Supporting Information

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SI Materials and Methods

Construct Preparation and Electrophysiology in *Xenopus laevis* Oocytes. All constructs were cloned into the pNB1 oocyte expression vector using the USER method (1). 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. The recordings were performed 2–3 d after injection, with a Cornerstone (Dagan) TEV-200 two-electrode voltage-clamp amplifier. Data analyses were performed using an Axon Instruments Digidata 1440A Low-Noise Data Acquisition System (Molecular Devices). The last 0.5 s of each voltage pulse were averaged using Clampfit 10.2 software for all current–voltage graphs. Oocytes were subjected to voltage pulses, with a holding potential of 0 mV, using a voltage protocol with a range of −180 mV to +40 mV in +20-mV increments, followed by a −120-mV voltage "tail" pulse. Data were low-pass-filtered at 20 Hz throughout all recordings. Oocytes were recorded in 75 mM NaCl, 20 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes (pH 7.4; titrated with Tris base), and osmolarity was balanced to 220–260 mmol osmotically active substances/kg with D-mannitol for the recordings. The oocytes were impaled with electrodes filled with 3 M KCl. For the oocyte groups with injected abscisic acid (ABA), 500 μM ABA was injected into each oocyte (to a final concentration of ∼50 μM ABA, assuming an approximate 500-nL total volume of oocytes). ABA microinjections were performed 15 min before voltage-clamp experiments. Error bars indicate SEM. Statistical analyses were performed using the Student's *t* test and two-way ANOVA (for datasets with a determined normal distribution) or the Mann–Whitney *U* test (for datasets with a nonnormal distribution). All experiments were performed at room temperature. The numbers (*n*) of individual oocytes analyzed in *n* batches are given in the figure legends. If not otherwise mentioned, data represent measurements of one representative batch of oocytes, as current magnitudes vary from batch to batch.

Oocyte Histology. Four days after fusion-protein [calcium-dependent protein kinase (CPK)6 and CPK23 coding sequences were fused to mTurquoise (2) in the pNB1 vector, and cRNA was prepared using the mMessage mMachine Transcription Kit (Ambion)] mRNA injection, three to five oocytes per freezing mold were submerged in 3 mL O.C.T. compound (Tissue-Tek). The samples were flash-frozen in liquid nitrogen and sliced into 100-μm sections using a cryostat microtome. The sections were collected on microscope slides, taking care to keep the sections from drying out by application of cool water vapor to the slide face containing the sections. Coverslips were immediately mounted on the slides with an airtight barrier formed by a thin ring of silicone vacuum grease (Beckman) sandwiched between the coverslip and the slide along the perimeter.

The prepared slides were temporarily stored in a dark, humidified chamber to be imaged within 1 h of sectioning. Each section was imaged on a Nikon Eclipse TE2000-U spinning disk confocal microscope with a 10× objective.

Site-Directed Mutagenesis, in Vitro Kinase Assays, Protein Expression, Isolation, and Western Blot Analyses. CPK6, Open Stomata 1 (OST1), ABI1, and SLAC1 N and C terminus coding sequences were cloned into a modified pGEX-6P1 (GE Healthcare) vector using the USER method (1). In the pGEX-6P1 vector, a USER cassette followed by a StrepII-tag was introduced. The PYR1 coding sequence was cloned into a modified pGEX-6P1 vector, resulting in an N-terminal StrepII-tag fusion. Plasmids were transformed into *Escherichia coli* Rosetta (DE3) pLysS (Novagen) and grown to $OD_{(A600)}$ ∼0.6. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM and carried out for 3 h at room temperature. For PYR1 protein isolation, the culture was incubated at 15 °C for 18 h. Harvesting the bacteria was achieved by centrifugation (4,000 × *g*; 15 min) and bacterial pellets were resuspended in buffer W (100 mM Tris, 150 mM NaCl, pH 8.0) supplemented with 1 mg/mL lysozyme and protease inhibitor mixture (Complete EDTA-free; Roche). After lysis of the cells by three 30-s sonication pulses (Heat Systems; Ultrasonics), insoluble cell debris was removed by centrifugation (25,000 × *g* for 30 min at 4 °C). CPK6, ABI1, PYR1, and SLAC1 N and C terminus were purified using Strep-Tactin MacroPrep (IBA) by gravity flow in Micro Bio-Spin chromatography columns (Bio-Rad) following instructions listed in the manufacturer's manual. Eluted recombinant proteins were supplemented with 10% (vol/vol) glycerol and stored at −20 °C. In vitro kinase assays were performed as previously described (3). The kinase buffer consisted of 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1× protease inhibitor (Complete EDTA-free; Roche), 2 mM DTT, 5 mM EGTA, 5 μCi [γ-^{32}P]ATP, and 4.87 mM $CaCl_2$ to gain a final free Ca^{2+} concentration of ∼3 μM (calculated with http://www.stanford.edu/∼cpatton/webmaxc/webmaxcE.htm) in most of the 20-μL reaction volumes. To remove free Ca^{2+} from the reaction buffer, no $CaCl_2$ was added and the reaction buffer was supplemented with 12.9 mM $MgCl_2$ to gain a final free Mg^{2+} concentration of 10 mM (calculated with http://www.stanford.edu/∼cpatton/webmaxc/webmaxcE.htm). For the in vitro reconstitution, either 5 μM ABA (dissolved in ethanol; lanes indicated with + ABA) or an equivalent amount of ethanol (lanes indicated with − ABA) was used. Using [γ-^{32}P]ATP, the reactions were started, carried out at room temperature for 10 min for most of the experiments, and subsequently stopped by adding 6× SDS-loading dye. To the reaction mixtures shown in lanes 2 and 3, components indicated by "10 min" were added after the initial 10-min incubation time and incubated for an additional 35 min before being stopped using 6× SDS-loading dye. After incubation at 98 °C for 5 min, proteins were separated by SDS/PAGE in 4–15% gradient gels (Mini Protean TGX; Bio-Rad) and subsequently stained using Page Blue (Fermentas). Radioactivity of ^{32}P was monitored using HyBlot CL autoradiography films (Denville Scientific).

Loading of proteins was controlled by performing Western blot analysis (Figs. 1*C* and 4*C*) as well as Coomassie staining. The reactions used for subsequent Western blot analysis were treated exactly the same way as those used for in vitro kinase assays with the only difference that no [γ-^{32}P]ATP was added. Proteins were separated by SDS/PAGE in 4–15% gradient gels (Mini Protean TGX; Bio-Rad) and subsequently blotted on Immobilon-P^{SQ} membranes (Millipore). GST-fusion proteins were detected using an anti–GST-HRP conjugate antibody (GE Healthcare), SuperSignal West Pico (Pierce), and BioMax MR film (Kodak). For Coomassie-stained loading controls, photographs of the gels were taken before detection of radioactivity by autoradiography.

P81 Filter Paper-Based Kinase Kinetics Assay. Recombinant SLAC1-NT (N terminus of SLAC1) used for kinase kinetics assays was concentrated using Amicon Ultra devices (Ultracell 30k; Millipore) and thereby the buffer was changed to 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 2 mM DTT. For determining concentrations of recombinant CPK6, CPK23, OST1, and SLAC1-NT, proteins were subjected to SDS/PAGE, Coomassie-stained, and dried

between two cellophane sheets to assess purity (Fig. S6). Subsequently, protein concentrations were measured using the BCA Protein Assay Kit (Pierce). To determine kinetic parameters, 20 nmol kinase was incubated in a buffer containing 75 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 3 mM DTT, 75 mM NaCl, and variable concentrations of SLAC1-NT (0.625–25 μM) in a 10-μL reaction for 2.5 min, 5.5 min, or 10 min at room temperature for CPK6, CPK23, or OST1, respectively. Note that for CPK6 and CPK23, the reaction buffer was supplemented with 4.87 mM $CaCl_2$ and 5 mM EGTA, resulting in ~3 μM free Ca^{2+} (calculated with http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm). After quenching the reactions by adding 90 μL 30% (vol/vol) acetic acid, 50 μL was spotted on P81-grade filter paper discs (Whatman) and subsequently washed in 0.5% (vol/vol) phosphoric acid five times for 5 min. After washing the filter discs in acetone for 2 min followed by air drying, ^{32}P incorporation was determined using scintillation fluid (Ecoscint; National Diagnostics) and a counter (Beckman). Using GraphPad Prism 5, kinetic constants were calculated.

***In Gel* Digest and LC-MS/MS.** Isolated recombinant SLAC1-NT protein was incubated with and without CPK6 protein for 10 min at room temperature using the same buffer conditions as used for in vitro kinase assays, with the only difference that instead of 5 μCi [γ-^{32}P]ATP, 100 μM ATP was present. After separation by SDS/PAGE and subsequent Coomassie staining, SLAC1-NT–corresponding bands were cut out. The gel slices were cut to 1-mm cubes and destained three times by first washing with 100 μL of 100 mM ammonium bicarbonate for 15 min, followed by addition of the same volume of acetonitrile (ACN) for 15 min. Samples were dried in a SpeedVac. Samples were then reduced by mixing with 200 μL of 100 mM ammonium bicarbonate, 10 mM DTT and incubated at 56 °C for 30 min. The liquid was removed and 200 μL of 100 mM ammonium bicarbonate, 55 mM iodoacetamide was added to the gel pieces and incubated at room temperature in the dark for 20 min. After the removal of the supernatant and one wash with 100 mM ammonium bicarbonate for 15 min, the same volume of ACN was added to dehydrate the gel pieces. The solution was then removed and samples were dried in a SpeedVac. For digestion, enough solution of ice-cold trypsin (0.01 μg/μL) in 50 mM ammonium bicarbonate was added to cover the gel pieces and set on ice for 30 min. After complete rehydration, the excess trypsin solution was removed, replaced with fresh 50 mM ammonium bicarbonate, and left overnight at 37 °C. The peptides were extracted twice by the addition of 50 μL of 0.2% (vol/vol) formic acid and 5% (vol/vol) ACN and vortex mixing at room temperature for 30 min. The supernatant was removed and saved. A total of 50 μL of 50% ACN/0.2% (vol/vol) formic acid was added to the sample, which was vortexed again at room temperature for 30 min. The supernatant was removed and combined with the supernatant from the first extraction. The combined extractions were analyzed directly by liquid chromatography (LC) in combination with tandem mass spectroscopy (MS/MS) using electrospray ionization. Trypsin-digested peptides were analyzed by HPLC coupled with tandem mass spectroscopy (LC-MS/MS) using nanospray ionization. The nanospray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo; Eksigent, ABSCIEX) using a 10 cm × 100 μm i.d. glass capillary packed with 5-μm C18 Zorbax beads (Agilent Technologies). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient [5–60% (vol/vol)] of ACN at a flow rate of 250 μL/min for 1 h. The buffers used to create the ACN gradient were buffer A [98% H_2O, 2% ACN, 0.2% formic acid, 0.005% TFA (vol/vol)] and buffer B [100% ACN, 0.2% formic acid, 0.005% TFA (vol/vol)]. MS/MS data were acquired in a data-dependent manner in which the MS1 data were acquired for 250 ms at an *m/z* of 400–1,250 Da and the MS/MS data were acquired from an *m/z* of 50–2,000 Da. For independent data acquisition (IDA) method parameters were as follows: a 250 ms time of flight survey scan (MS1-TOF) was followed by 50 product ion scans (MS2) of 25 ms each. For MS2 IDA criteria, ions that had reached the threshold of 200-counts and had the charge state +2, +3, or +4 where selected. Four-second exclusion criteria was chosen to limit the number of repetitive MS2 events on the same ion. Finally, the collected data were analyzed using Mascot (Matrix Science) and ProteinPilot 4.0 (AB SCIEX) for peptide identification. To further analyze phosphorylation of serine 59, product ion scan methods were constructed and carried out for the product of *m/z* = 765.4 [QVpSLETGFSVLNR (M + 2H)]. All LC conditions were identical to the above settings. For the product ion scans, the Q1 resolution was set to unit and the collision energy was set to 39 at an accumulation time of 200-ms scans.

1. Nour-Eldin HH, Hansen BG, Nørholm MHH, Jensen JK, Halkier BA (2006) Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. *Nucleic Acids Res* 34:e122.
2. Goedhart J, et al. (2010) Bright cyan fluorescent protein variants identified by fluorescence lifetime screening. *Nat Methods* 7(2):137–139.
3. Geiger D, et al. (2010) Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities. *Proc Natl Acad Sci USA* 107:8023–8028.

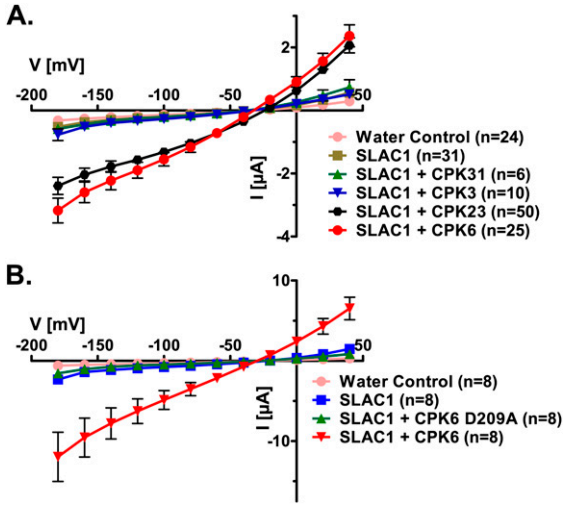

Fig. S1. Strong kinase activity-dependent activation of SLAC1 channel currents by the CPK6 protein kinase in *Xenopus* oocytes. (*A*) The average steady-state current–voltage relationships are shown in water-injected (pink circles), SLAC1-injected (brown squares), and SLAC1 + CPK-injected oocytes. The protein kinases CPK6 (red circles) and CPK23 (black hexagons) are able to increase SLAC1 activity, whereas CPK31 (green triangles), a homolog of CPK23, and CPK3 (blue triangles) did not significantly increase SLAC1 activity in oocytes. Representative data from one batch of oocytes are shown (CPK3, CPK31; of >3 batches), except for SLAC1 + CPK6 and SLAC1 + CPK23, which show average data from two to five independent oocyte batches. (*B*) SLAC1 was not activated by a kinase-inactive CPK6 mutant (D209A; green triangles), whereas coexpression of wild-type CPK6 (red triangles) together with SLAC1 resulted in a large anion current (n = 3 batches). Note that the degree of SLAC1 activation in *A* and *B* differed, as experiments were conducted in independent batches of oocytes, resulting in typical differences among oocyte batches. Therefore, the illustrated internal controls were always coinjected in the same batch of oocytes.

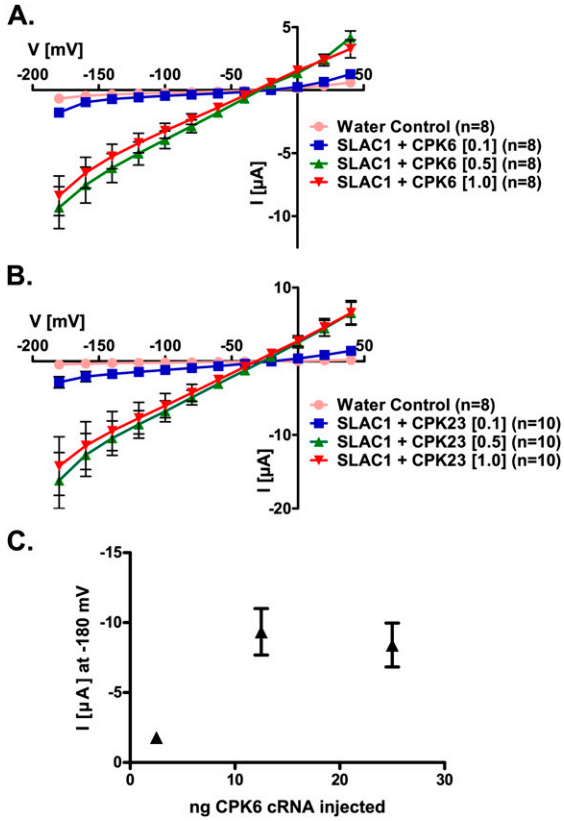

Fig. S2. CPK6 and CPK23 cRNA concentration-dependent activation of SLAC1 anion currents. (*A* and *B*) SLAC1 activity in response to injection of full cRNA content of CPK6 and CPK23 kinases (~25 ng per oocyte; red triangles) was compared with SLAC1 and 1/2 (~12.5 ng; green triangles) or 1/10 (~2.5 ng; blue squares) cRNA content of *CPK6*, to investigate the saturation point of SLAC1-mediated currents. Representative data from one batch of oocytes (of >2 batches) are shown in *A*. (*C*) Maximum SLAC1-mediated current activation as a function of injected CPK6 cRNA amount.

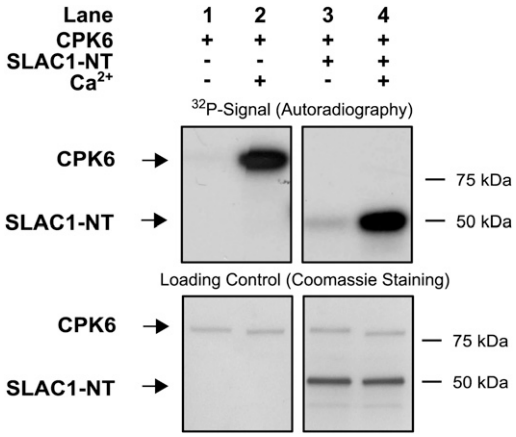

Fig. S3. SLAC1-NT phosphorylation is Ca^{2+}-dependent in vitro. In the absence of free Ca^{2+}, both CPK6 auto- (lane 1; *Upper*) and SLAC1-NT cross-phosphorylation (lane 3; *Upper*) are strongly decreased compared with the ^{32}P signal in the presence of Ca^{2+} (lanes 2 and 4; *Upper*). Note that the CPK6 migrating speed in the SDS/PAGE is Ca^{2+}-dependent (lanes 1–4; *Lower*), as previously reported for several CPKs (1–3).

1. Harmon AC, Putnam-Evans C, Cormier MJ (1987) A calcium-dependent but calmodulin-independent protein kinase from soybean. *Plant Physiol* 83:830–837.
2. Romeis T, Piedras P, Jones JDG (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defense response. *Plant Cell* 12:803–816.
3. Yoon GM, Cho HS, Ha HJ, Liu JR, Lee HS (1999) Characterization of NtCDPK1, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. *Plant Mol Biol* 39:991–1001.

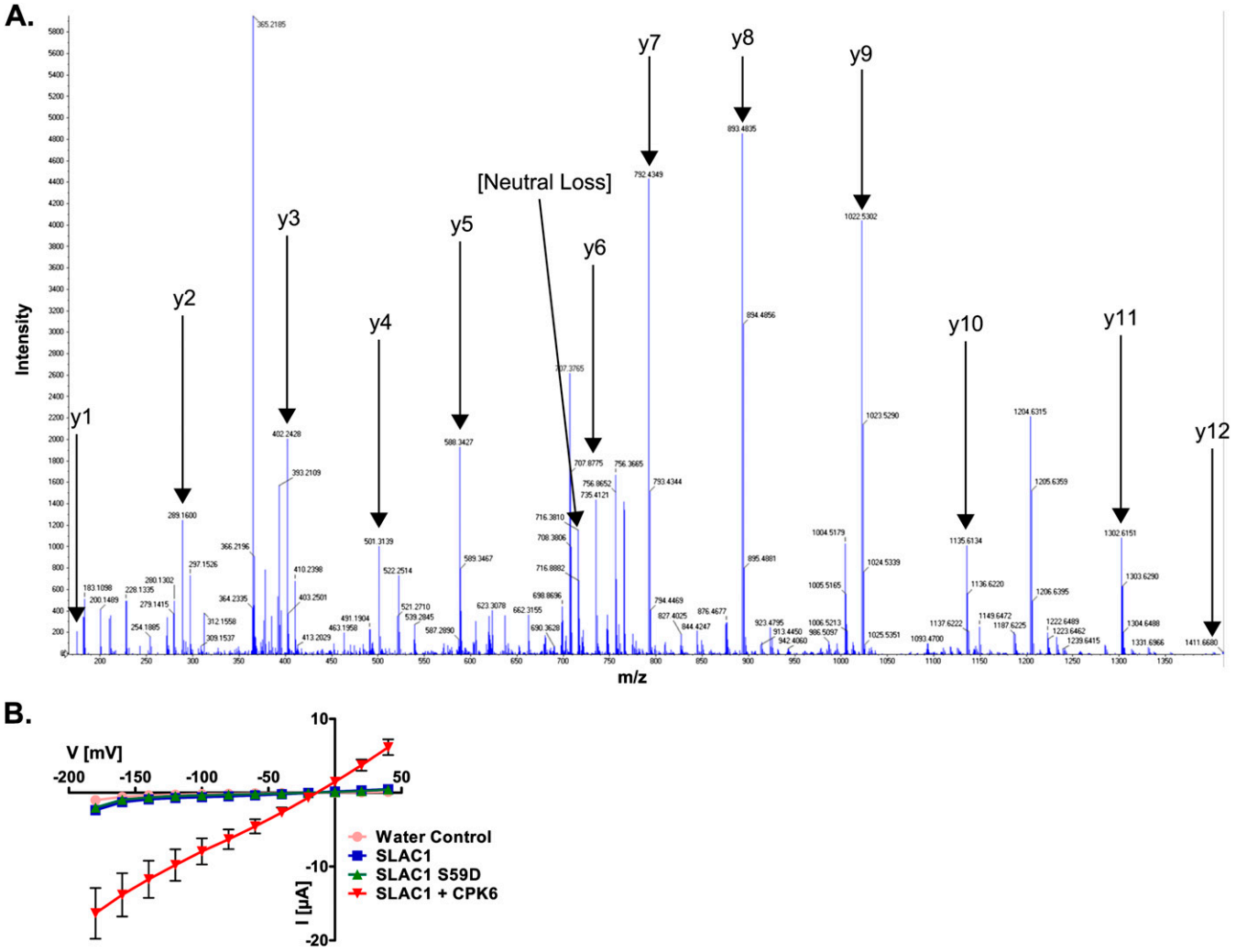

Fig. S4. LC-MS/MS identification of SLAC1-NT serine 59 phosphorylation by CPK6 and constitutive activation of SLAC1 by point mutation to aspartate (S59D). (*A*) Product ion scan spectrum for the peptide QVpSLETGFSVLNR [$(M + 2H)^+ = 765.4$]. Besides y ions, the peak for the neutral ion loss of 765.4, which is 716.4, is also labeled. (*B*) Additional control data from the same batch of oocytes as shown in Fig. 3*B*: Point mutation of serine 59 to aspartate (S59D) did not result in constitutive activation of SLAC1 in oocytes (SLAC1 S59D; green triangles). Blue squares represent wild-type SLAC1 without CPK6. CPK6 was able to activate SLAC1 (red triangles) ($n > 6$ oocytes per condition).

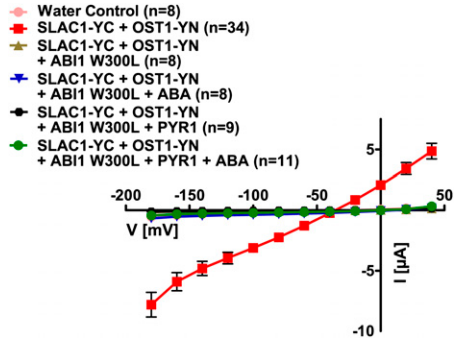

Fig. S5. The W300L point mutation in the PP2C phosphatase ABI1 disrupts ABA activation of SLAC1 ion currents mediated by OST1 in *Xenopus* oocytes. ABI1 W300L was able to inhibit SLAC1 activity similar to wild-type ABI1 (gold triangles), but exhibited an inability of PYR1 to enhance SLAC1-mediated inward currents in the presence of ABA (green circles). Data from one representative batch (of two batches) of oocytes are shown except for OST1 data without ABI1 (red squares; >3 batches averaged).

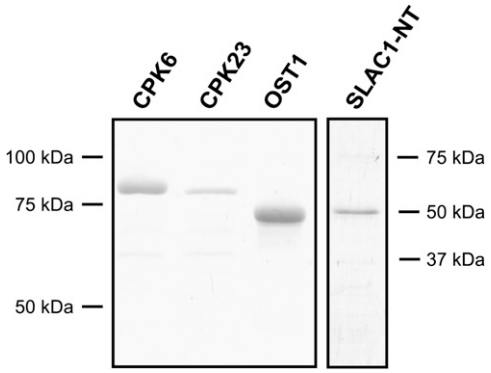

Fig. S6. Coomassie-stained SDS/PAGE of isolated recombinant proteins used for kinase kinetics assays.

Table S1. Statistical analysis of the identified peptide using Paragon algorithm/ProteinPilot

N	Unused	Total	% cov	% cov (50)	% cov (95)	Accessions	Confidence score (%)	Sequence	Modifications	dMass
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term	−0.00014535
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	0.00593622
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term	−0.00014535
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	0.00044304
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		0.00043076
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	0.00178582
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Phospho(S)@3	0.00126418
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	−0.00126594
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		−0.0005458
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		0.00030869
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		−0.00457413
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	0.00178582
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	−0.00419564
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		−0.0005458
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		0.00043076
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	−0.00126594
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	99.00000095	QVSLETGFSVLNR		−0.00457413
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	98.01999927	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	−0.00285286
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	96.96000218	QVSLETGFSVLNR	Phospho(S)@3	0.00626907
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	95.01000047	QVSLETGFSVLNR		−0.00274307
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	91.40999913	QVSLETGFSVLNR	Phospho(S)@3	0.00126418
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	90.8100009	QVSLETGFSVLNR		−0.0005458
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	89.37000036	QVSLETGFSVLNR	Gln→pyro-Glu@N-term; Phospho(S)@3	0.00044304
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	76.56999826	QVSLETGFSVLNR		−0.00457413
1	56.4	56.4	83.18	65.9	58.76	GST-SLAC1-NT-StrepII	42.6699996	QVSLETGFSVLNR		0.00030869

N: The rank of the specified protein relative to all other proteins in the list of detected proteins. Unused (ProtScore): A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher-scoring winning proteins. Total (ProtScore): A measure of the total amount of evidence for a detected protein. The Total ProtScore is calculated using all of the peptides detected for the protein. The Total ProtScore does not indicate the percent confidence for the identification of a protein. % cov (Coverage): The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence. % cov (50): The percentage of matching amino acids from identified peptides having confidence greater than or equal to 50% divided by the total number of amino acids in the sequence. % cov (95): The percentage of matching amino acids from identified peptides having confidence greater than or equal to 95% divided by the total number of amino acids in the sequence. Accessions: The accession number for the protein. A custom FASTA sequence entry was created for SLAC1 proteins with the name: "GST-SLAC1-NT-StrepII" and pasted into swissprot database for the protein pilot searches. Confidence score (%): The confidence for the peptide identification, expressed as a percentage. Sequence: Sequence of the identified peptide. Modifications: Modifications found by the search dMass (Delta Mass). dMass: The difference in mass between the precursor molecular weight (MW) and the theoretical MW of the matching peptide sequence (including modifications).