Supplemental materials and methods

Adi3 and *Pdk1* cDNA cloning

The sequence from the 776-bp *Adi3* Y3H cDNA fragment (Bogdanove and Martin, 2000) was used to search the tomato EST database (www.tigr.org) for a full-length *Adi3*. The 1781 bp contig *TC102958* matched *Adi3* but did not contain the 5' end of the cDNA. The remaining 5' end of *Adi3* was obtained by 5' RACE using the BD SMART RACE kit (BD Biosciences). The reverse primer 5'-AAATGCTTCCCAGGTTGTCGCTG TCG-3' based at nucleotide 1214 of the *Adi3* cDNA was used in the RACE reaction. A 5' RACE product of 1.6 kb was generated and sequence information confirmed an ATG start codon and 5' UTR. Primers based on the compiled *Adi3* sequence were used to amplify the *Adi3* cDNA from first strand cDNA. The resulting 2,829 bp cDNA was cloned into pCR2.1-TOPO (Invitrogen) and confirmed by sequencing (GenBank accession #AY849914).

Tomato *Pdk1* was identified by searching the tomato EST database (www.tigr.org) using the Arabidopsis *Pdk1* sequence (Deak et al, 1999). Sequencing confirmed that *cTOC6F17* (from contig *TC119429*) is a full-length *Pdk1* cDNA. The *Pdk1* cDNA was PCR-amplified and cloned into pCR2.1-TOPO (GenBank accession #AY849915).

Yeast two- and three-hybrid assays

The pEG202 vector was used for bait constructs and the pJG4-5 vector for prey constructs. *Pto* and *Bicoid* bait vectors and *AvrPto*, *Dorsal* and *Pto* prey vectors were described previously (Tang et al, 1996; Zhou et al, 1995). Constructs were transformed into yeast strain EGY48 containing the pSH18-34 reporter vector for yeast two-hybrid assays or EGY48 containing the AvrPto/pSH18-34 modified reporter vector for yeast three-hybrid assays (Bogdanove and Martin, 2000). Protein expression was confirmed by western blot. All other procedures for the yeast two- and three-hybrid assays followed (Golemis et al, 1996).

Protein expression

The *Pto* cDNA was previously cloned into pMAL-c2 (Loh and Martin, 1995). Site-directed mutagenesis was carried out using the QuickChange kit (Strategene). Constructs for protein expression were transformed into *E. coli* strain BL21 Star (DE3) (Invitrogen) and grown overnight in 2 ml LB at 37˚C. A 200 µl aliquot of the culture was added to 10 ml TB, grown at 37˚C to $O.D._{600}$ = 0.5, and protein expression induced with 100 µM IPTG for 3 hrs at 28˚C. Cells were harvested by centrifugation, lysed in 1 ml of extraction buffer (50 mM Tris [pH 8.0], 50 mM NaCl, 5 mM EDTA, 0.1 % Triton X-100, general protease inhibitors [Sigma]) by sonication, and debris pelleted by centrifugation. Proteins were purified from the supernatant by adding amylose resin (New England Biolabs) or α-FLAG M2 agarose (Sigma), incubating at 4˚C for 30 min with rotation, pelleting the resin by centrifugation, and washing three times with extraction buffer.

Kinase assays

For autophosphorylation assays, MBP translational fusion proteins were immobilized on 50 µl of amylose beads as above, washed three times with kinase buffer without ATP, and incubated with 5 µCi of [γ-^{32}P]ATP in 50 µl of kinase buffer. Pto kinase buffer: 50 mM Tris (pH 7.0), 1 mM DTT, 10 mM $MnCl_2$, 20 µM ATP. Adi3/Pdk1 kinase buffer: 10 mM Tris (pH 7.5), 150 mM NaCl, 1mM DTT, 10 mM $MgCl_2$, 20 µM ATP. Reactions were carried out at room temperature for 15 min and analyzed by 10% SDS-PAGE with phosphorimager (Molecular Dynamics) visualization and quantification. For cross-phosphorylation assays, MBP translational fusion proteins were purified separately on 50 µl of amylose resin, proteins left on the resin, samples mixed, and used in a kinase assay with the buffer of the enzyme carrying out the phosphorylation. Kinase assays were repeated a minimum of 3 times.

In vitro co-immunoprecipitation

MBP-Adi3 and Pdk1-FLAG proteins were produced as described above. Extracts of each protein were mixed in equal amounts and incubated at 4˚C for 30 min with rotation. Proteins were precipitated with α-FLAG M2 agarose at 4˚C for 30 min with rotation. The agarose was pelleted by centrifugation, washed 3 times with extraction buffer, split into two equal parts, separated by 10% SDS-PAGE, and analyzed by western blot using α-MBP antibody (New England Biolabs) at 1:10,000 or α-FLAG M2-HRP antibody (Sigma) at 1:1000.

Phosphoamino acid analysis and phosphopeptide mapping

Phosphoamino acid analysis and phosphopeptide mapping was carried out as described in (Van der Geer et al, 1993). Briefly, for phosphoamino acid analysis, MBP-Adi3 proteins were phosphorylated by MBP-Pdk1 with [γ-^{32}P]ATP as described above, separated by 10% SDS-PAGE, and transferred to PVDF membrane. The protein bands were excised from the PVDF, hydrolyzed in 6M HCl at 110˚C for 1 hour, evaporated to dryness in a centrifugal vacuum concentrator, and resuspended in pH 1.9 buffer (2.5% formic acid, 7.8% glacial acetic acid,) with 0.5 μg of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards. Hydrolyzed amino acids were separated by two dimensional electrophoresis TLC using pH 1.9 buffer for first dimension and pH 3.5 buffer (5% glacial acetic acid, 0.5% pyridine) for the second dimension. Unlabeled phosphoamino acid standards were visualized by spraying plates with 0.25% ninhydrin in acetone and radioactive phosphoamino acids visualized using a phosphorimager. Briefly, for phosphopeptide mapping, MBP-Adi3 proteins were phosphorylated by Pdk1 with [γ-^{32}P]ATP as described above, separated by 10% SDS-PAGE, and transferred to PVDF membrane, the protein bands excised from the PVDF, and digested with 20 μg of N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin in 50 mM ammonium bicarbonate at 37˚C for 4 hours. Peptides were separated in the first dimension by electrophoresis TLC in pH 1.9 buffer followed by ascending chromatography in phospho-chromatography buffer (37.5% *n*-butanol, 25% pyridine, 7.5% glacial acetic acid). Radiolabeled phosphopeptides were visualized using a phosphorimager.

Virus-induced gene silencing

A 937-bp cDNA fragment corresponding to the 687 5' bp of the *Adi3* ORF plus an additional 250 bp of the 3' UTR was cloned into the pTRV2 vector, and its identity confirmed by sequencing. Sequences for the tomato group VIIIa AGC kinase contigs *TC123326*, *TC144536*, and *TC138973* were obtained from www.tigr.org. Full length ESTs corresponding to *TC144536* (EST *cTOS8L12*) and *TC138973* (EST *cLEF42F14*), and 716 bp (bp 409-1207) of *TC147578* amplified from tomato gDNA were cloned into the pTRV2 vector. pTRV1 and pTRV2 derivatives were co-inoculated into 3-week-old tomato seedlings by *Agrobacterium*-mediated infiltration as described in Ekengren et al, (2003) and the phenotype was assayed 3 weeks later. Leaves were de-pigmented in 10% acetic acid, 30% chloroform, 60% EtOH to enhance visualization of cell death lesions. RT-PCR analysis was performed using leaf RNA 3 weeks after silencing as reported (Ekengren et al, 2003). Each silencing experiment was carried out a minimum of 3 times.

OSU-03012 inhibition of recombinant tomato Pdk1

Recombinant Pdk1-FLAG protein was expressed in *E. coli* as above and immobilized in wells of a 96 well plate coated with α-FLAG M2 antibody (Sigma) at 4˚C for 1 hour with rotation. Each well was washed three times with extraction buffer (see protein expression) followed by three washes with Pdk1 kinase buffer with out ATP (see kinase assays) plus desired concentrations of OSU-03012 or DMSO carrier. Fifty μl of Pdk1 kinase buffer plus desired concentrations of OSU-03012 or DMSO carrier with 5 μCi of [γ-^{32}P]ATP was placed in each well and the reaction carried out for 15 minutes at room temperature. The reaction mix was removed and each well washed with 200 μl 1x TBS. Pdk1-FLAG proteins were eluted from each well by adding 30 μl 1x SDS-PAGE buffer and heating at 100˚C for 5 minutes. Samples were separated by 10% SDS-PAGE, transferred to PVDF, analyzed by α-FLAG western blot, and imaged using a phosphorimager (Molecular Dynamics).

Protoplast isolation and treatment with OSU-03012

Tomato protoplast isolation was performed as described previously (Xing et al, 2001). 1.5 x 10^4 protoplasts were treated with OSU-03012 or DMSO to a final DMSO concentration of 0.1% and cell death analyzed over 24 hr. Cell viability was determined by treating protoplasts with 0.04% Evan's blue, incubating for 10 min, and counting dead cells out of a total of at least 200 cells under a light microscope. For attenuation of OSU-03012-induced cell death the vector pTEX was used for overexpression of Pdk1-FLAG and HA-Adi3 from the CaMV 35S promoter. 3.5 x 10^4 protoplasts were transfected by the PEG method with 40 μg of plasmid according to the protocol developed by Sheen (2002). Protein expression was carried out for 9 hr before the addition of OSU-03012 and incubation continued for an additional 12 hr before cell viability was determined. Expression of proteins was confirmed by western blot with α-HA antibody (Roche) and α-FLAG M2 antibody. Data shown are averages of 3 independent experiments.

References

Bogdanove AJ, Martin GB (2000) AvrPto-dependent Pto-interacting proteins and AvrPto-interacting proteins in tomato. *Proc Natl Acad Sci USA* **97**: 8836-8840

Deak M, Casamayor A, Currie RA, Downes CP, Alessi DR (1999) Characterization of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. *FEBS Lett.* **451**: 220-226

Golemis EA, Gyuris J, Brent R. (1996) Interaction trap/two-hybrid system to identify interacting proteins. In F.M. Ausubel, R.B., R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (ed.), *Current Protocols in Molecular Biology*. Greene Publishing Associates/John Wiley & Sons, New York, pp. 20.21.21 - 10.21.28.

Loh Y-T, Martin GB (1995) The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. *Plant Physiol.* **108**: 1735-1739

Sheen J (2002) A transient expression assay using Arabidopsis mesophyll protoplast. http://genetics.mgh.harvard.edu/sheenweb/

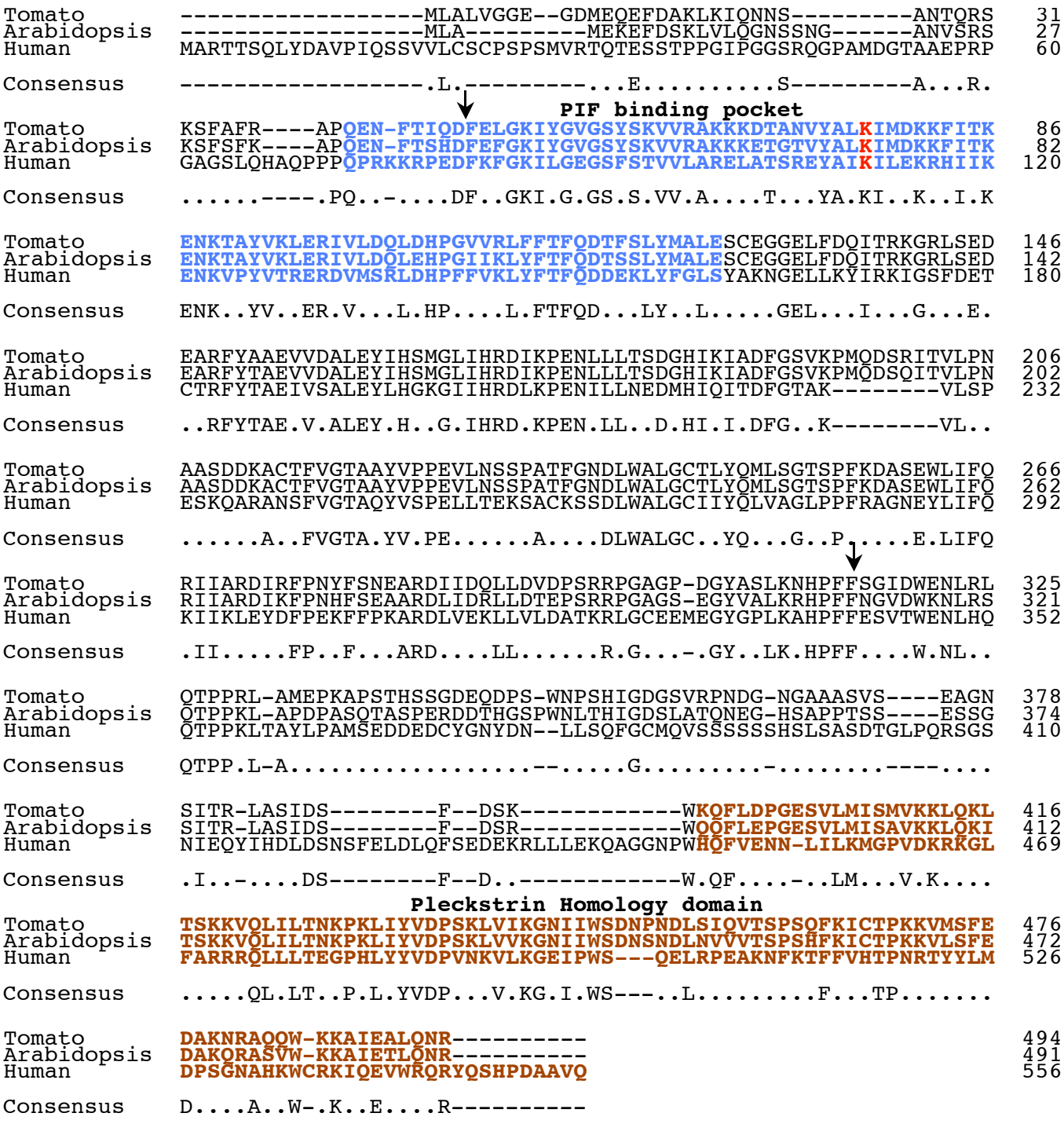
Tang X, Frederick RD, Zhou J, Halterman DA, Jia Y, Martin GB (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**: 2060-2063

Van der Geer P, Luo K, Sefton B, Hunter T. (1993) Phosphopeptide mapping and phosphamino acid analysis on cellulose thin-layer plates. In Hardie, D.G. (ed.), *Protein Phosphorylation*. IRL Press, Oxford, pp. 31-59.

Xing T, Malik K, Martin T, Miki BL (2001) Activation of tomato PR and wound-related genes by a mutagenized tomato MAP kinase kinase through divergent pathways. *Plant Mol Biol* **46**: 109-120

Zhou J-M, Loh Y-T, Bressan RA, Martin GB (1995) The tomato gene *Pti1* encodes a serine-threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell* **83**: 925-935

Supplemental Figure S1



Supplemental Figure S1. Alignment of Pdk1 proteins.

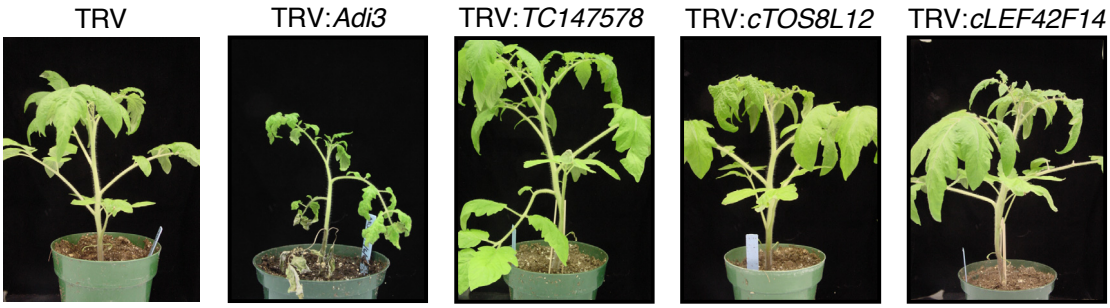
Pdk1 proteins from tomato (this study), Arabidopsis (Deak et al, 1999), and human (Alessi et al, 1997) were aligned using ClustalW. Numbers indicate the amino acid residues in the sequences. Gaps in the alignment are designated by dashes. Arrows indicate beginning and end of kinase domain. PIF binding pocket is shown in blue. ATP binding site Lys is shown in red. Pleckstrin homology domain is shown in brown.

References

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Curr Biol* **7**: 261-269

Deak M, Casamayor A, Currie RA, Downes CP, Alessi DR (1999) Characterization of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. FEBS Lett. **451**: 220-226

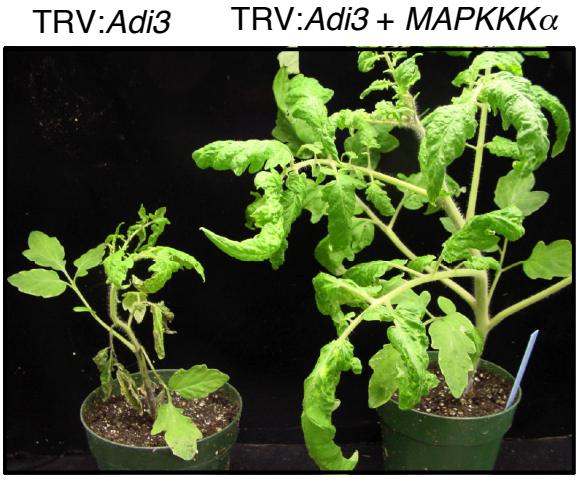
Supplemental Figure S2



Supplemental Figure S2. Silencing of Adi3-like genes in tomato does not affect plant stature.

Tomato plants were silenced for the indicated gene using the TRV VIGS vector as in Figure 4 and pictures were taken 3 weeks after silencing infiltrations.

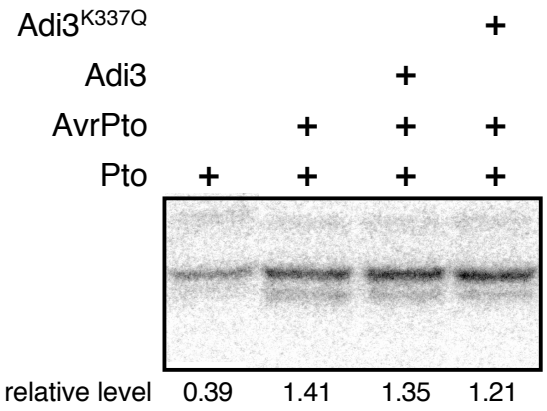
Supplemetal Figure S3



Supplemental Figure S3. Co-silencing of Adi3 and MAPKKKα restores plant stature.

Tomato plants were silenced for Adi3 and MAPKKKα using the TRV VIGS vector as in Figure 4 and pictures were taken 3 weeks after silencing infiltrations.

Supplemetal Figure S4



Supplemental Figure S4. Overexpression of Adi3 does not affect AvrPto induced MAPK activity.

Kinase-active Adi3 or kinase-inactive Adi3^{K337Q} was transiently expressed in *N. benthamiana* leaf tissue as in Figure 5D in the presence of Pto/AvrPto. AvrPto was expressed for 1 hour from a β-estradiol inducible promoter before samples were taken for analysis of MAPK activity by an in-gel kinase assay as previously described (del Pozo et al, 2004). Gels were visualized and quantified using a phosphorimager.

Reference

del Pozo O, Pedley KF, Martin GB (2004) MAPKKKα is a positive regulator of cell death associated with both plant immunity and disease. EMBO J **23**: 3072 - 3082