Interplays between nitric oxide and reactive oxygen species in cryptogein signaling

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Running Head: NO and ROS cross-talk in cryptogein signaling
ABSTRACT

The cellular messenger nitric oxide (NO) has many functions in plants. In this study, we investigated its interplays with Reactive Oxygen Species (ROS) in the defense responses triggered by the elicitin cryptogein produced by the oomycete *Phytophthora cryptogea*. The production of NO induced by cryptogein in tobacco cell suspensions was partly regulated through a ROS-dependent pathway involving the NADPH oxidase NtRBOHD. In turn, NO down-regulated the level of H$_2$O$_2$ derived from NtRBOHD activity. Both NO and ROS synthesis appeared to be under the control of two redundant isoforms of histone deacetylases of type 2 acting as negative regulators of cell death. Occurrence of an interplay between NO and ROS was further supported by the finding that cryptogein triggered a fast production of peroxynitrite (ONOO$^-$) resulting from the coupling reaction of superoxide (O$_2$•-) with NO. We provided evidence that ROS, but not NO, negatively regulate the intensity of activity of the protein kinase NtOSAK, a member of the SnRK2 protein kinase family. Furthermore, using a micro-array approach, we next identified fifteen genes early induced by cryptogein via NO. Interestingly, only a part of these genes was also modulated by ROS derived from NtRBOHD activity and encoded proteins showing sequence identity to ubiquitin ligases. Expression of those genes appeared to be negatively regulated by ONOO$^-$, suggesting that ONOO$^-$ mitigate the effects of NO and ROS in cell response to cryptogein. Finally, we provided evidence that NO required NtRBOHD activity for inducing cell death, thus confirming previous assumption that ROS channel NO through cell death pathways.

Keywords: cell death, cryptogein, defense responses, histone deacetylase, NADPH oxidase, nitric oxide, peroxynitrite, reactive oxygen species, signaling
INTRODUCTION

Nitric oxide (NO) is an endogenously produced ubiquitous free radical gas which plays key roles in various physiological processes in plants such as germination, root development, stomatal closure, flowering, hormone signaling or iron homeostasis (Besson-Bard et al. 2008a). Also, it has emerged as a molecule of interest in plant pathology (Bellin et al. 2013). Indeed, NO is rapidly produced in plant cells facing pathogen attack or elicited by MAMPs (Microbe-Associated Molecular Patterns) or DAMPs (Danger-Associated Molecular Patterns; Jeandroz et al. 2013). Its production involves nitrate reductase (Yamamoto-Katou et al. 2006; Perchepied et al. 2010; Rasul et al. 2012) and, according to several studies, a still unidentified enzyme displaying functional similarities with nitric oxide synthase (NOS), the main enzymatic source for NO in animals (Asai & Yoshioka, 2009; Corpas et al. 2009). Mounting evidences indicate that NO serves as a key messenger in plant defense. Supporting this statement, interplays between NO and major components of plant immune signaling pathways such as the second messengers Ca$^{2+}$, cyclic AMP and cyclic GMP, MAP (Mitogen Activated Protein) kinases, signaling lipids and the hormones salicylic acid, jasmonic acid or ethylene have been widely reported (Mur et al. 2008; Gaupels et al. 2011a; Yoshioka et al. 2011; Mandal et al. 2012; Yun et al. 2012). Through its signaling activity, NO seems to play a part in various stages of plant defense. It indeed contributes to early defense responses as well as to ultimate events including the hypersensitive response (HR) and systemic acquired resistance (SAR; Song & Goodman, 2001; Delledonne et al. 2003; Piterková et al. 2009). Although a role for NO in controlling disease resistance has been established in several pathosystems (Delledonne et al. 1998; Asai et al. 2009; Perchepied et al. 2010; Rasul et al. 2012), subtle processes underlying its functions remain poorly understood. Identification and functional analysis of NO-responsive genes provided significant progresses in understanding its role at a molecular level (Zago et al. 2006; Palmieri et al. 2008; Besson-Bard et al. 2009). Notably, these studies confirm the first hints (Delledonne et al. 1998; Durner et al. 1998) that NO regulates the expression of defense genes such as those encoding proteins related to secondary metabolism or pathogenesis-related proteins. Recently, these analysis were completed by the characterization of proteins regulated through NO-dependent processes at the post-translational level by S-nitrosylation and tyrosine nitration (Romero-Puertas et al. 2007; Lindermay & Durner, 2009; Vandelle & Delledonne, 2011; Astier et al. 2012a; Skelly & Loake, 2013). Of importance, several of them have important known implications in plant defense such as NPR1 (Nonexpresser of Pathogenesis-Related gene 1) and the NADPH oxidase AtRBOHD (Tada et al. 2008; Lindermayr et al. 2010; Yun et al. 2011), thus providing a new view of how NO impacts plant defense responses.

Several lines of research highlighted the existence of cross-talks operating between NO and reactive oxygen species (ROS) including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) which
production result mainly from the activity of NADPH oxidases and superoxide dismutases (SOD), respectively (Mittler et al. 2011). Basically, these species share several similarities, complementarities, but also show contrasting or independent effects. First, their productions occur simultaneously and constitute one of the earliest plant immune responses (Foissner et al. 2000; Delledonne et al. 2001). Second, both species display signaling functions and, for instance, were shown to regulate unique as well as common set of genes (Zago et al. 2006). Third, unregulated synthesis of these species has been implicated as causal or contributing to improper defense responses (see for instance Takahashi et al. 1997; Chamnongpol et al. 1998; Asai & Yoshioka, 2009; Rasul et al. 2012). Fourth, ROS and NO, as well as other reactive nitrogen species (RNS), have distinct reactivities and also abilities to freely cross membranes, depending on their chemical nature (Hughes, 2008; Ferrer-Sueta & Radi, 2009). Of particular importance here, NO is a relatively stable radical but reacts with dioxygen and other radicals (Hughes, 2008). Notably, NO reacts with \( \text{O}_2^- \) to form peroxynitrite (ONOO\(^-\)), a highly reactive oxidant produced in plant cells undergoing immune responses (Vandelle & Delledonne, 2011). The importance of the interplays between NO and ROS in plant defense has been the subject of particular investigations. Clearly, there is still confusion in understanding their interconnection and reciprocal influences. Indeed, in terms of production NO has been shown to favor or to suppress NADPH oxidase activity (Yun et al. 2011; Rasul et al. 2012) and evidences that ROS also control NO synthesis have been reported (Srivastava et al. 2009). Furthermore, both NO and ROS derived from NADPH oxidase activity were shown to account for HR. However, two distinct processes were proposed. In the first \( \text{H}_2\text{O}_2 \), but not \( \text{O}_2^- \), was the key ROS effector of HR and played a central role in channeling NO through the cell death pathway (Delledonne et al. 2001; Zago et al. 2006). According to the authors of these studies, ONOO\(^-\) was not a mediator of HR. In contrast and according to the situation encountered in animals, in the second process ONOO\(^-\) emerged as an essential intermediate of cell death not only during HR but also other physiological processes such as self-incompatible pollination (Alamillo & García-Olmedo, 2001; Serrano et al. 2012). Contrasting roles for NO and ROS in disease resistance have also been highlighted. In particular, Asai and Yoshioka (2009) demonstrated that NO and ROS had opposite effects in Nicotiana benthamiana plants infected by Botrytis cinerea, NO positively and ROS negatively regulating the basal resistance against the necrotrophic fungal pathogen.

Cryptogein is a 10 kDa elicitin produced by the oomycete Phytophthora cryptogea. Purified cryptogein causes defense responses in tobacco including HR and SAR against the black shank-causing agent Phytophthora parasitica var nicotianae as well as against other microbial pathogens (Bonnet et al. 1996). We and other research groups previously reported that cryptogein triggers NO production in leaf epidermal tobacco cells and/or in tobacco cell suspensions. The production of NO was assessed using different methods including 4,5 diamino-fluorescein (DAF)-based fluorescence
(Foissner et al. 2000; Lamotte et al. 2004; Besson-Bard et al. 2008b), electrochemistry (Besson-Bard et al. 2008b) and an oxidizer column NO detector relying on the ability of CrO₃ to oxidize NO to NO₂, NO₂ being subsequently captured by a Griess reagent trap (Vitecek et al. 2008). All these approaches gave consistent results: NO is produced at the intracellular level within few minutes and diffuses in the extracellular medium and in the gas phase of tobacco cell suspensions elicited by cryptogein. The enzymatic source for NO has not been identified but was shown to be sensitive to mammalian NOS inhibitors (Foissner et al. 2000; Lamotte et al. 2004; Besson-Bard et al. 2008b). Interestingly, the elicitor-induced NO production occurred simultaneously to those of H₂O₂ and O₂⁻ resulting from the activity of the plasma membrane NADPH oxidase NtRBOHD (Foissner et al. 2000; Simon-Plas et al. 2002; Lherminier et al. 2009).

Overall, the function of NO in cryptogein signaling is poorly understood but several arguments seem to support a signaling role. Indeed, it has been reported that NO acts as a Ca²⁺-mobilizing compound contributing to the increase in cytosolic free Ca²⁺ concentration mediated by the elicitor (Lamotte et al. 2004). Supporting an involvement of NO in Ca²⁺ signaling, we recently showed that cryptogein induces the S-nitrosylation of a calmodulin (CaM) tobacco isoform (Astier et al. 2012b; Jeandroz et al. 2013). Besides CaM, other proteins undergoing a fast S-nitrosylation upon cryptogein treatment of tobacco cells were identified. These NO target proteins include NtCDC 48 (cell division cycle), a member of the AAA+ ATPase (ATPase associated with various cellular activities) family displaying a chaperone-like activity (Astier et al. 2012b). In animals, CDC48 (named VCP/p97) governs important signaling pathways and, among other functions, helps to deliver protein substrates to the proteasome in quality control pathways (Meyer et al. 2012). A role for NO as an intermediate of cryptogein-triggered cell death has also been proposed as its scavenging reduced the rate of cell death conferred by the elicitor in tobacco cell suspensions (Lamotte et al. 2004). The function of NO in this process has not been elucidated. More generally, our understanding of the mechanisms underlying cryptogein-induced cell death is still faint. Recently, Bourque et al. (2011) demonstrated that NtHD2a and NtHD2b, two redundant isoforms of HDAC (histone deacetylase) of type II, negatively regulate cryptogein-triggered cell death. Impairment of their expression resulted in exacerbated cell death in cell suspension and in the formation of HR-like symptoms in distal leaves. The precise functions of NtHD2a/b in the regulation of cell death, as well as their functional link with NO, are still enigmatic.

The possibility that NO interacts with ROS in cryptogein signaling has been previously suggested (Foissner et al. 2000) but not investigated in details. More generally, the interactions between these species have rarely been assessed in a physiological context and, in most of the studies, rely on the use of NO and/or ROS exogenously applied. In the present study, we analyzed the interplays between NO and ROS derived from NtRBOHD in cryptogein signaling in tobacco cell
suspensions. Using a combination of pharmacological- and genetic-based approaches, we demonstrated that ROS partly control the production of NO while NO regulates $H_2O_2$ levels. Further supporting a functional link between NO and ROS, a fast $ONO_2^-$ synthesis was detected in elicited-tobacco cells. Furthermore, a transcriptomic analysis led to the identification of cryptogein-induced early genes commonly up-regulated by both NO and ROS but down-regulated by $ONO_2^-$. Finally, we provided evidence that NtRBOHD impairment compromises NO involvement in cell death, thus further supporting initial statements that $H_2O_2$ might channel NO through the cell death pathway (Delledonne et al. 2001; Zago et al. 2006). Taken together, our results provided physiological evidences that NO and ROS derived from NtRBOHD act together in mediating cryptogein signaling.
MATERIALS AND METHODS

Cell cultures and treatments

*Nicotiana tabacum* L. cv Xanthi were cultivated as previously described (Bourque et al. 2011). Briefly, cells were grown at 25°C on a rotary shaker (150 rpm) and under continuous light (photon flux rate 30-40 µmol.m\(^{-2}.s^{-1}\)) in Chandler’s medium (Chandler et al. 1972). Cells were sub-cultured every seven days. Same culture conditions were applied for the distinct cell lines.

For elicitor treatments, seven days-old cells were gently filtrated, washed and re-suspended in H10 buffer (175 mM mannitol, 0.5 mM CaCl\(_2\), 0.5 mM K\(_2\)SO\(_4\), 10 mM HEPES, pH 6.0) at a final concentration of 0.1 g/10 mL. Before treatments, cells were equilibrated at 25°C and 150 rpm in the same buffer for 2 or 3 h, depending on the experiments. With the exceptions of fluorescence measurement in which cells were kept in the dark, equilibration were performed under light condition.

Cryptogein was purified according to Bourque et al. (2011) and dissolved in water. The NO scavenger cPTIO ((4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was prepared in water and diphenyl iodonium (DPI) in DMSO. DPI and cPTIO were added to the cell suspensions 5 and 10 min before cryptogein, respectively. Control cells were treated with an equal volume of DMSO or water, respectively.

The NO donor diethylamine NONOate (DEA/NO) was prepared as previously described (Besson-Bard et al. 2008b). Briefly, a 0.01 M stock solution was prepared daily in NaOH and stored on ice. To initiate the release of NO, an aliquot of the stock solution was dissolved in 100 mM phosphate buffer, pH 7.2, at a final concentration of 2 mM. Few second after its dilution in the phosphate buffer, DEA/NO was applied to the cell suspensions to give a 200 µM final concentration. As a control, cells were treated with an equivalent concentration of diethylamine (DEA) prepared as described for DEA/NO.

Chemicals

All basic salts and chemicals were purchased from Sigma-Aldrich (Saint-Louis, USA) unless stated. The CuFL probe was from Strem Chemicals, Inc. (Bischheim, France). cPTIO and DEA/NO were from Alexis Biochemicals (San Diego, USA). Murashige & Skoog medium incl. Nitsch vitamins were from Duchefa Biochemie (Haarlem, The Netherlands). Complete Protease Inhibitors Cocktail was from Roche. γ-\(^{32}\)P] ATP was from Amersham Pharmacia Biotech AB.

NO production measurements
The detection of NO using 4,5-diaminofluorescein diacetate (DAF-2DA) was performed according to Lamotte et al. (2004). Filtrated and re-suspended cells were incubated for 1 h with 20 µM DAF-2DA. To remove excess of the probe, cells were washed three times with fresh H10 buffer and transferred into 24-well plates (1 mL/well, Costar, Corning Incorporated, Corning, NY, USA). After 30 min of incubation, cells were treated with cryptogein, cPTIO, DEA/NO, DPI or DMSO as detailed above. NO production was measured with a spectrofluorometer (Mithras, Berthold Technologies, Germany) using 485 nm excitation and 510 nm emission filters. Fluorescence was expressed as relative fluorescence units.

For NO detection with the CuFL probe, filtrated and equilibrated cells were transferred into 24-well plates (1 mL/well). Then, cells were pre-treated or not with cPTIO and exposed 40 min to cryptogein. Five minutes before ending cryptogein treatment, CuFL dissolved in DMSO was added to the cell suspensions with a final concentration of 5 µM. The fluorescence was measured by spectrofluorometry as described for DAF-based fluorescence.

**H$_2$O$_2$ production**

ROS production was determined by chemiluminescence as previously described (Pugin et al. 1997; Simon-Plas et al. 1997). After application of the treatments, triple aliquots of 250 µL cell suspensions were collected and transferred into vials. Then, cells were automatically supplemented with 300 µL of H50 buffer pH 6.5 (175 mM mannitol, 0.5 mM CaCl$_2$, 0.5 mM K$_2$SO$_4$, HEPES 50 mM) containing luminol at a final concentration of 13 µM. Chemiluminescence was measured using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). The concentration of H$_2$O$_2$ was calculated as previously described (Pugin et al. 1997; Simon-Plas et al. 1997) and expressed in nanomoles of H$_2$O$_2$ per gram of cells fresh weight.

**Peroxynitrite production and cellular localization**

The production of ONOO$^-$ was measured as followed: equilibrated tobacco cell suspensions kept in the dark were loaded with 5 µM aminophenyl fluorescein (APF) for one hour. After removing excess of the probe, cells were transferred into 24-well plates (1 mL per well) and incubated for 30 min before applying cryptogein or the ONOO$^-$ donor SIN-1 (3-morpholinosydnonimine hydrochloride) prepared in a 0.1 M phosphate buffer, pH 7.2.

Urate was used as a peroxynitrite scavenger. Urate was dissolved in 1 M NaOH and applied to cells at a final concentration of 1 mM. As controls, cells were supplemented with NaOH at a final concentration of 3.3 mM. A H50 buffer was used to stabilize the pH at 6.0. Fluorescence was measured as described previously for NO detection.
In order to determine the cellular localization of ONOO\(^-\), seven days-old cells were equilibrated for 2 h in 25\(^\circ\)C in the dark under continuous shaking at 150 rpm. Then, cells were treated with 100 nM cryptogein for 0, 20 or 40 min and stained with 15 \(\mu\)M APF for last 5 min of treatment. After three washes with H10 buffer, cells were immediately observed using a confocal laser scanning microscope (Leica TCS 4D; SP2; Leica Microsystems, Heidelberg, Germany) under the 40x NA1 oil immersion objective (HC PL APO CS 40x 0.75-1.25). The light source was a Ar-ArKr (488nm) beam laser and emission of APF fluorescence was pass-filtered between 510-545 nm. Chloroplast autofluorescence was pass-filtered between 665-705 nm. The unspecific background was removed with the median filter of Volocity\textsuperscript{\textregistered} 6.1.1 software (PerkinElmer, USA).

**Analysis of protein kinase activities**

Tobacco cells were grounded in liquid nitrogen and 2 mL powder samples were supplemented with 250 \(\mu\)L of protein extraction buffer (20 mM Tris-HCl pH 7.5; 2 mM EDTA; 2 mM EGTA; 50 mM -glycerophosphate; 250 mM sucrose; 10 mM \(\text{Na}_3\text{VO}_4\); 10 mM DTT, 1 mM PMSF; 1x Complete Protease Inhibitors Cocktail) or immunoprecipitation buffer (protein extraction buffer supplemented with 1% Triton X-100 and 150 mM NaCl). The extracts were centrifuged at 14 000 rpm for 30 min at 4\(^\circ\)C and supernatants were used for further analysis. The protein concentration was measured using the Protein Assay System described by Bradford (1976) using BSA as the reference for protein concentration.

**NtOSAK Immunoprecipitation assays:** Immunoprecipitation was performed as described previously (Kulik et al., 2012) with some minor modifications. Briefly, protein A-agarose beads (15 \(\mu\)L per sample) were washed three times with immunoprecipitation buffer and incubated for 4 hours with antibodies against the C-terminal domain of NtOSAK (24 \(\mu\)g) at 4\(^\circ\)C with gentle shaking. After incubation, agarose beads were pelleted by brief centrifugation and washed three times with 1 mL of immunoprecipitation buffer. Protein A-agarose portions with bounded antibodies were added to the protein extracts (200 \(\mu\)g per sample) and incubated for 4 hours at 4\(^\circ\)C with gentle shaking. Then, agarose beads-protein complexes were pelleted by brief centrifugation, washed three times with 1 mL of immunoprecipitation buffer, two times with 1 mL of 20 mM Tris-HCl pH 7.5 buffer and resuspended in 15 \(\mu\)L of the last buffer. Samples were supplemented with 3x concentrated Laemmli sample buffer (Laemmli, 1970), heated at 95\(^\circ\)C for 3 min with vigorous shaking and pelleted by brief centrifugation. The supernatant was analyzed by means of in-gel kinase activity assay (immunocomplex kinase activity assay) using MBP as a substrate.

In-gel kinase and Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent kinase activity assays were performed according to Zhang and Klessig (1997) and Szczegielniak et al. (2012). MAPK kinases phosphorylation
state was analyzed by the use of commercial Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (Cell Signalling Technology) according to procedure recommended by the manufacturer.

Cell death

Cell death was analyzed as previously described (Gauthier et al. 2007) with some modifications. Briefly, seven days-old cells were sub-cultured and incubated for 24 h in Chandler’s medium (Chandler et al. 1972). Then, cell suspensions pre-treated or not with cPTIO were exposed to 100 nM cryptogein. A 0.01% final concentration of neutral red was used as a vital dye accumulating in the acidic vacuole. Cells were observed under the light microscope and considered as dead if not accumulating neutral red. The experiment was repeated three times with five hundred cells counted for each assay.

Microarray analysis

The transcriptomic analysis was performed on wild type tobacco cells. For this purpose, cells pre-incubated or not with 500 µM cPTIO were treated with 100 nM cryptogein for 30 min. Samples were collected from three independent batches and three independent experiments were performed. Filtrated cells were immediately frozen in liquid nitrogen, then RNA were extracted with RNeasy Plant Mini Kit (Qiagen, Courtabeuf, France) and purity and concentrations estimated using Nanodrop1000 (Thermo Fisher Scientific, Waltham, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). RNA samples were hybridized on a 4x44K slides Tobacco Gene Expression Microarray, manufactured by Agilent Technologies (Santa Clara, USA), content sourced from TIGR Release 3, Unigene Build 11, TIGR PlantTA Release 5. The microarray slides contained 43 804 different tobacco test sequences and 1417 control sequences. Samples single color labeling (Low Input Quick Amp Labelling Kit, one color), hybridization (Microarray hybridization oven, Agilent) scanning (High resolution Microarray scanner G2505C, Agilent) and data extraction (Feature Extraction V10 software) were done by the Biopuce GenoToul Plateform (https://genomique.genotoul.fr/). The quintile normalization was done with GeneSpring. Ink 12.0 software (Agilent). To investigate the homogeneity between biological replicates, hierarchical clustering and principal component analysis were performed using the R Gui open-source software (R: A Language and Environment for Statistical Computing. R Core Team. R Foundation for Statistical Computing. Vienna, Austria. 2013. http://www.R-project.org). Differential expression between groups was assessed thanks to moderated paired t-test implemented in Limma package of Bioconductor (Smyth, 2004): genes were selected by Fold Change > 4 and False Discovery Rate (FDR) adjusted p-values < 0.01 taking into account multiple testing using Benjamini and Hochberg (1995) correction. Annotation implementation was performed for the best matched Nicotiana tabacum SGN
mRNA ESTs homologous to microarray sequences after translation to protein sequences. Functional analysis of genes with annotations to *Arabidopsis thaliana* was done with MapMan 3.5.1R2 free software (Max Planck Institute, Munich, Germany).

**qRT-PCR analyses**

RNAs were extracted from liquid nitrogen-preserved cells using Trizol reagent according to the manufacturer’s instructions (Invitrogen, Paisley, UK). Genomic DNA contamination was removed by treatment with DNase 1. The reverse transcription was performed on 500 ng of pure RNA samples using the ImpromII™ Reverse Transcriptase kit (Promega) with anchored oligo (dT15) (Promega) and 0.4 mM deoxynucleotide triphosphates. The resulting cDNAs were diluted ten times with water and 1 μl of each cDNA sample was assayed by qPCR in a ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA) using cGoTaq® qPCR Master Mix (Promega).

Expression levels were calculated relatively to the housekeeping genes *Ntubc2*, *L25* and *EF-1α* (Schmidt & Delaney, 2010) using the relative standard curve method. For each sample, target quantity of the gene of interest was determined by interpolating the value from the standard curve made from a cDNA pool which enables to take into consideration the efficiency of amplification. The value was then divided by the target quantity of the housekeeping gene.

To design primers for verifying the expression of the sequences used on microarray chips, the homolog ESTs were found on the [http://solgenomics.net/](http://solgenomics.net/) webpage by the search for *Nicotiana tabacum* SGN mRNA. All obtained sequences were aligned and contigs of sequences were prepared with Vector NTI Advance 11 (Life Technologies, Carlsbad, USA). Primers were designed as suitable to detect every sequence.

Primer sequences were as follows: *Ntubc2*-fw: 5’-CTGGACAGCAGACTGACATC -3'; *Ntubc2*-rev: 5’-CAGGATAATTTGCTGTAACAGATTA-3’; *L25*-fw: 5’-CCCCTCACCAAGAGTCTGC-3’; *L25*-rev: 5’-AAGGTTGTTGTTGCTCATAATCTT-3’; *EF-1α*-fw: 5’-TGGATGCACCACGAAGC-3’; *EF-1α*-rev: 5’-CCACATTGTACCAGGAAGT-3’; *NtrbohD*-fw: 5’-CTGACGCTAAAGCAGTAACAGATTCAAAAGT-3’; *A_95_P128872*-rev: 5’-CCACAGTCAATACAAAGAGTGATCCACATGC-3’; *A_95_P138477*-rev: 5’-CGGTCCAATTTTGCTGTAACAGATTA-3’; *A_95_P139122*-rev: 5’-CAGAATGGACGCGGCTTAAACATG-3’; *A_95_P11687-1α*-rev: 5’-CAGAATGGACGCGGCTTAAACATG-3’; *A_95_P121687*-rev: 5’-CGGAATTTGCTGTAACAGATTA-3’; *A_95_P139122*-rev: 5’-CCAATTTGCTGTAACAGATTA-3’; *A_95_P139122*-rev: 5’-CGGTCCAATTTTGCTGTAACAGATTA-3’; *A_95_P128872*-rev: 5’-CCACAGTCAATACAAAGAGTGATCCACATGC-3’; *A_95_P138477*-rev: 5’-CGGTCCAATTTTGCTGTAACAGATTA-3’.
CCAAAGATTGGTACAAGAGAACGACATGG-3’;  
NtrbohD-rev:  
5’-

CAGTTTTAAGTTGTCGCTGCAACCACCAAG-3’.

Statistical analysis

Significant differences between treatments were analyzed with Sigma Plot for Windows Version 11.0 (Systat Software Inc., Chicago, USA) by ANOVA test followed by stepwise multiple comparison procedure the Student-Neuman-Keuls (SNK) method (P < 0.05).
RESULTS

Nitric Oxide and ROS production: analysis of mutual regulation

NO production in tobacco cells exposed to cryptogein was monitored using the NO-sensitive fluorophore DAF-2DA as well as with the CuFL fluorescent probe. The DAF-2DA method is indirect and based on the measurement of RNS derived from NO autoxidation that nitrosate DAF-2 to yield to the fluorescent DAF-2 triazole (DAF-2T; Jourd’heuil, 2002). The CuFL dye consists in a fluorescein-based ligand (FL) complexed with Cu(II). NO induces the reduction of Cu(II) to Cu(I), forming NO•, which in turn nitrosates the ligand, thus giving the fluorescent FL-NO compound. CuFL allows the direct detection of NO with nanomolar sensitivity (Lim et al. 2006) and has been successfully used for NO detection in plants (Horchani et al. 2011; Rasul et al. 2012).

As we previously reported (Lamotte et al. 2004; Besson-Bard et al. 2008b), cryptogein triggered an increase in DAF-2T fluorescence which occurred within 10 min of treatment and was maintained for at least 80 min (Fig. 1A). The cryptogein-induced rise in fluorescence was almost completely suppressed by the membrane-permeable NO scavenger cPTIO. Although we cannot exclude the possibility that cPTIO might exert unspecific effects, its ability to act as a powerful NO scavenger has been widely reported (Foissner et al., 2000). Similarly, cryptogein treatment led to a significant increase of CuFL fluorescence (Supporting Information Fig. S1A). Here too, this process was deeply reduced by cPTIO. The effect of cPTIO, as well as the consistent results provided by the DAF-2DA and CuFL methods, further confirmed the ability of cryptogein to induce NO synthesis in tobacco cells.

To investigate the potential interplays between NO and ROS, in a first series of experiments NO synthesis was examined in gp15 cell suspensions. The gp15 cells are transformed with antisense constructs of the NADPH oxidase NtRBOHD, the major enzymatic source for \( \text{H}_2\text{O}_2 \) in cryptogein signaling (Simon-Plas et al. 2002). As shown in Fig. 1B, cryptogein mediated a fast and transient \( \text{H}_2\text{O}_2 \) production in wild-type (wt) tobacco cells which, as expected, was not observed in gp15 cells. Of interest, both \( \text{H}_2\text{O}_2 \) and NO production occurred within few minutes (Fig. 1A and 1B). When NO production was assessed in gp15 cell suspensions, compared to wt cells a reduction of nearly 40% was observed, whatever the dye used for NO detection (Fig. 1C and Supporting Information S1B). This reduction was not due to a lower permeability of gp15 cells to the probe (Fig. S2A). This data suggested that ROS derived from NtRBOHD activity could partly control NO production. To further support this assumption, the effect of the commonly used NADPH oxidase inhibitor diphenyliodonium (DPI) was also tested. DPI is a general inhibitor of flavine oxidoreductases previously shown to inhibit plant NADPH oxidases (Pugin et al., 1997). DPI, which severely blocked the cryptogein-induced \( \text{H}_2\text{O}_2 \) synthesis (Supporting Information Fig. S3), suppressed NO synthesis by...
40%. Although DPI might affect flavine oxidoreductases distinct than NtRBOHD, both the genetic and pharmacological impairment of $H_2O_2$ production partly and similarly impacted the elicitor-triggered NO production.

To better assess the cross-talk operating between NO and $H_2O_2$, the incidence of NO on $H_2O_2$ synthesis was also analyzed. For this purpose, $H_2O_2$ production was measured in tobacco cells exposed to cryptogein in the presence of the NO scavenger cPTIO. Scavenging of NO significantly increased the level of $H_2O_2$ by a 1.5 fold (Fig. 2), suggesting that NO influenced the rate of $H_2O_2$ in tobacco cells facing cryptogein treatment. Besides reinforcing the assumption that NO and $H_2O_2$ are closely linked, this result also indicated that cPTIO did not scavenge $H_2O_2$ as previously reported (Foissner et al. 2000).

To complete this work, the interplay between NO and $H_2O_2$ was also studied in CL5 cell suspensions. This stable silenced cell line is impaired in the expression of NtHD2a and NtHD2b, two redundant isoforms of HDAC acting as negative regulators of cryptogein-induced cell death (Bourque et al. 2011). Interestingly, compared to control cells, following cryptogein treatment the CL5 cells displayed a low production of $H_2O_2$ picking at 20 min (Fig. 3A). The cryptogein-induced NO synthesis was also significantly affected in the CL5 cell line, 50% to 60% of inhibition being observed depending on the method used to assess NO production (Supporting Information Fig. S1C and Fig. 3B, respectively). Here too, we checked that this reduction was not caused by a lower permeability of CL5 cells to the probe (Supporting Information Fig. S2B). Because ROS derived from NtRBOHD appeared to partly control NO production (Fig. 1), the lower NO synthesis measured in CL5 cells was expected. To further explore the functional relationship between NO and ROS, we also examined the level of NtRBOHD transcript in CL5 cells. We found that the accumulation of the corresponding mRNA was not statistically different between control and CL5 lines (data not shown), ruling out the hypothesis that the low $H_2O_2$ production observed in the CL5 line could be related to an impaired expression of NtRBOHD.

Collectively, data from these experiments highlight the occurrence of a functional link between NO and NtRBOHD-derived ROS in tobacco cells elicited by cryptogein. NO production appears to be partly dependent on ROS and NO impacts the level of $H_2O_2$.

**Cryptogein induces a production of peroxynitrite**

We next investigated whether cryptogein could induce a production of ONOO$. The generation of ONOO$ results from the coupling reaction of $O_2$ with NO in its radical form (NO$; Ferrer-Sueta & Radi, 2009). Supporting this initiative, as showed above the productions of NO and ROS derived from NtRBOHD activity occur simultaneously. For this purpose, we used the fluorescent
probe aminophenyl fluorescein (APF). APF reacts preferentially with ONOO\(^-\) and was successfully used to detect this reactive species in plants (Saito et al. 2006; Gaupels et al. 2011b; Begara-Morales et al. 2013). However, it also shows reactions with hypochlorite ("OCl") and hydroxyl radical ("OH; Setsukinai et al. 2003). To check the efficiency of this probe as an ONOO\(^-\) indicator, APF-loaded tobacco cells were first treated with the ONOO\(^-\) donor SIN-1 (Fig. 4A). A significant increase of APF fluorescence reaching a plateau after 2 hours was detected in SIN-1-treated cells. To further confirm that the observed increase in APF fluorescence was caused by ONOO\(^-\), a similar experiment was performed in presence of the ONOO\(^-\) scavenger urate as previously reported (Gaupels et al. 2011b).

Urate strongly suppressed SIN-1-induced rise of fluorescence, providing evidence that APF is a reliable tool to investigate ONOO\(^-\) generation in tobacco cell suspensions. It should be noticed that because urate was dissolved in NaOH, in all the assays cells were supplemented with an equivalent volume of NaOH at a final concentration of 3.3 mM, the pH of the culture media being stabilized at 6.

In this condition, the control cells showed a rise of fluorescence during the experiment (Fig. 4A and 4C).

Next, we applied a similar approach in cryptogein-treated cells. As shown Fig. 4B and 4E, the elicitor triggered a fast and pronounced rise in APF fluorescence which mainly occurred in chloroplasts and, to a lower extend, in the nucleus and along the plasma membrane. This increase was partially sensitive to urate (Fig. 4C), supporting the assumption that cryptogein triggered a production of ONOO\(^-\). The fact that ONOO\(^-\) generation results from the reaction between O\(_2\)\(^{\cdot}\) and NO also pushed us to check its production in gp15 and CL5 cells. As expected, depletion of NtRBOHD expression and, therefore, of O\(_2\)\(^{\cdot}\) production abrogated the elicitor-mediated increase of APF fluorescence (Fig. 4B and 4E). Similarly, only a slight rise in APF fluorescence was measured in cryptogein-treated CL5 cells producing reduced levels of ROS and NO (Fig. 4D). Taken together, these data strongly suggest that a production of ONOO\(^-\) occurs in tobacco cells exposed to cryptogein.

**NtRBOHD-derived ROS regulate the activity of NtOSAK, a member of the SnRK2 protein kinase family**

Previous studies highlighted the ability of cryptogein to induce activation of distinct protein kinases including Ca\(^{2+}\)-dependent protein kinases and mitogen-activated protein kinases (MAPK) including WIPK (wound-induced protein kinase) and SIPK (salicylic acid-induced protein kinase) (Klessig et al., 2000; Dahan et al., 2009). In order to provide a better view of the signaling functions of NO and ROS, their incidence on the regulation of cryptogein-induced protein kinases (PK) was examined. Therefore, protein extracts from wild type tobacco cells treated with cryptogein in the presence or not of cPTIO or from gp15 cells exposed to the elicitor were analyzed for PK activities by
in-gel kinase assays with MPB (myelin basic protein) as a substrate or by the use of commercial antibodies raised against phosphorylated residues of MAPKs.

Compared to wild type cells exposed to cryptogein, activities of the elicitor-induced MAPKs, Ca\textsuperscript{2+}-dependent and -independent kinases were not significantly changed in cells pre-treated with the NO scavenger cPTIO or in gp15 cells (data not shown). These data indicate that neither NO or ROS derived from NtRBOHD contribute to the activities of these PK in cryptogein signaling. To complete this work, we next analyzed the putative involvement of NtOSAK (Nicotiana tabacum Osmotic Stress-Activated protein Kinase), a tobacco serine/threonine protein kinase belonging to the SNF1 (Sucrose Non-Fermenting 1)-Related Kinases type 2 (SnRK2) family (Burza et al., 2006). Previous studies have shown that NtOSAK is rapidly and transiently activated in response to salt and osmotic stresses as well as in response to the toxic metal cadmium (Cd, Burza et al., 2006; Kulik et al., 2012).

Further supporting our interest for this PK, NO donors, H\textsubscript{2}O\textsubscript{2} as well as NO and/or ROS endogenously produced in response to salt and Cd were shown to contribute to NtOSAK activation (Wawer et al., 2010; Kulik et al., 2012). Based on these findings, we first investigated whether cryptogein could trigger the activation of NtOSAK. To check this possibility, proteins extracts from cryptogein-treated wild type tobacco cells were analyzed by immunocomplex-kinase activity assays using antibodies raised against NtOSAK. As shown in Fig. 5, the elicitor triggered a transient activation of NtOSAK, the maximum of activity being observed after 3 and 6 hours of elicitation. Next, NtOSAK activity was assessed in cryptogein-treated wild type cells in presence of cPTIO as well as in gp15 cells. The NO scavenger cPTIO did not affect the activation of this PK (data not shown). In gp15 cells, a slight activity of NtOSAK was already detected before cryptogein addition to the cell suspensions. Importantly, the intensity of its activity was exacerbated during the course of cryptogein treatment while its kinetic of activation was similar to those observed in wild type cells (Fig. 5). This observation suggested that the ROS derived from NtRBOHD might negatively regulate the intensity of NtOSAK activity.

**Identification of NO-regulated genes during cryptogein treatment**

To further study the interplays between NO and ROS, we checked whether NO and ROS could regulate the expression of common genes. First we looked for genes modulated by cryptogein through a NO-dependent process. An expression profiling of tobacco cell suspensions was performed using a tobacco gene expression microarray (Agilent Technologies) consisting of about 44,000 probes with an average length of 60 nucleotides. For this purpose, wt tobacco cell suspensions pre-treated or not for 10 min with the NO scavenger cPTIO were elicited with cryptogein for 30 min. Genes which
corresponding transcripts showed a fold change higher than 4 in response to cryptogein treatment, but lower than 4 in cells co-treated with the elicitor and cPTIO, were selected as NO target genes.

The microarray analysis revealed that compared to non-elicited control cells, 135 microarray probes displayed significant increased labeling with a fold change > 4 after 30 min of cryptogein treatment. In contrast, cryptogein did not trigger the down-regulation of genes. Using annotation tools, 71 of the 135 probes were linked to Arabidopsis thaliana genes (data not shown). Interestingly, in tobacco cells co-treated with cPTIO and cryptogein, among these 135 probes, 35 showed an altered expression, that is a fold change < 4. Fifteen of them matched to A. thaliana genes and, according to our selective criterion, were defined as NO target genes. The list of these genes, as well as their functional classes, are provided in Table 1. About 40 % of these genes encode proteins involved in signaling, notably protein kinases including CIPK 11, a member of the CIPK (Calcineurin B-like (CBL) Interacting Protein Kinase) family. CIPKs are related to yeast sucrose-non-fermenting protein kinases and animal AMP-activated protein kinases. Upon their activation by the Ca\(^{2+}\) sensors CBLs, CIPKs phosphorylate downstream targets and, consequently, mediate Ca\(^{2+}\) signaling (DeFalco et al. 2010). In A. thaliana, CIPK11 was shown to inhibit the activity of the plasma membrane proton pump H\(^{-}\)-ATPase AHA2 by abolishing the binding of 14-3-3 protein through a phosphorylation-dependent process (Fuglsang et al. 2007). According to the authors, CIPK11 is a key actor of the Ca\(^{2+}\)-dependent regulation of plasma membrane H\(^{-}\)-ATPase activity and extracellular acidification. Further supporting a link between NO and Ca\(^{2+}\) signaling, we identified two genes encoding CaM-binding protein including the tobacco orthologue of the A. thaliana transcription factor EDA39 (Embryo sac Development Arrested 39) previously shown to be induced by chitin and by the oomycete Peronospora parasitica (Eulgem et al. 2004; Libault et al. 2007). More generally, the identification of genes related to Ca\(^{2+}\) signaling fits well with our previous findings showing that Ca\(^{2+}\) and NO work together in mediating responses to pathogenic microorganisms and elicitors including cryptogein (Courtois et al. 2008; Rasul et al. 2012; Jeandroz et al. 2013). ZAT6 is another signaling-related gene of interest. In A. thaliana, it encodes a C2H2 zinc finger transcription factor previously shown to be inducible by chitin (Libault et al. 2007) and involved in the regulation of salt and osmotic stress responses (Liu et al. 2013), two stresses promoting NO synthesis (Gould et al. 2003). Besides signaling, three genes encoding proteins putatively involved in protein degradation were also found as NO-responsive genes. The A. thaliana orthologues correspond to PUB26, RHC2A and DUF1 displaying ubiquitin-ligase activities. This latter was shown to be involved in ABA and, once again, in chitin signaling (Libault et al. 2007; Kim et al. 2012). The other NO-dependent genes encode proteins related to hormone metabolism, vesicle transport and development. Intriguingly, only one NO-dependent gene, the tobacco orthologue of NUDX2, encodes a protein related to oxidative stress.
NUDX2 was indeed reported as being an ADP-ribose pyrophosphatase involved in tolerance to oxidative stress in A. thaliana (Ogawa et al. 2009).

Next, we checked whether the NO-dependent cryptogein-induced genes could be also regulated through ROS derived from NtRBOHD. For this purpose, we measured their level of expression by quantitative real-time PCR in wt and gp15 cells exposed to cryptogein for 30 min. Among the 15 NO-dependent genes, 4 were found as being both NO- and ROS-dependent including the tobacco orthologues of CIPK11, RHC2A, PUB26 and DUF1 (Table 2). Indeed, the cryptogein-induced accumulations of the corresponding transcripts were all found to be significantly reduced in cPTIO-treated cells as well as in gp15 cells. In contrast and as an example, expression of the tobacco orthologue of ZAT6 encoding a transcription factor appeared to be NO-dependent but ROS independent as the accumulation of the corresponding transcript was impaired in cPTIO-treated cells and unmodified in gp15 cells. Taken together, these results confirm the data described above indicating that part of the cryptogein-triggered NO production (around 40%) is under the control of NtRBOHD-derived species. Therefore, the NO target genes might be regulated through a ROS-dependent but also a ROS-independent pathway.

Since the expression of the tobacco orthologues of CIPK11, RHC2A, PUB26 and DUF1 appeared to be under the control of both NO and ROS in response to cryptogein treatment, we investigated whether the accumulation of the corresponding transcripts could be also regulated through ONOO⁻ (Fig. 6). When tobacco cell suspensions were pretreated with the ONOO⁻ scavenger urate, the cryptogein-induced accumulation of the transcripts of interest was significantly increased. This data suggested that ONOO⁻ mitigates the level of expression of these genes.

Analysis of NO and ROS involvement in cryptogein-triggered cell death

Nitric oxide and ROS are commonly designed as cell death mediators (Yoshioka et al. 2011). In the present study, we analyzed their respective involvement in the cell death triggered by cryptogein. As previously reported (Bourque et al. 2011), in wt tobacco cell suspensions cryptogein induced a significant cell death reaching 60% after 24 hours (Fig. 7). This percent was reduced to 13% in cells co-treated with the NO scavenger cPTIO, highlighting the involvement of NO, or NO-derived species, in the mechanisms leading to cell death. In gp15 cells, the percent of cell death mediated by the elicitor was reduced as compared to wt cells and reached only 36%, suggesting that ROS also play a role in this process. To better evaluate the contribution of NO in the cell death occurring in gp15 cells in response cryptogein treatment, we checked the effects of NO scavenging. Remarkably, cPTIO did not impact the level of cell death triggered by the elicitor in gp15 cells.
To complete this study, a similar analysis was performed in the CL5 cell line. As stated above, this line does not express NtHD2a and NtHD2b, two HDACs isoforms acting as repressors of cryptogein-induced cell death (Bourque et al. 2011). Furthermore, as described in Fig. 3 and S1, this line displayed a reduced production of NO and a low level of H$_2$O$_2$ when challenged by cryptogein. As shown in Fig. 7B, compared to control cells, the CL5 cells displayed a higher rate of cell death in response to cryptogein, confirming a role for NtHD2a and NtHD2b as negative regulators of cell death. This data also indicates that in contrary to gp15 cells, the reduced production of H$_2$O$_2$ observed in CL5 cells was not correlated to a lower occurrence of cell death. Regarding the incidence of NO on cryptogein-induced cell death in the CL5 line, its scavenging reduced by about 35% the rate of cell death.
In this work we investigated the interplays operating between NO and ROS derived from NtRBOHD activity in tobacco cells elicited by cryptogein. Our results indicated that these species modulate their respective production/levels, interact by forming peroxynitrite and influence the rate of cell death. Furthermore, NO appeared to function independently or in cooperation with ROS to regulate the expression of genes related to signaling and protein degradation amongst other functions.

Deficiency of ROS, through the impairment of NtRBOHD expression in gp15 cells or using DPI, resulted in decreasing NO production by about 40%. Accordingly, the cryptogein-mediated NO synthesis was partly suppressed in CL5 cells producing a low level of ROS. These data highlighted a key role for ROS in modulating NO production. Several studies also provided evidence that ROS act upstream of NO production. For instance, Srivastava et al. (2009) reported that NO synthesis occurs downstream of ROS production in Pisum sativum guard cells treated with chitosan. Similarly, using the NADPH oxidase-deficient double mutant AtrbohD/F, Bright et al. (2006) demonstrated that endogenous H$_2$O$_2$ production is required for ABA-induced NO synthesis in A. thaliana guard cells. In contrast, mechanisms in which NO promotes ROS production were also described. For example, in A. thaliana leaves exposed to oligogalacturonides, NO was shown to positively regulate AtrBOHD-mediated ROS synthesis (Rasul et al. 2012). In our study, the situation is rather complex as NO also controls ROS levels. Indeed, we observed that the scavenging of NO enhanced by a 1.5 fold the concentration of H$_2$O$_2$ in tobacco-elicited cells. Therefore, a bidirectional cross-talk occurs between both species: ROS appear as a step in the signaling cascade leading to NO production which further modulates the rate of H$_2$O$_2$.

The conclusion stated above raises the question of the mechanisms underlying this cross-regulation. Regarding the ROS-dependence of NO production, it has been previously shown that the synthesis of NO mediated by cryptogein requires Ca$^{2+}$ influx from the extracellular space (Lamotte et al. 2004). As H$_2$O$_2$ produced by NtRBOHD was assumed to contribute to this influx by promoting the activation of putative plasma membrane Ca$^{2+}$-permeable channels (Lecourieux et al. 2002), the resulting rise of cytosolic Ca$^{2+}$ could constitute a relay connecting H$_2$O$_2$ to NO. Concerning the regulation of ROS by NO, at least two scenarios might be envisaged. First, the enhancement of H$_2$O$_2$ concentration observed in cPTIO-treated cells following cryptogein exposure could reflect a negative regulation of NtRBOHD by NO. Supporting this hypothesis, Yun et al. (2011) found that NO abolishes AtrBHOD activity through S-nitrosylation of a critical Cys residue. This process destabilizes FAD or precludes its binding to the enzyme. According to the authors, S-nitrosylation of NtRBOHD serves to control ROS generation in cells facing pathogen attack and curbs their deleterious effects leading...
notably to excessive cell death. In a recent proteome-wide analysis aimed at identifying S-nitrosylated proteins in cryptogein signaling (Astier et al. 2012b), we were not able to identify NADPH oxidases as NO protein targets, thus questioning the possibility that NO could mitigate ROS production through NtRBOHD S-nitrosylation. However, a note of caution is required as the method used to selectively identify S-nitrosylated proteins is poorly efficient at targeting transmembrane proteins. Furthermore, NtRBOHD is a low abundant protein, reinforcing the difficulty in identifying this protein. Second, the enhanced H₂O₂ level observed in cPTIO-treated cells could be explained by a coupling reaction of NO and O₂•− leading to ONOO− formation. Indeed, if such mechanism occurs, the scavenging of NO by cPTIO might enhance O₂•− availability and, consequently, H₂O₂ concentration. Supporting this hypothesis, we demonstrated that cryptogein triggered a fast increase of APF fluorescence. This increase was markedly reduced in presence of the ONOO− scavenger urate and severely suppressed in gp15 cells impaired in NtRBOHD expression, confirming the assumption that APF fluorescence is indicative of ONOO− synthesis. More generally, the formation of ONOO− is very fast and the corresponding reaction between NO and O₂•− competes with other routes that consume O₂•−, notably the route involving SOD, its ultimate scavengers (Ferrer-Sueta & Radi, 2009). At least two factors promote its formation: the temporal occurrence of NO and O₂•− and the location of NO and O₂•−. Both criteria are fulfilled in the case of cryptogein signaling. Indeed, NO and ROS are produced simultaneously. Furthermore, similarly to NO (Foissner et al., 2000; Fig. S4), ONOO− was localized in the plastids, slightly in the nucleus and probably in the cytosol along the plasma membrane. Besides this work, several studies provided mounting evidence that ONOO− is generated in plant cells undergoing immune responses (reviewed by Vandelle & Delledonne, 2011). For instance, in their pioneer work, Saito et al. (2006) measured a fast ONOO− production in tobacco BY-2 cells exposed to INF1, the main elicitin secreted by the late blight pathogen Phytophthora infestans. Here too, ONOO− synthesis was detected using APF and the resulting increase of fluorescence was suppressed by urate. More recently, a urate-sensitive ONOO− accumulation was measured in A. thaliana leaves challenged with the avirulent pathogen Pseudomonas syringae pv tomato carrying the AvrB gene (Gaupeles et al. 2011b). The involvement of ONOO− in the plant immune response was further supported by the characterization/identification of proteins regulated by tyrosine nitration, a post-translation protein modification mediated by ONOO− (Saito et al. 2006; Romero-Puertas et al. 2007; Cecconi et al. 2009). According to Gaupeles et al. (2011b), tyrosine nitration might represent a major mean by which cells facing pathogen attack mediate the NO/ROS signal.

To investigate the incidence of NO and/or ROS on cryptogein signaling, we analyzed their respective involvement in cryptogein-induced PK activities and genes expression. The possibility that NO or ROS produced in the context of defense responses regulate the activity of PK has been
previously reported. These PKs include MAPK and CDPK (see for instance Grant et al., 2000; Yoshioka et al., 2011) but not SnRK2s. Through in gel kinase activity assays and western-blotting based approaches, we found that neither NO nor ROS controlled the activity of MAPK and Ca^{2+}-dependent/independent PK (data not shown). In contrast, ROS derived from NtRBOHD negatively regulated the intensity of the SnRK2 PK NtOSAK. Supporting this assumption, NtOSAK activity was remarkably increased in gp15 cells elicited by cryptogein as compared to wild type cells. In contrast, its kinetic of activation was similar in both genotypes. Interestingly, the NO scavenger cPTIO did not impact its kinetic as well as its intensity of activity in cryptogein-treated wild type cells. Collectively, these data highlight a specific action of ROS and not NO on cryptogein-induced NtOSAK activity. Importantly, they differ from previous findings indicating that the activity of this PK is up-regulated by NO and/or ROS in tobacco cells challenged by Cd or salt stresses (Wawer et al., 2010; Kulik et al., 2012). Therefore, although our study confirms the regulation of NtOSAK by ROS, it points out a distinct regulation of this PK by NO and/or ROS according the cellular conditions. More generally, although a role for SnRK2.4, the closest homolog of NtOSAK in A. thaliana, in the regulation of plant tolerance to cadmium has been reported (Kulik et al., 2012), its function in plant defense remains to be determined.

The microarray analysis allowed the identification of 15 early-modulated genes, which induction by cryptogein was suppressed by cPTIO. Because cPTIO reduced the elicitor-induced NO synthesis by almost 70% (Fig. 1), these genes were defined as NO target genes. Accordingly, several A. thaliana orthologues of these genes were also found as being induced by NO (At1G21380, At2G41380, At3G46620, At5G04340 and At5G47070; Parani et al. 2004; Ahlfors et al. 2009; Besson-Bard et al. 2009). Furthermore, the tobacco genes orthologues of At3G46620 and At5G47070 encoding a C3HC4-type RING finger protein and a putative protein kinase, respectively, were shown to be modulated in tobacco leaves infiltrated with the NO donor sodium nitroprusside (Zago et al. 2006). Interestingly, a significant part of these genes encode proteins related to signaling. This observation confirms our previous conclusion, as well as those of other teams, that NO displays a signaling function in plant immunity (see for instance Besson-Bard et al. 2008a; Gaupels et al. 2011b; Bellin et al. 2013; Skelly & Loake, 2013). The identification of the tobacco orthologue of CIPK11, a gene encoding CBL-interacting protein kinase in A. thaliana, is of particular interest. Indeed, as described in the results section, CIPK11 is a critical negative regulator of the plasma membrane H^{+}-ATPase that controls extracellular acidification (Fuglsang et al. 2007). According to these authors, a negative regulation of the plasma membrane H^{+}-ATPase by CIPK11 might be an advantage under conditions where H^{+}-ATPase activity has to be rapidly down-regulated, notably in response to fungal elicitors inducing plasma membrane depolarization and cytoplasmic acidification such as cryptogein (Pugin et al. 1997; Gauthier et al. 2007). Whether the NO-dependent induction of the tobacco
orthologue of CIPK11 is related to the regulation of plasma membrane H+-ATPase in cryptogein-induced effects remains to be investigated but warrants attention. Another NO-dependent gene of interest is the tobacco orthologue of AtNUDX2. The corresponding protein displays ADP-ribose pyrophosphatase activity (Ogawa et al. 2005). In animal cells, free ADP-ribose is a highly reactive compound molecule that causes the nonenzymatic mono-ADP-ribosylation of proteins and contributes to the activation of apoptosis during oxidative stress (Perraud et al. 2005). Ogawa et al. (2009) reported that the overexpression of AtNUDX2 in A. thaliana increased tolerance to oxidative stress caused by salinity or paraquat. This tolerance was correlated to the ability of the enzyme to metabolize potentially toxic ADP-ribose to AMP and ribose 5-phosphate. Here too, understanding the physiological significance of NUDX2 expression in tobacco, as well as its regulation by NO, will require further investigation.

Amongst the NO-dependent genes modulated in response to cryptogein, 4 (the tobacco orthologues of CIPK11, RHC2A, PUB26 and DUF1) were also found as being down-regulated in gp15 cells treated with the elicitor. This finding indicates that NO drives specific but also mutual signaling pathway(s) shared with ROS derived from NtRBOHD activity. A similar conclusion has been raised by Zago et al. (2006) who demonstrated that in tobacco NO and H2O2 act either individually or in partnership in regulating gene expression. Further supporting the involvement of both NO and ROS in regulating the expression of CIPK11, RHC2A, PUB26 and DUF1, we observed that the scavenging of ONOO− significantly increased the cryptogein-triggered accumulation of the corresponding transcripts. This data further support the hypothesis that ONOO− formation mitigates the effects of ROS and NO. It is noteworthy that 3 of the commonly-regulated genes, e.g. the tobacco orthologues of PUB26, RHC2A and DUF1, encode proteins displaying ubiquitin ligase activity. Notably, RHC2A and DUF1 have been characterized as RING domain-containing E3 ubiquitin ligases (Kim et al. 2012). Through their contribution in protein ubiquitination, ubiquitin ligases are main participants in protein degradation pathways (Guerra & Callis, 2012). The observation that NO and ROS commonly regulates genes predominantly related to protein ubiquitination is intriguing and, at this stage, we do not know the physiological meaning of this finding. More generally, this data complete a recent study showing that NO produced in response to cryptogein promotes the S-nitrosylation of the chaperone-like AAA+ ATPase CDC48 (Astier et al. 2012b). In animals and plants, CDC48 is involved in the targeting of ubiquitinylated proteins for degradation by the proteasome (Meyer et al. 2012). Importantly, CDC48 has been shown to be similarly regulated by NO and ROS, both compounds promoting the S-nitrosylation or oxidation on the same cysteine residue, respectively (Noguchi et al. 2005). Therefore, taken together these data suggest that NO and ROS might be part of the regulation processes of the ubiquitin system at the transcriptional and post-translational levels.
We previously provided first evidence that NO is one of the components involved in the mechanisms underlying cryptogein-triggered cell death (Lamotte et al., 2004). In the present study, we took advantage of the gp15 and CL5 cell suspensions to further investigate the role of both NO and ROS in that process. Compared to wild type cells, the reduction of cell death observed in cryptogein-treated gp15 cells indicates that ROS-derived from NtRBOHD activity might contribute to cell death. Concerning NO, in wt cells its scavenging by cPTIO correlated with a 70% reduction in cryptogein-triggered cell death. Scavenging of NO was also efficient in reducing cryptogein-induced cell death in CL5 cells; however, to a lower extend (35% of reduction). This data was expected as those cells generated 50% to 60% less NO as compared to control cells. Thus, the results obtained in wt and CL5 cells favors a role for NO in cell death. In gp15 cells, the finding that cPTIO was inefficient in suppressing the cell death mediated by the elicitor while these cells still generate NO suggests that NO requires NtRBOHD activity for inducing cell death. If so, the lower production of ROS detected in CL5 cells appears to be sufficient to forward NO into the cell death machinery. This hypothesis fits well with previous studies highlighting that NO alone is not able to kill cells buts needs to cooperate with ROS (Delledonne et al. 2001; de Pinto et al. 2002). More precisely, it has been proposed that NO requires well balanced H₂O₂ levels to be channeled into the cell death pathway (Delledonne et al. 2001; Zago et al. 2006). Accordingly, compared to wt cells, the involvement of NO in mediating cell death was minimized but still occurred in CL5 cells producing a low level of ROS.

Inevitably, our data raise the question of how NO and ROS derived from NADPH oxidase promote cell death. In animals, ONOO⁻ is known to act as a cytotoxic effector and mediator of cellular injuries (Ferrer-Sueta et al. 2009). However, such role in cryptogein signaling is doubtful as urate was previously reported to be ineffective in reducing the elicitor-mediated cell death (Lamotte et al. 2004). More generally, it is commonly recognized that ONOO⁻ is not an essential intermediate in the processes underlying plant cell death (Delledonne et al. 2001; Zago et al. 2006). Similarly, the list of genes regulated by both NO and ROS pointed out for a concerted action of both species in the control of genes encoding proteins displaying ubiquitin ligase activities, but did not allow the identification of genes previously reported as being involved in cell death such as Vacuolar Processing Enzymes (Hara-Nishimura et al. 2005). Functional analysis of these genes should provide a first view of the incidence of the synergism between NO and ROS derived from NtRBOHD. Finally, another issue of this study is that NO and ROS are not the sole intermediates of cryptogein-induced cell death. Indeed, the NO scavenger did not completely suppressed cell death in wt cells exposed to cryptogein. Furthermore, scavenging of NO in elicitor-treated gp15 and CL5 cells did not severely abolish the rate of cell death. Similarly, CL5 cells displayed a high rate of cells death while producing a low amount of H₂O₂. Identification of the NO- and ROS-independent intermediates will require further investigation.
To conclude, our study provide a detailed analysis of the interplays occurring between NO and ROS in a physiological context. Based on our data, we proposed the working model

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**TABLE 1. List of cryptogein-early induced genes regulated through a NO-depend process**

Tobacco cell suspensions were pre-incubated with 500 µM cPTIO and then treated with 100 nM cryptogein for 30 min. Genes are sorted according to functional classes and fold changes. Tobacco genes sequences used for microarray analysis were annotated to *A. thaliana* on a base of the best matched SGN EST. FC: Fold change.

<table>
<thead>
<tr>
<th>Probe number</th>
<th>Accession number</th>
<th>Gene name</th>
<th>Functional class</th>
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TABLE 2. List of cryptogein-early induced genes commonly regulated by both NO and ROS

Wild-type and gp15 cell suspensions were pre-incubated or not with 500 µM cPTIO and then treated with 100 nM cryptogein for 30 min. Levels of transcript accumulation was measured by qRT-PCR analysis. The data are presented as ratio of expression between cryptogein-treated and non-treated wt cells (wt), between cryptogein- and cryptogein + cPTIO-treated cells (cPTIO) and between cryptogein-treated and non-treated gp15 cells (gp15). The table presents results from three independent experiments. “-" means values with FC < 4. Statistical analysis (a,b) was done according to ANOVA followed by SNK test (P<0.05).

<table>
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<th>Probe number / Gene name</th>
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<td></td>
<td>wt</td>
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<tr>
<td>A_95_P128872 CIPK11</td>
<td>7.62 (a)</td>
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<td>20.76 (a)</td>
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<td>A_95_P121687 RHC2A</td>
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<td>A_95_P139122 DUF1</td>
<td>5.54 (a)</td>
</tr>
<tr>
<td>A_95_P082790 ZAT6</td>
<td>6.50 (a)</td>
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FIGURE LEGENDS

Figure 1. Cryptogein-induced NO production is partly regulated through a NtRBOHD-dependent process
A. NO production in cryptogein-treated cells. Tobacco cell suspensions were first pre-incubated with 500 µM cPTIO and then treated with 100 nM cryptogein. Graph present the time course of NO production measured using the intracellular NO-sensitive fluorophore DAF-2DA.
B. Time course of H₂O₂ production in cryptogein-treated cells. Wild type and gp15 cells were treated with 100 nM cryptogein. The concentration of H₂O₂ of was measured by luminescence.
C. Impact of NtRBOHD activity on cryptogein-mediated NO synthesis. Wild type and gp15 cells were pre-incubated or not with 5 µM DPI before cryptogein treatment (100 nM). Control cells were pre-treated with an equal volume of DMSO. The production of NO was measured after 40 min of cryptogein treatment using the intracellular NO-sensitive fluorophore DAF-2DA. The production of NO induced by cryptogein in wt cells in the absence of DMSO has been fixed at 100%.
For each figures, each value represents the mean ± SE of nine measurements (three replicates per experiment performed three times). Statistical analysis was performed by ANOVA followed by SNK test (P<0.05).

Figure 2. Incidence of NO on H₂O₂ level in cryptogein-treated cells
Tobacco cell suspensions were first pre-incubated with 500 µM cPTIO and then treated with 100 nM cryptogein. The production of H₂O₂ was measured by luminescence. The production of H₂O₂ induced by cryptogein in the absence of cPTIO has been fixed at 100%.
Each value represents the mean ± SE of fifteen measurements (three replicates per experiment performed five times). Statistical analysis was performed by ANOVA followed by SNK test (P<0.05).

Figure 3. Production of NO and H₂O₂ in CL5 cells elicited by cryptogein
A. Time course of H₂O₂ production in CL5 cells. Wild type and CL5 cells were treated with 100 nM cryptogein. The concentration of H₂O₂ was measured by luminescence.
B. Intracellular NO production in CL5 measured with the DAF-2DA probe. Cells were treated with 100 nM cryptogein for 40 min. The production of NO induced by cryptogein in wt cells has been fixed at 100%.
For each figure, each value represents the mean ± SE of nine measurements (three replicates per experiment performed three times). Statistical analysis was performed by ANOVA followed by SNK test (P<0.05).
Figure 4. Cryptogein triggers a peroxynitrite production in tobacco cell suspensions

A. Analysis of APF responsiveness to ONOO⁻. Wild type cells loaded with 5 μM APF were pre-incubated or not for 5 min with 1 mM of the ONOO⁻ scavenger urate (UA) and then with 2.5 mM SIN-1. Changes of APF fluorescence levels were monitored using a spectrofluorometer.

B. Time course of ONOO⁻ production in response to cryptogein-elicited cells. Wild type and gp15 cells loaded with 5 μM APF were treated with 100 nM of cryptogein. Changes of APF level of fluorescence were monitored as indicated in Fig. 4A.

C. Influence of urate on cryptogein-induced increase in APF fluorescence. Wild-type cells loaded with 5 μM APF were pre-incubated or not 5 min with 1 mM of the ONOO⁻ scavenger urate and then with 100 nM of cryptogein. Changes of APF level of fluorescence were monitored as indicated in Fig. 4A.

D. Cryptogein-induced ONOO⁻ production in CL5 cells. Wild type and CL5 cells loaded with 5 μM APF were treated 40 min with 100 nM of cryptogein. Changes of APF level of fluorescence were monitored as indicated in Fig. 4A. The production of ONOO⁻ induced by cryptogein in wt has been fixed at 100%.

E. Cellular localization of ONOO⁻. Wild type and gp15 cells were treated with cryptogein for 0, 20 and 40 min and stained for 5 min with 15 μM APF. Peroxynitrite localization (green fluorescence) was observed with a confocal laser scanning microscope under magnification 40x. Chloroplast autofluorescence appears in red. The Figure is representative of pictures from six independent replicates with a minimum of 8 cells observed per conditions in each experiment. Scale bar mean 50 μM. Arrows indicate the position of the nucleus.

For figures A-D, each value represents the mean ± SE of nine measurements (three replicates per experiment performed three times). For Fig. D, statistical analysis was performed by ANOVA followed by SNK test (P<0.05).

Figure 5. ROS negatively regulate NtOSAK activity

Seven days-old wild type and gp15 cells were treated with 100 nM cryptogein for up to 9h. After proteins extraction, NtOSAK activity was monitored by immunocomplex-kinase activity assay using specific anti-C-terminal NtOSAK antibodies. Representative results from three independent experiments are presented.

Figure 6. Peroxynitrite mitigated the expression of NO- and ROS-induced genes

Wild-type cell suspensions were pre-incubated or not with 1mM uric acid (UA) or 3.33 mM NaOH for 10 min and then treated with 100 nM cryptogein for next 30 min. Levels of transcript accumulation was measured by qRT-PCR. The data are presented as ratio of expression between cryptogein-treated and not-treated cells; between cryptogein- and cryptogein + UA-treated cells; between
cryptogein- and cryptogein + NaOH treated cells. Graphs present the results from three independent experiments. FC: fold change. Statistical analysis was done according to ANOVA followed by SNK test (P<0.05).

Figure 7. Nitric oxide and ROS involvement in cryptogein-induced cell death
A. Involvement of NO and ROS in cryptogein-induced cell death in wt and gp15 cells. Cell suspensions were pre-treated for 10 min with 500 µM cPTIO before the addition of 100 nM cryptogein. The percentage of dead cells was estimated at the indicated time by staining with neutral red.
B. Involvement of NO and ROS in cryptogein-induced cell death in CL5 cells. Cells were treated as detailed for Fig. 7A.
For both figures, each value represents the mean ± SE of 6 measurements (2 replicates per experiment performed 3 times). Statistical analysis was done by ANOVA followed by SNK test (P<0.05).

Figure 8. Interplay between NO and ROS derived from NtRBOHD activity in cryptogein signaling
The production of NO involves both NtRBOHD-dependent and -independent pathways. In the first one, both NO and ROS derived from NtRBOHD activity regulate a common set of genes including genes encoding proteins displaying putative ubiquitin ligase activities. Furthermore, NO involvement in cell death requires NtRBOHD activity. In turn, NO negatively regulates the level of H2O2, probably by forming peroxynitrite (ONOO−) through its coupling with superoxide (O2•−). In the NtRBOHD-independent pathway, NO regulates the expression of genes which products are related to signaling, hormone metabolism, vesicle transport and development. The regulation of NtOSAK activity in response to cryptogein specifically involves ROS but not NO. The role of NtOSAK is currently unknown.
SUPPORTING INFORMATION

Figure S1. NO production in cryptogein-treated cells tested by CuFL probe
NO production was measured after 40 min of cells treatment with 100 nM cryptogein. The production of NO induced by cryptogein has been fixed at 100%.

A. Nitric oxide production in tobacco cells first pre-incubated with 500 µM cPTIO and then treated with elicitor.
B. Incidence of NtRBOHD activity on cryptogein-mediated NO synthesis. The production of NO was measured in wt and gp15 cells after.
C. Incidence of NtHDAC2a/b on cryptogein-mediated NO synthesis. The production of NO was measured in wt and CL5 cells after.

Each value represents the mean ± SE of nine measurements (three replicates per experiment performed three times). Statistical analysis was performed by ANOVA followed by SNK test (P<0.05).

Figure S2. DEA/NO induced DAF-2T fluorescence in the distinct cell lines
A. Time course of DAF-2T fluorescence in wt and gp15 cell suspensions exposed to the NO donor DEA/NO. Cells loaded with DAF-2DA were treated with 200 µM of DEA/NO or 200 µM DEA as control.
B. Time course of DAF-2T fluorescence in wt and CL5 cell suspensions exposed to the NO donor DEA/NO. Cells loaded with DAF-2DA were treated with 200 µM of DEA/NO or 200 µM DEA as control.

Graphs present one representative experiment of three. Bars mean ± SE (n = 3).

Figure S3. Effect of DPI on cryptogein-induced H$_2$O$_2$ production
Cells were pre-incubated with 5 µM DPI (or equal volume of DMSO) for 5 min before cryptogein supply (100 nM). H$_2$O$_2$ levels were measured by luminescence. Each value represents the mean ± SE of 9 measurements (3 replicates per experiment performed 3 times).

Figure S4. Cellular localization of NO
After filtration, seven days-old cells were equilibrated in H10 buffer for 1 h in 25°C in the dark and then stained for 1 h with 5 µM DAF-2DA. After three washes with fresh buffer, cells were equilibrated for next 30 min in new buffer and treated with 100 nM cryptogein for 0, 20 or 40 min. Cells were observed with a confocal laser scanning with settings and analysis of images as described for APF in Fig. 4E. The Figure is representative of pictures from four independent replicates with a minimum of 8 cells observed per conditions in each experiment Scale bar mean 50 µM. Arrows indicate the position of the nucleus.
Figure 1

A

**DAF-2T fluorescence (rfu)**

- control
- cry
- cPTIO
- cPTIO+cry

B

**H$_2$O$_2$ (nmol/g FM)**

- wt control
- wt cry
- gp15 control
- gp15 cry

C

**DAF2T fluorescence (%)**

- wt control
- wt cry
- wt DPI cry
- wt DMSO cry
- gp15 control
- gp15 cry
Figure 2

H$_2$O$_2$ production (%)
Figure 3
Figure 4
**Figure 5**

**Figure 6**
Figure 7

1277

1278

A

B

Figure 7
Figure 8
Figure S1

A

B

C

CuFL fluorescence (%)

-20

0

20

40

60

80

100

120

140

wt control

wt cry

gp15 control

gp15 cry

wt control

CL5 control

CL5 cry

CuFL fluorescence (%)

0

20

40

60

80

100

120

140

120

100

80

60

40

20

0

b

b

c

c

a

a

c

c

b

b

a

a
Figure S2

Figure S3
**Figure S4**

A  | DAF-2T | chloroplasts | DIC + merge
---|---------|--------------|--------------
0' | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png)
20' | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png)
40' | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png)

B  | DAF-2T | chloroplasts | DIC + merge
---|---------|--------------|--------------
0' | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png)
20' | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png)
40' | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png)