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Additional Information

1 **Title:** Present knowledge and controversies, deficiencies and misconceptions  
2 on nitric oxide synthesis, sensing and signaling in plants.

3 **Short title:** Nitric oxide actions in plants

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9

1 **ABSTRACT**

2 **After thirty years of intensive work, nitric oxide (NO) has just started to be**  
3 **characterized as a relevant regulatory molecule on plant development and**  
4 **responses to stress. Its reactivity as a free radical determines its mode of action as**  
5 **an inducer of post-translational modifications of key target proteins through**  
6 **cysteine S-nitrosylation and tyrosine nitration. Many of the NO-triggered**  
7 **regulatory actions are exerted in tight coordination with phytohormone signaling.**  
8 **This review summarizes and updates the information accumulated on how NO is**  
9 **synthesized, sensed and transduced in plants, but also makes emphasis on**  
10 **controversies, deficiencies and misconceptions that are hampering our present**  
11 **knowledge on the biology of NO in plants. The development of non-invasive**  
12 **accurate tools for the endogenous NO quantitation as well as the implementation**  
13 **of genetic approaches that overcome misleading pharmacological experiments will**  
14 **be critical for getting significant advances in better knowledge of NO homeostasis**  
15 **and regulatory actions in plants.**

16

17 *Key words:* nitric oxide; synthesis; sensing; signaling; post-translational modifications;  
18 nitration; S-nitrosylation; phytohormones.

19

20 **INTRODUCTION**

21 Nitric oxide (NO) is a small gaseous free radical molecule that plays key roles in the  
22 physiology of living organisms. It has been particularly well studied in mammals, where  
23 its key regulatory role on physiological processes of vital importance such as

1 neurotransmission, inflammation response and cardiovascular diseases boosted research  
2 for around 40 years since 1980s (Palmer *et al.* 1987; Moncada & Higgs 1991; Schmidt  
3 & Walter, 1994). The first reports on NO production from plant tissues date back from  
4 the second half of the 1980s, being detected as a side product by *in vivo* nitrate  
5 reductase activity assays (Dean & Harper 1986; Klepper, 1987). However, the first  
6 reports on NO regulatory roles in plant-pathogen interactions were published at the end  
7 of the last century (Delledonne *et al.* 1998; Durner *et al.*, 1998). From that moment on,  
8 NO has been thoroughly studied in plants (for recent reviews see Astier *et al.* 2018;  
9 Hancock & Neill 2019; Del Castello *et al.* 2019). Although NO has been extensively  
10 reported as a relevant signaling molecule in plants (Domingos *et al.* 2015), neither its  
11 production nor its signal transduction mechanisms are fully elucidated (Astier *et al.*  
12 2018). Moreover, we have only few glimpses of the way NO is sensed in plants (Gibbs  
13 *et al.* 2014a). This panorama contrasts with the knowledge in mammals where it is well  
14 established that most of the NO is synthesized by NO synthases (NOS), sensed by  
15 guanylate cyclases (GC) and signaled through multiple intricate pathways. Most of  
16 these processes and components remain unidentified or mostly controversial in plant  
17 research (Wendehenne *et al.* 2001). This review aims to unravel some of these disputes  
18 by updating and summarizing the available information regarding NO production as  
19 well as sensing and further signaling in plants.

20

## 21 RELEVANT NO SYNTHESIS PATHWAYS IN PLANTS

22 The intermediate oxidation state of N in NO, between the abundant strongly oxidized  
23 forms such as nitrate or nitrite and fully reduced forms such as ammonium or the amino  
24 groups of amino acids, enables plants to produce NO either through reductive or

1 oxidative mechanisms (recently in-depth reviewed by Astier *et al.* 2018). Extensive  
2 reported data on NO production in different plant species and diverse biological  
3 situations pointed to the co-existence of multiple pathways likely functioning in either  
4 different tissues/organs and subcellular compartments or temporal patterns and  
5 environmental/developmental conditions. Figure 1 summarizes the most relevant NO  
6 production pathways operating in plants. Reductive pathways using nitrite as N source  
7 can be catalyzed by several reductases that include nitrate reductases (NR), Nitric  
8 Oxide-Forming Nitrite Reductase (NOFNiR) belonging to the mitochondrial Amidoxine  
9 Reducing Component (mARC) protein family, other Molybdenum Cofactor (MoCo)-  
10 dependent enzymes, or alternatively can use the mitochondrial electron transport chain  
11 (mETC) as reducing agent. Among those pathways, the NR-mediated and the mETC-  
12 dependent reduction of nitrite to NO are the most relevant sources of NO production in  
13 higher plants. The NR-mediated pathway is prevalent under normoxic conditions with  
14 high levels of nitrite and low nitrate or under acidic conditions (Yamasaki *et al.* 1999;  
15 Yamasaki & Sakihama 2000; Rockel *et al.* 2002). In turn, the mitochondrial pathway  
16 gains relevance under anaerobic/hypoxic conditions (Gupta *et al.* 2005) as a way to  
17 preserve respiration when oxygen is scarce by using nitrite as electron acceptor (Gupta  
18 & Igamberdiev 2011). Because the mETC-dependent reduction of nitrite to NO is very  
19 sensitive to the inhibition by O<sub>2</sub>, it is unlikely that this NO biosynthetic pathway  
20 functions under normoxia.

21           Although the interaction of NR with NOFNiR has been recently proposed as  
22 another reductive mechanism for the NO synthesis in eukaryotic algae (Chamizo-  
23 Ampudia *et al.* 2016; Fig. 1), the functionality of this pathway remains largely unknown  
24 in higher plants. While two genes coding for potential orthologues of the  
25 *Chlamydomonas* mARC proteins can be found in the *A. thaliana* genome and several

1 homologues were identified in other plants, to date, the NR:NOFNiR system has not  
2 been confirmed as functional in higher plants.

3       Regarding oxidative pathways for NO production, several evidences suggest that  
4 plants can synthesize NO by oxidation of reduced N-containing molecules. The  
5 oxidation of arginine to citrulline leading to the production of NO (Fig. 1), a reaction  
6 that is catalyzed in mammals by Nitric Oxide Synthases (NOSs), has been proposed to  
7 function also in plants (Durner *et al.* 1998; Barroso *et al.* 1999; Corpas & Barroso 2014;  
8 del Río *et al.* 2004). Moreover, the use of NOS inhibitors in pharmacological  
9 approaches as well as the heterologous expression of mammalian NOS in plants have  
10 demonstrated that the cofactors and conditions required for NOS activity indeed occur  
11 in plants, thus supporting the existence of plant NOS-like enzymes (Frunghillo *et al.*  
12 2014; Astier *et al.* 2018). Fluorescent analogues of arginine have been designed to try  
13 identifying and imaging potential NOS-like enzymes in tobacco (Chang *et al.* 2016).  
14 However, despite extensive recent efforts, the identification of the enzyme responsible  
15 for the NOS-like activity in higher plants did not succeed yet. The only NOS enzyme  
16 described to date from the plant kingdom belong to algal species (Foresi *et al.* 2015). A  
17 systematic search for NOS-like sequences in more than 1000 land plants and algae  
18 concluded that land plants do not express NOS enzymes (Jeandroz *et al.* 2016), and is  
19 thus unlikely that they produce NO through this oxidative mechanism. It is also feasible  
20 that despite putative plant NOS-like enzymes do not share overall sequence similarity  
21 with mammalian NOS, some key motifs or even single residues important for enzyme  
22 activity are conserved thus preserving the overall three-dimensional structure required  
23 for the enzyme activity. However, this is unlikely as bioinformatic searches in the  
24 Arabidopsis and rice genomes and proteomes did not yield positive identifications  
25 regarding conservation of short sequence motifs important for mammalian NOS

1 (Hancock & Neill 2019). Therefore, published data allowed shedding reasonable doubts  
2 about the existence of NOS enzymes in plants and, moreover, it has been also  
3 questioned whether the NO-cGMP signaling operates in plants as in animals (Astier *et*  
4 *al.* 2019). It has been proposed that another potential substrate of an oxidative NO  
5 synthetic pathway would be hydroxylamine (Rümer *et al.* 2009). However, in plants and  
6 cyanobacteria hydroxylamine seems to be reduced mainly to ammonium by class 1  
7 hemoglobins under hypoxia (Sturms *et al.* 2011).

8

9 *Pharmacological approaches supporting reductive and oxidative NO synthesis should*  
10 *be carefully assessed*

11 The use of NO donors or scavengers as well as inhibitors of nitrate reductase or NO  
12 synthase activities supported the involvement of NO in regulating physiological  
13 processes including the elongation and architecture of the root system (Pagnussat *et al.*  
14 2002; Correa-Aragunde *et al.* 2006; Singh & Bhatla 2018); the stomata closure (Hao *et*  
15 *al.* 2010) and senescence (Ji *et al.* 2016) in leaves; the embryo dormancy in apple seeds  
16 (Krasuska *et al.* 2016); or the pollen germination (He *et al.* 2007; Wang *et al.* 2009).  
17 However, these pharmacological approaches present several disadvantages and arouse  
18 controversies (Planchet & Kaiser 2006). Commonly used NO donors such as sodium  
19 nitroprusside (SNP) releases NO under light conditions but also generates cyanide as a  
20 side product (Bates *et al.* 1991). Cyanide, similarly to NO, has been reported to break  
21 seed dormancy and can be scavenged by compounds such as 2-phenyl-4,4,5-  
22 tetramethylimidazole-1-oxyl 3-oxide (PTIO) and its derivatives (Bethke *et al.* 2006),  
23 which are presumably specific scavengers of NO, thereby shedding doubts about  
24 usefulness of both donors and scavengers (D'Alessandro *et al.* 2013). Also this topic

1 remains controversial as it has been reported that SNP only releases cyanide under UV  
2 light irradiation but not under irradiation with visible light (Shishido & Ganzarolli de  
3 Oliveira 2001). It has been also reported that NO release from SNP requires cellular  
4 components, particularly thiol-containing molecules, which can trigger NO production  
5 in the absence of light (Grossi & D'Angelo 2005). Although alternative NO donors like  
6 S-nitroso-N-acetylpenicillamine (SNAP) or diethylenetriamine NONOate (DETA)  
7 avoid the production of active side products, caution with the interpretation of results  
8 derived from the use of different NO donors is advised. It has been reported that  
9 different NO donors have distinct photostabilities, both in aqueous solution and inside  
10 plant cells, and also that the release rate of NO depends on numerous endogenous  
11 factors (Floryszak-Wieczorek *et al.* 2006). Moreover, the pattern, timing and form of  
12 NO emitted has been reported to be quite different from donor to donor as well as the  
13 cell metabolic alterations detected upon treatments (Arasimowicz-Jelonek *et al.* 2011).  
14 Differential effects triggered by different NO donors were exemplified by processes  
15 such as cell death and suppression of ROS-scavenging systems, which were specifically  
16 altered by SNP and no other NO donor. In contrast, ferritin regulation was altered even  
17 in opposite ways by SNP and SNAP (Murgia *et al.* 2004; Ederli *et al.* 2009).

18 As for NO donors, the use of NR and NOS inhibitors to elucidate the source of  
19 NO in plants is not free from controversy either. Many of the major disadvantages come  
20 from the lack of specificity. Tungsten, applied mainly as sodium tungstate, has been  
21 extensively used in the NO research as an inhibitor of the enzyme nitrate reductase.  
22 However, the specificity of this inhibition has been questioned (Xiong *et al.* 2012;  
23 Adamakis *et al.* 2012). It has been reported that tungstate disrupts actin microfilaments  
24 and growth by targeting PIN auxin transporters (Adamakis *et al.* 2014a; b). Regarding  
25 oxidative NO biosynthesis, the use of N $\omega$ -nitro-L-arginine methylester (L-NAME) as a



1 NOS inhibitor has been widely used to support the NOS-mediated production of NO in  
2 plants. However, the specificity of this inhibitor is also debated as it actually impairs  
3 oligogalacturonide-induced NR activity without affecting the oligogalacturonide-  
4 triggered NO production in the Arabidopsis NR-deficient *nia1nia2* mutant, thus  
5 suggesting NR-mediated and L-arginine-dependent pathways of NO synthesis are not  
6 independent (Rasul *et al.* 2012). It should be also taken into account that the inhibitory  
7 activity of L-NAME requires the activity of endogenous esterases (Viteček *et al.* 2012),  
8 which might represent a limitation under some experimental conditions. In addition, L-  
9 NAME treatment altered growth and morphology of Arabidopsis roots through effects  
10 on microtubule organization (Krasylenko *et al.* 2017). Whether these effects are all due  
11 to specific effect on NOS-mediated NO synthesis or to unspecific effects remain to be  
12 deeply analyzed.

### 13 *Subcellular location of NO production*

14 Besides the controversy about NO biosynthesis pathways operative in plants, the  
15 subcellular location where NO is produced also remains to be analyzed in detail.  
16 Whereas the nitrate-dependent NO synthesis occurs in the cytoplasm catalyzed by  
17 cytosolic NRs both in vascular and non-vascular plants (Palavan-Unsal & Arisan 2009;  
18 Medina-Andrés *et al.* 2015), the arginine-dependent NO production catalyzed by NOS-  
19 like enzymes has been reported to occur in peroxisomes (Barroso *et al.* 1999; Corpas *et*  
20 *al.* 2004). In addition, it has been also reported a chloroplastic source of NO production  
21 (Jasid *et al.* 2006; Tewari *et al.* 2013). Although originally NO Associated 1 (NOA1)  
22 was proposed to be a mitochondrial protein (Guo & Crawford, 2005; Parihar *et al.*  
23 2008), the NO-deficiency of the Arabidopsis *noa1/rif1* mutant seems to be due to  
24 altered chloroplast function (Gas *et al.* 2009; Misra *et al.* 2014). NOA1-related  
25 production of NO does not occur in mitochondria, but this organelle has been

1 extensively reported as a relevant NO source in plants, sometimes producing other N  
2 oxides as a result of NO scavenging (Gupta & Kaiser, 2010; Gupta *et al.* 2010). In  
3 addition to the intracellular production of NO in different subcellular locations, NO can  
4 be produced from nitrite in the apoplast by non-enzymatic acidic conditions (Bethke *et*  
5 *al.* 2004; Fig. 1).

#### 6 *NO homeostasis depends on several enzyme activities and proteins*

7 Regardless of the source and cellular location, the intracellular levels of NO are the  
8 result of the balance between synthesis and metabolism or scavenging, which is strongly  
9 controlled by the action of several specific enzymes. NO can react with reduced  
10 glutathione (GSH) producing S-nitrosoglutathione (GSNO), which acts as a NO  
11 reservoir and as an efficient donor for protein nitrosylation (Jahnová *et al.* 2019) (Fig.  
12 2). However, GSNO is not restricted to be a NO reservoir as both molecules have been  
13 reported to exert additive functions in response to stress and development (Yun *et al.*  
14 2016). GSNO is metabolized to oxidized glutathione disulfide (GSSG) and ammonia by  
15 cysteine-rich GSNO Reductases (GSNOR), which regulate development and defense  
16 (Leterrier *et al.* 2011; Kwon *et al.* 2012; Xu *et al.* 2013). GSNOR is itself a target for S-  
17 nitrosylation thus representing an auto-regulatory loop (Guerra *et al.* 2016; Tichá *et al.*  
18 2017; Zhan *et al.* 2018). Another redox mechanism, based on the action of thioredoxins  
19 such as TRX h3 and h5, reducing S-nitrosylated proteins seems to be also relevant to  
20 control S-nitrosylation-related signaling specifically in plant immunity to pathogens  
21 (Tada *et al.* 2008; Kneeshaw *et al.* 2014). Thioredoxin-based regulation of S-  
22 nitrosylation releases the non-nitrosylated protein and NO, thus affecting NO  
23 homeostasis (recently reviewed by Mata-Perez & Spoel 2019). NO can be also  
24 scavenged by reacting with ROS. The reaction of NO and superoxide ( $O_2^-$ ) anion  
25 generates peroxynitrite ( $ONOO^-$ ), which is a powerful nitrating agent able to cause

1 tyrosine nitration of some proteins (Fig. 2) (Gaupels *et al.*, 2011; Begara-Morales *et al.*,  
2 2014). NO can react chemically with oxygen and generate nitrite and nitrate (Hancock  
3 2012) and, through a still unknown mechanism, can react with ROS and lipid peroxy  
4 radical (LOO·) to produce nitro-fatty acids (NO<sub>2</sub>-FAs) (Rubbo 2013) (Fig. 2). Finally,  
5 the homeostasis of NO can also be regulated through its oxidization to nitrate by  
6 nonsymbiotic and truncated hemoglobins (HB) (Fig. 2). HBs must be reduced to  
7 Fe(II)HB to dioxygenate NO (Chamizo-Ampudia *et al.* 2017). Nitrate has been  
8 proposed to regulate coordinately HB expression and NO homeostasis (Trevisan *et al.*  
9 2011). Importantly, it has been proposed that the limited production of NO via NR may  
10 be possibly due to the effect of the NO generated through NR reaction self-deactivating  
11 the enzyme by S-nitrosylation-mediated negative-feedback regulation (Fu *et al.* 2018).  
12 This is in agreement with the previously reported NO-triggered inhibition of NR  
13 activity in wheat leaves (Rosales *et al.* 2011). By contrast, it has been proposed that NO  
14 activates NR in *Brassica chinensis* presumably through post-translational modification  
15 (Du *et al.* 2008). Moreover, the use of NO donors, at different concentrations and  
16 developmental stages, on *Medicago truncatula* plants suggest that the effects on NRs  
17 and nitrate uptake are far more complex, with positive and negative regulation  
18 depending on the conditions tested (Antoniou *et al.* 2013).

19

## 20 **QUANTIFICATION OF ENDOGENOUS NO IS A CHALLENGING TASK**

21 The community working in different aspects of NO-related biology faces a systematic  
22 bottleneck in trying to quantify the endogenous levels of NO. Fluoresceins, such as  
23 diaminofluorescein (DAF), have been extensively used to detect NO (Foissner *et al.*  
24 2000) and in combination with flow cytometry allow quantifying NO in cell

1 suspensions (Kępczyński & Cembrowska-Lech 2018). However, DAF does not bind  
2 directly to NO but to N<sub>2</sub>O<sub>3</sub>, which is an oxidation product of NO (Kojima *et al.* 1998).  
3 It has been reported that DAF dyes also react with peroxidase and hydrogen peroxide  
4 (Ruemer *et al.* 2016) as well as ascorbic acid and dehydroascorbic acid (Stöhr &  
5 Stremlau 2006) to generate fluorescence similar to that of the N<sub>2</sub>O<sub>3</sub>-derived  
6 aminotriazole. In addition, the fluorescent aminotriazole formed during DAF- N<sub>2</sub>O<sub>3</sub>  
7 reaction is dependent on pH (Viteceka *et al.* 2008). All these data together entail a  
8 degree of uncertainty in DAF-based analysis of NO that should be kept in mind. As  
9 alternative fluorescent probes that directly bind NO, Fluorescent Nitric Oxide  
10 Cheletropic Trap (FNOCT) 8a and Cu derivative of 4-methoxy-2-(1H-naphthol[2,3-  
11 d]imidazol-2-yl)phenol (MNIP-Cu ) (Vandana *et al.* 2012; Jain *et al.* 2016) have been  
12 suggested as more specific probes for the analysis of endogenous NO. However, the  
13 validity of these probes has not been sufficiently proven to quantify NO under different  
14 conditions and experimental systems. While the simple use of fluorescent probes  
15 explains their extensive use, several other methodological approaches for NO assay in  
16 plants, including gas chromatography and mass spectrometry (Conrath *et al.* 2004),  
17 hemoglobin method by spectrophotometric measurement of the conversion of  
18 oxyhemoglobin to methemoglobin (Delledonne *et al.* 1998), laser photo-acoustic  
19 spectroscopy (Leshem & Pinchasov 2000; Mur *et al.* 2005), spin trapping of nitric oxide  
20 with electron paramagnetic resonance (EPR) (Pagnussat *et al.* 2002; Huang *et al.* 2004;  
21 Modolo *et al.* 2005; Calvo-Begueria *et al.* 2018), NO electrode (Yamasaki *et al.* 2001),  
22 chemiluminescent reaction involving ozone (Morot-Gaudry-Talarmain *et al.* 2002) and  
23 near-infrared fluorescent single wall carbon nanotubes (Giraldo *et al.* 2015) are also  
24 available. Many of these analytical techniques are suitable to measure NO emission but  
25 fail to detect the endogenously accumulated NO inside cells. EPR is by far more

1 specific and precise in determining NO than fluorescent probes (Mülsch *et al.* 1992;  
2 Kleschyov *et al.* 2007), but its use requires know-how and sophisticated equipment,  
3 which are not always available. Moreover, EPR-based quantification of NO is a  
4 severely limited technique for the analysis of large number of samples. Nevertheless,  
5 the combined EPR and fluorometric methods, whenever possible, should be used to  
6 draw reliable conclusions about NO production in plants (Calvo-Begueria *et al.* 2018).  
7 Pending the generation of new analytical tools allowing the quantitative measurement of  
8 endogenous NO, it seems advisable to use at least two analytical methods trying to rule  
9 out specific drawbacks of single techniques (Yamasaki *et al.* 2016).

10

## 11 **NO SENSING AND PHYTOHORMONE SIGNALING**

12 While the biosynthesis of NO still remains controversial, the way plants sense NO is  
13 even less known. NO perception in animals is performed through NO-inducible soluble  
14 Guanylate Cyclases (GC) that synthesize the second messenger 3',5'-cyclic guanosine  
15 monophosphate (cGMP) from guanosine triphosphate (GTP) (Friebe & Koesling 2003;  
16 Russwurm & Koesling 2004). Although a flavin monooxygenase called NO-dependent  
17 Guanylate Cyclase 1 (NOGC1), with higher affinity for NO than for molecular oxygen,  
18 was identified in *Arabidopsis* (Mulaudzi *et al.* 2011), it is not clear yet whether this  
19 enzyme produces enough cGMP to work as a truly NO receptor (Gross & Durner 2016).  
20 It is also unknown whether enzymes involved in cGMP degradation and downstream  
21 signaling, such as phosphodiesterases, are functional in plants (Gross & Durner 2016),  
22 which makes the functionality of a NO-cGMP signaling pathway in plants even more  
23 uncertain (Astier *et al.* 2019). In the absence of a GC receptor for NO, plants seem to  
24 sense NO mostly through chemical interaction with cofactor metals or with specific

1 amino acid residues of proteins that undergo NO-triggered post-translational  
2 modifications (PTMs; Astier & Lindermayr 2012). An alternative NO sensing  
3 mechanism involving the so called Cys-Arg/N-end rule proteolytic pathway have been  
4 reported in Arabidopsis (Gibbs *et al.* 2014a). This pathway consists in the specific  
5 oxidation of the C2 residue of transcription factors of the group VII of Ethylene  
6 Response Factors (ERF/AP2) family (ERFVIIs), which is strictly dependent on  
7 molecular oxygen and NO , and allows further arginylation, polyubiquitylation and  
8 proteasome-mediated degradation of ERFVIIs (Fig. 3) (Gibbs *et al.* 2014a). This  
9 pathway is essential for responses to low oxygen conditions (Gibbs *et al.* 2014b;  
10 Pucciariello & Perata 2017) and has been also proposed to function as a general sensor  
11 of abiotic stress (Vicente *et al.* 2017). ERFVIIs degradation through this pathway  
12 begins with the removal of the N-terminal methionine by a Methionine Aminopeptidase  
13 (MAP), thus exposing the tertiary destabilizing cysteine residue making it susceptible of  
14 direct oxidation (Fig. 3). The specificity of this reaction depends on Plant Cysteine  
15 Oxidase 1 (PCO1) and PCO2 enzymes, which catalyze the oxidation of the thiol group  
16 in the N-terminal Cys of the substrate to sulphinic acid using O<sub>2</sub> and NO as co-  
17 substrates (Fig. 3) (Gibbs *et al.* 2014a; Weits *et al.* 2014). Once it is oxidized, the Cys  
18 acts as a secondary destabilizing residue that becomes substrate for arginylation by Arg-  
19 tRNA Transferase (ATE) enzymes (Fig. 3). Transfer of an Arg to the N-terminal residue  
20 of the target protein constitutes an N-degron signal for the N-recognin E3 ubiquitin  
21 ligase Proteolysis6 (PRT6) to polyubiquitylate ERFVIIs previous to further  
22 degradation by the proteasome (Fig. 3). The N-end rule proteolysis of ERFVIIs takes  
23 place only under oxygen-rich conditions, being deactivated under low O<sub>2</sub> (Pucciariello  
24 & Perata 2017), but it is also strictly dependent on NR-mediated production of NO as  
25 ERFVIIs degradation in normoxia is blocked in *nialnia2* plants mutated in both

1 Arabidopsis NR proteins (Gibbs *et al.*, 2014a). ERFVIIIs act on ABI5 in NO triggered  
2 regulation of ABA signaling in seed germination and stomata closure (Gibbs *et al.*  
3 2014a), but also control photomorphogenesis-related processes such as hypocotyl  
4 elongation (Gibbs *et al.* 2014a) or apical hook opening (Abbas *et al.* 2015). This  
5 mechanism based on the turnover of regulatory proteins through focused proteolysis by  
6 the ATP-dependent proteasome machinery is a conserved regulatory mechanism in  
7 eukaryotes, and particularly in plant hormone signaling (Dreher & Callis 2007; Vierstra  
8 2009; Sadanandom *et al.* 2012; del Pozo & Manzano 2014; Gibbs *et al.* 2014b; Nagels-  
9 Durand *et al.* 2016).

#### 10 *NO-hormone functional interactions*

11 Besides ERFVIIIs role in sensing NO and regulating multiple aspects of plant  
12 physiology, other still not deciphered components are likely involved in mediating NO  
13 sensitivity and responsiveness. Many of the NO-regulatory effects are often exerted  
14 through interaction with phytohormone signaling pathways, including the five so-called  
15 classical plant hormones (gibberellins, auxins, abscisic acid, cytokinins and ethylene)  
16 and some of the most recently characterized (brassinosteroids, salicylic acid, jasmonates  
17 and strigolactones) both in development and defense against biotic and abiotic stresses  
18 (Durbak *et al.* 2012; Freschi 2013; Simontacchi *et al.* 2013; Fancy *et al.* 2017).

19 Numerous plant physiological processes such as seed dormancy and germination,  
20 skotomorphogenic and photomorphogenic vegetative development, root growth,  
21 stomatal closure, pollination, flowering, fructification or leaf senescence are somehow  
22 regulated through NO-hormone interactions (Beligni & Lamattina 2000; He *et al.* 2004;  
23 Bethke *et al.* 2006; Tsai *et al.* 2007; Qiao & Fan 2008; Prado *et al.* 2008; De Michele *et*  
24 *al.* 2009; Manjunatha *et al.* 2010; Lozano-Juste & Leon 2011; Arc *et al.* 2013; Liu &  
25 Guo 2013; Du *et al.* 2014). Moreover, several reports pointed to NO as a relevant

1 negative regulator of different components of the ABA signaling pathway acting either  
2 by promoting nitration/inactivation of the ABA receptors (Lozano-Juste & Leon 2010;  
3 Castillo *et al.* 2015) or the S-nitrosylation and degradation of positive regulators such as  
4 OST1/SnRK2.6 (Wang *et al.* 2015) or ABI5 (Albertos *et al.* 2015), thus suggesting the  
5 existence of tight regulatory interactions between NO and ABA. Similarly, it has been  
6 reported that the S-nitrosylation of ASK1 is essential for the assembly of the SCF<sup>TIR1</sup>  
7 complex required for auxin perception and signaling (Iglesias *et al.* 2018). Actually, the  
8 receptor TIR1 itself is positively regulated also by S-nitrosylation (Terrile *et al.* 2012).  
9 Also NPR1-triggered salicylate signaling is negatively regulated by S-nitrosylation  
10 favoring cytosolic NPR1 oligomerization (Tada *et al.* 2008; 2009; Lindermayr *et al.*  
11 2010).

12         It has been reported that plants transduce NO signal through S-nitrosylation of  
13 protein arginine methyl transferase 5 (PRMT5) involved in protein methylation  
14 machinery and the subsequent epigenetic regulation of pre-mRNA splicing, DNA  
15 damage repair, and mRNA translation (Blanc & Richard 2017) in response to  
16 environmental alterations (Hu *et al.* 2017). It is worth mentioning that the Polycomb  
17 Repressive Complex 2 (PRC2) subunit VRN2 is regulated, like ERFVIIIs, in an O<sub>2</sub>- and  
18 NO-dependent manner involving the Cys-Arg branch of the N-end rule proteolytic  
19 pathway (Gibbs *et al.*, 2018), thus representing an additional support for a functional  
20 link between NO signaling and epigenetic regulation. This regulatory mechanism is  
21 consistent with the reported role for NO as a repressor of the floral transition (He *et al.*,  
22 2004), as VRN2 promotes the transition from vegetative to reproductive development  
23 during vernalization by methylating and silencing the floral repressor gene  
24 *FLOWERING LOCUS C (FLC)* (Gendall *et al.*, 2001). Figure 4 summarizes different  
25 NO regulatory events based on NO-triggered post-translational modifications, including



1 cysteine oxidation and S-nitrosylation as well as tyrosine nitration, of hormone  
2 receptors, enzymes and transcription factors involved in signaling and regulation of  
3 multiple aspects of plant developmental and stress-activated responses.

4 NO sensing in *Arabidopsis* hypocotyls has been proposed to require the  
5 production, perception and downstream signaling of several hormones including  
6 ethylene, strigolactones and salicylates (Castillo *et al.* 2018). However, it remains  
7 unknown whether NO-hormone interactions are specific for each cell/tissue/organ and  
8 physiological process, or some of these interactions may be universal. Some of the  
9 regulatory effects of NO on hormone signaling are exerted by PTMs on early signaling  
10 components such as their receptors (Tada *et al.* 2008; Terrile *et al.* 2012; Castillo *et al.*,  
11 2015; Iglesias *et al.* 2018), what might be indicative of somehow early widespread  
12 regulation in contrast to more specific late effects on downstream components.  
13 Nevertheless, more work is required to substantiate the specificity of the NO exerted  
14 regulation on hormone signaling, especially when the targets of regulation belong to  
15 multigene families, such as in the case of ABA (Castillo *et al.* 2015).

16

## 17 **NO AS A REGULATOR OF DEVELOPMENT AND STRESS-RELATED** 18 **RESPONSES**

19 The exposure of plants to exogenous atmospheric or soil microorganism-  
20 produced NO or endogenously biosynthesized NO alters multiple aspects of their  
21 physiology. The major sources of NO in the atmosphere derive from industrial activity  
22 and car engines (Skalska *et al.* 2010). However, release from microbial-related activity  
23 in soils is also relevant (Pilegaard 2013). Levels of NO have been increasing  
24 continuously in the Earth atmosphere since industrial revolution started (Jaeglé *et al.*

1 2005). Considering this tendency, plants may be exposed in the future to relatively high  
2 NO concentrations with expected consequences for their growth and development. Due  
3 to the spontaneous conversion of NO to NO<sub>2</sub> under aerobic conditions, it is frequent to  
4 use the term NO<sub>x</sub> when NO is supplied in an oxygenated environment (Kasten *et al.*  
5 2017). This topic should be also taken into account when the experimental approaches  
6 are based on exogenous NO supply. Because the conversion of NO to NO<sub>2</sub> cannot be  
7 controlled under normoxic conditions, it is convenient to design experiments  
8 minimizing the exposure times and considering the effects triggered by nitrogen dioxide  
9 instead of NO.

#### 10 *Regulation of protein targets by post-translational modifications*

11 NO is able to act as a regulator mainly by triggering PTMs in proteins, which may alter  
12 their activity, subcellular localization, function, structure or stability. When the affected  
13 protein is a transcription factor, PTMs trigger transcriptome changes (Grun *et al.* 2006;  
14 Palmieri *et al.* 2008; Besson-Bard *et al.* 2009). A recent study on the effect of the NO  
15 donor S-nitrosocysteine on the Arabidopsis transcriptome pointed to around 700  
16 transcription factors potentially involved in multiple physiological processes including  
17 hormone signaling, protein degradation, development and responses to multiple stresses  
18 (Imran *et al.* 2018). Table 1 includes some examples of plant transcription factor  
19 regulation by NO and their functions in a wide array of developmental and stress-related  
20 processes. Most of the reports describing NO-exerted regulation on different targets are  
21 based on PTMs such as S-nitrosation (often referred as S-nitrosylation) of thiols and  
22 amines; nitration of tyrosine, tryptophan and phenylalanine, ubiquitylation of lysine,  
23 phosphorylation of serine, threonine and tyrosine or the oxidation of tyrosine and thiols  
24 (Gow *et al.* 2004; Hess & Stamler 2012). Among these PTMs, the S-nitrosylation of  
25 cysteines and the nitration of tyrosines (Fig. 2) are by far the most extensively studied

1 NO-related modifications (Astier & Lindermayr 2012). It has been also documented  
2 that NO can regulate gene expression through the interaction with and the modulation of  
3 secondary messengers such as cGMP, cADP-ribose, Ca<sup>2+</sup>, and notably with ROS  
4 (Durner *et al.* 1998; Lamotte *et al.* 2006; Astier *et al.* 2010; Mur *et al.* 2013). However,  
5 as mentioned above, the functional connection between NO signaling and cGMP and  
6 Ca<sup>2+</sup> regulatory components has been questioned (Astier *et al.*, 2019), thus suggesting  
7 that much of the work previously reported on this area has to be carefully re-evaluated.

8 S-nitrosylation is a redox modification consisting in the reversible covalent  
9 binding of NO to the thiol group of a cysteine residue in a target protein, leading to the  
10 formation of an S-nitrosothiol (SNO) (Fig. 2) (Astier *et al.* 2011). This modification is  
11 restricted to specific cysteine residues and it is completely dependent on the nature of  
12 the surrounding amino acids (Seth & Stamler 2011). However, and to our knowledge,  
13 no consensus primary sequences have been identified determining the potential of S-  
14 nitrosylation of cysteine residues. GSNO seems to be one of the main donors for the  
15 transnitrosylase activity in plants, modulating the total SNO content (Wang *et al.* 2006;  
16 Yu *et al.* 2012). Nitration of tyrosine residues of proteins is likely the main irreversible  
17 reaction caused by nitrating agents such as peroxynitrite (ONOO<sup>-</sup>). It results in the  
18 addition of a nitro group (-NO<sub>2</sub>) in the *ortho* position from the hydroxyl group of the  
19 aromatic ring of tyrosines, leading to the formation of 3-nitrotyrosine (3-NY) (Schopfer  
20 *et al.* 2003) (Fig. 2). Like S-nitrosylation, tyrosine nitration is also restricted to specific  
21 target tyrosine residues (Lozano-Juste *et al.* 2011), and can trigger conformational  
22 changes that lead to the activation or the inhibition of the target proteins (Bayden *et al.*  
23 2011). However, as for S-nitrosylation no consensus motifs in amino acid primary  
24 sequences have been reported to direct nitration of specific residues. NO-related PTMs  
25 and the corresponding conformational changes of the modified target proteins seem to

1 promote the polyubiquitylation and subsequent proteasomal degradation of the affected  
2 proteins (Castillo *et al.* 2015), thus representing an additional link between NO and  
3 ubiquitin-proteasome pathway. NO can also interact reversibly with the transition  
4 metals (iron, zinc or copper) at the heme center of metalloproteins to form metal-  
5 nitrosyl complexes through coordination chemistry (Ford 2010; Astier & Lindermayr  
6 2012). The bound NO group is then susceptible to further nucleophilic or less frequently  
7 electrophilic attacks, depending on the protein bounded (Astier & Lindermayr 2012;  
8 Toledo & Augusto 2012). The formation of the metal-nitrosyl complex can induce  
9 conformational changes that compromise the proper functioning of the affected protein  
10 (Ford, 2010; Toledo and Augusto, 2012). Plant hemoglobins (HB), currently called  
11 phytohemoglobins, are the best characterized plant proteins undergoing metal nitrosylation  
12 (Gupta *et al.* 2011). The oxidation of phytohemoglobins by NO produces nitrate, thus  
13 scavenging the NO in what is considered a general mechanism modulating NO  
14 bioavailability, regulation and detoxification of NO in plants (Gupta *et al.* 2011;  
15 Igamberdiev *et al.* 2011). Recently, the nitration of fatty acids by NO has also been  
16 demonstrated to be an important part of NO signaling in plants (Mata-Pérez *et al.* 2017)  
17 (Fig. 2). Several mechanisms have been described for nitrofatty acid synthesis but all of  
18 them involved radical  $\bullet\text{NO}_2$  (Rubbo 2013; Buchan *et al.* 2018). In plants, the regulatory  
19 roles of nitrofatty acids have been proposed to be related to their potential as NO donors  
20 and inducers of NO-related PTMs in plants (Mata-Pérez *et al.* 2017). It has been also  
21 suggested that nitrofatty acids reorganize lipid layers in membranes, thus resulting in  
22 altered structure and function of membrane associated proteins with diverse signaling  
23 functions (Franz *et al.* 2017).

24 *Regulation of metabolism*

1 In contrast to the increasing knowledge regarding the effect of NO on protein function  
2 and gene expression, our current knowledge on the NO impact on global metabolome of  
3 plants is scarce and limited to several stress-related processes. In stressed plants, ROS  
4 and NO are produced simultaneously. NO seems to alleviate the oxidative status  
5 through its antioxidant capacity, thus contributing to the redox homeostasis (Correa-  
6 Aragunde *et al.* 2015). However, extensive evidence suggest that NO may be involved  
7 in paradoxical processes exerting sometimes opposing regulatory functions. NO has  
8 been described to enhance or reduce the redox status of the plants depending on either  
9 acting in a chronic or acute mode (Groß *et al.* 2013). These effects could be due to  
10 multiple factors such as the relative NO cellular concentration, the location where it is  
11 produced or the complex interacting microenvironment. Among different subcellular  
12 localization where NO production is documented, peroxisomes have been characterized  
13 as subcellular locations where hydrogen peroxide and NO metabolisms are  
14 interconnected and may serve as a source of signaling molecules (Corpas *et al.* 2019).  
15 On the other hand, chloroplasts are not only a source of NO but an important site of NO  
16 action in regulating photosynthesis and oxygen evolving processes (Misra *et al.* 2014).  
17 Regarding primary metabolism, it has been reported that NO could modulate  
18 carbohydrate metabolism at the post-translational level and regulate glutathione and  
19 methionine metabolism at the transcriptional level (He *et al.* 2018). S-nitrosylation  
20 modifications led to a decrease in cellular glycolysis enzymes, ATP synthase activities,  
21 content of acetyl coenzyme A, ATP, ADP-glucose and UDP-glucose, which all together  
22 eventually inhibited polysaccharide-biosynthesis and caused monosaccharide  
23 accumulation (Zhang *et al.* 2017). Accordingly, NO-treated plants displayed less starch  
24 granules and increased sugar content (León *et al.* 2016). Regarding another primary  
25 metabolic pathway, nitrate assimilation seems to be also controlled by NO through

1 negative transcriptional regulation on NR and high affinity nitrate transporter encoding  
2 genes in wheat (Adavi & Sathee 2019), a process that seems to be dependent on the N  
3 compound source (Balotf *et al.* 2018). On the other hand, NR-mediated NO generation  
4 increases nitrogen uptake capacity by inducing lateral root formation under partial  
5 nitrate nutrition in rice (Sun *et al.* 2015). Also sulfur assimilation seems to be regulated  
6 by NO under stress conditions as mustard plants exposed to cadmium showed a NO-  
7 triggered stimulated S-assimilation and GSH production (Per *et al.* 2017). Besides  
8 involvement in regulating primary metabolism, NO also regulates numerous secondary  
9 metabolite specific pathways including terpenoid production in *Taxus chinensis* (Wang  
10 & Wu 2004); anthocyanin and flavonoid biosynthesis activated by brassinosteroids (Li  
11 *et al.* 2017a) or by cold acclimation (Costa-Broseta *et al.* 2018; 2019); and  
12 phenylpropanoid biosynthesis and triggered resistance to pathogens (Santos-Filho *et al.*  
13 2012; Li *et al.* 2017b). In addition, NO also regulates the production of secondary  
14 metabolites of high nutraceutical value (Zhang *et al.* 2012).

15

## 16 **CONCLUSIONS AND PERSPECTIVES**

17 The outstanding increase in plant NO research during the last three decades has opened  
18 many paths leading to a significant better knowledge on how NO is produced and  
19 sensed, and also on how and where NO exerts regulatory functions. However, a large  
20 fraction of the reported work on this topic should be contrasted by using more clear and  
21 direct genetic strategies instead of the unspecific and sometimes confusing  
22 pharmacological approaches. It is foreseeable that many data derived from  
23 pharmacological approaches are revisited in the near future likely helping to overcome  
24 some of the current inconsistencies and misunderstandings regarding NO production

1 and its mode of action. Particularly important will be the development of the proper  
2 tools allowing the true quantification of endogenous NO in plant cells. These analytical  
3 techniques should be straightforward and non-invasive to allow better and precise  
4 analysis of the endogenous NO in plants.

5

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9

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- 22

1 **FIGURE LEGENDS**

2 **Figure 1.** Reductive and oxidative NO biosynthesis pathways predominantly  
3 functioning under normoxic and hypoxic conditions.

4 mARC, mitochondrial Amidoxine Reducing Component; NR, nitrate reductase; NiR,  
5 nitrite reductase; NOFNiR, NO-forming nitrite reductase; mETC, mitochondrial  
6 electron transport chain; NOS, NO synthase; Fed, ferredoxin; THB1, truncated  
7 hemoglobin; XO, xanthine oxidoreductase; NiNOR, root nitrite-NO reductase.

8

9 **Figure 2.** Relevant processes and proteins involved in NO homeostasis.

10 FA, fatty acids; GLB, globin; GR, glutathione reductase; GSH, reduced glutathione;  
11 GSSG, glutathione disulfide; GSNO, nitrosoglutathione; GSNOR, GSNO reductase.

12

13 **Figure 3.** NO sensing based on the N-end rule pathway degradation of ERFVII  
14 transcription factors.

15 ERFVIIIs, Group VII of the Ethylene Response Factor family of transcription factors;  
16 MAPs, methionine aminopeptidases; PCOs, plant cysteine oxidases; ATEs, arginyl  
17 transferases; PRT6, proteolysis6 E3 ubiquitin ligase; Ubq, ubiquitin.

18

19 **Figure 4.** NO regulatory events based on NO-triggered post-translational modifications.

20 ERFVIIIs, Group VII of the Ethylene Response Factor family of transcription factors;  
21 PYR/PYL, pyrabactin resistant like; OST1/SnRK2.6, Open Stomata 1/sucrose non-

- 1 fermenting 1-related protein kinase 2-6; ABI5, ABA insensitive 5; PRMT5, type II
- 2 protein arginine methyltransferase 5; VRN2, PRC2 subunit VERNALIZATION 2;
- 3 NPR1, non expresser of pathogenesis related 1; TGAs, TGACG motif-binding protein
- 4 family; AHKs, Arabidopsis His kinases; AHPs, Arabidopsis His phosphotransfers;
- 5 PRRs, Response regulators; SCFTIR1/AFB, SKP-Cullin-F box Transport Inhibitor
- 6 Resistant1/Auxin signaling F-Box.

**Table 1.** Plant transcription factors and processes reported to be targets of NO regulation.

| Transcription factor       | Process  | Reference  |
|----------------------------|--|--|
| SRG1                       | S-nitrosylation relieves its transcriptional repression in plant immunity                      | Cui et al. 2018. Nat. Commun.  |
| VND7                       | S-nitrosylation promotes xylem vessel cell differentiation                                     | Kawave et al. 2018. Plant Cell Physiol.; Ohtani et al. 2018. Plant Signal Behav. |
| PIF1, HFR1                 | NO modulates PHYB-mediated seed germination by repressing PIF1 expression and stabilizing HFR1 | Li et al. 2018. Plant Physiol Biochem.   |
| SPCH, MUTE, SCRM2          | NO-promoted expression of these TFs initiate stomata development                               | Fu et al. 2016. Plant Sci.   |
| EIN3                       | NO-promoted seed germination is mediated by EIN3   | Li et al. 2016. Front. Plant. Sci.   |
| ERFVIIIs                   | Regulation of hypoxia-responsive gene expression   | Gasch et al. 2016. Plant Cell  |
| ERFVIIIs                   | NO-dependent Oxidation of ERFVIIIs is on the basis of NO sensing                               | Gibbs et al. 2014a. Mol. Cell  |
| ABI5                       | S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth      | Albertos et al. 2015. Nat. Commun.   |
| MYB30                      | S-nitrosylation of MYB30 alters its regulation on plant metabolism                             | Tavares et al. 2014. Biochim. Biophys. Acta                                      |
| ORE1/ AtNAC2/ ANAC092      | Mediates NO-induced cotyledon senescence   | Du et al. 2014. J. Exp. Bot.   |
| PIF3                       | NO induces PIF3 degradation to inhibit root elongation in light                                | Bai et al. 2014. Mol. Plant  |
| PIF3                       | NO inhibits hypocotyl elongation in darkness through DELLA accumulation and PIF3 scavenging    | Lozano-Juste and León. 2011. Plant Physiol.                                      |
| FIT                        | NO induces FIT degradation by the proteasome and alters Fe sensing and signaling               | Meiser et al. 2011. Plant Physiol.   |
| FIT, bHLH 38, 39, 100, 101 | GSNO mediates Fe-deficiency signaling  | Kailasam et al. 2018. Plant J.   |
| MYB2                       | NO-triggered S-nitrosylation inhibits its DNA binding activity                                 | Serpa et al. 2007. Biochem. Biophys. Res. Commun.                                |
| LeSPL                      | Contributes to cadmium tolerance by negatively regulating NO production                        | Chen et al. 2018. Planta   |
| Class I TCPs               | Negatively regulated by NO-triggered redox post-translational modification                     | Viola et al. 2013. Plant Physiol.  |









