The MAP Kinase Kinase MKK2 Affects Disease Resistance in Arabidopsis

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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; Tenh 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; Driollard et al. 2002; Ichimura et al. 2000; Kovalen et al. 2000; Nüse 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 lami

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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.

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

<table>
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<tr>
<th>AGI no.</th>
<th>Annotation</th>
<th>Ratio Mkk2-EE/Col-0</th>
<th>Classification</th>
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<td>Trypsin inhibitor</td>
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<td>At1g29430</td>
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<td>At1g21250</td>
<td>Wall-associated kinase 1</td>
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<td>Cellular communication</td>
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**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* ≤ 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.
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) (ACS6 and ACS10) 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.

**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 *P. syringae pv. tomato* DC3000 was evaluated. Bars represent the bacterial titers of three pooled leaf disks in six replicates (± standard error) obtained at different time points after infection. Three leaves disks were dipped in 10 mM MgSO₄ containing A, 8 × 10⁸ or B, 2 × 10⁹ 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.
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 evaluat-
ing the necrotic lesion area and fungal growth from the infected 
leaves (Fig. 4). In these assays, mkk2 null plants were as resis-
tant to A. brassicicola infection as wild-type Col-0, showing
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 (Table 1). 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 MAPK, 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 lipoxigenase 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.b1a.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 tripal 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 0.5x 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 22°C with 50-μE cool white fluorescent light on a 1:1 mixture of vermiculite and peat (Fimppeat; 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 Nol restriction site at the 5′ end and an NostI restriction site in front of the stop codon. The NostI restriction site at the 3′ end was used to introduce a c-MYC epitope as a NostI/NolI 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 Lacs buffer (25 mM Tris, pH 7.8, 75 mM NaCl, 10 mM MgCl2, 15 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO4, 15 mM β-glycero phosphate, 15 mM p-nitrophenylphosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin at 5 μg/ml, and aprotonin at 5 μg/ml). Protein extracts were prepared by grinding 200 mg of leaf material in 200 μl of Lacs 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 MgSO4 and 0.02% Silwet-77 to a concentration of 2 × 10^8 to 8 × 10^8 CFU ml^-1. Plants were infected by dipping and bacterial growth was assessed by plating dilution series of leaves ground in 10 mM MgSO4 on King’s B plates containing rifampicin at 25 μg ml^-1 as described (Weigel and Glazebrook 2000). 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 × 10^3 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 200°C, 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 [13C]5-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 230°C injector temperature. The column was held at 40°C for 1 min after injection, then heated by 15°C min^-1 to 250°C, held for 4 min, and heated by 20°C min^-1 to 300°C 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′-TCTTTGCTGCTTGGACCG-3′ and 5′-AAACCCTGACCATGTCC-3′ for PDF1.2 (AT5G44440) and 5′-TTCCATCCCTCGACAATGAA-3′ and 5′-AAGGCCACCATCAAACTCTCAGA-3′ for Tubulin α TUA4 (AT1G04820).

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LITERATURE CITED


