Supporting Information

SI materials and methods

Plant materials and growth conditions,

A. thaliana plants were grown in a chamber at 22°C with a 10 h light period and 60% relative humidity for 3 weeks and then in another chamber at 22°C with a 12 h light period and 60% relative humidity. The *A. thaliana* accession Col-0 was the background of all *A. thaliana* mutants used in this study. *A. thaliana aba2-12* (1), *mpk3-1* (SALK_151594) (2), *mpk6-2* (SALK_073907) (3), *hai1-2* (4), *hai1-2 aip1-1 hai3-1* (designated as *hai1 hai2 hai3* in this paper) (4), *abi2-2* (a loss-of function allele) (4), *myc2* (SALK_017005) (5), *myc2 myc3 myc4* (6) and *areb1 areb2 abf3* (7) were previously described. Estradiol-inducible AvrRpt2 transgenic lines (8) and dexamethasone-inducible MKK4DD transgenic lines (9) were described previously. The MYC2-GFP overexpression plants were obtained from Dr. Hironaka Tsukagoshi (Meijo University, JAPAN). Seedlings of *A. thaliana*, *C. rubella* (N22697) and *E. salsugineum* (Shandong) were grown on solidified half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose under a 10 h light period at 22°C.

Chemicals

ABA (A1049), MeJA (392705) and coronatine (C8115) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Flg22 was purchased from EZBiolab Inc. (Westfield, IN, USA).

Molecular cloning

Full length cDNAs of *HAI1, HAI2, HAI3, ABI2, MPK3 and MPK6* were amplified by PCR and cloned into the pENTR/D-TOPO vector following the

manufacturer's protocol (Life Technologies, Carlsbad, CA). For construction of BiFC vectors, the sequence of the myc-tagged N-terminal half of YFP was amplified from pBICmyc:nYFP (10) and cloned into the *Stu*I site of pBICAsc2 (10), producing pBICmyc:nYFPAsc2. Then, the PCR-amplified *HAI1, HAI2, HAI3* and *ABI2* sequences and the PCR-amplified *MPK3 and MPK6* sequences were cloned into the *Asc*I site of pBICmyc:nYFPAsc2 and pBICHA:cYFPAsc2 (11), respectively. For construction of plasmids used for subcellular localization assays, the Venus and tagRFP sequences were amplified by PCR and cloned into the *Stu*I site of pBICAsc2, producing pBICVenusAsc2 and pBICtagRFPAsc2, respectively. The PCR-amplified *HAI1, HAI2 and HAI3* sequences and the PCR-amplified *MPK3* and *MPK6* sequences were then cloned into the *Asc*I site of pBICtagRFPAsc2 and pBICVenusAsc2, respectively. For construction of plasmids used for recombinant protein production, the sequences of GST, MKK4DD (9) and *HAI1, HAI2, HAI3, MPK3* and *MPK6* were amplified by PCR and appropriate combinations of the amplified fragments were cloned into the pCold I vector (Takara-Bio, Shiga, Japan) using In-Fusion technology (Takara-Bio). The ATP binding pockets in the MPK3 and MPK6 constructs were mutated using QuikChange Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA) to block kinase activity (12). The PP2C domains of HAI1, HAI2 and HAI3 were amplified and cloned into the *Pst*I site of the expression vector for GST-fusion proteins. Primers used are listed in Table S1. All plasmids constructed in this study were verified by sequencing.

MAP kinase assays

MAP kinase assays were performed essentially as described previously (8, 13). For MAMP-triggered MAPK activation, 12-day-old *A. thaliana* seedlings grown on

half-strength MS medium were used. Treatment was carried out by transferring seedlings to fresh half-strength MS medium containing appropriate chemicals. For MAPK activation by DEX-inducible MKK4DD or during bacterial infection, 4-week-old *A. thaliana* plants grown on soil were used. *A. thaliana* tissues were frozen in liquid nitrogen and stored at -80°C. The frozen tissues were ground in liquid nitrogen and homogenized in MAPK extraction buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol (DTT), 10 mM NaF, 50 mM ß-glycerolphosphate, 10% glycerol, Complete proteinase inhibitor cocktail [Roche, Mannheim, Germany] and Phosstop phosphatase inhibitor cocktail [Roche]). The protein concentration was determined using Coomassie Protein Assay Kit (Life Technologies). Fifteen micrograms of protein was separated on a 10% polyacrylamide gel. Immunoblot analysis was performed using anti-phospho-p44/42 MAPK (1:5000, Cell Signaling Technology, Danvers, MA, USA; #9101), anti-AtMPK3 (1:2500, Sigma; M8318), anti-AtMPK6 (1:5000, Sigma; M7104) or anti-FLAG (1:5000, Sigma-Aldrich; F3165) as first antibodies and HRP-conjugated anti-rabbit IgG (1:10000, Sigma-Aldrich; A6154) or HRP-conjugated anti-mouse IgG (1:10000, Santa Cruz Biotechnology, Dallas, TX, USA; sc2005), as secondary antibodies.

BiFC and subcellular localization assays

Agrobacterium infiltration was carried out as described previously (10). *Agrobacterium* suspensions were mixed at a final OD_{600} of 0.4 for MAPK and PP2C constructs and OD_{600} of 0.2 for the p19 silencing suppressor (14). Three days after infiltration, leaves of *Nicotiana benthamiana* were observed under ZEISS LSM700 or LSM780 to visualize fluorescent proteins. Accumulation of HA- or myc-tagged proteins was confirmed by immunoblotting using anti-HA first antibody (1:5000, Roche; 3F10) and

anti-Rat IgG secondary antibody (1:5000, Santa Cruz Biotechnology; sc2032) or HRP-conjugated anti-myc antibody (1:2000, Roche; 9E10), respectively.

***In vitro* dephosphorylation of MAPK**

E. coli strain Rossetta 2 (DE3) (Novagen) was used for production of recombinant proteins as described in Mine et al (15). GST-tagged full-length HAI1, HAI2 and HAI3 proteins were not soluble. Therefore, we decided to express the PP2C domains of HAI1, HAI2 and HAI3. This significantly improved solubility of HAI1 but not HAI2 and HAI3. GST-fusion proteins and His-tagged proteins were purified using glutathione agarose (Life Technologies) and Dynabeads His-Tag Isolation and Pulldown (Life Technologies) according to the manufacturer's protocols. To prepare phosphorylated MPK3 and MPK6, 5 μg of purified His-tagged kinase-inactive MAPKs (12) were incubated with 1 μg of GST-MKK4DD at 30 °C for 1 h in kinase buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 200 μM ATP). The kinase reaction was terminated by removing ATP from the reaction using an Amicon Ultra-30K centrifugation unit (Millipore). To test phosphatase activity of HAI1, phosphorylated MAPKs (500 ng) were incubated with GST or GST-HAI1 (2 μg) at 30 °C for 1 h in phosphatase buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$). The reaction was terminated by adding concentrated Laemmli sample buffer, followed by boiling at 95 °C for 3 min. MAPKs were detected by immunoblotting as described above. GST-fusion proteins were detected by immunoblotting using HRP-conjugated anti-GST antibody (1:5000, Santa Cruz, sc138-HRP).

Statistical analysis

Statistical analysis was performed using the mixed linear model function, lmer, implemented in the package lme4 in the R environment. When appropriate, raw data

were log transformed to meet the assumptions of the mixed linear model. For the t-tests, the standard errors were calculated using the variance and covariance values obtained from the model fitting. The Benjamini-Hochberg method was applied to correct for multiple hypothesis testing when all pairwise comparisons of the mean estimates were made.

RNA extraction, cDNA synthesis and quantitative PCR

For bacterial infiltration, leaves of 4 to 5-week-old plants grown on soil were used. For chemical treatments, 12-day-old seedlings grown on half-strength MS medium were used. Total RNAs were isolated using TriFast (peqlab, Erlangen, Germany), followed by cDNA synthesis using superscript II (Life Technologies). Real-time PCR was performed using EvaGreen (Biotium, Hayward, CA, USA) on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) or the CFX Connect Real-Time PCR Detection System (Bio-Rad). Primers used are listed in Table S1. The following models were fit to the relative Ct value data compared to *Actin2*: $Ct_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY, genotype:treatment interaction, and random factors; R, biological replicate; e, residual; $Ct_{ytr} = YT_{yt}+R_r+e_{ytr}$, where YT, treatment:time interaction; $Ct_{gytr} = GYT_{gyt}+R_r+e_{gytr}$, where GYT, genotype:treatment:time interaction. The mean estimates of the fixed effects were used as the modeled relative Ct values, visualized as the relative log_2 expression values and compared by two-tailed t-test.

Stomatal aperture measurement

Stomatal aperture measurement was performed essentially as described previously (16). *A. thaliana* plants were kept for at least 3 hours under light to assure most stomata were open before starting experiments. Epidermal peals were prepared

from fully-expanded leaves of 4 to 5-week-old plants grown in a chamber at 22°C with a 12 h light period and 60% relative humidity and immersed in MES buffer (25mM MES-KOH [pH 6.15] and 10 mM KCl) or bacterial suspension (OD600 = 0.2 in MES buffer). Epidermal peels were observed under LSM700 (ZEISS). Stomatal aperture was measured by taking ratios of width and length of approx. 20 stomata in each experiment using ZEN software (ZEISS). The following model was fit to the stomatal aperture data: $StomatalAperture_{gytr} = GYT_{gyt}+R_r+e_{gytr}$, where GYT, genotype:treatment:time interaction, and random factors; R, biological replicate; e, residual.

Bacterial growth assay

Bacterial growth assays were performed essentially as described previously (13). Briefly, bacterial suspensions were infiltrated into leaves of 4 to 5-week-old plants using a needleless syringe. Log_{10}-transformed colony-forming units (cfu) per cm^2 leaf surface area were calculated and the following model was fit to the data; $CFU_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY, genotype:treatment interaction, and random factors; R, biological replicate; e, residual. The mean estimates of the fixed effects were used as the modeled bacterial titers, visualized as the relative log_2 expression values and compared by two-tailed t-test.

Chromatin immunoprecipitation

Tissue fixation and chromatin immunoprecipitation were carried out as described (17). Briefly, 2-week-old seedlings grown in liquid half-strength MS medium supplemented with 1% sucrose were treated with mock (water for MeJA and DMSO for coronatine), MeJA (100 μM) or coronatine (5 μM) for 3 h, followed by fixation in 1% formaldehyde solution. Fixed tissues were frozen in liquid nitrogen and stored at -80°C. Frozen tissues (~1 g) were ground in liquid nitrogen using a mortar and pestle and

suspended in 3 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 μM MG132 (Sigma), and complete protease inhibitor cocktails [Roche]). The suspension was sonicated twice on the Bioruptor sonication system (Diagenode, Seraing, Belgium) for 10 min at 4°C, followed by centrifugation at 20,000×g for 10 min at 4°C. The supernatant was used as the starting material for chromatin immunoprecipitation using anti-GFP antibody (Ab290; Abcam, Cambridge, UK). Aliquots of the supernatant were kept as input samples. The samples were analyzed by quantitative PCR using primers listed in Table S1. The percentage of input values of the ChIP DNA was further normalized over the value obtained for the *EIF4A1* promoter (AT3G13920). Fold enrichment was then calculated by taking ratios between normalized results from wild-type plants and from MYC2-GFP plants. The following model was fit to $\log_2$-transformed values of the normalized value data; $Ct_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY, genotype:treatment interaction and random factors; R, biological replicate; e, residual. The mean estimates of the fixed effects were compared by two-tailed t-tests.

Phylogenetic analysis

The complete protein sequences of *A. thaliana*, *A, lyrata, C. rubella, C. grandiflora*, *E. salsugineum, B. rapa*, tomato and rice were retrieved from Phytozome (18) and used for identification of putative orthologous groups using the OrthoMCL program (19). The proteins belonging to the same group as *A. thaliana* EDS5 were aligned using MUSCLE (20). A maximum likelihood phylogenetic tree was constructed using the MEGA6 software (21). To visualize conservation of G boxes, 500 bp upstream of the transcription start sites of the *Brassicaceae HAI1, HAI2 and HAI3* were retrieved from Phytozome and aligned using MUSCLE.

Accession numbers

The accession numbers for the genes discussed in this article are as follows: *Actin2* (At2g18780), *HAI1* (At5g59220), *HAI2* (At1g07430), *HAI3* (At2g29380), *VSP2* (At5g24770), *EsActin2* (Thhalv10020949m), *EsHAI1* (Thhalv10013567m), *EsHAI2* (Thhalv10007705m), *EsHAI3* (Thhalv10017797m), *CrActin2* (Carubv10013961m), *CrHAI1* (Carubv10026477m), *CrHAI2* (Carubv10009150m), *CrHAI3* (Carubv10024530m).

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