Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities

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In response to drought stress, the phytohormone abscisic acid (ABA) induces stomatal closure. Thereby the stress hormone activates guard cell anion channels in a calcium-dependent, as well as –independent, manner. Open stomata 1 protein kinase (OST1) and ABI1 protein phosphatase (ABA insensitive 1) represent key components of calcium-independent ABA signaling. Recently, the guard cell anion channel SLAC1 was identified. When expressed heterologously SLAC1 remained electrically silent. Upon coexpression with Ca^{2+}-independent OST1, however, SLAC1 anion channels appear activated in an ABI1-dependent manner. Mutants lacking distinct calcium-dependent protein kinases (CPKs) appeared impaired in ABA stimulation of guard cell ion channels, too. To study SLAC1 activation via the calcium-dependent ABA pathway, we studied the SLAC1 response to CPKs in the *Xenopus laevis* oocyte system. Split YFP-based protein–protein interaction assays, using SLAC1 as the bait, identified guard cell expressed CPK21 and 23 as major interacting partners. Upon coexpression of SLAC1 with CPK21 and 23, anion currents document SLAC1 stimulation by these guard cell protein kinases. Ca^{2+}-sensitive activation of SLAC1, however, could be assigned to the CPK21 pathway only because CPK23 turned out to be rather Ca^{2+}-insensitive. In line with activation by OST1, CPK activation of the guard cell anion channel was suppressed by ABI1. Thus the CPK and OST1 branch of ABA signal transduction in guard cells seem to converge on the level of SLAC1 under the control of the ABI1/ABA-receptor complex.

abscisic acid signaling | drought stress | guard cell | S-type anion channel

The drought hormone abscisic acid (ABA) triggers release of K^+ and anions from guard cells and thereby causes stomatal closure (1, 2). Recently, SLAC1, a guard cell anion channel, was identified (3–5). In guard cells of these ABA- and CO_2/O_3-insensitive mutant plants, anion currents appeared largely suppressed. When SLAC1 was expressed with the open stomata 1 protein kinase (OST1) in *Xenopus* oocytes, SLAC1-related anion currents, similar to those observed in guard cells, appeared (6). The presence of ABI1, however, prevented SLAC1 activation. This ABA pathway resembles the Ca^{2+}-independent activation of SLAC-type anion currents in guard cells. ABA signal transduction, however, has been shown to activate guard cell anion channels in a calcium-independent as well as -dependent manner (7–10). This became evident in *abi1-1* mutant plants, where anion channels do not respond to ABA anymore (11) but still activate with calcium (12). Furthermore the described mutant *growth controlled by abscisic acid* (*gca2*) (13–14), isolated from the *Arabidopsis* ecotype Landsberg *erecta*, was shown to be impaired in ABA-induced stomatal closure in a Ca^{2+}-dependent manner. Moreover $[Ca^{2+}]_{cyt}$ elevation was shown to result in activation of S-type anion channels via phosphorylation (12, 15), suggesting a role of phosphorylation events in $[Ca^{2+}]_{cyt}$ signaling.

CDPKs resemble Ca^{2+}-dependent Ser/Thr protein kinases, harboring an N-terminal kinase domain joined to a C-terminal CaM-like domain. A junction domain serves to stabilize and maintain the kinase in an auto-inhibited state (16–18). Ca^{2+} elevation renders these kinases active. CDPKs are encoded by a large multigene family (34 members) with possible redundancy and diversity in their function (16). Two members of this family, CPK3 and 6 have been identified by knock-out mutation as players in ABA-dependent regulation of guard cell S-type currents (19). Besides CPK3 and 6, CPK23 loss-of-function mutants exhibit a stoma phenotype when challenged with water stress (20). For this reason Ma and Wu (20) suggested that "the further identification and characterization of these CDPKs' downstream target(s) specifically expressed in stomatal guard cells will certainly increase our understanding in how these CDPKs act coordinately to transduce calcium signals in stomatal guard cells". The fact that (*i*) residual anion channel activities were observed in the OST1 loss-of-function compared to the *slac1-3* mutant (6) and (*ii*) stomata of OST1 mutant plants could close at least partially indicates that in the absence of OST1, SLAC1 is very likely activated by protein kinases of the CDPK family.

To study SLAC1 activation via the calcium-dependent ABA pathway, we studied the SLAC1 response to CPKs in the *Xenopus laevis* oocyte system. Split YFP-based protein–protein interaction assays, using SLAC1 as the bait, identified guard cell expressed CPK21 and 23 as interacting partners. Upon coexpression of SLAC1 with these CPKs, SLAC1-related anion currents document SLAC1 stimulation. Similar to OST1 action, CPK activation of the guard cell anion channel was suppressed by ABI1. In vitro kinase assays, however, revealed that CPK21 and CPK23 posses distinct Ca^{2+}-sensitivities. Thus the calcium-dependent (CPK21) and independent branch (OST1/CPK23) of ABA signal transduction in guard cells seem to converge on the level of SLAC1-ABI1 regulation.

Results

To study the role of ABA signaling components and to reconstitute SLAC1-based membrane-delimited ABA action, we expressed this guard cell anion channel together with CPK3, 6, 21, 23, 31, and PP2C protein phosphatases ABI1 and HAB1 in *X. laevis* oocytes. The interaction of those putative signaling components was visualized with the bimolecular fluorescence complementation technique (BiFC) (21). SLAC1 and several CPKs and phosphatases were fused to a complementary half of split YFP each (for illustration of constructs see Fig. S1). Various cRNA combinations were injected into *Xenopus* oocytes and analyzed by confocal microscopy. When expressed SLAC1::

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YFP^C alone or with YFP^N (Fig. S2*A*), no specific YFP fluorescence was emitted from oocytes. Upon coinjection of SLAC1::YFP^C and YFP^N fused to potential interaction partners YFP signals could be detected. Thereby strong and direct interaction between SLAC1 and CPK23 was identified via complementation of a functional YFP molecule (Fig. 1*A*). In contrast to SLAC1 interaction with CPK23, the YFP fluorescence between SLAC1 and CPK6 and 21 was weaker (Fig. S2*A*). BiFC-signals between SLAC1 and CPK3 were even fainter (Fig. S2*A*). To exclude the possibility that a high expression of two proteins in *Xenopus* oocytes leads to interactions already, we tested SLAC1 together with CPK31, a close homolog of CPK21 and 23 within the CDPK kinase family. Using this kinase no fluorescence complementation was observed (Fig. 1*B*). Coexpression of CPK21 or 23 together with ABI1 did generate a functional YFP molecule as well (Fig. 1*C* and Fig. S2*C*), indicating a direct interaction for these kinase-phosphatase pairs similar to that of OST1/ABI1 (6). In contrast, the PP2C-type phosphatase HAB1 (ABI1 homolog) (22) coexpressed with CPK23 did not restore YFP fluorescence in oocytes (Fig. 1*D*). Due to the fact that the discrimination between cytosolic and plasma membrane localization was not possible, BiFC experiments in oocytes were used to identify interacting partners, only. Because strongest BiFC signals were obtained between SLAC1 and CPK23 (Fig. 1*A*), in the following we focused on the action of CPK23 on the guard cell anion channel. To explore the interaction region between SLAC1 and the kinases we performed BiFC experiments between CPK23 and the N- and C-terminus of SLAC1 (amino acids 1–186 and 496–556, respectively). YFP complementation could only be monitored between the protein kinase and the SLAC1 N-terminus but not with the C-terminus (Fig. S2*B*). To confirm the channel-kinase interaction, we additionally performed BiFC experiments with *Arabidopsis* cells. The results gained from BiFC experiments in mesophyll protoplasts underpined those from the oocyte system and thus the notion that CPK23 directly interacts with SLAC1 (Fig. 1 *E* and *F*). Using the *Arabidopsis* protoplast system the YFP signal could be localized at the level of the plasma membrane, exclusively. To gain a measure for protein–protein interaction independent from BiFC assays, we conducted in vivo pull down experiments. Transient expression in *Arabidopsis* mesophyll protoplasts has recently been proven to be suitable for interaction studies between ABA signal transduction elements (23). Concerning the guard cell anion channel, N-terminal and C-terminal domains of SLAC1 fused to YFP were subjected to cotransfection with Strep-tagged CPK23 kinase and compared to kinase-free controls (Fig. 1*G*). After coexpression followed by the Strep-tag-based pull down step, SLAC1 NT::YFP protein could be detected with CPK23 (Fig. 1*G Lower*). In contrast, no protein enrichment of the C-terminal domain of SLAC1 was observed. These *in planta* data confirm those found with the heterologous oocyte expression system.

To study how protein–protein interaction between the anion channel SLAC1 and its potential regulators affects channel function, we explored in double-electrode voltage clamp (DEVC) experiments whether and how SLAC1 expression alone and coexpression with CPKs and ABI1 affects anion currents. Upon oocyte injection with SLAC1 or with CPK23 alone, no anion currents could be measured (Fig. 2 *A* and *B*). However, when SLAC1 was coexpressed with the kinase CPK23, typical SLAC-like anion currents could be elicited by membrane polarization (Fig. 2 *C* and *F*). Thereby the average current densities of

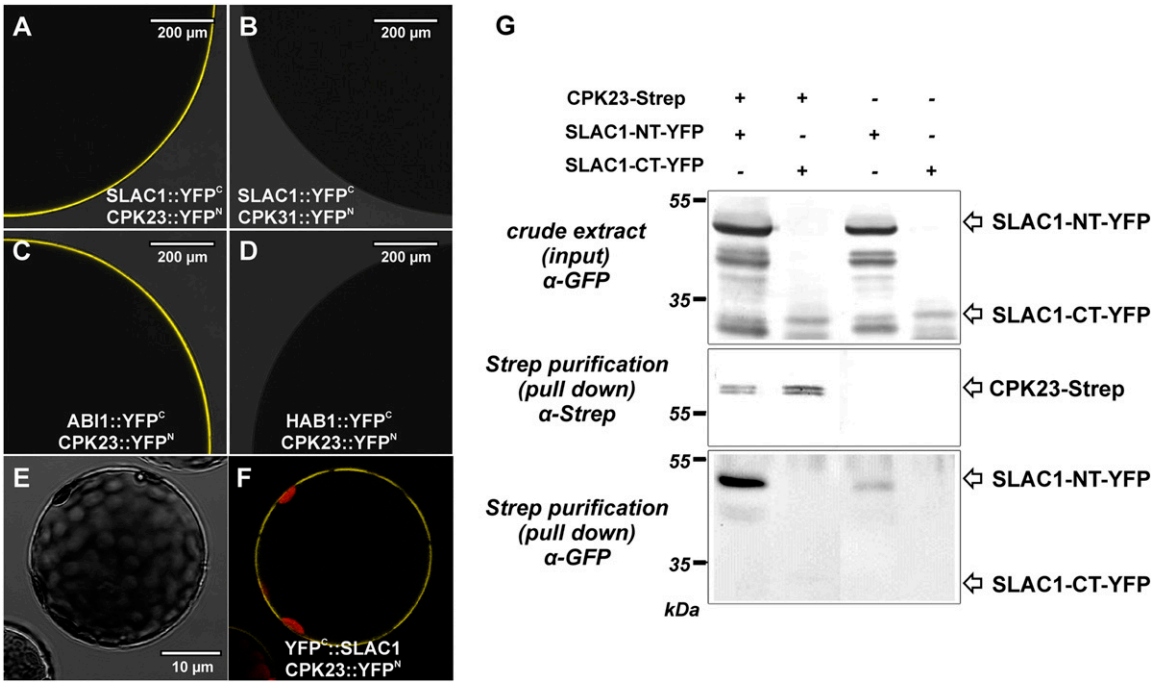

Fig. 1. Interaction studies among proteins involved in the ABA signaling pathway by bimolecular fluorescence complementation (BIFC) in *Xenopus* oocytes (*A–D*) and *Arabidopsis* mesophyll protoplasts (*E* and *F*). Pictures of oocytes, taken with a confocal laser scanning microscope, show a quarter of an optical slice of an oocyte. (*A*) SLAC1::YFP^C coexpressed with CPK23::YFP^N. (*B*) SLAC1::YFP^C coexpressed with CPK31::YFP^N. (*C*) ABI1::YFP^C coexpressed with CPK23::YFP^N. (*D*) HAB1::YFP^C coexpressed with CPK23::YFP^N. (*E* and *F*) Interaction of SLAC1 and CPK23 monitored in transiently transformed *Arabidopsis* mesophyll protoplasts by bimolecular fluorescence complementation. Plasmid combination was as follows: YFP^C::SLAC1 and CPK23::YFP^N. (*E*) Transmitted light. (*F*) Overlay of YFP and chlorophyll fluorescence. YFP fluorescence was emitted at the level of the protoplast plasma membrane. (*G*) CPK23 interacts with the N-terminus of SLAC1 *in planta*. *Arabidopsis* protoplasts were transfected with the indicated tagged constructs (+) and total protein extracts (input) were prepared. CPK23-Strep was affinity purified, and proteins were visualized using either anti-Strep detection system (*Middle*) or anti-GFP antibodies (*Upper* and *Lower*). NT-SLAC1-YFP and CT-SLAC1-YFP migrate at approximately 48 and 35 kDa, CPK23-Strep at about 60, respectively.

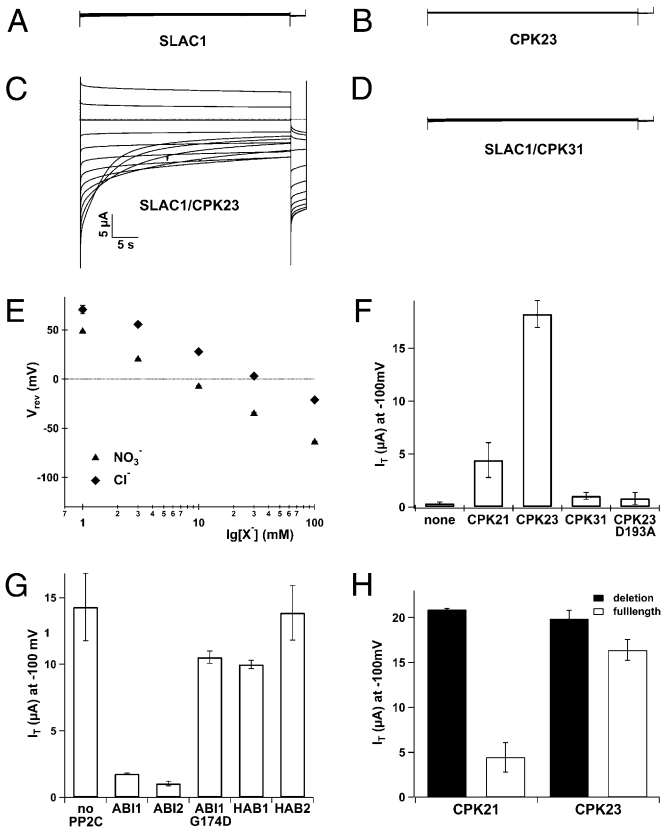

Fig. 2. Whole-oocyte current recordings in standard bath solution (30 mM Cl^-, pH 5.6). (*A–D*) Whole-oocyte current recordings upon 40-s-lasting voltage pulses ranging from +40 to −180 mV in 20 mV decrements followed by a 3 s voltage pulse to −120 mV. The holding potential was 0 mV. No current responses of ooyctes expressing CPK23 (*A*) or SLAC1 alone (*B*) could be recorded. Coexpression of CPK31 with SLAC1 mediated no anion currents either (*D*). Only coexpression of SLAC1 together with CPK23 (*C*) resulted in macroscopic anion currents slowly deactivating at negative membrane potentials. Representative cells are shown. (*E*) Reversal potentials for Cl^- and NO_3^- were shown as a function of the logarithmic external anion concentration. As expected for an anion-selective channel, the reversal potential shifted to more negative values with increasing anion concentrations ($n \geq 4$, mean ± SD). (*F*) Following a preactivation voltage pulse of 0 mV pronounced instantaneous anion currents (I_T) at −100 mV could be measured with SLAC1/CPK23-injected oocytes. No macroscopic currents were visible in oocytes coexpressing the kinase CPK31 together with SLAC1. SLAC1 activation by CPK21 was weaker than by CPK23. SLAC1 expression alone (indicated as none) as well as SLAC1 coexpression with CPK23 D193A did not result in macroscopic anion currents ($n \geq 4$, mean ± SD). (*G*) Inhibition of SLAC1 activity by coexpression of the protein phosphatases ABI1 and ABI2. Coinjection of the inactive ABI1 G174D, HAB1, and HAB2, however, could not prevent SLAC1/CPK23 mediated anion currents ($n \geq 3$, mean ± SD). (*H*) SLAC1 activation by a constitutive active C-terminal truncated Ca^{2+}-independent CPK23 (indicated as deletion) was slightly higher than activation by the wild-type CPK23 (full length). In the case of CPK21, this effect was more pronounced. Instantaneous anion currents (I_T) at −100 mV recorded in standard bath solution are shown ($n = 5$, mean ± SD).

SLAC1/CPK expressing oocytes reflected the different intensities of YFP fluorescence in the BiFC experiments (Fig. 1 *A* and *B*, and 2*F* and Fig. S2*A*). Coexpression of CPK31 together with SLAC1, for example, did not lead to any anion currents (Fig. 2 *D* and *F*). Due to the fact that CPK21 activated SLAC1 only moderately compared to CPK23 (Fig. 2*F*), we mainly focused on the electrical characterization of SLAC1 activated by CPK23. DEVC experiments with the functional SLAC1/CPK23 complex revealed that SLAC1 currents exhibited similar electrophysiological characteristics to that seen with OST1-activated SLAC1 (Fig. 2*E* and Fig. S3 and *SI Text 1*) (6). Note that for DEVC experiments, native constructs without any fused (half) YFPs were used.

Guard cells derived from *abi1-1* mutant lines that express a deregulated protein phosphatase 2C, lack SLAC-type anion channels upon ABA activation (11). To test whether ABI1 inhibit SLAC1 activation by CPKs as well, we coexpressed SLAC1, CPK23, and PP2Cs. When the functional anion channel/CPK complex was coexpressed with the protein phosphatase ABI1 or ABI2 (24, 25), anion currents were abolished (Fig. 2*G*). However, the PP2Cs HAB1 and HAB2 (22) could not prevent SLAC1 anion currents. BIFC experiments with CPK23::YFP^N and ABI1::YFP^C or HAB1::YFP^C (Fig. 1 *C* and *D*) are in agreement with the anion current recordings (Fig. 2*G*). Coexpression of CPK23 together with ABI1 restored YFP fluorescence, whereas CPK23 and HAB1 did not show BiFC-based protein interaction. Thus in the presence of ABI1, SLAC1 cannot be activated even when this guard cell anion channel is facing CPK23. Interaction with these protein kinases likely results in SLAC1 phosphorylation and renders the guard cell anion channel active. To test whether phosphorylation is essential for SLAC1 activity, we disrupted the kinase activity of CPK23 (*SI Text 2*). When coexpressed with SLAC1::YFP^C in the oocyte BiFC system, the CPK23 D193A::YFP^N mutant still complemented YFP fluorescence (Fig. S2*C*) but failed to activate SLAC1 mediated anion currents (Fig. 2*F*). Thus SLAC1 activation likely depends on phosphorylation by CPK23 (*SI Text 3*).

To examine whether SLAC1 activity in vivo is functionally correlated with the presence of CPK23, SLAC-type anion channel currents were quantified by patch clamp studies with guard cell protoplasts from wild-type plants, as well as *slac1-3* and *cpk23* loss-of-function mutants. Note that the same conditions (elevated cytosolic Ca^{2+} levels) were used to study guard cell anion channels in CPK3 and 6 mutants (19). In the absence of ABA and presence of 2 µM cytosolic free Ca^{2+}, S-type anion channels appeared active in wild-type cells (Fig. 3*A*). However, in the *cpk23* mutant the anion current density was prominently reduced (at −104 mV by 70%) in comparison with wild-type guard cells (Fig. 3*B*). Protoplasts of *slac1-3* loss of function mutants lacked anion currents.

To test whether these ABA signaling components are coexpressed with SLAC1 in *Arabidopsis* guard cells, we performed quantitative real time PCR of SLAC1, GORK, CDPKs, and ABI1 transcripts in wild-type guard cells in comparison with the respective mRNA levels in the *cpk23* knock out mutant (Fig. S4)

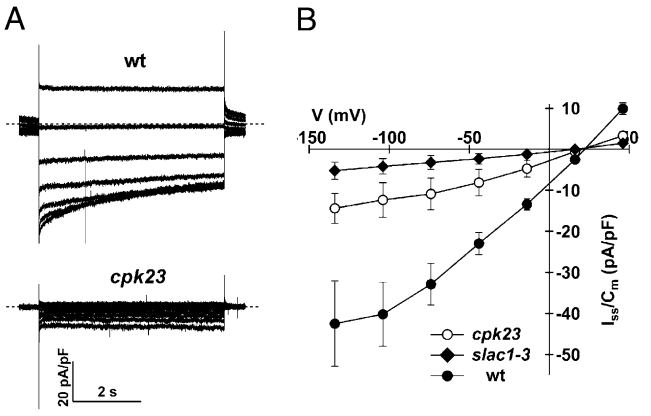

Fig. 3. (*A*) Macroscopic anion current recordings from *Arabidopsis thaliana* guard cell protoplasts in the presence of 2 µM cytosolic Ca^{2+}. Representative current responses from wild type and *cpk23 (Salk 007958)* to 7.5-s-lasting voltage pulses from +46 mV to −134 mV are shown. (*B*) Steady-state current densities plotted against the clamped voltages. Data points represent the mean ± SE. The number of at least three independent experiments were n = 7 for *cpk23* (open circles) and n = 6 for wild type (closed circles).

(26–27). For this purpose, we have chosen members of the CDPK kinase family that were shown to be involved in ABA-signaling in guard cells (19, 20, 28). qRT-PCR analysis showed that the transcript levels of SLAC1 and CPK3, 4, and 6, as well as the outward rectifying K^+ channel GORK, were not significantly altered ($P > 0.05$). Transcripts of CPK11 in guard cells of CPK23 knock-out mutant were found to be reduced by 40% relative to those in wild-type plants ($P < 0.05$). Expression of ABI1 and CPK21, however, was up-regulated by a factor of 2.5 ($P < 0.05$). Given the fact that the expression of CPK21—the closest homolog of CPK23—is up-regulated when CPK23 function is lost, we studied the effect of the CPK23 sister on SLAC1 in the oocyte system. Indeed, CPK21 was BiFC positive (Fig. S2*A*) and activated SLAC1 (Fig. 2*F*). Similar to the inhibition of CPK23-activated SLAC1 currents, ABI1 prevented anion currents of SLAC1/CPK21 coexpressing oocytes (88% inhibition for CPK23 and 85% for CPK21 at −100 mV).

In *Arabidopsis* guard cells, SLAC-type anion channels were reported to be sensitive to the protein kinase inhibitor K252a (12, 15). To study the ability of CPK23 to phosphorylate SLAC1 we performed in vitro kinase assays with recombinant CPK23 and either SLAC1 N- or C-termini. Using radio-labeled [γ^{32}]-ATP, we could show that CPK23 phosphorylates the N-terminus of SLAC1 exclusively (Fig. S5*A*). These results confirm our oocyte BIFC-experiments, in which an interaction of CPK23 with SLAC1 N-terminus but not with SLAC1 C-terminus was observed (Fig. S2*B*). In contrast, the kinase inactive CPK23 D193A mutant did not phosphorylate the SLAC1 NT (Fig. S5*B*). When performing in vitro kinase assays with the N-terminus of SLAC1 and the recombinant CPKs, we could observe similar phosphorylation activities for CPK21 and CPK23 in the presence of 3 μM Ca^{2+} (Fig. S5*C*). In contrast, no or only weak phosphorylation of the SLAC1 NT was observed in the presence of ABI1 with both CPKs, 21 and 23 (Fig. S5*D* and Fig. 4*A Upper*).

Recently the cytosolic RCAR1/PYR/PYL ABA receptor family has been identified and demonstrated to directly control the activity of PP2C protein phosphatases in an ABA-dependent manner (23, 29). To reconstitute the fast, posttranslational ABA signaling pathway of guard cells we tested whether the CPK23- and OST1-dependent phosphorylation of SLAC1 is also controlled by this ABA-receptor/phosphatase complex. Therefore, in vitro kinase assays were performed in the presence or absence of ABA with RCAR1, ABI1, CPK23, or OST1 and the N-terminus of SLAC1 (Fig. 4*A*). As a result, we could show that ABI1 (lane 3 and 4), rather than RCAR1 (lane 5 and 6), suppressed phosphorylation of SLAC1 NT. This inhibition of CPK23 (*Upper*) or OST1 action (*Lower*) appeared ABA-independent (Fig. 4*A*). The latter observation is in line with the inhibition of CPK23- or OST1-activated SLAC1 anion currents by ABI1 in oocytes (Fig. 2*G*) (6). In combination with RCAR1, ABI1, CPK23, or OST1 and the N-terminus of SLAC1, however, phosphorylation of SLAC1 NT appeared to be triggered by ABA only (Fig. 4*A*, lane 7 and 8). This result documents the successful reconstitution of the basic fast ABA-signaling pathway from ABA perception to anion channel phosphorylation/activation. This shows that the SLAC1 activating kinases CPK23 and OST1 are dictated by the ABA-dependent RCAR/ABI1 complex. In line with the inhibition of OST1 and CPK23, ABI1 suppressed CPK21 activity (Fig. S5*D*), indicating that ABI1 represents an inhibitor of the SLAC1 kinases CPK23/21 as well as OST1. Interestingly, CPK23 exhibited an enhanced autophosphorylation activity in the presence of ABI1 (Fig. 4*A*, lane 3 and 4 *Upper*). Moreover, in the presence of RCAR1, ABI1 appeared phosphorylated by CPK23 in the presence of ABA only (Fig. 4*A*, lane 7 and 8 *Upper*). In contrast to CPK23, OST1 autophosphorylation was inhibited by ABI1 (Fig. 4*A*, lane 3 and 4 *Lower*) and ABI1 was only phosphorylated by OST1 in the presence of RACR1 and ABA (Fig. 4*A*, lane 8 *Lower*).

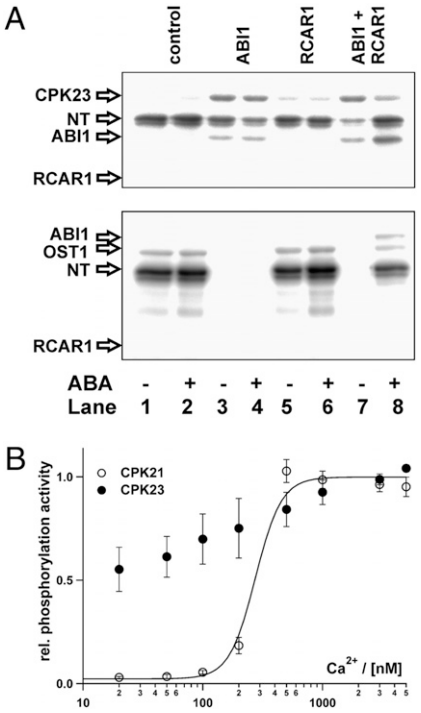

Fig. 4. In vitro kinase assays using recombinant proteins. (*A*) ABA-dependent phosphorylation of SLAC1 by CPK23 (*Upper*) and OST1 (*Lower*) is mediated by the ABA-receptor/PP2C phosphatase complex RCAR1/ABI1. (Lanes 1 and 2) ABA-independent SLAC1 NT phosphorylation by CPK23 or OST1. (Lanes 3 and 4) ABI1 inhibited the phosphorylation of SLAC1 NT by CPK23 or OST1 independent from the presence of ABA. (Lanes 5 and 6) RCAR1 was neither in the presence or absence of ABA capable to prevent SLAC1 phosphorylation by CPK23 or OST1. (Lanes 7 and 8) Combination of SLAC1 NT, CPK23 or OST1, ABI1 and RCAR1 in one reaction mixture: the phosphorylation of SLAC1 was dependent on the availability of ABA. The recombinant proteins SLAC1 NT, CPK23, and OST1 were GST-tagged whereas RCAR1 carried a His-tag. Note that recombinant ABI1 was His-tagged in *Upper* and GST-tagged in *Lower*. (*B*) In vitro phosphorylation activity of CPK21 and 23 as a function of various Ca^{2+} concentrations. The affinity toward Ca^{2+} of CPK21 could be best described by a Hill equation with a Hill factor of four. In contrast CPK23 exhibited a 60% core activity even at very low Ca^{2+} concentrations and was only weak Ca^{2+} dependent.

Anion currents from SLAC1/CPK23 expressing oocytes could be observed when SLAC1 was coexpressed with either full-length CPK23 or its truncated CPK23 variant, lacking its junction and calcium-binding domain. C-terminal truncation was shown to render CDPK kinases constitutively active and thus Ca^{2+}-insensitive (17, 18, 30). Interestingly, however, anion currents were only 20% higher with the truncated CPK23 version (Fig. 2*H*) documenting that resting $[Ca^{2+}]_{cyt}$ levels in oocytes seem to be sufficient for full-length CPK23 kinase activation (31). In oocyte experiments with coexpressed SLAC1 and wild-type CPK21, however, only weak anion currents were observed (Fig. 2 *F* and *H*). The Ca^{2+}-independent, constitutive active version of this CPK, however, exhibited SLAC1-derived anion currents similar to CPK23/SLAC1 expressing oocytes (Fig. 2*H*). These indications stimulated us to elucidate SLAC1 phosphorylation/activation under well defined Ca^{2+} and ATP levels using in vitro kinase assays. Ca^{2+} activity dependent SLAC1 NT phosphorylation was studied in the presence of CPK21 and CPK23. We recorded SLAC1 NT phosphorylation of high Ca^{2+} affinity with CPK21 whereas a low calcium affinity was observed with CPK23 (Fig. 4*B*). Reminiscent to four Ca^{2+}-binding EF-hands in the C-terminus of CDPK kinases (17), the Ca^{2+}-dependent phosphorylation activity of CPK21 could be best fitted by a Hill

equation with a Hill-constant of four (Fig. 4*B*). These in vitro kinase assays show that the Ca^{2+}-sensitivity of guard cell expressed CPK23 and 21 differ: (*i*) CPK21 is of high affinity ($K_{0.5} = 277 \pm 24$ nM), (*ii*) in contrast, CPK23 exhibits a composite calcium dependence with a Ca^{2+}-independent (60%) core activity and a Ca^{2+}-inducible component of low affinity. These results assign CPK23 to the weak Ca^{2+}-dependent ABA-signaling branch. The Ca^{2+} dependence of CPK21 is in line with our very recent quantification of calcium-dose-dependent SLAC-type channel activation of intact plants (32). In guard cells, half maximal S-type anion currents were activated by cytosolic Ca^{2+} concentrations in the 300- to 500-nM range.

Discussion

ABA-activation of guard cell anion channels in leaves of intact plants takes place under two different scenarios: one is Ca^{2+}-independent (8, 9), whereas the other is associated with changes in cytosolic Ca^{2+} levels (7, 33). Very recently we could show that the Ca^{2+} independent protein kinase OST1 activates SLAC1 derived anion currents upon ABA treatment in guard cells (6). OST1, in turn, is under the control of ABI1. Here we show that CDPK protein kinases are able to control the activation state of the slow guard cell anion channel in response to different Ca^{2+} concentrations as well as in a Ca^{2+}-independent manner. In line with the control of OST1 activity by ABI1, CPK23 and 21 turned out to be negatively regulated by ABI1 as well. One would thus predict plants with a constitutively low CPK but high ABI1 activity/density, or vice versa, that gain an ABA phenotype. The strong phenotype of ABA-insensitive *abi1-1* and *ost1* mutant plants (11, 34–37) and ABA-hypersensitive *abi1* knock out plants (38) are supporting this hypothesis.

Interestingly, in guard cell protoplasts a strong reduction of SLAC-like anion currents became evident in *cpk23* loss-of-function mutants, although the regulation of SLAC1 by CPK21 seems to compensate the CPK23 defect (Fig. S4). This observation becomes even more astonishing when considering the dual activation by OST1 and CPKs. Because ABI1 negatively regulates both types of protein kinases, it is tempting to speculate that a mutual equilibrium in OST1/ABI1- and CPK23-21/ABI1-kinase/phosphatase-dependent regulation exists, which fine tunes the activity of SLAC1. Upon perturbation (in respective kinase mutants), this equilibrium is distorted either due to the lack of activation by kinases and/or due to overcompensation of both pathways by ABI1. qRT PCR experiments with guard cells of *cpk23* knock out plants in comparison with wild-type plants confirmed this notion (Fig. S4). Disruption of CPK23 feeds back on the transcript level of CPK21 and most importantly resulted in the up-regulation of ABI1. Although CPK23 activates SLAC1 and ABI1 inhibits channel activity, in our hands the *cpk23* mutant did not exhibit a stoma phenotype. Under certain environmental conditions, this mutant seems to exhibit water stress phenotypes opposite to what one would expect from a loss of SLAC1 activation (20, 37). Such a behavior might be explained by a case-dependent misbalance between ABI1 and its interacting protein kinases leading to the inhibition of SLAC1 activation by ABA.

OST1/CPK23 and ABI1 seem to be associated with the calcium-independent steps in ABA signaling, whereas CPK21/ABI1 rather regulates SLAC1 in response to cytosolic calcium signals. Interaction of SLAC1 with protein kinases such as calcium-dependent CPK21 on one side and calcium-independent CPK23/OST1 on the other points to a bifurcated signal transduction pathway (Fig. S6). This is further corroborated by the fact that SLAC1 phosphorylation by CPK23 and OST1 are not identical (Fig. S3*F* and *SI Text 3*). Both pathways converge on the level of the anion channel SLAC1.

Based on our studies with SLAC1, ABI1 and CPK23/21 shown here and by Geiger et al. (6), we would suggest the following chain of events for ABA-induced stomatal closure (Fig. S6): (*i*) under water stress ABA is synthesized and transported to the stomata; (*ii*) ABA enters the guard cells and binds to the cytosolic PYR/PYL/RCAR-type ABA receptors (23, 29); (*iii*) receptor activation inactivates ABI1; (*iv*) OST1, CPK23, and CPK21 are relieved from ABI1 inhibition; (*v*) whereas OST1 and CPK23 are active at resting cytosolic Ca^{2+} concentrations, CPK21 requires a rise in cytosolic Ca^{2+}; (*vi*) finally, SLAC1 is activated by phosphorylation; (*vii*) SLAC1 opens, anions are released and depolarize the membrane potential; (*vi*) depolarization activates potassium release channel GORK (39); (*viii*) guard cells release osmotically active compounds (anions and K^+); and (*ix*) guard cell pairs deflate by concomitant water loss and subsequently the stomatal pore closes.

Methods

Real-Time PCR. Quantification of actin2/8 and SLAC1, OST1, ABI1, GORK, and CDPK-transcripts was performed by RT-PCR as described elsewhere (40). Rosette leaves of 6- to 8-week-old *Arabidopsis thaliana* ecotype Columbia (Col-0) and *cpk23* (Salk 007958) plants were harvested for RT-PCR analysis. Transcripts were each normalized to 10,000 molecules of actin2/8. Primers are listed in *SI Methods*.

Oocyte Recordings. The cDNA of SLAC1, CPK3, 6, 21, 23, 31, ABI1, ABI2, HAB1, and HAB2 were cloned into oocyte (BIFC) expression vectors by an advanced uracil-excision-based cloning technique described by Nour-Eldin et al. (41). Oocyte preparation and cRNA generation and injection have been described elsewhere (42). In two-electrode voltage-clamp studies (TEVC) oocytes after 2 or 3 days of expression were perfused with Kulori-based solutions. For detailed information regarding solutions, pulse protocols, and data analysis see *SI Methods*.

BIFC Experiments. The cDNA of SLAC1 and CPK23 were cloned into plant binary vectors (based on pCAMBIA vectors) as described by Nour-Eldin et al. (41). Transient protoplast expression was performed using the polyethylene glycol transformation method modified after (43); 16–28 h after transformation protoplast images were taken. For documentation of the oocyte and protoplast BIFC results, pictures were taken with a confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss Jena GmbH.) equipped with a Zeiss Plan-Neofluar 20×/0.5 objective for oocyte images and a Zeiss Plan-Neofluar 63×/1.25 oil objective for protoplasts. Images were processed (low-pass filtered and sharpened) identically with the image acquisition software LSM 5 Pascal (Carl Zeiss).

Cloning of *in Planta* Expression Constructs and in Vivo Pull Down Experiments. NT-SLAC1, CT-SLAC1, and CPK23 cDNAs were cloned in pJET1.2/blunt using CloneJETTMPCR Cloning Kit (Fermentas) and transferred via the introduced restriction sites into destination vectors: In case of CT-SLAC1 and NT-SLAC1 into pXCS-YFP (44) and in case of CPK23 into pXCS-HA-Strep (AY457636) (45) yielding pXCS-NT-SLAC1-YFP, pXCS-CT-SLAC1-YFP, and pXCS-CPK23-HA-Strep, respectively. For in vivo pull down experiments see *SI Methods*.

Patch-Clamp Experiments on Guard Cell Protoplasts. *A. thaliana* ecotype Columbia (Col-0), *cpk23 (Salk 007958)* and *slac1-3* mutants were grown on soil in a growth chamber at a 8/16 h day/night regime and 22/16 °C day/night temperature. Enriched protoplasts were stored on ice until aliquots were used for whole-cell patch clamp recordings of S-type anion currents, which were performed essentially as described by (19, 46, 47). For details of protoplast generation and patch clamp conditions see *SI Methods*.

Protein Purification and in Vitro Kinase Assays. OST1, CPK21, and 23, as well as ABI1, SLAC1 NT and CT, were subcloned into the recombinant expression vector pGEX 6P1 (GE Healthcare) and transformed into *Escherichia coli* (DE3) pLysS strain (Novagen). GST- or His-tagged recombinant proteins were purified as described by Belin et al. (48). Recombinant RCAR1 was purified as reported previously (23). In vitro kinase buffer was composed of 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 × protease inhibitor mixture (Roche), 2 mM DTT, 5 mM EGTA, and 5 μCi [γ-^{32}P] ATP (3,000 Ci/mmol). To obtain defined free Ca^{2+} concentrations, we calculated the amount of $CaCl_2$ to be added with the WEBMXC extended website (http://www.stanford.edu/~cpatton/maxc.html based on ref. 49). In vitro kinase assays in Fig. 4*A* and Fig. S5 *A–D* were performed in the presence of 3 μM free Ca^{2+}. The Ca^{2+} dependent phosphorylation activity of CPK21 and 23 was determined by counting radioactivity of excised gel slices harboring the SLAC1 NT. The affinity toward Ca^{2+} of CPK21 could be best described by a Hill equation with a Hill factor of four. For details see *SI Methods*.

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