The Arabidopsis Calcium-Dependent Protein Kinase, CPK6, Functions as a Positive Regulator of Methyl Jasmonate Signaling in Guard Cells

Shintaro Munemasa, Mohammad Anowar Hossain, Yoshimasa Nakamura, Izumi C. Mori, and Yoshiyuki Murata*

Graduate School of Natural Science and Technology, Okayama University, Tsushima-Naka, Okayama 700–8530, Japan (M.A.H., Y.N., Y.M.); and Institute for Plant Science and Bioresources, Okayama University, Kurashiki, Okayama 710–0046, Japan (I.C.M.)

Previous studies have demonstrated that methyl jasmonate (MeJA) induces stomatal closure dependent on change of cytosolic free calcium concentration in guard cells. However, these molecular mechanisms of intracellular calcium signal perception remain unknown. Calcium-dependent protein kinases (CDPKs) function as Ca\(^{2+}\) signal transducers in various plant physiological processes. It has been reported that four Arabidopsis (Arabidopsis thaliana) CDPKs, CPK3, CPK6, CPK4, and CPK11, are involved in abscisic acid signaling in guard cells. It is also known that there is an interaction between MeJA and abscisic acid signaling in guard cells. In this study, we examined the roles of these CDPKs in MeJA signaling in guard cells using Arabidopsis mutants disrupted in the CDPK genes. Disruption of the CPK6 gene impaired MeJA-induced stomatal closure, but disruption of the other CDPK genes did not. Despite the broad expression pattern of CPK6, we did not find other remarkable MeJA-insensitive phenotypes in the cpk6-1 mutant. The whole-cell patch-clamp analysis revealed that MeJA activation of nonselective Ca\(^{2+}\)-permeable cation channels is impaired in the cpk6-1 mutant. Consistent with this result, MeJA-induced transient cytosolic free calcium concentration increments were reduced in the cpk6-1 mutant. MeJA failed to activate slow-type anion channels in the cpk6-1 guard cells. Production of early signal components, reactive oxygen species and nitric oxide, in guard cells was elicited by MeJA in the cpk6-1 mutant as in the wild type. These results provide genetic evidence that CPK6 has a different role from CPK3 and functions as a positive regulator of MeJA signaling in Arabidopsis guard cells.

Guard cells, which form stomatal pores in the leaf epidermis of higher plants, can respond to various environmental stimuli, including light, drought, and pathogen infection (Israelsson et al., 2006; Melotto et al., 2006; Shimazaki et al., 2007). To regulate carbon dioxide uptake for photosynthesis, transpirational water loss, and innate immunity adequately, plants have developed a fine-tuned signal transduction system in guard cells.

The volatile phytohormone methyl jasmonate (MeJA) regulates various physiological processes, including pollen maturation, tendril coiling, and responses to wounding and pathogen attack (Liechti and Farmer, 2002; Turner et al., 2002). Similar to abscisic acid (ABA), MeJA plays a role in the induction of stomatal closure (Gehring et al., 1997; Suhita et al., 2003, 2004). Jasmonate-induced stomatal closure has been observed in various plant species, including Arabidopsis (Arabidopsis thaliana; Suhita et al., 2004; Munemasa et al., 2007; Saito et al., 2008), Hordeum vulgare (Tsonev et al., 1998), Commelina benghalensis (Raghavendra and Reddy, 1987), Vicia faba (Liu et al., 2002), Nicotiana glauca (Suhita et al., 2003), Paphiopedilum supersuk (Gehring et al., 1997), and Paphiopedilum tonsum (Gehring et al., 1997). These findings suggest that jasmonate-induced stomatal closure is one of the fundamental physiological responses in plants.

Calcium has been shown to serve as an important second messenger for the regulation of stomatal movement (Roelfsema and Hedrich, 2007; Kudla et al., 2010). ABA induces stomatal closure via the elevation of cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)). ABA activates guard cell plasma membrane nonselective Ca\(^{2+}\)-permeable cation (I\(_{ca}\)) channels, which mediate...
Ca\(^{2+}\) influx from the extracellular space (Hamilton et al., 2000; Pei et al., 2000), and also induces Ca\(^{2+}\) release from intracellular stores (Leckie et al., 1998; Grabov and Blatt, 1999; García-Mata et al., 2003; Lemtiri-Chlieh et al., 2003). I\(_{Ca}\) channels open on membrane hyperpolarization (Hamilton et al., 2000; Pei et al., 2000), and protein phosphorylation (Köhler and Blatt, 2002). ABA-induced Ca\(^{2+}\) release from intracellular stores is mediated by several second messengers, including nitric oxide (NO; García-Mata et al., 2003; Sokolovski et al., 2005).

It has been shown that MeJA-induced stomatal closure is inhibited by Ca\(^{2+}\) channel blockers and calmodulin inhibitors (Suhita et al., 2003, 2004). Additionally, our previous study revealed that MeJA activates guard cell plasma membrane I\(_{Ca}\) channels and that MeJA activation of I\(_{Ca}\) channels is abolished in the MeJA-insensitive mutant cotil (Munemasa et al., 2007). Activation of I\(_{Ca}\) channels has been proposed to contribute to [Ca\(^{2+}\)]\(_{cyt}\) elevation in guard cell ABA signaling (Hamilton et al., 2000; Pei et al., 2000). These findings suggest that cytosolic Ca\(^{2+}\) serves as an important second messenger in MeJA signaling in Arabidopsis guard cells.

Calcium-dependent protein kinases (CDPKs) are unique enzymes found in plants and some protozoa and are characterized as [Ca\(^{2+}\)]\(_{cyt}\) sensors in plants. Recently, Mori et al. (2006) and Zhu et al. (2007) suggested that four Arabidopsis CDPKs, CPK3, CPK6, CPK4, and CPK11, are involved in ABA-induced stomatal closure. There are functional redundancies between CPK3 and CPK6 (Mori et al., 2006) and between CPK4 and CPK11 (Zhu et al., 2007). CPK4 and CPK11 phosphorylate the ABA-responsive transcriptional factors ABF1 and ABF4 (AREB2) in vitro (Zhu et al., 2007). It was revealed that CPK3 and CPK6 are essential factors for ABA activation of I\(_{Ca}\) channels and slow-type (S-type) anion channels of guard cell plasma membrane, but downstream targets of CPK3 and CPK6 remain unknown (Mori et al., 2006). It has been reported that MeJA signaling and ABA signaling are partially overlapping and form a signaling network in guard cells (Suhita et al., 2003, 2004; Munemasa et al., 2007; Saito et al., 2008). These findings suggest that these CDPKs function as [Ca\(^{2+}\)]\(_{cyt}\) sensors in the MeJA signaling in guard cells.

In this study, we examined the roles of four CDPKs, CPK3, CPK6, CPK4, and CPK11, in MeJA-induced stomatal closure using a reverse genetic approach. We analyzed the stomatal phenotypes of these CDPK mutants and found that the cpk6 mutation impaired MeJA-induced stomatal closure. In CPK6 disruption mutants, MeJA activation of I\(_{Ca}\) channels and S-type anion channels was disrupted. We also addressed the roles of CPK6 in the production of early signal components, ROS and NO, in guard cell MeJA signaling. Our results suggest that CPK6 functions as a positive regulator in MeJA signaling in Arabidopsis guard cells.

**RESULTS**

Impairment of MeJA-Induced Stomatal Closure in cpk6 Mutants

To identify CDPKs that are involved in MeJA signaling in guard cells, the effect of MeJA on stomatal aperture was examined in four single Arabidopsis CDPK mutants, cpk3-1, cpk6-1, cpk4-1, and cpk11-2, and two double mutants, cpk3-1/cpk6-1 and cpk4-1/cpk11-2. Exogenous application of 10 μM MeJA induced stomatal closure in the single cpk3-1, cpk4-1, and cpk11-2 mutants and the double cpk4-1/cpk11-2 mutant similar to the wild type (Fig. 1A). The single cpk6-1 and double cpk3-1/cpk6-1 mutants showed MeJA insensitivity in stomatal closure. MeJA-induced stomatal closure was also impaired in another cpk6 mutant allele, cpk6-2 (Fig. 1B), suggesting that gene disruption in CPK6 confers the MeJA insensitivity. Mori et al. (2006) showed that CPK6 is expressed in mesophyll cells as well as in guard cells. We also found CPK6 expression in flowers, stems, cauline leaves, and roots (Supplemental Fig. S1A). In addition to stomatal movements, we checked the effects of MeJA on the expression of the MeJA-responsive genes VSP1 and VSP2 (Berger et al., 1995; Ellis and Turner, 2001; Liu et al., 2005) and on root growth (Staswick et al., 1992; Feys et al., 1994) in the cpk6-1 mutant. However, we could not find any remarkable phenotype of the cpk6-1 mutant in these observations (Supplemental Fig. S1, B and C).

**Figure 1.** MeJA-induced stomatal closure in Arabidopsis CPK3, CPK6, CPK4, and CPK11 gene disruption mutants. A, MeJA-induced stomatal closure in the wild type (WT) and cpk3-1, cpk6-1, cpk3-1/cpk6-1, cpk4-1, cpk11-2, and cpk4-1/cpk11-2 mutants. B, MeJA-induced stomatal closure in the cpk6-1 and cpk6-2 mutants. Twenty averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent se.
Roles of CPK6 in MeJA Signaling in Guard Cells

Impairment of Activation of $I_{\text{Ca}}$ Currents and Elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ by MeJA in cpk6-1 Guard Cells

Hyperpolarization-activated plasma membrane $I_{\text{Ca}}$ channels function in guard cell ABA signaling (Grabov and Blatt, 1998; Hamilton et al., 2000; Pei et al., 2000). ROS production (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003) and protein phosphorylation (Köhler and Blatt, 2002) are involved in the regulation of $I_{\text{Ca}}$ channel activity. The $I_{\text{Ca}}$ channels are also activated by exogenous application of MeJA (Munemasa et al., 2007). We examined the effects of the CPK6 disruption on MeJA activation of $I_{\text{Ca}}$ channels. MeJA activated $I_{\text{Ca}}$ currents in wild-type guard cell protoplasts (GCPs; $P < 0.05$ at $-180$ mV; Fig. 2, A and C) but did not activate $I_{\text{Ca}}$ currents in cpk6-1 GCPs ($P = 0.71$ at $-180$ mV; Fig. 2, B and D) and cpk6-2 GCPs ($P = 0.71$ at $-180$ mV; Supplemental Fig. S2A). Activation of $I_{\text{Ca}}$ channels contributes to the elevation of guard cell $[\text{Ca}^{2+}]_{\text{cyt}}$ (Hamilton et al., 2000; Pei et al., 2000). To assess the effect of impairment in MeJA activation of $I_{\text{Ca}}$ channels on $[\text{Ca}^{2+}]_{\text{cyt}}$ status in cpk6-1 guard cells, we conducted guard cell $[\text{Ca}^{2+}]_{\text{cyt}}$ imaging using a $\text{Ca}^{2+}$ reporter protein, yellow cameleon 3.6 (Nagai et al., 2004; Young et al., 2006). Three or more transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increments were observed in 52.9% of wild-type guard cells treated with 10 $\mu$M MeJA ($n = 9$ of 17 cells; Fig. 3, A and E), and no transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increment was observed in 17.6% of guard cells treated with 10 $\mu$M MeJA ($n = 3$ of 17 cells; Fig. 3, B and E). Compared with the wild type, a higher percentage of cells with no $[\text{Ca}^{2+}]_{\text{cyt}}$ transient was observed in cpk6-1 guard cells (61.1%; $n = 11$ of 18 cells; Fig. 3, D and E). Three or more $[\text{Ca}^{2+}]_{\text{cyt}}$ transients induced by MeJA were observed in only 16.7% of cpk6-1 guard cells ($n = 3$ of 18 cells; Fig. 3, C and E). We tested the effects of an $I_{\text{Ca}}$ channel blocker, LaCl$_3$, (Hamilton et al., 2000; Pei et al., 2000), on MeJA-induced transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increments. In wild-type guard cells pretreated with 50 $\mu$M LaCl$_3$, MeJA-induced transient $[\text{Ca}^{2+}]_{\text{cyt}}$ increments were not observed (100%; $n = 16$ of 16 cells; data not shown). These results suggest that CPK6 contributes to $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation by regulating $I_{\text{Ca}}$ channel activity during MeJA-induced stomatal closure.

Impairment of Activation of S-Type Anion Currents by MeJA in cpk6-1 Guard Cells

Our previous results demonstrated that activation of S-type anion channels is indispensable for MeJA-induced stomatal closure (Munemasa et al., 2007) and that CPK6 functions as a positive regulator of S-type anion channels in ABA signaling in guard cells (Mori et al., 2006). In this study, we examined MeJA activation of S-type anion channels in the cpk6-1 mutant using the whole-cell patch-clamp technique. MeJA activated S-type anion currents in wild-type GCPs ($P < 0.04$ at $-115$ mV; Fig. 4, A and C). In contrast, MeJA failed to activate S-type anion currents in cpk6-1 GCPs ($P = 0.48$ at $-115$ mV; Fig. 4, B and D) and cpk6-2 GCPs ($P = 0.53$ at $-115$ mV; Supplemental Fig. S2B). These results are consistent with the stomatal phenotype of the cpk6 mutants shown in Figure 1. In cpk3-1 GCPs, MeJA activated S-type anion currents similar to wild-type GCPs ($P < 0.05$ at $-115$ mV; Fig. 5). This result is consistent with the result that MeJA induces stomatal closure in the cpk3-1 mutant (Fig. 1A). Allen et al. (2002) and Siegel et al. (2009) indicated that the increased $[\text{Ca}^{2+}]_{\text{cyt}}$ was required for ABA or extracellular high $[\text{Ca}^{2+}]_{\text{cyt}}$ activation of S-type anion channels. In this paper, we also used a pipette solution with 2 $\mu$M $[\text{Ca}^{2+}]_{\text{cyt}}$ to measure S-type anion channel activity. Note that similar to several previous studies (Allen et al., 2002; Mori et al., 2006; Munemasa et al., 2007; Siegel et al., 2009), only small whole-cell currents were observed in MeJA-untreated GCPs.

Effects of the cpk6-1 Mutation on the Production of ROS and NO Induced by MeJA in Guard Cells

To clarify how CPK6 mediates MeJA signaling in guard cells, we evaluated the production of ROS and NO, which function as early signal components in guard cells. ROS produced by two NAD(P)H oxidases,
AtrbohD and AtrbohF, are important second messengers and function upstream of Ca\(^{2+}\) channel activation in Arabidopsis guard cells (Kwak et al., 2003). These two NAD(P)H oxidases are also involved in guard cell MeJA signaling (Suhita et al., 2004). Recently, it has been suggested that plant NAD(P)H oxidases are phosphorylated and activated by CDPKs (Kobayashi et al., 2007; Ogasawara et al., 2008). We tested the effects of CPK6 disruption on MeJA-induced ROS production in guard cells using the ROS-detection fluorescent dye, 2\#,7\#-dichlorodihydrofluorescein diacetate (H\(_2\)DCF-DA). We found that MeJA evoked ROS production in cpk6-1 guard cells as well as in wild-type guard cells (Fig. 6A).

NO also functions as an important second messenger in MeJA signaling in guard cells. It has been suggested that NO mediates Ca\(^{2+}\) release from intracellular stores during ABA-induced stomatal closure (Garcia-Mata et al., 2003; Sokolovski et al., 2005). To further understand the roles of CPK6 in MeJA signaling, we evaluated MeJA-induced NO production in cpk6-1 guard cells using the NO-detection fluorescent dye, 4,5-diaminofluorescein-2 diacetate (DAF-2DA). In wild-type guard cells, MeJA evoked NO production (Fig. 6A), which is consistent with our previous results (Munemasa et al., 2007). MeJA-induced NO production was also observed in cpk6-1 guard cells (Fig. 6B). The roles of CPK6 in ROS and NO production induced by ABA in guard cells have not yet been examined (Mori et al., 2006). We found that ROS and NO production induced by ABA were not impaired either in the cpk6-1 mutant (Fig. 6) or in the cpk3-1/cpk6-1 double mutant (data not shown), which show stronger ABA-insensitive phenotypes than the single cpk3 and cpk6 mutants (Mori et al., 2006).

DISCUSSION

The cpk6-1 Mutant Did Not Show MeJA-Induced Stomatal Closure

Previous studies have shown the similarity of ABA- and MeJA-signaling pathways that induce stomatal closure (Suhita et al., 2003, 2004; Munemasa et al., 2007; Saito et al., 2008; Islam et al., 2009, 2010). Although roles of second messengers, such as Ca\(^{2+}\)\(_{\text{cyt}}\), ROS, and NO, were elucidated in ABA signaling in guard cells, those in MeJA signaling have not been examined. Recent studies have shown that CDPKs play unambiguous roles in ABA signaling in guard cells (Mori et al., 2006; Zhu et al., 2007; Geiger et al., 2010). In this study, we present the involvement of CPK6 in MeJA-induced stomatal closure as well as in ABA signaling. This finding provides evidence for a common molecular mechanism in Ca\(^{2+}\) recognition in guard cells.

[Ca\(^{2+}\)\(_{\text{cyt}}\) elevation has been shown to be an essential step in early guard cell MeJA signaling (Suhita et al., 2003, 2004; Munemasa et al., 2007). Although pharmacological experiments have implied that CDPKs are involved in MeJA signaling in guard cells (Suhita et al., 2003, 2004), the mechanism by which elevated Ca\(^{2+}\)\(_{\text{cyt}}\) is linked to downstream signal components in guard cell MeJA signaling is still unclear. In this study, to identify CDPKs functioning in guard cell MeJA sig-

---

**Figure 3.** MeJA-induced [Ca\(^{2+}\)\(_{\text{cyt}}\) increments in wild-type (WT) and cpk6-1 guard cells. A, A representative fluorescence emission ratio (535:480 nm) showing transient [Ca\(^{2+}\)\(_{\text{cyt}}\) increments in 10 \(\mu\)M MeJA-treated wild-type guard cells (nine of 17 cells = 52.9%). B, A representative fluorescence emission ratio (535:480 nm) showing no transient [Ca\(^{2+}\)\(_{\text{cyt}}\) increment in 10 \(\mu\)M MeJA-treated wild-type guard cells (three of 17 cells = 17.6%). C, A representative fluorescence emission ratio (535:480 nm) showing transient [Ca\(^{2+}\)\(_{\text{cyt}}\) increments in 10 \(\mu\)M MeJA-treated cpk6-1 guard cells (three of 18 cells = 16.7%). D, A representative fluorescence emission ratio (535:480 nm) showing no transient [Ca\(^{2+}\)\(_{\text{cyt}}\) increment in 10 \(\mu\)M MeJA-treated cpk6-1 guard cells (11 of 18 cells = 61.1%). E, Stack column representation of the number of MeJA-induced transient [Ca\(^{2+}\)\(_{\text{cyt}}\) increments in wild-type guard cells (\(n = 17\)) and cpk6-1 guard cells (\(n = 18\)).
Roles of CPK6 in MeJA Signaling in Guard Cells

Figure 4. MeJA activation of S-type anion currents in wild-type GCPs and cpk6-1 GCPs. A, S-type anion currents in wild-type (WT) GCPs treated without MeJA (top trace) or 10 μM MeJA (bottom trace). B, S-type anion currents in cpk6-1 GCPs treated without MeJA (top trace) or 10 μM MeJA (bottom trace). C, Steady-state current-voltage relationships for MeJA activation of S-type anion currents in wild-type GCPs as recorded in A (white circles, control; black circles, 10 μM MeJA). D, Steady-state current-voltage relationships for MeJA activation of S-type anion currents in cpk6-1 GCPs as recorded in B (white circles, control; black circles, 10 μM MeJA). The voltage protocol was stepped up from +35 mV to −115 mV in 30-mV decrements (holding potential, +30 mV). GCPs were treated with 10 μM MeJA for 2 h before recordings. Note that [Ca2+]cyt was buffered to 2 mM. Each data point was obtained from at least seven GCPs. Error bars represent ± se.

Figure 5. MeJA activation of S-type anion currents in cpk3-1 GCPs. A, S-type anion currents in wild-type GCPs treated with 0 μM MeJA (top trace) or 10 μM MeJA (bottom trace). B, Steady-state current-voltage relationships for MeJA activation of S-type anion currents in wild-type GCPs as recorded in A (white circles, control; black circles, 10 μM MeJA). GCPs were treated with 10 μM MeJA for 2 h before recordings. Note that [Ca2+]cyt was buffered to 2 mM. Each data point was obtained from at least six GCPs. Error bars represent ± se.
mediated by plasma membrane $I_{Ca}$ channels, $Ca^{2+}$ release from intracellular stores contributes to $[Ca^{2+}]_{cyt}$ elevation in guard cells (Leckie et al., 1998; Grabov and Blatt, 1999; Garcia-Mata et al., 2003; Lemtiri-Chlieh et al., 2003). Our results here suggest that in $cpk6-1$ guard cells, $I_{Ca}$ channel activity is abolished but that these other $Ca^{2+}$ transport pathways may be still active and can release $Ca^{2+}$ to cytosol from intracellular stores. Several reports have shown that $Ca^{2+}$ release from intracellular stores in guard cells is mediated by NO (Garcia-Mata et al., 2003; Sokolovski et al., 2005). Previously, we reported that a NO-specific scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, partly inhibited MeJA-induced stomatal closure (Munemasa et al., 2007). However, pretreatment with 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide did not reduce transient increments of $[Ca^{2+}]_{cyt}$ induced by MeJA (Supplemental Fig. S3), indicating that NO production might not be involved in increments of $[Ca^{2+}]_{cyt}$ induced by MeJA.

**Impairment of MeJA Activation of S-Type Anion Channels in $cpk6-1$ Guard Cells**

ABA activation of S-type anion channels triggers a long-term plasma membrane depolarization, which is the primary driving force for $K^{+}$ efflux from guard cells (Schroeder et al., 1987; Schroeder and Keller, 1992; Schmidt et al., 1995; Pei et al., 1997). Similar to ABA, MeJA activates S-type anion channels, and the activation is disrupted in the MeJA-insensitive mutant $coi1$ (Munemasa et al., 2007). In this study, we showed that MeJA activation of S-type anion channels was disrupted in $cpk6-1$ and $cpk6-2$ GCPs (Fig. 4; Supplemental Fig. S2B). CPK6 is also implicated in the ABA activation of S-type anion channels (Mori et al., 2006).

These results indicate that CPK6 is closely associated with S-type anion channel activation in the phytohormone signaling network in guard cells. Arabidopsis SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) was identified as a guard cell plasma membrane protein that mediated S-type anion channel activity (Negi et al., 2008; Vahisalu et al., 2008). Recently, Geiger et al. (2010) showed that CPKs directly interact with SLAC1 and activate it. The activation was inhibited by protein phosphatase 2C (PP2C). They also confirmed direct interaction between CPK6 and SLAC1 using bimolecular fluorescence complementation analysis in Xenopus oocytes. Previously, we reported that MeJA failed to induce stomatal closure in the ABA-insensitive PP2C mutants $abi1-1$ and $abi2-1$ (Munemasa et al., 2007). Together with these findings, our results here imply that MeJA activates CPK6 via down-regulation of the PP2C activity, resulting in the activation of SLAC1. In the future, it should be elucidated how MeJA affects core components of early ABA signaling and ABA receptors PYR/PYL/RCAR, PP2Cs, SnRKs, and CDPKs. Note that in guard cell ABA signaling, S-type anion channels are also activated by a $[Ca^{2+}]_{cyt}$-independent pathway (Grabov et al., 1997; Levchenko et al., 2005; Geiger et al., 2009). The $[Ca^{2+}]_{cyt}$-independent pathway in guard cell MeJA signaling remains to be investigated.

We found that CPK3 gene disruption, which reduced ABA sensitivity in guard cells (Mori et al., 2006), did not affect the MeJA activation of S-type anion channels (Fig. 5). This result is consistent with the stomatal phenotype of the $cpk3-1$ mutant shown in Figure 1A, which provides evidence that CPK6 plays different roles from CPK3 in guard cell signaling.

**The Roles of CPK6 in MeJA Regulation of ROS and NO Production in Guard Cells**

ROS are key players during stomatal closure (Pei et al., 2000; Zhang et al., 2001; Suhita et al., 2004). In Arabidopsis guard cells, two NAD(P)H oxidases, AtRbohD and AtRbohF, catalyze the ROS production observed during ABA-induced and MeJA-induced stomatal closure (Kwak et al., 2003; Suhita et al., 2004). Recently, Kobayashi et al. (2007) showed that two
potato (Solanum tuberosum) CDPKs, StCDPK4 and StCDPK5, directly phosphorylated StrbohB in vitro and regulated the oxidative burst. The closest homolog of StCDPK4 and StCDPK5 in Arabidopsis is CPK6. Ogasawara et al. (2008) suggest that the activity of AtrbohD to produce ROS is regulated by phosphorylation. These previous findings led us to examine the effects of CPK6 disruption on ROS production in guard cells induced by MeJA. Similar to ROS, NO also functions as an important second messenger in MeJA signaling (Orozco-Cárdenas and Ryan, 2002; Munemasa et al., 2007) and ABA signaling (Desikan et al., 2002; Neill et al., 2002; Guo et al., 2003), while the mechanism of NO synthesis in guard cells is induced by MeJA. Similar to ROS, NO also functions as an important second messenger in MeJA signaling (Orozco-Cárdenas and Ryan, 2002; Munemasa et al., 2007) and ABA signaling (Desikan et al., 2002; Neill et al., 2002; Guo et al., 2003), while the mechanism of NO synthesis in guard cells remains controversial. Recent studies revealed the roles of \([\text{Ca}^{2+}]_{\text{cyt}}\) in NO production in plant cells (Gonugunta et al., 2008; Ma et al., 2008). In this study, we analyzed the effects of CPK6 disruption on NO production in guard cell MeJA signaling. Figure 6 shows that MeJA evoked the production of ROS and NO in the cpk6-1 guard cells, which is equivalent to ROS and NO production induced by MeJA in wild-type guard cells. These results suggest that CPK6 functions downstream of ROS and NO production in guard cell MeJA signaling.

Here, we clarified the roles of CPK6 in guard cell MeJA signaling. In guard cells, after perception by COF1 (Yan et al., 2009), MeJA induces the production of ROS and NO. The signal pathway of MeJA-induced ROS and NO production remains unclear. Although Suhita et al. (2004) suggested that \(
\text{Ca}^{2+}\) binding proteins (e.g. CDPKs and calmodulins) mediate MeJA-induced ROS production, our data indicate that CPK6 seems not to be involved in ROS production induced by MeJA. MeJA evokes \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation via both activation of \(I_{\text{Ca}}\) channels and \(\text{Ca}^{2+}\) release from intracellular stores (Suhita et al., 2003; Munemasa et al., 2007). Elevated \([\text{Ca}^{2+}]_{\text{cyt}}\) could activate CPK6. Interestingly, in the cpk6 mutants, MeJA activation of \(I_{\text{Ca}}\) channels was impaired, suggesting that the CDPK regulates \(I_{\text{Ca}}\) channel activity by a feedback loop as proposed by Morì et al. (2006). CPK6 is also involved in the regulation of S-type anion channel activity. We cannot exclude pleiotropic and indirect effects of cpk6 mutation on this ion channel regulation. However, the provided results give strong evidence that CPK6 functions as a positive regulator of MeJA signaling in Arabidopsis guard cells and has different roles from CPK3 in the phytohormone signaling network in guard cells.

**MATERIALS AND METHODS**

**Plant Material and Growth**

Throughout this study, we used the Arabidopsis (Arabidopsis thaliana) ecotype Columbia as the wild-type plant. Wild-type and cpk mutant plants were grown on a soil mixture of 70% (v/v) vermiculite (Asahi-kogyo) and 30% (v/v) Kureha soil (Kureha Chemical) in growth chambers at 22°C under a 16-h-light/8-h-dark photoperiod with photon flux density of 80 \(\mu\text{mol m}^{-2}\text{s}^{-1}\). The roots of Arabidopsis were grown in a soft agar gel of 1% (w/v) Phytagel (Wako Pure Chemical Industries). Plants were planted on Murashige and Skoog (MS) plates (Murashige and Skoog, 1962) containing MS salt mixture (Nihon Pharmaceutical), 2% (w/v) sucrose, 3 mg L\(^{-1}\) thiamine hydrochloride, 0.5 mg L\(^{-1}\) pyridoxine hydrochloride, 5 mg L\(^{-1}\) nicotinic acid, and 0.5% (w/v) Gellan gum (San-Ei Gen). Plates were kept at 4°C for 4 d and then transferred to growth chambers in a vertical orientation. Five-day-old seedlings were transferred to MS plates containing 10 µM MeJA. After 5 d, root length was measured.

**Electrophysiology**

For whole-cell patch-clamp recordings of \(I_{\text{Ca}}\) and S-type anion currents, Arabidopsis GCPs were prepared from rosette leaves of 4- to 6-week-old plants by the enzymatic method described previously (Pei et al., 1997). Whole-cell currents were recorded as described previously (Munemasa et al., 2007). For \(I_{\text{Ca}}\) 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). 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 mM), 10 mM ATP, and 10 mM HEPES-Tris (pH 7.1) in the pipette and 30 mM CsCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 10 mM MES-Tris (pH 5.6) in the bath. In both cases, osmolality was adjusted to 300 mmol kg\(^{-1}\) (pipette solutions) and 485 mmol kg\(^{-1}\) (bath solutions) with \(\text{D-sorbitol}\).

**Guard Cell [Ca\(^{2+}\)]\(_{\text{cyt}}\) Imaging**

Arabidopsis yellow camelone 3.6-expressing plants were used to examine \([\text{Ca}^{2+}]_{\text{cyt}}\) changes in guard cells as described (Allen et al., 1999; Young et al., 2006) with slight modifications. The abaxial side of excised leaves was softly mounted on a glass slide using a medical adhesive, followed by the removal of upper cell layers with a razor blade. The abaxial epidermal cells were kept in a solution containing 5 mM KCl, 50 µM CaCl\(_2\), and 10 mM MES-Tris (pH 6.15) under the light condition for 2 h. Then, the abaxial epidermal cells were treated with 10 µM MeJA by a peristatic pump after 5 min from the start of measurement. The cyan fluorescent protein and yellow fluorescent protein fluorescence in guard cells was captured and analyzed using the W-View System and AQUA COSMOS software (Hamamatsu Photonics).

**Detection of ROS and NO**

ROS production in guard cells was analyzed using H\(_2\)DCF-DA (Lee et al., 1999; Murata et al., 2001; Suhita et al., 2004). Epidermal cells were incubated...
for 3 h in medium containing 5 mM KCl, 50 mM CaCl₂, and 10 mM MES-Tris (pH 6.15), and then 50 mM H₂O₂-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. The dye-loaded tissues were treated with 10 μM MeJA or 10 μM ABA for 20 min, and then the fluorescence of guard cells was imaged and analyzed using AquaCOSMOS software. For NO detection in guard cells, 10 μM DAF-2DA was added instead of 50 μM H₂O₂-DA (Foisser et al., 2008; Neill et al., 2002; Huang et al., 2004).

Statistical Analysis
Significance of differences between data sets was assessed by Student’s t test analysis in this paper, except for in Figure 3 and Supplemental Figure S3. In these figures, x² analysis was performed. We regarded differences at the level of P < 0.05 as significant.

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: CPK3 (At4g23650), CPK6 (At2g17290), CPK4 (At4g09570), CPK11 (At1g35670). VSP1 (At5g24780), VSP2 (At5g24770), and Actin2 (At3g18780).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. CPK6 expression and cpk-1 mutant phenotype.

Supplemental Figure S2. IₐCa currents and S-type anion currents in cpkδ-2.

Supplemental Figure S3. Effect of cPTIO on MeJA-induced [Ca²⁺]ᵢ increments.

ACKNOWLEDGMENTS
Seeds of cpk/-1, cpk/-, and cpk/-/-/-2 were kind gifts from Dr. Da-Peng Zhang (China Agricultural University).

Received July 19, 2010; accepted October 25, 2010; published October 26, 2010.

LITERATURE CITED
Fey CBF, Benedetti CE, Penfold CN, Turner JJG (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
Köhler B, Blatt MR (2002) Protein phosphorylation activates the guard cell Ca²⁺ channel and is a prerequisite for gating by abscisic acid. Plant J 32: 185–194
Lemtiri-Chlief F, MacRobbie EAC, Webb AAR, Manison NF, Brownlee C, 560
Roles of CPK6 in MeJA Signaling in Guard Cells

from that of abscisic acid, g-substances, or methyl jasmonate. Plant Physiol 83: 732–734


