MAPK Signaling Regulates Nitric Oxide and NADPH Oxidase-Dependent Oxidative Bursts in *Nicotiana benthamiana*  

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Nitric oxide (NO) and reactive oxygen species (ROS) act as signals in innate immunity in plants. The radical burst is induced by INF1 elicitin, produced by the oomycete pathogen *Phytophthora infestans*. NO ASSOCIATED1 (NOA1) and NADPH oxidase participate in the radical burst. Here, we show that mitogen-activated protein kinase (MAPK) cascades MEK2-SIPK/NTF4 and MEK1-NTF6 participate in the regulation of the radical burst. NO generation was induced by conditional activation of SIPK/NTF4, but not by NTF6, in *Nicotiana benthamiana* leaves. INF1- and SIPK/NTF4-mediated NO bursts were compromised by the knockdown of NOA1. However, ROS generation was induced by either SIPK/NTF4 or NTF6. INF1- and MAPK-mediated ROS generation was eliminated by silencing Respiratory Burst Oxidase Homolog B (RBOHB), an inducible form of the NADPH oxidase. INF1-induced expression of RBOHB was compromised in SIPK/NTF4/NTF6-silenced leaves. These results indicated that INF1 regulates NOA1-mediated NO and RBOHB-dependent ROS generation through MAPK cascades. NOA1 silencing induced high susceptibility to *Colletotrichum orbiculare* but not to *P. infestans*; conversely, RBOHB silencing decreased resistance to *P. infestans* but not to *C. orbiculare*. These results indicate that the effects of the radical burst on the defense response appear to be diverse in plant–pathogen interactions.

INTRODUCTION

Rapid production of nitric oxide (NO) and reactive oxygen species (ROS), called the NO burst and the oxidative burst, respectively, plays a role in diverse physiological processes, such as resistance to biotic and abiotic stress, hormonal signaling, and development (Doke, 1983; Kwak et al., 2003; Bright et al., 2006; Grun et al., 2006; Takeda et al., 2008). Recently, NO has attracted attention as the radical that participates in innate immunity in plants. NO induces phytoalexin accumulation (Noritake et al., 1996), activation of the mitogen-activated protein kinase (MAPK; Clarke et al., 2000), and the expression of defense genes, such as Phe ammonia-lyase (PAL) and pathogenesis-related proteins (Durner et al., 1998). In animals, NO is produced by NO synthase (NOS). The sources of NO synthesis in plants include reduction in nitrite by nitrate reductase (NR), oxidation of Arg to citrulline by NO, and a nonenzymatic NO generation system (Bethke et al., 2004). Although evidence for Arg-dependent NO synthesis in plants has accumulated, no gene or protein that has a sequence similar to known mammalian-type NOS has been found in plants (Sagi et al., 2004; Garcia-Mata and Lamattina, 2003). Guo et al. (2003) identified a NOS-like enzyme from *Arabidopsis thaliana* (At NOS1) with a sequence similar to a protein that has been implicated in NO synthesis in the snail *Helix pomatia*. The At NOS1 protein has no NOS activity (Zemojtel et al., 2006); therefore, At NOS1 was renamed At NOA1 for NO ASSOCIATED1 (Crawford et al., 2006). However, *Arabidopsis* mutant noa1 is still useful for its phenotype, which shows reduced levels of NO in plant growth, fertility, hormonal signaling, salt tolerance, and plant-pathogen responses (Guo et al., 2003; He et al., 2004; Zeidler et al., 2004; Zhao et al., 2007). Knocking out or down NOA1 expression provides a powerful tool to analyze NO function.

ROS are also important signaling molecules in plant immunity. In animals, ROS are synthesized by the phagocyte enzymatic complex of NADPH oxidase, which consists of two plasma membrane proteins, gp91phox (phox for phagocyte oxidase) and p22phox. Cytosolic regulatory proteins, p47phox, p67phox, p40phox, and Rac2, translocate to the plasma membrane to form the active complex after stimulation (Lambeth, 2004). However, no homologs of the p22phox, p67phox, and p40phox regulators of the phagocyte NADPH oxidase were found in the *Arabidopsis* genome. However, a homolog of one component, human Rac GTPase, has been isolated from rice (*Oryza sativa*), and the constitutively active mutant of Rac activates ROS production (Wong et al., 2007). Plant NADPH oxidases designated as Respiratory Burst Oxidase Homolog (RBOH) have been identified as genes related to mammalian gp91phox in various plants (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998; Yoshioka et al., 2001, 2003; Sagi et al., 2004) and carry an N-terminal extension comprising two EF-hand motifs, suggesting that Ca$^{2+}$ regulates their activity. RBOHs are localized on the...
plasma membrane (Kobayashi et al., 2006). *Arabidopsis rbohD rbohF* double mutants show greatly decreased ROS production in response to infection with avirulent *Pseudomonas syringae pv tomato* DC3000 and *Hyaloperonospora parasitica* and in response to abscisic acid (Torres et al., 2002; Kwak et al., 2003). Analysis of the loss of function of *Nt RBOHD* in tobacco cells (*Nicotiana tabacum*) showed loss of ROS production by elicitor treatment (Simon-Plas et al., 2002). We also showed that silencing *Nb RBOHA* and *Nb RBOHB* in *Nicotiana benthamiana* plants leads to less ROS production and reduced resistance to infection by the potato pathogen *Phytophthora infestans* (Yoshioka et al., 2003). These reports suggest that RBOH is a key regulator of ROS production and has pleiotropic functions in plants.

The MAPK cascade is a major conserved signaling pathway used to transduce extracellular stimuli into intracellular responses in eukaryotes (MAPK Group, 2002; Nakagami et al., 2005). In the MAPK signal transduction cascade, a MAPK is activated by a MAPK kinase (MAPKK), which itself is activated by a MAPKK kinase (MAPKKK). Many studies have extensively characterized plant MAPKs, including tobacco wound-induced protein kinase (WIPK; Seo et al., 1999) and salicylic acid–induced protein kinase (SIPK; Zhang and Klessig, 1997) and their orthologs in other plant species (Lee et al., 2004; Katou et al., 2005; Pedley and Martin, 2005). WIPK and SIPK participate either in N gene–dependent resistance to tobacco mosaic virus (TMV; Zhang and Klessig, 1998; Jin et al., 2003) or in C9-dependent resistance to *Cladosporium fulvum*–derived elicitor Avr9 (Romeis et al., 1999) in a gene-for-gene specific way and in response to plant species–specific elicitors, such as elicitins (Zhang et al., 2000). MEK2, a tobacco MAPKK, is upstream of both WIPK and SIPK (Yang et al., 2001). Expression of *Nt MEK2DD*, a constitutively active mutant of MEK2, induces hypersensitive response (HR)-like cell death, defense gene expression, and generation of ROS, all of which are preceded by activation of endogenous WIPK and SIPK (Yang et al., 2001; Ren et al., 2002). We also cloned the potato (*Solanum tuberosum*) ortholog of tobacco MEK2, *St MEK2*, which was formerly described as *St MEK1* (Katou et al., 2003). Heterologous expression of *St MEK2DD* in *N. benthamiana* induces WIPK and SIPK activities (Katou et al., 2003) and then an oxidative burst accompanied by increased RBOHB expression (Yoshioka et al., 2003). MAPKKKα was identified as an upstream activator of MEK2 and SIPK in *N. benthamiana* (del Pozo et al., 2004). Silencing the MAPKKKα gene eliminated HR-like cell death caused by interaction between the *P. syringae* avirulence gene *AvrPto* and its cognate resistant gene *Pto* (del Pozo et al., 2004). Interestingly, HR-like cell death induced by MAPKKKα overexpression is reduced not only by silencing MEK2 or SIPK, but also by silencing MEK1 or NTF6 (del Pozo et al., 2004). NPK1-MEK1/NQK1-NTF6/NRK1 is a pivotal MAPK cascade in the regulation of cytokinesis (Soyano et al., 2003; Sasabe et al., 2006). Like WIPK or SIPK, silencing NPK1, MEK1, or NTF6 attenuates N- and Pto-mediated resistance against TMV (Jin et al., 2002; Liu et al., 2004) and *P. syringae* AvrPto, respectively (Ekenren et al., 2003). These studies indicated that MAPK cascades MEK2-WIPK/SIPK and NPK1-MEK1-NTF6 participate in disease resistance in plants. In addition, recent work has shown that tobacco NTF4, which shares 93.6% identity with SIPK, is also activated by MEK2, and ectopic expression of NTF4 induces HR-like cell death (Ren et al., 2006). However, the relationship between these MAPK cascades and the radical burst in response to pathogen signals is not clear.

In this study, we investigated the roles of MEK2-WIPK/SIPK/NTF4 and MEK1-NTF6 cascades in the regulation of NO and oxidative bursts in *N. benthamiana*. Gain-of-function and loss-of-function analyses showed that the MEK2-SIPK/NTF4 cascade controls the NOA1-mediated NO burst and that MEK2-SIPK/NTF4 and MEK1-NTF6 cascades regulate the NADPH oxidase-dependent oxidative burst. We also show that the NO burst and the oxidative burst have distinct effects on resistance to *P. infestans* and *Colletotrichum orbiculare* (syn. *C. lagenarium*) in *N. benthamiana*.

**RESULTS**

**St MEK2DD Activates MEK2-WIPK/SIPK Cascade, and Nb MEK1DD Activates MEK1-NTF6 Cascade**

To examine the function of MEK1-NTF6 cascade, we first isolated the tobacco MEK1 homolog from *N. benthamiana* using PCR with primers derived from a sequence of *Nt MEK1* using an *N. benthamiana* cDNA library as a template. Analysis of the deduced amino acid sequence of the PCR product showed that the product of this gene shares >99% identity with *Nt MEK1*, so it was designated *Nb MEK1* (*N. benthamiana* MEK1). In plants, MAPKKs are activated through phosphorylation of two Ser/Thr residues in a conserved S/TxxxS/T motif by MAPKKKs (MAPK Group, 2002). Replacement of two Ser/Thr residues with Gln or Asp results in constitutive activation of plant MAPKK (Yang et al., 2001; Ren et al., 2002). To examine the function of MEK1, we constructed *Nb MEK1DD*, a constitutively active mutant of *Nb MEK1*, in which the conserved Ser-219 and Thr-225 were converted to Asp. Nb MEK1DD was expressed under the control of the 35S promoter of *CaMV tumefaciens* using *Agrobacterium tumefaciens* infiltration of *N. benthamiana* leaves. Immuno-complex (IC) kinase assay using anti-NTF6 antibody showed no activation of NTF6 by Nb MEK1DD, probably because the amount of endogenous NTF6 was not sufficient to detect the activity by the antibody. Therefore, NTF6–HA (hemagglutinin) was co-expressed with *Nb MEK1DD–HA* or with *inf1*, an elicitin elicitor produced by *P. infestans* (Kamoun et al., 2003), by *Agrobacterium* infiltration in *N. benthamiana* leaves. As shown in Figure 1A, we confirmed that NTF6 is activated by *Nb MEK1DD* and INF1.

Our previous results showed that *Agrobacterium*–mediated expression of *St MEK2DD*, a constitutively active mutant of *St MEK2*, the potato ortholog of *Nt MEK2*, induces HR-like cell death accompanied by oxidative burst in *N. benthamiana* leaves, preceded by the activation of WIPK and SIPK (Katou et al., 2003; Yoshioka et al., 2003). In this study, we also found that *St MEK2DD* and INF1 activate WIPK and SIPK by IC kinase assay using anti-WIPK and SIPK antibodies (Figure 1A).

**INF1 and St MEK2DD Induce NOA1–Mediated NO Burst**

We reported previously that a NO burst is induced by INF1 in tobacco cell suspension culture (Yamamoto et al., 2004). To investigate whether MAPK cascades regulate the NO burst, St
MEK2DD and Nb MEK1DD were expressed using Agrobacterium infiltration in N. benthamiana leaves; inf1 and β-glucuronidase (GUS) were also expressed as positive and negative controls, respectively. The NO burst was detected by 4,5-diaminofluorescein diacetate (DAF-2DA)–mediated fluorescence (Figure 1B). Sodium nitroprusside, a NO generator, produced DAF-2DA–mediated fluorescence (Figure 1B), and the inducible fluorescence was eliminated by 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger (Figure 2), indicating that DAF-2DA detects NO. At 36 h after infiltration, the NO burst was markedly induced by the expression of St MEK2DD, but not by Nb MEK1DD (Figure 1B), suggesting that the NO burst is regulated by MEK2-WIPK/SIPK cascade. Time-course experiments showed that the DAF-2DA–mediated fluorescence was detected within 24 h after the infiltration and gradually increased until 36 h; thereafter, the leaf tissues showed a cell death phenotype. The timing of the NO burst was well matched with protein levels of St MEK2DD (see Supplemental Figure 1).
Next, we silenced MEK1 and MEK2 in N. benthamiana by virus-induced gene silencing (VIGS) to investigate the functions of these MAPKs. We cloned cDNA fragments of Nb MEK1 and Nb MEK2 into tobacco rattle virus (TRV) vector and introduced these into N. benthamiana by Agrobacterium infiltration (Ratcliff et al., 2001). Three weeks after the infiltration, the efficiency of the silencing was monitored using RT-PCR. The MEK1 and MEK2 transcripts decreased markedly in the silenced plants compared with the control plants infected with TRV vector alone (see Supplemental Figure 2 online). The silenced leaves were inoculated with A. tumefaciens containing inf1, St MEK2DD, or GUS as a control. The NO generation induced by INF1 and St MEK2DD was not compromised in MEK1-silenced plants compared with the NO generation in the TRV control-inoculated plants (Figure 1C). By contrast, MEK2-silenced plants showed a marked reduction in NO generation by INF1 (Figure 1C). NO production by St MEK2DD decreased to the level induced by GUS as a control because VIGS of Nb MEK2 also silenced the St MEK2DD transgene (see Supplemental Figure 2 online). These results indicate that the MEK2-WIPK/SIPK cascade, but not the MEK1-NTF6 cascade, participates in signaling pathways leading to the NO burst induced by INF1.

NOA1 participates indirectly in Arg-dependent NOS activity in plants (Guo et al., 2003; Zhao et al., 2007). To determine whether NOA1 contributes to the NO burst induced by INF1 and St MEK2DD, we isolated the NOA1 homolog from N. benthamiana (Kato et al., 2008) and silenced Nb NOA1 using the TRV-VIGS system. The transcripts in the silenced plants decreased compared with control plants infected with TRV alone (see Supplemental Figure 3A online). Silenced leaves were inoculated with A. tumefaciens harboring inf1, St MEK2DD or GUS as a control. The NO generation induced by INF1 and St MEK2DD significantly decreased in NOA1-silenced plants compared with TRV control-inoculated plants (Figure 2), suggesting that NOA1 participates in the NO burst induced by INF1 and St MEK2DD. Furthermore, we examined effects of some inhibitors on NO generation. L-NAME, the NOS inhibitor Nω-nitro-L-Arg-methyl ester, significantly reduced NO generation induced by INF1 and St MEK2DD in TRV control-plants but not in NOA1-silenced plants (Figure 2). By contrast, D-NAME, an inactive isomer of L-NAME, had no effect on NO burst induced by INF1 (see Supplemental Figure 4 online). The results suggested that NOA1 takes part in NO burst induced by INF1 and St MEK2DD and are in agreement with previous reports showing the involvement of NOA1 in Arg-dependent NOS activity (Guo et al., 2003; Zhao et al., 2007). Tungstate, an NR inhibitor, also significantly decreased NO generation in both TRV control- and NOA1-silenced plants (Figure 2), suggesting that NR is also involved in NO burst induced by INF1 and St MEK2DD.

SIPK and NTF4 Are Required for NO Burst Induced by INF1 and St MEK2DD

We evaluated whether the NO generation induced by INF1 and St MEK2DD depends on WIPK, SIPK, or both. We silenced WIPK and SIPK in N. benthamiana and confirmed a marked reduction in WIPK and SIPK transcripts in the silenced plants compared with control plants infected with TRV alone (Figure 3A). The NO generation induced by INF1 and St MEK2DD was slightly reduced in WIPK-silenced plants compared with that in TRV control plants (Figure 3B). By contrast, VIGS of SIPK and WIPK/SIPK markedly reduced the NO generation to near the control level (Figure 3B). These results indicate the involvement of SIPK in the INF1-induced NO burst.

Recently, NTF4 was shown to share the same upstream MAPK, MEK2, with SIPK and WIPK and to participate in disease resistance. Similar to SIPK and WIPK, NTF4 can also be activated by cryptogein, an elicitor elicitin derived from oomycete pathogen Phytophthora cryptogea, like INF1 (Ren et al., 2006). It is possible that NTF4 is also silenced in SIPK-silenced leaves because NTF4 shows high sequence identity to SIPK (see Supplemental Figure 5 online). We confirmed that VIGS of SIPK also silenced NTF4 using SIPK- and NTF4-specific primers (Figure 3A). To investigate the possibility that NTF4 is involved in NO generation, gain-of-function analyses of SIPK and NTF4 were done by Agrobacterium infiltration methods. The overexpression of SIPK and NTF4 resulted in the increases in SIPK and NTF4 activities, respectively (Figure 3C) as shown by Zhang and Liu (2001) and Ren et al. (2006). NO generation was induced by NTF4 as well as SIPK and compromised in NOA1-silenced plants (Figure 3D), suggesting that SIPK and NTF4 are involved in NO burst induced by INF1 and St MEK2DD.

Silencing SIPK Compromises the Expression of RBOHB Induced by St MEK2DD but Not by INF1

MAPK cascade participates in signaling to the oxidative burst (Yang et al., 2001; Ren et al., 2002). Our previous study showed that INF1 and St MEK2DD induce an oxidative burst accompanied by expression of RBOHB, which is an inducible form of the NADPH oxidase of N. benthamiana (Yoshioka et al., 2003). To assess whether the expression of RBOHB induced by INF1 and St MEK2DD depends on WIPK, SIPK, or both, we silenced the two MAPKs in N. benthamiana. Silenced leaves were inoculated with A. tumefaciens containing inf1 or St MEK2DD (Figure 4). We confirmed a reduction in accumulation of WIPK and SIPK transcripts in the silenced plants using RNA gel blot hybridization. The expression of RBOHB induced by St MEK2DD eliminated SIPK- and WIPK/SIPK-silenced plants (Figure 4), indicating that the MEK2-SIPK cascade regulates the expression of RBOHB. Unexpectedly, INF1-induced RBOHB expression was not compromised by the silencing of either WIPK or SIPK (Figure 4). These results suggest that INF1-induced expression of the RBOHB gene can be mediated by means of other signal pathways in addition to the MEK2-SIPK cascade.

Nb MEK1DD Induces RBOHB-Dependent Oxidative Burst

INF1 activated NTF6 and SIPK (Figure 1A). This finding prompted us to examine the possibility that Nb MEK1DD induces the oxidative burst accompanied by the expression of RBOHB. N. benthamiana leaves were inoculated with A. tumefaciens containing Nb MEK1DD, St MEK2DD, inf1, or GUS as control. At 24 h after inoculation with A. tumefaciens, the leaves were infiltrated with L-012 solution, a highly sensitive agent used to detect ROS
(Imada et al., 1999; Kobayashi et al., 2007), and were observed using a sensitive CCD camera. We confirmed that luminol detects not only ROS but also NO, whereas L-012 is highly ROS specific (see Supplemental Figure 6 online). Time-course experiments showed that L-012–mediated chemiluminescence peaked at 24 h after the agroinfiltration in agreement with protein levels of St MEK2DD (see Supplemental Figure 1 online) and with the expression profile of RBOHB (Figure 4). Therefore, ROS production was determined at 24 h in the following experiments. We found that Nb MEK1DD caused a modest oxidative burst compared with INF1 or St MEK2DD (Figure 5A). The expression of RBOHB was assessed at the indicated times after inoculation with A. tumefaciens and Agrobacterium infiltration buffer as a control. INF1 and St MEK2DD induced accumulation of RBOHB transcripts, and Nb MEK1DD also significantly activated the gene compared with GUS control (Figure 5B), which agreed with the profile of oxidative burst shown in Figure 5A. VIGS of NTF6 (see Supplemental Figure 3B online) compromised the expression of RBOHB induced by Nb MEK1DD (Figure 5C). These results suggest that the MEK1-NTF6 cascade participates in the RBOHB-dependent oxidative burst.

To confirm this possibility, we silenced RBOHA, which is a constitutively expressing form of the NADPH oxidase, as well as RBOHB in N. benthamiana. The effects of VIGS were monitored by RT-PCR, and both genes were well silenced (see Supplemental Figure 3B online). Silencing RBOHA slightly suppressed ROS generation induced by St MEK2DD, but knockdown of RBOHB eliminated the oxidative burst induced by INF1, St MEK2DD, and Nb MEK1DD to the control level (Figure 5D). These results strongly suggest that in addition to the MEK2-SIPK

**Figure 2.** Effects of VIGS of NOA1 and NOS or NR Inhibitors on NO Burst Induced by INF1 and St MEK2DD.

NO production was estimated as described in Figure 1B. A. tumefaciens–inoculated leaves were infiltrated with cPTIO (500 μM), L-NAME (5 mM), or tungstate (100 μM) for 1 h before infiltration of DAF-2DA. Data are means ± SE from four experiments. Bar = 1 mm.
cascade, the MEK1-NTF6 cascade also participates in the RBOHB-dependent oxidative burst.

Silencing SIPK and NTF6 Compromises the RBOHB-Dependent Oxidative Burst Induced by INF1

Both MEK2-SIPK and MEK1-NTF6 cascades might participate in the RBOHB-dependent oxidative burst (Figure 5). However, the downstream signaling pathways from INF1 to the oxidative burst are not clear. To evaluate the roles of SIPK and NTF6 in the RBOHB-dependent oxidative burst induced by INF1, we silenced SIPK and NTF6 in *N. benthamiana*. The effects of VIGS were monitored by RT-PCR, and each target gene was fully silenced (see Supplemental Figure 3C online). The silenced leaves were inoculated with *A. tumefaciens* containing inf1. The expression of downstream signaling pathways from INF1 to the oxidative burst are not clear. To evaluate the roles of SIPK and NTF6 in the RBOHB-dependent oxidative burst induced by INF1, we silenced SIPK and NTF6 in *N. benthamiana*. The effects of VIGS were monitored by RT-PCR, and each target gene was fully silenced (see Supplemental Figure 3C online). The silenced leaves were inoculated with *A. tumefaciens* containing inf1. The expression of...
examined the effects of silencing NOA1, RBOHB, or both on the basal defense to *P. infestans*, a potent pathogen of *N. benthamiana* (Kamoun et al., 1998), and *C. orbiculare* (syn. *C. lagenarium*), which is the causal agent of cucumber anthracnose disease and is also pathogenic to *N. benthamiana* (Takano et al., 2006). The effects of VIGS were monitored by RT-PCR, and each target gene was well silenced (see Supplemental Figure 3A online). INF1 derived from *P. infestans* induced NOA1-mediated NO and RBOHB-dependent oxidative bursts in *N. benthamiana* leaves, suggesting that NO and oxidative bursts were caused during *P. infestans* and *N. benthamiana* interactions. Supplemental Figure 7 online shows that *C. orbiculare* caused NOA1-mediated NO and RBOHB-dependent oxidative bursts in *N. benthamiana* leaves within 6 h after inoculation.

Zoospores of the virulent isolate of *P. infestans* were inoculated on the surface of the silenced leaves of *N. benthamiana*. NOA1- and NOA1/RBOHB-silenced plants showed yellowish leaves (Figure 7A), which were similar to the leaves of the *Arabidopsis* *noa1* knockout mutant (Guo et al., 2003). The phenotype is distinct from disease symptoms by pathogen infection. Although TRV control leaves showed modest disease symptoms (<20% in the whole leaf), NOA1- and RBOHB-silenced leaves showed mild (20 to 50%) and severe (>50%) disease symptoms, respectively. Double silencing of NOA1 and RBOHB also gave a high susceptibility to *P. infestans* (Figures 7B and 7D). Analysis of *P. infestans* biomass by real-time PCR showed that the growth rate of *P. infestans* matches the severity of the disease symptoms in the leaves (Figure 7D). Furthermore, to rule out the possibility that the insert sequences for VIGS also silenced unknown defense-related sequences, we used 5' terminal regions of the target genes for silencing. The phenotype of the silenced plants was similar to those in Figure 7 (see Supplemental Figure 8B online). These results indicated that RBOHB rather than NOA1 plays an important role in basal defense to *P. infestans*.

*C. orbiculare* conidia were sprayed on the silenced leaves. Small dark-colored spot lesions, which reflect susceptibility, not HR cell death, appeared on the leaves 6 d after the inoculation (Figures 7C and 7E). The number and size of disease spots on NOA1- or NOA1/RBOHB-silenced leaves increased approximately twofold compared with TRV control leaves. The number and size of spots on RBOHB-silenced leaves were similar to those of TRV control leaves. We also used 5' terminal regions of the target genes for silencing and confirmed the phenocopy in these silenced plants (see Supplemental Figure 8C online). These results indicate that NOA1, but not RBOHB, appears to participate in basal defense to *C. orbiculare*.

**DISCUSSION**

In this study, we performed *Agrobacterium*-mediated expression assays and VIGS as gain-of-function and loss-of-function analyses, respectively, and found that the MEK2-SIPK/NTF4 cascade controls the NOA1-mediated NO burst, and MAPK cascades MEK2-SIPK/NTF4 and MEK1-NTF6 regulate the NADPH oxidase-dependent oxidative burst as summarized in Figure 8. Knockdown of NOA1 and RBOHB showed that NO and oxidative bursts participate in defense responses to pathogens.
Figure 5. Nb MEK1DD Induces RBOHB-Dependent Oxidative Burst.

(A) Effects of the expression of inf1, St MEK2DD, and Nb MEK1DD on oxidative burst. N. benthamiana leaves were inoculated with A. tumefaciens containing inf1, St MEK2DD, Nb MEK1DD, or GUS as a control. These different inoculation sites on the same leaf were infiltrated with 0.5 mM L-012 solution (a reagent to detect ROS) 24 h after the inoculation and were monitored using a CCD camera. White circles, whose diameters are ~1.5 cm, indicate areas infiltrated with L-012. Chemiluminescence intensities were quantified by a program equipped with a photon image processor. Data are means ± SE from eight experiments.

(B) Profile of transcript accumulation of RBOHB induced by INF1, St MEK2DD, and Nb MEK1DD. Total RNA was isolated from leaves infiltrated with A. tumefaciens carrying the indicated gene expression constructs or infiltration buffer for infiltration control at the indicated times. Transcript accumulation was monitored by RNA gel blot hybridization; ethidium bromide staining of rRNA is shown as a loading control.

(C) VIGS of NTF6 compromised the expression of RBOHB induced by Nb MEK1DD. Total RNA was isolated from NTF6-silenced leaves infiltrated with A. tumefaciens containing Nb MEK1DD at the indicated times. Transcript accumulation was monitored by RNA gel blot hybridization; ethidium bromide staining of rRNA is shown as a loading control.

(D) Effects of VIGS of RBOHA and RBOHB on oxidative burst induced by INF1, St MEK2DD, or Nb MEK1DD. Silenced leaves inoculated with A. tumefaciens for 24 h were analyzed as described in (A). Data are means ± SE from four experiments.

NOA1 Is Required for NO Burst, and RBOH Is Required for Oxidative Burst in Defense Responses

Many studies have shown that NOS plays a pivotal role in NO synthesis in plants, as well as animals, and is responsible for various stress responses, development, and disease resistance (Guo et al., 2003; He et al., 2004; Zeidler et al., 2004; Zhao et al., 2007). Although arguments exist on the nature of NOA1 (Crawford et al., 2006; Zemotjel et al., 2006), the Arabidopsis knockout mutant noa1 impairs Arg-dependent NO synthesis (Guo et al., 2003; Zhao et al., 2007), increases disease susceptibility to the pathogen P. syringae pv tomato DC3000 (Zeidler et al., 2004), and is highly vulnerable to salt and oxidative stress (Zhao et al., 2007). In this study, we showed that VIGS of N. benthamiana NOA1 compromised INF1-, St MEK2DD-, and pathogen-induced NO bursts. Furthermore, L-NAME, a NOS inhibitor, did not reduce the NO burst in NOA1-silenced plants (Figure 2). Together, these findings show that this gene product can participate in the process of NO production by NOS activity. NOA1 gene expression is not induced by INF1 treatment in N. benthamiana (Kato et al., 2008), suggesting that posttranscriptional control of NOA1-influenced NO production is effected through the MEK2-SIPK/NTF4 cascade.

It has been shown that NR, a key enzyme of nitrogen assimilation, is another enzyme capable of producing NO in plants (Lamattina et al., 2003; Yamamoto et al., 2003). Here, we found that silencing NOA1 did not completely inhibit the NO burst induced by INF1 and St MEK2DD (Figure 2). Furthermore, the NO burst was significantly suppressed by tungstate, a NR inhibitor, in both TRV control- and NOA1-silenced plants (Figure 2). These results suggest that another factor, NR, also participates in the NO burst induced by INF1 and St MEK2DD in agreement with the previous finding that NR1 partially contributes to the INF1-induced NO burst in N. benthamiana (Yamamoto-Katou et al., 2006). However, the NOA1 silencing concomitant treatment with tungstate did not result in complete inhibition of the NO generation, suggesting that the nonenzymatic NO generation system also contributes to the NO burst, as reported by Bethke et al. (2004).

Defense-related RBOHs, which play a pivotal role in ROS production in response to pathogen signals, seem to be regulated at the transcriptional and posttranslational levels. We indicated previously that a calcium-dependent protein kinase (St CDK5) activates potato RBOHs by direct phosphorylation of the N-terminal regions (Kobayashi et al., 2007). CDPK appears to regulate a rapid and transient accumulation of hydrogen peroxide (H2O2; phase I burst) and a massive oxidative burst at 6 to 9 h after elicitor treatment (phase II burst; Yoshioka et al., 2001; Kobayashi et al., 2007). Liu et al. (2007) reported that conditional gain-of-function by St MEK2DD causes rapid shutdown of carbon fixation reaction in chloroplasts, which could lead to the generation of ROS in chloroplasts under illumination. They concluded that the chloroplast burst occurs earlier than the NADPH oxidase-dependent oxidative burst by MAPK (phase II burst) and that the chloroplast-generated ROS are only a facilitator that accelerates cell death because plants cells without mature chloroplasts die eventually. They suggested that mitochondria-generated ROS might be essential in accelerating the cell death.
process. Communication between chloroplasts and mitochondria exists in cells undergoing HR cell death (Yao et al., 2004). Mitochondrial dysfunction can be caused by MEK2DD. MEK2DD-mediated dysfunction can be prevented by Bcl-xL, which is a mammalian anti-apoptotic factor that can prevent mitochondrial dysfunction in plants, the same as in animals (Takabatake et al., 2007). We routinely use the chemiluminescence probe L-012 to detect RBOH-generated ROS in N. benthamiana leaves. The probe has been used for the analysis of ROS generated by membrane-bound NADPH oxidase in neutrophils (Imada et al., 1999). In this study, we failed to detect rapid chloroplast- and mitochondria-generated ROS in elicited plants after treatment with INF1, St MEK2DD, Nb MEK1DD (Figure 5D), and fungal infection (see Supplemental Figure 7 online), suggesting that our method is suitable to detect apoplastic ROS generated by membrane-bound RBOH in plant cells.

We propose that early chloroplast-generated ROS caused by Nt MEK2DD and phase I burst by elicitors might contribute to the influx of Ca\textsuperscript{2+} into cytoplasm and that the increased level of Ca\textsuperscript{2+} might result in activation of CDPK as an inducer of the phase II burst from NADPH oxidases localized in the plasma membrane.

**Figure 6.** SIPK, NTF4, and NTF6 Participate in the Oxidative Burst and the Expression of RBOHB Induced by INF1.

(A) N. benthamiana leaves were inoculated with the gene silencing constructs TRV:NTF6 (NTF6), TRV:SIPK/NTF6 (S/NTF6), or TRV alone as a control. Total RNA was isolated from silenced leaves infiltrated with A. tumefaciens containing inf1 at the indicated times. Accumulation of RBOHB mRNA was monitored by mRNA gel blot hybridization; ethidium bromide staining of rRNA is shown as a loading control.

(B) Effects of VIGS of SIPK (S), NTF6 (NTF6), or SIPK/NTF6 (S/NTF6) on the oxidative burst induced by INF1 or GUS as a control. Silenced leaves inoculated with A. tumefaciens for 24 h were analyzed as described in Figure 5A. Data are means ± SE from twelve experiments.

(C) Transcript accumulation of RBOHB induced by St MEK2DD, SIPK, and NTF4. Total RNA was isolated from leaves 24 h after inoculation with A. tumefaciens carrying expression constructs for the indicated proteins and was used for RT-PCR with specific primers for RBOHB.

(D) TRV control- and RBOHB-silenced leaves were inoculated with A. tumefaciens containing the indicated gene expression constructs. ROS were measured 24 h after the inoculation as described in Figure 5A. Data are means ± SE from four experiments.

**Crosstalk between Two MAPK Cascades and Radical Bursts**

The expression and activation of the *Arabidopsis* Ser/Thr kinase OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) are induced in response to H\textsubscript{2}O\textsubscript{2} (Rentel et al., 2004). OXI1 is required for activation of MAPKs (MPK3 and MPK6, orthologs of WIPK and SIPK, respectively, in *Arabidopsis*) after treatment with H\textsubscript{2}O\textsubscript{2} or an elicitor. In this study, we showed that in *N. benthamiana* the MEK2-SIPK cascade regulates the oxidative burst resulting from the induction of RBOHB expression (Figures 4 to 6). Together, we can assume the existence of a positive feedback circuit between SIPK and RBOHB. However, little is known about the crosstalk between MAPK cascades and NO production. In this study, we showed that the MAPK cascade MEK2-SIPK regulates the NO burst. NO also activates potential MAPKs, as shown by an in-gel kinase assay using MBP as a substrate (Clarke et al., 2000). SIPK may give a positive feedback between NO burst signals as well as oxidative burst signals.

A novel MAPKK (MKK1) has been identified as an effective inducer of HR-like cell death in *N. benthamiana*. MKK1 activates SIPK, and MKK1-mediated cell death is compromised by silencing SIPK (Takahashi et al., 2007). The transient expression of SIPK is sufficient to induce HR-like cell death and the expression of 3-hydroxy-3-methylglutaryl CoA reductase, a gene encoding a key enzyme in the phytoalexin biosynthesis pathway (Zhang and Liu, 2001). This study showed that SIPK regulates the expression of RBOHB and the NO burst. These results strongly indicate that SIPK plays a central role in multiple defense responses. However, our results also showed that silencing WIPK slightly compromises the NO burst induced by INF1 and St MEK2DD (Figure 3B). WIPK could function in accelerating SIPK-mediated cell death or in initiating a new pathway (Liu et al., 2003). WIPK might function as a factor to commit SIPK-mediated NO. NTF4 is a tobacco MAPK that reveals high sequence similarity to SIPK (93.6% identity) and a similar expression pattern and activation profile of SIPK (Ren et al., 2006). The conditional expression of
Figure 7. Effects of VIGS of NOA1 and RBOHB on Susceptibility to P. infestans and C. orbiculare.

(A) Effects of silencing of NOA1, RBOHB, or NOA1/RBOHB (N/B) on the phenotype of the leaves. Silenced leaves were photographed 3 weeks after initiation of VIGS by TRV infection with no additional challenge.

(B) Susceptibility to P. infestans in the silenced plants. Silenced leaves were inoculated with drops of a P. infestans zoospore suspension ($2 \times 10^5$ zoospores/mL). Inoculated leaves were photographed 4 d after the inoculation. Red boxes: close-up shown in lower photographs.
Figure 7. (continued).

(C) Susceptibility to C. orbiculare in the silenced plants. Silenced leaves were inoculated with C. orbiculare conidial suspension (1 × 10^6 conidia/mL) using a vaporizer. Inoculated leaves were photographed 6 d after the inoculation. Red boxes: close-up shown in lower photographs.

(D) Evaluation of disease symptom and biomass of P. infestans. Disease symptoms were evaluated by the following scoring system: disease symptom as percentage of whole leaf: 0, <20%; 1, 20 to 50%; 2, >50%. Data are means ± SE from seven experiments. To determine the biomass of P. infestans, real-time PCR was performed with P. infestans–specific primers using DNA isolated from inoculated leaves in (B). Data are means ± SE from four experiments.

(E) Number and size of lesion spots counted 6 d after inoculation with C. orbiculare. Diameter of lesion spots was recorded using the following scoring system: 0, <1 mm; 1, 1 to 2 mm; 2, >2 mm. Data are means ± SE from eight experiments.
diversified effects of ROS on disease resistance dependent on each host–parasite interaction. Phytophthora species, such as Phytophthora brassicae (Roetschi et al., 2001) and several isolates of Phytophthora cinnamomi (Robinson and Cahill, 2003), can infect Arabidopsis. On the other hand, P. infestans cannot infect Arabidopsis (Huitema et al., 2003) and instead induces HR cell death accompanied by an oxidative burst in the attacked cell (Kobae et al., 2006). Transgenic potato plants expressing a H2O2-generating glucose oxidase are resistant to P. infestans (Wu et al., 1995). This result supports the idea that ROS play an important role in disease resistance to P. infestans. We believe that each pathogen has diverse strategies for infection. For instance, recent work has shown that Ustilago maydis, a biotrophic pathogen of maize (Zea mays), uses a redox sensor protein that controls the H2O2 detoxification system for coping with early plant defense responses (Molina and Kahmann, 2007). In the barley (Hordeum vulgare)–Blumeria graminis interaction, silencing Hv RBOH A, a barley ortholog of Arabidopsis RBOHF, during the penetration process of B. graminis leads to increased penetration resistance (Trujillo et al., 2006), which could be due to a decrease in host cell wall softening usually induced by plant RBOH-mediated superoxide (O2•−).

ROS and NO are believed to play important roles independently or coordinately in plant innate immunity. ROS generated on the plasma membrane are released to the apoplast, inducing oxidative crosslinking of glycoproteins, strengthening the cell wall against secondary infection (Bradley et al., 1992) and simultaneously activating the Ca2⁺ channel to increase the level of cytosolic Ca2⁺ (Lecourieux et al., 2002). Ca2⁺ may function not only as an inducer of the oxidative burst but also as a signaling molecule downstream of the oxidative burst that causes various cellular responses, including defense (Torres and Dangl, 2005). However, NO signaling includes various messenger molecules, such as cGMP, cADP ribose, and Ca2⁺ (Durner et al., 1998; Wendehenne et al., 2001; Lamotte et al., 2004; Romero-Puertas et al., 2004), which both directly and indirectly modulate the expression of specific genes (Polverari et al., 2003; Parani et al., 2004). NO signaling pathways often include posttranslational modification of target proteins, such as NO-dependent Cys S-nitrosylation that can modulate the activity and function of different proteins (Sokolovski and Blatt, 2004; Feechan et al., 2005; Lindemayr et al., 2005; Romero-Puertas et al., 2007). NO can also react with O2•− to form the reactive molecule peroxynitrite (ONOO−). ONOO− can lead to formation of NO2 and the hydroxyl radical (OH•). OH• is a very strong oxidizing species that can rapidly attack biological membranes and all types of biomolecules, such as DNA and proteins, leading to irreparable damage, metabolic dysfunction, and cell death (del Rio et al., 2003). ONOO− is also responsible for Tyr nitration (Lammattina et al., 2003), which is the major toxic reactive nitrogen species in animal cells (Stamler et al., 1992). We reported previously that treatment of BY-2 tobacco cells with INF1 induces ONOO− generation, and we investigated Tyr nitration as a direct reaction of ONOO− (Saito et al., 2006); that study supported the idea that NO and O2•− are produced simultaneously through a convergent signaling pathway, that is, MAPK cascades in plants. ONOO− is relevant to HR and defense gene expression (Alamillo and García-Olmedo, 2001). One study emphasized that the combination of NO and H2O2, but not ONOO−, takes part in the induction of defense responses (Delledonne et al., 2001). NO or ROS are not essential for HR in plants but induce apoptosis in adjacent cells during the defense response (Tada et al., 2004). HR cell death may require a fine balance between NO and ROS (Delledonne et al., 2001). In this study, we showed that double silencing of RBOHB and NOA1 does not confer a synergistic effect on resistance to P. infestans and C. orbiculare compared with individual silencing of these genes (Figure 7; see Supplemental Figure 8 online), suggesting that ONOO− might not be required for full defense responses to at least these two pathogens.

In regulating gene expression, NO can induce the expression of PAL and chalcone synthase independently of ROS, and induction by NO of defense-related genes, such as glutathione S-transferase, depends on H2O2 (Grun et al., 2006). In abscisic acid signal transduction for stomatal closure, NO and H2O2 induced by abscisic acid individually inhibit stomatal opening specific to blue light (Bright et al., 2006; Zhang et al., 2007). However, the cooperative and individual roles of NO and ROS during disease resistance are unclear. Transgenic potato plants expressing St MEK2DD fused to a pathogenic-inducible promoter are resistant to both the biotrophic pathogen P. infestans and the necrotrophic pathogen Alternaria solani, which causes early blight of Solanaceae plants, such as potato and tomato (Solanum lycopersicum) (Yamamizo et al., 2006). However, our preliminary data indicate that transgenic potato plants carrying St CDPK5VK driven by the same pathogen-inducible promoter show high resistance to P. infestans but high susceptibility to A. solani. St CDPK5VK induces only ROS production (Kobayashi et al., 2007), and in this study, we showed that St MEK2DD induces both NO and ROS. These results support the results of this study that silencing NOA1 or RBOHB distinctly affects susceptibility to P. infestans and C. orbiculare. Plants may have obtained during evolution the signaling pathway regulating both NO and ROS production to adapt to a wide spectrum of pathogens.

**METHODS**

**Plant Growth Conditions**

Nicotiana benthamiana plants were grown at 25°C and 70% humidity under a 16-h photoperiod and an 8-h-dark period in environmentally controlled growth cabinets.

cDNA Cloning of Nb MEK1 and Site-Directed Mutagenesis

The cDNA of Nb MEK1 was amplified using PCR from an N. benthamiana cDNA library as a template (Yoshioka et al., 2003) using the following primers corresponding to the first 30 nucleotide sequences and 3′-terminal 30-nucleotide sequences of NT MEK1, respectively: forward 5′-ATGAGGAGCAGGAGGCATTTGAAGCATT-3′ and reverse 5′-TTATCTTTGAAAATTACGAGGGTTGCAG-3′. The PCR products were cloned into the TOPO plasmid vector (TOYOBO) and were sequenced. The constitutively active mutant of Nb MEK1, Nb MEK1DD, with the conserved Ser-219 and Thr-225 replaced by Asp, was generated using a Mutan-Super Express Km kit ( Takara). The sequences of the PCR products and Nb MEK1DD were verified by
sequencing with an ABI Prism BigDye Terminator Cycle Sequence kit (Perkin-Elmer).

**Agrobacterium tumefaciens**-Mediated Transient Expression in *N. benthamiana*

The cDNA fragment of constitutively active mutant Nb MEK1D0 was cloned into the pGreen binary vector (Hellens et al., 2000), in which a HA-tag was added to the C-terminal end. Nb MEK1D0, St MEK2D0 (Katou et al., 2003), and GUS, which was used as a control for the effect of *Agrobacterium* infiltration, were preceded by the 35S promoter of *Caulliflower mosaic virus*, a 5′-untranslated region was replaced with the ω sequence from the TMV, and the nopaline synthase terminator region was on the 3′-end of the gene (Jones et al., 1992).

Binary plasmids were transformed into *Agrobacterium* strain GV3101, which included the transformation helper plasmid pSoup (Hellens et al., 2000) by electroporation. The overnight culture was diluted 10-fold in LB/kanamycin/nitromycin/tetracycline and was cultured to OD600 0.6. The cells were harvested by centrifugation and were resuspended in 10 mM MES-NaOH, pH 5.6, and 10 mM MgCl2. The suspensions were adjusted to OD600 0.5, and acetylsyringone was added to 150 μM. The bacterial suspensions were incubated for 2 to 3 h at 22°C and then were infiltrated into leaves of 4- to 5-week-old *N. benthamiana* plants using a needleless syringe (Yoshioka et al., 2003). *A. tumefaciens* carrying inf1 was prepared as described by Kamoun et al. (2003).

**VIGS**

VIGS was done as described by Ratcliffe et al. (2001). The following primers were used to amplify cDNA fragments from *N. benthamiana* using the *N. benthamiana* cDNA library as a template (Yoshioka et al., 2003). Restriction sites were added to the 5′-end of forward or reverse primer for cloning into a TRV vector pTV00 (RNA2): Nb MEK1-F-ACcl (5′-CCGGTGCACCTCA-GAAACATAGGATAGACATTCTGC-3′) and Nb MEK1-R-Cal (5′-CCATCGAT-AAAACCTGCTTGCAAACAACTG-3′) (restriction sites are underlined), which produced a 363-bp fragment; Nb MEK2-F-Cal (5′-CCATCGATA-GATGTCGCCTGAGATG-3′) and Nb MEK2-R-Cal (5′-CGCACTGATGCTCAGTGTGCAAATCGA-3′), which produced a 261-bp fragment; Nb NOA1-F-BamHI (5′-CCGGATCCCTGCTTGCAAACAAAAAGATGACG-3′) and Nb NOA1-R-BamHI (5′-CCATCGATGTTGCTCTCTGATAGGACG-3′), which produced a 478-bp fragment; Nb WIPK-F-BamHI (5′-CCGGATCCCAAGAAGACCGTCAAACAAAAAGATGACG-3′) and Nb WIPK-R-BamHI (5′-CGCACTGATGCTCAGTGTGCAAATCGA-3′), which produced a 332-bp fragment; Nb SIPK-F-Clal (5′-CCATCGATTGATGTCGCCTGAGATG-3′) and Nb SIPK-R-Clal (5′-CCATCGCATGCTCCAGTGTGCAATCGA-3′), which produced a 500-bp fragment; Nb RBOHA-B-Clal (5′-CCAGAATCGATGATGTCGCCTGAGATG-3′) and Nb RBOHA-R-BamHI (5′-CGGGATCCATGATGTCGCCTGAGATG-3′), which produced a 500-bp fragment; and Nb NTf6-F-ACcl (5′-CCGGATCCCTGCTTGCAAACAAAAAGATGACG-3′) and Nb NTf6-R-Clal (5′-CCATCGATGCTCAGTGTGCAAATCGA-3′), which produced a 535-bp fragment.

NO Measurements

NO accumulation was monitored using NO-sensitive dye DAF-2DA (Daichi Pure Chemicals). Leaves were infiltrated with 200 mM sodium phosphate buffer at pH 7.4, including 12.5 μM DAF-2DA using a needleless syringe and were incubated for 1 h in the dark at room temperature before observation. Fluorescence from diaminotriazolofluorescein (DAF-2T), the reaction product of DAF-2DA, was used to obtain a fluorescent stereomicroscope (MZ FLII; Leica) equipped with a CCD camera. The excitation was at 470 nm, and the emission images at 525 nm were obtained at constant acquisition time. The fluorescence intensity of the digital image was determined by color histogram analysis of Photoshop version 7.0 (Adobe).

**ROS Measurements**

ROS measurements were done as described by Kobayashi et al. (2007). The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence. The 0.5 mM L-012 in 10 mM MOPS-KOH at pH 7.4 was infiltrated into *N. benthamiana* leaves using a needleless syringe. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera (Aquacosmos 2.5; Hamamatsu Photonics) and quantified using the U7501 program (http://jp.hamamatsucm.com/index).
RT-PCR
Total RNA from N. benthamiana leaves was prepared using TRizol reagent according to the procedure of the manufacturer (Invitrogen). RT-PCR was conducted using a commercial kit (ReverTra Ace -α-) TOYOBO). PCR was performed by ExTaq (Takara) with denaturing, annealing, and extension temperatures of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, respectively; for 26 cycles. Gene-specific primers of each sequence were as follows: Nb WIPK (5'-TACGGAGCATGAAAGTG-3') and 5'-CATGTGGAACATTTTCTGAG-3'), Nb NTF4 (5'-ACAGAACACAGCTCTCTCCTC-3'), and 5'-CATGTGGAATACTTTTCTTGCTTT-3'), as a control for equal cDNA amounts in each reaction. PCR was done with primers for EF-1α for 26 cycles (5'-CTCTAAAGGAAGTGATACAC-3' and 5'-CTCTTGGCTCATTAATGCTGTC-3'). The PCR products were separated on a 1.8% agarose gel and were visualized after ethidium bromide staining.

RNA Gel Blot Hybridization
Total RNA from N. benthamiana leaves was prepared as described for RT-PCR. RNA gel blot hybridization was done as described by Yamamizo et al. (2006). The probe for Nb WIPK was a 311-bp cDNA fragment derived from the primers used for RT-PCR. The probes for Nb SIPK and Nb RBOHB were 539-bp and 1350-bp cDNA fragments, respectively, derived from the following primers: Nb SIPK (5'-ACACGGATGATCTGATGCG-3') and 5'-ATGCAAAATCCTGAAACTCACGCACAC-3') and Nb RBOHB (5'-AGGAGCTGAAATCACCAGTATC-3') and 5'-AAAGTTTCCATCAAAAGGGAAGCACACAC-3'). Ethidium bromide-stained gels were used to show equal loads of RNA.

Fungal Strain, Media, and Conditions
Phytophthora infestans race 1.2.3.4 was maintained on susceptible potato (Solanum tuberosum) tubers. A suspension of P. infestans zoospores was prepared as described by Yoshioka et al. (1996). Detached zoospores were inoculated with a 1-mL drop of zoospore suspension (2 × 10^6 zoospores/mL) using a lens paper to disperse the zoospores. The inoculated leaves were kept at high humidity at 20°C.

Colletotrichum orbiculare strain 104-T was maintained on a potato dextrose agarose medium at 23°C in the dark. Susensions of the conidia at concentration 1 × 10^5 conidia/mL were sprayed onto attached N. benthamiana leaves. The inoculated plants were kept at high humidity in the dark at 23°C for 48 h and then under a 16-h photoperiod and an 8-h dark period at 23°C.

Determination of P. infestans Biomass
Quantitative real-time PCR was applied to determine the growth of P. infestans on N. benthamiana leaves. DNA was isolated from Nb infestans--inoculated leaves using the DNeasy Plant Mini Kit (Qiagen). The primers O8-3 (5'-GAAAGGCTAGGTGAGTGAG-3') and O8-4 (5'-TAACCGACAACTGATGAA-3') and EF-1α used for RT-PCR were used to amplify and detect a P. infestans--specific DNA sequence (Judelson and Tookey, 2000) and plant DNA, respectively, in the inoculated leaves. Quantitative PCR was performed using SYBR Premix Ex Taq (Takara) on a LightCycler 480 instrument (Roche Diagnostics). For each PCR, samples were prepared according to the procedure of the manufacturer. Samples were run for 40 cycles under the following thermal cycling protocol. Samples were preheated at 95°C for 10 s. Then, 40 amplification cycles were run: 5 s at 95°C and 20 s at 60°C. Fluorescence (520 nm) was detected at the end of the elongation phase for each cycle. Following the final amplification cycle, a melting curve was acquired by heating to 95°C, cooling to 65°C, and slowly heating to 95°C at 0.1°C/s with continuous measurement of fluorescence at 520 nm. Relative amounts of P. infestans DNA were determined by dividing plant DNA amounts derived from EF-1α in inoculated leaves. Therefore, pathogen biomass is given as relative units.

Accession Numbers
Sequence data from this article can be found in the GenBank/ EMBL/ DDBJ data libraries under accession numbers AB360634 (Nb NF6), AB360635 (Nb MEK1), AB360636 (Nb MEK2), AB373025 (Nb SIPK), and AB373026 (Nb NF4). The accession numbers for the other sequences mentioned in this article are as follows: St MEK2, AB091780; Nt SIPK, U94192; Nt NF4, X83880; Nb WIPK, AB098729; Nt WIPK, D61377; Nt NOA1, AB303300; Nb RBOHA, AB079498; Nb RBOHB, AB079499; At MPK6, D21842.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Time Course of Changes in NO and ROS after Inoculation with A. tumefaciens.
Supplemental Figure 2. VIGS of Nb MEK2 Suppressed the Transcript Accumulation of St MEK2 Transgene.
Supplemental Figure 3. Effects of VIGS on Nb NOA1, Nb RBOHB, Nb NF6, Nb RBOHA, and Nb SIPK.
Supplemental Figure 4. Effects of L-NAME and D-NAME on NO Burst Induced by INF1.
Supplemental Figure 5. Sequence Alignment between Nb NTF4 and Nb SIPK, and Phylogenetic Analysis of Related MAPKs.
Supplemental Figure 6. L-012 Is a Highly Sensitive Reagent for ROS Detection.
Supplemental Figure 7. Inoculation of C. orbiculare--Induced Nb NOA1–Mediated NO Burst and Nb RBOHB–Dependent Oxidative Burst in N. benthamiana.
Supplemental Figure 8. Effects of Silencing Nb NOA1 and Nb RBOHB on Disease Resistance to P. infestans and C. orbiculare.
Supplemental Data Set 1. Text File of Alignment Corresponding to the Phylogenetic Tree in Supplemental Figure 5.

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MAPK Signaling Regulates Nitric Oxide and NADPH Oxidase-Dependent Oxidative Bursts in *Nicotiana benthamiana*
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