Nitric Oxide as a Partner of Reactive Oxygen Species Participates in Disease Resistance to Necrotrophic Pathogen *Botrytis cinerea* in *Nicotiana benthamiana*

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Nitric oxide (NO) is an essential regulatory molecule in plant immunity in synergy with reactive oxygen species (ROS). However, little is known about the role of NO in disease resistance to necrotrophic pathogens. NO and oxidative bursts were induced during necrotrophic fungal pathogen *Botrytis cinerea* and *Nicotiana benthamiana* compatible interaction. Histochemical analyses showed that both NO and ROS were produced in adjacent cells of invaded areas in *N. benthamiana* leaves. Activation of salicylic acid-induced protein kinase, which regulates the radical burst, and several defense-related genes were induced after inoculation of *B. cinerea*. Loss-of-function analyses using inhibitors and virus-induced gene silencing were done to investigate the role of the radical burst in pathogenesis. We showed that NO plays a pivotal role in basal defense against *B. cinerea* and *PR-1* gene expression in *N. benthamiana*. By contrast, ROS function has a negative role in resistance or has a positive role in expansion of disease lesions during *B. cinerea*–*N. benthamiana* interaction.

Plants have evolved a variety of defense mechanisms to protect themselves against microbial colonization. Plants rely on basal defense by using a much less-specific recognition system that identifies pathogen-associated molecular patterns to prevent penetration and to restrict growth of pathogens (Jones and Dangl 2006). In response to pathogens that avoid, tolerate, or suppress basal defense, plants have evolved resistance (R) proteins that trigger a battery of inducible defense responses as typified by hypersensitive response (HR) upon pathogen recognition, hence gene-for-gene resistance. Cell death during HR is thought to deny nutrients to invading biotrophic pathogens, which can parasitize living plant cells, and to be dependent on balanced production of nitric oxide (NO) and reactive oxygen species (ROS) (Delledonne et al. 2001). NO and ROS are produced simultaneously through a mitogen-activated protein kinase (MAPK)-dependent pathway and play a pivotal role in basal defense (Asai et al. 2008), supported by the view that the MAPK cascade is a central signaling pathway in basal defense as well as in R protein-dependent resistance (Jones and Dangl 2006).

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NO and ROS are important signaling molecules that are rapidly generated after recognition of pathogens, called NO burst and oxidative burst, respectively (Hong et al. 2008; Torres et al. 2006; Wendehenne et al. 2004). In animals, NO is generated by NO synthase (NOS), which converts L-arginine (Arg) to L-citrulline and NO. In plants, two major routes are proposed for NO formation, reduction of nitrite to NO by nitrate reductase and oxidation of Arg to citrulline by NOS. 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 (Butt et al. 2003; Garcia-Mata and Lamattina 2003). Guo and associates (2003) identified a NOS-like enzyme from *Arabidopsis thaliana* (AtNOS1), based on homology to a hypothetical snail NOS. However, AtNOS1 protein has no NOS activity (Zemojtel et al. 2006); therefore, AtNOS1 was renamed AtNOA1 for NO ASSOCIATED1 (Crawford et al. 2006). Recent studies showed that AtNOA1 has circularly permuted GTPase activity (Moreau et al. 2008; Sudhamsu et al. 2008). The function of AtNOA1 remains to be resolved. 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 knocking down *NOA1* expression provides a powerful tool to analyze NO function.

Plasma membrane NADPH oxidase is a key generator of ROS (Kobayashi et al. 2006). Plant NADPH oxidases designated as respiratory burst oxidase homologs (RBOH) have been identified as genes related to mammalian gp91^{phox} (Torres and Dangl 2005; Yoshioka et al. 2001). Genetic evidences for RBOH function in the pathogen-induced oxidative burst have been provided by knocking out or knocking down *RBOH*. Double mutants of *Arabidopsis rbohD* and *rbohF* genes show greatly reduced ROS production in response to infection by avirulent *Pseudomonas syringae* pv. *tomato* DC3000 and *Hyaloperonospora parasitica* (Torres et al. 2002). Analysis of the loss of function of *NtRBOHD* in tobacco cells (*Nicotiana tabacum*) shows loss of ROS production by elicitor treatment (Simon-Plas et al. 2002). *NtRBOH*-silenced *N. benthamiana* plants show less ROS production in response to infection by the oomycete *Phytophthora infestans* (Yoshioka et al. 2003). These studies suggest that RBOH is a key regulator of ROS production during defense response.

Most experimental data available on the detection and roles of NO and ROS during plant-pathogen interactions are from

studies of infections by potential biotrophic pathogens, including bacteria and viruses. NO and oxidative bursts in response to avirulent *P. syringae* were observed in soybean and *Arabidopsis* suspension-cultured cells (Clarke et al. 2000; Delledonne et al. 1998). Infiltration of mammalian NOS inhibitors into leaves of *Arabidopsis* ecotype Col-0, which contains the *RPM1* resistance gene, leads to reduced accumulation of NO and increased growth of *P. syringae* pv. *maculicola* carrying the corresponding *avrRpm1* avirulence gene (Delledonne et al. 1998). The *Arabidopsis* *noal* knockout mutant shows reduction in NO production and basal defense in response to lipopolysaccharides, and an increase in susceptibility to the virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Zeidler et al. 2004). Silencing *NbrBOHA* and *NbrBOHB* in *N. benthamiana* reduces oxidative burst and disease resistance to *P. infestans* (Yoshioka et al. 2003). These results suggest that the radical burst participates in disease resistance to biotrophic pathogens. However, the roles of radical burst in plant defense responses against necrotrophic pathogens are not fully understood.

The necrotrophic pathogenic fungus *Botrytis cinerea*, which causes gray mold disease on many dicotyledon plants, secretes a spectrum of phytotoxins and lytic enzymes to kill cells from a wide spectrum of plants (van Kan 2006). Govrin and Levine (2000) proposed that cell death induced by *B. cinerea* is preceded by accumulation of ROS and is an important component of virulence. Increased levels of ROS in plants seem to cause more aggressive infection of *B. cinerea* and result in an accelerated colonization of host tissue (Govrin and Levine 2000; Tiedemann 1997). *B. cinerea* infection can lead to not only oxidative burst but also depletion of antioxidants in infected

and uninfected tissues (Muckenschnabel et al. 2002). Changes in redox balance correspond with a shift toward host cell death and, thus, increased susceptibility to *B. cinerea*. However, ROS production does not always result in increased susceptibility, because failure or success of infection by *B. cinerea* appears to depend on the timing and the intensity of ROS production (Asselbergh et al. 2007; Kunz et al. 2006). *B. cinerea* endo-polygalacturonase 1 (BcPG1), first known as a virulence factor (ten Have et al. 1998), elicits cell death and several defense responses, including production of NO and ROS and activation of MAPK (Kars et al. 2005; Poinssot et al. 2003; Vandelle et al. 2006). BcPG1-overproducing mutants showing decreased pathogenicity to grape and bean induce a significant and transient ROS production on the leaves (Kunz et al. 2006). Thus, there are several arguments about the role of ROS in defense responses during plants and *B. cinerea* interactions, and little is known about the role of NO as a counterpart of ROS in disease resistance to necrotrophic pathogens.

The MAPK tobacco salicylic acid (SA)-induced protein kinase (SIPK) simultaneously regulates production of NO and ROS in *N. benthamiana* (Asai et al. 2008). Activation of MAPK and the radical burst appear to be induced during disease development by *Botrytis* species (Ren et al. 2008; van Baarlen et al. 2004). In this study, we investigated the role of NO and oxidative bursts in basal defense against *B. cinerea* in *N. benthamiana*. Histochemical analysis showed that NO and ROS are produced and participate in symptom development of *B. cinerea* in *N. benthamiana*. We also showed that activation of SIPK and several defense-related genes are induced after inoculation of *B. cinerea*. Loss-of-function analyses using in-

Fig. 1. The radical burst during *Botrytis cinerea* and *Nicotiana benthamiana* interaction. *N. benthamiana* leaves were inoculated with a drop of *B. cinerea* conidia suspension (5×10^5 conidia/ml), and the results were photographed at **A** through **D**, 2 h postinoculation (hpi) or **E** through **L**, 2 days postinoculation (dpi). Inoculated leaves were stained with trypan blue (**A**, **F**, and **J**) or with 3,3'-diaminobenzidine (**B**, **G**, and **K**) and were treated with 4,5-diaminofluorescein diacetate (DAF-2DA) (**C**, **H**, and **L**). **C** and **D** are images at the same position of leaves treated with DAF-2DA, comparing diaminotriazolofluorescein fluorescence (**C**) with a differential interference contrast image (**D**). **I**, Autofluorescence on the DAF2-DA nontreated leaves shown in **H**. **J** through **L** show close-up images of the areas boxed in red in **F** through **H**. **A** through **D**, c = conidia; h = hyphae; ih = invasion hyphae; s = stoma. **E** through **I**, green bars indicate infected areas, yellow bars indicate invaded areas, and red bars indicate response areas. Scale bars = 50 μ m (**A** through **D** and **J** through **L**), 1 mm (**E** through **I**).

hibitors and virus-induced gene silencing (VIGS) showed that NO plays a key role, but ROS have an opposite effect in basal defense against *B. cinerea*.

RESULTS

Induction of NO and oxidative bursts during *B. cinerea*–*N. benthamiana* interaction.

We evaluated whether NO and ROS are induced by inoculation with *B. cinerea* in *N. benthamiana* leaves. To observe *B. cinerea* infection, the inoculated leaves were stained by trypan blue, which stains fungal hyphal structures as well as dead cells. At 12 h postinoculation (hpi), most *B. cinerea* conidia had germinated and some had penetrated into plant cells (Fig. 1A). Hydrogen peroxide (H_2O_2) and NO were detected by 3,3'-diaminobenzidine (DAB) staining and 4,5-diaminofluorescein diacetate (DAF-2DA)-mediated fluorescence, respectively. As a result, accumulation of H_2O_2 and NO was observed in cells that *B. cinerea* attempted to penetrate (Fig. 1B through D). Disease lesions appeared on leaves at 2 days postinoculation (Fig. 1E). Based on the observation of hyphal elongation of *B. cinerea*, and situation of plant cells, we divided disease lesions into three areas as shown in Figure 1E through I and Supplementary Figure 1. These areas were defined as i) infected areas, the center of lesions, indicating massive hyphae and dead cells resulting from infection; ii) invaded areas, edge of lesions, indicating the apical part of hyphae and progressive cell death; and iii) response areas, regions adjacent to lesion that indicated living cells. In infected areas, accumulation of H_2O_2 and NO was not observed (Fig. 1F through H). Fluorescence in infected areas and invaded areas without DAF-2DA indicates autofluorescence resulting from fungal infection (Fig. 1I). Hyphae of *B. cinerea* (Fig. 1J) and little accumulation of H_2O_2 and NO (Fig. 1G and H) were in invaded areas. Marked accumulation of H_2O_2 and NO was found in the response areas (Fig. 1K and L). These results indicate that NO and oxidative bursts are induced before *B. cinerea* invasion.

Inhibition of NO and oxidative bursts affects susceptibility to *B. cinerea*.

To evaluate the effects of the radical burst on susceptibility to *B. cinerea*, we used diphenylene iodonium (DPI), known as an inhibitor of not only NADPH oxidase (Govrin and Levine 2000) but also of NOS (Ma et al. 2008), and N^G -nitro-L-Arg-methyl ester (L-NAME), a NOS inhibitor. *B. cinerea* conidia were inoculated in drops onto leaf areas infiltrated with inhibitors or with H_2O as a control 1 h before inoculation. NO burst was detected by DAF-2DA-mediated fluorescence, and the inducible fluorescence was eliminated by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger (Fig. 2A and B), indicating that DAF-2DA detects NO. Timecourse experiments showed that the DAF-2DA-mediated fluorescence was slightly detected at 6 hpi and peaked at 12 hpi (Supplementary Fig. 2). We did not observe autofluorescence without DAF-2DA at this stage. Therefore, DAF-2DA-mediated fluorescence intensities were measured at 12 hpi in the following experiments. DPI treatment slightly suppressed NO burst induced by *B. cinerea* inoculation as compared with H_2O treatment (Fig. 2A and B). However, L-NAME-infiltrated leaf areas showed marked reduction of NO burst (Fig. 2A and B). D-NAME, an inactive isomer of L-NAME, had no effect on NO burst (Fig. 2A and B). These results suggest that NOS-like NO-generating activity might participate in NO burst induced by *B. cinerea* inoculation.

To detect ROS induced by *B. cinerea*, inoculated leaves were infiltrated with L-012 solution, a highly sensitive and

specific agent to detect ROS (Asai et al. 2008; Kobayashi et al. 2007), and were observed by using a sensitive CCD camera. Timecourse experiments showed that L-012-mediated chemiluminescence was detected at 12 hpi but not at 6 hpi; thereafter, much production of ROS was observed at 24 hpi (Supplemen-

Fig. 2. Effects of inhibitors on the radical burst during *Botrytis cinerea* and *Nicotiana benthamiana* interaction. *N. benthamiana* leaves were infiltrated with diphenylene iodonium (DPI) (50 μ M), D-NAME (5 mM), L-NAME (5 mM), or H_2O as a control 1 h before inoculation and were then inoculated with *B. cinerea* or potato dextrose broth (Control). **A**, At 12 h postinoculation (hpi), the inoculated leaf areas were infiltrated with 4,5-diaminofluorescein diacetate (DAF-2DA) solution and were then monitored 1 h later using fluorescence stereomicroscopy. In the case of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), inoculated leaf areas were infiltrated with cPTIO (500 μ M) 1 h before infiltration of DAF-2DA. Scale bars = 1 mm. **B**, Signal intensities were quantified by determining the mean green-channel values for the images with the histogram function. Data are means $\pm$ standard deviation (SD) from three experiments. Data were subjected to Student's *t*-test. **P* < 0.19 and ***P* < 0.05 versus the H_2O control treatment. **C**, Inoculated leaf areas were infiltrated with L-012 solution (a reagent to detect reactive oxygen species) 12 hpi and were monitored using a CCD camera. White circles indicate areas infiltrated with L-012. **D**, Chemiluminescence intensities were quantified. Data are means $\pm$ SD from five experiments. Data were subjected to Student's *t*-test. **P* < 0.17 and ***P* < 0.05 versus the H_2O control treatment.

tary Fig. 3). However, ROS production was determined at 12 hpi in the following experiments because some leaf tissues showed a cell-death phenotype at 24 hpi. *B. cinerea*-inducible oxidative burst markedly decreased with DPI treatment compared with the oxidative burst in leaf areas infiltrated with H₂O (Fig. 2C and D), suggesting that the oxidative burst induced by *B. cinerea* depends on NADPH oxidase. By contrast, leaf areas infiltrated with L-NAME showed slight increases in accumulation of ROS and D-NAME had no effect on the oxidative burst (Fig. 2C and D). These results suggest that *B. cinerea*-inducible oxidative burst is regulated negatively by NO.

We evaluated the effects of inhibitors on susceptibility to *B. cinerea*. Susceptibility to *B. cinerea* was assessed by measuring diameter of disease lesions and ion leakage as an index of cell death (Fig. 3). DPI-treated leaf areas showed slight reduction in disease symptoms compared with H₂O-treated leaf areas (Fig. 3). By contrast, leaf areas infiltrated with L-NAME, although not D-NAME, showed marked expanding disease lesions compared with leaf areas infiltrated with H₂O (Fig. 3). These results suggest that the oxidative burst induced by *B. cinerea* has a positive impact on expansion of disease lesions and that NO participates in resistance to *B. cinerea*. We also assessed the dose effects of inhibitors on growth of *B. cinerea* to rule out the possibility that the difference in disease lesions is due to inhibitory effects on growth of *B. cinerea* by inhibi-

tors. Although cycloheximide, an inhibitor of de novo protein synthesis, pronouncedly inhibited growth of *B. cinerea*, DPI, L-NAME, and D-NAME treatments at the concentrations that were used in susceptibility analysis in Figure 3 had no effect on growth of *B. cinerea* on potato dextrose agar (PDA) (Fig. 4).

SIPK and defense-related genes activated during *B. cinerea*-*N. benthamiana* interaction.

The MAPK cascade MEK2-SIPK regulates NbNOA1-mediated NO burst and oxidative burst accompanied by expression of *NbRBOHB*, which is an inducible form of NADPH oxidase of *N. benthamiana* (Asai et al. 2008). NO and oxidative bursts were induced during *B. cinerea*-*N. benthamiana* interaction (Figs. 1 and 2). Therefore, we evaluated if enzyme activity of SIPK and transcription of *NbRBOHB* are activated by *B. cinerea* inoculation. *N. benthamiana* leaves were inoculated with *B. cinerea* conidia. Activation of SIPK (Fig. 5A) and the expression of *NbRBOHB* (Fig. 5B) were markedly induced at 12 hpi, in agreement with the timing of NO and ROS induced after inoculation with *B. cinerea*. *NbNOA1* expression was decreased by inoculation with *B. cinerea* (Fig. 5B), as reported by Kato and associates (2008). These results indicate that activation of SIPK might participate in NO and oxidative bursts after recognition of *B. cinerea* in *N. benthamiana*.

Genetic approaches have showed that SA, jasmonic acid (JA), and ethylene (ET) signaling also participate in disease resistance to *B. cinerea* in *Arabidopsis thaliana* (Ferrari et al. 2003; van Baarlen et al. 2007). To evaluate if SA, JA, and ET signaling are induced after inoculation with *B. cinerea* in *N. benthamiana*, we assessed the expression of the SA-responsive gene *NbPR-1*, JA synthesis-related gene *NbLOX*, and ET-responsive gene *NbERF1* after inoculation. Semiquantitative reverse transcriptase-mediated polymerase chain reaction (RT-PCR) showed marked induction of the expression of *NbPR-1*, *NbLOX*, and *NbERF1* (Fig. 5B), suggesting induction of SA, JA, and ET signaling during *B. cinerea*-*N. benthamiana* interaction. We also evaluated if the expression of radical-responsive genes *NbGST* and *NbCAT1* (Zago et al. 2006; Vandenameele et al. 2003) are induced after inoculation with *B. cinerea*. At 12 hpi, both *NbGST* and *NbCAT1* were induced (Fig. 5B). The timing of *NbGST* and *NbCAT1* expression was well matched with the production of NO and ROS.

Fig. 3. Effects of inhibitors on susceptibility to *Botrytis cinerea*. *Nicotiana benthamiana* leaves infiltrated with inhibitors or H₂O were inoculated with *B. cinerea*. **A**, Necrotic lesions in *B. cinerea*-inoculated leaves at 3 and 4 days postinoculation (dpi). **B**, Average diameter of lesions formed on the leaves at 3 and 4 dpi. Data are means $\pm$ standard deviation (SD) from 10 experiments. Data were subjected to Student's *t*-test. **P* < 0.18 and ***P* < 0.01 versus the H₂O control treatment. **C**, Quantification of cell death at 0 and 3 dpi. Data are means $\pm$ SD from three experiments. Data were subjected to Student's *t*-test. **P* < 0.05 and ***P* < 0.01 versus the H₂O control treatment.

Fig. 4. Effects of inhibitors on growth of *Botrytis cinerea* on potato dextrose agar (PDA) plates. PDA plates including diphenylene iodonium (DPI) (50 μM or 250 μM), D-NAME (5 mM or 25 mM), L-NAME (5 mM or 25 mM), or cycloheximide (CHX) (1 mg/liter or 5 mg/liter) and intact PDA plates as a control were inoculated with *B. cinerea*. Colony diameters were measured at 3 days postinoculation. Data are means $\pm$ standard deviation from four experiments.

Silencing *NbNOA1* and *NbRBOHB* affects susceptibility to *B. cinerea*.

NOA1 participates in NO generation induced by abscisic acid, salt stress, and elicitors (Asai et al. 2008; Guo et al. 2003; Zhao et al. 2007). ROS generation induced by elicitors and after recognition of pathogens depends on NbRBOHB in *N. benthamiana* (Asai et al. 2008; Yoshioka et al. 2003). Pharmacological analyses in this study showed that NOS-like NO-generating activity and NADPH oxidase take part in *B. cinerea*-inducible NO and oxidative bursts, respectively, in *N. benthamiana* leaves (Fig. 2). To clarify the relationship between the radical burst and disease resistance to *B. cinerea*, we silenced either *NbNOA1*, *NbRBOHB*, or both in *N. benthamiana* by VIGS. Tobacco rattle virus (TRV) vector containing cDNA fragments of *NbNOA1* and *NbRBOHB* was introduced into *N. benthamiana* by *Agrobacterium* infiltration (Ratcliff et al. 2001). Three weeks after infiltration, the effectiveness of the silencing was monitored by using RT-PCR. *NbNOA1* and *NbRBOHB* transcripts markedly decreased in the silenced plants as compared with the control plants infected with TRV vector alone (Fig. 6A). *NbNOA1*- and *NbRBOHB*-silenced plants but not *NbRBOHB*-silenced plants showed significant reduction in NO burst induced by inoculation with *B. cinerea* compared with TRV control plants (Fig. 6B). However, oxidative burst induced by *B. cinerea* inoculation was eliminated in *NbRBOHB*- and *NbNOA1* and *NbRBOHB*-silenced plants (Fig. 6C). *NbNOA1*

Fig. 5. Activation of salicylic acid-induced protein kinase (SIPK) and defense-related genes by inoculation with *Botrytis cinerea*. Total protein and RNA were extracted from intact leaves (0) or from leaves inoculated with *B. cinerea* or potato dextrose broth (Control) at the indicated times (h). **A**, Kinase activities of the extracts were assayed by immunocomplex (IC) kinase assay with SIPK-specific antiserum using myelin basic protein (MBP) as a substrate. Immunoblot analyses were done using SIPK-specific antiserum. Protein loads were monitored by Coomassie blue (CBB) staining bands corresponding to the ribulose-1,5-bisphosphate carboxylase (Rubisco) large subunit. **B**, Total RNA was used for reverse transcriptase-mediated polymerase chain reaction with specific primers for *NbRBOHB*, *NbNOA1*, *NbPR-1*, *NbLOX*, *NbERF1*, *NbGST*, and *NbCAT1*. Equal loads of cDNA were monitored by amplification of constitutively expressed *NbEF-1α*.

silencing markedly induced oxidative burst compared with TRV control plants (Fig. 6C). These results suggest that *B. cinerea* causes *NbNOA1*-mediated NO burst and *NbRBOHB*-dependent oxidative burst in *N. benthamiana*.

Conidia of *B. cinerea* were inoculated in drops onto the surface of silenced leaves of *N. benthamiana*. *NbNOA1*- and *NbRBOHB*-silenced plants showed yellowish leaves (Fig. 7A), which were similar to leaves of the *Arabidopsis noa1* knockout mutant (Flores-Perez et al. 2008; Guo et al. 2003). The phenotype is distinct from disease symptoms by *B. cinerea* infection. *NbNOA1*-silenced leaves showed high susceptibility to *B. cinerea*, but *NbRBOHB*-silenced leaves showed marked reduction in the size of disease lesions compared with TRV control leaves (Fig. 7). Because marked increases in ROS generation were in *NbNOA1*-silenced leaves, these results suggest that ROS generated from plants participate in expansion of disease lesions by *B. cinerea* or negatively function in disease resistance to *B. cinerea*. *NbNOA1* and *NbRBOHB*-silenced leaves showed expansion

Fig. 6. Silencing *NbNOA1* and *NbRBOHB* compromises the radical burst induced by *Botrytis cinerea*. At 3 weeks after inoculation with Tobacco rattle virus (TRV) gene-silencing constructs TRV:*NbNOA1* (N), TRV:*NbRBOHB* (B), TRV:*NbNOA1*/*NbRBOHB* (N/B), and TRV as a control, silenced leaves were inoculated with *B. cinerea* or potato dextrose broth (Control). **A**, Total RNA extracted from silenced leaves at 12 h postinoculation (hpi) was used for reverse transcriptase-mediated polymerase chain reaction with specific primers for *NbRBOHB* and *NbNOA1*. Equal loads of cDNA were monitored by amplification of constitutively expressed *NbEF-1α*. **B**, Measurements of nitric oxide and **C**, reactive oxygen species were done at 12 hpi. Data are means $\pm$ standard deviation from four experiments. Data were subjected to Student's *t*-test. **P* < 0.05 and ***P* < 0.01 versus the TRV control.

of disease lesions compared with *NbRBOHB*-silenced leaves (Fig. 7), indicating that NO participates in disease resistance to *B. cinerea*.

SIPK activation and defense-related gene expression in *NbNOA1*- and *NbRBOHB*-silenced plants.

Activation of SIPK and the expression of defense-related genes are induced by inoculation with *B. cinerea* in *N. benthamiana* (Fig. 5). We evaluated the effects of silencing *NbNOA1*, *NbRBOHB*, or both on SIPK activation and defense-related gene expression. Using RT-PCR, we confirmed reduced accumulation of *NbNOA1* and *NbRBOHB* transcripts in silenced plants either mock-inoculated or inoculated with *B. cinerea* compared with corresponding control plants infected with TRV alone (Fig. 8). *NbNOA1*- and *NbNOA1* and *NbRBOHB*-silenced plants showed marked reduction of ribulose-1,5-bisphosphate carboxylase (Rubisco) large subunit protein levels (Fig. 8A), consistent with the decrease in mutants of *Arabidopsis noa1* (also called *rif1* for resistant to inhibition by FSM) (Flores-Perez et al. 2008). Knockout of *Atnoa1* influences ribosome activity resulting in defects in protein translation, eventually leading to decreased levels of plastome-encoded proteins (i.e., depletion of Rubisco levels) (Flores-Perez et al. 2008). We confirmed not only activation of SIPK but also its protein levels by immunoblot analysis in silenced plants. Silencing *NbNOA1*, *NbRBOHB*, or both had no effect on protein levels and activation of SIPK induced at 12 h after inoculation with *B. cinerea* conidia as compared with TRV control plants (Fig. 8A). Expression of *NbPR-1* but not *NbLOX* and *NbERF1* decreased in *NbNOA1*- and *NbNOA1* and *NbRBOHB*-silenced plants (Fig. 8B), suggesting the influence of NOA1-mediated NO in SA signaling. We also evaluated the expression of radical-responsive genes in silenced plants. Expression of *NbGST* and *NbCAT1* was compromised in *NbNOA1* and *NbRBOHB*-silenced plants but not in *NbNOA1*- and *NbRBOHB*-silenced plants (Fig. 8B). The results correspond to

studies in which *GST* and *CAT1* were induced by both NO and ROS (Xing et al. 2008; Zago et al. 2006).

DISCUSSION

This study showed that NO contributes to disease resistance against *B. cinerea* in *N. benthamiana*. By contrast, ROS function negatively in resistance or positively in expansion of disease lesions during *B. cinerea*-*N. benthamiana* interaction.

Plants are subject to attack by a wide variety of microbial pathogens. From the viewpoint of pathogen biology, the death of host cells, which is common as an effective plant defense mechanism during gene-for-gene resistance (i.e., HR), may be advantageous for necrotrophic pathogens feeding on dead matter, but it is disadvantageous for biotrophic pathogens feeding on living host tissue. Although the mechanisms of the genetic composition of the host and gene-for-gene dependencies in relation to necrotrophic pathogens remain to be clarified, many studies have investigated the relationship between HR cell death and necrotrophic pathogens. Studies of *A. thaliana* and tobacco suggested that *B. cinerea* may even need HR cell death to achieve full pathogenicity (Dickman et al. 2001; Govrin and Levine 2000). *A. thaliana* mutants with delayed or reduced cell-death response are generally more resistant to *B. cinerea* infection, but mutants in which cell death is accelerated are more susceptible (van Baarlen et al. 2007). HR cell death requires coordinated levels of NO and ROS (Delledonne et al. 2001). MAPK cascade MEK2-SIPK regulates NO burst and oxidative burst accompanied by induction of *NbRBOHB* expression in *N. benthamiana* (Asai et al. 2008). In this study, accumulation of NO and ROS in the same region was observed after infection by *B. cinerea* on *N. benthamiana* leaves (Fig. 1), in agreement with production of NO and ROS in compatible lily-*B. elliptica* interaction (van Baarlen et al. 2004). The radical burst was induced at 12 hpi, in agreement with the activation profile of SIPK (Fig. 5A) and with the expression pro-

Fig. 7. Effects of silencing *NbNOA1* (N), *NbRBOHB* (B), or *NbNOA1/NbRBOHB* (N/B) on susceptibility to *Botrytis cinerea*. Silenced leaves were inoculated with *B. cinerea*. **A**, Inoculated leaves were photographed at 3 days postinoculation (dpi). **B**, Average diameter of lesions formed on the leaves at 3 and 4 dpi. Data are means $\pm$ standard deviation (SD) from 10 experiments. **C**, Quantification of cell death at 0 and 3 dpi. Data are means $\pm$ SD from three experiments. Data were subjected to Student's *t*-test. **P* < 0.01 versus the *Tobacco rattle virus* (TRV) control. ***P* < 0.01 versus the *NbRBOHB*-silenced plants.

file of *NbRBOHB* (Fig. 5B). BcPG1 is a virulence factor of *B. cinerea* (ten Have et al. 1998) and induces the radical burst, MAPK activation, and cell death (Kars et al. 2005; Poinssot et al. 2003; Vandelle et al. 2006). *B. cinerea* may elicit defense responses of host plants (i.e., MAPK activation, NO and ROS production) to induce HR cell death for its own advantage. Paradoxically, a nonpathogenic mutant overproducing BcPG1 induces an HR-like reaction on grape and bean leaves in relation to a cessation of tissue colonization (Kunz et al. 2006), suggesting that HR cell death has an important role in *B. cinerea* virulence but may also be responsible for resistance, depending on the timing and strength of HR. In this study, we confirmed little accumulation of NO and ROS in invaded areas (Fig. 1, yellow bar) in which the hyphae of *B. cinerea* were observed. Depletion of the radical burst in invaded areas may be caused by *B. cinerea*. An oxidative burst-suppressing agent is included in the *B. cinerea* isolate B 1.12 suspension classified as aggressive but not in the isolate B 1.7 suspension classified as nonaggressive (Unger et al. 2005). In the abscisic acid-deficient tomato mutant, which is highly resistant to *B. cinerea*, accumulation of H_2O_2 was earlier and stronger than in the susceptible wild type at the site of infection (Asselbergh et al. 2007). Furthermore, accumulation of NO and ROS in response to *B. cinerea* was delayed in susceptible but not in resistant pelargonium (Floryszak-Wieczorek et al. 2007), suggesting development of disease or elimination of the pathogen is determined by speed and efficiency of the radical burst initiated by plants.

Genetic and pharmacological approaches showed that reduced endogenous NO levels lead to increased levels of ROS and oxidized lipids and proteins under ultraviolet and salt stresses (Shi et al. 2005; Zhao et al. 2007). Consistent with these studies, we showed that treatment with a mammalian NOS inhibitor and *NbNOA1* silencing compromise NO production and ROS accumulation increases after inoculation with *B. cinerea* (Figs. 2 and 6). NO can react very quickly with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) and thus decrease the amount of endogenous ROS. Interestingly, the radical burst was observed in tissues adjacent to disease lesions (i.e., response areas, red bar in Fig. 1). *B. cinerea* infection can lead to accumulation of free radicals in uninfected tissue (Muckenschnabel et al. 2001, 2003) and depletion of antioxidants (Muckenschnabel et al. 2002). These events that increase oxidative processes may be caused by *B. cinerea* to facilitate infection. Treatment of crop plants with various antioxidants and then by inoculation with *B. cinerea* results in markedly inhibited development of gray mold disease (Elad 1992). NO appears to act as an antioxidant of ROS in disease resistance to *B. cinerea*. Our results also showed that NO production during *B. cinerea* interaction was partially reduced by a mammalian NOS inhibitor and *NbNOA1* silencing, indicating that the sources of NO synthesis except NOS, such as nitrate reductase, might participate in NO production in response to *B. cinerea*. Plants may produce NO by more than one NO-producing system after recognition of *B. cinerea* to hold out against *B. cinerea*.

NO signaling includes complex processes that include increases in cytosolic Ca^{2+} concentration, cyclic GMP, cyclic ADP ribose, and protein kinases. NO also modulates protein activities directly by cysteine S-nitrosylation (Besson-Bard et al. 2008). The relationship between NO and SA, an important regulator of plant defense responses, has been studied (Klessig et al. 2000). S-nitrosylation of NPR1, a master regulator of SA, regulates its reaction between oligomer and monomer and maintains its homeostasis upon SA induction (Tada et al. 2008). SA signaling-deficient mutants of *A. thaliana* show high susceptibility to *B. cinerea* (Ferrari et al. 2003; van Baarlen et al. 2007), suggesting

that SA signaling takes part in disease resistance to *B. cinerea*. In this study, silencing of *NbNOA1* and both *NbNOA1* and *NbRBOHB* reduced the expression of the SA-responsive gene *NbPR-1* induced by inoculation with *B. cinerea* (Fig. 8). These results suggest that reduced basal defense against *B. cinerea* in *N. benthamiana* resulting from compromised endogenous NO production may be due to depletion of SA signaling.

The mechanism and site for NO and ROS syntheses in pathogenic microorganisms challenging the plants have been studied. NO accumulates in germinating conidia at all stages of *Colletotrichum coccodes* and is required for the regulation of germination and elongation depending on the developmental stages (Wang and Higgins 2005). NO has a role in appressorial maturation of *Blumeria graminis* (Prats et al. 2008). *B. cinerea* also appears to produce NO as reported by Floryszak-Wieczorek and associates (2007). On the other hand, knockout mutants of *B. cinerea* NADPH oxidase genes have great impact on penetration and colonization (Segmüller et al. 2008), indicating that NADPH oxidases are indispensable for pathogenicity of *B. cinerea*. Although inhibitor treatments had no effect on the growth of *B. cinerea* on a PDA plate (Fig. 4), they may affect the differentiation and pathogenicity of *B. cinerea* in plants. In this study, we did knockdown analyses by VIGS to find the contribution of NO produced from plants in

Fig. 8. Effects of silencing *NbNOA1* (N), *NbRBOHB* (B), or *NbNOA1/NbRBOHB* (N/B) on activation of salicylic acid-induced protein kinase (SIPK) and defense-related genes by inoculation with *Botrytis cinerea*. Total protein and RNA were extracted from silenced leaves inoculated with *B. cinerea* (B) and potato dextrose broth (C) as a control at 12 h postinoculation. **A**, Kinase activities of the extracts were assayed by immunocomplex (IC) kinase assay with SIPK-specific antiserum using myelin basic protein (MBP) as a substrate. Immunoblot analyses were done using SIPK-specific antiserum. Protein loads were monitored by Coomassie blue (CBB) staining bands corresponding to the ribulose-1,5-bisphosphate carboxylase (Rubisco) large subunit. **B**, Reverse transcriptase-mediated polymerase chain reaction with specific primers for *NbRBOHB*, *NbNOA1*, *NbPR-1*, *NbLOX*, *NbERF1*, *NbGST*, and *NbCAT1* was performed. Equal loads of cDNA were monitored by amplification of constitutively expressed *NbEF-1α*.

disease resistance against *B. cinerea* (Fig. 7). The function of NO and ROS derived from *B. cinerea* remains to be clarified. Further studies are needed to clarify the role of radicals in the cross-talk between plants and necrotrophic pathogens.

Recent studies indicated that the effects of the radical burst on defense response appear to be diverse in plant-pathogen interactions. In *N. benthamiana*, *NbRBOHB* silencing has a strong effect on resistance to the potential biotrophic pathogen *P. infestans* but not to the hemibiotrophic pathogen *C. orbiculare* (syn. *C. lagenarium*), whereas *NbNOA1* silencing induces high susceptibility to *C. orbiculare* but not to *P. infestans* (Asai et al. 2008). Our preliminary data indicate that transgenic potato (*Solanum tuberosum*) plants expressing a constitutively active form of the calcium-dependent protein kinase *StCDPK5* (*StCDPK5VK*), which activates *StRBOH* (Kobayashi et al. 2007), fused to a pathogen-inducible promoter show high resistance to *P. infestans* but high susceptibility to the necrotrophic pathogen *Alternaria solani* (M. Kobayashi, M. Yoshioka, S. Asai, and H. Yoshioka, *unpublished data*). *StCDPK5VK* induces production of ROS (Kobayashi et al. 2007) but not of NO. The results support the idea that ROS may play a negative role in disease resistance to *B. cinerea* or a positive role in expansion of disease lesions. Transgenic potato plants expressing a constitutively active form of MAPKK, *StMEK2* (*StMEK2^{DD}*) driven by the same pathogen-inducible promoter as for *StCDPK5VK*, are resistant to both *P. infestans* and *A. solani* (Yamamizo et al. 2006). *StMEK2^{DD}* activates SIPK and then induces both ROS and NO production (Asai et al. 2008). These results support the conclusions of this study that NO contributes to disease resistance against necrotrophic pathogens. NO may function as a key factor that adapts to a wide spectrum of pathogens.

MATERIALS AND METHODS

Plant growth conditions.

N. 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.

Fungal culture, pathogen inoculation, and assays.

Botrytis cinerea F-016-0001, which was originally isolated from strawberry, was obtained from Nihon Nohyaku Co., Ltd (Tokyo). *B. cinerea* was maintained on PDA at 39 g/liter (Difco, Detroit) at 20°C in the dark. The conidia were collected and were suspended in sterile potato dextrose broth at 12 g/liter (Difco). Detached *N. benthamiana* leaves were inoculated with 5- μ l droplets of conidia suspension of *B. cinerea* (1×10^5 or 5×10^5 conidia per milliliter). For analyses of NO and ROS, 20- μ l droplets of conidia suspension of *B. cinerea* (5×10^5 conidia per milliliter) were used. The inoculated leaves were kept at high humidity at 23°C with a 16-h photoperiod and an 8-h dark period. Lesions diameters were measured using calipers. For conductivity tests, two disks per leaf (16 mm diameter) were obtained from inoculated areas for each leaf and were floated in 10 ml of distilled water for 4 h at room temperature with gentle shaking. Conductivity was measured with a multifunctional meter D-54 (HORIBA, Kyoto, Japan).

Histochemical staining and microscopic observations.

Trypan blue and DAB staining was done as described by Yoshioka and associates (2003). Fungal hyphal structures and dead cells were observed by staining infected leaves with trypan blue. Infected leaves were transferred to a trypan blue solution (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10 ml of H₂O, and 10 mg of trypan blue) diluted in

ethanol 1:1 and were boiled for 1 to 2 min. The leaves were then destained overnight in chloral hydrate. To visualize H₂O₂ in the infection site of *B. cinerea*, DAB staining was done. Inoculated leaves were vacuum-infiltrated with a 1 mg/ml DAB solution (pH 3.9) and, then, were fixed on filter paper by immersion in a 3:1 solution of ethanol and acetic acid. Stained leaves were observed using a fluorescence microscope (Axio Imager, Carl Zeiss, Oberkochen, Germany) or a fluorescence stereomicroscope (MZ16FA, Leica, Wetzlar, Germany) equipped with a CCD camera (AxioCam HRc, Carl Zeiss).

Detection of NO.

NO accumulation was monitored by using NO-sensitive dye DAF-2DA (Daiichi Pure Chemicals, Tokyo) as described by Asai and associates (2008). Leaves were infiltrated with 200 mM sodium phosphate buffer at pH 7.4 and 12.5 μ M DAF-2DA, using a needleless syringe or in a vacuum using an aspirator and were incubated for 1 h in the dark at room temperature before observation. Fluorescence from diaminotriazolo-fluorescein (DAF-2T), the reaction product of DAF-2DA with NO, was captured at excitation 470/40 nm and emission 525/50 nm, using a fluorescence microscope (Axio Imager, Carl Zeiss) or a fluorescence stereomicroscope (MZ16FA, Leica) equipped with a CCD camera (Color 14 bit, AxioCam HRc, Carl Zeiss). To quantify fluorescence intensity, images were obtained using the fluorescence stereomicroscope at constant acquisition time. For acquirement of low magnification images in Figure 1H and I, the fluorescence images were obtained at approximately threefold exposure times compared with other images used for quantification of the fluorescence intensity. The long exposure was applied to detect autofluorescence. The fluorescence intensity of the scanned entire field of the image captured by a CCD camera was quantified by determining the mean green channel values for the images with the histogram function of Adobe Photoshop 7.0 (Adobe, Seattle), as described by Boccara and associates (2005). A fluorescence image was obtained from an inoculated area per leaf and at least three inoculated areas were analyzed as replicates for each treatment. To account for background fluorescence, the corresponding mean value for noninoculated leaves infiltrated with the fluorescent dye (19.4 ± 1.0) was subtracted.

Measurement of ROS.

ROS measurements were done as described by Kobayashi and associates (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 morpholinepropanesulfonic acid-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, Hamamatsu City, Japan) and was quantified using the U7501 program (Hamamatsu).

Immunoblotting.

Immunoblotting was done as described by Asai and associates (2008). The protein extracts (10 μ g) were separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, U.S.A.). After blocking in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk overnight at 4°C, the membranes were incubated with SIPK-specific antiserum diluted with TBS-T at room temperature for 1 h. After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Amersham,

Tokyo) diluted with TBS-T for 1 h at room temperature. The antibody-antigen complex was detected by using the ECL Western-blotting detection kit (Amersham) and Light-Capture (ATTO, Tokyo).

Immunocomplex kinase assay.

The immunocomplex kinase assay was done as described by Asai and associates (2008). The protein extracts (50 µg) were incubated with SIPK-specific antiserum in the immunoprecipitation buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM ethylene diamine tetraacetate, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1% Triton X-100) at 4°C for 2 h with shaking. The mixture was then incubated in a 40-µl aliquot of 50% (vol/vol) protein A-Sepharose (GE Healthcare, Little Chalfont, U.K.) at 4°C overnight with shaking. Protein A-Sepharose complexes were pelleted by brief centrifugation, were washed three times with 1 ml of the immunoprecipitation buffer, and then were washed three times with 1 ml of kinase reaction buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-KOH, pH 7.6, 10 mM MgCl₂ and 1 mM dithiothreitol). The kinase activity in the complex was assayed at 25°C for 30 min in a final volume of 15 µl containing 0.4 mg of myelin basic protein (MBP) per milliliter, 100 µM ATP, and 8 nCi of [γ-³²P] ATP per milliliter. The reaction was stopped by adding SDS-polyacrylamide gel electrophoresis sample loading buffer. After electrophoresis on 15% SDS-polyacrylamide gel, the phosphorylated MBP was visualized by autoradiography.

RT-PCR.

Total RNA from *N. benthamiana* leaves was prepared by using TRIzol reagent according to the procedure of the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). RT-PCR was carried out using a commercial kit (ReverTra Ace -α-, TOYOBO, Osaka, Japan). PCR was done with ExTaq (Takara, Tokyo) at denaturing, annealing, and extension temperatures 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s, respectively, for 24 to 26 cycles. Gene-specific primers of each sequence were:

NbRBOHB (5'-TTTTCTCTGAGGTTTGCACGCCACCA-3', 5'-GCCTTCATGTTGTTGACAATGTCTTT-3'),
NbNOA1 (5'-ATGGCGCCTAAACTCCTTGCTC-3', 5'-TTCA TTGTGGCCTCCACAACCC-3'),
NbPR-1 (5'-CATAACACAGCTCGTGCGAGATGTAG-3', 5'-A ACCACTGAGTATAGTGTCACAC-3'),
NbLOX (5'-CCTTAAGAGGAGATGGAAC-3', 5'-TCTAAG CTCATAAGCAATGG-3'),
NbERF1 (5'-GGCGAATTTTCCGGGAGACT-3', 5'-GGCTCC GATTTTACTTCGCC-3'),
NbGST (5'-GTGTTAGTGGCAGCTCTTATAGTCG-3', 5'-GC TTTCCATTGTGAATTAGCACTGG-3'), and
NbCAT1 (5'-AACAGTACCCAATTCCTTTTCGTGT-3', 5'-G TTTAATACTCCAACTGCAACTTC-3').

As a control for equal cDNA amounts in each reaction, PCR was done with primers for *NbEF-1α* for 26 cycles (5'-CCTCA AGAAGGTTGGATACAAC-3', 5'-TCTTGGGCTCATTAATC TGGTC-3'). The PCR products were separated on 1.0% agarose gel, were stained with ethidium bromide for visualization.

VIGS.

VIGS was done as described by Ratcliff and associates (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 the forward and reverse primer for cloning into TRV vector pTV00 (RNA2): *NbNOA1-F-BamHI* (5'-CGGGATCCCGGAGTCACAGATAG CTGACCG-3') and *NbNOA1-R-ClaI* (5'-CCATCGATGGCT

GTCCTCCAGCTTTGC-3') (restriction sites are underlined), which produced a 478-bp fragment, and *NbRBOHB-F-ClaI* (5'-CCATCGATGTTAAACAAACGAGGCGGCA-3') and *NbRBOHB-R-HindIII* (5'-CCCAGCTTTACATTCTCCAAA TTTGGCAC-3'), which produced a 500-bp fragment. *N. benthamiana* was infected with viruses by *Agrobacterium*-mediated transient expression of infectious constructs. pBINTRA6 (RNA1) and pTV00 containing the inserts (RNA2) were transformed by electroporation separately into *A. tumefaciens* GV3101, which includes the transformation helper plasmid pSoup (Hellens et al. 2000). A mixture of equal parts of *Agrobacterium* suspensions of RNA1 and RNA2 was inoculated into 2- to 3-week-old *N. benthamiana* seedlings. The inoculated plants were grown under a 16-h light and an 8-h dark cycle at 23°C. The upper leaves of the inoculated plants were used for assays 3 to 4 weeks after inoculation.

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