Endogenous Biosynthesis of S-Nitrosoglutathione From Nitro-Fatty Acids in Plants

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Nitro-fatty acids (NO2-FAs) are novel molecules resulting from the interaction of unsaturated fatty acids and nitric oxide (NO) or NO-related molecules. In plants, it has recently been described that NO2-FAs trigger an antioxidant and a defence response against stressful situations. Among the properties of NO2-FAs highlight the ability to release NO therefore modulating specific protein targets through post-translational modifications (NO-PTMs). Thus, based on the capacity of NO2-FAs to act as physiological NO donors and using high-accuracy mass-spectrometric approaches, herein, we show that endogenous nitro-linolenic acid (NO2-Ln) can modulate S-nitrosoglutathione (GSNO) biosynthesis in Arabidopsis. The incubation of NO2-Ln with GSH was analyzed by LC-MS/MS and the in vitro synthesis of GSNO was noted. The in vivo confirmation of this behavior was carried out by incubating Arabidopsis plants with 15N-labeled NO2-Ln throughout the roots, and 15N-labeled GSNO (GS15NO) was detected in the leaves. With the aim to go in depth in the relation of NO2-FA and GSNO in plants, Arabidopsis alkenal reductase mutants (aer mutants) which modulate NO2-FAs levels were used. Our results constitute the first evidence of the modulation of a key NO biological reservoir in plants (GSNO) by these novel NO2-FAs, increasing knowledge about S-nitrosothiols and GSNO-signaling pathways in plants.

Keywords: nitro-fatty acids, nitric oxide, S-nitrosoglutathione, S-nitrosothiols, NO-signaling, nitric oxide donor, Arabidopsis, alkenal reductase

INTRODUCTION

Nitric oxide (NO), a small, gaseous, and highly reactive molecule able to cross cell membranes, has been described as an important biological messenger both in animal and plant systems (Stamler et al., 1992; Yu et al., 2014). In the last few years, diverse studies have described NO as a regulator involved in disease resistance, the response to different abiotic stresses, and as a key molecule in plant physiological processes such as stomatal closure, seed germination, iron homeostasis or several developmental processes (Delledonne et al., 1998; Garcia-Mata et al., 2003; Valderrama et al., 2007; Chaki et al., 2009; Begara-Morales et al., 2013).
During the last few years, a growing body of studies has highlighted the relevance of fatty acid nitration in living systems. These molecules possess important biological properties, including the ability to release NO from NO2-FAs. Furthermore, a rearrangement in the structure of NO2-FAs with the putative release of this gaseous molecule has also been noted. On the other hand, NO2-FAs are also called nitroalkenes and have been proposed. In this sense, based on a modified Nef-reaction, the generation of a hydroxy-nitroso intermediate capable of producing NO has been postulated. For instance, a rise in the levels of SNO has been associated with greater susceptibility to pathogen infection and has also been proposed as a new wound signal in sunflower seedlings subjected to mechanical wounding.

Among different SNOs, highlight S-nitrosogluthathione (GSNO) constituting the S-nitrosated derivative of glutathione (GSH), the major intracellular antioxidant in plants. GSNO has been considered a major mobile biological reservoir of NO bioactivity and an essential component of NO-dependent signal transduction. GSNO has been located in vascular tissues, collenchyma cells and epidermal cells, pointing to this molecule as a mobile NO signal throughout the plant. In this sense, it bears mentioning that plasmolysis has the notable ability to propagate messengers such as different reactive oxygen and nitrogen species (ROS and RNS) during plant defence and nitrogen metabolism (Frungillo et al., 2014). In this scenario, knockout lines of this enzyme resulted in higher levels of GSNO and nitroalkene-related signaling pathways. In this line, AtAER, as the plant homologous of PGR-1, also regulates the cellular level of NO2-FAs in plants.
this study provide novel information concerning the SNO biosynthesis mechanisms, indicating that modulation of cellular levels of NO$_2$-FAs can directly influence the GSNO levels and, indirectly, SNOs. Thus, NO$_2$-FAs can be considered key players regulating the NO-dependent signaling pathways, highlighting the relevance of understanding the metabolism of GSNO in plant systems.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

*Arabidopsis thaliana* ecotype Columbia and aer mutant (SALK 005324C) plants were used in this study. The homocystegys of aer mutant (Alonso et al., 2003) was confirmed by PCR using the primers designed according to the Salk Institute Genomic Analysis Laboratory instructions (Table S1). For the different analyses, 7-day-old and 45-day-old *Arabidopsis (Arabidopsis thaliana)* plants were used. Both wild-type (WT) and mutant seeds were surface-sterilized for 5 min in 70% (v/v) ethanol containing 0.1% (w/v) SDS, placed for 20 min in sterile water containing 20% (v/v) SDS, and washed four times in sterile water. Then, seeds were grown up to 7 days in 0.8% phytoagar Petri plates under controlled conditions. The 45-day-old *Arabidopsis* plants were obtained by sowing seeds in tubes with 1% phytoagar and growing them in a culture chamber for 7 days under anaerobic conditions. Afterward, seeds were transferred to hydroponic cultures with a specific growth medium (Cellier et al., 2004) and aeration in controlled conditions (Day: 16 h, 22°C. Night: 8 h, 18°C. Light intensity: of 100 s$^{-1}$).

For treatments, 15NO$_2$-Ln was firstly synthesized and quantified as previously described (Mata-Pérez et al., 2016b) for the synthesis of NO$_2$-Ln but using 15NaNO$_2$ (Sigma-Aldrich, 490814) as a nitrating agent. Because NO$_2$-Ln is not commercially available, it was synthesized by a nitrosemilence-oxidation-hydroxylselenoxide elimination sequence as previously described (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2018) with minor modifications. Briefly, commercial linolenic acid (1.1 mmol) was incubated with solid mercury chloride (1.4 mmol), phenylselenyl bromide (1.1 mmol) and 15NaNO$_2$ (1.1 mmol) in a mixture of tetrahydrofuran-acetonitrile (1:1, v/v, 7.0 ml). This mixture was kept under Ar atmosphere for 4 h with continuous agitation. After removing solid suspension and solvent, the residue was dissolved in tetrahydrofuran (7.0 ml) and kept in a water-ice bath at 0°C. Then, a 30% hydrogen peroxide solution (11.0 mmol) was added dropwise and the mixture was maintained in the cooling bath for 20 min with continuous agitation. After allowing the sample to reach room temperature, the reaction crude was extracted with hexane (2 × 20 ml), washed with saturated aqueous sodium chloride, dry over anhydrous magnesium sulfate, filter and evaporate to dryness under reduced pressure. The residue was taken up in a hexane/ether/acetic acid mixture (5 ml, 80:20:1, v/v/v) and purified by flash column chromatography (silica gel 60, 230–400 mesh, Fluka, Buches, Switzerland) with a mixture of hexane/ether/acetic acid (80:20:1, v/v/v) and ensuring the purification of mononitrated linolenic acid. Finally, the fractions were analyzed by TLC, NMR and LC as described by Mata-Pérez et al. (2016b).

**Synthesis and Quantification of GSNO and GS$^{15}$NO Standards**

GS$^{15}$NO and 15N-labeled GSNO (GS$^{15}$NO) standards were prepared according to Hart (1985) by acid-catalyzed nitrosation of GSH (Sigma-Aldrich, G4251). Sodium nitrite (15N-labeled) (Sigma-Aldrich) was used to synthesize GS$^{15}$NO/GS$^{15}$NO, respectively. These compounds were quantified by measuring the absorbance at 334 nm (ε = 0.92 mM$^{-1}$·cm$^{-1}$).

**In Vitro Synthesis of GSNO From NO$_2$-Ln and GSH**

To study the formation of GSNO from NO$_2$-Ln and GSH, we incubated several concentrations of NO$_2$-Ln (0.1 and 1 mM) with 1 mM GSH in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM DTPA (diethylenetriaminepentaacetic acid) for 1 h at RT with a gentle agitation. Reactions were conducted in darkness. Formation of GSNO was analyzed by LC-ES/MS (Bruker Esquire 6000, HPLC Agilent 1100) in negative ion mode. The different analytes were separated in a Waters Spherisorb ODS2 C18 column (3 mm × 125 mm, 5 μm). The mobile-phase composition was water (A) and acetonitrile (B) both with 1% of formic acid at a flow rate of 0.6 ml min$^{-1}$. The gradient profile was as follows: 2–5% B (0–5 min); 40–95% B (6–22 min); and 95–2% B (22–25 min). MS/MS/MS (M3) analysis from GSNO was conducted in 0.40 V (335) and 0.60 V (305). The desolvation temperature was set at 400°C. In all cases, the data were collected, analyzed, and processed using Data Analysis Mass Spectrometry Software (Bruker, Daltonics).

**Detection of 15NO$_2$-Ln in *Arabidopsis* Leaves and NO$_2$-Ln in WT and aer Mutants Seedlings**

For this experimental design, and using 45-day-old *Arabidopsis* plants, the nutrient solution was removed and the root system was gently washed with distilled water (Begara-Morales et al., 2014a; Begara-Morales et al., 2014b). These plants were then incubated with 1 mM 15NO$_2$-Ln for 3 h, and this molecule was detected in leaves under non-stress conditions.

Lipid extracts from 45-day-old *Arabidopsis* leaves and 7-day-old WT and aer mutants seedlings were obtained using the Bligh and Dyer method (Bligh and Dyer, 1959) and prepared for LC-MS/MS detection of 15NO$_2$-Ln and NO$_2$-Ln, respectively, as it has been previously described (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2018).

**Detection of Endogenous GSNO and GS$^{15}$NO in *Arabidopsis***

For the endogenous detection of GSNO and GS$^{15}$NO, the method used was similar to that described elsewhere (Tsikas et al., 2013) with some modifications. All steps were performed under cooling or 4°C and darkness. The samples were worked up fresh and analyzed quickly to avoid the degradation of the GSNO. In this regard, *Arabidopsis* leaves and seedlings were
ground to a powder in a mortar with liquid nitrogen and suspended in an extraction buffer composed by 100 mM phosphate buffer, pH 7.8, containing 0.1 mM DTPA, 5 mM NEM (N-ethylmaleimide), 0.01 mM neocuprone (1/2, FW/V). Homogenates were centrifuged at 16,000xg for 10 min, 4°C and filtered using a standard 0.22-µm filter. Then, samples were ultra-filtered by centrifugation at 8,000xg, 30 min, 4°C using 5-kDa Vivaspin 2 HydroSart 2-ml cartridges. The ultra-filtered sample was placed into the precooled (4°C) autosampler and 25 µl were injected into LC-MS/MS instrument.

GSNO endogenous content was quantified by carrying out an internal standard calibration with GSNO and GS15NO. Next, GSNO or GS15NO were added to aliquots of the sample in a range of 0–6 nM just before being analyzed by LC-MS/MS. Because GSNO is very unstable, the loss of this molecule during sample processing was evaluated by spiking 75 nM GS15NO into the extraction buffer. This loss during sample work-up was estimated at about 80% from the initial spiked amount.

The analytes were separated using a Dionex Ultimate 3000 rapid separation liquid chromatograph (RSLC) (Thermo Scientific, USA) instrument. This was equipped with an Agilent Zorbax Rapid Resolution High-Definition (RRHD) Eclipse Plus C18 column (2.1 mm × 100 mm, 1.8-µm particle size). The mobile-phase composition was water (A) and acetonitrile (B), both of them with 1% of formic acid at a flow rate of 0.6 ml min⁻¹. The temperature of the column was 25°C and the injection volume was 20 µl. The gradient profile was as follows: 0 min, 0% B; 4 min, 100% B; 5 min, 100% B; 7 min, 0% B; 8 min, 0% B; 10 min.

The UHPLC system was connected to a TSQ Quantiva triple quadrupole (QqQ) (Thermo Scientific, USA) equipped with a heated electrospray ionization probe (HESI) operating in positive ion mode with the following operation parameters: spray voltage: 4,500 V; sheath gas 45; aux gas 5 arbitrary units; ion transfer tube temperature 150°C; vaporizer temperature 300°C; collision gas (CID), 1.5 mTorr. Multiple-reaction monitoring (MRM) transitions were optimized for each compound (Table S2). XCalibur software 3.0.63 (Thermo Fisher Scientific, San José, CA, USA) was used for method development and data analysis.

**Quantitative Real-Time Reverse Transcriptase-PCR (qRT-PCR)**

RNA isolation and gene expression of AER by qRT-PCR were performed in WT and aer 7-day-old Arabidopsis seedlings as previously described (Begara-Morales, 2014b RNA seq) using Actin 12 (AT3G46520) as internal standard. The specific primers used are listed in Table S1.

**Crude Extracts of Arabidopsis Seedlings and Immunodetection of AER**

Seven-day-old WT and aer Arabidopsis seedlings were ground to a powder in liquid nitrogen using a mortar and pestle, and the resulting powder was suspended in the extraction buffer (100 mM Tris-HCl buffer, pH 7.5, containing 0.1mM EDTA, 7% (w/v) PVPP, 5% Suc, 0.0005% Triton X-100, 1 mM PMSF, 15 mM DTT, and a commercial cocktail of protease inhibitors (AEBSF, 1,10-phenanthroline, pepstatin A, leupeptine, bestatin, and E-64 from Sigma-Aldrich; 1/2, FW/v)). Then, the crude extracts were centrifuged twice at 3,000xg for 6 min. Total protein content was analyzed by Bradford assay and separated by 10% SDS-PAGE and transferred to PVDF membranes (Immobilon P, Millipore, Bedford, MA, USA). For AER immunodetection, an specific antibody against Arabidopsis AER (Mano et al., 2005) was used at a dilution of 1:1000 and the immunoreactive band was detected using a photographic film (Hyperfilm, Amershams Pharmacia Biotech) with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech).

**Lipid Extraction and Fatty Acid Analysis**

Lipid extracts from 7-day-old WT and aer Arabidopsis plants were obtained by the Bligh and Dyer method (Bligh and Dyer, 1959) and the content of fatty acids was analyzed by gas mass spectrometry (Agilent 7890A) as previously described (Mata-Pérez, 2015). Briefly, the lipid fractions were evaporated under a stream of nitrogen and dissolved in benzene and Meth-Pre II (Alltech Chemicals Cat. No. 18007) GC reagent to perform the transesterification of the lipid fractions. Following the derivatization stage, a GC/MS analysis was carried out by injecting a 1-µl solution. Analyses were carried out in a 7890A GC system (Agilent, USA) equipped with an SP-2560 capillary column (100 m × 0.25 mm × 0.25 µm) and a Quattro micro GC mass spectrometer (Waters, USA). The GC column procedure was as follows: initial temperature 140°C, maintained for 5 min, increased at 4°C min⁻¹ to 250°C with a split ratio at injector port of 1:10. A standard oil mixture (Supelco ref. 18919-1AMP) was used to calibrate the gas chromatograph.

**Statistical Analysis**

To estimate the statistical significance between means, the data were analyzed by Student’s t-test. The differences were significant at p < 0.05. For each series of experiments, at least three independent biological replicates have been performed with three technical replicates per biological assay.

**RESULTS**

**In Vitro Synthesis of GSNO From GSH and NO2-Ln**

Based on the demonstrated ability of NO2-FAs acting as NO donors (Schnepfer et al., 2005; Gorczynski et al., 2007; Mata-Pérez et al., 2016a), the *in vitro* generation of GSNO from the reaction between GSH and NO2-Ln was analyzed by LC-ES/MS in negative ion mode (Figure S1).

In this sense, the full mass ion spectra (MS) of GSNO standard showed a major ion product with m/z of 335 corresponding to this low-molecular weight SNO when it was analyzed in negative ion mode (Figure S1A). Then the MS/MS (MS2) spectra displayed a major fragment with m/z of 305 (Figure S1B) corresponding to the homolytic dissociation of...
the S-nitroso group for generating the protonated glutathionyl radical ([GS+H]⁺) and the neutral radical NO (30 Da). The MS/MS/MS (MS3) fragmentation led to the detection of an ion fragment with m/z of 160 which confirmed GSNO occurrence (Figure S1C). Afterward, different concentrations of NO₂-Ln (0.1 and 1 mM) were incubated with 1 mM GSH and studied by LC-ESI/MS3 (Figure 1). Under these conditions, a chromatographic peak with MRM transition of 160 m/z was detected in both NO₂-Ln analyzed concentrations (Figures 1C, D). These peaks shared the same retention time as GSNO standard but not with GSH standard, thus confirming the formation of GSNO from the reaction between NO₂-Ln and GSH (Figures 1A, B). Additionally, product ions of the GSNO formed after reaction between NO₂-Ln and GSH showed the same fragmentation pattern as synthetic GSNO (Figure S2), and thereby confirmed the formation of GSNO.

Mobilization of NO₂-Ln Throughout Arabidopsis Plants

The capacity of NO₂-FAs to move through the plant was analyzed. For this, ¹⁵N-labeled NO₂-Ln was synthesized in order to show the presence of this NO₂-FA in the leaves and distinguish it from the endogenous NO₂-Ln. Thus, 1 mM ¹⁵NO₂-Ln was applied to the root system of 45-day-old Arabidopsis plants, as indicated in Materials and Methods. Then, the lipid fraction obtained from Arabidopsis leaves was studied by LC-MS/MS (Figure 2). The results showed a chromatographic peak with the MRM transition of 323/275 m/z (Figure 2B) sharing the same retention time as the ¹⁵NO₂-Ln standard (Figure 2A) and thus highlighting the mobilization of NO₂-FAs from the roots to the leaves of Arabidopsis plants.

Characterization of GSNO Synthesis From NO₂-Ln in Arabidopsis Leaves

The endogenous occurrence of GSNO was analyzed by LC-MS/MS in 45-day-old Arabidopsis plants. The MRM scan mode was used to display the presence of a peak with transitions of m/z 337/307 and 337/232 specific for the fragmentation of GSNO molecule and sharing the same retention time as GSNO standard (Figures 3A, B). According to the indications described in Materials and Methods—regarding the quantification of endogenous GSNO content, the concentration of this SNO in Arabidopsis leaves was 0.91 ± 0.23 pmol/mg protein (Table 1). These findings are consistent with previous data reported for low-mass SNO levels in Arabidopsis leaves (Feechan et al., 2005).

It is important to note that the presence of GS¹⁵NO was assessed by LC-MS/MS in Arabidopsis leaves after the plant roots were incubated with ¹⁵NO₂-Ln. This analysis showed a chromatographic peak sharing the same retention time as GS¹⁵NO standard with m/z 338/307 and 338/232 (Figures 3C, D) and thus confirming the observed peak corresponding to this low-molecular-weight SNO. Regarding the concentration of GS¹⁵NO detected in Arabidopsis leaves after the application of

![Figure 1](https://example.com/figure1.png)  
**FIGURE 1** | In vitro synthesis of GSNO from NO₂-Ln and glutathione (GSH). For the in vitro generation of GSNO, 1 mM GSH was incubated with 1 and 0.1 mM NO₂-Ln, as is described in Materials and Methods. (A, B) show GSH and GSNO standards, respectively. (C, D) display GSNO generated after the incubation of 1 mM GSH with 1 and 0.1 mM NO₂-Ln, respectively. Peaks refer to total ion intensity. Vertical dashed lines indicate peaks with the same retention time. m/z is mass-to-charge ratio. MS indicates full ion mass spectra. MS3 is MS/MS/MS ion fragmentation.

![Figure 2](https://example.com/figure2.png)  
**FIGURE 2** | Detection of ¹⁵NO₂-Ln in Arabidopsis leaves. 45-day-old Arabidopsis plants were incubated with 1 mM ¹⁵NO₂-Ln for 3 h and the lipid fraction from leaves was analyzed by LC-MS/MS as it is indicated in Materials and Methods. (A) ¹⁵NO₂-Ln standard with MRM transition of 323/275 m/z. (B) A chromatographic peak sharing the same retention time and m/z than ¹⁵NO₂-Ln standard in leaves from 45-day-old Arabidopsis plants incubated with ¹⁵NO₂-Ln. Peaks refer to a total ion intensity of 2.19 e4. Vertical dashed lines indicate peaks with the same retention time. MRM indicates multiple monitoring reaction. m/z indicates mass-to-charge ratio.
NO₂-Ln was 0.50 ± 0.15 pmol/mg protein (Table 1). These results confirm the potential of NO₂-Ln to generate GSNO in a significant amount, and the ability of NO₂-Ln to travel throughout the plant system.

NO₂-Ln Modulates the Endogenous Levels of GSNO

With the aim of support the previous demonstration about the relation between NO₂-FAs and GSNO in plants, Arabidopsis Alkenal Reductase (AER, ATG16970) deficient mutant lines were used. AER enzymatic activity modulates the unsaturated fatty acid levels since it is able to reduce unsaturated to saturated bonds. Therefore, this enzyme could have the potential to modulate NO₂-FA levels. To confirm this presumption, we used homozygous aer transgenic seedlings (SALK-005324C) and both transcript and protein levels were analyzed. Results show a decrease of about 35% on AER-transcript level (Figure 4A) and a concomitant reduction of approximately 60% in the AER-protein content (Figure 4B) compared to WT plants hence confirming the AER deficiency on these transgenic plants. To probe the connection between NO₂-FAs and GSNO content, we first observed that aer seedlings showed higher levels of unsaturated fatty acids as linolenic acid (Table 2). Consequently, the decrease in AER expression resulted in a three-fold increase of NO₂-Ln content (Figure 5A) importantly correlated to the observed two-fold increase of GSNO content (Figure 5B).

\[ \text{Endogenous GSNO in Arabidopsis leaves: } 0.91 \pm 0.23 \text{ pmol/mg prot, } 0.0018 \pm 0.00045 \text{ fmol/g FW} \]

\[ \text{GS}^{15}\text{NO} \text{ generated in leaves from Arabidopsis plants incubated with 1 mM NO}_2^2\text{-Ln for 3 h as described in Materials and Methods. Peaks refer to total ion intensity. Vertical dashed lines indicate peaks with the same retention time. m/z is mass-to-charge ratio.} \]
TABLE 2 | Composition of fatty acids (expressed as mg fatty acid / kg FW) in both 7’d wild-type and aer mutant seedlings detected by mass spectrometry techniques (GC-MS). 0.002% of Ln in WT and 0.004 % of Ln in aer mutants are nitrated in the form of NO$_2$-Ln.

| Fatty acids         | WT     | aer     |
|---------------------|--------|---------|
| Oleic acid (18:1)   | 5.86 ± 0.27 | 8.92 ± 0.59* |
| Linoleic acid (18:2)| 42.13 ± 1.99 | 61.18 ± 5.40* |
| Linolenic acid (18:3)| 115.00 ± 4.90 | 158.30 ± 14.20* |
| Stearic acid (18:0) | 11.21 ± 0.81 | 9.70 ± 0.41 |
| Linoleic acid (18:2) | 42.13 ± 1.99 | 61.18 ± 5.40* |
| Oleic acid (18:1)   | 5.86 ± 0.27 | 8.92 ± 0.59* |

Data are expressed as the mean ± SEM from at least three independent samples. Differences were significant at p<0.05 (*).

FIGURE 5 | Endogenous content of NO$_2$-Ln and GSNO of aer mutant seedlings. (A) The detection of endogenous NO$_2$-Ln levels in both wild type and aer mutant 7-day-old seedlings. Lipid extracts from 7-day-old seedlings were obtained as is indicated in Materials and Methods and analyzed by LC-MS/MS. (B) The levels of endogenous S-nitrosoglutathione (GSNO) in both wild type and aer mutant 7-day-old seedlings detected by mass spectrometry techniques. Data are expressed as the mean ± SEM from at least three independent samples. Differences from control values were significant at p < 0.05 (*).

DISCUSSION

For some time, the interest in the role and interaction of NO with biomolecules has significantly intensified. Most of the previous studies have mainly focused on the capability of NO to mediate several post-translational modifications (NO-PTM) such as the nitration and S-nitrosation of proteins. Nevertheless, in the last decade attention has focused on the ability of NO and NO-derived species to interact with non-saturated fatty acids yielding nitro fatty acids (NO$_2$-FAs) (Schopfer et al., 2011; Mata-Pérez et al., 2017). These molecules have emerged as novel signaling mediators in animal and plant systems. In this respect, NO$_2$-FAs can release NO in aqueous solutions and they are also able to mediate post-translational modifications of proteins through a mechanism called nitroalkylation (Lima et al., 2005; Schopfer et al., 2005; Gorczynski et al., 2007; Geisler and Rudolph, 2012; Mata-Pérez et al., 2016a; Aranda-Caño et al., 2019). These capacities confer to NO$_2$-FAs relevant anti-inflammatory, antioxidant, and pro-survival properties in animal systems (Schopfer et al., 2011; Delmastro-Greenwood et al., 2014). In this sense, nitro-oleic (NO$_2$-OA) and nitro-linoleic acids (NO$_2$-LA) blunt pro-inflammatory responses via alkylation of the p65 subunit of NF-κB and they also reduce the expression of vascular-cell adhesion molecule (VCAM)-1 (Cui et al., 2006). Moreover, nitro-conjugated linoleic acid (NO$_2$-cLA) is able to inhibit heme oxygenase 1 (HO-1), helping to resolve inflammation injuries (Bonacci et al., 2012). Beyond the well-defined properties of NO$_2$-FAs in animal systems, it has recently been demonstrated that NO$_2$-Ln is endogenously present in several plant species, including Arabidopsis thaliana, Pisum sativum or Oryza sativa (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2017). An RNA-seq analysis showed that the incubation of Arabidopsis cell cultures with this NO$_2$-FA promoted the induction of a large set of HSPs and several antioxidant systems such as ascorbate peroxidase (APX) or methionine sulfoxide reductase (MSRB) enzymes (Mata-Pérez et al., 2016b). In line with these results, a previous analysis with NO$_2$-OA in human endothelial cell cultures determined that this NO$_2$-FA was also able to prompt a defence response through greater expression of different HSPs (Kansanen et al., 2009), thus highlighting the beneficial responses which NO$_2$-FAs are able to promote. Moreover, a significant rise in the levels of these species has been reported under stress conditions such as inflammation and cardiac ischemia in animal systems (Nadtochiy et al., 2009; Rudolph et al., 2010) or under salinity, mechanical wounding or heavy metal stresses in plants (Mata-Pérez et al., 2016b). Therefore, these novel NO-derived species are important in animal and plant physiology because of their capability to set up a defence response against unfavorable conditions. In this regard, NO$_2$-Ln has been described to be able to regulate the function of APX to detoxify the H$_2$O$_2$ (Aranda-Caño et al., 2019).

Generation of GSNO From NO$_2$-Ln In Vitro and In Vivo

At present, it is well known that NO$_2$-FAs are NO donors in the cell environment (Gorczynski et al., 2007; Mata-Pérez et al., 2016a). Although the capacity of NO$_2$-FAs to release NO was firstly considered to be of minor significance in vivo and less than 1% in vitro, recent studies have shown that NO$_2$-FAs can generate NO in a similar way to GSNO, that is considered the major biological NO reservoir and a key regulator of a wide range of physiological and stress-related processes in plants (Mata-Pérez et al., 2016a; Begara-Morales et al., 2018).
Therefore, NO2-FAs, as NO2-Ln, provide a significant source of NO in plants and together with the high content of GSH in living systems, it could contribute to the total pool of GSNO and SNOs in cells. Based on this background, we investigated both the in vitro and in vivo capacity of NO2-Ln to modulate the generation of GSNO. In this regard, using different concentrations of this NO2-FA, the incubation of NO2-Ln with GSH was analyzed by mass spectrometry and the in vitro formation of GSNO was noted. The formation of GSNO was concentration-dependent, displaying the higher levels of this SNO after incubation of 1 mM of NO2-Ln with 1 mM GSH. Furthermore, to confirm whether NO2-Ln can modulate the levels of GSNO in vivo, we firstly studied the mobilization of 15NO2-FAs through the plant and, to achieve it, we undertook the synthesis of 15N-labeled NO2-Ln (15NO2-Ln). This labeled-NO2-FA was used to differentiate its action from endogenous NO2-Ln in Arabidopsis leaves (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2017). In this sense, 15NO2-Ln was applied to the root system and its occurrence was analyzed in the leaves of 45-day-old Arabidopsis plants. By using high-accuracy mass spectrometry approaches, we detected the presence of 15NO2-Ln in leaves from plants pre-incubated with this labeled NO2-FA. In line with these results, prior studies have shown the application of the fatty acid heptadecanoic acid (17:0) to the root system of several plant species including Glycine max, Zea mays or Lycopersicum esculentum, leading to its detection in leaves and, after the application in leaves, it was detected in both leaves higher on the plant and in roots. These results indicate translocation and the authors conclude that it could probably take place by the phloem (Terzaghi, 1989). Therefore, it has been shown that fatty acids or NO2-FAs can travel and exert their signaling actions throughout the whole plant.

After having shown that NO2-Ln can be mobilized across the plant and reach the shoots, the capability of 15NO2-Ln to modulate the levels of 15N-labeled GS15NO was studied. Firstly, using a LC-MS/MS approach similar to that described elsewhere (Tsikas et al., 2013) with some modifications, we assessed the endogenous occurrence of GSNO in Arabidopsis leaves. The endogenous GSNO level of 7-day-old plants (Figure 5B) detected is lower than that detected in 45 old-day plants (Table 1). Based on these results, GSNO levels appear to decrease during Arabidopsis development.

The endogenous GSNO content detected was consistent with levels previously reported by Feechan et al. (2005) in Arabidopsis after using a 5-kDa cut-off membrane in Arabidopsis leaf extracts and therefore detecting all low molecular weight SNOs. Nevertheless, this endogenous GSNO content is significantly lower than the nanomolar concentration described by Airaki et al. (2011) in Arabidopsis leaves. This apparent discrepancy could be a consequence of the acidic media used for protein extraction in Airaki et al. (2011). In that work, GSNO data at the nanomolar level are likely to be overestimated by an artifactual production of GSNO under these acidic extraction conditions when both nitrite and GSH are present (Broniowska et al., 2013). In addition, in the present work the GSNO detection was performed by LC-MS/MS that is a more sensitive technology that LC-MS to detect endogenous GSNO levels (Tsikas and Hanff, 2018). After showing that GSNO was endogenously present in Arabidopsis leaves, we incubated plants with 15NO2-Ln in the same way as previously described and we studied the occurrence of GS15NO. The results displayed the presence of GS15NO in the shoots after the treatment, thus confirming that NO2-Ln can modulate the generation of GSNO in vivo. We incubated plant roots with 1 mM of 15NO2-Ln and a concentration of GS15NO of 0.50 ± 0.15 pmol/mg was quantified in leaves. Different reasons emerge to explain this apparent low GS15NO detection. It was previously described that the use of 1 mM NO2-Ln can release NO in vitro in a ratio of 0.21 µM/min (Mata-Pérez et al., 2016a) or 12.6 µM/h. Therefore, during plant treatment with 1 mM 15NO2-Ln, the 15NO generated will be in the µM range. Consequently, the in vivo detection of 15NO2-Ln-dependent generation of GS15NO will be apparently low compared with the initial concentration of the labeled nitro fatty acid. In addition, NO2-FAs are more abundant esterified in complex lipids than in the free form (Fazzari et al., 2019). In this line, oral administration of dogs with NO2-OA confirmed that the main distribution of this NO2-FA is esterified in different complex lipids, especially triacylglycerides (TAGs) (Fazzari et al., 2019). Thus, a similar situation could be happening in Arabidopsis plants incubated with NO2-Ln, in which a substantial percentage of the initial amount of NO2-Ln could be esterified in complex lipids, therefore not being able to release NO and, ultimately, generate GSNO. In this regard, the total free NO2-Ln detected in Arabidopsis seedlings is around 4 pmol/g FW in control plants being increased about two-fold after different abiotic stresses (Mata-Pérez et al., 2016b). In addition, NO2-FAs are electrophile molecules that can mediate post-translational modification of proteins by nitroalkylation (Aranda-Caño et al., 2019) and therefore not all pool of NO2-Ln would not contribute to in vivo GSNO generation. Consequently, the GSNO concentration observed is consistent with the NO2-Ln capacity to release NO and its endogenous abundance.

It is worth noting that the exact mechanisms leading to GSNO formation remains unclear (Broniowska et al., 2013; Zafiagnini et al., 2016; Begara-Morales et al., 2018). Instead of a direct reaction of NO with GSH to generate GSNO, the most probably pathways to generate GSNO are the interaction of NO with the glutathionyl radical (GS·) or the formation of N2O3 as an intermediary (Figure 6) (Broniowska and Hogg, 2012; Broniowska et al., 2013; Kolesnik et al., 2013; Begara-Morales et al., 2018). However, it is possible the direct nitrosation of GSH by NO leading to GSNO at submicromolar concentrations of NO (Kolesnik et al., 2013). In this line, NO2-Ln is able to release NO at a rate of 0.21 µM/min at a physiological pH (Mata-Pérez et al., 2016a) and therefore it generates NO at a submicromolar levels. Consequently, the NO2-Ln-dependent generation of GSNO described in this work could be performed as a direct interaction of NO released from NO2-Ln and GSH (Figure 6).
The Modulation of Cellular Levels of NO\textsubscript{2}-Ln Directly Influences the GSNO Production

In order to clarify if the capacity of NO\textsubscript{2}-Ln to modulate GSNO levels could have physiological implications, we used mutant plants that are able to regulate endogenous levels of NO\textsubscript{2}-Ln. In this work, we have demonstrated the capacity of alkenal reductase (AER) enzyme to modulate endogenous levels of NO\textsubscript{2}-FAs, concretely NO\textsubscript{2}-Ln, as previously reported for its homologous in human and rats (prostaglandin reductase, PTGR-1) (Vitturi et al., 2013). A decrease of AER gene expression and protein content exhibited a three-fold increase of NO\textsubscript{2}-Ln and two-fold in GSNO levels, confirming the capacity of NO\textsubscript{2}-Ln to control the abundance of endogenous GSNO and therefore the NO-dependent signaling in plants (Begara-Morales et al., 2018). Furthermore, it is important to note that GS\textsubscript{15}NO\textsubscript{2} detected levels represent approximately 50% of those detected in the control situation, which taking into account the multiple possible targets of NO\textsubscript{2}-Ln, should be considered as a very important contribution. Regarding the mobility of NO and NO-derived molecules, GSNO have been detected in vascular bundles and epidermal cells of several plant species (Chaki et al., 2011a; Chaki et al., 2011b). Hence, it has been postulated that the phloem seems to be an active site for NO metabolism and also for GSNO generation (Gaufeps et al., 2017). All these results may suggest that the phloem can act as a key tissue location for the metabolism of NO\textsubscript{2}-FAs, NO and consequently GSNO.

On the other hand, SNOs and GSNO can mediate NO-PTMs like S-nitrosation of different protein targets hence triggering notorious consequences in their enzymatic activities or their protein functions. In this respect, GSNO and SNOs have been identified in numerous plant situations, highlighting the involvement of these molecules in diverse stressful situations. It is well documented that S-nitrosation plays a key role in plant immunity (Feechan et al., 2005; Romero-Puertas et al., 2008; Tada et al., 2008). For instance, knockout plants for the GSNO\textsubscript{1} enzyme (gsnor1) showed a high content of GSNO and indirectly of SNOs related to more disease susceptibility compared to WT plants (Feechan et al., 2005). Furthermore and regarding abiotic-stress conditions, a modulation in GSNO and SNO levels has also been reported in different plant species (Valderrama et al., 2007; Chaki et al., 2011a; Chaki et al., 2011b), supporting the contention that the abiotic-stress response can be mediated, at least in part, by S-nitrosation-signaling of key protein targets such as pea APX, which upregulates its activity during salt stress (Begara-Morales et al., 2014a).

Related to what has previously mentioned, NO\textsubscript{2}-FAs are lowly abundant in their free form (Tsikas et al., 2009; Mata-Pérez et al., 2016b). Actually, most of these nitro-derivatives are thought to be protein-adducted or putatively esterified with complex lipids being part of cell membranes (Rubbo, 2013). Certain conditions such as the nitro-oxidative burst taking place under several stress circumstances (for instance salt, heavy metal or wounding stresses in Arabidopsis (Mata-Pérez et al., 2016b), can prompt the release of free NO\textsubscript{2}-FAs from the pool of adducted proteins (Padilla et al., 2017) or certain other signals could be de-esterifying complex lipids from cell membranes with the subsequent liberation of free NO\textsubscript{2}-FAs. Bearing this in mind, we would like to highlight that a concomitant increase on the free NO\textsubscript{2}-FA pool together with the high abundance of GSH in living systems creates a perfect environment for the direct modulation of cellular levels of GSNO. This direct relation may have relevant consequences in plant physiology and it may facilitate the understanding about the modulation and control of the SNO-signaling pathway in plants.

Finally, this behavior can be exemplified in the proposed model for the modulation of GSNO-signaling pathway by NO\textsubscript{2}-Ln (Figure 7). NO\textsubscript{2}-FAs have recently been shown to be present in several organs and organelles of diverse plant species (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2017). In fact, these molecules can be mobilized from their cell locations through the plant organs and reach the shoots. This together with the fact that NO\textsubscript{2}-FAs have been described as physiological NO donors and the high abundance of the antioxidant GSH in living systems, may establish a proper cell environment for the formation of GSNO. The generation of this low-molecular-weight SNO from NO\textsubscript{2}-FAs can affect the SNO-signaling pathway by modulating the transport and storage of NO, the response to several (ab)iotic stress conditions, or the mediating
ability of SNOs to perform NO-PTMs. Thus, NO2-FAs can be considered new key modulators in the GSNO-dependent signaling cell response during physiological and stress conditions in plants.

CONCLUSIONS

Our study provides further relevant insights into the signaling mediated by NO2-FAs in plants. Data presented in this study provide novel information concerning the GSNO biosynthesis mechanisms, indicating that modulation of cellular levels of NO2-FAs can directly influence the GSNO levels. In fact, the key property of NO2-Ln to release NO allows to act as a powerful signaling molecule since it is able to induce functional changes mediated by NO or NO-related molecules including post-translational modifications such as S-nitrosation. Therefore, NO2-FAs can be considered key players regulating the NO-bioactivity, so that the study of the interactions between NO2-FAs and GSNO will increase the knowledge about SNO-signaling pathway in plants. On the basis of these results, the control of GSNO by NO2-FAs has emerged as an interesting regulation point of SNO-bioactivity in plant physiology and during (a)biotic stress processes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

This work was conceptualized by JB. Experiments were performed by all authors. The data were analyzed by CM-P, MP, JB-M, and JB. The paper was written by CM-P, MP, JB-M, RV, and JB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00962/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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