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 Ca2+ 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 Ca2+ 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.
Methyl Jasmonate and Abscisic Acid Signaling in 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 (H2DCF-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 H2O2-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...
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 ICa 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$_2$O$_2$ elicits ICa currents and [Ca$^{2+}$]$_{cyt}$ (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 s.e.

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**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 coi1 plants (gray bars). B, MeJA-induced stomatal closing in wild-type plants (white bars) but not in coi1 plants closing (Fig. 3). 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 s.e.
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 (Creeelman 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
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 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 Ca2+ (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.
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).

**Figure 5.** $I_C$ currents in wild-type GCPs and coi1 GCPs. A, Whole-cell recordings of $I_C$ 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 $I_C$ 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 $I_C$ 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 $I_C$ 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 $I_C$ 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 $I_C$ currents were recorded another 16 times. Each datum was obtained from at least $n = 6$ GCPs. Error bars represent s.e. 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 s.e.
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\(_2\)O\(_2\) (Lee et al., 1999; Zhang et al., 2001).

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

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**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
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 ab1-2-1 plants (Fig. 6A) but does induce production of ROS and NO in ab1-2-1 guard cells (Fig. 6B). These results indicate that MeJA induces stomatal closing via AB12-dependent signal pathway and suggest that the ab1-2-1 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 AB12 is sensitive to redox status (Meinhard et al., 2002). Therefore, the perception of redox signaling through redox sensors such as AB12 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, AB12, I_c channels, and S-type anion channels in this hormone signal cascade. COI1 functions upstream of the branch point of ABA signaling and MeJA signaling and AB12 functions downstream of the branch 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 coil 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 coil mutant (Columbia accession), and the ab1-2-1 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 coil mutant, which was used in all parts of this article, has an amber substitution in the Trp-44 of COI1 (TGG to TAG). Because coil mutants have the recessive sterile phenotype, this mutation was kept heterozygous.

Both ecotypes have the recessive sterile phenotype, this mutation was kept heterozygous. Additionally, the coil 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 coil seedlings were screened by root length, anthocyanin accumulation in cotyledons (Feyss 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 coil seedlings did not show inhibition of root elongation, anthocyanin accumulation in cotyledons, and luciferase expression induced by MeJA. The screened homozygous coil 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 blotted 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 (Foisser 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_c 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 1-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 Ca²⁺ 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_c current measurements, the pipette solution contained 10 mM BaCl_2, 0.1 mM dithiothreitol, 5 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 t-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.

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