CellPress
PARTNER JOURNAL

Molecular Plant
Review Article

Nitric Oxide: A Multitasked Signaling Gas in Plants

Patricia Domingos[1], Ana Margarida Prado[1], Aloysius Wong[2], Christoph Gehring[2] and Jose A. Feijo[1,3,*]

[1]Instituto Gulbenkian de Ciência, P-2780-156 Oeiras, Portugal

[2]Division of Biological and Environmental Sciences and Engineering, 4700 King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia

[3]Department of Cell Biology and Molecular Genetics, University of Maryland, 0118 BioScience Research Building, College Park, MD 20742-5815, USA

***Correspondence: Jose A. Feijo (jfeijo@umd.edu)**

http://dx.doi.org/10.1016/j.molp.2014.12.010

ABSTRACT

Nitric oxide (NO) is a gaseous reactive oxygen species (ROS) 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 the plant life cycle. NO has also been implicated as a signal in biotic and abiotic responses of plants to the environment. Remarkably, despite this plethora of effects and functional relationships, the fundamental knowledge of NO production, sensing, and transduction 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 especially 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 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 and sexual reproduction, namely in pollen tube guidance and embryo sac fertilization, pathogen defense, and responses to abiotic stress.

Key words: nitric oxide (NO), reactive oxygen species (ROS), plant sexual reproduction, cell communication, pollen

Domingos P., Prado A.M., Wong A., Gehring C., and Feijo J.A. (2015). Nitric Oxide: A Multitasked Signaling Gas in Plants. Mol. Plant. **8**, 506–520.

INTRODUCTION

Nitric oxide (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 hypothesized (Feelisch and Martin, 1995). The biological consequences of such evolutionary steps must have been far reaching: as NO does not require a carrier to cross membranes and reach intracellular targets, and diffuses very rapidly 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 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 vessel relaxation, immune defense responses, and participation in the fertilization process (Zhou and Zhu, 2009). ROS constitute a large group of molecules, and those that contain nitrogen (e.g. NO) form a subgroup that sometimes is also referred to as reactive nitrogen species (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 (Cardenas et al., 2008; Coelho et al., 2008; Šírová et al., 2011; 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

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.

Animals	References
NO synthase: calcium and calmodulin dependent	Bredt and Snyder, 1990
The three major NOS isoforms are cloned and purified	Janssens et al., 1992; Marsden et al., 1992; Charles et al., 1993; Geller et al., 1993; Nakane et al., 1993; Sherman et al., 1993; Hall et al., 1994
Plants—NOS-like evidence	
AtNOS1, later renamed AtNOA1-GTPase	Guo et al., 2003; Moreau et al., 2008
Inhibition of the NOS activity by macrophagic NOS inhibitors	Barroso et al., 1999
NOS detection by arginine-to-citrulline conversion	Durner et al., 1998; Foissner et al., 2000; Tischner et al., 2007
NOS-like gene, *Ostreococcus tauri*, single-celled green alga	Foresi et al., 2010
NO probes and sensors (some examples)	
Fluorescent dyes: DAF-2DA, DAF-FM	Kojima et al., 1998
Copper complex of a fluorescein modified with an appended metal-chelating ligand (FL)	Lim et al., 2006
NO-sensing H-NOX proteins: the primary receptor for NO, sGC; ATNOGC1; ATDGK4	Denninger and Marletta, 1999; Mulaudzi et al., 2011
Group VII ERF transcription factors	Gibbs et al., 2014

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.

addition, a tip-high ROS gradient produced by NADPH oxidases is required for pollen tube tip growth (Potocký et al., 2007; Wang et al., 2010a, 2010b; 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., 2010b).

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

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 nonenzymatic 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 nonenzymatic mechanism. The studies of NO on plant metabolism date back to the 1960s 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 nonenzymatic way through conversion of nitrogen dioxide to NO by carotenoids in the light (Cooney et al., 1994). Moreover, synthesis of NO on the apoplast has also been described by a nonenzymatic mechanism, whereby 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 with nitrogen assimilation, whose primary function is the reduction of nitrate to nitrite (Yamasaki and Sakihama et al., 2000). It can 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 NOS (Zhou and Zhu, 2009). Bursts of NO induced by auxins, ABA, other elicitors, or hydrogen peroxide (H_2O_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 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 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, which may create 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 has been found in the genomes of *Arabidopsis* or any other higher plant (Table 1) (Fröhlich and Durner, 2011). In the late 1990s, the palate of available tools to dissect NOS production was composed by several macrophagic NOS compound inhibitors, such as N^G-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) (Durner et al., 1998; Barroso et al., 1999; Foissner et al., 2000; del Ríoet 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, 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 versus 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 in which 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 the mitochondria (Guo et al., 2005). Unfortunately, the claim that this gene encoded a true NOS enzyme fell short after 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., 2006). 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. Interestingly a compensation mechanism in *Atnoa1* mutant Arabidopsis plants to reduce the negative effects of the mutation was suggested, these included photoprotection stimulation and SA and polyamine variations (Majláth et al., 2011). Moreover, the reason why this mitochondrial enzyme affects the global levels of NO production in the plant remains unknown (Table 1).

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 conversion from arginine to citrulline), 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)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide [cPTIO]),various mammalian pharmacologic approaches, and quantification of known effectors (e.g. cyclic guanosine monophosphate [cGMP] by ^{125}I-based radioimmunoassay or liquid chromatography–tandem mass spectrometry) (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 bioregulatory functions. Several reports have demonstrated the protective and toxic action of SNP as a signaling compound, depending on its concentration and the experimental system (Lamattina 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 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, and 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 cysteine 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 biding 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- to 9500-nm range.

NO IN PLANT SEXUAL REPRODUCTION

The search for the signals that drive pollen tube on a receptive stigma and inside the pistil toward 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 Franklin-Tong, 2013). This is not surprising, as the complex path and targeting of pollen tubes toward 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,

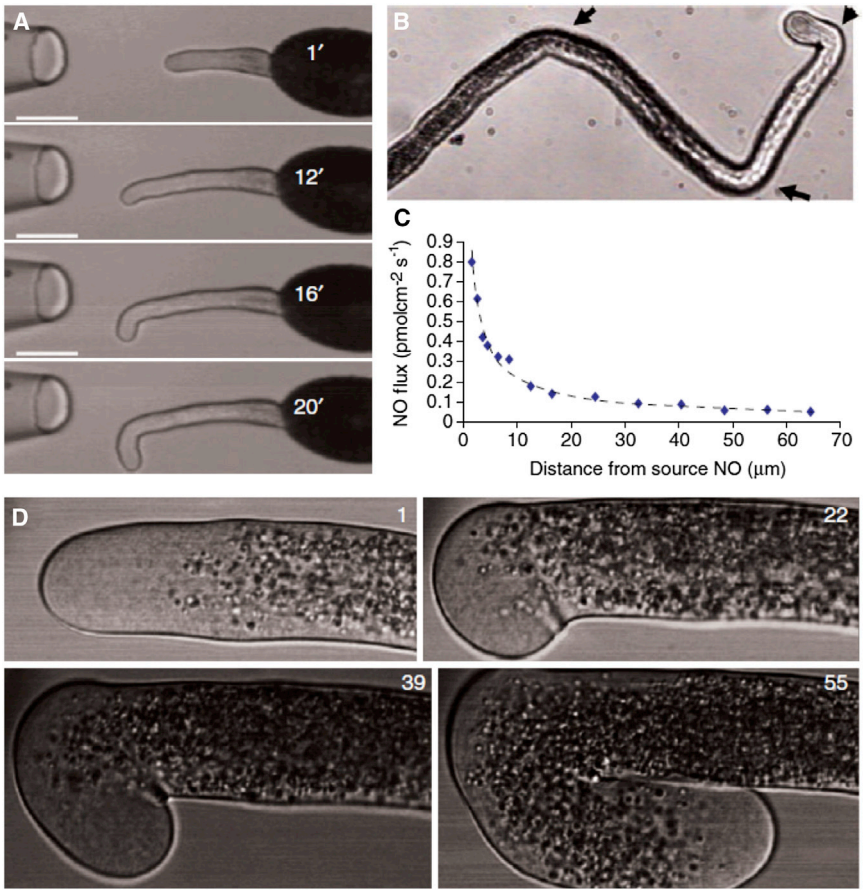

Figure 1. NO Acts as Negative Chemotropic Agent of Pollen Tube Growth.

(A) Time-lapse sequence of a *Lilium longiflorum* (Lily) growing pollen tube facing an NO point source (*S*-nitroso-acetylpenicillamine [SNAP]) (time is depicted in minutes).

(B and C) 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 on the order of 0.5 pmol/cm^2/s. **(C)**. Black arrows (**B**) show the NO point source relative location in reference to the growing pollen tube before the reorientation response.

(D) 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).

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 evidence from mutagenesis studies shows 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 this gas may have been co-opted evolutionarily for the task of cell–cell communication during the programming phase of sexual reproduction (Figure 1).

The implication of NO and other ROS in fertilization has also gained support in *Pyrus pyrifolia*, *Senecio squalidus*, and *Arabidopsis* (McInnis et al., 2006; Wang et al., 2010b). 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 suggest 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 the cGMP signaling pathway, in *Camellia sinensis* (Wang et al., 2012b).

NO and ROS were also proposed to participate in gamete fusion blockage, which does not occur in politubey mutants, characterized by fertilization of a single ovule 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 pollen grown *in vitro* does not show any inherent directionality (Wheeler et al., 2001). The role of NO in pollen tube directionality in *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 Ca^{2+} 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 an 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 filiform apparatus and synergid cells, still to be fully understood (Figure 2).

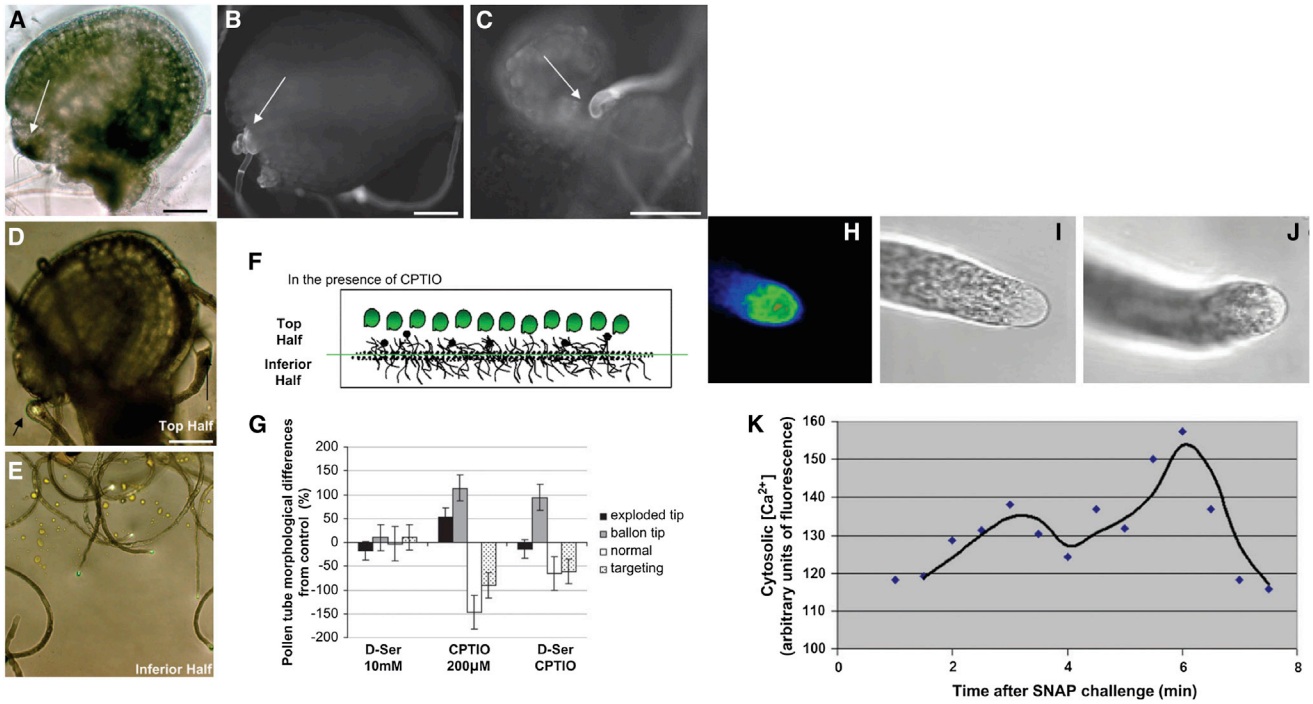

Figure 2. *Semi-vivo* Assays with Isolated Ovules and Pollen of *Lilium longiflorum*.

(A–G) Images of an isolated ovule **(A)** and pollen tube targeting **(B and C)** where arrows show micropyle entrance region. 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 halves 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), an 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 Ca^{2+} influx in pollen tubes by D-Ser partially rescued normal pollen tube morphology, suggesting that this pathway is also dependent on Ca^{2+} signaling **(H, I, and J)**. A role for NO in modulating Ca^{2+} signaling was further corroborated by direct imaging of the cytosolic free Ca^{2+} concentration **(H)** during NO-induced reorientation **(I, J, and K)** (adapted from Prado et al., 2008).

COMMUNICATING WITH NO: MULTITASKING IN PLANTS

In animals, NO is a vital signaling molecule involved in a diversity of important cellular functions including 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 defense, stomatal aperture, leaf senescence, flowering, and fertilization (Baudouin and Hancock, 2014; 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 cyclic ADP ribose (cADPR) 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 have been 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 ERF 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, as well as 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 (Sanz 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 (Fernández-Marcos et al., 2011, 2012). This signaling pathway is also observed in response to iron deficiency, where auxin induces an NO burst and ferric-chelate reductase (FCR) activity to enhance iron 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., 2010a), while the triple mutant *nia1nia2noa1* was later shown to produce less than 10% NO when compared with the wild-type (Lozano-Juste and León, 2010). Furthermore, these authors showed that MPK6 kinase activity is involved in the regulation of H_2O_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 NO and ethylene are synthesized as signaling molecules. Under these conditions, the plant hemoglobin 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 hemoglobin 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 cell 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, 1992; Allen et al., 2001; Li et al., 2009; Wang et al., 2012c). External calmodulin also triggers a significant increase in NO levels associated with stomata closure in wild-type *Arabidopsis*, but this effect is abolished in the *Atnoa1* and *gpa1* (Gα subunit of G protein) mutants (Li et al., 2009). The authors suggested an involvement of H_2O_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., 2012c). It was shown that the CAS transduces the $Ca^{2+}{}_{out}$ signal through the downstream action of NO and H_2O_2, which prolongs $Ca^{2+}{}_{in}$ oscillations. The crosstalk between NO and H_2O_2, two key intermediates in multiple stress responses (e.g. salt tolerance, hypoxia), is vital during guard cell stomata closure induced by ABA. A crosstalk between ABA and NO seems also to occur. Recently, NO was showed to negatively regulate ABA by inhibiting open stomata 1 (OST1)/sucrose nonfermenting 1 (SNF1)-related protein kinase 2.6 (SnRK2.6) through S-nitrosylation in guard cells (Wang et al., 2015). Gene disruption in the double mutant *AtrbohD/F* (reduced NADPH oxidase activity) impairs ABA-induced stomata closing, by disrupting ROS production and $Ca^{2+}{}_{in}$ increases, and the activation of plasma membrane Ca^{2+}-permeable channels in guard cells (Kwak et al., 2003). The double mutant *AtrbohD/F* was also used to demonstrate that ABA-mediated NO generation is dependent on ABA-induced H_2O_2 production (Bright et al., 2006). In essence, *AtrbohD/F* failed to generate NO in response to ABA, and H_2O_2-induced stomata closure was inhibited by the removal of NO with an NO scavenger, implying NO as a downstream effector of H_2O_2. A thermotolerance study demonstrated the activation of NO, which stimulated heat-shock (HS) DNA-binding factor activity and HS protein accumulation through H_2O_2 (Wang et al., 2014).

NO has been implicated in plant resistance to various abiotic stresses, including aluminum (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 *Atnoa1* 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., 2009b). During wheat resistance to Al-induced oxidative stress, the NR-mediated early NO burst maintained root function and enhanced antioxidant enzyme activities under Al toxicity (Sun et al., 2014b). The same group later showed that NO alleviated Al-induced root growth inhibition, through a regulatory relationship between NO and the ascorbate–glutathione cycle (Sun et al., 2014a).

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 $Ca^{2+}{}_{in}$ 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 cyclase 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 $Ca^{2+}{}_{in}$ elevation, which is regulated by a family of peptide signaling molecules, AtPeps, and their receptor (atPepR1)(Qi et al., 2010). $Ca^{2+}{}_{in}$ 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, Ma, 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_2O_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_2O_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* toxins in *Arabidopsis* (Yao et al., 2012). Moreover, NO production was almost completely blocked by supplementation with either diphenylene iodonium or dimethylthiourea (potent inhibitors of NADPH oxidase and H_2O_2 scavenger, respectively), suggesting that H_2O_2 may act upstream of NO during responses to *V. dahliae* 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 (cysteine 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, which 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 cysteine 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 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 CROSSTALK: 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 (Bolotina et al., 1994; Wilson and Garthwaite 2010; Wang et al., 2012a). NO can act indirectly through guanylate cyclases to activate cGMP-dependent cellular responses, e.g. through post-translational modification of proteins, by *S*-nitrosylation, and activation of phosphatases and protein kinases such as MAPKs. The *S*-nitrosylation biochemical reaction occurs when a nitrosyl group is added to the thiol side chain of cysteine residues to form SNO. All these events may lead to changes in tertiary structure and, subsequently, function of ion channels (e.g. Garcia-Mata et al., 2003; González et al., 2012; Wang et al., 2012a; 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 CO 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). Ca^{2+} has a pivotal role in NO-promoted stomatal closure. Garcia-Mata et al. (2003) established intracellular $Ca^{2+}{}_{in}$ concentration as principal effector of NO (<10 μM) in *Vicia faba* guard cells during ABA-induced response. The $Ca^{2+}{}_{in}$ rise leads to a subset of ABA-associated events, and the downstream inactivation of inward-rectifying K^+ channels ($I_{K,in}$) (Ca^{2+}-sensitive), to prevent K^+ uptake, and activation of outward-rectifying K^+ channels ($I_{K,out}$) and chloride (I_{Cl-}) channels at the plasma membrane, to facilitate solute efflux. By means of a patch clamp, the authors showed that a $Ca^{2+}{}_{in}$ elevation mediated by low nanomolar levels of NO was essential for normal inactivation of $I_{K,in}$ but not $I_{K,out}$, and activation of I_{Cl-} by ABA. Furthermore, NO did not promote a shift in the voltage sensitivity for Ca^{2+}-channel gating that is characteristic of ABA. In addition, NO-sensitive $Ca^{2+}{}_{in}$ release was blocked by ryanodine and 1-H-(1,2,4)-oxadiazole-[4,3-*a*]quinoxaline-1-one, antagonists of cADPR-sensitive Ca^{2+} channels and guanylate cyclase, respectively, suggesting that NO acts via cGMP and cADPR to sensitize endomembrane Ca^{2+} channels for internal Ca^{2+} 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 $I_{K,out}$ by *S*-nitrosylation. The effect of NO on the K^+ channel was mimicked by phenylarsine oxide, an oxidizing agent that crosslinks 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 Ca^{2+} release, implicating a target for protein kinase control in ABA signaling that would feed into an NO-dependent Ca^{2+} release (Sokolovski et al., 2005). Working downstream of H_2O_2, NO is involved on the ABA-inhibited blue light

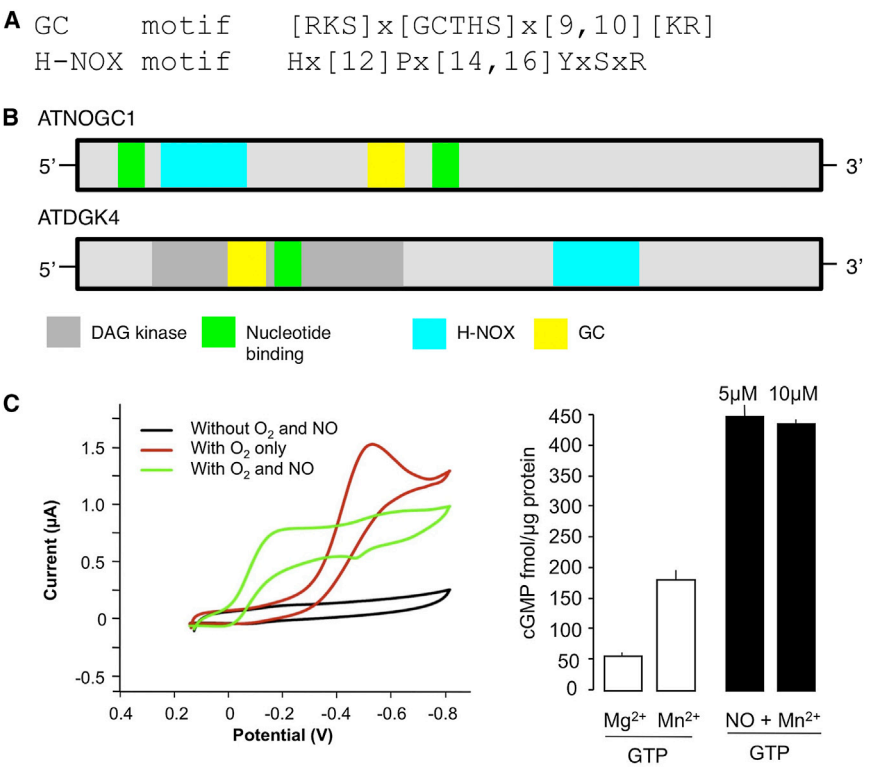

Figure 3. Identification and Characterization *Arabidopsis* H-NOX GC Candidate Molecules.

(A and B) The GC and H-NOX motifs **(A)** were used in PatMatch searches against the *Arabidopsis* proteome, 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).

(BL)-dependent stomatal opening by inducing the dephosphorylation of H^+-ATPase on the plasma membrane in *V. faba* guard cell protoplasts (Zhang et al., 2004, 2007). Recently, the same group showed that NO inhibits the BL-induced inward K^+ currents through Ca^{2+}, indicating that Ca^{2+} 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 crosstalk among Ca^{2+}, H_2O_2, and NO, and transcriptional activation of target gene through calmodulins and Ca^{2+}-dependent protein kinases was proposed (González et al., 2012). Furthermore, Ca^{2+}_{in} release through NAADP-, ryanodine-, and inositol-1,4,5-triphosphate-sensitive Ca^{2+} channels was also shown to be activated by NO and H_2O_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 B_6 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, thereby limiting spread of biotrophic pathogens from the initial site of infection, through the generation of NO and H_2O_2 mediated by Ca^{2+}_{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_2O_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 nonlinear interactive network and tight feedback control by both effectors.

THE QUEST FOR NO-SENSING MOLECULES IN PLANTS

In bacteria and animals, the *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 center leading to the generation of cGMP from guanosine triphosphate (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). In addition, 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

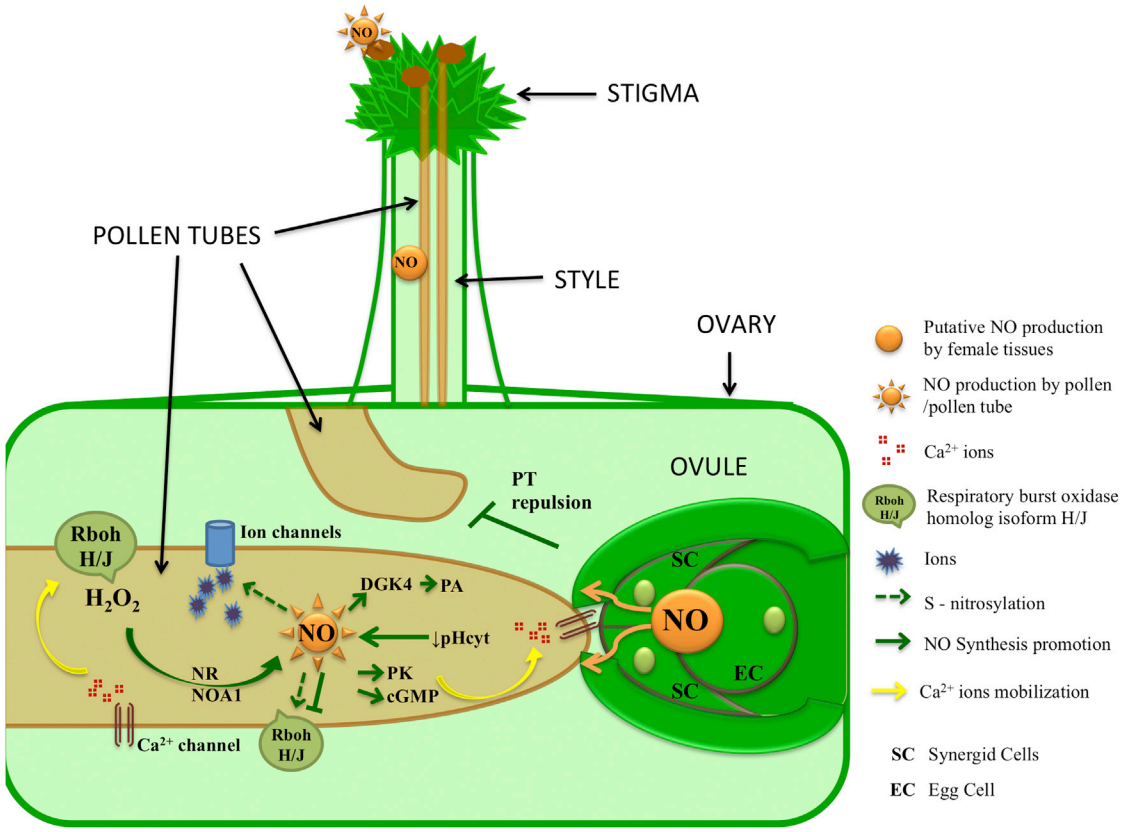

Figure 4. Model Depicting a Putative Feedback-Model of the NO/H_2O_2/Ca^{2+} Signaling Pathways During Sexual Reproduction in Flowering Plants.

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 RbohH/RbohJ are exclusively expressed in pollen and localize to the plasma membrane (PM) where they produce ROS, namely H_2O_2. The H_2O_2 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. Nonenzymatic 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 Ca^{2+} from intracellular stores; NO may also activate protein kinases (PK) enabling RbohH and RbohJ (Respiratory Burst Oxidase Homologue) to bind Ca^{2+}, triggering more ROS production, and cGMP, which can also activate Ca^{2+} channels at the PM. Possible *S*-nitrosylation of several proteins, such as ion channels (e.g. SLAH3, showed to be localized in the PM) (Gutermuth et al., 2013), 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 Ca^{2+} concentration. It is hypothesized that a fertilized ovule may exhibit an NO burst which will repulse incoming PTs (Pollen Tubes), promoting polyspermy blockage, through unknown mechanisms. Not drawn to scale.

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 at position 14, and retrieves more than 60 candidates (Supplemental 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) (Figure 3B and 3C). 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 (Potocký et al., 2003; 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, this would constitute a direct link between NO and Ca^{2+} signaling as has been proposed in, e.g., the pollen tubes of *Pinus bungeana* (Wang et al., 2009a). A microarray analysis, representing over 24,000 genes, verified changes in gene expression in response to NO in Arabidopsis. The study showed the up-regulation of several genes encoding disease-resistance proteins, transcription factors, zinc finger proteins, glutathione S-transferases, ABC transporters, kinases and biosynthetic genes of phytohormones, lignin and alkaloids, all potential NO-sensing molecules (Parani et al., 2004).

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 reorientation response and targeting to the ovule in the *L. longiflorum* model are modulated by NO (Prado et al., 2004, 2008; Wang et al., 2012b), it is conceivable that ATDGK4 has a role in NO-dependent directional growth of pollen tubes. In addition, in both *L. longiflorum* and *C. sinensis* systems the NO-induced inhibition of pollen tube growth and reorientation response are partially abrogated in the presence of the GC inhibitors (Prado et al., 2004; Wang et al., 2012b). 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 as well as the destruction of the ozone layer. Nowadays, NO is mostly appreciated as a molecule essential for innumerable functions in both animals and plants. It is a key signaling molecule that controls plant growth and development, 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 such as Ca^{2+} and cGMP seem to be able to act both upstream and downstream of NO, suggesting a complex nonlinear interactive network and tight feedback control. Thus far 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 in the understanding of NO sensing in plants.

New insights into the possible roles of NO during fertilization are discussed herein. Recent data show 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 upstream or downstream effectors of the NO signaling cascade, remain serious obstacles for further understanding its action mechanisms by genetic approaches.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

J.A.F.'s laboratory is funded by Fundação para a Ciência e Tecnologia (PTDC/BEX-BCM/0376/2012 and PTDC/BIA-PLA/4018/ 2012).

ACKNOWLEDGMENTS

P.D. acknowledges an FCT fellowship (SFRH/BD/69168/2010). No conflict of interest declared.

Received: October 3, 2014
Revised: December 11, 2014
Accepted: December 14, 2014
Published: December 23, 2014

REFERENCES

Agurla, S., Gayatri, G., and Raghavendra, A.S. (2014). Nitric oxide as a secondary messenger during stomatal closure as a part of plant immunity response against pathogens. Nitric Oxide **43C**:89–96.

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., von Bodman, S., and Berkowitz, G.A. (2007). Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell **19**:1081–1095.

Allen, G.J., Chu, S.P., Harrington, C.L., Schumacher, K., Hoffmann, T., Tang, Y.Y., Grill, E., and Schroeder, J.I. (2001). A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature **411**:1053–1057.

Barroso, J.B., Corpas, F.J., Carreras, A., Sandalio, L.M., Valderrama, R., Palma, J.M., Lupiáñez, J.A., and del Río, L.A. (1999). Localization of nitric oxide in plant peroxisomes. J. Biol. Chem. **274**:36729–36733.

Baudouin, E., and Hancock, J.T. (2014). Nitric oxide signaling in plants. Front Plant Sci. **4**:553.

Beale, K.M., Leydonm, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes entering an *Arabidopsis* ovule. Curr. Biol. **22**:1090–1094.

Bellamy, T.C., Wood, J., and Garthwaite, J. (2001). On the activation of soluble guanylyl cyclase by nitric oxide. Proc. Natl. Acad. Sci. USA **99**:507–510.

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

Boavida, L., Becker, J.D., Vieira, A.M., and Feijó, J.A. (2005). Gametophyte interaction and sexual reproduction: how plants make a zygote. Int. J. Dev. Biol. **49**:615–632.

Boavida, L.C., Borges, F., Becker, J.D., and Feijó, J.A. (2011). Whole genome analysis of gene expression reveals coordinated activation of signaling and metabolic pathways during pollen-pistil interactions in *Arabidopsis*. Plant Physiol. **155**:2066–2080.

Boisson-Dernier, A., Lituiev, D.S., Nestorova, A., Franck, C.M., Thirugnanarajah, S., and Grossniklaus, U. (2013). ANXUR receptor-like kinases coordinate cell wall integrity with growth at the pollen tube tip via NADPH oxidases. PLoS Biol. **11**:e1001719.

Bolotina, V.M., Najibi, S., Palacino, J.J., Pagano, P.J., and Cohen, R.A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature **368**:850–853.

Boon, E.M., and Marletta, M.A. (2005). Ligand specificity of H-NOX domains: from sGC to bacterial NO sensors. J. Inorg. Biochem. **99**:892–902.

Boon, E.M., Huang, S.H., and Marletta, M.A. (2005). A molecular basis for NO selectivity in soluble guanylate cyclase. Nat. Chem. Biol. **1**:54–59.

Bredt, D.S., and Snyder, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin requiring enzyme. Proc. Natl. Acad. Sci. USA **87**:682–685.

Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., and Neill, S.J. (2006). ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J. **45**:113–122.

Bright, J., Hiscocl, S.J., James, P.E., and Hancock, J.T. (2009). Pollen generates nitric oxide and nitrite: a possible link to pollen-induced allergic responses. Plant Physiol. Biochem. **47**:49–55.

Butt, Y.K.C., Lum, J.H.K., and Lo, S.C.L. (2003). Proteomic identification of plant proteins probed by mammalian nitric oxide synthase antibodies. Planta **216**:762–771.

Campbell, W.H. (1999). Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**:277–303.

Cardenas, L., Martınez, A., Sanchez, F., and Quinto, C. (2008). Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). Plant J. **56**:802–813.

Chandok, M.R., Ytterberg, A.J., van Wijk, K.J., and Klessig, D.F. (2003). The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. Cell **113**:469–482, Retraction in: Klessig, D.F., Ytterberg, A.J., and van Wijk, K.J. (2004). Cell 119, 445.

Chandok, M.R., Ekengren, S.K., Martin, G.B., and Klessig, D.F. (2004). Suppression of pathogen-inducible NO synthase (iNOS) activity in tomato increases susceptibility to *Pseudomonas syringae*. Proc. Natl. Acad. Sci. USA **101**:8239–8244, Retraction in: Klessig, D.F., Martin, G.B., and Ekengren, S.K. (2004). Proc. Natl. Acad. Sci. USA 101, 16081.

Charles, I.G., Palmer, R.M., Hickery, M.S., Bayliss, M.T., Chubb, A.P., Hall, V.S., Moss, D.W., and Moncada, S. (1993). Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proc. Natl. Acad. Sci. USA **90**:11419–11423.

Chen, W.W., Yang, J.L., Qin, C., Jin, C.W., Mo, J.H., Ye, T., and Zheng, S.J. (2010). Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in *Arabidopsis*. Plant Physiol. **154**:810–819.

Coelho, S.M., Brownlee, C., and Bothwell, J.H. (2008). A tip-high, Ca^{2+}-interdependent, reactive oxygen species gradient is associated with polarized growth in *Fucus serratus* zygotes. Planta **227**:1037–1046.

Cooney, R.V., Harwood, P.J., Custer, L.J., and Franke, A.A. (1994). Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. Environ. Health Perspect. **102**:460–462.

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

Correa-Aragunde, N., Graziano, M., Chevalier, C., and Lamattina, L. (2006). Nitric oxide modulates the expression of cell cycle regulatory genes during lateral root formation in tomato. J. Exp. Bot. **57**:581–588.

Correa-Aragunde, N., Lombardo, C., and Lamattina, L. (2008). Nitric oxide: an active nitrogen molecule that modulates cellulose synthesis in tomato roots. New Phytol. **179**:386–396.

Crawford, N.M., Galli, M., Tischner, R., Heimer, Y.M., Okamoto, M., and Mack, A. (2006). Response to Zemojtel et al.: plant nitric oxide synthase: back to square one. Trends Plant Sci. **11**:526–527.

Dai, Z., Farquhar, E.R., Arora, D.P., and Boon, E.M. (2012). Is histidine dissociation a critical component of the NO/H-NOX signaling mechanism? Insights from X-ray absorption spectroscopy. Dalton Trans. **41**:7984–7993.

del Río, L.A., Corpas, J.F., and Barroso, J.B. (2004). Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry **65**:783–792.

Delledonne, M., Xia, Y.J., Dixon, R.A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. Nature **394**:585–588.

Denninger, J.W., and Marletta, M.A. (1999). Guanylate cyclase and the .NO/cGMP signaling pathway. Biochim. Biophys. Acta **1411**:334–350.

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

Dordas, C., Hasinoff, B.B., Rivoal, J., and Hill, R.D. (2004). Class-1 hemoglobins, nitrate and NO levels in anoxic maize cell-suspension cultures. Planta **219**:66–72.

Dresselhaus, T., and Franklin-Tong, N. (2013). Male-female crosstalk during pollen germination, tube growth and guidance, and double fertilization. Mol. Plant **6**:1018–1036.

Dresselhaus, T., and Sprunck, S. (2012). Plant fertilization: maximizing reproductive success. Curr. Biol. **22**:R487–R489.

Duan, Q., Kita, D., Johnson, E.A., Aggarwal, M., Gates, L., Wu, H.M., and Cheung, A.Y. (2014). Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in *Arabidopsis*. Nat. Commun. **5**:3129.

Durner, J., Wendehenne, D., and Klessig, D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA **95**:10328–10333.

Fares, A., Rossignol, M., and Peltier, J.B. (2011). Proteomics investigation of endogenous S-nitrosylation in *Arabidopsis*. Biochem. Biophys. Res. Commun. **416**:331–336.

Feelisch, M., and Martin, J.F. (1995). The early role of nitric oxide in evolution. Trends Ecol. Evol. **10**:496–499.

Feijó, J.A., Costa, S., Prado, A.M., Becker, J.D., and Certal, A.C. (2004). Signaling by tips. Curr. Opin. Plant Biol. **7**:589–598.

Feijó, J.A. (2010). The mathematics of sexual attraction. J. Biol. **9**:18.

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

Fernández-Marcos, M., Sanz, L., and Lorenzo, O. (2012). Nitric oxide: an emerging regulator of cell elongation during primary root growth. Plant Signal. Behav. **7**:196–200.

Fewson, C.A., and Nicholas, D.J. (1960). Utilization of nitric oxide by micro-organisms and higher plants. Nature **188**:794–796.

Filippou, P., Antoniou, C., and Fotopoulos, V. (2012). The nitric oxide donor sodium nitroprusside regulates polyamine and proline metabolism in leaves of *Medicago truncatula* plants. Free Radic. Biol. Med. **56**:172–183.

Flores-Pérez, U., Sauret-Güeto, S., Gas, E., Jarvis, P., and Rodríguez-Concepción, M. (2008). A mutant impaired in the production of plastome-encoded proteins uncovers a mechanism for the homeostasis of isoprenoid biosynthetic enzymes in *Arabidopsis* plastids. Plant Cell **5**:1303–1315.

Foissner, I., Wendehenne, D., Langebartels, C., and Durner, J. (2000). *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J. **23**:817–824.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., et al. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422**:442–446.

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

Fröhlich, A., and Durner, J. (2011). The hunt for plant nitric oxide synthase (NOS): is one really needed? Plant Sci. **181**:401–404.

Frungillo, L., Skelly, M.J., Loake, G.J., Spoel, S.H., and Salgado, I. (2014). S-nitrosothiols regulate nitric oxide production and storage in plants through the nitrogen assimilation pathway. Nat. Commun. **5**:5401.

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L., and Blatt, M.R. (2003). Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proc. Natl. Acad. Sci. USA **100**:11116–11121.

Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., Di Silvio, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H., and Billiar, T.R. (1993). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc. Natl. Acad. Sci. USA **90**:3491–3495.

Gibbs, D.J., Md Isa, N., Movahedi, M., Lozano-Juste, J., Mendiondo, G.M., Berckhan, S., Marín-de la Rosa, N., Vicente Conde, J., Sousa Correia, C., Pearce, S.P., et al. (2014). Nitric oxide sensing in plants is mediated by proteolytic control of group VII ERF transcription factors. Mol. Cell **53**:369–379.

González, A., Cabrera Mde, L., Henríquez, M.J., Contreras, R.A., Morales, B., and Moenne, A. (2012). Cross talk among calcium, hydrogen peroxide, and nitric oxide and activation of gene expression involving calmodulins and calcium-dependent protein kinases in *Ulva compressa* exposed to copper excess. Plant Physiol. **158**:1451–1462.

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

Guo, F.Q., Okamoto, M., and Crawford, N.M. (2005). *Arabidopsis* nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. Plant Cell **17**:3436–3450.

Guo, K., Xia, K., and Yang, Z.M. (2008). Regulation of tomato lateral root development by carbon monoxide and involvement in auxin and nitric oxide. J. Exp. Bot. **59**:3443–3452.

Gupta, K.J., Igamberdiev, A.U., Manjunatha, G., Segu, S., Moran, J.F., Neelawarne, B., Bauwe, H., and Kaiser, W.M. (2011). The emerging roles of nitric oxide (NO) in plant mitochondria. Plant Sci. **181**:520–526.

Gutermuth, T., Lassig, R., Portes, M.T., Maierhofer, T., Romeis, T., Borst, J.W., Hedrich, R., Feijó, J.A., and Konrad, K.R. (2013). Pollen tube growth regulation by free anions depends on the interaction between the anion channel SLAH3 and calcium-dependent protein kinases CPK2 and CPK20. Plant Cell **11**:4525–4543.

Hall, A.V., Antoniou, H., Wang, Y., Cheung, A.H., Arbus, A.M., Olson, S.L., Lu, W.C., Kau, C.L., and Marsden, P.A. (1994). Structural organization of the human neuronal nitric oxide synthase gene (NOS1). J. Biol. Chem. **269**:33082–33090.

He, Y., Tang, R.H., Hao, Y., Stevens, R.D., Cook, C.W., Ahn, S.M., Jing, L., Yang, Z., Chen, L., Guo, F., et al. (2004). Nitric oxide represses the *Arabidopsis* floral transition. Science **305**:1968–1971.

Hebelstrup, K.H., van Zanten, M., Mandon, J., Voesenek, L.A., Harren, F.J., Cristescu, S.M., Møller, I.M., and Mur, L.A. (2012). Haemoglobin modulates NO emission and hyponasty under hypoxia-related stress in *Arabidopsis thaliana*. J. Exp. Bot. **63**:5581–5591.

Higashiyama, T., and Hamamura, Y. (2008). Gametophytic pollen tube guidance. Sex. Plant Reprod. **21**:17–26.

Hu, X., Neill, S.J., Tang, Z., and Cai, W. (2005). Nitric oxide mediates gravitropic bending in soybean roots. Plant Physiol. **137**:663–670.

Janssens, S.P., Shimouchi, A., Quertermous, T., Bloch, D.B., and Bloch, K.D. (1992). Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J. Biol. Chem. **267**:14519–14522, (published erratum in J. Biol. Chem. 1992;267, 22694).

Joudoi, T., Shichiri, Y., Kamizono, N., Akaike, T., Sawa, T., Yoshitake, J., Yamada, N., and Iwai, S. (2013). Nitrated cyclic GMP modulates guard cell signaling in *Arabidopsis*. Plant Cell **25**:558–571.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in *Arabidopsis*. Curr. Biol. **22**:1084–1089.

Kato, H., Takemoto, D., and Kawakita, K. (2012). Proteomic analysis of S-nitrosylated proteins in potato plant. Physiol. Plant **148**:371–386.

Kaya, H., Nakajima, R., Iwano, M., Kanaoka, M.M., Kimura, S., Takeda, S., Kawarazaki, T., Senzaki, E., Hamamura, Y., Higashiyama, T., et al. (2014). Ca^{2+}-activated reactive oxygen species production by *Arabidopsis* RbohH and RbohJ is essential for proper pollen tube tip growth. Plant Cell **26**:1069–1080.

Kim, B.H., Kim, C.H., Jung, K.Y., Jeon, B.H., Ju, E.J., and Choo, Y.K. (2004). Involvement of nitric oxide during *in vitro* fertilization and early embryonic development in mice. Arch. Pharm. Res. **27**:86–93.

Kneeshaw, S., Gelineau, S., Tada, Y., Loake, G.J., and Spoel, S.H. (2014). Selective protein denitrosylation activity of thioredoxin-h5 modulates plant immunity. Mol. Cell **56**:153–162.

Koh, S.D., Campbell, J.D., Carl, A., and Sanders, K.M. (1995). Nitric oxide activates multiple potassium channels in canine colonic smooth muscle. J. Physiol. **489**:735–743.

Kojima, H., Nakatsubo, N., Kikuchi, K., Urano, Y., Higuchi, T., Tanaka, J., Kudo, Y., and Nagano, T. (1998). Direct evidence of NO production in rat hippocampus and cortex using a new fluorescent indicator: DAF-2 DA. Neuroreport **9**:3345–3348.

Kolbert, Z., Bartha, B., and Erdei, L. (2008). Exogenous auxin-induced NO synthesis is nitrate reductase-associated in *Arabidopsis thaliana* root primordia. J. Plant Physiol. **165**:967–975.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. **22**:2623–2633.

Kwezi, L., Ruzvidzo, O., Wheeler, J.I., Govender, K., Iacuone, S., Thompson, P.E., Gehring, C., and Irving, H.R. (2011). The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signaling in plants. J. Biol. Chem. **286**:22580–22588.

Lamattina, L., Garcia-Mata, C., Graziano, M., and Pagnussat, G. (2003). Nitric oxide: the versatility of an extensive signal molecule. Annu. Rev. Plant Biol. **54**:109–136.

Lamotte, O., Courtois, C., Dobrowolska, G., Besson, A., Pugin, A., and Wendehenne, D. (2006). Mechanisms of nitric-oxide-induced increase of free cytosolic Ca^{2+} concentration in *Nicotiana plumbaginifolia* cells. Free Radic. Biol. Med. **40**:1369–1376.

Lassig, R., Gutermuth, T., Bey, T.D., Konrad, K.R., and Romeis, T. (2014). Pollen tube NAD(P)H oxidases act as a speed control to dampen growth rate oscillations during polarized cell growth. Plant J. **78**:94–106.

León, J., Castillo, M.C., Coego, A., Lozano-Juste, J., and Mir, R. (2014). Diverse functional interactions between nitric oxide and abscisic acid in plant development and responses to stress. J. Exp. Bot. **65**:907–921.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., and Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. **6**:427–437.

Li, J.H., Liu, Y.Q., Lü, P., Lin, H.F., Bai, Y., Wang, X.C., and Chen, Y.L. (2009). A signaling pathway linking nitric oxide production to heterotrimeric G protein and hydrogen peroxide regulates extracellular calmodulin induction of stomatal closure in *Arabidopsis*. Plant Physiol. **150**:114–124.

Li, Y., Wang, H., Tarus, B., Perez, M.R., Morellato, L., Henry, E., Berka, V., Tsai, A.L., Ramassamy, B., Dhimane, H., et al. (2012). Rational design of a fluorescent NADPH derivative imaging constitutive nitric-oxide synthases upon two-photon excitation. Proc. Natl. Acad. Sci. USA **109**:12526–12531.

Lim, M.H., Xu, D., and Lippard, S.J. (2006). Visualization of nitric oxide in living cells by a copper-based fluorescent probe. Nat. Chem. Biol. **2**:375–380.

Lindermayr, C., Saalbach, G., and Durner, J. (2005). Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. Plant Physiol. **137**:921–930.

Lombardo, M.C., and Lamattina, L. (2012). Nitric oxide is essential for vesicle formation and trafficking in *Arabidopsis* root hair growth. J. Exp. Bot. **63**:4875–4885.

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

Ma, W. (2011). Roles of Ca^{2+} and cyclic nucleotide gated channel in plant innate immunity. Plant Sci. **181**:342–346.

Ma, W., Smigel, A., Tsai, Y.C., Braam, J., and Berkowitz, G.A. (2008). Innate immunity signaling: cytosolic Ca^{2+} elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. Plant Physiol. **148**:818–828.

Ma, W., Smigel, A., Verma, R., and Berkowitz, G.A. (2009). Cyclic nucleotide gated channels and related signaling components in plant innate immunity. Plant Signal. Behav. **4**:277–282.

MacRobbie, E. (1992). Calcium and ABA-induced stomatal closure. Philos. Trans. R. Soc. Lond. B **338**:5–18.

Majláth, I., Szalai, G., Papp, I., Vanková, R., and Janda, T. (2011). Atnoa1 mutant Arabidopsis plants induce compensation mechanisms to reduce the negative effects of the mutation. J. Plant Physiol. **168**:1184–1190.

Marsden, P.A., Schappert, K.T., Chen, H.S., Flowers, M., Sundell, C.L., Wilcox, J.N., Lamas, S., and Michel, T. (1992). Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett. **307**:287–293.

Marton, M.L., and Dresselhaus, T. (2008). A comparison of early molecular fertilization mechanisms in animals and flowering plants. Sex. Plant Reprod. **21**:37–52.

McInnis, S.M., Desikan, R., Hancock, J.T., and Hiscock, S.J. (2006). Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signaling crosstalk? New Phytol. **172**:221–228.

Meier, S., Seoighe, C., Kwezi, L., Irving, H., and Gehring, C. (2007). Plant nucleotide cyclases: an increasingly complex and growing family. Plant Signal. Behav. **2**:536–539.

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

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

Monshausen, G.B., Bibikova, T.N., Messerli, M.A., Shi, C., and Gilroy, S. (2007). Oscillations in extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs. Proc. Natl. Acad. Sci. USA **104**:20996–21001.

Monteiro, D., Liu, Q., Lisboa, S., Scherer, G.E., Quader, H., and Malho, R. (2005). Phosphoinositides and phosphatidic acid regulate pollen tube growth and reorientation through modulation of $[Ca^{2+}]c$ and membrane secretion. J. Exp. Bot. **56**:1665–1674.

Moreau, M., Lee, G.I., Wang, Y., Crane, B.R., and Klessig, D.F. (2008). AtNOS/A1 is a functional *Arabidopsis thaliana* cGTPase and not a nitric oxide synthase. J. Biol. Chem. **283**:32957–32967.

Morimoto, T., Loh, P.C., Hirai, T., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. (2002). Six GTP-binding proteins of the Era/Obg family are essential for cell growth in *Bacillus subtilis*. Microbiology **148**:3539–3552.

Mulaudzi, T., Ludidi, N., Ruzvidzo, O., Morse, M., Hendricks, N., Iwuoha, E., and Gehring, C. (2011). Identification of a novel *Arabidopsis thaliana* nitric oxide-binding molecule with guanylate cyclase activity *in vitro*. FEBS Lett. **585**:2693–2697.

Mur, L.A., Mandon, J., Cristescu, S.M., Harren, F.J., and Prats, E. (2011). Methods of nitric oxide detection in plants: a commentary. Plant Sci. **181**:509–519.

Mur, L.A.J., Sivakumaran, A., Mandon, J., Cristescu, S.M., Harren, F.J., and Hebelstrup, K.H. (2012). Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. J. Exp. Bot. **63**:4375–4387.

Nakane, M., Schmidt, H.H., Pollock, J.S., Förstermann, U., and Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. FEBS Lett. **316**:175–180.

Neill, S., Barros, R., Bright, J., Desikan, R., Hancock, J., Harrison, J., Morris, P., Ribeiro, D., and Wilson, I. (2008). Nitric oxide, stomatal closure, and abiotic stress. J. Exp. Bot. **59**:165–176.

Olea, C., Jr., Boon, E.M., Pellicena, P., Kurriyan, J., and Marletta, M.A. (2008). Probing the function of heme distortion in the H-NOX family. ACS Chem. Biol. **3**:703–710.

Pagnussat, G.C., Lanteri, M.L., and Lamattina, L. (2003). Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol. **132**:1241–1248.

Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro. BMC Plant Biol. **6**:7–14.

Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327**:524–526.

Parani, M., Rudrabhatla, S., Myers, R., Weirich, H., Smith, B., Leaman, D.W., and Goldman, S.L. (2004). Microarray analysis of nitric oxide responsive transcripts in Arabidopsis. Plant Biotechnol. J. **4**:359–366.

Pellicena, P., Karow, D.S., Boon, E.M., Marletta, M.A., and Kuriyan, J. (2004). Crystal structure of an oxygen-binding heme domain related to soluble guanylate cyclases. Proc. Natl. Acad. Sci. USA **101**:12854–12859.

Penson, S.P., Schuurink, R.C., Fath, A., Gubler, F., Jacobsen, J.V., and Jones, R.L. (1996). cGMP is required for gibberellic acid-induced gene expression in barley aleurone. Plant Cell **8**:2325–2333.

Perazzolli, M., Dominici, P., Romero-Puertas, M.C., Zago, E., Zeier, J., Sonoda, M., Lamb, C., and Delledonne, M. (2004). *Arabidopsis* nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. Plant Cell **16**:2785–2794.

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

Potocký, M., Eliás, M., Profotová, B., Novotná, Z., Valentová, O., and Zárský. (2003). Phosphatidic acid produced by phospholipase D is required for tobacco pollen tube growth. Planta **217**:122–130.

Potocký, M., Jones, M.A., Bezvoda, R., Smirnoff, N., and Zarský, V. (2007). Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. New Phytol. **174**:742–751.

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

Prado, A.M., Colaço, R., Moreno, N., Silva, A.C., and Feijó, J.A. (2008). Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signaling. Mol. Plant **1**:703–714.

Qi, Z., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C.A., and Berkowitz, G.A. (2010). Ca^{2+}signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. PNAS **107**:21193–21198.

Ribeiro, E.A., Jr., Cunha, F.Q., Tamashiro, W.M., and Martins, I.S. (1999). Growth phase dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Lett. **445**:283–286.

Romero-Puertas, M.C., Campostrini, N., Mattè, A., Righetti, P.G., Perazzoll, M., Zolla, L., Roepstorff, P., and Delledonne, M. (2008). Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. Proteomics **8**:1459–1469.

Sanz, L., Fernández-Marcos, M., Modrego, A., Lewis, D.R., Muday, G.K., Pollmann, S., Dueñas, M., Santos-Buelga, C., and Lorenzo, O. (2014). Nitric oxide plays a role in stem cell niche homeostasis through its interaction with auxin. Plant Physiol. **166**:1972–1984.

Schmidt, P.M., Schramm, M., Schroder, H., Wunder, F., and Stasch, J.P. (2004). Identification of residues crucially involved in the binding of the heme moiety of soluble guanylate cyclase. J. Biol. Chem. **279**:3025–3032.

Sherman, P.A., Laubach, V.E., Reep, B.R., and Wood, E.R. (1993). Purification andcDNA sequence of an inducible nitric oxide synthase from a human tumor cell line. Biochemistry **32**:11600–11605.

Shi, H.T., Li, R.J., Cai, W., Liu, W., Wang, C.L., and Lu, Y.T. (2012). Increasing nitric oxide content in *Arabidopsis thaliana* by expressing rat neuronal nitric oxide synthase resulted in enhanced stress tolerance. Plant Cell Physiol. **53**:344–357.

Simpson, G.G. (2005). NO flowering. Bioessays **27**:239–241.

Šírová, J., Sedlářová, M., Piterková, J., Luhová, L., and Petřivalský, M. (2011). The role of nitric oxide in the germination of plant seeds and pollen. Plant Sci. **181**:560–572.

Sokolovski, S., and Blatt, M.R. (2004). Nitric oxide block of outward-rectifying K^+ channels indicates direct control by protein nitrosylation in guard cells. Plant Physiol. **136**:4275–4284.

Sokolovski, S., Hills, A., Gay, R., Garcia-Mata, C., Lamattina, L., and Blatt, M.R. (2005). Protein phosphorylation is a prerequisite for intracellular Ca^{2+} release and ion channel control by nitric oxide and abscisic acid in guard cells. Plant J. **43**:520–529.

Stone, J.R., and Marletta, M.A. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry **33**:5636–5640.

Stone, J.R., Sands, R.H., Dunham, W.R., and Marletta, M.A. (1995). Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cyclase. Biochem. Biophys. Res. Commun. **207**:572–577.

Straub, A.C., Lohman, A.W., Billaud, M., Johnstone, S.R., Dwyer, S.T., Lee, M.Y., Bortz, P.S., Best, A.K., Columbus, L., Gaston, B., et al. (2012). Endothelial cell expression of haemoglobin α regulates nitric oxide signaling. Nature **491**:473–477.

Sun, C., Liu, L., Yu, Y., Liu, W., Lu, L., Jin, C., and Lin, X. (2014a). Nitric oxide alleviates aluminum-induced oxidative damage through regulating the ascorbate-glutathione cycle in roots of wheat. J. Integr. Plant Biol. http://dx.doi.org/10.1111/jipb.12298.

Sun, C., Lu, L., Liu, L., Liu, W., Yu, Y., Liu, X., Hu, Y., Jin, C., and Lin, X. (2014b). Nitrate reductase-mediated early nitric oxide burst alleviates oxidative damage induced by aluminum through enhancement of antioxidant defenses in roots of wheat (*Triticum aestivum*). New Phytol. **201**:1240–1250.

Tada, Y., Spoe, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008). Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins. Science **321**:952–956.

Tischner, R., Galli, M., Heimer, Y.M., Bielefeld, S., Okamoto, M., Mack, A., and Crawford, N.M. (2007). Interference with the citrulline-based nitric oxide synthase assay by argininosuccinate lyase activity in *Arabidopsis* extracts. FEBS J. **274**:4238–4245.

Vandelle, E., Poinssot, B., Wendehenne, D., Bentéjac, M., and Alain, P. (2006). Integrated signaling network involving calcium, nitric oxide, and active oxygen species but not mitogen-activated protein kinases in BcPG1-elicited grapevine defenses. Mol. Plant Microbe Interact. **19**:429–440.

Wang, Y., Chen, T., Zhang, C., Hao, H., Liu, P., Zheng, M., Baluska, F., Samai, J., and Lin, J. (2009a). Nitric oxide modulates the influx of extracellular Ca^{2+} and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes. New Phytol. **182**:851–862.

Wang, H., Liang, X., Wan, Q., Wang, X., and Bi, Y. (2009b). Ethylene and nitric oxide are involved in maintaining ion homeostasis in *Arabidopsis* callus under salt stress. Planta **230**:293–307.

Wang, P., Du, Y., Li, Y., Ren, D., and Song, C.P. (2010a). Hydrogen peroxide-mediated activation of MAP kinase 6 modulates nitric oxide biosynthesis and signal transduction in *Arabidopsis*. Plant Cell **22**:2981–2998.

Wang, C.L., Wu, J., Xu, G.H., Gao, Y.B., Chen, G., Wu, J.Y., Wu, H.Q., and Zhang, S.L. (2010b). S-RNase disrupts tip-localized reactive oxygen species and induces nuclear DNA degradation in incompatible pollen tubes of *Pyrus pyrifolia*. J. Cell Sci. **123**:4301–4309.

Wang, J.Q., Chu, X.P., Guo, M.L., Jin, D.Z., Xue, B., Berry, T.J., Fibuch, E.E., and Mao, L.M. (2012a). Modulation of ionotropic glutamate receptors and Acid-sensing ion channels by nitric oxide. Front Physiol. **3**:164.

Wang, Y.H., Li, X.C., Zhu-Ge, Q., Jiang, X., Wang, W.D., Fang, W.P., Chen, X., and Li, X.H. (2012b). Nitric oxide participates in cold-inhibited *Camellia sinensis* pollen germination and tube growth partly via cGMP in vitro. PLoS One **7**:e52436.

Wang, W.H., Yi, X.Q., Han, A.D., Liu, T.W., Chen, J., Wu, F.H., Dong, X.J., He, J.X., Pei, Z.M., and Zheng, H.L. (2012c). Calcium-sensing receptor regulates stomatal closure through hydrogen peroxide and nitric oxide in response to extracellular calcium in *Arabidopsis*. J. Exp. Bot. **63**:177–190.

Wang, L., Guo, Y., Jia, L., Chu, H., Zhou, S., Chen, K., Wu, D., and Zhao, L. (2014). Hydrogen peroxide acts upstream of nitric oxide in the heat shock pathway in *Arabidopsis* seedlings. Plant Physiol. **164**:2184–2196.

Wang, P., Du, Y., Hou, Y.J., Zhao, Y., Hsu, C.C., Yuan, F., Zhu, X., Tao, W.A., Song, C.P., and Zhu, J.K. (2015). Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. Proc. Natl. Acad. Sci. USA. **112**:613–618.

Wedel, B., Humber, P., Harteneck, C., Foerster, J., Malkewitz, J., Bohme, E., Schulyz, G., and Koesling, D. (1994). Mutation of His-105 in the beta 1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. Proc. Natl. Acad. Sci. USA **91**:2592–2596.

Wendehenne, D., and Hancock, J.T. (2011). New frontiers in nitric oxide biology in plant. Plant Sci. **181**:507–508.

Wendehenne, D., Pugin, A., Klessig, D.F., and Durner, J. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends Plant Sci. **6**:177–183.

Wheeler, M.J., Franklin-Tong, V.E., and Franklin, F.C.H. (2001). The molecular and genetic basis of pollen-pistil interactions. New Phytol. **151**:565–584.

Wilkins, K.A., Bancroft, J., Bosch, M., Ings, J., Smirnoff, N., and Franklin-Tong, V.E. (2011). Reactive oxygen species and nitric oxide mediate actin reorganization and programmed cell death in the self-incompatibility response of papaver. Plant Physiol. **156**:404–416.

Wilkinson, J.Q., and Crawford, N.M. (1991). Identification of the *Arabidopsis* CHL3 gene as the nitrate reductase structural gene NIA2. Plant Cell **3**:461–471.

Wilson, G.W., and Garthwaite, J. (2010). Hyperpolarization-activated ion channels as targets for nitric oxide signaling in deep cerebellar nuclei. Eur. J. Neurosci. **31**:1935–1945.

Wilson, I.D., Neill, S.J., and Hancock, J.T. (2008). Nitric oxide synthesis and signaling in plants. Plant Cell Environ. **31**:622–631.

Wilson, I.D., Hiscock, S.J., James, P.E., and Hancock, J.T. (2009). Nitric oxide and nitrite are likely mediators of pollen interactions. Plant Signal. Behav. **4**:416–418.

Wudick, M.M., and Feijó, J.A. (2014). At the intersection: merging Ca^{2+} and ROS signaling pathways in pollen. Mol. Plant **7**:1595–1597.

Xia, J., Kong, D., Xue, S., Tian, W., Li, N., Bao, F., Hu, Y., Du, J., Wang, Y., Pan, X., et al. (2014). Nitric oxide negatively regulates AKT1-mediated potassium uptake through modulating vitamin B6 homeostasis in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **111**:16196–16201.

Yamamoto-Katou, A., Katou, S., Yoshioka, H., Doke, N., and Kawakita, K. (2006). Nitrate reductase is responsible for elicitin-induced nitric oxide production in *Nicotiana benthamiana*. Plant Cell Physiol. **47**:726–735.

Yamasaki, H., and Sakihama, Y. (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: *in vitro* evidence for the NR-dependent formation of active nitrogen species. FEBS Lett. **468**:89–92.

Yao, L.L., Pei, B.L., Zhou, Q., and Li, Y.Z. (2012). NO serves as a signaling intermediate downstream of H_2O_2 to modulate dynamic microtubule cytoskeleton during responses to VD toxins in *Arabidopsis*. Plant Signal. Behav. **7**:174–177.

Yoshioka, K., Moeder, W., Kang, H.G., Kachroo, P., Masmoudi, K., Berkowitz, G., and Klessig, D.F. (2006). The chimeric *Arabidopsis* CYCLIC NUCLEOTIDE GATED ION CHANNEL 11/12 activates multiple pathogen resistance responses. Plant Cell **18**:747–763.

Yu, M., Lamattina, L., Spoe, S.H., and Loake, G.J. (2014). Nitric oxide function in plant biology: a redox cue in deconvolution. New Phytol. **202**:1142–1156.

Yun, B.W., Feechan, A., Yin, M., Saidi, N.B., Le Bihan, T., Yu, M., Moore, J.W., Kang, J.G., Kwon, E., Spoel, S.H., et al. (2011). S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature **478**:264–268.

Zafra, A., Rodríguez-García, M.I., and Alché, Jde D. (2010). Cellular localization of ROS and NO in olive reproductive tissues during flower development. BMC Plant Biol. **10**:36.

Zeidler, D., Zahringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P., and Durner, J. (2004). Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. USA **101**:15811–15816.

Zemojtel, T., Frohlich, A., Palmieri, M.C., Kolanczyk, M., Mikula, I., Wyrwicz, L.S., Wanker, E.E., Mundlos, S., Vingron, M., Martasek, P., et al. (2006). Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. **11**:524–525.

Zhang, X., Wang, H., Takemiya, A., Song, C.P., and Shimazaki, K. (2004). Inhibition of blue light-dependent H^+ pumping by abscisic acid through hydrogen peroxide-induced dephosphorylation of the plasma membrane H^+-ATPase in guard cell protoplasts. Plant Physiol. **136**:4150–4158.

Zhang, X., Takemiya, A., Kinoshita, T., and Shimazaki, K. (2007). Nitric oxide inhibits blue light-specific stomatal opening via abscisic acid signaling pathways in *Vicia* guard cells. Plant Cell Physiol. **48**:715–723.

Zhao, Y., Schelvis, J.P., Babcock, G.T., and Marletta, M.A. (1998). Identification of histidine 105 in the beta1 subunit of soluble guanylate cyclase as the heme proximal ligand. Biochemistry **37**:4502–4509.

Zhao, M.G., Tian, Q.Y., and Zhang, W.H. (2007). Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. Plant Physiol. **144**:206–217.

Zhao, X., Qiao, X.R., Yuan, J., Ma, X.F., and Zhang, X. (2012). Nitric oxide inhibits blue light-induced stomatal opening by regulating the K^+ influx in guard cells. Plant Sci. **184**:29–35.

Zhao, X., Li, Y.Y., Xiao, H.L., Xu, C.S., and Zhang, X. (2013). Nitric oxide blocks blue light-induced K^+ influx by elevating the cytosolic Ca^{2+} concentration in *Vicia faba* L. guard cells. J. Integr. Plant Biol. **55**:527–536.

Zhou, L., and Zhu, D.Y. (2009). Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. Nitric Oxide **20**:223–230.