Guard Cell Salicylic Acid Signaling Is Integrated into Abscisic Acid Signaling via the Ca^{2+}/CPK-Dependent Pathway[1][OPEN]

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The phenolic hormone salicylic acid (SA) induces stomatal closure. It has been suggested that SA signaling is integrated with abscisic acid (ABA) signaling in guard cells, but the integration mechanism remains unclear. The Ca^{2+}-independent protein kinase Open Stomata1 (OST1) and Ca^{2+}-dependent protein kinases (CPKs) are key for ABA-induced activation of the slow-type anion channel SLAC1 and stomatal closure. Here, we show that SA-induced stomatal closure and SA activation of slow-type anion channel are impaired in the CPK disruption mutant *cpk3-2 cpk6-1* but not in the OST1 disruption mutant *ost1-3*. We also found that the key phosphorylation sites of SLAC1 in ABA signaling, serine-59 and serine-120, also are important for SA signaling. Chemiluminescence-based detection of superoxide anion revealed that SA did not require CPK3 and CPK6 for the induction of reactive oxygen species production. Taken together, our results suggest that SA activates peroxidase-mediated reactive oxygen species signal that is integrated into Ca^{2+}/CPK-dependent ABA signaling branch but not the OST1-dependent signaling branch in Arabidopsis (*Arabidopsis thaliana*) guard cells.

The stomatal aperture in the epidermis of plant leaves is formed by a pair of guard cells. Ion transport across the plasma and vacuolar membranes of guard cells regulates opening and closing the aperture (Jezek and Blatt, 2017). Guard cells can respond to a variety of stimuli such as light, drought, external Ca^{2+}, abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA) and, consequently, control gas exchange, transpirational water loss, and innate immunity (Murata et al., 2015; Melotto et al., 2017). To optimize growth under ever-changing environments in nature, plants have developed robust mechanisms that integrate the stress inputs and then output the optimal stomatal aperture in guard cells.

The phytohormone SA is a phenolic compound that triggers systemic acquired resistance (White, 1979; Ward et al., 1991; Uknes et al., 1992) and confers drought tolerance to plants (Miura et al., 2013; Okuma et al., 2014). It also has been reported that SA induces stomatal closure (Lee, 1998; Mori et al., 2001; Zeng and He, 2010; Khokon et al., 2011; Hua et al., 2012). The guard cell SA signaling and its cross talk with other signaling play key roles in stomatal immunity together with other signaling (Melotto et al., 2017), but the molecular mechanism remains to be clarified in detail.

Ca^{2+}-dependent protein kinases (CPKs) such as CPK3 and CPK6 (Mori et al., 2006) and the Ca^{2+}-independent protein kinase Open Stomata1 (OST1; Mustilli et al., 2002) function in ABA signal cascades in guard cells. It has been reported that CPK6 and OST1 also participate in MeJA signaling (Munemasa et al., 2011; Yin et al., 2016) and yeast elicitor signaling (Ye et al., 2013, 2015) in Arabidopsis (*Arabidopsis thaliana*) guard cells.

Slow anion channel-associated1 (SLAC1) is a plasma membrane anion transporter that is essential for guard cell slow-type (S-type) anion channel function and is involved in ABA-induced stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). Activation of the S-type anion channel triggers 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). When SLAC1 was coexpressed with CPKs or OST1 in *Xenopus laevis* oocytes, large anion currents similar to S-type anion currents in guard cells were observed (Geiger et al., 2009, 2010; Lee et al., 2009; Brandt et al., 2012). In the *X. laevis* oocyte system, CPKs and OST1 phosphorylate Ser-59 (S59) and Ser-120 (S120) of SLAC1 for the activation, respectively (Geiger et al., 2009, 2010; Brandt et al., 2012). A recent in planta analysis confirmed that the phosphorylation-dependent regulation

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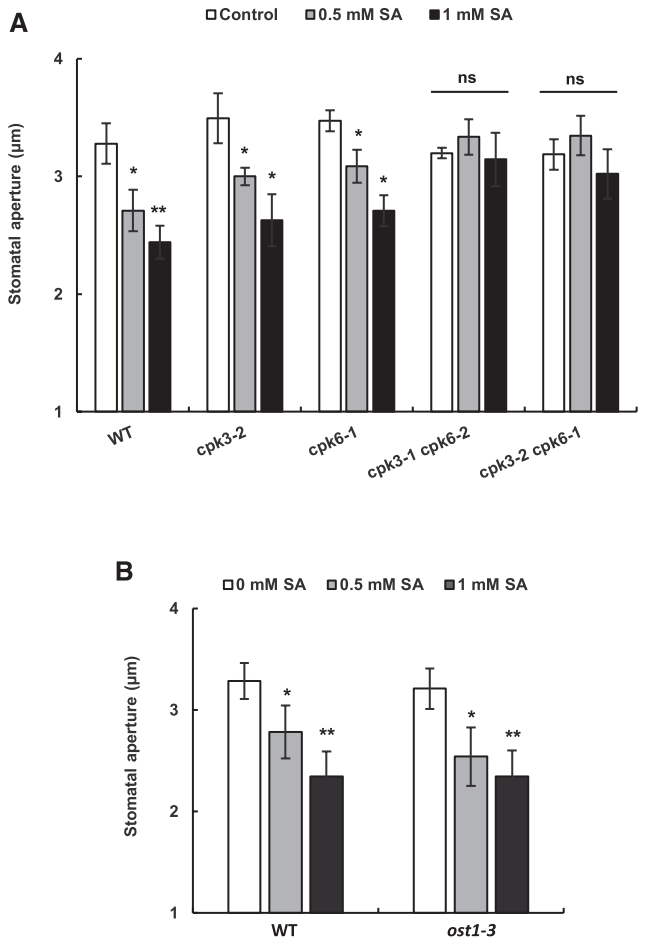

Figure 1. SA-induced stomatal closure responses in Arabidopsis *CPK* disruption mutants (A) and the *OST1* disruption mutant *ost1-3* (B). Averages of stomatal apertures from at least three independent experiments (total stomata per bar ≥ 60) are shown. Error bars represent SE. **, *, and ns indicate $P < 0.01$, $P < 0.05$, and $P > 0.05$, respectively. WT, Wild type.

of SLAC1 at both the two Ser residues is involved in ABA-induced S-type anion channel activation and stomatal closure (Brandt et al., 2015). Similar to ABA, SA activates the S-type anion channel in Arabidopsis guard cells (Khokon et al., 2017).

Reactive oxygen species (ROS) act as a second messenger in both ABA and SA signaling in guard cells. Plasma membrane NAD(P)H oxidases are responsible for ROS production in guard cell ABA signaling (Kwak et al., 2003), while salicylhydroxamic acid (SHAM)-sensitive peroxidases are major ROS sources in guard cell SA signaling (Mori et al., 2001). Guard cell plasma membrane Ca^{2+}-permeable cation (I_{Ca}) channels are activated by hyperpolarization and ABA (Grabov and Blatt, 1998, 1999; Hamilton et al., 2000, 2001; Pei et al., 2000). The I_{Ca} channel-mediated Ca^{2+} entry triggers Ca^{2+} release from intracellular Ca^{2+} stores, resulting in guard cell cytosolic Ca^{2+} elevation (Grabov and Blatt, 1998, 1999; Minguet-Parramona et al., 2016). ROS stimulate the I_{Ca} channels that mediate guard cell ABA signaling (Pei et al., 2000; Murata et al., 2001). In the NADPH oxidase double disruption mutant *rbohD/F*, I_{Ca} channels are activated by hydrogen peroxide (H_2O_2) but not by ABA, indicating that NADPH oxidase-derived ROS mediate the activation of I_{Ca} channels in guard cell ABA signaling (Kwak et al., 2003).

It has been shown that the second messengers ROS and Ca^{2+} are crucial for signal integration between ABA signaling and other signaling in guard cells (Mori et al., 2009; Song et al., 2014; Murata et al., 2015; Singh et al., 2017). The integration of SA and ABA signaling in guard cells also was proposed (Zeng and He, 2010), but the mechanism remains unclear.

In order to elucidate the integration mechanism of SA signaling with ABA signaling in guard cells, here we analyzed SA-induced stomatal closure using CPK3- and CPK6-disruption mutants and the OST1-disruption mutant. We found that, different from ABA, SA requires the CPK-dependent pathway but not the OST1-dependent pathway for the induction of stomatal closure. Whole-cell patch-clamp analysis revealed that SA activation of the S-type anion channel is impaired in *cpk3-2 cpk6-1* guard cell protoplasts (GCPs) but not in *ost1-3* GCPs. Both S59 and S120 of SLAC1 functioned in guard cell SA signaling as shown in ABA signaling. We also found that SA-mediated ROS production is not disrupted in the *cpk3-2 cpk6-1* mutant. Based on these results, we propose a new model for SA and ABA signaling integration in guard cells.

RESULTS

SA-Induced Stomatal Closure Is Impaired in *cpk3 cpk6* Double Mutants But Not in the *ost1* Mutant

As reported previously (Khokon et al., 2011; Hua et al., 2012; Issak et al., 2013), under our experimental conditions, SA significantly induced stomatal closure in a concentration-dependent manner in Arabidopsis (Supplemental Fig. S1). The impairment of ABA-induced stomatal closure in the *cpk3 cpk6* double mutant has been reported (Mori et al., 2006). We found that exogenous application of 0.5 and 1 mM SA significantly induced stomatal closure in the *cpk3-2* and *cpk6-1* single mutants but not in the *cpk3-2 cpk6-1* and *cpk3-1 cpk6-2* double mutants (Fig. 1A). These results suggest that CPK3 and CPK6 are involved in SA-induced stomatal closure as well as ABA-induced stomatal closure. Relative transcriptional levels of *CPK3* and *CPK6* were not changed by 0.5 mM SA treatment in guard cell-enriched epidermal tissues (Supplemental Fig. S2).

Next, we investigated the involvement of OST1 kinase in guard cell SA signaling. It has been reported that stomata of the *OST1* disruption mutant do not close in response to ABA (Mustilli et al., 2002), MeJA (Yin et al., 2016), yeast elicitor (Ye et al., 2015), and flg22 (Guzel Deger et al., 2015). However, we found that exogenous application of 0.5 and 1 mM SA significantly

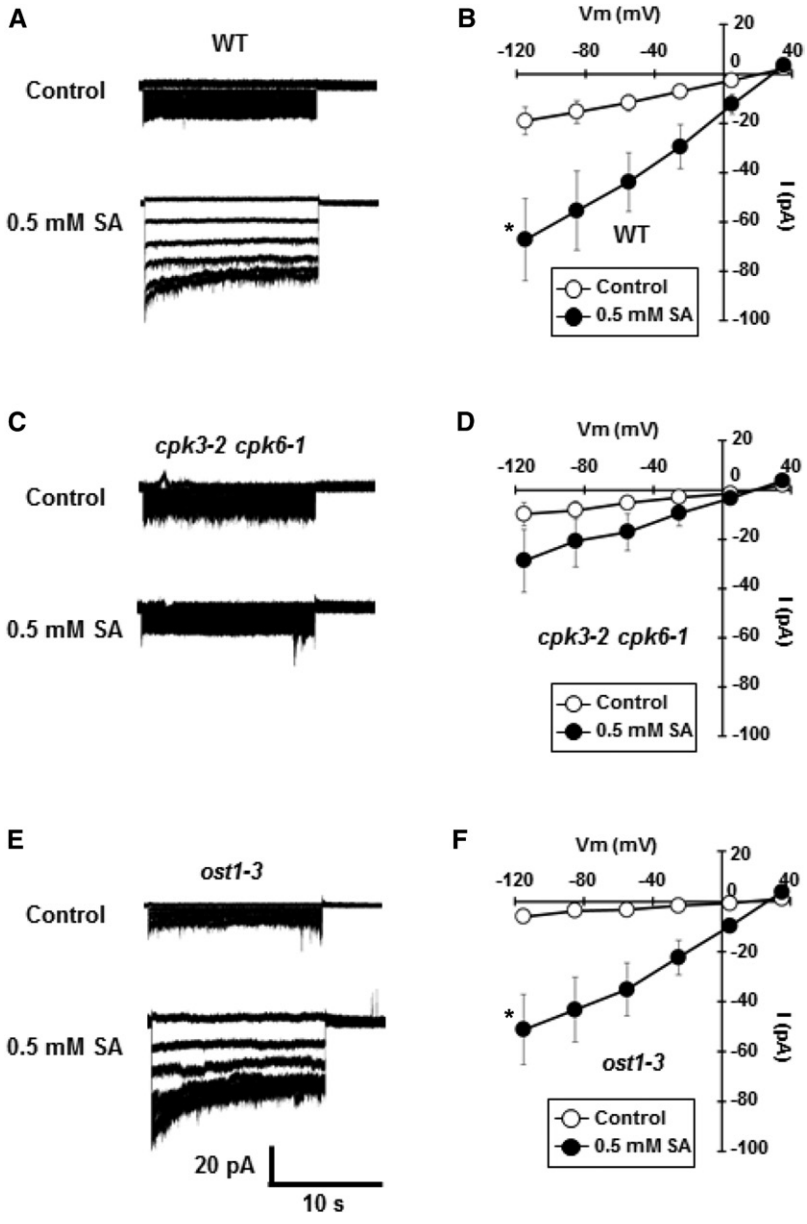

Figure 2. SA activation of S-type anion currents in wild-type (WT), *cpk3-2 cpk-6-1*, and *ost1-3* GCPs. A, C, and E, Representative whole-cell S-type anion currents of wild-type GCPs (A), *cpk3-2 cpk6-1* GCPs (C), and *ost1-3* GCPs (E) in the absence (top trace) or presence of 0.5 mM SA (bottom trace). B, D, and F, Average current-voltage curves of wild-type GCPs (B; $n = 6$ [control] and $n = 6$ [SA]), *cpk3-2 cpk6-1* GCPs (D; $n = 7$ [control] and $n = 6$ [SA]), and *ost1-3* GCPs (F; $n = 5$ [control] and $n = 7$ [SA]). White and black circles indicate the absence and presence of 0.5 mM SA, respectively. GCPs were pretreated with 0.5 mM SA for 10 min before starting patch-clamp analysis. Error bars represent SE. *, $P < 0.05$ (SA versus control).

induced stomatal closure in wild-type plants as well as in the *ost1-3* mutant (Fig. 1B).

Activation of S-Type Anion Channels by SA Is Impaired in the CPK3 CPK6 Disruption Mutant But Not in the OST1 Disruption Mutant

Similar to ABA, SA activates S-type anion channels in guard cells (Khokon et al., 2017), and stomata of the SLAC1-disruption mutants *slac1-1* and *slac1-3* failed to close in response to SA (Supplemental Fig. S3). These results indicate that SLAC1 S-type anion channels are essential for SA-induced stomatal closure. Next, we tested the SA activation of S-type anion channels in the *cpk3-2 cpk6-1* GCPs and *ost1-3* GCPs. As reported by Khokon et al. (2017), SA activates S-type anion channels in wild-type GCPs (Fig. 2A). We found that the SA activation of S-type anion channels was disrupted in the *cpk3-2 cpk6-1* GCPs (Fig. 2C) but not in *ost1-3* GCPs (Fig. 2E), indicating that CPK3 and CPK6, but not OST1, mediate SA signals for the activation of S-type anion channels.

SA-Induced Stomatal Closure Is Impaired in S59/S120 Double Disruption SLAC1 Mutants But Not in S59 and S120 Single Disruption SLAC1 Mutants

It has been reported that the amino acid residues S59 and S120 are the key phosphorylation sites of SLAC1 and are required for the activation of SLAC1 by CPKs and OST1 protein kinases, respectively (Geiger et al., 2009, 2010; Brandt et al., 2012). Brandt et al. (2015) reported that ABA-induced stomatal closure was impaired in the S59 and S120 double phosphosite

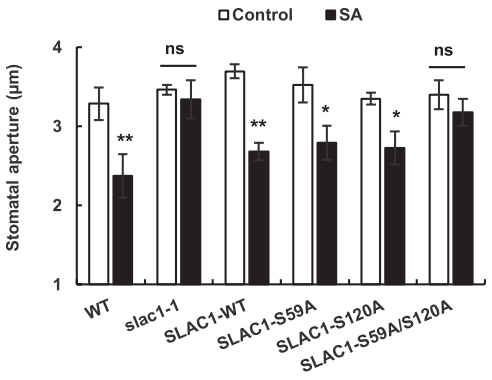

Figure 3. SA-induced responses of stomatal apertures in the *slac1-1* complementation lines *SLAC1-WT*, *SLAC1-S59A*, *SLAC1-S120A*, and *SLAC1-S59A/S120A*. Averages of stomatal apertures from at least three independent experiments (total stomata per bar ≥ 60) are shown. Error bars represent SE. **, *, and ns indicate $P < 0.01$, $P < 0.05$, and $P > 0.05$, respectively. SLAC1 complement lines are *slac1-1* mutants that express SLAC1 or SLAC1-phosphosite mutants fused with monomeric Venus (C terminally) under the control of the *SLAC1* promoter.

disruption mutant SLAC1-S59A/S120A (Ser is mutated to Ala) but not in the S59A or S120A single mutant, proving the in planta function of both phosphorylation sites. Next, to investigate the functions of the two phosphorylation sites S59 and S120 of SLAC1 in guard cell SA signaling, we performed stomatal bioassay analysis using *slac1-1* complementation lines with phosphosite-mutated SLAC1, *SLAC1-S59A*, *SLAC1-S120A*, and *SLAC1-S59A/S120A* (Brandt et al., 2015). *SLAC1-WT* complementation lines showed an SA-sensitive stomatal phenotype similar to that of wild-type plants (Fig. 3). We found that SA induced stomatal closure in the *SLAC1-S59A* and *SLAC1-S120A* single mutants but not in the *SLAC1-S59A/S120A* double mutant (Fig. 3).

SA Elicited ROS Production in the *cpk3 cpk6* Mutant as Well as in the Wild-Type Plants

It has been shown that SA triggers the accumulation of ROS such as superoxide anion (O_2^-) and H_2O_2 and that the ROS function as second messengers in the promotion of stomatal closure (Lee et al., 1999; Mori et al., 2001; Khokon et al., 2011, 2017). It is reported that SA elicits a SHAM-sensitive peroxidase-mediated ROS burst that triggers the activation of downstream SA signaling components in guard cells (Mori et al., 2001; Khokon et al., 2011). We examined the effects of SA on ROS production in the *cpk3-2 cpk6-1* mutants to investigate the positions of ROS production and the two CPKs in the SA signal pathway in guard cells.

We measured O_2^- production in guard cell enriched-epidermal tissues using CLA (*Cripridina* lucigenin-derived chemiluminescent agent: 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one; Kawano et al., 1998; Mori et al., 2001). The application of 0.5 mM SA evoked O_2^- production in wild-type guard cell-enriched epidermal tissues (Fig. 4), and the O_2^- production was inhibited by SHAM but not by the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), confirming that the SA-triggered O_2^- production is mediated mainly by SHAM-sensitive peroxidases (Mori et al., 2001; Khokon et al., 2011) but not by DPI-sensitive NADPH oxidases such as RBOHD/F (Kwak et al., 2003). Application of 0.5 mM SA also evoked O_2^- production in *cpk3-2 cpk6-1* guard cell enriched-epidermal tissues (Fig. 5). In previous studies, 3,3′-diaminobenzidine (DAB)-based apoplastic H_2O_2 measurement in whole leaves also was employed to dissect guard cell SA signaling (Khokon et al., 2011; Prodhan et al., 2017). The DAB staining analysis revealed that disruption of CPK3 and CPK6 did not affect SA-induced H_2O_2 production in whole leaves (Supplemental Fig. S4). Together, these results indicate that CPK3 and CPK6 protein kinases function downstream of ROS production in guard cell SA signaling.

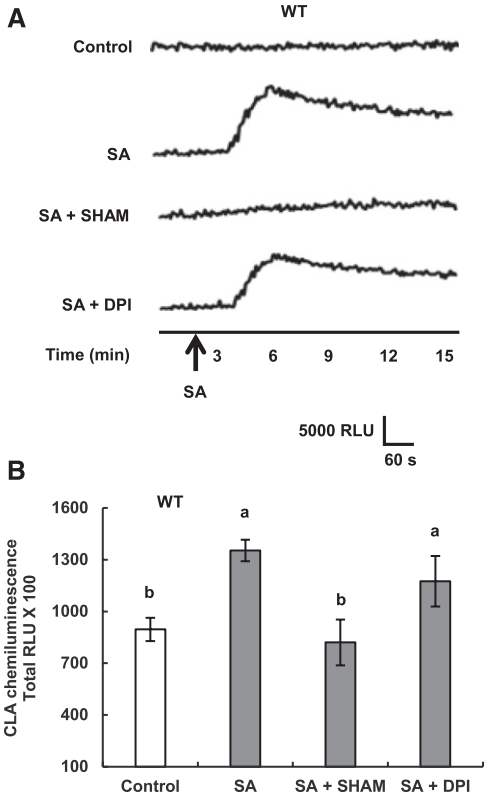

Figure 4. Effects of SHAM and DPI on SA-elicited O_2^- production in guard cell-enriched epidermal peels of Arabidopsis wild-type (WT) plants. A, Typical traces of the CLA chemiluminescence reflecting the production of O_2^- in the presence and absence of 2 mM SHAM (a peroxidase inhibitor) or 20 μM DPI (an NADPH oxidase inhibitor). SA at 0.5 mM was added at the time shown by the arrow. B, Total CLA chemiluminescence reflecting the production of O_2^- as recorded in A. Total RLU (relative luminescence units) is the sum of luminescence between 1 and 15 min. Each data point reflects the mean ($n = 3$). Error bars represent SE. Values indicated by the same letter do not differ significantly at the 5% level, as determined by Tukey's test.

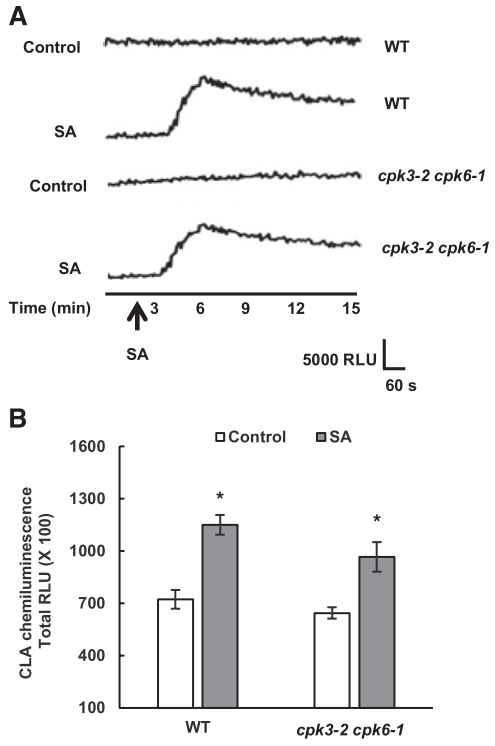

Figure 5. SA-elicited O_2^- production in wild-type (WT) and *cpk3-2 cpk6-1* mutant plants. A, Typical kinetics of CLA chemiluminescence reflecting O_2^- production in the presence or absence of SA in the wild type and the *cpk3-2 cpk6-1* mutant. Same WT traces are shown as in Fig. 4A. B, Total CLA chemiluminescence reflecting the production of O_2^- as recorded in A. Total RLU (relative luminescence units) is the sum of luminescence between 1 and 15 min.WT datasets include the same experimental replicates as Figure 4B and two additional experimental replicates. Each data point reflects the mean (n = 5). *, Significant difference ($P < 0.05$) from controls as assessed by Student's t test. Error bars represent SE.

DISCUSSION

SA and ABA induce stomatal closure in plants and, thus, play a crucial role in adaptation to stress conditions. Significant efforts have been devoted to identifying signaling components in guard cell responses to ABA. In comparison, very few studies have focused on SA signaling in guard cells. Although the roles of CPKs, OST1, and SLAC1 in ABA signaling in guard cells are elucidated, those in SA signaling remain to be examined. In this study, we present the involvement of CPKs and SLAC1 in guard cell SA signaling and elucidate the integration of SA signaling and ABA signaling in guard cells.

SA Requires CPK3 and CPK6 Protein Kinases But Not OST1 for the Induction of Stomatal Closure

It has been reported that SA regulates gene expression involved in the innate response via a Ca^{2+}-dependent pathway (Du et al., 2009; Wang et al., 2009; Coca and San Segundo, 2010). As well, Khokon et al. (2011) reported that the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker $LaCl_3$ significantly suppressed the SA-induced stomatal closure, suggesting the involvement of extracellular free Ca^{2+} in the modulation of SA-induced stomatal closure. However, the molecular identities of the Ca^{2+} sensors that are responsible for guard cell SA signaling were unknown. CPKs play a role as cytosolic Ca^{2+} concentration sensors in many aspects of plant physiological processes (Klimecka and Muszyńska, 2007). Several CPKs are important mediators of Ca^{2+}-dependent stomatal closure and S-type anion channel activation and play roles in ABA signaling in guard cells (Mori et al., 2006; Zhu et al., 2007; Geiger et al., 2010; Brandt et al., 2015). Mori et al. (2006) have reported that CPK3 and CPK6 positively regulate ABA-induced stomatal closure in Arabidopsis. In this study, we found that the disruption of CPK3 and CPK6 also impaired the SA-induced stomatal closure (Fig. 1A). Our findings along with the previous reports suggested that the CPK-dependent Ca^{2+} recognition could be key for an integration mechanism

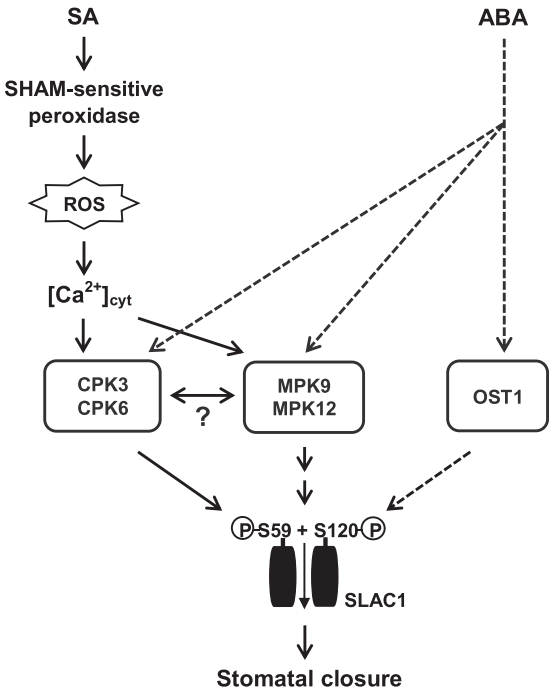

Figure 6. Proposed model of integration between SA signaling and ABA signaling in Arabidopsis guard cells. SA triggers a SHAM-sensitive peroxidase-mediated ROS signal that activates CPKs but not OST1 protein kinase. Then, the CPKs together with other unknown protein kinases (e.g. CIPKs) phosphorylate S59 and S120 of SLAC1 and activate it, whereas ABA requires OST1 as well as CPKs for the activation of SLAC1. These suggest that SA signaling integrates with ABA signaling via the Ca^{2+}/CPK-dependent signaling pathway in guard cells. The two MAPKs, MPK9 and MPK12, also function downstream of ROS and Ca^{2+} in guard cell SA signaling and regulate SLAC1 activity indirectly. The molecular mechanism underlying the interdependence of the CPK-dependent and MPK-dependent pathways for SLAC1 activation is unknown.

between SA signaling and ABA signaling in guard cells. It should be noted that, although pharmacological analysis (Khokon et al., 2011) and genetic analysis (this study) provide strong evidence that SA requires cytosolic Ca^{2+} signals to induce stomatal closure, so far, our Ca^{2+} imaging analyses using genetically encoded Ca^{2+} sensors could not detect any Ca^{2+} increase response in SA-treated Arabidopsis guard cells (yellow cameleon 3.6 [Khokon et al., 2011, 2017] and yellow cameleon 2.6 [data not shown]). Recently, de Jonge et al. (2017) reported that SA interferes with the fluorescence of several fluorescent proteins in planta; therefore, a Ca^{2+} imaging experiment using different types of Ca^{2+} biosensors or Ca^{2+}-sensitive fluorescent dyes is needed to validate the true effect of SA on cytosolic Ca^{2+} in the future. The other possible explanation is that SA does not significantly increase cytosolic Ca^{2+} but primes the Ca^{2+} sensitivity of the downstream targets, as proposed in the case of ABA signaling (for review, see Hubbard et al., 2012; Laanemets et al., 2013; Munemasa et al., 2015). Cytosolic Ca^{2+} regulation of S-type anion channels is primed by ABA in Arabidopsis guard cells (Brandt et al., 2015). It also was reported that phosphorylation enhances the sensitivity of the S-type anion current to cytosolic Ca^{2+} in *Vicia faba* guard cells (Chen et al., 2010). Future studies are needed to confirm whether or not SA has the Ca^{2+} sensitivity priming mechanism similar to that of ABA.

The OST1 protein kinase plays a key role in the ABA responses of Arabidopsis guard cells (Mustilli et al., 2002; Yoshida et al., 2002; Acharya et al., 2013; Imes et al., 2013). It has been reported that OST1 also can be activated independently of ABA (Yoshida et al., 2006), and the ABA-independent OST1 function is involved in stomatal closure induced by MeJA (Yin et al., 2016), CO_2 (Xue et al., 2011), and leaf-to-air vapor pressure deficit (Merilo et al., 2018; Pantin and Blatt, 2018). Different from these reports, here we found that SA-induced stomatal closure does not require OST1 (Fig. 1B).

Microbe-associated molecular patterns (MAMPs) such as yeast elicitor (Ye et al., 2015) and flg22 (Guzel Deger et al., 2015) also require OST1 to induce stomatal closure. It has been suggested that SA acts downstream of MAMP perception in MAMP-induced stomatal closure (Montillet et al., 2013; Montillet and Hirt, 2013). Different from the MAMPs, SA-induced stomatal closure does not require OST1 (Fig. 1B), suggesting that MAMPs activate the OST1-dependent pathway via an SA-independent manner in guard cells. The detailed cross talk mechanism for the SA-dependent pathway and the OST1-dependent pathway in guard cell MAMP signaling needs to be analyzed further. Recently, Khokon et al. (2017) showed that two mitogen-activated protein kinases (MAPKs), MPK9 and MPK12, are involved in guard cell SA signaling. The MPK9 and MPK12 double disruption mutant *mpk9 mpk12* is defective in SA-induced S-type anion channel activation and stomatal closure but not in ROS production (Khokon et al., 2017). Thus, similar to CPKs, the two MAPKs regulate guard cell SA signaling downstream of ROS production. Several studies report that CPKs directly phosphorylate and activate the SLAC1 S-type anion channel (Geiger et al., 2010; Brandt et al., 2012, 2015; Scherzer et al., 2012; Maierhofer et al., 2014). However, although two studies recently reported that MPK12 indirectly regulates the SLAC1 channel via phosphorylation of the protein kinase High Leaf Temperature1, which functions as a negative regulator of CO_2-induced stomatal movements (Hõrak et al., 2016; Jakobson et al., 2016), our current knowledge of the molecular mechanism of how MPK9 and MPK12 regulate SLAC1 activity in guard cell ABA and SA signaling is still limited. A detailed analysis of the underlying mechanisms that integrate MPK9 and MPK12 into the CPK-dependent SLAC1 regulation is an important topic for future research.

SLAC1 and Its Activation by the Phosphorylation of S59 and S120 Are Required for SA Signaling in Guard Cells

SLAC1 mediates S-type anion channel activity and stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). In this study, we found that the SLAC1 S-type anion channel is required for SA-induced stomatal closure (Supplemental Fig. S3), and the SA activation of S-type anion channels was impaired in the *cpk3-2 cpk6-1* GCPs (Fig. 2C) but not in *ost1-3* (Fig. 2E). Therefore, different from ABA, SA requires CPK3 and CPK6, but not OST1, for the activation of SLAC1 S-type anion channels.

Two-electrode voltage clamp analysis using *X. laevis* oocytes revealed that CPKs phosphorylate S59 and OST1 phosphorylates S120 of SLAC1, resulting in activation of the channel (Geiger et al., 2009, 2010; Brandt et al., 2012; Maierhofer et al., 2014). Moreover, in planta analysis concludes that both phosphorylation sites, S59 and S120, of SLAC1 are required for ABA-induced stomatal closure in Arabidopsis (Brandt et al., 2015). Similar to ABA (Brandt et al., 2015), SA-induced stomatal closure was impaired in the *S59A/S120A* double disruption SLAC1 mutant but not in the *S59A* and *S120A* single disruption SLAC1 mutants (Fig. 3). These results demonstrated that, in guard cell SA signaling, although OST1 kinase is absent (Fig. 1B), CPKs and other protein kinases (e.g. CIPKs; Maierhofer et al., 2014) can phosphorylate S59 and S120 of SLAC1 to activate the S-type anion channel, resulting in stomatal closure.

CPKs Function Downstream of ROS Production in the Guard Cell SA Signal Cascade

It has been shown that ROS function as important second messengers in guard cells during stomatal closure (Pei et al., 2000; Zhang et al., 2001; Suhita et al., 2004; Khokon et al., 2011). In guard cell ABA signaling, ROS production is mediated by plasma membrane NAD(P)H oxidases (Kwak et al., 2003), while ROS production in guard cell SA signaling is mediated by

SHAM-sensitive peroxidases (Mori et al., 2001; Khokon et al., 2011; Fig. 4).

It was suggested that CPK3 and CPK6 function downstream of ROS production in guard cell ABA signaling (Mori et al., 2006) and that CPK6 also functions downstream of ROS production in guard cell MeJA signaling (Munemasa et al., 2011). In this study, we found that SA elicited ROS production in *cpk3 cpk6* guard cell-enriched epidermis as well as in wild-type guard cell-enriched epidermis (Fig. 5). This result along with the previous reports indicate that the CPKs function downstream of SHAM-sensitive peroxidase-mediated ROS production as well as NAD(P)H oxidase-mediated ROS production in guard cell signaling. Therefore, the results presented as well as previous reports (Mori et al., 2006; Munemasa et al., 2011) provide evidence that the hormone-triggered ROS signals activate CPKs that function as signal integrators in guard cells. The mechanism by which SA-elicited ROS signal is transduced into the activation of guard cell CPKs remains unclear and needs to be investigated in the future.

Integration of SA and ABA Signaling Pathways in Arabidopsis Guard Cells

Based on the results discussed here, we propose a model (Fig. 6) showing the overlapping between SA signaling and ABA signaling in guard cells. Briefly, SA triggers SHAM-sensitive peroxidase-mediated ROS signal that activates not OST1 but CPKs and other protein kinases that phosphorylate S59 and S120 of SLAC1 and activate it, whereas ABA needs both OST1 and CPKs for the full activation of SLAC1 (Geiger et al., 2010; Brandt et al., 2015). Thus, the Ca^{2+}/CPK-dependent signaling branch is key for the integration of SA signaling with ABA signaling in guard cells. The involvement of the MAPKs MPK9 and MPK12 in guard cell SA signaling also is reported (Khokon et al., 2017). The two MAPKs function downstream of ROS and Ca^{2+} and mediate SLAC1 activation indirectly, but the molecular mechanism is unclear. The strong impairment of SA-induced S-type anion channel activation and stomatal closure is caused by the disruption of either the CPKs (this study) or MAPKs (Khokon et al., 2017), suggesting the unknown interdependent mechanism of CPK and MAPK signaling pathways. It has been reported that SA as well as ABA function as key mediators for biotic stress-triggered stomatal closure (Melotto et al., 2006; Zeng et al., 2010; Montillet et al., 2013). In addition, previous research reported an SA-independent biotic signaling that antagonizes Ca^{2+}-dependent but OST1-independent ABA signaling in guard cells (Kim et al., 2011). Therefore, the Ca^{2+}/CPK-dependent integration mechanism of SA signaling with ABA signaling described here might be one of innate immune responses for overcoming the pathogen-induced inhibition of Ca^{2+}-dependent ABA-induced stomatal closure.

MATERIALS AND METHODS

Plants and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used as wild-type plants. *cpk3-2 cpk6-1* (Mori et al., 2006), *ost1-3* (Yoshida et al., 2002), *slac1-1* and *slac1-3* (Vahisalu et al., 2008), *SLAC1-WT*, *SLAC1-S59A*, *SLAC1-S120A*, and *SLAC1-S59A/S120A* (Brandt et al., 2015) mutants were grown in controlled growth conditions in a 1:1 soil:vermiculite (v/v) mixture under a 16-h-light/8-h-dark photoperiod with photon flux density of 80 μmol m^{-2} s^{-1}. The temperature and relative humidity in the growth chamber were 21°C and 70%. The nutrient solution (0.1%; Hyponex) was provided to the plants twice per week. Four-week-old plants were used for the experiments. SLAC1 complement lines (Fig. 3) are *slac1-1* mutants that express SLAC1 or SLAC1-phosphosite mutants fused with monomeric Venus (C terminally) under the control of the SLAC1 promoter. More detailed information regarding the SLAC1 complement lines can be found in Brandt et al. (2015).

Analysis of Stomatal Apertures

Stomatal apertures were measured as reported by Khokon et al. (2011). Excised rosette leaves were blended in tap water for 30 s, and epidermal tissues were collected using a 100-μm nylon mesh. The collected epidermal tissues were dipped into stomatal assay buffer containing 50 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 5.5) for 2 h in the light to induce stomatal opening. Then, SA was added to the stomatal assay buffer and again incubated 2 h in the light. The epidermal tissues were collected, and 20 stomatal apertures were measured for each individual experiment. Acidification control experiments using HCl confirmed that the pH drop caused by SA in the buffer has no effect on stomatal apertures (data not shown).

Detection of ROS Production

The generation of O_2^- was measured by monitoring CLA chemiluminescence as described previously (Kawano et al., 1998; Mori et al., 2001). Guard cell-enriched epidermal tissues were prepared by blending the rosette leaves of Arabidopsis. SA at 0.5 mM was applied to epidermal tissues suspended in a medium consisting of 20 μM CLA, 50 mM KCl, 50 μM $CaCl_2$, and 10 mM MES (pH 5.5 with Tris). After the application of SA, the luminescence was measured between 1 and 15 min by a luminometer (AB2200; Atto). The chemiluminescence is presented as relative luminescence units.

The detection of apoplastic H_2O_2 production was measured using DAB by the method of Thordal-Christensen et al. (1997). The excised leaves were floated on buffer solution containing 50 mM KCl, 50 μM $CaCl_2$, and 10 mM MES (pH 5.5 with Tris) and incubated in the light for 3 h. Then, DAB (1 mg mL^{-1}) was added to the buffer solution and infiltrated gently in a vacuum for 2 h. After that, SA was applied to the buffer solution and again incubated for 2 h. The leaves were then decolorized by boiling in ethanol. ROS was visualized as a reddish-brown color and quantified using ImageJ software (National Institutes of Health).

Electrophysiology

Arabidopsis GCPs for whole-cell patch-clamp analysis were prepared enzymatically from rosette leaves as described previously (Pei et al., 1997). Whole-cell S-type anion currents were recorded as described previously (Munemasa et al., 2007). The patch-clamp solutions contained 150 mM $CsCl_2$, 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 $CsCl_2$, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.6) in the bath (Pei et al., 1997). Osmolarity was adjusted to 500 mmol kg^{-1} (pipette solution) and 485 mmol kg^{-1} (bath solution) using D-sorbitol. GCPs were pretreated with 0.5 mM SA in the bath solution for 10 min before starting patch-clamp experiments. The application of 0.5 mM SA changed the pH in the bath solution from 5.6 to 5.4, but the pH drop has no effect on S-type anion channel activity, which was confirmed by experiments using HCl (data not shown).

RNA Extraction and Real-Time PCR

Total RNA was isolated from guard cell-enriched tissues using Trizol reagent (Invitrogen), and cDNA was synthesized using PrimeScript RT

Master Mix (Perfect Real Time; Takara Bio) according to the manufacturer's instructions. Quantitative real-time PCR was performed using KOD SYBR qPCR Mix (Toyobo) and the Mx3005P Real-Time QPCR System (Stratagene). The levels of *CPK3* and *CPK6* transcript were normalized to that of *ACTIN2*. Relative quantification was performed using the comparative cycle threshold method. Primers used in gene-specific PCR amplification are as follows: for *CPK3* (At4g23650), 5′-AGCTGATATGGATGGAGATGG-3′ and 5′-ACGATCGGTGTCTACTTCAGC-3′; for *CPK6* (At2g17290), 5′-GTTCATTCTCCTACTACAGACC-3′ and 5′-ACCTGTGGCAATATCAGTACAC-3′; and for *ACTIN2* (At3g18780), 5′-AAAGGCCAACAGAGAGAAGATG-3′ and 5′-TGATGTCTCTTACAATTTCCCGC-3′.

Statistical Analysis

Unless stated otherwise, the significance of differences between mean values was assessed by Student's *t* test. Differences at the level of $P < 0.05$ were considered significant.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource (https://www.arabidopsis.org/) under accession numbers: CPK3 (At4g23650), CPK6 (At2g17290), OST1 (At4g33950), and SLAC1 (At1g12480).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Dose-dependent SA-induced stomatal closure in Arabidopsis wild-type plants.

Supplemental Figure S2. Effects of SA on *CPK3* and *CPK6* gene expression in guard cell-enriched tissues.

Supplemental Figure S3. SA-induced stomatal closure in the *SLAC1* disruption mutants *slac1-1* and *slac1-3*.

Supplemental Figure S4. SA-elicited apoplastic H_2O_2 production in wild-type and *cpk3-2 cpk6-1* plants.

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