Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action

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The plant hormone abscisic acid (ABA) is produced in response to abiotic stresses and mediates stomatal closure in response to drought via recently identified ABA receptors (pyrabactin resistance/regulatory component of ABA receptor; PYR/RCAR). *SLAC1* encodes a central guard cell S-type anion channel that mediates ABA-induced stomatal closure. Coexpression of the calcium-dependent protein kinase 21 (CPK21), CPK23, or the Open Stomata 1 kinase (OST1) activates SLAC1 anion currents. However, reconstitution of ABA activation of any plant ion channel has not yet been attained. Whether the known core ABA signaling components are sufficient for ABA activation of SLAC1 anion channels or whether additional components are required remains unknown. The Ca^{2+}-dependent protein kinase CPK6 is known to function in vivo in ABA-induced stomatal closure. Here we show that CPK6 robustly activates SLAC1-mediated currents and phosphorylates the SLAC1 N terminus. A phosphorylation site (S59) in SLAC1, crucial for CPK6 activation, was identified. The group A PP2Cs ABI1, ABI2, and PP2CA down-regulated CPK6-mediated SLAC1 activity in oocytes. Unexpectedly, ABI1 directly dephosphorylated the N terminus of SLAC1, indicating an alternate branched early ABA signaling core in which ABI1 targets SLAC1 directly (down-regulation). Furthermore, here we have successfully reconstituted ABA-induced activation of SLAC1 channels in oocytes using the ABA receptor pyrabactin resistant 1 (PYR1) and PP2C phosphatases with two alternate signaling cores including either CPK6 or OST1. Point mutations in ABI1 disrupting PYR1–ABI1 interaction abolished ABA signal transduction. Moreover, by addition of CPK6, a functional ABA signal transduction core from ABA receptors to ion channel activation was reconstituted without a SnRK2 kinase.

Arabidopsis | chloride channel

The perception of the phytohormone abscisic acid (ABA) is achieved by the recently discovered 14-member START protein family of ABA receptors named pyrabactin resistance (PYR), or regulatory component of ABA receptor (RCAR) (1, 2). PYR/RCARs have been shown to bind to clade A PP2Cs and inhibit the activity of these PP2Cs in the presence of ABA (1–5). Structural studies show that PYR1, PYL1, and PYL2 function as ABA receptors, with ABA binding in a protein cavity that locks down the ABA molecule (6–10).

ABA reduces transpirational water loss of plants by inducing stomatal closure (11). ABA can cause an increase in guard cell intracellular Ca^{2+} concentration (12–17), which leads to the down-regulation of inward-rectifying K^+ channels and activation of both slow-sustained (S-type) and rapid-transient (R-type) anion channels (18–20). Previous findings have led to the model that S-type anion channels play a key role in controlling stomatal closure (18, 21, 22). *slac1* mutant plants have greatly reduced S-type anion channel activity (23) and display impaired stomatal closure in response to ABA, elevated CO_2, ozone, reactive

oxygen species, calcium, and reduced humidity, underlining that SLAC1 represents a key component functioning downstream of these signals and is crucial for stomatal closure (23, 24).

The subclass III SnRK2 kinase OST1 (Open Stomata 1), also known as SnRK2.6, is required for ABA- and CO_2-induced stomatal closure (25–27). Several members of the PP2C family have been shown to directly interact with (3, 28, 29) and deactivate subclass III SnRK2 protein kinases (SnRK2.2, SnRK2.3, and SnRK2.6/OST1) through dephosphorylation in vitro (28–30).

S-type anion channels in guard cells are activated by phosphorylation events (31, 32). *Xenopus* oocyte electrophysiology experiments demonstrated that SLAC1 in the presence of the protein kinases OST1, calcium-dependent protein kinase 23 (CPK23), and, to a lesser extent, CPK21 can generate S-type anion channel activity (33–35). An S120A mutation in SLAC1 prevents channel activation by OST1, but CPK23 is still able to induce SLAC1 S120A-mediated currents in *Xenopus* oocytes (33, 34). SLAC1-mediated anion currents are down-regulated by the PP2C-type phosphatases ABI1, ABI2 (33, 34), and PP2CA (35). However, whether ABA activation of anion channels can be reconstituted in *Xenopus* oocytes with these core signaling components remains unknown (33–36).

The role and function of CPKs in the regulation of SLAC1 are still ambiguous. In vivo, CPK6 functions as a positive regulator of ABA control of S-type anion channels in guard cells (37). In *cpk3cpk6* double knockout mutant *Arabidopsis* plants, ABA- and Ca^{2+}-induced stomatal closure, as well as activation of S-type anion channel activity, was impaired in vivo, with CPK6 playing a more prominent role than CPK3 (37). CPK21 and CPK23 activate SLAC1 in *Xenopus laevis* oocytes (34), but *cpk21* and *cpk23* mutants show enhanced drought and salt tolerance (38, 39) rather than drought sensitivity. Furthermore, CPK6 was reported to weakly interact with SLAC1 in *Xenopus* oocytes (34). Here we functionally reconstitute activation of SLAC1 channels by ABA with either CPK6 or OST1 in the ABA signaling core and identify mechanisms of this signaling pathway. Interestingly, these findings further show that an alternate early ABA signaling core can be functionally reconstituted for ABA activation of

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a plant ion channel without inclusion of a SnRK2 kinase. Furthermore, in addition to the known dephosphorylation of OST1 by the ABI1 PP2C, we demonstrate that ABI1 directly dephosphorylates the N terminus of SLAC1, enabling tight control of anion channel activity.

Results

CPK6 Activates SLAC1 in *Xenopus* Oocytes and Phosphorylates the N Terminus of SLAC1. Two-electrode voltage-clamp experiments in *X. laevis* oocytes were performed to analyze SLAC1 regulation. When *SLAC1* cRNA was injected into oocytes alone, average inward currents were similar to those of water-injected control oocytes (Fig. 1 *A* and *B*). We investigated whether CPK6, which functions in S-type anion channel activation in vivo (37), can activate SLAC1 channels. Coexpression of SLAC1 and individual protein kinases, including CPK6 and CPK23, significantly activated SLAC1-dependent ion currents compared with those of SLAC1 alone (Fig. 1 *A* and *B* and Fig. S1*A*; $P = 0.001$ and $P = 0.04$ for CPK6 and CPK23, respectively; Mann–Whitney U test at

-180 mV). It was also confirmed that CPK6 was able to activate SLAC1 consistently throughout six of the tested batches of oocytes ($P < 0.001$; two-way ANOVA). Reversal potentials of SLAC1 and kinase-activated ion currents were close to the equilibrium potential for Cl⁻, consistent with the anion selectivity of S-type anion channels. Robust CPK6 activation of SLAC1 channels (Fig. 1 *A* and *B* and Fig. S1*A*) did not require experimentally imposed linkage of SLAC1 to the protein kinase using split yellow fluorescent protein (YFP-BiFC) fusions, as shown for OST1 (33). The calcium dependent protein kinases closely related to CPK6 and CPK23 [CPK12 and CPK31, respectively (40)] were not able to stimulate SLAC1 activity (Fig. 1 *A* and *B* and Fig. S1*A*; CPK12, $P = 0.305$; Student's t test; CPK31, $P = 0.234$; Mann–Whitney U test at -180 mV). Coexpressing the kinase-inactive variant of CPK6 (D209A) together with SLAC1 did not activate SLAC1 (Fig. S1*B*), indicating a kinase activity-dependent event. Comparison of SLAC1 activity in the presence of the tested kinases suggests that SLAC1 activations by CPK6 and CPK23 are statistically comparable (Fig. S1*A*; $P = 0.561$; Mann–Whitney U test at -180 mV), whereas CPK3 and CPK31 showed no significant activation of SLAC1 (Fig. S1*A*; $P = 0.363$ and $P = 0.321$ for CPK3 and CPK31, respectively; Mann–Whitney U test at -180 mV).

Localizations and abundances of CPK6 and CPK23 were analyzed in *Xenopus* oocytes. Confocal laser microscopy of cryomicrodissected CPK fusion protein (mTurquoise; mTq)-expressing *Xenopus* oocytes revealed that a significant proportion of the CPK6 and CPK23 proteins are located at the cell surface, with an additional weak fluorescence distributed throughout the cytoplasm (Fig. 2 *A* and *B*). Quantification of the fluorescence intensities showed that CPK6-mTq and CPK23-mTq abundance
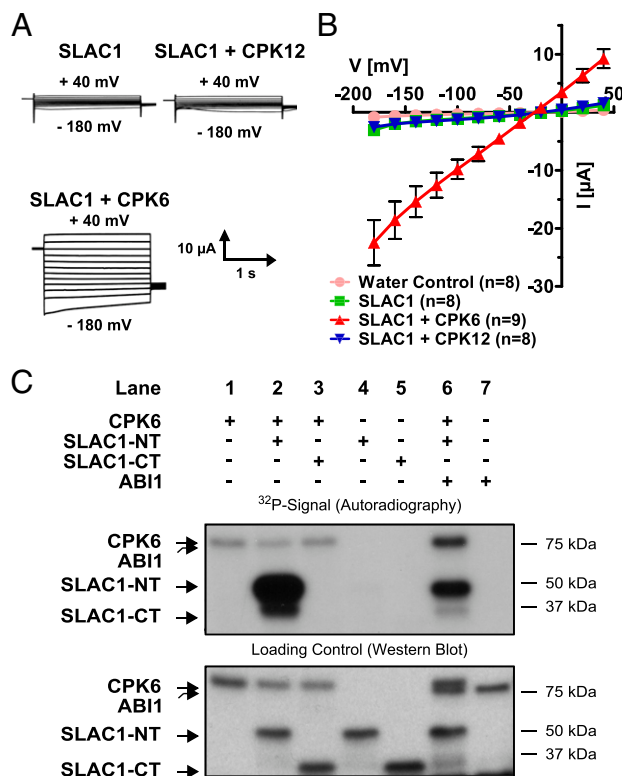

Fig. 1. Strong activation of SLAC1 channels by the CPK6 protein kinase in *Xenopus* oocytes and in vitro. (*A*) Whole-cell voltage-clamp recordings for oocytes expressing SLAC1, in the presence or absence of CPKs, show that coexpression of SLAC1 with CPK6 results in large Cl⁻ currents, as opposed to SLAC1 with CPK12. (*B*) The average steady-state current–voltage relationships are shown in water-injected (pink circles), SLAC1-injected (green squares), and SLAC1 + CPK-injected oocytes. The protein kinase CPK6 (red triangles) stimulates SLAC1 activity, whereas CPK12 (blue triangles), a homolog of CPK6, did not increase SLAC1 activity in oocytes. Representative data from one batch of oocytes are shown (of >3 batches tested). Error bars represent SEM. (*C*) Autoradiograph of in vitro kinase assays using recombinant proteins (*Upper*) and Western blot analysis detecting GST as loading control for the individual proteins (*Lower*). The kinase CPK6 (lane 1) is able to transphosphorylate the N terminus of SLAC1 (lane 2; SLAC1-NT) but not the C terminus (lane 3; SLAC1-CT). No signal could be detected upon incubation of SLAC1-NT (lane 4) or SLAC1-CT alone (lane 5). Coincubation of CPK6 with ABI1 decreased SLAC1-NT transphosphorylation (lane 6). Assaying ABI1 alone did not result in a ³²P signal (lane 7).
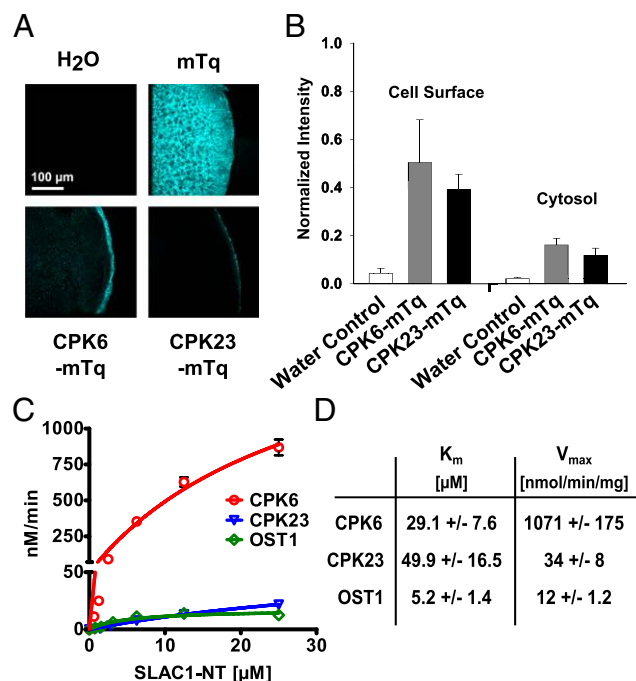

Fig. 2. CPK6 is expressed at the cell surface and high in vitro CPK6 activity toward SLAC1-NT phosphorylation. (*A*) Kinases CPK6 and CPK23 are localized at the cell surface, whereas mTurquoise (mTq) alone is uniformly spread throughout oocytes. (*B*) Analysis of fluorescence intensity (normalized to mTq) highlights the strong cell-surface localization of both CPK6 and CPK23 (data points represent mean ± SE; $n > 3$). (*C*) P81 filter-based protein kinase assays show kinetics of SLAC1-NT phosphorylation by CPK6 (red circles), CPK23 (blue triangles), and OST1 (green diamonds). Data points represent mean values ± SE ($n = 3$). (*D*) K_m values are given in μM SLAC1-NT, and maximum specific activities are in nmol PO₄ incorporated·min⁻¹·mg⁻¹ kinase.

did not significantly differ (Fig. 2B; cell surface, $P = 0.474$; cytosol, $P = 0.344$; Student's t test). To estimate a saturation point of CPK6 and CPK23-dependent SLAC1 activation, defined amounts of cRNA encoding the protein kinases CPK6 and CPK23 were injected into oocytes: a standard [*CPK6/23*] cRNA amount (~25 ng; [1.0]), an intermediate cRNA amount (~12.5 ng *CPK6/23*; [0.5]), or one-tenth cRNA amount (~2.5 ng *CPK6/23*; [0.1]). For these experiments, oocytes were coinjected with 25 ng *SLAC1* cRNA. Halving the injected cRNA amount resulted in no significant change in SLAC1 channel activity (Fig. S2; $P > 0.685$ at -180 mV; Student's t test). However, injecting one-tenth of the amount of cRNA resulted in a strong reduction of SLAC1-mediated anion currents (Fig. S2; $P < 0.01$ at -180 mV; Mann–Whitney U test), suggesting that the injected *CPK6/23* cRNA amount at 25 ng was near the saturation point when 25 ng *SLAC1* cRNA was coinjected.

In vitro protein kinase assays were performed to assess whether CPK6 can phosphorylate cytosolic domains of the SLAC1 channel. Wild-type recombinant CPK6 protein showed weak autophosphorylation (Fig. 1C, *Upper*, lane 1). The N terminus of SLAC1 (SLAC1-NT; amino acids 1–186), but not the C terminus (SLAC1-CT; amino acids 497–556), was strongly phosphorylated by CPK6 (Fig. 1C, *Upper*, lanes 2 and 3). The SLAC1 termini incubated alone did not show any signal (Fig. 1C, *Upper*, lanes 4 and 5). The strong CPK6-mediated SLAC1-NT phosphorylation was dependent on the presence of free Ca^{2+} in the reaction buffer (Fig. S3).

To quantify the degree of CPK6-mediated SLAC1 phosphorylation activity, P81-grade filter-based kinetic in vitro phosphorylation assays were performed (Fig. 2 C and D). The K_m and V_{max} values of the kinase substrate pairs reveal that CPK6 very efficiently and, to a lower extent, CPK23 and OST1, phosphorylates the SLAC1-NT in vitro (Fig. 2 C and D).

Identification and Characterization of Sites Phosphorylated by CPK6.
Due to activation of SLAC1-mediated ion currents by CPK6 (Figs. 1 and 3) and the in vivo function of CPK6 in guard cells (37), we focused on deciphering the properties of this regulation. To date, the only known amino acid that is crucial for SLAC1 activation by a protein kinase is serine 120 (33, 34, 41). To determine which residues may be relevant, trypsin-digested SLAC1-NT, phosphorylated by CPK6 in the presence of nonradioactive ATP, was analyzed by high-pressure liquid chromatography (HPLC) coupled to tandem mass spectroscopy (LC-MS/MS) using nanospray ionization. A SLAC1-NT phosphorylation site was identified at the amino acid serine 59 (Fig. 3A). Consequently, this amino acid was replaced by either alanine (which cannot be phosphorylated; SLAC1 S59A) or aspartate (putative phosphomimetic; SLAC1 S59D), and CPK6 activation of SLAC1 was analyzed in oocytes. Voltage-clamp recordings showed that SLAC1 S59A was not activated by CPK6 (Fig. 3B). SLAC1 S59D did not exhibit constitutive activation of anion current activity compared with wild-type SLAC1 (Fig. S4B), indicating that either this mutant is not a functional phosphomimetic or that additional phosphorylation sites are required. These findings indicate that serine 59 represents a crucial amino acid for CPK6-mediated activation of SLAC1.

Down-Regulation of CPK6-Activated SLAC1 Channels by Group A PP2Cs. We tested several PP2Cs that function in ABA signaling for their ability to inhibit CPK6-mediated SLAC1 activation. Experiments showed that the PP2C protein phosphatases ABI1 (42, 43), ABI2, and PP2CA (44–46) were able to almost completely down-regulate CPK6-activated anion currents in oocytes (Fig. 4 A and B).

In in vitro kinase assays, the phosphorylation of the SLAC1-NT by CPK6 (Fig. 1C, *Upper*, lane 2) was decreased when recombinant ABI1 protein was included in the reaction (Fig. 1C,
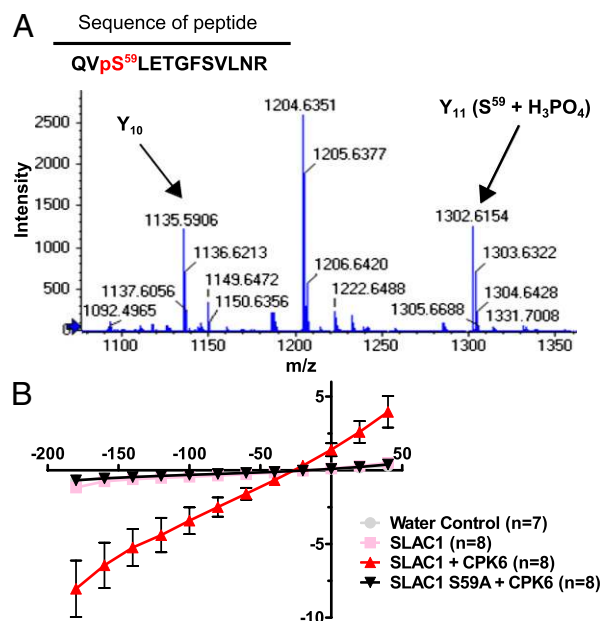

Fig. 3. Serine 59 of SLAC1 is crucial for CPK6-mediated activation of the anion channel. (A) Zoomed-in spectrum section of product ion scan for the peptide QVpSLETGFSVLNR [(M + 2H)$^+$ = 765.4]. The fragment ion (Y$_{11}$) corresponding to phosphorylated serine (S59) is labeled in the figure (Y$_{11}$ (S^{59} + H$_3$PO$_4$)). The Y$_{10}$ fragment is labeled to demonstrate that T62 and S65 are not phosphorylated for this ion. The complete spectrum and statistical parameters are presented in Fig. S4A and Table S1. (B) Average steady-state current–voltage relationships show that the point mutation of serine 59 to alanine (black triangles) abolishes anion channel activity due to CPK6 (red triangles). Representative data from one batch of oocytes are shown (of two batches tested).

Upper, lane 6 and Fig. 4C, *Upper*, lane 5). Data further indicate that ABI1 might also be phosphorylated by CPK6 (Fig. 1C, *Upper*, lane 6). ABI1 alone did not produce any signal on the autoradiogram (Fig. 1C, lane 7).

Experiments were pursued to address whether ABI1 is able to dephosphorylate amino acid residues in the N terminus of SLAC1 after phosphorylation by CPK6. The kinase inhibitor staurosporine strongly inhibited CPK6 transphosphorylation activity (Fig. 4C, *Upper*, lane 4). To probe the ability of the PP2C ABI1 to dephosphorylate SLAC1 directly, the SLAC1-NT was first phosphorylated by CPK6 (Fig. 4C, *Upper*, lane 1). Ten minutes later, either staurosporine alone (Fig. 4C, lane 3, 10 min) or staurosporine and recombinant ABI1 protein (Fig. 4C, lane 2, 10 min) were added, and the reaction was incubated for an additional 35 min. Ten-minute delayed addition of staurosporine alone did not change the phosphorylation state of the SLAC1-NT (Fig. 4C, *Upper*, lane 3), but coincubation with ABI1 strongly decreased the prior CPK6-mediated phosphorylation (Fig. 4C, *Upper*, lane 2). Thus, CPK6 phosphorylates the N terminus of SLAC1. Unexpectedly, ABI1 reduces this phosphorylation by directly dephosphorylating the SLAC1-NT, which differs from previously reported OST1–ABI1 interactions in which ABI1 did not directly dephosphorylate SLAC1 after OST1-mediated phosphorylation (33). This points to a mechanism for ABA-induced SLAC1 regulation (*Discussion*).

Can ABA Regulate CPK6- or OST1-Dependent SLAC1 Anion Channel Activity in Oocytes? In previous studies, reconstitution of ABA activation of SLAC1 channels has not been studied (33–36). To assess whether ABA activation of SLAC1 can be reconstituted, we coinjected cRNAs of *SLAC1*, *ABI1/ABI2*, the *PYR1* ABA receptor, and either *CPK6* or *OST1* into oocytes. In the absence
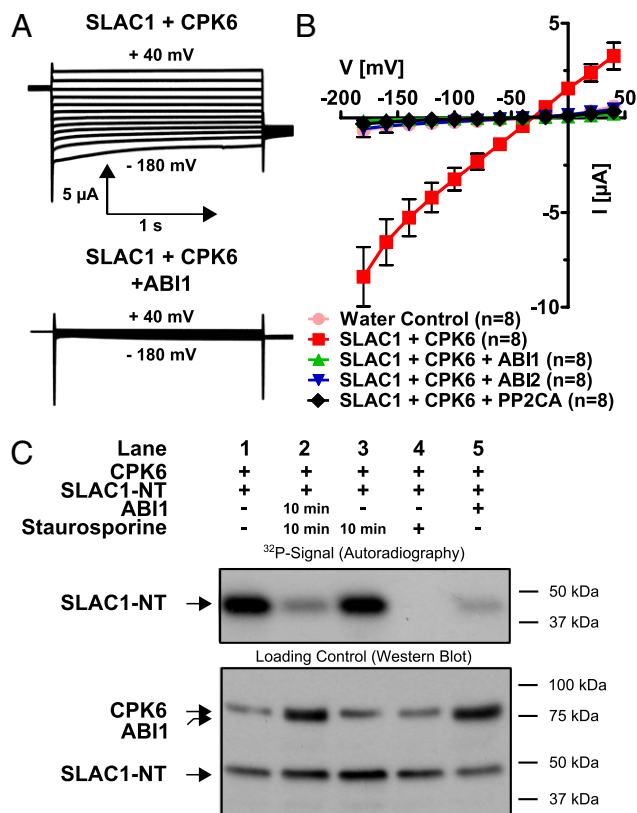
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Fig. 4. Strong inhibition of CPK6-mediated SLAC1 activation by PP2C protein phosphatases and direct SLAC1 N terminus dephosphorylation by ABI1. (*A*) Whole-cell current recordings for oocytes show that protein phosphatase ABI1 strongly inactivated CPK6-induced SLAC1 ion channel current. (*B*) Current–voltage relationships in oocytes containing SLAC1 and CPK6 demonstrate that the PP2Cs ABI1 (green triangles), ABI2 (blue triangles), and PP2CA (black diamonds) are capable of strongly inhibiting CPK6-mediated SLAC1 activity (red squares). Data from one representative batch of oocytes (of >3 batches) are shown. (*C*) (*Upper*) ^{32}P phosphorylation of the N terminus of SLAC1. (*Lower*) Loading controls of the individual proteins CPK6, ABI1, and SLAC1. After initial CPK6 exposure, the strong phosphorylation state of SLAC1-NT phosphorylated by CPK6 (*Upper*, lane 1) decreases if subsequently coincubated with ABI1 and staurosporine 10 min after CPK6 exposure (lane 2). However, phosphorylation of SLAC1-NT by preexposure to CPK6 is not reduced if the protein kinase inhibitor staurosporine alone is added 10 min after CPK6 exposure (lane 3). If staurosporine is added simultaneously with CPK6, SLAC1-NT transphosphorylation is strongly inhibited (lane 4). The ABI1 protein phosphatase added simultaneously with CPK6 inhibits phosphorylation of SLAC1-NT (lane 5).

Fig. 5. Functional reconstitution of ABA activation of SLAC1 channels in (*A* and *B*) CPK6- and (*C* and *D*) OST1-containing signaling pathways. Current traces (*A* and *C*) show examples of the ABA activation of SLAC1 channel currents in oocytes. An ABA-dependent signaling pathway was reconstituted in oocytes using the protein kinases CPK6 (*A* and *B*) and OST1 (*C* and *D*). Split YFP (BiFC) was used in the case of SLAC1 and OST1 coexpression (*C* and *D*). The PP2C phosphatase ABI2 (*A* and *B*) or ABI1 (*C* and *D*) is able to inhibit SLAC1 currents (blue triangles in *B* and *D*). In the absence of ABA, the ABA receptor PYR1 does not activate SLAC1 currents (black hexagons in *B* and *D*). However, in the presence of injected ABA, SLAC1 currents are strongly activated (red circles in *B* and *D*). Data from one representative batch of oocytes (of >3 batches) are shown in *D*. Data from two to five independent batches were averaged in *B*.

of ABA, PYR1 did not affect the small magnitude of anion currents in oocytes expressing the PP2Cs ABI2 or ABI1 with SLAC1 and CPK6 (Fig. 5*B*) or OST1 (Fig. 5*D*). Extracellular ABA application did not result in SLAC1 activation (*n* > 6). When ABA was injected into the cytoplasm of oocytes 15 min before the recording of oocyte currents, anion channel activity was dramatically enhanced in oocytes containing SLAC1, ABI1/ABI2, PYR1, and either CPK6 or OST1 (Fig. 5). Note that to enhance the activation of SLAC1 via OST1 (Fig. 5*D*), split YFP (BiFC) constructs were attached to the anion channel and kinase (33) to irreversibly link these proteins. In contrast, ABA activation of SLAC1 channels via CPK6 did not require imposed linkage of the expressed signaling proteins (Fig. 5 *A* and *B*). Therefore, the analyzed ABA signaling pathway components were sufficient to reconstitute ABA activation of SLAC1 currents with either the CPK6 or OST1-Yn protein kinases. Thus, a functional
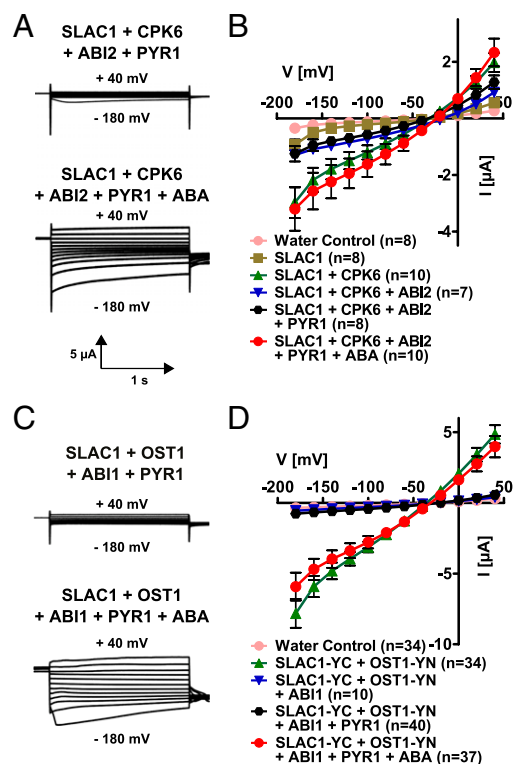
ABA signaling core was reconstituted with CPK6, enabling ABA activation of SLAC1 without a SnRK2 kinase (Fig. 5 *A* and *B*).

We attempted to reconstitute the ABA signaling core including PYR1, ABI1, CPK6, and SLAC1-NT in vitro. Inhibition of CPK6-mediated SLAC1-NT phosphorylation by ABI1 (Fig. 6*A*, *Upper*, lanes 5, 6, and 8) is decreased if the ABA receptor PYR1 and ABA are present (Fig. 6*A*, *Upper*, lane 7). Addition of PYR1 or ABA alone did not alter CPK6- and ABI1-dependent SLAC1-NT phosphorylation (Fig. 6*A*, *Upper*, lanes 1, 4, 5, and 8).

The tryptophan 300 residue of ABI1 was identified as being crucial for stabilizing the PYL1–ABA–ABI1 complex due to interaction of this residue with the narrow hydrophobic ABA-binding pocket (7, 10). When the ABA-dependent signaling pathway containing the ABI1 W300L mutant was expressed in oocytes, ABI1 W300L was able to inhibit SLAC1 ion currents similarly to wild-type ABI1 (Fig. S5). However, SLAC1 anion currents could not be activated upon injection of ABA when coexpressing PYR1, OST1, and ABI1 W300L (Fig. S5).

Discussion

In the present study, we show that the calcium-dependent protein kinase CPK6 activates SLAC1-dependent anion currents in *Xenopus* oocytes (Figs. 1, 3, and 4). In vitro kinase assays demonstrate that CPK6 phosphorylates the SLAC1 N terminus (Fig. 1*C*), as previously observed for CPK21, CPK23, and OST1 (33–
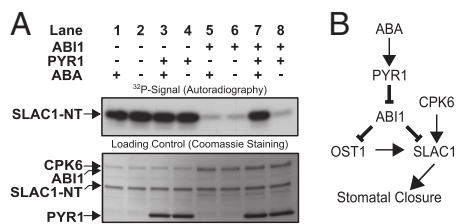
Fig. 6. (*A*) In vitro reconstitution of an ABA-dependent signal transduction pathway including PYR1, ABI1, CPK6, and SLAC1-NT protein. Independent of ABA, CPK6 phosphorylates SLAC1-NT (lanes 1 and 2). The presence of ABI1, but not PYR1, inhibits SLAC1-NT phosphorylation, with or without ABA (lanes 3–6). If CPK6, ABI1, and PYR1 are present, addition of ABA leads to the release of ABI1-mediated inhibition of SLAC1 phosphorylation (lane 7). (*B*) Model for ABA activation of the SLAC1 channel via the OST1 and CPK6 protein kinases and down-regulation by the ABI1 protein phosphatase. ABA binds to PYR1, which causes inhibition of PP2C protein phosphatases, including ABI1. This leads to ABA-induced activation of SLAC1 channels in *Xenopus* oocytes by CPK6, which functions in native guard cells in ABA activation of S-type anion channels (37). Note that the ABI1 protein phosphatase can directly dephosphorylate the SLAC1 N terminus, which represents a previously unknown target for ABI1 and a mechanism for tight negative SLAC1 regulation, in addition to the known down-regulation of OST1.

35, 41). Here, LC-MS/MS experiments revealed a phosphorylation site in SLAC1 (S59) that is crucial for SLAC1 channel activation by CPK6 in *Xenopus* oocytes (Fig. 3). This SLAC1 phosphorylation may lead to conformational changes in the channel, resulting in rearrangement of the phenylalanine residue 450, which is crucial for the gating of SLAC1 (47). Kinetic protein kinase quantification analyses reveal a high catalytic efficiency of CPK6 toward SLAC1-NT phosphorylation (Fig. 2 *C* and *D*), which is comparable to other plant kinases (48, 49).

A 1:10 cRNA ratio of channel versus activating kinase injection led to only very weak SLAC1 activation (Fig. S2 *A* and *B*), which appears to be puzzling, as protein kinases are potentially able to phosphorylate and thereby activate multiple channels. Potential reasons could include (*i*) direct CPK6-protein linkage to SLAC1, (*ii*) a lower expression level of the kinase compared with SLAC1, or (*iii*) that endogenous oocyte phosphatases might be able to remove the phosphate groups added by the kinases and thereby inhibit kinase-mediated SLAC1 activation until a certain threshold is reached. The effect of CPK3 expression on SLAC1 activity in the oocytes was statistically insignificant (Fig. S1*A*). Recent studies have shown differential effects of CPK3 and CPK6 on stomatal closing (50, 51). Consistent with these findings, CPK3 and CPK6 are not in the same CPK subfamily (37, 40). The CPK6 activation of SLAC1 in oocytes reported in this work and reduced ABA- and Ca^{2+}-induced S-type anion channel activation in guard cell protoplasts (37) are in line with the recently reported drought-resistant phenotype of CPK6-overexpressing *Arabidopsis* plants (52).

We have further observed strong inhibition of CPK6-activated SLAC1 channel activity in the presence of the three group A PP2Cs ABI1, ABI2, and PP2CA (Fig. 4 *A* and *B*). SnRK2 kinases have been shown to be directly regulated by PP2C phosphatases through dephosphorylation of the kinase-activation loop (28–30). Phosphorylation experiments at the N terminus of a SLAC1 homolog (SLAH3) by CPK21 suggested that ABI1 inhibits kinase activity, but does not dephosphorylate the N terminus of SLAH3 (36). Interestingly, the present findings provide biochemical evidence suggesting that the ABI1 protein phosphatase can directly dephosphorylate SLAC1 (Figs. 4*C* and 6*B*). Consequently, our results with CPK6 indicate an alternate ABA signaling pathway that is distinct from the linear PYR/RCAR–PP2C–SnRK2 (OST1)–SLAC1 such that, in addition to SnRK2 down-regulation, the PP2C ABI1 directly competes with CPK6

for (de)phosphorylation of the SLAC1 N terminus, which can add a mechanism for the tight regulation of S-type anion channels found in guard cells (20, 21) (Figs. 4*C* and 6*B*). The question of whether ABI1 additionally inhibits CPK6 activity will require further investigation.

We further demonstrate functional reconstitution of ABA activation of SLAC1 ion channel activity using either the CPK6 or OST1 protein kinase coexpressed with ABI1, ABI2, and PYR1 in *Xenopus* oocytes (Fig. 5). We show that ABA activation requires intracellular injection of ABA. Also, CPK6 mediates SLAC1-NT phosphorylation in vitro in response to ABA in the presence of PYR1 and ABI1 (Fig. 6*A*). An earlier study reported reconstitution of an ABA-dependent signaling pathway consisting of PYR/RCARs, group A PP2Cs, SnRK2 kinases, and the transcription factor ABF2 using a protoplast system with a luciferase reporter (53). Interestingly, the reconstituted ABA signal transduction pathway found here expands the present model for ABA signaling cores, by showing that the calcium-dependent protein kinase CPK6 can mediate an ABA-activated ion channel response in the absence of a SnRK2 protein kinase.

Conclusions

Our findings demonstrate the complete reconstitution of ABA activation of a plant ion channel, SLAC1, in a heterologous system including CPK6. Whereas strong activation of SLAC1 via the OST1 protein kinase required induced interaction of the OST1–SLAC1 pair via split YFP fusion, CPK6 strongly activates SLAC1 channels without any additional components that enhance protein–protein interactions. A regulatory phosphorylation site in CPK6 activation of SLAC1, S59, is identified (Fig. 3). The present study expands the current model for early ABA signaling mechanisms in which ABA binds to ABA receptors, including PYR1, leading to inhibition of PP2Cs and subsequent SnRK2 kinase activation. We show here that CPKs can replace SnRK2 kinases in ABA regulation of SLAC1 channels, suggesting that both of these protein kinase families function in parallel in vivo (Fig. 6*B*). These data further correlate with findings showing calcium dependence of ABA-induced stomatal closure (12–15, 17, 20) and parallel Ca^{2+}-independent mechanisms (20, 54). Moreover, data show that ABI1 directly dephosphorylates the N terminus of SLAC1, indicating a branched configuration: ABI1 deactivates SLAC1 by directly interacting with and down-regulating SnRK2s (28–30) and dephosphorylating the SLAC1 N terminus (Figs. 4*C* and 6*B*).

Materials and Methods

Construct Preparation, Electrophysiology, and Histology in *X. laevis* Oocytes. All constructs were cloned into the pNB1 oocyte expression vector using the USER method (55). cRNA was prepared using the mMessage mMachine Transcription Kit (Ambion). Approximately 25 ng of each tested cRNA, in a total volume of 50 nL, was injected into each oocyte for voltage-clamp recordings, if not otherwise stated. mTq fusion proteins were visualized in fixed sections using confocal laser microscopy. For information on instrument setup, solutions used, and analyses, see *SI Materials and Methods*.

Site-Directed Mutagenesis, Protein Expression, Isolation, in Vitro Kinase Assays, and Western Blot Analyses. CPK6, OST1, ABI1, PYR1, and SLAC1 N and C terminus coding sequences were cloned into modified pGEX-6P1 (GE Healthcare) vectors using the USER method (55), and the proteins were overexpressed in *Escherichia coli* Rosetta (DE3) pLysS (Novagen) and isolated using Strep-Tactin MacroPrep (IBA) (Fig. S6). In vitro kinase assays were performed as previously described (34). To assess phosphorylation kinetics, P81-grade filter paper (Whatman) and a scintillation counter were used. More detailed information for all methods is given in *SI Materials and Methods*.

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