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

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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 Cf-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 EDST 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-1 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 NFκB 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 pathways are activated by hormones (Kieber et al., 1993), biotic 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 (Ratcliffe et al., 2001) silencing systems have been employed to ascertain plant gene function.
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 Plt- 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
Transient 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; Azavedo 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 with 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.

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 OD600 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.
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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 OD600 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 OD600 0.1 and 0.3, and very weak HR was observed at OD600 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). 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 (Pstav), a pathogen of N. benthamiana, to replicate in the leaves of SGT1− and NPK1− plants. Pstav 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 Pstav, 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 Pstav strain rendered avirulent by expression of the recognized effector protein AvrRpt2 proliferates to a level 1000-fold less than does virulent Pstav on control and NPK1− plants (compare Figure 2F columns 1 and 2). In contrast, Pstav-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
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−

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**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 OD₆₀₀ 0.1, 0.3, and 0.6. Inoculation control, NPK1−, and SGT1− leaves with Agrobacterium (OD₆₀₀ 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 (OD₆₀₀ 0.5) carrying (1) 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.
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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− transplants 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. NOQ1, an MAPKK, communoprecipitates with NPK1 from yeast cells and is phosphorylated by NPK1 in vitro (Nishihama et al., 2001). Yet silencing of NOQ1 neither induces dwarving 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 (DNPK1, 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 DNPK1 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 indicate 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 pBiNTRAr6 and TRV RNA2 (Ratcliffe et al., 2001) were used. DNA fragments used for silencing were amplified from N. benthamiana cDNA by RT-PCR. Primers for NPK1: 5’-AAAA ACTCGAGGCTGGAAGATCCATGAAAGTGC3’ and 5’-AAATCGACTGAGTGGCTGTAAGGTGAAG-3’; primers for NbSGT1: 5’-AAA ACTCGAGGCTGGAAGATCCATGAAAGTGC3’ and 5’-AAATCGACTGAGTGGCTGTAAGGTGAAG-3’; primers for TC83317: 5’-AAAAC TCGAGGCTGGAAGATCCATGAAAGTGC3’ and 5’-AAATCGACTGAGTGGCTGTAAGGTGAAG-3’; primers used to amplify an N gene fragment from N18C cDNA plasmid (Witham et al., 1996): 5’- AAAACTCGAGTCCATGACATGTTGGAACCA-3’ and 5’-AAATCGACTGAGTGGCTGTAAGGTGAAG-3’.

Agrobacterium-Mediated Transient HR Assays

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 × 10⁵ 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 dT₅ adaptor was used for mRNA reverse transcription, and a primer (5’-ACGCCATACACAGCATACACCA-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’-TTTCCAGCACACCG-3’ and 5’-CGTACGTTTGGTGACCCG-3’), NB93317 (5’-CAGCCGATGAAAGGTTAAGGT-3’ and 5’-TGCGCT CGATACGTTTTTGAGAT-3’), and GFP (5’-GGCGCT CGATACGTTTTTGAGAT-3’ and 5’-GGCGCT CGATACGTTTTTGAGAT-3’) were used to detect target gene transcripts. The reference ubiquitin primers were 5’-GGCATGTTAAACACATGCA-3’ and 5’-GGGCTTGACCTGTTGTC-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.

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