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Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4

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Summary

Arabidopsis MPK4 has been implicated in plant defense regulation because *mpk4* knockout plants exhibit constitutive activation of salicylic acid (SA)-dependent defenses, but fail to induce jasmonic acid (JA) defense marker genes in response to JA. We show here that *mpk4* mutants are also defective in defense gene induction in response to ethylene (ET), and that they are more susceptible than wild-type (WT) to *Alternaria brassicicola* that induces the ET/JA defense pathway(s). Both SA-repressing and ET/JA-(co)activating functions depend on MPK4 kinase activity and involve the defense regulators EDS1 and PAD4, as mutations in these genes suppress de-repression of the SA pathway and suppress the block of the ET/JA pathway in *mpk4*. EDS1/PAD4 thus affect SA–ET/JA signal antagonism as activators of SA but as repressors of ET/JA defenses, and MPK4 negatively regulates both of these functions. We also show that the MPK4–EDS1/PAD4 branch of ET defense signaling is independent of the ERF1 transcription factor, and use comparative microarray analysis of *ctr1*, *ctr1/mpk4*, *mpk4* and WT to show that MPK4 is required for induction of a small subset of ET-regulated genes. The regulation of some, but not all, of these genes involves EDS1 and PAD4.

Keywords: hormone interactions, MAP kinase, pathogen responses.

Introduction

Plants are able to activate immune responses upon recognition of invading pathogens. Recognition may occur via gene-for-gene interactions in which a plant resistance (*R*) gene product interacts with or detects the action of a cognate pathogen avirulence (Avr) factor (Nimchuk *et al.*, 2003). R–Avr interactions induce rapid resistance responses at the infection site that are often mediated by salicylic acid (SA). Many virulent pathogens also induce basal defense responses that involve SA, and loss of basal defense causes hyper-susceptibility to virulent pathogens.

In addition to initiation of local defenses, R protein activation can lead to an immune state in systemic tissues termed systemic acquired resistance (SAR). SAR development in Arabidopsis correlates with expression of the pathogenesis-related (*PR*) genes *PR1*, *PR2* and *PR5*, and involves micro-oxidative bursts and SA accumulation in systemic tissues (Alvarez *et al.*, 1998; Malamy *et al.*, 1990; Uknes *et al.*, 1992). The role of SA in plant immunity is supported by the fact that exogenous SA, or high-level endogenous SA accumulation by expression of bacterial SA synthases, induce SAR-like resistance and *PR* gene expression (Verberne *et al.*, 2000). Conversely, SAR is impaired in the SA-deficient mutants *eds5* and *sid2* (Nawrath and Métraux, 1999). SA depletion by transgenic expression of

bacterial *nahG* salicylate hydroxylase also impairs SAR induction, although *nahG* expression has pleiotropic effects beyond SA catabolism (Heck *et al.*, 2003; van Wees and Glazebrook, 2003). Other defense-related hormones such as ethylene (ET) and jasmonic acid (JA) appear to be dispensable for SAR activation (Lawton *et al.*, 1995; Pieterse *et al.*, 1998).

Some signal transducers and transcriptional activators of SA-mediated responses have been identified. Many of these proteins are involved in local R-controlled responses, SAR, and maintenance of basal defenses, whereas others only have demonstrated roles in certain SA-mediated defense responses. Long-distance SAR signaling involves the activities of at least two apoplastic proteins. The non-specific lipid transfer-like protein DIR1 is required for an as yet undefined branch of SAR that is independent of systemic SA accumulation (Maldonado *et al.*, 2002), while the CDR1 protease is involved in triggering SA accumulation (Xia *et al.*, 2004). SA accumulation is negatively regulated by the MAP kinase MPK4 (Petersen *et al.*, 2000), and in many cases requires the aminotransferase ALD1 and the action of the interacting EDS1, PAD4 and SAG101 proteins that are essential components of basal resistance (Falk *et al.*, 1999; Feys *et al.*, 2001, 2005; Jirage *et al.*, 1999; Song *et al.*, 2004). EDS1 and PAD4 participate in a defense amplification loop that responds to SA and reactive oxygen intermediate-derived signals (Rusterucci *et al.*, 2001). Mechanisms of SA perception remain unclear, although a catalase, carbonic anhydrase and methylsalicylate esterase have been purified as SA-binding proteins (Forouhar *et al.*, 2005; Slaymaker *et al.*, 2002). The BTB/ankyrin repeat protein NPR1 is central to SA signal transduction, as *npr1* mutants are non-responsive to exogenous SA (Cao *et al.*, 1997). NPR1 translocates to the nucleus in the presence of SA and its actions include stimulation of the DNA-binding activity of the TGA family of leucine zipper transcription factors that bind to the *PR1* promoter to activate transcription (Fan and Dong, 2002; Johnson *et al.*, 2003). SA-dependent, NPR1- independent defense responses also exist, and may involve the transcription factor Why1 whose DNA-binding activity is induced by SA independently of NPR1 (Desveaux *et al.*, 2004).

SA-mediated defense responses provide protection from biotrophic fungi, oomycetes and bacteria such as *Erysiphe orontii*, *Peronospora parasitica* and *Pseudomonas syringae*. In contrast, defense against many necrotrophic fungi does not involve SA, but relies on ET and JA accumulation and signaling. Although it is unclear how necrotrophic fungi are recognized by plants, infection by these pathogens initiates a systemic defense system mediated by ET and JA, and associated with expression of the defensin PDF1.2 (Penninckx *et al.*, 1996). ET signaling involves a family of membrane-anchored receptors (ETR1, ETR2, EIN4, ERS1, and ERS2), the ETR1-associated protein kinase CTR1 that negatively regulates ET signaling, the family of labile EIN3-like transcription factors whose turnover is controlled by $SCF^{EBP1/EBP2}$ ubiquitin ligases, and other factors whose biochemical functions are unclear (Guo and Ecker, 2004). JA signaling is less well understood, but involves the ubiquitin ligase SCF^{COI1} and the JA-conjugating enzyme JAR1 (Devoto and Turner, 2003). ET and JA defense signaling converge on induction of the histone deacetylase HDA19 and the transcription factor ERF1. HDA19 is required for *Alternaria brassicicola* resistance, and its over-expression causes *ERF1* induction (Zhou *et al.*, 2005). *ERF1* overexpression in wild-type (WT), ET- and JA-insensitive genetic backgrounds is sufficient to induce *PDF1.2* expression and resistance to several necrotrophic fungi (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003; Solano *et al.*, 1998). The secreted lipase GLIP1 with anti-fungal activity is a physiologically relevant target of the ET/JA defense pathway, as *GLIP1* is induced by both hormones, and *glip1* mutants exhibit enhanced susceptibility to *A. brassicicola* infection (Oh *et al.*, 2005).

PDF1.2 serves as a useful marker for ET/JA pathway activation, but defense responses mediated by ET and JA also involve aspects distinct from *PDF1.2* induction. For example, the R2R3 Myb transcription factor BOS1 is induced in a JA-dependent manner by *Botrytis cinerea* infection, and is required for resistance to at least two necrotrophic fungi. Nonetheless, *PDF1.2* induction occurs normally in *bos1* mutants upon *B. cinerea* infection (Mengiste *et al.*, 2003).

While distinct, the SA-, ET- and JA-mediated defense systems interact in complex ways. Overlap in gene induction between SA, JA and ET treatments is significant (Schenk *et al.*, 2000), and the induction of some genes exhibits SA–JA and/or SA–ET synergism (Lawton *et al.*, 1994; Xu *et al.*, 1994), while some wound-related, JA-induced genes exhibit ET–JA antagonism (Norman-Setterblad *et al.*, 2000). A third systemic defense system, induced systemic resistance (ISR), is an example of the compatibility and independence of SA and ET/JA signaling, as ISR requires JA and ET signaling as well as NPR1, and can be induced with SAR to produce additive resistance effects (Pieterse *et al.*, 1998; van Wees *et al.*, 2000). Nonetheless, antagonistic interactions between signaling via SA and ET/JA are well documented. For example, the necrotroph-induced genes *ERF1*, *PDF1.2*, *b-CHI* and *PR4* are synergistically induced by ET and JA, but JA induction of *PDF1.2* can be inhibited by SA (Lorenzo *et al.*, 2003; Norman-Setterblad *et al.*, 2000). Mutual antagonism between SA and ET/JA was also evident from a microarray study of defense-related mutants infected with *P. syringae* pv. *maculicola* (Glazebrook *et al.*, 2003). This showed that expression of a cluster of SA-related genes, including *PR1*, was increased in ET- and JA-insensitive mutants, while ET/JA-related genes showed increased expression in SA pathway mutants. Inhibition of SA signaling by JA also occurs, as activation of JA signaling in

tomato enhances susceptibility to virulent *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000; Zhao *et al.*, 2003), while JA-insensitive mutants exhibit increased pathogen-induced SA levels and resistance in both Arabidopsis and tomato (Kloek *et al.*, 2001; Zhao *et al.*, 2003). *Pst* DC3000 uses the JA agonist coronatine as a virulence factor, and may thereby hijack antagonistic functions in the host to suppress the SA defense mechanism that combats its infection.

Despite evidence for SA–ET/JA antagonism, the underlying molecular mechanisms remain ill-defined. In Arabidopsis, genetic evidence suggests involvement of NPR1, the transcription factors ERF1 and WRKY70, and the MAP kinase MPK4 in the control of antagonism (Berrocal-Lobo *et al.*, 2002; Li *et al.*, 2004; Petersen *et al.*, 2000; Spoel *et al.*, 2003). Unsaturated fatty acid-derived signals may also play a role, as *ssi2* mutants, defective in a plastidic fatty acid desaturase, exhibit partially SA-dependent *PR1* expression and *Pst* DC3000 resistance, and strongly reduced, but oleic acid-rescuable, *PDF1.2* expression in response to JA (Kachroo *et al.*, 2001; Shah *et al.*, 2001). Formal genetic interpretations place NPR1 and WRKY70 as positive regulators of SA signaling, and as negative regulators of ET/JA signaling, while the opposite is true for ERF1 and MPK4. However, these observations do not clarify how antagonism is controlled, and, apart from a genetic interaction between WRKY70 and NPR1 in the suppression of *PDF1.2*, it is unclear how the actions of these factors are connected.

We showed previously that *mpk4* mutants constitutively express SA-mediated resistance responses but are blocked in defensin expression by JA (Petersen *et al.*, 2000). MAP kinases (MAPKs) are conserved in eukaryotic signal transduction where they orchestrate responses to extracellular stresses and developmental cues via phosphorylation of substrate proteins including transcription factors. In most cases, MAPK activity is controlled by sequential activation of three protein kinases, by which an MAPK kinase kinase (MAPKKK) activates an MAPK kinase (MAPKK) that in turn activates an MAPK by phosphorylation of conserved Thr and Tyr residues in the so-called MAPK T-loop (Madhani *et al.*, 1997). We have recently described the MPK4 substrate MKS1, a nuclear protein that interacts with two WRKY transcription factors (Andreasson *et al.*, 2005). The molecular phenotypes of plants over- or under-expressing MKS1 indicate that it mediates some effects of MPK4 on SA-mediated resistance responses but has little if any effect on responses mediated by JA.

Here we dissect the function of MPK4 in the SA–ET/JA defense network in further detail. We show that MPK4 kinase activity is central to both SAR repression and ET/JA defense induction, and that both processes involve EDS1 and PAD4 downstream of MPK4. Our data therefore place EDS1 and PAD4 as regulators of the antagonism between the SA- and ET/JA-mediated defense systems.

Results

MPK4 is required for defensin expression and resistance to Alternaria

The inducibility of *PDF1.2* mRNA accumulation by both ET and JA prompted us to test whether MPK4 is also required for ET-mediated *PDF1.2* expression. *mpk4/nahG* was included in this analysis to remove potential interference with the ET/JA pathway by high SA levels in *mpk4*. Both *mpk4* and *mpk4/nahG* exhibited strongly reduced PDF1.2 accumulation in response to ET compared with WT backgrounds (Figure 1a).

The ET/JA-regulated defense pathway is required for *PDF1.2* expression and resistance following infection by necrotrophic fungi including *Alternaria brassicicola* (Penninckx *et al.*, 1996; Thomma *et al.*, 1998). To test whether the block of *PDF1.2* expression in *mpk4* reflected a broader defect in ET/JA defense induction, the resistance of *mpk4* and *mpk4*/*nahG* to *A. brassicicola* was assessed. In contrast to L*er* and *nahG*, *mpk4* and *mpk4*/*nahG* developed clear disease symptoms and supported growth of fungal hyphae (Figure 1b). Increased susceptibility was also observed when plants had been pre-treated with methyl jasmonate (MeJA) to induce the ET/JA defense pathway (data not shown). Increased susceptibility was accompanied by reduced *PDF1.2* expression in non-infected leaves (Figure 1c). Thus, MPK4 is required for local resistance to *A. brassicicola* infection and systemic *PDF1.2* induction mediated by the ET/JA defense pathway. We note that the *mpk4*/*nahG* lines used are a mixed background between L*er* and Col-0, raising the possibility that the enhanced susceptibility of *mpk4*/*nahG* compared with *nahG* strains in Col-0 and L*er* may be due to genetic variation other than the *mpk4* mutation. This possibility is unlikely as *MPK4*/*nahG* lines from the same cross did not exhibit the hyper-susceptibility observed in *mpk4/nahG*.

To examine whether MPK4 is required for ET and JA signaling in a broader developmental context, we tested induction of two growth responses to these hormones in *mpk4* seedlings. *mpk4* exhibited both a seedling triple response to application of 50 μM of the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC), as well as inhibition of root growth by 1–100 μM MeJA (data not shown). This indicates that MPK4 is not required for all ET and JA responses.

MPK4 kinase activity is required for both SA and ET/JA pathway regulation

MAP kinases may regulate their targets by both kinase activity-dependent and -independent mechanisms (Bardwell *et al.*, 1998; Madhani *et al.*, 1997). It is therefore possible that the control of SA- and ET/JA-dependent defenses by MPK4 have different requirements for MPK4 kinase activity. To

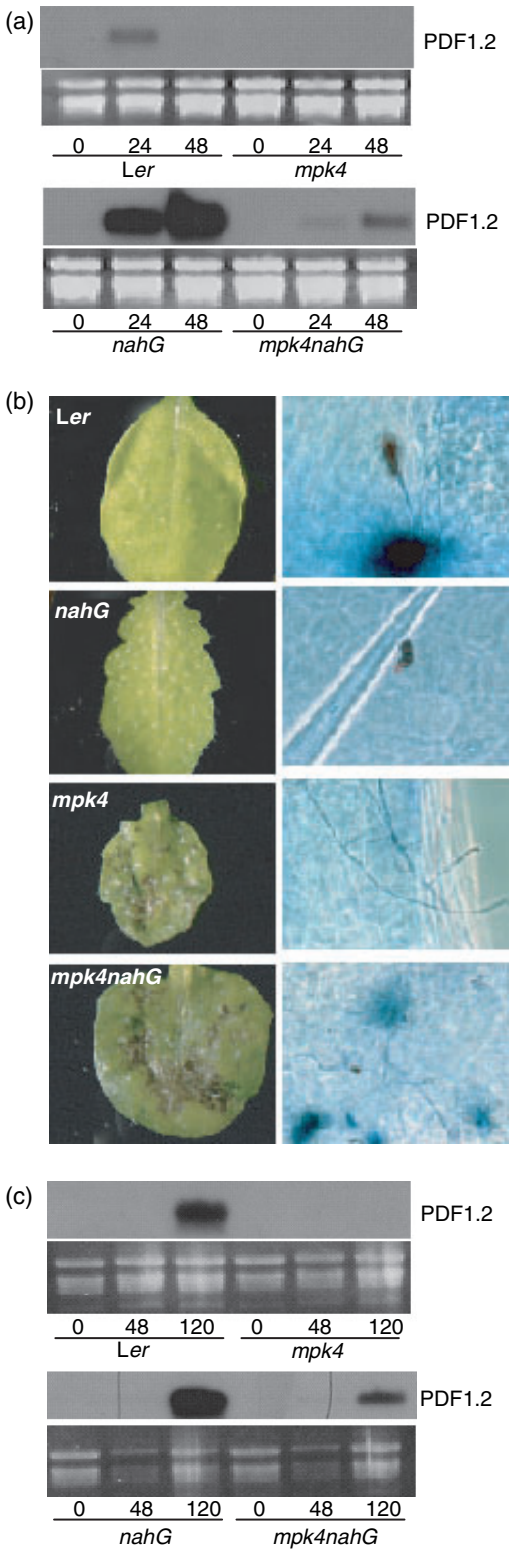

Figure 1. MPK4 is required for activation of the ethylene (ET)/jasmonic acid (JA) defense pathway.
(a) Induction of *PDF1.2* mRNA in response to ET. RNA from 3-week-old plants treated with 50 p.p.m. ET for 24 and 48 h was blotted and hybridized to ^{32}P-labeled labeled probes synthesized using a *PDF1.2* (At5g44420) cDNA fragment as template.
(b) Growth of *Alternaria brassicicola* on wild-type (WT) and *mpk4* leaves. Three droplets (15 μl) containing 2.5×10^5 spores per ml were placed on three leaves of 3-week-old plants. Leaves were examined 1 week after inoculation. The left panel shows symptoms of *A. brassicicola* leaf infections, the right panel shows staining of dead plant cells and fungal structures by trypan blue. The experiment was repeated twice with similar results.
(c) Induction of *PDF1.2* mRNA in non-infected, systemic leaves upon local infection with *A. brassicicola*. Non-infected leaf tissue was harvested at the times indicated. Spore inoculation was performed as in (b) and RNA analysis as in (a).

examine this possibility, we expressed two inactive, HA-epitope-tagged MPK4 mutants, mpk4^{AEF} and mpk4^{K72R}, in the *mpk4* null background. mpk4^{AEF}cannot be activated by T-loop phosphorylation, while mpk4^{K72R} is catalytically inactive but can be phosphorylated in the T-loop. In some MAP kinases, T-loop phosphorylation is important for both kinase activation and kinase-independent modulation of interactions with regulatory targets (Bardwell *et al.*, 1998). Western blotting and immunoprecipitation kinase assays confirmed that both mutant forms were expressed to the same levels as WT HA-epitope-tagged MPK4, and that they had no detectable kinase activity (Figure 2a).

We then examined the SA- and ET/JA-related phenotypes of these lines expressing mutant kinase forms. We previously showed that mpk4^{AEF} is unable to complement the dwarf and constitutive *PR1* expression phenotypes of the *mpk4* knockout mutant, suggesting that MPK4 kinase activity is required for repression of SA-dependent defenses (Petersen *et al.*, 2000). This was confirmed by analysis of mpk4^{K72R}, which also exhibited dwarfism, high-level accumulation of total SA (the sum of free and glucose-conjugated), and strong expression of *PR1* (Figure 2b,c). *PDF1.2* expression in mpk4^{K72R} and mpk4^{AEF} in response to ET and JA was then used to examine involvement of MPK4 kinase activity in the ET/JA pathway. In addition, *PDF1.2* induction in response to ET and JA was as severely blocked in mpk4^{K72R} and mpk4^{AEF}as in the *mpk4* null mutant (Figure 2d,e), and both mutants showed hypersusceptibility to *A. brassicicola* similar to the *mpk4* null mutants (Figure S1). This indicates that MPK4 kinase activity affects both the SA and ET/JA defense pathways.

To assess the impact of MPK4 kinase activity on the SA and JA/ET defense pathways more directly, we used a conditional loss-of-function MPK4 allele constructed according to a chemical–genetic system for protein kinases (Bishop *et al.*, 2000). In this system, a specific point mutation that enlarges the ATP-binding pocket is introduced into the kinase. This mutation sensitizes the kinase to inhibition by bulky C3–1′-naphtyl (NaPP1) or C3–1′-naphtylmethyl (NMPP1) derivatives of the Src tyrosine kinase family inhibitor PP1. NaPP1 and NMPP1 are not efficient inhibitors of WT protein kinases. The corresponding binding pocket residue in MPK4 is Y124. Therefore, HA-epitope-tagged MPK4^{Y124G}and MPK4^{Y124A} mutants were constructed and

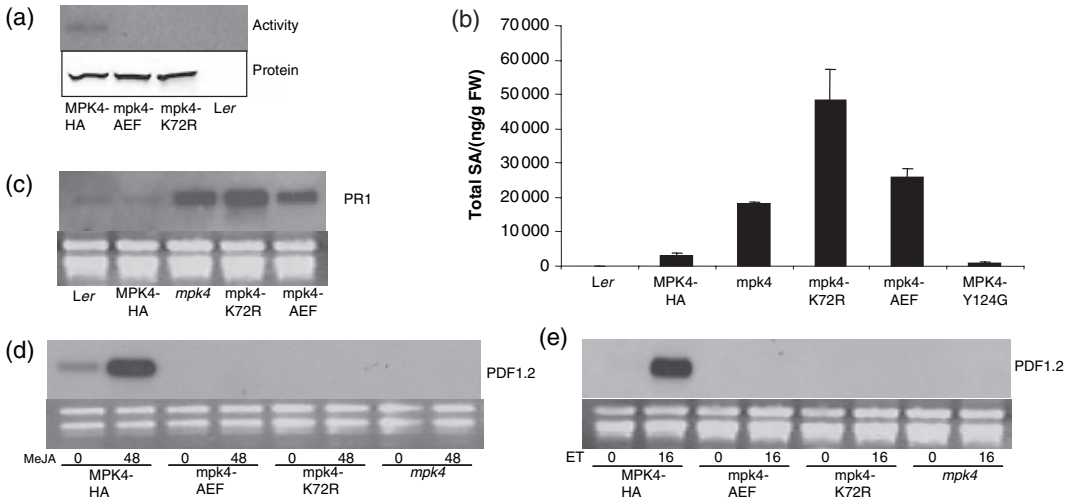

Figure 2. Analyses of kinase-dead MPK4 versions expressed in the *mpk4* background.
(a) Activity and expression level of MPK4 versions. HA-tagged MPK4 versions were immunoprecipitated from 200 μg of total protein extract, and the immunoprecipitates were divided for activity assay using ^{32}P-labeled ATP and myelin basic protein (MBP) as substrate, or Western analysis using anti-HA antibodies.
(b) Accumulation of total SA. Three-week-old leaves were subjected to metabolite extraction and glucosidase treatment, and the total SA (sum of free and glucose-conjugated) was quantified by comparison of UV-VIS absorption spectra with SA-spiked *sid2* controls following high performance liquid chromatography fractionation.
(c) Accumulation of *PR1* mRNA. RNA was extracted from 3-week-old leaves, and blots hybridized to ^{32}P-labeled, PR1-specific probes (At2g14610).
(d) Induction of *PDF1.2* mRNA in response to methyl jasmonate (MeJA). Three-week old plants were treated with 50 μM MeJA for 48 h.
(e) Induction of *PDF1.2* mRNA in response to ET. Three-week old plants were treated with 50 p.p.m. ET for 16 h.

expressed in the *mpk4* background. Both mutants fully complemented the morphological *mpk4* phenotypes, and had WT kinase activity levels when immunoprecipitated from naïve plants (data not shown). In addition, MPK4^{Y124G} had SA levels as low as *mpk4* mutants expressing transgenic WT MPK4 (Figure 2b). Both MPK4^{Y124G}and MPK4^{Y124A}, but not WT MPK4, were inhibited by NaPP1 (Figure 3a) and less potently by NMPP1 (not shown) in *in vitro* kinase assays with MPK4 versions immunopurified from total protein extracts. MPK4^{Y124G}showed stronger NaPP1 inhibition than MPK4^{Y124A} and was chosen for *in vivo* experiments.

The involvement of MPK4 kinase activity in SA-dependent defenses was investigated by spraying plants with NaPP1 and measuring *PR1* expression 20 h later. Compared with the *in vitro* assay described above, 100-fold higher NaPP1 concentrations were used for these *in vivo* experiments, as previously described in yeast (Bishop *et al.*, 2000). *PR1* mRNA accumulated specifically in MPK4^{Y124G} plants in an NaPP1-dose-dependent manner after 20 h (Figure 3b). To evaluate the role of MPK4 kinase activity in the ET/JA pathway, ET treatments for 16 h were performed in the presence or absence of NaPP1. In WT backgrounds, *PDF1.2* was induced regardless of the presence of NaPP1, while *PDF1.2* induction was strongly reduced by NaPP1 in MPK4^{Y124G} (Figure 3c). These results indicate that conditional loss of MPK4 kinase activity affects both SA and ET/JA responses over the relatively short time frames of 16–20 h.

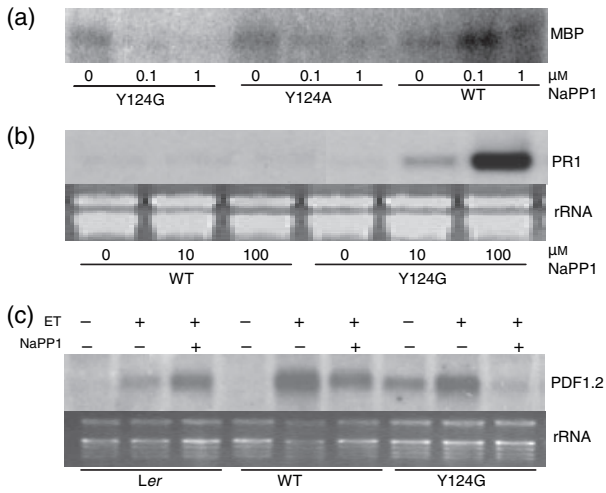

Figure 3. Analyses of inhibitor-sensitive MPK4 alleles.
(a) NaPP1 inhibition of kinase activities immunoprecipitated from total protein extracts. HA-tagged MPK4 versions were immunoprecipitated from 200 μg of total protein extract. Immunoprecipitates were incubated with a mock solution (0.01% DMSO), 100 nM NaPP1 or 1 μM NaPP1 for 10 min prior to in-solution phosphorylation reactions with MBP as substrate.
(b) Accumulation of *PR1* mRNA in response to NaPP1 application. Solutions containing either 1% DMSO, 10 μM or 100 μM NaPP1 in 1% DMSO were sprayed onto leaves of 3-week-old plants, and RNA was extracted 20 h later.
(c) Induction of *PDF1.2* mRNA in response to ET in the presence or absence of NaPP1. Three-week old plants were sprayed with mock or 100 μM NaPP1 solutions, and a 16 h treatment with 50 p.p.m. ET was started 1 h later.

EDS1 and PAD4 function downstream of MPK4 in SA-dependent defense regulation

Epistatic relationships between *mpk4* and other defense-related mutants were examined to assess the relative position(s) of MPK4 in the SA and ET/JA signaling networks. For analysis of the SA pathway, *eds1–2* and *pad4–2* (both in L*er*) were used because they exhibit attenuated SA accumulation and enhanced susceptibility to virulent pathogens including *Pst* DC3000 (Feys *et al.*, 2001). Both *mpk4/eds1–2* and *mpk4/pad4–2* partially suppressed dwarfism, and this suppression was more pronounced than that in *mpk4/nahG* (Figure 4a). In addition, *mpk4/eds1–2* and *mpk4/pad4–2* exhibited strong suppression of SA accumulation, *PR1* expression and resistance to *Pst* DC3000 (Figure 4b–d). Notably, *mpk4/eds1–2* showed nearly complete suppression of these phenotypes, while suppression in *mpk4/pad4* was less complete. The residual dwarfism, *Pst* DC3000 resistance and *PR1* expression were apparently not due to redundancy between EDS1 and PAD4 because *mpk4/pad4–2/eds1–2* triple mutants exhibited stronger morphological defects than either double mutant, and had resistance and *PR1* expression phenotypes similar to *mpk4/eds1–2* (data not shown). These data indicate that EDS1 and PAD4 act positively downstream of MPK4 in the control of SA levels and related defenses. Importantly, *mpk4*/*eds1* and *mpk4*/*pad4* exhibited stronger suppression of morphological defects, but weaker suppression of SA accumulation, than *mpk4*/*nahG*. This indicates that EDS1 and PAD4 can affect the morphological phenotype of *mpk4* via SA-independent mechanisms.

MPK4 acts downstream or independently of ERF1 in ET/JA defense regulation

To further analyse the relationship between MPK4 and the ET/JA signaling network, we examined its relationship to CTR1 and ERF1. This revealed that the high level of PDF1.2 accumulation in the *ctr1–2* mutant was completely suppressed in the *ctr1–2/mpk4* double mutants, while *ctr1–2* and *ctr1–2*/*mpk4* both accumulated similar levels of *ERF1* mRNA (Figure 5a). These results indicate that MPK4 functions downstream or independently of both CTR1 and ERF1.

Two approaches were used to confirm the disproportionate *ERF1* and *PDF1.2* expression in *mpk4* backgrounds. First, *ERF1* and *PDF1.2* mRNA accumulation following ET induction was examined in *mpk4* and *mpk4*/*nahG*. This showed that while *PDF1.2* expression was blocked in *mpk4* and *mpk4/nahG*, *ERF1* mRNA levels were either constitutively elevated (*mpk4*) or normally induced by ET (*mpk4/nahG*) (Figure 5b). Second, we transformed a 35S:ERF1 construct into *mpk4* heterozygotes and isolated several lines with constitutive ERF1 expression. The levels of ERF1 and *PDF1.2* mRNA were then examined in WT and *mpk4* dwarf plants segregating from these 35S:ERF1 transgenic lines. This

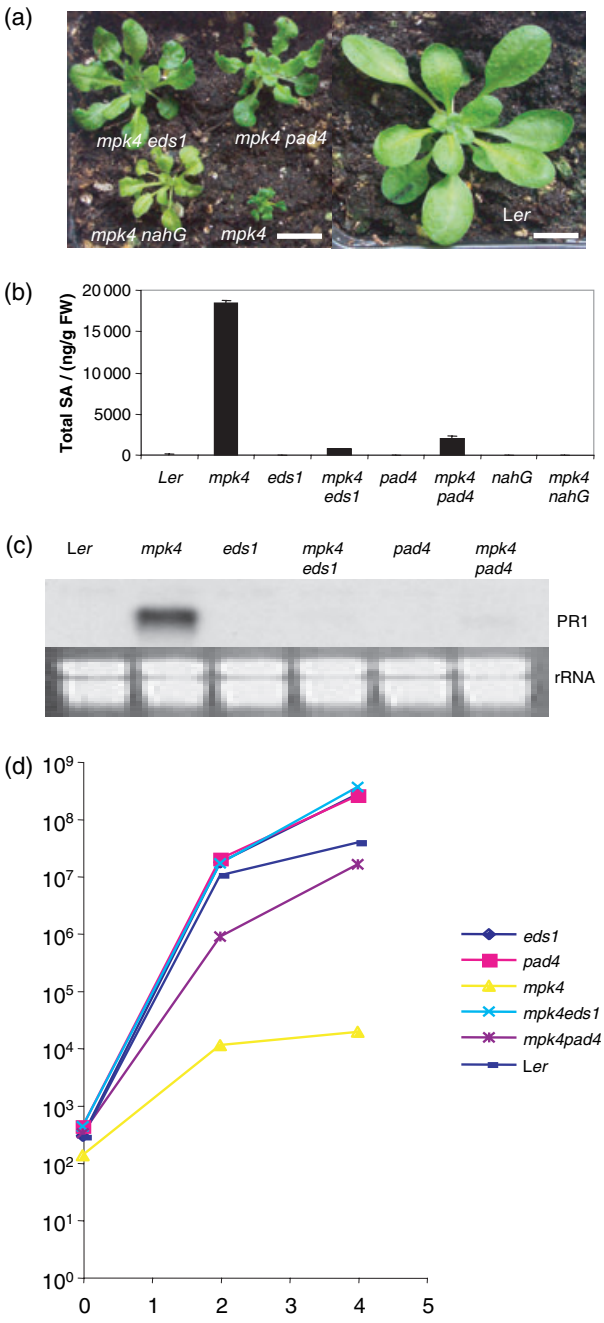

Figure 4. Mutations in *EDS1* and *PAD4* suppress SA-dependent defense activation in *mpk4*.
(a) Representative pictures of phenotypes of 3-week-old WT, *mpk4*, *mpk4/nahG*, *mpk4/pad4* and *mpk4/eds1* versus L*er* WT. Scale bar is 1 cm.
(b) Accumulation of total SA. Extraction and quantification were performed as in Figure 2(b).
(c) Accumulation of *PR1* mRNA in leaves of 18-day-old plants. Phosphorimager quantification of hybridization signals showed that the PR1 signal intensities in *mpk4/eds1* and *mpk4/pad4* were approximately 5% and approximately 10%, respectively, of that in *mpk4*.
(d) Growth of *Pst* DC3000 (CFU cm^{-2} leaf area). *Pst* DC3000 was vacuum-infiltrated at 10^5 CFU ml^{-1} into leaves and bacterial growth quantified by counting colony-forming units at the indicated time points. Each point is the average of triplicate samples and the entire experiment was repeated three times with similar results.

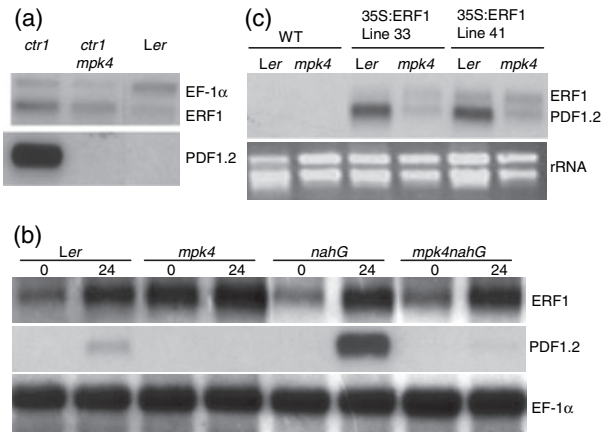

Figure 5. MPK4 regulates ET induction of PDF1.2 downstream or independently of CTR1 and ERF1.
(a) Accumulation of *PDF1.2* and *ERF1* mRNA in *ctr1*, *ctr1/mpk4* and Ler; 2 μg of polyA RNA was loaded per lane.
(b) Accumulation of *PDF1.2* and *ERF1* mRNA in response to 50 p.p.m. ET; 20 μg of total RNA was loaded per lane.
(c) Accumulation of *PDF1.2* and *ERF1* mRNA in *mpk4* and WT backgrounds over-expressing ERF1; 20 μg of total RNA was loaded per lane.

showed that accumulation of *PDF1.2* mRNA was significantly reduced (4–7-fold when quantified by Phosphorlmager analysis) in *mpk4* compared with WT siblings (Figure 5c). This result further suggests that MPK4 influences ET, and most likely JA, signaling downstream or independently of ERF1. Given the proximity of ERF1 to ET/JA transcriptional responses, ERF1 could be a target of MPK4. However, MPK4 and ERF1 did not interact in a yeast two-hybrid assay, and were not co-immunoprecipitated from total protein extracts (data not shown).

MPK4 effects on PDF1.2 *expression and* Alternaria *resistance are mediated by EDS1 and PAD4*

We next analysed whether MPK4 affects *PDF1.2* expression via a pathway contributing to the ET/JA defense network downstream of ERF1. If so, EDS1 and PAD4 could act as repressors in such a pathway as expression of *PDF1.2* in *cpr6–1/eds1* and *cpr6–1/pad4* was strongly enhanced compared with *cpr6–1*, while neither *pad4* nor *eds1* single mutants accumulated high levels of *PDF1.2* mRNA (Clarke *et al.*, 2001; Jirage *et al.*, 2001). We therefore tested whether induction of *PDF1.2* was restored in *mpk4/eds1–2* and *mpk4/pad4–2* double mutants. Significant *PDF1.2* mRNA accumulation was detected in *mpk4/eds1–2* in response to MeJA at 24 h after hormone application, whereas little *PDF1.2* mRNA was detected in *mpk4/pad4* and in SA-depleted *mpk4/nahG* (Figure 6a). Interestingly, *PDF1.2* mRNA accumulation was partially restored in *mpk4/pad4* at 7 h after MeJA application, indicating that the effects of *pad4* are, at least in part, epistatic to *mpk4* (Figure S2). This double mutant analysis indicates that EDS1 and PAD4 act as repressors of *PDF1.2* induction by MeJA downstream of MPK4, and suggests that EDS1 plays a more important role than PAD4 in such *PDF1.2* repression.

We also tested the involvement of EDS1 and PAD4 in ET signaling by monitoring *PDF1.2* mRNA accumulation in *ctr1–2* and *ctr1–2/mpk4* mutants into which the *eds1–2*, *pad4–2* and *eds1–2*/*pad4–2* alleles had been introduced. While the results of this analysis were more complex, they were consistent with a model in which EDS1 and PAD4 repress *PDF1.2* expression downstream of MPK4 (Figure 6b). First, PDF1.2 accumulation in the *ctr1–2* background was significantly increased in the absence of PAD4 or of both PAD4 and EDS1. Second, mutation of EDS1 bypassed the requirement of MPK4 for *PDF1.2* induction, while full *PDF1.2* induction was dependent on MPK4 in the *pad4* single mutant background. In contrast, the low PDF1.2 level in *ctr1*/*eds1* suggests that EDS1 has an activating as well as repressive role in ET-related induction of *PDF1.2*. Despite this exception, the results indicate that PAD4 and EDS1 act to repress *PDF1.2* downstream of MPK4 in ET/JA signaling.

To analyse the physiological relevance of the above differences in gene expression, we tested the resistance of *mpk4*/*pad4* and *mpk4*/*eds1* to *A. brassicicola* infection. Both double mutants were markedly less susceptible than *mpk4* single mutants and the SA-depleted *mpk4*/*nahG* line, although more hyphal growth and sporulation was observed on *mpk4/pad4* than on *pad4* and WT L*er* (Figure 6c).

Collectively, these results are consistent with a model in which *PDF1.2* expression and *A. brassicicola* resistance are regulated by a pathway requiring MPK4 activity. This pathway is mediated by the repressive effects of EDS1 and PAD4, and functions in addition to, or downstream of, the activating pathway mediated by ERF1 (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2004).

Enhanced EDS1 protein accumulation in mpk4 *mutants*

We examined whether MPK4 affects EDS1 protein accumulation by immunodetection in extracts of *mpk4* single and double mutants. All *mpk4* backgrounds tested, including SA-deficient *mpk4/nahG*, accumulated high levels of EDS1 (Figure 6d), although EDS1 levels were considerably higher in *mpk4* than in *mpk4*/*nahG* or *mpk4*/*pad4*. Thus, although SA may contribute to EDS1 accumulation in *mpk4* via increased *EDS1* mRNA accumulation, increased EDS1 protein levels in *mpk4* are not due solely to high SA levels (Falk *et al.*, 1999; Feys *et al.*, 2001). EDS1 may therefore be more directly regulated by MPK4. However, recombinant EDS1 was not an *in vitro* substrate of MPK4 immuno-purified from plant extracts (data not shown). Nonetheless, the correlation between high EDS1 levels and reduced *PDF1.2* induction (*mpk4* and *mpk4/nahG*), and the reversion of *PDF1.2* induction by the *eds1* mutation, suggest that EDS1 abundance or activity may be regulated by JA and/or ET via MPK4. We note

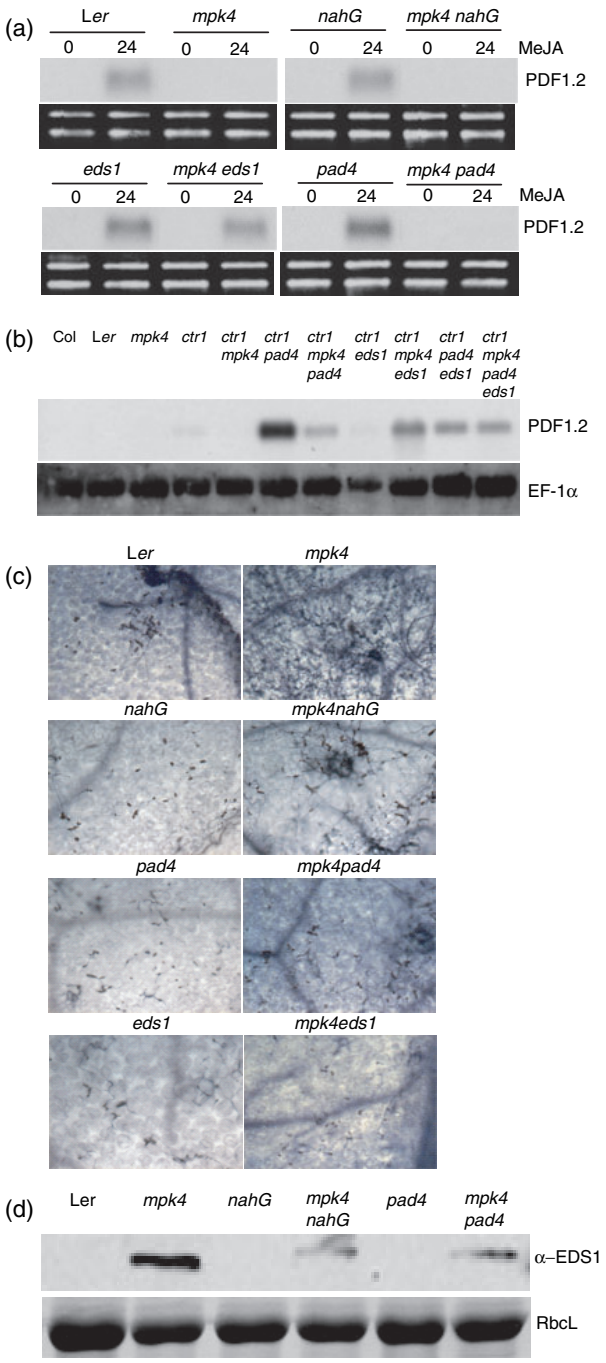

Figure 6. EDS1 and PAD4 function downstream of MPK4 in regulating PDF1.2 induction and *Alternaria brassicicola* resistance.
(a) Accumulation of *PDF1.2* mRNA in response to MeJA in *mpk4*, *mpk4/nahG*, *mpk4/eds1* and *mpk4/pad4*. Three-week-old plants were treated with 50 μM MeJA for 24 h.
(b) Accumulation of *PDF1.2* mRNA in *ctr1* backgrounds defective in EDS1, PAD4 and/or MPK4; 20 μg of total RNA was loaded per lane. To avoid saturation of the *ctr1 pad4* signal, the exposure was much shorter than the autoradiogram of Figure 5(a). This explains the apparent low intensity of the *PDF1.2* signal in *ctr1*.
(c) Growth of *A. brassicicola* on *mpk4*, *mpk4/nahG*, *mpk4/eds1* and *mpk4/pad4*. Leaves were infected with three 15 μl droplets of *A. brassicicola* spores at 2.5×10^5 spores per ml. Hyphal growth was revealed by trypan blue staining 7 days after inoculation. Six to eight plants of each genotype were infected, and there was little individual variation in hyphal growth among plants of a genotype.
(d) Accumulation of EDS1 protein in *mpk4*, *mpk4/nahG* and *mpk4/pad4*. At longer exposures, EDS1 protein was also detected in L*er*. The lower panel shows the RUBISCO large subunit (RbcL) from an identically loaded Coomassie-stained gel run in parallel with the Western blot.

that EDS1 or PAD4 protein levels were not affected by ET or MeJA, although EDS1 levels were significantly lower in *ctr1–2* than in WT Col-0 (not shown). In conclusion, high EDS1 protein levels may explain many of the SA, ET and JA defense defects observed in *mpk4* mutants, but mechanistic links between MPK4 activity and EDS1 accumulation remain unknown.

Global analysis of MPK4-dependent, ET-related genes

The action of MPK4, and possibly EDS1 and PAD4, in ET signaling was further characterized by comparing the transcriptomes of *ctr1–2*, *ctr1–2/mpk4*, *mpk4* and WT (L*er* and Col-0 samples). We used a two-factor ANOVA design with three replicates of each category yielding *P* values for differentially expressed genes in *ctr1* and *mpk4* and for interaction effects between *ctr1* and *mpk4*. *P*-value cut-offs of 0.005 for the two main effects, and 0.01 for interaction effects, resulted in only one predicted false positive (see Experimental procedures).

We focused on two classes of genes with significantly different expression levels among the four genotypes. Class-I represented the MPK4-dependent set of ET-related genes whose mRNAs over-accumulate in *ctr1–2* compared with WT, but where this difference is suppressed by *mpk4*. Of the 22 810 genes represented on the array, only 48 Class-I genes were identified (Table 1). Many (35) of these genes exhibited a pattern in which *mpk4* mutation alone led to significant under-expression relative to WT, such that expression in *ctr1/mpk4* became correspondingly lower than in *ctr1* (Figure 7a). Most of the Class-I genes have no known function. Apart from *PDF1.2*, only the bHLH transcription factor BEE1 has been associated with ET responses as it is induced by ACC (Friedrichsen *et al.*, 2002). We did not identify genes whose repression in *ctr1–2* versus WT required MPK4, indicating that MPK4 acts as an activator rather than a repressor of the induction of ET effectors.

The accumulation of mRNAs encoded by 78 Class-II genes was different from WT in both *ctr1–2* and *ctr1–2/mpk4*, but similar to WT in *mpk4* (Table 2). The accumulation of mRNAs of two of these genes (*EBP*, *b-CHI*) in the *ctr1* mutant backgrounds was shown to be independent of MPK4 by Northern blotting (Figure S3). This analysis also revealed that the mRNAs of these genes did not significantly

Table 1 Class-I genes with MPK4-dependent over-expression in *ctr1*

Gene	Description
At5g44420	Defensin PDF1.2a
At2g26020	Defensin PDF1.2b
At5g61160	Anthocyanin 5-aromatic acyltransferase-like
At1g73330	Protease inhibitor DR4
At1g18400	bHLH transcription factor BEE1
At3g14210	Putative myrosinase-associated protein
At5g65390	Arabinogalactan-protein AGP7
At1g78970	Lupeol synthase LUP1
At1g20190	Expansin EXP11
At3g60290	Oxidoreductase
At2g40670	Response regulator ARR16
At5g22460	Esterase/lipase/thioesterase family
At2g06850	Xyloglucan endotransglycosylase EXGT-A1
At2g36870	Putative xyloglucan endotransglycosylase
At1g44830	AP2 transcription factor
At4g02290	Endo-1,4-beta glucanase-like
At4g21410	Ser/Thr kinase-like
At4g37800	Putative xyloglucan endotransglycosylase
At1g65290	Acyl carrier protein family
At1g27460	Calmodulin-binding protein-like
At2g47880	Glutaredoxin
At5g10430	Arabinogalactan-protein AGP4
At5g60920	Phytochelatin synthetase-like COB
At3g16370	GDSL-motif lipase/hydrolase protein
At5g48900	Pectate lyase
At4g25260	Pectin esterase-like
At2g38180	GDSL-motif lipase/hydrolase protein

Functionally annotated genes upregulated in *ctr1* and suppressed by *mpk4* according to *P*-value criteria (see Experimental procedures). A full list of Class-I genes is given in Table S1.

over-accumulate in *ctr1/pad4* or *ctr1/eds1*. A relatively large group of MPK4-independent genes was expected because developmental defects typical of *ctr1–2* plants were retained in *ctr1–2*/*mpk4* double mutants. However, MPK4-independent genes also included known or putative defense-related genes such as *b-CHI* and several putative *R* genes (At5g17880, At5g17890, At5g36930, and At1g59124), indicating that MPK4 influences the expression of only a subset of ET-dependent defense genes.

PAD4 and EDS1 are involved in regulating some, but not all, MPK4-dependent genes

To determine whether the regulation of MPK4-dependent, ET-related genes generally involves PAD4 and EDS1, we used real-time RT-PCR to test the expression of some of the Class-I genes in the series of *ctr1* mutants into which *mpk4*, *pad4* and *eds1* alleles had been introduced. This analysis identified one

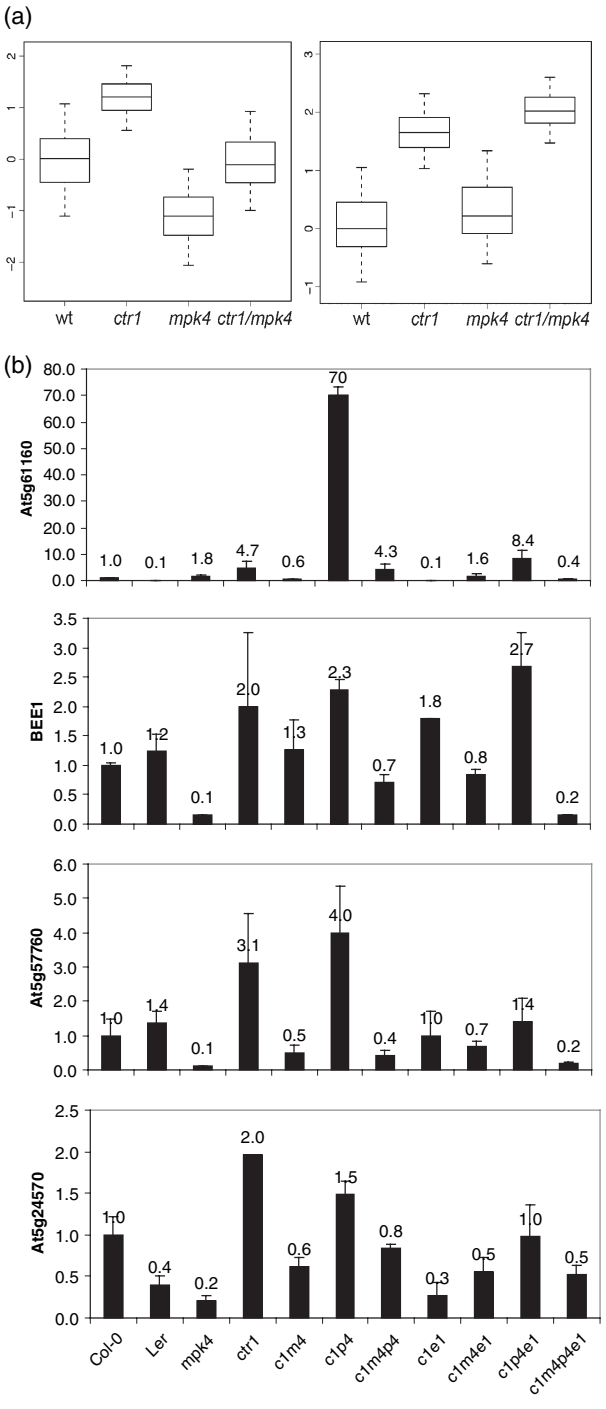

Figure 7. mRNA accumulation patterns in WT and mutants determined by transcriptomics and real-time quantitative PCR.
(a) Boxplots illustrating gene expression profiles of class I (left) and class-II (right) genes in WT (Col-0 and L*er*), *ctr1*, *mpk4* and *ctr1*/*mpk4* plants. Horizontal lines in boxes indicate median gene expression intensity in a given genotype, horizontal box edges indicate quartiles, and upper and lower bars indicate two standard deviations from median. The *y*-axes are scaled gene expression values and the unit is standard deviations from mean expression (*z*-score).
(b) Real-time PCR quantification of relative expression levels of MPK4-dependent genes whose regulation involves PAD4 and EDS1 (At5g61160) or proceeds independently of PAD4 and EDS1 (BEE1, At5g57760, and At5g24570). *c1*, *ctr1*; *m4*, *mpk4*; *p4*, *pad4*; *e1*, *eds1*. Error bars indicate standard deviations of triplicate, linearly transformed C_T data.

Table 2 Class-II genes with MPK4-independent over- or under-expression in *ctr1*

Gene	Description
At2g38530	Non-specific lipid transfer protein 2 (LTP 2)
At3g16770	AP2 transcription factor EBP
At4g30290	Putative xyloglucan endotransglycosylase
At1g62380	ACC oxidase ACO2
At5g17880	TIR-NBS-LRR class R-protein-like
At5g36930	TIR-NBS-LRR class R-protein-like
At1g63880	TIR-NBS-LRR class R-protein-like
At5g57240	Oxysterol-binding protein-like
At1g33790	Jacalin lectin family
At3g12500	Basic endochitinase PR3
At3g09260	Beta-glucosidase-like
At2g16060	Non-symbiotic hemoglobin AHB1
At3g22840	Early light-induced protein ELIP2
At5g17890	TIR-NBS-LRR class R-protein-like
At4g16260	Glucan endo-1,3-beta-glucosidase-like
At5g36220	Cytochrome P450 CYP91A1
At2g02850	Plastocyanin-like
At3g44970	Cytochrome P450
At5g02760	Protein phosphatase 2C
At4g14690	Early light-induced protein ELIP1
At5g05750	DnaJ protein family
At1g62770	Pectin esterase-like
At3g16430	Myrosinase binding protein-like
At5g64570	Glycosyl hydrolase
At1g73830	bHLH transcription factor BEE3
At5g67060	bHLH transcription factor bHLH088
At3g23150	Ethylene receptor-related ETR2
At2g30520	ROOT PHOTOTROPISM 2 RPT2
At1g79000	Acetyltransferase-related protein 2 PCAT2
At1g10480	C2H2-type zinc finger protein ZFP5
At5g50570	Squamosa promoter binding protein-like
At5g22510	Alkaline/neutral invertase
At3g14230	AP2 transcription factor
At5g25350	F-box LRR protein
At1g59700	Glutathione-*S*-transferase-like
At1g31580	CXC750
At3g18280	Lipid transfer protein/protease inhibitor family
At1g59124	CC-NBS-LRR class R-protein-like
At3g43600	Aldehyde oxidase 2 AAO2
At1g14210	Ribonuclease-like
At1g55920	Serine acetyltransferase SAT1
At1g69310	WRKY family transcription factor WRKY57
At3g17510	CBL-interacting protein kinase 1 CIPK1
At3g57410	VILLIN 3 VLN3
At4g34250	Fatty acid elongase 1 FAE1
At1g31600	Oxidoreductase
At2g27050	EIN3-like transcription factor EIL1
At5g25890	IAA28
At4g27300	S-locus protein kinase-like
At3g58550	Lipid transfer protein/protease inhibitor family

Functionally annotated genes differentially expressed in *ctr1* but not affected by MPK4. A full list of Class-II genes is given in Table S2.

additional mRNA with significant hyper-accumulation in *ctr1*/*pad4* (At5g61160, encoding an anthocyanin-5-aromatic acyl transferase-like protein, AACT). Similar to *PDF1.2*, *AACT* mRNA also accumulated to low levels in *ctr1*/*eds1*, but differed in that it exhibited a requirement for MPK4 in the *pad4*/*eds1* double mutant background (Figure 7b). In addition, the expression of three genes (bHLH transcription factor BEE1, and At5g57760 and At5g24570 encoding unknown proteins) was not stimulated by *pad4* and/or *eds1* mutation, although their full induction in *ctr1* was confirmed to depend on MPK4 (Figure 7b). This indicates that the set of MPK4-dependent genes does not constitute a regulon, but consists of differently regulated subgroups of genes. This is consistent with our inability to identify conserved promoter elements among all Class-I genes.

Discussion

Negative regulatory role of MPK4 activity in the SA defense pathway

We previously proposed that MPK4 negatively regulates SA-dependent defense responses via its basal kinase activity due to the activation of SA-dependent defenses in the *mpk4* knockout and in a kinase-inactive *mpk4* mutant (Petersen *et al.*, 2000). This model is consistent with protein kinase inhibitor studies in tobacco showing that Ser/Thr kinase inhibition led to accumulation of SA and to *nahG*-suppressible *PR1* expression (Conrath *et al.*, 1997). However, the dwarf stature of *mpk4* plants left open the possibility that deregulation of defenses was an indirect consequence of the loss of MPK4 kinase activity even though such phenotypes are common among mutants with constitutive expression of SA defenses, and their penetrance correlates with defense expression in mutants such as *cpr1* and *bon1* (Clarke *et al.*, 2001; Jirage *et al.*, 2001; Yang and Hua, 2004).

Here we examine the relationship between MPK4 activity and defense regulation in more detail. We can exclude the possibility that *PR1* expression arises solely as a consequence of developmental defects in *mpk4* mutants, because *PR1* was induced upon specific inhibition of MPK4 activity in plants with WT morphology (Figure 3). Although this indicates that MPK4 inactivation is sufficient to activate the SA defense pathway in a WT plant, it is still unclear whether such inactivation is required for activation of the pathway. Likewise, these results on conditional MPK4 inactivation do not exclude other indirect effects of MPK4 inhibition leading to activation of the SA pathway. A gain-of-function analysis of the requirement for MPK4 inactivation in SA-dependent defense activation would help address both questions, but our attempts to obtain constitutively active MPK4 variants have so far failed.

EDS1 and PAD4 in the SA-ET-JA defense network

In addition to its role as a negative regulator of the SA pathway, MPK4 is involved in regulating ET/JA-dependent defenses. We show that our initial report of reduced *PDF1.2*

mRNA induction in response to JA extends to the ET response, and that MPK4 kinase activity is required for *PDF1.2* induction by both JA and ET. A block of *PDF1.2* expression in *mpk4* is also seen in response to *A. brassicicola* infection. This reflects a physiologically important defect in induction of ET/JA-dependent defenses, because resistance to *A. brassicicola* is lost in *mpk4*, *mpk4/nahG* and, to some degree, in *mpk4*/*pad4* mutants.

The analysis of genetic interactions between *MPK4*, *EDS1* and *PAD4* supports a model of how MPK4 activity is required for both repression of SA and activation of ET/JA defenses. In this model, EDS1, and PAD4 to a lesser extent, are central to the antagonism between the SA and ET/JA defense pathways, acting as positive regulators of SA accumulation and negative regulators of ET/JA defense signaling. Both of these functions are negatively influenced, perhaps indirectly, by MPK4 activity. In the absence of MPK4 activity, EDS1 and PAD4 are effective as SAR activators mainly through SA amplification, and as ET/JA defense repressors via a function that does not rely on SA accumulation (Figure 8). Such an SA-independent function of PAD4 was previously suggested based on expression profiling experiments following bacterial infection of *pad4* mutants and mutants impaired in SA biosynthesis (Glazebrook *et al.*, 2003). This model is also consistent with the fact that *eds1*, *pad4* and *nahG* all suppress SA accumulation and *Pst* DC3000 resistance in *mpk4*, and that *eds1*, but not *nahG*, restores *A. brassicicola* resistance and *PDF1.2* inducibility by MeJA in *mpk4*. We note, however, that while our genetic data are consistent with this model, they do not exclude alternative scenarios for the actions of MPK4, EDS1 and PAD4 relative to each other in defense signaling.

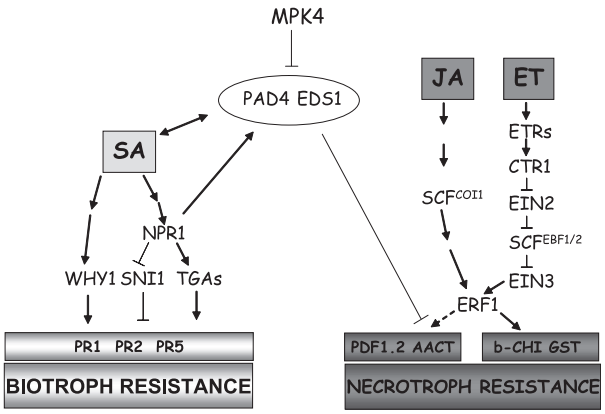

Figure 8. A model for MPK4 action in plant defense pathways.
Central points include:
EDS1 and PAD4 act as activators of the SA pathway and repressors of the ET/JA pathway
MPK4 negatively regulates both activities of EDS1 and PAD4
The repressive pathway acting in the ET/JA defense system is independent of known activating pathways converging on ERF1
The repressive pathway influences only a subset of known genes associated with the ET/JA defense pathway (such as PDF1.2 and anthocyanin-5-aromatic acyl transferase, AACT).

The strong reduction of *PDF1.2* expression in *mpk4* mutants that over-express transgenic ERF1 suggests that the repressive effects of MPK4–EDS1/PAD4 are mediated either downstream or independently of ERF1. As discussed below, given the poor overlap between ERF1-induced genes and MPK4-dependent, ET-related genes, this repression probably occurs independently of ERF1. Inhibition of the repressive effects on *PDF1.2* expression of EDS1/PAD4 clearly requires MPK4 activity, although it apparently does not involve induction of MPK4 activity above basal levels, because we have not detected enhanced MPK4 activity is response to MeJA, ET or in *ctr1* mutant backgrounds (P. Brodersen, unpublished results). Rather, it is likely to involve the action of other factors, as hyper-accumulation of *PDF1.2* mRNA is seen in *ctr1–2*/*pad4–2* and *ctr1–2*/*pad4–2*/*eds1–2* compared with *ctr1–2* that all have active MPK4.

A model in which EDS1 and PAD4 act as direct repressors of ET/JA signaling, in addition to more indirect effects via elevated SA levels, is consistent with the analysis of *PDF1.2* expression in *cpr6* mutants. *cpr6–1*/*eds1–2* exhibits strongly enhanced *PDF1.2* expression compared with *cpr6–1* even upon application of exogenous SA (Clarke *et al.*, 2001). In addition, as NPR1 is required for positive feedback induction of *EDS1* and *PAD4* by SA (Falk *et al.*, 1999; Jirage *et al.*, 1999), the failure of SA to repress JA induction of *PDF1.2* in *npr1–1* (Spoel *et al.*, 2003), as well as the hyper-induction of *PDF1.2* in *cpr6*/*npr1* (Clarke *et al.*, 2000), may be in part due to impaired *EDS1* and *PAD4* induction. Similarly, it is possible that NPR1-dependent repression of *PDF1.2* by WRKY70 (Li *et al.*, 2004) involves enhanced expression of *EDS1* and *PAD4*.

The EDS1 and PAD4 proteins both consist of multiple domains of unknown function. Taken together with their functions in both SA and ET/JA defense regulation, this raises the same question of genetic separability that we have attempted to address for MPK4. Answering this question and, if possible, assigning SA- or ET/JA-related functions to specific domains in EDS1 and PAD4, are goals for future research that could make use of the *mpk4*/*eds1* and *mpk4*/*pad4* mutants described here.

MPK4-dependent, ET-related genes

The MPK4-dependent set of ET response genes is narrow, and does not comprise all defense-related ET response genes. For example, the induction of *b-CHI* and several putative *R* genes was independent of MPK4. Some genes in the MPK4-dependent set, including *PDF1.2* and a few cell wall proteins or modifying enzymes, have known or possible defense-related functions, but their relationships to ET responses are unknown.

ERF1 induction is an important event in the activation of the ET/JA defense system that depends on activators such as EIN2, EIN3 and COI1 (Lorenzo *et al.*, 2003), and can be triggered by *ctr1* mutation (Solano *et al.*, 1998). Our microarray data suggest that, rather than acting downstream of ERF1 in the activating pathway, MPK4 acts in a repressive pathway independent of this activating pathway. First, the overlap of ET-related genes induced by ERF1 over-expression (Lorenzo *et al.*, 2003) with MPK4-dependent, ET-related genes is very limited. Second, the inducing effect of the *ctr1* mutation, and the repressive effect of the *mpk4* mutation, are largely additive for many of the MPK4-dependent, ET-related genes revealed by the microarray analysis.

For some of the MPK4-dependent genes, repression appears to be mediated at least in part by PAD4 and EDS1. This is the case for *PDF1.2* and *AACT*. Such genes may be involved in the defense response to necrotrophic fungi, because resistance to *A.brassicicola* in *mpk4* is largely restored by *pad4* or *eds1* mutation. *PDF1.2* is clearly associated with defense responses, and the same may be true for *AACT*, as it is one of a small set of genes that are hyper-induced by JA in the *jin1* mutant that exhibits enhanced resistance to necrotrophic fungi (Lorenzo *et al.*, 2004; R. Solano, CNB-CSIC, Madrid, Spain, personal communication).

The regulation of several other MPK4-dependent genes, including the BEE1 transcription factor, does not involve EDS1 and PAD4. It is currently unclear whether these genes are defense-associated, or involved in other MPK4-dependent, ET-regulated processes. Nonetheless, the fact that BEE1 is included in this set of genes suggests that their induction involves enhanced BEE1 expression. Significantly, the BEE family of transcription factors comprising the three closely related *BEE1*, *BEE2* and *BEE3* genes (Friedrichsen *et al.*, 2002) is required for ET induction of at least one gene in this set (P. Brodersen, J. Mundy, J. Nemhauser and J. Chory, Salk Institute, La Jolla, USA, unpublished data). The possible involvement of this gene set in the ET/JA defense pathway is currently under investigation.

Experimental procedures

DNA constructs

Triple C-terminally HA-tagged MPK4 versions were constructed as described previously (Petersen *et al.*, 2000). The Quick-Change kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis. A 35S–ERF1–nos construct in pROK2 obtained from Joseph Ecker (Solano *et al.*, 1998) was used as template in a PCR reaction with 5′-phosphorylated 35S and nos primers, and the product was cloned into the *Sma*I site of pCAMBIA3300.

Plant constructions

mpk4/eds1–2 *and* mpk4/pad4–2. *mpk4* heterozygotes were crossed to *eds1–2* and *pad4–2*. F_1 and F_2 plants were allowed to self, and families heterozygous for *mpk4* and homozygous for *eds1–2* or *pad4–2* were selected on kanamycin and by PCR with primers detecting the *eds1–2* deletion (Falk *et al.*, 1999) or the *pad4–2* frameshift after DNA sequencing (Jirage *et al.*, 1999). Double mutants segregating from these families were identified by phenotype, confirmed by PCR, amplified and used for subsequent analyses.

ctr1–2/mpk4/pad4–2 *and* ctr1–2/mpk4/eds1–2. *ctr1–2* plants heterozygous for *mpk4* were crossed to *eds1–2* and *pad4–2*. F_1 plants were kanamycin-selected and selfed. In F_2, kanamycin-resistant *ctr1–2* homozygotes were identified by phenotype and allowed to self. F_3 families homozygous for *eds1–2* or *pad4–2* were then selected by PCR, and triple mutants maintained as *mpk4* heterozygotes.

ctr1–2/mpk4/pad4–2/eds1–2 *and* mpk4/pad4–2/eds1–2. A *ctr1–2/mpk4/pad4–2* triple heterozygous plant (above) was crossed to *eds1–2*, and a quadruple heterozygote, identified in F_1 by kanamycin selection and PCR detecting the *ctr1–2* deletion (Kieber *et al.*, 1993) and the *eds1–2* and *pad4–2* alleles as described above, was allowed to self. Among 140 kanamycin-resistant F_2 progeny, a single *pad4/eds1* recombinant heterozygous for *ctr1–2* and *pad4–2*, but homozygous for *eds1–2*, was identified. Kanamycin-resistant F_3 progeny homozygous for *ctr1–2*, or lacking the *ctr1–2* allele, were identified by phenotype and PCR, and *pad4–2* homozygotes were selected by PCR, giving rise to *ctr1–2/mpk4/pad4–2/eds1–2* and *mpk4/pad4–2/eds1–2* families.

Plant treatments

Plants were grown in growth chambers under long days (16 h light/8 h darkness) for all treatments other than *P. syringae* infections for which short-day regimes were used (8 h light/16 h darkness). Day and night temperatures were 21 and 16°C, respectively.

For ET inductions, plants were kept in 11 l polycarbonate jars (Nalgene, Rochester, NY, USA) sealed with silicon grease, and 0.54 μl ET was injected with a 27G syringe through a rubber membrane. MeJA inductions were performed as previously described (Petersen *et al.*, 2000).

NaPP1 was synthesized as described previously (Bishop *et al.*, 2000) and dissolved in DMSO at 10 mM. For plant treatments, this stock was diluted to 100 μM in water containing 0.01% Silwet and sprayed onto leaves of 2–3-week-old plants. For mock treatments, 1% DMSO in water with 0.01% Silwet was used.

Pst DC3000 growth curves were determined as described by Petersen *et al.* (2000).

Alternaria brassicicola strain MUCL 20297 was grown on 0.5% potato dextrose for 14–20 days until sporulation was dense. Spores were suspended in water, filtered through Miracloth and their titre determined by Fuchs–Rosenthal cytometer counting. Spores were applied to leaves of 3-week-old plants in three 15 μl droplets per leaf at 2.5×10^5 spores per ml, and symptoms were evaluated 7 days later.

RNA analysis

Total RNA was extracted by Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Northern blotting and synthesis of radiolabeled probes was performed according to standard protocols. cDNA templates for PR1 and PDF1.2 were amplified by PCR as described previously (Petersen *et al.*, 2000). A cDNA fragment specific for ERF1 was amplified from 35S–ERF1 in pROK2 (Solano *et al.*, 1998), cloned in

antisense orientation in front of the T7 promoter (Promega, Madison, WI, USA) in pGEM-T-easy and used as a template for *in vitro* transcription incorporating radiolabeled ^{32}P-UTP.

For reverse transcription (RT) and quantitative PCR analysis, RNA samples were first treated with RQ1 DNase (Promega, Madison, WI, USA). RT reactions were done with 1 μg of RNA and 0.5 μg of $(dT)_{21}$ primer at 42°C with 0.1 unit of reverse transcriptase (Promega) and 2 units of RNasin (Promega) for 1 h in 20 μl reactions. Quantitative PCR was performed using the SYBR Green protocol (Applied Biosystems, Foster City, CA, USA) with 10 pmol of each primer and a 0.5 μl aliquot of RT reaction product in a 25 μl reaction. Quantitative PCR reactions were performed in triplicate and averaged for each line individually. Quantification of the threshold cycle (C_T) values obtained by quantitative PCR analysis was achieved by the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001) after verifying that the value C_T(ubiquitin)-C_T(target) remained constant for each of the target genes tested over a 100-fold cDNA dilution series.

SA measurements

Total SA was extracted and quantified as described by Newman *et al.* (2001).

Kinase assays

MPK4 versions were immunoprecipitated with 12CA5 anti-HA antibody as described previously (Petersen *et al.*, 2000). After three washes in immunoprecipitation buffer and one wash in kinase assay buffer, immunoprecipitates were incubated in 30 μl kinase buffer (20 mM Tris, pH 7.5, 2 mM EGTA, 30 mM $MgCl_2$, 1 mM Na_3VO_4, 50 μM ATP) with 5 μg myelin basic protein and 3 μCi of ^{32}P-ATP (3000 Ci mmol^{-1}) at 30°C for 30 min. Reactions were stopped by addition of SDS sample buffer and products resolved by SDS–PAGE. For inhibition assays with NaPP1 and NMPP1, immunoprecipitates were incubated with or without inhibitor in kinase assay buffer for 10 min on ice before addition of substrates. MPK4-HA and EDS1 Western blots were performed as described previously (Feys *et al.*, 2001; Petersen *et al.*, 2000).

Microarray hybridization and analysis

Total RNA was isolated from three independent replicates of *ctr1–2*, *mpk4*, *ctr1–2/mpk4* and WT (one Col-0 sample, two L*er* samples). The RNA was amplified and hybridized to 12 Affymetrix microarrays according to Affymetrix protocols (Affymetrix UK Ltd., High Wycombe, UK). Raw intensity data was normalized using R implementation of qspline (Gautier *et al.*, 2004; Workman *et al.*, 2002). An implementation of the logit-t method in the statistical language R (Lemon *et al.*, 2003), applying two-way ANOVA instead of a *t*-test, was used to calculate statistical significances of differential gene expression. False-positive rates were estimated by recalculating *P*-values with permuted sample categories. This procedure was repeated four times, generating four sets of 22 810 permuted *P*-values. The *P*-value cut-off was chosen so that only one permuted *P*-value was lower than the cut-off. The resulting *P*-value cut-offs were 0.005 for the main two effects and 0.01 for the interaction effect. Gene expression index values were calculated using perfect match-only implementation (Gautier *et al.*, 2004) of the method introduced by Li and Wong (2001). Gene expression profiles from significantly differentially expressed genes were clustered by partitioning around medoids (PAM) clustering ($k = 12$). Classes I and II correspond to PAM clusters 10 and 7, respectively. The data (raw and gene *P*-values) are publicly accessible from ArrayExpress under accession number E-MEXP-174 at http://www.ebi.ac.uk/arrayexpress/query/entry.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Mutants expressing inactive MPK4 proteins are hypersusceptible to *Alternaria brassicicola*.

Figure S2. Mutations in PAD4 or EDS1 suppress the block of PDF1.2 induction in *mpk4*.

Figure S3. MPK4-independent induction of two class-II genes.

Table S1 Full list of Class-I genes

Table S2 Full list of Class-II genes

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

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