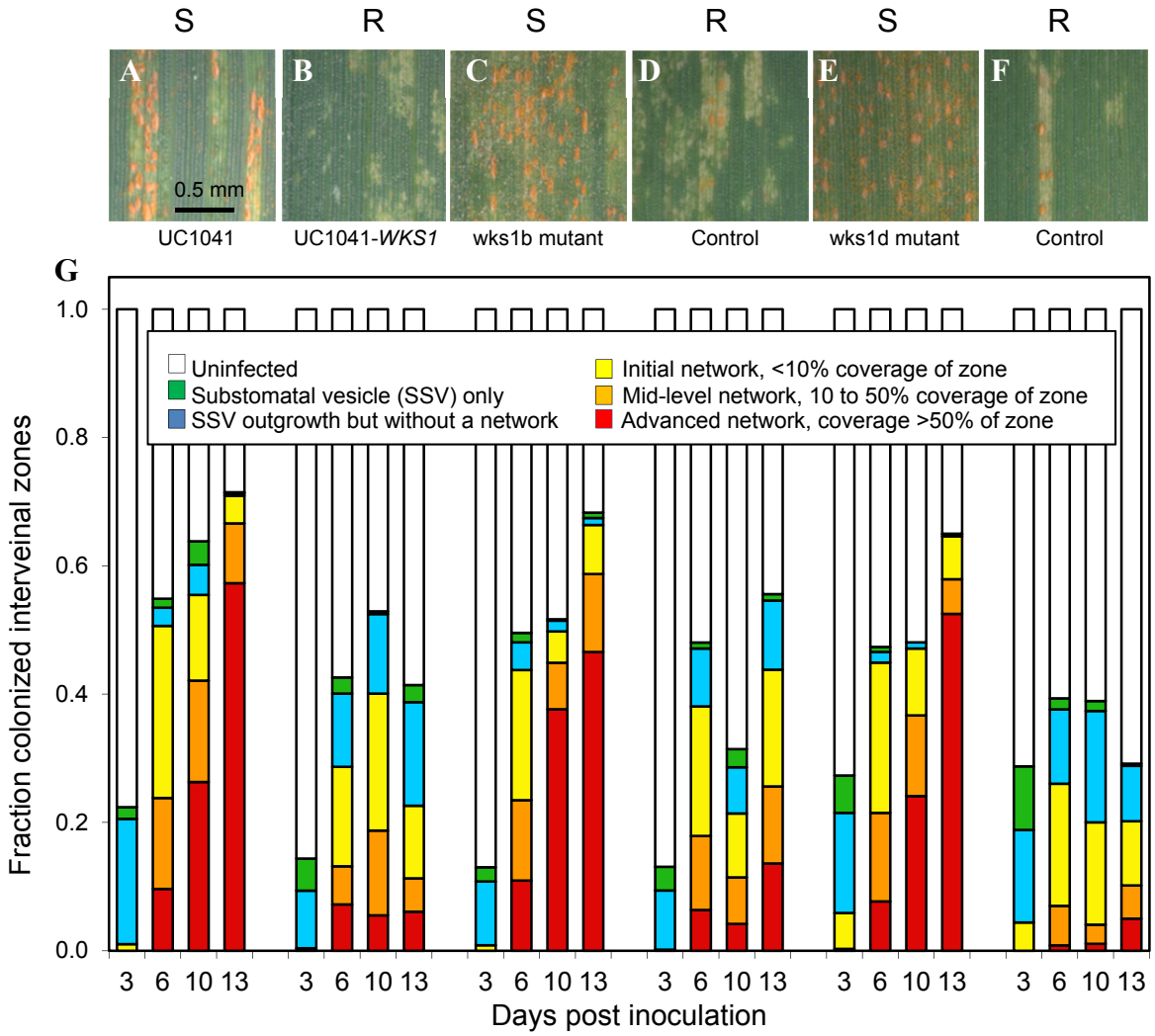
Supplemental Figures



Supplemental Figure 1. Pathogen Growth and Development in Interveinal Regions of Uvitex-Stained Leaves in Three Pairs of Hexaploid Wheat Isogenic Lines With and Without *WKS1*.

(A) UC1041 control (S= susceptible).

(B) UC1041 with wild type *WKS1* (R= resistant).

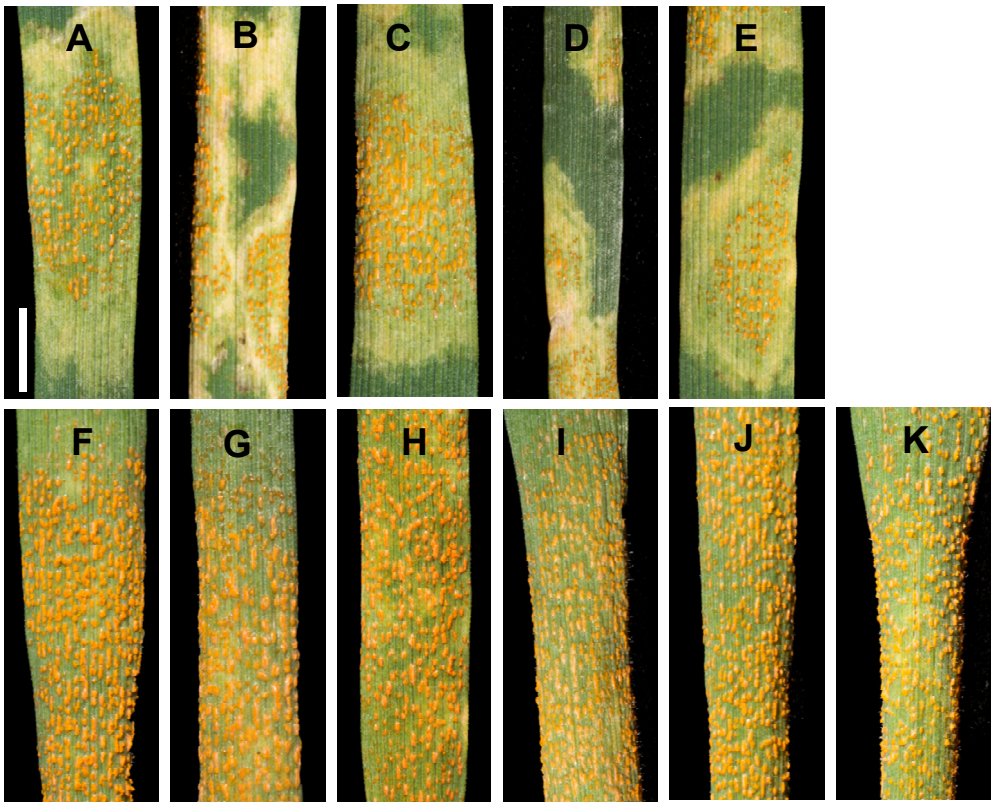
(C) wks1b susceptible mutant (mutation in kinase domain, Supplemental Figure 3A).

(D) Sister line of wks1b homozygous for wild type *WKS1* (resistant control).

(E) wks1d susceptible mutant (mutation in kinase domain, Supplemental Figure 3A).

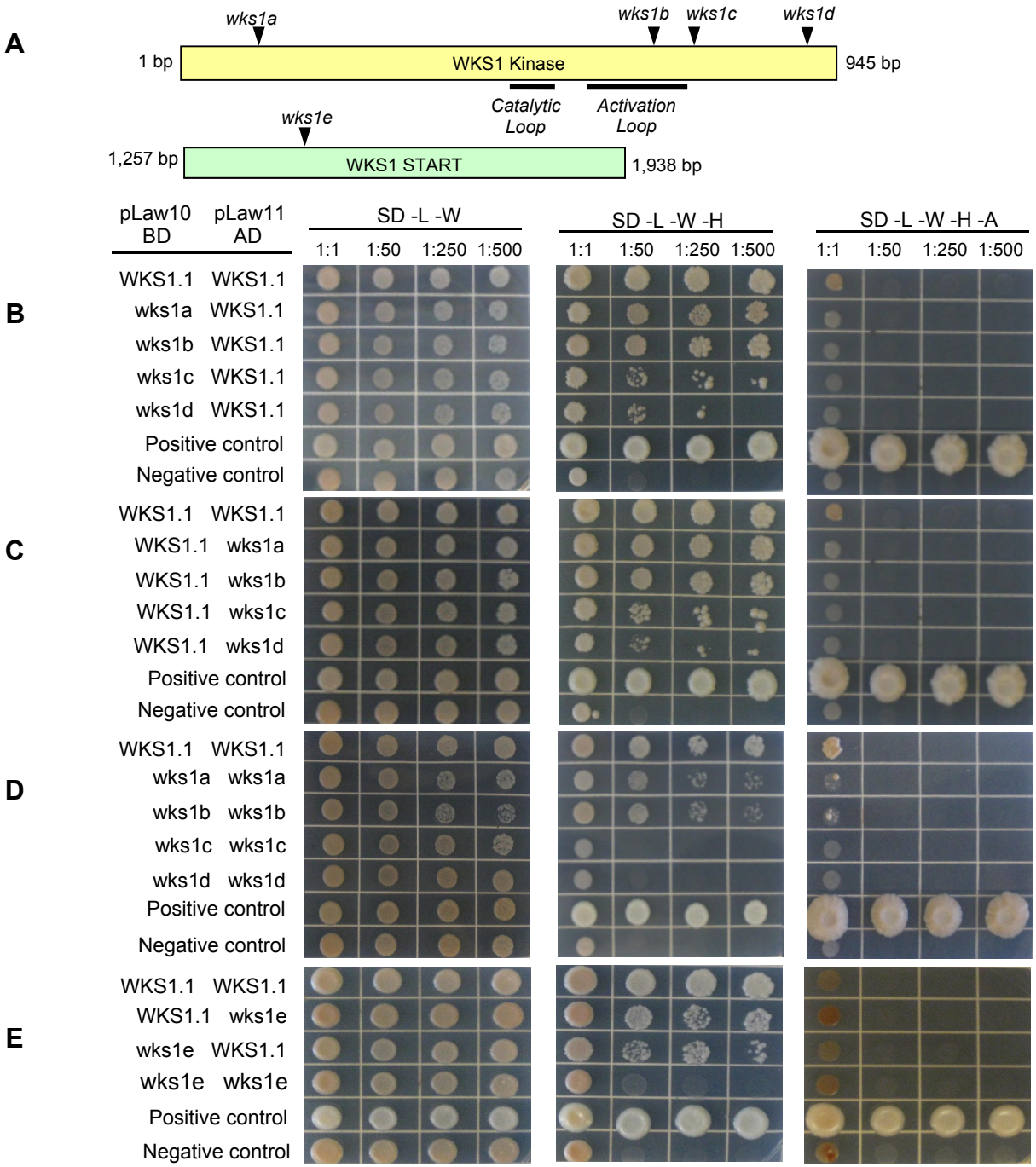
(F) Sister line of wks1d homozygous for wild type *WKS1* (resistant control).

(G) Pathogen growth and development in three pairs of isogenic lines with and without *WKS1*. Initial fungal development was similar, including urediniospore germination, germling penetration into stoma, and formation of substomatal vesicles and haustorial mother cells.



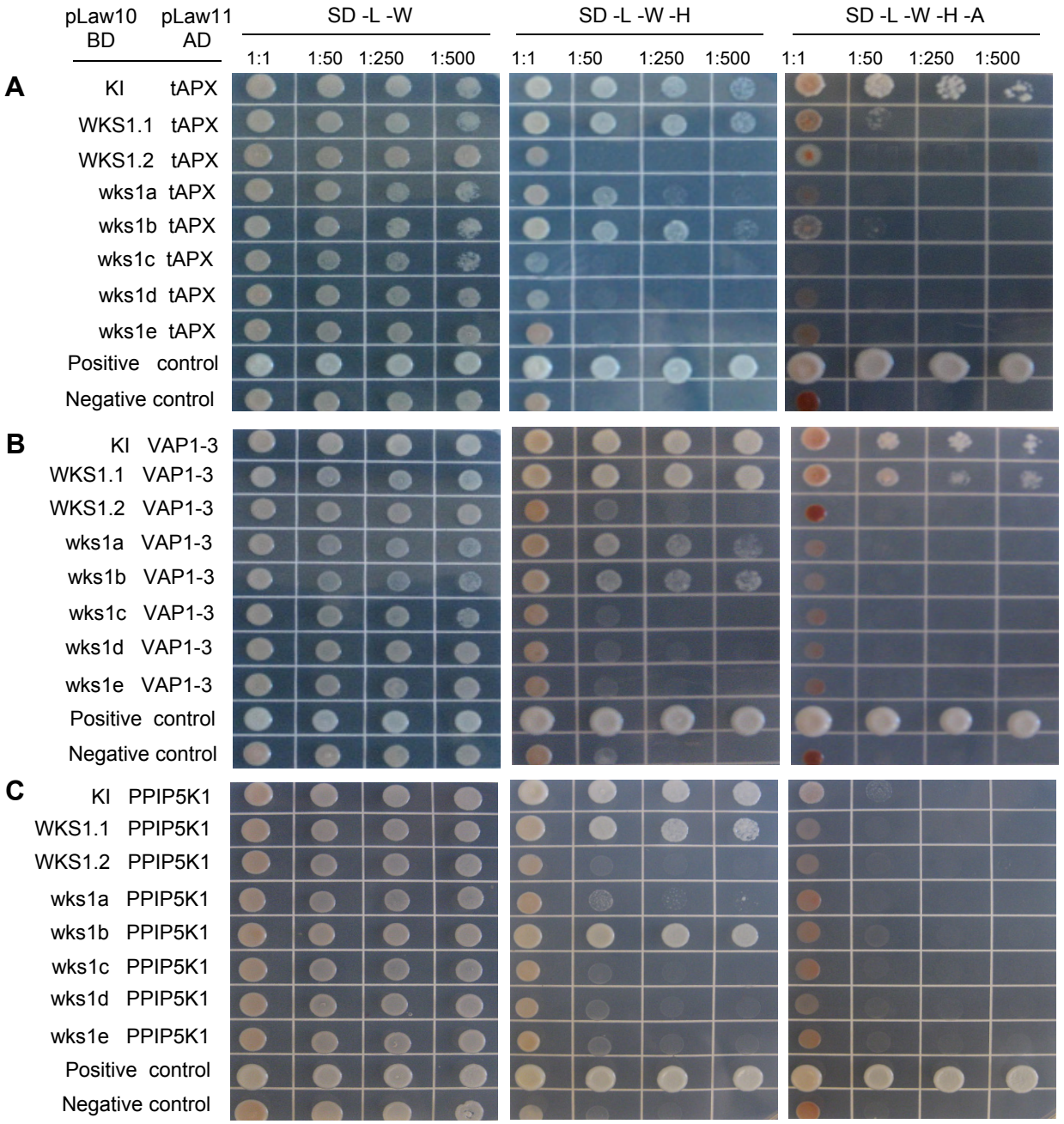
Supplemental Figure 2. Wheat Plants Transformed with NP:WKS1.1 or NP:WKS1.2 and Inoculated with *Pst* Race PST-08/21.

(A-E) NP:WKS1.1 transformed plants, events 1 through 5, respectively
(F-K) NP:WKS1.2 transformed plants, events 1 through 6, respectively.
Each panel shows a representative plant from independent events. Pictures were taken 15 days after inoculation. Scale: 0.5 cm.



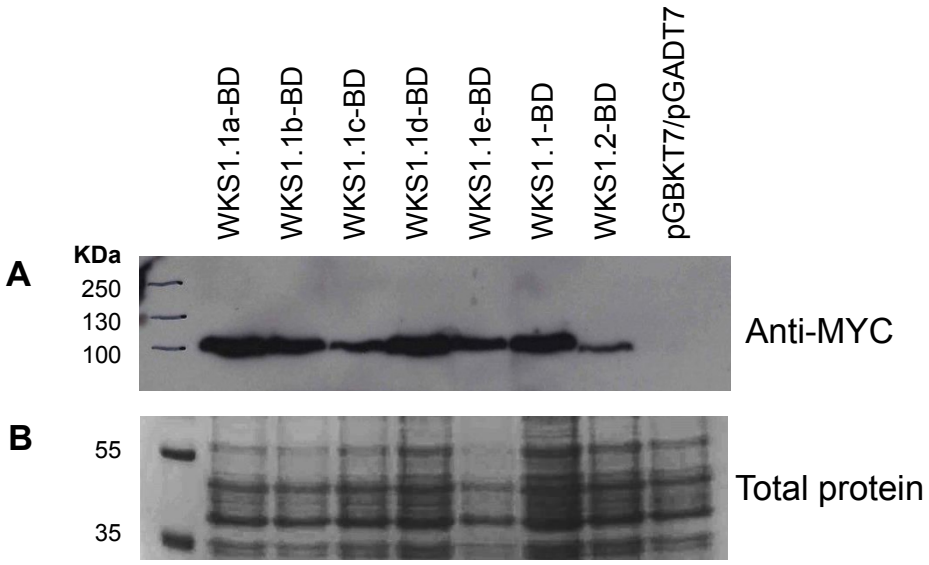
Supplemental Figure 3. Effect of WKS1.1 Mutations on Homodimer Formation.

(A) Location of WKS1.1 point mutations resulting in *Pst* susceptible plants. Numbers to the left and right of each rectangle refer to DNA co-ordinates in the WKS1.1 coding region.
(B-C) Interactions between mutant and wild-type WKS1.1 as activation (B) or binding domain (C).
(D) Homodimerization of wks1a-d kinase-mutants.
(E) Homodimerization of wks1e START-mutant and its interactions with the wild-type WKS1.1.
(B-E) Positive control = pGBKT7-53/pGADT7-T; Negative Control = pLAW10/pLAW11. BD= binding AD= activation domain. See also Supplemental Figure 5 for control Western Blots.



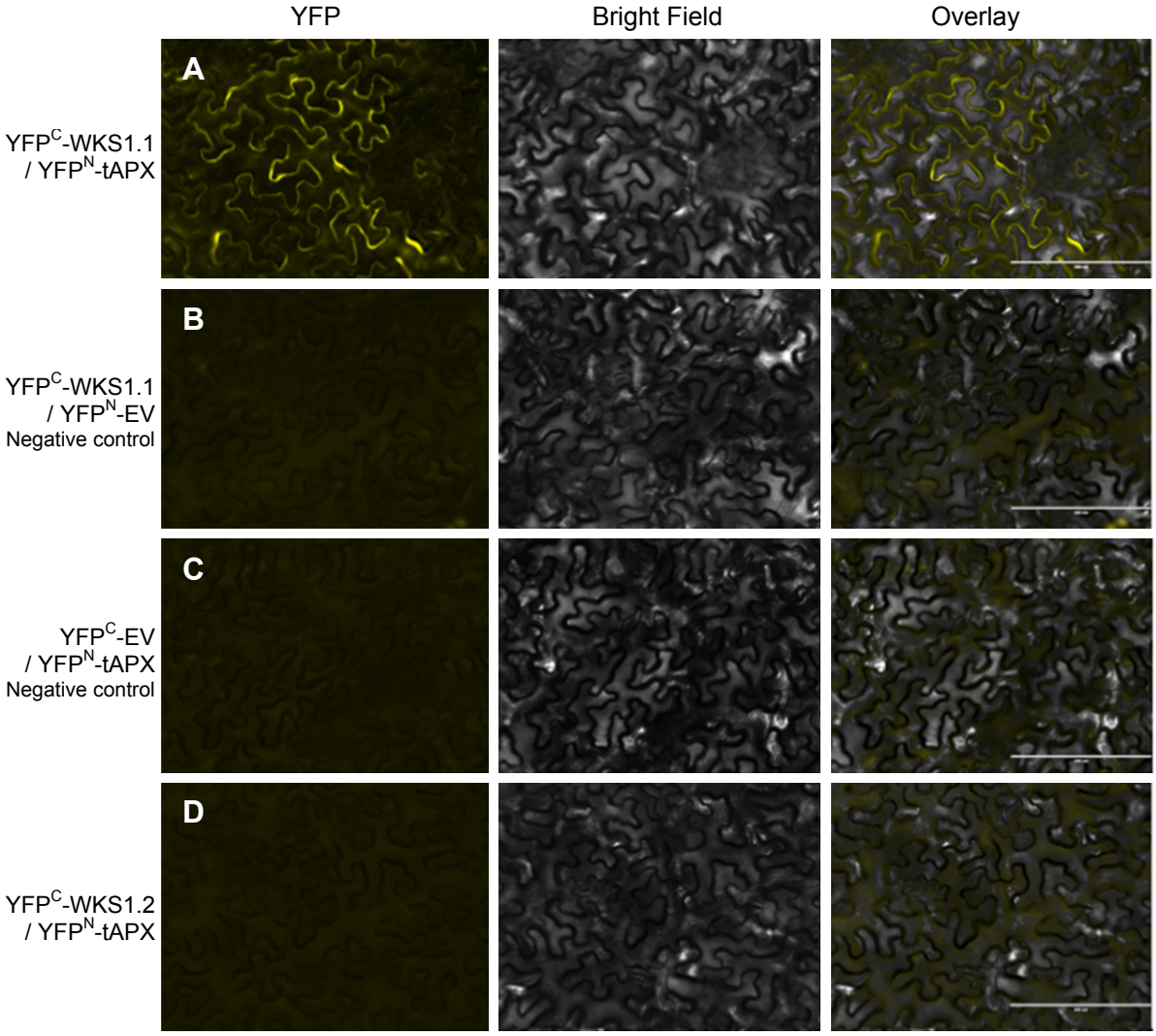
Supplemental Figure 4. Y2H Assays Between WKS1 Variants and Interacting Proteins tAPX, VAP1-3 and PPIP5K1-like.

(A) tAPX (clone identified in the Y2H screen including the last five exons)
(B) VAP1-3 (full length).
(C) PPIP5K1-like (clone identified in the Y2H screen including the last three exons).
(A-C) Positive control = pGBKT7-53/pGADT7-T; Negative Control = pLAW10/pLAW11. See Supplemental Figure 5 for control Western Blots. KI= Kinase-Inter-domain, wks1a-e= mutants.



Supplemental Figure 5. Western Blot Confirming that Mutant and Alternative Splice WKS1.2 Proteins Are Not Degraded in Negative Y2H Interactions in Figure 3 and Supplemental Figures 3 and 4.

(A) Western blot detection of mutant and alternative spliced WKS1 proteins using Myc Epitope Tag Antibody (Myc.A7) and a secondary goat anti-rabbit IgG peroxidase conjugate (see Supplemental Method 4). Mutant and alternative spliced WKS1 proteins were not degraded in negative Y2H interactions. BD=binding domain, pGBKT7/pGADT7= negative control.
(B) Total protein included as a loading control.



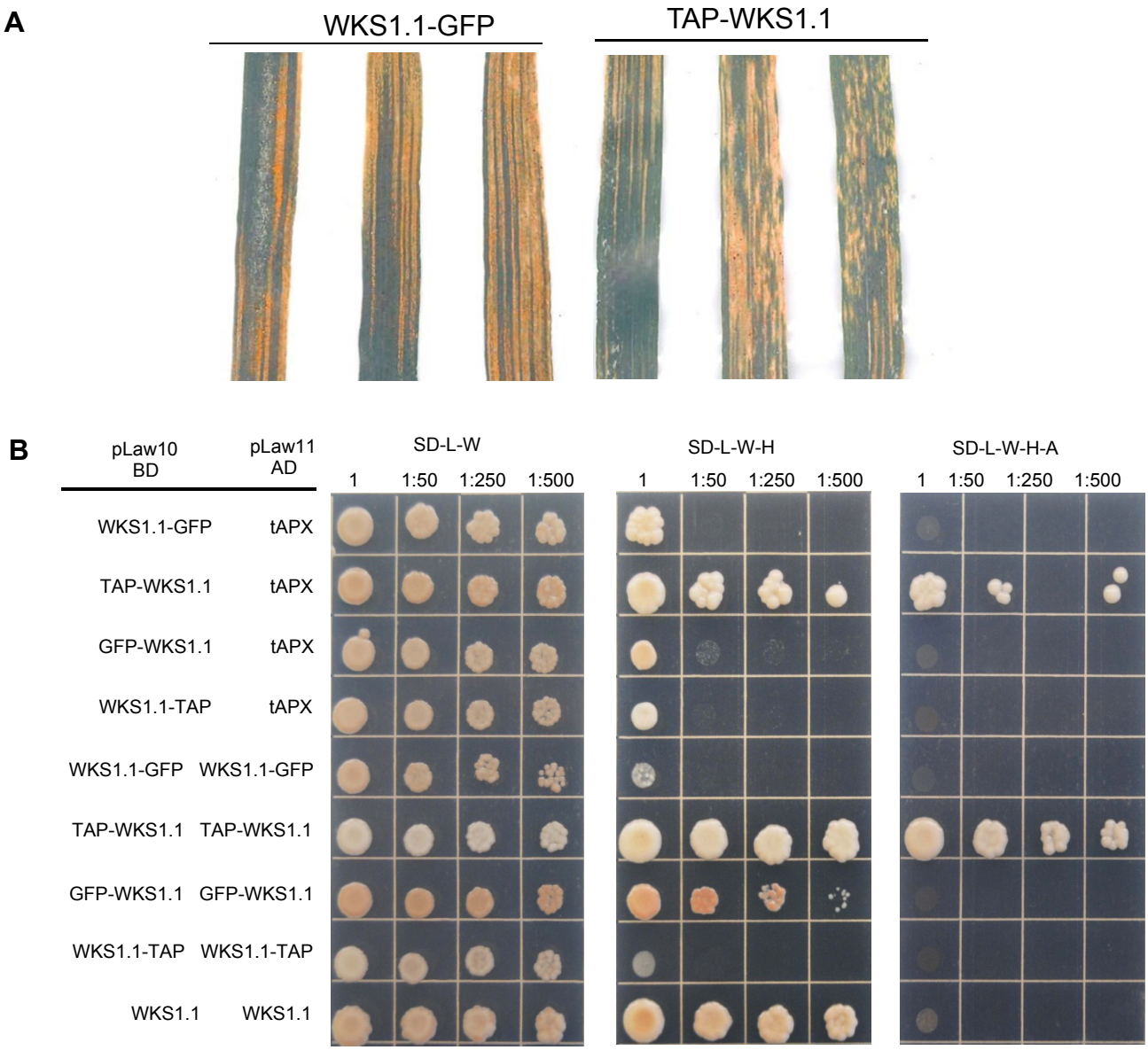
Supplemental Figure 6. Interaction Between tAPX and Splice Variants WKS1.1 and WKS1.2 by Bimolecular Fluorescence Complementation (BiFC).

(A) YFP^C-WKS1.1 and YFP^N-tAPX. YFP= YELLOW FLUORESCENCE PROTEIN, YFP^N= amino-terminal region, YFP^C= carboxy-terminal region, EV= empty vector. White bar indicates 200 µm in all pictures. Transient expression in *Nicotiana benthamiana* leaf epidermal cells followed protocols published before (Chang et al., 2013).

(B) YFP^C-WKS1.1 and YFP^N-EV. Abbreviations and methods are described in panel (A).

(C) YFP^C- EV and YFP^N-tAPX. Abbreviations and methods are described in panel (A).

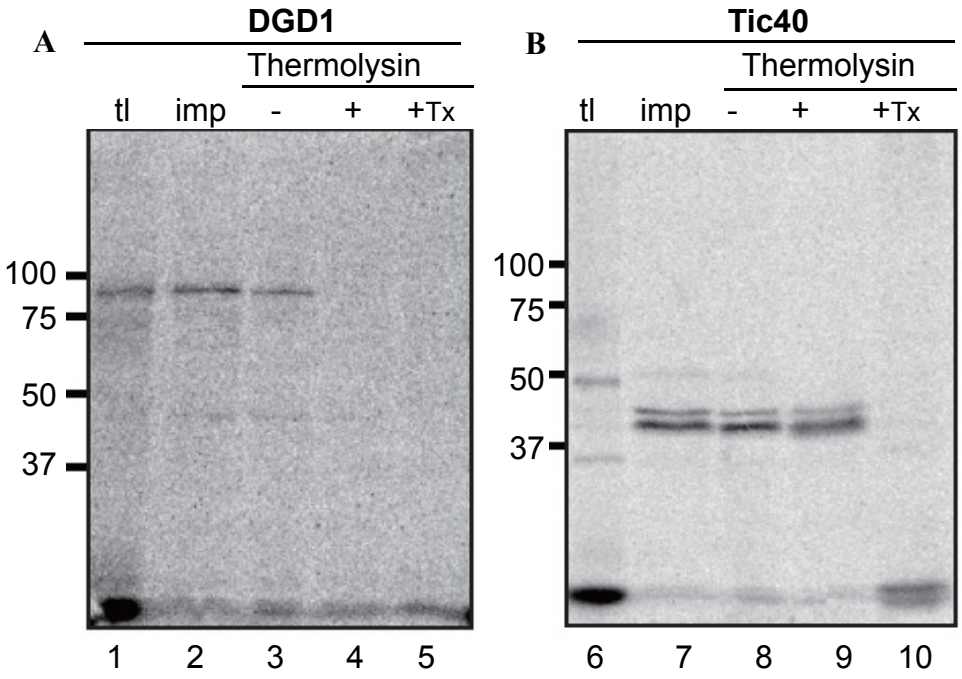
(D) YFP^C-WKS1.2 and YFP^N-tAPX. Abbreviations and methods are described in panel (A).



Supplemental Figure 7. Effect of WKS1.1 Fusions with Tags at the N- or C-Terminal Regions on Interactions with Full-length tAPX in Y2H Assays and on Resistance to *Pst*.

(A) Reaction to *Pst* race PST130 in transgenic wheat plants transformed with NP:WKS1.1-GFP (susceptible) and Ubi:TAP-WKS1.1 (partial resistance).

(B) Effect of GFP and TAP tags at the amino- and carboxy-terminus of WKS1.1 on the formation of homodimers and on the interactions with full-length tAPX. None of the constructs showed auto-activation when tested against an empty-bait or -prey vector.

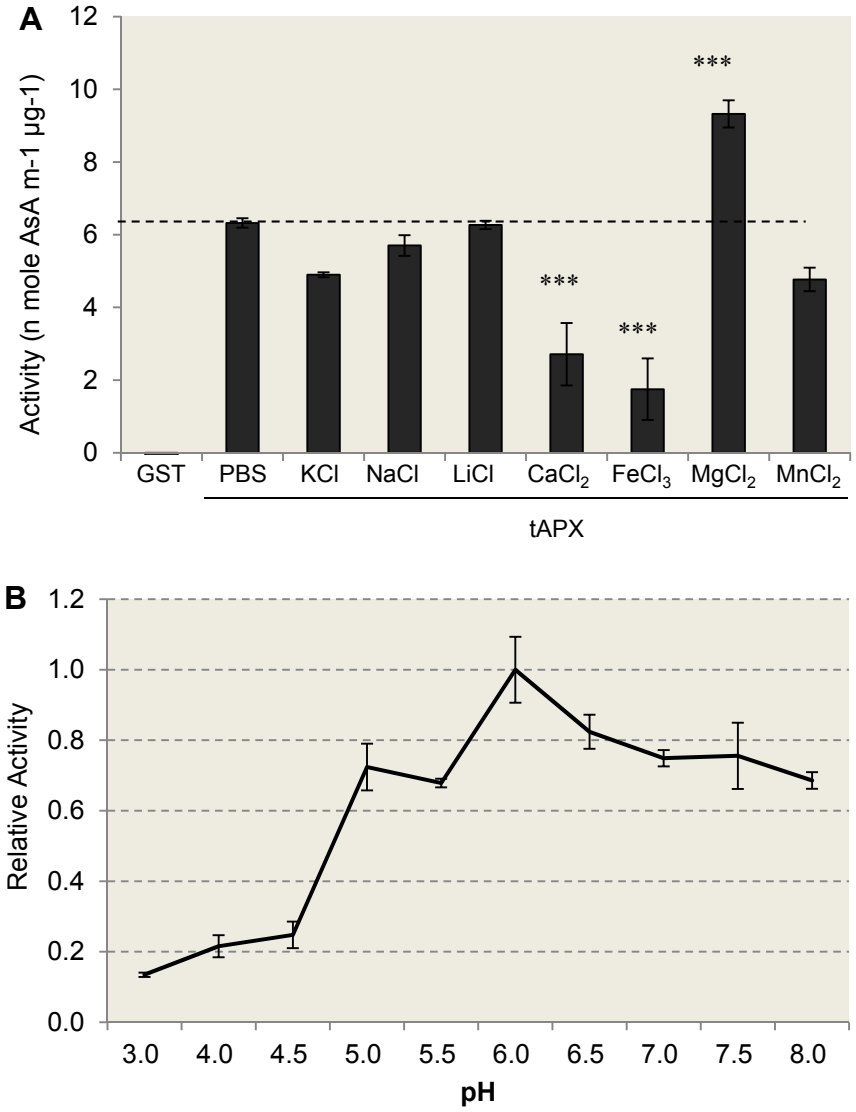


Supplemental Figure 8. Controls for the *in vitro* Chloroplast Import Assays.

(A-B) Radiolabeled DGD1 and Tic40 controls incubated with intact chloroplasts. tl = 10% of translation products, imp. = intact chloroplasts 20 min under import condition, thermolysin = chloroplasts incubated on ice for 30 min without (-) or with (+) thermolysin, or with thermolysin and 2% (v/v) Triton X-100 (Tx). Radiolabeled proteins were visualized using a phosphorimager. The sizes of molecular weight markers in kDa are shown to the left of each image

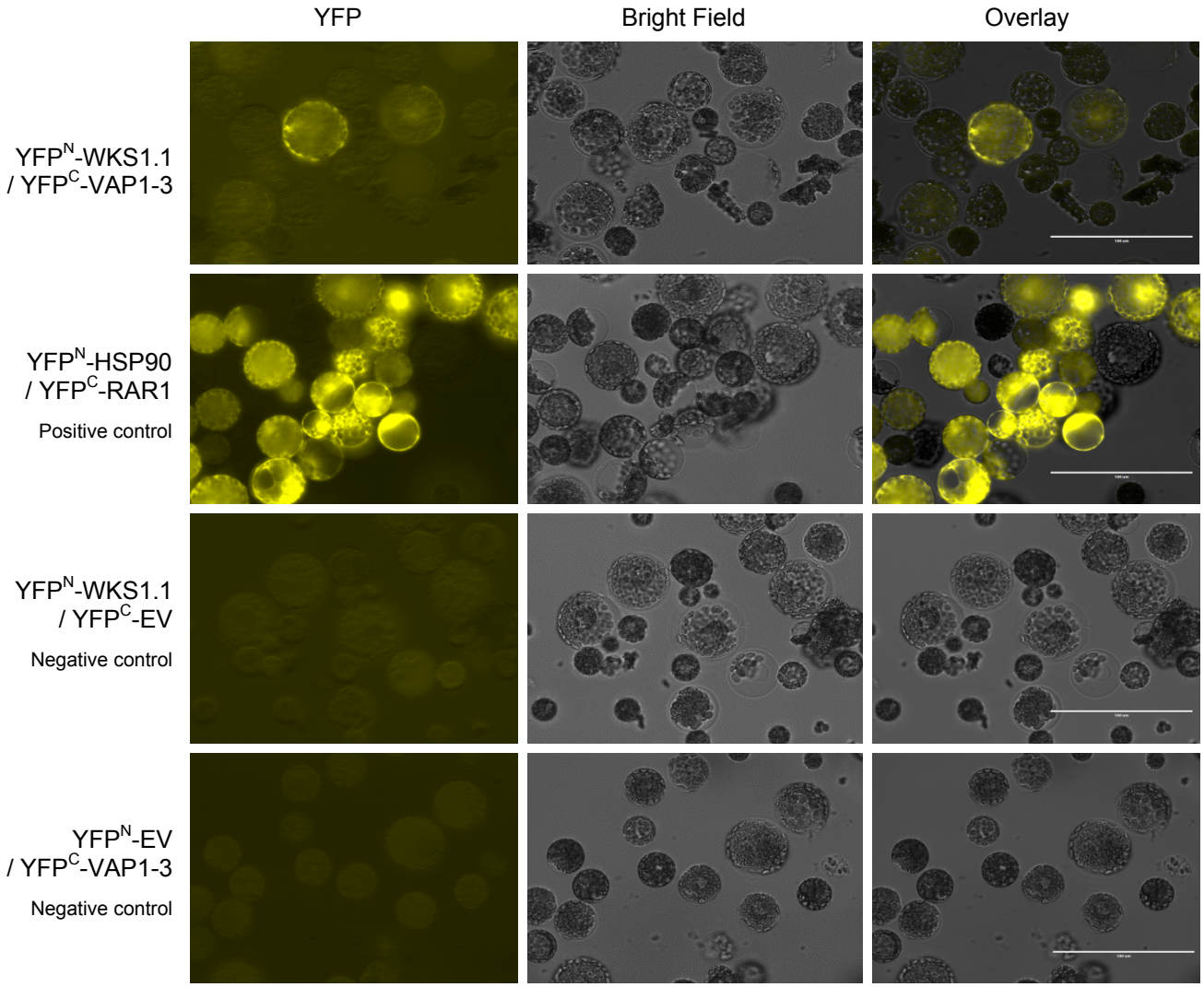
(A) DGD1 is an outer membrane protein (Froehlich et al., 2001) that is digested by the thermolysin treatment (lane 4).

(B) Tic40 is an inner membrane protein (Stahl et al., 1999) that is resistant to the thermolysin treatment (lane 9).



Supplemental Figure 9. Effect of Different Ions and pH on tAPX Activity *in vitro*.

(A) Effect of different ions on tAPX Activity. GST = no tAPX, PBS = indicates control buffer without ions. Concentrations of KCl and NaCl are 50 mM while other ions are at 5 mM (n = 4, *** = $P < 0.001$, Dunnett test vs. PBS control). Error bars indicate standard errors of the means.
(B) Effect of pH on tAPX activity. The specific enzymatic activity at pH6.0 was set as 1. Error bars indicate standard errors of the means (n = 4).



Supplemental Figure 10. Validation of the Interaction between VAP1-3 and WKS1.1 by Bimolecular Fluorescence Complementation (BiFC) in *N. benthamiana* Protoplasts.

YFP= YELLOW FLUORESCENCE PROTEIN, YFP^N= amino-terminal region, YFP^C= carboxy-terminal region. BiFC methods are as described before (Cantu et al., 2013). The positive control shows a stronger interaction between wheat HSP90 and RAR1 (Cantu et al., 2013). Negative controls include VAP1-3 and WKS1.1 combined with empty vector (EV). White bar indicates 100 µm.

Supplemental Tables

Supplemental Table 1. Average Resistance of Plants Transformed with NP:WKS1.1 and NP:WKS1.2 and Inoculated with *Pst* Race PST-08/21.

Background	Line or Event (N[1])		Figure[2]	Infection score[3] ± SE	Comments
Glasgow	Glasgow susceptible control (5)		Fig.1B	4.00 ± 0.00	Abundant sporulation, highly susceptible.
UC1041	UC1041 susceptible control (10)		Fig.1C	3.85 ± 0.10	Abundant sporulation, highly susceptible.
UC1041	UC1041+*WKS1* resistant control (10)		Fig.1D	1.28 ± 0.13	Clearly defined necrotic *Yr36* response, partial res.
Glasgow	NP:WKS1.1	Event 1 (10)	Fig.1F	1.30 ± 0.15	Clearly defined necrotic *Yr36* response, partial res.
		Event 2 (10)	Fig.1G	1.20 ± 0.13	Clearly defined necrotic *Yr36* response, partial res.
		Event 3 (10)	SF2a-e	1.40 ± 0.16	Clearly defined necrotic *Yr36* response, partial res.
		Event 4 (10)	SF2a-e	1.30 ± 0.15	Clearly defined necrotic *Yr36* response, partial res.
		Event 5 (5)	SF2a-e	1.20 ± 0.14	Clearly defined necrotic *Yr36* response, partial res.
Glasgow	NP:WKS1.2	Event 1 (7)	Fig.1E	4.00 ± 0.00	Abundant sporulation, no *Yr36* response, highly susceptible.
		Event 2 (7)	SF2f-k	3.86 ± 0.12	Abundant sporulation, no *Yr36* response, highly susceptible.
		Event 3 (7)	SF2f-k	3.86 ± 0.12	Abundant sporulation, no *Yr36* response, highly susceptible.
		Event 4 (7)	SF2f-k	3.86 ± 0.12	Abundant sporulation, no *Yr36* response, highly susceptible.
		Event 5 (7)	SF2f-k	3.71 ± 0.15	Abundant sporulation, no *Yr36* response, highly susceptible.
		Event 6 (7)	SF2f-k	4.00 ± 0.00	Abundant sporulation, no *Yr36* response, highly susceptible.

[1] Number of plants scored per line or event.
[2] Pictures shown in Figure 1 (Fig.1) or Supplemental Figure 2 (SF2) a-e= WKS1.1, f-k=WKS1.2.
[3] Infection evaluation: 0, no sporulation; 1, light sporulation; 2, intermediate sporulation; 3, moderate sporulation; 4, abundant sporulation.

Supplemental Table 2. Primers Used in This Study**.**

Primer Name	Primer Sequence	Objective
GST-WKS1 F	GCGGAATTCATGGAGCTCCCACGAAACAA	GST-WKS1.1 fusion
GST-WKS1.1 R	GCGCTCGAGTCAACTTTCACCACTTCCTG	
GST-WKS1 F	GCGGAATTCATGGAGCTCCCACGAAACAA	GST-WKS1.2 fusion
GST-WKS1.2 R	GCGCTCGAGTTAATTGTGGTATCTTGCAA	
ATG_F	CACCATGGAGCTCCCACGAAACAAAC	WKS1.1 clone for Y2H
Full_R	TCAACTTTCACCACTTCCTGA	
ATG_F	CACCATGGAGCTCCCACGAAACAAAC	WKS1.2 clone for Y2H
WKS1.2_R	GAGACTAGGACACATAACATTAATTG	
ATG_F	CACCATGGAGCTCCCACGAAACAAAC	WKS1 Kinase alone (KA) for Y2H
Kinase_R	AAGACGTTCTACAACATGATTCA	
ATG_F	CACCATGGAGCTCCCACGAAACAAAC	WKS1 Kinase + inter-domain (KI) for Y2H
KI_R	ATCATATGTCGTTCCCACAGGTC	
IS_F	CACCATGCGAATGCTTGGTAAAGATCAC	WKS1 Inter-domain + START (IS) for Y2H
Full_R	TCAACTTTCACCACTTCCTGA	
START_F	CACCATGTGGCGTCTTCTCGGATGCCAGAA	WKS1 START alone (SA) for Y2H
Full_R	TCAACTTTCACCACTTCCTGA	
TILL_569_F	GTGCCTTTGGTGAG**A**TTTTCCGAGGTTTTC	*wks1a* mutant (V55I) for Y2H
TILL_569_R	CTTTGCCAACATGAGTTCTATAGTTCTTAG	
TILL_312_F	CTAATAATGTAATA**A**GAAGCAGAGGTTAC	*wks1b* mutant (G199R) for Y2H
TILL_312_R	TAAAATAACTATTCTCCATAGAAAGCAACC	
TILL_480_F	AGAACACATTCAGA**T**TGGCCGGGTTGATC	*wks1c* mutant (T211I) for Y2H
TILL_480_R	GGATCCATGTAACCTCTGCTTCCTATTACA	
TILL_138_F	GGATATCAAGAAAC**A**TCCTGAAATGAATC	*wks1d* mutant (R205H) for Y2H
TILL_138_R	CTTCTGAGACACTCAATTGCCAGCTCTCC	
TILL_567_F	TATGAGTGG**A**ACTGCAGCTTCTCGT	*wks1e* mutant (D477N) for Y2H
TILL_567_R	ACGGCTACTGTCCATGCTCATGAGA	
UbiP-F1	TTTAGCCCTGCCTTCATACG	Validation of transgenic Ubi:NTAP-WKS1.1
TAP-F95	TCATCCAGAGCCTGAAGGAC	
TAP-R254	TTCAGGTTTGGGAGATGGAG	
START-pTNT-F	GCGGGTACCATGTGGCGTCTTCTCGGATGCCAGAA	Cloning START domain into GST-START for chloroplast import assay
WKS1R	GCGCCCGGGTCAACTTTCACCACTTCCTG	
tAPX-6B-F tAPC-6B-R	GCATTCTTGACGTCTCTGGTC CATCTTGCATGCCGACCAAT	Marker for the deletion of tAPX in chromosome arm 6BL
tAPX-BamH1-F	cgg*GGATCC*ATGGCGGCGTCGGAGGCCGCGCA	Recombinant tAPX protein purification for activity assay
tAPX-Xho1-R	cgg*CTCGAG*TTAGTTCCCGGCCAGAGACGTCAAG	

Supplemental Table 2. Continuation.

Primer Name	Primer Sequence	Objective
WKS1.1EcoRIF	cc*GAATTC*TATGGAGCTCCCACGAAACAA	Clone WKS1.1 kinase and kinase-linker into pET41b
WKS1.1KR	gtg*CTCGAG*AAGACGTTCTACAACATGATTCAT	
WKS1.1KIRXhoI	gtg*CTCGAG*TATGTCGTTCCCACAGGTCCAATC	
WKS1-for	GTACCATGGAGCTCCCACGAAACAA	Cloning of WKS1.1 and WKS1.2
WKS1.1-rev	AAAGCGCGCTACGTATCAACTTTCACCACTTCCTG	
WKS1.2-rev	AAAGCGCGCTACGTATCAATTGTGGTATCTTGCAA	
VAP1-3_F	CACCGAGGAAGCCACCATGAG	Full length VAP1-3 interactor
VAP1-3_R	CTACTCTTCTCCTATGTCTTCTTGA	
tAPX_F	ccATGGCGGAGCGCATCG	Full length tAPX interactor into pET28a
tAPX_R	CCTGGAAAATGGTTAATTACA	
PPIP5K-like_UF1	caCCCGTGGGCAGATCGG	Full length PPIP5K-like interactor into pENTRY
PPIP5K-like_SR1	GTTGCCACCAAATGTTAACAAAG	

All primers are written from 5' to 3' end. Start codons are underlined in the forward primers and stop codons are underlined in the reverse primers. For the five *wks1* mutant alleles generated by inverse-PCR the base changed in each allele is in bold and underlined in the forward primer. The amino acid change generated by each mutation and its position in the protein is indicated in parentheses. Lower case letters in the primers indicate added nucleotides to increase restriction efficiency

Supplemental Table 3. WKS1 Protein Interactors Detected in Y2H Screen and Their Y2H Interactions with Alternative Splice Variants WKS1.1 and WKS1.2 and Mutants (wks1a-e).

Closest plant protein	Thylakoid-associated ascorbate peroxidase	Vesicle-associated protein 1-3-like	Inositol hexakisphosphate & diphosphoinositol-pentakisphosphate kinase 1-like
Proposed abbreviation	tAPX	VAP1-3	PPIP5K1-like
GenBank accession	KJ614568	KJ614569	KJ614570
# of clones identified in Y2H	3 (2 FL, 1 KI) [1]	2 (KI) [1]	1 (KI) [1]
Validation	Full-length	Full-length	Partial (last 3 exons)
KI	Y	Y	Y
WKS1.1	Y	Y	Y
WKS1.2	N	N	N
wks1a	Y	Y	N
wks1b	Y	Y	Y
wks1c	N	N	N
wks1d	N	N	N
wks1e	N	N	N

[1] FL indicates clones detected with the full length WKS1.1 and KI clones detected with the Kinase + inter-domain region. Y = positive interaction and N = no interaction.

Supplemental Methods

Supplemental Method 1. Time Course of *Pst* Infection.

Six-week-old plants were inoculated with *Pst* as described before (Fu et al., 2009), harvested after 3, 6, 10 and 13 days, and fixed and stained in uvitex (Moldenhauer et al., 2006). One cm^2 leaf segments were evaluated with a Nikon Microphot SA fluorescence microscope with a UV-2A DM 400 filter (Nikon, Melville, NY, USA). Because *Pst* infections are limited by the parallel veins in the mature wheat leaves, fungal development in different interveinal zones generally arise from independent infection events. Interveinal zones in each of three different leaves from four replicates per time point were evaluated. Development of fungal growth in each inter-veinal zone in each leaf segment was recorded as either uninfected, only sub-stomatal vesicles, sub-stomatal vesicles with hyphal outgrowth but no fungal network, and different levels of fungal network development: < 10% ("initial network"), 10-50% ("mid-level network") and >50% (advanced network") of the inter-veinal zone. Formation of an extensive network in the mesophyll precedes the formation of the uredinia, which gives rise to the urediniospores.

Supplemental Method 2. Transgenic Lines.

NP:WKS1.1 and NP:WKS1.2 in Glasgow: NP:WKS1.1 and NP:WKS1.2 were initially cloned into the Entry vector pENTR/d-TOPO (Invitrogen, UK) without stop codons. Stop codons were incorporated by amplifying the WKS1.1 and WKS1.2 3' terminal regions with stop codon sequences included into the reverse primers, and replacing the respective *Spe*I-*Bss*HII fragments. NP:WKS1.1-stop and NP:WKS1.2-stop were then digested with *Not*I and *Sna*BI, and 5' termini filled in with T4 polymerase, prior to ligation into the *Pme*I site of binary vector pRLF10. The *A.tumefaciens* supervirulent strain EHA105 (Hood et al., 1993) was used for transformation of the T-DNAs into immature Glasgow wheat embryos using protocols described before (Risacher et al., 2009). Plantlets were transferred to Jiffy-7 peat pellets, and, following analysis by quantitative PCR for the copy number of the nptII gene, potted on, vernalized, and grown to maturity. Stable homozygous lines were identified by PCR and segregation ratio; phenotypic analysis was performed on T_4 plants. For stripe rust inoculation plants were infected at 10°C and kept 24 hour in dark and high humidity conditions. Afterwards, plants were transferred to a control environment room (CER) with a 19-14°C day/night temperature cycle (16h light/8h dark).

Ubi:TAP-WKS1.1 in Bobwhite: WKS1.1 was initially cloned into the Entry vector pENTR/d-TOPO (Invitrogen, Grace Island, NY, USA) and then incorporated into a Gateway binary destination vector Ubi-NTAP-1300 kindly provided by Michael Fromm (Rohila et al., 2006). Calluses derived from immature embryos of hexaploid spring variety Bobwhite were bombarded using a 1:1 molar ratio of Ubi:TAP-WKS1.1 and Ubi:BAR selectable marker plasmids (15.5 µg total) coated onto 1000 nm gold particles (Seashell Technology, La Jolla, CA, USA), according to the manufacturer's instructions. Transformants were selected as described before (Fu et al., 2009). Independent transgenic T_1 lines were obtained and positive plants were confirmed by PCR using primer pair TAP-F95 and TAP-R254 or by Western Blot

using TAP tag polyclonal antibody (Pierce, Thermo Fisher Scientific Inc. Rockford, IL). Phenotypic analysis was performed in T_3 and T_4 transgenic plants.

NP:WKS1.1-GFP in Bobwhite: Stable transgenic plants were generated in the spring wheat variety Bobwhite by bombardments using the same procedure described above (Table 1). Two independent transgenic events were generated with a construct encoding a fusion protein between WKS1.1 and GFP under the regulation of the 3.5 kb promoter region of WKS1 (NP:WKS1.1-GFP). As a control we developed an additional transgenic plant expressing GFP under the regulation of the same promoter region (NP:GFP) (Table 1). The presence of the GFP transgene was verified by PCR.

Fluorescence microscopy was performed using an Olympus FV1000 confocal laser scanning microscope (MCB LM Imaging Facility, Department of Molecular & Cellular Biology, University of California, Davis). For GFP the excitation wavelength was 488 nm and emission was 500 to 535 nm and for chlorophyll the excitation wavelength was 633 nm and emission was 645 to 745 nm.

Supplemental Method 3. *In vitro* Lipid Binding Assays.

To express the GST-fused WKS1.1 and WKS1.2 proteins in wheat germ extracts, we first cloned the two GST fusions into the pGEX-6P-1 vector (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) between the *Eco*R1 and *Xho*I sites (primers are described in Supplemental Table 1). The resulting pGEX clones were then subcloned into the pTNT vector (Promega, Madison, WI, USA) which was used for *in vitro* synthesis of protein using wheat germ extracts. To generate the pTNT GST-START$_{WKS1.1}$ clone, the START domain of WKS1.1 (without inter-domain region) was directly subcloned into the pTNT vector by using the START $_{WKS1.1}$ pTNT F and WKS1R primers (Supplemental Table 1), which contain an *Acc65*I site and *Xma*I site, respectively.

For the *in vitro* mRNA synthesis, the two pTNT plasmids were linearized with *Bgl*II. The plasmids constructs were then purified and transcribed *in vitro* using the Ribomax-T7 RNA production system (Promega, Madison, WI, USA). After extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), the mRNA was precipitated and washed with ethanol, dissolved in RNase-free water and stored at -80 ºC. The quality and quantity of the mRNA was determined by using Nanodrop (Thermo Fisher, Wilmington, DE, USA).

For the *in vitro* protein synthesis we used the wheat germ system following the manufacturers' instructions (Promega, Madison, WI USA). Expressed proteins were purified on GST SpinTrap purification columns (GE Healthcare, Piscataway, NJ, USA). Eluted proteins were dialyzed overnight in phosphate buffered saline (PBS) to remove glutathione and quantified by employing the BCA protein assay reagent (Pierce, Rockford, IL, USA). A 15 µl aliquot from the wheat germ reactions was combined with 3 µl of NuPAGE (Invitrogen, Grace Island, NY, USA) sample buffer, boiled for 3 min and separated by Nupage 4-12% Bis-Tris polyacrylamide gel electrophoresis prior to blotting onto nitrocellulose membranes (Bio-

Rad, Richmond, CA, USA) for immunoblots. Immunoblots on nitrocellulose were blocked with 5% nonfat milk in PBST (PBS with 0.05% Tween 20) for 1 h and washed in PBST.

For the lipid binding assays lipid strips and phosphatidylinositol (PIP) strips (Echelon Biosciences, Salt Lake City, UT USA) were blocked for 1 h with PBST, containing 3% (w/v) fatty acid free BSA. All subsequent incubations and washes included PBST/3% BSA. Membranes were incubated overnight at 4^{o} C with 9 µg/mL of each of the GST fusions separately, then washed four times over 45 min, and incubated for 2 h with rabbit anti-GST (Millipore, Billerica, MA, USA) at a 1:4,000 dilution. Membranes were then washed, incubated with anti-rabbit IgG-HRP at 1:10,000 dilution, and washed again before developing with SuperSignal West Pico Chemiluminescence substrate (Pierce Protein Biology Systems, Thermo Scientific, Rockford, IL, USA) and imaged using a Kodak Image Station 2000RT (Eastman Kodak, Rochester, NY, USA).

Supplemental Method 4. Yeast Two-hybrid (Y2H) Assays.

Bait/prey co-transformants generated by the lithium acetate method (Gietz and Woods, 2002) were selected on solid SD agar medium lacking leucine (L) and tryptophan (W). Positive transformants were then re-plated on SD medium lacking L, W, histidine (H) and adenine (A) to test for interaction. Three dilutions of 1:50, 1:250, and 1:500 were compared. pLAW10 and pLAW11 empty vectors were used as negative controls. A bait vector containing murine p53 (pGBKT7-53) and prey vector containing SV-40 large T-antigen (pGADT7-T) provided in the Matchmaker kit (Clontech Laboratories, Mountain View, CA, USA) were used as positive interaction controls. Auto-activation was tested by co-transforming bait and prey constructs with their reciprocal empty vector. Five point mutations previously shown to abolish WKS1 resistance (Fu et al., 2009) were introduced in the Y2H full length WKS1 construct using primers described in Supplemental Table 1 and the Phusion Site-Directed Mutagenesis kit (New England Biolabs, Ipswich, MA, USA). Full length WKS1 and WKS1 truncation constructs were cloned using primers described in Supplemental Table 1 and integrated into Y2H bait (pLAW10) and prey (pLAW11) vector backbones via Gateway recombination.

To test if the negative Y2H interactions of the WKS1.1 mutants and WKS1.2 were caused by the degradation of the modified WKS1 proteins, we co-transformed tAPX-plaw11 with WKS1.1a-, WKS1.1b-, WKS1.1c-, WKS1.1d-, WKS1.1e-, WKS1.1- or WKS1.2-plaw10 respectively into yeast AH109 gold. Total protein was extracted and approximately 20 µg protein was loaded in each lane. Proteins were detected in Western blots using a 1:1000 dilution of primary antibody against Myc Epitope Tag (Myc.A7, Thermo Scientific Catalog#: MA1-21316), and a 1:5000 dilution of goat anti-mouse IgG peroxidase conjugate secondary antibody (EMD Millipore's Calbiochem ® Billerica MA USA, catalogue # DC02L).

Supplemental Method 5. Wheat cDNA Y2H Library Screening and Validation.

We screened a cDNA Y2H library previously generated using RNA extracted from leaves of tetraploid wheat *Triticum turgidum* L. ssp. *durum* cv. Langdon infected with *Puccinia striiformis* race PST113 (Yang

et al., 2013). The library was cloned into prey vector pGADT7-Rec and transformed into strain Y187 (*MATα*). The compatible mating strain, Y2H Gold (*MAT*a), was transformed with pLAW10 bait vector containing either the full-length WKS1 or the kinase with the inter-domain region (KI). Mating procedures followed Matchmaker Gold Yeast Two-Hybrid System protocols (Clontech Laboratories, Mountain View, CA, USA). The mated library was initially plated on solid SD -L -W -H media, after which positive colonies were collected and transferred onto SD -L -W -H -A plates for more stringent selection. Plasmid DNA was extracted from positive yeast colonies using the QIAprep Spin Miniprep Kit (Qiagen, Redwood, City, CA, USA) and amplified in *E. coli* before sequencing using the Matchmaker AD LD-Insert Screening Amplimer Set (Clontech). To test for auto-activation, purified prey plasmids were co-transformed with empty bait vector, and were also re-transformed with the original bait construct to validate the positive growth obtained in the library screen.

Supplemental Method 6. *In vitro* Chloroplast Localization.

Radiolabeled precursors of WKS1.1 and WKS1.1-START domain (without any tag) were prepared using the TNT® T7 Coupled Wheat germ System (Promega, Madison, WI, USA) and L-[35S]-methionine (Amersham Biosciences, Piscataway, NJ, USA). Chloroplasts were prepared from pea seedlings grown in the greenhouse (Bruce et al., 1994). *In vitro* mRNA synthesis and protein synthesis were carried out using the TnT SP6 coupled reticulocyte lysate system (Promega, Madison, WI USA) followed the original manufacturers' instructions and was done on a 400 µl scale. After TnT reaction, radiolabeled proteins were incubated with isolated chloroplasts under light for 20 min, and then chloroplasts were re-isolated using 40% Percoll. After import for 20 min*, the reaction mixture was divided into four fractions. Intact chloroplasts in each fraction were re-isolated by a 40% Percoll cushion. The first fraction was saved as total import (imp).

Three other samples were resuspended to 0.5 µg chlorophyll/µL in import buffer containing 10 mM CaCl2 without (mock), or with 0.2 µg/µL thermolysin (+ t-lysin), or with 0.2 µg/µL thermolysin and 2 % Triton X-100 (+ t-lysin & TX100). After incubation for 30 min on ice, the protease activity was quenched by addition of EDTA to a final concentration of 10 mM. Chloroplasts were then re-isolated thorough a 40% Percoll Cushion containing 10 mM EDTA and resuspended in 100 µL of import buffer. Chlorohyll concentration of each sample was quantified by measuring absorbance at 652 nm and a sample containing 5 µg chlorophylls was loaded onto each lane.

For the time course of WKS1.1 *in vitro* chloroplast import assay, each reaction contained isolated chloroplasts (12.5 µg chlorophyll), 10 µL of the translation product and 3 mM Mg-ATP in import buffer. After incubation at room temperature under light, intact chloroplasts were re-isolated at different time points using 40% Percoll and were directly loaded onto 10% SDS-PAGE. The radiolabeled proteins were visualized by phosphor-imager analysis. Two Arabidopsis proteins, DGD1 and Tic40, were included as technical controls. Arabidopsis transcription factor DGD1 (Gabriele et al., 2010) is not transported into the

chloroplast (Figure 4C) and chloroplast inner envelope membrane translocon complex protein Tic40 is transported into chloroplast (Figure 4D) and is located in the stroma (Chou et al., 2003).

The presence of a chloroplast localization signal in WKS1 was tested using prediction programs ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), iPSORT (http://ipsort.hgc.jp/) , Predotar (https://urgi.versailles.inra.fr/predotar/predotar.html), and TargetP (http://www.cbs.dtu.dk/services/TargetP/).

Supplemental Method 7. Co-immunoprecipitation (CoIP).

For the WKS1-tAPX CoIP experiment, the supernatant of a total protein extract from leaves of Ubi:TAP-WKS1.1 transgenic plants was mixed with 100–150 µl of IgG Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA) and incubated at 4 ºC for 1 h with continuous rotation. After centrifugation at 200 g for 2–3 min, IgG supernatant was discarded and the collected IgG beads were washed in 30 ml extraction buffer lacking protease inhibitors. The washed beads were loaded onto a disposable chromatography column and washed with 10 ml Tobacco Etch Virus (TEV) cleavage buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% IGEPAL, 0.5 mM EDTA, 1 mM DTT). The TAP-tagged proteins were released by digestion with 30 µl TEV protease (300-500 U; Invitrogen) in TEV cleavage buffer containing 1 l M E-64 protease inhibitor for 1 h at 16 C. The TEV cleaved eluate was adjusted to 2 mM $CaCl_2$ and diluted in 3 volumes of calmodulin binding buffer (CBB: 10 mM b-mercaptoethanol; 10 mM Tris–HCl pH 8.0; 150 mM NaCl; 1 mM Mg-acetate; 1 mM imidazole; 2 mM $CaCl_2$; 0.1% IGEPAL) and incubated with 100 µl of calmodulin-agarose beads (Stratagene, La Jolla, CA, USA) for 1 h at 4 °C. The calmodulin-agarose beads were washed in 30 ml of calmodulin binding buffer and the proteins were eluted with buffer containing 10 mM beta -mercaptoethanol, 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM Mgacetate, 1 mM imidazole, 2 mM EGTA, 0.1% IGEPAL. The eluted proteins were precipitated with trichloroacetic acid and loaded onto a 4-15% gradient polyacrylamide gel for SDS-PAGE. After SDS-PAGE the proteins were transferred to a nitrocellulose membrane for Western blot, employing tAPX-specific antibodies (Agrisera, Vännäs, Sweden).

Supplemental Method 8. Recombinant WKS1-Kinase, tAPX and PPIP5K1-like Protein Purification.

The pET41b plasmids including the WKS1-kinase domain (KA) and the full-length *tAPX* and *PPIP5K1-like* genes were transformed into BL21 (DE3) plysS competent cells (Promega, Madison, WI USA). Bacteria was grown in TB medium to OD600 0.6~0.8 first at 37 °C and then at 25 °C before induction with 0.5 mM IPTG overnight. The cells were collected and suspended in 1/10 volume of B-PER Bacterial Protein Extraction Reagent (Pierce, Thermo Fisher Scientific Inc. Rockford, IL, USA) with Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics Corporation, Indianapolis, IN, USA). The cells were sonicated three times each for 30 s and incubated for 1 h with shaking in ice. After centrifugation at 18,000 rpm for 1 h, the supernatant was removed to a new tube, adjusted to 100 mM HEPES pH7.4, 500 mM NaCl, 1 mM ASA, 20 mM imidazole and mixed with 0.5 ml pre-equilibrated Ni-NTA resin (Pierce

Biotechnology Inc., Rockford, IL, USA) for 1 h in ice. The samples were then loaded into columns and washed with 20 volumes of binding buffer. Recombinant protein was mixed with 1 ml of Elution Buffer (100mM HEPES pH7.4, 500 mM NaCl, 1 mM ASA, 250 mM imidazole, 10% glycerol), held for 10 min, and then eluted. The protein was dialyzed overnight against dialysis buffer (100mM HEPES pH7.4, 500 mM NaCl, 1 mM ASA, 10% glycerol). Protein concentration was quantified with Qubit Protein Assay Kits (Molecular Probes, Life Technologies Corporation, Grand Island, NY, USA).

Supplemental Method 9. Phosphorylation Assays.

For the phospho-fluorescence assay, the reactions including 2 µg of each recombinant protein were stopped by adding 4 volumes of cold acetone and kept at -20 °C overnight. The proteins were pelleted by centrifugation at 15,000 rpm for 15 minutes and air-dried. Dried proteins were suspended in 200 µl of Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes ®, Life Tech. Grand Island, NY USA) in the dark for 1 h at room temperature. Proteins were pelleted again with cold acetone and washed three more times to remove the residual stain. Stained proteins were suspended in 200 µl of ddH_2O to quantify the fluorescence using a Tecan (Life Technologies, Grand Island, NY, USA) plate reader (excitation= 488 nm and emission= 595 nm).

For the *in vitro* phosphorylation assay, 5µl of γ-^{32}P-ATP (NEG502A001MC, 3000 Ci/mmol, 10mCi/ml, PerkinElmer Inc. Waltham, MA, USA) was mixed with 1 µg of WKS1.1 kinase protein and 5 µg of tAPX or PPIP5K1-like (control) recombinant protein in a kinase reaction buffer and kept for 30 minutes at room temperature. The samples were passed through a 7KMWCO Zeba Spin desalting column (Pierce, Thermo Fisher Scientific Inc. Rockford, IL) to remove the remaining free ATP. The samples were then separated in 10% SDS-PAGE and exposed to a phosphor-imager overnight. The IP image was collected with FUJI Film FIA-9000 (Suzhou, China).

For the *in vitro* and *in planta* gel retardation assays, ¼ volume of Laemmli Protein Loading Buffer Pack (Fermentas, Thermo Fisher Scientific Inc. Rockford, IL) was added to the samples, which were then boiled for 5 min. Protein samples were loaded onto 14% SDS PAGE with100 µM acrylamide-pendant Phos-tag and 100 µM of $MnCl_2$ (Wako Pure Chemical Industries, Ltd.). The proteins were run at 60 volts overnight in a 4 °C cold room. The gels were washed with 1 mM EDTA twice to remove the residual Mn^{2+} and twice with transfer buffer before electronic transfer and Western Blot detection with rabbit anti-tAPX antibody (Agrisera, Vännäs, Sweden, catalog number AS08 368).

Supplemental Method 10. tAPX Activity Assays.

tAPX Activity *in vitro* Assay: The recombinants tAPX protein was purified as described in Supplemental Method 8. For the activity assay, 2 µg of recombinant WKS1-KA protein was mixed with tAPX reaction buffer (50 mM PBS, 1 mM ascorbic acid, 10 mM $MgCl_2$ or other ions), to a total volume of 200 µl. Two µl of 1M H_2O_2 was added to start the reaction, and reads were taken at 290 nm every minute for 20 minutes

at room temperature. Activity was determined based on the absorbance decrease of L-ascorbate at 290 nm (using a standard curve for a linear range L-ascorbate measured also at 290 nm).

tAPX Activity *in planta* Assay: APX activity in wheat plants was measured in 2nd leaves from 6-week old plants. Tissue was ground into fine powder in liquid nitrogen and was mixed with 1.5 ml of extraction buffer (50 mM PBS pH7.0, 40 mM KCl, 10 mM $MgCl_2$, 1 mM AsA, 1% proteinase inhibitor, 1% phosphatase inhibitor and 1 mM PMSF) for 2 minutes at 60Hz in a high through-put grinder (Shanghai Wonhong Biotech Co. Ltd, Shanghai, China). Samples were filtered through miracloth (Millipore) into another 1.5 ml tube to remove cell wall debris. After centrifugation at 300 g for 10 m at 4°C, supernatants were transferred into 1.5 ml tubes and saved as “soluble fraction”. Pellets including the thylakoid fraction were washed thoroughly two times with 0.5 ml extraction buffer, suspended in 0.5 ml extraction buffer and saved as “thylakoid fraction”. To measure APX activity, 50 µl of soluble or thylakoid fractions were mixed with 1.95 ml reaction buffer (50 mMPBS pH7.0, 10 mM $MgCl_2$ and 0.5 mM AsA). Absorbance at 290 nm was measured for 0 minute with a spectrophotometer. 2 µl of 1 M H_2O_2 was added to start the reaction at room temperature and the samples were measured again at 5 minutes. APX activities were calculated based on the decrease of absorbance at 290 nm using a standard curve built with pure ascorbic acid. Four different plants per genotype were used as biological replicates. Protein amounts were determined using a BCA Protein Quantification Kit (Yeasen, Shanghai, China).

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