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Nitro-Oleic acid triggers ROS production via NADPH oxidase activation in plants: a pharmacological approach

Short running title: Exogenous application of Nitro-oleic acid triggers ROS production

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Graphical abstract

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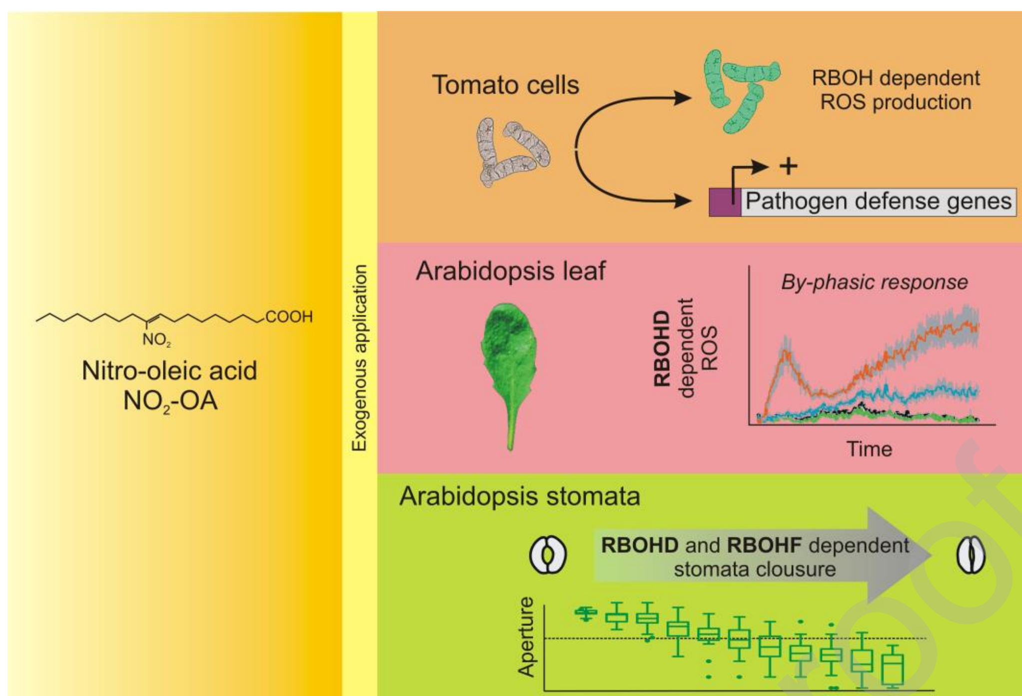

Highlight:

- A pharmacological approach to study the effect of nitrolipids on ROS production and stomatal closure via NADPH oxidases.

ABSTRACT

Nitrated fatty acids (NO_2-FAs) are important signaling molecules in mammals. NO_2-FAs are formed by the addition reaction of nitric oxide- and nitrite-derived nitrogen dioxide with unsaturated fatty acids double bonds. The study of NO_2-FAs in plant systems constitutes an interesting and emerging area. The presence of NO_2-FA has been reported in olives, peas, rice and Arabidopsis. To gain a better understanding of the role of NO_2-FA on plant physiology, we analyzed the effects of exogenous application of nitro-oleic acid (NO_2-OA). In tomato cell suspensions we found that NO_2-OA induced reactive oxygen species (ROS) production in a dose-dependent manner via activation of NADPH oxidases, a mechanism that requires calcium entry from the extracellular compartment and protein kinase activation. In tomato and Arabidopsis leaves, NO_2-OA treatments induced two waves of ROS production, resembling plant defense responses. Arabidopsis NADPH oxidase mutants showed that NADPH isoform D (RBOHD) was required for NO_2-OA-induced ROS production. In addition,

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on Arabidopsis isolated epidermis, NO_2-OA induced stomatal closure via RBOHD and F. Altogether, these results indicate that NO_2-OA triggers NADPH oxidase activation revealing its possible role in plants.

Keywords: nitro-oleic acid, tomato cell suspension, Arabidopsis, ROS, NADPH oxidase, stomatal closure, signaling.

Abbreviations:

•NO_2: nitrogen dioxide

•NO: nitric oxide

FA: fatty acid

H_2O_2: hydrogen peroxyde

NO_2-FA: nitro fatty acids

NO_2-Ln: nitro-linolenic acid

NO_2-OA: nitro-oleic acid

OA: oleic acid

ROS: reactive oxygen species

RBOH: respiratory burst oxidase homologs

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INTRODUCTION

Fatty acids (FA) not only provide structural integrity and energy for various metabolic processes to the plant cell but can also function as signal transduction mediators (Lim *et al.*, 2017). As an example, oxylipins are oxygenated FAs, many of which are electrophilic species involved in plant defense against biotic and abiotic stresses (Farmer & Mueller, 2013; Lim *et al.*, 2017).

Electrophilic nitro-fatty acids (NO_2-FAs) are formed by the addition reaction of nitric oxide ($^{\bullet}NO$)- and nitrite (NO_2^-)-derived nitrogen dioxide ($^{\bullet}NO_2$) to unsaturated fatty acids, in particular those containing conjugated double bonds (Schopfer *et al.*, 2011; Bonacci *et al.*, 2012). Electrophiles contain an electron-poor moiety, conferring attraction to electron-rich nucleophiles that donate electrons to form reversible covalent bonds via Michael additions (Chattaraj *et al.*, 2006). In this regard, the electrophilic reactivity of nitroalkenes facilitates reversible addition reactions with cellular nucleophilic targets (*e.g.,* protein Cys and His residues and reduced glutathione (GSH), Batthyany *et al.*, 2006; Baker *et al.*, 2007). This reactivity supports the post-translational modification of proteins, affecting their distribution and/or function. In addition, NO_2-FA has been reported to act as $^{\bullet}NO$ donors under certain conditions (Schopfer *et al.*, 2005; Gorczynsk *et al.*, 2007; Mata-Pérez *et al.*, 2016a).

The study of NO_2-FAs in plant systems constitutes an interesting and emerging area of investigation. The presence of nitro-conjugated linoleic acid (NO_2-cLA) nitroalkene in plants was first reported in extra-virgin olive oil and linked to the beneficial effects of the Mediterranean diet on human health (Fazzari *et al.*, 2014). In fresh olives, mass spectrometry analysis of acidic-hydrolized protein show presence of NO_2-OA-cysteins adducts (Fazzari *et al.*, 2014). In addition, NO_2-FAs were later detected in Pea (*Pisum sativum*) and Rice (*Oryza sativa*) (Mata-Pérez *et al.*, 2017). Likewise, in cell suspensions and in plants (seeds, seedlings and leaves) of the model plant *Arabidopsis thaliana*, Mata-Pérez *et al.*, (2016b) reported the presence of the nitroalkene nitro-linolenic acid (NO_2-Ln). The level of

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NO_2-Ln was modulated by both developmental stages and abiotic stresses (NaCl, low temperatures, cadmium or wounding). Moreover, transcriptomic analysis (RNA-seq) of Arabidopsis cell cultures treated with exogenous NO_2-Ln showed differential gene expression related to oxidative stress responses as well as up-regulation of several heat shock response genes (Mata-Pérez *et al.*, 2016b). In addition, in Arabidopsis roots and cell suspensions, NO_2-Ln treatments induced $^{\bullet}$NO production (Mata-Pérez *et al.*, 2016c).

Nitric oxide and reactive oxygen species (ROS) are signaling molecules involved in abiotic and biotic stress responses in plants. In this regard, tomato cell suspensions treated with pathogen-derived molecules, called elicitors like xylanase or chitosan displayed increased ROS and •NO production and induced plant-defense gene expression and cell death (Laxalt *et al.*, 2007; Raho *et al.*, 2011). During plant defense, NADPH oxidase activity of the Ca^{2+} and phosphorylation-dependent RBOHD (from respiratory burst oxidase homolog D) is upregulated, leading to increases in ROS production (Kadota *et al.*, 2015). In guard cell signaling, •NO and O_2^- production via RBOHD and F are second messenger formed during the ABA-mediated stomatal closure (García-Mata & Lamattina, 2002; Kwak *et al.*, 2003). Reaction of $^{\bullet}$NO and O_2^- produces peroxynitrite anion ($ONOO^-$), and its conjugated acid, peroxynitrous acid (ONOOH), promoting the generation of nitrogen dioxide which in turn nitrates unsaturated fatty acids, generating nitro lipids (O'Donnell *et al.*, 1999; Freeman *et al.*, 2008).

All major nitro lipids (NO_2-Ln, NO_2-LA and NO_2-OA) share the same electoprophilic center and similar reactivity properties, a characteristic that provides them with common mechanism of action and responses (Baker *et al.*, 2004). In animals, NO_2-OA has long been used as a surrogate to study and understand the regulation, signaling and metabolism of nitrated fatty acids given its additional stability and well developed synthetic routes (Freeman et al., 2008). Therefore, a pharmacological approach was applied to study the effect of exogenous application of NO_2-OA in tomato cell cultures and tomato and Arabidopsis leaves, with a particular focus on plant

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defense responses and stomatal closure.

MATERIALS AND METHODS

Lipids and nitrolipids preparation, application and measurements

NO_2-OA was synthesized and purified as previously described (Woodcock *et al.*, 2013; Bonacci *et al.*, 2011; respectively). The lipid conjugates were loaded on a C18 SPE column pre-equilibrated with 10% methanol and then eluted with methanol. Solvents used for extractions and mass spectrometric analyses were of HPLC grade or higher from Burdick and Jackson (Muskegon, MI). OA was purchased from Nu-Chek Prep (Elysian, MN).

Stock solutions of nitro fatty acids were made fresh in water (on glass containers) to reduce exposure of cell culture to solvents and then treatments were made in plastic containers with MS media. Given that the NO_2-FAs may decompose or absorb to container surface when prepared in water, as has been reported for other fatty acids (Schopfer *et al.*, 2005; Villacorta *et al.*, 2007; Mailman & Rose, 1990) we measured the net concentration of NO_2-OA dissolved in solution to account for losses to glass adsorption as well as decomposition. Only, 50% of NO_2-OA remain in solution (concentrations indicated for the treatments).

The quantification of NO_2-OA solutions was analyzed by HPLC-ESI-MS/MS using gradient solvent systems consisting of water containing 0.1% acetic acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B), and was resolved using a reverse phase HPLC column (100 × 2 mm x 5 μm C18 Luna column; Phenomenex) at a 0.65 ml/min flow rate. NO_2-OA was applied to the column at 30% B (0.3min) and eluted with a linear increase in solvent B (100% B in 14.7min). The NO_2-OA detection was performed using multiple reactions monitoring (MRM) on an AB5000 triple quadrupole mass spectrometer (Applied Biosystems, San Jose, CA) equipped with an electrospray ionization source. MS analyses for NO_2-OA used electrospray ionization in the negative ion mode with the collision gas set at 4 units, curtain gas 40, ion source gas #1 55 and #260, ion spray

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voltage −4500 V, and temperature 600 °C. The declustering potential was −100, entrance potential −5, collision energy −35, and the collision exit potential -18.4. MRM was used for sample analysis of nitrated fatty acids following the nitro group (m/z 46) upon collision-induced dissociation.

Tomato cell suspensions culture conditions

Tomato cell suspensions (*Solanum lycopersicum*, line Msk8) were grown at 25ºC in the dark in MS medium (Duchefa Biochemie, Haarlem, The Netherlands) as previously described (Laxalt *et al.*, 2007). Cells of four-day-old cultures were used for all experiments.

ROS and ˙NO production in cell suspension

Tomato cells (90 µl per well in 96-well microtitre plate, DeltaLab) were treated with 0.5, 5, 12.5, 25 or 50 µM of OA or NO_2-OA for 1, 4, 7 or 17 h. Plates were incubated at 25ºC in darkness. ROS production was detected by incubating cells with 4 µM H_2DCF-DA probe (Ubezio and Civoli, 1994; Molecular Probe, Eugene, OR, USA) during the last hour of each treatment. As an example, for 7 h treatment, at 6 h 4 µM of H_2DCF-DA was added and ROS production was measured as follow. Cells were immediately introduced in Fluoroskan Ascent microwell fluorometer (Thermo Electron Company, Vantaa, Finland) and fluorescence (ex 485nm, em 525nm) was recorded every 2 minutes for 60 minutes. The area under the curve (AUC, accumulated fluorescence) was calculated according to equation showed in Figure S5 and taken as an accumulated florescence value. For ˙NO determination 10 µM DAF-FM-DA was used as a probe (Kojima *et al.*, 1999, Molecular Probe, Eugene, OR, USA) and production was calculated as indicated above for H_2DCF-DA.

For observation of ROS production, 90 µl cells were treated with 50 µM of OA or NO_2-OA for 6 h and then incubated with 4 µM H_2DCF-DA for 1 h and visualized under the epifluorescence microscopy with an excitation filter of 495 nm and a barrier filter of 515 nm according to Gonorazky *et al.*, (2008).

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Hydrogen peroxide determination was carried out by Pyranine quenching assay according to Gonorazky et al., (2008, Pyranine Sigma-Aldrich, St. Louis, MO, USA). Briefly, five-day-old tomato cells were equilibrated in 50 mL assay buffer (5 mM Mes/ NaOH pH 5.7, 175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$) and allowed to equilibrate at 25 °C in the dark for 20 min on a rotary shaker (125 r.p.m.). This procedure was repeated twice after which the cells were allowed to equilibrate overnight with shaking. To measure oxidative burst, aliquots of 75 µL of cells equilibrated in assay buffer were pipetted into a 96-well microtiter plate. Then, 25 µL of a mix composed of assay buffer with 400 µg of pyranine in presence or absence of NO_2-OA or OA at the indicated concentrations. The quenching of pyranine fluorescence because of H_2O_2 production was recorded every 2 min for 60 min using an excitation wavelength of 405 nm and an emission wavelength of 525 nm in a Fluoroskan Acsent microwell fluorometer (Thermo Electron Company, Vantaa, Finland).

In situ hydrogen peroxide production was assayed by DAB staining. Briefly, 100 µl were treated with 50 µM of OA or NO_2-OA for 6 hours and then incubated overnight with 50 µl of 0.2% DAB solution (Sigma-Aldrich) prepared according to Daudi and O`Brien, (2012) and observed under light microscope.

Inhibition assays of ROS production in cell suspension

Tomato cell culture were treated in 96-well microtitre plate (90 µl per well) for 5 h with 50 µM of NO_2-OA and then incubated with different concentrations of NADPH oxidase inhibitor (DPI: 1, 5 or 10 µM, Sigma), calcium channel blocker ($LaCl_3$: 1, 5 or 10 mM, Sigma-Aldrich), extracellular calcium chelator (EGTA: 1, 5 or 10 mM, Sigma-Aldrich), protein kinase inhibitor (staurosporine: 0.2, 1 or 2 µM, Sigma-Aldrich) or $^{\bullet}$NO scavenger (cPTIO: 0.1, 0.5 or 1 mM, Invitrogen, Carlsbad, CA, USA) for an additional hour in presence of 4 µM H_2DCF-DA. Control cells (no treatment, negative control) and NO_2-OA-only treated cells (positive control) were incubated under the same conditions. As additional control, tomato cells were treated

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with 50 µM of OA and the higher concentration of inhibitors/blockers used (Figure S2E). Determination of ROS production was performed as indicated above.

qPCR analysis of gene expression

Tomato cells cultures (3 ml) were treated with 50 µM OA, 50 µM NO_2-OA or equivalent DMSO dilution (0.09% v/v, Merk, Darmstadt, Germany) as a control for 3 or 6 h. Cells were washed with phosphate buffer (pH 7.5, 50 mM), frozen in liquid nitrogen and total RNA was extracted using the Trizol method. cDNA was synthesized according to manufactured instruction using M-MLV enzyme (Invitrogen). Transcripts levels of *SlPR1a*, *SlHSR203J*, *SlPAL,* and *SlACT* (Actin) genes were analyzed by qPCR (StepOne, Thermo). Expression data are expressed as $\Delta\Delta C_t$ and *SlACT* was used a housekeeping gene. Primers used are listed in supplemental Table S1.

Plant material and growth conditions

Seeds from *Solanum lycopersicum*, line "Platense" (El Colono, Mar del Plata, Argentina) were germinated in Petri dish containing filter paper soaked in deionized water. After 5 days at 23ºC-25ºC in the dark, germinated seeds were transferred to soil (soil:pertite, 2:1) under 16h light / 8 h dark regime at 23ºC-25ºC.

Seeds from Arabidopsis (*Arabidopsis thaliana*) line wt Col-0, *rbohD* and *rbohF* mutants (Torres *et al.*, 2002) were germinated in soil (soil:vermiculite:perlite, 3:1:1) and kept at 4°C for 2 d. Then, they were grown at 25°C using 8 h light/16 h dark photoperiod.

ROS Detection in leaf discs

Leaf discs (5mm diameter) from full-expanded 4to and 5to leaf of tomato plants or 4-to 5-week-old Arabidopsis plants were placed on 96-well white plates floating in 200 µl of deionized water overnight at 25ºC without shaking. Water was replaced by 50 µl of 2X working solution (40

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mM luminol, Sigma-Aldrich, and 0.04 mg.mL^{-1} horseradish peroxidase, Sigma-Aldrich) and immediately treated with 50 µl of OA or NO_2-OA 1 µM, 10 µM or 50 µM to achieve final concentration of 0.5 µM, 5 µM or 25 µM. Control leaf disc were treated with 50 µl of water. Luminescence was measured with a luminometer (Thermo Scientific Luminoskan Ascent Microplate) over 6 h taking data every 2 minutes with an integration time of 1 s. For each treatment, 8 to 12 leaf disc from 8 to 12 different plants were used.

To assay EGTA and $LaCl_3$, leaf disc were treated from the beginning of the experiment with different concentrations as indicated in the Figure.

Ion leakage

Ion leakage experiments were performed using leaf disc from Arabidopsis wt Col-0 line from 4- to 5-week-old. Leaf disc were obtained as indicated above. Twelve discs were treated with OA or NO_2-OA at 5 µM, 25 µM or not treated as a control. Upon 6 h of treatment, ion conductivity was measured using a manual conductimeter (HI 8733, Hanna Instrument) as described by Anderson *et al.*, (2015) on a final volume of 18 ml. Assay was repeated 4 times with different plant batches.

Stomatal Assays

The stomatal aperture treatments were performed on epidermal strips excised from the abaxial side of fully expanded 3-weeks old Arabidopsis leaves. After stripping, the epidermal peels were floated in opening buffer (5 mM K-MES, pH 6.1, and 50 mM KCl) for 3 h in the light. The strips were subsequently maintained in opening buffer and exposed to different treatments. After 90 min, stomata were digitized using a Nikon DS-Fi 1 camera coupled to a Nikon Eclipse Ti microscope. Stomatal aperture width was measured on microphotograph using ImageJ analysis software (Schneider *et al.*, 2012).

Real time stomatal assay

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Epidermal strips were fixed to a perfusion chamber; opening buffer was perfused steadily with a flow rate of 1ml/min. The change of the stomatal opening was monitored with a microscope. After 3 h in light, the different reagents were added through the same perfusion line. The pore widths, of single stomata, were measured every two minutes using image analysis program ImageJ (Schneider *et al*., 2012). Opening values were normalized to the aperture values observed at the end of the pretreatment.

Statistical analysis of data

We use non-parametric Kruskal–Wallis test for analysis of tomato cells results. When we observed differences between groups ($p < 0.05$) we perform post hoc Dunn test or control Dunn test as indicated in Figures legend. Analysis of stomata apertures were perform running ANOVA with post hoc Tukey test using $p < 0.01$ as a significant diference. In both cases we use R program (R Core Team 2018)

RESULTS

NO_2-OA induces ROS and plant-defense gene expression in tomato cells

Exogenous aplication of NO_2-Ln to Arabidopsis cell suspensions induce gene expression (Mata-Perez *et al.*, 2016b). Bioinformatics analysis of RNAseq data revealed that a large number of NO_2-Ln-induced genes were related to oxidative stress response (Mata-Pérez *et al*., 2016b). In order to test whether NO_2-FA treatments were able to induce a physiological response, we tested if NO_2-OA could induce ROS production in tomato cell suspensions. As a control, we compared the response to oleic acid (OA), the corresponding non-nitrated fatty acid of NO_2-OA. Figure 1A shows an increase in the fluorescence signal of NO_2-OA-treated cells in a dose-dependent manner with significant differences starting at 7 h and 17 h of incubation. In the case of OA, none of the assayed conditions displayed any change in ROS production (Figure 1A). Fluorescence microscopy of tomato

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cells treated with 50 μM of NO_2-OA for 6 h showed a significant increased in the fluorescent signal (Figure 1B).

In order to validate ROS production in NO_2-OA-treated cells, we used two alternative methodologies. First, the method based on 3,3´-diaminobenzidine (DAB) staining to detect H_2O_2 formation was used (Daudi & O`Brien, 2012). NO_2-OA treated cells showed positive staining with DAB when compared to OA-treated tomato cells (Figure 1C). To further confirm this increase in ROS, H_2O_2 production was analyzed using the pyranine quenching assay (Gonorazky *et al.*, 2008). Figure 2D shows a rapid quenching of pyranine fluorescence in 50 μM NO_2-OA-treated cells. Altogether these results show that NO_2-OA but not OA triggers a dose- and time-dependent production of ROS in tomato cell suspensions.

Previous reports suggest that NO_2-FA could act as $^{\bullet}$NO donors in both, mammals (Lima *et al.*, 2005) and plants (Mata-Pérez *et al.*, 2016a, 2016b), being this one of mechanisms responsible for its physiological responses in cells. To test this hypothesis, tomato cells were treated for 1 and 6 h with NO_2-OA and $^{\bullet}$NO production analyzed using the fluorescent probe DAF-FM-DA. NO_2-OA was unable to induce $^{\bullet}$NO production in tomato cell suspensions at 1 h (data not shown) or 6 h of treatment (Supplemental Figure S1). These results indicate that under our experimental conditions NO_2-OA does not act as a $^{\bullet}$NO donor and/or induce $^{\bullet}$NO production.

In tomato cells, we reported a rapid ROS production associated with the induction of gene expression upon treatments with the fungal elicitor xylanase (Laxalt *et al.*, 2007; Gonorazky *et al.*, 2014b). Figure 2 shows the expression pattern of salicylic acid (SA)-dependent gene *SlPR1a*, a gene marker for hypersensitive response *SlHSR203J* and a jasmonic acid (JA)-dependent gene *SlPAL* upon treatment with NO_2-OA or OA. Gene expression increased significantly at 6 h of NO_2-OA treatment for *SlPR1a* and *SlHSR203J*, whereas PAL expression was increased already at 3 h post-treatment.

NADPH oxidase is involved in NO_2-OA-induced ROS production

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In plants, NADPH oxidase activation during plant defense is a key enzymatic source of ROS formation (Kadota *et al.*, 2015). To specifically evaluate the role of NADPH oxidases as a source of ROS production triggered by NO_2-OA, tomato cell suspensions were treated with the inhibitor diphenyleneiodonium (DPI). DPI treatments have been successfully used previously in cell suspensions and entire plant systems (Piedras *et al.*, 1998; Govrin & Levine, 2000; Orozco-Cárdenas *et al.*, 2001; De Jong *et al.*, 2004). In this regard, Figure 3 shows that the addition of DPI to NO_2-OA-treated cells decreased ROS production in a dose-dependent manner.

NADPH oxidase-dependent ROS production is finely tuned by several signaling components, among them Ca^{2+}, protein kinases and $^{\bullet}NO$-dependent posttranslational modifications (Kadota *et al.*, 2015; Yun *et al.*, 2011). Thus, we used a pharmacological approach to assess the role of these signaling mechanisms on NO_2-OA-induced ROS production. Both, the calcium channel blocker $LaCl_3$ and extracellular calcium chelator EGTA reduced ROS production triggered by NO_2-OA (Figure 3). Thus, we conclude that ROS production in response to NO_2-OA is triggered by Ca^{2+} entry from the extracellular compartment. Furthermore, the protein kinase inhibitor staurosporine decreased NO_2-OA-induced ROS production (Figure 3) highlighting the requirement of phosphorylation events for the NO_2-OA-dependent activation of NADPH oxidase. Finally, incubation of cells with the $^{\bullet}NO$ scavenger cPTIO did not affect NO_2-OA-induced ROS production (Figure 3). In aggregate, our results suggest that $^{\bullet}NO$ is not involved in signaling responses leading to increased ROS formation elicited by NO_2-OA in tomato cell suspensions.

We further studied whether NO_2-OA induced ROS production in tomato and Arabidopsis leaves. Apoplastic ROS levels were quantified by a luminol/peroxidase-based method in leaf discs. A representative experiment is shown in Figure 4A for tomato discs (an additional independent experiment is shown in Figure S2A). NO_2-OA induced a biphasic accumulation of ROS when a 25 μM dose was used, but a late and

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sustained ROS production at 5 μM (Figure 4A). In the case of OA, neither concentrations assayed induced ROS production (Figure S2A).

To confirm the pharmacological evidence that point to the participation of NADPH oxidases as the source of NO_2-OA-dependent ROS production, leaf disc from Arabidopsis wild type or from *rbohD* and *rbohF* single mutant were treated with NO_2-OA. As observed for tomato, wild type Col-0 leaf disc showed a biphasic ROS production at 25 μM NO_2-OA and a late and sustained ROS production at 5 μM (Figure 4B, left panel, additional independent experiments are shown in Figure S2B). Figure 4B shows that RBOHD but not RBOHF was required for NO_2-OA-induced ROS production. Additional independent assays are shown in Figure S2C. At least in our experimental conditions, no cell death was observed after 6 h of NO_2-OA treatment (Figure S3). Finally, as shown in tomato plant cell suspension assays, the use of calcium signaling blockers impacted negatively the ROS production in wt Col-0 Arabidopsis (Figure 4C, additional independent assays in Figure S2D).

NO_2-OA induces stomatal closure via RBOHD and RBOHF

Stomatal closure is a process regulated by a complex signaling network conformed by numeorous second messengers. ROS production is required for stomatal closure in response to different stimuli. To find out whether NO_2-OA regulates stomatal closure, we treated isolate epidermal peels (epidermal strips) from Arabidopsis leaves. Figure 5A shows that NO_2-OA induced stomatal closure in a dose dependent manner whereas OA have no effect.

Stomatal aperture assays shows the aperture of the stomatal population of the epidermal strips at the end of the treatement period. In order to study the dymanics of stomatal closure induction, we performed real time analysis of stomatal closure induction. Figure 5B shows the dynamic of a stomatal closure upon 5 μM of NO_2-OA. OA was used as a control and it is observed again that it has no effect on the induction of stomatal closure.

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The isoforms of RBOH that are expressed in guard cells are the RBOHD and RBOHF. It has been reported that RBOHF is required for ABA-induced stomatal closure, and RBOHD for the stomatal closure in response to pathogens (Kwak *et al.*, 2003; Zhang *et al.*, 2007). Accordingly, we analyzed NO_2-OA-induced stomatal closure in the *rbohD* and *rbohF* mutants. Figure 5C shows that in both *rbohD* and *rbohF* mutants the NO_2-OA-induced stomata closure was unpaired. These results indicated that NO_2-OA induces stomatal closure via NADPH oxidase activation.

DISCUSSION

Lipids function as signaling mediators in various plant processes with an important role in signal transduction. Signaling lipids in plants include a wide range of molecules such as glycerolipids, sphingolipids, fatty acids, oxylipins and sterols that participates in the response to different stresses like temperature, drought, wounding, nutrition starvation and pathogens among others (Wang, 2004). In this regard, NO_2-FAs represent a new class of lipid molecules involved in plant signaling. Sanchez-Calvo *et al*., (2013) proposed them to be novel mediators of $^{\bullet}$NO-dependent signaling pathways and metabolic processes in plant physiology. Later nitro-conjugated linoleic acid (9-NO_2-cLA and 12-NO_2-cLA isomers) were found for the first time in extra-virgin olive oil and NO_2-OA was identified in whole olives adducted to cysteines (Cys-NO_2-OA, Fazzari *et al*., 2014). In addition, NO_2-Ln was detected in Pea, Rice and Arabidopsis. In the later, its levels changed during development and abiotic stress and exogenous application of NO_2-Ln modulates gene expression as well (Mata-Pérez *et al*., 2016b; Mata-Pérez *et al*., 2017).

We first used tomato cells suspensions as a model system to study the effects of nitrolipids on plant physiology. As stated before, almost 50% of nitrolipids is lost during aqueous stock preparation in glass container. In addition, a rapid loss of nitro fatty acid through non-specific absorption to the plastic container during the treatment occurs and is due to the lack of

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fatty acid stabilizing proteins and the ionic strength of the tomato cell suspension media. We have previously measured that the rapid binding to the container walls accounts for up to 65% of the added nitro fatty acid, well in agreement with previous reports showing losses of up to 95% to the plastic container (Mailman & Rose, 1990). Thus, the significant drop in concentration has to be taken into account when considering the seemingly high treatment levels (up to 50 μM) as the effective concentration that the cells are exposed to would most likely not exceed 5 μM. This value is well in line with reports in animal cell cultures where serum proteins, in particular albumin, rapidly stabilize and deliver the nitro fatty acids into cells. Figure S4A show that NO_2-OA is associated to the tomato cells. Analysis of metabolic products of NO_2-OA in treated tomato cells revealed that NO_2-OA is internalized and metabolized (Figure S4B). In this regard, β-oxidation products and nitroalkene reduction products were detected. These metabolites are a consequence of enzymatic reactions that take place in the cytoplasm and mitochondria of cells. NO_2-OA induced ROS production in tomato cell suspension. This observation is in line with enhanced expression of several genes associated with H_2O_2 and ROS responses observed in Arabidopsis cell cultures (Mata-Pérez *et al.*, 2016b). In tomato cell suspensions, ROS burst can lead to the up-regulation of several defense genes (Gonorazky *et al.*, 2014a). Particularly, we have previously demonstrated that upon xylanase, a treatment that induces a ROS burst, there is an induction of plant-defense gene expression (Laxalt *et al.*, 2001; Laxalt *et al.*, 2007; Gonorazky *et al.*, 2008; Gonorazky *et al.*, 2014a). Exogenous addition of NO_2-OA triggered the expression of plant defense response genes (*SlPR1*, *SlHSR203J* and *SlPAL*). Thus, under this condition, NO_2-OA has a role in plant immune responses.

The inquiry of signaling downstream components of NO_2-OA but upstream to ROS production, led us to find that calcium and phosphorylation events are required for ROS production. In plants, Ca^{2+} regulates ROS formation by NAPDH oxidase, through direct interaction with the Ct region of the protein, or by modulation of its activity through the action of CDPks

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(Kadota *et al*., 2015; Sagi & Fluhr 2006). Our results show that ROS production is independent of •NO, and occurs via activation of the NADPH oxidase, which requires Ca^{2+} and phosphorylation events. The presence of both signaling components in plant resembles the signaling pathway described in mammalian cells for NO_2-FAs (Rudolph *et al*., 2010; Zhang *et al*., 2010).

Evidence found in tomato cell suspensions encouraged us to test if similar ROS responses could occur *in planta*. Both, tomato and Arabidopsis leaf discs showed ROS production upon NO_2-OA treatment. NO_2-OA triggered a first wave of ROS production followed by a second sustained formation of ROS, a response similar to LPS treatments in Arabidopsis discs (Shamg-Guan *et al*., 2018) or plant infection with a biotrophic pathogen (Baker & Orlandi, 1995; Lamb & Dixon, 1997). The lower concentration of NO_2-OA used triggers only the second ROS wave. Results obtained using extracellular calcium blockers in Arabidopsis leaf disc indicate that, although all ROS response depends on entry of calcium (since higher concentration of blockers inhibits ROS signal), first ROS wave was more sensible to calcium deficiency than the second ROS wave. These effects could indicate that the concentrations of calcium that promote the first and second ROS production waves are different.

Among NADPH oxidases present in Arabidopsis (10 members, RBOH A to I), specifically RBOHD is responsible for the rapid and strong production of ROS upon the perception of pathogen-associated molecular patterns (PAMPs) (Kadota *et al*., 2015). In guard cells, only RBOHD and F are expressed and are both involved in the regulation of stomatal movement (Sierla *et al*., 2016). RBOHD is required for PAMP-induced stomatal closure, a plant response that restric the microbial entry (Melotto *et al*., 2006; Mersmann *et al*., 2010; Kadota *et al*., 2014; Li *et al*., 2014). ROS production in the ABA-induced response depends mostly on RBOHF activity (Kwak *et al.,* 2003). In leaf discs, we found that biphasic ROS production was completely absent in *rbohD* mutant, indicating that RBOHD was the source of ROS. It is well known that ROS production in one subcellular

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compartment can affect ROS production in another one, as described for the hypersensitive response where apoplastic ROS affect choroplastic ROS production (Shapiguzov *et al.*, 2012). In Arabidopsis leaf discs treated with LPS, the second peak of ROS production was due to chloroplast activity (Shang-Guan *et al.*, 2018). Our results show that RBOHD is required for the first ROS peak, but taking into account the interplay between ROS sources inside the cell, we could not assert whether the second ROS wave was due to RBOHD activity or another source that is downstream of the RBOHD activation. In guard cells, we found that NO_2-OA induces stomatal closure, response that involves both RBOHD and RBOHF. The role of RBOHD and F acting downstream of NO_2-OA suggests that NO_2-FA has the ability to regulate ROS production via NADPH oxidases in stomatal closures responses suggesting NO_2-OA as a putative compoment of ABA and PAMP triggered response.

One mechanism of action of NO_2-FAs involves their reactivity as electrophiles through Michael addition reactions with cellular thiols (Freeman *et al*, 2008). NO_2-FA form adducts with GSH and proteins, thus generating post-translational modifications due to their electrophilic nature (Groeger & Freeman, 2010). We show clear evidence that NO_2-OA trigger ROS produccion through RBOH oxidase activation. RBOH is a plasma membrane protein that contains two EF-hands and several phosphorylation sites that are involved in activation (Suzuki *et al.*, 2011; Sierla *et al.*, 2016). Posttranslational regulation of RBOHD activation involves Ca^{2+} via direct binding to EF hand motifs and phosphorylation by Ca^{2+}-dependent and Ca^{2+}-independent protein kinases (Boudsocq *et al.*, 2010; Kadota *et al.*, 2014, 2015; Li *et al.*, 2014). The Cys890 amino acid residue of RBOHD is susceptible to modification by NO (S-nitrosylation), which reduces the activity of NADPHoxidase during the defense response to *Pseudomonas syringae* (Yun *et al.*, 2011). Another messenger that regulates RBOH in guard cells is phosphatidic acid (PA) that upon production via a phospholipase D, binds to RBOHD and RBOHF to induce ABA-dependent ROS production (Zhang *et al.*, 2009). Phospholipase C 2 associates with

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RBOHD and is required to sustain/reinforce the activity of RBOHD during the perception of the PAMP flagellin (D'Ambrosio *et al.,* 2017). How does NO_2-OA is regulating RBOH activation is not known. We could hypothesize that NO_2-OA form adducts with different proteins that participate in the signaling response leading to RBOH activation. For instance, NO_2-OA could interact directly with PAMPs receptors and/or signalling actors downstream that lead to modification of RBOHD activity. Similar scenarey could be envisage for ABA-induced RBOHF activation. Our group is working actively in solving these questions. Altogether we unravel the role of NO_2-FA in ROS production and RBOH D and F-dependen plant responses, such as plant immunity responses and stomatal closure.

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Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

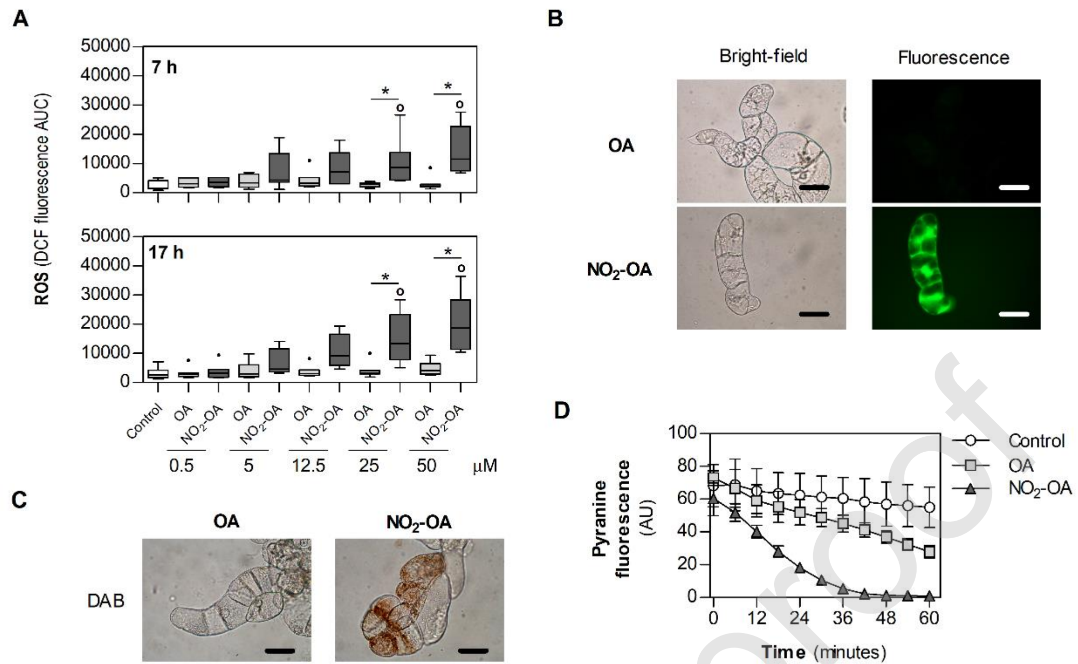

Figure 1. Reactive oxygen species (ROS) production in tomato cell suspensions treated with NO_2-OA.

(A) Tomato cell suspensions were treated with OA or NO_2-OA, or non-treated as a control. At 6 or 16 h of treatment 4 μM H_2DCF-DA was added and the fluorescence was measured for additional 1 h. The fluorescence was determined as the area under the curve (accumulated fluorescence within one hour). Data is represents by box-plot were the box is bound by 25th to 75th percentile, whiskers span follow Tukey method and the line in the middle is median of 4 to 8 independent experiments. * indicate a significant difference ($p<0.05$) between OA and NO_2-OA (nonparametric test Kruskal-Wallis, Dunn test post hoc). ° Indicate significant differences ($p<0.05$) relative to non-treated cells (nonparametric test Kruskal-Wallis, Dunn control test post hoc). Single dots denote outlier values. (B) ROS production on tomato cells suspensions treated for 6 h with 50 μM OA or NO_2-OA and then incubated with 4 μM H_2DCF-DA for 1 h. A representative light and epifluorescence microscope picture of experiments is shown. (C) H_2O_2 detection by DAB stain on tomato cells treated with 50 μM OA or NO_2-OA for 6h. (D) Oxidative burst. Cell suspensions were treated for 6 h with

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50 μM OA or NO_2-OA and then the quenching of pyranine fluorescence was recorded as a measure of the oxidative burst. Data represent media and error standard of 2 independent experiments. Bars= 5 μm in panels B and D.

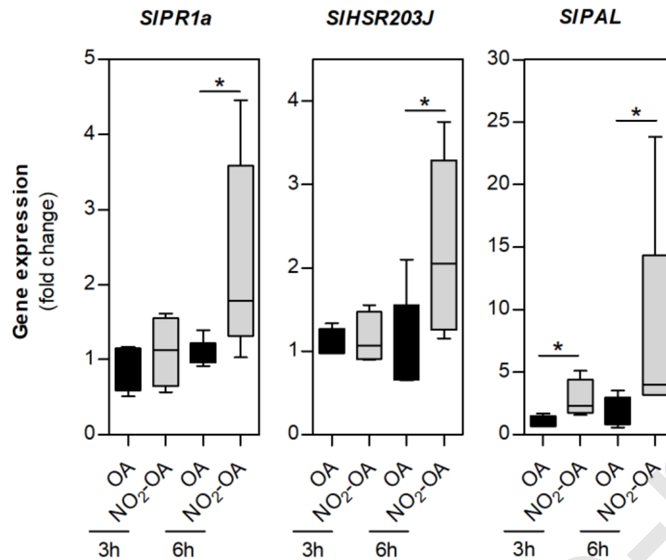

Figure 2. NO_2-OA induces plant defence gene expression.

Tomato cells suspensions were treated with 50 μM OA or NO_2-OA. Cells were incubated for 3 or 6 h and total RNA was extracted. Transcripts levels of *SlPR1a*, *SlHSR203J* and *SlPAL* were analyzed by qPCR. *SlACT* (Actin) was used as a housekeeping gene. Data were analyzed by $\Delta\Delta C_t$ method and fold change was calculated. Data is presented by box-plot were the box is bound by the 25th to 75th percentile, whiskers span follow Tukey method and the line in the middle is median of 4 or 5 experiments. * indicate significant differences ($p<0.05$, nonparametric test Kruskal-Wallis, Dunn test post hoc).

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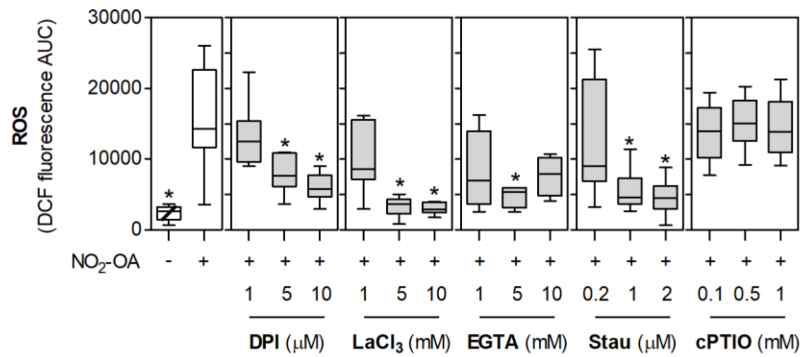

Figure 3. NO_2-OA induced ROS production requires NADPH oxidase, Ca^{2+} and phosphorylation events.

Tomato cell suspensions were incubated with 50 μM NO_2-OA for 6 hours (+) and as control, non-treated cells were incubated the same time (-). To 5 hours NO_2-OA treated cells, different concentrations of NADPH oxidase inhibitor (DPI), calcium channel blocker ($LaCl_3$), extracellular calcium chelator (EGTA), protein kinase inhibitor staurosporine (Stau) or $^{\bullet}NO$ scavenger (cPTIO) were added for another hour. Then, cells were incubated with 4 μM H_2DCF-DA and the accumulated fluorescence was determined. Data is presented by box-plot were the box is bound by the 25th to 75th percentile, whiskers span follow Tukey method and the line in the middle is median of 5 to 10 experiments. * indicates significant differences from NO_2-OA treated cells (nonparametric test Kruskal-Wallis, Dunn control test post hoc).

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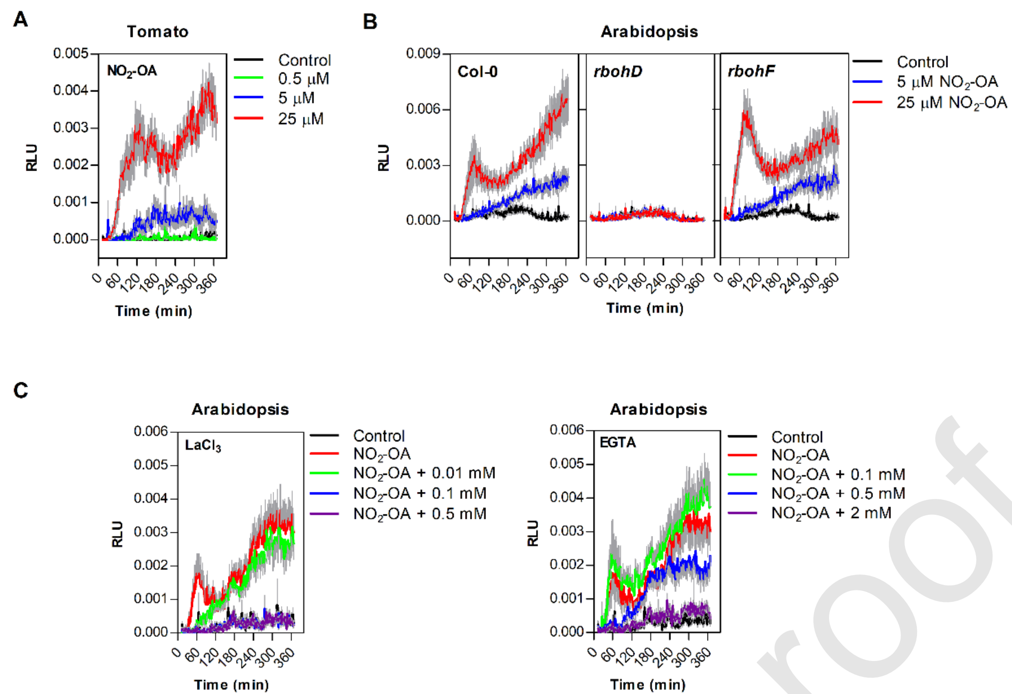

Figure 4. RBOHD and calcium are involved in NO_2-OA-induced ROS production *in planta*

Tomato leaf discs (A) or Arabidopsis Col-0, *rbohD* or *rbohF* leaf discs (B) were treated with different concentration of NO_2-OA and luminescence was measured with a luminometer (Thermo Scientific Luminoskan Ascent Microplate) over 6 h taking data every 2 minutes with integration time of 1 s. (C) Arabidopsis Col-0 leaf disc treated with water or 25 µM NO_2-OA in the presence of different concentrations of $LaCl_3$ or EGTA. ROS production was measured as indicated in (A) and (B).

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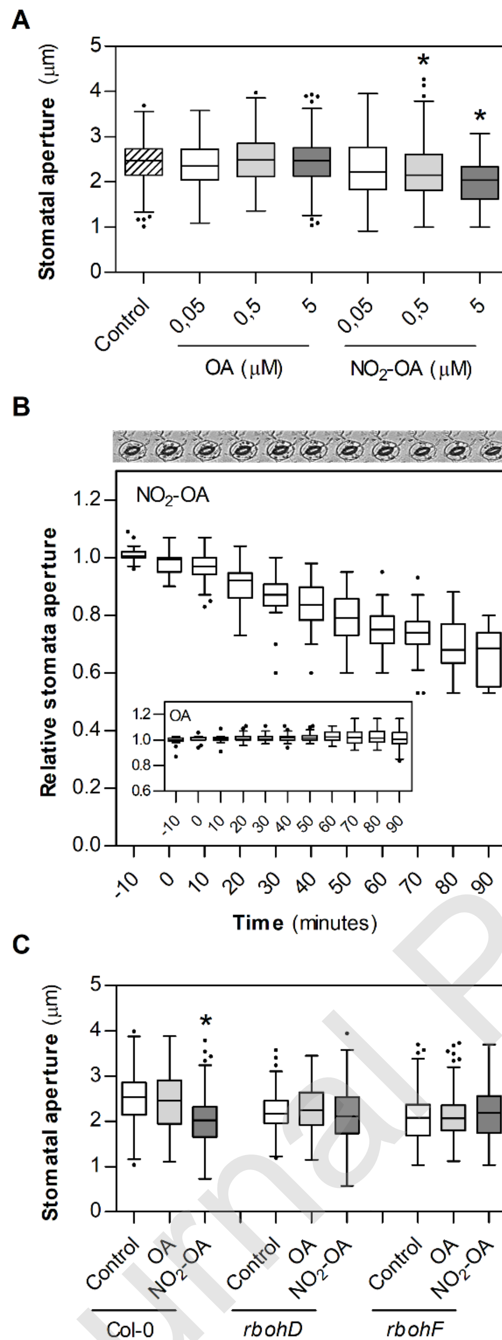

Figure 5. RBOHD and RBOHF are required for NO_2-OA-induced stomatal closure.

Epidermal strips from fully expanded Arabidopsis leaves were (A) pre-incubated for 3 h in opening buffer (5 mM K-MES, pH6.1, and 50 mM KCl) under light and subsequently treated with NO_2-OA or OA for 90 min. (B) Epidermal peels were mounted in a perfusion chamber and perfued with

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opening buffer for 2 h in light (perfusion rate 1ml/min). After pre incubation, 5 μM of NO_2-OA or OA was added to the perfusion buffer and individual stomatal aperture was recorded every two minutes for 90 min. Aperture values were normalized to the stomatal aperture average of each stomata during the last 10 min of pretreatment. (C) Epidermal strips were extracted from *rbohD*, *rbohF* or Col-0 plants, pre-incubated for 3 h in opening buffer and subsequently treated with 5 μM NO_2-OA or OA for 90 min. Epidermal strips were digitized using a digital camera coupled to an inverted microscope and pore width was measured using ImageJ. Aperture values are represents by box-plot were the box is bound by 25th to 75th percentile, whiskers span follow Tukey method and the line in the middle is median of 6 independent experiments. Single dots denote outlier values.

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