NITRIC OXIDE IN PLANTS

EDITED BY: Juan B. Barroso, Raquel Valderrama, Mounira Chaki,
Juan Carlos Begara-Morales and Marek Petrivalsky

PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-235-9
DOI 10.3389/978-2-88971-235-9

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

NITRIC OXIDE IN PLANTS

Topic Editors:
Juan B. Barroso, University of Jaén, Spain
Raquel Valderrama, University of Jaén, Spain
Mounira Chaki, University of Jaén, Spain
Juan Carlos Begara-Morales, University of Jaén, Spain
Marek Petrivalsky, Palacký University, Czechia

Citation: Barroso, J. B., Valderrama, R., Chaki, M., Begara-Morales, J. C., Petrivalsky, M., eds. (2021). Nitric Oxide in Plants. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-235-9

Table of Contents

frontiers in Plant Science

EDITORIAL
published: 25 June 2021
doi: 10.3389/fpls.2021.705157

Editorial: Nitric Oxide in Plants

Raquel Valderrama[1], Mounira Chaki[1], Juan Carlos Begara-Morales[1], Marek Petrivalský[2] and Juan B. Barroso[1]**

[1] Group of Biochemistry and Cell Signalling in Nitric Oxide, Department of Experimental Biology, Center for Advanced Studies in Olive Grove and Olive Oils, Faculty of Experimental Sciences, University of Jaén, Jaén, Spain, [2] Department of Biochemistry, Faculty of Science, Palacky University in Olomouc, Olomouc, Czechia

Keywords: nitric oxide signaling, phytohormone signaling, plant growth, seed development, fruit ripening, rhizobium-legume symbiosis, nitro-fatty acids, abiotic stress

Editorial on the Research Topic

Nitric Oxide in Plants

Nitric oxide (NO) is a multifunctional gaseous signaling molecule implicated in both physiological and pathological functions in plant systems. Since the first publication accounting for the presence of NO in plants in 1979, numerous milestone discoveries have revealed the complexity of its metabolic and signaling networks. This Research Topic on *Nitric Oxide in Plants* comprises 12 manuscripts and aims to provide new insights into the molecular networks of NO through both original articles and detailed reviews related to seed and plant development, abiotic stress responses, signaling, and beneficial plant-microbial interactions.

In the scope of plant development, the role of NO as a root growth regulator is investigated by Oláh et al. in an original article where the interplay between NO and strigolactones (SLs) in stress-free Arabidopsis plants is studied. Authors correlate the deficiency in SL synthesis or signaling with decreased *S*-nitrosoglutathione (GSNO) reductase (GSNOR) protein abundance and activity and conclude underlining the need for functional GSNOR to control NO/*S*-nitrosothiol levels during SL-induced primary root elongation. However, the authors specify that both SL and karrikin phytohormone signaling could have overlapping roles in their experimental design. Another big challenge for plant biotechnology is the improvement of crop productivity. It is well-established the positive correlation between the supply of nitrogen (*N*) fertilizers and the seed yield. In this context, the original article of Nejamkin et al. investigates the link between N availability and NO in the promotion of growth and seed production in transgenic tobacco plants transformed with the *Ostreococcus tauri* NOS gene (*OtNOS*). They show a severe attenuation in the *OtNOS*-promoted stimulation of growth and production in transgenic plants under conditions of nitrogen scarcity and emphasize the beneficial effects of the application of nitrate (NO_3^-)-containing fertilizers. The same kind of fertilizers is also recommended by Li et al. in their original article about Lycium fruits development and ripening in order to increase the endogenous NO content. They induced the silencing of the nitrate reductase (NR) gene in fresh fruits to demonstrate that NR-derived NO negatively regulated fruit coloration/ripening by suppressing anthocyanin *de novo* biosynthesis, as well as by redirecting the flavonoid biosynthetic pathway to proanthocyanidins production, a colorless taste factor. They also found the antagonistic effects of NO and abscisic acid (ABA) in the regulation of the coloration of Lycium fruits.

Two manuscripts go in-depth in the study of the role of NO in the nitrogen fixation by the symbiotic interaction between legume and rhizobium partners. Signorelli et al. reviews the current knowledge about the sources and fundamental roles of NO during the different stages of the interaction and discuss the connections between the metabolism of NO and cytokinins, auxin, and ABA signaling pathways. They also report dose- and time-dependent effects of NO on bacterial nitrogenase expression and activity in mature nodules and the protective role of leghemoglobins

OPEN ACCESS

Edited and reviewed by:
Vicent Arbona,
University of Jaume I, Spain

****Correspondence:***
Raquel Valderrama
ravalde@ujaen.es
Juan B. Barroso
jbarroso@ujaen.es

Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *04 May 2021*
Accepted: *01 June 2021*
Published: *25 June 2021*

Citation:
Valderrama R, Chaki M, Begara-Morales JC, Petrivalský M and Barroso JB (2021) Editorial: Nitric Oxide in Plants. Front. Plant Sci. 12:705157. doi: 10.3389/fpls.2021.705157

as reactive oxygen species (ROS)/ reactive nitrogen species (RNS)-scavengers which in turn could inactivate nitrogenase. Following the same thematic, the original article of Berger et al. investigates the contribution of NR, as the main source of NO in plants, for the functioning of the symbiosis. In this paper, a dual role in nodule functioning is attributed to NR: generating NO as a signal for gene regulation and metabolic adaptation, and contributing to the energy supply under the hypoxic conditions prevailing inside the nodule.

Seed germination is another relevant issue for biologists and agriculture and the critical role of NO in breaking dormancy has been extensively demonstrated. In this Topic, Wang et al. analyze the crosstalk between NO and the inhibitory effects of ABA in releasing potato seed dormancy. Researchers probe in their original article the ABA-dependent decrease of the NO content and the NOS-like and NR activities in potato during sprouting. On the other hand, the promotion by NO of ABA catabolism and the inhibition of ABA biosynthesis ultimately induced dormancy release and tuber sprouting. In the same line, Ciacka et al. summarize in their mini-review the current knowledge on NO and other RNS contribution in the modulation of crucial events related to the preservation of seed vigor and/or

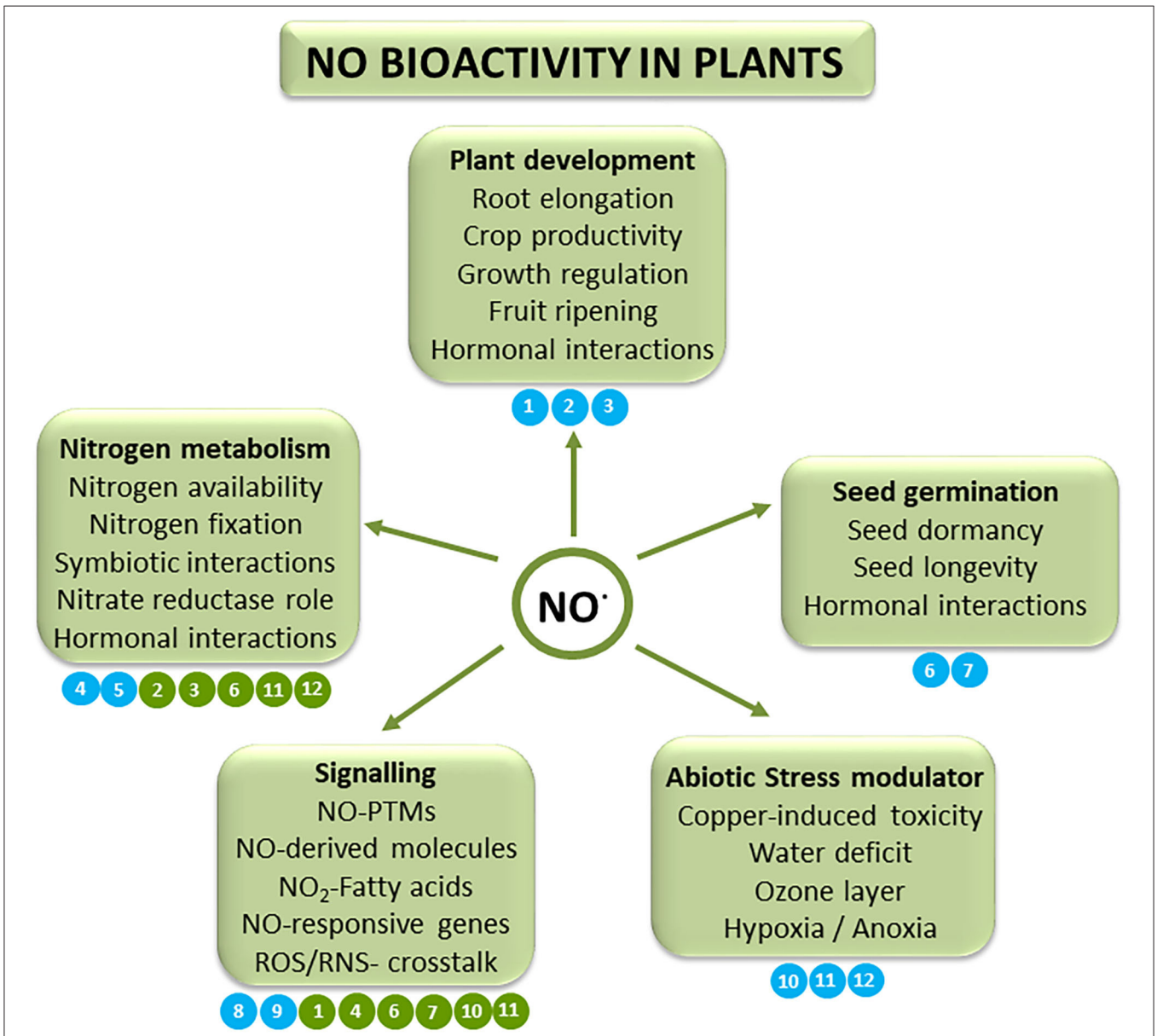

FIGURE 1 | Schematic model of the NO bioactivity in plants in the scope of plant development, seed germination, abiotic stress situations, signaling, and nitrogen metabolism. The numbers in blue indicate the main subject matter covered in each article in the Research Topic. Additionally, the numbers in green indicate the relationship to additional topics. [1] Oláh et al. [2] Nejamkin et al. [3] Li et al. [4] Signorelli et al. [5] Berger et al. [6] Wang et al. [7] Ciacka et al. [8] Mata-Pérez et al. [9] Nabi et al. [10] Rather et al. [11] Pissolato et al. [12] Timilsina et al..

regulation of seed longevity during aging. Authors highlight the interest of ROS-RNS cross-talk as NO counteracts ROS generation and stimulates the antioxidant system. Additionally, the authors indicate a concentration- and time-dependent effect of NO on ethylene, polyamines and ABA biosynthesis as well as the implication of the protein modification by *S*-nitrosylation in the regulation of the deterioration processes in seeds. Another kind of NO-derived modification, consistent with the nitration of unsaturated fatty acids, has been highlighted in the last few years in plant systems. This interaction results in the formation of nitro-fatty acids (NO_2-FAs). In this regard, the original article of Mata-Pérez et al. reports the ability of nitro-linolenic acid (NO_2-Ln) to move from roots to leaves in Arabidopsis plants. Moreover, given the potential of NO_2-Ln to release NO at physiological pH and temperature, it can modulate the *in vitro* and *in vivo* levels of GSNO, the major mobile biological reservoir of NO bioactivity. The manuscript provided by Nabi et al. analyzes the interaction of NO with biological molecules too. In particular, they contribute to this Topic with an original sequencing study aiming at the functional characterization of NO-responsive domains of unknown function (DUF)-containing genes in Arabidopsis leaves. They identified 231 upregulated and 206 down-regulated DUF genes. The study focuses on *AtDUF569* given its significant increase in expression and interesting interactions with other proteins. This gene negatively regulates biotic stress responses and differentially regulates plant shoot and root growth under nitro-oxidative stress conditions.

In the context of the implication of NO in the tolerance against abiotic stress situations, Rather et al. highlights in their review the major aspects of copper (Cu)-induced toxicity in plants and summarize two possible strategies for NO to mitigate damages: the upregulation of the enzymatic and non-enzymatic antioxidant systems or defense genes, and the NO-participation in the root exclusion and/or activation of metal-chelating ligands such as metallothioneins and phytochelatins. In the same scope of environmental injuries, Pissolato et al. evidenced in their original article an interesting strategy for alleviating the negative effects of water deficit on sugarcane plants. These researchers demonstrate that the increase in the NO_3 supply enhanced NO synthesis through NR which improves sugarcane performance under drought. Another environmental disaster is global warming and ozone layer depletion. Among others, nitrous oxide (N_2O) is a potent greenhouse gas. The review of Timilsina et al. describes the mitochondrial reduction of the NR-derived NO to N_2O under low oxygen conditions and proposes this route as a way to protect the mitochondrial and cellular integrity from the toxicity of NO accumulation under hypoxia and anoxia.

In summary, the variety of the work reported here (**Figure 1**) reinforces the already known relevance of NO in the biology of plant systems and highlights the complexity of its signaling roles. We hope that this Topic will encourage researchers to continue and initiate new studies in this exciting area where a plethora of molecular mechanisms remain unexplored.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This research was funded by ERDF grants co-financed by the Spanish Ministry of Economy and Competitiveness (Project PGC2018-096405-B-I00), the Junta de Andalucía (group BIO286), the R&D&I project within the framework Programme of FEDER Andalucía 2014–2020 (Reference 1263509), and the funding for the recruitment of researchers under Action 10 of the Research Support Plan of the University of Jaén (2019–2020; R.02/10/2020).

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 March 2020
doi: 10.3389/fpls.2020.00312

Nitrogen Depletion Blocks Growth Stimulation Driven by the Expression of Nitric Oxide Synthase in Tobacco

Andrés Nejamkin[1†], Noelia Foresi[1†], Martín L. Mayta[2], Anabella F. Lodeyro[2], Fiorella Del Castello[1], Natalia Correa-Aragunde[1], Néstor Carrillo[2] and Lorenzo Lamattina[1]*

[1] Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina, [2] Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Nitric oxide (NO) is a messenger molecule widespread studied in plant physiology. Latter evidence supports the lack of a NO-producing system involving a NO synthase (NOS) activity in higher plants. However, a NOS gene from the unicellular marine alga *Ostreococcus tauri* (*OtNOS*) was characterized in recent years. *Ot*NOS is a genuine NOS, with similar spectroscopic fingerprints to mammalian NOSs and high NO producing capacity. We are interested in investigating whether OtNOS activity alters nitrogen metabolism and nitrogen availability, thus improving growth promotion conditions in tobacco. Tobacco plants were transformed with *OtNOS* under the constitutive CaMV 35S promoter. Transgenic tobacco plants expressing *OtNOS* accumulated higher NO levels compared to siblings transformed with the empty vector, and displayed accelerated growth in different media containing sufficient nitrogen availability. Under conditions of nitrogen scarcity, the growth promoting effect of the *OtNOS* expression is diluted in terms of total leaf area, protein content and seed production. It is proposed that OtNOS might possess a plant growth promoting effect through facilitating N remobilization and nitrate assimilation with potential to improve crop plants performance.

Keywords: nitric oxide, nitric oxide synthase, *Nicotiana tabacum*, nitrogen, plant growth, seed production

OPEN ACCESS

Edited by:
Juan B. Barroso, University of Jaén, Spain

Reviewed by:
Oscar Lorenzo, University of Salamanca, Spain
Renaud Brouquisse, INRA Centres Provence-Alpes-Côte d'Azur, France

***Correspondence:**
Lorenzo Lamattina
lolama@mdp.edu.ar

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *12 September 2019*
Accepted: *03 March 2020*
Published: *20 March 2020*

Citation:
Nejamkin A, Foresi N, Mayta ML, Lodeyro AF, Del Castello F, Correa-Aragunde N, Carrillo N and Lamattina L (2020) Nitrogen Depletion Blocks Growth Stimulation Driven by the Expression of Nitric Oxide Synthase in Tobacco. Front. Plant Sci. 11:312. doi: 10.3389/fpls.2020.00312

INTRODUCTION

Crop productivity depends on strong nitrogen (N) fertilization, though plants use only 50% of the supplied nitrogen. For most plant species, NUE is defined by the plant capacity to extract inorganic nitrogen (N) from the soil, assimilate nitrate and ammonium, translocate, remobilize and recycle of organic N forms during the life cycle (Krstić and Sarić, 1983). Metabolic processes based on protein synthesis and N-containing biomolecules are critical for plant vegetative and reproductive growth and yield, and dependent on the adequate N supply (Sinclair and Rufty, 2012). Thus, improving NUE is a big challenge for plant biotechnology (Masclaux-Daubresse et al., 2010).

Both field and laboratory researches have demonstrated that increasing the supply of N fertilizers enhances growth and photosynthesis. The sensitivity for N fertilization is species specific and

Abbreviations: NO, nitric oxide; NUE, nitrogen use efficiency; *Ot*NOS, nitric oxide synthase from *Ostreococcus tauri*.

central for agriculture. Nitrogen scarcity results in a reduced leaf area and leaf energy production due to a reduced light interception for photosynthesis (Huber et al., 1989; Tóth et al., 2002; Dordas and Sioulas, 2008).

Given that nitrate reduction is the rate limiting step for N assimilation, nitrate reductase (NR) is considered a key enzyme in N acquisition. NR reduces nitrate to nitrite, but is also able to generate NO from nitrite (Kaiser et al., 2010). More recently, NR has been shown to play a role in NO homeostasis by supplying electrons from NAD(P)H through its diaphorase/dehydrogenase domain both to a truncated phytoglobins, which scavenges NO by its dioxygenase activity, and to the molybdoenzyme NO-forming nitrite reductase that can also generate NO from nitrite (Chamizo-Ampudia et al., 2017). In addition, a Nitrite:NO reductase was characterized as a membrane–bound enzyme that specifically produces NO from nitrite at pH 6 (Stöhr et al., 2001). It may reduce the apoplastic nitrite produced by NR playing a role in nitrate signaling via NO formation (Stöhr et al., 2001).

NO is a widespread signal molecule that participates in many physiological processes in all life kingdoms. In animals, NO is produced by the enzyme NO synthase (NOS; EC 1.14.13.39). All NOS make use of l-arginine and molecular oxygen as substrates and require the reduced cofactors nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH_4). Animal NOS is a bimodal enzyme, comprising an N-terminal oxygenase domain (NOSoxy) that binds protoporphyrin IX (heme) and C-terminal reductase domain (NOSred) that binds NADPH and the cofactors FMN and FAD. The two domains are connected by a calmodulin binding sequence (Griffith, 1995).

Genomic and functional analyses indicate that NOS enzymes are present in many organisms ranging from bacteria to humans (Gorren and Mayer, 2007). In higher plants, there are at least two enzymatic ways leading to NO production, the reductive and oxidative pathways: (1) NR, which reduces nitrate to nitrite, and then nitrite to NO (Yamasaki et al., 1999) and (2) a NOS-like enzymatic activity (Corpas et al., 2006). Nevertheless, no gene or protein with sequence similarity to animal or bacterial NOS has been yet identified in higher plants (Jeandroz et al., 2016). The first NOS of the plant kingdom was described in the photosynthetic unicellular Chlorophyte *Ostreococcus tauri* (Foresi et al., 2010; Weisslocker-Schaetzel et al., 2017). *O. tauri* is a single-celled green alga who shares a common ancestor with higher plants and is considered part of an early diverging class within the green plant lineage. Thus, it is an appropriate model system to study gene evolution and cellular processes in photosynthetic eukaryotes (Derelle et al., 2006).

In a previous work, we expressed *OtNOS* in Arabidopsis under the regulation of a stress-inducible promoter and showed that the transgenic lines displayed improved tolerance against salt and drought stresses (Foresi et al., 2015). The relevance of NO as a mediator of physiological and stress-related processes in plants has recently been reviewed (Begara-Morales et al., 2018). Meanwhile, other reports demonstrated that NO can regulate the first steps of N assimilation (Sanz-Luque et al., 2013) and also, through overexpression of phytoglobins, that NO can be oxidized to NO_3^- and enter into N assimilation pathways in dicots and monocots (Kuruthukulangarakoola et al., 2017; Zhang et al., 2019). In this work, based on the molecular characterization of *Ot*NOS activity and NO involvement in N metabolism, we generated transgenic tobacco plants expressing *OtNOS* under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter, and analyzed their growth and response to sufficient and deficient N conditions. Transgenic tobacco lines expressing *OtNOS* exhibited higher growth rates than plants transformed with the empty vector (EV) in nitrate sufficient condition. These findings extend our knowledge about the physiology of NO and nitrate in plants.

MATERIALS AND METHODS

Preparation and Characterization of *Nicotiana tabacum* Lines Expressing *OtNOS*

The *O. tauri* DNA sequence encoding NOS was synthesized, sequenced and cloned into the *Bam*HI and *Xba*I sites of pCHF3 (Jarvis et al., 1998) to give pCHF3:*OtNOS*. Plasmid pCHF3 is a binary vector carrying the CaMV 35S promoter and a pea (*Pisum sativum* L.) rubisco small subunit terminator. *Nicotiana tabacum* plants were transformed as described by Gallois and Marinho (1995) using *Agrobacterium tumefaciens* containing the plasmid of interest. Seedlings of *N. tabacum* cultivar Petit Havana (PH) from 6 to 8 weeks grown in Magenta boxes with MS-0, 0.8% (w/v) agar were used. After incubation and transformation process, stems of 1 to 2 cm long were transferred to Magenta boxes with MS-0, 0.8% (w/v) agar containing 0.1 mg ml^{-1} Kanamycin (Kan). Stems that formed roots were subsequently transferred to pots with soil.

Transgenic *35S:OtNOS* lines were selected on the basis of Kan resistance, and confirmed by genomic PCR and RT-PCR analyses. Primers used for RT-PCR are specified in **Supplementary Table S1** and amplified a 632-bp fragment. The primers for elongation factor 1α (*ef-1α*), a housekeeping gene used as a reference for mRNA levels between samples, are also indicated in **Supplementary Table S1**. For quantitative RT-PCR, total RNA was extracted using Trizol (Invitrogen). One μg of total RNA was used for first-strand cDNA synthesis with a M-MLV reverse transcriptase (Promega). PCR reactions were performed using Taq DNA polymerase (Invitrogen) with annealing temperature of 54°C and 35 cycles.

Plant Growth

Tobacco plants were grown in a culture chamber with long day photoperiod (16:8, light:darkness), temperature of 27°C/23°C (light/dark cycle), 150 μE m^{-2} s^{-1} of light intensity and 60% humidity. For all experiments, transgenic plants of T1 generation were selected by sowing and growing for 9 days in plates containing 0.1 mg.ml^{-1} Kan and 0.8% (w/v) agar supplemented with Hoagland solution (Hoagland and Arnon, 1938) or MS. For experiments under different N conditions, seedlings were transferred to plates containing a modified Hoagland/agar

solution without ammonium and with 10 mM NO_3^- (5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$) or 0.5 mM NO_3^- (KNO_3). Growth analysis was performed using different N concentrations (10, 3, 1.5, and 0.5 mM) in which 0.5 mM was established as a deficient N condition for tobacco. When nitrate was 0.5 mM, Hoagland solution was supplemented with KCl and $CaSO_4$ to maintain Ca^{+2} and K^+ levels. For experiments with more developed plants, seedlings were transferred to 200-cm^3 plastic pots with soil:perlite:vermiculite (1:1:1) and irrigated with water or 10 mM KNO_3. For seed production, seedlings were transferred to 5-lt pots containing perlite:vermiculite (1:1) and irrigated with modified Hoagland solution as previously described.

Detection of NO and Determination of Nitrate and Protein

Endogenous NO levels were estimated by using the NO-sensitive dye DAF-FM DA (Kojima et al., 1999; Moreau et al., 2008). Roots were observed by fluorescence microscopy and bright-field microscopy using an Eclipse E200 microscopy (Nikon). After Kan selection, seedlings were transferred for 7 days to plates containing agar and modified Hoagland solution as described above. For nitrate and protein determination, 100 mg of seedlings were ground in liquid N_2 and resuspended in 100 mM sodium phosphate pH 7.4. After centrifugation at 10,000 *g* for 15 min at 4°C, supernatants were used for nitrate determination as described by Cataldo et al. (1975). Samples were incubated with Salicylic acid (50 mg/ml) and reaction was stopped with 2N NaOH. Absorbance was measured at 410 nm. Protein content were determined by the Bradford method (Bradford, 1976).

Determination of Leaf Area and Root Length

For all treatments, seedlings were photographed, total whole leaf area per plant and total length of all roots per plant were measured using Image J software (Image J[1]).

Seed Germination

Transgenic tobacco seeds were sown and germinated in plates containing 0.8% (w/v) agar with Hoagland (10 mM NO_3^-), prior stratification. Germination was measured after 8 days, when the radicle pierced the seed coat (Bethke et al., 2006).

Oxygen Consumption

Oxygen consumption of adult leaves was analyzed using a Clark-type oxygen electrode. Detached leaves from tobacco *EV* and transgenic plants from two growth conditions (water and NO_3^-), were pre-incubated for 2 h in the dark in the reaction buffer (10 mM K_2HPO_4, pH 7.2, 10 mM KCl, 5 mM $MgCl_2$, 0.3 M mannitol) before measurements. Then, entire leaves were introduced into the chamber and oxygen consumption was measured for 10 min. Values were normalized to fresh weight and expressed relative to the plants with maximal O_2 consumption.

[1]http://imagej.nih.gov/ij/

Quantitative PCR Analysis

For quantitative RT-PCR, reactions were performed on a Step-one Real-time PCR machine from Applied Biosystems (California, United States) with Fast Universal SYBR Green Master Rox (Roche) to monitor the synthesis of double-stranded DNA. Software LinReg (Ruijter et al., 2009) was used to analyze data and relative transcript levels for each sample. Data were normalized against the levels of *ef-1α* cDNA. The primer sequences used are listed in **Supplementary Table S1**. Primers for NR were designed to amplify transcript of both NR isoforms (NIA1, NIA2).

Segregation Analysis

For estimation of loci number in which T-DNA was integrated, seeds of T1 were sown in Petri plates with MS-0 and Kan and were incubated in a phytotron for 10 days. After that, sensible and resistant plants were counted. It was assumed that if the segregation proportion of Kan resistant: sensible is 3:1, the T-DNA has been inserted in a unique site of the genome (Murgia et al., 2004).

Statistical Analysis

Results are expressed as means ± standard error. Data were analyzed using the Student's *t*-test for pairwise comparisons or ANOVA with *post hoc* Dunnett's method comparisons. We have developed a linear mixed-effects model, using the lme function from the nlme library in R software (version 3.1; R Foundation for Statistical Computing). Fixed effect was *OtNOS* expression, experiments and plates were treated as a random effect.

RESULTS

Expression of *OtNOS* Gene in Tobacco

We have previously showed that the expression of *OtNOS* with the control of a stress-inducible short promoter fragment (SPF) of the sunflower (*Helianthus annuus*) *Hahb-4* gene improved stress tolerance of Arabidopsis (Foresi et al., 2015). Based on those results, we tested whether a constitutive expression of *OtNOS* in tobacco plant could also generate a benefit on growth parameters. Thus, *OtNOS* under the control of the constitutive CaMV 35S promoter was cloned into the vector pCHF3 and used to transform tobacco (*Nicotiana tabacum* cv. *Petit Havana*) (**Supplementary Figure S1A**). Transgenic tobacco T_0 lines expressing *OtNOS* were screened by genomic PCR (**Supplementary Figure S1B**) and RT-PCR (**Supplementary Figure S1C**).

NOS-expressing lines were characterized by measuring fresh weight (FW) of tobacco plants growing in pots containing perlite:vermiculite:soil (1:1:1) irrigated with water or 10 mM nitrate. Transgenic *OtNOS* lines showed higher FW than *EV* plants after 26 and 60 days of irrigating with nitrate, while no significant differences were detected between lines when irrigated with water (**Figures 1A,B**).

Mitochondria are not only the cell organelles whose tasks are respiration and energy generation, but also they are sites

of NO production and target in the electron transport chain (ETC), altering the organelle physiology. The NO production and action on various mitochondrial complexes of the ETC play a major role in NO signaling, energy metabolism and growth of plants (Gupta et al., 2018). To assess whether *OtNOS* expression affects mitochondrial respiration rate, O_2 consumption was

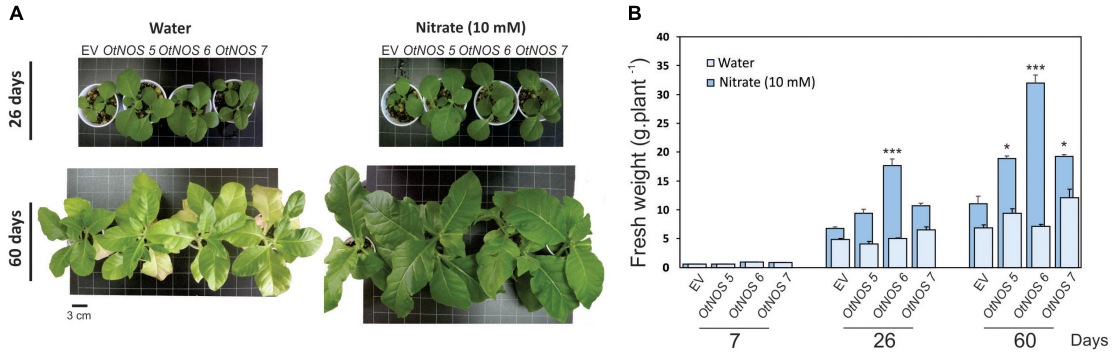

FIGURE 1 | Expression of *OtNOS* promotes growth of transgenic tobacco plants. **(A)** Representative photographs showing phenotypes of tobacco lines transformed with empty vector (*EV*) or *OtNOS* grown in plates containing MS with kanamycin (9 days) and then transferred to pots with substrate (perlite:vermiculite:soil, 1:1:1) and irrigated with water or 10 mM nitrate for the indicated times. **(B)** Fresh weight of *EV* and *OtNOS* transgenic lines was quantified as a measure of vegetative growth in these conditions. Values are means (± SE) of four to five biological replicates. Asterisks indicate statistically significant differences between *OtNOS* lines and *EV* (ANOVA *post hoc* Dunnett's method was used, $*p < 0.1$ and $***p < 0.001$).

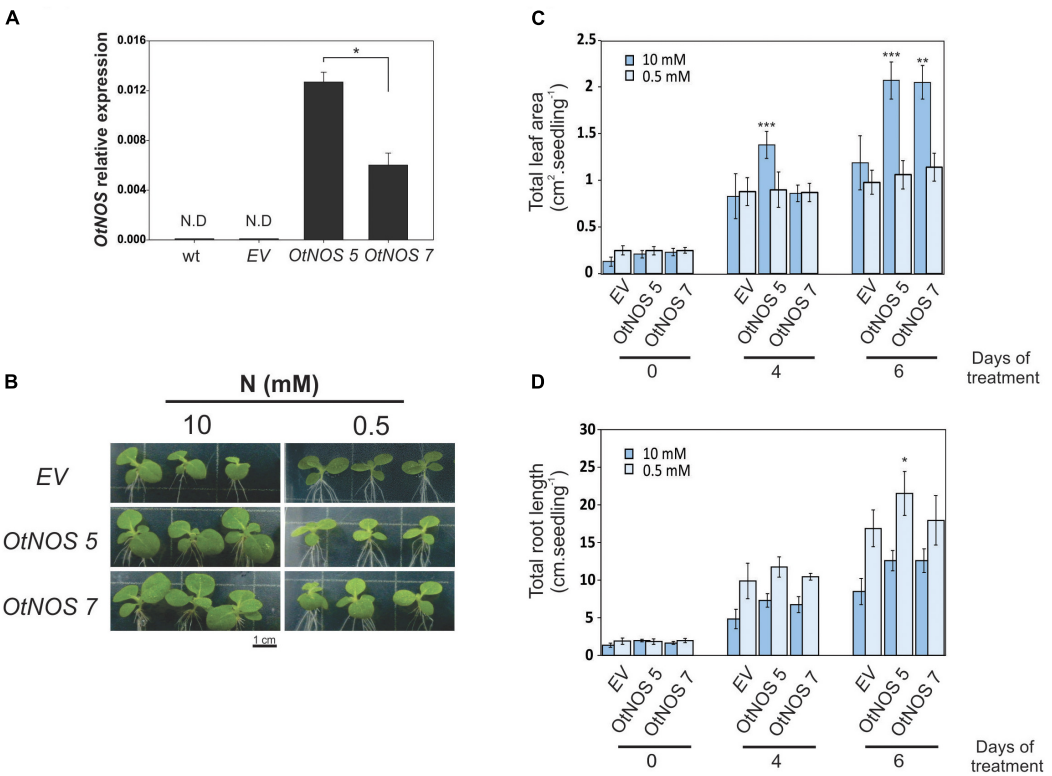

FIGURE 2 | Characterization of transgenic tobacco seedlings expressing *OtNOS* and growing under different N conditions. **(A)** Quantitative RT-PCR analysis of *OtNOS* expression in leaves of transgenic seedlings growing in plates containing Hoagland/agar (10 mM N) for 6 days (previously selected with kanamycin for 9 days). Values are means (± SE) of three independent experiments, each consisting of a biological replicate corresponding to a pool of two to three plants. Values were relativized using *ef1-α* as control. Asterisk indicates statistically significant differences between *OtNOS* lines (Student's *t*-test, $*p < 0.05$). N.D, no detection. **(B)** Representative pictures showing the phenotypes of aerial parts of 16-days-old seedlings after 6 days of growing in Hoagland medium containing 10 or 0.5 mM NO_3^-. Leaf area **(C)** and total root length **(D)** were measured during treatment using Image J software. Values are means (±SE) of three independent experiments, each consisting of at least three biological replicates. Asterisks indicate statistically significant differences between *OtNOS* lines and seedlings transformed with empty vector (*EV*) in same conditions (ANOVA *post hoc* Dunnett's method was used, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

analyzed. We found that leaves of water-watered EV plants had ∼40% more respiration than *OtNOS* lines in the same condition (**Supplementary Figure S2**). This difference of respiration rates between *OtNOS* and *EV* plants was independent of the age of the plant (40, 60, and 70-day old). In contrast, when plants were supplied with nitrate, less O_2 consumption was observed with the increasing of the plant age and irrespective of the genotype analyzed (**Supplementary Figure S2**).

OtNOS Expression Increases Tobacco Seedling Biomass Under N Sufficiency

Before continuing analyzing transgenic lines, we tested the number of T-DNA insertion (**Supplementary Table S2**). Since more than one insertion could generate confusing results, the following experiments were done only with the *OtNOS 5* and *OtNOS 7* tobacco transgenic lines. First, we checked *OtNOS* expression by quantitative RT-PCR (qPCR) and observed that *OtNOS 5* levels were two-fold higher than those of *OtNOS 7* (**Figure 2A**). Since we observed a different growth rate between transgenic and EV plants and considering that OtNOS may affect N metabolism since it uses arginine as substrate, we designed an experiment using Petri dishes containing different N concentrations (10 and 0.5 mM NO_3^-). Tobacco seedlings were grown in a modified Hoagland/agar medium in which NO_3^- was used as the sole N source. When seedlings were grown on sufficient NO_3^- concentration (10 mM), the OtNOS expressing seedlings exhibited growth advantages compared with EV (**Figure 2B**). However, when N was restricted (0.5 mM) this difference was abolished (**Figure 2B**). Statistical analyses indicate that *OtNOS* expression increased leaf biomass under nitrate-rich conditions and not under N-deficient conditions (**Figures 2B,C**). To get insights on root growth under contrasting nitrate availability, we measured development of the root system. Results shown in **Figure 2D** indicate that root length was, as expected, 50% larger under low N since seedling root system tries to explore the growing media seeking for more N (Guan et al., 2014). There are no significant differences in root system growth between EV and *OtNOS* plants except for the transgenic line *OtNOS 5* that displayed a slight increase of root growth under N deficiency at 0.5 mM nitrate (**Figure 2D**). It has been proved that treatments with exogenous NO donors or complete blockage of endogenous NO modifies root phenotype (Correa-Aragunde et al., 2004). Since no strong effects on root architecture was observed in the transgenic tobacco lines, it is considered that levels of NO present in roots of tobacco *OtNOS* lines are below concentrations required to induce a phenotype.

Levels of NO in *OtNOS* Transgenic Tobacco Roots

We analyzed if the expression of *OtNOS* leads to an increase in NO production in tobacco plants by using the DAF-FM DA, a cell-permeable fluorescent probe that reacts with the most oxidized forms of NO (NO^+ and N_2O_3). It is the most used probe for NO detection in plants (Guo et al., 2003; Planchet and Kaiser, 2006; Zottini et al., 2007; Lozano-Juste and León, 2010; Foresi et al., 2015). Roots of transgenic tobacco plants incubated with DAF-FM DA were analyzed by epi-florescence microscopy. After treatment, higher green fluorescence was observed in roots of both *OtNOS* transgenic tobacco lines compared to *EV* (**Figures 3A,B**). *Nicotiana tabacum* corresponds to pattern type 3 of radical hairs distribution, with rows of trichoblasts and trichomes alternating with rows of atrichoblasts (Kim et al., 2006). An increase in the intensity could be seen in the zone of differentiation corresponding to the root zone where trichoblasts and trichomes are generated. However, still the apex zone is observed with fluorescence, which is especially relevant, since NO is required for primary root elongation (Sanz et al., 2014).

Results indicate that constitutive expression of *OtNOS* effectively increased between 25 and 30% the NO production in tobacco root cells. To complement this observation and to get insights of the NO level in transgenic tobacco leaves, qPCR analysis of phytoglobin transcript level was performed considering that its transcript is induced by the increase of NO (Ohwaki et al., 2005; Shimoda et al., 2005; Kuruthukulangarakoola et al., 2017). Phytoglobins are

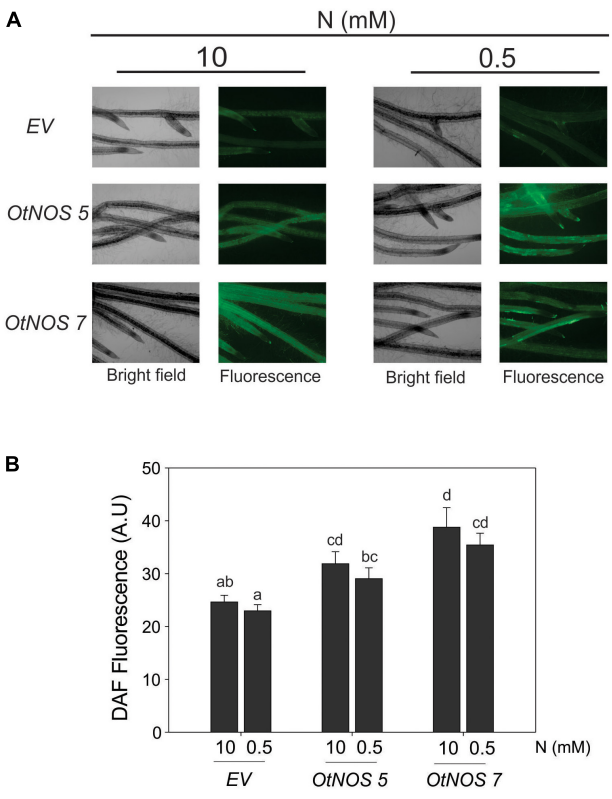

FIGURE 3 | Increased NO content in roots of transgenic tobacco lines expressing *OtNOS*. **(A)** NO content was determined using DAF-FM DA and detected by fluorescence microscopy in roots after 7 days of treatment with 0.5 or 10 mM NO_3^-. Images were taken at 40X. **(B)** Quantification of DAF-FM DA fluorescence using Image J software (AU, arbitrary units). Values are means (±SE) of two independent experiments each consisting of three biological replicates. Different letters indicate statistically significant differences (ANOVA, *post hoc* Tukey method was used, $p < 0.05$).

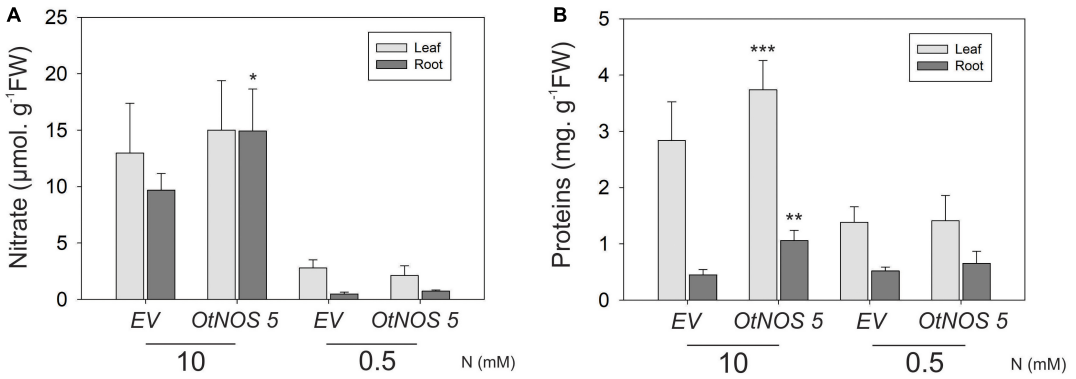

FIGURE 4 | Nitrate and protein content in transgenic tobacco line expressing *OtNOS*. Seedlings were grown in plates with Hoagland/agar medium containing 10 mM NO_3^- and kanamycin for 9 days. After selection, seedlings were transferred to plates with indicated N levels for 7 days for nitrate and protein determinations. Measurements were done using all leaves and root independently. **(A)** Nitrate was measured according to Cataldo et al. (1975). **(B)** Protein content was measured using Bradford's method (1976). Fresh weight (FW). Values are means (±SE) of at least three independent experiments each consisting of three to four biological replicates corresponding to a pool of two to three plants. Asterisks indicate statistically significant differences between *OtNOS 5* and *EV* (ANOVA, *post hoc* Dunnett's method was used, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

responsible of contributing to the control of high levels of NO in plants (Hebelstrup et al., 2013). In agreement with results obtained using the NO probe in roots, transcript levels of tobacco phytoglobin showed a slight increase in leaves of both transgenic tobacco lines *OtNOS 5* and *OtNOS 7* (**Supplementary Figure S3**).

Higher Expression of Nitrate Reductase (*NR*) in Tobacco *OtNOS* Transgenic Line

The growth phenotype of OtNOS transgenic tobacco plants prompted us to further explore N metabolite assimilation in those lines. Nitrate and protein content were measured from leaves and roots of *OtNOS 5* and *EV* tobacco lines. While no differences were found under low N condition, *OtNOS 5* line increases the levels of nitrate and protein compared to *EV* when grown in N sufficient condition (**Figure 4**). Accordingly, *OtNOS 7* line also increased protein content in leaves and roots during N sufficiency (**Supplementary Figure S4**).

To further investigate nitrate assimilation pathway, levels of NR transcript were studied in a short time of treatment since it is known that genes responding to nitrogen are highly dependent on N availability (Liu et al., 2017). Given that transgenic tobacco lines displayed similar growth phenotype compared to *EV* under low N conditions, we only analyzed NR expression under complete N supply. Results show that NR expression is higher in *OtNOS 5* and *7* compared to *EV* (**Figure 5**).

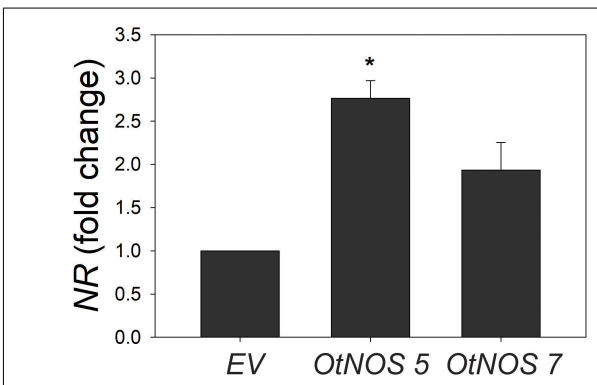

FIGURE 5 | Transcript levels of nitrate reductase (*NR*). Seedlings were grown in plates with Hoagland medium containing 10 mM NO_3^- and kanamycin for 9 days. After selection, seedlings were transferred to plates with the same N condition for 3 days and quantitative RT-PCR analysis of *NR* transcript level was perfomed. *NR* expression was relativized to *EV* plants. Values are means (±SE) of three independent experiments, each consisting of three biological replicates corresponding to a pool of two to three plants. Asterisk indicates statically significant differences compare to EV (Student's *t*-test, $*p < 0.05$).

OtNOS Expression Might Promote Tobacco Seed Yield When N Is Sufficient

It is well known that seed production is highly dependent on the plant N status (Sinclair and Rufty, 2012). To analyze whether N availability modifies differentially the number of flowers and seed production in transgenic *OtNOS* plants, transgenic and *EV* plants were grown until the end of life cycle. A positive effect of *OtNOS* expression could be observed in the number of flowers and seed yield under complete N condition in the line *OtNOS 5* (**Table 1**), that correlates with a high level of *OtNOS* expression. This effect was prevented when N supply was deficient (**Table 1**). Results indicate that N restriction resulted in a severe attenuation of the *OtNOS*-promoted stimulation of growth and production in tobacco.

DISCUSSION

Optimal plant growth fully relies on the availability of soil nutrients. Nitrogen (N) is a central resource required in large amounts to sustain the synthesis of organic molecules that constitute the plant. Arginine is not only a building block for protein biosynthesis, but also contains the highest nitrogen to carbon (N/C) ratio among amino acids, being a storage molecule

TABLE 1 | Transgenic tobacco line *OtNOS5* showed increased seed yield under complete nitrogen supply.

	Nitrogen (mM)	*EV*[a]	*OtNOS 5*[b]	Ratio [b/a]	*OtNOS 7*[c]	Ratio [c/a]
Flowers. plant^{-1}	10	30 ± 2	47 ± 4	1.5**	33	1.1
	0.5	16 ± 1	18 ± 2	1.1	15 ± 1	0.9
Total seed weight. plant^{-1} (g)	10	1.32 ± 0.2	2.38 ± 0.5	1.8*	1.0	0.8
	0.5	0.64 ± 0.02	0.71 ± 0.12	1.1	0.63 ± 0.01	0.9
Individual seed weight (mg)	10	0.067	0.079	1.2	0.094	1.4
	0.5	0.058	0.069	1.2	0.068	1.2
Germination (%)	10	96	98	1.02	78	0.81
	0.5	67	68	1.02	33	0.50

*Transgenic tobacco plants were grown in 5-lt pots containing perlite:vermiculite (1:1) and irrigated with Hoagland solution containing 10 mM NO_3^- for 40 days. After that, treatment was started by irrigation with Hoagland solution containing 10 mM or 0.5 mM NO_3^- until the end of the plant cycle. Individual area per seed was measured using ImageJ software. All flowers and seeds produced per plants were quantified. Values are means (±SE) of one experiments with two (OtNOS 7) and three to four (EV, OtNOS 5) biological replicates. Statistics were only done between EV and OtNOS 5 transgenic lines. Asterisks indicate statistically significant differences (Student's t-test, *p < 0.1, **p < 0.05).*

of organic N in plant cells. Thus, arginine metabolism possesses many physiological implications in higher plants (Winter et al., 2015). Plant arginases degrade arginine to produce ornithine and urea (Meng et al., 2015). Arginine is also a substrate for NO synthesis in plants through an activity of a yet undescribed protein named NO synthase-like (NOS-like) (Gas et al., 2009). We have previously shown that transgenic expression of *OtNOS* in Arabidopsis resulted in positive effects on germination, aerial growth and responses to water deficit mediated by changes in stomatal index and pore aperture (Foresi et al., 2015).

In this work, we show that transgenic tobacco plants expressing *OtNOS* are able to grow faster than siblings transformed with the *EV*. In addition, the transgenic line *OtNOS 5*, expressing higher level of *OtNOS* than *OtNOS 7* line is able to generate up to 80% more seeds than *OtNOS 7* and *EV* tobacco plants. In another work, transgenic tobacco plants that over expressed a mammalian NOS were generated (Chun et al., 2012). These transgenic plants are smaller compared to the wild type, exhibited enhanced resistance to biotic stress and contained high level of salicylic acid. Mammalian NOS uses the cofactor BH_4, but there are no biosynthetic pathways described for this cofactor in plants. Unlike OtNOS, that belongs to the plant kingdom and is capable of using tetrahydrofolate (THF) as cofactor, mammalian NOS activity was not detected with THF (Adak et al., 2002). Thereby, more work is necessary to understand how mammalian NOS activity is generating NO in the transformed tobacco plants. Here, it was demonstrated that *OtNOS* expression could bring potential benefits improving N metabolism in tobacco, a plant strongly dependent on N supply (Ruiz et al., 2006). Three experimental conditions were assayed to analyze the effects of *OtNOS* expression on tobacco growing under sufficient and deficient N supply: (*i*) pots with limited soil content supplemented or not with NO_3^-, (*ii*) plates containing 0.5 or 10 mM NO_3^- and (*iii*) pots containing perlite:vermiculite (1:1) and irrigated with Hoagland containing 0.5 or 10 mM NO_3^-. In all experimental models, *OtNOS* expression conferred tobacco an enhanced growth under sufficient N availability.

Mitochondria are tightly linked to N metabolism and assimilation in plants (Szal and Podgórska, 2012) and is also a source of NO generated from nitrite and cytochrome c oxidoreductase activity in complex III (Alber et al., 2017). Additionally, NO was shown to be able to partially inhibit mitochondrial respiration (Gupta et al., 2018). According to this, under low N condition, *OtNOS* transgenic plants display ∼40% inhibition of mitochondrial respiration respect to *EV* plants. In other reports, it has been shown that NO could inhibit aconitase, induce alternative oxidase (Kumari et al., 2019) and shift the metabolism toward amino acid and protein synthesis (Cvetkovska and Vanlerberghe, 2012; Gupta et al., 2012). We showed that transgenic tobacco lines have no different respiration rates under sufficient N availability. In this sense, it cannot be ascribed OtNOS phenotype to different respiration rates since the increase growth, nitrate and protein content in the *OtNOS* transgenic lines was observed only when plants where supplemented with nitrate.

NR expression was higher in the transgenic line *OtNOS 5* than in *OtNOS 7*, and it correlates with a higher *OtNOS* expression in *OtNOS 5*. NR expression and activity are regulated by many factors, nitrate, light, phytohormones, low temperature, drought, among others (Lillo et al., 2003; Lea et al., 2004; Park et al., 2011). Furthermore, NR is not only a key enzyme for N acquisition and assimilation in plants (Campbell, 2001), but also a key enzyme to modulate plant NO homeostasis (Tejada-Jimenez et al., 2019). The double mutant NR (*nia1 nia2*) has decrease nitrite levels and impaired NO synthesis (Modolo et al., 2006). These evidences indicate that there is a mutual regulation between NR activity and NO, hindering the interpretation of the results.

The NR substrate NO_3^- has also been shown to regulate flowering induction (Marín et al., 2011), crop growth and yield increments (Lawlor, 2002; Krapp et al., 2014). Moreover, the over-expression of tobacco *NR* increases seed protein content and weight in wheat without increasing the N supply (Zhao et al., 2013). We report here that the transgenic tobacco line that express more *OtNOS* transcript levels presented a positive correlation with protein content, NR expression and seed production. However, when N has been restricted, the promoter effect of OtNOS was blocked. Under N deficient conditions, it can be detected similar levels of *OtNOS* expression as in N sufficiency (**Supplementary Figure S5**) but only a slight decrease in NO content. The lack of correlation between lines for *OtNOS*

transcript levels, NO production and the growth phenotype may suggest that multiple factors are involved. Indeed, the effects observed in *OtNOS* lines may be not only due to the increment of NO, but also a result of NOS activity such as arginine depletion and/or increase of citrulline levels. Furthermore, NO homeostasis in plants is regulated through many oxidation processes and enzymatic activities (e.g. phytoglobin activity, chemical reaction with proteins and redox state of the cell).

As stated, NUE is an attractive target to modulate and improve growth and yield in crop plants. Recent findings in cyanobacteria highlight the importance of how N metabolism can be enriched through the activity of new and unexpected enzymes such as a singular NOS with a globin domain found in *Synechoccocus* PCC 7335 (*SyNOS*) (Correa-Aragunde et al., 2018) and arginine dihydrolase found in *Synechocystis* sp. PCC 6803 (Zhang et al., 2018). *SyNOS* and arginine dihydrolase metabolize arginine to generate mainly nitrate and ammonia, respectively, which are able to re-enter in the first steps of N assimilation and supply the elementary blocks required for the synthesis of macromolecules. Increasing the ability of plant cells for recycling macronutrients from storage molecules seems to be a promissory novel strategy to attain the goal of diminishing fertilization practices in crop fields without affecting yield.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

NF, AL, NC-A, NC, and LL are members of the research staff. AN and FD are graduates fellows and MM is a postdoctoral fellow from CONICET, Argentina.

FUNDING

This research was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICTs 2927/2015, PICT-2015-3828, and PICT-2014-2496 to LL, NC-A, and AL, respectively), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; PIP 0903/2011 to LL), and institutional grants from the Universidad Nacional de Mar del Plata, Argentina.

ACKNOWLEDGMENTS

We thank Dr. Cristina Lombardo (National University of Mar del Plata-CONICET), for the critical analysis of the tobacco root fluorescence images.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00312/full#supplementary-material

REFERENCES

Adak, S., Aulak, K. S., and Stuehr, D. J. (2002). Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*. *J. Biol. Chem.* 277, 16167–16171. doi: 10.1074/jbc.M201136200

Alber, N. A., Sivanesan, H., and Vanlerberghe, G. C. (2017). The occurrence and control of nitric oxide generation by the plant mitochondrial electron transport chain. *Plant Cell Environ.* 40, 1074–1085. doi: 10.1111/pce.12884

Begara-Morales, J. C., Chaki, M., Valderrama, R., Sánchez-Calvo, B., Mata-Pérez, C., Padilla, M. N., et al. (2018). Nitric oxide buffering and conditional nitric oxide release in stress response. *J. Exp. Bot.* 69, 3425–3438. doi: 10.1093/jxb/ery072

Bethke, P. C., Libourel, I. G. L., and Jones, R. L. (2006). Nitric oxide reduces seed dormancy in *Arabidopsis*. *J. Exp. Bot.* 57, 517–526. doi: 10.1093/jxb/erj060

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Campbell, W. H. (2001). Structure and function of eukaryotic NAD(P)H:Nitrate reductase. *Cell. Mol. Life Sci.* 58, 194–204. doi: 10.1007/PL00000847

Cataldo, D. A., Maroon, M., Schrader, L. E., and Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plant Anal.* 6, 71–80. doi: 10.1080/00103627509366547

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Chun, H. J., Park, H. C., Koo, S. C., Lee, J. H., Park, C. Y., Choi, M. S., et al. (2012). Constitutive expression of mammalian nitric oxide synthase in tobacco plants triggers disease resistance to pathogens. *Mol. Cells* 34, 463–471. doi: 10.1007/s10059-012-0213-0

Corpas, F. J., Barroso, J. B., Carreras, A., Valderrama, R., Palma, J. M., León, A. M., et al. (2006). Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. *Planta* 224, 246–254. doi: 10.1007/s00425-005-0205-9

Correa-Aragunde, N., Foresi, N., Del Castello, F., and Lamattina, L. (2018). A singular nitric oxide synthase with a globin domain found in *Synechococcus* PCC 7335 mobilizes N from arginine to nitrate. *Sci. Rep.* 8:12505. doi: 10.1038/s41598-018-30889-6

Correa-Aragunde, N., Graziano, M., and Lamattina, L. (2004). Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* 218, 900–905. doi: 10.1007/s00425-003-1172-7

Cvetkovska, M., and Vanlerberghe, G. C. (2012). Alternative oxidase modulates leaf mitochondrial concentrations of superoxide and nitric oxide. *New Phytol.* 195, 32–39. doi: 10.1111/j.1469-8137.2012.04166.x

Derelle, E., Ferraz, C., Rombauts, S., Rouzé, P., Worden, A. Z., Robbens, S., et al. (2006). Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11647–11652. doi: 10.1073/pnas.0604795103

Dordas, C. A., and Sioulas, C. (2008). Safflower yield, chlorophyll content, photosynthesis, and water use efficiency response to nitrogen fertilization under rainfed conditions. *Ind. Crops Prod.* 27, 75–85. doi: 10.1016/j.indcrop.2007.07.020

Foresi, N., Correa-Aragunde, N., Parisi, G., Caló, G., Salerno, G., and Lamattina, L. (2010). Characterization of a nitric oxide synthase from the plant kingdom: no generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. *Plant Cell* 22, 3816–3830. doi: 10.1105/tpc.109.073510

Foresi, N., Mayta, M. L., Lodeyro, A. F., Scuffi, D., Correa-Aragunde, N., García-Mata, C., et al. (2015). Expression of the tetrahydrofolate-dependent nitric oxide synthase from the green alga *Ostreococcus tauri* increases tolerance to

abiotic stresses and influences stomatal development in Arabidopsis. *Plant J.* 82, 806–821. doi: 10.1111/tpj.12852

Gallois, P., and Marinho, P. (1995). Leaf disk transformation using *Agrobacterium tumefaciens*-expression of heterologous genes in tobacco. *Plant Gene Transf. Expr. Protoc.* 49, 39–48. doi: 10.1385/0-89603-321-X:39

Gas, E., Flores-Pérez, Ú., Sauret-Güeto, S., and Rodríguez-Concepción, M. (2009). Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism. *Plant Cell* 21, 18–23. doi: 10.1105/tpc.108.065243

Gorren, A. C. F., and Mayer, B. (2007). Nitric-oxide synthase: a cytochrome P450 family foster child. *Biochim. Biophys. Acta Gen. Subj.* 1770, 432–445. doi: 10.1016/j.bbagen.2006.08.019

Griffith, O. (1995). Nitric oxide synthases: properties and catalytic mechanism. *Annu. Rev. Physiol.* 57, 707–736. doi: 10.1146/annurev.physiol.57.1.707

Guan, P., Wang, R., Nacry, P., Breton, G., Kay, S. A., Pruneda-Paz, J. L., et al. (2014). Nitrate foraging by *Arabidopsis* roots is mediated by the transcription factor TCP20 through the systemic signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* 111, 15267–15272. doi: 10.1073/pnas.1411375111

Guo, F. Q., Okamoto, M., and Crawford, N. M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* 302, 100–103. doi: 10.1126/science.1086770

Gupta, K. J., Kumari, A., Florez-Sarasa, I., Fernie, A. R., and Igamberdiev, A. U. (2018). Interaction of nitric oxide with the components of the plant mitochondrial electron transport chain. *J. Exp. Bot.* 69, 3413–3424. doi: 10.1093/jxb/ery119

Gupta, K. J., Shah, J. K., Brotman, Y., Jahnke, K., Willmitzer, L., Kaiser, W. M., et al. (2012). Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids. *J. Exp. Bot.* 63, 1773–1784. doi: 10.1093/jxb/ers053

Hebelstrup, K. H., Shah, J. K., and Igamberdiev, A. U. (2013). The role of nitric oxide and hemoglobin in plant development and morphogenesis. *Physiol. Plant.* 148, 457–469. doi: 10.1111/ppl.12062

Hoagland, D. R., and Arnon, D. I. (1938). *The Water-Culture Method for Growing Plants without Soil*. Berkeley, CA: University of California.

Huber, S. C., Sugiyama, T., and Alberte, R. S. (1989). Photosynthetic determinants of growth in maize plants: effects of nitrogen nutrition on growth, carbon fixation and photochemical features. *Plant Cell Physiol.* 30, 1063–1072. doi: 10.1093/oxfordjournals.pcp.a077846

Jarvis, P., Chen, L. J., Li, H. M., Peto, C. A., Fankhauser, C., and Chory, J. (1998). An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* 282, 100–103. doi: 10.1126/science.282.5386.100

Jeandroz, S., Wipf, D., Stuehr, D. J., Lamattina, L., Melkonian, M., Tian, Z., et al. (2016). Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. *Sci. Signal.* 9:re2. doi: 10.1126/scisignal.aad4403

Kaiser, W. M., Planchet, E., and Rümer, S. (2010). Nitrate reductase and nitric oxide. *Nitrogen Metab. Plants Post Genomic Era* 42, 127–145. doi: 10.1002/9781444328608.ch6

Kim, D. W., Lee, S. H., Choi, S. B., Won, S. K., Heo, Y. K., Cho, M., et al. (2006). Functional conservation of a root hair cell-specific cis-element in angiosperms with different root hair distribution patterns. *Plant Cell* 18, 2959–2970. doi: 10.1105/tpc.106.045229

Kojima, H., Urano, Y., Kikuchi, K., Higuchi, T., Hirata, Y., and Nagano, T. (1999). Fluorescent indicators for imaging nitric oxide production. *Angew. Chem. Int. Ed.* 38, 3209–3212. doi: 10.1002/(sici)1521-3773(19991102)38:21<3209::aid-anie3209>3.0.co;2-6

Krapp, A., David, L. C., Chardin, C., Girin, T., Marmagne, A., Leprince, A. S., et al. (2014). Nitrate transport and signalling in *Arabidopsis*. *J. Exp. Bot.* 65, 789–798. doi: 10.1093/jxb/eru001

Krstić, B., and Sarić, M. R. (1983). Efficiency of nitrogen utilization and photosynthetic rate in C3 and C4 plants. *Genet. Asp. Plant Nutr.* 106, 255–260. doi: 10.1007/978-94-009-6836-3_31

Kumari, A., Pathak, P. K., Bulle, M., Igamberdiev, A. U., and Gupta, K. J. (2019). Alternative oxidase is an important player in the regulation of nitric oxide levels under normoxic and hypoxic conditions in plants. *J. Exp. Bot.* 70, 4345–4354. doi: 10.1093/jxb/erz160

Kuruthukulangarakoola, G. T., Zhang, J., Albert, A., Winkler, B., Lang, H., Buegger, F., et al. (2017). Nitric oxide-fixation by non-symbiotic haemoglobin proteins in *Arabidopsis thaliana* under N-limited conditions. *Plant Cell Environ.* 40, 36–50. doi: 10.1111/pce.12773

Lawlor, D. W. (2002). Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *J. Exp. Bot.* 53, 773–787. doi: 10.1093/jxb/53.370.773

Lea, U. S., Ten Hoopen, F., Provan, F., Kaiser, W. M., Meyer, C., and Lillo, C. (2004). Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in high nitrite excretion and NO emission from leaf and root tissue. *Planta* 219, 59–65. doi: 10.1007/s00425-004-1209-6

Lillo, C., Lea, U. S., Leydecker, M. T., and Meyer, C. (2003). Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. *Plant J.* 35, 566–573. doi: 10.1046/j.1365-313X.2003.01828.x

Liu, K., Niu, Y., Konishi, M., Wu, Y., Du, H., Chung, H. S., et al. (2017). Discovery of nitrate-CPK-NLP signalling in central nutrient-growth networks. *Nature* 545, 311–316. doi: 10.1038/nature22077

Lozano-Juste, J., and León, J. (2010). Enhanced abscisic acid-mediated responses in *nia1nia2noa1-2* triple mutant impaired in NIA/NR- and AtNOA1-dependent nitric oxide biosynthesis in Arabidopsis. *Plant Physiol.* 152, 891–903. doi: 10.1104/pp.109.148023

Marín, I. C., Loef, I., Bartetzko, L., Searle, I., Coupland, G., Stitt, M., et al. (2011). Nitrate regulates floral induction in *Arabidopsis*, acting independently of light, gibberellin and autonomous pathways. *Planta* 233, 539–552. doi: 10.1007/s00425-010-1316-5

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., and Suzuki, A. (2010). Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* 105, 1141–1157. doi: 10.1093/aob/mcq028

Meng, Z., Meng, Z., Zhang, R., Liang, C., Wan, J., Wang, Y., et al. (2015). Expression of the rice arginase gene OsARG in cotton influences the morphology and nitrogen transition of seedlings. *PLoS One* 10:e0141530. doi: 10.1371/journal.pone.0141530

Modolo, L. V., Augusto, O., Almeida, I. M. G., Pinto-Maglio, C. A. F., Oliveira, H. C., Seligman, K., et al. (2006). Decreased arginine and nitrite levels in nitrate reductase-deficient *Arabidopsis thaliana* plants impair nitric oxide synthesis and the hypersensitive response to *Pseudomonas syringae*. *Plant Sci.* 171, 34–40. doi: 10.1016/j.plantsci.2006.02.010

Moreau, M., Gyu, I. L., Wang, Y., Crane, B. R., and Klessig, D. F. (2008). AtNOS/AtNOA1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric-oxide synthase. *J. Biol. Chem.* 283, 32957–32967. doi: 10.1074/jbc.M804838200

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S., and Soave, C. (2004). *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *Plant J.* 38, 940–953. doi: 10.1111/j.1365-313X.2004.02092.x

Ohwaki, Y., Kawagishi-Kobayashi, M., Wakasa, K., Fujihara, S., and Yoneyama, T. (2005). Induction of class-1 non-symbiotic hemoglobin genes by nitrate, nitrite and nitric oxide in cultured rice cells. *Plant Cell Physiol.* 46, 324–331. doi: 10.1093/pcp/pci030

Park, B. S., Song, J. T., and Seo, H. S. (2011). *Arabidopsis* nitrate reductase activity is stimulated by the E3 SUMO ligase AtSIZ1. *Nat. Commun.* 2:400. doi: 10.1038/ncomms1408

Planchet, E., and Kaiser, W. M. (2006). Nitric oxide (NO) detection by DAF fluorescence and chemiluminescence: a comparison using abiotic and biotic NO sources. *J. Exp. Bot.* 57, 3043–3055. doi: 10.1093/jxb/erl070

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37:e45. doi: 10.1093/nar/gkp045

Ruiz, J. M., Rivero, R. M., Cervilla, L. M., Castellano, R., and Romero, L. (2006). Grafting to improve nitrogen-use efficiency traits in tobacco plants. *J. Sci. Food Agric.* 86, 1014–1021. doi: 10.1002/jsfa.2450

Sanz, L., Fernández-Marcos, M., Modrego, A., Lewis, D. R., Muday, G. K., Pollmann, S., et al. (2014). Nitric oxide plays a role in stem cell niche homeostasis through its interaction with auxin. *Plant Physiol.* 166, 1972–1984. doi: 10.1104/pp.114.247445

Sanz-Luque, E., Ocaña-Calahorro, F., Llamas, A., Galvan, A., and Fernandez, E. (2013). Nitric oxide controls nitrate and ammonium assimilation in *Chlamydomonas reinhardtii*. *J. Exp. Bot.* 64, 3373–3383. doi: 10.1093/jxb/ert175

Shimoda, Y., Nagata, M., Suzuki, A., Abe, M., Sato, S., Kato, T., et al. (2005). Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant Cell Physiol.* 46, 99–107. doi: 10.1093/pci/pci001

Sinclair, T. R., and Rufty, T. W. (2012). Nitrogen and water resources commonly limit crop yield increases, not necessarily plant genetics. *Glob. Food Sec.* 1, 94–98. doi: 10.1016/j.gfs.2012.07.001

Stöhr, C., Strube, F., Marx, G., Ullrich, W. R., and Rockel, P. (2001). A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* 212, 835–841. doi: 10.1007/s004250000447

Szal, B., and Podgórska, A. (2012). The role of mitochondria in leaf nitrogen metabolism. *Plant Cell Environ.* 35, 1756–1768. doi: 10.1111/j.1365-3040.2012.02559.x

Tejada-Jimenez, M., Llamas, A., Galván, A., and Fernández, E. (2019). Role of nitrate reductase in NO production in photosynthetic eukaryotes. *Plants* 8:56. doi: 10.3390/plants8030056

Tóth, V. R., Mészáros, I., Veres, S., and Nagy, J. (2002). Effects of the available nitrogen on the photosynthetic activity and xanthophyll cycle pool of maize in field. *J. Plant Physiol.* 159, 627–634. doi: 10.1078/0176-1617-0640

Weisslocker-Schaetzel, M., André, F., Touazi, N., Foresi, N., Lembrouk, M., Dorlet, P., et al. (2017). The NOS-like protein from the microalgae *Ostreococcus tauri* is a genuine and ultrafast NO-producing enzyme. *Plant Sci.* 265, 100–111. doi: 10.1016/j.plantsci.2017.09.019

Winter, G., Todd, C. D., Trovato, M., Forlani, G., and Funck, D. (2015). Physiological implications of arginine metabolism in plants. *Front. Plant Sci.* 6:534. doi: 10.3389/fpls.2015.00534

Yamasaki, H., Sakihama, Y., and Takahashi, S. (1999). An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant Sci.* 4, 128–129. doi: 10.1016/S1360-1385(99)01393-X

Zhang, H., Liu, Y., Nie, X., Liu, L., Hua, Q., Zhao, G. P., et al. (2018). The cyanobacterial ornithine-ammonia cycle involves an arginine dihydrolase article. *Nat. Chem. Biol.* 14, 575–581. doi: 10.1038/s41589-018-0038-z

Zhang, J., Buegger, F., Albert, A., Ghirardo, A., Winkler, B., Schnitzler, J.-P., et al. (2019). Phytoglobin overexpression promotes barley growth in the presence of enhanced level of atmospheric nitric oxide. *J. Exp. Bot.* 70, 4521–4537. doi: 10.1093/jxb/erz249

Zhao, X. Q., Nie, X. L., and Xiao, X. G. (2013). Over-expression of a tobacco nitrate reductase gene in wheat (*Triticum aestivum* L.) increases seed protein content and weight without augmenting nitrogen supplying. *PLoS One* 8:e74678. doi: 10.1371/journal.pone.0074678

Zottini, M., Costa, A., De Michele, R., Ruzzene, M., Carimi, F., and Lo Schiavo, F. (2007). Salicylic acid activates nitric oxide synthesis in *Arabidopsis*. *J. Exp. Bot.* 58, 1397–1405. doi: 10.1093/jxb/erm001

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 08 April 2020
doi: 10.3389/fpls.2020.00311

NO and ABA Interaction Regulates Tuber Dormancy and Sprouting in Potato

Zhike Wang[1], Rui Ma[2,3,4], Mengshi Zhao[1], Fangfang Wang[1], Ning Zhang[1] and Huanjun Si[1,2]*

[1] College of Life Science and Technology, Gansu Agricultural University, Lanzhou, China, [2] Gansu Provincial Key Laboratory of Aridland Crop Science, Gansu Agricultural University, Lanzhou, China, [3] College of Agronomy, Gansu Agricultural University, Lanzhou, China, [4] Dingxi Academy of Agricultural Sciences, Dingxi, China

In plants, nitric oxide synthase (NOS)-like or nitrate reductase (NR) produces nitric oxide (NO), which is involved in releasing seed dormancy. However, its mechanism of effect in potato remains unclear. In this study, spraying 40 μM sodium nitroprusside (SNP), an exogenous NO donor, quickly broke tuber dormancy and efficiently promoted tuber sprouting, whereas 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), an NO scavenger, repressed the influence of NO on tuber sprouting. Compared with the control (distilled water), SNP treatment led to a rapid increase in NO content after 6 h and a decreased abscisic acid (ABA) content at 12 and 24 h. c-PTIO treatment significantly inhibited increase of NO levels and increased ABA production. In addition, N^G-nitro-L-arginine methyl ester, an NOS inhibitor, clearly inhibited the NOS-like activity, whereas tungstate, an NR inhibitor, inhibited the NR activity. Furthermore, NO promoted the expression of a gene involved in ABA catabolism (*StCYP707A1*, encoding ABA 8′-hydroxylase) and inhibited the expression of a gene involved in ABA biosynthesis (*StNCED1*, encoding 9-*cis*-epoxycarotenoid dioxygenase), thereby decreasing the ABA content, disrupting the balance between ABA and gibberellin acid (GA), and ultimately inducing dormancy release and tuber sprouting. The results demonstrated that NOS-like or NR-generated NO controlled potato tuber dormancy release and sprouting via ABA metabolism and signaling in tuber buds.

Keywords: nitric oxide, potato, tuber dormancy, sprouting, NO synthase, abscisic acid, nitrate reductase

OPEN ACCESS

Edited by:
Juan Carlos Begara-Morales, University of Jaén, Spain

Reviewed by:
Leon A. Terry, Cranfield University, United Kingdom
Michael Campbell, Penn State Erie, The Behrend College, United States

****Correspondence:***
Ning Zhang
ningzh@gsau.edu.cn

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *22 October 2019*
Accepted: *03 March 2020*
Published: *08 April 2020*

Citation:
Wang Z, Ma R, Zhao M, Wang F, Zhang N and Si H (2020) NO and ABA Interaction Regulates Tuber Dormancy and Sprouting in Potato. Front. Plant Sci. 11:311. doi: 10.3389/fpls.2020.00311

INTRODUCTION

The potato (*Solanum tuberosum* L.) is an important food crop and industrial raw material. Plant dormancy and subsequent germination are physiological processes of active adaptation to the environment. Potato tuber dormancy is defined as an inability to sprout under conditions that are favorable for sprouting (Liu et al., 2015). The dormancy and sprouting of potato tubers are very significant for potato cultivation, tuber production, and industrial processing (Aksenova et al., 2013). In potato cultivation, the dormancy degree of tubers as seed potatoes affects the emergence of seedlings in the field, uniformity, and yield (Sonnewald and Sonnewald, 2014), especially in the two season-cropping areas; excessive dormancy will prolong the sprouting and growth of tubers and finally affects the increase of yield. When tubers are used as food or raw materials for processing,

a long dormancy period is essential for transportation and storage, and dormancy release results in a large consumption of water and nutrients, decreasing commodity quality and value (Aksenova et al., 2013). Potato tubers exhibit a certain dormancy period after maturation, which ends with the occurrence of buds. The release of dormancy was essential for the sprouting of tubers, which was regulated by both internal and external factors (Koornneef et al., 2002). Tuber dormancy was also regulated by a variety of factors and was dependent on plant hormones (Suttle et al., 2012; Sonnewald and Sonnewald, 2014), genetic factors, tuber variety, storage temperature and conditions, and particular signaling molecules, such as nitric oxide (NO) (Noritake et al., 1996) and reactive oxygen species (ROS) (Peivastegan et al., 2019). In many plants, the balance between abscisic acid (ABA) and gibberellin acid (GA) is a major regulator of the dormancy state, in which GA promotes the progression from breaking to sprouting (Wróbel et al., 2017).

NO is a gaseous free radical that can easily diffuse through biofilms. NO plays a role in numerous plant physiological processes (Begara-Morales et al., 2019), such as transport (Sun et al., 2018), germination (Liu et al., 2010; Arc et al., 2013a), flowering (Khurana et al., 2011), metabolism (Hasanuzzaman et al., 2018), and senescence (Sun, 2018). Moreover, NO is a significant signaling molecule that regulates the plant response to various nonbiological and biological stresses (Begara-Morales et al., 2018), such as stomatal closure (Zhang et al., 2019), heat stress (Parankusam et al., 2017), disease (Srinivas et al., 2014), drought (Wang et al., 2016), and programmed cell death (Ma et al., 2010). Exogenous NO treatment was reported to break dormancy and promote germination in seeds of three warm-seasons C_4 grasses (Sarath et al., 2006).

In mammals, NO synthase (NOS) is the key enzyme that produces NO (Parankusam et al., 2017). There are numerous potential sources of NO in plants, which are predominantly mediated by NOS and nitrate reductase (NR) (Chamizo-Ampudia et al., 2017). In the presence of NADPH as an electron donor, NOS catalyzes the conversion of L-arginine to L-citrulline and NO (Stuehr et al., 2004), whereas NR reduces nitrates to NO (Gupta et al., 2011). Although the occurrence of NOS in plants has not yet been definitively demonstrated, some studies have indicated that NOS-like activities existed in many plants (Parankusam et al., 2017). These NOS-like activities appeared to be somewhat similar to mammalian NOS (Del Río et al., 2004). Moreover, N^G-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, inhibited NO synthesis in plants (Zhang et al., 2007). In addition, tungstate, an NR inhibitor, inhibited NO synthesis in *Arabidopsis* (Kolbert et al., 2010).

Sodium nitroprusside (SNP) is an NO donor that induces the production of NO. Exogenous SNP treatment was reported to break dormancy of barley (Bethke et al., 2004). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) is an NO scavenger that blocks the effects of NO donors and prolongs the dormancy in *Arabidopsis* (Liu et al., 2009). Some researchers have demonstrated interaction between NO signal transduction and ABA (Zhang et al., 2007; Arc et al., 2013a). For example, NO was involved in stomatal closure induced by ABA (Zhang et al., 2019). However, the crosstalk between NO and ABA in potato tuber dormancy and sprouting currently remains unclear. Therefore, this study focused on the influence of NO on potato tuber sprouting and investigated the relationship between ABA metabolism and NO signaling in tuber buds and the roles of NOS-like and NR in tuber sprouting.

MATERIALS AND METHODS

Plant Materials

The potato (*S. tuberosum* L.) cultivar "Favorita" was used in the study. The potatoes were planted in Dingxi Academy of Agricultural Sciences, Dingxi City, Gansu Province, China. The harvested tubers were stored at room temperature (25°C ± 1°C) (approximately 90% humidity) in the dark until fully mature. According to report, it takes approximately 1 week for harvested tubers to reach full maturity. When tubers mature completely, cell division is mainly concentrated in the epidermis and periderm cells. The inner cells of the tuber mainly accumulate and develop starch grains. The cell volume typically stops expanding, and few cells divide, whereas starch accumulation is very rich. The degree of vacuolation of cells continues to decrease and is becoming dormant (Teper-Bamnolker et al., 2012). SNP, L-NAME, tungstate, ABA, and c-PTIO were obtained from Beijing Biomarker Technology Company (Beijing, China).

Tuber Sprouting Test

Healthy tubers with similar size (70–80 mm in diameter) were selected. Dormant tubers were sprayed with distilled water (DW; control) or 10, 20, 40, 80, 160, or 320 μM SNP for 15 min. There were three repetitions per treatment and 50 tubers per repetition. The treated tubers were placed in a box and stored in a cool and well-ventilated place. The sprouting rate was counted every 10 days to determine the optimal SNP concentration. The tuber sprouting rate (%) = (sprouted tuber number/tuber total) × 100. The sprouted tuber means that the first bud of the tuber reaches 2 mm as the sprouting standard (Abbasi et al., 2015). This SNP concentration was then used in the subsequent combination assay, in which dormant tubers were sprayed with either DW (control), 40 μM SNP, 1 mM c-PTIO, or 40 μM SNP + 1 mM c-PTIO for 15 min. To investigate whether NO improves the tolerance to ABA, dormant tubers were sprayed with either DW (control), 100 μM ABA, 100 μM ABA + 40 μM SNP, or 100 μM ABA + 1 mM c-PTIO for 15 min. The bud eye tissues were sampled at 0, 10, 20, 30, 40, 50, and 60 days (Liu et al., 2015).

Determination of NO and ABA Contents

The NO content was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Purity and Biotechnology Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The ABA content was measured using an ELISA kit (Shanghai Jianglai Biotechnology Co., Ltd.,

Shanghai, China) according to the manufacturer's instructions, as described by Zhang et al. (2009).

Determination of NOS-Like and NR Activities

NOS-like activity was measured using a plant NOS ELISA Kit (Shanghai Xinyu Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions, as described by Diao et al. (2017). The NR activity was measured as described by Lu et al. (2014). Approximately 1.0 g of tuber bud eye tissues were ground in a mortar, and 10 mL of 50 mM phosphate buffer (pH 7.8) was added. The homogenate was centrifuged at 12,000 revolutions/min for 20 min. The supernatant was transferred to a new centrifuge tube, and the nitrite production was determined by measuring the absorbance at 540 nm using an ultraviolet-visible spectrophotometer.

RNA Extraction, Reverse Transcriptase–Polymerase Chain Reaction, and Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Fresh samples of RNA were extracted at various time points. The total RNA was isolated using an Easy Pure Plant RNA Kit (Quanshijin, Beijing, China). The extracted RNA was immediately stored at −80°C. RNA samples with an OD_{260}/OD_{280} ratio of between 1.98 and 2.0 were used in subsequent experiments. cDNA synthesis was performed using a TransScript One-Step gDNA Removal and cDNA Synthesis Super MixKit (Quanshijin). The cDNA solution was diluted eightfold with nuclease-free water. TransStart Green qPCR SuperMix (Quanshijin) was used to analyze the expression of key genes involved in potato dormancy release. Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) was performed using a Light Cycler 96 real-time PCR system (Roche, Basel, Switzerland) in a total reaction volume of 20 μL containing 10 μL 2 × TransStart Green qPCR SuperMix, 1.0 μL each primer (10 μmol/μL), 2.0 μL cDNA, and 6.0 μL double-distilled water. The reactions were mixed gently and incubated at 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 60 s. The qRT-PCR primer sequences were as follows: *StNOS-IP* (F5′-ACTTGTCCTGAAGGGAGGGA-3′; R5′-AGACCACGCAAACCTTGTCA-3′), *StNR* (F5′-AACGC TGAAGCATGGTGGTA-3′; R5′-CACCTCAACCTCGAGTGA CC-3′), *StEF1α* (F5′-CAAGGATGACCCAGCCAAG-3′; R5′-TT CCTTACCTGAACGCCTGT-3′), *StCYP707A1* (F5′-CAGGC TTTCAAGCCCGATTC-3′; R5′-TGAAGAGTGTACCGTGG AGA-3′), *StNCED1* (F5′-ACAGCCGGACACCATTTCTT-3′; R5′-CTAAACCGGCGTTTGCAACT-3′). The potato *StEF1α* gene was used as an internal reference gene. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of target genes (Livak and Schmittgen, 2001). All samples were carried out with three biological replicates and three technical replicates (Kundu et al., 2013).

Statistical Analysis

The data were analyzed using the IBM SPSS Statistics 20 software package (Amonk, New York). The different treatments were compared using Duncan multiple-range test with a significance level of $p < 0.05$. The charts were prepared using GraphPad Prism 8 (San Diego, United States).

RESULTS

Effects of SNP and c-PTIO on Tuber Sprouting

Spraying potato tubers with various concentrations of SNP led to sprouting rates of 71.18% (10 μM), 86.55% (20 μM), 93.54% (40 μM), 67.17% (80 μM), 56.12% (160 μM), 44.60% (320 μM), and 72.61% (control) at 60 days after treatment (**Figure 1A**). In the combination experiment, the tubers sprayed with 40 μM SNP had a sprouting rate of 56.23% at 20 days after treatment, whereas the c-PTIO–treated tubers had a sprouting rate of 3.04%, and the control group sprayed with DW had a sprouting rate of

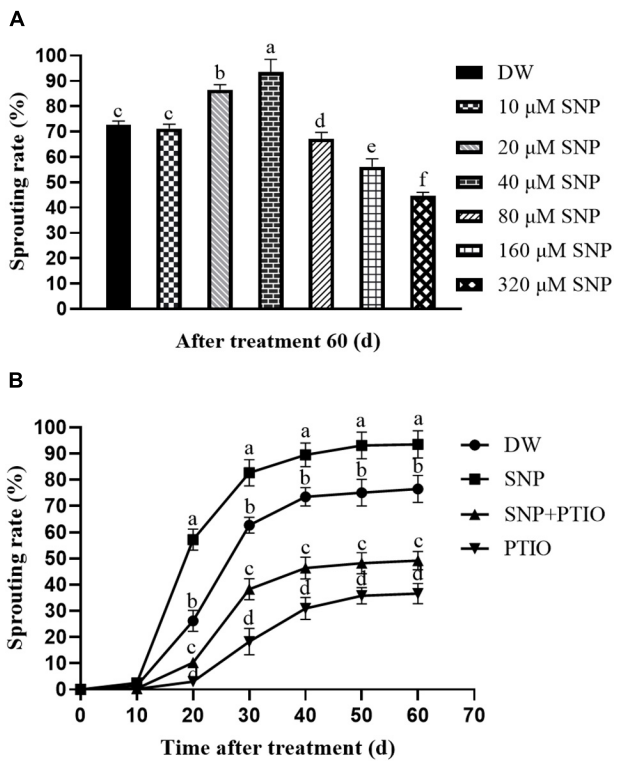

FIGURE 1 | Effects of SNP and c-PTIO on tuber sprouting. **(A)** Effect of different SNP concentrations on tuber dormancy release. Dormant tubers were sprayed with DW (control) or 10, 20, 40, 80, 160, or 320 μM SNP for 15 min. **(B)** Effect of SNP and the NO scavenger c-PTIO on tuber dormancy and sprouting. Dormant tubers were sprayed with DW (control), 40 μM SNP, 1 mM c-PTIO, or 40 μM SNP + 1 mM c-PTIO for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

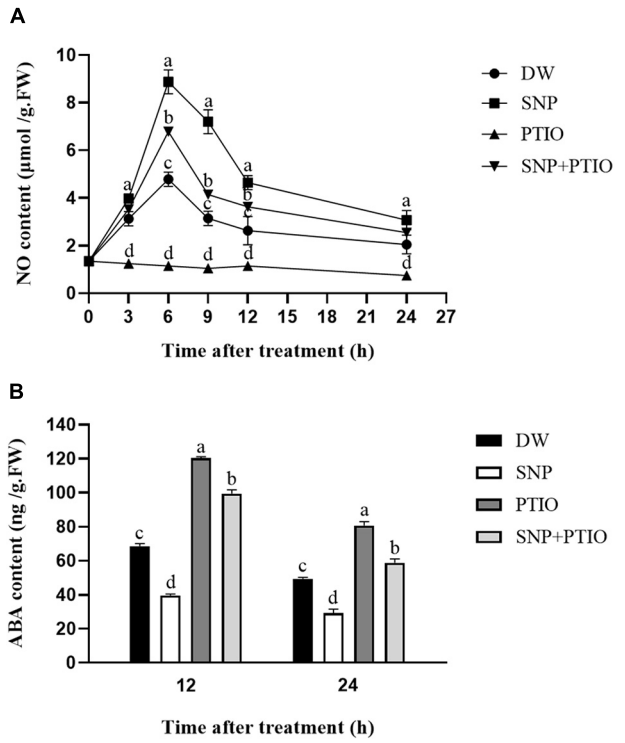

FIGURE 2 | Effects of SNP and c-PTIO on NO **(A)** and ABA **(B)** contents. Dormant tubers were sprayed with DW (control), 40 μM SNP, 1 mM c-PTIO, or 40 μM SNP + 1 mM c-PTIO for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

26.22% (**Figure 1B**). These results demonstrated that exogenous SNP treatment induced tuber dormancy release and accelerated sprouting. The NO scavenger c-PTIO markedly inhibited tuber sprouting, although combined treatment with both SNP and c-PTIO partially reversed this effect (**Figure 1B**).

Effects of SNP and c-PTIO on NO and ABA Contents

The NO and ABA contents were measured after the different treatments. As shown in **Figure 2A**, compared with the control, SNP treatment led to a rapid increase in the NO content during the first 6 h, which then gradually decreased. Treatment with c-PTIO alone strongly inhibited the increase in NO content, and c-PTIO also partially inhibited the increase in NO content induced by SNP, as shown in **Figure 2A**. As shown in **Figure 2B**, compared with the control, SNP treatment decreased the ABA content at 12 and 24 h after treatment, whereas c-PTIO treatment significantly increased the ABA content.

Influence of Enzyme Inhibitors on Relative Gene Expression

The relative expression levels of the *StNOS-IP* (encoding NOS-interacting protein) and *StNR* (encoding NR) genes after enzyme inhibitor treatment were analyzed via qRT-PCR. As shown in **Figure 3A**, treatment with L-NAME had little effects on the expression level of *StNOS-IP* gene. Similarly, the treatment with tungstate had no obvious influence on the expression level of *StNR* gene (**Figure 3B**).

To elucidate the role of NO in ABA metabolism in tuber buds, the expression of genes related to ABA metabolism (*StCYP707A1*, encoding ABA 8′-hydroxylase) and ABA biosynthesis (*StNCED1*, encoding 9-*cis*-epoxycarotenoid dioxygenase) was also examined. As shown in **Figure 3C**, SNP treatment significantly increased the expression of *StCYP707A1* compared with the control, and the highest expression occurred in 6 h after treatment. In contrast, as shown in **Figure 3D**, SNP treatment significantly decreased the expression of *StNCED1* compared with the control. These results demonstrated that NO promoted the expression of ABA catabolism gene (*StCYP707A1*) and inhibited the expression of ABA biosynthesis gene (*StNCED1*).

Influence of Enzyme Inhibitors on NOS-Like and NR Activities

To investigate the effect of the enzyme inhibitors on enzyme activity, the NOS-like and NR activities were measured. As shown in **Figure 4A**, L-NAME treatment markedly suppressed the NOS-like activity throughout the 60-day observation period compared with the control, and the highest NOS-like activity was observed at 20 days after treatment. A similar trend was observed for the NR activity after tungstate treatment (**Figure 4B**).

Influence of NO on ABA Tolerance of Potato During Sprouting

It is well known that ABA effectively inhibits the sprouting of tubers. As shown in **Figure 5**, the treatment of dormant tubers with 100 μM ABA significantly inhibited sprouting compared with the control. However, upon the addition of 40 μM SNP to ABA, the sprouting rate of the tubers was increased, whereas the addition of 1 mM c-PTIO to ABA caused the sprouting rate to further decrease. These results demonstrated that NO increased the ABA tolerance of potato during sprouting.

Influence of ABA Treatment on NO Content

To examine the effect of ABA on NO, the NO content was measured following ABA treatment. As shown in **Figure 6**, compared with the control, the NO content was decreased at 3 and 6 h after ABA treatment. The addition of SNP to ABA partially mitigated this decrease at both time points. These results demonstrated that ABA treatment decreased the NO content in potato during sprouting.

Influence of ABA on Relative Gene Expression

The effects of ABA on the relative expression of the *StNOS-IP*, *StNR*, *StCYP707A1*, and *StNCED1* genes were analyzed via qRT-PCR. As shown in **Figures 7A,B**, ABA treatment decreased the relative expression of the *StNOS-IP* and *StNR* genes after 12 h compared with the control, whereas ABA + SNP treatment

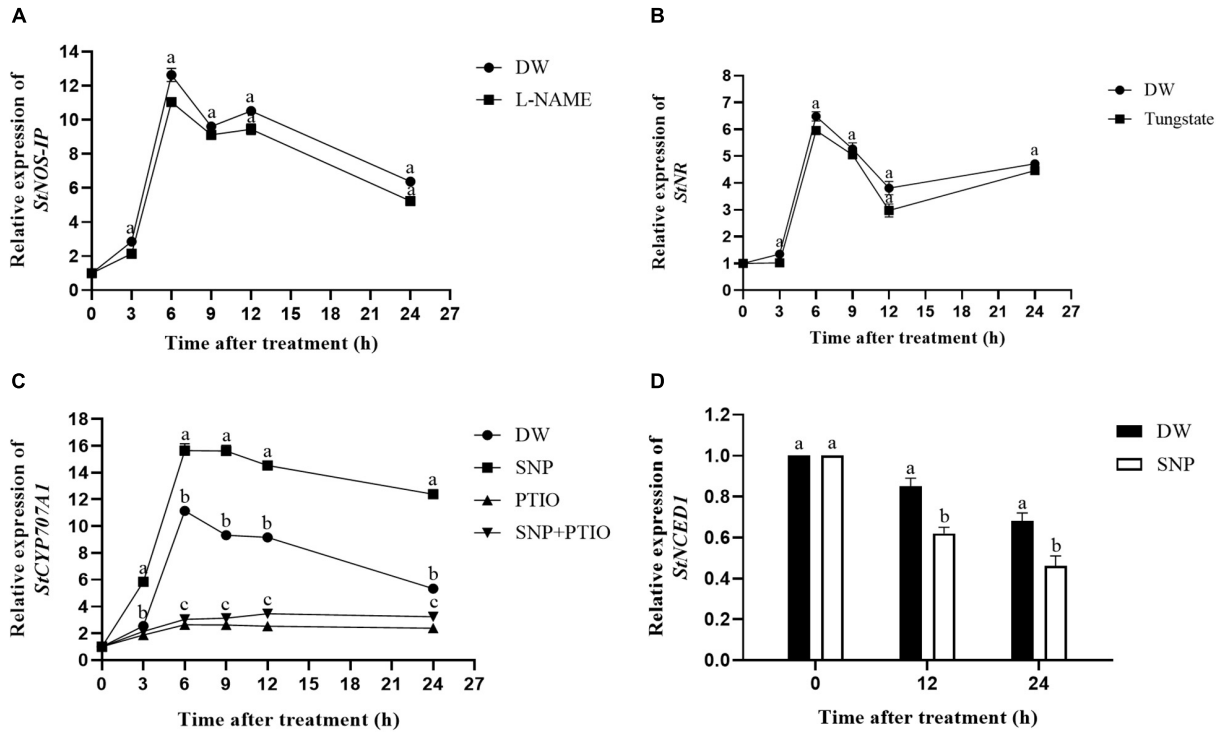

FIGURE 3 | Changes of the relative expression levels of genes (*StNOS-IP*, *StNR*, *StCYP707A1*, and *StNCED1*). **(A)** Effect of L-NAME treatment on the expression of *StNOS-IP* gene. Dormant tubers were sprayed with DW (control) or 1 mM L-NAME for 15 min. **(B)** Effect of tungstate treatment on the expression of *StNR* gene. Dormant tubers were sprayed with DW (control) or 1 mM tungstate for 15 min. **(C)** Effect of SNP treatment on the expression of *StCYP707A1* gene. Dormant tubers were sprayed with DW (control), 40 μM SNP, 1 mM c-PTIO, or 40 μM SNP + 1 mM c-PTIO for 15 min. **(D)** Effect of SNP treatment on the expression of *StNCED1* gene. Dormant tubers were sprayed with DW (control) or 40 μM SNP for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

partially mitigated this decrease for both genes. As shown in **Figures 7C,D**, ABA treatment had little effect on the expression level of the *StCYP707A1* gene, although it increased the relative expression of *StNCED1* gene after 12 h of treatment compared with the control.

Influence of ABA on NOS-Like and NR Activities

To investigate the effect of ABA on NO, the NOS-like and NR activities were measured following ABA treatment. As shown in **Figure 8**, the NOS-like and NR activities decreased at 3 and 6 h after ABA treatment compared with the control. The addition of SNP to ABA partially reversed this inhibitory effect for both enzymes. These results showed that ABA treatment inhibited the activities of NOS-like and NR in potato during sprouting.

DISCUSSION

NO has been demonstrated to play a critical role in dormancy breaking or germination in several plants, including wheat (Bethke et al., 2006a; Jacobsen et al., 2013), barley (Bethke et al., 2004), warm-season C_4 grass (Sarath et al., 2006), and lettuce (Beligni and Lamattina, 2000). Liu et al. (2009) reported that the NO donor SNP broke seed dormancy, whereas the NO scavenger c-PTIO blocked the effects of NO donors and extended dormancy in *Arabidopsis*. In this study, the exogenous 40 μM SNP treatment quickly broke the dormancy of tubers and promoted their rapid sprouting (**Figure 1A**), whereas the NO scavenger c-PTIO markedly delayed tuber sprouting (**Figure 1B**). However, combined treatment with SNP and c-PTIO partially reversed the effect of c-PTIO (**Figure 1B**). In plant research, the most common NO donors are SNP, S-nitrosopenicillamine (SNAP), S-nitrosoglutathione (GSNO), and diethylamine NONOate (DETA/NO). Different donors have different mechanisms of NO release. For example, SNP releases the nitrosonium cation (NO^+), whereas SNAP and GSNO typically release the NO radical ($^{\cdot}NO$). In aqueous solution, DETA/NO and SNAP produce instantaneous NO bursts lasting seconds to minutes, whereas the NO release effect of SNP is more prolonged (Planchet and Kaiser, 2006; Mur et al., 2013). SNP is one of the most extensively studied NO donors and can be used for the persistent production of NO (Mur et al., 2013). However, there are some shortcomings with the application of SNP. For example, the release of NO from SNP is accompanied by the production of the toxic gas hydrogen cyanide (Bethke et al., 2006b). According to the report of Oracz et al. (2009), SNP produced cyanide that was likely to induce ROS generation, for instance, hydrogen peroxide (H_2O_2). Because of time constraints, H_2O_2 content could not be measured in time in this study. In

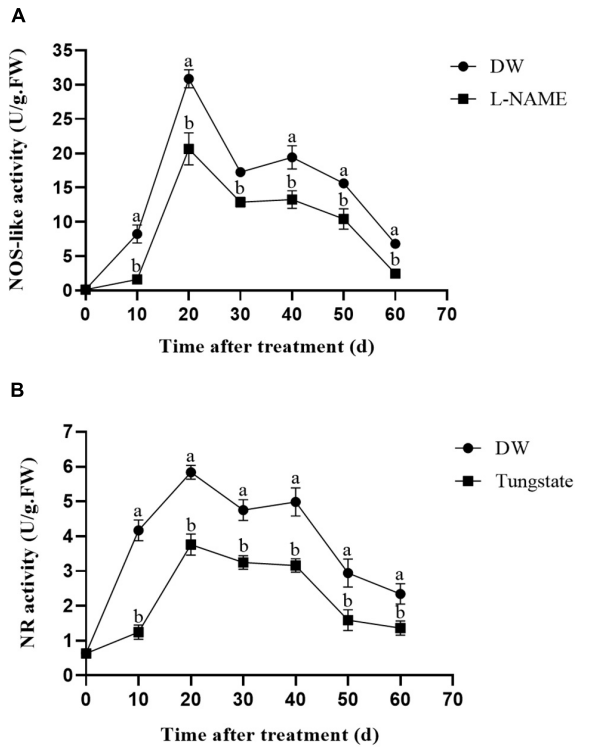

FIGURE 4 | Effect of enzyme inhibitors on NOS-like and NR activities. **(A)** NOS-like activity in potato tubers. Dormant tubers were sprayed with DW (control) or 1 mM L-NAME for 15 min. **(B)** NR activity in potato tubers. Dormant tubers were sprayed with DW (control) or 1 mM tungstate for 15 min. Data are presented as the means ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

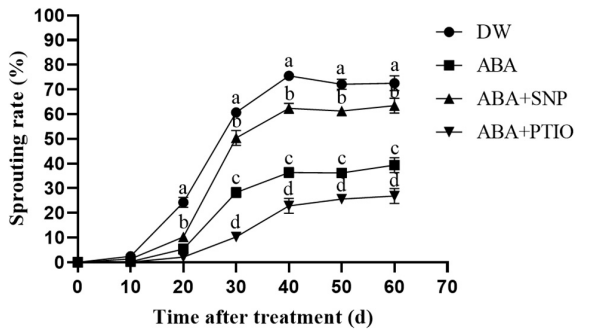

FIGURE 5 | Effect of NO on ABA tolerance of potato during sprouting. Dormant tubers were sprayed with DW (control), 100 μM ABA, 100 μM ABA + 40 μM SNP, or 100 μM ABA + 1 mM c-PTIO for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

Arabidopsis and barley seed, c-PTIO enhanced their dormancy (Bethke et al., 2004). In short, our findings are consistent with those reports for other species.

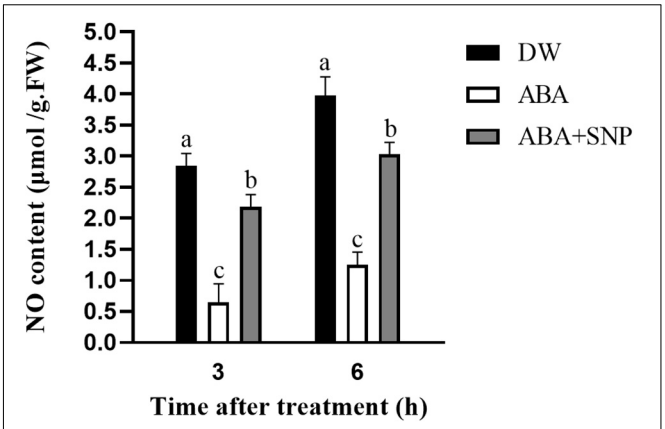

FIGURE 6 | Effect of ABA treatment on NO content. Dormant tubers were sprayed with DW (control), 100 μM ABA, or 100 μM ABA + 40 μM SNP for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

The recent plans to deregister Chlorpropham (CIPC) in European refer to germination inhibitors and herbicide CIPC have been banned use by the Council of Europe since January 1, 2020. The European Commission has published its 2019/989 implementing regulations on the nonextension of the approved active substance CIPC. According to this ordinance, the CIPC authorization has not been renewed. Member states should countermand their authorization for plant protection products involving CIPC as an active substance by January 8, 2020. CIPC is a highly efficient potato bud inhibitor (Frazier and Olsen, 2015). In the past, some export regulations required that potatoes must be treated with CIPC or other bud inhibitors as a plant disease prevention measure. However, CIPC is slightly toxic and remains in the potato tubers after application, which could cause harm to people and the environment (Abbasi et al., 2015). In an effort to effectively inhibit sprouting and reduce the occurrence of diseases during storage, tubers are typically stored at low temperature (Ou et al., 2014). Under the conditions of low-temperature storage, the content of reducing sugars in the tubers rapidly increases (Malone et al., 2006). During high-temperature processing, these reducing sugars react with free amino acids in tubers via the Maillard reaction (Claassen et al., 1993), which severely affects the color of fried tuber strips and flakes. Because the content of reducing sugars in potato chips is negatively correlated with the color of the chips, a higher reducing sugar content leads to decreased quality. The change in reducing sugar content in potato tubers is closely related to the temperature. When the storage temperature of tubers was lower than 10°C, the reducing sugar content increased, and the sucrose content also increased significantly. At 10°C, although the reducing sugar content increased slightly, the sucrose content remained essentially unchanged.

Detailed morphological analysis revealed that bud growth was not observed when tubers were dormant after harvest (Van Ittersum et al., 1992). At the molecular level, the

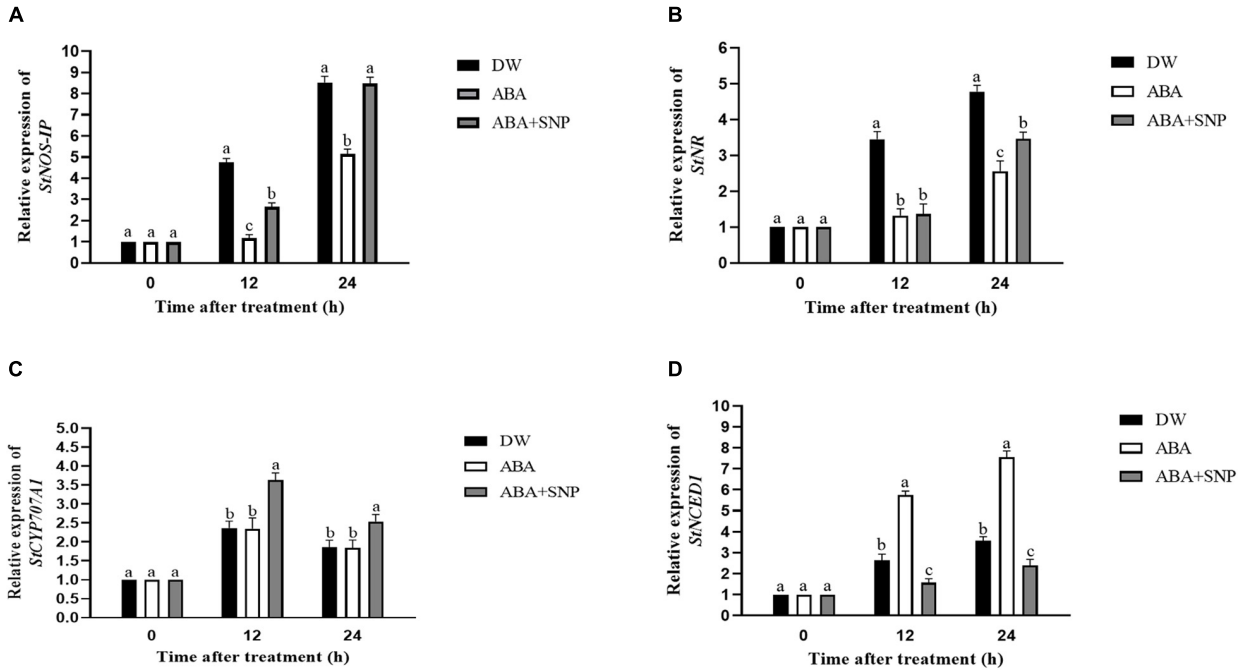

FIGURE 7 | Effect of ABA treatment on the relative expression of genes *StNOS-IP* **(A)**, *StNR* **(B)**, *StCYP707A1* **(C)**, and *StNCED1* **(D)**. Dormant tubers were sprayed with DW (control), 100 μM ABA, or 100 μM ABA + 40 μM SNP for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

meristem of the dormant tuber bud is rarely divided. When the buds begin to grow after the end of the dormancy (Leshem and Clowes, 1972), the cells in the middle stage of division are also increasing. Although mitosis is in a resting state, the resting meristem still possesses metabolic activity. Numerous studies have demonstrated that dormant meristems capture and incorporate precursors of RNA and proteins.

The NO and ABA contents were measured after the various treatments. Compared with the control, SNP treatment caused a rapid increase in the NO content at 6 h, which then gradually decreased, whereas c-PTIO treatment strongly inhibited the increase in NO content (**Figure 2A**). The addition of c-PTIO also partially mitigated the increase in the NO content caused by SNP (**Figure 2B**). Liu et al. (2009) reported that the fluorescence intensity was increased at 3 h before inhibition in *Arabidopsis* seeds and decreased after 6 h, while the fluorescence intensity was decreased after c-PTIO treatment. However, owing to technical and experimental constraints, we failed to detect NO using the fluorescent dye DAF-FMDA. The ABA content decreased at 12 and 24 h after treatment with SNP, whereas c-PTIO treatment significantly increased the ABA content (**Figure 2B**).

NOS-like and NR activities are two primary enzymatic sources of NO in plants (Parankusam et al., 2017). An increasing number of studies have demonstrated the presence of NOS-like activity in plants and its similarity to the mammalian enzyme (Del Río et al., 2004). Moreover, L-NAME, an NOS inhibitor, inhibits NO production in plants (Zhang et al., 2007), and tungstate, an NR inhibitor, was reported to inhibit NO production in *Arabidopsis* (Kolbert et al., 2010). To assess the effect of these enzyme inhibitors on enzymatic activity, the NOS-like and NR activities were measured. Compared with the control, L-NAME treatment markedly suppressed the NOS-like activity throughout the duration of the experiment, and the NOS-like activity reached its highest level at 20 days after treatment (**Figure 4A**). The same trend was observed for the NR activity following tungstate treatment (**Figure 4B**). Furthermore, the relative expression levels of the *StNOS-IP* and *StNR* genes were analyzed via qRT-PCR after enzyme inhibitor treatment. The results revealed that L-NAME treatment had no effect on the expression of the *StNOS-IP* gene (**Figure 3A**), and tungstate treatment had no effect on the expression of the *StNR* gene (**Figure 3B**). The *StNOS-IP* gene encoding NOS-interacting protein was obtained from the National Center for Biotechnology Information (NCBI) database[1] and was blasted in the Potato Genome Sequencing Consortium (PGSC) database[2]. The results revealed that this gene was predicted in the NCBI database (LOC102602935), but was a conserved gene of unknown function in the PGSC database (PGSC0003DMT400057534). We downloaded the sequence of this gene from the NCBI database and designed qRT-PCR primers. The gene possessed a total length of 1234 bp, a coding sequence length of 915 bp, and a translated protein length of 304 amino acids and contained two exons. However, the expression of the *StNOS-IP* gene had effect on the activity of NOS-like. How this gene influences the enzyme activity is unclear and will need further study.

[1]https://www.ncbi.nlm.nih.gov

[2]http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml

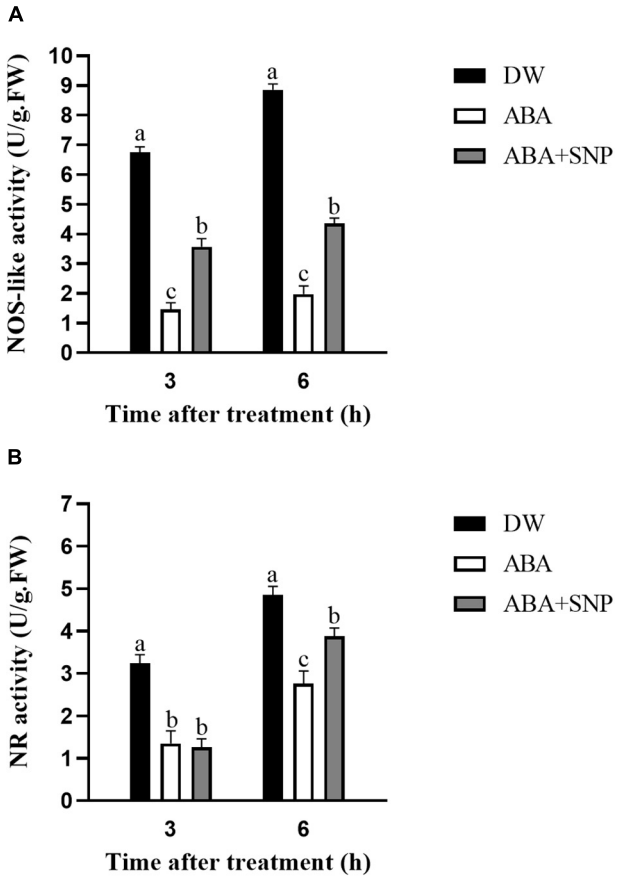

FIGURE 8 | Effect of ABA treatment on NOS-like **(A)** and NR **(B)** activities. Dormant tubers were sprayed with DW (control), 100 μM ABA, or 100 μM ABA + 40 μM SNP for 15 min. Data are presented as the mean ± SD for three replicates. When $p < 0.05$, the difference is significant according to Duncan multiple-range test. Values marked with the same letter indicate no significant difference.

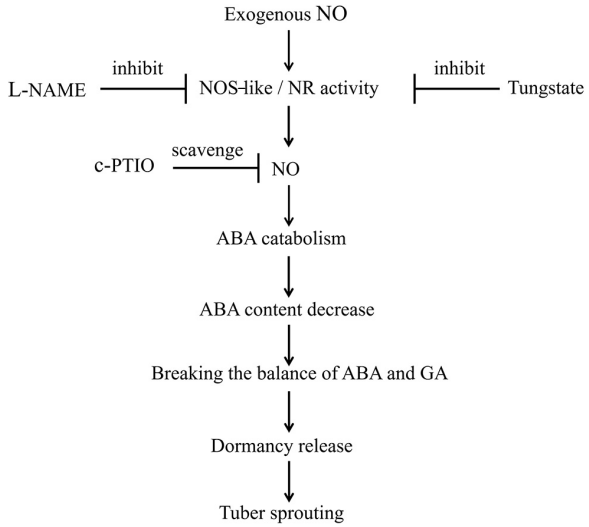

FIGURE 9 | Model depicting how NO, ABA, NOS-like, and NR control tuber dormancy and sprouting. Exogenous SNP spraying activates NOS-like or NR activity, which promotes NO production. Subsequently, NO promotes the catabolism of ABA and inhibits its biosynthesis. The resulting decreased ABA content disrupts the balance between ABA and GA, ultimately leading to dormancy release and tuber sprouting. In addition, L-NAME inhibits NOS-like activity, tungstate inhibits NR activity, and c-PTIO scavenges NO. Overall, the NOS-like or NR-generated NO controls potato tuber dormancy release and sprouting via ABA metabolism and signaling in tuber buds.

It has been suggested that NO is involved in mediating ABA catabolism. The *CYP707A* families of genes, which encode ABA 8′-hydroxylases, are responsible for regulating ABA catabolism (Sanz et al., 2015). The mechanism underlying the effect of NO on potato tuber dormancy remains unclear at present. To elucidate the role of NO in ABA metabolism in tuber buds, the expression of genes involved in ABA catabolism (*StCYP707A1*) and ABA biosynthesis (*StNCED1*) was also examined. The expression of *StCYP707A1* after SNP treatment was significantly higher than that in the control, and the highest expression occurred at 6 h after treatment (**Figure 3C**). In contrast, the expression of *StNCED1*after SNP treatment was significantly lower than that in the control (**Figure 3D**). These results demonstrated that NO promoted the expression of *StCYP707A1* and inhibited the expression of *StNCED1*.

It is well known that ABA inhibits seed germination. At present, it is generally believed that ABA is a positive regulator of dormancy induction, which is involved in the maintenance of dormancy and the decrease of endogenous ABA content during the release of dormancy (Campbell et al., 2008; Suttle et al., 2012). The use of the ABA inhibitors and the discovery of a synthetic mutant of ABA confirmed that ABA inhibits the release of dormancy, although this inhibitory effect can be reversed by GA. The crosstalk between ABA and NO regulating dormancy and germination in seed has been reported. Used exogenous SNP, an NO donor, promote NO production, which inhibits ABA synthesis and promotes ABA catabolism. According to the report of ABA crosstalk with ethylene and NO in seed dormancy and germination (Arc et al., 2013b), the signal transduction between ABA and NO was realized by PYR/PYL/RCAR receptor (Staszak et al., 2017). When ABA does not combine with the receptor, 2C protein phosphatase (PP2C) dephosphorylates sucrose nonfermenting 1–related protein kinase 2 (SnRK2) (Manohar et al., 2017). When ABA combining with the receptor, induces the formation of a protein complex with PP2C (Staszak et al., 2017) and releases inhibition of SnRK2 activity, which can phosphorylate downstream targets, containing ABA insensitive 5 (ABI5)-related transcription factors (Lim et al., 2015). Interactions between ABI3 and ABI5 mediate transcriptional regulation of ABA-responsive genes (Arc et al., 2013b). In our study, we suppose that treatment with exogenous SNP, an NO donor, activates NOS-like or NR activity, thereby promoting NO production. Subsequently, NO promotes the catabolism of ABA and inhibits its biosynthesis. Therefore, the ABA content is decreased, disrupting the balance between ABA and GA. Finally, dormancy release and tuber sprouting occur. However, the specific molecular mechanism of NO and ABA

interaction regulating the dormancy and sprouting of potato tubers remains unclear and will need further study.

The cotreatment of seeds with 200 μM SNP and 10 μM ABA was reported to promote the embryo root growth after 4 days, compared with treatment with ABA alone (Sarath et al., 2006). In addition, NO effectively improved the tolerance of *Arabidopsis* seeds to ABA (Liu et al., 2009). In our study, treatment of dormant tubers with 100 μM ABA significantly inhibited tuber sprouting compared with the control (**Figure 5**). However, upon adding 40 μM SNP to ABA, the sprouting rate of the tubers increased, and upon adding 1 mM c-PTIO to ABA, the sprouting rate of the tubers further decreased. These results demonstrated that NO improved the ABA tolerance of potato during sprouting.

To study the influence of ABA on NO, the NO content was measured following ABA treatment. As shown in **Figure 6**, compared with the control, the NO content decreased at 3 and 6 h after ABA treatment. The addition of SNP to ABA partially reversed this decrease at both time points. These results indicated that ABA treatment decreased the NO content in potato during sprouting. The NOS-like and NR activities were also measured following ABA treatment. Both activities decreased at 3 and 6 h after ABA treatment compared with the control, and the addition of SNP to ABA reversed the inhibitory effect of ABA on the activities of these two enzymes (**Figure 8**). These results showed that ABA treatment inhibited both the NOS-like and NR activities in potato tuber during sprouting.

The effects of ABA on the relative expression levels of the *StNOS-IP*, *StNR*, *StCYP707A1*, and *StNCED1* genes were analyzed by qRT-PCR. ABA decreased the relative expression of the *StNOS-IP* and *StNR* genes after 12 h of treatment (**Figures 7A,B**) compared with the control, whereas ABA + SNP treatment partially mitigated this decrease for both genes. ABA treatment had little influence on the expression level of the *StCYP707A1* gene, although it increased that of *StNCED1* gene after 12 h of treatment compared with the control (**Figures 7C,D**).

In summary, treatment with exogenous SNP, an NO donor, activates NOS-like or NR activity, thereby promoting NO production. Subsequently, NO promotes the catabolism of ABA and inhibits its biosynthesis. Therefore, the ABA content is decreased, disrupting the balance between ABA and GA. Finally, dormancy release and tuber sprouting occur. In addition, L-NAME and tungstate inhibit the activities of NOS-like and NR, respectively, and c-PTIO scavenges NO. Ultimately, the NOS-like or NR-generated NO controls potato tuber dormancy release and sprouting via ABA metabolism and signaling in tuber buds (**Figure 9**). However, the interactions between NO, GA, and ABA during the tuber dormancy release process are highly complex, and the underlying mechanisms require further study.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

NZ and HS conceived and designed the experiments. ZW, RM, MZ, and FW performed the laboratory experiments. ZW, MZ, and FW performed the data analysis and interpretation. ZW, NZ, and HS wrote the manuscript.

FUNDING

The National Natural Science Foundation of China (No. 31560413) and the Potato Industry Technology System of Gansu Province (GARS-03-P1) supported this research.

REFERENCES

Abbasi, K. S., Masud, T., Ali, S., Khan, S. U., Mahmood, T., and Qayyum, A. (2015). Sugar-starch metabolism and antioxidant potential in potato tubers in response to different antisprouting agents during storage. *Potato Res.* 58, 361–375. doi: 10.1007/s11540-015-9306-4

Aksenova, N. P., Sergeeva, L. I., Konstantinova, T. N., Golyanovskaya, S. A., Kolachevskaya, O. O., and Romanov, G. A. (2013). Regulation of potato tuber dormancy and sprouting. *Russ. J. Plant Physiol.* 60, 301–312. doi: 10.1134/S1021443713030023

Arc, E., Galland, M., Godin, B., Cueff, G., and Rajjou, L. (2013a). Nitric oxide implication in the control of seed dormancy and germination. *Front. Plant Sci.* 4:346. doi: 10.3389/fpls.2013.00346

Arc, E., Sechet, J., Corbineau, F., Rajjou, L., and Marion-Poll, A. (2013b). ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. *Front. Plant Sci.* 4:63. doi: 10.3389/fpls.2013.00063

Begara-Morales, J. C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M. N., and Barroso, J. B. (2019). The function of S-nitrosothiols during abiotic stress in plants. *J. Exp. Bot.* 70, 4429–4439. doi: 10.1093/jxb/erz197

Begara-Morales, J. C., Chaki, M., Valderrama, R., Sánchez-Calvo, B., Mata-Pérez, C., Padilla, M. N., et al. (2018). Nitric oxide buffering and conditional nitric oxide release in stress response. *J. Exp. Bot.* 69, 3425–3438. doi: 10.1093/jxb/ery072

Beligni, M. V., and Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyls elongation, three light-inducible responses in plants. *Planta* 210, 215–221. doi: 10.1007/PL00008128

Bethke, P. C., Gubler, F., Jacobsen, J. V., and Jones, R. L. (2004). Dormancy of *Arabidopsis* seeds and barley grains can be broken by nitric oxide. *Planta* 219, 847–855. doi: 10.1007/s00425-004-1282-x

Bethke, P. C., Libourel, I. G. L., and Jones, R. L. (2006a). Nitric oxide reduces seed dormancy in *Arabidopsis*. *J. Exp. Bot.* 57, 517–526. doi: 10.1093/jxb/erj060

Bethke, P. C., Libourel, I. G., Reinohl, V., and Jones, R. L. (2006b). Sodium nitroprusside, cyanide, nitrite, and nitrate break *Arabidopsis* seed dormancy in a nitric oxide-dependent manner. *Planta* 223, 805–812. doi: 10.2307/23389285

Campbell, M., Segear, E., Beers, L., Knauber, D., and Suttle, J. (2008). Dormancy in potato tuber meristems: chemically induced cessation in dormancy matches the natural process based on transcript profiles. *Funct. Integr. Genom.* 8, 317–328. doi: 10.1007/s10142-008-0079-6

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Claassen, P. A. M., Budde, M. A. W., and Calker, M. H. V. (1993). Increase in phophorylase activity during cold-induced sugar accumulation in potato tubers. *Potato Res.* 36, 205–217. doi: 10.1007/BF02360529

Del Río, L. A., Corpas, F. J., and Barroso, J. B. (2004). Nitric oxide and nitric oxide synthase activity in plants. *Phytochemistry* 65, 783–792. doi: 10.1016/j.phytochem.2004.02.001

Diao, Q., Song, Y., Shi, D., and Qi, H. (2017). Interaction of polyamines, abscisic acid, nitric oxide, and hydrogen peroxide under chilling stress in tomato (*Lycopersicon esculentum* Mill.) *seedlings*. *Front. Plant Sci.* 8:203. doi: 10.3389/fpls.2017.00203

Frazier, M. J., and Olsen, N. L. (2015). The effects of chlorpropham exposure on field-grown potatoes. *Am. J. Potato Res.* 92, 32–37. doi: 10.1007/s12230-014-9408-6

Gupta, K. J., Fernie, A. R., Kaiser, W. M., and van Dongen, J. T. (2011). On theorigins of nitric oxide. *Trends Plant Sci.* 16, 160–168. doi: 10.1016/j.tplants.2010.11.007

Hasanuzzaman, M., Oku, H., Nahar, K., Bhuyan, M. H. M. B., Mahmud, J. A., Baluska, F., et al. (2018). Nitric oxide-induced salt stress tolerance in plants: ROS metabolism, signaling, and molecular interactions. *Plant Biotechnol. Rep.* 12, 77–92. doi: 10.1007/s11816-018-0480-0

Jacobsen, J. V., Barrero, J. M., Hughes, T., Julkowska, M. M., Taylor, J. M., Xu, Q., et al. (2013). Roles for blue light, jasmonate and nitric oxide in the regulation of dormancy and germination in wheat grain (*Triticum aestivum* L.). *Planta* 238, 121–138. doi: 10.1007/s00425-013-1878-0

Khurana, A., Khurana, J. P., and Babbar, S. B. (2011). Nitric oxide induces flowering in the duckweed *Lemna aequinoctialis* Welw. (Syn. L. paucicostata Hegelm.) under noninductive conditions. *J. Plant Growth Regul.* 30, 378–385. doi: 10.1007/s00344-011-9199-7

Kolbert, Z., Ortega, L., and Erdei, L. (2010). Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of *Arabidopsis thaliana* L. roots. *J. Plant Physiol.* 167, 77–80. doi: 10.1016/j.jplph.2009.08.013

Koornneef, M., Bentsink, L., and Hilhorst, H. (2002). Seed dormancy and germination. *Curr. Opin. Plant Biol.* 5, 33–36. doi: 10.1016/S1369-5266(01)00219-9

Kundu, A., Patel, A., and Pal, A. (2013). Defining reference genes for qPCR normalization to study biotic and abiotic stress responses in *Vigna mungo*. *Plant Cell Repot* 32, 1647–1658. doi: 10.1007/s00299-013-1478-2

Leshem, B., and Clowes, F. A. L. (1972). Rates of mitosis in shoot apices of potatoes at the beginning and end of dormancy. *Ann. Bot.* 36, 687–691. doi: 10.1093/oxfordjournals.aob.a084624

Lim, C., Baek, W., Jung, J., Kim, J.-H., and Lee, S. (2015). Function of ABA in stomatal defense against biotic and drought stresses. *Int. J. Mol. Sci.* 16, 15251–15270. doi: 10.3390/ijms160715251

Liu, B. L., Zhang, N., Wen, Y. K., Jin, X., Yang, J. W., Si, H. J., et al. (2015). Transcriptomic changes during tuber dormancy release process revealed by RNA sequencing in potato. *J. Biotechnol.* 198, 17–30. doi: 10.1016/j.jbiotec.2015.01.019

Liu, Y. G., Shi, L., Ye, N. H., Liu, R., Jia, W. S., and Zhang, J. H. (2009). Nitric oxide-induced rapid decrease of abscisic acid concentration is required in breaking seed dormancy in *Arabidopsis*. *New Phytol.* 183, 1030–1042. doi: 10.1111/j.1469-8137.2009.02899.x

Liu, Y. G., Ye, N. H., Liu, R., Chen, M. X., and Zhang, J. H. (2010). H_2O_2 mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination. *J. Exp. Bot.* 61, 2979–2990. doi: 10.1093/jxb/erq125

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, S. Y., Zhuo, C. L., Wang, X. H., and Guo, Z. F. (2014). Nitrate reductase (NR)-dependent NO production mediates ABA- and H_2O_2-induced antioxidant enzymes. *Plant Physiol. Biochem.* 74, 9–15. doi: 10.1016/j.plaphy.2013.10.030

Ma, W., Xu, W., Xu, H., Chen, Y., He, Z., and Ma, M. (2010). Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. *Planta* 232, 325–335. doi: 10.1007/s00425-010-1177-y

Malone, J. G., Mittova, V., Ratcliffe, R. G., and Kruger, N. J. (2006). The response of carbohydrate metabolism in potato tubers to low temperature. *Plant Cell Physiol.* 47, 1309–1322. doi: 10.1093/pcp/pcj101

Manohar, M., Wang, D., Manosalva, P. M., Choi, H. W., Kombrink, E., and Klessig, D. F. (2017). Members of the abscisic acid co-receptor PP2C protein family mediate salicylic acid-abscisic acid crosstalk. *Plant Direct* 1, 1–14. doi: 10.1002/pld3.20

Mur, L. A., Mandon, J., Persijn, S., Cristescu, S. M., Moshkov, I. E., Novikova, G. V., et al. (2013). Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* 5:s052. doi: 10.1093/aobpla/pls052

Noritake, T., Kawakita, K., and Doke, N. (1996). Nitric oxide induces phytoalexin accumulation in potato tuber tissues. *Plant Cell Physiol.* 37, 113–116. doi: 10.1093/oxfordjournals.pcp.a028908

Oracz, K., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F., and Bailly, C. (2009). The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. *Plant Physiol.* 150, 494–505. doi: 10.1104/pp.109.138107

Ou, Y. B., Liu, X., Xie, C. H., Zhang, H. L., Lin, Y., Li, M., et al. (2014). Genome-wide identification of microRNAs and their targets in cold-stored potato tubers by deep sequencing and degradome analysis. *Plant Mol. Biol. Repor.* 33, 584–597. doi: 10.1007/s11105-014-0771-8

Parankusam, S., Adimulam, S. S., Bhatnagar-Mathur, P., and Sharma, K. K. (2017). Nitric oxide (NO) in plant heat stress tolerance: current knowledge and perspectives. *Front. Plant Sci.* 8:1582. doi: 10.3389/fpls.2017.01582

Peivastegan, B., Hadizadeh, I., Nykyri, J., Nielsen, K. L., Somervuo, P., Sipari, N., et al. (2019). Effect of wet storage conditions on potato tuber transcriptome, phytohormones and growth. *BMC Plant Biol.* 19:262. doi: 10.1186/s12870-019-1875-y

Planchet, E., and Kaiser, W. M. (2006). Nitric oxide production in plants: facts and fictions. *Plant Signal. Behav.* 1, 46–51. doi: 10.4161/psb.1.2.2435

Sanz, L., Albertos, P., Mateos, I., Sánchez-Vicente, I., Lechón, T., Fernández-Marcos, M., et al. (2015). Nitric oxide (NO) and phytohormones crosstalk during early plant development. *J. Exp. Bot.* 66, 2857–2868. doi: 10.1093/jxb/erv213

Sarath, G., Bethke, P. C., Jones, R., Baird, L. M., Hou, G., and Mitchell, R. B. (2006). Nitric oxide accelerates seed germination in warm-season grasses. *Planta* 223, 1154–1164. doi: 10.2307/23389359

Sonnewald, S., and Sonnewald, U. (2014). Regulation of potato tuber sprouting. *Planta* 239, 27–38. doi: 10.1007/s00425-013-1968-z

Srinivas, P., Wink, D., Mohanakumar, K. P., and Pillai, M. R. (2014). The legacy of nitric oxide: impact on disease biology. *Nitric Oxide* 43, 1–2. doi: 10.1016/j.niox.2014.09.005

Staszak, A. M., Guzicka, M., and Pawłowski, T. A. (2017). Signaling regulators of abscisic and gibberellic acid pathways are involved in dormancy breaking of Norway maple (*Acer platanoides* L.) *seeds*. *Acta Physiol. Plant.* 39, 241–251. doi: 10.1007/s11738-017-2544-0

Stuehr, D. J., Santolini, J., Wang, Z. Q., Wei, C. C., and Adak, S. (2004). Update on mechanism and catalytic regulation in the NO synthases. *J. Biol. Chem.* 279, 36167–36170. doi: 10.1074/jbc.R400017200

Sun, A. Z. (2018). The EPR method for detecting nitric oxide in plant senescence. *Methods Mol. Biol.* 1744, 119–124. doi: 10.1007/978-1-4939-7672-0_10

Sun, H. W., Feng, F., Liu, J., and Zhao, Q. Z. (2018). Nitric oxide affects rice root growth by regulating auxin transport under nitrate supply. *Front. Plant Sci.* 9:659. doi: 10.3389/fpls.2018.00659

Suttle, J. C., Abrams, S. R., Stefano-Beltrán, L. D., and Huckle, L. L. (2012). Chemical inhibition of potato ABA-8′-hydroxylase activity alters *in vitro* and *in vivo* ABA metabolism and endogenous ABA levels but does not affect potato microtuber dormancy duration. *J. Exp. Bot.* 63, 5717–5725. doi: 10.1093/jxb/ers146

Teper-Bamnolker, P., Buskila, Y., Lopesco, Y., et al. (2012). Release of apical dominance in potato tuber is accompanied by programmed cell death in the apical bud meristem. *Plant Physiol.* 158, 2053–2067. doi: 10.1104/pp.112.194076

Van Ittersum, M. K., Aben, F. C. B., and Keijzer, C. J. (1992). Morphological changes in tuber buds during dormancy and initial sprout growth of seed potatoes. *Potato Res.* 35, 249–260. doi: 10.1007/BF02357705

Wang, H., Huang, J., Li, Y., Li, C., Hou, J., and Liang, W. (2016). Involvement of nitric oxide-mediated alternative pathway in tolerance of wheat to drought stress by optimizing photo synthesis. *Plant Cell Rep.* 35, 2033–2044. doi: 10.1007/s00299-016-2014-y

Wróbel, S., Kęsy, J., and Treder, K. (2017). Effect of growth regulators and ethanol on termination of dormancy in potato tubers. *Am. J. Potato Res.* 94, 544–555. doi: 10.1007/s12230-017-9592-2

Zhang, A. Y., Jiang, M. Y., Zhang, J. H., Tan, M. P., and Hu, X. L. (2007). Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. *New Phytol.* 175, 35–60. doi: 10.2307/4641022

Zhang, L. S., Shi, X., Zhang, Y. T., Wang, J. J., Yang, J. W., et al. (2019). CLE9 peptide-induced stomatal closure is mediated by abscisic acid, hydrogen peroxide, and nitric oxide in *Arabidopsis thaliana*. *Plant Cell Environ.* 42, 1033–1044. doi: 10.1111/pce.13475

Zhang, Y., Tan, J., Guo, Z., Lu, S., Shu, W., and Zhou, B. (2009). Increased ABA levels in transgenic tobacco over-expressing 9-cis-epoxycarotenoid dioxygenase influences H_2O_2 and NO production and antioxidant defenses. *Plant Cell Environ.* 32, 509–519. doi: 10.1111/j.1365-3040.2009.01945.x

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.

frontiers in Plant Science

REVIEW
published: 29 May 2020
doi: 10.3389/fpls.2020.00675

Mechanisms and Role of Nitric Oxide in Phytotoxicity-Mitigation of Copper

Bilal A. Rather, Asim Masood, Zebus Sehar, Arif Majid, Naser A. Anjum and Nafees A. Khan*

Plant Physiology and Biochemistry Laboratory, Department of Botany, Aligarh Muslim University, Aligarh, India

Phytotoxicity of metals significantly contributes to the major loss in agricultural productivity. Among all the metals, copper (Cu) is one of essential metals, where it exhibits toxicity only at its supra-optimal level. Elevated Cu levels affect plants developmental processes from initiation of seed germination to the senescence, photosynthetic functions, growth and productivity. The use of plant growth regulators/phytohormones and other signaling molecules is one of major approaches for reversing Cu-toxicity in plants. Nitric oxide (NO) is a versatile and bioactive gaseous signaling molecule, involved in major physiological and molecular processes in plants. NO modulates responses of plants grown under optimal conditions or to multiple stress factors including elevated Cu levels. The available literature in this context is centered mainly on the role of NO in combating Cu stress with partial discussion on underlying mechanisms. Considering the recent reports, this paper: (a) overviews Cu uptake and transport; (b) highlights the major aspects of Cu-toxicity on germination, photosynthesis, growth, phenotypic changes and nutrient-use-efficiency; (c) updates on NO as a major signaling molecule; and (d) critically appraises the Cu-significance and mechanisms underlying NO-mediated alleviation of Cu-phytotoxicity. The outcome of the discussion may provide important clues for future research on NO-mediated mitigation of Cu-phytotoxicity.

Keywords: copper, nitric oxide, oxidative stress, photosynthesis, Cu-stress mitigation

OPEN ACCESS

Edited by:
Juan B. Barroso, University of Jaén, Spain

Reviewed by:
Youry Pii, Free University of Bozen-Bolzano, Italy
Masayuki Fujita, Kagawa University, Japan

****Correspondence:***
Asim Masood
asim.bot@gmail.com

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *07 October 2019*
Accepted: *29 April 2020*
Published: *29 May 2020*

Citation:
Rather BA, Masood A, Sehar Z, Majid A, Anjum NA and Khan NA (2020) Mechanisms and Role of Nitric Oxide in Phytotoxicity-Mitigation of Copper. Front. Plant Sci. 11:675. doi: 10.3389/fpls.2020.00675

INTRODUCTION

The increasing industrialization, hasty urbanization together with excessive use of chemical fertilizers and sewage sludge/water led to the severe contamination of soils with varied metals and metalloids (Nagajyoti et al., 2010; Brunetto et al., 2016; Asgher et al., 2018a,b). Among metals, copper (Cu) is an essential metal for plants, and promotes therein growth and development at 9.0 mg kg^{-1} (Havlin et al., 1999). The average content of Cu in plant tissues is 10 $\mu g\ g^{-1}$ dry weight; whereas, the precarious Cu concentration in nutrient media ranges between 10^{-14} and 10^{-16} M at which its deficiency has been noted (Baker and Senef, 1995). Cu is involved in the photosynthetic electron transport and redox reactions and it also acts as a cofactor in Cu/Zn–superoxide dismutase (Cu/Zn-SOD) (Bowler et al., 1994; Ouzounidou et al., 1995; Raven et al., 1999; Adrees et al., 2015). However, elevated Cu concentration can induce oxidative stress mainly through increased generation of reactive oxygen species (ROS) and thereby inhibit plant growth and productivity (Piotrowska-Niczyporuk et al., 2012; Thounaojam et al., 2012; Adrees et al., 2015; Chen et al., 2015).

Plants adopt different strategies to overcome elevated Cu concentration-caused toxicity. The list of these strategies includes the increased nutrient assimilation, induction in the antioxidant defense system, and the activation of biochemical and physiological processes such as increased signaling through associated plant growth regulators (PGRs). PGRs such as auxins (AUXs), cytokinins (CKs), gibberellins (GAs), brassinosteroids (BRs), ethylene (ET), jasmonic acids (JA), polyamines (PA), salicylic acid (SA), nitric oxide (NO), and strigolactones modulate physiological/biochemical and genetic processes and improve plant tolerance to major abiotic stresses including metals/metalloids (Maksymiec et al., 2007; Meng et al., 2009; Khan et al., 2012, 2015, 2016; Masood et al., 2012, 2016; Per et al., 2016, 2017a; Asgher et al., 2018a,b).

Earlier known as a vital signaling and effector molecule in animals, NO is known to occur also in plants, and act therein as a short-lived multifunctional gaseous signaling molecule (Astier et al., 2017). NO controls overall plant growth and the developmental processes starting from germination to senescence (Hu et al., 2007; Corpas and Barroso, 2015b). In particular, recent studies have shown that both production and signaling of NO are involved in the stress-acclimation processes in plants (Khan et al., 2012; Asgher et al., 2017; Kushwaha et al., 2019; Santisree et al., 2019). However, literature available in context with NO and plants is centered mainly on NO synthesis and its multitasking signaling in plants (Domingos et al., 2015; Astier et al., 2017). Additionally, literature in this context also reflected the role of exogenously applied NO in combating Cu stress with partial discussion on underlying potential mechanisms (Khan et al., 2012).

Given above, this paper is aimed to: (i) overview the aspects of the uptake, transport, and role of Cu in photosynthesis and nutrient-use-efficiency; (ii) highlight major aspects of Cu-phytotoxicity; (iii) discuss NO as a signaling molecule; and (iv) critically appraise the Cu-significance and mechanisms underlying NO-mediated alleviation Cu-toxicity in plants. Important clues for future research in this direction with the outcome of the facts are also discussed herein.

COPPER IN HIGHER PLANTS

Copper (Cu) is an essential trace element of most living organisms on the earth including plants, where >30 types of proteins are known to possess Cu as their structural constituent (Cohu and Pilon, 2010; Anjum et al., 2015b). As an essential micronutrient for plants, a minimum amount of Cu ensures different cellular functions. However, an excess uptake of Cu in plants may cause detrimental effects in metabolic functions and even risk to their survival (Adrees et al., 2015; Ambrosini et al., 2018; Marastoni et al., 2019b). In plants, Cu exists in two common oxidation states namely, Cu^{2+} and Cu^{+} ions. Cu^{2+} frequently prefers coordination with oxygen in aspartic and glutamic acid and with nitrogen in histidine side chains. On the other, Cu^{+} has a higher affinity with the sulfur in methionine or cysteine. The list of major Cu-containing proteins in plants includes plastocyanin, cytochrome-*c* oxidase (COX), ethylene receptors, Cu/Zn–superoxide dismutase (Cu/Zn SOD), tyrosinase, plantacyanin, phenol oxidase, laccase, ascorbate, and amine oxidase (**Table 1**). Cu mainly contributes in the transport of electrons in chloroplasts and mitochondria. Plastocyanin is one of the most abundant Cu proteins in photosynthetic tissues (Weigel et al., 2003). It is located in thylakoid lumen of chloroplasts and is responsible for the transport of electrons from cytochrome b6f complex to $P700^{+}$. Though in some algae this function can be served by a heme-containing cytochrome c6, information is scanty on the ortholog that can mimic the same function of plastocyanin in higher plants (Schubert et al., 2002; Wise and Hoober, 2007). Cu also serves as a critical co-factor in the mitochondria as a respiratory chain enzyme cytochrome *c* oxidase (COX). Composed of 12-14 sub-units, plant COX is the terminal enzymatic complex IV of the mitochondrial respiratory chain (Millar et al., 2004). Another major Cu-binding protein in plants is Cu/Zn-superoxide dismutase (Cu/Zn-SOD) that occurs in cytosol, chloroplasts, and peroxisomes. Arabidopsis possess three isoforms of CuZn-SOD, where CSD1 is located in the cytoplasm; CSD2 in chloroplast stroma, and CSD3 is a peroxisomal isoform having a minor activity (Kliebenstein et al., 1998). Another Cu-protein plantacyanin belongs to phytocyanin family of blue Cu containing proteins. Based on their magnetic and spectroscopic properties, plantacyanins are classified as Type I Cu proteins with size about 10–22 kDa (de Rienzo et al., 2000), and are primarily present in the cell wall. They are believed to act as transporters of electrons between a donor and acceptor. Plantacyanins are expressed in plants exposed to stresses such as high/low temperature, heavy metals, and high salinity, and are involved in plant-tolerance to these stresses (Maunoury and Vaucheret, 2011; Feng et al., 2013). Cu-dependent protein laccase belongs to the large group of multi-copper oxidases (MCOs) and is involved in polymerization (McCaig et al., 2005; Printz et al., 2016). Cu-dependent amine oxidases (CuAO) are among the amine oxidases and are associated with the cell wall, and flavin-containing polyamine oxidases. In plants, CuAO catalyzes the oxidation of putrescine that produces H_2O_2 involved in cell wall maturation, lignification, and programmed cell death (Cona et al., 2006). Polyphenol oxidase and ascorbate oxidases (AO) also belong to Cu-containing MCOs. Localized in the apoplast, AOs oxidize ascorbate into water and monodehydroascorbate, and thereby regulate its redox state (Kaur and Nayyar, 2014). AOs also modulate cell division and cell expansion via L-ascorbic acid (L-AA) redox repair (Kerk and Feldman, 1995; Kato and Esaka, 1999). Polyphenol oxidases are found in thylakoids, where these are involved in the defense mechanisms against pests and pathogens (Constabel and Barbehenn, 2008). In addition, Cu also plays an important role in cell signaling as the part of receptor proteins for ethylene sensing (Rodrıguez et al., 1999). Cu-homeostasis is mainly regulated by the transcription factor SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7). Through activating the transcription of plasma membrane COPT transporter genes (*COPT1*, *COPT2*, and *COPT6*), SPL7 modulates the Cu-uptake and homeostasis under Cu-deficiency (Yamasaki et al., 2009; Bernal et al., 2012). Thus, these requirements make Cu an ideal metal for normal functioning, growth and development in plants.

TABLE 1 | Summary of copper-associated proteins and their functions in plants.[1]

Type	Protein	Function
Cell Surface/Secretory Compartment Transporters and Receptors		
	P1B-Type ATPases	Proteins concerned with export of Cu^{+}
	Ctr (copper transporter)	Proteins involved in import of Cu^{+}
	Ethylene receptor	Cu acts as a cofactor and activates ethylene signaling
Electron transfer/Blue Cu proteins		
	Cytochrome *c* oxidase	Plays an important role in the last step of respiration
	Plastocyanin	Electron transfer during photosynthesis
Free Radical Scavenging		
	Cu/Zn SOD	Scavenger of free radicals
Oxidase		
	Laccase	Oxidative de-amination of polyamines
	Ascorbate oxidase	Regulates redox state of the cell
	Amine oxidase	Involved in cell wall maturation, lignification, Oxidizes diamines
	Polyphenol oxidase	Plays an important role in defensive mechanisms against pests and pathogens
Transcriptional regulators		
	Spl7	Transcriptional activator which gets activated in response to reduced Cu levels.
Chaperons/Storage		
	Atx1 (Antioxidant protein 1)	A metal chaperone carrying Cu to P-Type ATPases
	Ccs (Cu chaperone for superoxide dismutase)	Transports Cu to Cu/Zn SOD1

[1] *Based on the literature appraised in the paper.*

On the contrary, the condition of both Cu-deficiency and -elevation can bring severe consequences in plants (Yruela, 2005). Plants produced under Cu-deficiency showed alteration in the photosynthetic transport chain and reduction in the non-photochemical quenching, which is mainly due to inhibition in the function of plastocyanin (Abdel-Ghany and Pilon, 2008). On the otherside, Cu in excess causes significant toxicity and even the arrest of cellular metabolism in plants. In particular, photosynthetic electron transport is the main target under both Cu-deficiency and as well as in excess Cu. Therefore, it is essential to ensure adequate Cu-uptake and distribution in order to minimize its deleterious phytotoxic effects that in turn would regulate various homeostatic processes at cellular and whole plant levels.

COPPER UPTAKE AND TRANSPORT

Higher plants mainly take Cu in the form of Cu^{2+} ions from the rhizosphere, where the binding of Cu with various ligands facilitates the process (Welch et al., 1993). The studies on Cu uptake and transport into or within the cells are still in infancy. However, the successful implementation of advanced tools helped in uncovering transport process in yeast and other eukaryotic organisms (Nelson, 1999; Nevitt et al., 2012). Maintenance or correct regulation of Cu-homeostasis under Cu-regimes is governed by a complex system of metal-trafficking pathways available in higher plants. Plants possess a number of Cu-transporters (COPT; *COPT1-6*) involved in the uptake of Cu and secretion of metal ions (Puig et al., 2007; Andrés-Bordería et al., 2017; Andrés-Colás et al., 2018).

Current understandings on COPTs came into light as a result of having sequence homology with the eukaryotic Cu-transporters (named Ctr) and functional complementation in yeast (Puig and Thiele, 2002; Puig et al., 2007; Puig, 2014; Andrés-Bordería et al., 2017). All the members of this family contain three predicted transmembrane (TM) segments. The majority of the COPTs exhibit N-terminus methionine and histidine-rich putative metal-binding domains (Puig and Thiele, 2002; Klomp et al., 2003). In *Arabidopsis* genome, there occurs six COPT genes (*COPT 1-6*) encoding COPT transporters. COPT1, one of the most characterized members of the Cu-transporter family has been reported to permit the entry of Cu into the cells from outside to the cytoplasm (Kampfenkel et al., 1995; Sancenón et al., 2003). In addition, owing to its low Michaelis constant (KM) value, COPT1 transporter has also been reported to exhibit its high specificity for Cu^{2+} ion (Eisses and Kaplan, 2002; Sancenón et al., 2003). High specificity toward Cu^{2+} ions has also been reported for COPT2 and COPT 6 transporters (Jung et al., 2012; Garcia-Molina et al., 2013; Perea-García et al., 2013; Aguirre and Pilon, 2016). Potentially involved in the intracellular transport of Cu, COPT3 and COPT5 transporters possess one each of methionine and a histidine-rich box. Methionine residues and motifs vital for Ctr1 mediated high-affinity Cu-transport do not occur in COPT4 that has a non-direct role in Cu-transportation (Sancenón et al., 2004).

In addition to other processes, the transport of Cu^{2+} across the plasma membranes also involves P-type heavy metal ATPases (Williams and Mills, 2005; Takahashi et al., 2012; Yan et al., 2016). The transport of Cu into the cells may also be ascertained by the newly found cytosolic, soluble and low molecular weight heavy metal receptor proteins such as Cu chaperones (CCH), known

as metallo-chaperones (O'Halloran and Culotta, 2000; Huffman and O'Halloran, 2001). Cu chaperones including COX17 (Cu chaperone for COX), CCS (Cu chaperone for SOD), and two homologs of the yeast ATX1 (antioxidant protein 1) and CCH (ATX1-like Cu chaperone) were reported to be involved in the intracellular Cu transport in *Arabidopsis* (Casareno et al., 1998; Chu et al., 2005; Puig et al., 2007). The knowledge regarding the transport of Cu into the xylem is still in its infancy. In a recent study, compared to roots developed on different metal ions the roots developed on media with 50 μM Cu exhibited a huge decline in the levels of callose (O'Lexy et al., 2018). Additionally, Cu was observed to move through plasmodesmata by influencing plasmodesmata via regulating β-1,3-glucanases.

COPPER-INDUCED TOXICITY IN PLANTS

A large volume of literature is available on the impact of elevated Cu on major aspects in plants including germination and growth (López-Bucio et al., 2003; Lin et al., 2005; Mench and Bes, 2009; Potters et al., 2009; Bouazizi et al., 2010; Lequeux et al., 2010; Verma et al., 2011; Feigl et al., 2013; Gang et al., 2013; Muccifora and Bellani, 2013; Adrees et al., 2015; Marques et al., 2018), photosynthesis and related variables (Chatterjee and Chatterjee, 2000; Quartacci et al., 2000; Yruela, 2005; Küpper et al., 2009; Gonzalez-Mendoza et al., 2013; Mateos-Naranjo et al., 2013; Adrees et al., 2015; Feigl et al., 2015; de Freitas et al., 2015; Emamverdian et al., 2015; Sharma et al., 2017; Ambrosini et al., 2018), phenotypic changes (Barbosa et al., 2013; Feigl et al., 2013; Sánchez-Pardo et al., 2014; Adrees et al., 2015; Nair and Chung, 2015; Ali et al., 2016; Brunetto et al., 2016; Llagostera et al., 2016; Mwamba et al., 2016; Ambrosini et al., 2018; Shiyab, 2018; Marastoni et al., 2019b; Nazir et al., 2019; Shams et al., 2019), and nutrient-use-efficiency of plants (Chatterjee and Chatterjee, 2000; Ali et al., 2002; Keutgen and Pawelzik, 2008; Ivanova et al., 2010; Feigl et al., 2013; Azeez et al., 2015; Bankaji et al., 2015; Marastoni et al., 2019a). A brief discussion on the Cu-induced changes in germination and growth, photosynthesis and related variables, phenotypic changes, and nutrient-use-efficiency of plants are presented hereunder.

Germination and Growth

The effect of Cu was seen at different growth and developmental stages of plants from seed germination to the senescence. Contingent to Cu level and the growth stage of the test plant, excess Cu significantly affects Cu-sensitive plants. In germinating seeds, increasing Cu concentrations reduced the germination percentage of seeds in different plant species (Gang et al., 2013; Muccifora and Bellani, 2013). Similarly, germination in mungbean seeds was reported to decrease with increasing Cu concentrations (Verma et al., 2011). During an early stage of growth, elevated Cu concentrations inhibited leaf expansion but increased pigment content (Maksymiec et al., 1994; Maksymiec and Baszyński, 1996; Adrees et al., 2015). In addition to inhibition in growth and biomass, Cu toxicity in plants also includes bronzing/necrosis (Marschner, 1995; Mench and Bes, 2009; Marques et al., 2018). Increasing Cu-concentration reduces uptake of Fe, Zn, Mn, and Co (Marschner, 1995; Bouazizi et al., 2010; Feigl et al., 2013). Significant reductions in root and shoot biomass were found in *Arabidopsis* exposed to 2.5 and 5 μM Cu for 14 days (Lequeux et al., 2010). Elevated Cu can inhibit primary root growth and simultaneously stimulate lateral root formation and thereby remodel the root structure (López-Bucio et al., 2003; Potters et al., 2009; Lequeux et al., 2010). Excess Cu also causes overproduction of H_2O_2, which eventually weakens the cell wall-extensibility (Lin et al., 2005). Root growth is more severely affected by increased Cu than shoot growth that is obvious due to the retention of the major proportion of Cu taken up by plants.

Photosynthesis and Related Variables

Impacts of Cu on photosynthesis are well documented. The photosynthetic apparatus is susceptible to heavy metal toxicity, which in turn directly or indirectly significantly impact photosynthetic functions. Excess Cu has been reported to decrease the level of photosynthetic pigments such as chlorophyll (Küpper et al., 2009; Ambrosini et al., 2018). Cu concentrations were reported to decrease chlorophyll content in a number of plants including spinach (Ouzounidou et al., 1998), maize (Mocquot et al., 1996), cauliflower (Chatterjee and Chatterjee, 2000), and rapeseed and Indian mustard plants (Feigl et al., 2015). Cu excess can also impair chloroplast structure and thylakoid membrane composition (de Freitas et al., 2015; Sharma et al., 2017). Ciscato et al. (1997) found that the reduction in chlorophyll biosynthesis was mainly due to Cu-exposure mediated structural damages of chloroplast particularly at the thylakoid level. Disturbed metabolic activities like loss of chloroplast integrity, and change in plastid membrane composition and inhibition of photosynthetic electron transport have also been evidenced in plants exposed to elevated Cu levels (Quartacci et al., 2000; Adrees et al., 2015). Cu was found to inhibit both PS I and PS II, where PS II was found very sensitive to elevated Cu (Yruela, 2005). In another study, excess of Cu caused a reduction in photosynthesis mainly as a consequence of the higher photoinhibition (Mateos-Naranjo et al., 2013; Adrees et al., 2015). Cu in excess may also block the photosynthetic electron transport, inhibit photophosphorylation, and decrease membrane integrity (Maksymiec et al., 1994; Emamverdian et al., 2015). Cu-excess blocked the flow of electrons from Tyr z to $P680^+$ (Yruela, 2005). In *Avicennia germinans* elevated Cu-mediated 100% inhibition of net photosynthesis and reduction of chlorophyll fluorescence with damaged photosynthetic apparatus (Gonzalez-Mendoza et al., 2013). In a recent study on fibrous jute (*Corchorus capsularis*) plants, Saleem et al. (2020) reported heavy damage in the organelles of the leaves by exposure to soils having Cu-contaminated soil mixed with natural soil by 1:4 ratio. The authors also found a large number of chloroplast particles accumulated inside the cell wall and also outside the chloroplast in these plants.

Phenotypic Changes

Surplus level of Cu restricts plant growth and development (Shams et al., 2019). The impact of Cu toxicity is primarily on root growth and phenotype, which has a paramount significance

to the whole plant. In general, Cu accumulates mainly in roots rather than in shoots, although the different distribution and translocation of Cu depends on its concentration in the root-growing medium (Adrees et al., 2015). However, both shoots and roots exhibit specific symptoms of Cu-toxicity. High Cu concentrations in shoots induced pale green to white interveinal chlorosis on mature leaves, altered membrane permeability, enzyme activities and also reduced photosynthesis (Brunetto et al., 2016). In roots, excess Cu reduces root length and leads to darkening and thickening of root tips (Feigl et al., 2013). Cu stress has also been reported to decrease the area and expansions of leaves, and the size of stem in several plants (Barbosa et al., 2013; Feigl et al., 2013). In addition to reductions in shoot and root growth, elevated Cu-exposed plants exhibited phenotypic changes as toxicity symptoms, where roots showed intense dark color with increasing Cu concentration (Marastoni et al., 2019b). In several studies, elevated acquisition of Cu was culminated into chlorosis, leaf epinasty, decreased branching, thickening, and dark coloration (Nair and Chung, 2015; Ali et al., 2016) and also to the development of necrotic patches in leaf tips and margins (Llagostera et al., 2016). Altered surface root morphology, rolling of the leaf blade and reduced leaf area were also reported in plants under elevated Cu-exposure (Sánchez-Pardo et al., 2014; Nazir et al., 2019). Phytotoxic concentrations of Cu also impede the leaf proliferation, cell elasticity, and cell division and reduced the number and abundance of intercellular spaces and densely developed dark colored areas of xylem vessels (Ambrosini et al., 2018; Shiyab, 2018). Elevated Cu-accrued reduction in the leaf area resulted in reduced dry matter production (Mwamba et al., 2016). Therefore, phenotypic attributes may function as an effective bioindicator of Cu toxicity as well as for the characterization of the plants as resilient or sensitive to excess Cu.

Nutrient-Use-Efficiency of Plants

The accumulation of ions such as Na^+ and Cl^- accumulated in plant organs may compete with mineral nutrients and also disturb their uptake, translocation, and assimilation (Keutgen and Pawelzik, 2008). Higher concentrations of Cu reduced the content of N, P, and K in both shoot and root of maize; however, increased therein the concentration of Fe (Ali et al., 2002; Azeez et al., 2015). In sand culture grown cauliflower, the supply of 0.5 mM Cu for 30 days decreased Fe concentration (Chatterjee and Chatterjee, 2000). In both leaves and roots of *Brassica juncea* and *B. napus*, excess Cu impacted microelement homeostasis and decreased the concentrations of Zn, Fe, Mn, and Co (Feigl et al., 2013). Elevated Cu-mediated reduction in Zn in rapeseed has also been reported (Ivanova et al., 2010). In *Suaeda fruticosa*, Cu^{2+} increased K^+ contents in the shoots; however, Cu^{2+} showed no effect on the level of Mg^{2+} and Na^+ in the shoots (Bankaji et al., 2015). In a recent study on Cu-exposed oat cultivars (*Avena sativa* L. cv. Fronteira and cv. Perona), Marastoni et al. (2019a) reported a higher accumulation of Cu in the apoplasm which was argued to strongly reduce the available binding sites, leading eventually to a competitive absorption with Ca, Mn, and Zn.

NO as a Major Plant Signaling Molecule

As a key signaling component, NO is involved in various physiological and metabolic processes in plants including their adaptation to various stresses (Asgher et al., 2017; Fancy et al., 2017; Santisree et al., 2019). In plants, NO is synthesized both by enzymatic and non-enzymatic systems (Arasimowicz and Floryszak-Wieczorek, 2007; Hasanuzzaman et al., 2018) (**Figure 1**). Contingent to its concentration and the site of production NO shows both positive and negative effects. Further NO was seen to affect major metabolic pathways in plants, particularly of nutrient assimilation. Our recent research on NO suggests that salt stress effects on the photosynthetic performance are mitigated effectively when NO was applied along with the split application of both N and S, and the photosynthetic activity was stimulated through increased N and S assimilation and antioxidant system conferring tolerance against salt stress (Jahan et al., 2020). Similarly. NO was also shown to reverse glucose-mediated photosynthetic inhibition in *T. aestivum L.* under salt stress (Sehar et al., 2019). We have also shown that NO can improve S-assimilation and GSH production under Cd stress and prevent inhibitory photosynthesis in mustard (Fatma et al., 2016a,b; Per et al., 2017b). Usually, NO transmits its bioactivity by targeting proteins during post-translational modifications via cysteine *S*-nitrosylation that leads to the formation of *S*-nitrosothiols (SNOs). The reaction of NO with ROS such as superoxide anions also leads to the protein tyrosine nitration and yields nitrite (ONOO-). In fact, SNOs are the key signaling molecules largely involved in response to several stresses in plant biology (Begara-Morales et al., 2019). Generated as a result of the reaction of NO with reduced GSH, *S*-nitrosoglutathione (GSNO) is the most important among SNOs. This metabolite is considered as a major reservoir of NO. As a NO-reservoir, GSNO can be transported to other cells/tissues which confer NO as a long distance mobile signaling molecule. GSNO can also be converted into oxidized glutathione (GSSG) and NH_3 by GSNO reductase (GSNOR) (Fancy et al., 2017; Begara-Morales et al., 2018). In addition, a direct donation of the NO group of GSNO to other cellular thiols may occur via *S*-transnitrosylation reactions (Corpas et al., 2013). NO-dependent modifications in plant lipids such as nitro-fatty acids (NO_2-FA) have shown the importance of NO in cell signaling processes (Sánchez-Calvo et al., 2013; Fazzari et al., 2014). NO_2-FA such as linolenic acid has been reported to alleviate various abiotic stresses (Mata-Pérez et al., 2016). A NO-mediated pathway is also involved in the activation of mitogen-activated protein kinase (MAPK) signaling events (Pagnussat et al., 2004) and in the promotion of MPK6-mediated caspase-3-like activation (Ye et al., 2013).

NO IN PLANT GROWTH AND DEVELOPMENT

NO plays a vital role in regulating several processes related to plant growth and development, and qualifies the definition of plant growth regulators (Beligni and Lamattina, 2001; Xiong et al., 2009; Takahashi and Morikawa, 2014). The role of NO has been elucidated in growth and development of plants such

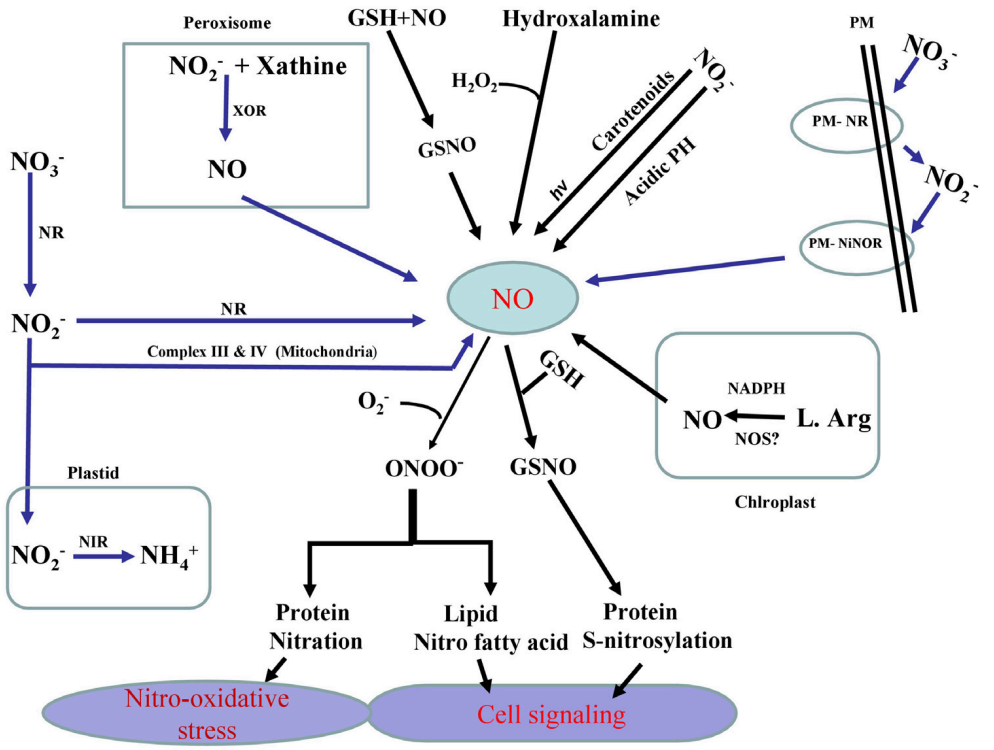

FIGURE 1 | Schematic representation of the pathways (enzymatic and non-enzymatic) and their major components involved in the generation of nitric oxide (NO) in plants. Enzymatic production of NO depends on NADPH dependent oxidation of L-Arg via NO synthase (NOS)-like activity and also nitrate reductase (NR) which converts nitrate (NO_3^-) to nitrite (NO_2^-) and later (NO_2^-) reduction into NO via NR itself or via electron transport chain in mitochondria. Specific plasma membrane-bound nitrate and nitrite: nitric oxide reductase (PM-NR/Ni:NOR) activity utilizes NO_2^- as a substrate to generate NO. Another possible route for NO formation is carried out by xanthine oxidoreductase (XOR). Non-enzymatic sources also result in the reduction of (nitrogen dioxide) NO_2 to NO by carotenoids. In non-enzymatic pathway of NO generation that operates under sufficiently acidic medium and NO_2^- gives rise NO and O_2. NO generated in various pathways reacts with reduced GSH to produce *S*-nitrosoglutathione (GSNO), a donor and major reservoir of NO. It is also clear that NO directly modifies target proteins through reacting with reactive oxygen species (ROS) including superoxide, to generate peroxynitrite (ONOO-) which in turn causes nitrosative stress via protein tyrosine nitration. On the other, NO can also directly control cellular thiols via *S*-transnitrosylation reaction.

as seed germination, senescence, root growth, leaf expansion, photo-morphogenesis, floral regulation, photosynthesis, root organogenesis, hypocotyl growth, and pathogen defense (Asgher et al., 2017; Corpas and Palma, 2018; Santisree et al., 2019), stomatal closure and the cytokinin signaling pathway (Tun et al., 2001; Desikan et al., 2004; Shi et al., 2015). Contingent to its concentration and the site of formation NO induces both positive and negative effects on plant metabolic processes. At lower concentrations, NO exhibited important positive effects in plants where it modulated germination, leaf expansion, and detoxification. On the other, several negative effects such as inhibition of photosynthesis, nitrosative stress, chlorophyll degradation were also noticed at much higher concentrations of NO (Zottini et al., 2002; Antoniou et al., 2013). In wheat seedlings, the rate of leaf expansion increased at lower concentrations of NO; however, no beneficial effect was observed at its higher concentrations (Tian and Lei, 2006). In maize, low concentration of NO promoted root growth, and its higher concentration inhibited the root growth (Lombardo et al., 2006). In a similar work, low concentration of NO (100 μM) stimulated growth of *Medicago truncatula*; whereas, a decreased growth was observed with higher concentration of NO (2.5 mM) (Filippou et al., 2013). The authors showed that 2.5 mM-mediated declines in photosynthetic rate, stomatal response, intracellular proline and putrescine accumulation and decreased *M. truncatula* growth (Filippou et al., 2013). Different dose of NO donors can differentially induce the elongation of root tips. To this end, an inhibited growth of hypocotyls in *Arabidopsis,* lettuce, and potato was observed with the treatment of 0.1 mM sodium nitroprusside (SNP), a NO-donor (Beligni and Lamattina, 2000). SNP was also reported to induce root development in *Zea mays* (Gouvea et al., 1997; Corpas and Barroso, 2015a). However, methylene blue, a NO-scavenger was reported to exhibit positive effects on the root development (Gouvea et al., 1997). Compared to GA_3, NO was reported to exhibit its significant role in breaking the dormancy of lettuce seeds (Beligni and Lamattina, 2000). NO can also control growth and re-orientation of pollen tubes (Prado et al., 2004), induce the lateral roots mediated by the plant growth- promoting *Rhizobacterium azospirillum* (Creus et al., 2005).

The role of NO in photosynthesis has rarely been investigated. However the NO-mediated moderate improvement in photosynthetic performance of *Solanum melongena* seedlings

was argued as a result of excessive quenching energy and an increase in quantum yield of PSII (Wu et al., 2013). NO with sulfur improved antioxidant defense system modulating stomatal behavior and sulfur assimilation in *Brassica juncea* (Fatma et al., 2016a). In cucumber seedlings, exogenous NO showed increased chlorophyll content, improved photosynthetic rate, transpiration rate and stomatal conductance (Fan et al., 2007). However, in *Phaseolus aureus*, NO reduced the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) and increased the content of PSII oxygen-evolving complex (Lum et al., 2005). In *Kalanchoe pinnata*, NO inhibited Rubisco activity by *S*-nitrosylation in a dose-dependent manner and also slowed down photosynthetic rate (Abat et al., 2008). In *Phaseolus vulgaris* guard cells, NO showed decreased activity of the H^+-ATPase (Ördög et al., 2013). At cellular level, NO breaks the chain reactions of oxidation and limits oxidative damage (Bakakina et al., 2014). NO can also prevent the generation of toxic hydroxyl radicals by binding with the superoxide radicals produced in the chloroplast and mitochondria during the process of electron transport chain (Arora et al., 2016). Due to its signaling nature NO was reported to upregulate the expression of certain genes to counteract oxidative damage (Jiao et al., 2016). NO also triggers the upregulation of genes such as that of chalcone synthase (CHS), glutathione-*S*-transferase (GST), alternative oxidase (AOX1a) and glutathione peroxidase (GPX) (Murgia et al., 2004). It has also been reported to deter gene expression of thylakoid ascorbate peroxidase (tAPX) controlling oxidative position of plant cell (Murgia et al., 2004).

NO-MEDIATED COPPER-TOLERANCE IN PLANTS

Role of NO in minimization of heavy metal stress in plants has been extensively studied (Laspina et al., 2005; Arasimowicz and Floryszak-Wieczorek, 2007; Xiong et al., 2009; Jhanji et al., 2012; Leterrier et al., 2012; Chmielowska-Bąk et al., 2014; Per et al., 2017b; Rizwan et al., 2018; Ahmad et al., 2018; Li et al., 2018) (**Table 2**). However, mechanisms underlying NO-mediated control of plant responses to elevated Cu-impacts are still elusive. There are two possible strategies that NO might use to mitigate heavy metal stress in plants. As the first approach, elevated Cu-exposed plants tend to upregulate their antioxidant enzymes activity or express genes involved in defense mechanism (Rizwan et al., 2018). Secondly, NO maintains the equilibrium of cellular free metal concentration either by excluding heavy metals through roots or by keeping a check on their cellular accumulation to a toxic level (Oz et al., 2015). The action mechanisms potentially involved in NO-mediated plant Cu-tolerance have been illustrated in **Figure 2**. The outcomes of the studies analyzing the role of NO-application in Cu-stressed plants revealed that NO reduces Cu-induced oxidative stress by increasing the activities of antioxidant enzymes; maintaining cellular redox homeostasis by elimination of ROS, and thereby promoting normal metabolic function (Cui et al., 2009; Zhang et al., 2009). The supply of sodium nitroprusside (SNP), a NO-donor to Cu-exposed *Panax ginseng*

TABLE 2 | Representative studies on the copper toxicity and nitric acid role on selected parameters in plants.

Plants	Copper concentration	Nitric oxide concentration	Time of nitric oxide supply	Parameters studied	Response	References
Panax ginseng	50 μM	100 μM		Antioxidant activity (SOD, POD, APX)	+	Tewari et al., 2008
Lycopersicon esculentum	1.0 μM	100 μM	21 days after sowing (DAS)	H2O2, MDA	–	Wang et al., 2010
Triticum aestivum	5.0 mM	100 μM	3 DAS	Germination percentage, Amylase activity	+	Hu et al., 2007
Oryza sativa	100 μM	200 μM	12 DAS	GSH, GSSG and phytochelatins	+	Mostofa et al., 2014
L. esculentum	50 μM	100 μM	8 DAS	SOD, POD, CAT, APX, H^+-ATPPase, H^+-PPase	+	Zhang et al., 2009
Catharanthus roseus	30 mg kg-1	50 μM	30 DAS	Phenylalanine ammonia-lyase activity and total soluble phenol content, leaf vincristine vinblastine and total alkaloid content	+	Liu et al., 2016
Lolium perenne	200 μM	100 μM		Seedlings growth	+	Dong et al., 2014
Lepidium sativum	50, 100, and 200 μM	50, 100 μM	20 DAS	Roots and shoots fresh weight, contents of chlorophyll *a*, *b*, total chlorophyll, and carotenoids	+	Raeisi et al., 2009
O. sativa	100 μM	200 μM	12 DAS	LOX activity, O_2^-, H_2O_2, MDA, and Proline content	–	Mostofa et al., 2014
O. sativa	10 mmol	100 mmol	12 DAS	NH_4^+ accumulation	–	Yu et al., 2005
Nicotiana tabacum	0.2 mM Cu	0.05 mM NO	20 DAS	Fresh weight and total chlorophyll contents, Rubisco and rubisco activase activity	+	Khairy et al., 2016

Symbols + and – indicate increase and decrease, respectively.

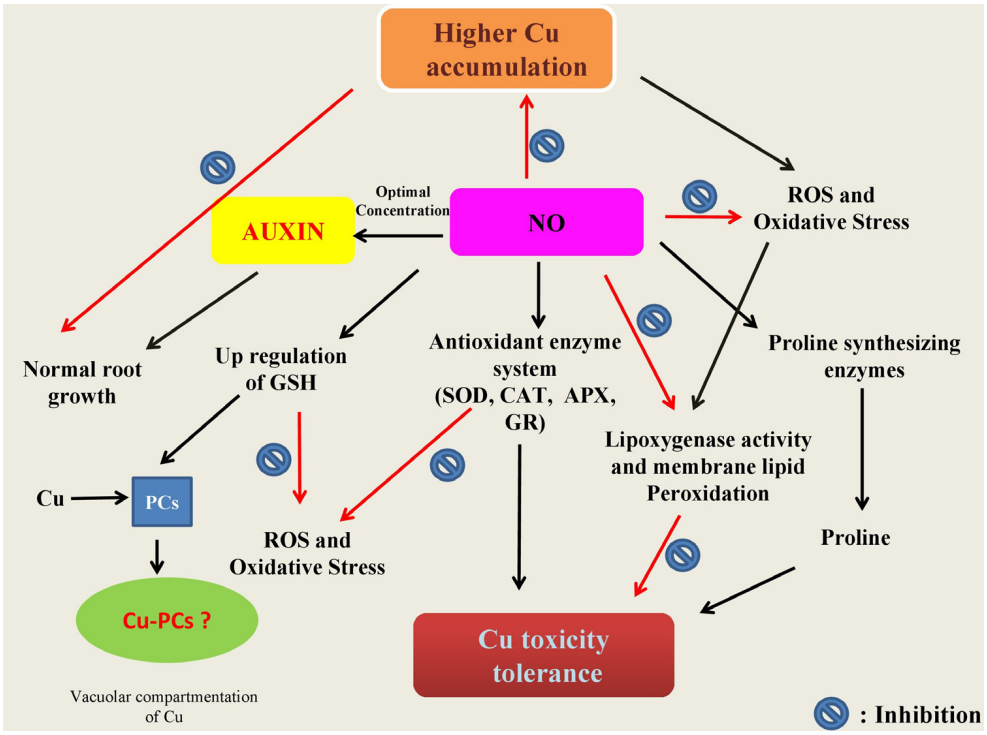

FIGURE 2 | Schematic representation of the potential mechanisms underlying protective roles of nitric oxide (NO) in plants. In summary: (a) NO is involved in cellular homeostasis under Cu stress by inducing/modulating enzymes (CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase) and non-enzyme including reduced glutathione (GSH) directly and/or indirectly involved the scavenging of reactive oxygen species (ROS); (b) NO can also be involved in the stimulation of the key enzymes of proline synthesis and thereby in modulation of the cellular proline and also in inhibition of lipoxygenase activity which in turn leads to membrane lipid peroxidation; and (c) once in cell, Cu ions make complex with phytochelatins (PCS) known to be induced by low molecular weight peptides (such as GSH) in the cytosol. The Cu-PC complexes are high molecular weight and are subsequently transported to the vacuole and thereby protect ill consequences of elevated Cu ions.

modulated the activity of H_2O_2-metabolizing enzymes including catalase (CAT), peroxidase, and ascorbate peroxidase (APX), and thereby increased the detoxification of H_2O_2 in roots (Tewari et al., 2008). Pre-treatment of *Triticum aestivum* with NO was reported to reverse the inhibitory effect of Cu stress by increasing the activity of superoxide dismutase (SOD) and CAT, and reducing the lipoxygenase activity and membrane lipid peroxidation (Hu et al., 2007). Besides inducing antioxidant system, supplied NO was also reported to promote the activity of H^+-ATPase and H^+-PPase in the plasma membrane or tonoplast which might play important role in tolerance to Cu stress by maintaining cytoplasmic pH (Cui et al., 2009; Zhang et al., 2009). The supplied NO-mediated escalation in the level of GSH has also been reported (Zhang et al., 2019). GSH has central role in plants in maintaining cellular redox potential (Anjum et al., 2014; Ahmad et al., 2020). Recently, Mostofa et al. (2014) reported alleviation of Cu-induced toxicity in *Oryza sativa* seedlings mainly as a result of interaction between NO and GSH. The authors revealed that the supply of SNP (200 μM) alone or in combination with GSH (200 μM) minimized Cu-impacts by reducing Cu-uptake and eased the Cu-induced oxidative damage by amending GSH production. Not only GSH content was increased but also increased the content of ascorbate (AsA), and the ratios of GSH/GSSG and AsA/DHA, which in turn strengthened antioxidant defense system improved Cu-tolerance. Moreover the contribution of different metal-chelating ligands, such as metallothioneins (MTs) and phytochelatins (PCs) are crucial players and plays pivotal role in conferring resistance to heavy metal tolerance in plants dealing with high concentrations of various metal inclusion (Cobbett and Goldsbrough, 2002; Anjum et al., 2015a; Chaudhary et al., 2018). Exogenous NO can also regulate the oxidation-reduction status of GSH-GSSG, control the GSH-PC metabolism, and also promote the vascular compartmentalization of excessive Cu in *Lemna esculentum* (Wang et al., 2018). In addition, MTs-responsive genes were induced by NO in *Solanum lycopersicum* and this NO-induced expression of MTs-related genes were reversed by NO scavenger [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazo-line-1-oxyl-3-oxide potassium salt (cPTIO)], NOS inhibitor [*N*-nitro-l-arginine methyl ester (L-NAME)] and NR inhibitor (tungstate), which confirmed the involvement of MTs in NO-mediated tolerance to Cu toxicity (Wang et al., 2010).

Proline, a multifunctional amino acid have diverse roles in response to stress conditions, such as in stabilization of proteins, subcellular structures and membranes and protecting cellular functions by scavenging ROS potentiate plant to survive under stress (Anjum et al., 2014; Kaur and Asthir, 2015;

Per et al., 2017b). The Cu-exposure accrued accumulation of proline has been found as a common response reported in several plant species (Zhang et al., 2008; Fidalgo et al., 2013; Mostofa et al., 2014). Cu-exposure caused increase in the endogenous NO production was found to modulate the cellular proline through stimulating the activity of the key proline-synthesizing enzymes such as pyrroline-5-carboxylate synthetase (Zhang et al., 2008). However, the correlation between NO content and Cu-induced proline accumulation in plants is not always apparent. For example proline content increased upon Cu-exposure but failed upon application of NO (Mostofa et al., 2014). Therefore, it indicates that the regulation of antioxidant system by NO is also dependent on the exposure conditions and the model plant. Photosynthetic functions are typically affected either directly or indirectly by elevated levels of Cu. In Cu-exposed grown *Chlorella*, NO application takes down the inhibition levels of O_2 fixation, O_2 evolution, and maximum quantum yield of PS II and also ominously diminished the oxidative burst (Kumar Singh et al., 2004). In another study, addition of 50, 100, 200 μM SNP protected *Lolium perenne* against Cu-toxicity as a result of increased chlorophyll content and photosynthesis, induced antioxidant enzyme activities, reduced Cu-induced oxidative damages, maintained intracellular ion equilibrium, and limited Cu translocation from roots to shoots (Dong et al., 2014). NO was found to scavenge ROS and control NH_4^+ accumulation in Cu-exposed *O. sativa* leaves (Yu et al., 2005). In Cu (50 μM)-exposed *Arabidopsis* seedlings, NO escalated the Cu-induced cotyledon expansion but alleviated cotyledon elongation processes (Petó et al., 2011).

In naked barley (without hull), Cu-tolerance involved nitrate reductase-mediated NO-production (Hu et al., 2015). Further NO-mediated strengthening of antioxidant defense system and the control of oxidative stress and cell death was also shown (Hu, 2016). In *Catharanthus roseus*, SNP ameliorated Cu-toxicity by decreasing the ROS-burst, promoting the contents of amino acids and the total phenolic in the roots, regulating mineral absorption and re-establishing ATPase activities (Liu et al., 2016). Yuan et al. (2013) have reported that the Cu stress in *Arabidopsis* affects root elongation by redistributing PIN1-mediated auxin (AUX). These witnessed phenotypic changes in Cu-effected roots are possibly due to AUX action, because in roots, the pattern of AUX distribution is vital for healthy root development. In addition to this Fernández-Marcos et al. (2011) showed that mutant cue1/nox1 changes NO levels, and high level of NO hampered the length of root apical meristem in *Arabidopsis*, and reduced transport of AUX and its response by altering the PIN1 levels. Therefore, the supply of optimal level of NO might be a responsible for maintaining the AUX concentration and its distribution when plants countered the heavy metal stress (**Figure 2**). In Arabidopsis, prolonged exposure of Cu impacted NO and AUX metabolism, and it was revealed that NO could improve Cu-induced inhibition of both root and stem growth by attenuating PIN1 induced AUX transport (Kolbert et al., 2012). In another report, Kolbert et al. (2015) established the relationship of the low Cu-sensitivity of nia1nia2noa1-2 mutant with the availability therein of low NO level and suggested that the contribution of the NR and NO associated 1-dependent pathways to NO synthesis. Thus, the above results pointed out that NO plays a vital role in response to Cu stress. However, there is still limitations in understanding the exact mechanism of NO action under Cu stress, and there is an utmost need to further investigate focusing more into molecular insights of NO action under Cu stress.

CONCLUSION AND PROSPECTS

This review appraised the literature available on Cu-induced toxicity and its NO-mediated amelioration and underlying mechanisms in plants. It is clear that NO is a diffusible gaseous molecule and plays a key role in performing a number of biological functions in plants. NO acts as a signaling molecule in inducing the antioxidant system during oxidative stress. There is a remarkable progress in our understanding on the biological role of NO in plants particularly in case of salt stress. However, least information is available on the response of NO on Cu stress. Insights are required into the signaling pathways and the direct targets of NO particularly in Cu-exposed plants. Examination of the Cu-induced modulation of the NO biosynthetic pathways and its involvement in the physiological roles of NO in plants would be imperative in this regard. Some of the biosynthetic pathways of NO in plants are well known but how these pathways are interconnected and what is the mode of action in each tissue and organ are required to be elucidated.

AUTHOR CONTRIBUTIONS

AM and NK conceived the idea. BR, ZS, and AM prepared the first draft. NA, AM, and NK corrected and improved the manuscript. All authors approved the final version for its publication.

REFERENCES

Abat, J. K., Mattoo, A. K., and Deswal, R. (2008). S-nitrosylated proteins of a medicinal CAM plant *Kalanchoe pinnata*–ribulose-1, 5-bisphosphate carboxylase/oxygenase activity targeted for inhibition. *FEBS J.* 275, 2862–2872. doi: 10.1111/j.1742-4658.2008.06425.x

Abdel-Ghany, S. E., and Pilon, M. (2008). MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*. *J. Biol. Chem.* 283, 15932–15945. doi: 10.1074/jbc.M801406200

Adrees, M., Ali, S., Rizwan, M., Ibrahim, M., Abbas, F., Farid, M., et al. (2015). The effect of excess copper on growth and physiology of important food crops: a review. *Environ. Sci. Pollut. Res.* 22, 8148–8162. doi: 10.1007/s11356-015-4496-5

Aguirre, G., and Pilon, M. (2016). Copper delivery to chloroplast proteins and its regulation. *Front. Plant Sci.* 6:1250. doi: 10.3389/fpls.2015.01250

Ahmad, M., Anjum, N. A., Asif, A., and Ahmad, A. (2020). Real-time monitoring of glutathione in living cells using genetically encoded FRET-based ratiometric nanosensor. *Sci. Rep.* 10:992. doi: 10.1038/s41598-020-57654-y

Ahmad, P., Ahanger, M. A., Alyemeni, M. N., Wijaya, L., and Alam, P. (2018). Exogenous application of nitric oxide modulates osmolyte metabolism, antioxidants, enzymes of ascorbate-glutathione cycle and promotes growth under cadmium stress in tomato. *Protoplasma* 255, 79–93. doi: 10.1007/s00709-017-1132-x

Ali, N. A., Bernal, M. P., and Ater, M. (2002). Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. *Plant Soil* 239, 103–111. doi: 10.1023/A:1014995321560

Ali, S., Rizwan, M., Ullah, N., Bharwana, S. A., Waseem, M., Farooq, M. A., et al. (2016). Physiological and biochemical mechanisms of silicon-induced copper stress tolerance in cotton (*Gossypium hirsutum* L.). *Acta Physiol. Plant.* 38:262. doi: 10.1007/s11738-016-2279-3

Ambrosini, V. G., Rosa, D. J., de Melo, G. W., Zalamena, J., Cella, C., Simão, D. G., et al. (2018). High copper content in vineyard soils promotes modifications in photosynthetic parameters and morphological changes in the root system of 'Red Niagara'plantlets. *Plant Physiol. Biochem.* 128, 89–98. doi: 10.1016/j.plaphy.2018.05.011

Andrés-Bordería, A., Andrés, F., Garcia-Molina, A., Perea-García, A., Domingo, C., Puig, S., et al. (2017). Copper and ectopic expression of the *Arabidopsis* transport protein COPT1 alter iron homeostasis in rice (*Oryza sativa* L.). *Plant Mol. Biol.* 95, 17–32. doi: 10.1007/s11103-017-0622-8

Andrés-Colás, N., Carrió-Seguí, A., Abdel-Ghany, S. E., Pilon, M., and Peñarrubia, L. (2018). Expression of the intracellular COPT3-mediated Cu transport is temporally regulated by the TCP16 transcription factor. *Front. Plant Sci.* 9:910. doi: 10.3389/fpls.2018.00910

Anjum, N. A., Aref, I. M., Duarte, A. C., Pereira, E., Ahmad, I., and Iqbal, M. (2014). Glutathione and proline can coordinately make plants withstand the joint attack of metal(loid) and salinity stresses. *Front. Plant Sci.* 5:662. doi: 10.3389/fpls.2014.00662

Anjum, N. A., Hasanuzzaman, M., Hossain, M. A., Thangavel, P., Roychoudhury, A., Gill, S. S., et al. (2015a). Jacks of metal/metalloid chelation trade in plants - an overview. *Front. Plant Sci.* 6:192. doi: 10.3389/fpls.2015.00192

Anjum, N. A., Singh, H. P., Khan, M. I. R., Masood, A., Per, T. S., Negi, A., et al. (2015b). Too much is bad–an appraisal of phytotoxicity of elevated plant-beneficial heavy metal ions. *Environ. Sci. Pollut. Res.* 22, 3361–3382. doi: 10.1007/s11356-014-3849-9

Antoniou, C., Filippou, P., Mylona, P., Fasoula, D., Ioannides, I., Polidoros, A., et al. (2013). Developmental stage-and concentration-specific sodium nitroprusside application results in nitrate reductase regulation and the modification of nitrate metabolism in leaves of *Medicago truncatula* plants. *Plant Signal. Behav.* 8:e25479. doi: 10.4161/psb.25479

Arasimowicz, M., and Floryszak-Wieczorek, J. (2007). Nitric oxide as a bioactive signalling molecule in plant stress responses. *Plant Sci.* 172, 876–887. doi: 10.1016/j.plantsci.2007.02.005

Arora, D., Jain, P., Singh, N., Kaur, H., and Bhatla, S. C. (2016). Mechanisms of nitric oxide crosstalk with reactive oxygen species scavenging enzymes during abiotic stress tolerance in plants. *Free Radic. Res.* 50, 291–303. doi: 10.3109/10715762.2015.1118473

Asgher, M., Khan, M. I. R., Anjum, N. A., Verma, S., Vyas, D., Per, T. S., et al. (2018a). Ethylene and polyamines in counteracting heavy metal phytotoxicity: a crosstalk perspective. *J. Plant Growth Regul.* 37, 1050–1065. doi: 10.1007/s00344-018-9823-x

Asgher, M., Per, T. S., Masood, A., Fatma, M., Freschi, L., Corpas, F. J., et al. (2017). Nitric oxide signaling and its crosstalk with other plant growth regulators in plant responses to abiotic stress. *Environ. Sci. Pollut. Res.* 24, 2273–2285. doi: 10.1007/s11356-016-7947-8

Asgher, M., Per, T. S., Verma, S., Pandith, S. A., Masood, A., and Khan, N. A. (2018b). Ethylene supplementation increases PSII efficiency and alleviates chromium-inhibited photosynthesis through increased nitrogen and sulfur assimilation in mustard. *J. Plant Growth Regul.* 37, 1300–1317. doi: 10.1007/s00344-018-9858-z

Astier, J., Gross, I., and Durner, J. (2017). Nitric oxide production in plants: an update. *J. Exp. Bot.* 69, 3401–3411. doi: 10.1093/jxb/erx420

Azeez, M., Adesanwo, O., and Adepetu, J. (2015). Effect of Copper (Cu) application on soil available nutrients and uptake. *Afr. J. Agric. Res.* 10, 359–364. doi: 10.5897/AJAR2014.9010

Bakakina, Y., Kolesneva, E., Dubovskaya, L., and Volotovski, I. (2014). "Cytoprotective role of nitric oxide under oxidative stress," in *Nitric Oxide in Plants: Metabolism and Role in Stress Physiology*, eds M. N. Khan, M. Mobin, F. Mohammad, and F. J. Corpas (Cham: Springer), 199–210. doi: 10.1007/978-3-319-06710-0_12

Baker, D. E., and Senef, J. P. (1995). "Copper," in *Heavy Metals in Soils*, ed. B. J. Alloway (London: Blackie Academic and Professional), 179–205.

Bankaji, I., Caçador, I., and Sleimi, N. (2015). Physiological and biochemical responses of *Suaeda fruticosa* to cadmium and copper stresses: growth, nutrient uptake, antioxidant enzymes, phytochelatin, and glutathione levels. *Environ. Sci. Pollut. Res.* 22, 13058–13069. doi: 10.1007/s11356-015-4414-x

Barbosa, R. H., Tabaldi, L. A., Miyazaki, F. R., Pilecco, M., Kassab, S. O., and Bigaton, D. (2013). Foliar copper uptake by maize plants: effects on growth and yield. *Ciênc. Rural* 43, 1561–1568. doi: 10.1590/S0103-84782013000900005

Begara-Morales, J. C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M. N., and Barroso, J. B. (2019). The function of S-nitrosothiols during abiotic stress in plants. *J. Exp. Bot.* 70, 4429–4439. doi: 10.1093/jxb/erz197

Begara-Morales, J. C., Chaki, M., Valderrama, R., Sánchez-Calvo, B., Mata-Pérez, C., Padilla, M. N., et al. (2018). Nitric oxide buffering and conditional nitric oxide release in stress response. *J. Exp. Bot.* 69, 3425–3438. doi: 10.1093/jxb/ery072

Beligni, M., and Lamattina, L. (2001). Nitric oxide in plants: the history is just beginning. *Plant Cell Environ.* 24, 267–278. doi: 10.1046/j.1365-3040.2001.00672.x

Beligni, M. V., and Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta* 210, 215–221. doi: 10.1007/PL00008128

Bernal, M., Casero, D., Singh, V., Wilson, G. T., Grande, A., Yang, H., et al. (2012). Transcriptome sequencing identifies *SPL7*-regulated copper acquisition genes *FRO4/FRO5* and the copper dependence of iron homeostasis in *Arabidopsis*. *Plant Cell* 24, 738–761. doi: 10.1105/tpc.111.090431

Bouazizi, H., Jouili, H., Geitmann, A., and El Ferjani, E. (2010). Copper toxicity in expanding leaves of *Phaseolus vulgaris* L.: antioxidant enzyme response and nutrient element uptake. *Ecotoxicol. Environ. Saf.* 73, 1304–1308. doi: 10.1016/j.ecoenv.2010.05.014

Bowler, C., Van Camp, W., Van Montagu, M., Inzé, D., and Asada, K. (1994). Superoxide dismutase in plants. *Crit. Rev. Plant. Sci.* 13, 199–218. doi: 10.1080/07352689409701914

Brunetto, G., Bastos de Melo, G. W., Terzano, R., Del Buono, D., Astolfi, S., Tomasi, N., et al. (2016). Copper accumulation in vineyard soils: rhizosphere processes and agronomic practices to limit its toxicity. *Chemosphere* 162, 293–307. doi: 10.1016/j.chemosphere.2016.07.104

Casareno, R. L. B., Waggoner, D., and Gitlin, J. D. (1998). The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. *J. Biol. Chem.* 273, 23625–23628. doi: 10.1074/jbc.273.37.23625

Chatterjee, J., and Chatterjee, C. (2000). Phytotoxicity of cobalt, chromium and copper in cauliflower. *Environ. Pollut.* 109, 69–74. doi: 10.1016/S0269-7491(99)00238-9

Chaudhary, K., Agarwal, S., and Khan, S. (2018). "Role of phytochelatins (PCs), metallothioneins (MTs), and heavy metal ATPase (HMA) genes in heavy metal tolerance," in *Mycoremediation and Environmental Sustainability*, ed. R. Prasad (Cham: Springer), 39–60. doi: 10.1007/978-3-319-77386-5_2

Chen, J., Shafi, M., Li, S., Wang, Y., Wu, J., Ye, Z., et al. (2015). Copper induced oxidative stresses, antioxidant responses and phytoremediation potential of Moso bamboo (*Phyllostachys pubescens*). *Sci. Rep.* 5:13554. doi: 10.1038/srep13554

Chmielowska-Bąk, J., Gzyl, J., Ruciñska-Sobkowiak, R., Arasimowicz-Jelonek, M., and Deckert, J. (2014). The new insights into cadmium sensing. *Front. Plant Sci.* 5:245. doi: 10.3389/fpls.2014.00245

Chu, C. C., Lee, W. C., Guo, W. Y., Pan, S. M., Chen, L. J., Li, H. M., et al. (2005). A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in Arabidopsis. *Plant Physiol.* 139, 425–436. doi: 10.1104/pp.105.065284

Ciscato, M., Valcke, R., Van Loven, K., Clijsters, H., and NavariIzzo, F. (1997). Effects of in vivo copper treatment on the photosynthetic apparatus of two *Triticum durum* cultivars with different stress sensitivity. *Physiol. Plant.* 100, 901–908. doi: 10.1111/j.1399-3054.1997.tb00016.x

Cobbett, C., and Goldsbrough, P. (2002). Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* 53, 159–182. doi: 10.1146/annurev.arplant.53.100301.135154

Cohu, C. M., and Pilon, M. (2010). "Cell biology of copper," in *Cell Biology of Metals and Nutrients*, eds R. Hell, and R.-R. Mendel (Berlin: Springer), 55–74. doi: 10.1007/978-3-642-10613-2_3

Cona, A., Rea, G., Angelini, R., Federico, R., and Tavladoraki, P. (2006). Functions of amine oxidases in plant development and defence. *Trends Plant Sci.* 11, 80–88. doi: 10.1016/j.tplants.2005.12.009

Constabel, C. P., and Barbehenn, R. (2008). "Defensive roles of polyphenol oxidase in plants," in *Induced Plant Resistance to Herbivory*, ed. A. Schaller (Berlin: Springer), 253–270. doi: 10.1007/978-1-4020-8182-8_12

Corpas, F., and Palma, J. (2018). Assessing nitric oxide (NO) in higher plants: an outline. *Nitrogen* 1, 12–20. doi: 10.3390/nitrogen1010003

Corpas, F. J., Alché, J. D. D., and Barroso, J. B. (2013). Current overview of S-nitrosoglutathione (GSNO) in higher plants. *Front. Plant Sci.* 4:126. doi: 10.3389/fpls.2013.00126

Corpas, F. J., and Barroso, J. B. (2015a). Functions of nitric oxide (NO) in roots during development and under adverse stress conditions. *Plants* 4, 240–252. doi: 10.3390/plants4020240

Corpas, F. J., and Barroso, J. B. (2015b). Nitric oxide from a "green" perspective. *Nitric Oxide* 45, 15–19. doi: 10.1016/j.niox.2015.01.007

Creus, C. M., Graziano, M., Casanovas, E. M., Pereyra, M. A., Simontacchi, M., Puntarulo, S., et al. (2005). Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. *Planta* 221, 297–303. doi: 10.1007/s00425-005-1523-7

Cui, X., Zhang, Y., Chen, X., Jin, H., and Wu, X. (2009). "Effects of exogenous nitric oxide protects tomato plants under copper stress," in *Proceedings of the 2009 3rd International Conference on Bioinformatics and Biomedical Engineering*, (Beijing: IEEE), 1–7. doi: 10.1109/ICBBE.2009.5162740

de Freitas, T. A., França, M. G. C., De Almeida, A.-A. F., De Oliveira, S. J. R., De Jesus, R. M., Souza, V. L., et al. (2015). Morphology, ultrastructure and mineral uptake is affected by copper toxicity in young plants of *Inga subnuda* subs. *luschnathiana* (Benth.) TD Penn. *Environ. Sci. Pollut. Res. Int.* 22, 15479–15494. doi: 10.1007/s11356-015-4610-8

de Rienzo, F., Gabdoulline, R., Menziani, M. C., and Wade, R. (2000). Blue copper proteins: a comparative analysis of their molecular interaction properties. *Protein Sci.* 9, 1439–1454. doi: 10.1110/ps.9.8.1439

Desikan, R., Cheung, M. K., Bright, J., Henson, D., Hancock, J. T., and Neill, S. J. (2004). ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. *J. Exp. Bot.* 55, 205–212. doi: 10.1093/jxb/erh033

Domingos, P., Prado, A. M., Wong, A., Gehring, C., and Feijo, J. A. (2015). Nitric oxide: a multitasked signaling gas in plants. *Mol. Plant* 8, 506–520. doi: 10.1016/j.molp.2014.12.010

Dong, Y., Xu, L., Wang, Q., Fan, Z., Kong, J., and Bai, X. (2014). Effects of exogenous nitric oxide on photosynthesis, antioxidative ability, and mineral element contents of perennial ryegrass under copper stress. *J. Plant Interact.* 9, 402–411. doi: 10.1080/17429145.2013.845917

Eisses, J. F., and Kaplan, J. H. (2002). Molecular characterization of hCTR1, the human copper uptake protein. *J. Biol. Chem.* 277, 29162–29171. doi: 10.1074/jbc.M203652200

Emamverdian, A., Ding, Y., Mokhberdoran, F., and Xie, Y. (2015). Heavy metal stress and some mechanisms of plant defense response. *Sci. World J.* 2015:756120. doi: 10.1155/2015/756120

Fan, H., Guo, S., Jiao, Y., Zhang, R., and Li, J. (2007). Effects of exogenous nitric oxide on growth, active oxygen species metabolism, and photosynthetic characteristics in cucumber seedlings under NaCl stress. *Front. Agric. Sci. China* 1, 308–314. doi: 10.1007/s11703-007-0052-5

Fancy, N. N., Bahlmann, A. K., and Loake, G. J. (2017). Nitric oxide function in plant abiotic stress. *Plant Cell Environ.* 40, 462–472. doi: 10.1111/pce.12707

Fatma, M., Masood, A., Per, T. S., and Khan, N. A. (2016a). Nitric oxide alleviates salt stress inhibited photosynthetic performance by interacting with sulfur assimilation in mustard. *Front. Plant Sci.* 7:521. doi: 10.3389/fpls.2016.00521

Fatma, M., Masood, A., Per, T. S., Rasheed, F., and Khan, N. A. (2016b). Interplay between nitric oxide and sulfur assimilation in salt tolerance in plants. *Crop J.* 4, 153–161. doi: 10.1016/j.cj.2016.01.009

Fazzari, M., Trostchansky, A., Schopfer, F. J., Salvatore, S. R., Sánchez-Calvo, B., Vitturi, D., et al. (2014). Olives and olive oil are sources of electrophilic fatty acid nitroalkenes. *PLoS One* 9:e84884. doi: 10.1371/journal.pone.0084884

Feigl, G., Kumar, D., Lehotai, N., Petõ, A., Molnár, Á., Rácz, É., et al. (2015). Comparing the effects of excess copper in the leaves of *Brassica juncea* (L. Czern) and *Brassica napus* (L.) seedlings: growth inhibition, oxidative stress and photosynthetic damage. *Acta Biol. Hung.* 66, 205–221. doi: 10.1556/018.66.2015.2.7

Feigl, G., Kumar, D., Lehotai, N., Tugyi, N., Molnár, Á., Ördög, A., et al. (2013). Physiological and morphological responses of the root system of Indian mustard (*Brassica juncea* L. Czern.) and rapeseed (*Brassica napus* L.) to copper stress. *Ecotoxicol. Environ. Saf.* 94, 179–189. doi: 10.1016/j.ecoenv.2013.04.029

Feng, H., Zhang, Q., Wang, Q., Wang, X., Liu, J., Li, M., et al. (2013). Target of tae-miR408, a chemocyanin-like protein gene (TaCLP1), plays positive roles in wheat response to high-salinity, heavy cupric stress and stripe rust. *Plant Mol. Biol.* 83, 433–443. doi: 10.1007/s11103-013-0101-9

Fernández-Marcos, M., Sanz, L., Lewis, D. R., Muday, G. K., and Lorenzo, O. (2011). Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN–FORMED 1 (PIN1)–dependent acropetal auxin transport. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18506–18511. doi: 10.1073/pnas.1108644108

Fidalgo, F., Azenha, M., Silva, A. F., de Sousa, A., Santiago, A., Ferraz, P., et al. (2013). Copper-induced stress in *Solanum nigrum* L. and antioxidant defense system responses. *Food Energy Sec.* 2, 70–80. doi: 10.1002/fes3.20

Filippou, P., Antoniou, C., and Fotopoulos, V. (2013). The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of *Medicago truncatula* plants. *Free Radic. Biol. Med.* 56, 172–183. doi: 10.1016/j.freeradbiomed.2012.09.037

Gang, A., Vyas, A., and Vyas, H. (2013). Toxic effect of heavy metals on germination and seedling growth of wheat. *J. Environ. Res. Dev.* 8, 206–213. doi: 10.4103/0976-9668.116964

Garcia-Molina, A., Andrés-Colás, N., Perea-García, A., Neumann, U., Dodani, S. C., Huijser, P., et al. (2013). The Arabidopsis COPT6 transport protein functions in copper distribution under copper-deficient conditions. *Plant Cell Physiol.* 54, 1378–1390. doi: 10.1016/j.ecoenv.2013.04.029

Gonzalez-Mendoza, D., Escoboza-Garcia, F., Santamaria, J. M., and Zapata-Perez, O. (2013). Copper stress on photosynthesis of black mangle (*Avicennia germinans*). *An. Acad. Bras. Ciênc.* 85, 665–670. doi: 10.1590/S0001-37652013000200013

Gouvea, C., Souza, J., Magalhaes, A., and Martins, I. (1997). NO●–releasing substances that induce growth elongation in maize root segments. *Plant Growth Regul.* 21, 183–187. doi: 10.1023/A:1005837012203

Hasanuzzaman, M., Oku, H., Nahar, K., Bhuyan, M. B., Al Mahmud, J., Baluska, F., et al. (2018). Nitric oxide-induced salt stress tolerance in plants: ROS metabolism, signaling, and molecular interactions. *Plant Biotechnol. Rep.* 12, 77–92. doi: 10.1007/s11816-018-0480-0

Havlin, J. L., Beaton, J. D., Tisdale, S. L., and Nelson, W. L. (1999). *Soil Fertility and Fertilizers—an Introduction to Nutrient Management*, 6th Edn. Upper Saddle River, NJ: Prentice Hall.

Hu, K. D., Hu, L.-Y., Li, Y. H., Zhang, F. Q., and Zhang, H. (2007). Protective roles of nitric oxide on germination and antioxidant metabolism in wheat seeds under copper stress. *Plant Growth Regul.* 53, 173–183. doi: 10.1007/s10725-007-9216-9

Hu, Y. (2016). Early generation of nitric oxide contributes to copper tolerance through reducing oxidative stress and cell death in hulless barley roots. *J. Plant Res.* 129, 963–978. doi: 10.1007/s10265-016-0841-0

Hu, Y., You, J., and Liang, X. (2015). Nitrate reductase-mediated nitric oxide production is involved in copper tolerance in shoots of hulless barley. *Plant Cell Rep.* 34, 367–379. doi: 10.1007/s00299-014-1715-3

Huffman, D. L., and O'Halloran, T. V. (2001). Function, structure, and mechanism of intracellular copper trafficking proteins. *Annu. Rev. Biochem.* 70, 677–701. doi: 10.1146/annurev.biochem.70.1.677

Ivanova, E., Kholodova, V., and Kuznetsov, V. V. (2010). Biological effects of high copper and zinc concentrations and their interaction in rapeseed plants. *Russ. J. Plant Physiol.* 57, 806–814. doi: 10.1134/S1021443710060099

Jahan, B., Tabish Rehman, M., Alajmi, M. F., and Khan, N. A. (2020). Treatment of nitric oxide supplemented with nitrogen and sulfur regulates photosynthetic performance and stomatal behavior in mustard under salt stress. *Physiol. Plant.* 168, 490–510. doi: 10.1111/ppl.13056

Jhanji, S., Setia, R., Kaur, N., Kaur, P., and Setia, N. (2012). Role of nitric oxide in cadmium-induced stress on growth, photosynthetic components and yield of *Brassica napus* L. *J. Environ. Biol.* 33, 1027–1032.

Jiao, C., Yang, R., Zhou, Y., and Gu, Z. (2016). Nitric oxide mediates isoflavone accumulation and the antioxidant system enhancement in soybean sprouts. *Food Chem.* 204, 373–380. doi: 10.1016/j.foodchem.2016.02.147

Jung, H. I., Gayomba, S. R., Rutzke, M. A., Craft, E., Kochian, L. V., and Vatamaniuk, O. K. (2012). COPT6 is a plasma membrane transporter that functions in copper homeostasis in Arabidopsis and is a novel target of SQUAMOSA promoter-binding protein-like 7. *J. Biol. Chem.* 287, 33252–33267. doi: 10.1074/jbc.M112.397810

Kampfenkel, K., Kushnir, S., Babiychuk, E., Inzé, D., and Van Montagu, M. (1995). Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. *J. Biol. Chem.* 270, 28479–28486. doi: 10.1074/jbc.270.47.28479

Kato, N., and Esaka, M. (1999). Changes in ascorbate oxidase gene expression and ascorbate levels in cell division and cell elongation in tobacco cells. *Physiol. Plant* 105, 321–329. doi: 10.1034/j.1399-3054.1999.105218.x

Kaur, G., and Asthir, B. J. B. P. (2015). Proline: a key player in plant abiotic stress tolerance. *Biol. Plant* 59, 609–619. doi: 10.1007/s10535-015-0549-3

Kaur, R., and Nayyar, H. (2014). "Ascorbic acid: a potent defender against environmental stresses," in *Oxidative Damage to Plants: Antioxidant Networks and Signaling*, ed. P. Ahmad (Amsterdam: Academic Press), 235–287. doi: 10.1016/B978-0-12-799963-0.00008-3

Kerk, N. M., and Feldman, N. (1995). A biochemical model for the initiation and maintenance of the quiescent center: implications for organization of root meristems. *Development* 121, 2825–2833.

Keutgen, A. J., and Pawelzik, E. (2008). Quality and nutritional value of strawberry fruit under long term salt stress. *Food Chem.* 107, 1413–1420. doi: 10.1016/j.foodchem.2007.09.071

Khairy, A. I. H., Oh, M. J., Lee, S. M., and Roh, K. S. (2016). Nitric oxide overcomes Cd and Cu toxicity in in vitro-grown tobacco plants through increasing contents and activities of rubisco and rubisco activase. *Biochim. Open* 2, 41–51. doi: 10.1016/j.biopen.2016.02.002

Khan, M. I. R., Nazir, F., Asgher, M., Per, T. S., and Khan, N. A. (2015). Selenium and sulfur influence ethylene formation and alleviate cadmium-induced oxidative stress by improving proline and glutathione production in wheat. *J. Plant Physiol.* 173, 9–18. doi: 10.1016/j.jplph.2014.09.011

Khan, N. A., Asgher, M., Per, T. S., Masood, A., Fatma, M., and Khan, M. (2016). Ethylene potentiates sulfur-mediated reversal of cadmium inhibited photosynthetic responses in mustard. *Front. Plant Sci.* 7:1628. doi: 10.3389/fpls.2016.01628

Khan, N. A., Nazar, R., Iqbal, N., and Anjum, N. A. (2012). *Phytohormones and Abiotic Stress Tolerance in Plants*. Heidelberg: Springer. doi: 10.1007/978-3-642-25829-9

Kliebenstein, D. J., Monde, R. A., and Last, R. L. (1998). Superoxide dismutase in Arabidopsis: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* 118, 637–650. doi: 10.1104/pp.118.2.637

Klomp, A. E., Juijn, J. A., Berger, R., and Klomp, L. W. (2003). The N-terminus of the human copper transporter 1 (hCTR1) is localized extracellularly, and interacts with itself. *Biochem. J.* 370, 881–889. doi: 10.1042/bj20021128

Kolbert, Z., Petõ, A., Lehotai, N., Feigl, G., and Erdei, L. (2012). Long-term copper (Cu^{2+}) exposure impacts on auxin, nitric oxide (NO) metabolism and morphology of *Arabidopsis thaliana* L. *Plant Growth Regul.* 68, 151–159. doi: 10.1007/s10725-012-9701-7

Kolbert, Z., Petõ, A., Lehotai, N., Feigl, G., and Erdei, L. (2015). Copper sensitivity of nia1nia2noa1-2 mutant is associated with its low nitric oxide (NO) level. *Plant Growth Regul.* 77, 255–263. doi: 10.1007/s10725-015-0059-5

Kumar Singh, A., Sharma, L., and Mallick, N. (2004). Antioxidative role of nitric oxide on copper toxicity to a chlorophycean alga, *Chlorella*. *Ecotoxicol. Environ. Saf.* 59, 223–227. doi: 10.1016/j.ecoenv.2003.10.009

Küpper, H., Götz, B., Mijovilovich, A., Küpper, F. C., and Meyer-Klaucke, W. (2009). Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator. *Plant Physiol.* 151, 702–714. doi: 10.1104/pp.109.139717

Kushwaha, B. K., Singh, S., Tripathi, D. K., Sharma, S., Prasad, S. M., Chauhan, D. K., et al. (2019). New adventitious root formation and primary root biomass accumulation are regulated by nitric oxide and reactive oxygen species in rice seedlings under arsenate stress. *J. Hazard. Mater.* 361, 134–140. doi: 10.1016/j.jhazmat.2018.08.035

Laspina, N., Groppa, M., Tomaro, M., and Benavides, M. (2005). Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. *Plant Sci.* 169, 323–330. doi: 10.1016/j.plantsci.2005.02.007

Lequeux, H., Hermans, C., Lutts, S., and Verbruggen, N. (2010). Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile. *Plant Physiol. Biochem.* 48, 673–682. doi: 10.1016/j.plaphy.2010.05.005

Leterrier, M., Airaki, M., Palma, J. M., Chaki, M., Barroso, J. B., and Corpas, F. J. (2012). Arsenic triggers the nitric oxide (NO) and *S*-nitrosoglutathione (GSNO) metabolism in *Arabidopsis*. *Environ. Pollut.* 166, 136–143. doi: 10.1016/j.envpol.2012.03.012

Li, Q., Huang, W., Xiong, C., and Zhao, J. (2018). Transcriptome analysis reveals the role of nitric oxide in *Pleurotus eryngii* responses to Cd2+ stress. *Chemosphere* 201, 294–302. doi: 10.1016/j.chemosphere.2018.03.011

Lin, C. C., Chen, L. M., and Liu, Z. H. (2005). Rapid effect of copper on lignin biosynthesis in soybean roots. *Plant Sci.* 168, 855–861. doi: 10.1016/j.plantsci.2004.10.023

Liu, S., Yang, R., Pan, Y., Ren, B., Chen, Q., Li, X., et al. (2016). Beneficial behavior of nitric oxide in copper-treated medicinal plants. *J. Hazard. Mater.* 314, 140–154. doi: 10.1016/j.jhazmat.2016.04.042

Llagostera, I., Cervantes, D., Sanmartí, N., Romero, J., and Pérez, M. (2016). Effects of copper exposure on photosynthesis and growth of the seagrass *Cymodocea nodosa*: an experimental assessment. *Bull. Environ. Contam. Toxicol.* 97, 374–379. doi: 10.1007/s00128-016-1863-y

Lombardo, M. C., Graziano, M., Polacco, J. C., and Lamattina, L. (2006). Nitric oxide functions as a positive regulator of root hair development. *Plant Signal. Behav.* 1, 28–33. doi: 10.4161/psb.1.1.2398

López-Bucio, J., Cruz-Ramırez, A., and Herrera-Estrella, L. (2003). The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* 6, 280–287. doi: 10.1016/S1369-5266(03)00035-9

Lum, H. K., Lee, C. H., Butt, Y. K. C., and Lo, S. C. L. (2005). Sodium nitroprusside affects the level of photosynthetic enzymes and glucose metabolism in *Phaseolus aureus* (mung bean). *Nitric Oxide* 12, 220–230. doi: 10.1016/j.niox.2005.03.002

Maksymiec, W., and Baszyński, T. (1996). Different susceptibility of runner bean plants to excess copper as a function of the growth stages of primary leaves. *J. Plant Physiol.* 149, 217–221. doi: 10.1016/S0176-1617(96)80198-2

Maksymiec, W., Russa, R., Urbanik-Sypniewska, T., and Baszyński, T. (1994). Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages. *Physiol. Plant.* 91, 715–721. doi: 10.1111/j.1399-3054.1994.tb03010.x

Maksymiec, W., Wojcik, M., and Krupa, Z. (2007). Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the presence or absence of jasmonate and ascorbate. *Chemosphere* 66, 421–427. doi: 10.1016/j.chemosphere.2006.06.025

Marastoni, L., Sandri, M., Pii, Y., Valentinuzzi, F., Brunetto, G., Cesco, S., et al. (2019a). Synergism and antagonisms between nutrients induced by copper toxicity in grapevine rootstocks: monocropping vs. *intercropping*. *Chemosphere* 214, 563–578. doi: 10.1016/j.chemosphere.2018.09.127

Marastoni, L., Sandri, M., Pii, Y., Valentinuzzi, F., Cesco, S., and Mimmo, T. (2019b). Morphological root responses and molecular regulation of cation transporters are differently affected by copper toxicity and cropping system depending on the grapevine rootstock genotype. *Front. Plant Sci.* 10:946. doi: 10.3389/fpls.2019.00946

Marques, D. M., Júnior, V. V., Da Silva, A. B., Mantovani, J. R., Magalhães, P. C., and De Souza, T. C. (2018). Copper toxicity on photosynthetic responses and root morphology of *Hymenaea courbaril* L.(*Caesalpinioideae*). *Water Air Soil Pollut.* 229:138. doi: 10.1007/s11270-018-3769-2

Marschner, H. (1995). *Mineral Nutrition of Higher Plants*, 2nd Edn. London: Academic Press.

Masood, A., Iqbal, N., and Khan, N. A. (2012). Role of ethylene in alleviation of cadmium-induced photosynthetic capacity inhibition by sulphur in mustard. *Plant Cell Environ.* 35, 524–533. doi: 10.1111/j.1365-3040.2011.02432.x

Masood, A., Khan, M. I. R., Fatma, M., Asgher, M., Per, T. S., and Khan, N. A. (2016). Involvement of ethylene in gibberellic acid-induced sulfur assimilation,

photosynthetic responses, and alleviation of cadmium stress in mustard. *Plant Physiol. Biochem.* 104, 1–10. doi: 10.1016/j.plaphy.2016.03.017

Mata-Pérez, C., Sánchez-Calvo, B., Padilla, M. N., Begara-Morales, J. C., Luque, F., Melguizo, M., et al. (2016). Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in Arabidopsis. *Plant Physiol.* 170, 686–701. doi: 10.1104/pp.15.01671

Mateos-Naranjo, E., Andrades-Moreno, L., Cambrollé, J., and Perez-Martin, A. (2013). Assessing the effect of copper on growth, copper accumulation and physiological responses of grazing species *Atriplex halimus*: ecotoxicological implications. *Ecotoxicol. Environ. Saf.* 90, 136–142. doi: 10.1016/j.ecoenv.2010.12.020

Maunoury, N., and Vaucheret, H. (2011). AGO1 and AGO2 act redundantly in miR408-mediated Plantacyanin regulation. *PLoS One* 6:e28729. doi: 10.1371/journal.pone.0028729

McCaig, B. C., Meagher, R. B., and Dean, J. F. (2005). Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. *Planta* 221, 619–636. doi: 10.1007/s00425-004-1472-6

Mench, M., and Bes, C. (2009). Assessment of ecotoxicity of topsoils from a wood treatment site. *Pedosphere* 19, 143–155. doi: 10.1016/S1002-0160(09)60104-1

Meng, H., Hua, S., Shamsi, I. H., Jilani, G., Li, Y., and Jiang, L. (2009). Cadmium-induced stress on the seed germination and seedling growth of *Brassica napus* L., and its alleviation through exogenous plant growth regulators. *Plant Growth Regul.* 58, 47–59. doi: 10.1007/s10725-008-9351-y

Millar, A. H., Eubel, H., Jänsch, L., Kruft, V., Heazlewood, J. L., and Braun, H. P. (2004). Mitochondrial cytochrome c oxidase and succinate dehydrogenase complexes contain plant specific subunits. *Plant Mol. Biol.* 56, 77–90. doi: 10.1007/s11103-004-2316-2

Mocquot, B., Vangronsveld, J., Clijsters, H., and Mench, M. (1996). Copper toxicity in young maize (*Zea mays* L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. *Plant Soil* 182, 287–300. doi: 10.1007/BF00029060

Mostofa, M. G., Seraj, Z. I., and Fujita, M. (2014). Exogenous sodium nitroprusside and glutathione alleviate copper toxicity by reducing copper uptake and oxidative damage in rice (*Oryza sativa* L.) seedlings. *Protoplasma* 251, 1373–1386. doi: 10.1007/s00709-014-0639-7

Muccifora, S., and Bellani, L. M. (2013). Effects of copper on germination and reserve mobilization in *Vicia sativa* L. seeds. *Environ. Pollut.* 179, 68–74. doi: 10.1016/j.envpol.2013.03.061

Murgia, I., De Pinto, M. C., Delledonne, M., Soave, C., and De Gara, L. (2004). Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. *J. Plant Physiol.* 161, 777–783. doi: 10.1016/j.jplph.2003.12.004

Mwamba, T. M., Li, L., Gill, R. A., Islam, F., Nawaz, A., Ali, B., et al. (2016). Differential subcellular distribution and chemical forms of cadmium and copper in *Brassica napus*. *Ecotoxicol. Environ. Saf.* 134, 239–249. doi: 10.1016/j.ecoenv.2016.08.021

Nagajyoti, P. C., Lee, K. D., and Sreekanth, T. (2010). Heavy metals, occurrence and toxicity for plants: a review. *Environ. Chem. Lett.* 8, 199–216. doi: 10.1007/s10311-010-0297-8

Nair, P. M., and Chung, I. M. (2015). Study on the correlation between copper oxide nanoparticles induced growth suppression and enhanced lignification in Indian mustard (*Brassica juncea* L.). *Ecotoxicol. Environ. Saf.* 113, 302–313. doi: 10.1016/j.ecoenv.2014.12.013

Nazir, F., Hussain, A., and Fariduddin, Q. (2019). Hydrogen peroxide modulate photosynthesis and antioxidant systems in tomato *(Solanum lycopersicum* L.) plants under copper stress. *Chemosphere* 230, 544–558. doi: 10.1016/j.chemosphere.2019.05.001

Nelson, N. (1999). Metal ion transporters and homeostasis. *EMBO J.* 18, 4361–4371. doi: 10.1093/emboj/18.16.4361

Nevitt, T., Öhrvik, H., and Thiele, D. J. (2012). Charting the travels of copper in eukaryotes from yeast to mammals. *Biochim. Biophys. Acta* 1823, 1580–1593. doi: 10.1016/j.bbamcr.2012.02.011

O'Halloran, T. V., and Culotta, V. C. (2000). Metallochaperones, an intracellular shuttle service for metal ions. *J. Biol. Chem.* 275, 25057–25060. doi: 10.1074/jbc.R000006200

O'Lexy, R., Kasai, K., Clark, N., Fujiwara, T., Sozzani, R., and Gallagher, K. L. (2018). Exposure to heavy metal stress triggers changes in plasmodesmatal permeability via deposition and breakdown of callose. *J. Exp. Bot.* 69, 3715–3728. doi: 10.1093/jxb/ery171

Ördög, A., Wodala, B., Rózsavölgyi, T., Tari, I., and Horváth, F. (2013). Regulation of guard cell photosynthetic electron transport by nitric oxide. *J. Exp. Bot.* 64, 1357–1366. doi: 10.1093/jxb/ers397

Ouzounidou, G., Èiamporová, M., Moustakas, M., and Karataglis, S. (1995). Responses of maize (*Zea mays* L.) plants to copper stress—I. Growth, mineral content and ultrastructure of roots. *Environ. Exp. Bot.* 35, 167–176.

Ouzounidou, G., Ilias, I., Tranopoulou, H., and Karataglis, S. (1998). Amelioration of copper toxicity by iron on spinach physiology. *J. Plant. Nutr.* 21, 2089–2101. doi: 10.1080/01904169809365546

Oz, M. T., Eyidogan, F., Yucel, M., and Öktem, H. A. (2015). "Functional role of nitric oxide under abiotic stress conditions," in *Nitric Oxide Action in Abiotic Stress Responses in Plants*, eds M. N. Khan, M. Mobin, F. Mohammad, and F. J. Corpas (Cham: Springer), 21–41. doi: 10.1007/978-3-319-17804-2_2

Pagnussat, G. C., Lanteri, M. L., Lombardo, M. C., and Lamattina, L. (2004). Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. *Plant Physiol.* 135, 279–286. doi: 10.1104/pp.103.038554

Per, T. S., Khan, N. A., Masood, A., and Fatma, M. (2016). Methyl jasmonate alleviates cadmium-induced photosynthetic damages through increased S-assimilation and glutathione production in mustard. *Front. Plant Sci.* 7:1933. doi: 10.3389/fpls.2016.01933

Per, T. S., Khan, N. A., Reddy, P. S., Masood, A., Hasanuzzaman, M., Khan, M. I. R., et al. (2017a). Approaches in modulating proline metabolism in plants for salt and drought stress tolerance: phytohormones, mineral nutrients and transgenics. *Plant Physiol. Biochem.* 115, 126–140. doi: 10.1016/j.plaphy.2017.03.018

Per, T. S., Masood, A., and Khan, N. A. (2017b). Nitric oxide improves S-assimilation and GSH production to prevent inhibitory effects of cadmium stress on photosynthesis in mustard (*Brassica juncea* L.). *Nitric Oxide* 68, 111–124. doi: 10.1016/j.niox.2016.12.012

Perea-García, A., Garcia-Molina, A., Andrés-Colás, N., Vera-Sirera, F., Pérez-Amador, M. A., Puig, S., et al. (2013). Arabidopsis copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. *Plant Physiol.* 162, 180–194. doi: 10.1104/pp.112.212407

Pető, A., Lehotai, N., Lozano-Juste, J., León, J., Tari, I., Erdei, L., et al. (2011). Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings. *Ann. Bot.* 108, 449–457. doi: 10.1093/aob/mcr176

Piotrowska-Niczyporuk, A., Bajguz, A., Zambrzycka, E., and Godlewska-Żyłkiewicz, B. (2012). Phytohormones as regulators of heavy metal biosorption and toxicity in green alga *Chlorella vulgaris* (Chlorophyceae). *Plant Physiol. Biochem.* 52, 52–65. doi: 10.1016/j.plaphy.2011.11.009

Potters, G., Pasternak, T. P., Guisez, Y., and Jansen, M. A. (2009). Different stresses, similar morphogenic responses: integrating a plethora of pathways. *Plant Cell Environ.* 32, 158–169. doi: 10.1111/j.1365-3040.2008.01908.x

Prado, A. M., Porterfield, D. M., and Feijó, J. A. (2004). Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* 131, 2707–2714. doi: 10.1242/dev.01153

Printz, B., Lutts, S., Hausman, J. F., and Sergeant, K. (2016). Copper trafficking in plants and its implication on cell wall dynamics. *Front. Plant Sci.* 7:601. doi: 10.3389/fpls.2016.00601

Puig, S. (2014). Function and regulation of the plant COPT family of high-affinity copper transport proteins. *Adv. Bot.* 2014:476917. doi: 10.1155/2014/476917

Puig, S., Andrés-Colás, N., García-Molina, A., and Penarrubia, L. (2007). Copper and iron homeostasis in *Arabidopsis*: responses to metal deficiencies, interactions and biotechnological applications. *Plant Cell Environ.* 30, 271–290. doi: 10.1111/j.1365-3040.2007.01642.x

Puig, S., and Thiele, D. J. (2002). Molecular mechanisms of copper uptake and distribution. *Curr. Opin. Chem. Biol.* 6, 171–180. doi: 10.1016/S1367-5931(02)00298-3

Quartacci, M. F., Pinzino, C., Sgherri, C. L., Dalla Vecchia, F., and Navari-Izzo, F. (2000). Growth in excess copper induces changes in the lipid composition and fluidity of PSII-enriched membranes in wheat. *Physiol. Plant.* 108, 87–93. doi: 10.1034/j.1399-3054.2000.108001087.x

Raeisi, M., Asrar, Z., and Pourseyedi, S. (2009). Interaction of sodium nitroprusside and copper on some growth and physiologic parameters of garden cress (*Lepidium sativum* L.). *Iran. J. Plant Biol.* 1, 55–76.

Raven, J. A., Evans, M. C., and Korb, R. E. (1999). The role of trace metals in photosynthetic electron transport in O 2-evolving organisms. *Photosynth. Res.* 60, 111–150.

Rizwan, M., Mostofa, M. G., Ahmad, M. Z., Imtiaz, M., Mehmood, S., Adeel, M., et al. (2018). Nitric oxide induces rice tolerance to excessive nickel by regulating nickel uptake, reactive oxygen species detoxification and defense-related gene expression. *Chemosphere* 191, 23–35. doi: 10.1016/j.chemosphere.2017.09.068

Rodrıguez, F. I., Esch, J. J., Hall, A. E., Binder, B. M., Schaller, G. E., and Bleecker, A. B. (1999). A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. *Science* 283, 996–998. doi: 10.1126/science.283.5404.996

Saleem, M. H., Ali, S., Irshad, S., Hussaan, M., Rizwan, M., Rana, M. S., et al. (2020). Copper uptake and accumulation, ultra-structural alteration, and bast fibre yield and quality of fibrous jute (*Corchorus capsularis* L.) plants grown under two different soils of China. *Plants* 9:404. doi: 10.3390/plants9030404

Sancenón, V., Puig, S., Mateu-Andrés, I., Dorcey, E., Thiele, D. J., and Peñarrubia, L. (2004). The Arabidopsis copper transporter COPT1 functions in root elongation and pollen development. *J. Biol. Chem.* 279, 15348–15355. doi: 10.1074/jbc.M313321200

Sancenón, V., Puig, S., Mira, H., Thiele, D. J., and Peñarrubia, L. (2003). Identification of a copper transporter family in *Arabidopsis thaliana*. *Plant Mol. Biol.* 51, 577–587.

Sánchez-Calvo, B., Barroso, J. B., and Corpas, F. J. (2013). Hypothesis: nitro-fatty acids play a role in plant metabolism. *Plant Sci.* 199, 1–6. doi: 10.1016/j.plantsci.2012.10.006

Sánchez-Pardo, B., Fernández-Pascual, M., and Zornoza, P. (2014). Copper microlocalisation and changes in leaf morphology, chloroplast ultrastructure and antioxidative response in white lupin and soybean grown in copper excess. *J. Plant Res.* 127, 119–129. doi: 10.1007/s10265-013-0583-1

Santisree, P., Adimulam, S. S., Sharma, K., Bhatnagar-Mathur, P., and Sharma, K. K. (2019). "Insights into the nitric oxide mediated stress tolerance in plants," in *Plant Signaling Molecules*, eds M. I. R. Khan, P. S. Reddy, A. Ferrante, and N. A. Khan (Amsterdam: Elsevier), 385–406. doi: 10.1016/B978-0-12-816451-8.00024-1

Schubert, M., Petersson, U. A., Haas, B. J., Funk, C., Schröder, W. P., and Kieselbach, T. (2002). Proteome map of the chloroplast lumen of *Arabidopsis thaliana*. *J. Biol. Chem.* 277, 8354–8365. doi: 10.1074/jbc.M108575200

Sehar, Z., Masood, A., and Khan, N. A. (2019). Nitric oxide reverses glucose-mediated photosynthetic repression in wheat (*Triticum aestivum* L.) under salt stress. *Environ. Exp. Bot.* 151, 277–289. doi: 10.1016/j.envexpbot.2019.01.010

Shams, M., Ekinci, M., Turan, M., Dursun, A., Kul, R., and Yildirim, E. (2019). Growth, nutrient uptake and enzyme activity response of Lettuce (*Lactuca sativa* L.) to excess copper. *Environ. Sustain.* 2, 67–73. doi: 10.1007/s42398-019-00051-7

Sharma, P., Sirhindi, G., Singh, A. K., Kaur, H., and Mushtaq, R. (2017). Consequences of copper treatment on pigeon pea photosynthesis, osmolytes and antioxidants defense. *Physiol. Mol. Biol. Plant* 23, 809–816. doi: 10.1007/s12298-017-0461-8

Shi, K., Li, X., Zhang, H., Zhang, G., Liu, Y., Zhou, Y., et al. (2015). Guard cell hydrogen peroxide and nitric oxide mediate elevated CO_2-induced stomatal movement in tomato. *New Phytol.* 208, 342–353. doi: 10.1111/nph.13621

Shiyab, S. (2018). Phytoaccumulation of copper from irrigation water and its effect on the internal structure of lettuce. *Agriculture* 8:29. doi: 10.3390/agriculture8020029

Takahashi, M., and Morikawa, H. (2014). Nitrogen dioxide is a positive regulator of plant growth. *Plant Signal. Behav.* 9:e28033. doi: 10.4161/psb.28033

Takahashi, R., Bashir, K., Ishimaru, Y., Nishizawa, N. K., and Nakanishi, H. (2012). The role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. *Plant Signal. Behav.* 7, 1605–1607. doi: 10.4161/psb.22454

Tewari, R. K., Hahn, E. J., and Paek, K. Y. (2008). Modulation of copper toxicity-induced oxidative damage by nitric oxide supply in the adventitious roots of *Panax ginseng*. *Plant Cell Rep.* 27, 171–181. doi: 10.1007/s00299-007-0423-7

Thounaojam, T. C., Panda, P., Mazumdar, P., Kumar, D., Sharma, G. D., Sahoo, L., et al. (2012). Excess copper induced oxidative stress and response of antioxidants in rice. *Plant Physiol. Biochem.* 53, 33–39. doi: 10.1016/j.plaphy.2012.01.006

Tian, X., and Lei, Y. (2006). Nitric oxide treatment alleviates drought stress in wheat seedlings. *Biol. Plant* 50, 775–778. doi: 10.1007/s10535-006-0129-7

Tun, N. N., Holk, A., and Scherer, G. F. (2001). Rapid increase of NO release in plant cell cultures induced by cytokinin. *FEBS Lett.* 509, 174–176.

Verma, J. P., Singh, V., and Yadav, J. (2011). Effect of copper sulphate on seed germination, plant growth and peroxidase activity of mung bean (*Vigna radiata*). *Int. J. Bot.* 7, 200–204. doi: 10.3923/ijb.2011.200.204

Wang, L., Yang, L., Yang, F., Li, X., Song, Y., Wang, X., et al. (2010). Involvements of H_2O_2 and metallothionein in NO-mediated tomato tolerance to copper toxicity. *J. Plant. Physiol.* 167, 1298–1306. doi: 10.1016/j.jplph.2010.04.007

Wang, Y., Hu, M., Cui, X., Lou, Y., and Zhuge, Y. (2018). Exogenous NO mediated the detoxification pathway of tomato seedlings under different stress of Cu and Cd. *J. Appl. Ecol.* 29, 4199–4207. doi: 10.13287/j.1001-9332.201812.031

Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F., et al. (2003). Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. *J. Biol. Chem.* 278, 31286–31289. doi: 10.1074/jbc.M302876200

Welch, R. M., Norvell, W. A., Schaefer, S. C., Shaff, J. E., and Kochian, L. V. (1993). Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: Does the root-cell plasmalemma Fe (III)-chelate reductase perform a general role in regulating cation uptake? *Planta* 190, 555–561. doi: 10.1007/BF00224795

Williams, L. E., and Mills, R. F. (2005). P1B-ATPases–an ancient family of transition metal pumps with diverse functions in plants. *Trends Plant Sci.* 10, 491–502. doi: 10.1016/j.tplants.2005.08.008

Wise, R. R., and Hoober, J. K. (eds) (2007). *The Structure and Function of Plastids*. Berlin: Springer. doi: 10.1007/978-1-4020-4061-0

Wu, X., Zhu, X., Chen, J., Yang, S., Ding, H., and Zha, D. (2013). Nitric oxide alleviates adverse salt-induced effects by improving the photosynthetic performance and increasing the anti-oxidant capacity of eggplant (*Solanum melongena* L.). *J. Hortic. Sci. Biotechnol.* 88, 352–360. doi: 10.1080/14620316.2013.11512976

Xiong, J., An, L., Lu, H., and Zhu, C. (2009). Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. *Planta* 230, 755–765. doi: 10.1007/s00425-009-0984-5

Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y., and Shikanai, T. (2009). *SQUAMOSA* promoter binding protein–like7 is a central regulator for copper homeostasis in *Arabidopsis*. *Plant Cell* 21, 347–361. doi: 10.1105/tpc.108.060137

Yan, J., Wang, P., Wang, P., Yang, M., Lian, X., Tang, Z., et al. (2016). A loss-of-function allele of OsHMA3 associated with high cadmium accumulation in shoots and grain of Japonica rice cultivars. *Plant Cell Environ.* 39, 1941–1954. doi: 10.1111/pce.12747

Ye, Y., Li, Z., and Xing, D. (2013). Nitric oxide promotes MPK6-mediated caspase-3-like activation in cadmium-induced *Arabidopsis thaliana* programmed cell death. *Plant Cell Environ.* 36, 1–15. doi: 10.1111/j.1365-3040.2012.02543.x

Yruela, I. (2005). Copper in plants. *Braz. J. Plant Physiol.* 17, 145–156. doi: 10.1590/S1677-04202005000100012

Yu, C. C., Hung, K. T., and Kao, C. H. (2005). Nitric oxide reduces Cu toxicity and Cu-induced NH^{4+} accumulation in rice leaves. *J. Plant Physiol.* 162, 1319–1330. doi: 10.1016/j.jplph.2005.02.003

Yuan, H. M., Xu, H. H., Liu, W. C., and Lu, Y. T. (2013). Copper regulates primary root elongation through PIN1–mediated auxin redistribution. *Plant Cell Physiol.* 54, 766–778. doi: 10.1093/pcp/pct030

Zhang, L. P., Mehta, S. K., Liu, Z. P., and Yang, Z. M. (2008). Copper-induced proline synthesis is associated with nitric oxide generation in *Chlamydomonas*

reinhardtii. *Plant Cell Physiol.* 49, 411–419. doi: 10.1093/pcp/pcn017

Zhang, P., Li, S., Guo, Z., and Lu, S. (2019). Nitric oxide regulates glutathione synthesis and cold tolerance in forage legumes. *Environ. Exp. Bot.* 167:103851. doi: 10.1016/j.envexpbot.2019.103851

Zhang, Y., Han, X., Chen, X., Jin, H., and Cui, X. (2009). Exogenous nitric oxide on antioxidative system and ATPase activities from tomato seedlings under copper stress. *Sci. Hortic.* 123, 217–223.

Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F., and Terzi, M. (2002). Nitric oxide affects plant mitochondrial functionality *in vivo*. *FEBS Lett.* 515, 75–78. doi: 10.1016/s0014-5793(02)02438-9

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.

frontiers in Plant Science

REVIEW
published: 03 June 2020
doi: 10.3389/fpls.2020.00521

The Role of Nitric Oxide in Nitrogen Fixation by Legumes

Santiago Signorelli[1,2,3], Martha Sainz[1], Sofía Tabares-da Rosa[1] and Jorge Monza[1]*

[1] Laboratorio de Bioquímica, Departamento de Biología Vegetal, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay, [2] The School of Molecular Sciences, Faculty of Science, The University of Western Australia, Crawley, WA, Australia, [3] Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley, WA, Australia

The legume-rhizobia symbiosis is an important process in agriculture because it allows the biological nitrogen fixation (BNF) which contributes to increasing the levels of nitrogen in the soil. Nitric oxide (·NO) is a small free radical molecule having diverse signaling roles in plants. Here we present and discuss evidence showing the role of ·NO during different stages of the legume-rhizobia interaction such as recognition, infection, nodule development, and nodule senescence. Although the mechanisms by which ·NO modulates this interaction are not fully understood, we discuss potential mechanisms including its interaction with cytokinin, auxin, and abscisic acid signaling pathways. In matures nodules, a more active metabolism of ·NO has been reported and both the plant and rhizobia participate in ·NO production and scavenging. Although ·NO has been shown to induce the expression of genes coding for NITROGENASE, controlling the levels of ·NO in mature nodules seems to be crucial as ·NO was shown to be a potent inhibitor of NITROGENASE activity, to induce nodule senescence, and reduce nitrogen assimilation. In this sense, LEGHEMOGLOBINS (Lbs) were shown to play an important role in the scavenging of ·NO and reactive nitrogen species (RNS), potentially more relevant in senescent nodules. Even though ·NO can reduce NITROGENASE activity, most reports have linked ·NO to positive effects on BNF. This can relate mainly to the regulation of the spatiotemporal distribution of ·NO which favors some effects over others. Another plausible explanation for this observation is that the negative effect of ·NO requires its direct interaction with NITROGENASE, whereas the positive effect of ·NO is related to its signaling function, which results in an amplifier effect. In the near future, it would be interesting to explore the role of environmental stress-induced ·NO in BNF.

Keywords: ·NO, reactive oxygen species, leghemoglobin, legumes, nitrogen fixation, reactive nitrogen species

OPEN ACCESS

Edited by:
Juan B. Barroso, University of Jaén, Spain

Reviewed by:
Alexandre Boscari, INRA Centre Provence-Alpes-Côte d'Azur, France
Oswaldo Valdes-Lopez, National Autonomous University of Mexico, Mexico

****Correspondence:***
Santiago Signorelli
ssignorelli@fagro.edu.uy

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *01 December 2019*
Accepted: *06 April 2020*
Published: *03 June 2020*

Citation:
Signorelli S, Sainz M, Tabares-da Rosa S and Monza J (2020) The Role of Nitric Oxide in Nitrogen Fixation by Legumes. Front. Plant Sci. 11:521. doi: 10.3389/fpls.2020.00521

INTRODUCTION

The biological nitrogen fixation (BNF) is the process of reducing atmospheric nitrogen (N_2) to ammonium (NH_4^+) catalyzed by the NITROGENASE enzyme. This process is carried out by a small group of bacteria, in either free-living condition, associated with different plants such as epiphytes or endophytes, or establishing endocellular symbiosis with legumes (Masson-Boivin et al., 2009). The rhizobium-legume symbiosis involves the exchange of carbon source produced

by the plant and ammonium fixed by the bacteria in specialized organs denominated nodules. This symbiosis helps legumes to naturally colonize nitrogen-poor soils. Thus, this symbiosis positively impacts on agriculture, not only for the savings of nitrogen (N) fertilizers but also due to the reduction of its negative impact on the environment, which is key to achieve sustainable agriculture. Particularly because of the importance of sustainable agriculture, the interest in the BNF has been revitalized in recent years (Sulieman and Tran, 2015), with an emphasis in the rhizobium-legume symbiosis involving the cultivation of food and fodder (Lindström et al., 2010; Laranjo et al., 2014). Moreover, researchers have done considerable efforts to introduce the ability to perform BNF into non-leguminous plants, either by introducing the NITROGENASE enzyme into plants through genetic engineering (Oldroyd and Dixon, 2014) or by using *Gluconacetobacter diazotrophicus*, a non-nodulating endophytic nitrogen-fixing bacterium (Cocking et al., 2006).

Nitric oxide (·NO) is a small free radical molecule, which is ubiquitous in plants and its production is often enhanced under stress conditions (Corpas et al., 2008, 2011; Signorelli et al., 2013, 2019). ·NO acts as a signaling molecule interacting with hormone signaling in plants (Klessig et al., 2000; Wang et al., 2015a) and regulating different developmental processes such as germination, root elongation, floral transitions, branching, and ripening (Lozano-Juste and Leon, 2011; Sanz et al., 2014; Chaki et al., 2015). The BNF is also modulated by ·NO at different stages of this process. In this review, we present and discuss the metabolism of ·NO in nodules, the different sources of ·NO, and its effect on nodule establishment, BNF and nodule senescence. With this, we attempt to provide clear views on what is currently know and highlight the outstanding questions that need to be investigated in this exciting research area.

·NO IN LEGUME-BACTERIA INTERACTION

The establishment of the legume-rhizobium symbiosis requires the recognition between the rhizobia and legume and the formation of nodules, the plant organ hosting the rhizobia and where the BNF takes place. The nodule provides a low oxygen (microoxic) environment which is necessary to prevent the inhibition of the NITROGENASE activity by oxygen (O_2). To cope with the lower O_2 availability, the cytochrome-pathway of respiration of mitochondria from nodules has a higher apparent affinity for O_2 than the equivalent of mitochondria from roots (Millar et al., 1995). This allows the nodule cells to produce ATP by oxidative phosphorylation which is used for the rhizobia as the energy source, together with other carbon sources, to fixate nitrogen. The fixated nitrogen is assimilated mostly in the cytosol of the nodule cells and taken up by the plant.

Two genetic pathways were shown to control the number of nodules produced in the root nodule symbiosis, one of them controlling the rhizobial infection and the other one controlling the nodule organogenesis (Penmetsa et al., 2003). Depending on the legume, the nodule can be indeterminate or determinate, which means respectively that they sustain or not meristematic activity (Hirsch, 1992). The nodulation process starts with the release of chemical signals by the root hairs, which attract rhizobia and trigger them to produce bacterial nodulation factors (Nod-factors) (**Figure 1** i). These Nod factors are perceived by the legume to promote root cell division and other downstream responses (Geurts and Bisseling, 2002). The rhizobia grow, induce a curl in the root hair (**Figure 1** ii) and a tubular and intracellular structure containing the bacteria, known as infection thread, is formed (**Figure 1** iii). As the rhizobia reproduce, the infection thread grows reaching first the base of root hair cell and later the nodule primordium (Geurts and Bisseling, 2002). The formation of this infection threat it also induced by the presence of the plant hormones cytokinin (CK) and auxin (AUX) (Roy et al., 2020). Afterward, the infection thread starts to release bacterial cells into the parenchyma cells of the developing nodule, where the bacterial cells differentiate into bacteroids, a transformation that requires morphological and physiological changes (**Figure 1** iv). For a detailed review of the nodule formation process, we recommend revising Gage (2004) or Roy et al. (2020).

In the different stages of the nodule formation, a crosstalk between the bacteria and the root cells occurs. Reactive oxygen species (ROS) are present among the molecules involved in this communication. ROS are produced at a high level in the nodule, mainly due to the high demand of respiration to support NITROGENASE activity and the autoxidation of oxygenated leghemoglobin (oxy-Lb) (Puppo et al., 1981). Therefore, the role of ROS on modulating the legume-rhizobia interaction, as well as the relevance of the antioxidant system to control them have been widely studied (Becana et al., 2010). Likewise, the presence of ·NO in the nodule is well documented (Baudouin et al., 2006; Nagata et al., 2008; del Giudice et al., 2011; Cam et al., 2012; Fukudome et al., 2016, 2018; Calvo-Begueria et al., 2018). Due to its reactive nature, ·NO and other reactive nitrogen species (RNS) can also interfere with this plant-rhizobia communication.

In the specific interactions *Lotus japonicus-Mesorhizobium loti* and *Medicago sativa-Ensifer meliloti*, ·NO was observed to be induced in the roots of plants 4 h post-inoculation (hpi), suggesting that ·NO participates at early stages of the plant-rhizobia interaction (Nagata et al., 2008). Interestingly, ·NO was only perceived at 4 hpi but not at 10 and 24 hpi, and this effect was not observed when the roots were inoculated with unspecific rhizobium (Nagata et al., 2008). In contrast, *L. japonicus* showed a higher and sustained accumulation of ·NO in roots when infected with different plant pathogens (Nagata et al., 2008). These observations suggest that the peak response observed at 4 hpi is a signal-recognition response, rather than a stress response. Different kinetics for ·NO accumulation were observed in the *Medicago truncatula–E. meliloti* symbiosis, but the results were consistent in the fact that ·NO is induced and necessary for the correct establishment of the symbiosis (del Giudice et al., 2011). In this symbiosis, the induction of ·NO was detected 2 days post-infection (dpi), during the curling of the root hair, and 6 dpi, when the infection thread is formed (del Giudice et al., 2011). Using reporter bacteria responsive to ·NO, the authors showed that the bacteria respond

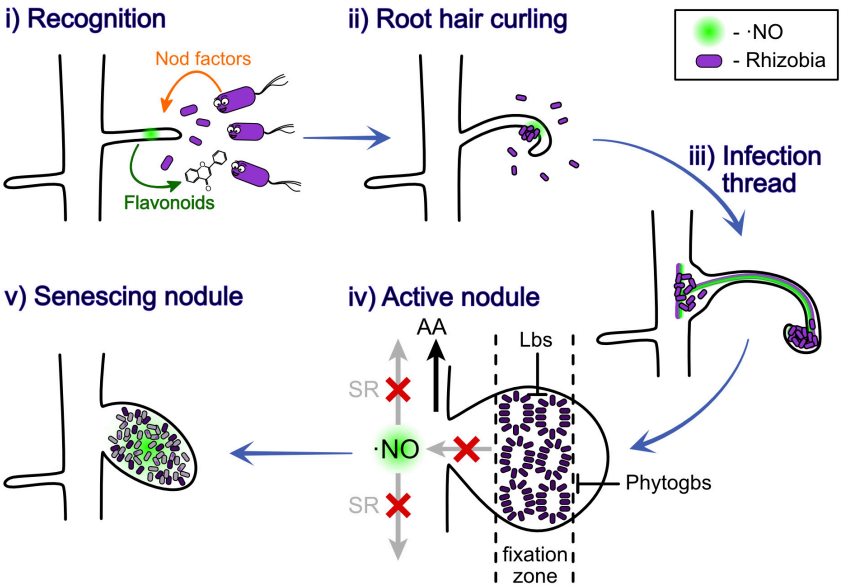

FIGURE 1 | Nitric oxide participates at different stages of the nodulation process. ·NO was observed to be involved at all the different stages of nodulation, plant-rhizobium recognition (i), root hair curling (ii), infection thread (iii), nitrogen fixation in active nodules (iv), and senescing nodules (v). The light purple indicates the bacteria; the dark purple indicates the bacteroids; the gray indicates dead bacteroids; and the diffuse light green indicates ·NO. AA, amino acids; Phytogbs, PHYTOGLOBINS; Lbs, LEGHEMOGLOBINS; SR, systemic response.

to the endogenously produced ·NO in the infection pocket, and when ·NO is specifically scavenged, the development of the nodule is delayed (del Giudice et al., 2011). Together, this evidence demonstrates that ·NO promotes the legume-bacteria interaction and nodule development, potentially acting as a signaling molecule (discussed in section "Signaling Role of ·NO in Nitrogen Fixation").

At later stages of this symbiosis ·NO was also shown to be present in *M. truncatula* nodules, both in developing (10 dpi) and mature nodules (30 dpi), in particular, in the fixation zone (Baudouin et al., 2006). Furthermore, ·NO was observed to locally induce nodule senescence in *M. truncatula* (Cam et al., 2012) and *L. japonicus* (Fukudome et al., 2018). These findings suggest the involvement of ·NO also during nitrogen fixation and nodule senescence (**Figure 1**).

Taking all together, the current evidence clearly shows the involvement of ·NO during the legume-rhizobia interaction. In many cases, a positive effect of ·NO on this interaction is observed, although the mechanism is not fully understood (discussed in section "Perspectives").

METABOLISM OF ·NO IN NODULES

Plants can produce ·NO by different ways, some of them non-enzymatic, as the non-enzymatic reduction of nitrite (Bethke et al., 2004), and others are dependent on enzymes such as the NITRATE REDUCTASE (NR), PLASMA MEMBRANE-BOUND NITRITE: ·NO REDUCTASE, mitochondrial-electron transport chain-dependent (mETC-dependent) nitrite-reducing activity, and the NOS-like activity (Stöhr et al., 2001; Planchet et al., 2005; Corpas et al., 2009; Astier et al., 2018). POLYAMINE OXIDASES and HYDROXYLAMINE OXIDASE have been also suggested to contribute to ·NO production (Tun et al., 2006; Rümer et al., 2009). Despite the several mechanisms involved in ·NO production, the NR and mETC-dependent reduction of nitrite are the better understood mechanisms to contribute to ·NO production, being the latter only relevant under microoxic and anoxic conditions (León and Costa-Broseta, 2020). Interestingly, under the nodulation process, the three genes encoding for NR of *M. truncatula* were shown to be induced (Damiani et al., 2016). In fact, the authors suggested a specific role of these enzymes as ·NO source in nodulation. Nonetheless, recent experiments coupling EPR (electron paramagnetic resonance) and DAF-2 (4,5-diaminofluorescein) to detect ·NO in bean and soybean nodules suggested that ·NO is also produced by nitrate- and arginine-independent pathways (Calvo-Begueria et al., 2018).

Under microoxic conditions, it is well known that nitrite can be used as final electron acceptor of the mETC to produce ·NO (Horchani et al., 2010; Gupta and Igamberdiev, 2011). Therefore, once the nodule is established, the microoxic condition is generated within the nodule promoting this extra source of ·NO. In this situation, ·NO is involved in the cycle named Phytoglobin-·NO (Phytogb-·NO), in which the ·NO produced from nitrite by the mETC diffuses into the cytosol where it is oxidized into nitrate by PHYTOGLOBINS (Phytogbs), and the resulting nitrate is reduced back to nitrite by NR and transported to the mitochondria where the cycle is repeated (**Figure 2**; Stoimenova et al., 2007; Igamberdiev et al., 2010; Gupta and Igamberdiev, 2011). This Phytogb-·NO cycle was suggested to function as

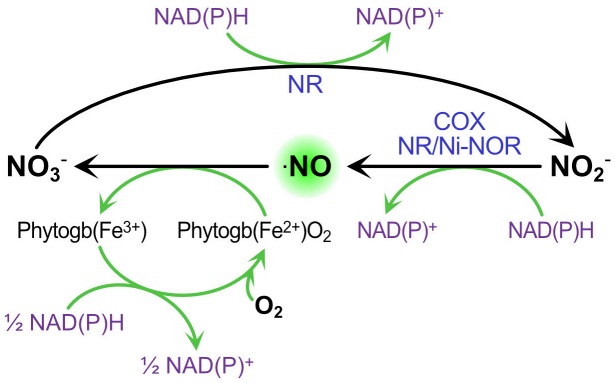

FIGURE 2 | The contribution of Phytogb/·NO cycle to ·NO production and NAD(P)H re-oxidation during hypoxia. ·NO is oxidized to nitrate by OXYPHYTOGLOBIN [Phytogb(Fe_2^+)O_2], which turns to METPHYTOGLOBIN [Phytogb(Fe^{3+})]. Nitrate is then reduced to nitrite through NITRATE REDUCTASE (NR), and nitrite is reduced to ·NO though NR, the PLASMA MEMBRANE NITRITE-·NO REDUCATSE (NI-NOR) or the CYTOCHROME OXIDASE (COX). NAD(P)H oxidation occurs in the reactions forming ·NO and its subsequent oxidation back to nitrate.

an alternative mechanism to the classic fermentation pathways (ethanol and lactate formation) for the re-oxidation of NAD(P)H to $NAD(P)^+$ during hypoxia (Igamberdiev and Hill, 2004). In microoxic cells, the energy production for short-term viability is achieved mainly through glycolysis. The Phytogb-·NO cycle would allow NADH re-oxidation during the reduction of NO_3^- to $\cdot NO_2^-$ and in the regeneration of reduced (Fe^{2+}) Phytogb (**Figure 2**), being the resulting NAD^+ available for the glycolytic process (Igamberdiev and Hill, 2004).

Besides the electron transport chain of the nodule cells, the electron transport chain of some rhizobia also contributes to ·NO production (Horchani et al., 2010) through the denitrification pathway, being the highest contribution for ·NO biosynthesis from the bacteroid side (Hichri et al., 2015). In the *Bradyrhizobium* genera, different strains have been shown to be able to denitrify under microoxic conditions, both in free-living conditions (Monza et al., 2006) and in symbiosis (Bedmar et al., 2005). Under flooding conditions however, in soybean nodules, the periplasmic NR and NiR of *Bradyrhizobium diazoefficiens* (also known as *Bradyrhizobium japonicum*) were suggested to be the main sources of ·NO (Sánchez et al., 2010). Although the contribution of ·NO by the bacteria is considered to be lower than that by the plants (Baudouin et al., 2006; Horchani et al., 2010), both partners are involved in the production of this molecule in mature nodules. Therefore, it is not surprising that some genes involved in both ·NO production and degradation have proven to be more expressed in mature nodule when compared to developing nodule (Berger et al., 2019). This indeed suggests that a more active metabolism of ·NO takes place in established nodules.

Finally, it is important to remark that ·NO is highly permeable to biological membranes (permeability coefficient in the membrane of 93 cm · s^{-1}, Signorelli et al., 2011), meaning that ·NO can diffuse in and out the bacteroids. Thus, the lack of a unique ·NO source together with its capacity to diffuse between the plant and the rhizobia set a complex scenario to elucidate the main source of ·NO in this interaction.

S-NITROSOTHIOLS IN NODULE

Nitric oxide can modulate the activity of different enzymes mainly through *S*-nitrosylation (also known as *S*-nitrosation). This reversible posttranslational modification of proteins is the covalent binding of a ·NO group to the thiol group of a cysteine residue leading to the formation of an *S*-nitrosothiol (SNO). Different mechanisms have been shown to mediate this modification, varying on the state of the cysteine (as radical or anion) and the nature of the ·NO group (as radical, anion, cation or transiently associated to other molecules) (Kovacs and Lindermayr, 2013). This modification produces structural changes in the protein, and when the modified residues are close enough to the active site, its activity is affected. The major effects of ·NO on biological processes are due to its capacity to induce post-translational modifications of key proteins involved in signaling cascade pathways, resulting in the up or down-regulation of downstream components such as transcription factors, which in turn affect the expression of a plethora of genes. For instance, ·NO is known to inactivate several proteins involved in the ABA signaling pathway that controls the activity of the transcription factor ABI5, resulting in the suppression of the ABA-mediated responses (Signorelli and Considine, 2018). Likewise, ·NO can target the group VII of ETHYLENE RESPONSE FACTORS (ERF) to proteasomal degradation (Gibbs et al., 2014), affecting the regulation of different process under regulation of these transcription factors, such as abiotic/biotic stress responses and developmental processes (Gibbs et al., 2015; Considine et al., 2017).

The inhibition of NITROGENASE by ·NO has been known since long time (Trinchant and Rigaud, 1982; Kato et al., 2010). Although this inhibition can be through the formation of metal-nitrosyl complex (Michalski and Nicholas, 1987), in the symbiosis *M. truncatula-E. meliloti* two NITROGENASE proteins (encoded by *nifK* and *nifH*) were reported to be *S*-nitrosylated (Puppo et al., 2013), which could present an alternative mechanism to post-translationally regulate the NITROGENASE activity in the bacteroids. In the same symbiosis, many proteins related to TCA cycle and carbohydrate metabolism were reported to be *S*-nitrosylated in both the legume and the partner rhizobium (Puppo et al., 2013). Moreover, in this legume three proteins involved in amino acid metabolism, the ASPARAGINE SYNTHETASE, GLUTAMINE SYNTHETASE, and S-ADENOSYLMETHIONINE SYNTHETASE were also reported as *S*-nitrosylated (Melo et al., 2011; Puppo et al., 2013). Although there have been some proteins identified as *S*-nitrosylated, in many cases the consequence of such modification on the enzymatic activity is still unknown. Conversely, the GLUTATHIONE PEROXIDASE 1 (GPX1) from nodules of *M. truncatula* is known to be reversibly inactivated by *S*-nitrosylation (Castella et al., 2017). As this enzyme participates in the transmission of redox signals, it was suggested that ·NO can exert part of its signaling function through the modulation of this protein (Castella et al., 2017).

Nevertheless, it is important to remark that ·NO levels do not always correlate with SNOs levels. This was clearly observed in non-nodulated roots of the model legume *L. japonicus* (Signorelli et al., 2019) and in nodules of *Arachis hypogaea* (Maiti et al., 2012). Thus, low levels of ·NO do not necessarily implicate that *S*-nitrosylation will not take place. Moreover, in *A. hypogaea* nodules it was observed that the levels of cytoplasmic SNOs were lower in the nodule than in the symbiotic bacteria; thus the authors suggested that the bacteria contribute to the protection of *S*-nitrosylation in the nodule and that this interaction might implicate the transfer of redox compounds between the bacteroids and the nodule cells (Maiti et al., 2012). Also, the number of SNOs in the nodule was observed to increase with the age of the nodule (Maiti et al., 2012).

Interestingly, a phytogb (AHb1) from *Arabidopsis thaliana* was the first protein of plants reported to be *S*-nitrosylated and the authors suggested this could be a mechanism to eliminate ·NO during hypoxic stress (Perazzolli et al., 2004). The authors also tested if the overexpression of AHb1 reduced the ·NO-mediated hypersensitive cell death in response to pathogens, but it was not the case (Perazzolli et al., 2004). Conversely, the detoxification of SNOs was proven to increase disease resistance upon infection with *Pseudomonas syringae* (Feechan et al., 2005). Therefore, increased SNO levels are believed to enhance the susceptibility to pathogens and change the redox status of the cells resulting in the activation of antioxidant responses (Begara-Morales et al., 2019 and references therein). In this sense, it would be interesting to understand if changes in SNOs, prior to rhizobium infection, promote or reduce the success of the infection. Understanding this could lead to the use of better management practices to promote rhizobia inoculation.

Together, the evidence demonstrates that SNOs occurs *in vivo* in legume nodules and, in some cases, the activity of the proteins is affected by this posttranslational modification. In the near future, it is expected that an increased number of *S*-nitrosylated proteins are going to be reported. For those proteins that have been already reported as *S*-nitrosylated, it would be important to understand the consequences on their activity.

ROS AND RNS HOMEOSTASIS IN THE NODULE

ROS and RNS Are Induced in the Establishment of the Legume-Rhizobia Interaction

Reactive oxygen species are formed due to partial reductions of O_2 and can react with cellular components leading to irreparable metabolic dysfunction or cell death. As ROS are ubiquitous in aerobic organisms, they tend to be controlled under normal conditions by different antioxidant systems. In such controlled conditions, ROS are essential for certain cellular functions. ROS include free radicals, such as superoxide radical ($O_2{\cdot}^-$) and hydroxyl radical (·OH), and non-radical compounds like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). The term RNS is used to designate ·NO and related molecules such as peroxynitrite ($ONOO^-$), nitrogen dioxide ($\cdot NO_2$), dinitrogen trioxide (N_2O_3) and SNOs. Like ROS, RNS can be moderately (e.g., ·NO, SNOs) or highly (e.g., $ONOO^-$, $\cdot NO_2$) reactive. RNS, directly or indirectly, participate in the post-translational modifications of proteins, which are involved in the cellular signaling process in both physiological and pathological conditions.

During the plant-rhizobium interaction, ROS play an important role and its production by the plants is known to be triggered, at least in part, by compatible Nod factors (Ramu et al., 2002). The silencing of the apoplastic-$O_2{\cdot}^-$-producing enzyme, RESPIRATORY BURST OXIDASE HOMOLOG (RBOH), negatively affected the symbiotic nitrogen fixation in different legumes (Marino et al., 2011; Arthikala et al., 2017). The plants also respond producing a peroxidase (RIP1) as a mechanism to prevent the excessive generation of H_2O_2 (Ramu et al., 2002). Therefore, both the ROS generation and the induction of antioxidant system to control ROS levels are essential for the correct establishment of the symbiosis (Ramu et al., 2002; Becana et al., 2010). Legumes count with the common antioxidant enzymes such as SUPEROXIDE DISMUTASE, CATALASE, and diverse PEROXIDASES, and non-enzymatic antioxidants such as ascorbate and glutathione as the first barrier to protect against ROS. Here the general antioxidant machinery will not be discussed. Instead, we describe those systems specific from the legume-rhizobia symbiosis.

Phytoglobins

Nodule function requires the protein LEGHEMOGLOBIN (Lb), which transport and deliver O_2 to the symbiosomes at a low but steady concentration that allows efficient bacteroid respiration while preventing NITROGENASE inactivation (Appleby and Bergersen, 1980). Besides Lbs, plants possess many other HEMOGLOBINS (Hbs), currently known as Phytogbs, which function is less clear in plants but have been linked to the control of RNS homeostasis (Dordas et al., 2003; Perazzolli et al., 2004; Nagata et al., 2008). Here, we introduce the different plant Hbs and their potential role in controlling ROS and RNS.

In vascular plants, Phytogbs can be divided into six types based on phylogenetic analyses and biochemical properties (Hill et al., 2016). Phytogb0 are localized in any organ of gymnosperms but also in algae and bryophytes, and have moderate to high affinity for O_2 (Garrocho-Villegas and Arredondo-Peter, 2008). Phytogbs1 have extremely high O_2 affinities, making them unsuitable for O_2 transport and delivery (Smagghe et al., 2009), and their main function seems to be related to the modulation of ·NO (Gupta et al., 2011b) and preserving cellular energy during hypoxia (Hill, 2012). In *L. japonicus*, the expression of *Ljphytogb1-1* gene increases in response to symbiosis with *M. loti*, but not when it interacts with pathogenic microorganisms so it has been proposed that Ljphytogb1-1 eliminates the ·NO produced in the initial response to the infection, allowing the establishment of symbiosis (Nagata et al., 2008). Very recently, it has been shown in *M. truncatula* that Mtphytogb1-1 regulates the concentration of ·NO both during early symbiosis steps and in mature nodules (Berger et al., 2020). Phytogbs2 display O_2 affinities that resemble those of Lbs (Dordas, 2009), and

their expression is known to be induced by CK treatments and cold conditions (Hunt et al., 2001). Vigeolas et al. (2011) proposed that this type of Phytogbs could facilitate the supply of O_2 to developing tissues. Phytogbs1 and Phytogbs2 are localized in any organ of angiosperms. Phytogbs3 represent a group with very low similarity to Phytogbs1 and Phytogbs2 and have moderate O_2 affinities (Watts et al., 2001). A biochemical property that defines Phytogbs3 is that they have a 2-on-2 α-helical sandwich secondary structure instead of the canonical 3-on-3 structure of other Phytogbs (Wittenberg et al., 2002). Although the functions of Phytogbs 3 are unknown, some of them are induced in nodules and mycorrhizal roots (Zhu et al., 2005) and might also interact with ·NO (Sanz-Luque et al., 2015). It has been suggested in *M. truncatula* that the function of two Phytogbs3 could be related to symbiosis by suppressing the initial defensive response of the plant due to the ability of these proteins to bind ·NO (Vieweg et al., 2005). In addition, some of their bacterial counterparts have been implicated in tolerance to nitrosative stress (Angelo et al., 2008). Both Lbs and SymPhytogb are proteins exclusively related to the symbiotic process facilitating a steady low O_2 supply to the bacterial microsymbionts (Smagghe et al., 2009). They present moderate to high O_2 affinity and are specifically localized in N_2-fixing nodules of legumes and actinorhizal or any other non-legume plant, respectively.

The Involvement of Plant Phytogbs in ROS and RNS Metabolism

In nodules, high respiration rates together with a high concentration of Lb and the abundance of catalytic Fe enhance, among other things, nodule capacity to generate ROS (Marino et al., 2009). Using leghemoglobin-RNA interference lines of *L. japonicus*, Günther et al. (2007) demonstrated that loss of Lb results in significantly lower H_2O_2 levels in nodules, which suggested a role of Lb in *in vivo* ROS production. Like other Hbs, oxy-Lb ($Lb^{2+}O_2$) auto-oxidizes spontaneously to form ferric (or meta) Lb (Lb^{3+}) and $O_2{\cdot}^-$, especially under the slightly acid pH of nodules (Puppo et al., 1981). The released $O_2{\cdot}^-$ can, in turn, oxidize other oxy-Lb molecules to Lb^{3+}, which enhances the inactivation of ferrous Lb (Lb^{2+}) and oxy-Lb. Oxy-Lb and Lb^{3+} can also be oxidized by H_2O_2 (Puppo et al., 1982). The reaction between H_2O_2 and oxy-Lb or ferric Lb, in equimolar proportion, forms ferryl Lb (Lb^{IV}), a stable but inactive form of Lb (Aviram et al., 1978; Sievers et al., 1978). When Lb and H_2O_2 are in a 1:2 ratio, radicals can be formed in the Tyr residues of the protein, which react forming 2 types of compounds: a green derivative, originated by the covalent binding of the heme with the apoprotein, and a dimeric Lb, originated by an intramolecular Tyr-Tyr bond (Moreau et al., 1995).

Leghemoglobins are synthesized by the plants when they are colonized by a symbiotic rhizobium to scavenge the excess of O_2 which can inhibit the NITROGENASE activity. Phytogbs exist in nodules but also in other organs like roots and leaves of all plants and their concentration range from 100 nM under normal conditions to 5–20 μM when induced by different kind of stresses or hormones (Gupta et al., 2011a). Instead, Lbs are found at millimolar concentrations in the nodules of legumes and are responsible for the typical red color of nodules (Gupta et al., 2011a).

All types of Phytogbs were shown to be highly expressed in *L. japonicus* nodules (Bustos-Sanmamed et al., 2011). Remarkably, LjPhytogb1-1, one protein of the Type 1 family, was proved to have an extremely high affinity for O_2 (K = 0.05 nM) (Sainz et al., 2013). Because of its high O_2 affinity, this protein remains oxygenated and active even in the presence of CO. This may be important in nodules, where CO can be formed in significant amounts as result of the Lb degradation by heme oxygenases (Baudouin et al., 2004; Sainz et al., 2013). As evidenced in the *L. japonicus* Phytogb1 overexpressing lines (LjPhytogb1-1), these proteins were shown to have a positive effect on the activity of the nodule during the *L. japonicus-M. loti* symbiosis and to delay senescence (Fukudome et al., 2018).

In the case of RNS, there is no specific enzymatic systems to scavenge them. Thus, any non-specific system can be relevant to attenuate the deleterious effect of RNS. This is the case of Phytogbs, which can directly scavenge RNS *in vitro* (Herold and Puppo, 2005) and *in vivo* in nodules of soybean and *L. japonicus* (Navascues et al., 2012; Sainz et al., 2015). Interestingly, the *L. japonicus* genes of these Phytogbs were shown to be induced by ·NO (Shimoda et al., 2005), suggesting a response to scavenge the excess of ·NO. As ·NO was shown to be high in the nitrogen fixation zone of nodules, the scavenging of ·NO by Lbs was suggested to be key to prevent inactivation of NITROGENASE activity by ·NO (Puppo et al., 2013). Because the nitration of Lbs requires low pH 5.5, which is more common in senescent nodules, the protection of Lbs against RNS may be more relevant in senescent nodules. Interestingly, both Lbs and Phytogbs can contribute to the generation of NO_3^- after scavenging ·NO and O_2, with the concomitant oxidation of $Hb^{2+}O_2$ to Hb^{3+}, by the cycle Phytogb-·NO mentioned above (Herold and Puppo, 2005; Berger et al., 2019).

Bacterial Contribution to ROS and RNS Scavenging

In the bacteria, a FLAVOPROTEIN and a SINGLE-DOMAIN HEMOGLOBIN from *B. japonicum* were shown to reduce cell death under the exposure to a ·NO-donor, suggesting that these proteins are relevant to detoxify the excess of ·NO formed as a by-product of NO_3^- assimilation (Cabrera et al., 2015). In *E. meliloti*, a FLAVOHEMOGLOBIN (HMP) was shown to be one of the most important enzymes to detoxify ·NO (Meilhoc et al., 2010; Cam et al., 2012).

Likewise, excessive ROS can be controlled by non-specific scavengers in the rhizobium. For instance, exopolysaccharides are produced in large amounts by *Rhizobium leguminosarum* bv. *trifolii* and play a significant protective role as a barrier against the ROS produced by the clover plants during the symbiotic interaction (Jaszek et al., 2014). Not only the ROS scavenging but also its production is a key factor to determine ROS cellular concentration. As mentioned above, ROS production in the legumes can be also regulated by the rhizobium through nodulation factors (Damiani et al., 2016). Understanding the

mechanisms controlling ROS and RNS concentration in the plant-rhizobium interaction has been key to produce and use genetic tools that allow manipulating ROS and RNS levels and unrevealing their importance in this process.

SIGNALING ROLE OF ·NO IN NITROGEN FIXATION

Given the evidence that ·NO regulates the transcription of genes encoding for different NITROGENASE activity and that it can also modulate NITROGENASE activity at posttranslational level, it is clear that ·NO can play a signaling role in nitrogen fixation. The evidence presented here up to now has been focused on NITROGENASE and close related genes. However, we know that signaling molecules interact with signaling cascade pathways involving phytohormones.

Although there is a strong body of evidence suggesting a role of ·NO in the legume-rhizobia interaction, it is still not clear how ·NO promotes this interaction. A potential mechanism includes the activation of CK signaling by ·NO. In the *M. truncatula–E. meliloti* symbiosis, the gene encoding for the CK receptor CRE1 (CYTOKININ RESPONSE 1) of this legume, which is the sole receptor mediating CK signaling to induce nodulation (Gonzalez-Rizzo et al., 2006), was observed to be induced by ·NO (Ferrarini et al., 2008; del Giudice et al., 2011). Downstream CK signaling, the transcription factors NODULE INCEPTION (NIN) and NODULATION SIGNALING PATHWAY 2 (NSP2) promote nodule development (Schauser et al., 1999; Murakami et al., 2007; Suzaki et al., 2012). Thus, the induction of CRE1 by ·NO illustrates a potential mechanism by which the ·NO produced soon after the infection could promote the establishment of the symbiosis at early stages (**Figure 3**).

Auxin accumulation is also important for nodule development, and both flavonoid and CK inhibit AUX transport to promote AUX accumulation in proliferating cortical cells (Suzaki et al., 2012). Based on evidence showing that ·NO down-regulate the expression of PIN AUX efflux carriers genes in arabidopsis and rice, Berger et al. (2019) suggested that ·NO can also contribute to the control of AUX transport by repressing the expression of PIN proteins to produce AUX accumulation and ultimately cell division in the nodule (**Figure 3**). Yet, it is not clear the mechanism by which ·NO can regulate PIN levels. A potential mechanism can be through the ·NO-induced CK signaling mentioned above (via CRE 1). CK was shown to control the PIN proteins and its degradation to redirect AUX efflux and establish local AUX accumulation (Plet et al., 2011; Marhavý et al., 2014). Through this mechanism, the ·NO-induced CK signaling could contribute to reducing PIN levels (**Figure 3**). In future research, it would be interesting to evaluate at protein level if CRE1 is induced by ·NO.

Abscisic acid (ABA) plays an important role in plant development and has been shown to negatively affect nodule development at different stages in several legumes (Phillips, 1971; Suzuki et al., 2004; Tominaga et al., 2009, 2010; Nagata and Suzuki, 2014). For instance, in the *L. japonicus* ABA-insensitive mutant *enf1* (enhanced nitrogen fixation 1), both

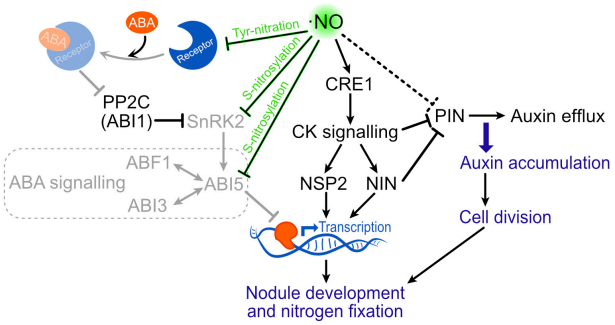

FIGURE 3 | Putative signaling effect of ·NO on nodule development. ·NO has been shown to induce the transcription of CRE1. Through this way, ·NO could promote CK signaling which is known to participate in nodule development. Moreover, ·NO is suggested to repress PIN expression resulting in auxin accumulation and cell division. Finally, the binding of ABA to the PYR/PYL/RCAR receptor can be avoided by tyrosine nitration, not allowing the inactivation of PP2C, which inhibits SnRK2 and the downstream ABA signaling through ABI5. ·NO is also known to directly inactivate both SnRK2 and ABI5 by *S*-nitrosylation, which also results in the suppression of ABA signaling. By these mechanisms, ·NO could attenuate ABA-mediated suppression of nodule development (shown in lower opacity). ABF1, ABA RESPONSIVE ELEMENT-BINDING FACTOR 1; ABI, ABA INSENSITIVE; CK, cytokinin; CRE1, CYTOKININ RESPONSE 1; NIN, NODULE INCEPTION; NSP2, NODULATION SIGNALING PATHWAY 2; PP2C, PROTEIN PHOSPHATASE OF THE TYPE IIC CLASS; SnRK2, SNF1-RELATED PROTEIN KINASE 2.

the nitrogen fixation and the number of nodules formed were almost double (Tominaga et al., 2009), suggesting that ABA inhibits nodule formation and nitrogen fixation. Accordingly, when *L. japonicus* WT plants were treated with an inhibitor of ABA synthesis, the number of nodule and nitrogen fixation activity was increased (Tominaga et al., 2010). This negative effect of ABA was related to its capacity to inhibit CK signaling, isoflavonoid synthesis and the calcium spiking produced after Nod-factor perception (Miwa et al., 2006; Ding et al., 2008; Tominaga et al., 2010; Nagata and Suzuki, 2014). *M. truncatula* lines over expressing the *A. thaliana abi1-1* allele, which codifies for the PROTEIN PHOSPHATASE OF THE TYPE IIC CLASS (PP2C) that is able to inhibit ABA signaling (**Figure 3**), showed hypernodulation phenotype (Ding et al., 2008). In this line, ABA was shown to suppress Nod-factor signal transduction and CK induction (Ding et al., 2008). The inhibition of ABA signaling by PP2C is through its capacity to dephosphorylate and inactivate the serine/threonine kinases SnRK2. In a similar way, ·NO was shown to be able to inactivate some family members of the SnRK2 kinases by *S*-nitrosylation (Wang et al., 2015a,b). Moreover, ·NO was shown to negatively regulate ABA signaling up- and down-stream SnrK2 (**Figure 3**; Albertos et al., 2015; Castillo et al., 2015; Wang et al., 2015a). Considering that ·NO production is enhanced during the legume-rhizobia interaction (**Figure 1**), it is expected that in such situation the ABA signaling pathway will be more prone to be affected by ·NO (**Figure 3**).

Besides affecting the response of hormones directly involved in nodule development (i.e., CK and AUX), ·NO also modulates

the response to salicylic acid (SA) (Klessig et al., 2000) and jasmonic acid (JA) (Huang et al., 2004), two hormones known to be involved in the innate immune response of plants (Betsuyaku et al., 2018; Tarkowski et al., 2020). Thus, it has been considered that by modulating the plant innate immune response ·NO could potentially modulate the establishment of the symbiosis (Tartaglia et al., 2019). In *M. truncatula* and *L. japonicus*, the SA-mediated plant defense pathways were shown to inhibit the formation of determinate- and indeterminate-type of nodules (Stacey et al., 2006). Regarding JA however, there is no clear evidence that it can modulate nodule development, in fact, transgenic plants over-expressing and silencing an enzyme of the JA biosynthesis (ALLENE OXIDE CYCLASE) were unable to affect development and function of nodules (Zdyb et al., 2011). It is not surprising that ·NO had a differential effect on SA and JA signaling, as SA and JA usually have a mutually antagonist effect (Betsuyaku et al., 2018). Further research would contribute to understand the importance of the innate immune response of plants in the legume-rhizobia interaction and how relevant ·NO is to this process.

PERSPECTIVES

The Effects of ·NO on Nitrogen Fixation

Different reports have suggested that ·NO can both promote and reduce nitrogen fixation. Although the conclusions in some cases are opposite, the results are not necessarily opposite. Furthermore, the effect of ·NO on nitrogen fixation depends on whether it acts directly on the NITROGENASE, or on upstream effectors, the concentration of ·NO and the time of the exposure. In this section, we will refer first to the evidence concluding a positive effect of ·NO on nitrogen fixation, followed by those suggesting a negative effect.

Positive Effects of ·NO on Nitrogen Fixation

As presented above in section "·NO in Legume-Bacteria Interaction", different works have concluded that ·NO is necessary for the correct establishment of the legume-rhizobia interaction and nodule development. This would ultimately result in better nitrogen fixation and thus can be considered as evidence supporting a positive effect of ·NO on BNF. Here, we discussed the potential mechanisms by which ·NO can have this effect, involving CK and AUX signaling pathways (**Figure 3**). Even before the legume-rhizobia interaction takes place, greater ·NO levels were suggested to repress plant defense reactions which in turn would promote the correct infection and nodule establishment (Berger et al., 2019). This would be another mechanism by which ·NO promotes BNF.

Nitric oxide levels can also modulate the NITROGENASE activity and in this way directly affect nitrogen fixation. Treatments of 0.1 mM sodium nitroprusside (SNP, ·NO donor) increased the NITROGENASE activity from *L. japonicus* nodules after 27 h by an unknown mechanism (Kato et al., 2010). As it is widely known that ·NO directly inhibits the NITROGENASE activity, we interpret these results as a positive effect on upstream effectors, that results in a greater NITROGENASE expression or activity (**Figure 4A**). Understanding this mechanism can shed light on key molecules controlling NITROGENASE activity.

Negative Effects of ·NO on Nitrogen Fixation

Some reports have suggested that ·NO can difficult the establishment of the legume-rhizobia interaction. For instance, *L. japonicus* plants treated with ·NO donors and lines having higher ·NO levels, due to a mutation in the *Phytogb1* gene, were shown to have problems to form the infection threat (Fukudome et al., 2016). Moreover, they observed lower number of nodule in the lines having increased ·NO levels (Fukudome et al., 2016). At first sight, this would be contradictory than other reports showing a

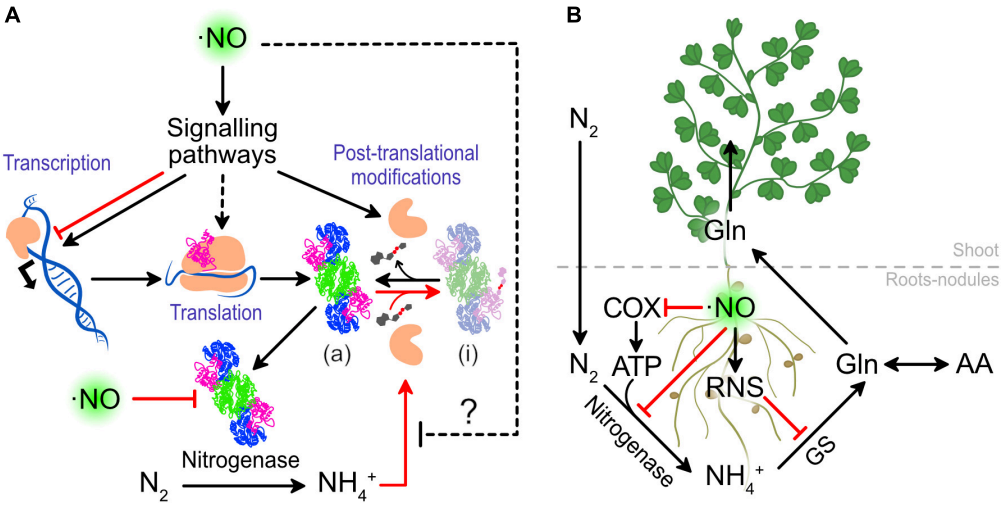

FIGURE 4 | The effect of ·NO on nitrogen fixation and assimilation. **(A)** Effects of ·NO on NITROGENASE activity. **(B)** Detrimental effects of ·NO and RNS on nitrogen fixation and assimilation. In red are presented the interactions that lead to a lower nitrogen fixation and assimilation. COX, CYTOCHROME OXIDASE; GS, GLUTAMINE SYNTHASE.

positive effect of the ·NO in the infection thread formation (Pii et al., 2007; del Giudice et al., 2011), but it may mean that altering the equilibrium of ·NO, either to more or less, always results in negative effect on the infection thread formation, which in some cases was interpreted as ·NO promoting the process and in other as inhibiting it.

The clearest negative effect of ·NO on nitrogen fixation is its capacity to inhibit NITROGENASE activity. The same authors who demonstrated in *L. japonicus* that ·NO enhances NITROGENASE activity when the nodules are treated with the 0.1 mM SNP, also showed that at higher concentrations (at 1 mM or above) SNP inhibits its activity (Kato et al., 2010). This was previously observed *in vitro* using NITROGENASE isolated from bacteroids of soybean nodules (Trinchant and Rigaud, 1982). This *in vitro* evidence demonstrates that ·NO can directly interact and inhibit NITROGENASE activity (**Figure 4**), independently of signaling events acting up-stream.

Besides the direct effect of ·NO on NITROGENASE, ·NO was also suggested to attenuate NITROGENASE activity at the transcriptional level in *B. japonicum* (Sánchez et al., 2010). In particular, nodules of soybean subjected to flooding increased the ·NO content due to a higher periplasmic NR (Nap) activity, and in such situation, a down-regulation of the expression of *B. japonicum nifH* gene, which encodes the Fe subunit of the NITROGENASE, was observed (Sánchez et al., 2010).

Because plant Hbs and bacterial HMP were shown to be important ·NO-detoxifying proteins in plants and rhizobia, respectively, different works used mutants and/or overexpressing lines for these proteins to control the endogenous levels of ·NO. From the plant side, Hbs overexpression in *L. japonicus* resulted in lower ·NO levels, a greater number of formed nodules and higher NITROGENASE activity, suggesting that basal levels of ·NO inhibit nitrogen fixation (Shimoda et al., 2009). From the bacterial side, Cam et al. (2012) used *E. meliloti Hmp* null mutant strain and *Hmp* overexpressing strain to infect *M. truncatula* nodules and manipulate the levels of ·NO. Through this approach, they showed that the *Hmp* null mutant had greater ·NO levels and a dramatic decrease in the NITROGENASE activity (Cam et al., 2012). They also found that nodule senescence was faster and greater in the *Hmp* null mutant, but smaller in the *Hmp* overexpressing strain (Cam et al., 2012). In this way, Cam et al. (2012) showed that keeping ·NO under control is not only important to protect NITROGENASE activity, but also to delay nodule senescence which ultimately will result in extended nitrogen fixation by the plant.

Together, the current evidence demonstrates how ·NO can regulate NITROGENASE activity not only at the transcriptional level but also at post-translational level (**Figure 4A**), and both the plants and the rhizobia play an important role controlling ·NO homeostasis.

The Effects of ·NO and RNS on Nitrogen Fixation and Assimilation

Beyond the effect of ·NO on NITROGENASE, ·NO is known to inhibit CYTOCHROME OXIDASE in different organisms including plants (Millar and Day, 1996). Thus, at high levels, ·NO can threat the ATP production which is necessary for large quantities for NITROGENASE activity (**Figure 4B**). In such a situation, the ALTERNATIVE OXIDASE (AOX) of nodules was suggested to play a role to allow nodule respiration (Millar et al., 1997), although ATP production would be still minimized.

Once the nitrogen is reduced to ammonium by the rhizobia, the plants assimilate the ammonium to transport the nitrogen in an organic form and reduce the levels of free ammonium which is toxic at high concentrations. The GLUTAMINE SYNTHETASE-GLUTAMATE SYNTHASE (GS-GOGAT) plays a major role in the assimilation of N to amino acids. The GLUTAMINE SYNTHASE 1 of legumes was shown to be nitrated, both in roots of *L. japonicus* under drought stress (Signorelli et al., 2019) and in nodules of *M. truncatula* (Melo et al., 2011), causing the inactivation of the enzyme (Melo et al., 2011). As protein nitration requires ·NO and ROS, we can assume that uncontrolled ·NO would not only inhibit NITROGENASE and cytochrome oxidase activity but also GLUTAMINE SYNTHETASE, compromising the total capacity of the legumes to reduce and assimilate atmospheric nitrogen (**Figure 4B**).

Our Conclusions on the Effect of ·NO on Nitrogen Fixation and Assimilation

Taking all together, the current evidence suggests that the positive effect of ·NO on nitrogen fixation is associated to the promotion of legume-rhizobia interaction and signaling pathways controlling the NITROGENASE activity (**Figures 1**, **3**, **4A**). On the other hand, the negative effects of ·NO on nitrogen fixation and assimilation seem to be mostly related to the direct inactivation of the main actors in the process of nitrogen fixation and assimilation by ·NO and RNS (**Figure 4**). Therefore, most of the evidence showing a negative effect of ·NO on BNF relay on the direct action of ·NO (or RNS) in a one to one ratio (e.g., CYTOCHOROME OXIDASE and NITROGENASE inactivation), whereas those linked to a positive effect seem to act on signaling pathways (e.g., CK, AUX, ABA) having an amplifier effect. Therefore, we speculate that, at physiological conditions, ·NO is more likely to have a positive effect on BNF.

Both plants and rhizobia ·NO-detoxifying systems were observed to be essential to control ·NO at basal levels and avoid NITROGENASE inactivation. The induction of early nodule senescence observed in different reports suggests that an early and uncontrolled increase of ·NO in the nodule can present a threat for plant productivity. In the experiments mentioned in this review, the levels of ·NO were manipulated to be abnormal, either using ·NO donors, mutant plants or rhizobium strains. However, we know that in wild type plants, levels of ·NO can be triggered by environmental factors. Therefore, it seems to be important to understand how to prevent the spikes of ·NO that can threat the biological fixation and nodule viability.

Alternative Signaling Pathways by Which ·NO Could Modulate BNF

The N-end rule pathway of proteolysis (NERP) is a mechanism by which O_2, and also ·NO, can act as signaling molecules to

promote protein degradation. In particular in plants, some ERF were shown to be subjected to proteasomal degradation under normoxia through this pathway (Gibbs et al., 2014; Meitha et al., 2018). As the nodule also has a low O_2 environment, ERF would be stable in such organ becoming a potential molecular switch that can be regulated by ·NO via the NERP. Therefore, evaluating the involvement of ERF in nodule seems to be a worth exploring area in the future.

Likewise, most research about ABA signaling has been focused on the control of dormancy in seeds or, at vegetative level, on stress responses, thus it is not clear yet which transcriptions factors could be involved downstream SnRK2 in nodulation. The basic leucine zipper transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5) is suggested to be the integrator of ABA and other phytohormone signaling during stress conditions and developmental processes (Skubacz et al., 2016). For example, CK is known to negatively regulate ABI5 protein level (Guan et al., 2014). Therefore, it would not be surprising that ABI5 also controls nodule formation. Currently, the role of ABI5 in nodule development is largely unexplored and further research in this direction would contribute to a better understanding of the molecular mechanism underpinning nodule development by phytohormones.

Finally, most of our knowledge about NITROGENASE regulation is limited to the transcriptional regulation of *nif* genes. However, in many nitrogen-fixing bacteria, the NITROGENASE is known to be regulated by post-translational modifications (Pope et al., 1985; Huergo et al., 2009; Heiniger et al., 2012). In particular, in the presence of ammonium, an ADP-ribosyltransfease (DraT2) transfers an ADP-ribose to a conserved arginine on DINITROGENASE REDUCTASE (NifH) to inactivate the NITROGENASE activity (Heiniger et al., 2012). This post-translational regulation involves several proteins such as NtrB, NtrC, GlnK2, DraG, which if inactivated by ·NO would promote the NITROGENASE activity by interfering with the inactivation of NITROGENASE in presence of ammonium (**Figure 4A**). Therefore, this seems worth exploring because understanding the effect of ·NO on these proteins could led to potential mechanisms by which ·NO could modulate the post-translational inhibition of the NITROGENASE activity.

What Is the Role of Abiotic Stress-Induced ·NO on BNF?

The establishment of the nodule and BNF are known to be extremely sensitive to modest drought conditions in many species (Serraj et al., 1999; Sinclair and Serraj, 1995). Because of that, there have been many reports focused on how drought and other environmental factors affect BNF. However, there are virtually no studies about the effect of endogenous abiotic stress-induced ·NO on BNF. It would be interesting to evaluate this because most environmental stresses result in the overproduction of ·NO and nitrosative stress. For instance, in the legumes *L. japonicus* and *Pisum sativum* an overproduction of ·NO in roots was reported when they are exposed to environmental stresses (Signorelli et al., 2013; Lehotai et al., 2016). However, in these studies, the plants were grown in a medium having high levels of nitrate (NO_3^-), which can be used by NR and contribute to ·NO overproduction. This does not represent a situation in which nodulation would take place, as it requires low levels of N in the soil, and perhaps in such conditions, there is no ·NO overproduction. Therefore, it would be important to evaluate if there is ·NO overproduction in nodulation and stress conditions, and what the effects of scavenging this ·NO are.

CONCLUSION

The involvement of ·NO throughout the whole process of legume-rhizobia interaction has been well documented, and most reports agree that ·NO is necessary for the correct establishment of the interaction. Both the bacteria and the plants have been shown to contribute to ·NO production and scavenging, and some findings points to the possibility that ·NO could promote the nodule formation by enhancing CK signaling pathway and interfere with AUX and ABA signaling pathways. Moreover, transcriptomic analyses have suggested that its metabolism is more active during nodule maturation. In mature nodules, many proteins have been identified as S-nitrosylated, including the thiol peroxidase GPX1 which is key for H_2O_2 sensing and transmission of oxidative signals. It is likely that this type of post-translational modification is the most responsible for the regulatory role of ·NO. To counteract this modification, legume plants possess different hemoglobins which play a significant role in ROS and RNS metabolism by contributing to ROS production and ·NO scavenging. In the near future, it is likely that we expand our knowledge about enzymes that are S-nitrosylated *in vivo* and the effect of such modification.

AUTHOR CONTRIBUTIONS

SS conceived the idea and structure of the manuscript, wrote the first draft of the manuscript, and illustrated all the figures. ST-D contributed to the section of metabolism of ·NO in nodules. MS contributed to the writing of the section about ROS and RNS homeostasis in the nodule and designed **Figure 2**. JM revised the whole manuscript.

REFERENCES

Albertos, P., Romero-Puertas, M. C., Tatematsu, K., Mateos, I., Sánchez-Vicente, I., Nambara, E., et al. (2015). S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. *Nat. Commun.* 6:8669. doi: 10.1038/ncomms9669

Angelo, M., Hausladen, A., Singel, D. J., and Stamler, J. S. (2008). Interactions of NO with hemoglobin: from microbes to man. *Methods Enzymol.* 436, 131–168. doi: 10.1016/S0076-6879(08)36008-X

Appleby, C. A., and Bergersen, F. J. (1980). "Preparation and experimental use of leghaemoglobin," in *Methods for Evaluating Biological Nitrogen Fixation*, ed. F. J. Bergersen (Chichester: Wiley), 315–335.

Arthikala, M. K., Montiel, J., Sánchez-López, R., Nava, N., Cárdenas, L., and Quinto, C. (2017). Respiratory burst oxidase homolog gene a is crucial for rhizobium infection and nodule maturation and function in common bean. *Front. Plant Sci.* 8:2003. doi: 10.3389/fpls.2017.02003

Astier, J., Gross, I., and Durner, J. (2018). Nitric oxide production in plants: an update. *J. Exp. Bot.* 69, 3401–3411. doi: 10.1093/jxb/erx420

Aviram, I., Wittenberg, B. A., Wittenberg, J. B., Wittenberg, A., and Wittenberg, J. B. (1978). The reaction of ferrous leghemoglobin with hydrogen peroxide to form leghemoglobin (IV). *J. Biol. Chem.* 253, 5685–5689.

Baudouin, E., Frendo, P., Le Gleuher, M., and Puppo, A. (2004). A Medicago sativa haem oxygenase gene is preferentially expressed in root nodules. *J. Exp. Bot.* 55, 43–47. doi: 10.1093/jxb/erh020

Baudouin, E., Pieuchot, L., Engler, G., Pauly, N., and Puppo, A. (2006). Nitric oxide is formed in Medicago truncatula-Sinorhizobium meliloti functional nodules. *Mol. Plant Microbe Interact.* 19, 970–975.

Becana, M., Matamoros, M. A., Udvardi, M., and Dalton, D. A. (2010). Recent insights into antioxidant defenses of legume root nodules. *New Phytol.* 188, 960–976. doi: 10.1111/j.1469-8137.2010.03512.x

Bedmar, E. J., Robles, E. F., and Delgado, M. J. (2005). The complete denitrification pathway of the symbiotic, nitrogen-fixing bacterium Bradyrhizobium japonicum. *Biochem. Soc. Trans.* 33, 141–144. doi: 10.1042/bst0330141

Begara-Morales, J. C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M. N., and Barroso, J. B. (2019). The function of S-nitrosothiols during abiotic stress in plants. *J. Exp. Bot.* 70, 4429–4439. doi: 10.1093/jxb/erz197

Berger, A., Boscari, A., Frendo, P., and Brouquisse, R. (2019). Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *J. Exp. Bot.* 70, 4505–4520. doi: 10.1093/jxb/erz159

Berger, A., Guinand, S., Boscari, A., Puppo, A., and Brouquisse, R. (2020). Medicago truncatula Phytoglobin 1.1 controls symbiotic nodulation and nitrogen fixation via the regulation of nitric oxide concentration. *New Phytol.* doi: 10.1111/nph.16462

Bethke, P. C., Badger, M. R., and Jones, R. L. (2004). Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* 16, 332–341. doi: 10.1105/tpc.017822

Betsuyaku, S., Katou, S., Takebayashi, Y., Sakakibara, H., Nomura, N., and Fukuda, H. (2018). Salicylic acid and jasmonic acid pathways are activated in spatially different domains around the infection site during effector-triggered immunity in Arabidopsis thaliana. *Plant Cell Physiol.* 59, 8–16. doi: 10.1093/pcp/pcx181

Bustos-Sanmamed, P., Tovar-Méndez, A., Crespi, M., Sato, S., Tabata, S., and Becana, M. (2011). Regulation of nonsymbiotic and truncated hemoglobin genes of Lotus japonicus in plant organs and in response to nitric oxide and hormones. *New Phytol.* 189, 765–776. doi: 10.1111/j.1469-8137.2010.03527.x

Cabrera, J. J., Salas, A., Torres, M. J., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2015). An integrated biochemical system for nitrate assimilation and nitric oxide detoxification in Bradyrhizobium japonicum. *Biochem. J.* 473, 297–309. doi: 10.1042/bj20150880

Calvo-Begueria, L., Rubio, M. C., Martínez, J. I., Pérez-Rontomé, C., Delgado, M. J., Bedmar, E. J., et al. (2018). Redefining nitric oxide production in legume nodules through complementary insights from electron paramagnetic resonance spectroscopy and specific fluorescent probes. *J. Exp. Bot.* 69, 3703–3714. doi: 10.1093/jxb/ery159

Cam, Y., Pierre, O., Boncompagni, E., Hérouart, D., Meilhoc, E., and Bruand, C. (2012). Nitric oxide (NO): a key player in the senescence of Medicago truncatula root nodules. *New Phytol.* 196, 548–560. doi: 10.1111/j.1469-8137.2012.04282.x

Castella, C., Mirtziou, I., Seassau, A., Boscari, A., Montrichard, F., Papadopoulou, K., et al. (2017). Post-translational modifications of Medicago truncatula glutathione peroxidase 1 induced by nitric oxide. *Nitric Oxide* 68, 125–136. doi: 10.1016/j.niox.2017.02.004

Castillo, M.-C., Lozano-Juste, J., Gonzalez-Guzman, M., Rodriguez, L., Rodriguez, P. L., and Leon, J. (2015). Inactivation of PYR/PYL/RCAR ABA receptors by tyrosine nitration may enable rapid inhibition of ABA signaling by nitric oxide in plants. *Sci. Signal.* 8:ra89. doi: 10.1126/scisignal.aaa7981

Chaki, M., Alvarez de Morales, P., Ruiz, C., Begara-Morales, J. C., Barroso, J. B., Corpas, F. J., et al. (2015). Ripening of pepper (Capsicum annuum) fruit is characterized by an enhancement of protein tyrosine nitration. *Ann. Bot.* 116, 637–647. doi: 10.1093/aob/mcv016

Cocking, E. C., Stone, P. J., and Davey, M. R. (2006). Intracellular colonization of roots of Arabidopsis and crop plants by Gluconacetobacter diazotrophicus. *In Vitro Cell. Dev. Biol. Plant* 42, 74–82. doi: 10.1079/IVP2005716

Considine, M. J., Diaz-Vivancos, P., Kerchev, P., Signorelli, S., Agudelo-Romero, P., Gibbs, D. J., et al. (2017). Learning to breathe: developmental phase transitions in oxygen status. *Trends Plant Sci.* 22, 140–153. doi: 10.1016/j.tplants.2016.11.013

Corpas, F. J., Chaki, M., Fernandez-Ocana, A., Valderrama, R., Palma, J. M., Carreras, A., et al. (2008). Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. *Plant Cell Physiol.* 49, 1711–1722. doi: 10.1093/pcp/pcn144

Corpas, F. J., Leterrier, M., Valderrama, R., Airaki, M., Chaki, M., Palma, J. M., et al. (2011). Nitric oxide imbalance provokes a nitrosative response in plants under abiotic stress. *Plant Sci.* 181, 604–611. doi: 10.1016/j.plantsci.2011.04.005

Corpas, F. J., Palma, J. M., Del Rio, L., and Barroso, J. B. (2009). Evidence supporting the existence of l-arginine-dependent nitric oxide synthase activity in plants. *New Phytol.* 184, 9–14. doi: 10.1111/j.1469-8137.2009.02989.x

Damiani, I., Pauly, N., Puppo, A., Brouquisse, R., and Boscari, A. (2016). Reactive oxygen species and nitric oxide control early steps of the legume – rhizobium symbiotic interaction. *Front. Plant Sci.* 7:454. doi: 10.3389/fpls.2016.00454

del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., et al. (2011). Nitric oxide is required for an optimal establishment of the Medicago truncatula-Sinorhizobium meliloti symbiosis. *New Phytol.* 191, 405–417. doi: 10.1111/j.1469-8137.2011.03693.x

Ding, Y., Kalo, P., Yendrek, C., Sun, J., Liang, Y., Marsh, J. F., et al. (2008). Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula. *Plant Cell* 20, 2681–2695. doi: 10.1105/tpc.108.061739

Dordas, C. (2009). Nonsymbiotic hemoglobins and stress tolerance in plants. *Plant Sci.* 176, 433–440. doi: 10.1016/j.plantsci.2009.01.003

Dordas, C., Hasinoff, B. B., Igamberdiev, A. U., Manac'h, N., Rivoal, J., and Hill, R. D. (2003). Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *Plant J.* 35, 763–770. doi: 10.1046/j.1365-313X.2003.01846.x

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Ferrarini, A., De Stefano, M., Baudouin, E., Pucciariello, C., Polverari, A., Puppo, A., et al. (2008). Expression of Medicago truncatula genes responsive to nitric oxide in pathogenic and symbiotic conditions. *Mol. Plant Microbe Interact.* 21, 781–790. doi: 10.1094/MPMI-21-6-0781

Fukudome, M., Calvo-Begueria, L., Kado, T., Osuki, K. I., Rubio, M. C., Murakami, E. I., et al. (2016). Hemoglobin LjGlb1-1 is involved in nodulation and regulates the level of nitric oxide in the Lotus japonicus-Mesorhizobium loti symbiosis. *J. Exp. Bot.* 67, 5275–5283. doi: 10.1093/jxb/erw290

Fukudome, M., Watanabe, E., Osuki, K.-I., Imaizumi, R., Aoki, T., Becana, M., et al. (2018). Stably transformed Lotus japonicus plants overexpressing phytoglobin LjGlb1-1 show decreased nitric oxide levels in roots and nodules as well as delayed nodule senescence. *Plant Cell Physiol.* 60, 816–825. doi: 10.1093/pcp/pcy245

Gage, D. J. (2004). Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* 68, 280–300. doi: 10.1128/mmbr.68.2.280-300.2004

Garrocho-Villegas, V., and Arredondo-Peter, R. (2008). Molecular cloning and characterization of a moss (Ceratodon purpureus) nonsymbiotic hemoglobin provides insight into the early evolution of plant nonsymbiotic hemoglobins. *Mol. Biol. Evol.* 25, 1482–1487. doi: 10.1093/molbev/msn096

Geurts, R., and Bisseling, T. (2002). Rhizobium nod factor perception and signalling. *Plant Cell* 14, s239–s249. doi: 10.1105/tpc.002451

Gibbs, D. J., Conde, J. V., Berckhan, S., Prasad, G., Mendiondo, G. M., and Holdsworth, M. J. (2015). Group VII ethylene response factors coordinate oxygen and nitric oxide signal transduction and stress responses in plants. *Plant Physiol.* 169, 23–31. doi: 10.1104/pp.15.00338

Gibbs, D. J., MdIsa, N., Movahedi, M., Lozano-Juste, J., Mendiondo, G. M., Berckhan, S., et al. (2014). Nitric oxide sensing in plants is mediated by proteolytic control of group VII ERF transcription factors. *Mol. Cell* 53, 369–379. doi: 10.1016/j.molcel.2013.12.020

Gonzalez-Rizzo, S., Crespi, M., and Frugier, F. (2006). The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. *Plant Cell* 18, 2680–2693. doi: 10.1105/tpc.106.043778

Guan, C., Wang, X., Feng, J., Hong, S., Liang, Y., Ren, B., et al. (2014). Cytokinin antagonizes abscisic acid-mediated inhibition of cotyledon greening by promoting the degradation of abscisic acid insensitive5 protein in Arabidopsis. *Plant Physiol.* 164, 1515–1526. doi: 10.1104/pp.113.234740

Günther, C., Schlereth, A., Udvardi, M., and Ott, T. (2007). Metabolism of reactive oxygen species is attenuated in leghemoglobin-deficient nodules of Lotus japonicus. *Mol. Plant Microbe Interact.* 20, 1596–1603.

Gupta, K. J., Hebelstrup, K. H., Mur, L. A. J., and Igamberdiev, A. U. (2011a). Plant hemoglobins: important players at the crossroads between oxygen and nitric oxide. *FEBS Lett.* 585, 3843–3849. doi: 10.1016/j.febslet.2011.10.036

Gupta, K. J., and Igamberdiev, A. U. (2011). The anoxic plant mitochondrion as a nitrite: NO reductase. *Mitochondrion* 11, 537–543. doi: 10.1016/j.mito.2011.03.005

Gupta, K. J., Igamberdiev, A. U., Manjunatha, G., Segu, S., Moran, J. F., Neelawarne, B., et al. (2011b). The emerging roles of nitric oxide (NO) in plant mitochondria. *Plant Sci.* 181, 520–526. doi: 10.1016/j.plantsci.2011.03.018

Heiniger, E. K., Oda, Y., Samanta, S. K., and Harwood, C. S. (2012). How posttranslational modification of nitrogenase is circumvented in Rhodopseudomonas palustris strains that produce hydrogen gas constitutively. *Appl. Environ. Microbiol.* 7, 1023–1032. doi: 10.1128/AEM.07254-11

Herold, S., and Puppo, A. (2005). Oxyleghemoglobin scavenges nitrogen monoxide and peroxynitrite: a possible role in functioning nodules? *J. Biol. Inorg. Chem.* 10, 935–945.

Hichri, I., Boscari, A., Castella, C., Rovere, M., Puppo, A., and Brouquisse, R. (2015). Nitric oxide: a multifaceted regulator of the nitrogen-fixing symbiosis. *J. Exp. Bot.* 66, 2877–2887. doi: 10.1093/jxb/erv051

Hill, R., Hargrove, M., and Arredondo-Peter, R. (2016). Phytoglobin: a novel nomenclature for plant globins accepted by the globin community at the 2014 XVIII conference on Oxygen-Binding and Sensing Proteins. *F1000Res.* 5:212. doi: 10.12688/f1000research.8133.1

Hill, R. D. (2012). Non-symbiotic haemoglobins-What's happening beyond nitric oxide scavenging? *AoB Plants* 2012:pls004. doi: 10.1093/aobpla/pls004

Hirsch, A. M. (1992). Developmental biology of legume nodulation. *New Phytol.* 122, 211–237. doi: 10.1111/j.1469-8137.1992.tb04227.x

Horchani, F., Prevot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2010). Both Plant and Bacterial Nitrate Reductases Contribute to Nitric Oxide Production in Medicago truncatula Nitrogen-Fixing Nodules. *Plant Physiol.* 155, 1023–1036. doi: 10.1104/pp.110.166140

Huang, X., Stettmaier, K., Michel, C., Hutzler, P., Mueller, M. J., and Durner, J. (2004). Nitric oxide is induced by wounding and influences jasmonic acid signaling in Arabidopsis thaliana. *Planta* 218, 938–946.

Huergo, L. F., Merrick, M., Monteiro, R. A., Chubatsu, L. S., Steffens, M. B. R., Pedrosa, F. O., et al. (2009). In vitro interactions between the PII proteins and the nitrogenase regulatory enzymes dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase-activating glycohydrolase (DraG) in Azospirillum brasilense. *J. Biol. Chem.* 284, 6674–6682. doi: 10.1074/jbc.M807378200

Hunt, P. W., Watts, R. A., Trevaskis, B., Llewelyn, D. J., Burnell, J., Dennis, E. S., et al. (2001). Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Mol. Biol.* 47, 677–692.

Igamberdiev, A. U., Bykova, N. V., Shah, J. K., and Hill, R. D. (2010). Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. *Physiol. Plant.* 138, 393–404. doi: 10.1111/j.1399-3054.2009.01314.x

Igamberdiev, A. U., and Hill, R. D. (2004). Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. *J. Exp. Bot.* 55, 2473–2482. doi: 10.1093/jxb/erh272

Jaszek, M., Janczarek, M., Kuczyński, K., Piersiak, T., and Grzywnowicz, K. (2014). The response of the Rhizobium leguminosarum bv. trifolii wild-type and exopolysaccharide-deficient mutants to oxidative stress. *Plant Soil* 376, 75–94.

Kato, K., Kanahama, K., and Kanayama, Y. (2010). Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in Lotus root nodules. *J. Plant Physiol.* 167, 238–241. doi: 10.1016/j.jplph.2009.08.006

Klessig, D. F., Durner, J., Noad, R., Navarre, D. A., Wendehenne, D., Kumar, D., et al. (2000). Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8849–8855. doi: 10.1073/pnas.97.16.8849

Kovacs, I., and Lindermayr, C. (2013). Nitric oxide-based protein modification: formation and site-specificity of protein S-nitrosylation. *Front. Plant Sci.* 4:137. doi: 10.3389/fpls.2013.00137

Laranjo, M., Alexandre, A., and Oliveira, S. (2014). Legume growth-promoting rhizobia: an overview on the Mesorhizobium genus. *Microbiol. Res.* 169, 2–17. doi: 10.1016/j.micres.2013.09.012

Lehotai, N., Lyubenova, L., Schröder, P., Feigl, G., Ördög, A., Szilágyi, K., et al. (2016). Nitro-oxidative stress contributes to selenite toxicity in pea (Pisum sativum L). *Plant Soil* 400, 107–122. doi: 10.1007/s11104-015-2716-x

León, J., and Costa-Broseta, Á. (2020). Present knowledge and controversies, deficiencies, and misconceptions on nitric oxide synthesis, sensing, and signaling in plants. *Plant Cell Environ.* 43, 1–15. doi: 10.1111/pce.13617

Lindström, K., Murwira, M., Willems, A., and Altier, N. (2010). The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. *Res. Microbiol.* 161, 453–463. doi: 10.1016/j.resmic.2010.05.005

Lozano-Juste, J., and Leon, J. (2011). Nitric oxide regulates DELLA content and PIF expression to promote photomorphogenesis in Arabidopsis. *Plant Physiol.* 156, 1410–1423. doi: 10.1104/pp.111.177741

Maiti, D., Sarkar, T. S., and Ghosh, S. (2012). Detection of S-nitrosothiol and nitrosylated proteins in Arachis hypogaea functional nodule: response of the nitrogen fixing symbiont. *PLoS One* 7:e45526. doi: 10.1371/journal.pone.0045526

Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., et al. (2014). Cytokinin controls polarity of PIN1-dependent Auxin transport during lateral root organogenesis. *Curr. Biol.* 24, 1031–3037. doi: 10.1016/j.cub.2014.04.002

Marino, D., Andrio, E., Danchin, E. G. J., Oger, E., Gucciardo, S., Lambert, A., et al. (2011). A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning. *New Phytol.* 189, 580–592. doi: 10.1111/j.1469-8137.2010.03509.x

Marino, D., Pucciariello, C., Puppo, A., and Frendo, P. (2009). The redox state, a referee of the legume-rhizobia symbiotic game. *Adv. Bot. Res.* 52, 115–151. doi: 10.1016/S0065-2296(10)52005-6

Masson-Boivin, C., Giraud, E., Perret, X., and Batut, J. (2009). Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? *Trends Microbiol.* 17, 458–466. doi: 10.1016/j.tim.2009.07.004

Meilhoc, E., Cam, Y., Skapski, A., and Bruand, C. (2010). The response to nitric oxide of the nitrogen-fixing symbiont Sinorhizobium meliloti. *Mol. Plant Microbe Interact.* 23, 748–759. doi: 10.1094/MPMI-23-6-0748

Meitha, K., Agudelo-Romero, P., Signorelli, S., Gibbs, D. J., Considine, J. A., Foyer, C. H., et al. (2018). Developmental control of hypoxia during bud burst in grapevine. *Plant Cell Environ.* 41, 1154–1170. doi: 10.1111/pce.13141

Melo, P. M., Silva, L. S., Ribeiro, I., Seabra, A. R., and Carvalho, H. G. (2011). Glutamine synthetase is a molecular target of nitric oxide in root nodules of Medicago truncatula and is regulated by tyrosine nitration. *Plant Physiol.* 157, 1505–1517. doi: 10.1104/pp.111.186056

Michalski, W. P., and Nicholas, D. J. D. (1987). Inhibition of nitrogenase by nitrite and nitric oxide in Rhodopseudomonas sphaeroides f. sp. denitrificans. *Arch. Microbiol.* 147, 304–308. doi: 10.1007/BF00463493

Millar, A. H., Day, D. A., and Bergersen, F. J. (1995). Microaerobic respiration and oxidative phosphorylation by soybean nodule mitochondria: implications for nitrogen fixation. *Plant Cell Environ.* 18, 715–726. doi: 10.1111/j.1365-3040.1995.tb00574.x

Millar, A. H., Finnegan, P. M., Whelan, J., Drevon, J. J., and Day, D. A. (1997). Expression and kinetics of the mitochondrial alternative oxidase in nitrogen-fixing nodules of soybean roots. *Plant Cell Environ.* 20, 1273–1282. doi: 10.1046/j.1365-3040.1997.d01-25.x

Millar, H. A., and Day, D. A. (1996). Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. *FEBS Lett.* 398, 155–158.

Miwa, H., Sun, J., Oldroyd, G. E. D., and Downie, J. A. (2006). Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of Lotus japonicus. *Mol. Plant Microbe Interact.* 19, 914–923.

Monza, J., Irisarri, P., Díaz, P., Delgado, M. J., Mesa, S., and Bedmar, E. J. (2006). Denitrification ability of rhizobial strains isolated from Lotus sp. *Antonie Van Leeuwenhoek* 89, 479–484.

Moreau, S., Davies, M. J., and Puppo, A. (1995). Reaction of ferric leghemoglobin with H_2O_2: formation of heme-protein cross-links and dimeric species. *Biochim. Biophys. Acta* 1251, 17–22. doi: 10.1016/0167-4838(95)00087-B

Murakami, Y., Miwa, H., Imaizumi-Anraku, H., Kouchi, H., Downie, J. A., Kawaguchi, M., et al. (2007). Positional cloning identifies Lotus japonicus NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. *DNA Res.* 13, 255–265. doi: 10.1093/dnares/dsl017

Nagata, M., Murakami, E., Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Suzuki, A., et al. (2008). Expression of a Class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in Lotus japonicus. *Mol. Plant Microbe Interact.* 21, 1175–1183.

Nagata, M., and Suzuki, A. (2014). "Effects of phytohormones on nodulation and nitrogen fixation in leguminous plants," in *Advances in Biology and Ecology of Nitrogen Fixation*, ed. T. Ohyama (Rijeka: InTech), 111–128. doi: 10.5772/57267

Navascues, J., Perez-Rontome, C., Gay, M., Marcos, M., Yang, F., Walker, F. A., et al. (2012). Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2660–2665. doi: 10.1073/pnas.1116559109

Oldroyd, G. E. D., and Dixon, R. (2014). Biotechnological solutions to the nitrogen problem. *Curr. Opin. Biotechnol.* 26, 19–54. doi: 10.1016/j.copbio.2013.08.006

Penmetsa, R. V., Frugoli, J. A., Smith, L. S., Long, S. R., and Cook, D. R. (2003). Dual genetic pathways controlling nodule number in Medicago truncatula. *Plant Physiol.* 131, 998–1008. doi: 10.1104/pp.015677

Perazzolli, M., Dominici, P., Romero-Puertas, M. C., Zago, E., Zeier, J., Sonoda, M., et al. (2004). Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *Plant Cell* 16, 2785–2794. doi: 10.1105/tpc.104.025379

Phillips, D. A. (1971). Abscisic acid inhibition of root nodule initiation in Pisum sativum. *Planta* 100, 181–190. doi: 10.1007/BF00387034

Pii, Y., Crimi, M., Cremonese, G., Spena, A., and Pandolfini, T. (2007). Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biol.* 7:21. doi: 10.1186/1471-2229-7-21

Planchet, E., Gupta, K. J., Sonoda, M., and Kaiser, W. M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. *Plant J.* 41, 732–743. doi: 10.1111/j.1365-313X.2005.02335.x

Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., et al. (2011). MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in Medicago truncatula. *Plant J.* 65, 622–633. doi: 10.1111/j.1365-313X.2010.04447.x

Pope, M. R., Murrell, S. A., and Ludden, P. W. (1985). Covalent modification of the iron protein of nitrogenase from Rhodospirillum rubrum by adenosine diphosphoribosylation of a specific arginine residue. *Proc. Natl. Acad. Sci. U.S.A.* 82, 3173–3177. doi: 10.1073/pnas.82.10.3173

Puppo, A., Dimitrijevic, L., and Rigaud, J. (1982). Possible involvement of nodule superoxide dismutase and catalase in leghemoglobin protection. *Planta* 50, 374–379.

Puppo, A., Rigaud, J., and Job, D. (1981). Role of superoxide anion in leghemoglobin autoxidation. *Plant Sci. Lett.* 22, 353–360. doi: 10.1016/0304-4211(81)90081-X

Puppo, A., Pauly, N., Boscari, A., Mandon, K., and Brouquisse, R. (2013). Hydrogen Peroxide and Nitric Oxide: Key Regulators of the Legume—Rhizobium and Mycorrhizal Symbioses. *Antioxid. Redox Signal.* 18, 2202–2019. doi: 10.1089/ars.2012.5136.

Ramu, S. K.,Peng, H.-M. and Cook, D. R. (2002). Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in Medicago truncatula. *Mol. Plant Microbe Interact.* 15, 522–528. doi: 10.1094/mpmi.2002.15.6.522

Roy, S., Liu, W., Nandety, R. S., Crook, A., Mysore, K. S., Pislariu, C. I., et al. (2020). Celebrating 20 years of genetic discoveries in legume nodulation and symbiotic nitrogen fixation. *Plant Cell* 132, 15–41. doi: 10.1105/tpc.19.00279

Rümer, S., Gupta, K. J., and Kaiser, W. M. (2009). Plant cells oxidize hydroxylamines to NO. *J. Exp. Bot.* 4, 853–855. doi: 10.1093/jxb/erp077

Sainz, M., Calvo-Begueria, L., Pérez-Rontomé, C., Wienkoop, S., Abián, J., Staudinger, C., et al. (2015). Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide-dependent mechanism. *Plant J.* 81, 723–735. doi: 10.1111/tpj.12762

Sainz, M., Pérez-Rontomé, C., Ramos, J., Mulet, J. M., James, E. K., Bhattacharjee, U., et al. (2013). Plant hemoglobins may be maintained in functional form by reduced flavins in the nuclei, and confer differential tolerance to nitro-oxidative stress. *Plant J.* 76, 875–887. doi: 10.1111/tpj.12340

Sánchez, C., Gates, A. J., Meakin, G. E., Uchiumi, T., Girard, L., Richardson, D. J., et al. (2010). Production of nitric oxide and nitrosylleghemoglobin complexes in soybean nodules in response to flooding. *Mol. Plant Microbe Interact.* 23, 702–711.

Sanz, L., Fernandez-Marcos, M., Modrego, A., Lewis, D. R., Muday, G. K., Pollmann, S., et al. (2014). Nitric oxide plays a role in stem cell niche homeostasis through its interaction with auxin. *Plant Physiol.* 166, 1972–1984. doi: 10.1104/pp.114.247445

Sanz-Luque, E., Ocaña-Calahorro, F., De Montaigu, A., Chamizo-Ampudia, A., Llamas, Á., Galván, A., et al. (2015). THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity. *Plant J.* 81, 467–479. doi: 10.1111/tpj.12744

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402, 191–195. doi: 10.1038/46058

Serraj, R., Sinclair, T. R., and Purcell, L. C. (1999). Symbiotic N2 fixation response to drought. *J. Exp. Bot.* 50, 143–155. doi: 10.1093/jxb/50.331.143

Shimoda, Y., Nagata, M., Suzuki, A., Abe, M., Sato, S., Kato, T., et al. (2005). Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in Lotus japonicus. *Plant Cell Physiol.* 46, 99–107. doi: 10.1093/pci/pci001

Shimoda, Y., Shimoda-Sasakura, F., Kucho, K. I., Kanamori, N., Nagata, M., Suzuki, A., et al. (2009). Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between Mesorhizobium loti and Lotus japonicus. *Plant J.* 57, 254–263. doi: 10.1111/j.1365-313X.2008.03689.x

Sievers, G., Ronnberg, M., and Rönnberg, M. (1978). Study of the pseudoperoxidatic activity of soybean leghemoglobin and sperm whale myoglobin. *BBA Protein Struct.* 533, 293–301.

Signorelli, S., and Considine, M. J. (2018). Nitric oxide enables germination by a four-pronged attack on ABA-induced seed dormancy. *Front. Plant Sci.* 9:296. doi: 10.3389/fpls.2018.00296

Signorelli, S., Corpas, F. J., Borsani, O., Barroso, J. B., and Monza, J. (2013). Water stress induces a differential and spatially distributed nitro-oxidative stress response in roots and leaves of Lotus japonicus. *Plant Sci.* 20, 137–146. doi: 10.1016/j.plantsci.2012.12.004

Signorelli, S., Corpas, F. J., Rodríguez-Ruiz, M., Valderrama, R., Barroso, J. B., Borsani, O., et al. (2019). Drought stress triggers the accumulation of NO and SNOs in cortical cells of Lotus japonicus L. roots and the nitration of proteins with relevant metabolic function. *Environ. Exp. Bot.* 161, 228–241. doi: 10.1016/j.envexpbot.2018.08.007

Signorelli, S., Möller, M. N., Coitińo, E. L., and Denicola, A. (2011). Nitrogen dioxide solubility and permeation in lipid membranes. *Arch. Biochem. Biophys.* 512, 190–196. doi: 10.1016/j.abb.2011.06.003

Sinclair, T. R., and Serraj, R. (1995). Legume nitrogen fixation and drought. *Nature* 378:344. doi: 10.1038/378344a0

Skubacz, A., Daszkowska-Golec, A., and Szarejko, I. (2016). The role and regulation of ABI5 (ABA-Insensitive 5) in plant development, abiotic stress responses and phytohormone crosstalk. *Front. Plant Sci.* 7:1884. doi: 10.3389/fpls.2016.01884

Smagghe, B. J., Hoy, J. A., Percifield, R., Kundu, S., Hargrove, M. S., Sarath, G., et al. (2009). Correlations between oxygen affinity and sequence classifications of plant hemoglobins. *Biopolymers* 91, 1083–1096. doi: 10.1002/bip.21256

Stacey, G., McAlvin, C. B., Kim, S. Y., Olivares, J., and Soto, M. J. (2006). Effects of endogenous salicylic acid on nodulation in the model legumes Lotus japonicus and Medicago truncatula. *Plant Physiol.* 141, 1473–1481. doi: 10.1104/pp.106.080986

Stöhr, C., Strube, F., Marx, G., Ullrich, W. R., and Rockel, P. (2001). A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* 212, 835–841. doi: 10.1007/s004250000447

Stoimenova, M., Igamberdiev, A. U., Gupta, K. J., and Hill, R. D. (2007). Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. *Planta* 226, 465–474.

Sulieman, S., and Tran, L. S. P. (2015). "Introduction," in *Legume nitrogen fixation in a changing environment: Achievements and challenges*, eds S. Sulieman and L. S. P. Tran (Cham: Springer International Publishing), 1–3.

Suzaki, T., Yano, K., Ito, M., Umehara, Y., Suganuma, N., and Kawaguchi, M. (2012). Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response. *Development* 139, 3997–4006. doi: 10.1242/dev.084079

Suzuki, A., Akune, M., Kogiso, M., Imagama, Y., Osuki, K. I., Uchiumi, T., et al. (2004). Control of nodule number by the phytohormone abscisic acid in the roots of two leguminous species. *Plant Cell Physiol.* 45, 914–922. doi: 10.1093/pcp/pch107

Tarkowski, Ł. P., Signorelli, S., and Höfte, M. (2020). GABA and related amino acids in plant immune responses: emerging mechanisms of action. *Plant Cell Environ.* doi: 10.1111/pce.13734 [Epub ahead of print].

Tartaglia, C., Gutierrez, P., Sabatini, O., Irisarri, P., Signorelli, S., Rebuffo, M., et al. (2019). "Caracterizacioòn de rizobios que nodulan Trifolium polymorphum," in *Cepas Nativas Para Desarrollar un Inoculante Rizobiano Competitivo y Eficiente en Treìbol Blanco*, ed. Unidad de Comunicación y Transferencia de Tecnología de INIA. (Montevideo: INIA), 33–45.

Tominaga, A., Nagata, M., Futsuki, K., Abe, H., Uchiumi, T., Abe, M., et al. (2009). Enhanced nodulation and nitrogen fixation in the abscisic acid low-sensitive mutant enhanced nitrogen fixation1 of Lotus japonicus. *Plant Physiol.* 151, 1965–1976. doi: 10.1104/pp.109.142638

Tominaga, A., Nagata, M., Futsuki, K., Abe, H., Uchiumi, T., Abe, M., et al. (2010). Effect of abscisic acid on symbiotic nitrogen fixation activity in the root nodules of Lotus japonicus. *Plant Signal. Behav.* 5, 440–443. doi: 10.4161/psb.5.4.10849

Trinchant, J. C., and Rigaud, J. (1982). Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids. *Appl. Environ. Microbiol.* 44, 1385–1388.

Tun, N. N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Segal Floh, E. I., et al. (2006). Polyamines induce rapid biosynthesis of nitric oxide (NO) in Arabidopsis thaliana seedlings. *Plant Cell Physiol.* 47, 346–354. doi: 10.1093/pcp/pci252

Vieweg, M. F., Hohnjec, N., and Küster, H. (2005). Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of Medicago truncatula. *Planta* 220, 757–766.

Vigeolas, H., Hühn, D., and Geigenberger, P. (2011). Nonsymbiotic hemoglobin-2 leads to an elevated energy state and to a combined increase in polyunsaturated fatty acids and total oil content when overexpressed in developing seeds of transgenic arabidopsis plants. *Plant Physiol.* 155, 1435–1444. doi: 10.1104/pp.110.166462

Wang, P., Du, Y., Hou, Y.-J., Zhao, Y., Hsu, C.-C., Yuan, F., et al. (2015a). Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. *Proc. Natl. Acad. Sci. U.S.A.* 112, 613–618. doi: 10.1073/pnas.1423481112

Wang, P., Zhu, J. K., and Lang, Z. (2015b). Nitric oxide suppresses the inhibitory effect of abscisic acid on seed germination by S-nitrosylation of SnRK2 proteins. *Plant Signal. Behav.* 10:e1031939. doi: 10.1080/15592324.2015.1031939

Watts, R. A., Hunt, P. W., Hvitved, A. N., Hargrove, M. S., Peacock, W. J., and Dennis, E. S. (2001). A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proc. Natl. Acad. Sci. U.S.A.* 98, 10119–10124.

Wittenberg, J. B., Bolognesi, M., Wittenberg, B., and Guertin, M. (2002). Truncated hemoglobins: a new family of hemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. *J. Biol. Chem.* 277, 871–874. doi: 10.1074/jbc.R100058200

Zdyb, A., Demchenko, K., Heumann, J., Mrosk, C., Grzeganek, P., Göbel, C., et al. (2011). Jasmonate biosynthesis in legume and actinorhizal nodules. *New Phytol.* 189, 568–579. doi: 10.1111/j.1469-8137.2010.03504.x

Zhu, L., Liu, X., Liu, X., Jeannotte, R., Reese, J. C., Harris, M., et al. (2005). Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of Medicago truncatula. *Planta* 220, 757–766.

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.

The handling editor declared a past co-authorship with several of the authors SS and JM.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fpls.2020.00970

Enhanced Nitric Oxide Synthesis Through Nitrate Supply Improves Drought Tolerance of Sugarcane Plants

*Maria Dolores Pissolato[1], Neidiquele Maria Silveira[2], Paula Joyce Carrenho Prataviera[2], Eduardo Caruso Machado[2], Amedea Barozzi Seabra[3], Milena Trevisan Pelegrino[3], Ladaslav Sodek[1] and Rafael V. Ribeiro[1]**

[1] Laboratory of Crop Physiology, Department of Plant Biology, Institute of Biology, University of Campinas, Campinas, Brazil, [2] Laboratory of Plant Physiology "Coaracy M. Franco", Center for Research & Development in Ecophysiology and Biophysics, Agronomic Institute, Campinas, Brazil, [3] Center for Natural and Human Sciences, Federal University of ABC, Santo André, Brazil

OPEN ACCESS

Edited by:
Juan Carlos Begara-Morales, University of Jaén, Spain

Reviewed by:
Fábio M. DaMatta, Universidade Federal de Viçosa, Brazil
Byung-Wook Yun, Kyungpook National University, South Korea

***Correspondence:**
Rafael V. Ribeiro
rvr@unicamp.br

Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *17 February 2020*
Accepted: *15 June 2020*
Published: *30 June 2020*

Citation:
Pissolato MD, Silveira NM, Prataviera PJC, Machado EC, Seabra AB, Pelegrino MT, Sodek L and Ribeiro RV (2020) Enhanced Nitric Oxide Synthesis Through Nitrate Supply Improves Drought Tolerance of Sugarcane Plants. Front. Plant Sci. 11:970. doi: 10.3389/fpls.2020.00970

Nitric oxide (NO) is an important signaling molecule associated with many biochemical and physiological processes in plants under stressful conditions. Nitrate reductase (NR) not only mediates the reduction of NO_3^- to NO_2^- but also reduces NO_2^- to NO, a relevant pathway for NO production in higher plants. Herein, we hypothesized that sugarcane plants supplied with more NO_3^- as a source of N would produce more NO under water deficit. Such NO would reduce oxidative damage and favor photosynthetic metabolism and growth under water limiting conditions. Sugarcane plants were grown in nutrient solution and received the same amount of nitrogen, with varying nitrate:ammonium ratios (100:0 and 70:30). Plants were then grown under well-watered or water deficit conditions. Under water deficit, plants exhibited higher root [NO_3^-] and [NO_2^-] when supplied with 100% NO_3^-. Accordingly, the same plants also showed higher root NR activity and root NO production. We also found higher photosynthetic rates and stomatal conductance in plants supplied with more NO_3^-, which was associated with increased root growth. ROS accumulation was reduced due to increases in the activity of catalase in leaves and superoxide dismutase and ascorbate peroxidase in roots of plants supplied with 100% NO_3^- and facing water deficit. Such positive responses to water deficit were offset when a NO scavenger was supplied to the plants, thus confirming that increases in leaf gas exchange and plant growth were induced by NO. Concluding, NO_3^- supply is an interesting strategy for alleviating the negative effects of water deficit on sugarcane plants, increasing drought tolerance through enhanced NO production. Our data also provide insights on how plant nutrition could improve crop tolerance against abiotic stresses, such as drought.

Keywords: nitrate reductase, nitrite, photosynthesis, reactive oxygen species, *S*-nitrosylation

INTRODUCTION

Nitric oxide (NO) is a diatomic radical gas and important signaling molecule in animals (Bogdan, 2015), fungi (Cánovas et al., 2016), bacteria (Crane et al., 2010), and plants (Mur et al., 2013). In plants, increasing evidence indicates NO as a key component of the signaling network, controlling numerous physiological and metabolic processes such as seed germination (Albertos et al., 2015), flowering (He et al., 2004), root growth (Fernandez-Marcos et al., 2011), respiration, stomatal conductance (Moreau et al., 2010; Wang et al., 2015), and adaptive responses to biotic and abiotic stresses (Shan et al., 2015; Fatma et al., 2016).

NO synthesis is increased in plants under drought and its role in promoting acclimation responses to cope with water deficit has been suggested (Cai et al., 2015; Silveira et al., 2017a). NO and NO-derived molecules play a critical role in intracellular redox signaling and in the activation of antioxidant defense mechanisms (Shi et al., 2014; Hatamzadeh et al., 2015; Silveira et al., 2015). For example, NO supply conferred drought tolerance to wheat seedlings, reducing membrane damage (García-Mata and Lamattina, 2001). Spraying S-nitrosogluthatione (GSNO)—a NO donor—on sugarcane plants resulted in higher photosynthesis under drought, promoting plant growth under stressful condition (Silveira et al., 2016).

The protective action of exogenous NO donors has been attributed to the elimination of superoxide ($O_2^{\bullet-}$) and enhancement of the antioxidant system in sugarcane plants under drought (Silveira et al., 2017b). In addition, one of the main downstream effects of NO is the post-translational regulation involving thiols (Hancock and Neill, 2019). S-nitrosylation is a redox modification consisting in the reversible attachment of NO to the thiol group of a cysteine residue in a target protein leading to the formation S-nitrosothiols (SNOs) (Astier et al., 2012; Fancy et al., 2016). Then, S-nitrosylation may cause a conformational change in proteins, changing their activity or function. On the other hand, NO can react with reduced glutathione (GSH), producing S-nitrosoglutathione (GSNO)—an endogenous NO reservoir and an efficient NO donor (Jahnová et al., 2019).

While the mechanisms of NO synthesis in animals have been well documented, NO synthesis and its regulation in plants are complex and poorly understood. In animals, NO is bio-synthesized through NO synthase (NOS), which oxidizes *L*-arginine and produces *L*-citrulline and NO (Alderton et al., 2001). Although some evidence indicates the presence of NOS-like activity in many plant species, genes encoding NOS have not yet been identified in higher plants (Santolini et al., 2017; Hancock and Neill, 2019). NO production in plant species and under diverse biological conditions point to the co-existence of multiple pathways, likely functioning in distinct tissues/organs and subcellular compartments (León and Costa-Broseta, 2019).

One of the most important pathways for NO production in land plants is through nitrate reductase (NR) (Gupta et al., 2011; Fancy et al., 2016; Chamizo-Ampudia et al., 2017; León and Costa-Broseta, 2019), a multifunctional enzyme that catalyzes NO_3^- reduction to NO_2^-, which is then reduced to NH_4^+ during the N assimilatory pathway (Heidari et al., 2011). Arasimowicz-Jelonek et al. (2009) reported low NO concentration in cucumber seedlings treated with a NR inhibitor, suggesting its role in NO synthesis. In rice roots, NO production through NR was increased in response to NO_3^- supply (Sun et al., 2015). Furthermore, low NO production by *Physcomitrella patens* occurred when it received a NR inhibitor (Andrés et al., 2015). Although there are data supporting the association between NR activity and NO production in plants (Mur et al., 2013), some authors have argued that NO production through NR represents only a small fraction (1–2%) of total NO_3^- reduction (Yamasaki et al., 1999; Rockel et al., 2002). However, the role of such a NO production pathway and its sensitivity to small changes in NO_3^- supply in plants under water deficit remain unknown.

Nitrogen is the most influential plant nutrient in sugarcane cultivation (Meyer et al., 2007). Nitrate (NO_3^-), ammonium (NH_4^+), and urea ($CO(NH_2)_2$) are the main forms of fertilizers and, thus, are the main sources of N for crops (Esteban et al., 2016). Some crops have a preference for NH_4^+ uptake (Malagoli et al., 2000), but most studies have reported stress symptoms associated with NH_4^+ toxicity (Barreto et al., 2018; Boschiero et al., 2019). While Robinson et al. (2011) reported the sugarcane preference for NH_4^+, Pissolato et al. (2019a) found that increasing NH_4^+ supply causes biomass reduction and photosynthesis impairment of sugarcane plants. Changing the N source, NO_3^- supply has been shown to increase the tolerance to abiotic stresses in maize (Rios-Gonzales et al., 2002) and grass species (Wang and Macko, 2011).

The literature concerning NO_3^- supply and stress tolerance, taken together, led us to hypothesize that the increased plant performance under limiting conditions could be related to NO production through NR activity. Here, our aim was to test the hypothesis that sugarcane plants that receive NO_3^- and no NH_4^+ as sources of nitrogen will have higher NR activity and thereby produce more NO, compared to plants receiving the same amount of nitrogen but as a mixture of NO_3^- (70%) and NH_4^+ (30%). As a consequence of NO production, oxidative damage will be reduced under water deficit, favoring photosynthetic metabolism and plant growth.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pre-sprouted sugarcane plants (*Saccharum* spp.) cv. IACSP95-5000 developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) were used. Six-week-old plants were transferred to plastic boxes (4 L) containing nutrient solution modified from De Armas et al. (1992): 5 mmol L^{-1} N (nitrate 90% + ammonium 10%); 9 mmol L^{-1} Ca; 0.5 mmol L^{-1} Mg; 1.2 mmol L^{-1} P; 1.2 mmol L^{-1} S; 24 μmol L^{-1} B; 16 μmol L^{-1} Fe; 9 μmol L^{-1} Mn; 3.5 μmol L^{-1} Zn; 1 μmol L^{-1} Cu; and 0.1 μmol L^{-1} Mo. Plants received this solution for 2 weeks until the establishment of treatments and the

nutrient solution was renewed every 3 days throughout the experimental period.

Electrical conductivity of nutrient solution was maintained between 1.8 and 2.0 mS cm^{-1} and pH at 5.9 ± 0.1. The pH was adjusted when necessary with 0.5 M citric acid or 0.5 M NaOH. Both variables were monitored on a daily basis using a portable electrical conductivity meter (mCA 150P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil) and a portable pH meter (mPA 210P, MS Tecnopon Instrumentação, Piracicaba SP, Brazil). The nutrient solution volume was also checked daily and completed with water when necessary. The nutrient solution was aerated continuously by using an air compressor (Master Super II, Master, São Paulo SP, Brazil).

The experiment was carried in a growth chamber (Instalafrio, Brazil), with a 12 h photoperiod, air temperature of 30/20°C (day/night), air relative humidity of 80% and photosynthetic photon flux density (PPFD) about 800 µmol m^{-2} s^{-1}.

Experiment I: Inducing NO Production Under Water Deficit Through NO_3^- Supply

Our previous study revealed that sugarcane plants can be supplied with 30% NH_4^+ in nutrient solution without compromising their photosynthesis and growth (Pissolato et al., 2019a). Thus, the NO_3^-:NH_4^+ ratios 100:0 and 70:30 were chosen to represent the treatments with more and less NO_3^-, while supplying the same amount of nitrogen and avoiding NH_4^+ toxicity. Plants were also subjected to varying water availability, according to the osmotic potential of nutrient solution: −0.15 MPa (reference, well-hydrated); and −0.75 MPa (water deficit, WD), totaling four treatments (2 NO_3^-:NH_4^+ ratios × 2 water treatments) with four biological replicates. The water deficit was induced by adding polyethylene glycol (Carbowax™ PEG-8000, Dow Chemical Comp, Midland MI, USA) to the nutrient solution, seven days after imposing NO_3^-:NH_4^+ ratios. To prevent osmotic shock, PEG-8000 was gradually added to the nutrient solution, reducing the osmotic potential of the solution by −0.20 MPa per day, i.e., −0.75 MPa was reached after three days (3^{th} day of the experiment). Plants were allowed to recover from water deficit after returning them to control conditions on the 7^{th} day of the experiment. They remained for four days under such conditions, when the experiment ended. For the biochemical analyzes, there were four biological replicates for both leaves and roots and samples were taken at the maximum water deficit (7^{th} day) and at the end of the rehydration period (11^{th} day). Samples were collected, immediately immersed in liquid nitrogen and then stored at −80°C. Biochemical analyzes were performed in technical triplicates.

Nitrate, Nitrite, and Ammonium

Fresh plant tissue samples (500 mg) were ground in liquid nitrogen and extraction medium containing methanol/chloroform/water (12:5:3 v/v). After centrifugation at 2,000 *g* for 5 min, the supernatants were collected, and chloroform and deionized water were added to them. The mixture was shaken vigorously and then centrifuged for 3 min at 2,000 *g* for phase separation. The upper aqueous phase was collected and maintained in a water bath at 37°C to remove traces of chloroform, and then the extracts were stored at −20°C (Bieleski and Turner, 1966).

For nitrate determination, an aliquot of the extract was pipetted into test tubes containing reaction medium (5% salicylic acid in conc. H_2SO_4). After 20 min, 2 N NaOH was added and the solution stirred. After cooling to room temperature, the absorbance was read in a spectrophotometer at 410 nm and the nitrate content calculated from a standard curve using KNO_3 (100–1000 nmol) (Cataldo et al., 1975). For nitrite, an aliquot of the extract was added to 1% sulfanilamide solution in 3 N HCl and 0.02% N-naphthyl ethylenediamine solution. The tubes were allowed to stand for 30 min in the dark at room temperature. Deionized water was added and nitrite content quantified after reading the absorbance at 540 nm (Hageman and Reed, 1980). For ammonium, the extract was added to microtubes, where solution A (1% phenol and 0.005% sodium nitroprusside) was added and followed by solution B (0.5% sodium hydroxide containing 2.52% sodium hypochlorite). The tubes were incubated for 35 min in a water bath at 37°C, and the absorbance read at 625 nm after cooling to room temperature (McCullough, 1967). A standard curve of $(NH_4)_2SO_4$ was used to estimate the ammonium content. Concentrations were corrected to sample water content and expressed on dry weight basis.

Nitrate Reductase Activity

Leaf and root nitrate reductase (NR, EC 1.7.1.1) activity was estimated as the rate of nitrite (NO_2^-) production (Cambraia et al., 1989). The enzyme extract was obtained from the macerate of 200 mg of fresh tissue with liquid nitrogen and homogenized with extraction medium containing 0.1 M tris-HCl buffer (pH 8.1), 4 mM $NiSO_4$, 20 mM reduced glutathione (GSH), deionized water, and 0.5 mM PMSF. Then, the crude extracts were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was collected and maintained on ice. The extract was added to the assay medium containing 100 mM Tris-HCl buffer (pH 7.5), 10 mM KNO_3, 0.05 mM NADH, and triton 1% X-100 (v/v), mixed and incubated at 30°C for 10 min. The reaction was quenched by adding 1% sulfanilamide solution in 1 M HCl and 0.01% N-naphthyl ethylenediamine. Nitrite production was determined by absorbance at 540 nm using a standard curve with KNO_2. The NR activity was expressed as µmol NO_2^- min^{-1} mg^{-1} protein.

S-Nitrosogluthatione Reductase Activity

Leaf and root S-nitrosogluthatione reductase (GSNOR, EC 1.2.1.1) activity was determined spectrophotometrically at 25°C by monitoring the oxidation of NADH at 340 nm, based on Rodríguez-Ruiz et al. (2017). Briefly, 200 mg of fresh tissue were grounded with liquid nitrogen, resuspended in 20 mM HEPES buffer (pH 8.0), 10 mM EDTA, 0.5 mM PMSF, and centrifuged for 10 min at 10,000 *g* and 4°C. The enzyme extract was added in to the assay medium (20 mM HEPES buffer pH 8.0 and 1.8 mM NADH) at 25°C, and maintained in the dark. The reaction was started by adding 4 mM GSNO (Silveira et al., 2016) and the GSNOR activity followed by NADH oxidation at 340 nm. Activity

was calculated using the NADH extinction coefficient (6.22 mM^{-1} cm^{-1} at 340 nm) and expressed as nmol NADH min^{-1} mg^{-1} protein.

S-Nitrosothiols Content

The total leaf and root proteins were extracted in deionized water and the resulting homogenate was used to estimate the S-nitrosothiol content through an amperometer, as described by Santos et al. (2016) and Zhang et al. (2000). Measurements were performed with the WPI amperometer TBR 4100/1025 (World Precision Instruments Inc., Sarasota FL, USA) and a specific nitric oxide (NO) sensor, ISO-NOP (2 mm). Aliquots of aqueous suspension were added to the sample compartment containing aqueous copper chloride solution (0.1 mol L^{-1}). This condition allowed the detection of free NO released from the S-nitrosothiols present in the leaf and root protein homogenate. The samples were run in triplicate, and the calibration curve was obtained with newly prepared GSNO solutions. The data were compared with the standard curve obtained and normalized against fresh weight. The SNO content was expressed as nmol NO mg^{-1} protein.

Intracellular NO Detection

NO was assayed in leaf and root segments according to Silveira et al. (2019a). For the roots, it was collected approximately 1 cm from the middle part of secondary root. For the leaves, a thin cross section was made with the aid of a scalpel. The segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM $CaCl_2$, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in solution of 10 μM DAF2-DA, mixing for 40 min in the dark at room temperature (Desikan et al., 2002; Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-DA, placed onto a glass slide and covered with a glass slip before observing fluorescence using an inverted confocal microscope set for excitation at 488 nm and emission at 515 nm (Model Zeiss LSM510, Carl Zeiss AG, Germany). Photographs were taken with a 10x magnification, 15 s exposure and 1× gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data were normalized by subtracting the values of the negative control (plants well-hydrated) and presented as mean pixel intensities.

Reactive Oxygen Species

The concentration of the superoxide anion ($O_2^{\bullet-}$) was determined in 50 mg of fresh tissue incubated in an extraction medium consisting of 100 μM EDTA, 20 μM NADH, and 20 mM sodium phosphate buffer, pH 7.8. The reaction was initiated by adding 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28°C under stirring for 5 min and the absorbance was read at 480 nm over a further 5 min (Mohammadi and Karr, 2001). $O_2^{\bullet-}$ production was assessed by the accumulation of adrenochrome using a molar extinction coefficient of 4.0×10^3 M^{-1} cm^{-1} (Boveris, 1984). $O_2^{\bullet-}$ concentration was expressed as μmol $O_2^{\bullet-}$ g^{-1} dry weight.

The quantification of hydrogen peroxide (H_2O_2) was performed following Alexieva et al. (2001). Homogenates were obtained from 100 mg of fresh tissue ground in liquid nitrogen with the addition of polyvinylpolypyrrolidone (PVPP) and 0.1% of trichloroacetic acid (TCA) solution (w/v). The extract was centrifuged at 10,000 *g* and 4°C for 15 min. The reaction medium consisted of 1 mM KI, 0.1 M potassium phosphate buffer (pH 7.5) and crude extract. The microtubes were left on ice in the dark for 1 h. After this period, the absorbance was read at 390 nm. A standard curve was obtained with H_2O_2, and the results were expressed as μmol H_2O_2 g^{-1} dry weight.

Lipid Peroxidation

The concentration of malondialdehyde (MDA) was measured and used as a proxy of lipid peroxidation. 200 mg of fresh tissue were macerated in extraction medium containing 0.1% TCA (w/v) and centrifuged at 10,000 *g* for 15 min. The supernatant was added to 0.5% thiobarbituric acid (w/v) in 20% TCA (w/v), and the mixture incubated at 95°C for 20 min (Cakmak and Horst, 1991). After this time, the reaction was stopped in an ice bath. Then a new centrifugation was performed at 10,000 *g* for 10 min, and after 30 min at room temperature the absorbance was read at 532 and 600 nm, and the non-specific absorbance at 600 nm was discounted. The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968) and results were expressed as μmol MDA g^{-1} dry weight.

Antioxidant Activity and Protein Extraction

The crude enzymatic extracts for the determination of superoxide dismutase activity (SOD), catalase (CAT) and ascorbate peroxidase (APX) were obtained from 100 mg of plant tissue in specific medium, followed by centrifugation at 12,000 *g* for 15 min at 4°C. The specific medium for CAT and SOD consisted of 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM PMSF and 1% PVPP, according to Peixoto et al. (1999). The specific medium for APX was composed of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, and 1 mM EDTA (Nakano and Asada, 1981).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to Giannopolitis and Ries (1977). The crude extract was added to the reaction medium consisting of 100 mM sodium phosphate buffer (pH 7.8), 50 mM methionine, 5 mM EDTA, deionized water, 100 μM riboflavin, and 1 mM nitro blue tetrazolium chloride (NBT). A group of tubes was exposed to light (fluorescent lamp, 30 W) for 10 min, and another group remained in darkness. The absorbance was measured at 560 nm and one unit of SOD defined as the amount of enzyme required to inhibit NBT photoreduction by 50%, and activity expressed as U min^{-1} mg^{-1} of protein.

Catalase (CAT, EC 1.11.1.6) activity was quantified following the procedure described by Havir and McHale (1987). The crude extract was added to the reaction medium consisting of 100 mM potassium phosphate buffer (pH 6.8), deionized water, and 125 mM H_2O_2. The reaction was carried out in a water bath at 25°C for 2 min, and CAT activity was assessed by the decrease in absorbance at 240 nm, using the molar extinction coefficient of 36 M^{-1} cm^{-1} and expressed activity as nmol min^{-1} mg^{-1} of protein.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was evaluated as described by Nakano and Asada (1981). The crude extract was added in reaction medium consisting of 100 mM potassium phosphate buffer (pH 6.8), deionized water, 10 mM ascorbic acid, and 10 mM H_2O_2. The reaction was carried out at 25°C for 2 min and APX activity quantified by the decrease in absorbance at 290 nm, using the molar extinction coefficient of 2.8 M^{-1} cm^{-1} and expressing activity as μmol min^{-1} mg^{-1} of protein.

The protein levels were determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The extract used for this analysis was the same as for SOD and CAT enzymes.

Leaf Gas Exchange

Gas exchange and chlorophyll fluorescence of the first fully expanded leaf with visible ligule were measured throughout the experimental period using an infrared gas analyzer (Li-6400, Li-Cor, Lincoln NE, USA) equipped with a modulated fluorometer (6400-40 LCF, Li-Cor, Lincoln NE, USA). Net CO_2 assimilation rate (A_n), stomatal conductance (g_s) and the effective quantum efficiency of photosystem II (ϕ_{PSII}) were measured under PPFD of 2000 μmol m^{-2} s^{-1} and air CO_2 concentration of 400 μmol mol^{-1}. The measurements were performed between 10:30 and 12:30 h, as carried out previously (Pissolato et al., 2019a). The vapor pressure difference between leaf and air (VPDL) was 2.1 ± 0.2 kPa, and leaf temperature was 30 ± 0.4°C during the evaluations.

Photosynthetic Enzymes

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was quantified in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM bicine-NaOH buffer (pH 7.8), 1 mM ethylenediaminetetraacetic (EDTA), 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μM leupeptin. The resulting solution was centrifuged at 14,000 *g* for 5 min at 4°C. An aliquot of leaf extract was incubated with the reaction medium containing 100 mM bicine-NaOH (pH 8.0) 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 3.5 mM ATP, 5 mM phosphocreatine, 0.25 mM NADH, 80 nkat glyceraldehyde-3-phosphate dehydrogenase, 80 nkat 3-phosphoglyceric phosphokinase, and 80 nkat creatine phosphokinase, for 10 min at 25°C. The oxidation of NADH was initiated by adding 0.5 mM ribulose-1,5-bisphosphate (RuBP) and total Rubisco activity was measured. The reduction of absorbance at 340 nm was monitored for 3 min (Sage et al., 1988; Reid et al., 1997).

The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was also evaluated in approximately 200 mg of leaves, which were macerated and homogenized in 100 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM PMSF, and centrifuged at 14,000 *g* for 25 min at 4°C. The supernatant was collected, and the reaction medium for PEPC activity contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 10 mM $NaHCO_3$, 33 nkat malic dehydrogenase, and 0.3 mM NADH. The reaction was initiated by adding 4 mM phosphoenolpyruvate at 30°C. The oxidation of NADH was monitored a 340 nm for 1 min (Degl'Innocenti et al., 2002).

Proteins were extracted from leaf samples with extraction buffer composed of 100 mM Tris, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, and separated by SDS-PAGE (Laemmli, 1970). The first gel was stained with Comassie Brilliant Blue, and the second was used for Western blot. SDS-PAGE electrophoresis was performed with equal amounts of protein per lane. Soluble proteins were denatured using SDS, and they were electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979). PEPC and Rubisco protein abundances were measured by detection of the PEPC subunit and Rubisco large subunit (RLS) using specific polyclonal antibodies (Agrisera Co, Sweden) according to the manufacturer's instructions.

Chlorophyll Content and Leaf Relative Water Content (RWC)

A chlorophyll meter (CFL 1030, Falker, Porto Alegre RS, Brazil) was used to assess the relative chlorophyll content (Chl). The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs, according to Jamaux et al. (1997): RWC=100×[(FW−DW)/(TW−DW)]. Leaf discs were collected during the morning (about 10:00 h) and measurements were taken at the maximum water deficit (7^{th} day), and four days after returning plants to the control condition (re-watering period, 11^{th} day).

Biometry

Leaf and root dry masses were quantified after drying samples in an oven (60°C) with forced-air circulation until constant weight. Leaf area of each plant was evaluated with a portable leaf area meter (model LI-3000, Li-Cor Inc., Lincoln NE, USA).

Experiment II: Using cPTIO to Offset the Benefits of NO in Plants Under Water Deficit

An additional experiment was performed to verify whether the benefits found in plants supplied with only NO_3^- and subjected to water deficit were in fact caused by NO. We used a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). cPTIO is a stable organic radical developed by Akaike and Maeda (1996), which has been widely used as a control as it oxidizes the NO molecule to form NO_2. In plants supplied with only NO_3^- as N source, the following treatments were evaluated: (a) well-watered condition, with an osmotic potential of the nutrient solution of −0.15 MPa; (b) water deficit, with an osmotic potential of nutrient solution of −0.75 MPa; and (c) same as b with 100 μM cPTIO.

First, plants were moved and roots placed in a moist chamber, where they were sprayed with cPTIO and remained in the dark for 1 hour. After this treatment, the plants were returned to the boxes with the original nutrient solution. This procedure was performed for four consecutive days from the moment the water deficit (−0.75 MPa) was installed. We also evaluated the production of intracellular NO, plant biomass, net CO_2 assimilation rate (A_n) and stomatal conductance (g_s) as described previously.

Experimental Design and Statistical Analyses

The experiment was carried out in a completely randomized design and two causes of variation were analyzed: water availability and nitrogen source. Data were analyzed using Bayesian statistics and we used the JASP software (https://jasp-stats.org/). When significant differences were detected, the mean values (n=4) were compared using Bayes Factor (BF_{10}). Our interpretation of Bayes Factor as evidence for alternative hypothesis (H_1) was based on Raftery (1995): when $1 < BF_{10} < 3$, there is a weak support to H_1; $3 < BF_{10} < 20$ indicates positive support to H_1; and $BF_{10} > 20$ indicates strong support to the alternative hypothesis.

RESULTS

Experiment I: Sugarcane Responses to Water Deficit as Affected by NO_3^- Supply

Nitrate, Nitrite, and Ammonium

We found no differences in leaf [NO_3^-] in plants subjected to water deficit (**Figure 1A**). Root [NO_3^-] was significantly higher in plants supplied with 100% NO_3^- under water deficit when compared to plants supplied with 70% NO_3^- under water deficit (**Figure 1B**). While leaf [NO_2^-] did not vary among treatments (**Figure 1C**), we found the highest root [NO_2^-] in plants supplied with 100% NO_3^- under water deficit (**Figure 1D**). We did not find significant changes in leaf and root [NH_4^+] due to NO_3^- supply under water deficit (**Figures 1E, F**). During the re-watering period, both previously stressed plants and the controls presented similar leaf and root [NO_3^-], [NO_2^-] and [NH_4^+] (**Figure 1**).

Nitrate Reductase, S-Nitrosoglutathione Reductase, and S-Nitrosothiols

Under low water availability, nitrate reductase (NR) activity was higher in plants supplied with 100% NO_3^- than those receiving 70% NO_3^-, regardless the plant organ (**Figures 2A, B**). While we did not notice differences among treatments for leaf NR activity during the re-watering period, root NR activity was higher under water deficit (**Figure 2B**). Under water deficit, plants supplied with 100% NO_3^- showed higher root GSNOR activity than those under 70% NO_3^- (**Figure 2D**). Non-significant differences were found in leaf SNO concentration while varying NO_3^- supply

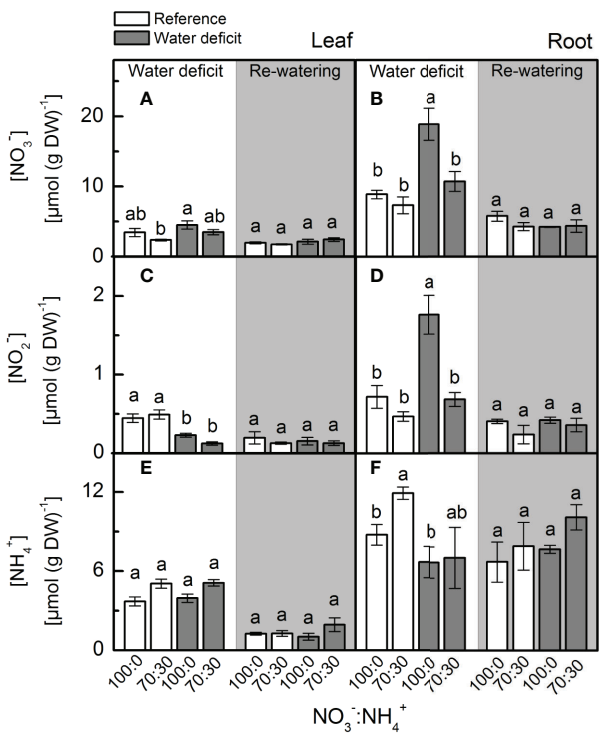

FIGURE 1 | Concentration of nitrate **(A**, **B)**, nitrite **(C**, **D)** and ammonium **(E**, **F)** in leaves **(A**, **C**, **E)** and roots **(B**, **D**, **F)** of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit, and the shaded area indicates the period of re-water. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference ($BF_{10} > 3$) among treatments in a given evaluation.

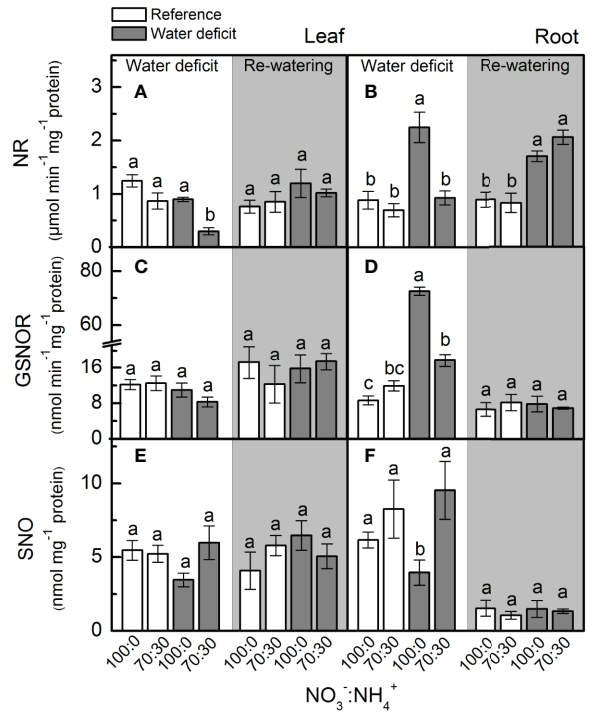

FIGURE 2 | Nitrate reductase activity [NR, in **(A, B)**], S-nitrosoglutathione reductase activity [GSNOR, in **(C, D)**] and S-nitrosothiol concentration [SNO, in **(E, F)**] in leaves **(A**, **C**, **E)** and roots **(B**, **D**, **F)** of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of re-water. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference ($BF_{10} > 3$) among treatments in a given evaluation.

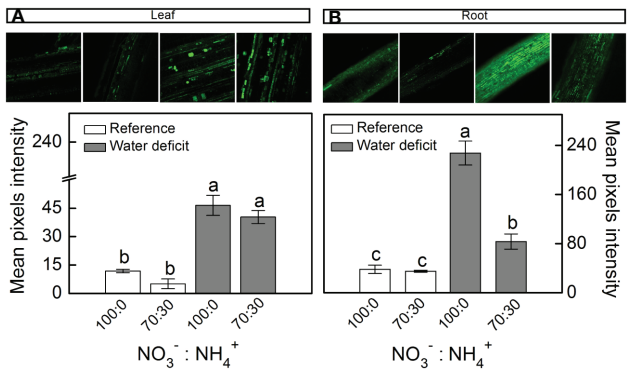

FIGURE 3 | Confocal microscopy images showing intracellular NO synthesis in leaves **(A)** and roots **(B)** of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. Mean pixel intensities are also shown. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference among treatments (BF_{10} > 3 for leaf; BF_{10} > 20 for root).

(**Figure 2E**). However, the lowest root S-nitrosothiols (SNO) concentration was observed in plants supplied with 100% NO_3^- under water deficit (**Figure 2F**).

Intracellular NO Synthesis

When plants were facing low water availability, the intracellular NO was increased in both leaves and roots (**Figure 3**). However, roots receiving 100% NO_3^- exhibited higher NO production than those supplied with 70% NO_3^- (**Figure 3B**). Such a response did not occur in leaves (**Figure 3A**).

Antioxidant Metabolism

Plants supplied with less NO_3^- presented higher leaf [$O_2^{\bullet-}$] when compared to ones supplied with 100% NO_3^- under water deficit (**Figure 4A**). When plants faced water deficit, the highest root [H_2O_2] was found under 70% NO_3^- supply (**Figure 4D**). Although showing higher accumulation of $O_2^{\bullet-}$ and H_2O_2 in leaves and roots, plants supplied with 70% NO_3^- did not show higher MDA content than those under 100% NO_3^- (**Figures 4E, F**).

At the maximum water deficit, we found no differences in leaf superoxide dismutase (SOD) activity due to changes in NO_3^- supply (**Figure 5A**), but the highest SOD activity was observed in roots supplied with 100% NO_3^- (**Figure 5B**). Plants supplied with 100% NO_3^- showed higher root ascorbate peroxidase (APX) and leaf catalase (CAT) activities under water deficit (**Figures 5D, E**), while root catalase (CAT) activity was not changed by NO_3^- supply and water deficit (**Figure 5F**).

Photosynthesis and Relative Water Content

Low water availability caused a large reduction in net CO_2 assimilation rate (A_n), however, plants supplied with more NO_3^- exhibited higher photosynthetic rates than those under NO_3^-:NH_4^+ 70:30 (**Figure 6A**). In addition, those plants showed

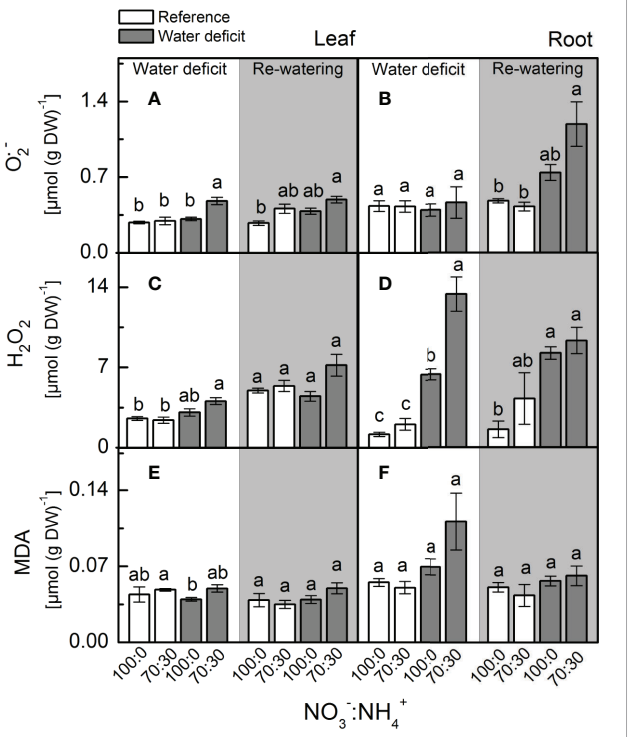

FIGURE 4 | Concentration of superoxide anion [$O_2^{\bullet-}$, in **(A, B)**], hydrogen peroxide (H_2O_2, in **(C, D)**] and malondialdehyde [MDA, in **(E, F)**] in leaves **(A, C, E)** and roots **(B, D, F)** of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of re-water. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference (BF_{10} > 3) among treatments in a given evaluation.

a faster recovery of A_n after re-watering when compared to ones receiving 70% NO_3^- (**Figure 6A**). Similar results were found for stomatal conductance (**Figure 6B**) and effective quantum efficiency of PSII (**Figure 6C**). A significant reduction in leaf relative water content was found under water deficit, as compared to well-watered conditions (**Figure 6D**). We did not observe any significant difference among treatments for the PEPC abundance and activity at maximum water deficit **(Supplementary Figures S1A, C)**. However, both Rubisco abundance and activity were decreased under water deficit, regardless of the variation in NO_3^- supply **(Supplementary Figures S1B, D)** The relative chlorophyll content was also reduced at the maximum water deficit, with no differences induced by NO_3^- supply (data not shown).

Plant Growth

The lowest values for shoot dry mass (**Figure 7A**) and leaf area (**Figure 7C**) were found in plants supplied with less NO_3^- under low water availability. In addition, the root dry mass of plants supplied with 70% NO_3^- was significantly reduced under water deficit (**Figure 7B**).

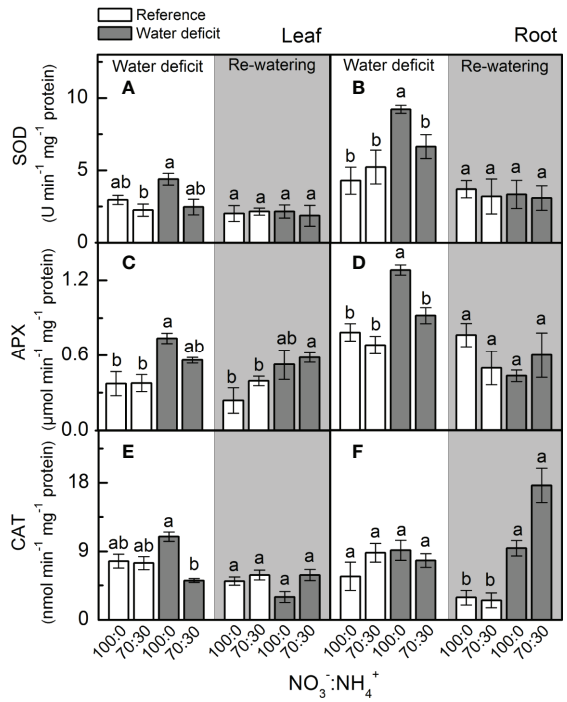

FIGURE 5 | Superoxide dismutase activity [SOD, in **(A, B)**], ascorbate peroxidase activity [APX, in **(C, D)**] and catalase activity [CAT, in **(E, F)**] in leaves **(A**, **C**, **E)** and roots **(B**, **D**, **F)** of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of re-water. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference ($BF_{10} > 3$) among treatments in a given evaluation.

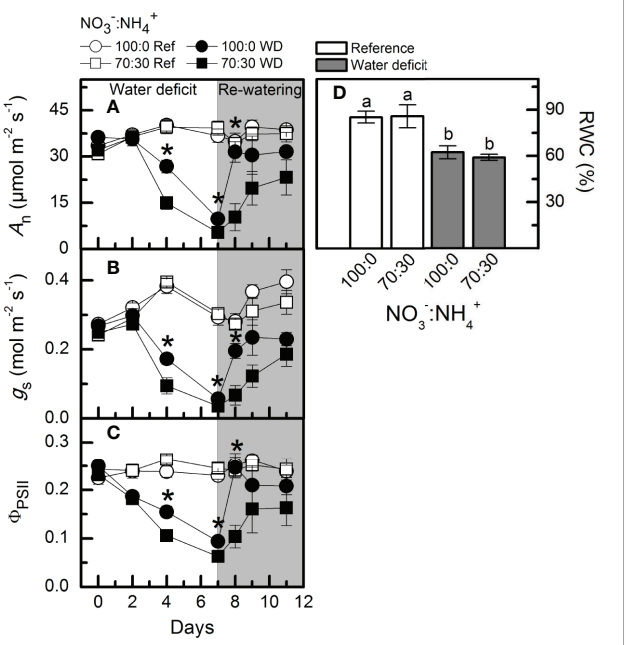

FIGURE 6 | Net CO_2 assimilation rate [A_n, in **(A)**], stomatal conductance [g_S, in **(B)**], effective quantum efficiency of PSII [Φ_{PSII}, in **(C)**] and leaf relative water content [RWC, in **(D)**] in sugarcane plants maintained well-hydrated (ref, white symbols and bars) or subjected to water deficit (WD, black symbols and gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. The white area indicates the period of water deficit and the shaded area indicates the period of re-water. Symbols and bars represent the mean value of four biological replicates ± se. Asterisks indicate significant differences between treatments under water deficit and different letters indicate statistical difference ($BF_{10} > 3$) among treatments in a given evaluation.

Experiment II: Offsetting the Benefits of NO Synthesis Induced by NO_3^- Supply

cPTIO—a NO scavenger—was sprayed on roots supplied with 100% NO_3^- and facing water deficit. As consequence, the intracellular NO synthesis was reduced in leaves and roots (**Figures 8A, B**) and plants showed lower photosynthetic rates and stomatal conductance under water deficit as compared to ones not sprayed with cPTIO (**Figures 9A, B**). As found in experiment I, plants presented decreases in root dry mass due to water deficit when cPTIO was sprayed (**Figure 9D**; **Supplementary Figure S2**).

DISCUSSION

Nitrate Supply Stimulates Root NO Production, Improving Photosynthesis and Antioxidant Metabolism of Sugarcane Under Water Deficit

Our findings revealed that nitrate reductase is an important enzymatic pathway for NO synthesis and also that sugarcane plants supplied with 100% NO_3^- presented enhancement of drought tolerance. Here, we found higher NO_3^- accumulation in roots under water deficit and receiving only NO_3^- as source of nitrogen (**Figure 1B**), which caused higher NO_2^- production when compared to roots exposed to 70% NO_3^- and 30% NH_4^+ (**Figure 1D**). Such findings are supported by higher root nitrate reductase (NR) activity (**Figure 2B**), which reduces NO_3^- to NO_2^- during the N assimilation pathway (Heidari et al., 2011). As an alternative reaction, NR may also reduce NO_2^- to NO (Fancy et al., 2016). In fact, the highest NO accumulation was found in roots under water deficit and supplied with only NO_3^- (**Figure 3B**) and it is known that NO_3^- and NO_2^- play a key role in NO synthesis through NR (Vanin et al., 2004; Yamasaki, 2005; Sun et al., 2015). In *Physcomitrella patens*, low NR activity was associated with drastic reductions in NO synthesis, further evidence that NR is an important pathway for NO production in plants (Andrés et al., 2015). It is worth noting that NO synthesis is low under non-limiting conditions, even in plants supplied with only NO_3^- (**Figure 3**). In general, increases in NO synthesis are expected under stressful conditions, when NO_2^- accumulation occurs (Mur et al., 2012). Not only NR is involved in NO synthesis but also a NO-forming nitrite reductase (NOFNiR) found recently in *Chlamydomonas reinhardtii*. When supplying such algae with NO_3^-, NO was synthetized through NOFNiR either *in vitro* or *in vivo* conditions (Chamizo-Ampudia et al., 2016; Chamizo-Ampudia et al., 2017). As NR

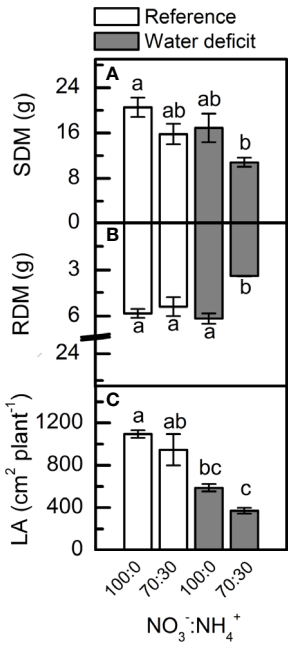

FIGURE 7 | Shoot [SDM, in **(A)**] and root [RDM, in **(B)**] dry mass and leaf area [LA, in **(C)**] of sugarcane plants maintained well-hydrated (reference, white bars) or subjected to water deficit (gray bars) and supplied with varying NO_3^-:NH_4^+ ratios: 100:0 and 70:30. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference among treatments (BF_{10} > 3 for SDM and LA; BF_{10} > 20 for RDM).

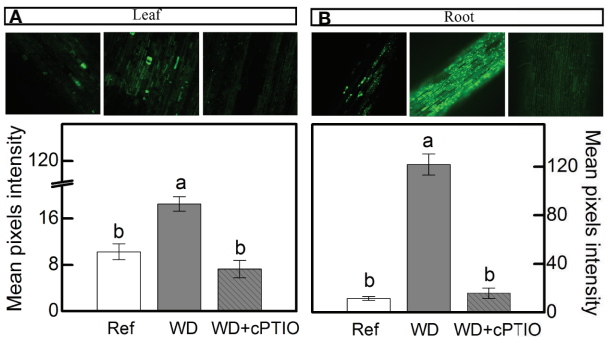

FIGURE 8 | Confocal microscopy images showing intracellular NO synthesis in leaves **(A)** and roots **(B)** of sugarcane plants supplied with only NO_3^- (100:0 NO_3^-:NH_4^+) and maintained well-hydrated (reference, white bars), subjected to water deficit (WD, gray bars) and subjected to water deficit and sprayed with cPTIO (WD+cPTIO, gray striped bars). Mean pixel intensities are also shown. Bars represent the mean value of four biological replicates ± se. Different letters indicate statistical difference among treatments (BF_{10} > 3 for leaf; BF_{10} > 20 for root).

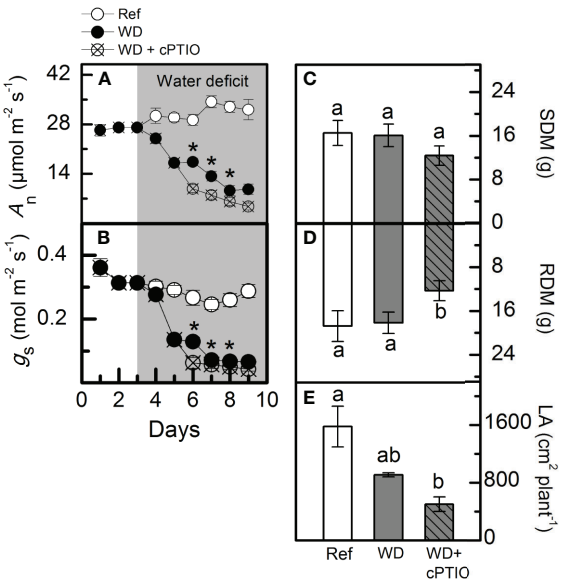

FIGURE 9 | Net CO_2 assimilation rate [A_n, in **(A)**], stomatal conductance [g_S, in **(B)**], shoot [SDM, in **(C)**] and root [RDM, in **(D)**] dry mass and leaf area [LA, in **(E)**] of sugarcane plants supplied with only NO_3^- (100:0 NO_3^-:NH_4^+) and maintained well-hydrated (reference, white symbols and bars), subjected to water deficit (WD, black symbols and gray bars) and subjected to water deficit and sprayed with cPTIO (WD+cPTIO, crossed symbols and gray striped bars). Symbols and bars represent the mean value of four biological replicates ± se. Asterisks indicate significant differences between treatments under water deficit **(A, B)** and different letters indicate statistical difference (BF_{10} > 3) among treatments in a given evaluation.

would have a significant role in NO synthesis under high NO_2^- and low NO_3^- availability (Kolbert et al., 2019), it is reasonable to suggest that NO synthesis could occur through the dual system NR : NOFNiR, a hypothesis to be tested in higher plants.

In the last decades, rapidly increasing evidence has indicated NO as an important player in plant responses to environmental constraining conditions by inducing the antioxidant defenses (Hatamzadeh et al., 2015; Silveira et al., 2017b). During cell detoxification, $O_2^{\bullet-}$ is dismuted to H_2O_2 by superoxide dismutase, which is rapidly eliminated by catalase and ascorbate peroxidase, producing H_2O and O_2 (Lázaro et al., 2013). Here, we observed higher superoxide dismutase activity in roots under water deficit and supplied with 100% NO_3^- (**Figure 5B**), with root [$O_2^{\bullet-}$] remaining similar among treatments (**Figure 4B**). Interestingly, there was lower $O_2^{\bullet-}$ accumulation in leaves under water deficit and supplied with only NO_3^- (**Figure 4A**), even with superoxide dismutase showing similar activity to the one found in plants supplied with 70% NO_3^- and 30% NH_4^+ (**Figure 5A**). As a possible explanation, such low leaf [$O_2^{\bullet-}$] may be related to the interaction of this radical with NO, generating peroxynitrite ($ONOO^-$), which in turn can lead to the formation of NO_2^- and the hydroxyl radical. $ONOO^-$ can also add a nitro group to tyrosine residues—a process known as tyrosine nitration (Wullf et al., 2009; Begara-Morales et al., 2014). Although tyrosine nitration was originally considered as indicative of stress conditions, recent evidence suggests its role in cell signaling (Mengel et al., 2013).

Root [H_2O_2] was lower in plants under water deficit that received 100% NO_3^- as compared to ones supplied with 70% NO_3^- and 30% NH_4^+ (**Figure 4D**), indicating an efficient detoxification through increased root ascorbate peroxidase

activity (**Figure 5D**). In fact, the activation of antioxidant mechanisms to maintain ROS homeostasis often involves NO (Hatamzadeh et al., 2015; Silveira et al., 2015). Many reports show that exogenous NO improves abiotic stress tolerance, causing decreases in [H_2O_2] and lipid peroxidation (Gross et al., 2013). Exogenous NO supply inhibits ROS accumulation in many plant species under stress conditions (Verma et al., 2013), such as cucumber and rice under drought (Farooq et al., 2009). Sugarcane plants supplied with GSNO—a NO donor—showed increases in the activity of antioxidant enzymes, such as superoxide dismutase in leaves and catalase in roots under water deficit (Silveira et al., 2017b). In addition, the S-nitrosylation has a role in mediating the interplay between NO and other reactive signaling mechanisms, such as those involving ROS. For instance, S-nitrosylation of RBOHD causes its inactivation, and thus reduces ROS formation through this pathway (Yu et al., 2012). Such findings revealed that NO has an important role in controlling endogenous ROS levels.

Higher superoxide dismutase and ascorbate peroxidase in roots facing water deficit and receiving only NO_3^- (**Figures 5B, D**) may be a consequence of S-nitrosylation. In pea (*Pisum sativum*), S-nitrosylation increased the activity of cytosolic ascorbate peroxidase (Begara-Morales et al., 2014). However, we noticed higher levels of S-nitrosothiols (SNOs) in roots under water deficit and supplied with NO_3^- and NH_4^+ (**Figure 2F**). At this point, one should consider that NO-mediated post-translational modifications on target proteins may be positive or negative (Nabi et al., 2019). Some of these modifications may alter signaling pathways mediated by other ROS (Holzmeister et al., 2014). According to Clark et al. (2000), S-nitrosylation can inhibit catalase activity, which implies that low level of S-nitrosylation can increase catalase activity during stress conditions, thus increasing ROS detoxification. In this way, higher [SNO] found in plants that received less nitrate (**Figures 2E, F**) is associated with changes in the antioxidant system that lead to increases in leaf [$O_2^{\bullet-}$] and root [H_2O_2] (**Figures 4A, D**). It has been proposed that S-nitrosylation can regulate [H_2O_2] in plants, controlling both the antioxidant defense system and the ROS-producing enzymes (Ortega-Galisteo et al., 2012; Yu et al., 2012).

Here, we found high GSNOR activity in roots under water deficit that received 100% NO_3^- and low accumulation of SNOs (**Figures 2D, F**). GSNOR can break down GSNO—a SNO, reducing GSNO levels and consequently decreasing the total cellular level of S-nitrosylation (Feechan et al., 2005). Thus, it indirectly controls the overall SNOs within cells (Feechan et al., 2005), suggesting that GSNOR may be crucial in regulating the cellular SNO pool. In fact, increases in GSNOR activity contributed to the reduction of S-nitrosylation in pea plants under salt stress (Camejo et al., 2013). As GSNO is an NO donor, we can argue that increases in root GSNOR activity under water deficit and supplied with only NO_3^- (**Figure 2D**) are related to the reduction of GSNO levels and linked to high NO synthesis in roots (**Figure 3B**). High levels of reactive nitrogen species (RNS) may be harmful to plants (Nabi et al., 2019), and the absence of GSNOR activity in plants results in a significant increase in levels of SNOs and impairment of plant immunity (Feechan et al., 2005), plant growth, and development (Kwon et al., 2012). Gong et al. (2015) demonstrated that absence of GSNOR activity increased the sensitivity of *Solanum lycopersicum* to alkaline stress due to the excessive accumulation of NO and SNOs, causing higher levels of endogenous S-nitrosylation and turning stomata insensitive to ABA.

Stomatal closure is the primary response of plants to water deficit, reducing the CO_2 supply for photosynthesis and then decreasing biomass production (Machado et al., 2009; Ribeiro et al., 2013). Although water deficit had reduced the stomatal conductance, higher NO_3^- supply alleviated such negative effects (**Figure 6B**). Due to higher stomatal conductance, sugarcane plants supplied with 100% NO_3^- showed an improvement in photosynthesis under water deficit (**Figure 6A**). By integrating CO_2 assimilation throughout the experimental period, plants supplied with only NO_3^- fixed about 1.5 times more carbon than those supplied with NO_3^- and NH_4^+. Such a response was also related to improvement of primary photochemistry, with plants showing higher conversion of light energy into chemical energy at the PSII level (**Figure 6C**).

Under water deficit, plants supplied with 70% NO_3^- and 30% NH_4^+ presented reduced root biomass as compared to those supplied with 100% NO_3^-, which were not affected by low water availability (**Figure 7B**). Such increase in root growth was associated with higher NO content (**Figure 6B**), as found by Silveira et al. (2016). At maximum water deficit, high NO synthesis was found in the root meristematic zone of plants supplied with 100% NO_3^- (**Supplementary Figure S3**). Several reports indicate that NO is involved in the regulation of root growth and developmental processes (Correa-Aragunde et al., 2004; Lombardo and Lamattina, 2012; Sun et al., 2015). The root system is able to perceive low water availability and to produce chemical signals that regulate the water flow from roots to shoots. NO is one of those chemical signals that stimulates root expansion and development (Silveira et al., 2016; Xu et al., 2017). Given the effects of NO on root growth, it is reasonable to assume a potential influence of NO mediating auxin signaling in roots. Correa-Aragunde et al. (2006) demonstrated that auxin-dependent cell cycle gene regulation was dependent on NO during lateral root formation in tomato plants. NO also modulates the auxin response during adventitious root formation in cucumber plants (Pagnussat et al., 2002) and *Arabidopsis thaliana* (Lombardo et al., 2006).

Overall, increases in NO content can trigger root development and improve water uptake, reducing the impact of low water availability on leaf water status and allowing higher stomatal conductance and photosynthesis, as noticed herein and also by Silveira et al. (2017b; 2019b). The novelty here is that we were able to induce NO synthesis in sugarcane plants by changing the nitrogen source. Such a finding has a practical consequence for sugarcane in the field as endogenous NO synthesis can be stimulated by increasing NO_3^- supply. Apart from economic issues, our data give insights on how stress tolerance can be managed by common practices in agricultural systems and further development on this technique should be carried out with field-grown plants, where interactions among nutrients, soil-root interactions, and soil nitrogen dynamics determine plant performance.

Is Sugarcane Performance Under Water Deficit Really Improved by NO?

Herein, we used 2-(4-carboxyphenyl)-4,4,5,5-tetramethy limidazoline-1-oxyl-3-oxide (cPTIO)—an endogenous NO scavenger (Akaike and Maeda, 1996)—to check if benefits induced by increasing NO_3^- supply were related to NO. cPTIO drastically reduced the DAF-2DA in plants under water deficit, indicating lower NO accumulation in both leaves and roots (**Figures 8A, B**). As consequence, plants showed even lower stomatal conductance and net CO_2 assimilation rate when compared to plants under water deficit and not supplied with cPTIO (**Figures 9A, B**). cPTIO sprays also reduced root growth (**Figure 9D**), as found previously (**Figure 7B**). Taken together, our data clearly show that the improved performance of sugarcane plants supplied with only NO_3^- was due to stimulation of NO synthesis under water deficit.

CONCLUSION

Sugarcane plants grown in nutrient solution containing only NO_3^- as nitrogen source were more tolerant to water deficit, and this response was associated with increased NO production and high nitrate reductase activity in roots. Herein, increasing NO_3^- supply was enough to stimulate NO synthesis and alleviate the effects of water deficit on sugarcane plants by increasing the activity of antioxidant enzymes, photosynthesis, stomatal conductance, and root growth. From a broad perspective, our data show that supplying more NO_3^- during nitrogen fertilization may improve sugarcane tolerance and be beneficial to field-grown sugarcane.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MDP executed the experiments, analyzed the data, and drafted the manuscript. NS assisted in the execution of biochemical analyzes, data analysis, and revised the manuscript. PP assisted in the analysis of antioxidant metabolism, nitrate reductase, and *S*-nitrosoglutathione. AS and MTP performed the analysis of *S*-nitrosothiols and nitrite. EM, LS, and RR edited and revised the manuscript.

FUNDING

This work was supported by the São Paulo Research Foundation (FAPESP, Grants #2017/11279-7, #2017/05029-8, and #2018/08194-2); the Coordination for the Improvement of Higher Education Personnel (CAPES, Grant Code Codes #001; #88882.317451/2019-01); and the National Council for Scientific and Technological Development (CNPq, Grants #302460/2018-7, #404815/2018-9, #313117/2019-5, and #311345/2019-0).

ACKNOWLEDGMENTS

The authors are also very grateful to the National Institute of Science and Technology of Photonics applied to Cell Biology (INFABIC) for guiding our analyses with the confocal microscope. This manuscript has been released as a pre-print at bioRxiv (Pissolato et al., 2019b) and contains part of the first author's Master thesis (Pissolato, 2019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00970/full#supplementary-material

REFERENCES

Akaike, T., and Maeda, H. (1996). Quantitation of nitric oxide using 2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). *Methods Enzymol.* 268, 211–221. doi: 10.1016/s0076-6879(96)68023-9

Albertos, P., Romero-Puertas, M. C., Tatematsu, K., Mateos, I., Sánchez-Vicente, I., Nambara, E., et al. (2015). S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. *Nat. Commun.* 6, 8669. doi: 10.1038/ncomms9669

Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001). Nitric Oxide Synthases: structure, function and inhibition. *Biochem. J.* 357, 593–615. doi: 10.1042/bj3570593

Alexieva, V., Sergiev, I., Mapelli, S., and Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ.* 24, 1337–1344. doi: 10.1046/j.1365-3040.2001.00778.x

Andrés, R. M., Peralta, A. S., Vázquez, J. P. S., Mendivil, S. N., Cabrera, J. A. P., Torres, M. E. S., et al. (2015). The nitric oxide production in the moss P*hyscomitrella patens* is mediated by nitrate reductase. *PloS One* 10, e0119400. doi: 10.1371/journal.pone.0119400

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., and Kubiś, J. (2009). Involvement of nitric oxide in water stress-induced responses of cucumber roots. *Plant Sci.* 177, 682–690. doi: 10.1016/j.plantsci.2009.09.007

Astier, J., Kulik, A., Koen, E., Besson-Bard, A., Bourque, S., Jeandroz, S., et al. (2012). Protein S-nitrosylation: what's going on in plants? *Free Radic. Biol. Med.* 53, 1101–1110. doi: 10.1016/j.freeradbiomed.2012.06.032

Barreto, R. F., Cruz, F. J. R., Gaion, L. A., Prado, R. M., and Carvalho, R. F. (2018). Accompanying ions of ammonium sources and nitrate: ammonium ratios in tomato plants. *J. Plant Nutr. Soil Sci.* 181, 382–387. doi: 10.1002/jpln.201700413

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., Jaramillo-López, J., et al. (2014). Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. *J. Exp. Bot.* 65, 527–538. doi: 10.1093/jxb/ert396

Bieleski, R. L., and Turner, N. A. (1966). Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatography. *Anal. Biochem.* 17, 278–293. doi: 10.1016/0003-2697(66)90206-5

Bogdan, C. (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends Immunol.* 36, 161–178. doi: 10.1016/j.it.2015.01.003

Boschiero, B. N., Mariano, E., Azevedo, R. A., and Trivelin, P. C. (2019). Influence of nitrate-ammonium ratio on the growth, nutrition, and metabolism of sugarcane. *Plant Physiol. Biochem.* 139, 246–255. doi: 10.1016/j.plaphy.2019.03.024

Boveris, A. (1984). Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods Enzymol.* 105, 429–435. doi: 10.1016/S0076-6879(84)05060-6

Bradford, M. N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Bright, J., Hiscoc, S. J., James, P. E., and Hancock, J. T. (2009). Pollen generates nitric oxide and nitrite: a possible link to pollen-induced allergic responses. *Plant Physiol. Biochem.* 47, 49–55. doi: 10.1016/j.plaphy.2008.09.005

Cánovas, D., Marcos, J. F., Marcos, A. T., and Strauss, J. (2016). Nitric oxide in fungi: is there NO light at the end of the tunnel? *Curr. Genet.* 62, 513–518. doi: 10.1007/s00294-016-0574-6

Cai, W., Liu, W., Wang, W. S., Fu, Z. W., Han, T. T., and Lu, Y. T. (2015). Overexpression of rat neurons nitric oxide synthase in rice enhances drought and salt tolerance. *PloS One* 10, e0131599. doi: 10.1371/journal.pone.0131599

Cakmak, I., and Horst, W. J. (1991). Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). *Physiol. Plant* 83, 463–468. doi: 10.1111/j.1399-3054.1991.tb00121.x

Cambraia, J., Pimenta, J. A., Estevao, M. M., and Sant'Anna, R. (1989). Aluminum effects on nitrate uptake and reduction in sorghum. *J. Plant Nutr.* 12, 1435–1445. doi: 10.1080/01904168909364048

Camejo, D., Romero-Puertas, M., Rodríguez-Serrano, M., Sandalio, L. M., Lázaro, J. J., Jiménez, A., et al. (2013). Salinity-induced changes in S-nitrosylation of pea mitochondrial proteins. *J. Proteomics* 79, 87–99. doi: 10.1016/j.jprot.2012.12.003

Cataldo, J. M., Haroom, M., Schrader, L. E., and Youngs, V. L. (1975). Rapid calorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun. Soil Sci. Plan.* 6, 71–80. doi: 10.1080/00103627509366547

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Ocaña-Calahorro, F., Mariscal, V., Carreras, A., et al. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. *Plant Cell Environ.* 10, 2097–2107. doi: 10.1111/pce.12739

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Clark, D., Durner, J., Navarre, D. A., and Klessig, D. F. (2000). Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Mol. Plant Microbe Interact.* 13, 1380–1384. doi: 10.1094/MPMI.2000.13.12.1380

Correa-Aragunde, N., Graziano, M., and Lamattina, L. (2004). Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* 218, 900–905. doi: 10.1007/s00425-003-1172-7

Correa-Aragunde, N., Graziano, M., Chevalier, C., and Lamattina, L. (2006). Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. *J. Exp. Bot.* 57, 581–588. doi: 10.1093/jxb/erj045

Crane, B. R., Sudhamsu, J., and Patel, B. A. (2010). Bacterial nitric oxide synthases. *Annu. Rev. Biochem.* 79, 445–470. doi: 10.1146/annurev-biochem-062608-103436

De Armas, R., Valadier, M. H., Champigny, M. L., and Lamaze, T. (1992). Influence of ammonium and nitrate on the growth and photosynthesis of sugarcane. *J. Plant Physiol.* 140, 531–535. doi: 10.1016/S0176-1617(11)80783-2

Degl'Innocenti, E., Guide, L., and Soldatini, G. F. (2002). Effect of chronic O_3 fumigation on the activity of some Calvin cycle enzymes in two poplar clones. *Photosynthetica* 40, 121–126. doi: 10.1023/A:1020127231198

Desikan, R., Griffiths, R., Hancock, J. T., and Neill, S. (2002). A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acidinduced stomatal closure in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* 99, 16314–16318. doi: 10.1073/pnas.252461999

Esteban, R., Ariz, I., Cruz, C., and Moran, J. F. (2016). Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Sci.* 248, 92–101. doi: 10.1016/j.plantsci.2016.04.008

Fancy, N. N., Bahlmann, A. K., and Loake, G. J. (2016). Nitric oxide function in plant abiotic stress. *Plant Cell Environ.* 40, 462–472. doi: 10.1111/pce.12707

Farooq, M., Basra, S. M. A., Wahid, A., and Rehman, H. (2009). Exogenously applied nitric oxide enhances the drought tolerance in fine grain aromatic rice (*Oryza sativa L.*). *J. Agro. Crop Sci.* 195, 254–261. doi: 10.1111/j.1439-037X.2009.00367.x

Fatma, M., Masood, A., Per, T. S., and Khan, N. A. (2016). Nitric oxide alleviates salt stress inhibited photosynthetic response by interacting with sulfur assimilation in mustard. *Front. Plant Sci.* 7, 521. doi: 10.3389/fpls.2016.00521

Feechan, A., Kwon, E., Yuri, B., Wang, Y., Pallas, J., and Loake, G. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. USA* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Fernandez-Marcos, M., Sanz, L., Lewis, D. R., Muday, G. K., and Lorenzo, O. (2011). Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED1 (PIN1)-dependent acropetal auxin transport. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18506–18511. doi: 10.1073/pnas.1108644108

García-Mata, C., and Lamattina, L. (2001). Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. *Plant Physiol.* 126, 1196–1204. doi: 10.1104/pp.126.3.1196

Giannopolitis, C. N., and Ries, S. K. (1977). Superoxide dismutase: Occurrence in higher plants. *Plant Physiol.* 59, 309–314. doi: 10.1104/pp.59.2.309

Gong, B., Wen, D., Wang, X., Wei, M., Yang, F., Li, Y., et al. (2015). S-nitrosoglutathione reductase modulated redox signaling controls sodic alkaline stress responses in *Solanum lycopersicum L. Plant Cell Physiol.* 1, 790–802. doi: 10.1093/pcp/pcv007

Gross, F., Durner, J., and Gaupels, F. (2013). Nitric oxide, antioxidants and prooxidants in plant defence responses. *Front. Plant Sci.* 4, 419. doi: 10.3389/fpls.2013.00419

Gupta, K. J., Fernie, A. R., Kaiser, W. M., and Dongen, J. T. V. (2011). On the origins of nitric oxide. *Trends Plant Sci.* 16, 160–168. doi: 10.1016/j.tplants.2010.11.007

Hageman, R. H., and Reed, A. J. (1980). Nitrate reductase from higher plants. *Methods Enzymol.* 69, 270–280. doi: 10.1016/S0076-6879(80)69026-0

Hancock, J. T., and Neill, S. J. (2019). Nitric Oxide: Its generation and interactions with other reactive signaling compounds. *Plants* 8, 41. doi: 10.3390/plants8020041

Hatamzadeh, A., Nalousi, A. M., Ghasemnezhad, M., and Biglouei, M. H. (2015). The potential of nitric oxide for reducing oxidative damage induced by drought stress in two turf grass species, creeping bent grass and tall fescue. *Grass Forage Sci.* 70, 538–548. doi: 10.1111/gfs.12135

Havir, E. A., and McHale, N. A. (1987). Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiol.* 84, 450–455. doi: 10.1104/pp.84.2.450

He, Y., Tang, R. H., Hao, Y., Stevens, R. D., Cook, C. W., Ahn, S. M., et al. (2004). Nitric oxide represses the *Arabidopsis* floral transition. *Science* 305, 1968–1971. doi: 10.1126/science.1098837

Heath, R. L., and Packer, L. (1968). Photoperoxidation in isolated chloroplast. I. kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 125, 189–198. doi: 10.1016/0003-9861(68)90654-1

Heidari, B., Matre, P., Nemie-Feyissa, D., Meyer, C., Rognli, O. A., Moller, S. G., et al. (2011). Protein phosphatase 2A B55 and A regulatory subunits interact with nitrate reductase and are essential for nitrate reductase activation. *Plant Physiol.* 156, 165–172. doi: 10.1104/pp.111.172734

Holzmeister, C., Gaupels, F., Geerlof, A., Sarioglu, H., Sattler, M., Durner, J., et al. (2014). Differential inhibition of Arabidopsis superoxide dismutases by peroxynitrite-mediated tyrosine nitration. *J. Exp. Bot.* 66, 989–999. doi: 10.1093/jxb/eru458

Jahnová, J., Luhová, L., and Petřivalský, M. (2019). S-nitrosoglutathione reductase—the master regulator of protein S-nitrosation in plant no signaling. *Plants* 8, 48. doi: 10.3390/plants8020048

Jamaux, I., Steinmetz, A., and Belhassen, E. (1997). Looking for molecular and physiological markers of osmotic adjustment in sunflower. *New Phytol.* 137, 117–127. doi: 10.1046/j.1469-8137.1997.00817.x

Kolbert, Z., Barroso, J. B., Brouquisse, R., Corpas, F. J., Gupta, K. J., Lindermayr, C., et al. (2019). A forty-year journey: The generation and roles of NO in plants. *Nitric. Oxide* 93, 53–70. doi: 10.1016/j.niox.2019.09.006

Kwon, E., Feechan, A., Yun, B. W., Hwang, B. H., Pallas, J. A., Kang, J. G., et al. (2012). AtGSNOR1 function is required for multiple developmental programs in Arabidopsis. *Planta* 236, 887–900. doi: 10.1007/s00425-012-1697-8

Lázaro, J. J., Jiménez, A., Camejo, D., Martí, M. C., Lázaro-Payo, A., Barranco-Medina, S., et al. (2013). Dissecting the integrative antioxidant and redox systems in plant mitochondria. Effect of stress and S-nitrosylation. *Front. Plant Sci.* 4, 460. doi: 10.3389/fpls.2013.00460

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

León, J., and Costa-Broseta, Á. (2019). Present knowledge and controversies, deficiencies, and misconceptions on nitric oxide synthesis, sensing, and signaling in plants. *Plant Cell Environ.* 43, 1–15. doi: 10.1111/pce.13617

Lombardo, M. C., and Lamattina, L. (2012). Nitric oxide is essential for vesicle formation and trafficking in *Arabidopsis* root hair growth. *J. Exp. Bot.* 63, 4875–4885. doi: 10.1093/jxb/ers166

Lombardo, M. C., Graziano, M., Polacco, J. C., and Lamattina, L. (2006). Nitric oxide functions as a positive regulator of root hair development. *Plant Signal. Behav.* 1, 28–33. doi: 10.4161/psb.1.1.2398

Machado, R. S., Ribeiro, R. V., Marchiori, P. E. R., Machado, D. F. S. P., Machado, E. C., and Landell, M. G. D. A. (2009). Biometric and physiological responses to water deficit in sugarcane at different phenological stages. *Pesq. Agropec. Bras.* 44, 1575–1582. doi: 10.1590/S0100-204X2009001200003

Malagoli, M., Canal, A. D., Quaggiotti, S., Pegoraro, P., and Bottacin, A. (2000). Differences in nitrate and ammonium uptake between Scots pine and European larch. *Plant Soil* 221, 1–3. doi: 10.1023/A:1004720002898

McCullough, H. (1967). The determination of ammonia in whole blood by a direct colorimetric method. *Clin. Chim. Acta* 17, 297–304. doi: 10.1016/0009-8981(67)90133-7

Mengel, A., Chaki, M., Shekariesfahlan, A., and Lidermayr, C. (2013). Effect of nitric oxide on gene transcription—S-nitrosylation of nuclear proteins. *Front. Plant Sci.* 4, 293. doi: 10.3389/fpls.2013.00293

Meyer, J. H., Schumann, A. W., Wood, R. A., Nixon, D. J., and Berg, M. V. D. (2007). Recent advances to improve nitrogen use efficiency of sugarcane in the South African sugar industry. *Proc. Int. Soc Sugar Cane Technol.* 26, 238–246.

Mohammadi, M., and Karr, A. L. (2001). Superoxide anion generation in effective and ineffective soybean root nodules. *J. Plant Physiol.* 158, 1023–1029. doi: 10.1078/S0176-1617(04)70126-1

Moreau, M., Lindermayr, C., Durner, J., and Klessig, D. (2010). NO synthesis and signaling in plants—where do we stand? *Physiol. Plant* 138, 372–383. doi: 10.1111/j.1399-3054.2009.01308.x

Mur, L. A., Sivakumaran, A., Mandon, J., Cristescu, S. M., Harren, F. J., and Hebelstrup, K. H. (2012). Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. *J. Exp. Bot.* 63, 4375–4387. doi: 10.1093/jxb/ers116

Mur, L. A., Mandon, J., Persijn, S., Cristescu, S. M., Moshkov, I. E., Novikova, G. V., et al. (2013). Nitric oxide in plants: an assessment of the current state of knowledge. *AoB Plants* 5, pls052. doi: 10.1093/aobpla/pls052

Nabi, R. B. S., Tayade, R., Hussain, A., Kulkarni, K. P., Imran, Q. M., Mun, B. G., et al. (2019). Nitric oxide regulates plant responses to drought, salinity, and heavy metal stress. *Environ. Exp. Bot.* 161, 120–133. doi: 10.1016/j.envexpbot.2019.02.003

Nakano, Y., and Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidases in spinach chloroplasts. *Plant Cell Physiol.* 22, 867–880. doi: 10.1093/oxfordjournals.pcp.a076232

Ortega-Galisteo, A. P., Serrano, M. R., Pazmiño, D. M., Gupta, D. K., Sandalio, L. M., and Puertas, M. C. R. (2012). S-Nitrosylated protein sinpea (*Pisum sativum* L.) leaf peroxisomes: changes under abiotic stress. *J. Exp. Bot.* 63, 2089–2103. doi: 10.1093/jxb/err414

Pagnussat, G. C., Simontacchi, M., Puntarulo, S., and Lamattina, L. (2002). Nitric oxide is required for root organogenesis. *Plant Physiol.* 129, 954–956. doi: 10.1104/pp.004036

Peixoto, P. H. P., Cambraia, J., Sant'ana, R., Mosquim, P. R., and Moreira, M. A. (1999). Aluminum effects on lipid peroxidation and on activities of enzymes of oxidative metabolism in sorghum. *Braz. J. Plant Physiol.* 11, 137–143.

Pissolato, M. D., Silveira, N. M., Machado, E. C., Zambrosi, F. C. B., Sodek, L., and Ribeiro, R. V. (2019a). Photosynthesis and biomass accumulation in young sugarcane plants grown under increasing ammonium supply in nutrient solution. *Theor. Exp. Plant Physiol.* 31, 401–411. doi: 10.1007/s40626-019-00154-w

Pissolato, M. D., Silveira, N. M., Prataviera, P. J., Machado, E. C., Seabra, A. B., Pelegrino, M. T., et al. (2019b). Enhanced nitric oxide synthesis through nitrate supply improves drought tolerance of sugarcane plants. *bioRxiv*. doi: 10.1101/860544

Pissolato, M. D. (2019). *Alleviating the effects of water deficit on sugarcane through nitrogen nutrition* (Master's thesis. Campinas (SP): University of Campinas).

Raftery, A. E. (1995). "Bayesian model selection in social research," in *Sociological Methodology*. Ed. P. V. Marsden (Cambridge: Blackwell), 111–196.

Reid, C. D., Tissue, D. T., Fiscus, E. L., and Strain, B. R. (1997). Comparison of spectrophotometric and radio isotopic methods for the assay of Rubisco in ozone-treated plants. *Physiol. Plant* 101, 398–404. doi: 10.1111/j.1399-3054.1997.tb01014.x

Ribeiro, R. V., Machado, R. S., Machado, E. C., Machado, D. F. S. P., Magalhães Filho, J. R., and Landell, M. G. A. (2013). Revealing drought-resistance and productive patterns in sugarcane genotypes by evaluating both physiological responses and stalk yield. *Exp. Agric.* 49, 212–224. doi: 10.1017/S0014479712001263

Rios-Gonzalez, K., Erdei, L., and Lips, S. H. (2002). The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. *Plant Sci.* 162, 923–930. doi: 10.1016/S0168-9452(02)00040-7

Robinson, N., Brackin, R., Vinall, K., Soper, F., Holst, J., Gamage, H., et al. (2011). Nitrate paradigm does not hold up for sugarcane. *PloS One* 6, e19045. doi: 10.1371/journal.pone.0019045

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W. M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. *J. Exp. Bot.* 53, 103–110. doi: 10.1093/jexbot/53.366.103

Rodríguez-Ruiz, M., Mioto, P., Palma, J. M., and Corpas, F. J. (2017). S-nitrosoglutathione reductase (GSNOR) activity is down-regulated during pepper (*Capsicum annuum* L.) fruit ripening. *Nitric. Oxide* 68, 51–55. doi: 10.1016/j.niox.2016.12.011

Sage, R. F., Sharkey, T. D., and Seemann, J. R. (1988). The in vivo response of the ribulose-1,5-bisphosphate carboxylase activation state and the pool sizes of photosynthetic metabolites to elevated CO_2 in *Phaseolus vulgaris* L. *Planta* 174, 407–416. doi: 10.1007/BF00959528

Santolini, J., André, F., Jeandroz, S., and Wendehenne, D. (2017). Nitric oxide synthase in plants: Where do we stand? *Nitric. Oxide* 63, 30–38. doi: 10.1016/j.niox.2016.09.005

Santos, M. C., Seabra, A. B., Pelegrino, M. T., and Haddad, P. S. (2016). Synthesis, characterization and cytotoxicity of glutathione-and PEG-glutathione-superparamagnetic iron oxide nanoparticles for nitric oxide delivery. *Appl. Surf. Sci.* 367, 26–35. doi: 10.1016/j.apsusc.2016.01.039

Shan, C., Zhou, Y., and Liu, M. (2015). Nitric oxide participates in the regulation of the ascorbate-glutathione cycle by exogenous jasmonic acid in the leaves of wheat seedlings under drought stress. *Protoplasma* 252, 1397–1405. doi: 10.1007/s00709-015-0756-y

Shi, H., Ye, T., Zhu, J. K., and Chan, Z. (2014). Constitutive production of nitric oxide leads to enhanced drought stress resistance and extensive transcriptional reprogramming in *Arabidopsis*. *J. Exp. Bot.* 65, 4119–4131. doi: 10.1093/jxb/eru184

Silveira, N. M., Oliveira, J. A. D., Ribeiro, C., Canatto, R. A., Siman, L., Cambraia, J., et al. (2015). Nitric oxide attenuates oxidative stress induced by arsenic in lettuce (*Lactuca sativa*) leaves. *Water Air Soil Poll* 226, 379. doi: 10.1007/s11270-015-2630-0

Silveira, N. M., Frungillo, L., Marcos, F. C., Pelegrino, M. T., Miranda, M. T., Seabra, A. B., et al. (2016). Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit. *Planta* 244, 181–190. doi: 10.1007/s00425-016-2501-y

Silveira, N. M., Hancock, J. T., Frungillo, L., Siasou, E., Marcos, F. C., Salgado, I., et al. (2017a). Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane. *Plant Physiol. Biochem.* 115, 354–359. doi: 10.1016/j.plaphy.2017.04.011

Silveira, N. M., Marcos, F. C., Frungillo, L., Moura, B. B., Seabra, A. B., Salgado, I., et al. (2017b). S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit. *Physiol. Plant* 160, 383–395. doi: 10.1111/ppl.12575

Silveira, N. M., Machado, E. C., and Ribeiro, R. V. (2019a). "Extracellular and intracellular NO detection in plants by diaminofluoresceins," in *Redox-mediated signal transduction: methods and protocols, methods in molecular biology*. Eds. J. T. Hancock and M. Conway (New York: Springer), 103–108.

Silveira, N. M., Seabra, A. B., Marcos, F. C., Pelegrino, M. T., Machado, E. C., and Ribeiro, R. V. (2019b). Encapsulation of S-nitrosoglutathione into chitosan nanoparticles improves drought tolerance of sugarcane plants. *Nitric. Oxide* 84, 38–44. doi: 10.1016/j.niox.2019.01.004

Sun, H., Li, J., Song, W., Tao, J., Huang, S., Chen, S., et al. (2015). Nitric oxide generated by nitrate reductase increases nitrogen uptake capacity by inducing lateral root formation and inorganic nitrogen uptake under partial nitrate nutrition in rice. *J. Exp. Bot.* 66, 2449–2459. doi: 10.1093/jxb/erv030

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350–4356. doi: 10.1073/pnas.76.9.4350

Vanin, A. F., Svistunenko, D. A., Mikoyan, V. D., Serezhenkov, V. A., Fryer, M. J., Baker, N. R., et al. (2004). Endogenous superoxide production and the nitrite/nitrate ratio control the concentration of bioavailable free nitric oxide in leaves. *J. Biol. Chem.* 279, 24100–24107. doi: 10.1074/jbc.M312601200

Verma, K., Mehta, S. K., and Shekhawat, G. S. (2013). Nitric oxide (NO) counteracts cadmium induced cytotoxic processes mediated by reactive oxygen species (ROS) in *Brassica juncea*: cross-talk between ROS, NO and antioxidant responses. *Biometals* 26, 255–269. doi: 10.1007/s10534-013-9608-4

Wang, L., and Macko, S. A. (2011). Constrained preferences in nitrogen uptake across plant species and environments. *Plant Cell Environ.* 34, 525–534. doi: 10.1111/j.1365-3040.2010.02260.x

Wang, P., Du, Y., Hou, Y. J., Zhao, Y., Hsu, C. C., Yuan, F., et al. (2015). Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. *Proc. Natl. Acad. Sci. USA* 112, 613–618. doi: 10.1073/pnas.1423481112

Wullf, A., Oliveira, H. C., Saviani, E. E., and Salgado, I. (2009). Nitrite reduction and superoxide-dependent nitric oxide degradation by *Arabidopsis* mitochondria: influence of external NAD(P)H dehydrogenases and alternative oxidase in the control of nitric oxide levels. *Nitric. Oxide* 21, 132–139. doi: 10.1016/j.niox.2009.06.003

Xu, X. T., Jin, X., Liao, W. B., Dawuda, M. M., Li, X. P., Wang, M., et al. (2017). Nitric oxide is involved in ethylene-induced adventitious root development in cucumber (*Cucumis sativus* L.) explants. *Sci. Hortic.* 215, 65–71. doi: 10.1016/j.scienta.2016.12.006

Yamasaki, H., Sakihama, Y., and Takahashi, S. (1999). An alternative pathway for nitric oxide production in plants: new features of an old enzyme. *Trends Plant Sci.* 4, 128–129. doi: 10.1016/S1360-1385(99)01393-X

Yamasaki, H. (2005). The NO world for plants: achieving balance in an open system. *Plant Cell Environ.* 28, 78–84. doi: 10.1111/j.1365-3040.2005.01297.x

Yu, M., Yun, B. W., Spoel, S. H., and Loake, G. J. (2012). A sleigh ride through the SNO: Regulation of plant immune function by protein S-nitrosylation. *Curr. Opin. Plant Biol.* 15, 424–430. doi: 10.1016/j.pbi.2012.03.005

Zhang, X., Cardosa, L., Broderick, M., Fein, H., and Davies, I. R. (2000). Novel calibration method for nitric oxide microsensors by stoichiometrical generation of nitric oxide from SNAP. *Electroanalysis* 12, 425–428. doi: 10.1002/(SICI)1521-4109(20000401)12:6<425::AID-ELAN425>3.0.CO;2-6

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fpls.2020.00962

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

*Capilla Mata-Pérez[1], María N. Padilla[1], Beatriz Sánchez-Calvo[1], Juan C. Begara-Morales[1], Raquel Valderrama[1], Mounira Chaki[1], Lorena Aranda-Caño[1], David Moreno-González[2], Antonio Molina-Díaz[2] and Juan B. Barroso[1]**

[1] Group of Biochemistry and Cell Signalling in Nitric Oxide, Department of Experimental Biology, Center for Advanced Studies in Olive Grove and Olive Oils, Faculty of Experimental Sciences, University of Jaén, Jaén, Spain, [2] Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, Jaén, Spain

Nitro-fatty acids (NO_2-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 NO_2-FAs trigger an antioxidant and a defence response against stressful situations. Among the properties of NO_2-FAs highlight the ability to release NO therefore modulating specific protein targets through post-translational modifications (NO-PTMs). Thus, based on the capacity of NO_2-FAs to act as physiological NO donors and using high-accuracy mass-spectrometric approaches, herein, we show that endogenous nitro-linolenic acid (NO_2-Ln) can modulate *S*-nitrosoglutathione (GSNO) biosynthesis in *Arabidopsis*. The incubation of NO_2-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 ^{15}N-labeled NO_2-Ln throughout the roots, and ^{15}N-labeled GSNO (GS^{15}NO) was detected in the leaves. With the aim to go in depth in the relation of NO_2-FA and GSNO in plants, *Arabidopsis* alkenal reductase mutants (*aer* mutants) which modulate NO_2-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 NO_2-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

OPEN ACCESS

Edited by:
Vasileios Fotopoulos, Cyprus University of Technology, Cyprus

Reviewed by:
Marcela Simontacchi, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Caroline Gaucher, Université de Lorraine, France

***Correspondence:**
Juan B. Barroso
jbarroso@ujaen.es

Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *18 April 2020*
Accepted: *11 June 2020*
Published: *30 June 2020*

Citation:
Mata-Pérez C, Padilla MN, Sánchez-Calvo B, Begara-Morales JC, Valderrama R, Chaki M, Aranda-Caño L, Moreno-González D, Molina-Díaz A and Barroso JB (2020) Endogenous Biosynthesis of S-Nitrosoglutathione From Nitro-Fatty Acids in Plants. Front. Plant Sci. 11:962. doi: 10.3389/fpls.2020.00962

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).

NO and NO-related molecules such as nitrogen dioxide (NO_2) or peroxynitrite ($ONOO^-$) can interact with biomolecules, resulting in several changes such as the nitration of fatty acids, proteins, and nucleic acids or the *S*-nitrosation of proteins. Among these modifications, protein tyrosine nitration and *S*-nitrosation have been widely studied in animal and plant systems (Bartesaghi et al., 2007; Abello et al., 2009; Foster et al., 2009; Astier et al., 2012; Radi, 2012). Nevertheless, in the last few years a growing body of studies have highlighted the relevance of fatty acid nitration in living systems (Schopfer et al., 2011; Mata-Pérez et al., 2017). In this regard, nitro-fatty acids (NO_2-FAs) result from the interaction of NO and NO-derived species with unsaturated fatty acids (Freeman et al., 2008). These molecules possess important biological properties, including the ability to release NO (Schopfer et al., 2005; Gorczynski et al., 2007; Mata-Pérez et al., 2016a) and the capacity to modify protein targets by a process specifically called nitroalkylation (Villacorta et al., 2007; Ichikawa et al., 2008; Bonacci et al., 2011; Kansanen et al., 2011; Geisler and Rudolph, 2012). However, the specific mechanism by which NO_2-FAs are able to release NO in aqueous solutions remains unknown to date although different ways 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 (Schopfer et al., 2005; Baker et al., 2009). Furthermore, a rearrangement in the structure of NO_2-FAs with the putative release of this gaseous molecule has also been noted (Lima et al., 2005; Gorczynski et al., 2007). On the other hand, NO_2-FAs are also called nitroalkenes given the ability of the adjacent carbon to the nitro (NO_2) group to act as a potent electrophile (Geisler and Rudolph, 2012). This carbon confers to NO_2-FAs the capacity to be a potential target of nucleophilic molecules such as the thiol groups of proteins with the subsequent modulation of conformation, location, and activity of these protein targets. Through these modifications, NO_2-FAs such as nitro-oleic (NO_2-OA) or nitro-linoleic acids (NO_2-LA) are able to promote vasodilator, antioxidant and anti-inflammatory effects in animal systems (Faine et al., 2010; Ambrozova et al., 2016; Mata-Pérez et al., 2016b; Kansanen et al., 2017; Turell et al., 2017; Verescakova et al., 2017). Recently, the endogenous occurrence of NO_2-Ln has been also described in several plant species such as *Arabidopsis thaliana*, *Pisum sativum*, and *Oryza sativa* (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2017). Moreover, this NO_2-FA is capable of launching a defence response through the induction of different heat-shock proteins (HSPs) and several antioxidant enzymes (Mata-Pérez et al., 2015; Mata-Pérez et al., 2016b) and hence the outstanding relevance of these signaling molecules both in animal and plant systems. In this transcriptomic analysis, the 2-alkenal reductase (AtAER, AT5G16970) was identified to be up-regulated by NO_2-Ln. This AtAER belongs to a NADPH-dependent reductases family that are involved in the detoxification of reactive carbonyls in plants (Mano et al., 2005; Yamauchi et al., 2011). In addition, AtAER appears to be phylogenetically related to animal's prostaglandin reductase-1 (PGR-1) that is also an alkenal one/reductase (AOR) with the capacity to reduce the double bond of α,β unsaturated 2-alkenals (Yamauchi et al., 2011; Vitturi et al., 2013; Mesa et al., 2015). Interestingly, this PGR-1 has also been described as a nitroalkene reductase enzyme that is able to reduce the double bond from α,β-unsaturated alkenes and therefore catalyze the conversion of the electrophilic nitroalkenes to the non-electrophilic nitroalkane (Vitturi et al., 2013). Consequently, PGR-1 regulates cellular levels of NO_2-FAs and mediates nitroalkene-related signaling pathways. In this line, AtAER, as the plant homologous of PGR-1, also regulates the cellular level of NO_2-FAs in plants.

Most NO signaling functions are transmitted by their ability to modify the cysteine residues of the target proteins. The resulting *S*-nitrosothiols (SNOs) can alter the function, location, conformation, and activities of proteins in numerous eukaryotic signaling pathways. The role of these molecules have been related to numerous processes including plant immune signaling (Feechan et al., 2005; Chaki et al., 2009; Spoel and Loake, 2011) or the implication in different adverse environmental conditions such as high light intensity, darkness, or salinity (Valderrama et al., 2007; Corpas et al., 2008; Corpas et al., 2016; Begara-Morales et al., 2018; Begara-Morales et al., 2019). For instance, a rise in the levels of SNO has been associated with greater susceptibility to pathogen infection (Feechan et al., 2005; Kneeshaw et al., 2014) and it has also been proposed as a new wound signal in sunflower seedlings subjected to mechanical wounding (Chaki et al., 2011a).

Among different SNOs, highlight *S*-nitrosoglutathione (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 (Espunya et al., 2012) 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 (Barroso et al., 2006; Chaki et al., 2011a; Chaki et al., 2011b). In this sense, it bears mentioning that phloem has the notable ability to propagate messengers such as different reactive oxygen and nitrogen species (ROS and RNS) during plant defence (Gaupels et al., 2017) and that GSNO is involved as a key molecule in the systemic response to wounding stress (Espunya et al., 2012). Thus, GSNO is currently considered a NO carrier throughout the plant, thereby giving NO the capacity of a long-distance signaling molecule. On the other hand, the levels of this low-molecular-weight SNO are controlled by the enzyme GSNO reductase (GSNOR1), decomposing it to oxidized glutathione (GSSG) and hydroxylamine (Frungillo et al., 2014). In this scenario, knockout lines of this enzyme resulted in higher levels of GSNO and *S*-nitrosated proteins (the addition of a NO moiety to a thiol group into a specific subset of cysteine residues in proteins), indicating that GSNOR1 indirectly governs the level of protein *S*-nitrosation in plants (Feechan et al., 2005).

Based on the capability of NO_2-FAs to release NO (Schopfer et al., 2005; Gorczynski et al., 2007; Mata-Pérez et al., 2016a) and the involvement of these molecules in key aspects of plant physiology, here we show that NO_2-Ln is able to move through the plant and also has the capacity to modulate the levels of GSNO both *in vitro* and *in vivo*. Thus, data presented in

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 ecotype Columbia and *aer* mutant (SALK 005324C) plants were used in this study. The homocygosis 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) bleach and 0.1% (w/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 100–120 μE m^{-2} s^{-1}).

For treatments, $^{15}NO_2$-Ln was firstly synthesized and quantified as previously described (Mata-Pérez et al., 2016b) for the synthesis of NO_2-Ln but using $^{15}NaNO_2$ (Sigma-Aldrich, 490814) as a nitrating agent. Because NO_2-Ln is not commercially available, it was synthesized by a nitroselenation-oxidation-hydroselenoxide 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 (1.4mmol), phenylselenyl bromide (1.1 mmol) and $^{15}NaNO_2$ (1.1 mmol) in a mixture of tetrahydrofuran-acentonitrile (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 keep 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

$GS^{14}NO$ and ^{15}N-labeled GSNO ($GS^{15}NO$) standards were prepared according to Hart (1985) by acid-catalyzed nitrosation of GSH (Sigma-Aldrich, G4251). Sodium nitrite ($^{14/15}N$-labeled) (Sigma-Aldrich) was used to synthesize $GS^{14}NO$/$GS^{15}NO$, respectively. These compounds were quantified by measuring the absorbance at 334 nm ($\epsilon = 0.92\ mM^{-1}{\cdot}cm^{-1}$).

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 (diethylenetetraminepentaacetic acid) for 1 h at RT[a] 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 $^{15}NO_2$-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 $^{15}NO_2$-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 $^{15}NO_2$-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 neucoproine (1/2, FW/V). Homogenates were centrifuged at 16,000*x*g for 10 min, 4°C and filtered using a standard 0.22-µm filter. Then, samples were ultra-filtered by centrifugation at 8,000*x*g, 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 $GS^{15}NO$. Next, GSNO or $GS^{15}NO$ 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 $GS^{15}NO$ 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-phenantroline, pepstatin A, leupeptine, bestatine, and E-64 from Sigma-Aldrich; 1/2, FW/v). Then, the crude extracts were centrifuged twice at 3,000*x*g 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, Amersham 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-Prep 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 NO_2-Ln

Based on the demonstrated ability of NO_2-FAs acting as NO donors (Schopfer et al., 2005; Gorczynski et al., 2007; Mata-Pérez et al., 2016a), the *in vitro* generation of GSNO from the reaction between GSH and NO_2-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 ($[GS+H]^{\cdot+}$) and the neutral radical $^{\cdot}NO$ (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 NO_2-Ln (0.1 and 1 mM) were incubated with 1 mM GSH and studied by LC-ES/MS3 **(Figure 1)**. Under these conditions, a chromatographic peak with MRM transition of 160 *m/z* was detected in both NO_2-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 NO_2-Ln and GSH **(Figures 1A, B)**. Additionally, product ions of the GSNO formed after reaction between NO_2-Ln and GSH showed the same fragmentation pattern as synthetic GSNO **(Figure S2)**, and thereby confirmed the formation of GSNO.

Mobilization of NO_2-Ln Throughout *Arabidopsis* Plants

The capacity of NO_2-FAs to move through the plant was analyzed. For this, ^{15}N-labeled NO_2-Ln was synthesized in order to show the presence of this NO_2-FA in the leaves and distinguish it from the endogenous NO_2-Ln. Thus, 1 mM $^{15}NO_2$-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 $^{15}NO_2$-Ln standard **(Figure 2A)** and thus highlighting the mobilization of NO_2-FAs from the roots to the leaves of *Arabidopsis* plants.

Characterization of GSNO Synthesis From NO_2-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 $GS^{15}NO$ was assessed by LC-MS/MS in *Arabidopsis* leaves after the plant roots were incubated with $^{15}NO_2$-Ln. This analysis showed a chromatographic peak sharing the same retention time as $GS^{15}NO$ 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 $GS^{15}NO$ detected in *Arabidopsis* leaves after the application of

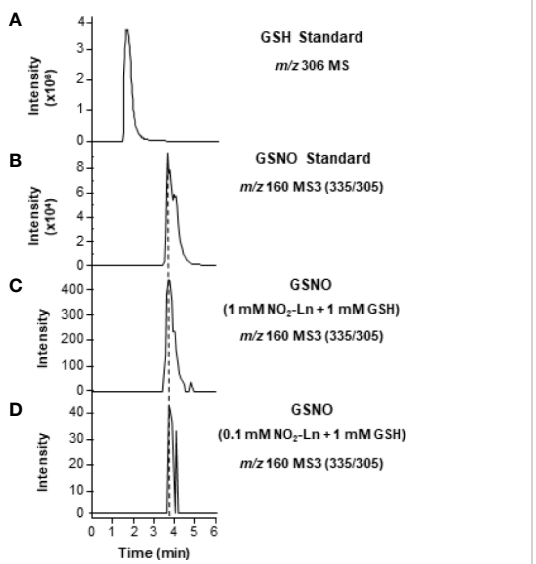

FIGURE 1 | *In vitro* synthesis of GSNO from NO_2-Ln and glutathione (GSH). For the *in vitro* generation of GSNO, 1 mM GSH was incubated with 1 and 0.1 mM NO_2-Ln, as is described in *Materials and Methods*. **(A, B)** show GSH and GSNO standards, respectively. **(C, D)** display GSNO generated after the incubation of 1 mM GSH with 1 and 0.1 mM NO_2-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.

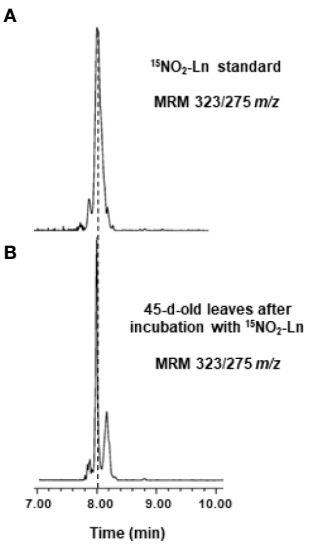

FIGURE 2 | Detection of $^{15}NO_2$-Ln in *Arabidopsis* leaves. 45-day-old *Arabidopsis* plants were incubated with 1 mM $^{15}NO_2$-Ln for 3 h and the lipid fraction from leaves was analyzed by LC-MS/MS as it is indicated in *Materials and Methods*. **(A)** $^{15}NO_2$-Ln standard with MRM transition of 323/275 *m/z*. **(B)** A chromatographic peak sharing the same retention time and *m/z* than $^{15}NO_2$-Ln standard in leaves from 45-day-old *Arabidopsis* plants incubated with $^{15}NO_2$-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.

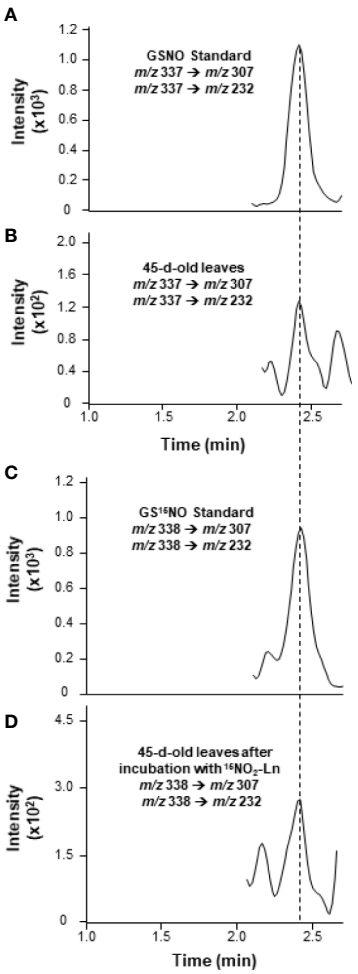

FIGURE 3 | Detection of endogenous GSNO and GS^{15}NO from $^{15}NO_2$-Ln in *Arabidopsis* leaves by LC-MS/MS in positive ion mode. **(A, B)** The detection of endogenous GSNO in leaves from 45-day-old *Arabidopsis* plants. **(C, D)** GS^{15}NO generated after the incubation of 45-day-old *Arabidopsis* plants with 1 mM $^{15}NO_2$-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 1 | Content of S-nitrosoglutathione (GSNO) in 45-d-old Arabidopsis leaves analysed by LC-MS/MS.

	pmol/mg prot	fmol/g FW
Endogenous GSNO in Arabidopsis leaves	0.91 ± 0.23	0.0018 ± 0.00045
GS^{15}NO generated in leaves from Arabidopsis plants incubated with $^{15}NO_2$-Ln	0.50 ± 0.15	0.0011 ± 0.00033

$^{15}NO_2$-Ln was 0.50 ± 0.15 pmol/mg protein (**Table 1**). These results confirm the potential of $^{15}NO_2$-Ln to generate GSNO in a significant amount, and the ability of $^{15}NO_2$-Ln to travel throughout the plant system.

NO_2-Ln Modulates the Endogenous Levels of GSNO

With the aim of support the previous demonstration about the relation between NO_2-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 NO_2-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 NO_2-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 NO_2-Ln content (**Figure 5A**) importantly correlated to the observed two-fold increase of GSNO content (**Figure 5B**).

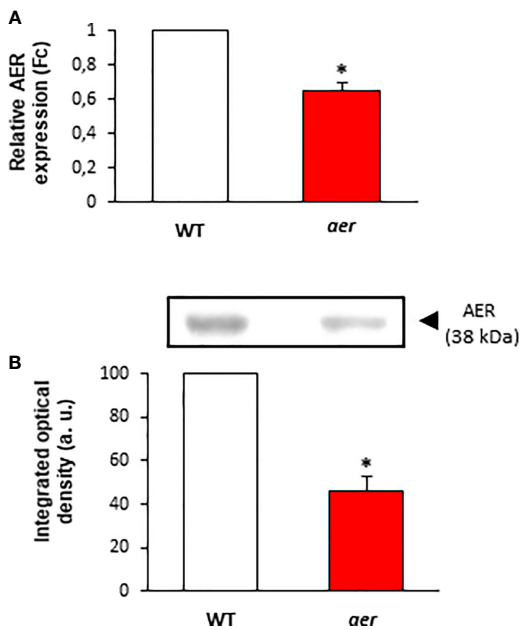

FIGURE 4 | Characterization of *aer* mutant seedlings. **(A)** Gene expression in *aer* mutant plants analyzed by real-time quantitative PCR. Actine was used as internal control. **(B)** The effect of mutation on AER protein content. Twenty microgram of wild and *aer* mutant seedling were subjected to SDS-PAGE. Proteins were electroblotted onto PVDF membranes and then incubated with an antibody against AER (1:1,000). The densitometry of the bands is expressed in arbitrary units (a.u.) of integrated optical density. Data are expressed as the mean ± SEM from at least three independent samples. Differences from control values were significant at $p < 0.05$ (*).

TABLE 2 | Composition of fatty acids (expressed at 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
Palmitic acid (16:0)	43.01 ± 2.53	40.00 ± 4.27

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

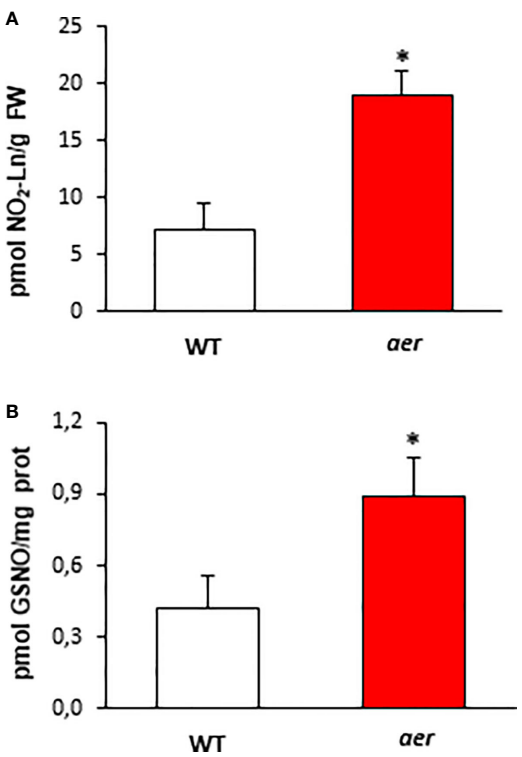

FIGURE 5 | Endogenous content of NO_2-Ln and GSNO of *aer* mutant seedlings. **(A)** The detection of endogenous NO_2-Ln levels in both wild type and *aer* mutant 7-day-old seedlings. Lipid extracts from 7-day-old seedlings were obtained as is indicated in *Materials and Methods* and analyzed by LC-MS/MS. **(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_2O_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, NO_2-FAs, as NO_2-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 NO_2-Ln to modulate the generation of GSNO. In this regard, using different concentrations of this NO_2-FA, the incubation of NO_2-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 NO_2-Ln with 1 mM GSH. Furthermore, to confirm whether NO_2-Ln can modulate the levels of GSNO *in vivo*, we firstly studied the mobilization of $^{15}NO_2$-FAs through the plant and, to achieve it, we undertook the synthesis of ^{15}N-labeled NO_2-Ln ($^{15}NO_2$-Ln). This labeled-NO_2-FA was used to differentiate its action from endogenous NO_2-Ln in *Arabidopsis* leaves (Mata-Pérez et al., 2016b; Mata-Pérez et al., 2017). In this sense, $^{15}NO_2$-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 $^{15}NO_2$-Ln in leaves from plants pre-incubated with this labeled NO_2-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 NO_2-FAs can travel and exert their signaling actions throughout the whole plant.

After having shown that NO_2-Ln can be mobilized across the plant and reach the shoots, the capability of $^{15}NO_2$-Ln to modulate the levels of ^{15}N-labeled GS^{15}NO 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 $^{15}NO_2$-Ln in the same way as previously described and we studied the occurrence of GS^{15}NO. The results displayed the presence of GS^{15}NO in the shoots after the treatment, thus confirming that NO_2-Ln can modulate the generation of GSNO *in vivo*. We incubated plant roots with 1mM of $^{15}NO_2$-Ln and a concentration of GS^{15}NO of 0.50 ± 0.15 pmol/mg was quantified in leaves. Different reasons emerge to explain this apparent low GS^{15}NO detection. It was previously described that the use of 1mM NO_2-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 1mM $^{15}NO_2$-Ln, the ^{15}NO generated will be in the μM range. Consequently, the *in vivo* detection of $^{15}NO_2$-Ln-dependent generation of GS^{15}NO will be apparently low compared with the initial concentration of the labeled nitro fatty acid. In addition, NO_2-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 NO_2-OA confirmed that the main distribution of this NO_2-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 NO_2-Ln, in which a substantial percentage of the initial amount of NO_2-Ln could be esterified in complex lipids, therefore not being able to release NO and, ultimately, generate GSNO. In this regard, the total free NO_2-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, NO_2-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 NO_2-Ln would not contribute to *in vivo* GSNO generation. Consequently, the GSNO concentration observed is consistent with the NO_2-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; Zaffagnini 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 N_2O_3 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, NO_2-Ln is able to release NO at a rate of 0.21 uM/min at a physiological pH (Mata-Pérez et al., 2016a) and therefore it generates NO at a submicromolar levels. Consequently, the NO_2-Ln-dependent generation of GSNO described in this work could be performed as a direct interaction of NO released from NO_2-Ln and GSH **(Figure 6)**.

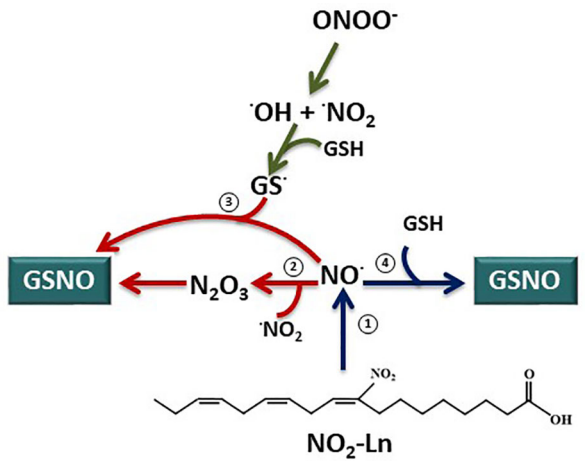

FIGURE 6 | GSNO biosynthesis from nitrolinolenic acid. Nitrolinolenic acid (NO_2-Ln) has the capacity to release nitric oxide (NO) at a physiological pH and temperature (1) (Mata-Pérez et al., 2016a). Therefore, this NO can contribute to the intracellular levels of S-nitrosoglutathione (GSNO). However, the exact mechanisms for GSNO generation remains to be elucidated. Instead of a direct reaction of NO and GSH, the formation of N_2O_3 as an intermediary (2) or the reaction of NO with the glutathionyl radical (GS.) (3) have been proposed as pathways for GSNO synthesis. Interestingly, the direct interaction of NO and GSH leading to GSNO formation appears to be possible at a submicromolar concentrations of NO (Kolesnik et al., 2013). Consequently, keeping in mind the submicromolar generation of NO from the NO_2-Ln (0.21 μM/min) (Mata-Pérez et al., 2016a), the NO_2-Ln-dependent formation of GSNO will take place by a direct reaction of NO released from the nitro fatty acid and GSH (4).

The Modulation of Cellular Levels of NO_2-Ln Directly Influences the GSNO Production

In order to clarify if the capacity of NO_2-Ln to modulate GSNO levels could have physiological implications, we used mutant plants that are able to regulate endogenous levels of NO_2-Ln. In this work, we have demonstrated the capacity of alkenal reductase (AER) enzyme to modulate endogenous levels of NO_2-FAs, concretely NO_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_2-Ln and two-fold in GSNO levels, confirming the capacity of NO_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 $GS^{15}NO$-detected levels represent approximately 50% of those detected in the control situation, which taking into account the multiple possible targets of NO_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 (Gaupels et al., 2017). All these results may suggest that the phloem can act as a key tissue location for the metabolism of NO_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 GSNOR1 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 it has previously mentioned, NO_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_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_2-FAs. Bearing this in mind, we would like to highlight that a concomitant increase on the free-NO_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 NO2-Ln (**Figure 7**). NO2-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 NO2-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 NO2-FAs can affect the SNO-signaling pathway by modulating the transport and storage of NO, the response to several (a)biotic stress conditions, or the mediating

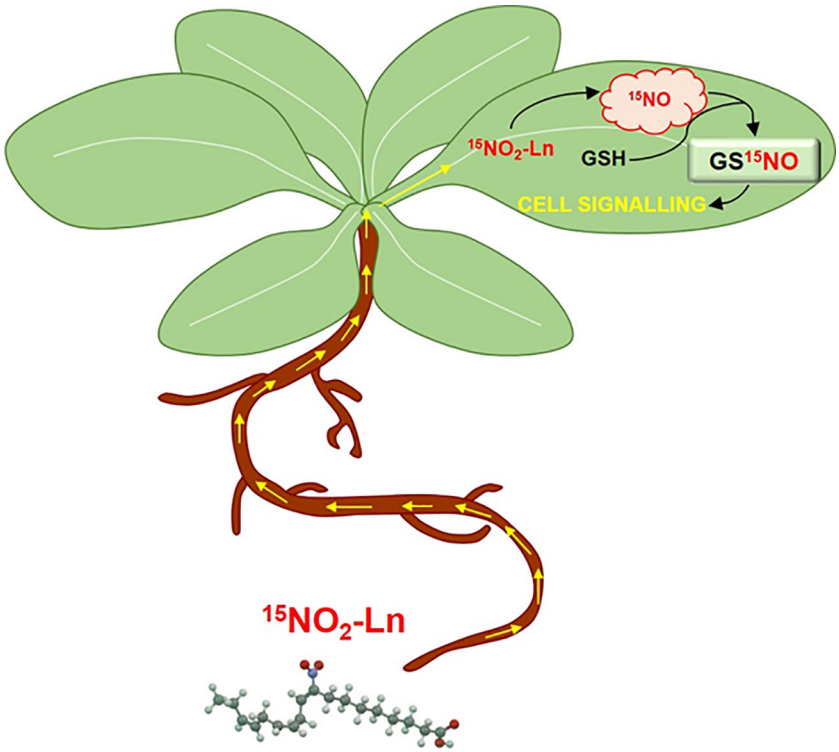

FIGURE 7 | Modulation of GSNO-signalling pathway by NO_2-Ln in *Arabidopsis* leaves. NO_2-Ln is up-taken by the root system and transported to plant leaves. Once in these organs, NO_2-Ln can release nitric oxide (NO) and mediate the S-nitrosation of abundant glutathione (GSH) present in plant leaves and leading to the formation of S-nitrosoglutathione (GSNO). The generation of this low-molecular weight S-nitrosothiol (SNO) from NO_2-Ln can affect the SNO-signaling pathway by modulating the transport and storage of NO, the response to several (a)biotic stress conditions or mediating the ability of SNO to perform post-translational modifications.

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 NO_2-FAs in plants. Data presented in this study provide novel information concerning the GSNO biosynthesis mechanisms, indicating that modulation of cellular levels of NO_2-FAs can directly influence the GSNO levels. In fact, the key property of NO_2-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, NO_2-FAs can be considered key players regulating the NO-bioactivity, so that the study of the interactions between NO_2-FAs and GSNO will increase the knowledge about SNO-signaling pathway in plants. On the basis of these results, the control of GSNO by NO_2-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.

ACKNOWLEDGMENTS

CM-P would like to thank the University of Jaén for funding the Ph.D. fellowship. This work was supported by an ERDF grant cofinanced by the Ministry of Economy and Competitiveness

(project PGC2018-096405-B-100), I+D+i Projects in the framework of the Andalusia 2014-2020 ERDF Operational Programme (Reference 1263509), Funding for the recruitment of researchers under the Action 10 of the Research Support Plan of the University of Jaén (2019-2020; R.02/10/2020) and the Junta de Andalucía (group BIO286) in Spain. LC-MS/MS technical and human support provided by CICT of Universidad de Jaén (UJA, MINECO, Junta de Andalucía, FEDER) is gratefully acknowledged. Authors would like to thank Dr. Mano from Yamaguchi University (Japan) for providing the antibody against *Arabidopsis* alkenal reductase.

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

REFERENCES

Abello, N., Kerstjens, H. A., Postma, D. S., and Bischoff, R. (2009). Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. *J. Proteome Res.* 8, 3222–3238. doi: 10.1021/pr900039c

Airaki, M., Sánchez-Moreno, L., Leterrier, M., Barroso, J. B., Plama, J. M., and Corpas, F. J. (2011). Detection and quantification of S-nitrosoglutathione (GSNO) in pepper (*Capsicum annuum* L.) plant organs by LC-ES/MS. *Plant Cell Physiol.* 52, 2006–2015. doi: 10.1093/pcp/pcr133

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., et al. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* 301, 653–657. doi: 10.1126/science.1086391

Ambrozova, G., Fidlerova, T., Verescakova, H., Koudelka, A., Rudolph, T. K., Woodcock, S. R., et al. (2016). Nitro-oleic acid inhibits vascular endothelial inflammatory responses and the endothelial-mesenchymal transition. *Biochim. Biophys. Acta (BBA)-General Subj.* 1860, 2428–2437. doi: 10.1016/j.bbagen.2016.07.010

Aranda-Caño, L., Sánchez-Calvo, B., Begara-Morales, J. C., Chaki, M., Mata-Pérez, C., Padilla, M. N., et al. (2019). Post-translational modification of proteins mediated by nitro-fatty acids in plants: nitroalkylation. *Plants (Basel)* 8, E82. doi: 10.3390/plants8040082

Astier, J., Kulik, A., Koen, E., Besson-Bard, A., Bourque, S., Jeandroz, S., et al. (2012). Protein S-nitrosylation: what's going on in plants? *Free Radical Biol. Med.* 53, 1101–1110. doi: 10.3390/ijms131115193

Baker, P. R., Schopfer, F. J., O'Donnell, V. B., and Freeman, B. A. (2009). Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids. *Free Radical Biol. Med.* 46, 989–1003. doi: 10.1016/j.freeradbiomed.2008.11.021

Barroso, J. B., Corpas, F. J., Carreras, A., Rodríguez-Serrano, M., Esteban, F. J., Fernández-Ocaña, A., et al. (2006). Localization of S-nitrosoglutathione and expression of S-nitrosoglutathione reductase in pea plants under cadmium stress. *J. Exp. Bot.* 57:, 1785–1793. doi: 10.1093/jxb/erj175

Bartesaghi, S., Ferrer-Sueta, G., Peluffo, G., Valez, V., Zhang, H., Kalyanaraman, B., et al. (2007). Protein tyrosine nitration in hydrophilic and hydrophobic environments. *Amino Acids* 32, 501–515. doi: 10.1007/s00726-006-0425-8

Begara-Morales, J. C., Chaki, M., Sánchez-Calvo, B., Mata-Pérez, C., Leterrier, M., Palma, J. M., et al. (2013). Protein tyrosine nitration in pea roots during development and senescence. *J. Exp. Bot.* 64, 1121–1134. doi: 10.1093/jxb/ert006

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., López-Jaramillo, J., et al. (2014a). Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and S-nitrosylation. *J. Exp. Bot.* 65, 527–538. doi: 10.1093/jxb/ert396

Begara-Morales, J. C., Sánchez-Calvo, B., Luque, F., Leyva-Pérez, M. O., Leterrier, M., Corpas, F. J., et al. (2014b). Differential transcriptomic analysis by RNA-seq of GSNO-responsive genes between Arabidopsis roots and leaves. *Plant Cell Physiol.* 55, 1080–1095. doi: 10.1093/pcp/pcu044

Begara-Morales, J. C., Chaki, M., Valderrama, R., Sánchez-Calvo, B., Mata-Pérez, C., Padilla, M. N., et al. (2018). Nitric oxide buffering and conditional nitric oxide release in stress response. *J. Exp. Bot.* 69, 3425–3438. doi: 10.1093/jxb/ery072

Begara-Morales, J. C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M. N., and Barroso, J. B. (2019). The function of S-nitrosothiols during abiotic stress in plants. *J. Exp. Bot.* 70, 4429–4439. doi: 10.1093/jxb/erz197

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/o59-099

Bonacci, G., Schopfer, F. J., Batthyany, C. I., Rudolph, T. K., Rudolph, V., Khoo, N. K., et al. (2011). Electrophilic fatty acids regulate matrix metalloproteinase activity and expression. *J. Biol. Chem.* 286, 16074–16081. doi: 10.1074/jbc.M111.225029

Bonacci, G., Baker, P. R., Salvatore, S. R., Shores, D., Khoo, N. K., Koenitzer, J. R., et al. (2012). Conjugated linoleic acid is a preferential substrate for fatty acid nitration. *J. Biol. Chem.* 287 (53), 44071–44082. doi: 10.1074/jbc.M112.401356

Broniowska, K. A., and Hogg, N. (2012). The chemical biology of S-nitrosothiols. *Antioxid. Redox Signaling* 17, 969–980. doi: 10.1089/ars.2012.4590

Broniowska, K. A., Diers, A. R., and Hogg, N. (2013). S-nitrosoglutathione. *Biochem. Biophys. Acta* 1830, 3173–3181. doi: 10.1016/j.bbagen.2013.02.004

Cellier, F., Conéjéro, G., Ricaud, L., Luu, D. T., Lepetit, M., Gosti, F., et al. (2004). Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K+ homeostasis. *Plant J.* 39, 834–846. doi: 10.1111/j.1365-313X.2004.02177.x

Chaki, M., Fernández-Ocaña, A. M., Valderrama, R., Carreras, A., Esteban, F. J., Luque, F., et al. (2009). Involvement of reactive nitrogen and oxygen species (RNS and ROS) in sunflower–mildew interaction. *Plant Cell Physiol.* 50, 265–279. doi: 10.1093/pcp/pcn196

Chaki, M., Valderrama, R., Fernández-Ocaña, A. M., Carreras, A., Gómez-Rodríguez, M. V., Pedrajas, J. R., et al. (2011a). Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in S-nitrosothiols in sunflower (Helianthus annuus) seedlings. *J. Exp. Bot.* 62, 1803–1813. doi: 10.1093/jxb/erq358

Chaki, M., Valderrama, R., Fernández-Ocaña, A. M., Carreras, A., Gómez-Rodríguez, M. V., López-Jaramillo, J., et al. (2011b). High temperature triggers the metabolism of S-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin–NADP reductase by tyrosine nitration. *Plant. Cell Environ.* 34, 1803–1818. doi: 10.1111/j.1365-3040.2011.02376.x

Corpas, F. J., Chaki, M., Fernández-Ocaña, A., Valderrama, R., Palma, J. M., Carreras, A., et al. (2008). Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. *Plant Cell Physiol.* 49, 1711–1722. doi: 10.1093/pcp/pcn144

Corpas, F. J., Chaki, M., Begara-Morales, J. C., Valderrama, R., Sánchez-Calvo, B., and Barroso, J. B. (2016). "Functional Implications of S-Nitrosothiols under Nitrooxidative Stress Induced by Abiotic Conditions," in *Nitric Oxide and Signalling in Plants. Advances in Botanical Research*, vol. 77. Ed. D. Wendehenne (London, UK: Elsevier Ltd.), 79–96.

Cui, T., Schopfer, F. J., Zhang, J., Chen, K., Ichikawa, T., Baker, P. R., et al. (2006). Nitrated fatty acids: endogenous anti-inflammatory signaling mediators. *J. Biol. Chem.* 28147, 35686–35698. doi: 10.1074/jbc.M603357200

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* 394:, 585–588. doi: 10.1038/29087

Delmastro-Greenwood, M., Freeman, B. A., and Wendell, S. G. (2014). Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids. *Annu. Rev. Physiol.* 76, 79–105. doi: 10.1146/annurev-physiol-021113-170341

Espunya, M. C., De Michele, R., Gómez-Cadenas, A., and Martínez, M. C. (2012). S-Nitrosoglutathione is a component of wound-and salicylic acid-induced systemic responses in Arabidopsis thaliana. *J. Exp. Bot.* 63, 3219–3227. doi: 10.1093/jxb/ers043

Faine, L. A., Cavalcanti, D. M., Rudnicki, M., Ferderbar, S., Macedo, S., Souza, H. P., et al. (2010). Bioactivity of nitrolinoleate: effects on adhesion molecules

and CD40–CD40L system. *J. Nutr. Biochem.* 21, 125–132. doi: 10.1016/j.jnutbio.2008.12.004

Fazzari, M., Vitturi, D. A., Woodcock, S. R., Salvatore, S. R., Freeman, B. A., and Schopfer, F. J. (2019). Electrophilic fatty acid nitroalkenes are systemically transported and distributed upon esterification to complex lipids. *J. Lipid Res.* 60, 388–399. doi: 10.1194/jlr.M088815

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Foster, M. W., Hess, D. T., and Stamler, J. S. (2009). Protein S-nitrosylation in health and disease: a current perspective. *Trends Mol. Med.* 15, 391–404. doi: 10.1016/j.molmed.2009.06.007

Freeman, B. A., Baker, P. R., Schopfer, F. J., Woodcock, S. R., Napolitano, A., and d'Ischia, M. (2008). Nitro-fatty acid formation and signaling. *J. Biol. Chem.* 283, 15515–15519. doi: 10.1074/jbc.R800004200

Frungillo, L., Skelly, M. J., Loake, G. J., Spoel, S. H., and Salgado, I. (2014). S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. *Nat. Commun.* 5, 5401. doi: 10.1038/ncomms6401

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L., and Blatt, M. R. (2003). Nitric oxide regulates K+ and Cl-channels in guard cells through a subset of abscisic acid-evoked signaling pathways. *Proc. Natl. Acad. Sci.* 100, 11116–11121. doi: 10.1073/pnas.1434381100

Gaupels, F., Durner, J., and Kogel, K. H. (2017). Production, amplification and systemic propagation of redox messengers in plants? The phloem can do it all! *New Phytol.* 214, 554–560.

Geisler, A. C., and Rudolph, T. K. (2012). Nitroalkylation—a redox sensitive signaling pathway. *Biochim. Biophys. Acta (BBA)-General Subj.* 1820, 777–784. doi: 10.1016/j.bbagen.2011.06.014

Gorczynski, M. J., Huang, J., Lee, H., and King, S. B. (2007). Evaluation of nitroalkenes as nitric oxide donors. *Bioorg. Med. Chem. Lett.* 17, 2013–2017. doi: 10.1016/j.bmcl.2007.01.016

Hart, T. W. (1985). Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Lett.* 26, 2013–2016. doi: 10.1016/S0040-4039(00)98368-0

Ichikawa, T., Zhang, J., Chen, K., Liu, Y., Schopfer, F. J., Baker, P. R., et al. (2008). Nitroalkenes suppress lipopolysaccharide-induced signal transducer and activator of transcription signaling in macrophages: a critical role of mitogen-activated protein kinase phosphatase 1. *Endocrinology* 149, 4086–4094. doi: 10.1210/en.2007-1639

Kansanen, E., Jyrkkänen, H. K., Volger, O. L., Leinonen, H., Kivelä, A. M., Häkkinen, S. K., et al. (2009). Nrf2-dependent and-independent responses to nitro-fatty acids in human endothelial cells identification of heat shock response as the major pathway activated by nitro-oleic acid. *J. Biol. Chem.* 284, 33233–33241. doi: 10.1074/jbc.M109.064873

Kansanen, E., Bonacci, G., Schopfer, F. J., Kuosmanen, S. M., Tong, K. I., Leinonen, H., et al. (2011). Electrophilic nitro-fatty acids activate NRF2 by a KEAP1 cysteine 151-independent mechanism. *J. Biol. Chem.* 286, 14019–14027. doi: 10.1074/jbc.M110.190710

Kansanen, E., Kuosmanen, S. M., Ruotsalainen, A. K., Hynynen, H., and Levonen, A. L. (2017). Nitro-oleic acid regulates endothelin signaling in human endothelial cells. *Mol. Pharmacol.* 92, 481–490. doi: 10.1124/mol.117.109751

Kneeshaw, S., Gelineau, S., Tada, Y., Loake, G. J., and Spoel, S. H. (2014). Selective Protein Denitrosylation Activity of Thioredoxin-h5 Modulates Plant Immunity. *Mol. Cell* 56, 153–162. doi: 10.1016/j.molcel.2014.08.003

Kolesnik, B., Palten, K., Schrammel, A., Stessel, H., Schmidt, K., Mayer, B., et al. (2013). Efficient nitrosation of glutathione by nitric oxide. *Free Radical Biol. Med.* 63, 51–64. doi: 10.1016/j.freeradbiomed.2013.04.034

Lima, É. S., Bonini, M. G., Augusto, O., Barbeiro, H. V., Souza, H. P., and Abdalla, D. S. (2005). Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation. *Free Radical Biol. Med.* 39, 532–539. doi: 10.1016/j.freeradbiomed.2005.04.005

Mano, J., Belles-Boix, E., Babiychuk, E., Inzé, D., Torii, Y., Hiraoka, E., et al. (2005). Protection against photooxidative injury of Tobacco leaves by 2-alkenal reductase. Detoxication lipid peroxide-derived reactive carbonyls. *Plant Physiol.* 139, 1773–1783. doi: 10.1104/pp.105.070391

Mata-Pérez, C., Sánchez-Calvo, B., Begara-Morales, J. C., Luque, F., Jimenez-Ruiz, J., Padilla, M. N., et al. (2015). Transcriptomic profiling of linolenic acid-responsive genes in ROS signaling from RNA-seq data in *Arabidopsis*. *Front. Plant Sci.* 6, 122. doi: 10.3389/fpls.2015.00122

Mata-Pérez, C., Sánchez-Calvo, B., Begara-Morales, J. C., Carreras, A., Padilla, M. N., Melguizo, M., et al. (2016a). Nitro-linolenic acid is a nitric oxide donor. *Nitric. Oxide* 57, 57–63. doi: 10.1016/j.niox.2016.05.003

Mata-Pérez, C., Sánchez-Calvo, B., Padilla, M. N., Begara-Morales, J. C., Luque, F., Melguizo, M., et al. (2016b). Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in Arabidopsis. *Plant Physiol.* 170:, 686–701. doi: 10.1104/pp.15.01671

Mata-Pérez, C., Sánchez-Calvo, B., Padilla, M. N., Begara-Morales, J. C., Valderrama, R., Corpas, F. J., et al. (2017). Nitro-fatty acids in plant signaling: new key mediators of nitric oxide metabolism. *Redox Biol.* 11, 554–561. doi: 10.1016/j.redox.2017.01.002

Mata-Pérez, C., Padilla, M. N., Sánchez-Calvo, B., Begara-Morales, J. C., Valderrama, R., Corpas, F. J., et al. (2018). Nitro-fatty acid detection in plants by high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. *Methods Mol. Biol.* 1747, 231–239. doi: 10.1007/978-1-4939-7695-9_18

Mesa, J., Alsina, C., Oppermann, U., Parés, X., Farrés, J., and Porté, S. (2015). Human prostaglandin reductase 1 (PGR1): Substrate specificity, inhibitor analysis and site-directed mutagenesis. *Chem.-Biol. Interact.* 234, 105–113. doi: 10.1016/j.cbi.2015.01.021

Nadtochiy, S. M., Burwell, L. S., Ingraham, C. A., Spencer, C. M., Friedman, A. E., Pinkert, C. A., et al. (2009). In vivo cardioprotection by S-nitroso-2-mercaptopropionyl glycine. *J. Mol. Cell. Cardiol.* 46, 960–968. doi: 10.1016/j.yjmcc.2009.01.012

Padilla, M. N., Mata-Pérez, C., Melguizo, M., and Barroso, J. B. (2017). In vitro nitro-fatty acid release from Cys-NO_2-fatty acid adducts under nitro-oxidative conditions. *Nitric. Oxide* 68, 14–22. doi: 10.1016/j.niox.2016.12.009

Radi, R. (2012). Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects. *Acc. Chem. Res.* 46, 550–559. doi: 10.1021/ar300234c

Romero-Puertas, M. C., Campostrini, N., Mattè, A., Righetti, P. G., Perazzolli, M., Zolla, L., et al. (2008). Proteomic analysis of S-nitrosylated proteins in Arabidopsis thaliana undergoing hypersensitive response. *Proteomics* 8, 1459–1469. doi: 10.1002/pmic.200700536

Rubbo, H. (2013). Nitro-fatty acids: novel anti-inflammatory lipid mediators. *Braz. J. Med. Biol. Res.* 46 (9), 728–734. doi: 10.1590/1414-431X20133202

Rudolph, V., Rudolph, T. K., Schopfer, F. J., Bonacci, G., Woodcock, S. R., Cole, M. P., et al. (2010). Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovasc. Res.* 85, 155–166. doi: 10.1093/cvr/cvp275

Schopfer, F. J., Baker, P. R., Giles, G., Chumley, P., Batthyany, C., Crawford, J., et al. (2005). Fatty acid transduction of nitric oxide signaling Nitrolinoleic acid is a hydrophobically stabilized nitric oxide donor. *J. Biol. Chem.* 280, 19289–19297. doi: 10.1074/jbc.M414689200

Schopfer, F. J., Cipollina, C., and Freeman, B. A. (2011). Formation and signaling actions of electrophilic lipids. *Chem. Rev.* 111, 5997–6021. doi: 10.1021/cr200131e

Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Curr. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007

Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science* 258, 1898–1902. doi: 10.1126/science.1281928

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., et al. (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science* 321, 952–956. doi: 10.1126/science.1156970

Terzaghi, W. B. (1989). Manipulating membrane fatty acid compositions of whole plants with tween-fatty acid esters. *Plant Physiol.* 91, 203–212. doi: 10.1104/pp.91.1.203

Tsikas, D., and Hanff, E. (2018). Measurement of S-nitrosoglutathione in plasma by liquid chromatography-tandem mass spectrometry. *Methods Mol. Biol.* 1747, 113–129. doi: 10.1007/978-1-4939-7695-9_10

Tsikas, D., Schmidt, M., Böhmer, A., Zoerner, A. A., Gutzki, F.-M., and Jordan, J. (2013). UPLC–MS/MS measurement of S-nitrosoglutathione (GSNO) in human plasma solves the S-nitrosothiol concentration enigma. *J. Chromatogr. B* 927, 147–157. doi: 10.1016/j.jchromb.2013.01.023

Tsikas, D., Zoerner, A. A., Mitschke, A., and Gutzki, F. M. (2009). Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC–MS/MS study. *Lipids* 44, 855–865. doi: 10.1007/s11745-009-3332-4

Turell, L., Vitturi, D. A., Coitiño, E. L., Lebrato, L., Möller, M. N., Sagasti, C., et al. (2017). The Chemical Basis of Thiol Addition to Nitro-conjugated Linoleic Acid, a Protective Cell-signaling Lipid. *J. Biol. Chem.* 292, 1145–1159. doi: 10.1074/jbc.M116.756288

Valderrama, R., Corpas, F. J., Carreras, A., Fernández-Ocaña, A., Chaki, M., Luque, F., et al. (2007). Nitrosative stress in plants. *FEBS Lett.* 581, 453–461. doi: 10.1016/j.febslet.2007.01.006

Verescakova, H., Ambrozova, G., Kubala, L., Perecko, T., Koudelka, A., Vasicek, O., et al. (2017). Nitro-oleic acid regulates growth factor-induced differentiation of bone marrow-derived macrophages. *Free Radical Biol. Med.* 104, 10–19. doi: 10.1016/j.freeradbiomed.2017.01.003

Villacorta, L., Zhang, J., Garcia-Barrio, M. T., Chen, X. L., Freeman, B. A., Chen, Y. E., et al. (2007). Nitro-linoleic acid inhibits vascular smooth muscle cell proliferation via the Keap1/Nrf2 signaling pathway. *Am. J. Physiol.-Heart Circulatory Physiol.* 293, H770–H776. doi: 10.1152/ajpheart.00261.2007

Vitturi, D. A., Chen, C. S., Woodcock, S. R., Salvatore, S. R., Bonacci, G., Koenitzer, J. R., et al. (2013). Modulation of nitro-fatty acid signaling. Prostaglandin reductase-1 is a nitroalkene reductase. *J. Biol. Chem.* 288, 25626–25637. doi: 10.1074/jbc.M113.486282

Yamauchi, Y., Hasegawa, A., Taninaka, A., Mizutani, M., and Sugimoto, Y. (2011). NADPH-dependent reductases involved in the detoxification of reactive carbonyls in plants. *J. Biol. Chem.* 286, 6999–7009. doi: 10.1074/jbc.M110.202226

Yu, M., Lamattina, L., Spoel, S. H., and Loake, G. J. (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. *New Phytol.* 202, 1142–1156. doi: 10.1111/nph.12739

Zaffagnini, M., de Mia, M., Morisse, S., di-Giacinto, N., Marchand, C. H., Maes, A., et al. (2016). Protein S-nitrosylation in photosynthetic organisms: a comprehensive overview with future perspectives. *Biochim. Biophys. Acta* 1864, 952–966. doi: 10.1016/j.bbapap.2016.02.006

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 July 2020
doi: 10.3389/fpls.2020.01019

Strigolactones Interact With Nitric Oxide in Regulating Root System Architecture of *Arabidopsis thaliana*

*Dóra Oláh, Gábor Feigl, Árpád Molnár, Attila Ördög and Zsuzsanna Kolbert**

Department of Plant Biology, University of Szeged, Szeged, Hungary

Both nitric oxide (NO) and strigolactone (SL) are growth regulating signal components in plants; however, regarding their possible interplay our knowledge is limited. Therefore, this study aims to provide new evidence for the signal interplay between NO and SL in the formation of root system architecture using complementary pharmacological and molecular biological approaches in the model *Arabidopsis thaliana* grown under stress-free conditions. Deficiency of SL synthesis or signaling (*max1-1* and *max2-1*) resulted in elevated NO and *S*-nitrosothiol (SNO) levels due to decreased *S*-nitrosoglutathione (GSNO) reductase (GSNOR) protein abundance and activity indicating that there is a signal interaction between SLs and GSNOR-regulated levels of NO/SNO. This was further supported by the down-regulation of SL biosynthetic genes (*CCD7, CCD8* and *MAX1*) in GSNOR-deficient *gsnor1-3*. Based on the more pronounced sensitivity of *gsnor1-3* to exogenous SL (*rac*-GR24, 2 μM), we suspected that functional GSNOR is needed to control NO/SNO levels during SL-induced primary root (PR) elongation. Additionally, SLs may be involved in GSNO-regulated PR shortening as suggested by the relative insensitivity of *max1-1* and *max2-1* mutants to exogenous GSNO (250 μM). Collectively, our results indicate a connection between SL and GSNOR-regulated NO/SNO signals in roots of *A. thaliana* grown in stress-free environment. As this work used *max2-1* mutant and *rac*-GR24 exerting unspecific effects to both SL and karrikin signaling, it cannot be ruled out that karrikins are partly responsible for the observed effects, and this issue needs further clarification in the future.

Keywords: ***Arabidopsis thaliana*, nitric oxide, root, *S*-nitrosoglutathione reductase, strigolactone**

OPEN ACCESS

Edited by:
Marek Petrivalsky,
Palacký University, Olomouc, Czechia

Reviewed by:
Guodong Wang,
Shaanxi Normal University, China
Koichi Yoneyama,
Ehime University, Japan

***Correspondence:**
Zsuzsanna Kolbert
kolzsu@bio.u-szeged.hu

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *29 January 2020*
Accepted: *22 June 2020*
Published: *03 July 2020*

Citation:
Oláh D, Feigl G, Molnár Á, Ördög A and Kolbert Z (2020) Strigolactones Interact With Nitric Oxide in Regulating Root System Architecture of Arabidopsis thaliana. Front. Plant Sci. 11:1019. doi: 10.3389/fpls.2020.01019

INTRODUCTION

Strigolactones (SLs) have been first identified as germination inducers of parasite plants in the 1960s (Cook et al., 1966) and since then, they have been found to be phytohormones due to their multiple roles in regulating growth and developmental processes of higher plants (Gomez-Roldan et al., 2008; Umehara et al., 2008; Zwanenburg and Blanco-Ania, 2018; Bouwmeester et al., 2019).

SLs as terpenoid lactones can be categorized as canonical SLs containing ABC ring and noncanonical SLs lacking such a ring (Al-Babili and Bouwmeester, 2015; Waters et al., 2017). SLs are synthetized from carotenoids in the plastids with the involvement of enzymes such as beta-carotene-isomerase (D27), two carotenoid cleavage dioxygenases (CCD7/MAX3 and CCD8/MAX4), cytochrome P450 (MAX1), and

LATERAL BRANCHING OXIDOREDUCTASE (Alder et al., 2012; Brewer et al., 2016). Following its transport into the cytoplasm, carlactone is converted into carlactonoic acid which is the common precursor of the naturally occurring SLs (Jia et al., 2019). Recently, the direct conversion of carlactonoic acid to orobanchol without passing through 4-deoxyorobanchol has been described (Wakabayashi et al., 2019). Moreover, a cytochrome P450 and a 2-oxoglutarate-dependent dioxygenase genes were identified being involved in SL synthesis in *Lotus japonicus* (Mori et al., 2020), and hydroxyl carlactone derivatives as relevant intermediaries in SL synthesis have been identified in *Arabidopsis* (Yoneyama et al., 2020). Despite the active research, our knowledge about the details of SL biosynthesis after carlactone is still limited (Bouwmeester et al., 2019). It has been shown that SLs are synthetized in both the root and the shoot and that the SL signal can spread from the root to the shoot system (Foo et al., 2001).

The perception of SLs involves the SL receptor DWARF14 (D14) protein having α/β fold hydrolase activity. The intact SL molecule promotes D14 activation which in turn deactivates bioactive SLs by the hydrolytic degradation following signal transmission (Seto et al., 2019). Consequently, the activated D14 can bind the MORE AXILLARY GROWTH2 (MAX2/D3) F-box type protein which assigns DWARF53 and SMXLs repressors for proteasomal degradation resulting in the induction of gene expression (Shabek et al., 2018; Bouwmeester et al., 2019). Recently, MAX2 was implicated as a regulator of karrikin (KAR) signaling (Nelson et al., 2011), and SMXL/D53, the downstream targets of MAX2 are responsible for the discrimination of SL and KAR signal pathways (Soundappan et al., 2015). The interference between SL and KAR signaling is further supported by the fact that *rac*-GR24 (racemic mixtures of GR24 stereoisomers) activates both signal pathways, thus exerts also non-SL-specific effects (Scaffidi et al., 2014; Li et al., 2016). The SL-induced gene expression manifests in physiological effects such as the inhibition of shoot branching, shaping of root system architecture, inducing leaf senescence (Pandey et al., 2016; Waters et al., 2017; Marzec and Melzer, 2018). Recently, Villaécija-Aguilar and co-workers (2019) added that root traits like root hair development, root skewing, straightness, and diameter are regulated by KAR signaling, while both KAR and SL pathways contribute to the regulation of lateral root density and epidermal cell length. Furthermore, SLs have been implicated in plant stress responses to diverse abiotic factors (reviewed by Mostofa et al., 2018) like nutrient deficiency (Kohlen et al., 2011), salinity and drought (Ha et al., 2014; Wang et al., 2019, reviewed by Mostofa et al., 2018) or chilling (Cooper et al., 2018).

Similar to SLs, research over the past 40 years has revealed that the gaseous signal molecule nitric oxide (NO) is a multifunctional growth regulator in plants (Kolbert et al., 2019a). While, the ability of SL synthesis is a unique feature of plants (Walker et al., 2019), any living organism is capable of the synthesis of NO. Algae utilize NO synthase (NOS)-like enzyme system for producing NO (Foresi et al., 2010; Foresi et al., 2015; Weisslocker-Schaetzel et al., 2017) while in higher land plants NOS gene homolog to animal gene has not been found (Jeandroz et al., 2016; Santolini et al., 2017; Hancock and Neill, 2019). The ability of NO liberation via NOS-system may be lost during the evolution of land plants (Fröhlich and Durner, 2011), which takes up high amounts of nitrate, and their physiological functions are greatly determined by nitrate acquisitions. A key process in nitrate-dependent NO synthesis of plants indirectly involves nitrate reductase (NR) activity which transfers electron from NAD(P)H to the NO-forming nitrite reductase (NOFNiR). This enzyme catalyzes the reduction of nitrite to NO (Chamizo-Ampudia et al., 2016; Chamizo-Ampudia et al., 2017). NO is synthetized endogenously within the plant body in a wide variety of tissues, and NO can also be taken up from the atmosphere or from the soil (Cohen et al., 2009). In biological systems, NO reacts with glutathione to form *S*-nitrosoglutathione (GSNO) being a less reactive and more stable molecule than NO. GSNO is able to release NO and can achieve long distance movement of NO signal *via* the xylem (Durner et al., 1999; Díaz et al., 2003; Barroso et al., 2006). Intracellular levels of GSNO are controlled by the activity of GSNO reductase (GSNOR) enzyme (Feechan et al., 2005; Lee et al., 2008; Chen et al., 2009) catalyzing the conversion of GSNO to GSSG and NH_3 in the presence of NADH (Jahnová et al., 2019).

Unlike SLs, the signal of NO isn't perceived by specific receptor, but the transfer of NO bioactivity is achieved by direct modification of target proteins. Cysteine *S*-nitrosation, tyrosine nitration, and metal nitrosylation are three major NO-dependent posttranslational modifications being physiologically relevant (Astier and Lindermayr, 2012). Additionally, the link between NO-related signaling and Ca^{2+}-, cGMP-, MAPK-, and PA-dependent signaling has also been revealed in diverse physiological processes (Pagnussat et al., 2004; Lanteri et al., 2008; Astier et al., 2011; Jiao et al., 2018). Like SLs, NO affects a range of physiological traits including seed development, vegetative and generative development like pollen tube growth, seed germination, root growth, gravitropism, flowering, fruit ripening (reviewed in Kolbert and Feigl, 2017). Additionally, NO also participates in responses of plants to abiotic stresses like salinity, drought, heavy metal, low oxygen availability, or temperature stresses (Fancy et al., 2017).

Based on the stimulating effect of NO on plant germination, vegetative growth or fruit ripening, NO-releasing substances such as nanoparticles could be effectively applied in agricultural practice (Rodríguez-Ruiz et al., 2019). Similarly, SLs and their agonists and antagonists may have a great potential for agricultural applications. Beyond plant protection, SLs may be used to improve the architecture of crops as well (Vurro et al., 2016; Takahashi and Asami, 2018).

It is sure that both NO and SL are important growth regulating signals of practical significance in plants. However, their interplay has been poorly examined. The majority of the few articles dealing with SL–NO interplay focus on the root system of crops like sunflower (Barthi and Bhatla, 2015), maize (Manoli et al., 2016), and rice (Sun et al., 2014) grown in the presence of different nutrient supplies. Collectively, these studies revealed that NO is an upstream regulator of SL signaling; however, the nature of the NO–SL relationship depends on the nutrient availability. During nitrate-induced root elongation, NO reduces SL biosynthesis thus resulting in alterations of PIN-

mediated auxin transport leading to cell elongation. Exogenous SL induces NO production suggesting negative feedback regulation of SL levels (Manoli et al., 2016). Low N and P availability triggers NO formation which in turn induces the proteasomal-degradation of D53 repressor protein and consequently intensifies SL signaling leading to root elongation (Sun et al., 2016). To clarify the role of SLs in root development, Marzec and Melzer (2018) recommended to perform experiments with plants grown during stress-free conditions. Because of the above reasons, this study aims to provide new evidence for the signal interplay between NO and SL in the formation of root system architecture using complementary pharmacological and molecular biological approaches in the model *Arabidopsis thaliana* grown under stress-free conditions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* wild-type (WT, Col-0), and their mutant lines *gsnor1-3* (Chen et al., 2009), *35S:FLAG-GSNOR1* (Frungillo et al., 2014), *max1-1*, *max2-1* (Stirnberg et al., 2002) were surfaced sterilized with 70% (v/v) ethanol for 1 min and with 30% sodium hypochlorite solution (1:3) for 15 min then washed five times with sterile distilled water. Seeds (approx. 30 seeds/Petri dish) were then transferred to half strength Murashige and Skoog medium (1% sucrose, 0.8% agar). Petri dishes were kept in a greenhouse under controlled conditions (photon flux density of 150 μmol m^{-2} s^{-1}, 12/12 h light and dark cycle, relative humidity of 55–60%, temperature of 25 ± 2°C) for 7 days.

Treatments

Stock solution of *rac*-GR24 and TIS108 (both purchased from Chiralix B.V., Nijmegen, Netherlands) was prepared in acetone or in DMSO, respectively. Appropriate volumes of stock solutions were added to the medium following sterilization through sterile syringe yielding 2 μM GR24 or 5 μM TIS108 concentrations in the media. These concentrations were chosen in pilot experiments using several doses (1, 2, 5 μM for GR24 and 1, 5, 10 μM for TIS108). Stock solutions of GSNO and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) were prepared in DMSO and were diluted to the final concentrations (250 μM GSNO and 800 μM cPTIO) with distilled water. Four days after placing the seeds on the media, GSNO and cPTIO solutions were added to the surface of the agar containing the root system. One milliliter of GSNO or cPTIO was added per Petri dish using 2-ml syringe and sterile filter.

Morphological Measurements

Primary root (PR) lengths of *Arabidopsis* seedlings were measured and expressed in mm. Lateral roots within the primary root (smaller than stage VII) were considered as lateral root primordia (LR_{prim}), whereas visible laterals which have already grown outside the PR were considered as emerged LRs (LR_{em}, larger than stage VII, Malamy and Benfey, 1997; Feigl et al., 2019). The number of LR_{prim} and LR_{em} was determined by using Zeiss Axiovert 200 inverted microscope and 20× objective (Carl Zeiss, Jena, Germany). LR density (number mm^{-1}) was calculated by dividing total number of LRs with PR length. The experiments were performed three times with 20 samples each (n = 60).

Detection of NO Levels

Levels of NO were detected with the fluorophore, 4-amino-5-methylamino-2′-7′-difluorofluorescein diacetate (DAF-FM DA). *Arabidopsis* seedlings were incubated in 10 μM dye solution for 30 min, in darkness, at room temperature and washed two times with TRIS-HCl buffer (10 mM, pH 7.4) according to Kolbert et al. (2012). Stained root samples were observed under Axiovert 200M (Carl Zeiss, Jena, Germany) fluorescent microscope equipped with digital camera (Axiocam HR) and filter set 10 (excitation 450–490 nm, emission 515–565 nm) Fluorescence intensities in the PRs were measured on digital images using Axiovision Rel. 4.8 software within circles of 38 μm radii. This analysis was carried out three times with 10 root tips examined (n = 10).

Determination of *S*-nitrosothiol Contents

The amount of SNO was quantified by Sievers 280i NO analyser (GE Analytical Instruments, Boulder, CO, USA) according to Kolbert et al. (2019b). Briefly, 250 mg of *Arabidopsis* seedlings was mixed with double volume of 1× PBS buffer (containing 10 mM N-ethylmaleimide and 2.5 mM EDTA, pH 7.4) and were grounded using Fast Prep ® Instrument (Savant Instruments Inc., Holbrook, NY). Samples were centrifuged twice for 15 min (20,000 g, 4°C). The supernatants were incubated with 20 mM sulphanilamide. 250 μl of the samples was injected into the reaction vessel filled with potassium iodide. SNO concentrations were quantified with the help of NO analysis software (v3.2). Measurement of SNO levels was performed on three separate plant generations with five technical replicates in each (n = 5).

Western Blot Analysis of GSNOR Protein Abundance

Whole *Arabidopsis* seedlings were grounded with extraction buffer (50 mM TRIS-HCl, pH 7.6–7.8) and centrifuged (4°C, 9300 g, 20 min). Protein extract was treated with 1% proteinase inhibitor and stored at −80°C. Protein concentrations were determined using the Bradford (1976) assay.

Fifteen microliters of denaturated protein extract was subjected to SDS-PAGE on 12% acrylamide gel. Proteins were transferred to PVDF membranes using the wet blotting procedure (25 mA, 16 h). After that, membranes were used for cross-activity assays with rabbit polyclonal antibody against GSNOR (1:2,000). Immunodetection was performed by using affinity, isolated goat anti-rabbit IgG-alkaline phosphatase secondary antibody at a dilution of 1:10,000, and bands were visualized by using the NBT/BCIP reaction. Protein bands were quantified by Gelquant software (provided by biochemlabsolutions.com). Western blot was carried out on three separate protein extracts from independent plant generations, at least two times per extract.

Spectrophotometric Measurement of GSNOR Activity

The specific activity of GSNOR was measured by monitoring the NADH oxidation in the presence of GSNO at 340 nm (Sakamoto et al., 2002). Plant homogenate was centrifuged (14,000 g, 20 min, 4°C), and 100 μg of protein extract was incubated in 1 ml reaction buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.2 mM NADH). Data are expressed as nmol NADH min^{-1} mg $protein^{-1}$. This measurement was performed on three separate plant generations with five technical replicates in each (n = 5).

Quantitative Real Time PCR Analysis

The expression rates of *Arabidopsis* genes *(NIA1, NIA2, GLB1, GLB2, GSNOR1, CCD7, CCD8, D14, MAX1, MAX2)* were determined by quantitative real-time reverse transcription PCR (RT-qPCR). RNA was purified from 90 mg of 7-day-old seedlings by using a NucleoSpin RNA Plant mini spin kit (Macherey-Nagel) according to the manufacturer's instruction. Furthermore, an additional DNAase digestion and purifying step was applied (ZYMO Research), and cDNA was synthetized using RevertAid reverse transcriptase. Primer3 software was used for designing primers. The primers used for RT-qPCR analyses are listed in **Table S1**. The expression rates of the NO- and SL associated genes were detected by quantitative real time PCR machine (qTOWER 2.0, Jena Instruments) using SYBR Green PCR Master Mix (Thermo Mix) (Gallé et al., 2009). Data were analyzed by using qPCRsoft3.2 software (Jena Instruments). Data were normalized to the transcript levels of the control samples; *ACTIN2* (At3918780) and *GAPDH2* (At1913440) were used as internal controls (Papdi et al., 2008). Each reaction was carried out in three replicates using cDNA synthesized from independently extracted RNAs. These analyses were performed on three separate plant generations with three technical replicates in each (n = 3).

Measurement of NO Liberation Capacity of GSNO

NO-sensitive electrode (ISO-NOP 2 mm, World Precision Instrument) was calibrated using a method of Zhang (2004). Donor solution (1 ml 250 μM GSNO in distilled water) was prepared and placed under illumination (150 μmol m^{-2} s^{-1}) in the greenhouse in order to stimulate conditions similar to treatment conditions. To ensure constant mixing of the solution magnetic stirrer was applied during the measurement. NO concentration (nM) was calculated from a standard curve. The standard curve and the results are presented in **Figure S2**. This measurement was carried out three times with three technical replicates in each (n = 3).

Statistical Analysis

All results are expressed as mean ± SE. Graphs were prepared in Microsoft Excel 2010 and in SigmaPlot 12. For statistical analysis, Duncan's multiple range test (one-way ANOVA, $P \leq 0.05$) was used in SigmaPlot 12. For the assumptions of ANOVA, we used Hartley's F_{max} test for homogeneity and the Shapiro–Wilk normality test.

RESULTS AND DISCUSSION

Root System of GSNOR- and SL Mutant *Arabidopsis* Seedlings

Compared to the wild-type (Col-0), the PR of *gsnor1-3* mutant was by 57% shorter; its root system contained very few LRs, and consequently its LR density was low (**Figure 1**) indicating that GSNOR activity is necessary for normal root development (Lee et al., 2008; Holzmeister et al., 2011; Kwon et al., 2012; Shi et al., 2015). Similarly, *35S:FLAG-GSNOR1* seedlings had shortened PRs and reduced numbers of laterals resulting in WT-like LR density, and the LR primordia to emerged LR ratio was similar to that of Col-0. As for the *max1-1* mutant, WT-like PR length was accompanied by increased number of emerged LRs and by consequently enhanced LR density compared to Col-0. The PR of *max2-1* mutant proved to be slightly (by 14%) shorter than in Col-0 and the LR number was significantly increased. The branched root systems of *max1-1* and *max2-1* suggest that MAX1-dependent SL biosynthesis and MAX2-associated SL-signaling inhibit LR development as was published previously by others (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). The LR_{prim} : LR_{em} ratio was similar in Col-0 and the mutants suggesting that SLs similarly influence both the initiation and the emergence of LRs. However, *max2-1* mutant has been proven to transmit both SL and KAR signals, thus the involvement of KAR in shaping root system architecture cannot be ruled out using this mutant (Villaécija-Aguilar et al., 2019).

Levels of NO and SNO in GSNOR- and SL Mutant *Arabidopsis* Seedlings

As shown in **Figure 2**, the level of NO and SNO in *gsnor1-3* was higher than in Col-0, while in *35S:FLAG-GSNOR1* plants, the increased endogenous NO level was accompanied by lower SNO levels than in the WT. The origin of the high NO level in the mutants is different. In *35S:FLAG-GSNOR1*, elevated nitrate content and nitrate reductase activity were observed which may result in the enhanced NO level (Frungillo et al., 2014), while in *gsnor1-3* the lack of GSNOR1 leads to enhanced SNO and consequently high NO contents. Based on these, applying *35S:FLAG-GSNOR1* mutant allows to draw conclusions about nitrate-derived NO while with the help of *gsnor1-3* mutant we can get information about the role of GSNOR-dependent NO removal. Moreover, the similar root system of the GSNOR mutants (**Figure 1**) can be explained by their high NO contents which are known to reduce auxin maximum and consequently cause PR shortening (Fernández-Marcos et al., 2011; Shi et al., 2015). In *max1-1* and *max2-1* significantly increased NO level and SNO content were detected compared to Col-0 (**Figure 2**).

Expressions of genes involved in NO metabolism (*NIA1, NIA2, GLB1, GLB2*) in *max1-1* mutants were similar to Col-0, but all examined genes were slightly down-regulated in *max2-1* (**Figure 3**). However, the changes were small and were not detectable in both *max* mutants, suggesting that these genes may not play a significant role in the regulation of NO in the absence of SLs.

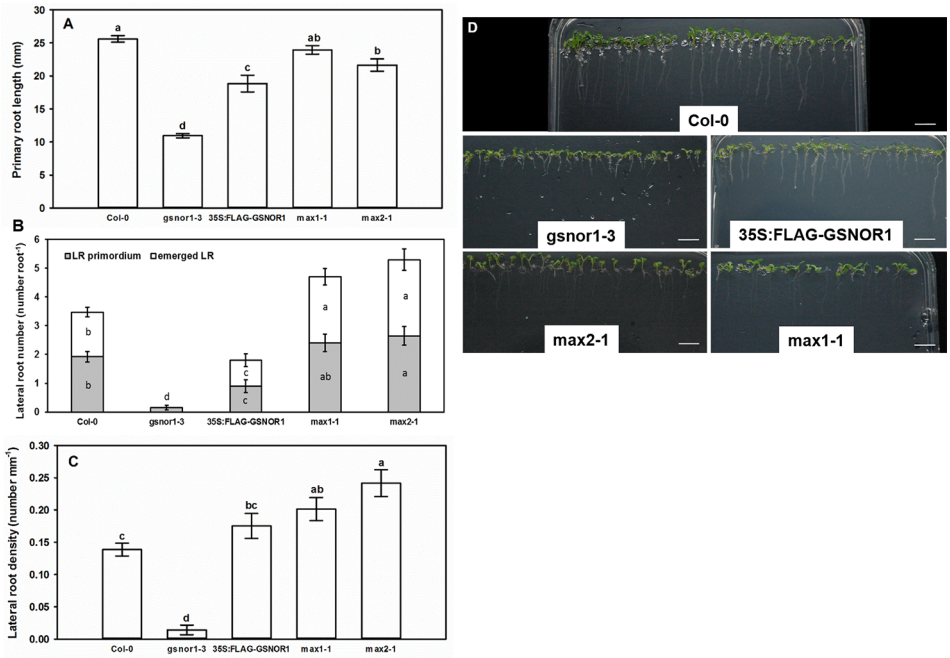

FIGURE 1 | Primary root length (mm, **A**), lateral root number (number root^{-1}, **B**) and lateral root density (number mm^{-1}, **C**) in 7-day-old Col-0, GSNOR- and SL mutant *Arabidopsis* lines grown during stress-free conditions. Different letters indicate significant differences according to Duncan's test (n = 60, P ≤ 0.05). **(D)** Representative photographs taken from 7-day-old *Arabidopsis* seedlings of different mutant lines grown on ½ MS medium. Bars = 1 cm.

Higher NO levels of the *max* mutants may be associated with higher SNO levels. GSNOR is a key regulator of SNO metabolism (Lindermayr, 2018), thus we assumed that *max* mutants show differences in association with GSNOR enzyme. Although, there were no relevant differences in the rates of *GSNOR1* expression in the plant lines (**Figure 4A**), the GSNOR protein abundance was significantly lower in *max* mutants compared to Col-0 (**Figures 4B, C**), and also the activity of the enzyme was decreased in *max1-1* and *max2-1* mutant seedlings (**Figure 4D**) which may provide the explanation for the elevated SNO and NO levels (**Figure 3**). These results indicate that SL (and/or possibly KAR) deficiency posttranscriptionally influence GSNOR enzyme resulting in decreased SNO/NO levels. As NO acts through SLs (and/or possibly KAR) to regulate root development, the effect of SL on GSNOR-regulated NO levels may be considered as compensatory feedback mechanism. Next, we examined the responses of GSNOR deficient and -overexpressing *Arabidopsis* lines to exogenous application of SL analog GR24 and SL synthesis inhibitor TIS108.

The Effect of SL Analog and Inhibitor on Root System and NO-Associated Genes in *Arabidopsis*

Similar to previously published results, GR24 treatment induced PR elongation in Col-0 *Arabidopsis* plants (Ruyter-Spira et al., 2011; Sun et al., 2014; Marzec, 2016), while TIS108 caused 50% inhibition of it (**Figure 5A**). To prove the SL-specific and non-toxic effect of TIS108 on *Arabidopsis* root, we applied GR24 together with TIS108 on Col-0 and we included *max1-1* mutant as a TIS108-resistant line (**Figure S1**). The *max1-1* mutant proved to be less sensitive to the root growth inhibiting effect of TIS108 compared to the wild-type (**Figure S1A**), and GR24 partly reversed the root shortening effect of TIS108 in Col-0 (**Figure S1B**). These indicate that the applied concentration of TIS108 is not toxic and exerts its biological effect through SLs. In case of *gsnor1-3*, SL analog did not trigger PR elongation and TIS108 reduced PR length by 67% compared to the control. These suggest that the root system of *gsnor1-3* is more sensitive to modifications of SL levels meaning that functional GSNOR enzyme is needed to control NO/SNO levels and to the positive effect of GR24 on PR elongation. Presumably, in case of GSNOR deficiency, NO/SNO levels are not properly regulated and high NO/SNO levels may cause PR shortening instead of elongation (Fernández-Marcos et al., 2011). The root elongation response of *35S:FLAG-GSNOR1* to SL analog or inhibitor did not differ from that of Col-0 indicating that overexpressing GSNOR enzyme or nitrate-derived NO has no effect on SL-induced elongation (**Figure 5A**). Treatment with GR24 resulted in reduced LR_{em} number and unchanged LR_{prim} number (**Figure 5B**) suggesting that SLs influence LR emergence but not LR initiation. In GSNOR overexpressing line, GR24-induced inhibition of LR emergence proved to be more pronounced than in Col-0. Additionally, in the stunted root system of *gsnor1-3*, the number of LR primordia was completely reduced by GR24. These results regarding the inhibitory effect of SL analog GR24 support previously published results (Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Arite et al., 2012; De Cuyper et al., 2015; Marzec, 2016). However, without using different GR24 stereoisomers we cannot exclude the possibility that

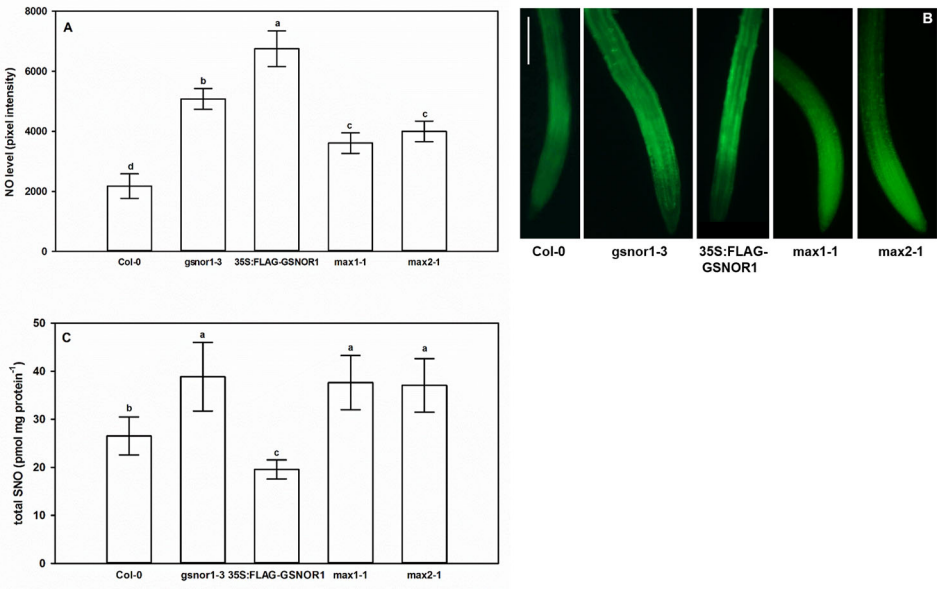

FIGURE 2 | Nitric oxide levels (pixel intensity, **A**) and SNO levels (pmol mg protein^{-1}, **C**) in Col-0, GSNOR- and SL mutant *Arabidopsis* seedlings grown during stress-free conditions for 7 days. Different letters indicate significant differences according to Duncan's test (n = 10 or 5, P ≤ 0.05). **(B)** Representative microscopic images showing DAF-FM DA-stained root tips of examined *Arabidopsis* lines. Bar = 100 μm.

rac-GR24 may interact with KAI2 thus interfering KAR signal transduction (Scaffidi et al., 2014) and consequently influencing root development (Villaécija-Aguilar et al., 2019). In Col-0 roots, TIS108 decreased the number of both staged-LRs, but in *35S:FLAG-GSNOR1* it increased the number of LR primordia. Based on these we can assume that in case of normal GSNOR level reduced SL level inhibits LR initiation, while in the presence of increased GSNOR activity or nitrate-derived NO SL inhibition leads to the

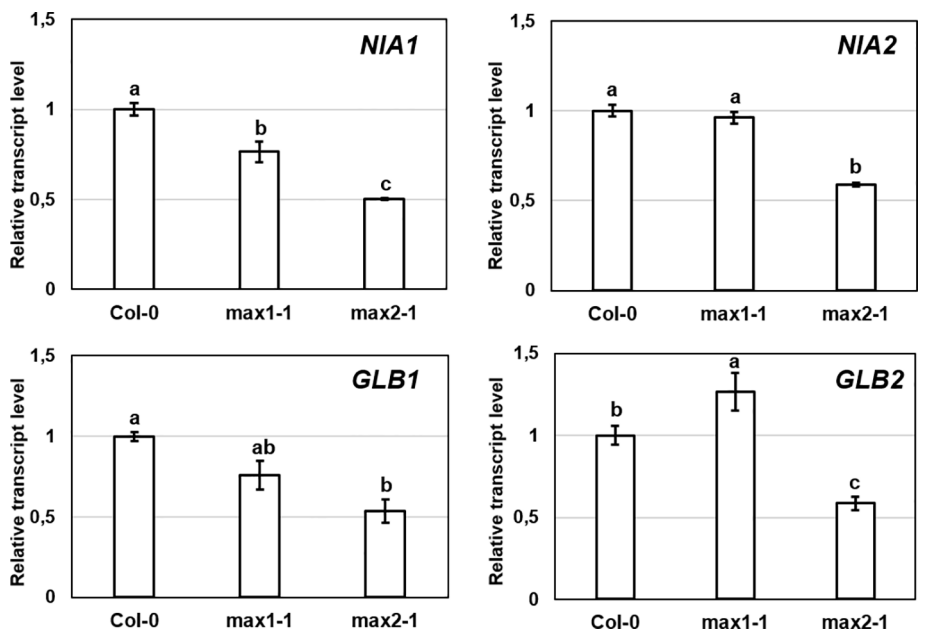

FIGURE 3 | Relative transcript level of selected NO-associated genes (*NIA1, NIA2, GLB1, GLB2*) in control Col-0, *max1-1* and *max2-1 Arabidopsis* seedlings. Different letters indicate significant differences according to Duncan's test (n = 3, P ≤ 0.05). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

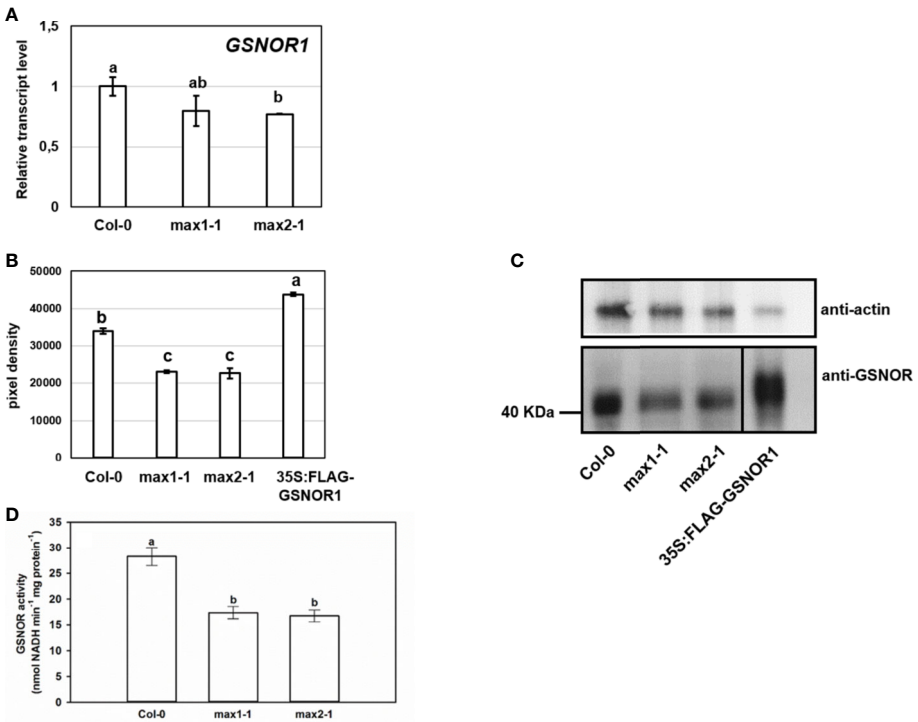

FIGURE 4 | Relative transcript level **(A)** of *GSNOR1* in Col-0, *max1-1* and *max2-1* seedlings. **(B, C)** Protein abundance of GSNOR in *max* mutants and *35S:FLAG-GSNOR1* (as a positive control). Anti-actin was used as a loading control. **(D)** GSNOR activity (nmol NADH min^{-1} mg $protein^{-1}$) in Col-0, *max1-1* and *max2-1* seedlings. Different letters indicate significant differences according to Duncan's test (n = 3 or 5, $P \leq 0.05$).

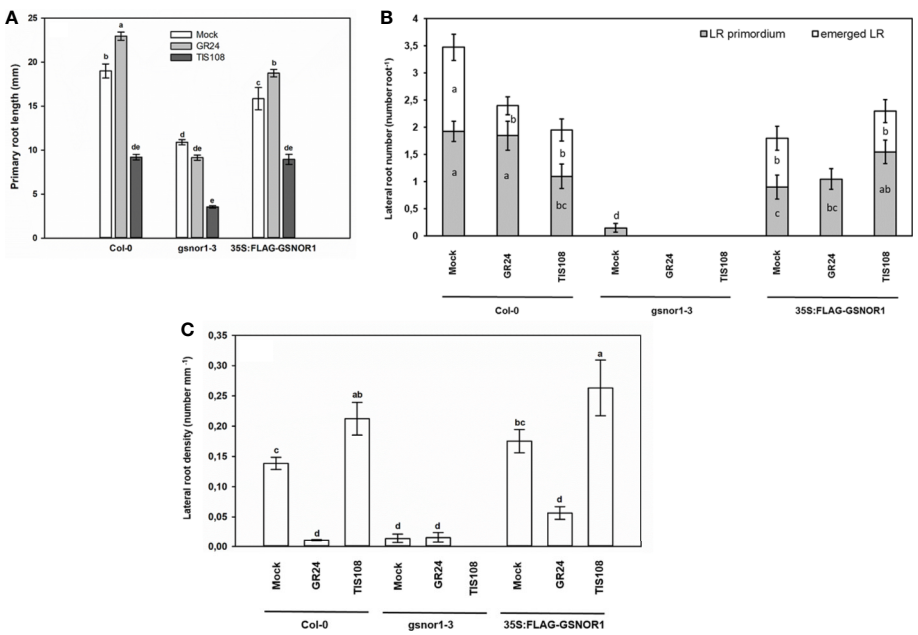

FIGURE 5 | Primary root length (mm, **A**), lateral root number (number $root^{-1}$, **B**) and lateral root density (number mm^{-1}, **C**) in Col-0, *gsnor1-3* and *35S:FLAG-GSNOR1 Arabidopsis* seedlings grown in the absence (Mock) or in the presence of GR24 (2 μM) or TIS108 (5 μM). Different letters indicate significant differences according to Duncan's test (n = 60, $P \leq 0.05$).

induction of LR initiation. These signal interactions may be complex and the knowledge of other contributing factors would be necessary to fully explain the observed effects. It can be a concern that the effect of the analog and the inhibitor is not always the opposite. At the same time, it is conceivable that an optimal SL level is needed for normal root growth. Increasing (by the addition of GR24) or lowering (by the addition of TIS108) the optimal SL level may result in similarly inhibited growth processes.

Treatment with GR24 resulted in significantly increased NO content in *Arabidopsis* roots (Kolbert, 2019). As for NO-associated genes, the expressions of *NIA1* and *NIA2* as well as *GSNOR1* didn't show any relevant modification in the presence of GR24 (**Figure 6**). In contrast, nitrogen regulatory protein P-II homolog (*GLB1*) and non-symbiotic hemoglobin 2 (*GLB2*) genes were upregulated by GR24. The *GLB* genes encode plant hemoglobins which may act as NO scavengers (Hebelstrup and Jensen, 2008; Hebelstrup et al., 2012; Mira et al., 2015). In this experimental system; however, *GLB1* and *GLB2* upregulation induced by GR24 did not lead to NO scavenging, but instead GR24 induced NO production (Kolbert, 2019). This seems to be an interesting contradiction that needs further research.

The Effect of NO Donor and Scavenger on SL-Associated Genes and Root System of *Arabidopsis*

We were interested also in reverse interplay, *i.e.*, whether under- or overproduction of GSNOR enzyme affects the expression of SL-associated genes (**Figure 7**). The examined genes (*CCD7, CCD8, MAX1*) involved in the synthesis of SLs showed down-regulation in GSNOR-deficient *Arabidopsis* compared to Col-0. This indicates that in case of low GSNOR activity, SL biosynthesis is inhibited. This further supports the interaction between GSNO metabolism and SL production in *Arabidopsis*. In addition, *CCD7* was down-regulated also in GSNOR overproducing *35S:FLAG-GSNOR1* seedlings. In contrast, the expressions of SL signaling genes (*D14* and *MAX2*) were not altered by GSNOR deficiency or overproduction. However, this was not supported by pharmacological treatments (GSNO or cPTIO), because we didn't observe relevant up- or downregulation of SL-associated genes (*CCD7, CCD8, MAX1, MAX2, D14*) in the presence of NO donor (GSNO) or scavenger (cPTIO) treatments (**Figure 8**). However, Castillo et al. (2018) observed larger induction in the expression of MAX1 and MAX2 in *Arabidopsis* seedlings due to NO treatment. From the applied 250 μM GSNO solution approx. 220 nM NO liberated over 15 min during the same circumstances as the plant treatments took place (**Figure S2**).

To further investigate this interaction, GSNO and cPTIO treatments were applied, and the responses of *max* mutants were examined (**Figure 9**). Exogenous GSNO treatment resulted in 50% root shortening in Col-0, whereas this effect was absent in *max* mutants suggesting that the examined SL (and KAR) mutants are GSNO-insensitive and that SLs (and/or possibly KAR) are needed for GSNO-induced root shortening. Similar results were obtained in *Arabidopsis* hypocotyls, where NO-triggered shortening was not observed in *max1*, *max2* and *max4* mutants (Castillo et al., 2018). According to Fernández-Marcos et al. (2011) GSNO inhibits root meristem activity through the reduction of PIN1-dependent auxin transport. Since SLs were proved to negatively regulate PIN proteins in *Arabidopsis* roots (Ruyter-Spira et al., 2011), we can assume that GSNO may exert its effect on PINs *via* inducing SL (and/or possibly KAR) synthesis and/or signaling; although the link between NO, PINs and SL (and KAR) should be clarified by future research. The NO scavenger cPTIO shortened PRs to a similar extent in all three plant lines (*Col-0, max1-1, max2-1*). Moreover, GSNO inhibited LR initiation and slightly increased LR emergence of Col-0, while cPTIO supplementation decreased the number of both types of LR. In *max1-1* and *max2-1* seedlings, LR emergence seemed to be insensitive to NO donor or scavenger. However, GSNO treatment caused reduction in the number of LR primordia of the *max1-1* mutant, and cPTIO treatment decreased LR initiation in both *max* mutants. Just like the matching effects of SL analog and inhibitor, the effects of NO donor and scavenger proved also to be often similar to each other, indicating the necessity of an optimal NO level for optimal root development.

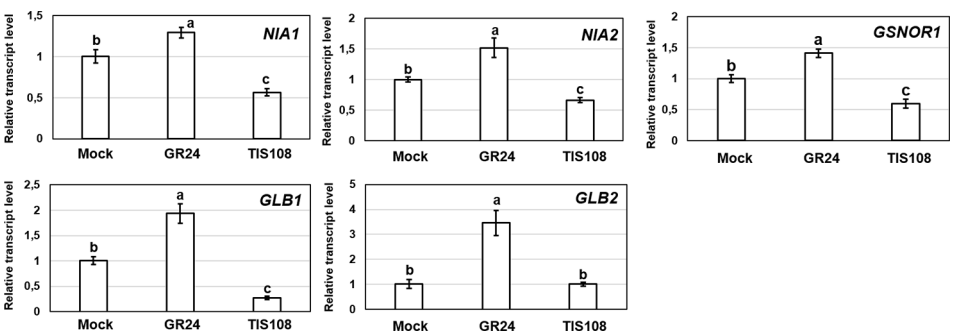

FIGURE 6 | Relative transcript level of selected NO-associated genes (*NIA1, NIA2, GSNOR1, GLB1, GLB2*) in Col-0 *Arabidopsis* grown under without (Mock) or with GR24 (2 μM) or TIS108 (5 μM). Different letters indicate significant differences according to Duncan's test ($n = 3$, $P \leq 0.05$). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

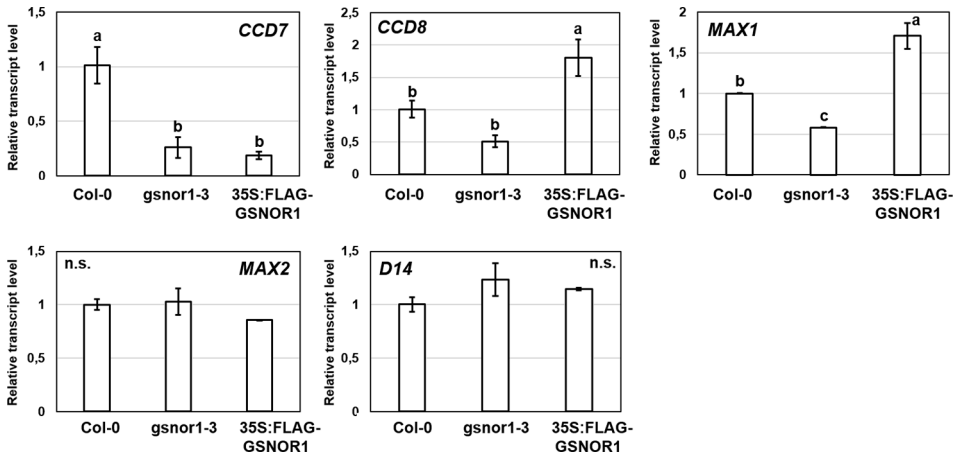

FIGURE 7 | Relative transcript level of selected SL-associated genes in Col-0, *gsnor1-3* and *35S:FLAG-GSNOR1 Arabidopsis* seedlings grown during stress-free conditions. Different letters indicate significant differences according to Duncan's test ($n = 3$, $P \leq 0.05$). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

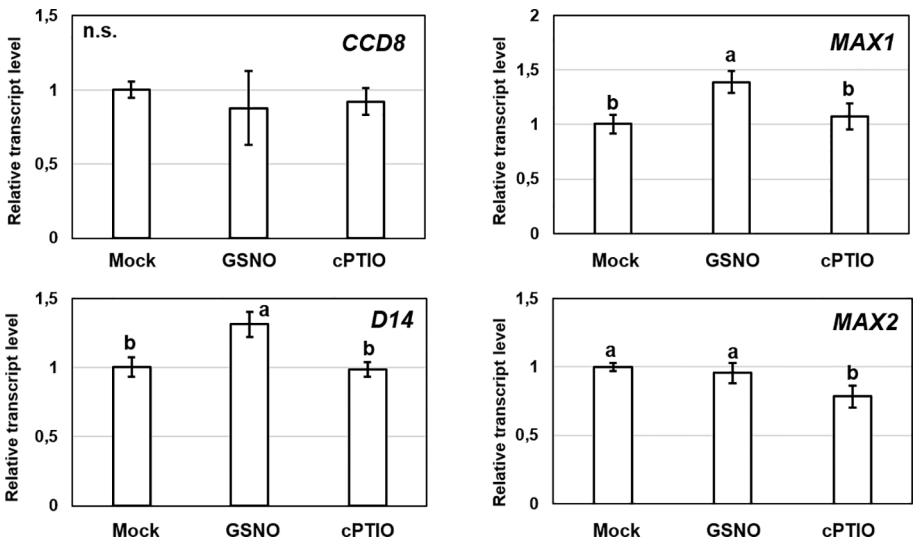

FIGURE 8 | Relative transcript level of selected SL-associated genes (*CCD7, CCD8, MAX1, MAX2, D14*) in Col-0 *Arabidopsis* grown in the absence (Mock) or in the presence of GSNO (250 μM) or cPTIO (800 μM). Different letters indicate significant differences according to Duncan's test ($n = 3$, $P \leq 0.05$). Data were normalized using the *A. thaliana ACTIN2* and *GAPDH2* genes as internal controls. The relative transcript level in Col-0 control samples was arbitrarily considered to be 1 for each gene.

CONCLUSION

The majority of the articles dealing with SL–NO interplay uses pharmacological approach and focuses on the root system of crops grown with special nutrient supply (excess nitrate or nitrogen- or phosphor deficiency). This study combines molecular biological and pharmacological approaches in order to reveal interactions between NO and SLs as growth regulating signals in the model plant *Arabidopsis thaliana* grown in stress-free conditions. As this study used *max2-1* mutant and rac-GR24, the observed effects might be non-specific to SL signaling, and the involvement of KAR signal pathway in this system cannot be ruled out. We observed for the first time that SL (and/or KAR)-deficiency resulted in elevated NO and SNO levels due to decreased GSNOR protein abundance and activity indicating that there is a signal interaction between SLs (and/or KAR) and GSNOR-regulated levels of NO/SNO. This was further supported by the down-regulation of SL biosynthetic genes (*CCD7, CCD8* and *MAX1*) in *gsnor1-3* containing elevated NO/SNO levels. Based on the more pronounced sensitivity of *gsnor1-3* to GR24, we suspected that functional GSNOR is needed to control

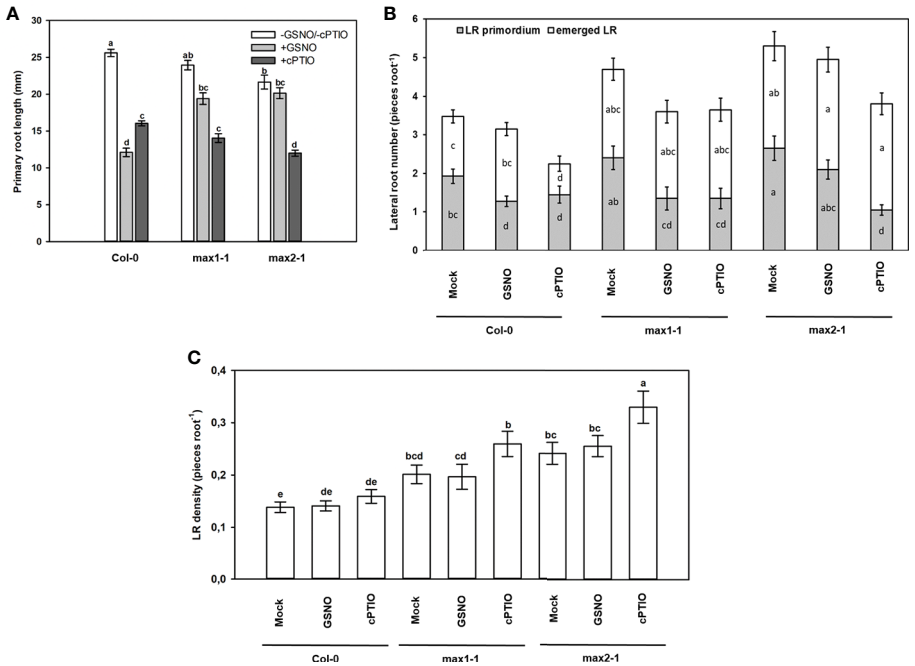

FIGURE 9 | Primary root length (mm, **A**), lateral root number (number root^{-1}, **B**) and lateral root density (number mm^{-1}, **C**) in Col-0, *max1-1, max2-1 Arabidopsis* seedlings grown in the absence (Mock) or in the presence of GSNO (250 μM) or cPTIO (800 μM) for 3 days. Different letters indicate significant differences according to Duncan's test (n = 20, P ≤ 0.05).

NO/SNO levels during SL (and/or KAR)-induced PR elongation. Furthermore, SLs (and/or KAR) may be involved in GSNO-regulated PR shortening as suggested by the relative insensitivity of *max1-1* and *max2-1* mutants to exogenous GSNO. Collectively, our results indicate for the first time a connection between SL (and/or KAR) and GSNOR-regulated NO/SNO signals in *Arabidopsis thaliana* roots. Future studies should reveal the SL- or KAR-specificity of interactions with NO using *d14* and *kai2* mutants and GR24 stereoisomers. In the future, the possible involvement of auxin signaling as a common interacting factor of NO and SL during root development should also be examined. Additional research efforts should focus on the possible role of NO-dependent posttranslational modifications (S-nitrosation, tyrosine nitration) in relation to SL-regulated plant development.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

DO performed the experiments and wrote the manuscript draft. GF performed the experiments and reviewed the manuscript. ÁM performed the experiments. AÖ performed experiments and reviewed the manuscript. ZK conceptualized the research, designed and directed the project, reviewed the manuscript draft, and wrote the final manuscript.

FUNDING

This work was financed by the National Research, Development and Innovation Fund [Grant no. NKFI-6, K120383]. ZK was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences [Grant no. BO/00751/16/8]. DO was supported by UNKP-19-3-SZTE-201 New National Excellence Program of the Ministry for Innovation and Technology. Some of the experiments were carried out by ZK during a 3-month-long visit at the Institute of Biochemical Plant Pathology, Helmholtz Zentrum München supported by TEMPUS Foundation in the frame of the Hungarian Eötvös Scholarship (MAEÖ-1060-4/2017).

ACKNOWLEDGMENTS

The authors thank Éva Kapásné Török and Elke Mattes for her valuable assistance during the experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01019/full#supplementary-material

REFERENCES

Al-Babili, S., and Bouwmeester, H. J. (2015). Strigolactones, a novel carotenoid-derived plant hormone. *Annu. Rev. Plant Biol.* 66, 161–186. doi: 10.1146/annurev-arplant-043014-114759

Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., et al. (2012). The path from beta-carotene to carlactone, a strigolactone-like plant hormone. *Science* 335, 1348–1351. doi: 10.1126/science.1218094

Arite, T., Kameoka, H., and Kyozuka, J. (2012). Strigolactone Positively Controls Crown Root Elongation in Rice. *J. Plant Growth. Regul.* 31, 165–172. doi: 10.1007/s00344-011-9228-6

Astier, J., and Lindermayr, C. (2012). Nitric oxide-dependent posttranslational modification in plants: An update. *Int. J. Mol. Sci.* 13, 15193–15208. doi: 10.3390/ijms131115193

Astier, J., Besson-Bard, A., Wawer, I., Parent, C., Rasul, S., Jeandroz, S., et al. (2011). "Nitric Oxide Signalling in Plants: Cross-Talk with Ca^{2+}, Protein Kinases and Reactive Oxygen Species," in *Annual Plant Reviews book series, Volume 42: Nitrogen Metabolism in Plants in the Post-genomic Era* (New York, USA: Wiley).

Barroso, J. B., Corpas, F. J., Carreras, A., Rodríguez-Serrano, M., Esteban, F. J., Fernández-Ocaña, A., et al. (2006). Localization of *S*-nitrosoglutathione and expression of *S*-nitrosoglutathione reductase in pea plants under cadmium stress. *J. Exp. Bot.* 57, 1785–1793. doi: 10.1093/jxb/erj175

Bharti, N., and Bhatla, S. C. (2015). Nitric oxide mediates strigolactone signaling in auxin and ethylene-sensitive lateral root formation in sunflower seedlings. *Plant Signal. Behav.* 10, e1054087. doi: 10.1080/15592324.2015.1054087

Bouwmeester, H. J., Fonne-Pfister, R., Screpanti, C., and De Mesmaeke, A. (2019). Strigolactones: Plant Hormones with Promising Features . *Angew. Chem. Int.* Ed. 58, 12778–12786. doi: 10.1002/anie.201901626

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Brewer, P. B., Yoneyama, K., Filardo, F., Meyers, E., Scaffidi, A., Frickey, T., et al. (2016). LATERAL BRANCHING OXIDOREDUCTASE acts in the final stages of strigolactone biosynthesis in Arabidopsis. *Proc. Natl. Acad. Sci.* 113, 6301–6306. doi: 10.1073/pnas.1601729113

Castillo, M.-C., Coego, A., Costa-Broseta, Á., and León, J. (2018). Nitric oxide responses in *Arabidopsis* hypocotyls are mediated by diverse phytohormone pathways. *J. Exp. Bot.* 69, 5265–5278. doi: 10.1093/jxb/ery286

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, Á., Ocaña-Calahorro, F., Mariscal, V., Carreras, A., et al. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*. *Plant Cell Environ.* 39, 2097–2107. doi: 10.1111/pce.12739

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trend. Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Chen, R., Sun, S., Wang, C., Li, Y., Liang, Y., An, F., et al. (2009). The Arabidopsis PARAQUAT RESISTANT2 gene encodes an *S*-nitrosoglutathione reductase that is a key regulator of cell death. *Cell Res.* 19 (2009), 1377–1387. doi: 10.1038/cr.2009.117

Cohen, M. F., Lamattina, L., and Yamasaki, H. (2009). "Nitric Oxide Signaling by Plant-Associated Bacteria," in *Nitric Oxide in Plant Physiology*. Eds. S. Hayat, M. Mori, J. Pichtel and A. Ahmad (Weinheim, Germany: Wiley-VCH Verlag GmbH & Co.), 161–172.

Cook, C. E., Whichard, L. P., Turner, B., Wall, M. E., and Egley, G. H. (1966). Germination of witchweed (*Striga lutea* Lour.): isolation and properties of a potent stimulant. *Science* 154, 1189–1190. doi: 10.1126/science.154.3753.1189

Cooper, J. W., Hu, Y., Beyyoudh, L., Yildiz Dasgan, H., Kunert, K., Beveridge, C. A., et al. (2018). Strigolactones positively regulate chilling tolerance in pea and in Arabidopsis. *Plant Cell Environ.* 41, 1298–1310. doi: 10.1111/pce.13147

Díaz, M., Achkor, H., Titarenko, E., and Martínez, M. C. (2003). The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. *FEBS Lett.* 543, 136–139. doi: 10.1016/S0014-5793(03)00426-5

De Cuyper, C., Fromentin, J., Yocgo, R. E., De Keyser, A., Guillotin, B., Kunert, K., et al. (2015). From lateral root density to nodule number, the strigolactone analogue GR24 shapes the root architecture of *Medicago truncatula*. *J. Exp. Bot.* 66, 137–146. doi: 10.1093/jxb/eru404

Durner, J., Gow, A. J., Stamler, J. S., and Glazebrook, J. (1999). Ancient origins of nitric oxide signaling in biological systems. *Proc. Nat. Acad. Sci. U.S.A.* 96, 14206–14207. doi: 10.1073/pnas.96.25.14206

Fancy, N. N., Bahlmann, A.-K., and Loake, G. J. (2017). Nitric oxide function in plant abiotic stress. *Plant Cell Physol.* 40, 462–472. doi: 10.1111/pce.12707

Feechan, A., Kwon, E., Yun, B. W., Wang, Y., Pallas, J. A., and Loake, G. J. (2005). A central role for *S*-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Feigl, G., Horváth, E., Molnár, Á., Oláh, D., Poór, P., and Kolbert, Zs. (2019). Ethylene-Nitric Oxide Interplay During Selenium-induced Lateral Root Emergence in Arabidopsis. *J. Plant Growth. Regul.* 38, 1481–1488. doi: 10.1007/s00344-019-09950-9

Fernández-Marcos, M., Sanz, L., Lewis, D. R., Muday, G. K., and Lorenzo, O. (2011). Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. *Proc. Natl. Acad. Sci. U. S. A.* 108, 18506–18511. doi: 10.1073/pnas.1108644108

Foo, E., Turnbull, C. G., and Beveridge, C. A. (2001). Long-distance signaling and the control of branching in the rms1 mutant of pea. *Plant Physiol.* 126, 203–209. doi: 10.1104/pp.126.1.203

Foresi, N., Correa-Aragunde, N., Parisi, G., Caló, G., Salerno, G., and Lamattina, L. (2010). Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. *Plant Cell* 22, 3816–3830. doi: 10.1105/tpc.109.073510

Foresi, N., Mayta, M. L., Lodeyro, A. F., Scuffi, D., Correa-Aragunde, N., García-Mata, C., et al. (2015). Expression of the tetrahydrofolate-dependent nitric oxide synthase from the green alga *Ostreococcus tauri* increases tolerance to abiotic stresses and influences stomatal development in *Arabidopsis*. *Plant J.* 82, 806–821. doi: 10.1111/tpj.12852

Fröhlich, A., and Durner, J. (2011). The hunt for plant nitric oxide synthase (NOS): is one really needed? *Plant Sci.* 181, 401–404. doi: 10.1016/j.plantsci.2011.07.014

Frungillo, L., Skelly, M. J., Loake, G. J., Spoel, S. H., and Salgado, I. (2014). *S*-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. *Nat. Commun.* 5, 5401. doi: 10.1038/ncomms6401

Gallé, Á., Csiszár, J., Secenji, M., Guóth, A., Cseuz, L., Tari, I., et al. (2009). Glutathione transferase activity and expression patterns during grain filling in flag leaves of wheat genotypes differing in drought tolerance: response to water deficit. *J. Plant Physiol.* 166, 1878–1891. doi: 10.1016/j.jplph.2009.05.016

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagès, V., Dun, E. A., Pillot, J. P., et al. (2008). Strigolactone inhibition of shoot branching. *Nature* 455 (7210), 189–194. doi: 10.1038/nature07271

Ha, C. V., Leyva-González, M. A., Osakabe, Y., Tran, U. T., Nishiyama, R., Watanabe, Y., et al. (2014). Positive regulatory role of strigolactone in plant responses to drought and salt stress. *Proc. Natl. Acad. Sci.* 111, 851–856. doi: 10.1073/pnas.1322135111

Hancock, J. T., and Neill, S. (2019). Nitric Oxide: its generation and interactions with other reactive signaling compounds. *Plants* 8, E41. doi: 10.3390/plants8020041

Hebelstrup, K. H., and Jensen, E. O. (2008). Expression of NO scavenging hemoglobin is involved in the timing of bolting in *Arabidopsis thaliana*. *Planta* 227, 917–927. doi: 10.1007/s00425-007-0667-z

Hebelstrup, K. H., van Zanten, M., Mandon, J., Voesenek, L. A. C. J., Harren, F. J. M., Cristescu, S. M., et al. (2012). Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in Arabidopsis thaliana. *J. Ex. Bot.* 63, 5581–5591. doi: 10.1093/jxb/ers210

Holzmeister, C., Fröhlich, A., Sarioglu, H., Bauer, N., Durner, J., and Lindermayr, C. (2011). Proteomic analysis of defense response of wildtype *Arabidopsis thaliana* and plants with impaired NO- homeostasis. *Proteom* 11, 1664–1683. doi: 10.1002/pmic.201000652

Jahnová, J., Luhová, L., and Petřivalský, M. (2019). *S*-Nitrosoglutathione Reductase—The Master Regulator of Protein *S*-Nitrosation in Plant NO Signaling. *Plants* 8, 48. doi: 10.3390/plants80200482019

Jeandroz, S., Wipf, D., Stuehr, D. J., Lamattina, L., Melkonian, M., Tian, Z., et al. (2016). Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. *Sci. Signal.* 9, re2. doi: 10.1126/scisignal.aad4403

Jia, K.-P., Li, C., Bouwmeester, H. J., and Al-Babili, S. (2019). "Strigolactone Biosynthesis and Signal Transduction," in *Strigolactones - Biology and Applications*. Eds. H. Koltai and C. Prandi (Switzerland: Springer Nature), 1–45.

Jiao, C., Yang, R., Wang, P., Tian, L., and Gu, Z. (2018). Mitogen-activated protein kinase mediates nitric oxide-induced isoflavone accumulation in soybean sprouts under UVB radiation. *Can. J. Plant Sci.* 98, 54–61. doi: 10.1139/cjps-2016-0369

Kapulnik, Y., Delaux, P. M., Resnick, N., Mayzlish-Gati, E., Wininger, S., Bhattacharya, C., et al. (2011). Strigolactones affect lateral root formation and root-hair elongation in *Arabidopsis*. *Planta* 233, 209–216. doi: 10.1007/s00425-010-1310-y

Kohlen, W., Charnikhova, T., Liu, Q., Bours, R., Domagalska, M. A., Beguerie, S., et al. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. *Plant Physiol.* 155, 974–987. doi: 10.1104/pp.110.164640

Kolbert, Zs., and Feigl, G. (2017). "Cross-talk of Reactive Oxygen Species and Nitric Oxide in Various Processes of Plant Development," in *Reactive Oxygen Species in Plants: Boon Or Bane - Revisiting the Role of ROS*. Eds. V. P. Singh, S. Singh, D. K. Tripathi, S. M. Prasad and D. K. Chauhan (Chichester, UK: John Wiley & Sons, Ltd), 261–289.

Kolbert, Zs., Pető, A., Lehotai, N., Feigl, G., Ördög, A., and Erdei, L. (2012). *In vivo* and *in vitro* studies on fluorophore-specificity. *Acta Biol. Szeged.* 56, 37–41.

Kolbert, Zs., Barroso, J. B., Brouquisse, R., Corpas, F. J., Gupta, K. J., Lindermayr, C., et al. (2019a). A forty-year journey: The generation and roles of NO in plants. *Nitric. Oxide* 93, 53–70. doi: 10.1016/j.niox.2019.09.006

Kolbert, Zs., Molnár, Á., Oláh, D., Feigl, G., Horváth, E., Erdei, L., et al. (2019b). *S*-Nitrosothiol Signaling Is involved in Regulating Hydrogen Peroxide Metabolism of Zinc-Stressed Arabidopsis. *Plant Cell Physiol.* 60, 2449–2463. doi: 10.1093/pcp/pcz138

Kolbert, Zs. (2019). Strigolactone-nitric oxide interplay in plants: The story has just begun. *Physiol. Plant* 165, 487–497. doi: 10.1111/ppl.12712

Kwon, E., Feechan, A., Yun, B.-W., Hwang, B.-H., Pallas, J. A., Kang, J.-G., et al. (2012). AtGSNOR1 function is required for multiple developmental programs in *Arabidopsis*. *Planta* 236, 887–900. doi: 10.1007/s00425-012-1697-8

Lanteri, M. L., Laxalt, A. M., and Lamattina, L. (2008). Nitric oxide triggers phosphatidic acid accumulation via phospholipase d during auxin-induced adventitious root formation in cucumber. *Plant Physiol.* 147, 188–198. doi: 10.1104/pp.107.111815

Lee, U., Wie, C., Fernandez, B. O., Feelisch, M., and Vierling, E. (2008). Modulation of nitrosative stress by *S*-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. *Plant Cell* 20, 786–802. doi: 10.1105/tpc.107.052647

Li, S., Chen, L., Li, Y., Yao, R., Wang, F., Yang, M., et al. (2016). Effect of GR24 stereoisomers on plant development in *Arabidopsis*. *Mol. Plant* 9, 1432–1435. doi: 10.1016/j.molp.2016.06.012

Lindermayr, C. (2018). Crosstalk between reactive oxygen species and nitric oxide in plants: Key role of *S*-nitrosoglutathione reductase. *Free Rad. Biol. Med.* 122, 110–115. doi: 10.1016/j.freeradbiomed.2017.11.027

Malamy, J. E., and Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Dev* 124, 33–44.

Manoli, A., Trevisan, S., Voigt, B., Yokawa, K., Baluška, F., and Quaggiotti, S. (2016). Nitric oxide-mediated maize root apex responses to nitrate are regulated by auxin and strigolactones. *Front. Plant Sci.* 6, 1269. doi: 10.3389/fpls.2015.01269

Marzec, M., and Melzer, M. (2018). Regulation of root development and architecture by strigolactones under optimal and nutrient deficiency conditions. *Int. J. Mol. Sci.* 19, 1887. doi: 10.3390/ijms19071887

Marzec, M. (2016). Perception and signaling of strigolactones. *Front. Plant Sci.* 7, 1260. doi: 10.3389/fpls.2016.01260

Mira, M. M., Adel, E.-S., and Stasolla, C. (2015). Ethylene is integrated into the nitric oxide regulation of Arabidopsis somatic embryogenesis. *J. Genet. Eng. Biotechnol.* 13, 7–17. doi: 10.1016/j.jgeb.2015.01.001

Mori, N., Nomura, T., and Akiyama, K. (2020). Identification of two oxygenase genes involved in the respective biosynthetic pathways of canonical and non-canonical strigolactones in *Lotus japonicus*. *Planta* 251, 40. doi: 10.1007/s00425-019-03332-x

Mostofa, M. G., Weiqiang Li, W., Nguyen, K. H., Fujita, M., and Phan Tran, L.-S. (2018). Strigolactones in plant adaptation to abiotic stresses: An emerging avenue of plant research. *Plant Cell Environ.* 41, 2227–2243. doi: 10.1111/pce.13364

Nelson, D. C., Scaffidi, A., Dun, E. A., Waters, M. T., Flematti, G. R., Dixon, K. W., et al. (2011). F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.* 108, 8897–8902. doi: 10.1073/pnas.1100987108

Pagnussat, G. C., Lanteri, M. L., Lombardo, M. C., and Lamattina, L. (2004). Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. *Plant Physiol.* 135, 279–286. doi: 10.1104/pp.103.038554

Pandey, A., Sharma, M., and Pandey, G. K. (2016). Emerging Roles of Strigolactones in Plant Responses to Stress and Development. *Front. Plant Sci.* 7, 434. doi: 10.3389/fpls.2016.00434

Papdi, C., Ábrahám, E., Joseph, M. P., Popescu, C., Koncz, C., and Szabados, L. (2008). Functional identification of *Arabidopsis* stress regulatory genes using the controlled cDNA overexpression system. *Plant Physiol.* 147, 528–542. doi: 10.1104/pp.108.116897

Rodríguez-Ruiz, M., Zuccarelli, R., Palma, J. M., Corpas, F. J., and Freschi, L. (2019). "Biotechnological Application of Nitric Oxide and Hydrogen Peroxide in Plants," in *Nitric Oxide and Hydrogen Peroxide Signaling in Higher Plants*. Eds. D. Gupta, J. Palma and F. Corpas (Cham: Springer), 245–270.

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., et al. (2011). Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in Arabidopsis: another belowground role for strigolactones? *Plant Physiol.* 155, 721–734. doi: 10.1104/pp.110.166645

Sakamoto, A., Ueda, M., and Morikawa, H. (2002). *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. *FEBS Lett.* 515, 20–24. doi: 10.1016/S0014-5793(02)02414-6

Santolini, J., Andre, F., Jeandroz, S., and Wendehenne, D. (2017). Nitric oxide synthase in plants: Where do we stand? *Nitric. Oxide* 63, 30–38. doi: 10.1016/j.niox.2016.09.005

Scaffidi, A., Waters, M. T., Sun, Y. K., Skelton, B. W., Dixon, K. W., Ghisalberti, E. L., et al. (2014). Strigolactone hormones and their stereoisomers signal through two related receptor proteins to induce different physiological responses in *Arabidopsis*. *Plant Physiol.* 165, 1221–1232. doi: 10.1104/pp.114.240036

Seto, Y., Yasui, R., Kameoka, H., Tamiru, M., Cao, M., Terauchi, R., et al. (2019). Strigolactone perception and deactivation by a hydrolase receptor DWARF14. *Nat. Commun.* 10, 191. doi: 10.1038/s41467-018-08124-7

Shabek, N., Ticchiarelli, F., Mao, H., Hinds, T. R., Leyser, O., and Zheng, N. (2018). Structural plasticity of D3-D14 ubiquitin ligase in strigolactone signalling. *Nature* 563 (7733), 652–656. doi: 10.1038/s41586-018-0743-5

Shi, Y.-F., Wang, D.-L., Wang, C., Hendrickson Culler, A., Kreiser, M. A., Suresh, J., et al. (2015). Loss of *GSNOR1* function leads to compromised auxin signaling and polar auxin transport. *Mol. Plant* 8, 1350–1365. doi: 10.1016/j.molp.2015.04.008

Soundappan, I., Bennett, T., Morffy, N., Liang, Y., Stanga, J. P., Abbas, A., et al. (2015). SMAX1-LIKE/D53 family members enable distinct MAX2-dependent responses to strigolactones and karrikins in *Arabidopsis*. *Plant Cell* 27, 3143–3159. doi: 10.1105/tpc.15.00562

Stirnberg, P., van De Sande, K., and Leyser, H. M. O. (2002). MAX1 and MAX2 control shoot lateral branching in Arabidopsis. *Dev* 129, 1131–1141.

Sun, H., Tao, J., Liu, S., Huang, S., Chen, S., Xie, X., et al. (2014). Strigolactones are involved in phosphate- and nitrate-deficiency-induced root development and auxin transport in rice. *J. Exp. Bot.* 65, 6735–6746. doi: 10.1093/jxb/eru029

Sun, H., Bi, Y., Tao, J., Huang, S., Hou, M., Xue, X., et al. (2016). Strigolactones are required for nitric oxide to induce root elongation in response to nitrogen and phosphate deficiencies in rice. *Plant Cell Environ* 39, 1473–1484.

Takahashi, I., and Asami, T. (2018). Target-based selectivity of strigolactone agonists and antagonists in plants and their potential use in agriculture. *J. Exp. Bot.* 69, 2241–2254. doi: 10.1093/jxb/ery126

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., et al. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature* 455, 195–200. doi: 10.1038/nature07272

Villaécija-Aguilar, J. A., Hamon-Josse, M., Carbonnel, S., Kretschmar, A., Schmidt, C., Dawid, C., et al. (2019). SMAX1/SMXL2 regulate root and root hair development downstream of KAI2-mediated signalling in Arabidopsis. *PloS Genet.* 15, e1008327. doi: 10.1371/journal.pgen.1008327

Vurro, M., Prandi, C., and Baroccio, F. (2016). Strigolactones: how far is their commercial use for agricultural purposes? *Pest Manage Sci.* 72, 2026–2034. doi: 10.1002/ps.4254

Wakabayashi, T., Hamana, M., Mori, A., Akiyama, R., Ueno, K., Osakabe, K., et al. (2019). Direct conversion of carlactonoic acid to orobanchol by cytochrome P450 CYP722C in strigolactone biosynthesis. *Sci. Adv.* 5, eaax9067. doi: 10.1126/sciadv.aax9067

Walker, C. H., Siu-Ting, K., Taylor,, O'Connell, A. M. J., and Bennett, T. (2019). Strigolactone synthesis is ancestral in land plants, but canonical strigolactone signalling is a flowering plant innovation. *BMC Biol.* 17, 70. doi: 10.1186/s12915-019-0689-6

Wang, Q., Ni, J., Shah, F., Liu, W., Wang, D., Yao, Y., et al. (2019). Overexpression of the Stress-Inducible SsMAX2 Promotes Drought and Salt Resistance via the Regulation of Redox Homeostasis in Arabidopsis. *Int. J. Mol. Sci.* 20, 837. doi: 10.3390/ijms20040837

Waters, M. T., Gutjahr, C., Bennett, T., and Nelson, D. C. (2017). Strigolactone signaling and evolution. *Annu. Rev. Plant Biol.* 68, 291–322. doi: 10.1146/annurev-arplant-042916-040925

Weisslocker-Schaetzel, M., Andre, F., Touazi, N., Foresi, N., Lembrouk, M., Dorlet, P., et al. (2017). The NOS-like protein from the microalgae *Ostreococcus tauri* is a genuine and ultrafast NO-producing enzyme. *Plant Sci.* 265, 100–111. doi: 10.1016/j.plantsci.2017.09.019

Yoneyama, K., Akiyama, K., Brewer, P. B., Mori, N., Kawano-Kawada, M., Haruta, S., et al. (2020). Hydroxyl carlactone derivatives are predominant strigolactones in *Arabidopsis*. *Plant Direct* 4, e00219. doi: 10.1002/pld3.219

Zhang, X. (2004). Real time and in vivo monitoring of nitric oxide by electrochemical sensors from dream to reality. *Front. Biosci.* 9, 3434–3446. doi: 10.2741/1492

Zwanenburg, B., and Blanco-Ania, D. (2018). Strigolactones: new plant hormones in the spotlight. *J. Exp. Bot.* 69, 2205–2218. doi: 10.1093/jxb/erx487

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.

frontiers in Plant Science

MINI REVIEW
published: 08 July 2020
doi: 10.3389/fpls.2020.01011

Effect of Nitrogen Reactive Compounds on Aging in Seed

Katarzyna Ciacka†, Urszula Krasuska†, Pawel Staszek†, Agnieszka Wal, Joanna Zak and Agnieszka Gniazdowska†*

Department of Plant Physiology, Institute of Biology, Warsaw University of Life Sciences—SGGW, Warsaw, Poland

Reactive nitrogen species (RNS) are universal compounds that are constantly present in plant cells. RNS function depends on their actual level (the "nitrosative door" concept), duration of plant exposure to RNS and the context of the exposure. RNS are involved in the nitration of nucleic acids and fatty acids, posttranslational protein modifications (nitration and *S*-nitrosylation), and modulation of reactive oxygen species metabolism. RNS are regulatory molecules of various physiological processes in plants, including seed formation, maturation, dormancy and germination. The free radical theory of aging, well documented for animals, indicated that RNS participate in the regulation of the life span. Some data point to RNS contribution in preservation of seed vigor and/or regulation of seed longevity. Seed aging is a problem for biologists and agriculture, which could be solved by application of RNS, as a factor that may potentially expand seed vitality resulting in increased germination rate. The review is focused on RNS, particularly nitric oxide contribution to regulation of seed aging.

Keywords: germination, nitric oxide, seed viability, aging, vigor

OPEN ACCESS

Edited by:
Marek Petrivalsky,
Palacký University, Olomouc, Czechia

Reviewed by:
Natalia V. Bykova,
Agriculture and Agri-Food Canada (AAFC), Canada
Petr Smýkal,
Palacký University, Olomouc, Czechia

***Correspondence:**
Urszula Krasuska
urszula_krasuska@sggw.edu.pl

***ORCID:**
Katarzyna Ciacka
orcid.org/0000-0003-1485-2901
Urszula Krasuska
orcid.org/0000-0001-8373-4509
Pawel Staszek
orcid.org/0000-0001-8532-5151
Agnieszka Gniazdowska
orcid.org/0000-0003-0077-3743

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *07 February 2020*
Accepted: *19 June 2020*
Published: *08 July 2020*

Citation:
Ciacka K, Krasuska U, Staszek P, Wal A, Zak J and Gniazdowska A (2020) Effect of Nitrogen Reactive Compounds on Aging in Seed. Front. Plant Sci. 11:1011. doi: 10.3389/fpls.2020.01011

INTRODUCTION

Climate change causes weather extremes that influence plant mortality therefore, impacts biodiversity. Seed quality has an important bearing on the fate of the whole plant, and its development and lifespan. Seeds are the basis of plant production, the ultimate source of all food for humans and animals. The quality of seeds strongly influences the growth of the mature plants and determines their survival under environmental stress conditions.

The typical length of time that a seed survives (lifespan) varies among plant species. Seed longevity describes the length of time that seeds can remain viable and is an important factor for seedbanks in soil, seeds stored in warehouses, and the industries of seed production and sale (Walters et al., 2010; Sano et al., 2016). Moreover, seed lifespan is imprinted in the genes and influences efforts to preserve gene diversity in seedbanks (Walters et al., 2010).

Seed longevity depends on internal and external factors (Sano et al., 2016). First of all, it is governed by the ability of seeds to withstand desiccation during maturation. *Recalcitrant*-type seeds are intolerant of water loss and are the most sensitive to aging, which is a major problem in their storage. *Orthodox*-type seeds are able to withstand low water content and are characterized by decreased metabolic activity. During long-term storage, seed longevity is determined by internal moisture content, external humidity, temperature, and oxygen pressure (Walters et al., 2010; Sano et al., 2016). Inappropriate storage conditions reduce seeds' viability, and/or ability to germinate. Therefore, seed aging is associated with a

reduction in longevity, mostly due to disturbances in metabolism and accumulation of harmful metabolites. Commonly, seed aging is associated with a loss of membrane integrity, modifications of nucleic acids, DNA degradation, impairment of protein and RNA synthesis, decreased energy metabolism (El-Maarouf-Bouteau et al., 2011 and citation therein; Corbineau, 2012). Furthermore, uncontrolled reactive oxygen species (ROS) generation, and inefficient antioxidant machinery are involved in the loss of seed vigor and viability (Bailly et al., 2008) (**Figure 1A**).

Seed aging is rather a slow process in the majority of plant species. Thus, artificial seed deterioration, including controlled deterioration treatment (CDT) or accelerated aging, are frequently applied in

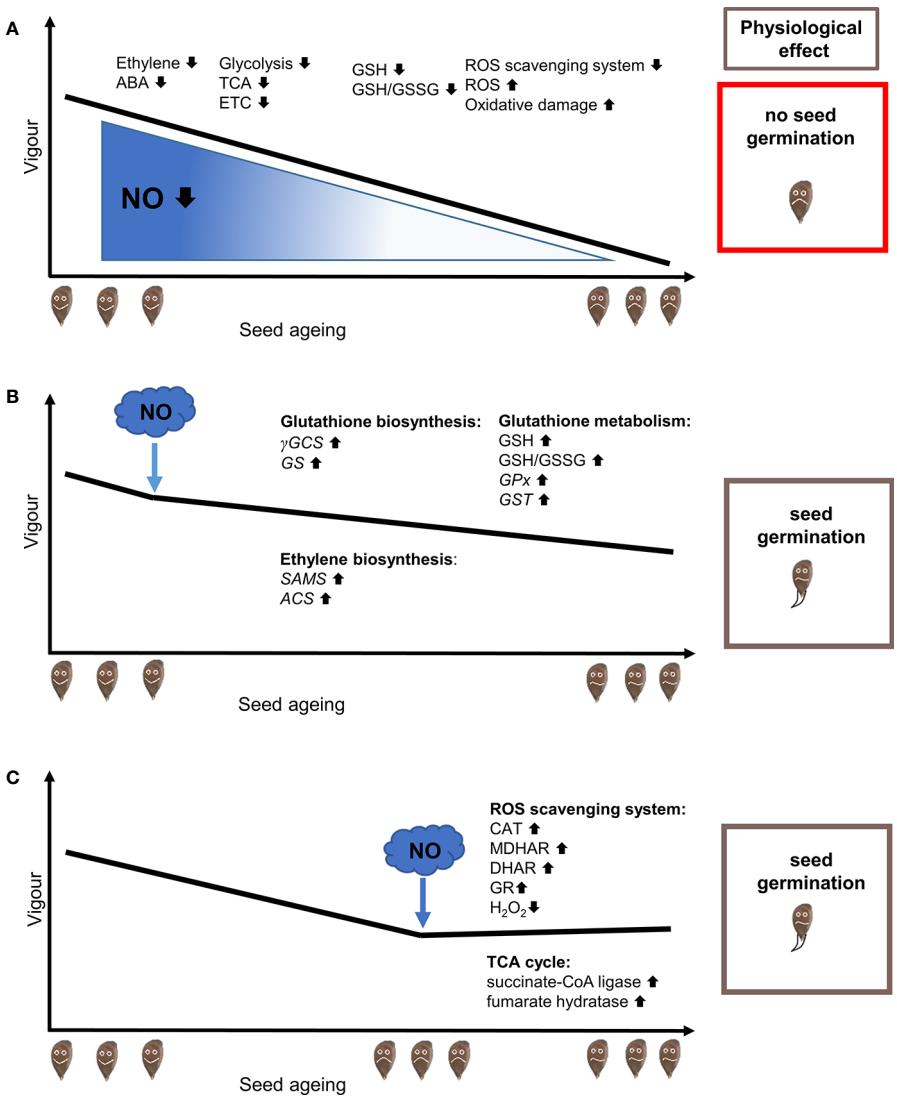

FIGURE 1 | RNS application to maintain seed quality in aged seeds subjected to controlled deteriorated treatment (CDT) mitigates reduction of endogenous NO level. **(A)** A decrease in seed vigor during the aging process is induced by CDT. Seed aging is linked to ROS accumulation, due to impaired ROS generation and ROS scavenging by the antioxidant system, decreased GSH pool and GSH/GSSG ratio (Bailly et al., 2008; Ratajczak et al., 2019), disturbances in phytohormonal balance—decreased ethylene and ABA level (Sano et al., 2016) and down-regulation of the proteins involved in glycolysis, tricarboxylic acid (TCA) cycle, the electron transport chain (ETC) and oxidative phosphorylation (Xin et al., 2011). **(B)** The application of nitric oxide (NO) or NO donors before CDT activates protecting mechanisms that prevent the reduction of seed vigor. NO pre-treatment of the seeds subjected to CDT improves their quality by (i) increasing of GSH level and GSH/GSSG ration, (ii) up-regulation of the expression of genes encoding γ-glutamylcysteine synthetase (γGCS), glutathione synthetase (GS), glutathione *S*-transferase (GST), glutathione peroxidase (GPx), (iii) stimulation of methionine metabolism due to up-regulation of the transcription of the genes encoding *S*-adenosyl-L-methionine synthetase (SAMS) and 1-aminocyclopropane-1-carboxylic acid synthase (ACS) enzymes of ethylene biosynthetic pathway (He et al., 2018). **(C)** The application of NO or NO donors during CDT improves seed vigor by inducing processes that lead to the initiation of repair of oxidative damages, mainly in mitochondria. NO stimulates (i) activity of enzymatic antioxidant system in mitochondria: catalase (CAT), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) (ii) leading to decreased H_2O_2 content in aged seeds, (iii) improves also mitochondrial function in aged seeds by enrichment of some proteins of TCA cycle: succinate-CoA ligase (ADP-forming) subunit and fumarate hydratase (Mao et al., 2018).

laboratory practice using established protocols (Bailly et al., 2008; El-Maarouf-Bouteau et al., 2011). Both treatments are based on application of sub-optimal conditions: elevated temperature and high humidity. The difference between CDT and accelerated aging is that during CDT seeds are imbibed to a precise water content prior to the warm temperature treatment (Corbineau, 2012). Such treatments are commonly performed to accelerate aging and produce uniformly aged seeds that are easy to examine and compare in scientific research. However, specific conditions of CDT cause some differences between artificially and naturally aged seeds. The present review addresses the role of reactive nitrogen species (RNS) in the regulation of aging in seeds, mostly in the context of CDT application.

A BRIEF INTRODUCTION INTO NITRIC OXIDE (NO) CELLULAR SOURCES AND BIOSYNTHESIS IN PLANTS

For a long time, NO was recognized mainly as a pollutant of anthropogenic origin. Thereafter, it was discovered to be important in many physiological processes. NO was hailed as the "Molecule of the Year" by the journal *Science* in 1992. Experiments to discover NO biochemistry and its mode of action in living organisms were intensified after the 1998 Nobel Prize in Physiology and Medicine awarded for work on NO as a signaling molecule in the cardiovascular system. It was demonstrated that NO is emitted by plants, a discovery that launched 40 years of intensive research. The fine scheme of the milestone publications related to NO study in plants was done by Kolbert et al. (2019) and Del Castello et al. (2019).

NO, and compounds that are formed from the interaction of NO with oxygen or superoxide anion ($O_2^{\bullet-}$) known as reactive nitrogen species (RNS), are generated in almost every cellular compartment (Durzan and Pedroso, 2002 and citation therein).

Biochemistry of NO is linked to the formation of its different redox forms: nitrosonium cation (NO^+), nitric oxide ($^{\bullet}NO$), and nitroxyl anion (NO^-). In aqueous solution $O_2^{\bullet-}$, which belongs to ROS, rapidly reacts with NO and gives rise to peroxynitrite ($ONOO^-$) and its protonated form—peroxinitrous acid (ONOOH) (Stamler et al., 1992 and citation therein; Durzan and Pedroso, 2002 and citation therein). NO also reacts with transition metal ions and other free radicals, and therefore it functions as an important regulator of metabolic processes (Stamler et al., 1992 and citation therein).

NO is synthesized *via* enzymatic and non-enzymatic pathways, which can be assigned to oxidative and reductive pathways. The best characterized and most acceptable enzymatic pathway of NO biosynthesis is a reaction catalyzed by nitrate reductase (NR) (Mohn et al., 2019). A recent report points on the primary function of the NIA1 isoform of NR as an NO-producing enzyme in Arabidopsis (Mohn et al., 2019). Moreover, some other molybdenum cofactor (Moco) containing enzymes are proposed to be involved in NO synthesis or homeostasis (Bender and Schwarz, 2018). An open question is whether higher plants possess an enzyme that is homologous to the mammalian NO synthase (NOS). The only known case of such an enzyme in the plant kingdom was found and extensively examined in a green alga, *Ostreococcus tauri* (Foresi et al., 2010). Although NOS-like activity (a reaction requiring all the cofactors and co-substrates of the mammalian NOS) has been measured in higher plants, no NOS homologues in genomes or transcriptomes of land plants were found in available databases (Kolbert et al., 2019). Doubts about the presence of L-arginine and the oxygen-dependent enzyme responsible for NO formation (like in mammalian NOS) led to the proposal that the term "NOS-like" be renamed "nitric oxide generating (NOG)" (Hancock and Neill, 2019).

The main non-enzymatic source of NO, and other reactive molecules derived from NO, is nitrite. Under acidic conditions, nitrite is protonated to nitrous acid (HNO_2), which undergoes decomposition into different nitrogen oxides (NOx), depending on the redox state of the local environment of the reaction (Yamasaki, 2000; Rocha et al., 2011). The rate of NO release from nitrite is increased in the presence of a mild reductants (e.g., ascorbic acid) (Yamasaki, 2000).

In seeds, only a few pathways of NO generation are discussed. During germination, NO production is thought to start shortly after imbibition and to correlate with oxygen depletion (Bykova et al., 2015). Under low oxygen conditions, mitochondria are one of the main producers of NO in plant cells (Gupta et al., 2011); therefore, the mitochondrial electron transport chain could be a potential source of NO in seeds at early stages of germination. The non-enzymatic reduction of nitrite to NO has been demonstrated in the apoplast of the aleurone layers of germinating barley (*Hordeum vulgare* L.) caryopsis (Bethke, 2004).

The lowered oxygen concentration in germinating seeds promotes NO formation by NR. This pathway was confirmed in sorghum (*Sorghum bicolor* L.) and tomato (*Solanum lycopersicum* L.) seeds (Simontacchi et al., 2004; Lara et al., 2014).

Another putative mechanism of NO synthesis in oxygen-limiting conditions is a reaction catalyzed by xanthine oxidoreductase (Godber et al., 2000). During the first stages of germination, due to limited oxygen access, the oxidative routes of NO biosynthesis (hydroxylamine oxidation, NOS-like pathway) are unlikely. However, after the radical protrusion these pathways can be prevailing.

NO—A CRUCIAL REGULATOR OF SEED GERMINATION AND PLANT SENESCENCE

NO plays a beneficial role as the universal regulatory molecule in plant physiology. In seed biology, its function is concentration-dependent and can be described by the model of the "nitrosative door" (Krasuska et al., 2015a), which is comparable to the concept of "oxidative window for germination" associated with critical range of ROS level (Bailly et al., 2008). Many previous studies have indicated the importance of NO in the regulation of seed dormancy and the transition from a dormant to non-dormant state (Šírová et al., 2011; Arc et al., 2013a; Bykova et al., 2015). The NO mode of action and its cross-talk with plant hormones in seeds have also been previously summarized (Arc et al., 2013b; Krasuska et al., 2015a; Sanz et al., 2015). Therefore, there is no doubt that NO is the modulator of seed germination,

and the positive effect of this molecule on dormancy breakage has been observed. NO and its donors were used to break seed dormancy, or accelerate germination and improve the vigor of developing seedlings (He et al., 2014; Shams et al., 2018). Exogenous NO is also used in seed stratification treatment (Liu et al., 2019) to promote dormancy alleviation and enable faster germination.

At the molecular level, RNS are responsible for *S*-nitrosylation, tyrosine and tryptophan nitration of proteins, as well as nitration of fatty acids and nucleic acids (Corpas et al., 2015; Mata-Pérez et al., 2017; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2019). Although such changes may constitute an integral signal transduction mechanism, in excess they cause degenerative processes linked to senescence, accelerated aging, or even cell death.

Evidence was provided that NO could be involved in the regulation of plant senescence. Treatment of soybean (*Glycine max* L.) cotyledons with NO deferred their aging *via* (among other effects) the stabilization of photosynthetic pigments (Jasid et al., 2009). In leaves, RNS contribute to the senescence process *via* interaction with ROS (Hung and Kao, 2003). Low level of NO caused by the expression of bacterial NO dioxygenase (NOD, the enzyme that converts NO into nitrate in the presence of oxygen) in Arabidopsis led to a senescence-like phenotype (Mishina et al., 2007). Fumigation of NOD-type plants with NO reduced the senescence phenotype, indicating that RNS delay senescence in leaves. There are no NODs in plants, nevertheless, the action of this enzyme is comparable to the hemoglobins' (Hbs) (Gardner et al., 1998). Plants nonsymbiotic Hbs (nHbs) decrease NO concentration (Perazzolli et al., 2004; Igamberdiev et al., 2011). There is a high probability that dioxygenase reaction of the system involving nHbs may also regulate plant senescence.

RNS IN SEED AGING

An imbalance in cellular homeostasis and the time-dependent persistent alterations in the structure and function of biomolecules lead to the accumulation of cellular damages. These changes are universal features of aging in all living organisms, and they have been observed in aged seeds. Seed deterioration is a progressive, irreversible decrease in seed longevity accompanied by alterations of the nucleic acid structure (DNA fragmentation, chromosomal aberration, telomere length change, DNA methylation), lower capacity of the antioxidant system, and loss of membrane integrity. Deterioration process is also linked to the protein inactivation due to a variety of mechanisms, including non-enzymatic glycation through Amadori–Maillard reactions, oxidation of sulfhydryl groups, conversion of amino acids within the protein leading to partial folding or unfolding, dissociation to monomers or subunits, and condensation to polymers (El-Maarouf-Bouteau et al., 2011; Hu et al., 2012; Fu et al., 2015).

The loss of seed vigor is manifested in a reduced germination rate, reduced number of seedlings, and increased number of abnormal seedlings. In laboratory conditions acceleration of seed aging is obtained through application of different aging treatments e.g. CDT. CDT was used in studies focused on establishing the role of RNS in the maintenance of seed quality. He et al. (2018) investigated NO action in the regulation of aging in elm (*Ulmus pumila* L.) seeds induced by CDT (37°C and 100% relative humidity). CDT decreased the vigor of seeds to 50% after 2 days, but application of sodium nitroprusside (SNP) before CDT supported high vigor (**Figure 1B**). Furthermore, the treatment of elm seeds with SNP before CDT significantly increased their germination rate. A burst of NO was observed at the beginning of the CDT aging of seeds, and the endogenous NO content decreased as CDT progressed (He et al., 2018).

Similar data were reported for apple (*Malus domestica* Borkh.) embryos isolated from warm stratified seeds (subjected to accelerated aging). The maximum level of NO emissions occurred after 21 days of the treatment and was followed by the decline of NO emissions to the 70th day (Dębska et al., 2013). In general, apple seeds require a long period (3 months) of cold stratification for dormancy alleviation. Apple embryos that germinated after seeds were aged by warm stratification formed fewer seedlings, which also had developmental malformations (Dębska et al., 2013). It was previously demonstrated that developmental abnormalities can be reversed after NO fumigation (Krasuska et al., 2015b).

Oxidative damages due to the excessive formation of ROS are widely accepted to be the major contributors to seeds deterioration leading to their aging (Bailly et al., 2008; Kurek et al., 2019). Detoxification mechanisms, including antioxidant metabolites and enzymes responsible for modulation of the ROS content, limit oxidative damages to proteins, lipids, and nucleic acids. Therefore, NO plays a role in the regulation of aging in seeds by counteracting ROS generation and stimulating the antioxidant system (**Figures 1B, C**). ROS have many deleterious effects on mitochondrial membranes, resulting in the release of cytochrome *c* into the cytosol to activate apoptotic cell death during the loss of seed viability (reviewed by Fu et al., 2015). Mitochondrial DNA is susceptible to ROS-induced damages, which lead to dysfunction of the organelles and are considered to be a major component of seed aging.

Mao et al. (2018) described the accumulation of H_2O_2 in mitochondria in artificially aged oat (*Avena sativa* L.) seeds that exhibited low vigor. The application of SNP to aged oat seeds had a protective effect, improving seed vigor and increasing ROS scavenging ability in mitochondria (**Figure 1C**). Higher activities of catalase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase in the ascorbate–glutathione (AsA–GSH) antioxidant system were also noticed. The activity of mitochondrial complex IV in the aged oat seeds decreased, but after application of NO, the activity increased to the level found in a non-aged caryopsis (Mao et al., 2018). This was a striking finding, because NO is considered an inhibitor of cytochrome *c* oxidase. Mao et al. (2018) suggested that alternative oxidase was stimulated by NO, which may partly explain the decline in ROS production. In addition, protein abundance levels of some tricarboxylic acid cycle (TCA) enzymes, succinyl-CoA ligase and fumarate hydratase, in mitochondria from aged oat seeds increased after SNP treatment (**Figure 1C**). Thus, it was proposed that the NO treatment of aged seeds, could increase the capacity of some reactions of the TCA cycle and also the AsA–GSH cycle, leading to a lowering of the mitochondrial H_2O_2 content (Mao et al., 2018).

GSH is a marker of seed vigor, and a low GSH/GSSG ratio is linked to seed aging and loss of vigor (Kranner et al., 2006) (**Figure 1A**). SNP pre-treatment of elm seeds subjected to CDT induced the expression of genes that encoded enzymes of the glutathione biosynthetic pathway and led to an increased level of GSH, which can protect seeds from oxidative damages resulting from ROS over-accumulation (He et al., 2018).

The vigor of aged seeds depends on ethylene emission and seeds' sensitivity to this hormone (Siriwitayawan et al., 2003). The physiological state and germination potential of seeds are linked to ethylene-NO cross-talk, ethylene biosynthesis, and signaling (Arc et al., 2013b). The interaction of NO and ethylene during dormancy release in apple embryos was demonstrated (Gniazdowska et al., 2010). Embryos sensitivity to this hormone (regarded as a beneficial germination factor) increased after short-time fumigation with NO. In the context of seed aging, it was shown that pre-treatment of elm seeds (before CDT) with NO donors (SNP, GSNO) prevented a drastic decrease of expression in genes encoding ethylene biosynthesis enzymes: *S*-adenosyl-L-methionine (SAM) synthetase and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (He et al., 2018) (**Figure 1B**). Taking into account that, on one hand, ethylene promoted germination of Arabidopsis seeds subjected to salinity stress (which induced oxidative stress), but on the other hand, SNP upregulated *ACS2* expression, resulting in a lower H_2O_2 level (Lin et al., 2013), it appears that the NO-ethylene synergistic interaction delays seed aging caused by oxidative stress.

In contrast, high ethylene emission typically occurs during senescence of leaves or petals and during fruit ripening (Li et al., 2013). ACC in the presence of NO can be converted *via* non-enzymatic reaction to ethylene (Gniazdowska et al., 2010). This raises the question whether an over-accumulation of RNS and ROS could stimulate non-enzymatic ethylene emission during seed aging (even during dormancy), which may accelerate deterioration.

SAM is the metabolite that links biosynthesis of NO, ethylene, and polyamines (PAs) (Krasuska et al., 2014; Krasuska et al., 2016). PAs are regulators of plant growth and development that also modulate seed aging. Their content generally decreases during seed aging (Matilla, 1996), and they are known to maintain vigor and viability during accelerated aging (Yalamalle et al., 2019). A reduction in the PAs level seems to be a significant prelude to senescence signals (Chen et al., 2019). In contrast, germination of apple embryos induced by NO fumigation was associated with increased activity of polyamine oxidase (PAO) (Krasuska et al., 2014). Conversion of spermine by PAO results in liberation of H_2O_2, the key molecule for seed dormancy breakage. Thus, NO-stimulated catabolism of PAs in seed aging is unwelcome, while NO-dependent H_2O_2 formation due to an enhancement of PAO activity may be beneficial during seed germination. However, prolonged NO action could be potentially destructive because it stimulates the generation of harmful ROS. So again, whether NO plays a positive or negative role in seed biology, including acceleration of aging, is concentration- and time-dependent.

Seed germination, vigor, and aging were linked to ABA control (Arc et al., 2013b). In dormant apple embryos, NO fumigation reduced sensitivity to ABA (Gniazdowska et al., 2007) as well as expression of the transcription factor abscisic acid insensitive 5 (*ABI5*) (Andryka-Dudek et al., 2019). Schausberger et al. (2019) used seeds of two lines of Chinese kale (*Brassica oleracea* L.) with different ABA content to show that a low endogenous ABA level increased sensitivity of seeds to artificial aging. In ABA-deficient or insensitive Arabidopsis mutants, reduction of seed longevity was clearly associated with a lack of dormancy (Sano et al., 2016).

NO participates in post-translational protein modifications (PTMs), including *S*-nitrosylation of proteins, which was suggested to play a beneficial role in seed germination. In Arabidopsis seeds, *S*-nitrosylation of ABI5 promoted germination by stimulating ABI5 protein degradation (Albertos et al., 2015). Furthermore, markedly decreased levels of SNO-proteins during prolonged seed aging can be recognized as a signal for selective protein degradation, similar to that observed for ABI5 (Albertos et al., 2015). Protein *S*-nitrosylation is a reversible PTM that was shown to enhance the activities of antioxidant enzymes, thereby reducing cellular ROS levels. Therefore, *S*-nitrosylation can indirectly protect against uncontrolled protein carbonylation (an irreversible PTM occurring as a reaction to oxidative stress) (Bai et al., 2011). It was demonstrated in recalcitrant *Antiaris toxicaria* Lesch. seeds, that pre-treatment with NO stimulated germination by causing *S*-nitrosylation of antioxidant enzymes, which modified their activities (Bai et al., 2011).

In elm seeds, *S*-nitrosylated proteins were accumulated at an early stage of aging (He et al., 2018). The study of He et al. (2018) showed that *S*-nitrosylation in elm seeds affected proteins of carbohydrate metabolism that participate in glycolysis, the mitochondrial TCA cycle, and pentose phosphate pathways. The authors concluded that *S*-nitrosylation, and therefore NO signaling, can be one of the mechanisms involved in regulation of deterioration process in seeds.

CONCLUSIONS

Aging leads to a decrease in the quality of seeds (**Figure 1A**), which limits not only agricultural production but also the preservation of global biodiversity. Studies aiming to understand the mechanisms underlying seed aging and the associated decrease in seed quality employ the artificial accelerated aging approaches that allow to obtain a pool of equally aged seeds. Treatments that effectively protect seeds against aging or prevent seed deterioration require further evaluation.

NO and other compounds belonging to the RNS family appear to mitigate the negative effects of seed aging. Treatment of seeds with NO or NO donors before induction of aging, or at the initial stages of aging (**Figures 1B, C**), activates the antioxidant system, which delays or prevents the initiation of mechanisms that induce aging. The application of NO donors at some stages of the aging process activates defence mechanisms (e.g., reversible redox PTMs, AsA–GSH cycle), which lead to an improvement in seed quality (**Figure 1C**), even if the aging process was activated earlier. Although evidence has been provided that NO can partially

prevent seed deterioration caused by aging, its application in the seed industry requires further research.

AUTHOR CONTRIBUTIONS

Conceptualization: UK, KC, PS, AG. Writing—original draft preparation: UK, KC, AG. Writing—editing and figure preparation: PS, JZ, AW. Supervision: AG. Funding acquisition: UK. All authors contributed to the article and approved the submitted version.

FUNDING

This work was performed during realization of the project financed by National Science Centre, Poland 2016/23/B/NZ9/03462 given to UK.

ACKNOWLEDGMENTS

The authors are sorry to all the colleagues whose papers were not cited in this mini-review due to space limitation.

REFERENCES

Albertos, P., Romero-Puertas, M. C., Tatematsu, K., Mateos, I., Sánchez-Vicente, I., Nambare, E., et al. (2015). *S*-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth. *Nat. Commun.* 6, 8669. doi: 10.1038/ncomms9669

Andryka-Dudek, P., Ciacka, K., Wiśniewska, A., Bogatek, R., and Gniazdowska, A. (2019). Nitric oxide-induced dormancy removal of apple embryos is linked to alterations in expression of genes encoding ABA and JA biosynthetic or transduction pathways and RNA nitration. *Int. J. Mol. Sci.* 20, 1007. doi: 10.3390/ijms20051007

Arasimowicz-Jelonek, M., and Floryszak-Wieczorek, J. (2019). A physiological perspective on targets of nitration in NO-based signaling networks in plants. *J. Exp. Bot.* 70, 4379–4389. doi: 10.1093/jxb/erz300

Arc, E., Galland, M., Godin, B., Cueff, G., and Rajjou, L. (2013a). Nitric oxide implication in the control of seed dormancy and germination. *Front. Plant Sci.* 4, 346. doi: 10.3389/fpls.2013.00346

Arc, E., Sechet, J., Corbineau, F., Rajjou, L., and Marion-Poll, A. (2013b). ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination. *Front. Plant Sci.* 4, 63. doi: 10.3389/fpls.2013.00063

Bai, X., Yang, L., Tian, M., Chen, J., Shi, J., Yang, Y., et al. (2011). Nitric oxide enhances desiccation tolerance of recalcitrant antiaris toxicaria seeds via protein *S*-nitrosylation and carbonylation. *PloS One* 6, e20714. doi: 10.1371/journal.pone.0020714

Bailly, C., El-Maarouf-Bouteau, H., and Corbineau, F. (2008). From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C. R. Biol.* 331, 806–814. doi: 10.1016/j.crvi.2008.07.022

Bender, D., and Schwarz, G. (2018). Nitrite-dependent nitric oxide synthesis by molybdenum enzymes. *FEBS Lett.* 592, 2126–2139. doi: 10.1002/1873-3468.13089

Bethke, P. C. (2004). Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* 16, 332–341. doi: 10.1105/tpc.017822

Bykova, N. V., Hu, J., Ma, Z., and Igamberdiev, A. U. (2015). "The role of reactive oxygen and nitrogen species in bioenergetics, metabolism, and signaling during seed germination," in *Reactive oxygen and nitrogen species signaling and communication in plants. signaling and communication in plants*. Eds. K. J. Gupta and A. U. Igamberdiev (Cham: Springer), 177–195.

Chen, D., Shao, Q., Yin, L., Younis, A., and Zheng, B. (2019). Polyamine function in plants: Metabolism, regulation on development, and roles in abiotic stress responses. *Front. Plant Sci.* 9, 1945. doi: 10.3389/fpls.2018.01945

Corbineau, F. (2012). Markers of seed quality: from present to future. *Seed. Sci. Res.* 22, S61–S68. doi: 10.1017/S0960258511000419

Corpas, F. J., Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., and Barroso, J. B. (2015). "Nitration and S-nitrosylation: Two post-translational modifications (PTMs) mediated by reactive nitrogen species (RNS) and their role in signalling processes of plant cells," in *Reactive oxygen and nitrogen species signaling and communication in plants, signaling and communication in plants, 23rd edn.* Eds. K. J. Gupta and A. U. Igamberdiev (Cham: Springer), 267–281.

Dębska, K., Krasuska, U., Budnicka, K., Bogatek, R., and Gniazdowska, A. (2013). Dormancy removal of apple seeds by cold stratification is associated with fluctuation in H_2O_2, NO production and protein carbonylation level. *J. Plant Physiol.* 170, 480–488. doi: 10.1016/j.jplph.2012.11.018

Del Castello, F., Nejamkin, A., Cassia, R., Correa-Aragunde, N., Fernández, R., Foresi, N., et al. (2019). The era of nitric oxide in plant biology: Twenty years tying up loose ends. *Nitric. Oxide* 85, 17–27. doi: 10.1016/J.NIOX.2019.01.013

Durzan, D. J., and Pedroso, M. C. (2002). Nitric oxide and reactive nitrogen oxide species in plants. *Biotechnol. Genet. Eng. Rev.* 19, 293–338. doi: 10.1080/02648725.2002.10648032

El-Maarouf-Bouteau, H., Mazuy, C., Corbineau, F., and Bailly, C. (2011). DNA alteration and programmed cell death during ageing of sunflower seed. *J. Exp. Bot.* 62, 5003–5011. doi: 10.1093/jxb/err198

Foresi, N., Correa-Aragunde, N., Parisi, G., Calo, G., Salerno, G., and Lamattina, L. (2010). Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. *Plant Cell* 22, 3816–3830. doi: 10.1105/tpc.109.073510

Fu, Y.-B., Ahmed, Z., and Diederichsen, A. (2015). Towards a better monitoring of seed ageing under *ex situ* seed conservation. *Conserv. Physiol.* 3, cov026. doi: 10.1093/conphys/cov026

Gardner, P. R., Gardner, A. M., Martin, L. A., and Salzman, A. L. (1998). Nitric oxide dioxygenase: An enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci.* 95, 10378–10383. doi: 10.1073/pnas.95.18.10378

Gniazdowska, A., Dobrzyńska, U., Babańczyk, T., and Bogatek, R. (2007). Breaking the apple embryo dormancy by nitric oxide involves the stimulation of ethylene production. *Planta* 225, 1051–1057. doi: 10.1007/s00425-006-0384-z

Gniazdowska, A., Krasuska, U., Czajkowska, K., and Bogatek, R. (2010). Nitric oxide, hydrogen cyanide and ethylene are required in the control of germination and undisturbed development of young apple seedlings. *Plant Growth Regul.* 61, 75–84. doi: 10.1007/s10725-010-9452-2

Godber, B. L. J., Doel, J. J., Sapkota, G. P., Blake, D. R., Stevens, C. R., Eisenthal, R., et al. (2000). Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *J. Biol. Chem.* 275, 7757–7763. doi: 10.1074/jbc.275.11.7757

Gupta, K. J., Igamberdiev, A. U., Manjunatha, G., Segu, S., Moran, J. F., Neelawarne, B., et al. (2011). The emerging roles of nitric oxide (NO) in plant mitochondria. *Plant Sci.* 181, 520–526. doi: 10.1016/j.plantsci.2011.03.018

Hancock, J. T., and Neill, S. J. (2019). Nitric oxide: Its generation and interactions with other reactive signaling compounds. *Plants* 8, 41. doi: 10.3390/plants8020041

He, H., De Souza Vidigal, D., Basten Snoek, L., Schnabel, S., Nijveen, H., Hilhorst, H., et al. (2014). Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. *J. Exp. Bot.* 65, 6603–6615. doi: 10.1093/jxb/eru378

He, Y., Xue, H., Li, Y., and Wang, X. (2018). Nitric oxide alleviates cell death through protein *S*-nitrosylation and transcriptional regulation during the ageing of elm seeds. *J. Exp. Bot.* 69, 5141–5155. doi: 10.1093/jxb/ery270

Hu, D., Ma, G., Wang, Q., Yao, J., Wang, Y., Pritchard, H. W., et al. (2012). Spatial and temporal nature of reactive oxygen species production and programmed cell death in elm (*Ulmus pumila* L.) seeds during controlled deterioration. *Plant Cell Environ.* 35, 2045–2059. doi: 10.1111/j.1365-3040.2012.02535.x

Hung, K. T., and Kao, C. H. (2003). Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. *J. Plant Physiol.* 160, 871–879. doi: 10.1078/0176-1617-01118

Igamberdiev, A. U., Bykova, N. V., and Hill, R. D. (2011). Structural and functional properties of class 1 plant hemoglobins. *IUBMB Life* 63, 146–152. doi: 10.1002/iub.439

Jasid, S., Galatro, A., Villordo, J. J., Puntarulo, S., and Simontacchi, M. (2009). Role of nitric oxide in soybean cotyledon senescence. *Plant Sci.* 176, 662–668. doi: 10.1016/j.plantsci.2009.02.007

Kolbert, Z., Barroso, J. B., Brouquisse, R., Corpas, F. J., Gupta, K. J., Lindermayr, C., et al. (2019). A forty year journey: The generation and roles of NO in plants. *Nitric. Oxide* 93, 53–70. doi: 10.1016/j.niox.2019.09.006

Kranner, I., Birtić, S., Anderson, K. M., and Pritchard, H. W. (2006). Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? *Free Radic. Biol. Med.* 40, 2155–2165. doi: 10.1016/j.freeradbiomed.2006.02.013

Krasuska, U., Ciacka, K., Bogatek, R., and Gniazdowska, A. (2014). Polyamines and nitric oxide link in regulation of dormancy removal and germination of apple (*Malus domestica* Borkh.) embryos. *J. Plant Growth Regul.* 33, 590–601. doi: 10.1007/s00344-013-9408-7

Krasuska, U., Ciacka, K., Andryka-Dudek, P., Bogatek, R., and Gniazdowska, A. (2015a). "Nitrosative Door" in seed dormancy alleviation and germination," in *Reactive oxygen and nitrogen species signaling and communication in plants, Signaling and communication in plants*, *1st edn.*, vol. 23 . Eds. K. J. Gupta and A. U. Igamberdiev (Cham: Springer), 215–237.

Krasuska, U., Dębska, K., Otulak, K., Bogatek, R., and Gniazdowska, A. (2015b). Switch from heterotrophy to autotrophy of apple cotyledons depends on NO signal. *Planta* 242, 1221–1236. doi: 10.1007/s00425-015-2361-x

Krasuska, U., Ciacka, K., Orzechowski, S., Fettke, J., Bogatek, R., and Gniazdowska, A. (2016). Modification of the endogenous NO level influences apple embryos dormancy by alterations of nitrated and biotinylated protein patterns. *Planta* 244, 877–891. doi: 10.1007/s00425-016-2553-z

Kurek, K., Plitta-Michalak, B., and Ratajczak, E. (2019). Reactive oxygen species as potential drivers of the seed aging process. *Plants* 8, 174. doi: 10.3390/plants8060174

Lara, T. S., Lira, J. M. S., Rodrigues, A. C., Rakocevic, M., and Alvarenga, A. A. (2014). Potassium nitrate priming affects the activity of nitrate reductase and antioxidant enzymes in tomato germination. *J. Agric. Sci.* 6, 72–80. doi: 10.5539/jas.v6n2p72

Li, Z., Peng, J., Wen, X., and Guo, H. (2013). Ethylene-insensitive3 is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. *Plant Cell* 25, 3311–3328. doi: 10.1105/tpc.113.113340

Lin, Y., Yang, L., Paul, M., Zu, Y., and Tang, Z. (2013). Ethylene promotes germination of Arabidopsis seed under salinity by decreasing reactive oxygen species: Evidence for the involvement of nitric oxide simulated by sodium nitroprusside. *Plant Physiol. Biochem.* 73, 211–218. doi: 10.1016/j.plaphy.2013.10.003

Liu, J., Xue, T., and Shen, Y. (2019). Effect of nitric oxide on seed germination and dormancy in empress trees in horttechnology. *Horttechnology* 29, 271–275. doi: 10.21273/HORTTECH04250-18

Mao, C., Zhu, Y., Cheng, H., Yan, H., Zhao, L., Tang, J., et al. (2018). Nitric oxide regulates seedling growth and mitochondrial responses in aged oat seeds. *Int. J. Mol. Sci.* 19, 1052. doi: 10.3390/ijms19041052

Mata-Pérez, C., Sánchez-Calvo, B., Padilla, M. N., Begara-Morales, J. C., Valderrama, R., Corpas, F. J., et al. (2017). Nitro-fatty acids in plant signaling: New key mediators of nitric oxide metabolism. *Redox Biol.* 11, 554–561. doi: 10.1016/j.redox.2017.01.002

Matilla, A. J. (1996). Polyamines and seed germination. *Seed. Sci. Res.* 6, 81–93. doi: 10.1017/S096025850000310X

Mishina, T. E., Lamb, C., and Zeier, J. (2007). Expression of a nitric oxide degrading enzyme induces a senescence programme in Arabidopsis. *Plant Cell Environ.* 30, 39–52. doi: 10.1111/j.1365-3040.2006.01604.x

Mohn, M., Thaqi, B., and Fischer-Schrader, K. (2019). Isoform-specific NO synthesis by *Arabidopsis thaliana* nitrate reductase. *Plants* 8, 67. doi: 10.3390/plants8030067

Perazzolli, M., Dominici, P., Romero-Puertas, M. C., Zago, E., Zeier, J., Sonoda, M., et al. (2004). Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *Plant Cell* 16, 2785–2794. doi: 10.1105/tpc.104.025379

Ratajczak, E., Małecka, A., Ciereszko, I., and Staszak, A. (2019). Mitochondria are important determinants of the aging of seeds. *Int. J. Mol. Sci.* 20, 1568. doi: 10.3390/ijms20071568

Rocha, B. S., Gago, B., Pereira, C., Barbosa, R. M., Bartesaghi, S. O., Lundberg, J., et al. (2011). Dietary nitrite in nitric oxide biology: a redox interplay with implications for pathophysiology and therapeutics. *Curr. Drug Targets* 12, 1351–1363. doi: 10.2174/138945011796150334

Sano, N., Rajjou, L., North, H. M., Debeaujon, I., Marion-Poll, A., and Seo, M. (2016). Staying alive: molecular aspects of seed longevity. *Plant Cell Physiol.* 57, 660–674. doi: 10.1093/pcp/pcv186

Sanz, L., Albertos, P., Mateos, I., Sánchez-Vicente, L., Lechón, I., Fernández-Marcos, M., et al. (2015). Nitric oxide (NO) and phytohormones crosstalk during early plant development. *J. Exp. Bot.* 66, 2857–2868. doi: 10.1093/jxb/erv213

Schausberger, C., Roach, T., Stöggl, W., Arc, E., Finch-Savage, W. E., and Kranner, I. (2019). Abscisic acid-determined seed vigour differences do not influence redox regulation during ageing. *Biochem. J.* 476, 965–974. doi: 10.1042/BCJ20180903

Shams, M., Yildirim, E., Agar, G., Ercisli, S., Ekinci, M., Dursul, L., et al. (2018). Nitric oxide alleviates copper toxicity in germinating seed and seedling growth of *Lactuca sativa* L. *Not. Bot. Hort. Agrobot. Cluj-Napoca* 46, 167–172. doi: 10.15835/nbha46110912

Simontacchi, M., Jasid, S., and Puntarulo, S. (2004). Nitric oxide generation during early germination of sorghum seeds. *Plant Sci.* 167, 839–847. doi: 10.1016/j.plantsci.2004.05.028

Siriwitayawan, G., Downie, A. B., and Geneve, R. L. (2003). Ethylene evolution is positively correlated with seed vigor in sweet corn and tomato seed lots with differing vigor levels but similar germination capacity. *J. Am. Soc. Hortic. Sci.* 128, 608–614. doi: 10.21273/jashs.128.4.0608

Šírová, J., Sedlářová, M., Piterková, J., Luhová, A., and Petřivalský, Y. (2011). The role of nitric oxide in the germination of plant seeds and pollen. *Plant Sci.* 181, 560–572. doi: 10.1016/j.plantsci.2011.03.014

Stamler, J., Singel, D., and Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science (80-)* 258, 1898–1902. doi: 10.1126/science.1281928

Walters, C., Ballesteros, D., and Vertucci, V. A. (2010). Structural mechanics of seed deterioration: Standing the test of time. *Plant Sci.* 179, 565–573. doi: 10.1016/j.plantsci.2010.06.016

Xin, X., Lin, X. H., Zhou, Y. C., Chen, X. L., Liu, X., and Lu, X. X. (2011). Proteome analysis of maize seeds: The effect of artificial ageing. *Physiol. Plant* 143, 126–138. doi: 10.1111/j.1399-3054.2011.01497.x

Yalamalle, V. R., Tomar, B. S., Jain, S. K., Arora, A., Kumar, A., and Munshi, A. D. (2019). Spermine induced protection of onion seed vigour and viability during accelerated ageing. *J. Environ. Biol.* 40, 1079–1083. doi: 10.22438/jeb/40/5/MRN-1047

Yamasaki, H. (2000). Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition in vivo. *Philos. Trans. R. Soc. B. Biol. Sci.* 355, 1477–1488. doi: 10.1098/rstb.2000.0708

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 July 2020
doi: 10.3389/fpls.2020.01041

Functional Insight of Nitric-Oxide Induced DUF Genes in *Arabidopsis thaliana*

Rizwana Begum Syed Nabi[1,2†], *Rupesh Tayade*[3†], *Qari Muhammad Imran*[1†], *Adil Hussain*[4], *Muhammad Shahid*[1] *and Byung-Wook Yun*[1*]

[1] Laboratory of Plant Functional Genomics, School of Applied Biosciences, Kyungpook National University, Daegu, South Korea, [2] Department of Southern Area Crop Science, National Institute of Crop Science, Rural Development Administration, Miryang, South Korea, [3] Laboratory of Plant Breeding, School of Applied Biosciences, Kyungpook National University, Daegu, South Korea, [4] Department of Agriculture, Abdul Wali Khan University, Mardan, Pakistan

Advances in next-generation sequencing technologies facilitate the study of plant molecular functions in detail and with precision. Plant genome and proteome databases are continually being updated with large transcriptomic or genomic datasets. With the ever-increasing amount of sequencing data, several thousands of genes or proteins in public databases remain uncharacterized, and their domain functions are largely unknown. Such proteins contain domains of unknown function (DUF). In the present study, we identified 231 upregulated and 206 downregulated DUF genes from the available RNA-Seq-based transcriptome profiling datasets of *Arabidopsis* leaves exposed to a nitric oxide donor, *S*-nitroso-L-cysteine (CysNO). In addition, we performed extensive *in silico* and biological experiments to determine the potential functions of *AtDUF569* and to elucidate its role in plant growth, development, and defense. We validated the expression pattern of the most upregulated and the most downregulated DUF genes from the transcriptomic data. In addition, a loss-of *AtDUF569* function mutant was evaluated for growth, development, and defense against biotic and abiotic stresses. According to the results of the study, *AtDUF569* negatively regulates biotic stress responses and differentially regulates plant growth under nitro-oxidative stress conditions.

Keywords: Arabidopsis, transcriptome, DUF569, differentially expressed genes, nitro-oxidative stress

OPEN ACCESS

Edited by:
Mounira Chaki,
University of Jaén, Spain

Reviewed by:
Pedro Carrasco,
University of Valencia, Spain
Juan De Dios Alché,
Consejo Superior de Investigaciones Científicas (CSIC), Spain

****Correspondence:***
Byung-Wook Yun
bwyun@knu.ac.kr

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Abiotic Stress, a section of the journal Frontiers in Plant Science

Received: *24 January 2020*
Accepted: *24 June 2020*
Published: *14 July 2020*

Citation:
Nabi RBS, Tayade R, Imran QM, Hussain A, Shahid M and Yun B-W (2020) Functional Insight of Nitric-Oxide Induced DUF Genes in Arabidopsis thaliana. Front. Plant Sci. 11:1041. doi: 10.3389/fpls.2020.01041

INTRODUCTION

Nitric oxide (NO) is a gaseous and highly reactive free radical involved as a signaling molecule in vital physiological processes in both animal and plant cells. Over the past few decades, various functional aspects of NO in animal and plant cell biology have been explored and described. NO in plants has increasingly emerged as an essential molecule involved in diverse plant functions, such as seed germination, growth, and development, plant defense, iron homeostasis, biotic and abiotic stresses, senescence, and cell death (reviewed by Domingos et al., 2015; Nabi et al., 2019) signaling pathways of indole acetic acid, abscisic acid (ABA), and other plant hormones (García-Mata and Lamattina, 2002; Pagnussat et al., 2003). Despite the importance and involvement of NO in diverse plant functions, its origin and mechanism of production in higher plants remain largely elusive.

One of the key roles of NO in the regulation of protein function is through posttranslational modifications such as *S*-nitrosation (or *S*-nitrosylation) and tyrosine nitration (Stamler et al., 1992; Greenacre and Ischiropoulos, 2001). Chief among them is *S*-nitrosation, the covalent attachment of NO to exposed cysteine residues of proteins to form *S*-nitrosothiols (Stamler et al., 1992). Such protein modification has been reported to play critical roles in cellular processes, enzyme pathways, protein–protein interactions, and protein localization (Tada, 2008; Wang et al., 2009; Yun et al., 2011).

In the current genomic era, advances in sequencing technologies are generating vast quantities of data at the transcriptomic and genomic levels. Although the genomes of several mammals, higher plants, and microbial species have been sequenced and annotated, several genes remain uncharacterized, and their biochemical and biological functions, in turn, remain unknown. Such uncharacterized proteins are deemed as proteins having domains of unknown function (DUF) and have been categorized in the Pfam database as a protein family with no functional annotation (Bateman et al., 2010; Finn et al., 2016; El-Gebali et al., 2019). Despite extensive research, DUF is one of the largest families in the Pfam database, representing more than 22% of the entire domains in the Pfam database, with approximately 4,000 DUFs that are poorly understood (Bateman et al., 2010; Finn et al., 2016).

Studies suggest that DUF domain-containing proteins play a vital role in plant stress responses. For instance, in wheat, the DUF622 domain-containing *TaSRG* (*Triticum aestivum* salt response gene) increased salt tolerance in overexpressed transgenic *Arabidopsis* and influenced gene expression levels under salt stress in rice (He et al., 2011). Similarly, in *Arabidopsis*, ABA and drought-induced ubiquitin ligase genes, *AtRDUF1* and *AtRDUF2* (RING-DUF1117E3), regulate ABA signaling and drought stress, while single and double knockout (KO) mutants of *AtRDUF1* and *AtRDUF2* exhibited reduced tolerance to ABA-mediated drought stress when compared with the wild type (WT) plants (Kim and Kim, 2013). Similarly, in another study on rice plant, Cui et al. (2016) reported that *DUF1645* was upregulated in response to various stress factors and conferred improved drought tolerance when overexpressed.

Furthermore, negative regulation of DUF genes has also been reported. For example, in rice, the *DUF966* domain-containing *OsDSR2* encodes a protein that negatively regulates responses to salt and simulated drought stress, along with ABA signaling (Luo et al., 2014). Recently, the functions and expression levels of various members of the rice *OsDUF866* family (*OsDUF866.1–4*) were analyzed (Li et al., 2017). Reports suggested that *OsDUF866.1* positively regulated heat stress tolerance, while *OsDUF866.2* expression decreased discernibly following exposure to drought conditions. *OsDUF866.3* was highly expressed in response to drought and cold stress and decreased under heat stress, while *OsDUF866.4* expression was upregulated under cold and heat stress and decreased under drought stress. In addition, the roles of other DUF genes in rice, such as *OsDUF872*, *OsDUF829*, and *OsDUF946*, have been described (Li et al., 2017; Li et al., 2018a; Li et al., 2018b).

Microarray and RNA-seq mediated transcriptome analyses have made it convenient to predict the global changes in gene expression in a particular genotype. A number of transcriptome analyses in response to various stress conditions have been conducted including H_2O_2-mediated oxidative stress (Desikan et al., 2001), ozone (Xu et al., 2015) cold, drought and oxidative stress (Sham et al., 2014). Nitrosative stress-induced changes in transcriptional regulation have been studied extensively. Microarray or RNA-Seq profiling has revealed several key genes with differential expression in response to 1 mM *S*-nitrosoglutathione (GSNO) and 0.1 and 1.0 mM sodium nitroprusside (SNP) in *Arabidopsis* (Parani et al., 2004; Begara-Morales et al., 2014). At the same time, such transcriptome data revealed several genes with unknown functions, which have exhibited differential expression in response to nitrosative stress. In our previous study involving *Arabidopsis* leaves, 1 mM *S*-nitroso-L-cysteine (CysNO)-mediated RNA-Seq-based transcriptome analysis revealed the differential expression of several thousand of genes (Hussain et al., 2016). In the present study, we identified approximately 440 DUF genes that exhibited differential responses to CysNO, including 235 upregulated and 205 downregulated genes in *Arabidopsis thaliana*, using high-throughput RNA-Seq data. We subsequently characterized them by *in silico* analysis to decipher any putative roles of the genes. In addition, we also studied the biological role of *AtDUF569* in plant growth, development, and defense, using a functional genomics study. Our aim was to identify and characterize NO-induced genes encoding DUFs, with an emphasis on *AtDUF569*.

MATERIALS AND METHODS

Transcriptome-Wide Identification of DUF Genes and Validation of RNA-Seq Data

In a previous study, we reported approximately 6436 differentially expressed genes (DEGs), among which 440 were identified as containing DUFs (Hussain et al., 2016). The DUF name is assigned based on the functional annotation information. When the function of the protein has not been determined, it is deposited in the Pfam database (El-Gebali et al., 2019). Such proteins/genes are commonly termed DUFs (Bateman et al., 2010; Punta et al., 2012). All the 440 DUF domain containing DEGs exhibited significant ($p \leq 0.05$) differential expression in response to 1 mM CysNO. The list of the DUF domain-containing DEGs was examined carefully for any duplicate values. To visualize the differential expression of the DUFs between the CysNO-treated and the untreated (control) samples, a heatmap with hierarchical clustering presented as a dendrogram was generated using *R* version 3.3.1.R (https://www.r-project.org/) using the fragments per kilobase of transcript per million mapped read (FPKM) values.

To validate the RNA-Seq data of the upregulated and downregulated DEGs qRT-PCR was performed as described previously (Imran et al., 2018a). Briefly, total RNA was extracted from *A. thaliana* leaf samples 6 h after 1 mM CysNO infiltration. The cDNA was synthesized using Biofact RT kit (BioFACT™, Korea) according to the manufacturer's standard protocol. A two-step qRT-PCR reaction was performed using 2× Real-Time PCR Master mix (including SYBR Green I; BioFACT™) in an Illumina Eco Real-Time PCR system (Illumina™, USA). The PCR conditions were initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 s and 60°C for 40 s. The fold change in transcript

accumulation of the selected genes was compared with that of the RNA-Seq data. Pearson's correlation coefficient (*R*) for both data sets was calculated using MS Excel 2016 (Microsoft Corp., Redmond, WA, USA). The primers used in the present study are listed in **Table S1**.

Functional Annotation and Gene Ontology Analysis of DUF Genes

To determine the NO-induced DUF genes, we used the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) to find the corresponding pathways and biological information. The CysNO-induced DUF domain-containing DEGs were analyzed for associated Gene Ontology (GO) terms that describe biological processes, molecular functions, and cellular components through the PANTHER v14.1 (http://pantherdb.org) web interface, using the applicable search field and *A. thaliana* selected as the organism. The "statistical overrepresentation test" was selected, and the rest of the analyses were performed using default settings. Only the GO terms with significant ($p < 0.05$) fold enrichment were selected, and pie charts were constructed for visualization using MS Excel 2016.

Promoter Analysis

To forecast the putative regulatory role of NO-responsive DUF genes, we obtained the promoter sequences 1 kb upstream of the transcription initiation site of the selected NO-responsive *Arabidopsis* DUF genes from The *Arabidopsis* Information Resource Center (TAIR) (https://www.arabidopsis.org/). The retrieved sequences were analyzed for potential cis-regulatory elements through the web-based MatInspector tool (https://omictools.com/matinspector-tool) with default settings. Only the transcription factor (TF) binding sites vital in plant regulatory responses to biotic and abiotic stresses were selected for visualization.

Phylogenetic Analysis

To examine the evolutionary relationship of the NO-responsive DUF DEGs, a list of 18 DUF DEGs including 10 upregulated and 8 downregulated (two downregulated genes were transposons) was used as query in Phytozome against rice (*Oryza sativa*), soybean (*Glycine max*), wheat (*Triticum aestivum*), maize (*Zea mays*) and tomato (*Solanum lycopersicum*). The protein sequences of these orthologs were retrieved from Phytozome web interface (https://phytozome.jgi.doe.gov/phytomine/begin.do) and aligned using ClustalW in Mega 7 (Kumar et al., 2016), the resultant alignment was used to make phylogenetic tree using maximum likelihood based on JTT matrix-based model (Jones et al., 1992) with bootstrap value of 1,000 replicates. All of the positions with less than 95% site coverage were eliminated; that is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. MEGA 7.0 (Kumar et al., 2016) was used for the analysis.

Plant Material and Growth Conditions

The seeds of WT *A. thaliana* (ecotype Col-0) and the mutant line *atduf*569 (AT1G69890) were obtained from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/). In addition, *atgsnor1-3* knockout mutant was used as disease susceptible control due to its established role in plant immunity (Feechan et al., 2005). Similarly, the *atsid2* mutant deficient in the SA pathway was used as a control for SA-mediated defense pathway, and *atcat2,* a high H_2O_2-producing mutant deficient in *AtCATALASE2* gene (AT1G74710; Hu et al., 2010) was used as susceptible controls for the nitro-oxidative stress assay and biotic stress-related experiments. Plants homozygous for T-DNA insertion were identified through genotyping using PCR with the T-DNA left border primers and gene-specific primers obtained from Signal Salk (http://signal.salk.edu/tdnaprimers.2.html; **Figure S1**, **Table S1**). The PCR conditions were an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The confirmed homozygous mutant line was used for experimental biological evaluation after collecting the seeds in bulk. All the seeds were surface-sterilized using 50% bleach (commercial bleach) with 1% (*v/v*) Triton X-100 added, for about 1–2 min. After rinsing five times, the sterilized seeds were incubated in sterile water and stratified overnight at 4°C for uniform germination before sowing either on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) or soil.

Nitro-Oxidative Stress Assay

The responses of *AtDUF569* (*AT1G69890*) to nitro-oxidative stress in plants were evaluated as described by Imran et al. (2016). Briefly, for nitro-oxidative stress conditions, Col-0 (WT), *atgsnor1-3* (*AT5G43940*), *atcat2* (AT1G74710), and *atduf*569 (AT1G69890) seeds were sterilized (See section *Plant material and growth conditions*) and grown on half-strength MS medium supplemented with 0.75 mM CysNO or GSNO as NO donors and 2 mM H_2O_2 or 1 μM MV as oxidative stress inducers, separately. The control and the treated plants were grown under 16/8 h light/dark conditions at 23 ± 2°C. The experiments were performed in triplicate and cotyledon development frequency (CDF) was measured after seven days, as described previously (Imran et al., 2018b). In addition, root and shoot lengths were measured 14 days after sowing.

Pathogen Inoculations and Pathogenicity Assessment

Plants were subjected to pathogen inoculation as described previously (Yun et al., 2011). Briefly, *Pseudomonas syringae* pv. *tomato* virulent (*Pst* DC3000) and avirulent strains (*Pst* DC30000 *avrB*) were used for the pathogenicity assays. Bacterial cultures were grown and maintained as described previously (Whalen et al., 1991). Briefly, a single colony was cultured in Luria–Bertani (LB medium) broth with respective antibiotics added and incubated at 28°C overnight with shaking. The bacterial cultures (1 ml each) were centrifuged at 8,000 rpm (LABOGene 1736R) for 3 min. The bacterial pellet was resuspended in 1 ml of 10 mM $MgCl_2$ solution. The prepared bacterial inoculum was then infiltrated into the abaxial side of the leaves *via* a needleless syringe at 5×10^5 CFU ml^{-1} for virulent *Pst* DC3000 and 0.002 at OD_{600} (1×10^6 CFU ml^{-1}) for avirulent *Pst* DC30000 *avrB*. Mock plants were infiltrated with the 10 mM $MgCl_2$ solution without bacteria. Leaf samples were collected over time for further gene expression analyses. In addition, leaf discs from *Pst DC3000*-inoculated leaves were crushed in 10 mM $MgCl_2$ and diluted 10 times. To identify the responses of

atduf569 and control genotypes to infection, the diluted samples were spread on LB agar plates containing the respective antibiotics. The development of symptoms was observed, and photos were captured using a digital camera (EOS 700D, Canon). Symptom development and pathogen growth in plant tissues were assessed 1, 2, and 3 d post-inoculation (dpi) by counting the bacterial CFU per leaf disc using the serial dilution method as described by Flors et al. (2008), and disease symptoms on the inoculated leaves were also observed.

Electrolyte Leakage

Cell death induced by pathogen infection was assessed through electrolyte leakage (Arasimowicz-Jelonek et al., 2009). Briefly, triplicate leaf discs (about 1 cm^2) with three leaf discs per replicate were collected from the plants inoculated with the avirulent bacterial pathogen (*Pst* DC3000 *avrB*) at an OD_{600} of 0.02 corresponding to 1×10^6 CFU ml^{-1}. The leaf discs were washed three times with deionized water and maintained in approximately 300 µl of deionized water/well in a six-well plate (SPL Life Sciences, Korea). Electrolyte leakage of each sample was recorded over several time points using a portable conductivity meter (Huriba Twin Cond B-173).

Statistical Analysis

The data were analyzed at $p \leq 0.05$ or $p \leq 0.01$ significance levels. A two-tailed *t*-test was performed to determine *p*-values using MS Excel 2016. The means and standard errors were calculated from at least three biological replicates and compared with the control.

RESULTS

Transcriptome-Wide Identification and Characterization of NO-Responsive DUF Genes

To identify NO-responsive DUF domain-containing genes, we analyzed CysNO-induced DEGs reported previously (Hussain et al., 2016). All the RNA-seq data can be found in the public repository for Gene Expression Omnibus (GEO) and Short Read Achieve (SRA) under accession numbers GSE81361 and SRP074890, respectively. Notably, a considerable number (437 DEGs) of NO-responsive DUF domain-containing genes were differentially expressed in response to 1 mM CysNO (**Supplementary Table S2**). A list of top 10 up- and down-regulated DEGs is given in **Table 1**. We analyzed all the NO-responsive DUFs for associating Gene Ontology (GO) terms. GO terms for biological process suggested that NO responsive DUFs were associated with chloroplast organization, pattern specification process, cellular metabolic process and gene expression *etc.* (**Figure 1A**). In GO terms for molecular functions majority of DUFs were associated with Iron ion transmembrane transporter activity (**Figure 1B**). In GO terms of cellular component majority of DUFs were associated with Golgi apparatus, Golgi subcomponent, and Endosomes *etc.* (**Figure 1C**). A heatmap with hierarchical clustering between the control and the CysNO-treated samples was constructed from the FPKM values in triplicates and shows the expression patterns of the DUF domain-containing DEGs (**Figure 2A**). Samples 1 to 3 represent three replicates from control (buffer only) while samples 4 to 6 represent three samples from CySNO treated plants. The red color in the treated samples indicates upregulated genes, while the intensity of the color represents the intensity of expression. The more the red color, the more expression after CySNO treatment (**Figure 2A**). Some samples with black color show very low expression approaching to zero (**Figure 2A**). Among the 437 DUF domain-containing DEGs, 53% (236 DEGs) were upregulated, while 47% (205 DEGs) were downregulated, with at least a two-fold change in their expression relative to the control plants (**Figure 2B**, **Supplementary Table S2**). Furthermore, we evaluated the role of the NO-induced *DUF569* (AT1G69890) in plant growth and defense against biotic and abiotic stresses. *AtDUF569* had a fold change of 57.29 in the transcriptome profile.

Validation of RNA-Seq Data Through qRT-PCR

The transcriptional changes in the expression of DUF domain-containing genes were validated by analyzing the 10 representative DEGs (five upregulated and five downregulated) through qRT-PCR. The genes were selected based on the highest fold change from both upregulated and downregulated genes in response to CysNO infiltration. The expression patterns in qRT-PCR corroborated the RNA-Seq results for all of the 10 analyzed *DUF* domain-containing DEGs. The RNA-Seq and qRT-PCR datasets were compared based on the Pearson's correlation coefficient (r = 0.905), revealing a high similarity between the datasets (**Figure 2C**).

Promoter Analysis for the Identification of the TF Binding Sites

We analyzed the promoter region of selected NO-induced DUF genes 1 Kb upstream of the transcription initiation site and the *cis*-elements or TF binding sites that were involved in plant stress were subsequently mapped (**Figure S2**). A detailed list of these TF binding sites is given in **Supplementary Table S3**. We identified the critical binding sites, including sites for the prolamin box (P-box), which was observed in 100% of the analyzed sequences. Similarly, two other elements, the W-box (WRKY TF family) and MAD-box (RIN), were found in 100% of the analyzed sequences. The AP2L ethylene-responsive TF was found in 54.1%, ETTIN (Auxin Response Factor 3) in 70.8%, ethylene-responsive TF ERF017 in 83.3%, ATMYB77-binding site in 83.3%, GT-box elements (GT2) in 70.8%, ANT (*Arabidopsis* protein AINTEGUMENTA) in 29.1%, SP8BF in 62.5%, HSFA1A in 95.8%, CDM1 in 29.6%, TATA-box in 91.6%, ANAC087 in 95.8%, and HBP1B in 91.6% of the analyzed sequences, respectively (**Figure S2**; **Table S3**). Among the above major elements, AP2L recognizes motifs within pathogenesis-related promoters, which may mediate the regulation of gene expression under biotic stress and components of stress signal transduction pathways (Xie et al., 2019). Similarly, other elements, such as ATMYB77 (an R2R3-type MYB TF), WRKY20, and ANAC087 (a NAC domain-containing protein), have been reported to play important roles in biotic and abiotic stresses. Therefore, the identification of regulatory elements and their modules is a prerequisite step for the understanding of gene expression and regulation (Nuruzzaman et al., 2013).

TABLE 1 | List of top 10 up- and downregulated NO-responsive DUF domain containing genes with their fold change as per transcriptomic analysis.

Accession No.	FPKM(1)	FPKM(2)	Fold change	p-value	Log2 (Fold change)	Annotation
AT4G10290	0.02000	27.65000	1382.50000	0.00006	10.70240	RmlC-like cupins superfamily protein; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF861, cupin-3 (InterPro : IPR008579), Cupin, RmlC-type (InterPro : IPR011051), RmlC-like jelly roll fold (InterPro : IPR014710); BEST *Arabidopsis thaliana* protein match is: RmlC-like cupins superfamily protein (TAIR : AT4G10280.1)
AT3G43250	0.11000	60.46000	549.63636	0.00000	9.12997	Family of unknown function (DUF572); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF572 (InterPro : IPR007590); BEST *Arabidopsis thaliana* protein match is: Family of unknown function (DUF572) (TAIR : AT2G32050.1)
AT1G62320	0.03000	3.99000	133.00000	0.00000	7.24010	ERD (early-responsive to dehydration stress) family protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: endomembrane system, membrane; EXPRESSED IN: pollen tube; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF221 (InterPro : IPR003864); BEST *Arabidopsis thaliana* protein match is: ERD (early-responsive to dehydration stress) family protein (TAIR : AT1G11960.1)
AT5G67210	0.82000	98.38000	119.97561	0.00000	6.90488	Encode a DUF579 (domain of unknown function 579) containing protein essential for normal xylan synthesis and deposition in the secondary cell wall.
AT5G41590	0.04000	4.76000	119.00000	0.00000	6.87891	Protein of unknown function (DUF567); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF567 (InterPro : IPR007612); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF567) (TAIR : AT2G38640.1)
AT2G14290	0.28000	23.59000	84.25000	0.00000	6.37693	CONTAINS InterPro DOMAIN/s: F-box domain, cyclin-like (InterPro : IPR001810), F-box domain, Skp2-like (InterPro : IPR022364), Protein of unknown function DUF295 (InterPro : IPR005174); BEST *Arabidopsis thaliana* protein match is: F-box family protein with a domain of unknown function (DUF295) (TAIR : AT5G25290.1); Has 351 Blast hits to 340 proteins in 13 species: Archae—0; Bacteria—2; Metazoa—0; Fungi—0; Plants—349; Viruses—0; Other Eukaryotes—0 (source: NCBI BLink).
AT1G54095	0.02000	1.63000	81.50000	0.00842	6.04292	Protein of unknown function (DUF1677); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF1677, plant (InterPro : IPR012876); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF1677) (TAIR : AT1G72510.2)
AT4G36820	0.12000	7.70000	64.16667	0.00000	6.01626	Protein of unknown function (DUF607); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF607 (InterPro : IPR006769); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF607) (TAIR : AT2G23790.1); Has 370 Blast hits to 370 proteins in 122 species: Archae—0; Bacteria—0; Metazoa—148; Fungi—54; Plants—129; Viruses—0; Other Eukaryotes—39 (source: NCBI BLink).
AT3G04300	0.08000	5.03000	62.87500	0.00000	5.96407	RmlC-like cupins superfamily protein; CONTAINS InterPro DOMAIN/s: Cupin, RmlC-type (InterPro : IPR011051), Protein of unknown function DUF861, cupin-3 (InterPro : IPR008579), RmlC-like jelly roll fold (InterPro : IPR014710); BEST *Arabidopsis thaliana* protein match is: RmlC-like cupins superfamily protein (TAIR : AT4G10300.1); Has 512 Blast hits to 512 proteins in 136 species: Archae—0; Bacteria—273; Metazoa—0; Fungi—0; Plants—140; Viruses—0; Other Eukaryotes—99 (source: NCBI BLink).
AT1G69890	1.92000	110.000	57.29167	0.00000	5.83694	CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF569 (InterPro : IPR007679), Actin cross-linking (InterPro : IPR008999); BEST *Arabidopsis thaliana* protein match is: Actin cross-linking protein (TAIR : AT1G27100.1)
AT4G14819	2.77000	0.03000	92.33333	0.00943	−6.71390	Protein of unknown function (DUF1677); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF1677, plant (InterPro : IPR012876); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF1677) (TAIR : AT3G22540.1); Has 35333 Blast hits to 34131 proteins in 2444 species: Archae—798; Bacteria—22,429; Metazoa—974; Fungi—991; Plants—531; Viruses—0; Other Eukaryotes—9,610 (source: NCBI BLink).
AT5G05840	0.48000	0.01000	48.00000	0.00258	−5.43983	Protein of unknown function (DUF620); CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF620 (InterPro : IPR006873); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF620) (TAIR : AT3G55720.1); Has 1807 Blast hits to 1807 proteins in 277 species: Archae—0; Bacteria—0; Metazoa—736; Fungi—347; Plants—385; Viruses—0; Other Eukaryotes—339 (source: NCBI BLink).
AT5G32621	0.42000	0.02000	21.00000	0.00140	−4.10636	transposable element gene; similar to unknown protein [*Arabidopsis thaliana*] (TAIR : AT3G15310.1); similar to hypothetical protein 24.t00017 [*Brassica oleracea*] (GB : ABD64939.1); contains InterPro domain Protein of unknown function DUF635; (InterPro : IPR006912); contains InterPro domain Bacterial adhesion; (InterPro : IPR008966)
AT3G54260	2.43000	0.12000	20.25000	0.00000	−4.37071	Encodes a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family containing a plant-specific DUF231 (domain of unknown function) domain. TBL gene family has 46 members, two of which (TBR/AT5G06700 and TBL3/AT5G01360) have been shown to be involved in the synthesis and deposition of secondary wall cellulose, presumably by influencing the esterification state of pectic polymers. A nomenclature for this gene family has been proposed (Volker Bischoff & Wolf Scheible, 2010, personal communication).

(Continued)

TABLE 1 | Continued

Accession No.	FPKM(1)	FPKM(2)	Fold change	p-value	Log2 (Fold change)	Annotation
AT5G25460	95.13000	6.13000	15.51876	0.00000	–3.95598	Protein of unknown function, DUF642; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: response to karrikin; LOCATED IN: plant-type cell wall; EXPRESSED IN: 22 plant structures; EXPRESSED DURING: 13 growth stages; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF642 (InterPro : IPR006946); BEST Arabidopsis thaliana protein match is: Protein of unknown function, DUF642 (TAIR : AT5G11420.1); Has 1,807 Blast hits to 1,807 proteins in 277 species: Archae—0; Bacteria—0; Metazoa—736; Fungi—347; Plants—385; Viruses—0; Other Eukaryotes—339 (source: NCBI BLink).
AT1G11700	39.37000	2.64000	14.91288	0.00000	–3.89880	Protein of unknown function, DUF584; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: chloroplast; EXPRESSED IN: 21 plant structures; EXPRESSED DURING: 11 growth stages; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF584 (InterPro : IPR007608); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function, DUF584 (TAIR : AT1G61930.1); Has 334 Blast hits to 333 proteins in 24 species: Archae—0; Bacteria—0; Metazoa—0; Fungi—4; Plants—328; Viruses—0; Other Eukaryotes—2 (source: NCBI BLink).
AT2G34170	10.84000	0.74000	14.64865	0.00000	–3.86850	Protein of unknown function (DUF688); FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; CONTAINS InterPro DOMAIN/s: Protein of unknown function DUF688 (InterPro : IPR007789); BEST *Arabidopsis thaliana* protein match is: Protein of unknown function (DUF688) (TAIR : AT1G29240.1)
AT4G12980	107.85	7.84000	13.75638	0.00000	–3.78144	Auxin-responsive family protein; CONTAINS InterPro DOMAIN/s: Uncharacterized conserved protein UCP037471 (InterPro : IPR017214), Cytochrome b561, eukaryote (InterPro : IPR004877), Protein of unknown function DUF568, DOMON-like (InterPro : IPR007613), DOMON related (InterPro : IPR005018), Cytochrome b561/ferric reductase transmembrane (InterPro : IPR006593); BEST *Arabidopsis thaliana* protein match is: Auxin-responsive family protein (TAIR : AT3G25290.2); Has 675 Blast hits to 673 proteins in 107 species: Archae—0; Bacteria—4; Metazoa—91; Fungi—93; Plants—473; Viruses—0; Other Eukaryotes—14 (source: NCBI BLink).
AT1G03300	2.99000	0.24000	12.45833	0.00000	–3.62457	Member of the plant-specific DUF724 protein family. Arabidopsis has 10 DUF724 proteins. Loss of function mutant has a WT phenotype
AT3G15310	25.97000	2.10000	12.36667	0.00000	–3.62950	transposable element gene; similar to unknown protein [*Arabidopsis thaliana*] (TAIR : AT5G32621.1); similar to hypothetical protein 24.t00017 [*Brassica oleracea*] (GB : ABD64939.1); contains InterPro domain Protein of unknown function DUF635; (InterPro : IPR006912); contains InterPro domain Bacterial adhesion; (InterPro : IPR008966)

Phylogenetic Analysis of *Arabidopsis At*DUF569 (AT1G69890)

To observe the ancestral evolutionary relationship of Arabidopsis DUF genes Top 10 upregulated and downregulated DEGs were used as query to identify their orthologs in agronomically important species such as rice, soybean, wheat, maize, and tomato. About 151 protein sequences from all the species were used to generate a phylogenic tree. From the analysis, we can predict that Arabidopsis DUFs are distributed almost in every clade. All the peptides can be divided into four major clades and subclades. From an evolutionary perspective, rice and Arabidopsis were distributed throughout the tree, interestingly, majority of the wheat proteins were grouped together with tomato and were evolutionarily distant from Arabidopsis, rice, and soybean (**Figure 3**) Together these results suggest that these species may share common ancestors and that NO-responsive DUF protein functions could be conserved in these species.

Interactome of CysNO-Induced DUF Gene (AT1G69890)

We have searched for interactions between the CysNO-induced *AtDUF569* (AT1G69890) and other proteins using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/). We observed some interesting interactions between the DUF569 and other proteins, and we identified 10 predicted functional partners, which included the uncharacterized protein, AT3G49790, known as a carbohydrate-binding protein. Its function is described as ATP-binding, but the underlying biological mechanism remains unknown. The PHLOEM PROTEIN 2-LIKE A10 is another carbohydrate-binding protein located in the mitochondria and found in several plant species and at different growth stages. The CYB-1 and ACYB-2 proteins are potentially transmembrane ascorbate ferrireductase 2, which contains two-heme-cytochrome (1 and 2) and is involved in the catalyzation of ascorbate-dependent transmembrane ferric-chelate reduction. Such interactions of DUF569 protein with other important proteins are illustrated in **Figure 4** and **Table S4**.

The *in silico* and experimental observations suggested that the AtDUF569 protein interacts with various proteins involved in cellular trafficking machinery and carbohydrate-binding and with glycine-rich proteins that participate in cellular stress responses and signaling (Czolpinska and Rurek, 2018).

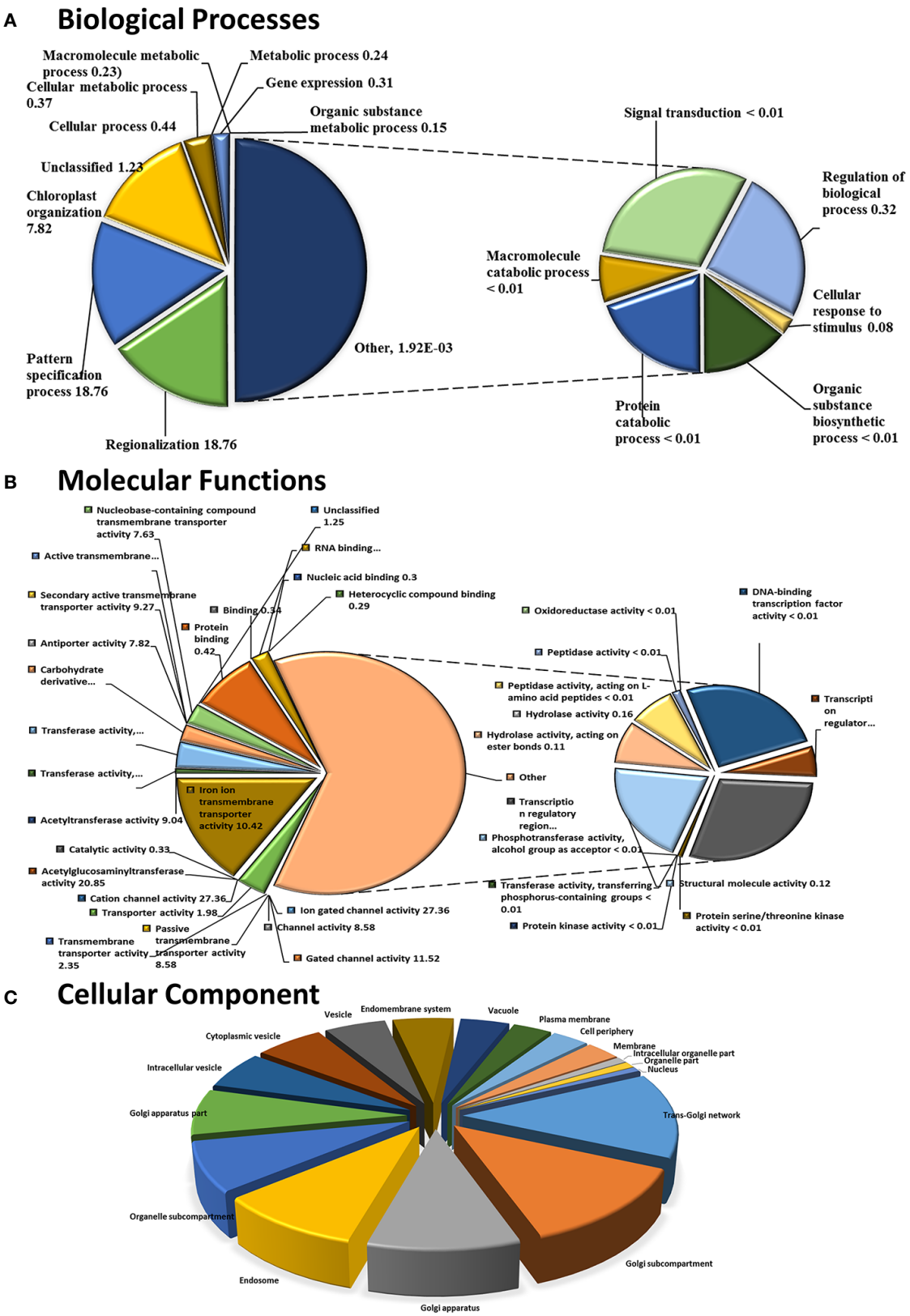

FIGURE 1 | Continued

FIGURE 1 | Functional GO term classification of CysNO-responsive DUF genes of *Arabidopsis* leaf transcripts. Gene ontologies for **(A)** Biological Processes **(B)** Molecular Functions and **(C)** Cellular Process were determined using *Arabidopsis thaliana* as a reference genome. Out of the 443 CySNO responsive genes, 440 were mapped with the reference genome, PANTHER version 14.1 (http://pantherdb.org) at $P < 0.05$ for the PANTHER GO-slim and the fold enrichment-values shown.

AtDUF569 Differentially Regulates Plant Shoot Growth and Negatively Regulates Root Growth Under Nitro-Oxidative Stress Conditions

We explored and applied the CDF as a development or fitness score for each plant. In the data recorded after seven days of treatment, our KO mutant *atduf569* exhibited a noticeably higher CDF compared with Col-0 during exposure to CysNO- and GSNO-induced nitrosative stress, while all plants shared similar CDF result 14 days post-treatment. However, regarding MV-induced oxidative stress, KO mutant exhibited markedly reduced CDF at both seven- and 14-days post-stress treatment. On the contrary, under H_2O_2 exposure, the CDF was markedly higher for the KO mutant than for the Col-0 plants at both seven and 14 days after stress treatment (**Figures 5** and **6**). The results suggested that the *atduf569* mutant was tolerant to CysNO- and

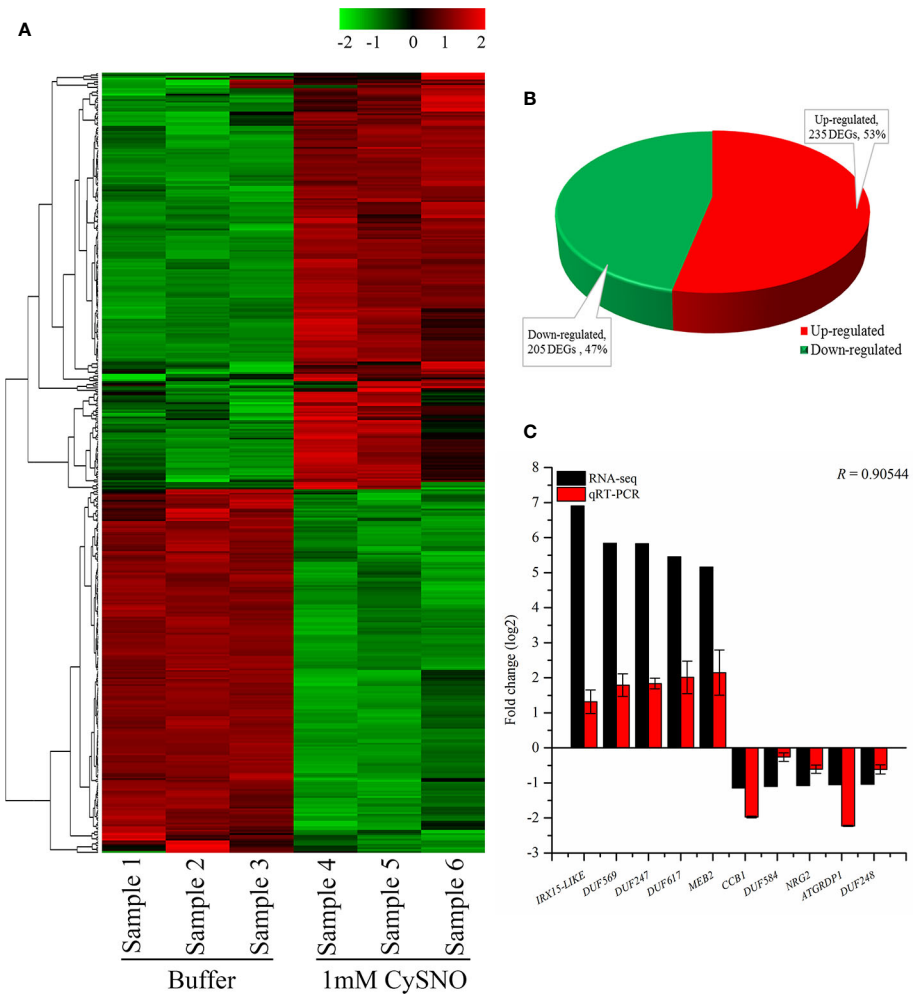

FIGURE 2 | Identification and analysis of NO-responsive DUF domain-containing genes. **(A)** Heatmap revealing the expression pattern of transcriptome-wide, DEGs in response to *S*-nitroso L cysteine (CySNO) and hierarchical clustering generated from the FPKM values of CysNO-responsive DUF domain-containing genes. Red and green colors indicate the expression values in the respective sample. Samples 1to 3 are buffer-treated while 4 to 6 are CySNO infiltrated. A color key representing the intensities of the expression values is also given. **(B)** Pie-chart illustrating the total 440 number of up- and downregulated NO-responsive DUF domain-containing DEGs; these gene were identified at a Q-value < 0.05 in the transcriptome. **(C)** qRT-PCR validation of RNA-Seq data. About 10 DUF domain-containing genes (five each from up- and downregulated DEGs) were selected based on their fold change for validation through qRT-PCR. The *R* value represents Pearson's correlation coefficient and was calculated among RNA-Seq and qRT-PCR datasets. The data points in C are the mean of three replicates while error bars represent standard error.

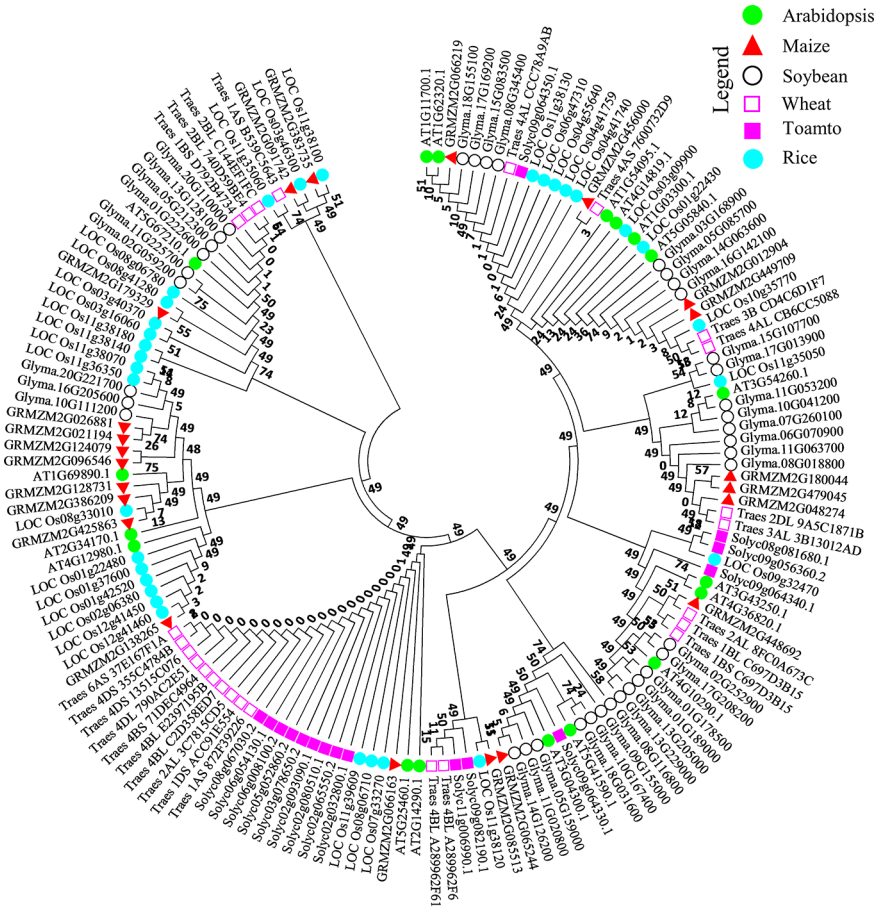

FIGURE 3 | Molecular phylogenetic analysis of the AtDUF569 protein with homologs from other plant species. About 18 NO-responsive DUF DEGs were used as query against rice, soybean, wheat, maize, and tomato databases in Phytozome. The best-hit orthologs were identified and sequences were retrieved and aligned using ClustalW in Mega 7. The resultant alignment was used to make phylogenetic tree using maximum likelihood method on JTT matrix-based model through which evolutionary history was inferred. The bootstrap value was 1,000 replicates and branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were eliminated. About 151 peptide sequences from Arabidopsis and various other crops mentioned above were used for this analysis. The positions with less than 95% site coverage were collapsed. The phylogenetic tree was constructed using Mega 7 software. Different species are labeled with different shapes and colors and can be found in the legend in the up-right corner of the figure.

GSNO-mediated nitrosative stress but was considerably tolerant and sensitive to H_2O_2 and MV, respectively. We also measured other plant attributes, such as shoot and root length, of the mutant in comparison to the WT (Col-0) and other designated control mutants. The KO mutant *atduf569* exhibited higher shoot length under oxidative stress (induced by H_2O_2 and MV) 14 days post stress treatment relative to the other plants. However, shoot and root length displayed differential patterns under different nitrosative stress conditions. In addition, shoot and root lengths of KO mutant *atduf569* were higher under CysNO, while the KO mutant under GSNO stress presented reduced length and increased shoot and root lengths, respectively (**Figures 5** and **6**). Overall, the results revealed that *AtDUF659* (AT1G69890) differentially regulates plant growth and development under nitro-oxidative stress conditions.

AtDUF569 Negatively Regulates Plant Basal Defense at Early Time Point

To examine the role of *AtDUF569* (AT1G69890) in plant basal defense, we observed the phenotypic response of the *atduf569* KO mutant line under the control conditions, as well as post pathogen inoculation as described elsewhere (Koornneef et al., 1991). The *atduf569* KO mutant line was in the Col-0 genetic background; therefore, WT Col-0 plants were used as a control, while *atgsnor1–3* KO mutant was used as a disease-susceptible control (Feechan et al., 2005). The loss-of-function mutant for *Salicylic acid induction-deficient 2* (*SID2*) inhibits pathogen-induced salicylic acid (SA) production and makes the plants highly susceptible to infection by pathogens. Therefore, *atsid2* was used as a second control for pathogenicity assessment. The pathogenicity assay revealed that the *atduf569* KO line exhibited

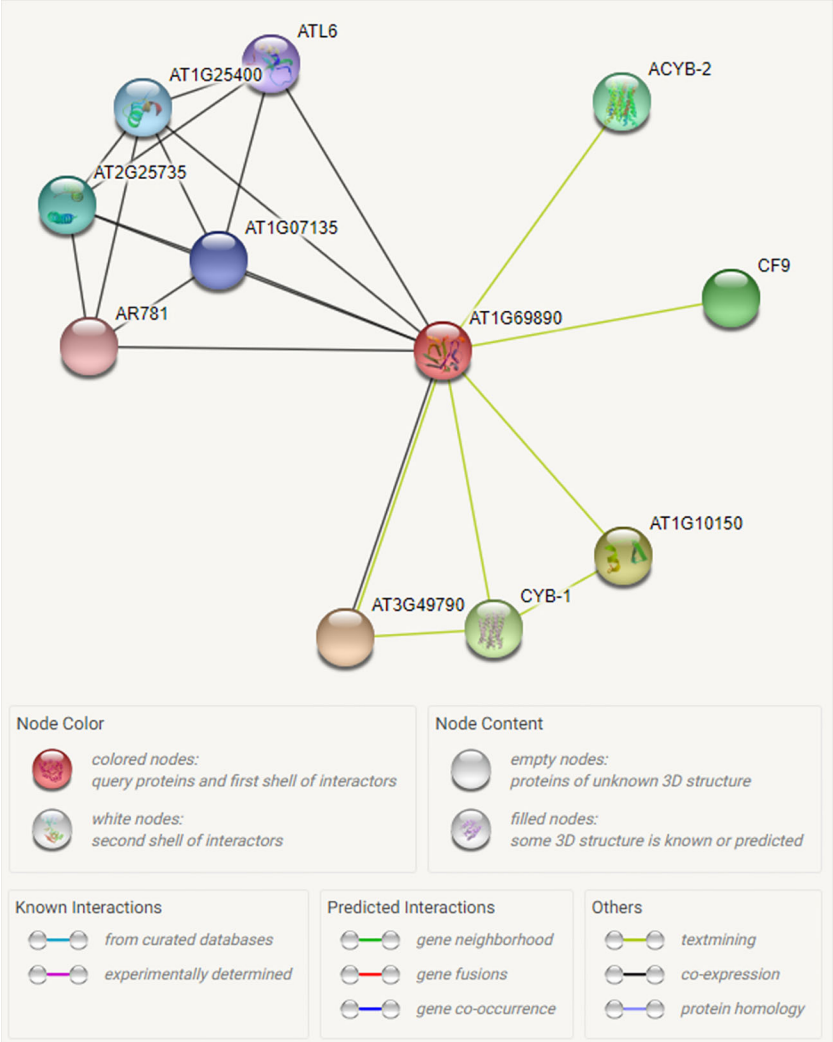

FIGURE 4 | Interactome of the CysNO-induced AtDUF569. To predict possible interaction of ATDUF569 with other proteins, the accession number of DUF569 was used as "Protein name" in the search option in online Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/) with default parameters. A list of interacting proteins with DUF569 is given in **Table S2** with relevant information. Different colors represent proteins or various types of interactions.

disease symptoms at 1 dpi, but in the later assessment, it was completely resistant toward the *Pst* DC3000 infection and exhibited no disease symptoms compared with Col-0 (WT), *atgsnor1–3*, and *atsid2* plants (**Figure 7A**). Similar results were observed for the bacterial CFU counts at 3 dpi (**Figure 7B**). Based on the results, we hypothesized that the enhanced resistance to pathogens in the *atduf569* line could have been due to the upregulation of the SA signaling pathway. Therefore, we performed an expression analysis for *Pathogenesis-related 1* (*PR1*) and *PR2* genes, which are regulators of SA-dependent gene expression. The qRT-PCR analysis revealed that the transcript accumulations of *PR1* and *PR2* were significantly ($P < 0.05$) lower in *atduf569* than in the WT (Col-0) but higher than those of recognized sensitive genotypes, *atgsnor1–3* and *atsid2*, 24 *h* post-inoculation (hpi) and 48 hpi, at which it displayed the highest expression. At 72 dpi, however, the expression level was lower in comparison to the expression level in WT, although the level was higher than in recognized sensitive genotypes *atgsnor1–3* and *atsid2* (**Figures 8A, B**).

AtDUF569 Study of Effector-Triggered Immunity or *R*-Gene Mediated Resistance

To examine whether the *AtDUF569* function is required for *R*-gene mediated resistance, we inoculated plants with *Pst* DC3000 *avrB*. Our results suggested early, high transcript accumulation of the in *atduf569* when compared to the WT plants (**Figure 9A**)

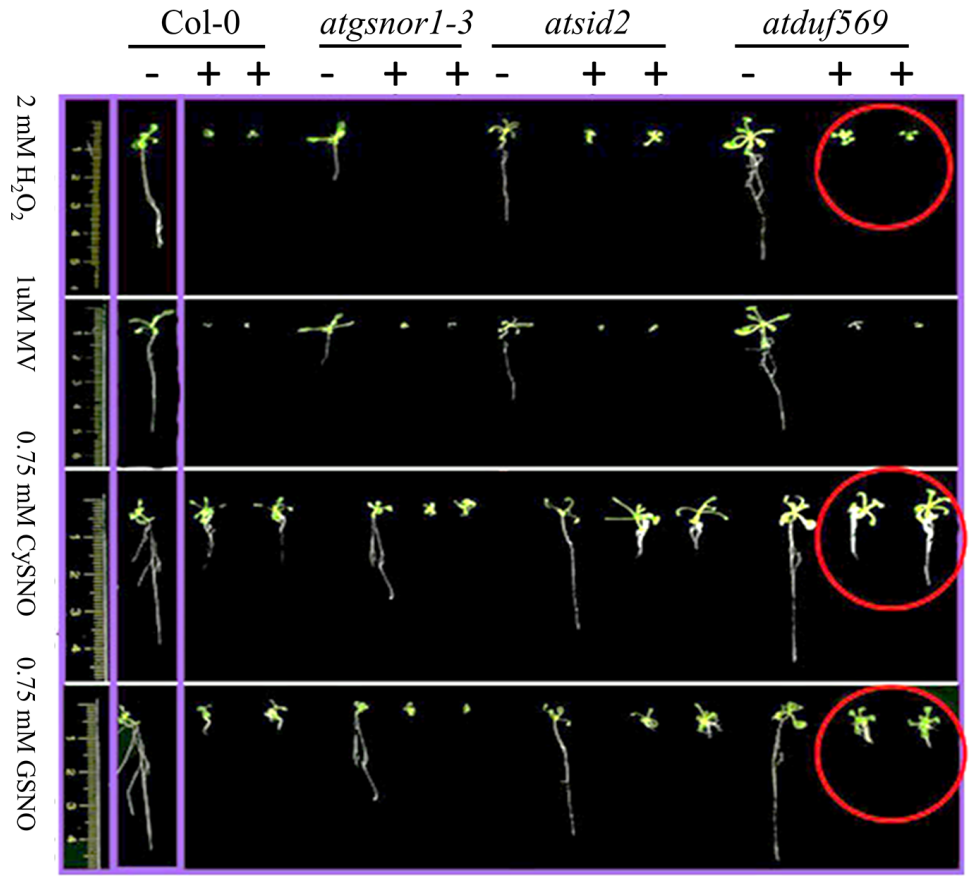

FIGURE 5 | Differential role of *AtDUF569* in plant development. Phenotypic evaluation of the genotypes under the control (–) and following exposure to various oxidative and nitrosative stresses (+). For the control genotype, a single plant is presented, whereas two plants are illustrated for the treatment.

at 0, 3, 6, and 12 hpi. On the contrary, *PR2* expression was significantly ($P < 0.05$) lower relative to the WT at 3 hpi with a gradual increase in expression at 6, 12, 24, and 48 hpi (**Figure 9B**). The observation indicated that *PR2* transcript accumulation might have a higher contribution to the control of pathogen growth at later time points. Furthermore, the electrolyte leakage assay, performed after *Pst* DC3000 *avrB* inoculation, revealed higher electrolyte leakage in the *atduf569* plants over time relative to the other genotypes except for *atgsnor1-3* (**Figure 9C**).

AtDUF569 Study for Systemic Acquired Resistance-Mediated Resistance

In plants, signaling molecules, such as azelaic acid, glycerol-3-phosphate, and SA-*PR1* and -*PR2*, are involved in mediating SAR in plants. The expression patterns of *PR1* and *PR2* revealed a significantly ($P < 0.05$) higher level of transcript accumulation in a*tduf569* at 0, 6, and 12 hpi but a drastically decreased level at 24 and 48 hpi in comparison to WT, *atgsnor1–3*, and *atsid2* plants (**Figures 10A, B**). In addition, the expression levels of *AZI* were significantly ($P \leq 0.05$) higher at 12 and 24 hpi and *G3pdh* at 0, 6, 12, 14, and 48 hpi (**Figures 10C, D**). Based on the expression pattern, the initial upregulation of *PR1* and *PR2* genes at 0, 6, and 12 hpi and the differential upregulation of *AZI* and *G3pdh* potentially contributed to the *atduf569* mutant plants' resistance to the *Pst* DC3000 infection. The results provide further evidence that the *atduf569*-resistant phenotype responded *via* the upregulation of SA-dependent *PR*s at the initial stage of pathogenicity, which controlled the effects of the pathogen and protected the mutant phenotype from later-stage disease development.

DISCUSSION

NO is a small, diatomic, and highly reactive gaseous molecule that orchestrates a plethora of physiological functions both in plants and animals. Although, its role has been well-explored in the mammalian system (Kerwin et al., 1995), NO opened a new

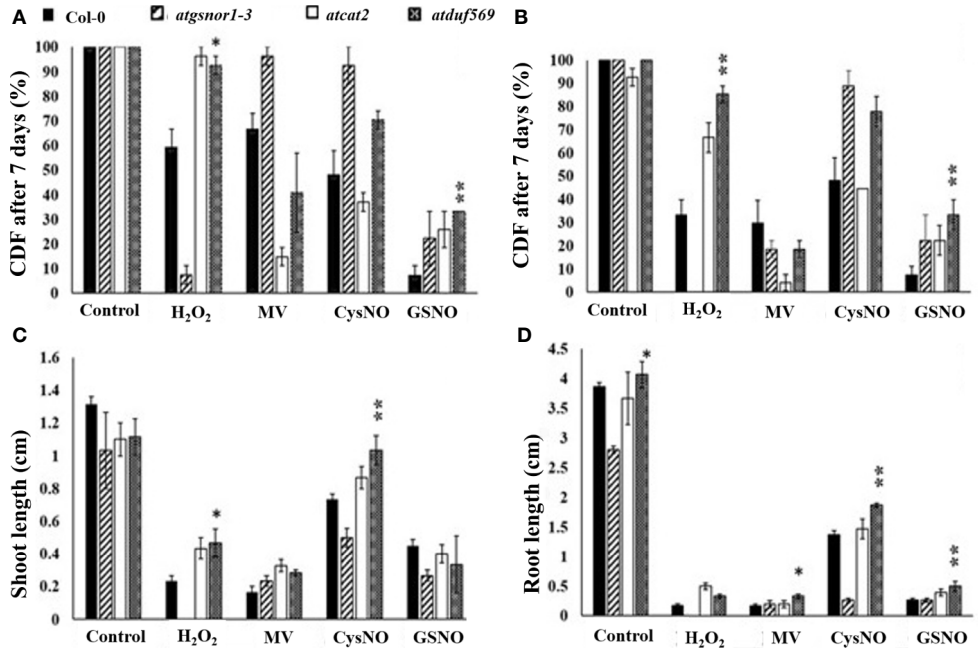

FIGURE 6 | Exogenous application of CysNO, GSNO, or H_2O_2 and MV mediated nitro-oxidative stress. Designated genotypes Col-0, *atgsnor1–3*, *atcat2*, and *atdfu569*, were grown on half-strength Murashige and Skoog (MS) medium, with and without supplementation with either H_2O_2 and methyl viologen (Medina-Rivera et al., 2015) or CysNO and *S*-nitrosoglutathione (GSNO) for oxidative and nitrosative stress conditions, respectively. **(A)** Cotyledon development frequency (CDF) of the designated genotypes seven days after sowing under indicated stress. **(B)** CDF of the designated genotypes 14 days after sowing under indicated stress. **(C)** Shoot lengths of the designated genotypes 14 days after indicated stress. **(D)** Root lengths of the designated genotypes 14 days after indicated stress. All data points represent the means of triplicates. The experiment was repeated three times, with similar results. Error bars represent the standard error. Significant interactions are indicated by an asterisk (Student's *t*-test with a 95% confidence level).

pandora-box in plant science when it was reported as a signaling molecule during plant defense (Delledonne et al., 1998; Durner et al., 1998). In plants, contrary to other signaling cascades, NO transfer its bio-activity *via* posttranslational modifications (PTMs) such as *S*-nitrosation (Stamler et al., 2001), tyrosine nitration (Ischiropoulos et al., 1992), and metal nitrosylation (Gupta et al., 2011). These PTMs are known to regulate protein function under various stress conditions; however, it used to be very difficult to predict that a particular protein may undergo NO-mediated PTMs. The evolution of *in silico* tools such as GPS-SNO (Xue et al., 2010) made it quite easy to predict if a protein may undergo S-nitrosation or not. Similar tools are available for other PTMs too. Global gene expression such as RNA-seq has made it easy to identify candidate genes that are responsive to a stimulus. In the last couple of decades, a number of transcriptomes have revealed a bulk of data that generated interesting information about NO-mediated transcriptional changes. For example, transcriptome analysis of Arabidopsis leaves and roots in response to 1 mM GSNO exogenous application differentially expressed about 3,263 genes after 3 h of treatment (Begara-Morales et al., 2014). Similarly, Parani et al. (2004) showed 422 DEGs in response to 0.1 mM sodium nitroprusside (SNP). Recently in RNA-seq mediated transcriptome analysis we reported more than six thousand genes (Hussain et al., 2016) and transcription factors (Imran et al., 2018a) that showed differential expression in response to 1 mM CySNO. In the present study, using a transcriptome-wide search, we identified a total of 440 NO-responsive DUF genes that showed differential expression in response to 1 mM CysNO. Among the 437 DUF domain-containing DEGs, 231 (53%) were upregulated, while 206 (47%) were downregulated, with at least a two-fold change in their expression, implying that the genes have a key role in transcriptional regulation of various physiological processes. The differential expression of such huge number of DUF genes suggests their involvement in NO biology and possibly in NO-mediated post-translational modifications. *In silico* analyses of DUF569 for targeting potential sites for phosphorylation suggest at least five different sites in DUF569 that could undergo phosphorylation (**Figure S3B**). Similarly, the 3D protein structure and simulation by GPS SNO for identification of potential sites for S-nitrosylation also suggested the presence of exposed Cysteine residues that could be potential target for S-nitrosylation (**Figures S3B, C**). DUF genes have also been identified in response to other stimuli. In Arabidopsis transcriptomic profiling in response to 0.1 and 1.0 mM SNP, 126 genes (98 upregulated and 28 downregulated) with

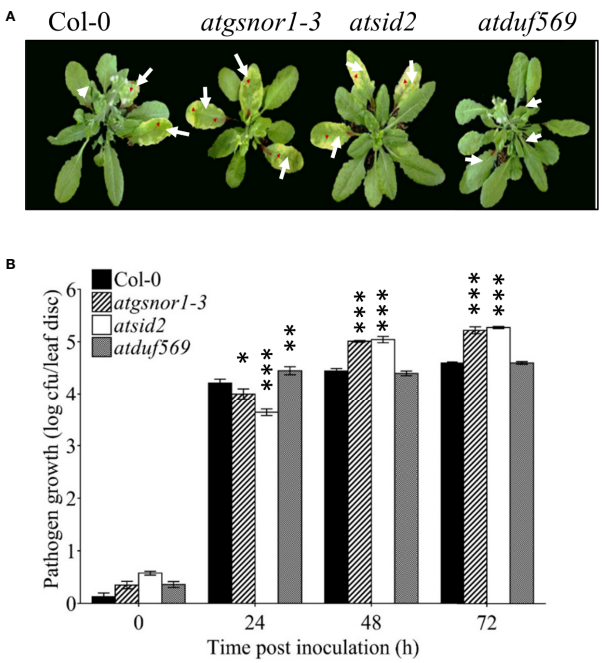

FIGURE 7 | *AtDUF569* negatively regulates basal defense. **(A)** Development of symptoms post-inoculation with virulent *Pst* DC3000, **(B)** Growth of the virulent *Pst* DC3000 in plants.

unknown functions were reported (Parani et al., 2004). Similarly, a transcriptomic study of *Arabidopsis* root exposed to 250 μM SNP revealed the largest category of genes with unknown and unclassified proteins known to date (Badri et al., 2008). More recently, in other plant species, such as upland cotton, transcriptome analysis based on Taq sequencing found 265 genes with uncharacterized proteins (Huang et al., 2018). Individual DUF genes have also been characterized. In a transcriptomic study of *Arabidopsis* plants exposed to SNP and O_3, there was a more than two-fold change in expression and the upregulation of *AtDUF569* (AT1G69890) (Ahlfors et al., 2009), suggesting that the *AtDUF569* participates in O_3-induced cell death and NO production.

We observed a significant increase in the expression of *AtDUF569* (57-fold) in response to NO donor application, which indicates relatively upstream and direct involvement of *AtDUF569* in NO-related responses in *A. thaliana*. The presence of the P-box binding site for prolamin box-binding factor 1 (pbf1) and W-box sequences in the promoters of all the DUF genes was particularly interesting because W-box sequences have been recognized as positive regulators of senescence in the Minghui 63 rice variety, in which they are bound by leaf senescence-specific proteins (Liu et al., 2016). Furthermore, the W-box is the cognate *cis*-element for WRKY proteins, which regulates essential cellular functions (Imran et al., 2018b). The presence of other *cis*-elements, such as RIN, the GT-box, ANT, HSF, and TATA, also indicates the involvement of DUF genes in key physiological processes. TFs are involved in plant development and defense, inducing or repressing the transcription of specific genes in signaling pathways that ultimately control the differential responses toward plant growth and defense (Jin et al., 2017). Reports have suggested that NO-responsive genes contain a significantly higher number of certain transcription factor binding sites (TFBS) in their promoter regions (Palmieri et al., 2008). Palmieri et al. (2008) using in silico approach analyzed 28447 Arabidopsis genes and suggested that several TFBS were found at least 15% more often in NO-induced genes. Furthermore, promoter analysis of other non-NO-responsive DUF genes may help scientists understand the full potential of the gene family. The functions of DUF genes in plants may well be highly similar and evolutionarily conserved, as indicated by the high homology observed during phylogenetic analysis of DUF genes from different plant species, which could be one of the reasons why the exact functions of the genes remain elusive, as one would expect different functions in different plant species; simple/model or complex.

Through the predicted interactome analysis, we observed that *AtDUF569* could interact with potentially important proteins. For example, out of ten proteins, DUF569 interacted with AT3G49790 which helps in ATP binding, therefore AtDUF569 is suggested to have important role during carbohydrate metabolism. Similarly, another interacting protein ATL6 E3 ubiquitin protein ligase is reported to be involved in regulating early steps of plant defense signaling, suggesting that AtDUF569 may regulate plant defense functions directly or by interacting with other proteins. Another interesting interacting protein is AR781 which is a pheromone receptor-like protein (DUF1645); if characterized fully, this gene might be a key in entomological research to attract a particular type of insects such as the honeybee. Thus, the interaction of AtDUF569 yields interesting and important information that can be further explored by an in-depth study.

We further sought to determine the role of DUF genes in the biological system using functional genomics approach. We, therefore, selected DUF569 which was among the top 10 DUFs that had the highest fold change in response to CysNO. Our results suggested that DUF569 negatively regulates shoot and root growth under nitro-oxidative stress, while in the case of pathogenicity, *duf569* showed sensitive phenotype at an early time point (24 h) followed by more pathogen growth while it showed a resistant phenotype afterward (**Figures 7A, B**). This was in accordance with the PR gene expression which showed low expression at 24 h while significantly higher expression at 48 h.

The trend indicates that the *atduf569*-resistant phenotype could be due to the upregulation of SA-dependent *PR* genes at the initial stage of pathogenicity, which influences the effect of the pathogen effect and, in turn, protects the mutant phenotype from later-stage toxicity and disease symptoms.

Collectively, our results suggested the possible involvement of DUF genes in plant metabolism and energy generation and/or turnover involving active trafficking between cellular organelles. Furthermore, biological data on *atduf569* KO plants suggested the involvement of the gene in regulating plant shoot and root

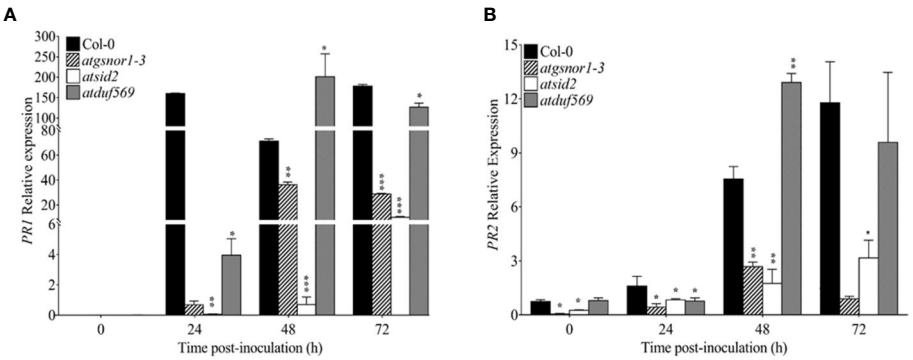

FIGURE 8 | Relative expression levels of *PR1* and *PR2* in basal defense. **(A)** Relative expression of *PR1* gene after *Pst* DC3000 (virulent) inoculation, **(B)** Relative expression of *PR2* gene after *Pst* DC3000 (virulent) inoculation. The experiments were conducted with three replicates and repeated four times, with similar results. Data were analyzed using the Student's *t*-test: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

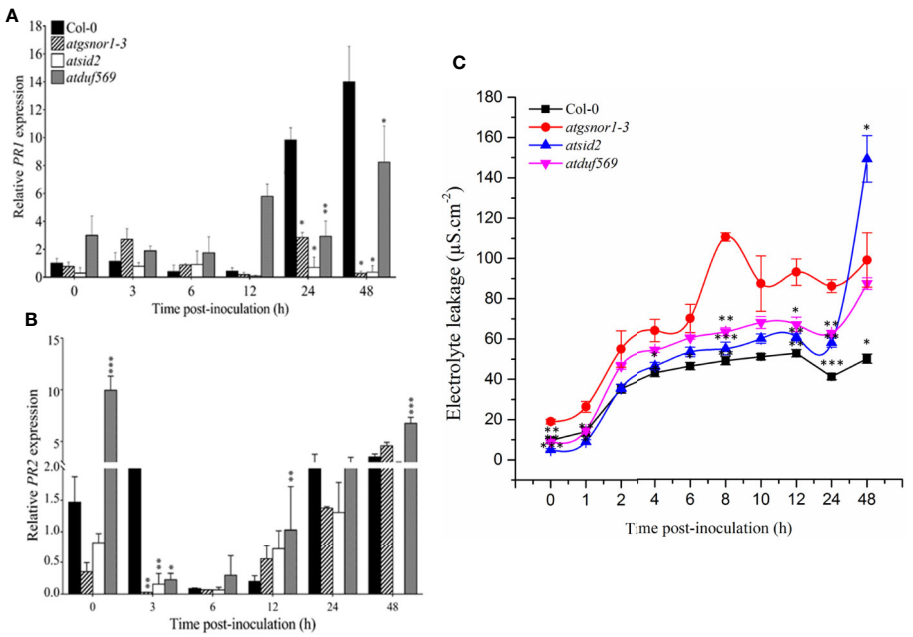

FIGURE 9 | Relative expression of *PR1* and *PR2* during evaluation of the genotypes for R-gene-mediated resistance. Plants were inoculated with the *Pst DC3000 avrB*, and symptoms were recorded for analyzing the gene expression of the pathogenesis-related *PR*s genes. **(A)** *PR1*, **(B)** *PR2* expression, **(C)** Electrolyte leakage over time. All the data points are the mean of three replicates. Data were analyzed using the Student's *t*-test: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

growth besides plant responses to oxidative as well as nitro-oxidative stress conditions.

CONCLUSION AND FUTURE PROSPECTS

The current study illustrates the role of *AtDUF569* in plant biology using a functional genomics approach. To the best of our knowledge, this is the first study attempting to functionally characterize NO-induced DUF genes in *Arabidopsis*. To date, no study has attempted to characterize NO-induced DUF genes in plant systems. Our study presents an exemplary model for future transcriptomic studies on similar DUF genes or proteins to elucidate molecular and functional aspects in detail in different plant species. This study motivates further studies to aid the

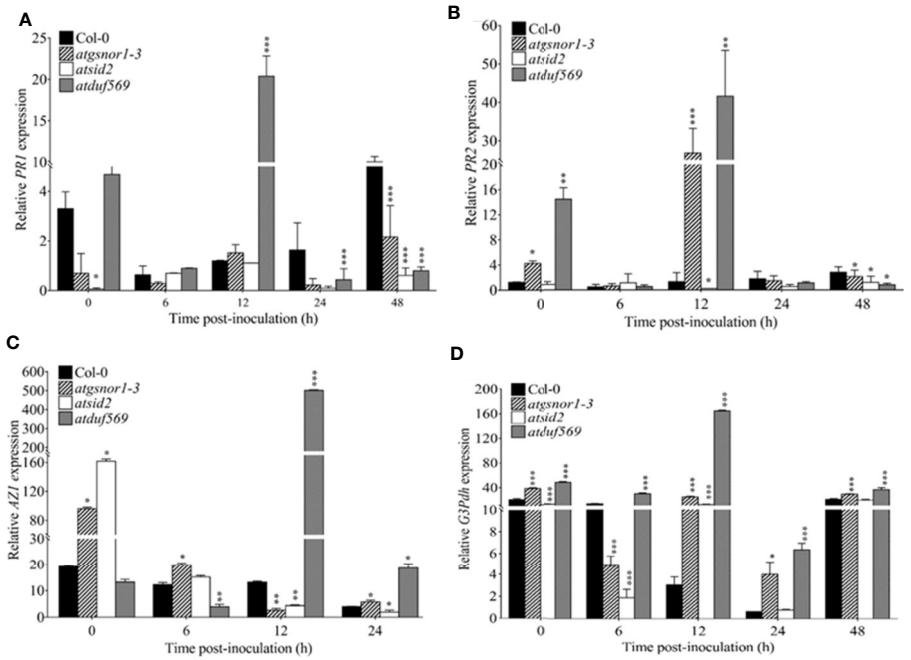

FIGURE 10 | Relative expression of *PR1*, *PR2*, *AZI*, and *G3DPH*. *AtDUF569* negatively regulates systemic acquired resistance. Plants were inoculated with the *Pst DC3000 avrB*, and symptoms were recorded to analyze the gene expression dynamics of the pathogenesis-related genes. All the data points are the mean of three replicates **(A)** *PR1*, **(B)** *PR2*, **(C)** *AZI*, and **(D)** *G3DPH*. Data were analyzed using the Student's *t*-test: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

comprehensive characterization of *AtDUF569* about its cellular functioning.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the the public repository for Gene Expression Omnibus (GEO) and Short Read Achieve (SRA) under accession numbers GSE81361 and SRP074890.

AUTHOR CONTRIBUTIONS

RN performed the experiments and analyzed the data. RN, RT, and QI drafted the manuscript. AH and MS helped with the conceptualization of the study. QI, MS, and RN prepared illustrations, figures, tables, and references. B-WY, AH, and QI edited the manuscript. B-WY contributed critical comments to the draft and approved the manuscript. All authors contributed to the article and approved the submitted version

FUNDING

This work was supported by Next-Generation Bio Green21 Program (SSAC, Grant No. PJ01342501) Rural Development Administration, South Korea.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01041/full#supplementary-material

REFERENCES

Ahlfors, R., Brosché, M., Kollist, H., and Jaakko, K. (2009). Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in Arabidopsis thaliana. *Plant J.* 58, 1–12. doi: 10.1111/j.1365-313X.2008.03756.x

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., and Kubiś, J. (2009). Involvement of nitric oxide in water stress-induced responses of cucumber roots. *Plant Sci.* 177, 682–690. doi: 10.1016/j.plantsci.2009.09.007

Badri, D. V., Loyola-Vargas, V. M., Du, J., Stermitz, F. R., Broeckling, C. D., Iglesias-Andreu, L., et al. (2008). Transcriptome analysis of Arabidopsis roots treated with signaling compounds: A focus on signal transduction, metabolic regulation and secretion. *New Phytol.* 179, 209–223. doi: 10.1111/j.1469-8137.2008.02458.x

Bateman, A., Coggill, P., and Finn, R. D. (2010). DUFs: Families in search of function. *Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun.* 66, 1148–1152. doi: 10.1107/S1744309110001685

Begara-Morales, J. C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., López-Jaramillo, J., et al. (2014). Dual regulation of cytosolic ascorbate peroxidase (APX) by tyrosine nitration and *S*-nitrosylation. *J. Exp. Bot.* 65, 527–538. doi: 10.1093/jxb/ert396

Cui, Y., Wang, M., Zhou, H., Li, M., Huang, L., Yin, X., et al. (2016). OsSGL, a novel DUF1645 domain-containing protein, confers enhanced drought tolerance in transgenic rice and arabidopsis. *Front. Plant Sci.* 7, 1–13. doi: 10.3389/fpls.2016.02001

Czolpinska, M., and Rurek, M. (2018). Plant glycine-rich proteins in stress response: An emerging, still prospective story. *Front. Plant Sci.* 9, 1–13. doi: 10.3389/fpls.2018.00302

Delledonne, M., Xia, Y., Dixon, R. A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* 394 (6693), 585–588. doi: 10.1038/29087

Desikan, R., Mackerness, S. A. H., Hancock, J. T., and Neill, S. J. (2001). Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiol.* 127 (1), 159–172. doi: 10.1104/pp.127.1.159

Domingos, P., Prado, A. M., Wong, A., Gehring, C., and Feijo, J. A. (2015). Nitric oxide: A multitasked signaling gas in plants. *Mol. Plant* 8, 506–520. doi: 10.1016/j.molp.2014.12.010

Durner, J., Wendehenne, D., and Klessig, D. F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc. Natl. Acad. Sci.* 95 (17), 10328–10333. doi: 10.1073/pnas.95.17.10328

El-Gebali, S., Mistry, J., Bateman, A., Eddy, S. R., Luciani, A., Potter, S. C., et al. (2019). The Pfam protein families database in 2019. *Nucleic Acids Res.* 47, D427–D432. doi: 10.1093/nar/gky995

Feechan, A., Kwon, E., Yun, B.-W., Wang, Y., Pallas, J., and Loake, G. J. (2005). A central role for S-nitrosothiols in plant disease resistance. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8054–8059. doi: 10.1073/pnas.0501456102

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., et al. (2016). The Pfam protein families database: Towards a more sustainable future. *Nucleic Acids Res.* 44, D279–D285. doi: 10.1093/nar/gkv1344

Flors, V., Ton, J., Van Doorn, R., Jakab, G., García-Agustín, P., and Mauch-Mani, B. (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against Pseudomonas syringae and Alternaria brassicicola. *Plant J.* 54, 81–92. doi: 10.1111/j.1365-313X.2007.03397.x

García-Mata, C., and Lamattina, L. (2002). Nitric oxide and abscisic acid cross talk in guard cells. *Plant Physiol.* 128, 790–792. doi: 10.1104/pp.011020

Greenacre, S. A. B., and Ischiropoulos, H. (2001). Tyrsosine nitration: localization, quantification, consequences for protein function and signal transduction. *Free Rad. Res.* 34, 541–581. doi: 10.1080/10715760100300471

Gupta, K. J., Hebelstrup, K. H., Mur, L. A. J., and Igamberdiev, A. U. (2011). Plant hemoglobins: Important players at the crossroads between oxygen and nitric oxide. *FEBS Lett.* 585 (24), 3843–3849. doi: 10.1016/j.febslet.2011.10.036

He, X., Hou, X., Shen, Y., and Huang, Z. (2011). TaSRG, a wheat transcription factor, significantly affects salt tolerance in transgenic rice and Arabidopsis. *FEBS Lett.* 585, 1231–1237. doi: 10.1016/j.febslet.2011.03.055

Hu, Y. Q., Liu, S., Yuan, H. M., Li, J., Yan, D. W., Zhang, J. F., et al. (2010). Functional comparison of catalase genes in the elimination of photorespiratory H2O2 using promoter- and 3'-untranslated region exchange experiments in the Arabidopsis cat2 photorespiratory mutant. *Plant Cell Environ.* 33, 1656–1670. doi: 10.1111/j.1365-3040.2010.02171.x

Huang, J., Wei, H., Li, L., and Yu, S. (2018). Transcriptome analysis of nitric oxide-responsive genes in upland cotton (Gossypium hirsutum). *PloS One* 13, 1–18. doi: 10.1371/journal.pone.0192367

Hussain, A., Mun, B.-G., Imran, Q. M., Lee, S.-U., Adamu, T. A., Shahid, M., et al. (2016). Nitric Oxide Mediated Transcriptome Profiling Reveals Activation of Multiple Regulatory Pathways in Arabidopsis thaliana. *Front. Plant Sci.* 7, 975. doi: 10.3389/fpls.2016.00975

Imran, Q. M., Falak, N., Hussain, A., Mun, B.-G., Sharma, A., Lee, S.-U., et al. (2016). Nitric oxide responsive heavy metal-associated gene AtHMAD1 contributes to development and disease resistance in Arabidopsis thaliana. *Front. Plant Sci.* 7. doi: 10.3389/fpls.2016.01712

Imran, Q. M., Hussain, A., Lee, S. U., Mun, B. G., Falak, N., Loake, G. J., et al. (2018a). Transcriptome profile of NO-induced Arabidopsis transcription factor genes suggests their putative regulatory role in multiple biological processes. *Sci. Rep.* 8, 1–14. doi: 10.1038/s41598-017-18850-5

Imran, Q. M., Hussain, A., Mun, B. G., Lee, S. U., Asaf, S., Ali, M. A., et al. (2018b). Transcriptome wide identification and characterization of NO-responsive WRKY transcription factors in Arabidopsis thaliana L. *Environ. Exp. Bot.* 148, 128–143. doi: 10.1016/j.envexpbot.2018.01.010

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., et al. (1992). Peroxynitrite-Mediated Tyrosine Nitration Catalyzed by Superoxide-Dismutase. *Arch. Biochem. Biophysics* 298 (2), 431–437. doi: 10.1016/0003-9861(92)90431-U

Jin, Y., Eser, U., Struhl, K., and Churchman, L. S. (2017). The Ground State and Evolution of Promoter Region Directionality. *Cell* 170, 889–898.e10. doi: 10.1016/j.cell.2017.07.006

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Bioinformatics* 8, 275–282. doi: 10.1093/bioinformatics/8.3.275

Kerwin, J. F.Jr., Lancaster, J. R.Jr., and Feldman, P. L. (1995). Nitric oxide: a new paradigm for second messengers. *J. Med. Chem.* 38 (22), 4343–4362. doi: 10.1021/jm00022a001

Kim, J. H., and Kim, W. T. (2013). The arabidopsis RING E3 ubiquitin ligase AtAIRP3/LOG2 participates in positive regulation of high-salt and drought stress responses. *Plant Physiol.* 162, 1733–1749. doi: 10.1104/pp.113.220103

Koornneef, M., Hanhart, C. J., and van der Veen, J. H. (1991). A genetic and physiological analysis of late flowering mutants in Arabidopsis thaliana. *MGG Mol. Gen. Genet.* 229, 57–66. doi: 10.1007/BF00264213

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Li, L., Xie, C., Ye, T., Xu, J., Chen, R., Gao, X., et al. (2017). Molecular characterization, expression pattern and function analysis of the rice OsDUF866 family. *Biotechnol. Biotechnol. Equip.* 31, 243–249. doi: 10.1080/13102818.2016.1268932

Li, L. H., Lv, M. M., Li, X., Ye, T. Z., He, X., Rong, S. H., et al. (2018a). The Rice OsDUF810 Family: OsDUF810.7 May be Involved in the Tolerance to Salt and Drought. *Mol. Biol. (Mosk).* 52, 567–575. doi: 10.1134/S0026898418040122

Li, L., Lv, M., Ye, T., He, X., Dong, Y., Rong, S., et al. (2018b). Genome-wide identification and analyses of the rice OsDUF639 family. *Int. J. Agric. Biol.* 20, 1117–1122. doi: 10.17957/IJAB/15.0611

Liu, L., Xu, W., Hu, X., Liu, H., and Lin, Y. (2016). W-box and G-box elements play important roles in early senescence of rice flag leaf. *Sci. Rep.* 6, 1–9. doi: 10.1038/srep20881

Luo, C., Guo, C., Wang, W., Wang, L., and Chen, L. (2014). Overexpression of a new stress-repressive gene OsDSR2 encoding a protein with a DUF966 domain increases salt and simulated drought stress sensitivities and reduces ABA sensitivity in rice. *Plant Cell Rep.* 33, 323–336. doi: 10.1007/s00299-013-1532-0

Medina-Rivera, A., Defrance, M., Sand, O., Herrmann, C., Castro-Mondragon, J. A., Delerce, J., et al. (2015). RSAT 2015: Regulatory sequence analysis tools. *Nucleic Acids Res.* 43, W50–W56. doi: 10.1093/nar/gkv362

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol. Plant* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Nabi, R. B. S., Tayade, R., Hussain, A., Kulkarni, K. P., Imran, Q. M., Mun, B. G., et al. (2019). Nitric oxide regulates plant responses to drought, salinity, and heavy metal stress. *Environ. Exp. Bot.* 161, 120–133. doi: 10.1016/j.envexpbot.2019.02.003

Nuruzzaman, M., Sharoni, A. M., and Kikuchi, S. (2013). Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front. Microbiol.* 4, 1–16. doi: 10.3389/fmicb.2013.00248

Pagnussat, G. C., Lanteri, M. L., and Lamattina, L. (2003). Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. *Plant Physiol.* 132, 1241–1248. doi: 10.1104/pp.103.022228

Palmieri, M. C., Sell, S., Huang, X., Scherf, M., Werner, T., Durner, J., et al. (2008). Nitric oxide-responsive genes and promoters in Arabidopsis thaliana: a bioinformatics approach. *J. Exp. Bot.* 59 (2), 177–186. doi: 10.1093/jxb/erm345

Parani, M., Rudrabhatla, S., Myers, R., Weirich, H., Smith, B., Leaman, D. W., et al. (2004). Microarray analysis of nitric oxide responsive transcripts in Arabidopsis. *Plant Biotechnol. J.* 2, 359–366. doi: 10.1111/j.1467-7652.2004.00085.x

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., et al. (2012). The Pfam protein families database. *Nucleic Acids Res.* 40, 290–301. doi: 10.1093/nar/gkr1065

Sham, A., Al-Azzawi, A., Al-Ameri, S., Al-Mahmoud, B., Awwad, F., Al-Rawashdeh, A., et al. (2014). Transcriptome Analysis Reveals Genes Commonly Induced by Botrytis cinerea Infection, Cold, Drought and Oxidative Stresses in Arabidopsis. *PloS One* 9 (11). doi: 10.1371/journal.pone.0113718

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., et al. (1992). S-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biologically active compounds. *Proc. Natl. Acad. Sci. U. S. A.* 89, 444–448. doi: 10.1073/pnas.89.1.444

Stamler, J. S., Lamas, S., and Fang, F. C. (2001). Nitrosylation: the prototypic redox-based signaling mechanism. *Cell* 106 (6), 675–683.

Tada, (2008). Plant Immunity Requires Conformational Charges of NPR1 via S-Nitrosylation and Thioredoxins. *Bone* 23, 1–7. doi: 10.1161/CIRCULATIONAHA.110.956839

Wang, Y. Q., Feechan, A., Yun, B. W., Shafiei, R., Hofmann, A., Taylor, P., et al. (2009). S-nitrosylation of AtSABP3 antagonizes the expression of plant immunity. *J. Biol. Chem.* 284, 2131–2137. doi: 10.1074/jbc.M806782200

Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. (1991). Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell* 3, 49–59. doi: 10.1105/tpc.3.1.49

Xie, Z., Nolan, T. M., Jiang, H., and Yin, Y. (2019). AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in Arabidopsis. *Front. Plant Sci.* 10, 1–17. doi: 10.3389/fpls.2019.00228

Xu, E. J., Vaahtera, L., and Brosche, M. (2015). Roles of Defense Hormones in the Regulation of Ozone-Induced Changes in Gene Expression and Cell Death. *Mol. Plant* 8 (12), 1776–1794. doi: 10.1016/j.molp.2015.08.008

Xue, Y., Liu, Z. X., Gao, X. J., Jin, C. J., Wen, L. P., Yao, X. B., et al. (2010). GPS-SNO: Computational Prediction of Protein *S*-Nitrosylation Sites with a Modified GPS Algorithm. *PloS One* 5 (6), ARTN e11290. doi: 10.1371/journal.pone.0011290

Yun, B.-W., Feechan, A., Yin, M., Saidi, N. B. B., Le Bihan, T., Yu, M., et al. (2011). S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. *Nature* 478, 264–268. doi: 10.1038/nature10427

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.

frontiers in Plant Science

REVIEW
published: 31 July 2020
doi: 10.3389/fpls.2020.01177

Potential Pathway of Nitrous Oxide Formation in Plants

Arbindra Timilsina[1,2]*, *Chuang Zhang*[1,2], *Bikram Pandey*[2,3], *Fiston Bizimana*[1,2], *Wenxu Dong*[1] and *Chunsheng Hu*[1,2]*

[1] Key Laboratory of Agricultural Water Resources, Hebei Key Laboratory of Soil Ecology, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, China, [2] University of Chinese Academy of Sciences, Beijing, China, [3] Key Laboratory of Mountain Ecological Restoration and Bio-resource Utilization and Ecological Restoration Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China

OPEN ACCESS

Edited by:
Juan B. Barroso,
University of Jaén, Spain

Reviewed by:
Maria J. Delgado,
Estación Experimental de Zaidín (CSIC), Spain
Francesco Di Gioia,
Pennsylvania State University (PSU), United States

***Correspondence:**
Arbindra Timilsina
arbintms@sjziam.ac.cn
Chunsheng Hu
cshu@sjziam.ac.cn

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 08 April 2020
Accepted: 20 July 2020
Published: 31 July 2020

Citation:
Timilsina A, Zhang C, Pandey B, Bizimana F, Dong W and Hu C (2020) Potential Pathway of Nitrous Oxide Formation in Plants. Front. Plant Sci. 11:1177. doi: 10.3389/fpls.2020.01177

Plants can produce and emit nitrous oxide (N_2O), a potent greenhouse gas, into the atmosphere, and several field-based studies have concluded that this gas is emitted at substantial amounts. However, the exact mechanisms of N_2O production in plant cells are unknown. Several studies have hypothesised that plants might act as a medium to transport N_2O produced by soil-inhabiting microorganisms. Contrarily, aseptically grown plants and axenic algal cells supplied with nitrate (NO_3) are reported to emit N_2O, indicating that it is produced inside plant cells by some unknown physiological phenomena. In this study, the possible sites, mechanisms, and enzymes involved in N_2O production in plant cells are discussed. Based on the experimental evidence from various studies, we determined that N_2O can be produced from nitric oxide (NO) in the mitochondria of plants. NO, a signaling molecule, is produced through oxidative and reductive pathways in eukaryotic cells. During hypoxia and anoxia, NO_3 in the cytosol is metabolised to produce nitrite (NO_2), which is reduced to form NO *via* the reductive pathway in the mitochondria. Under low oxygen condition, NO formed in the mitochondria is further reduced to N_2O by the reduced form of cytochrome c oxidase (CcO). This pathway is active only when cells experience hypoxia or anoxia, and it may be involved in N_2O formation in plants and soil-dwelling animals, as reported previously by several studies. NO can be toxic at a high concentration. Therefore, the reduction of NO to N_2O in the mitochondria might protect the integrity of the mitochondria, and thus, protect the cell from the toxicity of NO accumulation under hypoxia and anoxia. As NO_3 is a major source of nitrogen for plants and all plants may experience hypoxic and anoxic conditions owing to soil environmental factors, a significant global biogenic source of N_2O may be its formation in plants *via* the proposed pathway.

Keywords: anoxia, hypoxia, nitrate, nitrite, nitric oxide, nitrous oxide, mitochondrion

INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas, and its potential to increase global warming is approximately 300-fold higher than CO_2 (Tian et al., 2018). Globally, the primary sources that release N_2O into the atmosphere are soil, ocean, manure application, industries, and biomass burning (Thomson et al., 2012). The nitrification and denitrification processes, mainly mediated by certain groups of soil micro-organisms (Hu et al., 2015), account for more than two-thirds of its emission into the atmosphere (Thomson et al., 2012). These processes are considerably increased by human activities, leading to an increase in the production of N_2O in the soil, and thus, its concentration in the atmosphere. Increased level of N_2O in the atmosphere has significantly contributed to global warming (Tian et al., 2018); therefore, understanding the pathways of N_2O formation in various sources is essential for mitigating these effects.

Key pathways involved in N_2O production in microbes include nitrification, nitrifier denitrification, nitrification-coupled denitrification, and denitrification (Baggs, 2011; Hu et al., 2015; Tian et al., 2018). However, there seems to be a gap between source estimation and the global N_2O budget, leading to a high level of uncertainty in the budget estimations (Davidson and Kanter, 2014). This gap may be because not all sources of N_2O to the atmosphere are accounted for (Syakila and Kroeze, 2011). Therefore, it is necessary to understand all sources of N_2O and underlying mechanisms to elucidate its global budget. The production of N_2O in axenic microalgae (Weathers, 1984; Weathers and Niedzielski, 1986; Guieysse et al., 2013; Plouviez et al., 2017) and ascetically grown plants (Goshima et al., 1999; Hakata et al., 2003) indicates that it could be produced by higher organisms and that the processes might be different from those in micro-organisms. Algae and plants are not included as sources of N_2O (Syakila and Kroeze, 2011; Lenhart et al., 2019; Plouviez et al., 2019), but they might be the missing sources of N_2O, causing high uncertainties in the global budget.

The roles of plants in N_2O emission to the atmosphere are diverse. Plants can not only modify soil characteristics and subsequently influence N_2O production in the soil (Gao et al., 2019) but also produce it in significant amounts and release it to the atmosphere (Lenhart et al., 2019). Thus, understanding the pathway of N_2O formation and the contribution of plants to total emission is essential to accurately estimate the global N_2O budget. Several field-based studies have hypothesised that N_2O emitted by plants is produced in soil by microorganisms (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017). In this theory, plants are considered just a medium to transport N_2O produced by soil microorganisms; however, laboratory-based studies have provided clear evidence that plants produce and emit N_2O although the underlying mechanisms are unknown (Goshima et al., 1999; Hakata et al., 2003; Lenhart et al., 2019). Therefore, N_2O emitted by plants might originate from two sources, namely, the soil microorganisms and plants.

Studies, which have hypothesised that plant-emitted N_2O is produced by soil microorganisms, have only measured the fluxes from plants and concluded that plant-emitted N_2O might be produced by soil microorganisms. N_2O produced in plant cells might also use the same pathway, that is, transpiration, to release it to the atmosphere. This raises the question whether measuring the fluxes alone provides substantial evidence to prove the hypothesis, because flux measurement methods can just estimate the emission of N_2O and cannot distinguish the sources. More robust methods such as isotope studies would provide more insights to distinguish the sources of N_2O. For examples, injecting ^{15}N-N_2O into the root zone and measuring the subsequent fluxes would elucidate whether plants are a medium for N_2O transport or not. However, no study has injected ^{15}N-labeled N_2O into the soil zone and measured the subsequent N_2O emission from plants. Moreover, more powerful tools such as site preference (SP) measurement would provide insights to distinguish the sources of N_2O emitted by plants under field conditions.

In the natural environment, if plant emitted N2O constitute significant amount of both sources (soil micro-organisms and plant cells produced N2O), it will be highly challenging to distinguish the portion of the sources. A recent field experiment reported considerably lower N_2O concentrations in soil water than in tree stems (Ward et al., 2019). Similarly, plants exposed to NH_4 did not emit N_2O despite the high rate of N_2O production in the rhizosphere (Smart and Bloom, 2001), indicating that N_2O emitted by plants might not be produced by soil microorganisms and that N_2O emitted through transpiration might be a less significant process than N_2O production in plants. Furthermore, the hypothesis that plants are just a conduit for soil microorganisms-produced N_2O is not supported by a recent study of Lenhart et al. (2019). They provided new evidence that dual isotopocule fingerprints of N_2O emitted by plants differed from that produced by all known microbial or chemical processes, indicating that plant-emitted N_2O is produced in plant cells.

Although plants are known to produce N_2O and emit it to the atmosphere, the exact mechanisms of N_2O production in plant cells are unknown (Goshima et al., 1999; Hakata et al., 2003; Lenhart et al., 2019). This might be the reason that most studies on N_2O fluxes in plants (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017) have hypothesised that plant parts act as a conduit for soil-produced N_2O. Studies, which have claimed that plants could produce N_2O, have not elucidated a possible production pathway. Therefore, the main objective of this study was to review the possible pathway of N_2O formation in plant cells.

PATHWAY OF N_2O FORMATION IN PLANT CELLS

Nitrate (NO_3) as a Precursor for N_2O Formation in Plant Cells

Nitrogen (N) is an essential macronutrient influencing cell metabolism (O'Brien et al., 2016). Plants can use several forms

of N from the soil; however, NO_3 and ammonium (NH_4) are the major forms of inorganic N that are readily available for plant uptake (O'Brien et al., 2016; Hachiya and Sakakibara, 2017). NO_3 is a major source of N for plants in agricultural and natural soils (von Wirén et al., 2000), due to its high soil concentration and diffusion coefficients, making it readily available to plant roots (Miller and Cramer, 2005). After absorption, NO_3 is directly reduced in the root, transported to the leaf for reduction (Maathuis, 2009; Hachiya and Sakakibara, 2017), or stored in the vacuoles and remobilised when the external supply is limited (van der Leij, et al., 1998; Fan et al., 2007), making it an essential macronutrient in plant metabolism.

NO_3 is a major source for N_2O formation in both soil (Thomson et al., 2012) and plants (Goshima et al., 1999; Smart and Bloom, 2001; Hakata et al., 2003; Lenhart et al., 2015; Lenhart et al., 2019). Isotope labeling methods have demonstrated that plants as well as other eukaryotic organisms emit N_2O, only when supplied with NO_3. For example, when ^{15}N-labeled NO_3 was supplied as a source of N to various species of plants (Goshima et al., 1999; Smart and Bloom, 2001; Lenhart et al., 2019), lichens (Lenhart et al., 2015), and animals (Stief et al., 2009), ^{15}N-labeled N_2O was emitted, but when the N source was ^{15}N-labeled NH_4, there was no N_2O emission. This evidence clearly shows that NO_3 is the precursor of N_2O in lichens, higher plants, and animals. Therefore, if plants were just a medium of transportation of soil-produced N_2O as hypothesised by many studies (Chang et al., 1998; Rusch and Rennenberg, 1998; Machacova et al., 2013; Bowatte et al., 2014; Wen et al., 2017), aseptically grown plants would not have emitted N_2O when supplied with NO_3 (Goshima et al., 1999; Hakata et al., 2003). Similarly, if N_2O emitted by plants is produced by microorganisms (nitrifying and denitrifying bacteria), NH_4 supplementation should contribute to N_2O emission from plants and aseptically grown plants should not emit N_2O. As N_2O was not emitted when plants were supplied with NH_4 and aseptically grown plants emitted N_2O (Goshima et al., 1999; Hakata et al., 2003), we predicted that NO_3 metabolism in a cell might play a role in N_2O formation in plants. Moreover, the processes in the soil microbial communities and higher organisms may be different, or the denitrification process may be common between microorganism and plants, as NO_3 is the substrate for denitrification.

Nitrite (NO_2) Derived From NO_3 Reduction Is the Precursor of N_2O in Plant Cells

After the uptake of NO_3 by plant roots, it is reduced to NO_2 in plant cells by a cytosolic enzyme called nitrate reductase (NR) (Chamizo-Ampudia et al., 2017). However, in animals, NO_3 from food is reduced by bacteria in the digestive tracks (Lundberg et al., 2008). Moreover, germ-free mice are reported to possess NR activity, and the activity is catalysed by xanthine oxidoreductase, which is significantly high in the gastrointestinal tissues, compared with other tissues (Jansson et al., 2008). Under normal conditions, NO_3 absorbed by the roots is reduced to NO_2 by the NR, and then nitrite reductase (NiR) catalyses the reduction of NO_2 to NH_4, which is incorporated into amino acids (Oliveira and Sodek, 2013; Plouviez et al., 2017). However, under hypoxic and anoxic conditions, root NO_3 uptake increases with the activation level of NR (Botrel and Kaiser, 1997; Rockel et al., 2002; Morard et al., 2004; Horchani et al., 2010). Furthermore, NO_2 accumulates in the cytoplasm of cells (Allègre et al., 2004; Morard et al., 2004), as both hypoxia and anoxia suppress the reduction of NO_2 to NH_4 (Botrel and Kaiser, 1997). A ^{15}N isotope labeling study has showed that ^{15}N-NO_2 assimilation into amino acids is sharply reduced under hypoxic conditions (Oliveira and Sodek, 2013). The accumulated NO_2 in the cytoplasm enters the mitochondria with the help of proteins in the chloroplast (Sugiura et al., 2007; Gupta and Igamberdiev, 2011). Moreover, mitochondrial inner membrane anion channels may import NO_2 to the mitochondria (Gupta and Igamberdiev, 2011).

Not only NO_3, but also NO_2 is widely reported to be a precursor of N_2O in eukaryotic organisms. Using ^{15}N isotope labeling method, it has been demonstrated that NO_2 is another precursor of N_2O formation in plants and algal cells. For example, when ^{15}N-labeled NO_2 was supplied to aseptically grown tobacco plants (Goshima et al., 1999; Hakata et al., 2003) and algal systems (Weathers, 1984), they emitted ^{15}N-N_2O. Furthermore, axenic algae supplied with NO_2 produced N_2O (Guieysse et al., 2013; Plouviez et al., 2017). The enzyme NR has been proved to play a role in N_2O production in plants. For example, when tobacco plants were supplied with NO_3 and tungstate (NR inhibitor), N_2O production was inhibited in the plants (Goshima et al., 1999). As NO_2 also contributes to N_2O production in plants (Goshima et al., 1999; Hakata et al., 2003), NR might indirectly be involved in N_2O formation by catalysing the reduction of NO_3 to NO_2. A similar role of NR has been observed in algae (Plouviez et al., 2017). However, NiR-deficient transgenic plants and algae have been reported to produce N_2O when supplied with NO_2 (Hakata et al., 2003; Plouviez et al., 2017), and this suggests that the pathway of NO_2 reduction to NH_4 is not involved in N_2O production in plants and algal cells. Additionally, NO_3, NR, and NO_2 are involved in N_2O production in plants and algal cells, but NiR and NH_4 are not involved in the N_2O production pathway. This indicates that NO_2 has to be transported to other cell organelles rather than plastid. Overall, the available evidence indicates that exogenous NO_2 along with endogenous NO_2 derived through NO_3 reduction in the cytosol by NR plays a role in N_2O formation in plant cells.

Mitochondrial Reduction of Nitrite to NO

We previously discussed NO_3 reduction to NO_2 in the cytosol. NO has several essential roles in plant and animal cells (Wendehenne et al., 2001), and the conversion mechanisms of NO_2 to NO are well established in eukaryotic cells. In plants, NO can be produced in the chloroplast, peroxisomes, and mitochondria by either oxidative or reductive pathways (Rőszer, 2012). The oxidative pathway is dependent on L-arginine, polyamine, or hydroxylamine, whereas the reductive pathway is dependent on NO_3 and NO_2 (Benamar et al., 2008; Lundberg et al., 2008; Gupta et al., 2011; Gupta and Igamberdiev,

2011; Astier et al., 2018). The oxidative pathway of NO formation is dominant when the oxygen supply to cells is sufficient, whereas the reductive path is dominant under hypoxic conditions. By shifting from the oxidative to reductive pathway, the cells maintain the level of NO along with the physiological and pathological oxygen and proton gradients (Lundberg et al., 2008). It may be essential to shift processes, as plants may experience hypoxia due to soil environmental conditions.

In plant cells, NO_2 assimilation to NH_4 by the NiR enzyme is a well-known pathway of NO_2 metabolism. As NO_2 addition can lead to N_2O formation in plants (Goshima et al., 1999; Hakata et al., 2003) and NiR-deficient plants can produce N_2O (Hakata et al., 2003), we suggest that NO_2 is metabolised by another pathway in plants to produce N_2O. Although the mechanisms of NO_2 transport to the mitochondria are not precise, it is evident that the mitochondria are a site of reduction of NO_2 to NO. For example, the mitochondria have been reported to reduce NO_2 to NO under hypoxic and anoxic conditions in fungi (Kobayashi et al., 1996; Castello et al., 2006), algae (Tischner et al., 2004; Calatrava et al., 2017), plants (Gupta et al., 2005; Planchet et al., 2005; Benamar et al., 2008; Gupta and Kaiser, 2010), and animals (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Kozlov et al., 1999; Castello et al., 2006; Ascenzi et al., 2014). However, the enzymes involved in the mitochondrial reduction of NO_2 to NO are not clear. For example, mitochondria that lack NiR can reduce NO_2 to NO in animals and plants (Gupta and Igamberdiev, 2011). Nitric oxide synthases (NOS) have been reported to be present in plant (Guo and Crawford, 2005) and animal mitochondria (Giulivi et al., 1998). However, the NOS activity in the mitochondria of plants is questioned (Moreau et al., 2008; Gupta and Kaiser, 2010). Tischner et al. (2004) identified an alternative oxidase (AOX) in the mitochondria as a catalyser of the reduction of NO_2 to NO under anoxic conditions. The mitochondrial respiratory chain is responsible for NO production using NO_2 as the substrate under low pH, hypoxic, or anoxic conditions (Castello et al., 2006). Mitochondrial and bacterial electron transport chains (ETCs) are involved in NO production from NO_2 under hypoxic conditions than under normoxic conditions (Horchani et al., 2011). Under hypoxic conditions, NO_2 is reduced to NO at complex III in the mitochondria of pea plants (Benamar et al., 2008). Ascenzi et al. (2014) reported cytochrome c in horse heart cells and bovine heart reduced NO_2 to NO, and the activity was high under anoxic and acidic conditions (Basu et al., 2008). The mitochondrial molybdopterin enzymes in the reduced form catalyse the reduction of NO_2 to NO, and the rate was increased when the pH was decreased from 7.5 to 6.5 (Jakobs et al., 2014; Sparacino-Watkins et al., 2014; Maia and Moura, 2015; Bender and Schwarz, 2018). Furthermore, cytochrome reductase in tobacco plants can reduce NO_2 to NO (Alber et al., 2017). Although at the molecular level, the reductive pathway for NO formation is well documented, at the field scale, the emission of NO is less documented. For instance, when plants were supplied with NO_3, NO was emitted under anoxic conditions (Klepper, 1987; Rockel et al., 2002). The leaf NO_2 level and NO emission under anoxic conditions were significantly higher than those under normoxic conditions (Rockel et al., 2002). These findings suggest that NO_2 can be reduced to NO in the mitochondria; however, the involvement of various enzymes within the mitochondria raises the question whether these enzymes catalyse the reduction process simultaneously or they function differently under varied cell environment.

NO Conversion to N_2O in the Mitochondria

NO is a signaling molecule in cells, and several studies have focused on its formation in the mitochondria. However, studies on the reduction of NO to N_2O in the mitochondria are limited, although there is a strong indication that this process exists (Gupta and Igamberdiev, 2011). The inner membrane of the mitochondria has an enzyme called cytochrome c oxidase (CcO). The primary function of CcO is to reduce O_2 to H_2O (Collman et al., 2007; Blomberg and Ädelroth, 2018). Moreover, CcO has several other functions, such as the oxidisation of NO formed in the mitochondria to NO_2 (Brudvig et al., 1980; Zhao et al., 1995; Pearce et al., 2002; Taylor and Moncada, 2010). Furthermore, the reduced form of CcO can catalyse the reduction of NO to N_2O (Brudvig et al., 1980; Zhao et al., 1995). Thus, either oxidation or reduction of NO by CcO results in the metabolism of NO with safe end products. The similar properties of O_2 and NO facilitate the binding of NO to CcO, and this activity is pronounced under oxygen-limited conditions (Ghafourifar and Cadenas, 2005). The mitochondrial electron transport chain (ETC) in axenic algae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*) catalyses the reduction of NO to N_2O (Guieysse et al., 2013; Plouviez et al., 2017; Plouviez et al., 2019). CcO has some rudimentary nitric oxide reductase activity, and therefore, when NO is the substrate instead of O_2, two molecules of NO yield N_2O and H_2O (Brudvig et al., 1980; Zhao et al., 1995; Koivisto et al., 1997; Igamberdiev et al., 2010; Blomberg and Ädelroth, 2018; Poderoso et al., 2019). It has also been proven isotopically that NO is reduced to N_2O by CcO in higher organisms (Brudvig et al., 1980). As mitochondrial CcO has evolved from denitrifying enzymes, under hypoxic conditions in cells, the mitochondrial CcO can still reduce NO to N_2O (Saraste, 1994; Saraste and Castresana, 1994). Furthermore, another enzyme in the mitochondria, that is, quinone of the ETC catalyses NO reduction to N_2O (Alegria et al., 2004; Igamberdiev and Hill, 2009; Sanchez-Cruz and Alegría, 2009). Therefore, mitochondria can be a potent site of N_2O formation under oxygen-limited conditions, and it should be a focus of future research.

Similar to the observations in plants, macrofauna and earthworms are also found to emit N_2O when supplied with NO_3 and under O_2-limited conditions (Horn et al., 2003; Stief et al., 2009). Earthworms do not produce N_2O when supplied NH_4 (Horn et al., 2003). Moreover, in other studies, listed in **Table 1**, when ^{15}N-labeled NH_4 was used as a substrate, there was no N_2O emission. This shows that NO_3 metabolism at the cellular level produces N_2O in both plants and animals. As described above, the ETC in (**Figure 1**) mitochondria can reduce NO to N_2O under less oxic conditions, which suggests

that N_2O emitted by earthworms and macrofauna (Horn et al., 2003; Stief et al., 2009) might also be produced from hypoxic mitochondria. The gut of insects has a hypoxic environment (Johnson and Barbehenn, 2000), which may explain the higher level of N_2O production in the gut (Stief et al., 2009). Moreover, axenic algae supplied with NO_2 produced significantly higher levels of N_2O under dark conditions than under light conditions (Guieysse et al., 2013; Plouviez et al., 2017). The low emission of N_2O under light conditions may be due to the supply of photosynthetic O_2 to the cells.

Based on experimental evidence gathered from various studies, we propose that the reductive pathway of NO formation in the mitochondria and further reduction of NO by the mitochondrial ETCs contributes to the formation of N_2O (in eukaryotic cells, as presented in **Figure 1**). The process is catalysed by various enzymes, and it might be pronounced under hypoxic and anoxic conditions but not under normoxic conditions. The proposed pathway is further supported by the existence of a denitrifying pathway, and the associated enzymes and genes in *Globobulimina* species and the localisation of enzymes in the mitochondria (Woehle et al., 2018). As higher animals possess well developed respiratory and circulatory systems that transport O_2, they may not experience hypoxia. However, plants lack such sophisticated systems to transport O_2 (Voesenek et al., 2016), and therefore, may experience hypoxia and anoxia that favour N_2O formation. Field studies have reported high N_2O emission from plants under flooded conditions (Rusch and Rennenberg, 1998; Machacova et al., 2013), suggesting the role of hypoxia and anoxia in N_2O formation in plants.

SIGNIFICANCE OF N_2O FORMATION *VIA* THE NO_3-NO_2-NO PATHWAY IN PLANTS

NO_3 is not only a major nutrient in plant cells but also a signaling molecule (Zhao et al., 2018). Several studies have reported that NO_3 plays a role in hypoxia tolerance. For example, NO_3 maintains the growth of plants under oxygen-limited conditions, and its absence disturbs plant growth (Horchani et al., 2010). Anoxia tolerance of tomato plant is enhanced by nitrate reduction (Allègre et al., 2004). Moreover, anoxia strongly induces NR activity and the induced NR

TABLE 1 | Compilation of the substrates, mediums and products that used labeled N sources and their subsequent measurements of N_2O emissions.

	Substrate	Medium	Product	Reference
1.	^{15}N labeled NO_3 ^{15}N labeled NH_4	Aseptically grown tobacco plants	^{15}N labeled N_2O No N_2O emission	Goshima et al. (1999)
2.	^{15}N labeled NO_3 ^{15}N labeled NH_4	Lichen	^{15}N labeled N_2O No N_2O emission	Lenhart et al. (2015)
3.	^{15}N labeled NO_3 ^{15}N labeled NH_4	Wheat plant	^{15}N labeled N_2O No N_2O emission	Smart and Bloom (2001)
4.	^{15}N labeled NO_3	Soybean plant	^{15}N labeled NO and N_2O	Dean and Harper (1986)
5.	^{15}N labeled NO_3	Macro fauna	^{15}N labeled N_2O	Stief et al. (2009)
6.	^{15}N labeled NO_2	Tobacco plant	^{15}N labeled N_2O	Goshima et al. (1999)
7.	^{15}N labeled NO_2	Aseptically grown tobacco plant	^{15}N labeled N_2O	Hakata et al. (2003)
8.	^{15}N labeled NO_2	Axenic algae	^{15}N labeled N_2O	Weathers (1984)
9.	^{15}N labeled NO ^{14}N labeled NO	Reduced form of beef heart cytochrome c oxidase (CcO)	^{15}N labeled N_2O ^{14}N labeled N_2O	Brudvig et al. (1980)

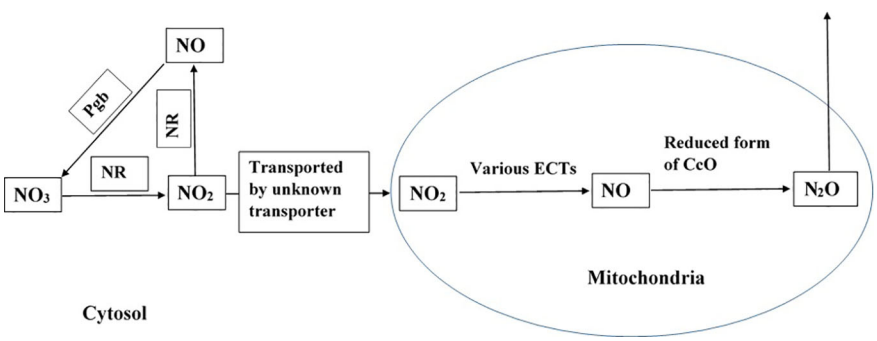

FIGURE 1 | Potential pathway of N_2O formation in plant cells. NR represents nitrate reductase and Pgb represents phytoglobin (Brudvig et al., 1980; Zhao et al., 1995; Goshima et al., 1999; Gupta and Kaiser, 2010; Guieysse et al., 2013; Plouviez et al., 2017). The pathway is active in presence of NO_3 and NO_2, and when plants experience hypoxia and anoxia.

activity prevents pH from dropping to life-threatening levels (Allègre et al., 2004). NO_3 nutrition in plants decreases the total respiration rate and reactive oxygen species levels (Wany et al., 2019), but increases ATP production under hypoxic conditions (Stoimenova et al., 2007; Wany et al., 2019). Under oxygen-limited conditions, NO_3 protects the ultrastructure of mitochondria (Vartapetian et al., 2003). The addition of NO_3 to the root zone of plants released significantly less amount of ethanol compared with roots supplied with NH_4 under hypoxic conditions (Oliveira et al., 2013). This suggests NO_3 plays an important role to decrease alcoholic fermentative metabolism in plants during hypoxia (Oliveira et al., 2013). Overall, these findings suggest that NO_3 and NR play an important role to maintain the integrity of plant cells under oxygen-limited conditions.

NO_2 is also reported to play important roles under oxygen-limited conditions in plants. Benamar et al. (2008) found that NO_2-dependent NO production in the mitochondria can regulate surrounding O_2 level. Moreover, plant mitochondria can synthesise ATP under anaerobic conditions when supplied with NO_2 (Stoimenova et al., 2007; Gupta et al., 2016). The supply of NO_2 decreased lipid peroxidation and reactive oxygen species formation (Gupta et al., 2016). The absence of NO_2 as a terminal acceptor for ETC during hypoxia leads to mitochondrial depolarisation (Gupta et al., 2016). NO_2 supplemented roots released significantly less amount of fermentative ethanol during hypoxia than NH_4-supplemented roots (Oliveira et al., 2012; Oliveira et al., 2013), suggesting the vital role of NO_2 in plants to survive under oxygen-limited conditions.

NO helps plants to cope under several environmental stresses. For example, NO is essential for the homeostasis of O_2 level in plants under oxygen-limited conditions (Gupta and Igamberdiev, 2011). NO production in the mitochondria has several implications in plants as illustrated in **Figure 2**. For example, NO can break seed dormancy and stimulate seed germination in plants (Beligni and Lamattina, 2000; Bethke et al., 2004). Similarly, under hypoxic stress, NO is vital for the formation of aerenchyma in the roots (Wany et al., 2017). NO production in the mitochondria under low-oxygen conditions can help in ATP synthesis, preventing excessive depletion of energy (Stoimenova et al., 2007). NO_3-NO_2-dependent NO production in plant roots decreases fermentative ethanol production during hypoxia (Oliveira et al., 2012; Oliveira et al., 2013).

Although NO has been well established as a signaling molecule, its high concentration in cells leads to cell death (Boscá and Hortelano, 1999; Brown and Borutaite, 2002). Therefore, it is critical to regulate its concentration in cells, as a higher amount of NO is formed under hypoxic and anoxic conditions. Two mechanisms are reported to occur in the

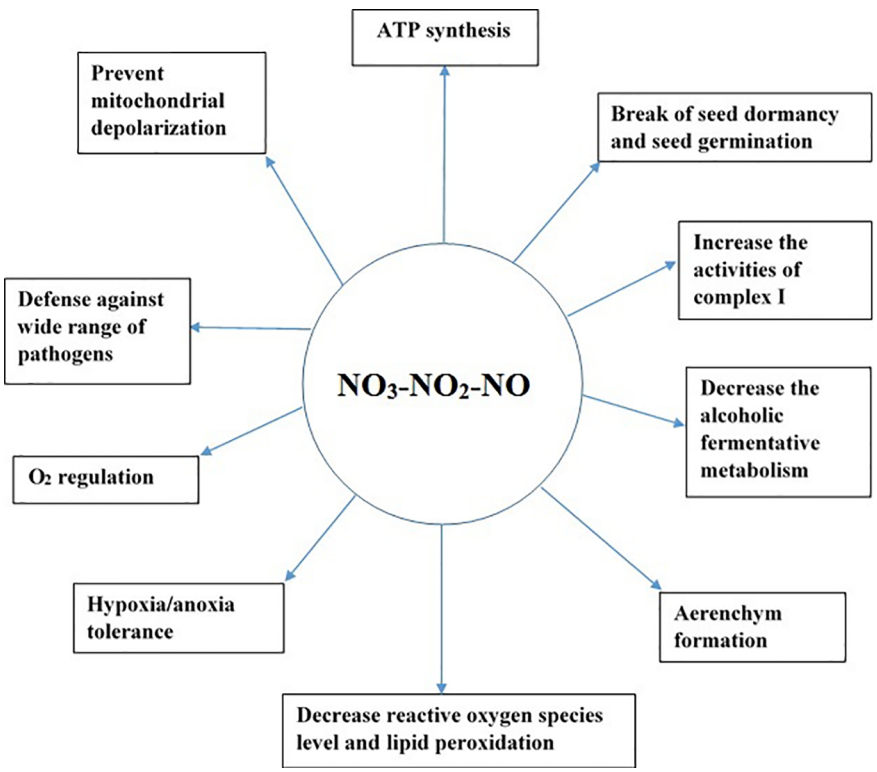

FIGURE 2 | Significance of the NO_3-NO_2-NO pathway in plants under hypoxic and anoxic conditions.

mitochondria to detoxify the high amount of NO formed, namely, oxidation of NO to NO_2 during normoxia (Cooper, 2002; Taylor and Moncada, 2010) and reduction of NO to N_2O during hypoxia (Cooper, 2002). As both products of NO metabolism in the mitochondria, that is, NO_2 and N_2O, are non-toxic, their formation might play a protective role in the mitochondria. If hypoxia-induced NO production in cells is high, it can cause DNA fragmentation, leading to cell death; however, if NO is scavenged, it can reduce DNA fragmentation (Wany et al., 2017). Therefore, scavenging of NO is essential to protect cells from high NO toxicity. Phytoglobins are reported to scavenge NO in the cytosol (Igamberdiev et al., 2010). Additionally, purified mitochondria have been reported to scavenge exogenous NO (Gupta et al., 2005; de Oliveira et al., 2008; Wulff et al., 2009; Gupta and Kaiser, 2010). Furthermore, the addition of NADH as an electron donor increased NO scavenging by the mitochondria (Gupta et al., 2005; de Oliveira et al., 2008; Wulff et al., 2009; Gupta et al., 2016), indicating that the mitochondria have a protective mechanism to detoxify the excess NO formed. NADH might act as an electron donor to reduce cytochrome c oxidase, leading to an increase in NO scavenging in purified mitochondria. As discussed in our proposed pathway of N_2O formation in the mitochondria, the conversion of NO to N_2O by the reduced form of CcO might be the potential pathway regulating excessive NO formed under oxygen-limited conditions in the mitochondria. Mitochondria are not only a source of NO, but also an important sink and target of NO (Igamberdiev et al., 2014), and long-term exposure of mitochondria to NO can lead to the dysfunction of mitochondria (Brown and Borutaite, 2002). Although the NO_3-NO_2-NO pathway has several roles (**Figure 2**) in plants during hypoxia and anoxia, NO accumulation at higher level is toxic to cells (Brown and Borutaite, 2002). Therefore, N_2O formation in the mitochondria *via* the NO_3-NO_2-NO pathway might be a strategy to protect cells and mitochondrial components from excessive NO formed under oxygen-limited conditions. Therefore, at molecular level, further research should focus on measuring NO and N_2O from isolated mitochondria to obtain more insights on mitochondria's role in scavenging excess NO during hypoxia and anoxia.

DO PLANT CELLS REDUCE N_2O TO N_2?

The last two enzymes of the denitrification process, namely, nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR), merged to form CcO (Saraste and Castresana, 1994; Stanton et al., 2018). Moreover, the copper site in bacterial N_2OR is similar to the CuA site in CcO (Kroneck, 2018). Many catalytic properties of CcO from denitrifying bacteria (*Paracoccus denitrificans*) and eukaryotic organisms are similar (Ludwig, 1987; Kadenbach et al., 1991). As eukaryotic mitochondrion is considered to be evolved from *P. denitrificans*, a denitrifying bacterium (John and Whatley, 1975), it may still possess rudimentary denitrification properties. Although during the evolution most of genes of the bacterium transferred to the nucleus, few remained in the mitochondrial DNA including genes of CcO (Kadenbach et al., 1991). Therefore, it may be possible that CcO of higher organisms might also possess similar properties like that of its ancestor, *P. denitrificans*. The significant negative relationship between N_2O consumption and CO_2 respiration rates in plants and lichens (Machacova et al., 2017) suggests that mitochondria are the possible site of N_2O consumption. This N_2O consumption observed in these eukaryotes might be at the site of CcO, as this enzyme is formed from the last two enzymes of denitrification. There are also reports of emission of ^{15}N-labeled N_2 from wheat crops supplied with ^{15}N-labeled NO_2 (Vanecko and Varner, 1955), suggesting that under certain cell conditions, mitochondria may also metabolise N_2O to N_2. However, to date, N_2 emission from plants is less reported. It may be due to the advanced systems of O_2 regulation in plants, and this might inhibit the complete process of denitrification. A recent study, which measured N_2O and N_2 emission from soil–plant systems, showed that N_2O and N_2 emitted by NO_3-rich soil–plant systems was three times higher than that by NH_4-supplemented soil–plant systems and bare soil (Senbayram et al., 2020), and this suggests that the possible role of N_2O and N_2 production in plants. Further experiments at the molecular level (mitochondria) are needed to explore the reason for the significant negative relation between N_2O emission and respiration rate in plants and lichen, as reported by Machacova et al. (2017).

CONCLUSIONS

To cope with the problems of global warming and ozone layer depletion, a good understanding of N_2O formation processes in various source is critical. Therefore, the N_2O formation process in plants is a matter of concern. The reductive pathway of NO formation in the mitochondria along with further reduction of NO by ETC is a possible pathway of N_2O formation in plants. Considering available evidence, we conclude that there is strong possibility that plant cells produce N_2O in the mitochondria under hypoxic and anoxic conditions. The theory that plants are only a conduit for N_2O produced by soil-inhabiting microorganisms might be an ambiguous explanation. The root zone may sense hypoxia and anoxia due to the soil environmental conditions, which may favour N_2O formation in the root mitochondria. As some studies have shown that N_2O emission from tree stems is higher than that from soils in natural habitats (Welch et al., 2019), the proposed pathway of N_2O formation in plants might play a significant role in understanding N cycling in eukaryotic organisms and the global N_2O budget. Furthermore, we have highlighted the reduction of NO to N_2O in the mitochondria, and therefore, it would be valuable to reassess the role of mitochondrial ETC under both hypoxic and anoxic conditions. Although, N_2O is a potent greenhouse gas ,its formation in the mitochondria might help to protect the integrity of the mitochondria and protect cells from the toxicity of NO accumulation during hypoxia.

AUTHOR CONTRIBUTIONS

AT wrote the manuscript. CH supervised the whole work. CZ, BP, FB, and WD commented on the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Natural Science Foundation of China (no. 41530859) and the National Key Research and Development Program of China (2016YFD0800102-4 and 2018YFC0213300-01).

ACKNOWLEDGMENTS

AT is grateful to CAS-TWAS President's Fellowship Program for granting the Ph.D. fellowship. We are thankful to Prof. Chengcai Chu from the Institute of Genetics and Developmental Biology, Beijing, China, for thoughtful comments. Moreover, we are grateful to Prof. Oene Oenema and Prof. Jiafa Luo for their valuable comments.

REFERENCES

Alber, N. A., Sivanesan, H., and Vanlerberghe, G. C. (2017). The occurrence and control of nitric oxide generation by the plant mitochondrial electron transport chain. *Plant Cell Environ.* 40, 1074–1085. doi: 10.1111/pce.12884

Alegria, A. E., Sanchez, S., and Quintana, I. (2004). Quinone-enhanced ascorbate reduction of nitric oxide: role of quinone redox potential. *Free Radical Res.* 38, 1107–1112. doi: 10.1080/10715760400009852

Allègre, A., Silvestre, J., Morard, P., Kallerhoff, J., and Pinelli, E. (2004). Nitrate reductase regulation in tomato roots by exogenous nitrate: a possible role in tolerance to long-term root anoxia. *J. Exp. Bot.* 55, 2625–2634. doi: 10.1093/jxb/erh258

Ascenzi, P., Marino, M., Polticelli, F., Santucci, R., and Coletta, M. (2014). Cardiolipin modulates allosterically the nitrite reductase activity of horse heart cytochrome c. *J. Biol. Inorg. Chem.* 19, 1195–1201. doi: 10.1007/s00775-014-1175-9

Astier, J., Gross, I., and Durner, J. (2018). Nitric oxide production in plants: an update. *J. Exp. Bot.* 69, 3401–3411. doi: 10.1093/jxb/erx420

Baggs, E. M. (2011). Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. *Curr. Opin. Env. Sust.* 3, 321–327. doi: 10.1016/j.cosust.2011.08.011

Basu, S., Azarova, N. A., Font, M. D., King, S. B., Hogg, N., Gladwin, M. T., et al. (2008). Nitrite reductase activity of cytochrome c. *J. Biol. Inorg. Chem.* 283, 32590–32597. doi: 10.1074/jbc.M806934200

Beligni, M. V., and Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. *Planta* 210 (2), 215–221. doi: 10.1007/PL00008128

Benamar, A., Rolletschek, H., Borisjuk, L., Avelange-Macherel, M. H., Curien, G., Mostefai, H. A., et al. (2008). Nitrite–nitric oxide control of mitochondrial respiration at the frontier of anoxia. *BBA-Bioenergetics* 1777, 1268–1275. doi: 10.1016/j.bbabio.2008.06.002

Bender, D., and Schwarz, G. (2018). Nitrite-dependent nitric oxide synthesis by molybdenum enzymes. *FEBS Lett.* 592, 2126–2139. doi: 10.1002/1873-3468.13089

Bethke, P. C., Gubler, F., Jacobsen, J. V., and Jones, R. L. (2004). Dormancy of Arabidopsis seeds and barley grains can be broken by nitric oxide. *Planta* 219, 847–855. doi: 10.1007/s00425-004-1282-x

Blomberg, M. R., and Ädelroth, P. (2018). Mechanisms for enzymatic reduction of nitric oxide to nitrous oxide-A comparison between nitric oxide reductase and cytochrome c oxidase. *BBA-Bioenergetics* 1859, 1223–1234. doi: 10.1016/j.bbabio.2018.09.368

Boscá, L., and Hortelano, S. (1999). Mechanisms of nitric oxide-dependent apoptosis: involvement of mitochondrial mediators. *Cell Signal.* 11, 239–244. doi: 10.1016/S0898-6568(98)00064-3

Botrel, A., and Kaiser, W. M. (1997). Nitrate reductase activation state in barley roots in relation to the energy and carbohydrate status. *Planta* 201, 496–501. doi: 10.1007/s004250050094

Bowatte, S., Newton, P. C., Theobald, P., Brock, S., Hunt, C., Lieffering, M., et al. (2014). Emissions of nitrous oxide from the leaves of grasses. *Plant Soil* 374, 275–283. doi: 10.1007/s11104-013-1879-6

Brown, G. C., and Borutaite, V. (2002). Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radical Bio. Med.* 33, 1440–1450. doi: 10.1016/S0891-5849(02)01112-7

Brudvig, G. W., Stevens, T. H., and Chan, S. II. (1980). Reactions of nitric oxide with cytochrome c oxidase. *Biochemistry* 19, 5275–5285. doi: 10.1021/bi00564a020

Calatrava, V., Chamizo-Ampudia, A., Sanz-Luque, E., Ocaña-Calahorro, F., Llamas, A., Fernandez, E., et al. (2017). How Chlamydomonas handles nitrate and the nitric oxide cycle. *J. Exp. Bot.* 68, 2593–2602. doi: 10.1093/jxb/erw507

Castello, P. R., David, P. S., McClure, T., Crook, Z., and Poyton, R. O. (2006). Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab.* 3, 277–287. doi: 10.1016/j.cmet.2006.02.011

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Chang, C., Janzen, H. H., Nakonechny, E. M., and Cho, C. M. (1998). Nitrous oxide emission through plants. *Soil Sci. Soc Am. J.* 62, 35–38. doi: 10.2136/sssaj1998.03615995006200010005x

Collman, J. P., Devaraj, N. K., Decréau, R. A., Yang, Y., Yan, Y. L., Ebina, W., et al. (2007). A cytochrome c oxidase model catalyzes oxygen to water reduction under rate-limiting electron flux. *Science* 315, 1565–1568. doi: 10.1126/science.1135844

Cooper, C. E. (2002). Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? *Trends Biochem. Sci.* 27, 33–39. doi: 10.1016/S0968-0004(01)02035-7

Davidson, E. A., and Kanter, D. (2014). Inventories and scenarios of nitrous oxide emissions. *Environ. Res. Lett.* 9:105012. doi: 10.1088/1748-9326/9/10/105012

de Oliveira, H. C., Wulff, A., Saviani, E. E., and Salgado, I. (2008). Nitric oxide degradation by potato tuber mitochondria: evidence for the involvement of external NAD(P)H dehydrogenases. *BBA-Bioenergetics* 1777, 470–476. doi: 10.1016/j.bbabio.2008.02.006

Dean, J. V., and Harper, J. E. (1986). Nitric oxide and nitrous oxide production by soybean and winged bean during the in vivo nitrate reductase assay. *Plant Physiol.* 82, 718–723. doi: 10.1104/pp.82.3.718

Fan, X., Jia, L., Li, Y., Smith, S. J., Miller, A. J., and Shen, Q. (2007). Comparing nitrate storage and remobilization in two rice cultivars that differ in their nitrogen use efficiency. *J. Exp. Bot.* 58 (7), 1729–1740. doi: 10.1093/jxb/erm033

Gao, G. F., Li, P. F., Zhong, J. X., Shen, Z. J., Chen, J., Li, Y. T., et al. (2019). *Spartina alterniflora* invasion alters soil bacterial communities and enhances soil N_2O emissions by stimulating soil denitrification in mangrove wetland. *Sci. Total Environ.* 653, 231–240. doi: 10.1016/j.scitotenv.2018.10.277

Ghafourifar, P., and Cadenas, E. (2005). Mitochondrial nitric oxide synthase. *Trends Pharmacol. Sci.* 26, 190–195. doi: 10.1016/j.tips.2005.02.005

Ghafourifar, P., and Richter, C. (1997). Nitric oxide synthase activity in mitochondria. *FEBS Lett.* 418, 291–296. doi: 10.1016/S0014-5793(97)01397-5

Giulivi, C., Poderoso, J. J., and Boveris, A. (1998). Production of nitric oxide by mitochondria. *J. Biol. Chem.* 273, 11038–11043. doi: 10.1074/jbc.273.18.11038

Goshima, N., Mukai, T., Suemori, M., Takahashi, M., Caboche, M., and Morikawa, H. (1999). Emission of nitrous oxide (N_2O) from transgenic tobacco expressing antisense NiR mRNA. *Plant J.* 19, 75–80. doi: 10.1046/j.1365-313X.1999.00494.x

Guieysse, B., Plouviez, M., Coilhac, M., and Cazali, L. (2013). Nitrous Oxide (N_2O) production in axenic Chlorella vulgaris microalgae cultures: evidence, putative

pathways, and potential environmental impacts. *Biogeosciences* 10, 6737–6746. doi: 10.5194/bg-10-6737-2013

Guo, F. Q., and Crawford, N. M. (2005). Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *Plant Cell* 17, 3436–3450. doi: 10.1105/tpc.105.037770

Gupta, K. J., and Igamberdiev, A. U. (2011). The anoxic plant mitochondrion as a nitrite: NO reductase. *Mitochondrion* 11, 537–543. doi: 10.1016/j.mito.2011.03.005

Gupta, K. J., and Kaiser, W. M. (2010). Production and scavenging of nitric oxide by barley root mitochondria. *Plant Cell Physiol.* 51, 576–584. doi: 10.1093/pcp/pcq022

Gupta, K. J., Stoimenova, M., and Kaiser, W. M. (2005). In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. *J. Exp. Bot.* 56, 2601–2609. doi: 10.1093/jxb/eri252

Gupta, K. J., Fernie, A. R., Kaiser, W. M., and van Dongen, J. T. (2011). On the origins of nitric oxide. *Trends Plant Sci.* 16, 160–168. doi: 10.1016/j.tplants.2010.11.007

Gupta, A. K., Kumari, A., Mishra, S., Wany, A., and Gupta, K. J. (2016). "The functional role of nitric oxide in plant mitochondrial metabolism," in *Advances in Botanical Research*, vol. 77. (Cambridge, MA: Academic Press), 145–163. doi: 10.1016/bs.abr.2015.10.007

Hachiya, T., and Sakakibara, H. (2017). Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. *J. Exp. Bot.* 68, 2501–2512. doi: 10.1093/jxb/erw449

Hakata, M., Takahashi, M., Zumft, W., Sakamoto, A., and Morikawa, H. (2003). Conversion of the nitrate nitrogen and nitrogen dioxide to nitrous oxides in plants. *Acta Biotechnol.* 23, 249–257. doi: 10.1002/abio.200390032

Horchani, F., Aschi-Smiti, S., and Brouquisse, R. (2010). Involvement of nitrate reduction in the tolerance of tomato (*Solanum lycopersicum* L.) plants to prolonged root hypoxia. *Acta Physiol. Plant* 32, 1113–1123. doi: 10.1007/s11738-010-0503-0

Horchani, F., Prévot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2011). Both plant and bacterial nitrate reductases contribute to nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules. *Plant Physiol.* 155, 1023–1036. doi: 10.1104/pp.110.166140

Horn, M. A., Schramm, A., and Drake, H. L. (2003). The earthworm gut: an ideal habitat for ingested N_2O-producing microorganisms. *Appl. Environ. Microb.* 69, 1662–1669. doi: 10.1128/AEM.69.3.1662-1669.2003

Hu, H. W., Chen, D., and He, J. Z. (2015). Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiol. Rev.* 39, 729–749. doi: 10.1093/femsre/fuv021

Igamberdiev, A. U., and Hill, R. D. (2009). Plant mitochondrial function during anaerobiosis. *Ann. Bot.* 103, 259–268. doi: 10.1093/aob/mcn100

Igamberdiev, A. U., Bykova, N. V., Shah, J. K., and Hill, R. D. (2010). Anoxic nitric oxide cycling in plants: participating reactions and possible mechanisms. *Physiol. Plantarum* 138, 393–404. doi: 10.1111/j.1399-3054.2009.01314.x

Igamberdiev, A. U., Ratcliffe, R. G., and Gupta, K. J. (2014). Plant mitochondria: source and target for nitric oxide. *Mitochondrion* 19, 329–333. doi: 10.1016/j.mito.2014.02.003

Jakobs, H. H., Froriep, D., Havemeyer, A., Mendel, R. R., Bittner, F., and Clement, B. (2014). The mitochondrial amidoxime reducing component (mARC): involvement in metabolic reduction of N-oxides, oximes and N-hydroxyamidinohydrazones. *ChemMedChem* 9, 2381–2387. doi: 10.1002/cmdc.201402127

Jansson, E.Å., Huang, L., Malkey, R., Govoni, M., Nihlén, C., Olsson, A., et al (2008). A mammalian functional nitrate reductase that regulates nitrite and nitric oxide homeostasis. *Nat. Chem. Biol.* 4, 411. doi: 10.1038/nchembio.92

John, P., and Whatley, F. R. (1975). *Paracoccus denitrificans* and the evolutionary origin of the mitochondrion. *Nature* 254, 495. doi: 10.1038/254495a0

Johnson, K. S., and Barbehenn, R. V. (2000). Oxygen levels in the gut lumens of herbivorous insects. *J. Insect Physiol.* 46, 897–903. doi: 10.1016/S0022-1910(99)00196-1

Kadenbach, B., Stroh, A., Hüther, F. J., Reimann, A., and Steverding, D. (1991). Evolutionary aspects of cytochromec oxidase. *J. Bioenerg. Biomembr.* 23 (2), 321–334. doi: 10.1007/BF00762225

Klepper, L. A. (1987). Nitric oxide emissions from soybean leaves during in vivo nitrate reductase assays. *Plant Physiol.* 85, 96–99. doi: 10.1104/pp.85.1.96

Kobayashi, M., Matsuo, Y., Takimoto, A., Suzuki, S., Maruo, F., and Shoun, H. (1996). Denitrification, a novel type of respiratory metabolism in fungal mitochondrion. *J. Biol. Chem.* 271, 16263–16267. doi: 10.1074/jbc.271.27.1626

Koivisto, A., Matthias, A., Bronnikov, G., and Nedergaard, J. (1997). Kinetics of the inhibition of mitochondrial respiration by NO. *FEBS Lett.* 417, 75–80. doi: 10.1016/S0014-5793(97)01258-1

Kozlov, A. V., Staniek, K., and Nohl, H. (1999). Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett.* 454, 127–130. doi: 10.1016/S0014-5793(99)00788-7

Kroneck, P. M. (2018). Walking the seven lines: binuclear copper A in cytochrome c oxidase and nitrous oxide reductase. *J. Biol. Inorg. Chem.* 23, 27–39. doi: 10.1007/s00775-017-1510-z

Lenhart, K., Weber, B., Elbert, W., Steinkamp, J., Clough, T., Crutzen, P., et al. (2015). Nitrous oxide and methane emissions from cryptogamic covers. *Global Change Biol.* 21, 3889–3900. doi: 10.1111/gcb.12995

Lenhart, K., Behrendt, T., Greiner, S., Steinkamp, J., Well, R., Giesemann, A., et al. (2019). Nitrous oxide effluxes from plants as a potentially important source to the atmosphere. *New Phytol.* 221, 1398–1408. doi: 10.1111/nph.15455

Ludwig, B. (1987). Cytochrome c oxidase in prokaryotes. *FEMS Microbiol. Rev.* 3 (1), 41–56. doi: 10.1111/j.1574-6968.1987.tb02451.x

Lundberg, J. O., Weitzberg, E., and Gladwin, M. T. (2008). The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discovery* 7, 156. doi: 10.1038/nrd2466

Maathuis, F. J. (2009). Physiological functions of mineral macronutrients. *Curr. Opin. Plant Biol.* 12, 250–258. doi: 10.1016/j.pbi.2009.04.003

Machacova, K., Papen, H., Kreuzwieser, J., and Rennenberg, H. (2013). Inundation strongly stimulates nitrous oxide emissions from stems of the upland tree Fagus sylvatica and the riparian tree *Alnus glutinosa*. *Plant Soil* 364, 287–301. doi: 10.1007/s11104-012-1359-4

Machacova, K., Maier, M., Svobodova, K., Lang, F., and Urban, O. (2017). Cryptogamic stem covers may contribute to nitrous oxide consumption by mature beech trees. *Sci. Rep.* 7, 13243. doi: 10.1038/s41598-017-13781-7

Maia, L. B., and Moura, J. J. (2015). Nitrite reduction by molybdoenzymes: a new class of nitric oxide-forming nitrite reductases. *J. Biol. Inorg. Chem.* 20, 403–433. doi: 10.1007/s00775-014-1234-2

Miller, A. J., and Cramer, M. D. (2005). "Root nitrogen acquisition and assimilation," in *Root Physiology: from Gene to Function.* (Dordrecht: Springer), 1–36.

Morard, P., Silvestre, J., Lacoste, L., Caumes, E., and Lamaze, T. (2004). Nitrate uptake and nitrite release by tomato roots in response to anoxia. *J. Plant Physiol.* 161, 855–865. doi: 10.1016/j.jplph.2003.11.003

Moreau, M., Lee, G.II, Wang, Y., Crane, B. R., and Klessig, D. F. (2008). AtNOS/AtNOA1 is a functional Arabidopsis thaliana cGTPase and not a nitric-oxide synthase. *J. Biol. Chem.* 283, 32957–32967. doi: 10.1074/jbc.M804838200

Oliveira, H. C., and Sodek, L. (2013). Effect of oxygen deficiency on nitrogen assimilation and amino acid metabolism of soybean root segments. *Amino Acids* 44, 743–755. doi: 10.1007/s00726-012-1399-3

Oliveira, H. C., Salgado, I., and Sodek, L. (2012). Involvement of nitrite in the nitrate-mediated modulation of fermentative metabolism and nitric oxide production of soybean roots during hypoxia. *Planta* 237, 255–264. doi: 10.1007/s00425-012-1773-0

Oliveira, H. C., Salgado, I., and Sodek, L. (2013). Nitrite decreases ethanol production by intact soybean roots submitted to oxygen deficiency: a role for mitochondrial nitric oxide synthesis? *Plant Signal. Behav.* 8, e23578. doi: 10.4161/psb.23578

O'Brien, J. A., Vega, A., Bouguyon, E., Krouk, G., Gojon, A., Coruzzi, G., et al. (2016). Nitrate transport, sensing, and responses in plants. *Mol. Plant* 9, 837–856. doi: 10.1016/j.molp.2016.05.004

Pearce, L. L., Kanai, A. J., Birder, L. A., Pitt, B. R., and Peterson, J. (2002). The catabolic fate of nitric oxide the nitric oxide oxidase and peroxynitrite reductase activities of cytochrome oxidase. *J. Biol. Chem.* 277, 13556–13562. doi: 10.1074/jbc.M109838200

Planchet, E., Gupta, K. J., Sonoda, M., and Kaiser, W. M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. *Plant J.* 41, 732–743. doi: 10.1111/j.1365-313X.2005.02335.x

Plouviez, M., Wheeler, D., Shilton, A., Packer, M. A., McLenachan, P. A., Sanz-Luque, E., et al. (2017). The biosynthesis of nitrous oxide in the green alga *Chlamydomonas reinhardtii*. *Plant J.* 91, 45–56. doi: 10.1111/tpj.13544

Plouviez, M., Shilton, A., Packer, M. A., and Guieysse, B. (2019). Nitrous oxide emissions from microalgae: potential pathways and significance. *J. Appl. Phycol.* 31, 1–8. doi: 10.1007/s10811-018-1531-1

Poderoso, J. J., Helfenberger, K., and Poderoso, C. (2019). The effect of nitric oxide on mitochondrial respiration. *Nitric. Oxide* 88, 61–72. doi: 10.1016/j.niox.2019.04.005

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W. M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J. Exp. Bot.* 53, 103–110. doi: 10.1093/jexbot/53.366.103

Rőszer, T. (2012). *The biology of subcellular nitric oxide*. Dordrecht: Springer. doi: 10.1007/978-94-007-2819-6

Rusch, H., and Rennenberg, H. (1998). Black alder (*Alnus glutinosa* (L.) Gaertn.) trees mediate methane and nitrous oxide emission from the soil to the atmosphere. *Plant Soil* 201, 1–7. doi: 10.1023/A:1004331521059

Sanchez-Cruz, P., and Alegría, A. E. (2009). Quinone-enhanced reduction of nitric oxide by xanthine/xanthine oxidase. *Chem. Res. Toxicol.* 22, 818–823. doi: 10.1021/tx800392j

Saraste, M., and Castresana, J. (1994). Cytochrome oxidase evolved by tinkering with denitrification enzymes. *FEBS Lett.* 341, 1–4. doi: 10.1016/0014-5793(94)80228-9

Saraste, M. (1994). Structure and evolution of cytochrome oxidase. *Anton. Leeuw. Int. J.* 65, 285–287. doi: 10.1007/BF00872214

Senbayram, M., Well, R., Shan, J., Bol, R., Burkart, S., Jones, D. L., et al. (2020). Rhizosphere processes in nitrate-rich barley soil tripled both N_2O and N_2 losses due to enhanced bacterial and fungal denitrification. *Plant Soil* 448, 509–522. doi: 10.1007/s11104-020-04457-9

Smart, D. R., and Bloom, A. J. (2001). Wheat leaves emit nitrous oxide during nitrate assimilation. *P. Natl. Acad. Sci.* 98, 7875–7878. doi: 10.1073/pnas.131572798

Sparacino-Watkins, C. E., Tejero, J., Sun, B., Gauthier, M. C., Thomas, J., Ragireddy, V., et al. (2014). Nitrite reductase and nitric-oxide synthase activity of the mitochondrial molybdopterin enzymes mARC1 and mARC2. *J. Biol. Chem.* 289, 10345–10358. doi: 10.1074/jbc.M114.555177

Stanton, C. L., Reinhard, C. T., Kasting, J. F., Ostrom, N. E., Haslun, J. A., Lyons, T. W., et al. (2018). Nitrous oxide from chemodenitrification: A possible missing link in the Proterozoic greenhouse and the evolution of aerobic respiration. *Geobiology* 16, 597–609. doi: 10.1111/gbi.12311

Stief, P., Poulsen, M., Nielsen, L. P., Brix, H., and Schramm, A. (2009). Nitrous oxide emission by aquatic macrofauna. *P. Natl. Acad. Sci.* 106, 4296–4300. doi: 10.1073/pnas.0808228106

Stoimenova, M., Igamberdiev, A. U., Gupta, K. J., and Hill, R. D. (2007). Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. *Planta* 226, 465–474. doi: 10.1007/s00425-007-0496-0

Sugiura, M., Georgescu, M. N., and Takahashi, M. (2007). A nitrite transporter associated with nitrite uptake by higher plant chloroplasts. *Plant Cell Physiol.* 48, 1022–1035. doi: 10.1093/pcp/pcm073

Syakila, A., and Kroeze, C. (2011). The global nitrous oxide budget revisited. *GGMM* 1, 17–26. doi: 10.3763/ghgmm.2010.0007

Taylor, C. T., and Moncada, S. (2010). Nitric oxide, cytochrome C oxidase, and the cellular response to hypoxia. *Arterioscl. Throm. Vas.* 30, 643–647. doi: 10.1161/ATVBAHA.108.181628

Thomson, A. J., Giannopoulos, G., Pretty, J., Baggs, E. M., and Richardson, D. J. (2012). Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philos. T. R. Soc B.* 367, 1157–1168. doi: 10.1098/rstb.2011.0415

Tian, H., Yang, J., Lu, C., Xu, R., Canadell, J. G., Jackson, R. B., et al. (2018). The global N_2O model intercomparison project. *B. Am. Meteorol. Soc.* 99, 1231–1251. doi: 10.1175/BAMS-D-17-0212.1

Tischner, R., Planchet, E., and Kaiser, W. M. (2004). Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga *Chlorella sorokiniana*. *FEBS Lett.* 576, 151–155. doi: 10.1016/j.febslet.2004.09.004

van der Leij, M., Smith, S., and Miller, A. (1998). Remobilisation of vacuolar stored nitrate in barley root cells. *Planta* 205, 64–72. doi: 10.1007/s004250050297

Vanecko, S., and Varner, J. E. (1955). Studies on nitrite metabolism in higher plants. *Plant Physiol.* 30:388. doi: 10.1104/pp.30.4.388

Vartapetian, B. B., Andreeva, I. N., Generozova, I. P., Polyakova, L.II, Maslova, I. P., Dolgikh, Y. II, et al. (2003). Functional electron microscopy in studies of plant response and adaptation to anaerobic stress. *Ann. Bot.* 91, 155–172. doi: 10.1093/aob/mcf244

Voesenek, L. A., Sasidharan, R., Visser, E. J., and Bailey-Serres, J. (2016). Flooding stress signaling through perturbations in oxygen, ethylene, nitric oxide and light. *New Phytol.* 209, 39–43. doi: 10.1111/nph.13775

von Wirén, N., Gazzarrini, S., Gojon, A., and Frommer, W. B. (2000). The molecular physiology of ammonium uptake and retrieval. *Curr. Opin. Plant Biol.* 3, 254–261. doi: 10.1016/S1369-5266(00)80074-6

Wany, A., Kumari, A., and Gupta, K. J. (2017). Nitric oxide is essential for the development of aerenchyma in wheat roots under hypoxic stress. *Plant Cell Environ.* 40, 3002–3017. doi: 10.1111/pce.13061

Wany, A., Gupta, A. K., Kumari, A., Mishra, S., Singh, N., Pandey, S., et al. (2019). Nitrate nutrition influences multiple factors in order to increase energy efficiency under hypoxia in Arabidopsis. *Ann. Bot.* 123, 691–705. doi: 10.1093/aob/mcy202

Ward, N. D., Indivero, J., Gunn, C., Wang, W., Bailey, V., and McDowell, N. G. (2019). Longitudinal gradients in tree stem greenhouse gas concentrations across six Pacific Northwest coastal forests. *J. Geophys. Res.- Biogeo.* 124, 1401–1412. doi: 10.1029/2019JG005064

Weathers, P. J., and Niedzielski, J. J. (1986). Nitrous oxide production by cyanobacteria. *Arch. Microbiol.* 146, 204–206. doi: 10.1007/BF00402352

Weathers, P. J. (1984). N_2O evolution by green algae. *Appl. Environ. Microb.* 48, 1251–1253. doi: 10.1128/AEM.48.6.1251-1253.1984

Welch, B., Gauci, V., and Sayer, E. J. (2019). Tree stem bases are sources of CH_4 and N_2O in a tropical forest on upland soil during the dry to wet season transition. *Global Change boil.* 25, 361–372. doi: 10.1111/gcb.14498

Wen, Y., Corre, M. D., Rachow, C., Chen, L., and Veldkamp, E. (2017). Nitrous oxide emissions from stems of alder, beech and spruce in a temperate forest. *Plant Soil* 420, 423–434. doi: 10.1007/s11104-017-3416-5

Wendehenne, D., Pugin, A., Klessig, D. F., and Durner, J. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. *Trends Plant Sci.* 6, 177–183. doi: 10.1016/S1360-1385(01)01893-3

Woehle, C., Roy, A. S., Glock, N., Wein, T., Weissenbach, J., Rosenstiel, P., et al. (2018). A novel eukaryotic denitrification pathway in foraminifera. *Curr. Biol.* 28, 2536–2543. doi: 10.1016/j.cub.2018.06.027

Wulff, A., Oliveira, H. C., Saviani, E. E., and Salgado, I. (2009). Nitrite reduction and superoxide-dependent nitric oxide degradation by Arabidopsis mitochondria: influence of external NAD (P) H dehydrogenases and alternative oxidase in the control of nitric oxide levels. *Nitric. Oxide* 21, 132–139. doi: 10.1016/j.niox.2009.06.003

Zhao, X. J., Sampath, V., and Caughey, W. S. (1995). Cytochrome c oxidase catalysis of the reduction of nitric oxide to nitrous oxide. *Biochem. Bioph. Res. Co.* 212, 1054–1060. doi: 10.1006/bbrc.1995.2076

Zhao, L., Liu, F., Crawford, N. M., and Wang, Y. (2018). Molecular regulation of nitrate responses in plants. *Int. J. Mol. Sci.* 19, 2039. doi: 10.3390/ijms19072039

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 August 2020
doi: 10.3389/fpls.2020.01215

LbNR-Derived Nitric Oxide Delays Lycium Fruit Coloration by Transcriptionally Modifying Flavonoid Biosynthetic Pathway

Gen Li[1], Beibei Qin[1], Shuodan Li[2], Yue Yin[3], Jianhua Zhao[3], Wei An[3], Youlong Cao[3] and Zixin Mu[1]**

[1] College of Life Sciences, Northwest A&F University, Yangling, China, [2] College of Grassland Agriculture, Northwest A&F University, Yangling, China, [3] National Wolfberry Engineering Research Center, Ningxia Academy of Agriculture and Forestry Sciences, Yinchuan, China

OPEN ACCESS

Edited by:
Raquel Valderrama,
University of Jaén, Spain

Reviewed by:
Yuepeng Han,
Wuhan Botanical Garden, Chinese Academy of Sciences, China
Marek Petrivalsky,
Palacký University, Olomouc, Czechia

***Correspondence:**
Youlong Cao
youlongchk@163.com
Zixin Mu
muzx@nwsuaf.edu.cn

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: 26 February 2020
Accepted: 27 July 2020
Published: 13 August 2020

Citation:
Li G, Qin B, Li S, Yin Y, Zhao J, An W, Cao Y and Mu Z (2020) LbNR-Derived Nitric Oxide Delays Lycium Fruit Coloration by Transcriptionally Modifying Flavonoid Biosynthetic Pathway. Front. Plant Sci. 11:1215. doi: 10.3389/fpls.2020.01215

Anthocyanin-derived fleshy fruit pigmentation has become an excellent system for studying the regulatory network underlying fruit ripening and quality. The transcriptional control of anthocyanin biosynthesis by MYB–bHLH–WDR complexes has been well established, but the intermediate signals through which the environmental or developmental cues regulate these transcription factors remain poorly understood. Here we found that nitric oxide (NO) production during Lycium fruit ripening decreased progressively presenting a negative relationship with anthocyanins. After cloning of the nitric reductase (*NR*) gene from *Lycium barbarum* (*LbNR*) plants, we demonstrated that *LbNR*-derived NO partially inhibited anthocyanin biosynthesis but enhanced proanthocyanidin (PA) accumulation, and delayed fruit coloration. Application of the NO donor, sodium nitroprusside (SNP), produced a similar effect. The endogenous or exogenous NO downregulated the transcripts both of the regulatory genes and the structural genes that related to anthocyanin biosynthesis, while upregulated both of those genes that related to PA biosynthesis. Given there is a significant negative relationship between the levels of anthocyanins and PAs during Lycium fruit ripening, NO not only inhibited anthocyanin *de novo* biosynthesis but redirected the flavonoid biosynthetic pathway from anthocyanins to PA production. Two types of LrMYB transcription factors of opposite nature, namely anthocyanin-specific and PA-specific, which belong to the R2R3-MYB subfamily and 1R-MYB subfamily, respectively, were identified from *L. ruthenicum* fruits. It was further found that NO acts by antagonizing the ABA signaling, a phytohormone we have previously shown playing a positive role in Lycium fruit coloration. Our results provided particularly novel information about NO–ABA–anthocyanin interplay during Lycium fruit development and ripening, which may fill a gap between the developmental cues and the transcriptional regulation of anthocyanin biosynthesis.

Keywords: Lycium, fruit ripening, nitric oxide (NO), nitric reductase (NR), anthocyanins, proanthocyanidins

INTRODUCTION

Nitric oxide (NO) was first identified as a unique, diffusible molecular messenger in animals, after which it emerged that NO in plants affects similar signal transduction pathways. Like that in mammalian systems, endogenous NO generation in plants occurs due to the catalytic action of two main enzymes, namely, NO synthase (NOS)-like and nitrate reductase (NR) (Chamizo-Ampudia et al., 2017; Del Castello et al., 2019; Kolbert et al., 2019). In general, the NOS family catalyzes the NADPH-dependent formation of NO, but there are no clear NOS homologs in plant genomes and no plant enzyme displaying NOS-like activity have been identified so far. Using nitrite as a substrate, plants generate NO by enzymatic and nonenzymatic routes. The former is mainly catalyzed by NR, a key enzyme involved in nitrogen assimilation (Chamizo-Ampudia et al., 2017; Del Castello et al., 2019). NR-derived NO is involved in many plant physiological processes ranging from growth regulation to the biotic and abiotic stress response.

Edible fleshy fruits, which provide a wide range of essential nutrients such as vitamins, antioxidants, and minerals, are natural and healthy foods (Gapper et al., 2013; Seymour et al., 2013). Fruit ripening is an important developmental event for fresh fruit-bearing plants because it determines the adaptation to the environment and the continuation of the species (Gapper et al., 2013; Seymour et al., 2013). Ripening time is also a very important agricultural trait for fleshy fruits because it satisfies people's demand for fruits in different seasons. Therefore, fruit ripening regulation is a hot research topic for horticulturists and fruit tree experts. The available data have established the role of NO in senescence delay, in which NO acts primarily but not solely by limiting ethylene emission, resulting in delayed ripening (Manjunatha et al., 2010; Manjunatha et al., 2012; Bodanapu et al., 2016; Yang et al., 2016; Palma et al., 2019). NO application can also improve fruit quality, cold resistance, secondary metabolite biosynthesis, shelf life, and disease resistance (Li et al., 2017; Corpas et al., 2018; Corpas and Palma, 2018; Palma et al., 2019). Moreover, factors that induce NO generation can suppress fruit softening (Guo et al., 2018). Although experiments focused on unveiling the involvement of NO in fruit ripening have provided some insights into the biochemical events of this process, its comprehensive genetic regulation is yet to be understood (Manjunatha et al., 2010).

Anthocyanidins and anthocyanins are colored pigments in food and pharmaceutical ingredients with potential health benefits (Khoo et al., 2017). Their antioxidant activity has been extensively explored in a great number of plant species as well as in different organs from the same plant (Giampieri et al., 2018). Fleshy fruits are one of the main natural sources of this type of pigments. Anthocyanins accumulate during fruit ripening, so an increase in anthocyanin levels is a sign of fruit maturation. Anthocyanins significantly contribute to the quality characteristics of fruits and are therefore the targets of many breeding programs. The modification of anthocyanin metabolism by the genetic engineering of fruit maturation has been reported to enhance quality and shelf life (Jaakola, 2013; Zhang et al., 2013; Zhang et al., 2014). The anthocyanin composition in ripe fruit is established *via* complicated metabolic networks regulated by genetic, developmental, and environmental factors (Jaakola, 2013). Currently, the anthocyanin biosynthetic pathway is well known, and the key regulatory genes in many species that control the pathway have been identified, but the intermediate signals by which the environmental or developmental cues regulate anthocyanin biosynthesis remain poorly understood (Jaakola, 2013; Xu et al., 2015). It is reported in animal cells that plant-sourced flavonoid compounds make their function through the NO–ROS or NO–cGMP pathway (Gao et al., 2014; Qian et al., 2017; Zhang et al., 2018). Given that both anthocyanins and NO are involved in the regulation of fruit ripening, quality, and shelf life (Manjunatha et al., 2010; Zhang et al., 2013), whether there is a direct link between the NO signaling and anthocyanin metabolism remains unclear (Wang et al., 2018).

Wolfberry or goji (fruits from *Lycium barbarum* L. and *L. chinense* Mill) have been used in China as food and medicine for millennia, and globally, they are increasingly consumed as health foods (Potterat, 2010; Olatunji et al., 2016; Yao et al., 2018). Goji was usually consumed in the form of its dry fruit; however, with the improvement of people's living conditions and increased health awareness, the market demand for fresh Lycium fruit is strongly increasing. Moreover, as with other bulk vegetables and fruits, the planting of Lycium indoors will be a trend in the future. These facts make the regulation of wolfberry ripening time and improvements in fresh fruit quality and shelf life a priority. However, basic research on the mechanisms behind the regulation of Lycium fruit ripening is very scarce. We recently demonstrated that phytohormone ABA enhanced Lycium fruit coloration/ripening by promoting anthocyanin biosynthesis (Li G. et al., 2019), at both genetic and pharmacological levels. In contrast, in the present work, we further found that *LbNR*-derived NO inhibited Lycium fruit coloration by suppressing anthocyanin *de novo* biosynthesis as well as by redirecting the flavonoid biosynthetic pathway from anthocyanin to proanthocyanidin (PA) production. Our results uncover a novel mechanism underlying developmental cues-mediated pigmentation regulation and suggest the possibility of engineering endogenous NO to control Lycium fruit ripening.

MATERIALS AND METHODS

Plant Materials

Lycium fruits with two distinct colors [black fruit (*L. ruthenicum* Murr), BF and yellow fruit (*Lycium barbarum* L. var. *auranticarpum*), YF] were collected from 5-year-old trees at the Wolfberry (Lycium) Germplasm Repository of Ningxia [Academy of Agriculture and Forestry Sciences, Ningxia Hui Autonomous Region, China (38°38′N, 106°09′E; altitude, 1,100 m)]. For analysis of fruit ripening process, fresh Lycium fruits were sampled at five ripening stages (S1–S5) in the natural state as described by Zhao et al. (2015) with some minor modifications. The ripening process was divided in detail into

the young fruit stage [S1, 9–10 days after anthesis (DAA)]; the green fruit stage (S2, 15–16 DAA); the early color breaker stage (S3, 20–22 DAA), the late color breaker stage (S4, 27–30 DAA); and the ripened fruit stage (S5, 30–34 DAA). The fruits that came from at least three different trees for the same ripening stage of each species were collected, immediately frozen in liquid nitrogen, and stored at −80°C prior to total RNA extraction and physiological analysis.

Exogenous SNP Treatment

To avoid strong transpiration, the experiment was performed before evening. The surface of the green healthy fruits (at the S2 stage, 15 DAA) was sprayed with 1 mM sodium nitroprusside (SNP, Sigma-Aldrich, as treatment) or ddH_2O (including 0.05% Tween 20, as a control), by a sprayer with a volume of 200 ml. The reagent was diluted with ddH_2O containing 0.05% Tween 20 (to increase adhesion on the blade surface). The whole tree or partial branches within one tree were sprayed according to the amount of fruits they bore. The reagent was sprayed on at least three trees with at least 200 healthy fruits per tree of each Lycium species. Our experimental design was based on a randomized complete block design (RCBD). Mature fruits (at the S5 stage) were sampled 15 days after spraying, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis.

Determination of NO Production

0.1 g sample powder was suspended in 1 ml NO extraction solution [50 mM Tris-HCl, pH 7.8, 0.2 mM EDTA, 0.2% (v/v) Triton X-100, 2% (w/v) PVP, 10% (v/v) glycerol, and 5 mM DTT]. The mixture was placed on ice and simultaneously shaken at 80 rpm for 30 min. After centrifugation at 14,000× g for 40 min at 4°C, 500 μl supernatant was utilized in the NO assay.

The NO release was determined according to the method of Vitecek et al. (2008) and Airaki et al. (2012) with some minor modifications. DAF FM-DA (D2321, Sigma) was added to a freshly prepared crude extract solution at a 5 μM final concentration. Then, the reaction mixtures were incubated at 37°C in the dark for 20 min, after which fluorescence was measured using a Hitachi F7000 spectrofluorimeter (Hitachi High-Technologies Corporation, Tokyo, Japan) at excitation and emission wavelengths of 485 nm and 515 nm, respectively. As control reaction mixtures, fruit samples were preincubated for 30 min with 1 mM sodium tungstate, a nitrate reductase inhibitor, before the fluorescent probe was added. The fluorescence produced (NO release) was expressed as arbitrary units per milligram of fresh weight (FW). All samples were analyzed in biological triplicate, in which each biological replicate had at least 30 fruits to be tested.

Anthocyanins Measurements

The amounts of anthocyanins in Lycium fruit extracts were determined spectrophotometrically as described by Li G. et al. (2019). The anthocyanin concentration was calculated according to a standard curve for which cyanidin-3-glucoside (626B021, Solarbio, China) was used as a standard. All samples were analyzed in biological triplicate, in which each biological replicate had at least 30 fruits to be tested.

Proanthocyanidins Determination

The proanthocyanidins (PAs) in Lycium fruits were extracted according to the method of Pang et al. (2008), with some minor modifications. Briefly, 0.1 g fruit sample was extracted using 1 ml of PA extract solution (75% acetone solution containing 1% glacial acetic acid) by vortexing and then sonicated at room temperature for 1 h. After centrifugation at 2,500 g for 10 min, the residues were reextracted twice as above. The pooled supernatants were then extracted three times with chloroform and three times with hexane. After the crude extract was lyophilized with a vacuum freeze dryer (LYOQUEST-85 plus) for 16 h, the dry powder was thoroughly dissolved in 1 ml of ultrapure water, and the liquid was then sterilized by passing through a 0.22 μm reinforced nylon membrane filter (Billerica, MA, USA).

PA level is determined by a method of High Performance Liquid Chromatography (HPLC) as described by Tian et al. (2017). For HPLC detection, an Agilent ZORBAX Eclipse Plus C18 column (4.6 × 100 mm, 1.8 μm) was used at the following chromatographic conditions: mobile phase A (water:formic acid: trifluoroacetic acid = 97.9:2:0.1): mobile phase B (acetonitrile: water:formic acid:trifluoroacetic acid = 48:49.9:2:0.1) = 8:2; a 280 nm detection wavelength; a 1.0 ml/min flow rate; a 10 μl injection volume; and a 24°C column temperature. The standard curve was drawn using the peak areas of different concentrations of (+)-catechin. The PA concentration was expressed as milligram (mg) PAs per gram (g) fresh weight. All samples were analyzed in biological triplicate, in which each biological replicate had at least 30 fruits to be tested.

ABA Extraction and Assay

Endogenous ABA was extracted from Lycium fruits according to the method described by Li G. et al. (2019), and ABA content was assayed using a Phytodetek Immunoassay Kit (PDK 09347/0096, Agdia, USA) according to the manufacturer's instructions. Briefly, 0.2 g of fruit sample was fully ground with 1 ml of precooled 80% methanol extract solution (containing 200 $mg{\cdot}L^{-1}$ 2,6-di-tert-butyl-p-cresol, and 500 $mg{\cdot}L^{-1}$ citric acid monohydrate). After overnight leaching at 4°C, the mixture was centrifuged at 10,000 rpm and 4°C for 15 min, and the above-described procedure was repeated with the supernatant. The twice-treated supernatant was then combined, and the ground fruit was concentrated and dried with a Visible Nitrogen Blower (KD200, ALLSHENG, China). Finally, 0.8 ml of precooled 80% methanol was added to the dry powder, and the mixture was mixed with a vortex shaker to form the crude extract of ABA. All samples were analyzed in biological triplicate, in which each biological replicate had at least 30 fruits to be tested.

Cloning the Full-Length Sequence of *LbNR* cDNA

Leaves from *L. barbarum* (collected from 5-year-old trees at the Wolfberry Germplasm Repository of Ningxia, China) were used for total RNA isolation with the TRIzol method (Invitrogen, Carlsbad, CA, USA), and the first strand of cDNA was synthesized from 1 μg total RNA using the reverse

transcriptase M-MLV (TaKaRa Biotechnology, Dalian, China) and oligo (dT) according to the manufacturer's instructions. According to the EST sequence alignment of *L. barbarum*, the primers of the intermediate fragment homologous to the *NR* gene of *Solanaceae* were designed, and the fragment was amplified. For rapid amplification of cDNA 3′-end (3′-RACE), the gene-specific primers (GSP), 3′SPI and 3′GSPII were designed, and F1 (3′GSPI), R1 (3′outer), F2 (3′GSPII), and R2 (3′inner) were subjected to nested PCR amplification. The PCR product was ligated into a pMD18-T vector for sequencing to obtain a 3′-end fragment. Simultaneously, the cDNA was treated with RNase H (TaKaRa Biotechnology, Dalian, China), dCTP, and Terminal Deoxynucleotidyl Transferase, and the capped 5′-end of the cDNA was used as a template for 5′-RACE. The nested PCR and multiple PCR procedures were referenced to 3′-RACE, and the PCR product was ligated into a pMD18-T vector for sequencing, to obtain a 5′-end fragment. Finally, the entire cDNA coding region was combined, reamplified, inserted into the pMD18-T vector, and sequenced and was hereby designated as *LbNR* (NCBI accession no. MK169415). The sequences of the universal primers 3′outer, 3′inner, 5′outer, and 5′inner as well as the sequences of the gene-specific primers 5′GSPI, 5′GSPII, 3′ GSPI and 3′GSPII are shown in the **Appendix** (**Table S1**).

Bioinformatic Analysis of *LbNR*

The phylogenetic tree of *LbNR* was constructed by MrBayes software and further beautified by the web page of https://itol.embl.de. The protein secondary structure was predicted by the web page of http://pfam.xfam.org/search.

Virus-Induced *LbNR* Gene Silencing

The CDS fragment of the *LbNR* gene carrying the adaptor sequence was obtained by a pair of amplification primers (5′-ATGGCTGCATCTGTTGAAAAT; 3′- AATTTATTACT GCAGATTGTTGTTA). The pTRV2 vector (Liu et al., 2002) was digested with PstI to not only linearize the vector, but also expose the adaptor sequence. Both the linearized vector and *LbNR* product were digested with T4 DNA polymerase to produce the sticky ends. The PCR product was ligated to the sticky end of the vector and then transformed into E. *coli* to screen for pTRV2-derivative colonies with the silenced target gene (pTRV2-*LbNR*). After pTRV1 and pTRV2 or pTRV2-*LbNR* was rapidly frozen by liquid nitrogen, they were transfected with *Agrobacterium tumefaciens* strain GV3101 by heat shock.

For infiltration, a single colony was selected, inoculated in 5 ml of Luria–Bertani medium containing appropriate antibiotics (50 mg L^{-1} rifampicin, 50 mg L^{-1} kanamycin), and grown overnight in a 28°C shaker. The next day, the agrobacterium cultures were harvested by centrifugation at 4,000× g for 15 min, and the cells were resuspended in the infiltration medium (10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone). After adjusting OD_{600} to 1.0, the cells were incubated at room temperature for 3–4 h. Subsequently, the pTRV1 and pTRV2 (Control) or pTRV2-derivative solutions were mixed at a ratio of 1:1 for injection. The VIGS analysis was performed in the green fruit stage (S2) for both of the species. The injection method was as described by Li G. et al. (2019) using a 1 ml syringe, and the needle was directed at the fruit growth point for pressure injection. The injection was conducted on at least three trees with at least 200 healthy fruits per tree of each treatment, for each species. For the fruit coloration rate assay, the fruits were collected at an interval of 24 h after injection, while for other analyses, the fruits were sampled 3 days after injection. All the sampled fruits were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Virus-Induced *LbNCED1* Gene Silencing

LbNCED1 (9-cis-epoxycarotenoid dioxygenase 1)-VIGS fruits were constructed as described by Li G. et al. (2019).

Determination of the Fruit Coloration Rate

The changes of fruit anthocyanin level were determined at 0, 1, 2, and 3 days after injection. The fruit coloration rate was reported as milligram anthocyanin (mg) per gram fresh weight fruit (g^{-1}FW) per day (d^{-1}).

Real-Time PCR

Total RNA was extracted from the fruit tissues using a RNAprep Pure Plant Kit (DP432, TianGen, Beijing, China), and the cDNA was synthesized through reverse transcription reaction by a PrimeScript RT reagent kit (Takara, Dalian, China). Genomic DNA was removed using a RNase Free DNase I kit (Takara, Dalian, China) as instructed by the manufacturer. For the relative gene expression analysis, the housekeeping gene *LbActin* (HQ415754) was implemented as an internal control as it exhibits a uniform expression pattern in Lycium plants (Zeng et al., 2014).

Quantitative PCR amplifications were conducted with the BioRad CFX96 Touch™ Real-Time PCR Detection System (USA) using the SYBR Premix Ex Taq (Takara, Dalian, China). Each 15 μl reaction contained 7.5 μl SYBR Premix Ex Taq mix, 1.0 μl cDNA template (containing 100 ng of cDNA), 1.2 μl primer mix (0.6 μl each of the forward and reverse primers), and 5.3 μl ddH_2O. The amplification program was as follows: one cycle of 30 s at 95°C, followed by 39 cycles of 5 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The primers used for RT-qPCR are described in **Table S2**.

Statistical Analysis

Statistical analyses were performed using SPSS version 19.0. Parameter differences among varied fruits' developmental stages or different treatments were determined using one-way ANOVA with appropriate *post hoc* analysis. The column figures were drawn by Origin 9.0.

RESULT

Cloning and Bioinformatic Analysis of NR Gene in Lycium Plants

A *NR* gene in L. *barbarum* (*LbNR*) was successfully cloned by RACE technology. The gene has 3,193 bases, of which 145 bases

are contained in the 5′ untranslated region (UTR), 312 bases are contained in the 3′ UTR, and 2,736 bases comprise the coding sequence (CDS). The *LbNR* CDS is translated to 911 amino acids. The cDNA sequence has been deposited in the NCBI nucleotide sequence database under accession number MK169415. A phylogenetic tree containing *LbNR* and some plants of the *Solanaceae* family (*Capsicum annuum*, *Solanum tuberosum*, *Nicotiana benthamiana*, *Solanum lycopersicum*) was constructed (**Figure 1A**). The tree was rerooted to the *Arabidopsis thaliana NR* gene *AtNIA1*. In the phylogenetic tree, the one with the highest homology to the gene *LbNR* is *NbNR* of *Nicotiana benthamiana* followed by *CaNR* of *Capsicum annuum*.

The amino acid sequence was used for secondary structure prediction (**Figure 1B**). The *LbNR* protein has five domains. Among them, the oxidoreductase molybdopterin binding domain (Oxidored molyb) and the Mo-co oxidoreductase dimerization domain (Mo-co dimer) bind to a molybdenum cofactor (MoCo). The cytochrome b5-like heme/steroid binding domain (Cyt-b5) binds to heme, and the oxidoreductase FAD-binding domain (FAD-binding-6) binds to FAD. When the FAD-binding domain receives electrons from NAD(P)H bound by the oxidoreductase NAD-binding domain (NAD-binding-1), the Cyt-b5 domain shuttles electrons to the Mo-co dimer, and the Mo-co dimer transfers the electrons to nitrate, completing the catalytic cycle.

Nitric Oxide and Proanthocyanidin Changes During Lycium Fruit Ripening

To explore the role of NO in fruit maturation, both the NO release and the *LbNR* mRNA level were examined in two different colors of Lycium fruits, black fruits in *L. ruthenicum* Murr (BF) and yellow fruits in *Lycium barbarum* L. var. *auranticarpum* (YF), during their ripening process. It is shown that both NO production and *LbNR* transcript amount decreased gradually during Lycium fruits ripening, and there is a significant positive relationship between them for each species (**Figure 2**).

We have previously investigated anthocyanin levels during the different stages of ripening (S1–S5) in those fruits (Li G. et al., 2019). The present work found that in contrast to anthocyanins, the PA content decreased progressively (**Figure 3**) as these two colors of Lycium fruits ripened. There is a significant positive relationship between PAs and NO for each species while a negative relationship between anthocyanins and NO (**Figure 3**). Similarly, both the PA biosynthesis-related structural genes, *e.g.*, *LbANR* and *LbLAR* (Chen et al., 2017), and the regulatory genes, *e.g.*, *LrMYB30* (Yan et al., 2017), a homolog of *AtTT2* (At5G35550), and *Lr TTG1-like* (Cluster-26021.71813) (Yan et al., 2019), a homolog of *AtTTG1* (AT5G24520.1) in *Arabidopsis*, were gradually downregulated during the ripening process (**Figure S1**).

Effect of Virus-Induced *LbNR* Gene Silencing on Lycium Fruit Coloration

To further study the effect of NR-derived NO on fruit ripening, we constructed virus-induced gene silencing (VIGS)-*LbNR*

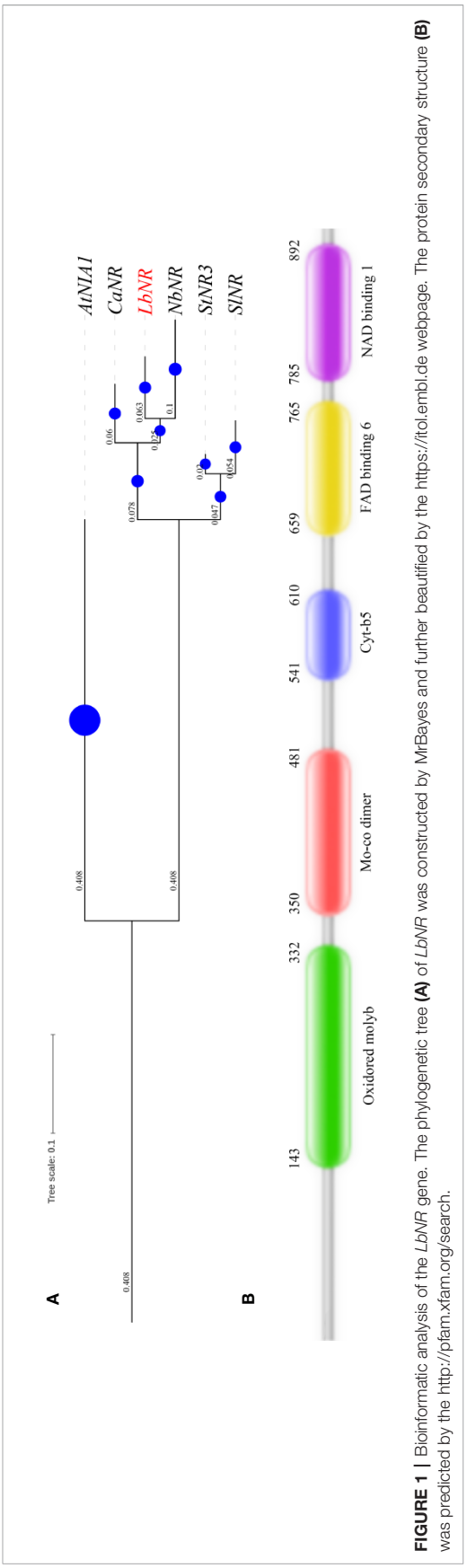

FIGURE 1 | Bioinformatic analysis of the *LbNR* gene. The phylogenetic tree **(A)** of *LbNR* was constructed by MrBayes and further beautified by the https://itol.embl.de webpage. The protein secondary structure **(B)** was predicted by the http://pfam.xfam.org/search.

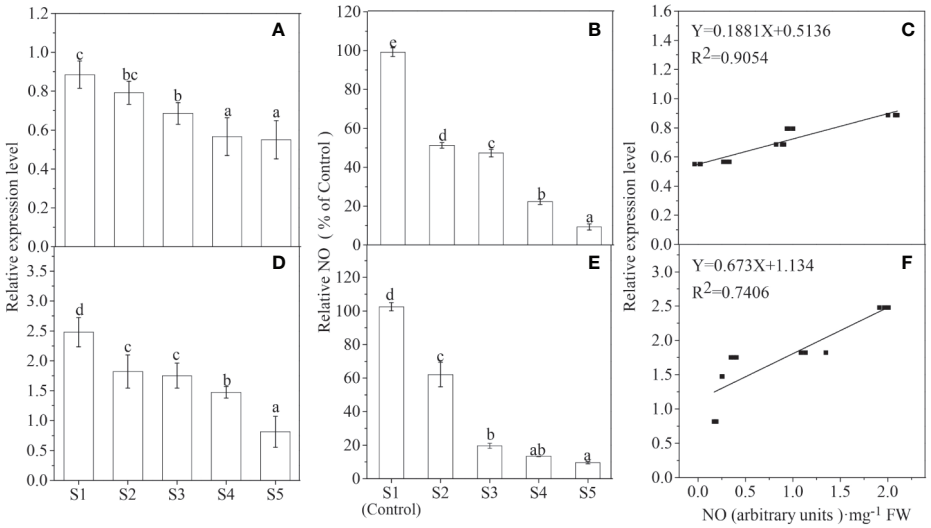

FIGURE 2 | Changes of *LbNR* gene expression **(A, D)** and NO release **(B, E)** as well as their relationship **(C, F)** during Lycium fruit ripening ($p < 0.05$). S1–S5 represents the different developmental stages of Lycium fruits as described in the section of *Materials and Methods*. The fruit samples were collected at the same time from the same trees that bear fruits at all five developmental stages, and at least three trees of YF **(A–C)** and BF **(D–F)**, respectively, were selected. In **(B, E)**, the NO release was converted to relative values, in which the value of the S1 stage was taken as 100%. The error bars represent the SDs of three independent replicates. Different letters on the bars for the same species indicate significant differences between the treatments ($p < 0.05$).

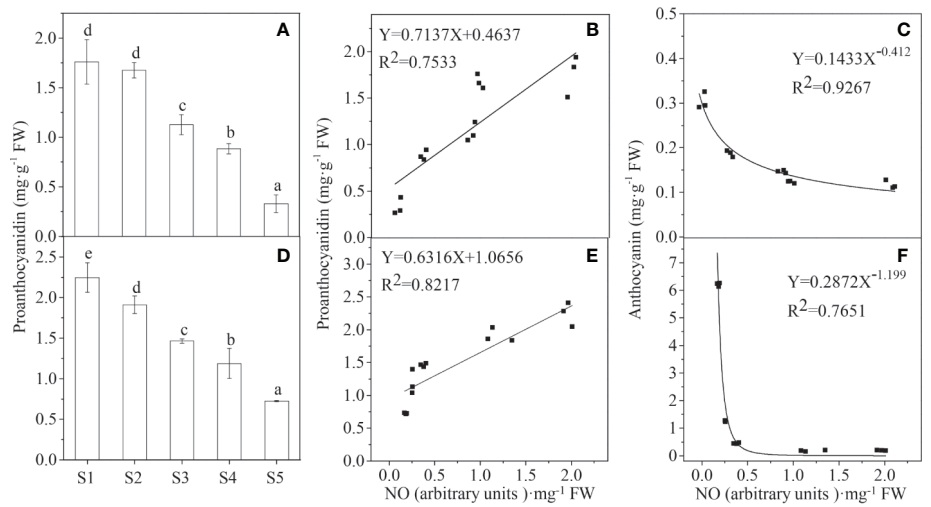

FIGURE 3 | The relationship between the NO release and the pigment content. Proanthocyanidin (PA) concentration declines progressively during the process of Lycium fruit ripening **(A, D)**, and there is a significant positive relationship between PA and NO **(B, E)** while a negative relationship between anthocyanin and NO **(C, F)**, both for YF **(A–C)** and BF **(D, F)**. For **(B, F)**, $p < 0.05$, and for **(C, E)**, $p < 0.01$. The error bars represent the SDs of three independent replicates. Different letters on the bars for the same species indicate significant differences between the treatments ($p < 0.05$).

fruits. Given that both the *LbNR* transcript abundance and NO release were significantly decreased in comparison with the control values (injected with empty vector), we successfully silenced the *LbNR* gene (**Figures 4A, B**). The anthocyanin content in *LbNR* gene-silenced fruits was higher than that in the control fruits. In contrast, *LbNR* silencing significantly decreased PA accumulation in mature Lycium fruits (**Figures 4C, D**). It is further validated from the molecular genetic level that NO negatively correlated with anthocyanins, but positively with PAs in Lycium fruits.

To compare the fruit ripening process in the control group (**Figures 5A, B**) with that in the VIGS group (**Figures 5C, D**), the

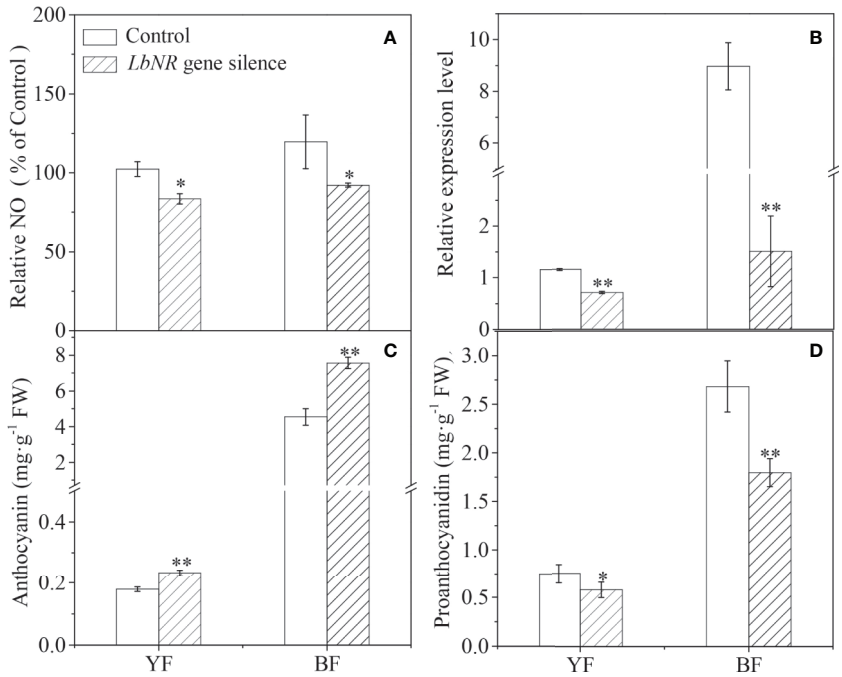

FIGURE 4 | Effect of virus-induced *LbNR* gene silencing on anthocyanin and PA accumulation in Lycium fruits. pTRV-*LbNR* vector construction and fruit injection were as described in *Materials and Methods*. After 3 days of Agrobacterium injection, the healthy modified YF or BF were sampled, immediately frozen in liquid nitrogen, and stored at −80°C to assay the NO release by the DAF FM-DA-mediated spectrofluorometric method **(A)**, to detect *LbNR* transcript by qRT-PCR **(B)**, to document anthocyanin and PA content by the spectrophotometric method **(C)**, or by HPLC method **(D)**, respectively. In **(A)**, the NO release was converted to relative values, in which the value of the empty vector-injected fruits was taken as 100%. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

fruit coloration rate was also surveyed. It is shown that *LbNR* silencing significantly facilitated fruit coloration both for the yellow and black color Lycium fruits (**Figures 5E, F**).

Effect of *LbNR*-Silencing on the Gene Expression of Flavonoid Biosynthetic Pathway

To explore the mechanism underlying the effect of NR-derived NO on anthocyanin biosynthesis during Lycium fruit ripening, the transcript abundance of genes in the flavonoid biosynthetic pathway was determined by qRT-PCR (**Figure 6**). *LbNR* silencing significantly upregulated the expression of several structural genes, *e.g.*, *LrCHS1* (chalcone synthase 1b, KC794742), *LrCHI2* (chalcone isomerase, KF031377), *LrF3H* (flavanone 3-hydroxylase, KC794744), *LrF3'H* (flavonoid 3-hydroxylase, KF732853), *LrF3'5 'H* (flavonoid 3, 5-hydroxylase), *LrDFR* (dihydroflavonol-4-reductase-like, KF031379), *LrANS* (anthocyanidin synthase, KC794745) and *LrUF3GT* (UDP glucose flavonoid 3-glucosyl transferase, KF768073). These genes have been isolated from *L. ruthenicum*, and their transcript amounts were positively correlated with anthocyanin content in this fruit, except *LrCHI* (Zeng et al., 2014). On the contrary, *LbNR* silencing significantly downregulated the structural genes involved in PA biosynthesis (**Figure S2**), *e.g.*, *LbANR* and *LbLAR* (Chen et al., 2017).

Similar to its effect on the structural genes, *LbNR* silencing also significantly upregulated the expression of the regulatory genes encoding MYB–bHLH–WD40 transcription factors (TFs) (**Figure 6**) specific to anthocyanin biosynthesis, including *LrAN2* (anthocyanin 2, KF768075) in the R2R3 MYB family, *LrJAF13* (KF768076) in the bHLH family, *LrAN11* (anthocyanin 11, KY131959) and *LrAN1b* (anthocyanin 1b, KF768077) in the WD40 family (Zeng et al., 2014). This effect is more obvious for BF than for YF because the former has a higher anthocyanin content. In addition to their genetic variation, a gene-specific sensitivity to endogenous NO was also observed in both the structural and the regulatory genes in Lycium fruits. On the contrary, *LbNR* silencing significantly downregulated genes encoding MYB–bHLH–WD40 TFs specific to PA biosynthesis (**Figure S2**), *e.g.*, *LrMYB30* and *LrTTG1-like*.

Exogenous SNP Reduced Anthocyanin Biosynthesis but Enhanced PA Biosynthesis

NO molecules released by a donor can either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency (Del Castello et al., 2019; Kolbert et al., 2019). Therefore, we examined the effect of spraying sodium nitroprusside (SNP), one of the most common NO donors, on Lycium trees on anthocyanin accumulation.

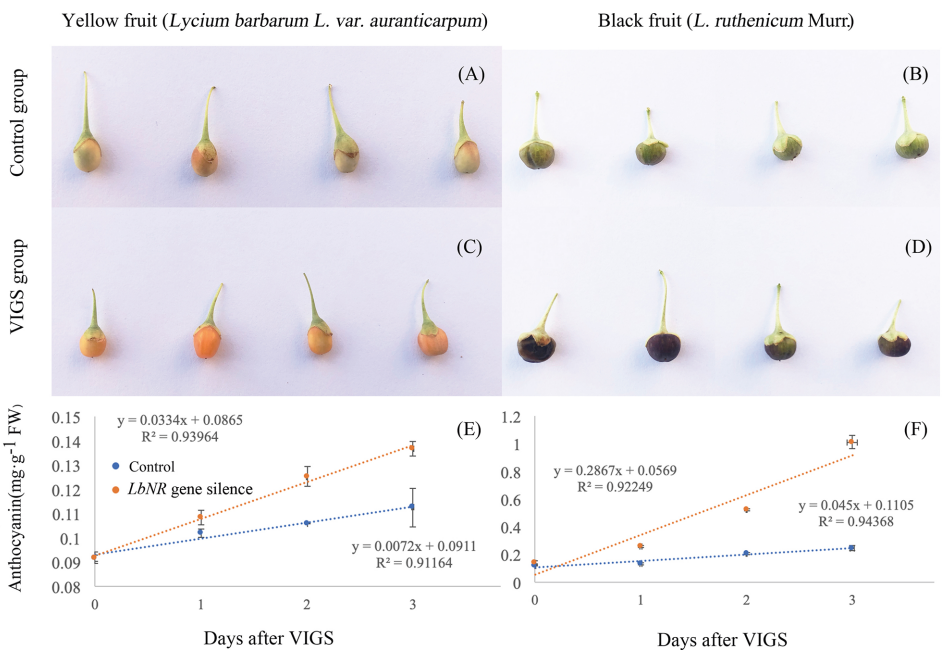

FIGURE 5 | The fruit phenotypes and coloration rates after virus-induced *LbNR* gene silencing. **(A, B)** Phenotypes of YF and BF at 2 d after injection with the pTRV empty vector, respectively. **(C, D)** Phenotypes of YF and BF at 2 d after *LbNR* gene silencing, respectively. **(E, F)** The coloration rates within 3 d after VIGS injection of YF and BF, respectively. For injection, the needle is inserted into the growing point of the green stage fruits (S2) for pressure injection. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments ($p < 0.01$).

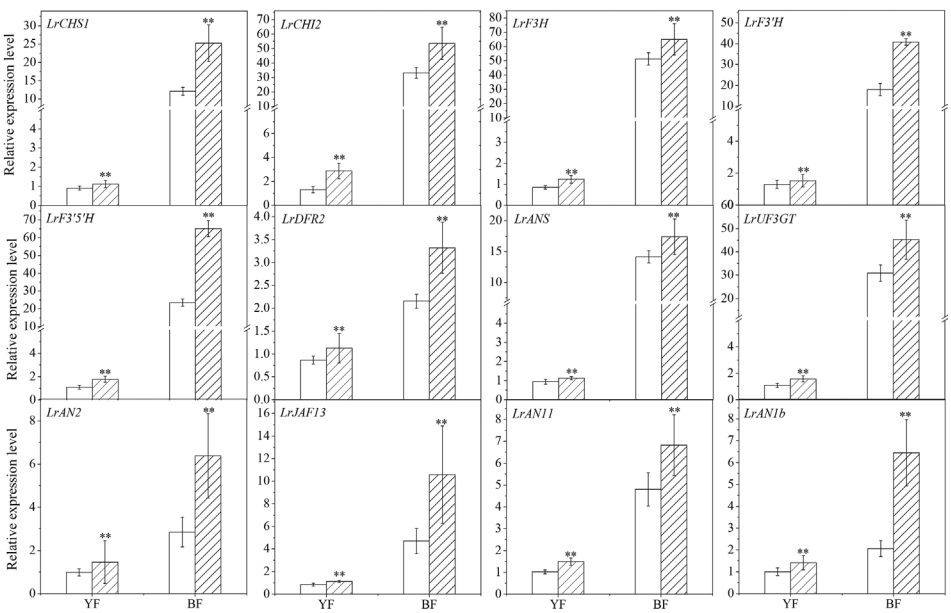

FIGURE 6 | Effect of virus-induced *LbNR* gene silencing on anthocyanin biosynthesis-related gene expression in Lycium fruits. Transcript levels of *LrCHS1*, *LrCHI2*, *LrF3H*, *LrF3'H*, *LrF3'5 'H*, *LrDFR2*, *LrANS*, and *LrUF3GT*, belonging to the structural genes, and transcript levels of *LrAN2*, *LrJAF13, LrAN11*, and *LrAN1b*, belonging to the MYB–bHLH–WD40 transcription factors, were shown. The gene expression differences between the two species were compared using YF (injected with empty vector) as the control for calculating the relative expression levels of the indicated genes. The data show the means ± SDs (n = 3). The asterisks on the bars for the same species indicate significant differences between the treatments. "**" indicates $p < 0.01$.

Exogenous SNP decreased anthocyanin levels in ripened black and yellow Lycium fruits (**Figures 7A, B**) but enhanced PA accumulation for both of the fruits compared to that observed in control fruits (**Figures 7C, D**).

SNP application significantly downregulated the transcription of both the structural genes *LrCHS1*, *LrCHI2*, *LrF3H*, *LrF3'H*, *LrF3'5 'H*, *LrDFR2*, *LrANS*, and *LrUF3GT* and the regulator genes *LrAN2*, *LrJAF13, LrAN11*, and *LrAN1b* in the flavonoid biosynthetic pathway (**Figure 8**). The implementation of SNP significantly upregulated genes involved in PA biosynthesis, both for the structural genes, *e.g.*, *LbANR* and *LbLAR* (**Figures S3A, B**), and the regulatory genes, *e.g.*, *LrMYB30* and *Lr TTG1-like* (**Figures S3C, D**). This transcriptional response was observed in both colors of fruits following SNP application, although there was a genotype- and gene-specific pattern. Therefore, we concluded that exogenous SNP decreased the transcripts of genes related to anthocyanin biosynthesis but enhanced that of genes related with PA biosynthesis.

Nitric Oxide Delays *L. ruthenicum* Fruit Coloration by Antagonizing ABA Action

Our previous studies have shown that ABA acts as one of the main positive regulators of Lycium fruit coloration/ripening (Li G. et al., 2019) by activating anthocyanin biosynthesis. In the present work, we further determined the interaction between ABA signaling and NO signaling in BF coloration. It is shown that *LbNR* silencing significantly promotes the accumulation of ABA in BF, while *LbNCED1* silencing clearly enhances the release of NO (**Figures 9A, B**). There is a significant negative relationship between the release of NO and the accumulation of ABA as well as between the transcripts of *LbNR* and *LbNCED1* during fruit ripening (**Figure S4**). Unlike NO enhanced PA biosynthesis, ABA inhibited it (**Figure S5**). Moreover, except *LrAN2* and *LrMYB30*, we identified the other MYB TFs in BF that were activated by NO but inactivated by ABA, *vice versa*, e.x. *LrMYB1*, *LrMYB3*, *LrMYB30*, *LrMYB44*, and *LrMYB73* (**Figures S6** and **S7**). Among these MYB TFs, *LrAN2*, *LrMYB1*, *LrMYB3*, and *LrMYB44* belong to the R2R3-MYB subfamily, while *LrMYB30* and *LrMYB73* belong to the 1R-MYB subfamily (Yan et al., 2017). Our results indicated that NO and ABA antagonized each other to regulate the coloration of Lycium fruits.

DISCUSSION

Fruit ripening is a highly regulated developmental process involving drastic internal transcriptional and biochemical modifications that coincide with seed maturation (Corpas et al., 2018). As a consequence of its action as an important

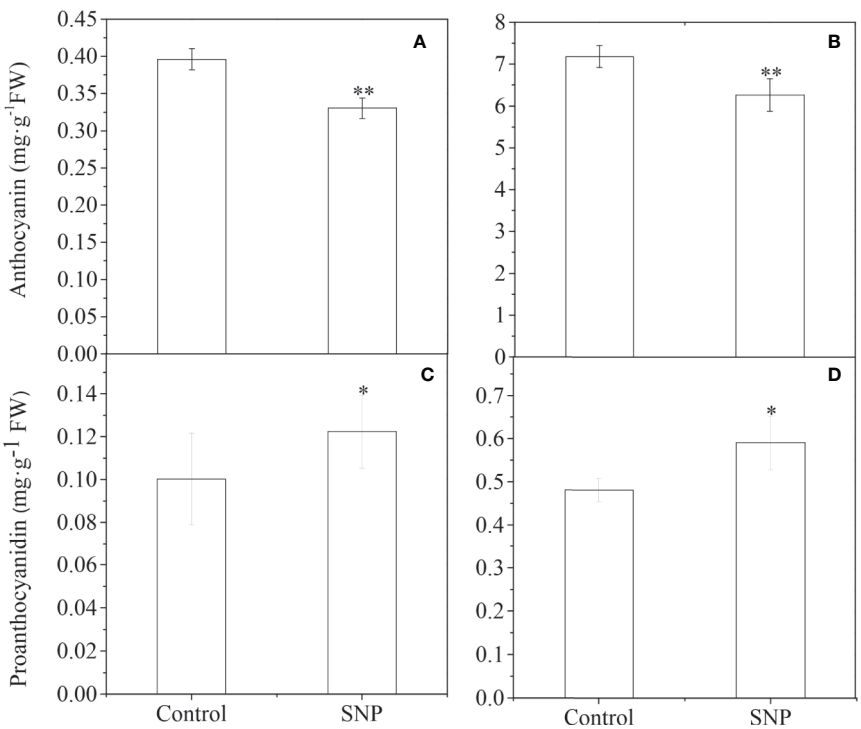

FIGURE 7 | Effect of exogenous SNP application on anthocyanin and PA accumulation in Lycium fruits. 1 mM SNP (soluted in 0.05% Tween 20) was utilized to spray the green stage fruits (S2) of YF and BF, on a sunny day after the sun went down. Fruits were sprayed with only a 0.05% Tween 20 solution as a control. After 15 days, the mature fruits (S5) were sampled to measure anthocyanin **(A, B)** and PA **(C, D)** content for both of YF **(A, C)** and BF **(B, D)**, respectively. The data show the means ± SDs (n = 3). The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

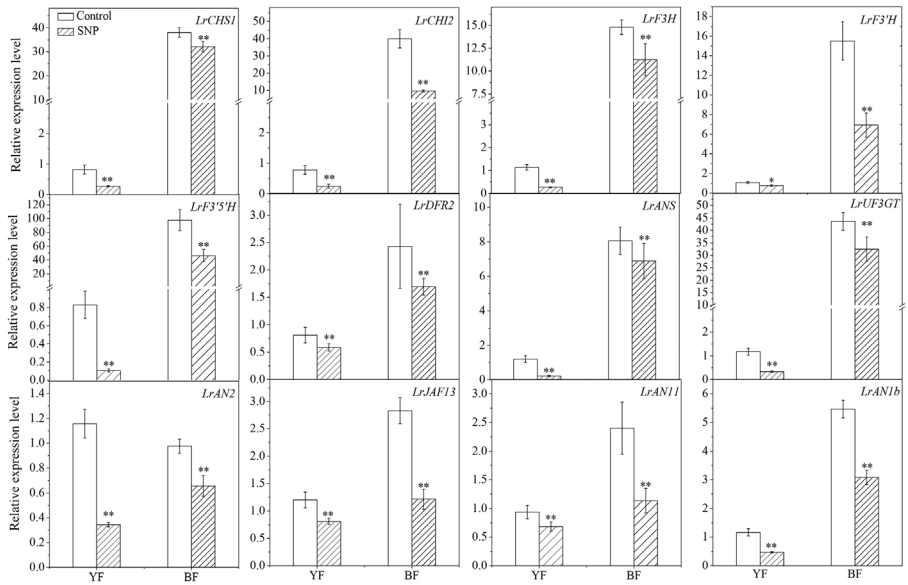

FIGURE 8 | Effect of exogenous SNP application on anthocyanin biosynthesis-related gene expression in Lycium fruits. Transcript levels of *LrCHS1*, *LrCHI2*, *LrF3H*, *LrF3'H*, *LrF3'5 'H*, *LrDFR2*, *LrANS*, *LrUF3GT LrAN2*, *LrJAF13*, *LrAN11*, and *LrAN1b* were shown. The gene expression differences between the two species were compared using YF (sprayed with ddH_2O) as the control for calculating the relative expression levels of the indicated genes. The data show the means ± SDs (n = 3). The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

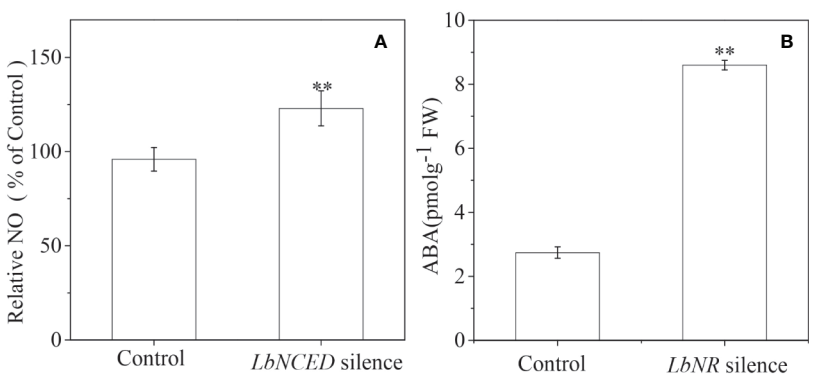

FIGURE 9 | Effect of *LbNR* silencing or *LbNCED1* silencing on ABA accumulation and NO release of *L. ruthenicum* fruits, respectively. ABA was assayed by an immunoassay method as described in the section of "*Materials and Methods*", and NO was determined as described in **Figure 2**. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments ($p < 0.01$).

gaseous signaling molecule in prokaryotes and eukaryotes, NO regulates critical developmental transitions and stress responses in plants (Del Castello et al., 2019; Kolbert et al., 2019). NO promotes leaf expansion, represses floral transition, stimulates light-dependent germination, promotes de-etiolation, inhibits maturation, and senescence, (Bruand and Meilhoc, 2019). Like in pepper, tomato, strawberry, and avocado plants (Gonzalez-Gordo et al., 2019; Palma et al., 2019), the present work indicated that the release of NO progressively declined during fruit ripening in Lycium plants, a medicinal and edible species. These results may show that it is a conserved event for endogenous NO production decline during fresh fruit ripening. In other words, NO has been demonstrated to delay fruit ripening generally, independently on whether fruits are climacteric or nonclimacteric (Palma et al., 2019). Integrating the results that the *LbNR* transcript amount is significantly positively correlated with the NO release during fruit ripening and that the VIGS-mediated *LbNR* gene silencing significantly decreased the NO production in ripe Lycium fruits, we could conclude that NR was partially responsible for NO biosynthesis

in Lycium plants. To our knowledge, this is the first report that NR-derived NO formation has been involved in fruit maturation of medicinal plant (Manjunatha et al., 2010; Palma et al., 2019).

Coloration of fresh fruits is a natural and excellent phenotypic marker for studying the mechanisms behind the regulation of fruit ripening and pigment metabolism. Anthocyanins and PAs (also known as condensed tannins) are two common flavonoid compounds in fruits. The former is an important pigment contributing to the coloration of many fruits, including that of *L. ruthenicum* in this study (Li G. et al., 2019), while the latter as a colorless metabolite, is an essential taste factor affecting astringency and bitterness of fruits (Khoo et al., 2017). It has been well established that the biosynthesis of anthocyanins and PAs shares most steps in the flavonoid pathway, and leucoanthocyanidins are the first branch point between these two biosynthesis pathways (Jaakola, 2013; Xu et al., 2015). Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) function at the branching points of the PA pathway, leading to catechin and epicatechin synthesis, respectively (Jaakola, 2013; Xu et al., 2015). Given *LbNR*-derived NO declined anthocyanin accumulation and downregulated the structural gene expression in the anthocyanin-biosynthesis pathway but elevated PA production and upregulated the transcripts of *LbLAR* and *LbANR*, it is proposed that NO delays Lycium fruit coloration by inhibiting *de novo* anthocyanin biosynthesis. Integrating the finding that there is a significant negative relationship between the levels of anthocyanins and PAs during Lycium fruit ripening (**Figure S8**), it is shown that NO also redirects the flavonoid biosynthetic pathway from anthocyanin to PA production, thus indirectly regulating Lycium fruit coloration. Our results may establish a direct relationship between the NO signaling and the flavonoid biosynthetic pathway in fresh fruits.

Flavonoid biosynthetic pathway is transcriptionally controlled mainly by a ternary complex of MYB–bHLH–WD40 TFs (termed the MBW complex), in which R2R3-MYB TFs function mainly to determine the specificity of gene activation for anthocyanin and PA biosynthesis (Jaakola, 2013; Xu et al., 2015; Allan and Espley, 2018). So far, mutiple flavonoid-related MYB activators have been identified in a great deal of plant species, among which some co-regulates both anthocyanin and PA accumulation, but others specific to one of them (Nesi et al., 2001; Bogs et al., 2007; James et al., 2017; An et al., 2018; Li C. et al., 2019). Besides acting as positive regulators, MYB TFs also act as negative regulators (or repressors) in the regulation of flavonoid biosynthesis. In the present work, four R2R3-MYB type TFs, e.x. *LrAN2*, *LrMYB1*, *LrMYB3*, and *LrMYB44* were inactivated by NO, while two 1R-MYB type TFs, e.x. LrMYB30 and LrMYB73 were activated by NO, at transcriptional level. So far there is not a report for 1R-MYB type TFs involved in flavonoid metabolism (Ma and Constabel, 2019), but the present work demonstrated in Lycium plants that it is related with PA biosynthesis. Except MYB TFs, both the examined bHLH TFs and WD40 proteins that were positively correlated with anthocyanins were negatively correlated with PAs. It is shown in the transcriptional level that PA-related MBW activators negatively correlated with anthocyanin-related MBW activators in Lycium plants (data not shown). Whether there is a direct antagonism between those R2R3-MYB type TFs and those 1R-MYB type TFs in Lycium fruits needs to be further investigated in the future. Moreover, genome-wide characterization of the MBW complex that coordinated regulation of PA and anthocyanin accumulation, or specific to each of them, is also urgently needed for Lycium plants.

It is well known that the fruit ripening process is regulated by various phytohormone interactions, in which ethylene and ABA make a positive effect, while IAA, CTK, and GA exert a negative one (Gapper et al., 2013; Guo et al., 2018). In consideration of the fact that there is neither ethylene release nor respiration rate increase during Lycium fruit ripening (Feng and Zhang, 2010), it is proposed that Lycium fruit belongs to the nonclimacteric group. We have previously documented that ABA acts as a ripening enhancing hormone in Lycium fruits both by genetic and pharmacological approaches (Li G. et al., 2019). In the present work, we further demonstrated that NO attenuated the coloration of Lycium fruits by antagonizing the action of ABA, thereby delaying the fruit ripening. The interaction between NO signaling and ABA signaling has been shown to be involved in many plant physiological processes, e.x. seed germination/dormancy, stomatal movement, leaf senescence, fruit ripening, abiotic stress response, *etc.* (Zhang et al., 2019). Within these physiological processes, NO and ABA have either synergetic or antagonistic functions (Prakash et al., 2019). Inhibition of ABA biosynthesis, promotion of ABA decomposition, and inhibition of ABA signal transduction may be behind the mechanisms why NO antagonizes ABA (Prakash et al., 2019). Of course, this needs to be further validated in Lycium fruits. It has been established in stomatal cells that NR-derived NO, which was induced by ABA, was involved in ABA-mediated stomatal movement (Kolbert et al., 2019). On the contrary, in the present work, we found that ABA inhibited NO production in Lycium fruits though the mechanism remains unclear. Maybe it is the ABA-induced reactive oxygen species accumulation which directly reacted with NO, thus declining the endogenous NO level (Prakash et al., 2019). Taken together, our results may provide particularly comprehensive information about NO–ABA–anthocyanins interplay during Lycium fruit development and ripening. Nevertheless, little is known about the interplay between NO and other regulators involved in fruit ripening of Lycium plants.

Although we highlighted the NO-mediated transcriptional modification of flavonoid biosynthetic pathway, it should be kept in mind that NO exerts a signaling function also by posttranslational modification of target proteins, *e.g.*, protein S-nitrosylation and nitration (Feng et al., 2019). In *Arabidopsis*, the addition of either of the NO donors SNP or GSNO inhibited the DNA-binding capacity of the minimal DNA-binding domain of the TF R2R3-MYB2 due to the S-nitrosylation of Cys53 or the S-nitrosylation of AtMYB30 at Cys49 and Cys53 (Serpa et al., 2007; Tavares et al., 2014). It is speculated, therefore, that NO regulates R2R3-MYB activity at both the transcriptional level and the posttranslational level, thus inhibiting anthocyanin biosynthesis

in Lycium fruits. However, the mechanism underlying the NO activation of PA-specific 1R-MYB TFs in these plants remains unclear. In the future, the NO-mediated posttranslational modification of the key enzymes involved in the biosynthesis of anthocyanins and PAs should also be addressed. Morover, as one of the metabolites of nitrate nutrients (Chamizo-Ampudia et al., 2017), the endogenous NO content can be fine-tuned by the regulation of NO_3^- application. Therefore, Lycium fruit coloration/ripening can also be manipulated by the application of fertilizers that contain NO_3^- (Jezek et al., 2018) as has been shown in *Arabidopsis*, grape berry and apple (Zhou et al., 2012; Habran et al., 2016; Wang et al., 2018). We look forward to this theme which will become a promising research project due to the versatile function of NO in regulating fresh fruit ripening, quality and shelf life.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding authors.

AUTHOR CONTRIBUTIONS

ZM, YC, and JZ designed the research. GL, BQ, and SL performed the experiments. GL and SL conducted the data analyses. YY, JZ, and WA conducted the field management work. ZM and GL wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was jointly supported by the National High-tech R&D Program (863 Program, 2013AA102606-04), the National Natural Science Foundation of China (31660220), the Project of Agricultural Breeding of New Wolfberry Varieties in Ningxia (2013NYYZ0101), and the Self-option and Foundation of Ningxia Academy of Agriculture and Forestry Sciences (NKYJ-18-16).

ACKNOWLEDGMENTS

We thank Prof. Yule Liu (the University of Tsinghua) for generously providing us pTRV1 and pTRV2 vector. We also appreciate Prof. Ren Chen (the University of Ningxia) for giving us the CDS sequence of *LrANS* gene, and Cuiping Wang (Ningxia Forestry Institute Key Laboratory of the Seedling Bioengineering) for gifting us the CDS sequence of *Lr TTG1-like* gene.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01215/full#supplementary-material

TABLE S1 | Primers utilized in LbNR full length cioning.

TABLE S2 | Primers utilized in qRT-PCR.

FIGURE S1 | Transcripts changes of PA biosynthesis-related genes during the process of YF (upper column) and BF (down column) ripening, respectively. The error bars represent the SDs of three independent replicates. Different letters on the bars for the same species indicate significant differences between the treatments ($p < 0.05$).

FIGURE S2 | Effect of *LbNR* silencing on PA biosynthesis-related gene expression, both for YF and BF. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments ($p < 0.01$).

FIGURE S3 | Effect of exogenous SNP on PA biosynthesis-related gene expression, both for YF and BF. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

FIGURE S4 | The relationship between the release of NO and the accumulation of ABA during the process of YF **(A)** and BF **(B)** ripening, respetively. $p < 0.01$.

FIGURE S5 | *LbNCED1* silencing enhanced PA accumulation, both for YF **(A)** and BF **(B)**. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

FIGURE S6 | Transcriptional response of LrMYB TFs to *LbNR* silencing in BF. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

FIGURE S7 | Transcriptional response of LrMYB TFs to *LbNCED1* silencing in BF. The error bars represent the SDs of three independent replicates. The asterisks on the bars for the same species indicate significant differences between the treatments. "*" indicates $p < 0.05$, and "**" indicates $p < 0.01$.

FIGURE S8 | The relationship between anthocyanins and PAs during the process of YF **(A)** and BF **(B)** ripening, respetively. $p < 0.01$.

REFERENCES

Airaki, M., Leterrier, M., Mateos, R. M., Valderrama, R., Chaki, M., Barroso, J. B., et al. (2012). Metabolism of reactive oxygen species and reactive nitrogen species in pepper (*Capsicum annuum* L.) plants under low temperature stress. *Plant Cell Environ.* 35, 281–295. doi: 10.1111/j.1365-3040.2011.02310.x

Allan, A. C., and Espley, R. V. (2018). MYBs drive novel consumer traits in fruits and vegetables. *Trends Plant Sci.* 23, 693–705. doi: 10.1016/j.tplants.2018.06.001

An, J. P., Li, R., Qu, F. J., You, C. X., Wang, X. F., and Hao, Y. J. (2018). R2R3-MYB transcription factor MdMYB23 is involved in the cold tolerance and proanthocyanidin accumulation in apple. *Plant J.* 96, 562–577. doi: 10.1111/tpj.14050

Bodanapu, R., Gupta, S. K., Basha, P. O., Sakthivel, K., Sadhana,, Sreelakshmi, Y., et al. (2016). Nitric oxide overproduction in tomato shr mutant shifts metabolic profiles and suppresses fruit growth and ripening. *Front. Plant Sci.* 7, 1714. doi: 10.3389/fpls.2016.01714

Bogs, J., Jaffe, F. W., Takos, A. M., Walker, A. R., and Robinson, S. P. (2007). The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiol.* 143, 1347–1361. doi: 10.2307/40065305

Bruand, C., and Meilhoc, E. (2019). Nitric oxide in plants: pro- or anti-senescence. *J. Exp. Bot.* 70, 4419–4427. doi: 10.1093/jxb/erz117

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate reductase regulates plant nitric oxide homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Chen, C., Xu, M., Wang, C., Qiao, G., Wang, W., Tan, Z., et al. (2017). Characterization of the *Lycium barbarum* fruit transcriptome and development of EST-SSR markers. *PLoS One* 12, e0187738. doi: 10.1371/journal.pone.0187738

Corpas, F. J., and Palma, J. M. (2018). Nitric oxide on/off in fruit ripening. *Plant Biol.* 20 (5), 805–807. doi: 10.1111/plb.12852

Corpas, F. J., Freschi, L., Rodriguez-Ruiz, M., Mioto, P. T., Gonzalez-Gordo, S., and Palma, J. M. (2018). Nitro-oxidative metabolism during fruit ripening. *J. Exp. Bot.* 69, 3449–3463. doi: 10.1093/jxb/erx453

Del Castello, F., Nejamkin, A., Cassia, R., Correa-Aragunde, N., Fernandez, B., Foresi, N., et al. (2019). The era of nitric oxide in plant biology: Twenty years tying up loose ends. *Nitric. Oxide* 85, 17–27. doi: 10.1016/j.niox.2019.01.013

Feng, M., and Zhang, N. (2010). Study on respiration characteristics of *Lycium barbarum*. *North. Hortic.* 34, 188–190.

Feng, J., Chen, L. C., and Zuo, J. R. (2019). Protein S-Nitrosylation in plants: Current progresses and challenges. *J. Integr. Plant Biol.* 61, 1206–1223. doi: 10.1111/jipb.12780

Gao, K., Liu, M., Cao, J., Yao, M., Lu, Y., Li, J., et al. (2014). Protective effects of *Lycium barbarum* polysaccharide on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. *Molecules* 20, 293–308. doi: 10.3390/molecules20010293

Gapper, N. E., McQuinn, R. P., and Giovannoni, J. J. (2013). Molecular and genetic regulation of fruit ripening. *Plant Mol. Biol.* 82, 575–591. doi: 10.1007/s11103-013-0050-3

Giampieri, F., Gasparrini, M., Forbes-Hernandez, T. Y., Mazzoni, L., Capocasa, F., Sabbadini, S., et al. (2018). Overexpression of the anthocyanidin synthase gene in strawberry enhances antioxidant capacity and cytotoxic effects on human hepatic cancer cells. *J. Agric. Food Chem.* 66, 581–592. doi: 10.1021/acs.jafc.7b04177

Gonzalez-Gordo, S., Bautista, R., Claros, M. G., Canas, A., Palma, J. M., and Corpas, F. J. (2019). Nitric oxide-dependent regulation of sweet pepper fruit ripening. *J. Exp. Bot.* 70, 4557–4570. doi: 10.1093/jxb/erz350

Guo, J., Wang, S., Yu, X., Dong, R., Li, Y., Mei, X., et al. (2018). Polyamines regulate strawberry fruit ripening by abscisic acid, auxin, and ethylene. *Plant Physiol.* 177, 339–351. doi: 10.1104/pp.18.00245

Habran, A., Commisso, M., Helwi, P., Hilbert, G., Negri, S., Ollat, N., et al. (2016). Roostocks/scion/nitrogen interactions affect secondary metabolism in the Grape Berry. *Front. Plant Sci.* 7, 1134. doi: 10.3389/fpls.2016.01134

Jaakola, L. (2013). New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends Plant Sci.* 18, 477–483. doi: 10.1016/j.tplants.2013.06.003

James, A. M., Ma, D., Mellway, R., Gesell, A., Yoshida, K., Walker, V., et al. (2017). Poplar MYB115 and MYB134 transcription factors regulate proanthocyanidin synthesis and structure. *Plant Physiol.* 174, 154–171. doi: 10.1104/pp.16.01962

Jezek, M., Zorb, C., Merkt, N., and Geilfus, C. M. (2018). Anthocyanin management in fruits by fertilization. *J. Agric. Food Chem.* 66, 753–764. doi: 10.1021/acs.jafc.7b03813

Khoo, H. E., Azlan, A., Tang, S. T., and Lim, S. M. (2017). Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. *Food Nutr. Res.* 61, 1361779. doi: 10.1080/16546628.2017.1361779

Kolbert, Z., Barroso, J. B., Brouquisse, R., Corpas, F. J., Gupta, K. J., Lindermayr, C., et al. (2019). A forty year journey: The generation and roles of NO in plants. *Nitric. Oxide* 93, 53–70. doi: 10.1016/j.niox.2019.09.006

Li, G., Zhu, S., Wu, W., Zhang, C., Peng, Y., Wang, Q., et al. (2017). Exogenous nitric oxide induces disease resistance against Monilinia fructicola through activating the phenylpropanoid pathway in peach fruit. *J. Sci. Food Agric.* 97, 3030–3038. doi: 10.1002/jsfa.8146

Li, C., Qiu, J., Huang, S., Yin, J., and Yang, G. (2019). AaMYB3 interacts with AabHLH1 to regulate proanthocyanidin accumulation in *Anthurium andraeanum* (Hort.)-another strategy to modulate pigmentation. *Hortic. Res.* 6, 14. doi: 10.1038/s41438-018-0102-6

Li, G., Zhao, J. H., Qin, B. B., Yin, Y., An, W., Mu, Z. X., et al. (2019). ABA mediates development-dependent anthocyanin biosynthesis and fruit coloration in *Lycium* plants. *BMC Plant Biol.* 19, 317. doi: 10.1186/s12870-019-1931-7

Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S. P. (2002). Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* 30, 415–429. doi: 10.1046/j.1365-313X.2002.01297.x

Ma, D., and Constabel, C. P. (2019). MYB repressors as regulators of phenylpropanoid metabolism in plants. *Trends Plant Sci.* 24, 275–289. doi: 10.1016/j.tplants.2018.12.003

Manjunatha, G., Lokesh, V., and Neelwarne, B. (2010). Nitric oxide in fruit ripening: trends and opportunities. *Biotech. Adv.* 28, 489–499. doi: 10.1016/j.biotechadv.2010.03.001

Manjunatha, G., Gupta, K. J., Lokesh, V., Mur, L. A., and Neelwarne, B. (2012). Nitric oxide counters ethylene effects on ripening fruits. *Plant Signal. Behav.* 7, 476–483. doi: 10.4161/psb.19523

Nesi, N., Jond, C., Debeaujon, I., Caboche, M., and Lepiniec, L. (2001). The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell* 13, 2099–2114. doi: 10.2307/3871430

Olatunji, O. J., Chen, H., and Zhou, Y. (2016). Lycium chinensis Mill attenuates glutamate induced oxidative toxicity in PC12 cells by increasing antioxidant defense enzymes and down regulating ROS and Ca^{2+} generation. *Neurosci. Lett.* 616, 111–118. doi: 10.1016/j.neulet.2015.10.070

Palma, J. M., Freschi, L., Rodriguez-Ruiz, M., Gonzalez-Gordo, S., and Corpas, F. J. (2019). Nitric oxide in the physiology and quality of fleshy fruits. *J. Exp. Bot.* 70, 4405–4417. doi: 10.1093/jxb/erz350

Pang, Y., Peel, G. J., Sharma, S. B., Tang, Y., and Dixon, R. A. (2008). A transcript profiling approach reveals an epicatechin-specific glucosyltransferase expressed in the seed coat of medicago truncatula. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14210–14215. doi: 10.1073/pnas.0805954105

Potterat, O. (2010). Goji (*Lycium barbarum* and L. *Chinense*): Phytochemistry, pharmacology and safety in the perspective of traditional uses and recent popularity. *Planta Med.* 76, 7–19. doi: 10.1055/s-0029-1186218

Prakash, V., Singh, V. P., Tripathi, D. K., Sharma, S., and Corpas, F. J. (2019). The Crosstalk between nitric oxide (NO) and abscisic acid (ABA) signalling molecules in higher plants. *Environ. Exp. Bot.* 161, 41–49. doi: 10.1016/j.envexpbot.2018.10.033

Qian, Y., Babu, P. V. A., Symons, J. D., and Jalili, T. (2017). Metabolites of flavonoid compounds preserve indices of endothelial cell nitric oxide bioavailability under glucotoxic conditions. *Nutr. Diabetes* 7, e286. doi: 10.1038/nutd.2017.34

Serpa, V., Vernal, J., Lamattina, L., Grotewold, E., Cassia, R., and Terenzi, H. (2007). Inhibition of AtMYB2 DNA-binding by nitric oxide involves cysteine S-nitrosylation. *Bioch. Biophys. Res. Commun.* 361, 1048–1053. doi: 10.1016/j.bbrc.2007.07.133

Seymour, G. B., Ostergaard, L., Chapman, N. H., Knapp, S., and Martin, C. (2013). Fruit development and ripening. *Ann. Rev. Plant Biol.* 64, 219–241. doi: 10.1007/978-981-13-2023-1_27

Tavares, C. P., Vernal, J., Delena, R. A., Lamattina, L., Cassia, R., and Terenzi, H. (2014). S-nitrosylation influences the structure and DNA binding activity of AtMYB30 transcription factor from *Arabidopsis thaliana*. *Bioch. Biophys. Acta-proteins Proteomics* 1844, 810–817. doi: 10.1016/j.bbapap.2014.02.015

Tian, J., Zhang, J., Han, Z., Song, T. T., Li, J. Y., Wang, Y. R., et al. (2017). McMYB12 transcription factors co-regulate proanthocyanidin and anthocyanin biosynthesis in malus crabapple. *Sci. Rep.* 7, 43715. doi: 10.1038/srep43715

Vitecek, J., Reinohl, V., and Jones, R. L. (2008). Measuring NO production by plant tissues and suspension cultured cells. *Mol. Plant* 1, 270–284. doi: 10.1093/mp/ssm020

Wang, X. F., An, J. P., Liu, X., Su, L., You, C. X., and Hao, Y. J. (2018). The nitrate-responsive protein MdBT2 regulates anthocyanin biosynthesis by interacting with the MdMYB1 transcription factor. *Plant Physiol.* 178, 890–906. doi: 10.1104/pp.18.00244

Xu, W., Dubos, C., and Lepiniec, L. (2015). Transcriptional control of flavonoid biosynthesis byMYB-bHLH-WDR complexes. *Trends Plant Sci.* 20, 176–185. doi: 10.1016/j.tplants.2014.12.001

Yan, L., Wang, C. P., Chen, J. W., Qiao, G. X., and Li, J. (2017). Analysis of MYB transcription factor family based on transcriptome sequencing in *Lycium*

ruthenicum Murr. *Sci. Agric. Sin.* 50, 3991–4002. doi: 10.3864/j.issn.0578-1752.2017.20.013

Yan, L., Chen, J. W., Wang, C. P., Tong, Q., Wang, C., Qiao, G. X., et al. (2019). Analysis of WD40 protein family based on transcriptome sequencing in *Lycium ruthenicum* Murr. *J. Nuclear Agric. Sci.* 33, 482–489. doi: 10.11869/j.issn.100-8551

Yang, Y., Zheng, Y., Liu, C., Chen, L., Ma, J., Sheng, J., et al. (2016). Inhibition of nitric oxide synthesis delayed mature-green tomato fruits ripening induced by inhibition of ethylene. *Sci. Hortic.* 211, 95–101. doi: 10.1016/j.scienta.2016.07.026

Yao, R., Heinrich, M., and Weckerle, C. S. (2018). The genus *Lycium* as food and medicine: A botanical, ethnobotanical and historical review. *J. Ethnopharmacol.* 212, 50–66. doi: 10.1016/j.jep.2017.10.010

Zeng, S., Wu, M., Zou, C., Liu, X., Shen, X., Hayward, A., et al. (2014). Comparative analysis of anthocyanin biosynthesis during fruit development in two *Lycium* species. *Physiol. Plant* 150, 505–516. doi: 10.1111/ppl.12131

Zhang, Y., Butelli, E., De Stefano, R., Schoonbeek, H. J., Magusin, A., Pagliarani, C., et al. (2013). Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. *Curr. Biol.* 23, 1094–1100. doi: 10.1016/j.cub.2013.04.072

Zhang, Y., Butelli, E., and Martin, C. (2014). Engineering anthocyanin biosynthesis in plants. *Curr. Opin. Plant Biol.* 19, 81–90. doi: 10.1016/j.pbi.2014.05.011

Zhang, B., Zhang, Z., Ji, H., Shi, H., Chen, S., Yan, D., et al. (2018). Grape seed proanthocyanidin extract alleviates urethral dysfunction in diabetic rats through modulating the NO-cGMP pathway. *Exp. Ther. Med.* 15, 1053–1061. doi: 10.3892/etm.2017.5499

Zhang, Q., Zhang, L., Geng, B., Feng, J., and Zhu, S. (2019). Interactive effects of abscisic acid and nitric oxide on chilling resistance and active oxygen metabolism in peach fruit during cold storage. *J. Sci. Food Agric.* 99, 3367–3380. doi: 10.1002/jsfa.9554

Zhao, J., Li, H., Xi, W., An, W., Niu, L., Cao, Y., et al. (2015). Changes in sugars and organic acids in wolfberry (*Lycium barbarum* L.) fruit during development and maturation. *Food Chem.* 173, 718–724. doi: 10.1016/j.foodchem.2014.10.082

Zhou, L. L., Shi, M. Z., and Xie, D. Y. (2012). Regulation of anthocyanin biosynthesis by nitrogen in TTG1-GL3/TT8-PAP1-programmed red cells of *Arabidopsis thaliana*. *Planta* 236, 825–837. doi: 10.1007/s00425-012-1674-2

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.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 September 2020
doi: 10.3389/fpls.2020.01313

Plant Nitrate Reductases Regulate Nitric Oxide Production and Nitrogen-Fixing Metabolism During the *Medicago truncatula–Sinorhizobium meliloti* Symbiosis

Antoine Berger[1,2], *Alexandre Boscari*[1], *Natasha Horta Araújo*[1], *Mickaël Maucourt*[3], *Mohamed Hanchi*[1], *Stéphane Bernillon*[4], *Dominique Rolin*[3,4], *Alain Puppo*[1] *and Renaud Brouquisse*[1]*

[1] Institut Sophia Agrobiotech, UMR INRAE 1355, Université Côte d'Azur, CNRS, Sophia Antipolis, France, [2] Department of Horticultural Science, University of Florida, Gainesville, FL, United States, [3] Univ. Bordeaux INRAE, UMR Biologie du Fruit et Pathologie, Villenave d'Ornon, France, [4] PMB-Metabolome, INRAE, Bordeaux Metabolome Facility, Villenave d'Ornon, France

Nitrate reductase (NR) is the first enzyme of the nitrogen reduction pathway in plants, leading to the production of ammonia. However, in the nitrogen-fixing symbiosis between legumes and rhizobia, atmospheric nitrogen (N_2) is directly reduced to ammonia by the bacterial nitrogenase, which questions the role of NR in symbiosis. Next to that, NR is the best-characterized source of nitric oxide (NO) in plants, and NO is known to be produced during the symbiosis. In the present study, we first surveyed the three NR genes (Mt*NR1*, Mt*NR2*, and Mt*NR3*) present in the *Medicago truncatula* genome and addressed their expression, activity, and potential involvement in NO production during the symbiosis between *M. truncatula* and *Sinorhizobium meliloti*. Our results show that Mt*NR1* and Mt*NR2* gene expression and activity are correlated with NO production throughout the symbiotic process and that MtNR1 is particularly involved in NO production in mature nodules. Moreover, NRs are involved together with the mitochondrial electron transfer chain in NO production throughout the symbiotic process and energy regeneration in N_2-fixing nodules. Using an *in vivo* NMR spectrometric approach, we show that, in mature nodules, NRs participate also in the regulation of energy state, cytosolic pH, carbon and nitrogen metabolism under both normoxia and hypoxia. These data point to the importance of NR activity for the N_2-fixing symbiosis and provide a first explanation of its role in this process.

Keywords: hypoxia, legumes, *Medicago truncatula*, nitric oxide, nitrogen-fixing symbiosis, nitrate reductase, nodules

OPEN ACCESS

Edited by:
Juan Carlos Begara-Morales, University of Jaén, Spain

Reviewed by:
Clare Gough, Centre National de la Recherche Scientifique (CNRS), France
Mounira Chaki, University of Jaén, Spain

***Correspondence:**
Renaud Brouquisse
renaud.brouquisse@inrae.fr

Specialty section:
This article was submitted to Plant Pathogen Interactions, a section of the journal Frontiers in Plant Science

Received: *14 February 2020*
Accepted: *11 August 2020*
Published: *04 September 2020*

Citation:
Berger A, Boscari A, Horta Araújo N, Maucourt M, Hanchi M, Bernillon S, Rolin D, Puppo A and Brouquisse R (2020) Plant Nitrate Reductases Regulate Nitric Oxide Production and Nitrogen-Fixing Metabolism During the Medicago truncatula–Sinorhizobium meliloti Symbiosis. Front. Plant Sci. 11:1313. doi: 10.3389/fpls.2020.01313

INTRODUCTION

In plants, yeasts, algae, and fungi, nitrate reductase (NR) is a key enzyme of the nitrogen (N) reduction and assimilation pathway. It catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is itself reduced to ammonia (NH_4^+) by nitrite reductase (NiR), before being assimilated into the amino acids and the nitrogen compounds of the cell (Campbell, 1999). Sixty-five million years ago, the legume family developed a beneficial mutual relationship with soil bacteria, the Rhizobia, which directly reduce atmospheric nitrogen (N_2) to ammonia (NH_4^+) through the activity of the nitrogenase under nitrogen deficiency situations (Wang et al., 2018). In exchange for NH_4^+, plants supply "board and lodging" for the bacteria, providing them with an ecological niche for their development and carbon nutrients for their functioning, in neoformed organs called nodules (Udvardi and Day, 1997; Terpolilli et al., 2012). Thus, in legume nodules, the bacterial nitrogenase substitutes for the NR–NiR pathway to produce NH_4^+ and supply the plant with reduced nitrogen. However, many studies have reported high NR expression and activity in symbiotic nodules (see Streeter, 1985a; Streeter, 1985b; Arrese-Igor et al., 1990; Silveira et al., 2001; Kato et al., 2003; Sanchez et al., 2010; Horchani et al., 2011, and references therein) and the question arose—and still arises—of what the NR can be used in the N_2-fixing symbiosis, where nitrogen reduction is ensured by nitrogenase.

The involvement of NR activity in NO production has been evidenced in many plant organs and tissues over the past 20 years (Dean and Harper, 1988; Yamasaki and Sakihama, 2000; Rockel et al., 2002; Sakihama et al., 2002; Gupta et al., 2005; Planchet et al., 2005; Kato et al., 2010; Kolbert et al., 2010). Until recently, NR was considered to produce directly NO *via* the reduction of NO_2^-, but another indirect mechanism of NO synthesis involving NR has been proposed (Chamizo-Ampudia et al., 2016; Chamizo-Ampudia et al., 2017). In this mechanism, through its diaphorase activity, NR transfers electron from NAD(P)H to a NO-forming nitrite reductase (NOFNiR) that catalyzes the reduction of NO_2^- to NO. Although the interaction between NR and NOFNiR has been argued in eukaryotic algae, the proof of concept in higher plant is still not demonstrated. NO is a reactive free radical gaseous molecule with a broad spectrum of regulatory functions in plant growth and development, and in response to abiotic and biotic factors (Kolbert et al., 2019). NO is particularly involved in the legume–Rhizobium symbiotic interactions (Hichri et al., 2015; Berger et al., 2019).

During the first hours after inoculation with the symbiotic partner, NO was observed in the roots of *Lotus japonicus*, *Medicago sativa*, and *Medicago truncatula* (Nagata et al., 2008; Fukudome et al., 2016; Hichri et al., 2016). Its production was also detected during the infection process along the infection thread and in the dividing cells of the *M. truncatula* nodule primordium (del Giudice et al., 2011). Similar results were observed in other Medicago species (Pii et al., 2007). In *M. truncatula* mature nodules, NO has been shown to accumulate particularly in the N_2-fixing zone (Baudouin et al., 2006; Hichri et al., 2016), and at the onset of nodule senescence a NO production was reported at the junction of the N_2-fixing and senescence zones (Cam et al., 2012). A recent study with *M. truncatula* showed that NO is produced throughout the whole symbiotic process, from infection with *Sinorhizobium meliloti* up to, at least, 8 weeks post-inoculation (wpi), exhibiting production peaks during the first hours of the symbiotic interaction, during early development of the nodule and when the nodule becomes mature (Berger et al., 2020). These observations suggest that NO performs specific signaling and/or metabolic functions during symbiosis. Indeed, two transcriptomic analyses led to the identification of NO-responsive genes either in 4 days post-inoculation (dpi) roots (Boscari et al., 2013) or in developing and mature nodules (Ferrarini et al., 2008). More than 400 plant genes are NO-regulated during the symbiotic process, including genes involved in nodule development normally induced by the symbiont, suggesting that NO participate in signal transduction in the plant–microorganism interaction (Ferrarini et al., 2008; Boscari et al., 2013). On the bacterial partner side, NO has also been shown to regulate a hundred genes, most of them being similarly regulated under microoxic conditions (Bobik et al., 2006; Meilhoc et al., 2010). The biological activity of NO is particularly mediated through redox-dependent protein modifications such as S-nitrosation, tyrosine nitration and metal nitrosylation (Besson-Bard et al., 2008; Hancock, 2019). Several key proteins involved in nodule primary metabolism or stress response were reported to be S-nitrosated, indicating a crucial role of NO in the energy, carbon, and nitrogen metabolism (Puppo et al., 2013). Among these proteins, enzymes such as glutathione peroxidase (Castella et al., 2017), glutamine synthetase (Melo et al., 2011; Seabra and Carvalho, 2015), and leghemoglobins (Mathieu et al., 1998; Navascues et al., 2012; Sainz et al., 2015; Becana et al., 2020) have been shown to be differently regulated by various NO-dependent modifications. Finally, NO has also been shown to play a metabolic function in the maintenance of energy status under hypoxic conditions, such as that prevailing in microoxic nodules (Igamberdiev and Hill, 2004; Igamberdiev and Hill, 2009). Indeed, NO is involved in a respiratory cycle, called Phytoglobin-NO respiration (PNR), allowing the regeneration of ATP under low oxygen concentrations. PNR is divided into four steps including: 1) NO_3^- reduction to NO_2^- by cytosolic NR, 2) NO_2^- transport from the cytosol to the mitochondrial matrix, 3) NO_2^- reduction to NO by the mitochondrial electron transfer chain, and 4) NO diffusion to the cytosol and oxidation to NO_3^- by Phytoglobins. Evidence indicates that PNR potentially functions and participates in the regeneration of ATP in N_2-fixing nodules (Horchani et al., 2011; Berger et al., 2020). Thus, the importance and diversity of NO functions in symbiosis, whether through the regulation of gene expression, the modulation of enzyme activity, or its involvement in energy metabolism, require that its production should be timely and tightly regulated during the symbiotic process. Considering that the NR pathway is the main NO production pathway in plants and that its activity is high in nodules, we hypothesized that NR is significantly involved in the production of NO during the symbiotic process.

The aim of this work was to test this hypothesis. To this end, we addressed the expression and activity of the three NR genes present in the *M. truncatula* genome, from the first hours of symbiotic interaction up to 8 wpi, at the onset of nodule senescence. Then, we investigated the impact of NR activity on NO production and energy regeneration during nodule development. Last, we studied its role in carbon and nitrogen metabolism in mature nodules. Based on our data, we discuss the potential roles of NR on NO homeostasis during the symbiotic process and more generally on the N_2-fixing process.

MATERIALS AND METHODS

Plants Growth and Inoculation Conditions

Medicago truncatula (cv Jemalong A17) were scarified, sterilized, and germinated as in del Giudice et al. (2011). Seedlings were cultivated and inoculated with *Sinorhizobium meliloti* 2011 strain either in Petri dishes as in del Giudice et al. (2011), or in planters as in Horchani et al. (2011). A basic intake of 0.2 mM KNO_3 is provided to plants on Petri dishes and planters. Cultures in Petri dishes were used for short-term experiments up to 14 days post-inoculation (dpi), while those in planters were used for long-term experiments up to 8 weeks post-inoculation (wpi). Roots and/or nodules were harvested at various times of the kinetics. For short-term experiments, 2 cm-long root segments corresponding to the infection zone (del Giudice et al., 2011) were harvested for gene expression and NO production. For long-term experiments only nodules were used.

NR Sequences Acquisition and Analyses

Protein sequences of NR were obtained from three genomic and protein databases: NCBI (www.blast.ncbi.nlm.nih.gov), Phytozome (www.phytozome.jgi.doe.gov), and Uniprot (www.uniprot.org). Local or multiple alignment search tools (BLAST from NCBI and Phytozome; Water and ClustalW from the European Bioinformatic Institute platform EBI) were used to analyze the sequence's quality, length, and uniqueness. Sequences kept were listed in **Supplementary Table S1**, where a nomenclature code was assigned to each protein in order to simplify data-reading in the phylogenetic tree. The code is made of the name of the species represented, the name allotted to the sequence on the literature or NCBI, and the number of amino acids composing each protein.

Construction of the Phylogenetic Tree

NR sequences were aligned using Muscle algorithm (Madeira et al., 2019), and their evolutionary history was inferred by Maximum Likelihood (ML) and the JTT matrix-based model (Jones et al., 1992) using MegaX software (Kumar et al., 2018). Initial trees calculated for the heuristic search were acquired automatically by applying Neighbor-Join (NJ) and BioNJ algorithms to a pair-wise distance matrix approximated using the JTT model. The number of distinct/identical residues between each pair of sequences in the multiple alignment was therefore calculated to construct the matrix, and the topology with a higher log likelihood value was selected. The tree was inferred by Nearest-Neighbor-Interchange (NIN) heuristic method, and to assess the reliability of the inference, the bootstrapping method was applied. The consensus tree deduced from 1,000 bootstrap repetitions was retained, and branches corresponding to divisions reproduced in less than 50% of the bootstrap repetitions were not considered. An outgroup, *Chlamydomonas reinhardtii* 's NR NIT1, was used to root the tree.

Construction of a Binary Vector for Hairy Root Transformation

For promoter transcriptional fusions, fragments of 1,647, 1,700, and 1,554 bp upstream of the start codon were amplified by PCR using the primers indicated in **Table S2** for Mt*NR1*, Mt*NR2*, and Mt*NR3* respectively. Each PCR fragment was first cloned into the pDONR207 donor vector and then into the plant expression vector pKGWFS7 (Karimi et al., 2002) using Gateway technology (Invitrogen, http://www.invitrogen.com). For the RNA interference (RNAi) construct, 432- and 441-bp fragments of Mt*NR1* (MtrunA17Chr3g0115151) and Mt*NR2* (MtrunA17Chr5g0424491) genes were amplified *via* polymerase chain reaction (PCR) with specific primers (**Table S2**). PCR products were independently ligated into pGEM-T easy vector (Promega) and subsequently subcloned into pENTR4 vectors in *Bam*HI–*Kpn*I restriction sites for Mt*NR1* and *Eco*RI and *Kpn*I restriction sites for Mt*NR2*. The pENTR4 vector carrying the Mt*NR1* or the Mt*NR2* fragment was recombined with pK7GWIWG5D(II) vector (Horchani et al., 2011) using the LR clonase enzyme mix (catalog no. 11791-019; Invitrogen) to create the RNAi expression vectors. As control, transgenic roots transformed with empty pK7GWIWG5D(II) vector and selected on the base of green fluorescent protein (GFP) marker expression were used. Constructs were checked by sequencing, introduced by electroporation into *Agrobacterium rhizogenes* strain ARqua1, and used for *M. truncatula* root transformation as described by Boisson-Dernier et al. (2001).

Measurement of NO Production

NO detection was performed as in Horchani et al. (2011) using the 4,5-diaminofluorescein probe (DAF-2, Sigma-Aldrich) with the following changes. Either nodules (20–30 mg fresh weight) or root segments (50–100 mg fresh weight) were incubated in the dark at 23°C in 1 ml of detection buffer (10 mM Tris-HCl pH 7.4, 10 mM KCl) in the presence of 10 μM DAF-2. As a control, NO production was measured in the same experimental system through the use of the Cu(II) fluorescein (CuFL) fluorescent probe (Strem Chemicals) instead of DAF-2 in the detection buffer as described in Horchani et al. (2011). Similar results were obtained with both probes. The production of NO was measured with a spectrofluorimeter-luminometer (Xenius, SAFAS, Monaco). Inhibitors are used as described in Horchani et al. (2011). The inhibitors are added to the reaction medium for the determination of NO at the concentration of 1 mM tungstate (Tg), 1 mM allopurinol, 1 mM propyl gallate, and 300 μM potassium cyanide (KCN). NO production was initiated 1 h after the addition of inhibitors. Three independent biological

replicates have been performed with three technical replicates per biological assay.

Nitrogen-Fixing Capacity Measurement

Nitrogenase activity of the nodules was determined *in vivo* by measuring acetylene reducing activity (ARA, Hardy et al., 1968). Nodulated roots were harvested and incubated at 30°C for 1 h in rubber-capped tubes containing 10% acetylene atmosphere. Ethylene concentrations were determined by gas chromatography (Agilent GC 6890N, Agilent Technologies) equipped with a GS-Alumina separating capillary column. Three independent biological replicates have been performed with five technical replicates per biological assay.

Phosphorus NMR

For each experiment, 0.9 to 1.1 g fresh weight of 4 wpi-old nodules (around 1,400 to 1,700 nodules) were harvested and incubated at ambient temperature in an aerated perfusion medium containing 1 mM KH_2PO_4, 1 mM $MgSO_4$,$7H_2O$, 0.25 mM K_2SO_4, 0.5 mM $CaCl_2$, 10 mM MES/KOH, pH 6.0, and 25 mM glucose. At the end of the preparation period of approximately 3 h, the nodules were placed between two filters into a 10-mm tightly closed NMR tube, part of a homebuilt perfusion system. The latter, evolved from experimental device described previously (Roby et al., 1987), allows circulation of the perfusion medium controlled in solute composition, temperature, and pH through the living nodule sample. The partial oxygen pressure in the perfusion medium was established by bubbling mixtures of oxygen and nitrogen (either 21:79 or 1:99% O_2:N_2) into the medium reservoir. At various time, effectors were added into the perfusion medium. Each series of *in vivo* biological experiment has been performed at least five times.

^{31}P NMR spectra were acquired at 202.47 MHz using a 500.16 MHz NMR spectrometer (Avance III, Bruker). For *in vivo* experiments, ^{31}P NMR spectra were recorded for 36 min using a 10 mm ATMA broadband observe probe. A solution of 500 mM Hexamethylphosphoramide (HMPA, ref H3380, Sigma) contained in a concentric capillary provides the chemical shifts and intensity references for the ^{31}P NMR spectra. 3,072 scans of 16 K data points were acquired with a 60° pulse angle, a spectral width of 14,204 Hz, acquisition time of 0.58 s and recycle delay of 0.1 s. Preliminary data processing was carried out with TOPSPIN 3.0 software (Bruker Biospin, Karlsruhe, Germany). Each Free Induction Decay (FID) was Fourier transformed (10 Hz line broadening), manually phased and baseline corrected. The resulting spectra were aligned by setting the HMPA signal to 30.73 ppm.

The resonance assignments were based on chemical shifts. ^{31}P chemical shifts were determined according to (Rolin et al., 1989). Subcellular pH was estimated by the use of a standard reference curve of pH as a function of chemical shift, which was obtained according to the method of Roberts et al. (1980).

Measurement of NR Activity

Tissue samples are ground with mortar and pestle in liquid nitrogen. The total proteins are extracted from 100 mg of powder using the following extraction buffer: 25 mM Tris HCl, pH 8.5, 1 mM EDTA, 20 μM FAD, 0.04% Triton X100, 10 μM $NaMO_4$, 1 mM DTT, 20 μM L-transepoxysuccinyl-leucylamido-[4-guanidino]butane (E64), 2 mM phenylmethylsulfonyl fluoride (PMSF). The extracts are centrifuged (15,000 g, 15 min). The NR activity is measured by quantifying the NO_2^- produced in the reaction mixture containing: the enzyme extract in 0.2 M HEPES, pH7.0, 15 mM KNO_3, and 250 μM of NADH (Miranda et al., 2001). The reaction is stopped after 30 min by boiling the samples for 3 min at 100°C. The nitrite produced is measured using Griess reagent (1% sulphanilamide in 1 M HCl and 0.01% NEDD [N-1-naphthylethylenediamine dihydrochloride] in water) and measured at 540 nm. Soluble proteins are assayed according to the method of Bradford (1976). For each series of experiments, at least three independent biological replicates have been performed with three technical replicates per biological assay.

RNA Isolation, Reverse Transcription and Gene Expressions

RNAs were isolated from 100 mg of frozen material ground in liquid N_2 using the RNAzol following the manufacturer's recommendations (Sigma-Aldrich). RNA quality was checked, and DNase treatment was carried out before the synthesis by GoScript reverse transcriptase (Promega) of the cDNAs. The RT-qPCR was made with Go-Taq qPCR master Mix kit according to manufacturer's instructions (Promega). RT-qPCR data analysis was carried out using RqPCRBase, an R package working on R computing environment for analysis of quantitative real-time PCR data (Hilliou and Tran, 2013). The expression of the different genes was normalized against two housekeeping genes *Mtc27* (Van de Velde et al., 2006) and *Mta38* (del Giudice et al., 2011). The reference value '1' was attributed to the first time when the cycle threshold (Ct) of the analyzed gene was significantly detectable. The data of comparative expression levels between genes are given on a logarithmic scale expressed as $40 - \Delta C_T$, where ΔC_T is the difference in qRT-PCR threshold cycle number between the respective gene and the reference gene; the number 40 was chosen because PCR run stops after 40 cycles (Bari et al., 2006; Truong et al., 2015; Berger et al., 2020). RT-qPCR analyses were carried out in triplicate using the primers reported in **Table S2**. Three independent biological replicates have been performed.

Enzymatic and HPLC Metabolite Analyses

Nodule metabolites (amino acids, organic acids, soluble sugars) were extracted by the alcoholic extraction method and resuspended in water as described in Brouquisse et al. (1991). Sucrose was determined enzymatically (Velterop and Vos, 2001) at 340 nm using a microplate spectrophotometer. Succinate and malate were determined by anion exchange HPLC (Dionex) with conductivity detection (Moing et al., 1998). Free amino-acids were analyzed by HPLC using the AccQ.Tag method from Waters (Milford, MA) with fluorescence detection (Moing et al., 1998). Three independent biological replicates have been performed with three technical replicates per biological assay.

Extraction and Measurement of Nodule Adenine Nucleotides

Adenine nucleotides were extracted and measured as in Horchani et al. (2011). Adenine nucleotides were measured in a Xenius spectrofluorimeter-luminometer using the ATPlite one-step assay system (Perkin-Elmer) according to the manufacturer's instructions. Three independent biological replicates have been performed with three technical replicates per biological assay.

β-Glucuronidase Detection In Planta

For β-glucuronidase detection, nodulated roots of composite plants were incubated at −20°C for 1 h in a mixture "acetone: phosphate buffer (Na_2HPO_4/NaH_2PO_4 0.1 M, pH 7.4)", 90%:10% (v/v), then washed twice with the phosphate buffer and incubated 3 h to overnight at room temperature in the dark in phosphate buffer containing potassium ferricyanide (0.5 mM) and X-gluc (0;5 $ng.ml^{-1}$). Nodulated roots or 80-µm-thick vibroslices, obtained with a HM560V Vibratome (Microm, http://www.microm.de) after embedding plant material in 4.5% low-melting-point agarose, were visualized with a Zeiss Axioplan II microscope (Carl Zeiss, http://www.zeiss.com) using dark-field optics. For each transformation experiment, 40 independent roots or nodules were transformed and individually analyzed for β-glucuronidase activity.

Statistical Analyses

Statistical analyses were performed using Student *t*-test or one-way analysis of variance (ANOVA) followed by a Fisher test. Data were considered as significantly different when $p < 0.05$.

RESULTS

NR Gene Expression During the Symbiosis Process

Research in three genomic and protein databases: NCBI (www.blast.ncbi.nlm.nih.gov), Phytozome (www.phytozome.jgi.doe.gov) and Uniprot (www.uniprot.org) revealed that all the plant genomes studied code for one to six NR sequences, with 76% of the species having between one and two NR (**Table S1**). Among the genomes of the Fabales group in **Table S1**, only three species seem to have more than two NR sequences: *Glycine max* and *Glycine soja*, with five and four sequences respectively and *M. truncatula* with three sequences. The three *M. truncatula NR* genes (Puppo et al., 2013; Roux et al., 2014), named Mt*NR1*, Mt*NR2*, and Mt*NR3*, respectively encode proteins of 902, 884, and 876 amino acids (**Figure S1A**). The three *NR* genes exhibit strong similarities (>70%) to each other and exhibit similar structure with four exons and three introns of different sizes (**Figure S1B**). Both Mt*NR1* and Mt*NR3* are closely present in chromosome 3, while Mt*NR2* is present in chromosome 5. To study the phylogenetic relationships operating on the NR sequences, a phylogenetic tree using NR from the Fabales family and different model plants was inferred (**Figure 1**). Forty seven non-redundant protein sequences from 21 species and belonging to the Solanales, Fabales, Brassicales, Malvales, Vitales, and Poales families were used (**Table S1**). The most striking result is the separation of the Fabales sequences into three distinct Hypothetical Taxonomic Units (HTU), named HTU1, HTU2, and HTU3 (**Figure 1**). MtNR2 is in the largest unit HTU1, whereas Mt*NR1* and Mt*NR3* are both in HTU3. For species with more than one sequence, we observe a distribution of these sequences over two HTUs.

As reported in the Affymetrix data (**Figure S2**), both Mt*NR1* and Mt*NR2* are significantly expressed in the roots and nodules, but Mt*NR3* is specifically expressed in the nodules. More precisely, the histochemical detection of GUS activity under the control of *NR* promoters at 4 dpi shows that the three NR genes are expressed in young developing nodules, *i.e.* in the controlled area (a block of cells contituted by pericycle, endodermis and dividing cortical cells, Xiao et al., 2014) of nodule primordium (**Figures 2A, C, E**). In 14 dpi-old nodules, Mt*NR1* expression is detected in the whole nodule (**Figure 2B**). *NR2* expression is also detected in the three zones but also at the periphery of the nodule in the vascular bundle (**Figure 2D**). In the case of Mt*NR3*, GUS staining appears mainly at the level of zones I and II and very slightly in zone III (**Figure 2F**). These results are consistent with Symbimics data (Roux et al., 2014; **Table 1**) which report a localization of the expression of the three NR genes close to that which we observe in 14 dpi-old nodules.

Then, we investigated their expression kinetics throughout the symbiotic process. To this end, we used two types of *M. truncatula* cultures: a short-term culture from 0 to 14 dpi and a long-term culture from 0 to 8 wpi. Both Mt*NR1* and Mt*NR2* are expressed at a significant level in non-inoculated roots (**Figures 3** and **4**). After inoculation with *Sinorhizobium meliloti*, Mt*NR1* expression significantly increases (30- to 45-fold) and exhibits three expression peaks at 10 hpi, 4 dpi, and 5 wpi (**Figures 3A, B**). Similarly, Mt*NR2* expression increases with two peaks at 10 hpi and 4 dpi and then reaches a plateau between 3 and 8 wpi (**Figures 3C, D**). Mt*NR3* expression is detected only from 4 dpi, increases to reach a plateau between 3 and 5 wpi, and then strongly increased (up to 300 times) after 6 wpi at the nodule senescence (**Figures 3E, F**). The expression level of *NR* as compared to each other, before inoculation and at four time-points in the symbiosis, is reported in **Figure 4**. Several features emerged from this analysis. 1) Mt*NR2* is the most highly expressed *NR* gene (30 to 100 times more than *NR1*) throughout the whole process. 2) The three *NR* genes are significantly expressed in N_2-fixing nodules. 3) Whereas the expression of Mt*NR1* decreases at the onset of nodule senescence, the expression of Mt *NR3* clearly increases and exceeds that of Mt*NR1* in 8 wpi nodules. By comparison with the other enzymes potentially involved in the synthesis of NO such as abscissic aldehyde oxidase (AAO), indole aldehyde oxidase (IAO), NO forming nitrite reductase (NOFNiR), sulfite oxidase (SOX), and xanthine dehydrogenase (XDH), it may be noted that the expression of *MtNR1* and *MtNR2* is significantly stronger than that of the other genes (**Figure S2**).

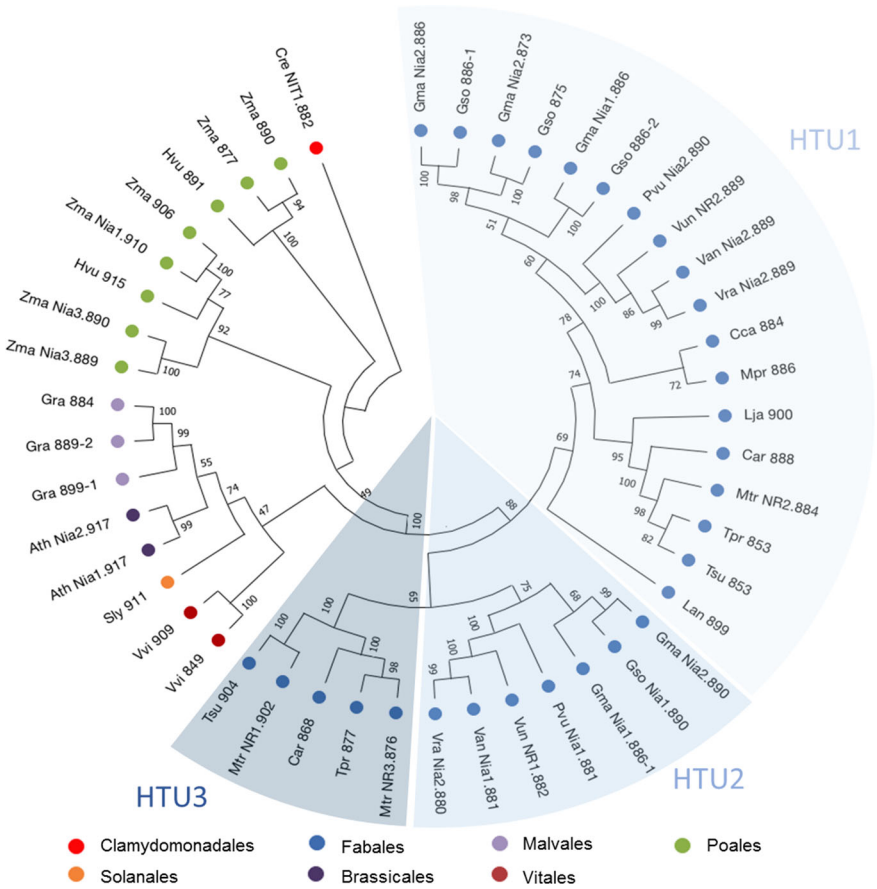

FIGURE 1 | Phylogenetic tree of NR protein sequences. 47 nitrate reductase sequences from model plants or belonging to the Fabaceae family were used in this analysis. The tree was inferred by maximum likelihood (ML) with MegaX (Kumar et al., 2018). Percentages displayed next to each branch represent the number of tree replicates in which the associated taxa were assembled together when performing 1,000 bootstraps. The lengths of the branches do not represent a phylogenetic distance.

NR Activity and NO Level During the Symbiotic Process

To assess the contribution of NR in NO production, the total NR activity was analyzed and compared to the NO production level during the symbiotic process (**Figure 5**). NR activity in non-inoculated roots is close to 4.2 ± 0.9 nmol.min^{-1}.g^{-1}FW. Following inoculation, NR activity exhibits a significant and reproducible 60–70% decrease within 4 hpi before returning close to its initial value at 10 hpi. After a new decrease at 24 hpi, NR activity strongly increases to 26.5 ± 1.1 nmol.min^{-1}.g^{-1}FW at 4 dpi, strongly decreases between 9 and 14 dpi, and then increases again to peak at 3–5 wpi. After the onset of nodule senescence, NR activity decreases to reach 5.1 ± 0.1 nmol.min^{-1}.g^{-1} FW at 8 wpi. As far as it is concerned, NO production level shows three transient production peaks, at 10 hpi, 4 dpi, and 3–4 wpi (**Figures 5** and **S3**; Berger et al., 2020). As a whole, the pattern of NR activity follows that of Mt*NR1* and *2* gene expression (**Figure 3**) and clearly shows a parallel with the production of NO (**Figure 5**). It particularly fits with the expression of Mt*NR1* and Mt*NR2* during the three first weeks of the symbiosis and with that of Mt*NR1* between 3 and 8 wpi, suggesting that NR1 is playing a major role in NO production. To check this hypothesis, a RNAi strategy was used. *M. truncatula* RNAi on the Mt*NR1*, Mt*NR2*, and double Mt*NR1-2* genes were constructed under the control of the zone III-specific promoter NCR001 (Mergaert et al., 2003; Horchani et al., 2011). Four wpi-old nodules were collected and analyzed for NR activity and NO production. As compared to the control, NR activity is decreased by 47 and 56%, respectively, in the RNAi::*NR1* and *NR2* nodules (**Figure 6A**), but the double RNAi::*NR1-2* does not make it possible to further reduce the total NR activity. Regarding NO production, it drops by 45% in RNAi::*NR1* nodules, but only by 18% in RNAi::*NR2* nodules (**Figure 6B**). The decrease in NO production in the nodules of double RNAi::*NR1-2* is of the same order of magnitude as that observed in RNAi::*NR1* nodules. These results clearly show that the decrease in NR activity is accompanied by a fall in NO production and that this fall is particularly related to NR1 activity rather than NR2.

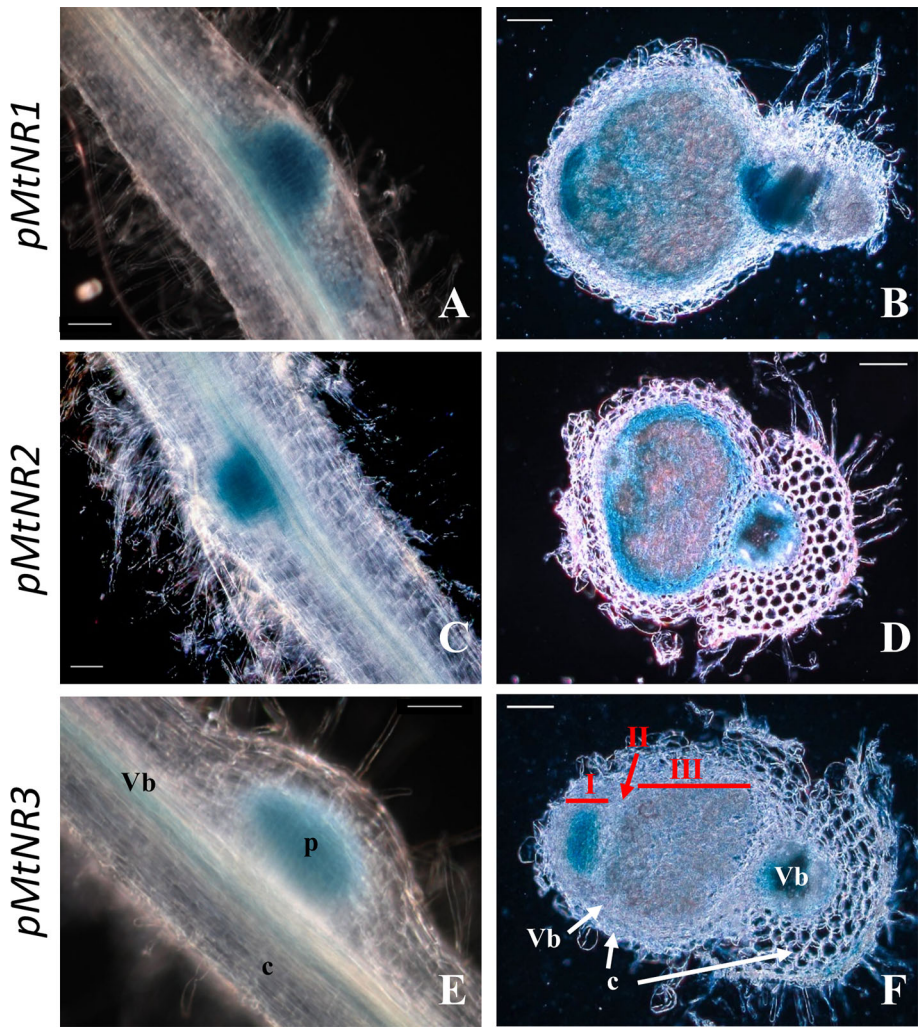

FIGURE 2 | Histochemical localization of *MtNRs* expression in *Medicago truncatula* roots. Localization of GUS activity in transgenic *M. truncatula* roots expressing the gusA reporter gene under the control of a 1.65 Kb *MtNR1* promoter fragment **(A, B)**, of a 1.7 kb *MtNR2* promoter **(C, D)** and of a 1.55 kb *pMtNR3* promoter **(E, F)**. Whole root segment 4 dpi with *S. meliloti* **(A, C, E)** and longitudinal section of a 2 wpi old nodules **(B, D, F)** were stained for 3, 5, or 16 h with X-gluc for the GUS activity for *pMtNR2, pMtNR1*, or *pMtNR3* respectively. Zones I, II, and III of the nodule are represented in red in picture F. p, nodule primordium; Vb, vascular bundles; c, cortex. Scale bars, 50 µm for **(A, C, E)**; 100 µm for **(B, D, F)**.

Involvement of NR Activity in NO Production and Energy Regeneration During Nodule Development

The concomitance of the peaks of the NR expression/activity and those of NO production suggests that NRs are involved in the production of NO. Thus, using an inhibitor approach, we investigated the participation of NR and other potential NO sources such as xanthine dehydrogenase and mitochondrial electron transport chain (ETC) in NO production in 10 hpi and 4 dpi roots and in 4 wpi nodules. As reported in **Figure 7**, NO production is 95% inhibited by KCN, used as a negative control. Allopurinol (AP), a specific inhibitor of xanthine dehydrogenase (XDH), moderately inhibits NO production (by 28%) in 10 hpi roots, whereas it is without effect in 4 dpi roots and 4 wpi nodules. In both 10 hpi and 4 dpi roots, propyl gallate (PG), an inhibitor of the mitochondrial alternative oxidase (AOX), inhibits NO production by 70–90%, indicating that the mitochondria are involved in this reaction. However, PG is ineffective in 4 wpi nodules. It can be noted that PG is also an inhibitor of polyphenol oxidases (Lin et al., 2013), but that the latter are not known to be directly or indirectly involved in the production of NO. The effects of PG on NO production can therefore be attributed to AOX inhibition. Interestingly, tungstate (Tg), a NR inhibitor, inhibits NO production by 88, 92, and 60% respectively in 10 hpi roots, 4 dpi roots, and 4 wpi nodules, and this inhibition is either partially (10 hpi and 4 dpi roots) or totally (4 wpi nodules) relieved by the addition of nitrite, the reaction product of NR (**Figure 7**). These data suggest that NR and ETC are involved in NO production.

To further investigate the potential involvement of NR and NO in energy metabolism, we analyzed the energy state (*i.e.* the

TABLE 1 | Access code and Symbimics expression of *Medicago truncatula* NR and NO-producing enzyme genes.

Genes	Code Affymetrix	Code gene Mt4.0	Code gene Mt5.0	Mt20120830-LIPM	DESEQ MEAN				
					FI	FIID	FIIP	IZ	ZIII
NR1	Mtr.42446.1.S1_at	Medtr3g073180	MtrunA17Chr3g0115151	Mt0006_00730	20.7	89.1	21.3	193.7	693.5
NR2	Mtr.10604.1.S1_at	Medtr5g059820	MtrunA17Chr5g0424491	Mt0008_10301	156.6	166.9	266.7	712.9	843
NR3	Mtr.31448.1.S1_at	Medtr3g073150	MtrunA17Chr3g0115131	Mt0006_00731	1.3	0.5	1.3	0	1.4
XDH1	Mtr.23395.1.S1_at	Medtr2g098030	MtrunA17Chr2g0328851	Mt0016_10367	80	52.3	37.3	39.2	52.7
AAO3	Mtr.29357.1.S1_at	Medtr5g087390	NC	Mt0010_10456	29.05	22.75	10.23	12.61	25.35
IAO3	Mtr.42638.1.S1_at	Medtr5g087410	NC	Mt0010_10457	4.95	9.36	4.05	19.45	62.19
NOFNiR1	Mtr.40060.1.S1_at	Medtr2g035460	MtrunA17Chr2g0296891	Mt0101_10060	43	29.2	4.1	18.8	125.4
NOFNiR2	Mtr.10348.1.S1_at	Medtr2g035470	MtrunA17Chr2g0296881	Mt0101_10060	43	29.2	4.1	18.8	125.4
NOFNiR3	Mtr.33463.1.S1_s_at	Medtr2g035480	MtrunA17Chr2g0296871	Mt0101_10060	43	29.2	4.1	18.8	125.4
SOX1	Mtr.28230.1.S1_at	Medtr6g023975	MtrunA17Chr6g0459641	Mt0033_10312	53	55.7	60.2	137.4	121.9
SOX2	NC	Medtr7g033410	MtrunA17Chr7g0227151	Mt0044_10132	3.3	1.5	2.6	4.6	9.2

Affimetrix gene codes were on (https://mtgea.noble.org/v3/), Mt4.0 gene codes were on (https://www.jcvi.org/medicago-truncatula-genome-database), Mt5.0 gene codes were on (https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/), Symbimics data were in Roux et al. (2014). NC, no code available; DESEQ MEAN corresponds to the expression value of the RNA-sequencing analysis in the different zones of the nodule; FI, meristematic zone; FIID, distal infection zone; FIIP, proximal infection zone; IZ, interzone; ZIII, N_2-fixing zone. Shades of red highlight the highest expression value for each gene. NR, nitrate reductase; XDH, xanthine dehydrogenase; AAO, abscissic aldehyde oxidase; IAO, indole aldehyde oxidase; NOFNiR, NO forming nitrite reductase; SOX, sulfite oxidase.

ATP/ADP ratio) in 10 hpi and 4 dpi roots and in 4 wpi nodules after a 4 h treatment in the presence of NR effectors (**Table 2**). In control and NO_2^--treated roots and nodules, ATP/ADP ratio is high, indicating that ATP-regenerating processes are not limited. In the presence of Tg, the ATP/ADP ratio is significantly decreased only in 4 wpi nodules, indicating that the inhibition of NR partially affects the energy state in mature nodules, but not in 10 hpi and 4 dpi roots. This decrease in the ATP/ADP ratio is not observed when 4 wpi nodules are incubated in the presence of both Tg and NO_2^-, which means that the supply of NO_2^- makes it possible to maintain the nodule energy state.

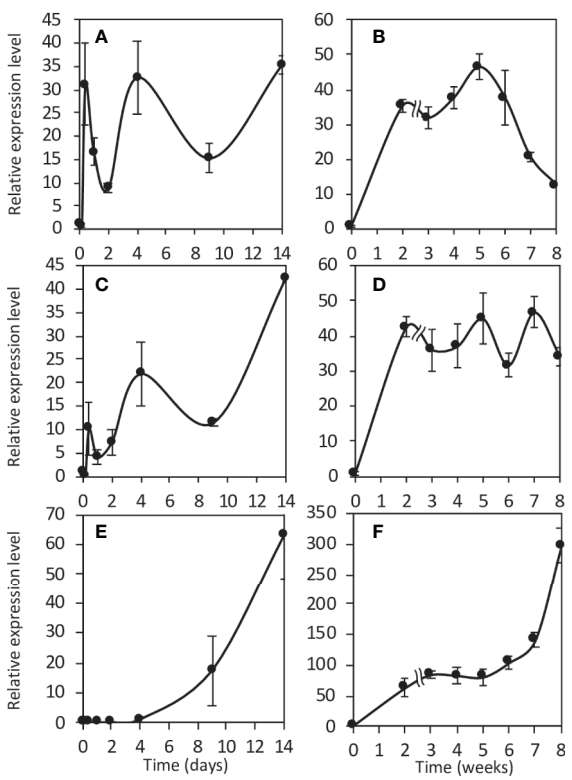

FIGURE 3 | Expression of *Medicago truncatula NR* genes during the symbiotic process. Expression analysis of the NR genes in roots and nodules during the symbiotic process. Short term kinetic 14 dpi **(A, C, E)**, long term kinetic 8 wpi **(B, D, F)**. Expression of NR1 **(A, B)**, NR2 **(C, D)** and NR3 **(E, F)**. Data are means ± SE of three biological replicates. Each measure was realized in three technical replicates. dpi, day post inoculation; wpi, week post inoculation.

Considered together, these results indicate that 1) both the NR and the mitochondrial ETC are involved in the production of NO, probably *via* the reduction of nitrate to nitrite by NR and the subsequent reduction of nitrite to NO at the mitochondrial ETC level, and 2) NR activity is linked to energy regeneration processes in mature nodules, but not during the first steps of the symbiosis.

Involvement of NR in Carbon and Nitrogen Metabolism in Mature Nodules

Based on the above results, it appeared important to investigate more precisely the role of NR activity in the energetic metabolism of mature nodules. To this end, using a perifusion system adapted to NMR spectrometer (Roby et al., 1987), we first followed the effects of *in vivo* transition from normoxia to hypoxia, and *vice versa*, on the energy metabolism of 4 wpi nodules. **Figure 8A** displays typical ^{31}P spectra obtained with living *M. truncatula* nodules during normoxia–hypoxia–normoxia transition experiments. The main resonance peaks are attributed to glucose-6-phosphate (G6P), cytosolic and vacuolar inorganic phosphate (Pi-cyt and Pi-vac, respectively), uridine di-phospho-glucose (UDPG), and to α, β and γ ATP. Transition from 21% O_2 (normoxia) to 1% O_2 (hypoxia) leads to an important reduction in ATP content and a significant acidification of cytoplasmic pH from 7.45 to 6.95 after 7 h of hypoxia, as measured by the shift of G6P and Pi-cyt resonances (**Figure 8A**). Back transition from 1 to 21% O_2 is accompanied by an increase in ATP and a progressive return of cytoplasmic pH to more alkaline values (7.2). In a second series of experiments, nodules were incubated at 21% O_2 in the

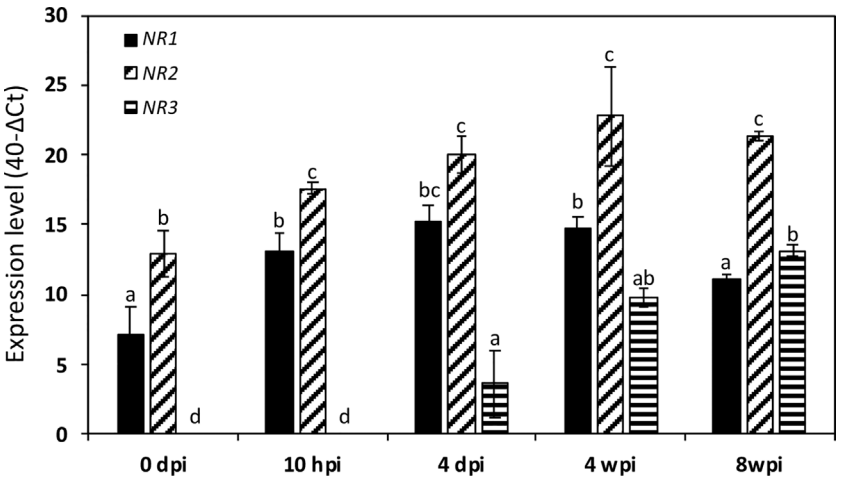

FIGURE 4 | Expression of *Medicago truncatula NR* genes at various times of the symbiotic process. The data are drawn from the same experiments as those presented in **Figure 3**. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test (P<0.05). dpi, day post inoculation; hpi, hour post inoculation; wpi, week post inoculation.

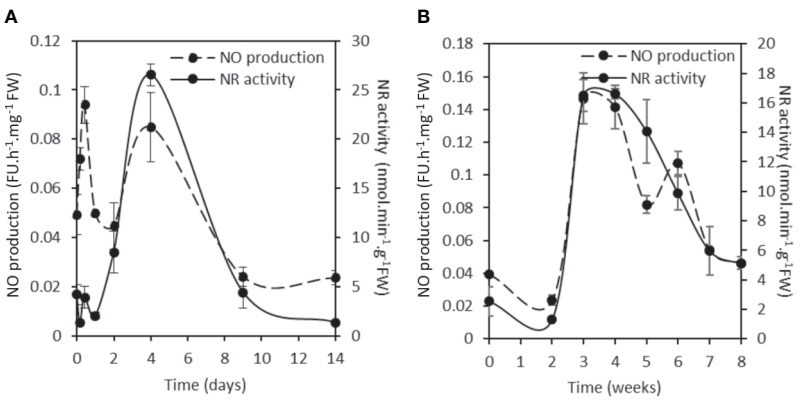

FIGURE 5 | NR activity and NO production during the *Medicago truncatula* symbiosis. Short term kinetic 14 dpi **(A)**, long term kinetic 8 wpi **(B)**. The nitrate reductase activity is expressed in nmol per min per g of fresh weight. The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. Data are means ± SE of three biological replicates. Each measure was realized in three technical replicates. dpi, day post inoculation; FU, fluorescence unit; wpi, week post inoculation.

presence of 1 mM Tg (**Figure 8B**). After 5 h of Tg treatment, ATP level decreases by a factor 2.5, and cytoplasmic pH decreases from 7.4 to 6.95, indicating a decrease in energy state and an acidification of the nodules. The addition of 1 mM NO_2^- triggers an increase in cytosolic pH to 7.15, indicating a progressive recovery of the cell metabolism (**Figure 8B**). Thus, the inhibition of NR by Tg and its subsequent relief by NO_2^- partially mimicks "normoxia–hypoxia–normoxia" transitions and support the involvement of NR in the energy metabolism of nodules.

Then, we investigated the potential involvement of NR in the N_2-fixing metabolism. To this end, mature nodules were incubated for 4 h in either the presence, or the absence, of 1 mM Tg, 10 mM NO_3^- and 1 mM NO_2^-. As reported in **Figure 9**, the nitrogenase activity, as measured by its acetylene reducing activity (ARA), is inhibited in the presence of either NO_3^- or Tg (or both) but is unaffected by the presence of NO_2^-, indicating that NR activity supports N_2 fixation. The changes in the content of various metabolites related to carbon and nitrogen metabolism of the nodules are consistent with this hypothesis. Thus, following NR inhibition by Tg (either in the presence or absence of NO_3^-), sucrose content increased, indicating a slowdown of its consumption by the nodules (**Figure 9B**). As a consequence, succinate and malate contents decreased (**Figures 9C, D**), indicating a lower supply of carbon nutrients to bacteroids, and asparagine content decreased (**Figure 9E**) as a result of the shortage of carbon substrate supply and ARA inhibition. Finally, alanine, which is a marker of hypoxia in

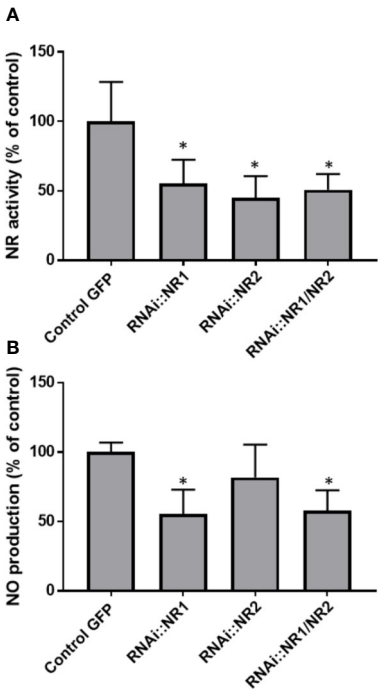

FIGURE 6 | NR activity and NO production in NR RNAi transformed *Medicago truncatula* nodules. **(A),** NR activity is expressed in nmol per min per g of fresh weight. **(B)**, The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. Data are means ± SE of three biological replicates. Each measure was realized in three technical replicates. Asteriks * indicate statistical difference when compared with the control GFP (transgenic roots transformed with empty pK7GWIWG5D(II) vector selected on GFP marker) at $P < 0.05$ according to Student's *t* test. FU, fluorescence unit.

plant tissues (Gibbs and Greenway, 2003), was found to increase in the presence of Tg (**Figure 9F**), indicating that NR inhibition mimics a hypoxia situation. The presence of NO_2^- reverses the effects of Tg (**Figure 9**).

DISCUSSION

Medicago truncatula NRs Are Involved in NO Production

The main objective of this study was to test the hypothesis that NR regulates NO production and controls N_2-fixing metabolism during the *M. truncatula–S. meliloti* symbiosis.

The Medicago genome encodes three *NR* genes, Mt*NR1*, Mt*NR2*, and Mt*NR3* (Puppo et al., 2013; Roux et al., 2014). Whereas most of the species analyzed possess between one and two sequences of NR, *M. truncatula* have three NRs. Mt*N3* and Mt*NR1* are both located on chromosome 3 separate from only 20 kb and are grouped in the same HTU on the phylogenetic tree, elements which are in favor of an event of duplication of Mt*NR1* to give birth to Mt*NR3*. This exhaustive search for NR sequences conducted to identify two potential orthologs to Mt*NR3* in species closed to *M. truncatula* such as *Trifolium pratense* and *Cicer arietanum*. However, we did not identify three NR sequences in *T. pratense* and *C. arietanum*. Mt*NR3* has been shown to be specifically expressed in symbiotic nodules, excluding other organs of the plant (http://mtgea.noble.org/v3/). Its expression from 4 dpi (**Figures 3** and **4**) and location in the nodule primordium as well as in zones I and II of the nodule (**Figure 2**, **Table 1**) suggest that MtNR3 plays a role in cell division and infection processes during nodule organogenesis. Similarly, the sharp increase in its expression beyond 6 wpi (**Figure 3F**) suggests that NR3 also plays an important role in nodule senescence process that will be worth investigating. However, its low expression level during symbiosis (**Figure 4**) and its expression pattern, very different from the NO production once (**Figures 3** and **5**), do not support, *a priori*, a potential involvement of NR3 in the production of NO, pointing to a specific role of this NR in nodule aging.

Mt*NR1* and Mt*NR2* correspond to inducible and/or constitutive forms that have been found in other higher plants such as *A. thaliana* and *G. max* (Santucci et al., 1995). Our results show that three concomitant peaks of Mt*NR1*/*NR2* expression and NR activity can be considered over the period analyzed (**Figures 3–5**): i) during the first hours of the symbiotic interaction at10 hpi; ii) during the early development of the nodule at 4 dpi; and iii) when the nodule becomes mature around 3–4 wpi. The most salient feature of this study is the parallel that can be drawn between the expression of Mt*NR1* and Mt*NR2* (**Figure 3**), the total NR activity (**Figure 5**), and the production of NO (**Figure 5**). During the infection phase, at 10 hpi, the increase in Mt*NR1* and Mt*NR2* expression is accompanied by a slight increase in NR activity, while the production of NO increases by a factor of 2, suggesting that NRs are probably not the only sources of NO. In contrast, later in the symbiotic process (at 4 dpi and 3–4 wpi), the correlation between gene expression, NR activity, and NO production suggests that NO production is directly related to NR. Moreover, the spatiotemporal expression of Mt*NR1* and Mt*NR2* (**Figure 2**) corresponds to the localization of NO production reported to occur in the nodule primordium (del Giudice et al., 2011) and in the N_2-fixing zone of the mature nodule (Baudouin et al., 2006). While keeping in mind that Mt*NR2* is much more expressed than Mt*NR1*, these observations strongly suggest that both NR1 and NR2 are involved in the production of NO during the symbiotic process. In a recent study dealing with the role of *M. truncatula* phytoglobins (Phytogbs) during N_2-fixing symbiosis we found that *Phytogb1.1* exhibits an expression pattern similar to that of NO production and is involved in its regulation during the different stages of the symbiosis (Berger et al., 2020). Thus, regardless of the potential mechanisms of post-translational regulation of Phytogbs and NRs activities, our results indicate that NRs probably work in coordination with Phytogb1.1 to regulate the level of NO at each stage of the symbiotic process.

The use of inhibitors of different NO sources (**Figure 7**) supports the hypothesis that at 10 hpi, 4 dpi, and 4 wpi, NRs are either directly, or indirectly, in combination with the mitochondrial ETC, involved in the production of NO. In fact,

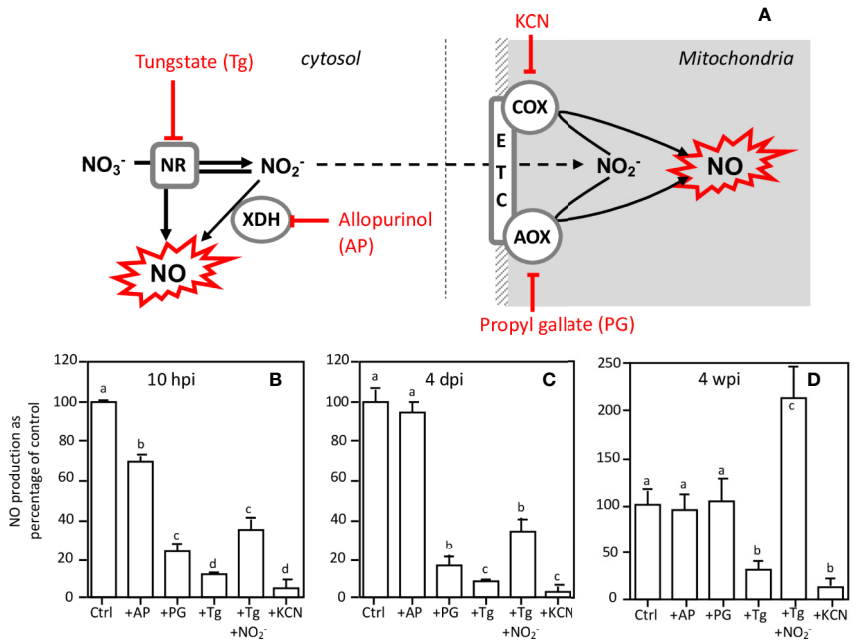

FIGURE 7 | Effects of inhibitors on NO production at various times of the *Medicago truncatula* symbiosis. **(A)**, schematic representation of inhibitor targets. NO production of 10 hpi-old **(B)** and 4 dpi-old **(C)** roots and of 4 wpi-old nodules **(D)** was measured after 4 h in the presence of either 1 mM tungstate (Tg), 1 mM allopurinol (AP), 1 mM propyl gallate (PG), 1 mM nitrite (NO_2^-), or 300 μM KCN. NO production is expressed as the percentage of the control values. Data are means ± SE of three biological replicates. Each measure was realized in three technical replicates. Values followed by different letters are significantly different according to one-way ANOVA analysis followed by a Fisher test ($P < 0.05$). dpi, day post inoculation; hpi, hour post inoculation; wpi, week post inoculation. AOX, alternative oxidase; COX, cytochrome oxidase; ETC, electron transfer chain; NR, nitrate reductase; XDH, xanthine dehydrogenase.

TABLE 2 | Effects of NR effectors on ATP/ADP ratio in *Medicago truncatula* roots and nodules.

	ATP/ADP ratio		
	10 hpi	**4 dpi**	**4 wpi**
Control	6.1 ± 1.1	6.3 ± 1.7	6.8 ± 1.2
+1 mM NO_2^-	5.7 ± 0.9	4.7 ± 0.4	6.4 ± 0.7
+1 mM Tg	5.2 ± 0.5	5.6 ± 3.3	4.4 ± 0.4 *
+1 mM NO_2^- +1 mM Tg	4.9 ± 0.6	4.5 ± 1.0	5.5 ± 0.7

*Either 10 hpi roots, 4 dpi roots, or 4 wpi-old nodules were incubated for 4 h in the presence of either 1 mM nitrite (NO_2^-), 1 mM tungstate (Tg), or both. Data are the means ± SE of three (10 hpi, 4 dpi) and four (4 wpi) biological replicates. Each measure was realized in three technical replicates. Asterisk * indicates statistical difference with the control at the same time point at $P < 0.05$ according to Student's t test. dpi, day post inoculation; hpi, hour post inoculation; wpi, week post inoculation.*

at these three stages the production of NO is inhibited by Tg. The use of Tg to inhibit NR activity has been successfully used in mature *M. truncatula* nodules to demonstrate the involvement of NR in NO production (Horchani et al., 2011). However, Tg is known to inhibit other molybdoenzymes than NR, including XDH, aldehyde oxidase, sulfite oxidase, and NO forming nitrite reductase (NOFNiR) (Mendel and Hänsch, 2002; Xiong et al., 2012, Chamizo-Ampudia et al., 2016), and the possibility that the Tg-dependent inhibition of NO production is linked to the inhibition of one of these other enzymes cannot be excluded. Notwithstanding the stability and the specific activity of these enzymes in the root and nodule cells, the weak expression level of their genes remains much lower than those of Mt*NR1* and Mt*NR2* (**Table 1**, **Figure S2**), and one can reasonably think that NRs remain the major molybdoenzymes in cells. The use of the inhibitor allopurinol (**Figure 7**) shows that XDH, of which expression remains significant during the symbiotic process (**Table 1**, **Figure S2**), is perhaps partially involved in the production of NO during the first hours of symbiosis, but neither during nodule organogenesis nor in the N_2-fixing nodule. Furthermore, the preferential expression of XDH in the infection zone of the nodules (**Table 1**) reinforces the hypothesis of its involvement in the defense responses which occur at the beginning of the symbiotic process. XDH is a peroxisomal enzyme capable of producing $NO^{\bullet}$ and superoxide anion ($O_2^{\bullet -}$) that can complex together to give peroxynitrite, $ONOO^-$ (del Río et al., 2004). $ONOO^-$ is known to play a key role in the induction of defense responses, particularly *via* tyrosine nitration of proteins (Saito et al., 2006), which is consistent with the involvement of NO production in defense responses at 10 hpi.

Otherwise, the partial or total reversion of Tg-dependent inhibition of NO production by nitrite (**Figure 7**) means that NRs at least produce the nitrite necessary for the production of NO. Indeed, the inhibition of NO production by mitochondrial ETC inhibitors (**Figure 7**) supports the hypothesis that NR

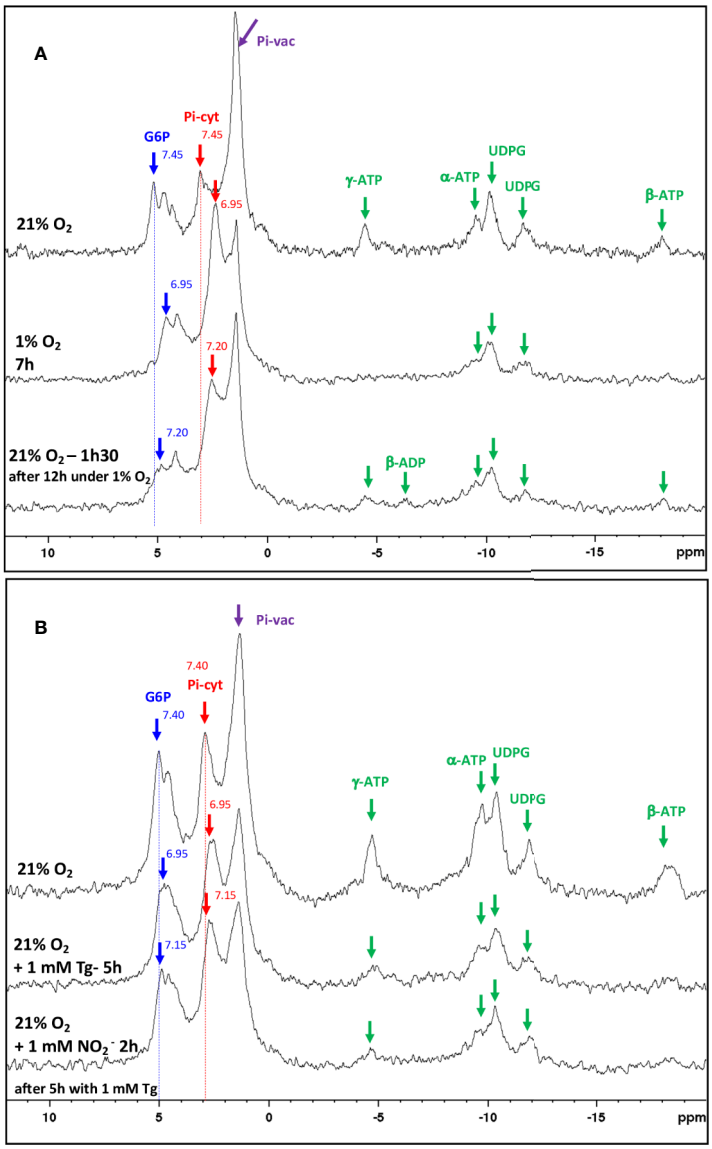

FIGURE 8 | *In vivo* ^{31}P-NMR study of metabolic transitions induced in *Medicago truncatula* nodules. **(A)** Proton-decoupled ^{31}P-NMR spectra of nodules in normoxia at 21°C, perifused with a nutritive medium at pH 6.0 with successively 21% O_2 (control), 1% O_2, and then 21% O_2. For each line, duration of the treatment is indicated up to the end of the spectrum. **(B)** Proton-decoupled ^{31}P-NMR spectra of nodules in normoxia at 21°C, perifused with a nutritive medium at pH 6.0 containing successively 21% O_2 (control), 1 mM tungstate (Tg) for 5 h, and then 1 mM nitrite (NO_2^-) for 2 h. For each line, duration of the treatment is indicated up to the end of the spectrum. Each spectrum series is drawn from a representative experiment of eight **(A)** and five **(B)** biological replicates. The values indicated next to the G6P and Pi-cyt resonances correspond to the cytosolic pH values. Exponential apodization and zero filling. Pi, inorganic phosphate; G6P, Glucose-6-phosphate; Pi-cyt, cytoplasmic Pi; Pi-vac, vacuolar Pi; UDPG, UDP-glucose.

activity is indirectly involved in NO production *via* the reduction of nitrate to nitrite and the subsequent nitrite reduction to NO by mitochondrial ETC. This indicates that from the beginning of the symbiotic process, a major part of NO is produced *via* a nitrite reducing pathway.

We should also keep in mind that NO in the nodule is not produced only by the plant partner. Indeed, the bacterial partner was shown to produce from 33 to 90% of NO in *M. truncatula* (Horchani et al., 2011) and soybean (Sanchez et al., 2010) nodules, respectively. If the bacterial denitrification pathway, including the periplasmic nitrate reductase (Nap) and nitrite reductase (Nir), has been described as the main enzymatic source of NO, additional genes encoding putative nitrate and nitrite reductase (called narB and NirN, respectively) have been recently identified that could also participate indirectly in NO synthesis (Ruiz et al., 2019). How the regulatory systems of the plant and the bacterial partners are coordinated to produce NO is one of the main issues to decipher the signaling and metabolic functions of NO at each stage of the symbiotic interaction.

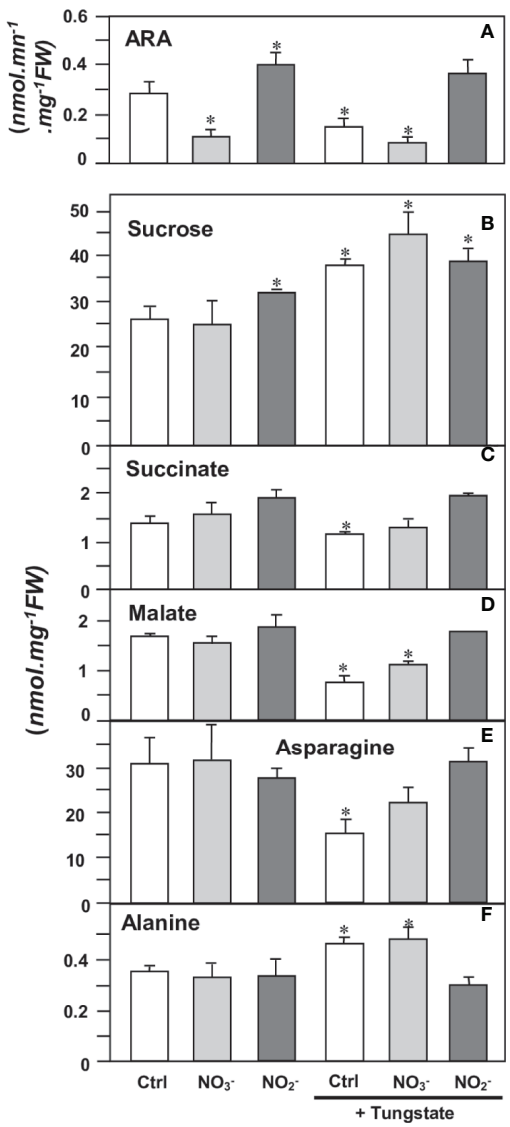

FIGURE 9 | Effects of NR effectors on nitrogenase activity and metabolite contents in *Medicago truncatula* nodules. 4 wpi-old nodules were incubated for 4 h in the presence of either 10 mM nitrate (NO_3^-), 1 mM nitrite (NO_2^-), or 1 mM tungstate (Tg). **(A)**, nitrogenase activity (estimated as ARA); **(B)**, sucrose; **(C)**, succinate; **(D)**, malate; **(E)**, asparagine; **(F)**, alanine. Data are means ± SE of three biological replicates. Each measure was realized in three technical replicates. Asterisks * indicate significant difference at $P<0.05$, when compared with the control (Ctrl) according to Student's *t* test.

NRs Regulate Energy State and Metabolism in Nitrogen-Fixing Nodules

The combined functioning of NR and mitochondrial ETC to produce NO has been associated with "Phytogb-NO" respiration and energy regeneration in hypoxic organs (Igamberdiev and Hill, 2009), and the role of NR in maintaining the ATP/ADP ratio has already been demonstrated in microoxic N_2-fixing nodules of *M. truncatula* (Horchani et al., 2011). Our results (**Table 2**) show that, contrary to what is observed in 4 wpi-old nodules, the inhibition of NRs at the beginning of the symbiotic process (10 hpi and 4 dpi) does not cause a decrease in the ATP/ADP ratio indicating that, at these stages, energy regeneration is not linked to the functioning of Phytogb-NO respiration. These observations were expected since, contrary to mature nodules, roots are normoxic organs, and the cellular energy is supposed to be regenerated by the O_2-dependent mitochondrial respiration. The role of mitochondrial ETC in NO production during the early days of the symbiotic process remains to be clarified.

The role of NR in energy, carbon, and nitrogen metabolism was investigated by using the NR inhibitor Tg. *In vivo* ^{31}P-NMR experiments (**Figure 8A**) showed that hypoxia leads to a fall in ATP and to the acidification of cytoplasmic pH inside the nodules. These data are consistent with earlier observations with maize root tips (Roberts et al., 1984), soybean nodules (Pfeffer et al., 1992) and sycamore maple cells (Gout et al., 2001). In the presence of Tg, the fall in ATP and the cytoplasm acidification resulting from the inhibition of NR and their partial reversion by the addition of nitrite (**Figure 8B**) confirm that NR activity is fully involved in the energy metabolism of nodules *via* "Phytogb-NO" respiration, as already observed by Horchani et al. (2011). Logically, the inhibition of energy metabolism triggers the inhibition of the carbon and nitrogen metabolism in nodules, with an accumulation of sucrose, the subsequent decrease in the supply of carbon substrates to bacteroids (succinate, malate), and ultimately, the decrease in nitrogen reduction (ARA) and assimilation (asparagine) activities (**Figure 9**). It is interesting to note that treatment of nodules with Tg leads to an increase in alanine content (**Figure 9F**). In hypoxic/anoxic conditions, alanine accumulates in tissues following the inhibition of O_2-dependent respiration and the induction of alanine aminotransferase (Limami et al., 2008, and references inside), and to date alanine is considered as one of the most prevalent and ubiquitous markers of hypoxia in plants (Gibbs and Greenway, 2003; Bailey-Serres and Voesenek, 2008; Limami et al., 2014). The accumulation of alanine, as well as the drop in the energy state (**Table 2**), is the demonstration that the inhibition of NR activity leads to the inhibition of the mitochondrial respiratory chain, the tricarboxylic acid cycle, and ultimately the carbon and nitrogen metabolism in the same way as the drop in O_2 content in hypoxic tissue.

This conclusion is reinforced by the results obtained in the presence of nitrite. Interestingly, our study shows that over a short period of time, *i.e.* 4 h, nitrite stimulates ARA, while nitrate inhibits it (**Figure 9**). The inhibitory effect of nitrate on nitrogen fixation is widely recognized, and the question arose as to whether this effect was potentially linked to nitrite. Indeed, nitrite is known to be a potent inhibitor of nitrogenase *in vitro* (Wong, 1980; Trinchant and Rigaud, 1982). However, several studies have shown that nitrite is not responsible for the *in vivo* inhibition of nitrogenase activity induced by nitrate (Streeter, 1985a; Streeter, 1985b; Becana et al., 1989; Arrese-Igor et al., 1997; Arrese-Igor et al., 1998). The present study confirms this point and shows that over a short period of time, nitrite not only

increases ARA, but even stimulates the energy, carbon, and nitrogen metabolism of nodules (**Figures 7–9**, **Table 2**). In a previous *in vivo* ^{31}P-NMR study carried out on anoxic maize roots, Libourel et al. (2006) reported that the addition of nitrite makes it possible to limit the drop in cytoplasmic pH following anoxic treatment. Here, we show that 1) similarly to hypoxia, NR inhibition induces a drop in cytoplasmic pH, and 2) this drop in cytoplasmic pH may be reversed by the addition of nitrite (**Figure 8**), confirming the key role of NR and PNR, in the functioning of microoxic nodules.

Considered together, our data first confirm that NR is a source of nitrite, and indirectly of NO, during the establishment and functioning of N_2-fixing symbiosis. Second, they support the hypothesis that NRs, in close collaboration with Phytogb1.1 (Berger et al., 2020), are strongly involved in the regulation of cellular energy and N_2-fixing metabolism through the functioning of the Phytogb-NO respiration. Finally, they show that NR activity is needed for the N_2-fixing symbiosis and constitutes a first attempt to explain its dual role in nodule functioning: 1) generating NO as a signal for gene regulation and metabolic adaptation, and 2) contributing to the energy supply under the hypoxic conditions prevailing inside the nodule.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

ABe, ABo, DR, AP, and RB planned and designed the research. ABe, ABo, MM, MH, NH, SB, DR, and RB performed the experiments. ABe, ABo, DR, AP, and RB analyzed and interpreted the data. ABe, ABo, and RB wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by the "Institut National de la Recherche en Agronomie", the "Centre National de la Recherche Scientifique", the Côte d'Azur University, and the French Government (National Research Agency, ANR) through the LABEX SIGNALIFE program (reference # ABR-11-LABX-0028-01) and the STAYPINK project (ANR-15-CE20-0005). NMR and HPLC analyses were performed at the Bordeaux Metabolome Facility that is partly supported by the national infrastructure for metabolomics and fluxomics MetaboHUB (ANR-11-INBS-0010) grant. Some of the results presented in this manuscript are part of the PhD thesis of ABe (Berger, 2019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01313/full#supplementary-material

REFERENCES

Arrese-Igor, C., García-Plazaola, J.II, Hernández, A., and Aparicio-Tejo, P. M. (1990). Effect of low nitrate supply to nodulated lucerne on time course of activites of enzymes involved in inorganic nitrogen metabolism. *Physiol. Plant* 80, 185–190. doi: 10.1111/j.1399-3054.1990.tb04394.x

Arrese-Igor, C., Minchin, F. R., Gordon, A. J., and Nath, A. K. (1997). Possible causes of the physiological decline in soybean nitrogen fixation in the presence of nitrate. *J. Exp. Bot.* 48, 905–913. doi: 10.1093/jxb/48.4.905

Arrese-Igor, C., Gordon, A. J., Minchin, F. R., and Denison, R. F. (1998). Nitrate entry and nitrite formation in the infected region of soybean nodules. *J. Exp. Bot.* 49, 41–48. doi: 10.1093/jxb/49.318.41

Bailey-Serres, J., and Voesenek, L. A. C. J. (2008). Flooding stress: acclimations and genetic diversity. *Annu. Rev. Plant Biol.* 59, 313–339. doi: 10.1146/annurev.arplant.59.032607.092752

Bari, R., Pant, B. D., Stitt, M., and Scheible, W.-R. (2006). PHO2, MicroRNA399, and PHR1 Define a Phosphate-Signaling Pathway in Plants. *Plant Physiol.* 141, 988–999. doi: 10.1104/pp.106.079707

Baudouin, E., Pieuchot, L., Engler, G., Pauly, N., and Puppo, A. (2006). Nitric oxide is formed in Medicago truncatula-Sinorhizobium meliloti functional nodules. *Mol. Plant-Microbe Interact.* 19, 970–975. doi: 10.1094/MPMI-19-0970

Becana, M., Minchin, F. R., and Sprent, J. II (1989). Short-term inhibition of legume N2 fixation by nitrate. *Planta* 180, 40–45. doi: 10.1007/BF02411408

Becana, M., Yruela, I., Sarath, G., Catalán, P., and Hargrove, M. S. (2020). Plant hemoglobins: a journey from unicellular green algae to vascular plants. *New Phytol.* 227, 1618–1635. doi: 10.1111/nph.16444

Berger, A., Boscari, A., Frendo, P., and Brouquisse, R. (2019). Nitric oxide signaling, metabolism and toxicity in nitrogen-fixing symbiosis. *J. Exp. Bot.* 70, 4505–4520. doi: 10.1093/jxb/erz159

Berger, A., Guinand, S., Boscari, A., Puppo, A., and Brouquisse, R. (2020). Medicago truncatula Phytoglobin 1.1 controls symbiotic nodulation and nitrogen fixation via the regulation of nitric oxide level. *New Phytol.* 227, 84–98. doi: 10.1111/nph.16462

Berger, A. (2019). *Contribution des phytoglobines et des nitrate réductase à la règulation de l''oxyde nitrique et de la fixation de l''azote dans la symbiose Medicago truncatula / Sinorhizobium meliloti. PhD thesis* (Nice (France): Université Côte d'Azur).

Besson-Bard, A., Pugin, A., and Wendehenne, D. (2008). New insights into nitric oxide signaling in plants. *Annu. Rev. Plant Biol.* 59, 21–39. doi: 10.1146/annurev.arplant.59.032607.092830

Bobik, C., Meilhoc, E., and Batut, J. (2006). FixJ: A major regulator of the oxygen limitation response and late symbiotic functions of Sinorhizobium meliloti. *J. Bacteriol.* 188, 4890–4902. doi: 10.1128/JB.00251-06

Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C., and Barker, D. G. (2001). Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant Microbe Interact.* 14, 695–700. doi: 10.1094/MPMI.2001.14.6.695

Boscari, A., del Giudice, J., Ferrarini, A., Venturini, L., Zaffini, A.-L., Delledonne, M., et al. (2013). Expression Dynamics of the Medicago truncatula Transcriptome during the Symbiotic Interaction with Sinorhizobium meliloti: Which Role for Nitric Oxide? *Plant Physiol.* 161, 425–439. doi: 10.1104/pp.112.208538

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Brouquisse, R., James, F., Raymond, P., and Pradet, A. (1991). Study of glucose starvation in excised maize root tips. *Plant Physiol.* 96, 619 LP – 626. doi: 10.1104/pp.96.2.619

Cam, Y., Pierre, O., Boncompagni, E., Hérouart, D., Meilhoc, E., and Bruand, C. (2012). Nitric oxide (NO): A key player in the senescence of Medicago truncatula root nodules. *New Phytol.* 196, 548–560. doi: 10.1111/j.1469-8137.2012.04282.x

Campbell, W. H. (1999). Nitrate reductase structure, function and regulation: Bridging the Gap between Biochemistry and Physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 277–303. doi: 10.1146/annurev.arplant.50.1.277

Castella, C., Mirtziou, I., Seassau, A., Boscari, A., Montrichard, F., Papadopoulou, K., et al. (2017). Post-translational modifications of Medicago truncatula glutathione peroxidase 1 induced by nitric oxide. *Nitric. Oxide* 68, 125–136. doi: 10.1016/j.niox.2017.02.004

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, Á, Ocaña-Calahorro, F., Mariscal, V., Carreras, A., et al. (2016). A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in Chlamydomonas. *Plant Cell Environ.* 39, 2097–2107. doi: 10.1111/pce.12739

Chamizo-Ampudia, A., Sanz-Luque, E., Llamas, A., Galvan, A., and Fernandez, E. (2017). Nitrate Reductase Regulates Plant Nitric Oxide Homeostasis. *Trends Plant Sci.* 22, 163–174. doi: 10.1016/j.tplants.2016.12.001

Dean, J. V., and Harper, J. E. (1988). The Conversion of Nitrite to Nitrogen Oxide(s) by the Constitutive NAD(P)H-Nitrate Reductase Enzyme from Soybean. *Plant Physiol.* 88, 389 LP – 395. doi: 10.1104/pp.88.2.389

del Giudice, J., Cam, Y., Damiani, I., Fung-Chat, F., Meilhoc, E., Bruand, C., et al. (2011). Nitric oxide is required for an optimal establishment of the medicago truncatula-sinorhizobium meliloti symbiosis. *New Phytol.* 191, 405–417. doi: 10.1111/j.1469-8137.2011.03693.x

del Río, L. A., Javier Corpas, F., and Barroso, J. B. (2004). Nitric oxide and nitric oxide synthase activity in plants. *Phytochemistry* 65, 783–792. doi: 10.1016/j.phytochem.2004.02.001

Ferrarini, A., De Stefano, M., Baudouin, E., Pucciariello, C., Polverari, A., Puppo, A., et al. (2008). Expression of Medicago truncatula genes responsive to nitric oxide in pathogenic and symbiotic conditions. *Mol. Plant Microbe Interact.* 21, 781–790. doi: 10.1094/MPMI-21-6-0781

Fukudome, M., Calvo-Begueria, L., Kado, T., Osuki, K.-I., Rubio, M. C., Murakami, E.-I., et al. (2016). Hemoglobin LjGlb1-1 is involved in nodulation and regulates the level of nitric oxide in the Lotus japonicus-Mesorhizobium loti symbiosis. *J. Exp. Bot.* 67, 5275–5283. doi: 10.1093/jxb/erw290

Gibbs, J., and Greenway, H. (2003). Review: Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Funct. Plant Biol.* 30, 1–47. doi: 10.1071/PP98095

Gout, E., Boisson, A.-M., Aubert, S., Douce, R., and Bligny, R. (2001). Origin of the Cytoplasmic pH Changes during Anaerobic Stress in Higher Plant Cells. Carbon-13 and Phosphorous-31 Nuclear Magnetic Resonance Studies. *Plant Physiol.* 125, 912 LP – 925. doi: 10.1104/pp.125.2.912

Gupta, K. J., Stoimenova, M., and Kaiser, W. M. (2005). In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. *J. Exp. Bot.* 56, 2601–2609. doi: 10.1093/jxb/eri252

Hancock, J. T. (2019). Considerations of the importance of redox state for reactive nitrogen species action. *J. Exp. Bot.* 70, 4323–4331. doi: 10.1093/jxb/erz067

Hardy, R. W., Holsten, R. D., Jackson, E. K., and Burns, R. C. (1968). The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. *Plant Physiol.* 43, 1185–1207. doi: 10.1104/pp.43.8.1185

Hichri, I., Boscari, A., Castella, C., Rovere, M., Puppo, A., and Brouquisse, R. (2015). Nitric oxide: A multifaceted regulator of the nitrogen-fixing symbiosis. *J. Exp. Bot.* 66, 2877–2887. doi: 10.1093/jxb/erv051

Hichri, I., Boscari, A., Meilhoc, E., Catalá, M., Barreno, E., Bruand, C., et al. (2016). "Nitric oxide: a multitask player in plant-microorganism symbioses," in *Gasotransmitters in Plants: The Rise of a New Paradigm in Cell Signaling*. Eds. L. Lamattina and C. Garcia-Mata (Switzerland: Springer International Publishing), 239–268. doi: 10.1007/978-3-319-40713-5_12

Hilliou, F., and Tran, T. (2013). RqPCRAnalysis: Analysis of quantitative real-time PCR data. *Bioinforma. 2013 - Proc. Int. Conf. Bioinforma. Model. Methods Algorithms*. (Science and Technology Publications, Lda), 202–211. doi: 10.5220/0004312002020211

Horchani, F., Prévot, M., Boscari, A., Evangelisti, E., Meilhoc, E., Bruand, C., et al. (2011). Both plant and bacterial nitrate reductases contribute to nitric oxide production in Medicago truncatula nitrogen-fixing nodules. *Plant Physiol.* 155, 1023–1036. doi: 10.1104/pp.110.166140

Igamberdiev, A. U., and Hill, R. D. (2004). Nitrate, NO and haemoglobin in plant adaptation to hypoxia: An alternative to classic fermentation pathways. *J. Exp. Bot.* 55, 2473–2482. doi: 10.1093/ixb/erh272

Igamberdiev, A. U., and Hill, R. D. (2009). Plant mitochondrial function during anaerobiosis. *Ann. Bot.* 103, 259–268. doi: 10.1093/aob/mcn100

Jones, D. T., Taylor, W. R., and Thornton, J. M. (1992). The rapid generation of mutation data matrices. *Comput. Appl. Biosci.* 8, 275282. doi: 10.1093/bioinformatics/8.3.275

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7, 193–195. doi: 10.1016/S1360-1385(02)02251-3

Kato, K., Okamura, Y., Kanahama, K., and Kanayama, Y. (2003). Nitrate-independent expression of plant nitrate reductase in Lotus japonicus root nodules. *J. Exp. Bot.* 54, 1685–1690. doi: 10.1093/jxb/erg189

Kato, K., Kanahama, K., and Kanayama, Y. (2010). Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in Lotus root nodules. *J. Plant Physiol.* 167, 238–241. doi: 10.1016/j.jplph.2009.08.006

Kolbert, Z., Barroso, J. B., Brouquisse, R., et al. (2019). A forty year journey: The generation and roles of NO in plants. *Nitric. Oxide* 93, 53–70. doi: 10.1016/j.niox.2019.09.006

Kolbert, Z., Ortega, L., and Erdei, L. (2010). Involvement of nitrate reductase (NR) in osmotic stress-induced NO generation of Arabidopsis thaliana L. roots. *J. Plant Physiol.* 167, 77–80. doi: 10.1016/j.jplph.2009.08.013

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35 (6), 15471549. doi: 10.1093/molbev/msy096

Libourel, I. G. L., Bethke, P. C., De Michele, R., and Jones, R. L. (2006). Nitric oxide gas stimulates germination of dormant Arabidopsis seeds: use of a flow-through apparatus for delivery of nitric oxide. *Planta* 223, 813–820. doi: 10.1007/s00425-005-0117-8

Limami, A. M., Glévarec, G., Ricoult, C., Cliquet, J.-B., and Planchet, E. (2008). Concerted modulation of alanine and glutamate metabolism in young Medicago truncatula seedlings under hypoxic stress. *J. Exp. Bot.* 59, 2325–2335. doi: 10.1093/jxb/ern102

Limami, A. M., Diab, H., and Lothier, J. (2014). Nitrogen metabolism in plants under low oxygen stress. *Planta* 239, 531–541. doi: 10.1007/s00425-013-2015-9

Lin, Y.-F., Hu, Y.-H., Lin, H.-T., Liu, X., Chen, Y.-H., Zhang, S., et al. (2013). Inhibitory Effects of Propyl Gallate on Tyrosinase and Its Application in Controlling Pericarp Browning of Harvested Longan Fruits. *J. Agric. Food Chem.* 61, 2889–2895. doi: 10.1021/jf305481h

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., et al. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* 47 (W1), W636W641. doi: 10.1093/nar/gkz268

Mathieu, C., Sophie, M., Frendo, P., Puppo, A., and Davies, M. (1998). Direct detections of radicals in intact soybean nodules: presence of nitric oxide-leghemoglobin complexes. *Free Radic. Biol. Med.* 24, 1242–1249. doi: 10.1016/S0891-5849(97)00440-1

Meilhoc, E., Cam, Y., Skapski, A., and Bruand, C. (2010). The response to nitric oxide of the nitrogen-fixing symbiont Sinorhizobium meliloti. *Mol. Plant Microbe Interact.* 23, 748–759. doi: 10.1094/MPMI-23-6-0748

Melo, P. M., Silva, L. S., Ribeiro, I., Seabra, A. R., and Carvalho, H. G. (2011). Glutamine Synthetase Is a Molecular Target of Nitric Oxide in Root Nodules of Medicago truncatula and Is Regulated by Tyrosine Nitration. *Plant Physiol.* 157, 1505–1517. doi: 10.1104/pp.111.186056

Mendel, R. R., and Hänsch, R. (2002). Molybdoenzymes and molybdenum cofactor in plants. *J. Exp. Bot.* 53, 1689–1698. doi: 10.1093/jxb/erf038

Mergaert, P., Nikovics, K., Kelemen, Z., Maunoury, N., Vaubert, D., Kondorosi, A., et al. (2003). A Novel Family in Medicago truncatula Consisting of More Than 300 Nodule-Specific Genes Coding for Small, Secreted Polypeptides with Conserved Cysteine Motifs. *Plant Physiol.* 132, 161–173. doi: 10.1104/pp.102.018192

Miranda, K. M., Espey, M. G., and Wink, D. A. (2001). A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. *Nitric. Oxide Biol. Chem.* 5, 62–71. doi: 10.1006/niox.2000.0319

Moing, A., Svanella, L., Rolin, D., Gaudillère, M., Gaudillère, J., and Monet, R. (1998). Compositional changes during the fruit development of two peach cultivars differing in juice acidity. *J. Amer. Soc Hortic. Sci.* 123, 770–775. doi: 10.21273/JASHS.123.5.770

Nagata, M., Murakami, E., Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Suzuki, A., et al. (2008). Expression of a class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in Lotus japonicus. *Mol. Plant-Microbe Interact.* 21, 1175–1183. doi: 10.1094/MPMI-21-9-1175

Navascues, J., Perez-Rontome, C., Gay, M., Marcos, M., Yang, F., Walker, F., et al. (2012). Leghemoglobin green derivatives with nitrated hemes evidence production of highly reactive nitrogen species during aging of legume nodules. *Proc. Natl. Acad. Sci.* 109, 2660–2665. doi: 10.1073/pnas.1116559109

Pfeffer, P., Rolin, D., Kumosinski, T., MacFall, J., and Schmidt, J. (1992). ^{31}P relaxation responses associated with N2/O2 diffusion in soybean nodule cortical cells and excised cortical tissue. *Plant Physiol.* 100, 1682–1690. doi: 10.1104/pp.100.4.1682

Pii, Y., Crimi, M., Cremonese, G., Spena, A., and Pandolfini, T. (2007). Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biol.* 7, 21. doi: 10.1186/1471-2229-7-21

Planchet, E., Jagadis Gupta, K., Sonoda, M., and Kaiser, W. M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. *Plant J.* 41, 732–743. doi: 10.1111/j.1365-313X.2005.02335.x

Puppo, A., Pauly, N., Boscari, A., Mandon, K., and Brouquisse, R. (2013). Hydrogen Peroxide and Nitric Oxide: Key Regulators of the Legume—*Rhizobium* and Mycorrhizal Symbioses. *Antioxid. Redox Signal.* 18, 2202–2219. doi: 10.1089/ars.2012.5136

Roberts, J. K. M., Ray, P. M., Wade-Jardetzky, N., and Jardetzky, O. (1980). Estimation of cytoplasmic and vacuolar pH in higher plant cells by 31P NMR. *Nature* 283, 870–872. doi: 10.1038/283870a0

Roberts, J. K., Callis, J., Wemmer, D., Walbot, V., and Jardetzky, O. (1984). Mechanisms of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. *Proc. Natl. Acad. Sci.* 81, 3379 LP – 3383. doi: 10.1073/pnas.81.11.3379

Roby, C., Martin, J. B., Bligny, R., and Douce, R. (1987). Biochemical changes during sucrose deprivation in higher plant cells. Phosphorus-31 nuclear magnetic resonance studies. *J. Biol. Chem.* 262, 5000–5007.

Rockel, P., Strube, F., Rockel, A., Wildt, J., and Kaiser, W. M. (2002). Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J. Exp. Bot.* 53, 103–110. doi: 10.1093/jexbot/53.366.103

Rolin, D. B., Boswell, R. T., Sloger, C., Tu, S.-I., and Pfeffer, P. E. (1989). In Vivo ^{31}P NMR Spectroscopic Studies of Soybean Bradyrhizobium Symbiosis. *Plant Physiol.* 89, 1238–1246. doi: 10.1104/pp.89.4.1238

Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., et al. (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J.* 77, 817–837. doi: 10.1111/tpj.12442

Ruiz, B., Le Scornet, A., Sauviac, L., Rémy, A., Bruand, C., and Meilhoc, E. (2019). The Nitrate Assimilatory Pathway in Sinorhizobium meliloti: Contribution to NO Production. *Front. Microbiol.* 10:1526. doi: 10.3389/fmicb.2019.01526

Sainz, M., Calvo-Begueria, L., Pérez-Rontomé, C., Wienkoop, S., Abian, J., Staudinger, C., et al. (2015). Leghemoglobin is nitrated in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide-dependent mechanism. *Plant J.* 81, 723–735. doi: 10.1111/tpj.12762

Saito, S., Yamamoto-Katou, A., Yoshioka, H., Doke, N., and Kawakita, K. (2006). Peroxynitrite Generation and Tyrosine Nitration in Defense Responses in Tobacco BY-2 Cells. *Plant Cell Physiol.* 47, 689–697. doi: 10.1093/pcp/pcj038

Sakihama, Y., Nakamura, S., and Yamasaki, H. (2002). Nitric Oxide Production Mediated by Nitrate Reductase in the Green Alga Chlamydomonas reinhardtii: an Alternative NO Production Pathway in Photosynthetic Organisms. *Plant Cell Physiol.* 43, 290–297. doi: 10.1093/pcp/pcf034

Sanchez, C., Gates, A. J., Meakin, G. E., Uchiumi, T., Girard, L., Richardson, D. J., et al. (2010). Production of nitric oxide and nitrosylleghemoglobin complexes in soybean nodules in response to flooding. *Mol. Plant-Microbe Interact.* 23, 702–711. doi: 10.1094/MPMI-23-5-0702

Santucci, D. M., Haas, B., and Smarrelli, J. (1995). Regulation of the inducible soybean nitrate reductase isoform in mutants lacking constitutive isoform(s). *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* 1247, 46–50. doi: 10.1016/0167-4838(94)00200-Z

Seabra, A. R., and Carvalho, H. G. (2015). Glutamine synthetase in Medicago truncatula, unveiling new secrets of a very old enzyme. *Front. Plant Sci.* 6, 578. doi: 10.3389/fpls.2015.00578

Silveira, J. A. G., Matos, J. C. S., Cecatto, V. M., Viegas, R. A., and Oliveira, J. T. A. (2001). Nitrate reductase activity, distribution, and response to nitrate in two contrasting Phaseolus species inoculated with Rhizobium spp. *Environ. Exp. Bot.* 46, 37–46. doi: 10.1016/S0098-8472(01)00082-X

Streeter, J. G. (1985a). Nitrate Inhibition of Legume Nodule Growth and Activity: II. Short Term Studies with High Nitrate Supply. *Plant Physiol.* 77, 325–328. doi: 10.1104/pp.77.2.325

Streeter, J. G. (1985b). Nitrate inhibition of legume nodule growth and activity: I. Long term studies with a continuous supply of nitrate. *Plant Physiol.* 77, 321–324. doi: 10.1104/pp.77.2.321

Terpolilli, J. J., Hood, G. A., Poole, P. S.R. K. B. T.-A (2012). Chapter 5 - What determines the efficiency of N2-fixing rhizobium-legume symbioses? *Adv. Microb. Physiol.* 60, 325–389.

Trinchant, J. C., and Rigaud, J. (1982). Nitrite and nitric oxide as inhibitors of nitrogenase from soybean bacteroids. *Appl. Environ. Microbiol.* 44, 1385–1388. doi: 10.1128/AEM.44.6.1385-1388.1982

Truong, H. N., Thalineau, E., Bonneau, L., Fournier, C., Potin, S., Balzergue, S., et al. (2015). The Medicago truncatula hypermycorrhizal B9 mutant displays an altered response to phosphate and is more susceptible to *Aphanomyces euteiches*. *Plant Cell Environ.* 38, 73–88. doi: 10.1111/pce.12370

Udvardi, M. K., and Day, D. A. (1997). Metabolite transport across symbiotic membranes of legume nodules. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 493–523. doi: 10.1146/annurev.arplant.48.1.493

Van de Velde, W., Guerra, J. C. P., De Keyser, A., De Rycke, R., Rombauts, S., Maunoury, N., et al. (2006). Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula. *Plant Physiol.* 141, 711–720. doi: 10.1104/pp.106.078691

Velterop, J. S., and Vos, F. (2001). A rapid and inexpensive microplate assay for the enzymatic determination of glucose, fructose, sucrose, L-malate and citrate in tomato (Lycopersicon esculentum) extracts and in orange juice. *Phytochem. Anal.* 12, 299–304. doi: 10.1002/pca.598

Wang, Q., Liu, J., and Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. *Front. Plant Sci.* 9:313. doi: 10.3389/fpls.2018.00313

Wong, P. P. (1980). Nitrate and Carbohydrate Effects on Nodulation and Nitrogen Fixation (Acetylene Reduction) Activity of Lentil (Lens esculenta Moench). *Plant Physiol.* 66, 78–81. doi: 10.1104/pp.66.1.78

Xiao, T. T., Schilderink, S., Moling, S., Deinum, E. E., Kondorosi, E., Franssen, H., et al. (2014). Fate map of Medicago truncatula root nodules. *Development* 141, 3517–3528. doi: 10.1242/dev.110775

Xiong, J., Fu, G., Yang, Y., Zhu, C., and Tao, L. (2012). Tungstate: is it really a specific nitrate reductase inhibitor in plant nitric oxide research? *J. Exp. Bot.* 63, 33–41. doi: 10.1093/jxb/err268

Yamasaki, H., and Sakihama, Y. (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett.* 468, 89–92. doi: 10.1016/S0014-5793(00)01203-5

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership