Review

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Nitric oxide function in plant biology: a redox cue in deconvolution

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Contents

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Summary

Nitric oxide (NO), a gaseous, redox-active small molecule, is gradually becoming established as a central regulator of growth, development, immunity and environmental interactions in plants. A major route for the transfer of NO bioactivity is S-nitrosylation, the covalent attachment of an NO moiety to a protein cysteine thiol to form an S-nitrosothiol (SNO). This chemical transformation is rapidly emerging as a prototypic, redox-based post-translational modification integral to the life of plants. Here we review the myriad roles of NO and SNOs in plant biology and, where known, the molecular mechanisms underpinning their activity.

I. Introduction

The gaseous compound nitric oxide (NO) was first described in 1772 as 'nitrous air' by Joseph Priestly, the English theologian, chemist, dissenting clergyman and educator. He was also the first to describe nitrous oxide (N_2O), which he termed 'nitrous air diminished'. Rather than focusing on his remit to heal the sick, Sir Humphry Davy concentrated on the uses of N_2O as a recreational drug with a series of his friends, including the romantic poets Shelly and Coleridge and many affluent ladies and gentleman of the period. Priestly's 'nitrous air' induced a sensation of mild drunkenness, often coupled with bouts of uncontrollable laughter.

Fast-forwarding to more recent times, NO has been demonstrated to orchestrate a plethora of physiological functions in mammals, was the subject of the Noble Prize in 1998 and was named 'molecule of the year' in 1992 by the journal *Science*. Despite the large amount of attention this small molecule has garnered within the animal field, the first report of NO generation within

biological systems was in plants (Klepper, 1979). Almost two decades later, a biological function for NO was identified when this small molecule was implicated as playing a role in plant immunity, initially in potato (*Solanum tuberosum*) (Noritake *et al.*, 1996) and then 2 yr later in Arabidopsis (Delledonne *et al.*, 1998; Durner *et al.*, 1998). To date, progress within the field of plant NO biology has been somewhat of a 'slow-burn'. However, the area is now gathering substantial momentum (Homem & Loake, 2013). In this context, a function for this gaseous free radical has recently been reported in a variety of processes integral to plant growth and development (Fernandez-Marcos *et al.*, 2011; Kwon *et al.*, 2012), in addition to numerous biotic (Hong *et al.*, 2008; Astier *et al.*, 2012b; Yun *et al.*, 2012) and abiotic stress responses (Cantrel *et al.*, 2011; Camejo *et al.*, 2013). Further, the application of new tools and technologies to this rapidly developing field is facilitating studies addressing the molecular mechanisms employed by NO to control a variety of key cellular processes (Spoel & Loake, 2011; Yu *et al.*, 2012). This review will explore the numerous sources proposed for NO synthesis, the myriad roles of NO and S-nitrosothiols (SNOs) and, where known, the molecular mechanisms underpining their function.

II. Routes of NO production

In mammals, NO is synthesized via an oxidative mechanism utilizing NO synthase (NOS), which consists of three well-characterized isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS)NOS (Alderton *et al.*, 2001). NOS isoforms may also be present in the mitochondria,including a constitutive(c-mtNOS) and an inducible (i-mtNOS)mitochondrial NOS (Lopez *et al.*, 2006; Escames *et al.*, 2007), which are thought to be derived from cytosolic nNOS and iNOS, respectively (Alderton *et al.*, 2001). Nevertheless, some studies have failed to locate mtNOS isoforms (Zaobornyj & Ghafourifar, 2012) which could be related to the different experimental designs and/or methods utilized in the NOS activity assays. NOS proteins catalyse the NADP-dependent oxidation of arginine (Arg) to NO and citrulline. However, genes encoding a structurally related enzyme have not been identified in higher plants despite the completion of numerous genome projects.

Seven sources have been proposed as possible routes for NO generation in plants (Gupta *et al.*, 2011; Mur *et al.*, 2013), which depend upon either reductive or oxidative chemistry (Fig. 1). Oxidative mechanisms include the production of NO from L-arginine (L-Arg), polyamines or hydroxylamines. By contrast, reductive routes are dependent upon nitrite as the primary substrate and include reduction via nitrate reductase (NR) and a plasma membrane-bound nitrite-NO reductase (NiNOR) and mitochondrial nitrite reduction.

III. Oxidative routes of NO synthesis

Despite the absence of an enzyme structurally related to that of mammalian NOS proteins, the production of NO and citrulline from L-Arg by higher plant extracts has been described and, further, established animal NOS inhibitors strikingly diminished this activity (Durner *et al.*, 1998; Delledonne *et al.*, 2001; Corpas *et al.*, 2006). Also, a loss-of-function mutant, *no overproducer 1* (*nox1*), has been reported to have several-fold greater concentrationsof L-Arg and, informatively, this plant line exhibits excessive NO and citrulline accumulation. These data therefore imply the existence of a plant NOS-like enzyme. Further, numerous studies in both Arabidopsisand tobacco (*Nicotiana tabacum*) have implicated such NOS-like activities as the source of reactive nitrogen intermediates (RNIs) during the nitrosative burst associated with plant immune function (Delledonne *et al.*, 1998; Durner *et al.*, 1998). However, no gene structurally analogous to mammalian *NOS* has been identified. Recently, a related protein has been discovered in *Ostreococcus tauri*, a single-celled green alga. *Ostreococcus tauri* NOS possessed 45% similarity to human NOS. This enzyme exhibited NOS activity *in vitro* and possessed similar properties to animal NOS proteins in terms of the K_m for L-Arg (12 μM) and the rate of NADPH oxidation (Foresi *et al.*, 2010). Unfortunately, this gene does not seem to be present in higher plants.

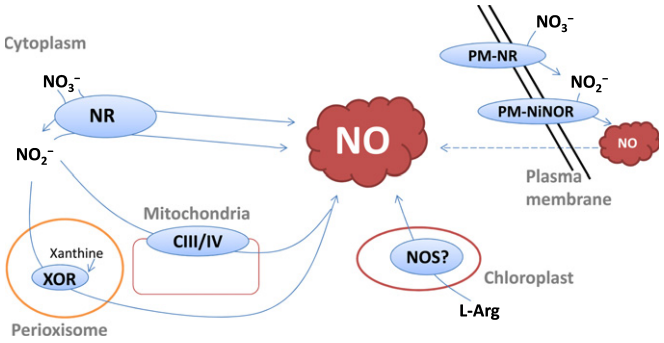

Fig. 1 Sources of nitric oxide (NO) production. The production of NO and citrulline from L-Arg by higher plant extracts has been described and animal NO synthase (NOS) inhibitors strikingly diminish this activity (Lea *et al.*, 2004; Lillo *et al.*, 2004; Corpas *et al.*, 2009). Nitrate reductase (NR), located in the cytosol, primarily catalyses the reduction of nitrate to nitrite. However, this enzyme can also catalyse the reduction of nitrite to NO (Yamamoto-Katou *et al.*, 2006). A plasma membrane (PM) -bound nitrite-NO reductase (NiNOR) activity has been uncovered in roots (Stohr *et al.*, 2001). The nitrite as substrate for NiNOR is probably provided by plasma membrane-bound NR in a coupled reaction. This enzyme generates extracellular NO and has been suggested to play a role in sensing nitrate availability (Meyer & Stohr, 2002).NO can also be generated by nitrite reduction in the mitochondrial inner membrane, probably via cytochrome c oxidase and/or reductase (Planchet *et al.*, 2005). The peroxisomal enzyme xanthine oxidoreductase (XOR) can also reduce nitrite to NO using NADH or xanthine as the reducing substrate (Godber *et al.*, 2000).

Interestingly, increases in the concentrationsof the polyamines spermine and spermidine induce NO release, but the actual reaction mechanism has not yet been resolved. Polyamine-mediated NO production has been proposed to be involved in root development and embryogenesis (Tun *et al.*, 2006), cadmium toxicity (Groppa *et al.*, 2008) and drought stress (Arasimowicz-Jelonek *et al.*, 2009). Another potential route for NO synthesis is via hydroxylamine-mediated synthesis. However, the location of hydroxylamine-mediated NO production is currently unknown, although hydroxylamine and reactive oxygen intermediates (ROIs) are known to act as substrates. This NO production pathway is thought to be involved in the regulation of ROI concentrations, especially during reoxygenation of anoxic tissues (Rumer *et al.*, 2009).

IV. Reductive routes of NO synthesis

NR, located in the cytosol, which primarily catalyses the reduction of nitrate to nitrite, is encoded by two genes in Arabidopsis, designated *Nitrate reductase [NADH] 1 (NIA1)* and *NIA2*, with *NIA2* encoding the enzyme responsible for the majority of NR activity (Wilkinson & Crawford, 1991). Significantly, this enzyme can also catalyse the reduction of nitrite to NO (Yamasaki & Sakihama, 2000; Rockel *et al.*, 2002). However, the efficiency of this reaction is low, and it requires small oxygen tensions, light and high nitrite concentrations (Rockel *et al.*, 2002; Planchet *et al.*, 2005). Nevertheless, a number of independent reports have suggested a role for NR in the generation of NO integral to numerous cellular processes including stomatal closure, osmotic stress, the plant defence response and auxin-induced lateral root formation (Kamoun *et al.*, 1998; Yamamoto-Katou *et al.*, 2006; Srivastava *et al.*, 2009).

A plasma membrane-bound NiNOR activity was first described in tobacco, with activity being limited to the roots (Stohr *et al.*, 2001). The nitrite as substrate for NiNOR is probably provided by plasma membrane-bound NR in a coupled reaction. This enzyme generates extracellular NO and has been suggested to play a role in sensing nitrate availability (Meyer & Stohr, 2002) and during interactions with mycorrhizal fungi (Moche *et al.*, 2010). Unfortunately, the identity of NiNOR still remains to be determined.

NO can also be generated by nitrite reduction in the mitochondrial inner membrane, probably via cytochrome c oxidase and/or reductase. However, this only occurs when the oxygen concentration drops below 20 mM (Planchet *et al.*, 2005). NAD(P)H provides electrons via ubiquinone and the mitochondrial electron transport chain. This process has also been reported to produce small amounts of ATP (Stoimenova *et al.*, 2007).

The peroxisomal enzyme xanthine oxidoreductase (XOR) can also reduce nitrite to NO. XOR has been shown to reduce nitrite to NO, using NADH or xanthine as the reducing substrate (Godber *et al.*, 2000). However, this reaction only occurs under anaerobic conditions. As peroxisomes are a major site for the generation of ROIs, this organelle may provide an important location for the interaction of these species with RNIs (del Rio *et al.*, 2004).

Clearly, the absence of a well-established route for NO biosynthesis, analogous to that demonstrated for mammals, may appear a significant impediment to the further development of the field. However, the emerging evidence suggests that NO generation could occur from multiple sources. This inherent redundancy might explain the failure, to date, of forward genetic screens to uncover sources of NO biosynthesis. Further, to best explore the phenotypic consequences of reduced NO synthesis, multiple systems for the production of this small molecule may need to be simultaneously disabled. Research programmes designed to shed additional light on the molecular machinery integral to NO biosynthesis should be a future priority. A key feature integral to the specificity of NO signalling in mammals is the widespread interaction of NOS isoforms with their target proteins, either directly (Kim *et al.*, 2005) or via scaffolding proteins such as Postsynaptic density protein 95 (PSD-95) (Lipton *et al.*, 2002) and C-Terminal PDZ Domain Ligand Of Neuronal Nitric Oxide Synthase (CAPON) (Fang *et al.*, 2000), facilitating their rapid and efficient S-nitrosylation. However, clearly there are many more S-nitrosylated substrates than NOS binding partners. Nevertheless, there are currently no examples of proposed plant NO-generating proteins interacting with either scaffolding proteins or their cognate S-nitrosylated targets. It may therefore be informative to search for such signalling specificity mechanisms.

V. Transfer of NO bioactivity

Central to the role of NO as a signalling molecule in biological systems are the mechanisms that underpin the translation of NO accumulation into biological function. Classical signal transduction networks are mediated largely by interactions based upon macromolecular shapes. Conversely, NO and related RNIs are thought to convey their bioactivity via chemical reactions with specific atoms of target proteins, which result in covalent modifications (Nathan, 2003). These redox signalling mechanisms, however, are still dependent upon extreme specificity to target the appropriate substrates. Further, any redox-driven post-translational modifications must be completely reversible to ensure transient signalling. Distinct from the vast majority of cysteine (Cys) amino acids embedded within their cognate proteins, a rare subset of these residues exhibit a low pKa sulphahydryl group which supports significant susceptibility to a range of redox-based post-translational modifications (Spadaro *et al.*, 2010) (Fig. 2). Significantly, the modifications of these highly reactive Cys residues by NO and related RNIs are reversible, except for sulphonic acid formation, the most highly oxidized modification. Chief among these redox-based post-translation modifications is S-nitrosylation, the addition of an NO moiety to a reactive Cys thiol to form an S-nitosothiol (SNO) (Spadaro *et al.*, 2010). This redox modification is a central route for NO bioactivity, as it can endow upon such Cys residues the ability to serve as a molecular switch, enabling the target protein to be directly responsive to changes in cellular redox status. S-nitrosylation has been shown to modulate enzyme activity

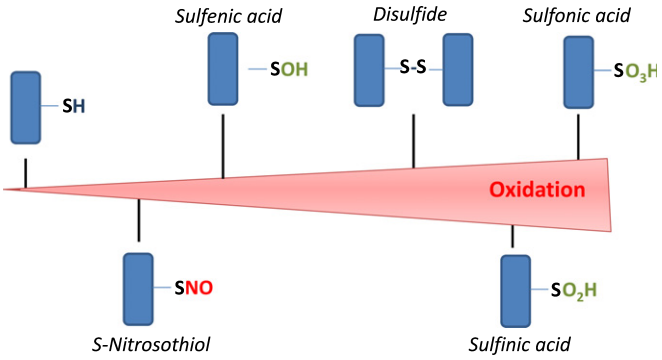

Fig. 2 Cysteine oxidative modifications. The redox code of modifications that can occur at rare, redox-active cysteine residues that exhibit a particularly low pKa value is shown. These range in the relative level of oxidation from cysteine thiols to the sulphonic acid residue. These modifications are all reversible except for sulphonic acid formation (Spadaro *et al.*, 2010). Chief among these redox-based post-translation modifications is S-nitrosylation, the covalent attachment of a nitric oxide (NO) moiety to a protein cysteine thiol to form an S-nitrosothiol (SNO) (Hong *et al.*, 2008; Yu *et al.*, 2012).

(Lindermayr *et al.*, 2005; Romero-Puertas *et al.*, 2007; Wang *et al.*, 2009; Yun *et al.*, 2011), protein localization (Tada *et al.*, 2008) and protein–protein interactions (Hara *et al.*, 2006). Therefore, this redox-based post-translational modification can be considered to be analogous to other more well-established protein modifications, for example, phosphorylation.

In animals, S-nitrosylation was initially implicated in the reaction of NO with cell-surface thiols associated with antimicrobial effects (Morris *et al.*, 1984), the modulation of ligand-gated receptor (NMDA) activity (Lei *et al.*, 1992) and alterations of smooth muscle cell function (Kowaluk & Fung, 1990). The first *in planta* biological function for S-nitrosylaltion emerged through a genetics approach, which uncovered a central role for SNOs in plant disease resistance (Feechan *et al.*, 2005). The exogenous addition of NO donors to plant protein extracts also demonstrated the *in vitro* formation of plant SNOs (Lindermayr *et al.*, 2005). The list of S-nitrosylated plant proteins is currently growing exponentially through the judicious application of the biotin-switch technique (Jaffrey *et al.*, 2001). For example, proteins specifically S-nitrosylated during plant imune function (Romero-Puertas *et al.*, 2007; Tada *et al.*, 2008; Wang *et al.*, 2009; Lindermayr *et al.*, 2010), cold treatment (Abat & Deswal, 2009), heavy metal exposure (De Michele *et al.*, 2009) and salt stress (Camejo *et al.*, 2013) have been described. Unfortunately, current strategies for the identification of Cys redox switches on a global scale are not straightforward and typically lack sensitivity. However, new techniques are evolving to help achieve this (Fomenko *et al.*, 2007; Weerapana *et al.*, 2010; Xue *et al.*, 2010).

Many Cys targets subject to S-nitrosylation are embedded within a proposed consensus motif (Stamler *et al.*, 1997), a situation similar to a variety of other distinct post-translational modifications. Additionally, hydrophobic regions can help drive Cys oxidative modifications because the reaction between NO and oxygen is promoted in such environments, producing species that support Cys modification (Liu *et al.*, 1998). Positively and negatively charged amino acids located within a distance of 6–8 Å in the tertiary protein structure may also stabilize S-nitrosylated Cys residues (Doulias *et al.*, 2010; Marino & Gladyshev, 2010). Although sites of S-nitrosylation can to some extent be predicted and computer programs have been developed to expediate this process (Xue *et al.*, 2010), the expanding list of plant S-nitrosylated proteins, together with that generated from other organisms, will enable the refinement of future *in silico*-based searches to facilitate the more accurate prediction of sites of SNO formation. A comparison of different computer programs enabling the prediction of S-nitrosylation sites has recently been discussed (Kovacs & Lindermayr, 2013).

Another pressing current limitation in this area is the sensitivity of the biotin-switch and associated mass spectrometry methodology. Currently, most proteins identified as being substrates for S-nitrosylation are relatively abundant,for example enzymes. It is proving difficult to identify more low-abundance targets, such as key regulatory proteins, because of these sensitivity issues. One short-term fix might be to employ cellular fractionation procedures to assay distinct cellular compartments, thereby enriching the concentrations of low-abundance regulators. Alternatively, new high-throughput techniques being developed to facilitate identification of Cys oxidative modifications (Fomenko *et al.*, 2007; Weerapana *et al.*, 2010; Xue *et al.*, 2010).

While S-nitrosylated proteins are being identified at an increasing rate, deep insights into how these modifications might regulate protein function at the angstrom level are only just beginning to be obtained within a plant biology context. A primer for these studies was the recent demonstration of how S-nitrosylation of an NADPH oxidase, Respiratory burst oxidase homolog D (RBOHD), modulates the function of this key enzyme (Yun *et al.*, 2011). Therefore, increasingly, NO-orientated research programmes may need to embrace structural biology-based approaches.

VI. NO function in plant immunity

A role for NO in plant immune function was first reported in potato, where treating tuber slices with NO donors was found to induce the accumulation of the potato phytoalexin rishitin. Further, this induction was blunted by the addition of NO scavengers (Noritake *et al.*, 1996). Collectively, these data implied that NO accumulation was sufficient to trigger accrual of a key antimicrobial molecule. Two years later it was suggested, following the application of NO donors, scavengers and NOS inhibitors, that NO could, in combination with ROIs, both engage the hypersensitive cell death response and activate the expression of *Phenylalanine ammonia-lyase (PAL)* and *Pathogenesis-related protein 1 (PR1)* genes (Delledonne *et al.*, 1998; Durner *et al.*, 1998).

The first genetic evidence for a role of SNO in the plant defence response came following a reverse genetics approach (Feechan *et al.*, 2005). A gene, *Arabidopsisthaliana* S-nitrosoglutathione (GSNO) reductase (*AtGSNOR1*), was identified which controlled total cellular concentrationsof GSNO (Feechan *et al.*, 2005). This metabolite is formed by the S-nitrosylation of the cellular antioxidant glutathione (GSH) and is thought to constitute a relatively stable store of NO bioactivity. Loss-of-function mutations in *AtGSNOR1* resulted in elevated total cellular SNO concentrations, while mutations that enhanced AtGSNOR1 activity promoted the turnover of these metabolites. Significantly, the absence of AtGSNOR1 function compromised nonhost, basal and *Resistance* (*R*) gene-mediated protection (Feechan *et al.*, 2005). Thus, changes in total SNO concentrations impinge upon multiple modes of plant disease resistance. Some mechanistic insight was uncovered when it was shown that changes in cellular SNO concentrations regulated both the accumulation of the plant immune activator salicylic acid (SA) (Loake & Grant, 2007) and expression of SA-dependent genes (Feechan *et al.*, 2005). These data suggested that NO via GSNO-mediated S-nitrosylation was a key regulator of SA-dependent defence responses and that excessive S-nitrosylation promoted disease susceptibility.

Cryptogein is a 10-kDa proteinaceous elicitor synthesized by the oomycete *Phytophthora cryptogea*. This immune activator induces a hypersensitive response (HR), resulting in death of the treated plant cells. Significantly, cryptogein has been shown to induce NO production in tobacco plants and in cell suspensions. NO production has been detected at both the intracellular and extracellular levels (Besson-Bard *et al.*, 2008) and has been implicated as a mediator of the increase in cytosolic free

Ca^{2+}concentrations induced by cryptogein in tobacco cells (Lamotte *et al.*, 2004). A series of proteins have recently been shown to be S-nitrosylated following cryptogein application. Cell Division Cycle 48 (CDC48), a member of the AAA+ ATPase family, was found to be among these NO targets. Analysis *in vitro* suggested that CDC48 was poly-S-nitrosylated. Further, Cys110, Cys526 and Cys664 were identified as the targets for SNO formation. Interestingly, Cys526 is located in the Walker A motif of the D2 domain of CDC48. This residue is thought to be involved in ATP binding and has been implicated as a target for oxidative modification in *Drosophila*. In tobacco, NO may abolish CDC48 ATPase activity and convey conformation changes in the vicinity of Cys526. Moreover, substitution of Cys526 by an alanine residue impacted CDC48 activity. Thus, CDC48 has been uncovered as a component in cryptogein-triggered NO signalling and Cys526 might function as a redox switch in the regulation of this protein (Astier *et al.*, 2012a). Moving forward, it will be interesting to uncover the role of CDC48 in disease resistance and how S-nitrosylation of Cys526 might modulate this activity.

SA has been shown to bind and modulate the activity of a number of proteins integral to the establishment of plant immunity (Chen *et al.*, 1993; Slaymaker *et al.*, 2002; Kumar & Klessig, 2003). In this context, SA-binding protein 3 (SABP3) shows a high affinity for SA and expresses carbonic anhydrase (CA) activity (Slaymaker *et al.*, 2002). Lipid-based molecules are integral to plant immunity and their functions have been linked to SA signalling (Kachroo *et al.*, 2001). Significantly, CA activity is thought to be required for lipid biosynthesis (Hoang & Chapman, 2002). Interestingly, SABP3 has recently been shown to be S-nitrosylated *in vivo* during the later stages of plant immune function (Wang *et al.*, 2009) (Fig. 3). S-nitrosylation of SABP3 at Cys280 was directly proportional to the intracellular concentrationof SNOs and the extent of this modification was controlled by AtGSNOR1 activity. Also, S-nitrosylation of SABP3 at Cys280 blunted both SA binding and CA activity. AtSABP3 was also found to be required for full host resistance. On the basis of these data, it was therefore proposed that inhibition of AtSABP3 CA function by S-nitrosylation might contribute to a negative feedback loop that modulates plant immunity. These findings further reinforce the connection between NO and SA function in the plant defence response.

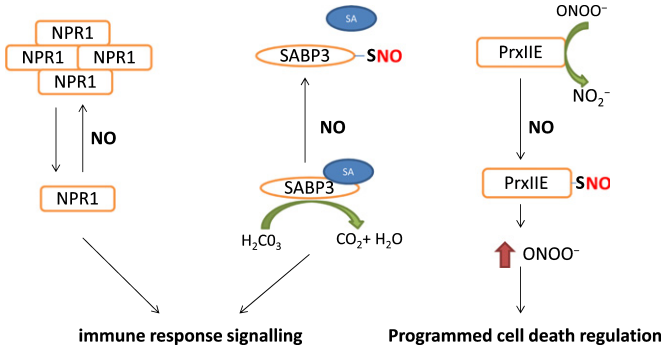

Fig. 3 Role of nitric oxide (NO) and associated S-nitrosylation in plant immunity and pathogen-triggered cell death. S-nitrosylation of the plant coactivator Non-expresser of pathogenesis-related genes 1 (NPR1) regulates the homeostasis of NPR1 monomer to multimer formation in the cytoplasm. This is significant for the establishment of plant immunity because only NPR1 monomers can move from the cytosol to the nucleus to activate salicylic acid (SA)-dependent gene expression (Tada *et al.*, 2008). S-nitrosylation of SA-binding protein 3 (SABP3) regulates both SA binding and the carbonic anhydrase activity of this enzyme and functions as part of a negative feedback loop in plant immunity (Wang *et al.*, 2009). Peroxiredoxin II E (PrxIIE) turns over peroxynitrite ($ONOO^-$). S-nitrosylation of this protein blunts its activity, increasing $ONOO^-$formation, which facilitates tyrosine nitration (Romero-Puertas *et al.*, 2007).

Non-expresser of pathogenesis-related genes 1 (NPR1) is a central regulator of the plant immune response and is thought to function as a co-activator (Fu & Dong, 2013). In the absence of attempted pathogen infection, NPR1 is largely sequestered in the cytoplasm as an oligomeric complex. The formation of this complex is mediated through the formation of disulphide bonds established by solvent-exposed Cys residues (Mou *et al.*, 2003). With the bulk of NPR1in this molecular form, the translocation of NPR1 to the nucleus is reduced, leading to only basal levels of SA-dependent gene expression (Fig. 3). Following the pathogen-triggered oxidative and nitrosative burst, a counterbalancing wave of cellular antioxidant activity has been proposed to promote reduction of the disulphide bonds required to maintain NPR1 homo-oligomer formation. The released NPR1 monomers, rather than being sequestered in the cytoplasm, are then translocated to the nucleus where they can subsequently help drive SA-dependent gene expression (Mou *et al.*, 2003). Thus, the dynamic equilibrium between NPR1monomers and oligomers in the cytosol is a key point of control in the development of plant immunity.

Superimposed upon changes in NPR1 disulphide bond status is the NO-mediated S-nitrosylation of this transcription co-activator. NO accrual following the pathogen-triggered nitrosative bursts promotes S-nitrosylation of NPR1 at Cys156, which is located at a predicted multimerization interface. This redox modification favours the formation of disulphide linkages between NPR1 monomers, resulting in the development of NPR1 oligomers. Informatively, mutation at Cys156, which precludes S-nitrosylation at this site, diminishes NPR1 multimerization. Collectively, these data imply that SNO-Cys156-mediated oligomerization is required to maintain NPR1 oligomer−monomer homeostasis, thereby facilitating a steady supply of monomer to maintain SA-dependent gene expression. In *atgsnor1-3* plants, however, where SNO concentrations are elevated, excessive S-nitrosylation of NPR1 might occur, thereby disturbing NPR1 oligomer−monomer homeostasis, leading to delayed and reduced SA-mediated gene expression (Feechan *et al.*, 2005; Tada *et al.*, 2008).

The genetic data relating to the control of NPR1 function apparently contrast with experiments employing Arabidopsisprotoplasts, where exogenous treatment with 100 μM GSNO has been reported to mediate NPR1 nuclear translocation (Lindermayr *et al.*, 2010).

Although this is probably attributable to the fact that NO donors induce accumulation of SA (Durner *et al.*, 1998), which triggers NPR1 nuclear localization, these differences might also reflect the physiology of protoplasts relative to plants and/or exogenous GSNO exposure as opposed to endogenous accumulation.

TGACG motif binding factor 1 (TGA1), a basic leucine zipper (bZIP) protein, has also been reported to be S-nitrosylated *in vitro*

at Cys260 and Cys266 (Lindermayr *et al.*, 2010). This regulatory protein is a member of a small group of bZIP proteins that are individually redundant but collectively essential for SA-dependent gene expression (Zhang *et al.*, 2003). TGA1 S-nitrosylation at Cys260 and Cys266 was proposed to protect this transcription factor from oxygen-mediated modification, promoting DNA binding to the *as-1* motif (Lindermayr *et al.*, 2010). Further, Cys172 and Cys287 have also been proposed to be integral to the DNA-binding activity of TGA1. In the TGA1 C260S C266S mutant, low-mobility proteins could be observed under oxidizing conditions, suggesting that disulfide bond formation might also occur between C172 and C287. Also, *tga1 tga4* mutant plants transformed with the TGA1 C172S C287S mutant showed hyperexpression of the defence-related genes *PR2* and *PR5*. Thesedata imply that reduction of these Cys residues is important for TGA1 activity, as the mutations mimic their reduced status. Collectively, the redox status of Cys172 and Cys287 may be important for the intramolecular structure of TGA1, opening of the disulfide bond and GSNO-dependent modification of these Cys residues appear to positively affect the DNA-binding activity of this transcription factor (Lindermayr *et al.*, 2010). However, thesedata contrast with previous findings that have suggested redox changes do not directly regulate DNA-binding activity of TGA1 (Despres *et al.*, 2003).

Collectively, these data clearly highlight an important role for NO function in SA signalling. Further, NO and associated S-nitrosylation appear to regulate multiple nodes of this signal pathway. Surprisingly, the key readouts from experiments conducted in different laboratories have been contrasting. It would be informative to explore these apparent differences to help resolve the associated issues.

VII. Role of NO in hypersensitive cell death

A conspicuous feature of the defence response following pathogen recognition is the development of an HR, a programmed execution of plant cells at sites of attempted infection (Greenberg & Yao, 2004). The accumulating evidence suggests that key drivers in cell death development are ROIs generated by NADPH oxidases and NO generated during the nitrosative burst (Delledonne *et al.*, 1998; Yun *et al.*, 2011). Interestingly, plant NADPH oxidases are related to those responsible for the pathogen-activated respiratory burst in mammalian phagocytes (Keller *et al.*, 1998; Torres *et al.*, 2002). Analysis of plant phenotypes resulting from mutations in *AtGSNOR1*suggestedthat the encoded enzyme controls global SNO concentrations during the development of HR cell death (Yun *et al.*, 2011). As SNO concentrations rise in *atgsnor1-3* plants relative to wildtype, the accumulation of SA and its β-glucoside (SAG) isstrikingly reduced. Nevertheless, HR cell death development in the *atgsnor1-3* line and in an *atgsnor1-3SA induction deficient 2* (*sid2*) double mutant, where SA concentrations are barely detectable, exhibits both accelerated kinetics and increased magnitude. Conversely, cell death in the *atgsnor1-1* line, where SNO concentrations are decreased, is both delayed and reduced, relative to wild-type plants. Therefore, surprisingly, SNOs promote cell death formation even in the presence of reduced SA concentrations (Yun *et al.*, 2011), a known activator of cell death (Shirasu *et al.*, 1997).

The relevance of these data to plant immunity were highlighted by the findings that *atgsnor1-3* and *atgsnor1-3 sid2* double mutants exhibited increased resistance to an avirulent oomycete *Hyaloperonospora arabidopsidis* isolate. This was unexpected because SA accumulation is ordinarily required for resistance against this pathogen (Wildermuth *et al.*, 2001) and both *atgsnor1-3* and *atgsnor1-3 sid2* plants have strikingly reduced SA concentrations. Therefore, the development of cell death with both accelerated kinetics and increased magnitude appears to be sufficient to convey resistance against a biotrophic pathogen in the presence of very low SA accumulation and, by extension, weak deployment of SA-dependent defence responses (Yun *et al.*, 2011).

Following pathogen recognition, SNO concentrations in *atgsnor1-3* plants were found to suppress apoplastic ROI accumulation, while diminished SNO concentrations in the *atgsnor1-1* line led to an increase in ROIs. Interestingly, neither transcript accumulation nor protein abundance of several NADPH oxidases (AtRBOH) were found to be regulated by changes in SNO concentrations. By contrast, AtRBOH activity was modulated by SNOs both *in vitro* and *in vivo*. Further, SNO was found to mediate these effects through *in vivo* S- nitrosylation of AtRBOHD at Cys 890 (Fig. 4). Further insight was provided by protein modelling which established that Cys890 is positioned close behind the conserved Phe 921 residue in AtRBOHD, which is thought to be crucial for binding of flavin adenine dinucleotide (FAD), an essential co-factor (Ingelman *et al.*, 1997). The model also indicated that S-nitrosylation of AtRBOHD at Cys 890 might disrupt the side-chain position of Phe 921 and impede FAD binding. These predictions were subsequently confirmed by experimentation (Yun *et al.*, 2011). Thus, Cys 890 S-nitrosylation during the expression of resistance suggested that this redox-based modification, manifested at relatively high SNO concentrations, might serve to reduce NADPH activity and subsequently diminish ROI accumulation, curbing the extent of cell death development

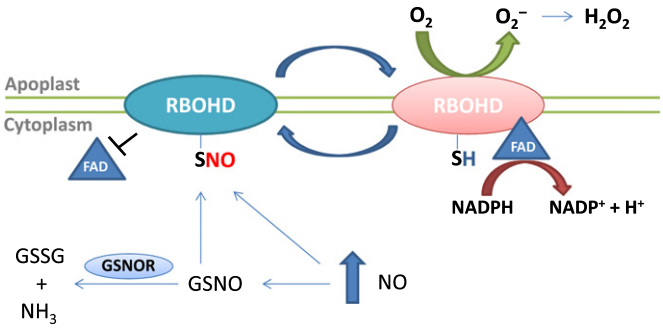

Fig. 4 S-nitrosylation regulates activity of the NADPH oxidase Respiratory burst oxidase homolog D (RBOHD) that contributes to the pathogen-activated oxidative burst. S-nitrosothiol (SNO) formation at Cys890 of RBOHD precludes binding of the essential co-factor flavin adenine dinucleotide (FAD), blunting synthesis of reactive oxygen intermediates (ROIs) (Yun *et al.*, 2011). This mechanism is conserved across kingdoms (Yun *et al.*, 2011) and may have significant implications for biomedicine (Qian *et al.*, 2012). The extent of SNO formation at Cys890 is indirectly controlled by *Arabidopsis thaliana*S-nitrosoglutathione reductase 1 (AtGSNOR1) activity (Yun *et al.*, 2011).

during the later stages of the HR. Significantly, Cys 890 is evolutionarily conserved and was also found to be S-nitrosylated in NADPH oxidases from humans and flies, implying that this regulatory mechanism may govern immune responses in both plants and animals (Yun *et al.*, 2011).

Peroxiredoxin II E (PrxII E) has also been shown to be S-nitrosylated during *R* gene-mediated resistance (Romero-Puertas *et al.*, 2007). This modification blunted the peroxynitrite ($ONOO^-$) detoxifying activity of this protein (Fig. 3). $ONOO^-$is formed in a diffusion-limited reaction between NO and O_2, is a potent oxidizing and nitrating species and can interfere with tyrosine (Tyr) kinase signalling in animals through nitration of Tyr residues (Klotz *et al.*, 2002). Therefore, it has been proposed that S-nitrosylation of PrxII E may blunt $ONOO^-$turnover by this enzyme and by extension might thus lead to a significant increase of $ONOO^-$-dependent nitrotyrosine generation at tyrosine residues that are susceptible to this modification. Hence, NO may control the impact of its own radicals during HR cell death development via S-nitrosylation of a key antioxidant enzyme, driving changes in Tyr kinase signalling.

In aggregate, these data clearly suggest a fundamental role for (S) NO in pathogen-triggered cell death. Further, these small molecules also appear to function in combination with ROIs generated from the oxidative burst. However, we are only just beginning to appreciate how these redox-active molecules interface with a growing cast of protein players. Uncovering further reactive Cys residues that are subject to oxidative modifications during the development of cell death will be an important future area for investigation.

VIII. NO and abiotic stress

NO has begun to emerge as an important endogenous signalling molecule in the adaptation of plants to abiotic stresses. The accruing data havesuggested a role for NO and in some cases SNOs in a variety of stress responses, including drought, salt, heat and cold stress. Water stress in its broadest sense encompasses both drought and salt stress. Most studies on water stress signalling have focused on salt stress primarily because plant responses to salt and drought are closely related and the mechanisms overlap.Salt stress afflicts plant agriculture in many parts of the world, particularly on irrigated land. Compared withsalt stress, the problem of drought is even more pervasive and economically damaging. Stomatal closure induced by the synthesis and redistribution of abscisic acid (ABA) is one of the important events during water stress (Seki *et al.*, 2007). Removal of NO has been shown to inhibit ABA-related stomatal responses using a combination of both chemical and genetic approaches. In this context, ABA has been proposed to mediate NO generation through H_2O_2 and H_2O_2-dependent stomatal closure, which can be inhibited by NO scavengers (Bright *et al.*, 2006) (Fig. 5). Further, stomatal closure in the NR double mutant *nia1nia2* is also impaired, suggesting NO potentially generated via this enzyme is required for regulating stomatal function. A requirement for Nitric Oxide Associated 1 (NOA1) has also been proposed (Desikan *et al.*, 2002). Further, high salinity seems to promote S-nitrosylation, leading to changes in the activity of some

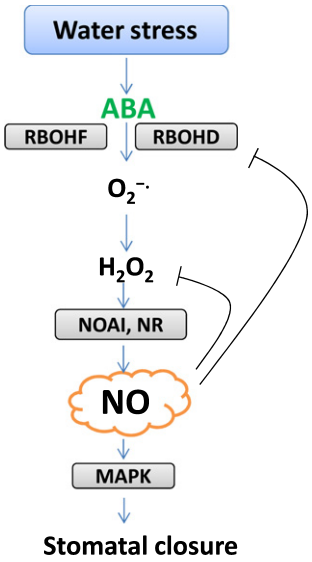

Fig. 5 Model showing key features integral to nitric oxide (NO) function during water stress signalling in plants. In response to water deficit, abscisic acid (ABA) accumulates, leading to the activation of the NADPH oxidases RBOHD and RBOHF (Respiratory burst oxidase homolog D and F), resulting in reactive oxygen intermediate (ROI) production (Kwak *et al.*, 2003; Bright *et al.*, 2006). H_2O_2 generated by this oxidative burst is thought to cue NO synthesis from nitrate reductase (NR), with also a possible requirement for *Arabidopsis thaliana* nitric oxide associated 1 (AtNOA1) (Desikan *et al.*, 2002; Bright *et al.*, 2006). NO has then been proposed to activate mitogen-activated protein kinase (MAPK) signalling cascades which then drive stomatal closure (Zhang *et al.*, 2007).

mitochondrial proteins in pea (*Pisum sativum*), for example, glycine dehydrogenase P subunit and F1 ATPase β subunit, indicating that abioticrelated respiratory and photorespiratory pathways could be regulated by this modification (Camejo *et al.*, 2013). NO has also been prostulated to activate mitogen-activated protein kinase (MAPK) signalling cascades which may then drive stomatal closure (Zhang *et al.*, 2007).

Cold stress is another environmental factor that significantly reduces crop yield. However, plants have evolved mechanisms to help ameliorate the effects of cold. Plants are thought to acquire freezing tolerance by a process termed cold acclimation, where prior exposure to low, but nonfreezing, temperatures significantly enhances survival in response to subsequent freezing temperatures. Cold acclimation correlates with a massive reprogramming of both gene expression and the metabolome (Thomashow, 2010). Some time ago it was suggested that exogenous NO increased cold tolerance in various plant species including wheat (*Triticum aestivum*), maize (*Zea mays*) and tomato (*Solanum lycopersicum*) (Neill *et al.*, 2003). This observation may be related to the fact that it is now well established that low temperatures promote oxidative stress. This is relevant because NO is thought to confer antioxidant activity (Beligni & Lamattina, 1999), and under some conditions this may occur by the NO-mediated suppression of peroxidative metabolism (Neill *et al.*, 2002). Thus, it is possible that NO may confer cold tolerance in part by functioning as an antioxidant.

In addition, NO has also been shown to specifically S-nitrosylate *Brassica juncea* proteins in response to cold (Abat & Deswal, 2009). More recently, it has been proposed that NR is a source of NO

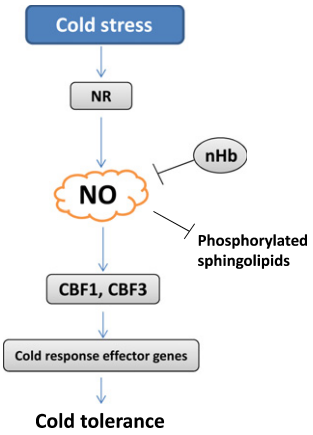

Fig. 6 Schematic showing the proposed role of nitric oxide (NO) during cold stress. Cold stress results in NO synthesis mediated by nitrate reductase (NR) (Cantrel *et al.*, 2011). Plant nonsymbiotic haemoglobins (nHbs) are able to function as scavengers of NO under cold stress conditions (Dordas *et al.*, 2003; Cantrel *et al.*, 2011). NO is thought to negatively regulate the accumulation of phosphosphingolipids, although the role of these molecules in cold stress remains to be established. Also, NR-generated NO was proposed to activate expression of the cold-induced master regulators *CBF1* (*CRT/DRE binding factor 1*) and *CBF3* (Cantrel *et al.*, 2011) which regulate a repertoire of cold-induced genes promoting tolerance to this stress.

following cold exposure (Cantrel *et al.*, 2011) (Fig. 6). Further, plant nonsymbiotic haemoglobins (nHbs) are known to scavenge NO (Dordas *et al.*, 2003). In this context, the up-regulation of nHb transcription was also uncovered in response to cold. Further, nHb over-expressing plant lines showed reduced expression of the cold-induced master regulators *CBF1* (*CRT/DRE binding factor 1*) and *CBF3* (Cantrel *et al.*, 2011). Finally, it was proposed that sphingolipids are transiently phosphorylated in response to cold exposure and that NO serves as a negative regulator of this modification (Cantrel *et al.*, 2011). Thus, the accumulating evidence suggests that NO is a central feature of cold adaptation, functioning in a variety of different ways to orchestrate this process.

Most of the world's crops are exposed to heat stress during some stages of their life cycle (Stone, 2001). Exposure to higher than optimal temperatures reduces yield and decreases crop quality. Furthermore, as climate change continues to drive increases in temperature, our appreciation of how plants respond to heat stress is becoming increasingly significant. In this context, NO is also emerging as a key player in heat acclimation. In a forward screen for mutations that blunt heat acclimation, lesions within the *HOT5* (*sensitive to hot temperatures 5*)/*AtGSNOR1* gene were uncovered. The *hot5* alleles were associated with increased nitrate and SNO concentrations and the corresponding mutant plants exhibited heat sensitivity. Further, heat sensitivity was enhanced in wild-type and *hot5* plants by NO donors and the heat sensitivity of these mutants could be rescued by an NO scavenger. Also, NO overproduction was found to result in defective thermotolerance. Collectively, these results reveal an important role for NO and SNOs in plant heat stress tolerance.

The accumulating data areestablishing an important role for NO in the signalling networks undrpinning a slew of plant stress responses. Thus, manipulating NO signal function within these contexts may offer novel opportunities for rational crop design to ameliorate abiotic stress impacts. Oxidative stress is thought to be a common denominator of stress responses (Apel & Hirt, 2004; Gechev *et al.*, 2006). Consequently, strategies aimed at improving stress resistance have often targeted the reduction of endogenous ROI accumulation (De Clercq *et al.*, 2013). However, it has recently been demonstrated that significant changes in the metabolism of RNIs can occur under low temperatures, promoting nitrosative stress leading to protein tyrosine nitration, a key marker of a nitrosative challenge and lipid peroxidation (Airaki *et al.*, 2012). Moreover, the development of nitrosative stress has also been reported for a number of other stress conditions (Corpas *et al.*, 2011). Therefore, the potential utility of approaches to ameliorate both accrual of RNIsand their potential deleterious interactions with ROIs are increasing in significance.

IX. NO function in plant development

In mammals, NO has a fundamental role in a plethora of physiological processes (Hirst & Robson, 2011). However, the role of this small molecule in developmental processes may not be so widespread. Deletion of individual *NOS* genes does not result in gross developmental perturbations (Huang *et al.*, 1993; Lee *et al.*, 2000). Nevertheless, a role for NO in heart development has been uncovered, with deficiency in eNOS resulting in congenital septal defects, cardiac hypertrophy and postnatal heart failure. In addition, eNOS is pivotal to the morphogenesis of major coronary arteries and myocardial capillary development (Liu & Feng, 2012). By contrast, the emerging evidence suggests that NO function in plants has a strikingly more pervasive role during development programmes than in the other kingdoms. Thus, NO is thought to modulate a variety of developmental processes such as germination (Beligni & Lamattina, 2000), flower development (Lee *et al.*, 2008; Kwon *et al.*, 2012), flowering time (He *et al.*, 2004; Kwon *et al.*, 2012) and apical dominance (Lee *et al.*, 2008; Kwon *et al.*, 2012). It is the influence of NO upon root growth and development, however, that has garnered most attention (Fernandez-Marcos *et al.*, 2011; Kwon *et al.*, 2012). In the last decade, a series of experiments have implicated NO as a central component in auxin-orchestrated root growth and development (Fig. 7). Further, the accumulating data suggest that NO might also modulate the interaction of roots with microorganisms in the rhizosphere (Correa-Aragunde *et al.*, 2004; Pagnussat *et al.*, 2004; Boscari *et al.*, 2013).

X. NO contributes to the balancing of growth with development in roots

A model for how NO might coordinate root growth and development is shown in Fig. 8. NO is able to induce adventitious root (AR) development in monot, dicot and gymnosperm plant species (Lanteri *et al.*, 2008). A number of secondmessengers involved in signalling cascades regulated by NO, implicated in AR development, have been uncovered. In this context, two parallel and independent pathways have been described: the first of these is thought to utilize cGMP through an NO-mediated activation of the enzyme guanylate cyclase (GC) (Pagnussat *et al.*, 2003).

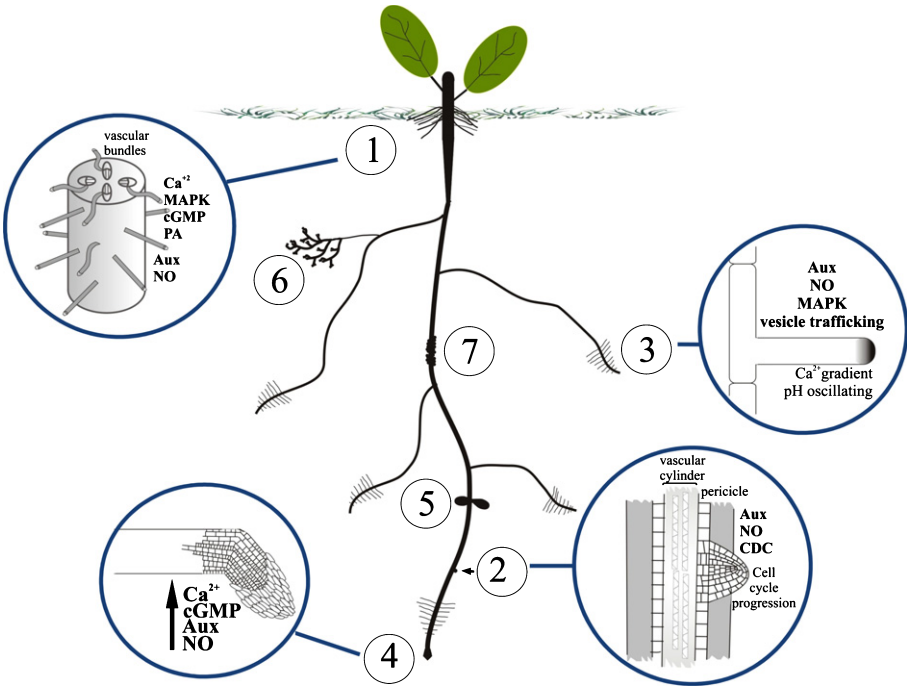

Fig. 7 Summary of key features determining root system architecture and functions where crosstalk between auxins and NO occurs. The schematic representation highlights the features occurring in an ideal functional root system: 1, adventitious root development; 2, lateral root formation; 3, root hair growth; 4, gravitropic response. The scheme also represents root symbiotic interactions and associations with rhizosphere-inhabiting microorganisms (Correa-Aragunde *et al.*, 2004; Pagnussat *et al.*, 2004; Boscari *et al.*, 2013); 5, nodulation with symbiotic bacteria; 6, mycorrhizal symbiosis; 7, association with plant growth promoting rhizobacteria (PGPR). Selected root growth and developmental processes that do not involve the interaction with other organisms are detailed in amplified snapshots.

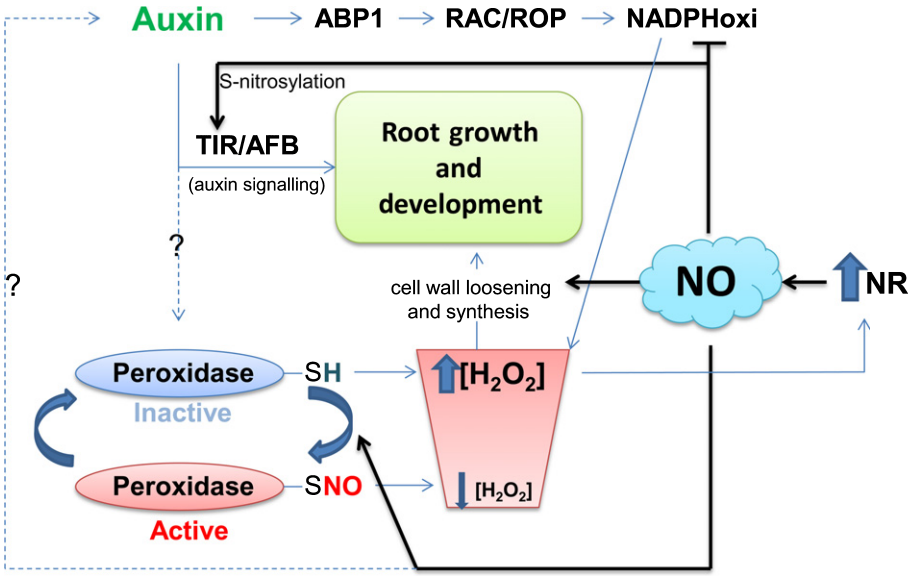

Fig. 8 Mutual regulation and balance between auxin, nitric oxide (NO) and reactive oxygen intermediates (ROIs) determine their biological activity. Auxin induces the production of reactive oxygen species (ROIs) through the activation of NADPH oxidase which is mediated by the binding of auxin to auxin-binding protein 1 (ABP1) and the activation of anRHOGTPase (RAC/ROP) (Duan *et al.*, 2010; Shi & Yang, 2011). The increase of NADPHoxidase-generated ROIs leads to: (1) activation of nitrate reductase (NR) and NO production, which in turn can blunt NADPH oxidase activity by S-nitrosylation, preventing excess ROI formation (Yun *et al.*, 2011); (2) the formation of the $OH^{\cdot}$ radical through apoplastic peroxidase functions in the cell expansion processes mediating cell wall loosening (Mueller *et al.*, 2009); (3) regulation by S-nitrosylation of the auxin receptor transport inhibitor response 1 (TIR1) (Terrile *et al.*, 2012). In parallel, auxin induces the partial inhibition of peroxidase through its denitrosylation, contributing to an increase in ROIs. The function of peroxidases can be reactivated by S-nitrosylation, as a result of increased NO production in a counterbalancing fashion. Additionally, fluctuating NO concentrations can also modulate cellulose synthase activity and cell wall biosynthesis contributing to cell growth. →, activation; ┤, inhibition; dashed lines and question mark, interaction not tested yet; filled lines, interaction demonstrated.

However, an NO-regulated GC still remains to be identified in plants. A secondpathway that encompasses an MAPK cascade (Pagnussat *et al.*, 2004) has also been reported. The engagement of both pathways seems to be required for a 'full' response. A third messenger derived from phospholipid signalling, phosphatidic acid (PA), is an agonist of AR development, probably acting downstream of both auxin and NO function (Lanteri *et al.*, 2008).

Conversely, NO has also been reported to inhibit root formation. Interestingly, utilizing chemical treatments and mutants with altered NO concentrations,it has been proposed that elevated

concentrations of NO reduce auxin transport and responses via a PIN-FORMED 1 (PIN1)-dependent mechanism. Polar auxin transport is impacted negatively by over-accumulationof NO because PIN1 protein levels appear to be reduceddramatically after delivery of exogenous NO. Consistent with NO-induced PIN1 disappearance,the *pin1* mutant is not resistant to NO. As PIN1 expression is not influenced by NO,the disappearance of PIN1 proteinmay be regulated post-translationally. However, NO-mediated PIN1 turnover appears to be via a proteasome-independent mechanism (Fernandez-Marcos *et al.*, 2011).Further, root meristematic activity maybe reduced concomitantly with these NO-mediated impacts, and the organization of the quiescent centre and surrounding cells of an NO over-producing mutant hasbeen reported to be distorted, thus suggesting a link between NO and auxin signalling in maintaining the integrity and activity of the root apical meristem (Fernandez-Marcos *et al.*, 2011).

Lateral root (LR) formation is an established model with which to study root branching capacity and the contribution of phytohormones to this process. LR formation is predominantly associated with auxin action and is generally linked to the inhibition of primary root (PR) elongation. NO is thought to be a downstream messenger in auxin signalling, promoting LR formation. Further, NO is able to induce LR formation even in the absence of auxin treatment (Correa-Aragunde *et al.*, 2004). Moreover, the inhibition of PR growth and the promotion of the branching process are completely blocked when endogenous NO is sequestered by scavengers (Correa-Aragunde *et al.*, 2004). Interestingly, thesedata contrast with the analysis of *AtGSNOR1* mutant plants, which exhibit differences in SNO concentrations (Feechan *et al.*, 2005). In this context, *atgsnor1-3* plants, which show elevated SNO concentrations, exhibited a lack of visible LR development. Also, both the *atgsnor1-1* plants, where the SNO concentration is reduced, and *atgsnor1-3* lines exhibited reduced PR length compared with wild type. PR length was reduced in *atgsnor1-1* and *atgsnor1-3* lines by 27.3% and 72.7%, respectively (Kwon *et al.*, 2012).

It has also been proposed that NO both acts at the pericycle establishing new founder cells and induces cell division and formation of new LR primordia. In this context, NO has been reported to activate the expression of cell cycle regulatory genes including cyclin D3 and, conversely, to repress the cyclin-dependent kinase inhibitor Kip Related Protein 2 (KRP2), collectively promoting the entry of cells into S phase (Correa-Aragunde *et al.*, 2006).

Cytokinin is a pivotal phytohormone in plant growth and development. Cytokinin signalling is thought to be mediated by a phosphorelay that sequentially transfers phosphoryl groups from the cytokinin receptors to histidine phosphotransfer proteins (AHPs) and response regulators (ARRs). Recently, it has been proposed that NO might negatively regulate cytokinin signalling by blunting phosphorelay activity through S-nitrosylation (Feng *et al.*, 2013). SNO formation at Cys115 of AHP1 has been reported to represses its phosphorylation and subsequent transfer of the phosphoryl group to ARR1. Further, a mutation of AHP1 that blocks S-nitrosylation partially relieves the inhibitory effect of NO cytokinin signalling. By contrast, a nitrosomimetic mutation within AHP1 decreased phosphorylation of AHP1 and ARR1 and partially disabled cytokinin signal transduction. These important findings uncover another mechanism whereby changes in cellular redox status modulate cytokinin signalling to coordinate plant growth and development (Feng *et al.*, 2013).

XI. NO action in root hair development and gravitropic responses

Root hairs are specialized root epidermal cells of higher plants whose functions are water absorption and anchorage. Root hairs exhibit a characteristic polarized growth shared with a number of other cells including fungal hyphae, pollen tubes and moss protonemata (Heath & Geitmann, 2000; Hepler *et al.*, 2001). Root hairs are formed from a differentiated root epidermal cell type termed trichoblasts. Significantly, NO has been shown to promote the differentiation of trichoblast cells in developing root hairs of both lettuce (*Lactuca sativa*) and Arabidopsis (Lombardo *et al.*, 2006). During root hair initiation, the trichoblasts are extensively vacuolated and the nucleus has been reported to migrate from a location in the centre of the trichoblast to the site of root hair formation (Klahre & Chua, 1999). Root hair growth is driven by the coordinated trafficking of secretory vesicles (Ovecka *et al.*, 2005). The root hair tip therefore is remarkably vesicle rich (Miller *et al.*, 1999) and vesicle trafficking is integral to root tip formation.

Recently, NO has been detected inside the vacuole in actively growing root hairs and the cytoplasm of more mature root hair cells. Depleting NO inArabidopsisroot hairs suggested that NO is required for endocytosis, vesicle formation and trafficking. However, NO bioactivity was not thought to be required for nuclear migration and vacuole development. Further, the NO generation mutant *nia1nia2* showed altered vesicle trafficking and shorter root hairs. Informatively, these phenotypes were restored with exogenous NO treatment. Thus, NO appears to function in vesicle formation and trafficking in root hairs (Lombardo & Lamattina, 2012). Further, mutations in *AtGSNOR1* also influenced root hair development. The *atgsnor1-1* line, which is reduced in SNO accumulation, exhibited strikingly elongated root hairs relative to wild-type plants. Conversely, in *atgsnor1-3* plants, where elevated SNO concentrations have been reported, root hairs were reduced in stature (Kwon *et al.*, 2012). It has also been determined that extracellular nucleotides can either stimulate or inhibit root hair growth through an NO- and ROS-regulated mechanism (Clark *et al.*, 2010).

At the tip of the PR, growth is under the influence of gravitational forces and responds through a complex mechanism combining multiple signalling molecules, cell-specific structures and hormonal cues. It has been proposed that NO participates in the auxin-regulated gravitropic response possibly through the activation of a GC and the subsequent induction of elevated concentrations of cGMP (Hu *et al.*, 2005).

XII. Signalling cross-talk in roots between NO and ROIs

Auxin has been proposed to drive increased concentrations of ROIs through at least two distinct mechanisms: activation of an

auxin-binding protein 1 (ABP1)-mediated RHO GTPase (RAC/ROPs) and the subsequent induction of NADPH oxidase activity (Duan *et al.*, 2010; Shi & Yang, 2011); and repression of peroxidase activity (Iglesias *et al.*, 2010; Lin *et al.*, 2011; Correa-Aragunde *et al.*, 2013) (Fig. 2). As a consequence, an elevated concentration of ROIs has been reported to result in the activation of NR (Wang *et al.*, 2010; Lin *et al.*, 2012). Subsequently, increased NR-mediated NO production might in turn regulate NADPH oxidase activity because this enzyme has been shown to be S-nitrosylated in leaves during attempted pathogen infection, resulting in reduced ROI synthesis (Yun *et al.*, 2011). Additionally, as recently demonstrated, increased NO production might activate ascorbate peroxidase 1 (APX1) activity (Correa-Aragunde *et al.*, 2013). Collectively, these responses could therefore function as components of a negative feedback loop, leading to a diminuition in the ROI concentration. However, such a network remains to be established in roots.

XIII. NO regulation of root iron homeostasis

NO is emerging as a central player controlling iron nutrition, metabolism and homeostasis in roots, facilitating the growth of both monot and dicot plants under low iron concentrations (Graziano *et al.*, 2002; Graziano & Lamattina, 2007). Indeed, NO possesses a high affinity for iron. The low-molecular-weight complexes formed between iron and NO are termed iron–nitrosyl complexes. These compounds consist of both mononitrosyl iron complexes (MNICs) and dinitrosyl iron complexes (DNICs) and their formation and interactions are central to NO biochemistry (Stamler *et al.*, 1992; Stamler & Feelish, 1996). NO can form iron–nitrosyl complexes *in vivo* with iron–sulphur and heme centres of proteins that are important for the biological activity of NO (Wink & Mitchell, 1998). As a result of iron−nitrosyl complex formation under high NO concentrations, iron mobility and availability areincreased, facilitating plant growth under low iron concentrations (Vanin *et al.*, 2004; Graziano & Lamattina, 2005).

It has also been suggested that NO regulates both iron reductase (FRO) and iron transporter (IRT) activities and turnover by activating the transcription factor FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) which regulates the expression of *FRO* and *IRT* gene expression (Meiser *et al.*, 2011; Meiser & Bauer, 2012). Significantly, FRO is related to the phagocyte NADPH oxidase gp91phox (Chanock *et al.*, 1994; Robinson *et al.*, 1999) and by extension to RBOHD and RBOHF which drive the oxidative burst in leaves following attempted pathogen infection (Grant & Loake, 2000). As NO has been shown to be a key regulator of NADPH oxidase activity via S-nitrosylation during imune function in plants (Yun *et al.*, 2011) and more recently in animals (Qian *et al.*, 2012), by extension a similar mechanism might control the action of FRO in root cells.

The recent demonstration of enhanced plant performance following the over-expression of an iron transporter (Schroeder *et al.*, 2013), together with the biotechnological approaches assayed to cope with iron fluctuations in soils (Darbani *et al.*, 2013), suggests that approaches to manipulate NO function in roots may provide new strategies to develop more robust crops with associated increased yields.

XIV. Future perspectives

It is now becoming increasingly apparent that NO exhibits a plethora of biological functions during the growth, development, environmental interactions and immune responses of plants. Thus, local changes in cellular and/or subcellular redox status impinge upon virtually every aspect of plant physiology, parallelling the situation in mammals. While there has been significant progress in our understanding of plant NO biology over the last 15 yr, many challenges remain. In addition to the thorny issue of NO synthesis, the flip-side of the coin is also a key area for futureexploration: how is NO signalling switchedoff or downgraded? Biological cues are typically transient in nature. In this regard, AtGSNOR1 has already been identified;however, this enzyme only controls S-nitrosylation indirectly by turning over GSNO (Feechan *et al.*, 2005) and therefore its activity presumably lacks the specificity to fine-tune (S) NO signalling. Additional enzymes may exist that reduce specific S-nitrosylated Cys residues back to the thiol, potentially leaving adjacent SNOs intact. Obviously, such denitrosylases may be important components of redox signal transduction. Hence, the identification and characterization of denitrosylases might shed further light on the molecular machinery integral to the transduction of NO bioactivity.

Another important issue to be addressed is how the necessary specificity connected with S-nitrosylation is achieved. As a comparison, there are large suites of kinases and E3 ligases, for example, to precisely regulate these alternative forms of post-translational modifications. Are there analogous enzymes connected with the transfer of NO bioactivity in plants? The accumulating evidence from other organisms suggests that a nascent set of nitrosylases might just be emerging (Kornberg *et al.*, 2010).

Nitrogen assimulation is essential to support plant cellular processes because this element is a key component of many macromolecules. Interestingly, the form of nitrogen fertilizers has long been speculated to impact upon plant disease and resistance (Huber & Watson, 1974; Gupta *et al.*, 2013). This may be connected to evidence that implies that the assimilation of nitrate, the primary source of nitrogen in soil, is linked via NR to the generation of NO (Lejay *et al.*, 1999; Yamasaki *et al.*, 1999; Munos *et al.*, 2004). Hence, understanding how NO assimulation, biosynthesis and possibly turnover might be interconnected is an area that may warrant some future attention.

Furthermore, the genetic tractability of and myriad molecular tools available for plant reference systems may enable NO-related discoveries that are transferable across kingdoms. A primer for this is the recent identification of a negative feedback loop that appears to regulate NADP oxidase function in plants, flies and mammals (Yun *et al.*, 2011; Qian *et al.*, 2012).

Finally, as our appreciation of plant NO biology develops it will become important to translate these findings into

commercial outputs.Thus, novel insights into the redox-based molecular machinery that controls NO function may help shape future breeding or rationaldesign strategies for a variety of key plant traits.

Acknowledgements

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