New insights into nitric oxide metabolism and regulatory functions

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Nitric oxide (NO) has been intensively studied to elucidate the role of this enigmatic signaling molecule in plant development, metabolism and disease responses. Many studies using pharmacological and biochemical tools have demonstrated that NO functions in hormone responses, programmed cell death, defense gene induction and signal transduction pathways. NO originates from two sources in plants: nitrite and arginine. Recent studies using mutants and transgenic plants have confirmed these key findings and have gone further to identify (i) a new mechanism to modulate NO bioactivity involving hemoglobin, (ii) a gene involved in arginine-dependent NO synthesis, and (iii) a novel function for NO signaling in flowering. These findings continue to elucidate the expanding role of NO in plant biology.

Roles for nitric oxide in plants

Nitric oxide (NO) serves as a signal in plants and animals. It functions in disease resistance, abiotic stress, cell death, respiration, senescence, root development, germination and hormone responses. It is an unusual signal in that it is a reactive, lipophilic and volatile free radical that can be cytotoxic. In plants, NO signaling involves cGMP, cADP ribose, Ca$^{2+}$, salicylic acid and protein kinases. There is also extensive overlap and cross talk with H$_2$O$_2$ signaling. Excellent reviews have been published recently that detail these and other aspects of NO signaling and synthesis [1–10].

Most of our understanding of NO synthesis and signaling is based on pharmacological studies that use NO donors and NO scavengers. In addition, NO is monitored by a variety of techniques including chemiluminescence, electron paramagnetic resonance and NO electrodes. For cellular studies, the NO-specific fluor- escein dyes 4,5-diaminofluorescein diacetate (DAF-2DA) and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) are used. Such studies, for example, have shown that abscisic acid (ABA) treatment of guard cells leads to an increase in NO levels (Figure 1) [11–14]. Treatment of guard cells with the NO scavenger cPTIO inhibits ABA-induced closure whereas treatment with NO donors induces stomatal closure. Similarly, auxin treatment elevates NO concentrations in hypocotyls, and NO induces adventitious and lateral root formation, whereas cPTIO inhibits auxin-induced responses in roots [15–18]. During hypersensitive responses, there is a burst of NO, that in turn induces defense genes [e.g. PAL (phenylalanine ammonia lyase) and PR-1] and, together with H$_2$O$_2$, cell death [8,9,19–22]. A burst of NO has also been observed in differentiating xylem cells, suggesting that NO is involved in xylem differentiation and cell death [23]. These and other experiments indicate that NO plays a key role in plant metabolism, signaling, defense and development. However, there are questions about NO that are still unanswered. For example, what are the physiologically relevant mechanisms for NO synthesis? What controls the level of NO in planta, and what insights can be obtained from mutants defective in NO synthesis or signaling? Recent publications over the past two years have provided some answers to these questions and new insights into NO metabolism and regulation.

Modulation of nitric oxide levels by hemoglobin

Animal studies have shown that NO and NO derivatives such as S-nitrosoglutathione regulate enzyme activity by modifying amino acid side chains of key metabolic or regulatory proteins [24,25]. For example, S-nitrosylation of a specific cysteine activates the oncoprotein GTPase p21$^{ras}$ and inhibits the transcription factor NF-$kappa$B [25]. Such reactions can occur without enzyme catalysis and are controlled by the concentration and redox state of NO and the availability and reactivity of target groups. In plants, programmed cell death (PCD) is affected by the level of NO; in particular, the ratio of NO to H$_2$O$_2$ is crucial for inducing cell death in cell culture systems [8,21]. Thus, modulating NO levels can regulate NO signaling and toxicity.

In animal and bacterial cells, hemoglobins play an important role in regulating NO. Bacteria have flavohemoglobins that detoxify NO, allowing them to evade host defense responses [26]. These enzymes convert NO to nitrate (aerobically) or nitrous oxide (anaerobically) using NADPH. In animals, NO can react with oxyhemoglobin to produce nitrate and methemoglobin (NO dioxygenase) or bind to the heme-Fe of deoxyhemoglobin to produce iron-nitrosyl-hemoglobin [27] (Figure 1). In addition, NO can react with specific cysteines to produce S-nitroso-hemo- globin [28,29] (Figure 1). Several recent papers have shown that plant hemoglobins also react with and modulate the level of NO.

Several types of hemoglobins are found in plants including symbiotic and nonsymbiotic hemoglobins.
Nonsymbiotic hemoglobins are further divided into class-1 proteins (Hb1), which have a high affinity for oxygen (1–3 nM), and class-2 proteins, which have a low affinity for oxygen [30,31]. Hb1 proteins are induced by hypoxia and help protect plants in low oxygen environments [31–34]. Hypoxia and anoxia also enhance NO emission [31,35]. It has been proposed that Hb1 proteins modulate the level of NO in plants [31].

Several laboratories have now reported that plant Hb1 proteins react with NO in vitro. Crude extracts from alfalfa roots or \textit{E. coli} cultures expressing a barley Hb1 degrade NO faster than do control cultures; this activity is inhibited by antibodies against plant Hb1 [36]. Purified \textit{Arabidopsis} AHb1 reacts with NO to produce nitrate and methemoglobin in the presence of oxygen [37]. The reaction to produce methemoglobin occurs stoichiometrically. To function catalytically, methemoglobin (Fe$^{3+}$) must be reduced back to hemoglobin (Fe$^{2+}$) to react again with NO. In bacteria and animals, this is accomplished by a separate flavin-containing reductase domain capable of catalytically degrade NO to nitrate. However, it is difficult to exclude the possibility that a separate flavoprotein is involved in AHb1 reduction \textit{in vivo} [34]. Indeed, a hemoglobin reductase system has been identified in alfalfa root cultures [36].

It is noteworthy that S-nitrosylation of AHb1 has also been found, indicating that AHb1 can scavenge NO through the production of S-nitrosohemoglobin [37]. S-nitrosylation is a key mechanism for NO signaling in animals but its role in plant signaling is unknown. An important advance was made recently when a proteomic analysis identified >50 S-nitrosylated proteins in \textit{Arabidopsis}. These proteins are implicated in processes that involve stress, redox, signaling, the cytoskeleton and metabolism [38]. In addition, there is evidence that NO regulates the activity of the K-channel in guard cells [39] and glyceraldehyde 3-phosphate dehydrogenase \textit{in vitro} [38] via S-nitrosylation.

To examine the relationship between NO and hemoglobin \textit{in vivo}, several groups have studied the effect of enhancing or suppressing Hb1 expression. This work has shown that Hb1 affects NO levels \textit{in vivo} and plant responses to hypoxic and nitrosative stress. Over-expression of Hb1 reduces NO levels under hypoxia whereas cells suppressed for Hb1 expression have enhanced levels of NO [34,37,40,41]. Over-expression of Hb1 results in better cell viability and stronger organ growth under hypoxia whereas the reverse suppresses organ growth [32,37,40]. Over-expression of a bacterial hemoglobin [42] or an alfalfa hemoglobin [43] produces more resistance to nitrosative stress. These findings establish a protective role for Hb1 that involves modulating NO levels during hypoxia.

Because NO is known to function in pathogen responses, several studies have examined the effects of Hb1 on the hypersensitive response but have produced conflicting results. In \textit{Arabidopsis}, Hb1 overexpression has little effect on NO production and cell death in plants infected with \textit{Pseudomonas syringae} (incompatible reaction) [37]. However, in tobacco, Hb1 overexpression results in fewer necrotic lesions when challenged with incompatible \textit{Pseudomonas syringae} or tobacco necrosis virus and produces more reactive oxygen species (ROS) and salicylic acid during the bacterial infection than do the control plants [43]. These lines also have higher levels of pathogenesis-related (PR-1a) transcript [34,44].

In summary, the findings described above demonstrate that class-1 nonsymbiotic hemoglobin protects plants during hypoxic stress and suppressed the increase in NO levels at low oxygen. The role of Hb1 in disease resistance is not yet clear; further work is required to elucidate the role. Nothing is known about its role in hormonal responses but it is likely to modulate such responses. In addition, there is a possibility that plant hemoglobins produce NO in addition to scavenging it. In mammalian systems, deoxyhemoglobin can act as a nitrite reductase (NiR) to produce NO, resulting in vasodilation [45]. This reaction occurs in the presence of \textmu M concentrations of nitrite at low oxygen levels. Such a reaction could occur in plants because hypoxia leads to an increase in nitrite levels and NO emission, particularly in the dark. This NO emission has been attributed to nitrate reductase and mitochondria (see below), but it is also possible that hemoglobin contributes. Class-1 hemoglobins are unlikely candidates because their high affinity for oxygen would generate few deoxyhemoglobins even at low oxygen levels. However, the class-2 hemoglobins have a lower affinity for oxygen and are better candidates to produce NO from nitrite at low oxygen levels. It would be interesting to see what effect overexpression or suppression of class-2 hemoglobins has on NO emission during hypoxia.

Another recent study used a bacterial flavohemoglobin (HMP) to study the role of NO during incompatible plant–pathogen interactions. The HMP gene, which acts as a NO dioxygenase (NOD), was introduced into \textit{Arabidopsis} to scavenge NO [46]. Transgenic plants expressing NOD emitted 50% less NO, degraded NO more quickly, and had an attenuated, pathogen-induced NO burst relative to wild-type plants. This approach is similar to that used to study salicylic acid signaling with the salicylic acid hydroxylase enzyme NahG [47].

When infected with avirulent \textit{Pseudomonas syringae}, NOD-expressing plants showed reduced hypersensitive cell death. A further reduction was obtained when...
NOD-expressing plants were infected with a transformed *Pseudomonas* expressing a related NOD gene from *Erwinia chrysanthemi* to deplete NO even further. The reduction in cell death was not associated with a substantial increase in pathogen growth in leaves. However, it was associated with a large decrease in H$_2$O$_2$ and PAL mRNA levels and a smaller decrease in salicylic acid levels. Furthermore, NOD-expressing plants have an increased ability to degrade H$_2$O$_2$ but this is not because of direct degradation of H$_2$O$_2$ by NOD. These results indicate that NO acts to increase H$_2$O$_2$ levels during an infection by inhibiting H$_2$O$_2$-degrading enzymes, which is consistent with a previous study showing that tobacco catalase and ascorbate peroxidase are inhibited by NO [48]. The decrease in H$_2$O$_2$ levels after infection in NOD-expressing plants was transient (up to four days); longer time points showed higher H$_2$O$_2$ levels consistent with a previous finding where constitutive expression of an alfalfa hemoglobin increased ROS levels in infected tobacco plants [43]. Taken together, these experiments support the conclusions from pharmacological experiments that NO is an important signaling molecule in plant defense responses.

**Synthesis of nitric oxide**

One of the mysteries about NO has revolved around its synthesis. Eight possible enzymatic sources for NO have been proposed [5] along with non-enzymatic mechanisms [49]. It has been known for many years that nitrate reductase (NR) can produce NO and N$_2$O from nitrite resulting in NO emission [35,50–52]. High NO emission rates correlate with high nitrite levels and NR activation, which occur during anoxia [35,53–55]. Under aerobic conditions, NO emission is low (usually less than 0.5 nmol g$^{-1}$ FW h$^{-1}$), but under anoxia in the dark, NO emission can reach high levels (150 nmol g$^{-1}$ FW h$^{-1}$) [35].

The role of NR in generating NO for signaling has been a puzzle. A recent review provides many reasons why NR is an important source for NO during ABA-induced stomatal closure [56]. In addition, analysis of an Arabidopsis NR mutant (G′4–3, an NR double mutant with low but detectable NR activity [57]) has shown that ABA-induced NO synthesis and stomatal closure requires NR [11]. However, other findings show no dependence on NR. NO accumulation in the G′4–3 NR mutant is similar to wild type during bacterial infection [58] and during mechanical stress or wounding [59]. In addition, plants do not always have access to nitrate [60]. This raises the question, how can such fundamental processes as stomatal movement depend on a signal whose synthesis requires access to nitrate? One explanation is that NR is one of several sources that contribute to NO-mediated responses [5]. These sources can be classified as nitrite-mediated and arginine-mediated (Figure 2). Nitrite-mediated sources include NR, mitochondria [61,62], the apoplast [49], Ni-NO reductase [63] and perhaps class-2 hemoglobin. The arginine-mediated source would be nitric oxide synthase (NOS, described below). A recent test of the multiple source model provides evidence for NR and NOS participating in NO production during sorghum germination. In the presence of 12 mM nitrate, germinating sorghum embryonic axes produced a strong and transient increase in NO accumulation that correlated with NR and NOS activity between 24 and 30 h of imbibition [64].

A source of NO production that does not rely on nitrite is NOS. NOS enzymes use arginine, O$_2$ and NADPH to produce NO and citrulline. These enzymes have been well characterized in animals and exist as three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) [65,66]. iNOS produces copious amounts of NO during immune and inflammatory responses and is induced transcriptionally by cytokines, lipopolysaccharides (LPS) and other signals. eNOS and nNOS, by contrast, are constitutively expressed (although there are exceptions [67]) and are activated by calmodulin (CaM) and calcium. eNOS and nNOS produce NO at a lower rate than iNOS does and are involved in hormonal and neuronal signaling. All three enzymes are large (130–150 kDa) and have two domains separated by a CaM binding site. The N-terminal oxygenase domain has the arginine-binding site with a heme–Fe cofactor that accepts electrons from flavin cofactors present in the C-terminal reductase domain. The N-terminal domain also uses tetrahydrobiopterin as a redox cofactor.

In plants, arginine-dependent NOS activity has been detected along with inhibition of NO production by NOS inhibitors [6,68–70]. However, no gene or protein with sequence similar to the large animal NOS proteins has been found even in the sequenced Arabidopsis genome. These results suggest that the NOS activity in plants comes from a different type of enzyme.

A recent paper provides evidence for a plant NOS that is distinct from known animal NOS proteins [14]. Arabidopsis has a gene with a sequence similarity to a gene from Helix pomatia (snail) that is implicated in NO synthesis. The snail gene encodes a 60 kDa protein that co-purifies with NO activity from snail ganglia extracts and, when expressed in E. coli, increases NO synthesis in crude extracts [71]. This protein cross-reacts with antibodies against nNOS but has no sequence similarity to known animal NOS enzymes. It is similar to a family of GTP binding and hydrolyzing proteins from bacteria. The investigators suggested that the snail gene might encode an NOS accessory or regulatory protein. When the corresponding gene (AtNOS1) was knocked-out in Arabidopsis, the resulting mutant had reduced NO accumulation in roots (as measured with DAF-2 DA dye) and reduced NOS activity in leaf extracts (as measured by citrulline production) [14]. Over-expression of AtNOS1 in Arabidopsis resulted in higher levels of NOS activity in leaf extracts.

Expression of AtNOS1 protein in E. coli as a glutathione-S-transferase fusion, and enrichment 80-fold by glutathione-affinity chromatography produced a protein fraction with enriched NOS activity as measured by citrulline and Greiss assays. The activity was dependent on Ca$^{2+}$, CaM and NADPH but not flavin or tetrahydrobiopterin. The specific activity of the protein was low (5 nmol min$^{-1}$ mg$^{-1}$), more similar to that of eNOS than iNOS in animals. Treating plants with hormones known to increase NO levels (e.g. ABA and auxin) had little effect on the level of AtNOS1 mRNA. Examination of mutant guard
Thus, AtNOS1 resembles eNOS and nNOS more than stomatal closure were strongly inhibited in the mutant. Cells showed that ABA-induced NO accumulation and NO synthesis from nitrite and arginine. Schematic diagram showing known mechanisms and mechanisms postulated to synthesize NO. Nitrite is reduced to NO via non-enzymatic methods in the apoplast, nitrite–nitric oxide reductase (NiNOR) in the plasma membrane, nitrate reductase (NR) in the cytosol, a postulated class-2 hemoglobin (Hb2), and mitochondria by mechanisms unknown. The main competition for these reactions is nitrite reduction to ammonium in the plastid. The arginine-dependent reaction involves nitric oxide synthase (NOS).

Cells showed that ABA-induced NO accumulation and stomatal closure were strongly inhibited in the mutant. Thus, AtNOS1 resembles eNOS and nNOS more than iNOS in that AtNOS1, eNOS and nNOS are constitutively expressed, reversibly regulated by Ca$^{2+}$ and CaM and produce NO for signaling functions.

The data described above link AtNOS1 to NO synthesis during hormonal signaling. A recent publication also implicates AtNOS1 in pathogen responses [72]. Key signaling molecules for animal innate immunity are LPS found in the outer membrane of Gram-negative bacteria. LPS induce defense responses in plants [72]. LPS treatments of Arabidopsis cell cultures and leaves induced a rapid NO burst (within minutes); this burst is 80% reduced in the atnos1 mutant. NO production was assayed with both DAF-FM DA and electron paramagnetic resonance (EPR). This mutant also shows a dramatic increase in susceptibility to the bacterial pathogen Pseudomonas syringae. LPS induces expression of many defense and stress-related genes including those that encode PR proteins, glutathione-S-transferase and cytochrome P450. Induction of these genes was almost completely blocked in the atnos1 mutant.

One mystery about AtNOS1 is how it produces NO. The current data indicate that AtNOS1 functions as an arginine-dependent NOS; however, the sequence of the protein provides no clue about the mechanism and AtNOS1 is much smaller (62 kDa) than the animal enzymes (130–150 kDa). Further work is needed to determine how AtNOS1 functions and if it interacts with other subunits. It is interesting that AtNOS1 is part of a family of proteins conserved in animals (e.g., humans, mouse, snail, Drosophila and Caenorhabditis elegans) and bacteria [73]. The function of these proteins in animals and bacteria is not known. At present, AtNOS1 is the only known enzyme associated with arginine-dependent NOS activity in plants.

**Novel regulatory function for nitric oxide**

A new role for NO has been identified: the control of flowering timing. Internal and external signals such as photoperiod, vernalization, gibberellins and the circadian clock induce reproductive development [74,75]. While investigating the effects of NO on vegetative growth, it was found that applying the NO donor sodium nitroprusside delayed the onset of flowering [76]. A genetic screen was then performed to find NO hypersensitive mutants. Among the mutants identified were six that had mutations at a single locus, nox1. Mapping and complementation analysis showed that NOX1 is allelic to CUE1, the chlorophyll a/b binding protein underexpressed 1 gene [77]. CUE1 encodes a chloroplast phosphoenolpyruvate/phosphate translocator [78]. nox1 (cue1) mutants have higher levels of arginine, citrulline and NO. The NO hypersensitivity of these mutants is because of the overproduction of NO, presumably from the higher levels of arginine. It is not clear how disruption of the phosphoenolpyruvate/phosphate translocator elevates arginine levels; however, the phenotype of nox1 further corroborates the finding that NO synthesis can occur in vivo via an arginine-dependent ROS activity.

Studies of these mutants have confirmed that NO affects flowering and have provided a mechanism for this effect [76]. nox1 (cue1) mutants flower later than wild-type plants; the atnos1 mutant, which has lower NO levels, flowered earlier in a closed system. Furthermore, elevated NO suppressed the floral meristem identity gene LFY and the floral promotion gene CO whereas high levels of NO enhanced the floral repressor FLC (Figure 3). CO expression was analyzed further because its expression is under circadian control. CO mRNA (as well as G1 mRNA, a regulator of CO) still oscillated with the same phase under diurnal rhythms in the nox1 and atnos1 mutants but the amplitudes were lower in nox1 and higher in atnos1 mutants. NO levels were found to oscillate to a small extent in a diurnal cycle but the oscillations (period and amplitude) of the central clock oscillators TOC1 and CCA1 were not affected in the nox1 mutant. Thus, NO suppresses the transition to flowering not by disrupting the circadian clock but by affecting the expression of regulatory genes in flowering pathways.

**Conclusion**

The past two years have marked several major developments in our efforts to understand NO regulation and metabolism. The involvement of non-symbiotic hemoglobins in modulating NO levels provides a new mechanism to regulate NO bioactivity in vivo. The discovery of an enzyme involved in arginine-dependent NO synthesis establishes NOS as a mechanism for NO production in signaling and disease responses in plants. These and other findings lead to further questions about NO metabolism and signaling. How does NO signaling occur during conditions of NO excess (e.g. anoxia and hypoxia)? What sources (e.g. NR versus NOS) are physiologically important for signaling, defense responses and development, and...
does NO regulate different targets depending on its source? How important is S-nitrosylation in mediating NO-mediated responses? How do NO and other reactive nitrogen species interact with reactive oxygen species to induce cell death and the hypersensitive response? What is the significance, if any, to the site (subcellular compartment) of NO synthesis? Further phenotypic analyses of NR, NOS activities and many other aspects of NO biology.

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