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

Short title: Nitric oxide actions in plants

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ABSTRACT

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

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

INTRODUCTION

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

RELEVANT NO SYNTHESIS PATHWAYS IN PLANTS

The intermediate oxidation state of N in NO, between the abundant strongly oxidized forms such as nitrate or nitrite and fully reduced forms such as ammonium or the amino groups of amino acids, enables plants to produce NO either through reductive or
oxidative mechanisms (recently in-depth reviewed by Astier et al. 2018). Extensive reported data on NO production in different plant species and diverse biological situations pointed to the co-existence of multiple pathways likely functioning in either different tissues/organs and subcellular compartments or temporal patterns and environmental/developmental conditions. Figure 1 summarizes the most relevant NO production pathways operating in plants. Reductive pathways using nitrite as N source can be catalyzed by several reductases that include nitrate reductases (NR), Nitric Oxide-Forming Nitrite Reductase (NOFNiR) belonging to the mitochondrial Amidoxine Reducing Component (mARC) protein family, other Molybdenum Cofactor (MoCo)-dependent enzymes, or alternatively can use the mitochondrial electron transport chain (mETC) as reducing agent. Among those pathways, the NR-mediated and the mETC-dependent reduction of nitrite to NO are the most relevant sources of NO production in higher plants. The NR-mediated pathway is prevalent under normoxic conditions with high levels of nitrite and low nitrate or under acidic conditions (Yamasaki et al. 1999; Yamasaki & Sakihama 2000; Rockel et al. 2002). In turn, the mitochondrial pathway gains relevance under anaerobic/hypoxic conditions (Gupta et al. 2005) as a way to preserve respiration when oxygen is scarce by using nitrite as electron acceptor (Gupta & Igamberdiev 2011). Because the mETC-dependent reduction of nitrite to NO is very sensitive to the inhibition by O$_2$, it is unlikely that this NO biosynthetic pathway functions under normoxia.

Although the interaction of NR with NOFNiR has been recently proposed as another reductive mechanism for the NO synthesis in eukaryotic algae (Chamizo-Ampudia et al. 2016; Fig. 1), the functionality of this pathway remains largely unknown in higher plants. While two genes coding for potential orthologues of the *Chlamydomonas* mARC proteins can be found in the *A. thaliana* genome and several
homologues were identified in other plants, to date, the NR:NOFNiR system has not been confirmed as functional in higher plants.

Regarding oxidative pathways for NO production, several evidences suggest that plants can synthesize NO by oxidation of reduced N-containing molecules. The oxidation of arginine to citrulline leading to the production of NO (Fig. 1), a reaction that is catalyzed in mammals by Nitric Oxide Synthases (NOSs), has been proposed to function also in plants (Durner et al. 1998; Barroso et al. 1999; Corpas & Barroso 2014; del Río et al. 2004). Moreover, the use of NOS inhibitors in pharmacological approaches as well as the heterologous expression of mammalian NOS in plants have demonstrated that the cofactors and conditions required for NOS activity indeed occur in plants, thus supporting the existence of plant NOS-like enzymes (Frungillo et al. 2014; Astier et al. 2018). Fluorescent analogues of arginine have been designed to try identifying and imaging potential NOS-like enzymes in tobacco (Chang et al. 2016). However, despite extensive recent efforts, the identification of the enzyme responsible for the NOS-like activity in higher plants did not succeed yet. The only NOS enzyme described to date from the plant kingdom belong to algal species (Foresi et al. 2015). A systematic search for NOS-like sequences in more than 1000 land plants and algae concluded that land plants do not express NOS enzymes (Jeandroz et al. 2016), and is thus unlikely that they produce NO through this oxidative mechanism. It is also feasible that despite putative plant NOS-like enzymes do not share overall sequence similarity with mammalian NOS, some key motifs or even single residues important for enzyme activity are conserved thus preserving the overall three-dimensional structure required for the enzyme activity. However, this is unlikely as bioinformatic searches in the Arabidopsis and rice genomes and proteomes did not yield positive identifications regarding conservation of short sequence motifs important for mammalian NOS.
(Hancock & Neill 2019). Therefore, published data allowed shedding reasonable doubts about the existence of NOS enzymes in plants and, moreover, it has been also questioned whether the NO-cGMP signaling operates in plants as in animals (Astier et al. 2019). It has been proposed that another potential substrate of an oxidative NO synthetic pathway would be hydroxylamine (Rümer et al. 2009). However, in plants and cyanobacteria hydroxylamine seems to be reduced mainly to ammonium by class 1 hemoglobins under hypoxia (Sturms et al. 2011).

Pharmacological approaches supporting reductive and oxidative NO synthesis should be carefully assessed. The use of NO donors or scavengers as well as inhibitors of nitrate reductase or NO synthase activities supported the involvement of NO in regulating physiological processes including the elongation and architecture of the root system (Pagnussat et al. 2002; Correa-Aragunde et al. 2006; Singh & Bhatla 2018); the stomata closure (Hao et al. 2010) and senescence (Ji et al. 2016) in leaves; the embryo dormancy in apple seeds (Krasuska et al. 2016); or the pollen germination (He et al. 2007; Wang et al. 2009). However, these pharmacological approaches present several disadvantages and arouse controversies (Planchet & Kaiser 2006). Commonly used NO donors such as sodium nitroprusside (SNP) releases NO under light conditions but also generates cyanide as a side product (Bates et al. 1991). Cyanide, similarly to NO, has been reported to break seed dormancy and can be scavenged by compounds such as 2-phenyl-4,4,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) and its derivatives (Bethke et al. 2006), which are presumably specific scavengers of NO, thereby shedding doubts about usefulness of both donors and scavengers (D’Alessandro et al. 2013). Also this topic
remains controversial as it has been reported that SNP only releases cyanide under UV
light irradiation but not under irradiation with visible light (Shishido & Ganzarolli de
Oliveira 2001). It has been also reported that NO release from SNP requires cellular
components, particularly thiol-containing molecules, which can trigger NO production
in the absence of light (Grossi & D'Angelo 2005). Although alternative NO donors like
S-nitroso-N-acetylpenicillamine (SNAP) or diethylenetriamine NONOate (DETA)
avoid the production of active side products, caution with the interpretation of results
derived from the use of different NO donors is advised. It has been reported that
different NO donors have distinct photostabilities, both in aqueous solution and inside
plant cells, and also that the release rate of NO depends on numerous endogenous
factors (Floryszak-Wieczorek et al. 2006). Moreover, the pattern, timing and form of
NO emitted has been reported to be quite different from donor to donor as well as the
cell metabolic alterations detected upon treatments (Arasimowicz-Jelonek et al. 2011).
Differential effects triggered by different NO donors were exemplified by processes
such as cell death and suppression of ROS-scavenging systems, which were specifically
altered by SNP and no other NO donor. In contrast, ferritin regulation was altered even
in opposite ways by SNP and SNAP (Murgia et al. 2004; Ederli et al. 2009).

As for NO donors, the use of NR and NOS inhibitors to elucidate the source of
NO in plants is not free from controversy either. Many of the major disadvantages come
from the lack of specificity. Tungsten, applied mainly as sodium tungstate, has been
extensively used in the NO research as an inhibitor of the enzyme nitrate reductase.
However, the specificity of this inhibition has been questioned (Xiong et al. 2012;
Adamakis et al. 2012). It has been reported that tungstate disrupts actin microfilaments
and growth by targeting PIN auxin transporters (Adamakis et al. 2014a; b). Regarding
oxidative NO biosynthesis, the use of \textit{N}ω-nitro-L-arginine methylester (L-NAME) as a
NOS inhibitor has been widely used to support the NOS-mediated production of NO in plants. However, the specificity of this inhibitor is also debated as it actually impairs oligogalacturonide-induced NR activity without affecting the oligogalacturonide-triggered NO production in the Arabidopsis NR-deficient nia1nia2 mutant, thus suggesting NR-mediated and L-arginine-dependent pathways of NO synthesis are not independent (Rasul et al. 2012). It should be also taken into account that the inhibitory activity of L-NAME requires the activity of endogenous esterases (Viteček et al. 2012), which might represent a limitation under some experimental conditions. In addition, L-NAME treatment altered growth and morphology of Arabidopsis roots through effects on microtubule organization (Krasylenko et al. 2017). Whether these effects are all due to specific effect on NOS-mediated NO synthesis or to unspecific effects remain to be deeply analyzed.

**Subcellular location of NO production**

Besides the controversy about NO biosynthesis pathways operative in plants, the subcellular location where NO is produced also remains to be analyzed in detail. Whereas the nitrate-dependent NO synthesis occurs in the cytoplasm catalyzed by cytosolic NRs both in vascular and non-vascular plants (Palavan-Unsal & Arisan 2009; Medina-Andrés et al. 2015), the arginine-dependent NO production catalyzed by NOS-like enzymes has been reported to occur in peroxisomes (Barroso et al. 1999; Corpas et al. 2004). In addition, it has been also reported a chloroplastic source of NO production (Jasid et al. 2006; Tewari et al. 2013). Although originally NO Associated 1 (NOA1) was proposed to be a mitochondrial protein (Guo & Crawford, 2005; Parihar et al. 2008), the NO-deficiency of the Arabidopsis noa1/rif1 mutant seems to be due to altered chloroplast function (Gas et al. 2009; Misra et al. 2014). NOA1-related production of NO does not occur in mitochondria, but this organelle has been
extensively reported as a relevant NO source in plants, sometimes producing other N
oxides as a result of NO scavenging (Gupta & Kaiser, 2010; Gupta et al. 2010). In
addition to the intracellular production of NO in different subcellular locations, NO can
be produced from nitrite in the apoplast by non-enzymatic acidic conditions (Bethke et
al. 2004; Fig. 1).

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

Regardless of the source and cellular location, the intracellular levels of NO are the
result of the balance between synthesis and metabolism or scavenging, which is strongly
controlled by the action of several specific enzymes. NO can react with reduced
glutathione (GSH) producing S-nitrosoglutathione (GSNO), which acts as a NO
reservoir and as an efficient donor for protein nitrosylation (Jahnová et al. 2019) (Fig. 2). However, GSNO is not restricted to be a NO reservoir as both molecules have been
reported to exert additive functions in response to stress and development (Yun et al.
2016). GSNO is metabolized to oxidized glutathione disulfide (GSSG) and ammonia by
cysteine-rich GSNO Reductases (GSNOR), which regulate development and defense
(Leterrier et al. 2011; Kwon et al. 2012; Xu et al. 2013). GSNOR is itself a target for S-
nitrosylation thus representing an auto-regulatory loop (Guerra et al. 2016; Tichá et al.
2017; Zhan et al. 2018). Another redox mechanism, based on the action of thioredoxins
such as TRX h3 and h5, reducing S-nitrosylated proteins seems to be also relevant to
control S-nitrosylation-related signaling specifically in plant immunity to pathogens
(Tada et al. 2008; Kneeshaw et al. 2014). Thioredoxin-based regulation of S-
nitrosylation releases the non-nitrosylated protein and NO, thus affecting NO
homeostasis (recently reviewed by Mata-Perez & Spoel 2019). NO can be also
scavenged by reacting with ROS. The reaction of NO and superoxide (O2⁻) anion
generates peroxynitrite (ONOO⁻), which is a powerful nitrating agent able to cause

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tyrosine nitration of some proteins (Fig. 2) (Gaupels et al., 2011; Begara-Morales et al., 2014). NO can react chemically with oxygen and generate nitrite and nitrate (Hancock 2012) and, through a still unknown mechanism, can react with ROS and lipid peroxyl radical (LOO·) to produce nitro-fatty acids (NO₂-FAs) (Rubbo 2013) (Fig. 2). Finally, the homeostasis of NO can also be regulated through its oxidization to nitrate by nonsymbiotic and truncated hemoglobins (HB) (Fig. 2). HBs must be reduced to Fe(II)HB to dioxygenate NO (Chamizo-Ampudia et al. 2017). Nitrate has been proposed to regulate coordinately HB expression and NO homeostasis (Trevisan et al. 2011). Importantly, it has been proposed that the limited production of NO via NR may be possibly due to the effect of the NO generated through NR reaction self-deactivating the enzyme by S-nitrosylation-mediated negative-feedback regulation (Fu et al. 2018). This is in agreement with the previously reported NO-triggered inhibition of NR activity in wheat leaves (Rosales et al. 2011). By contrast, it has been proposed that NO activates NR in Brassica chinensis presumably through post-translational modification (Du et al. 2008). Moreover, the use of NO donors, at different concentrations and developmental stages, on Medicago truncatula plants suggest that the effects on NRs and nitrate uptake are far more complex, with positive and negative regulation depending on the conditions tested (Antoniou et al. 2013).

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

The community working in different aspects of NO-related biology faces a systematic bottleneck in trying to quantify the endogenous levels of NO. Fluoresceins, such as diaminofluorescein (DAF), have been extensively used to detect NO (Foissner et al. 2000) and in combination with flow cytometry allow quantifying NO in cell
suspensions (Kępczyński & Cembrowska-Lech 2018). However, DAF does not bind
directly to NO but to N₂O₃, which is an oxidation product of NO (Kojima et al. 1998).
It has been reported that DAF dyes also react with peroxidase and hydrogen peroxide
(Ruemer et al. 2016) as well as ascorbic acid and dehydroascorbic acid (Stöhr &
Stremlau 2006) to generate fluorescence similar to that of the N₂O₃-derived
aminotriazole. In addition, the fluorescent aminotriazole formed during DAF- N₂O₃
reaction is dependent on pH (Vitecek et al. 2008). All these data together entail a
degree of uncertainty in DAF-based analysis of NO that should be kept in mind. As
alternative fluorescent probes that directly bind NO, Fluorescent Nitric Oxide
Cheletropic Trap (FNOCT) 8a and Cu derivative of 4-methoxy-2-(1H-napthol[2,3-
d]imidazol-2-yl)phenol (MNIP-Cu ) (Vandana et al. 2012; Jain et al. 2016) have been
suggested as more specific probes for the analysis of endogenous NO. However, the
validity of these probes has not been sufficiently proven to quantify NO under different
conditions and experimental systems. While the simple use of fluorescent probes
explains their extensive use, several other methodological approaches for NO assay in
plants, including gas chromatography and mass spectrometry (Conrath et al. 2004),
hemoglobin method by spectrophotometric measurement of the conversion of
oxyhemoglobin to methemoglobin (Delledonne et al. 1998), laser photo-acoustic
spectroscopy (Leshem & Pinchasov 2000; Mur et al. 2005), spin trapping of nitric oxide
with electron paramagnetic resonance (EPR) (Pagnussat et al. 2002; Huang et al. 2004;
Modolo et al. 2005; Calvo-Begueria et al. 2018), NO electrode (Yamasaki et al. 2001),
chemiluminescent reaction involving ozone (Morot-Gaudry-Talarmain et al. 2002) and
near-infrared fluorescent single wall carbon nanotubes (Giraldo et al. 2015) are also
available. Many of these analytical techniques are suitable to measure NO emission but
fail to detect the endogenously accumulated NO inside cells. EPR is by far more
specific and precise in determining NO than fluorescent probes (Mülsch et al. 1992; Kleschyov et al. 2007), but its use requires know-how and sophisticated equipment, which are not always available. Moreover, EPR-based quantification of NO is a severely limited technique for the analysis of large number of samples. Nevertheless, the combined EPR and fluorometric methods, whenever possible, should be used to draw reliable conclusions about NO production in plants (Calvo-Begueria et al. 2018).

Pending the generation of new analytical tools allowing the quantitative measurement of endogenous NO, it seems advisable to use at least two analytical methods trying to rule out specific drawbacks of single techniques (Yamasaki et al. 2016).

NO SENSING AND PHYTOHORMONE SIGNALING

While the biosynthesis of NO still remains controversial, the way plants sense NO is even less known. NO perception in animals is performed through NO-inducible soluble Guanylate Cyclases (GC) that synthesize the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) (Friebe & Koesling 2003; Russwurm & Koesling 2004). Although a flavin monoxygenase called NO-dependent Guanylate Cyclase 1 (NOGC1), with higher affinity for NO than for molecular oxygen, was identified in Arabidopsis (Mulaudzi et al. 2011), it is not clear yet whether this enzyme produces enough cGMP to work as a truly NO receptor (Gross & Durner 2016).

It is also unknown whether enzymes involved in cGMP degradation and downstream signaling, such as phosphodiesterases, are functional in plants (Gross & Durner 2016), which makes the functionality of a NO-cGMP signaling pathway in plants even more uncertain (Astier et al. 2019). In the absence of a GC receptor for NO, plants seem to sense NO mostly through chemical interaction with cofactor metals or with specific
amino acid residues of proteins that undergo NO-triggered post-translational modifications (PTMs; Astier & Lindermayr 2012). An alternative NO sensing mechanism involving the so called Cys-Arg/N-end rule proteolytic pathway have been reported in Arabidopsis (Gibbs et al. 2014a). This pathway consists in the specific oxidation of the C2 residue of transcription factors of the group VII of Ethylene Response Factors (ERF/AP2) family (ERFVIIs), which is strictly dependent on molecular oxygen and NO, and allows further arginylation, polyubiquitylation and proteasome-mediated degradation of ERFVIIs (Fig. 3) (Gibbs et al. 2014a). This pathway is essential for responses to low oxygen conditions (Gibbs et al. 2014b; Pucciariello & Perata 2017) and has been also proposed to function as a general sensor of abiotic stress (Vicente et al. 2017). ERFVIIs degradation through this pathway begins with the removal of the N-terminal methionine by a Methionine Aminopeptidase (MAP), thus exposing the tertiary destabilizing cysteine residue making it susceptible of direct oxidation (Fig. 3). The specificity of this reaction depends on Plant Cysteine Oxidase 1 (PCO1) and PCO2 enzymes, which catalyze the oxidation of the thiol group in the N-terminal Cys of the substrate to sulphinic acid using O2 and NO as co-substrates (Fig. 3) (Gibbs et al. 2014a; Weits et al. 2014). Once it is oxidized, the Cys acts as a secondary destabilizing residue that becomes substrate for arginylation by Arg-tRNA Transferase (ATE) enzymes (Fig. 3). Transfer of an Arg to the N-terminal residue of the target protein constitutes an N-degron signal for the N-recognin E3 ubiquitin ligase Proteolysis6 (PRT6) to polyubiquitinylate ERFVIIs previous to further degradation by the proteasome (Fig. 3). The N-end rule proteolysis of ERFVIIs takes place only under oxygen-rich conditions, being deactivated under low O2 (Pucciariello & Perata 2017), but it is also strictly dependent on NR-mediated production of NO as ERFVIIs degradation in normoxia is blocked in *nia1nia2* plants mutated in both
Arabidopsis NR proteins (Gibbs et al., 2014a). ERFVIIIs act on ABI5 in NO triggered regulation of ABA signaling in seed germination and stomata closure (Gibbs et al. 2014a), but also control photomorphogenesis-related processes such as hypocotyl elongation (Gibbs et al. 2014a) or apical hook opening (Abbas et al. 2015). This mechanism based on the turnover of regulatory proteins through focused proteolysis by the ATP-dependent proteasome machinery is a conserved regulatory mechanism in eukaryotes, and particularly in plant hormone signaling (Dreher & Callis 2007; Vierstra 2009; Sadanandom et al. 2012; del Pozo & Manzano 2014; Gibbs et al. 2014b; Nagels-Durand et al. 2016).

NO-hormone functional interactions

Besides ERFVIIIs role in sensing NO and regulating multiple aspects of plant physiology, other still not deciphered components are likely involved in mediating NO sensitivity and responsiveness. Many of the NO-regulatory effects are often exerted through interaction with phytohormone signaling pathways, including the five so-called classical plant hormones (gibberellins, auxins, abscisic acid, cytokinins and ethylene) and some of the most recently characterized (brassinosteroids, salicylic acid, jasmonates and strigolactones) both in development and defense against biotic and abiotic stresses (Durbak et al. 2012; Freschi 2013; Simontacchi et al. 2013; Fancy et al. 2017). Numerous plant physiological processes such as seed dormancy and germination, skotomorphogenic and photomorphogenic vegetative development, root growth, stomatal closure, pollination, flowering, fructification or leaf senescence are somehow regulated through NO-hormone interactions (Beligni & Lamattina 2000; He et al. 2004; Bethke et al. 2006; Tsai et al. 2007; Qiao & Fan 2008; Prado et al. 2008; De Michele et al. 2009; Manjunatha et al. 2010; Lozano-Juste & Leon 2011; Arc et al. 2013; Liu & Guo 2013; Du et al. 2014). Moreover, several reports pointed to NO as a relevant
negative regulator of different components of the ABA signaling pathway acting either
by promoting nitration/inactivation of the ABA receptors (Lozano-Juste & Leon 2010;
Castillo et al. 2015) or the S-nitrosylation and degradation of positive regulators such as
OST1/SnRK2.6 (Wang et al. 2015) or ABI5 (Albertos et al. 2015), thus suggesting the
existence of tight regulatory interactions between NO and ABA. Similarly, it has been
reported that the S-nitrosylation of ASK1 is essential for the assembly of the SCF\textsuperscript{TIR1}
complex required for auxin perception and signaling (Iglesias et al. 2018). Actually, the
receptor TIR1 itself is positively regulated also by S-nitrosylation (Terrile et al. 2012).
Also NPR1-triggered salicylate signaling is negatively regulated by S-nitrosylation
favoring cytosolic NPR1 oligomerization (Tada et al. 2008; 2009; Lindermayr et al.
2010).

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

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

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

NO AS A REGULATOR OF DEVELOPMENT AND STRESS-RELATED RESPONSES

The exposure of plants to exogenous atmospheric or soil microorganism-produced NO or endogenously biosynthesized NO alters multiple aspects of their physiology. The major sources of NO in the atmosphere derive from industrial activity and car engines (Skalska et al. 2010). However, release from microbial-related activity in soils is also relevant (Pilegaard 2013). Levels of NO have been increasing continuously in the Earth atmosphere since industrial revolution started (Jaegle et al. 2012).
Considering this tendency, plants may be exposed in the future to relatively high NO concentrations with expected consequences for their growth and development. Due to the spontaneous conversion of NO to NO$_2$ under aerobic conditions, it is frequent to use the term NOx when NO is supplied in an oxygenated environment (Kasten et al. 2017). This topic should be also taken into account when the experimental approaches are based on exogenous NO supply. Because the conversion of NO to NO$_2$ cannot be controlled under normoxic conditions, it is convenient to design experiments minimizing the exposure times and considering the effects triggered by nitrogen dioxide instead of NO.

Regulation of protein targets by post-translational modifications

NO is able to act as a regulator mainly by triggering PTMs in proteins, which may alter their activity, subcellular localization, function, structure or stability. When the affected protein is a transcription factor, PTMs trigger transcriptome changes (Grun et al. 2006; Palmieri et al. 2008; Besson-Bard et al. 2009). A recent study on the effect of the NO donor S-nitrosocysteine on the Arabidopsis transcriptome pointed to around 700 transcription factors potentially involved in multiple physiological processes including hormone signaling, protein degradation, development and responses to multiple stresses (Imran et al. 2018). Table 1 includes some examples of plant transcription factor regulation by NO and their functions in a wide array of developmental and stress-related processes. Most of the reports describing NO-exerted regulation on different targets are based on PTMs such as S-nitrosation (often referred as S-nitrosylation) of thiols and amines; nitration of tyrosine, tryptophan and phenylalanine, ubiquitylation of lysine, phosphorylation of serine, threonine and tyrosine or the oxidation of tyrosine and thiols (Gow et al. 2004; Hess & Stamler 2012). Among these PTMs, the S-nitrosylation of cysteines and the nitration of tyrosines (Fig. 2) are by far the most extensively studied.
NO-related modifications (Astier & Lindermayr 2012). It has been also documented that NO can regulate gene expression through the interaction with and the modulation of secondary messengers such as cGMP, cADP-ribose, Ca^{2+}, and notably with ROS (Durner et al. 1998; Lamotte et al. 2006; Astier et al. 2010; Mur et al. 2013). However, as mentioned above, the functional connection between NO signaling and cGMP and Ca^{2+} regulatory components has been questioned (Astier et al., 2019), thus suggesting that much of the work previously reported on this area has to be carefully re-evaluated.

S-nitrosylation is a redox modification consisting in the reversible covalent binding of NO to the thiol group of a cysteine residue in a target protein, leading to the formation of an S-nitrosothiol (SNO) (Fig. 2) (Astier et al. 2011). This modification is restricted to specific cysteine residues and it is completely dependent on the nature of the surrounding amino acids (Seth & Stamler 2011). However, and to our knowledge, no consensus primary sequences have been identified determining the potential of S-nitrosylation of cysteine residues. GSNO seems to be one of the main donors for the transnitrosylase activity in plants, modulating the total SNO content (Wang et al. 2006; Yu et al. 2012). Nitration of tyrosine residues of proteins is likely the main irreversible reaction caused by nitrating agents such as peroxinitrite (ONOO−). It results in the addition of a nitro group (-NO_{2}) in the ortho position from the hydroxyl group of the aromatic ring of tyrosines, leading to the formation of 3-nitrotyrosine (3-NY) (Schopfer et al. 2003) (Fig. 2). Like S-nitrosylation, tyrosine nitration is also restricted to specific target tyrosine residues (Lozano-Juste et al. 2011), and can trigger conformational changes that lead to the activation or the inhibition of the target proteins (Bayden et al. 2011). However, as for S-nitrosylation no consensus motifs in amino acid primary sequences have been reported to direct nitration of specific residues. NO-related PTMs and the corresponding conformational changes of the modified target proteins seem to
promote the polyubiquitylation and subsequent proteasomal degradation of the affected proteins (Castillo et al. 2015), thus representing and additional link between NO and ubiquitin-proteasome pathway. NO can also interact reversibly with the transition metals (iron, zinc or copper) at the heme center of metalloproteins to form metal-nitrosyl complexes through coordination chemistry (Ford 2010; Astier & Lindermayr 2012). The bound NO group is then susceptible to further nucleophilic or less frequently electrophilic attacks, depending on the protein bounded (Astier & Lindermayr 2012; Toledo & Augusto 2012). The formation of the metal-nitrosyl complex can induce conformational changes that compromise the proper functioning of the affected protein (Ford, 2010; Toledo and Augusto, 2012). Plant hemoglobins (HB), currently called phytoglobins, are the best characterized plant proteins undergoing metal nitrosylation (Gupta et al. 2011). The oxidation of phytoglobins by NO produces nitrate, thus scavenging the NO in what is considered a general mechanism modulating NO bioavailability, regulation and detoxification of NO in plants (Gupta et al. 2011; Igamberdiev et al. 2011). Recently, the nitration of fatty acids by NO has also been demonstrated to be an important part of NO signaling in plants (Mata-Pérez et al. 2017) (Fig. 2). Several mechanisms have been described for nitrofatty acid synthesis but all of them involved radical •NO₂ (Rubbo 2013; Buchan et al. 2018). In plants, the regulatory roles of nitrofatty acids have been proposed to be related to their potential as NO donors and inducers of NO-related PTMs in plants (Mata-Pérez et al. 2017). It has been also suggested that nitrofatty acids reorganize lipid layers in membranes, thus resulting in altered structure and function of membrane associated proteins with diverse signaling functions (Franz et al. 2017).

Regulation of metabolism
In contrast to the increasing knowledge regarding the effect of NO on protein function and gene expression, our current knowledge on the NO impact on global metabolome of plants is scarce and limited to several stress-related processes. In stressed plants, ROS and NO are produced simultaneously. NO seems to alleviate the oxidative status through its antioxidant capacity, thus contributing to the redox homeostasis (Correa-Aragunde et al. 2015). However, extensive evidence suggest that NO may be involved in paradoxical processes exerting sometimes opposing regulatory functions. NO has been described to enhance or reduce the redox status of the plants depending on either acting in a chronic or acute mode (Groß et al. 2013). These effects could be due to multiple factors such as the relative NO cellular concentration, the location where it is produced or the complex interacting microenvironment. Among different subcellular localization where NO production is documented, peroxisomes have been characterized as subcellular locations where hydrogen peroxide and NO metabolisms are interconnected and may serve as a source of signaling molecules (Corpas et al. 2019). On the other hand, chloroplasts are not only a source of NO but an important site of NO action in regulating photosynthesis and oxygen evolving processes (Misra et al. 2014).

Regarding primary metabolism, it has been reported that NO could modulate carbohydrate metabolism at the post-translational level and regulate glutathione and methionine metabolism at the transcriptional level (He et al. 2018). S-nitrosylation modifications led to a decrease in cellular glycolysis enzymes, ATP synthase activities, content of acetyl coenzyme A, ATP, ADP-glucose and UDP-glucose, which all together eventually inhibited polysaccharide-biosynthesis and caused monosaccharide accumulation (Zhang et al. 2017). Accordingly, NO-treated plants displayed less starch granules and increased sugar content (León et al. 2016). Regarding another primary metabolic pathway, nitrate assimilation seems to be also controlled by NO through...
negative transcriptional regulation on NR and high affinity nitrate transporter encoding genes in wheat (Adavi & Sathee 2019), a process that seems to be dependent on the N compound source (Balotf et al. 2018). On the other hand, NR-mediated NO generation increases nitrogen uptake capacity by inducing lateral root formation under partial nitrate nutrition in rice (Sun et al. 2015). Also sulfur assimilation seems to be regulated by NO under stress conditions as mustard plants exposed to cadmium showed a NO-triggered stimulated S-assimilation and GSH production (Per et al. 2017). Besides involvement in regulating primary metabolism, NO also regulates numerous secondary metabolite specific pathways including terpenoid production in Taxus chinensis (Wang & Wu 2004); anthocyanin and flavonoid biosynthesis activated by brassinosteroids (Li et al. 2017a) or by cold acclimation (Costa-Broseta et al. 2018; 2019); and phenylpronaoid biosynthesis and triggered resistance to pathogens (Santos-Filho et al. 2012; Li et al. 2017b). In addition, NO also regulates the production of secondary metabolites of high nutraceutical value (Zhang et al. 2012).

CONCLUSIONS AND PERSPECTIVES

The outstanding increase in plant NO research during the last three decades has opened many paths leading to a significant better knowledge on how NO is produced and sensed, and also on how and where NO exerts regulatory functions. However, a large fraction of the reported work on this topic should be contrasted by using more clear and direct genetic strategies instead of the unspecific and sometimes confusing pharmacological approaches. It is foreseeable that many data derived from pharmacological approaches are revisited in the near future likely helping to overcome some of the current inconsistencies and misunderstandings regarding NO production.
and its mode of action. Particularly important will be the development of the proper
tools allowing the true quantification of endogenous NO in plant cells. These analytical
techniques should be straightforward and non-invasive to allow better and precise
analysis of the endogenous NO in plants.

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Methods in Plant Nitric Oxide (NO) Research: Why Do We Always Need to Use

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Nitric oxide induces monosaccharide accumulation through enzyme S-
FIGURE LEGENDS

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

mARC, mitochondrial Amidoxine Reducing Component; NR, nitrate reductase; NiR, nitrite reductase; NOFNR, NO-forming nitrire reductase; mETC, mitochondrial electron transport chain; NOS, NO synthase; Fed, ferrredoxin; THB1, truncated hemoglobin; XO, xanthine oxidoreductase; NiNOR, root nitrite-NO reductase.

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

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

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

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

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

ERFVIIs, Group VII of the Ethylene Response Factor family of transcription factors; PYR/PYL, pyrabactin resistant like; OST1/SnRK2.6, Open Stomata 1/sucrose non-
fermenting 1-related protein kinase 2-6; ABI5, ABA insensitive 5; PRMT5, type II protein arginine methyltransferase 5; VRN2, PRC2 subunit VERNALIZATION 2; NPR1, non expresser of pathogenesis related 1; TGAs, TGACG motif-binding protein family; AHKs, Arabidopsis His kinases; AHPs, Arabidopsis His phosphotransfers; PRRs, Response regulators; SCFTIR1/AFB, SKP-Cullin-F box Transport Inhibitor Resistant1/Auxin signaling F-Box.
Table 1. Plant transcription factors and processes reported to be targets of NO regulation.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Process</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PIF1, HFR1</td>
<td>NO modulates PHYB-mediated seed germination by repressing PIF1 expression and stabilizing HFR1</td>
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<tr>
<td>SPCH, MUTE, SCRM2</td>
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<tr>
<td>EIN3</td>
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<td>ERFVIIIs</td>
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<tr>
<td>ABI5</td>
<td>S-nitrosylation triggers ABI5 degradation to promote seed germination and seedling growth</td>
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<tr>
<td>ORE1/AtNAC2/ANAC092</td>
<td>Mediates NO-induced cotyledon senescence</td>
<td>Du et al. 2014. J. Exp. Bot.</td>
</tr>
<tr>
<td>PIF3</td>
<td>NO induces PIF3 degradation to inhibit root elongation in light</td>
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<tr>
<td>FIT, bHLH 38, 39, 100, 101</td>
<td>GSNO mediates Fe-deficiency signaling</td>
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<tr>
<td>LeSPL</td>
<td>Contributes to cadmium tolerance by negatively regulating NO production</td>
<td>Chen et al. 2018. Planta</td>
</tr>
</tbody>
</table>
NORMOXIA

\[ \text{NH}_4^+ \rightarrow \text{Fed}_{\text{ox}} \rightarrow \text{NiR} \]

\[ \text{Fed}_{\text{red}} \rightarrow \text{NADH} \rightarrow \text{NAD}^+ \]

\[ \text{NO}_3^- \rightarrow \text{NR} \rightarrow \text{NO}_2^- \]

\[ \text{NADPH} \rightarrow \text{NADP}^+ \]

\[ \text{L-Arg} \rightarrow \text{L-Citrulline} \]

HYPOXIA

\[ \text{NO}_2^- \rightarrow \text{NO}_3^- \rightarrow \text{THB}1 \]

\[ \text{NOS-like?} \rightarrow \text{NADPH} \rightarrow \text{NADP}^+ \]

\[ \text{L-Citrulline} \rightarrow \text{NOS-like?} \]

\[ \text{NADPH} + \text{O}_2 \rightarrow \text{Ni:NOR} \]

\[ \text{Ni:NOR} \rightarrow \text{mETC} \]

\[ \text{NADPH} \rightarrow \text{NADP}^+ \]

\[ \text{NO}_2^- \rightarrow \text{NO} \]

\[ \text{NADPH} + \text{O}_2 \rightarrow \text{NADP}^+ \]

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