Supporting Information

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SI Materials and Methods

Screen for *hopZ-ETI deficient* Mutants. We infiltrated ~12,000 *Arabidopsis* ecotype Columbia (Col-0) ethylmethylsulfonate (EMS) mutants with a high inoculum of *Pseudomonas syringae* pathovar tomato DC3000 (*Pto*DC3000) carrying HopZ1a$_{PsyA2}$ [*Pto*DC3000 (*hopZ1a*)] and screened for plants that lacked a macroscopic hypersensitive cell death response (HR). After several rounds of screening, we identified 15 lines with a reproducible and heritable loss of the HopZ1a-induced HR (0.1% success rate). These 15 lines were crossed to the wild-type Col-0 and rescreened in the F_2 population. Of these 15 lines, 11 appeared to have a single recessive mutation because the segregation ratio was 3:1, showing an HR and not showing an HR, respectively. Because we had already demonstrated that ZAR1 is necessary for the recognition of HopZ1a (1), we carried out complementation analysis between the *zar1-1* tDNA insertion line and the recessive mutants. We also sequenced the upstream 2000-nt promoter and genic region of *ZAR1* in each noncomplemented mutant, and identified six mutants of *zar1*. We named the remaining nine non-*zar1* mutants, *hopZ-ETI-deficient* or *zed*.

To identify the mutated gene underlying the loss of the HopZ1a-induced HR, we created a mapping population of *zed1-2* crossed to *Arabidopsis* ecotype Landsberg *erecta* (L*er*). The F_2 population of the *zed1-2* × L*er* cross was rescreened for individuals that lacked the HopZ1a-induced HR. Consistent with our previous segregation analysis, ~25% of individuals lacked the HopZ1a-induced HR. We extracted pooled genomic DNA from a leaf of each individual lacking a HopZ1a-induced HR, sequenced the DNA by using an Illumina GAIIx, and used the next-generation mapping (NGM) methodology (2) to identify a peak of single-nucleotide polymorphisms on chromosome 3 that were not associated with L*er* or Col-0 (Fig. S1*A*). Within this peak, we identified three loci with C/T transitions, which are typically induced by EMS mutagenesis, and one locus with A/T and T/C changes (Fig. S1*B*). We tested tDNA insertion lines in the loci with C/T transitions to determine which locus was likely responsible for the recognition of HopZ1a (Fig. S1*C*). All tDNA insertion lines tested retained the HopZ1a-induced HR except for homozygous individuals of SALK_018065 (*zed1-6*; Fig. 1 *A* and *B* and Fig. S1*C*). Sanger sequencing of the other eight *zed* mutants identified additional residues in At3g57750 that contributed to the recognition of HopZ1a (Fig. 1*A*). Three *zed* mutants that do not appear to be mutated in *ZAR1* or *ZED1* remain to be characterized.

Bacterial Strains, Plasmids, and Routine Culture Conditions. HopZ1a was cloned from *P. syringae* pathovar *syringae* A2 and HopZ1b from *P. syringae* pathovar *glycinea* UnB647. *Escherichia coli* was grown in Luria-Bertani broth (LB) or Super Optimal broth with Catabolic repressor (SOC). *P. syringae* was grown in King's Broth (KB). Antibiotics were used at the following concentrations: for *E. coli*, 50 μg/mL kanamycin; for *P. syringae*, 50 μg/mL kanamycin, 50 μg/mL rifampicin, and 50 μg/mL cyclohexamide; for *Agrobacterium tumefaciens*, 100 μg/mL kanamycin and 100 μg/mL rifampicin.

Cloning. Unless otherwise indicated, Pfu polymerase (Fermentas) or Phusion polymerase (New England Biolabs) was used for all cloning, and all constructs were confirmed by sequencing. Sequence analysis was performed with BioEdit 7.0, CLC Genomics Workbench, and CLC Main Workbench. All constructs for *Pseudomonas* expression were expressed under their native promoters, and contained an in-frame HA tag at the C terminus, as described (3).

ZED1 was amplified by PCR using primers to add NheI and BamHI sites to the 5′ and 3′ ends, respectively, and was cloned into a modified pET28a vector (Novagen) with an in-frame N-terminal 6×His tag/TEV cleavage site. ZED1 mutants were cloned by a crossover PCR approach (4). The 5′ portion of the gene was amplified by PCR using a 5′ gene-specific primer, and a 3′ point mutation primer that introduced the mutation and spanned a ~30-nt region around the desired point mutation. The 3′ portion of the gene was amplified by PCR with a 5′ primer that was the reverse complement of the 3′ point mutation primer, and a 3′ gene-specific primer. These two PCR products were then mixed to use as template for the subsequent PCR. The full-length ZED1 cassette with the desired mutation was amplified by using 5′ and 3′ gene-specific ZED1 primers and cloned into the modified pET28a vector described above as for wild-type ZED1. ZED1, At3g57700, or HopZ1a (5) was amplified by PCR and cloned into the Gateway-compatible entry vector pDONR207 by a BP reaction, with a subsequent LR reaction into the pDEST15 N-terminal GST-fusion vector (Invitrogen).

To clone into the pEG202 bait vector (DupLEX-A Yeast Two Hybrid System; OriGene Technologies), ZAR1 was amplified by PCR using primers to add NotI sites to the 5′ and 3′ ends and a C-terminal HA tag to the 3′ end. We constructed five ZAR1 clones, one full-length (ZAR1$^{1\text{-}853}$) and four truncations fused to the DNA binding domain. ZAR1$^{1\text{-}144}$ includes the N-terminal coiled-coil (CC) domain and the EVILVD motif (ZAR1^{CC}). The EVILVD motif resembles the highly conserved acidic sequence EDVID, found in the potato Rx NB-LRR protein, that contributes to intramolecular interactions (6). ZAR1$^{1\text{-}391}$ includes the CC domain, the EVILVD motif, and the nucleotide-binding-site (NB) domain (ZAR1$^{CC\text{-}NB}$). ZAR1$^{145\text{-}391}$ has the NB domain alone (ZAR1^{NB}). ZAR1$^{1\text{-}609}$ includes the CC, EVILVD, NB, and the linker region between the NB and the leucine-rich repeats (LRR), but no LRRs (ZAR1$^{\Delta LRR}$). HopZ1a or HopZ1a$^{C/A}$ was amplified by PCR using primers to add unique BamHI and NotI sites to the 5′ and 3′ ends and a C-terminal HA tag to the 3′ end, and cloned into the pEG202 vector. HopF2 was amplified by PCR and cloned into the Gateway-compatible pDONR207 vector by a BP reaction, with a subsequent LR reaction into the pEG202 vector, and does not contain an HA tag. For the pJG4-5 prey vector that contains an in-frame HA tag (DupLEX-A Yeast Two Hybrid System; OriGene Technologies), HopZ1a or HopZ1a$^{C/A}$ was amplified by PCR using primers to add XhoI sites to the 5′ and 3′ ends. ZED1 was cloned into the pJG4-5 vector after amplification by PCR using primers to add unique EcoRI and XhoI sites to the 5′ and 3′ ends. ShcF2 was amplified by PCR and cloned into the Gateway-compatible pDONR207 vector by a BP reaction, with a subsequent LR reaction into the pJG4-5 vector.

To clone into the bimolecular fluorescence microscopy (BiFC) vectors (pBD-nYFP or pBD-cYFP), ZAR1 or ZED1 were amplified by PCR to contain XhoI restriction sites and were screened for directionality. HopZ1a$^{C/A}$ or MLO$^{\Delta 1-280}$ were amplified by PCR to contain a 5′ XhoI restriction site (and a start codon for MLO$^{\Delta 1-280}$) and a 3′ StuI restriction site. The 3′ primers were designed to maintain the reading frame of the C-terminal fusion. pBD-nYFP and pBD-cYFP were modified from pTA7002 (7) to add an HA tag and the N terminus of YFP (residues 1–155) or the C terminus of YFP (residues 156 to the stop codon) between the StuI and SpeI sites.

For the yeast integration experiments, ZED1 was amplified by PCR using primers to add attB1 and attB2 sites and cloned in-frame into the Gateway-compatible pDONR207 vector by a BP reaction, with a subsequent LR reaction into the BA350V vector. BA350V is a low-copy centromere (CEN)-based plasmid with a LEU2 selectable marker. ZED1 is expressed under the *GAL1* promoter and is fused in-frame with a C-terminal FLAG tag. HopZ1a or HopZ1a$^{C/A}$ was amplified by PCR using primers to add attB1 and attB2 sites and cloned in-frame into the Gateway-compatible pDONR207 vector by a BP reaction, with a subsequent LR reaction into the BA2262 vector. The integration vector BA2262 contains homothallic switching endonuclease (HO) sites for recombination into the HO locus and a nourseothricin (NAT) selectable marker. HopZ1a is expressed under the *GAL1* promoter and is fused in-frame with a C-terminal FLAG tag.

Illumina Sequencing. Illumina sequencing was performed with 37 cycle single reads by following the manufacturer's protocol on an Illumina GAIIx and pipelined by using the GA pipeline v1.4.

***P.syringae* HR, and in Planta Growth Assays.** For infiltrations, *P. syringae* was resuspended to an $OD_{600} = 0.1$ (~5×10^7 cfu/mL) for HR assays, or diluted to 1×10^5 cfu/mL for growth curves. Diluted inocula were hand infiltrated by using a needleless syringe as has been described (8). The HR was scored at 16–20 h. For growth assays, four disks (1 cm^2) were harvested, ground in 10 mM $MgCl_2$, and plated on KB with rifampicin and cycloheximide on day 0 and day 3 for colony counts. *Arabidopsis* plants were grown under 9 h of light (~130 $\mu E\ m^{-2} \cdot s^{-1}$) and 16 h of darkness, at 22 °C, in Sunshine #1 soil.

Protein Purification. To overexpress and purify 6×His:ZED1 or 6×His:ZED1 mutants (~41 kDa), the *E. coli* BL21 codon$^+$ strain was transformed with the appropriate ZED1 construct and cultured in LB at 37 °C with vigorous shaking. When the cell density reached an OD_{600} of 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce expression and the temperature was shifted to 18 °C overnight. Cells were harvested by centrifugation for 10 min at 5,000 × *g*, resuspended in binding buffer (50 mM Tris·HCl at pH 7.5, 500 mM NaCl, and 5 mM imidazole), and lysed by using the Emulsiflex C3 (Avestin) (ZED1) or a French press (ZED1 mutants) followed by sonication. Clarified lysates were passed over High Trap chelating columns (GE Healthcare) preloaded with nickel. Columns were washed with 25 column volumes of washing buffer (50 mM Tris·HCl at pH 7.5, 500 mM NaCl, and 30 mM imidazole), and 6×His:ZED1 or 6×His:ZED1 mutants were eluted with elution buffer (50 mM Tris·HCl at pH 7.5, 500 mM NaCl, and 250 mM imidazole). The 6×His:ZED1 protein was further purified by size-exclusion chromatography by using a Hi-Load 16/60 Superdex 200 prep grade column (Amersham Biosciences) in 20 mM Tris·HCl at pH 7.5 buffer with 150 mM NaCl at a flow rate of 1 mL/min. Aliquots were flash frozen in liquid nitrogen and stored at −80 °C for later use.

To overexpress and purify GST:HopZ1a (~70 kDa), the *E. coli* BL21(DE3) strain was transformed with the pDEST15-HopZ1a construct and cultured in LB at 37 °C with vigorous shaking. When the cell density reached an OD_{600} of 0.6, 1 mM IPTG was added to induce expression and the temperature was shifted to 18 °C overnight. Cells were harvested by centrifugation, resuspended in GST binding buffer [50 mM Hepes·KOH at pH 8.0, 500 mM NaCl, and 1% (vol/vol) Triton X-100], and lysed by using the Emulsiflex C3 (Avestin) followed by sonication. Extracts were clarified by centrifugation at 14,000 × *g* for 30 min. Clarified lysate was passed over glutathione Sepharose 4B resin (GE Healthcare), washed with 10 column volumes of GST binding buffer, 10 column volumes of GST wash buffer 1 [50 mM Hepes·KOH at pH 8.0, 100 mM NaCl, and 1% (vol/vol) Triton X-100] and 50 column volumes of elution buffer (50 mM Hepes·KOH at pH 8.0 and 100 mM NaCl). GST:HopZ1a was eluted with elution buffer plus 10 mM glutathione and concentrated in an Amicon UItra-15 10,000 Da NWML concentrator (Millipore).

To overexpress and purify GST:ZED1 (~64 kDa) or GST:At3g57700 (~65 kDa), the *E. coli* BL21(DE3) strain was transformed with the pDEST15-ZED1 or At3g57700 construct, and cultured in LB at 37 °C with vigorous shaking. Overnight cultures of BL21 (DE3) carrying ZED1 or At3g57700 in pDEST15 were used to inoculate 1 L of phosphate-buffered SOC medium at pH 7.0 and grown to an OD_{600} of 0.4–0.6 at 37 °C in 2.5 L of Ultra Yield Flasks (Thomson Instrument Company). Protein expression was induced with 0.5 mM IPTG at 18 °C for 18 h. Cells were pelleted by centrifugation, resuspended in GST binding buffer plus 1 mM PMSF and 5% (vol/vol) glycerol, and lysed by sonication. Clarified extract was passed over glutathione Sepharose 4B resin, washed with 10 column volumes of GST wash buffer 1 plus 5% (vol/vol) glycerol, and 20 column volumes GST elution buffer plus 5% (vol/vol) glycerol. GST-tagged proteins were eluted with GST elution buffer plus 10 mM glutathione and 5% (vol/vol) glycerol. Elution fractions were pooled and concentrated with Amicon Ultra-4 10,000 Da NWML (Millipore) centrifugal filters. During concentration, purified GST tagged proteins were exchanged into storage buffer [10 mM Hepes at pH 7.5, 100 mM NaCl, and 10% (vol/vol) glycerol].

Yeast Interaction. The MATα strain EGY48 (DupLEX-A Yeast Two Hybrid System; OriGene Technologies) was transformed with pJG4-5-HopZ1a, pJG4-5-HopZ1aC/A, pJG4-5-ZED1, or pJG4-5-ShcF, and transformants were selected on synthetic defined (SD) Glucose-Trp. The MAT A strain RFY206 (DupLEX-A Yeast Two Hybrid System; OriGene Technologies) was transformed with pEG202-ZAR1^{CC}, pEG202-ZAR1$^{CC\text{-}NBS}$, pEG202-ZAR1^{NBS}, pEG202-ZAR1$^{\Delta LRR}$, pEG202-full-length ZAR1, or pEG202-HopF2, and transformants were selected on SD Glucose-HisUra. Standard yeast matings were carried out in yeast extract/peptone/dextrone/adenine sulfate (YPAD) at 30 °C overnight. Diploids were selected twice on SD Glucose-HisUraTrp at 30 °C for 2–3 d. Protein–protein interactions were assayed by plating the diploid strain onto SD Glucose-UraLeuHisTrp and SD Galactose-UraLeuHisTrp. The reporter for protein interaction is LEU2.

Yeast Western Blots. EGY48α carrying pJG4-5 constructs was grown overnight in SD raffinose-Trp. RFY206A-carrying pEG202 constructs was grown overnight in SD glucose-UraHis. To induce protein expression for EGY48α strains, overnight cultures were used to inoculate new cultures at an $OD_{600} = 0.15$ in yeast extract/peptone (YP) galactose and grown for 5–7 h. For RFY206A strains, overnight cultures were used to inoculate new cultures at an $OD_{600} = 0.1$ in YPAD and grown for 5–7 h. Each culture was pelleted, resuspended in 250 μL of cracking buffer (1% β-mercaptoethanol and 0.25 M NaOH), and incubated on ice for 10 min. A solution of 50% TCA was added, and samples were incubated on ice for 10 min. After centrifuging for 10 min at 16,000 × *g* at 4 °C, the precipitated proteins were resuspended in 50 μL of Thorner buffer [8 M urea, 5% (wt/vol) SDS, 40 mM Tris·HCl at pH 6.8, 0.1 mM EDTA, 0.4 mg/mL bromophenol blue, and 1% (vol/vol) β-mercaptoethanol], and the pH was adjusted dropwise with 2 M unbuffered Tris until the samples were blue. Samples were resolved on 10% SDS/PAGE gels and transferred to nitrocellulose membrane for Western blot analysis. HA-tagged proteins were detected by using α-HA antibody (Roche).

***Agrobacterium* Transient Expression Assays, BiFC, and Western Blots.** *A. tumefaciens* GV2260 cultures were grown overnight at 28 °C in LB broth with kanamycin and rifampicin. The next day, the cultures

were washed twice in induction medium [50 mM Mes at pH 5.6, 0.5% (wt/vol) glucose, 1.7 mM NaH_2PO_4, 20 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$, and 200 μM acetosyringone] (9), and 2.6 mL was inoculated into 30 mL of fresh induction medium to grow overnight. The following day, cultures were spun down, washed twice in 10 mM Mes at pH 5.6 with 200 μM acetosyringone, and resuspended to an optical density of 0.3 at 600 nm. The culture containing each nYFP plasmid was mixed in equal volumes with a culture containing each cYFP plasmid. The underside of the leaves of 5- to 7-wk-old *Nicotiana benthamiana* plants were infiltrated by hand with a needleless syringe. Plants were sprayed with 20 μM dexamethasone (Sigma) 6–24 h after inoculation. Sections of leaves were imaged with a Leica SP3 microscope by using Leica software at 24–48 h after dexamethasone induction.

Surface Plasmon Resonance Technology. Binding and kinetic analyses between His-ZED1 or GST, and His-HopZ1a or GST-HopZ1a, were performed on a Biacore 3000 surface plasmon resonance (SPR) instrument (GE Healthcare). The CM5 sensor chip was primed three times with HPS-EP running buffer (10 mM Hepes at pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Surfactant P20) and preconditioned with three consecutive pulses of 10 μL each of 50 mM HCl, 50 mM NaOH, and 0.5% SDS, and water at a flow rate of 100 μL/min. The carboxymethyl dextran surface of the sensor chip was activated with *N*-ethyl-*N′*-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide. His-HopZ1a or GST-HopZ1a (30 μg/mL) in 10 mM Hepes pH 7.0 buffer was injected over the activated surface to ∼8,000 resonance units. Excess activated groups were blocked with ethanolamine. To subtract for bulk refractive index changes, blank surfaces were prepared.

Binding experiments were conducted at 25 °C in HPS-EP running buffer. Stock solutions of His-ZED1 or GST alone were diluted to 250 nM and injected over immobilized His-HopZ1a and GST-HopZ1a. For kinetic analyses, His-ZED1 was prepared by serial dilution from the stock solution to working concentrations (250 nM, 125 nM, 62.5 nM, 31.3 nM, 15.6 nM, 7.8 nM, and 3.9 nM) and injected over the surface of the sensor chip. After each binding and dissociation cycle, the sensor chip was regenerated with 15.5 mM NaOH. Several buffer blanks were also injected over the sensor chip surface for double referencing. Reference surface responses and blank responses were subtracted from the sensorgrams of His-ZED1 or GST alone during data processing. Control experiments were also done to test for mass transfer limitation. Binding and kinetic data were evaluated and fitted by using BIAevaluation version 4 (GE Healthcare). The kinetic data were fitted to a 1:1 model with mass transfer.

In Vitro Acetylation Assays. Purified His-ZED1 or His-ZED1 mutants, and/or GST-HopZ1a or GST (SignalChem), were incubated in 20 μL of acetylation buffer [50 mM Hepes·KOH at pH 8.0, 10% (vol/vol) glycerol, and 5 mM DTT], supplemented with 40 nCi of [^{14}C] acetyl-CoA (40–60 mCi/mmol; PerkinElmer) for 1 h at 30 °C. The reactions were stopped by adding 10× Laemmli buffer and separated on a 9% polyacrylamide gel. Gels were fixed in 50% (vol/vol) methanol and 10% (vol/vol) acetic acid for 30 min, followed by a 15-min incubation in Amplify (GE Heathcare). Gels were dried and placed in a phosphoimager cassette for 1 wk at −20 °C. The SDS/PAGE gel was run in duplicate and stained with Coomassie blue to visualize proteins.

In Yeast Acetylation Assay and Immunoprecipitation. The MATα strain Y7092 was transformed with 2–3 μg of NotI-digested *BA2261-hopZ1a* or *BA2262-hopZ1aC/A* integration constructs by following the standard PEG-lithium acetate protocol. Transformed yeast cells were plated on YPAD with 100 μg/mL nourseothricin (Werner Bioagents) and incubated at 30 °C for at least 3 d to select for yeast strains with integrated P_{GAL1}-*hopZ1a-FLAG::NAT^R* or P_{GAL1}-*hopZ1a$^{C/A}$-FLAG::NAT^R* at the *HO* locus. These strains were subsequently transformed with 1 μg of the centromeric plasmid *BA350V-zed1* (P_{GAL1}-*zed1-FLAG*), and the transformants were selected on SD glucose-Leu to create HopZ1a or HopZ1a$^{C/A}$ and ZED1 coexpression strains.

To generate the HopZ1a and ZED1 coexpression yeast strain, we first integrated the FLAG-tagged HopZ1a or HopZ1a$^{C/A}$ construct into the Y7092 yeast strain, and then transformed the strain with the FLAG-tagged ZED1 carried on a low-copy plasmid. FLAG-ZED1 was purified by using FLAG antibodies conjugated to agarose beads. We digested the immunoprecipitated proteins with trypsin and obtained 42% coverage of the ZED1 protein from liquid chromatography tandem mass spectrometry (LC-MS/MS). Yeast strains coexpressing the FLAG-tagged HopZ1a or HopZ1a$^{C/A}$, and ZED1 under the *GAL1* promoter, were grown overnight at 30 °C in 5 mL of SD raffinose-Leu. Overnight cultures were subsequently back diluted 1:50 into fresh SD raffinose-Leu the next morning and grown until midlog phase. Coexpression of HopZ1a or HopZ1a$^{C/A}$ and ZED1 was induced with 2% (wt/vol) galactose for 12 h. Induced cultures were pelleted and washed with ice-cold sterile ddH_2O. Whole-cell extracts were prepared by resuspending pelleted cells in 1 mL of cold IP buffer (50 mM Tris·HCl at pH 8.0, 150 mM NaCl, 1.5 mM MgOAc, 0.15% Nonidet P-40, 5 mM EDTA and complete protease inhibitor), added to equal volume of 0.5-mm beads, and lysed with bead beater. Cell debris was pelleted at 16,000 × *g* for 20 min at 4 °C. Supernatants containing FLAG-tagged HopZ1a or HopZ1a$^{C/A}$ and ZED1 proteins were incubated with anti-FLAG resin conjugated to agarose beads (Sigma) at 4 °C overnight and washed three times with 750 μL of cold IP buffer at 4 °C with rotation. Proteins were eluted with 200 μL of 150 ng/mL of FLAG peptide for 30 min at 4 °C with rotation.

Mass Spectrometry. LC-MS/MS analyses were carried out on immunoprecipitated FLAG-tagged ZED1 in the presence of HopZ1a or HopZ1a$^{C/A}$. Lyophilized protein samples were solubilized in 50 mM ammonium bicarbonate at pH 7.8, then subjected to reduction with DTT, alkylation with iodoacetamide, and overnight trypsin digestion at 37 °C. Tryptic-digested samples were treated with 1–3% (vol/vol) formic acid, lyophilized, zip tipped, and subjected to LC-MS/MS analyses. All measurements were performed on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray source coupled to a nanoLC system (Eksigent, AB SCIEX). Zip-tipped peptide digests (20 μL) were sampled with an autosampler (Eksigent, AB SCIEX) injected onto a trap column (Agilent; ZORBAX 300SB-C18, 5 μm, 5 × 0.3 mm), followed by an analytical column (Agilent; ZORBAX 300SB-C18, 3.5 μm, 150 × 0.75 mm) where the peptides were resolved under gradient elution for 90 min at a flow rate of 400 nL/min. The MS was run in data-dependent acquisition, with the top five multiply charged peptide ions selected for MS/MS analysis. Raw data files were converted to mzXML files and searched against a target database with the following parameters: (*i*) mass tolerance in MS mode: 20 ppm; (*ii*) mass tolerance in MS/MS mode: 0.5 Da; (*iii*) fixed modification: carbamidomethylation; (*iv*) variable modifications: oxidation and acetylation; (*v*) missed cleavages: 2. The output from the searches was validated by using Scaffold 3.6 (Proteome Software).

Kinase Assays. Purified GST-ZED1 or GST-At3g57700 protein (3–5 μg) were incubated in 15 μL of reaction buffer (20 mM Hepes·KOH at pH 7.5, 10 mM $MgCl_2$, and 10 mM $MnCl_2$) containing 2 μCi of [γ-^{32}P] ATP (6000 Ci/mmol; PerkinElmer). Reactions were incubated at room temperature for 60 min. Purified His-ZED1 protein was incubated in 20 μL of kinase buffer (50 mM Tris·HCl at pH 8.0, 10 mM $MnCl_2$ and/or 10 mM $MgCl_2$, and 5 μM ATP) supplemented with 1 μCi [γ-^{32}P]ATP

(6,000 Ci/mmol, PerkinElmer) for 30 min at room temperature. The reactions were stopped by adding 10× Laemmli buffer and separated on a 12% polyacrylamide gel. Incorporated radiolabel was visualized by autoradiography. Five micrograms of swine myelin basic protein (SignalChem) or calf thymus histone H1 (SignalChem) were used as generic substrates. The SDS/PAGE gel was run in duplicate and stained with Coomassie blue to visualize proteins.

Phylogenetic Analysis. Phylogenetic trees were generated on data produced by using NCBI BLASTP of ZED1 against *Arabidopsis thaliana* RefSeq dataset. All hits with an *e* value < 1*e*−30 and query coverage of at least 60% of the protein were recovered, resulting in 189 sequences. Full-length sequences were downloaded from National Center for Biotechnology Information (NCBI). Multiple sequence alignments were performed by using Muscle as implemented in MEGA 5 (10, 11) with 24 iterations and an unweighted pair group method with arithmetic means (UPGMB) clustering method (10, 12). Phylogenetic trees were reconstructed via neighbor-joining in MEGA 5, maximum likelihood in MEGA 5, and Bayesian methods by using Mr. Bayes v3.2.1. Only neighbor joining trees are shown, because all methods produced similar results. Neighbor-joining trees were constructed by using a Jones Taylor Thornton (JTT) substitution matrix and pairwise deletion for gap data. Bootstrapping (500 pseudoreplicates) was performed to assess phylogenetic confidence, and the values >50 are presented next to each node.

Protein Analysis. ZED1 was aligned to the reference sequence PKA-Cα by using CLC Genomics Workbench and annotated for kinase domains, loops, and key residues (13, 14). Protein sequences were analyzed by using ScanProsite (15) and Phyre 2.0 (16). PyMOL 1.1 (www.pymol.org) was used to view and manipulate the predicted 3D model.

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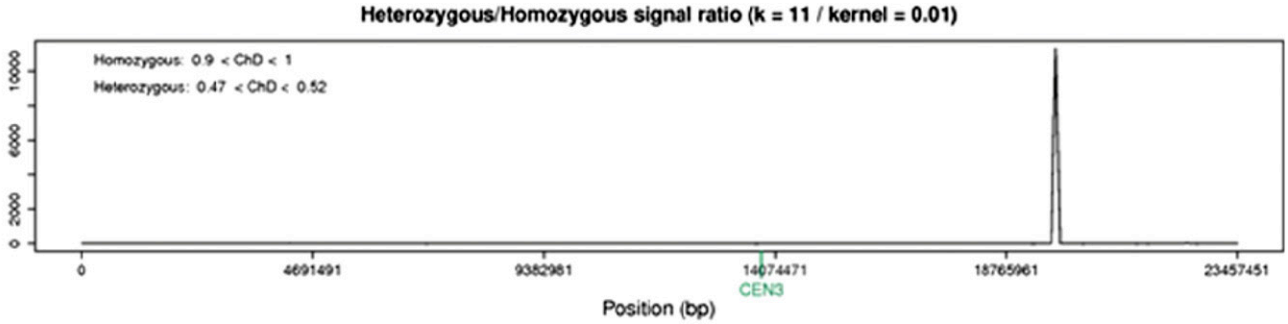
A



B

SNP annotations:

Chrom.	Position	Ref. base	SNP base	Depth	Discordant chastity	Accession	Tag	Strand	Ref. codon	SNP codon	AA change	BLOSUM 100
3	20795011	A	T	29	1.00	AT3G56040.1	CDS	-	GAT	GAA	D->E	2
3	20795012	T	C	29	1.00	AT3G56040.1	CDS	-	GAT	GGT	D->G	-4
3	21394263	C	T	30	1.00	AT3G57750.1	CDS	+	CAA	TAA	Q->*	-10
3	22100170	C	T	21	0.90	AT3G59820.1	CDS	-	AGA	AAA	R->K	3
3	23379267	C	T	33	0.91	AT3G63280.1	CDS	+	CAT	TAT	H->Y	1

C

Locus	T-DNA insertion line	Plants showing a HopZ1a-induced HR
At3g56040	not tested	not tested
At3g57750	Salk_018065[a]	16/22
At3g59820	GT_3_105710[a]	12/12
At3g63280	Salk_067558C[b]	3/3

[a]mixed genotype population, [b]confirmed homozygous

Fig. S1. NGM of *zed1*. (*A*) The peak indicates the region of the chromosome that is highly associated with single-nucleotide polymorphisms (SNPs) differing from the Col-0 or L*er* reference sequences. CEN3 is the position of the centromere on chromosome 3. (*B*) The peak in *A* consists of four genes, three of which have C/*T* transitions that are commonly associated with EMS mutagenesis. Depth indicates the number of reads with the base change. A discordant chastity statistic of 1.0 indicates that the observed SNP base is homozygous and always different from the reference, whereas a value of 0.0 indicates that the base is the same as the reference. (*C*) tDNA insertion lines in the genes identified in *B* were tested for the elicitation of the HopZ1a-induced HR.

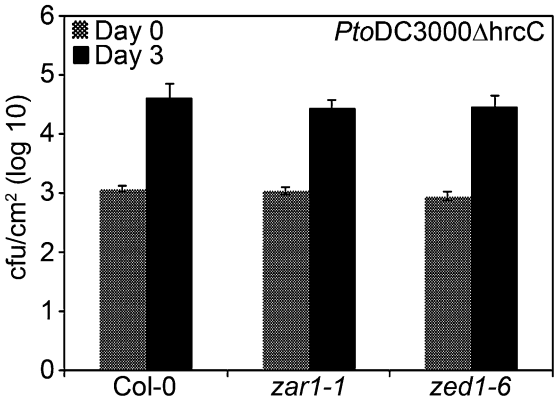


Fig. S2. *zed1* is not impaired in PAMP-triggered immunity. Col-0, *zar1-1*, or *zed1-6* were infiltrated with *Pto*DC3000ΔhrcC at 1×10^5 cfu/mL, and bacterial counts were determined one hour after infection (Day 0) and 3 d after infection (Day 3). Two-tailed homoschedastic *t* tests were performed to test for significant differences and none were found. Error bars indicate the SD from the mean of 10 samples. Growth assays were performed at least three times.

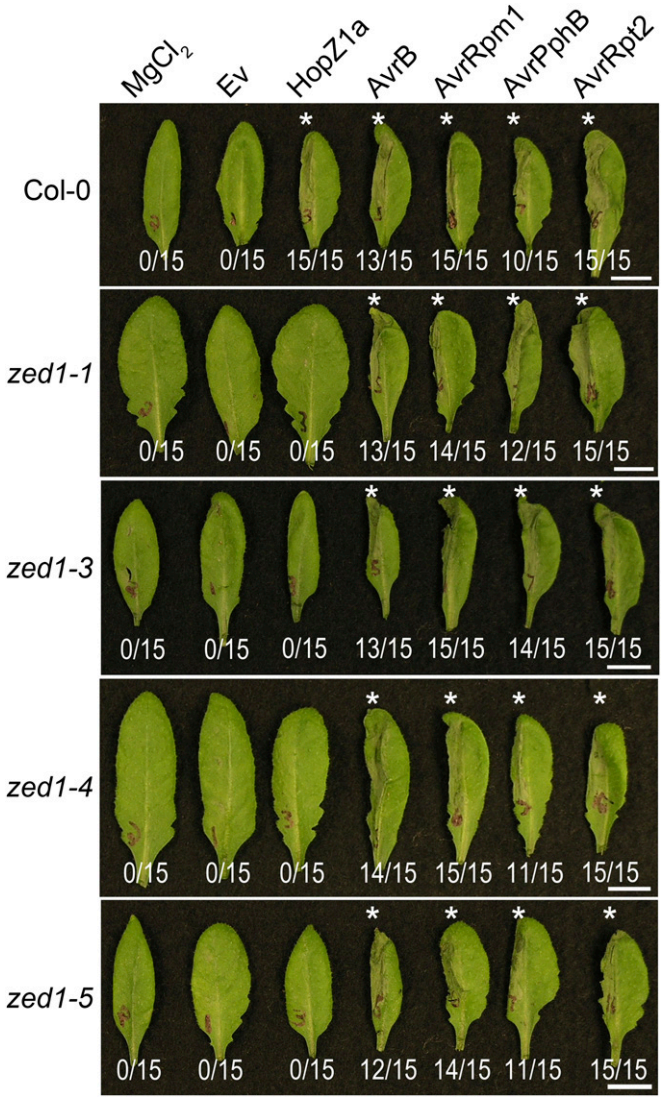


Fig. S3. Multiple alleles of *zed1* specifically lack recognition of HopZ1a. Half-leaves of Col-0, or additional *zed1* point mutants, were infiltrated with 10 mM $MgCl_2$ or *Pto*DC3000 carrying empty vector (Ev), HopZ1a, AvrB, AvrRpm1, AvrPphB, or AvrRpt2. The bacteria were pressure-infiltrated into the leaves at 5×10^7 cfu/mL. Photos were taken at 22 h after infiltration. The number of leaves showing an HR is indicated below the leaf. HRs are marked with an asterisk.

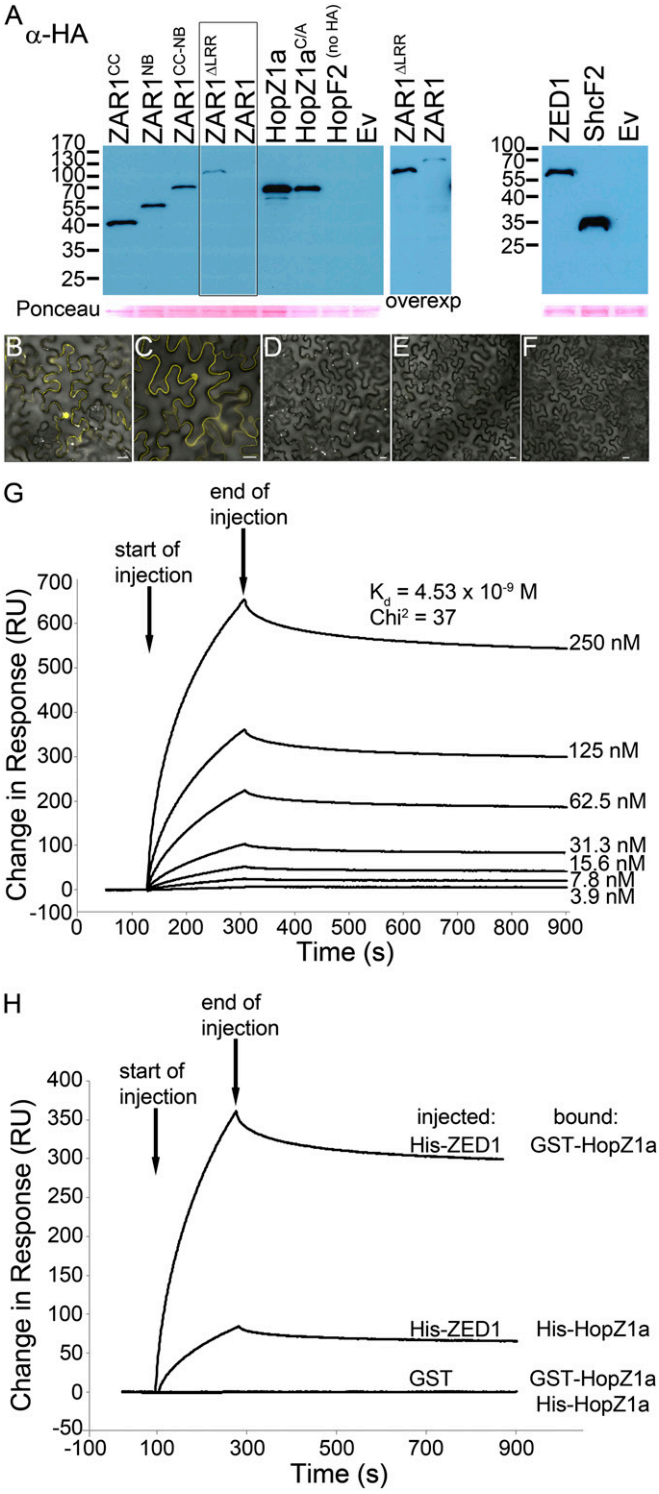


Fig. S4. ZED1 interacts with HopZ1a and ZAR1. (*A*) Immunoblot analysis of empty vector (Ev), or BD-fused and HA-tagged ZAR1, HopZ1a, or HopZ1a$^{C/A}$ in the yeast RFY206A strain (*Left*), and Ev, or activation-domain (AD)/HA-fused ZED1 or ShcF2 in the yeast EGY48α strain (*Right*). Equal amounts of proteins were resolved on 10% SDS/PAGE gels, blotted onto nitrocellulose, and probed with HA antibodies. *Center*, which is the same area as the boxed region, shows a 10x overexposure of the ZAR1$^{\Delta LRR}$ and ZAR1 lanes. HopF2 does not contain an HA tag. The Ponceau red stained blot was used as a loading control. The molecular masses of the BD-fused proteins are as follows: ZAR1^{CC}, 41.3 kDa; ZAR1^{NB}, 52.9 kDa; ZAR1$^{CC\text{-}NB}$, 69.7 kDa; ZAR1$^{\Delta LRR}$, 94 kDa; ZAR1, 121.5 kDa; HopZ1a or HopZ1a$^{C/A}$, 66.6 kDa. The molecular masses of the AD-fused proteins are as follows: ZED1, 50.2 kDa; ShcF2, 24.7 kDa. (*B–F*) BiFC of HopZ1a$^{C/A}$, ZED1, and ZAR1. *Agrobacterium* carrying HopZ1a$^{C/A}$::cYFP, ZAR1::cYFP, ZED1::nYFP, and MLO2$^{\Delta 1-280}$::nYFP or cYFP were mixed in equivalent optical densities and pressure-infiltrated into the leaves of *N. benthamiana*. HopZ1a$^{C/A}$ was used because HopZ1a causes an HR in *N. benthamiana*. Protein expression was induced by using 30 μM dexamethasone. Leaf sections were imaged by using a Leica SP3 confocal scanning microscope 24–48 h after induction. (Scale bar: 20 μm.) Coexpressed pairs are as follows. (*B*) ZED1::nYFP and ZAR1::cYFP. (*C*) ZED1::nYFP and HopZ1a$^{C/A}$-cYFP. (*D*) ZED1::nYFP and MLO2$^{\Delta 1-280}$::cYFP. (*E*) MLO2$^{\Delta 1-280}$::nYFP and ZAR1::cYFP. (*F*) MLO2$^{\Delta 1-280}$::nYFP and HopZ1a$^{C/A}$::cYFP. (*G*) Dose–response curves of His-ZED1 binding to GST-HopZ1a. GST-HopZ1a was immobilized to the

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surface of a Biacore CM5 sensor chip, and His-ZED1 was flowed over the bound surface at increasing protein concentration. ZED1 displays very high binding affinity (3–5 nM) to GST-HopZ1a. The start and end of His-ZED1 injection is indicated by arrows. (*H*) HopZ1a shows specific binding to ZED1. His-ZED1 or GST was flowed over immobilized GST-HopZ1a or His-HopZ1a. The injected protein ("injected") is shown above the corresponding line. The immobilized protein ("bound") is shown on the far right.

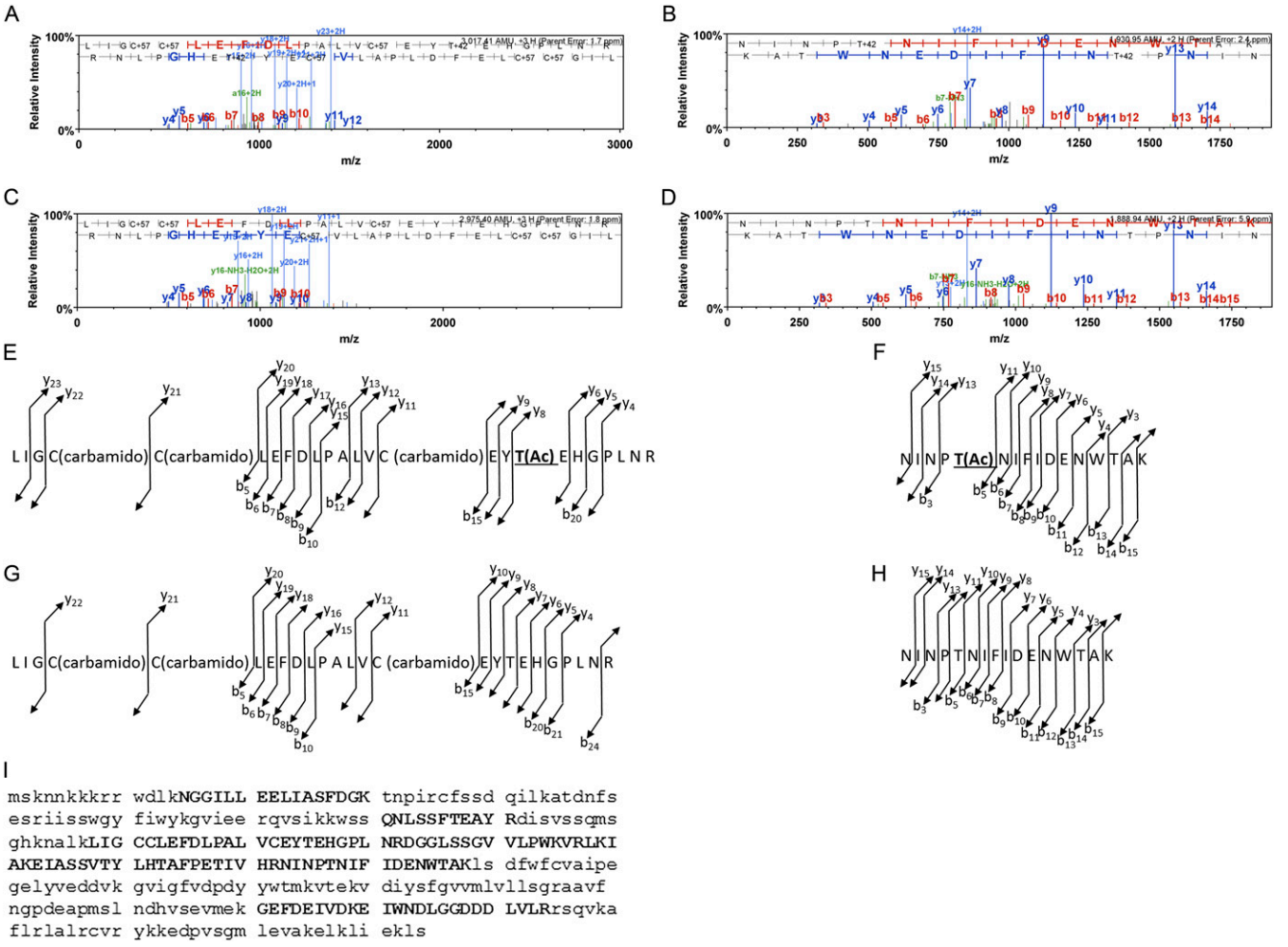

Fig. S5. HopZ1a acetylates ZED1 at threonines 125 and 177. Flag-tagged ZED1 was acetylated in yeast carrying HopZ1a (*A*, *B*, *E*, and *F*) or HopZ1a$^{C/A}$ (C, *D*, and *G*–*I*) and immunoprecipitated. ZED1 was digested with trypsin and analyzed by LC/MS. (*A* and *E*) MS/MS spectrum for acetylated ZED1 peptide A L^{108}IGCCLEFDLPALVCEYTEHGPLNR. The peptide measured 3,017.41 Da in the presence of HopZ1a. (*B* and *F*) MS/MS spectrum for acetylated ZED1 peptide B N^{173}INPTNIFIDENWTAK. The peptide mass was 1,930.95 Da in the presence of HopZ1a. (*C* and *G*) MS/MS spectrum lacks acetylation in peptide A L^{108}IGCCLEFDLPALVCEYTEHGPLNR. The peptide mass was 2,975.40 Da in the presence of HopZ1a$^{C/A}$. (*D* and *H*) MS/MS spectrum lacks acetylation in peptide B N^{173}INPTNIFIDENWTAK. The peptide measured 1,888.94 Da in the presence of HopZ1a$^{C/A}$. (*I*) ZED1 peptides recovered in LC-MS/MS analysis when coexpressed with HopZ1a$^{C/A}$ are shown in capital letters. No acetylated threonines were recovered.

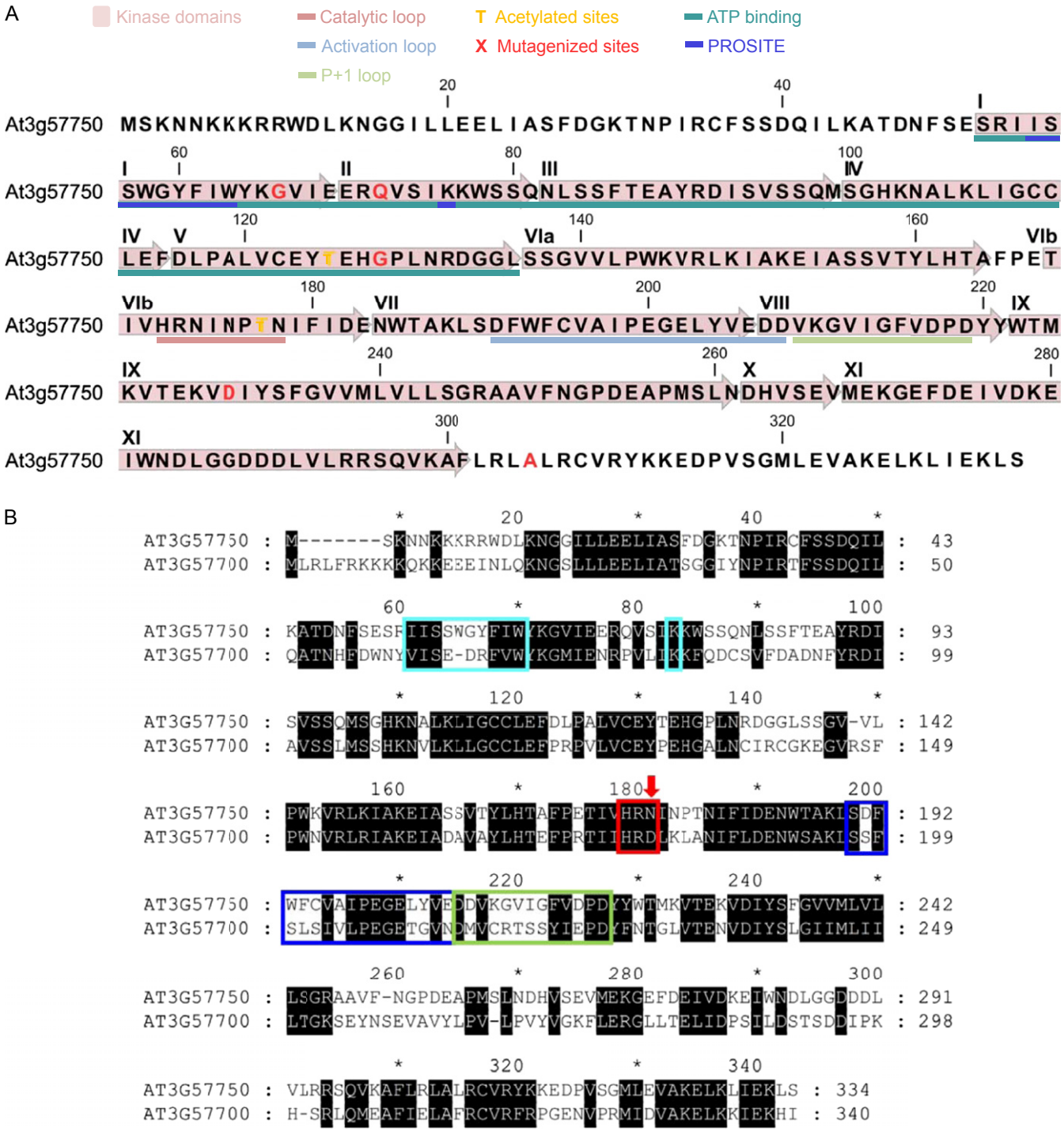

Fig. S6. Predicted functional domains of ZED1 pseudokinase. (*A*) The ZED1 protein sequence is annotated for kinase domains (pink box; ref. 1) from an alignment of ZED1 to the PKA-Cα reference kinase. Other functionally important kinase regions are underlined in various colors including the Prosite predicted ATP-binding residues. HopZ1a acetylation sites are in yellow, and mutations identified in the forward genetic screen are in red. (*B*) Pairwise global alignment of ZED1 (At3g57750) and ZRK10 (At3g57700). Boxed regions show the following: active sites predicted to be involved in ATP binding (light blue); HRD proton acceptor motif (red)—note that the proton accepting aspartate is missing in ZED1 (red arrow); activation loop (blue); and P+1 loop (light green). Black shaded regions indicate identical or similar residues.

1. Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J* 9(8):576–596.

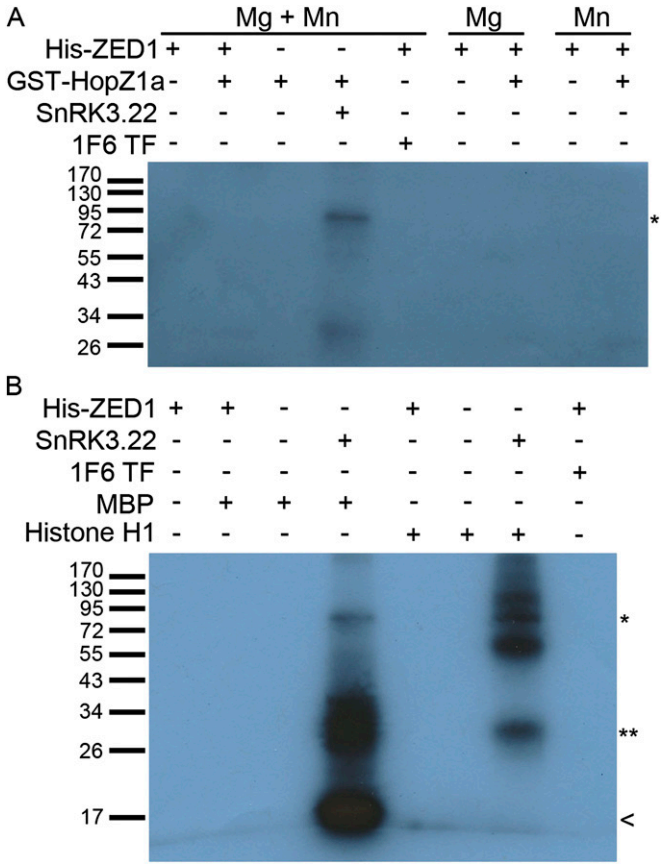

Fig. S7. ZED1 does not exhibit transphosphorylation activity. (*A*) Purified 6×His-ZED1 or SnRK3.22 was incubated with purified GST-HopZ1a, or 1F6 transcription factor (At1g52880) in the presence of [γ-^{32}P]ATP. Samples were separated on a 12% polyacrylamide gel and visualized by autoradiography. *, SnRK3.22. (*B*) Purified 6×His-ZED1 or SnRK3.22 was incubated with myelin basic protein (MBP), histone H1, or 1F6 transcription factor in the presence of [γ-^{32}P]ATP. Samples were separated on a 12% polyacrylamide gel and visualized by autoradiography. *, SnRK3.22; <, MBP (21.5 kDa); **, histone H1 (31–33 kDa).

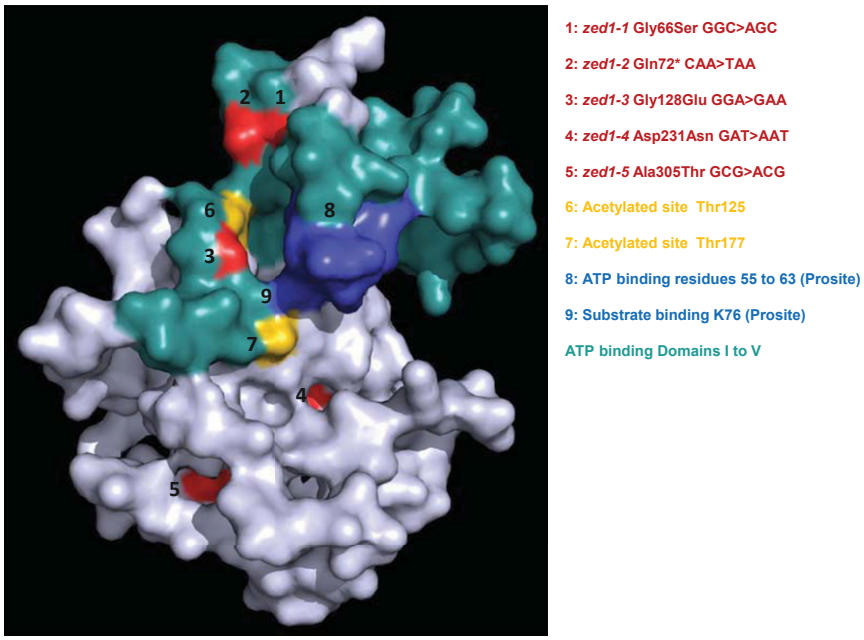

Fig. S8. Predicted 3D structure of ZED1 showing mutagenized sites, acetylated sites, and predicted functional sites. Structure was modeled by using Phyre 2.0 to identify the best structural match to ZED1 (Protein Data Bank ID code 2FO0) and visualized by using PyMOL 1.1. Residues 1–5 in red show EMS mutants identified from the genetic screen, with the wild-type and mutated codons, and corresponding amino acids. The acetylated sites in yellow were identified by mass spectrometry and by in vitro acetylation assays. Prosite-predicted ATP binding residues and substrate-binding residues are shown in bright blue. ATP binding domains in turquoise were annotated after aligning ZED1 to the reference kinase PKA-Cα (1, 2).

1. Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J* 9(8):576–596.
2. Scheeff ED, Eswaran J, Bunkoczi G, Knapp S, Manning G (2009) Structure of the pseudokinase VRK3 reveals a degraded catalytic site, a highly conserved kinase fold, and a putative regulatory binding site. *Structure* 17(1):128–138.

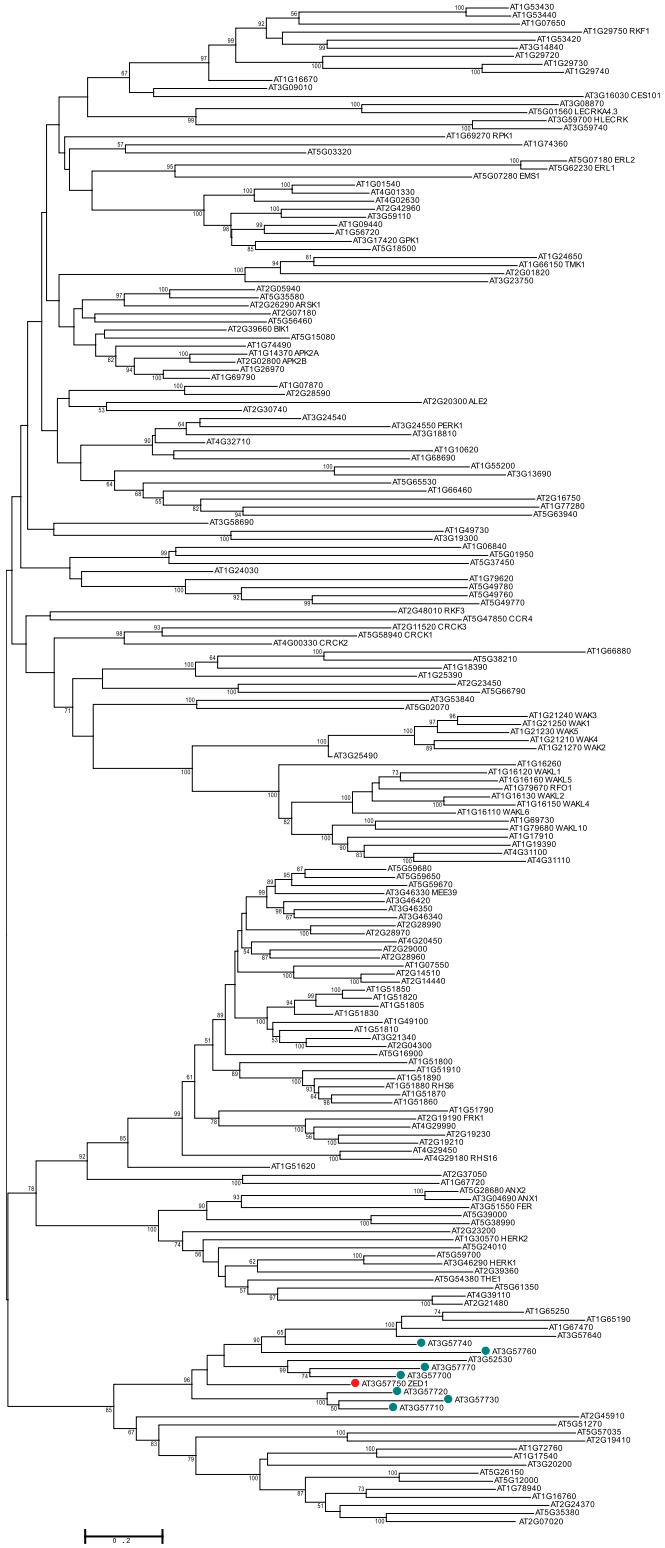

Fig. S9. Evolutionary relationships of ZED1-like kinases. The tree was generated by NCBI BLASTP of ZED1 against *A. thaliana* ecotype Col-0 and recovering all loci with at least 25% amino acid identity across at least 60% of the protein (corresponding to an *e* value of less than 10^{-23}), resulting in 199 sequences. The sequences were aligned by using Muscle as implemented in MEGA5 with 16 maximum iterations and a UPGMB clustering method (1, 2). The phylogenetic tree was constructed by using neighbor joining as implemented in MEGA5 (1, 3) using a JTT substitution matrix, a gamma distribution to correct for rate variation among sites (alpha = 1.5), and pairwise deletion for gap data. Bootstrapping (500 pseudoreplicates) was performed to assess phylogenetic confidence, and the values are presented next to each node.

1. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10):2731–2739.
2. Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32(5):1792–1797.
3. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4):406–425.