Supporting Information

Calcium-dependent protein kinase/NADPH-oxidase activation circuit is required for rapid defense signal propagation

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SI Materials and Methods

Plant material, growth conditions and treatment. *Arabidopsis thaliana* Col-0 wild-type and derived transgenic overexpressing and mutant plants were grown on soil with 8 h light/16 h dark cycle, 20 °C and 60 % relative humidity. *fls2* (SALK_093905), and 35S::NahG were kindly provided by B. Kemmerling (University Tübingen, Germany). The T-DNA insertion lines *rbohd* (SALK_070610), *cpk5* (SAIL_657_C06) and *cpk6* (SALK_025460C) were obtained from Nottingham Arabidopsis Stock Center (NASC). *Nicotiana benthamiana* plants were grown on soil in a greenhouse with 16 h light/8 h dark cycle.

Generation of constructs of CPK5 and RBOHD constructs. The coding region for full length CPK5 (At4g35310) was amplified from Col-0 cDNA with primers TR153 and TR164, truncated constitutive active kinase (CPK5-VK) lacking its regulatory domains with primers TR153 and TR232. Fragments were cloned into pENTR-D/TOPO (Invitrogen). RBOHD (At5g47910) was amplified from cDNA with primer TR2930 and TR3030 and cloned into pJET1.2/blunt Cloning Vector (Fermentas).

The kinase-deficient variants CPK5m and CPK5-VKm carrying the amino acid substitution D221A were generated by site-directed mutagenesis with primers TR376 and TR377. RBOHD phosphorylation mutants S39A, S148A, S162/3A and S343/7A were generated with TR3126/3127, TR3118/3119, TR3192/3193, TR3122/3123 and TR3190/3191 respectively. C-terminal Strep and YFP fusion constructs of CPK5 variants were generated by LR-Gateway recombination into pXCSG-Strep (1) or pXCSG-YFP (2). For N-terminal fusion with YFP RBOHD was cloned into pXNS2-YFP (3).

Transient protein expression in *Nicotiana benthamiana* and affinity purification. Transient expression in *N. benthamiana* was performed as described previously (1) but adjusting optical densities of Agrobacteria carrying *At*CPK5-Strep/*At*CPK5m-Strep or RBOHD constructs to 0.5 and of helper Agrobacterium with the silencing inhibitor construct to 0.25. After infiltration, plants were grown under short day (8 h light) conditions for 3 to 5 d. For enzyme purification,

0.75 g of leaf material was ground in liquid Nitrogen and thawed in 1.5 ml of extraction buffer (100 mM Tris, pH 8.0; 5 mM EGTA; 5 mM EDTA; 100 mM NaCl; 20 mM DTT; 0.5 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride); 2 µg/ml aprotinin; 2 µg/ml leupeptin; plant protease inhibitor cocktail (Sigma P9599, Taufkirchen, Germany), diluted 1:200; 0.5 % Triton X-100; and 100 µg/ml avidin). The extract was centrifuged at 21,000 x g, 15 min, 4 °C, and 40 µl of StrepTactin Macroprep (50 % suspension; IBA) was added to the supernatant. After incubation for 20 min (4 °C) in a rotation wheel, the affinity matrix was pelleted by centrifugation for 30 s at 700 x g and the supernatant discarded. The matrix was washed five times with 500 ml of wash buffer (50 mM Tris, pH 8.0; 2.5 mM EDTA; 100 mM NaCl; 2 mM DTT; 0.05 % Triton X-100), discarding the supernatant after each wash. Purified protein bound to the matrix was used for *in-vitro* kinase assay.

Transient protein expression in *Arabidopsis* leaf mesophyll protoplasts. Protoplast assays were performed as described previously (*5*). In brief: Leave stripes were incubated for 3 h in enzyme solution. Protoplasts were centrifuged after adding the same volume of W5-buffer. After counting and adjusting the cell density in W5-buffer to 2 x 10^5 cells/ml cells were rested on ice for 30 minutes. Transfection was performed with 30-40 % PEG-solution and a total amount of 20 µg plasmid-DNA. Cells were transferred to 6-well plates coated with 5 % calf-serum-albumin. Stress was applied 14 h after transfection to protoplasts carrying CPK5 full-length constructs. Protoplasts transformed with CPK5-VK constructs were harvested 6 h after transfection.

Protoplast viability test. Protoplasts were stained with 50 µg/ml Fluorescein diacetate (FDA) and 50 µg/ml Propidium iodide (PI) at indicated time points after transfection. For each time point 150-200 cells were counted using a fluorescence microscope (Nikon Eclipse 90i) and percentage of dead cells (red, stained with PI) was calculated.

Generation of transgenic plants. Transgenic Arabidopsis plants were generated using the floral-dip method. Briefly, flowering *Arabidopsis thaliana* accession Col-0 plants were dipped into *Agrobacterium tumefaciens* GV3101 pMP90RK carrying pXCSG-CPK5-YFP or pXCSG-

CPK5m-YFP constructs, and seeds were harvested to select independent transformants by BASTA selection.

Trypan blue staining. Cell death in unstressed plant tissue was stained with trypan blue in lactophenol and ethanol as described previously (4).

Bacterial growth assays. Bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 was grown in King's B medium at 28 °C overnight. For measuring bacterial growth, Arabidopsis leaves of six-week old plants were infiltrated with *Pst* DC3000 at 10^4 cfu/ml in 10 mM $MgCl_2$ using a needleless syringe. Three days after inoculation, bacterial growth was monitored by serial dilution plating of ground leaf discs.

ROS measurement. Reactive oxygen species production was monitored using a luminol-dependent assay. Flagellin-dependent oxidative burst in *A. thaliana* was conducted with six-week old plants grown under short day conditions. 0.3 cm leaf discs were floated overnight on 100 µl H_2O in a 96-well plate.

To determine NAD(P)H oxidase activity in *N. benthamiana* leaf discs, leaves of four-week old *N. benthamiana* plants (the 4^{th} true leaf) were infiltrated with *Agrobacterium tumefaciens* carrying RBOHD and CDPK expression constructs as well as silencing inhibitor P19. Two days after infiltration, 0.5 cm diameter leaf discs were cut out and floated on 100 µl H_2O in a 96-well plate overnight. On the following day, 100 µl assay solution (final concentration: 0,034 mg/ml luminol and 0,02 mg/ml horse radish peroxidase) with or without 200 nM flg22 was added to the leaf discs and luminescence was immediately measured over one hour in an LB941 microplate reader (Berthold technologies).

Nitroblue tetrazolium (NBT) staining. NBT staining was performed with six-week old Arabidopsis plants without prior treatment. After incubation of whole Arabidopsis rosettes in 0.1 % (w/v) NBT and 10 mM sodium azide for 18 h, plants were placed in ethanol to remove

chlorophyll and visualize NBT dye. Stained leaf area was quantified in 4 individuals per line using Image J (http://rsb.info.nih.gov/ij)

Gene expression by qRT-PCR analysis. To analyse transcript levels, RNA was extracted from leaf tissue using the Trizol method. 2 µg of RNA was treated with RNase-free DNase (Fermentas) and reverse transcribed with SuperscriptIII SuperMix (Invitrogen) according to the manufacturer's protocols. Real-time quantitative PCR analysis was performed in a final volume of 10 µl according to the instructions of Power SYBR Green PCR Master Mix (Applied Biosystems) using the CFX96 system (Bio-Rad). Amplification specificity was evaluated by post-amplification dissociation curves. *ACTIN2* (At3g18780) was used as the internal control for quantification of gene expression. Primer sequences are listed in Table S2.

Measurement of free salicylic acid. *Arabidopsis* leaf material was frozen in liquid nitrogen and ground in a Retsch mill. Free SA was extracted from 400 mg of this material by adding 1.6 ml ethyl acetate containing 80 ng 3-hydroxybenzoic acid (3HBA, FLUKA) as internal standard and incubation for 2 h 15 min in a shaker at 25 °C. After centrifugation (10 min, 13,800 x g) 1.3 ml of the ethyl acetate phase was transferred to a new test tube and the solvent was removed in a heated speed-vac. Samples were dried overnight with phosphorus pentoxide. The dried extract was resuspended in 70 µl of the silylation reagent N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Macherey-Nagel) and incubated for 1 h at 37 °C. Content of SA was measured by injecting 1 µl of the derivatized extract into GC/MS (TRACE GC Ultra/DSQII (Thermo Scientific)) using a 30 m x 0.25 mm capillary column with 0.25 µm film as stationary phase (FactorFour Column VF-17ms, Varian). The applied GC temperature program was as follows: 5 min at 70 °C, 70-280 °C with 5 °C/min, 10 min at 280 °C. Helium at a flow rate of 1.0 ml/min was used as carrier gas. For quantification Di(trimethylsilyl)-derivatives of SA and 3HBA were measured in the SIM-mode of the quadrupole MS. The m/z values monitored were 209, 233, 267 and 282. m/z = 267 was used for the quantification of SA normalized to the internal standard 3HBA. Salicylic acid (USP, Rockville) was used for creating a standard curve for 3HBA-normalized SA quantification.

***In-vitro* and in-gel protein kinase assays**. *In-vitro* kinase assay: *In-vitro* kinase assay was performed with affinity purified *At*CPK5 protein expressed in *N. benthamiana*. AtCPK5 bound to Strep-Tactin Macroprep resin was resuspended with 50 µl Buffer E (50 mM HEPES, pH 7.4; 2 mM DTT; 0.1 mM EDTA). 5 µl slurry was mixed with 20 µl Buffer E and 5 µl reaction mix (60 mM $MgCl_2$; 60 µM $CaCl_2$; 60 µM ATP; 60 µM Syntide 2 or 60 µM *At*RBOHD Peptide aa 143-152; 3 µCi [γ-^{32}P]-ATP). As negative control $CaCl_2$ was replaced by 12 mM EGTA. The reaction was performed for 10 min at RT and stopped with 3 µl 10% phosphoric acid. 20 µl of the reaction was spotted on P81 anion-exchange paper and washed 4 times with 1 % phosphoric acid and radioactivity was determined using scintillation mixture.

CDPK in-gel kinase assay: Protoplasts were harvested by centrifugation and frozen in liquid nitrogen. Protoplast pellets or frozen Arabidopsis leaf material samples were resuspended by vortexing in extraction buffer [100 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM DTT, 10 mM NaF, 10 mM NaVO4, 10 mM β-glycerole-phosphate, 0.5 mM AEBSF, 100 µg/mL avidin and 1 × protease inhibitor mixture (Sigma)]. Crude extracts were centrifuged at 20,000 × g for 5 min at 4 °C, and proteins were seperated by SDS-PAGE containing 0.25 mg/ml Histone III-S (Taufkirchen, Germany). The gel was washed two times 1h in wash-buffer (25 mM Tris-HCl, pH 7.5; 0.5 mM DTT; 5 mM NaF; 0.1 mM Na_3VO_4; 0.5 mg/ml BSA; 0.1 % Triton X-100). Protein-renaturation was performed by incubating the gel with renaturation buffer (25 mM Tris-HCl, pH 7.5; 0.5 mM DTT; 5 mM NaF; 0.1 mM Na_3VO_4) for 1h at room temperature, overnight at 4 °C and 1 h at room temperature. After equilibration of the gel for 30 min in the reaction buffer (25 mM Tris-HCl, pH 7.5; 1 mM DTT; 0.1 mM Na_3VO_4; 12 mM $MgCl_2$; 1 mM $CaCl_2$), the kinase reaction was performed for 1.5 h in the reaction buffer with 0.1 nM ATP and 50 µCi [γ-^{32}P]-ATP. The reaction was stopped and washed 6 times with 5 % TCA and 1 % phosphoric acid for 4 h. The gel was dried and visualized by autoradiography.

Targeted analysis of *in vivo* phosphorylation by selected reaction monitoring. Protein extracts were in-solution digested with trypsin. After acetone precipitation of 100 µg protein, pellets were resuspended in 6 M urea, 2 M thiourea, pH 8.0. Disulfide bonds in proteins were reduced by adding DTT and subsequently free cysteine residues were alkylated using iodoacetamide. Samples were then desalted over C18 tips (5). Tryptic peptide mixtures were analyzed by selected reaction monitoring using nanoflow HPLC (Easy nLC, Thermo Scientific)

and a triple quadrupole mass spectrometer (TSQ Quantum Discovery Max, Thermo Scientific) as mass analyzer. Peptides were eluted from a 75 µm analytical column (Easy Columns, Thermo Scientific) on a linear gradient running from 10 % to 30 % acetonitrile in 90 min and were ionized by electrospray directly into the mass spectrometer. The target ions were derived from phosphorylated and unphosphorylated forms of four specific *At*RBOHD peptides GAFSGPLGRPK, VFSR, TSSAAIHALK, and ILSQMLSQK. Selected reaction monitoring (SRM) was used to quantify the abundance of phosphorylated and unphosphorylated forms of the respective peptides within each sample. The quadrupole Q1 was set as a mass filter for the parent ion, while Q3 was set to monitor specific fragment ions, which had been determined experimentally based on synthesized standard peptides (AQUA Basic Kit, Thermo Scientifc). For each parent ion, at least three fragment ions were used. Mass width for Q1 and Q3 was 0.7 Da, scan time 5 ms. Data analysis merging of fragment ion information to peptide average and abundance quantification was done using the Pinpoint software v.1.0 (Thermo Scientific). For each phosphorylated and unphosphorylated peptide, a stable-isotope labeled synthetic standard peptide was used as an internal standard. Standard peptide VFSR was labeled with $^{15}N^{13}C_5$-valine, and standard peptide GAFSGPLGRPK with $^{15}N^{13}C_6$-Leucine. To each biological sample, 1 pmol of the two standard peptides were added prior to HPLC separation. For quantitative analysis, ion intensities of the light forms of the target peptides were normalized based on the ion intensities of the co-eluting standard peptide and normalized ion intensities sums of fragment ions in each sample were calculated.

Fragment ions for individual peptides:

pS39: GAF(pS)GPLGRPK (m/z 583.789; fragment ions m/z 534.8011, 667.4244, 724.4459, 244.165, 891.4443). pS162: T(pS)SAAIHALK (m/z 539.768; characteristic fragment ion m/z 723.45064) and pS163: TS(pS)AAIHALK (m/z 539.768; characteristic fragment ion m/z 890.449). pS148: VF(pS)R (m/z 294.63; fragment ions m/z 588.25436, 489.1852, 342.1168, 245.6423). pS343: IL(pS)QMLSQK (m/z 564.280, fragment ions m/z 515.2917, 734.386, 901.3843, 475.2869) and pS343_pS347: IL(pS)QML(pS)QK (m/z 604.263, fragment ions m/z 555.2748, 814.3523, 981.3507, 442.1692, 686.2938).

1. Witte CP, Noel LD, Gielbert J, Parker JE, & Romeis T (2004) Rapid one-step protein purification from plant material using the eight-amino acid StrepII epitope. *Plant molecular biology* 55(1):135-147.
2. Feys BJ, *et al.* (2005) Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. *The Plant cell* 17(9):2601-2613.
3. Cao FQ, *et al.* (2010) Identification and characterization of proteins involved in rice urea and arginine catabolism. *Plant physiology* 154(1):98-108.
4. Kemmerling B, *et al.* (2007) The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. *Current biology : CB* 17(13):1116-1122.
5. Rappsilber J, Ishihama Y, & Mann M (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Analytical chemistry* 75(3):663-670.

SI Figures

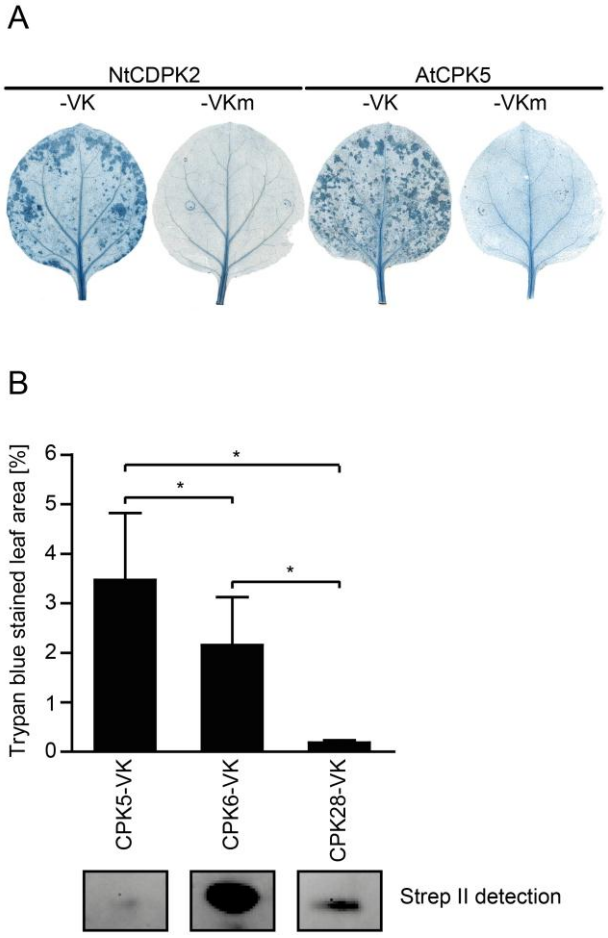

Fig. S1. Expression of constitutively active CPK5-VK leads to cell death symptoms. (*A*) CPK5-VK induces cell death in *N. benthamiana*. StrepII-tagged *Nt*CDPK2-VK and CPK5-VK protein kinase variants lacking the CDPK auto-inhibitory region and calmodulin-like domain, and respective kinase-deficient variants *Nt*CDPK2-VKm and CPK5-VKm, were transiently expressed in leaf mesophyll cells of *N. benthamiana*. Three days after infiltration, cell death symptoms were determined by staining with Trypan Blue. (*B*) Quantification of cell death symptoms in N. benthamiana plants transiently expressing Strep II tagged CPK5-VK, CPK6-VK and CPK28-VK. Cell death in N. benthamiana leaves was determined by Trypan Blue staining as in (A). Trypan Blue stained leaf area was quantified using imageJ. Error bars, SEM ($n \geq 3$); 1way ANOVA, Bonferroni Post Test, *, $p < 0.05$. Protein expression was verified by immunoblot analysis.

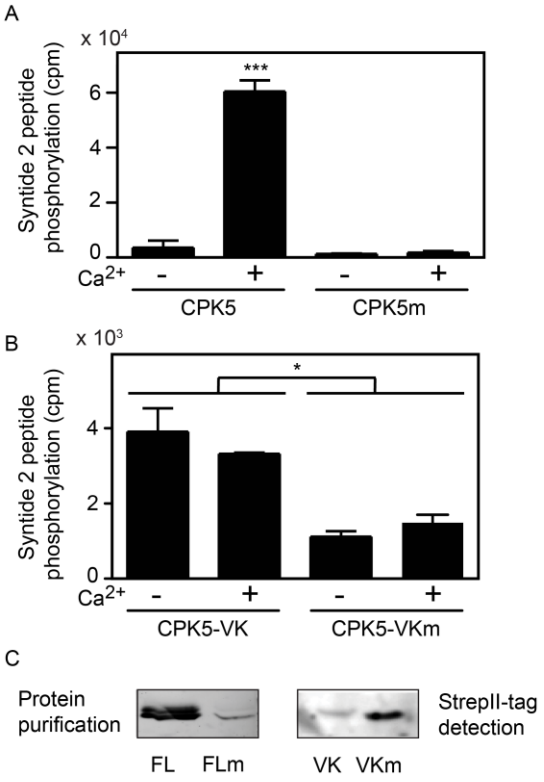

Fig. S2. *In vitro* kinase activity of purified CPK5 and CPK5-VK. StrepII-tagged full-length CPK5, and CPK5-VK (the constitutive active variant lacking the auto-inhibitory- and calmodulin-like domain), and the corresponding kinase-deficient variants CPK5m and CPK5-VKm were transiently expressed in *N. benthamiana.* The proteins were affinity purified using the StrepII tag. *In vitro* protein kinase assays in the absence or presence of 10 µM calcium with syntide2 as synthetic CDPK substrate were performed. (*A*) Increase in calcium-dependent kinase activity of full-length CPK5. Error bars, SD (n = 4); 1way ANOVA, Bonferroni Post Test, ***, $p < 0.0001$. (*B*) Reduced but calcium-independent kinase activity of truncated CPK5-VK. Error bars, SD (n = 3); 1way ANOVA, Bonferroni Post Test, *, $p < 0.05$; **, $p < 0.01$. (*C*) Control of protein purification by immunoblot analysis.

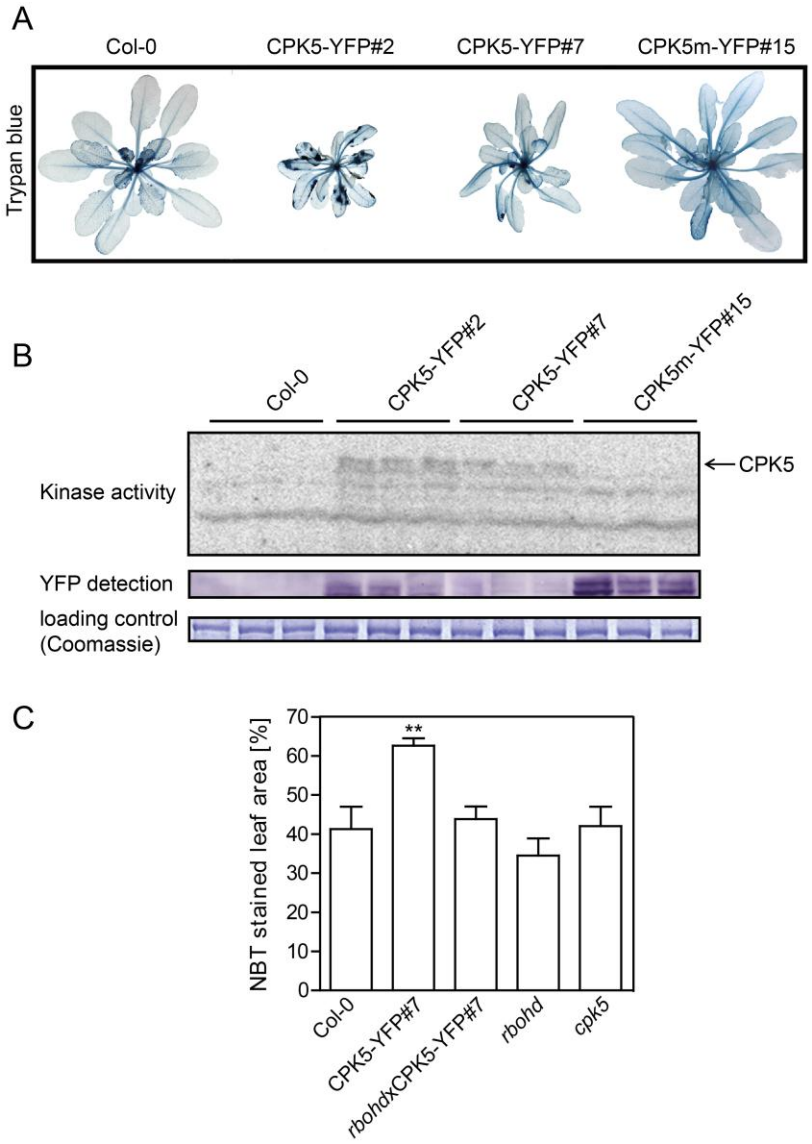

Fig. S3. Characterization of transgenic CPK5-expressing lines. (*A*) Growth and cell death development, determined by trypan blue staining, in four week old Col-0 were compared to plants expressing the following YFP-tagged CPK5 variants: *35S::CPK5-YFP#2* (strong overexpressor), *35S::CPK5-YFP#7* (moderate overexpressor), and *35S::CPK5m-YFP#15* (overexpressing kinase-deficient variant). (*B*) Transphosphorylation kinase activity of three individuals per line as in (*A*) was determined via in gel kinase assay (upper panel). Protein expression and equal loading was monitored by immunoblot analysis (middle panel) and Coomassie staining (lower panel). (*C*) Determination of superoxide production via NBT staining. Superoxide was detected in the absence of further stimulation over 18 h via NBT staining in Col-0 and *rbohd* and *cpk5* mutant plants as well as the transgenic CPK5-YFP#7 and *rbohd*xCPK5-YFP#7 lines. Error bars, SEM (n = 4); 1way ANOVA, Dunnett Post Test, **, $p < 0.01$.

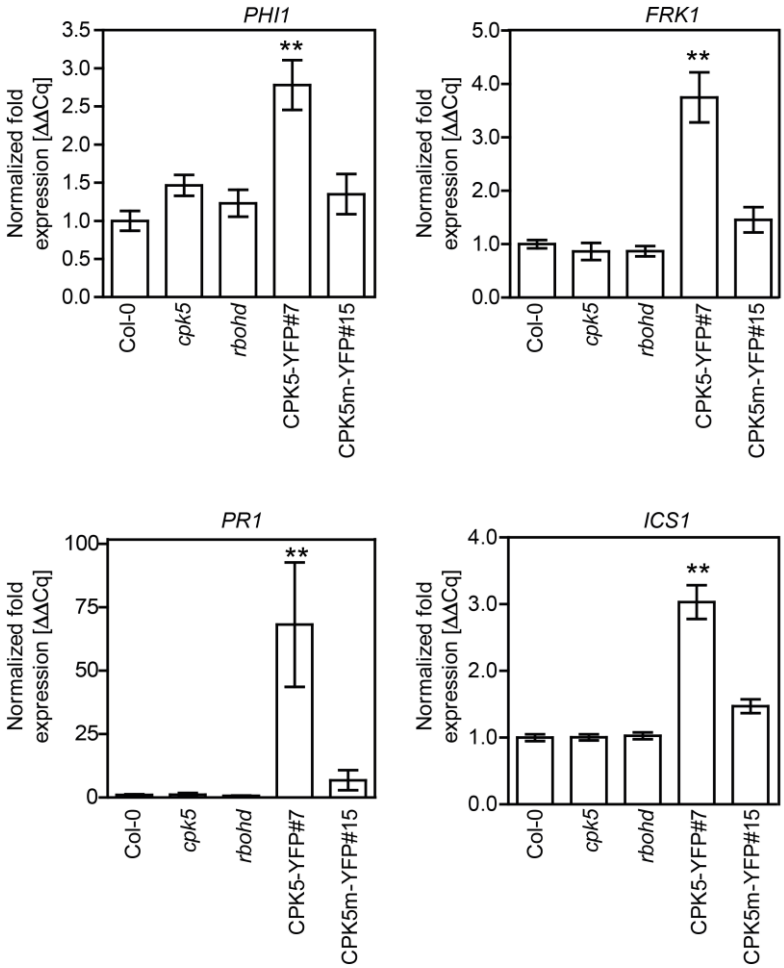

Fig. S4. CPK5-dependent defense gene expression. Basal gene transcription levels were determined by qRT-PCR in six week old plants in the absence of treatment. Expression of previously reported, rapidly flg22-induced defense genes *FRK1* and *PHI1*, as well as of the salicylic acid biosynthesis marker gene *ICS1* and the salicylic acid-dependent signaling gene *PR1* was compared in Col-0, *cpk5* and *rbohd* mutants and transgenic CPK5-YFP and CPK5m-YFP expressing lines. Error bars, SEM ($n \geq 11$); 1way ANOVA, Dunnett Post Test, **, $p < 0.01$.

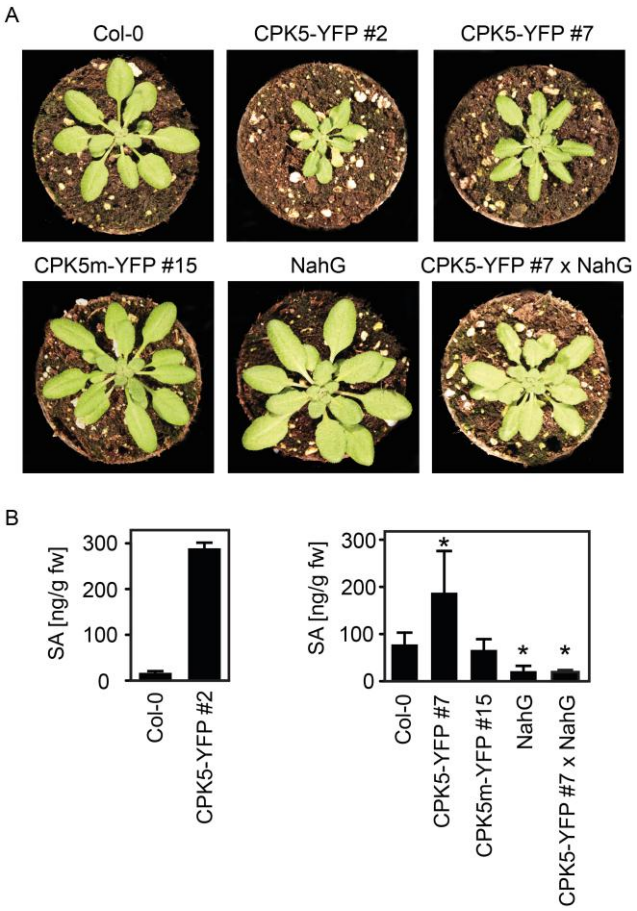

Fig. S5. CPK5 activates salicylic acid-dependent signaling pathways. (*A*) Phenotypic comparison of four week old Col-0, CPK5 and CPK5m overexpressing plants *35S::CPK5-YFP#2* (high overexpressor), *35S::CPK5-YFP#7* (moderate overexpressor), *35S::CPK5m-YFP#15* (expressing kinase-deficient variant), *35S::NahG* expressor, and of plants resulting from cross between *35S::CPK5-YFP#7x35S::NahG*. (*B*) Quantification of free salicylic acid (SA) content in rosette leaves of five week old plants of lines as described in (*A*). Error bars, SD ($n \geq 5$); Student's t-test, *, $p < 0.05$.

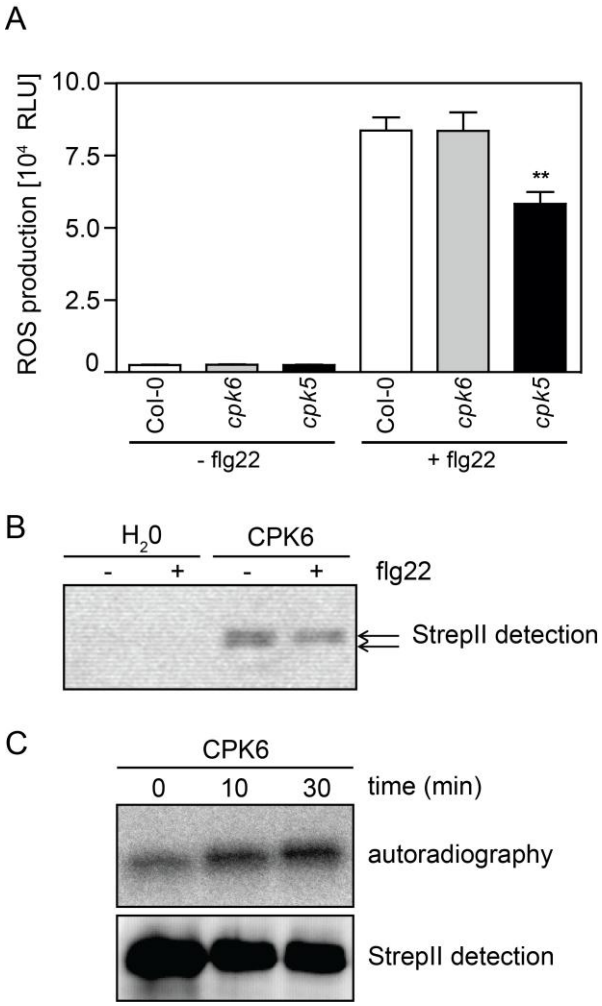

Fig. S6. Flg22 elicitation triggers CPK6 kinase activity. (*A*) CPK5, but not CPK6, is essential for flg22-induced ROS production. ROS production was determined over 60 min via a luminol-based assay with and without treatment with 200 nM flg22 in Col-0, *cpk5* and *cpk6* plants. Error bars, SEM ($n \geq 16$); 1way ANOVA, Dunnett Post Test, **, $p < 0.01$. (*B*) Flg22 induces biochemical modification of CPK6. Upon transient expression in Col-0 mesophyll protoplasts, full-length StrepII-tagged CPK6 showed biochemical modification detected 10 min after treatment with buffer (-) or 200 nM flg22 (+) by immunoblot analysis. (*C*) Increased CPK6 kinase activity after flg22 treatment. Histone in gel-kinase assay after CPK6 expression in Col-0 protoplasts. CPK6 protein kinase activation was analyzed 0, 10 and 30 min after treatment with 200 nM flg22 by histone in-gel kinase assay (upper panel) and immunoblot analysis (lower panel).

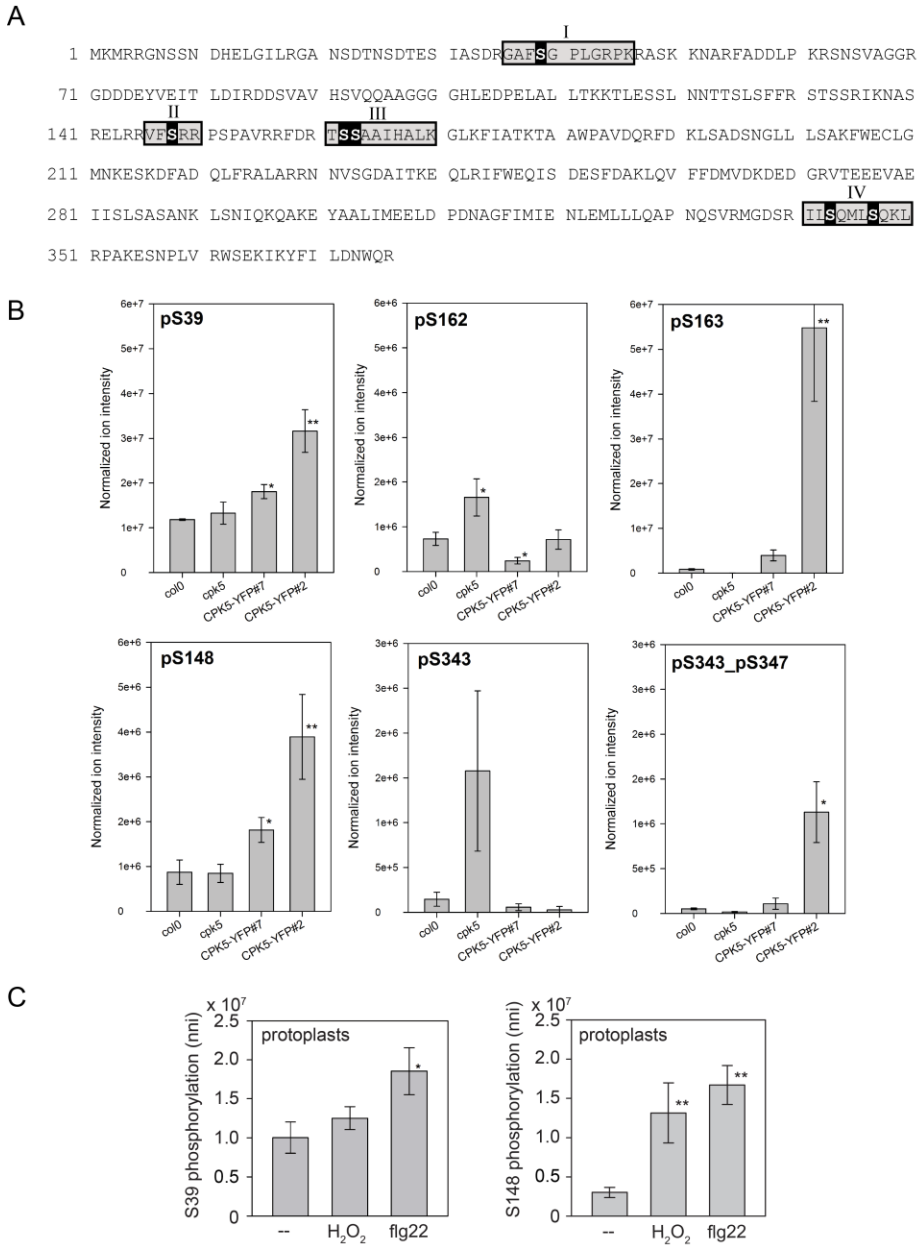

Fig. S7. SRM-analysis of CPK5-dependent *in vivo* phosphorylated distinct N-terminal serine residues of RBOHD. (*A*) N-terminal RBOHD peptides subject to CPK5-dependent phosphorylation *in vivo*. Protein sequence of RBOHD N-terminus. Identified *in vivo* phosphopeptides I – IV are shaded in grey, CPK5-dependent differential phosphorylation sites are shaded in black. (*B*) Rosette leaves of four week old plants from lines Col-0, *cpk5* mutant, *35S::CPK5-YFP#2*, and *35S::CPK5-YFP#7* were harvested, proteins extracted, and tryptic peptide mixtures analyzed by selected reaction monitoring using nanoflow HPLC and triple quadrupole mass spectrometry. Summed normalized fragment ion intensities of different phosphopeptides of RBOHD are shown. Error bar, SD (n = 3); 1way ANOVA, Holm-Sidak Post Test, **, $p < 0.01$; *, $p < 0.5$. (*C*) Quantification of CPK5-dependent phosphorylation of N-terminal serine residues S39 and S148 of RBOHD described in legend to Fig 3 B and C. Peptide phosphorylation in protoplasts in the absence of, or 10 min after, treatment with 200 nM flg22 or 200 µM H_2O_2. Error bar, SD (n = 3); 1way ANOVA, Holm-Sidak Post Test, **, $p < 0.01$; *, $p < 0.5$.

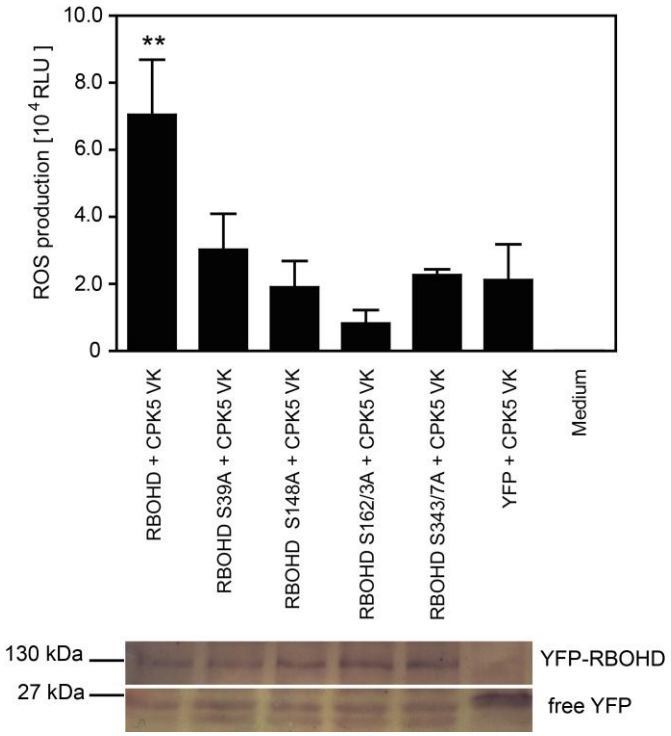

Fig. S8. *In vivo* differentially phosphorylated N-terminal serine residues of RBOHD are required for CPK5- and RBOHD-dependent ROS production. StrepII-tagged constitutively active CPK5-VK protein kinase was co-expressed in *N. benthamiana* with either YFP, wild type RBOHD fused to YFP, or with RBOHD variants in which identified CPK5-dependent differentially phosphorylated serine residues were substituted by alanine. ROS production was determined via a luminol-based assay in eight leaf discs per leaf per single measurement. Error bars, SEM (n = 3); 1way ANOVA, Dunnett Post Test, **, $p < 0.01$. YFP-fusion protein expression was monitored by immunoblot analysis.

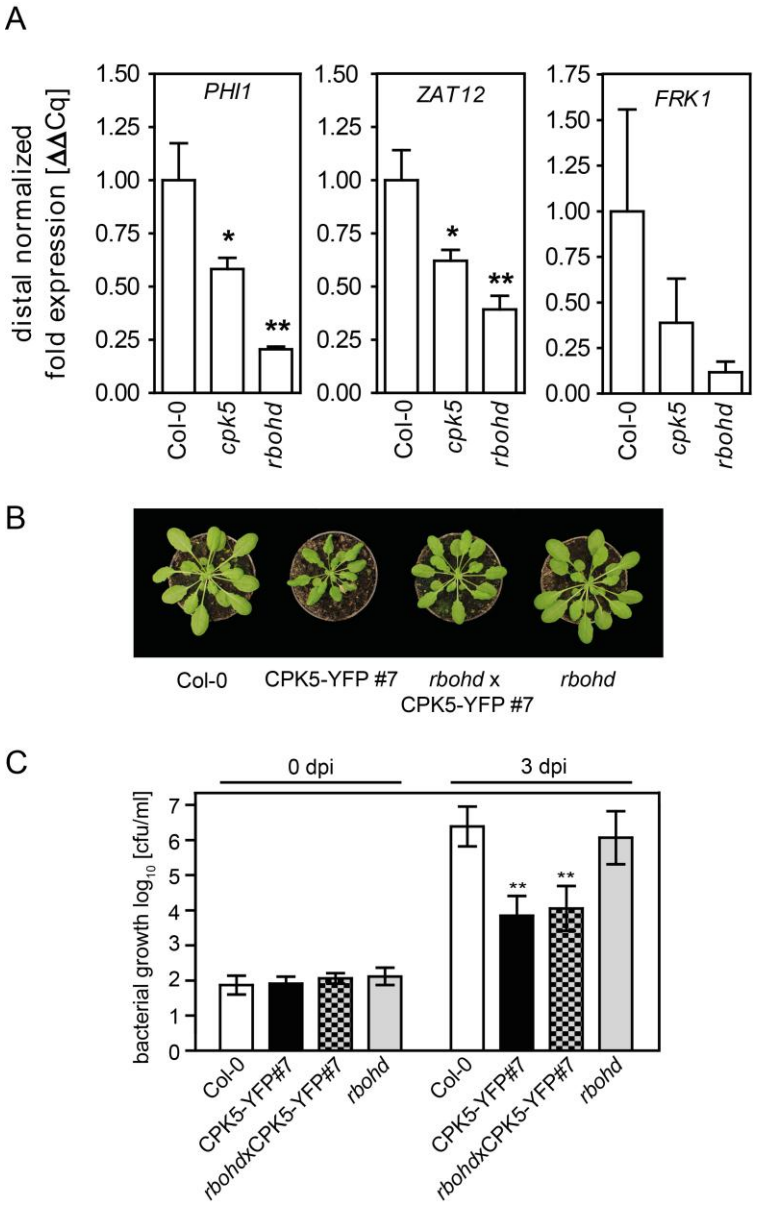

Fig. S9. CPK5 and RBOHD in distal defense responses. (*A*) Distal flg22-induced defense gene expression is dependent on CPK5 and RBOHD. 45 min after 200 nM flg22 injection into a lower rosette leaf of six week old Col-0, *cpk5* and *rbohd plant*s, gene expression of *PHI1*, *ZAT12* and *FRK1* was quantified by qRT-PCR in an upper leaf. Error bars, SEM ($n \geq 7$); 1way ANOVA, Dunnett Post Test, *, $p < 0{,}05$; **, $p < 0.01$. (*B*) Comparison of plant growth of the transgenic line *35S::CPK5-YFP#7* and *rbohd*x*CPK5-YFP#7*. (*C*) Resistance to the bacterial pathogen, *Pst* DC3000 in transgenic CPK5-overexpressing lines does not require RBOHD. Bacterial counts were taken in six week old Col-0, *rbohd*, *35S::CPK5-YFP#7* and *rbohd*x*35S::CPK5-YFP#7* plants at day 0 and day 3 after inoculation of *Pst* DC3000. Error bars, SD ($n \geq 20$); 1way ANOVA, Dunnett Post Test, **, $p < 0.01$.

SI Table

SI Table: Sequences of oligonucleotide primers

Construct	Sequence
cloning of CPK5, CPK5-VK and respective kinase deficient constructs and RBOHD	
CPK5 F	CACCATGGGCAATTCTTGCCGTG
CPK5 R	CGCGTCTCTCATGCTAATG
CPK5 VK F	CACCATGGGCAATTCTTGCCGTG
CPK5 VK R	AACACCATTCTCACAGATCCATG
CPK5m/VKm F	TGGTGTGATGCATAGAGCCTTGAAGCCTGAGAATTT
CPK5m/VKm R	AAATTCTCAGGCTTCAAGGCTCTATGCATCACACCA
RBOHD F	AAGAATTCATGAAAATGAGACGAGGCAA
RBOHD R	AAGGATCCCTAGAAGTTCTCTTTGTGGAAGTC
qRT-PCR	
NHL10 F	TTCCTGTCCGTAACCCAAAC
NHL10 R	CCCTCGTAGTAGGCATGAGC
PHI1 F	TTGGTTTAGACGGGATGGTG
PHI1 R	ACTCCAGTACAAGCCGATCC
FRK1 F	CGGTCAGATTTCAACAGTTGTC
FRK1 R	AATAGCAGGTTGGCCTGTAATC
PR1 F	CATGGGACCTACGCCTACC
PR1 R	TTCTTCCCTCGAAAGCTCAA
ICS1 F	TTCTCAATTGGCAGGGAGAC
ICS1 R	AAGCCTTGCTTCTTCTGCTG
Actin2 F	TCCCTCAGCACATTCCAGCAGAT
Actin2 R	AACGATTCCTGGACCTGCCTCATC
site-directed mutagenesis of RBOHD phosphorylation mutant constructs	
S39A F	CGTGGTGCCTTTGCCGGTCCGCTTGG
S39A R	CCAAGCGGACCGGCAAAGGCACCACG
S148A F	CGCCGCGTGTTCGCTAGACGTCCCTCC
S148A R	GGAGGGACGTCTAGCGAACACGCGGCG
S162/163A F	TTTGACCGCACGGCCGCCGCGGCCATCCAC
S162/163A R	GTGGATGGCCGCGGCGGCCGTGCGGTCAAA
S343A F	GACAGCAGGATACTTGCTCAGATGTTAAGTCAG
S343A R	CTGACTTAACATCTGAGCAAGTATCCTGCTGTC
S343/347A F	CTTGCTCAGATGTTAGCTCAGAAGCTTAGACCGGC
S343/347A R	GCCGGTCTAAGCTTCTGAGCTAACATCTGAGCAAG

F: forward; R: reverse