Molecular Plant • Volume 6 • Number 2 • Pages 528–538 • March 2013 RESEARCH ARTICLE

ABA Signaling in Guard Cells Entails a Dynamic Protein–Protein Interaction Relay from the PYL-RCAR Family Receptors to Ion Channels

Sung Chul Lee[a,b,1,2], **Chae Woo Lim**[b,2], **Wenzhi Lan**[a,c,2], **Kai He**[a] and **Sheng Luan**[a,d,1]

a Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
b School of Biological Sciences (BK21 Program), Chung-Ang University, Seoul, 156–756, Korea
c Plant Molecular Biology Institute and College of Life Sciences, Nanjing University, China
d WCU Program, Chonnam National University, Gwangju, Korea

ABSTRACT Plant hormone abscisic acid (ABA) serves as an integrator of environmental stresses such as drought to trigger stomatal closure by regulating specific ion channels in guard cells. We previously reported that SLAC1, an outward anion channel required for stomatal closure, was regulated via reversible protein phosphorylation events involving ABA signaling components, including protein phosphatase 2C members and a SnRK2-type kinase (OST1). In this study, we reconstituted the ABA signaling pathway as a protein–protein interaction relay from the PYL/RCAR-type receptors, to the PP2C–SnRK2 phosphatase–kinase pairs, to the ion channel SLAC1. The ABA receptors interacted with and inhibited PP2C phosphatase activity against the SnRK2-type kinase, releasing active SnRK2 kinase to phosphorylate, and activate the SLAC1 channel, leading to reduced guard cell turgor and stomatal closure. Both yeast two-hybrid and bimolecular fluorescence complementation assays were used to verify the interactions among the components in the pathway. These biochemical assays demonstrated activity modifications of phosphatases and kinases by their interaction partners. The SLAC1 channel activity was used as an endpoint readout for the strength of the signaling pathway, depending on the presence of different combinations of signaling components. Further study using transgenic plants overexpressing one of the ABA receptors demonstrated that changing the relative level of interacting partners would change ABA sensitivity.

Key words: abscisic acid; ABA receptor; protein kinase; protein phosphatase; SLAC1.

INTRODUCTION

Plants close their stomata to conserve water in response to a water-deficit condition. Plant hormone abscisic acid (ABA) plays a key role in the adaptation to water-deficit conditions through regulatory pathways that control gene expression and stomatal closure (Luan, 2002; Zhu, 2002; Wasilewska et al., 2008). The levels of ABA in plant tissues increase under drought conditions, and ABA down-regulates guard cell turgor pressure and thus triggers stomatal closure by modifying activities of a number of ion channels (Schroeder et al., 2001). The ion channels targeted by ABA signaling include K^+ channels and anion channels, which control ionic fluxes across the plasma membrane and tonoplast, thereby adjusting guard cell turgor pressure (Schroeder et al., 1987; Schroeder and Hagiwara, 1989; Lemtiri-Chlieh and MacRobbie, 1994; Negi et al., 2008; Vahisalu et al., 2008). The process starts with an increasing level of ABA activating anion efflux through anion channels thus inducing depolarization of guard cell plasma membrane. Depolarization drives K^+ efflux through outward-rectifying K^+ channels leading to water efflux, which reduces guard cell volume and leads to stomatal closure (Ache et al., 2000; Li et al., 2000; Ward et al., 2008).

To initiate ABA signaling, ABA receptors must exist in plant cells. Previous studies have identified several types of putative receptors that may mediate ABA functions (McCourt and Creelman, 2008; Cutler et al., 2010). Most recently, a family of START domain proteins, known as

[1] To whom correspondence should be addressed S.C.L.: E-mail sclee1972@cau.ac.kr, tel. 82–2–820–5207, fax 82–2–825–5206 S.L.: E-mail sluan@berkeley.edu, tel. (510) 642–6306, fax (510) 642–4995.

[2] These authors contributed equally to this work.

doi:10.1093/mp/sss078, Advance Access publication 30 August 2012

Received 28 June 2012; accepted 11 July 2012

PYR/PYLs-RCARs, were shown to function as ABA receptors (Fujii et al., 2009; Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Park et al., 2009). These ABA receptors interact with and inhibit the activity of group A protein phosphatase 2C (PP2C) (Ma et al., 2009; Santiago et al., 2009b; Szostkiewicz et al., 2010).

Genetic studies have identified several PP2C genes that are required for ABA signaling in *Arabidopsis*. Of these members, a group A PP2C, including ABI1, ABI2, HAB1, HAB2, AGH1, and PP2CA, generally function as negative regulators of the ABA response (Merlot et al., 2001; Nishimura et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Rubio et al., 2009). If PP2Cs function as negative regulators of the ABA signaling pathways, protein kinases are expected to act as positive regulators in the same pathways (Mustilli et al., 2002; Yoshida et al., 2006a; Fujii et al., 2007). OST1 is an *Arabidopsis* SnRK2-type protein kinase named SnRK2.6. Several other members in this family, such as SnRK2.2 and SnRK2.3, are also shown to function in ABA response (Belin et al., 2006; Yoshida et al., 2006a; Chae et al., 2007; Fujii et al., 2007). On the contrary to A-type PP2Cs, these three kinases are positive regulators of ABA signaling during seed development, germination, and in response to water stress (Fujii et al., 2007; Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009).

Downstream from the receptors, PP2Cs, and SnRKs are ion channels that control stomatal movements (Fujii et al., 2009; Geiger et al., 2009; Lee et al., 2009). Two groups identified the gene encoding the guard cell slow anion channel, named SLAC1, involved in stomatal regulation in *Arabidopsis* (Negi et al., 2008; Vahisalu et al., 2008). SLAC1 is required for stomatal closure induced by high CO_2 and ABA (Vahisalu et al., 2008) indicating that the SLAC1 protein may represent a critical component of guard cell anion channels that are responsible for stomatal closure induced by ABA or other signals. Indeed, recent studies have shown that SLAC1 serves as a substrate for and is activated by SnRK2.6/OST1 (Geiger et al., 2009; Lee et al., 2009). Furthermore, OST1 activity against the SLAC1 protein is inhibited by PP2Cs (Geiger et al., 2009; Lee et al., 2009).

In the present study, we addressed this question using the *Xenopus* oocyte expression system to assess SLAC1 activity and its regulation by ABA receptors (PYL-RCAR), kinases (SnRK2), and phosphatases (PP2C) in the ABA signaling pathway. The interactions among the ABA receptor, phosphatases, kinases, and the ion channel proteins was also examined using an electrophysiological method as well as yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assay. Our data provide evidence for a series of physical protein–protein interactions starting from ABA receptors that interact with and inhibit PP2CA. PP2CA regulates kinase activity by directly interacting with the OST1/SnRK2.6 kinase, which, in turn, interacts with and phosphorylates the SLAC1 channel protein.

RESULTS AND DISCUSSION

Members of the PYL/RCAR Family Physically Interact with A-Type PP2Cs and Form a Complex Network

Previous studies have shown that PYL/RCAR proteins interact with PP2C A-type family members and inhibit their activities (Ma et al., 2009; Park et al., 2009). We have shown previously that PP2CA, an A-type PP2C, interacts with and inhibits its SnRK2.6/OST1 activity thereby inhibiting the SLAC1 ion channel. To examine whether PP2CA mediates ABA signaling through PYL/RCAR-type ABA receptors and to further address the specificity and overlap of the functions of different family members in the PYL/RCAR family, we conducted a comprehensive interaction analysis of PP2CA and all PYL/RCAR proteins.

We first used the yeast two-hybrid system to screen for protein–protein interactions between PP2CA and all PYL/RCAR proteins. Figure 1 shows that PP2CA interacted with a majority of PYL/RCAR members except RCAR11, 12, and 14 without ABA. In previous reports, ABA is shown to promote interactions between some PYL members and PP2Cs (Ma et al., 2009; Santiago et al., 2009a); thus, it is possible that interactions between PP2CA and RCAR11, 12, and 14 would occur in the presence of ABA. Therefore, we added 10 μM ABA to the selection media, which enhanced the interactions between PP2CA with both RCAR12 and RCAR14, indicating that PP2CA interacts with these two RCAR members in an ABA-dependent manner. It is also possible that endogenous ABA from yeast might be sufficient to promote the RCARs–PP2CA interaction, because several fungi produce ABA (Hirai et al., 2000; Nambara and Marion-Poll, 2005).

We then used the BiFC procedure in plant cells to confirm the protein–protein interactions identified in the yeast two-hybrid assays. As shown in Figure 1B, co-expression of PP2CA and RCAR2 in epidermal cells of *Nicotiana benthamiana* generated yellow fluorescence protein (YFP) signals only in the nucleus identified by the arrows, indicating an interaction between the PP2CA and RCAR2. This result supports the yeast two-hybrid assay for a physical interaction between PP2CA and RCAR2 proteins. It also suggests that the PP2CA and RCAR proteins are both targeted to the nucleus, where they regulate gene expression (Yoshida et al., 2006b).

ABA Receptor RCAR2 Relieves PP2CA Inhibition of SLAC1 Activity

If an interaction between PYL/RCAR and PP2CA mediates ABA-dependent regulation of channels such as SLAC1, inhibition of SLAC1 by PP2CA, as reported previously (Lee et al., 2009), should be relieved by the presence of the PYL/RCAR proteins. We tested the functional relationship between RCAR2, PP2CA, and the SLAC1 ion channel in the *Xenopus* oocyte system by co-expressing different combinations of the proteins (Figure 2). We first expressed the SLAC1 channel with RCAR2, and found that SLAC1 activity was not

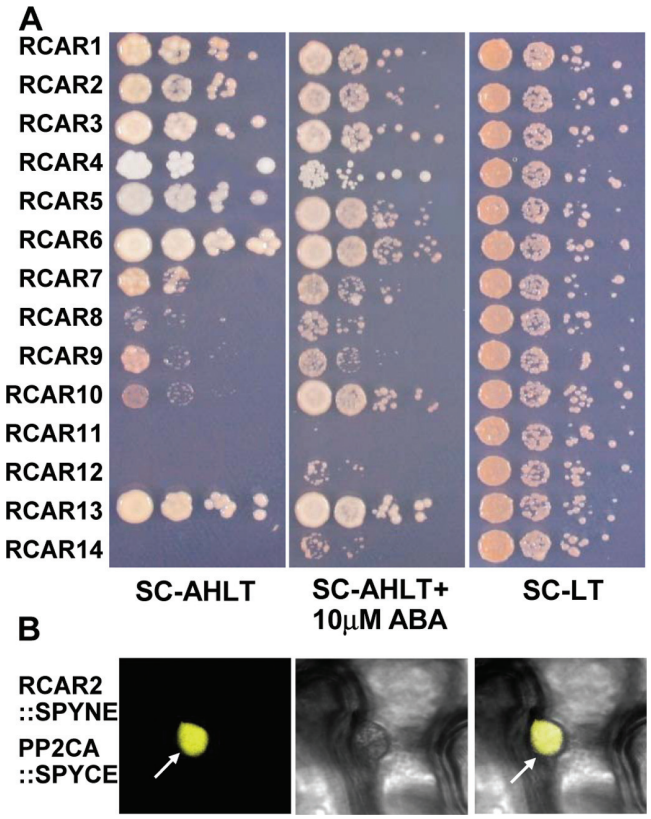

Figure 1. Physical Interactions among PP2CA and RCARs.

(A) Yeast two-hybrid assay of interactions between PP2CA and RCARs. Growth on the selection medium (SC-ALTH) was used as an indicator of interaction (left row) and with 10μM ABA (middle row). Growth in SC-LT was used as control (right row).

(B) Bimolecular fluorescence complementation (BiFC) assay of interactions among PP2CA and RCAR2. The fluorescence indicates interaction between the indicated partner proteins. The images were obtained from the YFP channel or bright field or a merged picture of the two. PP2CA-35S-SPYCE(M) co-expressed with RCAR2-35S-SPYNE(R)173.

changed significantly (Figure 2B). The SLAC1 channel was partially inhibited when co-expressed with the PP2CA. We then co-expressed the SLAC1 channel with both RCAR2 and PP2CA. The current recorded from these oocytes was larger than that recorded from oocytes expressing the SLAC1 ion channel with PP2CA, suggesting that RCAR2 counteracted the effect of PP2CA (Figure 2A). As an ABA receptor, RCAR2 interaction with PP2CA may be affected by presence of ABA (Ma et al., 2009; Santiago et al., 2009b). We tested whether SLAC1 channel activity in the oocyte was affected by ABA. Figure 2B shows that the currents generated by the oocytes co-expressing SLAC1, RCAR2, and PP2CA were not significantly changed by ABA. In addition, currents were unchanged by ABA when we used RCAR6, which is another RCAR member, instead of RCAR2. These results suggest that inhibition of SLAC1 channel activity by PP2CA can be restored partially by co-expressing RCAR2.

Structural studies of PYL/RCARs revealed two models that can account for the function of ABA in the ABA–receptor complex. One model suggests that binding of ABA to RCAR creates a recognition site for PP2C on the surface of RCAR to form a tertiary complex (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009). The other model suggests that PP2Cs stabilize the RCAR–ABA complex and promote binding affinity between RCAR and ABA (Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009). These models may apply to specific cases of PYL/RCAR interactions with PP2Cs, and our results here suggest that ABA does not change the interaction of RCAR2–PP2CA, which is consistent with the yeast two-hybrid result in Figure 1.

RCAR2 Inhibits PP2CA Activity and Recovers OST1 Kinase Activity towards the Channel Protein Substrates

According to previous studies, PYL/RCAR proteins inhibit the type-A sub-family of plant PP2Cs in the ABA signaling pathway (Ma et al., 2009; Park et al., 2009). In addition, RCAR family genes are associated with activating SnRK2-type kinases (Park et al., 2009). Several studies have shown that PYL/RCAR-mediated activation of SnRK2 kinases results from inhibition of PP2Cs (Fujii et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Our previous study also showed that PP2CA interacts with OST1 and inhibits its kinase activity towards its substrates such as SLAC1 (Lee et al., 2009). It is becoming clear that RCAR2 may relieve SLAC1 inhibition by PP2CA by activating SnRK2.6/OST1, which is normally inhibited by PP2CA. To confirm this, we first tested whether RCAR2 would inhibit PP2CA activity using an *in vitro* protein phosphatase assay. When expressed and purified from *E. coli*, phosphatase activity corresponding to full-length PP2CA was indeed inhibited by RCAR2 (Figure 3A). RCAR2 inhibited PP2CA activity with a half maximal inhibitory concentration (IC_{50}) value of 12.89 ng.

After finding that RCAR2 inhibits PP2CA activity, we determined whether RCAR2 affects OST1 kinase activity, which is normally inhibited by PP2CA using an *in vitro* kinase assay (Figure 3B). Based on our previous study on OST1 (Lee et al., 2009), this kinase displays autophosphorylation activity. We observed auto-kinase activity of OST1 without including any other substrate in the assay and this activity was abolished by adding PP2CA (Figure 3B). Interestingly, RCAR2 did not appear to affect OST1 inhibition by PP2CA, despite the observation that RCAR2 interacted with and inhibited PP2CA activity. When the OST1 substrate SLAC1 N-terminus was present, OST1 phosphorylation of SLAC1 was also abolished by the presence of PP2CA (Figure 3B). However, OST1 partially phosphorylated SLAC1 by addition of RCAR2 to the reaction containing PP2CA, OST1, and the SLAC1 N-terminus. In other words, RCAR2 did not seem to affect PP2CA inhibition of OST1 auto-kinase activity but partially alleviated the PP2CA inhibition of SLAC1 phosphorylation by OST1. The proportion of SLAC1 phosphorylation restored by RCAR2 was rather low, suggesting

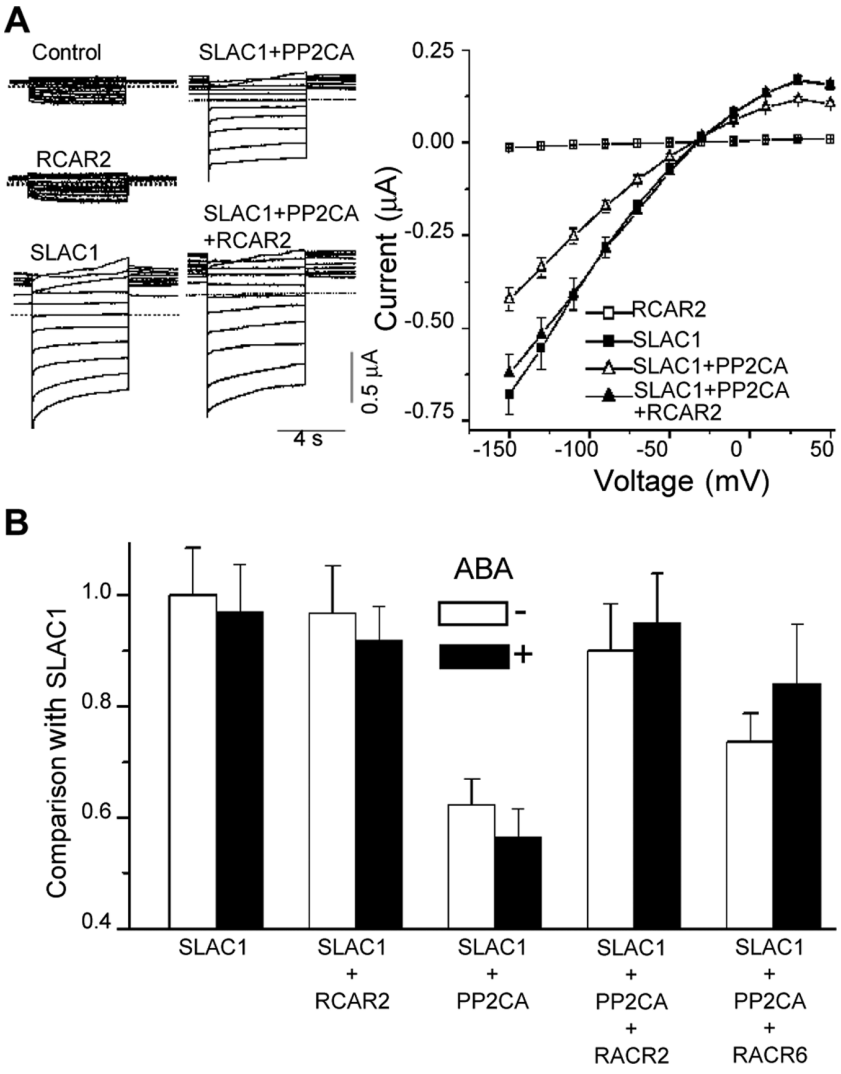

Figure 2. RCARs Regulate PP2CA Inhibition on SLCA1.

(A) RCAR2 recovers SLAC1 activity inhibited by PP2CA. (Left) Typical whole-cell current traces recorded from the oocytes injected with water (Control) or the oocytes injected with cRNA of RCAR2, SLAC1, SLAC1+PP2CA, or SLAC1+PP2CA+RCAR2. The current was recorded by the voltage steps of 50 to –150 mV (in 15-mV decrements, 7.5-s duration) with a 1.45-s prepulse to 0 mV. Dotted lines represent zero current level. (Right) The current–voltage relationship was deduced from the recordings of the control oocytes and the oocytes expressing RCAR2, SLAC1, SLAC1+PP2CA, and SLAC1+PP2CA+RCAR2.

(B) Exogenous ABA does not change SLAC1 activity regulated by PP2CA+RACR2 or by PP2CA+RCAR6. The relative value was calculated as summarized currents at –150 mV generated by various combinations/summarized currents at –150 mV generated by SLAC1. Mean current (±SE) at –150 mV recorded from the oocytes injected with various combinations of cRNA including SLAC1 alone, SLAC1+RCAR2, SLAC1+PP2CA, SLAC1+PP2CA+RCAR2, or SLAC1+PP2CA+RCAR6.

that the physical interaction between RCAR2 and PP2CA may be important to SLAC1 phosphorylation. On the contrary, PP2CA inhibition of OST1 activity against SLAC1 was very strong and PP2CA may use OST1 as a substrate for its phosphatase activity.

The question is whether PP2CA inactivates the kinase by dephosphorylating OST1, or by a simple physical interaction with the kinase without an enzyme–substrate relationship. Earlier studies seem to support both possibilities (Lee et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). Using a simple *in vitro* kinase assay, we determined whether PP2CA phosphatase activity is important in regulation of OST1 auto-kinase activity and activity against the SLAC1 N-terminus. Figure 3C shows that inclusion of PP2CA and its phosphatase-dead form, which is a PP2CA mutant protein with two amino acid mutations that eliminates PP2C activity, effectively abolished both auto-kinase activity (lanes 2 and 3) and activity towards the SLAC1 N-terminal domain (lanes 7 and 8). However, when we added PP2CA or its dead form after the kinase reaction, OST1 autophosphorylation was not altered by PP2CA (lanes 4 and 5), indicating that PP2CA does not dephosphorylate autophosphorylated OST1. In the case of SLAC1 phosphorylation by OST1, adding PP2CA after the kinase assay slightly reduced the level of SLAC1 phosphorylation (lane 9). Such a reduction seemed to be related to PP2CA phosphatase activity, because the phosphatase-dead form of PP2CA showed less of an effect on SLAC1 phosphorylation level (lane 10). Thus, PP2CA physically interacts with SLAC1 (Lee et al., 2009) and can dephosphorylate SLAC1 to a certain extent. Nevertheless, inhibition of OST1 activity by PP2CA does not seem to be associated with OST1 dephosphorylation in this *in vitro* assay system. These results suggest that PP2CA may interact directly and inhibit OST1 kinase activity. Taken together, our data suggest that the RCAR2 protein physically interacts with PP2CA, which may break the inhibitory bondage between PP2CA and OST1 leading to activation of OST1 kinase. The nature of 'inhibitory bondage' of PP2CA and OST1 may include both physical interaction and enzyme modification. Dynamic protein–protein interactions may determine the localization and function of signaling components in the ABA response.

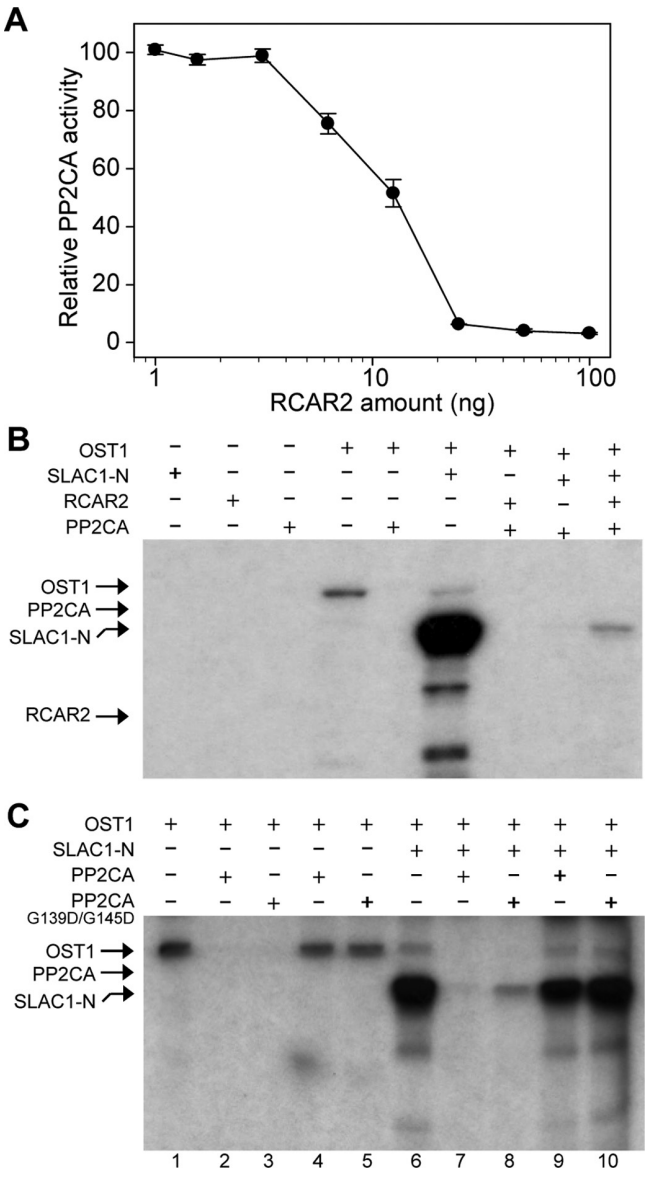

Figure 3. *In Vitro* Dephosphorylation and Phosphorylation Assay with OST1, SLAC1, PP2CA, and RCAR2.

(A) *In vitro* dephosphorylation assay of PP2CA with RCAR2. Increasing amounts of RCAR2 were added to a phosphatase reaction with 10 ng PP2CA. RCAR2 inhibits the protein phosphatase activity of PP2CA.
(B) RCAR2 affects SLAC1 phosphorylation via OST1 with PP2CA.
(C) PP2CA and PP2CA null mutant inhibits OST1 auto-kinase activity and activity against SLAC1. Following incubation for 30 min at 30°C, PP2CA or PP2CA null mutant was added and incubated for 30 min at 30°C in lane 4, 5, 9, or 10. The contents of the kinase assays are shown at the top of the autoradiography picture. The ^{32}P-labeled protein bands are indicated by arrows and names of the proteins at the left side. The presence and absence of the proteins are indicated by +/– labels, respectively.

Previous studies (Geiger et al., 2009; Lee et al., 2009) and results in this report have demonstrated the importance of physical interactions among the signaling components during ABA regulation of ion channel activity. Each component often interacts with multiple protein partners at the same time, and yet these components function in different plant cell compartments. How do these dynamic interactions among partner proteins change the subcellular location and potential function of various components? We tried to provide some clues for this question using multiple protein–protein interaction assays to dissect the relationships. First, we tested whether RCAR2 inhibits the OST1–PP2CA interaction in a multi-BiFC assay among RCAR2, PP2CA, and OST1. The interaction between RCAR2 and PP2CA or PP2CA and OST1 generated CFP and YFP signals in the nucleus, respectively (Figure 4A), indicating that RCAR2 may not have a major effect on the interaction between PP2CA and OST1.

In the case of PP2CA–OST1–SLAC1 interactions, it was interesting to observe both nucleus and plasma membrane localization of the fluorescence, depending on the partner (Figure 4B). While PP2CA and OST1 formed a complex in the nucleus, OST1 and SLAC1 interacted at the cell surface where SLAC1 is located. Both complexes formed simultaneously, indicating multiple subcellular locations for the OST1 protein. Along the same line, when OST1 and PP2CA each paired with SLAC1, both the phosphatase and the kinase were localized to the plasma membrane, forming a complex with the SLAC1 channel (Figure 4C). These results show that the direct interaction of PP2CA with OST1 localized in the nucleus but that the PP2CA–SLAC1–OST1 complex may be localized to the plasma membrane. It has become clear that multiple locations of individual signaling components can be achieved by interaction with different partner proteins and that such interactions can take place simultaneously in plant cells.

Overexpression of *RCAR2* Confers Transgenic Plants with ABA Hypersensitivity and Drought Tolerance

The presence of RCAR2 antagonized PP2CA inhibition of SnRK2 in the kinase and SLAC1 activation assays. To determine whether *RCAR2* overexpression *in planta* would alter ABA signaling and sensitivity, we generated transgenic plants overexpressing the coding sequence of the *RCAR2* gene under the control of a strong constitutive 35S promoter. *RCAR2* expression level was undetectable in wild-type plants under the conditions used in this study. The overexpressing lines showed significant transcripts levels (Figure 5A). The *RCAR2* transgenic lines and wild-type plants showed significantly different phenotypic growth (Figure 5B). The rosettes of the transgenic lines had slightly smaller stature under normal growth conditions without significant changes in flowering or seed production.

When we analyzed the ABA responses, the transgenic lines were ABA-hypersensitive during seed germination and early seedling growth (Figure 6). Germination rates of untreated seeds did not differ between wild-type and transgenic lines. On medium containing 0.5 μM ABA, 90% of the wild-type seeds germinated within 3 d, whereas the seeds from transgenic lines took 5–6 d to reach the same rate. Treatment

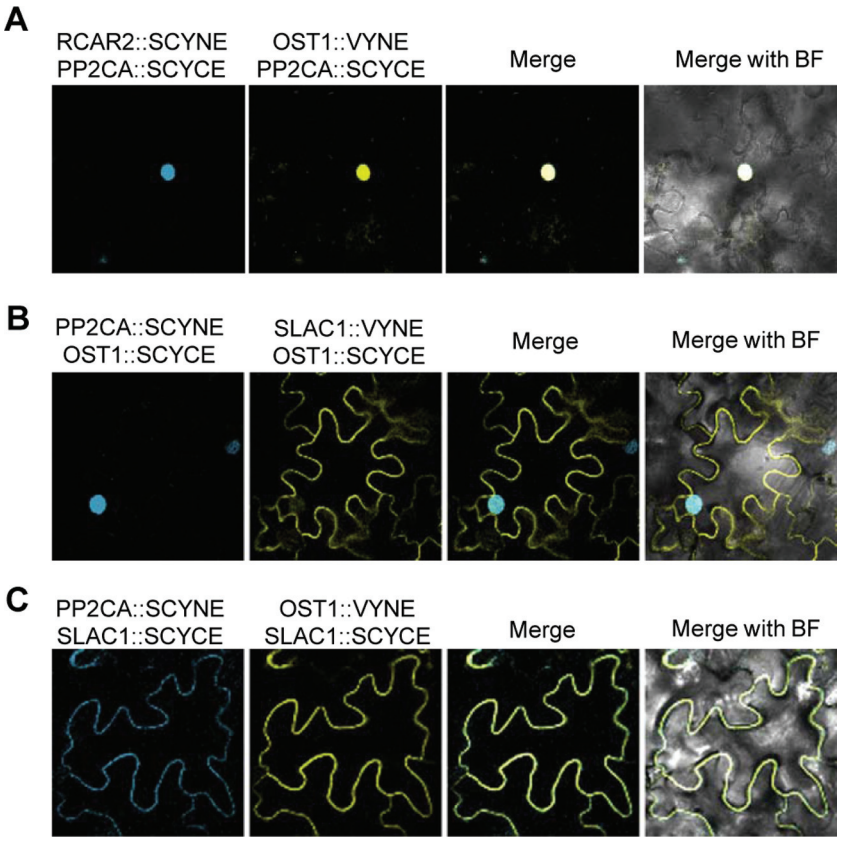

Figure 4. Multicolor BiFC Assay among RCAR2, PP2CA, OST1, and SLAC1.

(A) Simultaneous visualization of RCAR2/PP2CA (cyan) and OST1/PP2CA (yellow);
(B) PP2CA/OST1 (cyan) and SLAC1/OST1 (yellow);
(C) PP2CA/SLAC1 (cyan) and OST1/SLAC1 (yellow). The overlay of the cyan and yellow channels and the bright field image are depicted below the single-channel image.

with 1.0 μM ABA significantly inhibited seed germination in both wild-type and transgenic seeds. However, germination was again clearly more inhibited in the transgenic seeds (Figure 6A). In addition, the transgenic lines were also more sensitive to lower levels of ABA including 0.3 and 0.5 μM at the seed germination stage (Figure 6B). These results show that *RCAR2* overexpression increases ABA sensitivity in the germination stage.

We also examined the drought response of adult plants in the wild-type and transgenic lines (Figure 7). When grown under a well-watered condition, wild-type and transgenic plants did not show any significant phenotypic differences. However, after drought treatment by withholding watering, the transgenic lines exhibited enhanced drought tolerance compared to that in wild-type plants (Figure 7A). The transgenic plants became less wilted as compared to wild-type plants (Figure 7A), suggesting a decreased transpiration rate in transgenic plants. We determined the transpiration rate by measuring water loss from detached rosette leaves (Figure 7B). The fresh-weight loss of leaf tissues was greater in wild-type plants compared to that in the transgenic lines (Figure 7B).

To determine whether lower levels of transpiration rate resulted from ABA hypersensitivity in transgenic plants, we performed stomatal assay and found that the transgenic plants showed smaller stomatal apertures after plants were treated with ABA. Stomatal apertures in *RCAR2-1* and *RCAR2-2* were reduced to 66% and 58%, respectively, upon treatment with 10 μM ABA. In wild-type plants, the stomatal aperture was reduced to 77% (Figure 7C). These results indicate that *RCAR2* overexpression increases ABA sensitivity of

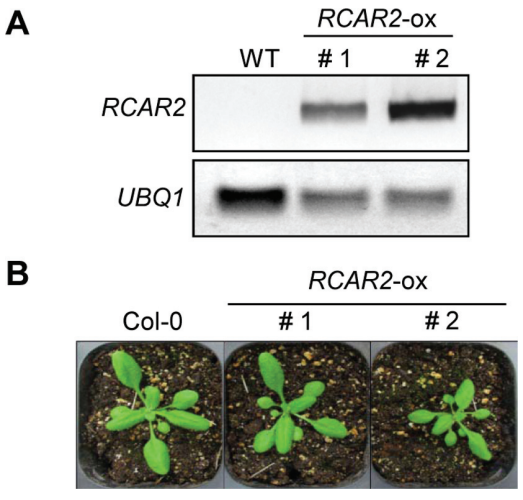

Figure 5. Construction of RCAR2-overexpressing Transgenic Plants.

(A) RT–PCR analysis of RCAR2 gene expression from wild-type (Col-0) and transgenic lines.

(B) Growth characteristics of the *Arabidopsis* transgenic plants overexpressing RCAR2. The plant phenotypes of 2-week-old wild-type (Col-0) and transgenic lines.

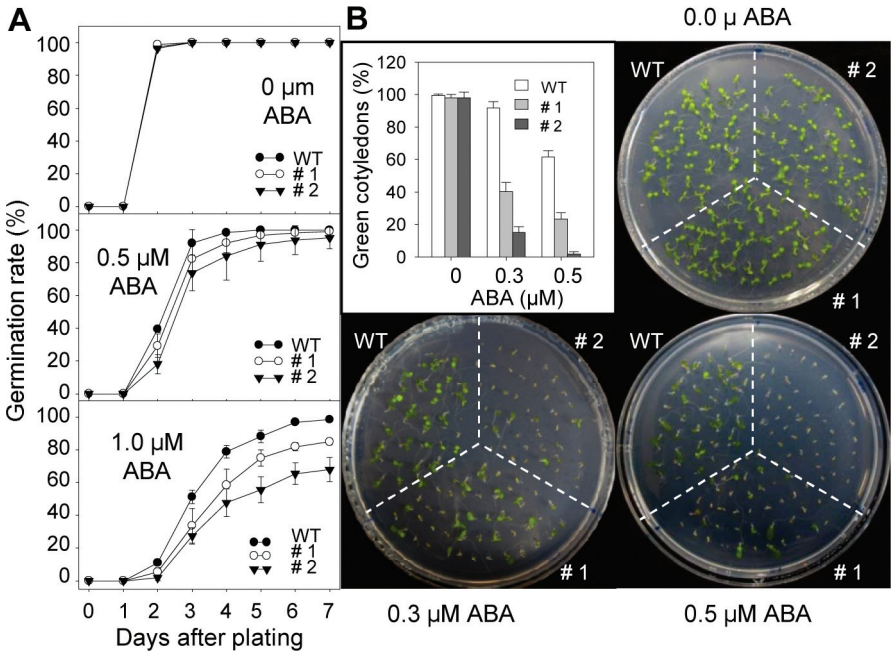

Figure 6. Enhanced Tolerance of the RCAR2-ox Transgenic Lines to Abscisic Acid (ABA).

(A) Seed germination of wild-type and transgenic lines exposed to ABA. Seeds were germinated on MS agar plates containing 0, 0.5, or 1.0 μM ABA. Data are the means ± standard deviation from three independent experiments each evaluating 100 seeds.

(B) Phenotype of wild-type and transgenic plants exposed to ABA. Photographs show plants after exposure to 0, 0.3, and 0.5 μM ABA for 7 d.

guard cells, leading to reduced water loss under drought conditions. The ABA responses in *RCAR2* transgenic plants were completely sensitive in seeds and vegetative tissues, indicating that RCAR2 may function as a positive regulator of ABA. Interestingly, these results are in agreement with the phenotypes of the triple *hab1-1 abi1-2 pp2ca-1* mutant (Rubio et al., 2009). These results again support the same principle: RCAR2 function serves to remove the brake to ABA signaling imposed by PP2CA. Taken together, these observations are consistent with earlier studies (Santiago et al., 2009b; Umezawa et al., 2009; Nishimura et al., 2010) and with the biochemical and electrophysiological work performed in this study: PYR/PYL/RCAR-type ABA receptors activate ABA responses by inhibiting PP2C A-type phosphatases, leading to activation of SnRK2-type kinases to phosphorylate downstream targets including SLAC1, which controls guard cell turgor and stomatal aperture.

Conclusions

Previous studies (Geiger et al., 2009; Lee et al., 2009) suggested that PP2C-type phosphatases lie upstream of the SnRK2-type kinase that, in turn, interact with and activate the SLAC1 channel to control the ABA response in guard cells. In this study, we added the ABA receptors to this regulation and reconstituted the complete pathway that leads ABA signal to ion channel regulation and stomatal closure as proposed in Figure 8. Under normal conditions, PP2CA inhibits SLAC1 channel activity by inactivation of OST1. When ABA levels are increased by environmental cues and PYL/RCAR levels are also increased, more ABA may bind to PYL/RCAR, inducing the formation of additional PYL/RCAR–PP2C complex and thus breaking the PP2C–OST1 complex to release active OST1 kinase. The OST1 kinase interacts with and activates the SLAC1 ion channel to mediate efflux of anions and decrease of turgor pressure.

Several questions remain unresolved in this model. First, it is still not clear how ABA triggers this signaling pathway. In studies to date, PYL/RCAR proteins directly associate with PP2Cs without ABA. It will be important to determine the condition and concentration dependence of ABA that modulates interaction of PYL/RCAR–PP2C. Second, the mechanism of OST1 inactivation by PP2Cs remains undetermined. Several reports have suggested that phosphatase activity is required for inactivation of SnRK2-type kinases (Umezawa et al., 2009; Vlad et al., 2009). Results from our studies suggest that both phosphatase activity and a physical interaction are important for PP2CA function in ABA signaling. Phosphatase activity is required for dephosphorylating and inactivating the SLAC1 ion channel (Figures 2 and 3). On the other hand, physical interactions may play a more important role in the inhibition of OST1 kinase activity. However, it is difficult to explain how physical interaction alone could effectively inactivate the kinase.

Considering the diversity of the PYL/RCAR family, which consists of 14 members and at least two subfamilies, functional redundancy and specificity are complex. Furthermore, group A PP2Cs and SnRK2-type kinases are also composed of multiple members. How these different phosphatases and kinases combine with PYL/RCAR proteins in a particular cell type or at a certain developmental stage of plants will provide a challenging area of research for years to come.

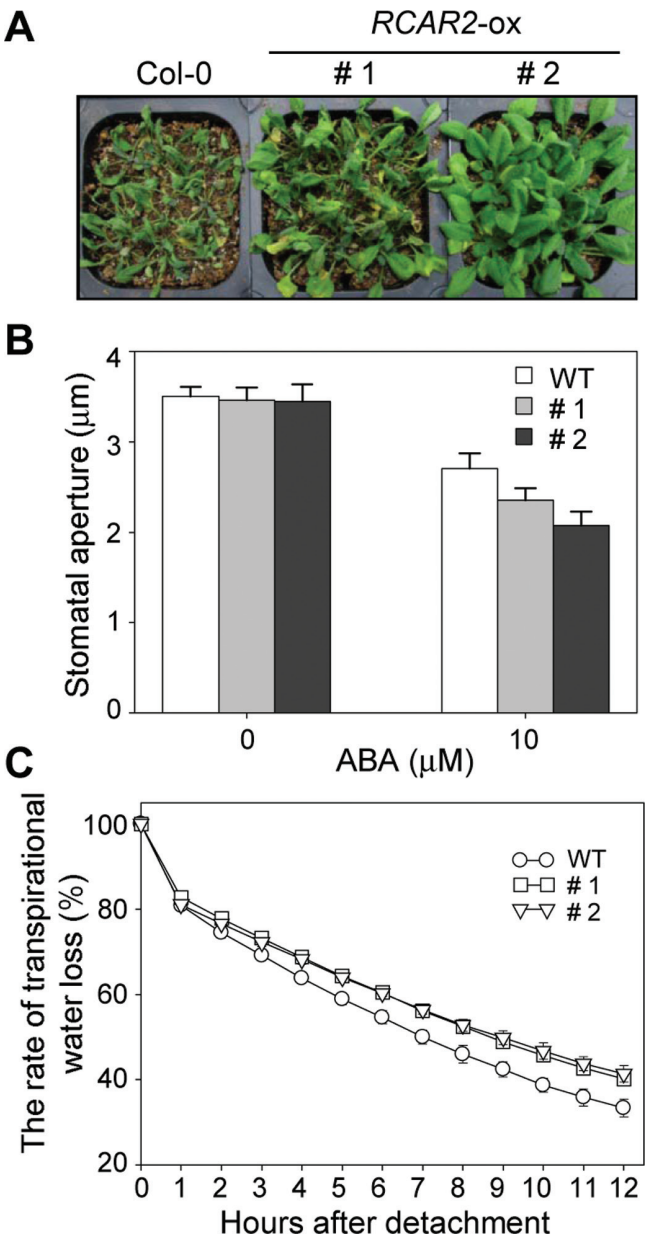

Figure 7. Enhanced Tolerance of the RCAR2-ox Transgenic Lines to Drought Stress.

(A) Growth of wild-type and transgenic plants after dehydration for 14 d.

(B) Stomatal movements in RCAR2-ox transgenic lines are hypersensitive to ABA. Stomatal apertures were measured under the microscope in wild-type and RCAR2-ox transgenic lines. Data are the means ± standard errors.

(C) Water loss from leaves of wild-type and transgenic plants at various times after detachment of leaves.

METHODS

Yeast Two-Hybrid Analysis

Each construct was built by cDNA fragments amplified by PCR and cloned into the pGBKT7 and pGADGH vectors. The lithium acetate method was used to introduce BD and AD plasmids into yeast strain AH109 (Ito et al., 1983). Yeast two-hybrid assays were performed as before (Li et al., 2006; Lee et al., 2007). Transformants were selected in SC-Leucine-Tryptophan media and transferred on the interaction selection media (SC-Adenine-Histidine-Leucine-Tryptophan) to score growth as an indicator of protein–protein interaction. For serial dilution assay, exponentially grown yeast cells were harvested and adjusted to OD_{600}=0.5 with sterilized double-distilled water and diluted to 1/10, 1/100, and 1/1000. Yeast cells, 2 μl, were spotted onto SC-Leucine-Tryptophan media and SC-Adenine-Histidine-Leucine-Tryptophan media with or without 10 μM ABA.

Bimolecular Fluorescence Complementation (BiFC) Assay

To generate the BiFC constructs, RCAR2, SLAC1, OST1, and PP2CA full-length cDNA with no stop codon were subcloned via *Spe*I/*Sal*I into 35S-SPYNE(R)173, 35S-SCYNE(R)173, 35S-VYNE, 35S-SPYCE(M), and 35S-SCYCE(M) vectors (Waadt et al., 2008). For transient expression, the *Agrobacterium tumefaciens* strain GV3101 carrying each construct was used together with the p19 strain for infiltration of 5-week-old *Nicotiana benthamiana* leaves. For microscopic analyses, leaf discs were cut 4 d after infiltration. The lower epidermis cells were analyzed by confocal microscopy (model Zeiss 510 UV/Vis Meta) operated with LSM Image Browser software.

Expression and Purification of GST-Fusion Proteins in *Escherichia coli* and Kinase Assay

To produce GST fusion proteins in *E. coli*, RCAR2, OST1, PP2CA, and the N-terminus of SLAC1 were cloned into pGEX4T-1 vector. All GST fusion constructs were transformed into *E. coli* strain BL21 (DE3) cells. Protein expression and purification of GST fusion protein were performed as described earlier (Li et al., 2006).

For the kinase assays, the buffer contained 20 mM Tris HCl (pH 7.5), 2.5 mM $MnCl_2$, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM DTT. Total volume of 40 μl included 7.5 μCi of [γ-^{32}P]ATP and the protein combinations (1 μg of the each protein) indicated in the figure legends. Following incubation for 60 min at 0°C, the reaction was stopped by adding 12.5 μl of 5X Laemmli buffer; 20 μl of the mixture was then separated by SDS/PAGE using a 10% (w/v) acrylamide gel. The gel was dried and ^{32}P was detected by autoradiography using a Typhoon 8600 imager (Molecular Dynamics, Piscataway, NJ).

Protein phosphatase activities of PP2CA were performed by using ProFluor Ser/Thr PPase assay kit (Promega, WI, USA) according to the manufacturer's protocol as described earlier (Santiago et al., 2009b). Briefly, phosphatase assays were performed in a 100-μl reaction volume containing 25 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 10 ng PP2CA protein.

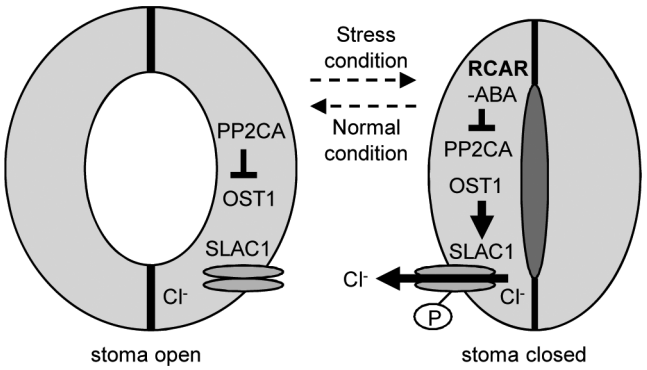

Figure 8. A Working Model for an ABA Signaling Pathway in the Regulation of Stomatal Movements.In the absence of ABA, PP2CA inhibit OST1 leading to stomata opening. Perception of ABA by RCAR leads to the inhibition of PP2CA and OST1 phosphorylates SLAC1 channel inducing stomata closure. –P stands for phosphorylation. This figure is modified from Lee et al. (2009).

Electrophysiological Procedure

The genes used in the electrophysiological assay were cloned into the pGEMHE oocyte expression vector. The preparation and determination of cRNA concentration prepared by the mMESSAGE mMACHINE T_7 RNA transcription kit were performed as previously described (Li et al., 2006; Lee et al., 2007). Freshly isolated *Xenopus* oocytes were injected with 23 nl of cRNA and used for voltage-clamp experiments 2 d after injection. Two-electrode voltage-clamp recordings were performed to measure SLAC1 currents. The pipette solution contained 3 M KCl. To test SLAC1 currents, we first used a modified ND96 solution in which NaCl was replaced by CsCl as the bath solution (containing 96 mM CsCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2 mM KCl, 5 mM HEPES, pH 7.5 adjusted with NaOH). The current was recorded by the voltage steps of 50 to –150 mV (in 15-mV decrements, 7.5-s duration) with a 1.45-s prepulse to 0 mV. The summarized data of the SLAC1 currents were generated from the pooled currents at 1.6 s of each voltage-clamp episode. Data are presented as representative recordings or as mean ± SE of *n* observations with three repetitions, in which *n* is the number of samples. Statistical comparisons were made using either Student's paired or unpaired *t*-tests as appropriate, and differences were considered to be significant at $p < 0.05$.

Plant Expression Vector Construction and *Arabidopsis* Transformation

To induce constitutive expression of the *RCAR2* gene under the control of the CaMV 35S promoter, the binary vector pBIN35S was used to generate a plasmid for *Arabidopsis* transformation. The full-length *RCAR2* cDNA sequence cloned into pBIN35S. The recombinant plasmids were verified by sequencing. The binary plasmids were introduced into the *Agrobacterium tumefaciens* strain GV3101 via electroporation. *Arabidopsis* transformation with the *RCAR2* gene was carried out using the floral dipping method (Clough and Bent, 1998). For selection of *RCAR2*-ox transgenic lines, seeds harvested from the putative transformed plants were plated on MS agar plates containing 50 μg ml^{-1} kanamycin.

Assays of Drought Tolerance

Three-week-old seedlings from the wild-type and *RCAR2*-ox transgenic lines were randomly planted in a tray containing soil mix (peat moss, perlite, and vermiculite, 9:1:1). Dehydration stress was imposed plants by withholding watering. To determine the drought tolerance in a quantitative manner, leaves were detached from each plant and placed in Petri dishes. The dishes were kept in a growth chamber with 40% relative humidity, and the loss of fresh weight was determined at the indicated times.

For stomatal aperture bioassays, four rosette leaves from 4-week-old plants were detached and floated in stomatal opening solution (SOS: 50 mM KCl and 10 mM MES-KOH, pH 6.15, 10 mM $CaCl_2$) in the light as described by Cheong et al. (2007). After 2.5 h, buffer was replaced with SOS containing ABA of various concentrations. After 2.5-h further incubation, 60 stomata were measured in each individual sample, and each experiment was performed in triplicate.

FUNDING

This work is supported by a grant from the Biogreen21 Program (PJ008222) Rural Development Administration and the Research Foundation of Korea (NFR) grant funded by the Korea government (No. 2011–0007600) (to S.C.L.), and the US National Science Foundation and Korean WCU Program of National Research Foundation (to S.L.). No conflict of interest declared.

REFERENCES

Ache, P., Becker, D., Ivashikina, N., Dietrich, P., Roelfsema, M.R., and Hedrich, R. (2000). GORK, a delayed outward rectifier expressed in guard cells of *Arabidopsis thaliana*, is a K^+-selective, K^+-sensing ion channel. FEBS Lett. **486**, 93–98.

Belin, C., de Franco, P.O., Bourbousse, C., Chaignepain, S., Schmitter, J.M., Vavasseur, A., Giraudat, J., Barbier-Brygoo, H., and Thomine, S. (2006). Identification of features regulating OST1 kinase activity and OST1 function in guard cells. Plant Physiol. **141**, 1316–1327.

Chae, M.J., Lee, J.S., Nam, M.H., Cho, K., Hong, J.Y., Yi, S.A., Suh, S.C., and Yoon, I.S. (2007). A rice dehydration-inducible SNF1-related protein kinase 2 phosphorylates an abscisic acid responsive element-binding factor and associates with ABA signaling. Plant Mol. Biol. **63**, 151–169.

Cheong, Y.H., Pandey, G.K., Grant, J.J., Batistic, O., Li, L., Kim, B.G., Lee, S.C., Kudla, J., and Luan, S. (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in *Arabidopsis*. Plant J. **52**, 223–239.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16**, 735–743.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010). Abscisic acid: emergence of a core signaling network. Annu. Rev. Plant Biol. **61**, 651–679.

Fujii, H., and Zhu, J.K. (2009). *Arabidopsis* mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proc. Natl Acad. Sci. U S A. **106**, 8380–8385.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y., Cutler, S.R., Sheen, J., Rodriguez, P.L., and Zhu, J.K. (2009). *In vitro* reconstitution of an abscisic acid signalling pathway. Nature. **462**, 660–664.

Fujii, H., Verslues, P.E., and Zhu, J.K. (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. Plant Cell. **19**, 485–494.

Fujita, Y., et al. (2009). Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in *Arabidopsis*. Plant Cell Physiol. **50**, 2123–2132.

Geiger, D., et al. (2009). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. Proc. Natl Acad. Sci. U S A. **106**, 21425–21430.

Hirai, N., Yoshida, R., Todoroki, Y., and Ohigashi, H. (2000). Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. Biosci. Biotechnol. Biochem. **64**, 1448–1458.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**, 163–168.

Kuhn, J.M., Boisson-Dernier, A., Dizon, M.B., Maktabi, M.H., and Schroeder, J.I. (2006). The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in *Arabidopsis*, and effects of abh1 on AtPP2CA mRNA. Plant Physiol. **140**, 127–139.

Lee, S.C., Lan, W., Buchanan, B.B., and Luan, S. (2009). A protein kinase–phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. Proc. Natl Acad. Sci. U S A. **106**, 21419–21424.

Lee, S.C., Lan, W.Z., Kim, B.G., Li, L., Cheong, Y.H., Pandey, G.K., Lu, G., Buchanan, B.B., and Luan, S. (2007). A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc. Natl Acad. Sci. U S A. **104**, 15959–15964.

Lemtiri-Chlieh, F., and MacRobbie, E.A. (1994). Role of calcium in the modulation of Vicia guard cell potassium channels by abscisic acid: a patch-clamp study. J. Membr. Biol. **137**, 99–107.

Li, J., Wang, X.Q., Watson, M.B., and Assmann, S.M. (2000). Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science. **287**, 300–303.

Li, L., Kim, B.G., Cheong, Y.H., Pandey, G.K., and Luan, S. (2006). A Ca^{2+} signaling pathway regulates a K^+ channel for low-K response in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **103**, 12625–12630.

Luan, S. (2002). Signalling drought in guard cells. Plant Cell Environ. **25**, 229–237.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science. **324**, 1064–1068.

McCourt, P., and Creelman, R. (2008). The ABA receptors—we report you decide. Curr. Opin. Plant Biol. **11**, 474–478.

Melcher, K., et al. (2009). A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. Nature. **462**, 602–608.

Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., and Giraudat, J. (2001). The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J. **25**, 295–303.

Miyazono, K., et al. (2009). Structural basis of abscisic acid signalling. Nature. **462**, 609–614.

Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J. (2002). *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell. **14**, 3089–3099.

Nakashima, K., et al. (2009). Three *Arabidopsis* SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant Cell Physiol. **50**, 1345–1363.

Nambara, E., and Marion-Poll, A. (2005). Abscisic acid biosynthesis and catabolism. Annu. Rev. Plant Biol. **56**, 165–185.

Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M., and Iba, K. (2008). CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature. **452**, 483–486.

Nishimura, N., et al. (2010). PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in *Arabidopsis*. Plant J. **61**, 290–299.

Nishimura, N., Yoshida, T., Murayama, M., Asami, T., Shinozaki, K., and Hirayama, T. (2004). Isolation and characterization of novel mutants affecting the abscisic acid sensitivity of *Arabidopsis* germination and seedling growth. Plant Cell Physiol. **45**, 1485–1499.

Park, S.Y., et al. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science. **324**, 1068–1071.

Rubio, S., Rodrigues, A., Saez, A., Dizon, M.B., Galle, A., Kim, T.H., Santiago, J., Flexas, J., Schroeder, J.I., and Rodriguez, P.L. (2009). Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. Plant Physiol. **150**, 1345–1355.

Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M.P., Nicolas, C., Lorenzo, O., and Rodriguez, P.L. (2004). Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. Plant J. **37**, 354–369.

Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S.Y., Jamin, M., Cutler, S.R., Rodriguez, P.L., and Marquez, J.A. (2009a). The abscisic acid receptor PYR1 in complex with abscisic acid. Nature. **462**, 665–668.

Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S.Y., Marquez, J.A., Cutler, S.R., and Rodriguez, P.L. (2009b). Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. Plant J. **60**, 575–588.

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

Schroeder, J.I., Kwak, J.M., and Allen, G.J. (2001). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature. **410**, 327–330.

Schroeder, J.I., Raschke, K., and Neher, E. (1987). Voltage dependence of K channels in guard-cell protoplasts. Proc. Natl Acad. Sci. U S A. **84**, 4108–4112.

Szostkiewicz, I., Richter, K., Kepka, M., Demmel, S., Ma, Y., Korte, A., Assaad, F.F., Christmann, A., and Grill, E. (2010). Closely related receptor complexes differ in their ABA selectivity and sensitivity. Plant J. **61**, 25–35.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009). Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **106**, 17588–17593.

Vahisalu, T., et al. (2008). SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature. **452**, 487–491.

Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P.L., Lauriere, C., and Merlot, S. (2009). Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in *Arabidopsis*. Plant Cell. **21**, 3170–3184.

Waadt, R., Schmidt, L.K., Lohse, M., Hashimoto, K., Bock, R., and Kudla, J. (2008). Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes *in planta*. Plant J. **56**, 505–516.

Ward, J.M., Maser, P., and Schroeder, J.I. (2008). Plant ion channels: gene families, physiology, and functional genomics analysis. Annu. Rev. Physiol. **71**, 59–82.

Wasilewska, A., Vlad, F., Sirichandra, C., Redko, Y., Jammes, F., Valon, C., Frey, N.F.d., and Leung, J. (2008). An update on abscisic acid signaling in plants and more. Mol. Plant. **1**, 198–217.

Yin, P., Fan, H., Hao, Q., Yuan, X., Wu, D., Pang, Y., Yan, C., Li, W., Wang, J., and Yan, N. (2009). Structural insights into the mechanism of abscisic acid signaling by PYL proteins. Nat. Struct. Mol. Biol. **16**, 1230–1236.

Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K. (2006a). The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in *Arabidopsis*. J. Biol. Chem. **281**, 5310–5318.

Yoshida, T., Nishimura, N., Kitahata, N., Kuromori, T., Ito, T., Asami, T., Shinozaki, K., and Hirayama, T. (2006b). ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among *Arabidopsis* protein phosphatase 2Cs. Plant Physiol. **140**, 115–126.

Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. **53**, 247–273.