CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector
Cell, Vol. 103, 1111–1120, December 22, 2000, Copyright ©2000 by Cell Press

Arabidopsis MAP Kinase 4 Negatively Regulates Systemic Acquired Resistance

Morten Petersen,[1,9] Peter Brodersen,[1,9] Henrik Naested,[1] Erik Andreasson,[1] Ursula Lindhart,[1] Bo Johansen,[2] Henrik B. Nielsen,[3] Michelle Lacy,[4] Mark J. Austin,[5] Jane E. Parker,[5] Sashi B. Sharma,[6] Daniel F. Klessig,[6] Rob Martienssen,[7] Ole Mattsson,[1] Anders B. Jensen,[1] and John Mundy[1,8]
[1]Institute of Molecular Biology
[2]Botanical Institute
Copenhagen University
Øster Farimagsgade 2A
1353 Copenhagen K
Denmark
[3]Center for Biological Sequence Analysis
Danish Technical University
2800 Lyngby
Denmark
[4]Monsanto Co.
800 North Lindbergh Boulevard
St. Louis, Missouri 63167
[5]Sainsbury Laboratory
John Innes Center
Colney
Norwich NR4 7U11
United Kingdom
[6]Waksman Institute
Rutgers University
Piscataway, New Jersey 08854
[7]Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, New York 11724

Summary

Transposon inactivation of *Arabidopsis* MAP kinase 4 produced the *mpk4* mutant exhibiting constitutive systemic acquired resistance (SAR) including elevated salicylic acid (SA) levels, increased resistance to virulent pathogens, and constitutive pathogenesis-related gene expression shown by Northern and microarray hybridizations. MPK4 kinase activity is required to repress SAR, as an inactive MPK4 form failed to complement *mpk4*. Analysis of *mpk4* expressing the SA hydroxylase NahG and of *mpk4/npr1* double mutants indicated that SAR expression in *mpk4* is dependent upon elevated SA levels but is independent of NPR1. *PDF1.2* and *THI2.1* gene induction by jasmonate was blocked in *mpk4* expressing NahG, suggesting that MPK4 is required for jasmonic acid–responsive gene expression.

[8]To whom correspondence should be addressed (e-mail: mundy@biobase.dk)
[9]These authors contributed equally to this work.

Introduction

Plant disease resistance is elicited by specific recognition of pathogen-derived molecules (Staskawicz et al., 1995). These interactions lead to rapid necrosis at the site of pathogen entry (the hypersensitive response, HR) and induction of a plant immune response known as systemic acquired resistance (SAR; Yang et al., 1997). SAR provides protection in uninfected parts of the plant against a spectrum of pathogens and is correlated with the expression of pathogenesis-related (PR) proteins, some with antimicrobial activity. The onset of SAR is also associated with increased levels of salicylic acid (SA) both at the infection site and systemically (Malamy et al., 1990). SA is necessary and sufficient for SAR induction, since exogenous SA application induces SAR and *PR* gene expression (Ward et al., 1991), while expression in plants of the bacterial salicylate hydroxylase (NahG) depletes SA and suppresses SAR (Gaffney et al., 1993).

Genetic approaches have been used in *Arabidopsis* to unravel plant defense pathways. Screens have identified recessive mutants affected in SA signaling that are also hypersusceptible to pathogens. For example, the *pad4*, *sid1*, and *sid2* mutations compromise SA accumulation in response to pathogen infection (Zhou et al., 1998; Nawrath and Metraux, 1999). The *eds1* mutation also operates upstream of SA-mediated plant defenses (Falk et al., 1999). In contrast, *npr1* mutants are able to accumulate SA but fail to mount SAR after pathogen infection or application of SA, implicating NPR1 in SA perception and downstream responses (Cao et al., 1994; Delaney et al., 1995). *PAD4* and *EDS1* encode lipase-like proteins (Falk et al., 1999; Jirage et al., 1999), whereas *NPR1* encodes an ankyrin repeat protein (Cao et al., 1997). NPR1 interacts with basic leucine zipper transcription factors that bind to *PR1* promoter elements, suggesting a direct link between NPR1 activity and regulation of *PR* gene expression (Zhang et al., 1999).

Other *Arabidopsis* mutations cause enhanced disease resistance. While many of these mutants exhibit HR-like lesions in the absence of pathogen challenge (so-called lesion-mimic mutants), there are only a few reports of constitutive defense mutants without necrotic lesions (*cpr1*, Bowling et al., 1994; *cpr6*, Clarke et al., 1998). Most, if not all, constitutive defense mutants accumulate elevated levels of SA and express *PR* genes constitutively. The presence of *nahG* in these mutants suppresses *PR* gene expression and distinct aspects of their enhanced resistance to bacteria and oomycete pathogens.

While these genetic analyses confirm the importance of SA and *NPR1* in regulating SAR, they also reinforce evidence for both *NPR1*- and SA-independent disease resistance pathways that are regulated by ethylene and jasmonic acid (JA; Pieterse and van Loon, 1999). For example, *PR* gene expression in *cpr6* requires SA but not NPR1, although NPR1 is necessary for bacterial resistance (Clarke et al., 1998). In contrast, the *ssi1* mutation completely bypasses *npr1* but depends on SA to

induce both *PR1* and expression of *PDF1.2*, a JA-responsive defensin (Shah et al., 1999). Thus, the CPR6 and SSI1 proteins may participate in signal communication between SA- and JA-dependent pathways. Such pathway cross-talk is consistent with studies demonstrating antagonism between SA and JA signaling in defenses against pathogens and insect herbivores (Felton et al., 1999; Gupta et al., 2000).

Molecular and biochemical analyses suggest that plant defense responses also involve MAP kinase (MAPK) activities (Ligterink et al., 1997; Zhang and Klessig, 1998; Romeis et al., 1999). Eukaryotic MAPKs act downstream of MAPK kinases (MAPKK) and MAPKK kinases (MAPKKK) in reversible phosphorylation cascades to transduce extracellular signals into cellular responses. While these events amplify specific signals, they also integrate different signals by cross-talk via higher-order complexes (Madhani and Fink, 1998). Consistent with this, a tobacco MAPK (SIPK) was found to be activated by SA, fungal elicitors, and viral infection and may be part of a cascade including the interacting SIPKK (Liu et al., 2000). Many important substrates for MAPKs are transcription factors that control the expression of downstream genes. Although *Arabidopsis* contains numerous MAPKs, their precise roles in perception of external stimuli and plant stress responses have not been determined (Mizoguchi et al., 1997).

Here we describe a recessive, transposon-tagged *Arabidopsis* mutant exhibiting constitutive defense responses without spontaneous necrotic lesions, including elevated SA levels and resistance to oomycete and bacterial pathogens. RNA blot and cDNA microarray hybridizations demonstrate that the mutant constitutively expresses *PR* genes normally induced by SA and fails to induce *PDF1.2* and *THI2.1* mRNA in response to JA. Molecular cloning, revertant analysis, and complementation studies demonstrate that the phenotype of the mutant (*mpk4*) is caused by loss of MPK4 activity. These data suggest a role of MPK4 in regulating plant defenses against pathogens.

Results

Analysis of the *mpk4* Mutant and *MPK4* Alleles

The *mpk4* mutant is a dwarf identified among stable transposant lines generated with a modified maize *Ds* element (Figure 1A; Sundaresan et al., 1995). *mpk4* has curled leaves and flowers with reduced pollen production and fertility. Microscopy revealed that *mpk4* dwarfism was caused by decreased cell size (Figure 1A). *mpk4* seed germinated with normal cotyledons and first exhibited dwarfism at the two- to three-leaf stage. No necrotic lesions were detectable on *mpk4*. In progeny of *mpk4* heterozygotes, the recessive dwarfing allele cosegregated with *Ds*-encoded kanamycin resistance. To identify this allele, genomic DNA flanking *Ds* was isolated, and sequencing revealed that *Ds* was integrated eight nucleotides upstream of the acceptor site of the first intron of *MPK4* (Figure 1B). Three approaches demonstrated that this insertion was responsible for the *mpk4* phenotype. First, revertants were generated by *Ds* excision following crosses to a line expressing *Ac* transposase. This identified wild-type F3 plants homozygous

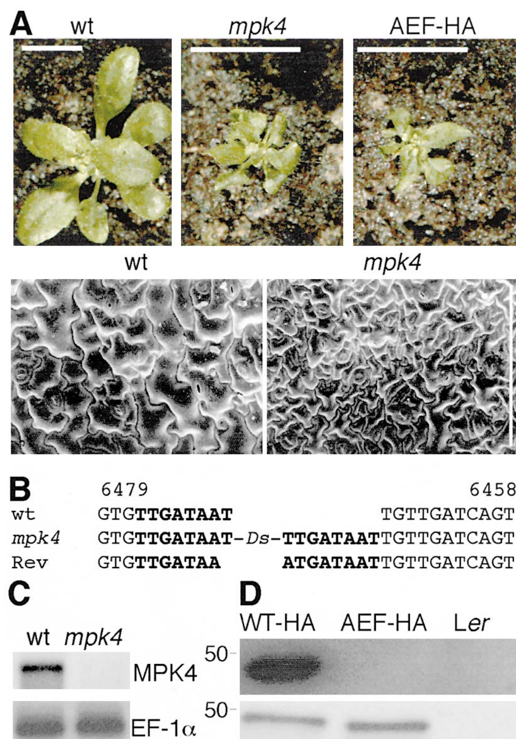

Figure 1. Phenotype of the *mpk4* Mutant and Inactivation of the *MPK4* Gene

(A) The upper panels show wild-type L*er*, *mpk4* homozygous, and *mpk4* expressing activation loop mutated MPK4 (T201A/Y203F; AEF-HA) at 21 days of growth in soil. Scale bar is 1 cm. The lower panels show a scanning electron micrograph of the adaxial leaf cells of the plants in the top panel. Scale bar is 100 μm.

(B) The top line shows the sequence of the *MPK4* first intron with acceptor site (AGT) from wild-type L*er*. The numbers above are base pairs from the same sequence of wild-type Col-0 (complement of GI:2191126; ABB61033). The middle line shows the 8 bp *Ds* target site insertion in *mpk4* (bold). The bottom line shows the 7 bp footprint with a single nucleotide change in the revertant produced by *Ds* excision.

(C) Northern blot of 10 μg total RNA from wild type (wt) and *mpk4* probed with radiolabeled MPK4 cDNA and EF-1α cDNA as a loading control.

(D) The upper panel shows kinase activities immunoprecipitated from *mpk4* expressing wild-type HA-tagged MPK4 (WT-HA) and mutated MPK4 (T201A/Y203F; AEF-HA). L*er* control is wild type without HA-tagged MPK4. The lower panel shows a Western blot of the same immunoprecipitates using anti-HA antibodies.

for kanamycin resistance. Genomic fragments were amplified from revertants, wild type, and *mpk4*. Sequencing revealed that *Ds* had created an 8 bp target site duplication on insertion in the *MPK4* intron and that a 7 bp footprint remained after *Ds* excision to restore the transcription unit (Figure 1B). Thus, transposition away from *MPK4* is linked to reversion of the dwarf phenotype. Second, RNA blot hybridization showed that *mpk4* homozygotes did not accumulate detectable MPK4 mRNA, in contrast to wild type (Figure 1C) as well as the revertant (not shown). Third, *mpk4* mutants were rescued by transformation with a 3.3 kbp fragment containing *MPK4* and 1150 bp of 5′ upstream and 506 bp of 3′ downstream sequence. In addition, *mpk4* was complemented with the same genomic fragment containing a

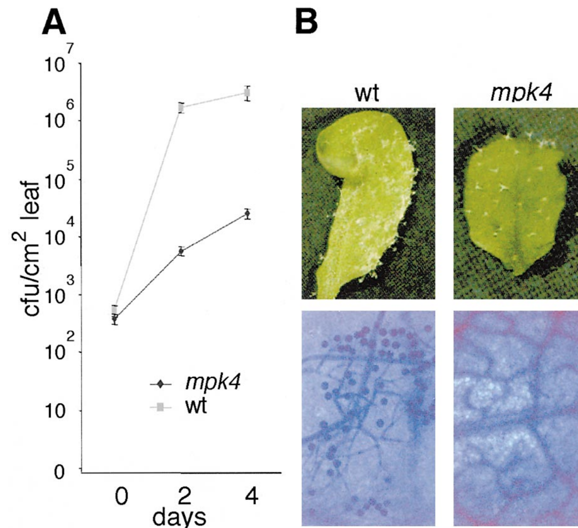

Figure 2. Resistance of *mpk4* to Bacterial and Oomycete Pathogens

(A) Four-week-old wild-type and *mpk4* plants were inoculated with the virulent strain DC3000 of *Pseudomonas syringae* pv. *tomato* at a concentration of 1×10^5 colony-forming units per ml (cfu/ml). Values represent average and standard deviations of cfu extracted from leaf disks in three independent samplings.

(B) Conidiospore suspensions of *P. parasitica* isolate Cala2 (4×10^5/ml) were sprayed onto 2-week-old wild-type and *mpk4* plants. Leaves were examined 3 and 7 days after inoculation macroscopically (left) and microscopically after lactophenol-trypan blue staining (right). Leaves shown here are from day 7. All pathology experiments were repeated at least twice with similar results.

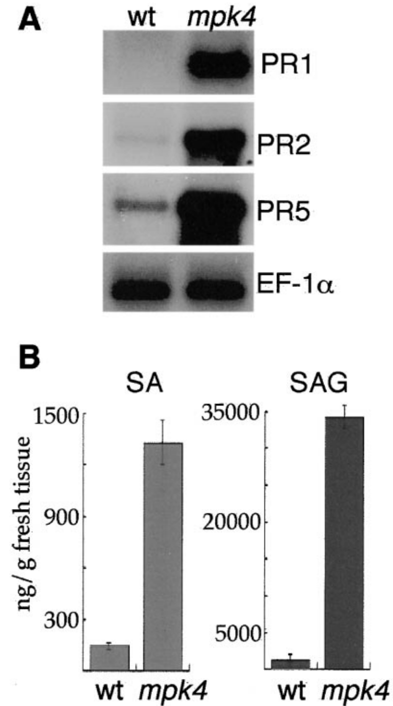

Figure 3. Accumulation of PR mRNAs and SA in Wild Type and *mpk4*

(A) RNA gel blots of 10 μg total RNA from wild type (wt) and *mpk4* probed with radiolabeled PR1, PR2, PR5, and EF-1α loading control.

(B) Leaves from 4-week-old plants grown in soil were harvested, and free SA and SAG contents (ng/g fresh weight) were quantified by HPLC.

triple HA epitope tag at the C terminus of MPK4. Western blotting and in-gel kinase assay showed that MPK4 is active in wild-type plants (Figure 1D). In contrast, equivalent levels of a catalytically inactive, HA-tagged MPK4 containing two mutations in activation loop residues (T201A/Y203F) had no effect on the *mpk4* phenotype (AEF-HA; Figures 1A and 1D). These results demonstrate that the *mpk4* phenotype is caused by loss of MPK4 kinase activity.

mpk4 Responds to Growth Regulators and Abiotic Stresses

Growth assays and RNA blot hybridization with target genes indicated that *mpk4* was not significantly impaired in responses to environmental stresses including desiccation, salt treatment, cold, or heat shock. *mpk4* responses to the phytohormones auxin, cytokinin, brassinosteroid, gibberellin, and abscisic acid were also normal (not shown). This suggests that the *mpk4* phenotype is not caused by defects in responses to any of these abiotic stresses and phytohormones. Although *mpk4* dwarfism was similar to that of ethylene constitutive triple response mutants (CTR; Kieber et al., 1993), *mpk4* did not exhibit a seedling CTR. In addition, *mpk4/ctr1-1* double mutants exhibited more extreme dwarfism than either mutant parent. Similarly, the double mutant *mpk4/ein2-2* (*ethylene-insensitive 2-2*; Johnson and Ecker, 1998) exhibited both *mpk4* dwarfism and *ein2-2* insensitivity to ethylene in the triple response assay (not shown). These data argue that MPK4 does not act in the ethylene response pathway between CTR1 and EIN2.

mpk4 Exhibits Increased Resistance to Pathogens

Constitutive defense response mutants such as *cpr1*, *ssi1*, and *lsd6* exhibit dwarfism and leaf curling similar to *mpk4* (Bowling et al., 1994; Weymann et al., 1995; Shah et al., 1999), so we examined resistance of *mpk4* to pathogens. We found that *mpk4* is highly resistant to a virulent bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Figure 2A), and to infection by a virulent isolate of the oomycete pathogen *Peronospora parasitica* (Cala2; Parker et al., 1996). This pathogen rapidly colonized and caused disease symptoms on wild-type plants but was undetectable in *mpk4* plants (Figure 2B). Thus, *mpk4* exhibits enhanced resistance to at least two unrelated types of pathogens.

mpk4 Expresses *PR* Genes Constitutively

Since *mpk4* exhibited resistance to pathogens, we compared the expression of *PR* genes in *mpk4* and wild type. RNA blots demonstrated that *PR1*, *PR2*, and *PR5*, which are normally induced during the development of SAR (Glazebrook, 1999), were constitutively expressed in *mpk4* (Figure 3A). This suggests that MPK4 negatively regulates the expression of these *PR* genes. In addition, *mpk4* expressing the inactive T201A/Y203F MPK4 form expressed *PR1* to the same level as the knockout mutant (not shown), indicating that MPK4 activity is required for the negative regulation of *PR* gene expression.

In order to identify a more complete set of downstream

Table 1. mRNAs Overexpressed in *mpk4* Seedlings

Fold[a] mpk4/wt	DB Number[b]	Gene or Homolog Description	Matrix 1 ls4[d]	Matrix 1 nt[c]	Matrix 2 ls10[d]	Matrix 2 nt
Northern						
	4454853	PR1	TTGACT	−706	GACTTTTC	−641
	6646759	PR5	TTGACT	−403	GACTAAAC	−398
Microarray[e]						
29.3	2288989	chitinase	TTGACT	−127	GACTTTTC	−566
23.8	1167961	extensin (EXT1)	TTGACT	−169c	GACTAACC	−203
19.2	166637	β-1,3-glucanase (BGL2/PR2)	TTGACT	−297	GACTTTTC	−400
11.6	553038	β-1,3-glucanase (BGL3)	TTGACT	−373	GACTTTTC	−474
11.3	1890156	glutathione S-transferase (ERD11)	TTGACT	−239	GACTATTC	−364
8.4	3461818	glutathione S-transferase	TTGACT	−206c	GACTAGTC	−208
7.5	6143882	monodehydroascorbate reductase	TTGACT	−119c	GACAATTC	−301
7.4	2160155	unknown				
6.9	903895	pectin methylesterase (PME1)	TTGATT	−84	GACGTTTC	−358
6.4	9758178	lipid transfer protein (MTE17.17)	TTGACT	−162	GACTTGAC	−163
6.2	4887748	LRR receptor kinase	TTGACT	−688c	GACTTATC	−705
6.2	2462835	hypothetical protein	TTGACT	−682	GACTAATC	−76c
5.4	7267528	LRR receptor kinase	TTGACT	−107c	GACTAACC	−451
5.3	3482931	oxalate oxidase-like (GLP5)	TTGACA	−191	GACTTTTC	−119c
5.3	7269612	stomatin-like	TTGACT	−73	GACTTTCC	−374
5.3	6686401	proline-rich, hypothetical protein	TTGACT	−106	GACTTTGC	−125c

[a] Fold mRNA accumulated in *mkp4* versus wild type determined by Northern or microarray hybridization.
[b] Protein database GI number; "unknown" is DNA GI number.
[c] Nucleotide position upstram of annotated ATG start codon; c denotes complementary strand.
[d] Sequences similar to *cis*-elements identified in the *PR1* promoter (linker scan 4 and 10, Lebel et al., 1998).
[e] mRNAs expressed more than 5-fold higher in *mpk4* than in wild type. Eighty percent (7864 of 9867) of the microarrayed *Arabidopsis* cDNAs hybridized significantly to *mpk4* and wild-type seedling cDNAs. In *mpk4* compared to wild type, 161 (2%) were between 2- and 4-fold underexpressed and 522 (6.6%) were greater than 2-fold overexpressed, while 7707 (98%) were between 3-fold over- and underexpressed.

genes in MPK4 signaling, we compared global gene expression in *mpk4* and wild-type seedlings by cDNA hybridization to a microarray of 9861 cDNAs expressed throughout *Arabidopsis* development (Ruan et al., 1998). This revealed that of the 7864 (80%) seedling cDNAs that hybridized, the majority hybridized at roughly equivalent levels in *mpk4* and wild type (7707 or 98% between 3-fold overexpressed and underexpressed). Only 16 cDNAs (≅0.2%) exhibited greater than 5-fold differences in hybridization levels between *mpk4* and wild type (Table 1). All 16 were more highly expressed in *mpk4*, suggesting that MPK4 is involved in the repression of a subset of genes. Database analysis showed that while the function of eight of these remain to be elucidated, eight encode well-known PR or wound-induced proteins (Glazebrook et al., 1997). These include chitinase and β-(1,3)-glucanases (PR2) that have antifungal activities, extensin and pectin methylesterase involved in cell wall modification (Merkouropoulos et al., 1999), and glutathione S-transferases, ascorbate reductase (Grantz et al., 1995), and oxalate oxidase (Zang et al., 1995), the latter potentially involved in oxidative cell wall cross-linking. In addition, lipid transfer proteins may contribute to plant defense (Molina and Olmedo, 1997), and LRR receptor kinases are involved in plant pathogen signaling (Glazebrook et al., 1997).

It has been reported previously that elicitor treatment of tobacco rapidly induces local and systemic expression of endoplasmic reticulum (ER)-resident folding chaperones including luminal binding protein (BiP), protein disulfide isomerase (PDI), and calreticulin (CRT; Jelitto-Van Dooren et al., 1999). This may prepare the ER for the massive upregulation of secreted PR proteins. The microarray analysis showed that *mpk4* also exhibits this response. cDNAs encoding homologues of the heat shock proteins HSP70 and HSP90 and of the reticuloplasmins BiP, PDI, CRT, and calnexin were between 3.5- to 4.5-fold more highly expressed in *mpk4* than in wild type (not shown).

The constitutive expression of the *PR* genes suggests that a pathway in which MPK4 participates may regulate the activity of a transcription factor or complex controlling *PR* gene expression. 5′ upstream sequences of 17 of these genes (15 from the microarray and PR1 and PR5) could be identified in the database. These sequences were searched for the occurrence of conserved sequence motifs that might be binding sites for common regulatory factors. Two consensus sequences were identified with statistically significant frequencies of occurrence (Table 1). One of these sequences, TTGACT ($p < 0.01$), is a negative regulatory element in the *Arabidopsis PR1* promoter (*LS4*; Lebel et al., 1998), and a similar element binds an elicitor-induced, WRKY transcription factor in the parsley *PR1* gene (W box; Eulgem et al., 1999). The other sequence (GACTWWHC, $p < 0.01$; W = A/T, H = A/T/C) is similar to a positive regulatory element in the *PR1* promoter (*LS10* or GGACTTTTC; Lebel et al., 1998). In contrast, the third *cis* element identified in the *PR1* promoter (*LS7* or G box; Lebel et al., 1998; Zhang et al., 1999) did not occur at a statistically significant frequency in these putative promoters.

SA-Dependent Signaling in *mpk4*

Since SA is necessary and sufficient for SAR, levels of SA and SA glucosides (SAG) were compared in wild type and *mpk4*. This showed that SA and SAG levels were

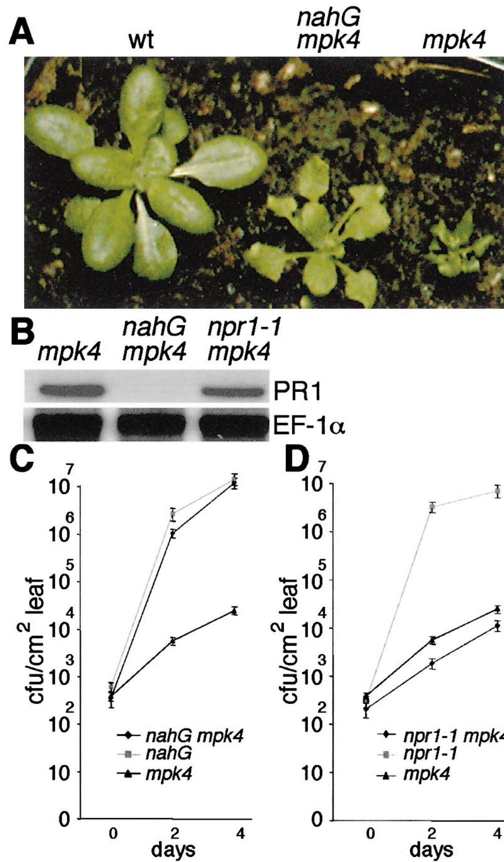

Figure 4. Phenotypes of *nahG/mpk4* and *npr1-1/mpk4* Plants

(A) Visible phenotypes of L*er* wild type (wt), a homozygous *mpk4* plant expressing NahG, and *mpk4*.

(B) RNA gel blot showing the accumulation of *PR1* mRNA in *mpk4*, homozygous *mpk4* expressing NahG, and the *npr1-1/mpk4* double mutant. EF-1α (bottom) is the loading control.

(C) Growth of the virulent strain DC3000 of *Pseudomonas syringae* pv. *tomato* after inoculation into *nahG/mpk4* and the parental *nahG* and *mpk4* lines. Experimental conditions were as described in Figure 2A.

(D) Similar experiment to (C) carried out on the *npr1-1/mpk4* double mutant and parental lines.

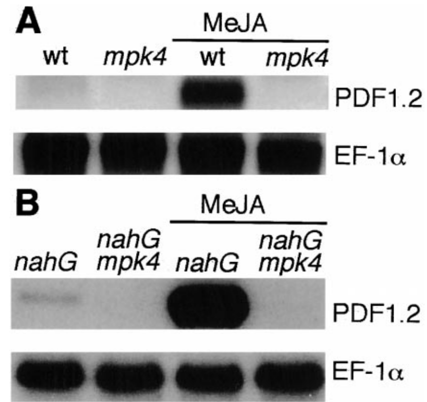

Figure 5. Accumulation of PDF1.2 mRNA in Wild Type, *mpk4* Mutant, and Plants Expressing *nahG*

(A) Northern blot showing the accumulation of the JA-inducible PDF1.2 mRNA in wild type (wt) and *mpk4*. EF-1α (bottom) is the loading control.

(B) Accumulation of PDF1.2 mRNA in wild type and *mpk4* mutants expressing *nahG*.

9- and 25-fold higher in *mpk4* (Figure 3B). The SA and SAG levels in *mpk4* are similar to those in *cpr1* (Bowling et al., 1994). Although both *CPR1* and *MPK4* are on chromosome 4, progeny analysis of crosses between *cpr1* and *mpk4* mutants demonstrated that they are not allelic (not shown).

Genetic approaches were used to determine whether the *mpk4* phenotype was SA dependent. Double heterozygous F1 progeny of crosses between homozygous *nahG* plants and plants heterozygous for *Ds* were identified by PCR. All dwarf F2 progeny were then shown to lack *nahG* by PCR. In addition, plants were identified with a partial suppression of *mpk4* dwarfism. All such plants were homozygous for *mpk4* and contained the *nahG* transgene (Figure 4A). The ability of *nahG* to suppress the *mpk4* phenotype was confirmed by measuring resistance toward *Pseudomonas syringae* pv. *tomato* DC3000 and the level of *PR1* expression in *mpk4/nahG* plants. This revealed that both bacterial resistance and *PR1* gene expression in *mpk4* were fully dependent upon SA (Figures 4B and 4C). Therefore, MPK4 functions upstream of SA in SAR signaling.

The *npr1-1* mutant is blocked in SA-mediated induction of *PR* genes (Cao et al., 1994). To examine whether *npr1-1* is epistatic to *mpk4*, the phenotypes of *mpk4/npr1-1* double mutants were examined. The double mutant fully retained *mpk4* dwarf stature, constitutively expressed *PR1*, and exhibited bacterial resistance as *mpk4* (Figures 4B and 4D). In addition, it exhibited the SA hypersensitivity typical of *npr1-1* seedlings (Bowling et al., 1997). Thus, either MPK4 and NPR1 participate in two different pathways leading to SAR, or MPK4 functions downstream of NPR1.

Gene Induction by Jasmonate Is Blocked in *mpk4*

PR gene overexpression in *mpk4* was the most striking difference revealed by the microarray analysis. However, eight genes hybridized >3 times less intensely to *mpk4* than wild-type cDNA. The most affected of these (GenBank Accession Number 4587541; <3.7-fold wild type) encodes a homolog of a myrosinase-associated protein from *Brassica napus* (MyAP; Taipalensuu et al., 1997). MyAP expression is induced by wounding and JA but is repressed by SA. JA is an important secondary signal in plant defense responses, and there is evidence for specific cross-talk between SA and the JA and ethylene signaling pathways (Pieterse and van Loon, 1999). We therefore compared the expression of *PDF1.2* and *THI2.1*, two JA-response genes, in wild type and *mpk4*. We found that *mpk4* does not express *PDF1.2* or *THI2.1* constitutively, unlike the *cpr* mutants *cpr5*, *cpr6*, and *ssi1* (Bowling et al., 1997; Clarke et al., 1998; Shah et al., 1999). More significantly, methyl jasmonate (MeJA) treatment failed to induce the expression in *mpk4* of *PDF1.2* (Figure 5A) and *THI2.1* (not shown). Since this could result from high SA levels antagonizing JA signaling (Felton et al., 1999; Gupta et al., 2000), PDF1.2 and THI2.1 mRNA accumulation after MeJA treatment was

examined in wild type and *mpk4* expressing *nahG*. This revealed that while PDF1.2 and THI2.1 mRNAs were induced by MeJA in *nahG* expressing wild type, they were not inducible in *mpk4/nahG* (Figure 5B; *THI2.1* not shown). These results indicate that MPK4 is required for *PDF1.2* and *THI2.1* expression in response to MeJA irrespective of the levels of SA in the plant.

Expression and Localization of *MPK4*

The expression pattern of *MPK4* was examined in transgenic plants carrying a transcriptional fusion between the GUS reporter and the same 1150 bp of 5′ upstream *MPK4* sequence used to drive the expression of the complementing genomic clones. In soil-grown plants, strong GUS activity was detected in the veins and stomatal guard cells of leaf plates, petioles, stem, and flowers, while leaf mesophyl cells showed weaker staining (Figure 6A). The leaf expression pattern was confirmed by in situ PCR with MPK4 cDNA specific primers which detected highest levels of MPK4 mRNA in phloem, leaf edges and stomata (Figure 6C). No signal was detected in non-reverse transcribed wild type leaves (Figure 6D), or in leaves of *mpk4* (not shown), confirming the specificity of the reaction. Vein and guard cell expression is shared by the SAR target gene *PR2* in a *cpr1* background (Bowling et al., 1994), and vein expression is shared by the putative SAR regulator, *SNI1* (*s*uppressor of *n*o *i*mmunity; Li et al., 1999). Curiously, in sterile-grown seedlings, GUS expression was largely confined to stomatal guard cells (Figure 6B). We do not yet know the basis for these apparent differences in MPK4 expression patterns.

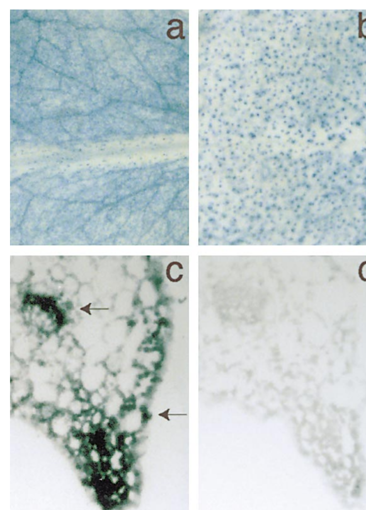

Figure 6. Expression Pattern of *MPK4*

Histochemical localization of the activity of the GUS reporter transcriptionally fused to 1150 bp of 5′ upstream sequence from *MPK4*.
(A) Leaf of 3-week-old soil-grown plant showing expression throughout the leaf, including veins and stomatal guard cells.
(B) Leaf of 10-day-old sterile-grown plant showing strong expression in guard cells.
(C) Localization of MPK4 mRNA by in situ PCR in soil-grown plants showing expression in leaf edges as well as guard cell and phloem (arrows).
(D) Serial section without reverse transcriptase added.

Discussion

Interactions between plants and pathogens involve recognition and signaling events that are distinct for different pathogen elicitors. However, many of these initial signals are integrated into convergent defense pathways (Yang et al., 1997; Glazebrook, 1999). One such pathway leads to the development of SAR, for which SA is a necessary and sufficient host signal.

Evidence reported here shows that the *mpk4* mutant exhibits constitutive SAR. Loss of *MPK4* function leads to increased SA levels, and similar to other SA-accumulating mutants, *mpk4* exhibits enhanced resistance to virulent pathogens. Furthermore, RNA blot analysis showed that *mpk4* constitutively expresses molecular markers of SAR. This was confirmed by microarray analysis that showed that mRNAs corresponding to 16 of the 7684 (0.2%) displayed cDNAs expressed in seedlings were statistically significantly more highly expressed in *mpk4* than in wild type. Eight of these 16 genes have been shown to be responsive to SA or induced by wounding and/or pathogen infection (Glazebrook et al., 1997). In addition, the 5′ upstream regions of these genes contain a consensus GACT WWHC motif and the W box (TTGACT) involved in the control of *Arabidopsis* and parsley *PR1* expression in response to elicitors and SA (Lebel et al., 1998; Eulgem et al., 1999). Thus, a specific set of effector genes involved in pathogen defense and SAR, presumably regulated via shared transcription factors, is constitutively expressed in *mpk4*.

Lesion-mimic mutants that constitutively express SAR and develop spontaneous necrotic lesions are common. Since coordinate activation of programmed cell death (PCD) and defense responses may result pleiotropically from disruption of cellular homeostasis (Mittler et al., 1995; Molina et al., 1999), the specific roles of genes defined by lesion-mimic mutations in defense signaling are uncertain. This raises the question of whether the constitutive SAR phenotype of *mpk4 is* a pleiotropic effect of disturbances of normal cell function in *mpk4*. However, several lines of evidence indicate that MPK4 specifically acts as a negative regulator of SAR. First, *mpk4* does not exhibit necrotic lesions and therefore does not fall into the common class of lesion-mimic mutants. The lack of spontaneous cell death in *mpk4* is critical, since disruption of normal cell function might be expected to turn on PCD pathways. Second, if the *mpk4* phenotype were a pleiotropic effect of an unbalanced biochemical state induced by the *mpk4* mutation, a general activation of defenses, including SA- and JA-dependent pathways, might be expected. Simultaneous activation of SA- and JA-dependent defense pathways is seen in *acd2*, *ssi1*, *cpr5*, and *cpr6* (Greenberg et al., 1994; Penninckx et al., 1996; Bowling et al., 1997; Clarke et al., 1998; Shah et al., 1999) as well as in tobacco plants expressing a bacterial proton pump (Mittler et al., 1995). However, in *mpk4* these two major defense pathways are oppositely affected, since SA-dependent defenses are constitutively expressed, while induction of JA-dependent defense genes is blocked. Third, microarray hybridization showed no other obvious differences than in defense related transcripts, suggesting that SAR expression is the only deviation from homeostasis in *mpk4*. In addition, *mpk4* responded normally

to a range of abiotic stresses and phytohormones, and MPK4 is, therefore, not involved in responses to these stimuli. Fourth, MPK4 is constitutively active under normal conditions and its activity is required to repress SAR, since the inactive MPK4 mutant (T201A/Y203F) failed to complement the dwarf and *PR1* expression phenotypes of *mpk4*. This argues that inappropriate cross-talk between MAPK isoforms in the *mpk4* mutant is not the cause of constitutive SAR expression. Thus, SAR is negatively regulated by MPK4 kinase activity.

NahG abolishes *PR* gene expression and bacterial resistance in *mpk4* and partially suppresses *mpk4* dwarfism. This indicates that MPK4 functions upstream of SA in SAR signaling. Also, since extreme dwarfism and constitutive expression of secreted PR proteins appear to be linked, the basis for the dwarfism of *mpk4* and other constitutive SAR mutants may include the metabolic cost of increased PR protein synthesis and maintenance of a secretory pathway tuned for massive protein secretion. Incomplete suppression of dwarfism by NahG is also observed in the *cpr1* and *dnd1* mutants (Bowling et al., 1994; Clough et al., 2000), suggesting that other targets that influence cell and resultant plant size are deregulated independently of SA in these mutants.

NPR1 has been shown to function downstream of SA accumulation in SA-mediated expression of *PR* genes and SAR (Delaney et al., 1995; Cao et al., 1997; Shah et al., 1999). In our studies, double *npr1-1/mpk4* mutants retain the dwarf, enhanced resistance, and constitutive *PR1* gene expression phenotypes of *mpk4* but also exhibit the SA hypersensitivity typical of *npr1-1*. This suggests that *mpk4* deregulates SA-mediated defenses independently of NPR1. This is consistent with the absence of the G box and the presence of the W box (TTGACT) and the 8-mer GACTWWHC motif in 5′ upstream regions of constitutively expressed genes in *mpk4*. While these three motifs are regulatory elements in the *PR1* promoter, the G box may be specifically involved in positive regulation of *PR1* by bZip factors interacting with NPR1 (Zhang et al., 1999). Several other genes affecting pathogen responses and SAR also act independently of, or partially through, NPR1. These include *CPR6* (Clarke et al., 1998), *ACD6* (Rate et al., 1999), *CPR5* (Bowling et al., 1997), and the *npr1* suppressor *SSI1* (Shah et al., 1999). In *cpr5* and in *lsd* mutants (lesions simulating disease resistance response; Dietrich et al., 1997), SAR is accompanied by the formation of spontaneous necrotic lesions. Since lesions are not observed in *mpk4*, MPK4 exerts a function downstream or independently of the HR in SAR development. Examining the epistatic relationship of *mpk4* to SA regulatory mutants such as *eds1* and *pad4* (Falk et al., 1999; Jirage et al., 1999) may help determine the hierarchy of these components in plant resistance.

Evidence from previous studies suggests that plant MAPKs participate in the integration of signals arising from diverse stress stimuli. For example, tobacco SIPK (*S*A-*i*nduced *p*rotein *k*inase) is activated by the tobacco mosaic virus (TMV)–resistance gene ***N*** interaction, fungal elicitors, nitric oxide, SA, and wounding (Romeis et al., 1999; Kumar and Klessig, 2000). Tobacco WIPK (*w*ound-*i*nduced *p*rotein *k*inase) is transcriptionally induced and activated by both wounding and the TMV–*N* interaction (Seo et al., 1995; Zhang and Klessig, 1998). Interestingly, overexpression of WIPK leads to elevated JA levels and constitutive expression of the JA-responsive gene *PI-II* (Seo et al. 1999). Furthermore, while wild-type plants accumulate JA and its target gene mRNAs in response to wounding, sense-suppressed *wipk* plants accumulate SA and express SAR target genes (Seo et al., 1995). Our microarray and RNA blot analyses show that induction of certain JA-responsive genes is blocked in *mpk4*. This effect is independent of SA levels, as *mpk4* mutants expressing *nahG* also fail to accumulate PDF1.2 and THI2.1 mRNA in response to MeJA. Thus, in addition to repressing SA-mediated defenses, MPK4 is required for JA-mediated gene expression. MPK4 may therefore be involved in integrating SA- or JA-dependent responses to selectively engage defenses against particular pathogen types or environmental stresses (Felton et al., 1999; Pieterse and van Loon, 1999).

The structure and function of MAPKs is broadly conserved among eukaryotic signaling pathways that transduce diverse extracellular stimuli into adaptive cellular responses. For example, the yeast *Saccharomyces cerevisiae* uses six MAPKs to regulate developmental pathways and environmental responses including filamentous growth, pseudohyphal development, mating, and growth in hypo- and hyperosmotica (Madhani and Fink, 1998). More than twenty MAP kinases have been cloned or genomically annotated in *Arabidopsis*, although their specific functions remain unclear (Mizoguchi et al., 1997). These MAPKs presumably act downstream of three major classes of putative MAPKKKs typified by CTR1, ANPs, and MEKK1. CTR1 negatively regulates responses to the gaseous hormone ethylene (Kieber et al., 1993), while one or more ANPs positively regulate oxidative stress responses and may negatively regulate responses to the hormone auxin via two MAPKs, MPK3, and MPK6 (Kovtun et al., 2000).

Yeast two-hybrid experiments show that MPK4, the MAPKKs, AtMKK2, AtMEK1, and the MAPKKK AtMEKK1 interact (Ichimura et al., 1998). They may, therefore, constitute a kinase cascade. AtMEK1 mRNA accumulates slowly after wounding (Morris et al., 1997), while AtMEKK1 mRNA accumulates in response to touch, cold, and salinity (Mizoguchi et al., 1996). These results suggest that MPK4 acts downstream of these kinases to mediate responses to these stress stimuli, although we have been unable to find data to support such a role for MPK4. Nonetheless, changes in MEKK1 levels in response to osmotic stress could affect MPK4 activity. Such cross-talk between pathogen and osmotic stress signaling may occur in tobacco, as SIPK is activated by both SA and osmotic stress (Hoyos and Zhang, 2000; Mikolajczyk et al., 2000). The expression pattern of *MPK4* indicates that such cross-talk may occur in guard cells, which exhibit specific responses to pathogen elicitors (Hammond-Kosack et al., 1994; Blatt et al., 1999), and that, as openings to the leaf interior, have long been considered to be important in pathology.

Experimental Procedures

Isolation and Characterization of *mpk4*

F3 progeny of transposant lines generated in Ecotype L*er* (Sundaresan et al., 1995) were examined for phenotypic mutants by growing 12 plants per line in soil. For scanning electron microscopy, leaves were fixed overnight at 5°C in 0.1 M phosphate buffered (pH 7.0)

2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in 1% OsO_4, dehydrated in acetone, critical-point dried via CO_2, gold sputter-coated, and examined in a Philips 515 scanning electron microscope.

mpk4 and *MPK4* Alleles

The genomic region of the *Ds* insertion was identified by Southern blotting of EcoR1 restricted *mpk4* genomic DNA probed with GUS carried on *Ds* (Sundaresan et al., 1995). A 4.5 kb hybridizing fragment including 2.1 kb flanking sequence was purified by gel electrophoresis, ligated to EcoRI-digested lambda gt11 arms, and the flanking region between *Ds* and the vector arm was amplified by PCR. Sequencing both strands on an Applied Biosystems ABI 310 identified the *Ds* insertion site in *mpk4*. Genomic DNA from wild type and *mpk4* revertants was used as template to amplify the region spanning the putative footprint, and these fragments were sequenced as above.

Pathogen Infection, SA Measurement, and MeJA Treatments

Four-week-old plants were infiltrated with a suspension of 1×10^5 cfu/ml of virulent *Pseudomonas syringae* pv. *tomato* DC 3000 strain, and bacterial growth was assayed (Parker et al., 1996). Inoculations with *P. parasitica* isolate Cala2 were performed on seedlings and monitored by staining with lactophenol-trypan blue (Parker et al., 1997). SA and SA glucoside were measured in leaves of 4-week-old plants (Bowling et al., 1994). For MeJA treatments, 4-week-old greenhouse plants were either sprayed with 50 μM MeJA solution containing 0.005% Silwet L-77 or water and 0.005% Silwet L-77 and harvested after 48 hr.

RNA Analyses

Total RNA was prepared for RNA gel blot hybridizations using standard protocols (RNAgents Total RNA, Promega). Probe templates were amplified by PCR from cDNAs or genomic DNA with primer sequences from *MPK4* (GI:457399), PR1 (GI:3810599), β-1,3-glucanase or PR2 (GI:166636), PR5 (GI:2435405), *PDF1.2* (GI: 4,759,674), *THI2.1* (GI: 1,181,530), and elongation factor 1α control (GI:16260). For cDNA microarray analysis, total RNA from 2 g of 18-day-old, soil-grown wild type and *mpk4* was extracted using Trizol Reagent (Life Technologies). Poly(A)$^+$ RNA was purified from 200 μg total RNA with 2 μg of Dynabeads Oligo(dT)$_{25}$ (Dynal). cDNA microarray production, preparation of fluorescent probes, and microarray hybridization and scanning have been described previously (Ruan et al., 1998). The hybridization experiment was performed twice using microarrays hybridized to cDNAs from two samples each of *mpk4* and wild-type mRNA.

Microarray Data Analyses

Analysis of microarray hybridization signals using the average $\log_2$ fold balanced difference between *mpk4* and wild-type signals and its standard deviation indicated that clones with $\geq$5-fold signals in *mpk4* than wild type were statistically significantly higher expressed in *mpk4* ($p < 0.01$). 5′ upstream sequences of *PR1*, *PR5*, and of the cDNAs shown by the microarray to be $\geq$5-fold higher expressed in *mpk4* than wild type were extracted from the database (Table 1). One of these (unknown) was not included because we could not determine the ORF start. The 17 putative promoter regions were used as input in a Gibb's sampler, which can detect short patterns or matrices that are not necessarily 100% conserved (Lawrence et al., 1993), to identify sequences that might be regulatory *cis*-elements. Searches were performed for elements ranging from 6 to 16 bp. The sampler repeatedly found Matrix 1 (TTGACT) and Matrix 2 (GACTWWHC) when searching for elements of 6 or 8 bp, respectively. The best matrices found for 7, 9, 10, 11, and 12 bp all had similarity to Matrix 2 but lower information content.

To estimate the statistical significance of these matrices or motifs, the nucleotides in the 17 promoter sequences were shuffled 300 times, producing 300 sets of 17 promoter sequences of conserved lengths and nucleotide compositions. Gibb's sampling was performed on each of the shuffled sequence sets for both 6 and 8 bp elements, and the total information content for the best matrix was collected. The information content for the 300 best matrices was approximately Gaussian-distributed, with a mean of 7.7 bit and 8.8 bit and a standard deviation of 0.27 bit and 0.25 bit, and with the highest information content found 8.5 bit (twice) and 9.6 bit (once) for the 6 and 8 bp matrices, respectively. The total information contents of 8.6 bit for Matrix 1 and 9.7 bit for Matrix 2 were therefore significantly ($p < 0.01$) higher than expected by random. Thus, there is less than 1% chance by random of finding any 6 bp or 8 bp motifs as conserved as Matrix 1 and 2 in sets of DNA sequences of the same length and nucleotide composition as the 17 promoters. Detailed protocols and microarray results are available upon request from the authors or at http://www.cell.com/cgi/content/full/103/7/1111/DC1.

Genetic Analyses

F1 progeny of crosses between *mpk4* and *npr1-1* plants were allowed to self-pollinate, and F3 seeds from 60 individual F2 plants were plated on MS with 250 μM SA. This allowed the identification of 26 *npr1* homozygotes by seedling hypersensitivity to SA (Bowling et al., 1997). Lines homozygous for *npr1* were tested for *Ds* in *mpk4* by PCR. Forty F3 seeds from plants homozygous for *npr1* and heterozygous for *mpk4* were grown in soil. All of these lines segregated for the *mpk4* dwarf phenotype, indicating that *mpk4* dwarfism was independent of *npr1.* All homozygous *npr1* dwarves examined expressed PR1 constitutively and were confirmed by sequencing as homozygous for *npr1-1*.

Plants homozygous for the *nahG* salicylate hydroxylase (from Karen Beasley, Novartis) were crossed to *mpk4* heterozygotes, and F1 progeny heterozygous for *Ds* in *mpk4* were identified by PCR. Three hundred F2 progeny were examined for *mpk4* homozygous dwarfism. Since the *nahG* hydroxylase is dominant, 75 dwarves would be expected if the activity of NahG did not suppress the *mpk4* phenotype. In contrast, 19 dwarves would be expected if *nahG* rescued *mpk4* dwarfism. Only 22 dwarves were identified, while 48 plants had a partially suppressed *mpk4* phenotype. PCR demonstrated that the *nahG* transgene was absent in all of the 22 dwarves, whereas plants exhibiting partially suppressed dwarfism were homozygous for *mpk4* and carried *nahG*.

HA Tagging, Immunodetection, and In-Gel Kinase Assay

A NotI linkered genomic MPK4 fragment including the 1150 bp promoter was amplified from La-0 genomic DNA and cloned into that site of pSLF172 (Forsburg and Sherman, 1997) to produce a C terminally triple HA-tagged MPK4. The activation loop mutant (T201A/Y203F) was made using the QuickChange kit (Stratagene). HA-tagged mutant and wild-type MPK4 were subcloned into pCAMBIA3300 and transformed into *mpk4* heterozygotes. Homozygous *mpk4* lines expressing HA-tagged MPK4 versions were identified in T2.

Protein extracts were prepared as described (Romeis et al., 1999) except that no buffer change was made prior to immunoprecipitation. One hundred micrograms of total protein was immunoprecipitated with 2 μg/ml monoclonal 12CA5 HA antibody (Boehringer) as described (Romeis et al., 1999). In-gel kinase assays were performed as previously described (Zhang and Klessig, 1998). Western blots were developed using alkaline phosphatase–conjugated anti-mouse antibody (Promega).

MPK4 Localization

An 1150 bp 5′ upstream fragment containing the *MPK4* promoter was isolated as a BamH1/HindIII–linkered PCR product and transcriptionally fused upstream of GUS in pCAMBIA3300. Plants were transformed by vacuum infiltration, and transgenics were selected with BASTA.

RT-ISPCR on FAA-fixed leaves was performed according to Johansen (1997) without pepsin and DNase treatment. MPK4 mRNA-specific primers spanning introns 3 and 5 were used for reverse transcription and PCR amplification. An anti-DIG-AP Fab fragment (Boehringer Mannheim) was used for detection.

Acknowledgments

Xinnian Dong is thanked for *npr1-1* and *cpr1* seed, and the *Arabidopsis* Biological Resource Center is thanked for DNA stocks. Ying Kaaring and Suksawad Vongvisutikkun are thanked for technical help. This work was supported by grants to J. M. from the European Union (QLG2-1999-00351) and Danish Biotechnology Program (9602416), to J. E. P. from the Gatsby Charitable Foundation, and

to D. F. K. from the U. S. Department of Agriculture (98353036664) and National Science Foundation (MCB9723952).

Received June 16, 2000; revised November 9, 2000.

References

Blatt, M.R., Grabov, A., Brearley, J., Hammond-Kozak, K., and Jones, J.D.G. (1999). K^+ channels of *Cf-9* transgenic tobacco guard cells as targets for *Cladisporium fulvum* Avr9 elicitor-dependent signal transduction. Plant J. *19*, 453–462.

Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X. (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. Plant Cell *6*, 1845–1857.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1997). The *cpr5* mutant of *Arabidopsis* expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell *9*, 1573–1584.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cel. *6*, 1583–1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell *88*, 57–63.

Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X. (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant *Arabidopsis cpr6–1* mutant. Plant Cell *10*, 557–569.

Clough, S.J., Fengler, K.A., Yu, I.-C., Lippok, B., Smith, R.K., and Bent, A.F. (2000). The *Arabidopsis dnd1* “defense, no death” gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. USA *97*, 9323–9328.

Delaney, T.P., Friedrich, L., and Ryals, J.A. (1995). *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA *92*, 6602–6606.

Dietrich, R., Richberg, M.H., Schmidt, R., Dean, C., and Dangl, J.L. (1997). A novel zinc finger protein is encoded by the *Arabidopsis* LSD1 gene and functions as a negative regulator of plant cell death. Cell *88*, 685–694.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K., and Somssich, I.E. (1999). Early nuclear events in plant defense signaling: rapid gene activation by WRKY transcription factors. EMBO J. *18*, 4689–4699.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E. (1999). *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. USA *96*, 3292–3297.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C., and Dixon, R.A. (1999). Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Curr. Biol. *9*, 317–320.

Forsburg, S.L., and Sherman, D.A. (1997). General purpose tagging vectors for fission yeast. Gene *191*, 191–195.

Gaffney, T., Friederich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessman, H., and Ryals, J. (1993). Requirement of salicylic acid for induction of systemic acquired resistance. Science *261*, 754–756.

Glazebrook, J. (1999). Genes controlling expression of defense responses in *Arabidopsis*. Curr. Opin. Plant Biol. *2*, 280–286.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1997). Use of *Arabidopsis* for genetic dissection of plant defense responses. Annu. Rev. Genet. *31*, 547–569.

Grantz, A.A., Brummell, D.A., and Bennett, A.B. (1995). Ascorbate free radical reductase mRNA levels are induced by wounding. Plant Physiol. *108*, 411–418.

Greenberg, J.T., Guo, A., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell *77*, 551–563.

Gupta, V., Willits, M.G., and Glazebrook, J. (2000). *Arabidopsis thaliana EDS4* contributes to salicylic acid (SA)-dependent expression of defense responses: evidence for inhibition of jasmonic acid signaling by SA. Mol. Plant Microbe Interact. *13*, 503–511.

Hammond-Kosack, K.E., Harrison, K., and Jones, J.G.D. (1994). Developmentally regulated cell death on expression of the fungal avirulence gene Avr9 in tomato seedlings carrying the disease-resistance gene *Cf-9*. Proc. Natl. Acad. Sci. USA *91*, 10445–10449.

Hoyos, M.E., and Zhang, S. (2000). Calcium-Independent Activation of Salicylic Acid-Induced Protein Kinase and a 40-Kilodalton Protein Kinase by Hyperosmotic Stress. Plant Physiol. *122*, 1355–1364.

Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998). Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis*. Biochem. Biophys. Res. Commun. *253*, 532–543.

Jelitto-Van Dooren, E.P.W.M., Vidal, S., and Denecke, J. (1999). Anticipating endoplasmic reticulum stress: a novel early response before pathogenesis-related gene induction. Plant Cell *11*, 1935–1944.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999). *Arabidopsis* thaliana *PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. USA *96*, 13583–13588.

Johansen, B. (1997). In situ PCR on plant material with sub-cellular resolution. Ann. Bot. *80*, 697–700.

Johnson, P.R., and Ecker, J.R. (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu. Rev. Genet. *32*, 227–254.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. Cell *72*, 427–441.

Kovtun, Y., Chiu, W.-L., Tena, G., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA *97*, 2940–2945.

Kumar, D., and Klessig, D.F. (2000). Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene, and jasmonic acid. Mol. Plant Microbe Interact. *13*, 347–351.

Lawrence, C.E., Altschul, S.F., Boguski, M.S., Liu, J.S., Neuwald, A.F., and Wootton, J.C. (1993). Detecting subtle sequence signals: a gibbs sampling strategy for multiple alignment. Science *262*, 208–214.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in *Arabidopsis*. Plant J. *16*, 223–233.

Li, X., Zhang, Y., Clarke, J.D., Li, X., and Dong, X. (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of npr1–1. Cell *98*, 329–339.

Ligterink, W., Kroj, T., Nieden, U.Z., Hirt, H., and Scheel, D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science *276*, 2054–2057.

Liu, Y., Zhang, S., and Klessig, D.F. (2000). Molecular cloning and characterization of a tobacco MAP kinase kinase that interacts with SIPK. Mol. Plant Microbe Interact. *13*, 118–124.

Madhani, H.D., and Fink, G.R. (1998). The riddle of MAP kinase signaling specificity. Trends Genet. *14*, 151–155.

Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I. (1990). Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science *250*, 1002–1004.

Merkouropoulos, G., Barnett, D.C., and Shirsat, A.H. (1999). The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic and salicylic acid and codes for a protein with unusual motifs. Planta *208*, 212–219.

Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F., and Dobrowolska, G. (2000). Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. Plant Cell *12*, 165–178.

Mittler, R., Shulaev, V., and Lam, E. (1995). Coordinated activation

of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. Plant Cell *7*, 29–42.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *93*, 765–769.

Mizoguchi, T., Ichimura, K., and Shinozaki, K. (1997). Environmental stress response in plants: the role of mitogen-activated protein kinases. Trends Biotechnol. *15*, 15–19.

Molina, A., and Olmedo, G.F. (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. Plant J. *12*, 669–675.

Molina, A., Volrath, S., Guyer, D., Maleck, K., Ryals, J., and Ward, E. (1999). Inhibition of protoporphyrinogen oxidase expression in *Arabidopsis* causes a lesion-mimic phenotype that induces systemic acquired resistance. Plant J. *17*, 667–678.

Morris, P.C., Guerrier, D., Leung, J., and Giraudat, J. (1997). Cloning and characterization of MEK1, an *Arabidopsis* gene encoding a homologues of MAP kinase kinase. Plant Mol. Biol. *35*, 1057–1064.

Nawrath, C., and Métraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell. *11*, 1393–1404.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of eds1, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. Plant Cell *8*, 2033–2046.

Parker, J.E., Coleman, M.J., Szabo, V., Frost, L.N., Schmidt, R., van der Biezen, E.A., Moores, T., Dean, C., Daniel, M.J., and Jones, J.G.D. (1997). The *Arabidopsis* downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6. Plant Cell *9*, 879–894.

Penninckx, I.A.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, J.-P., Manners, J.M., and Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell *8*, 2309–2323.

Pieterse, C.M.J., and van Loon, L.C. (1999). Salicylic acid-independent plant defense pathways. Trends Plant Sci. *4*, 52–58.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain of function *Arabidopsis acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell *11*, 1695–1708.

Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H., and Jones, J.D. (1999). Rapid Avr9- and Cf-9 -dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell *11*, 273–287.

Ruan, Y., Gilmore, J., and Conner, T. (1998). Towards *Arabidopsis* genome analysis: monitoring expression profiles of 1400 genes using cDNA microarrays. Plant J. *15*, 821–833.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995). Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science *270*, 1988–1992.

Seo, S., Sano, H., and Ohashi, Y. (1999). Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. Plant Cell. *11*, 289–298.

Shah, J., Kachroo, P., and Klessig, D.F. (1999). The *Arabidopsis* ssi1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. Plant Cell. *11*, 191–206.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G., and Jones, J.D.G. (1995). Molecular genetics of plant disease resistance. Science *268*, 661–667.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D.G., Dean, C., Ma, H., and Martienssen, R. (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. Genes Dev. *9*, 1797–1810.

Taipalensuu, J., Andreasson, E., Eriksson, S., and Rask, L. (1997). Regulation of the wound-induced myrosinase-associated protein transcript in *Brassica napus* plants. Eur. J. Biochem. *247*, 963–971.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J.A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell *3*, 1085–1094.

Weymann, K., Hunt, M., Uknes, S., Neuenschwander, U., Lawton, K., Steiner, H.Y., and Ryals, J. (1995). Suppression and restoration of lesion formation in *Arabidopsis* lsd mutants. Plant Cell *7*, 2013–2022.

Yang, Y., Shah, J., and Klessig, D.F. (1997). Signal perception and transduction in plant defense responses. Genes Dev. *11*, 1621–1639.

Zang, Z., Collinge, D.B., and Thordal-Christensen, H. (1995). Germin-like oxalate oxidase, an H_2O_2-producing enzyme, accumulates in barley attacked by the powdery mildew fungus. Plant J. *8*, 139–145.

Zhang, S., and Klessig, D.F. (1998). Resistance gene N-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. Proc. Natl. Acad. Sci. USA *95*, 7433–7438.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proc. Natl. Acad. Sci. USA *96*, 6523–6528.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998). PAD4 funtions upstream of salicylic acid to control defense responses in *Arabidopsis*. Plant Cell *10*, 1021–1030.