The Plant Journal (2006) **48**, 485–498 doi: 10.1111/j.1365-313X.2006.02888.x

The Arabidopsis MAP kinase kinase MKK1 participates in defence responses to the bacterial elicitor flagellin

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Received 2 July 2006; accepted 7 July 2006.

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Summary

Plants sense pathogens through both pathogen-associated molecular patterns and recognition of race-specific virulence factors, which induce basal defence or an accelerated defence (often manifest in the form of local cell death), respectively. A mitogen-activated protein kinase (MAPK) module in Arabidopsis was previously proposed to signal from perception of the bacterial elicitor flagellin to the activation of basal defence-related genes. Here, we present evidence for a parallel MAPK-signalling pathway involved in the response to flg22, a peptide corresponding to the most conserved domain of flagellin. The endogenous Arabidopsis MAP kinase kinase MKK1 is activated in cells treated with flg22, phosphorylates the MAPK MPK4 *in vitro*, and activates it *in vivo* in protoplasts. In *mkk1* mutant plants, the activation by flg22 of MPK4 and two other flg22-induced MAPKs (MPK3 and MPK6) is impaired. In the *mkk1* mutant, a battery of both flg22-induced and flg22-repressed genes show altered expression, indicating that MKK1 negatively regulates the activity of flagellin-responsive genes. Intriguingly, in contrast to the *mpk4* mutant, *mkk1* shows no morphological anomalies and is compromised in resistance to both virulent and avirulent *Pseudomonas syringae* strains. Thus, the MKK1 signalling pathway modulates the expression of genes responding to elicitors and plays an important role in pathogen defence.

Keywords: mitogen-activated protein kinase signalling, flagellin, pathogen resistance, defence-related genes, signalling networks, Arabidopsis.

Introduction

Plants are equipped with various defence mechanisms, some being constitutive barriers to pathogen spread, such as waxy cuticle or preformed antimicrobial compounds, while others rely on the recognition of pathogens and the induction of a variety of defence responses (Dangl and Jones, 2001; Jones and Takemoto, 2004). A broad range of pathogens is recognized by plants through so-called pathogen-associated molecular patterns (PAMPs), which are highly conserved molecular fragments of pathogenic molecules usually playing pivotal roles in microorganisms. Some known examples of this innate immune response are the recognition of a conserved 22-amino acid fragment derived from the bacterial flagellin flg22, of a 13-amino acid oligopeptide from a *Phytophthora sojae* 42-kDa glycoprotein (Pep13), and of the 22-kDa fungal protein xylanase (Boller, 2005). The contribution of PAMPs responses to disease resistance was demonstrated by spraying flg22 on Arabidopsis plants, which restricted bacterial invasion through a mechanism independent of the known systemic plant defence-signalling molecules salicylic acid (SA), jasmonic acid

(JA) and ethylene (ET; Kim *et al.*, 2005; Zipfel *et al.*, 2004). Signalling mechanisms and cellular responses acting downstream of the recognition of largely unrelated PAMPs are believed to be similar, and are known to include medium alkalinization, release of Ca^{2+}, generation of signalling phospholipids and activation of mitogen-activated protein kinases (MAPKs), as well as rapid production of reactive oxygen species (ROS; Nurnberger *et al.*, 2004). How precisely these signalling events are connected and function in a network is not well understood, but they are known to lead to an extensive defence-oriented transcriptional reprogramming and major changes in cellular metabolism (Navarro *et al.*, 2004).

Pathogens have evolved mechanisms to suppress or mask basal plant defence and redirect plant cell functions for their benefit. This process commonly involves secretion of race-specific virulence factors to the host cells. Some of these virulence factors, such as AvrPto, are potent suppressors of receptor function and MAPK activation (He *et al.*, 2006). Perhaps adapting from PAMPs recognition, plants are armed with specific R gene-encoded receptors that recognize avirulence (*avr*) gene-encoded factors from particular races of pathogens or plant proteins modified in response to Avr factors. The match of a particular combination of *R* gene and avirulence factor triggers the well known hypersensitive response (HR), restricting spread of the pathogen by induction of local cell death (Martin *et al.*, 2003). Transcription profiling experiments revealed a surprising overlap between the responses to flg22 and Avr factors (Navarro *et al.*, 2004). Moreover, signalling events downstream of PAMPs and *avr* products are known to share a number of signalling components, such as Ca^{2+}, phospholipids, ROS and MAPKs. Furthermore, the development of disease symptoms on infection by a virulent pathogen also follows some of the signalling events and gene-expression changes associated with resistance (del Pozo *et al.*, 2004). How specific responses are subsequently selected to trigger defence, HR or necrosis is not known.

MAPK cascades are used widely by eukaryotes to integrate signal inputs and connect them to specific cellular responses (Pedley and Martin, 2005). A MAPK signalling module consists of three protein kinases sequentially activated through phosphorylation by the upstream component: a MAP kinase kinase kinase (MAPKKK or MEKK); a MAP kinase kinase (MAPKK or MKK) and a MAP kinase (MPK). MAPK components are particularly abundant in plants: there are 80 putative MEKKs, 10 MKKs and at least 20 MPKs in Arabidopsis, but so far there is functional information for only few of these (Jonak *et al.*, 2002; Nakagami *et al.*, 2005).

Molecular, genetic and biochemical data demonstrated the involvement of MAPK pathways in defence responses (Pedley and Martin, 2005). A complete MAPK module in Arabidopsis transmitting signal from the flg22 elicitor through the FLS2 receptor was assembled based on over-expression experiments. This MAPK module is proposed to consist of MEKK1 connected to MKK4 or MKK5 and downstream to MPK3 or MPK6, and eventually leads to gene induction through the WRKY22 and WRKY29 transcription factors (Asai *et al.*, 2002). Transient overexpression of constitutively active MEKK1, MKK4, MKK5 or WRKY29 in Arabidopsis leaves causes enhanced resistance to bacterial and fungal pathogens, indicating that these MAPK-signalling components might trigger defence responses to a broad range of pathogens (Asai *et al.*, 2002). In variation from these results, interaction screens and functional complementation assays in yeast placed MEKK1 in a distinct MAPK module consisting of two closely related MKKs, MKK1 and MKK2, and the MAPK MPK4 (Ichimura *et al.*, 1998; Mizoguchi *et al.*, 1998). Strengthening these data, MPK4 is specifically phosphorylated and activated *in vitro* by MKK1 in response to various stress treatments, suggesting *in vivo* connection between these two kinases (Huang *et al.*, 2000; Matsuoka *et al.*, 2002; Teige *et al.*, 2004). Although MKK1 and MKK2 form complexes with identical upstream and downstream kinases, they appear to have distinct functions. MKK2 was shown to phosphorylate MPK4 in response to abiotic stress signals, while MKK1 was activated by pathogenic elicitors and ROS (Teige *et al.*, 2004). Consistently, the endogenous MPK4 was found to be activated both by biotic stresses, such as the bacterial elicitors flagellin or harpin, and by a variety of abiotic stresses (Desikan *et al.*, 2001; Droillard *et al.*, 2004; Ichimura *et al.*, 2000; Teige *et al.*, 2004). Genetic studies place MPK4 as negative regulator of pathogen responses, as the *mpk4* knockout mutant has elevated SA levels, constitutively expresses pathogenesis-related (PR) genes, and shows increased resistance to virulent biotroph pathogens. Expression of the SA hydroxylase NahG in *mpk4* plants abolishes PR gene expression and bacterial resistance, indicating that the *mpk4* phenotype requires SA. Furthermore, the *mpk4* mutant is impaired in the induction of JA- and ET-responsive genes, irrespective of SA levels (Brodersen *et al.*, 2006; Petersen *et al.*, 2000). Both the SA-repressing and the ET/JA-activating functions appear to depend on the defence regulators EDS1 and PAD4 (Brodersen *et al.*, 2006). A yeast two-hybrid screen recently identified a substrate for MPK4, designated MAP kinase 4 substrate 1 (MKS1). Analyses of transgenic plants and transcriptional profiling indicated that MKS1 is required for full SA-dependent resistance in *mpk4* mutants (Andreasson *et al.*, 2005). The tobacco orthologue of MPK4 has been isolated, and is activated by wounding. In NtMPK4-silenced tobacco plants, the induction by wounding of JA-responsive genes was inhibited (Gomi *et al.*, 2005).

While MPK4 is in a pathway including MKK1 and MKK2, the two other MAPKs implicated in flagellin response, MPK3 and MPK6, are activated by MKK4 and MKK5 (Asai *et al.*, 2002). Overexpression of a gain-of-function mutant form of MKK4, MKK5 or their closest tobacco orthologue NtMKK2

triggers HR-like lesions with a concomitant elevation of ROS and ET production (Jin *et al.*, 2003; Liu and Zhang, 2004; Liu *et al.*, 2003; Ren *et al.*, 2002). Consistently, silencing of NtMKK2, or of the tobacco orthologues of MPK6 and MPK3 (SIPK and WIPK, respectively), compromises N-gene-mediated resistance and HR (Jin *et al.*, 2003). Silencing of SIPK was also shown to enhance sensitivity to harpin-induced cell death (Samuel *et al.*, 2005), whereas silencing of MPK6 was reported to render Arabidopsis plants slightly more sensitive to both virulent and avirulent pathogens (Menke *et al.*, 2004).

It appears now that, rather than linear pathways, multiple interconnected MAPK pathways are required to transmit pathogen-derived signals and integrate defence responses (Pedley and Martin, 2005). Indeed, gene silencing and biochemical works in tomato identified multiple MAPK signalling components that are activated and required for resistance to bacterial infection on recognition of *Pseudomonas syringae* pv. *tomato* strains expressing AvrPto or AvrPtoB, and placed them in a complex network (del Pozo *et al.*, 2004). The individual contributions to defence responses of each distinct MAPK pathway are not well understood yet.

In this study, we investigate the role of MKK1 in defence responses using a loss-of-function *mkk1* mutant and studying *in vivo* interactions and activation of endogenous MAPK signalling components. We show that MKK1 is an integral component of flagellin responses in Arabidopsis and contributes to resistance to both virulent and avirulent bacterial pathogen strains.

Results

Flagellin activation of MPK4 is mediated by MKK1

A study based on transient overexpression of selected wild-type and gain-of-function MAPK-signalling components suggested a linear flagellin-induced pathway in Arabidopsis consisting of a MEKK1–MKK4/MKK5–MPK3/MPK6 module (Asai *et al.*, 2002). MPK4 was not included in this report, but later work clearly showed the activation of endogenous MPK4 on flagellin treatment (Droillard *et al.*, 2004). MPK4 was placed in a MAPK cascade downstream of MKK1 specifically on flagellin stimulation, based on their interaction and the activation of MPK4 when MKK1 was overexpressed (Teige *et al.*, 2004). Therefore, we decided to compare the respective roles played by MKK1 and MKK4 in the activation of the three known flagellin-induced MAPKs (MPK3, MPK4 and MPK6) using firstly transient transfection of Arabidopsis protoplasts.

To this end, protoplasts were transfected with the HA epitope-tagged MPKs, alone or co-expressed with myc epitope-tagged MKK1 or MKK4, and subsequently treated for 10 min with 500 nM flg22. After immunoprecipitation of the MPKs with anti-HA antibody, their activity was determined by *in vitro* kinase assays using myelin basic protein (MBP) as an artificial substrate. None of the MAP kinases was activated in the untreated conditions, whether expressed alone or in the presence of MKKs (Figure 1a). On flagellin treatment, MPK3 and MPK6, but not MPK4, showed activation without a co-expressed upstream MKK. Flagellin-induced activation of MPK3 and MPK6 was increased further in the presence of overexpressed MKK4, while MPK4 was activated only when co-expressed with MKK1. Surprisingly, overexpression of MKK1 also resulted in decreased MPK3 and MPK6 activation in flg22-treated protoplasts (Figure 1b). The relatively high endogenous levels of MKK4/5 are presumably sufficient for the activation of MPK3/6, which can be further increased by co-expression of MKK4. In contrast, based on publicly available microarray data, the expression level of *MKK1* is low and accordingly the endogenous MKK1 protein is scarce (Matsuoka *et al.*, 2002), hence MKK1 co-expression is needed for flg22-induced activation of overexpressed MPK4. In order to provide experimental data for this hypothesis, protoplasts were transformed with RNAi constructs specifically targeting MKK1 or its closest homologue, MKK2, to decrease the levels of potential upstream activators of MPK4. MKK2 was shown previously to be an upstream activator of MPK4, as well as MPK6, in response to cold and salt stress (Teige *et al.*, 2004). First, we tested the specificity of RNAi constructs by co-transforming protoplasts with different combinations of myc epitope-tagged MKK1, MKK2 and MKK1, MKK2 RNAi constructs. Immunoblot analysis demonstrated that the RNAi constructs specifically target the relevant MKK RNAs (Figure 1c). Next, protoplasts were transformed with MKK1 or MKK2 RNAi constructs to reduce the levels of endogenous MKK1 or MKK2, and the activity of the endogenous MPK4 was measured after immunoprecipitation with a specific antibody. We observed a reduced activation in response to flg22 treatment in protoplasts transfected with the RNAi constructs (Figure 1d). Furthermore, MPK4 induction by flg22 was significantly more decreased when the transfection was done with MKK1 RNAi compared with MKK2 RNAi. It should be kept in mind that the protoplast transfection has around 50% efficiency in our hands, and results not in a homogeneous population, but in a mixture of wild-type and transformed cells. Therefore, no complete abolishment of the MAPK activation can be expected, even if the MAPK-activating pathway is disrupted in transformed cells. Taken together, these data show that at least three MAPKs are involved in flagellin-induced signalling, and that the activation of MPK4 is dependent on MKK1.

MKK1 is activated in vivo *by flagellin elicitation and phosphorylates MPK4*

Previously it was shown that MKK1 interacts with, and phosphorylates in response to H_2O_2 specifically MPK4 but

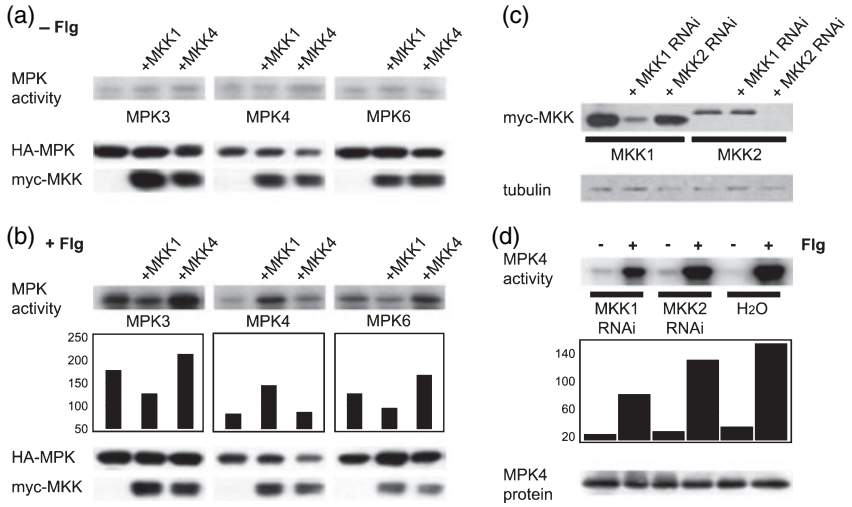

Figure 1. MKK1 mediates MPK4 activation in response to flagellin in Arabidopsis protoplasts.
(a, b) MPK3, MPK4 and MPK6 were transiently expressed alone or in co-expression with MKK1 or MKK4 in Arabidopsis protoplasts. Kinase activity of the immunoprecipitated MPKs was measured using MBP as an artificial substrate. MPK activity in untreated protoplasts is shown in (a); MPK activity in response to flg22 treatment is shown in (b). The signal detected by autoradiography has been quantified in (b) for better visualization (arbitrary units). Expression of MPKs and MKKs was detected by Western blot analysis with anti-HA (MPKs) or anti-c-myc (MKKs) antibody.
(c) Protoplasts were transformed with different combinations of myc-tagged MKK1 and MKK2 in co-transfection with RNAi constructs designed specifically to target MKK1 or MKK2. The effect and specificity of silencing were assessed by measuring the reduction in expression level of the targeted MKK. Western blotting using an antitubulin antibody is shown as a loading control.
(d) Effect of silencing MKK1 or MKK2 on MPK4 elicitation by flagellin. RNAi constructs targeting MKK1 or MKK2 were transfected into Arabidopsis protoplasts, and the activity of endogenous MPK4 in absence or presence of flg22 was measured using a specific antibody for immunocomplex kinase assays. The activities detected by autoradiography have been quantified for better visualization (arbitrary units). The expression of MPK4 was detected by Western blot using an MPK4-specific antibody.

not MPK3 and MPK6 (Teige *et al.*, 2004). To confirm that the endogenous MKK1 is activated by flagellin, and that MPK4 activation occurs through direct phosphorylation by MKK1, we expressed and purified recombinant kinase-dead GST fusion proteins of MPK3, MPK4 and MPK6, and used them as substrates to test MKK activity. Arabidopsis cells in suspension were treated for 10 min with 500 nM flg22. Endogenous MKK1 was immunoprecipitated using a commercial MKK1-specific antibody and subsequently tested for its ability to phosphorylate kinase-dead MPK3, MPK4 and MPK6 *in vitro*. The specificity of the commercial MKK1-specific antibody was tested on bacterially expressed MKK1 and MKK2 proteins tagged with GST and myc (Figure S1 in Supplementary Material). On flg22 treatment, phosphorylation of MPK4 and, to a lesser extent, of MPK6 increased, but the phosphorylation of MPK3 remained at basal level (Figure 2a,b). These results show that the endogenous MKK1 is activated *in vivo* by flg22 elicitation and confirms that its preferred substrate is MPK4.

mkk1 *mutant plants show reduced MPK3, MPK4 and MPK6 activation on flagellin elicitation*

To study the *in planta* function of MKK1 in flagellin-responsive MAPK pathways, we identified an *MKK1* mutant line. As no *mkk1* mutant was publicly available, we screened our library of Arabidopsis T-DNA insertion lines (Rios *et al.*, 2002), and isolated a mutant that carries a single insertion in the second intron of the *MKK1* gene (Figure 3a). The T-DNA insertion resulted in the complete loss of the corresponding mRNA expression, as found by RT–PCR analysis (Figure 3b). In contrast to the drastic dwarf phenotype of the previously described *mpk4* knockout mutant (Petersen *et al.*, 2000), detailed phenotypic analysis of homozygous *mkk1* plants revealed no obvious developmental defects under normal growth conditions (Figure 3c).

Next, we investigated the activation of endogenous MPK3, MPK4 and MPK6 in wild-type and *mkk1* mutant plants in response to flagellin elicitation *in vivo*. Wild-type and *mkk1* seedlings grown *in vitro* were incubated in liquid medium containing 1 μM flg22, and samples were collected at different time points. Protein extracts were used for in-gel kinase assays using MBP as a substrate. In wild-type plants, three MBP-phosphorylating kinases were activated within 5 min and their activity remained high until 20 min, and returned to near basal level after 60 min (Figure 4a, left panel). The three kinases of apparent molecular weights 44, 39 and 37 kDa correspond to MPK6, MPK3 and MPK4, respectively, as published previously (Droillard *et al.*, 2004) and as found by our experiments on *mpk3*, *mpk4* and *mpk6* knockout mutants where the corresponding bands were missing. In *mkk1* mutant seedlings, surprisingly the activity

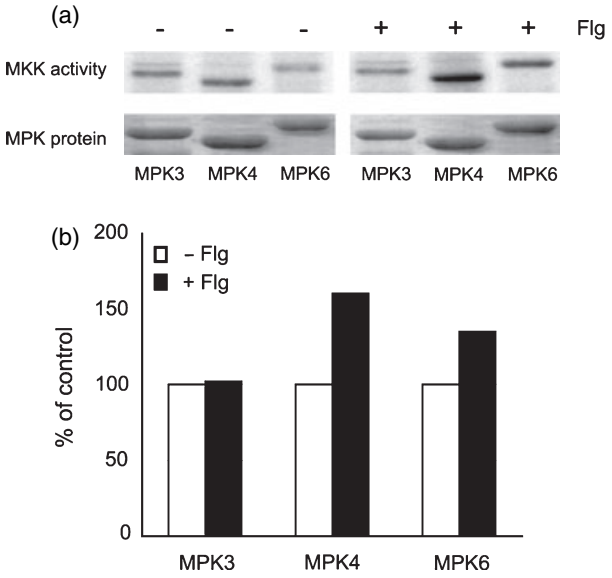

Figure 2. MKK1 is activated *in vivo* by flagellin and phosphorylates MPK4.
(a) Activity of endogenous MKK1 on purified kinase-dead MPKs. Arabidopsis cells were treated with flg22; MKK1 was then immunoprecipitated with a commercial MKK1-specific antibody and its activity determined by *in vitro* kinase assays using recombinant kinase-dead GST-MPK3, GST-MPK4 and GST-MPK6 as substrates (upper panel). Coomassie staining of a gel with equivalent amounts of the same protein samples is shown as a loading control (lower panel).
(b) Quantification of the signals detected by autoradiography in (a). The control signals, which represent basal phosphorylation activity in untreated conditions, were set to 100%.

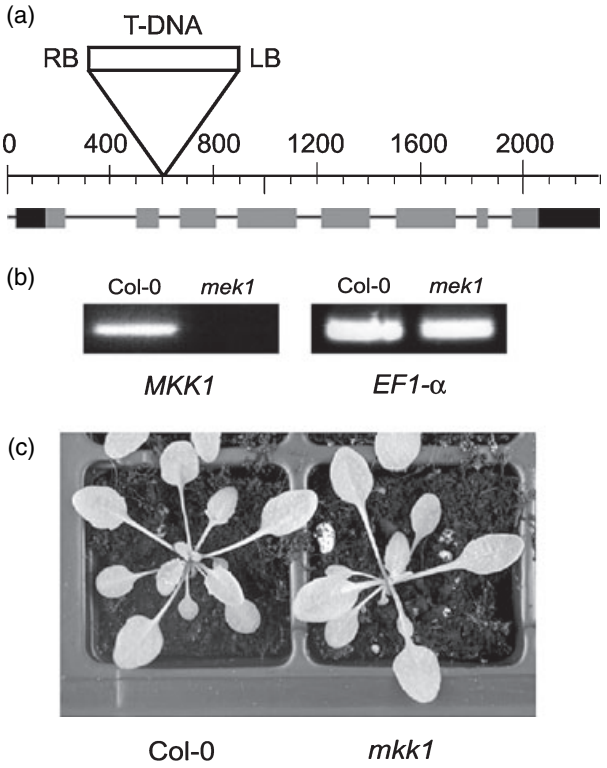

Figure 3. Isolation of an *mkk1* null mutant.
(a) Intro–exon structure of the *MKK1* gene according to the TIGR database and position of the T-DNA insertion in the *mkk1* line, determined by PCR and sequencing of the flanking regions as described in Methods.
(b) RT–PCR analysis of transcript levels in leaf tissue in wild-type Col-0 and *mkk1* mutant plants. The *MKK1* transcript is absent in *mkk1* plants.
(c) *mkk1* plants grown under normal conditions are morphologically indistinguishable from the wild type.

of all three MPKs was still detected, but to a significantly lower level (Figure 4a, right panel). Although the activation of MPK4 decreased considerably in the *mkk1* plants, it remained detectable, suggesting that MPK4 has more than one upstream activator, or that another MKK can substitute for MKK1 in *mkk1* mutants. It was also surprising that the *mkk1* mutation affects MPK3 and MPK6 responses to flg22 as well. Immunoblot analysis of the same protein extracts with MAPK-specific antibodies showed that the levels of all three kinases remained constant throughout the time course in both wild-type and mutant backgrounds, confirming that the MAPK activation occurs primarily through post-translational modifications (Figure 4b). Moreover, there was no detectable difference in protein levels between wild-type and mutant seedlings, indicating that the lower kinase activities observed in *mkk1* plants reflect reduced activation, not reduced levels of proteins.

To further confirm the identity of the activated kinases, we used specific antibodies raised against MPK3, MPK4 and MPK6 for immunocomplex kinase assays, using MBP as a substrate. In wild-type plants, all three MPKs showed a similar activation pattern on flg22 elicitation: they were activated within 5 min of treatment and remained high until 20 min (Figure 4c, left panel). In *mkk1* mutant seedlings, the activation of all three MPKs was lowered compared with wild-type (Figure 4c, right panel). The reduction in MPK3, MPK4 and MPK6 activities in the *mkk1* mutant is not dramatic, but was observed reproducibly in all our experiments. Two additional examples are provided in Figure S2 and S3.

Taken together, these results demonstrate that MKK1 is an integral component of MPK signalling pathways responding to flagellin, and contributes directly or indirectly to the activation of the three flagellin-induced MPKs.

The MKK1 mutation deregulates the expression of flagellin-responsive genes

Changes in gene expression are an important aspect of plant responses to a pathogenic infection. The early transcriptional response to flagellin elicitation has been studied extensively (Navarro *et al.*, 2004; Zipfel *et al.*, 2004). More than 1000 genes were found to be activated or repressed by flg22 application; a vast majority can be classified as being involved in signal transduction, transcriptional regulation

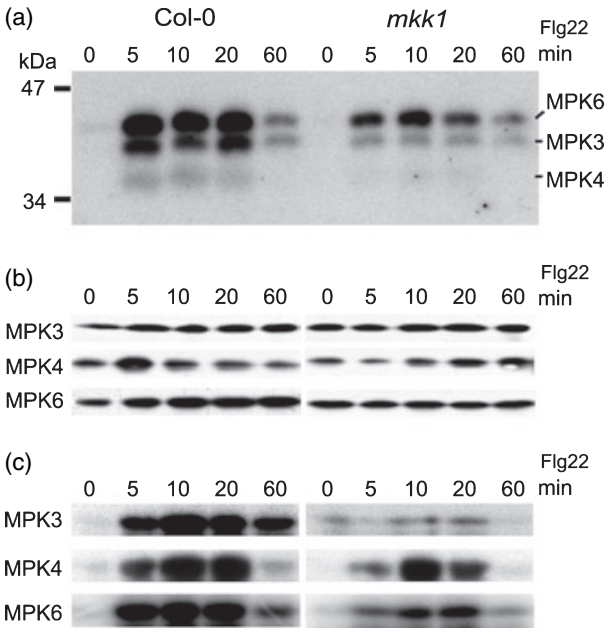

Figure 4. *mkk1* plants show decreased MPK3, MPK4 and MPK6 activation on flagellin treatment.
(a) Flagellin-induced activation of MPK3, MPK4 and MPK6 in wild-type versus *mkk1* mutant plants determined by in-gel kinase assays. Ten-day-old seedlings were treated with flg22 and collected at different time points. The assay showed activation by flg22 of three MBP-phosphorylating kinases of apparent molecular weight 44, 39 and 37 kDa, corresponding to MPK6, MPK3 and MPK4, respectively.
(b) Western blots showing protein levels of MPK3, MPK4 and MPK6 along the time course.
(c) Immunokinase assays performed on the same samples. MPK3, MPK4 and MPK6 were immunoprecipitated with specific antibodies and their activity was measured using MBP as a substrate.

and potential antimicrobial action (Zipfel *et al.*, 2004). To gain insight into the role of MKK1 in the regulation of these genes, we compared flg22-induced changes in wild-type and *mkk1* mutant seedlings. Ten-day-old seedlings (stage 1.02 according to Boyes *et al.*, 2001) were treated for 30 min with 1 μM flg22, and RNA was extracted and hybridized to full-genome *Arabidopsis thaliana* ATH1 chips (Affymetrix, Santa Clara, CA, USA).

Analysing the data from wild-type Col-0 plants showed that we could closely reproduce published results on early gene expression changes induced by flagellin treatment on young seedlings of Ler-0 ecotype (Zipfel *et al.*, 2004; Table S1). We then compared wild-type and *mkk1* mutant plants in the absence of flagellin (Table S2). Surprisingly, although *mkk1* plants do not show any morphological phenotypes, we found that the expression of a large number of genes representing a wide spectrum of functional categories was affected by the *MKK1* mutation. To delineate the role of MKK1 in flagellin response, we decided to focus on MKK1-dependent genes that changed in expression on flagellin treatment (Table S3). These genes belonged to various classes, including resistance-associated genes, signalling components (receptor kinases, transcription factors), and genes relating to cell wall structure and modification. We selected some representative genes from both flg22-repressed and flg22-induced categories, and performed quantitative RT–PCR analysis on RNA samples from wild-type versus *mkk1* mutant plants in control and flg22-treated conditions (Figure 5). A number of WRKY-type transcription factors are known to be induced by flagellin, including WRKY22 and WRKY29, which were placed downstream of MPK3 and MPK6 (Asai *et al.*, 2002). We found WRKY22, WRKY40 and WRKY53 to be strongly upregulated by flagellin treatment, as reported previously (Asai *et al.*, 2002; Navarro *et al.*, 2004; Zipfel *et al.*, 2004), and their expression was induced to slightly higher levels in *mkk1* plants. The MAP kinase MPK3, also known to be transcriptionally induced by flg22, was induced to comparable levels in Col-0 and *mkk1* (Figure 5a), consistent with the presence of similar amounts of MPK3 protein in mutant versus wild-type plants (Figure 4b). Among flagellin-induced genes, we tested the expression of three oxidative stress-related genes: two peroxidases (At5g39580 and At5g64120) and a blue copper-binding protein (At5g20230). These three genes showed more than 10-fold induction on flagellin application in Col-0 plants; in *mkk1* plants their expression level was much higher in control conditions compared with the wild type, and their expression was still induced by the treatment (Figure 5b). Similarly, we found three cell wall-related genes, encoding a pectinesterase (At4g02330), an extensin-like protein (At4g22470) and a xyloglucan endotransglycosylase (At4g25810), to be expressed at more than fivefold higher levels in *mkk1* plants (Figure 5b). Rapid repression of auxin-related genes occurs on flagellin treatment, possibly contributing to the growth inhibition observed in flg22-treated seedlings (Gomez-Gomez *et al.*, 1999; Navarro *et al.*, 2004, 2006). Three flagellin-repressed auxin-response genes (At2g21210, At4g38840, At4g38860) were expressed to significantly lower levels in *mkk1* compared with the wild type in control conditions, and their expression was decreased further on flagellin application (Figure 5c). A xylosidase (At5g49360) and an expansin (At1g69530) had a similar expression pattern, having lower expression in mutant plants and being further repressed by the elicitor (Figure 5c). The microarray data combined with the real time RT–PCR experiments indicated that MKK1 negatively regulates the expression of flagellin-responsive genes.

mkk1 *mutant plants are more susceptible to pathogen infection*

To assess whether impaired flagellin activation of MAPKs and deregulated gene expression in *mkk1* plants affect their resistance to pathogens, we studied their sensitivity to virulent and avirulent isolates of *P. syringae* pv. *tomato* by

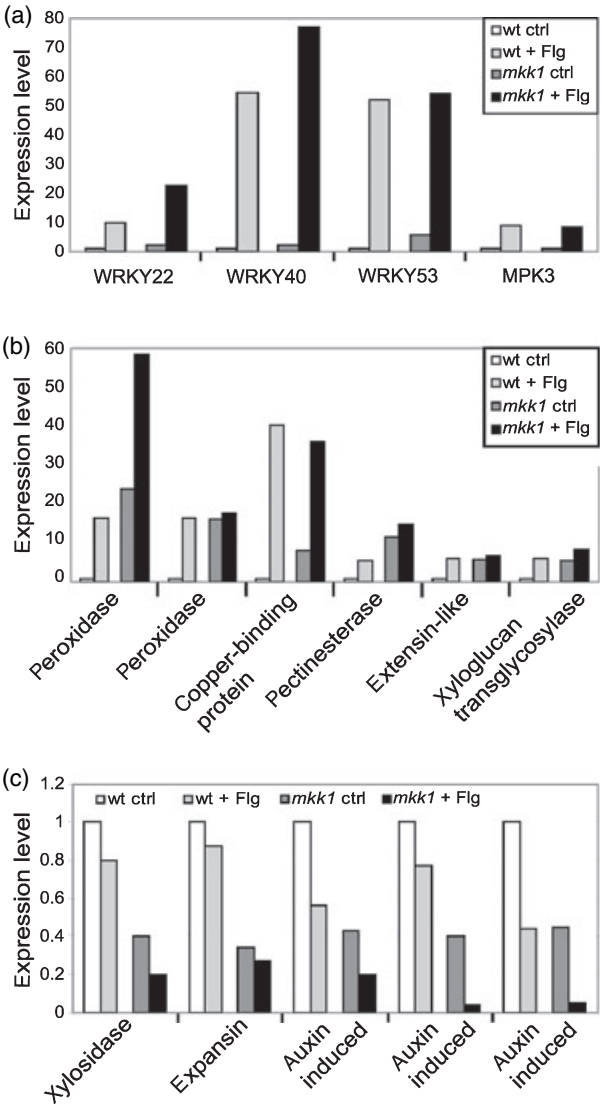

Figure 5. Quantitative RT–PCR analysis of flagellin-responsive genes.
Ten-day-old seedlings of Col-0 and *mkk1* were treated with flagellin for 30 min and RNA was extracted for microarray hybridization. Based on the microarray data, flagellin-responsive genes with altered expression in *mkk1* plants compared with Col-0 were identified and their expression tested using quantitative RT–PCR. Some other known flagellin-induced genes were tested to confirm that they were expressed normally in the absence of MKK1.
(a) Flagellin-induced genes, expression of which in *mkk1* is similar to wild-type.
(b) Flagellin-induced genes, expression of which is upregulated in *mkk1* in untreated conditions, and further upregulated on flagellin treatment.
(c) Flagellin-repressed genes, expression of which is downregulated in *mkk1* in control conditions, and further repressed by flagellin application.

measuring bacterial growth in infected leaves of soil-grown plants. By 3 days post-inoculation (dpi) with the virulent *P. s. tomato* strain DC3000, *mkk1* leaves contained an approximately 10-fold greater titre of bacteria than Col-0 control leaves (Figure 6a), suggesting that MKK1 plays a role in maintaining basal resistance. Inoculation with avirulent *P. s. tomato* strains expressing *AvrRpm1*, *AvrRpt2* or *AvrPphB* also resulted in significantly higher bacterial titres in the leaves of *mkk1* plants compared with the wild type (Figure 6b–d). This shows that race-specific resistance mechanisms are also compromised in *mkk1* plants, or that the defective basal resistance renders them more sensitive to avirulent pathogens. Both basal and race-specific resistance rely on the formation of ROS (Torres and Dangl, 2005). Therefore, we measured the production of ROS in wild-type and *mkk1* plants on flagellin application. We did not observe any difference, which indicates that the hypersensitivity to bacterial infection in the mutant plants is not caused by impaired production of ROS (Figure 6e,f).

Discussion

A MAPK module was delineated downstream of the FLS2 receptor in experiments using transient overexpression of various signalling components, and was proposed to consist of MEKK1, MKK4/5 and MPK3/6, which eventually activate the WRKY22 and WRKY29 transcription factors and presumably further downstream target genes (Asai *et al.*, 2002). In this study, we provided evidence for the existence of a parallel or alternative signalling module that transmits signal from flagellin elicitation and incorporates MKK1 and MPK4. Our conclusions are based on experiments showing the activation of endogenous MPK4 in addition to MPK3 and MPK6 on flagellin treatment. We confirm that MPK3 and MPK6 are activated when MKK4 is overexpressed, and show that MKK1 specifically activates MPK4. Furthermore, we find that the endogenous MKK1 is activated by flagellin and phosphorylates MPK4, while in the *mkk1* mutant the activation in response to flagellin of MPK4, as well as of MPK3 and MPK6, is compromised. Finally, we found that *mkk1* mutant plants are more sensitive to both virulent and avirulent bacterial pathogen strains.

As both upstream MKK-activating MEKKs and downstream MAPK targets considerably outnumber the MKKs, it is commonly believed that MKKs are convergence points within MAPK signalling in plants, while MEKKs and MAPKs provide specificity to upstream signals and downstream responses, respectively. Contradictory to this notion is the fact that very different signals can activate the same set of MAPKs, most commonly MPK3, MPK4 and MPK6 in Arabidopsis (Nakagami *et al.*, 2005). Likewise, the alfalfa MAPK MMK3 or its tobacco orthologue Ntf6 were implicated in cytokinesis, oxidative stress signalling and pathogenesis (Calderini *et al.*, 1998; Cardinale *et al.*, 2000; Nakagami *et al.*, 2004; del Pozo *et al.*, 2004). How such distinct signals are then transmitted by these MAPKs to lead to specific responses is not known. Similarly, although few MEKKs among the numerous existing in plants have been studied so far, several were proposed to have multiple roles. For instance AtANP1 and its tobacco orthologue NPK1 are

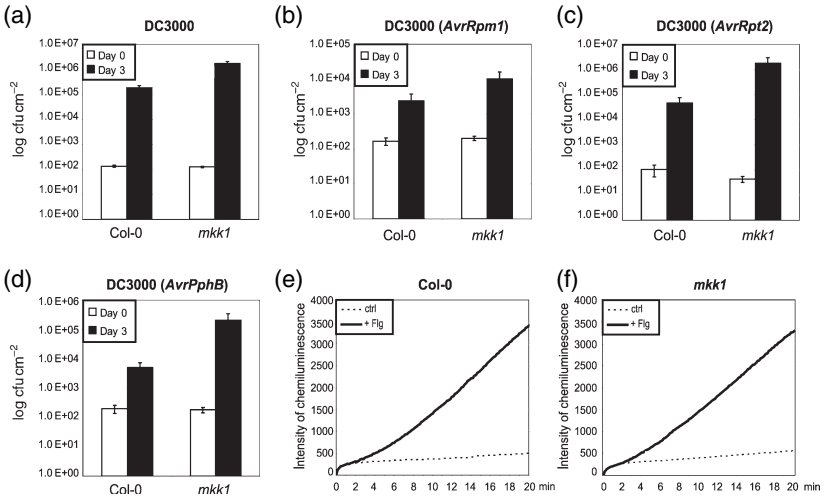

Figure 6. *mkk1* mutant plants display enhanced sensitivity to *Pseudomonas syringae* pv. *tomato*. Four-week-old plants were inoculated with various strains of *P. s. pv. tomato*, and leaf discs were harvested at 0 and 3 dpi. Each strain was tested at least twice. Bacterial growth is expressed as colony-forming units (cfu) cm^{-2}. The inoculum used was 10^5 cfu ml^{-1}, a concentration that does not lead to the appearance of HR-like lesions on the leaves within 3 days of inoculation.
(a–d) Inoculation with: (a) virulent *P. s. tomato*; (b) avirulent *P. s. tomato* expressing *AvrRpm1*; (c) avirulent *P. s. tomato* expressing *AvrRpt2*; (d) avirulent *P. s. tomato* expressing *AvrPphB*. Detached leaves of 6-week-old Col-0 and *mkk1* plants were treated with flagellin and the production of ROS was monitored for 20 min after elicitor application, using a luminol-based assay.
(e,f) Measurement of ROS production in (e) Col-0; (f) *mkk1*.

involved in regulating cytokinesis, oxidative stress and N-gene-mediated resistance (Jin *et al.*, 2002; Kovtun *et al.*, 2000; Soyano *et al.*, 2003). While MEKKs and MAPKs are used flexibly in multiple signalling events, MKKs in plants appear to be more stringent in function. MKK1 and MKK2 have been allocated to the same MAPK module, and are also the most similar in sequence among the Arabidopsis MKKs (62% identity); however, several experiments indicate that they differ in function and thus could provide specificity to the module. MKK1 and MKK2 have selective interactions with MPK4 and MPK6, and distinct potential to suppress yeast MAPK-signalling mutations (Mizoguchi *et al.*, 1998; Teige *et al.*, 2004). We found that endogenous MKK1 activated by flagellin treatment phosphorylated MPK4 *in vitro* and, to a lesser extent, MPK6. However, in our transient expression experiments we did not observe *in vivo* activation of MPK6 by overexpressed MKK1, suggesting that MKK1 and MPK6 are not part of an *in vivo* complex, although MPK6 can be a substrate for MKK1 to a certain extent *in vitro*. Correspondingly, MKK1 was also found to phosphorylate specifically MPK4, but not MPK3 and MPK6, in response to H_2O_2 (Teige *et al.*, 2004). According to previous additional biochemical and genetic data, MKK2 is specifically activated by salt and cold, while MKK1 is activated by pathogenic elicitors and H_2O_2; *mkk2* mutant is hypersensitive to salt and cold stress, while we find *mkk1* mutant to be compromised in pathogen resistance (Teige *et al.*, 2004). These results suggest that MKKs might be able to channel signalling by selective interactions with upstream activators and downstream MAPKs.

Although MKK1 was reported previously to be an upstream activator for MPK4, and this is largely confirmed by our present results, knockout mutations for MKK1 and MPK4 genes yield very different phenotypes. While *mpk4* knockout was severely dwarfed and pathogen-resistant, *mkk1* plants show no obvious growth defect, but are more sensitive for pathogen attack. Furthermore, biochemical analysis of flagellin-treated *mpk4* and *mkk1* seedlings revealed opposite changes in kinase activation in comparison with wild-type plants. In contrast to the impaired activation of MPK3, MPK4 and MPK6 in *mkk1* mutant plants, *mpk4* seedlings showed elevated MPK3 and MPK6 kinase activity (Figure S4 and S5). Our results are in conflict with those reported by Droillard *et al.* (2004), who found in a single time point that MPK3 and MPK6 are activated comparably in wild-type and *mpk4* mutant plants in response to flg22 and hypo-osmolarity. Increased MPK3 and MPK6 activities in the *mpk4* mutant are in agreement with a positive role for MPK3/6 and a repressor role for MPK4 in pathogen response, as reported (Asai *et al.*, 2002; Menke *et al.*, 2004; Petersen *et al.*, 2000). The results of our *mkk1* study appear to be in conflict with these data, but could be explained by a possible scaffolding function of Arabidopsis MEKK1. In budding yeast, the Ste11 MAPKKK participates in two different MAPK pathways; tethering Ste11 to the appropriate scaffold protein is sufficient to elicit the corresponding signalling pathway (Park *et al.*, 2003). Scaffolding could also control the specificity of plant MAPK modules, although these complexes still await identification. A recent publication showed that the closest known alfalfa homologue of MEKK1, OMTK1 directly inter-

acts with the MAPK MMK3, suggesting a possible scaffolding function for OMTK1. The truncation of the N-terminal regulatory domain of OMTK1 weakens the MAPK binding and compromises the capacity of OMTK1 to trigger cell death, underlining the importance of the regulatory domain (Nakagami *et al.*, 2004). We have not studied the MAPKKK layer of the MAPK module, but it was shown previously that MEKK1 interacts specifically through its C-terminal kinase domain with MKK1 and MKK2, while its N-terminal regulatory domain allows interaction with MPK4 (Ichimura *et al.*, 1998). Moreover, MKK1 specifically interacts with, and phosphorylates, MPK4 (Teige *et al.*, 2004). Thus, the catalytic interactions between MEKK1 and MKK1, and between MKK1 and MPK4, as well as the scaffolding function of the MEKK1 regulatory domain for MPK4, are consistent with the existence of a flagellin-induced MAPK module comprising MEKK1-MKK1 and MPK4. We find that in the *mkk1* mutant a number of early flagellin-induced genes are upregulated, consistent with other reports placing the MPK4 pathway as a negative regulator of pathogen response (Andreasson *et al.*, 2005; Petersen *et al.*, 2000).

The scaffolding would provide an insulated flg22-responsive MEKK1–MKK1–MPK4-signalling pathway. However, we know that in response to a number of biotic and abiotic stress stimuli, MPK4 is simultaneously activated with MPK3 and MPK6 (Droillard *et al.*, 2004) or with MPK6 (Desikan *et al.*, 2001; Ichimura *et al.*, 2000). We also find that, in the *mkk1* mutant, the activation not only of MPK4, but also of MPK3 and MPK6 is compromised in response to flg22. In the *mkk2* mutant, MPK6 activation is also lowered in response to cold (Teige *et al.*, 2004). What couples these two pathways together? MKK2 was suggested to activate both MPK6 and MPK4 in response to abiotic stresses, while in the yeast two-hybrid assay, MKK1 specifically interacted with MPK4 but not the other Arabidopsis MAPKs (Teige *et al.*, 2004). Furthermore, MKK1 appears specifically to activate MPK4, but not MPK6 and MPK3, when overexpressed in transfected protoplasts, and MKK1 cannot phosphorylate MPK3 *in vitro*, suggesting that the MPK4 and MPK3/6 pathways are separate. That these pathways are independent and can be uncoupled under certain conditions is also indicated by their different activation kinetics and different sensitivities to pharmacological perturbations, for example, their disruption by MEK inhibitors (Desikan *et al.*, 2001; Ichimura *et al.*, 2000). One way in which these pathways might be coupled is by sharing the upstream MAP3K, MEKK1, but with their activation kept sequential (Figure 7a). As we discussed, MEKK1 tethers MPK4 and MKK1 through its N- and C-terminal domains, respectively. This would prevent the association of MKK4/5 until MEKK1 is activated and dissociates the MKK1–MPK4 complex. In the *mkk1* mutant the inactive MPK4 might remain associated with MEKK1, thus blocking the activation of the MKK4/5–MPK3/6 pathway. Conversely, in the *mpk4* mutant the coupled activation mechanism would be lost, and MKK4/5 could freely associate, leading to inappropriately high MPK3/6 activation by flg22. Although this model can be reconciled with all currently available experimental evidence, it is unusual that a MAP3K, through its scaffolding function, couples two functionally distinct MAPK-signalling pathways, because scaffolds are meant to insulate rather than connect pathways. MEKK1 was described as the upstream activator for MKK4/5–MPK3/6 based on overexpression of a truncated form lacking the regulatory domain where MPK4 binds (Asai *et al.*, 2002). This truncated form could have an altered function, and might inappropriately activate downstream signalling events. Whether MKK4/5, and consequently MPK3/6, are then activated via MEKK1 or by an as-yet unknown MAP3K is not known, but could be addressed by analysing MPK3/6 activities in *mekk1* mutant plants.

If two distinct MAP3Ks regulate these pathways, what connects them, and what can account for the apparent contradiction that two components of the same pathway, MKK1 and MPK4, have opposing effects on the activity of MPK3/6 within the other pathway and on the pathogen response of the plant? The overexpression of a kinase-inactive mutant form of MEKK1 was shown to lower the

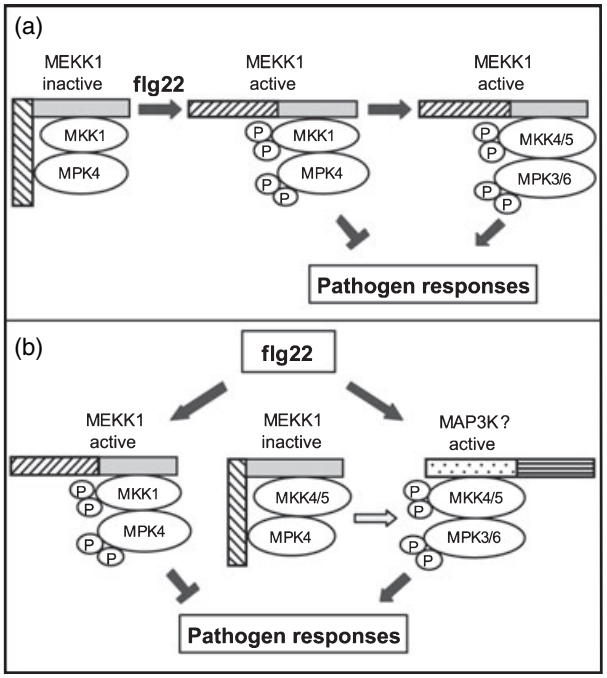

Figure 7. Proposed alternative working models for flagellin-induced MAPK signalling.
(a) Sequential model: activation of the two flg22-induced signalling pathways is coupled and sequential because they share the same MAP3K, MEKK1 that binds to MPK4, and restricts the activation of the other pathway until MKK1 and MPK4 are dissociated.
(b) Trapping model: the MEKK1–MPK4 complex restricts availability of MKK4/5 by trapping it in an inactive complex, thus MKK4/5 release and MPK3/6 activation depend on the presence and activity of MEKK1, MKK1 and MPK4.

activation of flagellin-responsive genes, and thus is able to interact with and block MKK4/5 activation (Asai *et al.*, 2002). This could provide an alternative model to explain how the two pathways are coupled (Figure 7b). Perhaps MEKK1 when bound to MPK4 also associates with MKK4/5, but as MKK4/5 is unable to activate MPK4, this complex would become trapped. The release of MKK4/5 would thus depend on the activity of MKK1 and on the presence of MPK4. This model would also explain the higher MPK3/6 activation in the *mpk4* mutant and the interdependence of the two pathways.

Both models depict two functions for MEKK1 and MPK4: they are part of a signalling cascade with MKK1 to repress pathogen-responsive gene expression, and they negatively regulate MKK4/5–MPK3/6, a pathway known to induce a number of pathogen responses. Although MPK4 is a central part of a flexible scaffolding module, we believe that it also directly mediates responses to stress stimuli by phosphorylating its target proteins. Indeed, the phenotype of the *mpk4* knockout mutant could not be rescued by complementation using a kinase-inactive version of MPK4 (Petersen *et al.*, 2000).

The MKK4/5–MPK3/6 pathway was shown to activate the expression of flagellin-responsive genes through the WRKY22/29 transcription factors (Asai *et al.*, 2002), while the MPK4 interacting proteins MKS1 and WRKY25/33 are part of a mechanism for repression of defence signalling (Andreasson *et al.*, 2005). Why do these two opposing pathogen-responsive pathways coexist? Both models implement the default state as the repression of pathogen response by the MPK4 pathway, which is rapidly tilted towards triggering the defence responses through the interconnection of the MPK4 and MPK3/6 pathways. Signal-dependent protein stability and chromatin remodelling were suggested to regulate the switch from gene repression to activation on pathogen elicitation (Andreasson *et al.*, 2005; Navarro *et al.*, 2004).

Interestingly, comparing gene expression between the *mkk1* mutant and the MKK2 overexpressor indicated an interchanging potential for MKK1 and MKK2. All three auxin-responsive genes studied were downregulated in both backgrounds, while the cell wall-related xyloglucan endotransglycosylase and pectinesterase, the WRKY22 and WRKY40 genes and the blue copper-binding protein gene were upregulated (Teige *et al.*, 2004). Similar changes in the expression of certain genes could indicate redirection of signalling modules relying on these two MKKs. As endogenous MKK1 protein levels are low, it might be that overexpressed MKK2 inadequately replaces MKK1, therefore preventing the formation of a functional MKK1 complex and, to some extent, mimicking the *mkk1* mutant in which MKK2 might substitute for MKK1. According to this interpretation, the observed changes in expression level could not be attributed distinctly to MKK1 or MKK2, illustrating the drawbacks of overexpressing and knockout mutant analysis.

We observed no constitutive expression of late PR genes in *mkk1* plants, such as genes encoding PR-1 or plant defensin protein (PDF1.4), but these were upregulated in the *mpk4* mutant. However, some early flagellin-responsive genes were upregulated, such as chitinases, disease-related proteins and receptor-like kinases (Table S3). Transcriptional analysis based on microarrays was performed on *mpk4* plants and revealed a surprisingly low number of genes affected by the mutation (Andreasson *et al.*, 2005; Petersen *et al.*, 2000). These data cannot be compared strictly with our experiments as they were performed on 18-day-old plants grown in soil, allowing possible exposure to various environmental stresses, while we used 10-day-old seedlings grown *in vitro*. It is worth noting, however, that both mutants showed upregulation of genes relating to cell wall synthesis or modification. The plant cell wall is not a simple physical barrier to pathogen invasion, but is also an important sensor and integrator of biotic stress (Vorwerk *et al.*, 2004). Surprisingly, a number of cell wall mutants are resistant to pathogens. A *pmr6* mutant, defective in a pectate lyase-like gene, is resistant to powdery mildew, independently of SA and of the ability to perceive JA or ET (Vogel *et al.*, 2002). In contrast, the resistance of a *cev1* cellulase mutant was attributed to constitutive JA signalling (Ellis *et al.*, 2002), while the callose synthesis mutant *pmr4* had SA-dependent disease resistance (Nishimura *et al.*, 2003). On the other hand, *mpk4* mutant bacterial resistance was dependent on SA (Petersen *et al.*, 2000). The *mkk1* mutant is hypersensitive to bacterial infection and does not appear to have elevated SA or JA levels, as none of the SA and JA responsive genes was found to be upregulated in our microarray data. Changes in cell wall composition can therefore result in different outcomes in terms of pathogen susceptibility, and the observed defect in pathogen resistance in *mkk1* plants might relate to alteration of the cell wall composition.

Flagellin and other elicitors are known to induce ROS production, and oxidative stress was previously reported to activate MAPK pathways (Kovtun *et al.*, 2000; Nakagami *et al.*, 2004; Teige *et al.*, 2004). Our measurements also demonstrated rapid increase of ROS in response to flagellin. Considering the ROS production, *mkk1* seedlings are indistinguishable from wild-type ones, suggesting that MKK1 functions downstream or independently of ROS. The respiratory burst oxidase homologue (*Rboh*) gene family plays an important role in ROS production in response to pathogen attack (Torres *et al.*, 2002). We have found that MAPK activation on flagellin treatment still occurs in an *atrbohd/f* mutant background (T.M., A.H. and L.B., unpublished data), indicating that RbohD/F-dependent ROS production might not be required. Similarly to

Pep13-induced activation of parsley MAP kinases (Kroj *et al.*, 2003), flg22 activation of MPK3, MPK4 and MPK6 in Arabidopsis might not rely on ROS signalling.

Our results show that MKK1 is a regulator of pathogen responses and modulates the activity of the previously reported parallel pathway comprising MKK4 and MKK5. Modularity and cross-talk between the numerous plant MAPK-signalling components have become increasingly evident over recent years. We now face the challenge of assigning them functions as components of intricate networks, not as separated signal transducers.

Methods

Cell culture, protoplasting and transfection

Arabidopsis cell-suspension culture was maintained as described (Mathur and Koncz, 1998). Protoplast isolation and polyethylene glycol-mediated transfection were performed as described by Anthony *et al.* (2004). MPK vector constructs were described by Kovtun *et al.* (2000). MKK and RNAi constructs were based on pK2GW7 and pK7GWIWG2(I) Gateway plasmids for overexpression and silencing, respectively (Karimi *et al.*, 2005). For transfection, 5 μg of each plasmid DNA was transfected into 4×10^5 protoplasts, which were cultured for 16 h before treatment and harvesting. The elicitor peptide flg22 was synthesized by Sigma Genosys (Sigma, St. Louis, MO, USA) and added in solution to cells or protoplasts to a final concentration of 500 nM.

Plant material

All Arabidopsis plants used in this study were of the Columbia (Col-0) ecotype, except for the *mpk4* mutant, which is of the Lansdberg (Ler) background. The *mkk1* mutant was identified by screening a T-DNA tagged population using gene- and T-DNA-specific primers (Rios *et al.*, 2002). The T-DNA insertion site was determined by DNA sequencing and found to be located in the second intron. Southern analysis confirmed the presence of a single insertion. Lines putatively homozygous for the T-DNA insertion were subjected to RT–PCR analysis and Southern blotting.

Plant treatments

After 48 h vernalization, seeds were sown on half-strength Murashige and Skoog medium (Duchefa M0255) supplemented with 1% sucrose, and grown under short-day condition (8 h light, 16 h dark at 22°C). Nine days after germination, seedlings at stage 1.02 were transferred to liquid germination medium (12 seedlings per 2 ml medium in 24-well plates). At 16 h after transfer the medium was supplied with flg22 peptide to a final concentration of 1 μM. The RNA samples for microarray analysis were produced from seedlings collected from different plates and treated independently before pooling to account for biological variation.

Antibody production

Polyclonal rabbit MAPK antibodies were raised against Imject Maleimide activated mcKLH (Pierce, Rockford, IL, USA)-coupled C-terminal amino acids of AtMPK3 (CALNPTYG), AtMPK4 (CETVKFNPQDSV) and AtMPK6 (CAHPYLNS). Cross-reactivity and specificity of the sera were tested by Western blot analysis using AtMPK3, AtMPK4 and AtMPK6 recombinant proteins expressed in *Escherichia coli*, and Arabidopsis crude protein extracts. The sera were precipitated with 40% $(NH_4)_2SO_4$, and pellets were dissolved in PBS and stored at −70°C. Cross-reactivity of the MKK1 aD-15 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was tested by immunoblotting using AtMKK1 and AtMKK2 recombinant proteins expressed in *E. coli*.

In vitro *kinase assays*

Protein extracts were prepared either from protoplasts by sonication in 100 μl Lacus buffer or from frozen cells/seedlings in 150 μl Lacus buffer with quartz sand as described by Bogre *et al.* (1999). In-gel kinase assays were carried out according to Shibuya *et al.* (1992) with modifications. Briefly, 25 μg total protein were fractionated on a 10% SDS–PAGE gel containing 0.5 mg ml^{-1} bovine brain MBP (Sigma). SDS was removed by three 20-min washes in 50 mM Tris pH 8.0, 20% 2-propanol and one wash in buffer A (50 mM Tris pH 8.0, 5 mM DTT). Proteins were denatured in 6 M guanidine hydrochloride, 20 mM DTT, 50 mM Tris–HCl pH 8.0 for 1 h at room temperature. Kinase renaturation was achieved overnight at 4°C in buffer A containing 0.04% Tween 40 with five changes of buffer. The phosphorylation assay was performed in 10 ml kinase reaction (50 mM HEPES–HCl pH 8.0, 2 mM DTT, 20 mM $MgCl_2$, 0.1 mM EGTA, 30 μM ATP and 50 μCi γ-^{32}P-ATP) in a sealed plastic bag at room temperature for 1 h. The gel was washed at room temperature for at least 5 h in 10% (w/v) trichloroacetic acid, 1% sodium pyrophosphate before drying and autoradiography. Immunocomplex kinase assays were performed as described by Meskiene *et al.* (2003). Samples were analysed by 15% acrylamide SDS–PAGE and subsequent autoradiography.

For measurement of MKK activity on purified MPKs, the cloning and expression of inactive GST-MPKs is described by Teige *et al.* (2004). A commercial MKK1 aD-15 antibody (Santa Cruz Biotechnology) was used for immunoprecipitation of endogenous MKK1 from 100 μg total protein extract, and 3 μg kinase-inactive MPK3, MPK4 and MPK6 were used as substrate.

Pathogen infection tests and ROS measurements

Maintenance of pathogens and pathogen assays were performed as described previously (Aarts *et al.*, 1998). Measurement of ROS was essentially as described by Gomez-Gomez *et al.* (1999). Briefly, leaf discs were cut into strips and incubated on water overnight. The solution was exchanged for 100 μl water containing 10 g ml^{-1} horseradish peroxidase, and 50 μM Luminol with and without 1 μM flg22. Real-time luminescence data were acquired using an intensified CCD camera (model HRPCS3) with a 16-mm F1.4 C mount lens and the manufacturer's software package IFS32, all from Photek Ltd (St Leonards on Sea, UK).

RNA isolation and quantitative RT–PCR

Treated seedlings were snap-frozen in liquid nitrogen. RNA was isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Concentration and purity of RNA were determined by measuring optical density at 260 and 280 nm.

cDNA was produced from 1 μg purified RNA using the RETROscript kit (Ambion, Austin, TX, USA) with provided random decamer

primers, following the manufacturer's two-step RT–PCR protocol. The cDNA solution was diluted eight times and 4 μl were used for each RT–PCR, except for At5g64120 and At5g20230, where double this amount was used. The PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) in 20 μl volume with 0.5 μM primer concentration. The following primers were used to detect target gene transcripts: At2g21210: 5′-GAGATTCGTGGTTCCAGTGA-3′ and 5′-AGAGGCGAGATCGATGAAGA-3′; At4g38840: 5′-AAGCAGATTCTCCGACAAG-3′ and 5′-GAAGGCTGGTCCAAGTACGA-3′, At4g38860: 5′-ACAATGCTACGACGAGGAA-3′ and 5′-TTCCTCTGCTTGTTGAAGAA-3′; At4g02330: 5′-CTTTTGCTGTTACATCGCCG-3′ and 5′-AAGCTTCGAAACTGCAACTA-3′; At5g39580: 5′-CTCTTGTTGGAGGAGGACAC-3′ and 5′-AGATCAACACGTGCTGATCC-3′, At5g64120: 5′-AAGGAACAGGCTGGCAAGTA-3′ and 5′-GTGTCCTCCGACGAGGACG-3′; At4g22470: 5′-AGCCATGTTGTTCCATCGTA-3′ and 5′-CTGCGACCACAAACCTTAAA-3′; At4g25810: 5′-TGGGAAACCTAAGTGGTGAT-3′ and 5′-CGGAGAATATGATGCGTTGT-3′; At1g69530: 5′-TGTCGTCACAGCCACAAAC-3′ and 5′-ACGGCACTCTTCGGTAAGC-3′; At5g20230: 5′-GCGACGAGCTCGAATTTGA-3′ and 5′-TGTGGTCCAGTGGTGTTTAG-3′; At4g23810: 5′-CCGAGCGTACAACTTATTCC-3′ and 5′-TGATGACTCTCGCTAGAACC-3′; At5g60390: 5′-GTGTTGTAACAAGATGGATGCC-3′ and 5′-CAGTCAAGGTTGGTTGACCTC-3′; At3g45640: 5′-GGAATCGTTTGCTCTGTGTTG-3′ and 5′-CATGATCAAGATGACGAAGAAGC-3′; At4g01250: 5′-CCATATCCAAGAGGATACTACAG-3′ and 5′-TGTCGGAGCTGGATGATTAT-3′; At5g49360: 5′-GTCTCAATGGGTACATTGTCTC-3′ and 5′-CTTCACTGCACCTTCCGTG-3′; At1g80840: 5′-AAGATCCACCGACAAGTGC-3′ and 5′-CCTCTCGGTTATGTTGCTCTTG-3′. The recommended PCR program for the iCycler machine was followed with an annealing temperature of 55°C. PCR efficiency was calculated using the DART program (Peirson *et al.*, 2003) and ratios of gene expression were determined with Genex (Bio-Rad, Hercules, CA, USA) using the At5g60390 transcript level for normalization.

Acknowledgements

The work in the laboratory of L.B. was supported by BBSRC and the EU–RTN project ACCY. T.M. was supported by Marie Curie Individual Fellowship HPMF-CT-2000-01119 and Marie Curie Reintegration Grant MERG-CT-2004-513623. S.C.P. and L.S. were supported by the Gatsby Charitable Foundation. We would like to thank Dr Jane Parker for sharing the *Pseudomonas* strains and Professor J. Sheen for providing the MPK constructs.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Specificity of the anti-MKK1 antibody.

Figure S2-3. Decreased activation of MPK3, MPK4 and MPK6 in response to flg22 in the *mkk1* mutant.

Figure S4-5. Increased MPK3 and MPK6 activation in response to flg22 in the *mpk4* mutant.

Table S1 Comparison of gene expression changes induced by flagellin (30 min treatment) between Zipfel *et al.* (2004) and this study

Table S2 Genes differentially expressed in Col-0 versus *mkk1* plants

Table S3 Flagellin-responsive genes differentially expressed in *mkk1* plants

Appendix S1 (Supplementary Method). Transcriptional analysis of flagellin response in wild-type and *mkk1* mutant plants.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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