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A high-throughput screen of cell-death-inducing factors in *Nicotiana benthamiana* identifies a novel MAPKK that mediates INF1-induced cell death signaling and non-host resistance to *Pseudomonas cichorii*

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Summary

A high-throughput overexpression screen of *Nicotiana benthamiana* cDNAs identified a gene for a mitogen-activated protein kinase kinase (MAPKK) as a potent inducer of the hypersensitive response (HR)-like cell death. NbMKK1 protein is localized to the nucleus, and the N-terminal putative MAPK docking site of NbMKK1 is required for its function as a cell-death inducer. *NbMKK1*-mediated leaf-cell death was compromised in leaves where *NbSIPK* expression was silenced by virus-induced gene silencing. A yeast two-hybrid assay showed that NbMKK1 and NbSIPK physically interact, suggesting that NbSIPK is one of the downstream targets of NbMKK1. *Phytophthora infestans* INF1 elicitor-mediated HR was delayed in *NbMKK1*-silenced plants, indicating that NbMKK1 is involved in this HR pathway. Furthermore, the resistance of *N. benthamiana* to a non-host pathogen *Pseudomonas cichorii* was compromised in *NbMKK1*-silenced plants. These results demonstrate that MAPK cascades involving NbMKK1 control non-host resistance including HR cell death.

Keywords: mitogen-activated protein kinase kinase, cell death, phytophthora infestans, nuclear localization, virus-induced gene silencing, non-host resistance.

Introduction

Programmed cell death is an essential physiological process occurring during plant development and in response to biotic and abiotic stress (Beers and McDowell, 2001). Defense mechanisms of plants against invading pathogens often include localized cell death, known as the hypersensitive response (HR). This process shares numerous characteristics with mammalian apoptosis (Lam *et al.*, 2001), and requires active transcription and translation (Greenberg, 1997). The activation of defense responses is initiated upon plant-pathogen recognition, mediated either by a gene-for-gene interaction between a plant resistance (*R*) gene and a pathogen avirulence (*Avr*) gene, or by the binding of a non-race-specific elicitor to the receptor (Baker *et al.*, 1997; Dangl and Jones, 2001; Hammond-Kosack and Jones, 1996; Martin, 1999). Extensive research on the signaling cascade leading to HR downstream of *R*-gene mediated pathogen recognition led to the identification of various genes required for *R*-gene-mediated HR, such as *Rar1* (Shirasu *et al.*, 1999), *Sgt1* (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Liu *et al.*, 2002b; Peart *et al.*, 2002), *Hsp90* (Hubert *et al.*, 2003; Liu *et al.*, 2004a; Lu *et al.*, 2004; Takahashi *et al.*, 2003a,b), *Ndr1* (Century *et al.*, 1995) and *Eds1* (Liu *et al.*, 2002a; Parker *et al.*, 1996). However, the information on the whole signaling network is still fragmentary, and many more important players involved in HR remain to be discovered.

In a previous study, we performed high-throughput overexpression screening of a plant cDNA library, and identified several genes that cause cell death in *Nicotiana benthamiana* leaves upon overexpression, including a gene for the class-II ethylene-responsive element binding factor

(ERF) (Nasir *et al.*, 2005). Here, we report on another cDNA identified in the screen, which codes for a novel mitogen-activated protein kinase kinase (MAPKK), a component of MAPK cascade.

The post-translational modification of proteins such as phosphorylation and de-phosphorylation plays an important role in cellular signaling. The MAPK cascade is one of the major pathways by which extracellular stimuli are transduced into intracellular responses in eukaryotic cells via the phosphorylation/dephosphorylation of signaling proteins (Davis, 2000; Herskowitz, 1995; Widmann *et al.*, 1999). MAPK cascades are composed of three sequentially acting kinase components, MAPKK kinase (MAPKKK), MAPKK and MAPK. MAPKs are activated via the dual phosphorylation of their Thr and Tyr residues within a TXY motif by MAPKKs as the upstream kinases, which, in turn, are activated by MAPKKKs. The most extensively characterized plant MAPKs are the tobacco salicylic-acid-induced protein kinase (SIPK; Zhang and Klessig, 1997) and the wound-induced protein kinase (WIPK; Seo *et al.*, 1995, 1999), and their orthologs in other plant species (Lee *et al.*, 2004; Pedley and Martin, 2005; Romeis, 2001; Zhang and Klessig, 2001). Both SIPK and WIPK are activated in a gene-for-gene-specific manner either in *N* gene-harboring tobacco plants exhibiting resistance to tobacco mosaic virus (TMV; Zhang and Klessig, 1998) or in Cf9-expressing tobacco responding to the *Cladosporium fulvum*-encoded elicitor Avr9 (Romeis *et al.*, 1999). A tobacco MAPKK NtMEK2 acts upstream of both SIPK and WIPK in pathways that lead to host cell death (Yang *et al.*, 2001). SIPK overexpression alone also resulted in host cell death and transcriptional activation of defense-related genes (Zhang and Liu, 2001). Moreover, gene silencing of *NtMEK2* compromised *R* gene-mediated resistance to viral pathogens (Jin *et al.*, 2003), and gene silencing of either *SIPK* or *WIPK* abrogated the defense of plants against a bacterial pathogen (Sharma *et al.*, 2003). Recently, del Pozo *et al.* (2004) identified a tomato MAPKKK, MAPKKKα, as a positive regulator of cell death associated with both plant immunity and disease. MAPKKKα gene silencing abrogated the HR-like cell death caused by the interaction between *Pseudomonas syringae* avirulence gene *AvrPto* and its cognate R gene *Pto*. MAPKKKα overexpression combined with the virus-induced gene silencing (VIGS) of various MAPKK annd MAPK genes showed that two MAPK cascades, MEK2 $\rightarrow$ SIPK and MEK1 $\rightarrow$ NTF6, are downstream components transducing the cell-death signals from MAPKKKα. Liu *et al.* (2004b)) showed that two MAPK cascades, MEK2 $\rightarrow$ SIPK/WIPK and MEK1/NQK1 $\rightarrow$ NTF6/NRK1, participate in the *N*-gene mediated resistance of *N. benthamiana* against TMV. Plants have more than 100 genes that encode MAPK-related proteins, yet relatively little is known about their function and contribution to different pathways. In the present study we first demonstrate that NbMKK1 is a positive regulator of cell death, and by employing the VIGS approach we show that NbMKK1 is involved in the regulation of the *Phytophthora infestans* INF1 elicitor-induced HR cell death and non-host resistance of *N. benthamiana* against *Pseudomonas cichorii*.

Results

Overexpression of NbMKK1 caused cell death

We performed a high-throughput *in planta* overexpression screening of 40 000 *N. benthamiana* cDNAs using the potato virus X (PVX) system, and identified several cDNAs, including *NbCD1* coding for a class-II ERF, that caused cell death upon overexpression (Nasir *et al.*, 2005). The DNA sequence of one such cDNA had a high similarity to *MAPKK* genes, and we named it *NbMKK1* (GenBank Accession No. AB243987). The predicted protein for *NbMKK1* is composed of 325 amino acids, and phylogenetic analysis classified NbMKK1 into the subfamily D of plant MAPKK (Ichimura *et al.*, 2002; Figure 1). The most closely related protein is LeMKK4 from tomato (Pedley and Martin, 2004; 87% amino acid sequence similarity). The amino acid sequence similarity of NbMKK1 to the well-studied MAPKKs was low: 27.4% similarity to NtMEK2 (belonging to subfamily C) and 30.1% similarity to NtMEK1 (subfamily A). NbMKK1 has putative phosphorylation sites (T209 and S215) and an invariant ATP binding site (K88) conserved in all MAPKKs.

Overexpression of *NbMKK1* resulted in a strong phenotype, causing cell death on *N. benthamiana* leaves 2 weeks after toothpick inoculation and 4 days after infiltration of *Agrobacterium tumefaciens* harboring *NbMKK1* in the pSfinx vector (Takken *et al.*, 2000; Figure 2). Next, we confirmed the correlation between the NbMKK1 protein production and cell death by employing the glucocorticoid-inducible expres-

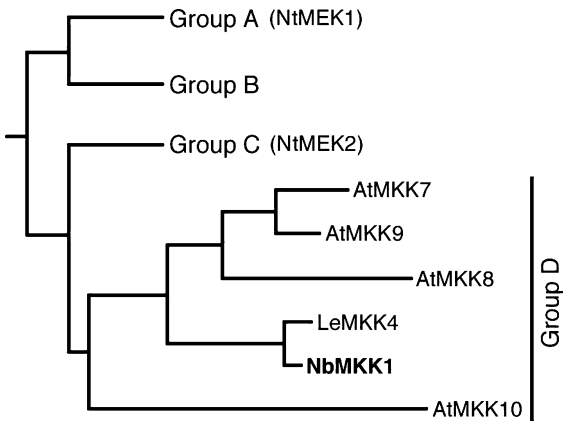

Figure 1. Phylogenetic tree of plant mitogen-activated protein kinase kinase (MAPKK) including *Nicotiana benthamiana* NbMKK1.
Relationships of the four groups of MAPKK (Groups A–D) are shown. Detailed phylogeny is given for MAPKKs in Group D. The species of origin of the MAPKKs are indicated by the abbreviation in front of the protein names; At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Nt, *Nicotiana tobacum*.

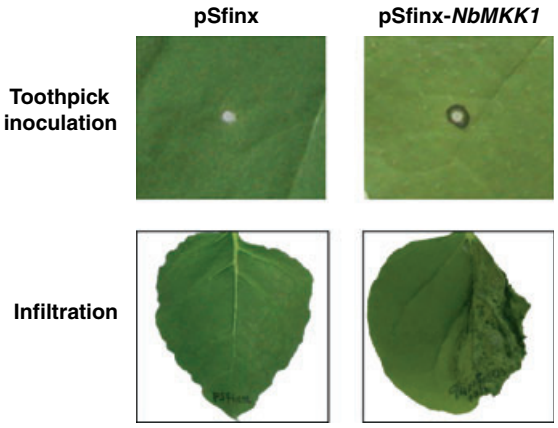

Figure 2. Cell-death phenotype in *Nicotiana benthamiana* leaves caused by *NbMKK1* overexpression.
Agrobacterium tumefaciens cells harboring either an empty pSfinx vector (left) or pSfinx-*NbMKK1* (right) were inoculated to *N. benthamiana* leaves either by toothpicks (top) or infiltrated into the right half of the leaf with a needleless syringe (bottom). Phenotypes of leaves 2 weeks post-inoculation (top) and 4 days post-agroinfiltration (bottom) are shown.

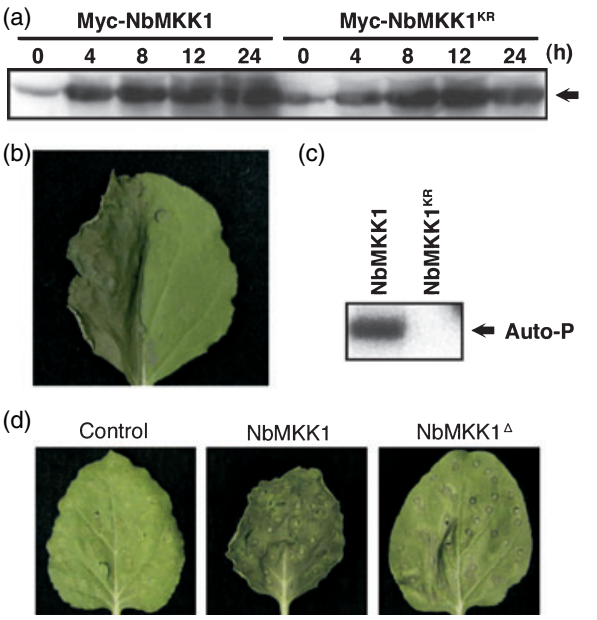

Figure 3. Protein expression, cell-death phenotype and autophosphorylation of NbMKK1 and NbMKK1^{KR}.
(a) Western blot analysis of NbMKK1 and NbMKK1^{KR} protein. NbMKK1 and its mutant construct NbMKK1^{KR} were transiently transformed into *Nicotiana benthamiana* leaves. Two days later, transgene expression was induced by the application of dexamethasone (DEX, 30 μM) and the expressed proteins were detected by c-myc-antibody.
(b) NbMKK1 caused cell death (left half of the leaf), but the kinase-dead mutant, NbMKK1^{KR}, did not (right half of the leaf). Pictures were taken 48 h after DEX treatment.
(c) NbMKK1 autophosphorylates, but the mutant NbMKK1^{KR} does not.
(d) Cell death caused by NbMKK1 lacking an N-terminal docking site (NbMKK1$^{\Delta}$, right) is delayed compared with that caused by the wild-type NbMKK1 (middle).

sion system GVG (Aoyama and Chua, 1997). Complementary DNA inserted into the GVG vector was modified so that the N-terminus of the protein was tagged with a triple c-myc epitope, resulting in c-myc-NbMKK1. *A. tumefaciens* harboring c-myc-NbMKK1 was infiltrated into *N. benthamiana* leaves by a needle-less syringe to establish the transient transformation of the leaves. Two days later, the glucocorticoid inducer dexamethasone (DEX) was infiltrated to induce c-myc-NbMKK1 expression. Production of c-myc-NbMKK1 protein was confirmed by western analysis using an anti-c-myc antibody. As shown in Figure 3(a), strong expression of c-myc-NbMKK1 protein became detectable 4 h after DEX treatment, and the protein was abundantly produced for up to 24 h. Leaf-cell death was clearly observed 48 h after DEX treatment (Figure 3b). This result demonstrates that the cell death is caused by the overproduction of NbMKK1.

To study whether kinase activity of NbMKK1 is necessary for causing cell death, a kinase-dead mutant of NbMKK1 was made by changing the amino acid K88 (lysine), required for ATP binding, into R (arginine), resulting in c-myc-NbMKK1^{KR}. The GVG vector-mediated inducible expression of c-myc-NbMKK1^{KR} did not cause leaf cell death even after 48 h of DEX treatment (Figure 3b), whereas the c-myc-NbMKK1^{KR} protein was abundantly expressed (Figure 3a). Furthermore, to confirm the link between NbMKK1 kinase activity and cell death, the autophosphorylation activity of NbMKK1 was tested *in vitro* using recombinant NbMKK1 and NbMKK1^{KR}. As expected NbMKK1 phosphorylated itself, whereas NbMKK1^{KR} did not (Figure 3c). These results clearly show that kinase activity of NbMKK1 is necessary to cause cell death in *N. benthamiana*. NbMKK1 has an N-terminal RERRQLNLRLPL sequence corresponding to the known MAPK docking site sequence (Ichimura *et al.*, 2002) that consists of a cluster of basic amino acid residues N-terminal to hydrophobic residues. The basic amino acid residues are supposed to interact electrostatically with acidic amino acid residues in the common docking domain of MAPKs (Tanoue *et al.*, 2000). To determine whether the MAPK docking site is important for the function of NbMKK1 in the plant, we deleted the N-terminal 40 amino acids residues from NbMKK1 (resulting in NbMKK1$^{\Delta}$) and expressed it in *N. benthamiana* leaves. As shown in Figure 3(d), the cell death induced by NbMKK1$^{\Delta}$ was much delayed compared with the cell death induced by NbMKK1. This result indicates that the N-terminal putative MAPK docking site of NbMKK1 is essential for the function of NbMKK1 as a rapid cell-death inducer.

H_2O_2 generation caused by NbMKK1 overexpression

We studied early cellular events leading to leaf cell death triggered by the expression of NbMKK1. In DEX-treated *NbMKK1*-transformed leaves, H_2O_2 generation started at

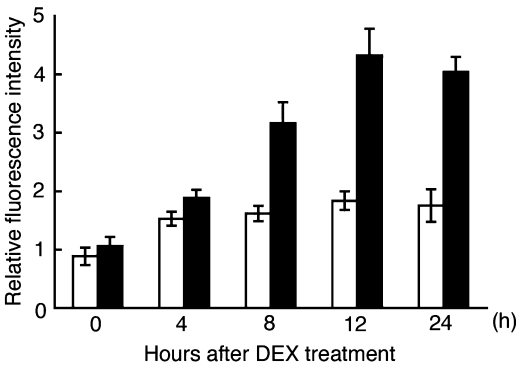

Figure 4. . NbMKK1 overexpression and H_2O_2 generation.
Hydrogen peroxide generation in either NbMKK1 (black bar) or NbMKK1^{KR} (white bar) overexpressing leaves. Data presented are the mean values of three independent experiments and error bars represent the standard deviation (DEX, dexamethasone).

4–8 h and continued until 24 h after the treatment (Figure 4), exactly corresponding to the timing of the c-myc-NbMKK1 protein accumulation in the leaf (Figure 3a). This H_2O_2 production is specifically induced by NbMKK1 kinase activity, as no H_2O_2 burst was observed in *NbMKK1*^{KR}-transformed leaves.

NbMKK1 is localized to the nucleus

To examine the subcellular localization of NbMKK1, jellyfish GFP cDNA was fused in frame to the 5′-end of NbMKK1 cDNA, and subsequently cloned in the GVG vector to generate GVG-GFP-NbMKK1. After expression of GFP-NbMKK1 in *N. benthamiana* cells GFP fluorescence was consistently observed in the nuclei, suggesting that NbMKK1 is localized to the nucleus (Figure 5a,b). This was confirmed by the fact that GFP-NbMKK1 localization exactly corresponded to the region stained by the DNA-specific stain 4′-6-Diamidino-2-phenylindole (DAPI) (Figure 5c). Moreover cell death was observed in GFP-NbMKK1-expressing leaves 48 h after DEX treatment (Figure 5b), suggesting that NbMKK1 may function in the nuclei to cause cell death.

NbSIPK acts downstream of NbMKK1 to trigger cell death

So far, intensive studies have been carried out to elucidate the roles of the two tobacco MAPKs, SIPK and WIPK, in plant-defense signaling (Romeis *et al.*, 1999; Seo *et al.*, 1995; Zhang and Klessig, 1998). In order to test whether NbSIPK and/or NbWIPK are downstream targets of NbMKK1, we first carried out an in-gel kinase assay with myeline basic protein (MBP) as substrate using leaves in which either NbMKK1 or NbMKK1^{KR} was overexpressed. It is known that MBP is a suitable substrate of SIPK and WIPK (Romeis *et al.*, 1999; Sharma *et al.*, 2003). The in-gel kinase assay showed that NbMKK1, but not NbMKK1^{KR}, overexpression strongly activated a 48-kDa kinase (Figure 6a). The sizes of SIPK and WIPK are known to be 48 and 46 kDa, respectively, so that we hypothesized that NbMKK1 activates NbSIPK.

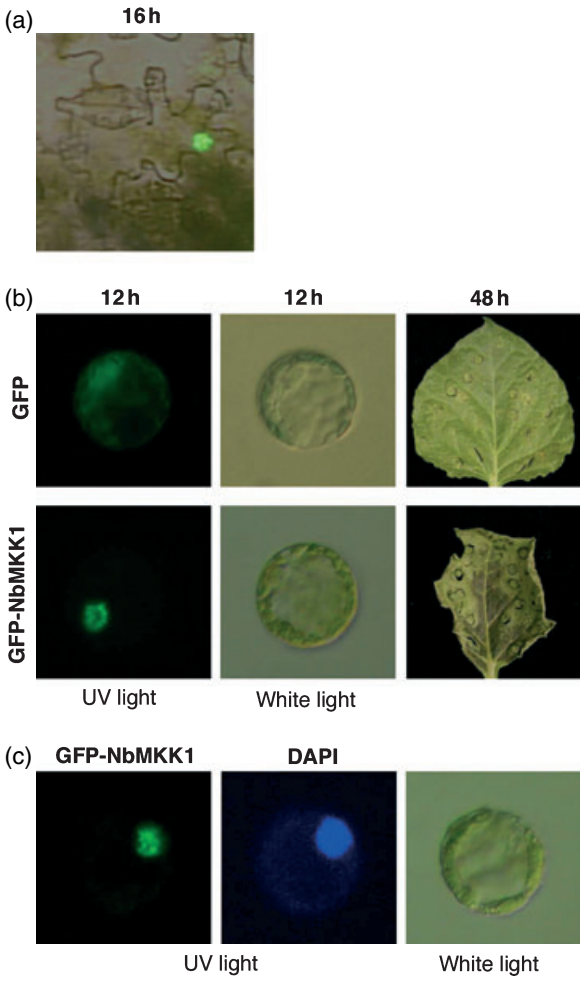

Figure 5. Localization of NbMKK1 in the nucleus.
(a) GFP-NbMKK1 localization in an epidermal cell observed with a fluorescence microscope.
(b) GFP-derived-fluorescence (left) and differential interference contrast (middle) images of protoplasts. Leaf phenotypes 48 h after dexamethasone (DEX) treatment are shown (right). GFP and GFP-NbMKK1 constructs in the pTA7001 vector were transiently transformed into *Nicotiana benthamiana* leaves. Twelve hours after DEX treatment, protoplasts were isolated and pictures were taken.
(c) Localization of GFP (left), nuclei stained by 4′-6-Diamidino-2-phenylindole (DAPI) (middle) and differential interference contrast (right) of a protoplast overexpressing GFP-NbMKK1.

Next, we designed an 'epistasis' experiment whereby the overexpression of *NbMKK1* was combined with VIGS of either *NbSIPK* or *NbWIPK*. The assumption is that the NbMKK1-mediated cell death would be compromised if the expression of a key downstream MAPK component was suppressed. As shown in Figure 6(b,c), the NbMKK1-mediated cell death is remarkably delayed in the *NbSIPK*-silenced plant, as compared with control, suggesting that NbSIPK is the MAPK transducing cell-death signal downstream of

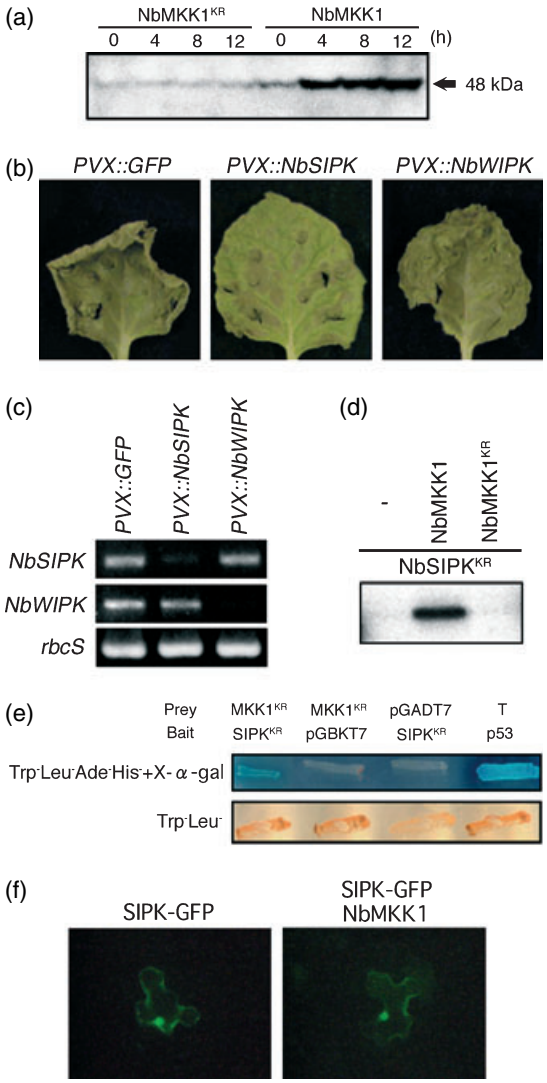

Figure 6. NbSIPK functions downstream of NbMKK1

(a) Myeline basic protein (MBP) in-gel kinase assay of leaves in which either NbMKK1^{KR} or NbMKK1 was overexpressed.

(b) NbMKK1-mediated cell death is delayed in *NbSIPK*-silenced plants. Overexpression of *NbMKK1* in *Nicotiana benthamiana* leaves where either the *NbSIPK* or the *NbWIPK* gene was silenced by virus induced gene silencing (VIGS). A potato virus X (PVX)-*GFP*-inoculated plant was used as a negative control. Pictures were taken 48 h after dexamethasone (DEX) treatment. The experiments were repeated three times with the same result.

(c) Confirmation of gene silencing of *NbSIPK* and *NbWIPK*. RT-PCR of *NbSIPK*, *NbWIPK* and *rbcS* genes in PVX-*GFP*, PVX-*NbSIPK* and PVX-*NbWIPK*-inoculated plants.

(d) NbMKK1 phosphorylates NbSIPK. Phosphorylation activities of *His*-NbMKK1 and *His*-NbMKK1^{KR} were determined by using the inactive mutant *His*-NbSIPK^{KR} as a substrate. Reaction in the absence (–) of mitogen-activated protein kinase kinase (MAPKK) was used as the control. The radioactivity of *His*-NbSIPK^{KR} was visualized by autoradiography following SDS-PAGE.

(e) NbMKK1 and NbSIPK interacted in yeast. A yeast two-hybrid assay was carried out using NbSIPK^{KR} as bait and NbMKK1^{KR} as prey. MKK1^{KR}-pGBK17 and pGADT7-SIPK^{KR} prey-bait combinations are negative controls and the T-p53 combination is a positive control.

(f) Salicylic-acid-induced protein kinase (SIPK)-GFP localization. SIPK-GFP was expressed either alone (left) or with NbMKK1 (right). SIPK-GFP was invariably localized both in the cytoplasm and the nuclei, irrespective of either the absence or the presence of NbMKK1.

NbMKK1. On the other hand, NbMKK1-mediated cell death did not alter in *NbWIPK*-silenced plants.

To test whether NbMKK1 is able to phosphorylate NbSIPK, an inactive His-NbSIPK^{KR} protein was used as a substrate. As His-NbSIPK^{KR} lacks autophosphorylation activity, it is an ideal substrate for assaying upstream MAPKK activities (Yang *et al.*, 2001). As shown in Figure 6(d), NbMKK1 phosphorylated His-NbSIPK^{KR}, whereas NbMKK1^{KR} did not.

These data suggest the presence of an NbMKK1-NbSIPK signaling pathway. To see whether these proteins physically interact, a yeast two-hybrid assay was carried out using NbSIPK^{KR} as bait and NbMKK1^{KR} as prey (Figure 6e). Both NbSIPK^{KR} and NbMKK1^{KR} are catalytically inactive, so they were expected not to interfere with yeast cellular signaling pathways. It is also thought that transient interaction can be stabilized by using inactive proteins (see Tanoue *et al.*, 1999). These two proteins indeed physically interacted in yeast. Taken together, *in vivo* and *in vitro* results demonstrate that NbMKK1 is an upstream kinase for NbSIPK, and that the NbMKK1 → NbSIPK cascade controls the observed HR-like cell death. To see the subcellular localization of NbSIPK NbSIPK-GFP fusion protein was overexpressed in *N. benthamiana* leaves, and its localization was observed in either the absence or the presence of NbMKK1 overexpression. NbSIPK-GFP was invariably localized both in the cytoplasm and the nuclei, and no change in its localization was observed as a result of the presence/absence of NbMKK1 (Figure 6f).

VIGS of NbMKK1 suppresses INF1-elicitor-mediated cell death

NbMKK1 exhibited a high basal expression, as studied by northern-blot analysis, and its expression did not show remarkable change by either treatments with *P. infestans* INF1 elicitin (Kamoun *et al.*, 1998) or inoculation of a non-host pathogen, *P. cichorii* (data not shown).

To evaluate the function of NbMKK1 in plant defense, *NbMKK1* expression was silenced by VIGS using PVX. A partial fragment of *NbMKK1* cDNA was cloned into PVX vector (pPC2S; Baulcombe *et al.*, 1995) in the anti-sense orientation, and RNA transcribed from this vector was inoculated to *N. benthamiana* leaves. The specificity of the *NbMKK1* partial fragment was tested by Southern analysis (Figure 7a). When used as a probe this fragment detected only one band in the *N. benthamiana* genomic DNA digested with *Hin*dIII, indicating that our VIGS is specific to *NbMKK1*. Twenty-one days after virus inoculation, gene silencing of *NbMKK1* in the 3–4 leaves above the inoculated leaf was confirmed by RT-PCR (Figure 7b). The overall appearance of *NbMKK1*-silenced plants was the same as the wild-type plants. To examine the response of *NbMKK1*-silenced plants to a pathogen HR elicitor, *P. infestans* INF1 elicitin (Kamoun *et al.*, 1998), which triggers the HR in *N. benthamiana*, was infiltrated to the leaves of *NbMKK1*-

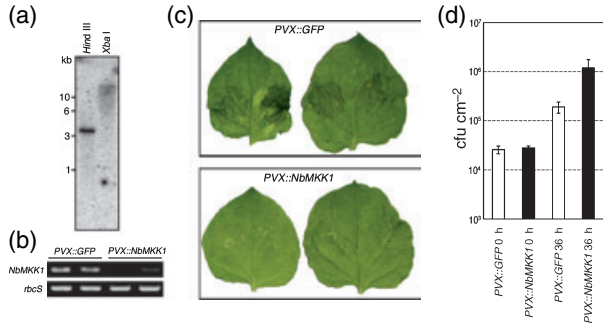

Figure 7. VIGS of *NbMKK1* delayed *Phytophthora infestans* (INF1)-mediated hypersensitive response (HR) development and attenuated resistance against a non-host pathogen *Pseudomonas cichorii*.
(a) Southern blot analysis of the NbMKK1 insert fragment used for VIGS. A single band was detected in the *Nicotiana benthamiana* genomic DNA digested with *Hin*dIII.
(b) RT-PCR of *NbMKK1* and *rbcS* genes in potato virus X (PVX)-*GFP*-infected and PVX-*NbMKK1*-infected plants.
(c) The HR caused by the INF1 elicitor was delayed in the *NbMKK1*-silenced plant (PVX-*NbMKK1*) compared with the PVX-*GFP* control. Pictures were taken 24 h after INF1 infiltration. The experiments were repeated three times with reproducible results.
(d) NbMKK1 gene silencing attenuated resistance against a non-host pathogen *P. cichorii*. White bars: control plants infected with PVX::GFP. Black bars: plants infected with PVX::NbMKK1. The vertical axis indicates the titer of *P. cichorii* in *N. benthamiana* leaves.

silenced and control plants. The timing of INF1-mediated cell death was consistently delayed in *NbMKK1*-silenced plants compared with control plants (Figure 7c). Twenty-four hours after INF1 treatment HR-like cell death was obvious in the control plants, whereas either no or only slight cell death was visible in *NbMKK1*-silenced plants. Forty hours after INF1 treatment both the control and *NbMKK1*-silenced plants developed similar levels of HR-like cell death (data not shown). The same results were obtained in three independent experiments.

To evaluate the role of NbMKK1 in host resistance against pathogens, we inoculated a non-host pathogen *P. cichorii* (Sharma *et al.*, 2003) to *N. benthamiana* plants whereby *NbMKK1* expression was silenced by VIGS. The *NbMKK1*-silenced plant allowed a higher growth of *P. cichorii* compared with the control 36 h after the inoculation (Figure 7d). The same results were obtained in three independent experiments. This result shows that a signaling pathway involving NbMKK1 is necessary for the full resistance of *N. benthamiana* against *P. cichorii*.

Discussion

We performed a high-throughput screen of cell-death-causing factors in *N. benthaminana*, and identified NbMKK1 as a key regulator of cell death associated with plant immunity. NbMKK1 overexpression resulted in the accumulation of the protein to nuclei and rapid cell death, confirming a positive role for NbMKK1 in cell-death regulation. Cell death caused by NbMKK1 overexpression was associated with the rapid generation of H_2O_2, displaying a similarity to pathogen-induced HR. Most importantly, the NbMKK1 loss-of-function study demonstrated that NbMKK1 is involved in the regulation of the HR caused by *P. infestans* elicitor INF1 and resistance against a non-host pathogen, *P. cichorii*.

NbMKK1 and MAPK cascades

The Arabidopsis genome encodes approximately 60 MAPKKKs, 10 MAPKKs and 20 MAPKs (Ichimura *et al.*, 2002). To date, functions of only a few complete plant MAPK cascades have been elucidated. An Arabidopsis cascade involving AtMEKK1, AtMKK4/5 and AtMPK3/6 functions downstream of FLS2 receptor-mediated flagellin perception and innate immunity (Asai *et al.*, 2002). The tomato LeMAPKKKα, LeMKK2 and LeMPK1/2/3 cascade transduces *Pto* resistance gene-mediated immunity (del Pozo *et al.*, 2004). Plant MAPKKs are classified to four groups, Groups A, B, C and D (Ichimura *et al.*, 2002), and sequence comparison placed NbMKK1 within Group D (Figure 1). Functions of several individual plant MAPKKs are known. The tobacco NtMEK1 of Group A is known to be involved in cell division and plant defense (Calderini *et al.*, 2001; Liu *et al.*, 2004b). Alfalfa PRKK is also classified in Group A and transmits an elicitor signal to downstream MAPKs (Cardinale *et al.*, 2002). Arabidopsis MKK3 and tobacco NPK2 of Group B mediate the nuclear transport of RAN-GDP (Quimby *et al.*, 2000). Tobacco NtMEK2 in Group C was found to be an upstream kinase of two plant MAPKs, SIPK and WIPK (Yang *et al.*, 2001). Overexpression of constitutively active NtMEK2 caused cell death (Yang *et al.*, 2001), and a VIGS study of *NtMEK2* indicated that NtMEK2 is involved in TMV resistance (Jin *et al.*, 2003). Arabidopsis AtMKK4 and AtMKK5, the orthologs of NtMEK2, showed similar effects as those observed with NtMEK2 (Ren *et al.*, 2002). However, until the report by Pedley and Martin (2004) there was no single report on the function of MAPKK belonging to Group D. Pedley and Martin (2004) showed that a tomato MAPKK, LeMKK4, causes cell death upon overexpression. Furthermore, they showed by an *in vitro* experiment that LeMKK4 activates downstream MAPKs, LeMPK2 (similar to tobacco SIPK) and LeMPK3 (similar to tobacco WIPK). Sequence analysis of NbMKK1 showed that NbMKK1 shared 87% amino acid homology to LeMKK4, suggesting that it is an ortholog of LeMKK4 in terms of sequence similarity and function.

We demonstrated that NbMKK1 is an upstream kinase of NbSIPK *in vivo* by a VIGS study (Figure 6b) as well as by *in vitro* studies (Figure 6d,e). This implies that there are at least two signal transduction pathways leading to SIPK activation: the NtMEK2-SIPK pathway (Yang *et al.*, 2001) and the NbMKK1-SIPK pathway (the present study). As SIPK overexpression also caused HR-like cell death (Zhang and Liu, 2001), it is highly probable that NbMKK1-mediated cell

death, as observed in the present study, was driven by the activation of NbSIPK. SIPK is activated rapidly in response to a variety of biotic and abiotic stresses including osmotic and salt stress, wounding, virus infection and treatment with non-race-specific elicitors from fungi and bacteria (Hoyos and Zhang, 2000; Lee *et al.*, 2001; Mikolajczyk *et al.*, 2000; Zhang and Klessig, 1998). Together, these data indicate that SIPK may be a convergence point for many different stress signal transduction pathways.

Nuclear localization of NbMKK1

Elucidating the subcellular localization of MAPK pathway components is a key to understanding how the specificity of signaling is maintained and the way in which signaling is further propagated (Cyert, 2001). MAPK and MAPKK localization has been studied considerably in mammalian and yeast systems under various conditions, and it was found that they change localization depending on cellular conditions (Chen *et al.*, 1992; Ferrigno *et al.*, 1998; Jaaro *et al.*, 1997; Lenormand *et al.*, 1998). The best studied localization of MAPK signaling cascades is that of the human extracellular signal-regulated kinase (ERK)1/2 cascade composed of ERK1/2 (MAPK), MEK1/2 (MAPKK) and Raf1 (MAPKKK) (Kondoh *et al.*, 2005; Pouyssegur *et al.*, 2002). A cytosolic localization of Raf1, MEK1/2 and ERK1/2 was demonstrated in quiescent cells. However, stimulation of cells caused a rapid translocation of Raf1 to the plasma membrane and the translocation of MEK1/2 and ERK1/2 to the nuclei. Whereas ERK1/2 is retained in the nucleus, MEK1 and 2 are rapidly transported back to the cytoplasm as a result of their nuclear export signal (Fukuda *et al.*, 1997; Jaaro *et al.*, 1997). Therefore, MAPKKs have generally been considered to be located in the cytoplasm most of the time. However, Raviv *et al.* (2004) recently reported that human MEK5, as well as its downstream MAPK, ERK5, are always localized to the nucleus irrespective of cellular conditions. In this case, the upstream MAPKKK, MEKK2, is shuttling between cytoplasm and nuclei. The nuclear localization of human MEK5 is similar to that of NbMKK1 in the present study. In plants, subcellular localization of MAPK components has been studied in several cases (Ahlfors *et al.*, 2004; Bögre *et al.*, 1999; Calderini *et al.*, 1998; Lee *et al.*, 2004; Ligterink *et al.*, 1997). Two Arabidopsis MAPKs, AtMPK3 and AtMPK6, were shown to translocate from the cytoplasm to the nuclei after ozone treatment (Ahlfors *et al.*, 2004). Similarly, in parsley cells two MAPKs, PcMPK3 and PcMPK6, translocate to nuclei following *phytophthora*-derived (Pep-13) elicitor treatment (Lee *et al.*, 2004). However, the upstream MAPKK of PcMPK3 and PcMPK6, PcMKK5, was retained in the cytoplasm after the stimulus. This observed localization of plant MAPK components is in accord with the pattern observed in human ERK1/2 localization. In this regard, nuclear localization of NbMKK1 is unique as it is always retained in the nucleus. However, no obvious nuclear localizing signal (NLS) was found in the NbMKK1 amino acid sequence. The mechanisms of nuclear localization of NbMKK1, and the regulation of its interaction with NbSIPK, should be addressed in future work.

NbMKK1 functions in INF1-mediated HR-like cell death and non-host resistance against P. cichorii

Phytophthora infestans INF1 elicitor-mediated HR-like cell death was remarkably delayed in *NbMKK1*-silenced *N. benthamiana* plants (Figure 7c). Furthermore, non-host resistance of *N. benthamiana* against a bacterial pathogen *P. cichorii* was attenuated in *NbMKK1*-silenced plants (Figure 7d). These loss-of-function results show that signals of INF1-induced cell death and non-host resistance against *P. cichorii* are transduced via a cascade involving NbMKK1. As overexpression of NbMKK1 caused HR-like cell death, whereas gene silencing of NbMKK1 attenuated HR and non-host resistance, we conclude that NbMKK1 is an important component of non-host resistance-related signaling. However, as INF1-mediated HR-like cell death was not totally abrogated in *NbMKK1*-silenced plants, there should be at least one more pathway downstream of INF1 leading to cell death. Possible candidates include the pathway involving NtMEK2 (Yang *et al.*, 2001) and those involving CDPK (Romeis *et al.*, 2001). In a previous study we showed that INF1-mediated HR was not affected by the gene silencing of *SIPK* and *WIPK*, and hypothesized that these two MAPKs are not major components in INF1 cell-death signal transduction (Sharma *et al.*, 2003). On the basis of the present study and that of Sharma *et al.* (2003), we predict that there is another unidentified MAPK other than SIPK/WIPK downstream of NbMKK1 that is involved in INF1 cell-death signaling. These target MAPKs and upstream MAPKKK of NbMKK1 should be identified in future studies.

Experimental procedures

Plant material and INF1 treatment

Nicotiana benthamiana plants were grown in a glasshouse at 23°C. INF1 elicitor (100 nM) was prepared according to Kamoun *et al.* (1998) and infiltrated to well-developed leaf blades. Leaves were collected at 0, 15, 30, 60, 120 and 240 min after infiltration and subsequently employed for isolation of RNA for cDNA library construction.

cDNA library construction in pSfinx vector and screening of cell-death-inducing factors

The cDNA library construction and the screening of cell-death-inducing factors by toothpick inoculation of *A. tumefaciens* clones was reported previously (Nasir *et al.*, 2005). Briefly, mRNA was isolated from total RNA by the use of an mRNA purification kit™ (Amersham Biosciences, http://www.amersham.com/), followed by

the synthesis of double-stranded cDNAs with the SuperScript Plasmid System™ (Invitrogen, http://www.invitrogen.com/). These cDNAs, with *Sal*I sites in the 5′-ends and *Not*I sites in the 3′-ends, were directionally cloned into a modified pSfinx vector (Takken *et al.*, 2000). The pSfinx library was transformed into *A. tumefaciens* strain MOG101 cells by electroporation. Cultured *A. tumefaciens* cells were lifted by toothpicks, and inoculated to *N. benthamiana* leaves.

Inducible expression of c-myc-NbMKK1 and GFP-NbMKK1

A DNA sequence corresponding to the triple c-myc tag (EFGEQKLISEEDLNGEQKLISEEDLNGEQKLISEEDLNGKL) was added onto the 5′-end of the open reading frame (ORF) of *NbMKK1* by PCR, resulting in c-myc-*NbMKK1* cDNA. This fragment was cloned into the *Xho*I and *Spe*I sites of the GVG-vector pTA7001 (Aoyama and Chua, 1997). Engineered GFP gene (mGFP; Haseloff and Amos, 1995) was fused to the 5′-end of *NbMKK1* cDNA, resulting in GFP-NbMKK1 and was cloned into pTA7001. These binary vectors were used for transformation of *A. tumefaciens* GV3101. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells to establish transient transformation. Two days after *A. tumefaciens* infiltration, DEX (30 μM in 0.1% ethanol) was infiltrated to induce transgene expression. *NbMKK1*^{KR}, a kinase-dead mutant of *NbMKK*, was generated by changing the nucleotides so that the conserved Lys88 residue was substituted by Arg. The mutant was generated following a procedure described previously (Yang *et al.*, 2001).

Measurement of H_2O_2

H_2O_2 generation was measured by using dichlorofluorescin diacetate (DCFH-DA) as described by Sanchez *et al.* (1990).

Protoplast preparation for the visualization of GFP-NbMKK1

Agrobacterium tumefaciens cells containing GFP-NbMKK1 vector were infiltrated into *N. benthamiana* leaves by a needleless syringe. Protoplasts were isolated 12 h after DEX treatment, by treating the leaf samples with a mixture of 1.4% cellulase Onozuka R-10 (Yakult, http://www.yakult.co.jp) and 0.4% macerozyme R-10 (Yakult).

Preparation of recombinant proteins and in vitro *phosphorylation assay*

An enterokinase site was introduced in both the 5′- and 3′-ends of the ORF of *NbMKK1*, *NbMKK1*^{KR} or *NbSIPK*^{KR} by PCR, and these were cloned in frame into the pET-46 Ek/LIC vector™ (Novagen, http://splash.emdbiosciences.com/). *Escherichia coli* cells (Origami B™[DE3]) were transformed with pET-46 Ek/LIC constructs, and protein production was induced with 0.5 mM IPTG at 25°C for 8 h. *His*-tagged proteins were purified using the MagneHis™ protein purification system (Promega, http://www.promega.com/), and desalted using Dialyzer™ (Spectrum, http://www.spectrapor.com/). An autophosphorylation assay was performed by incubating 0.5 μg of purified recombinant NbMKK1 or NbMKK1^{KR} in reaction buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 1 mM EGTA and 1 mM DTT) in the presence of 25 μM [γ-^{32}P]ATP at 30°C for 30 min. The reaction was stopped by the addition of SDS loading buffer, and kinase activity was detected by autoradiography following SDS-PAGE. The phosphorylation activity of NbMKK1s was determined by using the inactive mutant NbSIPK^{KR} (1 μg) as a substrate under the same conditions as the autophosphorylation assay, except that 0.1 μg of either NbMKK1 or NbMKK1^{KR} was used in the reaction.

Protein kinase assay

The in-gel kinase assay was performed as described by Takahashi *et al.* (2003a).

Yeast two-hybrid assay

A cDNA fragment corresponding to the NbSIPK^{KR} was cloned into pGBKT7 (Clontech, http://www.clontech.com/) resulting in pGBKT7-NbSIPK^{KR} (bait vector). A cDNA for NbMKK1^{KR} was cloned into pGADT7 (Clontech) resulting in pGADT7-NbMKK1^{KR} (prey vector). These two plasmids were co-transformed into a yeast strain AH109, and the transformed yeast cells streaked on selective agar plates containing minimal medium without Leu, Trp, His and Ade supplemented with 10 mM 3-amino-1,2,3-triazole (3-AT) and 40 mg l^{-1} X-α-gal.

Southern analysis

Genomic DNA (10 μg) of *N. benthamiana* isolated with DNeasy Plant Mini Kit (Qiagen, http://www1.qiagen.com/) was digested with restriction endonuclease *Hin*dIII and *Xba*I, respectively, and loaded on 0.8% agarose gel for electrophoresis. The separated DNA fragments were blotted onto nylon membrane (Hybond N+; Amersham) and hybridized with a ^{32}P-labeled complementary DNA fragment corresponding to the nucleotide positions 21–390 of the *NbMKK1* gene.

VIGS in N. benthamiana

A cDNA fragment corresponding to the nucleotide positions 21–390 of *NbMKK1*, whereby the first nucleotide of the first codon was set to position one, was cloned into the PVX vector pPC2S (Baulcombe *et al.*, 1995) in an anti-sense orientation resulting in pTXS.*NbMKK1*. pTXS.*NbMKK1* was linearized with the restriction endonuclease *Spe*I, and *in vitro* run-off transcripts were synthesized by T7 RNA polymerase. The transcripts were inoculated to *N. benthamiana* plants as described elsewhere (Saitoh *et al.*, 2001). Confirmation of the gene silencing of *NbMKK1* was made by RT-PCR using the primer pair 5′-CGCAACAAAATCCAAACG-3′ and 5′-AGTCGAGT CCGCTAAGTA-3′. *NbSIPK* and *NbWIPK* gene silencing was performed as described previously (Sharma *et al.*, 2003).

INF1 treatment and inoculation of P. cichorii

INF1 elicitor (100 nM) was prepared according to Kamoun *et al.* (1998) and infiltrated to well-developed leaf blades. *P. cichorii* SPC9001 (Hikichi *et al.*, 1998) was grown at 28°C in nutrient broth medium (Difco, http://www.vgdusa.com/DIFCO.htm) containing ampicillin (10 μg ml^{-1}) overnight. After centrifugation, bacterial cells were resuspended in 10 mM $MgCl_2$ (OD_{600} = 0.01). Bacterial suspensions were infiltrated into leaves using a needleless syringe. The increase in the numbers of bacteria was estimated in leaf discs. Further details are available in Sharma *et al.* (2003).

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