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NO signals in the haze
Nitric oxide signalling in plant defence

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Nitric oxide (NO) is gaining increasing attention as a regulator of diverse (patho-)physiological processes in plants. Although this molecule has been described as playing a role in numerous conditions, its production, turnover and mode of action are poorly understood. Recent studies on NO production have tended to highlight the questions that still remain unanswered rather than telling us more about NO metabolism. But regarding NO signalling and functions, new findings have given an impression of the intricacy of NO-related signalling networks. Different targets of protein S-nitrosylation have been characterised and enzymatic routes controlling this posttranslational modification are emerging, along with their physiological implications. Evidence is also accumulating for protein tyrosine nitration and cGMP as important components of NO-related signal transduction.

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Introduction

Nitric oxide (NO) is a small gaseous radical with diverse signalling functions. In plants, NO was first found to play a crucial role in mediating defence reactions against bacterial pathogens [1] and is now well known to influence numerous physiological processes throughout the entire plant life cycle. To name a few, NO is involved in germination, leaf expansion, lateral root development, flowering, stomatal closure, cell death and defence against biotic and abiotic stresses [2•]. Whereas descriptions of NO-mediated processes are accumulating, the plant signalling pathways governed by NO are still largely unknown. NO-related signalling can be attributed to various NO derivatives, collectively referred to as reactive

nitrogen species (RNS). RNS comprise not only the NO radical ($NO^•$) and its nitroxyl (NO^-) and nitrosonium (NO^+) ions, but also peroxynitrite ($ONOO^-$), S-nitrosothiols, higher oxides of nitrogen and dinitrosyl–iron complexes; in short, all NO derivatives that can effect NO-dependent modifications [3]. Hence, the term NO-related signalling is used here to summarise effects caused by all these RNS.

In principle, NO-related functions are subject to different levels of control. Production and turnover regulate NO bioavailability; once the NO level increases in the system, it can affect signalling either directly via protein modifications or indirectly via activation of second messengers. In animals, second messengers, such as cGMP, are well-characterised components of NO signal transduction, whereas studies of NO-dependent posttranslational modifications are more recent. However, in research conducted in plants, knowledge of the cGMP-dependent pathways is restricted to data gained using pharmacological approaches. By contrast, the impact of NO-dependent protein modifications, especially of S-nitrosylation, is the best studied mode of action in plants to date.

Among the diverse physiological processes affected by NO, available data predominantly explain signalling related to plant defence responses—which is the focus of this review.

Regulation of NO bioavailability
No simple answers to the question how NO is produced in plants

Three routes to yield NO have been described in plants: non-enzymatic conversion of nitrite to NO in the apoplast, nitrate reductase (NR)-dependent NO formation and NO synthase (NOS)-like activity, that is arginine-dependent NO formation. These pathways have been reviewed in detail [2•,4•]. In a nutshell: since the enzymatic source(s) of NO in plant stress responses remains elusive, unbiased genetic tools are still lacking for non-invasive manipulations of NO levels *in planta*. Despite numerous studies using pharmacological tools demonstrated NOS-like activity in plants, the identity of the enzymes involved remains unknown. The only postulated plant NOS (AtNOA1/RIF1) has recently been shown to have no NOS activity [5••]. Instead, it is a chloroplast-targeted GTPase essential for proper ribosome assembly [6•]. Mutation in this gene leads to reduced NO accumulation, probably because of its rapid

Table 1

Characterised mutants and transgenics with altered NO levels.

Gene	Description	Mod.	Species	NO level	related phenotype	Ref.
Noa1/Rif1	Nitric oxide associated1/resistant to fosmidomycin 1; chloroplastic GTPase	m	At	−	ROS accumulation	[5••,6•]
		s	At	+	−	[5••,6•]
Nia1 Nia2	Nitrate reductase	m	At	−	Reduced amino acid and nitrite levels	[8]
NiR	Nitrite reductase	as	Nt	+	Nitrite accumulation	[12]
Argah1 and 2	Arginine amidohydrolase; arginase	m	At	+	L-Arginine accumulation	[9]
Nox1/cue1	NO overproducer 1/chlorophyll a/b binding protein underexpressed 1; PEP phosphate translocator	m	At	+	L-Arginine accumulation	[10]
Rcd1	Radical-induced cell death1; unknown function.	m	At	+	ROS accumulation	[13]
Gsnor1/hot5	GSNO reductase	m	At	+	Accumulation of S-nitroso species and nitrate	[11•,19,20]
		as	At	+		
		s	At	−	−	[11•,19]
Vsr3	Vacuolar sorting receptor	as	At	+	ROS accumulation	[14]
Hb1	Ns haemoglobin	as	At, Ms	+	−	[17]
		s	At, Ms	−	Nitrate accumulation	[17]
Hmp	Bacterial flavohaemoglobin	s	At	−	Nitrate accumulation	[18]

as, antisense; At, Arabidopsis thaliana; GSNO, S-nitrosoglutathione; Mod., gene modification; Ms, Medicago sativa; m, mutant; Nt, Nicotiana tabacum; Ns, non-symbiotic; PEP, phosphoenolpyruvate; Ref., references; ROS, reactive oxygen species; s, sense.

reaction with the elevated amounts of ROS observed in the *Atnoa1* mutant [5••].

By contrast, NO can clearly be produced by NR activity [7]. However, NR deficiency also leads to impaired nitrogen assimilation, thus generally influencing primary and secondary metabolism. Indeed, Arabidopsis *nia1 nia2* double mutants display decreased levels of both nitrite and amino acids [8]. Hence, direct effects due to impaired NO biosynthesis are difficult to distinguish from those caused by metabolic alterations. In several other mutants, increased NO accumulation correlates with concentrations of putative substrates for NO biosynthesis (Table 1): (1) Arginase-deficient *Atargah 1-1* and *2-1* as well as *Atnox1/cue1*, defective in a phosphoenolpyruvate phosphate translocator, display elevated levels of L-arginine [9,10]; (2) *Atgsnor1-3/hot5-2*, defective in the *S*-nitrosoglutathione reductase, accumulates nitrate and (3) an antisense *NiR* (nitrite reductase) tobacco accumulates nitrite [11•,12]. Recently, the Arabidopsis *rcd1*, a mutant sensitive to ozone and *VSR3* (vacuolar sorting receptor 3) antisense transgenic were shown to overaccumulate NO and ROS, extending the list of genetically modified plants with altered NO and/or ROS homeostasis [13,14]. In summary, none of these NO-related mutants is exclusively affected in NO production. Their complex and pleiotropic phenotypes make genetic study of NO function difficult. The significance of using genetic tools for studying NO-related signalling *in vivo* is underlined by recent reports demonstrating the strict co-localisation of NO sources (i.e. NOSs) and *S*-nitrosylation in mammalian systems [15,16]. Therefore, the identification and

characterisation of NO-producing enzymes in plants, other than NR, remain equally challenging and mandatory tasks.

Different paths lead to RNS and their turnover

Relative to the elusive routes of NO production, mechanisms regulating bioavailable RNS are comparatively well characterised. As outlined above, different RNS contribute to NO-related signalling. Accordingly, a network of RNS transformation and turnover balances the bioavailability of these signal compounds. These pathways are illustrated here using three examples. NO can be metabolised to nitrate by non-symbiotic haemoglobins, like Arabidopsis Hb1, which acts as an NO dioxygenase using NADPH as an electron donor (Figure 1, [17]). However, the NO-reducing activity of Arabidopsis Hb1 is rather low, although physiologically relevant under hypoxic stress [17]. NO also reacts with GSH to form *S*-nitrosylated glutathione (GSNO), which can release NO or function as a transnitrosylating agent; it is thus considered a reactive nitrogen species (RNS) and natural reservoir of NO. The enzyme controlling GSNO levels is GSNO reductase (GSNOR). It reduces GSNO to oxidised glutathione and NH_3. Though highly specific for GSNO, GSNOR seems to also influence levels of protein *S*-nitrosylation [19]. In Arabidopsis, mutation of GSNOR (*gsnor1-3/hot5-2*) causes increased basal levels of NO and *S*-nitroso species. The mutant plants not only have impaired resistance to biotic and abiotic stresses, but also show reduced growth, fertility and reproduction. This complex phenotype underlines the vital role of functional RNS homeostasis in diverse physiological processes

Figure 1
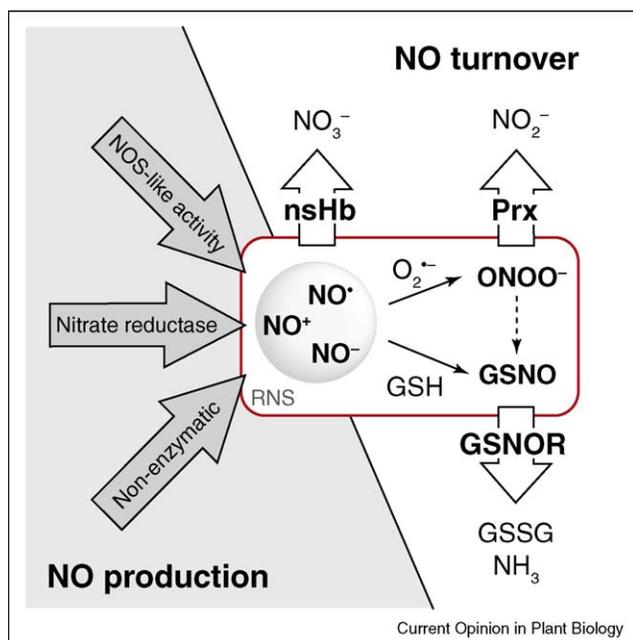
Routes of NO production and turnover. Three pathways of NO production are thought to exist in plants: NOS-like activity of (an) unidentified protein(s), NO production by nitrate reductase and non-enzymatic conversion of nitrite to NO under acidic conditions. Three main routes lead to NO turnover: NO scavenging by non-symbiotic haemoglobins, transformation of NO to GSNO and degradation by GSNOR and reaction of NO with superoxide to form peroxynitrite, which is detoxified by peroxiredoxins. Abbreviations: GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; NOS, nitric oxide synthase; nsHb, non-symbiotic haemoglobin; Prx, peroxiredoxin; RNS, reactive nitrogen species.
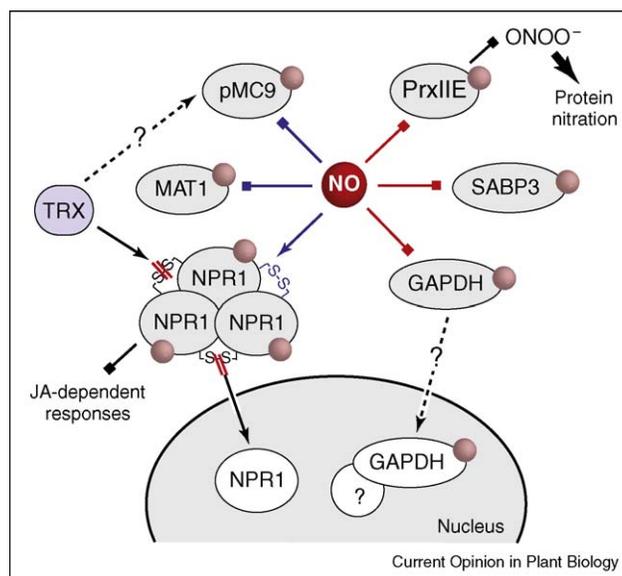
Figure 2

Characterised targets of protein S-nitrosylation in plants. S-nitrosylation under physiological conditions (basal NO tone, blue arrows) is assumed to inhibit MAT1 and pMC9 and to facilitate oligomer formation of NPR1. Induced NO production (i.e. defence-related NO burst; red arrows) leads to S-nitrosylation of GAPDH, SABP3 and PrxIIE. GAPDH activity is blocked upon S-nitrosylation and the protein is thought to translocate to the nucleus. S-nitrosylation of SABP3 leads to the loss of the enzyme's SA-binding capacity as well as inhibition of its carbonic anhydrase activity. Also PrxIIE activity is inhibited by S-nitrosylation, which leads to increased peroxynitrite levels and protein nitration. Under induced conditions, thioredoxins counteract S-nitrosylation of NPR1, leading to monomer release and translocation of this transcription co-factor to the nucleus. Dashed lines indicate hypothetical interactions, S-nitrosylation is symbolised by red bullets. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JA, jasmonic acid; MAT1, methionine adenosyltransferase 1; NPR1, non-expressor of pathogenesis-related protein 1; pMC9, prometacaspase 9; PrxIIE, peroxiredoxin II E; SABP3, salicylic acid binding protein 3; TRX, thioredoxin.

[11[•],20,21]. Finally, NO rapidly reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a potent oxidising and nitrating species. This non-enzymatic reaction is tightly controlled by the (enzymatic) formation of its precursors. $ONOO^-$ can, in turn, be detoxified by some peroxiredoxins. For example, Arabidopsis PrxIIE possesses peroxynitrite reductase activity (Figure 1) [22[••]] and therefore controls the bioavailability of $ONOO^-$.

Hence, a complex interplay between RNS becomes evident: by removing NO, other RNS can be formed, presumably with altered signalling functions ([23], cf. Figure 1 and below). Moreover, peroxiredoxin II E is inhibited by S-nitrosylation, causing increased $ONOO^-$-mediated protein tyrosine nitration. This demonstrates that enzyme regulation by NO can facilitate accumulation of another RNS.

NO-associated protein modifications

RNS can directly react with diverse biomolecules [3]. Amongst them, proteins can be modified by RNS through reactions with different amino acids or prosthetic groups.

More specifically, the main NO-associated protein modifications in the biological context are the covalent modifications of cysteine (S-nitrosylation) and tyrosine (tyrosine 3-nitration) residues and NO binding to transition metals (metal nitrosylation). To date, the best characterised of these is cysteine S-nitrosylation.

Protein S-nitrosylation

S-nitrosylation refers to the covalent attachment of an NO moiety to the thiol side chain of cysteine [24]. Although proteomic approaches identified numerous candidates in plants [25,26], only a few confirmed functional modifications have been reported (Figure 2). Nevertheless, beginning characterisation of modified proteins now reveals the physiological significance of S-nitrosylation.

S-nitrosylation contributes to gene regulation

NO-dependent gene regulation strongly depends on the plant organ and the inducing stimulus, reinforcing the

need to consider downstream regulatory networks [27]. Changes in gene expression have been intensively studied using different NO donors (reviewed by [28]), though the regulatory basis leading to these changes remains unknown. Common regulatory elements in differentially expressed genes could be identified using bioinformatics tools [29]. These theoretical considerations have recently been substantiated by experimental data that documented the control of NO over transcription factors [30,31••]. In particular, finding NPR1, a crucial component of disease resistance and signal cross-talk, to be regulated via S-nitrosylation added an important clue to understanding NO's signalling functions [31••]. The subcellular localisation of NPR1, and thus its transcription co-factor activity, is controlled by S-nitrosylation. Intermolecular disulphide bridges sustain NPR1 oligomers, which are retained in the cytosol. Intriguingly, S-nitrosylation facilitates oligomerisation of NPR1, whereas thioredoxins support reductive monomer release, a reaction that is boosted by SA (Figure 2). After identifying the crucial components, the next challenge will be to investigate how this intricate balance is achieved.

S-nitrosylation modulates phytohormonal signalling

NPR1 not only mediates defence gene expression, but also contributes to the suppression of JA-dependent responses in the cytosol in a so far unknown mode [32]. S-nitrosylation could thus contribute to the well-documented negative cross-talk between SA and JA signalling pathways. Moreover, allene oxide cyclase, a JA biosynthetic enzyme, has been shown to be S-nitrosylated during the hypersensitive response [26], which potentially represents another mechanism regulating oxylipin levels. In this connection, S-nitrosylation of salicylic acid binding protein 3 (SABP3) may also interfere with signal cross-talk by affecting lipid-derived signalling components as both carbonic anhydrase activity and SA-binding capacity of SABP3 are inhibited by S-nitrosylation [33]. Besides its apparent influence on SA/JA cross-talk, NO also affects ethylene, abscisic acid (ABA) and auxin signalling [34,35•,36]. S-nitrosylation of methionine adenosyltransferase 1 (MAT1; involved in ethylene biosynthesis) inhibits ethylene production [34], whereas NO is required for downstream responses to auxin and ABA [35•,36]. The interplay between different phytohormones and NO during innate immune reactions is an outstanding example of the emerging mutual influences and of how NO plays its regulatory role in vital functions of plants. ABA-dependent and SA-dependent stomatal closure upon pathogen recognition is mediated by NO; the virulence factor coronatine, a structural and functional mimic of JA, can reverse the effect and lead to stomatal reopening. These data implicate the involvement of at least three phytohormones (or mimics thereof) in pathogen-induced stomatal movements and suggest NO as a key mediator of these hormone responses [35•]. Although the role of NO in stomatal closure remains to be defined, S-nitrosylation of K+ outward rectifying chan-

nels supports a potential role of this posttranslational modification in modulating stomatal movements [37].

S-nitrosylation controls cell death

NO's control over Arabidopsis metacaspase 9, a potential executioner of programmed cell death [38•], is another intriguing example of how NO exerts its physiological functions through S-nitrosylation. Data indicate that this modification keeps the zymogen inactive under normal physiological conditions, inhibiting the autoproteolytic activity of prometacaspase 9. S-nitrosylation of Arabidopsis cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) could also play a role in the regulation of cell death, as it causes GAPDH inactivation and potentially induces translocation to the nucleus [39]. In animal systems, S-nitrosylated GAPDH binds to and stabilises Siah1 (an E3 ubiquitin ligase), followed by translocation to the nucleus, where Siah1 degrades nuclear proteins. These events finally lead to apoptosis [40]. A similar scenario, though conceivable, still remains to be demonstrated in plant cells. S-nitrosylation seems to have opposing roles in controlling programmed cell death: on the one hand, S-nitrosylation promotes the execution of programmed cell death by altering GAPDH function and on the other hand, S-nitrosylation of prometacaspase 9 keeps this putative executioner of cell death inactive. However, the kinetics of these signalling reactions suggests the involvement of enzymatic mechanisms [41]. Hence, activation of metacaspase 9 under conditions of increased NO production may depend on enzymatic denitrosylation. In this context, thioredoxins clearly deserve attention in the future ([42•], cf. NPR1).

This overview of S-nitrosylation already gives an impression of the emerging complexity of NO-dependent signalling in plants. But there is more to come: Arabidopsis PrxIIE has been found to be S-nitrosylated during the hypersensitive disease resistance response, resulting in not only inhibition of its hydroperoxide-reducing peroxidase activity, but also reduction of its ability to detoxify ONOO− [22••]. This inhibition leads to increased ONOO−-dependent nitrotyrosine formation. Thus, S-nitrosylation can modulate a central point of convergence for ROS-dependent and NO-dependent signalling pathways in response to several stresses by deactivating a crucial component of the cellular antioxidant system.

Protein tyrosine nitration

Protein tyrosine nitration results from a chemical reaction, in which ONOO− adds a nitro group in *ortho* position to the aromatic ring of tyrosine residues [43,44]. The added nitro group can alter protein function and conformation when placed on a relevant tyrosine residue and can increase the proteins' susceptibility to proteolysis [45,46].

In animals, nitration of tyrosine residues is often considered a marker for certain pathological conditions, but

has recently been suggested to be relevant to redox signalling [47•,48]. In plants, tyrosine nitration appears to be physiologically relevant during biotic and abiotic stresses [22••,49,50]. However, any physiological reaction must meet certain criteria to be considered a signalling process: (i) the signal compound is produced at strictly controlled rates; (ii) the induced reaction is reversible and/or the effects transient; (iii) the resulting modification (e.g. of a target protein) causes alterations of cell function(s). On the basis of these criteria, what arguments can be put forward to qualify tyrosine nitration as a signalling process in plants? (i) $ONOO^-$ production is not under direct enzymatic control, but nonetheless seems to be tightly regulated (see 'Regulation of NO bioavailability'). The transient increase of $ONOO^-$ levels in elicitor challenged tobacco cell culture supports this notion [50]. (ii) Recent studies report a transient increase of tyrosine-nitrated proteins during the disease resistance response [22••,50,51]. (iii) The only indications for functional modifications by tyrosine nitration come from the animal kingdom, where both enzyme inhibition and activation have been reported [52–54]. In plants, only one study provided an indirect proof of inhibition of glutathione *S*-transferase by RNS that correlated with increased nitro-tyrosine levels [55]. We have recently conducted a proteomic study to identify tyrosine nitrated proteins in Arabidopsis plants challenged with an incompatible bacterial pathogen [51]. Interestingly, several of the proteins with increased nitration levels during the hypersensitive resistance response are also nitrated in unchallenged plants. Thus, tyrosine nitration could play a role under physiological as well as stress conditions. Independently, we found that NtMEK2, a tobacco MAPKK, can be nitrated *in vitro*, resulting in loss of its activity (E Vandelle *et al.*, unpublished). In animal systems, $ONOO^-$ is known to interfere with phosphorylation cascades via nitration of relevant tyrosine residues in target proteins, mimicking or preventing tyrosine phosphorylation [47•]. Furthermore, several protein kinases, comprising all components of the MAPK module [47•], have been shown to be modulated by tyrosine nitration, being either activated [56,57] or inhibited [58,59]. On the basis of these findings, regulatory mechanisms controlling the MAPK module by tyrosine nitration are also plausible in plants, again pointing to RNS as crucial modulators of signal transduction.

Taken together, the above-mentioned data support our view that tyrosine nitration is a physiologically relevant posttranslational modification in plants.

Metal nitrosylation
NO can react with most transition metals, but its interactions with iron are probably the most relevant biologically [41,60]. The most prominent examples for modulation of enzymatic activity by NO in animals are associated with haem nitrosylation, such as inhibition of cytochrome *c* oxidase and NOS, regulation of diverse

cytochrome P450s and activation of soluble guanylate cyclase (sGC). sGC is the prototypic NO sensor and NO-modified protein in animals [61]. sGC is activated via haem nitrosylation and is responsible for the majority of physiological responses to NO. By contrast, virtually nothing is known about this NO-dependent modification in plants. The only exceptions are plant haemoglobins (cf. 'Regulation of NO bioavailability'). However, in this case the occurring reaction constitutes an NO scavenging mechanism and not a modification of enzymatic function. On the basis of chemical considerations, plant cytochrome P450s seem likely targets of metal nitrosylation. Given the functional diversity of plant cytochrome P450s, intriguing connections between NO-related signalling and different metabolic processes could emerge.

The missing link to cGMP
In animals, NO can initiate its biological effects through the activation of sGC and associated increase in the levels of the second messenger cGMP. Both a transient increase in cGMP and its involvement in several processes have also been demonstrated in plants [62]. Indeed, pharmacological and biochemical approaches showed that cGMP is involved in NO-dependent signalling, gene transcription modulation, root growth and gravitropism, pollen tube growth and orientation, hormone-dependent responses, stomatal opening and responses to biotic and abiotic stresses. Hence, the physiological role of this second messenger is unequivocally recognised. Although several potential GCs have been identified using bioinformatics tools, their relevance as cGMP sources still awaits demonstration. In fact, the overexpression of AtGC1 in *E. coli* [63] and activity analysis of the putative GC catalytic centre of the brassinosteroid receptor AtBRI1 *in vitro* [64] showed only low activity compared with the animal sGC [65], questioning their relevance as plant GCs. Supporting this observation, despite the capacity of these proteins to produce cGMP from GTP *in vitro*, our experiments did not reveal any increase in cGMP level in Arabidopsis plants overexpressing AtGC1 (J Hussain *et al.*, unpublished data). By contrast, we have produced Arabidopsis plants expressing the α-subunit and β-subunit of mammalian sGC that display an up to 40-fold increase in cGMP levels compared with wild-type plants (J Hussain *et al.*, unpublished). This has made a genetic tool available to study the impact of this second messenger in plants. The endogenous cGMP production systems, however, remain unclear.

Conclusions
We continue to gain new knowledge on NO-related signalling. However, the data available are still far from offering a comprehensive and consistent picture of NO function in plants. The lack of genetic tools substantially hinders research on NO production and functions. New findings, however, underline the importance of NO in plant cell physiology and the complexity of NO-related

signalling networks. Particularly, the impact of *S*-nitrosylation on protein function has been clearly demonstrated, plus different modes of NO-mediated signalling are gaining increasing attention. On the basis of the current status of research we can expect the functional relevance of other NO-mediated posttranslational modifications and/or second messengers to be demonstrated soon.

Acknowledgement

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