Nitric oxide synthesis and signalling in plants

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ABSTRACT

As with all organisms, plants must respond to a plethora of external environmental cues. Individual plant cells must also perceive and respond to a wide range of internal signals. It is now well-accepted that nitric oxide (NO) is a component of the repertoire of signals that a plant uses to both thrive and survive. Recent experimental data have shown, or at least implicated, the involvement of NO in reproductive processes, control of development and in the regulation of physiological responses such as stomatal closure. However, although studies concerning NO synthesis and signalling in animals are well-advanced, in plants there are still fundamental questions concerning how NO is produced and used that need to be answered. For example, there is a range of potential NO-generating enzymes in plants, but no obvious plant nitric oxide synthase (NOS) homolog has yet been identified. Some studies have shown the importance of NOS-like enzymes in mediating NO responses in plants, while other studies suggest that the enzyme nitrate reductase (NR) is more important. Still, more published work suggests the involvement of completely different enzymes in plant NO synthesis. Similarly, it is not always clear how NO mediates its responses. Although it appears that in plants, as in animals, NO can lead to an increase in the signal cGMP which leads to altered ion channel activity and gene expression, it is not understood how this actually occurs.

NO is a relatively reactive compound, and it is not always easy to study. Furthermore, its biological activity needs to be considered in conjunction with that of other compounds such as reactive oxygen species (ROS) which can have a profound effect on both its accumulation and function. In this paper, we will review the present understanding of how NO is produced in plants, how it is removed when its signal is no longer required and how it may be both perceived and acted upon.

Key-words: cGMP; nitrate reductase; nitric oxide synthase; S-nitrosylation.

INTRODUCTION

By the end of the 1980s, the physiological importance of nitric oxide (NO) in animal cells was becoming well-understood, and it was not long before the first synthesizing enzyme was characterized (Bredt & Snyder 1990). NO is now established as a key signalling molecule in many species, and in plants it is now known to orchestrate a wide range of processes. NO signals during disease resistance (Delledonne et al. 1998), regulates stomatal closure (Neill et al. 2002), represses flowering (He et al. 2004), inhibits the activity of certain enzymes (Clarke et al. 2000), activates mitogen-activated protein (MAP) kinase signalling pathways (Kumar & Klessig 2000; Pagnussat et al. 2004), modulates the expression of cell cycle genes (Correa-Aragunde et al. 2006), causes pollen tube re-orientation (Prado, Porterfield & Feijó 2004), reduces seed dormancy (Bethke, Libourel & Jones 2006) and stimulates germination (Beligni & Lamattina 2000). NO also regulates the expression of a number of genes involved in the synthesis of and response to jasmonic acid (Orozco-Cárdenas & Ryan 2002; Jih, Chen & Jeng 2003), pathogen responses (Durner, Wendehenne & Klessig 1998), the synthesis and signalling of ethylene, the phenylpropanoid pathway, protein antioxidation mechanisms, photosynthesis, cellular trafficking, cell death and other basic metabolic processes (Polverari et al. 2003) (for review, see Wendehenne, Durner & Klessig 2004). It has also been proposed that NO is functionally involved in other processes such as the reproductive mechanisms that operate during pollen recognition by the stigma (Hiscock et al. 2007). Additionally, in what may be part of its signalling mechanism, it also induces raised cGMP levels (Durner et al. 1998) and elevates the level of cytosolic free Ca²⁺ (Durner et al. 1998; Klessig et al. 2000; Garcia-Mata et al. 2003).

Much of this work has involved the pharmacological application of NO donors such as sodium nitroprusside (SNP) to emulate NO production, NO scavengers such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) to remove the signal [e.g. cPTIO + NO → 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl (cPTI) + NO₂] (Goldstein, Russo & Samuni 2003) and detection methods that have employed dyes such as 4,5-diaminofluorescein diacetate (DAF-2 DA) which fluoresces when bound to the N₂O₃ formed by the reaction of NO with NO₂. Such approaches have recently faced a degree of criticism (Planchet & Kaiser 2006; Arita et al. 2007) relating to the specificity of the fluorescent dyes used and the possibility that the compounds PTI or cPTI, formed when the relevant scavengers react...
with NO, may in fact be what actually inhibit apparent NO responses rather than its removal per se. However, while there may still be questions to answer concerning the absolute specificity of dyes such as DAF-2 DA which can only be partially answered by use of the NO-insensitive, negative control dye 4-aminofluorescein diacetate (4-AF DA), it would appear that PTI and cPTI have no affect on the ability of abscisic acid (ABA) to cause stomatal closure in Arabidopsis (Wilson et al., unpublished results), a process inhibited by the scavenging of NO with either PTIO or cPTIO. A number of plant studies have also used other techniques such as chemiluminescence and electron paramagnetic resonance (EPR) to measure NO in tissues, and as is discussed as follows, the use of specific mutants that fail to make NO in guard cells has further qualified its importance in eliciting fundamental biological responses.

In the gaseous state, NO is a free radical and is, therefore, inherently unstable and reactive with a very short half-life in air. Furthermore, the radical can gain or lose electrons to become the NO⁺ or NO⁻ species. Thus, the chemistry of NO and its interactions with other compounds is not simple, and when its movement between cells or through cells is mooted this chemistry needs to be considered.

For any compound to be considered as a cellular signalling molecule, it has to fulfil various generic criteria. A quick look through the list of known signalling molecules will initially show that they are usually relatively small or at least have the capacity to move easily from their site of activation or synthesis to their site of action. The movement of NO between cells has been suggested, and as a small, non-charged molecule its diffusion and movement through the lipid membrane environment are entirely feasible. In fact, it is likely that NO accumulates within membranes where it is probably biologically active. Signalling molecules are also often produced in a developmentally regulated, spatial manner. Thus, NO synthesis may be expected to result from the regulated activity of one or more dedicated enzymes which generate the signal at specific cellular sites as and when required. Animals possess a family of nitric oxide synthases (NOS), and similar enzymes might be expected to be found in plants. A signal such as NO must also be specifically perceived and defined, recognized responses elicited through the activity of one or more specific signalling pathways. Thereafter, a mechanism for its removal is also required if the signal is not to be propagated unnecessarily. In this review, we give an overview of each of these aspects of NO signalling in plants and discuss some of the techniques employed to study them.

**NO GENERATION**

In order for NO to developmentally activate one or more signalling pathways, it would be expected that, as and when required, its site-specific synthesis would be both rapid and efficient. Although NO can be generated chemically (Neill, Desikan & Hancock 2003), it is much more likely that such production results from enzyme activity. An overview of the potential routes of plant NO production which are described as follows is shown in Fig. 1. In animals, there is a family of synthases, two of which are present constitutively (eNOS and nNOS) and one which is inducible (named either mNOS or iNOS). In mammals, these are dimeric proteins comprising 130–160 kDa subunits. Using a short redox pathway, they convert L-arginine through a trapped intermediate, hydroxyl-arginine, to L-citrulline with the simultaneous release of NO. This reaction pathway has provided obvious methods to explore the likely parallels in plants. L-Arginine analogues such as N⁵-nitro-L-arginine methyl ester (L-NAME) are efficient inhibitors of animal NOS-mediated NO synthesis, and this and similar compounds have, therefore, been employed in plants to suggest the presence of such enzymes. This approach has been employed extensively and provided a body of evidence that NOS-like enzymes are present in plants (Crawford 2006). However, a note of caution is required here. Many plant investigators have failed to measure the production of citrulline in their assays, and there are doubts concerning the specificity of compounds such as L-NAME when used in plants. Thus, the underlying biochemistry is not always clear and at least one recent report has shown that argininosuccinate lyase interferes with the L-citrulline-based assay and has highlighted the need to verify the production of this compound when using such an approach to detect NOS activity (Tischner et al. 2007). Perhaps the definitive approach would be to measure the incorporation of radiolabel from radiolabelled L-arginine into L-citrulline as operational evidence of the correct NO synthesizing pathway.

![Figure 1. The various routes of nitric oxide (NO) production in plants cells. NO can be synthesized enzymatically from nitrite (NO₂⁻) by nitrate reductase (NR). There is also considerable evidence for L-arginine-dependent NO synthase (NOS) activity in plant cells, although the protein AtNOS1 is no longer considered to be a NOS and no other plant candidate for the role has been identified. Evidence also exists for the activity of a nitrite:NOR reductase in roots and for the ability of both chloroplasts and mitochondria to convert NO₂⁻ to NO.](image-url)
Early evidence for the presence of NOS-like enzymes in pea peroxisomes came from immunogold labelling (Del Rio et al. 2002). Later, by means of chemiluminescence and EPR, arginine-dependent NO generation was also determined in these organelles (Corpas et al. 2004). More recently, NOS activity has also been reported in the roots, stems and leaves of pea seedlings (Corpas et al. 2006), while Valderrama et al. (2007) have shown salinity-induced NOS activity in olives. However, the cloning of a NOS-encoding gene from pea has remained elusive, and the lack of direct homologs of mammalian NOS genes within the published Arabidopsis (Arabidopsis Genome Initiative 2000) and rice (Yu et al. 2002) genome sequences has shown that identification of the gene(s) for plant NOS enzyme(s) will be far from trivial. Using a snail sequence homolog, Guo, Okamoto & Crawford (2003) suggested the discovery of a NOS-encoding gene in Arabidopsis termed AtNOS1. Assayed by a commercial NOS assay, AtNOS1 appeared to have the ability to convert l-arginine to l-citrulline, and a T-DNA insertion mutant, Atnos1, showed reduced NO generation in guard cells and roots stimulated with ABA. Other studies also supported the notion that this was a true plant NOS gene (He et al. 2004; Zeidler et al. 2004; Bright et al. 2006), but recently this has been questioned as investigators have been unable to reproduce earlier results and detect the l-arginine-dependent formation of l-citrulline when using the commercial assay kit and working with either AtNOS1 or related enzymes from other species (Zemojtel et al. 2006). Other attempts to detect NO resulting from the activity of this enzyme have also failed (Crawford et al. 2006). Currently, the view is that although AtNOS1 may not be a NOS per se, it is, nonetheless, an important factor in NO synthesis or accumulation. Reflecting this, it has been suggested that the name of the protein be changed to Arabidopsis thaliana NO-associated 1 (AtNOA1) (Crawford et al. 2006). Although no such activity has been determined, part of the AtNOA1 sequence encodes a potential GTPase domain (Zemojtel et al. 2006). The protein has been shown to be targeted to the mitochondria (Guo & Crawford 2005), and it has been suggested that its function may be associated with mitochondrial ribosome biosynthesis. Presumably, impairment of this process in some way affects NO generation.

In reality, the current state of affairs leaves the field of plant NO biology without a cloned and definitively identified NOS protein. However, in animals, NOS activity comprises a short redox pathway involving flavins and haem groups. Here, despite the fact that theoretically each subunit individually possesses all the redox components necessary to sustain NO synthesis, electron flow actually occurs across both subunits of the dimer. Therefore, it is possible that plant NOS-like enzymes may comprise multiple yet unidentified subunits that each contains part of the redox machinery required for NO synthesis from l-arginine and that these cosegregate to form a holistic redox system when needed. It remains a speculation that much in the way GTPases are involved in the control of other enzymes that produce free radicals such as mammalian NAD(P)H oxidase (Miyano et al. 2006), proteins such as AtNOA1 may be involved in the control of such a mechanism.

Another source of plant NO results from the activity of the enzyme nitrate reductase (NR). This enzyme is usually associated with nitrogen assimilation, but can also generate NO from nitrite (Dean & Harper 1986; Rockel et al. 2002) in an NAD(P)H-dependent reaction. Such activity can be inhibited by tungstate, a characteristic that can be used to assess the involvement of NR in potential signalling pathways (Bright et al. 2006). NR is a molybdenum-containing enzyme, and it is assumed that tungstate in some way competitively interferes within the metal-binding site of the enzyme. Arabidopsis has two genes for NR, NIA1 and NIA2, which give rise to proteins that are very similar (83.5% identical at the amino acid level), but which have a number of independent sequence regions in, for example, the N-terminal region. Using mutants such as the double mutant nia1,nia2, it has been shown that NR is involved in ABA-induced NO generation in guard cells (Desikan et al. 2002). Initially, it was suggested that this might result from aberrant N metabolism in this double mutant leading to a reduced cellular arginine content and thus, reduced NOS activity as a result of diminished substrate availability (Crawford 2006; Modolo et al. 2006). However, comparative investigation of the single mutants, nia1 and nia2, showed that, despite being expressed in leaves at much lower levels than NIA2, the most influential NR enzyme in guard cell NO generation was that encoded by NIA1 (Bright et al. 2006). Supported by Yu, Sukumaran & Marton (1998), such data, where the nia2 mutant was little affected in its ability to make NO and where it is unlikely that the nia1 mutant had generally disrupted N metabolism, engender the idea that there is a specific role for NIA1 in NO signalling. NO is also produced by guard cells as stomata close during the transition from light to dark (She, Song & He 2004), and interestingly nia1 stomata fail in this respect while those of nia2 behave as wild type (Wilson et al., unpublished data). Such observations do support the concept that, in guard cells at least, the NR isoform, NIA1, has a definite role in the production of NO in response to stimuli that cause stomatal closure.

To be involved in signalling, NR activity would be expected to be controlled and indeed it has been found that NO generation from NR can be modulated by phosphorylation of a conserved serine (Ser534 in Arabidopsis) (Rockel et al. 2002) and the binding of 14-3-3 proteins (Lillo et al. 2004) which appear to modulate its proteolysis (Weiner & Kaiser 1999). Removal of the phospho-regulation by substitution of the serine results in increased NO production by leaves and roots. However, whether this occurs in a differential manner between the two NR isoforms in vivo during ABA-induced stomatal closure is unknown. Presumably, the different roles suggested for the NIA1 and NIA2 isoforms of NR would suggest their interaction within different protein complexes. Any specific increase in the transcription of the gene for and activation of NIA1 as opposed to NIA2 in guard cells in response to ABA would suggest, for example, that of the two isoforms NIA1 is that individually targeted by downstream components of the
ABA signalling pathway. Such questions await answers. NO generation by NR is also increased under hypoxia, and modulation of nitrite levels can influence the rate of this production (Morot-Gaudry-Talarmain et al. 2002; Rockel et al. 2002).

Other enzymes have been found in plants that can also generate NO. A plasma-membrane-bound enzyme, nitrite-NO oxidoreductase (Ni-NOR), has been found which is reported to be root specific (Stohr & Stremlau 2006). It appears to use cytochrome c as an electron donor in vitro, but it has yet to be cloned and fully identified. Animal xanthine oxidoreductase (XOR) is capable of producing NO under hypoxic conditions (Millar et al. 1998), but work with recombinant XOR by Planchet & Kaiser (2006) suggests that this enzyme is probably not relevant to NO signalling in plants. Organelle NO generation has also been reported by several groups. Planchet et al. (2005) showed reduction of nitrite to NO by tobacco mitochondria, while soy bean chloroplasts appear to use either arginine or nitrite to generate NO (Jasid et al. 2006). Bethke, Badger & Jones (2004) have also shown a non-enzymatic generation of NO from nitrite under low pH in barley aleurone layers.

**NO AND ITS INTERACTION WITH REACTIVE OXYGEN SPECIES (ROS)**

In addition to NO, other reactive compounds are also involved in signalling in plants. The best studied of these are the ROS such as superoxide anions and hydrogen peroxide (H$_2$O$_2$). Often, these ROS are both spatially and developmentally produced concomitant with NO, and in various organisms' enzymes such as XOR (Millar et al. 1998) are actually capable of producing both. Importantly, ROS and NO can react with each other. Superoxide anions will readily react with NO to produce the very potent compound peroxynitrite (ONOO$^-$). This is very toxic to many cells, but surprisingly plant cells seem to be somewhat resistant (Delledonne et al. 2001), and it has been suggested that ONOO$^-$ can act as a signal in its own right (Klotz et al. 2002; Minetti, Mallozzi & Di Stasi 2002). However, of more probable relevance here is the fact that the presence of superoxide anions can, therefore, influence the concentration of NO available for signalling. As a result, it has been suggested that the rates of NO synthesis in plant cells are often underestimated (Vanin et al. 2004). ROS can also increase the levels of NO by increasing the activity of NO-generating enzymes. In mung bean leaf cells, Lum, Butt & Lo (2002) found that H$_2$O$_2$-induced NO production via an activity which was inhibited by the addition of a NOS inhibitor, and Ca$^{2+}$ ions were implicated in the signalling mechanism that operated. Similar data were reported demonstrating H$_2$O$_2$ induction of NO accumulation in the guard cells of *Vicia faba* (She et al. 2004; He et al. 2005), and H$_2$O$_2$ generation has been shown to be required for ABA-induced NO generation in the guard cells of both *V. faba* and Arabidopsis (Dong et al. 2005; Bright et al. 2006). Removal of the H$_2$O$_2$ using antioxidants greatly reduced NO accumulation and subsequent stomatal closure. Similarly, NO accumulation was markedly reduced in guard cells of the NAD(P)H oxidase *rbohD/F* mutant or following the application of NAD(P)H oxidase inhibitors (Kwak et al. 2003).

Additionally, while other ROS can, as described earlier, directly affect NO synthesis and accumulation, they can also compete for potential NO binding sites. As is described, below NO-mediated S-nitrosylation of thiol groups in proteins is a likely mechanism by which NO signals. However, thiol groups are also subject to competitive interaction with other ROS. Figure 2 highlights the processes that potentially occur and which may thus, attenuate or modify NO signalling.

**NO REMOVAL**

As mentioned previously, there must also be an effective means of removing NO if its signal is not to be propagated *ad infinitum*. Figure 3 summarizes the various mechanisms described as follows by which NO may be removed in plants. Being a radical NO is inherently unstable and will react readily with O$_2$ to form both nitrite and nitrate. As discussed earlier, NO can also react with ROS which may react with compounds such as glutathione. Thus, there is most likely competition for thiol group availability in terms of NO signalling by S-nitrosylation.

![Figure 2](image)

**Figure 2.** Competition for thiol groups. Two cysteine thiol groups within a protein may interact to form a disulphide bond. However, single thiol groups may be either reversibly (Enz-SOH and Enz-SO$_2$H) and irreversibly (Enz-SO$_3$H) oxidized by reactive oxygen species (ROS) such as H$_2$O$_2$ or may be S-nitrosylated by nitric oxide (NO). Additionally, thiol groups may react with compounds such as glutathione. Thus, there is most likely competition for thiol group availability in terms of NO signalling by S-nitrosylation.
dependent on NAD(P)H. Reaction with haemoglobins to form nitrate in a reaction with GSNO reductase. Additionally, NO can be removed by its reaction with GSH to form S-nitrosylated glutathione (GSNO) which can then be reduced to GSSG and NH3 by the enzyme GSNO reductase. Additionally, NO can be removed by its reaction with haemoglobins to form nitrate in a reaction dependent on NAD(P)H.

Figure 3. The removal of nitric oxide (NO) and its interactions with reactive oxygen species (ROS). NO can react with ROS such as superoxide to form compounds such as peroxynitrite. It can also react with O2 in air to form nitrite and nitrate. NO can also react with GSH to form S-nitrosylated glutathione (GSNO) which can then be reduced to GSSG and NH3 by the enzyme GSNO reductase. Additionally, NO can be removed by its reaction with haemoglobins to form nitrate in a reaction dependent on NAD(P)H.

necessarily end its ability to mediate cell signalling and its reactions, and their resulting products such as peroxynitrite and nitrite may be important.

NO will react with thiol groups, and as discussed as follows, this is probably a major way in which it potentially modifies proteins and may mediate its signalling. However, in cells, there are also low-molecular-weight thiols that can partake in this reaction. Glutathione is a tripeptide which can typically be at very high concentrations (2–3 mM) in plant cells (Ball et al. 2004). The majority of cellular glutathione exists in the reduced state as GSH, but the equilibrium is reversible and the oxidized state, GSSG, can be formed. GSH is a major cell antioxidant and maintains the cytoplasm in a reduced state with a relatively low oxidative potential. Alteration of this reduced state has been implicated in programmed cell death mechanisms (Cai & Jones 1998; Kirlin et al. 1999). However, NO can react with GSH to form S-nitrosylated glutathione (GSNO) which can then be metabolized by the enzyme GSNO reductase (Diaz et al. 2003). As discussed further, such mechanisms have been shown to be important in plant defence mechanisms (Feechan et al. 2005) including those of a systemic nature (Rustérucci et al. 2007).

It has long been known that NO can react with the haem group in soluble guanylyl cyclase (sGC), and as discussed as follows, this is one of the major ways in which NO can potentially influence signalling pathways. However, a number of other haem-containing proteins can also react with NO. Probably the most important of these when considering NO removal is haemoglobin. Non-symbiotic haemoglobins (nsHbs) can react with NO to form nitrate in an NAD(P)H-dependent manner, and thus, lower its bio-availability. This has been shown in barley, alfalfa and Arabidopsis (reviewed by Perazzolli, Romero-Puertas & Delledonne 2006). nsHb is also expressed in the nodules of Alnus firma where it may act to remove NO (Sasakura et al. 2006). Further evidence of the involvement of Hbs in NO detoxification came from work involving the transgenic manipulation of Hb levels. For example, altering Ahb1 levels in Arabidopsis resulted in conversely altered NO emissions (Perazzolli et al. 2004). Similarly, Boccara et al. (2005) found that a flavohaemoglobin from Erwinia chrysanthemi, HmpX, could remove NO and that infection with a HmpX-deficient mutant of E. chrysanthemi caused high levels of NO production and the hypersensitive response, the complex, early defence response that causes cell death in order to restrict the growth of pathogens in the host plant. Clearly then, the accumulation of NO is not only dependent on an increase in its generation, but may also be caused by a decrease in its removal. Thus, the level of cellular NO available for signalling will be a balance between its rate of synthesis and removal. As yet, however, there has been little consideration of the developmental and spatial separation of these competing processes and as has been seen with cAMP and various other signalling molecules (Zaccolo 2006), their temporal and spatial separation may cause hot spots of increased NO concentration. Thus, particular cells or regions of cells may be responsive to NO, while other cells or areas of cells or organelles may remain unaffected. Consequently, much more detailed spatial and temporal measurements of cellular NO will be required to fully unravel such potential complexity.

NO PERCEPTION AND SIGNAL TRANSDUCTION

Plants definitely perceive and respond to NO, but the underlying mechanisms by which this is achieved remain unclear. There is currently much inherent research interest, but as no specific plant NO receptor has been identified, attempts to resolve such issues have shadowed the approaches used in studies of mammalian NO signalling. NO is particularly reactive and may interact with and modify numerous proteins. Thus, there may be multiple routes of NO perception and signalling to be unravelled. An overview of our current understanding of plant NO signalling which is described as follows is shown in Fig. 4.

A key player in animal NO signalling is sGC. Binding of NO to its haem domain results in increased cGMP levels which in turn activates a number of downstream signalling processes. The use of pharmacological inhibitors of mammalian sGC in plants has placed cGMP downstream of both guard cell ABA and NO signalling during stomatal closure (Neill et al. 2003). NO induces raised levels of cGMP in plants (Durner et al. 1998), and both ABA and the NO donor SNP induce a transient increase in cGMP in guard-cell-enriched preparations from Arabidopsis (Harrison et al., unpublished data). This can be prevented by the...
Figure 4. An overview of potential nitric oxide (NO) signalling mechanisms in plants. Stimuli-induced increases in the synthesis of NO that exceed its removal result in increased cGMP levels that induce increases in free Ca\(^{2+}\) levels via cADPR signalling. This and the activation of mitogen-activated protein (MAP) kinase signalling pathways can result in altered biochemistry and gene expression. NO may also signal via protein thiold group S-nitrosylation and protein Tyr nitration, and the activity of the ryanodine receptor (RyR) calcium channel may be regulated by S-nitrosylation. The availability of thiold groups for S-nitrosylation may also depend on the levels of other competing reactive oxygen species (ROS).

application of the sGC inhibitor 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or the NO scavenger PTIO. Thus, a similar mechanism of NO-induced cGMP synthesis and signalling may also operate in plants.

Immediately downstream of GMP is the key signalling intermediate cyclic ADP-ribose (cADPR) (Wendehenne et al. 2001) which, in animal cells, stimulates the release of Ca\(^{2+}\) through the intracellular ryanodine receptor calcium channel (RyR). It is feasible that a similar mechanism of NO signalling operates in plants. In tobacco, applied cADPR causes a rise in the level of accumulation of mRNAs encoding phenylalanine ammonia lyase (PAL) and the pathogenesis-related protein 1 (PR-1) (Durner et al. 1998). This can be prevented by the co-application of RyR inhibitors. The accumulation of both these mRNAs has also been shown to be NO regulated, and cADPR antagonists cause a reduction in the level of PR-1 transcripts (Klessig et al. 2000). NO is known to cause increases in the level of free Ca\(^{2+}\) (Durner et al. 1998; Garcia-Mata et al. 2003), and cGMP, cADPR and Ca\(^{2+}\) have all been shown to be involved in regulating stomatal movements in response to ABA (Garcia-Mata & Lamattina 2002; Neill et al. 2003). Removing the NO during this process effectively prevents stomatal closure and simultaneously inhibits the ABA-induced inactivation of the Ca\(^{2+}\)-dependent inward rectifying K\(^{+}\) channel and activation of the outward rectifying Cl\(^{-}\) channel (Garcia-Mata et al. 2003). Thus, NO and Ca\(^{2+}\) are both strongly implicated in the signalling cascade that must operate. Thus, it would seem very likely that responses to NO may be accomplished by signalling through cGMP, cADPR and Ca\(^{2+}\). In common with other plant hormones, NO is also known to activate MAP kinase

signalling pathways (Kumar & Klessig 2000; Pagnussat et al. 2004) which presumably results in altered gene expression.

Genes encoding sGCs which contain a NO-binding haem domain are present in the genomes of animals and many lower eukaryotes. Prokaryotic sGCs also contain a similar NO-binding H-NOX domain (Karow et al. 2004; Boon et al. 2006). However, no direct plant homologs of such NO-sensitive sGCs have yet been identified. The Arabidopsis guanylyl cyclase, AtGC1, does not appear to be activated by NO (Ludidi & Gehring 2003). Thus, exactly how NO induces increased cGMP levels in plants remains unresolved. However, it is very probable that the responsible NO-sensitive enzymes are significantly different from their animal counterparts. Recently, a domain with potential guanyl cyclase activity has been described for the Arabidopsis brassinosteroid receptor, BRI1 (Kwezi et al. 2007). Thus, additional novel plant guanylyl cyclases probably await discovery.

As mentioned previously, the redox chemistry of NO allows it to react with haem groups which are present in numerous proteins. However, NO may also signal by mechanisms such as the direct S-nitrosylation and indirect trans-nitrosylation of both protein cysteine residues and low-molecular-weight compounds such as glutathione. Additionally, the nitration of protein tyrosine residues may also constitute a NO signalling mechanism.

S-nitrosylation regulates a number of animal signalling processes, structural protein functions and metabolic pathways, and has become established as the prototype redox-based, post-translational protein modification in this kingdom (Wang et al. 2006). Evidence is now emerging that it may play a similarly important role in plant NO signalling.

When extracts of Arabidopsis cell cultures were treated with GSNO, a number of proteins apparently became S-nitrosylated (Lindermayr, Saalbach & Durner 2005). A wide range of cellular processes were implicated, but whether or not the proteins involved are S-nitrosylated in vivo and the biological significance of this if they remain to be determined. In vitro, the activity of a recombinant methionine adenosyl transferase isoform has been shown to be altered by S-nitrosylation in a manner dependent on the presence of a specific Cys residue (Lindermayr et al. 2006). Arabidopsis non-symbiotic haemoglobin AHB1 is S-nitrosylated in over-expressing transgenic plants (Perazzolli et al. 2004), and the activity of an Arabidopsis metacaspase is also similarly modulated by the S-nitrosylation of a particular Cys residue (Belenghi et al. 2007). Under reduced partial pressures of O\(_2\), the mammalian RyR1 calcium channel demonstrates specific calmodulin-dependent, Cys S-nitrosylation at levels of NO which are physiologically relevant (Eu et al. 2000). Such an occurrence in plants would certainly be of relevance to NO signalling, and there is some evidence suggesting that this may be the case in stomatal guard cells (Sokolovski & Blatt 2004).

Protein S-nitrosylation can also be mediated by GSNO (Wang et al. 2006) and thus, the degree to which it occurs may be reflected by the availability of this compound. Consequently, the biological activity of NO may also be...
reflected in the levels of GSNO that exist. An Arabidopsis GSNO reductase, AtGSNOR1, has been identified and has been shown to have importance in its role in the regulation of the signalling network controlled by the plant immune system activator, salicylic acid (Sakamoto, Ueda & Morikawa 2002; Diaz et al. 2003; Feechan et al. 2005). Loss-of-function mutations in AtGSNOR1 caused elevated S-nitrosylation levels and reduced resistance (R)-generated pathogenesis defence responses in the plants (Feechan et al. 2005). Conversely, gain-of-function mutant plants showed increased pathogen resistance. The activity of the enzyme and expression of its encoding gene are both reduced during Cd-induced stress in pea (Barroso et al. 2006). Thus, there is certainly a case for S-nitrosylation being involved in both stress- and pathogenesis-related signalling pathways. Presumably, this results from the elevated NO levels that are associated with these events, but how this is coordinated remains to be determined.

The study of plant S-nitrosylation is clearly in its infancy, and much investigation is needed to determine which specific proteins are involved in vivo during the different NO-regulated processes. However, there are also bioinformatics approaches that may facilitate this process. For example, various S-nitrosylation motifs have been determined from the appropriate regions of animal proteins that are so modified. An NO-targeted motif, [HKR]-C-[VILMFWC]-x-[DE], has been suggested (Wang et al. 2006) and the motif [GSTCYNQ]-[KRHDE]-C-[DE] has been suggested as that targeted by GSNO. Searches of Arabidopsis protein databases with these two motifs yield a few hundred hits which include a number of MAP kinases and other signalling proteins. Unfortunately, the list generated contains none of the proteins identified as being S-nitrosylated after GSNO exposure of Arabidopsis cell culture extracts (Lindermayr et al. 2005). Thus, laboratory-based investigation may be the only means to this end, a sentiment reiterated by Hao et al. (2006) who suggested that a machine learning approach had failed to predict a linear motif identifying potentially S-nitrosylated Cys residues in proteins. This said, a few of the more potentially interesting bioinformatically generated targets may be worth further study. Arabidopsis mutant complementation experiments where the supposedly S-nitrosylated Cys residue is either present or absent in the complementing transgenic protein would be relatively easy and could potentially open the door wide on NO signalling.

NO may also signal via the nitration of tyrosine residues, a process mediated by ROS such as the ONOO- anion and nitrogen dioxide (NO2) (Radi 2004). The ONOO- anion can cause tyrosine nitration in vitro. However, its role in this process in vivo has been questioned, and alternative mechanisms that depend on the formation of NO2 by haem peroxidases acting on nitrite have become predominant (Brennan et al. 2002). Various recombinant Arabidopsis haemoglobinins demonstrate peroxidase-like activity and have been shown to differentially mediate nitrite-dependent protein nitration in vitro (Sakamoto et al. 2004). Endogenous protein tyrosine nitration also occurs in mutant tobacco plants exhibiting greatly increased NO production (Morot-Gaudry-Talarmain et al. 2002). More recently, increases in protein tyrosine nitration have been shown to occur in the leaves of olives during salt stress (Valderrama et al. 2007), a process which increases NO synthesis and accumulation. However, the extent and biological significance of protein nitration in plants remain to be seen as does the mechanism by which what is essentially a non-reversible reaction acts as a signalling mechanism. Presumably, such a signalling pathway would involve some methods of protein recognition and turnover that could feasibly involve F-box proteins and the ubiquitin-mediated degradation pathway. Again, whether or not such mechanisms are important in NO signalling awaits clarification.

SUMMARY AND CONCLUSIONS

There is now little argument that NO plays a definitive role in regulating a number of fundamental biological processes in plants. However, many questions remain concerning exactly how NO is produced in a regulated manner and how this signal is perceived and propagated into defined biological responses. L-Arginine-dependent NOS-like activity has been measured in plants, but still no direct homologs of any of the animal enzymes have been found in any of the fully sequenced plant genomes. It is possible that multiple as yet uncharacterized subunits associate to form a functional plant NOS-like enzyme, but to date there are no data to indicate that this is the case. Clearly, there is much biochemical and genetic investigation required to resolve this issue. At present, the regulated synthesis of NO in guard cells by NR is perhaps the best understood at the genetic level, but even here data are scarce save for that concerning the role of the NIA1 isoform in NO synthesis during either ABA- or dark-induced stomatal closure. Little is known concerning how such synthesis is regulated and the guard cell signalling pathways that must presumably, therefore, specifically target the NIA1 isoform. Because ABA and H2O2 both induce guard cell NO synthesis during stomatal closure, a process inhibited in the nia1, but not the nia2 mutant, it is entirely feasible to assume that downstream components in the signalling pathways of these compounds impinge on both the gene and enzyme activity of the NIA1 isoform. However, such concepts require a better understanding of the developmental and spatial regulation of the transcriptional activity of both NIA1 and NIA2 during ABA-induced stomatal closure, the concomitant analysis of preferential NIA1 activation in guard cells and the identification of proteins that associate specifically with NIA1 during this process. Such data are presently lacking as is any clarity concerning the role of any other potential NO-synthesizing enzymes such as Ni-NOR and XOR.

The availability of NO for signalling is clearly a balance between its synthesis and removal. That NO is synthesized by plants is not disputed, but how it is removed is less well-understood. As discussed earlier, there are a number of ways that NO can potentially be either sequestered or removed, but perhaps the most convincing method
described in plants so far is that mediated by the haemoglobins. The evidence provided by transgenic experiments that modulate pathogen susceptibility is compelling. However, one certainly cannot rule out the involvement of molecules such as GSH in regulating NO levels. Interestingly, a number of the mechanisms by which NO might be removed result in the production of compounds which may either signal in their own right or which are substrates for the enzymes which produce NO. Thus, there are again issues of developmental and spatial activity that need to be resolved if we are to better understand how NO levels are modulated in a regulated manner.

How NO is perceived and how the resulting signal is transduced are also processes that are currently poorly understood in plants. However, in stomatal guard cells at least, this probably involves cGMP, cADPR and Ca\(^{2+}\) – calmodulin signalling mechanisms. The clarification as to how NO is initially perceived in plants requires further investigation. No specific receptor has yet been identified in plants, and the lack of any direct plant homolog of animal sGC leaves unanswered questions concerning how NO induces the initial rise in cGMP levels. Although it is likely to occur in plants, the role exacted by S-nitrosylation and tyrosine nitration in NO signalling also requires further study to answer questions concerning which proteins are actually affected and how their modification results in a biological response. There are also questions concerning thiol group availability and the competition between NO and other ROS for these that once again beg answers surrounding the developmental and spatial activity of these compounds within cells.

There is clearly much that we do not know concerning NO signalling, but this short review highlights the need for further investigations in what has emerged as an interesting and challenging area in plant biology.

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