Developmental Cell, Vol. 3, 291–297, August, 2002, Copyright ©2002 by Cell Press

# NPK1, an MEKK1-like Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Innate Immunity and Development in Plants

**Hailing Jin,[1,2] Michael J. Axtell,[1] Douglas Dahlbeck,[1] Obi Ekwenna,[1,2] Shuqun Zhang,[3] Brian Staskawicz,[1] and Barbara Baker[1,2,4]**

[1]Department of Plant and Microbial Biology
University of California, Berkeley
Berkeley, California 94720

[2]Plant Gene Expression Center
USDA-ARS
Albany, California 94710

[3]Department of Biochemistry
University of Missouri, Columbia
Columbia, Missouri 65211

## Summary

**Mitogen-activated protein kinase (MAPK) cascades are rapidly activated upon plant recognition of invading pathogens. Here, we describe the use of virus-induced gene silencing (VIGS) to study the role of candidate plant MAP kinase kinase kinase (MAPKKK) homologs of human *MEKK1* in pathogen-resistance pathways. We demonstrate that silencing expression of a tobacco MAPKKK, *Nicotiana Protein Kinase 1* (*NPK1*), interferes with the function of the disease-resistance genes *N*, *Bs2*, and *Rx*, but does not affect *Pto*- and *Cf4*-mediated resistance. Further, *NPK1*-silenced plants also exhibit reduced cell size, defective cytokinesis, and an overall dwarf phenotype. Our results provide evidence that *NPK1* functions in the regulation of *N*-, *Bs2*-, and *Rx*-mediated resistance responses and may play a role in one or more MAPK cascades, regulating multiple cellular processes.**

## Introduction

Plant recognition of pathogens is mediated by a large family of *R* gene-encoded receptor-like proteins and results in the activation of signaling cascades and the induction of defense and resistance responses (Dangl and Jones, 2001).

Genetic dissection of disease resistance has helped identify *R* gene pathway components. The *Arabidopsis NDR1* and *EDS1* genes were identified in screens for loss of race-specific resistance to, respectively, the bacterium *P. syringae* and the oomycete *P. parasitica* (Century et al., 1997; Falk et al., 1999). Both genes are required for multiple, but distinct, *R* functions. Likewise, barley *RAR1* is required for the function of some, but not all, barley *Mla* resistance genes. RAR1 is a zinc-coordinating protein, which, in higher metazoans, is fused to a protein domain homologous to the yeast SGT1 protein. SGT1 is involved in the SCF protein degradation complex and may regulate delivery of targets to the proteasome (Shirasu et al., 1999). Recent genetic evidence in *Arabidopsis* and gene silencing experiments in *N. benthamiana* suggest that plant homologs of SGT1 directly interact with RAR1 and are necessary for multiple *R* gene pathways (Austin et al., 2002; Azevedo et al., 2002; J. Peart and D. Baulcombe, personal communication).

The TIR-NBS-LRR subfamily of plant *R* genes encodes proteins with a conserved Toll/interleukin-1 receptor (TIR) domain, a nucleotide binding site (NBS), and a leucine-rich repeat (LRR) region (Baker et al., 1997). *N* was the first member identified in this subfamily, and the similarity of its N-terminal TIR domain to the cytoplasmic domain of the *Drosophila* Toll and human interleukin-1 receptors may indicate that *N* and other TIR-NBS-LRR resistance genes are evolutionarily related to receptors in the animal innate immune pathways (Whitham et al., 1994). The structural similarity between the N protein and the Toll and IL-1 receptor proteins led us to postulate that the TMV resistance pathway may share signaling components with Toll and IL-1R defense pathways (Whitham et al., 1994).

Due to their established regulatory role in activation of innate immunity, MAPK cascades were of particular interest as candidate signaling components in plant disease resistance. *MEKK1* encodes an MAPKKK that specifically phosphorylates and activates IκB kinase (IKK) in the animal innate immune pathway. The activated IKK complex in turn phosphorylates the inhibitor of NFκB, IκB, targeting IκB for degradation and releasing transcription factor NF-κB for translocation into the nucleus (Lee et al., 1997). MEKK1 is also important in regulating cell survival and apoptosis; its role changes from a survival-promoting kinase to an effector of cell death when cleaved by caspases (Widmann et al., 1998).

Many components of MAPK pathways, as well as some aspects of their regulation, are conserved throughout eukaryotic evolution (Schlesinger et al., 1998). Using highly conserved signature motif sequences, analysis of the *Arabidopsis* genome has revealed 23 putative MAPKs, 10 putative MAPKKs, and more than 25 MAPKKKs (Tena et al., 2001, Ren et al., 2002). It has been documented that plant MAPK cascades are activated by hormones (Kieber et al., 1993), abiotic stresses (Kovtun et al., 2000), pathogens (Zhang and Klessig, 1998), and pathogen-derived elicitors (Zhang et al., 1998; Asai et al., 2002) and are also activated at specific stages during the cell cycle (Nishihama et al. 2001). Considering the large number of plant MAPK cascade genes and their central role in cellular regulation, relatively few mutants in MAPK pathways have been isolated. One explanation for this limited collection of loss-of-function mutants may be embryonic lethality of mutations in MAPK cascade genes. Virus-induced gene silencing (VIGS) can generate loss-of-function mutations in essential genes in developed plants (Romeis et al., 2001), thus circumventing the otherwise lethal phenotype. Silencing of a host gene occurs after triggering the degradation of target RNA, and, in *N. benthamiana,* potato virus X (PVX)-based (Takken et al., 2000) and tobacco rattle virus (TRV)-based (Ratcliff et al., 2001) silencing systems have been employed to ascertain plant gene function.

[4]Correspondence: bbaker@socrates.berkeley.edu

Using this approach, we tested whether the silencing of several plant homologs of human *MEKK1* suppressed *R* gene-mediated resistance responses. Our results demonstrate that silencing the expression of one plant *MEKK1* homolog, *NPK1*, interferes with *N*-, *Bs2*-, and *Rx*-mediated resistance responses but does not affect the *Pto*- and *Cf4*-mediated hypersensitive response (HR). Furthermore, *NPK1*-silenced plants exhibit reduced cell size and an overall dwarf phenotype. These results suggest that NPK1 has a complex role in the regulation of multiple cellular processes.

## Results

### Identification of Solanaceous *MEKK1* Homologs

To begin investigating whether animal and plant defense pathways share MAPK signaling components, we conducted database searches for plant homologs of human *MEKK1* (Q13233). Searches of the GenBank databases using the BLASTP and TBLASTN programs identified homologs in the MEKK-like subfamily of plant MAPKKKs (30% identity and 50% amino acid similarity). All of the plant protein sequences identified contain regions homologous to the ~250-amino acid kinase domain of MEKK1. The plant genes encoding amino acid sequences showing greatest homology to the *MEKK1* kinase domain are tobacco *NPK1*, a tomato *NPK1* ortholog (BG125874), and *ANP1,* an *Arabidopsis NPK1* ortholog (Kovtun et al., 2000). Several additional tomato *MEKK1* homologs were identified, including TC93317 and TC88621, an AtMEKK1 ortholog (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/2/291/DC1). The *Raf*-like MAPKKK subfamily (e.g., *EDR1* and *CTR1*) was not identified in our searches. Solanaceous *MEKK1* homologs were PCR amplified from *N. benthamiana* cDNA (designated "NBxxxx"), and the PCR products were cloned into the PVX-based pGr106 vector for VIGS.

### *NPK1* Is Involved in *N*-Mediated TMV Resistance

Transgenic *N. benthamiana* plants carrying the *N* gene (*N. benthamiana::N)* were used to test the role of *MEKK1* homologs in *N*-mediated TMV resistance. As a positive control, a DNA fragment of the *N* gene was inserted into pGr106 (pGr106::*N*) and used for silencing the *N* gene itself. Another positive control was *SGT1* (Austin et al., 2002; Azevedo et al., 2002). pGr106::*SGT1* was constructed using a DNA fragment of the *N. benthamiana SGT1* gene (Experimental Procedures). Empty pGr106 vector was used as a negative control.

Attenuation of *N* gene-mediated resistance was monitored by the spread of GFP fluorescence following inoculation with TMV-GFP. *N*-silenced ($N^-$) and *SGT1*-silenced ($SGT1^-$) positive control as well as *NPK1*-silenced ($NPK1^-$) plants showed extensive GFP fluorescence on TMV-GFP-inoculated leaves and upper uninoculated leaves at 5–6 days post inoculation (dpi) (Figure 1A, b–d), indicating that systemic TMV-GFP spread to the young upper leaves. We observed that the spread of GFP fluorescence was greater in $SGT1^-$ and $N^-$ plants than in $NPK1^-$ plants. GFP fluorescence was not detected in plants treated with either pGr106 empty vector or pGr106 constructs containing NB93317 (Figure 1A, a), NB88621, or other *MEKK1* homologs (data not shown; see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/2/291/DC1). These experiments were repeated at least five times with total of 30–50 plants for each construction.

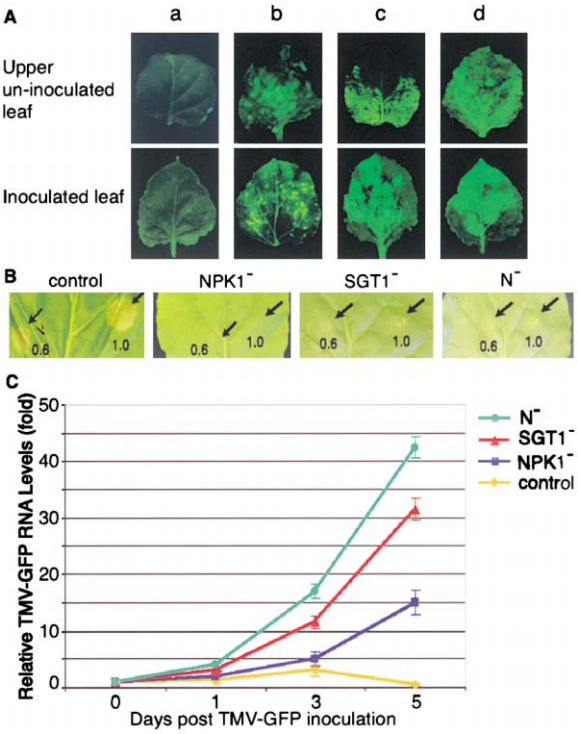


Figure 1. *N*-Mediated TMV Resistance Is Attenuated in $NPK1^-$ Plants

(A) GFP fluorescence in TMV-GFP-inoculated and upper uninoculated leaves of *N. benthamiana::N* plants silenced for (a) NB93317, (b) *NPK1*, (c) *SGT1*, and (d) *N*. Photographs were taken 5–6 days after TMV-GFP infection.

(B) TMV elicitor p50 HR assay in *N. benthamiana::N. Agrobacterium::p50* infiltrated into empty vector-treated (control), $NPK1^-$, $SGT1^-$, and $N^-$ leaves at concentrations of $OD_{600}$ 0.6 and 1.0. Photographs were taken 4 dpi.

(C) Relative levels of TMV-GFP RNA in $SGT1^-$, $N^-$, $NPK1^-$, and control plants.

Inoculation of *Agrobacterium* bearing a binary plasmid expressing TMV effector p50 induces *N*-mediated HR (Erickson et al., 1999). *Agrobacterium* carrying p50 was infiltrated at concentrations of $OD_{600}$ 0.6 and 1.0 onto silenced leaves and scored for HR after 2–3 dpi. Expression of p50 failed to induce obvious HR in $NPK1^-$, $N^-$, or $SGT1^-$ leaves (Figure 1B). In control plants, HR was induced by p50 at 2 dpi (Figure 1B). These results suggest that *NPK1* is required for *N* gene-mediated TMV resistance.

To confirm that the GFP fluorescence detected in TMV-GFP-inoculated plants was the result of TMV replication and spread, relative quantitative real-time RT-PCR was performed. TMV RNA levels were measured in $NPK1^-$, $SGT1^-$, $N^-$, and empty vector-treated plants (control) at 0, 1, 3, and 5 dpi (Figure 1C). We demonstrated the presence of abundant TMV-GFP RNA 5 days after TMV-GFP inoculation in $N^-$, $SGT1^-$, and $NPK1^-$ plants yet detected little TMV-GFP RNA in control plants.

By 5 dpi, SGT1$^-$ plants had less TMV-GFP RNA (approximately 82%) than N$^-$ plants, and the level of TMV-GFP RNA in NPK1$^-$ plants was about 47% of that in SGT1$^-$ plants and 36% of that in N$^-$ plants.

The disparities in TMV-GFP RNA levels between NPK1$^-$, SGT1$^-$, and N$^-$ plants could be interpreted as a result of different efficiencies of silencing. However, the relative reduction in the level of RNA of each silenced gene, as measured by real-time RT-PCR, was similar (85%–95%; data not shown), indicating comparable silencing efficiencies. These results suggest that, while *NPK1* plays a role in the *N* gene-mediated TMV resistance pathway, silencing *NPK1* has less of an effect on the pathway than does silencing *SGT1* or the *N* gene itself.

### *NPK1* Is Involved in the *Bs2* and *Rx* Signaling Pathways

To determine whether *NPK1* and other *MEKK1* homologs play a role in other solanaceous resistance gene pathways, we tested the effect of silencing these sequences on the *Bs2*-, *Rx*-, *Pto*-, and *RPS2*-mediated bacterial HR, as well as on the *Cf4*-mediated fungal HR.

*Bs2* was isolated from pepper and confers resistance to bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria (Xcv)* carrying *avrBs2*. When present as a transgene in *N. benthamiana* (*N. benthamiana::Bs2*), *Bs2* triggers an HR in response to the *AvrBs2* effector protein (Tai et al., 1999). Because *N. benthamiana* does not support the growth of *Xcv*, *avrBs2* was introduced into *N. benthamiana::Bs2* using *Agrobacterium*. *Agrobacterium*::*avrBs2* was infiltrated at concentrations of $OD_{600}$ 0.1, 0.3, and 0.6 onto silenced leaves and assayed for HR at 2 dpi (Figure 2A). HR developed in all three inoculation sites in control plants. No HR was observed on NPK1$^-$ leaves at $OD_{600}$ 0.1 and 0.3, and very weak HR was observed at $OD_{600}$ 0.6. SGT1$^-$ leaves also failed to give an HR at any inoculum level tested. These results demonstrate that *NPK1* and *SGT1* are necessary for the *Bs2*-mediated HR.

In order to test the role of NPK1 in *Rx*-mediated HR to PVX, we silenced *NPK1* and *SGT1* with TRV silencing vectors (Ratcliff et al., 2001). Inoculation of *Agrobacterium* carrying the full-length PVX genome either alone or with *Agrobacterium* carrying the *Rx* gene was performed on silenced leaves (Figure 2B). While expression of PVX alone did not induce HR, coexpression of *Rx* and PVX induced HR in control plants. However, in NPK1$^-$ and SGT1$^-$ plants, very weak or no HR was observed. This suggests that *NPK1* is involved in *Rx*/*PVX*-mediated HR signaling response.

*Pto*/*avrPto*- and *Cf4*/*avrCf4*-mediated HR signaling pathways were also tested on silenced plants by coexpression assays. *Pto* confers resistance to bacteria speck disease caused by *P. syringae* pv. *tomato* (Martin et al., 1993), and *Cf4* confers resistance to *Cladosporium fulvum* fungi (Jones and Jones, 1997). *Agrobacterium* harboring *avrPto* or *avrCf4* were infiltrated into silenced leaves either alone or in combination with *Pto* or *Cf4*. Plants infiltrated with *avr* genes alone did not exhibit an HR; however, both control and NPK1$^-$ plants infiltrated with a mixture of corresponding *avr* and *R* genes developed HR 2 dpi (Figures 2C and 2D). No HR was induced by *Pto*/*avrPto* in SGT1$^-$ or *Prf*-silenced control leaves, and no HR was induced by *Cf4*/*avrCf4* in SGT1$^-$ leaves. These results reveal that *NPK1* is not essential for either *Pto* or *Cf4* HR signaling, while SGT1 is required for both pathways.

The *Arabidopsis RPS2* gene confers resistance to *P. syringae* pv. *tomato* strains expressing the AvrRpt2 effector protein and exhibits an "overdose effect" on overexpression; the overdose effect is thought to mimic the normal activation of RPS2 by AvrRpt2 perception (Tao et al. 2000). Expression of (3 × 35S)-*RPS2-HA* or 35S-*RPS2-HA* in *N. benthamiana* results in an HR-like lesion due to the overdose effect (Figure 2E). The null alleles *rps2-204C-HA* and *rps2-205C-HA* (Axtell et al. 2001) do not cause a response. In addition, expression of 35S-*avrRpt2* also causes an HR in *N. benthamiana*, presumably due to recognition by an unknown *R* gene that may act as an *Rps2* ortholog (Mudgett and Staskawicz, 1999). We find that the silencing of *SGT1*, but not of *NPK1*, abolishes both the *RPS2* overdose response and the recognition of AvrRpt2 (Figure 2E). We tested the ability of *Pseudomonas syringae* pv. *tabaci* (*Pstab*), a pathogen of *N. benthamiana*, to replicate in the leaves of SGT1$^-$ and NPK1$^-$ plants. *Pstab* is able to multiply by six orders of magnitude within 4 days after inoculation. NPK1$^-$, SGT1$^-$, and control plants are equally susceptible to infection with *Pstab*, indicating that the developmental phenotype (see below) associated with NPK1 ablation does not interfere with bacterial pathogenesis in the absence of NBS-LRR recognition (Figure 2F, column 1). A *Pstab* strain rendered avirulent by expression of the recognized effector protein AvrRpt2 proliferates to a level 1000-fold less than does virulent *Pstab* on control and NPK1$^-$ plants (compare Figure 2F columns 1 and 2). In contrast, *Pstab*-avrRpt2 appears fully virulent on SGT1$^-$ plants, indicating that SGT1, but not NPK1, is required for recognition of bacteria expressing *AvrRpt2*. Similarly, we found that SGT1, but not NPK1, is required for nonhost resistance (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/2/291/DC1).

### VIGS of *NPK1* Is Specific and Efficient

We investigated whether attenuation of the *N*, *Bs2*, and *Rx* resistance responses by *NPK1* silencing resulted from silencing the *NPK1* gene alone or from silencing one or more related genes in addition to *NPK1*. Based on Southern blot analysis, *NPK1* is present as a single copy in the *N. benthamiana* genome (data not shown). The most closely related *N. benthamiana NPK1* homolog available is NB93317 (nearly 70% nucleic acid identity). We performed real-time RT-PCR on NPK1$^-$ plants to detect the mRNA levels of both *NPK1* and NB93317. The *NPK1* RNA level was reduced more than 90% 14 days following pGr106::*NPK1*-induced silencing compared to that of control plants and was not induced by TMV-GFP infection (Figure 3A). NB93317 mRNA was not reduced over this time course (Figure 3A). These data suggest that *NPK1* silencing is specific and does not lead to reduction in RNA levels of a closely related *MEKK1* homolog.

Gene silencing generates, and is thought to be mediated by, gene-specific 21–23 nucleotide RNAs (Hamilton

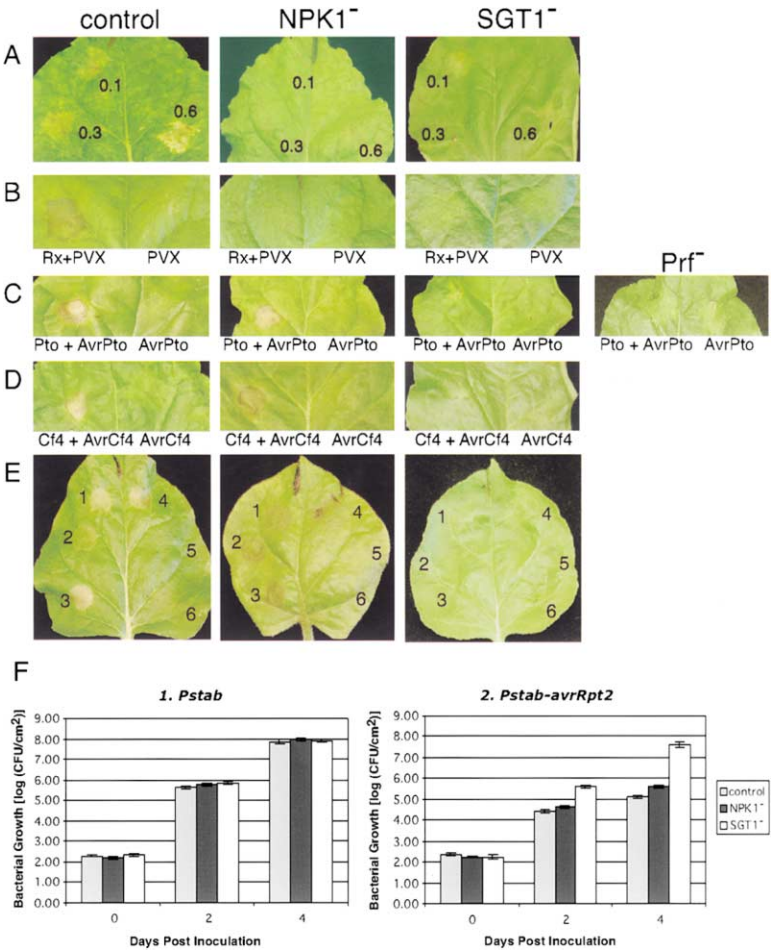


Figure 2. VIGS Inhibition of *R* and *avr* Gene-Induced HR in *N. benthamiana*

(A) *Agrobacterium*::*avrBs2* infiltrated into control, NPK1⁻, and SGT1⁻ *N. benthamiana:Bs2* leaves at concentrations of $OD_{600}$ 0.1, 0.3, and 0.6.

Inoculation control, NPK1⁻, and SGT1⁻ leaves with *Agrobacterium* ($OD_{600}$ 0.4) carrying (B) a mixture of *Rx* and *PVX* or *PVX* alone, (C) a mixture of *Pto* and *avrPto* or *avrPto* alone, and (D) a mixture of *Cf4* and *avrCf4* or *avrCf4* alone.

(E) Inoculation of *Agrobacterium* ($OD_{600}$ 0.5) carrying (1) (3 × 35S)-*RPS2-HA*, (2) 35S-*avrRpt2*, (3) a mixture of 35S-*RPS2-HA* and 35S-*avrRpt2*, (4) 35S-*RPS2-HA*, (5) *rps2-204C-HA*, and (6) *rps2-205C-HA*. All photographs were taken 3–4 dpi.

(F) Growth of bacteria after infiltration into control, NPK1⁻, and SGT1⁻ leaves. (1) *Pstab* (harboring empty vector) and (2) *Pstab-avrRpt2*.

and Baulcombe, 1999). To further verify that *NPK1* was silenced specifically, we assayed for gene-specific 21–23 oligonucleotides in NPK1⁻ and NB93317-silenced (NB93317⁻) plants (Johansen and Carrington, 2001). Twenty-one to 23 nt RNA hybridizing to *NPK1* was detected only in NPK1⁻ plants, but not in NB93317⁻ plants (Figure 3B). Conversely, the NB93317 probe detected 21–23 nt RNA only in NB93317⁻ plants (Figure 3B). In conjunction with our finding of specific reduction of *NPK1* RNA, the appearance of NPK1⁻-specific 21–23 nt RNA leads us to conclude that the attenuation of *N*, *Bs2*, and *Rx* resistance in NPK1⁻ plants is most likely the result of specifically silencing *NPK1*.

### NPK1⁻ Plants Are Defective in Growth and Development

NPK1⁻ plants are dramatically dwarfed in comparison to empty vector-treated control plants (Figure 4A) or plants silenced for other *MEKK1* plant homologs (data not shown). DAPI staining of epidermal cells revealed that many stomata guard cells from NPK1⁻ plants were binucleate or had no nucleus, and cytokinesis was incomplete (Figure 4B). These results provide corroborative evidence that NPK1 plays an important role in cytokinesis. Scanning electron microscopy (SEM) of longitudinal stem sections showed that cell elongation was suppressed in NPK1⁻ plants. The average length of NPK1⁻ stem cells was less than half the length of cells from control plants (Figures 4C and 4D). Leaf palisade cells from NPK1⁻ plants were approximately 80% of the length of control cells (Figures 4C and 4D). These results suggest a function of *NPK1* in normal cell growth and expansion.

## Discussion

We have demonstrated that suppression of *NPK1,* a plant MAPKKK gene, interferes with plant disease resistance and development. VIGS of *NPK1* reveals its functions in *N* gene-mediated TMV resistance, *Rx*- and *Bs2*-mediated HR signaling, and its role in cell growth and expansion.

While we identified *NPK1* independently, on the basis of strong kinase homology to the animal innate immune regulatory MAPKKK, *MEKK1*, previous work has shown that *NPK1* is involved in signaling in response to three other distinct stimuli. A constitutively active form of *NPK1* was able to suppress auxin-induced transcription in protoplasts (Kovtun et al., 1998); the *Arabidopsis* homolog of *NPK1*, *ANP1*, is required for oxidative-stress-induced transcription in protoplasts (Kovtun et al., 2000); NPK1 is necessary for cell plate formation (Nishihama et al., 2001, 2002). Defects in cell plate formation could contribute to the observed dwarf phenotype of NPK1⁻

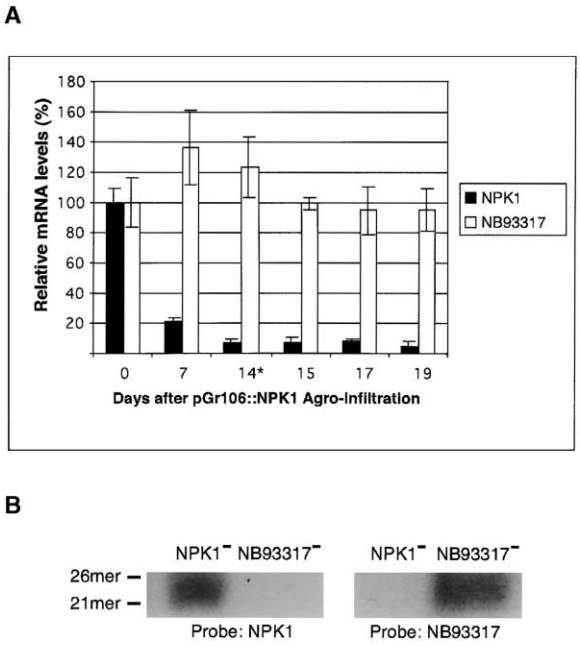


Figure 3. VIGS of *NPK1* Is Specific and Efficient

(A) Relative levels of endogenous *NPK1* and NB93317 RNAs at different time points after introduction of pGr106::*NPK1* silencing vector or an empty vector control. The asterisk indicates that the plants were subjected to TMV-GFP inoculation at 14 dpi of silencing.

(B) Northern blot analysis of LMW RNA extracted from NPK1$^-$ and NB93317$^-$ plants. The *NPK1* and NB93317 fragments in the silencing constructs were used as probes.

plants; however, defects in cytokinesis are predicted to generate multinucleate giant cells instead of the small cells we observed in NPK1$^-$ stem and leaf cells. The dwarf phenotype of NPK1$^-$ plants may result from defects in cell elongation, expansion, and division. This demonstrates a possible role for NPK1 in cell elongation and expansion in addition to its role in cytokinesis. Attempts to generate transgenic *NPK1*-silenced plants using duplex RNAi have not been successful; the failure to recover NPK1$^-$ transformants is an indication that *NPK1* is essential for plant development, and its absence results in lethality. Thus, NPK1 appears to be a "signal integrator" for many different biological "inputs", acting as a central regulator in plant signaling networks controlling plant defense, stress responses, hormone signaling, and cellular development.

In TMV resistance, *NPK1* silencing appears to have less effect than silencing *SGT1* or *N*. One interpretation of this observation is that N may initiate multiple signaling pathways, not all of which require NPK1, or that N may be able to utilize an alternate MAPKKK in the absence of NPK1. As a result, suppression of *NPK1* may leave other pathways intact, resulting in only partial loss of resistance. Another possibility is that the malformed cells that result from the reduction of *NPK1* could render NPK1$^-$ plants more susceptible to TMV-GFP and incompetent to induce the *Bs2/avrBs2*- and *Rx/PVX*-mediated HR. However, the specificity with which *NPK1* functions in pathogen-resistance signaling (affecting N, Rx, and Bs2, but not Pto, Cf4, the *RPS2* overdose response, or the *AvrRpt2* HR) argues against this interpretation. In addition, virulent bacterial pathogens are equally capable of replication in NPK1$^-$ tissue, as in control tissue, demonstrating that the altered cellular morphology does not affect pathogenesis. This specificity, in fact, suggests that NPK1 functions in either a unique pathway or in only a subset of *R* gene signaling cascades. *R* gene specificity could potentially be explained by structural differences between the *R* genes tested. The three genes affected by *NPK1*, *N*, *Rx*, and *Bs2*, are all predicted cytosolic NBS-LRR proteins without transmembrane domains; the Cf4 and Pto proteins, which do not require NPK1, are structurally distinct from the NBS-LRR class of disease-resistance proteins. However, we also found that the NBS-LRR protein RPS2 does not

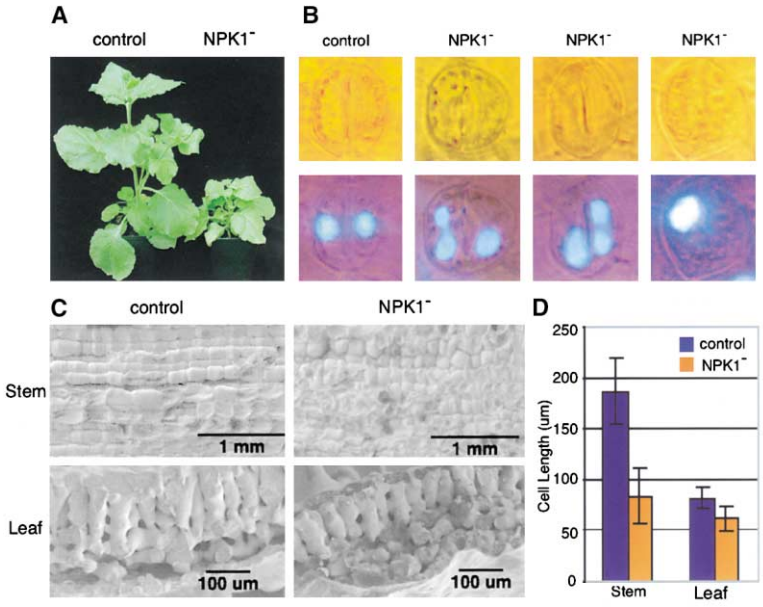


Figure 4. Developmental Defects in NPK1$^-$ Plants

(A) Whole plant phenotype of 3-week-old NPK1$^-$ and control *N. benthamiana* plants.

(B) DAPI staining of epidermal cells from young leaves of control and NPK1$^-$ plants observed in bright field (upper row) and dark field (lower row).

(C) SEM of longitudinal stem sections and freeze-fracture leaf sections from NPK1$^-$ and control plants.

(D) Cell size comparisons of stem and leaf cells between NPK1$^-$ and control plants.

require NPK1, at least, not for the overdose effect, suggesting that not all NBS-LRR proteins require NPK1 function. Determining the mechanistic basis for NPK1 requirement by *R* genes will be a goal for future research.

If *NPK1* functions through an MAPK cascade, its downstream MAPKK and MAPK have yet to be conclusively identified. NQK1, an MAPKK, coimmunoprecipitates with NPK1 from yeast cells and is phosphorylated by NPK1 in vitro (Nishihama et al., 2001). Yet silencing of *NQK1* neither induces dwarfing nor attenuates the *R* gene pathways tested (data not shown). *Arabidopsis ANP1* can be activated by oxidative stress to initiate an MAPK cascade involving two downstream MAPKs, *AtMPK3* and *AtMPK6* (Kovtun et al., 2000). Their tobacco orthologs, *SIPK* and *WIPK*, have been implicated in plant defense responses against TMV infection and other elicitors (Zhang and Klessig, 1998; Zhang et al., 1998). We found that the silencing of *SIPK, WIPK*, and *NtMEK2* (their upstream MAPKK) can attenuate *N*-mediated TMV resistance without affecting *Bs2*-, *Pto*-, or *Cf4*-mediated HR or plant morphology (data not shown). An in-gel kinase assay demonstrated that a constitutively active NPK1 (D*NPK1,* NPK1 with a deletion of the C-terminal regulatory domain [Kovtun et al., 1998]) appeared to only marginally activate SIPK and WIPK while transiently expressed in tobacco leaves (Yang et al., 2001); HA-tagged D*NPK1* also failed to phosphorylate NtMEK2 (data not shown). These data suggest that *NPK1* may not act via the NtMEK2-SIPK/WIPK cascade; however, it is possible that the regulatory domain deletion of *NPK1* perturbed interaction with NtMEK2 and subsequent activation of SIPK and WIPK. It therefore remains to be determined whether *NPK1* functions via a yet unverified MAPK cascade or by a non-MAPK pathway.

In this study we establish a role for NPK1 in the plant defense response and demonstrate that, as in barley and *Arabidopsis*, *N. benthamiana SGT1* is required for *R* gene function. Like its animal homolog MEKK1, NPK1 appears to be a regulatory gene with diverse functions. The pleiotropic developmental defects of NPK1$^-$ plants indicates the complexity of the NPK1 role in cellular signaling; the striking dwarf phenotype and abnormal cell morphology provide convincing evidence that NPK1 functions outside the realm of defense response. To date, *NPK1* is one of the best examples of a plant regulatory gene with roles in both defense and development.

## Experimental Procedures

### Plant Materials and Growth Conditions

Transgenic *N. benthamiana* plants used for gene silencing were grown at 24°C in a growth cabinet under a 16 hr light/8 hr dark cycle. *N. benthamiana::N* was transformed with the *N. glutinosa* genomic *N* clone as described previously (Whitham et al., 1994, 1996). *N. benthamiana::Bs2* was transformed with the *C. annum Bs2* genomic clone (Tai et al., 1999; B.S., unpublished data).

### Recombinant PVX and TRV Constructs for Virus-Induced Gene Silencing

The PVX binary vector pGr106 (Takken et al., 2000) and the TRV binary vectors pBINTRA6 and TRV RNA2 (Ratcliff et al., 2001) were used for silencing. DNA fragments used for silencing were amplified from *N. benthamiana* cDNA by RT-PCR. Primers for *NPK1*: 5′-AAA ACTCGAGGGTTGGAATACTTGCATAAGAATG-3′ and 5′-AAAATCGA TCATCAGAGTTTCCTAGTTTCCAG3-′; primers for *NbSGT1*: 5′-AAA ACTCGAGTTGGCCTGTATGAAGCTTGAAG-3′ and 5′-AAAATCGAT CCCATTAGATTCCACAAAGGAT-3′; primers for TC93317: 5′-AAAAC TCGAGGTGGTTTCAGATGATCAAACATC-3′ and 5′-AAAATCGATA CTGGCTCCAAAGGTGGCTTTG-3′; primers used to amplify an N gene fragment from N18C cDNA plasmid (Whitham et al., 1996): 5′-AAAACTCGAGTCCATCACATGTTCGGAACCA-3′ and 5′-AAAATCG ATCATTCTTCTCTATCAAATGCTTGTC-3′.

### *Agrobacterium*-Mediated Transient HR Assays

*Agrobacterium* strain GV2260 carrying the TMV effector gene (*p50*) in a binary plasmid (Erickson et al., 1999) was infiltrated into NPK1$^-$, SGT1$^-$, N$^-$, and control leaves of *N. benthamiana::N*. *Agrobacterium* carrying the *avrBs2* binary plasmid were infiltrated into *N. benthamiana::Bs2* NPK1$^-$ and SGT1$^-$ leaves (Tai et al., 1999). An *Agrobacterium*-mediated transient coexpression assay was performed for *Cf4*/*avrCf4*, wherein equal amounts of *Agrobacterium* carrying *Cf4* and *avrCf4* were mixed before inoculation (Van der Hoorn et al., 2000). The same protocol was used for *Pto* and *avrPto* coinoculations, *Rx* and *PVX* coinoculations, and *RPS2* and *avrRpt2* inoculations. These experiments were repeated three times, with a total of 20–24 plants for each silenced gene.

### Bacterial Growth Assays

The bacterial strain *Pseudomonas syringae* pv. *tabaci* carrying either empty plasmid pVSP61 or pVSP61 (*avrRpt2*) was used. Silenced leaves were infiltrated with a $5 \times 10^4$ CFU/ml bacterial suspension. Bacterial growth was monitored at 0, 2, and 4 dpi. The experiments were repeated at least three times.

### Real-Time RT-PCR

An Oligo $dT_{17}$ adaptor was used for mRNA reverse transcription, and a primer (5′-ACGCCATACCACAGTATACACCA-3′) annealed to the 3′ end of TMV CP coding region was used to amplify TMV-GFP RNA. Real-time PCR was performed with ABI PRISM 5700 using SYBR Green I (ABI). Primers were designed with Primer Expression 1.0 (ABI) to anneal to sequences in regions outside the region targeted for silencing to ensure that only the endogenous gene transcripts would be amplified. Primers for *NPK1* (5′-TGAATG TTTCCAGCACACCG-3′ and 5′-GCATACGTTTTGGTGACCTGC-3′), NB93317 (5′-CGCCGATGAACGGTTAAGTTT-3′ and 5′-TGCGCCT GCAGTTTTTGTG-3′), and GFP (5′-TCCATGGCCAACACTTGTCA-3′ and 5′-GGCATGGCACTCTTGAAAAAG-3′) were used to detect target gene transcripts. The reference ubiquitin primers were 5′-CGG CATGCTTAACACATGCA-3′ and 5′-AGCCGTTTCCAGCTGTTGTTC-3′. The comparative Ct method was applied (ABI User Bulletin #2). All the experiments were repeated at least three times.

### Scanning Electron Microscopy (SEM) and DAPI Assay

Fresh tissue was examined using an SEM (S-4700; Hitachi) with a cryostage. A Zeiss fluorescence microscope was used for examining DAPI-stained epidermal cells.

### Acknowledgments

We thank Jennifer Fletcher for valuable advice and critical reading of the manuscript, David Baulcombe for the PVX and the TRV vectors, Jen Sheen for the constitutively active *NPK1* plasmids and helpful discussions, Pierre De Wit for pCf4 and pAvrCf4 constructs, and Vanessa Handley and Miki Yamamoto for critical reading of the manuscript. This work was supported by USDA CRIS 5335-22000-005-00D and USDA-ARS/Syngenta CRADA 58-3K95-0-829.

Received: January 18, 2002
Revised: May 22, 2002

### References

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature *415*, 977–983.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D.G., and Parker, J.E. (2002). Regulatory role of SGT1 in early *R* gene-mediated plant defenses. Science *295*, 2077–2080.

Axtell, M.J., McNellis, T.W., Mudgett, M.B., Hsu, C.S., and Staskawicz, B.J. (2001). Mutational analysis of the Arabidopsis RPS2 disease resistance gene and the corresponding *Pseudomonas syringae avrRpt2* avirulence gene. Mol. Plant Microbe Interact. *14*, 181–188.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002). The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science *295*, 2073–2076.

Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S.P. (1997). Signaling in plant-microbe interactions. Science *276*, 726–733.

Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E., and Staskawicz, B.J. (1997). NDR1, a pathogen-induced component required for Arabidopsis disease resistance. Science *278*, 1963–1965.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826–833.

Erickson, F.L., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C., and Baker, B. (1999). The helicase domain of the TMV replicase proteins induces the N-mediated defence response in tobacco. Plant J. *18*, 67–75.

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

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950–952.

Johansen, L.K., and Carrington, J.C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. Plant Physiol. *126*, 930–938.

Jones, D.A., and Jones, J.D.G. (1997). The role of leucine-rich repeat proteins in plant defenses. Adv. Bot. Res. *24*, 89–167.

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

Kovtun, Y., Chiu, W.L., Zeng, W., and Sheen, J. (1998). Suppression of auxin signal transduction by a MAPK cascade in higher plants. Nature *395*, 716–720.

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

Lee, F.S., Hagler, J., Chen, Z.J., and Maniatis, T. (1997). Activation of the IκB α kinase complex by MEKK1, a kinase of the JNK pathway. Cell *88*, 213–222.

Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science *262*, 1432–1436.

Mudgett, M.B., and Staskawicz, B.J. (1999). Characterization of the Pseudomonas syringae pv. tomato AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. Mol. Microbiol. *32*, 927–941.

Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T., and Machida, Y. (2001). The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. Genes Dev. *15*, 352–363.

Nishihama, R., Soyano, T., Ishikawa, M., Araki, S., Tanaka, H., Asada, T., Irie, K., Ito, M., Terada, M., Banno, H., et al. (2002). Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. Cell *109*, 87–99.

Ratcliff, F., Martin-Hernandez, A.M., and Baulcombe, D. (2001). Technical Advance. Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J. *25*, 237–245.

Ren, D., Yang, H., and Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. J. Biol. Chem. *277*, 559–565.

Romeis, T., Ludwig, A., Martin, R., and Jones, J.D.G. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. *20*, 5556–5567.

Schlesinger, T.K., Fanger, G.R., Yujiri, T., and Johnson, G.L. (1998). The TAO of MEKK. Front. Biosci. *3*, d1181–d1186.

Shirasu, K., Lahaye, T., Tan, M.W., Zhou, F., Azevedo, C., and Schulze-Lefert, P. (1999). A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell *99*, 355–366.

Tai, T.H., Dahlbeck, D., Clark, E.T., Gajiwala, P., Pasion, R., Whalen, M.C., Stall, R.E., and Staskawicz, B.J. (1999). Expression of the Bs2 pepper gene confers resistance to bacterial spot disease in tomato. Proc. Natl. Acad. Sci. USA *96*, 14153–14158.

Tao, Y., Yuan, F., Leister, R.T., Ausubel, F.M., and Katagiri, F.M. (2000). Mutational analysis of the Arabidopsis nucleotide binding site-leucine-rich repeat resistance gene *RPS2*. Plant Cell *12*, 2541–2554.

Takken, F.L., Luderer, R., Gabriels, S.H., Westerink, N., Lu, R., de Wit, P.J., and Joosten, M.H. (2000). A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. Plant J. *24*, 275–283.

Tena, G., Asai, T., Chiu, W., and Sheen, J. (2001). Plant mitogen-activated protein kinase signaling cascades. Curr. Opin. Plant Biol. *4*, 392–400.

Van der Hoorn, R.A.L., Laurent, F., Roth, R., and De Wit, P.J.G.M. (2000). Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis. Mol. Plant Microbe Interact. *13*, 439–446.

Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). The product of the tobacco mosaic virus resistance gene N: similarity to Toll and the interleukin-1 receptor. Cell *78*, 1101–1115.

Whitham, S., McCormick, S., and Baker, B. (1996). The N gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. Proc. Natl. Acad. Sci. USA *93*, 8776–8781.

Widmann, C., Gerwins, P., Johnson, N.L., Jarpe, M.B., and Johnson, G.L. (1998). MEK kinase *1*, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. Mol. Cell. Biol. *18*, 2416–2429.

Yang, K.Y., Liu, Y., and Zhang, S. (2001). Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. Proc. Natl. Acad. Sci. USA *98*, 741–746.

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

Zhang, S., Du, H., and Klessig, D.F. (1998). Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora spp*. Plant Cell *10*, 435–449.