Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair

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In response to drought stress the phytohormone ABA (abscisic acid) induces stomatal closure and, therein, activates guard cell anion channels in a calcium-dependent as well as-independent manner. Two key components of the ABA signaling pathway are the protein kinase OST1 (open stomata 1) and the protein phosphatase ABI1 (ABA insensitive 1). The recently identified guard cell anion channel SLAC1 appeared to be the key ion channel in this signaling pathway but remained electrically silent when expressed heterologously. Using split YFP assays, we identified OST1 as an interaction partner of SLAC1 and ABI1. Upon coexpression of SLAC1 with OST1 in Xenopus oocytes, SLAC1-related anion currents appeared similar to those observed in guard cells. Integration of ABI1 into the SLAC1/OST1 complex, however, prevented SLAC1 activation. Our studies demonstrate that SLAC1 represents the slow, deactivating, weak voltage-dependent anion channel of guard cells controlled by phosphorylation/dephosphorylation.

ABA signaling | S-type anion channel | OST1/ABI1

Guard cells in the epidermis of plants balance the uptake of CO2 from the atmosphere and the concomitant loss of water from leaves (1–5). When water supply is limited, the drought hormone abscisic acid (ABA) triggers release of anions and K+ from guard cells (6–9). The decrease in guard cell osmotic pressure and volume results in stomatal closure, reducing transpirational loss of water from the leaf.

The initial steps in ABA signal transduction have been shown to activate guard cell anion channels in a calcium-dependent as well as-independent manner (10–13). Elements of these ABA signaling pathways, however, have been identified by genetic screens revealing ABA-insensitive and open-stomata plant mutants with deregulated guard cell volume control (14–17). Among them are protein phosphatases of the PP2C family [ABI1 (ABA insensitive 1) and refs. 2, 14, 18] and a Snf1-related protein kinase 2 [SnRK2.6 named open stomata 1 (OST1)], exhibiting the strongest phenotypes. The type-2C protein phosphatases ABI1 and ABI2 were identified initially on the basis of the ABA-insensitive abi1–1 and abi2–1 dominant mutations (14, 16, 18, 19). Guard-cell activity is impaired in these ABA-insensitive mutants, and, as a consequence, the stomata remain constitutively open even under drought (20). Characterization of loss-of-function alleles indicated that ABI1 and ABI2 are negative regulators of ABA action (21, 22). Using infrared thermography, the Arabidopsis mutants ost1–1 and ost1–2 (SnRK protein kinase family 2) appear “cold” under drought conditions due to their inability to limit their transpiration (17). These recessive ost1 mutations are disrupted in ABA-induced stomatal closure and inhibited in stomatal opening. The Snf1-related kinase 2 (SnRK2) proteins from several plant species have been implicated in ABA signaling pathways (e.g., ref. 15). In Arabidopsis guard cells, OPEN STOMATA 1 (OST1/SRK2E/SnRK2–6) has been described as a critical positive regulator of ABA signal transduction (17, 23). Moreover, OST1 activation in response to ABA is suppressed in the dominant abi1–1 mutant (17), indicating that the protein phosphatase ABI1 (14, 16, 19) negatively regulates ABA signal transduction upstream of OST1. In abi1–1 mutant plants, anion channels fail to respond to ABA (24). From the given information, it seems that the anion channels require phosphorylation for activity (25, 26).

Recently the first guard cell anion channel was identified. An ABA– and CO2/O3-insensitive mutant was shown to lack a gene encoding a putative guard cell anion transporter named SLAC1 (27, 28). In guard cells of these mutant plants, anion currents appeared largely suppressed. When expressed heterologously, SLAC1, however, remained electrically silent (27, 28). Thus to elicit the function of SLAC1 and its role in ABA signal transduction, we searched for partners interacting with this putative anion channel. Using protein–protein interaction assays we identified the protein kinase OST1 and the protein phosphatase ABI1 as regulators of SLAC1 within the ABA transduction pathway (14, 16, 17). When SLAC1 was expressed with OST1 in Xenopus oocytes, SLAC1-related anion currents similar to those observed in guard cells appeared (24, 29). The presence of ABI1, however, prevented SLAC1 activation. Our studies demonstrate that SLAC1 is controlled by OST1/ABI1-dependent phosphorylation/dephosphorylation.

Results

Using gas exchange measurements with intact Arabidopsis leaves, we could show that ost1–2 stomata during day-night transition close much slower than those of WT plants (Fig. S1a). In the light, ost1–2 stomata opened but could not properly adjust their stomatal aperture in response to ongoing water and turgor loss (Movie S1). Lack of OST1-dependent stomatal closure apparently gave rise to deregulated stomatal function and consequently permanent wilting (Fig. S1b and c, cf. refs. 15, 17, 30 and Movie S1). Previous studies characterized the involvement of ABI1 in regulation of slow guard cell anion channels (e.g., ref. 24). In contrast, such S-type anion channels have not yet been analyzed in the background of ost1 mutants. We therefore examined S-type anion channels in guard cells of ost1 mutant plants and assessed the interaction of SLAC1 with OST1 in vivo. In patch clamp experiments, guard cell protoplasts of Arabidopsis


The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912021106/DCSupplemental.
A. thaliana were loaded with 110 nM cytosolic free Ca\textsuperscript{2+} and stimulated with ABA. Under these conditions the macroscopic S-type anion currents in open-stomata mutant ost1–2 appeared largely suppressed compared to WT (Fig. 1A and B).

To test whether these previously identified ABA signaling components are coexpressed with SLAC1 in Arabidopsis guard cells, we performed quantitative real time PCR of SLAC1, OST1, and ABI1 transcripts in guard cells in comparison to the respective mRNA levels in mesophyll cells (Fig. S2; cf. refs. 31, 32). qRT-PCR analysis showed that SLAC1 and OST1 (17) expression appeared to be guard cell specific, while expression of ABI1 was found in both cell types.

Vahisalu et al. (28) showed that enzymatically isolated guard cell protoplasts from SLAC1 mutants exhibit a largely reduced S-type channel activity (Fig. 1B). We used Xenopus laevis oocytes, a well accepted heterologous expression system for animal, plant, or bacterial channels and transporters, to study whether the gene product of SLAC1 exhibits the S-type anion channel activity in dependence of OST1. The interaction of these putative signaling components was visualized with the bimolecular fluorescence complementation technique (BiFC, 33). SLAC1 and several protein kinases and phosphatases were fused to a complementary half of split YFP each (for illustration of constructs see Fig. S3). Various cRNA combinations were injected into Xenopus oocytes and analyzed by confocal microscopy (Fig. 2 and Fig. S5). Note, that BiFC experiments in oocytes identify only interaction of partners. Targeting of proteins to the oocyte plasma membrane or cytosol was not distinguished by these fluorescence measurements. When SLAC1, fused to the C-terminal half of the YFP (YFP\textsuperscript{C}), was expressed with the complementary N-terminal half of the YFP (YFP\textsuperscript{N}, Fig. 2B) no specific YFP fluorescence was emitted from oocytes. Upon coinjection of SLAC1::YFP\textsuperscript{C} and YFP\textsuperscript{N} fused to potential interaction partners, fluorescence signals could be detected between SLAC1 and OST1 (Fig. 2C) via complementation of a functional YFP molecule. To exclude the possibility that high
expression of 2 proteins in Xenopus oocytes leads to interactions already, we tested close homologues of OST1 (SnRK 2.2/2.3/2.8) together with SLAC1. In contrast to OST1, SnRK 2.2/2.3/2.8 interaction with SLAC1 appeared much weaker (Fig. S5c). In addition, it would be ruled out that the positive BIFC signals might represent nonspecific responses by using the K+ channel GORK together with OST1 as a negative control (Fig. S5b, see Supplemental Text 1). To explore the interaction site between SLAC1 and the kinase we performed BIFC experiments between OST1 and the N and C terminus of SLAC1 (amino acids 1 to 186 and 496 to 556, respectively). YFP complementation could only be monitored between the SLAC1 N terminus and the protein kinase (Fig. S5a). Coinjection of OST1::YFPN with AB1::YFPC caused YFP emission as well (Fig. 2D), indicating that OST1 directly interacts with AB1 (34). To investigate the in planta interactions between the ABA signaling components we performed BIFC experiments with Arabidopsis mesophyll protoplasts. After protoplast transformation with YFPC::SLAC1 and YFPN::OST1, SLAC1 currents appeared in oocytes (28), however, did not result in macroscopic anion currents with the split YFP-fused constructs. In two-electrode voltage clamp experiments of SLAC1 expression in oocytes (28), however, did not result in anion transport-competent cells. In two-electrode voltage clamp (TEVC) experiments we thus explored whether SLAC1 cRNA injection in Xenopus oocytes generates a functional anion transporter, when expressed with those ABA signaling components confirmed the data found with the heterologous oocyte expression system.

SLAC1 was annotated as a bacteria-like dicarboxylate carrier because of sequence similarities with the yeast MATE1 transporter (27, 28, 35, 36). Complementation and uptake experiments with a malate-transport deficient yeast mutant (27) and SLAC1 expression in oocytes (28), however, did not result in anion transport-competent cells. In two-electrode voltage clamp (TEVC) experiments we thus explored whether SLAC1 cRNA injection in Xenopus oocytes generates a functional anion transporter, when expressed with those ABA signaling components found to interact with the potential anion channel (cf. ref. 37). Oocyte injection with AB11, OST1 or SLAC1 alone did not result in macroscopic anion currents (Fig. 3A Upper). However, when coexpressed with OST1, currents of up to 50 μA appeared in chloride-based media (Fig. 3A Lower). OST1 activation of SLAC1, however, could be detected in about 25% of the oocyte batches only. In contrast, when using split YFP-fused constructs, SLAC1::YFPd and OST1::YFPN, SLAC1 currents appeared in each single oocyte. Note, that YFP-fusion did not affect the anion channel characteristics (Fig. S4) and that SLAC1 expression alone never led to macroscopic anion currents. Therefore we performed the biophysical characterization of SLAC1-mediated anion currents with the split YFP-fused constructs. Upon application of long lasting voltage pulses to negative membrane potentials, instantaneous SLAC1 currents were recorded, which were followed by a slow deactivation (Fig. 3A Lower, Fig. S6a), anion current characteristics reminiscent to S-type anion currents in intact guard cells (cf. ref. 38). Upon increasing the bath Cl− concentration from 10 to 100 mM the half-maximal activation voltage (V1/2) of SLAC1 channels shifted by 51 mV from −49.6 ± 5.8 mV to −100.4 ± 7.9 mV (Fig. S6c). This result indicates that the voltage dependency of SLAC1 is sensitive to the external chloride concentration. In agreement with an anion-permeable conductance, a reduction of the Cl− concentration in the bath shifted the reversal potential (Vrev) to positive membrane voltages (Fig. S6b and d). A 10-fold change of the external chloride concentration resulted in a 49.7 ± 1.6 mV shift of Vrev (Fig. S6d). In comparison the anions nitrate and thiocyanate similarly shifted Vrev by −51.4 ± 3.0 mV and −52.9 ± 1.9 mV, respectively, upon a 10-fold change of the external anion concentration (Fig. S6d). Replacement of Na+ by K+ or Li+, however, had no effect on SLAC1 currents and reversal potentials (Fig. S7a). To estimate the permeability of SLAC1 for physiologically relevant anions relative to chloride, the halide was replaced by NO3−, SO42−, HCO3− and the dicarboxylate malate and the shift in the respective reversal potentials was determined (Fig. B). The derived relative anion permeability sequence of I− (16.92 ± 0.28) > NO3− (8.19 ± 0.44) > Br− (4.02 ± 0.309) > Cl− (1 ± 0.00) > HCO3− (0.05 ± 0.01) > malate− (0.04 ± 0.00) > SO42− (0.04 ± 0.00) characterized SLAC1 as an anion-selective channel with preference to NO3− and Cl− (cf. ref. 39). SLAC1 was isolated in a screen for mutants with defects in stomatal CO2 sensitivity. The impermeability for HCO3− and the lack of HCO3−-induced activity changes of SLAC1 indicate that the anion channel does not sense the CO2 concentration directly. To exclude that acidic bicarbonate or malate buffers could have side-affected the measurements of the SLAC1 conductance, the pH dependence of SLAC1 was analyzed in 100 mM chloride solution. Changing the bath pH from 5 to 6 and 7, SLAC1 activity remained unaffected (Fig. S7b), indicating that protons do not drive chloride transport (cf. 40, 41). In patch clamp studies with plant protoplasts, the anion channel blocker DIDS has previously been shown in plants to inhibit anion currents (42–44). Addition of 100 μM DIDS or...
SLAC1::YFPC in the oocyte BiFC system, OST1 D140A::YFPN aspartate (see also essential for SLAC1 activity, we disrupted the kinase activity of guard cell anion channel active. To test whether phosphorylation is kinase likely results in SLAC1 phosphorylation and renders the BIFC experiments with OST1::YFP N and ABI1::YFP C or b YFP complementation with HAB1 (Fig. S5 to activate SLAC1 mediated anion currents (Fig. 3 [2 hours of incubation with recombinant OST1 and radiolabeled \([\gamma^{32}P]\) ATP, we could identify 3 regions of the SLAC1 N terminus, which were phosphorylated by OST1 (position from R41 to L60, from S71 to F90 and from T101 to D130, Table S1). To further confirm this observation, we used a site-directed mutagenesis approach. Thereby all highly predicted serine/threonine phosphorylation sites in the SLAC1 N terminus (predicted by NetPhos2.0; http://www.cbs.dtu.dk/services/NetPhos/ or Scansite motif Scan; http://scansite.mit.edu/motifscan.seq.phtml) were mutated. Testing the respective SLAC1 mutants, we identified residue Ser-120 as an important OST1 target. Replacement of serine 120 by alanine did not affect YFP complementation (Fig. S5b), but SLAC1 could not be activated by OST1 anymore (Fig. 3c). When Ser-120 was substituted by aspartate, to mimic phosphorylation, SLAC1, however, was not active in the absence of OST1. This indicates that Ser-120 represents a critical amino acid residue but its phosphorylation seems to be not sufficient for SLAC1 activation. The specificity of SLAC1 activation by OST1 was tested by using close homologues of OST1. Coexpression of the OST1 homologues SnRK2.2/2.3 and 2.8 activated SLAC1 to a much weaker extent than OST1 (Fig. 3c). These findings are well in line with the results obtained by our BiFC experiments (Fig. S5c).

ABI1 was supposed to act upstream of OST1, repressing OST1 kinase activity upon ABA treatment (17). To test whether potential negative regulators inhibit SLAC1 activation by OST1, we coexpressed SLAC1 and OST1 as well as a set of PP2Cs. When the functional anion channel/kinecomplex was coexpressed with the protein phosphatase ABI1 or ABI2 (18, 22), anion currents were abolished (Fig. 3d). In contrast, neither the PP2C HAB1 nor HAB2 (47), could prevent SLAC1 anion currents. Coexpression of OST1 together with ABI1 generated YFP fluorescence (Fig. 2 D and F), whereas OST1 did not show YFP complementation with HAB1 (Fig. S5b). These results of BIFC experiments with OST1::YFPN and ABI1::YFPc or HAB1::YFPc (Fig. 2D and Fig. S5b) are thus supported by the anion current recordings in oocytes (Fig. 3d). Thus ABI1 is capable to inactivate the OST1 pathway by which the guard cell anion channel SLAC1 is activated.

To study the ability of OST1 to phosphorylate SLAC1, we asked whether the recombinant kinase was able to phosphorylate either SLAC1 N or C terminus by in vitro kinase assays. Using radio-labeled \([\gamma^{32}P]\) ATP, we could show that OST1 phosphorylates the N terminus of SLAC1 exclusively (Fig. 4a). Kinase-inactive OST1 D140A neither showed autophosphorylation nor phosphorylation of SLAC1 NT (Fig. 4a). These results corroborate our oocyte and Arabidopsis protoplast experiments, in which an interaction of OST1 with SLAC1 N terminus but not with SLAC1 C terminus was observed (Fig. S5a). In contrast, no or only a faint phosphorylation of SLAC1 NT by OST1 was observed in the presence of ABI1 (Fig. 4b, compare lane 1 and 2). Consequently, we elucidated, whether ABI1 regulates the activity of OST1 or directly dephosphorylates the N terminus of SLAC1. To prevent phosphorylation of the SLAC1 NT by OST1 we used the ATP analogue ATPγS in surplus (3 mM) relative to ATP (100 µM) (Fig. 4b lane 3). The resulting SLAC1-thio-phosphate-ester is resistant to hydrolysis by phosphatases and would therefore be removed as target of OST1 phosphorylation from the reaction mixture. To elucidate if ABI1 is capable to dephosphorylate the N terminus of SLAC1, we initially phosphorylated SLAC1 NT by OST1 in the presence of \([\gamma^{32}P]\) ATP. Subsequently, we added ATPγS and ABI1 to the reaction mixture (Fig. 4b lane 4). Although ATPγS was present to prevent further phosphorylation (cf. Fig. 4b lane 3), ABI1 was not capable to dephosphorylate SLAC1 NT (Fig. 4b lane 4). This indicates that ABI1 acts as a negative regulator of OST1 rather than of the SLAC1 channel.

Discussion

We could demonstrate by functional expression in Xenopus oocytes that guard cell expressed SLAC1 encodes a weak voltage-dependent, anion-selective plasma membrane channel rather than a malate transporter (27, 28). Furthermore SLAC1 shares neither structural nor functional similarities to AtALMT1, the Arabidopsis orthologue to the wheat aluminum-induced malate channel (48). Thus SLAC1 represents a novel type of inorganic anion channel with unknown tertiary structure, but kinetic properties very similar to the slow anion channel first described with Xanthium strumarium and Vicia faba guard cells (49, 50). Permeability studies with this channel type in V. faba guard cell protoplasts point to a relative malate to chloride permeability of 0.24 (39) which is 6-fold higher than

![Fig. 4. In vitro kinase activity of native recombinant GST1-tagged proteins. (A) Phosphorylation of SLAC1 termini (NT and CT) by OST1 was tested by using radio-labeled \([\gamma^{32}P]\) ATP (Left: Coomassie stained SDS PAGE; Right: radioautogram of the gel; the presence of proteins in the reaction assay was indicated by \(\gamma^{32}P\)). Only SLAC1 NT was phosphorylated by OST1. In contrast OST1 WT, the OST1 mutant D140A did not phosphorylate SLAC1 NT. Arrowheads indicate the position of recombinant proteins. (B) In vitro kinase assays with native recombinant proteins revealed that OST1 activity was prevented by ABI1 and the ATP analogue ATPγS. When SLAC1 NT was phosphorylated before adding ABI1 together with ATPγS (indicated by the black box, lane 4) we could not detect any dephosphorylation activity of ABI1 within 45 min of incubation at RT. The molecular weight of the protein ladder is indicated in kDa.](http://www.pnas.org/cgi/content/doi/10.1073/pnas.0912021106)
the ratio calculated for SLAC1 expressed in oocytes (Fig. 3B). The putative elevated relative malate permeability in planta likely results from malate transporters expressed in addition to SLAC1 in guard cells such as AtALMT-type carriers or AtABCB14 (48, 51, 52).

In Arabidopsis guard cells, S-type anion channels were reported to be sensitive to the protein kinase inhibitor K252a (26). Furthermore, in guard cells derived from abi1–1 mutant lines that express a deregulated protein phosphatase 2C, S-type anion channels lack ABA activation (24). Here we could show that SLAC1 anion channel interacts with and is activated by the protein kinase OST1. ABI2 functions as a negative regulator of OST1-dependent phosphorylation of SLAC1. Therefore, we suggest that the ABI2 phosphatase targets the OST1 activation rather than the channel dephosphorylation. Recently it was reported that an Arabidopsis triple mutant, with a disruption of 3 strongly ABA-activated protein kinases (SnRK2.2, 2.3 and OST1), is completely insensitive to ABA regarding seed dormancy, germination, seedling growth, and plant transpiration (53, 54). Although the close homologues SnRK2.2 and 2.3 (55) of OST1 were shown to activate SLAC1 as well (to some lesser extent, Fig. 3C), the distinct spatiotemporal expression of these kinases (especially SnRK2.2 and 2.3) likely contributes to their different physiological function.

Several ABA binding proteins (protein families) are currently discussed as putative ABA receptors (56–61). Our previous in planta guard cell studies have shown that cytosolic application of ABA is activating SLAC1-like anion channels instantaneously, whereas external stimulation delays this process (11). This observation points to an ABA importer and internal ABA receptor. Using independent approaches, recently 2 labs identified a new family of ABA-binding proteins (57, 59). These proteins interact with ABI1 and could represent the predicted cytoplasmic ABA receptor (11). In line with our findings, it was shown that the ABA-receptor/ABI1 complex interacts and activates SnRK2 kinases upon ABA-treatment. This fills the gap between ABA perception and SLAC1-mediated membrane depolarization. Based on these findings future reconstitution experiments to decipher perceptive (constitutive) and slow (transcriptionally-induced) ABA signaling pathways may now appear increasingly feasible (1).

Methods

Real-Time PCR. Quantification of actin2/8 and SLAC1/OST1/ABI1-transcripts was performed by real-time PCR as described elsewhere (62). Transcripts were each normalized to 10,000 molecules of actin 2/8. Primers are listed in Table S1 Methods.

Protein purification and in Vitro Kinase Assays. OST1, ABI1, SLAC1 NT and CT were cloned into the recombinant expression vector pTEVEX 6P1 (GE Healthcare) and transformed into Escherichia coli (DE3) pLysS strain (Novagen). GST-tagged recombinant proteins were purified as described by Belin et al. (68). In vitro kinase buffer was composed of 20 mM Hepes, pH 7.5, 0.5% (vol/vol) Triton X-100, 2 mM MnCl2, protease inhibitor mixture (Roche), 10 mM NaF, 5 mM β-glycerolphosphate, and 5 mM [γ-32P] ATP (3,000 Ci/mmol) (cf. ref. 68). Reactions were carried out for 15 min at RT and then stopped by adding 6 × SDS loading buffer and heating to 90 °C for 5 min. Proteins were separated by SDS-PAGE, detected by autoradiography and quantified by ImageJ.

Acknowledgments. We thank Gregory Harms for critical reading and comments on the manuscript. We gratefully acknowledge H. H. Nour-Eldin for providing full-length and plant vectors. This work was supported by grants from the Deutsche Forschungsgemeinschaft within the research group FOR 964 (to R.H. and T.R.) and GK 1342 (to R.H.) and a King Saud University grant (to R.H. and K.A.S.R.).
Supporting Information

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SI Text

Supplemental Text 1. To rule out that interactions arise from simple over-expression we performed control experiments with Guard cells Outward Rectifying K+ channel (GORK). GORK is, like SLAC1, involved in stomatal closure. Channel function and expression of GORK is ABA sensitive (1, 2). In contrast to SLAC1 in coexpression experiments with oocytes, GORK together with OST1 did not show any fluorescence complementation in BIFC experiments (Fig. S5b).

Supplemental Text 2. Replacing aspartate by alanine impairs the OST1 kinase activity. This position has been shown to play a crucial role in the active site as proton acceptor in protein kinases (consensus motif: HRDLKxxN; for review see ref. 3).

SI Methods

Real-Time PCR. Quantification of actin2/8 and SLAC1/OST1/ABI1-transcripts was performed by real-time PCR as described elsewhere (4). Transcripts were each normalized to 10,000 molecules of actin2/8. Primers used: AtACT2/8fwd 5'-GGT GAT GGT GTG TCT-3', AtACT2/8rev 5'-ACT GAG CAC AAT GTT AC-3', SLAC1fwd 5'-CCG GCC TGC ACT CA-3', SLAC1rev 5'-TCA GTG ATG CGA CTC TT-3', ABI1fwd 5'-CTG CAA TAA CCA ATA CTC-3', ABI1rev 5'-TCT TCT TCT CGC TAG TAA-3', OST1fwd 5'-AGC ATA ACA CGA TGA C-3', OST1rev 5'-TCC TGT GAG GTA ATG G-3'.

Cloning and cRNA Generation. The cDNA of SLAC1, OST1, SnRK2.2, 2.3, 2.8, ABI1, ABI2, HAB1 and HAB2 were cloned into oocyte (BIFC-) expression vectors (based on pGEM vectors) by an advanced uracil-excision-based cloning technique described by Nour-Eldin et al. (5). Site-directed mutations were introduced with the quick-change site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene/Agilent Technologies). For functional analysis cRNA was prepared using the mMessage mMachine T7 Transcription Kit (Ambion). Oocyte preparation and cRNA injection have been described elsewhere (6).

Oocyte Recordings. In TEVC experiments oocytes were perfused with Kulori-based solutions. The standard solution contained 10 mM Mes/Tris pH 5.6, 1 mM CaCl2, 1 mM MgCl2, 2 mM KCl, and 24 mM NaCl as well as 70 mM Na-gluconate. Solutions for selectivity measurements were composed of 50 mM Cl−, HCO3−, SO42−, NO3−, or malate− sodium salts, 85 mM Na-gluconate, 1 mM Ca-gluconate2, 1 mM Mg-gluconate2, 1 mM K-gluconate, as well as 10 mM Tris/Mes pH 5.6. Osmolarity was adjusted to 220 mOsmol/kg using D-sorbitol. Steady state currents (Iss) were extracted at the end of 15 s voltage pulses starting from a holding potential (Vh) of 0 mV and ranging from +60 to −180 mV in 10 mV decrements. The relative open probability P0 was determined from current responses to a constant voltage pulse to −120 mV subsequent to different test pulses. These currents were normalized to the saturation value of the calculated Boltzmann distribution. The half-maximal activation potential (V1/2) and the apparent gating charge (z) were determined by fitting the experimental data points with a single Boltzmann equation. Instantaneous currents (I) were extracted right after the voltage jump from the holding potential to test pulses (500 ms) ranging from +60 to −180 mV in 10 mV decrements.

BIFC Experiments. Transient protoplast expression was performed using the polyethylene glycol transformation method modified after (7). Sixteen to 24 h after transformation protoplast images were taken. For documentation of the oocyte and protoplast BIFC (8) results, pictures were taken with a confocal laser scanning microscope (LSM 5 Pascal Carl Zeiss Jena GmbH), equipped with a Zeiss Plan-Neofluar 20x/0.5 objective for oocyte images and a Zeiss Plan-Neofluar 63x/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).

Patch Clamp Experiments on Guard Cell Protoplasts. Arabidopsis thaliana ecotype Columbia (Col-0), ost1–2 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. Rosette leaves of 6–8 week-old plants were blended in ice-cold water 3 times for 20 s and washed with water through a 300-μm nylon mesh. The residual tissue was incubated for 18 h at 16–18 °C in enzyme solution composed of 0.65% (wt/vol) cellulase Onozuka-R10 (Serva), 0.35% (wt/vol) macerozyme Onozuka-R10 (Serva), 0.25% (wt/vol) bovine serum albumine (Serva), 0.05 mM KCl, 0.05 mM CaCl2, 5 mM ascorbic acid, 0.05% (wt/vol) kanamycin sulfate (Fluka) and adjusted to pH 5.5/Tris and to an osmolality of 400 mosmol/kg with D-sorbitol. Afterward the enzyme treatment was continued at room temperature on a rotary shaker (75 rpm) for further 30 min. Then, the suspension was filtered through a 300-μm and 20-μm nylon mesh, washed with wash solution (400 mM sorbitol and 1 mM CaCl2) and centrifuged at 100 × g and 4 °C for 12 min. The 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 (9, 10). The standard bath solution was composed of (in mM) 30 CsCl, 2 MgCl2, 0.5 LaCl3, and 10 Mes pH 5.6/Tris. For ABA activation of S-type currents the bath solution additionally contained 25 μM ABA, and protoplasts were preincubated in ABA (25 μM)-containing wash solution 20 to 30 min before patch clamping. The pipette solution consisted of (in mM) 150 TEA-Cl, 2 MgCl2, 5 Mg-ATP, 5 Tris-GTP, 10 Hepes pH 7.1/Tris. To obtain a free Ca2+ concentration of 110 nM, the pipette solution additionally contained 10 mM EGTA plus 3 mM CaCl2. The osmolality of the pipette and bath media was adjusted to 440 and 400 mosmol/kg, respectively, with D-sorbitol. Anion currents were measured 7 min after whole cell access and finally normalized off-line to the membrane capacitance (Cm) of the respective protoplast. For recording of ABA activated S-type anion currents, 7.5-s-lasting voltage pulses were applied from a holding voltage of +9 mV in the range from +44 to −136 mV in 30-mV decrements, followed by a 100-ms voltage pulse to −191 mV. The clamped voltages were corrected off-line for the liquid junction potential (11).

Protein Purification and in Vitro Kinase Assays. OST1, ABI1, and SLAC1 NT and CT were subcloned into the recombinant expression vector pGEX 6P1 (GE Healthcare) and transformed into E. coli (DE3) pLysS strain (Novagen). Bacteria were grown to OD 0.8 to 1.2 at 600 nm and production of GST-tagged proteins was induced by 0.4 mM isopropylthio-β-galactoside for 4 h at 25 °C. Cells were collected by centrifugation and then lysed...
(in 50 ml PBS) by 3 20 s lasting sonication steps. To remove insoluble bacterial fractions the lysate was centrifuged for 20 min at 15,000 × g. Native purification using Glutathion-Sepharose 4 B beads (Amersham Biosciences) was performed in a batch procedure according to manufacturer’s instructions; 50 mM Tris/HCl with 10 mM reduced glutathione was used for protein elution. Lysis and wash buffers were composed of 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4 (pH 7.3). Proteins were dialyzed and stored in 50 mM Tris/HCl. In vitro kinase buffer was composed of 20 mM Hepes, pH 7.5, 0.5% (vol/vol) Triton X-100, 2 mM MnCl2, 1 x protease inhibitor mixture (Roche), 10 mM NaF, 5 mM β-glycerophosphate and 5 mCi [γ-32P] ATP (3,000 Ci/mmol) (cf. ref. 9). Reactions were carried out for 15 min at room temperature and then stopped by adding 6 x SDS loading buffer and heating to 90 °C for 5 min.

Proteins were separated by SDS-Gel electrophoresis using a 8 to 16% gradient acrylamide gel (Precise™ protein gel, Thermo Scientific) and detected by coomassie blue stain and autoradiography.

CelluSpots peptide arrays were ordered from Intavis Bioanalytical Instruments; 20 aa long peptides with 10 aa overlap at each end of SLAC1 N and C terminus were coupled to microscope slides by acetylation. To prevent nonspecific binding, the arrays were blocked by immersing the slides in 1 mg/ml BSA solution for 2 h at room temperature. The phosphorylation reaction was carried out for 2 h at room temperature by the use of 2 µg kinase, 5 mCi [γ-32P] ATP (3,000 Ci/mmol) and the in vitro kinase buffer described above. Subsequently slides were washed 3 to 4 times with PBS buffer containing 0.05% Tween-20. Phosphorylation was detected by autoradiography.

Fig. S1. ost1–2 plants are unable to control stomatal closure. (a) Reduction in transpiration of ost1–2 leaves upon light to dark transition is delayed. White and black bars indicate light and dark periods, respectively. Representative experiments are shown. (b) Wilting of ost1–2 leaves in the light. Please note, that water feeding via the petiole does not prevent wilting of excised ost1–2 leaves. The same leaves are shown at the indicated time points (See also Movie S1). (c) Time course of ost1–2 leaves wilting compared to WT. The position of the leaf tip (see arrow in b) relative to the leaf base was plotted against time (after excision). Data from 3 individual ost1–2 and 2 WT leaves each from different plants are shown.
Fig. S2. Quantification of SLAC1, ABI1, and OST1 transcript levels in guard cells in comparison to mesophyll cells. Transcripts were normalized to 10,000 molecules of actin2/8 (n = 4, mean ± SD).
Fig. S3. Illustration of BiFC fusion constructs used in this study. (a) Fusion proteins used for oocytes BiFC recordings. (b) Fusion proteins used for interaction studies in Arabidopsis protoplasts by BiFC. (a and b) Abbreviations used in this study: YFP, yellow fluorescent protein; YFP<sup>C</sup>, C-terminal half of YFP (amino acid 156 to 239); YFP<sup>N</sup>, N-terminal half of YFP (amino acid 1 to 155); SLAC1 NT harbors amino acid 1 to 186; SLAC1 CT exhibits amino acid 496 to 556;

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<th>YFP CT</th>
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<td>OST1 cDNA</td>
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Fig. S4. Whole oocyte currents of a representative SLAC1::YFP<sup>C</sup> OST1::YFP<sup>N</sup> coexpressing oocyte (Left) compared to an oocyte expressing the native proteins (SLAC1 and OST1, Right) without split-YFP fusion. Note, electrical properties of the resulting currents were independent from the constructs (with or without split-YFP fusion) used for oocyte injection.
Fig. S5. A series of BiFC experiments in Xenopus oocytes identified interacting proteins. Pictures showing a quarter of an optical slice of an oocyte were taken with a confocal laser scanning microscope. The coinjected plasmids combinations are indicated in the figure. The C-terminal half of the YFP was fused to SLAC1 (termini of SLAC1 in a) whereas the N-terminal half of the YFP was fused to the kinases/phosphatases. Representative images are shown. (a) The N terminus of SLAC1 showed strong interaction with OST1, whereas SLAC1 CT OST1 coexpression lacked YFP fluorescence. OST1::YFP<sup>N</sup> and HAB1::YFP<sup>C</sup> did not result in YFP fluorescence. (b) Neither the disruption of the kinase activity of OST1 (D140A) nor the use of the SLAC1 mutant, S120A, prevented interaction with SLAC1 or OST1, respectively. BiFC control experiments with GORK coexpressed with OST1 did not result in YFP fluorescence. (c) Coexpression studies of SLAC1 with different members of the SnRK kinase family. Note that strongest BiFC signals were obtained with OST1.
Fig. S6. Deactivation characteristics and anion dependency of SLAC1 currents. (a) A single 15 s voltage pulse to −180 mV starting from the holding potential of 0 mV in 30 mM external Cl⁻ was applied. The deactivation kinetics could be fitted with a double exponential equation, resulting in the indicated time constants.

(b) SLAC1 steady-state currents (Iₚₛₛ) normalized to the value at +30 mV in 100 Cl⁻ were plotted against the membrane voltage. Note the reduction of Cl⁻ efflux at negative membrane potentials (n = 4, mean ± SD).

(c) The relative open probability (rel. Pₒ) of SLAC1 in 10 and 100 mM Cl⁻ was plotted against the membrane potential. The half-maximal activation potential (V₁/₂) shifted to more negative potentials with increasing external Cl⁻ concentrations. Data points were fitted with a single Boltzmann equation (solid lines, n = 4, mean ± SD).

(d) Reversal potentials for Cl⁻, NO₃⁻ and SCN⁻ in the bath 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 = 4, mean ± SD). Experiments with SLAC1 activated by OST1 were performed with oocytes expressing SLAC1::YFP² and OST1::YFP¹.
Fig. S7. (a and b) SLAC1 mediated instantaneous currents ($i_t$) were independent from the nature of external cations and the external proton concentration ($n=3\pm SD$). $i_t$ currents were normalized to currents at $-100$ mV at pH 5 and 100 mM KCl (in a) or 100 mM KCl, pH 5.5 (in b). (c) Percentage of SLAC1 current inhibition in oocytes by the anion channel inhibitors DIDS and SITS at the indicated inhibitor concentrations (open bars, $n=4$). The black bar shows the inhibition of SLAC1-like currents in Arabidopsis guard cell protoplasts (WT) by 100 μM DIDS ($n=3$). Current inhibition was determined in oocytes at $-100$ mV and in protoplasts at $-96$ mV. Experiments with SLAC1 activated by OST1 were performed with oocytes expressing SLAC1::YFP$^c$ and OST1::YFP$^a$. 
Excised leaves from 6 to 8 week old WT (Ler) and ost1–2 plants were put into a 1.5 ml reaction cup filled with water. The movie spanned 6 h in the light. In contrast to WT leaves, ost1–2 leaves could not properly adjust their stomatal aperture in response to ongoing water- and turgor loss. This resulted in premature wilting of ost1–2 leaves relative to WT leaves.
Table S1. CelluSpots peptide arrays were used to identify putative phosphorylation sites within the N- and C-terminal part of SLAC1. Thereby phosphorylation by OST1 was detected by the use radio-labeled [$\gamma^{32}$P] ATP and subsequent autoradiography. + indicates peptides which were (weakly) phosphorylated. ++ indicates peptides which showed high phosphorylation signals.

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