

Running head: MKK1 and MKK2 mediated defense signalling in Arabidopsis

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***Arabidopsis* MAP Kinase Kinases MKK1 and MKK2 have overlapping functions in defense signalling mediated by MEKK1, MPK4 and MKS1**

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Abstract

The *Arabidopsis thaliana* MKK1 and MKK2 MAP kinase kinases have been implicated in biotic and abiotic stress responses as part of a signalling cascade including MEKK1 and MPK4. Here, the double loss-of-function mutant (*mkk1/2*) of *MKK1* and *MKK2* is shown to have marked phenotypes in development and disease resistance similar to those of the single *mek1* and *mpk4* mutants. Since *mkk1* or *mkk2* single mutants appear wild type, basal levels of MPK4 activity are not impaired in them, and MKK1 and MKK2 are in part functionally redundant in unchallenged plants. These findings are confirmed and extended by biochemical and molecular analyses implicating the kinases in jasmonate- and salicylate-dependent defense responses, mediated in part via the MPK4 substrate MKS1. In addition, transcriptome analyses delineate overlapping and specific effects of the kinases on global gene expression patterns demonstrating both redundant and unique functions for MKK1 and MKK2.

Introduction

Eukaryotic mitogen-activated protein kinase (MPK) cascades act downstream of receptors or sensors to transduce extracellular stimuli, including abiotic and biotic stresses, into adaptive, intracellular responses. The basic assembly of a MPK cascade is a three-kinase module.

MPK, the last kinase in the cascade, is activated by dual phosphorylation of Thr and Tyr residues in its kinase catalytic activation loop. This phosphorylation is mediated by a MPK kinase (MPKK or MEK), which is activated by a MPKK kinase (MPKKK or MEKK).

Multiple members for each of these three tiers of kinases may be expressed in a cell. This may contribute to the specificity of transmitted signals, and permit the integration of signals due to interactions between a kinase of one tier with more than one kinase of another tier (Madhani and Fink, 1998). DNA-binding transcription factors are targets of MPK cascades whose activities are directly or indirectly regulated by MPK phosphorylation. MPK cascades may thereby regulate gene expression in response to extracellular signals. In plants, MAP kinase signalling has been associated *inter alia* with abiotic stress (Ichimura et al., 2000; Teige et al., 2004; Alzwi and Morris, 2007) and with basal and systemic acquired resistance (SAR) to pathogens (Asai et al., 2002; Mészáros et al., 2006; Brader et al., 2007; Loake and Grant, 2007; Zhang et al., 2007).

Genetic analyses indicate that *Arabidopsis* MPK4 acts as a negative regulator of salicylic acid (SA)-mediated defense against biotrophic pathogens, as illustrated by enhanced resistance to biotrophs and expression of the *pathogenesis-related 1 (PR1)* gene in *mpk4* loss-of-function mutants. Conversely, MPK4 is required for ethylene (ET)- and jasmonic acid (JA)-mediated defense against necrotrophic pathogens, as typified by enhanced susceptibility to necrotrophs and reduced expression of the antifungal plant *defensin 1.2 (PDF1.2)* gene in *mpk4* mutants (Petersen et al., 2000; Brodersen et al., 2006). MPK4 kinase activity is enhanced by pathogen-derived elicitors (Desikan et al., 2001), and also by abiotic stresses such as cold and wounding (Ichimura et al., 2000).

MPK4 is predominantly localized in nuclei where it interacts with a substrate protein, MKS1. As may be expected for a kinase substrate, *MKS1* overexpression partially phenocopies the *mpk4* mutant (dwarfism, enhanced SA levels and constitutive *PR1* gene expression), while *MKS1* under-expression partially suppresses these *mpk4* mutant phenotypes (Andreasson et al., 2005). MKS1 can be multiply phosphorylated by MPK4 (Caspersen et al., 2007), and may function as an adaptor linking MPK4 activity to the two transcription factors WRKY25 and WRKY33. Both of these factors may be involved in regulating gene expression in response to pathogens: both appear to negatively regulate certain SA-mediated responses while WRKY33 may activate certain ET- and JA-mediated responses (Zheng et al., 2006; Zheng et al., 2007).

Arabidopsis MKK1 (Morris et al., 1997) and MKK2 (Ichimura et al., 1998) both interact with MPK4 in yeast two-hybrid assays (Ichimura et al., 1998; Mizoguchi et al., 1998), and MPK4 can be phosphorylated and activated both *in vitro* and *in vivo* by MKK1 (Huang et al., 2000; Matsuoka et al., 2002). MKK1 and MKK2 have also both been associated with biotic and abiotic stresses in several studies. For example, *MKK1* gene expression is wound-induced (Morris et al., 1997), and transiently expressed MKK1 is activated by pathogen-derived elicitors such as flagellin and laminarin, as well as by hydrogen peroxide (Teige et al., 2004). In addition, an *MKK1* T-DNA insertion mutant exhibits a small reduction in MPK4 activation in response to flagellin treatment, and is slightly more sensitive to infection by *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Mészáros et al., 2006).

Following transient overexpression in protoplasts, MKK2 kinase activity and MPK4 activation have been shown to be enhanced by salt and cold treatment, but not significantly so by elicitor treatment. An *MKK2* T-DNA insertion mutant was found to be hypersensitive to cold and salt treatment, with much reduced MPK4 activity in response to cold stress, whereas plants expressing a constitutively active version of MKK2 showed enhanced cold and salt tolerance (Teige et al., 2004). In addition, these latter plants were found to exhibit enhanced resistance to biotrophic pathogens but compromised resistance to necrotrophs (Brader et al., 2007). Interestingly, the single *mkk1* or *mkk2* mutants do not exhibit the phenotypic characteristics of *mpk4*. This genetic evidence indicates that MKK1 and MKK2 have partly redundant functions in the activation of MPK4, since complete loss of MPK4 kinase activity results in the severe developmental phenotype of the *mpk4* single mutant (Petersen et al., 2000).

Yeast two-hybrid analyses indicated that MPK4, MKK1 and MKK2 also interact with the stress-induced MPKKK, MEKK1 (Ichimura et al., 1998; Mizoguchi et al., 1998), and MEKK1 can activate both MKK1 (Hadiarto et al., 2006) and MKK2 (Teige et al., 2004). Mutations in *MEKK1* (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007) result in plants morphologically similar to *mpk4* mutants including dwarfed growth, enhanced PR1 gene expression and reduced MPK4 activity induction by hydrogen peroxide or flagellin. *mekk1* mutants show some differences to *mpk4* in root phenotype and premature senescence of primary leaves, and the *mekk1-1/mpk4* double mutant has a more severe phenotype than either single mutant, including an absence of root and shoot growth. This suggests that MEKK1 and MPK4 function in more than one pathway, a shared one regulating pathogen responses and (an)other(s) important for seedling development (Su et al., 2007). However, genetic data has not previously been presented to identify which MPKK(s) function(s) between MEKK1 and MPK4.

In order to further investigate the roles of MKK1 and MKK2 in plant stress and disease responses, T-DNA insertion mutants of both genes were isolated and analyzed as single and double mutants. Our data show that the morphological, molecular and defense-related phenotypes of the *mkk1/2* double mutant resemble those of the *mpk4* and *mekk1* single mutants. This indicates that MPK4 is controlled by MEKK1 through the partially redundant activities of MKK1 and MKK2.

RESULTS

The *mkk1/2* double mutant is severely dwarfed

T-DNA insertion mutants of *MKK1* (Salk line 027645) (Alonso et al., 2003) and *MKK2* (SAIL line 511_H 01) (Sessions et al., 2002) were backcrossed to wild type Col-0. Plants homozygous for the insertions were isolated by PCR screening with gene-specific and T-DNA left border primers. Sequence analysis of the left border PCR products showed an insertion 1,332 bp after the initiation ATG of the major ORF in intron 5 of *mkk1*, and at 1,387 bp, also in intron 5, for *mkk2* (Fig. 1A). Since two previous insertion mutations have already been described for *MKK1* (Mészáros et al., 2006; Xing et al., 2007), this new allele may be designated *mkk1-3*, but is referred to simply as *mkk1* below. The *mkk2-1* allele is identical to that analysed by Teige et al., (2004). RT-PCR of RNA from the homozygous *mkk1/mkk2* double mutant (hereafter referred to as *mkk1/2*) mutants did not detect full length transcripts for either *MKK1* or *MKK2* (Fig. 1B). Neither single mutant showed any obvious morphological or developmental abnormality compared to wild type Col-0 plants (Fig. 1C), including flowering time under long or short days, root growth, root hair length and morphology, as well as guard cell spacing and morphology (data not shown). Germination and seedling growth of the single mutants were not significantly different from wild type after treatments with auxin, cytokinin, 1-aminocyclopropane-1-carboxylic acid (ACC), gibberellic or JA, nor was etiolation or de-etiolation affected (data not shown). The *mkk2* mutant, but not *mkk1*, exhibited slightly enhanced ABA and salt sensitivity in germination tests, confirming previous findings (Fig. S1; Teige et al., 2004). In contrast to the single mutants, *mkk1/mkk2* was severely dwarfed with curled, dark green leaves and exhibited early senescence of the cotyledons and the first few true leaves (Fig. 1C).

Wild type *MKK1* or *MKK2* cDNA sequences driven by the CaMV 35S promoter were used to transform wild type-like plants heterozygous for *mkk1* and homozygous for *mkk2*. Of twelve primary transformants shown to contain the *MKK1* cDNA by PCR, five were found to be homozygous for *mkk1/2* and these were all fully wild type in morphology. Similarly, of twelve *MKK2* transformed lines, four were found to be homozygous for *mkk1/2* and also wild type in morphology (Fig. 1D). These results confirm that the dwarfism and premature senescence phenotypes of *mkk1/2* are due to loss of function of both *MKK1* and *MKK2*.

To extend this comparative phenotypic analysis at the molecular level, gene expression profiling was also performed on *mkk1*, *mkk2* and *mkk1/2*. As summarised in a Venn diagram, of the most significantly differentially regulated genes compared to wild type (Fig. 1E and Table SI), most of the changes in transcript levels in the *mkk1/2* double mutant were not

shared by *mkk1* or *mkk2* single mutants. The large overlap between genes differentially regulated in *mkk1* and *mkk2* of almost 30 % (Fig. 1E) indicates that a common function of basal levels of MKK1 or MKK2 is required for wild type expression of these shared genes. In keeping with the morphological phenotypes of the single and double mutants, this indicates that MKK1 and MKK2 have partially redundant functions.

***mkk1/2* and the Defense-related Hormones SA, ET and JA**

Both single *mkk1* and *mkk2* mutants were found to have close to wild type levels of free SA, whereas *mkk1/2* exhibited more than a 20-fold increase in SA levels compared to wild type (Fig. 2A). The *sid2* mutant prevents SA accumulation during the development of SAR (Wildermuth et al., 2001), and the *mkk1/2/sid2* triple mutant showed much reduced levels of SA (Fig. 2A). In keeping with these findings, the dwarf habit, but not the early senescence phenotype, of *mkk1/2* was partially alleviated by *sid2* mutation (Fig 2C). This indicates that the severe growth phenotype of *mkk1/2* is in part dependent upon SA, as is the case both for the *mpk4* (Petersen et al., 2000) and the *mekk1* mutants (Suarez-Rodriguez et al., 2007).

The *mkk1/2* double mutant also superficially resembles the dark green, dwarf phenotype of the constitutive ET response mutant *ctr1* (Kieber et al., 1993), and of plants over-producing ET (Guzman and Ecker, 1990). ET measurements showed that levels produced by *mkk1/2* were some 4-fold higher than in wild type (Fig. 2B). However, several lines of evidence indicate that *mkk1/2* dwarfism and early senescence are not ET-related. First, no plants were found to exhibit a constitutive triple response amongst a large population (>200) segregating for *mkk1/2* doubly homozygous seedlings. Second, although the triple mutants *mkk1/2/ein2* and *mkk1/2/etr1-1* were ET insensitive, as judged by the absence of a triple response when dark-germinated in the presence of the ET precursor ACC, they were morphologically indistinguishable from *mkk1/2* mutants when grown in the light (Fig. 2D). Similar experiments have shown that the morphological phenotype of the *mpk4* mutant is independent of ET signalling (Petersen et al., 2000).

To further compare *mkk1/2* responses with those to SA or ET treatments, gene expression profiling of *mkk1/2* was compared with that of wild type treated with the salicylic acid analogue BTH or the ET precursor ACC. As shown in a Venn diagram (Fig. 2E and Table SII), only 23 genes (4.6%) from the 500 most differentially regulated genes were shared between ACC-treated wild type and *mkk1/2* mutants, which is comparable to that shared between ACC-treated and BTH-treated wild type plants (27 genes, 5.4%). In contrast, 109 genes (21.8%; p -value $\sim 10^{-78}$) were shared by BTH-treated wild type and *mkk1/2* mutants. Significantly, these genes include the ‘classic’ pathogenesis related proteins PR1, PR2, two

PR3-type chitinases, and PR5 (Table SII). A gene ontology analysis of the differentially regulated genes further supports this, as defense related terms in this overlap were over represented (Table SIV). These results indicate that the molecular phenotype of the *mkk1/2* mutant is independent of ET but dependent upon SA and affects, like *mpk4*, the expression of genes required for systemic acquired resistance.

Endogenous JA levels were also determined in leaves of mutant plants. Basal JA levels were slightly higher in *mkk1*, *mkk2* and *mkk1/2* plants in comparison to the wild type. JA levels were also observed to increase after wounding in the single mutants and *mkk1/2* plants, in both wounded leaves and systemically (Fig. S2). Thus wound-induced JA biosynthesis is not impaired, either in the single mutants, or in the *mkk1/2* double mutant.

Real-time PCR was used to examine the expression of the SA- and ET/JA-responsive genes *PR1* and *PDF1.2* in response to treatment with BTH or JA respectively. mRNA of the SA-responsive *PR1* gene (Uknes et al., 1992) was barely detectable in wild type and *mkk1* plants, while *PR1* mRNA levels were slightly raised in *mkk2*, and markedly so in untreated *mkk1/2* double mutants (Fig. 3A). BTH treatment enhanced *PR1* expression to higher levels in all genotypes. As the *mkk1/2* mutant exhibited constitutive *PR1* expression, this suggests that these two genes function redundantly as negative regulators of SAR. However, *PR1* mRNA accumulation was still increased by BTH in *mkk1/2*. This is in keeping with earlier findings that signalling through MPK4 primarily affects basal *PR1* expression levels (Andreasson et al., 2005).

PDF1.2 is an antifungal protein whose expression is induced by JA and ET (Manners et al., 1998). PDF1.2 expression was strongly enhanced by JA in wild type and in *mkk2* plants, but much less so in the *mkk1* and *mkk1/2* backgrounds (Fig. 3B). Given that JA levels are normal in all mutant lines, this suggests that MKK1 is an intermediary in JA signalling. Consistent with this, wounding induced systemic *VSP1* mRNA accumulation in wild type and in *mkk2*, but much less so in *mkk1* and *mkk1/2* plants (Fig. S3). These results indicate that the insensitivity of *mkk1/2* to JA and wounding is mainly due to loss-of-function of *MKK1*.

Disease Responses of *mkk1/2*

The response of both single and double mutants to biotic stress was investigated. *mkk1* and *mkk2* showed no difference to wild type with respect to bacterial growth five days after challenge with a virulent strain of *Pst* DC3000 (Fig. 4A). However, *mkk1/2* and *mpk4* supported significantly less bacterial growth, whereas the positive controls NahG (Ryals et al., 1995) and *atgsnor1-3* (Feechan et al., 2005) showed higher levels of bacterial growth. The

resistance of *mkk1/2* to *Pst*DC3000 can be partly attributed to the enhanced ET production of the double mutant, but more obviously by its SA content since both the *mkk1/2/ein2* (ET insensitive) and the *mkk1/2/sid2* (reduced SA production) triple mutants carried a greater bacterial load than *mkk1/2* alone (Fig. 4B).

The mutants were also challenged with the virulent oomycete pathogen *Hyaloperonospora parasitica* (Noco2). At eight days post inoculation, extensive hyphal growth and oospore production was seen on the wild type and the single mutants, increased fungal growth for NahG plants and *atgsnor1-3* was observed, but very little fungal development on leaves of *mkk1/2* could be detected (Fig. 4C, D). Spore counts on the triple mutants *mkk1/2/ein2* and *mkk1/2/sid2* showed that the enhanced SA level of *mkk1/2* is a major determinant of resistance.

Pst DC3000 is semi-biotrophic and *H. parasitica* is a biotrophic pathogen. Thus, the resistance of *mkk1/2* to the necrotrophic fungal pathogen *Botrytis cinerea* was also tested by quantifying the number of outgrowing lesions following infection. As shown in Fig. 4E, the resistance to *B. cinerea* of *mkk1* and *mkk2* was not significantly different than wild type, whilst *mkk1/2* was statistically significantly more susceptible than wild type.

MKK1 and MKK2 Function Upstream of MPK4

Since the *mkk1/2* double mutant resembles *mpk4* both morphologically and at the molecular level, MPK4 kinase activity was monitored in the single and double MKK mutants. After flagellin elicitor peptide flg22 treatment, MPK4 was strongly activated in the wild type, *mkk1* and *mkk2*. However, the signal for MPK4 activity in the *mkk1/2* double mutant was similar to that of the untreated control, and to *mpk4*; western blotting showed that MPK4 protein levels remained constant after flg22 treatment and in the MKK mutants (Fig. 5A). After treatment with the SA analogue BTH, no difference was seen in MPK4 kinase activity between the wild type and the single *mkk1* mutant. In contrast, neither *mkk2* nor the *mkk1/2* double mutant showed induction in MPK4 activity, although MPK4 levels stayed constant (Fig. 5B). These results indicate that the requirement of MKK1 and MKK2 for the enhancement of MPK4 activity is dependent on the upstream stimulus. In at least one instance both MKKs are required, but in another MKK2 will suffice.

It has been reported that MPK3 and MPK6 activation by flg22 is also modified in an *mkk1* mutant (Mészáros et al. 2006) and that MPK6 activity is reduced in the *mkk2* mutant when cold-stressed (Teige et al. 2004). Therefore MPK3 and MPK6 activity was also monitored in the same samples used for flg22-induced MPK4 activity assays (Fig S5). Both MPK3 and

MPK6 kinase activities were reduced in *mkk1*, *mkk2* and in *mkk1/2* relative to that seen for flg22-treated wildtype plants. However as no significant difference in MPK3 or MPK6 activity was seen between the single and double mutants, it is unlikely that either of these two kinases are involved in the differences in phenotype observed between the single mutants and *mkk1/2*.

Since the *mpk4* mutant is partially rescued by knockdown of the MPK4 substrate MKS1 (Andreasson et al., 2005), an *mks1* knockout mutant (transposon insertion line GT5.108403; J-L. Qiu, unpublished results) was crossed into the *mkk1/2* double mutant. *mkk1/2/mks1* triple mutants selected by genotyping exhibited partial alleviation of the dwarf phenotype, but retained the early senescence phenotype (Fig. 5C). This partial suppression provides genetic evidence confirming that MPK4 and MKS1 act downstream of MKK1 and MKK2.

MEKK1, MKK1/2 and MPK4 Exhibit a Gradient of Phenotypic Severities

The *mkk1/2* double mutant morphologically resembles not only *mpk4* but also the *mekk1* mutant (Fig. 6A), in that both *mekk1* and *mkk1/2* are severely dwarfed, show premature senescence of cotyledons and leaves, have stubby root hairs, and are practicably infertile when grown at 24°C although *mkk1/2* pollen was viable when used in crosses. Both *mkk1/2* dwarfism and infertility were partially rescued by growth at temperatures above 30°C (Fig. S4A), as has been reported for *mekk1* and *mpk4* (Petersen et al., 2000; Andreasson et al., 2005; Ichimura et al., 2006; Nakagami et al., 2006; Su et al., 2007; Suarez-Rodriguez et al., 2007). However, the dwarf phenotype of *mekk1* is more pronounced than that of *mkk1/2* and, in contrast to *mekk1*, the root length and branching pattern of *mkk1/2* appeared normal, as did that of *mpk4* (Figs. S4B and C). Thus, our data appears to show that mutations in genes encoding proteins that are biochemically linked in signalling cascades show decreasing complexities of phenotype ‘down’ through the pathway.

This relationship was further assessed by comparative analysis of differentially regulated genes among *mekk1*, *mkk1/2*, and *mpk4* (Fig. 6B and Table SIII). This revealed that more than 10% of genes overlap for all three mutants, while 20-30% of genes are shared by any two of the mutants. Significantly, the same *PR1*, *PR3* chitinase and *PR5* genes with shared differential expression in *mkk1/2* and BTH treatment (Fig. 2E) were among the 54 genes differentially expressed in the three mutants. In addition, three enzymes implicated in synthesis of the phytoalexin camalexin (*PAD3*, *CYP71A13* and *CYP79B2*; Nafisi et al., 2007), and three genes involved in the synthesis or maintenance of SA (*ICS1*, *EDS1* and *PAD4*) are shared by *mekk1* and *mpk4* and are also up-regulated in *mkk1/2* although they are not among the 500 most significantly differentially regulated genes in *mkk1/2*. Gene ontology analysis of

the overlapping genes in *mkk1/2*, *mekk1* and *mpk4* revealed ‘systemic acquired resistance’ to be the most significantly over represented term among many other terms implicated in defense response (Table SIV). Thus, these data confirm the strong phenotypic harmony of the mutants and indicate that they are all involved in signalling defense responses despite the distinct transcriptome signatures of each mutant.

To further assess the relationships between *mkk1*, *mkk2* and *mkk1/2* relative to other mutants, their global transcriptional profiles were compared to those of other mutants as described previously (Nielsen et al., 2007). To this end, the gene expression signature of each mutant, consisting of their 1200 most significantly differentially expressed genes versus wild type, was compared to a compendium of 44 public, mutant transcriptome profiles (Nielsen et al., 2007). Figure 6C illustrates these comparisons as a graphic, wherein the thickness of the lines (edges) denotes the relative strengths of the intersections between transcriptional signatures of the mutants. The *mkk1* and *mkk2* single mutants both have moderate transcriptional phenotypes and do not associate strongly to other mutants. In contrast, the transcriptional phenotype of the *mkk1/2* double mutant deviates strongly from wild type and Fig. 6C shows that their signatures intersect strongly with those of the *mekk1*, *cpr5* and *mpk4* mutants containing 468, 419 and 365 genes, respectively. With respect to the MPK4 substrate MKS1, the intersection is weaker for both *mkk1/2* (225 genes) and *mekk1* (274 genes) than for *mpk4* (454 genes). This is consistent with the function of MKS1 as a direct MPK4 substrate.

DISCUSSION

The *Arabidopsis* MAP kinase MPK4 has been reported to function as a negative regulator of SA-dependent systemic acquired resistance (Petersen et al., 2000), as a positive regulator of ET and JA mediated defences (Brodersen et al., 2005), and has also been implicated in responses to abiotic stress (Teige et al., 2005). Biochemical and yeast two-hybrid evidence suggests that MPK4 is part of signalling cascades including the MAP kinase kinases MKK1 and MKK2 and the MAP kinase kinase kinase MEKK1 (Ichimura et al., 1998; Ichimura et al., 2006). Here, we present genetic, molecular and biochemical evidence confirming this model. In addition, the results presented show that each kinase has specific functions in regulating other defence-related, morphological and developmental processes.

A key genetic observation is that while the *mkk1* and *mkk2* single mutants appear morphologically normal, the *mkk1/2* double mutant is similar to the *mpk4* mutant: both *i.* are dark green dwarves with curled leaves, *ii.* constitutively overproduce SA and exhibit enhanced resistance to biotrophic pathogens and insensitivity to JA, and *iii.* have enhanced basal expression of *PR1* and other SA-dependent defense genes. Importantly, the *mkk1/2* phenotype is partially complemented by loss-of-function of the MPK4 substrate MKS1, indicating that MKS1 lies downstream of MPK4, MKK1 and MKK2. In addition, the *mkk1/2* double mutant also resembles the *mekk1* mutant (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007), as both are more severely dwarfed than *mpk4*, both are infertile, have curled, prematurely senescing leaves and stubby root hairs, and both can be in part rescued by high temperature. However, *mekk1* is even more severely dwarfed than is *mkk1/2*, has a greater incidence of short root hairs and exhibits much reduced root branching, not seen in *mkk1/2* or *mpk4* (Nakagami et al., 2006; Su et al., 2007). Thus, the order of severity of morphological phenotypes is *mekk1* > *mkk1/2* > *mpk4*, which supports their proposed functional order in a signalling cascade, consistent with a hypothesis that other pathways branch off at each level of signalling, although other mechanisms might also explain these phenotypic differences.

The morphological similarities between *mekk1*, *mkk1/2* and *mpk4* mutants are mirrored in their transcriptome profiles. While the single *mkk1* and *mkk2* mutants have modest transcriptional phenotypes relative to wild type plants, *mkk1/2* exhibits a gene expression signature more similar to both *mekk1* and *mpk4*. This close relationship is confirmed by comparison of the transcript profiles with those of more than 40 other mutants and transgenics. In this analysis, *mekk1*, *mkk1/2*, *mpk4* and transgenics overexpressing MKS1 associate closely. Interestingly, although both are wild type in appearance, the transcriptome

profiles of the single *mkk1* and *mkk2* mutants differ significantly, as they share less than 30 % of genes differentially expressed versus wild type. This provides molecular evidence that MKK1 and MKK2 have both overlapping and specific functions.

These genetic and transcriptional observations concur with biochemical and physiological data presented here and previously. Although MPK4 activity in untreated wild type Col-0 is barely detectable by *in vitro* kinase assays, the *mpk4* mutant shows a pronounced phenotype. This phenotype cannot be complemented by expressing an inactive (kinase-dead) MPK4 transgene in *mpk4*, indicating that a basal level of MPK4 activity is necessary to maintain the wild type phenotype (Petersen et al., 2000). Since the *mkk1* and *mkk2* mutants appear morphologically wild type, this indicates that in these single mutants, basal level MPK4 activity is not impaired, and that in the unchallenged condition MKK1 and MKK2 are at least in part functionally redundant. This is supported by the finding that for *mkk1/2*, SA levels are high, and there is enhanced resistance to the biotrophs *Pst* DC3000 and *H. parasitica*. Consistent with MPK4's role as a positive regulator of ET and JA mediated defences, *mkk1/2* is less resistant to the necrotrophic pathogen *B. cinerea*. Since both the *mkk1* single mutant and *mkk1/2* were found to be less sensitive to JA, but *mkk1* was not significantly more susceptible to *B. cinerea* than the wild type, this suggests that resistance to *B. cinerea* is mediated by factors other than just by JA.

As well as maintaining ground-state MPK4 activity, the data presented here show that MKK1 and MKK2 are both involved in the enhancement of MPK4 activity by elicitor treatment. Treatment with the flg22 elicitor results in strong MPK4 activation in wild type, *mkk1* and *mkk2*, but not in *mkk1/2*. In this case, both MKK1 and MKK2 appear to be required to activate MPK4. In contrast, BTH treatment resulted in MPK4 activation in wild type and in *mkk1*, but not in *mkk2*. This suggests that different stimuli can induce MPK4 activation by different degrees of upstream activation of MKK1 and/or MKK2. This hypothesis might explain why a small reduction in MPK4 activity at certain time points in flagellin-treated *mkk1* mutants was noted by Mészáros et al., (2006), since only 1 μ M flg22 was used in their experiments whereas 10 μ M flg22 was used here. As treatment with the flg22 elicitor does not activate MPK4 in *mekk1* (Suarez-Rodriguez et al., 2007), this is consistent with MEKK1 functioning upstream of both MKK1 and MKK2.

As noted above, phenotypic complexity appears reduced in mutants at each level of the MPK4 signalling pathway, which suggests that other signalling pathways branch off at these points. This is also indicated by analysis of triple mutants, as a knockout of the gene encoding the MPK4 substrate MKS1 only rescues the dwarf phenotype, not the early senescence

phenotype. Thus, MPK4 affects only one subset of a pathway downstream of MEKK1, MKK1 and MKK2. Similarly, the *sid2* mutant only partially complements *mkk1/2*, as the triple *mkk1/2/sid2* mutant is still relatively small and exhibits early senescence. This indicates that MKK1 and MKK2 affect signalling pathways independently of MPK4 and SA, and is consistent with our findings that BTH can still induce *PR1* expression in the *mkk1/2* and *mpk4* mutants, indicating that MKK1, MKK2 and MPK4 are not the sole players regulating SA-mediated plant defense (Fig. 3A). Evidence for distinct functions for MKK1 and MKK2, some of which may be associated with MPK4 activation, but others of which might be linked to activation of alternative MPKs, is also presented here and elsewhere (Teige et al., 2004; Mészáros et al., 2006; Brader et al., 2007; Xing et al., 2007). For example, the *mkk1* mutant exhibited JA insensitivity, a trait shared by *mpk4* but not by *mkk2*. In contrast to *mkk1*, the *mkk2* mutant showed salt and ABA insensitivity, traits not shared with *mpk4*, which may be associated with the activation of MPK6 by MKK2.

There are interesting parallels, but also some differences, in the regulation of defence signalling in other species such as *Nicotiana tabacum*. Biotic and abiotic stress in this tobacco will activate SIPK and WIPK, orthologs of *Arabidopsis* MPK3 and MPK6 respectively, resulting in JA accumulation and hypersensitive responses (Yang et al., 2001). Plants in which *SIPK* or *WIPK* are silenced show reduced wound-induced JA production, and SA levels after wounding are enhanced (Seo et al., 2007). This is in contrast to the situation in *Arabidopsis* in which JA levels are not affected in the mutants studied here, whereas SA levels are enhanced in *mkk1/2* in the unchallenged state. The tobacco ortholog of MPK4 is NtMPK4, which is also activated by wounding and is required for expression of JA-responsive genes (Gomi et al., 2005). As in *Arabidopsis*, stress-related MAP kinase activation in tobacco involves multiple upstream regulators; SIPKK will activate NtMPK4 and SIPK, but not WIPK (Gomi et al., 2005) whereas MEK2 will activate both SIPK and WIPK (Yang et al., 2001).

In summary, several lines of evidence provided here strongly support a pathway including MEKK1, MKK1, MKK2 and MPK4 in defense against pathogens. Some 60 MAPKKs, but only 10 MKKs and 20 MAPKs, are predicted to be encoded in the *Arabidopsis* genome (Ichimura et al., 2002). While it therefore seems likely that multiple MPKs can be activated by one MKK, it is more surprising to find that a single MAPK is regulated by more than one MKK. This might be explained if different combinations of MKKs maintain the specificity of different stimuli to certain responses. While such signalling specificity may be effected in individual cells, it is also likely that different cell type and temporal expression or activation

patterns of the three tiers of kinases contribute to the integration of signalling specificities within plants.

MATERIALS AND METHODS

Plant Materials and Treatments

Plants were grown in a controlled environment room at 22°C constant temperature and a 16 h light, 8 h dark photoperiod (200 mE m² s⁻¹). For treatment prior to RT-PCR or immunoprecipitation, plants were sprayed with BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester, (Bion 50WG, 50 % active ingredient)), or methyl jasmonate (Sigma) as 100 µM solutions in water. *Pseudomonas syringae* pv *tomato* strain DC3000 inoculations were conducted by spraying four-week old plants with virulent bacteria at 6 x 10⁸ cfu per ml and assaying for bacterial growth after 5 days. *Hyaloperonospora parasitica* (Noco2) inoculations were conducted spraying with conidia at 4 x 10⁴ per ml and conidial production was assayed after 10 days. *Botrytis cinerea* infections were performed as described by Bessire et al., (2007). Treatments with plant growth substances during germination and wounding of leaves were as described previously (Alzwi and Morris, 2007).

T-DNA Insertional Mutants, Crosses and Complementation

T-DNA insertion mutants were for *mkk1* (At4g26070) (Salk line 027645; (Alonso et al., 2003) and *mkk2* (At4g29810) (SAIL line 511_H 01; (Sessions et al., 2002). Mutants were back-crossed to Columbia (Col-0) and homozygous mutants isolated from the F2 progeny by means of PCR analysis using gene-specific and T-DNA specific primers. These were as follows:

MKK1-126 5'-GGAAGCTTATGCCCTAATCCC-3'

MKK1-1022 5'-CACCATTGCGAGATGAAGGAG-3'

PROK-LB 5'-GCGTGGACCGCTTGCTGCAACT-3'

MKK2-2 5'-GCCAAGAACGAAGTAGACGAA-3'

MKK2-1889 5'-GAAGTAGGACGCGAGATTGAT-3'

Garlic-LB 5'-TAGCATCTGAATTTTCATAACCAATCTCGATACAC-3'

MKK1 and *MKK2* are closely linked; to isolate the double mutant, F2 plants were screened on kanamycin to select the *MKK1* specific T-DNA, and 200 resistant seedlings treated with Basta to select for the *MKK2* specific T-DNA. Two doubly resistant plants were found and

the *mkk1/2* genotype confirmed by PCR analysis. These plants were heterozygous for *mkk1*, and segregated out homozygous *mkk1/mkk2* plants in the F3 in an approximate 1:3 ratio.

Triple mutants were produced by using wild type looking plants heterozygous for *mkk1* and homozygous for *mkk2*. The ethylene insensitive *mkk1/2/ein2* and *mkk1/2/etr1-1* mutants were selected by germination of the F2 on ACC-containing media, selection of ethylene-insensitive seedlings and PCR analysis for *mkk1/2*. The *mkk1/2/sid2* mutants were selected by PCR analysis using the following *SID2* specific primers:

SID2-F 5'-ACATCTATATTCTCAATTGGC-3'

SID2-R 5'-CCAATAGGTCCCGCATAACAT-3'

The *mkk1/mkk2/msk1* triple mutant was also selected for by PCR analysis using the following *msk1* specific primers:

MKS1gt-For 5'-GGTACATTGATAGGAGGAAGACTTGGAAC-3'

MKS1gt-Rev 5'-TCCTTCCGATCAACAGAACCAGAAG-3'

DS1 5'-GTTTTTCGTTTCCGTCCCGCAAG-3'

The *mkk1/2* mutant was complemented by transforming plants heterozygous for *mkk1* and homozygous for *mkk2* with constructs based on pGPTV-HPT (Becker et al., 1992) and bearing CaMV 35S promoter-driven cDNAs for *MKK1* or *MKK2*, and selecting for transformants with hygromycin. Transformed plants were analysed by PCR.

SA, ET and JA Measurements

ET was measured by gas chromatography as described by Locke et al., (2000). SA and JA were measured by GC-MS as described by Engelberth et al., (2003) and Lee et al., (2004), using propyl paraben and dihydrojasmonic acid as internal standards.

RNA isolation, RT-PCR and real-time RT-PCR

Total RNA was extracted by Tri-Reagent based RiboPure Kit (Ambion, Austin, TX, USA). RT-PCR was performed as before (Qiu et al., 2002) using the following primers:

MKK1-F 5'-AGCGAGTTCTTCGAGGTCTTTG-3'

MKK1-R 5'-AGCAAGTGGGGGAATCAAAGATC-3'

MKK2-F 5'-CATCCCTGACTCCTATCTTTCTG-3'

MKK2-R 5'-CGATCCTGCATCTGTGAAGTAG-3'

ACT7-F 5'-GTGAGGATATTCAGCCACTTGTCTG-3'

ACT7-R 5'-TGTGAGATCCCGACCCGCAAGATC-3'

For quantitative PCR analysis RNA samples were first treated with RQ1 DNase (Promega, Madison, WI, USA). Quantitative RT-PCR was performed using the Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) with 10 pmol of each primer and approximately 100 ng total RNA in a 20 µl reaction. Reactions were run on icycler IQ (Bio-Rad, Hercules, CA, USA). Quantitative PCR reactions were performed in triplicate for each individual line and quantification of the threshold cycle (CT) values obtained by quantitative PCR analysis was achieved by calculating means of normalized expressions using the Q-gene software (Muller et al. 2002). Primers for RT PCR were as follows:

ACT2-F 5'-GGTAACATTGTGCTCAGTGGTGG-3'

ACT2-R 5'-AACGACCTTAATCTTCATGCTGC-3'

PR1-F 5'-GTGGGTTAGCGAGAAGGCTA-3'

PR1-R 5'-ACTTTGGCACATCCGAGTCT-3'

PDF1.2-F 5'-TGGTGGAAGCACAGAAGTTG-3'

PDF1.2-R 5'-GATCCATGTTTGGCTCCTTC-3'

Northern blotting with Digoxigenin labelled *VSP* probes was carried out as described by Morris and Alzwy (2007).

Immunoprecipitation and Western blotting

Two mg total protein extract in immunoprecipitation buffer (40mM Tris/HCl, pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 10mM β-glycerophosphate, 0.5% Triton, 0.5% NP-40, 1mM DTT, phosphatase inhibitor cocktail and protease inhibitor cocktail from Roche) were

incubated with 3 µg antibodies for 3 hours at 4°C followed by precipitation with protein A–Sepharose CL-4B or protein G-agarose beads (GE-healthcare) for 2 hours. After washing the beads four times with immunoprecipitation buffer, the proteins were eluted by boiling in 40µl Laemmli loading buffer for 10 min. Samples were subsequently processed by Western blotting, using the One-Step IP-Western kit from Genscript (Piscataway, NJ 08854, USA), following the protocol described by the manufacturer.

***In Vitro* Kinase Assay**

Arabidopsis plants were treated with 10 µM flg22 and 100 µM BTH for 30 min. Total proteins were extracted and immunoprecipitated with anti-MPK3, anti-MPK4 and anti-MPK6 (Sigma) as described above, and kinase assays were performed as previously described (Caspersen et al., 2007) by combining immunoprecipitations with 5µg MBP, 3 µCi [³²P] ATP and kinase assay buffer (50 mM ATP, 80 mM Tris/HCl, pH 7.5, 4 mM EGTA, 120 mM MgCl₂, 4 mM Na₃VO₄, 4 mM DTT), incubated with shaking for 30 min at 30 °C. The reactions were stopped by adding Laemmli loading buffer and heating at 100°C for 10 min. The resultant supernatants were resolved on 12% SDS-PAGE gels, and dried gels were exposed using phosphoimaging screens.

Microarray and Data Analysis

Total RNA was extracted from three independent batches of plants using Trizol Reagent (Ambion, Austin, TX, USA). From each batch of wild type (Col-0) and mutants *mkk1*, *mkk2* and *mkk1/2*, whole plants were harvested before and 24 hours after spraying with 100 µM BTH. Total RNA from the resulting 12 samples were amplified and labelled using the MessageAmp™ II-Biotin Enhanced Kit (Ambion). 15 µg of RNA for each sample was fragmented, labeled and hybridized to Affymetrix ATH1 GeneChips according to the standard Affymetrix protocol. The microarray data was pre-processed by RMA (Irizarry et al., 2003a; Irizarry et al., 2003b), and annotated according to the Arabidopsis genome TAIR7 version (released April 11, 2007). Venn diagrams were generated with the top 500 genes sorted by p-value (*mkk1*, *mkk2*, *mkk1/2*, *mpk4* and BTH treatment) or fold change (*mek1*). Gene expression signatures consisting of the top most significant genes as assessed by *t*-statistics vs. wild type (Col-0) plants or extracted from the FARO-compendium generated by Nielsen et al. (2007) were generated for the Venn diagrams (top 500 genes) in Figures 1D, 2E and 6B, and the FARO graph (top 1209) in Figure 6C. The FARO genotype graph (Figure 6C) includes gene expression signatures from *mkk1*, *mkk2* and *mkk1/2* and a subset the FARO-compendium, consisting of all mutants and overexpressors (*n*=47, of which 5 exist as

duplicates assayed by two independent laboratories). The graph was drawn using Cytoscape 2.5.1 (Shannon et al., 2003). Only gene expression signature intersections exceeding 250 genes are drawn. The thickness of the edges indicates the size (in number of common genes) of the intersection. The nodes have been manually placed in respect to the intersection sizes. All the microarray data are deposited at NCBI GEO with the accession number GSE10646 (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE10646). GO analysis was done using the BINGO plug-in (Maere et al., 2005) for Cytoscape (Shannon et al., 2003).

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Figure Legends

Figure 1. Mutant phenotypes. A) Gene structure of *MKK1* and *MKK2*. Exons are represented by black boxes, and introns and untranslated regions by black lines. Triangles indicate T-DNA insertions; the T-DNA was located 1,332 bp downstream of the translational initiation codon in *mkk1*, and 1,387 bp downstream of the initiation codon in *mkk2*. Primers used for RT-PCR in B are indicated by arrows. B) *MKK1* and *MKK2* mRNA levels in wild type, *mkk1* and *mkk2* detected by RT-PCR. C) Morphology of Col-0 wild type, *mkk1*, *mkk2*, and the *mkk1/2* double mutants. D) Complementation of *mkk1/2* by CaMV 35S promoter driven-*MKK1* or *MKK2* cDNA. E) Venn diagram of global gene expression signatures of *mkk1*, *mkk2* and *mkk1/2* mutants compared to wild type. Here intersections are between top-500 most significantly differentially expressed genes as measured by the Affymetrix GeneChip, ATH1 (22810 probe sets). If at random, 11 genes are expected to overlap between any two gene sets, and ~0,2 genes between 3 gene sets.

Figure 2. Hormone levels and growth phenotypes of *mkk1/2/sid2* and *mkk1/2/ein2* triple mutants. A) Free salicylic acid and B) ethylene levels. Error bars represent standard error of the mean. C) Phenotypes of *mkk1/2/sid2* and D) *mkk1/2/ein2* triple mutants. E) Venn diagram showing overlapping gene expression signatures between BTH- or ACC-treated wild type plants and the *mkk1/2* double mutant. Overlap is between the top-500 most significantly differentially expressed genes as measured by the ATH1 Affymetrix GeneChip.

Figure 3. Defense gene expression in the mutants. A) *PR1* and B) *PDF1.2* mRNA levels were monitored by real-time RT-PCR after treatment of the mutants with BTH or jasmonic acid respectively. Means \pm SD are shown.

Figure 4. Disease resistance in mutants. A) Response to infection by *Pseudomonas syringae* of wild type Col-0, *mkk1*, *mkk2* and *mkk1/2* compared to *nahG* transgenic. B) Response to *P. syringae* of *mkk1/2* compared to *mkk1/2/ein2* and *mkk1/2/sid2* triple mutants, *atgsnor1-3* and *mpk4*. C) Growth of *Hyaloperonospora parasitica* detected by trypan blue staining and D) corresponding conidial counts per mg tissue. E) Growth of *Botrytis cinerea* in mutants assayed as the number of outgrowing lesions developed 3 days after inoculation. Experiments were repeated three times with similar results and error bars represent standard error of the means.

Figure 5. MKK1 and MKK2 function upstream of MPK4. A) and B) MPK4 kinase activity in single and double mutants. MPK4 protein was immunoprecipitated out of total protein extracts from plants treated for 30 minutes with 10 μ M flg22 (A) or 100 μ M BTH (B) and

used in an in-gel kinase assay with MBP as substrate. Duplicate gels were subjected to western blotting to monitor MPK4 protein levels. C) Phenotype of *mkk1/2/mks1* triple mutant in comparison to *mkk1/2* and wild type.

Figure 6. MEKK1, MKK1/2 and MPK4 function in the same pathway. A) Morphological phenotypes of Ler wild type, *mekk1*, *mkk1/2* and *mpk4*. B) Venn diagram showing overlapping gene expression changes in *mekk1*, *mkk1/2* and *mpk4*. Overlap is between the top-500 most significantly differentially expressed genes as measured by the ATH1 Affymetrix GeneChip. C) 'Functional Association(s) by Response Overlap' (FARO) genotype graph, illustrating the intersections between gene expression signatures of a series of mutants and overexpressors. The thickness of the edges illustrates the size of the intersections between transcriptional signatures of the mutants (nodes). Highlighted in red are *mkk1*, *mkk2*, *mkk1/mkk2*, *mekk1*, *mpk4* and the MKS1 overexpressor. Only response signature intersections exceeding 250 genes are drawn. Mutants assayed multiple times by independent laboratories (i.e. *det2*, *ein2*, *ga1*, *zat12 oex.* and *lfy12*), are represented by a node for each assay.

Supplemental Figure Legends

Figure S1, The *mkk2* mutant shows enhanced salt and ABA sensitivity. Percentage germination after one week on media supplemented with NaCl or abscisic acid (ABA) of Col-0 wild type, *mkk1* and *mkk2* mutants. Error bars indicate standard error of the mean.

Figure S2, Jasmonic acid content of wild type, *mkk1*, *mkk2* and *mkk1/2* in control, local, and systemic leaves 2 hours after wounding.

Figure S3. Northern blot of *VSP1* gene expression in wild type, *mkk1*, *mkk2* and *mkk1/2* in control, local and systemic leaves 2 hours after wounding.

Figure S4. Morphological phenotypes of mutants. A, *mkk1/2* plants grown at normal or high temperature. B, root morphology of vertically grown two weeks old seedlings; 1) Col-0, 2) *mpk4*, 3) *mkk1/2*. C, root hair morphology of *mpk4* and *mkk1/2* (200 X magnification, bar = 100 μ m)

Figure S5. MPK3 and MPK6 kinase activity in wild type, *mkk1*, *mkk2* and *mkk1/2* backgrounds. MPK3 and MPK6 protein was immunoprecipitated out of total protein extracts

from plants treated for 30 minutes with 10 μ M flg22 and used in an in-gel kinase assay with MBP as substrate. Duplicate gels were subjected to western blotting to monitor MPK3 and MPK6 protein levels.

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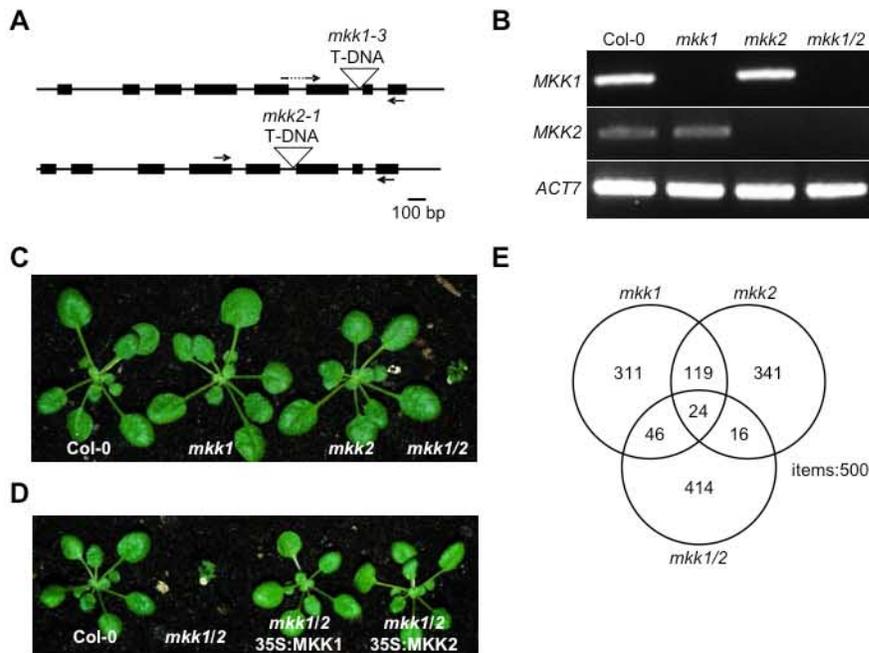


Figure 1. Mutant phenotypes. A) Gene structure of *MKK1* and *MKK2*. Exons are represented by black boxes, and introns and untranslated regions by black lines. Triangles indicate T-DNA insertions; the T-DNA was located 1,332 bp downstream of the translational initiation codon in *mkk1*, and 1,387 bp downstream of the initiation codon in *mkk2*. Primers used for RT-PCR in B are indicated by arrows. B) *MKK1* and *MKK2* mRNA levels in wild type, *mkk1* and *mkk2* detected by RT-PCR. C) Morphology of Col-0 wild type, *mkk1*, *mkk2*, and the *mkk1/2* double mutants. D) Complementation of *mkk1/2* by CaMV 35S promoter driven-*MKK1* or *MKK2* cDNA. E) Venn diagram of global gene expression signatures of *mkk1*, *mkk2* and *mkk1/2* mutants compared to wild type. Here intersections are between top-500 most significantly differentially expressed genes as measured by the Affymetrix GeneChip, ATH1 (22810 probe sets). If at random, 11 genes are expected to overlap between any two gene sets, and ~0,2 genes between 3 gene sets.

Fig 2

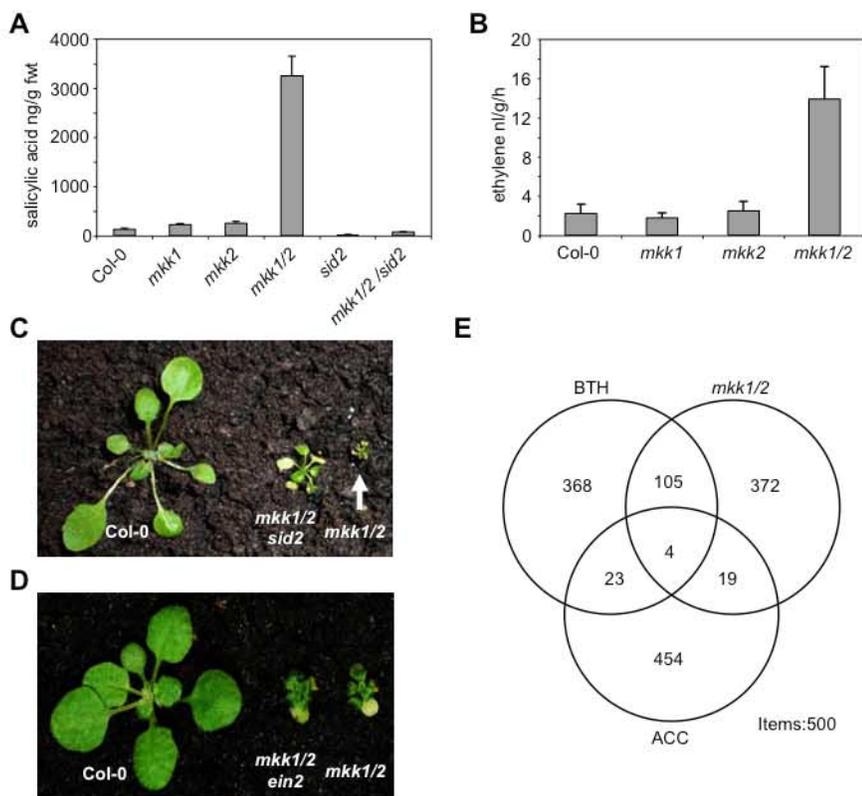


Figure 2. Hormone levels and growth phenotypes of *mkk1/2/sid2* and *mkk1/2/ein2* triple mutants. A) Free salicylic acid and B) ethylene levels. Error bars represent standard error of the mean. C) Phenotypes of *mkk1/2/sid2* and D) *mkk1/2/ein2* triple mutants. E) Venn diagram showing overlapping gene expression signatures between BTH- or ACC-treated wild type plants and the *mkk1/2* double mutant. Overlap is between the top-500 most significantly differentially expressed genes as measured by the ATH1 Affymetrix GeneChip.

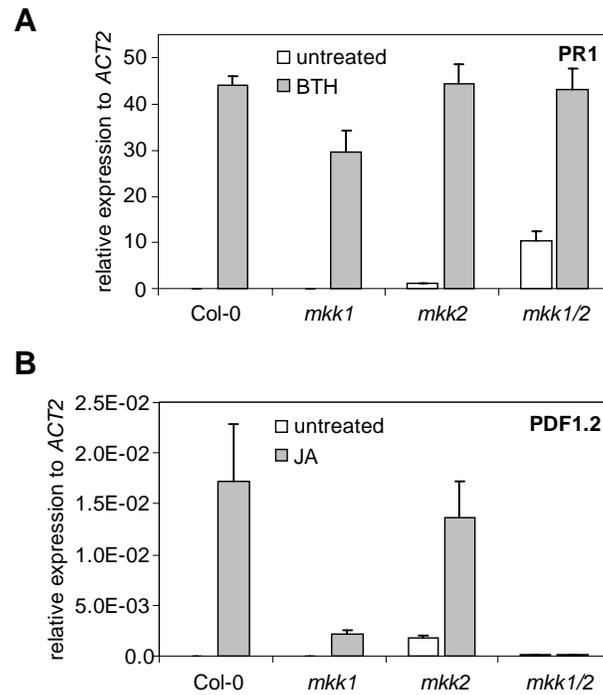


Figure 3. Defense gene expression in the mutants. A) *PR1* and B) *PDF1.2* mRNA levels were monitored by real-time PCR after treatment of the mutants with BTH or jasmonic acid respectively. Error bars represent standard error of the mean.

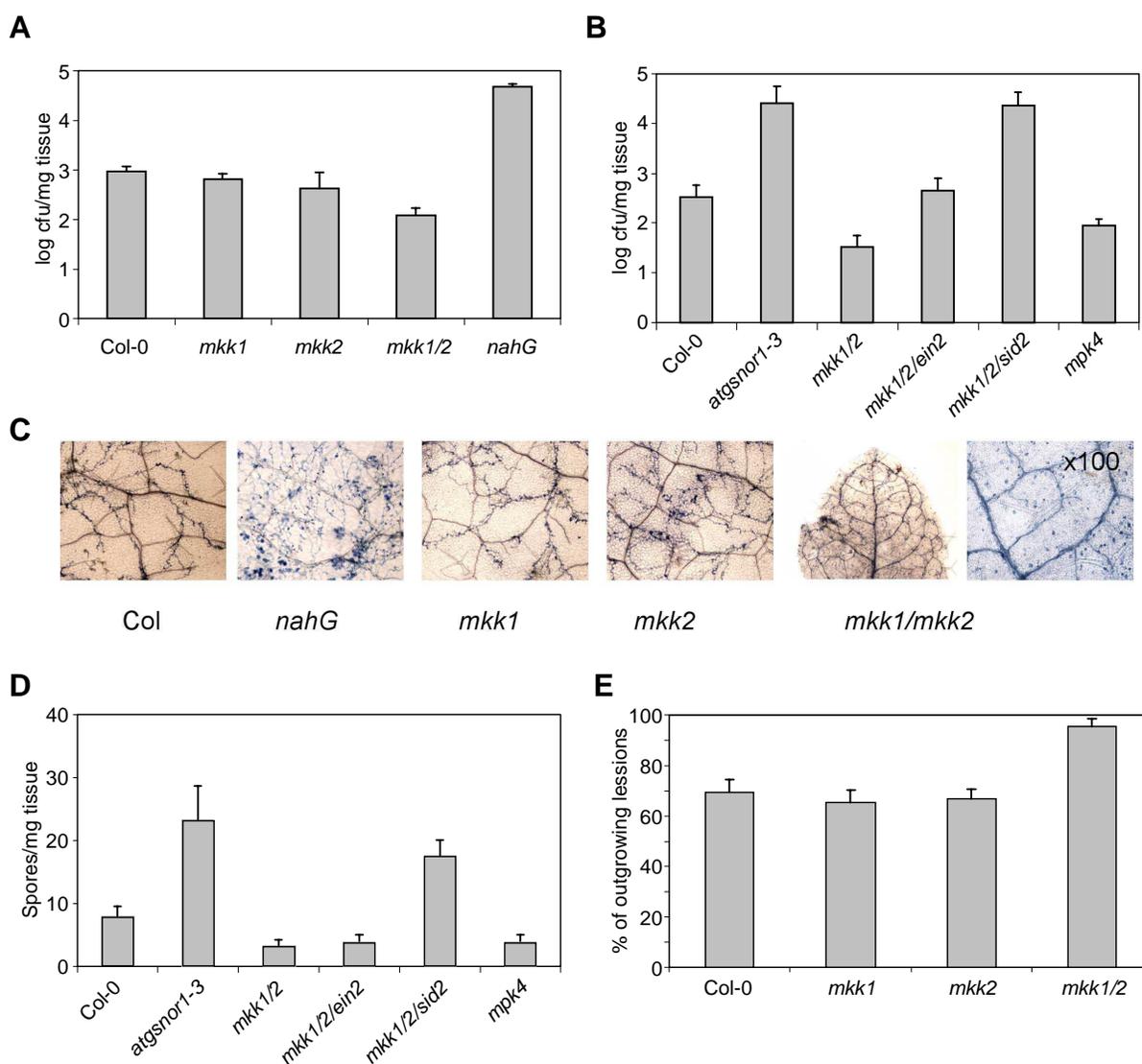


Figure 4. Disease resistance in mutants. A) Response to infection by *Pseudomonas syringae* of wild type Col-0, *mkk1*, *mkk2* and *mkk1/2* compared to *nahG* transgenic. B) Response to *P. syringae* of *mkk1/2* compared to *mkk1/2/ein2* and *mkk1/2/sid2* triple mutants, *atgsnor1-3* and *mpk4*. C) Growth of *Hyaloperonospora parasitica* detected by trypan blue staining and D) corresponding conidial counts per mg tissue. E) Growth of *Botrytis cinerea* in mutants assayed as the number of outgrowing lesions developed 3 days after inoculation. Experiments were repeated three times with similar results and error bars represent standard error of the means.

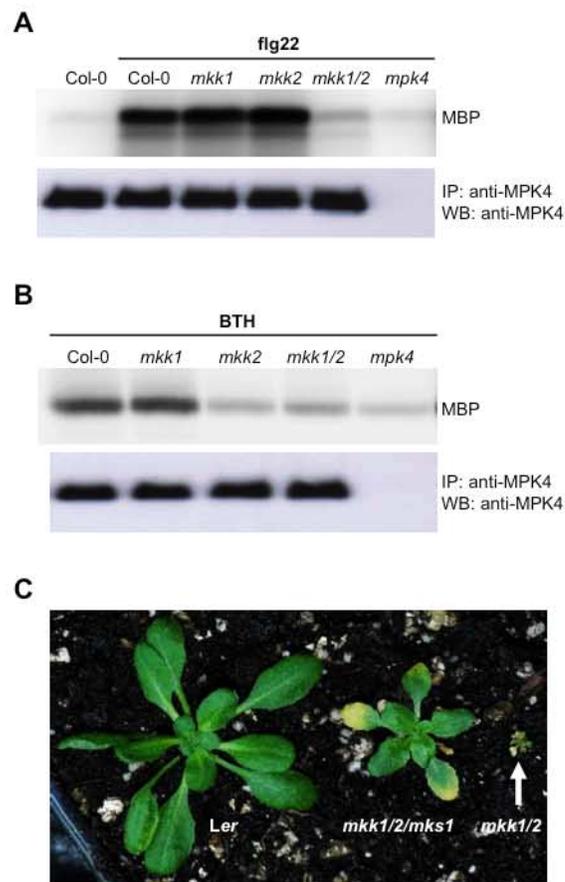


Figure 5. MKK1 and MKK2 function upstream of MPK4. A) and B) MPK4 kinase activity in single and double mutants. MPK4 protein was immunoprecipitated out of total protein extracts from plants treated for 30 minutes with 10 μ M flg22 (A) or 100 μ M BTH (B) and used in an in-gel kinase assay with MBP as substrate. Duplicate gels were subjected to western blotting to monitor MPK4 protein levels. C) Phenotype of *mkk1/2/mks1* triple mutant in comparison to *mkk1/2* and wild type.

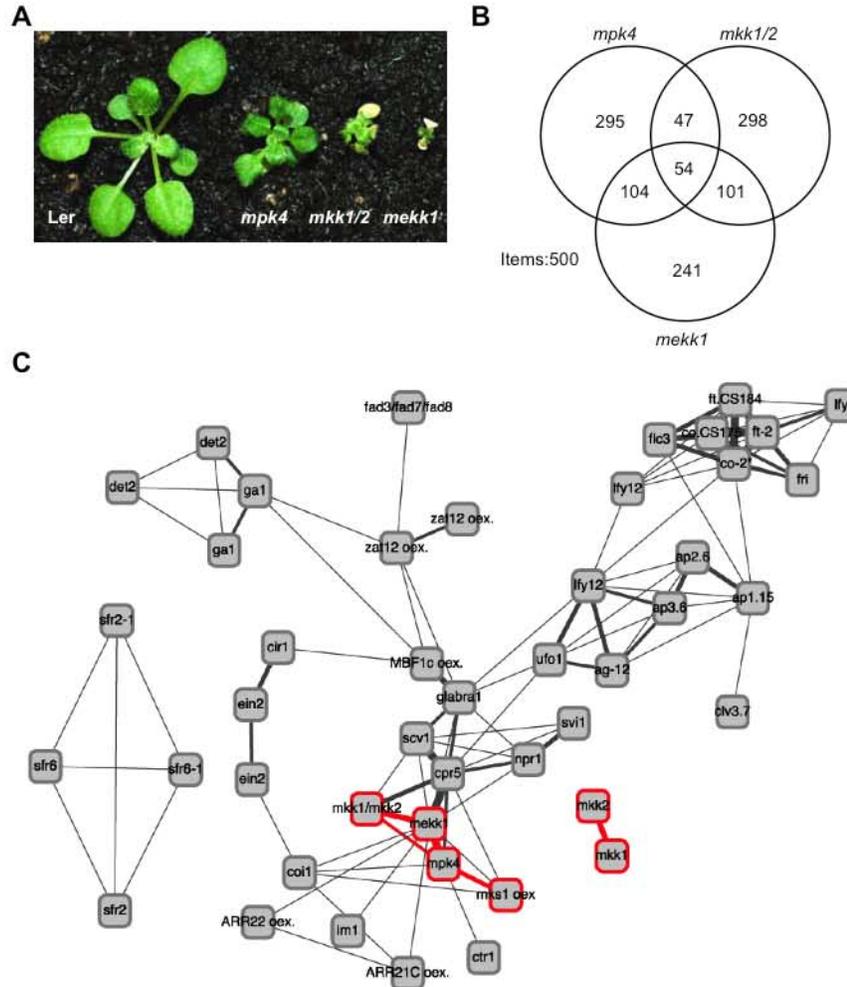


Figure 6. MEKK1, MKK1/2 and MPK4 function in the same pathway. A) Morphological phenotypes of Ler wild type, *mekk1*, *mkk1/2* and *mpk4*. B) Venn diagram showing overlapping gene expression changes in *mekk1*, *mkk1/2* and *mpk4*. Overlap is between the top-500 most significantly differentially expressed genes as measured by the ATH1 Affymetrix GeneChip. C) Functional Association(s) by Response Overlap (FARO) genotype graph, illustrating the intersections between gene expression signatures of a series of mutants and overexpressors. The thickness of the edges illustrates the size of the intersections between transcriptional signatures of the mutants (nodes). Highlighted in red are *mkk1*, *mkk2*, *mkk1/mkk2*, *mekk1*, *mpk4* and the MKS1 overexpressor. Only response signature intersections exceeding 250 genes are drawn. Mutants assayed multiple times by independent laboratories (i.e. *det2*, *ein2*, *ga1*, *zat12 oex.* and *lfy12*), are represented by a node for each assay.