Molecular Plant, Volume *13*

Supplemental Information

Phosphorylation of the *Pseudomonas* Effector AvrPtoB by *Arabidopsis* SnRK2.8 Is Required for Bacterial Virulence

Lei Lei, Danielle M. Stevens, and Gitta Coaker

Phosphorylation of the Pseudomonas effector AvrPtoB by Arabidopsis SnRK2.8 is required for Bacterial Virulence

Running Title: Phosphorylation of the AvrPtoB effector by SnRK2.8

Authors and Affiliations: Lei Lei[1], Danielle M. Stevens[1], Gitta Coaker[1*]

[1]Department of Plant Pathology, University of California, Davis, Davis, CA, USA

*Corresponding author: Gitta Coaker; Email: glcoaker@ucdavis.edu

Supplemental methods

Yeast two-hybrid screen

The GAL4 based Matchmaker yeast two-hybrid system was used for the AvrPtoB-kinase interaction screen (Clonetech). *AvrPtoB* and a *mCherry* negative control were cloned into the pGADT7 vector fused to the GAL4 activation domain and HA tag. *Arabidopsis SnRK*s and *CDPKs* were cloned into the pGBKT7 vector, which contains the GAL4 DNA binding domain and N-terminal Myc epitope tag. In order to detect interactions with CDPKs, their C-terminal Ca^{2+} regulatory and auto-inhibitory domains were removed prior to clone into the pGBKT7 vector. Primers are listed in Table S2. The pGADT7-*AvrPtoB* and pGADT7-*mCherry* plasmids were separately co-transformed with each pGBKT7-*SnRK/CPKΔC* plasmid into the yeast strain AH109, colonies were selected on SD -Leu/-Trp dropout media and tested for interactions on SD -Leu/-Trp/-His dropout media containing X-α-Gal. Yeast transformation and media preparation were performed per manufacturer instructions (Clontech). To confirm protein expression, yeast proteins were extracted as described previously and subjected to anti-HA HRP (Roche #12013819001; 1:2,000) and anti-Myc (Clontech #631206; 1: 2,000) immunoblotting (Zhang et al., 2011b).

Plant protein extraction and immunoblotting

Plant tissues were ground in liquid nitrogen and homogenized in Protein Extraction Buffer [(PEB: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.5% IGEPAL, 5% glycerol, 1 mM PMSF, 3 mM DTT, 1 × CPI (Thermo Fisher Scientific), 1× PPI (Thermo Fisher Scientific), 50 mM MG132 (Sigma-Aldrich)] (Chen et al., 2017). The homogenate was cleaned by centrifuging at 14,000 rpm for 15 min at 4 ℃, and boiled with 5 × SDS buffer (250 mM Tris-HCl pH 6.8, 6% SDS, 0.5 M DTT, 30% glycerol, 0.08% bromophenol blue) for 10 min.

Co-immunoprecipitation assays

To confirm the association between AvrPtoB and SnRK/CDPKs *in planta*, kinase dead variants were generated and tested for their ability to associate with wild-type AvrPtoB after transient expression in *N. benthamiana. SnRK1.1-KD* (K71A), *SnRK2.6-KD* (K50A), *SnRK2.8-KD* (K33A), *CPK4ΔC-KD* (K54A) and *CPK5ΔC-KD* (K126A) kinase dead variants were generated by PCR-based site-directed mutagenesis and fused with a C-terminal HA tag in the binary vector pGWB414 (Nakagawa et al., 2007). Primers are listed in Table S3. *AvrPtoB* fused with a C-terminal GFP tag was cloned into the dexamethasone (Dex)-inducible binary vector pTA7001 (Gu and Innes, 2011). Binary vectors were transformed into *Agrobacterium tumefaciens* GV3101. *Agrobacterium* suspensions were co-infiltrated into *N. benthamiana* leaves at an OD600 = 0.4 for AvrPtoB and an OD600 = 0.6 for each kinase. Twenty-four hours post-inoculation, 15 μM DEX and 0.01% Silwet L-77 were sprayed to induce the expression of AvrPtoB-GFP. Two grams of leaf tissue per sample were collected three hours post-DEX application.

For immunoprecipitations, *N. benthamiana* leaf tissues were ground in liquid nitrogen and re-suspended in 2 mL IP buffer (50mM Tris-HCL ph7.5, 150mM NaCl, 0.1% Triton, 0.2% NP-40, 1× complete protease inhibitor (Thermo Fisher Scientific #A32963) and 1× phosphatase inhibitor (Thermo Fisher Scientific #A32957), 1mM DTT, 40uM MG132, 0.5% PVP). Samples were centrifuged at 14,000 rpm for 15 min and filtered to remove debris using a poly-prep chromatography column (10 mL, Bio-Rad). The supernatant was incubated with 25 μL of anti-GFP agarose beads at 4℃ for 1.5 hr. Beads were washed once with IP buffer by centrifuging at 3000 rpm for two min and twice by filtration through a pierce centrifuge column (0.8 mL, Bio-Rad). Proteins were eluted from the beads by boiling in 2× Laemmli buffer for five min. Proteins were separated by SDS-PAGE and immunoblotted with anti-HA HRP (Roche #12013819001; 1:2,000) and anti-GFP HRP (Miltenyi Biotec #130-091-833; 1:3,000).

To test the ability of AvrPtoB phosphorylation mutants to disrupt FLS2-BAK1 complex formation, *AvrPtoB* phospho-null mutants (S258A and S205AS210AS258A) were generated by PCR-based site-directed mutagenesis. Primers are listed in Table S2. *AvrPtoB* variants were fused with C-terminal 3× FLAG tag in binary vector pTA7001, and *FLS2* and *BAK1* were separately fused with C-terminal GFP tag and HA tag in binary vector pEarleyGate 103 and pGWB14 (Earley et al., 2006; Nakagawa et al., 2007). The binary vectors were transformed into

Agrobacterium tumefaciens GV3101. *FLS2*, *BAK1*, and *AvrPtoB* variants *Agrobacterium* suspensions were co-infiltrated into *N. benthamiana* leaves, 15 μM DEX and 0.01% Silwet L-77 were sprayed to induce the expression of AvrPtoB at 24 hpi for three hours, two grams of leaf tissue for each sample were collected after 15 min treatment with 5 mM $MgCl_2$ or 10 μM flg22. Immunoprecipitation was performed as described above.

Recombinant protein purification

AvrPtoB and *SnRK2.8* were cloned in *E.coli* expression vector pDEST15 (Invitrogen) fused with N-terminal GST tag, the constructs were transformed into *E.coli* BL21 (DE3). 200 mL of *E.coli* culture was grown at 28 ℃ until OD600 = 0.5. Protein expression was induced with 0.5mM IPTG at 16 ℃ for 12 hr. Cells were harvested by centrifuging at 5000 g 4 ℃ for 10 min and washed once with buffer A (0.1 M Tris-HCl pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 × CPI, 10 mM DTT and 10 uM MG132). Cell pellets were resuspended in 3 mL of buffer A with 15 μg/mL lysozyme and incubated on ice for 30 min. Total protein was released by sonication and incubated with Glutathione Sepharose 4B (GE Healthcare #GE17-0756-01) at 4 ℃ for one hour. Agarose beads were washed three times with buffer A by centrifuging at 5000 g for 5 min. Proteins were eluted by incubating with buffer B (50 mM Tris-HCl pH8, 10 mM reduced Glutathione) for 10 min at room temperature (RT).

Kinase activity assay

An *in vitro* kinase activity assay was performed with recombinant proteins, 3 μg of GST-AvrPtoB and 0.3-1 μg GST-SnRK2.8 were mixed in kinase buffer (20 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM ATP, 1 mM DTT). The kinase reaction was performed at 30 ℃ for 30 min and stopped by 3 × Laemmli buffer. Protein samples were separated in SDS-PAGE and immunoblotted with anti-pSer antibody (Sigma #P3430; 1:1000) and anti-GST antibody (Sigma; 1: 3000).

Phosphorylation site identification and quantification

To identify AvrPtoB phosphorylation sites *in vivo*, *AvrPtoB-YFP* in the pBluescript vector was transiently expressed in Col-0 and *snrk2.8* protoplasts, protoplast preparation and transient transformation were performed as previously described (Yoo et al., 2007). 1 mL of protoplasts was transfected with 100 μg of plasmid and collected in 9 hr. Protein was released in IP buffer (without 0.5% PVP) and subject to GFP-IP as described above. Protein peptides were generated by in-solution trypsin digest as previously described and subjected to LC-MS/MS run by Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) (Minkoff et al., 2013). LC-MS/MS data was analyzed by software MaxQuant (Tyanova et al., 2016).

To quantify the phosphorylated peptides, an inclusion list of phosphopeptide and control peptides, including the Mono-isotopic precursor (m/z) and charge state (z), was generated by software Skyline based on previous MS data, as shown in Table S4 (MacLean et al., 2010). The peptide samples were scanned by Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) with a parallel reaction monitoring (PRM) method. The PRM data were analyzed by MaxQuant and Skyline, the peptides peak areas were exported for the quantification analysis.

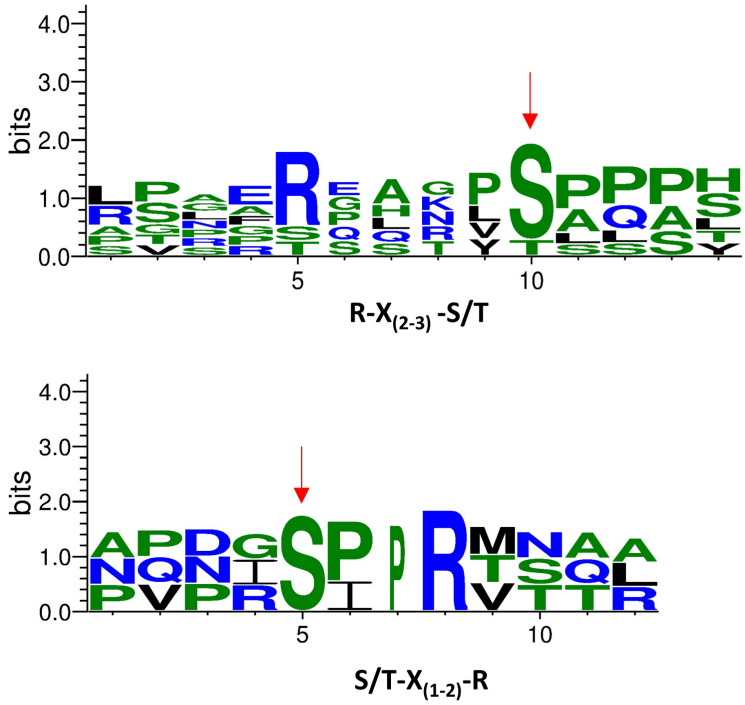
NPR1 and FLS2 accumulation

To test the ability of AvrPtoB to inhibit NPR1 and FLS2 accumulation in *Arabidopsis* Col-0 and the *snrk2.8* knockout. DC3000 -/- carrying the empty vector (EV) or a plasmid expressing wild-type *AvrPtoB* were syringe infiltrated into Col-0 and *snrk2.8* at a concentration of 1×10^8 CFU mL^{-1} and proteins extracted after 4h (NPR1) and 8h (FLS2). The immunoblot was performed by anti-NPR1 (Agrisera #AS12 1854; 1:1000) and anti-FLS2 (Agrisera #AS12 1857; 1:5000) primary antibody followed by anti-rabbit-HRP (BioRad #170-5046; 1:3000) secondary antibody.

To test the ability of AvrPtoB phosphorylation mutants to inhibit NPR1 and FLS2 accumulation, *AvrPtoB* phospho-null mutants (S258A and S205AS210AS258A) and *AvrPtoB* phospho-mimic mutants (S258D and S205DS210DS258D) were generated by PCR-based site-directed mutagenesis. Primers are listed in Table S2. *AvrPtoB* variants were fused with C-terminal 3× FLAG tag in binary vector pTA7001, and *NPR1* and *FLS2* were separately fused with C-terminal HA tag and GFP tag in binary vector pEarleyGate 103 and pGWB14 (Earley et al., 2006; Nakagawa et al., 2007). The immunoblot was performed by anti-HA HRP (Roche

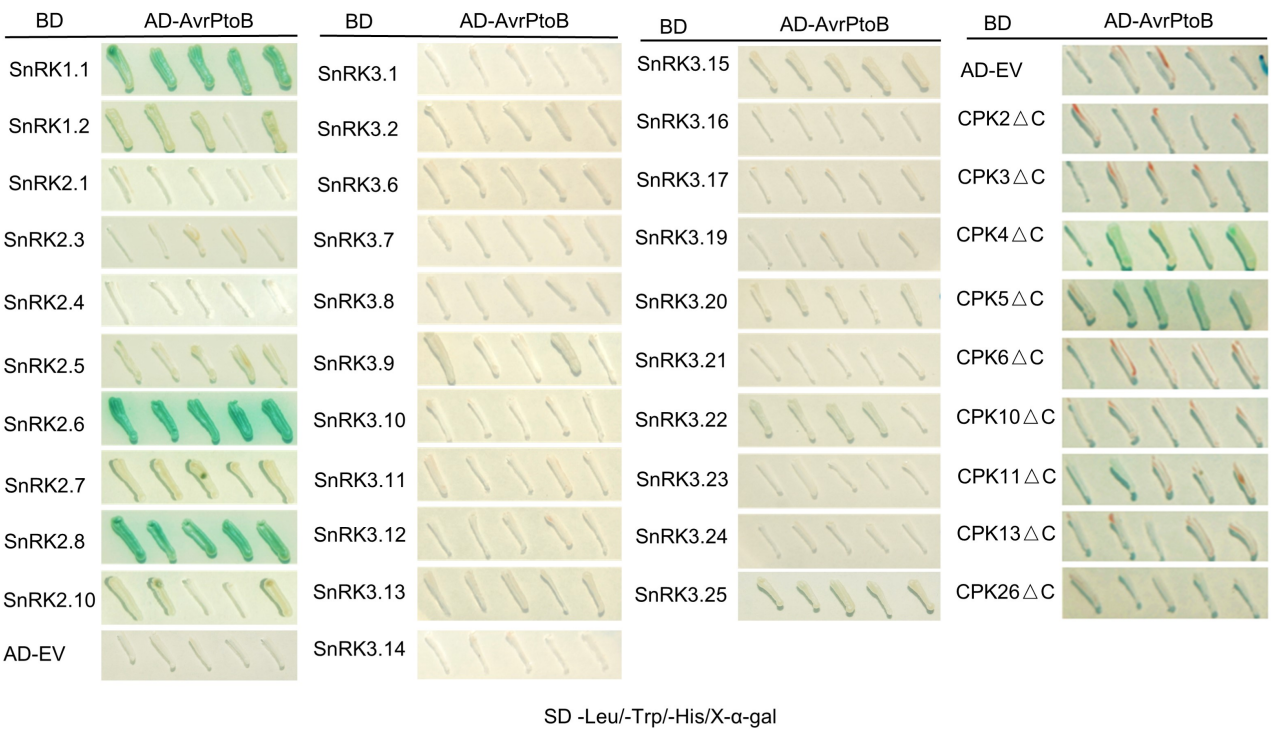
#12013819001; 1:2,000), anti-FLAG (Sigma #A8592; 1:3000), and anti-GFP HRP (Miltenyi Biotec #130-091-833; 1:3,000).

Supplemental figure



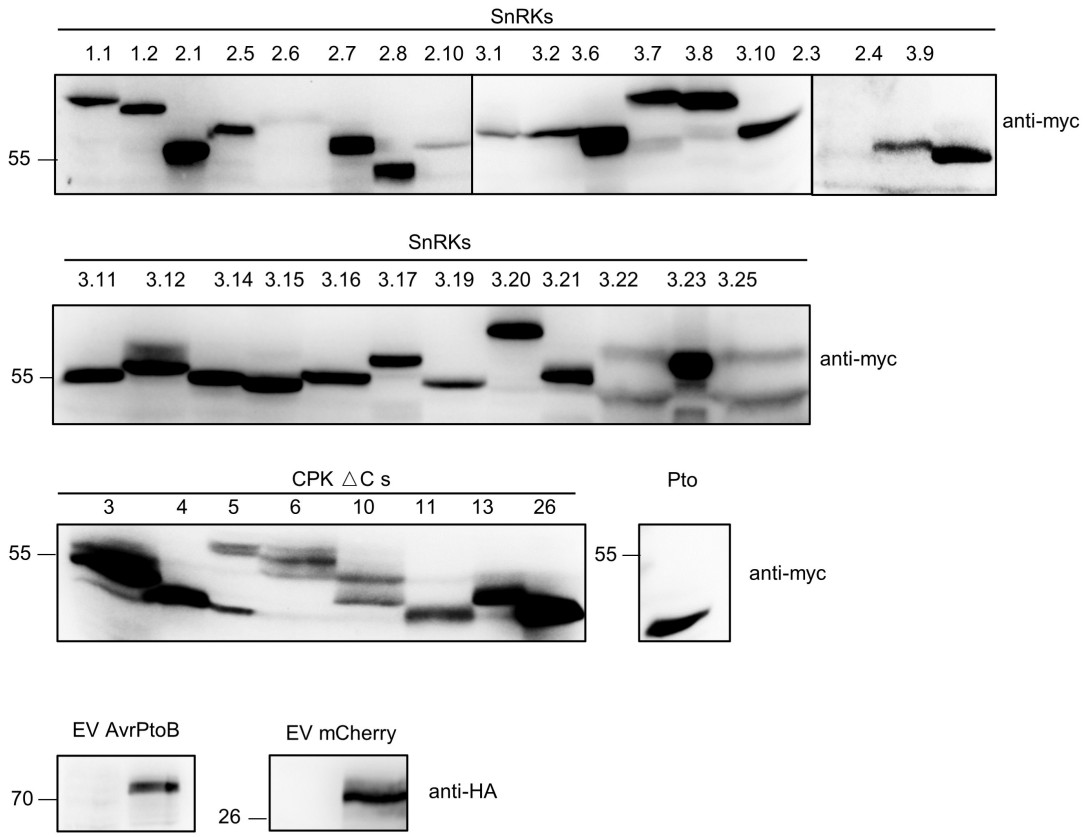
Supplemental Figure 1. Amino acid conservation surrounding known effector phosphorylation sites. Related to Figure 1.

WebLogo alignment of the effector phosphorylation sites shown in Table S1. Red arrows point the phosphorylated residues. Amino acid size correlates with degree of conservation. The sequence logo was created by the WebLogo 3 (Crooks et al., 2004).



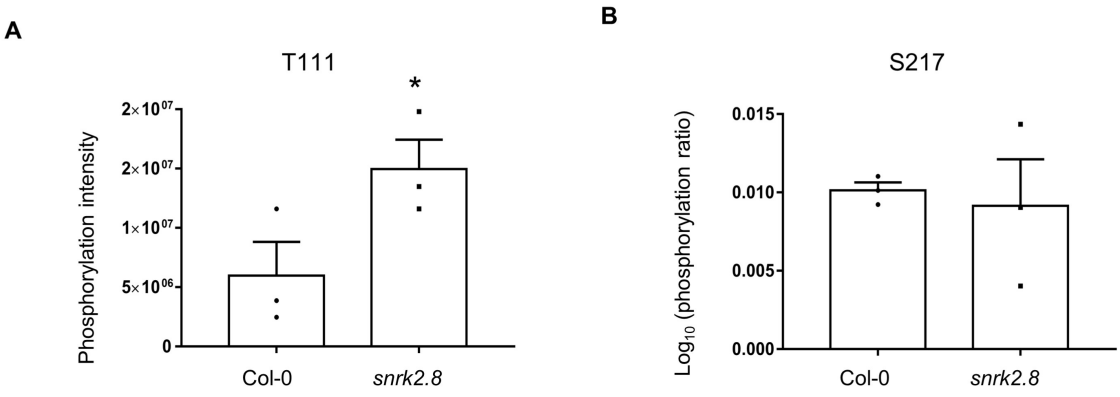
Supplemental Figure 2. Yeast-two hybrid screening of interactions between the *Pseudomonas syringae* effector AvrPtoB and SnRK or CDPK members. Related to Figure 1.

Yeast two-hybrid (Y2H) assay of AvrPtoB and SnRK-CDPKs in the Matchmaker GaL4 system. AvrPtoB was expressed from the pGADT7 vector, while SnRKs and C-terminal deletions of CPKs (CPKs△C) were expressed from the pGBKT7 vector in *Saccharomyces cerevisea.* The pGBKT7 empty vector (EV) was included as a negative control. Blue colonies on SD -Leu/-Trp/-His/X-α-gal media indicate protein-protein interactions. AD = activation domain vector pGADT7, BD = binding domain vector pGBKT7.



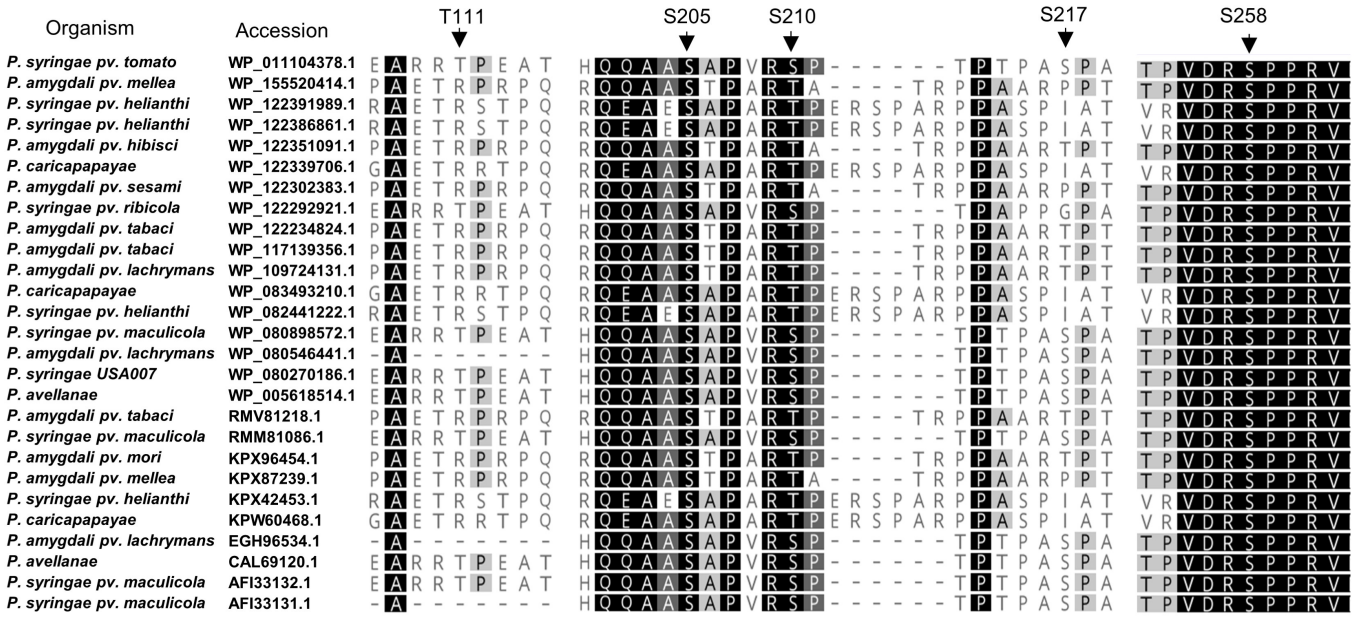
Supplemental Figure 3. Protein expression of *SnRKs*, *CPKs*, and *AvrPtoB* used in the Y2H assay. Related to Figure 1.

Yeast proteins were extracted and SnRK and CPK proteins were detected by anti-myc immunoblot and AvrPtoB was detected by anti-HA immunoblot. Expression of the positive (Pto) and negative (mCherry) controls used in Figure 1 are shown.



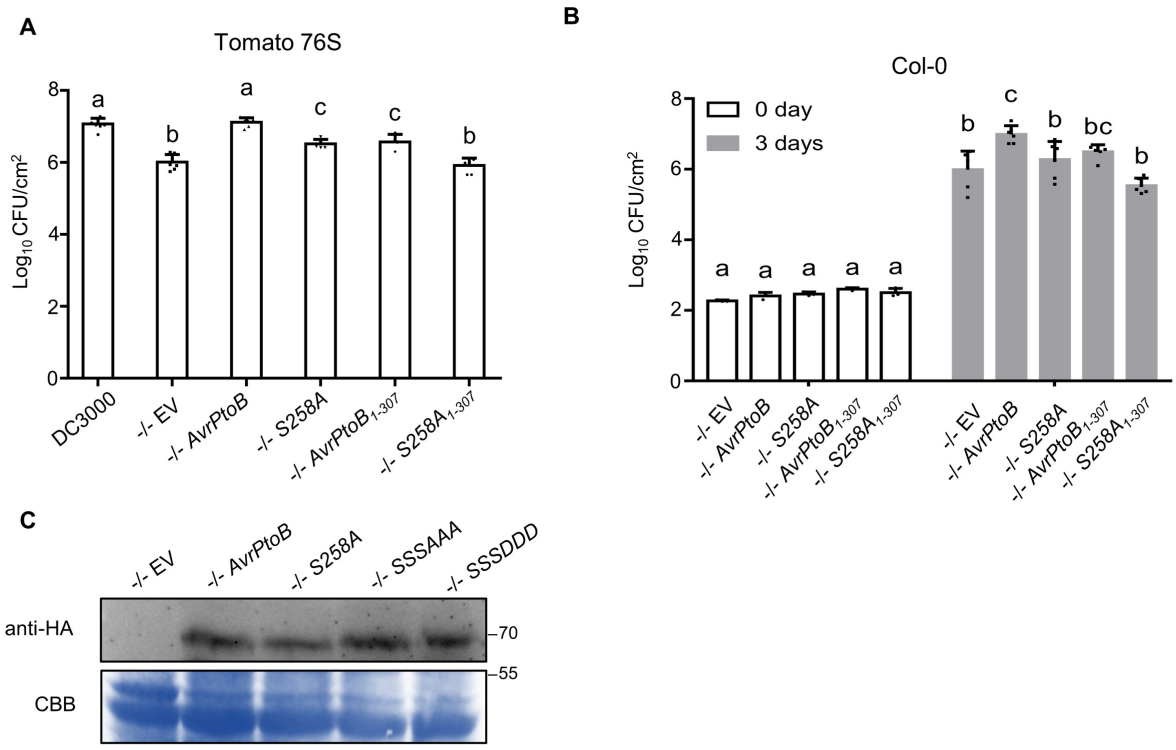
Supplemental Figure 4. AvrPtoB T111 and S217 phosphorylation levels in the *snrk2.8* knockout compared to wild-type Col-0. Related to Figure 2.

AvrPtoB-GFP was expressed in Col-0 and *snrk2.8* protoplasts, total proteins were subjected to anti-GFP IP followed by tryptic digestion. Phosphorylated peptides were detected by LC-MS/MS with the parallel reaction monitoring method. The peptide phosphorylation intensity of T111 (A) and the phosphorylation ratio of S217 (B) were determined using Skyline software. Data are means ± SE of three biological replicates (separate transfections). Asterisks indicate significant differences (Student's t-test, *$p<0.05$).



Supplemental Figure 5. Conservation of phosphorylated residues in different AvrPtoB homologs. Related to Figure 2.

AvrPtoB phosphorylated residues identified by mass spectrometry were analyzed for their conservation across 27 AvrPtoB homologs from *P. syringae*. The translated sequence of avrptoB were used in BLAST searches against the NCBI nr database on January 5th, 2020 (*Pseduomonas syringae* group, taxid: 136849; default parameters otherwise) to identify homologs. Hits were filtered based on a minimum 65% sequence identity, a minimum 85% query coverage and an E-value of 0. Sequences were downloaded and aligned in Genious using Genious alignment (default parameters).The percentage of identity is shown by gradient white to black (white means less than 60% similar, gray means 60-80% similar, dark gray means 80-100%, and black means 100% similar). AvrPtoB phosphorylation sites identified *in vivo* are shown with arrows.



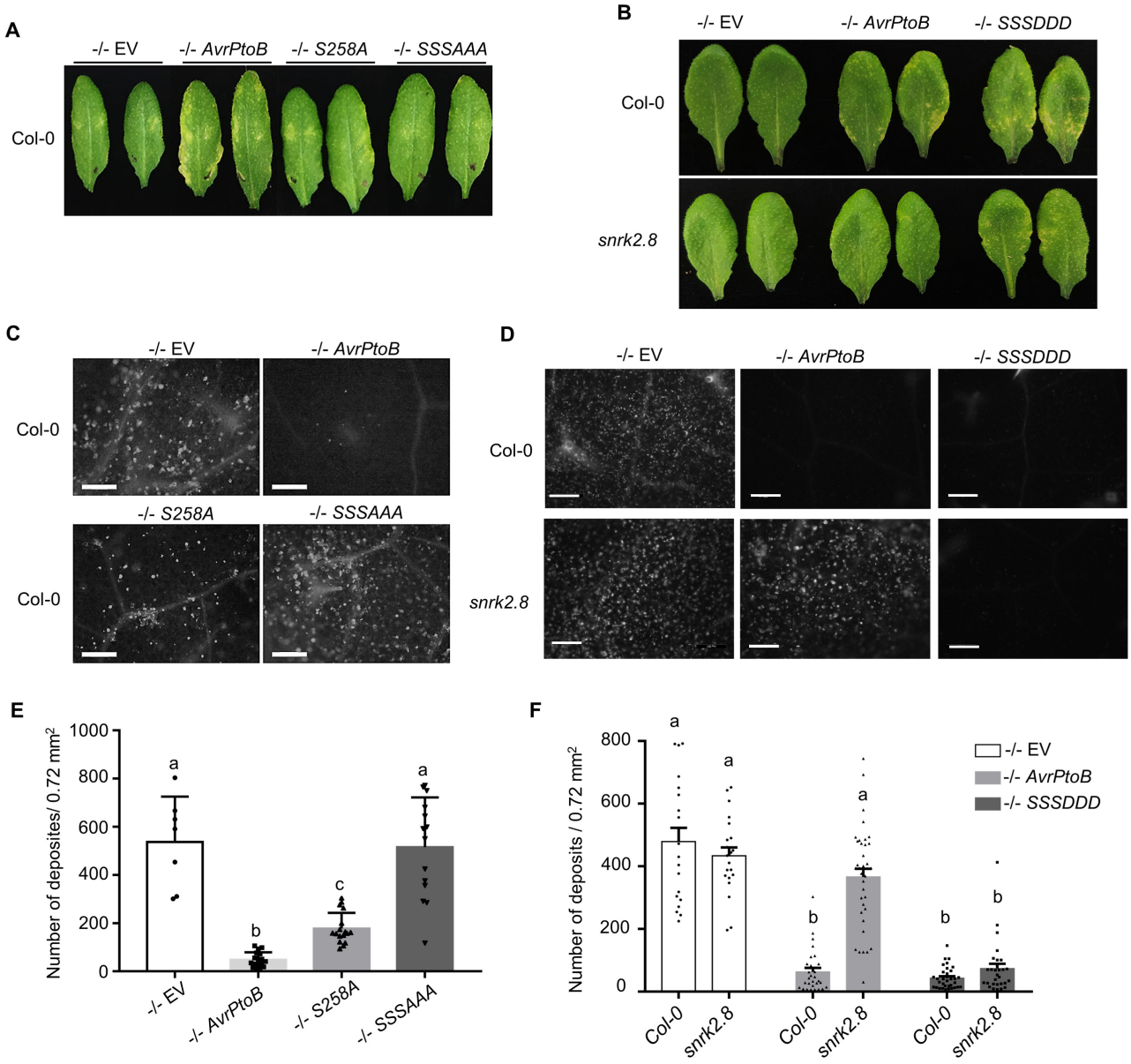
Supplemental Figure 6. AvrPtoB S258 is required for virulence in tomato and *Arabidopsis*.

Related to Figure 3.

(A) Bacterial populations in the susceptible tomato genotype Rio Grande 76S five days post-inoculation with *P. syringae* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* (-/-) variants. DC3000 -/- carrying empty vector (EV) or plasmids expressing wild-type *AvrPtoB*, $AvrPtoB_{S258A}$, $AvrPtoB_{1-307}$, and $AvrPtoB_{1-307S258A}$ were dip inoculated on tomato at a concentration of 2×10^8 CFU mL^{-1} with 0.005% Silwet. Log_{10} CFU/cm^2 = log_{10} colony-forming units per cm^2 of leaf tissue. Data are means ± SD (n ≥ 6 plants). Different letters indicate significant differences (one-way ANOVA, Tukey's test, $p < 0.05$).

(B) Bacterial populations in *Arabidopsis* Col-0 three days post-inoculation with DC3000 -/- variants. DC3000 -/- variants as described in (A) were syringe infiltrated in Col-0 at a concentration of 2×10^5 CFU mL^{-1}. Data are means ± SD (n = 4 plants for day 0, n = 6 plants for day 3). Different letters indicate significant differences (two-way ANOVA, Tukey's test, $p < 0.05$).

(C) Expression of AvrPtoB-HA and its derivatives from DC3000 -/- . Bacteria were grown in minimal media at 18 °C. Anti-HA western blotting was performed to detect the expression of AvrPtoB in bacterial pellets.



Supplemental Figure 7. Phosphorylated AvrPtoB serine residues are required for virulence. Related to Figure 3.

(A) Disease symptoms of *Arabidopsis* Col-0 after inoculation with *P. syringae* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* (-/-) variants. DC3000 -/- carrying empty vector (EV) or plasmids expressing wild-type *AvrPtoB*, $AvrPtoB_{S258A}$, or $AvrPtoB_{S205AS210AS258A}$ (*SSSAAA*) were syringe infiltrated into *Arabidopsis* Col-0 at a concentration of 2×10^5 CFU mL^{-1}. Disease symptoms were observed 4 days post-inoculation (dpi).

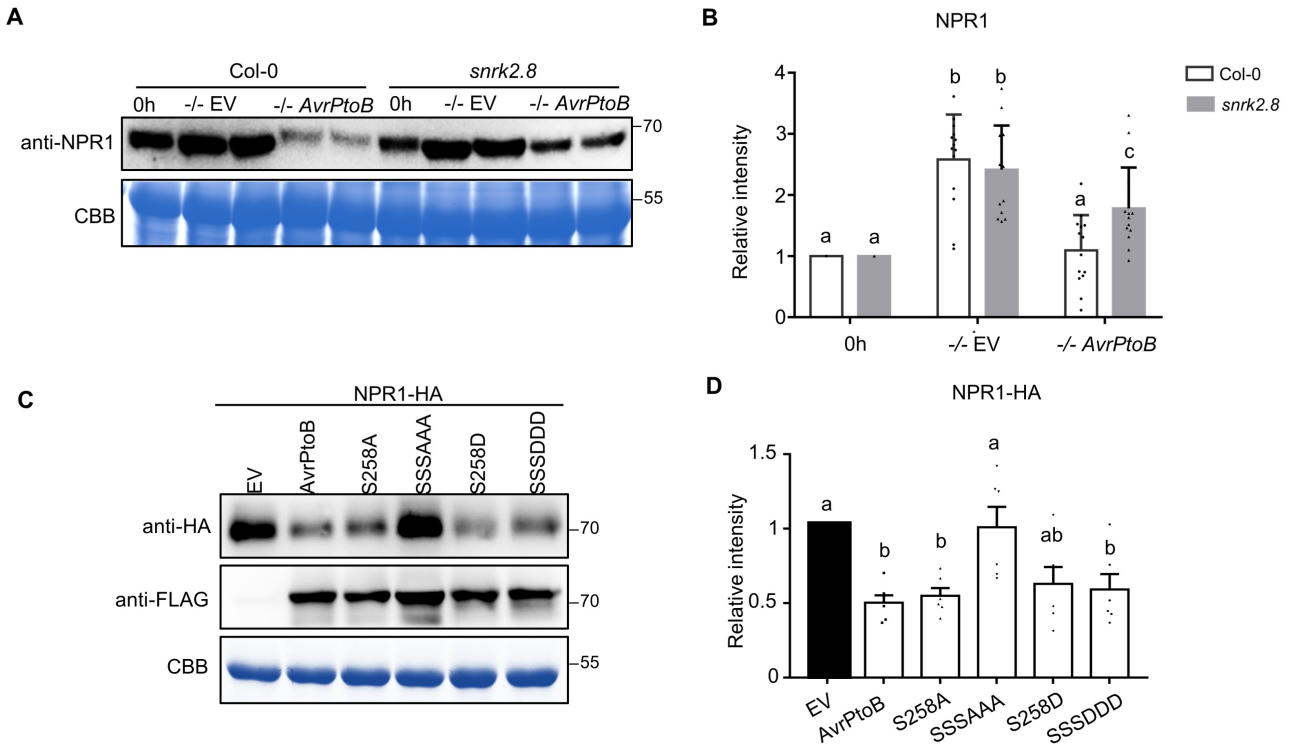
(B) Disease symptoms of *Arabidopsis* Col-0 after inoculation with *P. syringae* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* (-/-) variants. DC3000 -/- carrying empty vector (EV) or plasmids expressing wild-type *AvrPtoB*, or $AvrPtoB_{S205DS210DS258D}$ (*SSSDDD*). Plant were treated as described in (A).

(C) Callose deposition in *Arabidopsis* Col-0 after inoculation with -/- carrying EV, *AvrPtoB*, *S258A*, and *SSSAAA*. Leaves were inoculated with *P. syringae* at a concentration of 1×10^8 CFU mL^{-1} and harvested 16h later. Leaves were stained by 1% aniline blue and imaged by fluorescence microscopy. Scale bar, 100 µm.

(D) Callose deposition in *Arabidopsis* Col-0 after inoculation with -/- carrying EV, *AvrPtoB*, and *SSSDDD*. Leaves were inoculated and callose visualized as described in (C).

(E) Quantification of callose deposits. Data are means ± SD (n = 8 images from 8 leaves for -/- EV, n = 18 images from 18 leaves for rest variants). Different letters indicate significant differences (one-way ANOVA, Tukey's test, $p < 0.05$).

(F) Quantification of callose deposits. Data are means ± SD (n = 21 images from 21 leaves for -/- EV, n = 30 images from 30 leaves for rest variants). Different letters indicate significant differences (one-way ANOVA, Tukey's test, $p < 0.05$).



Supplemental Figure 8. AvrPtoB mediated degradation of NPR1 requires the plant kinase SnrK2.8 and phosphorylated residues. Related to Figure 4.

(A) NPR1 accumulation in *Arabidopsis* Col-0 and the *snrk2.8* knockout after inoculation with *P. syringae* pv. *tomato* DC3000Δ*avrPto*Δ*avrPtoB* (-/-) variants. DC3000 -/- carrying the empty vector (EV) or a plasmid expressing wild-type *AvrPtoB* were syringe infiltrated into Col-0 and *snrk2.8* at a concentration of 1×10^8 CFU mL^{-1} and proteins extracted after four hours. Protein extracts were subjected to anti-NPR1 immunoblotting. Coomassie Brilliant Blue (CBB) staining shows equal protein loading.
(B) Quantification of NPR1-HA band intensity in (C). NPR1-HA bands intensities were quantified by Image Lab 6.0.1 (BIO-RAD). The values were normalized first by Rubisco bands and subsequently by the intensities of "EV" treatment. Data are means ± SD (n = 6 leaves). Different letters indicate significant differences (one-way ANOVA, Tukey's test, $p < 0.05$).
(C) NPR1 accumulation in the presence of AvrPtoB phosphorylation mutants in *N. benthamiana* after Agrobacterium-mediated transient expression. *35S::NPR1-HA* was co-expressed with FLAG-tagged Dex-inducible *AvrPtoB*, $AvrPtoB_{S258A}$, $AvrPtoB_{S205AS210AS258A}$ (*SSSAAA*), $AvrPtoB_{S258D}$ or $AvrPtoB_{S205DS210DS258D}$ (*SSSDDD*). The Dex inducible EV was used as a control. The expression of AvrPtoB-FLAG phosphorylation variants was induced by 15 µM DEX for 5 hours 24h post-Agrobacterium infiltration. Protein extracts were subjected to anti-HA and anti-FLAG immunoblotting. CBB staining shows protein loading.
(D) Quantification of NPR1 band intensity in (A). NPR1 bands intensities were quantified by Image Lab 6.0.1 (BIO-RAD). The values were normalized first by Rubisco bands and subsequently by the intensities of "0h" bands. Data are means ± SD (n = 14 leaves). Different letters indicate significant differences (one-way ANOVA, Tukey's test, $p < 0.05$).

Supplemental Table 1. The phosphorylation of bacterial type III effectors and their phosphorylation sites are identified by mass spectrometry. Related to Figure 1.

Data were from the references in the table. Red indicates the phosphorylated residue.

Effector	Pathogen	Phosphorylation site	Host kinase	Required for virulence	Required for avirulence	Reference
AvrPto	*P. syringae*	NPNGS_{149}IRMTL	unknown	Yes	Yes	Anderson et al., 2006 Yeam et al., 2009
AvrPtoB	*P. syringae*	PVDRS_{258}PPRVNQR	unknown	Yes	Yes	Xiao et al., 2007
		QFSQLRT_{450}ISKAD	Pto/Fen (*in vitro*)	unknown	Yes	Ntoukakis et al., 2009
HopQ1	*P. syringae*	PVLERSKS_{51}APAL	SnRK (*in vitro*)	Yes	unknown	Li et al., 2013 Giska et al., 2013
AvrB	*P. syringae*	unknown	RIPK (*in vitro*)	unknown	Yes	Desveaux et al., 2007 Chung et al., 2014
AvrBsT	*X. campestris*	unknown	CaPIK1 (*in vitro*)	unknown	Yes	Kim et al., 2014
XopN	*X. campestris*	APPRREHVS_{688}APSSP	unknown	Yes	unknown	Taylor et al., 2012
XopE2	*X. euvesicatoria*	RGRFRQPT_{66}LQPH LSNGRSATYS_{133}SLSY AQPIS_{334}PRTTAA	unknown	unknown	unknown	Dubrow et al., 2018
NopL	*Rhizobium*	RSGPSQAGLS$_{139}$PSAT SPSATPLLNPS$_{148}$PPP H LTAERGRS$_{198}$PQPS	SIPK (*in vitro*)	Yes	unknown	Zhang et al., 2011a Ge et al., 2016
NopP	*Rhizobium*	unknown	unknown	Yes	unknown	Skorpil et al., 2005

Supplemental Table 2. Related to Figure 2 and supplemental figure 4.

AvrPtoB was expressed in Col-0 and *snrk2.8* protoplasts and total proteins were subjected to anti-GFP IP followed by tryptic digestion. Phosphorylated peptides were detected by LC-MS/MS with the parallel reaction monitoring method. Peptide phosphorylation ratios were determined using Skyline software. Phoshorylation intensity is reported for residue T111 as no unphosphorylated peptides were detected by MS. Data are means ± SE of three biological replicates (separate transfections).

	Phosphorylation ratio		**SE**	
Phosphorylated residue	**Col-0**	***snrk2.8***	**Col-0**	***snrk2.8***
S205	93.88	10.20	± 46.05	± 5.12
S210	173.85	17.90	± 85.40	± 8.81
S258	40263.92	4272.50	± 25060.68	± 2455.38
S217	0.01	0.01	± 0.001	± 0.003
	Phosphorylation intensity		**SE**	
	Col-0	***snrk2.8***	**Col-0**	***snrk2.8***
T111	3880000	13500000	± 2837677.1	± 2478126.5

Supplemental Table 3. Primers used in this study. Related to Methods.

Name	Sequence 5' to 3'	Purpose
SnRK1.1-LP	CACCATGTTCAAACGAGTAGATGAGTTT	Full-length SnRK1.1 CDS
SnRK1.1-RP	GAGGACTCGGAGCTGAGCAAG	
SnRK1.2-LP	CACCATGGATCATTCATCAAATAGATTTGGC	Full-length SnRK1.2 CDS
SnRK1.2-RP	GATCACACGAAGCTCTGTAAGAAAGG	
SnRK1.3-LP	CACCATGGATGGATCATCGGAAAAAAC	Full-length SnRK1.3 CDS
SnRK1.3-RP	GAGGACACCTAGCTCTCTGAGAAACG	
SnRK2.1-LP	CACCATGGACAAGTATGACGTTGTC	Full-length SnRK2.1 CDS
SnRK2.1-RP	AGCTTTGTCAGACTCTTGACAAGACTG	
SnRK2.3-LP	CACCATGGATCGAGCTCCGG	Full-length SnRK2.3 CDS
SnRK2.3-RP	GAGAGCGTAAACTATCTCTCCGCTAC	
SnRK2.4-LP	CACCATGGACAAGTACGAGCTGG	Full-length SnRK2.4 CDS
SnRK2.4-RP	ACTTATTCTCACTTCTCCACTTGCGTG	
SnRK2.5-LP	CACCATGGACAAGTATGAGGTTGTGAA	Full-length SnRK2.5 CDS
SnRK2.5-RP	AGCTTTGGGAGGCTCTTGACAAGAATG	
SnRK2.6-LP	CACCATGGATCGACCAGCAGTGA	Full-length SnRK2.6 CDS
SnRK2.6-RP	CATTGCGTACACAATCTCTCCGCTACTG	
SnRK2.7-LP	CACCATGGAGAGATACGACATCTTAAGAG	Full-length SnRK2.7 CDS
SnRK2.7-RP	TAGAGCACATACGAAATCACCATTTCTC	
SnRK2.8-LP	CACCATGGAGAGGTACGAAATAGTG	Full-length SnRK2.8 CDS
SnRK2.8-RP	CAAAGGGGAAAGGAGATCAGCG	
SnRK2.9-LP	CACCATGGAGAAGTATGAGATGGTG	Full-length SnRK2.9 CDS
SnRK2.9-RP	TGCGTAATCATCATACCATTCTTCATCA	
SnRK2.10-LP	CACCATGGACAAGTACGAGCTTGTTAA	Full-length SnRK2.10 CDS
SnRK2.10-RP	ACTGACTCGGACTTCTCCCATG	
SnRK3.1-LP	CACCATGGAGAAGAAAGGATCTGTGT	Full-length SnRK3.1 CDS
SnRK3.1-RP	GTGCCAAGCTAATACAAAGTCGATCAA	
SnRK3.2-LP	CACCATGGAGAACAAACCAAGTGTATT	Full-length SnRK3.2 CDS
SnRK3.2-RP	TGATGGTTCTTGCTCTCCTTGTTCATC	
SnRK3.6-LP	CACCATGGATAAAAACGGCATAGTTT	Full-length SnRK3.6 CDS
SnRK3.6-RP	ATGTATCACTTCAATCTTCTCATTGTTGC	
SnRK3.7-LP	CACCATGGCTCAAGTACTATCTACAC	Full-length SnRK3.7 CDS
SnRK3.7-RP	CTGTTCAATTTCAGGTGGCAAACA	
SnRK3.8-LP	CACCATGGAAAATAAGCCAAGTGTTTTG	Full-length SnRK3.8 CDSS
SnRK3.8-RP	AAACTTCAATGGTTCTTCCTGTTCTTG	
SnRK3.9-LP	CACCATGGCGGAGAAAATCACG	Full-length SnRK3.9 CDS
SnRK3.9-RP	TTCAGTGTCAGACGGCAAGAAAGAAATG	

Name	Sequence 5' to 3'	Purpose
SnRK3.10-LP	CACCATGGAATCACTTCCCCAG	Full-length SnRK3.10 CDS
SnRK3.10-RP	CATGATGTCATTGTGCCATGAAAGAAC	
SnRK3.11-LP	CACCATGACAAAGAAAATGAGAAGAGTGG	Full-length SnRK3.11 CDS
SnRK3.11-RP	AAACGTGATTGTTCTGAGAATCTCTGAC	
SnRK3.12-LP	CACCATGAGTGGAAGCAGAAGGAAG	Full-length SnRK3.12 CDS
SnRK3.12-RP	TTGCTTTTGTTCTTCAGCGGCTG	
SnRK3.13-LP	CACCATGGTGGTAAGGAAGGTG	Full-length SnRK3.13 CDS
SnRK3.13-RP	ACGTCTTTTACTCTTGGCCTTGGTG	
SnRK3.14-LP	CACCATGGTCGGAGCAAAACC	Full-length SnRK3.14 CDS
SnRK3.14-RP	AGCAGGTGTAGAGGTCCAGAAAATG	
SnRK3.15-LP	CACCATGGTAGATTCTGACCCGG	Full-length SnRK3.15 CDS
SnRK3.15-RP	CGACGTCGTATGTACTTGAGTTGGTTC	
SnRK3.16-LP	CACCATGGTGAGAAGGCAAGAG	Full-length SnRK3.16 CDS
SnRK3.16-RP	AGTTACTATCTCTTGCTCCGGCGAG	
SnRK3.17-LP	CACCATGTTGATCCCCAACAAAAAATTA	Full-length SnRK3.17 CDS
SnRK3.17-RP	CTTTGCTGTTTCTTTCTTAACTTCGTTAT	
SnRK3.19-LP	CACCATGTCTTTTACAATTCCTAGACTG	Full-length SnRK3.18 CDS
SnRK3.19-RP	CGGTTTGTCAGGAACTTTATAAACCG	
SnRK3.20-LP	CACCATGGCTCAAGCCTTGG	Full-length SnRK3.20 CDS
SnRK3.20-RP	TTCAGTATCAGATGGCAAATACAATGCTTC	
SnRK3.21-LP	CACCATGGTGATAAAGGGAATGCG	Full-length SnRK3.21 CDS
SnRK3.21-RP	CGCTAAAAGCTCCTGTACTTGTGATG	
SnRK3.22-LP	CACCATGCCAGAGATCGAGATTGC	Full-length SnRK3.22 CDS
SnRK3.22-RP	AATAGCCGCGTTTGTTGACGACG	
SnRK3.23-LP	CACCATGGCTTCTCGAACAACG	Full-length SnRK3.23 CDS
SnRK3.23-RP	TGTCGACTGTTTTGCAATTGTCCG	
SnRK3.24-LP	CACCATGGAGGAAGAACGG	Full-length SnRK3.24 CDS
SnRK3.24-RP	ACAATCCTCGGAAGAAGTGTTATTATTA	
SnRK3.25-LP	CACCATGGGATCCAAACTTAAACTTTAC	Full-length SnRK3.25 CDS
SnRK3.25-RP	GCAGTCACTACCAGAATTTTCATCAC	
CPK2ΔC-LP	CACCATGGGTAATGCTTGCGTT	CPK2 C-terminal deleted CDS
CPK2ΔC-RP	CACACCGTCAATCTGTACCCAT	
CPK3ΔC-LP	CACCATGGGCCACAGACACAGC	CPK3 C-terminal deleted CDS
CPK3ΔC-RP	CTCCCCATCTTCTCTAATCCACGG	
CPK4ΔC-LP	CACCATGGAGAAACCAAACCCT	CPK4 C-terminal deleted CDS
CPK4ΔC-RP	AGCATGTTCATCAACAATCCAAG	
CPK5ΔC-LP	CACCATGGGCAATTCTTGCC	CPK5 C-terminal deleted CDS
CPK5ΔC-RP	AACACCATTCTCACAGATCCATG	

Name	Sequence 5' to 3'	Purpose
CPK6ΔC-LP	CACCATGGGCAATTCATGTCGT	CPK6 C-terminal deleted CDS
CPK6ΔC-RP	AACTCCATTCTCACAGATCCATG	
CPK10ΔC-LP	CACCATGGGTAACTGTAACGCC	CPK10 C-terminal deleted CDS
CPK10ΔC-RP	TTTCTTTGCATTCTGTATCCATGGG	
CPK11ΔC-LP	CACCATGGAGACGAAGCCAAAC	CPK11 C-terminal deleted CDS
CPK11ΔC-RP	TGCTTGTTCATCGACAATCCATG	
CPK13ΔC-LP	CACCATGGGAAACTGTTGCAGA	CPK13 C-terminal deleted CDS
CPK13ΔC-RP	TTTCTTTGCGTTCTGAATCCATGG	
CPK26ΔC-LP	CACCATGAAGCACAGCGGTGG	CPK26 C-terminal deleted CDS
CPK26ΔC-RP	AACTCCATTTTCACAGATCCAAGG	
SnRK1.1 KA-LP	CATAAGGTTGCTATCGCGATCCTCAATCGTC	Generate SnRK1.1 kinase dead mutant
SnRK1.1 KA-RP	GACGATTGAGGATCGCGATAGCAACCTTATG	
SnRK2.6 KA-LP	CTTGTTGCTGTTGCATATATCGAGAG	Generate SnRK2.6 kinase dead mutant
SnRK2.6 KA-RP	CTCTCGATATATGCAACAGCAACAAG	
SnRK2.8 KA-LP	GAGCTTTTCGCTGTTGCGTTCATCGAGCGAG	Generate SnRK2.8 kinase dead mutant
SnRK2.8 KA-RP	CTCGCTCGATGAACGCAACAGCGAAAAGCTC	
CPK4ΔC KA-LP	CTAATTACGCTTGCGCATCAATCCCAAAAC	Generate CPK4ΔC kinase dead mutant
CPK4ΔC KA-RP	GTTTTGGGATTGATGCGCAAGCGTAATTAG	
CPK5ΔC KA-LP	GACTACGCTTGTGCGTCAATATCCAAG	Generate CPK5ΔC kinase dead mutant
CPK5ΔC KA-RP	CTTGGATATTGACGCACAAGCGTAGTC	
AvrPtoB-LP	CACCATGGCGGGTATCAATGGAGC	Clone Full-length AvrPtoB
AvrPtoB-RP	GGGGACTATTCTAAAAGC	
AvrPtoB 307-RP	TACATGTCTTTCAAGGGCCGTG	Clone $AvrPtoB_{1-307}$
AvrPtoB S258A -LP	CCGGTCGACAGGGCCCCGCCACGCG	Generate AvrPtoB S258A mutant
AvrPtoB S258A-RP	CGCGTGGCGGGGCCCTGTCGACCGG	
AvrPtoB S258D-LP	CCGGTCGACAGGGACCCGCCACGCG	Generate AvrPtoB S258D mutant
AvrPtoB S258D-RP	CGCGTGGCGGGTCCCTGTCGACCGG	
AvrPtoB S205S210A-LP	CAACAGGCGGCGGCAGCGCCAGTGAGGGCGCCCACGCCAAC	Generate AvrPtoB S205AS210AS258A mutant
AvrPtoB S205S210A-RP	GTTGGCGTGGGCGCCCTCACTGGCGCTGCCGCCGCCTGTTG	
AvrPtoB S205DS210D-LP	CAACAGGCGGCGGATGCGCCAGTGAGGGATCCCACGCCAAC	Generate AvrPtoB S205DS210DS258D mutant
AvrPtoB S205DS210D-RP	GTTGGCGTGGGATCCCTCACTGGCGCATCCGCCGCCTGTTG	
snrk2.8 SALK_073395-LP	ATTTTCCAAAGAGCTTTTCGC	snrk2.8 T-DNA line genotyping
snrk2.8 SALK_073396-RP	GGTGATAGGTTTCCGAGCTTC	

Supplemental Table 4. Isolation list for PRM. Related to Figure 2.

Modified and unmodified peptide sequences used to quantify phosphorylation by PRM are noted. CID = collision induced dissociation, m/z = mass to charge, z = charge state.

Peptide sequence	Peptide modified sequence	m/z	z
AEARRTPEATADASAPR	AEARRT[+80]PEATADASAPR	617.2899	3
RAVHQQAASAPVR	RAVHQQAASAPVR	464.2603	3
RAVHQQAASAPVRSPTPTPASPAASSSGSSQR	RAVHQQAASAPVRSPTPTPASPAASSSGSSQR	786.9028	4
	RAVHQQAASAPVRSPTPTPASPAASSSGSSQR	629.7237	5
	RAVHQQAAS[+80]APVRSPTPTPASPAASSSGSSQR	806.8944	4
	RAVHQQAASAPVRS[+80]PTPTPASPAASSSGSSQR	806.8944	4
AVHQQAASAPVR	AVHQQAASAPVR	617.8362	2
	AVHQQAASAPVR	412.2265	3
AVHQQAASAPVRSPTPTPASPAASSSGSSQR	AVHQQAASAPVRSPTPTPASPAASSSGSSQR	996.8343	3
	AVHQQAASAPVRSPTPTPASPAASSSGSSQR	747.8775	4
	AVHQQAAS[+80]APVRSPTPTPASPAASSSGSSQR	767.8691	4
	AVHQQAASAPVRS[+80]PTPTPASPAASSSGSSQR	1023.49	3
	AVHQQAASAPVRS[+80]PTPTPASPAASSSGSSQR	767.8691	4
SPTPTPASPAASSSGSSQR	SPTPTPASPAASSSGSSQR	886.9241	2
	SPTPTPASPAASSSGSSQR	591.6185	3
	SPTPTPAS[+80]PAASSSGSSQR	926.9073	2
SSNTAASQTPVDRSPPR	SSNTAASQTPVDRSPPR	590.9625	3
	SSNTAASQTPVDRS[+80]PPR	925.9233	2
	SSNTAASQTPVDRS[+80]PPR	617.6179	3
	PSNTPPSNAPAPPPTGR	828.411	2
	PSNT[+80]PPSNAPAPPPTGR	868.3942	2
AALDPIASQFSQLR	AALDPIASQFSQLR	757.9023	2
	AALDPIASQFS[+80]QLR	797.8855	2

Supplemental references

Anderson, J. C., Pascuzzi, P. E., Xiao, F., Sessa, G., and Martin, G. B. (2006). Host-Mediated Phosphorylation of Type III Effector AvrPto Promotes Pseudomonas Virulence and Avirulence in Tomato. *Plant Cell* **18**:502–514.

Chen, H., Chen, J., Li, M., Chang, M., Xu, K., Shang, Z., Zhao, Y., Palmer, I., Zhang, Y., McGill, J., et al. (2017). A Bacterial Type III Effector Targets the Master Regulator of Salicylic Acid Signaling, NPR1, to Subvert Plant Immunity. *Cell Host Microbe* **22**:777-788.e7.

Chung, E.-H., El-Kasmi, F., He, Y., Loehr, A., and Dangl, J. L. (2014). A Plant Phosphoswitch Platform Repeatedly Targeted by Type III Effector Proteins Regulates the Output of Both Tiers of Plant Immune Receptors. *Cell Host Microbe* **16**:484–494.

Crooks, G. E., Hon, G., Chandonia, J.-M., and Brenner, S. E. (2004). WebLogo: A Sequence Logo Generator. *Genome Res* **14**:1188–1190.

Desveaux, D., Singer, A. U., Wu, A.-J., McNulty, B. C., Musselwhite, L., Nimchuk, Z., Sondek, J., and Dangl, J. L. (2007). Type III Effector Activation via Nucleotide Binding, Phosphorylation, and Host Target Interaction. *Plos Pathog* **3**:e48.

Dubrow, Z., Sunitha, S., Kim, J.-G., Aakre, C. D., Girija, A. M., Sobol, G., Teper, D., Chen, Y. C., Ozbaki-Yagan, N., Vance, H., et al. (2018). Tomato 14-3-3 Proteins Are Required for Xv3 Disease Resistance and Interact with a Subset of Xanthomonas euvesicatoria Effectors. *Mol Plant-microbe Interactions Mpmi* **31**:1301–1311.

Earley, K. W., Haag, J. R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C. S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* **45**:616–629.

Ge, Y.-Y., Xiang, Q.-W., Wagner, C., Zhang, D., Xie, Z.-P., and Staehelin, C. (2016). The type 3 effector NopL of Sinorhizobium sp. strain NGR234 is a mitogen-activated protein kinase substrate. *J Exp Bot* **67**:2483–2494.

Giska, F., Lichocka, M., Piechocki, M., Dadlez, M., Schmelzer, E., Hennig, J., and Krzymowska, M. (2013). Phosphorylation of HopQ1, a Type III Effector from Pseudomonas syringae , Creates a Binding Site for Host 14-3-3 Proteins. *Plant Physiol* **161**:2049–2061.

Gu, Y., and Innes, R. W. (2011). The KEEP ON GOING protein of Arabidopsis recruits the ENHANCED DISEASE RESISTANCE1 protein to trans-Golgi network/early endosome vesicles. *Plant Physiol* **155**:1827–38.

Kim, D. S., Choi, H. W., and Hwang, B. K. (2014). Pepper mildew resistance locus O interacts with pepper calmodulin and suppresses Xanthomonas AvrBsT-triggered cell death and defense responses. *Planta* **240**:827–839.

Li, W., Yadeta, K. A., Elmore, J. M., and Coaker, G. (2013). The Pseudomonas syringae Effector HopQ1 Promotes Bacterial Virulence and Interacts with Tomato 14-3-3 Proteins in a Phosphorylation-Dependent Manner. *Plant Physiol* **161**:2062–2074.

MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., and MacCoss, M. J. (2010). Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinform Oxf Engl* **26**:966–8.

Minkoff, B. B., Burch, H. L., and Sussman, M. R. (2013). Methods in Molecular Biology. *Methods Mol Biology Clifton N J* **1062**:353–379.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R., Kawai, T., Tanaka, K., Niwa, Y., et al. (2007). Improved Gateway Binary Vectors: High-Performance Vectors for Creation of Fusion Constructs in Transgenic Analysis of Plants. *Biosci Biotechnology Biochem* **71**:2095–2100.

Ntoukakis, V., Mucyn, T. S., Gimenez-Ibanez, S., Chapman, H. C., Gutierrez, J. R., Balmuth, A. L., Jones, A. M. E., and Rathjen, J. P. (2009). Host Inhibition of a Bacterial Virulence Effector Triggers Immunity to Infection. *Science* **324**:784–787.

Skorpil, P., Saad, M. M., Boukli, N. M., Kobayashi, H., Ares-Orpel, F., Broughton, W. J., and Deakin, W. J. (2005). NopP, a phosphorylated effector of Rhizobium sp. strain NGR234, is a major determinant of nodulation of the tropical legumes Flemingia congesta and Tephrosia vogelii: NopP of Rhizobium sp. NGR234. *Mol Microbiol* **57**:1304–1317.

Taylor, K. W., Kim, J.-G., Su, X. B., Aakre, C. D., Roden, J. A., Adams, C. M., and Mudgett, M. B. (2012). Tomato TFT1 Is Required for PAMP-Triggered Immunity and Mutations that Prevent T3S Effector XopN from Binding to TFT1 Attenuate Xanthomonas Virulence. *Plos Pathog* **8**:e1002768.

Tyanova, S., Temu, T., and Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* **11**:2301–2319.

Xiao, F., Giavalisco, P., and Martin, G. B. (2007). Pseudomonas syringae Type III Effector AvrPtoB Is Phosphorylated in Plant Cells on Serine 258, Promoting Its Virulence Activity. *J Biol Chem* **282**:30737–30744.

Yeam, I., Nguyen, H. P., and Martin, G. B. (2009). Phosphorylation of the Pseudomonas syringae effector AvrPto is required for FLS2/BAK1-independent virulence activity and recognition by tobacco. *Plant J Cell Mol Biology* **61**:16–24.

Yoo, S.-D., Cho, Y.-H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc* **2**:1565–1572.

Zhang, L., Chen, X.-J., Lu, H.-B., Xie, Z.-P., and Staehelin, C. (2011a). Functional Analysis of the Type 3 Effector Nodulation Outer Protein L (NopL) from Rhizobium sp. NGR234: SYMBIOTIC EFFECTS, PHOSPHORYLATION, AND INTERFERENCE WITH MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING. *J Biol Chem* **286**:32178–32187.

Zhang, T., Lei, J., Yang, H., Xu, K., Wang, R., and Zhang, Z. (2011b). An improved method for whole protein extraction from yeast Saccharomyces cerevisiae. *Yeast Chichester Engl* **28**:795–8.