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The MEKK1-MKK1/MKK2-MPK4 Kinase Cascade Negatively Regulates Immunity Mediated by a Mitogen-Activated Protein Kinase Kinase Kinase in *Arabidopsis* Ⓒ Ⓦ

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In *Arabidopsis thaliana*, the MEKK1-MKK1/MKK2-MPK4 mitogen-activated protein (MAP) kinase cascade represses cell death and immune responses. In *mekk1*, *mkk1 mkk2*, and *mpk4* mutants, programmed cell death and defense responses are constitutively activated, but the mechanism by which MEKK1, MKK1/MKK2, and MPK4 negatively regulate cell death and immunity was unknown. From a screen for suppressors of *mkk1 mkk2*, we found that mutations in *suppressor of mkk1 mkk2 1* (*summ1*) suppress the cell death and defense responses not only in *mkk1 mkk2* but also in *mekk1* and *mpk4*. *SUMM1* encodes the MAP kinase kinase kinase MEKK2. It interacts with MPK4 and is phosphorylated by MPK4 in vitro. Overexpression of *SUMM1* activates cell death and defense responses that are dependent on the nucleotide binding–leucine-rich repeat protein SUMM2. Taken together, our data suggest that the MEKK1-MKK1/MKK2-MPK4 kinase cascade negatively regulates MEKK2 and activation of MEKK2 triggers SUMM2-mediated immune responses.

INTRODUCTION

Plants use a large repertoire of immune receptors to sense attacks by microbial pathogens and trigger downstream defense responses. One type of immune receptor recognizes conserved microbial components collectively known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (Boller and Felix, 2009). PAMP receptors are usually transmembrane receptor-like kinases (RLKs) or receptor-like proteins that directly interact with PAMPs. The other type of intracellular immune receptors known as Resistance (R) proteins recognizes effector molecules secreted by pathogens (Eitas and Dangl, 2010). This recognition can be either direct or indirect. Most R proteins belong to the nucleotide binding domain and leucine-rich repeat–containing (NB-LRR) protein family.

In plant defense responses, mitogen-activated protein kinase (MAPK) cascades play important roles in transducing signals from upstream receptors to the downstream targets (Pitzschke et al., 2009a). A MAPK cascade is a signaling module usually consisting of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase, and a MAPK. Activation of MAPKKKs by upstream signals results in sequential phosphorylation of their downstream MAPKKs and MAPKs. Perception of PAMP signals by PAMP receptors leads to the activation of at least two MAPK cascades composed of MEKK1-MKK4/MKK5-MPK3/MPK6 or MEKK1-MKK1/MKK2-MPK4 (Boller and Felix, 2009).

Without challenges from pathogens, plant defense responses mediated by different plant immune receptors have to be kept under tight control to prevent autoimmunity. Mutants with autoactivated immune responses often exhibit dwarf morphology, accumulate high levels of the defense hormone salicylic acid (SA), and constitutively express immunity marker genes, such as *PR1* and *PR2*. Gain-of-function mutations in immune receptors can lead to autoimmunity, and loss-of-function mutations in negative regulators that prevent autoimmune responses can also result in constitutive activation of defense responses. For example, a number of mutations in NB-LRR–type *R* genes, such as *snc1*, *ssi4*, *slh1*, *Rx*, *uni-1D*, and *chs3-3D*, have been found to constitutively activate downstream defense responses (Bendahmane et al., 2002; Shirano et al., 2002; Zhang et al., 2003a; Noutoshi et al., 2005; Igari et al., 2008; Bi et al., 2011). Gain-of-function mutations in the RLK SNC4 and receptor-like protein SNC2 also activate downstream defense responses (Bi et al., 2010; Zhang et al., 2010). Additionally, recessive mutations in *SRFR1* and *CPR1* lead to increased accumulation of R proteins SNC1 and RPS2 and constitutive activation of R protein–mediated immune responses, suggesting that negative regulation of R protein accumulation is important for preventing autoimmunity (Kim et al., 2010; Li et al., 2010; Cheng et al., 2011; Gou et al., 2012). Furthermore, loss of function of the RLK BIR1 causes activation of immunity mediated by another RLK SOBIR1 (Gao et al., 2009).

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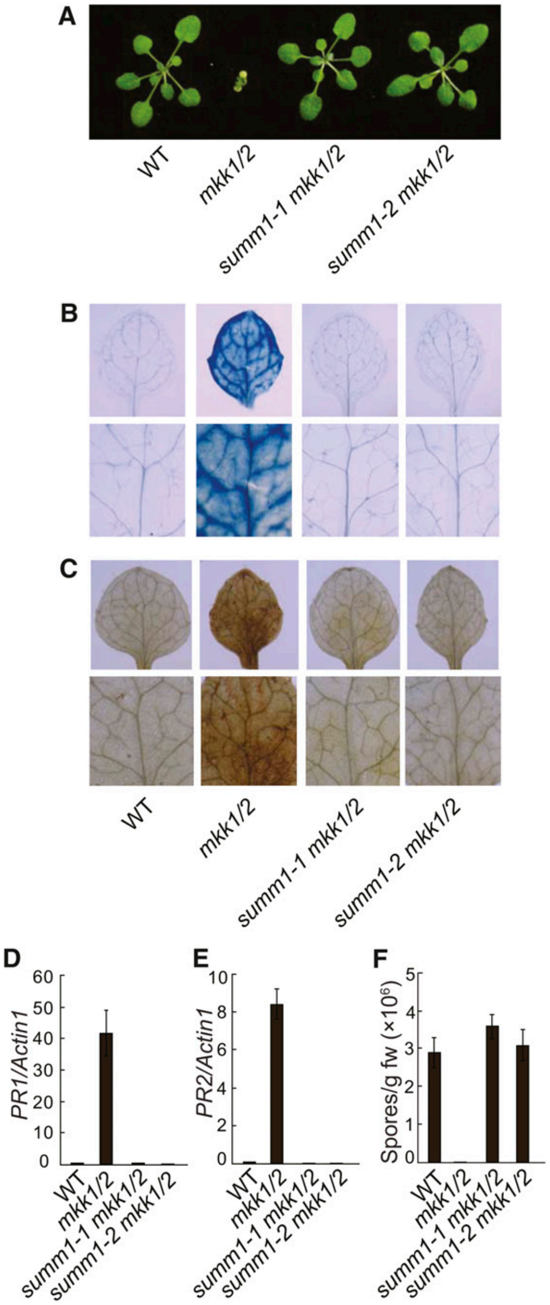


Figure 1. Suppression of *mkk1 mkk2* Mutant Phenotypes by *summ1-1* and *summ1-2*.

(A) Morphology of the wild type (WT), *mkk1 mkk2* (*mkk1/2*), *summ1-1 mkk1 mkk2*, and *summ1-2 mkk1 mkk2*. The photograph shows 4-week-old soil-grown plants.

(B) and **(C)** Trypan blue **(B)** and DAB **(C)** staining of wild-type, *mkk1 mkk2* (*mkk1/2*), *summ1-1 mkk1 mkk2*, and *summ1-2 mkk1 mkk2* seedlings.

(D) and **(E)** *PR1* **(D)** and *PR2* **(E)** expression in wild-type, *mkk1 mkk2* (*mkk1/2*), *summ1-1 mkk1 mkk2*, and *summ1-2 mkk1 mkk2* seedlings. Values were normalized to the expression of *ACTIN1*. Error bars represent ±SD of three replicates.

(F) Growth of *H.a.* Noco2 on the wild type, *mkk1 mkk2* (*mkk1/2*), *summ1-1 mkk1 mkk2*, and *summ1-2 mkk1 mkk2*. Three-week-old seedlings were sprayed with *H.a.* Noco2 spores (5 × 10^4 spores/mL). Infections were scored 7 d after inoculation by counting the number of resuspended conidia spores per gram of leaf samples. Error bars represent ±SD of three replicates. This experiment was repeated three times with similar results.

About a decade ago, it was reported that knocking out *Arabidopsis thaliana MPK4* results in constitutive defense responses (Petersen et al., 2000). The *mpk4* mutant plants are dwarf, accumulate high levels of SA, and exhibit enhanced pathogen resistance. The dwarf phenotype of *mpk4* can be partially suppressed by silencing *MAP kinase 4 substrate1* (*MKS1*) (Andreasson et al., 2005). Later studies showed that *mekk1* single mutants and *mkk1 mkk2* double mutants also exhibit similar phenotypes like *mpk4* (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009b). Analysis of *mekk1 sid2* and *mkk1 mkk2 sid2* mutant plants revealed that the elevated SA levels contribute very little to the mutant phenotypes in *mekk1* and *mkk1 mkk2* (Ichimura et al., 2006; Qiu et al., 2008).

MKK1 and MKK2 interact with MEKK1 and MPK4 in vivo, and activation of MPK4 by flg22 requires MEKK1 as well as MKK1 and MKK2, suggesting that MEKK1, MKK1/MKK2, and MPK4 form a MAPK cascade to negatively regulate plant immune responses. However, the mechanism on how this kinase cascade regulates plant immunity is unclear. In this study, we show that the autoimmunity phenotypes in *mpk4*, *mekk1*, and *mkk1 mkk2* mutant plants are caused by activation of defense responses mediated by *SUMM1* (for *SUPPRESSOR OF mkk1 mkk2 1*). *SUMM1* encodes the MAPKKK MEKK2, which is directly targeted by MPK4.

RESULTS

Identification and Characterization of *summ1* Mutants

To understand the mechanism of how the MEKK1-MKK1/MKK2-MPK4 kinase cascade negatively regulates plant immunity, a screen for suppressors of *mkk1 mkk2* was performed. Seeds of *mkk1-1 mkk2-1* were obtained by growing the mutant plants at 28°C, as high temperature partially suppresses the mutant phenotype of *mkk1 mkk2* (Gao et al., 2008). The *mkk1-1 mkk2-1* seeds were subsequently mutagenized with ethyl methanesulfonate (EMS), and the M1 plants were grown at 28°C to maturity. To identify mutants that suppress the seedling lethality phenotypes of *mkk1 mkk2*, M2 plants were grown on soil at 23°C. A total of ~50 *summ* mutants were identified. Two alleles of *summ1*, *summ1-1* and *summ1-2*, were characterized in detail.

As shown in Figure 1A, *summ1-1 mkk1 mkk2* and *summ1-2 mkk1 mkk2* exhibited wild type–like morphology. Trypan blue staining revealed that the extensive cell death observed in the *mkk1 mkk2* double mutant was completely suppressed in *summ1-1 mkk1 mkk2* and *summ1-2 mkk1 mkk2* (Figure 1B). 3,3′-diaminobenzidine (DAB) staining showed that accumulation of hydrogen peroxide (H_2O_2) in *mkk1 mkk2* was also blocked in the triple mutants (Figure 1C). In *mkk1 mkk2*, defense responses are constitutively activated. As shown in Figures 1D and 1E, constitutive expression of defense marker genes *PR1* and *PR2* was also completely suppressed in the *summ1-1 mkk1 mkk2* and *summ1-2 mkk1 mkk2* triple mutants.

To determine whether pathogen resistance in *mkk1 mkk2* is affected by the *summ1* mutations, seedlings of *summ1-1 mkk1 mkk2* and *summ1-2 mkk1 mkk2* were challenged with the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2. While *H.a.* Noco2 failed to grow on the *mkk1 mkk2* double mutant, growth of the pathogen on *summ1-1 mkk1 mkk2* and *summ1-2 mkk1 mkk2* plants was comparable to that on the wild type, suggesting that constitutive pathogen resistance in *mkk1 mkk2* was suppressed by the *summ1* mutations (Figure 1F).

Positional Cloning of *SUMM1*

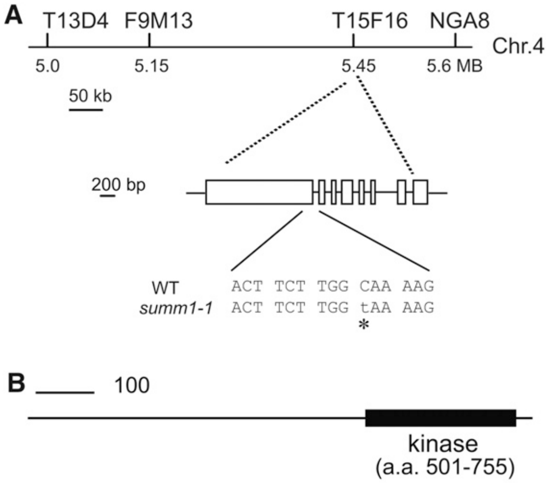
To map the *summ1-1* mutation, *summ1-1 mkk1 mkk2* (in the Columbia [Col] background) was crossed with Landsberg *erecta* (L*er*). F2 plants homozygous for *mkk1 mkk2* were selected for linkage analysis. Crude mapping using 48 such plants revealed that *summ1-1* was flanked by markers T13D4 and T15F16 on chromosome 4 (Figure 2A). Further mapping using progeny of plants that were homozygous for *mkk1 mkk2* but heterozygous for *summ1* indicated that the *summ1-1* mutation is between markers F9M13 and T15F16, a region of ~300 kb. We reasoned that *SUMM1* may be induced by pathogen infections. To identify the *summ1-1* mutation, candidate genes in this region whose expression is induced by pathogen infections were identified using the microarray database at The Arabidopsis Information Resource and sequenced. Sequencing of candidate genes revealed a C-to-T mutation in the coding region of *At4g08480*. The mutation introduced an early stop codon in *At4g08480*, which encodes the MAPKKK MEKK2, also known as MAPKKK9 (Figure 2A).

Sequence analysis of *At4g08480* in *summ1-2* revealed that it also contains a mutation in *MEKK2*. This mutation changes Pro-740 to Leu. To determine whether other *summ* mutants also contain mutations in *At4g08480*, *At4g08480* was amplified from the other 48 mutants by PCR and sequenced. Seventeen additional *summ* mutants were found to contain mutations in *At4g08480* (Figures 2C; see Supplemental Figure 1 online). Most of the mutations result in amino acid changes in the kinase domain. These results suggest that *SUMM1* is *At4g08480*.

Suppression of Cell Death and Defense Responses of *mpk4* by *summ1-1*

MPK4 has previously been shown to function downstream of MKK1 and MKK2 (Gao et al., 2008; Qiu et al., 2008). Like the *mkk1 mkk2* double mutant, *mpk4* mutants in Col background are also seedling lethal. To determine whether the mutant phenotypes in *mpk4-3* can be suppressed by *summ1-1*, the *summ1-1 mpk4-3* double mutant was created by crossing *mpk4-3* (in Col) and *summ1-1 mkk1 mkk2*. As shown in Figure 3A, the dwarf morphology of *mpk4-3* was completely suppressed by *summ1-1*, and no obvious cell death was observed in the double mutant.

Trypan blue staining showed that the massive cell death observed in *mpk4-3* was largely suppressed in the *summ1-1 mpk4-3* double mutant (Figure 3B). However, there is still some staining in the double mutant, suggesting the existence of microscopic cell death in *summ1-1 mpk4-3*. DAB staining showed that the H_2O_2 level in *summ1-1 mpk4-3* was much lower than that in *mpk4-3* but still modestly higher than in the wild type (Figure 3C). Analysis of *PR* gene expression indicated that the expression of *PR1* and *PR2* in *summ1-1 mpk4-3* is dramatically reduced compared to those in *mpk4-3*, but still higher than the wild type (Figures 3D and 3E). These data suggest that the activation of cell death and defense responses in *mpk4* is mainly dependent on SUMM1.



C

Alleles	Mutation	a.a. changes
summ1-1	C1504 to T	Q502 to Stop
summ1-2	C2219 to T	P740 to L
summ1-3	G1568 to A	G523 to E
summ1-4	G547 to A	D183 to N
summ1-5	G1999 to A	E667 to K
summ1-6	G2236 to A	A746 to T
summ1-7	C1805 to T	S602 to F
summ1-8	G1931 to A	G644 to E
summ1-9	G530 to A	G177 to D
summ1-10	C337 to T	R113 to Stop
summ1-11	G2143 to A	G715 to S
summ1-12	G2059 to A	G687 to R
summ1-13	C157 to T	R53 to W
summ1-14	G1023 to A	W341 to Stop
summ1-15	Intron C to T	NA
summ1-16	G1882 to A	A628 to T
summ1-17	G1831 to A	G611 to S
summ1-18	G1993 to A	A665 to T
summ1-19	G1588 to A	E530 to K

Figure 2. Map-Based Cloning of *SUMM1*.

(A) Map position and the mutation in *summ1-1*. WT, the wild type.

(B) Protein structure of SUMM1/MEKK2. a.a., amino acids.

(C) Mutations identified in the *summ1* alleles and the consequences of mutations to SUMM1/MEKK2 protein.

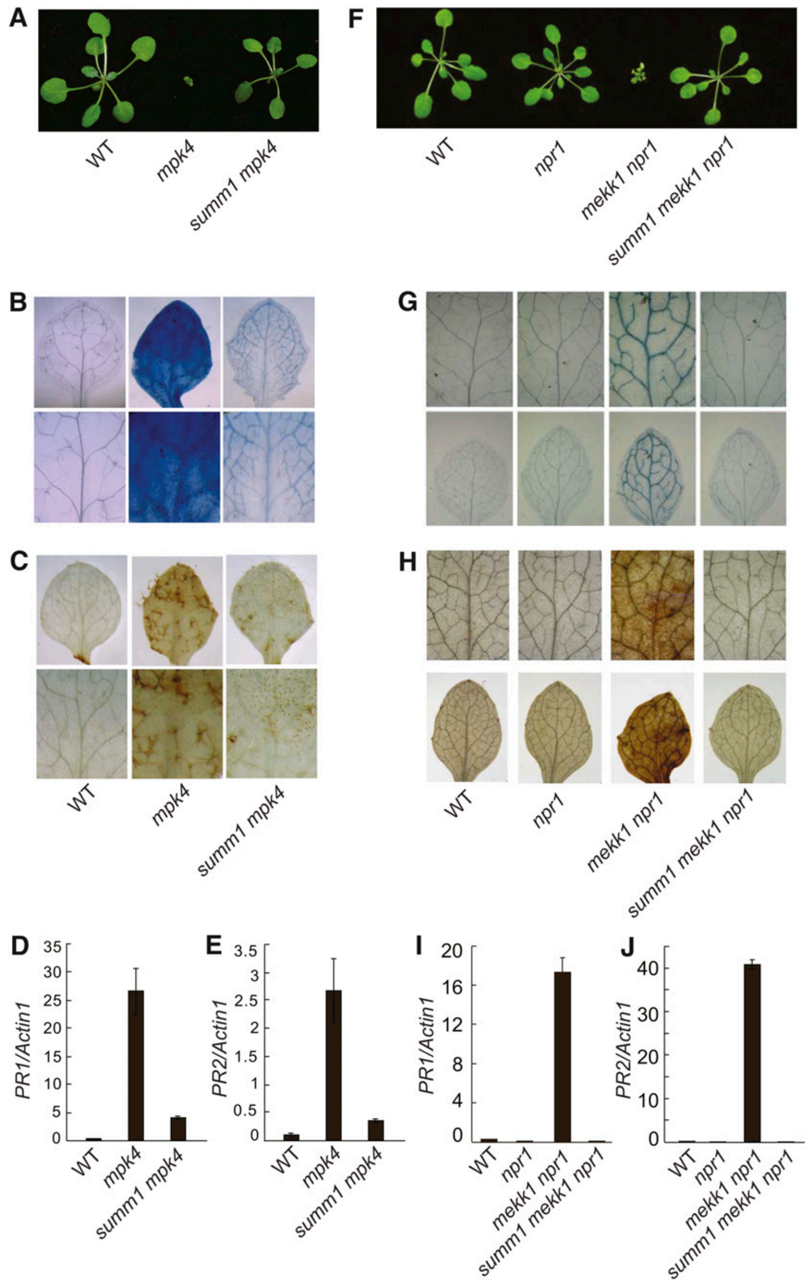


Figure 3. Suppression of *mpk4-3* and *mekk1-4* Mutant Phenotypes by *summ1* Mutants.

(A) Morphology of the wild type (WT), *mpk4-3* (*mpk4*), and *summ1-1 mpk4-3* (*summ1 mpk4*). The photograph shows 4-week-old soil grown plants.

(B) and **(C)** Trypan blue **(B)** and DAB **(C)** staining of wild-type, *mpk4-3*, and *summ1-1 mpk4-3* seedlings.

(D) and **(E)** *PR1* **(D)** and *PR2* **(E)** expression in wild-type, *mpk4-3*, and *summ1-1 mpk4-3* seedlings. Values were normalized to the expression of *ACTIN1*. Error bars represent ±SD of three replicates.

(F) Morphology of the wild type, *npr1-1* (*npr1*), *mekk1-4 npr1-1* (*mekk1 npr1*), and *summ1-3 mekk1-4 npr1-1* (*summ1 mekk1 npr1*). The photograph was taken on 4-week-old soil-grown plants.

(G) and **(H)** Trypan blue **(G)** and DAB **(H)** staining of wild-type, *npr1-1*, *mekk1-4 npr1-1*, and *summ1-3 mekk1-4 npr1-1* seedlings.

(I) and **(J)** *PR1* **(I)** and *PR2* **(J)** expression in the wild type, *npr1-1*, *mekk1-4 npr1-1*, and *summ1-3 mekk1-4 npr1-1*. Values were normalized to the expression of *ACTIN1*. Error bars represent ±SD of three replicates.

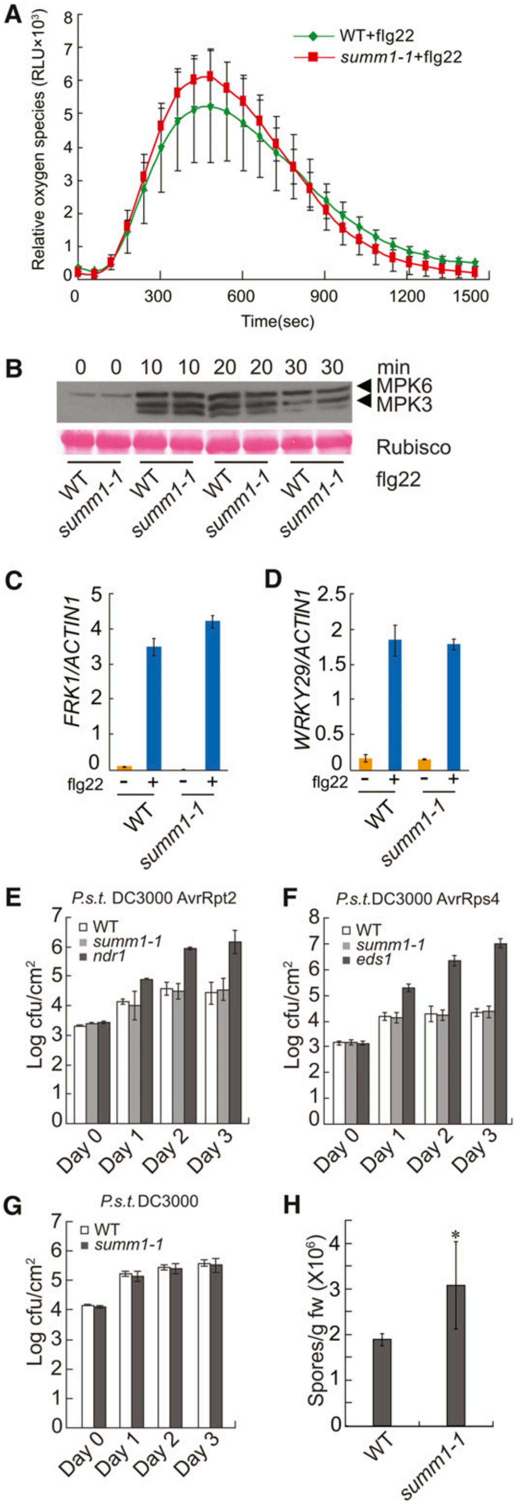


Figure 4. Analysis of Different Immune Responses in *summ1-1*.

(A) flg22-induced oxidative burst in *summ1-1*. Leaf slices were treated with 1 μM flg22 before ROS was measured. Error bars represent the SD of 12 independent samples. RLU, relative luminescence units; WT, wild type.

(B) flg22-induced MAPKs activation. Two-week-old seedlings grown on half-strength MS medium were treated with 10 μM of flg22. Samples were collected at 0, 10, 20, and 30 min and analyzed by immunoblots using an anti-Erk antibody (Cell Signaling; #4370S).

(C) and **(D)** Real-time RT-PCR analysis of the induction of *FRK1* **(C)** and *WRKY29* **(D)** in the wild type and *summ1-1* by flg22. Two-week-old seedlings grown on half-strength MS plates were sprayed with 10 μM of flg22 4 h before samples were taken. Error bars represent ±SD of three replicates.

(E) to **(G)** Growth of *P.s.t.* DC3000 *avrRpt2* **(E)**, *P.s.t.* DC3000 *avrRps4* **(F)**, and *P.s.t.* DC3000 **(G)** on the indicated genotypes. Five-week-old plants grown under short-day conditions were infiltrated with *P.s.t.* DC3000 at a concentration of $OD_{600} = 0.002$ and *P.s.t.* DC3000 *avrRpt2* and *P.s.t.* DC3000 *avrRps4* at a concentration of $OD_{600} = 0.001$. Samples were taken at 0 h (Day 0), 24 h (Day 1), 48 h (Day 2), and 72 h (Day 3) after inoculation, respectively. Error bars represent ±SD of six replicates.

(H) Growth of *H.a.* Noco2 on the wild type and *summ1-1*. Four-week-old plants were sprayed with spores of *H.a.* Noco2 at a concentration of 50,000 spores/mL. Error bars represent SD of three replicates. *$P < 0.05$, statistical difference from the wild type.

All experiments in this figure were independently repeated three times with similar results.

[See online article for color version of this figure.]

Suppression of Cell Death and Defense Responses in *mekk1* by *summ1-3*

MEKK1 functions upstream of MKK1 and MKK2, and mutations in *MEKK1* result in similar mutant phenotypes as the *mkk1 mkk2* double mutant (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007). We therefore speculated that *mekk1* activates SUMM1-dependent defense responses, and a double mutant of *mekk1* and *summ1* would help us to test our hypothesis. Because *SUMM1* and *MEKK1* are closely linked and the distance between the two genes is only ~12 kb, it is not feasible to obtain the *summ1 mekk1* double mutant by crossing *summ1* and *mekk1*. To address this problem, we mutagenized *mekk1-4 npr1-1* (Gao et al., 2008) with EMS and screened for mutants that suppressed the seedling lethality phenotype of the mutant. The mutants were subsequently sequenced to determine whether they contained mutations in *SUMM1*.

One of the mutants that completely suppressed the morphological phenotypes of *mekk1-4 npr1-1* was found to contain a mutation in *SUMM1* (Figure 3F). The mutation is the same as the *summ1-3* mutation identified from the suppressor screen of *mkk1 mkk2* (Figure 2C). When a construct expressing *SUMM1* with a C-terminal 3xFLAG tag under its own promoter was transformed into *summ1-3 mekk1-4 npr1-1*, all transgenic plants displayed a seedling-lethal phenotype, suggesting that suppression of the seedling-lethal phenotype of *mekk1-4 npr1-1* was caused by the *summ1-3* mutation. Trypan blue staining showed that cell death in *mekk1-4 npr1-1* was suppressed by *summ1-3* (Figure 3G). DAB staining indicated that the elevated H_2O_2 level in *mekk1-4 npr1-1* was reduced to the wild-type level in *summ1-3 mekk1-4 npr1-1* (Figure 3H). Analysis of defense gene expression showed that expression of *PR1* (Figure 3I) and *PR2* (Figure 3J) in *summ1-3 mekk1-4 npr1-1* was also comparable to that in wild-type plants. These data indicate that *summ1-3* completely suppress the cell death and constitutive defense responses in *mekk1-4 npr1-1*.

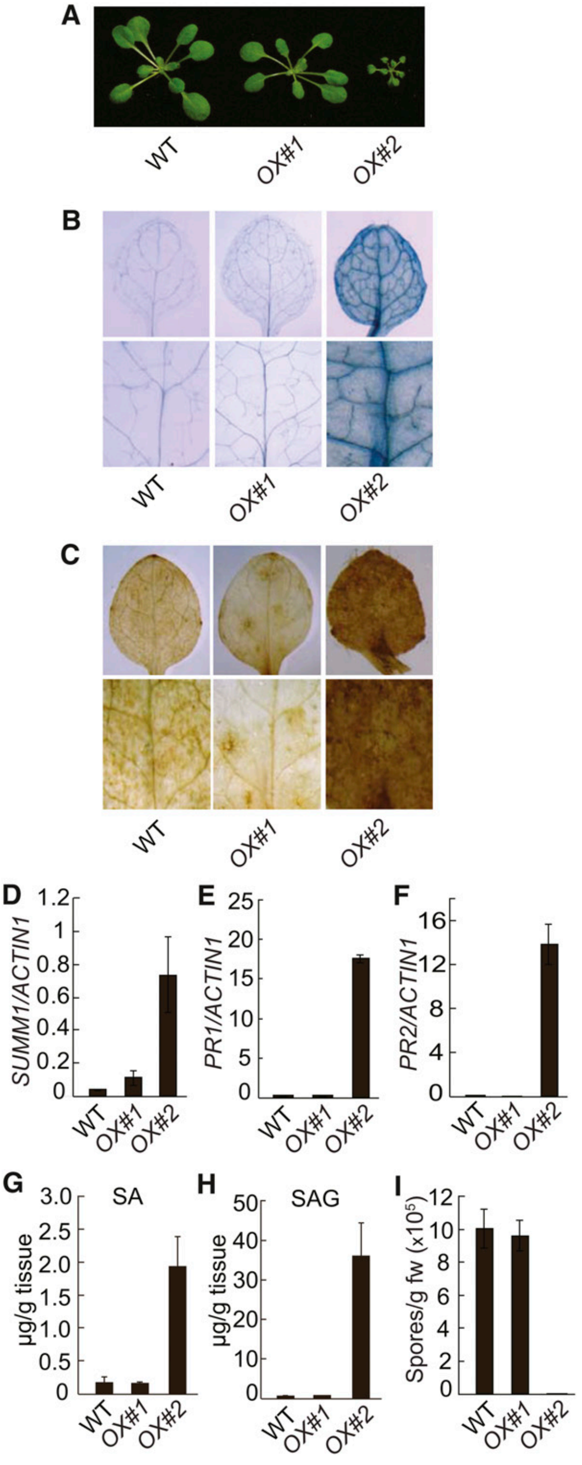


Figure 5. Overexpression of *SUMM1* Leads to Activation of Cell Death and Defense Responses.

(A) Morphology of the wild type (WT) and *SUMM1-FLAG* transgenic lines OX#1 and OX#2. The photograph shows soil-grown plants ~4 weeks after planting.

(B) and **(C)** Trypan blue **(B)** and DAB **(C)** staining of the seedlings of the wild type and the *SUMM1-FLAG* transgenic lines OX#1 and OX#2.

(D) Real-time RT-PCR analysis of *SUMM1* expression in the wild type and the *SUMM1-FLAG* transgenic lines OX#1 and OX#2. Error bars represent ±SD of three replicates.

(E) and **(F)** *PR1* **(E)** and *PR2* **(F)** expression in the wild type and the *SUMM1-FLAG* transgenic lines. Error bars represent ±SD of three replicates.

(G) and **(H)** Free SA **(G)** and SAG **(H)** levels in the wild type and the *SUMM1-FLAG* transgenic lines. This experiment was repeated twice with similar results. Error bars represent ±SD of four replicates.

(I) Growth of *H.a.* Noco2 on the wild type and the *SUMM1-FLAG* transgenic lines. Inoculation of the pathogen and scoring of the infection were performed as shown in Figure 1F. Error bars represent ±SD of three replicates. This experiment was repeated three times with similar results. fw, fresh weight.

flg22-Induced PAMP Responses Are Not Affected in *summ1-1*

MEKK1 was previously shown to be required for PAMP-induced activation of MPK4 (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007). Because SUMM1/MEKK2 shares high sequence similarity with MEKK1, we tested whether SUMM1 is required for flg22-induced reactive oxygen species (ROS) production, activation of MAPKs, and upregulation of *FRK1* and *WRKY29*. As shown in Figure 4A, induction of ROS by flg22 was comparable in the wild type and *summ1-1*. Activation of MPK3 and MPK6 was not affected in *summ1-1* either (Figure 4B). Real-time RT-PCR showed that induction of *FRK1* and *WRKY29* was also comparable in the wild type and *summ1-1* (Figures 4C and 4D). These data indicate that flg22-induced PAMP responses are intact in *summ1-1*.

SUMM1 Is Not Required for Resistance Mediated by *RPS2* and *RPS4*

Next, we tested whether resistance mediated by *R* genes such as *RPS2* and *RPS4* is affected in *summ1-1*. As shown in Figures 4E and 4F, growth of *Pseudomonas syringae* pv *tomato (P.s.t.)* DC3000 carrying *avrRpt2* or *avrRps4* was comparable in the wild type and *summ1-1*, suggesting that resistance mediated by *RPS2* and *RPS4* was not affected by *summ1-1* and SUMM1 is not a general defense regulator downstream of plant immune receptors. We further tested whether SUMM1 is required for basal resistance against virulent pathogens. As shown in Figure 4G, growth of the virulent *P.s.t.* DC3000 on wild-type and *summ1-1* plants was similar. Interestingly, when *summ1-1* was challenged with *H.a.* Noco2, it supported significantly higher growth of the oomycete pathogen (Figure 4H), suggesting that *summ1-1* affects basal resistance against *H.a.* Noco2.

Overexpression of *SUMM1* Activates Cell Death and Defense Responses

When *SUMM1* with a C-terminal 3xFLAG-tag was expressed in wild-type plants under its own promoter, about half of T1 transgenic plants exhibited dwarf morphology, suggesting that defense responses might be activated in these plants. Two representative *SUMM1-FLAG* lines with SUMM1-FLAG expressed at different levels were characterized in detail. As shown in Figure 5A, line #2 was much smaller than the wild type and line #1. Trypan blue staining showed that there was extensive cell death in line #2 (Figure 5B). Line #2 also accumulated high levels of H_2O_2 (Figure 5C) compared with the wild type and line #1. Real-time RT-PCR showed that the expression of *SUMM1* in line #2 was about 10-fold higher than in line #1 (Figure 5D). In addition, both *PR1* (Figure 5E) and *PR*2 (Figure 5F) were constitutively expressed in line #2. Analysis of SA levels showed that both free and total SA accumulated more in line #2 compared with the wild type and line #1 (Figures 5G and 5H). Furthermore, line #2 exhibited strongly enhanced resistance to *H.a.* Noco2 (Figure 5I). These data suggest that overexpression of *SUMM1* leads to constitutive activation of cell death and defense responses.

SUMM1/MEKK2 Interacts with MPK4

The epistatic relationship between *SUMM1* and all members of the MEKK1-MKK1/MKK2-MPK4 MAPK cascade suggests that MPK4 might be a negative regulator of SUMM1. However, whether MPK4 and SUMM1 physically interact with each other is unclear. Thus, we first tested whether SUMM1/MEKK2 and MPK4 interact with each other in yeast two-hybrid assays. As shown in Figure 6A, MEKK2 interacted with MPK4 but not MPK6 or the empty vector. MEKK2 contains two distinct domains. The N-terminal domain is quite divergent from other MEKKs and its function is unknown. The C terminus contains the kinase domain, which is highly conserved in MEKKs. To determine which region of MEKK2 interacts with MPK4, we expressed the N-terminal part of MEKK2 and the C-terminal kinase domain separately. As shown in Figure 6B, the N-terminal but not the C-terminal domain of MEKK2 interacted with MPK4 in the yeast two-hybrid assay, suggesting that the N terminus of MEKK2 contains the interface of interaction with MPK4.

To test whether MEKK2 and MPK4 associate with each other in vivo, we performed coimmunoprecipitation (co-IP) analysis using transgenic plants expressing the MEKK2-3xFLAG fusion protein under its own promoter. As shown in Figure 6C, MPK4 coimmunoprecipitated with 3xFLAG-tagged MEKK2 from total

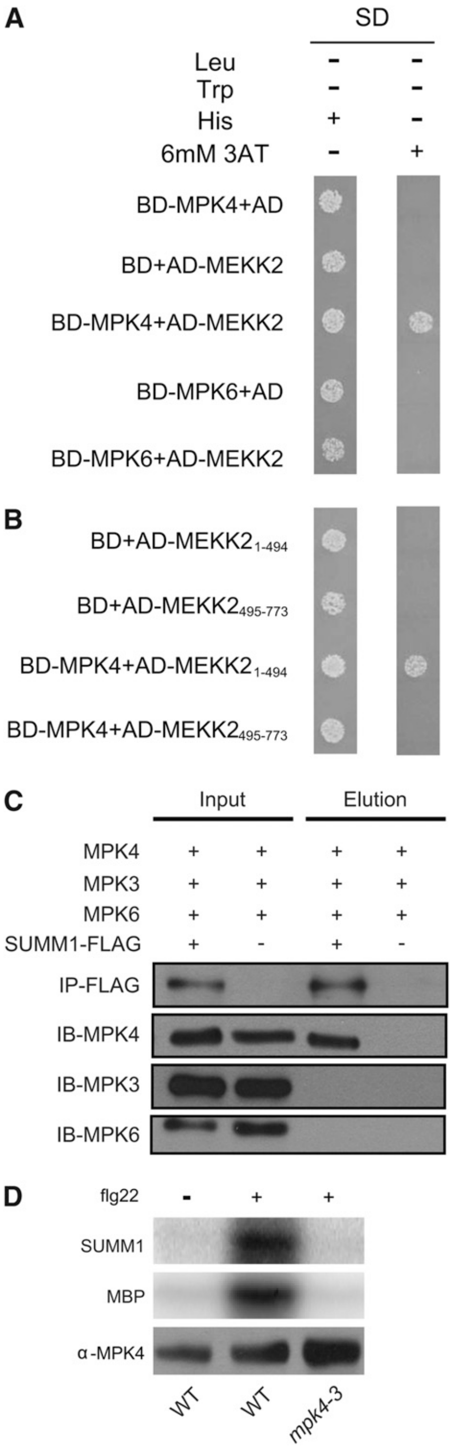


Figure 6. MPK4 Interacts with MEKK2 and Phosphorylates the N Terminus of MEKK2.

(A) Yeast two-hybrid analysis of the interaction between MPK4 and MEKK2.

(B) Yeast two-hybrid analysis of the interaction between MPK4 and the N-terminal ($MEKK2_{1-494}$) and C-terminal ($MEKK2_{495-773}$) domains of MEKK2.

(C) Co-IP of MPK4 with MEKK2-3xFLAG in total proteins extracts from *SUMM1-3xFLAG* transgenic plants. Total protein extracts were subjected to immunoprecipitation with anti-FLAG Sepharose beads. Crude lysates (left panel, Input) and immunoprecipitated proteins (right panels, Elution) were detected with anti-FLAG, anti-MPK4, anti-MPK3, and anti-MPK6 antibodies, respectively. Wild-type plants without the *SUMM1-3xFLAG* transgene were used as a negative control. This experiment was repeated three times with similar results.

(D) Phosphorylation of the N terminus of SUMM1/MEKK2 by MPK4. MPK4 was immunoprecipitated from the wild type (WT) and *mpk4-3*. After incubation with [γ-^{32}P]ATP and the immunoprecipitated MPK4 or mpk4-3 mutant protein in protein kinase buffer, the *E. coli*–expressed N-terminal domain of SUMM1 was separated on 10% SDS-PAGE. The autoradiograph of the gel is shown in the top panel, and immunoblot analysis of MPK4 levels is shown in the bottom panel. This experiment was repeated four times with similar results.

protein extracts of the transgenic plants but not from the protein extract of wild-type plants without the transgene. Immunoblot analysis of the immunoprecipitated proteins using anti-MPK3 and anti-MPK6 antibodies showed that MPK3 and MPK6 were not coimmunoprecipitated with MEKK2. These data suggest that MEKK2 interacts with MPK4 but not MPK3 and MPK6 in planta.

Phosphorylation of the N-Terminal Domain of SUMM1/MEKK2 by MPK4

The direct physical interaction between MPK4 and MEKK2/SUMM1 prompted us to test whether MEKK2 is a substrate for MPK4. To test whether MPK4 can phosphorylate MEKK2, the N-terminal domain of MEKK2 ($MEKK2_{1-500}$) with a 6×His-tag was expressed in *Escherichia coli* and purified using Ni^{2+}-nitrilotriacetate chromatography. MPK4 was purified from wild-type or *mpk4-3* mutant plants by immunoprecipitation using anti-MPK4 antibodies. In vitro kinase assays were subsequently performed using the $MEKK2_{1-500}$ and MPK4 proteins. As shown in Figure 6D, $MEKK2_{1-500}$ was phosphorylated by MPK4 from the flg22-treated wild-type plants but not the mutant protein from *mpk4-3* plants, suggesting that MEKK2 is indeed a substrate of MPK4.

To identify the amino acid(s) in $MEKK2_{1-500}$ that was phosphorylated by MPK4, MPK4-treated *E. coli*–expressed $MEKK2_{1-500}$ was analyzed by mass spectrometry. As shown in Table 1, Ser-365 was phosphorylated in the protein. When we analyzed MEKK2-3xFLAG immunoprecipitated from transgenic plants expressing the fusion protein, Ser-365 was also found to be phosphorylated in vivo (Table 1), suggesting that Ser-365 in MEKK2 is most likely a target site of MPK4. Several additional phosphorylation sites were also identified in the MEKK2-3xFLAG protein (Table 1). It remains to be determined which kinase(s) is responsible for the phosphorylation of these sites.

Root Development Defects in *mpk4-3* Are Not Suppressed by *summ1-1*

In addition to negatively regulating plant defense responses, MPK4 is also important for cytokinesis and plant development (Beck et al., 2010; Kosetsu et al., 2010; Takahashi et al., 2010). The *mpk4-2* mutant exhibits increased root width and has abnormal root hairs. To test whether *summ1-1* suppresses the root development phenotypes of *mpk4*, we measured the root width in *mpk4-3* and *summ1-1 mpk4-3* and found that both *mpk4-3* and *summ1-1 mpk4-3* have increased root width compared with the wild type and *summ1-1* (see Supplemental Figure 2A online). In addition, abnormal root hairs were also observed in both *mpk4-3* and *summ1-1 mpk4-3* but not in the wild type and *summ1-1* (see Supplemental Figure 2B online), suggesting that *summ1-1* cannot suppress the developmental phenotypes of *mpk4* roots.

We also measured the root width and examined the morphology of root hairs in *mekk1-4 npr1-1*, *summ1-3 mekk1-4 npr1-1*, *mkk1 mkk2*, and *summ1-1 mkk1 mkk2*. As shown in Supplemental Figures 3A to 3D online, the root width of these mutants is comparable to that in wild-type plants and root hairs developed normally in the mutant plants. We also analyzed the roots of the *SUMM1-FLAG* transgenic line OX#2. No difference was observed between wild-type and the transgenic plants in root width and root hair morphology (see Supplemental Figures 3E and 3F online). These data suggest that MEKK1, MKK1/MKK2, and SUMM1 do not function in *Arabidopsis* root development as MPK4 does.

SUMM1/MEKK2 Functions Upstream of SUMM2

Our study on another suppressor mutant of *mkk1 mkk2*, *summ2-1*, revealed that *SUMM2* encodes a coiled-coil NB-LRR R protein whose activity is negatively regulated by the MEKK1, MKK1/MKK2, and MPK4 kinase cascade (Zhang et al., 2012). To determine whether *SUMM2* is required for activation of defense responses by overexpression of *SUMM1*, we transformed *summ2-8*, a T-DNA knockout mutant of *SUMM2*, with the construct expressing SUMM1-FLAG fusion protein under its own promoter. None of the transgenic plants exhibited a dwarf phenotype.

Three transgenic lines in *summ2-8* background and two transgenic lines in wild-type background with similar SUMM1-FLAG protein levels were analyzed further. As shown in Figure 7A, the transgenic lines in *summ2-8* background displayed wild-type morphology, whereas the transgenic lines in wild-type background exhibited dwarf morphology. Trypan blue staining and DAB staining revealed extensive cell death (Figure 7B) and high level of H_2O_2 (Figure 7C) in the transgenic lines in wild-type background but not in transgenic lines in *summ2-8* background. Analysis of the expression of *PR1* (Figure 7D) and *PR2* (Figure

Table 1. Phosphopeptides from MEKK2

Assay	Amino Acid No.	Peptide Sequence
In vitro	362–375	GVTpSPVLNLRPTDK
In vivo	77–96	SNpSSENKIPNEDISVSTSSR
	150–156	pSLDFPNR
	362–375	GVTpSPVLNLRPTDK
	362–386	GVTpSPVLNLRPTDKEVVDSGTVENR
	757–772	RPLPSSGSGSTpSPLIR

Phosphopeptides were identified by mass spectrometry analysis as previously described (Tang et al., 2009). A lowercase p indicates phosphorylation of the Ser residue that follows.

7E) showed that they were also constitutively expressed in the transgenic lines in wild-type background but not in transgenic lines in *summ2-8* background. These data suggest that SUMM1 functions upstream of SUMM2 to regulate plant defense responses.

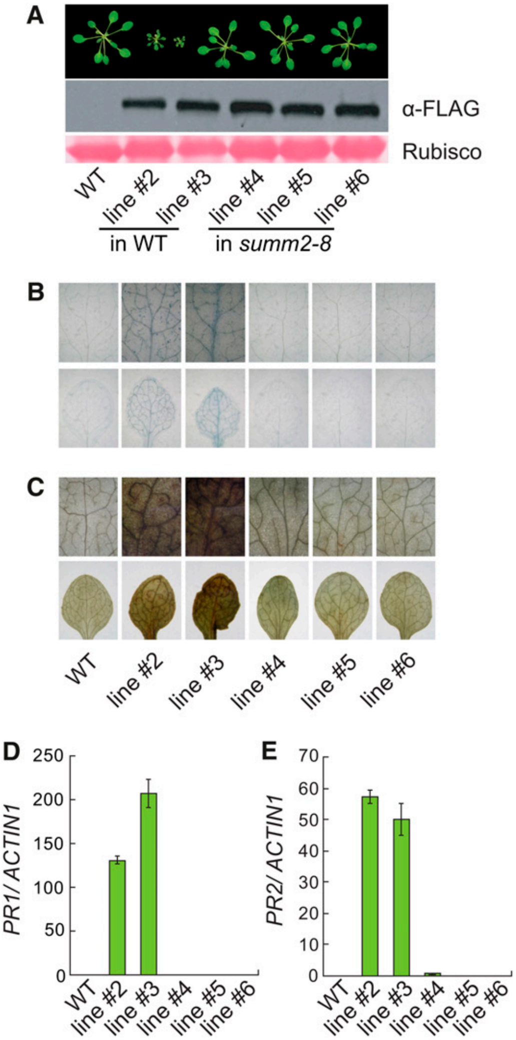


Figure 7. *SUMM2* Is Required for Defense Responses Activated by *SUMM1*.

(A) Morphology of ~3-week-old *SUMM1-FLAG* transgenic lines and SUMM1-FLAG expression levels. Line #2 and #3 are two representative *SUMMI1-FLAG* transgenic lines from the transformation of wild-type plants. Line #4, #5, and #6 are three representative *SUMM1-FLAG* transgenic lines from the transformation of *summ2-8* plants. Rubisco, ribulose-1,5-bis-phosphate carboxylase/oxygenase; WT, the wild type.

(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 quantitative PCR. Values were normalized relative to the expression of *ACTIN1*. Error bars represent SD of three measurements.

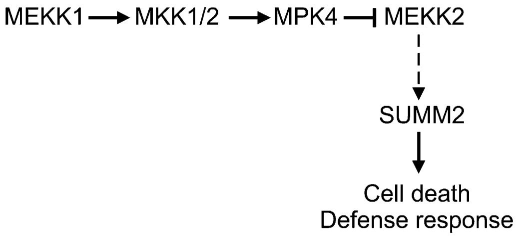


Figure 8. A Working Model for Repression of MEKK2-Mediated Immunity by the MEKK1-MKK1/2-MPK4 MAPK Cascade.

MEKK2 functions as a positive regulator of the NB-LRR R protein SUMM2, and its activity is negatively regulated by the MEKK1-MKK1/2-MPK4 kinase cascade. As shown by the dashed arrow, the mechanism by which MEKK2 activates SUMM2 remains to be determined.

DISCUSSION

Our analysis of suppressor mutants of *mkk1 mkk2* identified MEKK2 as a positive regulator of plant immunity. Not only do mutations in MEKK2 suppress the dwarf phenotype and the constitutive defense responses in *mekk1*, *mkk1 mkk2*, and *mpk4* mutant plants, overexpression of MEKK2 is also sufficient to activate defense responses mediated by the NB-LRR R protein SUMM2. Our data suggest that the MEKK1, MKK1/MKK2, and MPK4 kinase cascade negatively regulates MEKK2 and MEKK2 functions as a positive regulator of SUMM2-mediated plant immunity (Figure 8).

Although *mekk1*, *mkk1 mkk2*, and *mpk4* mutants exhibit similar morphology, *mekk1* knockout mutants and *mpk4* alleles in Col background are more severely dwarfed than *mkk1 mkk2* (Gao et al., 2008), suggesting that functions of MKK1 and MKK2 can be partially compensated for by another MKK with overlapping functions. Probably due to the more severe phenotypes in *mpk4-3*, suppression of cell death, accumulation of H_2O_2, and upregulation of *PR* genes in *summ1-1*, *mpk4-3* is not as complete as that in the *summ1-1 mkk1 mkk2* triple mutant. The incomplete suppression of defense responses in *mpk4-3* by *summ1-1* suggests that MPK4 regulates additional defense components in addition to MEKK2. Previously, MKS1 was also identified as a substrate of MPK4 (Andreasson et al., 2005). Silencing *MKS1* partially suppresses the *mpk4* mutant morphology. It is possible that MEKK2 and MKS1 function independently and the residual cell death and defense responses in *summ1-1 mpk4-3* are results of activation of MKS1-mediated defense responses.

Both yeast two-hybrid and co-IP analyses showed that MPK4 interacts with MEKK2. In addition, MPK4 can phosphorylate MEKK2 in vitro. These data suggest that MEKK2 is a direct substrate of MPK4 and MPK4 most likely suppresses defense responses through inactivating MEKK2 by phosphorylation. Since MAPKKKs usually function upstream of MAPKs, it is unexpected that MEKK2 serves as a target of MPK4. Previously it was shown that MEKK1 interacts with MPK4 in yeast two-hybrid assays (Ichimura et al., 1998). It is unclear whether MPK4 negatively regulates the activity of MEKK1 by phosphorylation and whether phosphorylation of

MAPKKKs by their downstream MAPKs is used as a mechanism of negative feedback regulation of MAPK cascades.

MEKK2 contains an N-terminal domain with unknown functions and a C-terminal kinase domain. The N-terminal domain interacts with MPK4 and can be phosphorylated by MPK4 in vitro, indicating that it may have a regulatory function. Most of the *mekk2* mutations found to suppress the *mkk1 mkk2* mutant phenotypes are located in the kinase domain, suggesting that the kinase domain of MEKK2 is important for activation of defense responses. It remains to be determined whether MEKK2 functions like traditional MAPKKKs, which transduce signals through downstream MAPKKs and MAPKs.

In addition to its function in plant defense, MPK4 also plays an important role in regulating cytokinesis (Beck et al., 2010; Kosetsu et al., 2010). Due to defect in cytokinesis, *mpk4* mutants also exhibit various root development phenotypes. Interestingly, *summ1-1* suppresses the autoimmune phenotypes but not the root development phenotypes of *mpk4-3*, suggesting that the constitutive defense responses and defects in root development can be uncoupled. This is also supported by findings that overexpressing SUMM1 results in cell death and activation of defense responses, but not defects in root development. Similarly, the *mpk4-1* mutant in L*er* background displays constitutive defense responses but has no visible defects in cytokinesis and root development (Kosetsu et al., 2010). Our results suggest that MPK4 is a multifunctional MAPK regulating at least two separate biological processes. On one hand, it functions together with ANP2/ANP3 and MKK6 to regulate microtubule organization and cytokinesis. On the other hand, it forms a kinase cascade together with MEKK1 and MKK1/MKK2 to negatively regulate MEKK2-mediated defense responses.

Studies on bacterial effectors revealed that HopAI1 inactivates MAPKs by removing the phosphate group from phosphothreonine through a unique phosphothreonine lyase activity to suppress PAMP responses (Zhang et al., 2007), whereas HopF2 suppresses PAMP-mediated immunity by inhibiting MAPKKs (Wang et al., 2010), suggesting that targeting MAPK cascades downstream of plant immune receptors plays important roles in bacterial virulence. Since MEKK2 functions upstream of the NB-LRR R protein SUMM2, it is likely that MEKK2 evolved to sense the attack of the MEKK1-MKK1/MKK2-MPK4 kinase cascade by microbial pathogens. Disruption of the activity of MEKK1, MKK1/MKK2, or MPK4 leads to activation of MEKK2, which triggers SUMM2-mediated immune responses.

One important question is how MEKK2 activates SUMM2-mediated defense responses. We were not able to detect any direct interaction between MEKK2 and SUMM2. One possibility is that MEKK2 functions as a component of a MAPK cascade and activates defense responses through its downstream MAP kinase kinase and MAPK. Alternatively, MEKK2 may not function as a traditional MAPKKK. In this scenario, it may directly activate SUMM2-mediated defense responses by phosphorylation of its target protein(s), which is recognized by SUMM2. Future studies on other *summ* mutants may help us identify genes that function between *MEKK2* and *SUMM2* and lead to better understanding about how MEKK2 regulates plant immune responses.

METHODS

Plant Materials, Mutant Screen, and Characterization

Mutants *mkk1-1 mkk2-1* (*mkk1 mkk2*), *mpk4-3*, *mekk1-4 npr1-1*, and *summ2-8* were reported previously (Gao et al., 2008; Zhang et al., 2012). The *summ1-1* single mutant was obtained by backcrossing *summ1-1 mkk1 mkk2* to Col wild-type plants. The *summ1-1 mpk4-3* double mutant was obtained by crossing *summ1-1 mkk1 mkk2* with *mpk4-3*. The *summ1-3 mekk1-4 npr1-1* triple mutant was identified from an EMS-mutagenized mutant population of *mekk1-4 npr1-1*.

For gene expression analysis, RNA was purified from 2-week-old seedlings grown on half-strength Murashige and Skoog (MS) plates. Reverse transcription was performed using the M-MLV RTase cDNA synthesis kit from Takara. Real-time PCR was performed on the cDNA reverse transcribed from three independent RNA samples using Takara SYBR Premix Ex Taq II. Primers used for real-time PCR analysis of *PR1*, *PR2*, and *Actin1* were described previously (Zhang et al., 2003b). The primers used for real-time PCR analysis of *MEKK2* are RT-MEKK2-F and RT-MEKK2-R (see Supplemental Table 1 online). All experiments on gene expression analysis were repeated at least three times. For cell death and H_2O_2 analysis, 12-d-old seedlings grown on half-strength MS plates were stained with trypan blue and DAB according to procedures previously described (Parker et al., 1996; Thordal-Christensen et al., 1997). ROS were measured using a luminol-dependent assay (Trujillo et al., 2008). *H a.* Noco2 infection was performed by spraying 2-week-old seedlings with spore suspensions in H_2O at a concentration of 50,000 spores per mL and scored as previously described (Bi et al., 2010). SA was extracted and measured using HPLC as previously described (Li et al., 1999).

Map-Based Cloning of *summ1-1*

The markers used for mapping were designed using the Monsanto *Arabidopsis* polymorphism and L*er* sequence collections (Jander et al., 2002). All primer sequences are listed in Supplemental Table 1 online. T13D4 and NGA8 are based on Indel polymorphisms. F9M13 and T15F16 are based on single nucleotide polymorphisms. For marker F9M13, primers F9M13-F and F9M13-Col-R were used to detect the presence of the Col allele, and primers F9M13-F and F9M13-Ler-R were used to detect the presence of the L*er* allele. For marker T15F16, primers T15F16-F and T15F16-Col-R were used to detect the presence of the Col allele, and primers T15F16-F and T15F16-Ler-R were used to detect the presence of the L*er* allele.

For testing whether the *summ1-3* mutation is responsible for the suppression of *mekk1-4* mutant phenotypes in *summ1-3 mekk1-4 npr1-1*, the promoter region of *SUMM1* was amplified by PCR using primers SUMM1-Promoter-F and SUMM1-Promoter-R, and the coding region of *SUMM1* was amplified by PCR using primers SUMM1-F and SUMM1-R from wild-type genomic DNA. The two PCR fragments were sequentially cloned into a modified pCAMBIA1305 vector to obtain pCAMBIA1305-SUMM1-FLAG for expressing the MEKK2-3xFLAG fusion protein under its own promoter. pCAMBIA1305-SUMM1-FLAG was transformed into *summ1-3 mekk1-4 npr1-1* and wild-type plants by the floral dip method (Clough and Bent, 1998).

Yeast Two-Hybrid Assays

To create the MPK4 and MPK6 bait plasmids, *MPK4* cDNA was amplified by primers MPK4-F and MPK4-R, whereas *MPK6* cDNA was amplified using MPK6-F and MPK6-R and cloned into pBI880. The *MEKK2* cDNA was amplified using primers MEKK2-pBI881-F and MEKK2-pBI881-R and cloned into the prey vector pBI881. The N-terminal fragment of MEKK2 was amplified using primers MEKK2-pBI881-F and MEKK2-N-pBI881-R, whereas the C-terminal fragment of MEKK2 was amplified using primers MEKK2-C-pBI881-F and MEKK2-pBI881-R. Both fragments were cloned into pBI881. All primer sequences are listed in Supplemental Table 1 online. For yeast two-hybrid assays, bait and prey plasmids were cotransformed into yeast strain Y1348. Yeast strains containing the bait and prey plasmids were cultured in SD-Trp-Leu liquid medium overnight and diluted to OD_{600} = 0.005 using double distilled water. Ten microliters of the diluted culture was plated on SD-Trp-Leu-His with 6 mM 3-amino-1,2,4-triazole and SD-Trp-Leu dropout plates.

Co-IP

For the co-IP experiment, 2-week-old-seedlings of Col-0 and MEKK2-3×FLAG transgenic plants on half-strength MS plates were used. About 0.9 g tissue was ground in liquid nitrogen and suspended in 0.9 mL grinding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM PMSF, and 1× Protease Inhibitor Cocktail from Roche). The samples were spun at 21,000*g* for 10 min at 4°C followed by incubation with 40 μL of protein G beads (GE Healthcare; 17-0618-01) for 30 min with rotation. After the beads were removed by centrifugation, 40 μL anti-FLAG M2 agarose (Sigma-Aldrich; 087K6001) was added to supernatant and incubated at 4°C for 2 h with rotation. The beads were spun down and washed three times using grinding buffer. For elution, the beads were incubated with 100 μg/mL FLAG peptide at 4°C with rotation for 25 min. The supernatant was collected by centrifugation, and the immunoprecipitated proteins were detected by immunoblotting. The primary antibodies used for immunoblotting were mouse anti-FLAG monoclonal antibody (Sigma-Aldrich) and rabbit anti-MPK4, anti-MPK3, and anti-MPK6 (Sigma-Aldrich).

MPK4 Kinase Assays

To express the N-terminal domain of MEKK2 (amino acids 1 to 500) with a 6×His-tag, the cDNA fragment of *MEKK2* was amplified using primers MEKK2-N-pET24c-F and MEKK2-N-pET24c-R (see Supplemental Table 1 online) and cloned into pET-24c. The plasmid was transformed into *Escherichia coli* strain BL21 for expressing the protein and the protein was purified using Ni^{2+}-nitrilotriacetate chromatography.

To isolate MPK4 protein for kinase assays, 12-d-old seedlings of the wild type and *mpk4-3* were sprayed with 10 μM flg22. About 0.5 g of tissue from each sample was harvested in liquid nitrogen 10 min later. One milliliter 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, and 1 mM Protease Inhibitor) was added to resuspend the sample. After centrifugation, the supernatant was collected and incubated with 2 μL anti-MPK4 antibodies (Sigma-Aldrich) for 1 h with constant rotation at 4°C. Next, 20 μL of Protein A beads was added into each sample and incubated for another 3 h at 4°C. After centrifugation, the beads were washed with 1 mL of extraction buffer three times and with 1 mL of kinase buffer (50 mM HEPES 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, and 10 μM ATP) once. The beads were spun down and resuspended in 15 μL of kinase buffer.

For the kinase assay, 9 μL MPK4 was incubated with ~1 μg of $MEKK2_{1\text{-}500}$ protein or 0.5 μg of MBP, 0.5 μL ATP (200 μM), 10 μCi [γ-^{32}P] ATP, and kinase reaction buffer (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, and 10 μM ATP) in a total volume of 15 μL at 30° C for 30 min. The reaction was ended by adding SDS loading buffer. After separation by SDS-PAGE, phosphorylation of $MEKK2_{1\text{-}500aa}$ and MBP was detected by autoradiography.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At4g08480 (SUMM1), AT4G01370 (MPK4), At2g14610 (PR1), At3g57260 (PR2), and At2g37620 (Actin1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Morphological Phenotypes of the Wild Type, *mkk1-1 mkk2-1*, and Different *summ1* Mutant Alleles.

Supplemental Figure 2. Development Defects in the Roots of *mpk4-3* Are Not Suppressed by *summ1-1*.

Supplemental Figure 3. *MEKK1*, *MKK1/MKK2*, and *SUMM1* Are Not Required for Root Development.

Supplemental Table 1. Primer Sequences.

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AUTHOR CONTRIBUTIONS

Q.K., M.G., and Y.Z. designed the research. Q.K., N.Q., M.G., Z.Z., X.D., F.Y., Y.L., and O.X.D. performed research. S.C., X.L., and Y.Z. analyzed data. Q.K., X.L., and Y.Z. wrote the article.

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