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The MAP kinase substrate MKS1 is a regulator of plant defense responses

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Arabidopsis **MAP kinase 4 (MPK4) functions as a regulator of pathogen defense responses, because it is required for both repression of salicylic acid (SA)-dependent resistance and for activation of jasmonate (JA)-dependent defense gene expression. To understand MPK4 signaling mechanisms, we used yeast two-hybrid screening to identify the MPK4 substrate MKS1. Analyses of transgenic plants and genome-wide transcript profiling indicated that MKS1 is required for full SA-dependent resistance in *mpk4* mutants, and that overexpression of MKS1 in wild-type plants is sufficient to activate SA-dependent resistance, but does not interfere with induction of a defense gene by JA. Further yeast two-hybrid screening revealed that MKS1 interacts with the WRKY transcription factors WRKY25 and WRKY33. WRKY25 and WRKY33 were shown to be *in vitro* substrates of MPK4, and a *wrky33* knockout mutant was found to exhibit increased expression of the SA-related defense gene *PR1*. MKS1 may therefore contribute to MPK4-regulated defense activation by coupling the kinase to specific WRKY transcription factors.**

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Introduction

Plant disease resistance is induced via host recognition of pathogen elicitors, which may be either general to a large class of pathogens or specific to a race of pathogen. Bacterial flagellin is an example of a general elicitor that induces resistance through interaction with a plasma membrane-localized receptor kinase (Gomez-Gomez and Boller, 2000; Zipfel *et al*, 2004). This mode of pathogen recognition resembles pathways of innate immunity induction upon perception of pathogen-associated molecular patterns in insects and vertebrates. In addition, plants use a second recognition mode to perceive specific factors produced by the pathogen to promote infection. While normally acting as virulence factors, these race-specific elicitors activate resistance in plant hosts harboring specific, matching resistance (*R*) genes. Race-specific elicitors are therefore referred to as avirulence (Avr) factors. Recognition of Avr factors by R proteins may occur predominantly via 'surveillance' of host proteins manipulated by Avr activity rather than by direct R–Avr interaction, although examples of both scenarios have been reported (Dangl and Jones, 2001). At the infection site, responses to R–Avr interactions include Ca^{2+} influx (Scheel, 1998), production of reactive oxygen species (Delledonne *et al*, 1998) and expression of pathogenesis-related *PR* genes, some of which have antimicrobial activity (Narasimhan *et al*, 2005). Several protein kinases are implicated in local resistance signaling (Romeis *et al*, 2001), and signal transduction components may be shared between resistance responses induced by general elicitors and Avr factors. For example, the MAP kinases WIPK and SIPK are activated upon the Cf-9/Avr9 R–Avr interaction in tobacco, and their *Arabidopsis* orthologs MPK3/6 function in flagellin-induced resistance activation as well as in resistance mediated by specific *R* genes (Romeis *et al*, 1999; Asai *et al*, 2002; Menke *et al*, 2004).

Upon R protein activation, immunity may also be promoted in uninfected tissues by the induction of systemic acquired resistance (SAR) dependent upon salicylic acid (SA). Thus, impairment of SA biosynthesis by mutation of the isochorismate synthase ICS1/SID2 or the putative transporter EDS5 abrogates activation of SAR (Nawrath and Métraux, 1999; Wildermuth *et al*, 2001). In response to some infections, the interacting proteins of unknown biochemical function EDS1 and PAD4 are requisite, positive regulators of SA biosynthesis (Feys *et al*, 2001). As SA itself may not be a systemic signal (Forouhar *et al*, 2005), genes such as *DIR1* (Maldonado *et al*, 2002) and *CDR1* (Xia *et al*, 2004) are necessary for the production or transmission of the signal(s) required for SAR.

A well-characterized component in SA signaling is the BTB/ankyrin repeat protein NPR1. Following SA application, monomeric NPR1 released by reduction from cytosolic, disulfide-bound oligomers translocates to the nucleus, where it interacts with and induces the binding of TGA transcription factors to *PR* promoters (Johnson *et al*, 2003; Mou *et al*, 2003). BTB proteins function as substrate receptors for cullin-3-based ubiquitin E3 ligases in plants and animals (Furukawa *et al*, 2003; Dieterle *et al*, 2005), but no direct substrates of NPR1-mediated degradation have been identified as yet. Despite the importance of NPR1, NPR1-independent pathways

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exist (Clarke *et al*, 1998; Petersen *et al*, 2000). These may involve the WHY1 DNA-binding protein (Desveaux *et al*, 2004) and WRKY transcription factors that bind to W-boxes in *PR* genes (Eulgem *et al*, 2000; Maleck *et al*, 2000; Petersen *et al*, 2000).

In addition to the SA-dependent defense system, defense pathways mediated by ethylene (ET) and jasmonates (JA) exist. Such pathways provide resistance to herbivores and to pathogens distinct from those that induce SA-dependent defenses (Turner *et al*, 2002; Wang *et al*, 2002). While overlapping gene sets are induced by SA, JA and ET (Schenk *et al*, 2000), SA and ET/JA responses exhibit clear antagonisms (Norman-Setterblad *et al*, 2000; Glazebrook *et al*, 2003), and the PDF1.2 defensin is a prototype ET/JA target gene negatively regulated by SA. The significance of the antagonistic relationship between these two plant defense pathways is unknown, and its molecular basis also remains poorly characterized. For example, genetic studies in *Arabidopsis* have placed the positive regulators of SA signaling NPR1 and WRKY70 in the same pathway negatively regulating PDF1.2 expression (Spoel *et al*, 2003; Li *et al*, 2004).

We have previously shown that knockout of *Arabidopsis* MAP kinase 4 (*mpk4*) leads to accumulation of SA and activation of SA-dependent resistance, as well as a block of PDF1.2 induction by JA (Petersen *et al*, 2000), and hypersusceptibility to herbivore feeding and infection by an ET/JA pathway-inducing fungus (E Andreasson and P Brodersen, unpublished data, 2004). This suggests that MPK4 functions in the control of pathogen defense pathway antagonism. Together with MPK6, MPK4 is also activated by a range of abiotic stresses and, since the MAP kinase kinase MKK2 is required for both MPK4 activation and appropriate abiotic stress adaptation, MPK4 may also have a role in such stress signaling (Huang *et al*, 2000; Ichimura *et al*, 2000; Teige *et al*, 2004). Similar complexities of MAP kinase function have been described in yeast and animal cells. A key to understanding such complexity is the identification and characterization of kinase substrates. For example, mating in *Saccharomyces cerevisiae* is induced by activation of the MAP kinases Fus3 and Kss1, while specific induction of filamentous growth involves Kss1 activation. Fus3 activation ensures specificity of the mating response because it phosphorylates the Tec1 transcription factor required for expression of filamentation genes, thereby targeting Tec1 for degradation (Bao *et al*, 2004).

Arabidopsis contains 20 MAP kinases, like MPK4 of the ERK type, and only five typical MPK phosphatases (MAPK Group, 2002). Therefore, numerous plant MAP kinase pathway specificity determinants can be expected. To date, the only known MAP kinase substrates are two isoforms of ET biosynthetic enzymes that are phosphorylated and activated by MPK6, explaining the effect of MPK6 activation on ET accumulation (Liu and Zhang, 2004). Here, we report the identification and characterization of an MPK4 substrate, designated MAP kinase 4 substrate 1 (MKS1). MKS1 acts downstream of MPK4 in the SA-dependent pathway, but does not appear to be involved in the ET/JA pathway. MKS1 interacts with the transcription factors WRKY 25 and WRKY33, and may thereby couple the MAP kinase to regulation of specific transcription factors.

Results

MPK4 interacts in yeast and in vitro with MKS1

To identify intermediates in the signaling pathway(s) mediated by MPK4, we isolated MPK4 interacting proteins by yeast two-hybrid (Y2H) cDNA library screening with full-length MPK4 as bait. Initial immunoblotting with anti-MPK4 antibody (Huang *et al*, 2000) showed that the GAL4BD-MPK4 bait construct in plasmid pGBKT7 was expressed in the yeast strain PJ69-4A, and Y2H assays showed that this construct, alone or transformed with the empty pACT prey vector, did not activate expression of selectable markers. A total of 2×10^7 clones were then screened representing 6.6-fold redundancy of the Clontech library used. Four clones encoding full-length MKS1 protein were identified by prototrophy for adenine and histidine. The interaction between MPK4 and MKS1 was confirmed by selectable marker and β-galactosidase reporter activation upon cotransformation of MPK4 and MKS1 in bait and prey vectors, and *vice versa*. Directed Y2H assays indicated that this interaction was specific, as MKS1 did not interact with MAP kinases closely (MPK5) or more distantly (MPK17) related to MPK4 (MAPK Group, 2002), or with MPK3 or MPK6 involved in innate immunity responses (Asai *et al*, 2002). The MKS1 and MPK4 interaction was confirmed by *in vitro* binding assays. This showed that recombinant, N-terminal 6xHis-tagged MKS1 from *Escherichia coli* could pull down labeled MPK4, but not a control human lamin (Figure 1A).

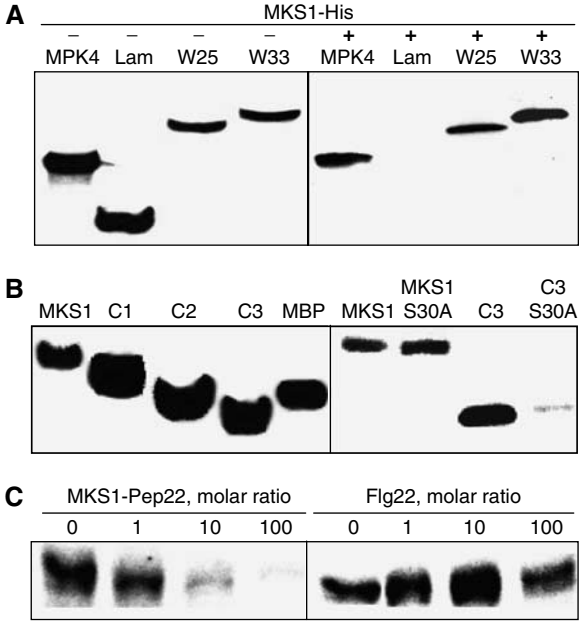


Figure 1 MKS1 interactions and phosphorylation *in vitro*. (**A**) MKS1 interactions with MPK4, W25 and W33 used N-terminal 6xHis-tagged MKS1 (His-MKS1) purified from *E. coli*. ^{35}S-methionine-labeled MPK4, W25 or W33 was incubated without (−) or with (+) nickel-agarose-coupled His-MKS1. Human lamin (Clontech) was a negative control. (**B**) Left: Phosphorylation of full-length recombinant MKS1, C-terminal MKS1 truncations (C1–C3) and myelin basic protein (MBP) as positive control. HA-tagged MPK4 (HA-MPK4) was immunoprecipitated (IP) from complemented *mpk4* transgenic plants. Right: *In vitro* kinase assays with mutated full-length MKS1 (MKS1 S30A) and mutated C3 (C3 S30A). (**C**) Phosphorylation of full-length MKS1 and increasing molar ratios of a 22-amino-acid, MKS1-derived peptide, Pep22 (left; Supplementary Figure S1) and the 22-amino-acid flagellin elicitor Flg22 (right).

MKS1 homologs are found in diverse plant species

MKS1 is a 222-amino-acid protein (predicted mass of 23 851 and isoelectric point of 6.0) that lacks predicted subcellular targeting sequences. MKS1 is a member of an apparently plant-specific family including proteins from monocots and dicots (Figure 2). Family members share a conserved VQ motif of unknown function (Protein Family PF05678; Figure 2, domain II), while those most similar to MKS1 include a less conserved, N-terminal region (domain I). In contrast, the C-terminal regions are divergent although they contain numerous Ser-Pro residues that are potential MAP kinase phosphorylation sites (Sharrocks *et al*, 2000; Liu and Zhang, 2004). MKS1 contains 12 Ser-Pro sites, one each in domains I and II and 10 in the C-terminal region.

Two family members have been partially characterized: Sib1, a nuclear-encoded, chloroplast targeted protein that interacts with plastid-encoded, plastid RNA polymerase σ-factor Sig1 (Morikawa *et al*, 2002), and tomato ACRE169 whose mRNA is rapidly induced following activation of *Cf-9*-dependent resistance by the race-specific elicitor *Avr9* (Durrant *et al*, 2000). This indicates that MKS1 family members may be involved in transcriptional regulation and in responses to pathogens.

MPK4 phosphorylates MKS1 in vitro

We have previously shown that the expression of a wild-type MPK4 gene including a C-terminal, 3× HA epitope tag complements the *mpk4* loss-of-function mutant and produces immunoprecipitable MPK4 that phosphorylates the standard MAP kinase substrate myelin basic protein *in vitro* (Petersen *et al*, 2000). We therefore used this system to demonstrate that immunoprecipitated, HA-tagged MPK4 from plants phosphorylated recombinant His-tagged MKS1 and three C-terminal MKS1 truncations purified from *E. coli* (C1–3; Figures 1B and 2). This indicates that MKS1 is an *in vitro* substrate of MPK4, and that MKS1 Ser30 and/or Ser72, both contained in the C3 truncation, may be phosphorylation sites. This was confirmed by demonstrating that although MPK4 phosphorylated full-length MKS1 carrying a Ser30Ala substitution, phosphorylation of the C3 truncation carrying this substitution was severely reduced (Figure 1B).

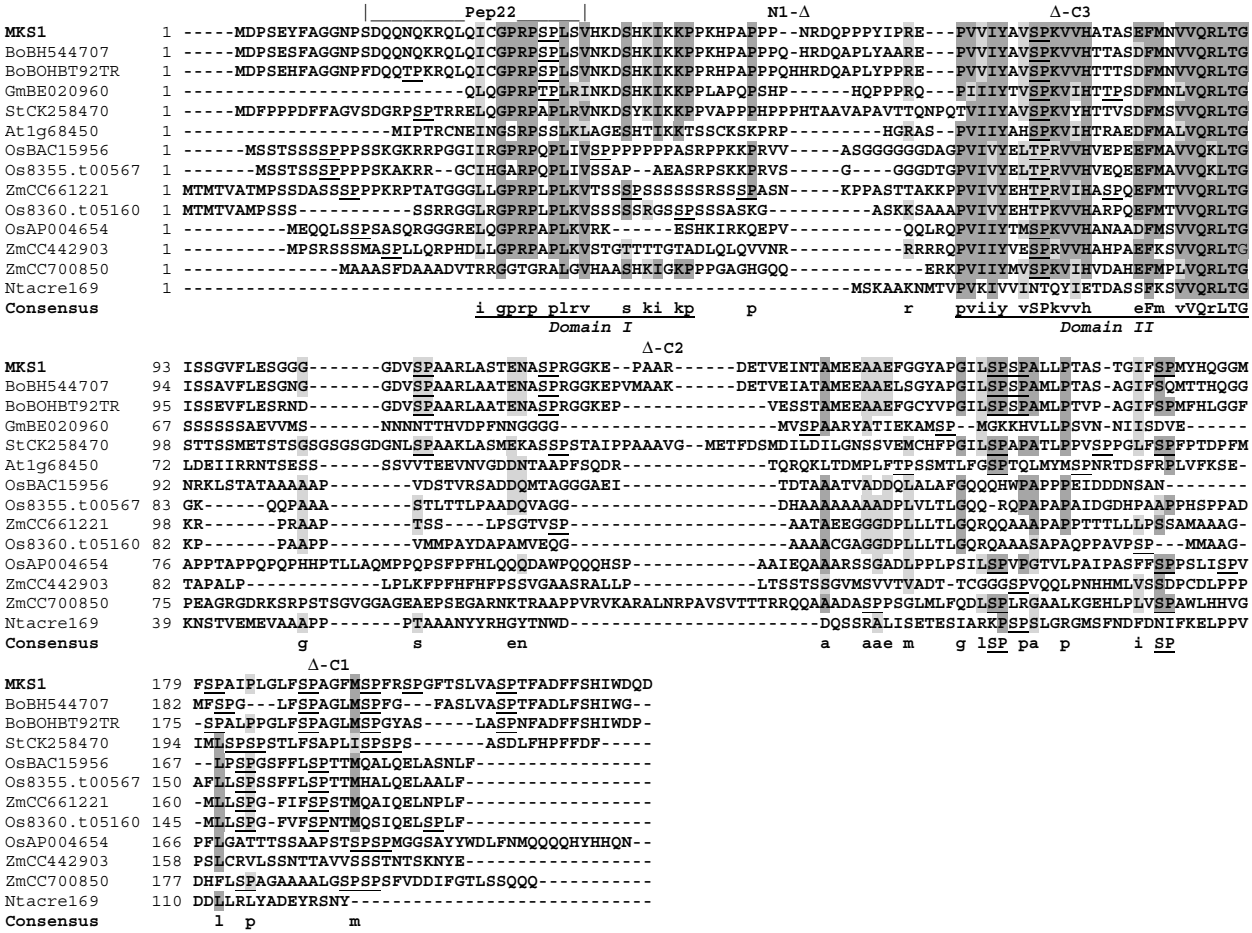


Figure 2 MKS1 and homologs. DNA and amino-acid sequences of MKS1 (At3g18690) were used to query databases at www.ncbi.nlm.nih.gov/BLAST for proteins similar to MKS1. Protein sequences of selected accessions were aligned at clustalw.genome.jp and identical/similar residues highlighted at www.ch.embnet.org/software/BOX_form.html. Putative MAP kinase phosphorylation sites (S/TP) are underlined. The sequence of the Pep22 peptide is indicated by an overbar. The ends of an N-terminally truncated (N1) and three C-terminally truncated (C1–3) MKS1 versions described in the text are noted above the MKS1 sequence. Putative domains I and II are underlined in the consensus. Species abbreviations are as follows: At: *Arabidopsis thaliana*; Bo: *Brassica oleracea*; Gm: *Glycine max*; St: *Solanum tuberosum*; Os: *Oryza sativa*; Nt: *Nicotiana tabacum*; Zm: *Zea mays*. Similar plant proteins not aligned here include BM340911, CAD40925, CC613160, CC635639, Al390921, AL138658, T46022, AP004654, AP003260, AC143340 and *Arabidopsis* At1g21326, At2g41180, At2g44340, At2g42140 and At3g56710 (Sig1; Morikawa *et al*, 2002).

MPK4 phosphorylation of full-length MKS1 was competed at a 10 molar ratio, and abolished at a 100 molar ratio, by a synthetic 22-residue peptide derived from MKS1 (pep22; Figures 1C and 2). This peptide (SDQQNQKRQLQICGPRP SPLSV) was synthesized because it included Ser30 and because the sequence upstream is reminiscent of MAP kinase substrate docking domains (Sharrocks *et al*, 2000). In contrast, the unrelated, 22-residue Flg22 peptide (Gomez-Gomez and Boller, 2000; Asai *et al*, 2002) did not affect MKS1 phosphorylation. This indicates that MKS1-derived pep22 specifically interacts with MPK4. Furthermore, the C1–3 MKS1 truncations interacted with MPK4 in Y2H experiments, while an N-terminal truncation, N1, failed to interact (Figure 2). These results indicate that MPK4 interacts with the N-terminal region of MKS1 including pep22 and domain I, and that this interaction is necessary for MPK4 phosphorylation of MKS1.

MKS1 interacts with MPK4 in vivo

A selected monoclonal antibody raised in mice against MKS1 pep22 was used to detect recombinant MKS1 extracted from *E. coli* and endogenous MKS1 from plants, both with the expected mobility in SDS–PAGE (Figure 3A). Monoclonal anti-pep22 antibodies also co-immunoprecipitated from plants MKS1 with MPK4 immunodetected by the HA tag (Figure 3B). In addition, the levels of phosphorylated MKS1 detected with a phosphoserine/phosphothreonine-specific antibody were markedly higher in wild-type plants than in *mpk4* mutants (Figure 3C). These results indicate that MKS1 and MPK4 interact *in vivo*, and that MPK4 is the major kinase activity that phosphorylates MKS1 under default conditions.

Transgenic plants expressing gene fusions between the green fluorescent protein (GFP) and MPK4 or MKS1 were produced to examine their subcellular localization. The MPK4-GFP gene fusion fully complemented the *mpk4* mutant phenotype, indicating that it provided functional MPK4 activity. Western analysis with a GFP antibody detected a single band of the expected size of the MPK4-GFP fusion protein (data not shown), indicating that the intact fusion, and not a cleavage product liberating

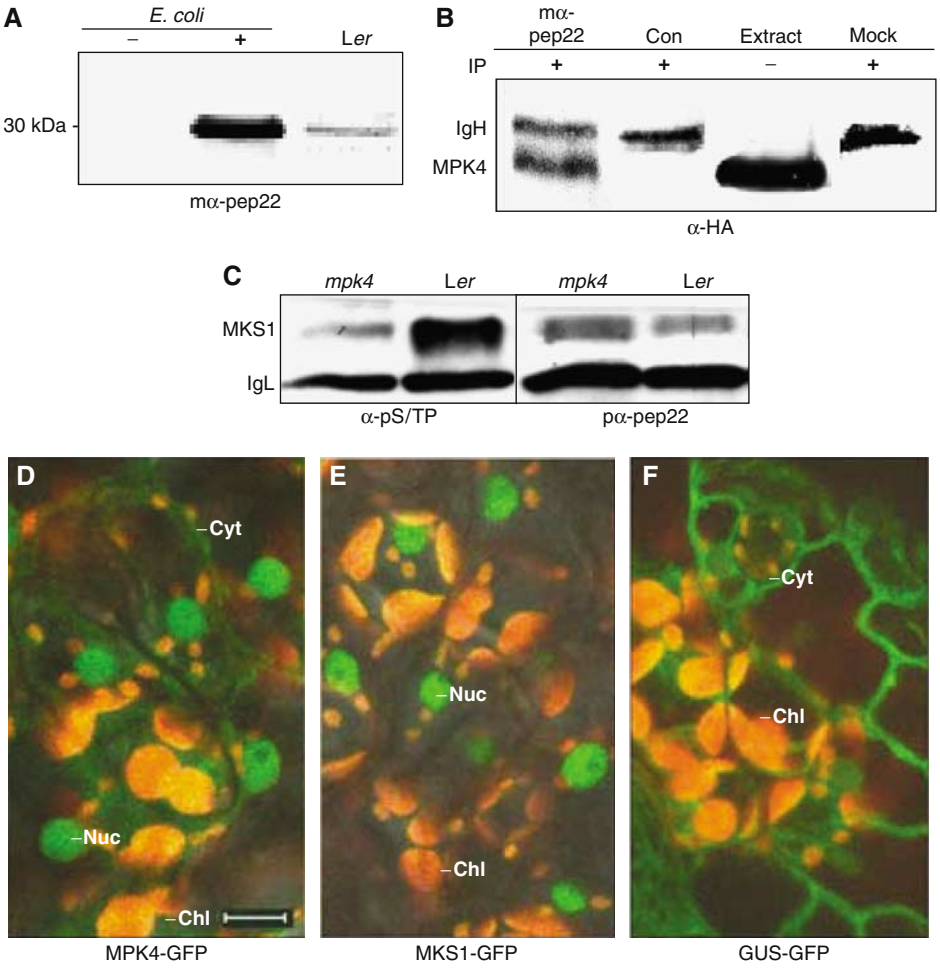


Figure 3 MKS1 interaction, phosphorylation and localization *in vivo*. (**A**) Immunodetection of MKS1 in *E. coli* extracts before (−) and after (+) IPTG induction, and from extracts of leaves (L*er*) by a monoclonal anti-Pep22 antibody (mα-pep22) recognizing a protein of the predicted size of MKS1 (~28 kDa). (**B**) Immunodetection of HA-MPK4 by anti-HA antibody (α-HA) in immunoprecipitates from plant extracts with mα-pep22 (lane 1), negative control monoclonal antibody (Con; lane 2) or in mock immunoprecipitation lacking extract (lane 4). MPK4 from plant extracts is shown (raw extract; lane 3). MPK4 and immunoglobin heavy chain (IgH) are indicated. (**C**) Immunodetection of MKS1 following immunoprecipitation with mα-Pep22 from L*er* or *mpk4* extracts. MKS1 is detected with an anti-phosphoserine/phosphothreonine antibody (α-pS/TP) and polyclonal anti-Pep22 antibody (pα-pep22). MKS1 and immunoglobin light chain (IgL) are indicated. *In planta* localization in mesophyll cells of (**D**) MPK4-GFP, (**E**) MKS1-GFP and (**F**) GUS-GFP fusion proteins. Cyt: cytoplasm; Nuc: nuclei; Chl: chloroplast (orange autofluorescent); — 10 μm size bar.

MPK4, was responsible for complementation. The MPK4-GFP fusion produced weak but readily detectable GFP fluorescence in cytoplasmic strands and strong fluorescence in nuclei (Figure 3D). Similarly, the MKS1-GFP fusion under control of the cauliflower mosaic virus (CaMV) 35S promoter or 1.9 kbp of MKS1 5′ upstream sequence produced strong fluorescence only detectable in nuclei (Figure 3E). The MKS1-GFP fusion was apparently functional, as its overexpression via the 35S promoter produced the same phenotype as overexpression of MKS1 alone (see below, data not shown). In contrast, a control GUS-GFP fusion from pCAMBIA3300, under control of the 35S CaMV promoter, produced strong fluorescence in cytoplasmic strands (Figure 3F). These results indicate that MKS1 is primarily localized in nuclei and that the presence of both MKS1 and MPK4 in nuclei is consistent with their ability to interact *in vivo*.

Altered MKS1 expression affects defense responses and mpk4 phenotypes

MKS1 function was addressed in transgenic plants that overexpressed immunodetectable MKS1 from the constitutive CaMV 35S promoter (35S-*MKS1*), or that underexpressed MKS1 by RNA interference (*MKS1*-RNAi). Compared to wild type, 35S-*MKS1* plants were semi-dwarfed (Figure 4A and B), accumulated increased levels of PR1 mRNA (Figure 4C) and of SA (~13 500 ng SAG/g fresh weight leaf tissue in 35S-*MKS1* compared to ~3500 ng in wild type), and were more resistant to the virulent biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Figure 4D). Analysis of independent 35S-*MKS1* lines indicated that such characteristics of heightened defense signaling were correlated with MKS1 expression (data not shown). All of the 35S-*MKS1* lines examined were fully fertile and the expression of the MKS1 transgene was stable over at least four generations. In

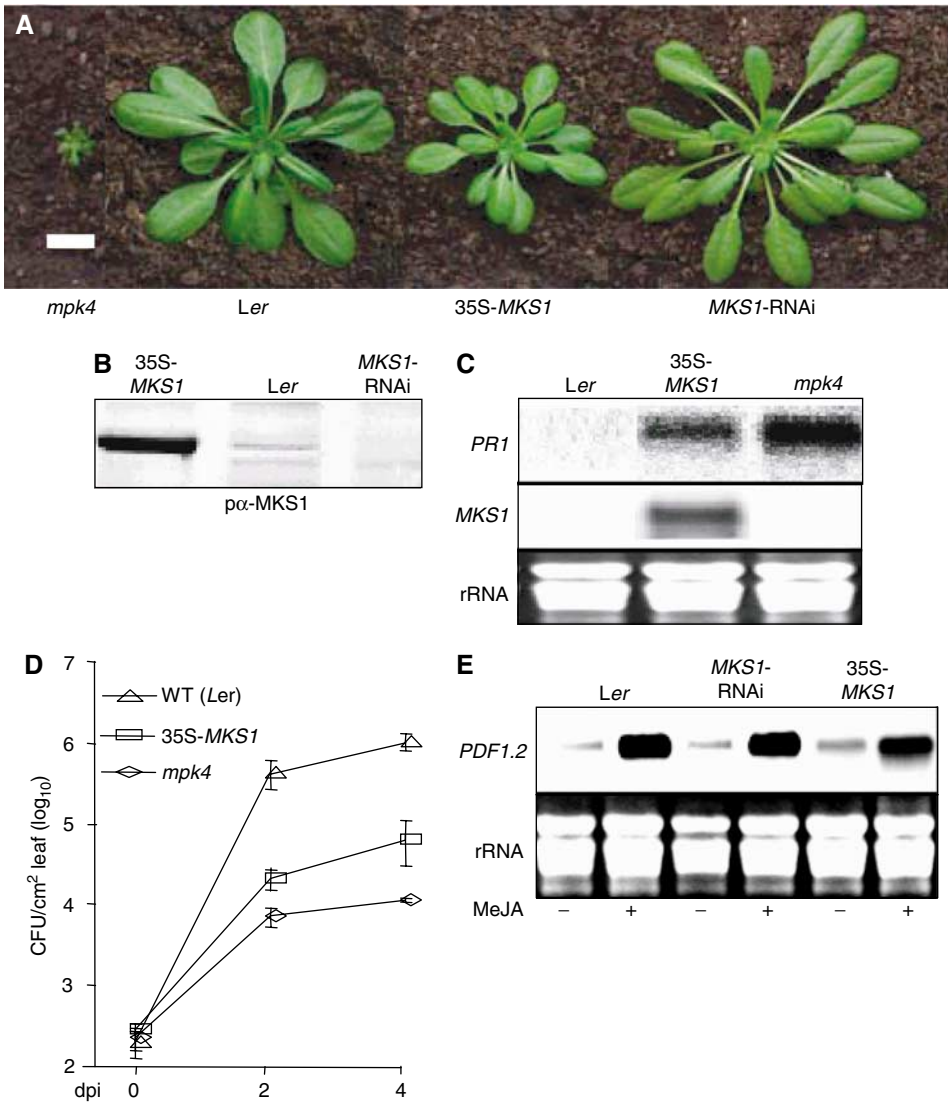


Figure 4 Effects of MKS1 over- and underexpression. (**A**) Phenotypes of *mpk4*, wild type (L*er*) and transgenic (Col) overexpressing MKS1 (35S-*MKS1*) or underexpressing MKS1 (*MKS1*-RNAi). The size bar is 2 cm. (**B**) Immunodetection with pα-Pep22 antibody of MKS1 in extracts from 35S-*MKS1*, wild type (L*er*) and *MKS1*-RNAi. (**C**) RNA blot detection of *PR1* mRNA in 35S-MKS1, *mpk4* and wild-type L*er*. (**D**) Growth of *P. syringae* pv. *tomato* DC3000 in *mpk4*, 35S-*MKS1* and L*er*. (**E**) Detection of *PDF1.2* mRNA in wild type (Col), *MKS1*-RNAi and 35S-*MKS1* following MeJA (+) and mock (−) treatments.

contrast, *MKS1*-RNAi plants did not exhibit observable growth phenotypes (Figure 4A). These results provide functional links between MKS1 and MPK4, as *mpk4* mutants exhibit dwarfism and constitutive defense signaling (Petersen *et al*, 2000).

As noted above, *PDF1.2* defensin gene induction by JA is blocked in *mpk4*, suggesting that MPK4 mediates JA-dependent responses to necrotrophic pathogens (Pieterse and van Loon, 1999). In contrast, MKS1 under- or overexpression had little, if any effect, on *PDF1.2* mRNA levels (Figure 4E), indicating that MKS1 is not involved in JA-responsive gene expression downstream of MPK4.

To position genetically MKS1 in MPK4 signaling, *mpk4/MKS1*-RNAi mutant plants were constructed. These plants exhibited partial suppression of *mpk4* dwarfism and a clear reduction in *PR1* mRNA levels (Figure 5A), again indicating that MKS1 acts downstream of MPK4. Furthermore, *mpk4/MKS1*-RNAi plants exhibited decreased resistance to *P. syringae* DC3000 compared to *mpk4* (Figure 5B). This partial suppression of the *mpk4* phenotype by *MKS1*-RNAi indicates that components other than MKS1 perform functions downstream of MPK4.

Gene expression profiling of 35S-MKS1 and mpk4

Comparative transcript profiling revealed that of 22 810 genes probed, 800 (3.5%) exhibited significantly differential expression patterns between wild-type, *mpk4* and 35S-*MKS1* plants (Supplementary Figure S1, clusters A–G). mRNAs of PR genes including *PR1* and *PR2* and the SA biosynthetic enzyme ICS1 (encoded by *SID2*) were increased in both *mpk4* and 35S-*MKS1* plants compared to wild type (Supplementary Figure S1, cluster A). This suggests that MPK4 and MKS1 participate in regulating the expression of these defense-related genes. Analyses of 5′ upstream regions of putative target genes revealed significant occurrences of two sequence motifs TAGACT and TGACTT, the latter a putative WRKY binding site or W-box (Lebel *et al*, 1998; Eulgem *et al*, 2000; Petersen *et al*, 2000). This suggests that WRKY factors participate in regulating genes downstream of MPK4 and MKS1.

To further analyze the overlap between genes affected by the *mpk4* mutation and the 35S-*MKS1* transgene, the two were tested individually against wild type using *t*-tests. Because the *t*-test has substantially less power than the ANOVA, only the 800 most significant genes in the ANOVA test were considered. This revealed that *mpk4* and 35S-*MKS1* significantly affected 350 and 156 genes, respectively, of which 76 genes were affected by both *mpk4* and 35S-*MKS1*. Considering the total number of genes measured ($n = 22\,810$), the overlap of 76 genes is highly significant ($P = 6e{-}99$). This supports the finding that MPK4 depends on MKS1 in the

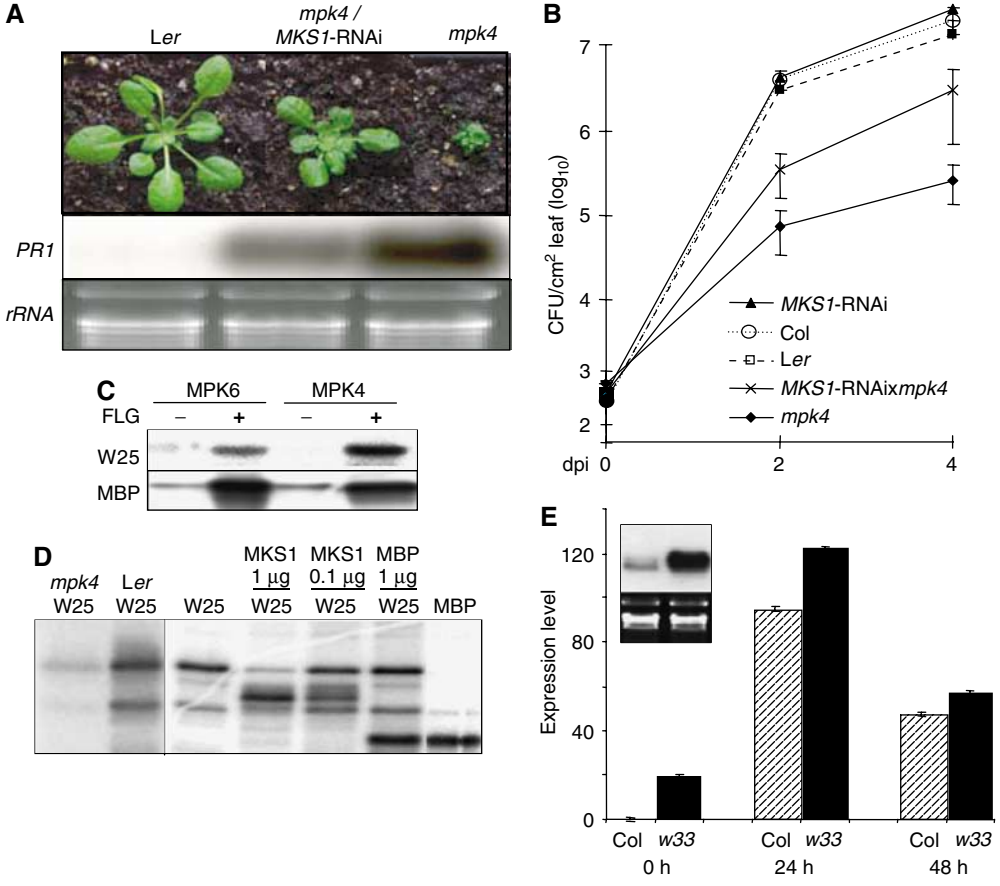


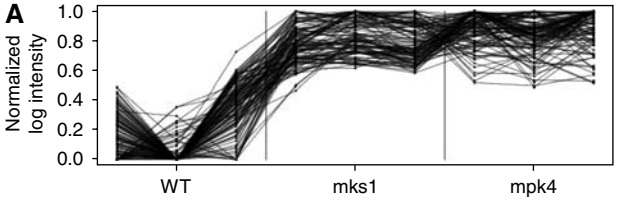
Figure 5 Suppression of *mpk4* by *MKS1*-RNAi, WRKY phosphorylation and *w33* phenotype. (**A**) Top: Phenotypes of wild type (L*er*), *mpk4* carrying *MKS1*-RNAi (*mpk4/MKS*1-RNAi) and *mpk4*. Bottom: Detection of *PR1* mRNA in wild type (L*er*), *mpk4/MKS1*-RNAi and *mpk4* versus rRNA loading control. (**B**) Growth of *P. syringae* pv. *tomato* DC3000 in wild types *L*er and *Col*, *mpk4*, *MKS1*-RNAi and *mpk4/MKS1*-RNAi. (**C**) Phosphorylation of full-length W25 by immunoprecipitation of MPK6 or MPK4 from *Arabidopsis* cells without (−) or with (+) treatment with flagellin. (**D**) Phosphorylation of N-terminal region of W25 with MPK4 immunoprecipitation from *mpk4* or L*er* (left) and of W25, MKS1 and MBP with immunoprecipitation of MPK4-HA (right). (**E**) RNA blot (inset) and real-time PCR detection of PR1 mRNA in Col and *w33* before (0 h and inset) or after infiltration with DC3000.

regulation of a set of genes. Of the 76 genes affected by both *mpk4* and 35S-*MKS1*, only one gene was affected in the opposite direction, further supporting a relation between MPK4 and MKS1 in regulating a subset of genes. However, this analyses also confirmed that *mpk4* affects the expression of other genes independently of 35S-*MKS1*, as can be seen in clusters B, E and F that contain 112, 169 and 173 genes, respectively. Among these are a number of genes that are implicated in defense and stress responses: WRKYs 30, 32, 46, 48, 60 and 75, basic chitinase (*PR3*) and *EDS1*. This and the partial suppression of the *mpk4* phenotype by *MKS1*-RNAi (Figure 5A) indicate that other effects of MPK4 are mediated by effectors other than MKS1. Of particular interest are genes overexpressed in both *mpk4* and 35S-*MKS1* (Figure 6A, cluster A), as well as genes underexpressed only in the *mpk4* background (Figure 6B, cluster B). This latter group includes the JA-inducible *PDF1.2* gene, which was more than 10-fold underexpressed in the *mpk4* background compared to both 35S-*MKS1* and wild type. This confirms the RNA blot results (Figure 4E), and indicates that MKS1 function downstream of MPK4 does not affect defense responses mediated by JA.

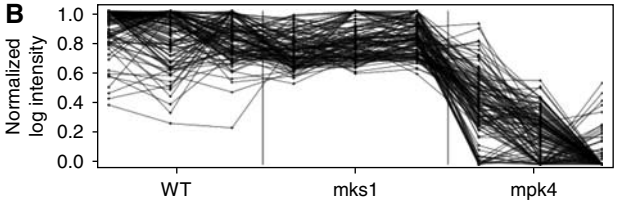
MKS1 interacts in yeast and in vitro with WRKY25 and WRKY33

To further investigate MKS1 function, we conducted a second Y2H library screen to identify additional MKS1 interacting proteins. Of 6×10^7 clones screened, four encoding WRKY25 (W25) and 11 encoding WRKY33 (W33) were identified and confirmed by β-galactosidase assays. The interactions of MKS1 with W25 and W33 were also confirmed *in vitro* using His-tagged MKS1 (Figure 1A). These interactions were apparently specific, as Y2H assays with MKS1 as bait did not permit the growth of yeast expressing the closest homolog W26, or yeast expressing W29 involved in flagellin responses (Asai *et al*, 2002). Furthermore, W33 was found to interact in yeast with the truncated MKS1 versions N1, C1 and C2, but not with C3 (Figure 2). This suggests that MKS1 domain II including the VQ motif is involved in the interaction between MKS1 and W33.

W25 and W33 contain N-terminal D-domains with Ser-Pro residues that may be MAP kinase phosphorylation sites (Eulgem *et al*, 2000; Liu and Zhang, 2004). Although we did not detect interactions between MPK4 and W25 or W33 in directed Y2H assays, recombinant W25 and W33 were phosphorylated by MPK4 *in vitro*. Kinase assays with MPK6 or MPK4 immunoprecipitated from plant cells showed that full-length W25 was preferentially phosphorylated by MPK4 (Figure 5C). In addition, the anti-MPK4 antibody preferentially precipitated, from wild type versus *mpk4*, MPK4 that phosphorylated the N-terminal regions of W25 and W33 (Figure 5D, left panel and data not shown). Interestingly, MKS1, but not the standard MAP kinase substrate MBP, inhibited W25 or W33 phosphorylation by MPK4 (Figure 5D, right). These results suggest that MKS1 may be involved in modulating the phosphorylation of the WRKYs. Two additional lines of evidence indicate a role for W33 in regulating gene expression downstream of MPK4. First, as for *mpk4* and 35S-*MKS1*, plants of an available insertion line with a T-DNA in the first intron of the *W33* gene accumulated significantly more *PR1* mRNA under normal conditions and following infection with *P. syringae* DC3000 (Figure 5E). Second, these *w33* mutants were significantly smaller than



Fold	Locus	TAIR annotation Feb. 2004
95.5	At1g58025	BROMO-domain protein family
13.2	At2g43570	Chitinase
11.1	At2g14610	PR-1
9.5	At3g22600	Lipid transfer protein (LTP)
8.1	At1g75040	PR-5
7.8	At1g33960	AIG1, avirulence-responsive
7.2	At3g53150	Glucosyltransferase
6.0	At1g74710	Isochorismate synthase 1 (SID2)
5.8	At1g19610	Plant defensin protein (PDF1.4)
5.6	At3g52430	Phytoalexin-deficient 4 (pad4)
4.9	At1g08450	Calreticulin 3 (CRT3)
4.7	At3g45290	7-transmembrane MLO (MLO3)
4.2	At3g57260	PR-2
4.1	At5g14930	Senescence-associated (SAG101)
3.2	At5g47220	Ethylene response 2 (EREBP-2)
3.0	At4g01720	WRKY47 transcription factor
2.5	At5g37780	Calmodulin 4
2.2	At1g22070	bZIP transcription factor, TGA3



Fold	Locus	TAIR annotation Feb.2004
29.7	At4g01370	MAP kinase 4 (MAPK4)
20.6	At5g44420	Plant defensin (PDF1.2a)
5.4	At3g25760	Allene oxide cyclase
4.1	At1g20190	Expansin, putative (EXP11)
3.8	At4g26530	Fructose-bisphosphate aldolase
2.7	At1g12090	Lipid transfer protein (LTP)
2.4	At5g06290	2-cys peroxiredoxin-related
2.2	At4g09010	Ascorbate peroxidase
2.0	At3g16140	Photosystem I subunit VI
1.9	At4g21280	Oxygen-evolving protein 16
1.9	At1g67740	Photosystem II complex (psbY)
1.9	At1g34310	Auxin response factor 1

Figure 6 Expression profiles of genes differentially expressed between wild-type, *mpk4* and 35S-*mks1* plants. Two clusters A and B of significantly differentially expressed genes are shown. (**A**) Top: Expression profiles of genes in cluster A through triplicates of the three genotypes (*x*-axis) versus normalized log expression index values. Vertical lines mark the borders between the genotypes. Bottom: List of selected genes from cluster A. (**B**) as in (A) but for cluster B genes. See Supplementary Figure S1 for a comprehensive list of clustered genes.

wild type when grown under short-day (8 h) conditions. Of the 30 plants examined, the total leaf area ($n = 80$ each) of *w33* plants was 45% (±6%) and the area of the two largest

leaves was 34% (±11%) of wild type. Such reduced growth phenotypes are typical of a number of constitutive defense mutants (Clarke *et al*, 1998; Xia *et al*, 2004).

Discussion

Using Y2H screening with MPK4 as bait, we identified the MPK4 interacting protein MKS1. *In vitro* and i*n vivo* interaction and phosphorylation assays, together with the low level of phosphorylated MKS1 in the *mpk4* mutant, confirmed that MKS1 is an MPK4 substrate. In addition, analyses of transgenic plants showed that over- or underexpression of MKS1 oppositely affect pathogen resistance and *PR* gene expression. For example, compared to wild type, plants that overexpressed MKS1 from a constitutive promoter (35S-*MKS1*) were smaller, accumulated increased total SA and exhibited increased resistance to the virulent pathogen *P. syringae DC3000*, as well as increased expression of *PR* genes. This indicates that plants that accumulate excess levels of unphosphorylated MKS1 exhibit similar phenotypes to plants lacking MPK4 kinase activity, and is consistent with a model in which default phosphorylation of MKS1 represses SA signaling. These data, combined with partial suppression of SA-dependent defense activation in *mpk4* mutants by reduced expression of MKS1 via *MKS1*-RNAi, indicate that MKS1 is required downstream of MPK4 to effect defense responses regulated by the SA pathway.

These results were confirmed and extended by comparative gene expression profiling of *mpk4* and 35S-*MKS1* plants. While this revealed a significant overlap in genes differentially regulated in *mpk4* and 35S-*MKS1* compared to wild type, it showed that loss of MPK4 function affected the expression of other genes independently of MKS1. This is consistent with the expectation that MPK4 phosphorylates substrates other than MKS1. For example, other MPK4 substrates affecting JA signaling may exist because loss of MPK4 activity reduces basal and JA/ET-inducible *PDF1.2* mRNA accumulation (Petersen *et al*, 2000; P Brodersen, unpublished data, 2004), while *PDF1.2* mRNA levels are not significantly affected by MKS1 over- or underexpression. This indicates that MKS1 overexpression produces plants with increased resistance to biotrophic pathogens, while maintaining a functional JA defense pathway.

Additional evidence for a role of MKS1 in defense signaling comes from our identification by Y2H screening of the WRKY transcription factors W25 and W33 as specific MKS1 interactors. Of the more than 70 *Arabidopsis* WRKY factors, W33 is most similar to W25 and to parsley WRKY1 involved in responses to pathogen elicitors (Turck *et al*, 2004). As shown here, W25 and W33 can be phosphorylated by MPK4 *in vitro*, presumably in what has been referred to as their D-domains (Eulgem *et al*, 2000), which contain the only Ser-Pro sites in the proteins:

W25 ^{67}YLDSPLLLSSSHSLISPTTGT^{84}
W33 ^{47}SISSPSLVSPSTCFSPSLFLDSPAFVSSSANVLASPTTGA^{84}.

The several Ser-Pro sites in such domains suggest that the WRKYs may be multiply phosphorylated, as we have shown here for MKS1 and as has been recently demonstrated for the ET biosynthetic enzyme ACS6 by MPK6 (Liu and Zhang, 2004).

Preliminary functional characterization of W33 is provided by our demonstration that a *w33* reduced or loss-of-function mutant shares at least two characteristics with *mpk4* and 35S-*MKS1* plants. First, they are significantly smaller than wild type, as seen for other constitutive defense mutants (Clarke *et al*, 1998; Xia *et al*, 2004). Second, *w33* accumulates heightened basal levels of *PR-1* mRNA. Although these phenotypes are tightly linked with the T-DNA insert in GABI KAT line 324B11, complementation of this allele with wild-type *W33* sequences and analyses of other reduced or loss-of-function w33 alleles are required to further assess W33 function. Given the potential redundancy of W25 and W33 function and the complexity of WRKY auto- and inter-regulation (Turck *et al*, 2004), analyses of *w25*/*w33* double mutants and of W25 and W33 target genes are required to substantiate their function(s) downstream of MPK4 and MKS1.

MKS1 is a member of a novel family of plant proteins that contain a conserved VQ motif in what we here refer to as domain II. MKS1 and other members also contain an N-terminal region, here termed domain I, which includes Ser30 that is phosphorylated by MPK4 *in vitro*. We have shown that this is not the only site of *in vitro* phosphorylation, indicating that MKS1 may be phosphorylated by MPK4 and other MAP kinases *in vivo* on one or more of 12 Ser-Pro sites. Nonetheless, the MKS1 N-terminal region, including domain I, is important for interaction with MPK4, as shown by directed Y2H assays and reduced MKS1 phosphorylation in the presence of pep22 derived from domain I. The reduced levels of phosphorylated MKS1 in *mpk4* plants also argue for the specificity of *in vivo* phosphorylation. In contrast, the Y2H assays with MKS1 truncations indicated that domain II is required for WRKY interaction with MKS1. This suggests that the VQ motif may represent the core of a protein–protein interaction domain, and is consistent with the interaction between another VQ motif protein with an RNA polymerase σ-factor (Morikawa *et al*, 2002). Such interactions may explain the nuclear localization of MKS1, which lacks predicted nuclear localization signals.

MKS1 may function as an MPK4 adaptor or coupling protein that affects the activities of WRKY factors and perhaps other proteins. Such activities may be determinants of the specificity of MAP kinase pathways (Baker *et al*, 2001), and suppression of the *mpk4* phenotype by *MKS1*-RNAi indicates this role for MKS1. Consistent with MKS1 nuclear localization and the interaction of different MKS1 domains with MPK4 and the WRKYs, these proteins may be part of transcription or chromatin remodeling complexes, as described in yeast (de Nadal *et al*, 2004; Edmunds and Mahadevan, 2004). Models of an MPK4 pathway must account for the ability of MPK4 and W33 to default repress, and of MKS1 to mediate defense signaling. Our observations are consistent with a binary interaction model in which MPK4–MKS1 complexes are largely distinct from MKS–WRKY complexes. If so, MKS1 phosphorylation by MPK4 could be modulated by sequestration of MKS1 in complexes with WRKYs or other proteins. Such a model would explain our inability to detect interactions between MPK4 and W25 or W33 in yeast, although we cannot exclude transient interactions between MPK4 and the WRKYs, as noted for other MAP kinase substrates (Manning and Cantley, 2002). Alternatively, MKS1 forms a ternary or larger complex with MPK4 and W25 and/or W33 that regulates their activities. In this case, MKS1

overexpression may effect the inhibition of MPK4 activity toward W25 and W33 seen here *in vitro*. Overall, the results presented here indicate that plants have a mechanism for repression of defense signaling, and that controlled overexpression of MKS1 provides a potential tool for sustainable agriculture by inhibiting this signaling.

Materials and methods

Yeast two-hybrid assays and cloning

MKS1 (NM112755) is encoded by At3g18690, WRKY 25 (AAL13040) by At2g30250 and WRKY33 (AAM34736) by At2g38470. Y2H screening was performed with full-length *MPK4* and *MKS1* cDNA baits and the *Arabidopsis* MATCHMAKER library according to the manufacturer (Clontech).

Molecular biology and biochemistry

In vitro interaction assays were performed with His-tagged MKS1 purified from *E. coli* and ^{35}S-methionine-labeled MPK4, WRKY33, WRKY25 and control lamin generated using a T7 polymerase-coupled reticulocyte lysate system (Promega). A 10 µl portion of ^{35}S-labeled protein was mixed with 200 µl 1% BSA in binding buffer (BB: 50 mM KPO_4, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 2 µg/ml leupeptin, 1 mM AEBSF, 2 µg/ml antipain, 2 µg/ml aprotinin), incubated on ice for 15 min and then centrifuged for 10 min at 4°C. The supernatant was added to 1 µg His-MKS1 protein bound to Ni beads in 200 µl 1% BSA in BB and incubated for 2 h at 4°C. Beads were washed three times with 1 ml wash buffer (50 mM KPO_4, pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 5% Triton X-100). Purification of active HA-tagged MPK4 was as described previously (Petersen *et al*, 2000). Phosphorylation assays were performed by combining MPK4, substrate protein (1 µg unless specified), 3 µCi [^{32}P-γ]ATP and kinase buffer (200 µM ATP, 80 mM Tris–HCl, pH 7.5, 8 mM EGTA, 120 mM $MgCl_2$, 4 mM Na_3VO_4, 4 mM DTT). Samples were incubated for 60 min at 30°C. The N-terminal deletions used were Met1–Gln220 for W25 and Met1–Asn220 for W33. For detection of MKS1, polyclonal sera and monoclonal (HYB 330-01, Danish Serum Institute, Copenhagen) antibody were used. A 1 mg portion of extract protein was used for co-immunoprecipitation experiments, as described previously (Feys *et al*, 2001). pS/TP was detected with 2321L antibody (Cell Signaling). Antibodies against MPK4 and MPK6 and flagellin treatments of *Arabidopsis* cells (L*er*) have been described previously (Nühse *et al*, 2000). Free and glycosylated SA were analyzed, as described previously (Newman *et al*, 2001). GFP expression in mesophyll cells of young leaves of stably transformed lines was visualized with a Zeiss LSM 510 microscope. RNA was prepared for gel blot analysis, as described previously (Petersen *et al*, 2000). Probe templates were amplified by PCR from cDNAs or genomic DNA with primer sequences from *PR1* (At2g14610), *PDF1.2* (At5g44420) and *MKS1*. For RT–PCR and Q-PCR analysis, RNA samples were incubated with 1 U of DNase according to the manufacturer (Promega, Madison, WI). RT reactions were carried out with 1 µg of RNA and 0.5 µg of random hexamer primer at 42°C with 0.1 U of RT (Promega) and 2 U of RNasin (Promega) for 1 h in 20 µl reactions. Product aliquots were used as template for RT–PCR and Q-PCR analysis. Q-PCR was performed using the SYBR Green protocol (Applied Biosystems, Foster City, CA) with 10 pmol of each primer and 0.5 µl aliquot of RT reaction product in a 25 µl reaction. A standard curve was made by determining Ct (threshold cycle) values for a dilution series of the RT reaction product for each primer pair. Using this standard curve, the relative quantification for each reaction was calculated from its Ct value due to a linear relationship between Ct value and log. Q-PCR reactions were in triplicate and averaged for each line individually. *PR1* expression was standardized relative to 18S rRNA expression for each data point. The relative quantity for the control sample (Col) at $t = 0$ was arbitrarily set to 0. Expression levels of all other samples were expressed relative to this control sample. Primers were as follows: *PR1*: forward 5′-gtgggttagcgagaaggcta-3′, reverse 5′-actttggcacatccgagtct-3′; *18S*: forward 5′-cggctaccacatccaa ggaa, reverse 5′-gctgcaattaccgcggct-3′.

Genetics and transgenics

The CaMV 35S-*MKS1* transgene was stably introduced into ecotype L*er* with pCAMBIA1301, and *MKS1*-RNAi construct was introduced into ecotype Col via plasmids SLJ1382B1 (A Ludwig and JDG Jones, The Sainsbury Laboratory, Norwich) and pCAMBIA3300. The W33 insertional mutant was GABI KAT line 324B11 (Rosso *et al*, 2003). RT–PCR was used to show that it did not accumulate detectable W33 mRNA. The MKS1-GFP gene fusion under control of 1.9 kb 5′ upstream *MKS1* sequence was made, as previously described for producing the MPK4-GFP fusion (Petersen *et al*, 2000). Bacterial resistance assays were performed as described previously (Parker *et al*, 1996; Petersen *et al*, 2000). Plants were infiltrated with 1×10^5 colony forming units per ml (CFU/ml). Values represent average and standard deviations of CFU extracted from leaf disks 0, 2 and 4 days after inoculation in three independent samplings. Plants were grown under 8 h light and 16 h darkness at 22°C for up to 5 weeks.

Microarray hybridization and analysis

Total RNA was isolated from three replicates of wild type, *mpk4* and 35S-*MKS1* grown in a chamber with 16 h light (21°C) and 8 h dark (16°C). RNA was amplified according to the standard Affymetrix protocol and hybridized to the Affymetrix ATH1 oligonucleotide microarray (accession # E-MEXP-173, ArrayExpress database, EBI). Raw intensity data were normalized using R implementation of qspline (Workman *et al*, 2002; Gautier *et al*, 2004). An implementation in the statistical language R of the logit-t method (Lemon *et al*, 2003) applying one-way ANOVA was used to calculate statistical significance for differential gene expression. Likewise, logit-t was applied for the comparison between wild type and both *mpk4* and S35-*MKS1*. Genes with *P*-value less than 0.01 were considered significant. Hypergeometric statistic was used to access the significance of the overlap of genes significantly affected by *mpk4* and S35-*MKS1*. Gene expression index values were calculated using the perfect match only implementation method (Li and Wong, 2001). Gene expression profiles for significantly differentially expressed genes were clustered by k-means. For promoter analysis, 800 bp upstream regions from all genes profiled in the microarray experiments were extracted. All patterns from 5 to 12 bp in length were tested for significant over- or under-representation between upstream regions of genes included in one of the seven clusters (Supplementary Figure S2) and all 22 810 genes. Hypergeometric statistics was applied, as described previously (Jensen and Knudsen, 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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