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MAPKKKα is a positive regulator of cell death associated with both plant immunity and disease

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Many plant pathogens cause disease symptoms that manifest over days as regions of localized cell death. Localized cell death (the hypersensitive response; HR) also occurs in disease-resistant plants, but this response appears within hours of attempted infection and may restrict further pathogen growth. We identified a MAP kinase kinase kinase gene (*MAPKKKα*) that is required for the HR and resistance against *Pseudomonas syringae*. Significantly, we found that *MAPKKKα* also regulates cell death in susceptible leaves undergoing *P. syringae* infection. Overexpression of *MAPKKKα* in leaves activated MAPKs and caused pathogen-independent cell death. By overexpressing *MAPKKKα* in leaves and suppressing expression of various *MAPKK* and *MAPK* genes by virus-induced gene silencing, we identified two distinct MAPK cascades that act downstream of *MAPKKKα*. These results demonstrate that signal transduction pathways associated with both plant immunity and disease susceptibility share a common molecular switch.

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Introduction

Localized cell death occurs in both susceptible and immune plants during pathogen attack. In susceptible plants, disease symptoms referred to as 'specks', 'spots' or 'blights' appear over the course of many days. In 'gene-for-gene'-based immunity, a host resistance (R) protein recognizes a pathogen avirulence (Avr) protein leading to host defense responses that are induced within hours. The immune response is frequently accompanied by a form of localized programmed cell death (PCD), termed the 'hypersensitive response' (HR; Goodman and Novacky, 1994), which shares morphological and mechanistic features with animal apoptosis (Lam *et al*, 2001).

The HR can be elicited as soon as 2–3 h after the contact of a single bacterium with a plant cell. However, for practical purposes, we define here the HR as macroscopic cell death, which occurs when a minimum of 25% of the cells in contact with the pathogen die and develop confluent cell death (Goodman and Novacky, 1994). Many host responses precede the HR, including proteolysis, changes in ion fluxes, production of reactive oxygen and nitrogen species, and activation of MAPK cascades. The HR may inhibit or delay further spread of the pathogen, but its biological significance has been difficult to ascertain due to the lack of plant mutants affected solely in cell death. In some host–pathogen interactions, timely activation of cell death is necessary for pathogen containment, whereas in others cell death is either not essential or not sufficient for disease resistance (Lam *et al*, 2001). Therefore, a requirement for cell death might rely on the particular host–pathogen interaction, which determines the rapidity of activation of defense responses. It has been proposed that cell death acts as a second layer of defense if a certain threshold of defense stress signals is reached.

Much less is known about the molecular events associated with cell death that occur in susceptible plant tissues. Recent studies suggest that cell death in different plant tissues also involves host-controlled PCD (Greenberg and Yao, 2004). In the case of biotrophic pathogens, early activation of host PCD would likely limit pathogen proliferation. These pathogens therefore appear to have developed strategies to avoid recognition or to actively suppress host defenses. In contrast, facultative necrotrophic pathogens benefit from host cell death and kill the host by injecting toxins or activating host PCD. Thus, host-encoded PCD processes appear to play a critical role in determining both disease progression and immunity.

As a model system to investigate plant disease resistance and susceptibility, we study the interaction between tomato leaves and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Pedley and Martin, 2003). In susceptible tomato plants, *P. s.* pv. *tomato* enters the apoplastic space where it multiplies and, over the course of several days, causes bacterial speck disease. Typical symptoms of speck disease include localized cell death (1 mm necrotic 'specks') surrounded by chlorotic haloes. In severe infections, entire leaves die and the plant becomes defoliated. In resistant tomato plants, a ser/thr kinase encoded by the *R* gene *Pto* confers resistance to *P. s.* pv. *tomato* by recognizing and interacting with the pathogen type III effector proteins AvrPto or AvrPtoB, which are translocated and act inside the plant cell. This recognition event leads to a variety of defense responses including an HR at the site of pathogen attack. Prf, a protein with a nucleotide-binding site and a region of leucine-rich repeats is required for Pto-mediated resistance. Several substrates of Pto have been identified including a ser/thr protein kinase, Pti1, and three ERF-like transcription factors, Pti4/5/6 (Pedley and Martin, 2003). In a recent study using virus-induced gene silencing (VIGS), nine additional genes were found to contribute to Pto-mediated resistance in tomato. Two of these genes encode MAP kinase kinases (MAPKKs; *LeMEK1* and *LeMEK2*) and two encode

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MAPKs (*LeNTF6* and *LeWIPK*) thereby implicating for the first time MAPK signaling cascades in Pto-mediated resistance (Ekengren *et al*, 2003).

MAPKs are activated by several *R* gene-mediated pathways and pathogen-derived elicitors and play an essential role in defense (Zhang and Klessig, 2001). Recently, a complete MAPK cascade has been implicated in the innate immune response of *Arabidopsis* to bacterial flagellin (Asai *et al*, 2002). Pathways involving the tobacco orthologs of *LeMEK1/LeNTF6*, *LeMEK2/LeWIPK* and *LeMEK2/LeSIPK* have been studied by both biochemical methods and gain- and loss-of-function approaches. In a MAPK cascade regulating plant cytokinesis, the MAPKKK NPK1 activates the MAPKK NtMEK1, which lies upstream of the MAPK NtNTF6 (Soyano *et al*, 2003). NPK1 also mediates resistance in *Nicotiana benthamiana* to tobacco mosaic virus (Jin *et al*, 2002). NtMEK2 acts upstream of both WIPK and SIPK in a pathway(s) leading to host cell death (Yang *et al*, 2001). SIPK overexpression also results in host cell death and transcriptional activation of defense-related genes (Zhang and Liu, 2001), whereas silencing of either SIPK or WIPK, or both, compromises *R* gene-mediated resistance to viral and bacterial pathogens (Jin *et al*, 2003; Sharma *et al*, 2003). Placing these kinases in context with other defense signaling pathways in plants is contingent on the identification of MAPKKKs that act as upstream activators of HR-based host cell death. However, achievement of this goal has been complicated by the presence of a large number of *MAPKKK* genes in plant genomes (>60 in *Arabidopsis*; Ichimura *et al*, 2002).

Although Pto was isolated from tomato, it also functions in the wild tobacco species, *N. benthamiana*, where it recognizes AvrPto and induces the HR. Thus, Prf and certain downstream components of this signaling pathway are conserved in this species. *N. benthamiana* offers experimental advantages because of its large leaves and amenability to VIGS (Lu *et al*, 2003). We took advantage of the *N. benthamiana*/Pto-AvrPto system and silenced a large number of random genes originating from a cDNA library prepared from leaf tissues exposed to various pathogens and elicitors. Here we report the identification and characterization of the *MAPKKKα* gene, which is involved in regulation of host cell death during both immune and susceptible plant–pathogen interactions. We present evidence that the signal transduction pathway initiated by MAPKKKα involves two distinct MAPK cascades.

Results

Identification of NbMAPKKKα from a genetic screen for plants impaired in Pto-mediated cell death

To identify components of the Pto-mediated cell death signaling pathway, we conducted a screen of random cDNAs using VIGS in *N. benthamiana*. Over 2400 cDNAs were tested from a normalized, size-selected, cDNA *N. benthamiana* mixed elicitor library (cNbME) made in a Potato Virus X-based vector (see Supplementary Methods). Each clone was used to infect two *N. benthamiana* plants and, after silencing had occurred, two leaves were infiltrated with *Agrobacterium tumefaciens* (Agro-infiltrated) carrying either 35S::*Pto*/35S::*avrPto* or a 35S::*Pto* gene encoding a constitutive active form of the kinase (35S::*Pto[Y207D]*; Rathjen *et al*, 1999). Approximately 3% of the silenced clones affected Pto-mediated cell death (see Supplementary Methods). Silencing with PVX clone cNbME28C8 (data not shown) showed among the strongest and most reproducible inhibition of cell death triggered by Pto/AvrPto or Pto(Y207D) and it was therefore chosen for further characterization.

The insert in cNbME28C8 matched a single tomato contig (with two ESTs, GenBank Nos. AW036651 and BF050299) with homology to *AtMAPKKKα*; we refer to the *N. benthamiana* and tomato orthologs as *NbMAPKKKα* and *LeMAPKKKα*. To elicit stronger and more uniform silencing, we cloned the cNbME28C8 insert into a tobacco rattle virus vector (TRV; Liu *et al*, 2002) and used this construct for silencing in *N. benthamiana*. Complete inhibition of cell death caused by expression of Pto/AvrPto or Pto(Y207D) (Figure 1A-5 and -6) occurred in 87 and 38% of the HR assays, respectively, whereas partial inhibition occurred in 8 and 33% of the cases, respectively. No cell death was observed when Pto alone was expressed in leaves (Figure 1A-2). The abundance of endogenous *NbMAPKKKα* transcripts was greatly decreased in silenced leaves, demonstrating successful silencing (Figure 1B).

To support the likelihood that silencing was specific to the *MAPKKKα* gene, we performed VIGS experiments with two additional cDNA fragments derived from unique regions of the *NbMAPKKKα* gene. VIGS using the 5′-regulatory domain and a 3′-UTR fragment in *N. benthamiana* also resulted in inhibition of Pto-mediated cell death (Supplementary Figure S1A and B; data not shown for 3′-UTR). In addition, endogenous transcript levels of *NbNPK1* (the most closely related *Nicotiana* spp gene available in the database) remained at wild-type levels in *MAPKKKα*-silenced *N. benthamiana* plants (Supplementary Figure S1C). These data, combined with the observation that in tomato *LeMAPKKKα* is a single-copy gene (Supplementary Figure S2), indicate that the cell death inhibition phenotype is due to specific silencing of *MAPKKKα*.

By measuring electrolyte leakage, we quantified suppression of cell death in *N. benthamiana* leaves silenced for *NbMAPKKKα*, *Prf*, or infected with TRV-only (Figure 1C). The development of the HR in TRV-only-infected plants expressing *Pto/AvrPto* began 2.5–3 days after Agro-infiltration and was associated with an increase in ion leakage (Figure 1C). Leaves silenced for *NbMAPKKKα* showed no or few visual symptoms of cell death and had reduced ion leakage compared to TRV-only-infected plants. Ion leakage in TRV::*NbMAPKKKα* plants was slightly higher than that observed for TRV::*Prf*-silenced plants, which showed no cell death (Figure 1C). These results are consistent with the possibility that NbMAPKKKα lies genetically downstream of Prf and that other signaling pathway(s) also contribute to cell death.

NbMAPKKKα plays a role in multiple plant cell death pathways

We next examined whether NbMAPKKKα is a specific component of the Pto/AvrPto signaling pathway (Figure 1A). *N. benthamiana* leaves silenced for *NbMAPKKKα* were infiltrated with *Agrobacterium* strains carrying the following genes expressed by the CaMV 35S promoter: the tomato *R* gene *Cf9* and the *Cladosporium fulvum* avirulence gene *avr9* (Figure 1A-1; Van der Hoorn *et al*, 2000); *avrPto,* which is weakly recognized by a *Pto*-like gene in *N. benthamiana* (Figure 1A-3; He *et al*, 2004); *avrPtoBΔ6*, which is recognized

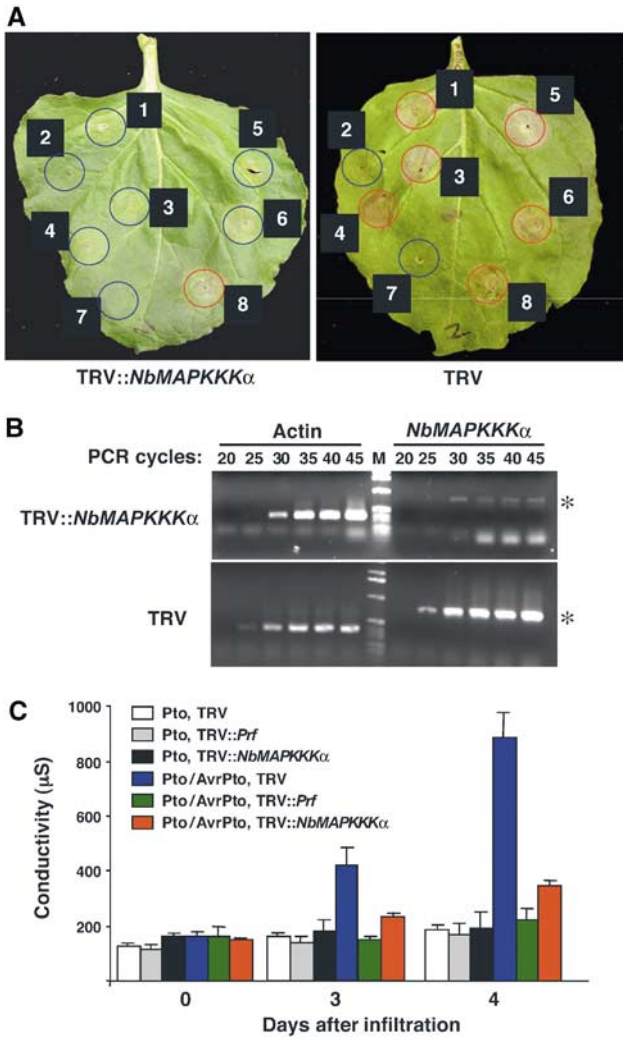

Figure 1 Cell death is suppressed in *N. benthamiana* plants silenced for MAPKKKα. (**A**) Inhibition of cell death on TRV::*NbMAPKKKα* leaves compared with TRV-only leaves after Agro-infiltration with (1) Cf9/Avr9, (2) Pto, (3) AvrPto, (4) AvrPtoBΔ6, (5) Pto(Y207D), (6) Pto/AvrPto, (7) BaxΔC and (8) Bax. Cultures were mixed 1:1 (single transgenes were mixed with an *Agrobacterium* strain carrying an empty vector). Photographs were taken 5 days after Agro-infiltration. The blue circles indicate no cell death and the red circles indicate cell death. Similar results were obtained in five independent experiments, each consisting of 6–8 plants with 2–3 leaves infiltrated per plant. In these experiments, inhibition of Pto/AvrPto and Pto(Y207D) HR was scored based on the parameters described in Figure 3A and presented as % in the text. (**B**) *NbMAPKKKα* transcript abundance is reduced in TRV::*NbMAPKKKα*-silenced plants compared to TRV-only plants as assessed by RT–PCR. First strand cDNA was prepared from leaf tissue surrounding the infiltrated areas and used in RT–PCR with primers specific for *NbMAPKKKα* (right panel) or actin (left panel). PCR products were sampled from each cycle indicated, separated on an agarose gel and stained with ethidium bromide. Lane M shows 1 kb DNA ladder (Promega Co., Madison, WI). Asterisks indicate *NbMAPKKKα* PCR product. (**C**) Electrolyte leakage is reduced in TRV::*NbMAPKKKα* and TRV::*Prf* plants compared to TRV-only plants. Leaves were Agro-infiltrated with Pto/AvrPto or Pto/empty vector as described in (A). Data presented are means of three plants/line and error bars represent standard deviations. The experiment was repeated three times with similar results.

by the *N. benthamiana Rsb* gene (Figure 1A-4; Abramovitch *et al*, 2003); or the mouse proapoptotic effector gene *Bax*, whose expression triggers cell death in plants (Figure 1A-8; Lacomme and Santa Cruz, 1999); and *BaxΔC,* which does not cause cell death in plants (Figure 1A-7). Cell death was suppressed in the areas expressing *Cf9/avr9*, *avrPtoBΔ6*, *avrPto* and *Pto(Y207D)*. Occasionally, Bax-induced cell death proceeded slower in some leaves silenced for *NbMAPKKKα* compared to TRV-only-infected leaves, but this reduction was less consistent than that observed in the *R/avr* treatments. No cell death was observed in any leaf tissue transiently expressing *BaxΔC* or Cf9 alone (not shown for Cf9). NbMAPKKKα thus appears to be a convergent point for multiple *R* gene signaling pathways activated by bacteria and fungi.

MAPKKKα belongs to the A2 subgroup of MEKKs

We cloned full-length *NbMAPKKKα* and *LeMAPKKKα* cDNAs (GenBank Nos. AY500155 and AY500156). The *NbMAPKKKα* ORF encodes a kinase catalytic domain flanked by N- and C-terminal putative regulatory domains (Supplementary Figure S1A). Alignment of the insert from cNbME28C8 with the full-length *NbMAPKKKα* sequence showed that it derives from the C-terminal regulatory domain (Supplementary Figure S1A). A search of the tomato EST database with the full-length *N. benthamiana* and tomato *MAPKKKα* cDNA sequences identified another tomato EST (BI423417) originating from the 5′ end of the *LeMAPKKKα* gene. Alignment of the amino-acid sequences deduced from the *NbMAPKKKα* and *LeMAPKKKα* cDNA sequences revealed 85.3% identity overall and 93% within the kinase domain (Figure 2A). MAPKKKα belongs to the MEKK class or 'group A' of plant MAPKKKs (Ichimura *et al*, 2002). The minimal signature motif for this group is conserved in LeMAPKKKα but differs slightly in NbMAPKKKα (Figure 2A; Tena *et al*, 2001). Both *N. benthamiana* and tomato MAPKKKα proteins have a serine-rich region at the N-terminus that is present in some but not all plant MAPKKKα orthologs (Ichimura *et al*, 2002).

In *Arabidopsis*, there are 12 MEKKs with only five of them having a known function: *AtMAPKKKα-1* (*YODA*), the *ANP* genes (*ANP1*, *ANP2* and *ANP3*) and *MEKK1*. *YODA,* the MAPKKK with the most closely related kinase domain, is required for embryonic cell fate regulation (Lukowitz *et al*, 2004). *ANP1*, *ANP2* and *ANP3* and their tobacco ortholog, *NPK1,* play a role in plant cytokinesis as well as in auxin, abiotic and biotic responses (Tena *et al*, 2001; Jin *et al*, 2002). *MEKK1* regulates innate immune responses to bacterial flagellin (Asai *et al*, 2002). We aligned the amino-acid sequences from the kinase domains of *MAPKKKα* genes from *N. benthamiana*, tomato, *Arabidopsis*, rice and *Brassica napus* along with the sequences from *Arabidopsis* members from subgroup A2, and MEKK1 and NtNPK1 (Figure 2B). AtMAPKKKα is closely related to MAPKKKα proteins from other plant species, supporting an orthologous relationship. Finally, the high degree of conservation among the MAPKKKα amino-acid sequences of rice and various solanaceous and brassica species suggests that the *MAPKKKα* gene arose before monocots and dicots diverged.

MAPKKKα positively regulates cell death associated with host resistance to two P. syringae pathogens

To investigate the possible role of MAPKKKα in an incompatible host–pathogen interaction, we silenced *NbMAPKKKα* in *N. benthamiana* plants expressing *Pto* from a CaMV 35S promoter and challenged them with an HR-causing inoculum

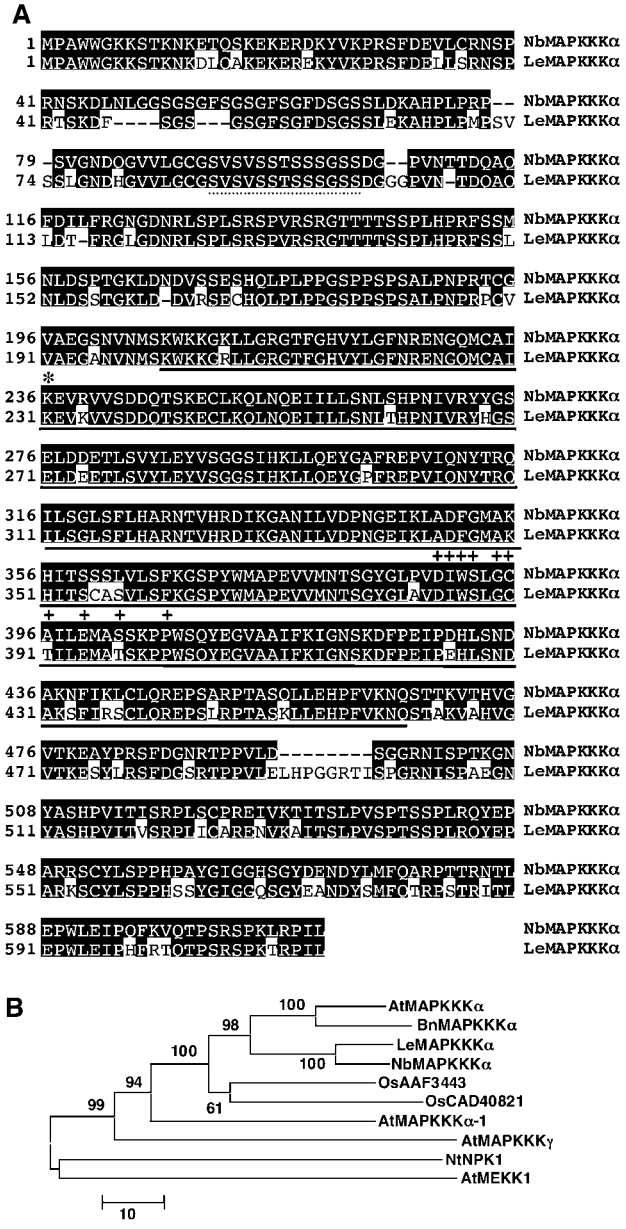

Figure 2 Sequence of MAPKKKα and relationship to MAPKKKα from other plant species. (**A**) Alignment of NbMAPKKKα and LeMAPKKKα predicted protein sequences using Clustal W method. Numbers on the left indicate amino-acid positions. Identical residues are shaded in black. The serine-rich domain is marked with a dotted line. The deduced kinase domain is underlined with a solid black line. The catalytic ATP-binding lysine is marked with an asterisk (*) and the signature motif for the plant MEKK family is indicated with crosses (+). (**B**) Neighbor-joining tree using MEGA.2.1 method (see Supplementary Methods) based on the deduced catalytic kinase domain of NbMAPKKKα (AY500155), LeMAPKKKα (AY500156) and orthologs (BnMAPKKKα [CAA08995], *B. napus*; AtMAPKKKα [At1g53570], *A. thaliana*; OsAAF34436, OsCAD40821, *Oryza sativa*), with MEKK A2 subgroup members (AtMAPKKKα-1 [At1g63700], AtMAPKKKγ [At5g66850]) and other plant MEKKs (AtMEKK1 [At4g08500]; NtNPK1 [A48084], *Nicotiana tabacum*) involved in disease signaling. Sequences were obtained from the NCBI database. Scale below represents amino-acid substitutions and numbers on the tree represent bootstrap scores.

of 10^7 CFU/ml of avirulent *P. s.* pv. *tabaci* (*avrPto*). The HR did not develop, or was much reduced, in *NbMAPKKKα*-silenced leaves, whereas leaves infected with TRV-only showed a full HR (Figure 3A). No HR was observed in *Prf*-silenced plants (Figure 3A). At 1 week after inoculation with a lower titer of *P. s.* pv. *tabaci* (*avrPto*) (10^6 CFU/ml, which does not cause an HR), *Prf*-silenced plants developed disease symptoms, consistent with the requirement of Prf for Pto-mediated resistance (Figure 3A). As expected, no disease symptoms were evident in TRV-only plants due to Pto-mediated recognition of AvrPto. Unexpectedly, disease symptoms did not form on *NbMAPKKKα*-silenced leaves. This observation suggested that MAPKKKα might also play a role in disease-associated cell death (Figure 3A; see next section).

To avoid possible confounding effects due to overexpression of *Pto* in *N. benthamiana*, we also carried out silencing experiments in tomato plants expressing Pto from its native promoter (RG-PtoR). We generated a tomato TRV::*LeMAPKKKα* clone similar to the *N. benthamiana* cNbME28C8 to silence *LeMAPKKKα* in RG-PtoR tomato plants. After 3–4 weeks, the silenced leaves were infiltrated with an HR-inducing titer (5×10^7 CFU/ml) of *P. s.* pv. *tomato* (*avrPto*) DC3000 (hereafter DC3000). Treatment with Trypan blue, which stains dead cells, revealed that tomato *LeMAPKKKα*-silenced leaves showed significantly less cell death 18 h after bacterial infiltration compared to TRV-only-infected plants, which showed confluent and intense Trypan blue staining (Figure 3B). These observations were quantitated and supported by reduced electrolyte leakage in leaves silenced for *LeMAPKKKα* (or for *Prf*) (Figure 3C). Therefore, MAPKKKα plays an important role in both *N. benthamiana* and tomato in modulating the immune response to avirulent *P. syringae* strains carrying the *avrPto* gene.

MAPKKKα positively regulates cell death associated with disease susceptibility

To test the hypothesis that MAPKKKα might also play a role in disease-associated cell death, we silenced *NbMAPKKKα* in *N. benthamiana* and inoculated the leaves with a disease-causing titer (5×10^5 CFU/ml) of the virulent pathogen *P. s.* pv. *tabaci* (Figure 4A). No disease symptoms were observed in any *NbMAPKKKα*-silenced leaves 4 days after *P. s.* pv. *tabaci* inoculation, whereas disease-associated cell death developed in all nonsilenced plants (Figure 4A).

To analyze the effect of *MAPKKKα* loss of function on bacterial speck disease, we carried out a similar experiment in tomato plants (RG-prf3), which are susceptible to DC3000. *MAPKKKα*-silenced RG-prf3 tomato leaves were infiltrated with DC3000 (10^4 CFU/ml) and scored for disease symptoms 4 days later. RG-prf3 leaves silenced for *LeMAPKKKα* displayed milder disease symptoms, with speck size greatly reduced compared to TRV-only-infected plants. (Figure 4B). We examined the effect of *LeMAPKKKα* silencing on bacterial growth in leaves. At 4 days after infection, *LeMAPKKKα*-silenced leaves displayed ~80-fold less bacteria compared to TRV-only-infected plants (Figure 4C). The degree of silencing correlated with the reduction of bacterial growth and reduced speck symptoms (Supplementary Figure S3). Thus, we conclude that host-mediated cell death during the compatible interaction plays an important role in allowing full growth of DC3000.

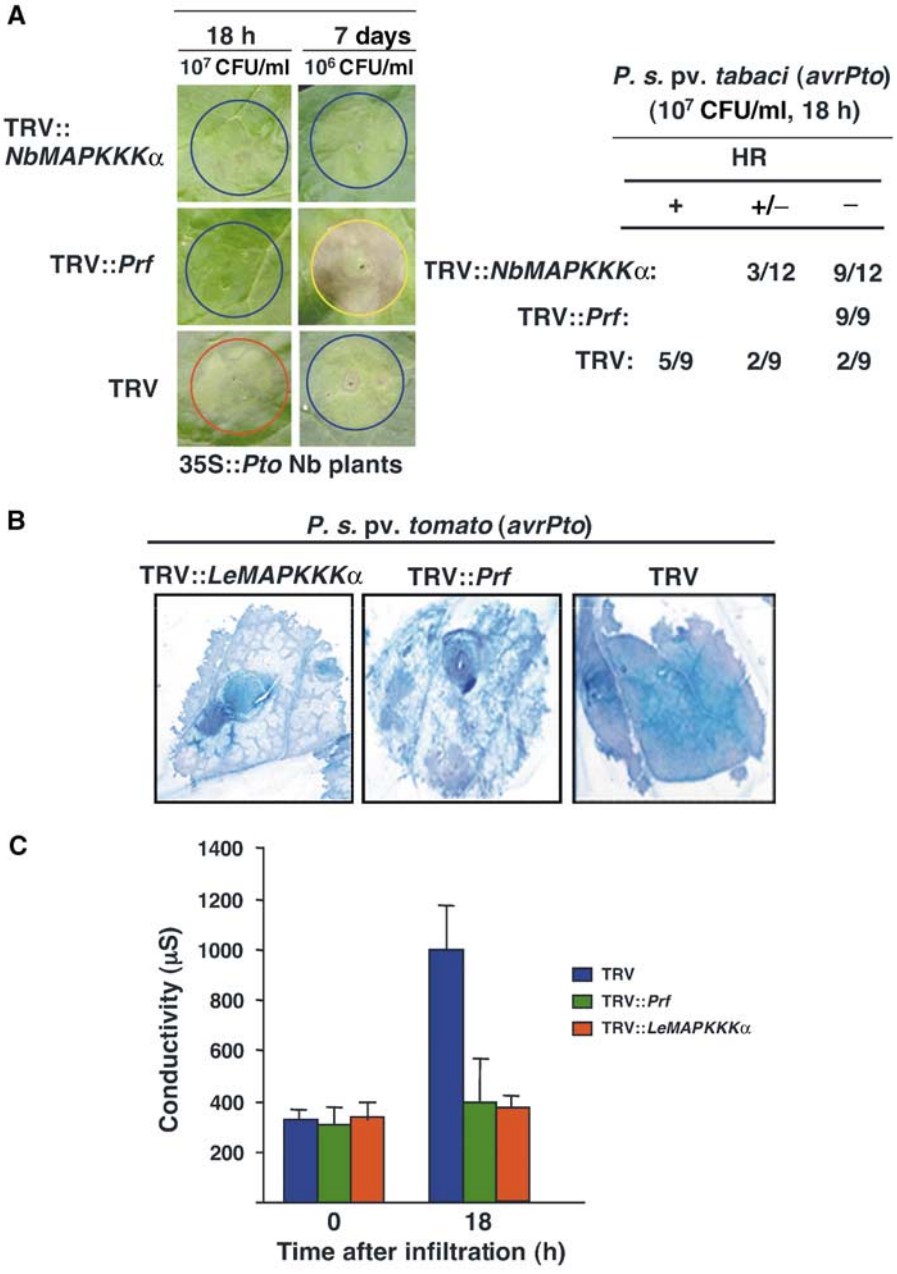

Figure 3 MAPKKKα is required for the Pto-mediated HR. (**A**) *N. benthamiana* 35S::*Pto* plants silenced for *NbMAPKKKα* or *Prf* showed less or no HR compared to TRV-only plants 18 h after inoculation with *P. s.* pv. *tabaci* (*avrPto*) at 10^7 CFU/ml (photo panel on left). Results of HR assays are shown in the table on the right. HR occurred in >80% of the infiltrated area (+); HR cell death occurred in 20–80% of the infiltrated area (+/−); no cell death was observed (−). Number in each category is shown over total scored. Disease symptoms were not evident in TRV::*NbMAPKKKα* or TRV-only plants but developed in TRV::*Prf* plants 7 days after infection, at 10^6 CFU/ml (photo panel on right). The blue circle indicates no cell death, the yellow circle indicates disease-associated cell death and the red circle indicates HR. (**B**) Detection of HR cell death by Trypan blue staining. RG-PtoR tomato leaves silenced for *LeMAPKKKα* or *Prf* were compared to TRV-only leaves 18 h after infiltration of 5×10^7 CFU/ml of *P. s.* pv. *tomato* DC3000 (*avrPto*). The experiment was repeated three times with similar results. For each experiment, positive and negative controls were analyzed in parallel: TRV::*Prf* (no HR observed), TRV-only (confluent blue area indicates HR-associated cell death). (**C**) Quantification of the HR cell death inhibition in tomato leaves shown in (B) by ion leakage of leaf discs following the procedure described in Figure 1C.

Overexpression of MAPKKKα activates MAPKs and causes pathogen-independent cell death

Because the silencing of MAPKKKα blocked cell death, we reasoned that its overexpression might cause cell death. We developed estradiol-inducible constructs to express either full-length LeMAPKKKα protein (LeMAPKKKα) or just the kinase domain (LeMAPKKKα^{KD}) and transiently overexpressed them in leaves. Both LeMAPKKKα and LeMAPKKKα^{KD} resulted in cell death in *N. benthamiana* and in tomato (data not shown) leaves within 36 and 24 h after estradiol application, respectively (Figure 5A). No cell death was observed following expression in *N. benthamiana* leaves of an LeMAPKKKα protein with a point mutation disrupting the essential lysine in the ATP-binding site

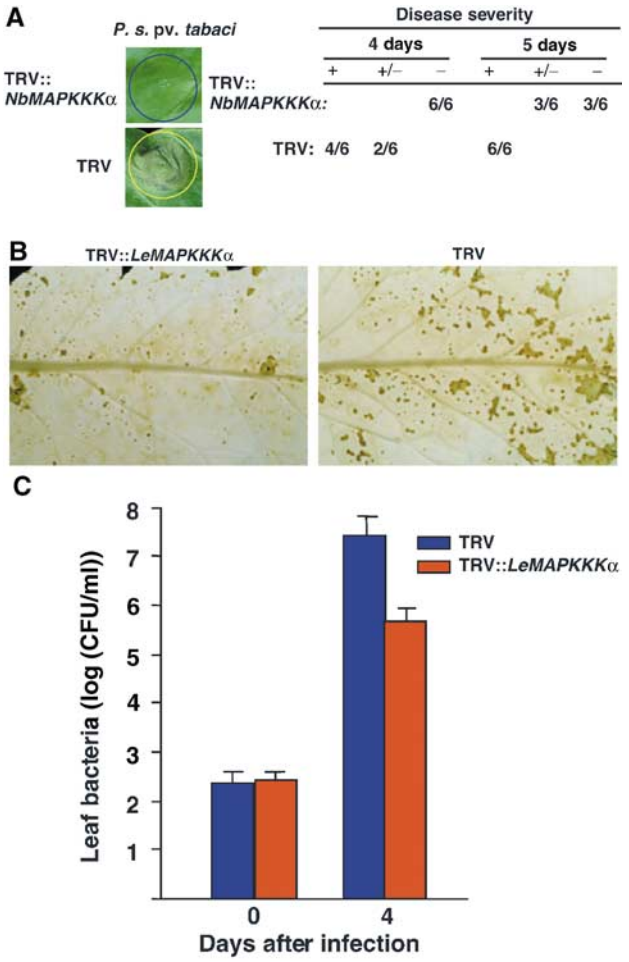

Figure 4 MAPKKKα plays a role in development of bacterial disease symptoms. (**A**) Symptoms in *N. benthamiana* plants silenced for *NbMAPKKKα* or TRV-only inoculated with 5×10^5 CFU/ml of *P. s.* pv. *tabaci* 5 days after infection (left panel). Disease severity (at 4 or 5 days after infection) is shown in the table. Disease symptoms occurred in >80% of the infiltrated area (+); disease symptoms occurred in 20–80% of the infiltrated area (+/−); no disease symptoms were observed (−). The experiment was performed with three plants per line in which two leaves were inoculated per plant. The blue circle indicates no cell death and the yellow circle indicates disease-related cell death. (**B**) Tomato leaves susceptible to bacterial speck disease (RG-prf3) were silenced for TRV::*LeMAPKKKα* or TRV-only were inoculated with 10^4 CFU/ml DC3000. After 4 days, leaves were destained and photographed. TRV-only leaves developed more lesions than TRV::*LeMAPKKKα*-silenced leaves. (**C**) Growth of DC3000 on TRV::*LeMAPKKKα* tomato leaves (RG-prf3) is reduced ~80-fold compared to TRV-only leaves 4 days after bacterial inoculation. A total of three TRV-only and seven TRV::*LeMAPKKKα*-infected RG-prf3 tomato plants were vacuum-infiltrated with 10^4 CFU/ml DC3000. For bacterial growth measurements, 3 leaf discs/plant (1 cm^2) were obtained from silenced leaves. Tissue was collected from the same areas for RT–PCR to monitor silencing efficiency (see Supplementary Figure S3) and bacterial counts from plants that did not show satisfactory silencing were excluded. Data presented are the means of three plants and error bars represent the standard deviation.

(LeMAPKKKα^{KD-}) indicating that kinase activity of MAPKKKα is required for the cell death phenotype.

To determine if cell death was associated with the activation of a MAPK cascade, we performed an in-gel kinase assay using myelin basic protein as a substrate for MAPK activity (Figure 5B). Cells expressing either LeMAPKKKα or

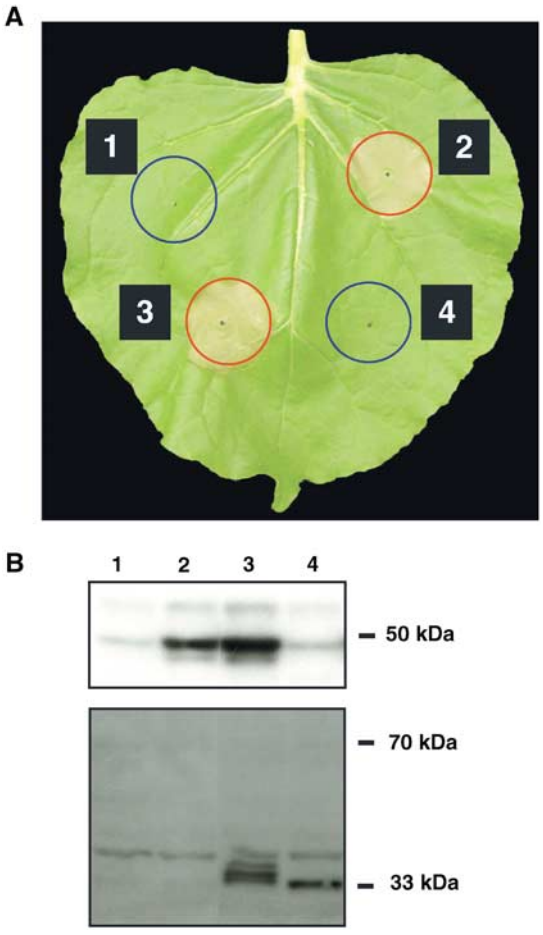

Figure 5 Overexpression of LeMAPKKKα in leaves causes cell death and stimulates MAPK activity. (**A**) Assessment of cell death in *N. benthamiana* leaves after infiltration of *Agrobacterium* strains carrying (1) empty vector, (2) *LeMAPKKKα*, (3) *LeMAPKKKα^{KD}* (*LeMAPKKKα$^{202-458}$*) or (4) *LeMAPKKKα^{KD-}* (*LeMAPKKKα$^{202-458/K231M}$*). All genes were tagged with the double hemagglutinin (HA) epitope and expressed from an estradiol-inducible promoter. The blue circle indicates no cell death and the red circle indicates cell death. Photograph was taken 36 h after application of estradiol. (**B**) Expression of LeMAPKKKα activates MAPK activity in leaf tissue. Protein extracts from *N. benthamiana* leaves transformed with the constructs described in (A) were assayed for MAPK activity using the in-gel kinase assay (top panel) 6 h after application of estradiol. Expression of the transiently expressed proteins was monitored by immunoblot analysis with anti-HA antibody (bottom panel). Lanes are numbered corresponding to (A).

LeMAPKKKα^{KD} showed increased MAPK activity at 6 h after estradiol application. Expression of LeMAPKKKα^{KD-}, which did not cause cell death, showed no increased MAPK activity. Immunoblot analysis confirmed the expression of the proteins (Figure 5B). LeMAPKKKα^{KD} migrated as three bands, which is likely an indication of differential phosphorylation, since the kinase inactive mutant (LeMAPKKKα^{KD-}) migrated as a single band (Figure 5B). Although wild-type LeMAPKKKα was not detectable by immunoblots, host cell death and activation of MAPKs indicated that it was expressed.

Two MAPK cascades act downstream of MAPKKKα to trigger cell death

To investigate the MAPK cascade activated by MAPKKKα, we designed epistasis experiments that combined a gain-of-func-

tion cell death assay (by overexpression of LeMAPKKKα^{KD}) with a loss-of-function approach based on VIGS of various MAPKKs and MAPKs involved in plant defense (Zhang and Klessig, 2001; Ekengren *et al*, 2003). The assumption was that MAPKKKα-mediated cell death would not occur if the expression of key downstream MAPKK or MAPK components was suppressed.

N. benthamiana plants were silenced for the MAPKKs, *MEK1, MEK2* or *SIPKK*, or for the MAPKs, *SIPK, WIPK, NTF4* or *NTF6*. As controls, we included plants infected with TRV-only and silenced for *Prf*. LeMAPKKKα^{KD} or LeMAPKKKα^{KD-} was transiently expressed in the silenced leaves via Agro-infiltration (Figure 6A). As expected, cell death was not observed in any leaf area expressing LeMAPKKKα^{KD-}. In TRV-infected plants expressing LeMAPKKKα^{KD}, total collapse of the tissue occurred by 48 h. Silencing of two MAPKK genes (*MEK1* and *MEK2*) and two MAPK genes (*SIPK* and *NTF6*) suppressed cell death normally caused by expression of LeMAPKKKα^{KD} (Figure 6). However, silencing of three other genes (*SIPKK*, *WIPK* (Figure 6) or *NTF4* (data not shown)) had no effect on LeMAPKKKα-mediated cell death. Silencing of *Prf* did not affect LeMAPKKKα-mediated cell death, indicating that MAPKKKα lies downstream of Prf in the Pto pathway. We confirmed a decrease in transcript abundance for each of the genes targeted for silencing (Supplementary Figure S4).

NtMEK1 and NtMEK2 have been reported to act upstream of NTF6 and SIPK, respectively (Yang *et al*, 2001; Soyano *et al*,

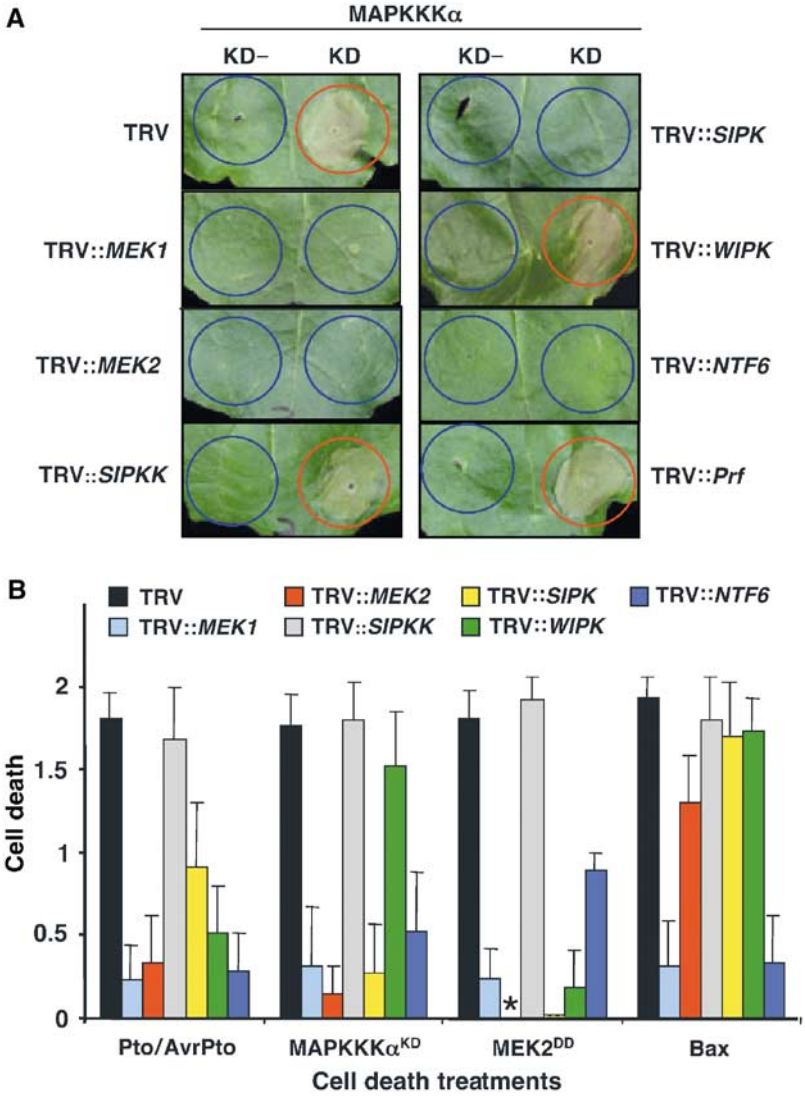

Figure 6 Identification of two MAPK cascades acting downstream of MAPKKKα. (**A**) Overexpression of LeMAPKKKα^{KD} and LeMAPKKKα^{KD-} using an estradiol-inducible promoter in *N. benthamiana* leaves silenced for the MAPKK and MAPK genes shown, and for *Prf* and in TRV-only leaves. The blue circle indicates no cell death and the red circle indicates cell death. The photographs were taken 48 h after induction with estradiol. (**B**) Overexpression of LeMAPKKKα^{KD}, LeMEK2^{DD}, Pto/AvrPto or Bax (and their respective controls; LeMAPKKKα^{KD-}; LeMEK2; Pto/empty vector; BaxΔC) via Agro-infiltration (as described in Figure 1A) in *N. benthamiana* leaves silenced for the genes indicated or in TRV-only leaves. Expression of the transgenes was induced by estradiol 48 h after Agro-infiltration. Cell death was recorded 48 h after estradiol application (i.e., 4 days after bacteria infiltration) by using the following scoring system: 2 for full HR; 1 for partial HR; and 0 for complete inhibition of HR. Shown are the means of four independent experiments for LeMAPKKKα^{KD}, Pto/AvrPto and Bax and three independent experiments for LeMEK2^{DD}. Four to six silenced plants per construct (with 2–3 leaves infiltrated in each) were used in each experiment. Error bars represent the standard deviation. *Not performed.

2003). Therefore, our data suggest that two different MAPK cascades participate in the MAPKKKα-dependent cell death pathway. To examine the relationship of these two MAPK cascades, we again used epistasis experiments but this time we triggered cell death at different 'entry points' into the cell death pathway. *N. benthamiana* leaves were silenced for *MEK1, MEK2, SIPK, WIPK, NTF6* or, as a negative control, *SIPKK* (Figure 6B). We then expressed Pto/AvrPto, LeMAPKKKα^{KD}, LeMEK2^{DD}, a constitutive active form of LeMEK2 (expression of NtMEK2^{DD} causes cell death; Yang *et al*, 2001), or Bax, along with the corresponding negative controls (Pto; LeMAPKKKα^{KD-}; LeMEK2; and BaxΔC) (Figure 6B).

Pto/AvrPto-mediated cell death was prevented in leaves silenced for *MEK1, MEK2, SIPK* (intermediate), *NTF6* and *WIPK* whereas LeMAPKKKα^{KD}-mediated cell death was prevented in *MEK1-, MEK2-, SIPK-* or *NTF6*-silenced leaves (Figure 6B). Pto/AvrPto- and LeMAPKKKα^{KD}-mediated cell death developed normally in TRV-only- or *SIPKK*-silenced controls (Figure 6B). No cell death was detected in any of the areas infiltrated with negative controls (data not shown). These results suggest that SIPK and not WIPK acts downstream of MEK2 in a MAPKKKα-dependent cell death pathway. Interestingly, cell death caused by expression of NtMEK2^{DD} was fully suppressed in leaves silenced for *SIPK, WIPK* or *MEK1* and partially in leaves silenced for *NTF6* (Figure 6B). This observation is consistent with MEK2 being the upstream MAPKK of SIPK and WIPK and suggests that the MEK1/NTF6 cascade acts downstream or in parallel with the MAPKKKα/MEK2/SIPK cascade. Bax-induced cell death was compromised in plants silenced for *MEK1* and *NTF6* but developed normally in plants silenced for *MEK2, SIPK* or *WIPK.* This observation implies that Bax-induced cell death is downstream of the MAPKKKα/MEK2/SIPK cascade and upstream of MEK1/NTF6.

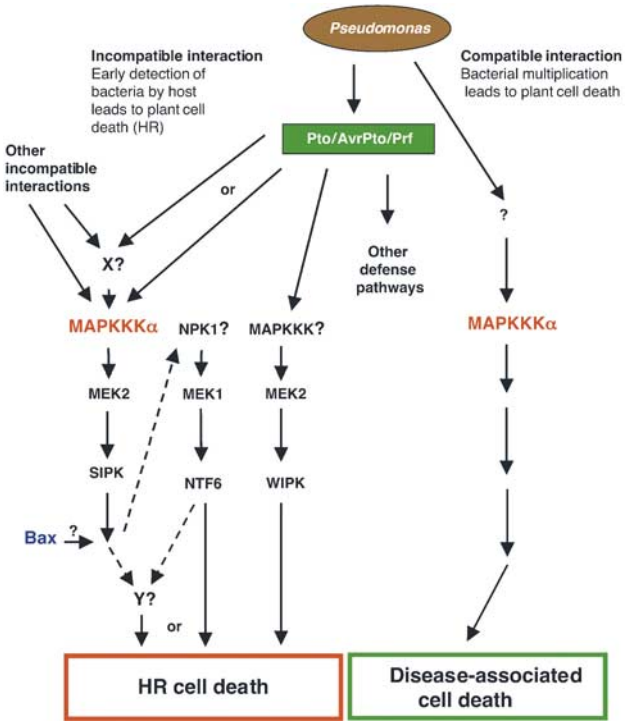

Figure 7 Model for MAPKKKα-mediated cell death signaling. Model based on epistasis analysis combining cell death assays with *N. benthamiana* plants silenced for various MAPKK and MAPK genes. Two MAPK cascades are shown acting downstream of Pto in a MAPKKKα-mediated cell death signaling pathway. A MEK2/WIPK cascade might also contribute to cell death via a MAPKKKα-independent pathway. Depiction of a role for MAPKKKα in disease-associated cell death is based on reduced bacterial speck disease symptoms in *N. benthamiana* or in tomato silenced for *MAPKKKα.* 'X' indicates a possible upstream activator of MAPKKKα lying between Pto and MAPKKKα. Dotted lines indicate the possibility of the two MAPK cascades acting either sequentially or in a parallel co-dependent manner. 'Y' indicates a possible integrator of signals if the pathways act in parallel.

Discussion

We have identified MAPKKKα as a key regulator of cell death associated with plant immunity and disease susceptibility (Figure 7). Silencing of *MAPKKKα* suppressed the host immune response toward two avirulent *P. syringae* strains and inhibited the progression of disease symptoms and bacterial growth in susceptible plants. MAPKKKα overexpression resulted in pathogen-independent cell death, confirming a positive role for MAPKKKα in cell death regulation. Two MAPK cascades, acting in either a sequential or a co-dependent parallel manner, appear to act downstream of MAPKKKα in this cell death signaling pathway. Therefore, we propose a role for MAPKKKα as a shared molecular switch underlying both immunity and disease susceptibility in response to yet unknown signals originating from pathogen attack.

Two other members of the MEKK group of plant MAPKKKs have assigned roles in defense signaling. MEKK1 mediates the innate immune response to bacterial flagellin, whereas NPK1 participates in several *R* gene responses (Asai *et al*, 2002; Jin *et al*, 2002). In contrast to MAPKKKα, overexpression of either full-length or constitutive active MEKK1 or NPK1 in leaves did not result in plant cell death, suggesting that they regulate different aspects of defense responses (Tena *et al*, 2001; Asai *et al*, 2002). Two Raf-like plant MAPKKKs (EDR1 and CTR1) and a MAPK (MPK4) have been identified in genetic screens as negative regulators of salicylic acid-dependent defense and ethylene-dependent responses (Jonak *et al*, 2002). Interestingly, MAPKKKα seems to share downstream MAPKK and MAPK components with MEKK1 and CTR1 (Asai *et al*, 2002; Ouaked *et al*, 2003). A goal for the future will be to integrate the MAPKKKα-mediated cell death pathway with other MAPK cascades operating in plant defense responses.

MAPKKKα-mediated cell death requires two MAPK cascades

MAPK signaling cascades have recently been implicated in the Pto pathway. Notably, VIGS of *MEK1*, *MEK2* and *NTF6,* which compromised Pto-mediated disease resistance in tomato (Ekengren *et al*, 2003), also compromised MAPKKKα-mediated cell death in *N. benthamiana*. However, SIPK but not WIPK appears to play a role in MAPKKKα-mediated cell death, whereas WIPK but not SIPK was implicated in Pto-mediated disease resistance in tomato. Because SIPK activation precedes and is required for WIPK activation (Liu *et al*, 2003), it is possible that residual SIPK from incomplete silencing in tomato cells was sufficient to activate WIPK and mount a resistance response. However, we cannot rule

out that (i) SIPK plays a role in cell death signaling and not in Pto-mediated resistance or (ii) SIPK is the downstream MAPK in the MAPKKKα cascade signaling for cell death in disease, contributing to speck formation and bacterial growth. Future biochemical analysis, more efficient silencing in tomato, and testing the role of SIPK in disease will shed light on these questions.

We confirmed, as shown by others (Yang *et al*, 2001; Ekengren *et al*, 2003), that WIPK is a component of Pto-mediated HR signaling and MEK2 is its likely upstream MAPKK. However, our epistasis experiments indicate that WIPK does not play a direct role in MAPKKKα-mediated cell death. This conclusion is based on our experiments in which WIPK-silenced leaves showed inhibition of cell death due to expression of Pto/AvrPto or $MEK2^{DD}$, but not to MAPKKKα. It has been suggested that WIPK could function in accelerating SIPK-mediated cell death or initiating a new pathway (Zhang and Liu, 2001; Liu *et al*, 2003). We found no delayed onset of MAPKKKα-mediated cell death in *WIPK*-silenced leaves compared to unsilenced plants. It is possible that MAPKKKα overexpression causes sustained SIPK activity in the presence of reduced WIPK in *WIPK*-silenced leaves, leading to cell death. Our observations support that WIPK might act in a MAPK cascade (involving MEK2) activated by an unknown MAPKKK that contributes to Pto-mediated HR and resistance. Several MAPKKKs can initiate cascades sharing the same MAPKKs in animals (Widmann *et al*, 1999). The presence of >60 MAPKKKs and only 10 MAPKKs in *Arabidopsis* implies similar convergence in plants (Ichimura *et al*, 2002).

The identity of a plant MAPKKK that receives the input signals for cell death induction during biotic interactions was previously unknown. MAPKKKα-mediated cell death requires two MAPK cascades. The first cascade, MAPKKKα/MEK2/SIPK, emerges as a pro-cell death module with all three components shown to play a positive role in cell death signaling (Yang *et al*, 2001; Zhang and Liu, 2001; Ren *et al*, 2002). It is remarkable that a modest reduction in MEK2 expression still resulted in MAPKKKα-mediated cell death inhibition (Supplementary Figure S4). SIPK and WIPK are transiently activated following several stresses. However, in incompatible interactions that result in an HR, SIPK activation is long-lasting and precedes a delayed activation of WIPK (Zhang and Klessig, 2001). We hypothesize that MAPKKKα activation and association with MEK2 and SIPK result in enhanced SIPK activity during the HR.

We also show for the first time that a second cascade, involving MEK1 and NTF6, is important for PCD signaling in plants. Silencing of *MEK1* and *NTF6* in *N. benthamiana* suppressed cell death induced by expression of Pto/AvrPto, MAPKKKα, $MEK2^{DD}$, Bax or C9/Avr9 (Figure 6B and data not shown). These observations suggest that MEK1 and NTF6 are signaling components of convergent cell death pathways and lie genetically downstream or in a co-dependent parallel pathway with MAPKKKα/MEK2/SIPK. The NPK1/MEK1/NTF6 cascade plays an important role in cell division (Soyano *et al*, 2003) and NPK1 also participates in the immune response to TMV but not in Pto-mediated HR (Jin *et al*, 2002). Jin *et al* (2002) also noted that *SIPK*, *WIPK* and *MEK2* silencing did not affect Pto-mediated HR. These results do not agree with our observations or those of Ekengren *et al* (2003). It is possible that this discrepancy is due to the weaker silencing system (PVX versus TRV) and the stronger HR assay used by Jin *et al* (2002). In the light of this observation, we believe that it remains a possibility that NPK1 is the MAPKKK upstream of MEK1 and NTF6 signaling for cell death.

Bax expression defines two distinct cascades signaling for cell death

The fact that Bax-mediated cell death was completely inhibited in leaves silenced for *MEK1* and *NTF6*, but not in leaves silenced for *MAPKKKα*, *MEK2* or *SIPK*, allowed us to differentiate two cascades: MAPKKKα/MEK2/SIPK and MEK1/NTF6. These results were supported by the overexpression of constitutive active $LeMEK2^{DD}$, which did not lead to cell death in leaves silenced for *SIPK, WIPK, MEK1* or *NTF6*. Because Bax also induces cell death in yeast and, as in plants, requires the carboxy-terminal transmembrane domain that docks it in the mitochondrion, it is plausible that mitochondria-related events account for Bax-mediated cell death in both systems (Lacomme and Santa Cruz, 1999). In animal apoptosis, the mitochondria are considered the sensors of cellular homeostasis disturbances as well as integrators and amplifiers of cell death signals.

MAPKKKα is a signaling component mediating both immunity and disease susceptibility

A role for MAPKKKα in cell death underlying both immunity and disease susceptibility supports the emerging theme that common signaling pathway(s) operate in *R* gene-mediated immunity and in basal resistance. This theme is reinforced by several recent findings. First, in comparisons of compatible and incompatible *P. syringae* interactions by using gene expression profiling, biochemical and microscopic analysis, Tao *et al* (2003) and Yao *et al* (2002) found that responses and features in early incompatible interactions were similar to those in late compatible interactions, with most of the differences being quantitative. Second, manipulation of plant PCD pathways by ectopic expression of the antiapoptotic *p35* gene from baculovirus reduced the HR and disease resistance to incompatible pathogens (del Pozo and Lam, 2003) and also decreased susceptibility to *P. s.* pv. *tomato* and necrotrophic fungal pathogens (Lincoln *et al*, 2002). Thus, it appears likely that the same target affected by the p35 protein operates in the HR and in disease-related cell death. Our results strongly support the notion that cell death events involved in immunity and in disease susceptibility are mechanistically related at the level of the MAPKKKα switch.

Our results also indicate that MAPKKKα is a necessary host component for bacterial parasitism, and support the hypothesis that virulent bacterial pathogens like *P. syringae* induce host cell death pathways late during infection, possibly as nutrients become scarce (Stone *et al*, 2000). However, we cannot rule out that MAPKKKα might be regulating other susceptibility-related pathways in addition to controlling cell death. Perturbation of these pathways could also lead to inhibition of bacterial growth.

Model for MAPKKKα participation in Pto/AvrPto pathway

We illustrate our working model for the role of MAPKKKα in Pto-mediated signaling in Figure 7 (*Arabidopsis thaliana*

homologs are presented in Supplementary Table ST2). In the presence of Prf, Pto recognition of the bacterial effector AvrPto initiates signaling pathways leading to disease resistance. Other components of the Pto pathway, in addition to the nine genes described by Ekengren *et al* (2003), are Pti1, a ser/thr kinase that is thought to have a positive role in HR signaling, and Pti4, Pti5 and Pti6, which activate transcription of defense-related genes (Pedley and Martin, 2003). Pto, Pti1 or another unknown downstream kinase could phosphorylate and activate MAPKKKα. However, because MAPKKKα appears to be a convergent point for signaling by multiple R genes, a common second messenger or stress sensor (represented by 'X' in Figure 7) detecting early R-mediated signaling events might activate MAPKKKα. MAPKKKα would then activate MEK2, which in turn activates SIPK. Activation of downstream effectors of this cascade results in the production of ethylene, transcription of defense-related genes and generation of phase II ROS (Ren *et al*, 2002; Kim *et al*, 2003; Ouaked *et al*, 2003). ROS could lead to the activation of a MAPKKK, possibly NPK1 (Tena *et al*, 2001), which in turn might activate MEK1/NTF6. WIPK might play a role in cell death amplification participating in a different cascade. We propose that both MAPK cascades required for MAPKKKα-mediated cell death signaling (MAPKKKα/MEK2/SIPK) and (NPK1?/MEK1/NTF6) cooperate, through a yet unknown molecular mechanism balancing pro-cell death stimuli versus cell proliferation cues (shown as dashed lines and 'Y' in Figure 7). The integration of these inputs might decide whether the ultimate fate of the cell is death or proliferation.

In the future, it will be important to identify activating molecules and downstream effectors in the MAPKKKα-mediated cascade, to determine which MAPKK and MAPK components of the MAPKKKα cascade act in disease susceptibility, and ultimately to identify the molecular link(s) between both cascades. Comparisons of changes in gene expression and protein phosphorylation in wild type, MAPKKKα loss-of-function and MAPKKKα-overexpressing plant cells should also help identify components of plant PCD. Finally, it is possible that manipulation of the MAPKKKα loss-of-function phenotype might be usefully deployed in certain crops to confer enhanced tolerance to pathogens in the field.

Materials and methods

Plant material and bacterial strains

N. benthamiana or transgenic derivatives expressing a 35S::*Pto* transgene, resistant tomato (*Lycopersicon esculentum*) line Rio Grande-PtoR (RG-PtoR; *Pto/Pto*, *Prf/Prf*) or susceptible line Rio Grande-prf3 (RG-prf3; *Pto/Pto, prf/prf*) were used for silencing experiments. *A. tumefaciens* strains GV2260 and GV3101 were used for VIGS in *N. benthamiana* and in tomato, respectively. GV2260 was used for Agro-infiltration in leaves. *P. syringae* pv. *tabaci* (strain 11528R) or *P. syringae* pv. *tabaci* (*avrPto*) were used for pathogen assays in *N. benthamiana* and *P. syringae* pv. *tomato* (*avrPto*) strain DC3000 was used in tomato.

Construction of a cDNA library in a PVX vector, inoculation and monitoring HR

cNbME library was made from *N. benthamiana* leaf tissue treated with abiotic and biotic elicitors, cloned into a PVX-based vector and transformed into GV2260. Two *N. benthamiana* plants were inoculated per clone, and 3 weeks later the fourth and fifth leaves above the initially infected ones were tested for HR development by Agro-infiltration (see details in Supplementary Methods).

TRV vector and derivatives, inoculation and HR and disease assays

TRV vector was described in Liu *et al* (2002). TRV derivatives were transformed into *Agrobacterium* strains and prepared for infection as described in Liu *et al* (2002) at $OD_{600} = 0.25$ (*N. benthamiana*) or 0.5 (tomato). For silencing in tomato, seedlings with two emerging leaflets were syringe infiltrated in the cotyledons (primers for TRV2 derivatives are described in Supplementary Methods). *P. syringae* strains were infiltrated into leaves with a vacuum or a syringe. We were unable to measure reliably bacterial growth in RG-PtoR plants silenced for *LeMAPKKKα* because, unlike in the study by Ekengren *et al* (2003), the reduced lesion development in these plants (as discussed in Results) made it difficult to identify silenced tissue.

Agro-infiltration assay

GV2260 cultures ($OD_{600} = 0.12$) carrying *Avr* or *R* genes were grown and prepared for infiltration following a similar procedure as described in He *et al* (2004).

RNA isolation and RT–PCR analysis

RNA extraction, first strand cDNA synthesis, RT–PCR and RT–PCR-specific primers used are described in Supplementary Methods and Supplementary Table ST1.

Cell death measurements

Staining with Trypan blue was performed as described in He *et al* (2004). For conductivity tests, 5 discs/plant (1 cm diameter) were obtained from infiltrated areas for each plant and floated in 5 ml ddH_2O water for 4 h at room temperature with gentle shaking and conductivity was measured with an Acorn Con 5 meter (Oakton Instruments, Vernon Hills, IL).

Cloning of N. benthamiana and tomato MAPKKKα sequences

Full-length *N. benthamiana* and tomato *MAPKKKα* cDNA sequences were obtained using Smart RACE amplification kit (BD Bioscience-Clontech, Palo Alto, CA). At least three clones were sequenced to confirm cloning.

LeMAPKKKα and LeMEK2^{DD} mutagenesis and cloning

LeMEK2 was obtained from the tomato EST collection (cLER2B17). LeMAPKKKα^{KD-} and LeMEK2 constitutive active (LeMEK2^{DD}) generation and cloning into pER8 are described in Supplementary Methods.

Protein expression, protein extraction and in-gel kinase assay

LeMAPKKKα, *LeMAPKKKα^{KD}* and *LeMAPKKKα^{KD-}* were cloned into pER8 and transformed into GV2260. At 48 h after Agro-infiltration (at $OD_{600} = 0.06$, except for LeMAPKKKα^{KD}, at $OD_{600} = 0.015$), 1 cm leaf disks were removed floated on 20 mM HEPES (pH 7.5), treated with 17-β-estradiol (5 μM) (after 2 h) and frozen in liquid N_2. Protein extraction, immunoblot analysis and in-gel kinases assay are described in Supplementary Methods.

Supplementary data

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

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