The *Nicotiana benthamiana* Mitogen-Activated Protein Kinase Cascade and WRKY Transcription Factor Participate in Nep1_{Mo}-Triggered Plant Responses

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Many bacterial, fungal, and oomycete species secrete necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLP) that trigger programmed cell death (PCD) and innate immune responses in dicotyledonous plants. However, how NLP induce such immune responses is not understood. Here, we show that silencing of the MAPKKK α -MEK2-WIPK mitogen-activated protein kinase (MAPK) cascade through virus-induced gene silencing compromises hydrogen peroxide accumulation and PCD induced by Nep1_{Mo} from *Magnaporthe oryzae*. WIPK interacts with NbWRKY2, a transcription factor in *Nicotiana benthamiana*, in vitro and in vivo, suggesting an effector pathway that mediates Nep1_{Mo}-induced cell death. Unexpectedly, salicylic acid-induced protein kinase (SIPK)- and NbWRKY2-silenced plants showed impaired Nep1_{Mo}-induced stomatal closure, decreased Nep1_{Mo}-promoted nitric oxide (NO) production in guard cells, and a reduction in Nep1_{Mo}-induced resistance against *Phytophthora nicotianae*. Expression studies by real-time polymerase chain reaction suggested that the MEK2-WIPK-NbWRKY2 pathway regulated Nep1_{Mo}-triggered NO accumulation could be partly dependent on nitrate reductase, which was implicated in NO synthesis. Taken together, these studies demonstrate that the MAPK cascade is involved in Nep1_{Mo}-triggered plant responses and MAPK signaling associated with PCD exhibits shared and distinct components with that for stomatal closure.

Plants have evolved multiple defense mechanisms against phytopathogens, including the hypersensitive response (HR) and stomatal closure (Dangl et al. 1996; Lam et al. 2001; Melotto et al. 2006; Mur et al. 2008). HR is considered a form of localized programmed cell death (PCD) that results in the formation of necrotic lesions around infection sites (Greenberg and Yao 2004; Hammond-Kosack and Jones 1996). The PCD program is initiated upon plant-pathogen recognition. In addition to classic gene-for-gene interactions, bindings of certain selected pathogen-associated molecular patterns (PAMPs) (also called microbe- or danger-associated molecular patterns) and nonhost-

specific elicitors to their receptors can trigger HR (Nürnberger et al. 2004; Schwessinger and Zipfel 2008; Zhang et al. 1998). Plants recognize invading microbes by sensing PAMPs and activate downstream signaling cascades, leading to PAMP-triggered immunity (PTI) (Schwessinger and Zipfel 2008).

Necrosis and ethylene-inducing peptide 1 (Nep1) is secreted by taxonomically diverse organisms, including bacteria, fungi, and oomycetes (Cechin et al. 2008). NLP (Nep1-like protein) from different pathogens play distinct roles in pathogen virulence. In some cases, microbial NLP are considered as positive virulence factors accelerating disease and pathogen growth in host plants through disintegration. The virulence could be increased by transformation of *Colletotrichum coccodes* with *Fusarium oxysporum* and the spectrum of hosts could be infected (Amsellem et al. 2002). Also, NLP-deficient mutants of *Erwinia carotovora* were reduced in virulence on both rubbers and stems of potato (Pemberton et al. 2005). Transformation of the NLP-deficient *Pectobacterium carotovorum* subsp. *carotovorum* strain with NLP_{pya}- or NLP_{pp}-encoding sequences showed partial restoration (30 to 40%) in bacterial virulence and potato tuber maceration (Ottman et al. 2009). However, the deletion of NLP in fungal pathogens *Botrytis elliptica*, *Mycosphaerella graminicola*, and *Verticillium dahliae* did not attenuate virulence, suggesting that NLP are not virulence determinants in these plant pathogen interactions (Motteram et al. 2009; Staats et al. 2007; Zhou et al. 2012).

In many different dicotyledonous plants, the recognition of Nep1 results in cell death (Gijzen and Nürnberger 2006; Pemberton and Salmond 2004; Schwessinger and Zipfel 2008). It is suggested that NLP may associate with the outer surface of the plasma membrane to trigger HR (Qutob et al. 2006; Schouten et al. 2008), and global gene expression analyses also revealed an overlap between the responses to NLP and other elicitors leading to ethylene and active oxygen production (Bae et al. 2006; Garcia-Brugger et al. 2006). The mitogen-activated protein kinase (MAPK) cascade is known to play important roles in mediating cellular membrane stress and cell wall-integrity pathways and, therefore, could be important in studying NLP-induced plant immune responses.

The MAPK cascades are major pathways that transduce and amplify extracellular and intracellular stimuli to a wide range of overlapping or specific intracellular responses in eukaryotic cells (Chen and Thorner 2007). The MAPK pathway contains a functionally three-tiered kinase cascade comprising a MAPK kinase kinase (MAPKKK, also called MAP3K, MEKK, or MKKK), a MAPK kinase (MAPKK, also called MAP2K, MEK, or MKK), and a MAPK. Plant MAPK cascades have

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been implicated in diverse biological processes, including cell division, hormone signaling, cell-wall biosynthesis, cell growth and differentiation, abiotic stress, and biotic stress (Andreasson et al. 2005; Ludwig et al. 2005; Takahashi et al. 2004; Teige et al. 2004). Recent studies demonstrated that MAPK are rapidly activated in different eukaryotic pathogens and pathogen-derived elicitors or PAMP-triggered plant defense (Asai et al. 2008; del Pozo et al. 2004; Liu et al. 2008; Nakagami et al. 2005; Oh et al. 2009; Pedley and Martin 2005; Ren et al. 2006; Tena et al. 2001; Zhang and Klessig 2001).

Increasing evidence has also revealed that MAPK play key roles in various inducer-triggered hypersensitive cell deaths. Two key MAPK isolated from tobacco (i.e., wound-induced protein kinase [WIPK] and salicylic acid-induced protein kinase [SIPK]) are associated with elicitors and *N*- and *Cf9*-mediated defense (Jin et al. 2003; Romeis et al. 1999; Zhang and Klessig 1998; Zhang et al. 2000). In the absence of pathogens, expression of NtMEK2^{DD}, a constitutively active mutant of MEK2, triggers HR-like cell death, defense gene expression, and active oxygen species (AOS) generation, all of which are preceded by activation of endogenous WIPK and SIPK (Jin et al. 2003). Similarly, heterologous expression of potato (*Solanum tuberosum*) StMEK2^{DD} in *Nicotiana benthamiana* leads to the activation of WIPK and SIPK (Katou et al. 2003) and generation of AOS (Yoshioka et al. 2003). Potential MAPKKK upstream of NtMEK2–SIPK/Nt4/WIPK include orthologs of *Arabidopsis* MEKK1 and tobacco MAPKKK α (Asai et al. 2002; del Pozo et al. 2004). Silencing of components in the MAPKKK α –MEK2–SIPK cascade prevents AvrPto and Pto-mediated hypersensitive cell death (HCD), and MAPKKK α overexpression-triggered HR-like cell death is attenuated not only by silencing MEK2 or SIPK, but also by silencing MEK1 or NTF6, providing loss-of-function evidence that this MAPK pathway regulates the HR cell-death process (del Pozo et al. 2004; Oh et al. 2009). Consistent with WIPK and SIPK, silencing of *NPK1*, *MEK1*, or *NTF6* attenuates *N*- and *Pto*-mediated resistance to *Tobacco mosaic virus* (Jin et al. 2002, 2003; Liu et al. 2003; del Pozo et al. 2004) and *Pseudomonas syringae*, respectively (Ekengren et al. 2003). Treatment of *N. benthamiana* with elicitor INF1 (derived from *Phytophthora infestans*) activates the MEK2–SIPK/NTF4 cascade to control the NOA1-mediated nitric oxide (NO) burst, and MAPK cascades (MEK2–SIPK/NTF4 and MEK1–NTF6) regulate the NADPH oxidase-dependent oxidative burst by gain-of-function and loss-of-function analyses, respectively (Asai et al. 2008). Additionally, several WRKY transcription factors have been shown to be phosphorylated by MAPK and to be involved in plant immunity (Eulgem and Somssich 2007; Kim and Zhang 2004; Menke et al. 2005; Popescu et al. 2009).

We previously demonstrated the involvement of respiratory burst oxidase homolog (RBOH), vacuolar processing enzyme (VPE), and G proteins in elicitor-triggered plant immunity by *Potato virus X* (PVX)-based virus-induced gene silencing (VIGS) (Zhang et al. 2009, 2010, 2012). In the present study, we characterized the roles of the MAPK cascade and WRKY transcription factor in the regulation of Nep1_{M0}-induced responses using VIGS. This study will expand our understanding of the signaling networks involved in plant responses to Nep1_{M0} and other elicitors.

RESULTS

Silencing of MAPKKK α , MEK2, and WIPK compromises the Nep1_{M0}-triggered HR.

The use of PVX for VIGS in *N. benthamiana* has been demonstrated previously in studies of RBOH, VPE, and G proteins (Zhang et al. 2009, 2010, 2012). Previous studies sug-

gested that there are MAPKKK α –MEK2–SIPK/WIPK and MAPKKK α –MEK1–NTF6 pathways in *N. benthamiana* (Ekengren et al. 2003; del Pozo et al. 2004; Pedley and Martin 2004). Complementary DNA fragments corresponding to MAPKKK α , MEK1, MEK2, WIPK, SIPK, and NTF6 were isolated from *N. benthamiana* and were cloned into PVX binary vector pGR107 (PVX-MAPKKK α , PVX-MEK1, PVX-MEK2, PVX-WIPK, PVX-SIPK, or PVX-NTF6) and were then introduced into *N. benthamiana* by *Agrobacterium* infiltration (Ekendren et al. 2003; del Pozo et al. 2004; Liu et al. 2004). Three to four weeks after PVX infection, RNA was isolated from leaf tissue collected from the upper part of each of the silenced plants. For each gene-silenced and PVX-only infected plant, the relative abundance of transcripts for the targeted gene was determined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 1A). As expected, the transcript of each gene tested decreased markedly in the silenced plants compared with the control plants infected with the PVX vector alone. Moreover, both real-time PCR and Western immunoblot analysis confirmed that silencing of MEK1, MEK2, WIPK, SIPK, and NTF6 was specific with no co-silencing occurring (Supplementary Fig. S1).

We next investigated the possible involvement of each of the signaling genes in Nep1_{M0}-mediated cell death. Three to four weeks after viral infection, the silenced leaves verified by RT-PCR were inoculated with Nep1_{M0} solution or phosphate-buffered saline (PBS) buffer as a control, and the reactions of different plants to Nep1_{M0} were compared (Supplementary Fig. S2). Infiltration of the Nep1_{M0} solution into PVX control-plant leaves resulted in strong necrotic effects, as it did in the MEK1-, SIPK-, and NTF6-silenced plants. Infiltration of MAPKKK α -, MEK2-, and WIPK-silenced plants failed to lead to a reaction, even 72 h after the onset of elicitation (Fig. 1B). No necrotic effects were observed with the PBS treatment. The Nep1_{M0} protein with a mutated heptameric peptide motif displayed no necrosis-inducing activity and did not elicit defense responses and was no longer used in follow-up assays. These results indicated that the MAPKKK α –MEK2–WIPK cascade participates in signaling that leads to cell death induced by Nep1_{M0}.

MAPKKK α –MEK2–WIPK cascade regulates Nep1_{M0}-induced H₂O₂ accumulation.

Studies focused on MAPK and reactive oxygen species (ROS) signaling in plants (Pitzschke and Hirt 2006) have suggested that cell death mediated by MAPK is associated with hydrogen peroxide (H₂O₂) production (Ren et al. 2002). To test whether Nep1_{M0}-triggered H₂O₂ accumulation is dependent on MAPK, we examined H₂O₂ production by diaminobenzidine (DAB) staining. As shown in Figure 2A, the reddish brown-colored precipitants of oxidized DAB, an indication of H₂O₂ production, were visible in leaves from MEK1- and SIPK-silenced and control plants treated with Nep1_{M0}. Light staining was observed in MAPKKK α -, MEK2-, WIPK-, and NTF6-silenced leaves after Nep1_{M0} treatment (Fig. 2). As with the controls, Nep1_{M0} mutant failed to trigger H₂O₂ generation, and no H₂O₂ generation was detected in silenced or control plants treated with PBS. These results suggested that the MAPKKK α –MEK2–WIPK cascade might include conserved signaling components that regulate Nep1_{M0}-activated H₂O₂ accumulation and contribute to Nep1_{M0}-triggered cell death.

Silencing of SIPK impairs Nep1_{M0}-activated stomatal closure.

Stomata play an active role in the innate immune system (Melotto et al. 2006, 2008). It has been reported that elicitors triggered RBOH-, VPE-, and G protein-dependent stomatal closure in *N. benthamiana* (Zhang et al. 2009, 2010, 2012), but

whether MAPK contribute to elicitor-induced stomatal closure remained unclear. *Nep1_{Mo}* induced stomatal closure in *MAPKKKα*-, *MEK1*-, *MEK2*-, *WIPK*-, and *NTF6*-silenced plant leaves incubated with *Nep1_{Mo}* solution showed stomatal closure in comparison with controls. However, *SIPK*-silenced leaves responded weakly to this treatment, and *Nep1_{Mo}* mutant failed to induce stomatal closure. Consequently, *Nep1_{Mo}*-induced stomatal aperture analyses were performed with all the gene-silenced plants, and *SIPK*-silenced plants showed a markedly reduced response to *Nep1_{Mo}* (Fig. 3). Compared with control plants, none of the gene-silenced plants showed detectable differences in stomatal phenotype after treatment with PBS. This is an indication that *SIPK* silencing compromises *Nep1_{Mo}*-induced stomatal closure and that not all MAPK are involved in stomatal closure.

MEK2, WIPK, and SIPK are required for NO burst induced by *Nep1_{Mo}*.

MEK2–SIPK has been reported to mediate NO burst induced by INF1 (Asai et al. 2008; Yamamoto et al. 2004) and NO is known as a key signaling molecule of abscissic acid (ABA)-induced stomatal closure (Bright et al. 2006; Garcia-Mata and Lamattina 2002; Neill et al. 2002). In our previous study, cPTIO (a NO scavenger) restricted the *Nep1_{Mo}*-induced stomatal closure, demonstrating that NO is an essential factor (Zhang et al. 2012). To investigate whether MAPK regulate NO burst, we compared NO generation in guard cells isolated from control, *MAPKKKα*-, *MEK1*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NTF6*-silenced *N. benthamiana* plants 3 h after *Nep1_{Mo}*-treatment. The NO generation induced by *Nep1_{Mo}* was slightly affected in *MAPKKKα*-, *MEK1*-, and *NTF6*-silenced plants compared with that in PVX control plants (Fig. 4A). In contrast, silencing of *MEK2*, *WIPK*, and *SIPK* markedly reduced NO genera-

tion to near control levels (Fig. 4). *Nep1_{Mo}* mutant failed to induce NO accumulation. These results strongly implicated the involvement of MEK2–WIPK/SIPK in the *Nep1_{Mo}*-induced NO burst in the guard cells.

Silencing of *MAPKKKα*, *MEK1*, *MEK2*, *WIPK*, *SIPK*, and *NTF6* does not affect *Nep1_{Mo}* activation of Ca^{2+} generation in guard cells.

Since elicitor-treatment elevates $[Ca^{2+}]_{cyt}$ profile (Garcia-Brugger et al. 2006; Tavernier et al. 1995), we examined whether $[Ca^{2+}]_{cyt}$ is also regulated by MAPK. The $[Ca^{2+}]_{cyt}$ in the guard cells was monitored by cell-permeable Fura-2/AM. *Nep1_{Mo}* induced a marked rise in $[Ca^{2+}]_{cyt}$ in the guard cells of control plants, in contrast to PBS control. Cytosolic Ca^{2+} fluorescence in the guard cells of both the controls and gene-silenced plants was altered slightly after *Nep1_{Mo}* treatment for 3 h (Fig. 5).

Silencing of *NbWRKY2* interferes with *Nep1_{Mo}*-triggered HR.

Previous studies also indicated that the MAPK cascade participates in the cell-death signaling mediated by the WRKY transcription factor (Ekengren et al. 2003; Menke et al. 2005). We found that *MAPKKKα*-, *MEK1*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NTF6*-silenced plants had decreased transcription of *NbWRKY2* in response to *Nep1_{Mo}* (Supplementary Fig. S3). To determine whether *NbWRKY2* contributes to the cell death induced by *Nep1_{Mo}*, we silenced *NbWRKY2* using the PVX-VIGS system. *NbWRKY1* and *NbWRKY2* share 95% amino acid sequence identity and are the most abundant WRKY genes in *Nicotiana* species. Silencing efficiency was verified by quantitative (q)RT-PCR, using unique primers to distinguish *NbWRKY2* from *NbWRKY1*, and the results showed that *NbWRKY2* transcripts

Fig. 1. Virus-induced gene silencing-mediated silencing and induction of hypersensitivity responses with *Nep1_{Mo}* (50 nM). **A**, First-strand cDNA was generated from total RNA obtained from *Potato virus X* (PVX)-only plants (left panel) or from plants silenced for the genes shown on the right. Reverse transcription-polymerase chain reaction was performed with the cDNA and specific primers to the targeted gene and *EF1α* as an endogenous control. **B**, Leaves (representative of three replicate treatments) from control PVX, *MAPKKKα*-, *MEK1*-, *MEK2*-, *WIPK*-, *SIPK*-, *NTF6*-silenced *Nicotiana benthamiana* were infiltrated with *Nep1_{Mo}* solution. The red and black circles indicate cell death and no cell death, respectively. Leaves were removed from plants after 2 days of treatment (upper row) and were bleached in ethanol (lower row).

decreased specifically in the silenced plants (Fig. 6A). Cell death induced by *Nep1_{Mo}* was compromised in *NbWRKY2*-silenced plants compared with PVX control-inoculated plants (Fig. 6B). In addition, DAB staining showed light staining in *NbWRKY2*-silenced leaves after *Nep1_{Mo}* treatment (Fig. 6C). These results suggested that *NbWRKY2* functions downstream of the MAPKKK α -MEK2-WIPK cascade to regulate cell death induced by *Nep1_{Mo}*.

***NbWRKY2* functions directly downstream of the MAPKKK α -MEK2-WIPK cascade.**

As SIPK activates WRKY through phosphorylation, it appears that SIPK and WIPK transcription factors could be functionally related (Menke et al. 2005). To test the mechanism by which MAPK and *NbWRKY2* regulate *Nep1_{Mo}*-triggered HCD, we tested the interaction between WIPK and *NbWRKY2* using yeast two-hybrid assay. Only yeast hosts coexpressing *WIPK* and *NbWRKY2* were able to grow in selective medium plus 1 mM 3-amino-1,2,4-triazole and X-gal (Fig. 7A). Control strains expressing the strongly interacting pGADT7-RecT and pGBKT7-53 and noninteracting pGADT7-RecT and pGBKT7-Lam were included as controls (Fig. 7A). To further validate this interaction, we tested the association in living cells between WIPK and *NbWRKY2*, using a bimolecular fluorescence complementation (BiFC) approach with WIPK fused to the C-terminus of the yellow fluorescent protein (YFP) (WIPK-YFP^C) and *NbWRKY2* fused to the N-terminus of YFP (*NbWRKY2*-YFP^N). YFP was localized to the nucleus of *Arabidopsis* meso-

phyll protoplasts (Fig. 7B). As a negative control, WIPK-YFP^C was co-transformed with the YFP^N vector, and no YFP fluorescence was observed (Fig. 7B). Co-transformation of *NbWRKY2*-YFP^N and the YFP^C vector into protoplasts also did not result in any visible fluorescence. These results sug-

Fig. 3. Stomatal aperture measurements show that *Nep1_{Mo}*-induced stomatal closure is reduced in *SIPK*-silenced *Nicotiana benthamiana*. Stomatal aperture was measured 3 h after incubation in phosphate buffered saline (10 mM) and *Nep1_{Mo}* (50 nM). Values represent means $\pm$ standard error from three independent experiments; $n = 50$ apertures per experiment. Data were compared using the Student's *t*-test at the 95% significance level.

Fig. 2. In situ detection of hydrogen peroxide using diaminobenzidine staining on *MAPKKK α* -, *MEK1*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NTF6*-silenced *Nicotiana benthamiana* leaves in response to *Nep1_{Mo}*. **A**, *Nicotiana benthamiana* leaves silenced for *MAPKKK α* -, *MEK1*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NTF6* were compared with *Potato virus X* (PVX)-only leaves 6 h after infiltration of phosphate buffered saline (10 mM) or *Nep1_{Mo}* (50 nM). Elicitation with the elicitor was conducted on plants by infiltrating an equivalent elicitor solution of 25 μ l. **B**, Quantitative scoring of staining in leaves of the control and gene-silenced plants with elicitor treatment. The analysis was repeated for three sets of independently silenced plants in each experiment; the values shown were the means $\pm$ standard deviation of duplicate assays. The experiment was repeated twice with similar results.

Fig. 4. Effects of silencing of *MAPKKK α* , *MEK1*, *MEK2*, *WIPK*, *SIPK*, and *NTF6* on nitric oxide (NO) burst in guard cells in response to Nep1_{Mo}. **A**, In all cases, NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2DA) was loaded into cells of the epidermal peels, and fluorescence was measured after addition of phosphate buffered saline (10 mM) and Nep1_{Mo} (50 nM). For each treatment, fluorescence and bright field images were shown. Results from several experiments were compiled in this figure. Experiments were repeated at least three times, and representative images were shown. Green indicates NO burst. **B**, Quantitative analysis of in vivo NO generation monitored using DAF-2DA fluorescence. Results were presented as the mean ($n \geq 3$) fluorescence intensity per pixel.

gested that WIPK interacts directly with NbWRKY2 and NbWRKY2 functions downstream of the MAPK cascade in Nep1_{Mo}-signaling.

Nep1_{Mo} induces NbWRKY2-mediated stomatal closure and NO burst in guard cells.

The above findings prompted us to examine the possibility that both Nep1_{Mo}-induced stomatal closure and NO accumulation in the guard cells are NbWRKY2-dependent. Consistently, NbWRKY2-silenced plants significantly attenuated Nep1_{Mo}-induced stomatal closure (Fig. 8A), whereas NO generation by Nep1_{Mo} decreased to the level induced by PBS in controls (Fig. 8B). These results indicated that, in addition to the MEK2–WIPK cascade, NbWRKY2 might also participate in the Nep1_{Mo}-induced production of NO in the guard cells.

SIPK interacts with NbWRKY2.

The hyposensitivity to Nep1_{Mo} promotion of stomatal closure in SIPK- and NbWRKY2-silenced plants also prompted us to investigate whether SIPK physically interacts with NbWRKY2. Again, only yeast cells coexpressing SIPK and NbWRKY2 were able to grow in selective medium, while the controls did not (Fig. 9A). In BiFC, fluorescence within the nucleus can be observed in *N. benthamiana* leaves coexpressing SIPK and NbWRKY2. In contrast, no fluorescence can be detected in the

leaves coexpressing NbWRKY2 and p2YC vector, the p2YN vector and SIPK, or the negative control p2YN and p2YN vector (Fig. 9B). To further validate this interaction, a coimmunoprecipitation assay was performed. The ability of NbWRKY2 anti-His antibody to precipitate SIPK in the presence of recombinant NbWRKY2 protein but not in its absence (Fig. 9C) was demonstrated. The observations confirmed that SIPK is an interacting partner of NbWRKY2 in stomatal closure.

MAPKKKα–MEK2–WIPK/SIPK cascade and NbWRKY2 participate in Nep1_{Mo}-triggered resistance of *N. benthamiana* to *P. nicotianae*.

Since elicitors can induce systemic resistance in plants (Garcia-Brugger et al. 2006; Yang et al. 2009), we tested whether silencing of MAPKKKα, MEK2, WIPK, SIPK, and NbWRKY2 had any effects on Nep1_{Mo}-triggered disease resistance. Silenced leaves were infiltrated with 10 μl of Nep1_{Mo} at one spot and, 4 h later, the transcript level of pathogenesis-related protein (*PR2b*, X53600) was found approximately 30-fold higher after Nep1_{Mo} treatment in *N. benthamiana* (Supplementary Fig. S4). The leaf surfaces opposite the Nep1_{Mo}-infiltrated sides were inoculated with 2 × 2 mm mycelial plugs of *P. nicotianae*. Systemic resistance was determined 48 h after *P. nicotianae* inoculation by comparing and measuring the size of lesions. The typical water-soaked *Phytophthora* lesions appeared

Fig. 5. Nep1_{Mo} activation of Ca²⁺ generation in guard cells of control and MAPKKKα-, MEK1-, MEK2-, WIPK-, SIPK-, and NTF6-silenced plants. **A**, Leaf epidermal peels prepared from the control or gene-silenced plants were loaded with the Ca²⁺ dye Fura-2 AM prior to incubation in phosphate buffered saline (10 mM) and Nep1_{Mo} (50 nM). In each case, corresponding fluorescence and bright field images are shown. The areas of the peel subjected to analysis are greater than those shown in the figure. This experiment was repeated three times. Representative cells from one of three experiments are shown. In each experiment, a minimum of three epidermal peels were used as treatment replicates. **B**, Quantitative analysis of in vivo Ca²⁺ generation monitored using Fura-2 AM fluorescence as shown in A. Results are presented as the mean (n ≥ 3) fluorescence intensity per pixel.

within 24 h postinoculation, and the expanding disease lesions around the inoculated spots were observed in all silenced plants at 48 h postinoculation. However, Nep1_{M0} treatment significantly inhibited the expansion of lesions in PVX control-inoculated leaves of *N. benthamiana* (Fig. 10). These findings suggested that *MAPKKKα*, *MEK2*, *WIPK*, *SIPK*, and *NbWRKY2* are all required for Nep1_{M0}-induced systemic resistance to *P. nicotianae*.

***MAPKKKα*, *MEK2*-, *WIPK*-, *SIPK*-, and *NbWRKY2*-silenced plants show altered gene expression related to AOS homeostasis and transcription.**

Compromised cell death in *MAPKKKα*-, *MEK2*-, *WIPK*-, and *NbWRKY2*-silenced plants, hyposensitivity to Nep1_{M0} promotion of stomatal closure in *SIPK*- and *NbWRKY2*-silenced plants, increased susceptibility to *P. nicotianae* in *MAPKKKα*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NbWRKY2*-silenced plants after Nep1_{M0} treatment, and altered accumulation of H₂O₂ and NO all suggested dysregulation of gene function is associated with defense-related redox control and transcription. To address this possibility, the kinetics of the expression of five selected genes after Nep1_{M0} infiltration was examined by qRT-PCR in gene-silenced and control plants (Fig. 11). The transcript levels of acidic pathogenesis-related protein (*PR1a*, X06930) and *PR2b* (X53600) showed no significant difference ($P > 0.05$) in *MAPKKKα*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NbWRKY2*-silenced and control plants with PBS treatment but were three- to 33-fold lower in gene-silenced plants. The transcript level of *HSR203J* (AB091430), a HR marker gene, was approximately 1.7- to threefold lower after Nep1_{M0} treatment in *MAPKKKα*-, *MEK2*-, *WIPK*-, and *NbWRKY2*-silenced plants than in control plants, which was coincident with necrotic lesion formation (Fig. 1B). In *MAPKKKα*-, *MEK2*-, *WIPK*-, *SIPK*-, and

NbWRKY2-silenced and control plants, the expression of a disease resistance protein (*EDS1*, AF479625) was induced by Nep1_{M0} compared with PBS treatment and was down-regulated 1.5- to 4.5-fold in gene-silenced plants compared with control plants upon Nep1_{M0} treatment. Compared with the control plants, nitric reductase (*NR*; AB245431) expression showed no obvious difference in *MAPKKKα*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NbWRKY2*-silenced plants with PBS treatment (Fig. 11). It was approximately 2.8- to 5.5-fold lower in gene-silenced plants than in control plants after Nep1_{M0} treatment. In summary, an overlapping set of genes related to plant defense and transcription was found to show significant changes in expression in *MAPKKKα*-, *MEK2*-, *WIPK*-, *SIPK*-, and *NbWRKY2*-silenced plants compared with control plants.

DISCUSSION

We determined that the MAPK cascade and WRKY transcription factor are functionally required for Nep1_{M0} signaling. Based on the phenotypes observed, we developed a model that involves three of these genes in the Nep1_{M0}-induced HR and stomatal closure (Fig. 12). First, our experiments confirmed the role of a MAPK cascade in Nep1_{M0}-induced HR. Based on previous reports (del Pozo et al., 2004), the *MAPKKKα*-*MEK2*-*WIPK* cascade most likely positively regulates HR mediated by Nep1_{M0}. Second, as *NbWRKY2* has been shown to physically interact with *WIPK*, we tentatively placed *NbWRKY2* downstream of the *MAPKKKα*-*MEK2*-*WIPK* cascade in regulating HR mediated by Nep1_{M0}. Our data further defined the pathways from the elicitor recognition event and provided important new tools for characterizing elicitor signaling. Third, the *SIPK* interacted with *NbWRKY2* to control Nep1_{M0}-induced stomatal closure via the suppression of NO

Fig. 6. Nep1_{M0}-mediated cell death is suppressed in *Nicotiana benthamiana* plants silenced for *NbWRKY2*. **A**, *N. benthamiana* leaves were inoculated with *Potato virus X* (PVX)-*NbWRKY2* and PVX as a control. Total RNA was isolated from leaves 3 weeks after inoculation with *Agrobacterium tumefaciens* containing PVX-*NbWRKY2* and was used for quantitative reverse transcription-polymerase chain reaction with specific primers for *NbWRKY1* and *NbWRKY2*. **B**, Inhibition of cell death on PVX-*NbWRKY2* leaves compared with PVX-only leaves after infiltration of Nep1_{M0} solution. **C**, H₂O₂ accumulation is reduced in *NbWRKY2*-silenced plants compared with PVX-only plants.

Fig. 7. WIPK and NbWRKY2 interaction. **A**, Yeast two-hybrid interaction of WIPK with NbWRKY2. WIPK was coexpressed with NbWRKY2 in yeast strain AH109 and was grown on synthetic dropout nutrient (SD) medium lacking Leu and Trp (upper) or SD medium lacking Ade, His, Leu, and Trp (lower). pAD-WIPK = WIPK subcloned in the prey vector pGADT7; pBD-NbWRKY2 = NbWRKY2 subcloned in the bait vector pGBKT7. The pAD-RecT+pBD-53 construct served as a positive control. The pAD-RecT+pBD-Lam construct served as a negative control. **B**, WIPK associates with NbWRKY2 in vivo by bimolecular fluorescence complementation analysis. *Arabidopsis* protoplasts coexpressing WIPK and NbWRKY2 resulted in detectable yellow fluorescent protein (YFP) fluorescence in the nuclei. YFP fluorescence was excited at 488 and imaged at 518 to 540 nm by confocal microscopy.

Fig. 8. NbWRKY2 participates in nitric oxide (NO)-mediated stomatal closure induced by Nep1_{Mo}. **A**, The stomatal aperture was measured after 3 h of incubation in light after Nep1_{Mo} treatment. Values represent means $\pm$ 1 standard error (SE) from three independent experiments; n = 50 apertures per experiment. Data were compared using the Student's t -test at the 95% significance level. **B**, NO-sensitive dye 4,5-diaminofluorescein diacetate was loaded into cells of the epidermal peels, and fluorescence was measured after addition of phosphate buffered saline (10 mM) and Nep1_{Mo} (50 nM). Data are means $\pm$ SE from three independent experiments.

accumulation in guard cells. Our observations also suggested that the function of many defense-signaling components is conserved among different pathogen–plant interactions.

NbWRKY2 functions downstream of a MAPK cascade to positively regulate Nep1_{Mo}-induced cell death and resistance against *P. nicotianae*.

In different interaction systems, there may be subtle (but perhaps essential) differences between PAMP-triggered immunity and cell death or immunity triggered by various elicitors. MAPK

cascades are known as major pathways by which extracellular stimuli are transduced into intracellular responses in plants (Asai et al. 2002). The requirement of these kinases in defense-related signaling has been demonstrated previously in the *Pto*, *N*-mediated, gene-for-gene interaction and in the FLS2-mediated response to flagellin (Asai et al. 2002; Zhang and Klessig 1998, 2001). Silencing of *SIPK* leads to hypersensitivity of the Harpin-mediated HR and higher levels of ROS (which is different for Nep1_{Mo} elicitation) (Samuel et al. 2005). Similarly, VIGS of *WIPK* and *SIPK* genes reduced the resistance to a bacterial

Fig. 9. SIPK and NbWRKY2 interaction. **A**, Yeast two-hybrid interaction of SIPK with NbWRKY2. pAD-SIPK, SIPK subcloned in the prey vector pGADT7; pBD-NbWRKY2, NbWRKY2 subcloned in the bait vector pGBKT7. The pAD-RecT+pBD-53 construct served as a positive control. The pAD-RecT+pBD-Lam construct served as a negative control. **B**, Bimolecular fluorescence complementation visualization of SIPK and NbWRKY2 interaction in agrobacterium-infiltrated *Nicotiana benthamiana* leaves. Confocal and bright field images of epidermal leaf cells from *N. benthamiana* infiltrated with a mixture of *Agrobacterium* suspensions harboring constructs encoding the indicated fusion proteins. **C**, Co-immunoprecipitation of NbWRKY2 with SIPK. NbWRKY2 or SIPK protein was incubated with NbWRKY2 anti-His antibody in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of NbWRKY2, and complexes were pulled down with protein A agarose and were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% gel. Ponceau S staining shows the amount of input protein for NbWRKY2 and SIPK, respectively. Size markers are shown on the left of the panel.

pathogen but had no effect on INF1-induced hypersensitive response in *Nicotiana benthamiana* (Sharma et al 2003). Furthermore, SIPK-suppression has differential effects on some hrpZ and β -megaspermin-induced defense responses in tobacco (Hall et al 2007). In the study, WIPK and SIPK or their orthologs were activated and were found necessary for the defense response. Notably, two entire MAPK cascades have been identified downstream of FLS2 and Pto, consisting of MEKK1, MAPKKK α , MAPKK, MEK4/MEK5, MEK2, and MAPK, MPK3/MPK6, and WIPK, respectively (Asai et al. 2002; del Pozo et al. 2004). WIPK and SIPK are the direct substrates for MEK2 mediating N-response signaling in *N. benthamiana* (Jin et al. 2003). Here, we showed that cell death and systemic

resistance against *P. nicotianae* from Nep1_{Mo} is compromised in MAPKKK α -, MEK2-, and WIPK-silenced plants (Figs. 1 and 9), suggesting that Nep1_{Mo}-induced PCD and systemic resistance are mediated by a MAPK cascade. Our findings further reinforced previous observations that signaling events initiated by diverse pathogens converge at the point of MEK2–WIPK kinases. Additionally, Nep1_{Mo}-induced and *Pto*- and *N* gene-mediated cell death may share a similar HCD mechanism that depends on the MAPKKK α –MEK2–WIPK cascade.

NbWRKY2 was found to interact with WIPK by yeast two-hybrid and BiFC analyses (Fig. 7). To date, several transcription factors, including TGA, WRKY, Myb, and EREBP, have been shown to act downstream of various MAPK (Asai et al.

Fig. 10. MAPKKK α -, MEK2-, WIPK-, SIPK-, and NbWRKY2-silenced plants display enhanced sensitivity to *Phytophthora nicotianae*. **A**, For the control and gene-silenced plants, fully expanded leaves collected 4 h after Nep1_{Mo} treatment (10 μ l, black circle) were inoculated with a 2 $\times$ 2 mm hyphal plug on the left leaf surfaces opposite the Nep1_{Mo}-infiltrated sides. Then, the leaves were placed in petri dishes containing filter paper saturated with sterilized distilled water and were kept under a 16-h-day and 8-h-night regime at 25°C. Pictures of the lesions were taken at 48 h postinoculation and the lesion diameter (red circle) was measured. **B**, Resistance evaluation based on diameter of lesion spots. The inhibition rate = (diameter of necrosis with phosphate buffered saline treatment – diameter of necrosis with Nep1_{Mo} treatment)/diameter of control. Data are means $\pm$ standard error from eight experiments.

Fig. 11. Expression analysis of genes associated with redox control and transcription in MAPKKK α -, MEK2-, WIPK-, SIPK-, and NbWRKY2-silenced plants by quantitative reverse transcription-polymerase chain reaction. At 6 h after treatment with or without Nep1_{Mo} (50 nM), leaf samples were harvested from the inoculation site, the lower, and the upper leaves; *N. benthamiana* EF-1 α expression is used to normalize the expression value in each sample, and relative expression values were determined against buffer or *Potato virus X* (PVX)-infected plants using the comparative cycle threshold method ($2^{-\Delta\Delta C_t}$). Bars represent mean (three biological replicates) $\pm$ standard deviation.

Silencing of components in the MAPKKK α -MEK2-WIPK cascade compromises Nep1_{Mo}-induced H₂O₂ accumulation.

oxidase, contributes to H₂O₂ accumulation to a greater degree than AtrbohF, plant cell death was more severely inhibited in an AtrbohF mutant than in an AtrbohD mutant after treatment with avirulent *Pseudomonas syringae* DC3000 expressing the AvrRpm1 elicitor (Torres et al. 2002). Similarly, in cryptogein-treated tobacco plants, H₂O₂ plays an essential role in plant cell death by provoking AOS-mediated lipid peroxidation in the light (Montillet et al. 2005) but not in the dark (Rustérucci et al. 1999).

***SIPK*- and *NbWRKY2*-silenced plants show a reduced stomatal closure response to *Nep1_{M0}* and resistance against *P. nicotianae*.**

An increasing number of studies in plants have strongly confirmed the importance of stoma function in plant immunity, and stomatal closure observed is an output of PTI. Our results showed the induced resistance against *P. nicotianae* from *Nep1_{Mo}* was also compromised in *SIPK*- and *NbWRKY2*-silenced plants (Fig. 9), suggesting that *SIPK* and *NbWRKY2* are key regulators of plant immunity to regulate stomatal apertures, inhibiting the entry of pathogens into the plant leaf during infection.

In this study, silencing of *MEK2*, *WIPK*, *SIPK*, and *NbWRKY2* was found to suppress NO accumulation in the guard cells triggered by Nep1_{Mo}; however, only *SIPK* and *NbWRKY2* silencing compromised Nep1_{Mo}-induced stomatal closure. This result suggested that stomatal movement is associated with NO accumulation, but reduced NO accumulation may not result in inhibition of stomatal closure. NO, serving as a signaling molecule, not only regulates plant physiological processes, but also coordinates the HR and plant innate immunity (Ali et al. 2007; Dangl 1998).

Cross talk between MAPK cascades, Ca^{2+} , H_2O_2 , and NO.

NO, H₂O₂, and calcium are essential signaling components during cell death induced not only by elicitors but also by biotic and abiotic stress. The present study highlights that NO accumulation by a fungal elicitor such as Nep1_{Mo} is also mediated by the MAPK. Other studies have also shown that the MEK2–SIPK/NTF4 cascade controls the INF1-mediated NO burst (Asai et al. 2008). Our results support the theory that common signaling components such as NO, H₂O₂, and Ca²⁺ participate in the transduction of diverse signals emanating from biotic or abiotic stress, including UV-B or ozone stress (da Silva et al. 2011; Fujita et al. 2006; Holley et al. 2003).

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graph TD
    Elicitor --> MAPKKK
    MAPKKK --> MAPKK
    MAPKK --> MAPK
    MAPK --> Target
    Target --> Response

    Nep1Mo --> MAPKKK_alpha[MAPKKKα]
    MAPKK_alpha --> MEK2
    MEK2 --> WIPK
    WIPK --> NbWRKY2_1[NbWRKY2]
    NbWRKY2_1 --> HR

    Nep1Mo --> Q1[?]
    Q1 --> Q2[?]
    Q2 --> SIPK
    SIPK --> NbWRKY2_2[NbWRKY2]
    NbWRKY2_2 --> Stomatal_closure[Stomatal closure]
  
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Vol. 25, No. 12, 2012 / 1649

et al. 2006). Using pharmacological experiments, apoplastic Ca^{2+} influx was demonstrated to be important in the oxidative burst, and Ca^{2+} was shown to activate an AOS in vitro in tobacco and tomato (Miura et al. 1995, 1999; Sagi and Fluhr 2001). In this study, we found that silencing of *SIPK* inhibited Nep1_{Mo} -induced NO accumulation in guard cells, which concurs with previous findings that *SIPK* is required for the NO burst induced by INF1 (Asai et al. 2008). The MEK2–WIPK/*SIPK* cascade regulates the NO burst triggered by Nep1_{Mo} as well as HR cell death, which suggests that a balance of NO and AOS determines the fate of plants.

***Nep1*_{Mo} alters gene expression targeting signal transduction.**

We found that silencing of *NbWRKY2* in *N. benthamiana* blocked cell death induced by Nep1_{Mo} (Fig. 6), and WIPK was able to physically interact with *NbWRKY2* transcription factors (Fig. 7). Therefore, we tentatively place *NbWRKY2* downstream of WIPK. To date, several transcription factors, including WRKY family members, have been shown to act downstream of various MAPK (Liu et al. 2004). A study demonstrated that WRKY1 also interacts with *SIPK* in the hypersensitive response-like cell death in tobacco (Menke et al. 2005). This interaction occurs because activated *SIPK* binds to the W box sequence (Menke et al. 2005).

Nep1_{Mo} triggered a complex immune response in *Arabidopsis*, including MAPK activation, production of NO, ethylene, camalexin, and callose, and extensive reprogramming of the transcriptome (Motteram et al. 2009; Qutob et al. 2006). qRT-PCR analyses showed that the transcript expression of *PR1a*, *PR2b*, and *EDS1*, which are associated with resistance signaling and plant defense execution, is up-regulated by Nep1_{Mo} and is down-regulated in gene-silenced plants upon Nep1_{Mo} treatment. These results suggested that the genes encoding putative disease resistance proteins are involved in Nep1_{Mo} -induced cell death and plant defense.

NO can be synthesized in plants by nitrite reduction with nitrate reductase, oxidation of arginine to citrulline by napoline synthase, and a nonenzymatic NO generation system (Crawford 2006). Here, qRT-PCR analyses showed that silencing of *MAPKKKα*, *MEK2*, *WIPK*, and *NbWRKY2* appears to decrease the expression of *NR* after Nep1_{Mo} treatment. The decreased NO accumulation in *MEK2*-, *WIPK*-, and *SIPK*-silenced plants indicates that NR may participate in Nep1_{Mo} -induced NO production. Further characterization of Nep1_{Mo} -mediated cell death signaling in plants will enhance our understanding of biological processes that lead to susceptibility in plant-pathogen interactions and improve our ability to control plant diseases.

***Nep1*_{Mo} triggers plant defense.**

In this study, we comprehensively characterized the immune response to Nep1_{Mo} . Results from this study and previous work allowed us to conclude that Nep1_{Mo} is responsible for a complex immune response. This response includes MAPK activation, production of NO, Ca^{2+} , and H_2O_2 , extensive reprogramming of the transcriptome, and plant resistance. Cell death and tissue necrosis terminate this massive defense response. The effects of Nep1_{Mo} resemble those triggered by interaction of AvrPto and Pto, suggesting that both signals are perceived as equivalent determinants of microbial nonself recognition by the plant and trigger similar activation of the plant surveillance system. These findings also facilitate the study of the molecular architecture of merging signaling pathways and possible synergisms between them in one plant. In comparison to the activation of *Pto*-mediated plant cultivar-specific resistance, relatively little is known about the signaling pathways mediating non-cultivar specific plant resistance. Our isolation of fungus-derived Nep1_{Mo} and characterization of its defense-inducing

potential in *N. benthamiana* now provide suitable tools for such an approach, to determine its relationship with toxin signaling and PAMP signaling.

Furthermore, regarding the presence of *Nep1*-encoding genes in the genomes of both monocot-infecting fungal pathogens and dicot-infecting plant pathogens, determining whether *Nep1*-triggered plant pathogen interaction from different systems shares the same downstream factors or whether diversified components have evolved will also be interesting.

MATERIALS AND METHODS

Plant materials, elicitors, and treatment protocols.

N. benthamiana plants were grown in a growth chamber under a 16-h-light and 8-h-dark cycle at 25°C. A needleless syringe was used to inject 25 μl of *Magnaporthe oryzae* Nep1_{Mo} (50 nM) into tiny cuts on the underside of the leaf, thereby flooding the apoplastic space. To prepare Nep1_{Mo} , overnight cultures of *Escherichia coli* BL21 cells carrying pET32b harboring the *Nep1*_{Mo} gene (GenBank accession number MGG_08454) were diluted (1:100) in Luria-Bertani medium containing ampicillin (50 $\mu\text{g/ml}$) and were incubated at 37°C. When the optical density at 600 nm of the culture reached 0.6, Nep1_{Mo} secretion into the culture medium was induced via the addition of 0.4 mM isopropyl-beta-D-thiogalactopyranoside for 6 h. Comparison of NLP primary sequences found in public databases revealed strong conservation of a central hepta-peptide motif, GHRHDWE (Ottmann et al. 2009). The site-directed mutations within the GHRHDWE site of Nep1_{Mo} , located at amino acids 123 to 129, were constructed using a two-step amplification procedure to create a Nep1_{Mo} mutant 1 and a mutant 2 by two pairs of primers (F1: GAATTCATGCTTCCCAAGT TCTTCACTCTCC, R1: ACCTCCACCTCCACCTCCACCC ATGCCCCGCGACGGCGAGTC; F2: GGTGGAGGTGGAG GTGGAGGTAACGTCATCATCTGG, R2: AAGCTTTCATT GGAAAACCTTGGCCAGGTTG) and then, GHRHDWE was individually exchanged for glycine by overlap PCR. Nep1_{Mo} and Nep1_{Mo} mutant were expressed as a His tag fusion protein. Proteins were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein purification was performed with the Ni-nitrilotriacetic acid resin (Qiagen, Valencia, CA, U.S.A.), and the purified proteins were dialyzed against a phosphate-buffered saline (PBS) buffer (pH 7.4) and were stored at –20°C prior to use. Protein concentrations were determined using the Bradford reagent (Qutob et al. 2006), and concentrated stock solutions (500 nM) were prepared.

DNA constructs and seedling infection for virus-induced gene silencing.

VIGS for the *MAPKKKα* (GenBank accession number AY500155), *MEK1* (GenBank accession number AJ302651), *MEK2* (GenBank accession number AF325168), *WIPK* (GenBank accession number AB098729), *SIPK* (GenBank accession number AB098730), *NTF6* (GenBank accession number AY547494), and *NbWRKY2* (GenBank accession number TC12974) genes in *N. benthamiana* was performed using potato virus X (PVX), as previously described by Zhang and associates (2009, 2010, 2011). The *MAPKKKα*, *MEK1*, *MEK2*, *WIPK*, *SIPK*, *NTF6*, and *NbWRKY2* inserts were 276, 257, 267, 262, 262, 274, and 323 bp, respectively, which were derived from the 3' termini of their respective open reading frames and were inserted into the PVX vector separately in the antisense direction to generate PVX-*MAPKKKα*, PVX-*MEK1*, PVX-*MEK2*, PVX-*WIPK*, PVX-*SIPK*, PVX-*NTF6*, and PVX-*NbWRKY2*. The constructs containing the inserts were transformed into *Agrobacterium tumefaciens* GV3101. Bacterial suspensions were applied to the undersides of *N.*

benthamiana leaves, using a 1-ml needleless syringe. Plants exhibited mild mosaic symptoms 3 weeks after inoculation. The third or fourth leaf above the inoculated leaf in which silencing was most consistently established was used for further analyses.

DAB staining.

Following the methods of Gan and associates (2009), leaves were harvested 6 h after Nep1_{M0} treatment and were immediately vacuum-infiltrated for 20 min with PBS (pH 7.4) containing 0.5% (wt/vol) DAB. The leaves were placed in light for 10 h and were then boiled for 20 min in 80% ethanol. The intensity and pattern of DAB staining was assessed visually. Quantitative scoring of H₂O₂ staining in leaves was analyzed using Quantity One software (Bio-Rad, Segrate, Italy).

RNA isolation, semiquantitative RT-PCR, and qRT-PCR.

As described in Zhang and associates (2010), real-time RT-PCR was performed using the cDNA and gene-specific primers (Supplementary Table 1). Each cDNA was amplified by quantitative PCR using SYBR PrimeScript RT-PCR kit (TaKaRa, Dalian, China) and the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.). To ensure that similar amounts of cDNA were used for silenced and nonsilenced plants, we ran parallel reactions with elongation factor 1 α (*EF1 α*) primers as controls. *N. benthamiana* *EF- α* expression was used to normalize the expression value in each sample, and relative expression values were determined against buffer or PVX-infected plants using the comparative cycle threshold method ($2^{-\Delta\Delta C_t}$) (Zhang et al. 2010).

Stomatal aperture measurements.

Stomatal apertures were measured as described by Chen and associates (2004) and Zhang and associates (2009, 2010). Leaves were derived from various plants silenced for *MAP-KKK α* , *MEK1*, *MEK2*, *WIPK*, *SIPK*, *NTF6*, *NbWRKY2*, and for controls. After a 3-week inoculation, samples were harvested from the third and fourth leaves above the inoculation site. Abaxial (lower) epidermis was peeled off and floated in 5 mM KCl, 50 mM CaCl₂, and 10 mM MES-Tris (pH 6.15) in light for at least 2 h to open the stomata fully before experimentation, to minimize the effects of other factors in stomatal response, because the mesophyll signals can also significantly influence stomatal behavior. The epidermal strips were then followed by Nep1_{M0} treatment for 3 h to induce a stomatal response. The maximum diameter of stomata was measured under an optical microscope. At least 50 apertures in each treatment were obtained. The experiments were repeated three times.

NO measurement in guard cells.

NO accumulation was determined using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich, St. Louis) according to Ali and associates (2007). Epidermal strips were prepared from control and gene-silenced plants and were incubated in 5 mM KCl and 10 mM MES-Tris (pH 6.15) in light for 2 h, followed by incubation in 20 μ M DAF-2DA for 1 h in the dark at 25°C, and finally, were rinsed three times with 10 mM Tris-HCl (pH 7.4) to wash off excess fluorophore. Images of guard cells were obtained 3 h after Nep1_{M0} treatment under a fluorescence microscope (excitation wavelength, 470 nm; emission wavelength, 515 nm; Leica DMR, Wetzlar, Germany). Fluorescence emission from guard cells was analyzed using Quantity One software.

Measurement of Ca²⁺ in guard cells.

Following Chen and associates (2004), the epidermal strips were peeled gently and incubated in 5 mM fura-2/acetoxyl-

methyl ester (Dojin Biochemicals, Kumamoto, Japan) for 60 min at 37°C in darkness. After aspiration of the fura-2/acetoxyl-methyl ester solution, the glass slides were rinsed. Guard cell images were taken 3 h after Nep1_{M0} treatment by a fluorescence microscope and were analyzed with the software Quantity One.

Yeast two-hybrid and BiFC assays.

The yeast two-hybrid was carried out in an AH109 yeast host as described previously (James et al. 1996). *WIPK*, *SIPK*, and *NbWRKY2* were amplified by RT-PCR from *N. benthamiana* total cDNA. The *NbWRKY2* gene was cloned into pGBKT7 as bait, and the *WIPK* and *SIPK* genes were cloned into pGADT7 as prey, respectively. Yeast transformation was performed by the LiAc and polyethylene glycol method, as described in the Yeast Protocols Handbook (Clontech, Palo Alto, CA, U.S.A.). Yeast transformants were screened on synthetic dropout nutrient medium with galactose and X-gal but lacking uracil, histidine, and tryptophan. Blue colonies indicate a positive interaction between two proteins in yeast.

Co-immunoprecipitation.

For co-immunoprecipitation experiments, *NbWRKY2* was subcloned in pET32a, was expressed in *E. coli* BL21, and was purified by standard nickel-affinity chromatography (Novagen, Madison, WI, U.S.A.). *SIPK* was cloned to p2YC, and the construct was transiently expressed through agrobacterium-mediated transformation into *N. benthamiana*. Protein extracts were prepared by grinding 400 mg of leaf tissue in 1 ml RIPA lysis and extraction buffer (Beyotime, Jiangsu, China) in the presence of 0.1 mM phenylmethylsulfonyl fluoride (Beyotime). Purified *NbWRKY2*, *SIPK* protein, and 2 μ l of anti-hemagglutinin antibody were co-incubated overnight at 4°C. Proteins from the sample lysate were fractionated using SDS-PAGE, were transferred to an Immobilon-PSQ polyvinylidene difluoride membrane, and were then blocked using PBS (pH 7.4). Mouse anti-His monoclonal antibodies (Sigma-Aldrich) were added at a ratio of 1:5,000 and were incubated at room temperature for 90 min. The membrane was then incubated with a goat anti-mouse IRDye 800CW (Odyssey, Lincoln, NE, U.S.A.), were processed, and signals were detected using ODYSSEY LI-COR, with excitation set at 780 and 800 nm.

Disease resistance assay.

A *Phytophthora parasitica* var. *nicotianae* strain (Pp016) was maintained on LBA medium (60 g of lima bean, 20 g of agar, 1,000 ml of H₂O) at 25°C for 5 days. In the detached-leaf inoculation assay, fully expanded leaves collected 4 h after Nep1_{M0} treatment (10 μ l) were inoculated with a 2 $\times$ 2 mm hyphal plug on the left leaf surfaces opposite the Nep1_{M0}-infiltrated sides. Then the leaves were placed in petri dishes containing filter paper saturated with sterilized distilled water and were kept under a 16-h-day and 8-h-night regime at 25°C. Pictures of the lesions were taken at 48 h postinoculation and the lesion diameter was measured. The inhibition rate = (diameter of necrosis with PBS treatment – diameter of necrosis with Nep1_{M0} treatment)/diameter of control.

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