Defense induced by a bis-aryl methanone compound leads to resistance in potato against *Phytophthora infestans*

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Abstract Plants recognize certain microbial compounds as elicitors of their active defense mechanisms. In the present study, NUBS-4190, a synthetic bis-aryl-methanone compound elicited NO and ROS generation in potato suspension cultured cells and intact potato leaves. Hypersensitive cell death was found in these cultured cells and in potato leaves without the accumulation of phytoalexins in the tubers. Defense-related genes such as *StrbohB*, *StrbohC*, *StNR1*, *StNR5*, *Sthsr203J* and *StPR1* were expressed in potato suspension cultured cells treated with NUBS-4190. Resistance against *Phytophthora infestans* also increased in NUBS-4190-treated potato leaves.

Keywords Bis-aryl methanone · Nitric oxide · *Phytophthora infestans* · Potato · Reactive oxygen species · Resistance

Introduction

Early recognition of a pathogen by a plant is essential for the plant to activate the available biochemical and structural defenses against the pathogen. Once a particular plant molecule recognizes and reacts with bioactive compound(s), alarm signals are transduced to host cell molecules and to nuclear genes to activate the production of protective substances against the pathogen. Early responses involving ion influx, production of nitric oxide (NO) and reactive oxygen

species (ROS), such as H_2O_2 and O_2^- , then transduce the elicitor signal to downstream defense responses as signalling molecules (Zhao et al. 2005). These early events are followed by other defense responses including induction of HR-like cell death, limiting pathogen spread (Apel and Hirt 2004), and further reinforcement of the cell walls and increased expression of defense-associated genes (Mur et al. 2008).

In plants, NO is known to participate in several responses, including germination, flowering, stomatal closure and pathogen defense (Besson-Bard et al. 2008; Delledonne et al. 1998; Durner et al. 1998; Wilson et al. 2008). NO participates in the complex interplay of defense-related signalling pathways controlling disease resistance (Perchevied et al. 2010). NO and target genes of NO contribute to the oligogalacturonide-triggered responses against *Botrytis cinerea* (Rasul et al. 2012), NO participates as a partner of ROS in disease resistance to *B. cinerea* in *Nicotiana benthamiana* (Asai and Yoshioka 2009). This involvement of NO in plant disease resistance is related to its interplay with ROS, and cell death during hypersensitive response (HR) may result from the simultaneous and balanced production of NO and ROS (Zaninotto et al. 2006). NO and ROS exert reciprocal control on each other (Arasimowicz-Jelonek et al. 2009; Cecconi et al. 2009; Clarke et al. 2000). In the context of plant–pathogen interactions, NO is involved in the modulation of salicylic acid, jasmonic acid, and ethylene synthesis and possibly other response mechanisms in plants (Delledonne 2005; Huang et al. 2002; Parani et al. 2004).

Another feature of disease resistance in many plants is the rapid collapse and death of challenged cells in the HR, a form of programmed cell death (PCD) in plants (Dangl et al. 1996; Lam et al. 2001). Hypersensitive cell death depends on O_2^- and H_2O_2 generation, is a key player accompanying the defense (Heath 2000; Torres 2010; Zurbirriggen et al. 2009).

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H₂O₂ involvement is also reported in many types of PCD induced by abiotic stress. Indeed, H₂O₂ is involved in the PCD induced by heat shock in tobacco BY-2 cells (Locato et al. 2008; Vacca et al. 2004) and in the hypoxia-induced lysigenous aerenchyma formation in *Arabidopsis* (Mühlenbock et al. 2007). Treatment with NO generators contradictorily results in diminishing ROS effects (Noritake et al. 1996; Orozco-Cárdenas and Ryan 2002) or in increasing them (Delledonne et al. 2001; Murgia et al. 2004). The balance between ROS and NO has also been proposed to be a critical aspect in inducing PCD (Delledonne et al. 1998, 2001).

Many elicitors have been isolated from various organisms, including bacteria, viruses, oomycetes and fungi. Typical examples of general elicitors are chitin (Felix et al. 1993), bacterial flagellin (Felix et al. 1999; Zipfel et al. 2004), elicitors from *Phytophthora* species such as cryptogein in their apoplast (Billard et al. 1988) or oomycete Pep-13 (Nürnberg et al. 1994). Chemical or synthetic compounds also can have elicitor activities in plants. Cheong et al. (1991) showed that chemically synthesized oligo-8-glucosides, ranging in size from hexamer to decamer, induced phytoalexin accumulation in soybean. Benzothiadiazole (BTH) and 2,6-dichloroisonicotinic acid (INA) induced resistance in different plant species as a preventive measure against pathogen growth (Goellner and Conrath 2008; Perazzolli et al. 2008; Vallad and Goodman 2004). A synthetic harmless chemical, β -aminobutyric acid induced resistance against numerous plant diseases in various pathosystems (Jakab et al. 2001).

These facts clearly show that a great diversity of elicitors, inducing various biological effects, are present in plants and crops. In the present study, we used NUBS-4190, a bis-aryl methanone (3,5-difluorophenyl-[3-methyl-4-(methylsulfonyl)isoxazol-5-yl]-methanone) to study induced resistant reactions (Fig. 1). NUBS-4190 is a synthetic compound previously reported to elicit NO and induced resistance in the model plant *N. benthamiana* against *Phytophthora infestans* (Monjil et al. 2013). Authors studied the defense role against pathogen, particularly to examine the effect of NUBS-4190 to ascertain early reactions and to evaluate the induced resistant reactions in potato plants and suspension cultured

potato cells. This study also examines the defense strategy in potato-*P. infestans* interactions and defense activities in potato in response to the NUBS-4190.

Materials and methods

Test plants

Suspension cultured potato cells, leaves and tubers of cultivar Sayaka, which has resistant genes *R1* and *R3*, were used: suspension cultured cells are very sensitive to early responses to NUBS-4190 treatment; intact potato leaves were used to observe elicitor responses in more natural conditions; tubers are the best material for examining phytoalexin production (unpublished data by M. S. Monjil and K. Kawakita). Suspension cultured cells were prepared from callus obtained from potato tuber discs in callus-inducing solid medium [1 mL/L B5-vitamin (100 g/L myo-inositol, 2.5 g/L glycine, 500 mg/L thiamine-HCl, 500 mg/L pyridoxine-HCl, 5 g/L nicotinic acid, 50 mg/L biotin, 50 mg/L folic acid), 2 mg/L naphthalene acetic acid (NAA), 0.5 mg/L kinetin, 3 % w/v sucrose, 0.2 % w/v Phytigel and Murashige-Skoog basal medium], in the dark. Suspension cultured cells were shake-cultured at 100 rpm at 23 °C in 80 mL of MS broth supplemented with 1 mL/L vitamin complex (100 g/L myo-inositol, 2.5 g/L glycine, 500 mg/L thiamine-HCl, 500 mg/L pyridoxine-HCl, 5 g/L nicotinic acid, 50 mg/L biotin, 50 mg/L folic acid), 2 mg/L NAA, 0.5 mg/L kinetin, 3 % sucrose and Murashige-Skoog basal medium. Cells were subcultured every week and used for experiments 4–5 days after the subculturing. Potato plants were grown at 23 °C and 70 % humidity with a 16 h photoperiod in environmentally controlled growth cabinets, and the leaves were removed for the experiments.

NUBS-4190 and other elicitors

NUBS-4190, a synthetic compound from the chemical compound library of the Plant Pathology Laboratory, Graduate School of Bioagricultural Sciences, Nagoya University, Japan was tested as an elicitor, and two other elicitors, INF1 (*infl* gene product) and MEM (methanol extract of mycelia from *P. infestans*), were used as positive controls in this study.

Elicitor INF1 was prepared according to the method of Yamamoto et al. (2004); overnight cultures of *Escherichia coli* cells, carrying a chimeric plasmid (pFB53) with the *infl* gene (Kamoun et al. 1997), were diluted (1:100) in Luria-Bertani broth containing 50 μ g/mL of ampicillin and incubated at 37 °C. When the OD₆₀₀ of cultures reached 0.6, production of INF1 was induced by adding 0.4 mM isopropyl

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

β -D-thiogalactopyranoside. After incubating for 3–4 h, the culture was centrifuged, and the resultant supernatant was dialyzed against water in SnakeSkin dialysis tubing (7 kDa molecular mass cutoff, Pierce Biotechnology, Rockford, IL, USA) overnight at 4 °C. This preparation was then used as INF1 elicitor.

MEM was prepared as follows. Mycelia collected from *P. infestans* were frozen in liquid nitrogen and ground with a mortar and pestle. The ground mycelia were transferred to a Falcon tube (50 mL) containing 10 mL of methanol per 1 g mycelia, then homogenized using a polytron type homogenizer (HG30, Hitachi Koki, Tokyo, Japan) for 2 min. After centrifugation at 4 °C, 3000×g for 30 min, the supernatant was collected and dried with an evaporator for use.

NUBS-4190 treatment

The suspension cultured potato cells, the intact potato leaves and the potato tubers differ in their sensitivity to NUBS-4190, so they were treated with different concentrations. The suspension cultured cells are the most sensitive and were treated with 1 µg/mL NUBS-4190, and leaves were treated with 10 µg/mL. For cell death experiments, a higher concentration (50 µg/mL) was applied so that cell death at the leaf surface could be clearly viewed. For phytoalexin studies in tubers, 50 µg/mL was used.

Pathogenic isolate of *P. infestans*

Pathogenic isolate (*P. infestans*) race 1.2.3.4 was used throughout the research. Zoosporeangia were collected, and zoospore production was induced by the methods of Doke (1975). Zoosporeangia suspensions were prepared following the method of Monjil et al. (2013); the isolate was subcultured on rye medium for 7–10 days, then 20 mL of water was added to the surface of the colonies, which were then rubbed with a cotton swab to release the zoosporeangia. NUBS-4190-pretreated intact leaves were inoculated with a 1 mL sample 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, and then moved to a growth room at 23 °C.

Measurement of NO production

NO was detected using the method described by Monjil et al. (2013). Suspension cultured potato cells were collected and washed with assay buffer (5 mM MES-KOH,

pH 5.7, containing 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄). They were resuspended in assay buffer and equilibrated for 1 h at 100 rpm at 23 °C. Cells were then treated with elicitors and incubated at 100 rpm at 23 °C for 3 h. NO was measured using the NO indicator diaminofluorescein-2 (DAF-2 DA; Daiichi Pure Chemicals, Tokyo, Japan). The cells were incubated with 10 µM DAF-2 DA for 1 h, then fluorescence from DAF-2T, the reaction product of DAF-2 with NO, was measured with a fluorescence spectrophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) at 30 °C. The excitation and emission wavelengths for DAF-2T are 495 nm and 515 nm (band paths 3 nm and 3 nm), respectively.

Potato leaves were infiltrated with 200 mM sodium phosphate buffer at pH 7.4 and 12.5 µM DAF-2DA, using a needleless syringe and were incubated for 1 h in the dark at room temperature before observation. Fluorescence from DAF-2T was captured using a fluorescence stereomicroscope (MZ16FA, Leica, Heerbrugg, Switzerland) equipped with a CCD camera (Color 14 bit, AxioCam HRc, Carl Zeiss, Göttingen, Germany). The fluorescence intensity of the scanned field of the image captured by a CCD camera was quantified by determining the mean values for the green channel for the images with the histogram function of Adobe Photoshop 7.0 (Adobe, Seattle, WA, USA) (Boccaro et al. 2005). A fluorescence image was obtained from 1 inoculated area per leaf, and at least 3 inoculated areas were analyzed as replicates for each treatment.

Measurement of O₂[−] production

Suspension cultured potato cells (50 mg/mL) were washed with the assay buffer (175 mM mannitol, 50 mM MES-KOH, 0.5 mM CaCl₂ and 0.5 mM K₂SO₄, pH 5.7) twice to remove liquid culture medium. For the detection of O₂[−] produced by cultured cells, the cells were resuspended in assay buffer, equilibrated for 1 h at 100 rpm at 23 °C. Cells were then treated with elicitors and incubated at the same condition for 3 h. After the treatment, the relative intensity of O₂[−] generation was measured by counting photons from the chemiluminescence of 20 mM L-012 (8-amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H) dione sodium salt)-mediated using a chemiluminescence reader Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany).

Plant leaf O₂[−] was measured as described by Kobayashi et al. (2007); potato leaves were infiltrated with 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) via a needleless syringe. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in the dark chamber at 20 °C (Aquacosmos

2.5; Hamamatsu Photonics, Shizuoka, Japan) and quantified using the U7501 program (Hamamatsu Photonics).

H₂O₂ in situ detection

Leaf discs of potato plants infiltrated with elicitors were removed with a cork borer at 3, 6, 12 and 24 h after infiltration. H₂O₂ was detected using the DAB staining method described by Thordal-Christensen et al. (1997). Leaf discs were placed in 1 mg/mL 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO, 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 phosphate-buffered saline/glycerol (50 %) and kept at 4 °C for further examination. H₂O₂ was visualized as a reddish brown coloration.

Detection of cell death

Opposite surfaces of leaves were infiltrated with elicitors using a needleless syringe. Death areas that developed on

the infiltrated leaf surface were monitored 6, 12, 24 and 48 h after treatment with elicitors. Cell death was also checked using an electrolyte leakage method, adapted from the method of Yeom et al. (2011). Leaves were infiltrated with elicitors under the leaf surface, and leaf discs (1 cm in diameter) were collected from the leaf 3, 6 and 12 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).

Detection of phytoalexins

Potato phytoalexins produced in potato tubers were extracted using ethyl acetate following the method described previously (Noritake et al. 1996) with a little modification. Concave holes (diameter 15 mm, depth 7 mm) were drilled in the parenchyma of the sliced tuber about 10–15 mm thick with an electric drill. After aging at 20 °C for 24 h in dark humid chamber, 500 µL of each treatment was added to the concave hole and was

Fig. 2 NO generation activities of NUBS-4190 in potato. **a** NO generation in potato suspension cultured cells at 3 h after treatment with 10 µg/mL of NUBS-4190 with or without 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO). DMSO (0.2 %, v/v) and *inf1* gene product (INF1) (150 nM) were used as negative and positive control, respectively. The cells were incubated with 10 µM 4,5-diaminofluorescein diacetate (DAF-2DA) for 1 h before measurement with a fluorescence spectrophotometer using 495 nm excitation and 515 nm emission wavelengths (band paths 3 nm and 3 nm, respectively). Each value represents the mean and standard deviation of 3 replicates. **b** Time course of NUBS-4190-induced NO generation experiments in suspension cultured potato

cells up to 6 h. **c** Potato leaves were infiltrated with 10 µg/mL NUBS-4190 and incubated for 12 h before NO measurement. The treated leaf areas were infiltrated with DAF-2DA solution and were monitored 1 h later using fluorescence stereomicroscopy. DMSO (1 % v/v) and INF1 (150 nM) were used as negative and positive control, respectively. In a separate experiment (lower panel), treated leaf areas were infiltrated with 1 mM cPTIO 1 h before infiltration with DAF-2DA. **d** 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 and ****P* < 0.001 vs DMSO control treatment

Fig. 3 O_2^- inducing activity of NUBS-4190 in potato. **a** NUBS-4190-induced O_2^- generation was measured in suspension cultured potato cells treated with 1/10 volume of NUBS-4190 for a final concentration of 1 μ g/mL. DMSO (0.2 %, v/v) was used as a negative control and methanol extract of mycelia from *Phytophthora infestans* (MEM) as a positive control. The O_2^- was measured in a 96-well microtiter plate with L-012 (a reagent to detect O_2^-) using a chemiluminescence plate reader, Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany). **b** O_2^- in potato leaves was measured by treating with 10 μ g/mL NUBS-4190. Treated leaf areas were infiltrated with L-012 solution 12 h after treatment and monitored using a CCD camera. Circles indicate areas infiltrated with L-012. **c** Chemiluminescence intensities of **b** were quantified with a photon image processor. Data were analyzed with a Student's *t* test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (vs DMSO control)

incubated under the same conditions for 48 h. Phytoalexins were extracted from collected liquid from treated holes with an equal amount of ethyl acetate. The extract was separated on TLC plates (TLC aluminum sheet of silica gel 60, Merck, Whitehouse Station, NJ, USA), which were developed with cyclohexane:ethylacetate (1:1, v/v) and visualized by spraying with 75 % sulfuric acid containing 0.5 % (w/v) vanillin followed by heating at 120 °C.

RNA preparation and RT-PCR

Expression of potato genes after the treatment of elicitors was analyzed by RT-PCR (Kato et al. 2008). RT-PCR was conducted using a commercial kit (ReverTra-Plus-; Toyobo Co., Osaka, Japan). The cDNA was synthesized from total RNA (1 μ g) 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. Gene-specific primers for each sequence were as follows: *StEF-1 α* forward primer, 5'-ATTGTGCTCATTGGCCACG-3'; *StEF-1 α* reverse primer, 5'-CCAGTCGAGGTTGGTAGACC-3'; *StrbohB* forward primer, 5'-ATCGAAAACACGAGGG ATTC-3'; *StrbohB* reverse primer, 5'-GCACCAGACTT ACTCCTATC-3'; *StrbohC* forward primer, 5'-CGGAAA ATCATCACCCGCAC-3'; *StrbohC* reverse primer, 5'-C GGCTCCGGCACTAGTTCCG-3'; *StPR1* forward primer, 5'-GTGCTAGAGTCAAGTGCAAC-3'; *StPR1* reverse primer, 5'-GAATCAAAGTCTGGTTGCTC-3'; *StNR 1 α* forward primer, 5'-TCAATTCACGATACTAGAGACG-3'; *StNR 1 α* reverse primer, 5'-GCATAAGTCGAGCCAA AGGG-3'; *StNR5* forward primer, 5'-TACAACA TTGGCATTGGCTTG-3'; *StNR5* reverse primer, 5'-AACA TTTTATACCTAAAGTTAACA-3'; *Stshr203 J* forward primer, 5'-AAGCTGATTGGTACATGTACTATGC-3'; *Stshr203 J* reverse primer, 5'-TAGCTCCGATTTACTTCG CTG-3'; *StPAL 1 α* forward primer, 5'-CTGGTCGGCCTA ATTAAAG-3'; and *StPAL 1 α* reverse primer, 5'-GGTTGC AGAACGGATGAC-3'.

Resistance-inducing activity against pathogen infection

Potato leaves were grown and were sprayed with 10 μ g/mL NUBS-4190 in DMSO (1 %, v/v) using a hand sprayer. DMSO (1 %, v/v) treatment was used as a control. After 24 h of treatment, leaves were inoculated with *P. infestans* (2×10^5 zoospores/mL), then kept in dark, humid conditions for 18 h.

Microscopic observations

Leaves of potato were stained with lactophenol trypan blue (Takemoto et al. 1999) with a minor modification to view cell death and colonization by *P. infestans*. Briefly, infected leaves were cleared in methanol overnight, then the cleared tissue was boiled for 2 min in lactophenol trypan blue stain (10 mL of H₂O, 10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue). After the leaves cooled at room temperature for 1 h, the stain was replaced with aqueous chloral hydrate (1 g/mL). Stained leaves were monitored using a light microscope (Olympus BX51, Tokyo, Japan).

Fig. 4 H_2O_2 accumulation in potato leaves treated with NUBS-4190. **a** Accumulation of H_2O_2 in potato leaves 3, 6, 12 and 24 h after treatment with by NUBS-4190 (10 $\mu\text{g}/\text{mL}$) and incubation with the dye 3,3'-diaminobenzidine (DAB). Methanol extract of mycelia from *Phytophthora infestans* (MEM) elicitor (500 $\mu\text{g}/\text{mL}$) was used as positive control, 1 % (v/v) DMSO as negative control. Reddish brown color indicates H_2O_2 . Bars 50 μm . **b** H_2O_2 in leaf tissues was quantified using ImageJ software. Each value represents the mean and standard deviation of 3 replicates. Data were analyzed with Student's *t*-test, * $P < 0.05$, ** $P < 0.01$ vs DMSO control

Results

NO generation

NO generation in a time-course experiment was assayed in suspension cultured cells of potato treated with 10 $\mu\text{g}/\text{mL}$ NUBS-4190-treated using DAF-2DA-mediated fluorescence (Fig. 2a). To scavenge NO responsive events in cells, a potent NO scavenger, cPTIO was used. NO generation in the cultured cells treated with NUBS-4190 was eliminated by cPTIO. Higher NO generation was found in the experiment from 1 to 6 h and peaked 3 h after treatment (Fig. 2b). NO accumulation in NUBS-4190-treated potato leaves was detected using DAF-2DA-mediated fluorescence with a spectrofluorometric assay. The leaf areas were treated with NUBS-4190 and infiltrated with DAF-2DA solution, and NO production was monitored 1 h later using fluorescence stereomicroscopy. An increase in fluorescence indicative of NO production was observed

when leaves were exposed to 10 $\mu\text{g}/\text{mL}$ NUBS-4190 (Fig. 2c, d). The addition of cPTIO suppressed elicitor-induced NO generation indicating that the increase of fluorescence was due to NO production. Increased NO generation was not found in 1 % (v/v) DMSO-treated potato leaves, rather basal fluorescence was detected by DMSO and did not differ statistically from the cPTIO treatment.

O_2^- generation

To study the role of NUBS-4190 on O_2^- induction in potato, we added NUBS-4190 to suspension cultured potato cells and O_2^- was measured by using the O_2^- -unique luminous reagent L-012. NUBS-4190 induced significantly higher O_2^- generation in suspension cultured cells (Fig. 3a) and a similar reaction in sites on potato leaves that had been infiltrated with 10 $\mu\text{g}/\text{mL}$ of NUBS-4190 (Fig. 3b, c).

Fig. 5 Induction of cell death by NUBS-4190 in potato. **a** NUBS-4190 induced hypersensitive cell death in potato suspension cultured cells (Sayaka) after 3 h treatment. Methanol extract of mycelia from *Phytophthora infestans* (MEM) elicitor (500 µg/mL) was used as the positive control, 1 % (v/v) DMSO as the negative control. Dead cells were quantified by adding 0.05 % (w/v) Evans to cell suspensions for 15 min, and 500 µL of the cell suspension was washed five times with 1 mL of distilled water each time to remove excess stain. Dye bound to dead cells was then solubilized in 1 mL of 50 % methanol that contained 1 % (w/v) SDS for 30 min at 50 °C, and extracted solution was then quantified by monitoring the absorbance at 600 nm in spectrophotometer. **b** Potato leaves were infiltrated with 50 µg/mL NUBS-4190 and incubated for 48 h. Hypersensitive cell death began 8–12 h after elicitor treatment as a wet area on the leaf surface, which increased within a few hours; by 24 h, distinct dead areas were visible. Data were analyzed with Student's *t* test, **P* < 0.05, ***P* < 0.01 vs DMSO control

H₂O₂ generation

The effect of NUBS-4190 on H₂O₂ accumulation in potato leaves is shown in Fig. 4. H₂O₂ generation was detected in NUBS-4190-treated leaves stained with DAB. DAB captures H₂O₂ and forms a reddish-brown color at sites of peroxidase activity. H₂O₂ production was observed in potato leaves at 3, 6, 12 and 24 h after elicitor treatment. Within 3 h after treatment, NUBS-4190 induced a high level of H₂O₂ and profound reddish brown colors were observed in DAB stained leaves (Fig. 4a, b).

Fig. 6 Gene expression after NUBS-4190 treatment of potato. Total RNAs were isolated from suspension cultured potato cells treated with 1/10 volume of NUBS-4190 or *inf1* gene product (INF1) and methanol extract of mycelia from *Phytophthora infestans* (MEM) as positive control for final concentration of 1, 100 µg/mL and 15 nM, respectively, for 3 h. DMSO (1 %, v/v) was used as a negative control. RNAs were analyzed by RT-PCR using specific primers for *StrbohB*, *StrbohC*, *StNR1*, *StNR5*, *Sthsr203J*, *StPAL* and *StPR1*. Equal loads of cDNA were monitored by amplification of constitutively expressed *StEF1α*

Induction of cell death

When suspension cultured potato cells were treated with 1 µg/mL NUBS-4190 and stained with the vital stain Evans blue, increased cell death was observed within 3 h of the treatment (Fig. 5a). Infiltration of potato leaves with 50 µg/mL NUBS-4190 resulted in rapid macroscopic changes; the first symptoms appeared within 18–24 h after the treatment. Leaf tissues in the infiltrated areas gradually died, and dead and dried areas clearly appeared after 48 h at the infiltrated site (Fig. 5b).

Fig. 7 NUBS-4190 induced resistance against *Phytophthora infestans* in potato. **a** NUBS-4190-treated potato leaves and DMSO (0.2 %, v/v)-treated control leaves were kept in an incubator for 24 h, then inoculated with spores of *P. infestans*. Photographs were taken from 6 days post inoculation. **b** Appearance of disease symptoms showing differences in severity representative of the five classifications used in **c**. **c** Percentages of potato leaves with disease symptom severities in each of the five classes represented in **b**. Percentage disease severities on treated potato leaves from 1 to 6 days post inoculation with *P. infestans*. At least 10 inoculated leaves from each plant were counted

Expression of defense-related genes

To investigate whether defense-related genes are induced by NUBS-4190, we extracted total RNAs from NUBS-4190-treated potato suspension cultured cells for analysis by RT-PCR (Fig. 6). INF1 and MEM were used in this experiment as positive controls, i.e., as NO and ROS (O_2^- and H_2O_2) inducers, respectively, in potato. NUBS-4190 induced expression of *StrbohB* and *StrbohC*, suggesting that generation of ROS was induced in potato cells. The expression of the hypersensitive response marker gene *Sthsr203J* indicated that NUBS-4190 induced HR reaction. Pathogenesis-related 1 (PR1) gene is salicylic-acid

responsive and is a useful molecular marker for systemic acquired resistance (SAR) response. High expression of *StPRI* was found after the NUBS-4190 treatment. Nitrate reductase genes *StNR1* and *StNR5* were also expressed by NUBS-4190.

Induced disease resistance in potato against *P. infestans*

NUBS-4190 (10 μ g/mL) was sprayed on excised leaves, which were inoculated with *P. infestans* 1 day later; excised leaves pretreated with DMSO (2 %) served as a negative control. Within 3 days, DMSO-treated plant leaves developed water-soaked lesions on leaves (Fig. 7a, c), which

Fig. 8 Phytoalexins did not accumulate after 40 µg/mL NUBS-4190 treatment of potato tubers. Phytoalexins, extracted 48 h after treatment, and 25 µg of purified rishitin were separated and developed on a TLC plate. 1, DMSO; 2, 1 mg/mL methanol extract of mycelia from *Phytophthora infestans* (MEM) elicitor; 3, 40 µg/mL NUBS-4190 and R, rishitin

expanded to nearly cover the leaves within 7–8 days. In contrast, few spots developed on leaves treated with NUBS-4190. Resistance against *P. infestans* was not recorded in 0.2 % DMSO-treated potato leaves.

Phytoalexins accumulation

Potato tubers were treated with NUBS-4190 or MEM elicitor as a positive control or 0.4 % DMSO as a negative control. No phytoalexins were found in the TLC separation of extracts from NUBS-4190-treated potato tubers (Fig. 8).

Discussion

Elicitor-induced secondary metabolite production has been characterized as mediated by endogenous signaling. Among signal molecules, NO has been reported to play an important role (Xu 2007; Zhao et al. 2005). In the present situation concerning the NO-eliciting synthetic bis-aryl methanone compound, NUBS-4190, NO generation was triggered in potato (Fig. 2) similar to our previous study, in which NUBS-4190 induced NO-mediated

resistance against *P. infestans* in *N. benthamiana* (Monjil et al. 2013). NO induction by NUBS-4190 in potato is sharp and reproducible, and NUBS-4190 could be a good synthetic compound for NO induction in potato. Although there has been criticism about the specificity of DAF-fluorescence for detecting NO (Rümer et al. 2012), we confirmed NO generation by showing high expression of nitrate reductase genes *StNR1* and *StNR5* in NUBS-4190-treated potato (Fig. 6).

The oxidative burst, one of the most rapid defense reactions to pathogen attack, which constitutes the production of ROS, primarily O_2^- and H_2O_2 , at the site of attempted invasion (Apostol et al. 1989). In the present study, ROS was induced in NUBS-4190-treated leaves and suspension cultured cells of potato (Figs. 3, 4). In our previous study (Monjil et al. 2013), NUBS-4190 induced NO but not ROS in *N. benthamiana*. These results indicate that potato and *N. benthamiana* and/or the signal pathways responsible for ROS production may differ in their sensitivity to NUBS-4190. When NUBS-4190 induced NO in *N. benthamiana*, signals for ROS production might not be transduced and/or NO suppressed the production of ROS. Reducing the endogenous NO level using cPTIO or plant mutants impaired in inducible NO production enhanced H_2O_2 accumulation (Asai et al. 2008; Tada et al. 2004), suggesting that part of the O_2^- produced by NADPH oxidase is scavenged by NO. Yun et al. (2011) found that NO abolished *AtrbohD* activity through S-nitrosylation.

An HR-like cell death was observed in NUBS-4190-treated potato leaves and suspension cultured cells (Fig. 5). When O_2^- and H_2O_2 levels increase in plants in response to pathogen attack, a HR results in the death of host cells (Apel and Hirt 2004). NO also induces plant defense responses including cell death (Wendehenne et al. 2004). It is reported that NO and ROS together, but not individually, are required to induce cell death (Delledonne et al. 1998). A balanced production of NO and O_2^- results in production of $ONOO^-$ and is important for inducing HR-like cell death (Saito et al. 2006). On the other hand, NO has been reported as not necessary to induce HR-like cell death (Wang et al. 2010). Da Silva et al. (2011) reported that dihydrosphingosine-induced NO production in tobacco BY-2 cells is not necessary for the induction of HR-like cell death. These reports suggest that NUBS-4190 induced both ROS and NO production in potato, which is responsible for leading to cell death.

In potato, a group of defense response-related genes, namely *StrbohB*, *StrbohC*, *StPR1*, *StNR1* and *StNR5* were expressed after NUBS-4190 treatment (Fig. 6), indicating that NUBS-4190 plays an important role in defense-related gene expression. To examine whether defense activities such as ROS, NO and HR-like cell death are effective against the pathogen, NUBS-4190-treated potato leaves

were inoculated with *P. infestans*, and a higher level of resistance in potato was stimulated at the stage susceptible to *P. infestans* (Fig. 7). Moreover, *StPR1* gene expression provided the systemic nature of this resistance.

After elicitor treatment, ROS generation is not essential to induce phytoalexin accumulation in plants. We showed that NUBS-4190 induced ROS and NO in potato but the level was not sufficient to induce phytoalexin accumulation. Doke (1983) reported that O_2^- generation is closely connected with phytoalexin production by hyphal wall components from *P. infestans*. In contrast, it had been reported that *N,N*-dimethylsphingosine induces phytoalexin production and hypersensitive cell death of Solanaceae plants without generation of ROS (Uruma et al. 2009). Devlin and Gustine (1992) reported that induction of phytoalexins was not dependent on ROS in white clover suspension cultures treated with avirulent *Pseudomonas corrugata* or with mercuric chloride. Treatment with DPI, a flavoprotein inhibitor of NADPH oxidase, blocks ROS generation but does not interfere with the induction of phytoalexins (Hahlbrock et al. 1995). Thus, whether ROS is involved in phytoalexins accumulation may depend on the plant species.

NUBS-4190 is a synthetic compound that elicits the HR in potato leaves and induces the production of multiple signaling molecules such as ROS and NO and the expression of the PR1 gene to enhance systemic resistance. These results indicate that NUBS-4190 has potential as an activating agent of potato plant defense.

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