MPMI Vol. 20, No. 5, 2007, pp. 589–596. doi:10.1094/MPMI-20-5-0589. © 2007 The American Phytopathological Society

The MAP Kinase Kinase MKK2 Affects Disease Resistance in *Arabidopsis*

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Submitted 28 August 2006. Accepted 18 December 2006.

The *Arabidopsis* mitogen-activated protein kinase (MAPK) kinase 2 (MKK2) was shown to mediate cold and salt stress responses through activation of the two MAP kinases MPK4 and MPK6. Transcriptome analysis of plants expressing constitutively active *MKK2* (MKK2-EE plants) showed altered expression of genes induced by abiotic stresses but also a significant number of genes involved in defense responses. Both MPK4 and MPK6 became rapidly activated upon *Pseudomonas syringae* pv. *tomato* DC3000 infection and MKK2-EE plants showed enhanced levels of MPK4 activation. Although MKK2-EE plants shared enhanced expression of genes encoding enzymes of ethylene (ET) and jasmonic acid (JA) synthesis, ET, JA, and salicylic acid (SA) levels did not differ dramatically from those of wild-type or *mkk2*-null plants under ambient growth conditions. Upon *P. syringae* pv. *tomato* DC3000 infection, however, MKK2-EE plants showed reduced increases of JA and SA levels. These results indicate that MKK2 is involved in regulating hormone levels in response to pathogens. MKK2-EE plants were more resistant to infection by *P. syringae* pv. *tomato* DC3000 and *Erwinia carotovora* subsp. *carotovora*, but showed enhanced sensitivity to the fungal necrotroph *Alternaria brassicicola*. Our data indicate that MKK2 plays a role in abiotic stress tolerance and plant disease resistance.

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 stress. Mitogen-activated protein kinase (MAPK) 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 MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and finally the MAPK. Different MAPK pathways respond to a variety of external stimuli and have been characterized in yeast, animals, and plants (Chang and Karin 2001; Davis 2000; 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 (Gustin et al. 1998; Herskowitz 1995; Hohmann 2002; O'Rourke et al. 2002). In contrast, plants have approximately 20 MAPKs, but relatively little is known about the function and composition of the different pathways (Jonak et al. 2002; MAPK group 2002; Tena et al. 2001; Zhang and Klessig 2001). The MAPKs investigated so far were involved mainly in stress responses (Jonak et al. 2002). In the genetic model plant *Arabidopsis*, MPK3, MPK4, and MPK6 are activated by a diverse set of stresses, including pathogens, osmotic, cold, and oxidative stress (Asai et al. 2002; Desikan et al. 2001; Droillard et al. 2002; Ichimura et al. 2000; Kovtun et al. 2000; Nühse et al. 2000; Petersen et al. 2000).

Compared with our current knowledge of the 20 plant MAPKs, much less is known on the functions of the 10 MAPKKs or of the more than 60 putative MAPKKKs (MAPK group 2002). 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 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 (Huang et al. 2000; Matsuoka et al. 2002; Mizoguchi et al. 1998).

Our recent biochemical and genetic analysis (Teige et al. 2004) indicated that MKK2 is an upstream activator of MPK4 and MPK6 and plays a critical role in the cold and salt stress response in *Arabidopsis*, but does not mediate activation of these MAPKs by the elicitors flagellin and laminarin. However, transcriptome profiling of plants overexpressing wild-type and constitutively active MKK2 revealed significant changes in the expression of a number of genes encoding proteins involved in transcriptional regulation, defense, signaling, and metabolism. Although many of the affected genes overlapped with those found in cold and salt stress (Fowler and Thomashow 2002; Kreps et al. 2002; Seki et al. 2002), a significant number was linked to plant pathogen defense responses. Therefore, we investigated whether MKK2 also could play a role in the signal transduction of pathogen defense. In this work, we show that MPK4 and MPK6 are rapidly activated upon *Pseudomonas syringae* pv. *tomato* DC3000 infection, whereas plants expressing constitutively active MKK2 (MKK2-EE plants) have enhanced levels of MPK4 activation. MKK2-EE plants are more resistant to a virulent strain of *P. syringae* pv. *tomato* DC3000 and *Erwinia carotovora* subsp. *carotovora* SCC1, but hypersensitive to *Alternaria brassicicola*. In contrast, no differences in sensitivity were observed of *mkk2* plants against *E. carotovora* subsp. *carotovora* SCC1 or *A. brassicicola*. Hormone analysis

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revealed that MKK2-EE plants were compromised in the production of jasmonic acid (JA) and salicylic acid (SA) upon infection by *P. syringae* pv. *tomato* DC3000. In summary, our data indicate that MKK2 is involved in both abiotic and biotic stress responses in plants.

RESULTS

A number of defense-related genes are upregulated in plants expressing constitutively active MKK2.

In our previous work (Teige et al. 2004), we produced several independent lines stably expressing constitutively active *MKK2* under the 35S promoter of *Cauliflower mosaic virus* (CAMV) (MKK2-EE plants). The *MKK2-EE* mutant gene was tagged with an myc epitope for easier detection and isolation from plant material. Kinase activity measurements of several independent MKK2-EE lines by immunocomplex kinase assays using a MAPK-specific antibody revealed increased activity of both target MAPKs (MPK4 and MPK6) in these plants (Teige et al. 2004). For 152 genes, comparative transcript profiling using *Arabidopsis* ATH1 DNA microarrays from Affymetrix of wild-type plants and MKK2-EE plants revealed greater than threefold expression difference (Teige et al. 2004). Of this set of genes, 92 could be attributed to known functions and 27% of these were related to pathogen defense (Table 1). These results suggested to us that MKK2 also might function in plant pathogen defense.

Mkk2 null plants and MKK2-EE lines are oppositely affected in sensitivity to *P. syringae* pv. *tomato* DC3000.

To investigate whether MKK2 might alter pathogen responses in *Arabidopsis*, we first compared the sensitivity of wild-type Col-0 plants with lines expressing constitutively active MKK2 and *mkk2* knock-out plants to the hemibiotrophic pathogen *P. syringae* pv. *tomato* DC3000. For this purpose, the previously characterized *mkk2* null T-DNA insertion line was compared with two lines expressing the constitutively active *MKK2-EE* allele expressed as myc-tagged versions under control of the constitutive 35S CaMV promoter. It should be noted that *mkk2* null and MKK2-EE plants showed no obvious phenotype under normal ambient experimental conditions. After infection with *P. syringae* pv. *tomato* DC3000, no differences were observed in the development of visible disease symptoms. However, quantification of the infection process showed significant differences among the tested plant lines. MKK2-EE plants displayed significantly less bacterial growth compared with Col-0 wild-type plants 48 h postinoculation (Fig. 1). In comparison, *mkk2* null plants showed only slightly higher bacterial numbers in independent assays. Using a lower inoculum, *mkk2* plants allowed faster bacterial growth within 24 to 48 h after infection (Fig. 1B), but the data is less significant ($P \leq 0.1$, Student's t test) than with the MKK2-EE lines. These data indicate that MKK2 also might be involved in basal resistance in biotrophic plant–pathogen interactions.

Table 1. Prominent up- and downregulated genes in *MKK2-EE* lines

AGI no.	Annotation	Ratio Mkk2-EE/Col-0	Classification
Upregulated			
At1g72920	Disease resistance protein (TIR-NBS class)	4,02	Cell rescue, defense
At2g34930	Disease resistance protein family	3,20	Cell rescue, defense
At2g40000	Nematode-resistance protein	3,83	Cell rescue, defense
At1g72940	Disease resistance protein (TIR-NBS class)	3,97	Cell rescue, defense
At1g66090	Disease resistance protein (TIR-NBS class)	3,86	Cell rescue, defense
At4g01250	WRKY family transcription factor	3,53	Transcription
At3g50060	myb DNA-binding protein (MYB77)	5,16	Transcription
At5g47220	Ethylene responsive element binding factor 2 (EREBP-2)	7,83	Transcription
At4g37260	myb DNA-binding protein (AtMYB73)	3,70	Transcription
At2g40140	CCCH-type zinc finger protein	4,21	Transcription
At1g80840	WRKY family transcription factor	5,91	Transcription
At1g32640	bHLH protein (RAP-1); identical to bHLH protein	3,31	Transcription
At3g55980	Zinc finger transcription factor (PEI1)	4,90	Transcription
At1g33760	Transcription factor TINY	10,11	Transcription
At4g31800	WRKY family transcription factor	5,90	Transcription
At5g61600	DNA binding protein EREBP-4	9,70	Transcription
At1g73500	MAP kinase kinase 5	5,67	Signaling
At2g30020	Protein phosphatase 2C (PP2C)	4,88	Signaling
At4g11280	ACC synthase AtACS-6	7,85	Cell communication
At1g72520	Lipoxygenase	4,18	Cell communication
At2g06050	12-Oxophytodienoate reductase (OPR3)	3,55	Cell communication
At1g73540	Diphosphoinositol polyphosphate phosphohydrolase	4,83	Cell communication
At5g61160	Anthocyanin 5-aromatic acyltransferase	6,89	Metabolism
At4g23600	Tyrosine transaminase	1,60	Metabolism
At5g24780	Vegetative storage protein Vsp1	3,97	Metabolism
At1g52400	β-Glucosidase (BG1)	8,63	Cell rescue, defense
At2g43510	Trypsin inhibitor	1,77	Cell rescue, defense
At1g60590	Polygalacturonase	2,22	Cell rescue, defense
At5g44420	Plant defensin protein (PDF1.2a)	3,93	Cell rescue, defense
At2g17840	Senescence-associated protein 12	3,17	Unknown role
At1g54040	Jasmonate inducible protein	3,35	Cell rescue, defense
At3g16390	Lectin	3,79	Cell rescue, defense
Downregulated			
At2g32680	Disease resistance protein family	0,75	Cell rescue, defense
At4g38860	Auxin-induced protein	0,30	Transcription
At1g29430	Auxin-induced protein	0,31	Transcription
At4g38840	Auxin-induced protein	0,27	Transcription
At1g21250	Wall-associated kinase 1	0,75	Cellular communication

MKK2-EE plants show enhanced MPK4 activation upon *P. syringae* pv. *tomato* DC3000 infection.

To assay directly for a dependency of the activation of MPK4 and MPK6 upon pathogen infection in vivo, *Arabidopsis* plants were infiltrated with *P. syringae* pv. *tomato* DC3000 and protein extracts were prepared at 0, 20, and 30 min after infiltration. Endogenous MPK4 and MPK6 kinases were immunoprecipitated from these extracts with specific antibodies followed by determination of their protein amounts by Western analysis and kinase activities using MBP as a substrate. In wild-type plants, MPK4 and MPK6 both become rapidly activated within 20 min after infection (Fig. 2A). The activation clearly was a post-translational regulation, because MPK4 and MPK6 protein amounts did not change under these conditions (Fig. 2A).

When MKK2-EE plants were analyzed for MPK4 and MPK6 activation by *P. syringae* pv. *tomato* DC3000, we observed activation of both kinases within 20 min (Fig. 2B, 20 min). When compared with Col-0 plants, levels of MPK6 activation were quite comparable upon *P. syringae* pv. *tomato* DC3000 infection, but those of MPK4 clearly exceeded those seen in wild-type plants (Fig. 2A and 2B, *Pst* DC3000). The enhanced MPK4 activation in MKK2-EE plants could be partially explained by the fact that increased MPK4 kinase activity levels already were observed in untreated plants (Fig. 2B, 0 min). As shown by comparative Western analysis with Col-0 plants (Fig. 2A and 2B, 0 min), the enhanced kinase activity of MPK4 in MKK2-EE plants clearly is not due to increased MPK4 protein levels.

When *mkk2* null mutant plants were analyzed, MPK4 and MPK6 activity levels, as well as protein amounts in untreated plants, were similar to those found in wild-type Col-0 plants (Fig. 2C and A, respectively). Upon infection of *mkk2* mutant plants by *P. syringae* pv. *tomato* DC3000, the activation profiles of MPK4 was reduced at 30 min but not at 20 min, whereas MPK6 kinase activation was comparable with wild-type Col-0 plants (Fig. 2C and A, respectively). The data suggest that another MAPKK is present in *mkk2* mutants that can still mediate the pathogen-induced activation of the two MAPKs.

To control whether the infiltration treatment might be sufficient to activate the MAPKs, we also carried out mock infections with buffer alone. Under these conditions, MPK4 and MPK6 showed only limited activation (Fig. 2A through C, mock), indicating that the mechanical and osmotic stress components in the *P. syringae* pv. *tomato* DC3000 infection process were not responsible for the changes observed in MAPK activities.

Altered levels of JA in MKK2-EE plants.

Transcriptome analysis of MKK2-EE plants indicated upregulation of genes involved in synthesis of ethylene (ET) (ACC synthase *ACS6*) and JA (lipoxygenase and 12-oxophytodienoate reductase *OPR3*). These data suggested to us that these plants might accumulate increased levels of ET and JA. In line with these expectations, MKK2-EE lines showed enhanced transcript levels of the ET/JA marker gene *PDF1.2*. Accumulation of SA, JA, and ET has been implicated as a signal for activation of plant defense responses and plant pathogen resistance; therefore, we characterized the levels of SA, JA, and ET in the different lines before and after infection with *P. syringae* pv. *tomato* DC3000. SA levels in mock-treated *mkk2* null and MKK2-EE lines were comparable with those in wild-type plants (Fig. 3A). After 48 h of infection by *P. syringae* pv. *tomato* DC3000, accumulation of SA was observed in all lines, although MKK2-EE lines clearly showed 30 to 40% lower amounts of SA 48 h postinoculation (hpi) (Fig. 3A). Although MKK2-EE lines showed strong upregulation of *ACS6*, ET production before and after infection by *P. syringae* pv. *tomato* DC3000 was comparable with that seen in wild-type plants (Fig. 3B). In *mkk2* null plants, however, lower ET levels were observed before and after infection, but other independent experiments did not show such a clear correlation. When JA levels were determined, no major differences were observed between *mkk2* null and wild-type plants either before or after infection with *P. syringae* pv. *tomato* DC3000 (Fig. 3C). Despite the fact that MKK2-EE plants have increased lipoxygenase and *OPR3* levels, these plants showed even slightly lower JA levels in the uninfected state. Interestingly, upon infection with the bacterial pathogen, the accumulation of JA was strongly compromised in MKK2-EE plants when compared with Col-0 wild-type and *mkk2* plants, and JA levels 48 hpi were almost back to the same levels as in the mock-treated MKK2-EE plants. These results indicate that MKK2 is involved in modulating hormone levels in response to pathogen infection.

MKK2-EE plants are impaired in resistance against infection by the necrotrophic fungus *A. brassicicola*.

JA is an important regulator of defense and, here, especially against nectrotrophic pathogens. In *Arabidopsis*, resistance to *A. brassicicola* relies on JA-dependent defense mechanisms and the capability to produce the phytoalexin camalexin (Thomma et al. 1999). Mutants incapable of synthesizing JA, such as those defective in three fatty acid desaturases (*triple fad*), or insensitive to JA, such as *coi1* as well as *pad3* unable to accumulate camelexin, are susceptible to infection by this necrotrophic pathogen (Thomma et al. 1998, 1999). To investi-

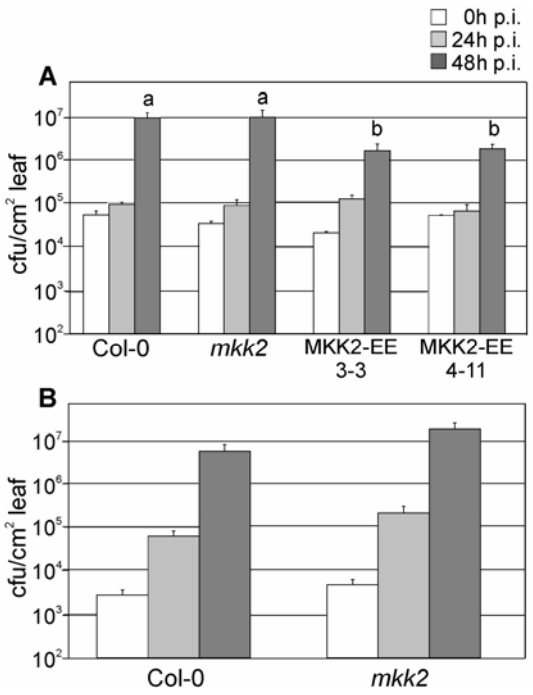

Fig. 1. Differentially affected sensitivity of mitogen-activated protein kinase kinase 2 (*mkk2*) null and MKK2-EE plants to infection by *Pseudomonas syringae* pv. *tomato* DC3000. Growth of *P. syringae* pv. *tomato* DC3000 in wild-type, MKK2-EE, and *mkk2* null plants after infection by dipping. Bars represent the bacterial titers of three pooled leaf disks in six replicates (± standard error) obtained at different time points after infection. Plants were dipped in 10 mM $MgSO_4$ containing **A,** 8×10^6 or **B,** 2×10^5 CFU/ml; h p.i. = h postinoculation. Significant differences 48 h p.i. ($P < 0.05$) were calculated by one-way analysis of variance with a least significant difference test and are indicated by different letters.

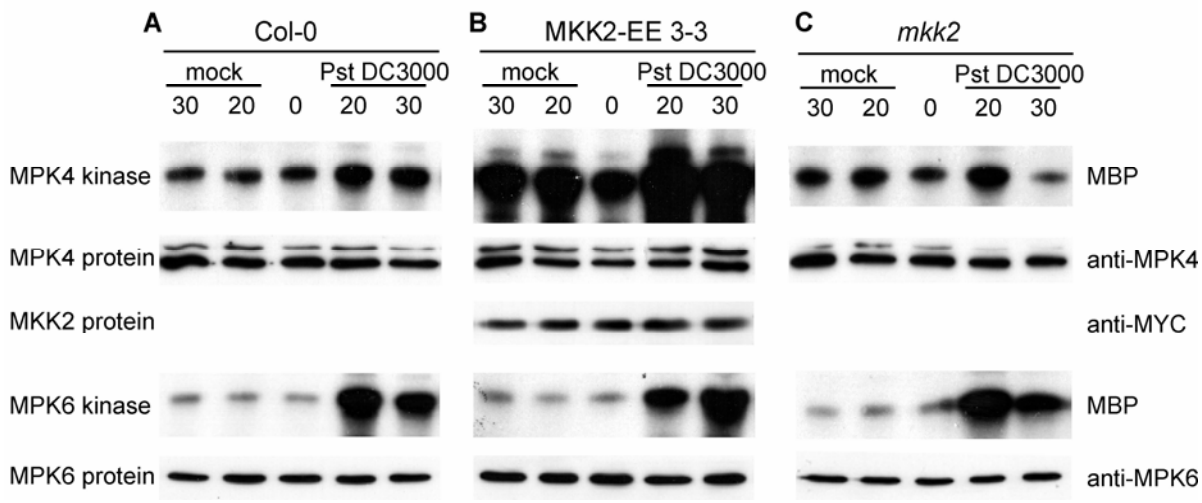

Fig. 2. Enhanced activation of mitogen-activated protein (MAP) kinase 4 (MPK4) by *Pseudomonas syringae* pv. *tomato* DC3000 infection in MAP kinase kinase 2 (MKK2-EE) plants. Kinetics of MPK4 and MPK6 activation in **A,** wild-type Col-0; **B,** MKK2-EE 3-3; and **C,** *mkk2* null plants in response to *P. syringae* pv. *tomato* DC3000 infection. MPK4 and MPK6 were immune precipitated with specific antibodies from leaves of *P. syringae* pv. *tomato* DC3000-infected plants as described in the experimental procedures. MPK activity was measured in immunocomplex kinase assays using MBP as substrate and levels of MPK4 and MPK6 proteins were detected in Western blots.

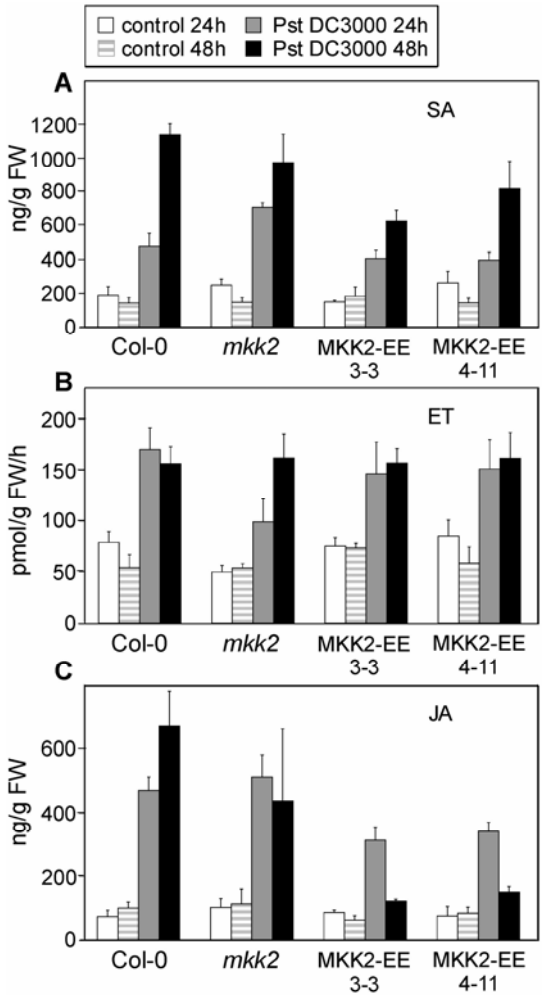

Fig. 3. Determination of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) in mitogen-activated protein kinase kinase 2 (MKK2-EE) plants after infection by *Pseudomonas syringae* pv. *tomato* DC3000. **A,** SA; **B,** ET; and **C,** JA were determined in wild-type Col-0, *mkk2* null, and two different MKK2-EE lines 24 and 48 h after infection with *P. syringae* pv. *tomato* DC3000 (8×10^6 CFU/ml; Pst DC3000) or mock inoculation with 10 mM $MgSO_4$ containing 0.02% Silwet-20 (control). The values represent the average of three replicates ± standard error.

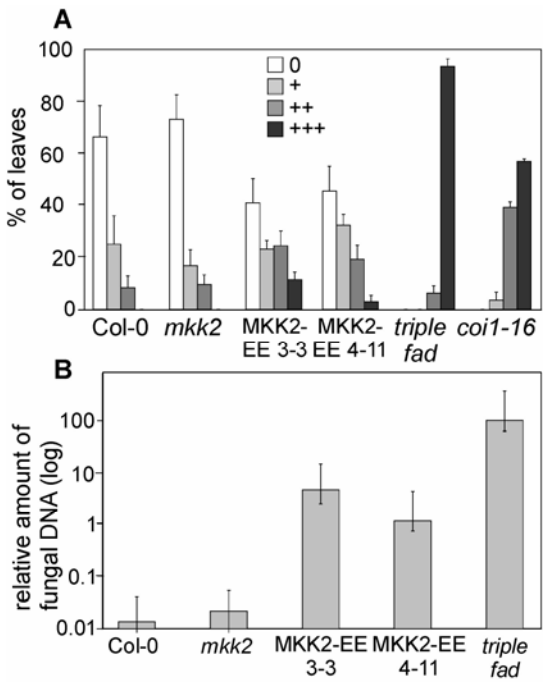

Fig. 4. Mitogen-activated protein kinase kinase 2 (MKK2-EE) plants are impaired in resistance against infection by *Alternaria brassicicola.* Both *mkk2* null and MKK2-EE plants were compared with wild-type Col-0, *coi1-16*, and *triple fad* mutants upon infection with *A. brassicicola.* **A,** Necrotic lesions 7 days postinoculation (dpi). **B,** Evaluation of symptoms ± standard error of three (*coi1-16* and *triple fad*) to five experiments 7 dpi, where 0 = no symptoms or less than 5% of the leaf area with symptoms, + = 5 to 25%, ++ = 25 to 50%, and +++ = more than 50% of the leaf area with necrotic lesions. **C,** Quantification of fungal growth 7 dpi by quantitative polymerase chain reaction. Bars represent the average amount of fungal DNA in relation to plant DNA and are the means of ten replicates ± standard error.

gate whether MKK2 may affect tolerance to a necrotroph, *mkk2* null and MKK2-EE plants were compared with wild-type, *coi1-16,* and *triple fad* mutants upon infection with *A. brassicicola*. The state of infection was determined by evaluating the necrotic lesion area and fungal growth from the infected leaves (Fig. 4). In these assays, *mkk2* null plants were as resistant to *A. brassicicola* infection as wild-type Col-0, showing

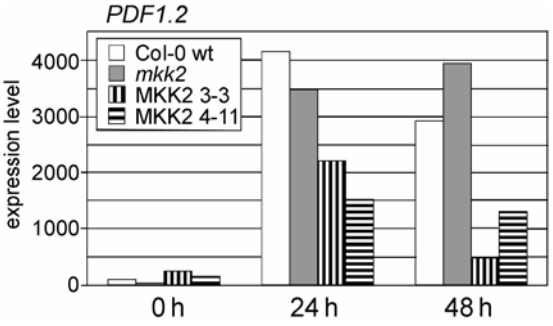

Fig. 5. *Alternaria brassicicola*-induced gene expression in mitogen-activated protein kinase kinase 2 (*mkk2*) null and MKK2-EE plants. Local leaf samples were collected from 4-week-old Col-0 wild-type, *mkk2* null, and MKK2-EE plants at 0, 24, and 48 h after inoculation with 4 × 5 µl of *A. brassicicola* spore suspension (10^6 spores ml^{-1} in water). Total RNA was extracted and analyzed by quantitative RT-PCR for *PDF1.2* expression. The data were normalized to *Tubulin* α expression and set to 100 for Col-0 at 0 h. Values correspond to the average of two biological replicates.

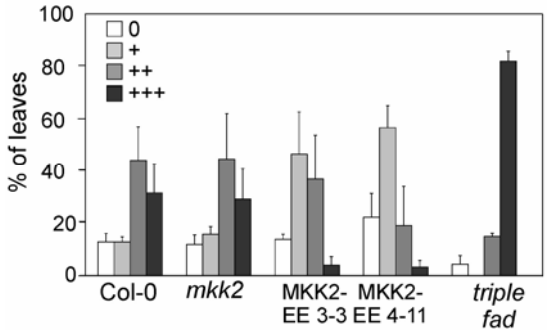

Fig. 6. Enhanced tolerance of mitogen-activated protein kinase kinase 2 (*MKK2-EE*) lines to *Erwinia carotovora*. Both *mkk2* null and MKK2-EE lines, as well as wild-type Col-0 plants, were inoculated with *E. carotovora* subsp. *carotovora* SCC1. Disease symptom development 48 h after infection symptoms ± standard error of three experiments was monitored and qualified as following: 0 = no symptoms, infection dried out at inoculation site; + = infection stopped soon after spreading, less than 50% of the leaf area macerated; ++ = infection spreads to more than 50% of the leaf area; and +++ = infection spreads systemically, infected leaf completely macerated.

only limited disease symptoms. Although MKK2-EE lines were considerably more sensitive to infection by the fungal pathogen, these plants still were less sensitive than *coi1-16, triple fad* (Fig. 4), or *pad3* mutants (data not shown).

Despite the increased sensitivity of MKK2-EE lines against *A. brassicicola*, the JA marker gene *PDF1.2* is already elevated in noninduced MKK2-EE plants (Table1). Therefore, we determined *PDF1.2* levels by reverse-transcription polymerase chain reaction (RT-PCR) 24 and 48 h after infection with *A. brassicicola*. *PDF1.2* transcript amounts strongly increased in wild-type and *mkk2* mutants after infection, but the induction was clearly diminished in MKK2-EE lines (Fig. 5). These results show that the MKK2-EE plants are compromised in their resistance against the necrotrophic pathogen *A. brassicicola* and that the enhanced susceptibility is correlating with lower *PDF1.2* levels upon *A. brassicicola* infection.

Enhanced tolerance of *MKK2-EE* lines to *E. carotovora*.

Because the resistance of *Arabidopsis* plants against infection by the necrotrophic fungus *A. brassicicola* strongly relies on JA signaling, we employed *E. carotovora* subsp. *carotovora* strain SCC1 as another pathogen model where disease resistance relies on both SA- and JA-dependent defense responses (Kariola et al. 2003; Li et al. 2004). To determine the contribution of MKK2 to resistance of *Arabidopsis* toward *E. carotovora* subsp. *carotovora* SCC1, *mkk2* null and MKK2-EE lines as well as wild-type plants were inoculated with *E. carotovora* subsp. *carotovora* SCC1 and disease symptom development was monitored. Whereas *mkk2* null plants displayed disease symptoms and bacterial growth comparable with those of wild-type plants (Fig. 6), MKK2-EE lines showed increased resistance to *E. carotovora* subsp. *carotovora* SCC1 infection, such as decreased tissue maceration and spreading of the disease 48 h after inoculation (Fig 6). These results indicate that *MKK2* function also contributes to resistance against *E. carotovora*.

DISCUSSION

MAPK pathways mediate cellular responses to a great variety of different extracellular signals in plants. We had shown previously that MKK2 functions in abiotic stress signaling and is an upstream regulator of MPK4 and MPK6. In this work, we have analyzed the function of MKK2 in innate immunity against three different pathogens: the bacterial pathogens *P. syringae* pv. *tomato* DC3000 and *E. carotovora* subsp. *carotovora*, as well as the fungal pathogen *A. brassicicola.* We found that *MKK2-EE* lines are more resistant to *P. syringae* pv. *tomato* DC3000 and *E. carotovora* subsp. *carotovora* SCC1, but more sensitive to *A. brassicicola.* These data demonstrate that MKK2 is involved in transducing both abiotic and biotic signals in *Arabidopsis*.

Plants expressing constitutively active MKK2 show enhanced resistance against *P. syringae* pv. *tomato* DC3000 and *E. carotovora* subsp. *carotovora* SCC1.

By yeast two-hybrid analysis, MPK4 and MPK6 were identified as the strongest interactors with MKK2, and both MPK4 and MPK6 were activated by MKK2 in vitro and in vivo (Teige et al. 2004). MPK4 and MPK6 are among the most prominent kinases and can be activated by both abiotic and biotic stresses (Nakagami et al. 2005). Interestingly, whereas *mpk4* mutants show enhanced resistance (Petersen et al. 2000), *mpk6*-silenced lines are compromised in their resistance against infection by *P. syringae* pv. *tomato* DC3000 (Menke et al. 2004). In our present work, we found that plants with constitutive MKK2 activity showed increased resistance against *P. syringae* pv. *tomato* DC3000 and *E. carotovora* subsp. *carotovora* SCC1, suggesting that MKK2 also is involved in mediating innate immunity. However, *mkk2* null plants were hardly more sensitive to bacterial infection by *P. syringae* pv. *tomato* DC3000. On the other hand, *mkk2* null plants are clearly hypersensitive to abiotic stresses such as freezing and high salt concentrations (Teige et al. 2004), which indicates that another MAPKK can functionally substitute for MKK2 deficiency in pathogen defense but not in abiotic stress responses. A possible candidate for this function could be the closely related MAPKK MKK1, which is activated in response to various abiotic stresses (Matsuoka et al. 2002), but cross-talk with other MAPK pathways also might be a possible scenario, because lines expressing constitutively active MKK2 show sixfold increased expression levels of MKK5, leaving it an open question as to which MAPKK can truly substitute for MKK2 upon pathogen interactions.

Upregulation of genes for synthesis of ET and JA is not correlated with increased levels of hormones in plants expressing constitutively active MKK2.

MKK2-EE plants showed upregulation of ACC synthase *ACS6*, lipoxygenase, and *OPR3* (Table 1), suggesting that these

plants might accumulate increased levels of ET and JA. For this purpose, we decided to determine the amounts of SA, ET, and JA in *mkk2* null, MKK2-EE, and wild-type plants. The amounts of SA, ET, and JA were not strongly different between *mkk2* null, MKK2-EE, and wild-type control plants under ambient growth conditions. ET amounts increased in all plant lines at 48 h after infection by *P. syringae* pv. *tomato* DC3000. JA an SA levels also increased in wild-type and *mkk2* null mutants upon bacterial infection, but MKK2-EE lines showed lower SA and clearly less JA accumulation, suggesting that MKK2 functions in modulating JA synthesis in response to pathogen infection. The increased levels of lipoxygenase and OPR3 transcripts in MKK2-EE lines could be a consequence of a defective feedback pathway. Whether JA signaling also affects gene expression of *ACS6* is not clear, but might be a consequence of cross-talk between the JA and ET pathways. Moreover, enhanced basal levels of JA-responsive genes do not always correlate with increased JA levels (Li et al. 2004) and might reflect alterations in modulation of JA perception or cross-talk with other signaling pathways.

Plants expressing constitutively active MKK2 are compromised in resistance against infection by the necrotrophic fungus *A. brassicicola*.

If JA synthesis is impaired in response to pathogen infection in MKK2-EE plants, it was expected that these plants should be compromised in their resistance against necrotrophic pathogens. Whereas *mkk2* null plants were as resistant to *A. brassicicola* infection as wild-type plants, MKK2-EE lines were considerably more sensitive to infection by the fungal pathogen. However, MKK2-EE plants were still more resistant than *coi1-16*, *triple fad* (Fig. 4), or *pad3* mutants (not shown). The increased sensitivity of MKK2-EE plants to *A. brassicicola* is accompanied by lower induction of *PDF1.2* 24 and 48 hpi (Fig. 5). *PDF1.2* expression in *Arabidopsis* is enhanced after pathogen and superoxide anion-generating paraquat treatment and requires functional ET and JA signaling (Penninckx et al. 1998). Therefore, our results suggest that the impaired JA synthesis or signaling may underlie the sensitivity of MKK2-EE plants against infection by the necrotrophic pathogen *A. brassicicola*. However, we cannot rule out the possibility that other signaling pathways modulating ET perception or oxidative stress signaling are affected in the MKK2-EE lines as well.

Interestingly, MKK2-EE plants were more resistant to another necrotroph, the bacterial pathogen *E. carotovora* (Fig. 6). Recent publications point to a differential regulation of defense against *E. carotovora* than to *A. brassicicola* and show that partial suppression of JA defense and increased oxidative stress and SA signaling enhances resistance against *E. carotovora* strain SCC1 (Kariola et al. 2005; Li et al. 2006).

Does MKK2 function in integrating abiotic and biotic stress signaling?

Overall, MPK4 and MPK6 were identified as in vivo targets of MKK2, showing that MKK2 mediates cold and salt stress signaling via MPK4 and MPK6 (Teige et al. 2004). Whereas MPK6 has been accepted to function as a general mediator of biotic and abiotic stresses, the role of MPK4 is less clear. Petersen and associates (2000) postulated that MPK4 is a negative regulator of systemic acquired disease resistance. Multiple studies have shown that MPK4 and MPK6 are both involved in mediating recognition of pathogen-associated molecular patterns and wounding, as well as various abiotic stresses (Asai et al. 2002; Ichimura et al. 2000; Nakagami et al. 2006; Teige et al. 2004). MPK4 and MPK6 can be activated by MKK2 in response mainly to abiotic stresses and by MKK1 upon bacterial and fungal elicitors (Teige et al. 2004). However, bacterial infection with *P. syringae* pv. *tomato* DC 3000 instead of elicitors alone also triggers enhanced activation of MPK4 in MKK2-EE lines (Fig. 2). These results suggest that MKK1 and MKK2 might play largely different roles in stress signaling. In agreement with this model, it was found recently that *mkk1* mutants are compromised in resistance to *P. syringae* pv. *tomato* DC 3000 (Meszaros et al. 2006). In contrast, we show here that *mkk2* plants are hardly affected in resistance to three different pathogen systems, including *P. syringae* pv. *tomato* DC 3000. These results suggest that MKK1 and MKK2 contribute to a large degree to different signaling modules with specialized roles. Therefore, the identification of the components constituting different MKK modules should be an important future task for furthering our understanding of these important signaling pathways.

MATERIALS AND METHODS

Plant material.

The *Arabidopsis* ecotype Columbia (Col-0) was used as genetic background. The *mkk2* T-DNA null line (Garlic_511_H01.b.1a.Lb3Fa) was obtained from the Syngenta *Arabidopsis* Insertion Library, Torrey Mesa Research Institute (San Diego, CA, U.S.A.) and characterized as reported (Teige et al. 2004). Plants expressing constitutively active MKK2-EE were produced using the binary expression vector pGreenII 0029 (Hellens et al. 2000) under control of the 35S promoter and transformed as an MYC-epitope-tagged MKK2 version using the floral dipping method (Clough and Bent 1998) into Col-0 wild-type plants. Seed of transformed plants were selected and characterized as described (Teige et al. 2004). Seed of the fatty acid desaturase *triple fad* (*fad3-2/fad7-2/fad8*) mutant were provided by J. Browse (Washington State University, Pullman, U.S.A.), and seed of JA-insensitive *coi1-16* by J. Turner (University of East Anglia, Norwich, U.K.). Seed were germinated in 0.5× Murashige Skoog (MS) medium (Sigma, St. Louis), and plants were grown under 50-μE cool white fluorescent light and long day conditions (16 h of light and 8 h of dark). *Arabidopsis* protoplasts were prepared from a suspension culture as described (Cardinale et al. 2000; Mathur and Koncz 1997). For hormone analysis and pathogen treatment, 3- to 4-week-old plants were cultivated under a 12-h light period at 22ºC with 50-μE cool white fluorescent light on a 1:1 mixture of vermiculite and peat (Finnpeat; B2 Kekkilo Oyj, Tuusula, Finland).

Molecular cloning and construction of expression vectors.

The open reading frames of MPK4, MPK6, and MKK2 originally were amplified from a cDNA library (Minet et al. 1992) with an *Nco*I restriction site at the 5′ end and an *Not*I restriction site in front of the stop codon. The *Not*I restriction site at the 3′ end was used to introduce a c-MYC epitope as a *Not*I/*Not*I cassette. The constitutively active MKK2 allele MKK2-EE was generated by changing the putative phosphorylation sites from threonine to glutamic acid residues (T220E and T226E).

Protein extracts from *Arabidopsis* leaves.

Protein extracts were prepared in Lacus buffer (25 mM Tris, pH 7.8, 75 mM NaCl, 10 mM $MgCl_2$, 15 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM $NaVO_3$, 15 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin at 5 μg/ml, and aprotinin at 5 μg/ml). Protein extracts were prepared by grinding 200 mg of leaf material in 200 μl of Lacus buffer and sea sand (Bögre et al. 1999).

Immunocomplex kinase assays.

Immunocomplex kinase assays were done according to Cardinale and associates (2002).

Pathogen treatments of plants.

P. syringae pv. *tomato* DC3000 was cultivated in King's B medium, and the cells were pelleted, resuspended, and diluted in 10 mM $MgSO_4$ and 0.02% Silwet-77 to a concentration of 2×10^5 to 8×10^6 CFU ml^{-1}. 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 rifampicin at 25 μM ml^{-1} as described (Weigel and Glazebrook 2002). *E. carotovora* subsp. *carotovora* SCC1 (Pirhonen and Palva 1988) was cultured overnight at 28°C in Luria-Bertani medium. Bacteria were harvested by centrifugation, resuspended in 50 mM NaCl, diluted to 10^6 CFU ml^{-1}, and applied as 5-μl droplets on leaves on plants kept at >95% relative humidity after infection. Symptoms were monitored 48 hpi and the bacterial growth was determined by homogenizing the infected plants in 10 ml of 0.9% NaCl and plating serial dilutions on Luria plates containing ampicillin at 50 μg ml^{-1}. *A. brassicicola* (strain 567.77; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was maintained on potato carrot extract agar and spore suspensions (conidial spores at 5 to 10×10^5 ml^{-1} in potato dextrose broth) were applied on leaves as 5-μl drops after making a small wound with a pipette tip. After inoculation, plants were kept at >95% relative humidity and symptoms were assessed 7 days postinoculation. For assessing fungal growth, single treated and control leaves were frozen in liquid nitrogen in 10 replicates on the seventh day after infection. Relative fungal biomass was determined by quantitative PCR on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) as described (Kariola et al. 2005).

Plant hormone measurements.

ET emission was quantified as described (Vahala et al. 2003) by putting whole rosettes of 2-week-old plants grown on MS/2 medium with wet filter paper in a 25-ml airtight glass vial. After 2 h of incubation in the dark, a 1-ml sample was taken with a disposable syringe and analyzed with a gas chromatograph (Varian 3700, Palo Alto, CA, U.S.A.) equipped with a poropax Q column (80 to 100 mesh, 1 m by 3.2 mm) and flame ionization detection. Carrier gas was helium (flow rate: 30 ml min^{-1}) and column, injector, and detector temperatures were 40, 150, and 200ºC, respectively.

SA and JA were analyzed by using the vapor-phase extraction method described by Schmelz and associates (2003). Internal standards were 40 ng of $^{13}C_1$-SA and 20 ng of dihydrojasmonic acid (Montesano et al. 2005) in each sample. Gas chromatography–mass spectrometry (GC-MS) analysis was performed on a Trace-DSQ (Thermo) in the single ion monitoring mode on a ZB-35 capillary GC column (35% phenyl- and 65% methylpolysiloxane, 30 m by 0.25 mm by 0.25 μm) with splitless injection and 230ºC injector temperature. The column was held at 40ºC for 1 min after injection, then heated by 15ºC min^{-1} to 250ºC, held for 4 min, and heated by 20ºC min^{-1} to 300ºC final temperature (kept for 3 min) with helium as carrier gas (flow, 1 ml min^{-1}).

RNA analyses with real-time PCR.

Gene expression was quantified by RT-PCR as described previously (Brader et al. 2006) using specific primers 5′-TCTTTGCTGCTTTCGACG-3′ and 5′-AAACCCCTGACCATGTCCC-3′ for *PDF1.2* (AT5G44420) and 5′-TCCATCCTCCTCGACAATGAA-3′ and 5′-AAGGCACCATCAAACCTCAGA-3′ for Tubulin α *TUA4* (AT1G04820).

ACKNOWLEDGMENTS

This work was supported by grants from the Austrian Science Foundation, the Vienna Science and Technology Fund, the European Community, the Academy of Finland, and from Biocenter Helsinki. We thank J. Turner for kindly providing us *coi1-16* and J. Browse for *triple fad* mutant seed.

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