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

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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
products are known to share a number of signalling components downstream of PAMPs and avr factors (Navarro et al., 2004). Moreover, signalling events downstream of PAMPs and avr factors trigger the well known hypersensitive local cell death (Martin et al., 2000). 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 biotrophic 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 MKK1–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$_2$O$_2$ specifically MPK4 but
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 M KK1 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 M KK1 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). In mkk1 seedlings, surprisingly the activity of these kinases decreased to near basal level after 20 min, and remained low until 60 min (Figure 4a, right panel). These results show that the endogenous MKK1 is activated in vivo by flg22 elicitation and confirms that its preferred substrate is MPK4.
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
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 increased 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 xylanase (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
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
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$_2$O$_2$ (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$_2$O$_2$; 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 MAPKK 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 (Andreason 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 mkk4 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 MAPK3/6 activities in mkk1 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
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 interacting potential for MKK1 and MKK2. All three auxin-responsive genes studied were downregulated in both backgrounds, while the cell wall-related xylglucan 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 and MKK2 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., 2002). For transfection, 5 pg of each plasmid DNA was transfected into 4 x 10^6 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 Landsberg (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 isolated using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) 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 (CETVFKNPDDSV) and AtMPK6 (CAHYLYNS). 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% (NH₄)₂SO₄, 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 pg 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 x 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 MgCl₂, 0.1 mM EGTA, 30 μM ATP and 50 μCi γ⁻³²P-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. (2000). 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 HPVCS3) 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 At1g564120 and At1g20230, 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'-ACCGACAAGTGC-3' and 5'-CATGATCAAGATGACGAAGAAGC-3'; At1g69530: 5'-ACCTAAGTGGTGAT-3' and 5'-TGTTGAAGAA-3'; At4g23840: 5'-AAGGCTGGTCCAAGTACA-3' and 5'-GGAATCGTTTGCTCTG-3'; At1g80840: 5'-AAGATCC-3' and 5'-GATGATTAT-3'. Specificity of the anti-MKK1 antibody.

The recommended PCR program for the iCycler machine was a pre-incubation of 15 min at 95°C; 40 cycles of denaturation (15 s at 95°C), annealing (30 s at 60°C), and extension (1 min at 72°C); and a final elongation of 10 min at 72°C. 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'-ACCGACAAGTGC-3' and 5'-CATGATCAAGATGACGAAGAAGC-3'; At1g69530: 5'-ACCTAAGTGGTGAT-3' and 5'-TGTTGAAGAA-3'; At4g23840: 5'-AAGGCTGGTCCAAGTACA-3' and 5'-GGAATCGTTTGCTCTG-3'; At1g80840: 5'-AAGATCC-3' and 5'-GATGATTAT-3'. Specificity of the anti-MKK1 antibody.

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

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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.


