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Function of a mitogen-activated protein kinase pathway in *N* gene-mediated resistance in tobacco

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Summary

The active defense of plants against pathogens often includes rapid and localized cell death known as hypersensitive response (HR). Protein phosphorylation and dephosphorylation are implicated in this event based on studies using protein kinase and phosphatase inhibitors. Recent transient gain-of-function studies demonstrated that the activation of salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), two tobacco mitogen-activated protein kinases (MAPKs) by their upstream MAPK kinase (MAPKK), NtMEK2 leads to HR-like cell death. Here, we report that the conserved kinase interaction motif (KIM) in MAPKKs is required for NtMEK2 function. Mutation of the conserved basic amino acids in this motif, or the deletion of N-terminal 64 amino acids containing this motif significantly compromised or abolished the ability of NtMEK2^{DD} to activate SIPK/WIPK *in vivo*. These mutants were also defective in interacting with SIPK and WIPK, suggesting protein–protein interaction is required for the functional integrity of this MAPK cascade. To eliminate *Agrobacterium* that is known to activate a number of defense responses in transient transformation experiments, we generated permanent transgenic plants. Induction of *NtMEK2^{DD}* expression by dexamethasone induced HR-like cell death in both T_1 and T_2 plants. In addition, by using PVX-induced gene silencing, we demonstrated that the suppression of all three known components in the NtMEK2–SIPK/WIPK pathway attenuated *N* gene-mediated TMV resistance. Together with previous report that SIPK and WIPK are activated by TMV in a gene-for-gene-dependent manner, we conclude that NtMEK2–SIPK/WIPK pathway plays a positive role in *N* gene-mediated resistance, possibly through regulating HR cell death.

Keywords: mitogen-activated protein kinase, salicylic acid-induced protein kinase, wounding-induced protein kinase, *N* gene, tobacco mosaic virus.

Introduction

Plants deploy a battery of active defense responses when they are challenged by pathogens. These responses often include the generation of reactive oxygen species (ROS), the activation of a complex array of defense genes, the production of antimicrobial phytoalexins, and the rapid and localized hypersensitive response (HR) cell death (Lamb and Dixon, 1997; Scheel, 1998; Torres *et al.*, 2002). One or the combination of these defense mechanisms will eventually kill or stop the spread of pathogens in an incompatible interaction. Several lines of evidence suggest that HR cell death during plant disease resistance is a form of programmed cell death because it requires active transcription and translation and is genetically defined (Dangl *et al.*, 1996; Greenberg, 1997; Lam *et al.*, 2001). The activation of these defense responses is initiated by the plant recognition of pathogens, which is 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 its receptor (Baker *et al.*, 1997; Dangl and Jones, 2001; Hammond-Kosack and Jones, 1996; Martin, 1999). Signals generated from such interactions are transduced into cellular responses via several interlinked

pathways (Hammond-Kosack and Jones, 1996; Martin, 1999; Scheel, 1998).

Several distinct MAPK cascades have been implicated in the regulation of plant disease resistance, either positively or negatively (Frye *et al.*, 2001; Innes, 2001; Petersen *et al.*, 2000; Tena *et al.*, 2001; Zhang and Klessig, 2001). Infection of resistant tobacco plants that carry the *N* resistance gene with tobacco mosaic virus (TMV) leads to the activation of salicylic acid-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK), two tobacco MAPKs (Zhang and Klessig, 1998). In addition, both MAPKs are activated by Avr9, a fungal *Avr* factor from *Cladosporium fulvum* in a Cf-9-dependent manner (Romeis *et al.*, 1999). Other non-host-specific elicitors such as elicitins from *Phytophthora* spp., fungal cell wall-derived elicitors, xylanase from *Trichoderma viride* and bacterial elicitor harpin also activate these two MAPKs (Lee *et al.*, 2001; Suzuki *et al.*, 1999; Zhang *et al.*, 1998, 2000), suggesting that these two MAPKs are converging points after the perception of different pathogens and pathogen-derived elicitors. The activation of SIPK is very rapid, which potentially allows it to control multiple defense responses either directly or indirectly. The orthologs of SIPK and WIPK in other plant species have also been shown to be activated by various elicitors (Cardinale *et al.*, 2000; Desikan *et al.*, 2001; Ligterink *et al.*, 1997; Nühse *et al.*, 2000; Stratmann and Ryan, 1997). Pharmacological studies using general or specific kinase inhibitors suggested that SIPK and WIPK activation is involved in the regulation of both defense gene activation and HR-like cell death (Lee *et al.*, 2001; Zhang *et al.*, 1998, 2000). Furthermore, by adding inhibitors at different times after the elicitin treatment, it was shown that the prolonged activation of SIPK and/or delayed activation of WIPK are required for tobacco cells to commit to cell death program (Zhang *et al.*, 2000). Prolonged activation of a MAPK was also associated with HR-like cell death of tobacco cells treated with fungal xylanase and *Arabidopsis* cells treated with bacterial harpin (Desikan *et al.*, 2001; Suzuki *et al.*, 1999).

More direct evidence for the role of SIPK and WIPK in HR cell death came from a conditional gain-of-function study using NtMEK2^{DD}, a constitutively active mutant of NtMEK2 (Yang *et al.*, 2001). In tobacco leaves transiently transformed with *NtMEK2^{DD}* under the control of a steroid-inducible promoter, induction of *NtMEK2^{DD}* expression by the application of dexamethasone (DEX) leads to HR-like cell death, which is preceded by the activation of endogenous SIPK and WIPK. The magnitude and the kinetics of endogenous SIPK/WIPK activation by NtMEK2^{DD} are similar to those induced by pathogens or pathogen-derived elicitors (Zhang and Klessig, 1998a; Zhang *et al.*, 2000). To eliminate the potential side-effects of *Agrobacterium* that is known to induce plant defense responses (Ditt *et al.*, 2001), we generated permanent transgenic tobacco expressing *NtMEK2* or its mutants under the control of steroid-inducible promoter. There is a strict correlation between the expression of *NtMEK2^{DD}* and the activation of endogenous SIPK and WIPK, which is followed by the HR-like cell death phenotype in both T_1 and T_2 plants. In addition, we identified the kinase interacting motif (KIM) in the N-terminus of NtMEK2 that is required for its *in vivo* function. Mutation of this motif blocked the interaction between NtMEK2 and SIPK/WIPK, which also abolished the ability of NtMEK2^{DD} to activate SIPK and WIPK *in vivo* and the induction of HR-like cell death by NtMEK2^{DD}. More importantly, using the PVX virus-induced gene silencing technique, we demonstrated that the suppression of all three known components in the NtMEK2–SIPK/WIPK pathway attenuated the *N* gene-mediated resistance against TMV, which provided one of the first loss-of-function evidence to support a positive role of this MAPK pathway in plant disease resistance.

Results

Expression of NtMEK2^{DD} *activates downstream MAPKs and leads to HR-like cell death in both T_1 and T_2 transgenic plants*

Our recent study revealed that the expression of *NtMEK2^{DD}*, an active mutant of *NtMEK2* induced multiple defense responses including defense gene activation and HR-like cell death. The activation of these defense responses is preceded by the activation of endogenous SIPK and WIPK, two downstream MAPKs of NtMEK2 (Yang *et al.*, 2001). To exclude possible influence from *Agrobacterium* used in these transient assays, we generated permanent transgenic tobacco plants. Wild-type *NtMEK2*, its inactive mutant *NtMEK2^{KR}*, in which the catalytic essential Lys111 (K) was replaced with an Arg (R), and the constitutively active mutant *NtMEK2^{DD}*, in which the Thr227 and Ser233 were substituted with Asp (D) were transformed into tobacco. Again, pTA7002 steroid-inducible vector was used (Aoyama and Chua, 1997). To facilitate the detection of transgene expression, a Flag tag was added to the N-terminus of the transgene. More than 30 independent lines were generated for each construct. As another negative control, 16 lines with empty pTA7002 vector were also generated.

Transgene inducibility in T_1 plants was examined by treating detached leaves with DEX (30 μM). The petioles of the detached leaves were kept in water to prevent dehydration. Leaf discs were collected 12 h after DEX treatment and transgene expression was determined by immunoblot analysis using anti-Flag antibody. Cell death phenotype was scored at 24 and 48 h after induction. As shown in Table 1, 8 out of 33 *NtMEK2^{WT}* lines and 14 out of 33 *NtMEK2^{KR}* lines showed transgene expression after DEX

Table 1 Correlation between the inducibility of *NtMEK2^{DD}* expression and HR-like cell death in T_1 transgenic tobacco plants

Constructs	Total lines generated[1]	Lines showed transgene inducibility[2]	Lines showed HR-like cell death[3]
NtMEK2^{WT}	33	8	0
NtMEK2^{DD}	47	19	19
NtMEK2^{KR}	33	14	0
pTA7002	16	0	0

[1]Transgenic tobacco plants were generated by *Agrobacterium*-mediated leaf-disc transformation and selected on hygromycin plates.
[2]Transgene inducibility was examined using leaf discs harvested 12 h after the application of DEX (30 μM) by immunoblot analysis with anti-Flag antibody.
[3]HR-like cell death was scored 2 days after DEX infiltration of detached leaves whose petioles were kept in water.

treatment. However, neither SIPK/WIPK activation nor cell death phenotype was observed. By contrast, all 19 out of the 47 *NtMEK2^{DD}* transgenic lines that showed transgene expression gave HR-like cell death phenotype. Again, the expression of *NtMEK2^{DD}* resulted in the activation of endogenous SIPK and WIPK in these 19 lines, which preceded the appearance of HR-like cell death phenotype. For the lines that showed weaker induction of NtMEK2^{DD} such as lines *NtMEK2^{DD}*-22 and *NtMEK2^{DD}*-7, the cell death phenotype was delayed.

A detailed time-course analyses were also performed on selected T_1 plants. As shown in Figure 1(a), expression of transgene in both *NtMEK2^{DD}*-2 line and *NtMEK2^{KR}*-29 line was detectable at the protein level by immunoblot analysis using anti-Flag antibody between 2 and 4 h after DEX treatment. However, SIPK and WIPK activation occurred only in the *NtMEK2^{DD}*-2 plant as determined by an in-gel kinase activity assay with myelin basic protein (MBP) as a substrate (Figure 1a, lower panel), which was followed by the appearance of HR-like cell death phenotype (Figure 1b). In permanent transgenic plants, the induction of transgene expression was much lower, which, on average, was only about 5–10% of that in the transient transformation (data not shown). Nonetheless, this very low level of NtMEK2^{DD} protein was sufficient to activate downstream endogenous SIPK and WIPK and induce HR-like cell death. Among the 19 *NtMEK2^{DD}* T_1 lines that showed transgene inducibility, all with the exception of two lines, *NtMEK2^{DD}*-1 and *NtMEK2^{DD}*-2 set seeds. These two lines were maintained by

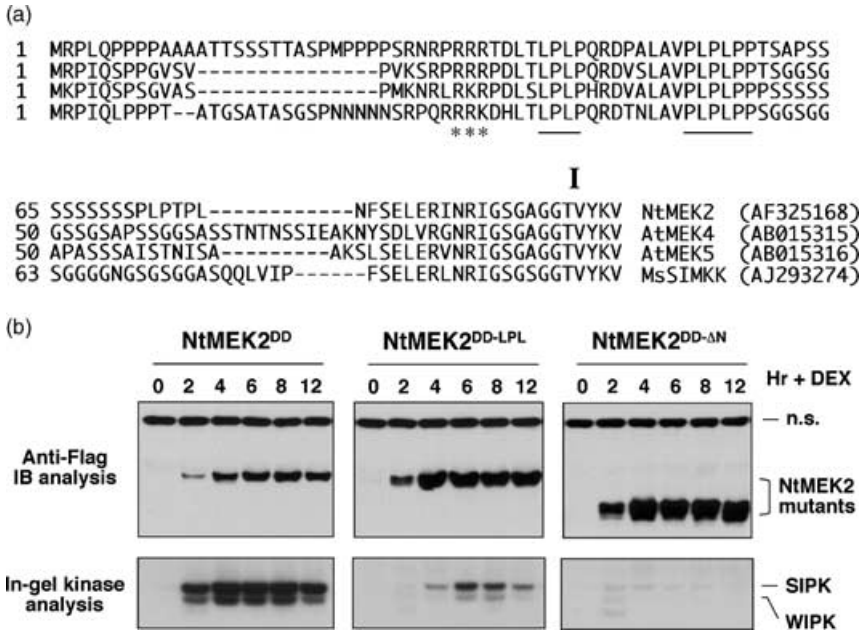


Figure 2. Kinase interacting motif (KIM) in the N-terminus of NtMEK2 is required for its ability to activate SIPK and WIPK *in vivo*.
(a) The N-termini of tobacco NtMEK2 and its orthologs from other plant species contain a basic amino acid cluster followed by a Leu/Pro-rich region similar to the KIM domain that is evolutionarily conserved in MAPKKs from animals and yeast. The conserved basic amino acid cluster was indicated with asterisks. The Leu- and Pro-rich region was underlined. Roman number indicated the subdomain I of the kinase catalytic domain. The accession number for each MAPKK was listed in parenthesis.
(b) Two mutants were generated by either substituting the conserved RRR with LPL (NtMEK2$^{DD\text{-}LPL}$) or deleting the N-terminal 64 amino acids (NtMEK2$^{DD\text{-}\Delta N}$) that contains the basic amino acid cluster and Leu/Pro-rich region. The resulted mutants were transiently transformed into tobacco. Transgene expression was monitored by immunoblot (IB) analysis with anti-Flag antibody (upper) after DEX treatment. The activity of SIPK and WIPK was determined by in-gel kinase assay with MBP as a substrate (lower). A non-specific (n.s.) band detected by anti-Flag antibody was shown as a reference for equal loading and the sizes of Flag-tagged proteins.

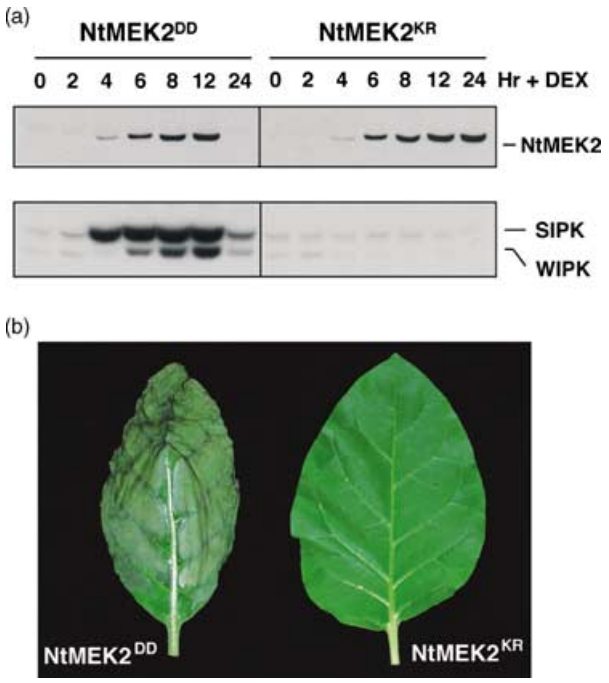
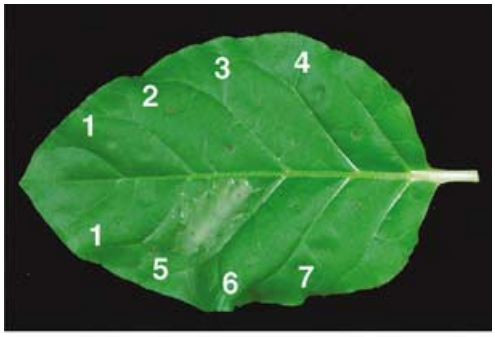


Figure 1. Expression of *NtMEK2*^{DD} activates SIPK/WIPK and induces HR-like cell death in permanent transgenic tobacco plants.
(a) Induction of *NtMEK2*^{DD} expression activates endogenous SIPK and WIPK. Detached leaves from T_1 transgenic tobacco, *NtMEK2*^{DD}-2 and *NtMEK2*^{KR}-29 were treated with DEX (30 μM) and leaf discs were taken at indicated times. The expression of transgene was assayed by immunoblot analysis using anti-Flag antibody (upper). The activation of endogenous MAPKs was determined by an in-gel kinase assay with MBP as a substrate (lower).
(b) Activation of endogenous SIPK and WIPK leads to HR-like cell death in *NtMEK2*^{DD} transgenic plants. Detached leaves were treated with DEX (30 μM). The picture was taken 24 h after DEX infiltration.



	Constructs	+DEX	-DEX
1.	Vector	-	-
2.	NtMEK2^{WT}	-	-
3.	NtMEK2^{WT-LPL}	-	-
4.	NtMEK2$^{WT-\Delta N}$	-	-
5.	NtMEK2^{DD}	HR	-
6.	NtMEK2^{DD-LPL}	- *	-
7.	NtMEK2$^{DD-\Delta N}$	-	-

Figure 3. The conserved KIM domain of NtMEK2 is required for its function. *NtMEK2* and its mutant constructs in pTA7002 vector were transiently transformed into different sections of tobacco leaves. Two days later, the transgene expression was induced by the application of DEX (30 μM). In control plants (–DEX), 0.1% ethanol, the solvent for DEX, was used. Photo was taken 24 h after DEX treatment. 'HR' denotes the development of HR-like cell death, and '–' indicates no visible phenotype. Asterisk indicates that the patchy cell death appeared on leaf sections infiltrated with *NtMEK2*$^{DD-LPL}$ after 48 h.

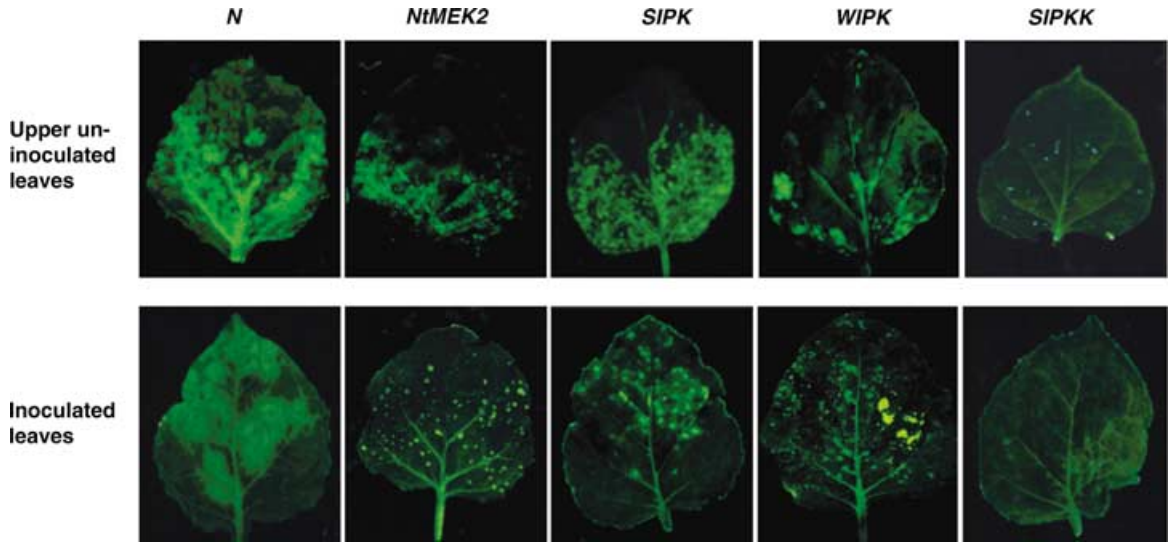


Figure 6. Suppression of *NtMEK2*, *SIPK*, or *WIPK* expression via PVX virus-induced gene silencing compromised *N* gene-mediated resistance.
N. benthamiana::NN plants were infiltrated with *Agrobacterium* carrying binary PVX constructs with fragment of *N* (a positive control), *NtMEK2*, *SIPK*, *WIPK*, or *SIPKK* (a negative control). Fourteen days later, the upper leaves that show the silencing of target gene were inoculated with GFP-tagged TMV. The presence/spreading of the TMV virus in both the inoculated leaf and the upper uninoculated leaf was determined 5 days later by visualizing the green fluoresces under UV light.

vegetative propagation. None of the other 17 *NtMEK2^{DD}* lines with inducible *NtMEK2^{DD}* transgene showed 3 : 1 segregation in T_2 generation, suggesting multiple insertions. HR-like cell death phenotype was observed in all the offspring that expressed *NtMEK2^{DD}* after DEX treatment, which was again preceded by the activation of endogenous SIPK and WIPK (data not shown). The strict correlation between *NtMEK2^{DD}* expression and SIPK/WIPK activation as well as HR-like cell death phenotype in both T_1 and T_2 plants confirmed our previous results from transient transformation experiments (Yang *et al.*, 2001). In addition, the potential complication of agrobacterial cells in transient transformation was ruled out.

The N-terminal kinase interactivity motif (KIM) is essential for NtMEK2 function in vivo

The specificity and fast responsiveness of MAPK activation could be attributed to the organization of the three kinases in a MAPK cascade, which frequently form a complex either through a scaffold protein or through direct protein–protein interactions (Kolch, 2000; Tanoue *et al.*, 2000). The common docking (CD) domain, which was first identified in MAPKs from yeast and mammals, is conserved in plant MAPKs as well (Zhang and Liu, 2001). This domain contains two invariable acidic amino acid residues and is involved in the interaction of MAPKs with their upstream kinases, substrates and negative regulators, MAPK phosphatases (Kolch, 2000; Tanoue *et al.*, 2000).

The KIM domain in MAPKKs that is responsible for interacting with the CD domain of MAPKs is also present in plant MAPKKs (Figure 2a). This domain consists of a basic amino acid center with hydrophobic residues on one or both sides. The basic amino acid residues interact electrostatically with the acidic amino acid residues in the CD domain of MAPKs (Kolch, 2000; Tanoue *et al.*, 2000). The other feature in the N-termini of this subfamily of plant MAPKKs, including NtMEK2, AtMEK4, AtMEK5, and MsSIMKK is the Leu and Pro-rich region following the basic amino acid center (Figure 2a). To determine whether the KIM domain and the Leu/Pro-rich region are important for the NtMEK2 function in plants, the conserved basic amino acid cluster, RRR in the NtMEK2^{DD} were replaced with LPL, which was named NtMEK2$^{DD\text{-}LPL}$, or the N-terminal 64 amino acids residues were deleted, the resulting mutant was named NtMEK2$^{DD\text{-}\Delta N}$. The ability of these two mutants to activate SIPK and WIPK was tested *in vivo* by transient transformation assays. As shown in Figure 2(b) (lower panel), the activation of SIPK and WIPK by NtMEK2$^{DD\text{-}LPL}$ was delayed and very weak, whereas the NtMEK2$^{DD\text{-}\Delta N}$ was completely inactive, even the steady-state levels of both proteins were similar to or even higher than that of NtMEK2^{DD} (Figure 2b, upper panel). With the reduced ability to activate SIPK and WIPK, cell death induced by NtMEK2$^{DD\text{-}LPL}$ was much delayed, from the normal 12 h to 48 h or longer. In addition, the cell death caused by NtMEK2$^{DD\text{-}LPL}$ was patchy (data not shown). NtMEK2$^{DD\text{-}\Delta N}$, which lost the ability to activate endogenous SIPK and WIPK, failed to induce HR-like cell death (Figure 3). In permanently transformed tobacco plants, even NtMEK2$^{DD\text{-}LPL}$ failed to activate downstream SIPK/WIPK and HR-like cell death (data not shown). This is likely caused by lower level expression of NtMEK2$^{DD\text{-}LPL}$ in permanent transgenic plants, which was again only 5–10% of that in the transient transformation experiment (data not shown). These results suggest that the conserved KIM domain plays a crucial role in plants and both the basic amino acid residues and the Leu/Pro-rich region are involved in the activation of downstream SIPK and WIPK.

In addition to NtMEK2$^{DD\text{-}LPL}$ and NtMEK2$^{DD\text{-}\Delta N}$ mutants, we also constructed LPL and ΔN mutants in the wild-type NtMEK2 background. In the transient transformation assay, the expression patterns of NtMEK2^{WT}, NtMEK2$^{WT\text{-}LPL}$ and NtMEK2$^{WT\text{-}\Delta N}$ were similar to those of NtMEK2^{DD}, NtMEK2$^{DD\text{-}LPL}$ and NtMEK2$^{DD\text{-}\Delta N}$, respectively (Figure 4a). Similar to NtMEK2^{WT}, expression of neither NtMEK2$^{WT\text{-}LPL}$ nor NtMEK2$^{WT\text{-}\Delta N}$ led to MAPK activation and HR-like cell death (Figures 3 and 4a) (Yang *et al.*, 2001). As a result, we could not assess the activities of NtMEK2$^{WT\text{-}LPL}$ and NtMEK2$^{WT\text{-}\Delta N}$ in reference of NtMEK2^{WT} using the activation of endogenous SIPK and WIPK as a marker. Previously, we found that although basal level activity of NtMEK2 is present in cells as determined by the immune complex kinase assay, it is not sufficient to activate SIPK or WIPK (Yang *et al.*, 2001). This is likely because of the MAPK phosphatase that could counteract the basal level activity of NtMEK2 to keep the activities of SIPK and WIPK at very low levels in cells. For this reason, it would be more accurate to estimate the kinase activity of these mutants *in vitro* by using the immune complex kinase assay in the absence of the phosphatase activity.

Flag-tagged NtMEK2 and its mutants were immunoprecipitated from cell extracts using anti-Flag antibody and the kinase activity was determined using recombinant SIPK^{KR} and WIPK^{KR}, the inactive mutants of SIPK and WIPK as substrates. The use of inactive SIPK and WIPK recombinant proteins prevents the autophosphorylation activity of recombinant MAPKs that will interfere with the determination of MAPKK activities. As shown in Figure 4(b), NtMEK2^{DD} showed the highest activity towards both SIPK and WIPK. The activity of NtMEK2$^{DD\text{-}LPL}$ was significantly attenuated, while the activity of NtMEK2$^{DD\text{-}\Delta N}$ was barely detectable. These results are consistent with their ability to activate SIPK and WIPK *in vivo* (Figures 4a and 2b). NtMEK2^{WT} showed basal level activity towards both SIPK and WIPK as previously reported, although this low activity was not sufficient to activate SIPK and WIPK *in vivo* due to the presence of counteracting phosphatase(s) (Figure 4a, lower panel) (Yang *et al.*, 2001). Mutation of the basic amino

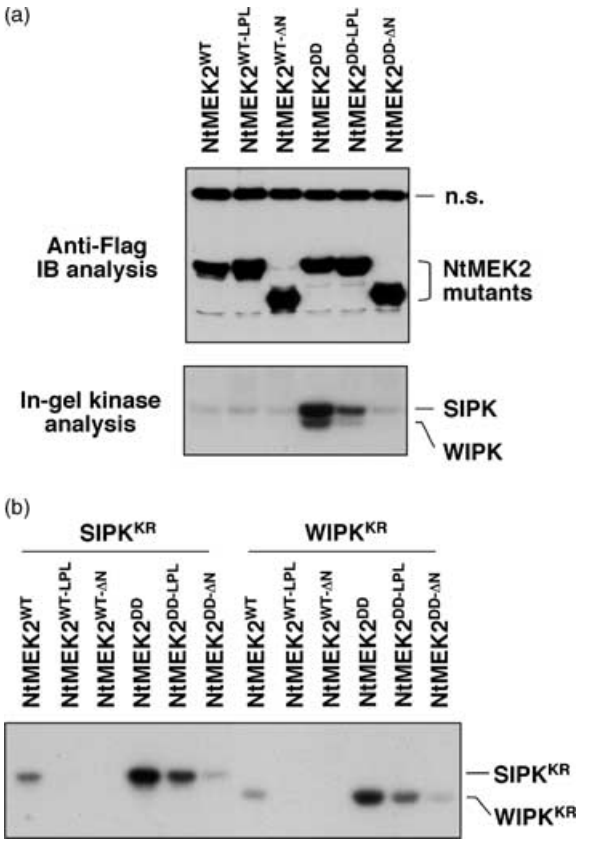


Figure 4. Direct assay of the MAPKK activity of LPL and ΔN mutants in NtMEK2^{WT} and NtMWK2^{DD} background.
(a) Activation of endogenous SIPK and WIPK by LPL and ΔN mutants *in vivo*. LPL or ΔN mutants in both NtMEK2^{WT} and NtMEK2^{DD} background were transiently transformed into tobacco leaves. Protein extracts were prepared from tissues harvested 6 h after the DEX treatment. The expression of transgene was monitored by immunoblot (IB) analysis using anti-Flag antibody (upper). A non-specific (n.s.) band detected by anti-Flag antibody was shown as a reference for equal loading and the molecular weight of the mutants. The activation of endogenous MAPKs was determined by an in-gel kinase assay using MBP as a substrate (lower).
(b) The MAPKK activity of LPL and ΔN mutants as determined by immune complex kinase assay using recombinant SIPK and WIPK. Flag-tagged NtMEK2^{WT} or its mutant proteins were immunoprecipitated from protein extracts described in (a) with anti-Flag antibody. The MAPK phosphorylation activity in the immune complex was determined *in vitro* using HisWIPK^{KR} and HisSIPK^{KR} as substrates (0.05 μg μl^{-1}) in the presence of γ-^{32}P-ATP. The reaction was stopped by the addition of SDS-sample buffer. Phosphorylated MAPK was visualized by autoradiography after SDS–PAGE.

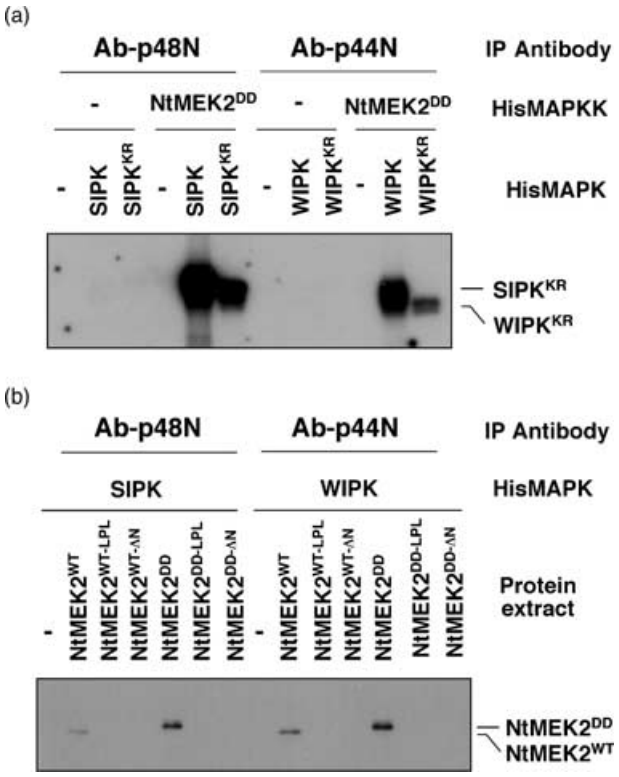


Figure 5. The N-terminal KIM domain of NtMEK2 is involved in its interaction with SIPK and WIPK.
(a) Recombinant NtMEK2 interacts with SIPK and WIPK *in vitro*. Recombinant His-tagged SIPK, SIPK^{KR}, WIPK, or WIPK^{KR} (1 μg) was incubated alone or with recombinant His-tagged NtMEK2^{DD} (1 μg) in 50 μl immunoprecipitation buffer for 1 h at 4°C. SIPK- or WIPK-specific antibodies (Ab-p48N or Ab-p44N, 2 μg) were added to reaction mixtures containing SIPK or WIPK, respectively. Protein A-agarose was then used to pull down SIPK or WIPK together with their interacting protein. The presence of NtMEK2^{DD} is determined by its kinase activity towards SIPK and WIPK in the presence of [γ-^{32}P]-ATP. Phosphorylated protein was visualized by autoradiography after SDS–PAGE.
(b) The N-terminal KIM domain is required for the interaction of NtMEK2 with SIPK and WIPK. NtMEK2^{WT} or its mutants were transiently transformed into tobacco leaves. Protein extracts were prepared from tissues harvested 6 h after the DEX treatment. After the addition of 2.5 μg recombinant SIPK or WIPK and incubation at 4°C for 30 min, Ab-p48N or Ab-p44N was used to immunoprecipitate SIPK or WIPK, together with their interacting protein. The presence of NtMEK2 or its mutants in the immune complex was determined by immunoblot (IB) analysis using anti-Flag antibody.

acid cluster (NtMEK2$^{WT\text{-}LPL}$) or the deletion of N-terminal 64 amino acids (NtMEK2$^{WT\text{-}\Delta N}$) completely abolished the low level activity of NtMEK2 (Figure 4b). These results demonstrated that the N-terminal KIM domain is essential for the MAPKK activity of NtMEK2 and its *in vivo* function. It should be pointed out here that the low MAPKK activity detected in NtMEK2^{WT} tissue was not because of the basal enzymatic activity of NtMEK2^{WT}, but rather a very small percentage of NtMEK2^{WT} protein was in the active state. This conclusion is based on our previous observation that the basal enzymatic activity of recombinant NtMEK2^{WT} is undetectable, even when microgram levels of protein were used (Yang *et al.*, 2001).

The KIM domain of NtMEK2 is required for its interaction with SIPK and WIPK

To provide direct evidence for the involvement of KIM domain in the interaction between NtMEK2 and SIPK/WIPK, we performed co-immunoprecipitation experiments using

both recombinant proteins and total cellular extracts. In the first experiment, we mixed 1 μg of recombinant NtMEK2^{DD} with 1 μg of recombinant SIPK or WIPK, or their inactive mutants, SIPK^{KR} and WIPK^{KR}. SIPK- or WIPK-specific antibody was then used to immunoprecipitate the MAPKs. The presence of NtMEK2^{DD} in the complex was detected by in-solution MAPKK activity assay. As shown in Figure 5(a), immunoprecipitated SIPK and WIPK can be strongly phosphorylated, indicating the presence of NtMEK2^{DD} in the complex. The use of SIPK^{KR} and WIPK^{KR} excluded the possibility of autophosphorylation. In the absence of NtMEK2^{DD}, little or no phosphorylation activity was detected (Figure 5a, lanes 2, 3, 8, and 9). The higher labeling of SIPK and WIPK (Figure 5a, lanes 5 and 11) is likely caused by higher autophosphorylation activity after SIPK and WIPK were activated by NtMEK2^{DD}. In contrast, SIPK^{KR} and WIPK^{KR} do not have kinase activity even after they were phosphorylated by NtMEK2^{DD} (Yang *et al.*, 2001).

To compliment the *in vitro* experiments using purified recombinant proteins and to determine if the KIM domain is required for the interaction, SIPK and WIPK were used to pull-down Flag-tagged NtMEK2 or its mutants from total cellular extracts. Equal amount of total protein (100 μg) from leaves transformed with *NtMEK2* or its mutants was incubated with 2.5 μg recombinant SIPK or WIPK for 30 min at 4°C. SIPK- or WIPK-specific antibody (5 μg) was then used to pull down SIPK or WIPK, together with their interacting proteins. The presence of NtMEK2 or its mutants in the immune complex was determined by immunoblot analysis using anti-Flag antibody. As shown in Figure 5(b), NtMEK2 and NtMEK2^{DD} can be pulled down by both anti-SIPK (Ab-p48N) and anti-WIPK (Ab-p44N) antibodies. However, neither antibody could pull down Flag-tagged LPL- or ΔN-mutant of NtMEK2 or NtMEK2^{DD}. These results demonstrated that the N-terminus of NtMEK2, especially the basic amino acid cluster in the N-terminal KIM domain is essential for its interaction and activation of downstream SIPK and WIPK. We consistently observed that NtMEK2^{DD} was pulled down more efficiently by SIPK or WIPK, suggesting that either the active form of NtMEK2 has higher affinity with downstream MAPKs or the complex is more stable.

Suppression of NtMEK2, SIPK and WIPK expression attenuates the N *gene-mediated resistance to TMV*

Although mounting correlative and gain-of-function evidence implicated SIPK and WIPK in the positive regulation of plant defense responses, loss-of-function evidence is lacking. Attempts to use antisense and dominant negative mutants to suppress the activation of SIPK and WIPK by pathogens have so far failed (Liu, Y., and Zhang, S., unpublished results). As a result, we attempted PVX virus-induced gene silencing (VIGS) technology, which has been proven to be a powerful tool to acquire loss-of-function data in a number of cases (Baulcombe, 1999; Romeis *et al.*, 2001; Ruiz *et al.*, 1998; Takken *et al.*, 2000). Complimentary DNA fragments corresponding to *NtMEK2*, *SIPK*, and *WIPK* were isolated from *Nicotiana benthamiana* by RT–PCR and cloned into PVX binary vector pGr106. To initiate gene silencing, *Agrobacterium* containing pGr106 construct was infiltrated into young *N. benthamiana* plants that carry *N*-resistance transgene (*N. benthamiana*::*NN*). Fourteen days post-infiltration, green fluorescence protein (GFP)-tagged TMV (TMV-GFP) was inoculated on silenced leaves. The multiplication and spreading of TMV, which indicates the loss of resistance, were scored by detecting the green fluorescence from TMV-GFP and viral RNA directly. Plants infiltrated with empty PVX vector displayed little green fluorescence on inoculated leaves and no fluorescence on the upper uninoculated leaves (Jin *et al.*, 2002). However, when *N* gene was silenced, large patches of green florescence, an indication of the presence of TMV, were found in both inoculated leaves and the upper uninoculated leaves after 5 days (Figure 6). The systemic spreading of TMV indicated the loss of *N* gene-mediated resistance. Silencing of *NtMEK2*, *SIPK*, or *WIPK* all led to the attenuation of resistance (Figure 6), although the symptom was less severe than that in plants with *N* gene silenced. In contrast, suppression of *SIPKK*, a tobacco MAPKK identified as SIPK interacting protein (Liu *et al.*, 2000), did not compromise the *N*-mediated resistance (Figure 6). This is consistent with our previous finding that SIPKK is not upstream of SIPK or WIPK based on evidence from both

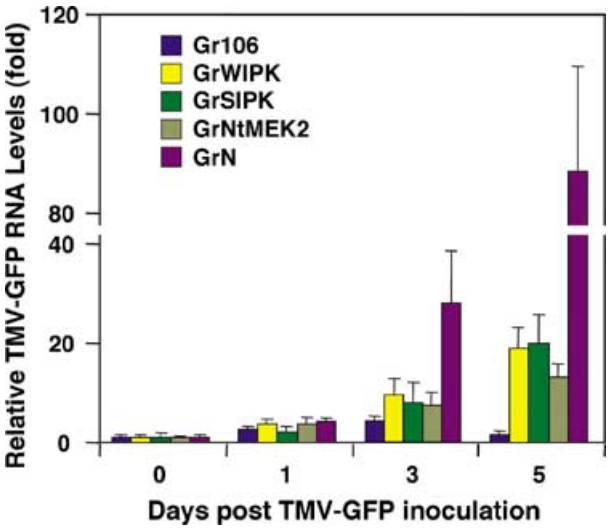


Figure 7. Quantification of viral RNA in silenced tissues after TMV-GFP inoculation.
The multiplication and spread of TMV were quantified by real-time RT–PCR. Leaf tissue was harvested at various times after TMV-GFP inoculation and RNA was extracted with Trizol reagent (Life Technologies). After being treated with DNase I, the total RNA was reverse transcribed and PCR amplified using TMV-specific primers. Relative levels of viral RNA were calculated using ubiquitin as an internal standard as described in Methods. Standard deviations are indicated by vertical error bars for each time point.

in vitro and *in vivo* studies (Yang *et al.*, 2001). In addition to the direct visualization of green fluorescence under UV light, the multiplication and spread of TMV were also quantified by real-time RT–PCR. As shown in Figure 7, there was about 20-fold increase in viral RNA in plants with *NtMEK2*, *SIPK*, or *WIPK* being silenced. In contrast, when *N* gene was suppressed, an 80-fold increase of viral RNA was detected.

The efficiency of *NtMEK2*, *SIPK*, or *WIPK* gene silencing was determined by real-time RT–PCR. As shown in Figure 8, the transcripts of *NtMEK2*, *SIPK*, and *WIPK* were reduced by more than 80% in plants 14 days after the infiltration of *Agrobacterium* carrying PVX construct. The more substantial loss of resistance when *N* gene was silenced in comparison to when *NtMEK2*, *SIPK*, or *WIPK* was silenced could be a result of the loss of all defense responses when *N* gene is repressed. In contrast, MAPK pathway may control only a subset of defense responses, which will leave other defense signaling pathways functional therefore a partial loss of resistance. One potential such pathway is the NtCDPK2 pathway reported recently (Romeis, 2001). Suppression of NtCDPK2 and/or closely related subfamily members compromised the Cf-9/Avr9- and Cf-4/Avr4-mediated activation of HR and wilting phenotype. At this stage, there is no evidence indicating that NtCDPK2 and NtMEK2–SIPK/WIPK are in a linear pathway. Alternatively, it could be that although the levels of transcript were suppressed to a similar level (Figure 8) (Jin *et al.*, 2002), the decrease of protein was to different levels due to the difference in the stability of proteins, which may lead to the varying degrees of loss of resistance. The sequence similarity between different MAPKKs is very low. For instance, *NtMEK2* and *SIPKK* only share 29% identity at DNA level, which makes cross suppression unlikely. *SIPK* and *WIPK*, two closely related MAPKs in tobacco share 52% identity at the DNA level. Even so, the suppression of *WIPK* expression did not affect the levels of *SIPK* transcript (Figure 9), demonstrating the specificity of pGrWIPK construct. However, suppression of SIPK did block the TMV-induced WIPK accumulation. This is because TMV-induced *WIPK* gene activation is mediated by SIPK (Liu *et al.*, in preparation).

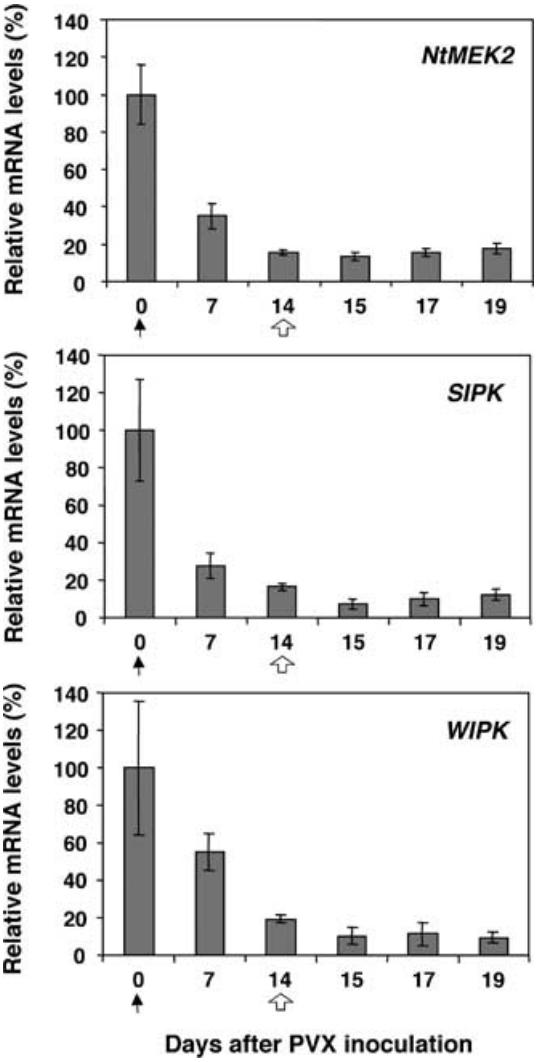


Figure 8. Suppression of *NtMEK2*, *SIPK*, and *WIPK* expression by PVX virus-induced gene silencing.
The efficiency of *NtMEK2*, *SIPK*, or *WIPK* gene silencing was determined by real-time RT–PCR. Leaf tissue was harvested from silenced plants and RNA was extracted with Trizol reagent (Life Technologies). After being treated with DNase I, the total RNA was reverse transcribed and PCR amplified using gene-specific primers. Relative quantification of mRNA was calculated using ubiquitin as an internal standard as described in Experimental procedure. Standard deviations are indicated by vertical error bars for each time point. Arrows indicate the time that binary PVX constructs in *Agrobacterium* were infiltrated, and block arrows indicate the time of TMV-GFP inoculation.

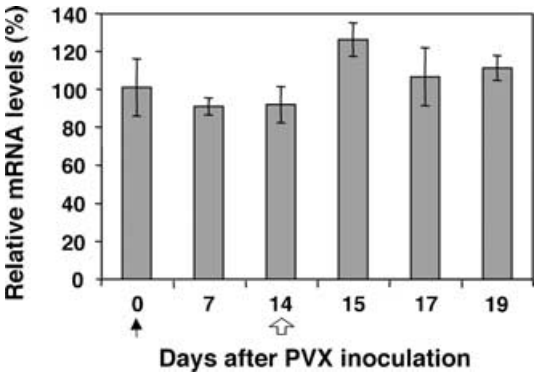


Figure 9. Suppression of *WIPK* expression by PVX virus-induced gene silencing does not affect the levels of *SIPK* transcript.
To determining the specificity of gene silencing, the levels of *SIPK* transcript in plants inoculated with *Agrobacterium* carrying pGrWIPK construct were monitored by real-time RT–PCR. The same RNA samples as in Figure 8, bottom panel were used. Standard deviations are indicated by vertical error bars for each time point. Arrows indicate the time that binary PVX constructs in *Agrobacterium* were infiltrated, and block arrows indicate the time of TMV-GFP inoculation.

Discussion

Permanent transgenic tobacco plants with *NtMEK2^{WT}*, *NtMEK2^{DD}*, and *NtMEK2^{KR}* under the control of steroid-inducible promoter were generated. There is a strict correlation between the inducibility of *NtMEK2^{DD}* expression and HR-like cell death in both T_1 and T_2 generations. In contrast, expression of *NtMEK2^{WT}* and *NtMEK2^{KR}* failed to activate endogenous MAPKs and defense responses. Mutation and deletion analyses demonstrated that the conserved KIM domain at the N-terminus of NtMEK2 is involved in its interaction with downstream SIPK and WIPK, which is essential for the *in vivo* function of NtMEK2. In addition to the gain-of-function evidence, we found that the suppression of all three known components in the NtMEK2–SIPK/WIPK pathway compromised the *N* gene-mediated resistance against TMV in *Nicotiana benthamiana*::*NN* plants. Together with our previous report that SIPK and WIPK are activated in tobacco by TMV infection in a gene-for-gene-dependent manner (Zhang and Klessig, 1998), we concluded that NtMEK2–SIPK/WIPK pathway in tobacco plays positive roles in plant disease resistance signaling, possibly via regulating the HR cell death as indicated by the gain-of-function evidence. Consistent with this, it was reported recently that transient transformation of the active mutant of *AtMEK4*, an ortholog of tobacco *NtMEK2*, enhanced the resistance of *Arabidopsis* against both bacteria and fungal pathogens (Asai *et al.*, 2002). The permanent *NtMEK2^{DD}* transgenic plants behave like a wild-type tobacco before DEX treatment. After the application of DEX, HR-like cell death developed rapidly (Figure 1). As a result, we cannot test the resistance of these plants against TMV infection after the induction of *NtMEK2^{DD}* expression.

HR cell death is often associated with disease resistance (Dangl *et al.*, 1996; Goodman and Novacky, 1994; Greenberg, 1997). It has long been speculated that the HR cell death and associated dehydration are directly responsible for limiting pathogen growth and development. All pathogens, including fungi, bacteria, and viruses require a water environment to multiply and acquire nutrients from plant cells. In the case of viral pathogens, cell death/dehydration also affects the plasmodesmata through which the viruses spread to other cells. The quick dehydration of the tissue undergoing HR cell death therefore will prevent the proliferation of pathogens and restrict them to a small area. In contrast, cell death in a compatible interaction is slow and not associated with quick dehydration. Recently, there were several reports demonstrating that plant resistance to viruses can be uncoupled from HR cell death (Cole *et al.*, 2001; Cooley *et al.*, 2000). Although these observations do not rule out that HR plays an important role in resistance, it does suggest that disease resistance can be achieved by other mechanisms or only a subset of defense responses in the absence of cell death. *N* gene transgenic *N. benthamiana* plants (*N. benthamiana*::*NN*) gained full resistance to TMV infection. However, HR cell death after TMV infection is variable and difficult to score quantitatively, especially in the presence of background symptom after PVX infection (Jin, H. and Baker, B., unpublished results). As a result, it is difficult to obtain the loss-of-function evidence for the role of NtMEK2–SIPK/WIPK pathway in HR cell death in response to TMV using this system.

Our attempts to generate stable loss-of-function transgenic plants by either antisense approach or overexpression of the negative SIPK or NtMEK2 mutants have so far failed. A recent study using RNA interference (RNAi) revealed that the suppression of SIPK lead to higher sensitivity to ozone, similar to the overexpression of SIPK (Samuel and Ellis, 2002). It is unknown whether the mechanisms of ultra-sensitivity of these two opposing transgenic plants to ozone are the same. One possible interpretation of the data is that SIPK is not required for ozone-induced cell death. Ozone may cause cell death directly by inducing ROS that also activate SIPK. Although both ROS/ozone and pathogen-related stimuli activate SIPK, the kinetics of SIPK activation is different. Ozone and exogenously applied H_2O_2 induce a very transient activation of SIPK, whereas SIPK activation by avirulent pathogens or elicitors that induce cell death is long lasting (Samuel and Ellis, 2002; Suzuki *et al.*, 1999; Zhang and Klessig, 2001), suggesting that the activation of SIPK by ROS/ozone and pathogens are mechanistically different. Furthermore, the rapid generation of H_2O_2 is not required for the elicitor/Avr factor-induced activation of SIPK (Romeis *et al.*, 1999). These results suggest that ROS is not a signal for the activation of SIPK by pathogens or pathogen-derived elicitors. In contrast, our recent study demonstrated that MAPK-activation induced HR-like cell death is associated with the generation of ROS (Ren *et al.*, 2002), indicating that ROS could function downstream of SIPK in the cell death pathway.

As signaling molecules, the components in a MAPK cascade exist at a very minute amount in cells. It was estimated that SIPK is only of 0.004% of the total soluble proteins (Zhang and Klessig, 1997) and NtMEK2 is likely to exist at a similar amount based on results from animal systems (Huang and Ferrell, 1996). The rapid and very efficient activation of MAPK cascades partly relies on the interactions between MAPK, MAPKK, and MAPKKK, or the binding of all three kinases by a scaffold protein in a particular MAPK cascade (Davis, 2000; Kolch, 2000; Tanoue *et al.*, 2000; Widmann *et al.*, 1999). These interactions also contribute to the specificity of MAPK activation and multi-functional facet of a single MAPK or MAPKK. Depending on which MAPKK or MAPKKK that a MAPK forms a complex with, this portion of a MAPK will respond to a different stimuli, resulting in that a MAPK plays multiple functions in a cell (Chang and Karin, 2001; Widmann *et al.*, 1999). The CD

domain in MAPKs and the KIM domain in MAPKKs are involved in the interaction between MAPKs and MAPKKs in yeast and animal systems (Kolch, 2000; Tanoue *et al.*, 2000). In this report, we demonstrated that the KIM domain, which is also conserved in plant MAPKKs, is essential for the NtMEK2 function *in vivo*. We detected a direct interaction between recombinant NtMEK2 and SIPK or WIPK by co-immunoprecipitation experiments (Figure 5a). After the concentration of SIPK or WIPK in the total cell extracts was increased by the addition of recombinant proteins, anti-SIPK or anti-WIPK antibody pulled down endogenous NtMEK2 and NtMEK2^{DD}, but not their mutants (Figure 5b), demonstrating the involvement of KIM domain in the interaction between NtMEK2 and SIPK/WIPK. The fact that higher concentration of SIPK or WIPK was needed to co-immunoprecipitate NtMEK2 or NtMEK2^{DD} also suggests that the complex might be dynamically unstable in the cell extracts where the proteins are much more diluted. The elevation of the concentration of one of the interactors favors the formation of MAPKK/MAPK complex.

The MAPKKK(s) upstream of NtMEK2 is currently unknown. There are two likely candidates, NPK1 and the tobacco ortholog of *Arabidopsis* AtMEKK1. NPK1 was shown previously to activate AtMPK6 and AtMPK3, the orthologs of tobacco SIPK and WIPK, respectively, in *Arabidopsis* protoplast transient transformation assays (Kovtun *et al.*, 2000). Furthermore, the suppression of *NPK1* also compromises the *N* gene-mediated resistance of TMV in *N. benthamiana::NN* plants. However, in *Agrobacterium*-mediated transient transformation experiments, the active NPK1 deletion mutant, ΔNPK1 only weakly activate SIPK or WIPK in tobacco (Jin *et al.*, 2002). One likely reason is that the level of Δ*NPK1* expression was not high enough to overcome the effect of phosphatases that act on NtMEK2 or SIPK/WIPK. Recently, AtMEKK1 was reported to mediate the Flg22-induced AtMPK6/AtMPK3 activation via AtMEK4 and AtMEK5 in *Arabidopsis* protoplasts (Asai *et al.*, 2002). However, the suppression of a tobacco ortholog of AtMEKK1 had no effect on the *N* gene-mediated TMV resistance (Jin *et al.*, 2002), suggesting that MEKK1 may not form a cascade with NtMEK2–SIPK/WIPK in the *N* resistance signaling pathway. Despite these facts, it is still possible that both NPK1 and MEKK1, and possibly other MAPKKKs, can function as the upstream MAPKKK in the NtMEK2–SIPK/WIPK pathway, depending on what is the stimulus. In mammalian cells, there are ample precedents that multiple MAPKKKs can feed into one MAPK. As a result, signals generated from different stimuli converge and induce a shared set of responses (Davis, 2000; Widmann *et al.*, 1999). This speculation is consistent with the higher number of MAPKKKs in the *Arabidopsis* genome (MAPK Group, 2002).

In yeast and animal systems, MAPK cascades play central roles in converting the signals generated from receptors/sensors into nuclear/cellular responses. A single MAPK can phosphorylate and activate multiple downstream substrates in both cytoplasmic and nuclear compartments therefore control multiple responses (Chang and Karin, 2001; Davis, 2000; Widmann *et al.*, 1999). On the other hand, multiple stimuli can feed into one MAPK through multiple upstream components including MAPKKKs, which allows a single MAPK to regulate the shared responses elicited by multiple stimuli. It appears that the NtMEK2–SIPK/WIPK pathway has similar mechanisms of actions in tobacco. Besides the roles of SIPK in biotic stress responses, this MAPK is also implicated in plant responses to environmental/abiotic stresses, such as wounding, high/low osmolarity, drought, salinity, ozone, and UV irradiation (Tena *et al.*, 2001; Zhang and Klessig, 2001). The activation of SIPK by these environmental stimuli is transient and may lead to the transient activation of defensive or protective responses that are shared with those elicited by pathogens. SIPK orthologs in other species are responsive to similar stresses, suggesting the functional conservation in different plant species (Desikan *et al.*, 2001; Ichimura *et al.*, 2000; Munnik *et al.*, 1999; Nühse *et al.*, 2000). The conditional system used in this study results in prolonged activation of SIPK and delayed activation of WIPK, which mimics the activation of these two MAPKs in plants challenged by avirulent pathogens, including TMV (Zhang and Klessig, 1998). We speculate that whether SIPK functions in a general defensive/protective pathway or in disease resistance depends on the kinetics of SIPK activation. A transient activation of SIPK may enhance the general defense of a plant by turning on the protective mechanism. In contrast, a long-lasting activation of SIPK and delayed activation of WIPK can cause HR cell death, which is part of the resistance response. We observed a positive correlation between the levels of NtMEK2^{DD} expression and the levels of SIPK/WIPK activation, suggesting that the activity of NtMEK2 is crucial in determining the activation of SIPK/WIPK in cells. To fully understand how the kinetics of SIPK/WIPK activation is controlled, we will need to identify both the upstream kinase of NtMEK2 and the negative regulators of this pathway, including the phosphatases that inactivate NtMEK2, SIPK, and WIPK. The identification of the upstream MAPKKK(s) and downstream MAPK substrate(s) will also help us understand how SIPK and WIPK respond to multiple stimuli and regulate multiple defense responses.

Experimental procedures

Mutagenesis and preparation of pTA7002 constructs

Substitution mutants were generated by QuickChange site-directed mutagenesis (Stratagene). Deletion mutants were generates by high fidelity PCR using primers flanking the deleted sequence. After ligation, the plasmid was transformed into XL-1 blue *E. coli*

cells. The mutations and deletion were confirmed by sequencing. After each PCR, the constructs were sequenced to ensure that no error was introduced. *NtMEK2* and its mutants with a Flag epitope at their N-termini were then inserted into the *Xho*I–*Spe*I sites of the steroid-inducible pTA7002 binary vector (Aoyama and Chua, 1997). The 5′-untranslated region of *NtMEK2* was replaced with the Ω sequence from tobacco mosaic virus.

Agrobacterium*-mediated transformation of tobacco*

Tobacco plants (*N. tabacum* cv. Xanthi nc [NN]) were grown at 25°C in a growth room programmed for 14-h light/10-h dark cycle. Tobacco plants, 5–7-week-old, were used for experiments. *Agrobacterium tumefaciens*-mediated transient transformation experiments were performed as described previously (Yang *et al.*, 2001). Permanent transgenic tobacco plants were generated by *Agrobacterium*-mediated leaf-disc transformation (Horsch *et al.*, 1985) and selected by hygromycin resistance. Transgenic plants were moved into soil and transgene inducibility was tested 3–4 weeks later using detached leaves. The T_1 plants were then allowed to set seeds in a greenhouse. Either vegetatively propagated T_1 plants or selected T_2 plants with similar level of transgene expression were used for experiments.

Protein extraction, immunoblot analysis, and kinase assays

Protein was extracted from leaf tissue and stored at −80°C (Zhang and Klessig, 1998). The concentration of protein extracts was determined using the Bio-Rad protein assay kit with BSA as the standard. Immunoblot analysis, in-gel kinase activity assay, and immune complex (IC) kinase activity assay were performed as described previously (Yang *et al.*, 2001).

Co-immunoprecipitation of NtMEK2 using Ab-p48N or Ab-p44N antibody

For co-immunoprecipitation of recombinant NtMEK2, 1 μg of $HisNtMEK2^{DD}$ protein was mixed with 1 μg of HisSIPK, $HisSIPK^{KR}$, HisWIPK, or $HisWIPK^{KR}$ in 50 μl of 20 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Tween-20. After incubation at 4°C for 1 h, 2 μg of Ab-p48N or Ab-p44N was added to tube with SIPK or WIPK, respectively. After the addition of Protein A-agarose (15 μl of packed volume), the mixture was incubated for 2 h at 4°C on a rocker. The beads were collected by brief centrifugation and washed twice with the same buffer (1.5 ml each) and then twice with kinase reaction buffer (1 ml each). The presence of $NtMEK2^{DD}$ in the immune-complex was determined by detecting its MAPKK activity.

For coimmunoprecipitation of Flag-tagged NtMEK2 from total extracts prepared from leaves transiently transformed with *NtMEK2* or its mutants, 2.5 μg of HisSIPK or HisWIPK was added to 100 μg of protein extract. After incubation for 30 min at 4°C, SIPK or WIPK was immunoprecipitated with Ab-p48N or Ab-p44N antibody. The presence of NtMEK2 or its mutants in the immune complex was determined by immunoblot analysis using anti-Flag antibody as described above.

PVX virus-mediated gene silencing

Transgenic *Nicotiana benthamiana* plants carrying *N* gene (*N. benthamiana::NN*) used for gene silencing were grown at 24°C in a growth cabinet under a 16 h:8 h light:dark cycle as previously described (Jin *et al.*, 2002).

Binary PVX vector pGr106 (Takken *et al.*, 2000) was used for constructing recombinant virus for gene silencing. The following primers were used to amplify corresponding cDNA fragments from *N. benthamiana*. Restriction site *Xho*I or *Cla*I was added at the 5′-end of forward or backward primer for cloning into pGr106. Primers for *NtMEK2*: 5′AAAATCGATCAATCATGCGACCTCTTCAACCAC3′, 5′AAAACTCGAGCTGACTCTTTAGGGATGTGGATC3′, producing a 530-bp fragment; primers for *SIPK*: 5′AAAACTCGAGTAAAGGTGCTTACGGCATCGTTTG3′, 5′AAAATCGATTGGCGGATGTATCGTTTTGCATTC3′, producing a 690-bp fragment; primers for *WIPK*: 5′AAAATCGATACATACATTCCGCGAATGTTCTTC3′, 5′AAAACTCGAGTAGGAAAGTAGATACTCCAGATC3′, producing a 720-bp fragment; primers for *SIPKK*: 5′AAAACTCGAGCCATCAGACAACCAGTTATG3′, 5′AAATCGATACTTGGTGCTGGTTGGTCAAC3′, producing a 700-bp fragment. *Agrobacterium* strain GV2260 carrying recombinant PVX constructs were infiltrated into 4–5-week-old *N. benthamiana::NN* plants as previously described (Jin *et al.*, 2002).

Real-time RT–PCR to determine the efficiency of gene silencing and viral RNA level

Leaf tissue was harvested from silenced plants and RNA was extracted with Trizol (Gibco), the total RNA was treated with DNase I and reverse transcribed as described (Jin *et al.*, 2000). Oligo dT17 adapter was used for reverse transcription, and primers for *NtMEK2* (5′ACAGCTGTTGCGTCATCCATT3′, 5′TTGATGTGCTGGCTGATGAAC3′), *SIPK* (5′TTAGTGACGAGCCCATTTGCA3′, 5′TGCAAGCGACTCCCTGTAAATC3′) and *WIPK* (5′GATTTTCTTGCTCGGACCGA3′, 5′AATCAAGGTGCCCACAAGCTA3′) were used to PCR-amplify the target gene transcripts. These primers anneal to sequences outside the regions of the genes targeted for silencing therefore ensured that only the endogenous gene transcript was detected. Primers for amplifying ubiquitin internal control were 5′CGGCATGCTTAACACATGCA3′ and 5′AGCCGTTTCCAGCTGTTGTTC3′. TMV viral RNA level was determined as described previously (Jin *et al.*, 2002). The comparative Ct method was applied to calculate relative quantification of sample RNA compared to control RNA from empty-vector-treated plants at various time points (ABI User Bulletin #2). All the experiments were repeated at least thrice.

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