Specificity in nitric oxide signalling

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Abstract

Reactive nitrogen species (RNS) and their cognate redox signalling networks pervade almost all facets of plant growth, development, immunity and environmental interactions. The emerging evidence implies that specificity in redox signalling is achieved by a multi-layered molecular framework. This encompasses the production of redox cues in the locale of the given protein target and protein tertiary structures that convey the appropriate local chemical environment to support redox-based, post-translational modifications (PTMs). Nascent nitrosylases have also recently emerged that mediate the formation of redox-based PTMs. Reversal of these redox-based PTMs, rather than their formation, is also a major contributor of signalling specificity. In this context, the activity of S-nitrosoglutathione (GSNO) reductase and Thioredoxin h5 (Trxh5) are a key feature. Redox signalling specificity is also conveyed by the unique chemistries of individual RNS which is overlaid on the structural constraints imposed by tertiary protein structure in gating access to given redox switches. Finally, the interactions between RNS and ROS (Reactive oxygen species) can also indirectly establish signalling specificity through shaping the formation of appropriate redox cues. It is anticipated that some of these insights might function as primers to initiate their future translation into agricultural, horticultural and industrial biological applications.

Introduction

Reactive nitrogen species (RNS) are central to the regulation of a plethora of cellular processes in plants integral to development(Foreman *et al.*, 2003; Kwon *et al.*, 2012; Homem and Loake, 2013; Kaya *et al.*, 2014; Rodríguez-Ruiz *et al.*, 2017), immunity (Torres *et al.*, 2002) and abiotic stress (Wrzaczek *et al.*, 2013; Vermeirssen *et al.*, 2014; Fancy *et al.*, 2016; Mata-Pérez *et al.*, 2017). Significantly, these small, redox-active molecules are key components of cellular signal transduction networks (del Río *et al.*, 2006; Yun *et al.*, 2012; Skelly and Loake, 2013). However, the molecular details underpinning exactly how signalling specificity is achieved by these small, redox active molecules remains to be fully established. Most of the recent insights in this area have been achieved in mammals, where NO signalling is the subject of intensive investigation due to its links with both health and disease. Thus, in this review we also highlight some of the recent mammalian literature in this area, as a primer for research in plant biology. In a protein-centric signalling pathway, the complementarity of macromolecular shapes largely guides non-covalent ligand binding to an associated receptor. Conversely, RNS predominantly transmit signals via their inherent chemistries, targeting specific atoms of proteins resulting in redox-based, post-translational modifications (PTMs) (Nathan, 2003). Therefore, RNS molecular recognition is conveyed at the atomic rather than the macromolecular level. Herein, we will discuss some of the features of RNS signalling, focusing on NO, which convey specificity onto this pivotal redox-based cue.

NO synthesis in plants

RNS is a collective term used to describe both free radicals with a short half-life and non-radicals, which either serve as oxidizing/reducing agents or become transformed into radicals. RNS include: nitric oxide (NO), peroxynitrite ($ONOO^-$), *S*-nitrosothiols (SNOs), higher oxides of nitrogen (NO_X) and dinitrosyl-iron complexes. These molecules are largely derived from NO, which in mammals is synthesised via an oxidative mechanism utilizing NO synthase (NOS), which consists of three well characterized isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Alderton *et al.*, 2001). NO is produced by the

NADPH-dependent oxidation of L-Arginine, producing L-citrulline and NO (Palmer *et al.*, 1988; Knowles and Moncada, 1994; Correa-Aragunde *et al.*, 2013). A NOS has also been reported to function in the green alga, *Ostreococcustauri* (Foresi *et al.*, 2010). However, a total of 1087 transcriptome sequences of land plants have been interrogated for the presence of canonical NOS sequences but none were found (Jeandroz *et al.*, 2016). Hence, unless plant NOS enzymes are assembled from multi-polypeptides (Mata-Pérez *et al.*, 2017), land plants generate NO by one or more mechanisms distinct from mammals. In this context, Nitrate reductase (NR) is a key enzyme for NO production in plants and NR also facilitates NO homeostasis (Chamizo-Ampudia *et al.*, 2016). Moreover, various other sources for NO production have been proposed in higher plants including: a NOS-like activity, xanthine oxidoreductase and apolyamine/hydroxylamine-mediated NO synthesis. Thus, the generation of NO still remains to be fully established in higher plants(Durner *et al.*, 1998; Stöhr and Stremlau, 2006; Stoimenova *et al.*, 2007; Gupta *et al.*, 2011; Yu *et al.*, 2014).

In order to acknowledge the biological significance of NO, in vivo synthesis of *S*-nitrosothiols (SNO) must be known. Multiple in vitro studies have highlighted various mechanisms for SNO formation. NO as a free radical (NO) can lose or gain electrons to generate oxidized nitrosonium cation (NO^+) or a reduced nitroxyl anion (NO^-) or NO can react with superoxide (O_2^-) and oxygen (O_2) to generate peroxynitrite ($ONOO^-$) and NO oxides (N_2O_3/NO_X), which all can act as S-nitrosylating agents (Figure 1) (Arnelle and Stamler, 1995; Broniowska and Hogg, 2012). Furthermore, NO radicals can react directly with thiyl radicals (RS) or metal–NO complexes (M–NO) or protein thiols to generate SNO. The thiol groups and thiyl radicals are either be part of a protein or glutathione. Furthermore, hydrogen peroxide, organic hydroperoxides and peroxynitrite oxidizes reactive cysteine thiols (R-SH) to form sulfenic acids or sulfenamides (R-SNR) or sulfonamides (R-SONR), and sulfonamides ($R\text{-}SO_2NR$) (Klomsiri *et al.*, 2011).

A redox code of cysteine residue modifications?

Rare, highly reactive protein cysteine (Cys) thiols (SH) embedded within protein structures, are key atomic targets for RNS dependent signalling (Boehning and Snyder, 2003; Poole *et al.*, 2004). These Cys residues are solvent exposed with a low pK_a sulphahydryl group, which facilitates their efficient oxidation by RNS(Meng *et al.*, 2002; Yu *et al.*, 2014). Redox-based Cys modification is influenced by the ionization state of the reactive Cys: thiolate anions ($-S^-$) have a greater tendency to donate electrons and become polarized relative to their correspondent protonated counterparts.

Cys thiols undergo a redox continuum of modifications generating an array of possible outcomes including: S-nitrosylation, the addition of a nitric oxide (NO) moiety to form an S-nitrosothiol (S-NO), S-glutathionylation (S-SG), S-sulphenation (S-OH), S-thiolation (S-S, i.e. disulphide formation) and S-sulphination ($S\text{-}O_2H$) (Figure 2) (Spadaro *et al.*, 2010; Spoel and Loake, 2011, Klomsiri *et al*., 2011). A key feature of specificity in redox signalling is the translation of different redox-based modifications into discrete protein functions to generate different cellular outcomes. This has been proposed to provide the basis of a redox code that translates protein structure into function. For example, a single Cys residue in OxyR, a transcriptional activator of *Escherichia coli*, is modified to generate four stable, redox-related modifications (S-H, S-OH, S-SG, or S-NO) (Kim *et al.*, 2002). Each modification resulted in a differential conformational change in OxyR structure, conveying unique DNA binding affinities, promoter activities and co-operative properties. These findings imply that fine-grained transcriptional regulation might therefore be controlled by exploiting distinct Cys modifications.

Local chemical environments conducive to redox-based PTMs

S-nitrosylation has emerged as a prototypic redox-based, PTM across phylogenetic kingdoms (Foster *et al.*, 2009; Yun *et al.*, 2011). In plants, protein-SNO formation has been shown to help shape growth, development, immunity and abiotic interactions (Lee *et al.*, 2008; Kwon *et al.*, 2012). Several studies have been undertaken to uncover a potentially conserved sequence motif for S-nitrosylation of protein targets, utilizing high-throughput proteomic approaches in conjunction with bioinformatic interrogation. Various factors have been reported to contribute to the selectivity of Cys residues for protein *S*-nitrosylation including: solvent accessibility, the presence of acidic-basic groups in their proximity (within 6 to 8 angstroms), the incidence of adjacent hydrophobic amino acids, increased nucleophilicity and the presence of local α-helical domains (Marino and Gladyshev, 2010; Doulias *et al.*, 2010; Chen *et al.*, 2010; Seth and Stamler, 2011). The motif I/L-X-C-X_2-D/E carries a Cys residue with a high nucleophilicity, surrounded by a hydrophobic residue, I/L, and an acidic residue D/E.This motif thereforecontains most, if not all, of the characteristics thought to be necessary for S-nitrosylation of a target Cys. In this context, this motif is associated with iNOS dependent S-nitrosylation of S100A8/A9 (calprotectin/calcium binding protein complex) and was present in 19 additional candidate SNO-proteins (Jia *et al.*, 2012). However, analysis of 445 human proteins with 810 SNO-peptides revealed only 15 peptides present in 15 proteins contain the I/L-X-C-X_2-D/E motif (Jia *et al.*, 2012; Lee *et al.*, 2012).

Hence, results based on large scale data sets have cast doubt on the reliability of a specific motif based on a primary amino acid sequence underpinning S-nitrosylation.

Interestingly, a computational tool, GPS-SNO 1.0, has been developed for the prediction of S-nitrosylation sites. This can provide a convenient and rapid strategy to generate useful information for subsequent experimental verification (Xue *et al.*, 2010). The developers utilised 504 experimentally verified S-nitrosylation sites in 327 unique proteins. The developed GPS-SNO 1.0 tool, predicted at least one potential S-nitrosylation site in 359 out of 485 S-nitrosylated proteins, with a previously unknown S-nitrosylation site. Further, a set of 46 proteins with 53 S-nitrosylation sites experimentally identified in *Arabidopsis*, were interrogated by GPS-SNO 1.0. A total of 60 S-nitrosylation sites were identified amongst these proteins, but only 11 of these sites had been confirmed experimentally (Chaki *et al.*, 2014). Thus, simple motif recognition/computational tools may also be of only limited value as they only reveal information associated with common features responsible for specificity and patterns implemented in predictive algorithms. Consequently, these bioinformatics approaches have largely failed to identify a specific consensus sequence that directs site-selective S-nitrosylation.

As the overall tertiary structure of proteins largely defines the activity of a given cognate amino acid residue, it may be naive to attempt the identification of target Cys residues based on a motif with only primary sequence information. In addition to protein three-dimensional structure, future bioinformatic tools should also consider the given redox cue, for example, NO, GSNO or even oxidised lipids (Mata-Pérez *et al.*, 2017), the likely concentrations of the given RNS and the physiological conditions under which the given PTM occurs, because all these variables can also influence Cys target specificity.

Proximity based RNS production and associated Cys modification

Another potential mechanism thought to be integral for conveying specificity in redox signalling is the proximity of the target Cys to the source of either RNS. Thus, the sub-cellular organization of the source of redox active molecules within cells alongside the spatial and temporal existence of given protein targets maybe of prime importance to trigger a particular redox signalling pathway (Derakhshan *et al.*, 2007). For example, in mammalian cells, eNOS and nNOS co-exist in cardiac myocytes but are located within distinct subcellular compartments: while eNOS is located in the plasma membrane, nNOSis found in the sarcoplasmic reticulum. Spatial confinement of these NO sources enables this redox-

active molecule to trigger discriminate signalling pathways which determine distinct downstream effects. Thus, NO generated by eNOS limits myocardial contractility, whereas NO generated by nNOS increases contractility (Barouch *et al.*, 2002).

An alternative biological strategy for achieving specificity in redox signalling is through proximity of both the given RNS source and the cognate target Cys thiol. For example, eNOS binds with Heat Shock Protein 90 (Hsp90) directly which results in *S*-nitrosylation of Hsp90 at Cys597, affecting its ATPase activity (Martínez-Ruiz *et al.*, 2005). Moreover, Cycloxygenase-2 (COX-2) becomes activated following direct binding of iNOS, which results in the selective S-nitrosylation of COX-2 on Cys526 (Kim, 2005).

A variation on this theme is the deployment of a scaffold protein to link the redox molecule generator with the target protein. In this context, NMDAR (*N*-methyl-D-aspartate receptor) binds indirectly with nNOS via the scaffold-protein PSD95 (postsynaptic density protein). The NMDAR-PSD95-nNOS ternary complex is essential for NMDAR activation which ultimately also increases nNOS activity (Brenman *et al.*, 1996; Christopherson *et al.*, 1999). Interestingly, another adapter protein, *Carboxy*-terminal *PDZ* ligand *o*f nNOS (CAPON), competes with PSD95 for binding with nNOS, positioning NO generation to drive S-nitrosylation of Dexras (Ras superfamily gene induced by dexamethasone) at Cys11, which facilitates the exchange of bound GDP (guanosine diphosphate) for GTP (guanosine triphosphate) (Jaffrey *et al.*, 1998, 2002).

While now well established in mammalian systems, this proximity based production of redox cues has not to date been demonstrated in plants. At least in the case of NO and other RNS, this has been impeded by the continuing controversy regarding the source of NO production in plants(Corpas *et al.*, 2006; Jeandroz *et al.*, 2016; Mata-Pérez *et al.*, 2017; Zemojtel *et al.*, 2017).

Specific protein *trans*-nitrosylation

Interestingly, the accumulating evidence from mammalian systems suggests that some proteins might function to transfer their NO group linked to the protein Cys sulfur to specific protein substrates, effectively functioning as nitrosylase “enzymes”, akin to more established enzymes in PTMs such as protein kinases (Hess *et al.*, 2005). To date, only a small number of *S*-nitrosylases have been reported in animals, but this list is steadily growing (Nakamura and Lipton, 2013).

Glyceraldehyde 3-phosphate dehydrogenase GAPDH, a classic glycolytic enzyme, has been proposed to function as a nitrosylase. Upon apoptotic stimulation, mammalian GAPDH is modified at Cys150 by NO, generated by NOS. Subsequently, SNO-GAPDH is thought to bind with Siah1, an E3 ubiquitin ligase, which is then translocated to nucleus (Hara *et al.*, 2005). SNO-GAPDH binding with Siah1 stabilizes this protein against turnover and in the nucleus Siah1 degrades its target substrates and also enhances p300/CBP-associated acetylation of nuclear proteins, including p53, which cause cell death (Sen *et al.*, 2008). SNO-GAPDH-Siah1 complex also mediate nuclear translocation of mutant Huntingtin (mHtt) protein which is associated with Huntington's disease (Bae *et al.*, 2006). Further, after nuclear translocation, SNO–GAPDH also transfers its NO group at Cys150 to specific cysteine residues within targeted protein thiols including SIRT1, a histone deacetylase-2 (HDAC2) and DNA-activated protein kinase (DNA-PK) (Kornberg *et al.*, 2011). Hence, SNO-GAPDH essentially also functions as an S-nitrosylase, transferring its NO to Cys thiols embedded in target proteins. However, under low levels of NO availability GOSPEL (GAPDH's competitor Of Siah Protein Enhances Life) is S-nitrosylated and competes with Siah1 for GAPDH binding, preventing the formation of a GAPDG-Siah1 complex (Sen *et al.*, 2009).

The S-nitrosylation of haemoglobin has been proposed to regulate human blood pressure (Jia *et al.*, 1996). The central iron molecule of the heme group in hemoglobin can bind oxygen facilitating the transfer of this molecule from the lungs to the rest of the body. Binding of NO at this heme group rapidly facilitates auto-*S*-nitrosylation of haemoglobin at its highly conserved Cysβ93 residue, by the transfer of the bound NO moiety, freeing the heme iron to bind with oxygen. In human hypoxia, hemoglobin-SNO has been proposed to function as a nitrosylase, where the NO group is transferred from SNO-Cysβ93 to the anion exchange protein (AE1/band3) in human red blood cells, following binding to the red blood cell membrane. Subsequently, SNO-AE1 is thought to release NO which can subsequently diffuse into smooth muscle cells, promoting the relaxation of blood vessels (Pawloski *et al.*, 2001). In this scenario, allosteric regulation of haemoglobin is employed to propagate a vasodilatory signal via the *trans*-nitrosylative transfer of NO. Thus, protein *trans*-nitrosylation, constitutes an additional strategy to convey specificity to NO-related redox signalling in biological systems.

Removal of redox-based PTMs

An important feature of the addition of PTMs to their protein targets associated with cellular

signalling is their selective reversal to disengage the given signal networks. It is highly likely that specificity in redox signalling is achieved largely by rates of reversal of Cys modifications, rather than by their formation, as rapidly degraded redox modifications would likely have far less impact than more persistent ones (Derakhshan *et al.*, 2007). In this context, it has been demonstrated that different protein-SNOs can have widely diverse biological lifetimes(Seth and Stamler, 2015).While a proportion of this can be attributed to the innate chemical stability of a given protein-SNO, this property is also influenced by potential non-enzymatic breakdown, for example, by either of the key cellular antioxidant molecules, ascorbate or glutathione (Masella *et al.*, 2005; Feechan*et al.*, 2005; Benhar *et al.*, 2008; Kneeshaw *et al.*, 2014). In green alga *Chlamydomonasreinhardtii*, hemoglobin affects the nitrogen assimilation pathway by simultaneously modulating NO levels and NR activity (Sanz-Luque *et al.*, 2015). Interestingly, non-symbiotic plant hemoglobins (phytoglobins) have also been proposed to function as NO scavengers in plants, with their activity possibly impacting plant developmental programmes (Hebelstrup *et al.*, 2013). However, these proteins are not thought to control the consequences of the pathogen-triggered, nitrosative burst and associated host cell death during plant immunity (Perazzolli *et al.*, 2004). Ascorbic acid can reduce phytoglobins in support of NO scavenging, generating nitrate and monodehydroascorbate. The monodehydroascorbate is recycled back to ascorbic acid by monodehydroascorbate reductase using NADH. Phytoglobins scavenge NO by forming S-nitrosophytoglobin, however, how this is recycled back to the reduced phytoglobin remains to be determined, but recent evidence suggests this does not involve ascorbate *in planta* (Wang and Hargrove, 2013).

Significantly, in more established signal transduction mechanisms, such as phosphorylation, signalling and its associated specificity is the result of a delicate poise between the activities of kinase and phosphatase activities. The conceptual equivalent of protein phosphatases associated with redox signalling has recently started to come into sharper focus.

Indirect and selective protein denitrosylation

It is now well established that potential non-enzymatic mechanisms make a key contribution to protein-SNO homeostasis (Liu *et al.*, 2001; Feechan *et al.*, 2005). The antioxidant tripeptide glutathione (GSH) has been shown to access sites of S-nitrosylation in protein-SNOs, reducing the SNO group back to a Cys thiol and forming S-nitrosoglutathione (GSNO), GSH S-nitrosylated at its Cys residue, as a consequence (Corrales *et al.*, 1999; Romero & Bizzozero, 2009). *Arabidopsis* GAPDH is S-nitrosylated at Cys149 and is

denitrosylated selectively by GSH (Zaffagnini *et al.*, 2013). However, in animals, SNO-GAPDH binding with Siah1, an E3 ubiquitin ligase, forms a complex which changes the conformation of SNO-GAPDH, precluding GAPDH-SNO-Cys149 from GSH, abolishing GSH mediated denitrosylation (Paige *et al.*, 2008).

Importantly, GSNO can function as a natural NO donor driving S-nitrosylation of reactive Cys thiols by *trans*-nitrosylation, resulting in specific S-nitrosylation of the target protein (Hess *et al.*, 2005). GSNO is relatively stable and can therefore function as a cellular reservoir of NO bioactivity. Significantly, GSNO can be turned over by GSNO reductase (Feechan *et al.*, 2005; Lee *et al.*, 2008; Chen *et al.*, 2009). Thus, S-nitrosylated proteins maybe in dynamic equilibrium with their de-nitrosylated counterparts controlled by the activity of GSNOR.

A growing body of evidence has demonstrated that GSNOR mediated denitrosylation has essential roles in both plant immunity and development. Loss or gain-of-function mutants in *GSNOR1* in *Arabidopsis* result in either reduced or increased denitrosylation respectively. While reduced denitrosylation promotes enhanced disease susceptibility, increased denitrosylation leads to enhanced disease resistance (Feechan *et al.*, 2005; Tada *et al.*, 2008). Further, the control of denitrosylation has also been shown to regulate pathogen-triggered cell death by controlling the extent of S-nitrosylation at Cys890 of the Respiratory Burst Oxidase Homolog D (RBOHD) and this mechanism maybe evolutionary conserved across kingdoms (Yun *et al.*, 2011). In a similar fashion, dysregulation of denitrosylation also appears to impact a number of key plant developmental programmes (Kwon *et al.*, 2012; Lee *et al.*, 2008; Lea *et al.*, 2004).

But how is specificity in GSNOR function established? The emerging evidence suggests that tertiary protein structure restricts the ability of GSH to function as a non-enzymatic denitrosylase to a sub-set of reactive proteins Cys thiols (Foster *et al.*, 2009; Yun *et al.*, 2016). Thus, GSH might not be able to denitrosylate all possible cellular protein-SNOs, forming the free protein thiol and GSNO in the process. Consequently, only a sub-set of total cellular S-nitrosylated proteins will be under the indirect control of the protein denitrosylase, GSNOR.

Direct and selective protein denitrosylation

Thioredoxins (Trxs) are present in all living organisms and their activity can be recycled by

NADPH-dependent thioredoxin reductase (TrxR). Trx/TrxR mediated denitrosylation has emerged as an important mechanism to control redox regulation. In animals Trx mediateddenitrosylation of Casepas-3 results in the activationof this enzyme (Benhar *et al.*, 2008).Other targets for Trx mediated selective denitrosylation in animals are caspase-9, protein tyrosine phosphatase 1B and GAPDH (Benhar *et al.*, 2008).

In plant immunity, Trxh5has been proposed to selectively denitrosylatethe transcriptional co-activator,NPR1(Kneeshaw *et al.*, 2014). NPR1 is S-nitrosylatedin response to increasing GSNO levels, leading to NPR1 oligomerization. Thus, sequestering this co-activator in the cytosol, preventing its nuclear translocation and the subsequent activation of *Pathogenesis Related* (*PR*) gene expression (Tada *et al.*, 2008; Yu *et al.*, 2014). Trxh5 and TrxR have been proposed to directly denitrosylate NPR1, leading to the release of NPR1 monomers, their translocation to the nucleus and the subsequent engagement of *PR* gene transcription (Kneeshaw *et al.*, 2014). Thus, selective denitrosylation of NPR1 is an important feature of the plant immune response. However, it is noteworthy that NPR1 function is also impacted by GSNOR activity (Feechan et al., 2005; Tada et al., 2008), suggesting the S-nitrosylation status of this transcriptional co-activator is regulated directly by Trxh5 but indirectly by GSNOR. The emerging biochemical and genetic evidence suggests that Trxh5 and TrxR can denitrosylate a sub-set of the plant SNO proteome directly and selectively *in vivo*(Kneeshaw *et al.*, 2014). Thus, the regulation of denitrosylation at other key protein Cys thiols is also likely to be under the control of Trxh5 and TrxR. Further, Trxs are comprised of a large gene family in *Arabidopsis*, therefore it is possible other Trx family members might also function in combination with TrxR as direct and selective denitrosylases of a range of possible substrates.

Interestingly, in mammals, two Trx-mimetic (TXM) peptides derived from Trxs conserved active site (CXXC) have been documented to act as denitrosylating catalysts (Kronenfeld *et al.*, 2015). TXMs with TrxR can effectively reduce both low-molecular-weight SNOs and protein SNO. GSNO is reduced to GSH through *trans*-nitrosylation resulting in S-nitrosylated TXM, which are cyclized by TrxR or other endogenous thiol reductases. TXMs have ability to denitrosylate multiple classes of SNO proteins. TXM also protects TrxR from SNO-mediated loss of activity which is important to conserve its function in redox signalling. Collectively, the current state-of-the-art suggests that Trx and TrxR can function as direct and selective denitrosylases to regulate a subset of S-nitrosylated proteins across kingdoms.

Discrete activities of given RNS

Recent studies have shown that NO and SNO might have discrete or overlapping protein targets in relation to redox signalling (Yun *et al.*, 2016), providing an additional layer of RNS signalling specificity. NO has been proposed to have a more extensive range of biological activity relative to GSNO (Figure 3). In addition to protein S-nitrosylation, NO is thought to be largely responsible for metal-nitrosylation, the most rapid known reaction of NO with proteins. NO forms metal-nitrosyl complexes (M-NO) with metals embedded within proteins, for instance zinc (Zn^{2+}), iron (Fe^{2+} or Fe^{3+}), or copper (Cu^{2+}) (Leitner *et al.*, 2009). Mammalian soluble guanylate cyclase is a prototypic example of a protein that is regulated by this type of PTM(Derbyshire and Marletta, 2012). NO can also nitrosylate amino acid side chains to form so-called N-NO (Stamler *et al.*, 1992). However, NO cannot directly oxidize amino acid side chains at any significant biological rate. Hence, NO mediates N-NO via auto oxidation to nitrogen dioxide, dinitrogen trioxide or peroxynitrite. In contrast, GSNO is thought to principally mediate protein S-nitrosylation (Foster *et al.*, 2009; Yun *et al.*, 2016).

A microbial Flavohaemoglobin (FHb) has been shown to turnover NO in a number of bacteria and may function as a virulence factor to counter antimicrobial NO production by potential mammalian hosts produced via iNOS (Poole *et al.*, 1996; Poole and Hughes, 2000).Loss-of-function mutations in a *FHb* present in the human pathogens *Cryptococcus neoformans*or *Salmonella typhimurium* reduced their virulence in mice. However, virulence was restored in mice lacking an iNOS (Bang *et al.*, 2006; de Jesús-Berríos *et al.*, 2017). Informatively, double mutantslacking both FHb and GSNOR function further reduced the virulence of *Cryptococcus neoformans*,consistent with an additive antimicrobial function of NO and GSNO (Liu *et al.*, 2004), suggesting these RNS might have separable functions. Thus, NO and GSNO might target a different spectrum of microbial protein Cys thiols.

Similarly, in *Saccharomyces cerevisiae* a *FHb* knock-out mutant strain was deficient in NO turnover as compared to wild-type or *GSNOR* knock-out strains. Further, exogenous application of NO retarded the growth of the *fhb* mutant but had no impact on either wild-type or a *gsnor* mutant strain. However, exogenous application of GSNO had greater impact on the *fhb gsnor* double mutant relative to either of the single mutant strains. Thus, GSNOR activity conveys greater protection against GSNO-induced nitrosative stress, rather than nitrosative stress established by NO(Foster *et al.*, 2009). This is consistent with the posit that NO and GSNO may have distinct and overlapping activities.

Genetic evidence for this dichotomy between the biological activities of NO and GSNO has also recently been established in plants(Yun *et al.*, 2016). The *Arabidopsis* mutants, *no-over*

producer 1-1(*nox1-1*) and *gsnor1-3*exhibit increased levels of either NO or SNO *in vivo*,respectively (He, 2004; Feechan *et al.*, 2005; Yun *et al.*, 2016). Informatively, the double mutant, *gsnor1-3 nox1-1*, appears to be genetically additive, as it shows greater susceptibility to pathogens than either of the single mutants alone. Further, *GSNOR1* over expression in *gsnor1* plants restored wild-type levels of immunity, however, this was not the case in *nox1-1* plants. This may reflect the notion that NO can drive both M-NO and N-NO formation in addition to protein S-nitrosylation. In contrast, GSNO predominately favours the generation of protein-SNOs. Interestingly, genetic analysis suggests that high *in vivo* SNO concentrations can facilitate pathogen-triggered hypersensitive response (HR) cell death independently of ROS synthesized by the NADPH oxidases, AtRBOHD and AtRBOHF, responsible for the extracellular oxidative burst (Yun et al., 2011). While NO was previously reported to require superoxide (O_2^-) in order to trigger the HR (Delledonne *et al.*, 2001) (see next section). Collectively, these data suggest that NO and SNO might have discrete or overlapping functions in the context of redox signalling during the establishment of plant immunity (Yun *et al.*, 2016) and perhaps other plant molecular systems, providing addition scope for specificity in redox regulation of cellular function.

Specificity conveyed by interactions between RNS and ROS

Chemical interactions between RNS and ROS is a prominent feature of redox signalling networks. These interactions have been proposed to indirectly modulate the activity of a series of proteins (Hausladen and Fridovich, 1994; Romero *et al.*, 2003; Wang *et al.*, 2013)and by extension, this presents another way of achieving specificity in redox signalling. For example, NO is unable to influence the activity of the tricarboxylic acid cycle enzyme, aconitase, in *Escherichia coli*. However, the interaction of NO with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), generates a powerful inhibitor of aconitase activity (Hausladen and Fridovich, 1994).

In plant biology, interaction between NO and hydrogen peroxide (H_2O_2) has been reported to be a key facet associated with the development of the hypersensitive response (HR), leading to the programmed execution of pathogen challenged plant cells (Mur *et al.*, 2008).NO production following pathogen recognition has been posited to beinsufficient to activate HR cell death. Rather, the HR has been proposed to be triggered only by a balanced production of NO and ROS(Delledonne *et al.*, 2001; Lin *et al.*, 2012). Thus, HR cell death is activated after interaction of NO not with superoxide (O_2^-), presumably produced by RBOHD and RBOHF (Torres *et al.*, 2002), but with H_2O_2 generated from O_2^- by superoxide dismutase (Delledonne

et al., 2001). Increasing the level of O_2^- reduced NO-mediated HR cell death, by converting more NO into peroxynitrate ($ONOO^-$), which was proposed to have low levels of toxicity towards plant cells. However, this is counterintuitive, because it is well established that $ONOO^-$ exhibits an extremely high level of cellular toxicity (Pacher and Szabo, 2008). Perhaps plants can more effectively turnover $ONOO^-$?

In a similar fashion, both NO and GSNO have been proposed to be important mediators in the process of H_2O_2-induced leaf cell death in rice (Lin *et al*., 2012) and in response to the fungal elicitor, cryptogein, the production of NO and H_2O_2 in tobacco appears to be reciprocally regulated (Kulik*et al.*, 2015). Also, S-nitrosylation of RBOHD at Cys890 curbs ROS production limiting pathogen-triggered cell death development (Yun *et al.*, 2011). An interaction between NO and H_2O_2has also been reported to be required for stimulation of stomatal closure in *Arabidopsis* in response to ultraviolet-B exposure. An increase in H_2O_2 scavenging activity or an inhibition of H_2O_2 synthesis prevented NO generation and subsequent stomatal closure (He *et al.*, 2013).

In aggregate, this work demonstrates that either direct or indirect interactions between distinct redox molecules can shape the specificity of signalling outputs.

Conclusions and future prospects

The prevalence of redox signalling in the biology of plants and other organisms is being increasingly appreciated. However, there are many potential molecular switches, including but not limited to highly reactive Cys thiols and chemically co-ordinated metal co-factors.So how is specificity in redox regulation achieved? As discussed above, the emerging model suggests that redox specificity is mediated via a multi-layered molecular framework. This might encompass the production of the signalling redox molecule(s) in the locale of the protein target. Tertiary protein structures subsequently provide a chemical environment compatible with oxidation/reduction of the target redox switch. Superimposed upon this molecular landscape, *trans*-nitrosylation, by a nascent but growing repertoire of nitrosylases, can function as an additional mechanism to deliver and then transfer an NO moiety to a target protein thiol. Reversal of redox modifications, rather than their formation, is also a major contributor to signalling specificity. In this context, selective, indirect protein denitrosylation conveyed by GSNOR-dependent GSNO turnover is a prominent feature. This mechanism is augmented by the selective and direct denitrosylation mediated by Trxh5 and TrxR.

Signalling specificity is also mediated by the unique chemistries of individual RNS which is overlaid on the structural constraints imposed by tertiary protein structure gating access to given redox switches. Finally, the direct or indirect interactions between RNS and ROS can also indirectly establish signalling specificity through shaping the formation of appropriate redox cues.

The emerging evidence suggests redox signalling pervades almost all aspects of plant growth, development, immunity and abiotic environmental interactions(Besson-Bard *et al.*, 2008; Yu *et al.*, 2012, 2014).A deeper understanding of the fundamental mechanisms underpinning this mode of regulatory control is now an urgent future priority. In biomedicine, insights into free radical biology and associated redox signalling are already being actively translated into blockbuster pharmaceuticals, especially within the areas of cardiovascular disease, sexual dysfunction, pain, neuroprotection, asthma, and anti-inflammatories (Janero, 2000; Foster *et al.*, 2003).As the plant redox field moves forward, similar opportunities will emerge in relation to agriculture, horticulture and industrial biotechnology. An exciting future rich in "radical" activity lies ahead.

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Figure 1. Generation of S-nitrosothiols.

Nitric oxide (NO) can lose or gain an electron to form an oxidized nitrosonium cation (NO^+) or a reduced nitroxyl anion (NO^-), respectively. NO can also react with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$). Further, NO can react with oxygen (O_2) to form higher order nitrogen oxides (NO_x), such as dinitrogen trioxide (N_2O_3). NO radicals can also react directly with transition metals in metal containing proteins to form metal-nitrosyl complexes (M–NO). Each of the described molecules can react with either thiol groups, typically as a thiolate (RS^-) or thiyl ($RS^\cdot$) associated with either proteins or glutathione to generate an S-nitrosothiol (SNO).

Figure 2. Schematic overview of redox-based Cysteine (Cys) modifications. Cys thiol (SH), S-nitrosothiol (SNO), sulphenic acid (SOH), disulphide (S–S), S-glutathionylation (SSG), sulphinic acid (SO2H) and sulphonic acid formation (SO3H). Molecules in this sequence are formed following increasing oxidation. The formation of all these function groups is thought to be reversible, except for sulphonic acid formation, which is irreversible. (Modified from Spadaro *et al.*, 2010).

Figure 3. Schematic model showing discrete and overlapping activities of NO and GSNO associated with cellular signalling. NO can target metals embedded in proteins (1), amino acid side chains (2), GSH to form GSNO, which is reversible (3) and; proteins, to form S-nitrosothiols (4). NO can also S-nitrosylate GAPDH (5). NO can be metabolized by microbial FHb into NO_3/N_2O (6). GSNO can *trans*-nitrosylate selected protein targets (7). GSH can reverse S-nitrosylation at a sub-set of proteins generating the denitrosylated protein and GSNO (8). GAPDH-SNO can function as a nitrosylase (in mammals) to S-nitrosylate target proteins (9). GSNO can S-nitrosylate GAPDH forming GAPDH-SNO and GSH (10). GSH can denitrosylate GAPDH-SNO in *Arabidopsis* to generate GAPDH and GSNO (11). Trx (Trxh5 in *Arabidopsis*) can directly and selectively denitrosylate a subset of protein-SNO (12).