The *coronatine-insensitive 1* Mutation Reveals the Hormonal Signaling Interaction between Abscisic Acid and Methyl Jasmonate in Arabidopsis Guard Cells. Specific Impairment of Ion Channel Activation and Second Messenger Production[1][OA]

Shintaro Munemasa, Kenji Oda, Megumi Watanabe-Sugimoto, Yoshimasa Nakamura, Yasuaki Shimoishi, and Yoshiyuki Murata*

Graduate School of Natural Science and Technology, Okayama University, Tsushima-Naka, Okayama 700–8530, Japan (S.M., Y.N., Y.S., Y.M.); Research Institute for Biological Sciences OKAYAMA, Kibichuo-cho, Okayama 716–1241, Japan (K.O.); and Department of Agriculture, Okayama University, Tsushima-Naka, Okayama 700–8530, Japan (M.W.-S.)

Methyl jasmonate (MeJA) elicits stomatal closing similar to abscisic acid (ABA), but whether the two compounds use similar or different signaling mechanisms in guard cells remains to be clarified. We investigated the effects of MeJA and ABA on second messenger production and ion channel activation in guard cells of wild-type Arabidopsis (*Arabidopsis thaliana*) and MeJA-insensitive *coronatine-insensitive 1* (*coi1*) mutants. The *coi1* mutation impaired MeJA-induced stomatal closing but not ABA-induced stomatal closing. MeJA as well as ABA induced production of reactive oxygen species (ROS) and nitric oxide (NO) in wild-type guard cells, whereas MeJA did not induce production of ROS and NO in *coi1* guard cells. The experiments using an inhibitor and scavengers demonstrated that both ROS and NO are involved in MeJA-induced stomatal closing as well as ABA-induced stomatal closing. Not only ABA but also MeJA activated slow anion channels and Ca^{2+} permeable cation channels in the plasma membrane of wild-type guard cell protoplasts. However, in *coi1* guard cell protoplasts, MeJA did not elicit either slow anion currents or Ca^{2+} permeable cation currents, but ABA activated both types of ion channels. Furthermore, to elucidate signaling interaction between ABA and MeJA in guard cells, we examined MeJA signaling in ABA-insensitive mutant *ABA-insensitive 2* (*abi2-1*), whose ABA signal transduction cascade has some disruption downstream of ROS production and NO production. MeJA also did not induce stomatal closing but stimulated production of ROS and NO in *abi2-1*. These results suggest that MeJA triggers stomatal closing via a receptor distinct from the ABA receptor and that the *coi1* mutation disrupts MeJA signaling upstream of the blanch point of ABA signaling and MeJA signaling in Arabidopsis guard cells.

Stomatal pores that are formed by a pair of guard cells respond to various stimuli, including plant hormones and elicitors. Methyl jasmonate (MeJA), which mediates various plant defense responses (Liechti and Farmer, 2002; Turner et al., 2002), has been reported to induce stomatal closing (Gehring et al., 1997; Suhita et al., 2003; Suhita et al., 2004). It has been suggested that there are several components common to both abscisic acid (ABA)-induced and MeJA-induced stomatal closing (e.g. reactive oxygen species [ROS] and cytosolic alkalization [Suhita et al., 2004]). Nitric oxide (NO) as well as ROS functions as an important second messenger in ABA-induced stomatal closing signal transduction (Desikan et al., 2002; Neill et al., 2002; Bright et al., 2006). It has been shown that NO is also involved in the defense signaling mediated by MeJA (Orozco-Cárdenas and Ryan, 2002; Huang et al., 2004). Moreover, a recent finding suggests that ABA induces NO production via hydrogen peroxide (H_2O_2) synthesis (Bright et al., 2006). However, the role of NO in MeJA-induced stomatal closing remains to be clarified. These second messengers have been shown to mediate ion channels in the plasma membrane of guard cells. For example, ROS and NO modulate the activation of K^+ channels (Garcia-Mata et al., 2003; Köhler et al., 2003; Sokolovski and Blatt, 2004; Sokolovski et al., 2005), Cl^-channels (Garcia-Mata et al., 2003; Sokolovski et al., 2005), and Ca^{2+} channels (Allen et al., 2000; Pei et al., 2000; Murata et al., 2001; Köhler et al., 2003; Kwak et al., 2003) in guard cells of fava bean (*Vicia faba*) or Arabidopsis (*Arabidopsis thaliana*).

[1] This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (grants for Scientific Research on Priority Areas to Y.M.).

* Corresponding author; e-mail muta@cc.okayama-u.ac.jp; fax 81–86–251–8388.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yoshiyuki Murata (muta@cc.okayama-u.ac.jp).

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.106.091298

The activation of several types of ion channels in the plasma membrane of guard cells is an important process for ABA-induced stomatal closing. Activation of slow (S-type) anion channels depolarizes the plasma membrane of guard cells (Schroeder and Keller, 1992; Schmidt et al., 1995; Pei et al., 1997), and the activation of S-type anion channels by ABA is impaired in many ABA-insensitive mutants (Pei et al., 1997; Li et al., 2000; Wang et al., 2001; Kwak et al., 2002). These results indicate that activation of S-type anion channels is essential for ABA-induced stomatal closing.

Ca^{2+} permeable cation (I_{Ca}) channels, which are activated by H_2O_2, contribute to elevation of cytosolic-free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in guard cells (Pei et al., 2000). I_{Ca} channels are also activated by ABA and fungal elicitors only in the presence of cytosolic NAD(P)H (Murata et al., 2001; Klüsener et al., 2002), and activation of I_{Ca} channels by ABA is abolished in the Arabidopsis ABA-insensitive mutants *gca2* (Pei et al., 2000) and *ABA-insensitive 2* (*abi2-1*; Murata et al., 2001). In addition, both ABA-induced ROS production and ABA-induced cytosolic NAD(P)H-dependent activation of I_{Ca} channels are impaired in the Arabidopsis double mutant of catalytic subunits of plasma membrane NAD(P)H oxidases *atrboh D/F* (Kwak et al., 2002). These studies revealed that NAD(P)H oxidase-mediated ROS production is necessary for activation of I_{Ca} channels by ABA.

It has been demonstrated that K^+ efflux across the plasma membrane of guard cells is stimulated by long-term depolarization of the plasma membrane (Schroeder et al., 1987) and that cytosolic alkalization activates outward rectifying K^+ currents (Blatt and Armstrong, 1993). Recently, it was demonstrated that MeJA activates outward potassium currents in guard cell protoplasts (GCPs) of fava bean (Evans, 2003). However, to our knowledge, no study has elucidated whether MeJA regulates other ion channels in the plasma membrane of guard cells.

In this study, we used the MeJA-insensitive mutant, *coronatine-insensitive 1* (*coi1*), which fails in various jasmonate-mediated responses, including inhibition of root growth and expression of defense genes (Feys et al., 1994; Benedetti et al., 1995; Penninckx et al., 1996; Xie et al., 1998). The *coi1* mutation disrupts an F-box protein that functions in the E3 ubiquitin ligase involved in the 26S proteasome-mediated protein degradation pathway (Xie et al., 1998; Xu et al., 2002). However, the role of F-box proteins in signal transduction in guard cells remains unknown.

In this article, to elucidate the signaling interaction between ABA and MeJA in guard cells, we examined ROS production, NO production, activation of S-type anion channels, and activation of I_{Ca} channels induced by MeJA and also tested the effects of MeJA on stomatal aperture and second messenger production in the *abi2-1* mutant. Based on our results, we present a new model of hormonal signaling interaction in Arabidopsis guard cells.

RESULTS

Impairment of MeJA-Induced Stomatal Closing in *coi1* Mutant

To clarify MeJA signaling in guard cells, we examined stomatal movements of the MeJA-insensitive mutant *coi1*. ABA induced stomatal closing in *coi1* plants as well as wild-type plants (Fig. 1A). Both plants responded to ABA in a similar dose-response manner, suggesting that ABA signal cascade in the *coi1* mutant could not be disrupted. However, MeJA induced stomatal closing in wild-type plants but did not induce stomatal closing in *coi1* plants (Fig. 1B). Time courses of stomatal closing in wild-type plants and *coi1* plants are shown in Figure 1, C and D, respectively. Application of 1 μM ABA or 1 μM MeJA reduced stomatal apertures by 18% at 30 min and by 21% at 120 min in wild-type plants (Fig. 1C). The similar time course of stomatal movements was observed in *coi1* plants treated with ABA, but there was no change in stomatal aperture of *coi1* plants treated with MeJA (Fig. 1D). Note that reverse transcriptional-PCR analysis with highly purified GCPs (>80%) showed that *COI1* is expressed in guard cells (data not shown). This result indicates that COI1 can be involved in MeJA signaling in guard cells. Taken together, our results indicated that the *coi1* mutation specifically impaired MeJA-induced stomatal closing but not ABA-induced stomatal closing.

Impairment of ROS Production and NO Production in *coi1* Mutant

To elucidate that ROS and NO act as second messengers in MeJA signaling pathway in guard cells, we examined effects of the *coi1* mutation on ROS production and NO production induced by MeJA using the ROS detection fluorescence dye 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), and the NO detection fluorescence dye 4,5-diaminofluorescein-2 diacetate (DAF-2DA). As shown in Figure 2A, ABA induced ROS production ($P < 0.01$) and NO production ($P < 0.05$) in wild-type guard cells, which is consistent with previous results (Pei et al., 2000; Murata et al., 2001; Neill et al., 2002; Garcia-Mata et al., 2003). MeJA also promoted both ROS production ($P < 0.001$) and NO production ($P < 0.01$) in wild-type guard cells. ABA stimulated ROS production ($P < 0.02$) and NO production ($P < 0.04$) in *coi1* guard cells as well as wild-type guard cells, whereas MeJA did not induce either ROS production ($P = 0.55$) or NO production ($P = 0.69$) in *coi1* guard cells unlike wild-type guard cells (Fig. 2B).

To confirm that ROS and NO function as second messengers on MeJA-induced stomatal closing, we evaluated the effects of the NAD(P)H oxidase inhibitor diphenylene iodonium chloride (DPI), the H_2O_2-specific scavenger catalase, and the NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) on MeJA-induced stomatal

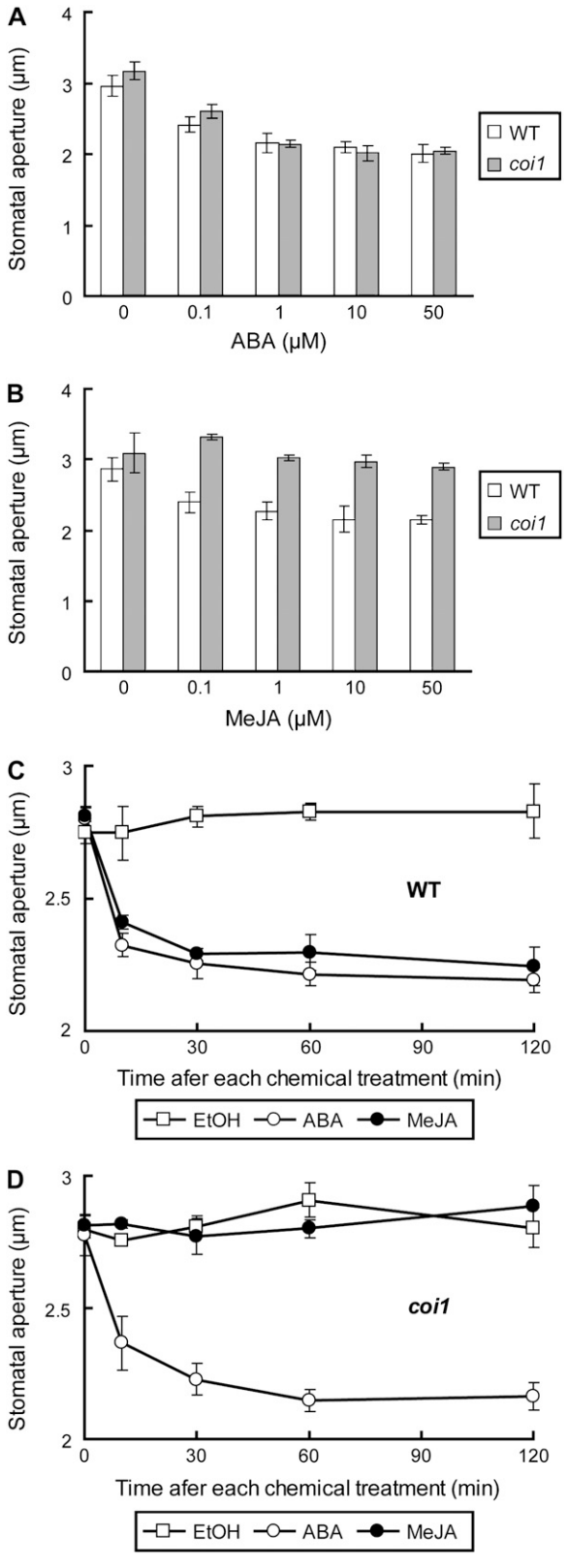

Figure 1. ABA-induced and MeJA-induced stomatal closing in wild-type plants and *coi1* plants. A, ABA-induced stomatal closing in wild-type plants (white bars) and in *coi1* plants (gray bars). B, MeJA-induced stomatal closing in wild-type plants (white bars) but not in *coi1* plants (gray bars). C, The time course of stomatal apertures in wild-type plants treated with 0.1% ethanol (white squares), 1 μM ABA (white circles), and 1 μM MeJA (black circles). D, The time course of stomatal movements of *coi1* plants treated with 0.1% ethanol (white squares), 1 μM ABA (white circles), and 1 μM MeJA (black circles). Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SES.

closing (Fig. 3). Treatment with 25 μM DPI, 100 units/mL catalase, or 100 μM cPTIO significantly prevented ABA-induced stomatal closing, as shown in previous reports (Pei et al., 2000; Zhang et al., 2001; Neill et al., 2002). Treatment with each compound also inhibited MeJA-induced stomatal closing in wild-type plants. These results suggest that MeJA as well as ABA induces stomatal closing via ROS production and NO production in guard cells.

Activation of S-Type Anion Currents and I_{Ca} Currents by MeJA in Wild-Type Guard Cells and Impairment of Activation of These Ion Channels in *coi1* Guard Cells

Many reports have demonstrated that the activation of S-type anion channels in the plasma membrane of guard cells is important for ABA-induced stomatal closing. However, no report demonstrates activation of S-type anion channels in the plasma membrane of guard cells induced by MeJA. Thus, we examined whether MeJA activates S-type anion channels. ABA activated S-type anion currents in wild-type GCPs ($P < 0.03$ at -115 mV; Fig. 4, A and C), which is consistent with previous reports (Pei et al., 1997; Wang et al., 2001; Kwak et al., 2002). Pretreatment with MeJA elicited S-type anion currents in wild-type GCPs ($P < 0.01$ at -115 mV; Fig. 4, A and C). These results suggest that S-type anion channels may play an important role in MeJA-induced stomatal closing as well as ABA-induced stomatal closing. ABA elicited S-type anion currents in *coi1* GCPs ($P < 0.04$ at -115 mV; Fig. 4, B and D) as well as wild-type GCPs, whereas MeJA did not activate S-type anion currents in *coi1* GCPs ($P = 0.57$ at -115 mV; Fig. 4, B and D) unlike wild-type GCPs. These results are consistent with the results of stomatal movements, ROS production, and NO production shown in Figures 1 and 2.

It has been suggested that signals to induce stomatal closing (i.e. ABA and extracellular high Ca^{2+} condition) modulate priming of Ca^{2+} sensors, including calcium-dependent protein kinases in guard cells, which can response elevation of $[Ca^{2+}]_{cyt}$ and then activate S-type anion channels (Allen et al., 2002; Mori et al., 2006). We tested whether the *coi1* mutation affects activation of S-type anion channels by Ca^{2+}. Preincubation of GCPs with 40 mM $CaCl_2$ evoked S-type anion currents both in wild-type GCPs and *coi1* GCPs when the concentration of $[Ca^{2+}]_{cyt}$ was 2 μM (Fig. 4E).

Elevation of $[Ca^{2+}]_{cyt}$ in guard cells occurs during stomatal closing (McAinsh et al., 1995), and application of ABA and H_2O_2 elicits I_{Ca} currents and $[Ca^{2+}]_{cyt}$

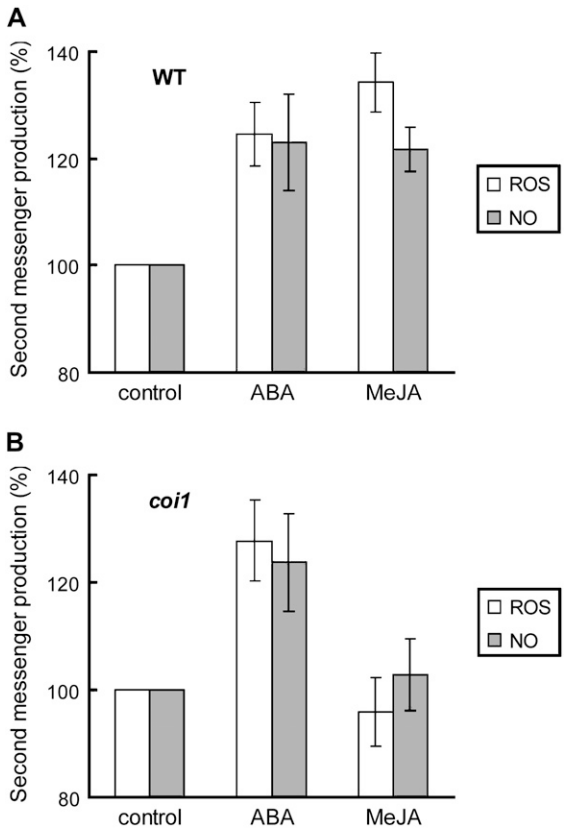

Figure 2. ABA-induced and MeJA-induced production of ROS and NO in wild-type guard cells and *coi1* guard cells. A, Effects of ABA (50 μM) and MeJA (50 μM) on ROS production ($n = 4$, white bars) and NO production ($n = 4$, gray bars) in wild-type guard cells. B, Effects of ABA (50 μM) and MeJA (50 μM) on ROS production ($n = 4$, white bars) and NO production ($n = 5$, gray bars) in *coi1* guard cells. The vertical scale represents the percentage of H_2DCF-DA fluorescence levels (ROS) and DAF-2DA fluorescence levels (NO) when fluorescent intensities of ABA- or MeJA-treated cells are normalized to control value taken as 100% for each experiment. Each data was obtained from >60 total guard cells. Error bars represent SES.

oscillation in guard cells (Allen et al., 1999b, 2000; Hamilton et al., 2000; Pei et al., 2000; Köhler et al., 2003). Thus, we examined whether MeJA activates I_{Ca} channels. MeJA ($P < 0.04$ at -180 mV) as well as ABA ($P < 0.05$ at -180 mV) elicited typical spiky I_{Ca} currents in wild-type GCPs (Fig. 5, A and C). However, in *coi1* GCPs, MeJA ($P = 0.60$ at -180 mV) did not elicit I_{Ca} currents in contrast with ABA ($P < 0.01$ at -180 mV; Fig. 5, B and D). We also examined activation of I_{Ca} currents by H_2O_2 in the *coi1* mutant. Application of 1 mM H_2O_2 to bath solution elicited I_{Ca} currents both in wild-type GCPs and in *coi1* GCPs (Fig. 5E). Additionally, treatment with H_2O_2 induced stomatal closing both in wild-type plants and *coi1* plants (Fig. 5F).

Impairment of MeJA-Induced Stomatal Closing by the *abi2-1* Mutation

To clarify the signaling interaction between ABA and MeJA in Arabidopsis guard cells, we examined whether MeJA induces stomatal closing, ROS production, and NO production in the ABA-insensitive protein phosphatase 2C mutant, *abi2-1* (Meyer et al., 1994; Leung et al., 1997), of which the mutation disrupts ABA signaling downstream of ROS production and NO production in guard cells (Murata et al., 2001; Desikan et al., 2002). Neither ABA nor MeJA induced stomatal closing in *abi2-1* plants unlike in wild-type plants (Fig. 6A). MeJA as well as ABA elicited ROS production and NO production in *abi2-1* guard cells (Fig. 6B).

DISCUSSION

MeJA-Induced Stomatal Closing

Similar to ABA, MeJA accumulates in planta under drought conditions (Creelman and Mullet, 1995). As shown in Figure 1, MeJA induces stomatal closing in a dose- and time-dependent manner similar to that of ABA-induced stomatal closing. Furthermore, we also found that, similar to ABA, MeJA regulates production of second messengers and activation of ion channels in Arabidopsis guard cells. Taken together, these findings suggest that MeJA could play a physiological role in inducing stomatal closing to accommodate the drought condition as ABA.

Specific Impairment of MeJA-Induced Stomatal Closing in *coi1* Mutant

Suhita et al. (2004) investigated the signal interaction between ABA and MeJA using ABA-insensitive mutant *ost1* and MeJA-insensitive mutant *jar1* and reported that the *ost1* mutation and the *jar1* mutation impaired both ABA-induced stomatal closing and MeJA-induced stomatal closing. These results showed some signal interactions between ABA and MeJA but failed to clarify MeJA-specific signaling or ABA-specific

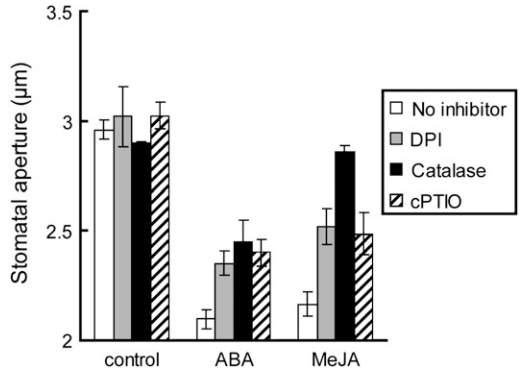

Figure 3. ABA-induced and MeJA-induced stomatal closing inhibited by DPI, catalase, and cPTIO. Rosette leaves of wild-type plants were treated with 20 μM DPI (gray bars), 100 units/mL catalase (black bars), or 100 μM cPTIO (hatched bars). Then, rosette leaves pretreated with each inhibitor for 30 min were treated with 1 μM ABA or 1 μM MeJA. Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SES.

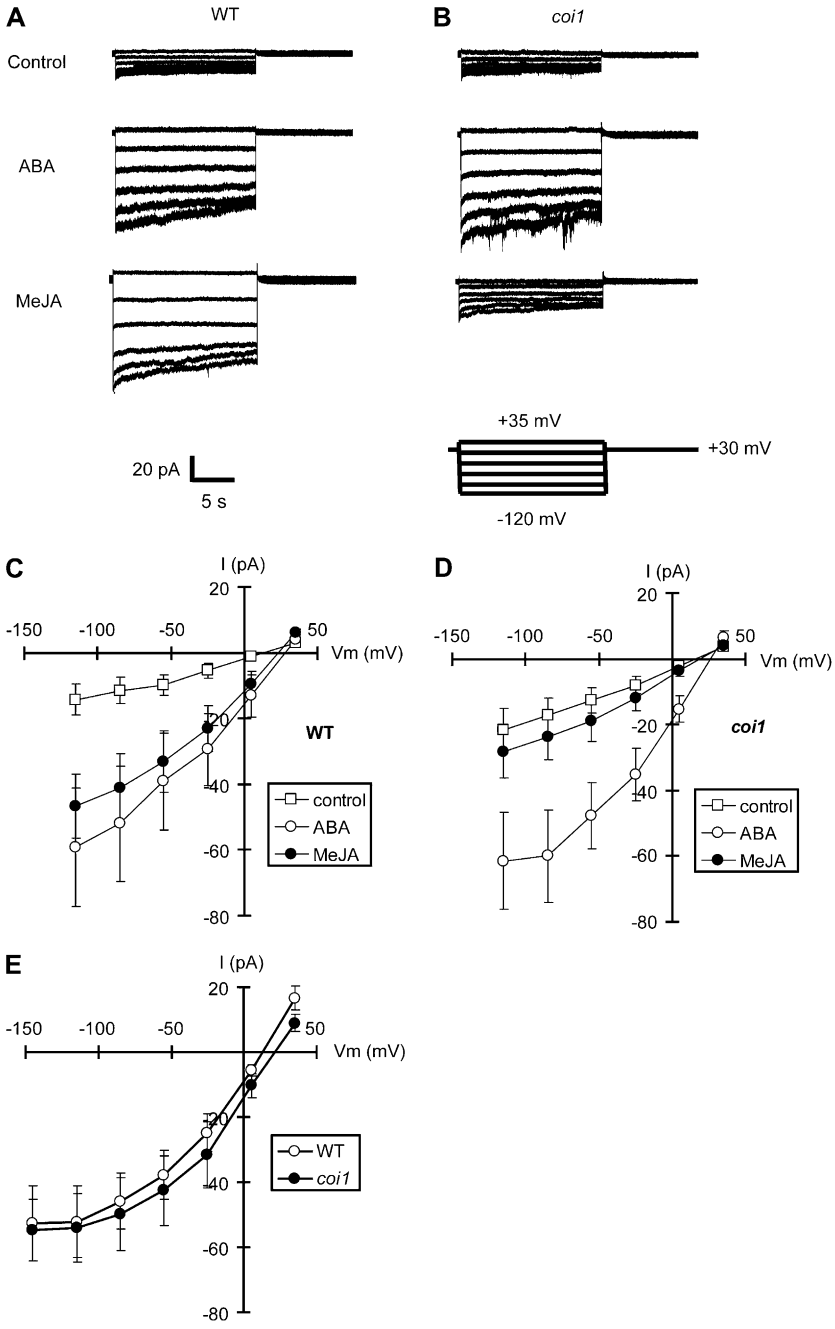

Figure 4. S-type anion currents in wild-type GCPs and *coi1* GCPs. A, Whole-cell recordings of S-type anion currents in wild-type GCPs treated with no hormones (top trace), 50 μM ABA (middle trace), or 50 μM MeJA (bottom trace). B, Whole-cell recordings of S-type anion currents in *coi1* GCPs treated with no hormones (top trace), 50 μM ABA (middle trace), or 50 μM MeJA (bottom trace). C, Steady-state current-voltage relationships for ABA and MeJA activation of S-type anion currents in wild-type GCPs as recorded in A (white squares, control; white circles, 50 μM ABA; black circles, 50 μM MeJA). D, Steady-state current-voltage relationships for ABA and MeJA activation of S-type anion currents in *coi1* GCPs as recorded in B (white squares, control; white circles, 50 μM ABA; black circles, 50 μM MeJA). E, Steady-state current-voltage relationships for extracellular high Ca^{2+} (40 mM) activation of S-type anion currents in wild-type GCPs (white circles) and in *coi1* GCPs (black circles). The voltage protocol was stepped up from 35 mV to −115 mV in 30-mV decrements (holding potential, 30 mV). GCPs were treated with 50 μM ABA or 50 μM ABA MeJA for 2 h before recordings. Each datum was obtained from at least $n = 6$ GCPs. Error bars represent SES.

signaling. In this article, the *abi2-1* (Fig. 6A) and *abi1-1* (data not shown) mutations impaired both ABA-induced stomatal closing and MeJA-induced stomatal closing. These data also indicated that there are some interactions between ABA signaling and MeJA signaling but could not give us any valuable information about ABA-specific signaling and MeJA-specific signaling.

The *coi1* mutants exhibit male sterility, susceptibility to insect attack and pathogen infection, and insensitivity to jasmonates, which are involved in root growth and defense gene expression (Feys et al., 1994; Benedetti et al., 1995; Penninckx et al., 1996; McConn et al., 1997; Xie et al., 1998). In this article, we investigated MeJA signaling and the signaling interaction between ABA and MeJA using the *coi1* mutant. The *coi1* mutation impaired MeJA-induced stomatal closing but did not impair ABA-induced stomatal closing (Fig. 1). These results indicate that, in contrast with the *jar1* mutation, the *coi1* mutation disrupts MeJA signaling upstream of a blanch point between ABA signaling and MeJA signaling in Arabidopsis guard cells, suggesting that COI1 could be an early signaling component in MeJA signaling and that COI1 might be closely involved in perception of MeJA. In future experiments, the *coi1* mutant could be more

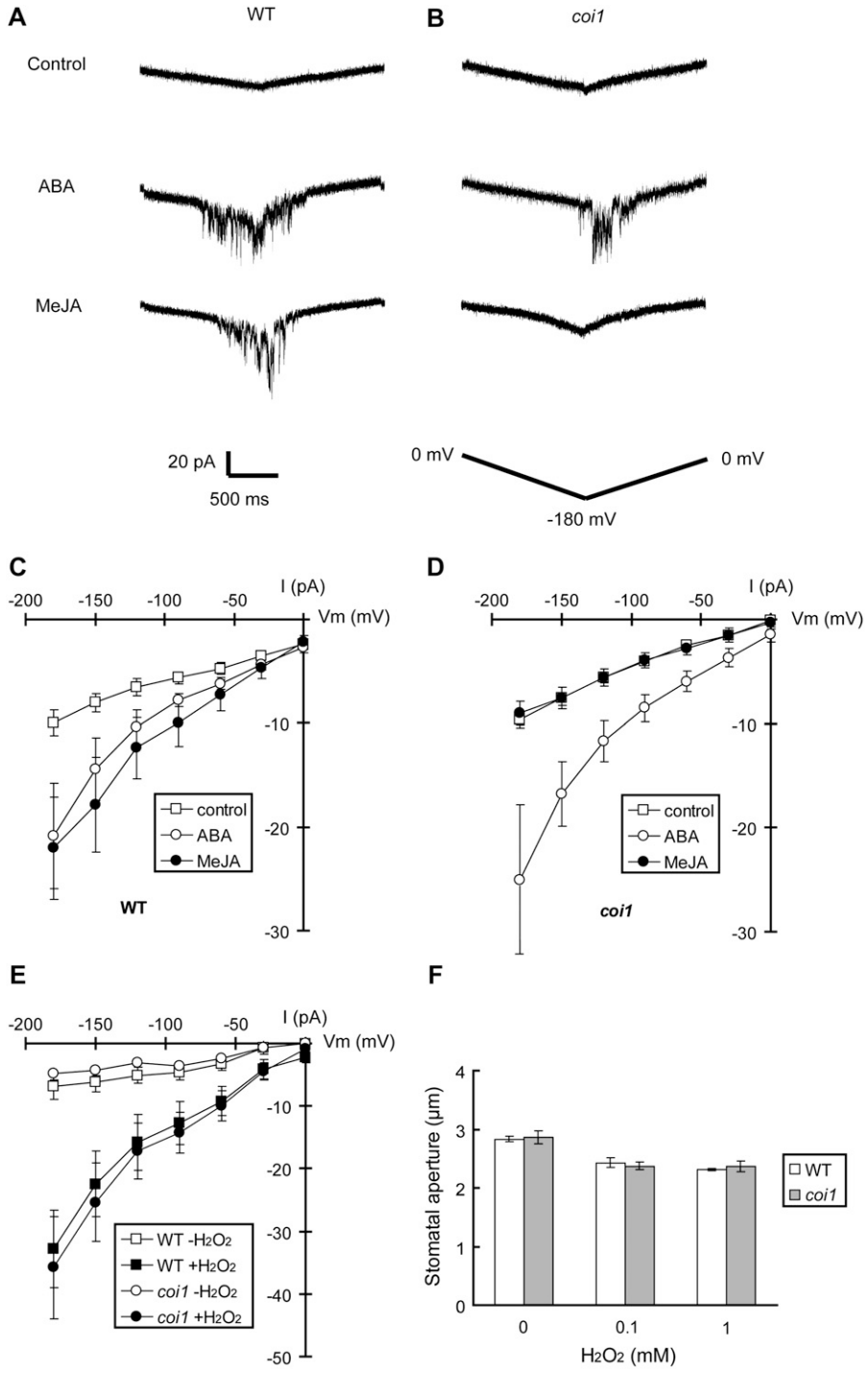

Figure 5. I_{Ca} currents in wild-type GCPs and *coi1* GCPs. A, Whole-cell recordings of I_{Ca} currents in wild-type GCPs treated with no hormones (top trace), 50 μM ABA (middle trace), or 50 μM MeJA (bottom trace). B, Whole-cell recordings of I_{Ca} currents in *coi1* GCPs treated with no hormones (top trace), 50 μM ABA (middle trace), or 50 μM MeJA (bottom trace). C, Current-voltage relationships for ABA and MeJA activation of I_{Ca} currents in wild-type GCPs as recorded in A (white squares, control; white circles, 50 μM ABA; black circles, 50 μM MeJA). D, Current-voltage relationships for ABA and MeJA activation of I_{Ca} currents in *coi1* GCPs as recorded in B (white squares, control; white circles, 50 μM ABA; black circles, 50 μM MeJA). E, Current-voltage relationships for H_2O_2 (1 mM) activation of I_{Ca} currents in wild-type GCPs and *coi1* GCPs (white squares, wild-type $-$ H_2O_2; black squares, wild-type + H_2O_2; white circles, *coi1* $-$ H_2O_2; black circles, *coi1* + H_2O_2). A ramp voltage protocol from 20 to -180 mV (holding potential, 0 mV; ramp speed, 200 mV/s) was used. After making the whole-cell configuration, control currents were recorded 16 times for each GCP as control data. Then, 50 μM ABA, 50 μM MeJA, or 1 mM H_2O_2 was applied to bath solution and I_{Ca} currents were recorded another 16 times. Each datum was obtained from at least $n = 6$ GCPs. Error bars represent SEs. F, H_2O_2-induced stomatal closing in wild-type plants (white bars) and in *coi1* plants (gray bars). Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SEs.

available to elucidate different roles of MeJA from ABA in guard cell response to various environmental stresses.

Involvement of ROS and NO Production in MeJA Signaling in Guard Cells

Both ROS and NO play important roles in MeJA-induced defense signal transduction (Orozco-Cárdenas et al., 2001; Orozco-Cárdenas and Ryan, 2002; Huang et al., 2004), but roles of ROS and NO in MeJA-induced stomatal closing remain unclear. In this study, we found that MeJA as well as ABA induces both ROS production and NO production in wild-type guard cells (Fig. 2) and that ROS and NO actually function as second messengers in the signal pathway of MeJA-induced stomatal closing (Fig. 3). However, in contrast with ABA, MeJA did not induce either ROS production or NO production in *coi1* guard cells (Fig. 2). These results suggest that the *coi1* mutation disrupts

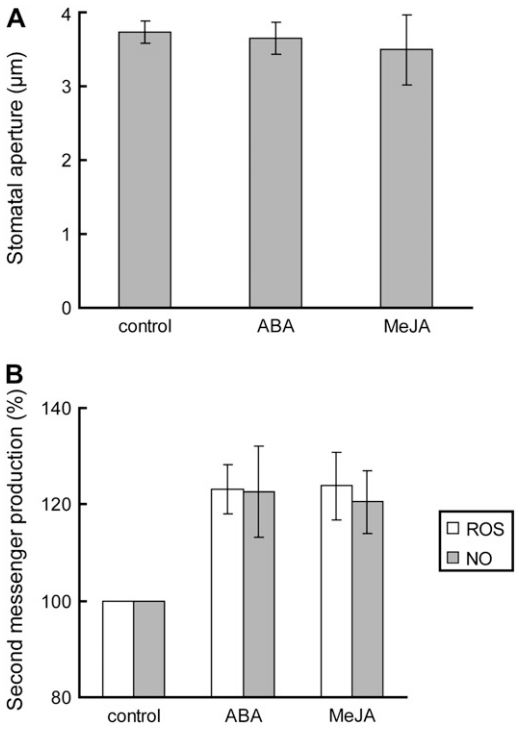

Figure 6. ABA-induced and MeJA-induced stomatal closing in the *abi2-1* mutant. A, Stomatal closing caused by 10 μM ABA and 10 μM MeJA in *abi2-1* plants. Note that the concentration of each chemical was sufficient to induce stomatal closing in wild-type plants. Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SES. B, ROS production (white bars) and NO production (gray bars) caused by 50 μM ABA and 50 μM MeJA. The vertical scale represents the percentage of H_2DCF-DA fluorescence levels (ROS) and DAF-2DA fluorescence levels (NO) when fluorescent intensities of ABA- or MeJA-treated cells are normalized to control value taken as 100% for each experiment. Each datum was obtained from >60 total guard cells. Error bars represent SES.

MeJA signaling upstream of production of ROS and NO, while the *abi2-1* mutation disrupts MeJA signaling downstream of production of ROS and NO in Arabidopsis guard cells (Fig. 6).

DPI, catalase, and cPTIO partly inhibited ABA-induced and MeJA-induced stomatal closing (Fig. 3). Interestingly, catalase showed stronger inhibitory effects on MeJA-induced stomatal closing than ABA-induced stomatal closing (Fig. 3). This finding suggests that, in addition to the production of NAD(P)H oxidase in the guard cell plasma membrane, apoplastic production of ROS by epidermal cells and/or mesophyll cells adjacent to guard cells might be involved in MeJA-induced stomatal closing, because catalase is a cell-impermeable scavenger of H_2O_2 (Lee et al., 1999; Zhang et al., 2001).

Activation of Ion Channels in the Plasma Membrane of Guard Cells by MeJA

It has been shown that the activation of S-type anion channels, which triggers long-term plasma membrane depolarization that results in K^+ efflux via outward K^+ channels, is the primary driving force causing K^+ efflux in guard cells (Schroeder et al., 1987; Schroeder and Keller, 1992; Schmidt et al., 1995; Pei et al., 1997); that is, the activation of S-type anion channels is essential for ABA-induced stomatal closing. In this study, we found that MeJA as well as ABA activates S-type anion channel in wild-type GCPs (Fig. 4, A and C).

S-type anion channels are activated via cytosolic Ca^{2+} dependent and phosphorylation events (Schroeder and Hagiwara, 1989; Schmidt et al., 1995; Allen et al., 1999a, 2002; Mori et al., 2006). Although activation of S-type anion currents by MeJA was impaired in *coi1* GCPs (Fig. 4, B and D), pretreatment with extracellular high Ca^{2+} elicited S-type anion currents both in wild-type GCPs and in *coi1* GCPs (Fig. 4E). This result suggested that COI1 could function upstream of Ca^{2+} sensor priming and $[Ca^{2+}]_{cyt}$ elevation to activate S-type anion channel activation.

We also observed activation of I_{Ca} channels by MeJA in wild-type GCPs but not in *coi1* GCPs (Fig. 5). Suhita et al. (2003) showed that Ca^{2+} channel blockers inhibit MeJA-induced stomatal closing. These findings suggested that the activation of I_{Ca} channels is necessary for Ca^{2+}-dependent signal transduction in MeJA-induced stomatal closing. We also found the *coi1* mutation had no effect on activation of I_{Ca} channels by H_2O_2 (Fig. 5E) and disrupted production of ROS and NO caused by MeJA (Fig. 2). These results also support the conclusion that ROS and NO function as second messengers to activate the plasma membrane ion channels on MeJA signaling in guard cells.

Involvement of ABI2 Protein Phosphatase 2C in MeJA-Induced Stomatal Closing

ABI2 is a protein phosphatase 2C (Meyer et al., 1994; Leung et al., 1997) and the *abi2-1* mutation disrupts

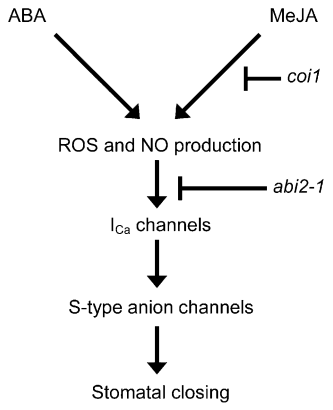

Figure 7. A simple model of the signaling interaction between ABA and MeJA in Arabidopsis guard cells. Similar to ABA, MeJA also induces ROS production and NO production and activates I_{Ca} channels and S-type anion channels. The *coi1* mutation disrupts MeJA signaling upstream of the blanch point of ABA signaling and MeJA signaling. The *abi2-1* mutation disrupts both ABA signaling and MeJA signaling downstream of production of ROS and NO.

ABA signaling downstream of ROS production and NO production in guard cells (Murata et al., 2001; Desikan et al., 2002). MeJA does not induce stomatal closing in *abi2-1* plants (Fig. 6A) but does induce production of ROS and NO in *abi2-1* guard cells (Fig. 6B). These results indicate that MeJA induces stomatal closing via *ABI2*-dependent signal pathway and suggest that the *abi2-1* mutation could disrupt MeJA signaling downstream of production of ROS and NO in Arabidopsis guard cells. It has been shown that the protein phosphatase activity of ABI2 is sensitive to redox status (Meinhard et al., 2002). Therefore, the perception of redox signaling through redox sensors such as ABI2 could be indispensable for the stomatal closing caused by MeJA.

The Physiological Significance of *COI1* in MeJA Signaling in Arabidopsis Guard Cells

Based on our results, we presented a simple model of the signaling interaction between ABA and MeJA in Arabidopsis guard cells in Figure 7. This model positions *COI1*, production of ROS and NO, *ABI2*, I_{Ca} channels, and S-type anion channels in this hormone signal cascade. *COI1* functions upstream of the blanch point of ABA signaling and MeJA signaling and *ABI2* functions downstream of the blanch point.

COI1 encodes one of the F-box proteins that function in E3 ubiquitin-ligase complexes, which are involved in the 26S proteasome-mediated protein degradation pathway (Xie et al., 1998; Xu et al., 2002). In this article, we found that the *coi1* mutation impaired production of second messengers and activation of ion channels induced by MeJA in guard cells, suggesting that the ubiquitin/proteasome pathway could regulate production of second messengers and activation of ion channels in plants.

MATERIALS AND METHODS

Plant Material and Growth

Throughout this study, we used the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia as the wild-type plant. Columbia, the *coi1* mutant (Columbia accession), and the *abi2-1* mutant (Landsberg *erecta* accession) were grown in growth chambers (22°C, 8,000 Lux under a 16-h-light/8-h-dark regime). Both ecotypes showed similar stomatal response to MeJA and ABA (data not shown). The *coi1* mutant, which was used in all parts of this article, has an amber substitution in the Trp-44 of COI1 (TGG to TAG). Because *coi1* mutants have the recessive sterile phenotype, this mutation was kept heterozygous. Additionally, the *coi1* mutant has the JA-responsive *VSP1*::*luciferase* reporter system, as previously described (Ellis and Turner, 2001). F2 seeds were sown on standard Murashige and Skoog plates. Plates were subjected to vernalization treatment at 4°C for 4 d and then transferred to growth chambers in a vertical orientation. Five-day-old seedlings were transferred to Murashige and Skoog plates containing 50 μM MeJA. Plates were further incubated in growth chambers for another 5 d. After that, homozygous *coi1* seedlings were screened by root length, anthocyanin accumulation in cotyledons (Feys et al., 1994), and luciferase activities (Ellis and Turner, 2001). For measurements of luciferase activities, the solution containing 3 mM luciferin (Promega) and 0.01% Triton X-100 was sprayed on detached leaves. Homozygous *coi1* seedlings did not show inhibition of root elongation, anthocyanin accumulation in cotyledons, and luciferase expression induced by MeJA. The screened homozygous *coi1* seedlings were transferred to the soil condition as wild-type plants.

Stomatal Aperture Measurements

Stomatal aperture measurements were performed as described previously (Pei et al., 1997; Murata et al., 2001). Excised rosette leaves were floated on medium containing 5 mM KOH, 50 μM $CaCl_2$, and 10 mM MES-Tris, pH 6.15, for 2 h in the light (8,000 Lux) to induce stomatal opening followed by the addition of MeJA or ABA. Then, stomatal apertures were measured after 2-h incubation. Leaves were blended for 30 s and epidermal peels were collected. Twenty stomatal apertures were measured on each epidermal peel. For time course experiments, stomatal apertures were measured at each pointed time after application of ethanol, ABA, or MeJA.

Detection of ROS and NO

ROS and NO production in guard cells was analyzed by using H_2DCF-DA (Lee et al., 1999; Murata et al., 2001; Suhita et al., 2004) and DAF-2DA (Foissner et al., 2000; Neill et al., 2002; Huang et al., 2004), respectively. In the case of the ROS detection, epidermal peels were incubated for 3 h in medium containing 5 mM KOH, 50 μM $CaCl_2$, and 10 mM MES-Tris, pH 6.15, and then 50 μM H_2DCF-DA was added to this medium. The epidermal tissues were incubated for 30 min at room temperature, and then the excess dye was washed out with distilled deionized water. The dye-loaded tissues were treated with 50 μM ABA or 50 μM MeJA for 20 min, and then fluorescence of guard cells was imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics). For NO detection, 10 μM DAF-2DA was added instead of 50 μM H_2DCF-DA.

Electrophysiology

For whole-cell patch-clamp recordings of S-type anion and I_{Ca} currents, Arabidopsis GCPs were prepared from rosette leaves of 4- to 6-week-old plants with the digestion solution containing 1.0% cellulase R10, 0.5% macerozyme R10, 0.5% bovine serum albumin, 0.1% kanamycin, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol, pH 5.5, with KOH, as previously described (Pei et al., 1997). Whole-cell currents were recorded using a CEZ-2200 patch clamp amplifier (Nihon Kohden). The resulting values were corrected for liquid junction potential, and leak currents were not subtracted. For data analysis, pCLAMP 8.1 software (Molecular Devices) was used. For S-type anion current measurements, the patch-clamp solutions contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5.58 mM $CaCl_2$ (free Ca^{2+} concentration, 2 μM), 5 mM ATP, and 10 mM HEPES-Tris, pH 7.1, in the pipette and 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ (40 mM $CaCl_2$ in Fig. 4E only), and 10 mM MES-Tris, pH 5.6, in the bath (Pei et al., 1997). For I_{Ca} current measurements, the pipette solution contained 10 mM $BaCl_2$, 0.1 mM dithiothreitol, 3 mM NADPH, 4 mM EGTA, and 10 mM HEPES-Tris, pH 7.1. The bath solution contained 100 mM $BaCl_2$, 0.1 mM dithiothreitol, and 10 mM MES-Tris, pH 5.6 (Pei et al., 2000; Murata et al., 2001). In both cases, osmolarity was adjusted to 500 mmol/kg (pipette solutions) and 485 mmol/kg (bath solutions) with D-sorbitol.

Statistical Analysis

Significance of differences between data sets was assessed by Student's *t*-test analysis in all parts of this article. We regarded differences at the level of $P < 0.05$ as significant.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: *COI1*, At2g39940; and *ABI2*, At5g57050.

ACKNOWLEDGMENTS

We thank Koji Shimizu for his help in obtaining patch-clamp data and Masanori Nishiyama for his assistance in obtaining fluorescence data. We also thank Dr. Shigeko Utsugi for providing the *abi2-1* and *abi1-1* seeds.

Received October 12, 2006; accepted December 28, 2006; published January 12, 2007.

LITERATURE CITED

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF, et al (2000) Alteration of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. Science **289:** 2338–2342

Allen GJ, Kuchitsu K, Chu SP, Murata Y, Schroeder JI (1999a) Arabidopsis *abi1-1* and *abi2-1* phosphatase mutations reduce abscisic acid-induced cytoplasmic calcium rises in guard cells. Plant Cell **11:** 1785–1798

Allen GJ, Kwak JM, Chu SP, Llopis J, Tsien RY, Harper JF, Schroeder JI (1999b) Cameleon calcium indicator reports cytoplasmic calcium dynamics in *Arabidopsis* guard cells. Plant J **19:** 735–747

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI (2002) Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the Arabidopsis farnesyltransferase mutant *era1-2*. Plant Cell **14:** 1649–1662

Benedetti CE, Xie D, Turner JG (1995) COI1-dependent expression of an Arabidopsis vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiol **109:** 567–572

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

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ (2006) ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. Plant J **45:** 113–122

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA **92:** 4114–4119

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

Ellis C, Turner JG (2001) The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell **13:** 1025–1033

Evans NH (2003) Modulation of guard cell plasma membrane potassium currents by methyl jasmonate. Plant Physiol **131:** 8–11

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759

Foissner I, Wendehenne D, Langebartels C, Durner J (2000) *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J **23:** 817–824

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

Gehring CA, Irving HR, McConchie R, Parish RW (1997) Jasmonates induce intracellular alkalinization and closure of *Paphiopedilum* guard cells. Ann Bot (Lond) **80:** 485–489

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

Huang X, Stettmaier K, Michel C, Hutzler P, Mueller MJ, Durner J (2004) Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. Planta **218:** 938–946

Klüsener B, Young JJ, Murata Y, Allen GJ, Mori IC, Hugouvieux V, Schroeder JI (2002) Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. Plant Physiol **130:** 2152–2163

Köhler B, Hills A, Blatt MR (2003) Control of guard cell ion channels by hydrogen peroxide and abscisic acid indicates their action through alternate signaling pathways. Plant Physiol **131:** 385–388

Kwak JM, Moon JH, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI (2002) Disruption of a guard cell-expressed protein phosphatase 2A regulatory subunit, *RCN1*, confers abscisic acid insensitivity in Arabidopsis. Plant Cell **14:** 2849–2861

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J **22:** 2623–2633

Lee S, Choi H, Suh S, Doo I-S, Oh K-Y, Choi EJ, Taylor SAT, Low PS, Lee Y (1999) Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reaction oxygen species from guard cells of tomato and *Commelina communis*. Plant Physiol **121:** 147–152

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis *ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9:** 759–771

Li J, Wang XQ, Watson MB, Assmann SM (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science **287:** 300–303

Liechti R, Farmer EE (2002) The jasmonate pathway. Science **296:** 1649–1650

McAinsh MR, Webb AAR, Taylor JE, Hetherington AM (1995) Stimulus-induced oscillations in guard cell cytosolic free calcium. Plant Cell **7:** 1207–1219

McConn M, Creelmann RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA **94:** 5473–5477

Meinhard M, Rodriguez PL, Grill E (2002) The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. Planta **214:** 775–782

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science **264:** 1452–1455

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, et al (2006) CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca^{2+}-permeable channels and stomatal closure. PLoS Biol **4:** e327

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

Neill SJ, Desikan R, Clarke A, Hancock JT (2002) Nitric oxide is a novel component of abscisic acid signaling in stomatal guard cells. Plant Physiol **128:** 13–16

Orozco-Cárdenas ML, Narváez-Vásquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin and methyl jasmonate. Plant Cell **13:** 179–191

Orozco-Cárdenas ML, Ryan CA (2002) Nitric oxide negatively modulates wound signaling in tomato plants. Plant Physiol **130:** 487–493

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

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406:** 731–734

Penninckx IAMA, Eggermont K, Terras FRG, Thomma BPHJ, De Samblanx GW, Buchala A, Métraux JP, Manners JM, Broekaert WF (1996) Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. Plant Cell **8:** 2309–2323

Schmidt C, Schelle I, Liao YJ, Schroeder JI (1995) Strong regulation of slow anion channels and abscisic acid signaling in guard cells by phosphorylation and dephosphorylation events. Proc Natl Acad Sci USA **92:** 9535–9539

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

Schroeder JI, Keller BU (1992) Two types of anion channel currents in guard cells with distinct voltage regulation. Proc Natl Acad Sci USA **89:** 5025–5029

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

Sokolovski S, Blatt MR (2004) Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. Plant Physiol **136:** 4275–4284

Sokolovski S, Hills A, Gay R, Garcia-Mata C, Lamattina L, Blatt MR (2005) Protein phosphorylation is prerequisite for intracellular Ca^{2+} release and ion channel control by nitric oxide and abscisic acid in guard cells. Plant J **43:** 520–529

Suhita D, Kolla VA, Vavasseur A, Raghavendra AS (2003) Different signaling pathways involved during the suppression of stomatal opening by methyl jasmonate or abscisic acid. Plant Sci **164:** 481–488

Suhita D, Raghavendra AS, Kwak JM, Vavasseur A (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol **134:** 1536–1545

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell **14:** S153–S164

Wang XQ, Ullah H, Jones AM, Assmann SM (2001) G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. Science **292:** 2070–2072

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an *Arabidopsis* gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094

Xu L, Liu F, Lechner E, Genschik P, Crosby WL, Ma H, Peng W, Huang D, Xie D (2002) The SCF^{COI1} ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell **14:** 1919–1935

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