The Plant Cell, Vol. 19: 3266–3279, October 2007, www.plantcell.org © 2007 American Society of Plant Biologists

The *Arabidopsis* Mitogen-Activated Protein Kinase Kinase MKK3 Is Upstream of Group C Mitogen-Activated Protein Kinases and Participates in Pathogen Signaling [W]

Róbert Dóczi,[a] Günter Brader,[b] Aladár Pettkó-Szandtner,[a] Iva Rajh,[a] Armin Djamei,[a] Andrea Pitzschke,[a] Markus Teige,[a] and Heribert Hirt[a,c,1]

[a] Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, A-1030 Vienna, Austria
[b] Viikki Biocenter, Department of Biological and Environmental Sciences, Division of Genetics, Faculty of Biosciences, University of Helsinki, FIN-00014 Helsinki, Finland
[c] Unité de Recherche en Génomique Végétale, Plant Genomics Research Unit, F-91057 Evry, France

Although the *Arabidopsis thaliana* genome contains genes encoding 20 mitogen-activated protein kinases (MAPKs) and 10 MAPK kinases (MAPKKs), most of them are still functionally uncharacterized. In this work, we analyzed the function of the group B MAPK kinase, MKK3. Transgenic *ProMKK3:GUS* lines showed basal expression in vascular tissues that was strongly induced by *Pseudomonas syringae* pv *tomato* strain DC3000 (*Pst* DC3000) infection but not by abiotic stresses. The growth of virulent *Pst* DC3000 was increased in *mkk3* knockout plants and decreased in *MKK3*-overexpressing plants. Moreover, *MKK3* overexpression lines showed increased expression of several *PR* genes. By yeast two-hybrid analysis, coimmunoprecipitation, and protein kinase assays, MKK3 was revealed to be an upstream activator of the group C MAPKs MPK1, MPK2, MPK7, and MPK14. Flagellin-derived flg22 peptide strongly activated MPK6 but resulted in poor activation of MPK7. By contrast, MPK6 and MPK7 were both activated by H_2O_2, but only MPK7 activation was enhanced by MKK3. In agreement with the notion that MKK3 regulates the expression of *PR* genes, *ProPR1:GUS* expression was strongly enhanced by coexpression of MKK3-MPK7. Our results reveal that the MKK3 pathway plays a role in pathogen defense and further underscore the importance and complexity of MAPK signaling in plant stress responses.

INTRODUCTION

Due to their sessile life cycle, plants have developed sophisticated mechanisms to rapidly sense a changing environment and protect themselves from environmental biotic and abiotic stresses. Mitogen-activated protein kinase (MAPK or MPK) cascades are common mechanisms to translate external stimuli into cellular responses in all eukaryotes, including higher plants. These protein kinase cascades consist of three subsequently acting protein kinases, a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK or MKK), and finally the MAPK. Different MAPK pathways respond to a variety of external stimuli and have been characterized in yeast, animals, and plants (Davis, 2000; Chang and Karin, 2001; Hohmann, 2002; Jonak et al., 2002).

The genome of the yeast *Saccharomyces cerevisiae* encodes six different MAPKs, and cellular functions for five of these MAPKs have been established (Herskowitz, 1995; Gustin et al., 1998; Hohmann, 2002; O'Rourke et al., 2002). By contrast, plants have ~20 MAPKs, but relatively little is known about the function and composition of the different pathways (Tena et al., 2001; Zhang and Klessig, 2001; MAP Kinase Group, 2002; Jonak et al., 2002). The MAPKs investigated to date were mainly involved in stress responses (Jonak et al., 2002). In the genetic model plant *Arabidopsis thaliana*, MPK3, MPK4, and MPK6 are activated by a diverse set of stresses, including pathogens, osmotic, cold, and oxidative stresses (Ichimura et al., 2000; Kovtun et al., 2000; Nühse et al., 2000; Petersen et al., 2000; Desikan et al., 2001; Asai et al., 2002; Droillard et al., 2002).

Compared with our current knowledge of the 20 plant MAPKs, much less is known of the functions of the 10 MAPKKs or of the >60 putative MAPKKKs (MAP Kinase Group, 2002). The 20 MAPKs can be divided into four groups. Best studied are MPK3 and MPK6 of group A and MPK4 of group B, whereas to date there is no functional information on groups C and D. Based on sequence alignment, plant MAPKKs can also be divided into four groups. MKK1 and MKK2 of group A and MKK4 and MKK5 of group C MAPKKs mainly appear to be involved in stress responses. By contrast, little is known about the function of group B and D MAPKKs. A recent article by Takahashi et al. (2007) provides evidence that MKK3, the only member of group B MAPKKs, plays a role in jasmonate (JA)-mediated developmental signaling. With the exception of MKK7, which was recently shown to be an inhibitor of polar auxin transport (Dai et al., 2006), there is no information on the function of group D MAPKKs.

A broad range of pathogens are recognized by plants through pathogen-associated molecular patterns (PAMPs), which are

[1] Address correspondence to heribert.hirt@univie.ac.at.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Heribert Hirt (heribert.hirt@univie.ac.at).
[W] Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.106.050039

highly conserved molecular structures of pathogenic microbes (Nürnberger et al., 2004). PAMP perception systems in plants and animals are mechanistically comparable, and PAMP recognition by receptors triggers nitric oxide, reactive oxygen species (ROS), and MAPK cascades that induce innate immune responses (Nürnberger et al., 2004). In particular, plants possess a specific recognition system for a conserved, 22–amino acid motif (flg22) of the bacterial flagellin (Felix et al., 1999). Recognition of flg22 in *Arabidopsis* occurs via a host protein complex that contains the FLS2 Leu-rich repeat receptor kinase (Gomez-Gomez et al., 2001). Recognition of flg22 by FLS2 leads to the production of ROS, medium alkalinization, and induction of pathogen-responsive genes (Felix et al., 1999; Gomez-Gomez et al., 1999; Nühse et al., 2000). flg22 activates multiple MAPKs, and Asai et al. (2002) identified a MAPK cascade that consists of the MEKK1-MKK4/MKK5-MPK3/MPK6 module and functions in innate immunity by the combination of transient expression analyses with biochemical and genetic approaches. MPK4 is also activated by flg22, and recent genetic evidence suggests that MEKK1 is upstream of MKK1 and MPK4 in flagellin and ROS signaling (Ichimura et al., 2006; Mészáros et al., 2006; Nakagami et al., 2006; Su et al., 2007; Suarez-Rodriguez et al., 2007). Recently, it was shown that both flg22 and another bacterial PAMP, called elf18 (Zipfel et al., 2006), result in activation of the MPK3/6 pathway, indicating that different elicitor-derived signals converge to activate the same MAPK cascade. Activation of MPK3/6 results in the induction of WRKY transcription factor genes (Asai et al., 2002), and *MPK6*-silenced *Arabidopsis* plants are compromised in resistance to different pathogens (Menke et al., 2004).

A unique group of plant MAPKKs is group B, which contains only one gene, *MKK3*, in all three sequenced plant genomes. Interestingly, all currently known MKK3 orthologs contain a C-terminal extension encoding a nuclear transfer factor domain (Hamel et al., 2006). Nuclear Transport Factor2 (NTF2) is a small protein that mediates the nuclear import of RAN-GDP and binds to both RAN-GDP and FxFG repeat–containing nucleoporins (Quimby et al., 2000). Interestingly, the only MKK encoded in the *Chlamydomonas* genome also belongs to the MKK3 structural class, including the C-terminal NTF2-like domain, indicating that this chimeric arrangement has had a long and successful evolutionary history in the lineage of photosynthetic eukaryotes (Hamel et al., 2006). The combination of a MAPKK and an NTF2-like domain in plants appears to be unique among eukaryotic taxa. However, there are other examples for modular architecture of proteins with NTF2-like domains, such as TAP, a vertebrate nuclear mRNA export factor, or Ras-GAP SH3 domain binding protein (Suyama et al., 2000).

Recently, it was found that *Arabidopsis* root growth inhibition by JA is negatively regulated by MKK3 (Takahashi et al., 2007). Accordingly, a JA marker gene (*VEGETATIVE STORAGE PROTEIN2* [*VSP2*]) is also negatively regulated by the pathway. On the other hand, *PLANT DEFENSIN1.2* (*PDF1.2*), a JA-regulated pathogen marker gene, along with some other JA-responsive genes (e.g., *LIPOXYGENASE2* [*LOX2*] and *VSP1*) are positively regulated by MKK3. Here, we provide evidence that *Arabidopsis* MKK3 is part of a novel pathogen-signaling MAPK pathway. We show that MKK3 is an activator of group C MPKs and acts as a positive regulator of *PR* gene expression.

RESULTS

MKK3 Expression Is Induced upon *Pseudomonas syringae* Infection

Gene expression is often indicative of gene function. As MAPK pathways have been implicated in the signaling of various stresses, we tested whether the *MKK3* gene is induced in response to different stress stimuli. For this purpose, a 1997-bp fragment upstream of the *MKK3* translational start codon was fused to the β-glucuronidase (*GUS*) reporter gene and transgenic lines were generated with the fusion construct. Six single-insertion lines (as judged by selection marker segregation) were used. All of these lines showed the same expression pattern. Histochemical GUS assays of untreated 16-d-old seedlings showed basal GUS expression in leaf vascular tissues (Figure 1A). This expression pattern did not change when different abiotic stress conditions, such as cold, salt, and drought, were applied. However, a strong induction was detected in leaves that were infiltrated with *Pseudomonas syringae* pv *tomato* strain DC3000 (*Pst* DC3000) (Figures 1B and 1C). To rule out the possibility that this staining is caused by a bacterial enzyme, ecotype Columbia (Col-0) plants were also infiltrated with this strain, but in this case no staining was detected (data not shown). Publicly available microarray data accessed via Genevestigator (Zimmermann et al., 2004) also showed that of all tested stress stimuli, *MKK3* was induced only by *Pseudomonas*. This suggested that MKK3 might have a role in pathogen signaling.

MKK3 Positively Regulates *PR* Gene Expression and Plays a Role in Defense against *Pst* DC3000

To analyze the function of *MKK3* (At5g40440) in *Arabidopsis*, we isolated a T-DNA insertion line in the Col-0 background from seed material obtained from the Salk Institute Genomic Analysis Laboratory collection. Genomic analysis of the T-DNA insertion line indicated that these plants carry a T-DNA insertion at 1058 of 1563 bp downstream of the start codon within exon 7 of the *MKK3* gene (Figure 1D), leading to an mRNA null phenotype (Figure 1E). This mutant was designated *mkk3-1* by Takahashi et al. (2007); thus, for clarity, we also use this nomenclature.

In addition to the *mkk3* mutant line, we also generated *MKK3*-overexpressing lines in the Col-0 background. We used either the wild-type cDNA (*MKK3-WT*) or a constitutively active mutant form that was generated by changing both putative phosphorylation sites to Glu residues (S235E and T241E: *MKK3-EE*). Both gene forms were expressed as myc epitope–tagged versions under the control of the constitutive cauliflower mosaic virus 35S promoter (Figure 1E). Three homozygous lines of both constructs were used in subsequent experiments. *MKK3*-overexpressing plants showed no obvious morphological phenotype under normal ambient experimental conditions.

To complement *mkk3-1*, the 35S:MKK3-WT:myc expression cassette was transformed into this mutant background (see Supplemental Figure 1A online). Two homozygous sublines were used in subsequent experiments.

One of the best-characterized molecular markers associated with pathogen response is the gene *PR1*. To investigate the

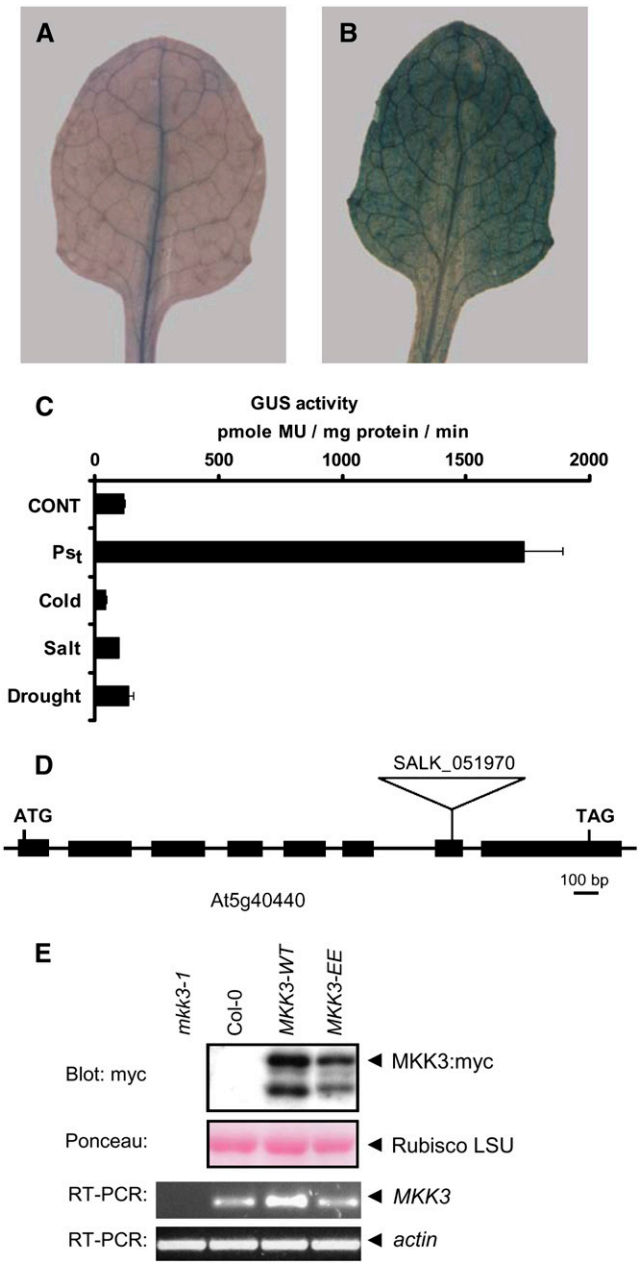

Figure 1. Analysis of *ProMKK3:GUS*, *mkk3-1*, and *MKK3*-Overexpressing Lines.

(A) and **(B)** Histochemical analysis of GUS activity in *ProMKK3:GUS* plants in response to *Pst* DC3000 infiltration. Mock-infiltrated seedlings **(A)** and seedlings infiltrated with 10^6 colony-forming units (cfu)/mL *Pst* DC3000 **(B)** are shown.

(C) Effect of treatments on GUS activity of short-day-grown, 16-d-old transgenic *Arabidopsis* seedlings carrying the –1997 *ProMKK3:GUS* construct. CONT, untreated control; Pst, infiltrated with 10^6 cfu/mL *Pst* DC3000; Cold, 4°C in dark; Salt, 400 mM NaCl; Drought, lid of the Petri dishes removed. The duration of all treatments was 20 h. Similar results were obtained with five independent transgenic lines. Specific GUS activity is expressed as pmol 4-methylumbelliferyl-β-D-glucuronide·mg^{-1} protein·min^{-1}. Values obtained with untransformed *Arabidopsis* seedlings were subtracted. Error bars show SE. GUS staining and flourimetric assays were repeated three times with similar results.

(D) Genomic organization of the *MKK3* gene and the position of the T-DNA insertion within *mkk3-1* as determined by PCR and sequencing of the flanking regions. Bar = 100 bp.

(E) Analysis of MKK3:myc expression and *MKK3* transcript levels from 16-d-old seedlings. MKK3 protein was detected using anti-myc antibody, and the large subunit (LSU) of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) is shown as a loading control for equal protein amounts in wild-type Col-0 and plants overexpressing wild-type or constitutively active MKK3-EE. The *mkk3* null line (*mkk3-1*), wild-type Col-0, and *MKK3*-overxpressing plants were analyzed by RT-PCR. *MKK3* was amplified using gene-specific primers, and the *ACT3* actin gene was used as a control for equal cDNA amounts. Samples were analyzed from three plants per line with similar results.

possible role of *MKK3* in regulating *PR1* expression, we determined *PR1* transcript levels by quantitative RT-PCR analysis. We found that basal *PR1* expression levels were enhanced in the overexpression lines (Figure 2A), suggesting that MKK3 has a positive regulatory role in *PR1* expression. Similarly, we also found a set of other *PR* genes (*PR2*, *PR3*, and *PR4*) to be induced by *MKK3* overexpression (Figure 2A).

Transcriptional induction of the *MKK3* gene by *Pst* DC3000 infection and the identification of *PR* genes as transcriptional targets suggested that MKK3 might alter pathogen responses in *Arabidopsis*. Thus, we first compared the sensitivity of *mkk3-1* and MKK3-overexpressing lines with wild-type Col-0 plants upon infection with the hemibiotrophic pathogen *Pst* DC3000. For this purpose, bacterial growth in Col-0 control plants was compared with that in *mkk3-1* and transgenic lines expressing myc-tagged versions of either the wild type or constitutively active *MKK3-EE*. Quantification of the infection process showed significant differences among the tested plant lines. As shown in Figure 2B, *MKK3-EE* overexpressor lines displayed significantly less bacterial growth compared with wild-type plants at 48 and 72 h after inoculation. Overexpressing wild-type *MKK3* did not have a significant effect on bacterial growth rate. On the contrary, *mkk3-1* plants showed at 72 h after inoculation significantly higher bacterial numbers than in wild-type plants. The bacterial growth rate in *mkk3-1*/*MKK3* complementation lines was reduced compared with that in the mutant genotype, although not completely down to wild-type levels (see Supplemental Figure 1B online).

These data indicate that *MKK3* plays a role in regulating basal resistance in plant–pathogen interactions.

MKK3 Interacts with Group C MAPKs

In a typical MAPK pathway, MKKs function as activators of specific MAPKs. To identify possible downstream MAPK targets of MKK3, we tested for direct interaction in a pair-wise yeast two-hybrid analysis with 14 different MPKs, representing all four groups of *Arabidopsis* MAPKs. To this end, the MPKs were cloned into the GAL4 activation domain vector pGAD. The interactions of MPKs with MKK3, which was fused to the LexA binding domain in the pBTM vector, were subsequently determined

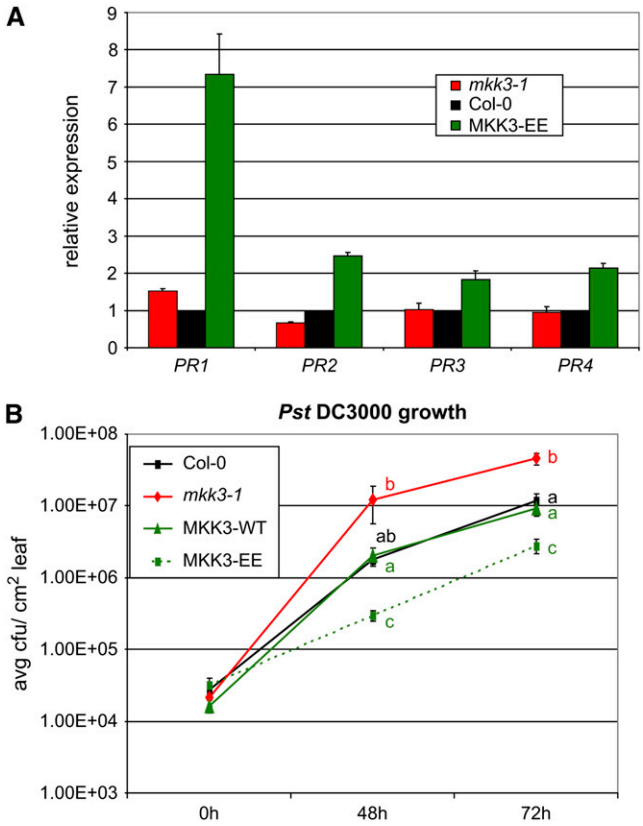

Figure 2. Characterization of MKK3 in Pathogen Response.

(A) Analysis of *PR* gene expression in long-day-grown *mkk3-1*, Col-0, and *MKK3-EE*–overexpressing plants by quantitative RT-PCR. *PR* genes were amplified using gene-specific primers, and the *UBQ4* gene was used as an internal control. The experiment was repeated two times with similar results, and the averages of the two independent biological repeats are shown. Error bars represent SE.

(B) Growth of *Pst* DC3000 in *mkk3-1*, wild-type Col-0, and *MKK3*-overxpressing plants (MKK3-WT and MKK3-EE) after infection by dipping. Growth curves represent the bacterial titers of three pooled leaf discs in six replicates at different times after infection on a logarithmic scale. The experiment was repeated three times with similar results, and the averages of all experiments are shown. Different letters at the 48- or 72-h data points indicate significant differences ($P < 0.05$) calculated with data from all replicates at each time point with one-way analysis of variance and Tukey's honestly significant difference test. Error bars represent SE.

in quantitative β-galactosidase assays. MKK3 showed the strongest interaction with MPK7 and to a lower degree also with MPK1 and MPK2 (Figure 3A). These MAPKs belong to group C MPKs. Interaction of the four group C MPKs with all 10 *Arabidopsis* MKKs was also tested in a similar pair-wise yeast two-hybrid analysis. MPK1, MPK2, and MPK7 only interacted with MKK3 but with no other MKK in yeast (Figures 3B to 3D). Interestingly, MPK14, the fourth member of this group, did not interact with MKK3 in the original setup (i.e., *MKK3* in bait vector and *MAPK*s in prey vector). However, when they were cloned in the opposite vectors, a weak interaction of MKK3 with MPK14 was observed (data not shown).

Compared with the other *Arabidopsis* MKKs, MKK3 has an unusual structural feature consisting of a NTF2 domain in its extended C-terminal region. In order to determine whether this domain has any effect on the interaction properties of MKK3, a truncated version of MKK3 was generated lacking the NTF2-like domain (*MKK3ΔNTF2*). Loss of this domain resulted in a largely reduced interaction capability of MKK3: interaction with MPK7 was strongly reduced, and the weak interactions with MPK1 and MPK2 were completely lost (see Supplemental Figure 2 online). These results suggest that MKK3 is upstream of group C MPKs and that interaction requires a proper MKK3 structure as well.

MKK3 Activates Group C MAPKs

To test whether MKK3 is capable of activating MPKs in *Arabidopsis*, protoplasts were transformed with hemagglutinin (HA) epitope–tagged versions of different MPKs, representing all four groups of *Arabidopsis* MAPKs. This set of MPKs was also co-transformed with either myc epitope–tagged wild-type *MKK3* or constitutively active *MKK3*. All constructs were expressed under the control of the cauliflower mosaic virus 35S promoter. After immunoprecipitation of the MPKs, their activities were determined by in vitro kinase assays using myelin basic protein (MBP) as an artificial substrate. In contrast with all other MPKs, only group C MAPKs (i.e., MPK1, MPK2, MPK7, and MPK14) were activated by the constitutively active MKK3-EE (Figure 4A). On the other hand, the basal activities of the other kinases tested were not affected by MKK3 coexpression, suggesting that MKK3 is not an activator of these MAPKs. As shown by protein gel blotting, the enhanced activity of group C MPKs by coexpression with MKK3-EE was not due to increased protein levels compared with coexpression with MKK3-WT (Figure 4A, middle panels). In the case of group C MPKs, however, an accumulation of the MPK proteins occurred upon *MKK3* coexpression compared with single transformation. Possibly, these kinases are protected from degradation when forming a complex with MKK3. Together, these results show that MKK3 is an activator of group C MPKs.

MKK3 Phosphorylates MPK7

As MPK7 is the strongest interactor with MKK3 and because of its apparent coregulation with MKK3 in microarray data (see below), we focused our further work on this interaction. To investigate whether MKK3 can directly phosphorylate MPK7, we expressed and purified recombinant kinase-inactive glutathione *S*-transferase (GST) fusion proteins of MPK7 and MPK6 as a control. MKK3 wild type and constitutively active MKK3-EE were immunoprecipitated from transiently transformed protoplasts and tested for their ability to phosphorylate GST-MPK6 and GST-MPK7 in vitro. Although GST-MPK7 was only weakly phosphorylated by wild-type MKK3 (Figure 4B), a strong increase in MPK7 phosphorylation was detected when constitutively active MKK3-EE was used (Figure 4B). By contrast, GST-MPK6 was not phosphorylated by either the wild type or the constitutively

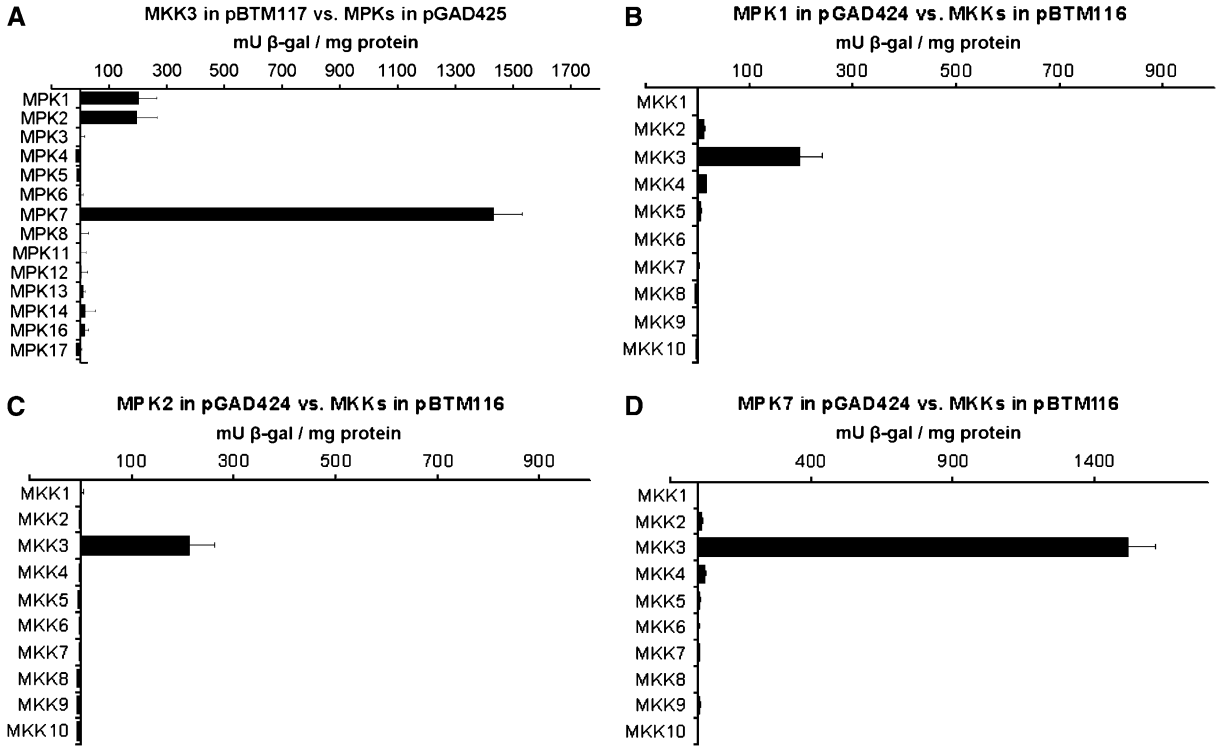

Figure 3. Identification of Downstream MAPKs of MKK3 by Directed Two-Hybrid Analysis.

(A) Quantitative yeast two-hybrid analysis of pBTM116-MKK3 with 14 different pGAD424-MPKs, representing all MAPK groups in the *Arabidopsis* genome.

(B) to **(D)** Quantitative yeast two-hybrid analysis of the MKK3-interacting MPKs with all 10 MKKs of the *Arabidopsis* genome.

(B) pGAD424-MPK1 with pBTM116-MKKs.

(C) pGAD424-MPK2 with pBTM116-MKKs.

(D) pGAD424-MPK7 with pBTM116-MKKs.

Interaction is expressed as specific β-galactosidase activity in units per milligram of protein. For each plasmid combination, the background values of both constructs measured with the corresponding empty vector were subtracted. The error bars represent the SD from three technical repeats. Two biological repeats were performed, with similar results.

active MKK3 variant when immunoprecipitated from protoplasts (Figure 4B).

MKK3 Interacts with MPK7 in Planta

To confirm that MPK7 associates with MKK3 in planta, coimmunoprecipitation assays from plants were performed. Plant extracts from Col-0 and myc-tagged MKK3-WT overexpressor plants were immunoprecipitated with an anti-MPK7 antibody that was raised in rabbit against an MPK7 C-terminal oligopeptide (see Supplemental Figure 3 online). As a negative control, the reactions were performed without the addition of antibody. The samples were subsequently subjected to immunoblot analysis with anti-myc antibody. As Figure 4C shows, MKK3 could be detected from anti-MPK7 immunopreciptates of MKK3:myc-expressing plant samples. This indicates that the two proteins participate in a protein complex in vivo.

To confirm this result, coimmunoprecipitation was also performed in the other direction using transiently transformed protoplasts. HA-tagged *MPK7* and myc-tagged *MKK3* were cotransformed and protein extracts were immunoprecipitated either with anti-myc antibody or without antibody. Protein gel blot analysis with anti-HA antibody revealed that MKK3 pulled down MPK7 (Figure 4D).

Together, the physical interaction, activation, and phosphorylation analyses convincingly indicate that MKK3 is an upstream activator of group C MPKs.

H_2O_2, but Not flg22, Activates MPK7 in an MKK3-Mediated Manner

In order to justify that MPK7 is a downstream MAPK of MKK3 in pathogen-responsive signaling, we tested whether MPK7 is activated in response to flagellin, a PAMP known to activate MAPK pathways, and in response to H_2O_2, a secondary signal that is produced in plant cells after pathogen recognition events. For this purpose, we transiently expressed the HA-tagged MAPKs MPK6 and MPK7 in *Arabidopsis* protoplasts in the

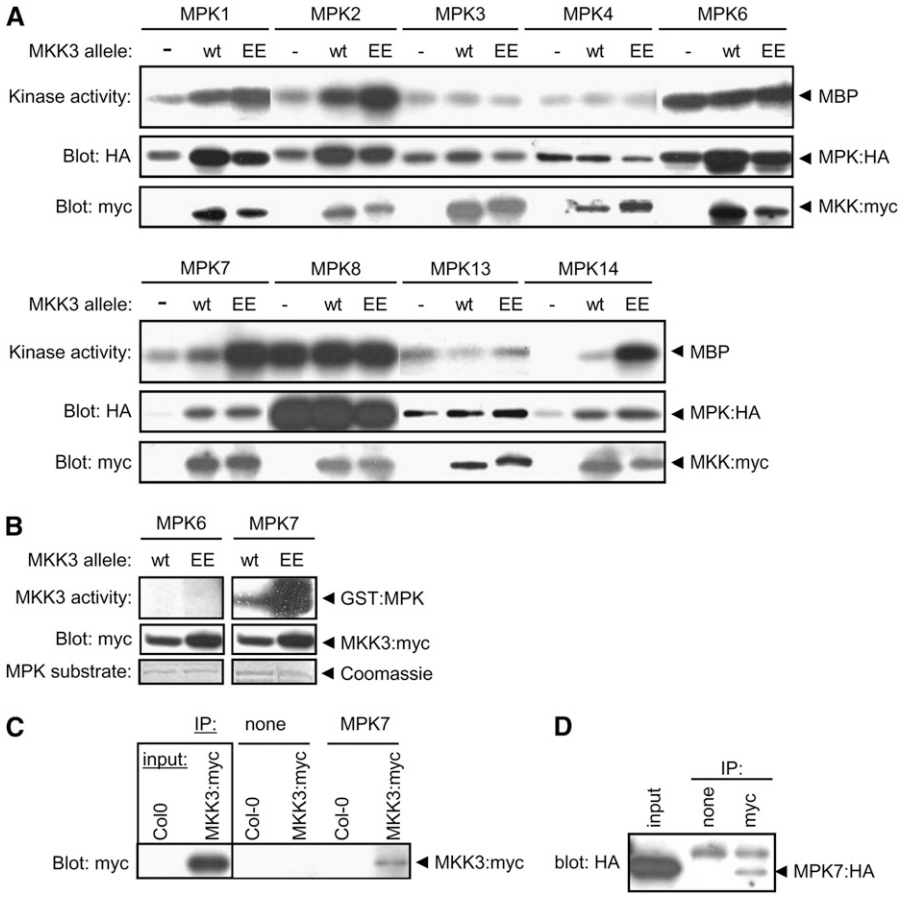

Figure 4. Activation of Downstream MAPKs by MKK3 and Complex Formation of MPK7 and MKK3 in Planta.

(A) Activation of different MPKs by MKK3. MPKs were transiently transformed into *Arabidopsis* protoplasts. Activation of MPKs was tested by coexpression of the MPKs with either wild-type MKK3-WT or constitutively active MKK3-EE. The kinase activity of immunoprecipitated MPKs was determined with MBP as an artificial substrate and autoradiography after SDS-PAGE. Expression of the MPKs and MKK3 was detected by protein gel blot analysis with anti-HA (MPKs) or anti-myc (MKK3) antibody.

(B) In vitro phosphorylation of MPK7 by MKK3. Wild-type (wt) and constitutively active (EE) myc epitope–tagged MKK3 were immunoprecipitated from *Arabidopsis* protoplasts and subsequently used for the phosphorylation of recombinant kinase-inactive GST-MPK6 and GST-MPK7. Phosphorylation of MPKs was analyzed by autoradiography after SDS-PAGE. MKK3 protein was detected using anti-myc antibody; a Coomassie blue stain of the MPK substrates is shown in the lower panel.

(C) MKK3 coimmunoprecipitates with MPK7 from plant extracts. Protein extracts from seedlings of wild-type Col-0 (Col-0) and myc-tagged MKK3-overexpressing (MKK3:myc) plants were immunoprecipitated (IP) either with anti-MPK7 antibody or without antibody addition. Protein gel blot analysis shows the presence of MKK3:myc in the crude protein extracts (input) or in the immunoprecipitated protein complexes.

(D) MPK7 coimmunoprecipitates with MKK3 from protoplast extracts. Protein extracts from protoplasts transiently transformed with HA-tagged MPK7 and myc-tagged MKK3 were immunoprecipitated either with anti-myc antibody or without antibody addition. Protein gel blot analysis shows the presence of MPK7:HA in the crude protein extract (input) or in the immunoprecipitated protein complexes. The MPK activation experiments were repeated five times and the coimmunoprecipitation experiments were repeated three times, with similar results.

presence or absence of MKK3 or MKK4. After immunoprecipitation, MAPK activity was determined by in vitro kinase assays using MBP as a substrate. We found that MPK7 was activated by H_2O_2 and that this activation was strongly enhanced when coexpressed with MKK3-myc, leading to activity levels comparable to the activity when coexpressed with constitutively active MKK3 (Figure 5A). On the other hand, flg22 treatment only caused minor activation of MPK7, even when coexpressed with MKK3 (Figure 5B).

MPK6 was also activated by H_2O_2, and this activation was not influenced by coexpressing it with MKK3 (Figure 5C). Coexpression of its known upstream activator, MKK4, was sufficient for a moderate increase in MPK6 activity without external stimuli. In contrast with MKK3, however, MKK4 coexpression enhanced MPK6 activation in response to H_2O_2 (Figure 5C). The failure to activate MPK7 with flg22 was not due to the inability of cells to recognize and respond appropriately to this PAMP, as MPK6 was strongly activated by flg22 (Figure 5D).

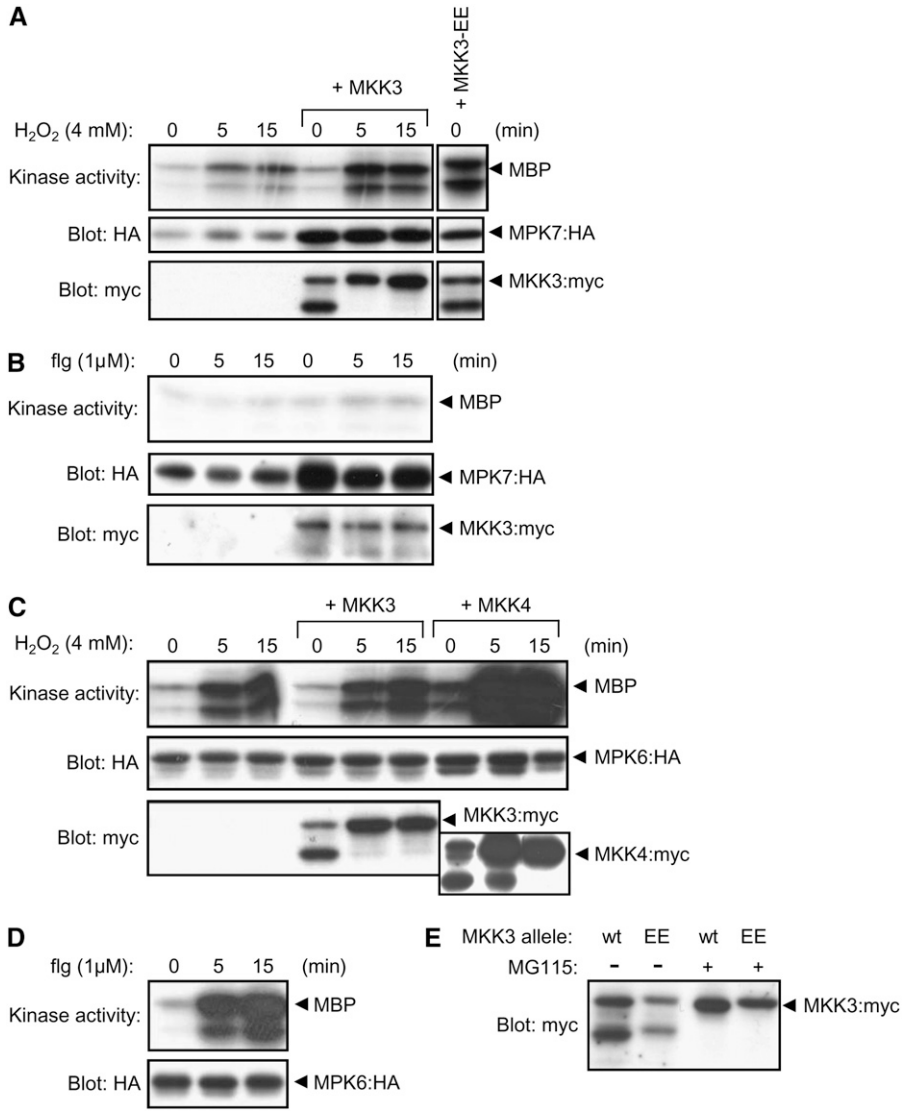

Figure 5. MKK3-Mediated Activation of MPK7 and MPK6 by H_2O_2 and flg22.

(A) to **(D)** *Arabidopsis* protoplasts were transiently transformed with MPK7 (**[A]** and **[B]**) or with MPK6 (**[C]** and **[D]**) alone or in the presence of MKK3 or MKK4. Protoplasts were treated with either H_2O_2 (**[A]** and **[C]**) or flg22 (**[B]** and **[D]**) for the indicated periods. The kinase activity of immunoprecipitated MPKs was determined with MBP as an artificial substrate and by autoradiography after SDS-PAGE. Expression of MPK7 and MPK6 and of MKK3 and MKK4 was detected by protein gel blot analysis with anti-HA (MPKs) or anti-myc (MKKs) antibody. These activation assays were repeated three times for MPK7 and twice for MPK6.

(E) Stabilization of MKK3 by MG115. Wild type (wt) and constitutively active (EE) myc epitope–tagged MKK3 from nontreated and MG115-treated protoplasts was detected by protein gel blot analysis with anti-myc antibody. This experiment was repeated twice.

The expressed MPK7 and MKK3 proteins were detected by protein gel blotting. Interestingly, we found that MPK7 protein accumulated to a higher level when coexpressed with MKK3 (Figures 5A and 5B). This accumulation, however, did not result in enhanced kinase activation, as judged from MBP phosphorylation levels. Bands of MKK3 migrating at lower molecular mass than predicted for the full-length MKK3:myc were also detected (Figures 1E and 5). These lower bands disappeared upon H_2O_2 treatment (Figures 5A and 5C). To test whether this is a proteasome-mediated process, we treated protoplasts with MG115, a potent proteasome inhibitor. As shown in Figure 5E, MG115 treatment resulted in the accumulation of full-length-sized MKK3 protein. By contrast, MPK6 levels were constant in all experiments (Figures 5C and 5D). H_2O_2 treatment had an effect on MKK4 similar to MKK3 (Figure 5C). These findings show that MKK3 is an upstream activator of MPK7 that responds to H_2O_2 and that this pathway probably functions independently of flagellin signaling.

MKK3 and MPK7 Induce the *PR1* Promoter

Since quantitative RT-PCR analysis showed an induction of *PR1* expression in *MKK3*-overexpressing lines, we also tested whether *PR1* is a transcriptional target of MPK7 and MKK3 by detecting *PR1* promoter–dependent reporter activity. For this purpose, a *ProPR1:GUS* construct was transformed into *Arabidopsis* protoplasts in the presence or absence of different MAPK signaling components. Coexpressing *MPK7* alone was sufficient to induce the *PR1* promoter, and the promoter activity could be further enhanced when MKK3-EE was also coexpressed (Figure 6). Coexpression of MPK6 resulted in a moderate increase in reporter activity, which was slightly increased further by coexpression of MKK4-EE but not by coexpression of MKK3-EE. Coexpression of the active forms of MKK3 and MKK4 with the *ProPR1:GUS* construct in the absence of MPKs was sufficient only for a moderate induction. There is a great difference between promoter induction by MKK3-EE alone and with MPK7 coexpression. This failure of MKK3-EE to induce the promoter is probably due to low levels of endogenous MPK7, which is probably a bottleneck in this system. NPR1 was used as a positive control, and its coexpression resulted in an induction of the *PR1* promoter–dependent reporter activity similar to MPK7 + MKK3-EE coexpression. These results strongly support the notion that MKK3 and MPK7 are part of a signaling pathway that regulates *PR1* expression.

DISCUSSION

The *Arabidopsis* genome encodes 20 MAPKs and 10 MAPK kinases, most of which are still functionally unknown. We set out to functionally analyze MKK3, the only member of group B MAPK kinases. We demonstrate that the susceptibility of *Arabidopsis* plants to infection by virulent *Pst* DC3000 growth correlates with *MKK3* expression and activity levels. While *MKK3* itself is transcriptionally induced upon *Pst* DC3000 infection, *MKK3* is also a positive regulator of *PR* gene expression. In addition, MKK3 interacts with and activates MPK1, MPK2, MPK7, and MPK14, which are the four members of group C MAPKs, for which no functional information has been available to date.

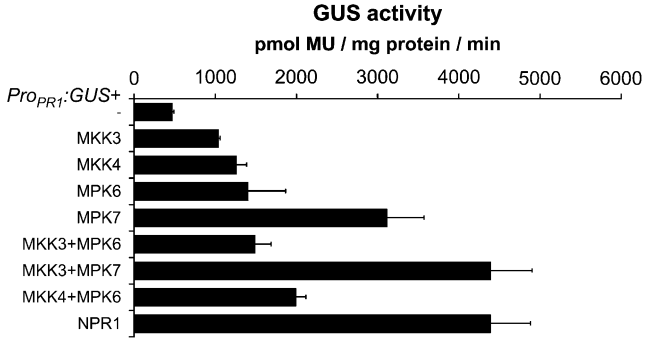

Figure 6. Induction of the *PR1* Promoter by the MKK3-MPK7 Module.

Arabidopsis protoplasts were transformed with either a *ProPR1:GUS* fusion construct alone or in combination with different MAPKs and constitutively active MAPK kinases. GUS activity is expressed as pmol 4-methylumbelliferyl-β-D-glucuronide·mg^{-1} protein·min^{-1}. The error bars represent SE from three measurements. Values obtained from untransformed *Arabidopsis* protoplasts were subtracted.

In order to explore the potential role of *MKK3*, its expression was studied using *Arabidopsis* lines transformed with a *ProMKK3:GUS* fusion construct. Whereas basal expression was observed mainly in vascular tissues, widespread expression of *MKK3* was strongly induced by *Pst* DC3000 infection but not by different abiotic stimuli. This suggested that *MKK3* could have a role in pathogen signaling. Since constitutively active MKK3 results in MAPK activation of group C MPKs (Figure 4A), this pathway is probably regulated at both the posttranslational and transcriptional levels, the latter of which might be part of a positive feedback loop for amplifying the signal. The involvement of feedback loops appears to be a relatively common theme in plant pathogen-signaling networks for fine-tuning defense responses (Shah, 2003). Microarray data (Zimmermann et al., 2004) show that besides induction by *Pseudomonas* infection, *MKK3* is also induced upon senescence. We confirmed this observation in our *ProMKK3:GUS* lines (data not shown). Furthermore, microarray data show the identified downstream MAPK *MPK7* to be regulated in a very similar fashion. Like *MKK3*, *MPK7* is induced during senescence and by *Pseudomonas* infection.

Besides its own transcriptional induction by *Pst* DC3000, we also found MKK3 to be a signaling component regulating *PR* gene expression. Our genetic studies support this notion, as bacterial growth assays showed that *Pst* DC3000 growth is effectively retarded in plant lines overexpressing *MKK3-EE*, whereas *mkk3-1* knockout plants were more susceptible to bacterial invasion. These findings strongly suggest that MKK3 plays a role in *Arabidopsis* defense signaling. Complementation of *mkk3-1* by MKK3-WT expression in this background resulted in a reduction of bacterial growth, although not completely to wild-type values (see Supplemental Figure 1 online). The remaining difference in resistance might be explained by the lack of the observed positive feedback loop mechanism in this system.

In a prototypical MAPK cascade, MAPK kinases act through phosphorylating and thereby activating downstream MAPKs. To identify potential MPK partners of MKK3, a directed pair-wise yeast two-hybrid analysis was performed, revealing that MKK3 selectively interacts with MPK1, MPK2, and MPK7 and to some extent with MPK14 in yeast. These MAPKs belong to the group C MAPKs. In protoplasts, an active version of MKK3 could activate MPK1/2/7 and MPK14, all four members of the group C MAPKs. Because *MKK3* and *MPK7* are transcriptionally coregulated by *Pst* DC3000, we focused our further analyses on the MKK3–MPK7 interaction. We found that MPK7 is stabilized by MKK3 at the protein level. Interestingly, this accumulation of MPK7 did not lead to an increase of MPK7 activity, which, however, could be achieved either by H_2O_2 treatment or by coexpressing constitutively active MKK3. These data suggest that complex formation with MKK3 might protect MPK7 from degradation. Indeed, by coimmunoprecipitation experiments, we could show that MKK3 and MPK7 form a complex with each other in planta.

Protein degradation also plays a role in MKK3 regulation. Bands of lower molecular mass than full-length MKK3 were detected both in seedlings (Figure 1D) and in protoplasts (Figure 5), and we also observed that the lower bands disappeared upon

treatment with the proteasome inhibitor MG115. In fact, H_2O_2 treatment had the same effect, suggesting that protein stabilization plays a role in MKK3 and MPK7 signaling. The role of protein stabilization, however, must be indirect, since the proteasome completely destroys target proteins; here, however, we observed distinct bands of MKK3. In the absence of H_2O_2, constitutively active MKK3-EE was also detected as multiple bands, indicating that this effect is separable from activation. Nonetheless, MAPK kinase rearrangement by H_2O_2 may be a more general phenomenon, as a very pronounced accumulation of full-length MKK4 protein was also observed following H_2O_2 treatment.

MPK7 is activated by H_2O_2, and this activation is clearly enhanced by coexpression with MKK3 (Figure 5A). Furthermore, *PR1* promoter–dependent *GUS* expression was strongly induced by coexpression of MPK7 with MKK3 (Figure 6A). These data indicate that MKK3 and MPK7 are part of a pathogen-signaling MAPK pathway that regulates *PR1* gene expression. Using the *ProPR1:GUS* construct, we could narrow down the transcriptional activation of the pathway to an 833-bp promoter region of *PR1*. Functional analysis of this promoter sequence revealed five regulatory motifs (Lebel, 1998). Of these five regulatory elements, three are perfectly conserved within the *MKK3* promoter and are binding sites for GCN4 (TGACTG: −695 to −690) and WRKY (TTGACT: −1491 to −1486, −1106 to −1101, −223 to −218, and −35 to −30) transcription factors. Moreover, an 8-bp site similar to the nuclear factor κB binding site is also conserved in both promoters (GGACTTTTC: −138 to −130 in *ProMKK3*). The GCN4 and WRKY sites also occur in the *MPK7* promoter (−1548 to −1553 and −1058 to −1053, respectively), although the GCN4 site is inverted. We also analyzed a 2000-bp sequence upstream of *MKK4*, another *Arabidopsis* MAPK kinase that is involved in innate immunity responses (Asai et al., 2002), but none of the *PR1* promoter elements was found within this region of the *MKK4* promoter. Considering this fact, the conservation of such elements in *PR1* and *MKK3* promoters is highly remarkable.

Pathogens in higher eukaryotes are recognized by multiple receptors that recognize PAMPs (Nürnberger et al., 2004). The production of ROS is an important early component of the innate immune system in animals and plants (Nürnberger and Scheel, 2001). The recognition of flg22 in *Arabidopsis* occurs via the FLS2 receptor (Gomez-Gomez et al., 2001) and leads to the production of ROS, medium alkalinization, the activation of MPK3/6 via MKK4/5 or MPK4 via MKK1, and the induction of pathogen-responsive genes (Felix et al., 1999; Gomez-Gomez et al., 1999; Nühse et al., 2000; Asai et al., 2002; Mészáros et al., 2006). Surprisingly, flg22 was not an activator of the MKK3-MPK7 module, although a minor increase in MPK7 activity could be observed following flg22 treatment. This low degree of activation could be a consequence of some ROS accumulation after flg22 recognition. Therefore, we assume that the MKK3-MPK7 pathway might be activated by a recognition system other than that of flagellin that may use ROS as a secondary signal or, alternatively, the applied H_2O_2 in our experiments causes a physiological change that initiates the signaling.

A recent article by Takahashi et al. (2007) demonstrates that *Arabidopsis* root growth inhibition by JA is negatively regulated by MKK3. Accordingly, a JA marker gene (*VSP2*) is also negatively regulated by the pathway. JA is not only a key regulator of development in plants but also of biotic and abiotic stress responses (reviewed in Browse, 2005). According to Takahashi et al. (2007), some JA-responsive genes (e.g., *LOX2* and *VSP1*) along with *PDF1.2*, a JA-regulated pathogen marker gene, are positively regulated by MKK3. As we found MKK3 to be a positive regulator of pathogen response, it is reasonable to speculate that MKK3-mediated pathogen signaling is also part of the JA signaling network. Interestingly, JA signaling is also linked to ROS. For example, JA induces the accumulation of antioxidant pathways, and in a JA-deficient *Arabidopsis* mutant (*opr3*) the induction of these pathways upon ozone exposure is abolished (Sasaki-Sekimoto et al., 2005), and *oji1*, a JA-insensitive mutant, is hypersensitive to O_3 treatments (Kanna et al., 2003). On the other hand, JA generates H_2O_2, an agent we found to activate MPK7, as a secondary signal for the activation of defense genes (Orozco-Cardenas and Ryan, 1999; Orozco-Cardenas et al., 2001).

Takahashi et al. (2007) place MPK6 downstream of MKK3 in JA signaling, whereas we did not observe an activation of MPK6 by MKK3. This apparent contradiction probably can be assigned to differences in biochemical approaches. Yeast two-hybrid assays in both laboratories showed no interaction between MKK3 and MPK6 (cited as data not shown in Takahashi et al., 2007). Takahashi et al. (2007) used GST-purified recombinant kinases to show both phosphorylation and activation of MPK6 by MKK3 in vitro. To this end, we used kinases immunoprecipitated from protoplasts; thus, activation of MAPKs took place in living cells. The use of high concentrations of purified kinases in vitro might result in unspecific activities; in addition, removal of the kinase from the cell often results in a loss of its physiological regulatory mechanisms (reviewed in Manning and Cantley, 2002). Nevertheless, a weak activation of MPK1 and MPK2 by MKK3 was also observed in this setup, and MPK7 was not tested with active MKK3.

A strong activation of MPK6 upon constitutively active MKK3DD induction is shown by Takahashi et al. (2007), supporting the idea that MPK6 is downstream of MKK3 in planta. As no physical interaction was shown between MKK3 and MPK6, an indirect effect cannot be completely ruled out. As at least four distinct MAPK kinases activate MPK6 in cascades regulating different biological responses, scaffold proteins may play a key role in each pathway, and their absence in yeast can explain the failure to detect MKK3–MPK6 interaction by the two-hybrid system.

As indicated by a number of biochemical studies, the presence of only 10 MAPKKs is compatible with the notion that these kinases serve as entry routes to many upstream signals as well as bifurcation points for the activation of multiple downstream MAPKs (Cardinale et al., 2002; Jin et al., 2003). In *Arabidopsis*, MKK4 and MKK5 can activate both MPK3 and MPK6 (Asai et al., 2002), whereas MPK4 can be activated by MKK1 and MKK2 (Mizoguchi et al., 1998; Huang et al., 2000; Matsuoka et al., 2002). Moreover, MKK2 can target MPK4 and MPK6 (Teige et al., 2004). In agreement with this concept, our data show that MKK3 is an activator of all four group C MAPKs.

The common docking (CD) domain of the consensus sequence [LH][LHY]Dxx[DE]xx[DE]EPxC in MAPKs functions as a docking site for MAPKKs, phosphatases, and protein substrates

(Tanoue et al., 2000). *Arabidopsis* MPKs belonging to groups A and B possess an evolutionarily conserved CD domain in their C-terminal extension (MAP Kinase Group, 2002). Interestingly, the corresponding CD domains in group C MPKs appear to be modified in the hydrophobic residues (VP[IV][SD]L[DE][IV]-DE[ND][ML]xx[DE]) and might explain their (at least in yeast) specific interaction with MKK3. Interestingly, MPK14 is a very weak interactor of MKK3. MPK7 and MPK14 are highly homologous proteins, and perhaps MPK14 is only able to fully interact with MKK3 in planta in the presence of a third component and/or under certain conditions, in order to avoid promiscuous interactions. Deletion of the C-terminal NTF2-like domain of MKK3 significantly reduces its capability to interact with MAPKs, suggesting that a proper structure is important for proper binding.

On the basis of these results, we suggest a role for MKK3 function in pathogen signaling. Accordingly, plants can detect the presence of *Pst* DC3000 by PAMPs, which are recognized by an array of specific receptors on the plant cells. As a consequence, secondary signals, such as ROS, are triggered. JA can also trigger ROS production. ROS can activate the MKK3-MPK7 module that induces target genes such as *PR1*, thereby activating defense responses. In this pathway, transcriptional feedback loops are also at work, as *MKK3* and *MPK7* gene expression is induced upon *Pst* DC3000 infection. On the other hand, JA can activate MPK6 in an MKK3-dependent manner, leading to root growth arrest via the AtMYC2 transcription factor, and the expression of defense genes (e.g., *PDF1.2*).

An interesting question that remains open is the role of the other three group C MAPKs, which should be clarified by further investigations in the future. It was recently shown that members of the C1 subgroup, MPK1 and MPK2, are activated by diverse stress signals, such as wounding, JA, abscisic acid, and H_2O_2 (Ortiz-Masia et al., 2007). Therefore, it is very likely that this group of MPKs have redundant functions in stress signaling. In addition, a remarkable coregulation of these genes is shown in microarray data in different organs (see Supplemental Figure 4 online). With the exception of *MPK2*, all four *MPK*s appear to be expressed at a relatively low basal level in all organs and tissues. Interestingly, all four group C MPKs are highly expressed in xylem and cork tissues. Coinciding with this expression pattern, *MKK3* also has a strong basal expression in vascular tissues, suggesting that *MKK3* and its downstream MAPK targets in these cell types may have a role other than stress signaling.

METHODS

Plant Materials

Arabidopsis thaliana Col-0 was used as genetic background. Seeds were germinated on 0.5× Murashige and Skoog (MS) medium (Sigma-Aldrich), and plants were grown at 24 to 26°C, 30% RH, and ~50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ cool-white light under long-day (16 h of light/8 h of dark) conditions. *Arabidopsis* protoplasts were prepared from a suspension culture as described (Cardinale et al., 2000).

Analysis of the *mkk3* T-DNA Insertion Line

The *mkk3* T-DNA insertion line was isolated from a seed stock obtained from the SALK T-DNA collection (SALK_051970). We screened plants germinated on 0.5× MS medium, without kanamycin, with a T-DNA–specific (LBa1, 5′-TGGTTCACGTAGTGGGCCATCG-3′) and a gene-specific (LP, 5′-GGGCCTGTTAATCTTATGTTGCAG-3′) primer pair for the presence of the insertion. For further mapping of the T-DNA insertion site, we also used the T-DNA–specific primer RB (5′-TTCAACGTTG-CGGTTCTGTCAGTT-3′) and the gene-specific primer RP (5′-AATCT-AAGTTTGTAATATAAAGCTCTTGC-3′). The LP-Lba1 PCR product was cloned and verified by sequencing.

Pairs of primers were used to determine whether the plants were homozygous or heterozygous for the T-DNA insertion (MKK3FlankFwd, 5′-GTCTGGTTAAGAATTGGATTCTTTC-3′; MKK3FlankRev, 5′-TCATC-AAATCCATCAAAGAGGGAG-3′).

For RT-PCR, the following primers were used: for *actin3* (At2g37620), ACT3Fwd, 5′-ATGGTTAAGGCTGGTTTTGC-3′, and ACT3Rev, 5′-AGC-ACAATACCGGTAGTACG-3′; for *MKK3* (At5g40440), MKK3Fwd, 5′-TGG-CTATGTGTGCTACTTTTG-3′, and MKK3Rev, 5′-GGTGAGCATATCAG-CTAAATC-3′.

Molecular Cloning and Construction of Expression Vectors

Open reading frames of the different MPKs and MKKs were amplified from a cDNA library (Minet et al., 1992) with a *Nco*I restriction site at the 5′ end and a *Not*I restriction site in front of the stop codon. The truncated version of *MKK3* (bases 1 to 1095, *MKK3ΔNTF2*), lacking the NTF2-like domain, was amplified the same way. The *Not*I restriction site was used to introduce either a triple HA epitope or a c-myc epitope. The constitutively active allele of MKK3 was generated by changing both putative phosphorylation sites to Glu residues (S235E and T241E). These point mutations resulted in a constitutively active kinase (MKK3-EE). The *GUS* gene with polyadenylation signal was subcloned as an *Xho*I-*Kpn*I fragment into the binary vector pGreenII 0029 (Hellens et al., 2000) after removing the restriction sites that were also present in the MCS of pGreenII 0029. The resulting plasmid was designated pGreenII 0029-GUS. A 1997-bp region upstream of the *MKK3* translational start was amplified from Col-0 genomic DNA using the PCR primers PMKK3Fwd (5′-CTTTG-TTTGCATTTCAGGCTAGTC-3′) and PMKK3Rev (5′-GATAACTACTTTT-TCTGTAACACAGAC-3′). A 5′ *Eco*RI and a 3′ *Xho*I was attached and thereby was subcloned into pGreenII 0029-GUS. An 833-bp region upstream of the *PR1* (At2g14610) translational start was amplified from genomic DNA using the PCR primers introducing a *Spe*I (PPR1Fwd, 5′-CAATTTAAACTGCGTACTAGTGTTTGG-3′) and a *Nco*I (PPR1rev, 5′-AATcCATggTTCTAAGTTGATAATGG-3′) restriction sites. This PCR product was subcloned into pGreenII 0029-GUS.

Generation of Transgenic Plants

Transgenic *Arabidopsis* lines were generated using the floral dipping method (Clough and Bent, 1998) with Col-0 wild-type plants. Wild-type or constitutively active MKK3 (EE) was cloned into the binary plant expression vector pGreenII 0029 (Hellens et al., 2000) under the control of the 35S promoter and transformed as myc epitope–tagged versions. Transformed plants were selected by growth on kanamycin-containing medium. Expression of the transgenic constructs was verified by protein gel blotting, and selected lines of the second generation after transformation were used for the experiments.

RNA Isolation from *Arabidopsis* and RT-PCR

Twelve-day-old *Arabidopsis* seedlings were frozen in liquid nitrogen. One hundred milligrams of plant material was processed in one sample. RNA was isolated according to the manufacturer's instructions using the RNeasy Plant Mini kit (Qiagen). The concentration and purity of RNA were determined by measuring OD_{260} and OD_{280}. RT-PCR for the data

presented in Figure 1E was performed according to Romeis et al. (2001). PCR products were separated on 1.5% agarose gels.

For the quantitative real-time RT-PCR data presented in Figure 2A, total RNA was isolated using the TRI reagent method (Chomczynski and Sacchi, 1987) optimized for plant RNA extraction. First-strand cDNA was synthesized from 2 μg of total RNA using the Moloney murine leukemia virus H-minus reverse transcriptase system (Fermentas) with oligo(dT) primer. The cDNAs were diluted 1:4 with nuclease-free water. Aliquots of the same cDNA sample were used for all primer sets during real-time PCR, and amplification reactions were performed with all primer sets during the same PCR run. Gene-specific primers designed for the sequences corresponding to *UBQ4* (forward, 5′-GCTTGGAGTCC-TGCTTGGACG-3′; reverse, 5′-CGCAGTTAAGAGGACTGTCCGGC-3′) were used as an internal control. Reactions were performed in a volume of 20 μL containing 150 nM of each primer, 2 μL of cDNA sample, and RealMasterMix SYBR ROX. Real-time PCR was performed with the Mastercycler ep realplex on a 96-well reaction plate using the parameters recommended by the manufacturer.

The specific primers for the examined genes were as follows: *PR1* (forward, 5′-AAGGGTTCACAACCAGGCAC-3′; reverse, 5′-CACTGCA-TGGGACCTACGC-3′); *PR2* (forward, 5′-GGGACGGCTCTCGTGGCT-ACC-3′; reverse, 5′-CGCGCGTTATCGAAACTCGCGG-3′); *PR3* (forward, 5′-GACGCCGACCGTGCCGCCGGG-3′; reverse, 5′-CGGCGACTCTC-CCGTCTTGGCC-3′); and *PR4* (forward, 5′-GGACCAATGCAGCAACG-GAGGC-3′; reverse, 5′-GGCTGCCCAATGAGCTCATTGCC-3′). Each PCR was performed in triplicate, and controls without template were included. Two independent experiments were performed using independent biological materials. The $2^{-\Delta\Delta Ct}$ method was used to analyze the real-time PCR data (Livak and Schmittgen, 2001). The expression of the examined genes was normalized to the endogenous control.

GUS Activity Assays

The 5-bromo-4-chloro-3-indolyl-β-glucuronic acid substrate was used for histochemical staining as described (Jefferson, 1987). For fluorimetric assays, crude extracts were prepared and GUS activity was tested as described (Jefferson, 1987). Extracts were standardized based on protein concentration as determined by the method of Bradford (1976).

Treatments of Plants for GUS Assays

For GUS assays, *Pseudomonas syringae* pv *tomato* DC3000 was cultivated in NYG medium (5 g peptone, 3 g yeast extract, and 20 g glycerol per liter), and the cells were pelleted, resuspended, and diluted in 10 mM $MgSO_4$ and 0.02% Silwet-20 to a concentration of 10^6 cfu/mL. Sixteen-day-old seedlings, grown under short-day conditions on 0.5× MS medium, were vacuum-infiltrated with the bacterial suspension, and GUS staining was performed after incubating the seedlings overnight in Petri dishes on wet Whatman paper. Mock inoculation was performed by infiltrating 10 mM $MgSO_4$ and 0.02% Silwet-20. Similarly, seedlings on wet Whatman paper in Petri dishes were used for cold treatment. For salt treatment, Whatman paper discs were soaked with 400 mM NaCl solution. For drought treatment, the lids of the Petri dishes used for seedling growth were removed.

Pseudomonas Growth Assays

Pst DC3000 was cultivated in King's B medium, and the cells were pelleted, resuspended, and diluted in 10 mM $MgSO_4$ and 0.02% SilwetL-77 to a concentration of 10^6 cfu/mL. Plants were infected by dipping, and bacterial growth was assessed by plating dilution series of leaves ground in 10 mM $MgSO_4$ on King's B plates containing 25 μM rifampicin/mL as described (Weigel and Glazebrook, 2002). Statistical analysis was performed with SPSS 10.07. After log transformation of the data of all replicates, data were normally distributed and were analyzed by one-way analysis of variance followed by Tukey's honestly significant difference posthoc test for multiple comparisons between the means.

Yeast Two-Hybrid Assays

Quantitative yeast two-hybrid assays were performed as described (Teige et al., 2001) using the yeast two-hybrid strain L40 and the vectors pBTM116 (Vojtek et al., 1993) for the LexA-BD fusions of MKKs and pGAD424 (Clontech) for the Gal4-AD fusions of the MPKs.

Transient Expression Assays

The open reading frames of MPKs and of MKK3 were cloned into the plant expression vector pRT100 (Topfer et al., 1987; Kiegerl et al., 2000) and fused at their C-terminal end either to a triple HA epitope (MPKs) or to a c-myc epitope (MKKs). *Arabidopsis* protoplast transient expression assays were done as described (Ouaked et al., 2003).

Expression and Purification of GST Fusion Proteins

Escherichia coli strain BL-21 codon plus (Stratagene) was transformed with the expression constructs cloned into the pGEX4-T1 vector (Amersham Pharmacia Biotech) and expressed as GST fusion proteins. Growth of bacteria and isolation of recombinant GST fusion proteins were done according to Matsuoka et al. (2002).

Protein Extracts from *Arabidopsis* Protoplasts and Seedlings

Protein extracts were prepared either from protoplasts as described (Cardinale et al., 2002) or from 200 mg of frozen seedlings in 200 μL of Lacus buffer and sea sand according to Bögre et al. (1999). For coimmunoprecipitation, samples were extracted in 50 mM Tris, pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and proteinase inhibitor mix (Roche).

Coimmunoprecipitation from Plant Extracts

Protein extracts (500 μg) were precleared with 50 μL of protein A–Sepharose beads for 2 h at 4°C, then immunoprecipitated overnight in the presence of antibodies with 50 μL of beads. Samples were washed three times with wash buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, and 0.1% Tween 20) and subjected to immunoblotting. The anti-MPK7 antibody was raised in rabbits by Eurogentec against the C-terminal peptide H2N-LYYHPEAEISNA-COOH.

In Vitro Kinase Assays

Recombinant GST-MPKs (1 μg) were incubated in 15 μL of kinase reaction buffer (50 mM Tris, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 0.1 mM ATP, and 3 μCi of [γ-^{32}P]ATP) with immunoprecipitated myc epitope–tagged wild-type or constitutively active alleles of MKK3 from protoplasts. All kinase reactions were performed at room temperature for 30 min and stopped after 30 min by adding SDS loading buffer and heating for 2 min at 95°C. Reaction products were analyzed by SDS-PAGE, autoradiography, and Coomassie Brilliant Blue R 250 staining.

Immunocomplex Kinase Assays

Immunocomplex kinase assays were performed according to Cardinale et al. (2002). Briefly, protein extracts of equal protein amounts were immunoprecipitated with 30 μL of protein A–Sepharose beads. The beads were washed two times with wash buffer (50 mM Tris, pH 7.4,

250 mM NaCl, 5 mM EGTA, 5 mM EDTA, and 0.1% Tween 20) and with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM EGTA, and 1 mM DTT). Kinase reactions of the immunoprecipitated proteins were performed in 15 μL of kinase buffer containing 5 μg of MBP, 0.1 mM ATP, and 3 μCi of [γ-^{32}P]ATP. Phosphorylation of MBP was visualized by autoradiography after separation by 15% SDS-PAGE.

Immunoblotting

For protein gel blotting, equal protein amounts from extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and probed with HA or myc monoclonal antibodies. Alkaline phosphatase–conjugated anti-mouse IgG (Sigma-Aldrich) was used as a secondary antibody, and the reaction was visualized by fluorography using CDP-Star (Amersham Pharmacia Biotech) as a substrate.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At4g26070 (*MKK1*), At4g29810 (*MKK2*), At5g40440 (*MKK3*), At1g51660 (*MKK4*), At3g21220 (*MKK5*), At5g56580 (*MKK6*), At1g18350 (*MKK7*), At3g06230 (*MKK8*), At1g73500 (*MKK9*), At1g32320 (*MKK10*), At1g10210 (*MPK1*), At1g59580 (*MPK2*), At3g45640 (*MPK3*), At4g01370 (*MPK4*), At4g11330 (*MPK5*), At2g43790 (*MPK6*), At2g18170 (*MPK7*), At1g18150 (*MPK8*), At1g01560 (*MPK11*), At2g46070 (*MPK12*), At1g07880 (*MPK13*), At4g36450 (*MPK14*), At5g19010 (*MPK16*), At2g01450 (*MPK17*), At1g64280 (*NPR1*), At2g14610 (*PR1*), At3g57260 (*PR2*), At3g12500 (*PR3*), At3g04720 (*PR4*), At2g37620 (*actin3*), and At5g20620 (*UBQ4*).

Supplemental Data

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

Supplemental Figure 1. Complementation of *mkk3-1*.

Supplemental Figure 2. Interaction of MAPKs with a Truncated MKK3 Form Lacking the C-Terminal NTF2-Like Domain in a Directed Two-Hybrid Analysis.

Supplemental Figure 3. Characterization of the Anti-MPK7 Antibody.

Supplemental Figure 4. Digital RNA Gel Blot Showing Organ Distribution of Group C MAPK Expression.

ACKNOWLEDGMENTS

We thank Anneliese Auer and Andrey Belokurow for technical assistance. We thank the Academy of Finland and the Biocenter Helsinki. This work was supported by projects of the Austrian Science Fund, the Vienna Science and Technology Fund, and the European Union Research Training Network (HPRN-CT-2002-00249).

Received January 1, 2007; revised September 21, 2007; accepted September 27, 2007; published October 12, 2007.

REFERENCES

Asai, T., Tena, G., Plotnikova, J., Willman, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Asubel, F.M., and Sheen, J. (2002). MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415:** 977–983.

Bögre, L., Calderini, O., Binarova, P., Mattauch, M., Till, S., Kiegerl, S., Jonak, C., Pollaschek, C., Barker, P., Huskisson, N.S., Hirt, H., and Heberle-Bors, E. (1999). A MAP kinase is activated late in plant mitosis and becomes localized to the plane of cell division. Plant Cell **11:** 101–113.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.

Browse, J. (2005). Jasmonate: An oxylipin signal with many roles in plants. Vitam. Horm. **72:** 431–456.

Cardinale, F., Jonak, C., Ligterink, W., Niehaus, K., Boller, T., and Hirt, H. (2000). Differential activation of four specific MAPK pathways by distinct elicitors. J. Biol. Chem. **275:** 36734–36740.

Cardinale, F., Meskiene, I., Ouaked, F., and Hirt, H. (2002). Convergence and divergence of stress-induced mitogen-activated protein kinase signaling pathways at the level of two distinct mitogen-activated protein kinase kinases. Plant Cell **14:** 703–711.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature **410:** 37–40.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Dai, Y., Wang, H., Li, B., Huang, J., Liu, X., Zhou, Y., Mou, Z., and Li, J. (2006). Increased expression of MAP KINASE KINASE7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in Arabidopsis. Plant Cell **18:** 308–320.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell **103:** 239–252.

Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K., and Neill, S.J. (2001). Harpin induces activation of the Arabidopsis mitogen-activated protein kinases AtMPK4 and AtMPK6. Plant Physiol. **126:** 1579–1587.

Droillard, M., Boudsocq, M., Barbier-Brygoo, H., and Lauriere, C. (2002). Different protein kinase families are activated by osmotic stresses in *Arabidopsis thaliana* cell suspensions. Involvement of the MAP kinases AtMPK3 and AtMPK6. FEBS Lett. **527:** 43–50.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18:** 265–276.

Gomez-Gomez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell **13:** 1155–1163.

Gomez-Gomez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J. **18:** 277–284.

Gustin, M.C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **62:** 1264–1300.

Hamel, L.P., et al. (2006). Ancient signals: Comparative genomics of plant MAPK and MAPKK gene families. Trends Plant Sci. **11:** 192–198.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., and Mullineaux, P.M. (2000). pGreen: A versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. **42:** 819–832.

Herskowitz, I. (1995). MAP kinase pathways in yeast: For mating and more. Cell **80:** 187–197.

Hohmann, S. (2002). Osmotic adaptation in yeast—Control of the yeast osmolyte system. Int. Rev. Cytol. **215:** 149–187.

Huang, Y., Li, H., Gupta, R., Morris, P.C., Luan, S., and Kieber, J.J. (2000). ATMPK4, an Arabidopsis homolog of mitogen-activated protein kinase, is activated in vitro by AtMEK1 through threonine phosphorylation. Plant Physiol. **122:** 1301–1310.

Ichimura, K., Casais, C., Peck, S.C., Shinozaki, K., and Shirasu, K. (2006). MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. **281:** 36969–36976.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K. (2000). Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. Plant J. **24:** 655–665.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. **5:** 387–405.

Jin, H., Liu, Y., Yang, K.Y., Kim, C.Y., Baker, B., and Zhang, S. (2003). Function of a mitogen-activated protein kinase pathway in N gene-mediated resistance in tobacco. Plant J. **33:** 719–731.

Jonak, C., Okresz, L., Bogre, L., and Hirt, H. (2002). Complexity, cross talk and integration of plant MAP kinase signalling. Curr. Opin. Plant Biol. **5:** 415–424.

Kanna, M., Tamaoki, M., Kubo, A., Nakajima, N., Rakwal, R., Agrawal, G.K., Tamogami, S., Ioki, M., Ogawa, D., Saji, H., and Aono, M. (2003). Isolation of an ozone-sensitive and jasmonate-semi-insensitive Arabidopsis mutant (oji1). Plant Cell Physiol. **44:** 1301–1310.

Kiegerl, S., Cardinale, F., Siligan, C., Gross, A., Baudouin, E., Liwosz, A., Eklof, S., Till, S., Bogre, L., Hirt, H., and Meskiene, I. (2000). SIMKK, a mitogen-activated protein kinase (MAPK) kinase, is a specific activator of the salt stress-induced MAPK, SIMK. Plant Cell **12:** 2247–2258.

Kovtun, Y., Chiu, W.L., Tena, G., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA **97:** 2940–2945.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. Plant J. **16:** 223–233.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods **25:** 402–408.

Manning, B.D., and Cantley, L.C. (2002). Hitting the target: Emerging technologies in the search for kinase substrates. Sci. STKE **2002:** PE49.

MAPK Group (2002). Mitogen-activated protein kinase cascades in plants: A new nomenclature. Trends Plant Sci. **7:** 301–308.

Matsuoka, D., Nanmori, T., Sato, K., Fukami, Y., Kikkawa, U., and Yasuda, T. (2002). Activation of AtMEK1, an Arabidopsis mitogen-activated protein kinase kinase, in vitro and in vivo: Analysis of active mutants expressed in *E. coli* and generation of the active form in stress response in seedlings. Plant J. **29:** 637–647.

Menke, F.L., van Pelt, J.A., Pieterse, C.M., and Klessig, D.F. (2004). Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in Arabidopsis. Plant Cell **16:** 897–907.

Mészáros, T., Helfer, A., Hatzimasoura, E., Magyar, Z., Serazetdinova, L., Rios, G., Bardoczy, V., Teige, M., Koncz, C., Peck, S., and Bogre, L. (2006). The Arabidopsis MAP kinase kinase MKK1 participates in defence responses to the bacterial elicitor flagellin. Plant J. **48:** 485–498.

Minet, M., Dufour, M.E., and Lacroute, F. (1992). Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J. **2:** 417–422.

Mizoguchi, T., Ichimura, K., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998). Identification of a possible MAP kinase cascade in *Arabidopsis thaliana* based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants. FEBS Lett. **437:** 56–60.

Nakagami, H., Soukupová, H., Schikora, A., Zárský, V., and Hirt, H. (2006). A mitogen-activated protein kinase kinase kinase mediates reactive oxygen species homeostasis in Arabidopsis. J. Biol. Chem. **281:** 38697–38704.

Nühse, T.S., Peck, S.C., Hirt, H., and Boller, T. (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. J. Biol. Chem. **275:** 7521–7526.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: Striking similarities and obvious differences. Immunol. Rev. **198:** 249–266.

Nürnberger, T., and Scheel, D. (2001). Signal transmission in the plant immune response. Trends Plant Sci. **6:** 372–379.

O'Rourke, S.M., Herskowitz, I., and O'Shea, E.K. (2002). Yeast go the whole HOG for the hyperosmotic response. Trends Genet. **18:** 405–412.

Orozco-Cardenas, M., and Ryan, C.A. (1999). Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA **96:** 6553–6557.

Orozco-Cardenas, M.L., Narvaez-Vasquez, J., and Ryan, C.A. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell **13:** 179–191.

Ortiz-Masia, D., Perez-Amador, M.A., Carbonell, J., and Marcote, M.J. (2007). Diverse stress signals activate the C1 subgroup MAP kinases of Arabidopsis. FEBS Lett. **581:** 1834–1840.

Ouaked, F., Rozhon, W., Lecourieux, D., and Hirt, H. (2003). A MAPK pathway mediates ethylene signaling in plants. EMBO J. **22:** 1282–1288.

Petersen, M., et al. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103:** 1111–1120.

Quimby, B.B., Wilson, C.A., and Corbett, A.H. (2000). The interaction between Ran and NTF2 is required for cell cycle progression. Mol. Biol. Cell **11:** 2617–2629.

Romeis, T., Ludwig, A.A., Martin, R., and Jones, J.D. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. **20:** 5556–5567.

Sasaki-Sekimoto, Y., et al. (2005). Coordinated activation of metabolic pathways for antioxidants and defence compounds by jasmonates and their roles in stress tolerance in Arabidopsis. Plant J. **44:** 653–668.

Shah, J. (2003). The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6:** 365–371.

Su, S.H., Suarez-Rodriguez, M.C., and Krysan, P. (2007). Genetic interaction and phenotypic analysis of the Arabidopsis MAP kinase pathway mutations mekk1 and mpk4 suggests signaling pathway complexity. FEBS Lett. **581:** 3171–3177.

Suarez-Rodriguez, M.C., Adams-Phillips, L., Liu, Y., Wang, H., Su, S.H., Jester, P.J., Zhang, S., Bent, A.F., and Krysan, P.J. (2007). MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Physiol. **143:** 661–669.

Suyama, M., Doerks, T., Braun, I.C., Sattler, M., Izaurralde, E., and Bork, P. (2000). Prediction of structural domains of TAP reveals details of its interaction with p15 and nucleoporins. EMBO Rep. **1:** 53–58.

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2007). The mitogen-activated protein kinase cascade MKK3–MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell **19:** 805–818.

Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat. Cell Biol. **2:** 110–116.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L., and Hirt, H. (2004). The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. Mol. Cell **15:** 141–152.

Teige, M., Scheikl, E., Reiser, V., Ruis, H., and Ammerer, G. (2001). Rck2, a member of the calmodulin-protein kinase family, links protein synthesis to high osmolarity MAP kinase signaling in budding yeast. Proc. Natl. Acad. Sci. USA **98:** 5625–5630.

Tena, G., Asai, T., Chiu, W.L., and Sheen, J. (2001). Plant mitogen-activated protein kinase signaling cascades. Curr. Opin. Plant Biol. **4:** 392–400.

Topfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.H. (1987). A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Res. **15:** 5890.

Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. (1993). Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:** 205–214.

Weigel, D., and Glazebrook, J. (2002). Arabidopsis: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.).

Zhang, S., and Klessig, D.F. (2001). MAPK cascades in plant defense signaling. Trends Plant Sci. **6:** 520–527.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. **136:** 2621–2632.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125:** 749–760.