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Nitric oxide production mediates oligogalacturonide-triggered immunity and resistance to *Botrytis cinerea* in *Arabidopsis thaliana*

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ABSTRACT

Nitric oxide (NO) regulates a wide range of plant processes from development to environmental adaptation. In this study, we investigated the production and/or function of NO in *Arabidopsis thaliana* leaf discs and plants elicited by oligogalacturonides (OGs) and challenged with *Botrytis cinerea*. We provided evidence that OGs triggered a fast and long lasting NO production which was Ca^{2+} dependent and involved nitrate reductase (NR). Accordingly, OGs triggered an increase of both NR activity and transcript accumulation. NO production was also sensitive to the mammalian NO synthase inhibitor L-NAME. Intriguingly, we showed that L-NAME affected NO production by interfering with NR activity, thus questioning the mechanisms of how this compound impairs NO synthesis in plants. We further demonstrated that NO modulates RBOHD-mediated reactive oxygen species (ROS) production and participates in the regulation of OG-responsive genes such as anionic peroxidase (PER4) and a β-1,3-glucanase. Mutant plants impaired in PER4 and β-1,3-glucanase, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea.* Taken together, our investigation deciphers part of the mechanisms linking NO production, NO-induced effects and basal resistance to *B. cinerea*.

Keywords: calcium; nitrate reductase; plant defence; reactive oxygen species.

INTRODUCTION

Defence responses of plants against microbial attack are initiated by signal molecules released during the infection process and mediated by a number of signalling components. These components include nitric oxide (NO), a widespread molecule in living organisms involved in major physiological processes (Torreilles 2001; Besson-Bard, Pugin & Wendehenne 2008a). In mammals, NO is mainly synthesized from L-arginine by NO synthase (NOS). In plants, NO is also derived from nitrogen-containing precursors but these precursors are more diverse than in animals. In broad outline, NO is thought to be generated in plants from two enzymatic pathways: an L-arginine-dependent pathway and a nitrite-dependent pathway (Besson-Bard *et al*. 2008a). Many studies highlighted the occurrence of an enzymatic process in which NO is produced from L-arginine by an unidentified enzyme sensitive to mammalian NOS inhibitors (also named NOS-like enzyme) although there is no obvious homolog of mammalian NOS in the plant genomes sequenced so far (Cueto *et al*. 1996; Modolo *et al*. 2002; del Río *et al*. 2004; Corpas *et al*. 2006). Tun *et al*. (2008) have reported the putative involvement of a polyamine (PA)-dependent production of NO related to L-arginine metabolism, this amino acid being one key precursor of PAs. *In vivo* and *in vitro* studies also highlighted that NO is a by-product of nitrate reductase (NR) activity when nitrite is used as a substrate (Yamasaki 2000; Sakihama, Nakamura & Yamasaki 2002). NO production from NR occurs in specific context in which the cytosolic nitrite reaches high concentrations, that is in the mM range. NR was shown to be involved in NO production in several physiological situations, such as abscisic acid (ABA)-induced stomatal closure, hypoxia, cold stress or in nitrogen-fixing nodules (Desikan *et al*. 2002; Dordas *et al*. 2004; Zhao *et al*. 2009; Cantrel *et al*. 2011; Horchani *et al*. 2011). In *Arabidopsis thaliana*, two cytosolic isoforms of NR (NR1 and NR2) have been identified. These isoforms have a high degree of coding sequence similarity and are 83.5% identical at the amino acid level. NR2 represents ~90% of the total NR activity regarding nitrate reduction (Wilkinson & Crawford 1991). Comparative analysis using the single mutants *nia1* and *nia2* suggested that the most committed NR isoform to NO production in response to ABA or cold stress was encoded by *NR1* (Bright *et al*. 2006; Zhao *et al*. 2009). Finally, it has also been reported that NO synthesis from nitrite occurs in mitochondria associated with mitochondrial electron transport (Planchet *et al*. 2005; Horchani *et al*. 2011).

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In plants, NO was found, among different roles, to be involved in mediating defence reactions against microbial pathogens (Delledonne *et al*. 1998; Leitner *et al*. 2009). Firstly, NO was reported to be rapidly generated in several plant-pathogen models and using different detection methods (Vandelle & Delledonne 2008). A rapid and intense intracellular NO production was detected in tobacco epidermal cells and cell suspensions treated with the *Phytophthora cryptogea* elicitin cryptogein using diaminofluorescein diacetate (DAF-2DA), a cell permeable NO specific fluorescent probe (Foissner *et al*. 2000) and by electrochemistry (Besson-Bard *et al*. 2008b). In response to the necrotrophic fungus *Botrytis cinerea*, *Pelargonium peltatum* leaves also initiated a near-immediate NO burst but, interestingly, its generation was dependent on the genetic make-up of the host plant. Precisely, in resistant genotypes, a subsequent wave of NO generation was correlated with the resistance to *B. cinerea*, whereas in susceptible cultivar, the second NO burst was absent (Floryszak Wieczorek *et al*. 2007). Recently, Piterkova *et al*. (2009) also reported a systemic NO production in adjacent and distant uninoculated leaves of tomato plants challenged with the biotrophic fungus *Oidium neolycopersici*. Secondly, it was shown that pathogen-induced NO production or exogenous treatment with NO donors affects plant defence molecular responses, such as the expression of defence-related genes or phytoalexins biosynthesis and, therefore, could participate in plant disease resistance (Delledonne *et al*. 1998; Durner, Wendehenne & Klessig 1998). More recently, it was observed that the NR-deficient double mutant (*nia1nia2*), which shows substantially reduced NO production after bacterial or fungal pathogen inoculation, showed no hypersensitive response and was hypersusceptible to *Pseudomonas syringae* (Modolo *et al*. 2006; Oliveira *et al*. 2009) and to the necrotrophic fungal pathogen *Sclerotinia sclerotiorum* (Perchepied *et al*. 2010). Although Modolo *et al*. (2006) have noticed that the levels of amino acids, and particularly L-arginine, are strongly reduced in *nia1nia2 A. thaliana* leaves, NO emission by *nia1nia2* leaves did not increase in the amino acid-recovered mutants (Oliveira *et al*. 2009). These results suggest that the susceptibility to pathogen is a consequence of the reduced ability to synthesize NO. Similarly, plants affected in *AtNOA1* (NO-associated protein 1) expression exhibited a reduced endogenous NO level and were more susceptible to the virulent bacteria *P. syringae* pv tomato DC3000, to the fungi *Colletotrichum orbiculare*, *S. sclerotiorum* and *B. cinerea* (Zeidler *et al*. 2004; Asai, Ohta & Yoshioka 2008; Asai & Yoshioka 2009; Perchepied *et al*. 2010).

Research conducted over the past years has revealed that NO mediates part of its effect through modulation of protein kinase activities, post-translational modifications of target proteins, and mobilization of free Ca^{2+} and other second messengers (Besson-Bard *et al*. 2008a). Particularly, it has been reported that the involvement of NO in plant immunity is related to its interplay with reactive oxygen species (ROS) and cell death occurring during hypersensitive response (HR) might result from the simultaneous and balanced production of NO and ROS (Zaninotto *et al*. 2006). NO and ROS exert reciprocal control on each other, directly (reaction of NO and ROS to form peroxynitrite anion $ONOO^-$; Cecconi *et al*. 2009) or indirectly (e.g. through the NO-dependent inhibition of catalase and ascorbate peroxidase, two major H_2O_2-scavenging enzymes by NO; Clarke *et al*. 2000; Arasimowicz *et al*. 2009).

In spite of these numerous evidence providing indirect evidence associating NO to plant defence reactions, little is known about the mechanisms that link NO production to its physiological activity. In order to further investigate the role of NO in a pathophysiological context, in the present study we analysed its functions in the plant defence responses triggered by oligogalacturonides (OGs) in *A. thaliana* plants. OGs are structural components of the pectin homogalacturonan chains of plant cell wall. In *A. thaliana*, they are released during the interaction with pathogens, such as the necrotrophic fungus *B. cinerea*, which secretes polygalacturonase (PG) as part of their cell wall degrading enzyme arsenal. OGs, with a degree of polymerization (DP) between 10 and 25, are considered as endogenous elicitors as they are released from plant cell wall in response to the damage caused by invading pathogens. In *A. thaliana* or grapevine (*Vitis vinifera*), OG treatment induces a variety of defence responses including accumulation of phytoalexins, β-1,3-glucanase and chitinase. Moreover, OGs have been shown to contribute to triggered immunity against fungal pathogens including *B. cinerea* (Aziz *et al*. 2007; Ferrari *et al*. 2007). About half of the *A. thaliana* genes affected by OG treatment display a similar pattern of expression after *B. cinerea* infection, suggesting that at least part of the responses activated by *B. cinerea* are mediated, directly or indirectly, by OGs (Ferrari *et al*. 2007). Therefore, OGs represent a valuable tool to analyse the mechanisms involved in plant-pathogen interaction.

In this study, using genetic, biochemical and pharmacological approaches, we provided evidence that OGs induced a NR-dependent NO production together with an increased NR activity and NR transcript accumulation. In addition, NO production was sensitive to the mammalian NOS inhibitor L-NAME. Intriguingly, L-NAME also impaired OG-induced NR activity, thus questioning the mode of action of this widely used compound. Analysis of the signalling pathway involving NO showed that its production was Ca^{2+} dependent and occurred downstream of the putative plasma membrane cyclic nucleotide-gated channel CNGC2. Furthermore, NO production modulated AtRBOHD-mediated ROS production and the transcriptional activation of defence-related genes encoding the anionic peroxidase PER4 and a β-1,3-glucanase. Finally, by pharmacological and reverse genetic approaches, we provided evidence that NO, as well as its target genes, contributes to the OG-triggered immunity against *B. cinerea*. Taken together, our data reinforce the concept that NO is a key mediator of plant defence responses.

MATERIALS AND METHODS

Plant material and growth conditions

The *dnd1* (CNGC2) mutant line (Ali *et al*. 2007) was a gift from Dr Berkowitz (University of Connecticut, USA). The *rboh*D mutant was kindly provided by Dr Torres (Universidad Politecnica de Madrid, Spain). The double NR *nia1nia2* mutant (NR1-Ds and NR2 deletion mutant) was kindly provided by S. Neill (University of the West of England-UK). Mutants impaired in anionic peroxidase PER4 (*At1g14540*) were *Per4-1* (N655479/SALK_544730) and *Per4-2* (N661 085/SALK_110617), and in β-1-3-glucanase (*At3g55430*) was *Glu* (N642531/SALK_142531). Seeds of these mutant lines and of the wild-type Col-0 were obtained from Nottingham Arabidopsis Stock Center (NASC). All mutant lines used in this work are in the Col-0 background.

The plants were cultivated in commercial soil (Jiffy-7, Puteaux, France) in a climate-controlled growth chamber (KBW, Binder, Germany) with a 10 h light (175 μE s^{-1} light intensity), 14 h dark cycle with the following settings: 20 °C light, 18 °C dark; 70% relative humidity light/95% dark. Plants were fertilized every week with a modified Hoagland nutrient solution containing 1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$. As the *nia1nia2* double mutant could not assimilate nitrate and is affected in its development, we restored normal growth by watering the mutants with a medium supplemented with 10 mM ammonium citrate (Cantrel *et al*. 2010). Under these conditions, no significant differences of growth were observed between 4-week-old Col-0 wild type and *nia1nia2* mutants; the average shoot biomasses being 196 mg [standard deviation (SD) = 76] for Col-0 and 230 mg (SD = 80) for *nia1nia2*.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) except L-NAME (N^G-nitro-L-arginine-méthyl ester) which was from ALEXIS Biochemicals (San Diego, CA, USA). Sodium tungstate, lanthanum chloride, L-NAME and cPTIO ((4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) were dissolved in water. DAF-2DA (4,5-diaminofluorescein diacetate) was received as a stock solution of 5 mM in dimethyl sulfoxide (DMSO). Diphenylene iodonium (DPI) was dissolved in DMSO.

OGs, with an average DP of 25, were obtained from GOEMAR (Saint Malo, France). OGs were dissolved in water at a working concentration of 2.5 mg mL^{-1}.

NO measurements

NO production was monitored using 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma-Aldrich), a membrane-permeable derivative of the NO-sensitive fluorophore 4,5-diaminofluorescein. This method is indirect and relies on the measurement of reactive nitrogen species (notably N_2O_3 and NO^+) derived from NO auto-oxidation that nitrosate DAF-2 to yield the highly fluorescent DAF-2 triazole (DAF-2T; Jourd'heuil 2002).

Detection of DAF2 fluorescence in leaf discs (8 mm diameter) was performed according to Cantrel *et al*. (2011). Briefly, leaf discs excised from plants were infiltrated under vacuum for 3 min with an aqueous solution of 20 μM DAF-2DA and 2.5 mg mL^{-1} of OGs in 50 mM Tris-HCl (pH 7.5). For control treatment, OGs were replaced by an equivalent volume of water. L-arginine supplementation of *nia1nia2* double mutant (and Col-0 as control) was performed by infiltrating the leaf discs with 1 mM L-arginine. After infiltration, the discs were washed with Tris-HCl (50 mM, pH 7.5). Each disc was put in separate well of 96 well plates (Microtest ^{Tm} flatbottom; Becton Dickinson Europe, Meylan, France) in 200 μL Tris-HCl (50 mM, pH 7.5) with OGs or water. The increase of DAF-2T fluorescence triggered by OGs was measured using a spectrofluorometer (Mithras; Berthold Technologies, Bad WildBad, Germany). Fluorescence was measured with a 485 nm excitation and a 535 nm emission filters for 12 h. Eight leaf discs were used for each treatment. Fluorescence was expressed as relative fluorescence units (arbitrary units: au).

Importantly, because the leaf discs have been vacuum infiltrated and kept in buffer solution for up to 12 h in the dark, we could not exclude the possibility that they became hypoxic during the experiment. Because constant oxygen availability is important for ROS formation and NO auto-oxidation resulting in the formation of DAF-reactive forms, hypoxia might alter the rate of H_2O_2 and DAF-2T synthesis. To reduce this risk, the plates were shaken during the experiment. Furthermore, we found that when leaf discs were vacuum infiltrated 24 h before adding OGs in the solution in which leaf discs were fluttering, the production of H_2O_2 and increase of DAF-2T fluorescence were still occurring, minimizing the influence of hypoxia in the measurement (data not shown).

Gene expression analysis by quantitative real-time qPCR

Treated leaves were immediately frozen in liqud nitrogen. Total RNA was isolated with Tri reagent (Molecular Research Centre Inc., Cincinnati, OH, USA). Nucleic acid concentration was estimated at 260 nm. Purity of total RNA was assessed by determining the 260/280 ratio and the integrity was checked by agarose gel electrophoresis.

First-strand cDNA templates were produced by using the cDNA synthesis kit (Superscript^{Tm} III Reverse Transcriptase; Invitrogen, Carlsbad, CA, USA) from 2 μg of total RNA treated with DNaseI (Sigma-Aldrich) as recommended by the manufacturer. Reactions were performed in a final volume of 20 μL containing 8 μL of cDNA (dilution 1/80) sample, 200 nM of forward and reverse primers, and 10 μL of real-time SYBR Green mix (Abgene; Thermo Scientific, Surrey, UK). qPCR was conducted with a Mastercycler (Sequence Detection System; Applied Biosystem, Foster City, CA, USA) in 96 well plates (Optical Reaction Plate with Barcode; Applied Biosystem). Gene-specific

primers were as follows: *PER4-At1g14540* (5′-cactggttcaga tggacaaa-3′ and 5′-aacaaacgaattatcgctgc-3′), *PLP2-At2g265 60* (5′-aagaaaagaagatccgagac-3′ and 5′-attcaaacgtacaagtga cc-3′), *CHI-IV-At3g54420* (5′-tgttgactcccaccattt-3′ and 5′-cggtcgatccaactctac-3′) and β-1,3-glucanase-*At3g55430* (5′-cttcgctggaactggtatct-3′ and 5′-gcagaatctcatttccgact-3′). The specificity of the primers was tested by sequencing the PCR products. PCR amplification efficiency of target DNA was examined to measure linearity of dilution. For each gene, a standard curve was generated with serial dilutions of plasmids containing the respective PCR product. These standard curves showed similar slopes demonstrating an efficiency of more than 90%.

Expression levels were calculated relative to the housekeeping gene (HK) *UBQ10- At4g05320* (5′-ctatatgctc gctgctgagc-3′ and 5′-aagccaggcagagacaactc-3′) using the comparative threshold cycle method, where Ct represents the threshold cycle for target amplification: $\Delta\Delta Ct = \Delta Ct_{\text{gene of interest}} - \Delta Ct_{HK}$. The $2^{-\Delta\Delta CT}$ method was used to analyse the relative changes in gene expression from real-time quantitative PCR experiments (Livak & Schmittgen 2001).

In vivo NR activity

In vivo NR activity was assayed by the protocol of Yu, Sukumaran & Marton (1998) with minor modifications. NR activity was measured in a spectrophotometric assay by determining the amount of NO_2^- released from the tissue. Leaf discs were weighed, vacuum infiltrated with or without OGs in Tris-HCl for 3 min and incubated for 4 h within the respected solutions. After incubation, leaf discs were transferred into 1 mL of reaction buffer [40 mM KNO_3, 0.08 mM Na_2HPO_4, 0.02 mM NaH_2PO_4 and 4% (v/v) *n*-propanol, pH 7.5] and incubated in the dark for 2 h. The reaction was stopped by the addition of 200 μL of 1% sulphanilamide (dissolved in 3 N HCl) and 200 μL of 0.05% *N*-(1-napthyl)ethylenediamine hydrochloride. The concentration of NO_2^- was determined by measuring the optical density (OD) of the solution at 540 nm.

H_2O_2 measurements

H_2O_2 was measured using a luminol-dependent assay (Keppler, Baker & Atkinson 1989). Two millimetre leaf discs of 4-week-old Arabidopsis plants were excised and five discs per condition were incubated in 200 μL of H_2O overnight in assay tubes. Then, 4 μL of 3 mM luminol solution (final concentration 60 μM) was added to the tubes 5 min before treatment. Luminescence was measured in a luminometer (Lumat LB 9507; Berthold Technologies) for a total time of 50 min. Measurements were integrated over 10 s periods.

B. cinerea culture and infection method

B. cinerea strain BMM (Zimmerli *et al.* 2001) was grown on Petri plates containing potato dextrose agar (PDA; DIFCO, Lawrence, KS, USA) for 10–12 d (light 10 h, 20 °C; dark 14 h, 18 °C). Spores were harvested in water and subsequent filtration through glass wool to remove hyphae. Spores were quantified under a microscope. About 1 h before infection, spores were diluted in one-fourth potato dextrose broth (PDB; DIFCO) to obtain a final concentration of 5×10^4 spores mL^{-1}. For assessment of symptom in plants, droplets of 6 μL of spore suspension (5×10^4 spores mL^{-1}) were deposited on six leaves of 4-week-old plants (wild type and mutants). Plant infections were performed in covered plastic box to maintain high humidity and returned to the growth chamber. Lesion diameters were measured after 72 h of inoculation using callipers. Results were analysed by one-way analysis of variance (ANOVA) on ranks, followed by Dunnett's test ($P > 0.05$), using the SigmaPlot software (Jandel Scientific, Chicago, IL, USA).

RESULTS

OGs induce NO production in *A. thaliana* (Col-0) leaf discs

The ability of OGs to induce NO production in Col-0 was investigated by monitoring the time course of DAF-2T fluorescence accumulation in OG-treated leaf discs pre-infiltrated with DAF-2DA (Fig. 1). A similar method was recently used to measure NO production in response to cold stress (Cantrel *et al.* 2011). DAF-2T is a fluorescent compound resulting from the nitrosation of DAF-2 by reactive nitrogen species derived from NO auto-oxidation such as dinitrogen trioxide N_2O_3 (Besson-Bard *et al.* 2008b). OGs triggered an increase in fluorescence which occurred within 30 min (Fig. 1, Supporting Information Figs S2 & S3). Increase of fluorescence remained constant thereafter and reached almost 190% of the control value at 6 h (Fig. 1B). A slight increase in DAF fluorescence was also observed in control leaf discs (Fig. 1A). This background production might be related to constitutive NO production and/or to the wounding triggered during leaf disc preparation. To argue that the OG-induced rise of fluorescence was related to NO production, a similar experiment was performed in the presence of cPTIO. This compound is a stable organic radical that oxidizes the NO molecule to form the $^{\bullet}NO_2$ radical (Akaike & Maeda 1996). The ability of cPTIO to scavenge NO exogenously applied or endogenously produced in biological samples has been reported in many studies (see, for instance, Bright *et al.* 2006; Planchet and Kaiser, 2006; Zottini *et al.* 2007; Besson-Bard *et al.* 2008b). As shown in Fig. 1, fluorescence increase in both OG-treated and control leaf discs was markedly suppressed by cPTIO.

The DAF-2-based assay for NO detection has been shown to be a suitable technique for the detection of NO (Vandelle & Delledonne 2008). However, as specified above, this method is indirect as it allows the measurement of NO-derived species (Besson-Bard *et al.* 2008b). Furthermore, doubts have arisen over the specificity of DAF-2 for NO-related species (for review, see Mur *et al.* 2011). Therefore, to strengthen our assumption that OGs trigger NO

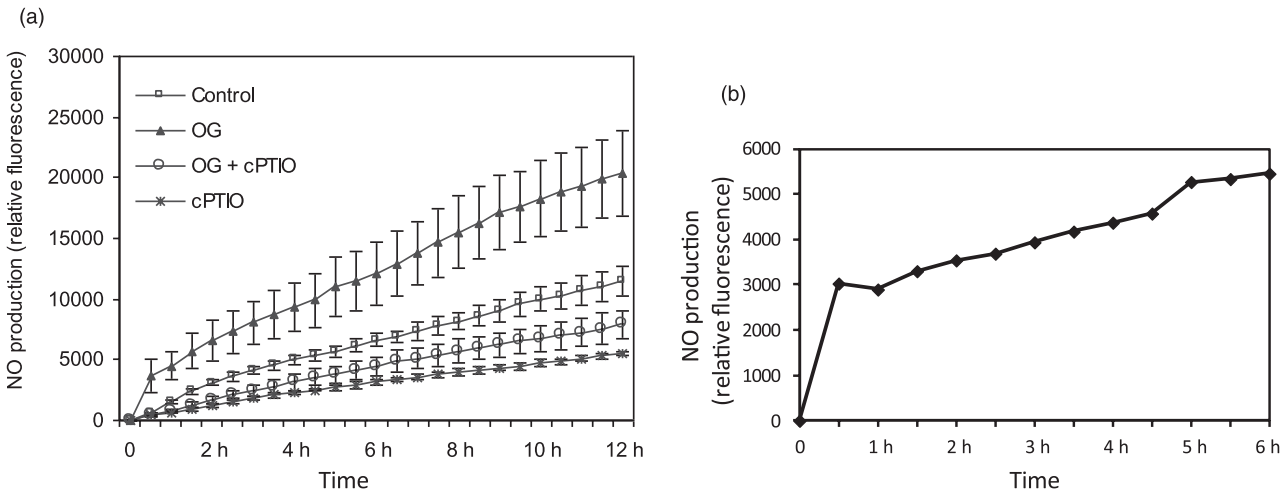

Figure 1. (a) Time course of nitric oxide (NO) production in oligogalacturonide (OG)-treated Col-0 leaf discs. NO production was monitored using diaminofluorescein diacetate (DAF-2DA) (20 μM) in the absence (control) or presence of OGs (2.5 mg mL^{-1}), with or without NO scavenger (cPTIO 500 μM). Each value represents the mean of 8 measurements ± standard deviation. This experiment is representative of 10 independent experiments using independent biological material. (b) Time course of OG-induced NO production between 1 and 6 h. Fluorescence values of control (water treatment) were subtracted from values obtained in response to OGs.

synthesis, we used a second approach for NO measurement based on the use of the CuFL fluorescent probe. The CuFL probe reacts directly and rapidly with NO and is believed to be more sensitive than DAF-2A (Supporting Information Fig. S1; Lim, Xu & Lippard 2006). However, NO detection with CuFL is less convenient than DAF-2DA as freshly prepared probe is poorly stable and could not be used after 1 h (manufacturer's instructions). Compared with control, OGs triggered a significant increase of CuFL fluorescence (Supporting Information Fig. S2). As expected, this fluorescence increase was completely suppressed by cPTIO, thus further supporting our hypothesis that OGs trigger NO synthesis.

OG-induced NO production is L-NAME sensitive

Several studies have suggested that the production of NO from L-arginine in plants could involve an unidentified NOS-like enzyme in which activity is sensitive to mammalian NOS inhibitors (Corpas *et al*. 2006, 2011; Cueto *et al*. 1996; Modolo *et al*. 2002; del Río *et al*. 2004). To investigate the putative involvement of a NOS-like enzyme, we checked the sensitivity of OG-induced NO production to L-NAME, a widely used mammalian NOS competitive inhibitor. L-NAME was reported to suppress NO synthesis, as well as NOS-like activities, in plant tissues and cell suspensions exposed to various stimuli. L-NAME reduced OG-induced NO production in Col-0 leaf discs by 40% after 12 h of treatment (Fig. 2). This inhibition was observed at earlier time point (Supporting Information Fig. S3a).

NR is involved in OG-induced NO production

The potential involvement of NR as a NO source was investigated using a combination of genetic and pharmacological approaches. This analysis was completed by following the incidence of OGs on NR activities and transcript accumulation.

We first investigated the putative contribution of NR as an enzymatic source for NO production during OG treatment using the NR *nia1nia2* double mutant (Fig. 3a and Supporting Information Fig. S3b). OG-induced increase of DAF-2T fluorescence was partially reduced by about 50% in *nia1nia2* as compared with wild-type Col-0 leaf discs. The NO scavenger cPTIO completely suppressed the increase of fluorescence triggered by the elicitor in *nia1nia2*, indicating that the remaining fluorescence measured in the mutant was also due to OG-induced NO production. Interestingly,

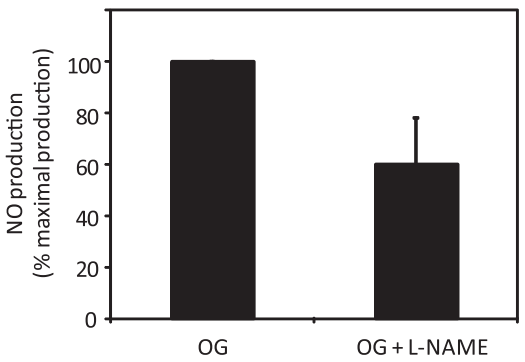

Figure 2. Effect of L-NAME on oligogalacturonide (OG)-induced nitric oxide (NO) production in Col-0 leaf discs. Arabidopsis leaf discs from wild-type (Col-0) plants loaded with diaminofluorescein diacetate (DAF-2DA) (20 μM) were treated by OGs (2.5 mg mL^{-1}) with or without a pretreatment with L-NAME (5 mM). NO accumulation was determined after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± standard deviation of three independent experiments.

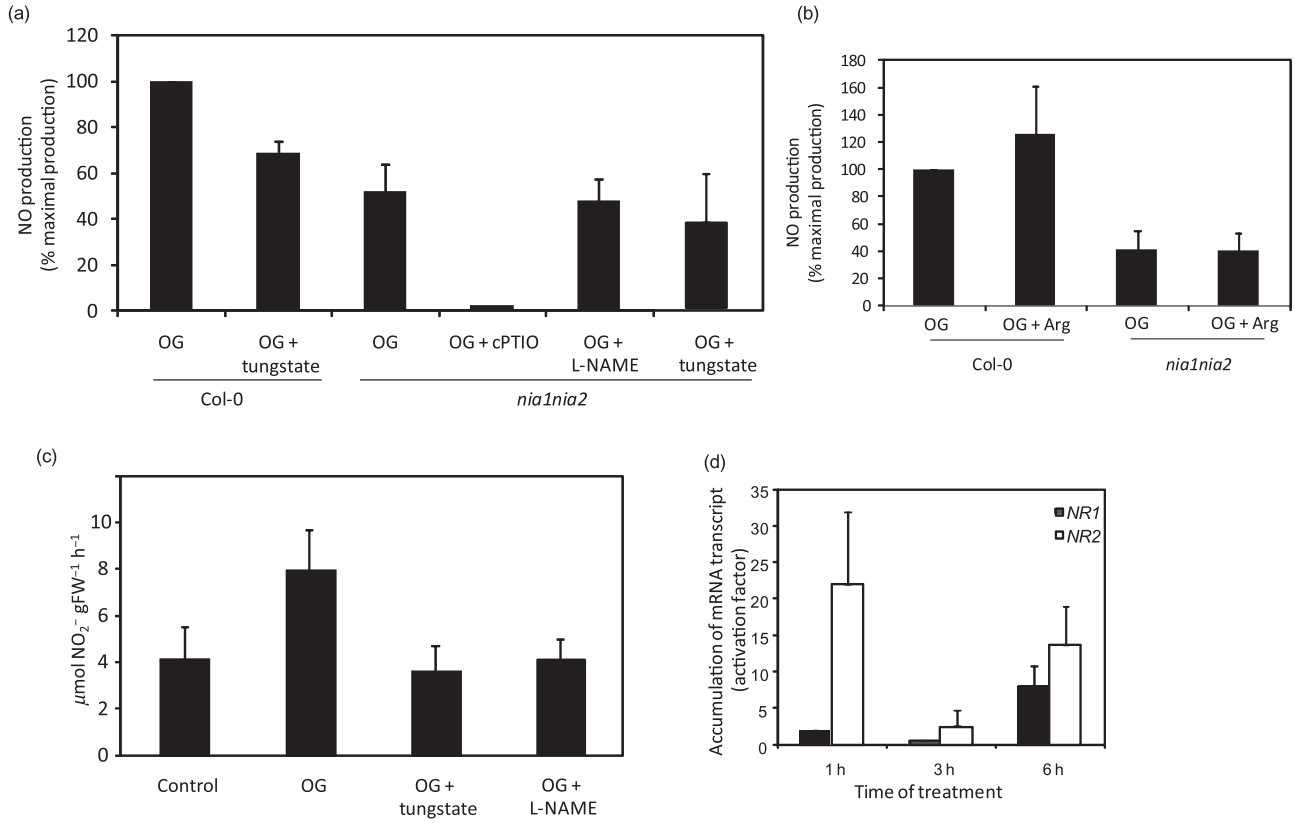

Figure 3. Involvement of nitrate reductase (NR) in oligogalacturonide (OG)-induced nitric oxide (NO) production. (a) Diaminofluorescein diacetate (DAF-2DA)-infiltrated (20 μM) Arabidopsis leaf discs from wild type (Col-0), and *nia1nia2* plants were treated by OGs (2.5 mg mL^{-1}) or water. The effect of tungstate (0.1 mM) and/or L-NAME (5 mM) on OG-induced NO production was analysed on Col-0 and *nia1nia2* leaf discs. Bar graph is the mean of five independent experiments using independent biological material (eight replicates per experiment). NO accumulation determined after 12 h of treatment is expressed as a percentage of the maximal response measured in wild-type Col-0 leaf discs treated with OGs, after subtracting background fluorescence of corresponding control. (b) Effect of L-arginine on OG-induced NO production in Col-0 and *nia1nia2* leaf discs. Arabidopsis leaf discs loaded with DAF-2DA (20 μM) were treated by OGs (2.5 mg mL^{-1}) with or without L-arginine (1 mM). NO accumulation was determined after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± standard deviation (SD) of three independent experiments. (c) *In vitro* NR activity in wild-type (Col-0) leaf discs treated with water, OGs, OGs + tungstate (0.1 mM) and OGs + L-NAME. Each value represents the mean ± SD of three independent experiments performed using independent biological materials. Tungstate and L-NAME alone did not modify significantly NR activity measured in control. (d) Time course of accumulation of *NR1* and *NR2* mRNA transcripts in Col-0 leaf discs treated with OGs or water. Transcribed mRNAs were analysed by real-time quantitative RT-PCR. Levels of transcripts were calculated using $2^{-\Delta\Delta CT}$ method. This experiment is representative of three independent experiments using independent biological material. Bars indicate average expression (±SD) of three replicates.

L-NAME did not reduce the remaining NO production observed in the NR mutant (Fig. 3a). To further confirm the involvement of NR as a NO source, we also examined the effect of the NR inhibitor tungstate on NO production. Pretreatment of Col-0 leaf discs with tungstate reduced OG-triggered NO production by almost 30% (Fig. 3a and Supporting Information Fig. S3b). In contrast, pretreatment of *nia1nia2* with tungstate did not modify significantly the reduced NO production observed in the double mutant in response to OGs alone, suggesting that the remaining NO production in *nia1nia2* is probably not due to residual NR activity (Fig. 3a).

Modolo *et al.* (2006) demonstrated that the *nia1nia2* double mutant has much lower L-arginine content in leaves (almost 10 times lower) as compared with wild-type plants, suggesting that the L-arginine-dependent pathway for NO synthesis could be down-regulated in the *nia1nia2* mutant. To check this possibility, leaf discs were co-infiltrated with OGs and L-arginine. We observed that L-arginine supply did not restore NO production in the *nia1nia2* mutant (Fig. 3b), strongly suggesting that the reduced NO production in the *nia1nia2* mutant was first related to the impaired expression of the corresponding genes.

To complete this analysis, we next investigated whether the involvement of NR as a NO enzymatic source in response to OGs was correlated with changes in NR activity and transcript accumulation. Firstly, we quantified *in vivo* NR activity in *A. thaliana* Col-0 leaf discs. As shown in Fig. 3b, OG treatment induced a twofold increase of NR activity as compared with control after 4 h of treatment. As

expected, OG-induced NR activity was completely suppressed by tungstate (Fig. 3b). In the *nia1nia2* mutant, a residual activity representing 2–3.3% of the activity measured in Col-0 was detected (Supporting Information Fig. S7). In order to better understand the link between Arg- and NR-dependent pathways for NO production, we monitored NR activity in the presence of L-NAME (Fig. 3b). Surprisingly, L-NAME inhibited OG-induced NR activity to a similar extent than tungstate. Secondly, we estimated *NR1* and *NR2* mRNA transcript accumulation by quantitative real-time RT-PCR. A rise in the accumulation of both transcripts occurred in response to OG (Fig. 3c). However, *NR2 m*RNA showed noticeable higher and faster accumulation than *NR1* mRNA (Fig. 3c). Furthermore, *NR2* transcripts showed a biphasic accumulation, the lowest accumulation being observed at 3 h post-treatment. Taken together, these results indicate that OGs elicit a NR-dependent NO production together with an increase of NR activity and transcript accumulation.

Ca^{2+} influx modulates NO production in response to OGs

The Ca^{2+} dependency of NO production has been previously shown in several studies (Courtois *et al*. 2008). In particular, Ali *et al*. (2007) identified CNGC2, a plasma membrane cyclic nucleotide-gated channel (CNGC) member, as a key Ca^{2+}-permeable channel linking lipopolysaccharide (LPS)-triggered Ca^{2+} influx to downstream NO production in *A. thaliana*. Furthermore, OGs have been shown to trigger a fast and transient elevation in cytosolic-free Ca^{2+} in *A. thaliana*, this process being suppressed by the calcium channel blocker lanthanum (La^{3+}; Moscatiello *et al*. 2006). Based on these data, we tested whether CNGC2 could act upstream of OG-evoked NO generation using the *dnd1* (*defence no death1*) mutant impaired in functional CNGC2. In control leaf discs, Col-0 and *dnd1* mutant showed approximately similar level of basal DAF-2T fluorescence (data not shown). In response to OGs, *dnd1* leaf discs displayed a lower increase of DAF-2T fluorescence as compared with Col-0. Indeed, NO production in *dnd1* leaf discs reached 49% of the value measured in Col-0 leaf discs (Fig. 4). Accordingly, a significant decrease in OG-induced NO production occurred also in Col-0 leaf discs pretreated with La^{3+} (Fig. 4).Therefore, the reduced NO production in both *dnd1* and La^{3+}-treated Col-0 plants suggests a role for Ca^{2+} influx in mediating the OG signal to NO production.

NO modulates OG-triggered ROS production in *A. thaliana* leaf discs

Oxidative burst is a common early response of plant cells to pathogen attack and elicitor treatment. Galletti *et al*. (2008) demonstrated that OG treatment induced an oxidative burst mediated by the NADPH oxidase AtRBOHD. In order to analyse a potential functional link between ROS

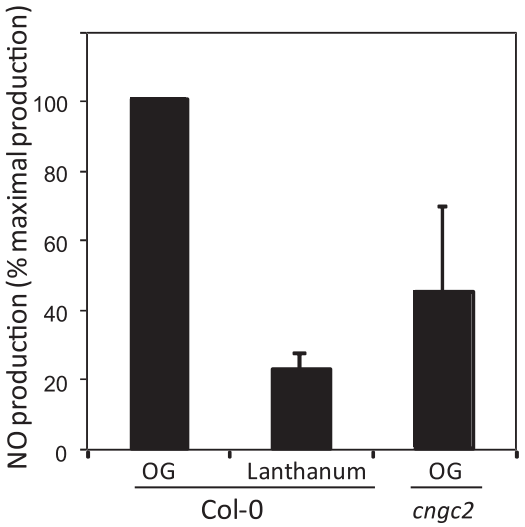

Figure 4. Involvement of Ca^{2+} influx in oligogalacturonide (OG)-induced nitric oxide (NO) production. OG-induced NO production was measured in Col-0 or *cngc2* leaf discs loaded with diaminofluorescein diacetate (DAF-2DA) (20 μM). Leaf discs were treated by OGs (2.5 mg mL^{-1}) in the presence or absence of lanthanum (5 mM). Histogram represents the NO production measured after 12 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Each value is a mean ± standard deviation of three independent experiments.

and NO production in response to OGs, the role of NO in controlling the oxidative burst was first studied. ROS production was measured in leaf discs using a luminol-based assay. In response to OGs, ROS burst was detectable approximately 3 min after elicitation, reached a maximum after 10 min and declined thereafter (Fig. 5a). These data fit well with our recent report showing that OG induces a fast and transient ROS production in *A. thaliana* cell suspensions (Dubreuil-Maurizi *et al*. 2011). It also indicates that both ROS and NO production occur simultaneously (Fig. 1, Supporting Information Figs S2 & S3). As expected, the ROS production was sensitive to the NADPH oxidase inhibitors DPI (Fig. 5a) and also quinacrine, a general inhibitor of oxidases (not shown). Furthermore, it was completely abolished in the *RbohD* mutant as reported by Galletti *et al*. (2008; Fig. 5b). Pretreatment of leaf discs with cPTIO, L-NAME or La^{3+} 10 min prior to OG treatment led to an inhibition of OG-stimulated ROS elevation (Fig. 5b). Surprisingly, the *nia1nia2* NR mutant, which displayed a reduced NO synthesis (Fig. 2), exhibited a similar increase of ROS production in response to OGs as compared with Col-0 (Fig. 5b).

To complete this analysis, we also verified NO level in response to OGs in *AtRbohD*. NO production in *rbohD* mutant was only 20% lower than Col-0 at 12 h (Fig. 5c). Compared with Col-0, and taking into account variability, we considered this difference poorly significant, indicating that ROS produced by AtRBOHD do not, or slightly, control OG-induced NO production.

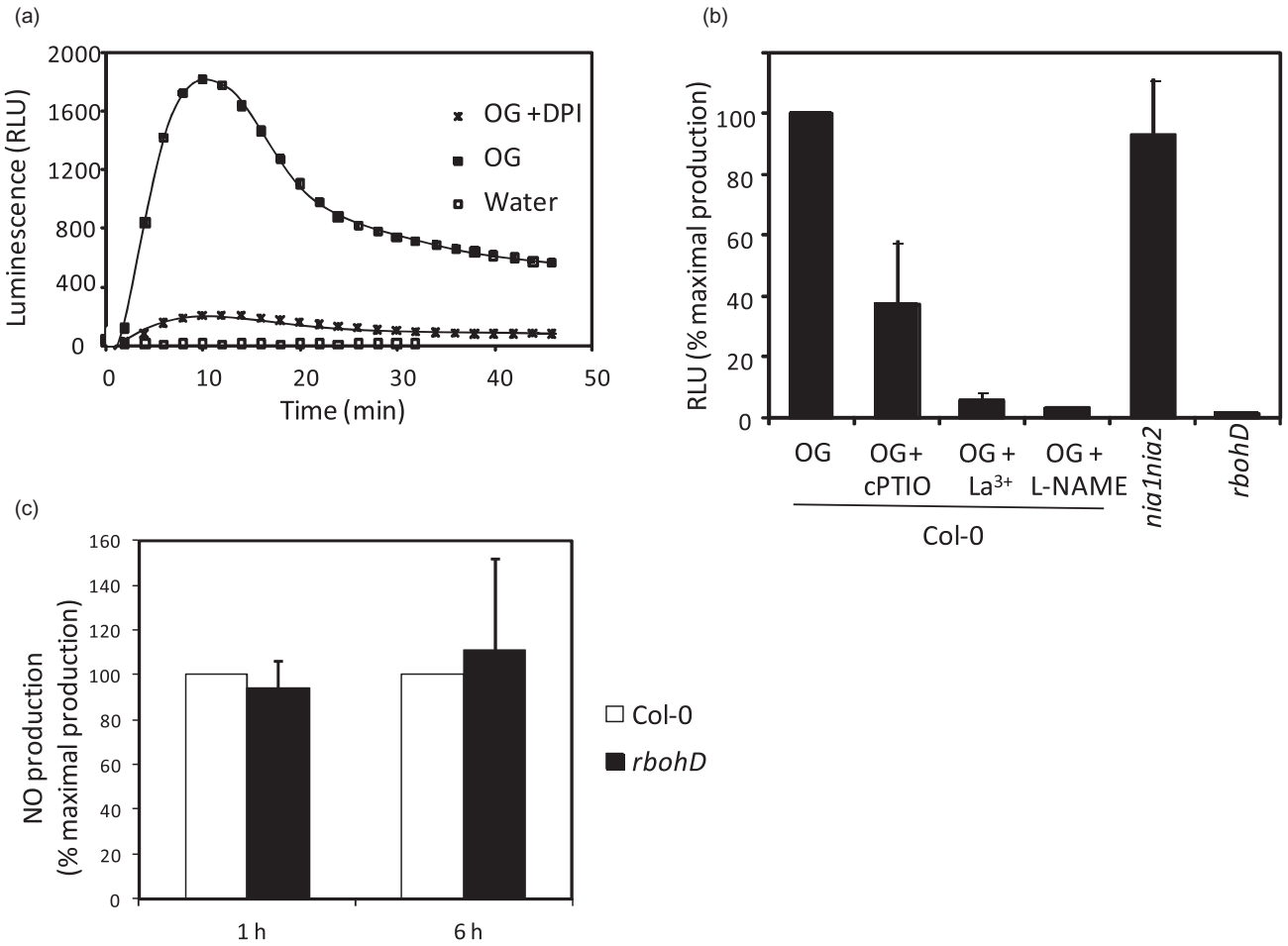

Figure 5. Relationships between reactive oxygen species (ROS) and nitric oxide (NO) production in plants elicited by oligogalacturonides (OGs). (a) Accumulation of extracellular H_2O_2 in response to OGs. The production of ROS was measured with a luminol-based assay. Leaf discs from Col-0 plants were treated with water (control) or OGs (2.5 mg mL^{-1}). Diphenylene iodonium (DPI) (10 μM) was added 10 min before treatment. Treatment with DPI alone did not induce luminescence. The data are representative of three experiments. (b) Effect of NO on H_2O_2 production in response to OGs. H_2O_2 production was measured as described in (a) and was represented as a percentage of the maximum ROS production observed in leaf discs of Col-0 and mutants elicited by OGs after subtracting background fluorescence of corresponding control. Luminescence values were recorded at 10 min of treatment (peak maximum). cPTIO (500 μM), L-NAME (5 mM) and lanthanum (La^{3+}; 5 mM) were added 10 min before treatment. (c) OG-induced NO production in Col-0 and *rbohD* leaf discs. NO accumulation was determined after 1 and 6 h of treatment and expressed as a percentage of the maximal response after subtracting background fluorescence of corresponding control. Values are a mean ± standard deviation of three independent experiments. RLU, relative luminescence units.

NO regulation of OG-responsive genes

To demonstrate the involvement of NO in the plant defence responses induced by OGs, we monitored the expression of a set of four genes, selected according to previous studies, in leaf discs co-treated with cPTIO or L-NAME. The four genes correspond to *At1G14540* encoding an anionic peroxidase (PER4), *At2G26560* encoding a phospholipase A2 (PLP2), *At3G54420* encoding a chitinase IV (CHI-IV) and *At3G55430* encoding a β-1,3-glucanase. These four genes showed global transcriptional changes when *A. thaliana* plants were treated by OGs (Ferrari *et al.* 2007) and were found to be up-regulated upon *B. cinerea* infection, a fungal pathogen known to release OGs from plant cell walls (La Camera *et al.* 2005; Doxey *et al.* 2007; Ferrari *et al.* 2007). Furthermore, *CHI-IV and* β-*1,3-glucanase* were identified as putative NO-responsive genes after analysis of publicly available expression data literature (Besson-Bard *et al.* 2009).

Compared with the control, a clear increase in *PER4* and *PLP2* transcript levels was observed at 1 h and, to a lower extent, 3 h after OG treatment (Fig. 6a). The increase of β-*1,3-glucanase* transcript level occurred after 3 h and decreased, while remaining significant, after 6 h of treatment (Fig. 6a). In contrast, the expression of *CHI-IV* gene was not clearly up-regulated in our experiment (data not shown).

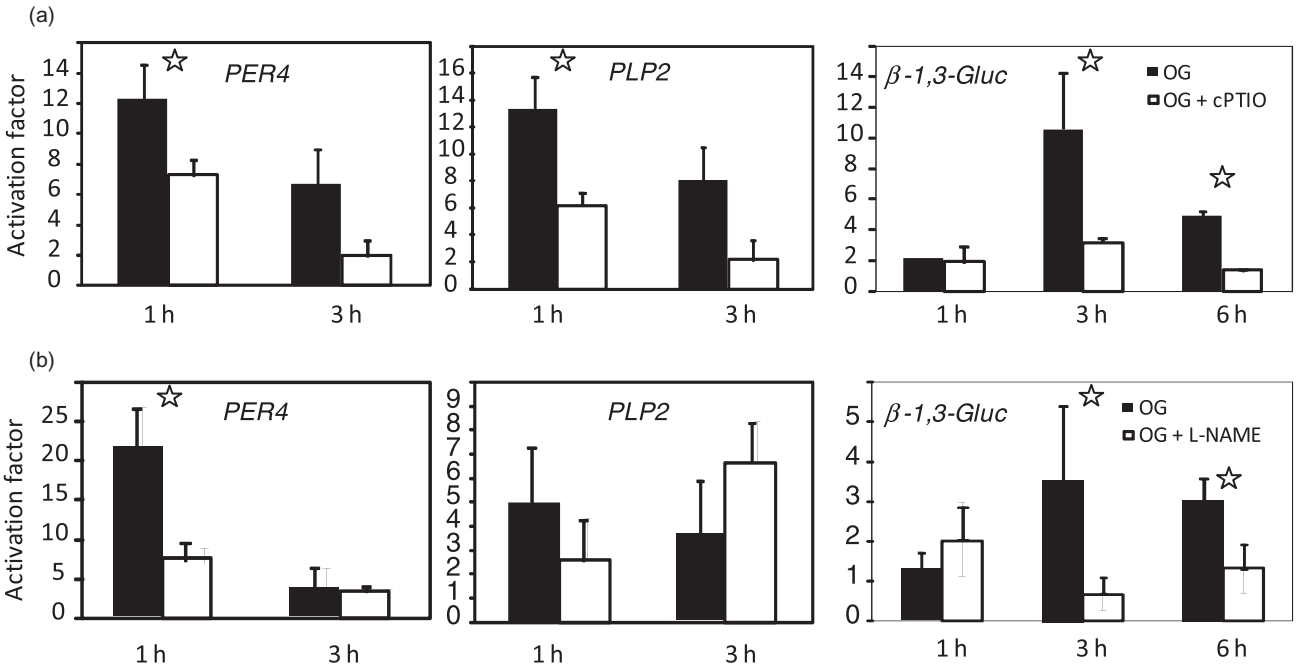

Figure 6. Role of nitric oxide (NO) on transcript accumulation of oligogalacturonide (OG)-responsive genes. (a) Effect of cPTIO treatment on transcript accumulation of OG-responsive genes. Col-0 leaf discs were infiltrated with water or OGs (2.5 mg mL^{-1}) in the presence or absence of cPTIO (500 μM) and harvested for analysis after different time intervals. This experiment is representative of three independent biological experiments. Bars indicate average expression [±standard deviation (SD)] of three replicates. (b) Effect of L-NAME on transcript accumulation of OG-responsive genes. Col-0 leaf discs were infiltrated with water or with OGs (2.5 mg mL^{-1}) in the presence or absence of L-NAME (5 mM) and were harvested for analysis after different time intervals. This experiment is representative of three independent biological experiments. Bars indicate average expression (±SD) of three replicates. Statistically significant comparisons are indicated by a star (Student's *t*-test, $P < 0.05$).

cPTIO reduced the OG-induced up-regulation of these genes (Fig. 6a). However, only *PER4* and β-*1,3-glucanase* transcript accumulation was affected by L-NAME treatment (Fig. 6b). Interestingly, the level of *PLP2* transcript was not significantly different in *nia1nia2* compared with Col-0 (Supporting Information Fig. S4). This result indicates that PLP2 expression, although cPTIO sensitive, is neither regulated by NR-dependent pathway nor by Arg-dependent pathway.

NO participates to basal resistance to the fungal pathogen *B. cinerea*

We used the *A. thaliana*/*B. cinerea* interaction model to determine the role of NO and NO/OG-responsive genes in plant defence. *B. cinerea* is a necrotizing fungus causing several pre- and post-harvest diseases in more than 200 plant species. It was previously demonstrated that OGs are released and accumulate during *B. cinerea* infection. OG release results from PG activity, this activity being required for pathogen virulence (Ten Have *et al*. 1998; Hahn, Darvill & Albersheim 2001). Arabidopsis leaves were infiltrated with water or different concentrations of cPTIO prior to inoculation with *B. cinerea*. The average diameter of necrotic lesions of plants pretreated with 500 μM cPTIO was significantly larger than in control plants (Fig. 7a). To rule out the possibility that the difference in disease lesions was due to an effect of cPTIO on fungal growth, we assessed the effect of cPTIO on the growth of *B. cinerea in vitro*. cPTIO has no effect on growth at the concentrations used in susceptibility analysis (Supporting Information Fig. S5).

Finally, we investigated *A. thaliana* basal resistance to *B. cinerea* in the different genetic background of interest by estimating macroscopic symptoms (e.g. diameter of lesions inflicted by the fungus). We used the double NR mutant *nia1nia2* affected in OG-induced NO production and T-DNA insertion mutants impaired in the expression of the OGs/NO-inducible genes *PER4* and β-*1,3-glucanase* genes (*per4-1*, *per4-2* and *glu*, respectively). Mutants were challenged with *B. cinerea* and disease symptoms were assessed 3 d post-inoculation. Our results indicate that *per4-1*, *per4-2* and *glu* were more susceptible than Col-0, indicating that these two proteins are involved in basal plant resistance to *B. cinerea* (Fig. 7b). *Nia1nia2* double mutant was also more susceptible to *B. cinerea* (Fig. 7b).

DISCUSSION

OG-induced NO production

Although many studies highlight a role for NO in plant physiological processes, its detection and quantification is still a major challenge. Several methods for investigating

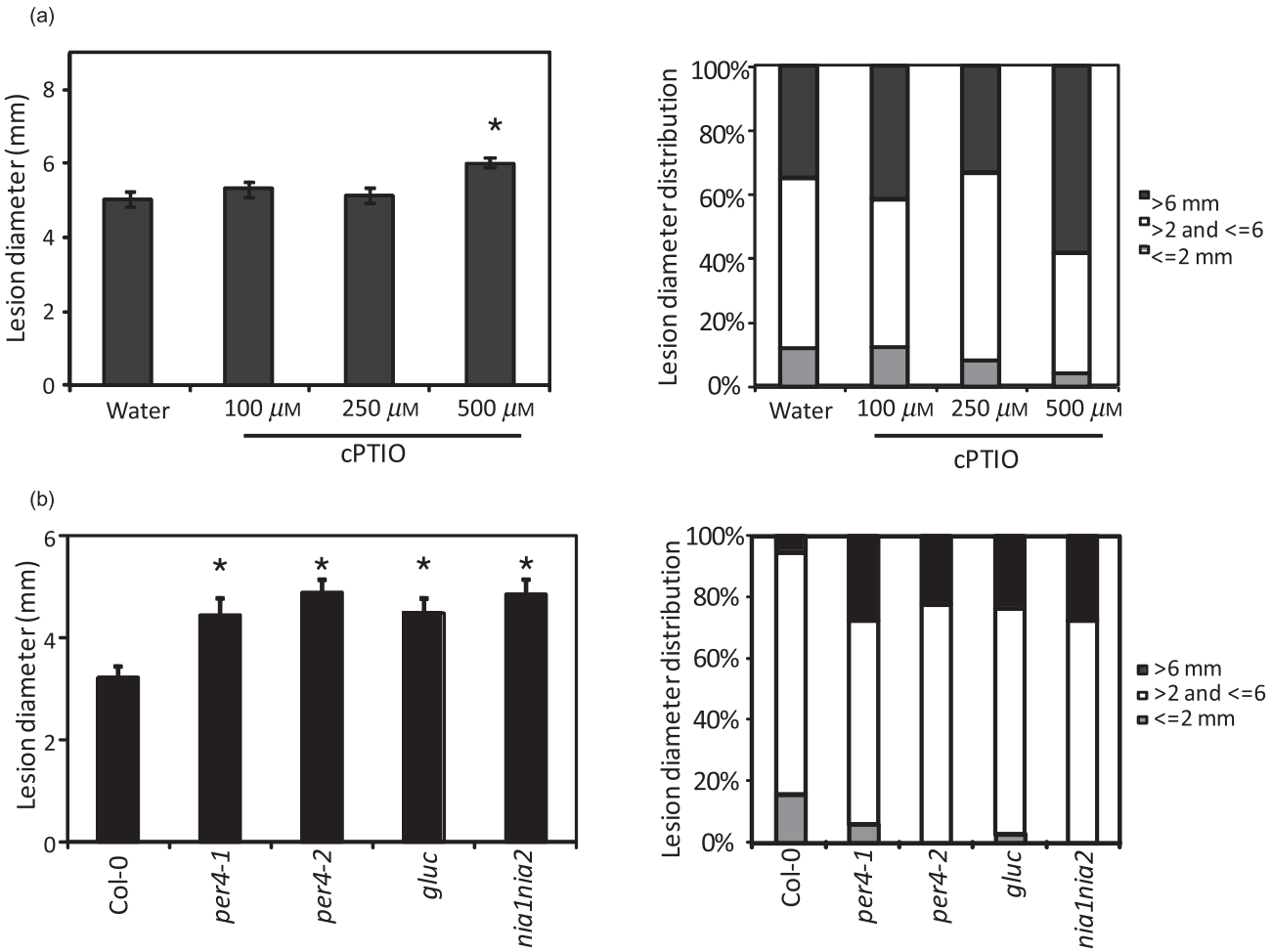

Figure 7. Role of nitric oxide (NO) in basal resistance to *Botrytis cinerea.* (a) Exogenous application of cPTIO decreases resistance against *B. cinerea* in *Arabidopsis thaliana.* Col-0 leaves were infiltrated with different concentration of cPTIO or water and then inoculated with *B. cinerea* spores (6 μL droplets; $5{\cdot}10^4$ spores mL^{-1}). Lesion diameters were measured 3 d after inoculation (left panel) and were grouped into three classes according to their sizes, and the percentage of lesion distribution is shown (right panel). Data are the means (±SE) of three independent experiments performed on 10 plants for each genotype with four lesions per plant. Asterisks indicate statistically significant differences between wild type (WT) and cPTIO treatment (see Materials and Methods). (b) Basal resistance to *B. cinerea* in nitrate reductase (*nia1nia2*), peroxidase 4 (*per4-1* and *per4-2*) and β-1,3-glucanase (*glu*) mutant lines. Four-week-old plants were inoculated on leaves with a 6 μL droplet ($5{\cdot}10^4$ spores mL^{-1}) and symptoms were scored 3 d later (left panel). Lesion diameters were grouped into three classes according to their sizes and the percentage of lesion distribution is shown (right panel). This experiment was performed on 10 plants for each genotype (four inoculations per plant) and was representative of three independent biological experiments. Data are the mean ± SE. Asterisks indicate statistically significant differences between WT and mutant lines (see Materials and Methods).

NO production in plant tissues and cell suspensions have been developed, each offering not only individual advantages but also disadvantages, including the lack of sensitivity and/or the risk of interference by NO-unrelated molecules (for review, see Mur *et al.* 2011). To face the risk of misinterpretation, it is recommended to measure NO production using more than one technique. In the present study, NO production was first monitored using the DAF-2-based assay. This well-used method is indirect and relies on the measurement of reactive nitrogen species derived from NO auto-oxidation, notably N_2O_3. The possibility that DAF-2 also reacts with NO-unrelated molecules, thus generating compounds having fluorescence emission profiles similar to that of DAF-2T, has been raised in several studies (Mur *et al.* 2011). In the present investigation, we showed that OG treatment of leaf discs triggered an increase of fluorescence. This increase was not observed in the presence of the NO scavenger cPTIO, thus supporting the assumption that it is probably related to NO. To further support this hypothesis, NO production was also investigated using the fluorescence probe CuFL. This probe reacts directly with NO, is more sensitive, and was shown to detect NO specifically over other (reactive) species present in biological systems, including not only H_2O_2, peroxynitrite and HNO but also nitrite and nitrate (Lim *et al.* 2006). We observed that OGs induced a significant rise of CuFL fluorescence in

leaf discs, this process being sensitive to cPTIO. Therefore, both the DAF-2 and CuFL methods gave consistent results and support the hypothesis that OGs promote NO production in *A. thaliana* leaf discs.

Our data provide several lines of evidence implicating NR as a major source for NO in response to OGs. OG-induced NO production was reduced in the *nia1nia2* mutant and partly suppressed in wild-type leaf discs treated with the NR inhibitor tungstate. At the biochemical and molecular levels, the NR-dependent NO production was correlated with enhanced NR activity and up-regulation of *NIA1* and *NIA2* gene expression. Involvement of NR in NO production has been reported in interactions between plants and pathogen/elicitors such as necrotrophic fungal pathogen (Asai *et al*. 2008; Perchepied *et al*. 2010), bacteria (Modolo *et al*. 2006; Oliveira *et al*. 2009), chitosan (Li *et al*. 2009) and the elicitin INF1 (Yamamoto-Katou *et al*. 2006). However, Modolo *et al*. (2006) demonstrated that this double mutant has much lower L-arginine content in leaves (almost 10 times lower) as compared with wild-type plants, suggesting that Arg-dependent pathway for NO synthesis could be down-regulated in the *nia1nia2* mutant. We observed that L-arginine supply did not restore NO production in the *nia1nia2* mutant. This result confirms the previous observations of Oliveira *et al*. (2009) showing that no significant increase of NO emission was measured in response to inoculation with pathogen in *nia1nia2* plants treated with L-arginine. Furthermore, it seems to exclude the possibility that the lower level of NO observed in *nia1nia2* in response to OGs is related to an L-arginine deficiency in the leaves. Induction of NR activity and/or up-regulation of NR genes have also been demonstrated in potato infected by *Phytophthora infestans* or in *A. thaliana* infected by *Sclerotinia sclerotorium* (Yamamoto *et al*. 2003; Perchepied *et al*. 2010). Interestingly, coincident NO production and NR activity induction have been also reported in response to other stimuli like cold stress (Zhao *et al*. 2009; Cantrel *et al*. 2011), H_2O_2 treatment (Wang *et al*. 2010) or the *Verticillium dahliae* toxin (VD toxin; Shi & Li 2008). Thus, our study strengthens the possibility that NR-mediated NO production participates in the mechanisms underlying the plant adaptive response to biotic and abiotic stresses through transcriptional regulation, *de novo* NR biosynthesis and enzyme activation.

In addition to NR, we provided data suggesting that OG-induced NO synthesis might also involve an L-arginine-dependent process. Indeed, NO production was partially suppressed by the mammalian NOS inhibitor L-NAME. The ability of this compound to reduce NO synthesis, as well as NOS-like activities in plant tissues and cell suspensions, has been widely reported (for review, see Corpas *et al*. 2011). However, one must be cautious on interpretation based on L-NAME. Firstly, the molecular target(s) of L-NAME are unknown in plants. Secondly, previous studies performed in animal biological models showed that beside NOS, L-NAME could also interact with iron and, accordingly, was reported to inhibit the reduction of ferric cytochrome C by ferrous iron (Peterson *et al*. 1992). Thirdly, as discussed by Besson-Bard *et al*. (2008a), the possibility that this compound could also affect the activities of other L-arginine metabolizing enzyme should not be excluded. Notably, the inhibitory effect of mammalian NOS inhibitors on NO production could be related to their ability to suppress enzyme activities including enzymes related to PA synthesis (such as arginase and arginine decarboxylase) and catabolism (PA oxidases). Because the PAs spermine and spermidine were reported to trigger NO synthesis in *A. thaliana* (Tun *et al*. 2006; Wimalasekera *et al*. 2011), the suppression of NO synthesis by mammalian NOS could reflect an inhibition of PA synthesis rather than a direct effect on a putative NOS-like enzyme. We tested the involvement of polyamine oxidases (PAOX) in OG-induced NO synthesis using *AtPaox* mutants. Compared with Col-0, non-significant modification in NO production was observed, minimizing the involvement of PAs in our biological model (data not shown). Finally, we should not rule out the possibility that L-NAME treatment also triggers metabolic disorders impacting NO synthesis through an unspecific process. The identification of the enzymatic source producing NO from L-arginine and a detailed analysis of the molecular mechanism underlying L-NAME effect, notably on nitrogen metabolism, are therefore priority questions that remained to be answered.

Taken together, basically our results suggest that both a NR-dependent and a L-NAME sensitive pathway could mediate NO production in response to OGs. If both processes are unique sources for NO and act independently, the application of L-NAME in the NR double mutant *nia1nia2* is expected to lead to an additive effect, that is a complete inhibition of OG-induced NO production. In contrast, we observed that L-NAME did not further affect the remaining OG-induced NO production in the *nia1nia2* mutant. Moreover, we observed that L-NAME inhibits OG-induced NR activity but not clearly NR gene expression. These data are intriguing and suggest at least two scenarios. Firstly, based on the likely poor specificity of L-NAME, they strongly favour the hypothesis that NR is the main source for NO in response to OGs and that L-NAME effect on NO synthesis and NR activity could reflect unspecific action of the NOS inhibitor. In this regard, to reduce the risk of misinterpretation of data based on L-NAME, we recommend to potential users of this compound to include controls showing the impacts of L-NAME on NR transcript accumulation and activity. Secondly, based on studies showing that L-NAME inhibits NOS-like activities in plants (Corpas *et al*. 2011), these results might also suggest that two pathways, that is the L-arginine and NR pathways, are co-involved in NO production and do not work independently: L-NAME-sensitive NO production could affect NR-dependent NO production. Supporting this assumption, two studies reported that NO donors and the NO scavenger cPTIO modulate NR activity in *Brassica chinensis* and *Solanum lycocarpum*, thus leading to the hypothesis that NR activity may be self-regulated rapidly by its product NO or by NO produced *via* other sources (Du *et al*. 2008; Jin *et al*. 2009). The effect of NO was shown to be

dependent on N concentration (Jin *et al*. 2009). Based on these data, the authors hypothesized that NO can stimulate NR activity at the post-translational level through a direct interaction or, alternatively, by affecting the activity of proteins involved in NR regulation including protein kinases or phosphatases. However, it cannot be excluded that part of the NO produced by Arg-dependent pathway could be oxidized to nitrite, thus providing substrate for NR-triggered NO synthesis.

Whatever the scenario, using L-NAME and/or approaches to impair NR expression and activity, we were not able to completely suppress OG-induced NO synthesis. In contrast, the NO scavenger cPTIO strongly blocked the production of NO triggered by OGs. Therefore, although we cannot exclude the hypothesis that the remaining NO production is related to a residual NR activity in the *nia1nia2* mutant or in leaf discs treated with tungstate, these observations suggest the involvement of an alternative route to the NR- and Arg-dependent pathways. This route could be involved in OG-induced accumulation of PLP2 transcripts which appeared cPTIO sensitive but not affected by L-NAME or in the *nia1nia2* mutant. Numerous possible sources for NO have been proposed (Neill, Desikan & Hancock 2003; Planchet *et al*. 2005; Tun *et al*. 2008). Apart from NR and a potential L-arginine-dependent pathway, so far we do not have experimental evidence for the occurrence of another route for NO synthesis (data not shown).

OG-induced NO production depends on Ca^{2+} influx

Moscatiello *et al*. (2006) previously reported that OG effects in *A. thaliana* cell suspensions are mediated through a La^{3+}-sensitive transient elevations of cytosolic Ca^{2+}. Interestingly, we measured a lower OG-induced NO production in the *dnd1* mutant impaired in the expression of the Ca^{2+}-permeable channel *CNGC2*. The reduced NO production observed in *dnd1* suggests a role for CNGC2 and Ca^{2+} in the transduction of OG signal leading to NO production. The inhibition of OG-induced NO production by La^{3+}, a calcium channel blocker, corroborates this conclusion. Interestingly, La^{3+} did not block *in vivo* OG-induced NR activity, allowing us to suggest that calcium influx controls only Arg-dependent NO production (Supporting Information Fig. S6). In support of our findings, CNGC2 and associated Ca^{2+} influx were shown to act upstream of LPS-induced Arg-dependent NO production in *A. thaliana* guard cells (Ali *et al*. 2007). Similarly, pharmacological-based strategies pointed out the importance of Ca^{2+} influx in elicitor-triggered NO production (Courtois *et al*. 2008). However, the effect of lanthanum on NR activity should be interpreted cautiously: whereas calcium influx is important for NO production, it is also known to contribute to NR inhibition through calcium-dependent protein kinase phosphorylation and binding to 14-3-3 proteins (Buchanan, Gruissem & Jones 2000 for review). Therefore, the observation that lanthanum did not affect NR activity might also reflect inhibitory role of Ca^{2+} on NR activity.

NO production modulates AtRBOHD-mediated oxidative burst

One of the earliest characterized events of defence mechanisms is the oxidative burst corresponding to the generation of ROS including H_2O_2 and the superoxide anion, $O_2^{\bullet-}$ (Lamb & Dixon 1997; Wojtaszek, Stobiecki & Bolwell 1997). There is considerable evidence that the elicitor- and pathogen-induced oxidative bursts in *Arabidopsis* are mediated by AtRBOH proteins, homolog of the gp91 subunit of mammalian NADPH oxidase (Torres, Dangl & Jones 2002). Our results show that the OG-induced oxidative burst in *A. thaliana* leaf discs was abolished using DPI and in the *Atrboh*D mutant (Fig. 5a,c), confirming that plasma membrane NADPH oxidase was responsible for the OG-induced oxidative burst as previously observed by Galletti *et al*. (2008).

We observed that DAF fluorescence was not significantly affected in *AtrbohD* mutant indicating that H_2O_2 was not sufficient for OG-induced NO generation. This finding diverges from other studies assuming that ROS production acts upstream of NO production. Indeed, H_2O_2 was shown to be required for ABA- and UV-B-induced NO production in guard cells of both *Vicia faba* and *A. thaliana* (Dong *et al*. 2005; He *et al*. 2005; Bright *et al*. 2006) and for chitosan-response in *Pisum sativum* (Srivastava *et al*. 2009). In contrast, we observed that the application of the cPTIO or L-NAME diminished the oxidative burst in response to OGs (Fig. 5b). Although a direct scavenging of ROS by cPTIO could not be excluded, these data, together with L-NAME effect, suggest a positive role for NO in regulating ROS production. These findings indicate that the induction of H_2O_2 accumulation may be a downstream component of OG-induced NO production. These results are in agreement with previous observations made in the context of the interaction between *B. cinerea* and *V. vinifera* which showed that NO, whether produced in response to BcPG1 or released by a NO donor, leads to ROS production in grapevine cells (Vandelle *et al*. 2006). In contrast, other studies reported that reducing endogenous NO level using cPTIO or plant mutants impaired in inducible NO production (e.g. NR double mutant) enhanced H_2O_2 accumulation (Tada *et al*. 2004; Asai *et al*. 2008), suggesting that part of the $O_2^{\bullet-}$ produced by NADPH oxidase is scavenged by NO. Furthermore, recently Yun *et al*. (2011) found that NO abolishes AtRBOHD activity through S-nitrosylation.

Importantly, in contrast to cPTIO or L-NAME treatments, we observed that the *nia1nia2* mutant behaves like wild-type plants in terms of OG-induced ROS generation, indicating that NR-mediated NO production is not essential for oxidative burst. These data support the hypothesis that NO production might involve at least two enzymatic sources, NO resulting from the Arg-dependent pathway being involved in the control of the oxidative burst.

However, as discussed above, this interpretation is hindered by the limited specificity of L-NAME.

OG-induced NO-dependent genes are involved in *A. thaliana* basal resistance to *B. cinerea*

We analysed, using real-time qPCR, transcript accumulation of candidate genes in response to OGs in the presence of cPTIO and L-NAME. The reduced transcript level of *PLP2*, *PER*4 and β-*1,3-glucanase* observed in leaf discs co-treated with cPTIO or L-NAME and OGs indicates that their expression is, at least partly, dependent on NO. As *PER4* and β-*1,3-glucanase* genes were also induced by *B. cinerea*, we investigated the role of the corresponding proteins in the resistance to the pathogen using mutant lines. The different mutant lines impaired in *PER4* and β-*1,3-glucanase* were more susceptible to *B. cinerea*, indicating that *PER4* and β-*1,3-glucanase* expression is required for *A. thaliana* resistance against *B. cinerea*. Because NO regulates the expression of these genes in response to OGs, these data also suggest that NO is involved in the processes controlling resistance through gene regulation. Accordingly, a higher susceptibility to *B. cinerea* was observed for Col-0 plants treated with cPTIO and in the *nia1nia2* mutant. Importantly, impairment of *PER4* and β-*1,3-glucanase* expression, as well as the suppression of NO synthesis by cPTIO, had a weak effect on plant resistance to *B. cinerea*. Indeed, disease susceptibility was only increased by 20% in those genotypes or in cPTIO-co-treated Col0 plants. However, we should mention that plant resistance to necrotrophic pathogens such as *B. cinerea* has a quantitative and complex genetic basis involving probably numerous genes. Indeed, 23 quantitative trait loci (QTL) with significant influence on *B. cinerea* lesion size or camalexin accumulation have been identified in *A. thaliana* (Rowe & Kliebenstein 2008).

Our observations corroborate previous studies suggesting a role for NO in disease resistance to necrotrophic pathogen (Asai & Yoshioka 2009; Perchepied *et al*. 2010). Notably, our investigation demonstrates that the effect of endogenously produced NO could be explained by the regulation of genes activated during fungal colonization or elicitor treatment. These genes encode proteins related to plant defence. The anionic peroxidase PER4 is described as a lignin-forming peroxidase putatively located in the endomembrane system. This protein is related to the PR9 family (van Loon, Rep & Pieterse 2006) and closely similar to prxA3a involved in both lignin content and composition in hybrid aspen (Li *et al*. 2003). PR-9 is a specific type of peroxidase that could act in cell wall reinforcement and then enhance resistance to pathogens. *β*-1,3-glucanase is also referred to as a PR protein of the PR2 family (Doxey *et al*. 2007). This protein, putatively localized in plant cell wall, could affect mycelium growth by hydrolysing the protective glucan sheath on the surface of the fungus such as *B. cinerea* (Gil-ad, Bar-Nun & Mayer 2001).

In conclusion, we proposed a role for NO in mediating OG-induced effects in *A. thaliana*, including ROS production and the expression of genes encoding *PER4* and a *β*-1,3-glucanase (Fig. 8). The control of the expression of those genes by NO might constitute one of the mechanisms underlying *A. thaliana* basal resistance to *B. cinerea*.

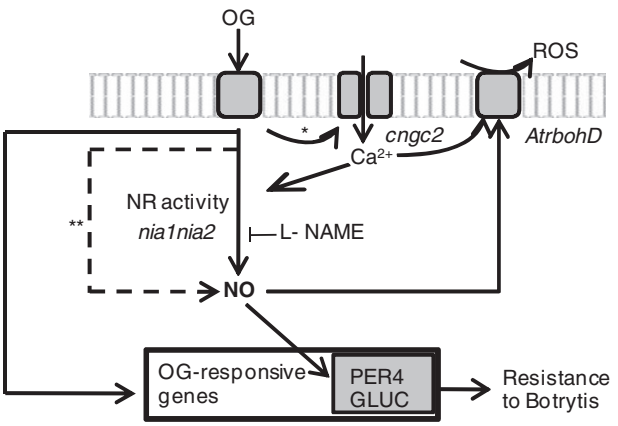

Figure 8. Schematic illustration of the signalling network involving nitric oxide (NO) that modulates oligogalacturonide (OG)-triggered immunity against *Botrytis cinerea* in *Arabidopsis thaliana*. NO production involves a nitrate reductase (NR) pathway, sensitive to L-NAME. OG-induced Arg-dependent NO production was Ca^{2+} dependent and modulated RBOHD-mediated reactive oxygen species (ROS) production. NO produced by Arg and NR pathways participates in the regulation of OG-responsive genes such as anionic peroxidase (PER4) and a *β*-1,3-glucanase. Mutant plants impaired in PER4 and *β*-1,3-glucanase, as well as Col-0 plants treated with the NO scavenger cPTIO, were more susceptible to *B. cinerea.* (*): OGs elicit a rapid elevation of cytosolic-free calcium in *A. thaliana* cells (Moscatiello *et al*. 2006). (**): not characterized alternative route for NO production.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparative analysis of NO detection using DAF-2 and CuFL fluorescent probes. The DAF-2 probe was added at a final concentration of 5 μM in 200 μL Tris-HCl (50 mM, pH 7.5). The CuFL probe (Strem Chemicals, Bischheim, France) was freshly prepared by adding 1:1 FL solution (1.0 mM) to the copper (II) solution (1.0 mM) and added at a final concentration of 5 μM in 200 μL Tris-HCl (10 mM, pH 7.5) KCl (10 mM). The increase of fluorescence triggered by GSNO (200 μM) was measured using a spectrofluorometer (Mithras, Berthold Technologies, Germany) with a 485 nm excitation and a 535 nm emission filters. A second addition of GSNO (200 μM) was performed after 120 min (arrows). Fluorescence was expressed as relative fluorescence units (arbitrary units: au) after subtracting background fluorescence of time zero (t0) and of the corresponding control (probe without GSNO). This experiment is representative of 3 independent experiments.

Figure S2. NO detection in *A. thaliana* leaf discs by CuFL probe. NO production was monitored after 1 hour of treatment in the absence (control) or presence of OGs (2.5 mg·mL^{-1}), with or without NO scavenger (cPTIO 500 μM). Each value represents the mean of 8 measurements ± SD. Leaf discs excised from plants were infiltrated under vacuum for 3 min with an aqueous solution of OGs. For control treatment, OGs were replaced by an equivalent volume of water. After infiltration, discs were incubated for one hour with respective solutions and then each disc was put in separate well of 96 well plates (Microtest flatbottom, Becton Dickinson, Europe) in 200 μL Tris-HCl (10 mM, pH 7.5) KCl (10 mM). The CuFL probe (Strem Chemicals, Bischheim, France) was freshly prepared by adding 1:1 FL solution (1.0 mM) to the copper (II) solution (1.0 mM) and added in each well (final concentration of 5 μM). The increase of fluorescence triggered by OGs was measured using a spectrofluorometer (Mithras, Berthold Technologies, Germany) with a 485 nm excitation and a 535 nm emission filter. Fluorescence was expressed as relative fluorescence units (arbitrary units: au) after subtracting background fluorescence of time zero (t0). This experiment is representative of 3 independent experiments using independent biological material.

Figure S3. Effect of L-NAME (a) or tungstate (b) on OG-induced NO production in Col-0 leaf discs. NO production was monitored as described in Fig. 1 and visualized in the first 3 hours of treatment after subtracting fluorescence of corresponding control. Each value represents the mean of 8 measurements. This experiment is representative of at least 3 independent experiments using independent biological material.

Figure S4. *PLP2* transcript accumulation in response to OGs in the *nia1nia2* double mutant. Col-0 and *nia1nia2* leaf discs were infiltrated with water or OGs (2.5 mg·mL^{-1}) and harvested for analysis after different time intervals. Bars indicate average expression (±SD) of three biological replicates.

Figure S5. Effect of cPTIO on *Botrytis cinerea* growth. *Botrytis cinerea* strain BMM (Zimmerli *et al.* 2000) was grown on Petri plates containing PDA (potato dextrose agar, DIFCO) for 10–12 days. Spores were harvested in water and were quantified under a microscope. To verify the effect of cPTIO on *B. cinerea* growth, spores were diluted to obtain the final concentration of 5×10^4 spores mL^{-1}. 12 μL

of spore suspension (5×10^4 spores mL^{-1}) with cPTIO or water in PDB medium was placed on glass slides and allowed to grow under high humidity for different time. Fungal growth was observed under microscope.

Figure S6. Effect of cPTIO treatment on transcript accumulation of NR genes. Col-0 leaf discs were infiltrated with water or OGs (2.5 $mg \cdot mL^{-1}$) in the presence or absence of L-NAME (5 mM) and harvested for analysis after different time intervals. Transcribed mRNAs were analysed by real-time quantitative RT-PCR. Levels of transcripts were calculated using $2^{-\Delta\Delta CT}$ method. This experiment is representative of 2 independent experiments using independent biological material.

Figure S7. *In vitro* NR activity in wild-type (Col-0) leaf discs treated with water (control), OGs/OGs + lanthanum (5 mM) and in *nia1nia2* NR mutant. Each value represents the mean ± SE of three independent experiments performed using independent biological materials.

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