Difference in Abscisic Acid Perception Mechanisms between Closure Induction and Opening Inhibition of Stomata[1][W][OPEN]

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Abscisic acid (ABA) induces stomatal closure and inhibits light-induced stomatal opening. The mechanisms in these two processes are not necessarily the same. It has been postulated that the ABA receptors involved in opening inhibition are different from those involved in closure induction. Here, we provide evidence that four recently identified ABA receptors (PYRABACTIN RESISTANCE1 [PYR1], PYRABACTIN RESISTANCE-LIKE1 [PYL1], PYL2, and PYL4) are not sufficient for opening inhibition in Arabidopsis (*Arabidopsis thaliana*). ABA-induced stomatal closure was impaired in the *pyr1/pyl1/pyl2/pyl4* quadruple ABA receptor mutant. ABA inhibition of the opening of the mutant's stomata remained intact. ABA did not induce either the production of reactive oxygen species and nitric oxide or the alkalization of the cytosol in the quadruple mutant, in accordance with the closure phenotype. Whole cell patch-clamp analysis of inward-rectifying K^+ current in guard cells showed a partial inhibition by ABA, indicating that the ABA sensitivity of the mutant was not fully impaired. ABA substantially inhibited blue light-induced phosphorylation of H^+-ATPase in guard cells in both the mutant and the wild type. On the other hand, in a knockout mutant of the SNF1-related protein kinase, *srk2e*, stomatal opening and closure, reactive oxygen species and nitric oxide production, cytosolic alkalization, inward-rectifying K^+ current inactivation, and H^+-ATPase phosphorylation were not sensitive to ABA.

The phytohormone abscisic acid (ABA), which is synthesized in response to abiotic stresses, plays a key role in the drought hardiness of plants. Reducing transpirational water loss through stomatal pores is a major ABA response (Schroeder et al., 2001). ABA promotes the closure of open stomata and inhibits the opening of closed stomata. These effects are not simply the reverse of one another (Allen et al., 1999; Wang et al., 2001; Mishra et al., 2006).

A class of receptors of ABA was identified (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). The sensitivity of stomata to ABA was strongly decreased in quadruple and sextuple mutants of the ABA receptor genes *PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR* (*PYR/PYL/RCAR*; Nishimura et al., 2010; Gonzalez-Guzman et al., 2012). The PYR/PYL/RCAR receptors are involved in the early ABA signaling events, in which a sequence of interactions of the receptors with PROTEIN PHOSPHATASE 2Cs (PP2Cs) and subfamily 2 SNF1-RELATED PROTEIN KINASES (SnRK2s) leads to the activation of downstream ABA signaling targets in guard cells (Cutler et al., 2010; Kim et al., 2010; Weiner et al., 2010). Studies of *Commelina communis* and *Vicia faba* suggested that the ABA receptors involved in stomatal opening are not the same as the ABA receptors involved in stomatal closure (Allan et al., 1994; Anderson et al., 1994; Assmann, 1994; Schwartz et al., 1994). The roles of PYR/PYL/RCAR in either stomatal opening or closure remained to be elucidated.

Blue light induces stomatal opening through the activation of plasma membrane H^+-ATPase in guard cells that generates an inside-negative electrochemical gradient across the plasma membrane and drives K^+ uptake through voltage-dependent inward-rectifying K^+ channels (Assmann et al., 1985; Shimazaki et al., 1986; Blatt, 1987; Schroeder et al., 1987; Thiel et al., 1992). Phosphorylation of the penultimate Thr of the plasma membrane H^+-ATPase is a prerequisite for blue light-induced activation of the H^+-ATPase (Kinoshita and Shimazaki, 1999, 2002). ABA inhibits H^+-ATPase activity through dephosphorylation of the penultimate

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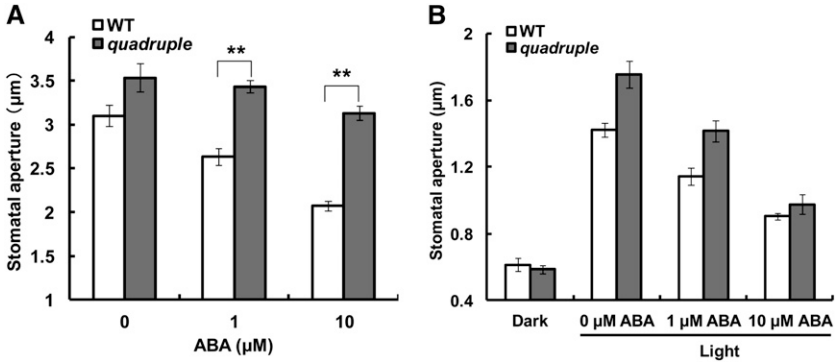

Figure 1. Induction of stomatal closure and inhibition of light-induced stomatal opening by ABA in the wild type (WT) and the *pyr/pyl1/py2/pyl4* quadruple mutant (*quadruple*). A, ABA-induced stomatal closure in the wild type and the quadruple mutant. Averages from five independent experiments ($n = 5$; a total of 100 stomata) are shown. B, ABA inhibition of stomatal opening in wild-type and quadruple mutant plants. Dark represents fully dark-adapted stomata; 0 μM ABA indicates 2.5-h light treatment after the dark adaption with 0.1% ethanol as the solvent control; 1 and 10 μM ABA represent an ABA addition at the same time as the initiation of light treatment. Averages from three independent experiments are shown ($n = 3$; 60 total stomata). Asterisks indicate significant differences (**$\alpha = 0.01$) by Student's *t* test. A data set obtained by Y.Y. is shown out of the results carried out by three independent examiners, with Y.Y., Y.A., and I.C.M. obtaining essentially the same results. Error bars represent SE.

Thr in the C terminus of the H^+-ATPase in guard cells, resulting in prevention of the opening (Goh et al., 1996; Zhang et al., 2004; Hayashi et al., 2011). Inward-rectifying K^+ currents (I_{Kin}) of guard cells are negatively regulated by ABA in addition to through the decline of the H^+ pump-driven membrane potential difference (Schroeder and Hagiwara, 1989; Blatt, 1990; McAinsh et al., 1990; Schwartz et al., 1994; Grabov and Blatt, 1999; Saito et al., 2008). This down-regulation of ion transporters by ABA is essential for the inhibition of stomatal opening.

A series of second messengers has been shown to mediate ABA-induced stomatal closure. Reactive oxygen species (ROS) produced by NADPH oxidases play a crucial role in ABA signaling in guard cells (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003; Sirichandra et al., 2009; Jannat et al., 2011). Nitric oxide (NO) is an essential signaling component in ABA-induced stomatal closure (Desikan et al., 2002; Guo et al., 2003; Garcia-Mata and Lamattina, 2007; Neill et al., 2008). Alkalization of cytosolic pH in guard cells is postulated to mediate ABA-

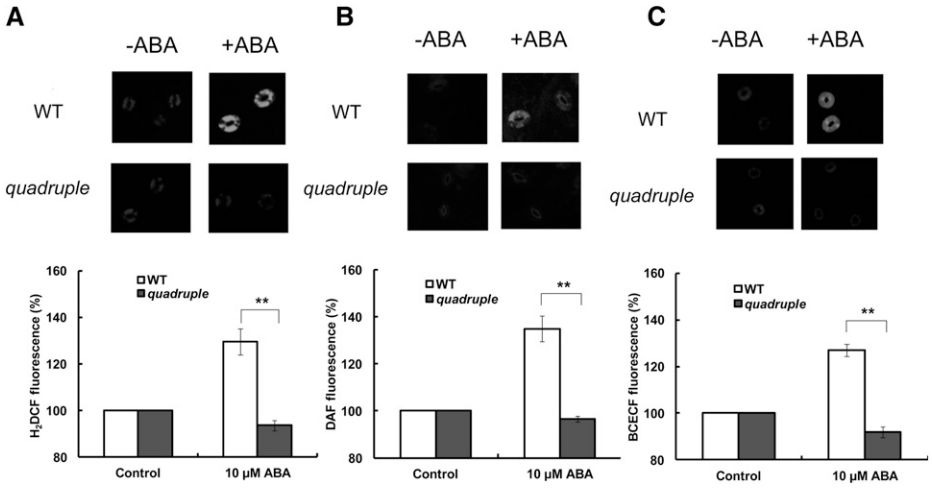

Figure 2. ABA-induced ROS and NO production and cytosolic alkalization in wild-type (WT) and *pyr1/pyl1/pyl2/pyl4* quadruple mutant (*quadruple*) guard cells. A, ROS production. Representative grayscale H_2DCF fluorescence images (top panel) and the percentage of H_2DCF fluorescence levels (bottom panel) are shown. B, NO production. Representative grayscale diaminofluorescein (DAF) fluorescence images (top panel) and DAF fluorescence levels (bottom panel) are shown. C, Cytosolic alkalization. Representative BCECF images (top panel) and BCECF fluorescence levels (pH; bottom panel) are shown. Fluorescence intensities are normalized to the control value taken as 100% for each experiment. The epidermis was preloaded with fluorescent dyes before treatments. Ten micromolar ABA or 0.1% ethanol (solvent control) was added to the suspension of epidermal preparations for 20 min. Bars indicate averages of five independent experiments ($n = 5$; a total of 100 guard cells per bar). Asterisks indicate significant differences (**$\alpha = 0.01$) by Student's *t* test. Error bars represent SE.

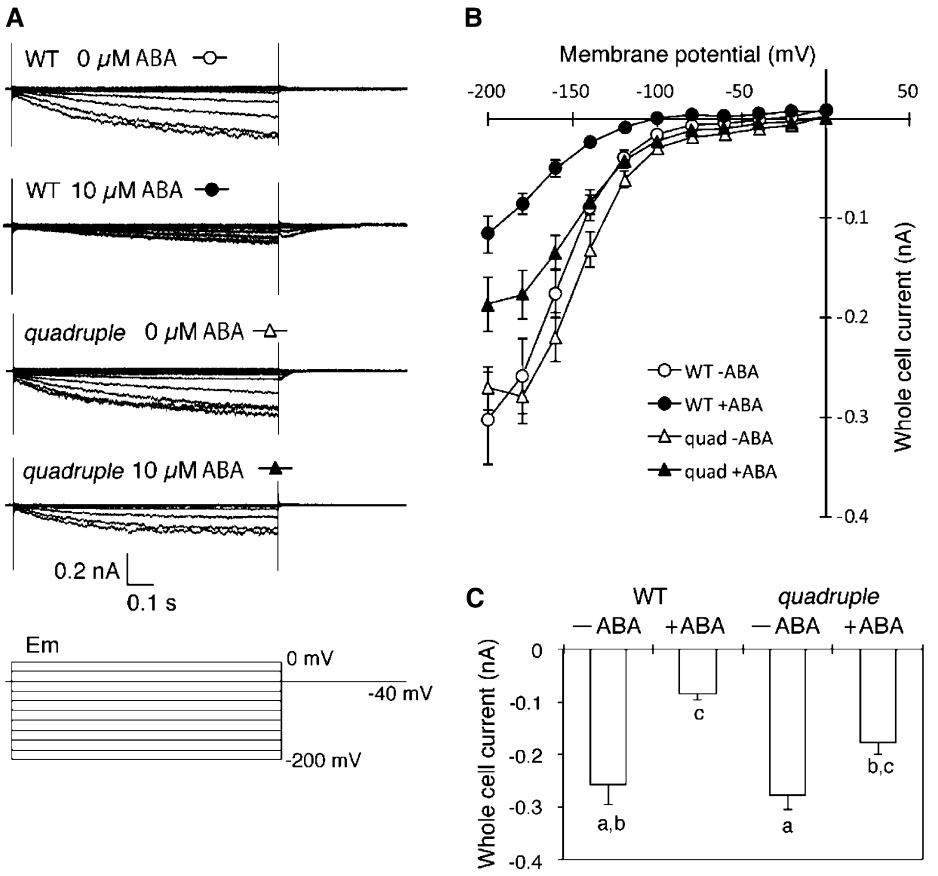

Figure 3. Inactivation of I_{Kin} by ABA in wild-type (WT) and *pyr1/pyl1/pyl2/pyl4* quadruple mutant (*quadruple*) GCPs. A, Typical raw traces of I_{Kin} of the wild type and the quadruple mutant with (+) and without (−) 10 μM ABA. The bottom panel indicates the applied step pulse membrane voltage (Em) protocol. B, Current-voltage curve of I_{Kin}. White circles, the wild type without ABA (n = 12); black circles, the wild type with 10 μM ABA (n = 7); white triangles, the quadruple mutant without ABA (n = 20); black triangles, the quadruple mutant with 10 μM ABA (n = 14). C, Whole cell current at −180 mV represented from B. Letters below bars show subgroups indicated by ANOVA with Tukey's honestly significant difference posthoc analysis. Different letters indicate significantly different means (α = 0.05).

induced stomatal closure in Arabidopsis (*Arabidopsis thaliana*) and *Pisum sativum* and *Paphiopedilum* species (Irving et al., 1992; Gehring et al., 1997; Grabov and Blatt, 1997; Suhita et al., 2004; Gonugunta et al., 2008). These second messengers transduce environmental signals to ion channels and ion transporters that create the driving force for stomatal movements (Ward et al., 1995; MacRobbie, 1998; Garcia-Mata et al., 2003).

In this study, we examined the mobilization of second messengers, the inactivation of I_{Kin}, and the suppression of H^+-ATPase phosphorylation evoked by ABA in Arabidopsis mutants to clarify the downstream signaling events of ABA signaling in guard cells. The mutants included a quadruple mutant of *PYR/PYL/RCARs*, *pyr1/pyl1/pyl2/pyl4*, and a mutant of a SnRK2 kinase, *srk2e*.

RESULTS

Differential Responses of the *pyr1/pyl1/pyl2/pyl4* Quadruple Mutant in ABA-Induced Stomatal Closure and ABA-Inhibited Stomatal Opening

The effects of exogenous ABA on stomatal movements were examined in the wild type and the *pyr1/pyl1/pyl2/pyl4* quadruple ABA receptor knockout mutant. Stomatal closure was induced when ABA was externally applied to fully open stomata of the wild type. By contrast, ABA-induced stomatal closure was incomplete in the quadruple mutant (Fig. 1A; Supplemental Fig. S1), essentially as reported previously (Nishimura et al., 2010). Aperture width of the preopened stomata in the light was wider in the quadruple mutant than in the wild type. This indicates that ABA sensitivity was different between stomata of the wild type and the mutant with respect to the degree of closure induction.

We next examined the inhibition of light-induced stomatal opening. Exogenously applied ABA to the dark-adapted epidermis strongly inhibited subsequent light-induced stomatal opening in the wild type (Fig. 1B). ABA inhibited the opening of the mutant's stomata to a similar extent, in contrast to its effects on closure induction (Fig. 1B). The mean aperture of the mutants was wider than that of the wild type in the absence of ABA.

In the quadruple mutant, the ABA responsivity of stomatal movements appeared to be different between opening and closure. The above results suggest that different receptors are involved in ABA-induced closure and ABA-inhibited opening.

Impairment of ROS and NO Production, and Cytosolic Alkalization in the Quadruple Mutant

ABA-induced stomatal closure is accompanied by the production of ROS and NO and an increase of cytosolic pH in guard cells (Irving et al., 1992; Blatt and Armstrong,

1993; Garcia-Mata and Lamattina, 2002; Neill et al., 2002; Kwak et al., 2003). ROS and NO have been shown to act as second messengers (Pei et al., 2000; Neill et al., 2002). Exogenous ABA induced an increase of hydrogen peroxide (H_2O_2), as shown by 2′,7′-dichlorofluorescin fluorescence, in wild-type guard cells (Fig. 2A), in accordance with previous results (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003; Munemasa et al., 2007). Unlike the wild type, the quadruple mutant showed no elevation of H_2O_2 level (Fig. 2A). NO production in guard cells, as shown by diaminofluorescein fluorescence, was induced by ABA in the wild type but not in the mutant (Fig. 2B). ABA elicited cytosolic alkalization, as shown by an increase of 2′,7′-Bis(carboxyethyl)-4 or 5-carboxyfluorescein (BCECF) fluorescence, in guard cells in the wild type but not in the mutant (Fig. 2C).

ABA Inhibition of I_{Kin} in Guard Cells

Inactivation of inward-rectifying K^+ channels is one of the key events in the repression of stomatal opening (Blatt and Thiel, 1994; Schwartz et al., 1994; Eisenach et al., 2012). Here, we employed the whole cell patch-clamp technique to examine ABA inactivation of I_{Kin} in isolated guard cell protoplasts (GCPs). Without ABA treatment, no significant difference in the amplitude of I_{Kin} was observed between wild-type and quadruple mutant GCPs (Fig. 3). Treatment of GCPs with 10 μM ABA for 2 h significantly reduced I_{Kin} in the wild type. I_{Kin} was significantly but partially reduced in the quadruple mutant. This indicates that PYR1, PYL1, PYL2, and PYL4 ABA receptors are partially involved in the inactivation of I_{Kin}. However, these receptors are not the only factors upstream of I_{Kin} regulation.

Inhibition of Blue Light-Induced Phosphorylation of Plasma Membrane H^+-ATPase in Guard Cells by ABA

We examined the inhibition of phosphorylation of H^+-ATPase in the guard cells of the quadruple mutant by immunohistochemical staining (Hayashi et al., 2011). In red light, the fluorescence intensity, an indication of the phosphorylation of H^+-ATPase, was low in the guard cells of the quadruple mutant and the wild type (Fig. 4A, left panels). The addition of blue light over the background red light increased the phosphorylation level both in the wild type and the mutant to a similar extent (Fig. 4A, middle panels). When 10 μM ABA was included, the blue light-induced H^+-ATPase phosphorylation was substantially inhibited in the wild type, as reported previously (Hayashi et al., 2011), as well as in the mutant (Fig. 4A, right panels). Figure 4B shows the result of semiquantitative analysis of the fluorescence images. The fluorescence was increased five to eight times by blue light treatment. ABA inhibited the phosphorylation, and a significant difference was not observed. Together, these results suggest that

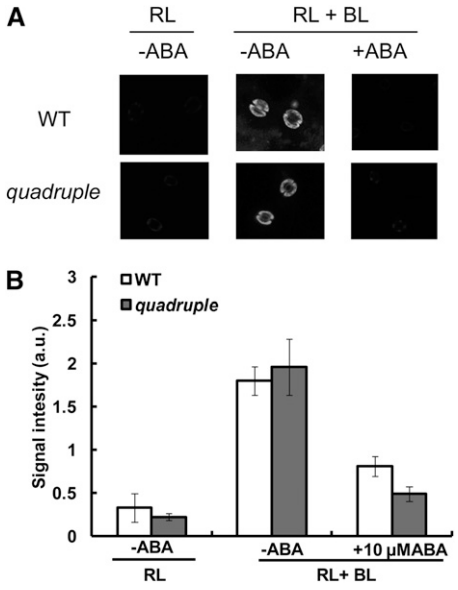

Figure 4. Inhibition of blue light-induced phosphorylation of H^+-ATPase by ABA in wild-type (WT) and *pyr1/pyl1/pyl2/pyl4* quadruple mutant (*quadruple*) guard cells. A, Typical fluorescence images of stomata from the wild type and the quadruple mutant. B, Quantification of fluorescence images of stomata. The fluorescence intensity of guard cells visualized with anti-pThr and Alexa Fluor 488-conjugated secondary antibody was quantified as described in "Materials and Methods." Isolated epidermal fragments were illuminated with red light (RL) for 20 min and successively illuminated with red light or blue light with red light (RL + BL) for 2.5 min. Where indicated, 10 μM ABA was added immediately before the RL + BL treatment. Bars indicate averages of four independent experiments ($n = 4$; 120 total guard cells per bar). Error bars represent SE. a.u., Arbitrary units. We detected no significant difference between the wild type and the quadruple mutant by Student's t test ($\alpha = 0.05$).

ABA inhibition of stomatal opening in the mutant was intact.

ABA Responses of Guard Cells in the *srk2e* Mutant

A key node in the ABA signaling network includes OST1/SnRK2.6/SRK2E, which is an ABA-activated protein kinase predominantly expressed in Arabidopsis guard cells, PP2Cs, and the PYR/PYL/RCAR ABA receptors (Yoshida et al., 2006; Hirayama and Shinozaki, 2007; Cutler et al., 2010; Nishimura et al., 2010; Weiner et al., 2010). We examined stomatal phenotypes, second messengers, I_{Kin} inactivation, and H^+-ATPase phosphorylation in an *OST1/SnRK2.6/SRK2E* null mutant.

ABA-induced stomatal closure was abolished in the presence of 1 and 10 μM ABA in *srk2e* (Fig. 5A) as well as in the *pyr1/pyl1/pyl2/pyl4* quadruple mutant (Fig. 1A). Light-induced stomatal opening was not inhibited by 1 or 10 μM ABA in *srk2e* (Fig. 5B), which is different from the result with the quadruple mutant (Fig. 1B).

ABA-induced stomatal closure is accompanied by ROS production (Kwak et al., 2003). Ten micromolar ABA did not induce ROS production in *srk2e* (Fig. 6) or

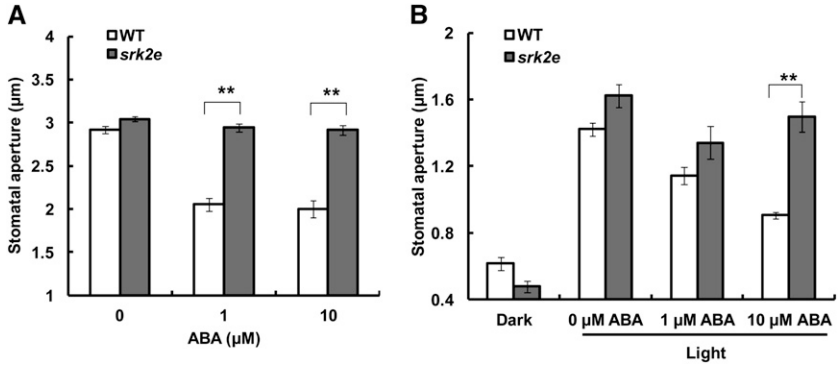

Figure 5. Induction of stomatal closure and inhibition of light-induced stomata opening by ABA in the wild type (WT) and the *srk2e* mutant. A, ABA-induced stomatal closure in wild-type and *srk2e* plants. Averages from eight independent experiments ($n = 8$; 160 stomata per bar) are shown. B, ABA inhibition of stomatal opening in wild-type and *srk2e* plants. Dark represents fully dark-adapted stomata; 0 μM ABA indicates 2.5-h light treatment after the dark adaption with 0.1% ethanol as the solvent control; 1 and 10 μM ABA represent an ABA addition at same time as the initiation of light treatment. Averages from three independent experiments are shown ($n = 3$; 60 stomata per bar). Asterisks indicate significant differences (**$\alpha = 0.01$) by Student's *t* test. Error bars represent SE.

pyr1/pyl1/pyl2/pyl4 (Fig. 2), indicating that SRK2E kinase, like the PYR1, PYL1, PYL2, and PYL4 ABA receptors, is involved in ABA-induced stomatal closure.

Pretreatment of GCPs with 10 μM ABA for 2 h did not significantly inhibit I_{Kin} in *srk2e* (Fig. 7) but inhibited I_{Kin} in *pyr1/pyl1/pyl2/pyl4* (Fig. 3), suggesting that SRK2E, unlike PYR1, PYL1, PYL2, and PYL4, is involved in the ABA inhibition of stomatal opening. The amplitude of I_{Kin} of *srk2e* was smaller than that of the wild type in the absence of ABA (-279 ± 27 and -146 ± 28 pA at -180 mV, respectively; $n = 20$ for Columbia-0 and $n = 5$ for *srk2e* [$P < 0.01$]; Figs. 3 and 7). This may be attributed to the regulation of expression or posttranslational modification of inward-rectifying K^+ channels by the impairment of OST1-mediated ABA signaling.

srk2e, unlike the wild type and the quadruple mutant, did not show ABA inactivation of light-induced phosphorylation of H^+-ATPase (Fig. 8). Together, these results show that the H^+-ATPase phosphorylation level is well correlated with stomatal opening inhibition by ABA in all examined plant lines: the wild type, the quadruple mutant, and *srk2e*.

DISCUSSION

In this study, we examined the differences in ABA signaling components during open inhibition and closure induction in the quadruple *pyr/pyl/rcar* ABA receptor mutant of Arabidopsis. Our results demonstrate that the ABA receptors PYR1, PYL1, PYL2, and PYL4, which control ABA-induced stomatal closure, are not strongly involved in the inhibition of stomatal opening by ABA. This suggests that one or more other ABA receptors are involved in the inhibition of stomatal opening. This difference should make it easier to identify the ABA receptors involved in the inhibition of stomatal opening by ABA.

ABA Receptors Involved in Opening Inhibition Have Not Been Identified

In 2009, PYR/PYL/RCAR proteins, which constitute a 14-member family, were identified as ABA receptors in Arabidopsis (Ma et al., 2009; Park et al., 2009). All of them except PYL13 were able to activate ABA-responsive gene expression in protoplast transfection assays (Fujii

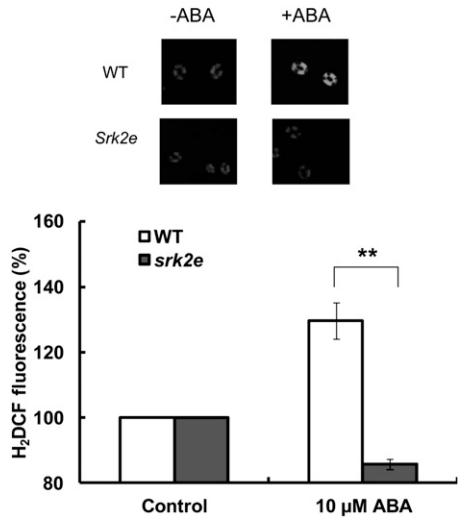

Figure 6. ABA-induced ROS production in wild-type (WT) and *srk2e* guard cells. Representative grayscale H_2DCF fluorescence images (top panel) and percentage of H_2DCF fluorescence levels (bottom panel) are shown. Fluorescence intensities of ABA-treated cells are normalized to the control value taken as 100% for each experiment. Ten micromolar ABA or solvent control (0.1% ethanol) was added to the epidermal preparation for 20 min. Bars indicate averages of five independent experiments ($n = 5$; 100 total guard cells per bar). Asterisks indicate significant differences (**$\alpha = 0.01$) by Student's *t* test. Error bars represent SE.

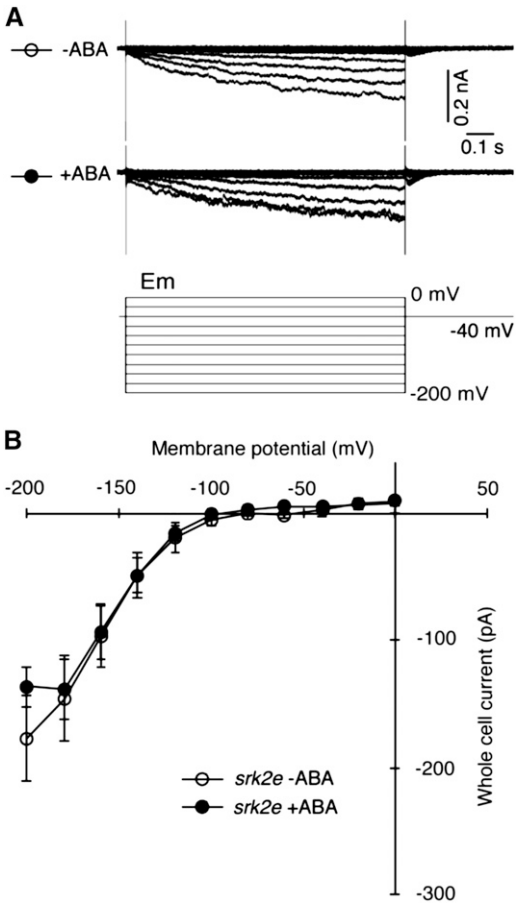

Figure 7. Inactivation of I_{Kin} by ABA in *srk2e* GCPs. A, Representative current in the absence (−ABA) and presence (+ABA) of 10 μM ABA. The bottom panel (Em) indicates the applied step pulse protocol. B, Current-voltage curve of I_{Kin}. GCPs of *srk2e* were treated with 0.1% ethanol (solvent control; white symbols; n = 5) or 10 μM ABA (black symbols; n = 6). Error bars indicate SE.

and Zhu, 2009). PYR1, PYL1, PYL2, and PYL4 appear to be predominantly expressed in guard cells, and their disruption resulted in insensitivity to ABA in the stomata (Nishimura et al., 2010). The involvement of other ABA receptors in opening inhibition is deduced.

Stomata of a plasma membrane ABA uptake transporter mutant, *abcg40*, opened faster than the wild type in the presence of 1 μM ABA (Kang et al., 2010), indicating that ABA was transported to the insides of the cells that functioned in opening inhibition. This suggests that the unidentified ABA receptor localizes in the cells. Moreover, an excised inside-out patch-clamp analysis of Ca^{2+}-permeable channels in *V. faba* GCPs revealed that the ABA perception sites were on the cytosolic side of the patched membrane (Hamilton et al., 2000). Hence, some of the other 10 PYR/PYL/RCAR members, which are relatively low in abundance compared with the four PYR/PYL/RCARs in guard cells and localized inside of the cell, might be involved in the opening inhibition.

Microinjection experiments have suggested that the extracellular ABA perception sites mainly function in opening inhibition, while intracellular sites are essential for closure induction (Anderson et al., 1994; Assmann, 1994; Schwartz et al., 1994). One can assume that the four Arabidopsis PYR/PYL/RCARs are the intracellular ABA receptors and that an unidentified extracellular ABA receptor remains functional in the quadruple mutant.

ABA Responses of Stomatal Movements: Opening and Closure Are Regulated Differentially

Based on studies in which plants were exogenously treated with NO and H_2O_2, both compounds were reported to be involved in the signaling process of inhibition of light-induced stomatal opening (Garcia-Mata and Lamattina, 2007; Yan et al., 2007). We found that ABA did not induce the production of ROS or NO or cytosolic alkalization in the quadruple mutant guard cells (Fig. 2), suggesting that the production of ROS and NO and cytosolic alkalization in guard cells function downstream of PYR1, PYL1, PYL2, and PYL4. ROS and

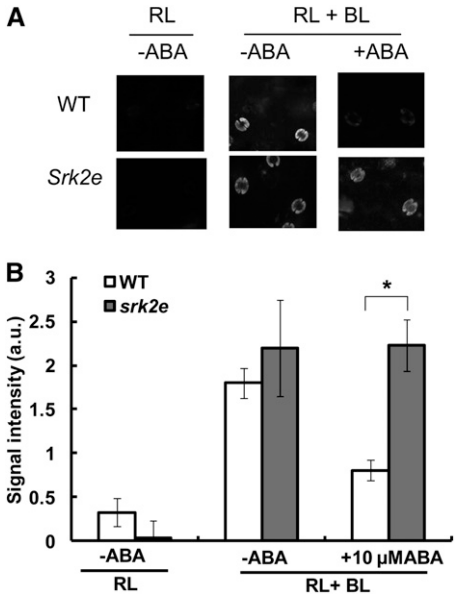

Figure 8. ABA inhibition of blue light-induced phosphorylation of H^+-ATPase in wild-type (WT) and *srk2e* guard cells. A, Typical fluorescence images of stomata using anti-H^+-ATPase in the epidermis from the wild type and the *srk2e* mutant. B, Quantification of fluorescence images of stomata. The fluorescence intensity of guard cells visualized with anti-pThr and Alexa Fluor 488-conjugated secondary antibody was quantified. Isolated epidermal fragments were illuminated with red light (RL) for 20 min and subsequently illuminated with red light or blue light with red light (RL + BL) for 2.5 min. Where indicated, 10 μM ABA was added immediately before the RL + BL treatment. Bars indicate averages of four independent experiments (n = 4; 120 total guard cells per bar). The asterisk indicates a significant difference (*α = 0.05) by Student's t test. Error bars represent SE. a.u., Arbitrary units.

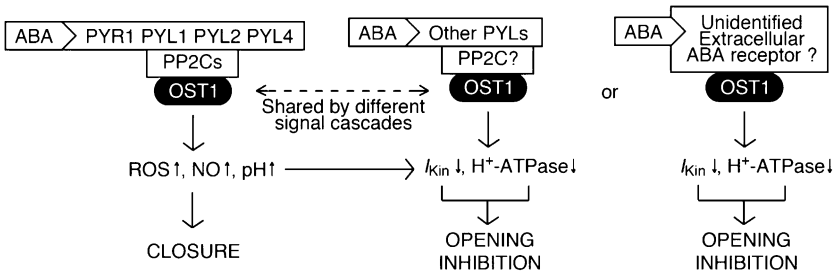

Figure 9. A simple model of the difference in ABA perception mechanisms between closure induction and opening inhibition of stomata in Arabidopsis guard cells.

NO production and cytosolic alkalization may only partly function in the inhibition of stomatal opening. The partial inactivation of I_{Kin} in the quadruple mutant (Fig. 3) may be due to NO or ROS, which are known to inhibit I_{Kin} (Garcia-Mata et al., 2003; Köhler et al., 2003). Alkalization may also be involved in the regulation of I_{Kin} (Blatt, 1992; Blatt and Armstrong, 1993; Grabov and Blatt, 1997; Wang et al., 2012). In this study, the partial inactivation of I_{Kin} in the mutant could not have been caused by a pH change, because the pipette solution was buffered in our patch-clamp experiments. Moreover, the fact that ABA partially inactivates I_{Kin} in the quadruple mutant, which lacks the alkalization, indicates that the inactivation of I_{Kin} (Fig. 3) is not caused by a pH change. However, the possibility that pH is involved in the inactivation of inward-rectifying K^+ channels under the four PYR/PYL/RCAR receptors could not be excluded. The finding that H^+-ATPase was inactivated in the quadruple mutant (Fig. 4) indicates that the regulation of H^+-ATPase is not a downstream event of H_2O_2 production, NO production, and alkalization.

Nonratiometric measurements of BCECF fluorescence revealed that cytosolic pH was not increased by ABA in the quadruple mutant (Fig. 2). This suggests that ABA-induced alkalization is impaired in the mutant or that the cytosolic pH in the mutant is constitutively higher than that in the wild type due to the derepression of ABA signaling.

The modulation of I_{Kin} is closely related to the kinetics of stomatal opening (Eisenach et al., 2012), and I_{Kin} decreases with increasing cytosolic pH (Grabov and Blatt, 1997; Wang et al., 2012). On the other hand, ABA inhibition of stomatal opening did not appear to be affected in the quadruple mutant (Fig. 1B), in which cytosolic pH varies little (Fig. 2). These results indicate that the inhibition of stomatal opening is mainly caused by the suppression of H^+-ATPase activity and, coincidentally, by the inactivation of I_{Kin} by cytosolic alkalization.

In the *abi1-1* mutant, ABA does not induce stomatal closure in spite of NO production (Desikan et al., 2002). On the other hand, ABA-induced H_2O_2 accumulation was observed in *abi1-1* but not in *abi2-1* (Murata et al., 2001). These complicated facts suggest that PYR/PYL/RCAR in guard cells interact with PP2Cs in such a way that PP2Cs function in differentiated signal branches in ABA signaling. Another possibility is that the complicated phenotype is caused by abnormal specificities of substrate of the semidominant mutants *abi1-1* and *abi2-1*.

The apertures of open stomata in Figure 1B were even narrower than those of closed stomata in Figure 1A. This is attributed to the difference in the methods of sample preparation between the closure assay and the opening assay. For the closure assay, whole leaves were incubated in the opening buffer (see "Materials and Methods") and then blended immediately before the observation. On the other hand, the blended epidermal fragment was obtained first and then incubated in the opening buffer in order to examine opening inhibition.

In the Arabidopsis quadruple mutant, the sensitivity to ABA of the light-induced stomatal opening was impaired (Nishimura et al., 2010), which is inconsistent with our finding that the sensitivity was intact in the quadruple mutant (Fig. 1B). Stomata exhibit hysteresis in their light response: after light illuminates a leaf, stomatal conductance increases and subsequent dark treatment cannot fully reduce stomatal conductance to the original dark level (Ng and Jarvis, 1980). Our preliminary experiments show that irradiation for 1 h makes stomata partially open even after a 3-h dark treatment. The incomplete closure is likely a hysteresis in the response of stomata to light. In order to avoid this artifact, we used dark-adapted plants. The difference may also be due to the difference of the growth environments in the two laboratories.

PYR/PYL/RCAR Receptors and OST1

OST1 kinase is reported to play a key role in phosphorylating many substrates in the ABA responses of Arabidopsis stomata (Mustilli et al., 2002; Yoshida et al., 2002; Fujii and Zhu, 2009; Nakashima et al., 2009), and AAPK has a similar function in *V. faba* guard cells (Li and Assmann, 1996; Mori and Muto, 1997; Li et al., 2000). It was proposed that the activity of OST1 kinase is regulated by a complex of PYR/PYL/RCAR receptors and PP2C (Cutler et al., 2010; Kim et al., 2010; Weiner et al., 2010). We examined the ABA inhibition of stomatal opening of a mutant of OST1/SnRK2.6/SRK2E. In this study, *srk2e*, which is a T-DNA insertion mutant of the Columbia-0 ecotype (Yoshida et al., 2002), was used instead of *ost1-2*. *ost1-2* is a mutant derived from Landsberg *erecta* (Mustilli et al., 2002). The quadruple mutant was generated from three Columbia-0

background mutants and one Landsberg background mutant (Park et al., 2009).

In contrast to the quadruple mutant, the *srk2e* mutant was insensitive to ABA in the inhibition of opening. These results suggest that the functions of PYR1, PYL1, PYL2, and PYL4 in ABA-induced stomatal closure and ABA inhibition of stomatal opening in Arabidopsis are different from those of OST1, as shown in Figure 9. PYR1, PYL1, PYL2, and PYL4 bind ABA and activate OST1 kinase through the inhibition of PP2C (Cutler et al., 2010). One of the functions of the activated OST1 kinase is to induce the production of ROS and NO and the alkalization of cytosolic pH (Figs. 6 and 7). These signaling events activate the stomatal closure machinery. At the same time, to inhibit opening, other PYR/PYL/RCAR members or an unidentified extracellular ABA receptor might bind ABA and activate OST1 through an unknown mechanism. The OST1 kinase activated by the unknown mechanism regulates the activities of the inward-rectifying K^+ channel and H^+-ATPase, independently of the PYR1/PYL1/PYL2/PYL4-dependent pathway.

Several substrates of OST1/AAPK have been reported. NADPH oxidase is a substrate (Sirichandra et al., 2009). ROS production was revealed to function downstream of the four PYR/PYL/RCAR receptors (Fig. 2). SLAC1 slow anion channel (Negi et al., 2008; Vahisalu et al., 2008) and ALMT12 rapid anion channel (Meyer et al., 2010; Sasaki et al., 2010) are substrates of OST1/AAPK (Li et al., 2000; Geiger et al., 2009; Imes et al., 2013). These anion channels are involved in stomatal closure and most likely function under the PYR/PYL/RCAR-dependent pathway. The inward-rectifying K^+ channel is also a substrate of OST1/AAPK (Mori et al., 2000; Sato et al., 2009). The phosphorylation of the inward-rectifying K^+ channel inactivates I_{Kin} (Sato et al., 2009). This agrees with the lack of I_{Kin} regulation in the *srk2e* mutant (Fig. 7). Other OST1/AAPK substrates, which are involved in H^+-ATPase activity regulation, would play roles under the unknown ABA receptor(s) in opening inhibition.

In plants other than Arabidopsis, guard cell SnRK2 kinases may be regulated by other factors, such as SnRK2-interacting Ca^{2+} sensor protein (Bucholc et al., 2011), glyceraldehyde-3-phosphate dehydrogenase (Wawer et al., 2010), and phosphatidic acid (Testerink et al., 2004). These factors may be involved in PYR1/PYL1/PYL2/PYL4-independent (or -dependent) ABA signaling in Arabidopsis guard cells or may interact with unknown ABA receptors.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) wild type (ecotype Columbia-0), *pyr1/pyl1/pyl2/pyl4* quadruple mutant (Park et al., 2009), and *srk2e* mutant (Yoshida et al., 2002; Umezawa et al., 2009) were grown in plastic pots filled with 70% (v/v) vermiculite (Asahi-kogyo) and 30% (v/v) Kureha soil (Kureha Chemical) in a growth chamber (80 μmol m^{-2} s^{-1} photon flux with white fluorescent tubes under a 16-h-light/8-h-dark regime). Temperature and relative humidity in the growth chamber were controlled at 22°C ± 2°C and 60% ± 10%, respectively.

Measurement of Stomatal Aperture

Stomatal Closing Assay

Stomatal aperture was examined as described previously (Uraji et al., 2012). In brief, excised rosette leaves from 4- to 6-week-old plants were floated on the opening buffer containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 6.15) for 2 h in the light (80 μmol m^{-2} s^{-1}) to preopen stomata. Subsequently, the leaves were chopped with a Waring blender, and epidermal fragments were collected on a piece of a nylon net (mesh size, 200 μm). Aperture width was microscopically measured after a 2-h incubation under illumination in the presence of ABA or the solvent control (0.1% ethanol).

Stomatal Opening Assay

Rosette leaves of 4- to 6-week-old dark-adapted plants (overnight in the dark, 8 h) were blended with a Waring commercial blender before the light period and suspended in the opening buffer. After a succeeding 2-h incubation in the dark, the widths of stomatal aperture were measured, which served as the dark control. The blended leaf specimens were subsequently incubated in the light for 2.5 h in the presence of ABA or the solvent control (0.1% ethanol). Twenty randomly selected stomatal apertures were measured per leaf. Typically, three to five independent biological repeats were conducted. The reproducibility of the experimental results was independently ensured by one or two other experimenter(s) unassociated with the presented data.

Measurement of ROS and NO Production

The production of ROS and NO in guard cells was estimated by the methods described by Munemasa et al. (2007) utilizing the fluorescence indicators 2′,7′-dichlorodihydrofluorescein (H_2DCF) diacetate and diaminofluorescein 2-diacetate (CAS 205391-02-2), respectively.

Measurement of the Cytosolic pH of Guard Cells

A pH-sensitive fluorescent dye, BCECF-acetoxymethyl ester (BCECF-AM; CAS 117464-70-7), was used to examine the change in the cytosolic pH of guard cells as described previously (Islam et al., 2010). In order to avoid artifacts from the variation of dye-loading efficiency and/or deesterification by time, the same amount of the solvent (0.1% ethanol) was added for the same period as the 10 μM ABA treatment. Percentage increase of fluorescence from the control of the same plant line was shown as pH change.

Whole Cell Patch-Clamp Recording of I_{Kin}

Patch-clamp experiments were carried out essentially as described previously (Munemasa et al., 2007; Saito et al., 2008). GCPs were enzymatically isolated from rosette leaves of 4- to 6-week-old plants. Whole cell currents were measured using a patch-clamp amplifier (model CEZ-2200; Nihon Kohden). Data were acquired and analyzed with pCLAMP 8.2 software (Molecular Devices). The pipette solution contained 30 mM KCl, 70 mM potassium-Glu, 2 mM $MgCl_2$, 3.35 mM $CaCl_2$, 6.7 mM EGTA, and 10 mM HEPES adjusted to pH 7.1 with Tris, and the bath solution contained 30 mM KCl, 2 mM $MgCl_2$, 40 mM $CaCl_2$, and 10 mM MES titrated to pH 5.5 with Tris. Osmolarity of the pipette solution and the bath solution was adjusted with D-sorbitol to 500 and 485 mmol kg^{-1}, respectively. In order to examine the effect of ABA, GCPs were treated with 10 μM ABA (for the solvent control, 0.1% ethanol) for 2 h before gigaohm seal establishment.

Phosphorylation of the Penultimate Thr of the Plasma Membrane H^+-ATPase in Guard Cells

Blue light-induced phosphorylation of the penultimate Thr in the C terminus of plasma membrane H^+-ATPase in guard cells was detected immunohistochemically using a specific antibody against the phosphorylated penultimate Thr of H^+-ATPase (anti-pThr) according to a previous report (Hayashi et al., 2011). In brief, epidermis prepared by blending rosette leaves from dark-adapted plants was incubated under background red light for 20 min. ABA or ethanol (solvent control) was added to the incubation medium, followed by a 2.5-min blue light treatment. The specimens were fixed with paraformaldehyde and stuck on a coverslip. After permeabilizing the cells, phosphorylated

H^+-ATPases were visualized with anti-pThr and the Alexa Fluor488-labeled secondary antibody. The fluorescence of Alexa Fluor488 was imaged with a fluorescence microscope, and the intensity of the fluorescence of guard cells was quantified with ImageJ software (for details, see Hayashi et al., 2011).

Statistical Analysis

The significance of differences between data sets was assessed by Student's *t* test analysis in all parts of this article unless otherwise stated. Difference at the level of $P < 0.05$ was regarded as significant.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: PYR1 (At4G17870), PYL1 (At5g46790), PYL2 (At2g26040), PYL4 (At2g38310), and OST1/SRK2E (At4g33950).

Supplemental Data

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

Supplemental Figure S1. Induction of stomatal closure and inhibition of light-induced stomatal opening by ABA in wild type and *pyr1 pyl1 py2 pyl4* quadruple mutant in %.

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