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Abscisic Acid Activation of Plasma Membrane Ca^{2+} Channels in Guard Cells Requires Cytosolic NAD(P)H and Is Differentially Disrupted Upstream and Downstream of Reactive Oxygen Species Production in *abi1-1* and *abi2-1* Protein Phosphatase 2C Mutants

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The hormone abscisic acid (ABA) regulates stress responses and developmental processes in plants. Calcium-permeable channels activated by reactive oxygen species (ROS) have been shown recently to function in the ABA signaling network in Arabidopsis guard cells. Here, we report that ABA activation of these I_{Ca} Ca^{2+} channels requires the presence of NAD(P)H in the cytosol. The protein phosphatase 2C (PP2C) mutant *abi1-1* disrupted ABA activation of I_{Ca} channels. Moreover, in *abi1-1*, ABA did not induce ROS production. Consistent with these findings, in *abi1-1*, H_2O_2 activation of I_{Ca} channels and H_2O_2-induced stomatal closing were not disrupted, suggesting that *abi1-1* impairs ABA signaling between ABA reception and ROS production. The *abi2-1* mutation, which lies in a distinct PP2C gene, also disrupted ABA activation of I_{Ca}. However, in contrast to *abi1-1*, *abi2-1* impaired both H_2O_2 activation of I_{Ca} and H_2O_2-induced stomatal closing. Furthermore, ABA elicited ROS production in *abi2-1*. These data suggest a model with the following sequence of events in early ABA signal transduction: ABA, abi1-1, NAD(P)H-dependent ROS production, abi2-1, I_{Ca} Ca^{2+} channel activation followed by stomatal closing.

INTRODUCTION

The plant hormone abscisic acid (ABA) regulates a range of physiological processes, including seed maturation, control of vegetative growth, and promotion of dormancy, as well as tolerance of plants to adverse environmental conditions such as drought, cold, and salinity (Koornneef et al., 1998; Leung and Giraudat, 1998). In response to drought, ABA causes closing of stomatal pores, which are formed by pairs of guard cells in the epidermis of leaves and other aerial tissues. Stomatal closing results in a reduction of plant transpirational water loss. ABA induces an increase in cytosolic Ca^{2+} in guard cells, which precedes the reduction in stomatal aperture (McAinsh et al., 1990). Cytosolic Ca^{2+} elevation in turn activates slow (S-type) anion channels and downregulates inward K^+ channels in guard cells (Schroeder and Hagiwara, 1989), resulting in net ion release and turgor reduction leading to stomatal closing. ABA increases Ca^{2+} by inducing both Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular space (Schroeder and Hagiwara, 1990; Grabov and Blatt, 1998; Leckie et al., 1998; Staxen et al., 1999; Hamilton et al., 2000; MacRobbie, 2000; Pei et al., 2000). ABA activation of plasma membrane Ca^{2+} influx also is required in Arabidopsis suspension culture cells, suggesting that ABA activation of Ca^{2+} influx is a more general component of ABA signaling in plants (Ghelis et al., 2000b). More than one type of plasma membrane Ca^{2+} channel may exist in guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000). The second messengers inositol 1,4,5-trisphosphate, cyclic ADP ribose, and calcium have been suggested to cause Ca^{2+} release via different endomembrane Ca^{2+} channels in response to ABA in guard cells (Gilroy et al., 1990; Ward and Schroeder, 1994; Parmar and Brearley, 1995; Lee et al., 1996; Leckie et al., 1998; Bewell et al., 1999; Staxen et al., 1999). Furthermore, a

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recent study also implicates inositol hexakisphosphate in Ca^{2+}-dependent stomatal movements (Lemtiri-Chlieh et al., 2000).

ABA triggers Ca^{2+} influx via nonselective Ca^{2+}-permeable channels in *Vicia* guard cells (Schroeder and Hagiwara, 1990). Cellular mechanisms that activate guard cell plasma membrane Ca^{2+} channels have been identified. Studies show that membrane hyperpolarization causes cytosolic Ca^{2+} increases in guard cells (Gilroy et al., 1991; Grabov and Blatt, 1998; Allen et al., 2000). Hyperpolarization-activated Ca^{2+} (I_{Ca}) channels were identified in Arabidopsis and *Vicia* guard cells (Hamilton et al., 2000; Pei et al., 2000). In Arabidopsis the I_{ca} currents were shown to be carried by non-selective cation channels (Pei et al., 2000). In *Vicia* guard cells, intracellular ABA transiently enhanced the activity of hyperpolarization-activated Ca^{2+} channels (Hamilton et al., 2000). In Arabidopsis guard cells, H_2O_2 and ABA stimulated hyperpolarization-activated Ca^{2+}-permeable I_{Ca} channels (Pei et al., 2000).

ABA was shown to induce the production of reactive oxygen species (ROS) in Arabidopsis guard cells (Pei et al., 2000). H_2O_2 activation of I_{Ca} channels and H_2O_2-induced stomatal closing were abolished in the ABA-insensitive mutant *gca2* (Himmelbach et al., 1998), providing genetic evidence for roles of ROS and I_{Ca} channels in ABA signaling (Pei et al., 2000). Interestingly, in maize embryos and *Vicia* guard cells, ABA was shown recently to increase H_2O_2 levels (Guan et al., 2000; Zhang et al., 2001), indicating that ABA-induced ROS production may be of more general importance for ABA signaling. Diphenylene iodonium chloride (DPI), an inhibitor of NAD(P)H oxidases, partially inhibited ABA-induced stomatal closing (Pei et al., 2000). DPI also can inhibit other flavoenzymes (O'Donnell et al., 1993); therefore, further analyses are required to determine whether NAD(P)H contributes to the guard cell ABA response.

The dominant mutations *abi1-1* and *abi2-1* lie in two distinct type 2C protein phosphatases (PP2Cs) (Koornneef et al., 1984; Leung et al., 1994; Meyer et al., 1994; Grill and Himmelbach, 1998; Leung and Giraudat, 1998). The *abi1-1* and *abi2-1* mutations reduce ABA-induced cytosolic Ca^{2+} increases in guard cells (Allen et al., 1999a). Furthermore, experimentally imposing cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevations bypasses these mutants and restores S-type anion channel activation and stomatal closing (Allen et al., 1999a), demonstrating that *abi1-1* and *abi2-1* disrupt early ABA signaling at the level of, or upstream of, ABA-induced $[Ca^{2+}]_{cyt}$ increases. However, it remains unknown where these PP2C mutants act in the early signaling cascade and whether they affect ABA activation of I_{Ca} channels.

In this article, we investigate the link of ABA signaling to ROS production and I_{Ca} activation, and we analyze whether ABA activation of I_{Ca} depends on cytosolic NAD(P)H. Furthermore, we analyze whether *abi1-1* and *abi2-1* affect this newly recognized branch of ABA signaling, and if so, at what points in the signaling pathway. The results show a requirement of NAD(P)H in ABA activation of I_{Ca} and demonstrate, via several independent analyses, that *abi1-1* and *abi2-1* differentially disrupt ABA activation of I_{Ca}.

RESULTS

NAD(P)H Requirement for ABA Activation of Ca^{2+} Channels

To examine whether NAD(P)H may play a role in the ABA stimulation of hyperpolarization-activated calcium-permeable channels, we analyzed ABA effects on I_{Ca} in the presence or absence of cytosolic NADPH. ABA and H_2O_2 were applied to patch-clamped guard cells, and responses were recorded. The presence of 0.1 mM DTT in the patch clamp pipette and bath solutions inhibited the spontaneous activation of I_{Ca} currents, as reported previously (Pei et al., 2000). In the absence of DTT, some guard cells showed constitutive I_{Ca} activity (Pei et al., 2000), which supports the findings that oxidative processes activate these Ca^{2+} channels.

Arabidopsis guard cells (Landsberg *erecta* ecotype) were patch clamped for 10 min in the whole-cell mode before extracellular ABA application. When NADPH, the cytoplasm of guard cells, was not included in the patch clamp pipette solution which dialyzes, ABA did not activate I_{Ca} currents, as shown in Figures 1A and 1B ($n = 10$). However, when 5 mM NADPH was added to the pipette solution, ABA activated I_{Ca} currents ($n = 13$), as reported previously (Figures 1C and 1D) (Pei et al., 2000). Addition of the NADPH oxidase inhibitor DPI (12.5 μM) to the pipette solution inhibited the ABA activation of I_{Ca} ($n = 3$; data not shown). ABA also activated I_{Ca} in the presence of 1 mM NADPH ($P < 0.01$) (Figures 1E and 1F; $n = 8$). The average amplitudes of ABA-activated whole-cell I_{Ca} currents at -198 mV were statistically similar at 1 μM and 5 mM NADPH ($P > 0.11$).

In additional sets of experiments, 5 mM cytosolic NADPH caused an activation of I_{Ca} in the absence of added ABA in some cells, whereas with 1 mM cytosolic NADPH, ABA activation of I_{Ca} occurred (I.C. Mori, G.J. Allen, and J.I. Schroeder, data not shown). NADPH oxidation has been reported to be similar to NADH oxidation; therefore, not only NADPH but also NADH functions as a substrate of peroxidases to produce ROS in higher plants (Bestwick et al., 1998). When 5 mM NADPH was replaced with 5 mM NADH in the pipette solution, ABA activated I_{Ca} to a lesser extent, with an average amplitude of -7.8 ± 1.6 pA at -198 mV ($n = 9$; data not shown).

abi1-1 and *abi2-1* PP2C Mutants Disrupt the ABA Activation of I_{Ca}

To determine whether the *abi1-1* and *abi2-1* mutations affect the ABA activation of I_{Ca}, guard cell protoplasts of the *abi1-1* and *abi2-1* mutants were first patch clamped for ~10 min in

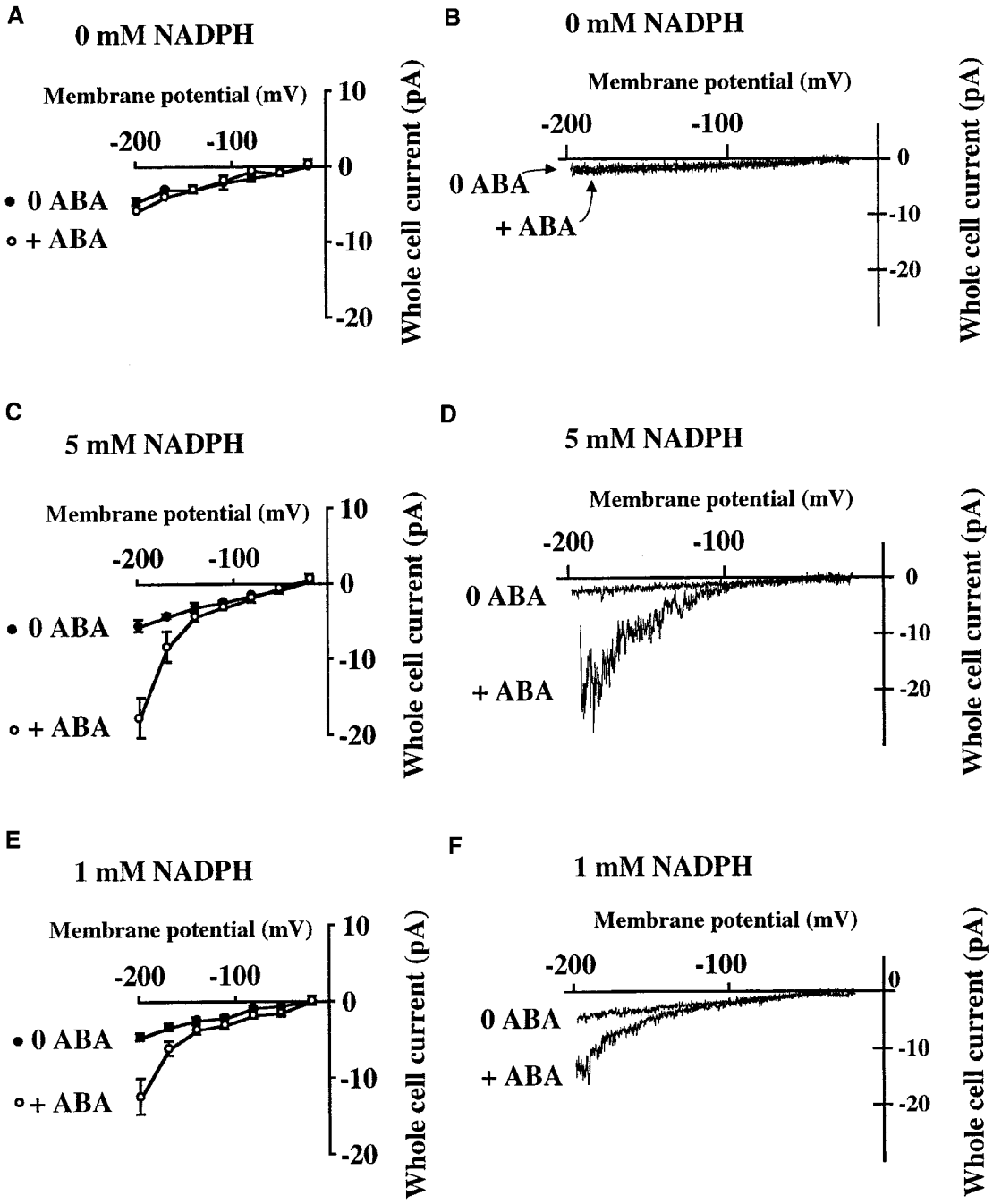

Figure 1. ABA Activation of Ca^{2+}-Permeable I_{Ca} Currents in Arabidopsis Guard Cell Protoplasts Requires Cytosolic NADPH.

(A) and **(B)** ABA (50 μM) did not activate I_{Ca} calcium channels when the pipette solution did not include NADPH or NADH. **(B)** shows two overlapping traces from a guard cell before and after ABA application.

(C) and **(D)** ABA (50 μM) activated I_{Ca} calcium currents when 5 mM NADPH was added to the pipette solution.

(E) and **(F)** ABA activated I_{Ca} when 1 mM NADPH was added to the pipette solution.

(A), **(C)**, and **(E)** show average responses, and **(B)**, **(D)**, and **(F)** show responses in individual cells before and 5 min after ABA application. ABA was added ~10 min after establishing whole-cell recordings, and whole-cell currents were measured before ABA application and in the same cells 5 min after extracellular ABA application in all recordings. The numbers of cells averaged are given in the text. Closed circles, before ABA addition to batch solution; open circles, 5 min after ABA addition to the same cells. Error bars represent SEM.

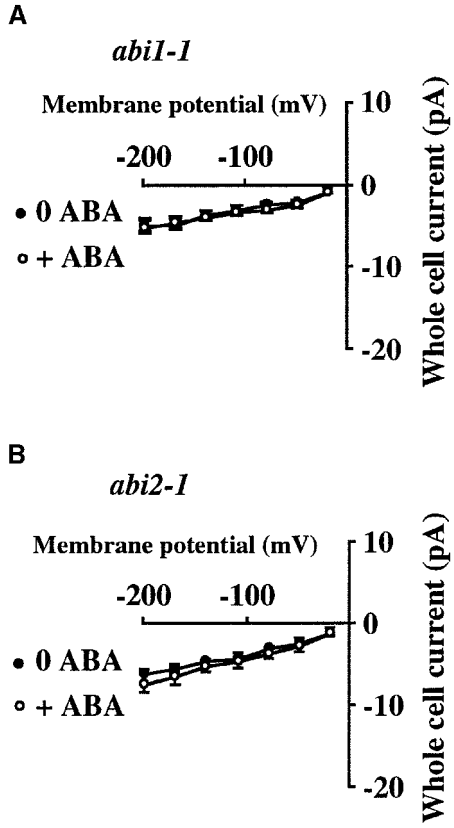

Figure 2. ABA Failed to Activate Ca^{2+} Channel Currents in *abi1-1* and *abi2-1* PP2C Mutant Guard Cells.

(A) ABA (50 μM) did not activate I_{Ca} in *abi1-1* guard cells with 5 mM NADPH included in the pipette solution ($n = 5$).
(B) ABA (50 μM) did not activate I_{Ca} in *abi2-1* guard cells with 5 mM NADPH added to the pipette solution ($n = 5$).
Experiments were performed as described in Figure 1. Error bars represent SEM.

the whole-cell mode. Subsequently, the same cells were treated with ABA by bath perfusion. Figure 2 shows the effects of *abi1-1* and *abi2-1* on the ABA activation of I_{Ca}. ABA did not activate I_{Ca} in either mutant in the presence of 5 mM cytosolic NADPH ($n = 5$, $P > 0.72$ for *abi1-1*; $n = 5$, $P > 0.35$ for *abi2-1*).

H_2O_2-Induced Responses Are Impaired in *abi2-1* but Not in *abi1-1*

To examine whether the *abi1-1* and *abi2-1* mutations impair the activation of I_{Ca} upstream or downstream of or parallel to ROS production, we first analyzed H_2O_2 activation of the hyperpolarization-activated Ca^{2+} channel currents in *abi1-1* and *abi2-1*. H_2O_2 (100 μM) clearly activated I_{Ca} in *abi1-1* guard cells, as shown in Figures 3A and 3B ($n = 6$, $P < 0.04$). Interestingly, however, H_2O_2 failed to activate I_{Ca} in *abi2-1* guard cells (Figures 3C and 3D) ($n = 5$, $P > 0.84$).

Exposure of stomates to H_2O_2 induces $[Ca^{2+}]_{cyt}$ increases and partial stomatal closing in *Commelina* and Arabidopsis (McAinsh et al., 1996; Pei et al., 2000). To further analyze H_2O_2-mediated signal transduction in *abi1-1* and *abi2-1*, we performed stomatal closing assays in wild-type, *abi1-1*, and *abi2-1* leaves. As reported previously, extracellular Ca^{2+} is required for H_2O_2 induction of stomatal closing and $[Ca^{2+}]_{cyt}$ increases (Pei et al., 2000). When 0.1 mM $CaCl_2$ was added to the bath solution, partial stomatal closing occurred (DeSilva et al., 1985; Allen et al., 1999a). However, buffering the total free Ca^{2+} concentration to ~0.1 mM in the cell wall space of epidermal strips with a bath solution containing 0.1 mM EGTA and 0.2 mM Ca^{2+} minimized Ca^{2+}-induced stomatal closing and allowed analysis of H_2O_2 responses (Pei et al., 2000). H_2O_2 at 100 μM triggered a reduction in stomatal aperture in the wild type (Landsberg *erecta* ecotype), as illustrated in Figure 4A ($n = 3$ experiments, $P < 0.01$). The partial H_2O_2 response compared with the ABA response (Figure 4A) is consistent with the proposed model of parallel branches together mediating early ABA signaling (see Figure 5f in the article by Pei et al., 2000). H_2O_2 also triggered a reduction in stomatal aperture in the *abi1-1* mutant (Figure 4B; $n = 3$, $P < 0.02$). However, stomatal aperture measurements showed that H_2O_2-induced stomatal closing was impaired in the *abi2-1* mutant (Figure 4C; $n = 3$, $P > 0.46$). Stomatal movement results were confirmed in additional control and blind experiments (see Methods).

ABA Enhances ROS Levels in the Wild Type and *abi2-1* but Not in *abi1-1*

To analyze ABA-dependent ROS production in *abi1-1* and *abi2-1*, ROS levels were analyzed in populations of wild-type, *abi1-1*, and *abi2-1* guard cells using the fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA), which reports changes in the oxidative state of guard cells (Ohba et al., 1994; Lee et al., 1999; Pei et al., 2000). As shown in Figure 5, the relative fluorescence emission increased after treatment of wild-type guard cells with 50 μM ABA (four experiments, $P < 0.02$). However, ROS measurements showed that 50 μM ABA did not increase the relative fluorescence emission in *abi1-1* guard cells (six experiments). A slight ABA-induced decrease in ROS levels was observed in *abi1-1* guard cells, which was not significant in all data sets ($P = 0.02$ to 0.053) (Figure 5). Conversely, ABA at 50 μM increased the relative fluorescence emission in *abi2-1* guard cells (nine experiments, $P < 0.001$) (Figure 5). Impairment of ABA-induced ROS production in *abi1-1* was significant compared with that in the wild type ($P < 0.001$) and *abi2-1* ($P < 0.001$). Differential ABA-induced fluorescence responses in

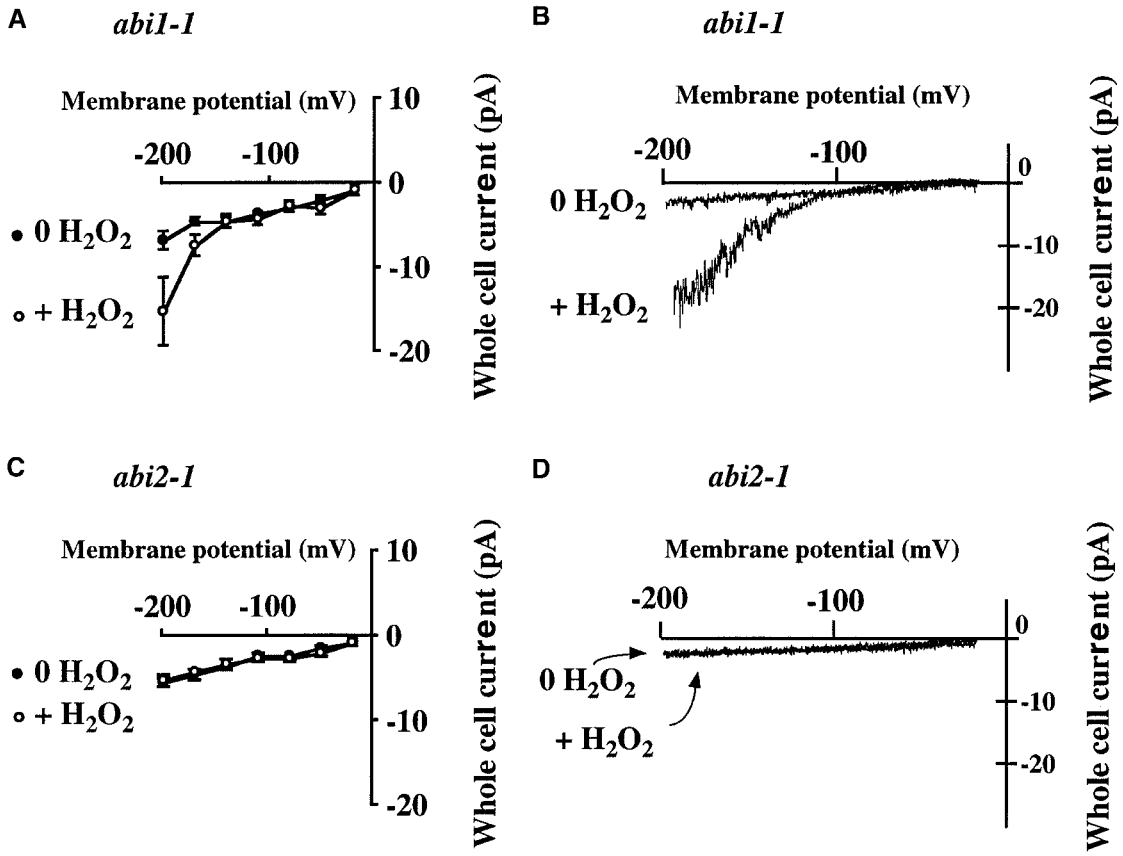

Figure 3. H_2O_2 Activates Ca^{2+}-Permeable Currents in *abi1-1* but Not *abi2-1* Arabidopsis Guard Cell Protoplasts.

(A) and **(B)** H_2O_2 (100 μM) activates I_{Ca} in *abi1-1* guard cells ($n = 6$).
(C) and **(D)** H_2O_2 (100 μM) did not activate I_{Ca} in *abi2-1* guard cells ($n = 5$). **(D)** shows two overlapping traces.
Experiments were performed as described in Figure 1 except that H_2O_2 was used instead of ABA. Error bars represent SEM.

abi1-1 and *abi2-1* were confirmed in additional blind experiments (see Methods).

DISCUSSION

ABA induces an increase in $[Ca^{2+}]_{cyt}$, leading to a reduction in stomatal aperture. Recent studies of Arabidopsis mesophyll suspension culture cells showed that ABA induction of *Rab18* gene expression requires ABA activation of plasma membrane Ca^{2+} influx followed by S-type anion channel activation, suggesting that the analyzed early signaling mechanisms in guard cells may be components of ABA signaling in many plant cell types (Ghelis et al., 2000a, 2000b). ABA stimulates hyperpolarization-activated I_{Ca} Ca^{2+} channels via ROS production, suggesting a new branch in early ABA signaling (Pei et al., 2000). ABA also increases the endogenous level of ROS in maize embryos, suggesting that H_2O_2 functions in ABA regulation of seed maturation (Guan et al., 2000). Furthermore, recent studies have linked ABA with oxidative responses (Bueno et al., 1998; Gong et al., 1998). These studies suggest that ROS may be of more general importance for ABA signal transduction in plants.

Cytosolic NADPH Is Necessary for the ABA Activation of I_{Ca}

ROS is a term for radicals and other reactive species derived from oxygen. ROS have been implicated in numerous signal transduction pathways in both plant and animal cells (Lamb and Dixon, 1997; Rhee et al., 2000). In plants, ROS, including the superoxide radical and H_2O_2, act as important second messengers in defense responses triggered by pathogens and elicitors (Levine et al., 1994; Lamb and Dixon,

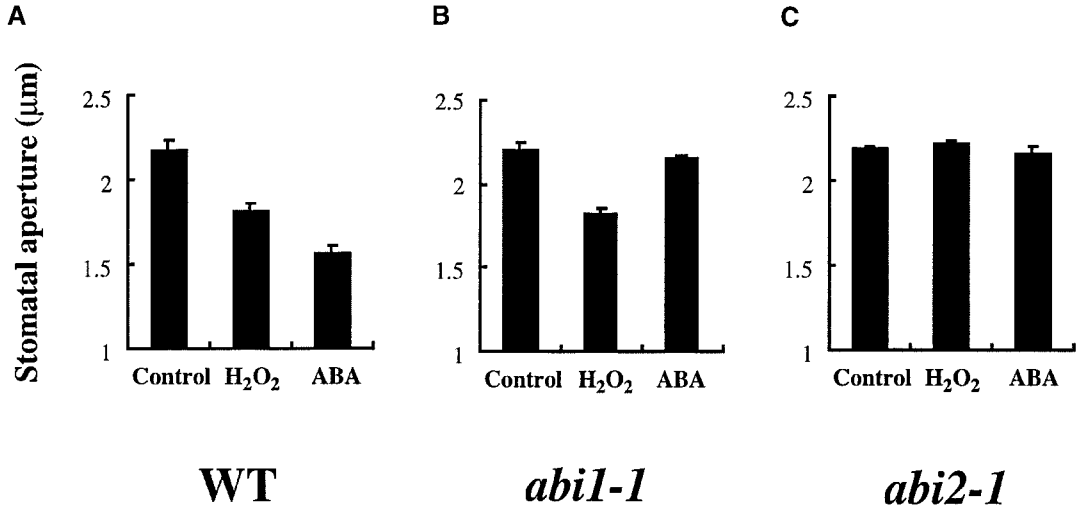

Figure 4. Differential Effects of H_2O_2 on Stomatal Apertures of *abi1-1* and *abi2-1*.

(A) Both ABA (50 μM) and H_2O_2 (100 μM) induced stomatal closing in the wild type (Landsberg *erecta* ecotype).
(B) ABA did not cause stomatal closing, but H_2O_2 elicited partial stomatal closing in *abi1-1*.
(C) Neither ABA nor H_2O_2 elicited stomatal closing of *abi2-1*.
Averages from n = three leaf epidermal experiments are shown (60 stomates per bar). Error bars represent SEM.

1997). Many enzymes can produce ROS in plant cells. Activation of these enzymes is closely associated with oxidative bursts (Lamb and Dixon, 1997). A plasma membrane oxidase generates superoxide (Keller et al., 1998), a peroxidase produces H_2O_2 directly, and an oxalate oxidase also generates H_2O_2 (Baker and Orlandi, 1995). DPI, an inhibitor of neutrophil NADPH oxidases (Cross and Jones, 1986, 1991), partially inhibits ABA-induced stomatal closing (Pei et al., 2000). The elicitors oligogalacturonic acid and chitosan reduce stomatal aperture and induce the production of ROS in tomato and *Commelina* guard cells (Lee et al., 1999). The ROS activation of I_{Ca} channels has been proposed to represent a possible joint branch of multiple stress signaling pathways (Pei et al., 2000; Schroeder et al., 2001).

Previous studies have shown that the ABA activation of Ca^{2+} channels in *Vicia* guard cells is transient and attenuated (Schroeder and Hagiwara, 1990; Hamilton et al., 2000). In the present study of Arabidopsis guard cells, we obtained ABA activation of I_{Ca} in patch-clamped whole cells by adding NADPH or NADH via the patch pipette to the cytosol of guard cells. However, no ABA response was found in the relatively small Arabidopsis guard cells when no NAD(P)H was added to the patch pipette. The requirement of NADPH or NADH, ABA-induced ROS production, and the inhibitory effects of DPI suggest that NAD(P)H oxidases and/or redox control of sulfhydryl groups contributes to ABA signal transduction. NAD(P)H is formed by the reduction of NAD(P) via light-supplied energy in guard cell chloroplasts (Shimazaki et al., 1989). A previous study showed that ABA-induced stomatal closing requires guard cell metabolism (Weyers et al., 1982). In this respect, the results presented here indicate a possible link between guard cell metabolism and ion channel regulation. ABA signaling requires hydrolyzable ATP in guard cells (Schmidt et al., 1995) and phosphorylation events as positive transducers of stomatal closing, which also would require intact guard cell metabolism.

Plasma Membrane Ca^{2+} Channel Activity in Guard Cells

Guard cells show spontaneous activity of hyperpolarization-induced Ca^{2+} increases (Gilroy et al., 1991; Grabov and Blatt, 1998; Allen et al., 1999b) and spontaneous activity of plasma membrane Ca^{2+} currents (Hamilton et al., 2000; Pei et al., 2000). Whether the spontaneous Ca^{2+} influx and I_{Ca} are mediated by the same Ca^{2+} channel remains unknown. Interestingly, the spontaneous activity of hyperpolarization-induced Ca^{2+} currents was inhibited by adding DTT to the patch clamp pipette (cytosolic) solution and bath solutions (Pei et al., 2000; this study).

Fungal elicitors have been shown to induce hyperpolarization-activated Ca^{2+} channels in tomato suspension culture cells (Gelli et al., 1997). These Ca^{2+} channels show a similar I_{Ca}-like activation by hyperpolarization and a more pronounced time-dependent activation. Interestingly, in some tomato cells, spontaneous activity of hyperpolarization-activated Ca^{2+} channels was observed, which also was inhibited by 1 mM DTT (A. Gelli and E. Blumwald, personal

communication). Pathogenic elicitors cause ROS production in plants, with Ca^{2+} influx occurring both before and after ROS production (Knight et al., 1991; Price et al., 1994; Lamb and Dixon, 1997; Kawano et al., 1998), suggesting that more than one Ca^{2+} channel or activation mechanism may contribute to this response. I_{Ca}-like Ca^{2+} channels may contribute to the secondary response that follows ROS production.

Interestingly, hyperpolarization-activated Ca^{2+} channels also have been identified in Arabidopsis cells from the cortical elongation zone of roots and the epidermis of the growing root tip, but not in mature epidermis or in pericycle cells (Kiegle et al., 2000) or in the apex of Arabidopsis root hair cells (Very and Davies, 2000). These studies suggest that hyperpolarization-activated Ca^{2+} channels may contribute to various signal transduction and growth processes in plants, and their opposite voltage dependence compared with depolarization-activated Ca^{2+} channels (Huang et al., 1994; Marshall et al., 1994; Thuleau et al., 1994) suggests activation during different signaling processes.

Differential Disruption by *abi1-1* and *abi2-1* PP2Cs

Stomata of the *abi1-1* and *abi2-1* mutants are insensitive to ABA (Finkelstein and Somerville, 1990; Roelfsema and Prins, 1995; Pei et al., 1997). ABA regulation of K^+ channels is impaired by *abi1-1* expression (Armstrong et al., 1995), and ABA activation of S-type anion channels is impaired in *abi1-1* and *abi2-1* (Pei et al., 1997). The Arabidopsis *abi1-1* and *abi2-1* mutations impair ABA-induced cytoplasmic Ca^{2+} increases in guard cells (Allen et al., 1999a). ABA induces both Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular space. However, it remained unknown which Ca^{2+} increase mechanisms were affected by *abi1-1* and *abi2-1*. The identification of ROS and I_{Ca} as early ABA signaling intermediates has allowed a direct analysis of the effects of the *abi* PP2C mutations on early signal transduction mechanisms.

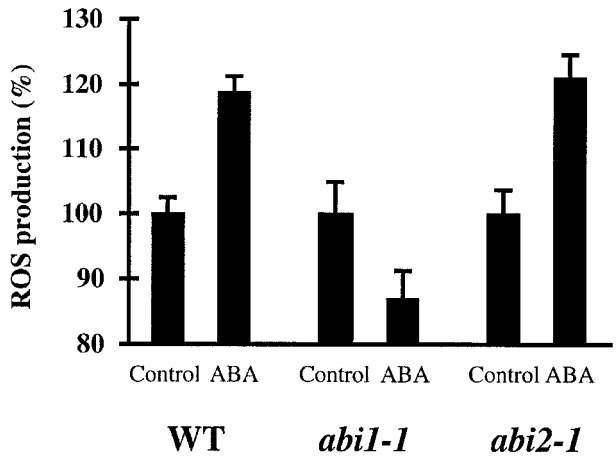

Figure 5. Differential Production of ROS in *abi1-1* and *abi2-1* Guard Cells Treated with ABA.

ABA (50 μM) increased ROS production in the wild type (WT) (four experiments, $n = 161$ cells before ABA treatment, $n = 123$ cells after ABA treatment) and *abi2-1* (nine experiments, $n = 221$ cells before ABA treatment, $n = 207$ cells after ABA treatment). ABA did not increase ROS production in *abi1-1* (six experiments, $n = 110$ cells before ABA treatment, $n = 150$ cells after ABA treatment). Changes in ROS levels were analyzed by measuring H_2DCF fluorescence levels in guard cells in response to ABA or solvent (0.1% ethanol) control applications. Error bars represent SEM.

Here, we show that the PP2C mutations *abi1-1* and *abi2-1* both disrupt the ABA activation of I_{Ca}. ABA did not induce the production of ROS in the *abi1-1* mutant. H_2O_2-activated I_{Ca} and H_2O_2-induced stomatal closing were not impaired in *abi1-1*. These findings suggest that the *abi1-1* mutation disrupts ABA signaling upstream of ROS production, as illustrated in Figure 6. In contrast, ABA elicited ROS production in the *abi2-1* mutant, but H_2O_2 did not activate I_{Ca} and did not induce stomatal closing in *abi2-1*. These data suggest that the *abi2-1* mutation impairs ABA signaling downstream of ROS production (Figure 6). These data lead to a simple model for the positioning of I_{Ca}, ROS production, and the abi1-1 and abi2-1 protein phosphatases in the ABA signal transduction cascade in Arabidopsis guard cells, as illustrated in Figure 6. Note that ABA may function by downregulating ROS-scavenging enzymes such as catalase. The data further suggest that abi1-1 interacts with early signal transduction mechanisms upstream of ROS production. Previous studies have suggested that *abi1-1* and *abi2-1* have distinct functions, even though they both disrupt ABA signaling in general (Gilmour and Thomashow, 1991; Vartanian et al., 1994; Gosti et al., 1995; Bruxelles et al., 1996; Söderman et al., 1996; Pei et al., 1997; Strizhov et al., 1997). Our data are consistent with these findings and provide a working model for the ABA signal transduction pathway to explain the differential effects of *abi1-1* and *abi2-1* (Figure 6).

It remains unknown whether abi1-1 and abi2-1 additionally affect Ca^{2+} release from intracellular stores and a possible parallel Ca^{2+}-independent pathway (Allan et al., 1994; Allen et al., 1999a). Ca^{2+} release can occur parallel to Ca^{2+} influx at low ABA concentrations (MacRobbie, 2000), and perhaps also downstream of I_{Ca} activation, based on Ca^{2+} activation of plant phospholipase C isoforms (Staxen et al., 1999) and on the proposed Ca^{2+}-induced Ca^{2+} release via vacuolar slow vacuolar (SV) channels (Ward and Schroeder, 1994; Bewell et al., 1999). If abi1-1 impairs an ABA receptor, parallel mechanisms could be accounted for by one effect of *abi1-1*. Note that the abi1-1 and abi2-1 PP2Cs may interact with more than one protein. Future research is needed to analyze the effects of *abi1-1* and *abi2-1* on Ca^{2+} release mechanisms.

In summary, the present study provides strong support for the new model that ABA-induced ROS production and

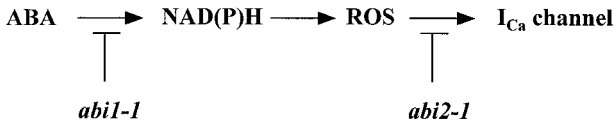

Figure 6. Model Summarizing the Differential Disruption of ABA Activation of I_{Ca} Ca^{2+} Channels by the Two *abi1-1* and *abi2-1* PP2C Mutants.

ABA activation of Ca^{2+} channels required cytosolic NAD(P)H, was inhibited by DPI, and was accompanied by ROS production.

I_{Ca} Ca^{2+}-permeable channel activation are important components of ABA signal transduction. The cytosolic NAD(P)H requirement for ABA activation of I_{Ca} channels, together with DTT and DPI inhibition of I_{Ca}, suggests that NAD(P)H oxidases and/or redox control of sulfhydryl groups contributes to early ABA signaling. Furthermore, abi1-1 and abi2-1 interact with important and distinct transducers of this early ABA signaling branch upstream and downstream of ROS production, respectively.

METHODS

Isolation of Arabidopsis Guard Cells

Arabidopsis thaliana wild type (Landsberg *erecta* ecotype) and the *abi1-1* and *abi2-1* mutant lines were used in this study. The mutant genotypes and homozygosity were confirmed using a previously described polymerase chain reaction method (Leung et al., 1997). The abscisic acid (ABA) insensitivity was further confirmed in seed germination assays (Pei et al., 1997; Allen et al., 1999a). Note that during the course of this work, a separate set of *abi1-1* and *abi2-1* seed was obtained from the Arabidopsis stock center (Ohio State University, Columbus). However, seed germination and polymerase chain reaction tests showed that these consisted of a mixed population. The stock center was informed, and these seed were not used further. Plants were grown in soil in plant growth chambers with a 16-hr-light (80 μE light fluence rate) and 8-hr-dark regimen and watered with deionized water every day. Arabidopsis guard cell protoplasts were isolated enzymatically from leaf epidermal strips of 4- to 6-week-old plants. Arabidopsis rosette leaves were blended in a commercial blender in deionized water three times for 5 sec each and collected using a nylon mesh (pore size, 62 μm). The collected epidermal tissue was incubated in 10 mL of medium containing 1% Cellulase R-10, 0.5% Macerozyme R-10 (Yakult, Japan), 0.5% BSA, 0.5 M mannitol, 0.1 mM KCl, 0.1 mM $CaCl_2$, 10 mM ascorbic acid, and 0.1% kanamycin sulfate, pH 5.5 (with KOH), for 15 to 17 hr at 24°C on a shaker. Isolated guard cell protoplasts were collected and washed twice as described previously (Pei et al., 1997).

Patch Clamp and Data Acquisition

Whole-cell patch clamp recordings from Arabidopsis guard cells were made using Axopatch 200 and 200A amplifiers (Axon Instruments, Union City, CA) that were connected to microcomputers via interfaces as described (Pei et al., 1997). Seal resistances were >10 GΩ. Liquid junction potentials were corrected (Ward and Schroeder, 1994). Initially, upon establishment of whole-cell recordings, a current was observed in some guard cells. This current, however, disappeared within 30 sec to 4 min after establishing whole-cell recordings in 90% of the guard cells analyzed. The frequency of occurrence of this initial current varied from cell preparation to cell preparation. After the initial current had vanished and ~10 min after establishing whole-cell recordings, ABA and H_2O_2 were applied by bath perfusion to patch-clamped guard cells and responses were recorded. pClamp software (Axon Instruments) was used to acquire and analyze whole-cell currents. The standard voltage protocol ramped from −18 to −198 mV (ramp speed, 180 mV/sec). The interpulse period was 1 min. Whole-cell currents were not leak subtracted. Data were analyzed using Axograph software (Axon Instruments, Inc., Foster City, CA). The bath solution used in patch clamp experiments contained 100 mM $BaCl_2$, 0.1 mM DTT, and 10 mM Mes titrated to pH 5.6 with Tris, and the pipette solution was composed of 10 mM $BaCl_2$, 0.1 mM DTT, 4 mM EGTA, 10 mM Hepes adjusted to pH 7.1, and Tris. To investigate the effects of ABA on a Ca^{2+}-permeable, non-selective cation current (I_{Ca}), NADPH or NADH was added to the pipette solution at the indicated concentrations in the text. To analyze the hyperpolarization-activated currents, we used Ba^{2+} ions, which are permeable to I_{Ca} channels (Pei et al., 2000).

Stomatal Aperture Measurements

Stomatal movement analyses were performed as described previously (Pei et al., 2000). Rosette leaves from 4- to 6-week-old plants were exposed to white light (125 μE fluence rate) while floating in a solution containing 10 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM EGTA (free extracellular Ca^{2+} concentration buffered to ~0.1 mM), and 10 mM Mes titrated to pH 6.15 with KOH. Subsequently, 100 μM H_2O_2 and 50 μM ABA were added to the bath solution as indicated in the Figures and text. After treatment for 2 hr in white light (125 μE fluence rate), leaves were blended and stomatal apertures were measured by focusing on the inner lips of stomates (away from the focal plane of guard cells) as described (Ichida et al., 1997). In each epidermal peel experiment, 20 stomatal apertures were measured at each condition. In additional control and blind experiments, the ability of H_2O_2 to cause stomatal closing in wild-type and *abi1-1* leaves, but not *abi2-1* leaves, was reproduced ($n = 3$ experiments per line; wild type ± H_2O_2, $P < 0.03$; *abi1-1* ± H_2O_2, $P < 0.02$; *abi2-1* ± H_2O_2, $P > 0.36$). Standard errors were determined relative to the square root of the number of epidermal strip experiments, as in previous studies (Ichida et al., 1997; Pei et al., 2000). All statistical analyses were performed using the TTEST program in Excel 5.0 software (Microsoft, Redmond, WA). Values of $P < 0.05$ were considered to show statistically significant differences.

Reactive Oxygen Species Detection in Guard Cells

Reactive oxygen species (ROS) production in guard cells was analyzed using 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) (Ohba et al., 1994; Lee et al., 1999). This nonfluorescent compound is permeable to the plasma membrane and is converted to dichlorofluorescin (H_2DCF), which is impermeable. H_2DCF can be oxidized by peroxi-

dases and H_2O_2. Epidermal tissues were isolated from 6-week-old plants with a commercial blender. The epidermal tissues were incubated in 30 mM KCl and 10 mM Mes-KOH, pH 6.15, in the light at room temperature for 2 hr (Pei et al., 2000). Note that immediately after epidermal tissue isolations, guard cells showed increased ROS levels, likely as a result of mechanical perturbation from epidermis excision. However, after 2-hr incubations of epidermal tissues in white light (125 μE), ROS levels decreased. Fifty micromolar H_2DCF-DA was added to the incubation medium and then either 0.1% ethanol (control) or 50 μM ABA was added to the incubation medium after 20 to 30 min of dye loading. The epidermal tissues were collected using a nylon mesh and washed with distilled water twice after 20 to 30 min of ABA or ethanol control treatments. Guard cells were observed under a fluorescence microscope equipped with a cooled charge-coupled device camera. Note that prolonged exposure of H_2DCF-loaded guard cells to excitation light led to a transient increase in ROS and subsequent bleaching of the dye. Therefore, to compare fluorescence responses in control and ABA-treated samples, excitation light exposure was reduced using neutral density filters and limited to a 10-sec exposure, and only one image was captured per sample. All experiments reported here (Figure 5) were confirmed in additional blind experiments (*abi2-1* ± ABA, five experiments, $n = 234$ guard cells, $P < 0.01$). *abi1-1* showed a slight but statistically insignificant ABA-induced reduction in ROS (five experiments, $n = 152$ guard cells, $P = 0.053$). Images were acquired and the fluorescence emission of guard cells was analyzed using Adobe Photoshop 5.0 (Mountain View, CA).

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