

# Constitutive Expression of Mammalian Nitric Oxide Synthase in Tobacco Plants Triggers Disease Resistance to Pathogens

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Nitric oxide (NO) is known for its role in the activation of plant defense responses. To examine the involvement and mode of action of NO in plant defense responses, we introduced calmodulin-dependent mammalian neuronal nitric oxide synthase (*nNOS*), which controls the *CaMV35S* promoter, into wild-type and *NahG* tobacco plants. Constitutive expression of *nNOS* led to NO production and triggered spontaneous induction of leaf lesions. Transgenic plants accumulated high amounts of H<sub>2</sub>O<sub>2</sub>, with catalase activity lower than that in the wild type. *nNOS* transgenic plants contained high levels of salicylic acid (SA), and they induced an array of SA-, jasmonic acid (JA)-, and/or ethylene (ET)-related genes. Consequently, *NahG* co-expression blocked the induction of systemic acquired resistance (SAR)-associated genes in transgenic plants, implying SA is involved in NO-mediated induction of SAR genes. The transgenic plants exhibited enhanced resistance to a spectrum of pathogens, including bacteria, fungi, and viruses. Our results suggest a highly ranked regulatory role for NO in SA-, JA-, and/or ET-dependent pathways that lead to disease resistance.

## INTRODUCTION

Nitric oxide (NO) is a ubiquitous biological messenger that is involved in a wide range of functions such as regulation of vascular tone, neuronal communication, and immune response to infection in animal cells (Schmidt and Walter, 1994). In 1998, NO was shown to have a role in plants (Delledonne et al., 1998; Durner et al., 1998), followed by detection of nitric oxide synthase (NOS)-like activity in various plants (Barroso et al., 1999; Corpas et al., 2009; Wendehenne et al., 2001). Several studies have shown that NO may also function as an important signaling molecule during various aspects of plant growth and development, such as photomorphogenesis, leaf expansion,

root growth, senescence, programmed cell death, and cytokinin signaling (Beligni and Lamattina, 2000; Beligni et al., 2002; Gupta et al., 2011; Leshem, 1996; Leshem et al., 1998; Tun et al., 2001).

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NO, along with salicylic acid (SA) and reactive oxygen species (ROS), also plays a pivotal role in signaling and initiation of plant defense responses to microbial pathogens (Durner and Klessig, 1999; Van Camp et al., 1998; Wendehenne et al., 2001; Yoshioka et al., 2008; 2011). When NO is exogenously applied to tobacco and soybean suspension cells, it triggers the expression of several defense-related genes and potentiates ROS-induced cell death (Delledonne et al., 1998; 2001; Durner et al., 1998;). Infection with either avirulent bacterial pathogens or elicitors triggers an NO burst in suspension-cultured *Arabidopsis* and tobacco cells (Clarke et al., 2000; Foissner et al., 2000). A NOS-like enzyme (AtNOS1) has been identified in *Arabidopsis thaliana* but is not directly involved in NO synthesis; therefore, AtNOS1 was renamed AtNOA1 for NO ASSOCIATED1 (Crawford et al., 2006; Guo et al., 2003; Zemojtel et al., 2006). Recently, AtNOA1 showed GTPase activity and was needed in chloroplast biogenesis (Flores-Pérez et al., 2008; Moreau et al.,

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2008; Sudhamsu et al., 2008; Van Ree et al., 2011). Although supported by a growing body of evidence that suggests the existence of NOS-like activities in higher plants, no genuine plant NOS gene has been cloned to date.

Plant cells evoke multiple responses to defend themselves against pathogenic infections, such as the hypersensitive response (HR) and systemic acquired resistance (SAR). During plant defense against microbial pathogens, SA and NO play key roles as second messengers. In addition to SA-dependent defense responses, either plant hormones or growth regulators, such as jasmonic acid (JA) and ethylene (ET), may function as alternative signals that induce resistance against necrotrophic pathogens and regulate a subset of pathogenesis-related (PR) genes, including *PR-3*, *PR-4* (also called as *H $\theta$* ) and *PDF1.2* (Dong, 1998; Thomma et al., 2001). The reported contribution by NO to disease resistance, against necrotrophic pathogens, might imply that NO functions as a key factor in plant adaptation to a wide spectrum of pathogens (Asai and Yoshioka, 2009).

Relationships between NO, SA, and ROS during the establishment of disease resistance have been studied. Similar to HR, NO acts synergistically with ROS to potentiate cell death in soybean suspension cells (Delledonne et al., 1998). NO also functions independently from ROS to induce defense-related gene expression. NOS inhibitors have been shown to compromise the HR in *Arabidopsis* and tobacco (Delledonne et al., 1998; 2001; Durner et al., 1998). In addition, NO appears to activate defense responses through an SA-dependent signaling pathway: NO treatment of tobacco leaves led to a significant increase in endogenous SA, as well as in defense-related gene expression, but it failed to increase these same responses in *NahG* transgenic tobacco plants (Durner et al., 1998). NO-releasing compounds also induce disease resistance against tobacco mosaic virus (TMV) in tobacco, and NO is required for the development of SAR, which is induced by SA in TMV-infected tobacco (Song and Goodman, 2001). NO, therefore, may act synergistically with ROS and SA to transduce plant defense signals. Furthermore, S-nitrosylation is an important route for the transfer of NO bioactivity, and it is significantly involved in plant defense signaling (Feechan et al., 2005; Yu et al., 2012). In S-nitrosylation, a NO moiety is covalently attached to a protein cysteine thiol to form an S-nitrosothiol, which is recently emerging as a key regulatory process during the establishment of plant disease resistance (Spadaro et al., 2010; Tada et al., 2008; Yun et al., 2011).

To understand the involvement of NO in plant defense responses in more detail, we generated transgenic tobacco plants that over-express mammalian NOS cDNA. NOS transgenic tobacco plants exhibited HR-like lesions and accumulated both SA and ROS. In addition to SA-responsive genes, we also observed up-regulation of JAVET-responsive gene expression, including *PIN2*, *EREBP*, and *ACC oxidase*, in the transgenic tobacco plants. Most importantly, the over-expression of mammalian NOS cDNA conferred broad-spectrum resistance against bacterial, fungal, and viral pathogens. The results suggest that, together with SA and ROS, JA and/or ET participate in NO-mediated plant defense signaling.

## MATERIALS AND METHODS

### Construction of transgenic plants, plant materials, and growth conditions

For the construction of transgenic plants, rat brain NOS cDNA was first ligated into a plant binary vector (*pCambia1300*) that

harbors *hpt* as a selection marker, which was placed under the control of the *CaMV35S* promoter for sense orientation. The recombinant plasmid was introduced into either wild-type tobacco plants (*Nicotiana tabacum* cv Xanthi-nc) or *NahG-10* tobacco plants (gift from J. Ryals, Paradigm Genetics, Research Triangle Park, NC), via the *Agrobacterium tumefaciens*, (EHA101)-mediated, leaf disc, transformation method (Horsch et al., 1985). Either the neuronal NOS (*nNOS*) or *nNOS/NahG* transformants were selected on Murashige-Skoog medium that contained either hygromycin (20 mg L<sup>-1</sup>) or hygromycin/kanamycin (100 mg L<sup>-1</sup>), respectively. T<sub>2</sub> progeny, of transgenic plants that expressed *nNOS*, were used for the experiments and maintained at 25/20°C (day/night) in a growth chamber, with a 16-hr photoperiod and 65% relative humidity.

### NO detection by fluorescence analysis

Intracellular NO, in leaves of tobacco transgenic plants, was detected with a fluorescent dye: 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (DAF-FM-DA, Alexis Biochemicals; Guo et al., 2003). Samples were incubated in a solution with a NO probe (10  $\mu$ M DAF-FM-DA), for 30 min, washed three times with AT3 medium, and mounted on microscopic slides. Fluorescence was estimated from a confocal laser scanning microscopic image (Nikon PCM2000; excitation wavelength 488 nm; emission wavelength 515 to 530 nm).

### Histochemistry and microscopy

The accumulation of autofluorescent materials was detected, using methods described by Dietrich et al. (1994). Briefly, leaves were cleared by boiling them in lactophenol, rinsing them in 50% ethanol, and rinsing them in water. We observed autofluorescence with an ultraviolet epifluorescence microscope. Staining for the presence of H<sub>2</sub>O<sub>2</sub> via the diaminobenzidine (DAB) uptake method was performed as described in Thordal-Christensen et al. (1997).

### Antioxidant enzyme assays

Activities of catalase (CAT) and peroxidase (POX) were measured following the methods of Rao et al. (1997). Soluble protein (1 g) was extracted from leaves of 4-week-old tobacco plants by grinding tissues in a mortar with 3 ml of 100 mM potassium phosphate buffer (pH 7.5) that contained 2 mM Na<sub>2</sub>-EDTA, 1% (w/v) polyvinylpyrrolidone-40, and 1 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged for 15 min at 12,000  $\times$  g and desalted by gel filtration (PD-10 column). CAT activity was determined after consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm for 5 min in 3 ml reaction mixture that contained 100 mM potassium phosphate buffer (pH 7.5) and 10 ml 30% H<sub>2</sub>O<sub>2</sub>. POX activity was determined at 470 nm for 5 min in 3 ml reaction mixture that contained 100 mM potassium phosphate buffer (pH 6.5), 16 mM guaiacol, and 10 ml 10% H<sub>2</sub>O<sub>2</sub>. All enzyme assays were performed at 25°C on 20 to 50 mg of protein. All experiments described above were repeated twice, with three replicates per experiment.

### Measurement of SA

Plant material (usually 1.2 to 1.5 g young leaf) was grounded up and frozen in liquid nitrogen. Briefly, each tissue was extracted in 6 ml of ice-cold methanol at 4°C for 24 h and added to 3.6 ml ice-cold water and 3 ml chloroform, with 10 mM of a 3,4,5-trimethoxy-*trans*-cinamic acid as internal standard. After vortexing, the samples were kept at 4°C for 12 h. The combined supernatants were dried in a speed vacuum. The residue was re-suspended in 0.6 ml ice-cold water/methanol (1:1 v/v)

and analyzed by high-performance liquid chromatography (HPLC), as described by Meuwly and Métraux (1993).

### RNA extraction and RNA blot analysis

Isolation of total RNA and Northern hybridizations were performed as described by Park et al. (2004). The tobacco PR cDNA probes were provided by J. Ryals (Paradigm Genetics, Research Triangle Park, USA) and the phenylalanine ammonia-lyase cDNA probe was donated by C. Lamb (John Innes Centre, UK). Tobacco cDNA clones, one encoding ACC oxidase and one encoding PIN2, were provided by W.T. Kim (Yonsei University, Korea) and S.H. Lee (Pohang University of Science and Technology, Korea), respectively. Tobacco *EREBP* cDNA and *actin* cDNAs were amplified by polymerase chain reaction using specific primers that were based on published sequences. All probes were radiolabeled with [ $\alpha$ - $^{32}$ P]dATP, using a random-primed labeling method.

### Bioassays of transgenic plants

A bacterial pathogen, *Pseudomonas syringae* pv *tabaci*, was grown in King's B (KB) medium supplemented with 50 mg L<sup>-1</sup> rifampicin. The bacterial culture was washed with 10 mM MgCl<sub>2</sub> and re-suspended in 10 mM MgCl<sub>2</sub>. Bacterial density was determined by measuring OD<sub>600 nm</sub>. The bacteria were diluted to the desired concentrations in 10 mM MgCl<sub>2</sub> and inoculated into the leaves of mature tobacco with a needleless 1-ml syringe. Leaf discs (5 mm in diameter) were taken from the infection site at different time points, ground in 10 mM MgCl<sub>2</sub>, diluted, and plated on a KB agar plate that contained 50 mg L<sup>-1</sup> rifampicin. Bacterial growth was monitored by counting colony-forming units (cfus). Measurements for each time point were performed in triplicate. Inoculation of tobacco with *P. parasitica* was conducted under aseptic conditions. To assay resistance to the infection by the oomycete, wild-type and transgenic tobacco plants were inoculated with agar plugs that contained hyphae of *P. parasitica*. In addition, fully-expanded young leaves were inoculated with TMV in TET buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.0) by gently rubbing leaves with both carborundum and 10 mg-per-leaf TMV. Inoculated plants were maintained at 22°C, and the size and the number of HR lesions were monitored at different time points.

## RESULTS

### Expression of the mammalian *nNOS* gene in tobacco plants

The initial research that explored the role of NO in the plant defense system indicates that infiltration of mammalian NOS enzyme into plant cells successfully triggers both NO production and activation of plant defense-related gene expression (Durner et al., 1998). Based on those results, we tested whether constitutive expression of the mammalian NOS gene in a plant could activate a plant defense system against pathogens. We did this by expressing rat brain NOS (*nNOS*) (Bredt et al., 1991) cDNA, which was under the control of the constitutive Cauliflower Mosaic Virus (*CaMV*) 35S promoter in tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc). We also generated transgenic tobacco plants that co-expressed *nNOS* and *NahG* genes, which encode bacterial salicylate hydroxylase (named *nNOS/NahG*), since SA plays crucial roles in NO-mediated defense signaling (Durner et al., 1998; Klessig et al., 2000). The transgenic tobacco T<sub>0</sub> lines that expressed *nNOS* and *nNOS/NahG* were initially screened by Northern blot analysis (Supplementary Fig. S1). Based on the transcript level of *nNOS*, we selected two independent transgenic lines that showed

strong *nNOS* expression out of about 15 T<sub>0</sub> *nNOS* and *nNOS/NahG* plants. We further selected homozygous T2 progeny from the T<sub>0</sub> *nNOS* and *nNOS/NahG* lines and used them for further analyses.

Interestingly, *nNOS* transgenic plants formed spontaneous necrotic lesions on their leaves (Figs. 1A and 3). The lesions were observed on all plant leaves and at all developmental stages, with the exception of the latest-developing top leaves. Untransformed wild-type and empty, vector-transformed, transgenic, control plants, both grown under identical conditions, did not show these symptoms. In addition, the *nNOS* transgenic plant showed a smaller-sized, morphological phenotype than that in both wild-type and *NahG* plants (Fig. 1A). In contrast, *nNOS/NahG* transgenic plants showed a phenotype similar to wild-type and *NahG* plants, implying NO involvement in the SA-related, plant defense signaling. We also measured shoot and root fresh weights. The growth of *nNOS* transgenic plants decreased by 3- and 8-fold in shoot and root, respectively, when compared to the growth of wild-type, *NahG*, and *nNOS/NahG* plants (Figs. 1B and 1C).

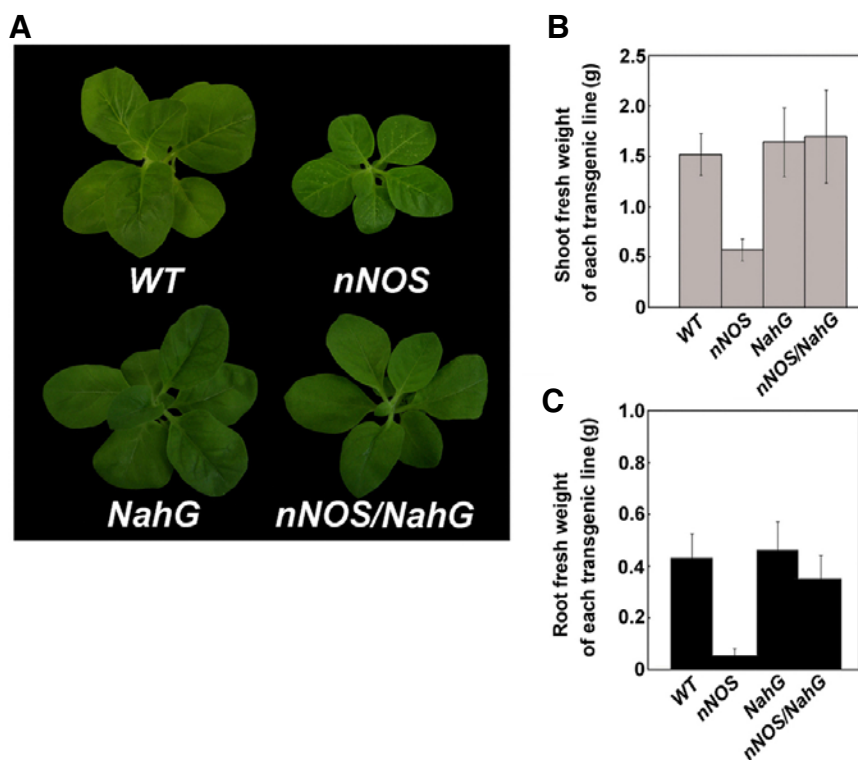
### Identification of NO in the *nNOS* transgenic tobacco plants

We first tested if expression of mammalian *nNOS* led to the production of NO in tobacco transgenic plants by using DAF-FM-DA. DAF-FM DA is cell-permeable and reacts with the more oxidized NO forms (e.g., NO<sup>+</sup> and N<sub>2</sub>O<sub>3</sub>), and it has been widely used to detect NO levels in plants (Guo et al., 2003; Lozano-Juste and Leon, 2010; Planchet and Kaiser, 2006; Zottini et al., 2007). Since epidermal peels of transgenic tobacco plants can be effectively loaded with DAF-FM-DA, we did so and analyzed the peels with confocal laser-scanning microscopy. After treatment, strong green fluorescence was observed in a number of leaf epidermal cells, indicating NO production in the leaves of both *nNOS* and *nNOS/NahG* transgenic tobacco plants, but not in those of wild-type and *NahG* plants (Fig. 2). The results indicated that constitutive expression of mammalian NOS effectively triggered NO production in plant cells. In addition, integration of NO production with the previously observed *NahG* phenotype tentatively showed that SA is in a position downstream of *nNOS*-mediated NO production.

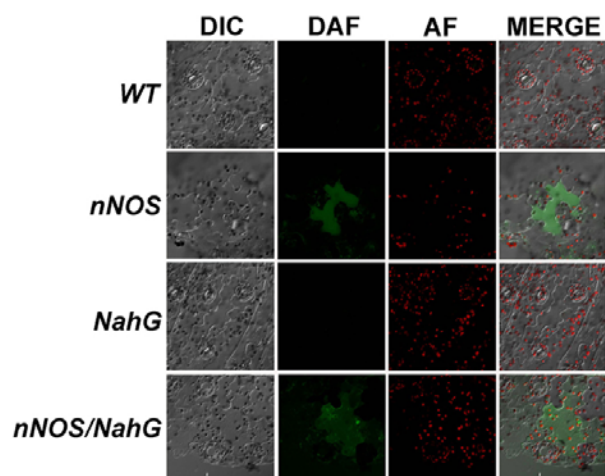
In animals, NO is primarily generated by a NOS enzyme that converts L-arginine to both L-citrulline and NO (Marletta, 1994). Furthermore, injection of recombinant mammalian NOS enzymes into tobacco plants induces the expression of defense-related genes, such as *PR-1* and *PAL*; and, like a mammalian system, NOS activity in tobacco plants is also dependent on both a NOS substrate (arginine) and cofactors (Durner et al., 1998). Based on previous reports, we speculate that expression of rat *nNOS* gene in transgenic tobacco plants may produce NO through the same mechanism known in animals.

### HR-like lesion formation and H<sub>2</sub>O<sub>2</sub> accumulation in *nNOS* transgenic tobacco plants

The *nNOS* plants showed reduced shoot growth (Fig. 1B), reduced root growth (Fig. 1C), and reduced fertility (data not shown). The most obvious phenotype of *nNOS* transgenic plants was the development of spontaneous lesions on their leaves (Figs. 1 and 3). The cell death phenotype was more obvious on the lower (older) leaves than on the upper (younger) leaves of *nNOS* transgenic tobacco plants. The phenotypes of *nNOS/NahG* transgenic tobacco plants, however, were similar to those of wild-type plants, indicating important roles for SA in both the formation of lesions and the developmental defects caused by constitutive expression of *nNOS*.



**Fig. 1.** Phenotypes of transgenic tobacco plants that constitutively express mammalian neuronal nitric oxide synthase (*nNOS*). (A) Formation of spontaneous disease, lesion-like, necrotic regions in the *NOS* transgenic plants. The whole plant morphology of a 6-week-old representative of a *NOS* transgenic plant (*nNOS*) showed that lesions only appeared in the older leaves. Wild-type plant (*WT*), *NahG* transgenic plant (*NahG*), and *nNOS/NahG* transgenic plant (*nNOS/NahG*) of similar ages are shown. (B) Shoot fresh weight of each transgenic line. The fresh weight was calculated by shoot fresh weight/plant. Error bars represent standard deviations ( $n = 12$ ). (C) Root fresh weight of each transgenic line. The fresh weight was calculated by root fresh weight/plant. Error bars represent standard deviations ( $n = 12$ ).



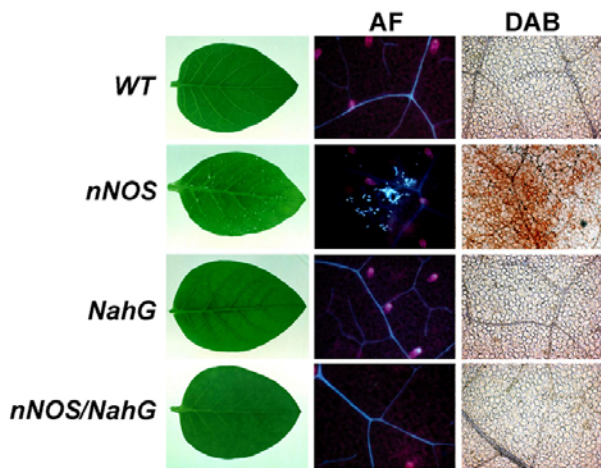
**Fig. 2.** NOS activity in *nNOS* transgenic tobacco plant. NOS activities were measured, using fluorescence microscopy and intracellular DAF-FM-DA signals (DAF), on epidermal cells from wild-type (*WT*), *nNOS* transgenic (*nNOS*), *NahG* transgenic (*NahG*), and *nNOS/NahG* transgenic (*nNOS/NahG*) plants. The epidermis of transgenic tobacco leaves was loaded with 10  $\mu$ M DAF-FM-DA. The images were obtained with a confocal laser scanning microscope (Nikon PCM2000). DIC, differential interference contrast; DAF, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) treatments; AF, autofluorescent materials; MERGE, overlapping images of DIC, DAF, and AF.

To determine whether the lesions mimicked a pathogen-induced HR, we tested for the presence of cellular and bio-

chemical markers associated with HR (Hammond-Kosack and Jones, 1996). Substantial accumulations of autofluorescent material and  $H_2O_2$  production (revealed by brown color development after staining with DAB) were detected on the leaves of *nNOS* transgenic tobacco plants. In contrast, *nNOS/NahG* transgenic plants did not exhibit spontaneous cell death, autofluorescence, or  $H_2O_2$  accumulation in their leaves (Fig. 3). These results suggest that the expression of mammalian *nNOS* in tobacco plants generates a signal that leads to HR-like cell death and the commonly associated oxidative burst.

#### Differential regulation of antioxidant enzymes in *nNOS* transgenic tobacco plants

Previous reports had shown transiently stimulated NO production, which was achieved by either infiltration of the mammalian NOS enzyme or the application of NO donors into plant cells, induces both SA accumulation and the expression of defense-related genes. Moreover, activated cell death, concurrent with  $H_2O_2$ , has been demonstrated (Delledonne et al., 1998; Durner et al., 1998). To further understand the role(s) of SA in NOS-mediated cell death and oxidative burst, we measured the activities of antioxidant enzymes in *nNOS* transgenic plants. Previous reports suggested that SA inhibits CAT and ascorbate peroxidase (APX) activities, leading to an amplification of the oxidative burst (Chen and Klessig, 1991; Durner and Klessig, 1995; 1996). Indeed, the *nNOS* transgenic tobacco plants exhibited markedly decreased catalase activity, whereas this did not occur in *nNOS/NahG* transgenic plants (Fig. 4A). This result indicates that elevated levels of SA, induced by *nNOS* activity, exert an inhibitory effect on endogenous catalase. As a result,  $H_2O_2$  accumulated in *nNOS* transgenic plants; however, no significant difference in APX activity was observed to distinguish wild-type from transgenic plants (data not shown). In



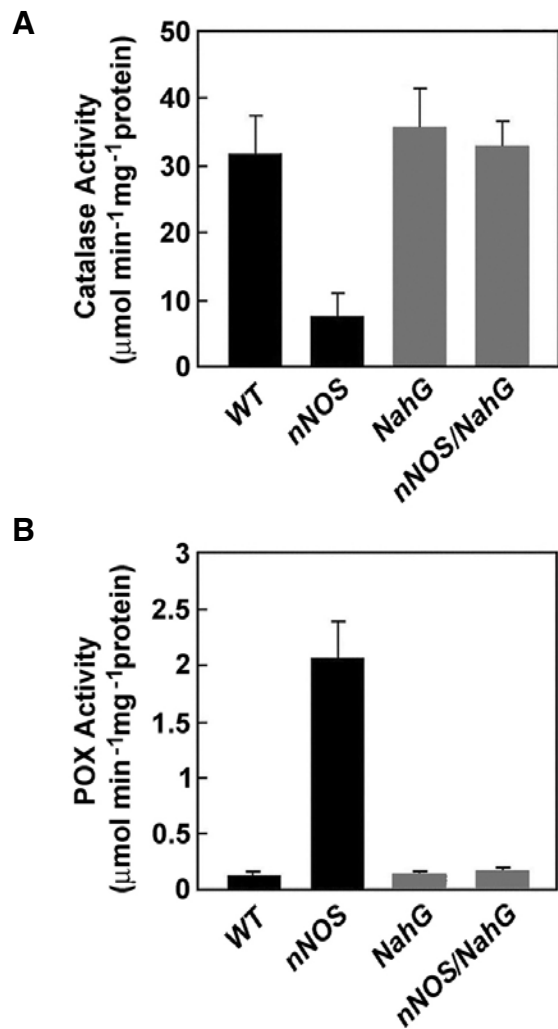
**Fig. 3.** Analysis of cell death phenotype in *nNOS* transgenic tobacco plants. Autofluorescent materials (AFs) were visualized with an ultraviolet epifluorescence microscope.  $H_2O_2$  accumulation was revealed by DAB staining (DAB). *WT*, wild-type tobacco plant; *nNOS*, *nNOS* transgenic plant; *NahG*, *NahG* transgenic plant; *nNOS/NahG*, *nNOS/NahG* transgenic plant.

contrast, the activity of guaiacol POX, which is not affected by SA (Dumer and Klessig, 1995), increased by approximately 10-fold in *nNOS* transgenic plants (Fig. 4B). Upregulation of POX activity in *nNOS* transgenic plants is therefore caused by SA-dependent  $H_2O_2$  accumulation. Based on these results, we propose that elevated levels of SA mediate  $H_2O_2$  accumulation at least partly via the inhibition of CAT activity. Subsequently, increased  $H_2O_2$  level can act synergistically with NO to trigger HR-like cell death in *nNOS* transgenic tobacco plants.

#### Accumulation of SA, and constitutive expression of defense-related genes, in *nNOS* transgenic tobacco plants

SA is a critical signal for activation of SA-dependent plant defense responses (Dumer et al., 1997; Ryals et al., 1996). In several studies of transgenic plants and mutants with spontaneous lesion-mimic phenotypes, elevated levels of endogenous SA were observed and the plants frequently exhibited induction of defense genes (Alvarez, 2000; Dangl et al., 1996; Ryals et al., 1996). We analyzed the endogenous levels of total SA (free and glucose-conjugated SA forms) in *nNOS* transgenic tobacco plants. Total SA levels in *nNOS* transgenic plants were approximately 2  $\mu\text{g}$  per gram fresh weight and were 23-fold higher than in wild-type plants (Fig. 5). Co-expression of the bacterial *NahG* gene was similar to that in wild-type plants that had *nNOS*-triggered SA accumulation.

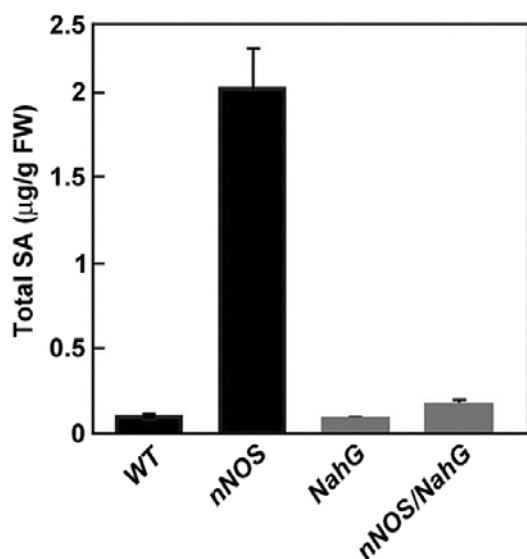
Next, we determined whether spontaneous lesion formation and high accumulation of SA in *nNOS* transgenic plants is accompanied by transcriptional activation of defense-related genes. Total RNA was isolated from the leaves of axenically-grown, *nNOS*, transgenic plants; and then we looked for the expression of defense-related genes that encode PAL and PR proteins. We found that the transcription of five *PR* genes, which are known to be enhanced during SAR in tobacco (Ryals et al., 1996), increased in response to *nNOS* expression, in a SA-dependent manner (Fig. 6). The *PAL* gene was also induced in *nNOS* transgenic plants, but in contrast to *PR* genes, appeared to be SA-independent. These results suggest that constitutive expression of mammalian *nNOS* is both effective



**Fig. 4.** Differential regulation of antioxidant enzyme activities in *nNOS* transgenic tobacco plants. The specific activities of catalase (CAT) (A) and guaiacol peroxidase (POX) (B) were measured in soluble crude proteins extracted from leaves of 4-week-old plants, as described in "Materials and Methods". All experiments were repeated twice, with three replicates per experiment, and error bars represent standard deviations.

and sufficient for the induction of plant defense signaling pathways.

Plants use multi-faceted signaling cascades such as SA- or JA/ET-dependent pathways to promote resistance against pathogens (Dong, 1998). To determine whether over-expression of mammalian *nNOS* triggers SA- and JA/ET-dependent plant defense pathways, we analyzed the expression of known, JA/ET-responsive, defense genes in *nNOS* transgenic tobacco plants. Interestingly, the expression of JA/ET-responsive defense genes, including *EREBP* (Ohme-Takagi and Shinshi, 1995), *ACC oxidase* (Kim et al., 1998), and *Pin2*, was induced in transgenic tobacco plants. The expression of these genes also increased significantly in *nNOS/NahG* plants, in which SA accumulation was abolished (Fig. 6). Taken together, these results provide biological evidence that JA and/or ET, along with SA, function as signaling molecules in NO-mediated plant de-



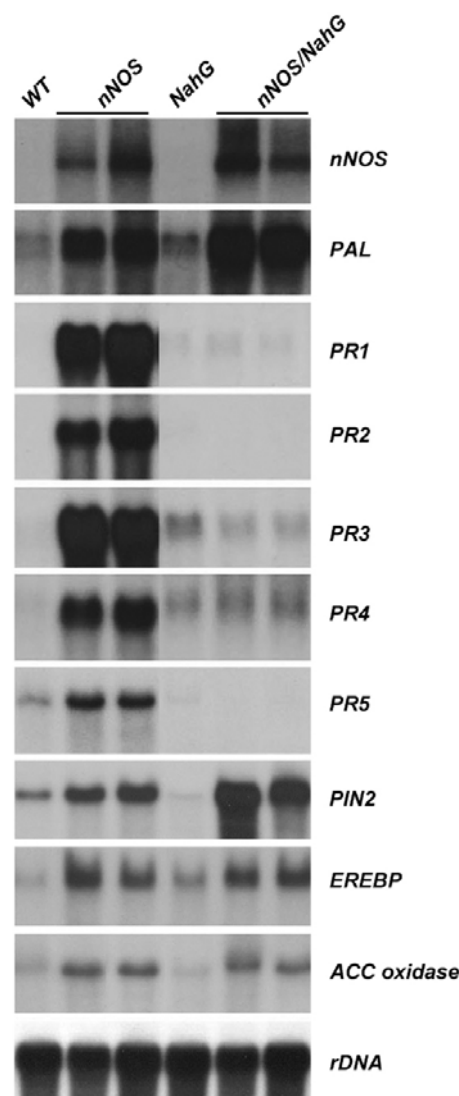
**Fig. 5.** SA levels in wild-type plant (WT), *nNOS* transgenic plant (*nNOS*), *NahG* transgenic plant (*NahG*), and *nNOS/NahG* transgenic plant (*nNOS/NahG*). Total SA levels (free SA plus glucose-conjugated SA) were determined in transgenic tobacco plants and analyzed in leaves from 4-week-old plants using HPLC. Values represent the mean and standard deviations obtained from three replicates per sample.

fense responses.

#### Overexpression of mammalian *nNOS* confers resistance to a broad spectrum of pathogens

Spontaneous cell death, and accumulation of SA, H<sub>2</sub>O<sub>2</sub>, and *PR* gene transcripts, in *nNOS* transgenic plants, prompted us to test the resistance of these plants to pathogens. We first infiltrated *nNOS* transgenic plants with a virulent bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* (Pst). Transgenic *nNOS* plants exhibited a significantly elevated level of resistance to Pst. Inhibition of pathogen growth in *nNOS* transgenic plants was observed as early as 24 h and for five days after inoculation. Bacterial growth in these plants was inhibited 50-fold more than wild-type plants (Fig. 7A).

We then tested the resistance of these transgenic plants to *Phytophthora parasitica* var. *nicotianae*, an oomycete pathogen that causes black shank disease in tobacco. *nNOS* transgenic and wild-type tobacco plants were inoculated with agar plugs that contained hyphae of *P. parasitica* and we monitored the development of disease symptoms. Five days post-inoculation, disease symptoms appeared on the wild-type plants but not on transgenic plants. By 8 days post-inoculation, wild-type plants showed severe leaf wilting and stem rot symptoms. They eventually died at 10 days post-inoculation (Fig. 7B); however, *nNOS* transgenic plants remained healthy without appreciable disease symptoms (Fig. 7B). We also tested for the resistance of *nNOS/NahG* transgenic plants to bacterial and fungal pathogens, but *nNOS/NahG* transgenic plants did not show resistance to the pathogens, indicating that SA plays a critical role in NO-mediated defense signaling in plants (Figs. 7A and 7B). In addition, transgenic *nNOS* plants exhibited enhanced resistance against an avirulent viral pathogen, TMV (Figs. 7C and 7D). The transgenic plants developed TMV-induced HR lesions approximately 12 h earlier than wild-type plants. The TMV-

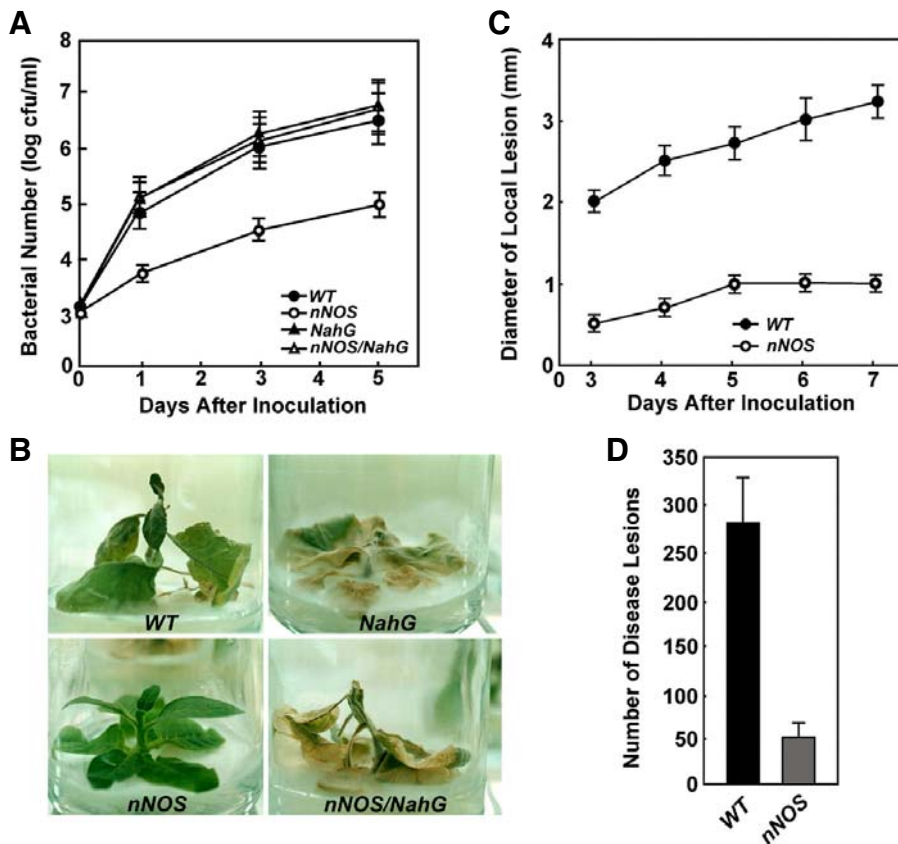


**Fig. 6.** Overexpression of mammalian *nNOS* in tobacco plants increased defense-related gene expressions. Total RNA was isolated from axenically-grown wild-type (WT), *nNOS* transgenic (*nNOS*), *NahG* transgenic (*NahG*), and *nNOS/NahG* transgenic (*nNOS/NahG*) plants. The endogenous levels of defense-related gene expression were analyzed by Northern blot analysis. The rDNA transcript was used as a loading control.

induced average lesion size in the transgenic plants was on average 70% smaller than in wild-type plants (Fig. 7C). Moreover, the transgenic plants showed fewer (~5-fold less) TMV-induced disease events (Fig. 7D). These two characteristics that reduce lesion size and lesion number are therefore associated with enhanced SAR in response to TMV.

#### DISCUSSION

In plants, NO both signals and regulates many physiological processes, including germination, flowering, seed dormancy, pollen tube growth, root development, leaf senescence, and stomatal movements. In addition, the NO signals help orches-



**Fig. 7.** Broad-spectrum disease resistance of transgenic tobacco plants that constitutively expressed the mammalian neuronal nitric oxide synthase (*nNOS*). (A) *In planta* bacterial growth. The bacterial pathogen, *Pseudomonas syringae* pv *tabaci*, was inoculated onto leaves of wild-type (WT), *nNOS*, *NahG*, and *nNOS/NahG* transgenic tobacco plants, at  $10^5$  cfu/ml, and bacterial growth was monitored over 5 days. Measurements for each time point were performed in triplicate. (B) Disease response to a virulent fungal pathogen, *Phytophthora parasitica* var. *nicotianae*. Two agar plugs, containing hyphae of *P. parasitica*, were placed on agar media and incubated in a culture bottle, together with 1-month-old wild-type (WT), *nNOS*, *NahG*, or *nNOS/NahG* transgenic tobacco plants, for 12 days. (C, D) Enhanced resistance of *nNOS* transgenic plants to tobacco mosaic virus (TMV) infection. Fully-expanded young leaves of wild-type (WT) and transgenic (*nNOS*) plants were inoculated with TMV. Lesion size was measured over 7 days (C), and lesion number was counted 5 days after inoculation (D).

trate responses to a wide array of stresses, such as temperature extremes, salinity, drought, heavy metals, and pathogens (Baudoin, 2011). Despite the growing body of evidence implicating NO, SA, and ROS in plant-pathogen interactions, including *PR* gene expression and HR, detailed mechanisms of NO function in plant defense responses are still missing. This might be either partially or entirely due to methodological restrictions. Because plant genes that encode a genuine NO-synthase have yet to be found, only indirect approaches have been used to study NO-mediated, plant, defense signaling, such as infiltration of mammalian NOS protein directly into plant tissues and pharmacological approaches that employ either NO-releasing compounds or NOS inhibitors (Delledonne et al., 1998; Durner et al., 1998). In this study, by analyzing stable plant transformants that expressed mammalian *nNOS*, we demonstrated that NO communicates with JA and/or ET pathways (and SA and ROS pathways) during defense responses to pathogen attacks.

NO potentiates induction of hypersensitive cell death and defense-related gene expression by acting synergistically with ROS in both soybean cells and *Arabidopsis* (Delledonne et al., 1998). In addition, application of either recombinant mammalian NOS or NO donors to both tobacco plants and suspension cells triggers SA accumulation and the expression of defense-related genes, such as *PR1* and *PAL*, in an SA-dependent manner (Durner et al., 1998). These findings imply that NO, SA, and ROS act cooperatively to activate plant defense signaling. Here, transgenic tobacco plants that express mammalian *nNOS* cDNA showed HR-like spontaneous necrotic lesion formation on their leaves, accumulation of  $H_2O_2$  and SA, and elevated *PR*

gene expression (Figs. 3 and 5). Interestingly, these NO-triggered events were completely abolished in *NahG* plants that expressed an SA-degrading enzyme. These results suggest that elevated SA levels triggered by NO can potentiate both the NO-mediated oxidative burst and the associated hypersensitive cell death in *NOS* transgenic tobacco plants. We propose that increased NO induced by over-expression of mammalian *nNOS* triggers SA biosynthesis through a cGMP and cADP ribose-dependent pathway (Durner et al., 1998). In turn, SA elevates  $H_2O_2$  accumulation by inhibiting antioxidant enzymes, such as CAT. Finally, NO and  $H_2O_2$  synergistically participate in hypersensitive cell death of *NOS* transgenic tobacco plants.

To confirm our hypothesis on  $H_2O_2$  accumulation, we measured the activities of the endogenous antioxidant enzymes, CAT, APX, and POX. In *nNOS* transgenic plants (but not in *nNOS/NahG* transgenic plants), endogenous CAT activity decreased approximately 3-4x, as compared to wild-type tobacco plants (Fig. 4A), thereby supporting our hypothesis. We could not, however, observe any significant differences in APX activity. One possible explanation for this discrepancy may be the existence of different  $K_d$  values for the SA binding to CAT and APX, which have been previously reported as 14 mM and 78 mM, respectively (Chen and Klessig, 1991; Durner and Klessig, 1995). Clark et al. (2000) suggest that NO binds directly to CAT and APX, and inhibits their activities. However, SA more likely plays a more critical role in NO-triggered ROS generation because we did not observe inhibition of CAT activity in *nNOS/NahG* transgenic plants. In contrast to CAT, the activity of SA-insensitive POX increased in *nNOS* transgenic plants, but did not increase in wild-type and *nNOS/NahG* transgenic plants

(Fig. 4B). POX activity may have increased as a means to scavenge accumulated H<sub>2</sub>O<sub>2</sub> in *nNOS* transgenic plants.

Plants use multiple signaling cascades to evoke broad-spectrum disease resistance; therefore, induction of both SA-dependent and SA-independent (or JA/ET-dependent) defense response pathways is required (Dong, 1998). To test whether the over-expression of *nNOS* can effectively induce the JA/ET-dependent pathway, in addition to the SA-dependent pathway, we monitored the expression levels of SA-responsive and JA/ET-responsive genes in *nNOS* transgenic tobacco plants. We observed an induction of the *PAL* gene in a NO-dependent fashion; an induction of SA-responsive genes, such as *PR1*, *PR2* and *PR5*; and elevated expressions of JA/ET-responsive genes, such as *PIN2*, *EREBP*, and *ACC oxidase* (Fig. 6). Interestingly, *nNOS/NahG* transgenic plants also showed elevated expression of these JA/ET-responsive genes. The latter result is in agreement with data that NO treatment of *NahG* plants results in activation of JA-responsive genes (Huang et al., 2004). These results suggest that overexpression of *nNOS* may sufficiently induce both the JA/ET-dependent pathway and the SA-dependent pathway.

To summarize, our results strongly suggest that the crucial participants in animal NO signaling also function in plants. In addition, NO production seems to always be tightly connected to various signals within plant cells. We also provide evidence for the critical involvement of NO in plant defense responses, both by interacting with SA and ROS pathways, and interacting with pathways that involve JA and ET. Identification of either NOS or other NO producers in higher plants may provide unique opportunities to understand the regulation of various metabolic pathways for which NO is the signaling molecule. Further investigations into the regulation of NO production and its downstream effects in plants should involve studies on both the biochemical analysis of NO production and the NO scavenging pathways of plant defense signaling. It will be important to study how other enzymes, such as those involved in nitrate reduction, can be recruited to provide NO signaling. Such information will be crucial for our understanding of NO signaling pathways that occur during development and stress responses. Such knowledge will also provide a better understanding of the roles that NO-related mechanisms play during plant defense responses.

*Note: Supplementary information is available on the Molecules and Cells website ([www.molcells.org](http://www.molcells.org)).*

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