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Bis-aryl methanone compound is a candidate of nitric oxide producing elicitor and induces resistance in *Nicotiana benthamiana* against *Phytophthora infestans*

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ABSTRACT

Nitric oxide (NO) is important in some physiological responses of plants and plays a crucial role in the regulation of both defense responses and inducing resistance to fungal pathogens. NUBS-4190, a new bis-aryl-methanone compound elicited NO production and defense responses in *Nicotiana benthamiana* against *Phytophthora infestans*. NUBS-4190 induced resistance in *N. benthamiana* to *P. infestans*, without association of reactive oxygen generation and hypersensitive cell death. Callose induction was reduced in NUBS-4190-treated *N. benthamiana* leaves after challenge inoculation of *P. infestans* indicating the penetration resistance. Involvement of pathogenesis-related 1a (*NbPR1a*) and nitric oxide associated 1 (*NbNOA1*) genes in the induced resistance to *N. benthamiana* against *P. infestans* was found to be associated with resistance. Increased susceptibility in *NbPR1a*- and *NbNOA1*-silenced plants correlated with the constitutive accumulation of *PR1a* transcripts and NO associated salicylic acid. Moreover, reduced NO generation in *NOA1* silenced *N. benthamiana* plants treated with NUBS-4190 indicated that *NbNOA1* is involved in NUBS-4190-mediated NO production and is required for defense responses.

Introduction

Nitric oxide (NO) is a conserved signaling molecule common to plants and animals [1,2]. In plants, NO is known to participate in several responses, including germination, flowering, stomatal closure, and pathogen defense [2–6]. NO participates in the complex interplay of defense-related signaling pathways controlling disease resistance [7]. In the context of plant-pathogen interactions, NO is involved in the modulation of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) synthesis and possibly other response mechanisms in plants [8–10].

Elicitors are signaling molecules that are involved in plant responses by triggering various defense reactions. Elicitors have been isolated from fungi, bacteria and oomycete pathogens and can be proteins, peptides, glycoproteins, lipids and oligosaccharides [11]. Many studies have demonstrated that NO plays important roles in fungal elicitor-induced secondary metabolite production of plant cells [12–15]. NO participates as an integrative signal of diverse endogenous plant hormones including ABA-regulated stomatal closure [16], auxin-mediated lateral root formation [17], jasmonic acid and cytokinin signaling [18,19]. Alkamides induce NO accumulation during adventitious root formation in *Arabidopsis* shoot explants [20,21]. Besides these natural elicitors or compounds, many researchers have used NO donors and scavengers in experiment to elucidate NO signaling [22–25] namely sodium nitroprusside (SNP), a compound that is likely to generate NO^+ [26], *S*-nitrosoglutathione (GSNO), a compound that releases NO but that may have other effects [27] and SNAP (*S*-nitroso-*N*-acetylpenicillamine) [4]. To clarify NO signaling pathways in plants, it is necessary to search for synthetic compounds which can induce NO generation in plants besides natural extracts or elicitors.

The wild *Nicotiana* sp., *Nicotiana benthamiana*, is a Solanaceous model plant which can be applied for functional analysis of genes by manipulating expression level of genes of interest using *Agrobacterium* sp.-mediated transient gene expression or virus-induced gene silencing (VIGS) [28]. *N. benthamiana* is used as a model plant to analyze resistance reactions of Solanaceous plants against *Phytophthora infestans*. *N. benthamiana* plants have age-related resistance against *P. infestans*. Shibata et al. [29] proved that *P. infestans* produced disease symptoms on 20- to 25-day-old *N. benthamiana* plants but mature plants were resistant to all isolates.

We searched for the compounds which elicit NO production in plants and defense response in *N. benthamiana* against *P. infestans*. As a candidate, we found NUBS-4190, 3,5-difluorophenyl-[3-methyl-4-(methylsulfonyl)isoxazol-5-yl]-methanone (Fig. 1)

Abbreviations: cPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; DAF-2DA, diaminofluorescein-2 diacetate; DMSO, dimethyl sulfoxide; L-012, 8-amino-5-chloro-7-phenylpyridol[3,4-*d*]pyridazine-1,4(2*H*,3*H*) dione sodium salt; MES, 2-(*N*-morpholino)ethanesulfuric acid; MOPS, 3-(*N*-morpholino)propanesulphonic acid; MS medium, Murashige–Skoog basal medium; NAA, napthalene acetic acid; HR, hypersensitive response; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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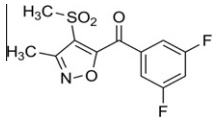

Fig. 1. Chemical structure of NUBS-4190 (3,5-difluorophenyl-[3-methyl-4-(methylsulfonyl)isoxazol-5-yl]-methanone).

elicits NO among chemical compounds library in our laboratory (unpublished). We examined the effect of the compound in *N. benthamiana* and characterized the plant responses to NUBS-4190 in particular, (1) to know whether NUBS-4190 induces resistance in *N. benthamiana* against *P. infestans*, (2) to explain the relationship between NUBS-4190-induced NO generation and reactive oxygen generation (ROS) and hypersensitive response like (HR-like) cell death, and (3) to identify the genes responsible for NUBS-4190-induced resistance in *N. benthamiana* against *P. infestans* infection.

Materials and methods

Chemical material

NUBS-4190 is a synthetic compound from chemical compounds library in our laboratory. The chemical formula is 3,5-difluorophenyl-[3-methyl-4-(methylsulfonyl)isoxazol-5-yl]-methanone. NUBS-4190 is a highly pure compound because any contaminant was not detected in ^{1}H-NMR spectrum. In preliminary research it was found that NUBS-4190 generated NO in tobacco suspension cultured cells (unpublished).

Biological materials, growth conditions and inoculation

Seeds of *N. benthamiana* were provided by the Leaf Tobacco Research Center (Japan Tobacco Inc., Tokyo). *N. benthamiana* plants were grown at 23 °C and 70% humidity under a 16 h photoperiod and an 8 h dark period in environmentally controlled growth

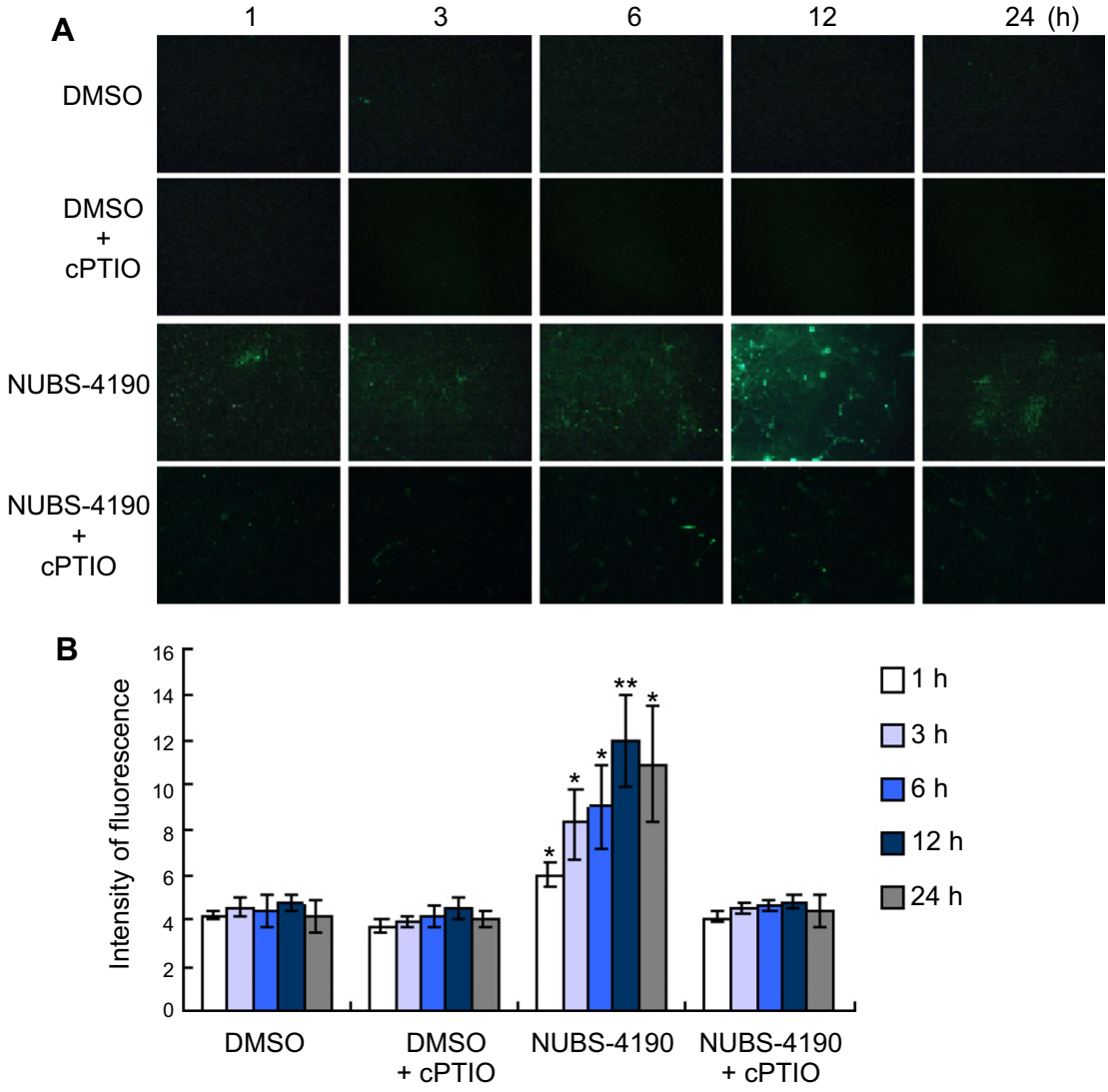

Fig. 2. NO generation activities of NUBS-4190 in *N. benthamiana*. (A) *N. benthamiana* leaves were infiltrated with 20 μg/ml NUBS-4190 and incubated for 24 h after treatment. The treated leaf areas were infiltrated with 4,5-diaminofluorescein diacetate (DAF-2DA) solution and were monitored 1 h later using fluorescence stereomicroscopy. NUBS-4190 was dissolved in DMSO. DMSO (0.2%) was used as a negative control. Treated leaf areas were infiltrated with 500 μM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) 1 h before infiltration of DAF-2DA. (B) Signal intensities were quantified by determining the mean channel values for the images with the histogram function. Each value represents the mean and standard deviation of 5 replicates. Data were subjected to Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus DMSO treated control treatment.

cabinets. The pathogenic isolate [*P. infestans* (Mont.) De Bary], race 1.2.3.4 was used throughout the research. Collection of zoosporangia and induction of zoospore production from *P. infestans* was performed [30]. Zoosporangia suspensions from the *P. infestans* isolates were prepared as follows. *P. infestans* isolates were sub-cultured on rye-media for 7–10 days. 20 ml of water was added to the surface of the *P. infestans* colonies, which were then rubbed with a cotton swab to release the zoosporangia. Leaves of *N. benthamiana* plants were inoculated with 1 ml aliquots of *P. infestans* zoospores (2×10^5 zoospores/ml) and covered with lens paper to keep the suspension of zoospores on the surface of the leaves. The inoculated plants were kept at high humidity at 20 °C for 1 day post inoculation, and then moved to a growth room at 23 °C.

In this study, wild-type *Colletotrichum orbiculare* isolate 104-T was also used. *C. orbiculare* was provided by Dr. Y. Takano at Kyoto University. *C. orbiculare* cultures were maintained at 24 °C on potato dextrose agar medium. For inoculation, 20–25 days-old *N. benthamiana* plants were sprayed with 2 ml of conidial suspension (1×10^6 conidia/ml) and incubated in a humid plastic box at 24 °C [31]. For pathogenicity tests, inoculated leaves were observed daily basis for symptoms.

Preparation of INF 1 elicitor

INF1 elicitor (*inf1* gene product) was prepared according to the method of Yamamoto et al. [32] as overnight cultures of *Escherichia coli* cells, carrying a chimeric plasmid (pFB53) with the *inf1* gene [33], were diluted (1:100) in Luria–Bertani (LB) medium containing 50 μg/ml of ampicillin and incubated at 37 °C. *E. coli* preparations of INF1 and pFB53 vector were provided by Dr. S. Kamoun at Sainsbury Laboratory. When the OD_{600} of cultures reached 0.6, production of INF1 was induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3–4 h. The culture was centrifuged and the resultant supernatant was dialyzed against water with SnakeSkin dialysis tubing (7 kD molecular mass cutoff, Pierce Biotechnology) overnight at 4 °C. This preparation was then used as INF1 elicitor.

Measurement of NO production

N. benthamiana leaves were infiltrated with 200 mM sodium phosphate buffer at pH 7.4 and 12.5 μM DAF-2DA, using a needle-less syringe and were incubated for 1 h in the dark at room

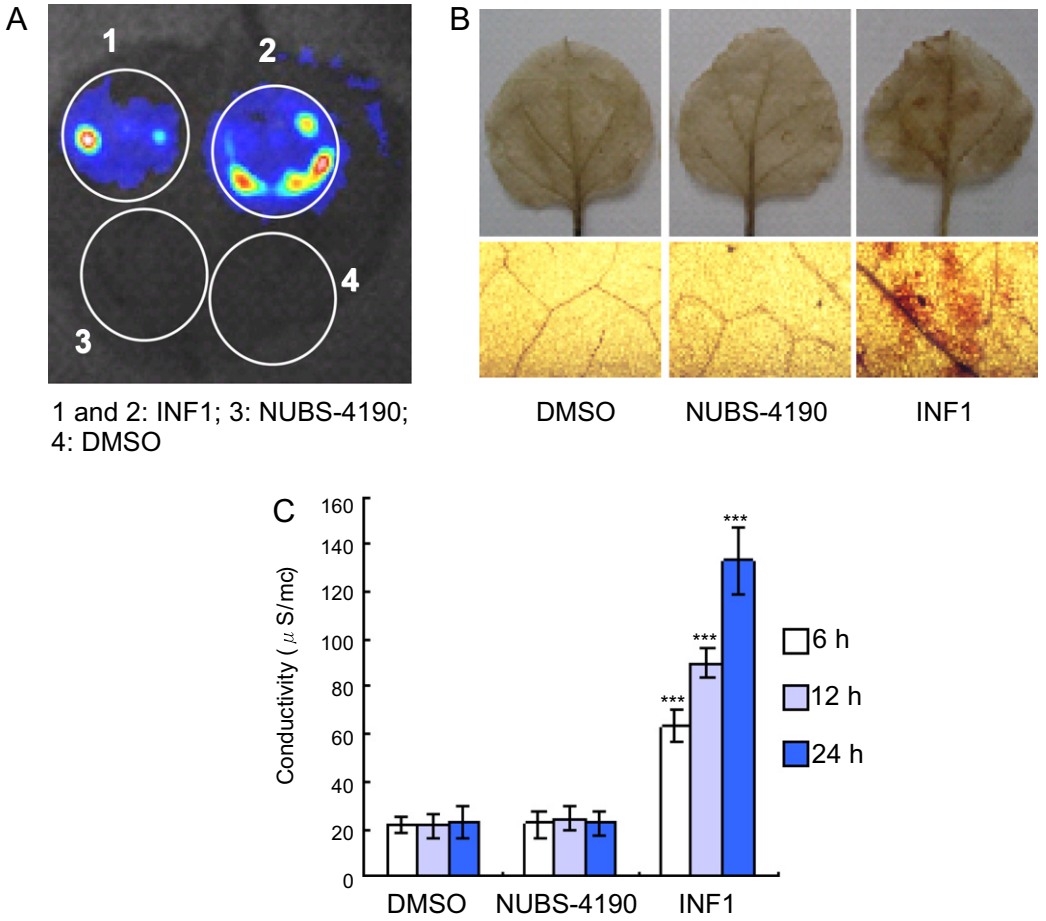

Fig. 3. O_2^- and H_2O_2 production and HR-like cell death activities induced by NUBS-4190 in *N. benthamiana* plants. (A) O_2^- inducing activity of NUBS-4190 on *N. benthamiana* plants. Plant leaves were treated with 20 μg/ml of NUBS-4190, and O_2^- producing activity of *N. benthamiana* leaves was detected. NUBS-4190 treated leaf areas were infiltrated with L-012 solution 12 h after treatment and were monitored using a CCD camera. Circles indicate the areas infiltrated with L-012. (B) H_2O_2 accumulation. Accumulation of H_2O_2 by NUBS-4190 (20 μg/ml) in *N. benthamiana* leaves after incubation of tissues with the dye DAB were determined 12 h after treatment. INF1 was used at the concentration of 150 nM as a positive control, where 0.2% DMSO was used as a negative control. Reddish brown coloration indicates the H_2O_2 accumulation. (C) Electrolyte leakage experiment of cell death in *N. benthamiana* leaves by NUBS-4190. After treatment with NUBS-4190 by infiltration into leaves, leaf discs were floated on distilled water and shaken for 2 h at room temperature. Electrical conductivity was measured using a conductivity meter. Data were subjected to Student's *t*-test. $^{***}P < 0.001$ versus DMSO treated control treatment.

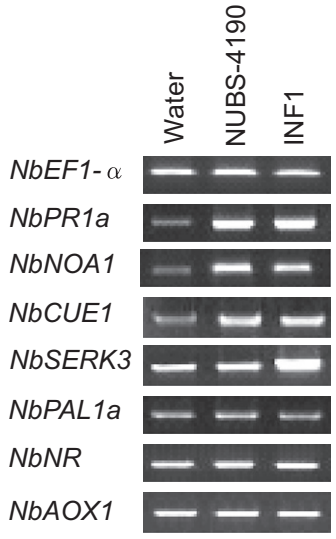

Fig. 4. Gene expression by NUBS-4190 in *N. benthamiana* leaves. Total RNA was isolated from *N. benthamiana* leaves treated with NUBS-4190 for 24 h. INF1 elicitor was used as a positive control and 0.2% DMSO was used as a negative control. RT-PCR analysis was done using specific primers for *NbPR1*, *NbNOA1*, *NbCUE1*, *NbSERK3*, *NbPAL1a*, *NbNR*, and *NbAOX1*. Equal loads of cDNA were monitored by amplification of constitutively expressed *NbEF1α*.

temperature before observation. Fluorescence from DAF-2T, the reaction product of DAF-2DA with NO, was captured using a fluorescence stereomicroscope (MZ16FA, Leica) equipped with a CCD camera (Color 14 bit, AxioCam HRc, Carl Zeiss). 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) [34]. 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 non-inoculated leaves infiltrated with the fluorescent dye (10.0 ± 1.0) was subtracted.

Measurement of ROS production

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence. L-012 (Wako, Osaka, Japan) is a luminol derivative that is highly sensitive to superoxide anion (O_2^-) [35]. To detect the ROS production in *N. benthamiana* leaves, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was allowed to infiltrate to the intercellular space of leaves via a syringe without a needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20 °C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan), and quantified using the U7501 program (Hamamatsu Photonics).

H_2O_2 in situ detection

Leaves of *N. benthamiana* plants were infiltrated with elicitor including NUBS-4190 and leaf discs were removed with a cork borer at 24 h after infiltration. H_2O_2 detection was performed following the 3,3′ diaminobenzidine-HCl (DAB) staining method [36]. Treated intact leaves and leaf discs were placed in 1 mg/ml DAB (Sigma, St. Louis, USA) and incubated at room temperature overnight. DAB reactions were examined in leaves cleared in boiling ethanol (96%) for 10 min. The samples were then stored in ethanol (96%) at room temperature or mounted in PBS (phosphate-buffered saline)/glycerol (50%) and kept at 4 °C for further examination. H_2O_2 was visualized as a reddish brown coloration.

Detection of cell death

To detect the cell death in the plant, elicitors were infiltrated by using a syringe without a needle from the opposite surface of the leaves. Cell death was checked by an electrolyte leakage method, which was adapted from the method described by Yeom et al. [37]. Leaves were infiltrated by elicitors under the leaf surface, and leaf discs (1 cm in diameter) were collected from the leaf 6, 12 and 24 h after treatment. Seven leaf discs were floated on 7 ml of distilled water for 2 h at room temperature and electrical conductivity was measured using a conductivity meter (Horiba, Kyoto, Japan).

RT-PCR

Expression of *N. benthamiana* genes after the treatment of compounds was analyzed by RT-PCR [31]. Total RNAs from *N. benthamiana* leaves were prepared by using TRIzol reagent according to the procedure of the manufacturer (Invitrogen, Carlsbad, CA, USA.). RT-PCR was conducted using a commercial kit (ReverTra-Plus; Toyobo Co., Osaka, Japan). The cDNA was synthesized from total RNA (1 μg) with an oligo (dT) primer. After the cDNA synthesis reaction, the PCR was performed with denaturing, annealing, and extension temperatures of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, respectively, for 21–27 cycles. Gene-specific primers for each sequence were as follows: *NbEF-1α* forward primer, 5′-CTGATTATTGACTCCACCACTG-3′, *NbEF-1α* reverse primer, 5′-CATCTTGTTACAGCA GCAAATC-3′, *NbPR1a* forward primer, 5′-CACTCTTGCCGTGCCC-3′, *NbPR1a* reverse primer, 5′-CACGAACCGAGTTACGC-3′, *NbNOA1* forward primer, 5′-ACACCTCGGAAGTGGATG-3′, *NbNOA1* reverse primer, 5′-GACAGTAGGCGACATCGTC-3′, *NbCUE1* forward primer, 5′-AAGCTTGATACTTTGGTGCTCGGA-3′, *NbCUE1* reverse primer, 5′-GG ATCCACCATAAACTTCTTGCTG-3′, *NbSERK3* forward primer, 5′-GT CCAATCCCTGACACACTG-3′, *NbSERK3* reverse primer, 5′-GAGGC GGAGGCGGAGAAGC-3′, *NbPAL1a* forward primer, 5′-CTGGTCGG CCTAATTCTAAAG-3′, *NbPAL1a* reverse primer, 5′-GGTTGCAGAACG-GATGAC-3′, *NbNR* forward primer, 5′-GTTCAGTCACCTAGAAGCCG-3′, *NbNR* reverse primer, 5′-TGGAAATTAGTACTGTGGGACG -3′, and *NbAOX1* forward primer, 5′-ATGATGACACGTGGAGTG-3′, *NbAOX1* reverse primer, 5′-TTGTACGTCTCCCATGGC-3′.

Resistance-inducing activity against pathogen infection

N. benthamiana plants were grown and used for resistance induction study. NUBS-4190 at the concentration of 20 μg/ml was infiltrated into *N. benthamiana* leaves using a needleless syringe. DMSO treatment was used as a control. After 24 h of treatment, challenge inoculation was performed by *P. infetans* (2×10^5 zoospores/ml) and plants were kept in dark and humid condition for 18 h. The first three large leaves of *N. benthamiana* plants from below were selected for challenge inoculation study because young-aged, lower leaves are easily infected by *P. infestans*.

Microscopic observation

To visualize plant cell death and colonization of *P. infestans*, leaves of *N. benthamiana* were stained with lactophenol trypan blue [38] with a minor modification. Briefly, infected leaves were cleared in methanol overnight, and then the cleared tissue was boiled for 2 min in lactophenol trypan blue stain (10 ml of H_2O,

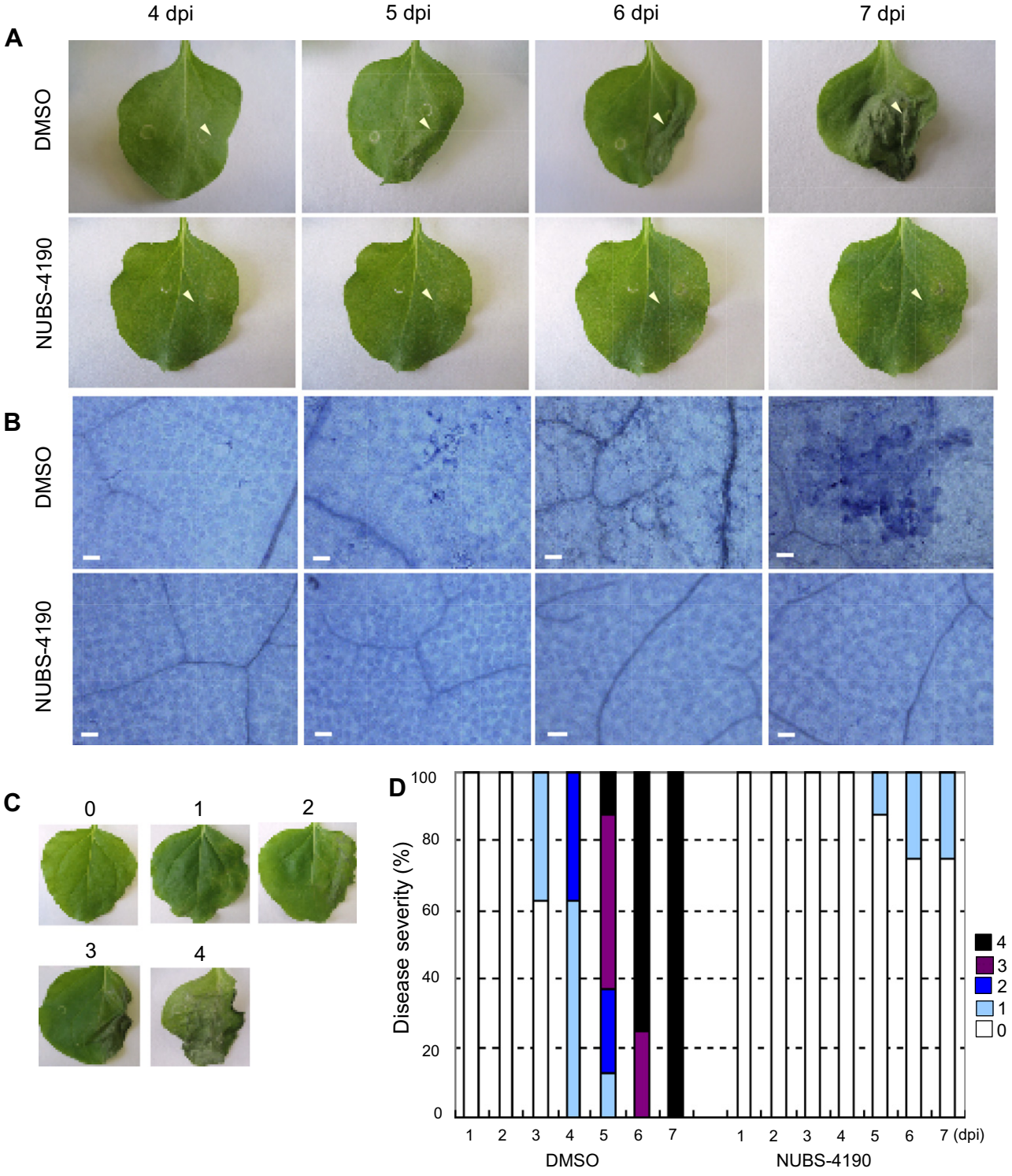

Fig. 5. NUBS-4190 induced resistance in *N. benthamiana* against *P. infestans*. (A) 0.2% DMSO treated control plant leaves (upper panel) and NUBS-4190-treated plant leaves were kept in an incubator for 24 h and then were inoculated with the spores of *P. infestans*. Arrowheads indicate the point of inoculation. Photographs were taken from 4 to 7 days post inoculation (from starting lesion to final damage). (B) Microscopic observation of *N. benthamiana* leaves from 4 to 7 days post inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol trypan blue to visualize dead plant cells. Bar = 50 μm. (C) Appearance of disease symptoms showing differences in severity representative of the five classifications used in D. (D) Plots showing percentages of *N. benthamiana* leaves with disease symptom severities in each of the five classes represented in D, for leaves of DMSO and NUBS-4190 treated plant leaves inoculated with *P. infestans* from 1 to 7 days post inoculation. At least 15 inoculated leaves from each plant were counted.

10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue). After the leaves had been allowed to cool at room temperature for 1 h, the stain was replaced with chloral hydrate at the concentration of 1 g/ml. Stained leaves were monitored using a microscope (Olympus BX51, Tokyo, Japan).

Preparation of silencing constructs and virus-induced gene silencing (VIGS)

Partial cDNAs of *NbNOA1* and *NbPR1a* were amplified from *N. benthamiana* DNA using gene-specific primers. Amplified

fragments were ligated into the *Sma*I site of the pTV00 vector [28] to generate pTV00:NOA1 and pTV00:PR1a. pTV00 and pBINTRA6 vectors were provided by Dr. D. C. Baulcombe (University of Cambridge, Cambridge, MA, USA.). For all constructs, the inserts were amplified and sequenced to confirm cloning of the correct gene fragment. The pTV00 vectors were transformed into *Agrobacterium tumefaciens* (strain GV3101 with helper plasmid pSoup) by electroporation with a MicroPulser electroporator (Bio Rad) and transformants were selected on LB media supplemented with 50 μg/ml rifampicin, 50 μg/ml kanamycin and 2.5 μg/ml tetracycline.

Induction of VIGS was performed as previously reported [28]. *A. tumefaciens* GV3101 carrying the binary TRV RNA 1 construct pBINTRA6, and the TRV RNA2 vector pTV00 or pTV00 derivatives, were cultured to saturation in LB media. Bacterial suspensions were then collected by centrifugation at 16,000*g* for 1 min. The bacterial cells were then resuspended in 10 mM MES–NaOH (pH 5.6), 10 mM $MgCl_2$ and 150 μM acetosyringone (final OD_{600} = 0.5), and incubated at room temperature for 2 h. The cultures were mixed in a 1:1 ratio (RNA1/RNA2), and used to infiltrate into the intercellular space of leaves of *N. benthamiana* from the backside of the leaf using a syringe without a needle by placing a finger from on the other side of the leaf. After 3–4 weeks of infiltration, the upper leaves of the inoculated plants were used for experiments. Plants inoculated with *A. tumefaciens* carrying pBINTRA6 and pTV00 were used as negative controls for the experiments.

Callose staining

To visualize callose deposition, leaves were treated and stained in 0.1% aniline blue as described by Currier and Strugger [39] with a minor modification. In brief, discs of leaf tissue with a radius of 4 mm, were fixed in a solution of 1% gluteraldehyde, 5 mM citric acid, 90 mM Na_2HPO_4, pH 7.4, decolorized by 100% ethanol overnight and then stained with 0.1% (w/v) water soluble aniline blue in 67 mM phosphate buffer adjusted to pH 12 with 1 N KOH. Fluorescence due to callose was examined with a microscope (Olympus BX51TRF, Tokyo, Japan) equipped with fluorescence optics.

Results

NO producing activity

To examine the effect of NUBS-4190 on NO production *N. benthamiana* leaves were infiltrated with NUBS-4190 at the concentration of 20 μg/ml. NO production was detected using DAF-2DA-mediated fluorescence, and the fluorescence was eliminated by cPTIO, an NO scavenger (Fig. 2A and B). Since NUBS-4190 was dissolved in 0.2% DMSO, 0.2% DMSO was used as a negative control. INF1, a proteinacious elicitor from *P. infestans*, was used as a positive control, since INF1 induces NO in *N. benthamiana* [40]. Visibility of green fluorescence indicating NO generation was high in NUBS-4190-treated leaves 12 h after infiltration and lower fluorescence was detected in c-PTIO-treated leaves. Quantification of fluorescence intensity was measured by determining the mean green channel values for the images with the histogram function of Adobe Photoshop (Fig. 2B). These results suggest that NUBS-4190 induces NO generation in *N. benthamiana* plants.

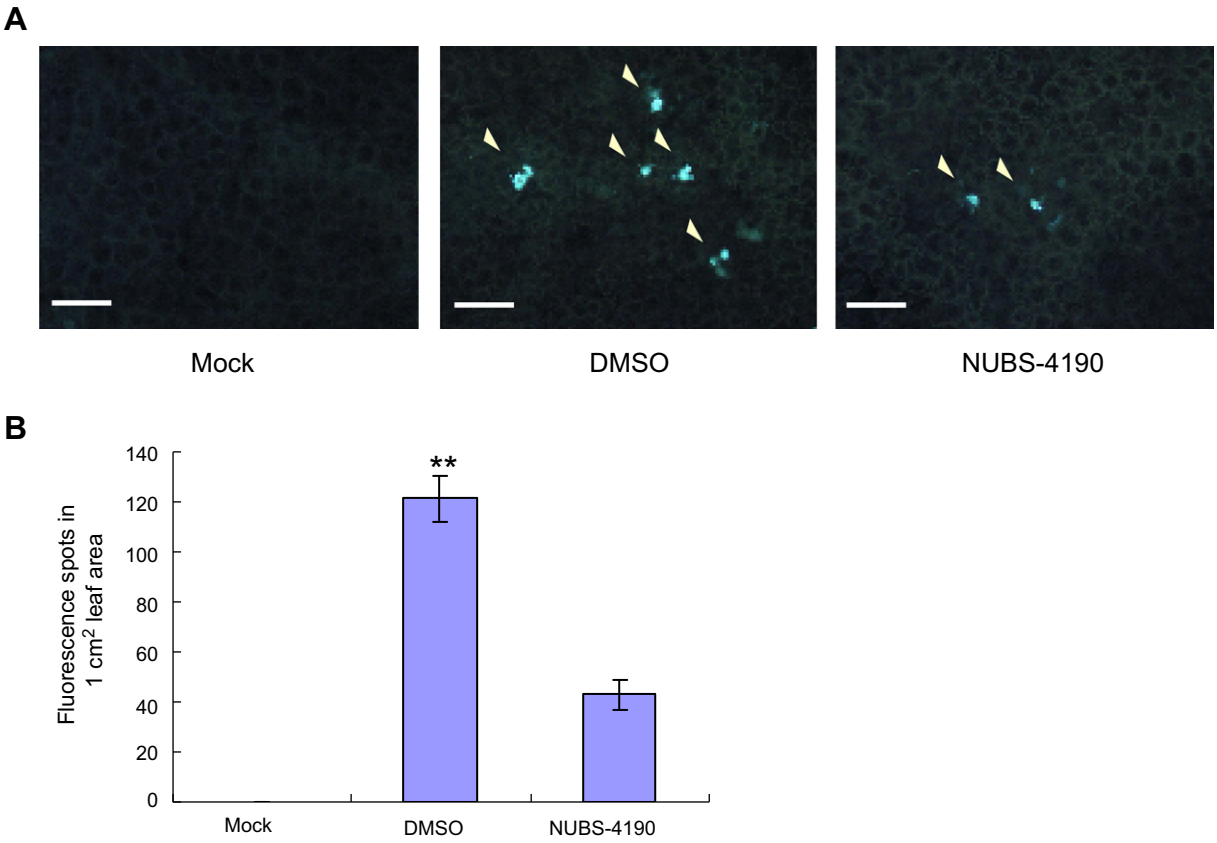

Fig. 6. Role of NUBS-4190 in *N. benthamiana* contributing to penetration resistance against *P. infestans*. (A) Callose staining of *P. infestans* penetration sites. NUBS-4190- or DMSO-treated control plants were inoculated with *P. infestans* and penetration sites (callose depositions, arrowheads) were stained with aniline blue 24 h after inoculation. Bar = 50 μm). (B) Penetration sites by *P. infestans* were detected with aniline blue staining and number of spots were counted in 5 leaf discs per treatment. Data were subjected to Student's *t*-test. $^{**}P < 0.01$ versus NUBS-4190 treated elicitor treatment.

ROS production and hypersensitive cell death are independent of NUBS-4190

O_2^- producing activity of *N. benthamiana* was measured by treating with NUBS-4190 using O_2^- unique luminous reagent L-012. NUBS-4190 did not generate O_2^- in *N. benthamiana* leaves (Fig. 3A). Moreover, DAB staining of *N. benthamiana* leaves were done to detect H_2O_2 (Fig. 3B). H_2O_2 generation was not found in the leaves treated with 20 μg/ml of NUBS-4190 or 0.2% DMSO, while reddish brown coloration was found in the leaves treated with 150 nM INF1 as a positive control.

HR-like cell death was also examined in *N. benthamiana* leaves. Ion leakage has been observed as an indicator of plant cell death [41]. Similar amounts of ion leakage was detected in NUBS-4190-treated leaves 6, 12 and 24 h after treatment compared to DMSO-treated *N. benthamiana* leaves (Fig. 3C), indicating that no cell death activity is involved in NUBS-4190-treated plants. On the contrary, the INF1 elicitor induced higher degrees of cell death. These results show that neither ROS nor HR-like cell death producing activities participate with NO generation by NUBS-4190.

Expression of defense-related genes

Several defense-related gene primers for *NbPR1a*, *NbNOA1*, *NbCUE1*, *NbSERK3*, *NbPAL1a*, *NbNR* and *NbAOX1* were used to investigate the NUBS-4190-induced gene expression on *N. benthamiana*. Total RNAs were extracted from NUBS-4190-treated *N. benthamiana* leaves and were analyzed by RT-PCR using the *NbEF1α* gene as an internal standard (Fig. 4). Gene expression in 0.2% DMSO- and INF1-treated leaves were used as a negative and a positive control, respectively. Expression of *NbPR1a* (pathogenesis-related 1a), a marker gene of disease resistance, was induced in NUBS-4190 treated *N. benthamiana* leaves. *NOA1* participates in NO generation induced by elicitors [31,42,43]. Expression of *NbNOA1* (nitric oxide associated 1) was markedly induced in NUBS-4190-treated *N. benthamiana* leaves after 24 h treatment. NUBS-4190 induced expression of *NbCUE1* (chlorophyll a/b binding protein (CAB) underexpressed 1) and *NbSERK3* (somatic embryogenesis receptor kinases 3) genes which are involved in elevation of endogenous NO [5] and basal resistance against *P. infestans* in *N. benthamiana* [44], respectively. Higher expression of *NbAOX* (Alternative oxidase), *NbNR* (Nitrate reductase) and *NbPAL1a* (Phenylalanine amonialyase 1a) was not found in NUBS-4190-treated leaves. These results indicate that *NbPR1* and *NbNOA1* are mostly involved in NUBS-4190-induced defense activities in *N. benthamiana*.

NUBS-4190 induced disease resistance in N. benthamiana against P. infestans

To investigate the resistance activities during interaction between NUBS-4190-treated *N. benthamiana* and *P. infestans*, *N.*

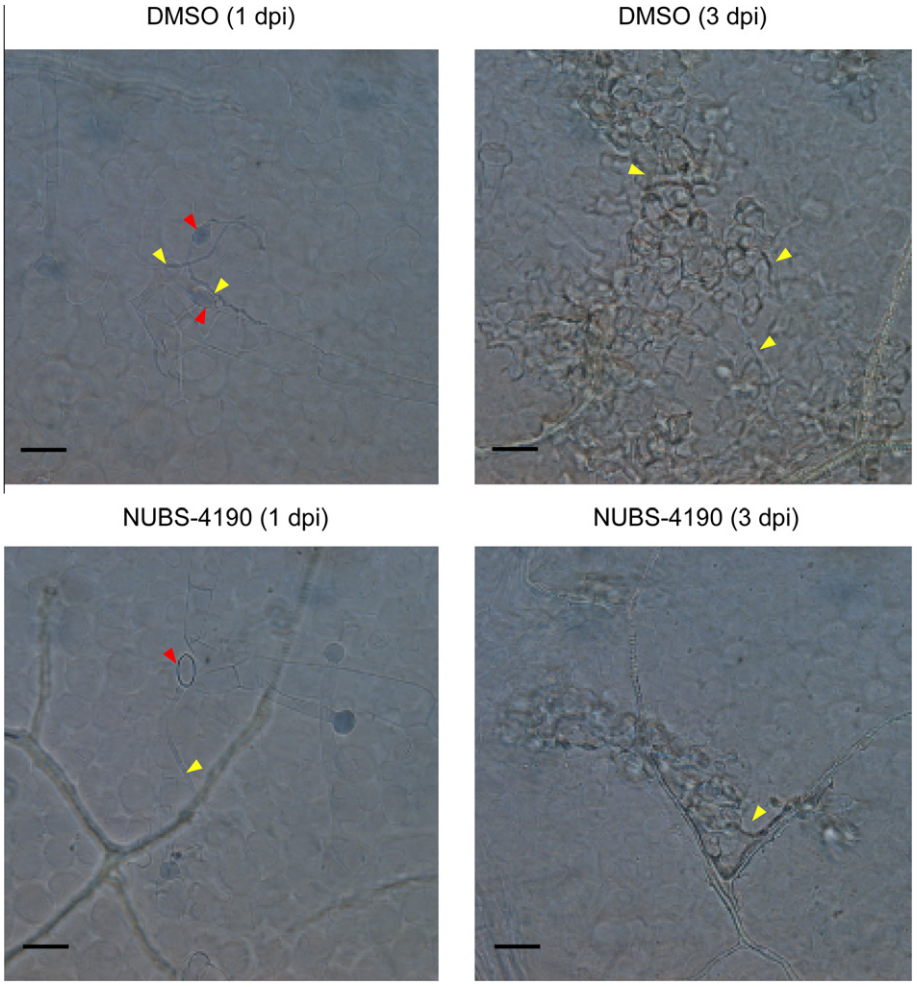

Fig. 7. Inhibition of *P. infestans* infection in NUBS-4190 treated *N. benthamiana* leaves. NUBS-4190- or DMSO-treated leaves were inoculated with *P. infestans* and hyphal growth and infection were observed microscopically. Inoculated leaves were stained with lactophenol trypan blue to visualize hyphae of *P. infestans*. Yellow arrowheads and red arrowheads indicate hyphae and zoosporangia of *P. infestans*, respectively. Bars = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

benthamiana plants were inoculated with *P. infestans*. Within 5 days after the inoculation of *P. infestans*, 0.2% DMSO treated plants (20–25 days from the date of sowing) showed water-soaked disease symptoms on inoculated areas of leaves (Fig. 5A), and the disease lesions almost extended over entire leaves within 7–8 days. In contrast, no symptoms or merely light brown discolorations were observed in same-aged leaves treated with NUBS-4190 (Fig. 5A and D). These results indicate that NUBS-4190 induces resistance in *N. benthamiana* plants against *P. infestans*. The interaction between *N. benthamiana* and *P. infestans* was examined using light microscopy of inoculated leaves after staining with lactophenol trypan blue which is indicative of cell death. As a result, densely stained epidermal and mesophyll cells were observed in the DMSO treated leaves from 5 days after inoculation. By 7 days after inoculation, only dark-stained, dead areas remained (Fig. 5B). On the other hand, no stained area developed in NUBS-4190-treated leaves. These results confirm that NUBS-4190 induces resistance in *N. benthamiana* plants against *P. infestans*.

NUBS-4190-treated plants display penetration resistance

Callose constitutes an important factor contributing to penetration resistance against invading pathogens. As a part of the defense responses of plants against *P. infestans*, callose is deposited at the penetration sites [45,46]. To detect the accumulation of callose, *N. benthamiana* plant leaves were treated with NUBS-4190 and inoculated with *P. infestans*. Callose was visualized by aniline blue staining of inoculated plant leaves. NUBS-4190-treated leaves showed fewer number of dot like calloses, whereas 0.2% DMSO-treated control plant showed many callose structures as an indication of successful hyphal penetration (Fig. 6A and B). These data suggest that NUBS-4190-treated plants show resistance against *P. infestans* and protect plants from hyphal penetration.

Inhibition of P. infestans in NUBS-4190-treated N. benthamiana plants

To observe the degree of *P. infestans* infection, *N. benthamiana* plants pre-treated with NUBS-4190 were inoculated with *P. infestans*, and then stained with lactophenol trypan blue (Fig. 7). After germination of cystspores of *P. infestans*, the hyphal growth and infection were suppressed in NUBS-4190 treated plant leaves. After 3 days of inoculation, few hyphae were observed in leaf surface treated with NUBS-4190, while normal hyphal growth of *P. infestans* was observed in DMSO treated plant leaves.

NO- and SA-mediated signaling in NUBS-4190-induced resistance of N. benthamiana against P. infestans

To investigate signaling pathways in NUBS-4190-induced resistance of *N. benthamiana* against *P. infestans*, VIGS was applied to knockdown genes involved in NO- and SA-mediated signaling pathways. Gene fragments of *N. benthamiana* homologues of *A. thaliana* NO induction *NOA1* and SA-mediated *PR1a* were isolated by reverse-transcription polymerase chain reaction (RT-PCR) and cloned into the vector for tobacco rattle virus (TRV)-mediated VIGS, pTV00 [28]. It was confirmed that expression of target genes was suppressed in VIGS-induced plants treated with NUBS-4190 (Fig. 8A). *NbPR1a*-silenced plants showed normal growth compared with the control TRV-infected plant, while the *NbNOA1*-silenced plant showed a chlorosis phenotype. Leaves of *NbPR1a* and *NbNOA1*-silenced *N. benthamiana* were treated with 0.2% DMSO and NUBS-4190. The treated plants were kept in an incubator for 24 h and then were inoculated with the spores of *P. infestans*. Over 7 days of observation after the inoculation, *NbNOA1*-silenced plants showed the development of disease symptoms after 5 days post inoculation and *NbPR1a*-silenced plants showed those

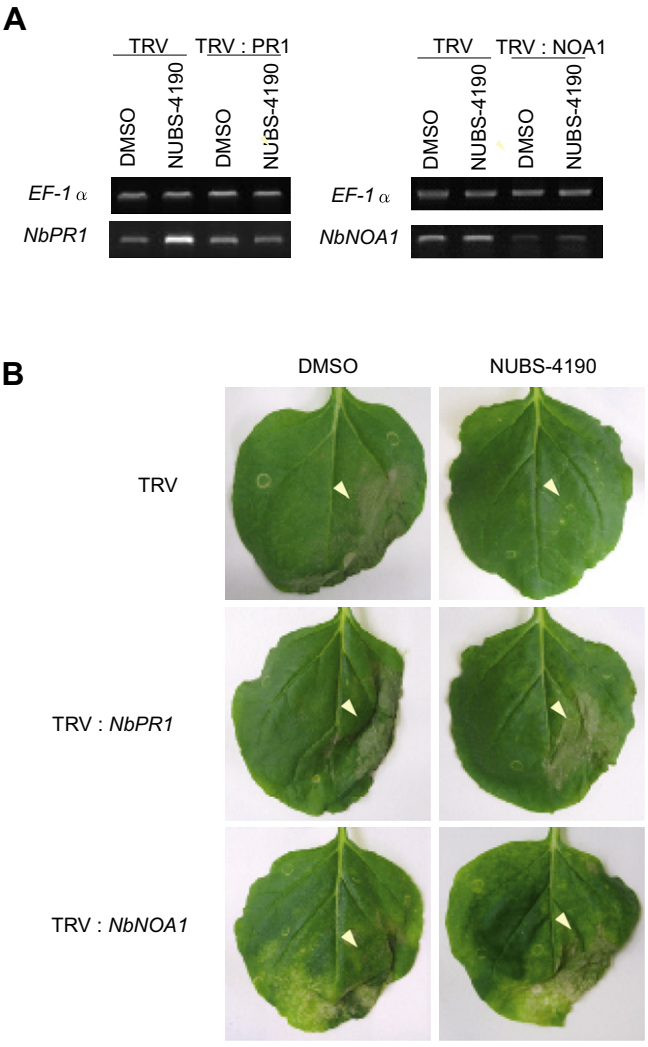

Fig. 8. Both *NbPR1* and *NbNOA1* are essential for NUBS-4190 induced resistance of *N. benthamiana* to *P. infestans*. (A) *N. benthamiana* was inoculated with Tobacco rattle virus (TRV), TRV:PR1 or TRV:NOA1 and total RNA was isolated from *N. benthamiana* leaves after the treatment with 20 μg/ml of NUBS-4190 or with DMSO as a control for 24 h. Silencing of target genes was confirmed by reverse-transcription polymerase chain reaction with gene specific primers from at least three separate leaves. (B) Leaves of *NbPR1* and *NbNOA1*-silenced *N. benthamiana* were treated with water containing DMSO and NUBS-4190 and treated plants were kept in an incubator for 24 h and then were inoculated with the spores of *P. infestans*. Arrowheads indicate the point of inoculation. Photographs were taken 5 days post inoculation.

symptoms after 6 days post inoculation (Fig. 8B). These data indicated that both *NbPR1a* and *NbNOA1* genes are involved in NUBS-4190-induced *N. benthamiana* resistance against *P. infestans*.

NbNOA1 associated NO generation by NUBS-4190 in N. benthamiana

To examine whether the induction of NO generation by NUBS-4190 depends on *NbNOA1* expression, *NbNOA1* silenced *N. benthamiana* plants were used. After 20 days of inoculation with the silencing constructs, *N. benthamiana* leaves were infiltrated with NUBS-4190 at the concentration of 20 μg/ml and incubated for 24 h after treatment. DAF-2DA-mediated low visibility of green fluorescence was detected in TRV:NOA1 *N. benthamiana* leaves treated with NUBS-4190, indicating suppression of NO (Fig. 9A and B). TRV plants treated with NUBS-4190 generated higher NO.

Therefore, it is suggested that *NbNOA1* is associated with NUBS-4190-induced NO generation in *N. benthamiana*.

Resistance against C. orbiculare infection

In Fig. 5, it was shown that NUBS-4190 induced disease resistance against late blight pathogen *P. infestans*. To know whether NUBS-4190 induced resistance against other pathogens or not, a hemi-biotrophic fungal pathogen, *C. orbiculare* (syn. *Colletotrichum lagenarium*) was selected for this experiment. NUBS-4190 and DMSO-treated control plants were kept in an incubator for 24 h and then conidia of *C. orbiculare* were inoculated by spraying onto the whole leaf. NUBS-4190 inducing resistance reactions successfully reduced *C. orbiculare* infection and disease development in *N. benthamiana* (Fig. 10). In NUBS-4190-treated leaves, development of disease spots began after one day compared to that in DMSO treated plants (Fig. 10C). Initially developed spots in NUBS-4190-treated *N. benthamiana* did not further spread and remained as a small and dot-like spot on the leaf surface (Fig. 10A).

Discussion

NUBS-4190 as a candidate for NO elicitor

It has been characterized that the elicitor-induced secondary metabolite production is mediated by the endogenous signaling among which NO has been reported to play an important role [47,48]. Elicitors derived from fungal or plant cell walls induce defense responses in plants by means of complex signaling mechanisms that include ion fluxes across the plasma membrane, reversible protein phosphorylation and generation of ROS and NO [49,50]. In the present situation involving an NO eliciting synthetic compound, we showed that a new bis-aryl methanone compound NUBS-4190 triggered NO generation in *N. benthamiana* leaves when it was detected using DAF2-DA. Since NO induction by

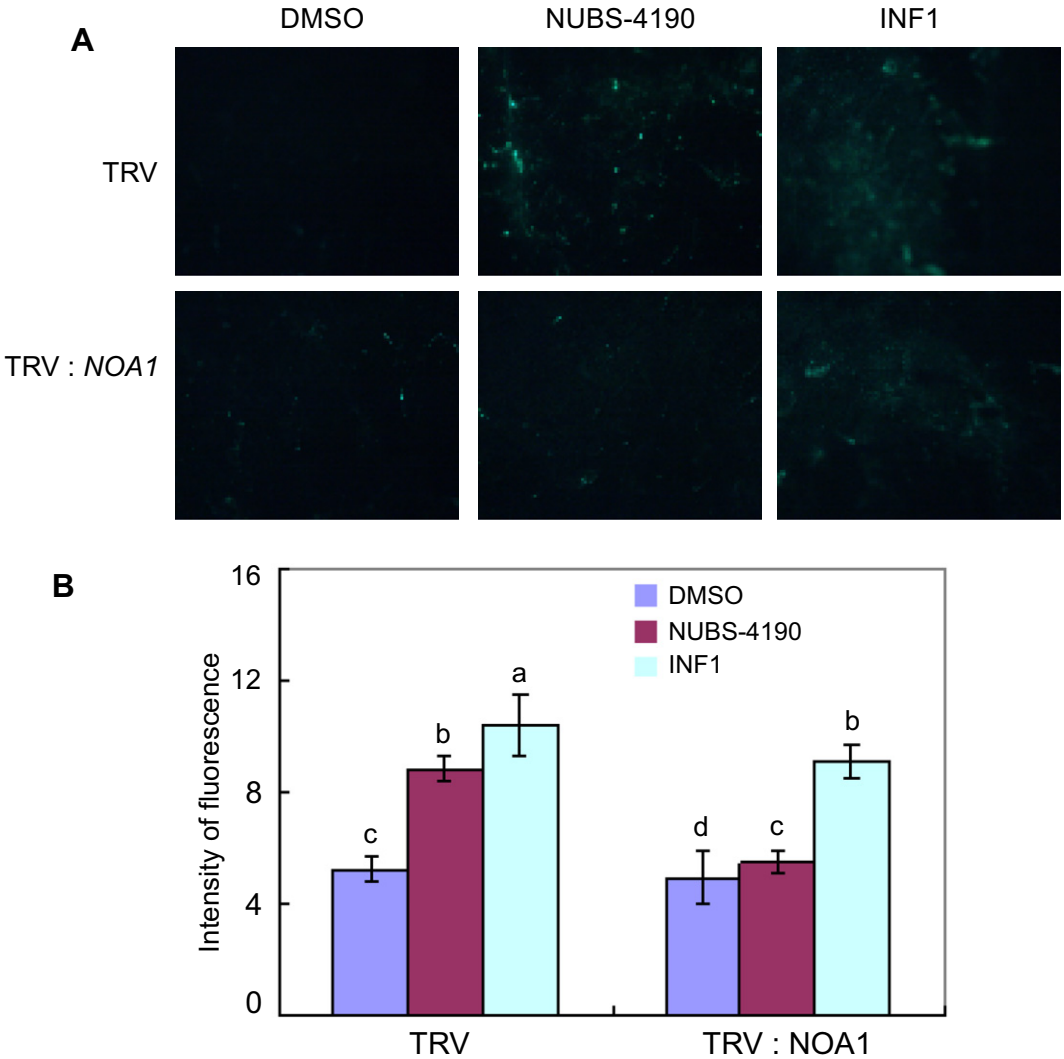

Fig. 9. *NbNOA1* associated NO generation by NUBS-4190 in *N. benthamiana*. (A) After 20 days of inoculation with gene silencing construct TRV:NOA1 or TRV, silenced *N. benthamiana* plant leaves were infiltrated with 20 μg/ml of NUBS-4190 and incubated for 24 h after treatment. The treated leaf areas were infiltrated with DAF-2DA solution and NO was determined by monitoring 1 h later using fluorescence stereomicroscopy. INF1 elicitor (150 nM) and 0.2% DMSO were used as a positive and a negative control, respectively. (B) Signal intensities were quantified by determining the mean channel values for the images with the histogram function. Each value represents the mean and standard deviation of three replicates. Different letters designate statistically different fluorescence intensity (ANOVA, $^{***}P < 0.001$).

NUBS-4190 in *N. benthamiana* is sharp and reproducible, NUBS-4190 could be a good candidate for a NO elicitor. However, further investigation is still needed to convince that the induced molecule should be NO.

Relationship among NO, ROS and HR

As shown in Fig. 3A and B, ROS (O_2^- and H_2O_2) induction was not detected in NUBS-4190-treated *N. benthamiana*. Ion leakage indicating cell death was not induced by NUBS-4190 in *N. benthamiana* leaves (Fig. 3C). It is unique that NUBS-4190-induced resistance is dependent on a NO, non-ROS and non-HR-like cell death in *N. benthamiana*. These results provide two possibilities. Firstly, NUBS-4190 may not induce ROS completely. It is possible that NO and ROS together, but not individually, are required to induce cell death [3], and balanced production of NO and O_2^- resulting in production of $ONOO^-$ is important to induce hypersensitive response (HR)-like cell death [51]. Secondly, NO production may suppress ROS production. Some studies reported that reducing endogenous NO level using cPTIO or plant mutants impaired in inducible NO production enhanced H_2O_2 accumulation [52,53], suggesting that part of the O_2^- produced by NADPH oxidase is scavenged by NO. Yun et al. [54] found that NO abolished *AtrbohD* activity through S-nitrosylation. NO is not necessary to induce HR-like cell death. Actually, dihydrosphingosine-induced NO production in tobacco BY-2 cells is not necessary for the induction of HR-like cell death [55].

Resistance in N. benthamiana against P. infestans

To examine the defense role of NUBS-4190, NUBS-4190-treated *N. benthamiana* leaves were inoculated with *P. infestans*. It was found that a higher level of resistance in *N. benthamiana* was stimulated at the stage susceptible to *P. infestans* (Fig. 5). These results were not caused by antifungal activity by NUBS-4190 because no inhibition of *P. infestans* was recorded by NUBS-4190 in rye-seed nutrient media (Supplementary Fig. 1). Several studies support our findings. Elicitors from *Phytophthora* spp. could activate a wide variety of protective mechanisms involved in preventing pathogen replication and spread in *Nicotiana* species [15]. Similar results were also found in *Taxus chinensis* suspension cells induced by elicitor from *Penicillium citrinum* [56], soybean cotyledons induced by elicitor from *Diaporthe phaseolorum* f. sp. *meridionalis* [57] and *Hypericum perforatum* suspension cells induced by elicitors from *Aspergillum niger* [14]. Elicitors and consequent NO play crucial roles in the regulation of both defense responses and resultant resistance to fungal pathogens [11,58]. Zheng et al. [59] reported that NO production induced by an

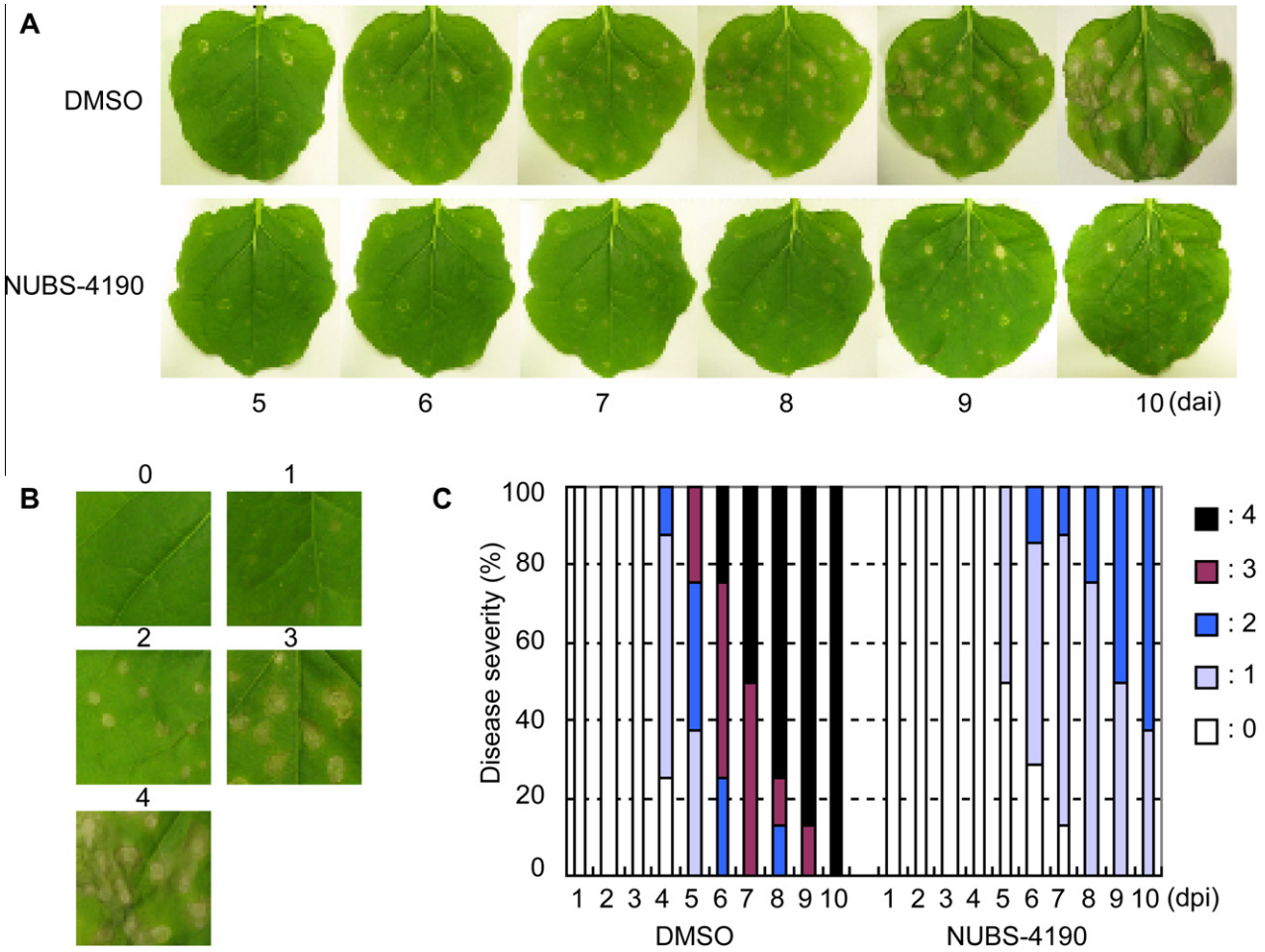

Fig. 10. NUBS-4190 induced resistance against *C. orbiculare* in *N. benthamiana* plants. (A) DMSO (0.2%) treated control plant leaves and NUBS-4190 treated plant leaves were kept in an incubator for 24 h and then conidia of *C. orbiculare* were inoculated by spraying into the whole leaf. (B) Appearance of disease symptoms showing differences in severity representative of the five classifications used in C. (C) Plots showing percentages of *N. benthamiana* leaves with disease symptom severities in each of five classes. Treated leaves were inoculated with *C. orbiculare* and severities of disease symptoms were evaluated from 1 to 10 days after inoculation. Five inoculated leaves from each treatment were counted.

elicitor from *Botrytis cinerea* plays an important role in disease resistance in tomato fruit.

Synthesis of secondary metabolite calloses plays a marginal role in penetration resistance [60]. NUBS-4190-treated *N. benthamiana* leaves showed the number of calloses to be reduced after challenge inoculation of *P. infestans* indicating that the compound induced the penetration resistance or inability to produce infection (Fig. 6). The Arabidopsis mutants *powdery mildew resistant 4* (*pmr4*) or *glucan synthase-like 5* (*gsl5*), which are defective in gene encoding callose synthase responsible for the callose depositions at wound and penetration sites, were more resistant against powdery mildew infection [60,61]. The enhanced resistance against otherwise virulent pathogens suggests that callose is not required for resistance and that, in fact, the loss of callose synthase is responsible for resistance [62]. Additionally, we investigated infection of *P. infestans* in NUBS-4190 treated *N. benthamiana* and we found delay or inhibition of *P. infestans* growth (Fig. 7). Taken together, these results suggest that NUBS-4190 triggers penetration resistance in *N. benthamiana* and *P. infestans* interactions.

NbPR1a and NbNOA1 are essential for the resistance to P. infestans

NUBS-4190 induced the expression of *NbPR1a* and *NbNOA1* as shown in Fig. 4. Salicylic acid functions as a key signal in regulating disease resistance by inducing *PR* gene expression [63]. Eschen-Lippold et al. [62] reported that the increased resistance in potato transgenic plants correlated with the constitutive accumulation of salicylic acid and *PR1* transcripts. Furthermore, defense responses in plants and *PR1* gene expression are activated by NO [64]. These reports suggest that NO induced SA-mediated signaling pathway resulting in *PR1a* expression.

In addition, we showed that NO generation was reduced in *NOA1*-silenced *N. benthamiana* treated with NUBS-4190 (Fig. 8). NOA1 in plant cells was initially considered to be functionally similar to mammalian NO synthase [64], but was shown to have GTPase rather than NO synthase activity [65]. NO production and its regulation still remain controversial. Recently, Van Ree et al. [66] suggested that NOA1 has a primary role in chloroplast function and indirectly affects NO accumulation. Mandal et al. [67] reported that oleic acid regulates NO synthesis by regulating NOA1 levels. Our study revealed that leaves of *NbPR1a* and *NbNOA1*-silenced *N. benthamiana* treated with NUBS-4190 showed the development of disease symptoms after challenge inoculation of *P. infestans* (Fig. 8B), indicating a significant reduction in resistance against *P. infestans*. Kato et al. [31] showed that silencing *NbNOA1* decreased INF1-induced NO production as well as suppressed INF1-induced *PR1a* gene expression and increased susceptibility to *C. lagenarium*. It may be possible that NO levels increased by NOA1 activate disease resistance against plant pathogen via expression of PR1a.

Resistance against C. orbiculare infection

Our experiment showed induction of substantial resistance by NUBS-4190 in *N. benthamiana* to *P. infestans*. NUBS-4190 also lead to enhanced resistance against *C. orbiculare*, which possibly resulted in the NO generation and *PR1a* gene induction by NUBS-4190 in *N. benthamiana*. Some early infections were observed in NUBS-4190 treated leaves, but the infected areas were not increased and leaves developed normally (data not shown). This suggested that NUBS-4190 and resultant NO induced basal resistance.

All of these results by NUBS-4190 provide a better understanding of the NO-mediated defense mechanisms in *N. benthamiana*. The bioassay of NUBS-4190 in *N. benthamiana* thus revealed the role of NO for defense against *P. infestans*. In particular, in *NbNOA1*- and *NbPR1a*-silenced *N. benthamiana* plants defense ability against *P. infestans* was reduced suggesting their involvement in resistant reactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.niox.2012.12.004.

References

[1] D. Wendehenne, A. Pugin, D.F. Klessig, J. Durner, Nitric oxide: comparative synthesis and signaling in animal and plant cells, Trends in Plant Science 6 (2001) 177–183.
[2] A. Besson-Bard, A. Pugin, D. Wendehenne, New insights into nitric oxide signaling in plants, Annual Review of Plant Biology 59 (2008) 21–39.
[3] M. Delledonne, Y.J. Xia, R.A. Dixon, C. Lamb, Nitric oxide functions as a signal in plant disease resistance, Nature 394 (1998) 585–588.
[4] J. Durner, D. Wendehenne, D.F. Klessig, Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose, Proceedings of the National Academy of Sciences of the United States of America 95 (1998) 10328–10333.
[5] Y.K. He, R.H. Tang, Y. Hao, R.D. Stevens, C.W. Cook, S.M. Am, L.F. Jing, Z.G. Yang, L.G. Chen, F.Q. Guo, F. Fiorani, R.B. Jackson, N.M. Crawford, Z.M. Pei, Nitric oxide represses the *Arabidopsis* floral transition, Science 305 (2004) 1968–1971.
[6] I.D. Wilson, S.J. Neill, J.T. Hancock, Nitric oxide synthesis and signalling in plants, Plant Cell and Environment 31 (2008) 622–631.
[7] L. Perchepied, C. Balague, C. Riou, C. Claudel-Renard, N. Riviere, B. Grezes-Besset, D. Roby, Nitric oxide participates in the complex interplay of defense-related signaling pathways controlling disease resistance to *Sclerotinia sclerotiorum* in *Arabidopsis thaliana*, Molecular Plant–Microbe Interactions 23 (2010) 846–860.
[8] M. Delledonne, NO news is good news for plants, Current Opinion in Plant Biology 8 (2005) 390–396.
[9] X. Huang, U. von Rad, J. Durner, Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells, Planta 215 (2002) 914–923.
[10] M. Parani, S. Rudrabhatla, R. Myers, H. Weirich, B. Smith, D.W. Leaman, S.L. Goldman, Microarray analysis of nitric oxide responsive transcripts in *Arabidopsis*, Plant Biotechnology Journal 2 (2004) 359–366.
[11] T. Nurnberger, Signal perception in plant pathogen defense, Cellular and Molecular Life Sciences 55 (1999) 167–182.
[12] X.Y. Hu, S.J. Neill, W.M. Cai, Z.C. Tang, Nitric oxide mediates elicitor-induced saponin synthesis in cell cultures of *Panax ginseng*, Functional Plant Biology 30 (2003) 901–907.
[13] J.W. Wang, J.Y. Wu, Involvement of nitric oxide in elicitor-induced defense responses and secondary metabolism of *Taxus chinensis* cells, Nitric Oxide 11 (2004) 298–306.
[14] M.J. Xu, J.F. Dong, M.Y. Zhu, Nitric oxide mediates the fungal elicitor-induced hypericin production of *Hypericum perforatum* cell suspension cultures through a jasmonic-acid-dependent signal pathway, Plant Physiology 139 (2005) 991–998.
[15] Y.H. Zhang, X.F. Yang, Q. Liu, D.W. Qju, Y.L. Zhang, H.M. Zeng, J.J. Yuan, J.J. Mao, Purification of novel protein elicitor from *Botrytis cinerea* that induces disease resistance and drought tolerance in plants, Microbiological Research 165 (2010) 142–151.
[16] R. Desikan, R. Griffiths, J. Hancock, S. Neill, A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*, Proceedings of the National Academy of Sciences of the United States of America 99 (2002) 16314–16318.
[17] G.C. Pagnussat, M. Simontacchi, S. Puntarulo, L. Lamattina, Nitric oxide is required for root organogenesis, Plant Physiology 129 (2002) 954–956.
[18] X. Huang, K. Stettmaier, C. Michel, P. Hutzler, M.J. Mueller, J. Durner, Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*, Planta 218 (2004) 938–946.
[19] N.N. Tun, M. Livaja, J.J. Kieber, G.F.E. Scherer, Zeatin-induced nitric oxide (NO) biosynthesis in *Arabidopsis thaliana* mutants of NO biosynthesis and of two-component signaling genes, New Phytologist 178 (2008) 515–531.
[20] J.C. Campos-Cuevas, R. Pelagio-Flores, J. Raya-Gonzalez, A. Mendez-Bravo, R. Ortiz-Castro, J. Lopez-Bucio, Tissue culture of *Arabidopsis thaliana* explants

reveals a stimulatory effect of alkamides on adventitious root formation and nitric oxide accumulation, Plant Science 174 (2008) 165–173.
[21] A. Mendez-Bravo, J. Raya-Gonzalez, L. Herrera-Estrella, J. Lopez-Bucio, Nitric oxide is involved in alkamide-induced lateral root development in *Arabidopsis*, Plant and Cell Physiology 51 (2010) 1612–1626.
[22] T. Noritake, K. Kawakita, N. Doke, Nitric oxide induces phytoalexin accumulation in potato tuber tissues, Plant and Cell Physiology 37 (1996) 113–116.
[23] I. Murgia, M.C. de Pinto, M. Delledonne, C. Soave, L. de Gara, Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells, Journal of Plant Physiology 161 (2004) 777–783.
[24] L. Ederli, L. Reale, L. Madeo, F. Ferranti, C. Gehring, M. Fornaciari, B. Romano, S. Pasqualini, NO release by nitric oxide donors in vitro and in planta, Plant Physiology and Biochemistry 47 (2009) 42–48.
[25] M.N. Khan, M.H. Siddiqui, F. Mohammad, M. Naeem, Interactive role of nitric oxide and calcium chloride in enhancing tolerance to salt stress, Nitric Oxide 27 (2012) 210–218.
[26] J.S. Stamler, D.J. Singel, J. Loscalzo, Biochemistry of nitric oxide and its redox-activated forms, Science 258 (1992) 1898–1902.
[27] N. Hogg, Biological chemistry and clinical potential of S-nitrosothiols, Free Radical Biology and Medicine 28 (2000) 1478–1486.
[28] F. Ratcliff, A.M. Martin-Hernandez, D.C. Baulcombe, Tobacco rattle virus as a vector for analysis of gene function by silencing, Plant Journal 25 (2001) 237–245.
[29] Y. Shibata, K. Kawakita, D. Takemoto, Age-related resistance of *Nicotiana benthamiana* against Hemibiotrophic pathogen *Phytophthora infestans* requires both ethylene- and salicylic acid-mediated signaling pathways, Molecular Plant–Microbe Interactions 23 (2010) 1130–1142.
[30] N. Doke, Prevention of the hypersensitive reaction of potato cells to infection with an incompatible race of *Phytophthora infestans* by constituents of the zoospores, Physiological Plant Pathology 7 (1975) 1–7.
[31] H. Kato, S. Asai, A. Yamamoto-Katou, H. Yoshioka, N. Doke, K. Kawakita, Involvement of NbNOA1 in NO production and defense responses in INF1-treated *Nicotiana benthamiana*, Journal of General Plant Pathology 74 (2008) 15–23.
[32] A. Yamamoto, S. Katou, H. Yoshioka, N. Doke, K. Kawakita, Involvement of nitric oxide generation in hypersensitive cell death induced by elicitin in tobacco cell suspension culture, Journal of General Plant Pathology 70 (2004) 85–92.
[33] S. Kamoun, P. vanWest, A.J. deJong, K.E. deGroot, V. Vleeshouwers, F. Govers, A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato, Molecular Plant–Microbe Interactions 10 (1997) 13–20.
[34] M. Boccara, C.E. Mills, J. Zeier, C. Anzi, C. Lamb, R.K. Poole, M. Delledonne, Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host, Plant Journal 43 (2005) 226–237.
[35] M. Kobayashi, I. Ohura, K. Kawakita, N. Yokota, M. Fujiwara, K. Shimamoto, N. Doke, H. Yoshioka, Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase, Plant Cell 19 (2007) 1065–1080.
[36] H. ThordalChristensen, Z.G. Zhang, Y.D. Wei, D.B. Collinge, Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction, Plant Journal 11 (1997) 1187–1194.
[37] S.-I. Yeom, H.-K. Baek, S.-K. Oh, W.-H. Kang, S.J. Lee, J.M. Lee, E. Seo, J.K.C. Rose, B.-D. Kim, D. Choi, Use of a secretion trap screen in pepper following *Phytophthora capsici* infection reveals novel functions of secreted plant proteins in modulating cell death, Molecular Plant–Microbe Interactions 24 (2011) 671–684.
[38] D. Takemoto, D.A. Jones, A.R. Hardham, GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of *Arabidopsis* by oomycete pathogens, Plant Journal 33 (2003) 775–792.
[39] H.B. Currier, S. Strugger, Analine blue and fluorescence microscopy of callose in bulb scales of *Allium cepa* L, Protoplasma 45 (1956) 552–559.
[40] A. Yamamoto-Katou, S. Katou, H. Yoshioka, N. Doke, K. Kawakita, Nitrate reductase is responsible for elicitin-induced nitric oxide production in *Nicotiana benthamiana*, Plant and Cell Physiology 47 (2006) 726–735.
[41] L. Rizhsky, H.J. Liang, R. Mittler, The combined effect of drought stress and heat shock on gene expression in tobacco, Plant Physiology 130 (2002) 1143–1151.
[42] D. Zeidler, U. Zahringer, I. Gerber, I. Dubery, T. Hartung, W. Bors, P. Hutzler, J. Durner, Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes, Proceedings of the National Academy of Sciences of the United States of America 101 (2004) 15811–15816.
[43] E. Gas, U. Flores-Perez, S. Sauret-Gueto, M. Rodriguez-Concepcion, Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism, Plant Cell 21 (2009) 18–23.
[44] A. Chaparro-Garcia, R.C. Wilkinson, S. Gimenez-Ibanez, K. Findlay, M.D. Coffey, C. Zipfel, J.P. Rathjen, S. Kamoun, S. Schornack, The receptor-like kinase SERK3/BAK1 is required for basal resistance against the late blight pathogen *Phytophthora infestans* in *Nicotiana benthamiana*, Plos One 6 (2011) e16608.
[45] V. Vleeshouwers, W. van Dooijeweert, F. Govers, S. Kamoun, L.T. Colon, The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*, Planta 210 (2000) 853–864.
[46] V.A. Halim, L. Eschen-Lippold, S. Altmann, M. Birschwilks, D. Scheel, S. Rosahl, Salicylic acid is important for basal defense of *Solanum tuberosum* against *Phytophthora infestans*, Molecular Plant–Microbe Interactions 20 (2007) 1346–1352.
[47] J. Zhao, L.C. Davis, R. Verpoorte, Elicitor signal transduction leading to production of plant secondary metabolites, Biotechnology Advances 23 (2005) 283–333.
[48] M.J. Xu, Nitric oxide: a potential key point of the signaling network leading to plant secondary metabolite biosynthesis, Progress in Natural Science 17 (2007) 1397–1404.
[49] C. Lamb, R.A. Dixon, The oxidative burst in plant disease resistance, Annual Review of Plant Physiology and Plant Molecular Biology 48 (1997) 251–275.
[50] J. Durner, D.F. Klessig, Nitric oxide as a signal in plants, Current Opinion in Plant Biology 2 (1999) 369–374.
[51] S. Saito, A. Yamamoto-Katou, H. Yoshioka, N. Doke, K. Kawakita, Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells, Plant and Cell Physiology 47 (2006) 689–697.
[52] Y. Tada, T. Mori, T. Shinogi, N. Yao, S. Takahashi, S. Betsuyaku, M. Sakamoto, P. Park, H. Nakayashiki, Y. Tosa, S. Mayama, Nitric oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defense response of oat, Molecular Plant–Microbe Interactions 17 (2004) 245–253.
[53] S. Asai, K. Ohta, H. Yoshioka, MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*, Plant Cell 20 (2008) 1390–1406.
[54] B.W. Yun, A. Feechan, M.H. Yin, N.B.B. Saidi, T. Le Bihan, M. Yu, J.W. Moore, J.G. Kang, E. Kwon, S.H. Spoel, J.A. Pallas, G.J. Loake, S-nitrosylation of NADPH oxidase regulates cell death in plant immunity, Nature 478 (2011). 264-U161.
[55] D. Da Silva, C. Lachaud, V. Cotelle, C. Brière, S. Grat, C. Mazars, P. Thuleau, Nitric oxide production is not required for dihydrosphingosine-induced cell death in tobacco BY-2 cells, Plant Signaling and Behavior 6 (2011) 736–739.
[56] M.J. Xu, J.F. Dong, M.Y. Zhu, Involvement of NO in fungal elicitor-induced activation of PAL and stimulation of taxol synthesis in *Taxus chinensis* suspension cells, Chinese Science Bulletin 49 (2004) 1038–1043.
[57] L.V. Modolo, F.Q. Cunha, M.R. Braga, I. Salgado, Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp. *meridionalis* elicitor, Plant Physiology 130 (2002) 1288–1297.
[58] Y.J. Yuan, C. Li, Z.D. Hu, J.C. Wu, A.P. Zeng, Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxol production, Process Biochemistry 38 (2002) 193–198.
[59] Y. Zheng, L. Shen, M.M. Yu, B. Fan, D.Y. Zhao, L.Y. Liu, J.P. Sheng, Nitric oxide synthase as a postharvest response in pathogen resistance of tomato fruit, Postharvest Biology and Technology 60 (2011) 38–46.
[60] A.K. Jacobs, V. Lipka, R.A. Burton, R. Panstruga, N. Strizhov, P. Schulze-Lefert, G.B. Fincher, An *Arabidopsis* callose synthase, GSL5, is required for wound and papillary callose formation, Plant Cell 15 (2003) 2503–2513.
[61] M.T. Nishimura, M. Stein, B.H. Hou, J.P. Vogel, H. Edwards, S.C. Somerville, Loss of a callose synthase results in salicylic acid-dependent disease resistance, Science 301 (2003) 969–972.
[62] L. Eschen-Lippold, R. Landgraf, U. Smolka, S. Schulze, M. Heilmann, I. Heilmann, G. Hause, S. Rosahl, Activation of defense against *Phytophthora infestans* in potato by down-regulation of syntaxin gene expression, New Phytologist 193 (2012) 985–996.
[63] J. Durner, J. Shah, D.F. Klessig, Salicylic acid and disease resistance in plants, Trends in Plant Science 2 (1997) 266–274.
[64] F.Q. Guo, M. Okamoto, N.M. Crawford, Identification of a plant nitric oxide synthase gene involved in hormonal signaling, Science 302 (2003) 100–103.
[65] M. Moreau, G.I. Lee, Y. Wang, B.R. Crane, D.F. Klessig, AtNOS/AtNOA1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric-oxide synthase, Journal of Biological Chemistry 283 (2008) 32957–32967.
[66] K. Van Ree, B. Gehl, E.W. Chehab, Y.-C. Tsai, J. Braam, Nitric oxide accumulation in Arabidopsis is independent of NOA1 in the presence of sucrose, Plant Journal 68 (2011) 225–233.
[67] M.K. Mandal, A.C. Chandra-Shekara, R.D. Jeong, K.S. Yu, S.F. Zhu, B. Chanda, D. Navarre, A. Kachroo, P. Kachroo, Oleic acid-dependent modulation of NITRIC OXIDE ASSOCIATED 1 protein levels regulates nitric oxide-mediated defense signaling in *Arabidopsis*, Plant Cell 24 (2012) 1654–1674.