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REVIEW PAPER

Nitric oxide production in plants: an update

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Abstract

Nitric oxide (NO) is a key signaling molecule in plant physiology. However, its production in photosynthetic organisms remains partially unresolved. The best characterized NO production route involves the reduction of nitrite to NO via different non-enzymatic or enzymatic mechanisms. Nitrate reductases (NRs), the mitochondrial electron transport chain, and the new complex between NR and NOFNiR (nitric oxide-forming nitrite reductase) described in *Chlamydomonas reinhardtii* are the main enzymatic systems that perform this reductive NO production in plants. Apart from this reductive route, several reports acknowledge the possible existence of an oxidative NO production in an arginine-dependent pathway, similar to the nitric oxide synthase (NOS) activity present in animals. However, no NOS homologs have been found in the genome of embryophytes and, despite an increasing amount of evidence attesting to the existence of NOS-like activity in plants, the involved proteins remain to be identified. Here we review NO production in plants with emphasis on the presentation and discussion of recent data obtained in this field.

Keywords: Arginase, arginine, copper amine oxidase, nitrate reductase, nitric oxide, nitric oxide production, plant.

Introduction

Nitric oxide (NO) is a small molecule that possesses a wide range of physiological functions in living organisms. Although this radical gas was originally described as an air pollutant, it has since been shown to be involved in signaling functions in all living organisms. In plants, NO has been shown to be involved in many physiological processes, such as germination, flowering, or leaf senescence, and in response to environmental stresses (Mur *et al.*, 2013).

The mechanism of action of NO in plants has been investigated for more than two decades. In plants, NO donors or endogenous NO are able to modulate the expression of several genes involved in hormonal signaling, primary metabolism, or stress responses (Besson-Bard *et al.*, 2009; Grün *et al.*, 2006). Acting as a molecular messenger, NO has been shown to interact with the signaling pathways dependent on cGMP, Ca^{2+}, and notably with reactive oxygen species (ROS) (Astier *et al.*, 2010; Mur *et al.*, 2013). Several lines of evidence demonstrated that NO impacts the signaling of many phytohormones involved in developmental as well as in defense processes (Freschi, 2013). At the molecular level, NO has been shown to be responsible for three main specific post-translational modifications (PTMs) of proteins. It can reversibly bind the thiol group of cysteinyl residues leading to their *S*-nitrosation in a so-called *S*-nitrosylation process,

Abbreviations: ADC, arginine decarboxylase; AO, aldehyde oxidase; ARC, amidoxime reducing component; AS, argininosuccinate; AtNOA1, nitric oxide associated 1; BH_4, tetrahydrobiopterin; CaM, calmodulin; eNOS, endothelial NOS; GSNOR, *S*-nitrosoglutathione reductase; iNOS, inducible NOS; mETC, mitochondrial electron transport chain; Moco, molybdenum cofactor; Ni-NR activity, nitrite:NO reductase activity; nNOS, neuronal NOS; NO, nitric oxide; NOFNiR, nitric oxide-forming nitrite reductase; NOS, nitric oxide synthase; NR, nitrate reductase; PA, polyamine; PTM, post-translational modification; ROS, reactive oxidative species; SO, sulfite oxidase; TH_4, tetrahydrofolate; THB1, truncated hemoglobin 1; XO, xanthine oxidase.

impacting the conformation, the activity, or the localization of the target protein. NO can also nitrosylate tyrosine residues in a so-called tyrosine nitration reaction, forming 3-nitrotyrosine, a process which is mainly irreversible. Finally, NO can also interact reversibly with the heme center of metalloproteins, generally resulting in conformational changes that will impact their activity (Astier and Lindermayr, 2012). 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). Similarly to any signaling, the NO pathway in plants is tightly controlled through the action of specific enzymes. The turnover of NO messaging is, for example, dependent on the action of non-symbiotic hemoglobins that can oxidize it to nitrate, as well as on the activity of *S*-nitrosoglutathione reductase (GSNOR) that control the GSNO content in the cell, a major reservoir of NO (Mur *et al.*, 2013).

Despite intensive studies that revealed its variety of functions and reactivity in photosynthetic organisms, NO production in plants is still not fully understood and remains one of the most challenging issues of the field. NO synthesis in plants can be schematically achieved via two main routes defined by their chemical properties, one reductive and one oxidative. The reductive pathway is based on the reduction of nitrites to NO, while the oxidative route relies on the oxidation of aminated molecules.

In this review, we provide an update about NO production in plants in general, reviewing the overall and recent data available concerning this question.

Reduction of nitrite

NO can originate from different routes and substrates in living organisms. One of the synthesis routes concerns the reduction of nitrite. To date, this reductive route is the most firmly described and evidenced synthesis pathway for NO in plants.

Non-enzymatic reduction of nitrite

The reduction of nitrites to NO can occur non-enzymatically in particular conditions, such as low pH or highly reducing environments, when high concentrations of nitrate are present. These specific situations can happen, for example, in the apoplast of barley aleurone layers. Although such conditions are rarely encountered, they can lead to an efficient and rapid production of NO from nitrite (Bethke *et al.*, 2004).

Nitrate reductase

In addition to this non-enzymatic reduction, several proteins have been described to catalyze the production of NO from nitrites.

Nitrate reductase (NR) is a multifunctional cytoplasmic enzyme involved in nitrogen assimilation and metabolism. It is responsible for the first rate-limiting step of nitrate assimilation by catalyzing the reduction of nitrate to nitrite using NADH as an electron donor. The active enzymatic homodimeric complex requires the presence of molybdopterin, heme, and FAD as cofactors (Campbell, 2001). Interestingly, in addition to this primary NR activity, this enzyme has also been described to possess a nitrite:NO reductase activity (Ni-NR activity; Yamasaki *et al.*, 1999; Yamasaki and Sakihama, 2000; Rockel *et al.*, 2002). This second reaction is relatively low and represents only 1% of the nitrate-reducing capacity of NR in normal conditions. Whereas the NR K_m for nitrite is notably higher than that for nitrate (100 µM compared with 10 µM), the Ni-NR activity requires a nitrite accumulation to occur and is efficiently inhibited by nitrates (K_i 50 µM). However, the reaction can be promoted by specific conditions such as anoxic or acidic environments, which leads to a substantial production of NO, from 2 nmol gFW^{-1} h^{-1} to 200 nmol gFW^{-1} h^{-1} *in vivo* (Rockel *et al.*, 2002; Meyer *et al.*, 2005). Despite these specific requirements, the importance of NO production by NR in plant physiology has been clearly demonstrated using both pharmacological and genetic approaches (Wilson *et al.*, 2008; Mur *et al.*, 2013). For example, the use of *Arabidopsis thaliana* NR-impaired lines *nia1*, *nia2*, or the double mutant *nia1/nia2*, revealed the crucial role of NR-dependent NO production in various processes, such as stomatal movements (Desikan *et al.*, 2002; Hao *et al.*, 2010), hormone responses (Kolbert and Erdei, 2008; Hao *et al.*, 2010), salt, osmotic, or cold stress responses (Zhao *et al.*, 2009; Kolbert *et al.*, 2010; Xie *et al.*, 2013), and floral or root development (Seligman *et al.*, 2008; Méndez-Bravo *et al.*, 2010; Lombardo and Lamattina, 2012).

The new nitric oxide-forming nitrite reductase

The crucial role of NR in NO signaling in plants has been reinforced by recent works. Indeed, another NO-producing mechanism by NR has been unraveled in *Chlamydomonas reinhardtii*. In this unicellular alga, it has been shown that NR can interact with the partner protein NOFNiR (nitric oxide-forming nitrite reductase) to produce NO from nitrite. NOFNiR belongs to the amidoxime reducing component (ARC) protein family. Although ARC proteins were initially described from the animal field for reducing amidoxime prodrugs to their corresponding amino form *in vitro*, their exact physiological role *in vivo* is not fully understood (Havemeyer *et al.*, 2006). In *C. reinhardtii*, it has been demonstrated that NOFNiR can reduce nitrite to NO in an NAD(P)H reaction using electrons provided by the diaphorase activity of NR. Interestingly, this NO-producing activity occurs in normoxia and is not inhibited by nitrate, in contrast to the Ni-NR activity. In addition, the gene expression patterns and enzymatic activity of the two components of this system have been shown to correlate (Chamizo-Ampudia *et al.*, 2016, 2017). Interestingly, the *A. thaliana* genome contains two genes for ARC protein (Chamizo-Ampudia *et al.*, 2016), one of them presenting an NO-producing activity *in vitro* (Yang *et al.*, 2015). The determination of the existence of an NR:NOFNiR system in higher plants, similar to what is found in *C. reinhardtii*, would provide some information about the ubiquity

of this system in the green lineage and could better explain the crucial role of NR observed in plant NO production.

In parallel, the same team demonstrated that in addition to NOFNiR, NR could also associate with the truncated hemoglobin 1 (THB1) of *C. reinhardtii*. NR reduces THB1 through its diaphorase activity, which becomes active and can efficiently convert NO to nitrate in the presence of oxygen (Sanz-Luque *et al.*, 2015). This apparent contradictory role of NR in NO signaling, participating both in the production and the turnover of this signaling molecule, is actually coherent when considered in the light of the complex regulation of the nitrate cycle of the alga (reviewed by Calatrava *et al.*, 2017). However, this complexity highlights the importance of defining further the precise physiological role of NO produced by this specific system, considering its potential involvement in developmental or defense processes.

Plasma membrane-bound NR

In addition to the involvement of the cytoplasmic NR, the participation of a membrane-bound nitrite reductase (Ni:NOR) in the production of NO in plants has been reported. Using membrane fractions from tobacco roots, nitrite-dependent NO production was measured and attributed to a putative Ni:NOR that is yet to be identified (Stöhr *et al.*, 2001). This membrane-bound protein would be exclusively found in roots, and produces NO from nitrite in the apoplasm of the cells, using NAD(P)H as electron donor. Its activity is dependent on low oxygen pressure and it would function together with an apoplastic membrane-bound NR that would provide nitrite from nitrate (Stöhr and Ullrich, 2002; Stöhr and Stremlau, 2006). Further work suggested a role for this Ni-NOR-produced NO in the mycorrhizal colonization of tobacco roots (Moche *et al.*, 2010).

Role of other molybdoenzymes

Both NR and NOFNiR display the presence of a molybdenum cofactor (Moco) in their structural features. In plants, other Moco-containing enzymes exists, namely xanthine oxidases (XOs), aldehyde oxidases (AOs), and sulfite oxidases (SOs), and they have been shown potentially to possess an NO-producing activity from nitrite.

XO is a highly conserved enzyme described initially in mammals as being responsible for purine catabolism, and hydroxylating hypoxanthine to xanthine and xanthine to urea. In plants, two XOs have been shown to contribute to ROS homeostasis during biotic stress, by generating both superoxide anions that contribute to the ROS burst and ureic acid involved in H_2O_2 removal in chloroplast (Yesbergenova *et al.*, 2005; Ma *et al.*, 2016). The potential nitrite reduction capacity of mammalian isoforms under anaerobic conditions has been documented *in vitro* for several years (Maia and Moura, 2015). In white lupine roots, a pharmacological approach using allopurinol, an inhibitor of XO, resulted in an inhibition of NO accumulation during development, suggesting a potential role for XO in this mechanism *in vivo* (Wang *et al.*, 2010). However, the data concerning a potential *in vivo* role for XO in NO production in plants are scarce, and no NO emission from recombinant protein could be evidenced *in vitro* (Planchet *et al.*, 2005).

A structurally close enzyme related to XO is AO. AOs are cytoplasmic enzymes that generally catalyze the oxidation of aldehydes to carboxylates, producing superoxide anions. Its nitrite reduction activity has also been confirmed under anaerobiosis for several mammalian homologs *in vitro* (Maia and Moura, 2015). In plants, AOs participate in the synthesis of phytohormones such as abscisic acid (ABA) or indole-3-acetic acid (IAA), and contribute to ROS production (Zarepour *et al.*, 2012; Yergaliyev *et al.*, 2016), therefore being important for developmental processes and defense responses. However, no information is available about their NO-producing capacity *in vivo* in plants.

A last member of the Moco-containing enzyme family in plants is SO. SO is also a conserved enzyme, found in the peroxisomes, that catalyzes the oxidation of sulfite to sulfate, by an O_2-dependent mechanism (Eilers *et al.*, 2001). Its capability to reduce nitrite to NO has been quite recently demonstrated *in vitro* for the human isoform under anoxia, but this reaction requires more specific conditions and is less potent than the one observed for mammalian XO and AO (Wang *et al.*, 2015). In plants, the role of SOs is mainly assumed to concern the removal of toxic sulfite in the cell (Yarmolinsky *et al.*, 2013). Similarly to AOs, its involvement in nitrite reduction *in planta* has not been addressed yet.

Mitochondrial electron transport chain

In addition to the mechanisms described above, NO can be produced from nitrite through the action of the mitochondrial electron transport chain (mETC) in plants. After pioneer works demonstrating that nitrite-dependent NO formation could be prevented by mETC inhibitors in algae and tobacco (Tischner *et al.*, 2004; Planchet *et al.*, 2005), mETC-dependent NO production has been demonstrated in various species of plants such as pea, tobacco, and barley (Gupta *et al.*, 2005; Gupta and Kaiser, 2010). This reaction was located to the membrane of the mitochondria, involving mainly complex III and IV. It is determined by the availability in nitrite (K_m of 175 µM) and requires anaerobic conditions, as oxygen can readily inhibit the reaction (K_i of 0.6 µM). This reaction therefore is restricted to tissues exposed to hypoxia such as roots, and its occurrence can be explained by the requirement for an electron acceptor to preserve respiration, when oxygen is lacking (Gupta and Igamberdiev, 2011). In addition to metabolism preservation and allowing a correct functioning of mitochondria, this mETC-dependent NO production has also been suggested to be involved in signaling regulation processes (Palmieri *et al.*, 2010).

NOS-like activity in plants

In addition to the reductive pathway from nitrite, several lines of evidence demonstrate the existence of an oxidative route for NO production in plants, similar to the main pathway described in animals.

In mammals, although a reductive route by molybdenum-containing enzymes or non-enzymatically has recently been highlighted under acidic/reducing environments (Maia and Moura, 2015), the production of NO is principally achieved through the enzymatic activity of specialized enzymes: the nitric oxide synthases (NOSs).

These enzymes catalyze the formation of L-citrulline and NO from L-arginine through double mono-oxygenation. They work as homodimers, and contain schematically two main parts, the N-terminal domain possessing an oxidative activity and the C-terminal domain presenting a reducing activity. These two domains are linked to a calmodulin (CaM)-binding site. The binding of CaM triggers a structural change of the homodimer required for the enzymatic activity. This activity also needs the presence of several cofactors: FMN, FAD, and tetrahydrobiopterin (BH_4). Additionally, the reaction requires electrons from NADPH and the presence of oxygen (Förstermann and Sessa, 2012).

Several NOS isoforms have been characterized in animals, which possess specific features. In humans, three main isoforms have been studied. Two of them are constitutive, the endothelial NOS (eNOS) and the neuronal NOS (nNOS), with the other one being inducible (iNOS). The eNOS and nNOS activity requires the presence of a Ca^{2+}-loaded CaM. Their activation leads to a quick, short, and relatively small release of NO (pmol min^{-1} mg^{-1} NOS), classically associated with NO signaling-dependent cellular processes. The iNOS does not require the presence of a Ca^{2+}-loaded CaM for activation, and leads to a stronger and long-lasting release of NO (nmol min^{-1} mg^{-1} NOS). Its activation is involved generally in immune responses or pathology, where NO acts as a cytotoxic agent (Förstermann and Sessa, 2012).

NOS in plants

With the identification of NO as a crucial mediator of physiological processes in plants in the late 1990s, several studies sought to determine NO sources in the plant kingdom, primarily aiming to identify and characterize NOS homologs. Two main candidates have been described. The biochemical purification of a NOS-like activity from kilograms of tobacco leaves led to the identification of a P variant of the glycine decarboxylase complex. Unfortunately, further studies demonstrated that this protein does not produce NO; consequently, the corresponding articles were retracted. Another approach undertaken in *A. thaliana* led to the identification of a candidate that presented homology to an enzyme implicated in NO synthesis in the snail *Helix pomatia* (Guo *et al.*, 2003). Investigations demonstrated that the corresponding mutant displays an impaired NO content. However, the enzyme, initially named AtNOS1 (nitric oxide synthase 1), was further characterized as a functional small GTPase and therefore renamed AtNOA1 (nitric oxide associated 1; Moreau *et al.*, 2008). It is noteworthy that even if the mechanism underlying its impaired NO production is unclear, the *Atnoa1* mutant is used as a general NOS-like impaired tool, leading to an increasing amount of data referring to the study of a NOS-like activity in plant. These two unsuccessful examples of plant NOS identification drove the community to question seriously their existence in the green lineage (Zemojtel *et al.*, 2006; Fröhlich and Durner, 2011).

However, in the beginning of 2010, the first NOS from the plant kingdom was characterized in the green algae *Ostreococcus tauri* (Foresi *et al.*, 2010). This enzyme was identified by sequence homology to the human NOS, with ~43% similarity to the eNOS sequence. The cofactor-binding sites for FAD, FMN, BH_4, and CaM are present, as well as L-arginine and an NADPH-binding site. This enzyme was shown to produce NO from L-arginine similarly to the animal NOSs. Its importance in light irradiance stress responses was also demonstrated in the *O. tauri* model (Foresi *et al.*, 2010). These results demonstrated the possibility of the presence of an endogenous and functional NOS in plants, with an actual role in plant physiology.

The first description of a canonical NOS from the plant kingdom was recently completed with an extensive analysis of the transcriptomes and genomes of >1300 species of plants, looking for the presence of NOS homologs (Jeandroz *et al.*, 2016). These authors screened the 1000 Plants (1KP) international multidisciplinary consortium's transcriptome database and the publicly available algal genome sequences, using the OtNOS and nNOS from human as templates. They could highlight 15 complete sequences presenting enough similarity with templates to be identified as NOS, all belonging to algal species. The identified sequences contain the key features of NOS, and the binding sites for NOS cofactors are conserved. The oxidative domain, especially in its N-terminal part, presents some diversity in the different candidates identified that could impact the dimerization of the enzyme or the binding of BH_4. This hypothesis is reinforced by the fact that OtNOS uses tetrahydrofolate (TH_4) instead of BH_4 to accomplish the enzymatic reaction *in vitro* and *in vivo* (Foresi *et al.*, 2010, 2015), and that the screen of 1KP database reveals the absence of the enzymes responsible for BH_4 synthesis in plants. First structural and phylogenic analyses of these plant NOS candidates show that the activity is likely to be achieved independently of Ca^{2+}, and demonstrate the presence of a diversity of structures that may result in a variety of functions (Jeandroz *et al.*, 2016; Santolini *et al.*, 2017). These observations raise the question of their role in algal physiology and constitute a promising new aspect of research to better understand the role of NO in the plant kingdom in general.

If these recent data confirmed the existence of NOS in several photosynthetic organisms, they also show that no homologs of NOS sequence can be found in any of the >1000 transcriptomes of land plants screened (Jeandroz *et al.*, 2016). These results, together with the unsuccessful attempts to purify candidates, tend to demonstrate that canonical NOSs probably do not exist in embryophytes. According to the phylogeny, it is likely that the NOS gene was transmitted from a common ancestor before the formation of the eukaryotic supergroup, and was later lost in land plants, the NOS from algae being the remaining testimony of these events (Jeandroz *et al.*, 2016; Santolini *et al.*, 2017).

The confirmation of the absence of canonical NOS in land plants raises the question of the relevance of a NOS activity in plants. Indeed, several studies carried out in plants suggested the existence of an oxidative route for NO production, so-called NOS-like activity.

Measurements of NOS-like activity in plants

Originally, the assumption that NOS would be present in plants comes from the measurement of NOS-like activity in plant tissues. Pioneer studies carried out in the mid-/late 1990s attested to the presence of this NOS-like activity in several plant models, such as maize, pea, tobacco, and lupine (Cueto *et al.*, 1996; Ninnemann and Maier, 1996; Durner *et al.*, 1998; Barroso *et al.*, 1999; Ribeiro *et al.*, 1999). It is noteworthy that these original works use the same technique to measure NOS activity: the citrulline-based assay. The principle of this technique is to follow the conversion of radiolabeled arginine provided as a substrate to radiolabeled citrulline (Bredt and Snyder, 1989). The reaction mixture generally contains all the common NOS cofactors and, after incubation, is applied to a cation exchange chromatography column that will retain the positively charged arginine but not citrulline. The radioactivity in the flowthrough is then assumed to refer to the converted citrulline, and its count theoretically directly correlates with the NOS activity present in the sample. However, this assay does not identify citrulline as a product, and its relevance to follow NOS-like activity in plants was seriously questioned (Tischner *et al.*, 2007). It was actually demonstrated that the arginine-dependent activity measured from *A. thaliana* leaf extracts using the citrulline assay in normal conditions was mainly producing argininosuccinate (AS) rather than citrulline. Indeed, primary metabolism in plants differs from that in animals, and arginine can be metabolized in several different pathways, including through the action of AS lyase resulting in the measurement of AS formation (Fig. 1). These results highlight the caution needed in the transposition and interpretation of techniques used from other fields.

Nevertheless, the presence of NOS-like activity was later confirmed in plants using other techniques that directly measure the production of NO, such as chemiluminescence assay (Corpas *et al.*, 2004, 2006; Valderrama *et al.*, 2007; Chaki *et al.*, 2009) or EPR (Caro and Puntarulo, 1999; Pagnussat *et al.*, 2002; Dordas *et al.*, 2004; Simontacchi *et al.*, 2004; Jasid *et al.*, 2006, 2008). These activities were referred to as NOS-like activity as they were reported to be strictly dependent on the presence of arginine and NADPH, and several NOS co-factors. The localization of this NOS-like activity has been proposed, such as in chloroplasts or peroxisomes (Barroso *et al.*, 1999; Jasid *et al.*, 2006; Corpas and Barroso, 2014), but a clear picture is yet to be obtained regarding the enzymatic activity. More importantly, the corresponding enzymes remain to be identified.

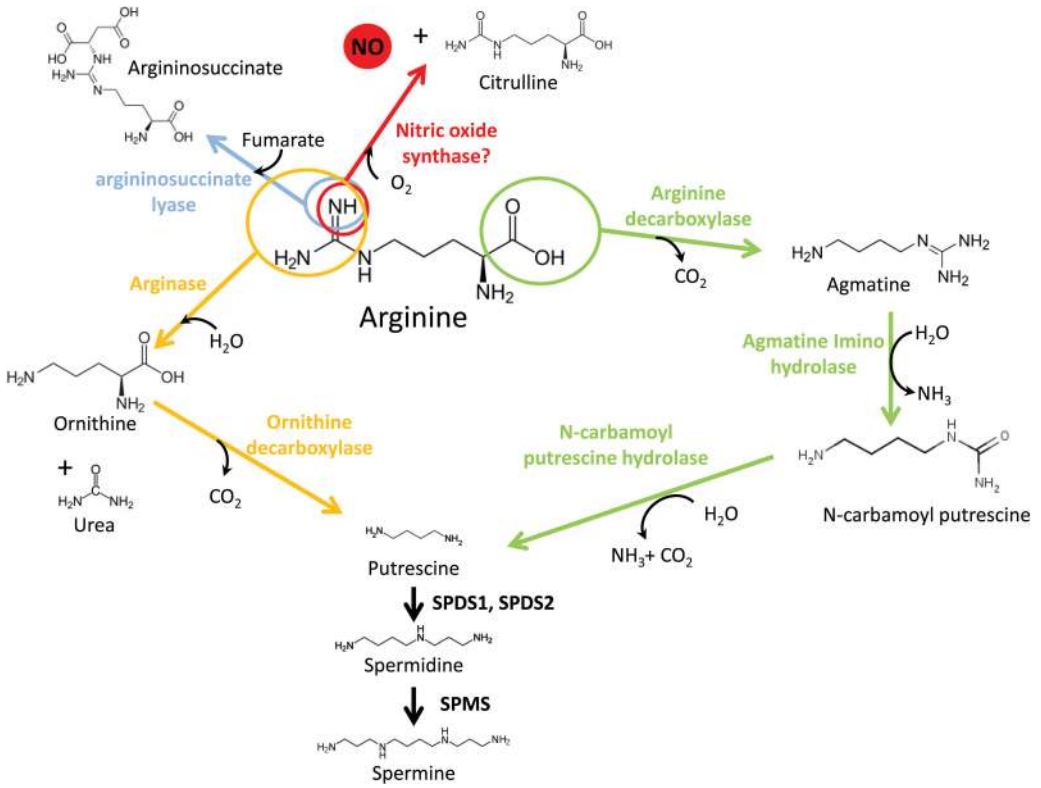

Fig. 1. Schematic representation of the principal arginine metabolism pathways in plants. Arginine can be the substrate for several enzymes. Argininosuccinate lyase can generate argininosuccinate from arginine and fumarate. The guanylyl group of arginine can also be processed by arginase to ornithine and urea, or possibly by a NOS-like activity to citrulline. Arginine decarboxylase is another enzyme using arginine as a substrate, metabolizing it to agmatine. Arginine is the precursor of the principal polyamines in plants, such as putrescine, spermidine, and spermine. SPDS, Spermidine synthase; SPMS, Spermine synthase.

Table 1. *Summary of principal reports using NOS inhibitors in plant systems*

Organism	Tissue/cell type	Inhibitors applied (concentration μM)	NO production inhibition (%)	Technique used for NO production monitoring	Reference
Arabidopsis thaliana	Root	L-NAME (100)	75	DAF	Tossi *et al.* (2013)
Arabidopsis thaliana	Leaf	L-NAME (25)	75	DAF	Hao *et al.* (2010)
Arabidopsis thaliana	Leaf/root	L-NAME (200–1000)	50–80	Citrulline assay/DAF	Guo *et al.* (2003)
Arabidopsis thaliana	Root	L-NAME (5000); AG (2000)	Strong (not quantified)	DAR-4MAM	Corpas *et al.* (2009)
Arabidopsis thaliana	Leaf	L-NAME (3000); L-NNA (300)	100; 100	DAF	Ji *et al.* (2016)
Arabidopsis thaliana	Leaf	L-NAME (300); L-NNA (300)	100; 100	DAF	Zhao *et al.* (2009)
Arabidopsis thaliana	Root	L-NMMA (1000)	0	DAF	Kolbert *et al.* (2010)
Arabidopsis thaliana	Leaf	L-NNA (10000)	85	Citrulline assay/DAF	Zhao *et al.* (2007)
Brassica rapa	Root	L-NMMA (200)	90		Chen *et al.* (2014)
Chorispora bungeana	Cell suspension	L-NAME (300)	100	Oxyhemoglobin assay/ Greiss assay	Liu *et al.* (2010)
Cucurbita maxima × *C. moschata*	Seedling	L-NAME (200)	80	DAF	Li *et al.* (2017)
Elymus nutans	Leaf	L-NNA (150)	100		Fu *et al.* (2015)
Glycine max	Chloroplast	L-NAME (5000); L-NNA (5000)	100; 100	EPR	Jasid *et al.* (2006)
Glycine max	Cotyledon	L-NIL (3000)	30	Citrulline assay	Modolo *et al.* (2002)
Helianthus annuus	Hypocotyl	AG (5000); L-NMMA (1000)	100; 100	Ozone chemiluminescence	Chaki *et al.* (2011)
Hibiscus moscheutos	Root	L-NNA (10000)	40	Citrulline assay/DAF	Tian *et al.* (2007)
Lupinus albus	Root	L-NAME (1000); L-NMMA (1000)	50; 50	Citrulline assay	Cueto *et al.* (1996)
Lycopersicon esculentum	Seedling	L-NAME (200)	70	DAF	Diao *et al.* (2016)
Malus domestica	seed	L-NAME (300)	100	Oxyhemoglobin assay/DAF	Krasuska *et al.* (2016)
Nicotiana benthamiana	Leaf	L-NAME (200)	50	DAF	Deng *et al.* (2016)
Nicotiana tabacum	Leaf	L-NAME (5000)	100	DAF	Zhang *et al.* (2011)
Nicotiana tabacum	Cell suspension	L-NAME (10000)	55	DAF	Lamotte *et al.* (2004)
Nicotiana tabacum *Xanthi*	Cell suspension. Extracts	L-NMMA (1000)	37	Citrulline assay	Durner *et al.* (1998)
Nicotiana tabacum	Leaf	L-NMMA (na)	50	DAF	Foissner *et al.* (2000)
Olea europaea	Leaf	AG (1000)	100	Ozone chemiluminiscence	Valderrama *et al.* (2007)
Paulownia tomentosa	Pollen tube	L-NAME (50)	100		He *et al.* (2007)
Pennisetum glaucum	Seedling	L-NAME (10000)	50	DAF	Manjunatha *et al.* (2009)
Pinus bungeana	Pollen tube	L-NNA (45)	40	DAF	Wang *et al.* (2009)
Pisum sativum	Leaf extract	AG (1000)	70	Ozone chemiluminescence	Corpas *et al.* (2008)
Pisum sativum	Plant	L-NAME (1000); AG (2000)	55; 85	DAF/EPR	Corpas *et al.* (2004)
Pisum sativum	Extract	L-NAME (1000); AG (1000); L-NMMA (1000); L-NIL (1000)	90; 100; 88; 59	Citrulline assay	Barroso *et al.* (1999)
Scutellaria baicalensis	Cell suspension	L-NNA (100)	100	DAF	Zhang *et al.* (2014)
Solanum lycopersicum	Root	L-NAME (20)	50	DAF	Negi *et al.* (2010)
Solanum lycocarpum	Root	L-NAME (500)	90		Jin *et al.* (2011)
Vicia faba	Leaf	L-NAME (1000)	100		Garcia-Mata and Lamattina (2007)
Vicia faba	Leaf	L-NAME (25)		DAF	Yan *et al.* (2007)
Zea Mays	Seedling	L-NAME (100)	35	DAF	Tossi *et al.* (2009*a*)
Zea mays	Leaf	L-NAME (200)	80	DAF	Sang *et al.* (2008*b*)
Zea mays	Leaf	L-NAME (na)	70		Tossi *et al.* (2009*b*)
Zea mays	Leaf/root	L-NAME (3000); AG (3000)	30; 30	Citrulline assay	Ribeiro *et al.*, 1999)
Zea mays	Leaf	L-NAME (200)	71		Sang *et al.* (2008*a*)

L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NNA, L-N^{ω}-nitroarginine; L-NMMA, N^{G}-monomethyl- L-arginine; L-NIL, N^{6}-(1-iminoethyl)- L-lysine; AG, aminoguanidine; DAF, diaminofluorescein; DAR, diaminorhodamine.

Pharmacological approaches: use of inhibitors

Another substantial part of the work providing evidence for the presence of a NOS-like activity in plants comes from the analyses of NOS inhibitor effects in plant systems. NOS inhibitors are mainly arginine analogs, which compete for the active site of the enzyme. For the last two decades, NOS inhibitors were used in various conditions on various plant models. The compilation of the data available concerning the use of NOS inhibitors in plants reveals a very strong variation in the effect observed (Table 1). The discrepancy between the different

concentrations used (from 25 µM to up to 10 mM) and the effectiveness of the inhibition recorded (from 0 to 100% inhibition, sometimes considering the same model) highlights the complexity of studying the NO-producing system in plants.

Another concern to be raised in the use of these NOS inhibitors is regarding their specificity. It is well defined that analogs of arginine can impact the activity of several enzymes (Víteček *et al.*, 2012). As an example, aminoguanidine can efficiently inhibit amine oxidase enzymes in plants (Planas-Portell *et al.*, 2013). Considering also that the main results characterizing the NOS-like activity in plants are obtained using complex systems such as crude extract, it cannot be excluded that the observed NO production impairment comes from an indirect enzymatic mechanism. Moreover, in the absence of their target(s), inhibitors can display enhanced off-target effects. The determination of the precise inhibitor target(s) is therefore a prerequisite for the correct interpretation of their observed effects. For all these reasons, these pharmacological approaches must be interpreted with caution.

Nevertheless, these pharmacological approaches share a consistency in the potential of a NOS inhibitor to prevent NO production in general, in different plant species and plant cell types. They also show that using different NO-monitoring techniques favors the existence of NOS-like activity in plants.

Heterologous expression of NOS and hydroxylamine oxidation in plants

In addition to the direct measurement of NOS-like activity in plants, additional approaches provide hints confirming its existence.

Genetic constructs aiming to express NOS in plants have been generated. The expression of recombinant nNOS from rat resulted in higher NO content, observed in *A. thaliana*, tobacco, and rice, correlated with higher resistance to biotic and abiotic stresses (Chun *et al.*, 2012; Shi *et al.*, 2012; Cai *et al.*, 2015). Similarly, expression of the OtNOS for algae in *A. thaliana* resulted in a functional enzyme producing NO *in planta*, correlated with a better germination and a tolerance to salt, oxidative, and drought stresses (Foresi *et al.*, 2015). Taken together, these approaches demonstrate that the cofactors and conditions required for a functional NOS activity are present in plants, arguing in favor of the existence of the oxidative NO production route.

In the same direction, *in vitro* experiments conducted on tobacco cell suspensions demonstrated that plants possess the ability to oxidize hydroxylamines to NO (Rümer *et al.*, 2009). However, the occurrence of this substrate in the natural physiology of plants is questionable and the involved enzyme has yet to be identified.

Arginine metabolism and NO oxidative production route in plant

Measurements of NOS-like activity and the use of NOS inhibitors have suggested an arginine-dependent NO production pathway in plants. Several other works have also linked arginine-dependent metabolism with NO signaling in photosynthetic organisms. As an example, the commonly used mutant *Atnox1*, impaired in the expression of a chloroplast phosphoenolpyruvate/phosphate translocator, presents elevated levels of arginine correlated with a constitutive overproduction of NO (He *et al*, 2004; Frungillo *et al.*, 2014). In plants, the arginine pool depends on the activity of several enzymes that use it as a substrate (Fig. 1).

Arginases are enzymes that catalyze the conversion of arginine into ornithine and urea reacting with the guanidyl group of the amino acid. Two isoforms are found in *A. thaliana*; both have been localized to the mitochondria (Flores *et al.*, 2008). Interestingly, genetic approaches demonstrated that *A. thaliana* mutants impaired in arginase expression display an increased NO content correlated with higher putrescine and spermine levels (Flores *et al.*, 2008; Shi *et al.*, 2013). Conversely, overexpression of arginase led to a decreased NO production and putrescine and spermine levels, which correlated to a susceptibility to abiotic stresses (Shi *et al.*, 2013). Recently, the involvement of a higher arginase activity impacting the arginine pool was found to be responsible for the impaired NO production and developmental phenotype observed in the *A. thaliana* mutant for the copper amine oxidase 8 (CuAO8), an enzyme involved in polyamine (PA) catabolism (Groß *et al.*, 2017). Similar results were obtained in cotton where an increased arginase activity due to the overexpression of the rice arginase gene resulted in decreased NO production that correlated with the developmental phenotype in roots (Meng *et al.*, 2015).

Arginine decarboxylases (ADCs) are enzymes responsible for the formation of agmatine through the decarboxylation of arginine. This constitutes the first step of the unique PA synthetic route in *A. thaliana.* Two isoforms are also found in *A. thaliana*, both being chloroplastic (Borrell *et al.*, 1995). Interestingly, transient overexpression of the pepper ADC1 resulted in an increased NO accumulation in tobacco cells, together with an accumulation of PAs (Kim *et al.*, 2013). Accordingly, the *A. thaliana* mutant *adc2.1* was impaired in melatonin- or iron deficiency-induced NO accumulation, correlated with PA accumulation deficiency (Zhou *et al.*, 2016)

PAs are found in all living kingdoms. The most common PAs found in plants are putrescine, spermine, and spermidine containing two, three, and four amine groups, respectively. These molecules have been shown to be involved in a wide range of physiological mechanisms in plants, from development to stress responses (Tiburcio *et al.*, 2014; Liu *et al.*, 2015). Over the last 15 years, several works reported that exogenous application of PAs results in NO production in several plant models (Tun *et al.*, 2006; Yang *et al.*, 2014; Diao *et al.*, 2016; Zhou *et al.*, 2016). In agreement with these data, *A. thaliana* mutants impaired in the expression of two different enzymes regulating PA catabolism, CuAO1 and CuAO8, presented an altered NO production (Wimalasekera *et al.*, 2011*a*, *b*; Groß *et al.*, 2017). It is important to note that arginine is the precursor of PA synthesis, connecting their metabolism (Fig. 1).

Taken together, these data strengthen the link existing between arginine metabolism and NO in plants, favoring

the existence of an oxidative NO production route in higher plants, even if the enzymes responsible for this potential activity remain to be identified.

Concluding remarks

The determination of NO sources in plants has clearly been and remains a challenging issue of the field. The intensive studies carried out over the last decade depict the emergence of a complex system where several players are involved. It is now apparent that nitrite reduction is the main source of NO. The recent findings concerning the nitrite reduction through the association of NR and NOFNiR proteins in *C. reindhartii* open up an interesting aspect of research to determine if this mechanism is also present in higher plants. More generally, the in-depth characterization of the other Moco-containing proteins could provide information on their role in the reductive NO production route.

The amount of data regarding the oxidative NO production pathway in plants has also accumulated in recent years. The identification of a dozen NOSs restricted to the algal genome is surprising and interrogative. The characterization of their activity and the corresponding impact on algal metabolism could help to better define the physiological role of NO in these models. On the other hand, it is now clear that no canonical NOSs are present in embryophyte transcriptomes. However, several pieces of evidence reported in this review are in favor of the existence of NOS-like activity. This activity is dependent on arginine, or at least the arginine metabolic pathways. The apparent contradiction between the measurement of NOS-like activity and the absence of NOS in higher plants could be explained by the requirement for protein complex formation to bring different polypeptides required to reconstitute a full NOS activity into close proximity, similarly to what is observed for NR:NOFNiR, as recently suggested (Corpas and Barroso, 2017). The identification and characterization of the proteins involved and the precise substrate/cofactors needed are a prerequisite for a better understanding of NO formation in plants.

Taken together, the data available on NO production in plants reveal a deep complexity and diversity. The specificity of each source needs clarification as well as a better determination of the enzymes involved in its production. These constitute an important and promising aspect of research for a better comprehension of NO physiological function in plants.

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