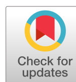

A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells

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Contributed by Bob B. Buchanan, September 16, 2009 (sent for review May 18, 2009)

The plant hormone abscisic acid (ABA) serves as a physiological monitor to assess the water status of plants and, under drought conditions, induces stomatal pore closure by activating specific ion channels, such as a slow-anion channel (SLAC1) that, in turn, mediate ion efflux from the guard cells. Earlier genetic analyses uncovered a protein kinase (OST1) and several 2C-type phosphatases, as respective positive and negative regulators of ABA-induced stomatal closure. Here we show that the OST1 kinase interacts with the SLAC1 anion channel, leading to its activation via phosphorylation. PP2CA, one of the PP2C phosphatase family members acts in an opposing manner and inhibits the activity of SLAC1 by two mechanisms: (1) direct interaction with SLAC1 itself, and (2) physical interaction with OSTI leading to inhibition of the kinase independently of phosphatase activity. The results suggest that ABA signaling is mediated by a physical interaction chain consisting of several components, including a PP2C member, SnRK2-type kinase (OST1), and an ion channel, SLAC1, to regulate stomatal movements. The findings are in keeping with a paradigm in which a protein kinase-phosphatase pair interacts physically with a target protein to couple a signal with a specific response.

anion transport | protein dephosphorylation | protein phosphorylation | signal transduction

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Abscisic acid (ABA) has been found to act as a signal in modulating the activity of both K^+ and anion channels of the plasma membrane of guard cells (1–3). Based on inhibitor studies, protein kinases and phosphatases constitute an interface between ABA and the ion channel (4–8). Several protein kinases and phosphatases that regulate ABA signaling have been identified in Arabidopsis with genetic approaches. For instance, ABI1 and ABI2 are closely related protein phosphatase 2C (PP2C) members that negatively regulate ABA response (9–11). In a later study, two other PP2C members related to ABI1 and ABI2 were identified in seed germination mutants showing a hypersensitive response to ABA (12). In another line of investigation, reverse genetic procedures have led to the identification of additional PP2C members including PP2CA, HAB1, and HAB2 that play a role in ABA signaling (13–15). Each of these ABA-signaling members belongs to the "A type" PP2C phosphatase family (16).

An opposing participant, notably an ABA-activated protein kinase (AAPK), was uncovered early on with *Vicia faba* (17). In subsequent work, a mutant of the AAPK homologue was identified in Arabidopsis as Open Stomata mutant *ost1* (18). The *ost1* mutant is ABA-insensitive and keeps its stomata open, even under drought conditions. OST1 is a member of the Arabidopsis SnRK2-type protein kinase family that includes other members functional in the ABA response (19–22). Each of these SnRK2 kinases is involved in the ABA response and is activated upon ABA treatment.

Collectively, previous work has demonstrated that SnRK2-type kinases and PP2C-type phosphatases play a major role in ABA signaling, leading to stomatal closure and inhibition of seed germination. Moreover, several studies based on channel analysis have connected the ABA-linked kinases and phosphatases to ion channel regulation in guard cells. For example, ABI1 and ABI2 were reported to regulate anion channel activity in guard cells with specific mutants (*abi1* and *abi2*) that displayed defective patterns of channel activation following ABA treatment (23). The OST1 homologue in *Vicia faba*, AAPK, is also a critical modulator of anion channel activity in guard cells (24).

Despite this progress, anion channels of guard cells had not been characterized at the molecular level until quite recently. In the past year, two groups have identified the same gene encoding an anion transporter functional in stomatal response to high CO_2 in Arabidopsis (25, 26). The gene, named *SLAC1* for *SLOW ANION CHANNEL 1*, is required for stomatal closure induced by either high CO_2 or ABA. Further, a loss of function mutation in the *SLAC1* gene eliminated ABA-induced anion channel activity in guard cell protoplasts (25). In highlighting the SLAC1 protein as a critical component of guard cell anion channels, these results raise interesting questions. Is SLAC1 an anion channel? Does it serve as a target for ABA signaling components such as the protein kinases and phosphatases described above? Do the kinases and phosphatases modify SLAC1 channel activity directly or indirectly? Finally, what is the relationship between the kinases and the phosphatases?

In the present study, we have addressed these questions using the *Xenopus* oocyte expression system to assess the potential ion channel activity of SLAC1 and its regulation by the kinases and phoshatases in the ABA signaling pathway. The interaction among the channel, kinase and phosphatase proteins was also examined in yeast and plant cells. Our data provide evidence that the OST1 kinase interacts directly with the SLAC1 channel and enhances its activity by phosphorylation. The phosphatase, PP2CA, on the other hand, can inactivate the channel by inhibiting OST1 kinase activity. These findings raise the possibility that phosphatase-kinase complexes provide a general mechanism for control of signaling pathways in plants.

Results and Discussion

PP2CA and OST1 Physically Interact with SLAC1. In an earlier study on an inward K^+-channel (27), we showed that AKT1 (for Arabidopsis K^+ transporter 1), is regulated by a network of CIPKs and a PP2C member, AIP1. We demonstrated that the PP2C protein phosphatase physically interacts with a protein kinase and the channel, providing a mechanism for specifying the interaction of particular kinases and phosphatases in the regulation of channel activity. Recognizing that this general protein interaction-based system may be applicable to other ion

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Author contributions: S.C.L., W.L., and S.L. designed research; S.C.L. and W.L. performed research; S.C.L., W.L., B.B.B., and S.L. analyzed data; and S.C.L., W.L., B.B.B., and S.L. wrote the paper.

The authors declare no conflict of interest.

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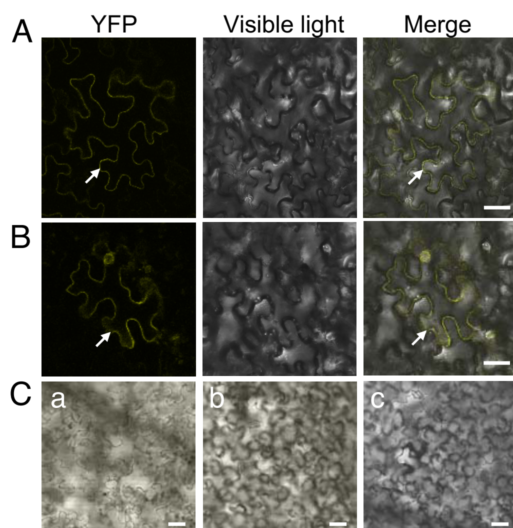

Fig. 1. Physical interaction of SLAC1 with OST1 (*A*) and PP2CA (B) as analyzed by BiFC in *Nicotiana benthamiana*. The fluorescence indicates interaction between the indicated partner proteins. SLAC1–35S-SPYCE(M) construct was coexpressed with OST1–35S-SPYNE(R)173 (A) or with PP2CA-35S-SPYNE(R)173 (*B*). The images were obtained from the YFP channel or bright field or a merged picture of the two. (*C*) Negative controls depicting the overlay of the YFP channel and bright field images. (*a*) SLAC1–35S-SPYCE(M) coexpressed with 35S-SPYNE(R)173; (*b*) OST1–35S-SPYNE(R)173 with 35S-SPYCE(M); (*c*) PP2CA-35S-SPYNE(R)173 with 35S-SPYCE(M). The arrows point to the fluorescence displayed by the three interacting partner proteins. (Scale bar, 50 μm.)

channels, we searched for a broader physical interaction network among ion channels, protein kinases, and phosphatases. For several reasons we focused on the SnRK-type protein kinases, PP2C family, the K^+-channels, and the more recently identified SLAC family.

First, the CIPK family kinases, earlier shown to regulate AKT1, by physical interaction, are categorized into a subgroup in the SnRK family (28). Secondly, OST1, known to be important for ion channel regulation and ABA signaling in guard cells, belongs to another SnRK subfamily (28). Thirdly, the AIP1 phosphatase that regulates AKT1 is a PP2C family member and, moreover, several PP2C members are involved in ion channel regulation in guard cells (5, 23). We expected that, by identifying a complete interaction network of members among the SnRKs, PP2Cs, and channels, we could possibly understand a general molecular mechanism for the control of guard cell turgor and ABA-induced stomatal closure.

Using yeast two hybrid screening, we have identified a number of interaction partners among the SnRK kinases, PP2C phosphatases, and ion channels. We focus here on the SnRK2 member OST1, PP2C member PP2CA, and SLAC1, a possible anion channel-all these proteins were found to play a role in ABA signaling in earlier genetic studies. We confirmed protein–protein interactions identified in the yeast two hybrid assays using the bimolecular fluorescence complementation (BiFC) procedure in plant cells. Fig. 1 shows that SLAC1 interacts with OST1 and PP2CA in the epidermal cells of *Nicotiana benthamiana*. The interaction between SLAC1 and OST1 (Fig. 1*A*) or between SLAC1 and PP2CA (Fig. 1*B*) generates the yellow fluorescence at the plasma membrane that is identified by arrows. The respective negative controls (target proteins fused to half of YFP coexpressed with the other half of the YFP) did not yield detectable YFP signals (Fig. 1*C*).

OST1 Activates, PP2CA Inhibits SLAC1 Activity. Using physical interactions as a guide, we sought to determine the functional relationships among OST1, PP2CA, and SLAC1 by coexpressing different combinations of the proteins in the *Xenopus* oocyte system. We first expressed the SLAC1 protein alone to examine its possible activity as an anion channel. Although earlier studies (25, 26) had demonstrated that SLAC1 functions as an essential part of the guard cell anion channel, SLAC1 expression in the *Xenopus* oocytes did not appear to produce anion channel current in those studies. It is possible that SLAC1 is only one subunit of a multisubunit channel, requiring other subunits to constitute a functional channel. Alternatively, SLAC1 is a channel *per se* but requires a regulator to be activated, as shown earlier for another ion channel AKT1 (27, 29, 30). It is also possible that SLAC1 channel activity was not sufficiently large under the conditions used in earlier studies. In particular, previous studies [e.g., Kowdley et al. (31)] showed that *Xenopus* oocytes produce endogenous voltage-dependent Cl^- currents that may interfere with the characterization of the SLAC1 channel. Indeed, we observed such a current in both control and SLAC1-injected oocytes when we used 100 mM CsCl in the regular bath solution, making it difficult to distinguish endogenous and SLAC1-generated currents. We subsequently used a revised bath solution containing mannitol with low pH–conditions shown to suppress this endogenous Cl^- current (32) and facilitate measurement of anion channel activity in guard cells (25, 33). In this way, we observed a significantly reduced endogenous current in the control oocytes (Fig. 2*A*). Under these conditions, the endogenous calcium-activated chloride channel was not active (34).

In the SLAC1-injected oocytes, we recorded a new, time-independent instantaneous Cl^- current, clearly different from the endogenous current (Fig. 2*A*). However such a small current made it difficult to characterize further. Since guard cell anion channels have high permeability to NO_3^- (33), we expected that SLAC1-associated current would be larger if NO_3^- was used as a carrier. As in earlier studies (35), outward anion channels expressed in the oocytes are highly sensitive to different anions in the bath solution. Indeed, we recorded a much larger current in SLAC1-injected oocytes when the bath solution contained 30 mM $CsNO_3$ (Fig. 2 *A* and *B*). The reversal potential of currents generated by SLAC1 expressed in the oocytes perfused with 30 mM $CsNO_3$ or CsCl was -47 ± 4 or -5 ± 2 mV, respectively (Fig. 2*B*). The permeability ratio of NO_3^- to Cl^- is 5.4, which is similar to the values observed earlier for anion channels in guard cells (36). SLAC1 current was also sensitive to extracellular CsBr and Cs_2-malate with a reversal potential of -32 ± 2.1 and 16 ± 1.5 mV, respectively. SLAC1 thus displayed the similar selectivity sequence of $NO_3^- > Br^- > Cl^- > malate^{2-}$ as shown for anion channels in guard cells (33, 36). We also injected various amounts of SLAC1 cRNA and recorded a dose-dependent current with both Cl^- and NO_3^- (Fig. 2*C*). In addition, we applied several typical anion channel blockers to the bath solution and observed inhibition of the SLAC1 currents (Fig. 2 *A* and *D*). These results confirmed that SLAC1 can constitute an anion channel with properties similar to the slow anion channel in guard cells, in keeping with results from an earlier study showing no slow anion current in the guard cells of *slac1* mutant plants (25).

The SLAC1 channel currents were enhanced significantly when SLAC1 was coexpressed with the OST1 kinase (Fig. 3*A*, upper right). By contrast, when coexpressed with the PP2CA phosphatase, SLAC1 channel activity was inhibited (Fig. 3*A*, lower left). OST1 or PP2CA expressed alone did not significantly increase oocyte endogenous channel activity (Fig. 3*C*). When the three components (OST1, PP2CA, and SLAC1) were coexpressed, the current was smaller than that recorded with oocytes expressing the ion channel alone (Fig. 3*A*, lower right), suggesting that PP2CA more than fully counteracted the stimulatory effect of OST1. Based on these results, the mechanism control-

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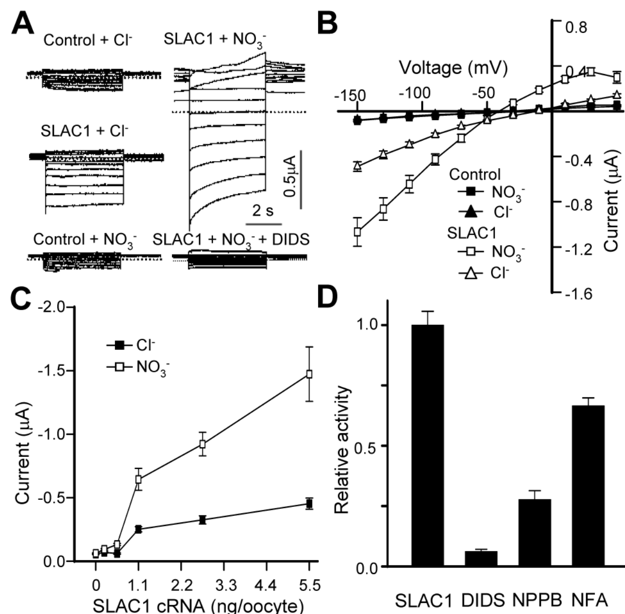

Fig. 2. Currents generated by SLAC1 expressed in the *Xenopus* oocytes. (*A*) Typical whole-cell current traces were recorded from the oocytes injected with water perfused with 30 mM CsCl (Control+Cl^-) or 30 mM $CsNO_3$ (Control+NO_3^-), or from the oocytes injected with 23 nL of 125 μg/mL SLAC1 cRNA perfused with 30 mM CsCl (SLAC1+Cl), 30 mM $CsNO_3$ (SLAC1+ NO_3^-), or 30 mM $CsNO_3$ and 0.1 mM DIDS (SLAC1+ NO_3^-+DIDS). The membrane potential was stepped from a 1.45 s holding potential of 0 mV to a potential of 50 to −150 mV (in 15mV decrements, 7.5 s duration) with a 4.05 s at 0 mV. Dotted lines represent zero current level. (*B*) The current-voltage relationship is shown for the control oocytes injected with water or oocytes injected with 23 nL of 125 μg/mL SLAC1 cRNA, perfused with 30 mM $CsNO_3$, or CsCl. (*C*) The relationship between SLAC1 activity and the amount of SLAC1 cRNA injected into the oocytes, perfused with 30 mM CsCl, or $CsNO_3$. The current value was taken at 1.6 s of each voltage-clamp episode at −150 mV. (*D*). Effects of anion channel blockers on the currents generated by SLAC1 at −150 mV. The relative activity was calculated as the current generated by SLAC1 in the oocytes perfused with blockers/the current generated by SLAC1 in the oocytes perfused without blockers. Niflumic acid (NFA), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), or 5-nitro-2-(3-phenylpropylalanine) benzoate (NPPB) was added to bath solution at 0.1 mM. All perfusion buffers contain the carrier salts (30 mM CsCl or Cs NO_3) plus 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Mes/Tris, pH 5.6, 220 mosmol/kg adjusted with *D*-mannitol.

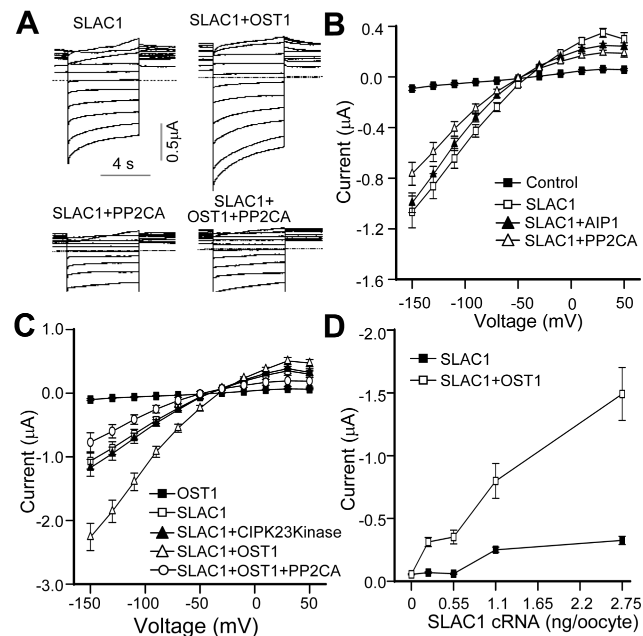

Fig. 3. PP2CA and OST1 regulate SLAC1 channel activity in the *Xenopus* oocytes. (*A*) Typical whole-cell current traces were recorded from the oocytes injected with cRNA of SLAC1, SLAC1+PP2CA, SLCAC1+OST1, or SLAC1+OST1+PP2CA. (*B*) The current-voltage relationship is shown for control, SLAC1, SLAC1+AIP1 (an A-type PP2C inhibiting *Shaker*-type K^+ channel AKT1), or SLAC1+PP2CA. (*C*) The current-voltage relationship is shown for OST1, SLAC1, SLAC1+CIPK23kinase (a CIPK23 truncated form with kinase domain only), SLAC1+OST1, or SLAC1+OST1+PP2CA. The perfusion solution consisted of 30 mM $CsNO_3$ in the buffer system described in Fig. 2. (*D*) OST1 enhances the SLAC1 current in the oocytes injected with different amount of cRNA, perfused with 30 mM CsCl in the buffer system as in Fig. 2.

ling the phosphorylation status of the SLAC1 channel protein appears to be more complex than a simple kinase-phosphatase balance. Coexpression with CIPK23 or AIP1, the kinase-phosphatase pair that regulates AKT1 activity reported earlier (27), had no effect on the activity of SLAC1 (Fig. 3 *B* and *C*). An experiment in which we injected increasing amounts of SLAC1 cRNA with and without OST1 revealed consistent activation of SLAC1, indicating that SLAC1 activation by OST1 is a property of SLAC1 and not an artifact due to overexpression of SLAC1 itself (Fig. 3*D*). The finding that SLAC1 channel activity was strongly dependent on OST1 coexpression may explain why earlier studies (25, 26) failed to reveal SLAC1 activity in oocytes.

PP2CA Physically Interacts with OST1 and Inhibits Its Kinase Activity Toward the SLAC1 Channel. According to genetic analyses, OST1 appears to be located downstream from ABI1, a PP2C in the ABA signaling pathway (11). A separate study showed that ABI1 interacts with a SnRK2 type kinase and controls its ABA-induced activity (19). These results suggest that ABI1-like PP2Cs (such as PP2CA in this study) interact with SnRK2-like protein kinases (such as OST1) and regulate kinase activity and, in this way, control ABA-linked responses, such as ion channel activity and stomatal closure. Based on this evidence, we reasoned that PP2CA regulation of SLAC1 may be mediated, at least in part, by its ability to alter the activity of OST1.

To follow up, we first tested whether OST1 and PP2CA physically interact with each other in yeast two-hybrid assays. Using OST1 fused to the bait vector and several PP2Cs in the activation vector, we cotransformed yeast cells with individual pairs of PP2C-OST1 and observed that PP2CA, and several other PP2C members tested, strongly interacted with OST1 (Fig. 4*A*). All these PP2C members, including ABI1, ABI2, and PP2CA, were earlier shown to function in ABA signaling (see *Introduction*). HAB1 and another PP2C member (At5g59220) also interacted with OST1 albeit with lower affinity (Fig. 4*A*).

Using an in vitro kinase assay, we then determined whether PP2CA affects OST1 kinase activity. Based on previous studies with SnRK3-type kinases [CIPKs, (37)] and SnRK2 family members (19), these enzymes have autokinase activity. We observed self-phosphorylation activity of OST1 in the absence of other added substrates (Fig. 4*B*). Since SLAC1 cytosolic domains physically interact with OST1 and since SLAC1 activity can be enhanced by OST1 coexpressed in *Xenopus* oocytes that contain no other plant proteins, we surmised that OST1 may act directly on the channel protein by phosphorylation. When expressed and purified from *E. coli*, the recombinant proteins corresponding to the N- and C-terminal cytosolic domains of the SLAC1 channel were, indeed, phosphorylated by OST1 (Fig. 4*B*). As a control, the C-terminal cytosolic domain of AKT1 was not phosphorylated (Fig. 4*B*). The strongest phosphorylation was found to be associated with the SLAC1 N terminus (Fig. 4*B*). The mutated inactive form of OST1 (mutation in the ATP-binding domain residue K50) showed no activity (Fig. 4*B*).

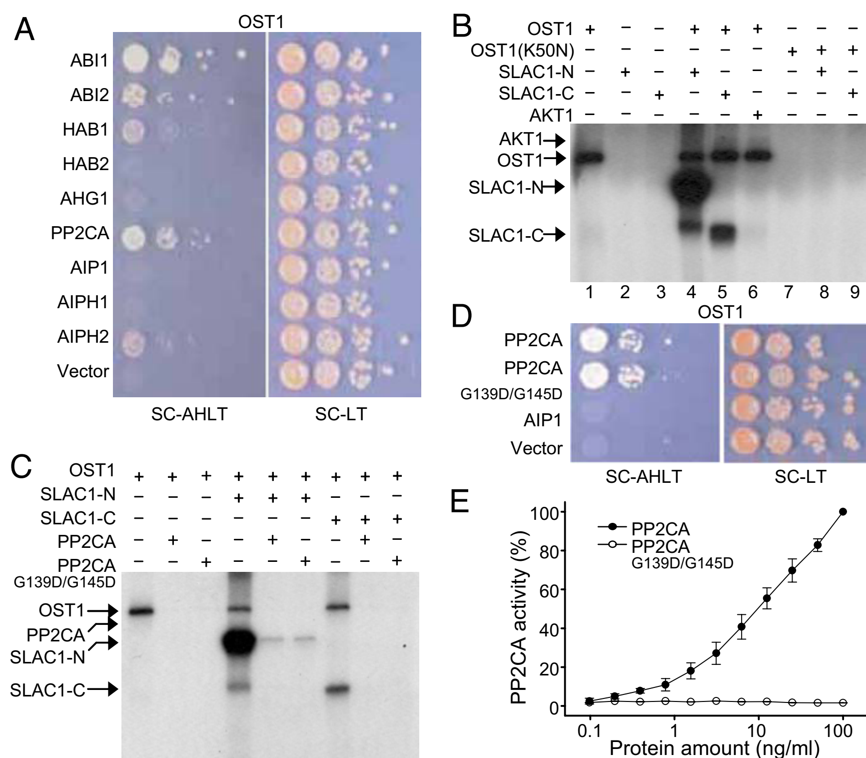

Fig. 4. OST1 phosphorylates SLAC1 N-terminal and C-terminal domains and is inactivated by PP2CA. (*A*) OST1 interacted with PP2CA and several other PP2C members. OST1 was cloned into the pGBTKT7 and the PP2Cs were in pGAD.GH. Yeast cells were as described in *Materials and Methods* and in Fig. 1. AIPH1: AIP1 homologue 1 (At2g29380); AIPH2: AIP1 homologue 2 (At5g59220). (*B*) Phosphorylation of SLAC1 by OST1. The contents of the kinase assays were 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. (*C*) PP2CA and PP2CA null mutant inhibit OST1 autokinase activity and activity against SLAC1. The assays are presented in the same manner as described in (*B*). (*D*) OST1 interacted with PP2CA and PP2CA null mutant in a yeast two hybrid assay described in (*A*). The PP2CA null mutant, as well as PP2CA, was in pGAD.GH. (*E*) In vitro dephosphorylation assay of PP2CA and PP2CA null mutant (see *Materials and Methods*)

After finding that SLAC1 protein domains serve as substrates for OST1 kinase, we tested whether PP2CA regulates OST1 activity against SLAC1. Figure 4*C* shows that inclusion of PP2CA essentially abolished both autokinase activity and activity toward the SLAC1 N- and C-terminal domains. PP2CA may inhibit OST1 by simply binding to the kinase domain or by dephosphorylating the OST1 protein, or, alternatively, by reducing net phosphorylation through dephosphorylating the substrates. To test these possibilities, we generated a PP2CA mutant protein with two amino acid mutations, previously shown to eliminate PP2C activity (38). This mutant still interacted with OST1 in the yeast two hybrid system (Fig. 4*D*) but lacked phosphatase activity (Fig. 4*E*). To our surprise, including the inactive form of PP2CA in the kinase assay, also inhibited the OST1 kinase activity (Fig. 4*C*), suggesting that PP2CA blocks the activity of OST1 simply by forming a PP2CA-OST1 complex.

To address the relationship of OST1 and PP2CA in a functional assay, we coexpressed the SLAC1 channel with OST1 and either the wild type or inactive mutant form of PP2CA in *Xenopus* oocytes (Fig. 5*A*). We found that both the active and phosphatase-inactive forms of PP2CA eliminated activation of SLAC1 by OST1 (Fig. 5*A*). However, the inactive form of OST1 failed to activate SLAC1 (Fig. 5*A*), suggesting that phosphorylation of the channel is essential for channel activation by OST1.

When coexpressed with the SLAC1 channel in the absence of OST1, only the active, and not the inactive, phosphatase inhibited channel activity (Fig. 5*A*). This result suggests that PP2CA may interact directly and dephosphorylate SLAC1 channel leading to inhibition of the channel. We suggest, therefore, that PP2CA regulates SLAC1 activity by two mechanisms: (*i*) by interacting with OST1 and blocking its kinase activity, and (*ii*) by dephosphorylating the SLAC1 protein.

To explore whether OST1 or PP2CA regulates SLAC1 channel by increasing its expression or localization, we injected cRNA coding for GFP-SLAC1 fusion protein into oocytes so that we could monitor the expression and localization of the channel. The oocytes expressing GFP-SLAC1 fusion in the presence or absence of the OST1 or PP2CA all displayed green fluorescence at the plasma membrane (Fig. 5*B*), indicating that the kinase or phosphatase does not affect the expression or location of SLAC1 channel. The SLAC1 current was also recorded from the oocytes that express the GFP-SLAC1 fusion protein, indicating that fusion to GFP did not disrupt SLAC1 function.

Concluding Remarks. Results from genetic analyses are consistent with the view that protein phosphatases (2C type) and a protein kinase (OST1) function between the ABA signal and the target channel protein in ABA-induced stomatal closure. Our results in this study show that both the kinase and phosphatase directly interact with SLAC1 in regulating channel activity. In addition, PP2C interacts directly with OST1 and decreases associated kinase activity through formation of a complex, independently of its activity as a phosphatase. Because PP2C type phosphatases lie upstream of the OST1 protein kinase and act as negative regulators of ABA signaling, we propose the signaling pathway shown in Fig. 6. Under normal conditions, when ABA levels are low, PP2C members interact with OST1 and inhibit its kinase activity, leaving the SLAC1 anion channel with basal activity. When ABA levels increase, ABA binds to its receptors and

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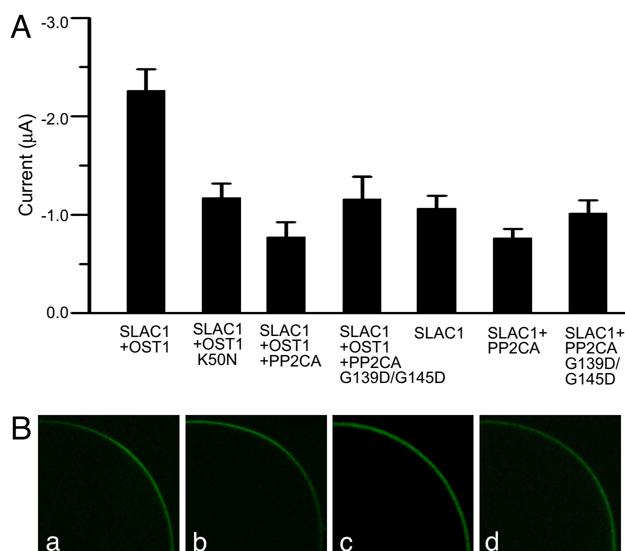

Fig. 5. (*A*) SLAC1 regulation by the OST1 and PP2CA involves more than one mechanism. Mean current (± SE) at −150 mV recorded from the oocytes injected with various combinations of cRNA including SLAC1 alone, SLAC1+OST1, SLAC1+(OST1K50N, kinase null mutant), SLAC1+OST1+PP2CA, SLAC1+OST1+(PP2CAG139D/G145D, phosphatase null mutant), SLAC1+PP2CA, SLAC1+PP2CAG139D/G145D. (*B*) Expression and localization of GFP-SLAC1 fusion protein in the presence or absence of kinase or phosphatase regulator. The photographs depict fluorescence in the plasma membrane of oocytes. (*a*) SLAC1-GFP, (*b*) OST1 + SLAC1-GFP, (*c*) PP2CA + SLAC1-GFP or (*d*) OST1 + PP2CA + SLAC1-GFP.

inhibits PP2C activity as recently reported (39, 40). Alternatively, the ABA receptor may interact with the PP2C protein and dissociate it from the OST1-PP2C complex. The latter series of events leads to activation, first of the OST1 kinase and then of the SLAC1 channel, releasing anions and depolarizing the membrane. This depolarization also activates the GORK channel that mediates the efflux of cations from the guard cell, leading to a further drop of turgor and closure of stomatal pores.

This study raises a number of interesting questions. One, central to ABA-controlled ion transport, relates to the mechanism by which the OST1 kinase phosphorylates SLAC1 to regulate channel activity. The situation is complicated by the finding of at least two phosphorylation sites, one each at the C- and N-termini of the channel, that are separated by six transmembrane helices. A detailed understanding of the significance of this dual phosphorylation must await further work, including a structural analysis of both the channel and the kinase. Another question concerns the mechanism by which PP2C inactivates

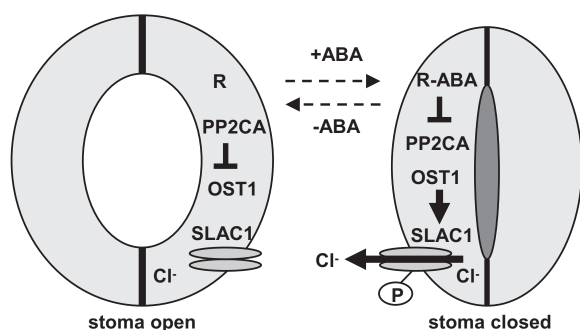

Fig. 6. A working model for an ABA signaling pathway in the regulation of stomatal movements. R, ABA receptors. −P stands for phosphorylation. Details are described in *Concluding Remarks*.

OST1. Current results suggest that phosphatase activity is not required for this inactivation and that physcial interaction between these two proteins is sufficient. Further work should elucidate the details of this regulatory mechanism.

The work also prompts a comparison with an earlier study in which the potassium channel AKT1 (27) is regulated by a seemingly parallel signaling pathway that centers on a protein kinase of the SnRK type and a protein phosphatase in the 2C family. However, although the participants are similar, the processes regulated are different: the earlier pathway controls cell expansion by regulation of the inward K-channel, AKT1, functional in ion uptake, whereas the latter pathway affects cell shrinkage and stomatal closure by regulating ion efflux via the anion channel SLAC1. That such diverse transport processes can be regulated by these kinase-phosphatase pairs is suggestive of a much larger network of signaling kinases and phosphatases that regulate multiple physiological processes in plants. While details await further exploration, pathways of this type would seem especially suitable in enabling plants to respond to environmental challenges.

Materials and 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 (41). Yeast two-hybrid assays were performed as before (27, 29). Transformants were selected in SC-Leucin-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 1/10, 1/100 and 1/1000. Yeast cells, 2 μL, were spotted onto SC-Leucin-Tryptophan media and SC-Adenine-Histidine-Leucine-Tryptophan media.

Bimolecular Fluorescence Complementation (BiFC) Assay. To generate the BiFC constructs, SLAC1, OST1, and PP2CA full length cDNA with no stop codon were subcloned via SpeI/SalI into 35S-SPYNE(R)173 and 35S-SPYCE(M) vectors (42). For transient expression, the *Agrobacterium tumefaciens* strain GV3101 carrying each construct was used together with the p19 strain for infiltration of 5-week-old *N. benthamiana* leaves. For microscopic analyses, leaf discs were cut 4 days after infiltration. The lower epidermis cells were analyzed by confocal microscopy (model Zeiss 510 UV/Vis Meta) operated with LSM Image Browser software.

Construction of Activity-Null OST1 and PP2CA Mutants. To make the OST1 kinase-null mutant, the ATP-binding Lys at the 50th amino acid position was changed to Asn. The glycines at the 159th and 165th amino acid positions of the catalytic domain were changed into Asp for making the phosphatase-null PP2CA mutant. The primer pairs for generating these mutants follow. OST1 mutant: F5′- AGCTTGTTGCTGTT**AAC**TATATCGAGAGA-3′; R5′-TCTCTCGATATA**GTT**AA CAGCAACAAGCT-3′; PP2CA mutant: F5′- CTAC**GAT**GTCTTTGAC**GAC**CATGG-3′; R5′-CCATG**GTC**GTCAAAGAC**ATC**GTAG-3′. For the yeast-two hybrid assay, the kinase assay and electrophysiological experiments, we cloned activity-null OST1 PP2CA mutants into pGAD.GH, pGEX4T-1, and pGEMHE vectors, respectively. All constructs were verified by sequencing.

Electrophysiological Procedure. The preparation and determination of cRNA concentration were performed as previously described (27, 29). Freshly isolated *Xenopus* oocytes were injected with 23 nL of cRNA and used for voltage-clamp experiments 2 days after injection. Two-electrode voltage clamp recordings were performed to measure SLAC1 currents. The pipette solution contained 3 M KCl. To reduce the endogenous currents, we developed a new bath solution that contained 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Mes/Tris (pH 5.6) and 30 mM anions as cesium salt ($CsNO_3$, Cs_2-malate, CsBr, CsCl); osmolality was adjusted with *D*-mannitol to 220 mmol/kg. Voltage steps of 50 to −150 mV (in 15 mV decrements, 7.5 s duration) with a 1.45 s prepulse to 0 mV were applied to record SLAC1 activity. Histograms of SLAC1 were generated from the pooled current reached after 1.6 s of each voltage-clamp episode. The Goldman-Hodgkin-Katz equation was applied to estimate permeability ratios for the different anions (X) with respect to Cl^- (P_X/P_{Cl}) by using the formula:

$P_X/P_{Cl} = ([Cl^-]_o \exp(-\Delta E_{rev}F/RT) - [Cl^-]_t)/[X]_o$, where $[Cl^-]_o$ and $[Cl^-]_t$ are the

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external Cl^- concentrations in control and test anion-substituted solutions, respectively. ΔE_{rev} is the difference in reversal potential value when using those two solutions. $[X^-]_o$ is the external concentration of the substituting anion, *F* and *R* the Faraday and gas constants, and T the absolute temperature. Data are presented as representative recordings or, in some cases, as the mean ± SE of at least six recordings.

Expression and Purification of GST-Fusion Proteins in *Escherichia coli* and Kinase Assay. To produce GST fusion proteins in *E. coli*, OST1, PP2CA, and both the N- and C 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 (29).

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 indicated in the figure legends. Following incubation for 30 min at 30 °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% (wt/vol) acrylamide gel. The gel was dried and ^{32}P was detected by autoradiography using a Typhoon 8600 imager (Molecular Dynamics).

Protein phosphatase assays of PP2CA and PP2CA null mutant were performed by using ProFluor Ser/Thr PPase assay kit (Promega) according to the manufacturer's protocol.

ACKNOWLEDGMENTS. We thank Dr. E.Y. Isacoff (UC Berkeley) for providing oocytes used in this study. This research was supported by the National Science Foundation, the U.S. Department of Agriculture, and the California Agricultural Experiment Station (to S.L. and B.B.B.).

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