

REVIEW PAPER

Role of electrophilic nitrated fatty acids during development and response to abiotic stress processes in plants

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Abstract

Nitro-fatty acids are generated from the interaction of unsaturated fatty acids and nitric oxide (NO)-derived molecules. The endogenous occurrence and modulation throughout plant development of nitro-linolenic acid (NO₂-Ln) and nitro-oleic acid (NO₂-OA) suggest a key role for these molecules in initial development stages. In addition, NO₂-Ln content increases significantly in stress situations and induces the expression of genes mainly related to abiotic stress, such as genes encoding members of the heat shock response family and antioxidant enzymes. The promoter regions of NO₂-Ln-induced genes are also involved mainly in stress responses. These findings confirm that NO₂-Ln is involved in plant defense processes against abiotic stress conditions via induction of the chaperone network and antioxidant systems. NO₂-Ln signaling capacity lies mainly in its electrophilic nature and allows it to mediate a reversible post-translational modification called nitroalkylation, which is capable of modulating protein function. NO₂-Ln is a NO donor that may be involved in NO signaling events and is able to generate S-nitrosoglutathione, the major reservoir of NO in cells and a key player in NO-mediated abiotic stress responses. This review describes the current state of the art regarding the essential role of nitro-fatty acids as signaling mediators in development and abiotic stress processes.

Keywords: Abiotic stress, gene expression, nitroalkylation, nitro-fatty acids, post-translational modifications, signaling, S-nitrosoglutathione.

Introduction

Fatty acids and lipids are fundamental cellular components that perform essential functions, such as serving as energy reserves or providing structural integrity, and can also act as intracellular and extracellular signal transduction mediators (Hou *et al.*, 2016; Lim *et al.*, 2017). In this regard, the peroxidation

of polyunsaturated fatty acids generates oxidized lipid products called reactive lipid species that exhibit electrophilic capacity and can therefore react with nucleophiles and mediate cell signaling processes (Schopfer *et al.*, 2011; Higdon *et al.*, 2012; Beavers *et al.*, 2017). One particular kind of reactive

lipid species that results from the interaction of unsaturated fatty acids with nitric oxide (NO) and derived species, such as nitrogen dioxide ($\cdot\text{NO}_2$) and peroxyntirite (ONOO^-), are called nitro-fatty acids (NO_2 -FAs), nitrolipids, or nitroalkenes (Schopfer *et al.*, 2011; Buchan *et al.*, 2018; Schopfer and Khoo, 2019). Although the means by which NO_2 -FAs form remains unknown, different mechanisms have been proposed (Fig. 1). The generation of NO_2 -FAs is a non-enzymatic process. NO_2 -FAs are endogenously formed by redox reactions of NO and NO-derived molecules such as ONOO^- , formed through a reaction of NO and superoxide anion ($\text{O}_2^{\cdot-}$), that promote the generation of $\cdot\text{NO}_2$, which in turn nitrates unsaturated fatty acids (Woodcock *et al.*, 2013; Delmastro-Greenwood *et al.*, 2015). Thus, ONOO^- mediates fatty acid nitration by oxidation via homolysis of peroxyntirous acid (ONOOH) to $\cdot\text{NO}_2$ and $\cdot\text{OH}$. Furthermore, ONOO^- also reacts with CO_2 to form nitrosoperoxocarbonate (ONOOCO_2), and this compound can undergo homolytic scission to form $\cdot\text{NO}_2$.

Nitration of fatty acids takes place when a NO_2 group is added to an alkyl chain. At present, two mechanisms are considered responsible for the nitration of unsaturated fatty acids (Buchan *et al.*, 2018; Schopfer and Khoo, 2019). The first involves a direct radical-radical reaction between $\cdot\text{NO}_2$ and an alkyl radical, and has no biological relevance (Buchan *et al.*, 2018). The nitration reactions of unsaturated fatty acids that have biological relevance consist of the direct addition of $\cdot\text{NO}_2$ to the double bond to generate a carbon-centered radical. This radical can react with another $\cdot\text{NO}_2$ molecule to form an unstable nitro-nitrite or dinitro compound that will decompose, releasing HNO_2 and forming an electrophilic NO_2 -FA. These molecules contain an electrophilic carbon, and proteins and

low-molecular-weight thiols can be reversibly adducted by the Michael addition reaction to the β -carbon in a process called nitroalkylation.

In animals, NO_2 -FAs have been related to anti-inflammatory and antioxidant processes (Cui *et al.*, 2006; Ferreira *et al.*, 2009; Kansanen *et al.*, 2009; Freeman *et al.*, 2018; Rom *et al.*, 2018; Schopfer *et al.*, 2018; Schopfer and Khoo, 2019), and have been proposed to act as signaling molecules in the kidney (Jobbagy and Tan, 2018) and in cardiovascular diseases (Mollenhauer *et al.*, 2018) and breast cancer (Woodcock *et al.*, 2018). Oral administration of nitro-oleic acid (NO_2 -OA) to rats and dogs has shown that this electrophilic nitrated fatty acid is more abundantly esterified in complex lipids, especially triacylglycerides (Fazzari *et al.*, 2015, 2019). NO_2 -FAs can then be released by the action of lipase and can thus mediate signaling processes in remote tissues (Buchan *et al.*, 2018). As a result of their electrophilic capacity, a significant proportion of these molecules is adducted with nucleophiles, mainly proteins (Schopfer *et al.*, 2009). In this line, the presence of an electron-withdrawing nitro ($-\text{NO}_2$) substituent in β -carbon confers these molecules potent electrophile reactivity and, consequently, the capacity to react with nucleophiles such as cysteine (Cys) and histidine (His) residues by the reversible post-translational modification (PTM) called nitroalkylation (Batthyany *et al.*, 2006; Rudolph *et al.*, 2009; Aranda-Caño *et al.*, 2019). This process can therefore modulate the function of the adducted protein and even affect its structure or subcellular localization (Baker *et al.*, 2007; Rudolph *et al.*, 2009). To act as a molecular cue, the modification has to be transient and reversible. Accordingly, NO_2 -FAs can be

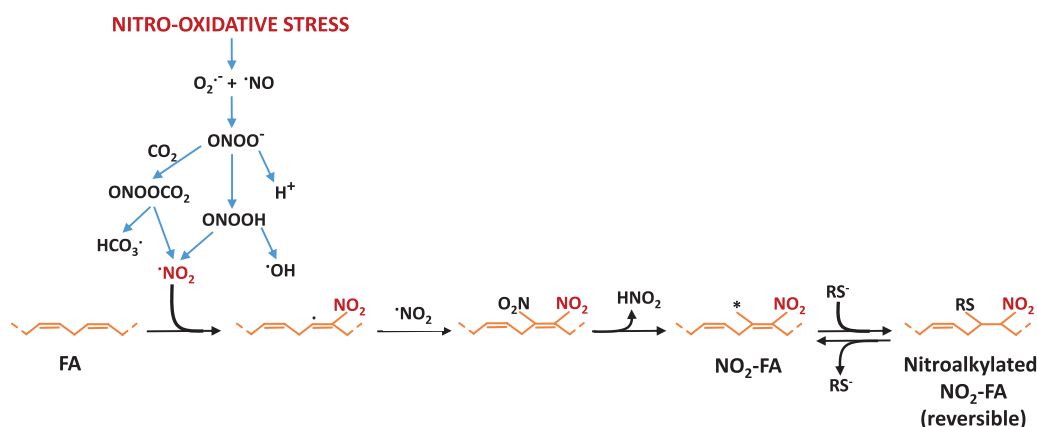


Fig 1. Nitro-fatty acid (NO_2 -FA) formation. Under nitro-oxidative stress conditions, NO_2 -FAs are endogenously formed by redox reactions of nitric oxide (NO) and NO-derived molecules such as peroxyntirite (ONOO^-), formed through a reaction of NO and superoxide anion ($\text{O}_2^{\cdot-}$), that promote the generation of nitrogen dioxide ($\cdot\text{NO}_2$), which in turn nitrates unsaturated fatty acids (FAs). ONOO^- mediates nitration of FAs by oxidation via homolysis of peroxyntirous acid (ONOOH) to $\cdot\text{NO}_2$ and $\cdot\text{OH}$. ONOO^- also reacts with CO_2 to form nitrosoperoxocarbonate (ONOOCO_2), which can undergo homolytic scission to form $\cdot\text{NO}_2$. Nitration of FAs takes place when a NO_2 group is added to an alkyl chain. The main nitration reaction of FAs consists of the direct addition of $\cdot\text{NO}_2$ to the double bond to generate a carbon-centered radical. This radical can react with another $\cdot\text{NO}_2$ molecule to form an unstable nitro-nitrite or dinitro compound that will decompose, releasing HNO_2 and forming an electrophilic NO_2 -FA. These molecules contain an electrophilic carbon (*), and protein and low-molecular-weight thiols can be reversibly adducted by the Michael addition reaction to the β -carbon in a process called nitroalkylation.

released from an adducted protein by a significant rise in the levels of hydrogen peroxide (H_2O_2) and $ONOO^-$ generated in a nitro-oxidative stress situation (Padilla *et al.*, 2017). This is particularly important because the anti-inflammatory and antioxidant signaling properties of these molecules determined in mice and endothelial human cells (Cui *et al.*, 2006; Ferreira *et al.*, 2009; Kansanen *et al.*, 2009) have been attributed to free NO_2 -FAs. In this regard, NO_2 -OA and nitro-linoleic acid (NO_2 -LA) have been proposed to be capable of modulating the function of the proteins PPAR- γ , NF κ B, and Nrf2/Keap1 by nitroalkylation and, consequently, of promoting anti-inflammatory responses in animals (reviewed by Schopfer *et al.*, 2011; Delmastro-Greenwood *et al.*, 2014; Schopfer and Khoo, 2019).

Another signaling pathway of NO_2 -FAs is related to their well-characterized capacity to act as NO donors in animals and plants (Schopfer *et al.*, 2005; Gorczynski *et al.*, 2006; Mata-Pérez *et al.*, 2016a; Vollár *et al.*, 2020). It has been proposed that NO_2 -FAs release NO in its radical form by two possible pathways based on a modified Nef reaction or a rearrangement of nitroalkene to a nitrite ester (Lima *et al.*, 2005; Schopfer *et al.*, 2005). However, the exact mechanism by which NO_2 -FAs release radical NO remains to be elucidated. A good electrophilic capacity allows these molecules to rapidly react with nucleophiles and, consequently, the ability to release NO has been considered secondary to NO_2 -FAs (Batthyany *et al.*, 2006; Baker *et al.*, 2007; Schopfer *et al.*, 2009). Nevertheless, the

NO released from NO_2 -FAs has been described as a modulator of key cellular processes. For instance, NO_2 -LA mediates the S-nitrosation of CD40 that leads to its inactivation and the triggering of an anti-inflammatory response in rats and in human leukemia cells (Faine *et al.*, 2010). So, the possibility that these molecules perform important cellular signaling functions via NO release cannot be ruled out. Indeed, it has recently been described how nitro-linolenic acid (NO_2 -Ln) is able to release NO (Mata-Pérez *et al.*, 2016b), synthesize S-nitrosoglutathione (GSNO) from glutathione (GSH), and modulate GSNO levels *in vitro* and *in vivo* in Arabidopsis plants (Mata-Pérez *et al.*, 2020).

Although the presence and biological functions of NO_2 -FAs have been well established in animals, knowledge about these molecules in plants is still scarce. To date, NO_2 -Ln and NO_2 -OA have been endogenously detected in different plant species (Table 1). In a first study, nitro-conjugated linoleic acid (NO_2 -cLA) was detected in extra virgin olive oil (Fazzari *et al.*, 2014). Later, NO_2 -Ln occurrence was described during development and stress-response situations in Arabidopsis plants (Mata-Pérez *et al.*, 2016c), where it mediates defense mechanisms (Mata-Pérez *et al.*, 2016c, 2017; Aranda-Caño *et al.*, 2019). Subsequently, NO_2 -Ln was also detected in different organs and subcellular compartments of other plant species, such as *Pisum sativum* and *Oryza sativa* (Mata-Pérez *et al.*, 2017). The endogenous presence of NO_2 -OA has recently been described in *Brassica napus* (Vollár *et al.*, 2020).

Table 1. Endogenous concentration and biological function of NO_2 -FAs detected in different plant species

Plant species	Organ/subcellular fraction	NO_2 -FA in planta	Concentration (pmol g ⁻¹ FW)	Biological function	References
<i>Arabidopsis thaliana</i>	Seeds	NO_2 -Ln	11.18	Induction of HSR and anti-oxidant response	Mata-Pérez <i>et al.</i> (2016c)
	14-day-old seedlings	NO_2 -Ln	3.84		
	30-day-old leaves	NO_2 -Ln	0.36		
	45-day-old leaves	NO_2 -Ln	0.54		
	9-day-old ACSC	NO_2 -Ln	0.28		
<i>Pisum sativum</i>	Roots	NO_2 -Ln	0.072	ND	Mata-Pérez <i>et al.</i> (2017)
	Mitochondria	NO_2 -Ln	0.084		
	Peroxisomes	NO_2 -Ln	0.282		
<i>Oryza sativa</i>	Leaves	NO_2 -Ln	0.748	ND	Mata-Pérez <i>et al.</i> (2017)
<i>Brassica napus</i>	Seeds	NO_2 -OA	1700	Induce seed germination	Vollár <i>et al.</i> (2020)
	2-day-old seedlings	NO_2 -OA	370		
	7-day-old seedlings	NO_2 -OA	626		
	7-day-old shoots	NO_2 -OA	347		
	7-day-old roots	NO_2 -OA	426		
<i>Solanum lycopersicum</i>	Cell culture	NO_2 -OA ^a	ND	Activation of NADPH oxidase Induction of the stomatal closure via NADPH oxidase D and F	Di Palma <i>et al.</i> (2020)
<i>Arabidopsis thaliana</i>	Leaves	NO_2 -OA ^a	ND		
<i>Arabidopsis thaliana</i>	Epidermis	NO_2 -OA ^a	ND		
<i>Arabidopsis thaliana</i>	5-day-old seedlings	NO_2 -OA ^a	ND	Inhibition of primary root growth by inhibition of mitotic process	di Fino <i>et al.</i> (2020)

ACSC, Arabidopsis cell suspension cultures (modified from Vollár *et al.* (2020); FW, fresh weight; ND, not determined.

^a Not detected *in planta*, but in a pharmacological approach using exogenous treatment with NO_2 -OA.

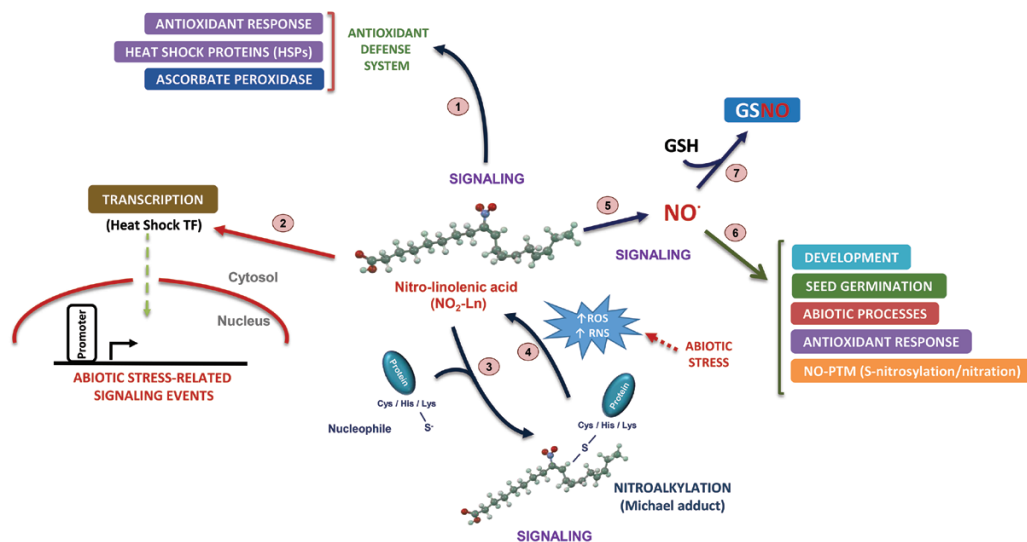


Fig. 2. Biological actions of nitro-linolenic acid ($\text{NO}_2\text{-Ln}$) in plant cells. $\text{NO}_2\text{-Ln}$ is able to prompt the expression of the chaperone network and antioxidant systems, such as ascorbate peroxidase, which can mediate stress-related responses in plants (pathways 1 and 2). $\text{NO}_2\text{-Ln}$ can regulate protein function via nitroalkylation (pathway 3), a post-translational modification that can be reverted by uncontrolled ROS and RNS overproduction generated in a stress situation (pathway 4). $\text{NO}_2\text{-Ln}$ acts as a NO donor (pathway 5) and can therefore be involved in NO-mediated signaling events (pathway 6). Finally, NO released from $\text{NO}_2\text{-Ln}$ is able to generate GSNO, the major NO reservoir in cells, *in vivo* (pathway 7). Therefore, $\text{NO}_2\text{-Ln}$ contributes to the maintenance of NO storage in cells.

This endogenous presence in different plant species highlights the ubiquitous distribution of $\text{NO}_2\text{-FAs}$ in the plant kingdom (Mata-Pérez *et al.*, 2017). Besides this *in vivo* detection, different pharmacological approaches have suggested that $\text{NO}_2\text{-OA}$ triggers the production of reactive oxygen species (ROS) in tomato (*Solanum lycopersicum*) and Arabidopsis leaves (di Palma *et al.*, 2020) and inhibits root growth (di Fino *et al.*, 2020).

Based on this background, the analysis of the function of $\text{NO}_2\text{-FAs}$ in plants is a novel emerging area that has made significant progress in the past 5 years. This review describes the current state of the art concerning the essential role of $\text{NO}_2\text{-FAs}$ as signaling mediators in plant biology.

Modulation of electrophilic nitrated fatty acid levels during plant development

The presence of $\text{NO}_2\text{-FAs}$ and their potential signaling events has been well addressed in animals in the past decade (Schopfer and Khoo, 2019). By comparison, this area has been studied only relatively recently in plants, where a growing body of evidence indicates a key role of these electrophilic molecules in plant biology (Fig. 2). First, $\text{NO}_2\text{-FAs}$ were detected in extra virgin olive oil and fresh olives (Fazzari *et al.*, 2014) from mature olive trees. Conjugated nitro-linoleic acid ($\text{cNO}_2\text{-LA}$) was detected in extra virgin olive oil, and its presence has been linked to the anti-inflammatory properties of olive oil, an essential component of the Mediterranean diet (Fazzari *et al.*, 2014). More recently, a significant advance has been made

with regard to the presence and potential signaling capacity of $\text{NO}_2\text{-FAs}$ in plants.

In a pioneer study in the field of NO in plants, Mata-Pérez *et al.* (2016c) quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) the endogenous occurrence of $\text{NO}_2\text{-Ln}$ in 9-day-old cell cultures and 14-day-old seedlings of the model plant *Arabidopsis thaliana* (Table 1). The presence of other $\text{NO}_2\text{-FAs}$, such as $\text{NO}_2\text{-OA}$ and $\text{NO}_2\text{-LA}$, was also investigated, and it was concluded that they were not present in Arabidopsis (Mata-Pérez *et al.*, 2016c). This result is associated with the major presence of the precursor of $\text{NO}_2\text{-Ln}$, linolenic acid, in Arabidopsis, relative to the content of oleic and linoleic acids (Mata-Pérez *et al.*, 2016c). Then, to investigate the potential role of $\text{NO}_2\text{-Ln}$ in plants, a first analysis of its modulation throughout plant development was performed. $\text{NO}_2\text{-Ln}$ content decreases during development and senescence, with higher concentrations detected in seeds and the lowest concentrations in 30-day-old and 45-day-old leaves of Arabidopsis (Table 1) (Mata-Pérez *et al.*, 2016c). A similar result has recently been reported in *B. napus*, in which Vollár *et al.* (2020) analyzed the endogenous presence of $\text{NO}_2\text{-OA}$, as this plant exhibits a high content of its precursor oleic acid. In this study, the highest content of $\text{NO}_2\text{-OA}$ was also observed in seeds, with a ~4-fold reduction in 2-day-old seedlings (Table 1). Interestingly, the range of concentrations detected in the two studies differed, with $\text{NO}_2\text{-OA}$ being present at a concentration ~150-fold higher in *Brassica* seeds in relation to the concentration of $\text{NO}_2\text{-Ln}$ in Arabidopsis seeds. The results of these studies suggest a potential role of $\text{NO}_2\text{-FAs}$ as a signaling molecule in the first stage of plant development. In fact, $\text{NO}_2\text{-FAs}$ have been

proposed to act as NO donors (Lima *et al.*, 2005; Schopfer *et al.*, 2005; Górczynski *et al.*, 2006; Mata-Pérez *et al.*, 2016a; Vollár *et al.*, 2020), and it is well known that NO strongly influences seed germination (Gibbs *et al.*, 2014; Albertos *et al.*, 2015; Castillo *et al.*, 2015; Wang *et al.*, 2015; Signorelli and Considine, 2018). Therefore, future research should identify targets of NO₂-FAs during seed germination to look for NO₂-FA-mediated NO and nitroalkylation signaling pathways.

In order to extend the analysis of NO₂-FAs to other plant species, different organs and subcellular compartments of *P. sativum* and *O. sativa* were analysed (Table 1). Similarly to Arabidopsis, NO₂-Ln was the only NO₂-FA detected in pea roots and rice leaves (Table 1) (Mata-Pérez *et al.*, 2017). NO₂-Ln was also detected in different subcellular compartments, such as mitochondria and peroxisomes of pea plants (Table 1) (Mata-Pérez *et al.*, 2017). The presence of only NO₂-Ln in these plant species suggests the ubiquitous presence of this NO₂-FA in the plant kingdom. Furthermore, the exogenous application of NO₂-OA to Arabidopsis seedlings inhibits primary root growth and cell division, probably by interfering with the mitotic process (di Fino *et al.*, 2020). On the other hand, NO₂-OA was detected in *B. napus*, but other NO₂-FAs, such as NO₂-Ln and NO₂-LA, were not investigated in this species (Vollár *et al.*, 2020). Nevertheless, to extend our knowledge about whether NO₂-FAs are universal in plants or whether these molecules show a plant-specific presence, future research will be needed to determine the roles of different NO₂-FAs across plant species. In this vein, the studies of di Palma *et al.* (2020) and di Fino *et al.* (2020) were unable to detect endogenous NO₂-OA in Arabidopsis and tomato, which suggests that this type of NO₂-FA might not be present in these plants, as previously observed by Mata-Pérez *et al.* (2016c, 2017) for Arabidopsis.

Nitro-fatty acids mediate pleiotropic signaling events during abiotic stress in plants

Plants are continuously exposed to various environmental attacks that can affect their viability and therefore impair crop

yields. One of the main challenges in plant science is understanding how plants cope with these difficult situations, in an attempt to limit the negative impact of abiotic stresses on their productivity. Different signaling molecules, such as ROS and reactive nitrogen species (RNS), have been postulated to act as mediators of redox signaling events that lead to plant responses to stress, with Cys residues acting as molecular sensors of abiotic stress-related signaling processes (Begara-Morales *et al.*, 2016). Given that NO₂-FAs are NO donors and are able to modulate Cys residues via nitroalkylation, and that NO₂-FA-mediated anti-inflammatory and antioxidant responses in animal systems have been described (Nadtochiy *et al.*, 2009; Schopfer *et al.*, 2009; Rudolph *et al.*, 2010), the potential role of these signaling molecules in plant responses to adverse environmental conditions has been investigated. As a result, increased NO₂-Ln has been observed in response to different abiotic stress situations (Mata-Pérez *et al.*, 2016c). Treatment of Arabidopsis cell suspension cultures (ACSC) with 100 mM NaCl for 5 min increased the NO₂-Ln content more than 3-fold (Table 2) (Mata-Pérez *et al.*, 2016c). Prolonging this stress up to 30 minutes maintained a higher NO₂-Ln concentration relative to the control, but significantly lower than that observed after 5 minutes of stress (Table 2) (Mata-Pérez *et al.*, 2016c). This result suggests a quick response of NO₂-Ln-mediated signaling during the plant response to salinity that is attenuated when saline stress is prolonged. Moreover, induction of NO₂-Ln content has been observed in 14-day-old Arabidopsis plants under different stress situations (Table 2). In response to wounding and cadmium stress, the NO₂-Ln content increased ~2-fold compared with basal conditions, whereas the NO₂-Ln content increased 1.5-fold under low temperature stress (Table 2).

A specific justification for this general induction of NO₂-FAs has recently been postulated, as it was shown that different abiotic stress situations can generate uncontrolled ROS and RNS production that can lead to the release of NO₂-FAs from the nitroalkylated adducts established with proteins (Fig. 2, pathway 4) (Padilla *et al.*, 2017). Therefore, during the nitro-oxidative stress process that derives from an abiotic stress situation, the oxidation of Michael adducts can lead to the release of free NO₂-FAs that, in turn, may mediate stress-related signaling processes (Fig. 2, pathways 1, 2, and 6). In contrast, the endogenous

Table 2. Concentration of nitro-linolenic acid under different abiotic stress conditions in Arabidopsis

Plant material	Basal concentration (pmol g ⁻¹ FW)	Stress	Concentration under stress (pmol g ⁻¹ FW)	References
14-day-old seedlings	3.84	Wounding (3 h)	7.46	Mata-Pérez <i>et al.</i> (2016c)
		150 μM CdCl ₂	6.62	
		Low temperature (8 °C, 48 h)	5.75	
9-day-old ACSC	0.28	100 mM NaCl (5 min)	0.96	Mata-Pérez <i>et al.</i> (2016c)
		100 mM NaCl (30 min)	0.52	

ACSC, Arabidopsis cell suspension cultures FW, fresh weight.

content of NO₂-OA in *B. napus* under different stress situations has not been analyzed (Vollár *et al.*, 2020). These data would be of great importance because they would allow determination of the behavior of different NO₂-FAs under these adverse conditions. Moreover, a pharmacological approach has proposed that NO₂-OA is able to induce ROS production via NADPH oxidase in tomato cells and Arabidopsis and tomato leaves (di Palma *et al.*, 2020). Unfortunately, NO₂-OA has not been endogenously detected in Arabidopsis or tomato, which makes it difficult to establish the importance of this NO₂-OA-mediated signaling for plant physiology.

In any case, the involvement of NO₂-Ln in stress-related signaling events has been demonstrated by transcriptomic analyses using RNA-seq and mass spectrometry, which is discussed in the following sections. Accordingly, all these data suggest that NO₂-Ln could act as a mediator of signaling events during the plant response to stress.

NO₂-Ln induces changes in the transcription of abiotic stress-related genes

The signaling pathways mediated by ROS and RNS under physiological and stress conditions have been widely addressed in the literature (Begara-Morales *et al.*, 2019), and the transcriptomic profile of linolenic acid-responsive genes in ROS signaling from RNA-seq data has been studied in Arabidopsis (Mata-Pérez *et al.*, 2015). However, very little information exists about NO₂-FA-mediated signaling processes in plants. As stated above, different stress situations increase free NO₂-Ln levels, which, in turn, could be related to the signaling events that lead to an effective response to stress situations. In line with this, NO₂-Ln is able to regulate gene expression in a concentration-dependent manner within the 1–100 μM range (Mata-Pérez *et al.*, 2016c). In addition, an RNA-seq analysis has revealed the signaling capacity of this NO₂-FA in Arabidopsis after treating ACSC with 10 and 100 μM NO₂-Ln (Mata-Pérez *et al.*, 2016c). It is noteworthy that NO₂-Ln has been endogenously detected in Arabidopsis, which means that transcriptomic profiling results have a functional implication in plant biology. A total of 1308 genes were differentially expressed, with 437 up-regulated and 871 down-regulated genes, after treatment with 100 μM NO₂-Ln (Mata-Pérez *et al.*, 2016c). This transcriptional reprogramming is similar to that observed with other signaling molecules, such as H₂O₂ and GSNO (reviewed by Begara-Morales *et al.*, 2019), and therefore confirms that NO₂-Ln is a molecule that can mediate signaling events in plants. NO₂-Ln induces the expression of genes related mainly to abiotic stress (Fig. 2, pathway 2), such as high light intensity, temperature, and oxidative stress-related processes, with a gene ontology overrepresentation in H₂O₂, ROS, and oxygen-containing compound responses (Mata-Pérez *et al.*, 2016c). These findings indicate the potential capacity of this NO₂-FA as a regulator of plant responses to abiotic stress processes.

Interestingly, ~40% of NO₂-Ln-induced genes are related to heat shock response (HSR) family genes, which highlights small heat-shock proteins (sHSPs), some heat-shock transcription factors (HSFA2 and HSFA7B), and members of the HSP40, HSP60, HSP70, and HSP90 families (Mata-Pérez *et al.*, 2016c). The most induced gene was *HSP21*, with a fold change of 3995, with a concomitant increase in protein content in response to NO₂-Ln (Mata-Pérez *et al.*, 2016c). Furthermore, the importance of the induction of *HSP21* for chloroplast development under heat stress has been described in Arabidopsis (Zhong *et al.*, 2013). This NO₂-FA-induced HSR appears to be a conserved mechanism, as similar behavior has been observed in human endothelial cell cultures pre-incubated with NO₂-OA (Kansanen *et al.*, 2009) and conjugated nitro-linoleic acid (Lu *et al.*, 2019), where the anti-inflammatory and antioxidant functions of NO₂-FA-regulated genes were described. Similarly, the antioxidant capacity of NO₂-Ln has also been proposed in plants (Fig. 2, pathway 1) since the treatment of ACSC with 100 μM NO₂-Ln induces some antioxidant enzymes, such as ascorbate peroxidase (APX) or methionine sulf-oxide reductase. APX is a key antioxidant enzyme within the ascorbate–GSH cycle that is involved in detoxifying H₂O₂ by using ascorbate as an electron donor (Asada, 1992; Noctor and Foyer, 1998). This enzyme is localized in different subcellular compartments, where it has been related to responses to stresses including high light and high temperature (Pandey *et al.*, 2017). Two of these NO₂-Ln induced genes, *HSFA2* and *APX*, have been described as interactors under stressful situations such as high temperature and light-intensity stress (Nishizawa *et al.*, 2006). MSR is a family of enzymes considered to be regulators of antioxidant defense, as they catalyze the reduction of methionine sulfoxide, generated under a stress situation, to methionine. In this line, MSRs have been related to the plant response to oxidative stress (Dos Santos *et al.*, 2005; Rouhier *et al.*, 2006; Li *et al.*, 2012).

To delve into the signaling pathways orchestrated by NO₂-Ln in plants, the MEME (Multiple Em for Motif Elicitation) software for discovering conserved motifs in the promoter regions of NO₂-Ln-induced genes was used. A conserved motif, AGGCCCATTA, was identified in these genes, which is related to the stress response, cell cycle, and protein synthesis. Given that an important set of NO₂-Ln-responsive genes belongs to HSR families, the promoter regions of these genes has also been analyzed, and the conserved motif TGGGC(C/T)TT present in light-induced promoters was identified (Mata-Pérez *et al.*, 2018). All these data reinforce the capacity of NO₂-Ln to act as a modulator of stress-related signaling processes.

NO₂-Ln-mediated nitroalkylation regulates protein function in Arabidopsis

As mentioned above, NO₂-FAs are electrophilic molecules that mediate the nitroalkylation PTM (Fig. 2, pathway

3) (Delmastro-Greenwood *et al.*, 2014; Aranda-Caño *et al.*, 2019; Schopfer and Khoo, 2019). Consequently, they can modulate the function, or even the subcellular localization, of protein targets (Baker *et al.*, 2007; Rudolph *et al.*, 2009; Aranda-Caño *et al.*, 2019; Schopfer and Khoo, 2019). In animal systems, NO₂-FAs have been proposed to mediate pleiotropic signaling actions by nitroalkylation (reviewed by Schopfer and Khoo, 2019). In line with this, different protein targets of this PTM have been identified, which highlights those related to anti-inflammatory and antioxidant properties or exerting protective actions in neurodegenerative diseases (Schopfer *et al.*, 2011; González-Perilli *et al.*, 2017; Aranda-Caño *et al.*, 2019; Chavarría *et al.*, 2019; Schopfer and Khoo, 2019). However, information about the potential capacity of NO₂-FAs to mediate signaling events via this modification in plants is starting to be explored. To our knowledge, only one study conducted in plants has shown the capacity of a NO₂-FA to mediate electrophilic reactions (Aranda-Caño *et al.*, 2019). Thirty-seven proteins adducted with NO₂-Ln were endogenously identified in Arabidopsis cell cultures by LC-MS/MS, which suggests the importance of this modification in plant physiology. Moreover, the number of these nitroalkylated proteins increased to 342 after treating cell cultures with 100 μM NO₂-Ln. One of these NO₂-Ln adducted proteins is cytosolic APX2 (Aranda-Caño *et al.*, 2019). Interestingly, the enzyme activity of recombinant APX from Arabidopsis is significantly inhibited by 10 μM NO₂-Ln (Aranda-Caño *et al.*, 2019).

To investigate whether this behavior is a consequence of the nitroalkylation PTM, an LC-MS/MS analysis was performed, and His-43 and His-163 were identified as the preferred nitroalkylation sites. With the help of an *in silico* analysis, it was also determined that His-43 and His-163 were located at the active site and metal-binding site, respectively, which suggests that the nitroalkylation of these residues impairs APX enzyme activity and therefore regulates its protein function (Aranda-Caño *et al.*, 2019). This finding confirms that the inhibition of APX by NO₂-Ln is a consequence of nitroalkylation, and not of its capacity to release NO and mediate NO signaling events. Thus, in stress situations with increased cellular oxidative states, nitroalkylated APX can be oxidized and nitro-fatty acid can be released. Next, the protein recovers its activity in an attempt to control the redox state at the beginning of the stress situation. Furthermore, these free NO₂-FAs can induce gene expression related to abiotic stress and oxidative stress-related responses, such as the induction of APX itself. It is worth noting that APX is also regulated at the transcriptional and protein levels by NO₂-Ln (Mata-Pérez *et al.*, 2016c); thus, the modulation of this antioxidant enzyme appears to be an essential regulatory point in the NO₂-Ln-mediated response to oxidative stress. The function of APX is therefore multi-regulated, as it also has a gain in function through S-nitrosation of Cys-32 by NO (Yang *et al.*, 2015). In summary, this finding shows for the first time in plants the specific capacity of NO₂-FAs to modulate, by nitroalkylation,

the function of an early key antioxidant system in plants, and therefore opens up a promising research field in the study of physiological and stress processes in plants.

NO₂-FAs release NO that generates endogenous GSNO in plants

In addition to their ability to mediate signaling events via nitroalkylation, NO₂-FAs are also NO donors (Fig. 2, pathway 5) (Lima *et al.*, 2005; Schopfer *et al.*, 2005; Gorczynski *et al.*, 2006; Mata-Pérez *et al.*, 2016a; Zatloukalova *et al.*, 2019; Vollár *et al.*, 2020) and can therefore contribute to NO-mediated signaling in physiological and stress situations in plants. First, treatment of 30 day-old Arabidopsis plants with 1 mM NO₂-Ln increased the NO content in roots and leaves (Sánchez-Calvo *et al.*, 2013). Subsequently, a lower NO₂-Ln concentration (100 μM) also increased the *in vivo* NO content in 7 day-old Arabidopsis roots (Mata-Pérez *et al.*, 2016a, b) and 9 day-old ACSC (Mata-Pérez *et al.*, 2016a). Moreover, the capacity of NO₂-Ln to release NO was well characterized *in vitro* by different methodological approaches. A pH-dependent release of NO from NO₂-LA and NO₂-Ln was shown, with maximum NO generation at physiological pH (Mata-Pérez *et al.*, 2016a). Interestingly, the exogenous treatment of *B. napus* with 100 μM NO₂-OA increased the NO levels and also improved seed germination (Vollár *et al.*, 2020). This confirms the idea that the high level of NO₂-FAs in early stages of development (Mata-Pérez *et al.*, 2016c; Vollár *et al.*, 2020) might be related to prompting seed germination, due to the capacity of NO₂-FAs to act as NO donors.

Given their capacity to act as NO donors, NO₂-FAs could be involved in NO signaling in plants, for example, in development, plant responses to stress, or mediating different NO-PTMs, such as S-nitrosation or tyrosine nitration (Fig. 2, pathway 6). The relevance of NO₂-Ln in NO signaling has gone one step further because its ability to generate GSNO has been demonstrated very recently *in vitro* and *in vivo* (Fig. 2, pathway 7) (Mata-Pérez *et al.*, 2020). Although the exact mechanism of GSNO formation remains unclear, the direct interaction of GSH with submicromolar concentrations of NO has previously been reported as an efficient pathway to generate GSNO *in vivo* (Kolesnik *et al.*, 2013). Bearing in mind that NO₂-Ln is a physiological NO donor that releases NO at submicromolar levels (Mata-Pérez *et al.*, 2016a), the direct interaction of this NO and GSH could take place (Mata-Pérez *et al.*, 2020). The *in vitro* generation of GSNO from the reaction between GSH and NO₂-Ln has been determined by LC-ES/MS. In addition, the incubation of Arabidopsis roots with ¹⁵NO₂-Ln allowed the detection of this labeled NO₂-FA and GS¹⁵NO in leaves, which therefore confirmed that NO₂-Ln is able to travel through the plant and directly contribute to GSNO generation *in vivo* (Mata-Pérez *et al.*, 2020). Due to the high NO release ratio from NO₂-Ln (Mata-Pérez *et al.*, 2016a), this NO₂-FA contributes significantly and quantitatively to the

total GSNO pool in plant cells. This is supported by the fact that the endogenous GSNO concentration in 45-day-old *Arabidopsis* leaves was 0.91 pmol mg⁻¹ protein and that exogenous NO₂-Ln was able to generate 0.50 pmol mg⁻¹ protein in the same plants (Mata-Pérez *et al.*, 2020).

To analyze the potential contribution of NO₂-Ln to GSNO generation, an *Arabidopsis* mutant line with defective expression of the enzyme alkenal reductase (AtAER) was used. This enzyme forms part of a family of NADPH-dependent reductases involved in the detoxification of reactive carbonyls in plants (Mano *et al.*, 2005; Yamauchi *et al.*, 2011). It is also phylogenetically related to animal prostaglandin reductase-1 (PGR1), which is an alkenal one/reductase (AOR) (Yamauchi *et al.*, 2011; Vitturi *et al.*, 2013; Mesa *et al.*, 2015) able to act as a nitroalkene reductase enzyme that catalyzes the conversion of electrophilic nitroalkenes into non-electrophilic nitroalkane (Vitturi *et al.*, 2013). Similarly, the *Arabidopsis aer* mutant, which has low AER transcript and protein levels, exhibits a 3-fold increase of NO₂-Ln content, which therefore confirms that AtAER regulates the cellular level of NO₂-FAs in plants. This higher NO₂-Ln level is accompanied by a 2-fold increase in GSNO content in *Arabidopsis aer* plants (Mata-Pérez *et al.*, 2020). Hence, this result confirms that NO₂-Ln contributes to the *in vivo* generation of GSNO and its potential contribution to the generation of S-nitrosothiols in plants. These findings are extremely important in the field of NO in plants, as GSNO is the main reservoir of NO, from which NO is released to orchestrate NO-mediated signaling events, especially during stress responses (Begara-Morales *et al.*, 2018). Therefore, the *in vivo* generation of GSNO from NO₂-Ln highlights the relevance of this NO₂-FA for plant biology because it can act as an important regulator of NO-mediated signaling events during plant responses to abiotic stress.

Conclusions and future perspectives

NO₂-FAs are produced by the interaction of unsaturated fatty acids with NO and NO-derived molecules, such as •NO₂ and ONOO⁻. In plant systems, the endogenous presence and biological function of these molecules are now starting to be explored. Despite this being a novel emerging research area, significant advances have been made in recent years. In this line, NO₂-Ln has been endogenously detected in various plant species, such as *Arabidopsis*, pea, and rice (Mata-Pérez *et al.*, 2016c, 2017), and NO₂-OA has been detected *in vivo* in *B. napus* (Vollár *et al.*, 2020). Both of these NO₂-FAs exhibit the highest levels in the first developmental stage, which has been linked to the promotion of seed germination.

NO₂-Ln has been proposed to mediate the pleiotropic signaling pathways summarized in Fig. 2. NO₂-Ln is able to induce defense mechanisms via the induction of the chaperone network and different antioxidant systems, such as APX, which is a key enzyme of the ascorbate-GSH cycle involved in the

detoxification of H₂O₂ (pathway 1). NO₂-Ln also mediates the induction of heat shock transcription factors that can ultimately regulate the expression of abiotic stress-related genes (pathway 2). The electrophilic nature of NO₂-FAs allows them to interact with nucleophiles such as Cys, His, and Lys residues in proteins, which leads to the formation of the Michael adduct via the reversible nitroalkylation PTM (pathway 3). This PTM has been proposed to modulate the structure and function of different cellular targets, such as APX. The uncontrolled overproduction of ROS and RNS in abiotic stress situations, which takes place with nitro-oxidative stress, leads to the oxidation of the Michael adduct and therefore releases free NO₂-Ln that is able to mediate different signaling mechanisms (pathway 4).

It is well established that NO₂-Ln acts as a NO donor (pathway 5) and can therefore be involved in NO-mediated signaling in plants, for example, in development, abiotic stress-response processes, or regulation of specific targets such as antioxidant systems by the NO-PTMs of S-nitrosation and tyrosine nitration (pathway 6). Finally, it has recently been proposed that NO₂-Ln, via NO release, is able to generate GSNO *in vivo* (pathway 7) and thus contributes to maintaining the major NO reservoir in cells. In this way, NO₂-Ln could also be involved in GSNO-dependent signaling mechanisms and probably contributes to S-nitrosothiol homeostasis in plant cells.

Although significant advances have been made regarding the presence and signaling role of NO₂-FAs in plants, future research is necessary to examine in depth the potential capacity of these molecules to mediate important functions in plant biology. Hence, the endogenous identification of different NO₂-FAs in distinct plant species is very important for determining the plant-specific presence of these NO₂-FAs. In this regard, exogenous treatment with NO₂-FAs without their endogenous identification would make it difficult to determine the functional importance of these molecules in plant biology. The modulation of NO₂-Ln has been well characterized in development and abiotic stress situations, but other NO₂-FAs, such as NO₂-OA, have not been analyzed in stress-related processes. More data on the modulation of other NO₂-FAs in stress situations in different plant species are therefore needed.

Future research should focus on the signaling properties of NO₂-Ln, to identify the cellular targets of this NO₂-FA. As the endogenous nitroalkylation of proteins has been demonstrated under physiological conditions and it has been shown that this modification increased in abiotic stress situations, the identification of nitroalkylated proteins should be investigated in order to investigate the repercussions of this PTM in abiotic stress situations. To decipher the involvement of NO₂-FAs in the signaling events that lead to plant responses to stress, the use of mutant plants that show differences in NO₂-FA content could be helpful. At the same time, the capacity of NO₂-Ln to release NO and to generate GSNO *in vivo* is another relevant area for future research, given the wide range of processes regulated by NO/GSNO.

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Author contributions

JB and JB-M conceptualized this work and wrote the manuscript. All authors contributed to drafting the work, supervised the revision of the manuscript, and validated the final submission.

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