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

Peter Brodersen1, Morten Petersen1, Henrik Bjørn Nielsen2, Shijiang Zhu1, Mari-Anne Newman3, Kevan M. Shokat4, Steffen Rietz5, and John Mundy1,*

1Institute of Molecular Biology, Copenhagen University, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark, 2Center for Biological Sequence Analysis, BioCentrum-DTU, Building 208, Technical University of Denmark, DK-2800 Lyngby, Denmark, 3Institute of Plant Biology, Royal Veterinary and Agricultural University, Thorvaldsenvej 40, DK-1871 Frederiksberg, Denmark, 4Department of Cellular and Molecular Pharmacology, University of California San Francisco, CA 94143-0450, USA, and 5Department of Plant–Microbe Interactions, Max-Planck Institute for Plant Breeding Research, Carl-von-Linné Weg 10, 50829 Cologne, Germany

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 have only 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 (Maltonado 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 Erwinia ichtorii, 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 defense PDF1.2 (Pennisetckx 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 SCFEBP1/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 SCFCO1 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 over-expression 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 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 defense 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 Ler 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 Ler and Col-0, raising the possibility that the enhanced susceptibility of mpk4/nahG compared with nahG strains in Col-0 and Ler 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-aminoacyclopropane-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.

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
examine this possibility, we expressed two inactive, HA-epitope-tagged MPK4 mutants, mpk4AEF and mpk4K72R, in the mpk4 null background. mpk4AEF cannot be activated by T-loop phosphorylation, while mpk4K72R 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 mpk4AEF 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 mpk4K72R, 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 mpk4K72R and mpk4AEF 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 mpk4K72R and mpk4AEF 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 MPK4Y124G and MPK4Y124A mutants were constructed and
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<sup>Y124G</sup> had SA levels as low as mpk4 mutants expressing transgenic WT MPK4 (Figure 2b). Both MPK4<sup>Y124G</sup> and MPK4<sup>Y124A</sup>, but not WT MPK4, were inhibited by NaPP1 (Figure 3a) and less potently by NMPP1 (not shown) in <i>in vitro</i> kinase assays with MPK4 versions immunopurified from total protein extracts. MPK4<sup>Y124G</sup> showed stronger NaPP1 inhibition than MPK4<sup>Y124A</sup> and was chosen for <i>in vivo</i> 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 <i>in vitro</i> assay described above, 100-fold higher NaPP1 concentrations were used for these <i>in vivo</i> experiments, as previously described in yeast (Bishop et al., 2000). PR1 mRNA accumulated specifically in MPK4<sup>Y124G</sup> 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<sup>Y124G</sup> (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 2](image1)

**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 <sup>32</sup>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 rRNA controls following high performance liquid chromatography fractionation.
(c) Accumulation of PR1 mRNA. RNA was extracted from 3-week-old leaves, and blots hybridized to <sup>32</sup>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.

![Figure 3](image2)

**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 Ler) 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/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 disproportion-ate *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
showed that accumulation of PDF1.2 mRNA was significantly reduced (4–7-fold when quantified by PhosphorImager 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/induced JA signaling downstream or independently of ERF1. If so, EDS1 could act as 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 ctrl–2 and ctrl–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 ctrl–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 ctrl/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 Ler (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; Feyes 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
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 (Ler 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 mpk4/ctr1–2/mpk4 became correspondingly lower than in mpk4 (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
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.

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g44420</td>
<td>Defensin PDF1.2a</td>
</tr>
<tr>
<td>At2g26020</td>
<td>Defensin PDF1.2b</td>
</tr>
<tr>
<td>At5g61160</td>
<td>Anthocyanin 5-aromatic acyltransferase-like</td>
</tr>
<tr>
<td>At1g73330</td>
<td>Protease inhibitor DR4</td>
</tr>
<tr>
<td>At1g18400</td>
<td>bHLH transcription factor BEE1</td>
</tr>
<tr>
<td>At3g14210</td>
<td>Putative myrosinase-associated protein</td>
</tr>
<tr>
<td>At5g65390</td>
<td>Arabinogalactan-protein AGP7</td>
</tr>
<tr>
<td>At1g78970</td>
<td>Lupeol synthase LUP1</td>
</tr>
<tr>
<td>At1g20190</td>
<td>Expansin EXP11</td>
</tr>
<tr>
<td>At3g60290</td>
<td>Oxidoreductase</td>
</tr>
<tr>
<td>At2g40670</td>
<td>Response regulator ARR16</td>
</tr>
<tr>
<td>At5g22460</td>
<td>Esterase/lipase/thioesterase family</td>
</tr>
<tr>
<td>At2g06850</td>
<td>Xyloglucan endotransglycosylase EXGT-A1</td>
</tr>
<tr>
<td>At2g38670</td>
<td>Putative xyloglucan endotransglycosylase</td>
</tr>
<tr>
<td>At1g44830</td>
<td>AP2 transcription factor</td>
</tr>
<tr>
<td>At4g02290</td>
<td>Endo-1,4-beta glucanase-like</td>
</tr>
<tr>
<td>At5g65390</td>
<td>Esterase/lipase/thioesterase family</td>
</tr>
<tr>
<td>At1g78970</td>
<td>Lupeol synthase LUP1</td>
</tr>
<tr>
<td>At1g27460</td>
<td>Calmodulin-binding protein-like</td>
</tr>
<tr>
<td>At2g47880</td>
<td>Glutaredoxin</td>
</tr>
<tr>
<td>At5g10430</td>
<td>Arabinogalactan-protein AGP4</td>
</tr>
<tr>
<td>At5g60920</td>
<td>Phytochelatin synthetase-like COB</td>
</tr>
<tr>
<td>At3g16370</td>
<td>GDSL-motif lipase/hydrolase protein</td>
</tr>
<tr>
<td>At5g48900</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td>At4g25260</td>
<td>Pectin esterase-like</td>
</tr>
<tr>
<td>At2g38180</td>
<td>GDSL-motif lipase/hydrolase protein</td>
</tr>
<tr>
<td>At5g24570</td>
<td>GDSL-motif lipase/hydrolase protein</td>
</tr>
</tbody>
</table>

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.

### 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 Ler), 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.

**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
MPK4 regulation of defenses via EDS1 and PAD4

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>At2g38530</td>
<td>Non-specific lipid transfer protein 2 (LTP 2)</td>
</tr>
<tr>
<td>At3g16770</td>
<td>AP2 transcription factor EBP</td>
</tr>
<tr>
<td>At4g30290</td>
<td>Putative xyloglucan endotransglycosylase</td>
</tr>
<tr>
<td>At1g62380</td>
<td>ACC oxidase AC02</td>
</tr>
<tr>
<td>At5g17880</td>
<td>TIR-NBS-LRR class R-protein-like</td>
</tr>
<tr>
<td>At5g36930</td>
<td>TIR-NBS-LRR class R-protein-like</td>
</tr>
<tr>
<td>At1g63880</td>
<td>TIR-NBS-LRR class R-protein-like</td>
</tr>
<tr>
<td>At5g57240</td>
<td>Oxysterol-binding protein-like</td>
</tr>
<tr>
<td>At1g33790</td>
<td>Jaccalin lectin family</td>
</tr>
<tr>
<td>At3g12500</td>
<td>Basic endochitinase PR3</td>
</tr>
<tr>
<td>At3g02600</td>
<td>Beta-glucosidase-like</td>
</tr>
<tr>
<td>At2g16060</td>
<td>Non-symbiotic hemoglobin AHB1</td>
</tr>
<tr>
<td>At3g22840</td>
<td>Early light-induced protein ELIP2</td>
</tr>
<tr>
<td>At5g17890</td>
<td>TIR-NBS-LRR class R-protein-like</td>
</tr>
<tr>
<td>At4g16260</td>
<td>Glucan endo-1,3-beta-glucosidase-like</td>
</tr>
<tr>
<td>At5g36220</td>
<td>Cytochrome P450 CYP91A1</td>
</tr>
<tr>
<td>At2g02850</td>
<td>Plastocyanin-like</td>
</tr>
<tr>
<td>At3g44970</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>At5g02760</td>
<td>Protein phosphatase 2C</td>
</tr>
<tr>
<td>At4g14690</td>
<td>Early light-induced protein ELIP1</td>
</tr>
<tr>
<td>At5g05750</td>
<td>DnAJ protein family</td>
</tr>
<tr>
<td>At1g62770</td>
<td>Pectin esterase-like</td>
</tr>
<tr>
<td>At3g16430</td>
<td>Myrosinase binding protein-like</td>
</tr>
<tr>
<td>At5g64570</td>
<td>Glycosyl hydrolase</td>
</tr>
<tr>
<td>At1g73830</td>
<td>bHLH transcription factor BEE3</td>
</tr>
<tr>
<td>At5g67060</td>
<td>bHLH transcription factor bHLH088</td>
</tr>
<tr>
<td>At3g23150</td>
<td>Ethylene receptor-related ETR2</td>
</tr>
<tr>
<td>At2g30520</td>
<td>ROOT PHOTOTROPISM 2 RPT2</td>
</tr>
<tr>
<td>At1g79000</td>
<td>Acetyltransferase-related protein 2 PCAT2</td>
</tr>
<tr>
<td>At1g10480</td>
<td>C2H2-type zinc finger protein ZFP5</td>
</tr>
<tr>
<td>At5g05700</td>
<td>Squamosa promoter binding protein-like</td>
</tr>
<tr>
<td>At5g22510</td>
<td>Alkaline/neutral invertase</td>
</tr>
<tr>
<td>At3g14230</td>
<td>AP2 transcription factor</td>
</tr>
<tr>
<td>At5g25350</td>
<td>F-box LRR protein</td>
</tr>
<tr>
<td>At1g58700</td>
<td>Glutathione-S-transferase-like</td>
</tr>
<tr>
<td>At1g31580</td>
<td>CXC750</td>
</tr>
<tr>
<td>At3g18280</td>
<td>Lipid transfer protein/protease inhibitor family</td>
</tr>
<tr>
<td>At1g59124</td>
<td>CC-NBS-LRR class R-protein-like</td>
</tr>
<tr>
<td>At3g43600</td>
<td>Aldehyde oxidase 2 AAO2</td>
</tr>
<tr>
<td>At1g14210</td>
<td>Ribonuclease-like</td>
</tr>
<tr>
<td>At1g55520</td>
<td>Serine acetyltransferase SAT1</td>
</tr>
<tr>
<td>At1g69310</td>
<td>WRKY family transcription factor WRKY57</td>
</tr>
<tr>
<td>At3g17510</td>
<td>CBL-interacting protein kinase 1 CIPK1</td>
</tr>
<tr>
<td>At3g57410</td>
<td>VILIN 3 VLN3</td>
</tr>
<tr>
<td>At4g34250</td>
<td>Fatty acid elongase 1 FAE1</td>
</tr>
<tr>
<td>At1g31600</td>
<td>Oxidoreductase</td>
</tr>
<tr>
<td>At2g27050</td>
<td>EIN3-like transcription factor EIL1</td>
</tr>
<tr>
<td>At5g25890</td>
<td>IAA28</td>
</tr>
<tr>
<td>At4g27300</td>
<td>S locus protein kinase-like</td>
</tr>
<tr>
<td>At3g58550</td>
<td>Lipid transfer protein/protease inhibitor family</td>
</tr>
</tbody>
</table>

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.

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 (Spelo 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 hyperinduced 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 3SS–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 Smal site of pCAMBIA3300.

Plant constructions

mpk4/eds1–2 and mpk4/pad4–2. mpk4 heterozygotes were crossed to eds1–2 and pad4–2. F1 and F2 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 deletion 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.

ctrl1–2/mpk4/pad4–2 and ctrl1–2/mpk4/eds1–2. ctrl1–2 plants heterozygous for mpk4 were crossed to eds1–2 and pad4–2. F1 plants were kanamycin-selected and selfed. In F2, kanamycin-resistant ctrl1–2 homozygotes were identified by phenotype and allowed to self. F2 families homozygous for eds1–2 or pad4–2 were then selected by PCR, and triple mutants maintained as mpk4 heterozygotes.

ctrl1–2/mpk4/pad4–2/eds1–2 and mpk4/pad4–2/eds1–2. A ctrl1–2/mpk4/pad4–2 triple heterozygous plant (above) was crossed to eds1–2, and a quadruple heterozygote, identified in F1 by kanamycin selection and PCR detecting the ctrl1–2 deletion (Kieber et al., 1993) and the eds1–2 and pad4–2 alleles as described above, was allowed to self. Among 140 kanamycin-resistant F2 progeny, a single pad4/eds1 recombinant heterozygous for ctrl1–2 and pad4–2, but homozygous for eds1–2, was identified. Kanamycin-resistant F2 progeny homozygous for ctrl1–2, or lacking the ctrl1–2 allele, were identified by phenotype and PCR, and pad4–2 homozygotes were selected by PCR, giving rise to ctrl1–2/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 applied to leaves of 3-week-old plants in three 15 l droplets per leaf. Spores were suspended in water, filtered through Miracloth and their titre was determined by Fuchs–Rosenthal cytometer counting. Spores were applied to leaves of 2–3-week-old plants. For mock treatments, 1% DMSO in water containing 0.01% Silwet was used. For ET treatments, the stock was diluted to 100 µl in water containing 0.01% Silwet 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 32P-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 (Ct) values obtained by quantitative PCR analysis was achieved by the 2-Ct method (Livak and Schmittgen, 2001) after verifying that the value C (ubiquitin)-C (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 MgCl2, 1 mM Na3VO4, 50 μM ATP) with 5 μg myelin basic protein and 3 μCi of 32P-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 (Feyes et al., 2001; Petersen et al., 2000).

**Microarray hybridization and analysis**

Total RNA was isolated from three independent replicates of ctr-1-2, mpk4, ctr-1-2/mpk4 and WT (one Col-0 sample, two Ler 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 (Bardwell et al., 2000) 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 matching 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.

**Acknowledgements**

We thank J. Ecker for providing the 3S–ERF1 plasmid, and W. Brokaert for A. brassicicola. L. Navarro and Z. Nimchuk are thanked for critical reading of the manuscript. This work was supported by grants to P.B. from the Faculty of Science, University of Copenhagen, and to J.M. from the Danish Research Councils (23–01–0145) and the European Union (hprrct20000093).

**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

**References**


