REVIEW

Nitro-fatty acids: electrophilic signaling molecules in plant physiology

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Abstract

Main conclusion **Nitro fatty acids (NO2-FA) have relevant physiological roles as signaling molecules in biotic and abiotic stress, growth, and development, but the mechanism of action remains controversial. The two main mechanisms involving nitric oxide release and thiol modification are discussed.**

Abstract Fatty acids (FAs) are major components of membranes and contribute to cellular energetic demands. Besides, FAs are precursors of signaling molecules, including oxylipins and other oxidized fatty acids derived from the activity of lipoxygenases. In addition, non-canonical modified fatty acids, such as nitro-fatty acids (NO_2-FAs), are formed in animals and plants. The synthesis NO_2-FAs involves a nitration reaction between unsaturated fatty acids and reactive nitrogen species (RNS). This review will focus on recent findings showing that, in plants, NO_2-FAs such as nitro-linolenic acid (NO_2-Ln) and nitro-oleic acid (NO_2-OA) have relevant physiological roles as signaling molecules in biotic and abiotic stress, growth, and development. Moreover, since there is controversy on mechanisms of action of NO_2-FAs as signaling molecules, we will provide evidence showing why this aspect needs further evaluation.

Introduction

Nitro-fatty acids (NO_2-FAs) are the product of the reaction between nitric oxide (NO)- and nitrite (NO_2^-)-derived reactive nitrogen species (RNS) and unsaturated fatty acids. The formation of nitro-lipids was initially proposed based on the observation that NO inhibited lipid oxidation propagation reactions (Rubbo et al. 1994). The resulting species remained elusive, but the incorporation of a NO_2 group into linoleic acid was reported (O'Donnell et al. 1999). Nitrated linoleic acid was then reported in humans (Baker et al. 2004; Lima et al. 2002). NO_2-FAs were later detected in plants (Fazzari et al. 2014) and proposed as signal molecules

involved in oxidative stress responses, plant development, and defense responses (Mata-Perez et al. 2016a; Arruebarrena Di Palma et al. 2020; Vollár et al. 2020; Di Fino et al. 2020).

Mechanisms of NO_2-FA formation

The signaling events triggered by NO_2-FAs have been extensively studied, contrasting with the lower number of reports evaluating the formation and characterization of endogenous species. In animals, reactions in the gastric compartment promoted by dietary or salivary NO_2^- are the major contributor to systemic levels (Delmastro-Greenwood et al. 2015). A second mechanism includes NO-driven nitration reactions during inflammation (Bonacci et al. 2012; Vitturi et al. 2013; Villacorta et al. 2018). A third mechanism includes the reaction of NO_2^--derived species during ischemia/reperfusion events. Independent of the mechanism, the nitration of fatty acids occurs through an initial addition of the nitrogen dioxide radical ($^\bullet NO_2$) to a double bond with the formation of a β-nitroalkyl radical (Trostchansky and Rubbo 2008;

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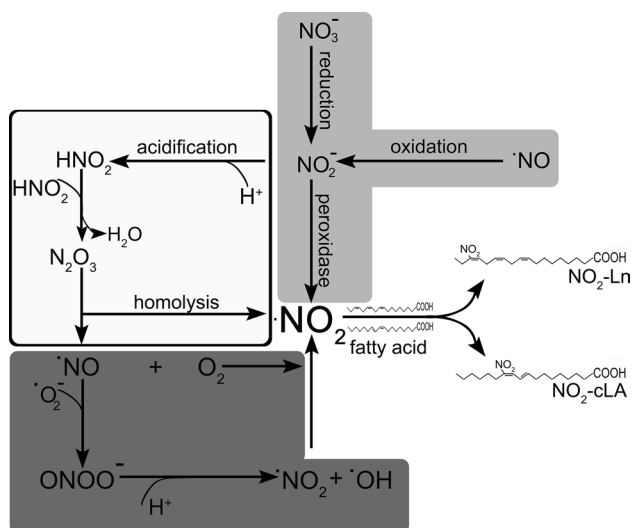   University of Pittsburgh, Pittsburgh, PA, USA

Fig. 1 Role of reactive nitrogen species in fatty acid nitration. Nitration of fatty acids occurs via the addition of $^\bullet NO_2$ (nitrogen dioxide radical) to a double bond. NO-dependent nitration (dark grey boxes). Under oxidative conditions with concomitant formation superoxide anion ($O_2^{\bullet-}$) and $^\bullet NO$, these molecules react at diffusion-limited reaction rates to yield peroxynitrite ($ONOO^-$). Through protonation and then homolysis, $ONOO^-$ produces $^\bullet NO_2$. Under conditions with high local NO concentrations and the presence of oxygen, the auto-oxidation of NO leads to the formation of nitrogen dioxide. Heme peroxidase-catalyzed formation (light grey boxes). NO_2^-, the proximal product of $^\bullet NO$ oxidation and nitrate (NO_3^-) reduction, can be oxidized to $^\bullet NO_2$ in a one-electron oxidation reaction catalyzed by heme peroxidases. Acid-catalyzed formation (white boxes). The protonation of NO_2^- to HNO_2 under acidic conditions (pKa 3.4) leads to its dimerization to form dinitrogen tetroxide ($H_2N_2O_4$), which, upon dehydration, forms N_2O_3. This species is unstable and undergoes homolysis to form $^\bullet NO$ and $^\bullet NO_2$

Schopfer et al. 2019). Figure 1 summarizes the chemical mechanisms of NO_2-FA formation. Thus, the different biological scenarios in which NO_2-FA are formed are defined by the mechanism involved in the formation of $^\bullet NO_2$. This has important consequences, as the different mechanisms involve reaction milieus that differ on hydrophobicity, the rate and level of different nitrogen oxides formation, oxygen tension, and contributions of other oxidants and antioxidants. These factors will modulate the rates, yields, and products formed during the nitration reactions. The main nitration pathways relevant to biological conditions are depicted in different colours in Fig. 1:

- NO-dependent nitration: NO plays a central role in the formation of nitrated fatty acids. Under oxidative conditions with concomitant formation of superoxide anion ($O_2^{\bullet-}$) and $^\bullet NO$, these molecules react at diffusion-limited reaction rates to yield peroxynitrite ($ONOO^-$). Upon protonation to peroxynitrous acid at physiological pH values, ONOO—can undergo homolytic cleavage to form ($^\bullet NO_2$ and $^\bullet OH$). Under conditions with high local $^\bullet NO$ concentrations and the presence of oxygen, the auto-oxidation of NO leads to the formation of nitrogen dioxide.

- Heme peroxidase-catalyzed formation of $^\bullet NO_2$: NO_2^-, the proximal product of $^\bullet NO$ oxidation and nitrate (NO_3^-) reduction, can be oxidized to $^\bullet NO_2$ in a one-electron oxidation reaction catalyzed by heme peroxidases (van der Vliet et al. 1997).

- Acid-catalyzed formation of $^\bullet NO_2$: The protonation of NO_2^- to HNO_2 under acidic conditions (pKa 3.4) leads its dimerization to form dinitrogen tetroxide ($H_2N_2O_4$), which, upon dehydration, forms N_2O_3. This species is unstable and undergoes homolysis to form NO and $^\bullet NO_2$ (Vitturi et al. 2015).

The nitration of fatty acids proceeds through the addition of the $^\bullet NO_2$ radical to a double bond present in fatty acids. This is an extremely fast reaction, but results in an unstable product and the $^\bullet NO_2$ is consequently eliminated with the reformation of the double bond (Pryor et al. 1981; Gallon and Pryor 1993). This futile reaction can lead to double bond isomerization, as demonstrated for arachidonic acid (Balazy and López-Fernández 2003). When the addition reaction occurs on a conjugated double bond, the intermediary product is stabilized by resonance, providing on opportunity for other reactions to occur. Through a series of complex reactions, this radical is finally oxidize to a nitroalkene (Fazzari et al. 2014). Consequently, nitration yields are largely increased ($> 10^5$ fold) in the presence of conjugated double bonds (Bonacci et al. 2012). In plants, similar scenarios can be foreseen; however, it is unclear whether any of the mechanisms mentioned above play a role or if these reactions take place in specific cellular or subcellular compartments.

NO_2-FAs detection

Nitrated fatty acids were initially described in plants as cysteine adducts of nitro-oleic acid (NO_2-OA) (50 pmol NO_2-OA-Cys/g FW) and as conjugated nitro-linoleic acid (NO_2-cLA) (9- and 12-NO_2-cLA isomers), as described in olive fruit and extra virgin olive oil (EVOO), respectively (Fazzari et al. 2014). Later, in *Arabidopsis thaliana,* nitro-linolenic acid (NO_2-Ln) was detected in seeds (11 pmol/g FW), 14-old days seedling (3.8 pmol/g FW) and in leaves of 45-day old plants (0.54 pmol/g FW) (Mata-Pérez et al. 2016a). The same group later reported the presence of NO_2-Ln in rice (*Oryza sativa*) and Pea (*Pisum sativum*). However, some methodological details were missing in the manuscript (e.g., growth plant condition, stage of the plants, techniques for isolation and detection of NO_2-FAs), making the interpretation of the results more difficult (Mata-Pérez

et al. 2017). Recently, another group reported the presence of NO$_2$-OA in *Brassica napus* seeds (1.69 nmol/g FW) and 7-day-old seedling plants (0.66 nmol/g FW) (Vollár et al. 2020). The published data makes it clear that the highest content of NO$_2$-FAs as free fatty acid (non-esterified) is found in seeds, likely due to the high fatty acid content found in seeds (Mata-Pérez et al. 2016a; Vollár et al. 2020). In mammals, the levels of esterified NO$_2$-FAs account for over 95% of total circulating levels, with accumulation in the adipose tissue (Fazzari et al. 2017). Thus, these reports provide support to the presence and regulation of NO$_2$-FAs levels in plants.

Despite these findings, the detection, characterization, and quantification of NO$_2$-FAs in plants have proven extremely challenging. Several factors have contributed to this fact and are analyzed below:

Formation of Michael addition products: NO$_2$-FAs form reversible covalent adducts with nucleophilic amino acids of proteins, particularly cysteine and histidine (Rudolph et al. 2009). The cellular cytoplasm is a highly reducing environment and, in animals, the cysteine content in proteins amounts to 10–50 mM and glutathione (GSH) levels are between 4 and 12 mM (Rudolph et al. 2009; Turell et al. 2013). In animal-derived cells, it has been calculated that approximately 99% of NO$_2$-cLA is adducted, leaving only a small proportion of the molecule free for detection (Turell et al. 2017). Moreover, the proportion of free fatty acids in plant cells is low compared to the total fraction of fatty acid esterified to membranes phospholipids and triglycerides. Thus, to account for the presence of NO$_2$-FA in membranes, investigators treated the lipid extracts with a lipase cocktail (Mata-Pérez et al. 2016a, 2018).

Matrix effects: plant samples are very rich in pigments of lipidic nature, such as chlorophylls and carotenoids, that can affect extraction yields and further reduce detection of NO$_2$-FAs by dampening ionization when measured by liquid chromatography with tandem mass spectrometry (LC–MS/MS) (Mata-Pérez et al. 2018). Although specific experiments to address this problem have not been conducted, the high content of these pigments might be partially responsible for the low concentration detected in pigmented tissues compared to non-pigmented tissues.

Currently, the number of identified nitrated lipid species in plants (NO$_2$-OA, NO$_2$-Ln and NO$_2$-cLA) is smaller than those reported in humans (Salvatore et al. 2020). NO$_2$-cLA is the most abundant NO$_2$-FA detected in human urine and plasma, followed closely by NO$_2$-cLn (Salvatore et al. 2020; Bonacci et al. 2012). In vitro assays revealed that conjugated LA (cLA) yielded 10^5 greater nitration products than LA (Bonacci et al. 2012). The presence of NO$_2$-cLA in olive is intriguing given the scarcity of cLA when compared to other fatty acids (Fazzari et al. 2014). Apart from the cLA isomers, the isomers of α-linolenic acid (Ln) containing conjugated dienes and trienes are the second most prevalent species in humans (Salvatore et al. 2020). Conjugated linolenic acid (cLn) isomers are present at high levels in certain plant species and are the primary natural source of conjugated trienes (Hennessy et al. 2016), providing an interesting and abundant pool of nitration targets. Fatty acids with conjugated triene systems present in plants are mainly represented by (i) eleostearic acid (9-*cis*,11-*trans*,13-*trans*-octadecatrienoic) found in a tung plant *Aleurites fordii* (Takagi and Itabashi 1981) and chinese bitter gourd *Momordica charantia* (Liu et al. 1997); (ii) Calendic acid (*trans*-8,*trans*-10,*cis*-12-octadecatrienoic acid) found throughout the genus *Calendula* (Qiu et al. 2001) and (iii) Punicic acid (*cis*-9,*trans*-11,*cis*-13-octadecatrienoic acid) found in pomegranate seeds (*Punicia granatum*) (Takagi and Itabashi 1981) and in *Trichosanthes*, *Mormodica balsamina, Fevillea trilobata* oil and *Ecballium elaterium* (Hennessy et al. 2016). Thus, the presence of cLn in different plant species makes them attractive candidates for searching and identifying new nitro-fatty acids in plant species.

Mechanisms of NO$_2$-FA action

Nitro-lipids are electrophilic molecules that participate in reversible Michael addition with proteins involved in cell signaling, metabolism, and transcription (Schopfer et al. 2019). The main intracellular targets are soft nucleophiles such as cysteine present in proteins and/or GSH, but proteomic approaches also revealed the formation of histidine adducts (Schopfer et al. 2011). In animals and humans, the mechanism of action of NO$_2$-FAs involves nitro-alkylation, a post-translational modification that regulates stability, activity, and localization of the target proteins (Rubbo and Radi 2008; Rudolph and Freeman 2009; Geisler and Rudolph 2012). Nitro-alkylation is a reversible process with the ability to regulate physiological processes by modulating the activity of transcription factors and enzymatic reactions. In plants, the first evidence of nitro-alkylation was reported in olive fruit, where NO$_2$-OA was found adducted to cysteine residues (Fazzari et al. 2014). The significance of nitro-alkylation reactions in plants was later established by the targeting of cytosolic Ascorbate peroxidase (APX) in Arabidopsis (Aranda-Caño et al. 2019).

Nitro-alkylation resembles the mode of action of reactive electrophile species (RES) produced by plant cells under different stresses (Farmer and Davoine 2007; Farmer and Mueller 2013). Interestingly, transcriptional and physiological responses are shared between RES and NO$_2$-FA. First, the RES-oxilipins OPDA (12-oxophytodienoic acid) (Muench et al. 2016) and electrophilic isothiocyanates (Øverby et al. 2015; Ferber et al. 2020) induced the activation of heat shock factors and transcription of several heat shock (HS)

proteins as described for NO_2-Ln (Mata-Pérez et al. 2016a). In addition, inhibition of primary root growth and cellular cycle was reported for RES-oxylipins, electrophilic isothiocyanates (Almeras et al. 2003; Mueller et al. 2008; Pauwels et al. 2008; Åsberg et al. 2015; Urbancsok et al. 2017, 2018) and NO_2-OA treatments in Arabidopsis (Di Fino et al. 2020). Considering the similarities between these reactive electrophilic species, future studies should focus on evaluating possible common signaling and/or mechanisms of action on plant physiology and divergent activities between these reactive species. More research is necessary to expand our understanding of target proteins and shed light on the signaling pathway in which NO_2-FA participate.

Another mechanism of action reported for NO_2-FAs relates to their ability to act as NO donors. This mechanism was first described in biochemical systems, cell culture, ex vivo and in vitro, and later translated to plant physiology (Schopfer, et al. 2005; Lima et al. 2003; Gorczynski et al. 2007; Mata-Pérez et al. 2016b, c; Grippo et al. 2020; Sánchez-Calvo et al. 2013). Nonetheless, the mechanism(s) whereby NO_2-FAs release NO have not been definitively established and remain controversial. A possible mechanism may include a modified Nef reaction, where, under neutral aqueous conditions, NO_2-FAs form a nitronate anion, that upon a sequence of protonation and deprotonation steps forms a nitroso intermediate characterized by a weak C–N bond that decomposes to release NO (Schopfer, et al. 2005). Using electron paramagnetic resonance (EPR) spectroscopy, NO_2-cLA, NO_2-LA and NO_2-OA have been shown to decompose and generate NO in aqueous solutions. By comparing the EPR signal intensities of spin-adducts, NO_2-cLA generated a significantly greater yield of NO than NO_2-LA and NO_2-OA (Grippo et al. 2020). In vitro, NO_2-LA releases less than 0.1% of NO in an aqueous solution. The NO release was inhibited in the presence of non-ionic detergent micelles as hydrophobic environments stabilize NO_2-LA (Schopfer et al. 2005). Other studies demonstrated that both NO_2-OA isomers yields, under optimal conditions, less than 0.1% of NO, with a 46 times lower release compared to NO donor SNP when using a 50 times higher concentration of NO_2-OA (Gorczynski et al. 2007). In an aqueous solution, NO_2-Ln releases NO with a peak within the first hour but 6 times lower than the rate reported for the NO donor GSNO (Mata-Pérez et al. 2016b; Ederli et al. 2009). The deprotonation of the bisallylic carbon is the first step in releasing NO; consequently, NO_2-Ln and NO_2-LA display higher NO release rates than NO_2-OA. Nonetheless, the rates and yields observed in biochemical systems remain very low. These results clearly show that nitro-alkenes have an objectively lower ability to release NO than SNP or GSNO donors. More importantly, NO_2-FA NO release reactions compete against in vivo alkylation reactions. The high intracellular thiol concentration, the rapid formation of GSH and protein adducts, and the increased stability by incorporation into phospholipids and glycerolipids further reduces their ability to release NO (Schopfer et al. 2019). However, subcellular compartmentalization and/or enzymatic reactions could promote NO release under specific environments and cellular contexts.

NO formation was detected in vivo in Arabidopsis leaves and roots treated with 1 mM NO_2-Ln (Sánchez-Calvo et al. 2013), and in primary root treated with 100 μM of NO_2-Ln (Mata-Pérez et al. 2016c). Curiously, those NO_2-Ln treatments triggered different physiological responses than those triggered by NO donors (further discussed in the next section). A dual response was reported in Brassica seeds treated with NO_2-OA: at 50 μM, NO_2-OA did not release NO; however, at 100 μM, NO_2-OA NO was formed, while 500 μM NO_2-OA did not produce NO (Vollár et al. 2020). In contrast, we failed to detect NO release from Arabidopsis roots or tomato culture cells treated with NO_2-OA (Arruebarrena Di Palma et al. 2020; Di Fino et al. 2020). In aggregate, while all nitro-alkenes are electrophilic and react with cellular nucleophiles, some of them might release NO under specific conditions. The elucidation of the mechanism of action for NO_2-FA under physiological conditions will require considering NO release and Michael addition reactions and incorporate new approaches to dissect the individual contributions of these pathways to the biological response.

Differentiation from NO-dependent activities

In higher plants, NO is involved in a wide range of biological processes, including germination, plant metabolism, senescence, cellular death, stomatal movement, photosynthesis, gravitropism, and primary root growth (Del Castello et al. 2020; Lamattina et al. 2003). Some molecular targets of NO_2-FAs are chemically similar to those described for NO signaling, including redox-sensitive cysteine residues. This signaling crossover complicates the analysis of the responses obtained with NO donors and NO_2-FAs and the evaluation of the mechanism responsible for the biological actions.

NO participates in seed germination regulation by interacting with the ABA signaling during seed dormancy (Liu and Zhang 2009). For instance, pharmacological approaches demonstrated that most known NO donors promote dormancy-releasing and subsequent germination, while NO scavengers favour dormancy (Bethke et al. 2007). It was reported that in *Brassica napus*, NO_2-OA releases NO, which in turn increases germination rate (Vollár et al. 2020). However, this effect was only observed when seeds were treated with 100 μM NO_2-OA, with higher or lower concentrations failing to increase NO levels and affect germination.

Further work will be necessary to elucidate the mechanisms of action and the signaling pathway involved in this process.

NO upregulates APX activity through S-nitrosation of the conserved cysteine 32 residue (Correa-Aragunde et al. 2013; Begara-Morales et al. 2014; Yang et al. 2015). Conversely, in vitro NO$_2$-Ln nitro-alkylate APX's histidine residues inhibiting its enzymatic activity (Aranda-Caño et al. 2019). However, although NO$_2$-Ln has been proposed to act through NO release (Mata-Pérez et al. 2016b), S-nitrosation of the APX cysteine residue upon in vitro NO$_2$-Ln treatments has not been reported (Aranda-Caño et al. 2019). The overlapping activities as an NO donor and alkylation agent highlight the complication of establishing a mechanism of action and remind us to be cautious when considering NO$_2$-Ln exclusively as a NO donor.

NO inhibits primary root growth in *Arabidopsis thaliana* (Fernández-Marcos et al. 2011). Seedlings treated with NO donors show an imbalance in auxin levels (decrease in auxin response reporter DR5:GUS activity) due to NO-dependent alteration of the auxin efflux protein PIN-FORMED1 localization in the cellular membrane of meristematic cells (Fernández-Marcos et al. 2011). Arabidopsis seedlings treated with NO$_2$-OA showed primary root length inhibition without affecting endogenous NO levels and auxin homeostasis (Di Fino et al. 2020). In contrast, NO$_2$-OA reduced root growth by inhibiting the cell cycle in the meristem zone (Di Fino et al. 2020). This provides further evidence that NO and NO$_2$-FAs can induce the same morphological responses through independent signaling pathways.

RNAseq analysis of Arabidopsis cell suspensions treated with NO$_2$-Ln showed that several upregulated genes are related to the heat shock response (Mata-Pérez et al. 2016a). This response was first reported in NO$_2$-FAs-treated endothelial cells (Kansanen et al. 2009). To study whether NO$_2$-Ln modulates gene expression via exclusive NO production, we compared RNAseq data from NO$_2$-Ln (Mata-Pérez et al. 2016a) vs. NO treated Arabidopsis leaves (Hussain et al. 2016) (Fig. 2). Among the most representative shared genes, heat shock protein (HSPs) expression was strongly up-regulated while RNA transcription factors were down-regulated (Fig. 2, a complete set of data is shown in Supplemental Table S1, and a complete list of GO terms for biological processes in Supplemental Table S2). Despite this similarity, it is worth mentioning that only 122 genes are shared between both treatments and that the expression of 194 genes regulated by NO$_2$-Ln was unaffected by the NO treatment under the analyzed conditions. Altogether, the evidence presented above points towards a NO$_2$-FA-specific mechanism of action in different plant physiological systems.
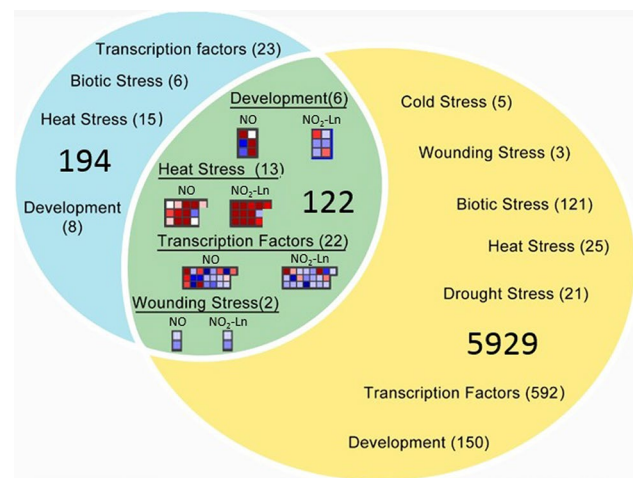

Fig. 2 Overlap of genes whose expression is affected by treatments with NO$_2$-Ln and NO. Venn Diagram showing the overlap of differential expressed genes (*p*-value < 0.05 and log2 ≥ 1 or ≤ − 1) between treatment with NO$_2$-Ln (blue circle) and treatment with NO (yellow circle) was performed with MapMan Software. On the overlapping area are the principal groups of genes shared between the treatments. Those groups of genes were extracted from the BIN analysis performed with MapMan Software. On the unshared area genes related to stress, development and transcription whose expression is only affected in one of the two treatments. A complete list can be found in Supplemental Table 1. RNA seq data was obtained from: Arabidopsis cells treated with from 100 μM of NO$_2$-Ln for 1 h (Mata-Pérez et al. 2016a) and Arabidopsis leaves infiltrated with 1 mM CysNO for 6 h (Hussain et al. 2016)

NO$_2$-OA as regulators of ROS and Ca^{2+}

A recent report showed that exogenous application of NO$_2$-OA induced ROS production on both tomato cell suspensions and tomato and Arabidopsis leaves (Fig. 3, Arruebarrena Di Palma et al. 2020). Pharmacological and reverse genetic experiments showed that NO$_2$-OA-induced ROS were generated specifically by NADPH oxidase RBOH isoform D. The NADPH oxidase RBOHD is responsible for the rapid and robust production of ROS upon pathogen-associated molecular patterns perception (Kadota et al. 2015). In line with the activation of RBOHD, we tested the expression of genes related to pathogen responses observing up-regulation of *SlPR1a*, *SlHSR203J* and *SlPAL* (Arruebarrena Di Palma et al. 2020). Multilayered regulations tightly control RBOHD activity. It is mainly activated via direct binding of Ca^{2+} to EF-hand motifs, by Ca^{2+}-dependent and independent protein kinases, and by the signal lipid phosphatidic acid (PA) (Kadota et al. 2015; Zhang et al. 2009). No Nitric Oxide was detected under these experimental conditions, suggesting that NO$_2$-FA-derived NO was not a primary contributor to the observed biological responses. Moreover, as RBOHD was reported to be negatively regulated
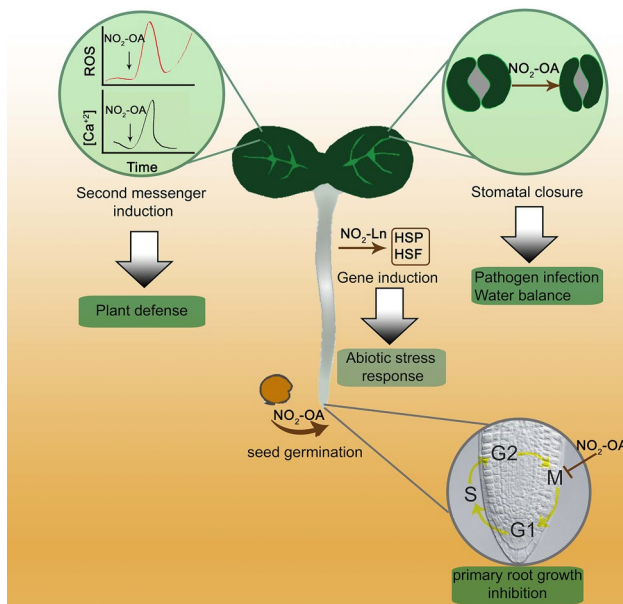
Fig. 3 Schematic representation of the physiological responses induced by NO$_2$-FAs in Arabidopsis. Exogenous application of nitro-oleic acid (NO$_2$-OA) induces both reactive oxygen species (ROS) and cytosolic calcium levels in Arabidopsis leaf discs (Arruebarrena Di Palma et al. 2020). In addition, NO$_2$-OA induces stomatal closure (Arruebarrena Di Palma et al. 2020), seed germination (Vollár et al. 2020), and cell cycle regulation (Di Fino et al. 2020). Another NO$_2$-FA, nitro-linolenic acid (NO$_2$-Ln) induces a specific set of genes that codify for heat shock proteins (HSP) and heat shock factors (HSF) in Arabidopsis cell cultures (Mata-Peréz et al. 2016a). The green box shows the biological process involved. Arrow indicates induction

by NO via S-nitrosation of Cys890 (Yun et al. 2011), a NO role is unlikely as we observed enzymatic activation upon treatment with NO$_2$-OA (Arruebarrena Di Palma et al. 2020). In addition, RBOHD and F were required for NO$_2$-OA-dependent induction of stomatal closure (Fig. 3, Arruebarrena Di Palma et al. 2020). However, the mechanism involved in the NADPH oxidase activation by NO$_2$-OA remains unclear, and it could be hypothesized that NO$_2$-FA form adducts directly with RBOHD protein regulating its activity. Nevertheless, we found that calcium and protein kinase activation are required to induce maximal ROS production upon NO$_2$-OA treatments (Arruebarrena Di Palma et al. 2020). Thus, it might be possible that NO$_2$-FA directly regulate protein kinases and/or Ca^{2+} channels and, therefore, Ca^{2+}-dependent protein kinases.

Cytosolic-free calcium elevation is an early signal in the developmental process and response to abiotic and biotic stresses in plants. Membrane-localized Ca^{2+} permeable ion channels modulate the influx of Ca^{2+}, leading to elevation in cytosolic Ca^{2+} concentrations (Kong et al. 2020). Results obtained in our lab using leaf discs that express the cytosolic calcium sensor aequorin showed that [Ca^{+2}]cyt increases

within 60–90 min in a dose-dependent manner between 5 and 25 μM NO$_2$-OA treatments (unpublished result). The specific target and the mechanistic regulation of NO$_2$-FA on calcium intracellular concentrations have not been explored, but cys residues in target proteins might be key molecular targets. Recently, the H$_2$O$_2$-induced Ca^{2+} increase 1 (HPCA1, a leucine-rich-repeat receptor kinase) was shown to act as an extracellular H$_2$O$_2$ sensor (Wu et al. 2020). Interestingly, HPCA1 contains four Cys residues acting as conserved redox-active sites. The molecular mechanism of H$_2$O$_2$ sensing involves the oxidation of these cysteines that induce a conformational change of the receptor. Nitrolipid targeting and regulation of HPCA1 might hold the key to understanding the relationship between Ca^{2+} and NO$_2$-FA.

Redox regulators as targets of NO$_2$-FAs?

Activation or deactivation of signaling pathways and enzymatic activities mediated by NO$_2$-FA involves the reaction with cysteines of various cellular targets (Schopfer et al. 2019).

A well-described signaling pathway regulated by NO$_2$-FAs in mammals is Nfr2/Keap1 (nuclear factor (erythroid-derived 2)-like 2)/kelch-like ECH-associated protein 1). Keap1 functions as a negative regulator of Nrf2 by keeping Nrf2 in the cytoplasmic compartment, promoting ubiquitination and downstream degradation by the proteasome. Under metabolic and inflammatory stress conditions, the formation of NO$_2$-FA leads to the nitro-alkylation of Keap1 and destabilization of Nfr2-Keap1 complex. This inhibits Nrf2 proteasomal degradation and causes nuclear translocation and accumulation of newly synthesized Nrf2 protein, inducing the gene transcription of antioxidant and detoxification enzymes that protect against oxidative stress, inflammation, and drug toxicity (Magesh et al. 2012; Suzuki et al. 2013; Joshi et al. 2012; Kansanen et al. 2011). The central redox regulator NPR1 (non expressor of pathogenesis-related genes 1) is the plant counterpart of the mammalian Nfr2-Keap1 pathway, with which they share a common redox sensing mechanism (González-Bosch 2018). Both are activated by modifications (oxidation or alkylations) of their thiol groups that promote their nuclear translocation and activation of defense genes. In both pathways, proteasomal degradation prevents their activation and transcription activity (González-Bosch 2018). Arabidopsis NPR1 has 17 cysteines, with 10 of them being highly conserved among different species (Mou et al. 2003). Upon pathogen infection or accumulation of salicylic acid (SA), changes in cellular redox potential lead to the reduction of cysteines through the activity of thioredoxins (TRX-h3 and TRX-h5), promoting NPR1 translocation to the nucleus (Withers and Dong 2016). Unlike Nrf2, NPR1 does not contain a DNA-binding domain

but acts as a transcriptional coactivator by interacting with several transcriptional factors leading to the expression of pathogenesis-related (PR) proteins (Withers and Dong 2016). Indeed, Farmer and Mueller (Farmer and Mueller 2013) proposed NPR1 as a possible target of RES. Considering the similarities between both signaling mechanisms, it will be interesting to focus future research on NPR1 as a possible target of NO_2-FA action in plants.

Another relevant cellular target reported in animals is glutathione (GSH) (Schopfer et al. 2019). Through enzymatic and non-enzymatic reactions, GSH plays an active role in plant cell redox control systems. Glutathione protects cells against oxidative damage induced by environmental challenges (Hernández et al. 2017). The concentration of GSH in Arabidopsis leaf cells is between 0.05 mM in vacuole to near 15 mM in mitochondria (Gill et al. 2013), making it a likely target of NO_2-FA in plants. Exogenous treatment of tomato cell suspension with NO_2-OA results in the formation of GS-NO_2-OA adducts, with a concomitant reduction in GSH cellular concentration (Arruebarrena Di Palma et al. 2018). Also, it was reported that the release of ^{15}N-labeled NO from exogenously applied $^{15}NO_2$-Ln (1 mM) induces the generation of GS^{15}NO (Mata-Pérez et al. 2020). The authors indicated that the formation of NO from NO_2-Ln probably led to the modulation of the intracellular GSH pool, although the exact mechanism of GSNO formation from NO_2-Ln and GSH remains to be elucidated. Moreover, GSH was reported to interplay with different plant hormones like JA, MeJA, SA, and ethylene (Hasanuzzaman et al. 2017). Although GSH has multiple roles in plant physiology, the pathways or mechanisms involved in transducing GSH-induced responses have only been partially addressed (Hasanuzzaman et al. 2017). Given the concentration and reactivity of GSH with NO_2-FA, it would be important to establish the role that adduct formation plays in modulating plant signaling and physiology.

Final considerations

NO_2-FA has emerged as new molecules regulating physiological processes in plants. Different NO_2-FA were identified and detected in a variety of species. Our group has expanded the knowledge about this molecule on plant physiology, studying both their role in development (Di Fino et al. 2020) as well as signaling (Arruebarrena Di Palma et al. 2020) (Fig. 3). Depending on the nitrolipid, the exogenous application may exert a role as an electrophile (NO_2-OA), an NO donor (NO_2-Ln), or both. The identification of 342 protein adducted to NO_2-FA (working as an electrophile) has been reported, but unfortunately, their identities have not

been revealed so far (Aranda-Caño et al. 2019). The study of nitrolipid function in non-model plants and the identification of the cellular targets of NO_2-FA will shed light on its mechanism of action and the plant physiological responses that they might regulate.

Author contribution statement AML and LDF conceived the review. LDF wrote the initial draft. AADP wrote about the physiological role of NO2-FA. EAP performed the RNAseq analysis. CGM, AML and FJS contributed to writing and manuscript editing.

Declarations

Conflict of interest FJS acknowledges financial interest in Creegh Inc. No other competing financial interests are noted.

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