanisms of the early ABA signaling cascade remain to be identified, and the sequence of early intracellular coupling events is a topic of current interest.

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Biophysical, cell biological, pharmacological, and second messenger regulation studies have suggested that activation of slow anion channels in guard cells can function as a rate-limiting step in stomatal closing (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992; Schmidt et al., 1995). When slow (S-type) anion channels are activated, the resulting sustained efflux of anions from guard cells would produce long-term depolarization (Schroeder and Keller, 1992). ABA has been shown to cause long-term depolarizations in guard cells (Ishikawa et al., 1983; Thiel et al., 1992). Depolarization in turn can activate outward-rectifying K^+ channel currents, which mediate K^+ efflux (Schroeder et al., 1987). Furthermore, the activity of outward-rectifying K^+ channel currents is enhanced by ABA via cytosolic alkalization (Blatt, 1990, 1992; Lemtiri-Chlieh and MacRobbie, 1994). The resulting simultaneous efflux of K^+ and anions lowers the turgor and the volume of guard cells, resulting in closure of stomatal pores (Schroeder and Hedrich, 1989; Hetherington and Quatrano, 1991).

Patch–clamp studies with broad bean guard cells have shown that slow anion channels are strongly activated by elevation in cytosolic Ca^{2+} and by phosphorylation events (Schroeder and Hagiwara, 1989; Schmidt et al., 1995). The close correlation of slow anion channel and K^+ channel regulation and ABA signaling in guard cells by phosphorylation events (Armstrong et al., 1995; Schmidt et al., 1995) and the identification of the *ABI1* gene as a protein phosphatase 2C

(Leung et al., 1994; Meyer et al., 1994) suggest that phosphorylation and dephosphorylation events are important components of early ABA signaling. The rapid (R-type) guard cell anion channels, which activate transiently, are stimulated in a different manner: stimulation is by cytosolic nonhydrolyzable nucleotides (Hedrich et al., 1990). The observed large dynamic range of S-type anion channel regulation in guard cells from near complete inactivity to stimulation of 200 to >1000 channels per cell corresponds to the strong upregulation and downregulation predicted during stomatal movements (Schroeder and Hagiwara, 1989; Schmidt et al., 1995). However, direct evidence for ABA activation of slow anion channels is lacking; therefore, the question of whether slow anion channels are stimulated during ABA signaling remains unanswered. Furthermore, the sequence of events in the upstream signaling cascade remains unknown. The ability to use Arabidopsis signaling mutants together with high resolution patch–clamp analysis of ion channels in guard cells that are proposed targets of early signaling mechanisms would provide a potent system to elucidate upstream events.

Mutations in two Arabidopsis loci, *abi1* and *abi2*, produce ABA insensitivity with respect to all studied ABA responses, suggesting that these mutations could affect early ABA signal transduction events (Koornneef et al., 1984; Finkelstein and Somerville, 1990). The structure and roles of the *abi2* locus remain unknown. The *ABI1* gene was shown to encode a protein phosphatase 2C–type homolog (Leung et al., 1994; Meyer et al., 1994). Transgenic tobacco expressing the Arabidopsis *abi1-1* dominant mutant cDNA showed a two- to sixfold reduction in outward K^+ channel currents and an insensitivity of K^+ currents to ABA (Armstrong et al., 1995). K^+ channel regulation was partially restored by protein kinase inhibitors (Armstrong et al., 1995). However, in transgenic tobacco, no differences in the rate-limiting anion current were observed between control and *abi1-1*–transformed plants (Armstrong et al., 1995). Direct patch–clamp analysis of Arabidopsis guard cells has been hampered by their small size and inaccessibility. Therefore, we developed an approach that reproducibly allows patch–clamp recordings in Arabidopsis guard cells, and here, we reveal major differences in the mechanisms underlying ABA control of slow anion channel signaling in the wild type and *abi1* and *abi2* mutants. Based on results presented here, new elements and sequences of the early ABA signal transduction cascade are proposed.

RESULTS

K^+ Channels in Wild-Type and *abi* Arabidopsis Guard Cells

Patch–clamp techniques were developed for Arabidopsis guard cells, and results from 247 cells are reported here. Initial patch–clamp experiments were performed with Arabidopsis guard cells by using K^+ salt solutions to determine whether inward-rectifying K^+ (K^+_{in}) channel currents exist similar to those identified in guard cells of broad bean (Schroeder et al., 1987). Figure 1A shows typical, large K^+_{in} channel currents recorded in wild-type Arabidopsis guard cells (Figure 1; $n = 62$). The presence of saturating concentrations of ABA (50 μM), in both cytosolic and bath solutions in the same cells, did not greatly affect the magnitude of steady state K^+_{in} channel currents under the imposed conditions for the reasons discussed below (Figures 1B and 1C). Patch–clamp analysis of guard cells isolated from both the *abi1* and *abi2* mutants (Koornneef et al., 1984) also showed typical K^+_{in} channel currents, although *abi2* guard cells consistently showed smaller K^+_{in} channel currents under the imposed conditions (Figures 1D and 1G).

On average there was no significant ABA regulation of K^+_{in} channels in both *abi1* and *abi2* guard cells under the conditions used in this study (Figures 1D to 1I). Small differences in the time courses of K^+_{in} current activation fall within the observed experimental variation. However, we have observed regulation of both inward- and outward-rectifying K^+ channels by cytosolic Ca^{2+} and cytosolic pH in Arabidopsis guard cells (Z.-M. Pei and J.I. Schroeder, unpublished data; $n = 15$) (Schroeder and Hagiwara, 1989; Blatt, 1992). Note that the pipette solution used in Arabidopsis guard cell experiments sufficiently buffered cytosolic Ca^{2+} and pH levels in the small patch-clamped Arabidopsis guard cells to suppress ABA regulation, as demonstrated in broad bean guard cells by Lemtiri-Chlieh and MacRobbie (1994). High concentrations of Ca^{2+} and pH buffers were used here (see Methods). Therefore, these findings correlate with previous studies on ABA regulation of K^+ channels showing abolition of this ABA response by strong cytosolic buffering, suggesting the need for cytosolic Ca^{2+} and pH changes during ABA regulation of K^+ channels (Lemtiri-Chlieh and MacRobbie, 1994).

K^+_{in} channel current magnitudes were consistently smaller in *abi2* guard cells when compared with wild-type and *abi1* guard cells under the imposed conditions (Figures 1G to 1I). This finding in *abi2* guard cells appears to lie in contrast to the enhanced stomatal opening phenotype of ABA-insensitive mutants (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Roelfsema and Prins, 1995). However, previous studies have suggested that K^+_{in} current magnitudes are sufficient but usually not rate limiting for stomatal movements (Schroeder et al., 1987; Kelly et al., 1995). Average rates of physiological K^+ influx during stomatal opening correspond to ~8 pA of K^+_{in} current in broad bean, whereas absolute K^+_{in} current activities in guard cells usually lie within a range from 100 to >500 pA in broad bean and Arabidopsis guard cells (Figure 1).

We conclude that patch–clamp studies with Arabidopsis guard cells are feasible, that K^+_{in} channel currents similar to those identified and characterized in broad bean guard cells also prevail in Arabidopsis (Figure 1), and that experimental

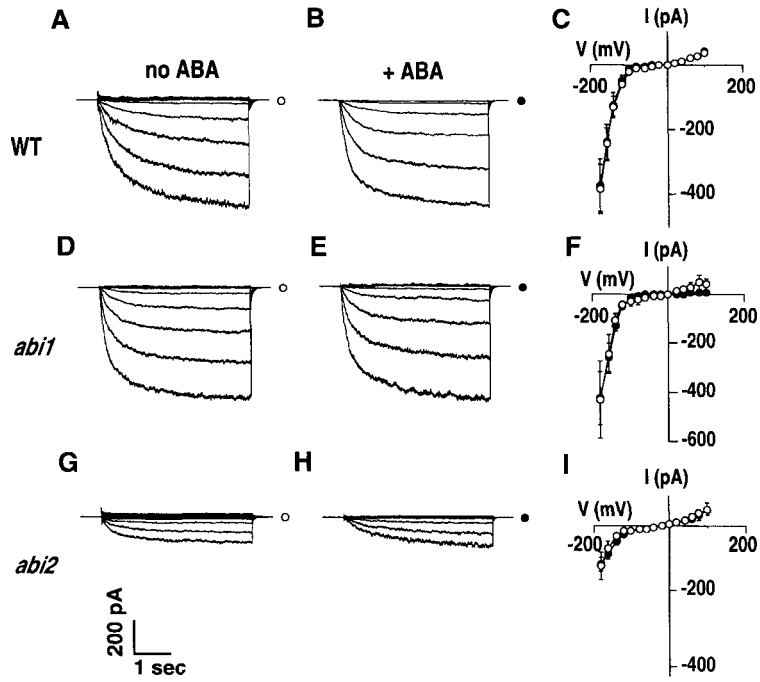

Figure 1. Patch–Clamp Recordings of K^+_{in} Channel Currents from Arabidopsis Wild-Type, *abi1*, and *abi2* Guard Cells in the Absence and Presence of ABA.

(A), **(D)**, and **(G)** K^+_{in} channels recorded in the absence of ABA (no ABA) in wild-type (WT), *abi1*, and *abi2* guard cells, respectively.
(B), **(E)**, and **(H)** K^+_{in} channels recorded in the presence of 50 μM ABA (+ABA) in the cytosol and bath in wild-type, *abi1*, and *abi2* guard cells, respectively.
(C), **(F)**, and **(I)** Average current–voltage relationships as recorded in the absence (○) and presence (●) of 50 μM ABA in wild-type, *abi1*, and *abi2* guard cells, respectively.
In all recordings, membrane potentials were stepped from a holding potential of −40 mV to potentials ranging from +100 to −180 mV in 20-mV increments. K^+ salt solutions were used for K^+_{in} channel current recordings (see Methods). Data are the mean ± SE (n = 5 to 14 for each condition; data are from a total of 62 cells).

conditions with weaker cytosolic buffering are needed to study ABA regulation of K^+_{in} channels in Arabidopsis guard cells, as has been demonstrated to be necessary for regulation in broad bean guard cells (Blatt, 1992; Lemtiri-Chlieh and MacRobbie, 1994; Schwartz et al., 1994; Kelly et al., 1995). We emphasize that the results shown in Figure 1 do not contradict studies showing ABA regulation of K^+ channels but allow a comparison with ABA coupling to anion channels in Arabidopsis guard cells under these strong cytosolic buffering conditions, as presented in the following.

ABA Activates Anion Channel Currents in Wild-Type Arabidopsis Guard Cells

The activity of S-type anion channels in guard cells has been proposed to be essential for stomatal closing (Schroeder and Hagiwara, 1989). The dynamic range of upregulation or downregulation of these anion channels is very large. However, direct evidence for activation of guard cell anion channels by ABA is lacking. To address this question directly, the effects of ABA on anion channels were analyzed in Arabidopsis guard cells in both the wild type and the ABA-insensitive mutants *abi1* and *abi2* (n = 170).

To determine whether anion channel currents similar to those described in broad bean guard cells could also be found in Arabidopsis guard cells, initial experiments were conducted using experimental conditions that produce activation of large slow anion currents in broad bean guard cells (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992). The membrane potential was held at the anion equilibrium potential and subsequently stepped to membrane voltages ranging from −145 to +35 mV, as shown in Figure 2A (inset at top). However, under these recording conditions, only a small, nearly linear background conductance was found in Arabidopsis guard cells (Figures 2A and 2C; n = 37). These data showed that significant anion currents were not activated under the imposed conditions (Figure 2A).

Subsequent experiments were performed with guard cells under the same conditions as above; however, ABA was

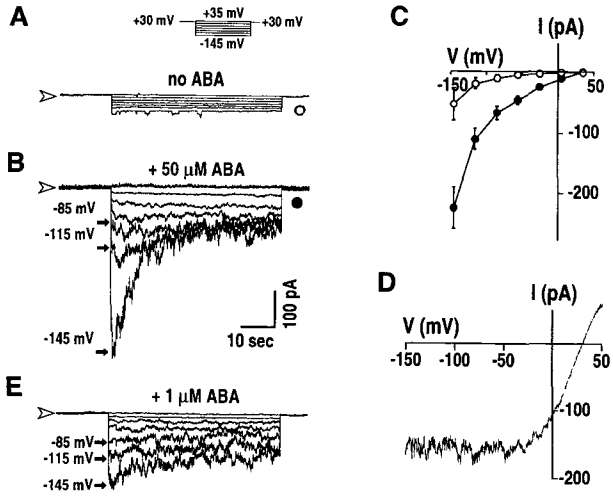

Figure 2. ABA Activates Slow Anion Channel Currents in Wild-Type Arabidopsis Guard Cells.

(A) Whole-cell recordings of currents in a wild-type Arabidopsis guard cell in the absence of ABA (no ABA).

(B) Whole-cell recording of currents in a wild-type Arabidopsis guard cell in the presence of 50 μM ABA (+ 50 μM ABA).

(C) Average peak current–voltage relationships from experiments as recorded in **(A)** (○; n = 12) and **(B)** (●; n = 15). Data are the mean ± SE.

(D) Steady state voltage dependence of ABA-activated Arabidopsis slow anion channel currents. The voltage ramp was from +50 to −150 mV at 3 mV/sec.

(E) Whole-cell recording of currents in a wild-type Arabidopsis guard cell in the presence of 1 μM ABA (+1 μM ABA).

Open arrows show zero current levels. The voltage protocol for **(A)**, **(B)**, and **(E)** is shown in the top inset in **(A)** and was used for all anion channel current measurements in this study. ABA was applied to both the cytosol and bath solutions in Figures 1 to 7. For anion channel current recordings in this study, CsCl solutions were used as given in Methods, unless indicated otherwise.

added to both the bath and cytosolic solutions. In the presence of ABA on both sides of the membrane, large whole-cell currents were observed at physiological membrane potentials (Figures 2B and 2C). Both peak currents and steady state currents were consistently increased by ABA (Figures 2B and 2C). When the membrane was stepped to potentials more negative than −100 mV, whole-cell currents showed a slow relaxation (Figure 2B). The slow relaxation at hyperpolarized membrane potentials (Figure 2B) and steady state current voltage properties (Figure 2D) were analogous to properties described for slow anion channels in broad bean guard cells (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992).

Experiments were designed to determine whether the ABA-activated whole-cell currents (Figures 2B and 2C) were carried by anions. Guard cell currents activated by ABA reversed at +29.3 ± 4.2 mV (n = 6; Figure 2D), which corresponded closely to the imposed equilibrium potential for Cl^- ions (E_{Cl^-} = +32.5 mV). When the Cl^- equilibrium potential was shifted to E_{Cl^-} = 0 mV, the reversal potential of guard cell currents shifted to 0 mV (n = 6; data not shown). These data showed that the whole-cell currents in Arabidopsis guard cells were carried by Cl^- ions. Furthermore, these data provide direct evidence that ABA activates slow anion channels in guard cells (Figures 2A to 2C).

Activation of anion channels in Arabidopsis guard cells was found for ABA concentrations in the range of 1 (n = 7) to 50 (n = 21) μM ABA. An example of ABA activation of slow anion channels in Arabidopsis guard cells at 1 μM ABA is shown in Figure 2E. At both 1 and 50 μM ABA, a slow decline in guard cell anion channel activity was observed after gaining access to the cytosol with patch–clamp electrodes. ABA-induced peak currents decreased to 79.2 ± 8.7% (n = 5) of initial levels during 30-min recordings, indicating effects of slowly diffusible factors (Pusch and Neher, 1988).

Activation of Slow Anion Channels by ABA Is Strongly Impaired in *abi1* and *abi2* Mutants

The two Arabidopsis mutant loci *abi1* and *abi2* produce ABA insensitivity with respect to many ABA responses (Koornneef et al., 1984; Finkelstein and Somerville, 1990). To determine whether ABA activation of slow anion channels in guard cells is affected by these mutations, patch–clamp recordings of guard cell protoplasts isolated from *abi1-1* and *abi2-1* plants were pursued. To saturate the ABA response when studying ABA-insensitive mutants, we performed experiments in the presence of 50 μM ABA in both the cytosolic and extracellular solutions in the same cells. In response to steady state ABA application over physiological exposure time, only small background currents were found in *abi1* guard cells both in the absence (Figure 3A; n = 7) and presence (Figure 3B; n = 7) of ABA. Average current–voltage curves of seven guard cells for each condition showed that no ABA activation of guard cell currents was found under these conditions (Figure 3C). Similar results to *abi1* were found in *abi2* guard cells in the absence (Figure 3D; n = 7) and presence (Figure 3E; n = 5) of ABA. Interestingly, the finding that exposure of *abi2* guard cells to 50 μM ABA also did not activate slow anion currents (Figure 3F) provides cell biological evidence that *abi2* affects the very early ABA signaling cascade. These studies show that ABA activation of anion channel currents is strongly repressed in both *abi1* and *abi2* mutants.

A recent study on ABA signaling in transgenic tobacco plants carrying the Arabidopsis *abi1-1* mutant transgene suggested that *abi1* may not effect anion channel activities in these transformants (Armstrong et al., 1995). However, ABA-induced anion efflux current densities in control wild-type tobacco guard cells were only 6 μA cm^{-2} at −60 mV when using 200 mM K^+ acetate-filled electrodes (Armstrong

et al., 1995). These currents correspond to only ~9% of the slow anion channel density observed at −60 mV in Arabidopsis guard cells (~64 μA cm^{-2}; Figure 2). Use of acetate in microelectrodes has been previously shown to abolish Ca^{2+}-activated sustained depolarizations in guard cells (see Figure 1 and pages 276 and 278 in Blatt, 1987), which had suggested to us that cytosolic acetate may inhibit the activity of the Ca^{2+}-dependent S-type anion channels in guard cells (Schroeder and Hagiwara, 1989). To test this possibility, we perfused Arabidopsis guard cells with pipette solutions containing 120 mM CsCl plus 30 mM Cs–acetate, as shown in Figure 4 (n = 3). Under these conditions, the ABA-activated slow anion channels were abolished, demonstrating that in the presence of cytosolic acetate, resolution of guard cell anion channel activities is limited (Figure 4A). Note that cytosolic acetate did not influence K^+ channels, as demonstrated by replacing cesium in the bath solution with K^+ in the same cells (Figure 4B). The lack of large anion channel activities in tobacco and broad bean guard cells by using acetate in electrodes may therefore be attributable to acetate inhibition. Not accounting for the acetate inhibition could lead to models that might underestimate the relative importance of anion channels in guard cell signaling.

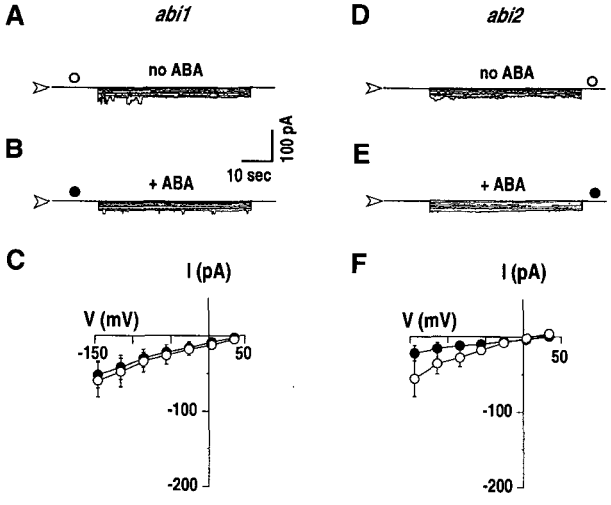

Figure 3. ABA Does Not Activate Slow Anion Channels in Either *abi1* or *abi2* Guard Cells.

(A) Whole-cell recording of currents in an *abi1* guard cell in the absence of ABA (no ABA).

(B) Whole-cell recording of currents in an *abi1* guard cell in the presence of 50 μM ABA (+ ABA).

(C) Average peak current–voltage relationships for *abi1* guard cells in the absence (○; n = 7) and the presence (●; n = 7) of 50 μM ABA. Recordings were performed as given in **(A)** and **(B)**.

(D) Whole-cell recording of currents in an *abi2* guard cell in the absence of ABA.

(E) Whole-cell recording of currents in an *abi2* guard cell in the presence of 50 μM ABA.

(F) Average peak current–voltage relationships of *abi2* guard cells recorded in the absence (○; n = 7) and presence (●; n = 5) of 50 μM ABA. Recordings were performed as given in **(D)** and **(E)**. Open arrows show zero current levels. Data in **(C)** and **(F)** are the mean ± SE.

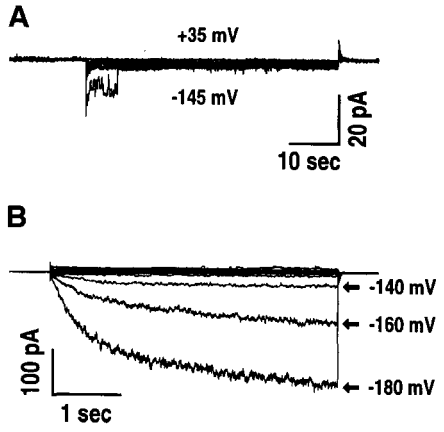

Figure 4. Cytosolic Acetate Inhibits Slow Anion Channel Activity in Wild-Type Arabidopsis Guard Cells.

(A) Acetate inhibits ABA-activated slow anion channel currents in wild-type Arabidopsis guard cells. The pipette solution contained 120 mM CsCl, 30 mM Cs-acetate, and 50 μM ABA. Other conditions and components in both pipette and bath solutions were identical to those given in Figure 2B (see Methods).

(B) Acetate does not affect K^+_{in} channels. After whole-cell current recordings in **(A)** were completed, 30 mM CsCl in the bath solution was replaced by bath perfusion with K^+ salt solution in Figure 1 (see Methods). Note that similar to cesium in broad bean guard cells, cesium in the Arabidopsis cytosol does not abolish inward K^+ channel currents, because cytosolic Cs^+ only abolishes unidirectional outward K^+ channel currents. The voltage protocol is as given in the legend to Figure 1.

The strong degree of impairment of ABA-induced activation of anion channels in both *abi1* and *abi2* mutants (compare Figures 2B, 3B, and 3E) provides evidence for important roles of these mutations in affecting early ABA signaling. In addition, these findings constitute genetic support for the proposed rate-limiting role of slow anion channel regulation for stomatal closing (Schroeder and Hagiwara, 1989; Schroeder et al., 1993). This strong impairment in ABA regulation should provide an effective response to characterize further mechanisms by which either mutant disrupts ABA signaling.

Phosphorylation and Dephosphorylation Modulators Differentially Affect ABA Signaling in the Wild Type and in *abi* Mutants

The *abi1* gene was shown to encode a protein phosphatase 2C homolog (Leung et al., 1994; Meyer et al., 1994). Guard

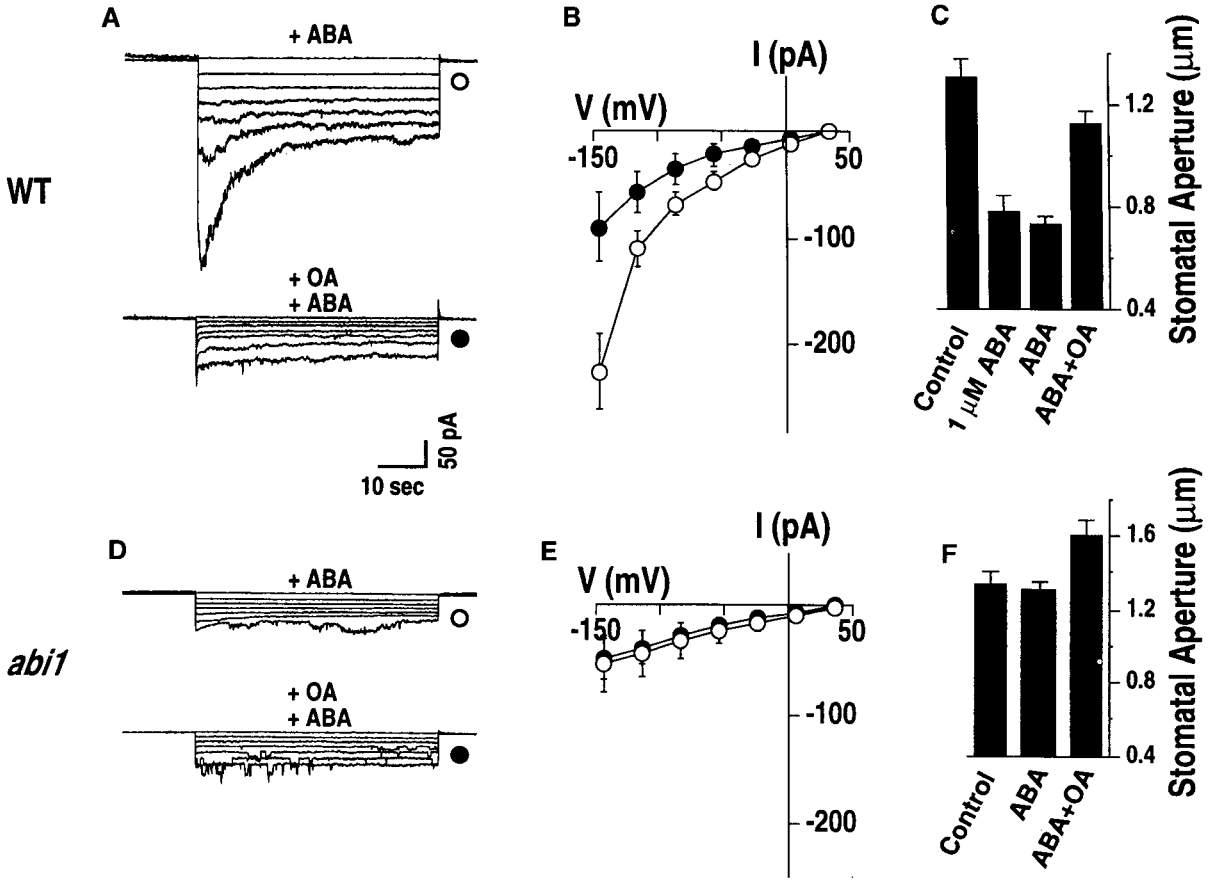

Figure 5. Okadaic Acid Partially Inhibits ABA-Activated Anion Currents and ABA-Induced Stomatal Closing.

(A) ABA activation of slow anion channels is partially inhibited by exposure to 1 μM okadaic acid. At top are shown currents in the presence of 50 μM ABA (+ABA) and in the absence of okadaic acid. At bottom are shown currents in the presence of both 50 μM ABA (+ABA) and 1 μM okadaic acid (+OA). WT, wild type.

(B) Average peak current–voltage relationships for experiments are as given in **(A)** in the absence (○) and presence (●) of 1 μM okadaic acid.

(C) ABA-induced stomatal closing is partially inhibited by 1 μM okadaic acid in the presence of 100 μM ABA in wild-type Arabidopsis (ABA + OA).

(D) In *abi1* guard cells, the lack of ABA activation of anion channel currents (Figures 3B and 3C) is not affected by exposure to 1 μM okadaic acid. Shown are whole-cell currents recorded with 50 μM ABA but in the absence of okadaic acid (+ABA, top) and presence of 1 μM okadaic acid (+OA, +ABA, bottom).

(E) Average peak current–voltage relationships are illustrated for *abi1* experiments in the absence (○) and presence (●) of 1 μM okadaic acid. Conditions are as given in **(D)**.

(F) Exposure of *abi1* stomata to 1 μM okadaic acid and 100 μM ABA enhances stomatal opening (ABA + OA).

All anion current recordings were performed in the presence of 50 μM ABA in the cytosol and bath. Data in **(B)** and **(E)** are the mean ± SE (n = 6 to 15 for each condition; n = 44 total cells). The numbers of illustrated averaged stomatal apertures and epidermal strip experiments are given in the text. Stomatal apertures were measured after treatment with control buffer (Control), 100 μM ABA (ABA), or 100 μM ABA plus 1 μM okadaic acid (ABA + OA) for 2 hr in **(C)** and **(F)**. Treatment with 1 μM ABA in wild-type Arabidopsis (1 μM ABA) is shown in **(C)**.

cells from transgenic tobacco expressing the Arabidopsis mutant *abi1-1* gene show that protein kinase and phosphatase inhibitors affect K^+ channel regulation (Armstrong et al., 1995). Furthermore, recent studies with broad bean guard cells have shown that slow anion channels are activated by protein phosphorylation events and completely inactivated by dephosphorylation events and that a close correlation to ABA-regulated stomatal movements exists (Schmidt et al., 1995; Esser et al., 1997). In broad bean guard cells, the kinase inhibitor K-252a completely abolishes both

slow anion channel activity and ABA-induced stomatal closing, whereas the phosphatase inhibitor okadaic acid enhances both responses (Schmidt et al., 1995; Esser et al., 1997). Therefore, the effects of okadaic acid on Arabidopsis guard cell anion currents in the wild-type line were analyzed. Interestingly, in contrast to the effect observed in broad bean guard cells, okadaic acid consistently inhibited ABA activation of anion channels in Arabidopsis guard cells (Figures 5A and 5B; $n = 27$). In *abi1* ($n = 9$) and in *abi2* ($n = 6$) guard cells, the ABA insensitivity of anion currents was not significantly affected by okadaic acid (Figures 5D and 5E; see below for *abi2*). This was to be expected, given that anion channels could not be activated by ABA in these mutants.

The finding that the protein phosphatase inhibitor okadaic acid suppressed ABA activation of anion channels in Arabidopsis guard cells lies in juxtaposition to results with broad bean and Commelina guard cells. Broad bean and Commelina showed enhancement of ABA-induced stomatal closing and stimulation of anion channels by okadaic acid or by the addition of cytosolic ATP (Schmidt et al., 1995; Esser et al., 1997). Okadaic acid inhibition of slow anion channel activation in Arabidopsis guard cells (Figures 5A and 5B) suggested that ABA-induced stomatal closing should also be inhibited by okadaic acid in wild-type Arabidopsis leaves, if the observed anion channel regulation controls stomatal movements. In wild-type Arabidopsis leaves, both 1 and 100 μM ABA closed preopened stomata (Figure 5C; for 1 μM ABA, $n = 60$ stomata in four experiments, $P < 0.001$; for 100 μM ABA, $n = 105$ stomata in six experiments, $P < 0.001$).

Okadaic acid at 1 μM partially inhibited ABA-mediated stomatal closing in Arabidopsis (Figure 5C). This is consistent with okadaic acid inhibition of anion channels (Figures 5A and 5B). Other protein phosphatase inhibitors such as calyculin A also inhibited the stomatal response to ABA (data not shown). Note that two different techniques were used for ABA and inhibitor applications to Arabidopsis stomata and that blind experiments were also conducted for this study (see Methods), confirming results reported here. Thus, in wild-type Arabidopsis leaves, protein phosphatase inhibition counteracted both ABA activation of anion channels and ABA induction of stomatal closing (Figures 5A to 5C).

Additional experiments were performed at the high concentration of 100 μM ABA, which illustrates the robust phenotypes of the *abi1-1* and *abi2-1* mutants. ABA-induced stomatal closing was abolished in the *abi1-1* mutant (Figure 5F; $n = 130$ stomata in nine experiments). This result is in close correlation with the strong suppression of slow anion channel activation by ABA in *abi1-1* (Figures 3A to 3C). In addition, when *abi1* mutants were treated with okadaic acid in the presence of ABA, stomatal opening was enhanced (Figure 5F). Enhanced stomatal opening has been observed in broad bean by complete anion channel downregulation or blockage (Schroeder et al., 1993; Schmidt et al., 1995; Schwartz et al., 1995). Note that the ABI1 protein is classified by homology as an okadaic acid–insensitive protein phosphatase type 2C and has indeed been shown to be okadaic acid insensitive (Leung et al., 1994; Meyer et al., 1994; Bertauche et al., 1996, M.B. Leube and E. Grill, personal communication), suggesting that at least two different phosphatases participate critically in ABA signaling in Arabidopsis guard cells (see Discussion).

The data presented above suggest that ABA activation of (non-ABI1) protein phosphatases may play an important role in the propagation of the ABA signal in Arabidopsis (Figures 5A and 5C). Therefore, experiments were designed to determine whether inhibition of protein kinases could rescue the *abi1* or *abi2* defects. The exposure of *abi1* guard cells to both ABA and the kinase inhibitor K-252a (1 to 2 μM) dramatically suppressed the *abi1* deficiency in ABA-induced anion channel regulation (Figures 6A and 6B; $n = 20$). However, the kinase inhibitor alone, in the absence of ABA, could not activate slow anion channels in *abi1* guard cells, as summarized in Figure 7B (compare bars 1 and 5). The average stimulation of the ABA–plus–K-252a–activated anion currents in *abi1* guard cells corresponded to 57% of the average activation of the ABA response in wild-type guard cells. Furthermore, in stomatal movement assays, the inability of ABA to produce stomatal closing in the *abi1* mutant was also partially overcome by exposure of Arabidopsis leaves to the kinase inhibitor K-252a, showing correlation to anion channel regulation properties (Figure 6C; $n = 80$ stomata in five experiments, $P < 0.004$) and K^+ channel properties (Armstrong et al., 1995).

In contrast to results from experiments with the *abi1* mutant, the kinase inhibitor K-252a did not rescue the impairment of the anion channel response to ABA in *abi2* guard cells (Figures 6D and 6E; $n = 17$). Furthermore, ABA-induced stomatal closing was also not rescued by kinase inactivation in *abi2* (Figure 6F; $n = 25$ stomata in two experiments). These data provide evidence for a substantial difference in the functions of *abi1* and *abi2* during ABA signaling and support a central role of protein kinase and phosphatase regulation during ABA signaling.

Figure 7 illustrates averaged results of anion channel regulation in wild-type, *abi1*, and *abi2* guard cells. In wild-type guard cells, okadaic acid inhibited ABA signaling (Figure 7A, bars 2 and 4). K-252a caused only a small average enhancement of ABA signaling, possibly because ABA already inhibited a K-252a–sensitive kinase (Figure 7A, bars 2 and 6). In addition, K-252a alone, with no ABA exposure, also did not activate anion channels in *abi1* and *abi2* guard cells (Figures 7B and 7C, bars 1 and 5). However, in the presence of ABA, K-252a rescued signal transduction in *abi1* guard cells but not in *abi2* guard cells (Figures 7B and 7C, bars 2 and 6).

A simple epistatic model to explain these findings would be that *ABI1* affects events upstream of an ABA-inactivated (K-252a–sensitive) protein kinase. In *abi1* guard cells, the ABA inactivation of this kinase would be impaired and could be rescued by K-252a (Figure 6A and Figure 7B, bars 2 and 6; see Discussion). The *abi2* mutation in turn would not affect

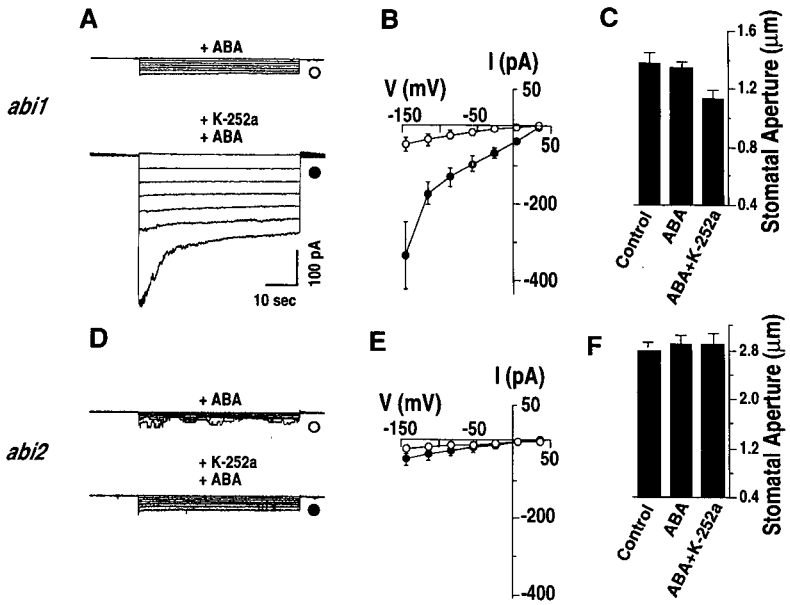

Figure 6. Kinase Inactivation Restores ABA-Activated Slow Anion Currents and ABA-Induced Stomatal Closing in *abi1-1* but Not *abi2-1* Guard Cells.

(A) The protein kinase inhibitor K-252a rescues ABA activation of slow anion channels in *abi1* guard cells. Experiments were performed in the presence of 50 μM ABA (+ABA) and in the absence of K-252a (top) and in the presence of both 50 μM ABA (+ABA) and 2 μM K-252a (+K-252a and +ABA, bottom).

(B) Average peak current–voltage relationships are illustrated for *abi1* guard cells exposed to 50 μM ABA in the absence (○) and presence (●) of 2 μM K-252a.

(C) K-252a (1 μM) partially rescues ABA-induced stomatal closing in the presence of 100 μM ABA in *abi1* mutants.

(D) K-252a could not rescue ABA activation of anion channels in *abi2* guard cells. Experiments were performed in the presence of 50 μM ABA (+ABA) and in the absence of K-252a (top) and in the presence of both 50 μM ABA and 2 μM K-252a (+K-252a and +ABA, bottom).

(E) Average peak current–voltage relationships for experiments in the absence (○) and presence (●) of K-252a are shown. Recordings were performed as given in **(D)**. In **(B)** and **(E)**, data are mean ± SE (n = 5 to 9 for each condition).

(F) K-252a (1 μM) did not rescue ABA-induced stomatal closing in the presence of 100 μM ABA in *abi2* mutants. All other conditions are as given in Figure 5.

reactions between *ABI1* and the kinase because K-252a could not rescue the *abi2* mutation (Figures 6D to 6F and Figure 7C, bars 2 and 6).

DISCUSSION

ABA functions in the regulation of plant growth and development and in responses to environmental stresses, including drought (Zeevaart and Creelman, 1988; Mansfield et al., 1990; Hetherington and Quatrano, 1991). ABA-induced stomatal closing is a physiological response to drought stress that limits water loss from leaves through stomatal pores. The mechanism of stomatal closing requires the efflux of anions and K^+ from guard cells, resulting in a loss of guard cell volume and turgor that produces stomatal closure (Raschke, 1979; MacRobbie, 1981). Plasma membrane anion channels have been suggested to function as a central control mechanism in this response (Schroeder and Hagiwara, 1989; Hedrich et al., 1990). Analysis of the regulation of guard cell ion channels that mediate anion and K^+ efflux during stomatal closing provides high-resolution assays to identify upstream events during ABA signal transduction.

The Arabidopsis *abi1* and *abi2* mutants could have defects in early ABA signal transduction events because they impair diverse responses to ABA (Koornneef et al., 1984; Finkelstein and Somerville, 1990; Armstrong et al., 1995). However, what roles the *abi2* mutation plays and whether it affects very early or later signaling mechanisms remain unknown. Direct quantitative cell biological and biophysical analyses of Arabidopsis guard cells provide powerful approaches to address these questions but have been hampered mainly by the relatively small size of these cells. In this study, we have developed and applied patch–clamp techniques and physiological stomatal movement assays to study upstream signaling events in wild-type and ABA-insensitive mutants of Arabidopsis.

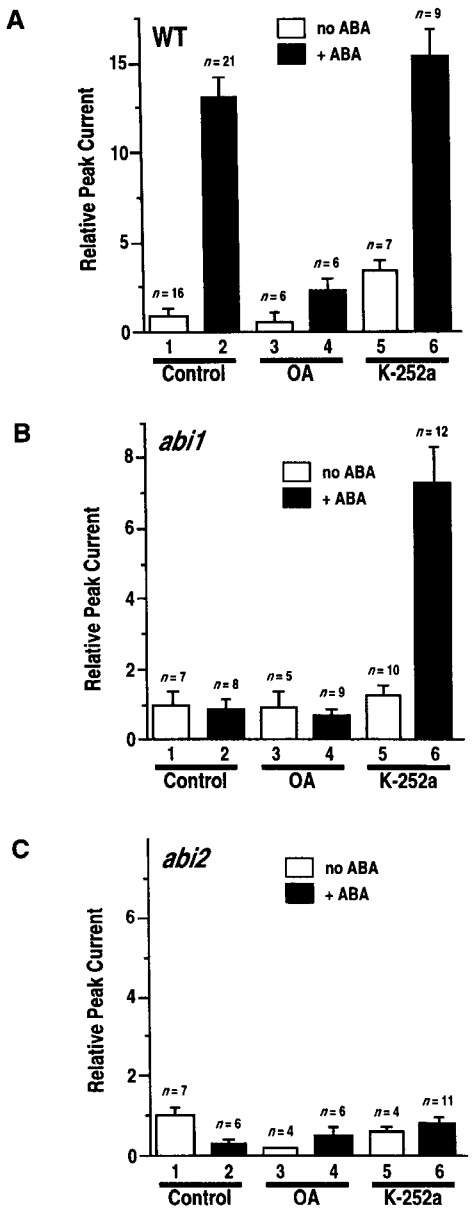

Figure 7. Average Effects of ABA, Okadaic Acid, and K-252a on Anion Channel Currents Recorded in Arabidopsis Wild-Type, *abi1-1*, and *abi2-1* Guard Cells.

(A) In wild-type (WT) Arabidopsis guard cells, the addition of ABA (+ABA) activates anion channels (bar 2), and the activation is inhibited by the phosphatase inhibitor okadaic acid (OA) (bar 4).
(B) In *abi1* guard cells, ABA fails to activate anion channels (bar 2), and the kinase inhibitor K-252a restores ABA activation of anion channels only when ABA is applied at the same time (bars 5 and 6).
(C) In *abi2* guard cells, ABA fails to activate anion channels (bar 2), and K-252a could not restore ABA activation of anion channels (bar 6).
Whole-cell peak currents were recorded as given in Figure 2B at a membrane potential of −145 mV. Anion currents were normalized with respect to data in the absence of ABA (bar 1) in **(A)**, **(B)**, and **(C)**. The number of cells averaged (± SE) is shown at the top of each bar.

Our results support several novel conclusions and working hypotheses, which are discussed in more detail later and are briefly listed here. (1) We now provide direct evidence that ABA activates slow anion channels. (2) *abi2-1* disrupts early ABA signal transduction. (3) The inhibition of an okadaic acid–sensitive protein phosphatase, which is distinct from the ABI1 protein phosphatase, reduces both ABA activation of slow anion channels in guard cells and ABA-induced stomatal closing. (4) Our data suggest the hypothesis that during ABA signaling, a negatively regulating protein kinase activity is inhibited. This kinase suppression is necessary, but not sufficient without ABA, to activate the guard cell slow anion channels and induce stomatal closing. (5) Kinase suppression rescues the *abi1* but not the *abi2* mutant phenotype, demonstrating differential effects of these two mutations. These findings are discussed in detail below and can be interpreted in new testable working models for ABA signaling pathway segments that propose relative positions and roles of *abi1*, *abi2*, phosphorylation events, and anion channels in the signaling cascade.

ABA Activation of Slow Anion Channels in Guard Cells

Previous studies have suggested that slow anion channels function as a central controller of stomatal closing and have shown a close correlation of anion channel regulation to ABA signaling (Schroeder and Hagiwara, 1989; Schmidt et al., 1995). However, whether ABA directly activates slow anion channels had remained unknown. Here, we demonstrate that in wild-type guard cells, ABA strongly activates slow anion channels. The large, average 13-fold stimulation of anion channel currents (Figure 7A) is an underestimate because an Arabidopsis guard cell background conductance was not subtracted (e.g., see Figure 2A). This supports the hypothesis that slow anion channel activation is required for stomatal closing and demonstrates that anion channels are strongly upregulated in response to the physiological ABA signal.

Arabidopsis guard cells showed similar K^+_{in} channel currents to those identified in broad bean (Schroeder et al., 1987), maize (Fairley-Grenot and Assmann, 1993), potato (Müller-Röber et al., 1995), and tobacco (Armstrong et al., 1995). The lack of a clear K^+ channel regulation shown here does not contradict previous studies showing K^+ channel regulation by ABA (Blatt, 1990; Lemtiri-Chlieh and MacRobbie, 1994; Schwartz et al., 1994; Armstrong et al., 1995), because of the large pH and Ca^{2+} buffering capacities imposed on small Arabidopsis guard cells in our study. Consistent with our findings, in broad bean, such Ca^{2+} and pH buffering capacities have also been shown to disrupt K^+ channel regulation by ABA (Lemtiri-Chlieh and MacRobbie, 1994). Therefore, based on current models and previous reports, the imposed buffering capacities should preclude K^+ channel regulation (see Blatt, 1992; Lemtiri-Chlieh and MacRobbie, 1994; Kelly et al., 1995). The same strong experimental buffering

capacities did not interfere with the large dynamic range in ABA regulation of anion channels (Figures 2 to 7). These data indicate that ABA regulation of anion channels may occur via more direct or more strongly modulated pathways than K^+ channel regulation and that possibly both Ca^{2+}-dependent (Schroeder and Hagiwara, 1989; McAinsh et al., 1990) and Ca^{2+}-independent (MacRobbie, 1992; Allan et al., 1994) pathways regulate slow anion channels. In addition, the large dynamic range of ABA regulation of slow anion channels provides an excellent response to probe intermediate steps in ABA signal transduction.

The protein phosphatase inhibitor okadaic acid inhibited the ABA activation of slow anion channels in wild-type Arabidopsis guard cells and reduced ABA-induced stomatal closing (Figures 5A to 5C). Based on homology of the ABI1 phosphatase with protein phosphatase 2C–type enzymes and the insensitivity of recombinant ABI1 protein to okadaic acid (Bertauche et al., 1996; M. Leube and E. Grill, personal communication), we propose that a second protein phosphatase, in addition to ABI1, has a role in ABA signaling. By cytosolic loading of immunosuppressants, data have suggested that a calcineurin-type protein phosphatase (PP2B), which would be okadaic acid insensitive, can function in K^+ channel regulation in broad bean guard cells (Luan et al., 1993). Okadaic acid also inhibits ABA induction of the *PHAV1* gene in barley aleurone (Kuo et al., 1996).

Suppression of Anion Channel Activation in *abi1* and *abi2* Mutants

ABA activation of anion channel currents in the *abi1* and *abi2* mutants was abolished (Figures 3, 7B, and 7C). The finding that the *abi2-1* mutant disrupts ABA signaling places the effect of this mutation in the early ABA signaling cascade. Our study does not distinguish whether the ABI2 protein directly or indirectly affects signaling, which will require *ABI2* gene isolation and further functional analyses. The repression of ABA signaling in *abi1* and *abi2* was also observed when large, 50-μM concentrations of ABA were applied to both sides of the membrane, illustrating the robust phenotypes of *abi1* and *abi2.* These findings are consistent with the complete lack of ABA-induced stomatal closing in both *abi1* and *abi2* mutants even at 100 μM ABA (Figures 6C and 6F; Roelfsema and Prins, 1995) and underscore the role of slow anion channels as a central regulator. If anion channels had been activated by ABA to a degree similar to wild-type guard cells, a significant ABA-induced stomatal closing would be expected in *abi1* and *abi2.*

abi1, *abi2*, and Protein Kinase Regulation

In the *abi1* but not in the *abi2* mutant, the kinase inhibitor K-252a partially restored ABA regulation of slow anion channels and ABA-induced stomatal closing (Figure 6). The effects of kinase inhibitors in *abi1* and *abi2* suggest the following. (1) Mutations at the *abi1* and *abi2* loci affect ABA signaling differentially. (2) *abi2* affects signaling between ABA receptors and slow anion channels. (3) The rescue of the *abi1* phenotype could be explained by a simple epistatic hypothesis. ABA signaling leads to the inhibition of a kinase, and this inhibition is necessary for slow anion channel activation and stomatal closing. These data suggest that a protein kinase acts as a central negative regulator of ABA signaling in Arabidopsis guard cells. Similarly, signal transduction in response to the hormone ethylene may also occur through the downregulation of a central serine/threonine kinase activity. CTR1, a Raf kinase homolog, is a negative regulator of ethylene signal transduction, and null mutations in the *CTR1* gene produce constitutive ethylene responses (Kieber et al., 1993). Interestingly, the recent molecular cloning of the *era1* mutant in Arabidopsis also indicates that ABA signaling in seeds includes a rate-limiting negatively regulating component (Cutler et al., 1996). The *abi2* mutant was not rescued by protein kinase inhibitors, suggesting that *abi2* affects responses downstream of the negatively regulating kinases, in a parallel pathway, or upstream of a branch point before ABI1 but not between ABI1 and the negatively regulating kinase.

The finding that the K-252a rescue of ABA-induced stomatal closing in *abi1* is small though significant is consistent with models in which different protein kinases could play counteracting roles in either stomatal closing or stomatal opening (Shimazaki et al., 1992; Schmidt et al., 1995; Pei et al., 1996). Inhibition of counteracting protein kinases by K-252a may explain the partial rescue in the stomatal closing response found here.

Because the suppression of kinase activity by K-252a alone, without coapplication of ABA, did not produce ABA responses, other ABA-induced signals are also required to activate slow anion channels (Figures 7A and 7B, bars 1, 5, and 6). This provides evidence that ABA signal transduction may proceed through a branched pathway. The finding that K^+_{in} current magnitudes are affected by the *abi2* mutation provides further evidence for branch points in ABA signaling (Figures 1A and 1G). In tobacco, the twofold stimulation of K^+ efflux channels by ABA is disrupted by transgenic expression of the mutant Arabidopsis *abi1-1* gene (Armstrong et al., 1995). Results showing that kinase inhibitors partially restore ABA regulation of K^+ channels and stomatal closing in transgenic tobacco (Armstrong et al., 1995) correlate closely to findings on slow anion channels reported here. In branched models, *abi1* would affect one branch, whereas signaling mechanisms required in the other branch remain unknown. A branched pathway for ABA signal transduction in guard cells has also been proposed based on the observations of Ca^{2+}-dependent and Ca^{2+}-independent stomatal closing (MacRobbie, 1992; Allan et al., 1994). Our study provides insights into elements of signaling pathways and can be used to propose testable sequences in ABA transduction pathways.

Species-Dependent Differences in ABA Signaling

It is of interest that in guard cells of Arabidopsis and transgenic tobacco, ABA signaling is modulated in a manner opposite that found in broad bean and Commelina (Figures 5 to 7; Armstrong et al., 1995; Schmidt et al., 1995; Esser et al., 1997). Consistent with this observation, patch–clamp conditions that strongly activate slow anion channels in broad bean guard cells (Schroeder and Hagiwara, 1989; Schmidt et al., 1995) do not produce slow anion channel currents without the addition of ABA in Arabidopsis (Figure 2A and bar 1 in Figure 7A). In broad bean, cytosolic hydrolyzable ATP is required as a substrate for central stimulatory protein kinases (Schmidt et al., 1995), whereas in Arabidopsis, negatively regulating kinases are central to the response and could be stimulated by ATP addition. This difference in different species could provide a basis for the differential regulation among plant types. Interestingly, ABA regulation similar to that reported here for Arabidopsis guard cells was consistently observed for other ABA responses in Arabidopsis (K. Kuchitsu and J.I. Schroeder, unpublished data), suggesting that the present results apply generally to ABA signaling in Arabidopsis.

The observed differences among species may provide initial insights into plant-specific differences in guard cell and ABA signaling mechanisms and will be interesting for further comparative analyses. It is also conceivable that different elements of the same signaling pathway become rate limiting in different species, resulting in the differences revealed here. These data highlight that many additional components, pathways, and/or cross-talk among components will need to be characterized and that cross-species experiments require careful analysis.

In Arabidopsis hypocotyl cells, depletion of cytosolic ATP was recently shown to lead to activation of slow anion channels (Thomine et al., 1995). This indicates that in Arabidopsis, kinase activity may also negatively regulate S-type anion channels in cell types other than guard cells and may provide a basis for the differences observed in Arabidopsis when compared with broad bean and Commelina guard cells.

Anion Channels in Tobacco Guard Cells and Acetate

In tobacco guard cells carrying the mutant *abi1* transgene, ABA activation of anion channels identical to the wild-type response has been indicated (Armstrong et al., 1995), which differs from the regulation of guard cell slow anion channels in the Arabidopsis *abi1-1* mutant analyzed here. This may be attributable to the different plant types investigated. For example, RNA gel blot analysis showed no *ABI1* mRNA homologs in tobacco, suggesting putative significant structural differences in the native tobacco gene. Therefore, it is possible that the Arabidopsis *abi1-1* transgene may not be sufficiently conserved to reliably transduce all signals in tobacco. Other plant-specific differences in the signaling cascade may also play roles.

In addition to the question of plant specificity, the use of 200 mM acetate in the microelectrodes (Armstrong et al., 1995), which inhibits slow anion channel activity (Figure 4), may explain why guard cell microelectrode studies have consistently failed to show large anion channel activities in broad bean and tobacco. Guard cells impaled with electrodes containing 200 mM acetate showed a repression of Ca^{2+}-induced sustained depolarizations (reminiscent of S-type depolarizations) (Figure 1 and pages 276 and 278 in Blatt, 1987). This suggested to us that Ca^{2+}-dependent S-type anion channel activity (Schroeder and Hagiwara, 1989) may be inhibited by acetate. Therefore, we directly tested and showed here that cytosolic loading with 30 mM acetate, even with the addition of 120 mM cytosolic CsCl, completely suppresses slow anion channels (Figure 4). These data show that acetate-containing solutions are suited for characterizing K^+ channels because other ion channel currents are repressed, as has been shown for glutamate-containing solutions (Schroeder et al., 1987). The acetate inhibition shown here could also lead to models in which the relative contribution of cytosolic anion channel regulation to stomatal movements might be underestimated.

The finding that anion efflux current densities in tobacco guard cells by using acetate solutions corresponded to $<10\%$ (Armstrong et al., 1995) of the characterized guard cell slow anion channel activity indicates that a small subset of guard cell anion channels functions in the presence of acetate, which may be regulated differently in tobacco from the major slow anion channel activities reported here and previously (Schroeder and Hagiwara, 1989; Schmidt et al., 1995). The question of whether the reported effects of the Arabidopsis *abi1-1* transgene on anion currents in transgenic tobacco is related to the use of acetate and/or Arabidopsis versus tobacco plant specificity would require detailed investigation.

In the present study, experiments with *abi* mutants were directly performed using Arabidopsis guard cells. If ABA could activate slow anion channel currents in the Arabidopsis *abi1* mutant comparable to the wild type, this would result in at least a partial stomatal closing response because of residual K^+ channel activities observed in all guard cell K^+ channel studies reported to date. However, no ABA-induced stomatal closing response was found in the Arabidopsis *abi1* (and the *abi2*) mutant at up to 100 μM ABA (Figures 6C and 6F; Roelfsema and Prins, 1995). In addition, the simultaneous rescue of the *abi1* anion channel and stomatal regulation phenotypes by protein kinase inactivation in Arabidopsis further supports the ABA insensitivity of slow anion channels in the Arabidopsis *abi1-1* mutant allele, as demonstrated in this study.

Conclusions and Working Models for ABA Signaling

In conclusion, we directly demonstrate here that ABA strongly activates slow anion channels in guard cells. This result is consistent with the suggested rate-limiting role of

slow anion channels in controlling stomatal closing. We have also shown that both *abi1* and *abi2* strongly and distinctly repress early ABA signaling and anion channel activation. The complete lack of ABA-induced stomatal closing in the *abi1* and *abi2* mutants (Figures 6C and 6F) is closely correlated with the observed strong lack of anion channel activation by ABA in these mutants (Figures 3, 7B, and 7C). Data reported here suggest a function in ABA-induced signaling for an okadaic acid–sensitive protein phosphatase that is distinct from ABI1. Furthermore, a K-252a–sensitive protein kinase is proposed to function as a negative regulator of ABA signaling.

New sequential working models for ABA signal transduction in Arabidopsis can be proposed and tested from these findings. Here, we delineate simple models that represent different examples of a scheme as depicted in Figure 8, because such simplified models will be testable: (1) ABA reception is followed by (2) ABI1 modulation, which in turn downregulates a (3) negatively regulating K-252a–sensitive protein kinase. An okadaic acid–sensitive protein phosphatase (4) is activated by ABA either in parallel to kinase inhibition (Figures 8A and 8B) or upstream of ABI1 (Figure 8B). In addition, the (5) *abi2* locus disrupts ABA signaling but not between ABI1 and the negatively regulating kinase (Figures 8A and 8B). A (6) parallel signaling pathway is required, because kinase inactivation alone (without ABA) is not sufficient to produce anion channel activation and stomatal closing. As examples, alternatives for branched signaling models are shown in Figures 8A and 8B. ABA signal transduction results in strong stimulation of (7) slow anion channels and in enhancement of (8) outward-rectifying K^+ channel activity (Armstrong et al., 1995), leading to stomatal closing.

We emphasize that alternative, related derivatives of the illustrated models can be proposed and are not meant to be excluded here, for example, models with constitutively active protein kinases or protein phosphatases feeding into the ABA signaling pathway from the side. The relative positions of Ca^{2+}, pH, and other regulatory mechanisms in these models remain to be determined. In summary, significant advances have been made in understanding early ABA signal transduction events. The ability to simultaneously apply Arabidopsis molecular genetics and high-resolution patch–

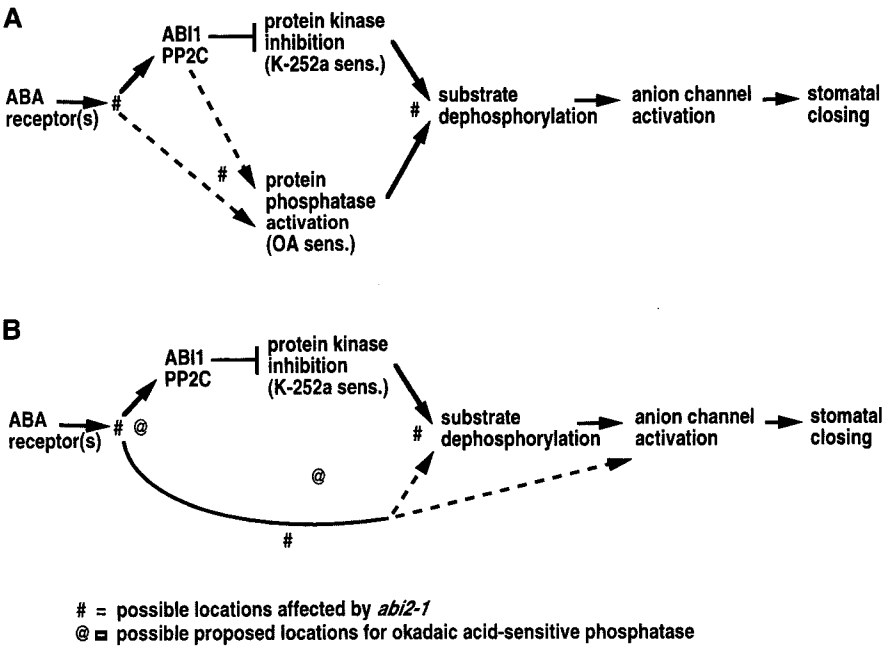

Figure 8. Examples of Hypothetical Branched Working Models for Locations and Roles of ABA Signaling Components during Anion Channel Regulation in Arabidopsis Guard Cells.

(A) and **(B)** show iterations of related models discussed in the text.

The depicted models include the demonstrated disruption of signaling by the *abi1* and *abi2* mutants, a negatively regulating protein kinase that is influenced by the ABI1 protein phosphatase 2C (PP2C) and a positively regulating okadaic acid–sensitive (sens.) protein phosphatase. Although certain locations of *abi2* effects and an okadaic acid–sensitive protein phosphatase can be excluded from the findings reported here (see text), several alternative locations remain within the framework of the discussed models and are indicated. Other related models are also possible (see text). Dashed arrows represent alternative (nonexclusive) paths. Note that other second messengers and parallel regulation of other plasma membrane and vacuolar membrane ion channels are not shown here for simplicity (for proposed channel model, see Schroeder and Hedrich, 1989; Ward et al., 1995).

clamp analysis as described here will allow testing, further specification, and extension of the proposed models and will further expand our understanding of early ABA signal transduction in plants.

METHODS

Isolation of *Arabidopsis thaliana* Guard Cells

Arabidopsis seeds of the *abi1-1* (*abi1*) and *abi2-1* (*abi2*) mutant alleles (Koornneef et al., 1984) were used in this study. Both mutants are ethyl methanesulfonate–induced mutants derived from the Landsberg *erecta* ecotype, which was used as the wild-type line throughout this study. 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) with a 16-hr-light and 8-hr-dark cycle at 20°C at a photon fluency rate of 100 μmol m^{-2} sec^{-1} and watered with deionized water every 3 days. Arabidopsis guard cell protoplasts were isolated from 4- to 6-week-old plants by enzymatic digestion of leaf epidermal strips, as previously described (Kruse et al., 1989; Schroeder and Keller, 1992). Briefly, two Arabidopsis rosette leaves (2 cm long) were blended in a blender (Waring, New Hartford, CT) in cold (4°C) deionized water three times, 20 sec each, and collected between blendings on a 292-μm mesh. The epidermal tissue was then incubated in 10 mL of medium containing 1.3% cellulase R10 and 0.7% macerozyme R10 (Yakult Honsha, Tokyo, Japan), 0.5% BSA, 0.5 M D-mannitol, 0.1 mM KCl, 0.1 mM $CaCl_2$, 10 mM ascorbic acid, and 0.1% kanamycin sulfate at pH 5.5 (with Tris) for 16 hr at 22 ± 2°C on an orbital shaker (70 rpm; Bellco Glass, Vineland, NJ). Isolated guard cell protoplasts were then collected and washed twice as described previously (Schroeder and Keller, 1992). The typical yield was on the order of 10,000 guard cells per two leaves. The typical purity was 60 guard cell protoplasts per 100 total protoplasts in this isolation, with other protoplasts being mesophyll protoplasts. Guard cell protoplasts with diameters between 4 and 5 μm were used in all patch–clamp experiments reported here. Arabidopsis leaves have a small fraction of large stomata with larger guard cell protoplasts, which were not analyzed here.

Patch–Clamp and Data Acquisition

Patch–clamp pipettes were prepared from soft glass capillaries (Kimax 51; Kimble, Toledo, OH) and pulled on a multistage programmable puller (model P-87; Sutter Instrument, Novato, CA). Giga ohm seals between electrode and plasma membrane (>15 GΩ) were obtained by suction and usually appeared within 2 to 3 min (Hamill et al., 1981). Cells were pulled up to the bath solution surface to reduce stray capacitance. Whole-cell configurations were established by applying increased suction to the interior of the pipette.

Protoplasts were voltage clamped using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Voltage pulses were applied, and data were stored and analyzed as described previously (Schroeder and Hagiwara, 1989). Illustrated anion channel recordings were recorded either 2 to 3 min or 12 to 15 min (Figure 7A) after establishing whole-cell configurations. For bar graphs (Figure 7), an average whole-cell background resistance of 10 GΩ was subtracted from whole-cell currents, as determined when using K^+-glutamate solutions. No leak subtractions were applied to all of the other illustrated data and current–voltage curves (Figures 1 to 6). Note that the degree of actual anion channel regulation by ABA and other modulators is a lower estimate, because nonleak background ionic currents typically found in Arabidopsis guard cells (Figure 2A) were not subtracted.

Solutions

The solutions used in patch–clamp experiments for anion channels were composed of 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 3.35 mM $CaCl_2$, and 10 mM Hepes-Tris, pH 7.1, in the pipette medium and 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM Mes-Tris, pH 5.6, in the bath medium (Schroeder and Keller, 1992), unless indicated otherwise. Five-millimolar concentrations of Mg–ATP and Tris–GTP were added to the pipette solution before use. The cytosolic K^+ salt solution for K^+ current measurements was composed of 30 mM KCl, 70 mM K-glutamate, 2 mM $MgCl_2$, 6.7 mM EGTA, 3.35 mM $CaCl_2$, 5 mM ATP, and 10 mM Hepes-Tris, pH 7.1. The bath solution contained 30 mM KCl, 40 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Mes-Tris, pH 5.5. Fifty micromolar abscisic acid ([±]-*cis*,*trans*-abscisic acid [ABA]; Sigma) was freshly added to the bath and pipette solutions to study mutants at saturating ABA concentrations, unless indicated otherwise. Osmolalities of all solutions were adjusted to 485 mmol/kg for bath solutions and 500 mmol/kg for pipette solutions by addition of D-sorbitol. Guard cell protoplasts were treated with ABA, 1 μM okadaic acid (Calbiochem, La Jolla, CA), or 1 to 2 μM K-252a (Calbiochem) in 500 mM D-mannitol, 0.1 mM $CaCl_2$, and 0.1 mM KCl at 22 ± 2°C for 1.5 hr before recordings to show the robust phenotypes of *abi1-1* and *abi2-1* at steady state. K-252a stocks were prepared in DMSO. The final concentration of DMSO in the treatment solution was 0.1% (v/v), and DMSO controls showed no effects.

Stomatal Aperture Measurements

ABA as well as kinase and phosphatase modulators were applied to intact Arabidopsis leaves through the transpirational stream as follows. Side branches of Arabidopsis stems with fully expanded stem leaves were clipped, and the cut ends were immediately placed in 1.5-mL tubes containing 1 mL of distilled water containing ABA and modulators and exposed to light under the normal growth conditions as described above. An alternative approach (see Schwartz et al., 1995) was used in parallel, in which intact stem or rosette leaves were detached from plants and floated in solutions containing either 20 mM KCl, 5 mM Mes-KOH, pH 6.15, and 1 mM $CaCl_2$ or distilled water and exposed to light at a fluency rate of 150 μmol m^{-2} sec^{-1}. Subsequently, ABA was added to the solution at 1 to 100 μM to assay for stomatal closing. Results from the two stomatal assay methods consistently showed similar results (K. Kuchitsu, D. Metzger, J. Mori, D. Hussey, and J.I. Schroeder, unpublished data), and for clarity, only results using the former technique are illustrated in Figures 5 and 6.

Leaves of similar size and age were used in similar experiments to compare controls and various treatments. After 2 hr of treatment, two leaves were removed and briefly blended. Results were confirmed in blind assays. The epidermis was collected on a nylon mesh (292 μm) and placed on a microscope slide. Stomatal apertures were observed with a digital video camera (model VCB-3524; Sanyo, Tokyo) attached to an inverted microscope (Diaphot 300; Nikon, Tokyo). The width of the stomatal aperture as well as the height of stomata were

measured using image analysis computer software (Scion Image; Scion Corporation, Frederick, MD; and National Institutes of Health, Bethesda, MD). The inner edges of guard cells whose height was between 16 and 22 μm were focused, and the apertures of usually 15 to 20 stomata were measured. Data analyses including statistical *t* tests were performed using Microsoft Excel software (version 5.0; Microsoft Corporation, Redwood, CA). Data were also analyzed by plotting the ratio of stomatal aperture to height (Roelfsema and Prins, 1995) as a function of treatments that showed similar results to the illustrated stomatal aperture results.

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