Mitogen-Activated Protein Kinases 3 and 6 Are Required for Full Priming of Stress Responses in Arabidopsis thaliana

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In plants and animals, induced resistance (IR) to biotic and abiotic stress is associated with priming of cells for faster and stronger activation of defense responses. It has been hypothesized that cell priming involves accumulation of latent signaling components that are not used until challenge exposure to stress. However, the identity of such signaling components has remained elusive. Here, we show that during development of chemically induced resistance in Arabidopsis thaliana, priming is associated with accumulation of mRNA and inactive proteins of mitogen-activated protein kinases (MPKs), MPK3 and MPK6. Upon challenge exposure to biotic or abiotic stress, these two enzymes were more strongly activated in primed plants than in nonprimed plants. This elevated activation was linked to enhanced defense gene expression and development of IR. Strong elicitation of stress-induced MPK3 and MPK6 activity is also seen in the constitutive priming mutant edr1, while activity was attenuated in the priming-deficient npr1 mutant. Moreover, priming of defense gene expression and IR were lost or reduced in mpk3 or mpk6 mutants. Our findings argue that prestress deposition of the signaling components MPK3 and MPK6 is a critical step in priming plants for full induction of defense responses during IR.

INTRODUCTION

Upon infection by a pathogen, plants can develop enhanced resistance to subsequent infections by a broad spectrum of pathogens. This type of induced resistance (IR) requires the endogenous plant hormone salicylic acid (SA) and is known as systemic acquired resistance (SAR) (Ryals et al., 1996; Durrant and Dong, 2004). In addition to pathogen attack, resistance can also be induced by treatment with certain natural or synthetic compounds (Ryals et al., 1996; Beckers and Conrath, 2007). Although it is best known for its protection against pathogens (Ryals et al., 1996; Durrant and Dong, 2004), IR can be effective against abiotic stress as well (Janda et al., 1999; Senaratna et al., 2000; Kohler et al., 2002). In plants, IR is frequently associated with the accumulation of antimicrobial pathogenesis-related (PR) proteins (Van Loon et al., 2006) and with the so-called priming of cells (Kohler et al., 2002). Priming is the phenomenon that enables cells to respond to much lower levels of a stimulus in a more rapid and robust manner than nonprimed cells (Conrath et al., 2002; Conrath et al., 2006). Thus, plants primed by treatments that induce resistance show a faster and/or stronger activation of defense responses when subsequently challenged by pathogens or abiotic stresses (Conrath et al., 2002; Conrath et al., 2006).

For example, inoculation of Arabidopsis thaliana leaves with Pseudomonas syringae pv tomato (Pst) strain DC3000 expressing the avrRpt2 avirulence gene was shown to enhance defense responses that were manifested throughout the plant upon subsequent Pst DC3000 infection, wounding with forceps, or infiltration of water into leaves (Kohler et al., 2002). Similar observations of enhanced defense responses were made when priming and resistance were induced in plants by previous colonization of the roots with certain beneficial rhizobacteria (Verhagen et al., 2004) or upon treatment with the nonprotein amino acid β-amino butyric acid (Zimmerli et al., 2000), SA, or its functional analogs (Kohler et al., 2002). Among the latter, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) has attracted much attention (Ryals et al., 1996) because it is a potent inducer of the primed state (Kohler et al., 2002) and IR (Lawton et al., 1996; Ryals et al., 1996) at moderate rates of application (100 to 300 μM) and since it also provides protection against a broad spectrum of diseases in various crops in the field (Kessmann et al., 1994).

Although priming has been known as a component of IR responses in plants (Kuč, 1987; Zimmerli et al., 2000; Verhagen et al., 2004) and mammals (Hayes et al., 1991) for several years, and recently has been associated with an adaptive immune
response in *Drosophila melanogaster* as well (Pham et al., 2007), very little is known about the molecular mechanism(s) of priming. It has been proposed that priming is associated with increased accumulation of inactive cellular signaling proteins that play an important role in signal amplification (Conrath et al., 2006). Subsequent exposure to stress could activate these dormant signaling proteins, thereby initiating signal amplification and lead to faster and/or stronger activation of defense responses and IR (Conrath et al., 2006). However, the identity of these hypothetical proteins has remained obscure.

Mitogen-activated protein kinase (MPK) cascades are three-tiered signaling kinase modules that can be found in all eukaryotes (Herskowitz, 1995; Ichimura et al., 2002). They function downstream of sensors/receptors and transmit extracellular stimuli into intracellular responses while at the same time amplifying the transducing signal (Herskowitz, 1995; Ichimura et al., 2002). Signal amplification is achieved by a MPK cascade of three hierarchically arranged, interacting types of kinases. MPK activity is induced upon phosphorylation by MPK kinases (MPKKs, MAPKKs, or MEKs), which are in turn phosphorylation activated by MPKK kinases (MPKKKs, MAPKKKs, or MEKKs). In *Arabidopsis*, there are 20 MPKs, 10 MPKKs, and ~80 MPKKKs (Colcombet and Hirt, 2008). Because of their important role in cellular signal amplification, MPKs, MPKKs, and MPKKKs are excellent candidates for cellular signaling enzymes that mediate priming.

Analysis of publicly available microarray data to identify regulatory nodes in the transcriptional network of IR revealed that BTH induces the expression of MPK3 more than twofold in *Arabidopsis* (Wang et al., 2006). This kinase had not been previously associated with IR or priming, but rather was linked to direct responses to pathogen infection, mechanical, osmotic, and oxidative stresses, as well as microbe-associated molecular pattern and abscisic acid signaling (Mizoguchi et al., 1996; Nakagami et al., 2005; Ren et al., 2008). Therefore, we reasoned that the BTH-induced accumulation of MPK3 mRNA might lead to stronger activation of these responses upon subsequent exposure of the BTH-pretreated plants to biotic and abiotic stress. Here, we identify MPK3, and the functionally redundant MPK6, as important components required for full priming in *Arabidopsis* and show that the prestress deposition of these inactive kinases is a possible mechanism of priming during development of IR.

**RESULTS**

Induction of Priming and IR Correlates with MPK3 Expression

To investigate whether MPK3 could play a role in the development of priming and IR, we first assessed whether the resistance-inducing bacterial strains of Pst DC3000 harboring the avirulence gene *avrRpt2* and *Pseudomonas syringae pv phaseolicola* (Psp) carrying *avrB* could activate MPK3 gene expression (Figure 1A). In addition, the ability to induce MPK3 expression of various SA analogs that differ in their capacity to prime plants for IR was investigated (Figure 1B). Halogenated SA derivatives, such as 4-chloro-SA and 3,5-dichloro-SA, were previously shown to prime parsley cell cultures (Thuilke and Conrath, 1998) and *Arabidopsis* plants (Kohler et al., 2002) for augmented defense gene activation and to induce resistance to tobacco mosaic virus in tobacco (*Nicotiana tabacum*; Conrath et al., 1995). By contrast, 3-hydroxybenzoic acid was found to be inactive in these assays. As is shown in Figure 1, there was activation of the MPK3 gene in *Arabidopsis* upon infection with either of the two avirulent strains of bacteria (Figure 1A) as well as after treatment with BTH, SA, and 4-chloro-SA (Figure 1B). By contrast, MPK3 was not induced after treatment with 3-hydroxybenzoic acid (Figure 1B). This strong correlation between the ability of avirulent bacteria and various SA-related compounds to activate MPK3 gene expression and their capability to prime plants for augmented defense gene activation and IR (Conrath et al., 1995; Thuilke and Conrath, 1998; Kohler et al., 2002) supports our hypothesis that MPK3 plays a role in priming *Arabidopsis* for IR.

Priming for IR Is Associated with Accumulation of MPK3 and MPK6 Transcripts and Proteins

As priming and IR are time-dependent processes (Ryals et al., 1996; Thuilke and Conrath, 1998; Durrant and Dong, 2004), we
next examined the kinetics of MPK3 mRNA accumulation using BTH as the inducer of priming and resistance. At the same time, we analyzed whether the BTH-induced accumulation of MPK3 mRNA was associated with accumulation of MPK3 proteins. We found that treatment of Arabidopsis with 100 μM BTH was associated with biphasic accumulation of transcript for MPK3 as well as gradual accumulation of MPK3 protein (Figure 2A). However, the accumulation of MPK3 transcript and protein was not associated with dual phosphorylation of the TEY amino acid motif within the activation loop of MPK3 (Figure 2A), which is required for kinase activity (Ray and Sturgill, 1988). Similar observations were made for MPK6 (Figure 2B); MPK6 and its closest homolog MPK3 show 75% amino acid identity. However, the BTH-induced accumulation of MPK6 transcript and protein was less pronounced (Figure 2B). Together, these results suggest that MPK3 and MPK6 accumulate in an inactive form during priming of Arabidopsis with BTH. Therefore, these two proteins appear to be good candidates for cellular signaling components that contribute to the primed state in plants.

Enhanced Activation of MPK3 and MPK6 in Primed Plants

To investigate whether MPK3 and MPK6 might indeed display quantitatively greater activity in primed and subsequently challenged Arabidopsis plants, BTH-treated leaves with enhanced levels of MPK3 and MPK6 (Figure 2) were exposed to stress. After BTH treatment, leaves were dip-inoculated with virulent Ps pv maculicola strain ES4326 (Dong et al., 1991). At various times after infection, an aliquot of leaves was analyzed for dual TEY phosphorylation. As is shown in Figure 3A, activation-associated phosphorylation of MPK3 and MPK6 was induced upon bacterial infection of nonprimed and BTH-primed leaves. In both types of leaves, dual TEY phosphorylation of MPK3 and MPK6 was strongest at the 50-min time point after infection, but for MPK3 it was more intense and lasted longer in primed than in non-primed plants (Figure 3A).

In a second set of experiments, simple infiltration of water was used as an abiotic stress after the priming treatment with BTH. This stimulus was selected because MPK3 was initially described as a wound-induced protein kinase (WIPK; Seo et al., 1995; the WIPK is the tobacco ortholog of Arabidopsis MPK3), which can be activated by the infiltration of water into leaves (Zhang and Klessig, 1998). Infiltration of water also elicits a cell collapse response (Roebuck et al., 1978) that includes the expression of wound-responsive genes (Young et al., 1996; Kohler et al., 2002). In addition, changes in water potential have been associated with the presence of bacteria in Arabidopsis leaves (Wright and Beattie, 2004); thus, water infiltration may mimic bacterial infection to some extent. Figure 3B shows that dual TEY phosphorylation of MPK3 and MPK6 was induced upon infiltration of water into nonprimed and primed leaves. In both types of leaves, dual TEY phosphorylation was strongest at the 10-min time point after infiltration, and, as seen for MPK3 after Ps pv maculicola infection, the phosphorylation response was more intense and lasted longer in primed than in nonprimed leaves (Figure 3B). For inoculation with bacteria or water infiltration, the enhancement of TEY phosphorylation was seen only for MPK3 or it was more pronounced for MPK3 than for MPK6, respectively (Figure 3).

**Figure 2.** BTH Induces Accumulation of MPK3 and MPK6 Transcripts and Proteins but Does Not Elicit Dual TEY Motif Phosphorylation.

(A) Accumulation of MPK3 transcript and MPK3 protein. Leaves were harvested at various times after treatment of plants with 100 μM BTH (+; dark vertical bars) or a wettable powder carrier control (−; light vertical bars). An aliquot of leaf tissue was used for RNA extraction and quantitative RT-PCR (qRT-PCR) analysis to examine the abundance of MPK3 transcript normalized to that for ACTIN2. Another aliquot of leaf tissue was used for protein extraction and SDS-PAGE followed by immunodetection of MPK3 protein and dual TEY phosphorylation with polyclonal antibodies. Immunodetection of a loaded doubly phosphorylated human ERK2 (pERK2) kinase served as a positive control for blotting and immunodetection.

(B) Accumulation of MPK6 transcript and MPK6 protein. The experimental setup and analyses described in (A) were used to assess the abundance of MPK6 transcript and to immunodetect MPK6 and pTEpY. The experiments were performed four times with similar results. The values shown are means + SD (n = 4). Bars above diagrams give light/dark periods. hpt, h post-treatment; WB, protein gel blot. Prior to immunodetection, the blots were stained with Ponceau S to assess whether gel loading was equal. For quantification of immunodetection signals, see Supplemental Figure 1 online.
Attenuation of Priming in mpk3 and mpk6 Mutants

The above results point to MPK3 and, perhaps, MPK6 as possible important priming components for the enhanced stress response in Arabidopsis. To elucidate whether these two enzymes indeed are crucial for priming, and to provide genetic evidence for this, we included knockout mutants and transgenic plants in our studies. The expression of two genes, one encoding the PR1 pathogenesis-related protein and another encoding Phe ammonia-lyase 1 (PAL1), were chosen as markers for the activation of plant defense responses. These two genes were selected since they have been identified previously as reliable marker genes for the enhanced induction of defense responses (Kohler et al., 2002) and IR (Ryals et al., 1996; Durrant and Dong, 2004). The PAL enzyme plays a rate-limiting role in the phenylpropanoid pathway with important functions in the overall plant defense response (Hahlbrock and Scheel, 1989), while members of the PR1 family of proteins have been shown to exert inhibitory effects against phytopathogenic oomycetes (Alexander et al., 1993). In wild-type (Columbia-0) Arabidopsis, treatment with 100 μM BTH did not induce the PAL1 gene and only slightly induced PR1 (Figure 4). Infiltration of water into leaves elicited some PAL1 expression but did not induce PR1 (Figures 4A and 4B). However, when plants were primed with BTH for 3 d and then stimulated by water infiltration, we observed enhanced expression of both PAL1 and PR1 (Figures 4A and 4B).

When compared with wild-type plants, an mpk3 deletion mutant (Miles et al., 2005; Figure 4A) and an mpk6 T-DNA knockout plant (Figure 4B) at the 2-h time point following stimulation were only slightly affected in PAL1 activation by infiltration alone. Similarly, PR1 expression was not altered by water infiltration in the mpk3 T-DNA insertion mutant at the time point assayed (Figure 4B). However, in the two mpk3 mutants, priming for enhanced PAL1 expression was almost absent (Figures 4A and 4B) and primed PR1 expression was reduced (Figure 4B). This result contrasts with the direct activation by BTH of the SAR marker genes PR1, PR2, PR5, and GST1. Their activation was unaffected or even increased in the mpk3 and mpk6 T-DNA insertion mutants (Figure 4C). These findings indicate that MPK3 and MPK6 are associated with priming and not simply activation of defense genes during IR.

Similar to the mpk3 mutants, PAL1 activation by infiltration alone was unaffected in nonprimed plants in which the expression of MPK6 was repressed by RNA interference (RNAi) (Miles et al., 2005; Figure 4A). The same finding was made in an mpk6 T-DNA knockout plant (Figure 4B). In mpk6, PR1 expression by water infiltration alone was also unchanged (Figure 4B). However, in MPK6 RNAi plants (Figure 4A) and the mpk6 T-DNA mutant (Figure 4B), priming-mediated enhancement of stress-induced PAL1 expression was still observed, but to a lesser extent than in the wild type. The same was true for the accumulation of PR1 transcripts in mpk6 (Figure 4B). By contrast, direct activation (as opposed to priming) by BTH of PR1, PR2, PR5, and GST1 were either not affected or even enhanced also in the mpk6 T-DNA knockout mutant (Figure 4C). In sum, these results suggested that MPK3 is a major component in BTH-induced priming of defense gene activation, while MPK6 appears to serve a lesser role.

TEY Phosphorylation and Enhanced PAL1 Expression Are Affected in Known Priming Mutants

The above results argue that the BTH-induced prestress accumulation of inactive MPK3 and MPK6 and the enhanced induction of their activation-associated phosphorylation by stress causally contribute to enhanced inducibility of defense genes and to IR. If this proposition is true, one would expect that the BTH priming of stress-induced TEY phosphorylation of MPK3 and MPK6 would be enhanced in a permanently primed Arabidopsis mutant, while it would be lower, or even absent, in Arabidopsis plants that are defective for priming and for IR. To address this issue, we first used npr1-3, a mutant in the Arabidopsis Nonexpressor of PR1 (NPR1) gene. This mutant (subsequently referred to in this work as npr1) accumulates high endogenous levels of SA (Durrant and Dong, 2004) but does not express SA/BTH-induced priming for enhanced activation of defense genes after either Pst DC3000 infection, wounding with forceps, or water infiltration (Kohler et al., 2002). npr1 is also defective in development of IR induced by either chemicals or pathogen infection (Durrant and Dong, 2004). Analysis of the

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**Figure 3.** Dual TEY Phosphorylation Is Enhanced in BTH-Primed Leaves after Dip Inoculation with Bacteria or Infiltration with Water.

(A) Primed plants challenged by dipping leaves into bacterial suspension. Plants were treated with 100 μM BTH (+) or wettable powder carrier (−) for 3 d. Leaves were then challenged by dipping into a suspension of *Pst pv maculicola* strain ES4326 (5 × 10⁶ cfu mL⁻¹) in 10 mM MgCl₂ containing 0.01% Silwet L-77 (time point zero), harvested at the times indicated, and analyzed for dual phosphorylation of the TEY motif by SDS-PAGE, protein gel blotting, and immunodetection with polyclonal antibodies.

(B) Primed plants challenged by water infiltration. The same experimental setup as described in (A) but challenging leaves by infiltration of water. The experiments were done three times with similar results. Prior to immunodetection, the blots were stained with Ponceau S to assess whether gel loading was equal. For quantification of immunodetection signals, see Supplemental Figure 1 online.
above-mentioned microarray data to identify regulatory nodes in the transcriptional network of IR (Wang et al., 2006) revealed that BTH induced the accumulation of MPK3 and, to a lesser extent MPK6, transcripts in wild-type Arabidopsis, but did not do so in npr1. In agreement with this, the BTH-mediated enhancement of stress-induced dual phosphorylation of the TEY activation motif in MPK3 and MPK6 (Figures 3 and 5), and the associated boost in defense gene activation (Figures 4 and 5), which were seen in the wild type, were reduced in the priming-deficient and IR-deficient npr1 mutant (Figure 5).

Complementary results were obtained with the enhanced disease resistance1-1 (edr1-1) mutant (subsequently referred to in this work as edr1). EDR1 encodes a CTR1-like MPKKK that is a negative regulator of SA-inducible defense responses in Arabidopsis (Frye et al., 2001). edr1 exhibits constitutively enhanced resistance to fungal and bacterial pathogens, but not constitutive expression of the defense genes PR1 or β-1,3-GLUCANASE (Frye and Innes, 1998; Frye et al., 2001). However, upon pathogen attack, expression of these genes is stronger in edr1 than in wild-type plants (Frye and Innes, 1998). After pathogen attack, edr1 also shows stronger induction of other defense responses, such as the hypersensitive response and callose deposition. Thus, edr1 is constitutively primed for defense responses (Van Hulten et al., 2006). Interestingly, in comparison to wild-type plants, the endogenous level of MPK3 was enhanced about twofold in edr1 (Figure 5; see Supplemental Figure 1 online). In addition, dual TEY phosphorylation and PAL1 expression were elevated in water-infiltrated edr1 compared with the wild type, even in the absence of BTH pretreatment; they were further enhanced upon pretreatment with BTH (Figure 5). These results suggest that the enhanced dual TEY phosphorylation of MPK3, and to a lesser extent MPK6, contributes to the primed defense response phenotype of edr1.

IR and SAR Are Affected in mpk3 and mpk6 Mutants

The results presented in Figures 3 to 5 suggest that predeposition of inactive MPK3 and MPK6 might play a crucial role in priming for enhanced activation of defense responses in carrier or BTH for 3 d. Leaves were then left untreated or infiltrated with water and assayed for PAL1 expression after 2 h. Asterisks indicate significant differences (Student’s t test, n = 4, P < 0.05).

(B) Reduced defense gene activation in mpk3 and mpk6 T-DNA mutants. Same experimental setup as in (A) but using mpk3 and mpk6 T-DNA insertion mutants, assaying PR1 gene expression after 24 h, and determining MPK activity 10 min after infiltration using an in-gel kinase assay. Asterisks indicate significant differences (Student’s t test, n = 4, P < 0.05). For quantification of immunodetection signals, see Supplemental Figure 1 online.

(C) BTH-induced SAR gene expression in mpk3 and mpk6 T-DNA mutants. Wild-type, mpk3, and mpk6 plants were treated with wettable powder carrier (-) or BTH (+). Three days later, leaf tissue was harvested and analyzed for the accumulation of transcript for PR1, PR2, PR5, and GST1. GST1, glutathione S-transferase.

The experiments were performed three times with similar results. The values shown are means ± SD (n = 4).
were reduced in DC3000 in wild-type plants. In comparison, resistance levels shown in Figure 6A, treatment with BTH induced resistance to and by examining inoculated leaves for disease symptoms. As development of BTH-IR by determining the amount of bacterial growth as well as two sets of experiments were performed. First, wild-type plants the development of BTH-induced pathogen resistance and SAR, Arabidopsis.

Wild-type plants or npr1 or edr1 mutants were treated with wettable powder carrier or BTH (100 µM) for 3 d. Leaves were then left untreated or infiltrated with water and assayed for presence of MPK3 and dual phosphorylation of the TEY motif in MPK3 and MPK6 at the 10-min time point or for PAL1 expression at the 2-h time point after infiltration. The experiment was performed three times with similar results. The values shown are means ± sd (n = 4). Asterisks indicate significant differences (Student’s t test, n = 4, P < 0.05). WB, protein gel blot. Prior to immunodetection, the blots were stained with Ponceau S to assess whether gel loading was equal. For quantification of immunodetection signals, see Supplemental Figure 1 online.

Arabidopsis. To test whether the two proteins also play a role in the development of BTH-induced pathogen resistance and SAR, two sets of experiments were performed. First, wild-type plants as well as mpk3 and mpk6 mutants were pretreated with BTH or wettable powder carrier for 3 d and then infected with virulent Pst DC3000 bacteria. Four days after infection, aliquots of leaves from wild-type, mpk3, or mpk6 plants were assessed for development of BTH-IR by determining the amount of bacterial growth and by examining inoculated leaves for disease symptoms. As shown in Figure 6A, treatment with BTH induced resistance to Pst DC3000 in wild-type plants. In comparison, resistance levels were reduced in mpk3 and attenuated to a lesser extent in mpk6 (Figure 6).

In the second set of experiments, wild-type plants as well as mpk3 and mpk6 mutants were infected on three lower leaves with Pst DC3000 harboring the avirulence gene avrRpt2. Three days later, two upper leaves were challenged with virulent Pst DC3000. Three days after the challenge infection, aliquots of leaves from wild-type, mpk3, or mpk6 plants were assessed for development of SAR by determining bacterial growth. As shown in Figure 6B, the primary infection with Pst DC3000 avrRpt2 induced SAR to Pst DC3000 in wild-type plants and the mpk6 mutant, but did not do so in mpk3, although this mutant still retained resistance mediated by avrRpt2 perception (see Supplemental Figure 2 online). In wild-type plants and the mpk6 mutant, SAR development was preceded by more intense TEY phosphorylation of MPK3, but not MPK6, 2 h after challenge infection of preinoculated plants (Figure 6C).

DISCUSSION

Priming of cells for faster and stronger activation of defense upon a stress stimulus plays an important role in various forms of IR in plants and animals (Hayes et al., 1991; Conrath et al., 2006; Pham et al., 2007). Until now, the identity of hypothetical signaling components that would accumulate during priming but be employed only upon exposure to challenge stress has been obscure. Here, we show that prestress deposition of two members of the MPK family of signaling enzymes, MPK3 and MPK6, in an inactive form likely plays an important role in priming in Arabidopsis. Hyperactivation of one or more factor(s) upstream of MPK3 or MPK6 is an alternative, but not mutually exclusive, explanation for our findings.

In Arabidopsis and some other plants, MPK3 has been associated with direct responses to various stresses, including wounding (Seo et al., 1995; Mizoguchi et al., 1996; Nakagami et al., 2005; Ren et al., 2008). In the two mpk3 mutants, BTH-induced priming, manifested as enhanced inducibility of defense gene expression by water infiltration, BTH-IR, and SAR to Pst DC3000, was absent or markedly reduced at the time points assayed (Figures 4A, 4B, 6A, and 6B), while the direct activation by BTH of SAR marker genes was unaffected (Figure 4C). In comparison, in MPK6-silenced plants and the mpk6 mutant, the priming-mediated enhancement of infiltration-induced defense gene activation (Figures 4A and 4B), BTH-IR (Figure 6A), and SAR (Figure 6B) were little or not affected compared with that in the wild type, as was direct activation by BTH of SAR marker genes in mpk6 (Figure 4C).

Therefore, it is likely that MPK3 is a major component in BTH-induced priming of enhanced inducibility of defense gene activation and resistance in Arabidopsis, while MPK6 probably serves a more minor role. Because hyperactivation of MPK6 doesn’t correlate with enhanced PAL1 or PR1 expression (Figure 4B), and since hyperphosphorylation of the TEY motif in MPK6 doesn’t coincide with SAR in the mpk3 mutant (Figures 6B and 6C), a commanding role of MPK6 in priming and SAR seems unlikely. The latter conclusion is supported by Menke et al. (2004) who demonstrated that MPK6-silenced plants were not affected in their ability to develop SAR.

However, as priming for enhanced PAL1 and PR1 expression in many cases was somewhat affected in MPK6 RNAi plants and/or the mpk6 mutant (Figures 4A and 4B), both MPK3 and MPK6 seem to be important for full priming in Arabidopsis. This may explain why knockout of only one of the two genes encoding MPK3 or MPK6 doesn’t always attenuate IR (Figures 6A and 6B).

Indeed, physiological interaction (Miles et al., 2005), substrate overlap (Feilner et al., 2005; Merkouropoulos et al., 2008), and overlapping functions (Wang et al., 2008) have been shown for MPK3 and MPK6. Moreover, the tobacco orthologs of Arabidopsis MPK3 and MPK6, WIPK and SA-induced protein kinase, interact with each other in planta (Liu et al., 2003). Also, preexisting WIPK can enhance the cell death response induced by expression of a constitutively active MPK kinase (Nt MEK2DD) (Liu et al., 2003). Unfortunately, because simultaneous knockout of MPK3 and MPK6 is embryo-lethal (Wang et al., 2007), such double knockout plants are not available to more rigorously address the role(s) of MPK3 and MPK6 in priming, which is likely to be at least partially redundant.
In our laboratories, several approaches to overexpress the MPK3 gene in *Arabidopsis* under control of constitutive and inducible promoters failed (see Supplemental Information online). However, overexpression of MPK3 orthologs in other plants enhanced their resistance to pathogens. For example, preinoculation of cucumber (*Cucumis sativus*) roots with the fungal biocontrol agent *Trichoderma asperellum* systemically activates the gene for *Trichoderma*-induced protein kinase (TIPK), which is orthologous to *Arabidopsis* MPK3 and induces resistance to the leaf pathogen *Ps pv lachrymans* (Shoresh et al., 2006). Overexpression of the TIPK gene in cucumber plants enhanced their resistance to *Ps pv lachrymans* even in the absence of *Trichoderma* preinoculation, while TIPK antisense plants displayed increased susceptibility to pathogen attack (Shoresh et al., 2006). Thus, the systemic expression of TIPK upon *Trichoderma* infection of cucumber roots seems to be responsible for systemic immunity to *Ps pv lachrymans* (Shoresh et al., 2006).

Similarly, transformation of rice (*Oryza sativa*) plants with MK1, the pepper (*Capsicum annuum*) ortholog of *Arabidopsis* MPK3, resulted in constitutive expression of the transgene, constitutive accumulation of the MK1 protein, as well as enhanced resistance to rice blast disease (Lee et al., 2004). Together, these findings demonstrate that priming and enhanced resistance to subsequent infections can be conferred by induced or constitutive expression of genes that are orthologous to *Arabidopsis* MPK3.

Based on these reports and on our findings, we propose that MPK3 and, to a lesser extent MPK6, play an important role in development of IR to biotic and abiotic stress in plants. This assumption is supported by microarray analyses in which MPK3 expression was associated with chemically and biologically IR in *Arabidopsis* (Schenk et al., 2000, 2003; Wang et al., 2006). Interestingly, a recent report demonstrated that HopA11, an effector that contributes to *Pst* virulence in plants, acts as a phosphotheorein lyase that dephosphorylates and thus inactivates MPKs (Li et al., 2007). HopA11 directly interacts with *Arabidopsis* MPK3 and MPK6 (Zhang et al., 2007). It also suppresses MPK3 and MPK6 activation by bacterial flagellin (a microbe-associated molecular pattern) and dampens the associated immune response (Zhang et al., 2007). Thus, upregulation of MPK3 and MPK6 levels seems to enhance, and downregulation of MPK3/MPK6 activity reduce, defense in *Arabidopsis*.

Interestingly, the plant pathogen *Agrobacterium tumefaciens* even in the absence of *Trichoderma* preinoculation, while TIPK antisense plants displayed increased susceptibility to pathogen attack (Shoresh et al., 2006). Thus, the systemic expression of TIPK upon *Trichoderma* infection of cucumber roots seems to be responsible for systemic immunity to *Ps pv lachrymans* (Shoresh et al., 2006). Similarly, transformation of rice (*Oryza sativa*) plants with MK1, the pepper (*Capsicum annuum*) ortholog of *Arabidopsis* MPK3, resulted in constitutive expression of the transgene, constitutive accumulation of the MK1 protein, as well as enhanced resistance to rice blast disease (Lee et al., 2004). Together, these findings demonstrate that priming and enhanced resistance to subsequent infections can be conferred by induced or constitutive expression of genes that are orthologous to *Arabidopsis* MPK3.

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also suppresses MPK3-mediated defense responses during delivery of its infectious T-DNA into the host plant (Djamei et al., 2007). The finding that the so-called lethal factor, which is a major virulence factor secreted by *Bacillus anthracis*, cleaves MPKs and leads to ineffective priming of T cells (Agrawal and Pulendran, 2004) suggests that MPKs might also have a role in priming in mammals.

It has been known for several years that priming is part of IR in animals (Gifford and Lohmann-Matthes, 1987; Koerner et al., 1987; Pham et al., 2007) and plants (Kuc’, 1987; Zimmerli et al., 2000; Verhagen et al., 2004). However, little was known about the molecular mechanisms of priming. Here, we propose that the elevated accumulation of inactive MPK3 and MPK6 is a critical step in the priming mechanism of plants that leads to enhanced inducibility of defense responses upon a stress stimulus and to IR. It will be interesting to see whether prestress deposition of inactive proteins corresponding to *Arabidopsis* MPK3 and MPK6 also plays a role in animal priming and immunity.

**METHODS**

Plant Material, Growth Conditions, and Plant Treatment

*A. thaliana* wild-type plants and mutants were grown on soil at 8 h light/16 h dark, 20°C, and 60 to 70% relative humidity. Wild-type plants are the Arabidopsis accession Columbia-0 obtained from the European Arabidopsis Stock Center (Nottingham, UK). The *Arabidopsis* mutant referred to as *npr1* was *npr1-3* and was kindly provided by K. Dong (Duke University, Durham, NC). *edr1* was *edr1-1* and was a courtesy of R. Innes (Indiana University, Bloomington, IN). Seeds for the *mpk3* deletion mutant and *MPK6* RNAi plants were a gift from B. E. Ellis (University of British Columbia, Canada). *MPK3* and *MPK6* T-DNA insertion mutants were *mpk3-1* and *mpk6-2* (Wang et al., 2007). Five-week-old plants were infiltrated on three to four lower leaves with avirulent *Pseudomonas syringae* pv tomato DC3000 (10^8 cfu mL^{-1}) harboring the avirulence gene avrRpt2 or *Pseudomonas syringae* pv phaseolicola carrying avrRpt2 in 10 mM MgCl_2. Alternatively, the plants were entirely sprayed with 100 μM BTH (Syngenta), 300 μM SA (Sigma-Aldrich), 300 μM 4-chloro-SA (Sigma-Aldrich), or 300 μM 3-hydroxybenzoic acid (Sigma-Aldrich). All these compounds were dissolved in a solution of a wettable powder carrier (Syngenta). Control plants were left untreated, infiltrated on three to four lower leaves with MgCl_2 in the absence of bacteria, or sprayed with wettable powder carrier. Three days later, upper leaves were harvested and analyzed for the accumulation of MPK3 transcripts or left untreated, pressure infiltrated with tap water, or challenge-infected by infiltration or dipping into a suspension of either PST DC3000 (5 × 10^8 cfu mL^{-1}) or *Ps* pv *maculicola* strain ES4326 (5 × 10^6 cfu mL^{-1}) in 10 mM MgCl_2 containing 0.01% TEY phosphorylation or gene expression. When done, bacterial titer and disease symptoms were estimated 3 or 4 d after infection. For the determination of bacterial leaf titer, leaf discs were harvested from infected leaves, homogenized in 10 mM MgCl_2, and then serially diluted and spread on agar plates containing King’s medium B. Colonies were counted to determine bacterial leaf titer.

RNA Isolation and Real-Time qRT-PCR

Total RNA was isolated from leaves using TRI Reagent (Molecular Research Center). One microgram of total RNA was incubated with 1 unit of DNasel (Fermentas) in a total volume of 10 μL at 37°C for 15 min. After inactivation of DNasel by heating to 70°C for 15 min, RNA was reversely transcribed using 200 units of Revert Aid M-MuLV reverse transcriptase (Fermentas), and 2.5 μM random nanomer primers in a sample volume of 20 μL. Diluted cDNA was used as template for real-time qRT-PCR with an ABI PRISM 7000 sequence detector system (Applied Biosystems). Each 10-μL reaction mixture contained 5 μL SYBR Green Master mix (Applied Biosystems) and 0.2 μM (final concentration) gene-specific primers (see Supplemental Table 1 online). Real-time DNA amplification was analyzed using the ABI PRISM 7000 SDS 1.0 software (Applied Biosystems). The Ct (cycle at the threshold) value is set constant throughout the study and corresponds to the log linear range of PCR amplification. The normalized amount of target reflects the relative amount of target transcripts with respect to the endogenous reference gene ACTIN2. To detect and exclude nonspecific amplions, the melting curves of all PCR products were analyzed, all final products were visualized by agarose gel electrophoresis to ensure amplification of a single product of the correct size, and products were fully sequenced. Results of qRT-PCR analyses were verified by at least three independent experiments each with four biological replicates (means + SD, n = 4 biological replicates).

Protein Extraction, SDS-PAGE, Protein Gel Blot Analysis, Immunodetection, and In-Gel Kinase Assay

Total protein was extracted from frozen leaf tissue, subjected to SDS-PAGE, transferred to a nitrocellulose membrane (Whatman), and used for immunodetection as described (Conrath et al., 1997). Equal loading was checked by Ponceau S staining. Primary rabbit antibodies against MPK3 and MPK6 were from Daniel Klessig (Boye Thompson Institute, Ithaca, NY). The anti-phospho-p44/42 MPK (Thr202/Tyr204) antibody, which detects doubly phosphorylated MPK3 and MPK6 (Heese et al., 2007), was from New England Biolabs. Antibiot-antibody complexes were detected with horseradish peroxidase–conjugated anti-rabbit secondary antibody (New England Biolabs) followed by chemiluminescence detection with SuperSignal West Pico chemiluminescent substrate (Pierce). In-gel kinase assay was performed as described (Zhang and Klessig, 1997).

Statistical Analyses

The experiments shown were done at least three times with similar results. Where necessary, means of acquired data were compared using Student’s t test ([n = 4 to 10 biological replicates] [leaves or plants] in a single experiment, P < 0.05), and significant differences are indicated with asterisks.

Accession Numbers

Locus information and sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank databases under the following accession numbers: ACTIN2, At3g18780; GST1, At1g02930; MPK3, At3g45640; MPK6, At2g43790; PAL1, At2g37040; PR1, At2g14610; PR2, At3g57260; PR-5, At1g75040; and N. tabacum WIPK, D61377. Germplasm identification numbers for the mpk mutants used are as follows: mpk3-1, SALK_151594; mpk6-2, SALK_073907.

Supplemental Data

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

**Supplemental Figure 1.** Quantification of Immunodetection Signals in Figures 2, 3, 4B, 5, and 6C.

**Supplemental Figure 2.** mpk3 and mpk6 Mutants Retain Resistance Mediated by avrRpt2 Perception.
Supplemental Table 1. List of Gene-Specific Forward and Reverse Primers Used for Real-Time qRT-PCR Analysis.

Supplemental Information. Summary of Attemps to Overexpress the MPK3 and MPK6 Genes in Arabidopsis.

Supplemental References.

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