



## Nitro-fatty acids in plant signaling: New key mediators of nitric oxide metabolism



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### ABSTRACT

Recent studies in animal systems have shown that NO can interact with fatty acids to generate nitro-fatty acids (NO<sub>2</sub>-FAs). They are the product of the reaction between reactive nitrogen species and unsaturated fatty acids, and are considered novel mediators of cell signaling based mainly on a proven anti-inflammatory response. Although these signaling mediators have been described widely in animal systems, NO<sub>2</sub>-FAs have scarcely been studied in plants. Preliminary data have revealed the endogenous presence of free and protein-adducted NO<sub>2</sub>-FAs in extra-virgin olive oil (EVOO), which appear to be contributing to the cardiovascular benefits associated with the Mediterranean diet. Importantly, new findings have displayed the endogenous occurrence of nitro-linolenic acid (NO<sub>2</sub>-Ln) in the model plant *Arabidopsis thaliana* and the modulation of NO<sub>2</sub>-Ln levels throughout this plant's development. Furthermore, a transcriptomic analysis by RNA-seq technology established a clear signaling role for this molecule, demonstrating that NO<sub>2</sub>-Ln was involved in plant-defense response against different abiotic-stress conditions, mainly by inducing the chaperone network and supporting a conserved mechanism of action in both animal and plant defense processes. Thus, NO<sub>2</sub>-Ln levels significantly rose under several abiotic-stress conditions, highlighting the strong signaling role of these molecules in the plant-protection mechanism. Finally, the potential of NO<sub>2</sub>-Ln as a NO donor has recently been described both *in vitro* and *in vivo*. Jointly, this ability gives NO<sub>2</sub>-Ln the potential to act as a signaling molecule by the direct release of NO, due to its capacity to induce different changes mediated by NO or NO-related molecules such as nitration and S-nitrosylation, or by the electrophilic capacity of these molecules through a nitroalkylation mechanism. Here, we describe the current state of the art regarding the advances performed in the field of NO<sub>2</sub>-FAs in plants and their implication in plant physiology.

### 1. Introduction

Electrophilic nitro-fatty acids (NO<sub>2</sub>-FAs) are endogenously formed by redox reactions of nitric oxide (NO) and NO-derived molecules that promote the generation of nitrogen dioxide (NO<sub>2</sub>); which in turn nitrates unsaturated fatty acids [1–3]. The first report describing the endogenous occurrence of lipid nitrated derivatives was defined both in normolipidemic and hyperlipidemic donors [4]. Numerous studies have since reported a selective nitration of fatty acids under physiological conditions, highlighting the greater susceptibility to nitration of conjugated fatty acids than methyl-interrupted dienes or polyenes in animal systems [5]. Most of these analyses have shown that these

molecules trigger pleiotropic signaling actions including a remarkable anti-inflammatory and antioxidant response [6–9]. A measurable increase in the levels of NO<sub>2</sub>-FAs has been shown under different pathological conditions such as ischemia reperfusion injury or after LPS injection in the peritoneum [1,10].

Currently, the formation mechanisms of NO<sub>2</sub>-FAs remain unknown although different ways have been proposed, such as by NO<sub>2</sub>, peroxyxynitrite (ONOO<sup>-</sup>) or protonation of nitrite (NO<sub>2</sub><sup>-</sup>) yielding nitrous acid (HNO<sub>2</sub>) and subsequent nitrating and nitrosating species [11]. By contrast, several ways of inactivating nitro-fatty acid signaling have recently been described. In this regard, the NADPH-dependent enzyme prostaglandin reductase-1 (PGR-1) is involved in the inactivation of

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NO<sub>2</sub>-FAs in the liver through a saturation mechanism [12]. Moreover, conjugation of these molecules to reduced glutathione (GSH) and subsequent  $\beta$ -oxidation and excretion through urine has also been noted as a mechanism of inactivation of NO<sub>2</sub>-FAs [13]. Furthermore, due to the hydrophobic nature of these molecules, cell membranes and/ or lipoproteins may constitute a natural source of NO<sub>2</sub>-FAs [14,15]. Based on the electrophilic capacity of nitro-fatty acids, an important part of the endogenous content of these molecules could also be found adducted with different nucleophiles, mainly with proteins [16]. In fact, the intravenous injection of nitro-oleic acid (NO<sub>2</sub>-OA) in mice has shown that this NO<sub>2</sub>-FA is rapidly adducted to plasma thiol-containing proteins and glutathione, and this being only a small portion of free NO<sub>2</sub>-OA in plasma [1]. Furthermore, it has been recently described the release of NO<sub>2</sub>-FAs from Cys-adducted nitroalkenes under nitro-oxidative stress situations [17]. Representative ROS and RNS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and ONOO<sup>-</sup> respectively, are able to initiate the release of free nitroalkenes from protein-adducted NO<sub>2</sub>-FAs. These nitro-oxidative conditions promote the formation of a  $\beta$ -nitrosulfoxide intermediate and consequently releasing free NO<sub>2</sub>-FAs. This behavior may have relevance under several stressful situations both in animal and plant systems such as inflammation or under different adverse environmental conditions in which an increase of ROS and RNS has been noted [18–22]. Actually, the anti-inflammatory and antioxidant signaling properties of these molecules have been attributed to free NO<sub>2</sub>-FAs [23–27].

The beneficial properties of NO<sub>2</sub>-FAs described above have been attributed mainly to the electrophilic capacity of the  $\beta$ -carbon of the reactive nitroalkenyl substituent from NO<sub>2</sub>-FAs, which readily undergoes a reversible Michael addition [2,28]. This reaction is known specifically as nitroalkylation and occurs primarily with functionally significant Cys and His residues on transcription-regulating proteins and enzymes (Fig. 1) [2,16,28]. Through this post-translational alteration, NO<sub>2</sub>-FAs can reportedly exert pleiotropic signaling actions, particularly an anti-inflammatory and antioxidant response [2,29]. In this respect, electrophilic nitroalkenes such as nitro-oleic (NO<sub>2</sub>-OA) or nitro-linoleic (NO<sub>2</sub>-LA) acids have been identified as antagonists of the nuclear lipid receptor peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) [30,31], the Keap1-Nrf2 signaling pathway [6,32], and the heat-shock response [26]. Furthermore, the antagonistic properties of NO<sub>2</sub>-FAs on NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) also promote a strong anti-inflammatory response [23].

On the other hand, the ability of NO<sub>2</sub>-FAs to release NO has been thoroughly described [33–35], but the precise mechanism of NO release has not yet been identified. Nevertheless, [34] postulated that NO released from NO<sub>2</sub>-LA may be based on a modified Nef reaction [34]. In this sense, deprotonation of the carbon  $\gamma$  to the nitro group leads to the formation of an unstable nitroso intermediate that rapidly degrades to release either HNO, the predicted Nef reaction product, or <sup>•</sup>NO, a product considered likely due to the weak C-N bond present on the hydroxyl-nitroso intermediate. An alternative mechanism to the modified Nef reaction was also hypothesized based on a rearrangement of the nitroalkene to a nitrite ester, followed by N-C bond hemolysis to form <sup>•</sup>NO and a lipid radical [36]. The release of NO from NO<sub>2</sub>-FAs has been considered a very minor component of the diverse biological actions of these molecules inasmuch as the  $\beta$ -carbon adjacent to the nitro group is strongly electrophilic and reacts covalently with protein and thiols [16,37,38]. However, NO release from NO<sub>2</sub>-LA mediates the S-nitrosylation of the pro-inflammatory member CD40 with subsequent inactivation, thereby triggering an anti-inflammatory response [39]. Therefore, we cannot rule out that this NO release can have important consequences for key cellular targets.

Although the occurrence and the biological actions of NO<sub>2</sub>-FAs have been widely explored in animal systems, the study of these molecules in plant systems constitutes an interesting and emerging area. In this regard, the present review describes the current state of the art with

respect to the advances in the study of NO<sub>2</sub>-FAs in plant systems and their implications in plant physiology.

## 2. Characterization of the endogenous occurrence of NO<sub>2</sub>-FAs in plant systems

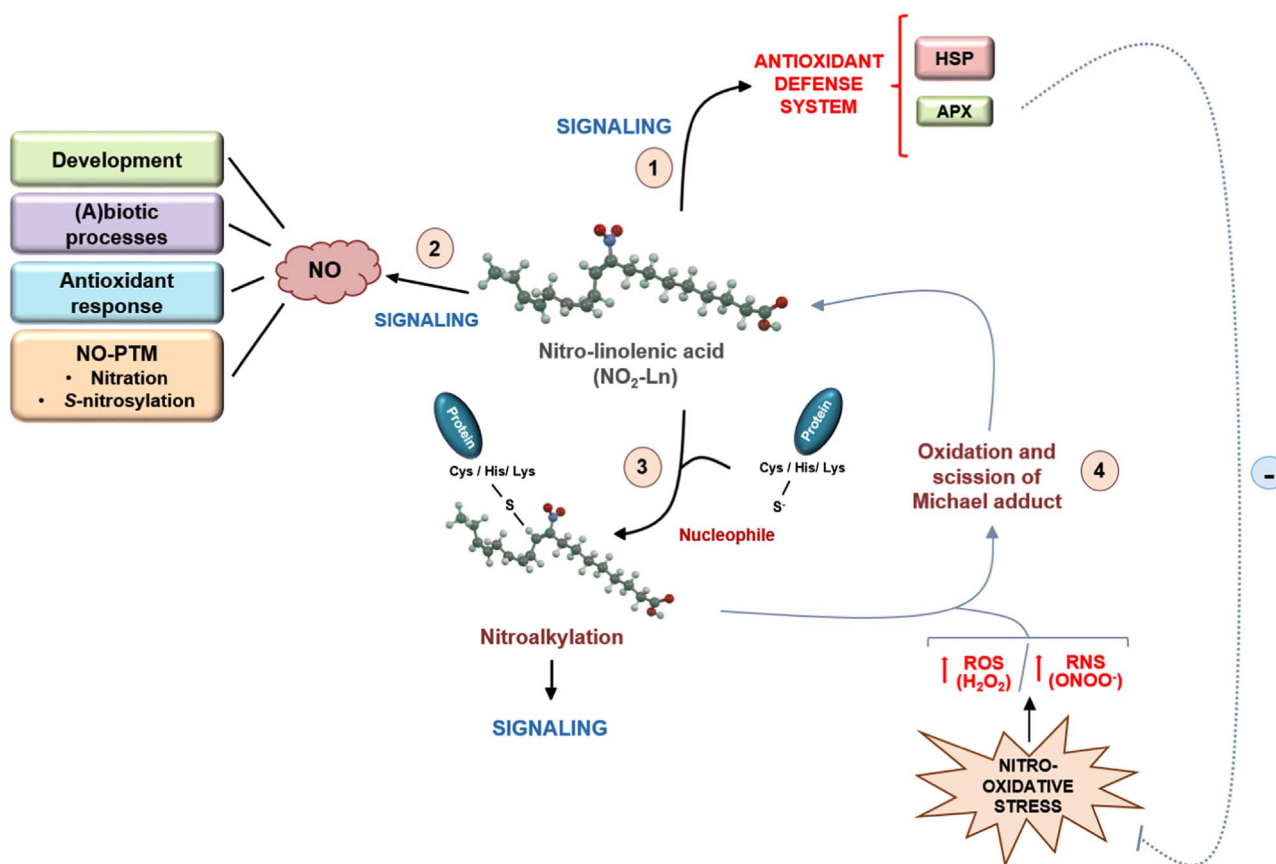
### 2.1. Extra-virgin olive oil (EVOO) and olives

The Mediterranean diet constitutes a key source of unsaturated fatty acids with extra-virgin olive oil (EVOO) providing health benefits to humans. The Mediterranean diet is associated with different anti-inflammatory and anti-hypertensive effects leading to a reduced risk of cardiovascular morbidity and mortality [40–42]. Based on the previously reported anti-inflammatory properties attributed to NO<sub>2</sub>-FAs, an initial study analyzing the endogenous occurrence of these molecules was made in EVOO and fresh olives [43]. In this respect, the authors noted the presence of nitrated derivatives of conjugated linoleic acid (NO<sub>2</sub>-cLA) in EVOO, specifically of 9- and 12-NO<sub>2</sub>-cLA isomers. Moreover, the Mediterranean diet is also characterized by the high intake of leafy vegetables and cured meats, which are high in nitrates (NO<sub>3</sub><sup>-</sup>) and nitrites (NO<sub>2</sub><sup>-</sup>). NO<sub>3</sub><sup>-</sup> can be reduced by bacteria in the saliva to NO<sub>2</sub><sup>-</sup> and the latter can form nitrous acid (HNO<sub>2</sub>) in the stomach being thereby broken down to NO<sub>2</sub>. Furthermore, oils, fish, several seeds, and dairy products such as milk or cheese are rich sources of  $\omega$ -3 and  $\omega$ -6 fatty acids, including oleic, linoleic and linolenic acids. These factors together with acidic conditions of digestion may lead to the gastric generation of NO<sub>2</sub>-FAs. In this regard, the incubation of EVOO with gastric juice artificial and <sup>15</sup>N-labelled sodium nitrite (Na[<sup>15</sup>N]O<sub>2</sub>) results in the detection of different NO<sub>2</sub>-FAs such as 9- and 10-NO<sub>2</sub>-OA; 9/10-, 12- and 13-NO<sub>2</sub>-LA and 8-, 9-, 11- and 12-NO<sub>2</sub>-cLA [43]. Finally, NO<sub>2</sub>-OA-cysteine adducts have been endogenously detected in acid-hydrolyzed proteins from the peel and mesocarp of freshly picked whole olives of different cultivars. For example, NO<sub>2</sub>-OA-cysteine levels were higher in Picual olive cultivars and more abundant in the peel from these olives [43], underscoring that EVOO and olives are both a source and metabolic reserve of NO<sub>2</sub>-FAs.

Importantly, the *ex vivo* detection of NO<sub>2</sub>-OA and NO<sub>2</sub>-LA in EVOO may be responsible in part for the numerous beneficial properties attributed to this important source of fatty acids in the Mediterranean diet. Furthermore, nitrated derivatives of oleic and linoleic acids not only inhibit leukocyte and platelet activation [44], vascular smooth-muscle proliferation [6], and lipopolysaccharide-stimulated macrophage cytokine secretion [23], but also activate PPAR $\gamma$  receptor [45] and induce endothelial heme oxygenase 1 expression [46]. These NO<sub>2</sub>-FAs also strongly modulate Nrf2/Keap1 [6,23] and NF- $\kappa$ B-regulated inflammatory signaling [23], as previously mentioned. Therefore, the presence of nitrated derivatives of oleic, linoleic or conjugated linoleic acids may contribute to the cardiovascular benefits associated with the Mediterranean diet.

### 2.2. The model plant *Arabidopsis thaliana*

Previous findings on the endogenous occurrence of NO<sub>2</sub>-FAs in EVOO and olives prompted the analysis of the presence of these molecules in the model plant *Arabidopsis thaliana*. The study was performed using mass spectrometric approaches in 9-d-old *Arabidopsis* cell-suspension cultures (ACSC) and in 14-d-old seedlings [27]. Firstly, lipid composition analysis of 9-d-old ACSC and 14-d-old seedlings revealed that linolenic acid (18 $\Delta$ 3) was the most abundant unsaturated fatty acid in both samples followed by linoleic (18 $\Delta$ 2) and oleic (18 $\Delta$ 1) acids. This background motivated the study of the presence of nitrated derivatives of the most abundant unsaturated fatty acids, and therefore an analysis was made of the presence of NO<sub>2</sub>-OA, NO<sub>2</sub>-LA and nitro-linolenic acid (NO<sub>2</sub>-Ln). Synthesis of these different standards by a nitroselenation procedure made it possible to



**Fig. 1. Schematic model of  $\text{NO}_2\text{-Ln}$  signaling.** Pathway 1 indicates that  $\text{NO}_2\text{-Ln}$  is able to induce a defense mechanism through the induction of the chaperone network and the increase in the expression of ascorbate peroxidase enzyme. This latter may be involved in the alleviation of the oxidative stress generated by the overproduction of ROS such as  $\text{H}_2\text{O}_2$ . Furthermore,  $\text{NO}_2\text{-Ln}$  is a NO donor being therefore implicated in the wide range of actions in which this molecule is involved (pathway 2). The electrophilic ability of this nitro-fatty acid could contribute to the signaling actions involving  $\text{NO}_2\text{-FAs}$  (pathway 3) and, under nitro-oxidative stress conditions, the oxidation of Michael adducts may occur with subsequent nitroalkene release (pathway 4) and the observed antioxidant properties of these molecules. NO: nitric oxide; HSP: heat shock protein; APX: ascorbate peroxidase.

**Table 1**

Endogenous detection of nitro-linolenic acid ( $\text{NO}_2\text{-Ln}$ ) in several plant species and in different subcellular fractions. ACSC, Arabidopsis cell suspension cultures.

Plant species	Organ/Subcellular fraction	pmol $\text{NO}_2\text{-Ln}$ / g FW
<i>Arabidopsis thaliana</i>	14-D-old seedlings	3.84
	9-D-old ACSC	0.28
Pea ( <i>Pisum sativum</i> )	Roots	0.072
	Leaves	0.084
Rice ( <i>Oryza sativa</i> )	Mitochondria	0.282
	Peroxisomes	0.748

identify the only endogenous occurrence of  $\text{NO}_2\text{-Ln}$  by comparing the retention time of the chromatographic peak of  $\text{NO}_2\text{-Ln}$  standard to those observed in both 9-D-old ACSC and 14-D-old seedlings [27]. As confirmation that the peak detected corresponded to electrophilic  $\text{NO}_2\text{-Ln}$ , a nitroalkylation reaction with  $\beta$ -mercaptoethanol ( $\beta$ -ME) of the lipid extract was carried out by seeking the covalent nitroalkylated adduct formed by the reaction of  $\text{NO}_2\text{-Ln}$  and the thiol of  $\beta$ -ME. After incubation, the retention time of the peaks detected changed, confirming the electrophilic capacity of  $\text{NO}_2\text{-Ln}$  and showing the endogenous presence of this nitro-fatty acid in arabidopsis. Furthermore, the  $\text{NO}_2\text{-Ln}$  content was analyzed in both samples (Table 1) and the results showing  $0.28 \pm 0.04$  pmol/g FW in 9-D-old ACSC and  $3.84 \pm 0.44$  pmol/g FW in 14-D-old seedlings [27]. These low levels were consistent with previous results found in the field of animal systems

for other nitro-fatty acids such as  $\text{NO}_2\text{-OA}$  [47] and  $\text{NO}_2\text{-LA}$  suggesting these molecules could act as important signaling components of plant physiology. Moreover, the presence of other nitrated derivatives from oleic, linoleic and conjugated-linoleic acids was also analyzed. In all studied samples, the endogenous occurrence of nitro-oleic acid (MRM transition 326/279  $m/z$ ) was not observed due to no chromatographic peaks were detected with this MRM transition. Regarding the presence of nitro-linoleic or nitro-conjugated linoleic acids with MRM transition 324/277  $m/z$ , a chromatographic peak with this MRM transition was observed. Nevertheless, the incubation of lipid extracts from all the analyzed samples with the electrophile  $\beta$ -ME did not make disappear those chromatographic peaks assuring they did not correspond to any of these  $\text{NO}_2\text{-FAs}$ .

### 2.3. Nitro-fatty acid detection in other plant species

Besides showing the endogenous presence of  $\text{NO}_2\text{-Ln}$  in the arabidopsis, the detection of  $\text{NO}_2\text{-FAs}$  in other plant species was also analyzed (Table 1). In line with the approaches followed in arabidopsis, nitrated derivatives from the most abundant unsaturated fatty acids were found. This analysis resulted in the endogenous detection of  $\text{NO}_2\text{-Ln}$  in roots from pea (*Pisum sativum*) plants and in leaves from rice (*Oryza sativa*) plants, the levels of this nitro-fatty acid being 0.072 and 0.748 pmol/g FW, respectively (Table 1). Mitochondrial and peroxisomal fractions from pea plants were also subjected to the analysis of the different  $\text{NO}_2\text{-FAs}$ , revealing  $\text{NO}_2\text{-Ln}$  in both samples. The content of this molecule was about 0.084 and 0.282 pmol/g FW, in the mitochondrial and peroxisomal fractions, respectively (Table 1). In all analyzed samples was only possible to detect the endogenous

occurrence of NO<sub>2</sub>-Ln, but not other nitrated derivatives from major fatty acids such as NO<sub>2</sub>-OA or NO<sub>2</sub>-LA, suggesting the higher susceptibility of linolenic acid to be endogenously nitrated and the relevance of this NO<sub>2</sub>-FA in plant systems. All these results also highlight a ubiquitous distribution of NO<sub>2</sub>-FAs in plant kingdom and the potential signaling actions of these molecules in plant systems.

### 3. Involvement of nitro-fatty acids at development and under abiotic stress

In light of the outcomes of the *A. thaliana* analyses, the study concerning the levels of NO<sub>2</sub>-Ln was extended throughout plant development and under different stress conditions, specifically abiotic stress. Furthermore, based on the low levels of NO<sub>2</sub>-Ln in plant systems, a transcriptomic analysis with RNA-seq technology helped to elucidate the potential signaling role of this molecule in plant physiology.

#### 3.1. Modulation of NO<sub>2</sub>-Ln content throughout the development of *Arabidopsis*

As mentioned above, the endogenous occurrence of NO<sub>2</sub>-Ln was observed in 9-d-old ACSC and in 14-d-old seedlings. These results prompted an analysis of the content of NO<sub>2</sub>-Ln throughout the developmental process of *Arabidopsis*. In this sense, different *Arabidopsis* plant materials such as seeds, 14-d-old seedlings, and leaves from 30- and 45-d-old plants, with clear symptoms of senescence the latter, were used for this study. Higher levels of NO<sub>2</sub>-Ln were found at the beginning of the development, specifically in seeds ( $11.18 \pm 1.68$  pmol/g FW) and in 14-d-old seedlings ( $3.84 \pm 0.44$  pmol/g FW). Nevertheless, this content significantly declined with the progress of plant development and with senescence in leaves from 30- ( $0.36 \pm 0.04$  pmol/g FW) and 45-d-old plants ( $0.54 \pm 0.06$  pmol/g FW) [27]. Nitro-fatty acids have been demonstrated to be *in vitro* and *in vivo* NO donors [33–35,48]. NO has a major role at the beginning of plant development because this molecule triggers seed germination [49], among other key processes in plant physiology. The higher content of NO<sub>2</sub>-Ln detected in seeds may imply that this nitro-fatty acid acts as a NO donor at this stage and therefore favors germination and the onset of vegetative development. In this respect, it has been recently shown that *S*-nitrosylation of the ABA-related transcription factor, ABI5 promotes the interaction with CUL4-based and KEG E3 ligases, which are subsequently degraded by the proteasome, promoting seed germination [50]. Therefore, it cannot be ruled out that NO<sub>2</sub>-Ln could release NO in seeds and mediate a *S*-nitrosylation mechanism of key transcription factors such as ABI5, thereby stimulating seed germination.

#### 3.2. Raising of NO<sub>2</sub>-Ln levels under abiotic-stress situations

Besides the involvement of NO and other NO-related molecules in several physiological processes such as germination, growth, development, and senescence [22,49,51–56], this gaseous molecule is also involved in the response to several (a)biotic stress conditions such as pathogen infection, symbiotic interactions, mechanical wounding, salinity, UV-radiation, and heavy-metal stress [21,57–67]. In relation to this implication, the levels of NO<sub>2</sub>-Ln were analyzed under different abiotic-stress conditions. With 9-d-old ACSC exposure to 100-mM NaCl, a significant rise of NO<sub>2</sub>-Ln content was observed after 5 min of treatment ( $0.96 \pm 0.12$  pmol/g FW) compared to steady-state levels ( $0.28 \pm 0.04$  pmol/g FW), which tripled the levels of this nitro-fatty acid. Nevertheless, after 30 min of salt stress the levels of NO<sub>2</sub>-Ln fell [27]. Moreover, in 14-d-old seedlings subjected to mechanical wounding, heavy-metal and low-temperature stress, a noteworthy rise in NO<sub>2</sub>-Ln levels was recorded, values being  $7.46 \pm 1.20$ ,  $6.62 \pm 0.98$  and  $5.75 \pm 0.79$  pmol/g FW, respectively, with regard to control seedlings ( $3.84 \pm 0.44$  pmol/g FW) [27]. These results indicate the involvement of

NO<sub>2</sub>-Ln in the response to different abiotic-stress situations.

## 4. Signaling role of NO<sub>2</sub>-Ln in plant physiology

Because the NO<sub>2</sub>-Ln content was modulated throughout plant's development and under different stressful situations together with the low observed levels of this molecule in plants systems, a transcriptomic analysis was performed to define the potential signaling actions of this molecule in plant physiology. Furthermore, based on the implications of NO<sub>2</sub>-Ln in a wide range of actions, the ability to release NO both *in vitro* and *in vivo* was also assessed.

#### 4.1. Transcriptomic analysis of NO<sub>2</sub>-Ln-responsive genes

To establish the potential signaling mechanisms in which NO<sub>2</sub>-Ln was involved, a transcriptomic analysis by RNA-seq technology was performed by analyzing 10 μM and 100 μM concentrations of this nitro-fatty acid in 9-d-old ACSC [27]. The use of these concentrations of NO<sub>2</sub>-Ln indicated a clear signaling response of this molecule in plant physiology, in agreement with concentrations used in previous studies in animal systems [13,47]. Importantly, the application of NO<sub>2</sub>-Ln to ACSC prompted a dose-dependence response ranging from 1 μM to 100 μM NO<sub>2</sub>-Ln which was shown by qRT-PCR and by DNASTAR software [27], this having been previously described for NO<sub>2</sub>-OA or NO<sub>2</sub>-cLA in animal systems [5,26].

Firstly, with the use of the Blast2GO suite, a Gene Ontology term-enrichment analysis of NO<sub>2</sub>-Ln-responsive genes showed that over-expressed genes were related to stress response, but specifically with the response to abiotic stress and to oxidative stress-related processes. By contrast, down-regulated genes were involved in some biological processes and especially with the biosynthesis of several cell compounds [27]. Therefore, these results suggest a close relationship between NO<sub>2</sub>-Ln and the involvement in different abiotic-stress conditions. A subset of genes modulated by NO<sub>2</sub>-Ln in ACSC were selected for a transcriptional analysis in other stages of plant development, further confirming the signaling capacity of this molecule in plant physiology. Results from this analysis show that NO<sub>2</sub>-Ln application to 14- and 45-d-old *Arabidopsis* plants followed the same trend both in up- and down-regulated genes, thus corroborating the signaling capacity of NO<sub>2</sub>-Ln throughout the overall development of *Arabidopsis* [27].

An in-depth analysis of genes modulated by NO<sub>2</sub>-Ln indicated that up-regulated genes were involved mostly in protein folding and the response to heat and H<sub>2</sub>O<sub>2</sub> [27]. In line with these results, a network graph analysis with GeneMANIA resource showed that around a 40% of the total genes induced by NO<sub>2</sub>-Ln treatment corresponded to members of the heat-shock response (HSR). Among these were numerous small heat-shock proteins (sHSP), but also some heat-shock transcription factors (HSFA2 and HSFA7B) as well as members of HSP40, HSP60, HSP70, and HSP90 families. The only transcriptomic analysis made to date with NO<sub>2</sub>-FAs in animal systems has been a microarray study with NO<sub>2</sub>-OA in human endothelial cell cultures [26] showing a noteworthy activation of the HSR in an Nrf2-independent manner. This activation of the HSP pathway contributes to cell protection through signaling actions involved in mainly anti-inflammatory processes. Outcomes derived from the study of the involvement of NO<sub>2</sub>-Ln in plant physiology resulted in a similar response to that previously observed with NO<sub>2</sub>-OA in animal systems, therefore suggesting a conserved mechanism of action of NO<sub>2</sub>-FAs both in animal and in plant systems. At this point, it is also important to bear in mind that incubation of ACSC with other NO<sub>2</sub>-FAs such as NO<sub>2</sub>-OA and NO<sub>2</sub>-LA generated a similar transcriptional response to that of the same subset of genes for NO<sub>2</sub>-Ln, suggesting a universality of the signaling mechanism that these molecules are able to trigger [27].

As mentioned above, NO<sub>2</sub>-Ln was involved in the response to several abiotic-stress conditions such as the response to heat, salinity,

heavy metals, low temperature and mechanical wounding [27]. A general phenomenon underlying abiotic-stress situations is oxidative stress [68,69]. Based on GO-term analysis, NO<sub>2</sub>-Ln has been associated with the response to H<sub>2</sub>O<sub>2</sub>, to reactive oxygen species (ROS), and to oxygen-containing compounds [27]. Among genes related to these processes, the high induction of cytosolic ascorbate peroxidase 2 (APX2) was noted together with other members involved in different redox processes [27]. APX is a key component of plant-defense responses against oxidative stress because this enzyme is able to detoxify the H<sub>2</sub>O<sub>2</sub> generated under different stressful situations using ascorbate as an electron donor. Furthermore, the close interaction between the HSF2 transcription factor and APX2 enzyme under stressful situations such as high temperature and light-intensity stress has been reported [70]. This nitro-fatty acid is able to act as a signaling mediator in the plant-defense mechanism in abiotic-stress situations, setting up a defense response against cell damage arising as a result of stress and mediated mainly by HSP induction. Therefore, these results suggest a close relationship with high temperature, abiotic stress in general, and oxidative stress—with both stress processes tightly regulated by NO<sub>2</sub>-Ln.

Finally, a high percentage of down-regulated genes were involved in the metabolism and decrease in sugar biosynthesis, in cell-wall components, and in chlorophyll, together with a decline in genes associated with photosynthesis, the electron-transport chain, and metabolite transport [27]. This phenomenon leads to a metabolic reconfiguration with a down-regulation in the biosynthesis of new components, counteracting energy costs and indicating is required to maintain a balance between the continuation of cell function and survival [71,72].

#### 4.2. NO<sub>2</sub>-Ln is a NO donor

All the results previously described show a significant involvement of NO<sub>2</sub>-Ln in plant physiology and specifically in the defense response to adverse abiotic conditions. However, the mechanisms by which this nitro-fatty acid is able to launch this plant-defense response remain unknown. As has been described for NO<sub>2</sub>-OA or NO<sub>2</sub>-LA in animal systems, the ability of NO<sub>2</sub>-Ln to modulate the generation of NO has also been assessed in the Arabidopsis in order to define how NO<sub>2</sub>-Ln sets up this antioxidant mechanism.

Firstly, several *in vitro* approaches were carried out to assess the capacity of NO<sub>2</sub>-Ln to generate NO. An initial study was performed using a spectrophotometric assay with DAF-2 fluorophore in phosphate buffer and in the presence of 5 μM of NO<sub>2</sub>-Ln. Outcomes derived from this analysis showed that this molecule was able to boost fluorescence due to NO release, this being reduced with the application of the NO scavenger, cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide) [35]. In this regard, a progressive increase in the NO release was registered up to 20 min and afterwards a decrease, this coinciding with a fall in the NO<sub>2</sub>-Ln content analyzed by HPLC-MS/MS [35]. In line with these results, the analysis of the ability of S-nitrosoglutathione (GSNO) to release NO has previously been described, and that GSNO has been shown to have a maximum of NO release at 30 min with a drastic reduction in this generation from this moment [73]. Therefore, both the biological NO donor GSNO and NO<sub>2</sub>-Ln have almost identical patterns of NO release in aqueous solutions. Due to pitfalls of NO detection with fluorophores such as DAF, the oxidation of oxyhemoglobin to methemoglobin due to NO release was also assessed with NO<sub>2</sub>-Ln. The incubation of this nitro-fatty acid with oxyhemoglobin led to typical changes in absorbance such as a decrease at 580 nm and 543 nm and a rise in the absorbance at 630 nm and 503 nm, confirming the ability of NO<sub>2</sub>-Ln to release NO by this approach [35]. Finally, this capacity was also quantified by ozone chemiluminescence such that both NO<sub>2</sub>-Ln (100 and 1000 μM) and NO<sub>2</sub>-LA (80 μM) reached a maximum of NO release at a neutral pH (pH 7.4) and a minimum at acidic conditions (pH 5.4 and 6.4) with

an amount of NO release ranging from 10 to 210 nM/min [35]. These findings highlight that NO release from NO<sub>2</sub>-FAs is not favored under acidic conditions and this could have great relevance in cell compartments and organelles such as mitochondria, peroxisomes, and cytosol, where the pH values are neutral or basic [74].

Notably, although it has been proposed that NO release from NO<sub>2</sub>-FAs is less than 1% and is of minor significance *in vivo* [28], it might be worth considering that NO released.

in plant systems could have a significant importance due to the ability of NO<sub>2</sub>-Ln to release NO is 4.7–40-fold higher than this capacity analyzed in animal systems [35], which it is not surprising given that plant systems are the natural source of these fatty acids. Furthermore, it has been proposed that NO<sub>2</sub>-FA are not free in the cell under basal conditions, but part of cell membranes and/or adducted with proteins [14,16]. In these situations, the ability to release NO is very low under physiological conditions. Nevertheless, the higher content of NO<sub>2</sub>-FAs detected under different abiotic stressful situations in plants such as wounding, low temperature, salinity or heavy metals [27], could occur with a parallel increase in the content of NO and may act as an important signaling mechanism under these adverse conditions.

On the other hand, it has been also shown that NO<sub>2</sub>-Ln is able to release NO *in vivo*. A preliminary study on the capacity of NO<sub>2</sub>-Ln to be a NO donor *in vivo* was performed in leaves and roots from 30-d-old arabidopsis plants pre-incubated with 1 mM NO<sub>2</sub>-Ln [75]. The result of this incubation significantly augmented green fluorescence due to NO release in both organs. Furthermore, the pre-treatment with cPTIO dramatically reduced fluorescence, thus confirming that NO<sub>2</sub>-Ln releases NO inside leaves and roots from 30-d-old arabidopsis plants. Afterwards, this ability was also checked in 7-d-old arabidopsis seedlings pre-incubated with 100 μM NO<sub>2</sub>-Ln, and a rise in fluorescence was also detected in roots from these plantlets and with a reduced fluorescence observed after cPTIO application [48]. Finally, with the use of 9-d-old ACSC, the same behavior resulted using 100 μM NO<sub>2</sub>-Ln [35]. In this respect, it is important to bear in mind that NO was found inside cells, where the pH is neutral. Accordingly, the capacity of NO<sub>2</sub>-Ln to be a NO donor seems to be important in organelles and cell compartments where the pH is neutral or basic. All these results underscore the capability of NO<sub>2</sub>-Ln to be a NO donor and the possible implications of this molecule in a wide range of signaling actions in plant physiology.

Fig. 1 shows a schematic model of NO<sub>2</sub>-Ln signaling. This nitro-fatty acid can establish a defense mechanism and an antioxidant response against adverse environmental conditions through the induction in the expression of a large set of heat-shock proteins (HSPs) and several antioxidant systems such as ascorbate peroxidase (APX), among others (pathway 1). Furthermore, it has recently been shown that NO<sub>2</sub>-Ln is a NO donor (pathway 2). Based on the key role of NO in a wide range of physiological and stress-response processes, the ability of NO<sub>2</sub>-Ln to act as a NO donor may have great relevance in plants. In fact, given that the content of NO<sub>2</sub>-Ln suffers a significant increase under several abiotic stress situations such as mechanical wounding, salinity, low temperature or cadmium stresses and the initial stage of Arabidopsis development [27], the NO release from increased NO<sub>2</sub>-Ln could be mediating diverse signaling actions that supporting these processes. Therefore, the ability of NO<sub>2</sub>-Ln to act as a NO donor may be able to support the NO implication in plant development, different biotic or abiotic processes, the antioxidant response or even mediating several post-translational modifications (NO-PTMs) as nitration and S-nitrosylation through which NO transmits its bioactivity [76–78]. In the latter case, NO released from NO<sub>2</sub>-Ln could bind to specific cysteine thiol groups of proteins thus promoting the formation of S-nitrosothiols (SNOs) and therefore be they implied in processes such as plant immunity [79,80] or the response to abiotic stress conditions [21,64,81]. In fact, most of the S-nitrosylated proteins involved in the response to abiotic stress situations are redox-related proteins, such as ROS generating enzymes or antioxidant systems, suggesting a role of S-

nitrosylation as a key regulator of the redox homeostasis during adverse environmental conditions. In this regard, results derived from RNA-seq analysis showed that ascorbate peroxidase is highly induced by NO<sub>2</sub>-Ln treatment [27]. Very recently, the S-nitrosylation of this enzyme was observed both *in vitro* and *in vivo* under salt stress and thus promoting this PTM an increase in the activity of this enzyme [61]. Therefore, NO released from NO<sub>2</sub>-Ln could be mediating an S-nitrosylation process of APX enzyme and hence increasing the enzyme activity, thus preventing the oxidative stress generated under this adverse condition. At this point, it could be important not to rule out that, based on the ability of NO<sub>2</sub>-Ln to be a NO donor, this molecule could be binding to free GSH on plants and generating S-nitrosoglutathione (GSNO), contributing to increase the levels of low molecular weight SNO in plant systems. Finally, NO derived from NO<sub>2</sub>-Ln could be reacting with superoxide anion (O<sub>2</sub><sup>-</sup>), commonly produced under several stress situations [69], thus generating the most powerful oxidant peroxynitrite (ONOO<sup>-</sup>) [82]. This RNS could be mediating the nitration of specific tyrosine residues from key cellular targets thus affecting concrete signaling pathways or, by contrast, provoking an increase in the protein nitration content.

The electrophilic capacity of nitroalkenes is also a feasible signaling pathway with important components of plant signaling (pathway 3). Results obtained in animal systems have shown that nitroalkylation of specific anti-inflammatory and antioxidant-related proteins leads to the modification of their activities, thus promoting important beneficial mechanisms [2,7]. Due to there is no information available concerning this PTM in plants, analyzing this capacity with key components of antioxidant pathways could be useful to understand the mechanism of action of NO<sub>2</sub>-FAs in plant systems. In close relation to this, it is also plausible that under nitro-oxidative stress conditions, in which a rise in the content of ROS and RNS take place, the oxidation of the Michael adduct and subsequent release of free nitroalkenes could occur (pathway 4). This could have important signaling implications under stress conditions, because free NO<sub>2</sub>-FAs may be able to activate the relevant antioxidant properties that these molecules possess. Finally, the observed increase in the expression of APX could help alleviate the oxidative stress generated under stressful conditions by detoxifying the H<sub>2</sub>O<sub>2</sub> produced under these circumstances.

## 5. Conclusions and future perspectives

A significant advance in understanding the interaction between NO and NO-derived molecules with unsaturated fatty acids has been achieved in animal systems. This interaction has started to be studied very recently in plant systems with the endogenous occurrence of nitrated derivatives of linolenic acid in the model plant *Arabidopsis*. Although an antioxidant response and a defense mechanism against several abiotic-stress conditions is activated by NO<sub>2</sub>-Ln and the ability of this molecule to release NO has also been reported, the precise mechanisms by which this nitro-fatty acid is able to induce these responses remain unknown. In this regard, future research should be focused on analyzing the electrophilic ability of NO<sub>2</sub>-FAs with specific plant-cell targets and their involvement in plant signaling. It is also important to take into account the capacity of NO<sub>2</sub>-Ln to release NO as well as the further implications of this molecule in a wide range of actions in plant physiology. Moreover, other mechanisms involving NO<sub>2</sub>-FAs could be considered which will hopefully offer insight into how NO<sub>2</sub>-Ln signaling regulates the important antioxidant and defense mechanisms that this molecule is able to set up. Finally, current mass spectrometry approaches have achieved a higher sensitivity allowing the routine and suitable level detection of these important signaling molecules. Therefore and in view of this landscape, NO<sub>2</sub>-FAs could be considered as new key mediators of NO metabolism in both physiological and stress conditions.

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