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Disruption of PAMP-Induced MAP Kinase Cascade by a *Pseudomonas syringae* Effector Activates Plant Immunity Mediated by the NB-LRR Protein SUMM2

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SUMMARY

Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) serves as a primary plant defense response against microbial pathogens, with MEKK1, MKK1/MKK2, and MPK4 functioning as a MAP kinase cascade downstream of PAMP receptors. Plant Resistance (R) proteins sense specific pathogen effectors to initiate a second defense mechanism, termed effector-triggered immunity (ETI). In a screen for suppressors of the *mkk1 mkk2* autoimmune phenotype, we identify the nucleotide-binding leucine-rich repeat (NB-LRR) protein SUMM2 and find that the MEKK1-MKK1/MKK2-MPK4 cascade negatively regulates SUMM2-mediated immunity. Further, the MEKK1-MKK1/MKK2-MPK4 cascade positively regulates basal defense targeted by the *Pseudomonas syringae* pathogenic effector HopAI1, which inhibits MPK4 kinase activity. Inactivation of MPK4 by HopAI1 results in activation of SUMM2-mediated defense responses. Our data suggest that SUMM2 is an R protein that becomes active when the MEKK1-MKK1/MKK2-MPK4 cascade is disrupted by pathogens, supporting the hypothesis that R proteins evolved to protect plants when microbial effectors suppress basal resistance.

INTRODUCTION

Plants use two different strategies to detect microbial pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). The first detection strategy is mediated by pattern recognition receptors (PRRs) at the plasma membrane that recognize pathogen-associated molecular patterns (PAMPs) and establish PAMP-triggered immunity (PTI). PAMPs are typically conserved microbial molecules, such as bacterial flagellin and translation elongation factor Tu (EF-Tu) (Boller and Felix, 2009). The second detection strategy is mediated by plant Resistance (R) proteins that sense specific pathogen effectors, also known as avirulence proteins, to initiate effector-triggered immunity (ETI). These two layers of defense constitute the plant immune system which protects plants from pathogen attack. Most plant R proteins belong to the nucleotide-binding site leucine-rich repeat (NB-LRR) class (Caplan et al., 2008a), which shares structural similarity to animal innate immune receptors, such as NOD1 and NOD2. NB-LRR R proteins contain either a Toll-interleukin 1-like receptor (TIR) domain or a coiled-coil (CC) domain at their N termini.

Recognition of pathogen effectors by plant R proteins can be either direct or indirect. In a few cases, direct interactions have been observed between R proteins and their cognate effectors (Deslandes et al., 2003; Dodds et al., 2006; Jia et al., 2000; Krasileva et al., 2010). However, in most cases plant R proteins indirectly recognize effectors by detecting their effects on plant target proteins. This model, in which R proteins are activated by modifications to independent effector target proteins, is known as the guard hypothesis (Van der Biezen and Jones, 1998). One of the most extensively studied examples of a "guardee" is RPM1 interacting protein 4 (RIN4). RIN4 associates with two NB-LRR proteins, RPM1 and RPS2 (Mackey et al., 2002). Two unrelated *Pseudomonas syringae* effectors, AvrB and AvrRpm1, can promote phosphorylation of RIN4 and activate RPM1-mediated immune responses (Chung et al., 2011; Liu et al., 2011). RPM1-induced protein kinase 1 (RIPK1) is partially responsible for RIN4 phosphorylation (Liu et al., 2011). A third *Pseudomonas* effector, AvrRpt2, cleaves RIN4 through its cysteine protease activity, and the degradation of RIN4 activates RPS2-mediated immunity (Axtell and Staskawicz, 2003; Mackey et al., 2003). Additional studies on *Arabidopsis* PBS1, tomato protein kinase Pto and protease Rcr3, and a chloroplastic sulfurtransferase NRIP1 in *Nicotiana benthamiana* emphasize the indirect recognition of effectors by plant R proteins (Caplan et al., 2008b; Mucyn et al., 2006; Rooney et al., 2005; Shao et al., 2003).

Mitogen-activated protein (MAP) kinase cascades play important roles in plant immunity. A MAP kinase cascade consists of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MKK), and a MAP kinase (MPK) (MAPK-Group, 2002). Signals from upstream receptors are transduced and amplified through the MAP kinase cascade. In *Arabidopsis*, at least two MAP kinase cascades are activated downstream of PAMP receptors. One leads to activation of MPK3 and MPK6 (Asai et al., 2002). The other leads to activation of MPK4 (Gao et al., 2008; Qiu et al., 2008b). Activation of MPK4 requires MEKK1 and

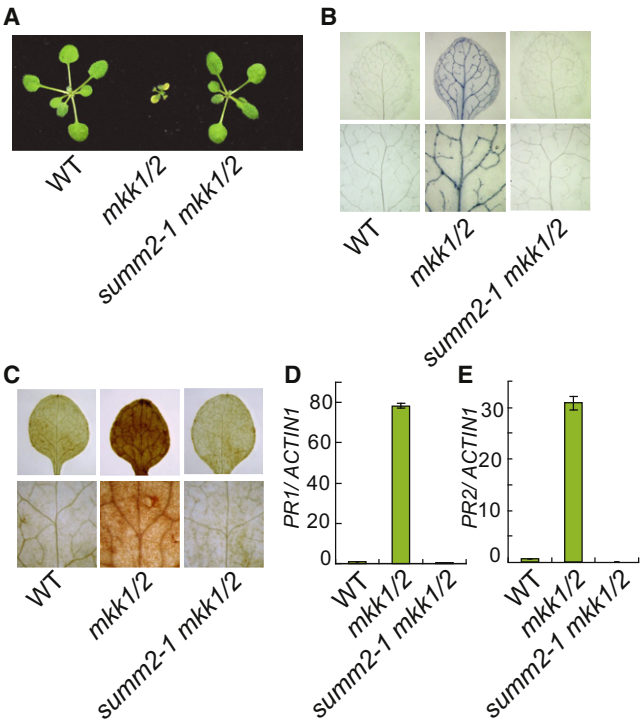

Figure 1. Characterization of *summ2-1 mkk1 mkk2*

(A) Morphological phenotypes of Col (WT), *mkk1 mkk2* (*mkk1/2*), and *summ2-1 mkk1 mkk2*. Photos were taken on 3-week-old soil-grown plants.

(B and C) Trypan blue staining (B) and DAB staining (C) of true leaves of the indicated genotypes.

(D and E) Gene expression levels of *PR1* (D) and *PR2* (E) determined by qPCR. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations from three measurements.

MKK1/MKK2 (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006; Qiu et al., 2008b; Suarez-Rodriguez et al., 2007).

MPK4 was originally identified as a negative regulator of plant immunity (Petersen et al., 2000). The *mpk4* mutant exhibits autoimmune phenotypes characterized by dwarf morphology, spontaneous cell death, and constitutive defense responses. Later studies showed that *mekk1* and *mkk1 mkk2* mutant plants have phenotypes similar to those of *mpk4* and MKK1 and MKK2 are functionally redundant (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006; Qiu et al., 2008b; Suarez-Rodriguez et al., 2007). MKK1 and MKK2 interact with both MPK4 and MEKK1 in yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays (Gao et al., 2008; Mizoguchi et al., 1998; Teige et al., 2004), suggesting that MEKK1, MKK1/MKK2, and MPK4 form a kinase cascade. This is supported by observations that MPK4 phosphorylation is impaired in *mekk1* and *mkk1 mkk2* mutant plants treated with the flagellin-derived peptide flg22 (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006; Qiu et al., 2008b; Suarez-Rodriguez et al., 2007). Interestingly, unlike MPK4, the kinase activity of MEKK1 is not required for either the *mekk1* mutant phenotypes or flg22-induced activation of MPK4, suggesting that MEKK1 may serve as a scaffold to facilitate interactions between MKK1/MKK2 and MPK4 (Suarez-Rodriguez et al., 2007). Downstream of MPK4, MAP kinase 4 substrate 1 (MKS1) was identified as a direct substrate of MPK4 (Andreasson et al., 2005). Silencing MKS1 has a modest effect on the mutant morphology of *mpk4*. The detailed mechanism of the negative regulation of plant immunity by the MEKK1-MKK1/MKK2-MPK4 kinase cascade remains largely unclear.

To overcome PTI, pathogens deliver a large repertoire of effectors into plant cells to subvert defense responses and promote pathogenesis. MAP kinase pathways are one of the major targets of pathogen effectors. *Pseudomonas syringae* HopAI1 is an ortholog of *Shigella* effector OspF, which irreversibly inactivates mammalian MAP kinase Erk1/2, c-Jun N-terminal kinase, and p38 by cleaving phosphate groups from phosphothreonines (Li et al., 2007). In *Arabidopsis*, HopAI1 targets MPK3 and MPK6 and inactivates their kinase activities to suppress plant defense responses (Zhang et al., 2007). Another example of an effector targeting the MAP kinase pathway is HopF2 from *Pseudomonas syringae* (Wang et al., 2010). HopF2 ADP-ribosylates MKK5 and blocks its kinase activity. Other pathogen effector proteins such as AvrB from *Pseudomonas syringae* and VirE2 from *Agrobacterium* were also reported to manipulate plant MAP kinase pathways to promote virulence (Cui et al., 2011; Djamei et al., 2007). These findings highlight the importance of MAP kinase pathways in host-pathogen interactions.

It has been proposed that R proteins evolved to protect plants from microbial effectors that suppress basal resistance (Chisholm et al., 2006; Jones and Dangl, 2006). In this study, we showed that the MEKK1-MKK1/MKK2-MPK4 kinase cascade is required for basal defense and that it is protected by the plant CC-NB-LRR R protein SUMM2. Disruption of the kinase pathway leads to activation of immunity mediated by SUMM2.

RESULTS

Identification and Characterization of *summ2-1 mkk1 mkk2* Triple Mutant

To understand the molecular mechanism underlying the autoimmune phenotypes of the *mkk1 mkk2* double mutant, a genetic screen was carried out to identify suppressors of *mkk1 mkk2*. Seeds of *mkk1 mkk2* were mutagenized with EMS, and the M2 plants were screened for mutants with wild-type morphology. About 50 mutants were obtained from the screen. One of the mutants, *summ2-1* (suppressor of *mkk1 mkk2*, *2-1*), completely suppresses the extreme dwarfism of *mkk1 mkk2* (*mkk1/2*) (Figure 1A).

In *mkk1 mkk2*, cell death is constitutively activated (Gao et al., 2008; Qiu et al., 2008b). To check whether *summ2-1* suppresses cell death in *mkk1 mkk2*, *summ2-1 mkk1 mkk2* seedlings were stained with trypan blue. Extensive cell death was observed in *mkk1 mkk2*, but not in *summ2-1 mkk1 mkk2* (Figure 1B). Since *mkk1 mkk2* accumulates reactive oxygen species, we performed 3,3′-diaminobenzidine (DAB) staining on the mutant plants to examine H_2O_2 accumulation. As shown in Figure 1C, the elevated H_2O_2 levels in *mkk1 mkk2* were reduced to wild-type level in *summ2-1 mkk1 mkk2*.

To determine whether constitutive defense responses in *mkk1 mkk2* were also suppressed by *summ2-1*, the expression levels of two defense marker genes, *Pathogenesis-Related 1* (*PR1*) and *PR2*, were determined by qPCR. The constitutive expression of *PR1* and *PR2* in *mkk1 mkk2* was completely abolished by *summ2-1* (Figures 1D and 1E). Taken together, these data

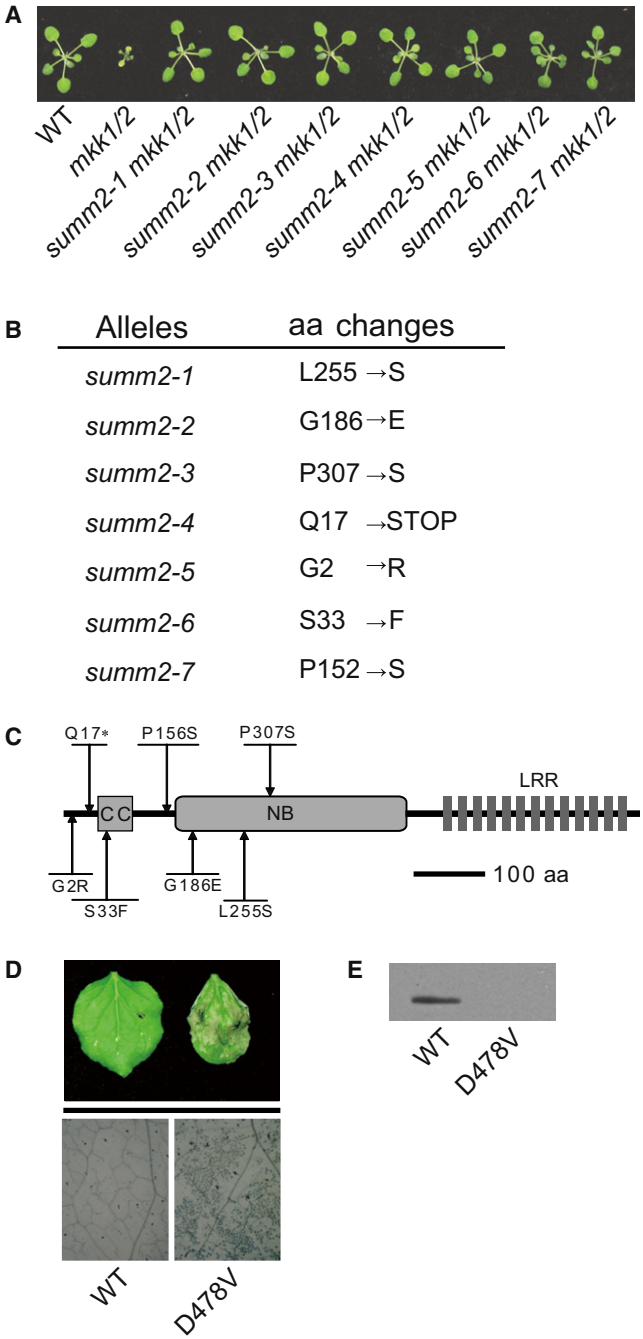

Figure 2. Identification of *SUMM2*

(A) Morphology of the seven *summ2 mkk1/2* mutants and Col (WT).

(B) The effect of each *summ2* mutation on the amino acid sequence.

(C) Protein structure of SUMM2. CC, coiled-coil domain; NB, nucleotide binding domain; LRR, leucine-rich repeat domain. The position of the affected amino acid in each *summ2* mutant is indicated.

(D) *Agrobacterium*-mediated transient expression of the D478V mutant of SUMM2 in *N. benthamiana* results in HR-like cell death. (Top panel) Photos of *N. benthamiana* leaves infiltrated with *Agrobacterium* carrying constructs expressing the indicated proteins. (Lower panel) Trypan blue staining of leaves infiltrated with *Agrobacterium* carrying constructs expressing the indicated proteins. WT, wild-type; D478V, Asp478 substituted for Val. All proteins were expressed with a C-terminal HA tag.

(E) Western blot analysis of wild-type SUMM2 and the D478V mutant in *N. benthamiana* leaves infiltrated with *Agrobacterium* carrying constructs expressing the indicated proteins with a C-terminal HA tag using an anti-HA antibody. See also Figure S1.

indicate that the *summ2-1* mutation suppresses the autoimmune phenotypes of *mkk1 mkk2*.

SUMM2 Encodes a CC-NB-LRR Protein

To map *summ2-1*, we crossed *summ2-1 mkk1 mkk2* (in the genetic background of the Columbia [Col-0] ecotype) with wild-type Landsberg *erecta* (L*er*) to generate a segregating F2 population. Plants homozygous for *mkk1 mkk2* were identified by PCR genotyping and used for linkage analysis. Crude mapping showed that *summ2-1* is located in a region flanked by markers F22O13 and T20H2 on chromosome 1 (see Figure S1A available online). Fine mapping narrowed the mutation to a region of ~300 kb between F12F1 and F13K23. Sequencing candidate genes in the region revealed a G to A point mutation in *At1g12280* (GenBank accession number NM_101100), which results in an amino acid change from Leu255 to Ser (Figure S1A).

To test whether other *mkk1 mkk2* suppressors carry mutations in *At1g12280*, we sequenced *At1g12280* in the rest of *mkk1 mkk2* suppressors and identified six additional alleles of *summ2* (Figure 2). To further confirm that suppression of the autoimmune phenotypes in *summ2-1 mkk1 mkk2* was indeed caused by loss of function of *At1g12280*, a T-DNA insertion mutant of *At1g12280*, designated *summ2-8*, was obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *summ2-8* T-DNA insert is in the coding region of the gene. The expression level of *At1g12280* was dramatically reduced in *summ2-8* (Figure S1B). When *summ2-8* was crossed into *mkk1 mkk2*, the *summ2-8 mkk1 mkk2* triple mutant exhibited wild-type morphology (Figure S1C). In addition, activation of cell death, accumulation of H_2O_2, and constitutive expression of *PR1* and *PR2* in *mkk1 mkk2* were also suppressed in *summ2-8 mkk1 mkk2* (Figures S1D–S1G). These data indicate that loss of *At1g12280* function suppresses the autoimmune phenotypes of *mkk1 mkk2*.

Sequence analysis revealed that *At1g12280* encodes a CC-NB-LRR protein. Among the seven *summ2* alleles obtained by EMS mutagenesis, six carry missense mutations in the CC or NB domains, and one carries an early nonsense mutation (Figures 2B and 2C). The closest homolog of SUMM2 in *Arabidopsis* is RPS5 (Warren et al., 1998). SUMM2 and RPS5 share ~65% identity and 80% similarity at amino acid level. *SUMM2* is present in all 80 *Arabidopsis* ecotypes that have been sequenced (Cao et al., 2011). In four of these ecotypes it contains single base pair deletions that cause frameshifts leading to truncation of the protein.

Substitution of the Asp to Val in a conserved MHD motif of the NB domain was found to constitutively activate several CC-NB-LRR R proteins without pathogen interaction (Bendahmane et al., 2002; Howles et al., 2005; Tameling et al., 2006). To test whether SUMM2 is truly an R protein, we generated a construct expressing a SUMM2 mutant with Asp478 in the MHD motif changed to Val. *Agrobacterium*-mediated transient expression of the D478V mutant in *N. benthamiana* resulted in hypersensitive response (HR)-like cell death (Figure 2D).

Cell death in the infiltrated leaves was confirmed by trypan blue staining (Figure 2D). In the wild-type SUMM2 control, we did not observe HR-like cell death. Western blot analysis detected the wild-type SUMM2, but not the D478V mutant protein (Figure 2E), suggesting that the mutant protein is probably turned over rapidly.

Constitutive Activation of Cell Death and Defense Responses in *mekk1* Is Dependent on SUMM2

Since MEKK1 acts upstream of MKK1/MKK2 in a MAP kinase cascade, we tested whether the autoimmune phenotypes of *mekk1* are also mediated by SUMM2. To obtain *summ2 mekk1* double mutants, we crossed *summ2-4* and *summ2-8* with *mekk1-1* and identified double mutants in the F2 population by PCR genotyping. As shown in Figure 3A, the extreme dwarf morphology of *mekk1* was completely suppressed by the *summ2* mutations. Trypan blue staining showed that cell death in *mekk1-1* was also completely suppressed by *summ2-4* and *summ2-8* (Figure 3B). In addition, H_2O_2 accumulation in *mekk1-1* was blocked by the *summ2* mutations (Figure 3C). Furthermore, the constitutive expression of *PR1* and *PR2* in *mekk1-1* was abolished in the *mekk1 summ2* double mutants (Figures 3D and 3E). Collectively, these data indicate that SUMM2-dependent immunity is activated in *mekk1* mutants.

mpk4 Activates SUMM2-Mediated Cell Death and Defense Responses

MPK4 acts downstream of MKK1/MKK2 and MEKK1. To test whether cell death and defense response activation in *mpk4* are dependent on SUMM2, we performed double mutant analysis. Double mutants were obtained by crossing *mpk4-3* with *summ2-4* and *summ2-8* and screening the F2 population using genotyping markers specific to the mutations of interest. As shown in Figure 3F, the sizes of the double mutants were slightly smaller than wild-type plants but much bigger than *mpk4-3*. Cell death and H_2O_2 accumulation in *mpk4-3* were also dramatically reduced in the *summ2 mpk4-3* double mutants (Figures 3G and 3H). In addition, constitutive *PR1* and *PR2* expression in *mpk4-3* was mostly suppressed in the *summ2 mpk4-3* double mutants (Figures 3I and 3J). These data suggest that activation of defense responses in *mpk4-3* is largely dependent on SUMM2. Unlike the *summ2 mekk1* and *summ2 mkk1 mkk2* mutant plants, constitutive cell death, accumulation of H_2O_2, and *PR* gene expression are not completely blocked in *summ2 mpk4-3* double mutants, suggesting that MPK4 is most likely also involved in the negative regulation of immune responses that are independent of SUMM2.

To test whether SUMM2 associates with MPK4, MKK1/MKK2, and MEKK1 in vivo, we analyzed their interactions using a BiFC approach (Walter et al., 2004). As shown in Figure S2A, no interaction was observed between SUMM2 and MPK4, MKK1/MKK2, or MEKK1. As a positive control, *Arabidopsis* mesophyll protoplasts were cotransformed with constructs expressing MKK1-YFP^N and MPK4-YFP^C, and YFP fluorescence was observed on the plasma membrane of the transformed protoplasts. We also tested whether SUMM2 interacts with MKS1 and did not find any interaction between them either (Figure S2A).

To test whether SUMM2 is required for MPK4 activation, we analyzed the kinase activity of immunoprecipitated MPK4 from wild-type and *summ2-8* plants using myelin-basic protein (MBP) as the substrate. As shown in Figure S2B, activation of MPK4 by flg22 treatment was not affected in *summ2-8*. MKS1 and WRKY33 are two downstream target proteins of MPK4 that work together to regulate the expression of the calmalexin synthesis gene *PAD3* (Qiu et al., 2008a). Consistent with the observation that SUMM2 is not required for the activation of MPK4 by flg22, SUMM2 is not required for flg22-induced upregulation of *PAD3* (Figure S2C).

MKK1/MKK2 and MEKK1 Are Required for Basal Resistance in *Arabidopsis*

Activation of the MEKK1-MKK1/MKK2-MPK4 kinase cascade by PAMPs suggests that kinase cascades may also play important roles in the positive regulation of plant immunity. This positive role in immunity could be masked by the activation of SUMM2 in the *mekk1*, *mkk1 mkk2*, and *mpk4* mutant backgrounds. To test whether MKK1 and MKK2 are required for basal defense, we inoculated *summ2 mkk1 mkk2* with the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2. As shown in Figure 4A, *summ2 mkk1 mkk2* exhibited enhanced susceptibility to *H.a.* Noco2, suggesting that MKK1 and MKK2 positively regulate basal defense. We also challenged the *summ2 mkk1 mkk2* triple mutants with the bacterial pathogen *Pseudomonas syringae pv tomato* (*P.s.t.*) DC3000. As shown in Figure 4B, growth of *P.s.t.* DC3000 in *summ2 mkk1 mkk2* is about 5-fold higher than in wild-type, further supporting a positive role of MKK1 and MKK2 in basal defense.

To determine whether MEKK1 is also required for basal defense, we challenged *mekk1 summ2* mutant plants with *H.a.* Noco2 and *P.s.t.* DC3000, respectively. As shown in Figures 4C and 4D, the *summ2-4 mekk1* and *summ2-8 mekk1* double mutant also exhibited enhanced susceptibility to these pathogens, suggesting that MEKK1 is also a positive regulator of basal defense.

Next we tested whether MKK1/MKK2 and MEKK1 are required for PAMP-induced oxidative burst and callose deposition. As shown in Figures S3A and S3B, flg22-triggered oxidative burst was not affected in *summ2-8 mkk1 mkk2* and *summ2-8 mekk1* mutant plants. Callose deposition induced by flg22 was also unaffected in *summ2-8 mkk1 mkk2* and *summ2-8 mekk1* (Figures S3C and S3D). *FRK1* is a defense marker gene strongly induced by flg22. As shown in Figure S3E, induction of *FRK1* by flg22 was not affected in either *summ2-8 mkk1 mkk2* or *summ2-8 mekk1*. Interestingly, induction of *FMO1*, a gene required for both basal resistance and systemic acquired resistance (Bartsch et al., 2006; Jing et al., 2011; Mishina and Zeier, 2006), was compromised in both *summ2-8 mkk1 mkk2* and *summ2-8 mekk1* (Figure S3F).

HopAI1 Activates SUMM2-Mediated Immunity

Because loss of MEKK1, MKK1/MKK2, or MPK4 function activates SUMM2-mediated defense responses, we tested whether disruption of the MEKK1-MKK1/MKK2-MPK4 kinase cascade by microbial pathogens can also trigger SUMM2-mediated immunity. Since HopAI1 has been shown to target MPKs (Zhang et al., 2007), we examined whether the expression of HopAI1 in transgenic plants activates SUMM2-mediated defense responses. Wild-type and *summ2-8* plants were transformed with a construct expressing HopAI1-FLAG fusion protein

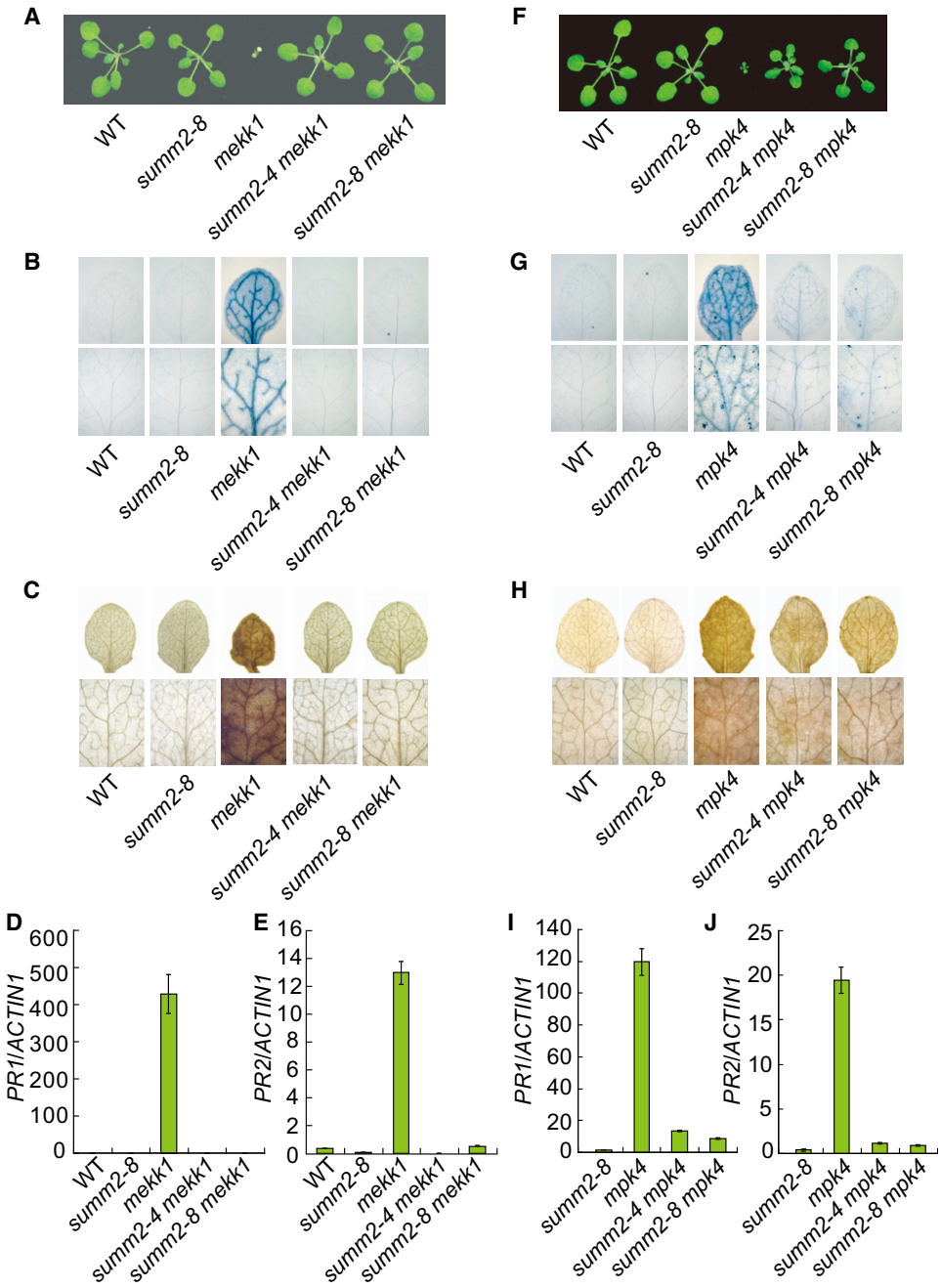

Figure 3. Suppression of the Autoimmune Phenotypes of *mekk1* and *mpk4* by *summ2* Mutations

(A) Morphology of 3-week-old soil-grown WT (wild-type), *summ2-8*, *mekk1-1*, *summ2-4 mekk1-1*, and *summ2-8 mekk1-1* plants.

(B and C) Trypan blue staining (B) and DAB staining (C) of true leaves of WT, *summ2-8*, *mekk1-1*, *summ2-4 mekk1-1*, and *summ2-8 mekk1-1*.

(D and E) Expression levels of *PR1* (D) and *PR2* (E) in WT, *summ2-8*, *mekk1-1*, *summ2-4 mekk1-1*, and *summ2-8 mekk1-1* as determined by qPCR. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations of three measurements.

(F) Morphology of 3-week-old soil grown WT, *summ2-8*, *mpk4-3*, *summ2-4 mpk4-3*, and *summ2-8 mpk4-3*.

(G and H) Trypan blue staining (G) and DAB staining (H) of true leaves of WT, *summ2-8*, *mpk4-3*, *summ2-4 mpk4-3*, and *summ2-8 mpk4-3*.

(I and J) Expression levels of *PR1* (I) and *PR2* (J) in WT, *summ2-8*, *mpk4-3*, *summ2-4 mpk4-3*, and *summ2-8 mpk4*-3 as determined by qPCR. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations of three measurements. See also Figure S2.

under the control of an estradiol-inducible promoter (Li et al., 2005). About 20% of transgenic lines in the wild-type background exhibited dwarf morphology without treatment of estradiol, and most of the dwarf plants died at seedling stage. In contrast, none of the transgenic plants in *summ2-8* background displayed the dwarf phenotype.

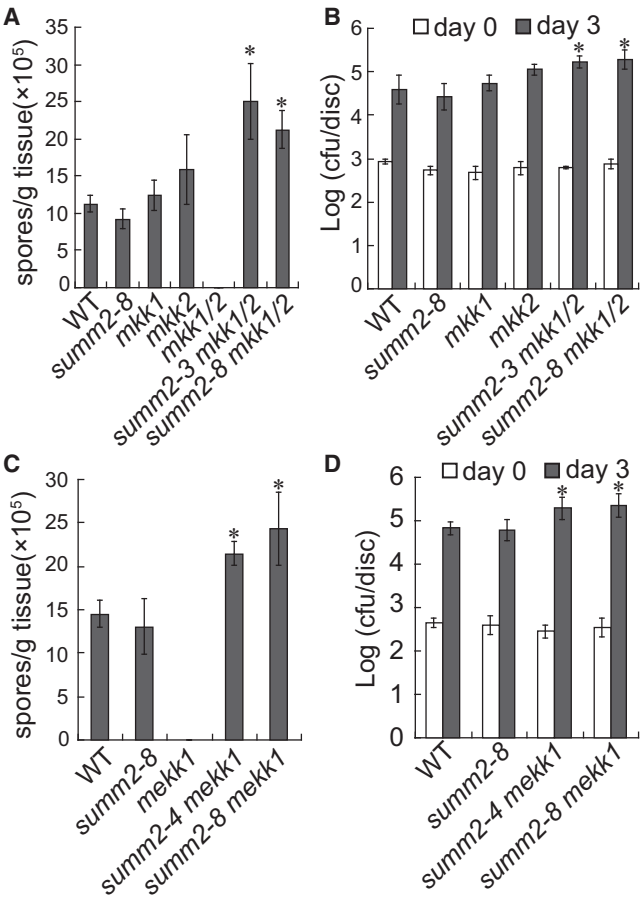

Figure 4. MKK1/MKK2 and MEKK1 Positively Regulate Basal Resistance

(A) Growth of *H.a.* Noco2 on Col (WT), *summ2-8*, *mkk1-1*, *mkk2-1*, *summ2-3 mkk1 mkk2* (*mkk1/2*), and *summ2-8 mkk1 mkk2* plants. Eighteen-day-old plants were treated with *H.a.* Noco2 at 5 × 10^4 spores/ml. Spores were collected and scored 7 days after inoculation. Error bars represent standard deviations of three replicates. *p < 0.01, statistical difference from wild-type.

(B) Growth of *P.s.t.* DC3000. Plants were infiltrated with *P.s.t.* DC3000 at a concentration of OD_{600} = 5 × 10^{-4}. Error bars represent standard deviations of bacterial growth from five different plants. *p < 0.01, statistical difference from wild-type.

(C) Growth of *H.a.* Noco2. Error bars represent standard deviations of three replicates. *p < 0.05, statistical difference from wild-type.

(D) Growth of *P.s.t.* DC3000. The plants were infiltrated with *P.s.t.* DC3000 at a concentration of OD_{600} = 5 × 10^{-4}. Error bars represent standard deviations of five replicates. *p < 0.05, statistical difference from wild-type. See also Figure S3.

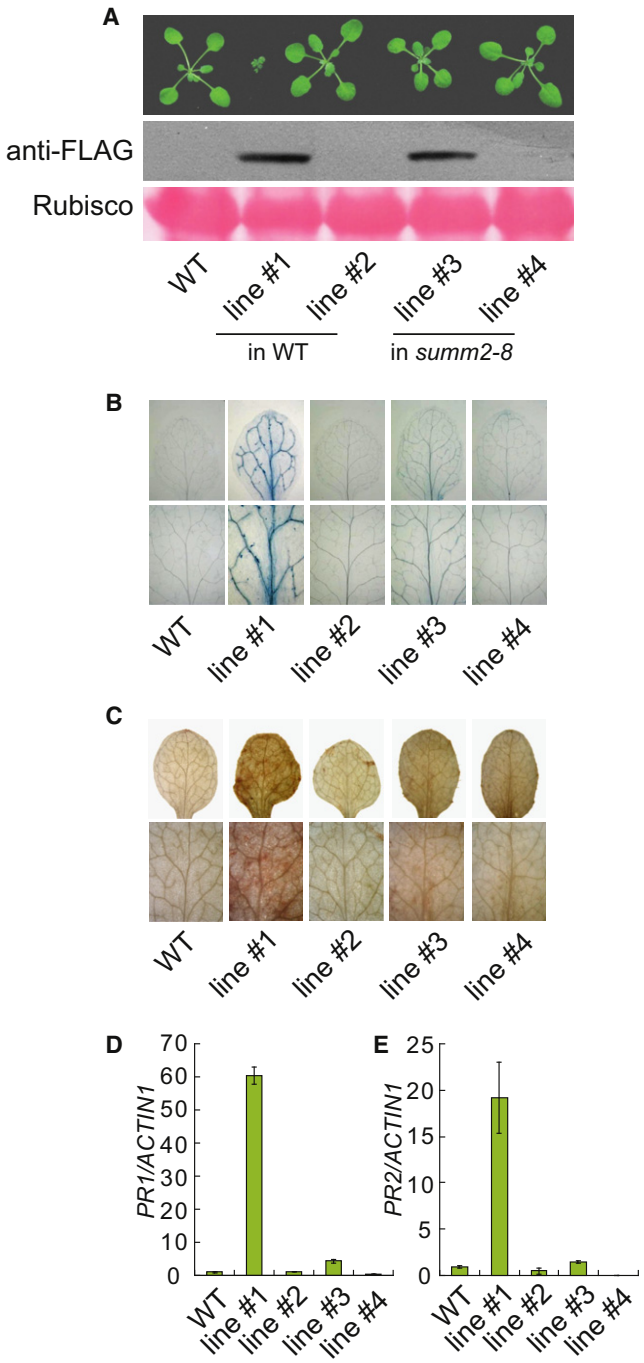

Figure 5. SUMM2 Is Required for HopAI1-Triggered Defense Responses

(A) Morphology of 3-week-old soil-grown *HopAI1-FLAG* transgenic lines and HopAI1-FLAG expression levels. Lines #1 and #2 are two representative *HopAI1-FLAG* transgenic lines from the transformation of wild-type plants. Lines #3 and #4 are two representative *HopAI1-FLAG* transgenic lines from the transformation of *summ2-8* plants.

(B and C) Trypan blue staining (B) and DAB staining (C) of the true leaves of indicated genotypes.

(D and E) Expression levels of *PR1* (D) and *PR2* (E) as determined by qPCR. Values were normalized relative to the expression of *ACTIN1*. Error bars represent standard deviations of three measurements. See also Figure S4.

Two representative HopAI1-FLAG transgenic lines in the wild-type background were chosen for further analysis. Western blot analysis showed that there is leaky expression of HopAI1-FLAG protein in line #1, but not in line #2 when the plants were not treated with estradiol (Figure 5A). Two HopAI1-FLAG transgenic lines (lines #3 and #4) in *summ2-8* background were used as controls. Line #3 showed similar leaky expression as line #1 in wild-type background, while line #4 has no detectable expression of the fusion protein. Among the transgenic lines, only line #1 exhibited dwarf morphology. Extensive cell death (Figure 5B) and high level of H_2O_2 (Figure 5C) were also observed

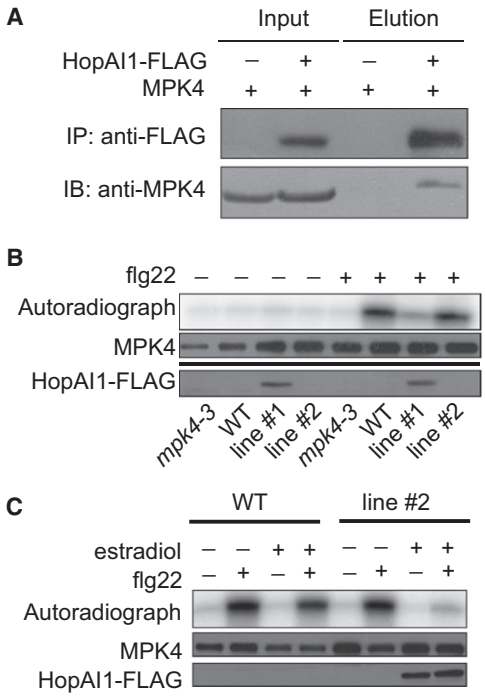

Figure 6. HopAI1 Targets MPK4 In Vivo

(A) MPK4 interacts with HopAI1 in vivo. Two-week-old seedlings were pretreated with 50 μM estradiol. Soluble proteins were exacted from nontransformed wild-type and *HopAI1* transgenic plants and immunoprecipitated with an agarose-conjugated anti-FLAG antibody. Crude lysates (Input) and immunoprecipitated proteins (Elution) were detected with anti-MPK4 and anti-FLAG antibody, respectively. Two independent experiments were performed with similar results.

(B and C) Suppression of MPK4 kinase activity in HopAI1-FLAG transgenic line #1 (B) and estradiol-treated line #2 (C). Two-week-old seedlings were treated with or without 10 μM flg22 for 10 min to induce the activation of MPK4. Line #2 was treated with 50 μM of estradiol to induce the expression of HopAI1-FLAG in (C). Total protein was extracted and MPK4 was immunoprecipitated with anti-MPK4 antibodies. The kinase activity (autoradiograph) of MPK4 was detected using MBP as a substrate.

in line #1, but not other transgenic lines. Analysis of the expression of *PR1* (Figure 5D) and *PR2* (Figure 5E) showed that both genes were constitutively expressed in line #1, but not other transgenic lines. These data suggest that leaky expression of HopAI1 activates SUMM2-mediated defense responses.

Next we analyzed cell death and defense gene expression in lines #2 and #4 after treatment with estradiol. Estradiol induced the expression of HopAI1-FLAG in both line #2 and line #4 (Figure S4A). As shown in Figures S4B–S4D, treatment with estradiol resulted in activation of cell death and upregulation of *PR1* and *PR2* expression in line #2, but not line #4, confirming that expression of HopAI1 activates SUMM2-dependent defense responses.

MPK4 Is a Target of HopAI1

Although HopAI1 is known to interact with MPK3 and MPK6 (Zhang et al., 2007), whether HopAI1 interacts with MPK4 was not previously tested. To determine whether MPK4 is a target of HopAI1, coimmunoprecipitation experiments were performed using HopAI1-FLAG transgenic plants. As shown in Figure 6A, MPK4 coimmunoprecipitated with HopAI1-FLAG, suggesting that HopAI1-FLAG associates with MPK4 in vivo. Next we tested whether the kinase activity of MPK4 is affected in the HopAI1-FLAG transgenic plants. MPK4 was immunoprecipitated with anti-MPK4 antibodies and assayed for kinase activity using MBP as a substrate. In HopAI1-FLAG line #1, a clear reduction of MPK4 activity was observed (Figure 6B). We also analyzed the MPK4 kinase activity in HopAI1-FLAG line #2 after treatment with estradiol. As shown in Figure 6C, treatment of estradiol induced the expression of HopAI1-FLAG and resulted in reduced activation of MPK4 by flg22 in HopAI1-FLAG line #2. These data suggest that HopAI1 targets MPK4 to block its kinase activity.

DISCUSSION

MAP kinase cascades are involved in signal amplification during diverse biological processes (MAPK-Group, 2002). It has previously been shown that the MEKK1-MKK1/MKK2-MPK4 kinase cascade negatively regulates cell death and defense responses (Gao et al., 2008; Qiu et al., 2008b). Loss-of-function mutations to *MEKK1*, *MKK1/MKK2*, and *MPK4* all lead to extreme dwarfism and enhanced resistance to pathogens (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006; Petersen et al., 2000; Qiu et al., 2008b; Suarez-Rodriguez et al., 2007), but the underlying mechanism causing these phenotypes has not been uncovered. Here we report that the autoimmune responses caused by loss of the MAP kinase cascade function are mediated by the CC-NB-LRR protein SUMM2. Mutations in SUMM2 suppress the dwarf morphology as well as constitutive defense responses in *mekk1*, *mkk1 mkk2*, and *mpk4* mutant plants, suggesting that SUMM2 serves as a molecular sensor of the activity of the MEKK1-MKK1/MKK2-MPK4 kinase cascade. Disturbance of this MAP kinase cascade would activate SUMM2-mediated defense responses.

In support of this hypothesis, the *Pseudomonas* effector protein HopAI1 is capable of targeting MPK4 to inhibit its kinase activity, and activating SUMM2-mediated defense responses. Expression of HopAI1 in wild-type plants, but not in *summ2-8* plants, leads to dwarfism and activation of immune responses. These data suggest that SUMM2 protects the MEKK1-MKK1/MKK2-MPK4 kinase cascade from disruption by microbial pathogens. In *P.s.t.* DC3000, HopAI1 is inactivated by an insertion in its promoter region (Lindeberg et al., 2006), which explains the lack of resistance to *P.s.t.* DC3000 in wild-type plants carrying *SUMM2*. It remains to be determined whether there are additional pathogen effectors that also target the MEKK1-MKK1/MKK2-MPK4 kinase cascade and activate SUMM2-mediated immunity.

When the D478V mutant of SUMM2 was overexpressed in *N. benthamiana* by *Agrobacterium*-mediated transient expression, cell death was observed in the whole infiltrated area. In contrast, cell death in the *mekk1*, *mkk1 mkk2*, and *mpk4* mutant plants appears predominantly in cells along the vascular tissue. Whether this is caused by tissue-specific expression of SUMM2 or an unknown factor required for activation of SUMM2 in these areas remains to be determined.

R proteins are often directly associated with their guardees (Jones and Dangl, 2006). Since we did not observe interactions

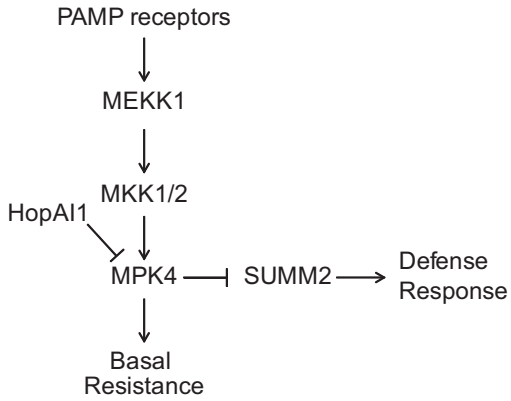

Figure 7. A Model for Dual Functions of the MEKK1-MKK1/MKK2-MPK4 Kinase Cascade in Plant Immunity

The MEKK1-MKK1/MKK2-MPK4 kinase cascade functions downstream of PAMP receptors to positively regulate basal resistance. It also negatively regulates immunity specified by the NB-LRR protein SUMM2. Disruption of the MAP kinase cascade leads to activation of SUMM2 and its downstream defense responses.

between MPK4 and SUMM2, SUMM2 probably does not guard MPK4 through direct protein-protein interaction. The requirement of MPK4 activity for suppressing cell death, defense responses, and the activation of SUMM2-mediated defense responses by HopAI1 indicates that SUMM2 senses changes in MPK4 activity. The guardee of SUMM2 is most likely a protein that functions downstream of MPK4. Future identification of the guardee that SUMM2 directly interacts with will help improve our understanding of how MEKK1-MKK1/MKK2-MPK4 kinase cascade disruption leads to the activation of SUMM2-mediated immune responses.

In addition to negatively regulating plant defense responses, MPK4 also functions downstream of MKK6/ANQ to regulate cytokinesis and plant development (Beck et al., 2010; Kosetsu et al., 2010; Takahashi et al., 2010), suggesting that MPK4 is a multifunctional MAP kinase. We found that suppression of constitutive defense responses in *summ2 mpk4* double mutants is not as complete as in the *summ2 mekk1* and *summ2 mkk1 mkk2* mutant plants. The *summ2 mpk4* double mutant still exhibited residual cell death and enhanced *PR* gene expression. In contrast, *summ2* mutations completely suppressed cell death and defense gene expression in *mekk1* and *mkk1 mkk2*. It is likely that MPK4 also functions in other MAP kinase cascades to negatively regulate SUMM2-independent immune responses, while MEKK1 and MKK1/MKK2 do not participate in those cascades.

Surprisingly, in addition to their function in repressing SUMM2-mediated immunity, MEKK1 and MKK1/MKK2 were found to be required for basal resistance to *H.a.* Noco2 and *P.s.t.* DC3000. Enhanced susceptibility to *H.a.* Noco2 and *P.s.t.* DC3000 was observed in *summ2 mekk1* and *summ2 mkk1 mkk2* plants. Because constitutive defense responses in *mpk4* are not completely suppressed by mutations in *SUMM2*, we were not able to analyze the role of MPK4 in basal defense. As MEKK1, MKK1/MKK2, and MPK4 form a kinase cascade that is activated during PTI, it is likely that MPK4 functions together with MEKK1 and MKK1/MKK2 to positively regulate basal defense.

Based on our study, a working model was proposed in Figure 7. The MEKK1-MKK1/MKK2-MPK4 kinase cascade has dual functions in plant immunity. It positively regulates basal resistance and negatively regulates immunity mediated by the NB-LRR protein SUMM2. Inactivation of the kinase cascade by bacterial effector proteins such as HopAI1 leads to the activation of immune responses mediated by SUMM2. It was hypothesized that R protein-mediated immunity was evolved to neutralize attacks by pathogen effectors which target PTI to promote virulence (Chisholm et al., 2006; Jones and Dangl, 2006). Our study provides clear evidence of evolution of a plant NB-LRR protein to protect PTI from disruption by pathogens.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Plants were grown at 23°C under 16 hr light/8 hr dark on soil or 1/2 MS medium. *mekk1-1*, *mkk1-1 mkk2-1* (*mkk1 mkk2*), and *mpk4-3* mutants (all in Col background) were described previously (Gao et al., 2008; Ichimura et al., 2006; Nakagami et al., 2006). *mkk1 mkk2* is temperature sensitive and seedling lethal at 23°C but can complete its life cycle at 28°C. Seeds of *mkk1 mkk2* were obtained by growing the plants at 28°C. To screen for suppressors of *mkk1 mkk2*, *mkk1 mkk2* seeds were mutagenized with EMS, and the M2 population was screened for mutants with wild-type-like morphology at 23°C.

The *summ2-4* single mutant was obtained by backcrossing *summ2-4 mkk1 mkk2* with wild-type Col plants. The *summ2-4 mekk1-1* and *summ2-4 mpk4-3* double mutants were generated by crossing *summ2-4* with *mekk1-1* and *mpk4-3*. *summ2-8* (SAIL_1152_A06) was obtained from ABRC. The *summ2-8 mekk1-1* and *summ2-8 mpk4-3* double mutants were generated by crossing *summ2-8* with *mekk1* or *mpk4-3*.

The construct expressing HopAI1-FLAG has been previously described (Li et al., 2005). The construct was introduced into *Agrobacterium* and used to transform wild-type and *summ2-8* plants.

Mutant Characterization

To measure gene expression, total RNA was extracted from 12-day-old seedlings grown on 1/2 MS medium using RNAiso reagent (Takara). Reverse transcription was conducted with M-MLV reverse transcriptase (Takara), and qPCR was carried out using the SYBR Premix Ex TaqII kit (Takara). The primers used to amplify *PR1*, *PR2*, and *Actin1* have been previously described (Zhang et al., 2003). The primers used for amplification of *SUMM2* are 5′-AGCATC TACTGGCAACCTC-3′ and 5′-ATCATTTCATTGCTTCAGCTC-3′. The primers used for amplification of *PAD3* are 5′-TACTTGTTGAGATGGCATTGTTGAA-3′ and 5′- CTTCCTCCTGCTTCGCCAAT-3′. The primers used for amplification of *FRK1* are 5′-TCTGAAGAATCAGCTCAAGGC-3′ and 5′-TGTTGGCTTCA CATCTCTGTG-3′. The primers used for amplification of *FMO1* are 5′-GGAGA TATTCAGTGGCATGC-3′ and 5′-TTTGGTTAGGCCTATCATGG-3′.

Trypan blue and DAB staining were carried out on 2-week-old seedlings grown on 1/2 MS medium following previously described procedures (Parker et al., 1996; Thordal-Christensen et al., 1997). Measurement of oxidative burst was performed on leaf strips of 5-week-old plants grown under short day conditions using a luminal-dependent assay (Trujillo et al., 2008). For the callose deposition assay, 5-week-old short-day grown plants were infiltrated with 1 μmol flg22 and stained for callose deposition 24 hr later as previously described (Hauck et al., 2003).

Pathogen Infections

For *H.a.* Noco2 infections, 2-week-old seedlings were sprayed with *H.a.* Noco2 spores (5×10^4 spores/mL). Seven days postinfection, spores from approximately ten seedlings per line were collected and counted using a hemocytometer as previously described (Bi et al., 2010). For *P.s.t.* DC3000 infections, 5-week-old seedlings grown at 23°C under 12 hr light/12 hr dark

were infiltrated with bacterial suspensions ($OD_{600} = 5 \times 10^{-4}$). Bacterial growth in the inoculated leaves was determined 3 days after inoculation.

Map-Based Cloning of *SUMM2*

To map *summ2-1*, the *summ2-1 mkk1 mkk2* triple mutant in Col background was crossed with wild-type L*er*. Plants homozygous for *mkk1 mkk2* in the F2 population were identified by PCR and used for linkage analysis. The markers used for mapping *summ2-1* were designed based on the Monsanto *Arabidopsis thaliana* polymorphism and L*er* sequence collection (Jander et al., 2002). Marker primers used include F22O13, 5′-TCGCTTTCCACAAAGGCGAG-3′ and 5′-CACTTCCAACCATCAGAGGC-3′; F13K23, 5′-TTTATTTCACACATAG TGCAG-3′ and 5′-GGAGATTTAGGGGATTACGAGATCG-3′; and T20H2, 5′-GATAAACCTGTTACAGCCTGA-3′ and 5′-CATGACATTGAAGTCCCTGA-3′. Markers F22O13, F13K23, and T20H2 were designed using Indel polymorphisms. Marker F12F1 is based on a SNP polymorphism. The common primer is 5′-TAGTCTCTCGACTAACCACC-3′; Col-specific primer is 5′-TAATAGAC CCGCAAAGGCCT-3′; and the L*er*-specific primer is 5′-TAATAGACCCGC AAAGGCC-3′.

Agrobacterium-Mediated Transient Expression in *N. bethamiana*

For expression of full-length SUMM2, the cDNA of *SUMM2* was amplified by PCR using primers 5′-GTCATGGTCGACATGGGAGCTTGTTTAACAC TCT-3′ and 5′-CCGACTAGTGCCATAAAGACCCACAATCT-3′ and cloned into pCambia1300-3HA, a modified pCambia1300 vector containing an in-frame 3 × HA tag at the C terminus. Overlapping PCR was used to introduce the D478V mutation into SUMM2.

The primers for making the mutation are 5′-GTTAAAATGCATGTTGTGG TTCGG-3′ and 5′-CCGAACCACAACATGCATTTTAAC-3′. *Agrobacterium*-mediated transient expression in *N. bethamiana* was carried out as previously described (Bendahmane et al., 2002). Bacterial suspensions (OD_{600} = 1.0) were infiltrated into leaves of 4- to 5-week-old *N. bethamiana*. Cell death and protein expression were analyzed 3 days after infiltration.

Coimmunoprecipitation

For coimmunoprecipitation experiments, 2-week-old Col and *HopAI1-FLAG* transgenic plants grown on 1/2 MS media were sprayed with 50 nM estradiol. Twelve hours later, tissue was collected, ground in liquid nitrogen, and resuspended in 1 volume of grinding buffer (50 mM Tris-HCl 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 0.1% NP40, 1 mM PMSF, 1× Protease Inhibitor Cocktail from Roche). The samples were spun at 13,200 rpm for 10 min at 4°C, and the supernatant was incubated with 20 μl of protein G beads (GE Healthcare) for 30 min with constant rotation. After the beads were pelleted by centrifugation, the supernatant was incubated with an agarose-conjugated anti-FLAG antibody (Sigma) for 2 hr at 4°C with rotation. The beads were collected by centrifugation, washed three times with grinding buffer, and eluted by incubation with 50 μl of 100 μg/mL FLAG peptide for 30 min. The eluted proteins were subsequently analyzed by western blot using anti-MPK4 (Sigma) or anti-FLAG antibody (Sigma).

Immunocomplex Kinase Assays

To immunoprecipitate MPK4, 2-week-old seedlings grown on 1/2 MS media were pretreated with flg22 for 10 min. About 0.5 g of tissue was harvested, ground in liquid nitrogen, and resuspended in 1 ml of extraction buffer (50 mM HEPES 7.4, 50 mM NaCl, 10 mM EDTA, 0.1%Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM PI). After centrifugation at 13,200 rpm for 15 min, the supernatant was collected and incubated with 2 μl anti-MPK4 antibody (sigma) for 1 hr at 4°C with constant rotation. Protein A beads (20 μl) were then added into the samples and incubated for another 3 hr. The beads were spun down and washed three times with 1 ml extraction buffer and once with 1 ml kinase buffer (50 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 μM ATP). Beads were collected by centrifugation and resuspended in 15 μl of kinase buffer. To determine the kinase activity of MPK4, 10 μl of the beads were incubated with a solution containing 1 μl MBP, 1 μl ATP (200 μM), 10 μCi [γ-^{32}P] ATP, and 2.5 μl kinase buffer (total volume 15 μl) at 30°C for 30 min. The reactions were terminated with the addition of SDS loading buffer. Samples were separated by SDS-PAGE, and phosphorylation of MBP was detected by autoradiography.

ACCESSION NUMBERS

The GenBank accession number for the *SUMM2* sequence reported in this paper is NM_101100.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.chom.2012.01.015.

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