Supporting Information

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SI Experimental Procedures

Plant Growth Condition and Generation of Transgenic Plants. *Arabidopsis* plants were grown in soil (Metro Mix 366, Sun Gro Horticulture) in a growth room at 23 °C, 65% relative humidity and 75 $\mu E \cdot m^{-2} \cdot s^{-1}$ light with a 12-h photoperiod for approximately 4 wk before protoplast isolation or bacterial inoculation. To grow *Arabidopsis* seedlings, the seeds were surface sterilized with 50% (vol/vol) bleach for 10 min, and then placed on the plates with half-strength Murashige and Skoog medium containing 0.5% sucrose, 0.8% agar and 2.5 mM 2-(N-morpholino)ethanesulfonic acid at pH 5.7. The *bik1* mutant plants, WT Col-0 plants, *pCB302* empty vector (EV) transgenic plants (in Col-0 background), and *pBIK1::BIK1-HA* transgenic plants in *bik1* mutant background were reported previously (1). The *pBAK1::BAK1-GFP* transgenic plants in Col-0 background were obtained from J. Li (Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI). The *Agrobacterium*-mediated transformation was used to introduce *pCB302-pBIK1::BIK1-HA* into *pBAK1::BAK1-GFP* transgenic plants, and the double transgenic plants were selected with Basta resistance and immunoblot using α-HA (Roche) and α-GFP (Roche) antibodies. To generate *pBIK1::BIK1^{Y150F}-HA*, *pBIK1::BIK1^{Y243F}-HA*, or *pBIK1::BIK1^{Y250F}-HA* transgenic plants in the *bik1* mutant background, we introduced individual point mutations into *pCB302-pBIK1::BIK1-HA* by site-directed mutagenesis kit. The *BIK1* coding region for each derivative was fully sequenced to confirm the proper mutation. The transformants were selected with Basta resistance, α-HA immunoblot, the lines with comparable protein expression level with *pBIK1::BIK1-HA* transgenic plants were used to produce homozygous plants for further analysis. The *BIK1km* transgenic plants were generated with *35S::BIK1-HA* construct. At least two homozygous lines for each transgenic line were analyzed.

Bimolecular Fluorescence Complementation Assay and Reporter Assay. For bimolecular fluorescence complementation (BiFC) assay, BAK1 and BIK1 were subcloned into the modified BiFC vectors from pHBT-BAK1-HA or pHBT-BIK1-HA vector with BamHI and StuI digestion. For protoplasts transfection, 200 μL protoplasts were transfected with various BiFC constructs as indicated in Fig. 1. Fluorescent signals in the protoplasts were examined with a confocal microscope (Olympus FV1000 Confocal Microscope) 12 h after transfection. The filter sets used for excitation and emission are as follows: yellow fluorescence protein was excited at 515 nm, and the emission was collected between 520 and 550 nm; chlorophyll was excited at 488 nm, and the emission was collected between 560 and 650 nm; and bright field was at 633 nm. Images were captured in multichannel mode, and analyzed and processed with OLYMPUS FLUOVIEW Version 3.0 Viewer. For the protoplast transfection procedure, 100 μL of protoplasts at a density of 2×10^5 per mL were transfected with *pBIK1::BIK1-HA* or its mutants for flg22-induced BIK1 phosphorylation assay, and 50 μL of protoplasts were transfected with *pBIK1::BIK1-HA* or its mutants and *pFRK1::LUC/UBQ-GUS* for *FRK1* promoter activity assay. For in vivo tyrosine phosphorylation assay, 500 μL of protoplasts were transfected with *pBIK1::BIK1-GFP*, and treated with 1 μM flg22 for 10 min.

In Vitro Pull-Down Assay. For GST glutathione agarose pull-down assay, GST and GST–*Botrytis*-induced kinase 1 (BIK1) were individually expressed in the *Escherichia coli* BL21 strain and purified as the form of fusion proteins immobilized with glutathione agarose following standard protocols. Five micrograms of maltose-binding protein (MBP) or MBP-brassinosteroid insensitive 1-associated kinase 1 (BAK1)CD (tagged with HA) fusion proteins were preincubated with 5 μL prewashed glutathione agarose beads in 150 μL incubation buffer (10 mM Hepes at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% Triton X-100) at 4 °C for 1 h with gentle shaking. After centrifuging at 12,000 × *g* for 5 min, the supernatant was transferred and incubated with prewashed GST or GST-BIK1 glutathione agarose beads at 4 °C for another 1 h. The beads were collected and washed 4× with immunoprecipitation (IP) washing buffer and once with 50 mM Tris·HCl at pH 7.5. The immunoprecipitated proteins were analyzed by Western blot with an α-HA antibody.

For MBP amylose agarose pull-down assay, MBP and MBP-BAK1CD amylose agarose beads were used as bait against GST or GST-BIK1 fusion proteins for IP assay following a similar protocol as above. The immunoprecipitated proteins were analyzed by Western blot with an α-GST antibody.

In Vitro Phosphorylation Assay. Expression and affinity purification of the GST and MBP fusion proteins were performed as the standard protocol. The protein concentration was determined with the BioRad Quick Start Bradford Dye Reagent and confirmed by the NanoDrop ND-1000 Spectrophotometer. For in vitro kinase assay, kinase reactions were performed in 30 μL kinase buffer (20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM EGTA, 100 mM NaCl, and 1 mM DTT) containing 10 μg fusion proteins with 0.1 mM cold ATP and 5 μCi [^{32}P]-γ-ATP at room temperature for 3 h with gentle shaking. The reactions were stopped by adding 4× SDS loading buffer. The phosphorylation of fusion proteins was analyzed by autoradiography after separation with 10% SDS/PAGE.

Seedling Coimmunoprecipitation Assay. Approximately 5 g 10-d-old transgenic seedlings grown from half-strength Murashige and Skoog medium agar plates were ground in liquid N_2, and further lysis with 10 mL ice-cold extraction buffer. After vortexing vigorously for 30 s, the samples were centrifuged at 12,470 × *g* for 10 min at 4 °C. The 30-μL supernatant was used for Western input control (α-HA and α-GFP) assay, and the remainder was incubated with 10-μL α-GFP antibody for 2 h and then incubated with 20 μL protein-G-agarose for another 2 h at 4 °C with gentle shaking. The beads were collected and washed 3× with IP washing buffer and 1× with 50 mM Tris·HCl, pH 7.5. The immunoprecipitated proteins were analyzed by Western blot with an α-HA or α-GFP antibody.

MS Analysis. The reaction was performed for 3 hr at room temperature with gentle shaking and stopped by adding 4× SDS loading buffer. Six individual reactions were combined and separated by 10% (wt/vol) SDS-PAGE gel. The gel was stained with Thermo GelCode Blue Safe Protein Stain and washed with double distilled water (ddH_2O). The corresponding bands were sliced for MS analysis, which was performed according to Gao et al. (2). Briefly, proteins were separated by 10% SDS-PAGE gel. The gel was stained with Thermo GelCode Blue Safe Protein Stain and washed with dH_2O. The corresponding bands were sliced and gel bands were in-gel digested with trypsin overnight, and phosphopeptides were enriched for liquid chromatography-MS/MS analysis with a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). The MS/MS spectra were analyzed with Mascot (Matrix Science; version 2.2.2), and the identified phosphorylated peptides were manually inspected to ensure

confidence in phosphorylation site assignment. To detect Y243 as a phosphorylation site, T242A mutant was used for MS assay since T242 was heavily phosphorylated.

Pathogen Infection Assays. *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 and *P. syringae maculicola* (*Psm*) ES4326 strains were grown overnight at 28 °C in King's B medium with 50 μg/mL rifampicin or streptomycin respectively. Bacteria were collected, washed, and diluted to the desired density with ddH_2O. For flg22-mediated protection assay, leaves from 4-wk-old soil-grown plants were preinoculated with 100 nM flg22 or H_2O and 24 h later, the same leaves were infiltrated with *Pst* at the concentration of 5×10^5 cfu/mL using a needleless syringe. For the *Psm* infection assay, the leaves were directly infiltrated with *Psm* at the concentration of 5×10^5 cfu/mL. Bacterial counting was performed from six leaves of different plants as three repeats at 2- and 3-d postinoculation (dpi). Two leaf discs were ground in 100 μL ddH_2O and serial dilutions were plated on Tryptic Soy Agar medium with appropriate antibiotic. Bacterial cfu were counted 2 d after incubation at 28 °C. Each data point is shown as triplicates. The disease symptom was recorded from the representative infected leaves at the indicated time points.

For the *Botrytis cinerea* infection assay, *B. cinerea* strain BO5-10 was cultured on potato dextrose agar (PDA) (Difco) and incubated at room temperature. Conidia were collected and resuspended in 1/4 PDA with 0.5% Knox Gelatin. The suspension was passed through Mirocloth. The conidia density was adjusted to 2.5×10^5 spores per mL, and 10 μL spore suspension was dropped on 5-wk-old soil-grown detached leaves. The infected leaves were covered with a dome and at least 30 leaves for each line were assayed.

Reactive Oxygen Species Production Assay. Four to five leaves from each 5-wk-old plant were excised into leaf discs of 0.25 cm^2, following an overnight incubation in 96-well plate with 100 μL ddH_2O to eliminate the wounding effect. H_2O was replaced by 100 μL reaction solution containing 50 μM luminol and 10 μg/mL horseradish peroxidase (Sigma) supplemented with 100 nM flg22. The measurement was conducted immediately after adding the solution with a luminometer (Perkin-Elmer; 2030 Multilabel Reader, Victor ×3), with a 1-min interval reading time for a period of 30 min. The measured value for reactive oxygen species (ROS) production from 36 leaf discs per treatment was indicated as means of relative light units.

Point Mutation Primers Used in This Study (Mutated Sites Are Underlined).

BIK1S236A-F: 5′-GAGTTATGTTGCTACAAGGGTCATGG-3′

BIK1S236A-R: 5′-CCATGACCCTTGTAGCAACATAACTC-3′

BIK1T237A-F: 5′-GAGTTATGTTAGTGCAAGGGTCATGG-3′

BIK1T237A-R: 5′-CCATGACCCTTGCACTAACATAACTC-3′

BIK1S236A/T237A-F: 5′-GAGTTATGTTGCTGCAAGGGTCATGG-3′

BIK1S236A/T237A-R: 5′-CCATGACCCTTGCAGCAACATAACTC-3′

BIK1Y23F-F: 5′-AGAGCAGCGATCTTTTTGGTCTAAGTCTC-3′

BIK1Y23F-R: 5′-GAGACTTAGACCAAAAAGATCGCTGCTCT-3′

BIK1Y150F-F: 5′-CGTCTTCTAGTCTTCGAGTTTATGCAAAAAGG-3′

BIK1Y150F-R: 5′-CCTTTTTGCATAAACTCGAAGACTAGAAGACG-3′

BIK1Y234F-F: 5′-GGTGATTTGAGTTTTGTTAGTACAAGG-3′

BIK1Y234F-R: 5′-CCTTGTACTAACAAAACTCAAATCACC-3′

BIK1Y243F-F: 5′-CATGGGTACTTTTGGGTACGCCG-3′

BIK1Y243F-R: 5′-CGGCGTACCCAAAAGTACCCATG-3′

BIK1Y245-F: 5′-CATGGGTACTTATGGGTTCGCCGCGCCTGAG-3′

BIK1Y245-R: 5′-CTCAGGCGCGGCGAACCCATAAGTACCCATG-3′

BIK1Y250F-F: 5′-CGCGCCTGAGTTCATGTCATCAGG-3′

BIK1Y250F-R: 5′-CCTGATGACATGAACTCAGGCGCG-3′

BIK1Y316F-F: 5′-GCTAGACACACAGTTCCTACCTGAAGAAGC-3′

BIK1Y316F-R: 5′-GCTTCTTCAGGTAGGAACTGTGTGTCTAGC-3′

BIK1T242A-F: 5′-GGGTCATGGGTGCTTATGGGTACGCC-3′

BIK1T242A-R: 5′-GGCGTACCCATAAGCACCCATGACCC-3′

BIK1Km-F: 5′-GTCATCGCCGTTGCAGCGCTTAACCAAGAA-3′

BIK1Km-R: 5′-TTCTTGGTTAAGCGCTGCAACGGCGATGAC-3′

BAK1CDT450A-F: 5′-ACACACATGTGACAGCCGCAGTGCGT-3′

BAK1CDT450A-R: 5′-ACGCACTGCGGCTGTCACATGTGTGT-3′

BAK1CDT455N-F: 5′-GCAGTGCGTGGGAACATTGGTCATATA-3′

BAK1CDT455N-R: 5′-TATATGACCAATGTTCCCACGCACTGC-3′

BAK1CDKm-F: 5′-CTTTAGTGGCCGTTATGAGGCTAAAAGAG-3′

BAK1CDKm-R: 5′-CTCTTTTAGCCTCATAACGGCCACTAAAG-3′

1. Lu D, et al. (2010) A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc Natl Acad Sci USA* 107(1): 496–501.

2. Gao X, et al. (2013) Bifurcation of Arabidopsis NLR immune signaling via Ca^{2+}-dependent protein kinases. *PLoS Pathog* 9(1):e1003127.

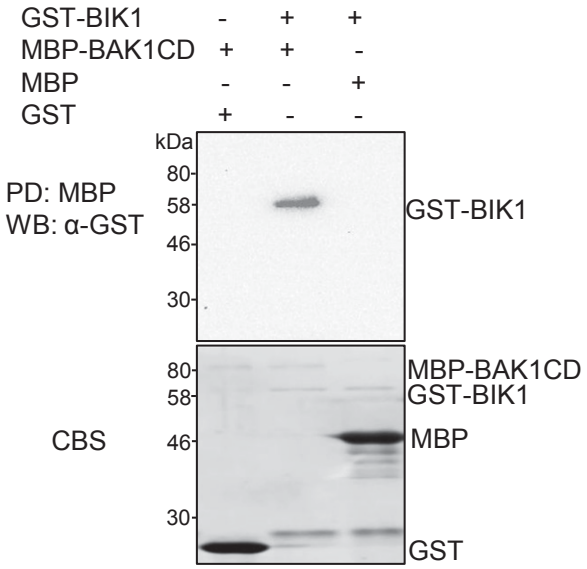

Fig. S1. BIK1 interacts with BAK1 cytosolic domain in vitro. MBP was the control for MBP-BAK1 protein. GST was the control for GST-BIK1 protein. MBP and MBP-BAK1CD were incubated with MBP beads, and then incubated with GST or GST-BIK1. The beads were collected and washed for Western blot of immunoprecipitated proteins with an α-GST antibody.

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BIK1   RNFRPDSVIGEGGFGCVFKGWLDESTLTPTKPGTGLVIAVKKLNQ---EGFQG-HREWLTEINYLGQLSHPNLVKLIGYC 140
PBL1   RNFRSDSVVGEGGFGCVFRGWLDETTLTPTKSSSGLVIAVKRLNP---DGFQG-HREWLTEINYLGQLSHPNLVKLIGYC 171
BAK1   DNFSNKNILGRGGFGKVYKGRLAD----------GTLVAVKRLKE---ERTQGGELQFQTEVEMISMAVHRNLLRLRGFC 353
BRI1   NGFHNDSLIGSGGFGDVYKAILKD----------GSAVAIKKLIH---VSGQG-DREFMAEMETIGKIKHRNLVPLLGYC 946
IRAK1  HNFSEELKIGEGGFGCVYRAVMRN----------TVYAVKRLKENADLEWTAVKQSFLTEVEQLSRFRHPNIVDFAGYC 304

BIK1   LEDEHRLLVYEFMQKGSLENHLFRRG-AYFKPLPWFLRVNVALDAAKGLAFLHSDP-VKVIYRDIKASNILLDADYNAKL 218
PBL1   LEDEQRLLVYEFMHKGSLENHLFANGNKDFKPLSWILRIKVALDAAKGLAFLHSDP-VKVIYRDIKASNILLDSDFNAKL 250
BAK1   MTPTERLLVYPYMANGSVASCLRERP-ESQPPLDWPKRQRIALGSARGLAYLHDHCDPKIIHRDVKAANILLDEEFEAVV 432
BRI1   KVGDERLLVYEFMKYGSLEDVLHDPK-KAGVKLNWSTRRKIAIGSARGLAFLHHNCSPHIIHRDMKSSNVLLDENLEARV 1025
IRAK1  AQNGFYCLVYGFLPNGSLEDRLHCQT-QACPPLSWPQRLDILLGTARAIQFLHQDS-PSLIHGDIKSSNVLLDERLTPKL 382

BIK1   SDFGLARDGPMGDLS--------YVSTRVMGTYGYAAPEYMSSGHLNARSDVYSFGVLLLEILSGKRALDHN-------RP 284
PBL1   SDFGLARDGPMGEQS--------YVSTRVMGTFGYAAPEYVSTGHLNARSDVYSFGVVLLELLCGRQALDHN-------RP 316
BAK1   GDFGLAKLMDYKDTH---------VTTAVRGTIGHIAPEYLSTGKSSEKTDVFGYGVMLLELITGQRAFDLA-------RL 497
BRI1   SDFGMARLMSAMDTH--------LSVSTLAGTPGYVPPEYYQSFRCSTKGDVYSYGVVLLELLTGKRPTDSP-------DF 1091
IRAK1  GDFGLARFSRFAGSSPSQSSMVARTQTVRGTLAYLPEEYIKTGRLAVDTDTFSFGVVVLETLAGQRAVKTHGARTKYLKD 462

BIK1   AKEEN--LVDWARPYLTSKRKVLLIVDNRLDTQYLPEEAVRMASVAVQCLSFEPKSRPTMDQVVRALQQLQDN------L 356
PBL1   AKEQN--LVDWARPYLTSRRKVLLIVDTRLNSQYKPEGAVRLASIAVQCLSFEPKSRPTMDQVVRALVQLQDS------V 388
BAK1   ANDDDVMLLDWVKGLLKEKKLEALVDVDLQGN-YKDEEVEQLIQVALLCTQSSPMERPKMSEVVRMLEGDGLAERWEEWQ 576
BRI1   GDNN---LVGWVKQHAKLRISDVFDPELMKEDPALEIELLQHLKVAVACLDDRAWRRPTMVQVMAMFK----------E 1157
IRAK1  LVEEEAEEAGVALRSTQSTLQAGLAADAWAAPIAMQIYKKHLGQLACCCLHRRAKRRPPMTQENSYVSSTGRAHSGAAPW 542
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Fig. S2. Alignment of BIK1 kinase domain with related kinases. Red box indicates that Y150 and Y250 in BIK1 are highly conserved, blue box indicates that Y316 in BIK1 is conserved in PBL1 and BAK1, and purple box indicates that T237 in BIK1 corresponds to T450 in BAK1.

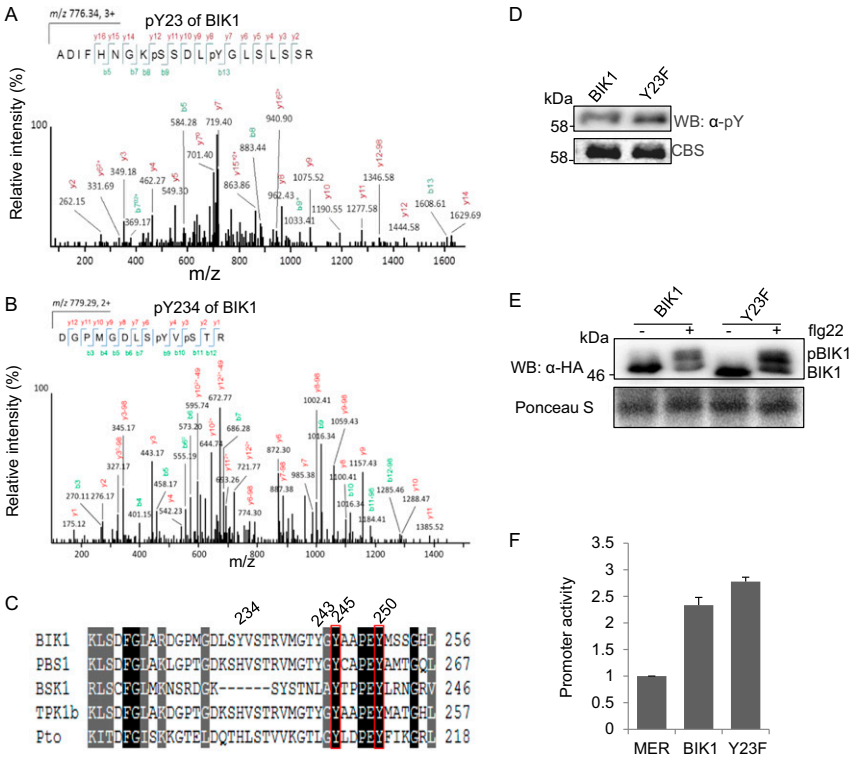

Fig. S3. Y23 and Y234 are phosphorylated but Y23 mutation does not affect the function in flg22 signaling. (*A*) BIK1 Y23 is autophosphorylated with MS analysis. (*B*) BIK1 Y234 is autophosphorylated with MS analysis. (*C*) Amino acid sequence alignment of the kinase activation loop of BIK1, PBS1, BSK1, TPK1b, and Pto. The number on the top indicates the position of amino acid in BIK1. (*D*) Y23F does not affect BIK1 autophosphorylation on tyrosine residues in vitro. The fusion proteins of GST-BIK1 and GST-BIK1Y23F were used in the in vitro phosphorylation assay and tyrosine phosphorylation was detected by immunoblotting with an α-pY antibody (*Upper*). The protein loading was shown by CBS (*Lower*). (*E*) Y23F does not affect flg22-induced BIK1 phosphorylation. BIK1 or BIK1Y23F were expressed in WT protoplasts for 8 h followed by 1 μM flg22 treatment for 10 min, and subjected with immunoblotting with an α-HA antibody. The flg22-mediated BIK1 phosphorylation is indicated by the mobility shift (*Upper*) and the protein loading is shown by Ponceau S staining of the membrane (*Lower*). (*F*) Y23F does not affect *pFRK1::LUC* activation by BIK1. The *pFRK1::LUC* was coexpressed with BIK1, BIK1Y23F, or a vector control (MER) in protoplasts for 6 h. UBQ10-GUS was included as a transfection control and the luciferase activity was normalized with GUS activity. The above experiments were repeated three times with similar results.

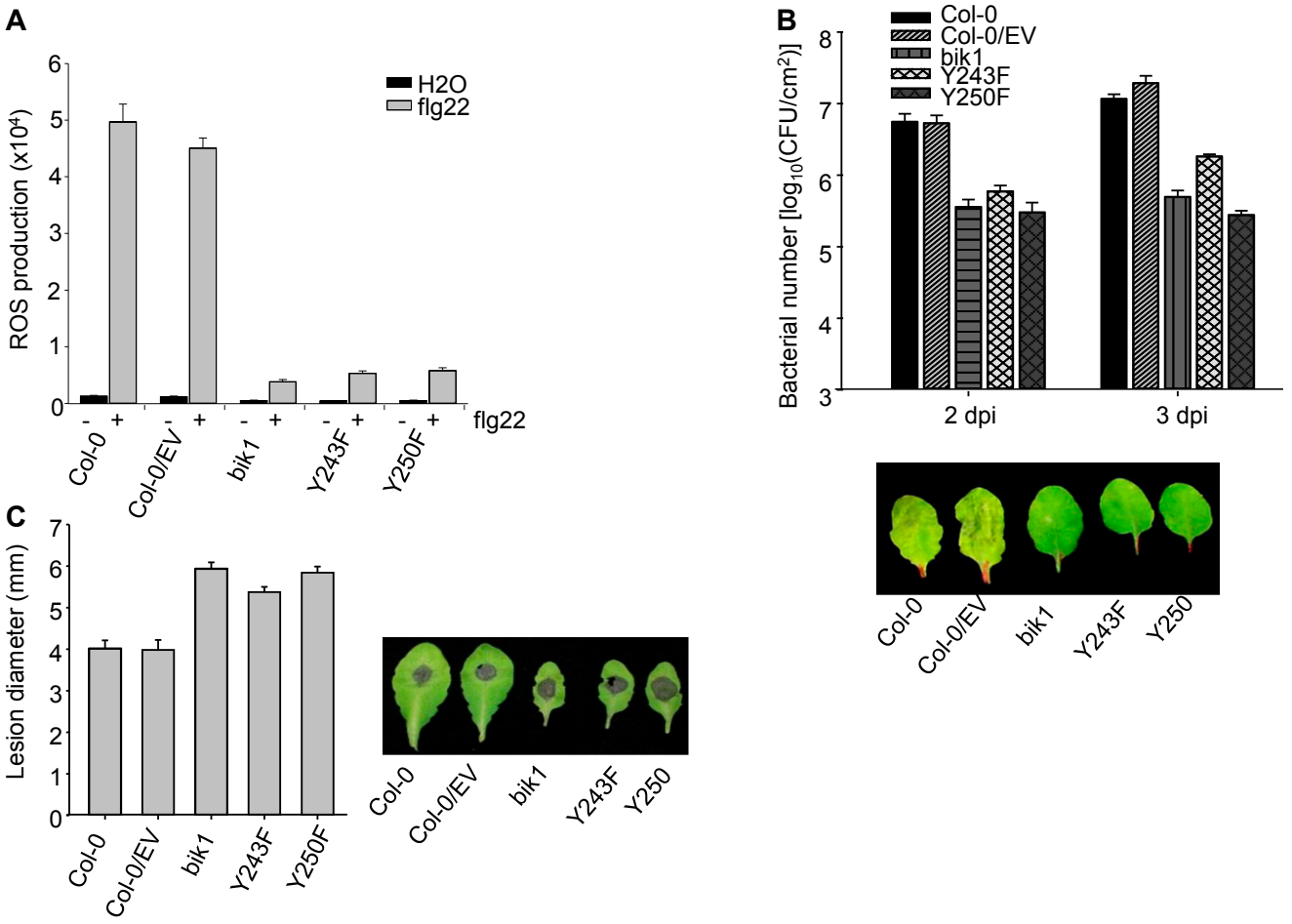

Fig. S4. BIK1 tyrosine residues are important for its function in plant immunity. (*A*) flg22-triggered ROS production in Col-0, *bik1* mutant and complementation transgenic plants (*pBIK1::BIK1*^{Y243F}-HA line D-2, *pBIK1::BIK1*^{Y250F}-HA line A2). ROS production in response to 100 nM flg22 from leaf discs of 5-wk-old plants was measured and presented as total photon counts during 30 min of treatment. Values presented are mean ± SE (n = 36). (*B*) Bacterial growth of *Psm* infection. Leaves from 4-wk-old Col-0, *bik1* mutant and complementation transgenic plants (*pBIK1::BIK1*^{Y243F}-HA line D-2, *pBIK1::BIK1*^{Y250F}-HA line A2) were hand inoculated with *Psm* at 5×10^5 cfu/mL and the bacterial growth was measured at 2 and 3 dpi. The data are shown as mean ± SE of three repeats. The picture was taken at 3 dpi. (*C*) Disease assay of *B. cinerea* infection. Leaves from 4-wk-old plants (Col-0, *pBIK1::BIK1*^{Y243F}-HA line D-2, *pBIK1::BIK1*^{Y250F}-HA line A2) were deposited with *B. cinerea* strain BO5 at a concentration of 2.5×10^5 spores per mL. Disease symptom was recorded 3 dpi. The lesion diameter was measured at 2 dpi. The data are shown as mean ± SE of at least 30 leaves.

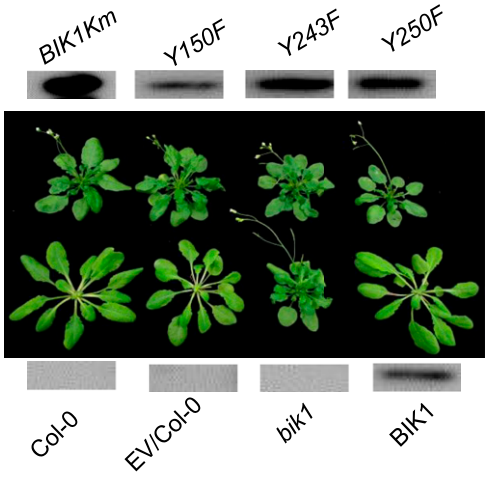

Fig. S5. Y150, Y243, and Y250 are required for BIK1 functions in growth and development. BIK1^{Y150F}, BIK1^{Y243F}, and BIK1^{Y250F} complementation plants fail to rescue *bik1* growth defects and early flowering phenotypes. The protein expression of transgene is shown by Western blot.