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Nitric oxide: a multitasked signaling gas in plants

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Short Summary: Nitric oxide (NO) is a gaseous reactive oxygen species that has evolved as a signaling hormone in many physiological processes in plants. Despite all these effects, the fundamental knowledge of NO production, sensing and transducing in plants remains inadequately characterized. In this review we cover the current understanding of NO production, perception and action, with a special focus on the importance of NO in cell-cell communication during developmental processes, sexual reproduction, pathogen defense and other abiotic responses.
Abstract

Nitric oxide (NO) is a gaseous reactive oxygen species that has evolved as a signaling hormone in many physiological processes in animals. In plants it has been demonstrated to be a crucial regulator of development, acting as a signaling molecule present at each step of plant life cycle. NO has also been implicated as a signal in biotic and abiotic responses of plants to environment. Remarkably, despite this plethora of effects and functional relationships, the fundamental knowledge of NO production, sensing and transducing in plants remains largely unknown or inadequately characterized. In this review we cover the current understanding of NO production, perception and action in different physiological scenarios. We specially address the issues of enzymatic and chemical generation of NO in plants, NO sensing and downstream signaling, namely the putative cGMP and Ca\(^{2+}\) pathways, ion channel activity modulation, gene expression regulation, and the interface with other reactive oxygen species (ROS) which can have a profound effect on both NO accumulation and function. We also focus on the importance of NO in cell-cell communication during developmental processes, sexual reproduction, namely in pollen tube guidance and embryo sac fertilization, pathogen defense and other abiotic responses.

Keywords: Nitric Oxide (NO), Reactive Oxygen Species (ROS), Plant Sexual Reproduction, Cell Communication, pollen
Introduction

NO is thought to be an ancient molecule in the history of life on earth and its involvement in counteracting the rise of atmospheric levels of $O_2$ and increased levels of ozone ($O_3$) has been hypothesised (Feelisch and Martin, 1995). The biological consequences of such evolutionary step must have been far reaching: as NO does not require a carrier to cross membranes and reach intracellular targets, and diffuses very fast due to its gaseous nature, it is possible that a cellular signaling system between cells could have evolved before the existence of canonical cellular receptors. Regarding the biological origin of NO, it is possible that the pathway for its production derived from mechanisms of denitrification or nitrification. The very fundamental nature of these facts is perhaps suggestive that the ubiquity of NO functions in prokaryotic and eukaryotic life organization might have been one of the first biological signaling mechanisms (Feelisch and Martin, 1995). Being a free radical, or reactive oxygen species (ROS), NO functions as a gasotransmitter diffusible multitasked messenger, that was first described in mammals, where it plays variable functions ranging from neurotransmission, blood vessels relaxation, immune defense responses and participating in the fertilization process (Zhou and Zhu, 2009). ROS are a large group of molecules and those that contain nitrogen (e.g. NO) form a sub-group that sometimes is also referred to as reactive nitrogen species or RNS.

Several reports suggest that a moderate amount of NO production is essential for animal fertilization and early embryo development (e.g. Kim et al., 2004). In plants, both NO and other ROS have been implicated in mediating signaling responses in tip-growing cells, namely in pollen tubes. ROS are involved in regulating polarity and growth in tip-growing cells (Cárdenas et al., 2008; Coelho et al., 2008; Wudick and Feijó, 2014). *Arabidopsis* root hairs exhibit high apical levels of ROS, which could modulate root hair tip growth by activating a $Ca^{2+}$ channel (Foreman et al., 2003; Monshausen et al., 2007). In addition, a tip-high ROS gradient produced by NADPH oxidases is required for pollen tube tip growth (Potocký et al., 2007; Wang et al., 2010; Boisson-Dernier et al. 2013; Lassig et al. 2014; Kaya et al. 2014), and is essential for pollen tube rupture (Duan et al. 2014). Moreover, ROS may also be involved in the *Pyrus pyrifolia* self-incompatibility response (Wang et al., 2010).

This review will focus on the importance of NO in cell communication, particularly female-male talk during fertilization, and discuss recent data concerning this theme. An overview of the NO
synthase-like (NOS-like) controversy will also be addressed, as well as the tools that are currently being used to detect NO production and NO-dependent signaling cascades.

**NOSynthase (NOS): the missing Holy Grail?**

For the past 40 years of plant research a dense signaling network orchestrated by NO has been described in various aspects, but the source of its production is still by and large a matter of discussion. Both enzymatic and non-enzymatic pathways have been described, but there is no consensus at sight on the central source of NO in plants, and even less its regulation.

NO is involved in plant metabolism, and the nitrification/denitrification cycle provides NO as a by-product of nitrous oxide oxidation into the atmosphere by means of a non enzymatic mechanism. The studies of NO on plant metabolism date back to the sixties when Fewson and Nicholas(1960) addressed the recruitment of NO by microorganisms and higher plants. NO was suggested to be a key intermediate in the metabolism of inorganic nitrogen compound in higher plants and nitrogen fixing organisms. It was only in 1994 that NO was proved to be endogenously produced in a non-enzymatic way through conversion of nitrogen dioxideto NO by carotenoids in the light (Cooney et al., 1994). Moreover, synthesis of NO on the apoplast has also been described by non-enzymatic mechanism, where nitrite is converted to NO under acid conditions in response to abscisic acid (ABA) and gibberellins (Bethke et al., 2004).

The best described enzymatic source of NO in plants is the NAD(P)H-dependent nitrate reductase (NR), a cytosolic enzyme associated withinitrogen assimilation, whose primary function is the reduction of nitrate to nitrite (Yamasaki and Sakihama et al., 2000). Itcan further reduce nitrite to NO by a mitochondrial electron transport dependent reductase (Planchet et al., 2005), which uses arginine as a substrate, following a reaction similar to that observed for the well characterized animal NOSs(Zhou and Zhu, 2009). Bursts of NO induced by auxins, ABA, other elicitors, or hydrogen peroxide (H$_2$O$_2$) seem all to be dependent on NR activity (Bright et al., 2006;Yamamoto-Katou et al., 2006;Kolbert et al., 2008; León et al., 2014;). Arabidopsis has two known Nitrate reductase (NR) genes, NIA1 and NIA2 (Campbell, 1999). Comparative studies of individual and double mutants, nia1/nia2, showed a significant reduction in NO
synthesis and different contribution to the synthesis of NO in different tissues (Bright et al., 2006; Modolo et al., 2006).

In animals, there are three different isoforms of the NO Synthase (NOS), which operate in distinct localizations (Zhou and Zhu, 2009). All NOSs’ are active as homodimers, converting L-arginine to L-citrulline and NO. When the availability of L-arginine is reduced, these enzymes also produce superoxide anion and NO, that may originate peroxynitrite (Wendehenne et al., 2001). A recent paper reported the presence of a NOS in algae (Foresi et al., 2010). However, to-date no direct ortholog of these canonical NOS was found in the genomes of Arabidopsis or any other higher plant (Table 1). In the late 90’s, the palette of available tools to dissect NOS production was composed by several mNOS compounds inhibitors, such as NG-monomethyl-L-arginine (NMMA) and arginine analogs, and assays for arginine-to-citrulline conversion were used to detect the presence of NOS-like enzymes in different plant tissues (e.g. roots, leaves and stems) and organelles (e.g. peroxisomes) (Barroso et al., 1999; Durner et al., 1998; Foissner et al., 2000; del Río et al., 2004). A later approach used mammalian antibodies that were raised against NOS-like epitopes, which detected immunoreactive proteins in plants in different organelles (Barroso et al., 1999; Ribeiro et al., 1999). This approach reached a dead end when, in a proteomic study in maize, cross-reactivity was demonstrated to be due to binding to many polypeptides apparently unrelated to NOS proteins, that were unspecifically recognized by the mammalian antibodies (Butt et al., 2003).

Various claims of a genetic characterization of an inducible-NOS also ended up being refuted (Chandok et al., 2003 and 2004). However, Guo et al.(2003), using a sequence similar to a protein that has been implicated in NO synthesis in the snail Helix pomatia and a commercial NOS assay, claimed the identification of a NOS-like enzyme from Arabidopsis thaliana, originally baptized as AtNOS1. The enzyme activity was indirectly determined by measurement of NO contents in wild-type vs. mutant plants, the latter showing reduced NO generation. In addition, Atnos1 plants showed a growth phenotype that could be rescued by the application of NO donor compounds. Subsequent studies where NO specific electrodes or the NO-specific fluorescent dye (DAF-2) were used, also confirmed the reduced NO content in the Atnos1 mutant (He et al., 2004; Zeidler et al., 2004). Furthermore, isolated mitochondria from leaves of Atnos1, were defective in L-arginine based NO production, and presented elevated levels of hydrogen peroxide, superoxide anion, oxidized lipid, and oxidized proteins, implying that AtNOS1
protein is targeted to mitochondria (Guo et al., 2005). Sadly, the claim that this gene encoded a true NOS enzyme felt shortafter several other groups failed to reproduce the originally reported NOS activity with recombinant AtNOS1 and other purified recombinant proteins from rice and maize (Zemojtel et al., 2006a). Consequently, this led to the renaming of the protein to AtNOA1 (Crawford et al., 2006). Moreover, the closest homolog of AtNOA1, the Bacillus subtilis YqeH, has been shown to participate in ribosome assembly and stability (Morimoto et al., 2002), implying that AtNOA1 is a GTPase that binds to ribosomes and consequently plays a role in their proper assembly and/or stability (Flores-Pérez et al., 2008; Moreau et al., 2008). AtNOA1 was later shown to be plastid-targeted where it appears to be required for proper organelle biogenesis (Flores-Pérez et al., 2008). Despite the frustrated hunt for a bona fide NOS in Arabidopsis, Atnoa1 remains to date the only functional mutant with reduced levels of NO, and as such continues to be used as an experimental tool. And the reason why this mitochondrial enzyme affects the global levels of NO production in the plant remains unknown.

(Table 1 here)

Uncovering NO signal transduction pathways, mechanisms and functions

The lack of a true NOS subsequently slowed down the discovery of NO downstream signaling processes. Nevertheless, several different methods have been consistently used to elucidate NO-dependent processes. These include assays for NOS activity (e.g. by arginine to citrulline conversion), NO-binding fluorescent dyes (4,5-diaminofluorescein diacetate- DAF-2DA; and 4-amino-5-methylamino-2,7-difluorofluorescein- DAF-FM), NO donors (Sodium nitroprusside- SNP; and S-nitroso-N-acetylpenicillamine- SNAP), NO-scavengers (2-(4-carboxyphenyl)-4,5,5-tetramethylimidazoline-1-oxyl-3-oxide- cPTIO), various mammalian pharmacologic approaches, and quantification of known effectors (e.g. cGMP by e.g. 125I-based radioimmunoassay or LC-MS/MS) (e.g. Durner et al., 1998; Prado et al., 2004; Joudoi et al., 2013). However, many of these compounds elicit pleiotropic responses and the assays can give rise to artifacts (Mur et al., 2011). The NO donor, SNP, has been widely used to exploit the diverse NO bio-regulatory functions. Several reports have demonstrated the protective and toxic
action of SNP as a signaling compound, depending on its concentration and on the experimental system (Lammatina et al., 2003; Filippou et al., 2012). SNP was recently demonstrated to regulate the production of endogenous proline and polyamine metabolites in time-, concentration- and development-dependent manners (Filippou et al., 2012).

In parallel, other genomic and proteomic approaches have been described. Expression of a rat neuronal NOS (nNOS) in Arabidopsis resulted in an overall improved drought tolerance and enhanced disease resistance, affecting the level of water loss and stomatal aperture, altering metabolic content, as well as delaying flowering (Shi et al., 2012). Another approach made use of the post-translational protein modification process of S-nitrosylation, a redox modification of a cystein thiol group by NO (e.g. Lindermayr et al., 2005; Romero-Puertas et al., 2008; Fares et al., 2011). Kato et al. (2012) identified proteins regulated by S-nitrosylation in potato tissues. In these experiments a modified and optimized biotin switch assay and nano liquid chromatography combined with mass spectrometry was applied, and this modified method promises to better understand the signal transduction pathway elicited by NO transient signals.

Interestingly, a unique prototype of NOS inhibitor was designed, termed nanoshutter (NS1), which targets the NADPH site of NOS, and produces specific fluorescence enhancement upon binding to constitutive NOS (Li et al., 2012). The authors propose that NS1 is a promising tool for noninvasive imaging of NOS in living tissues, with two-photon excitation in the 800-950 nm range.

**NO in plant sexual reproduction**

The search for the signals that drive pollen tube on a receptive stigma and inside the pistil towards the micropyle have allowed the characterization of many chemotropic molecules, but by and large, our understanding of the whole process remains affected by numerous gaps (Boavida et al., 2005; Higashiyama and Hamamura, 2008; Marton and Dresselhaus, 2008; Dresselhaus and Tong, 2013). This is not surprising as the complex path and targeting of pollen tubes towards the ovules involves interactions with more than one tissue, and dramatically different conditions, ranging from the open air of the stigma, to near anoxia inside the ovary, and invasive growth through the style (Feijó, 2010). Likely these diverse cell-cell environments have led to various
communication processes and accordingly a number of different molecules have been described to possess various sorts of attraction or repulsion properties in modulating pollen tube growth. Several lines of evidence point to a role of chemotropic cues during pollen tube navigation, and genetic evidences from mutagenesis studies show the existence of genes associated with long and short-range chemotropic cues that enable pollen tube–pistil communication (Palanivelu and Preuss, 2006). NO was firstly proposed to be involved on growth and steering by its negative chemotropic effect on Lily pollen tube growth (Feijó et al., 2004; Prado et al., 2004)(Figure 1). The fact that pollen itself was demonstrated to produce NO (Bright et al. 2009) makes it possible that the this gas may have co-opted evolutionarily for the task of cell-cell communication during the programic phase of sexual reproduction.

The implication of NO and othertypes of ROS in fertilization have also gained support in Pyrus pyrifolia, Senecio squalidus and Arabidopsis (McInnis et al., 2006; Wang et al., 2010). A rapid and transient increase in ROS and NO, each showing a distinctive “signature”, was recently demonstrated during Self-Incompatible (SI) fertilization in Papaver rhoeas (Wilkins et al. 2011). Moreover these authors showed that ROS and NO act upstream and mediate key SI events, namely the formation of SI-induced actin punctate foci and the activation of a DEVDase/caspase-3-like activity, previously shown to be involved in the execution of SI-programmed cell death. Interestingly, by using electron paramagnetic resonance (EPR) and the diamino-rhodamine (DAR) probe it was possible to demonstrate that the generation of NO occurs just after the landing of pollen on the stigma in Brassica napus (Wilson et al., 2009). The proposed mechanism sets pollen grain hydration as the signaling cue that triggers an initial constitutive NO release. Studies in olive reproduction tissues also revealed the presence of NO and ROS during pollen-pistil interaction (Zafra et al., 2010), showing that these signaling molecules are produced in a tissue and stage specific manner during flower development. Stigma and anthers of olive also seem to accumulate NO and ROS, in particular at the pollen grain apertures. More recent data also suggests that NO production from an unknown NOS-like enzyme activity decreases the cold-responsive pollen germination, inhibits tube growth and
reduces proline accumulation, partly via cGMP signaling pathway in *Camellia sinensis* (Wang et al., 2012).

NO and ROS were also proposed to participate in gamete fusion blockage, which does not occur in politubey mutants, characterized by fertilization of a singleovule by more than one pollen tube (Beale et al., 2012; Dresselhaus and Sprunck, 2012; Kasahara et al., 2012). Arguments for pollen tube directionality arise from the observation that *in-vitro* grown pollen does not show any inherent directionality (Wheeler et al., 2001). The role of NO in pollen tube directionality *in vivo* and *semi-vivo* conditions was further dissected by Prado et al. (2008), to show a possible role of NO on ovule targeting, and the possible involvement of cytosolic Ca²⁺ on the process. Moreover, transcriptomic data on pollen-pistil interactions (Boavida et al. 2011) indicated a time-course-specific modulation of *AtNOA1* and *NR1* and *NR2* transcripts which putatively may trigger a NO signaling pathway (Figure 2). It is therefore hypothesized that NO may directly affect the targeting of pollen tubes to the ovule’s micropyle by modulating the action of its diffusible factors. The politubey phenotype is also suggestive of a role on the final steps of pollen tube penetration and discharge along the filliform apparatus and synergid cells, still to be fully understood.

(figure 2 here)

**Communicating with NO: multitasking in Plants**

In animals, NO is a vital signaling molecule involved in a diversity of important cellular functions, from regulation of blood flow and arterial pressure, immune response, neurotransmission and cell differentiation (Zhou and Zhu, 2009). Most of these interactions occur by simple diffusion from its site of production to target sites. It is now well established that NO is a key player in the regulation of different plant developmental processes, including photomorphogenesis, plant defence, stomatal aperture, leaf senescence, flowering and fertilization (Wendehenne and Hancock, 2011; Yu et al., 2014). Early studies showed that NO could promote expression of defense genes in barley aleurone, through cGMP and cADP-ribose in tobacco (Penson et al., 1996; Durner et al., 1998). There is no evidence to-date of DNA promoter sequences, or other elements, from eukaryotic genes that
directly bind or react to NO. Yet NO can influence the activity of transcription factors and intervene in upstream signaling cascades, mRNA stability and translation (Lamattina et al., 2003). NO represses flowering in Arabidopsis and NO-sensitive features in the circuitry of flowering time control were identified, repressing the amplification of gene expression that is dependent on the circadian clock and thereby promoting the accumulation of mRNA encoding a key repressor of flowering FLOWERING LOCUS C (He et al., 2004; Simpson, 2005). Interestingly, for the first time, a central mechanism for the sensing of NO in plants was recently identified, the plant-specific group VII ethylene response transcription factors (ERFs), which act as 'master sensors'; the study demonstrates a direct modulation of ERFs stability throughout the plant life cycle, specifically on seed germination, stomatal closure, and hypocotyl elongation (Gibbs et al., 2014).

NO generation has also been shown to interfere with various auxin-dependent responses such as root development (Pagnussat et al., 2003; Correa-Aragunde et al., 2004) and auxin-mediated gravitropism (Hu et al., 2005). Recently NO was also postulated to interact with auxins to regulate the homeostasis of the stem cell niche (Sanz et al. 2014). NO also appears to influence root development through the initiation of cell cycle genes and patterns of cellulose synthesis (Correa-Aragunde et al., 2006, 2008). Interestingly, NO seems to accumulate in cortex/endodermis stem cells in Arabidopsis root tips (Fernández-Marcos et al., 2011). A recent study demonstrated that a depletion of NO in NO-deficient mutants caused reduced primary root elongation, and small root meristems with abnormal divisions, and disturbance of auxin biosynthesis, transport and signaling. Remarkably, NO also accumulated in cortex/endodermis stem cells and their immediate progeny, generating endodermal and cortical tissues, implicating NO as a key player in the regulation of stem cell decisions through its interaction with auxin (Lusan et al., 2014). During Arabidopsis cell growth in the root apical meristem, PIN1 dependent acropetal auxin transport was reduced when high levels of NO were applied, which has been interpreted as a result of the inhibition of polar auxin transport, and may imply that NO acts downstream of auxin (Fernandez-Marcos et al., 2011, 2012). This signaling pathway is also observed in response to iron (Fe) deficiency, where auxin induces an NO burst and ferric-chelate reductase (FCR) activity to enhance Fe uptake at the root plasma membrane (Chen et al., 2010).

In tomato, NO reduces primary root growth and promotes lateral root development (Correa-Aragunde et al., 2004; Guo et al., 2008).
In Arabidopsis the NR double mutant *nia1-2nia2-5* still retains 30% NO biosynthesis during lateral root development (Wang et al. 2010), while the triple mutant *nia1nia2noa1* was later shown to produce less than 10% NO when compared to the wild type (Lozano-Juste and León, 2010). Furthermore, these authors showed that MPK6 kinase activity is involved in the regulation of H$_2$O$_2$ induced NO synthesis through phosphorylation of NIA2 during Arabidopsis root development. Depletion of NO was shown to be required for endocytosis, vesicle formation, and trafficking in Arabidopsis root hairs (Lombardo and Lamattina 2012).

In seedlings, the isoform of nitrite-reductase NIA1, although less abundant and active than NIA2, has a definitive role in the production of NO during stomatal closure induced by ABA (Wilkinson and Crawford, 1991; Bright et al., 2006; Wilson et al., 2008). In response to oxygen depletion in roots both signaling molecules NO and ethylene are synthesized. Under these conditions, the plant haemoglobin (pHb) has been shown to act in roots and shoots, to scavenge NO to form nitrate, indicating its role in NO detoxification (Dordas et al., 2003, 2004; Perazzolli et al., 2004; Hebelstrup et al., 2012). In animals, NO diffusion and signaling was shown to be regulated by the oxidation state of the haemoglobin α protein within endothelial cells, blocking its diffusion to the smooth muscle (Straub et al., 2012). The mitochondrial NO production from nitrite, also contributes to hypoxic survival by maintaining a minimal ATP formation (Gupta et al., 2011).

In leaves, guard cells aperture is controlled by phytohormones, e.g ABA, and various environmental signals, e.g. light, CO$_2$ and temperature. Stomata closure can be promoted by extracellular Ca$^{2+}$, through intracellular calcium ([Ca$^{2+}$]$_{in}$) oscillations (MacRobbie et al., 1992; Allen, 2001; Li et al., 2009; Wang et al., 2012). External calmodulin also triggers a significant increase in NO levels associated with stomata closure in the wild type of Arabidopsis, but this effect is abolished in the *Atnoa1* and *gpa1* (G-alpha-subunit of G protein) mutants (Li et al. 2009). The authors suggested an involvement of H$_2$O$_2$ production that would lead to AtNOA1 and NO accumulation, and stomata closure. Furthermore, the Ca$^{2+}$-sensing receptor (CAS), a protein localized in the Arabidopsis chloroplast thylakoid membranes also regulates Ca$^{2+}_{out}$-induced Ca$^{2+}_{in}$ transients and stomata closure (Wang et al., 2012). It was shown that the CAS transduces the Ca$^{2+}_{out}$ signal through the downstream action of NO and H$_2$O$_2$ which prolongs Ca$^{2+}_{in}$ oscillations. The cross-talk between NO and H$_2$O$_2$, two key intermediates in multiple stress responses (e.g. salt tolerance, hypoxia), is vital during guard cell stomata closure induced by
ABA. Gene disruption in the double mutant \textit{AtrbohD/F} (reduced NADPH oxidase activity) impairs ABA-induced stomata closing, by disrupting ROS production and Ca$^{2+}$ increases, and the activation of plasma membrane Ca$^{2+}$-permeable channels in guard cells (Kwak et al., 2003). The double mutant \textit{AtrbohD/F} was also used to demonstrate that ABA mediated NO generation is dependent on ABA-induced H$_2$O$_2$ production (Bright et al. 2006). In essence, \textit{AtrbohD/F} failed to generate NO in response to ABA, and H$_2$O$_2$-induced stomata closure was inhibited by the removal of NO with a NO-scavenger, implying NO as a downstream effector of H$_2$O$_2$. Athermotolerance study demonstrated the activation of NO which stimulated heat-shock (HS) DNA-binding factors activity and HS protein accumulation through H$_2$O$_2$ (Wang et al., 2014).

NO has been implicated in plant resistance to various abiotic stresses, including Aluminium (Al) stress, and salt tolerance (Yu et al., 2014). In Arabidopsis the gas enhances plant tolerance to drought and contributes to stomata closure evoked by the water stress phytohormone ABA (Neill et al. 2008). Analysis of the mutant \textit{Attnoa1} showed a greater Na$^+$ to K$^+$ ratio in shoots in relation to the wild type, and NaCl increase was rescued by the addition of NO donor SNP (Zhao et al., 2007). The cytosolic NaCl increase in Arabidopsis wild type callus induced by salt stress was attenuated by the addition of an ethylene biosynthesis inhibitor, indicating that ethylene and NO may cooperate in stimulating plasma membrane H$^+$-ATPase activity to modulate ion homeostasis for salt tolerance (Wang et al., 2009). During wheat resistance to Al-induced oxidative stress, thenitrate reductase-mediated early NO burst, maintained root function and enhanced antioxidant enzyme activities, under Al toxicity (Sun et al., 2014). The same group later showed that NO alleviated Al-induced root growth inhibition, through a regulatory relationship between NO and the ascorbate-glutathione (AsA-GSH) cycle (Sun and Liu et al., 2014).

\textbf{NO in plant defense}

Plants have developed complex innate and induced immune responses, called the hypersensitive response (HR), to protect themselves against microbial pathogens and herbivorous insects which can culminate in systemic acquired resistance (SAR) (Yu et al., 2014). An early important event is the Ca$^{2+}$ elevation, through plasma membrane Cyclic Nucleotide-Gated ion Channels (CNGC) (Ali et al. 2007). This is consistent with a role for cGMP in plants, and indeed,
several putative guanylate cyclases activity proteins have been identified in Arabidopsis, one of which is the phytosulfokine receptor (PSKR) (Meier et al., 2007). In PSKR, the cytosolic guanylate cyclase domain is activated specifically by its biologically active ligand, a sulfonated phytosulfokine (Kwezi et al., 2011). Another study points to the importance of the cGMP-activated channel CNGC2 for an inward Ca\(^{2+}\) conductance that leads to Ca\(^{2+}\)\(_{\text{in}}\) elevation, which is regulated by a family of peptide signaling molecules AtPeps and their receptor (atPepR1)(Qi et al., 2010). Ca\(^{2+}\)\(_{\text{in}}\) increase stimulates the generation of salicylic acid (SA), NO and ROS which trigger programmed cell death in the vicinity of the infection thereby limiting pathogen growth (Durner et al., 1998; Ma et al., 2009, 2011). Ca\(^{2+}\) sensors and downstream targets of the Ca\(^{2+}\) signal, Calmodulin (CaM) and CaM-like proteins, were suggested to be involved in Pathogen-Associated Molecular Pattern (PAMP)-induced NO-synthesis (Ma et al., 2008). Several studies report that NO has a determinant role in the HR (e.g. Delledonne et al., 1998; Durner et al., 1998; Mur et al., 2012). Furthermore, cooperativity between NO and ROS, namely H\(_2\)O\(_2\), seems to be essential to fully activate the HR, as a ROS burst was shown to be insufficient to elicit a strong disease resistance (Delledonne et al., 1998). NO function was proposed as a partner in the defense response promoting its potentiation, by controlling the biphasic ethylene formation during the HR in plants subjected to pathogens (Mur et al., 2012). The synergistic effects of NO and H\(_2\)O\(_2\) have been proposed as a point of convergence in the activation of MAPKs and hence in the transcriptional activation of a set of target genes (Lamattina et al., 2003; Wendehenne and Hancock, 2011). This interaction was shown to act as an upstream signaling cue to modulate the dynamic microtubule cytoskeleton during defense responses to *Verticillium dahliae* (VD) toxins in Arabidopsis (Yao et al., 2012). Moreover, NO production was almost completely blocked by supplementation with either DiPhenylene Iodonium (DPI) or Dimethylthiourea (DMTU) (potent inhibitors of NADPH oxidase and H\(_2\)O\(_2\) scavenger, respectively), suggesting that H\(_2\)O\(_2\) may act upstream of NO during responses to VD-toxins in Arabidopsis. Pathogen attack triggers redox changes and gene regulation in plant immunity. Several targets of protein S-nitrosylation during the HR have been characterized in *A. thaliana* (Romero-Puertas et al., 2008). The increase in SA and NO molecules leads to the regulation of the conformation of NPR1 (Nonexpressor of Pathogenesis-Related genes) by direct binding through Cys\(^{521/529}\), and/or by S-nitrosylation of Cys\(^{156}\), respectively, which maintains protein homeostasis (Tada et al., 2008). Later on, a molecular framework for S-nitrosothiols (cystein thiols modified by NO,
SNOs) function was established during the HR and SAR (Yun et al., 2011). The work showed that oxidative and nitrogen bursts, and S-nitrosoglutathione reductase (GSNOR) activity, lead to a raise in total cellular SNOs, that in turn regulate the rate of cell death. On the other hand, SNO signaling suppresses both nitrate uptake and GSNOR which is S-nitrosylated by NO derived from nitrate assimilation, to fine tune nitrate homeostasis (Frungillo et al., 2014). Furthermore, when concentrations of SNOs were high, NO function also governed a negative feedback loop limiting the HR mediated by S-nitrosylation of NADPH oxidase (Melo et al., 2011). Interestingly, a conserved cystein at the C-terminal portion, Cys$^{890}$, in NADPH oxidase suggests that this mechanism may govern immune responses in both animals and plants (Yun et al., 2011). Moreover, the oxidoreductase Thioredoxin-h5 (TRXh5) was shown to reverse SNO modifications by acting as a selective protein-denitrosylation reductase, demonstrating that SNOs can act as specific, reversible signaling cues (Kneeshaw et al., 2014).

NO and ion channel cross-talk – from localization to action

NO has long been associated with ion transport and the regulation of ion channels in mammalian tissues, while in plants the realization that such mechanisms may operate is gaining momentum. In animals, the first evidence of direct regulation was proposed when it was determined that the hyperpolarization response to NO activates multiple potassium channels in canine colonic smooth muscle, directly and via cGMP-mediated mechanisms (Koh et al. 1995). Since then, several reports have strengthened the pivotal role of NO on ion channel modulation in numerous physiological processes and in certain neurological diseases (Boilotina et al., 1994; Wilson and Garthwaite 2010; Wang et al., 2012). NO can act indirectly through guanylate cyclases to activate cGMP-dependent cellular responses e.g. through post-translational modification of proteins, by S-nitrosylation, activation of phosphatases and protein kinases, like MAPKs. The S-nitrosylation biochemical reaction occurs when a nitrosyl group is added to the thiol side-chain of cystein residues to form SNO. All these events may lead to ion channel changes in tertiary structure and, subsequently, function (e.g. Garcia-Mata et al., 2003; González et al., 2012; Wang et al., 2012; Zhao et al., 2012; Joudoi et al., 2013).
In plants direct evidence of NO ion channel regulation comes mainly from electrophysiological studies in guard cells. Biotic and abiotic stressors, such as pathogen attack, high CO2 concentrations, drought and ABA-associated stomata closure, induce NO synthesis (Durner et al., 1998; Bright et al., 2006; Guo et al., 2008; Agurla et al., 2014). Ca2+ has a pivotal role in nitric oxide (NO)-promoted stomatal closure. Garcia-Mata and colleagues (2003) established intracellular Ca2+in concentration as principal effector of NO (<10 µM) in Vicia faba guard cells during ABA induced response. The Ca2+in rise leads to a subset of ABA-associated events, and the downstream inactivation of inward-rectifying K+ channels (IK,in) (Ca2+-sensitive), to prevent K+ uptake, and activation of outward-rectifying K+ channels (IK,out) and chloride (ICl-) channels at the plasma membrane, to facilitate solute efflux. By means of patch clamp, the authors showed that a Ca2+in elevation mediated by a low nanomolar levels of NO, was essential for normal inactivation of IK,in, but not IK,out, and activation of ICl by ABA. Furthermore, NO did not promote a shift in the voltage sensitivity for Ca2+-channel gating that is characteristic of ABA. Furthermore, NO-sensitive Ca2+in release was blocked by ryanodine and 1-H-(1, 2, 4)-oxadiazole-[4,3-a]quinolxalin-1-one (ODQ), antagonists of cADPR-sensitive Ca2+ channels and guanylate cyclase, respectively, suggesting that NO acts via cGMP and cADPR to sensitize endomembrane Ca2+-channels for internal Ca2+ release.

In a subsequent work in the same cell system, it was shown that when NO is elevated from approximately 10 µM to 20 µM, it directly modifies the plasma membrane IK,out by S-nitrosylation. The effect of NO on the K+ channel was mimicked by phenylarsine oxide, an oxidizing agent that cross-links vicinal thiols (Sokolovski and Blatt, 2004). Based on physiological data, it was proposed that NO sensitivity of outward-rectifying K+ channels could represent either a response to oxidative stress or an imbalance between nitrosylation and denitrosylation imposed by the presence of exogenous NO. NO-dependent signals can also be modulated through protein phosphorylation upstream of intracellular Ca2+ release, implicating a target for protein kinase control in ABA signaling that would feed into an NO-dependent Ca2+ release (Sokolovski et al., 2005). Working downstream of H2O2, NO is involved on the ABA-inhibited blue light (BL)-dependent stomatal opening by inducing the dephosphorylation of H+-ATPase on the plasma membrane in Vicia faba guard cell protoplasts (Zhang 2004, 2007). Recently, the same group showed that NO inhibits the BL-induced inward K+ currents through Ca2+, indicating that Ca2+ plays a dual and distinctive role in the crosstalk between BL and NO.
signaling in guard cells, mediating both the BL-induced K\(^+\) influx as an activator at a lower concentration and the NO-blocked K\(^+\) influx as an inhibitor at a higher concentration (Zhao et al. 2012, 2013).

NO and ion channel communication have been implicated in several other different physiological functions including responses to plant acclimation to environmental changes, exposure to heavy metals and metalloids, and plant innate immunity. Recently, copper-induced cross talk among Ca\(^{2+}\), H\(_2\)O\(_2\) and NO, transcriptional activation of target gene through calmodulins and Ca\(^{2+}\)-dependent protein kinases was proposed (González et al., 2012). Furthermore, Ca\(^{2+}\)\(_{\text{in}}\) release through NAADP-, ryanodine-, and IP\(_3\)-sensitive Ca\(^{2+}\) channels, was also shown to be activated by NO and H\(_2\)O\(_2\), either by oxidation and/or nitrosylation of thiol groups present in these proteins. A genetic approach provided evidence that NO lowers K\(^+\) channel AKT1-mediated K\(^+\) absorption, by modulating vitamin B6 biosynthesis, implying a role of NO in the control of high-K\(^+\) content conditions in plants (Xia et al., 2014).

Pathogen perception may culminate in programmed cell death (PCD) thereby limiting spread of biotrophic pathogens from the initial site of infection, through the generation of NO and H\(_2\)O\(_2\) mediated by Ca\(^{2+}\)\(_{\text{in}}\) elevation. Several reports have implicated plasma membrane CNGC in this response (e.g. Yoshioka et al., 2006; Ali et al., 2007). However, there is evidence that NO and H\(_2\)O\(_2\) synthesis can also act upstream from cytosolic Ca\(^{2+}\) elevation during HR, due to the activation of plasma membrane and intracellular membrane Ca\(^{2+}\) channels (Levine et al., 1996; Kwak et al., 2003; Lamotte et al., 2006; Vandelle et al., 2006). Taken together, this suggests a complex non-linear interactive network and tight feedback control by both effectors.

The quest for NO sensing molecules in plants

In bacteria and animals, the \textit{in vivo} target for NO is the soluble guanylyl cyclase (GC) (Denninger and Marletta, 1999). Specifically, NO binds to the heme group that is localized at a domain termed Heme Nitric oxide/OXygen (H-NOX) and this binding activates the GC catalytic leading to the generation of cGMP from GTP (Palmer et al., 1987; Bellamy et al., 2001). H-NOX domains have a unique protein fold that is different from other known heme-binding proteins, and this structural uniformity is observed across species ranging from bacteria to animals (Boon...
and Marletta, 2005). Mutational and structural studies have assigned the histidine (H) residue as a proximal ligand for docking to the iron core of the heme porphyrin moiety (Wedel et al., 1994; Zhao et al., 1998). This binding results in a 5-coordinate complex (Stone et al., 1995) which becomes a nitrosyl complex when bound to NO (Stone and Marletta, 1994), thus severing the proximal histidine-iron bond (Stone et al., 1995) leading to a subsequent displacement of the heme moiety (Dai et al., 2012). Meanwhile, the conserved ‘YxSxR’ signature (Figure 3A) downstream of the histidine residue stabilizes heme propionates through hydrogen bonding with the side chains of the heme group (Pellicena et al., 2004) and importantly, this motif and in particular the ‘Y’ and ‘R’ residues, are crucial for heme binding and for transducing changes in the heme geometry in association with NO, as well as the concomitant activation and alteration of the catalytic rate of the soluble GC (Schmidt et al., 2004). Additionally, the proline (P) residue at position 14 of the H-NOX motif (Figure 3A) contributes to the structural ‘flattening’ of the otherwise distorted heme domain leading to an increased affinity for oxygen as demonstrated in the H-NOX domain of *Thermoanaerobacter tengcongensis* (Olea et al., 2008). Molecular selectivity for NO has also been established where a tyrosine residue further downstream of the ‘YxSxR’ signature causes the exclusion of oxygen – an attribute that is crucial for the survivability of obligate anaerobes (Boon et al., 2005).

In a quest to identify candidate NO-binding molecules with H-NOX motifs in plants, an H-NOX motif was extracted from the alignment of soluble GCs across different species and used in a pattern matching search against the Arabidopsis proteome. This extended H-NOX motif (Hx[12]Px[14,16]YxSxR) (Figure 3A) retrieves four candidate molecules and the relaxed motif that omits the conserved but functionally less important proline (P) at position 14, retrieves more than 60 candidates (Supplementary File 1). Indeed, one of the four H-NOX candidates, the ATNOGC1 (AT1G62580), is annotated as a monooxygenase and has been reported to bind gas with greater preference for NO over oxygen. Incidentally, this molecule also harbors a GC catalytic domain that is stimulated *in vitro* by NO donors (Mulaudzi et al., 2011) (Figures 3B-C).

Another H-NOX candidate is the Arabidopsis DIACYLGLYCEROL KINASE 4 (ATDGK4; AT5G57690) (Figure 3B). Diacylglycerol kinases catalyze the phosphorylation of diacylglycerol to phosphatidic acid which in turn causes the release and mobilization of Ca\(^{2+}\) from intracellular stores. For example, cytosolic Ca\(^{2+}\) has been shown to modulate the regulation of pollen tube growth of *Agapanthus umbellatus* by phosphoinositides and phosphatidic acid (Potocky et al.,
2004; Monteiro et al., 2005). If indeed it turns out that NO binding to ATDGK4 does occur and
causes a change in catalytic activity of the kinase, then this would constitute a direct link
between NO and Ca\(^{2+}\) signaling as has been proposed in for e.g. the pollen tubes of *Pinus
bungeana* (Wang et al., 2009).

Furthermore, microarray data have revealed that the ATDGK4 is specifically expressed in
pollen. Since pollen tube growth and development in *Lilium longiflorum* and *Camelia sinensis*,
and the re-orientation response as well as targeting to the ovule in the *Lilium longiflorum* model
are modulated by NO (Prado et al., 2004; Wang et al., 2012; Prado et al., 2008), it is conceivable
that ATDGK4 has a role in NO-dependent directional growth of pollen tubes. Additionally, in
both *Lilium longiflorum* and *Camelia sinensis* systems, the NO-induced inhibition of pollen tube
growth and re-orientation response are partially abrogated in the presence of the GC inhibitors
(Prado et al., 2004; Wang et al., 2012). These findings are consistent with a candidate GC
catalytic center in ATDGK4.

Conclusions

NO was once regarded as a poisonous air pollutant, responsible for the formation of
photochemical smog and acid rain leading to the destruction of the ozone layer. Today is mostly
appreciated as a molecule essential to innumerable functions in both animals and plants. It is a
key signaling molecule that controls e.g. root development and stomata movement, but when
concentrations of this simple gas molecule are too high and/or the spatial generation patterns are
disrupted, it is toxic to cells. This toxicity was eventually co-opted during evolution to allow NO
to serve as a central plant immune defense mechanism to pathogens. Curiously, known effectors
like Ca\(^{2+}\) and cGMP, seem to be able to act both up- and downstream of NO, suggesting a
complex non-linear interactive network and tight feedback control. To this date there are no
available methods that accurately quantify the kinetics of NO production and its spatial patterns
but further elucidation of the biochemistry and cell biology of H-NOX containing plant
molecules promises new insights into the role of NO in plant signaling, and may constitute the
next step into the understanding of NO-sensing in plants.
New insights into the possible roles of NO during fertilization were discussed. Recent data, shows that NO is one of the first signaling cues being produced following pollen hydration on the stigma, suggesting a possible role in the regulation of the onset of germination. This exciting hypothesis needs further substantiation, as well as the putative generation of NO from nitrite. The involvement of NO in the politubey phenotypes also predicts functions at the later stages of fertilization. The existence of many pieces of evidence for a role of other ROS in the process makes it plausible that NO and ROS collaborate in the process in a coordinated way (figure 4). Many questions remain to be answered, but centrally, the identification of new enzymes that catalyze the production of NO, and of key up- or downstream effectors of NO-signaling cascade remains a serious obstacle to further understanding of its mechanisms by genetic approaches.

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References


is mediated by proteolytic control of group VII ERF transcription factors. Mol Cell. 53(3), 369-79.


Yao, L.L., Pei, B.L., Zhou, Q., and Li, Y.Z. (2012). NO serves as a signaling intermediate downstream of H$_2$O$_2$ to modulate dynamic microtubule cytoskeleton during responses to VD toxins in Arabidopsis. Plant Signal Behav. 7(2), 174-7.


Table 1

Brief summary and description of data supporting the existence of a NOS-like protein in higher plants: known NO probes and probable sensors.

<table>
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<th>Animals</th>
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<td>NO synthase - calcium and calmodulin dependent</td>
<td>Bredt and Snyder, 1990</td>
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<td>The three major NOS isoforms are cloned and purified</td>
<td>Nakane et al., 1994; Hall et al., 1994; Geller et al., 1993; Sherman et al., 1993; Charles et al., 1993; Janssens et al., 1992; Marsden et al., 1992</td>
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<td>AtNOS1, latter renamed AtNOA1 - GTPase</td>
<td>Guo et al., 2003; Moreau et al., 2008</td>
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<td>Inhibition of the NOS activity by mNOS inhibitors</td>
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<td>NOS detection by arginine-to-citrulline conversion</td>
<td>Dumer et al., 1998; Foissner et al., 2000; Tischner et al., 2007</td>
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<td>NOS-like gene, <em>Ostreococcus tauri</em>, single-celled green alga</td>
<td>Foresi et al., 2010</td>
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<th>NO probes and sensors (some examples)</th>
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<td>Fluorescent dyes - DAF-2DA, DAF-FM</td>
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<td>Copper complex of a fluorescein modified with an appended metal-chelating ligand (FL)</td>
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<td>NO-sensing H-NOX proteins: the primary receptor for NO, sGC, ATNOGC1, ATDGK4</td>
<td>Denninger and Marletta, 1999; Mulandzi et al., 2011</td>
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<td>Group VII ERF transcription factors</td>
<td>Gibbs et al., 2014</td>
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Figure Legends

Figure 1- NO acts as negative chemotropic agent of pollen tube growth, as shown in this timelapse sequence of a *Lilium longiflorum* (lily) growing pollen-tube facing an NO point-source (s-nitroso-acethylpenicilamine [SNAP]) (a)(time is depicted in minutes). Subsequent challenges with SNAP produce similar effects (b). The turning reaction is dependent on the sensing of a critical NO flux that we have quantified to be in the order of 0.5 pmol/cm²/sec. (c). Inhibition of putative phosphodiesterase type V activity by sildenafil citrate potentiates the turning response, to an angle of up to 180° (adapted from Feijó et al., 2004).

Figure 2- Semi-vivo assays with isolated ovules and pollen of *Lilium longiflorum*. a-c images of an isolated ovule (a) and pollen tubes targeting (b-c). Evidence that NO is necessary for micropyle targeting to occur is given by experiments in which tubes are either challenged with ovules, or freely growing (top- and inferior- half in (f)). Under these conditions, tubes can develop ballooned tips (d) or grow normally (e). Yet by addition of 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), a NO scavenger, the proportions of pollen tube growth patterns are altered (g). This resulted in abrogation of pollen tube polar growth and subsequent formation of balloon tips in pollen tubes facing ovules. Interestingly activation of Ca²⁺ influx in pollen tubes by D-Ser partially rescued normal pollen tube morphology, suggesting that this pathway is also dependent on Ca²⁺ signaling. A role for NO in modulating Ca²⁺ signaling was further corroborated by direct imaging of the cytosolic free Ca²⁺ concentration during NO-induced re-orientation (adapted from Prado et al., 2008).

Figure 3- Identification and characterization Arabidopsis H-NOX GC candidate molecules. The GC and H-NOX motifs (a) were used in PatMatch searches against the Arabidopsis proteome and of which, only two molecules harbor both the GC and H-NOX domains (b). (c) Cyclic voltammetry studies have determined that the ATNOGC1 binds NO with greater preference than oxygen and importantly, the presence of NO activates the GC catalytic center (reproduced from Mulaudzi et al., 2011).

Figure 4- Model depicting a putative feedback-model of the NO/H₂O₂/Ca²⁺ signaling pathways during sexual reproduction in flowering plants. Upon landing on a receptive stigma, the pollen grain hydrates and becomes activated. NO is thought to be one of the first signaling cues being produced, suggesting a possible role in the regulation of the onset of germination. NADP(H) oxidases RobhH/J are exclusively expressed in pollen and localize to the plasma membrane (PM) where they produce ROS, namely H₂O₂. The H₂O₂ excess would promote NO synthesis through NR and/or NOA1, which in turn could block NADPH oxidase activity by S-nitrosylation, preventing excess ROS formation. Non-enzymatic formation of NO may also occur at acidic pH values, when nitrite dismutates to NO and nitrate. NO can then bind to DGK4, catalyzing the phosphorylation of diacylglycerol to phosphatidic acid which causes the release and mobilization of Ca²⁺ from intracellular stores; NO may also activate protein kinases enabling Robh’s to bind Ca²⁺, triggering more ROS production, and cGMP which can also activate Ca²⁺ channels at the PM. Possible S-nitrosylation of several proteins, such as ion channels (e.g. SLAH3), links NO to volume regulation. External cues, such as polyamines (acting through ROS), peptides or ROS secreted from the ovules may also increase NO and cytosolic Ca²⁺ concentration. It is hypothesized that a fertilized ovule
may exhibit a NO burst which will repulse incoming PTs promoting polyspermy blockage, through unknown mechanisms (not drawn to scale).
Figure 1
Figure 2
A GC motif \([RKS][GCHS][9,10][KR]\)
H-NOX motif \([Hx][12]Px[14,16]YxSxR\)

B ATNOGC1

\[
\begin{array}{c}
5' \quad \text{DAG kinase} & \text{Nucleotide binding} & \text{H-NOX} & \text{GC} & 3' \\
\end{array}
\]

ATDGK4

\[
\begin{array}{c}
5' \quad \text{DAG kinase} & \text{Nucleotide binding} & \text{H-NOX} & \text{GC} & 3' \\
\end{array}
\]

C

- **Current (µA)**
  - Without O$_2$ and NO
  - With O$_2$ only
  - With O$_2$ and NO

- **cGMP (fmol/µg protein)**
  - Mg$^{2+}$
  - Mn$^{2+}$
  - NO + Mn$^{2+}$

**Potentials (V):**
- 0.4
- 0.2
- 0
- -0.2
- -0.4
- -0.6
- -0.8