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# Regulating the regulator: nitric oxide control of post-translational modifications

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45
46 **Key words: Nitric oxide, phosphorylation, *S*-nitrosation, SUMOylation, S-nitrosylation,**
47 **persulfidation, reactive nitrogen species, reactive oxygen species**
48
49
50

**SUMMARY**

Nitric oxide (NO) is perfectly suited for duties as a redox signalling molecule. A key route for NO bioactivity occurs via protein *S*-nitrosation, the addition of a NO moiety to a protein cysteine (Cys) thiol (-SH) to form a *S*-nitrosothiol (SNO). This process is thought to underpin a myriad of cellular processes in plants linked to development, environmental responses and immune function. Here we collate emerging evidence showing that NO bioactivity regulates a growing number of diverse post-translational modifications (PTMs) including SUMOylation, phosphorylation, persulfidation and acetylation. We provide examples of how NO orchestrates these processes to mediate plant adaptation to a variety of cellular cues.

80 **Introduction**
81 More than two hundred reversible protein post-translational modifications (PTMs) have been
82 identified to date, massively expanding the proteome and by extension enabling a plethora of
83 protein functions (Minguez *et al.*, 2012), providing an escape from genetic incarceration.
84 Typically, PTMs target amino acid residues embedded within conserved motifs (Tompa *et al.*,
85 2014). In this context, redox signalling is rapidly emerging as a key regulator of plant protein
86 function associated with a myriad of plant processes. The small gaseous molecule, nitric oxide
87 (NO), is a central player in redox signal transmission, mediating its redox functions
88 predominantly through *S*-nitrosation / *S*-nitrosylation: the addition of a NO moiety to a cysteine
89 (Cys) sulfhydryl/thiol to form an *S*-nitrosothiol (SNO) (Lindermayr *et al.*, 2005; Besson-Bard
90 *et al.*, 2008b; Leterrier *et al.*, 2011; Yun *et al.*, 2016). This redox-based modification has been
91 shown to regulate development, environmental responses and plant immunity. The emerging
92 evidence suggests that NO orchestrates some of these processes through regulating the
93 deployment of diverse PTMs. Here, we highlight some of these recent developments.
94
95 **SUMOylation**
96
97 SUMOylation, the covalent attachment of the small ubiquitin-like modifier (SUMO) to target
98 proteins is emerging as a key modulator of eukaryotic immune function. In plants, a SUMO1/2-
99 dependent process has been proposed to control the deployment of host immunity (Lee *et al.*,
100 2008a; van den Burg *et al.*, 2010; Saleh *et al.*, 2015). Recently, a key role for *S*-nitrosation in
101 the control of SUMOylation has emerged (Skelly *et al.*, 2019). Following the pathogen
102 triggered nitrosative burst, increasing NO levels were shown to drive *S*-nitrosation of the
103 *Arabidopsis* SUMO E2 enzyme, SCE1, at Cys139. The SUMO-conjugating activities of both
104 SCE1 and its human homologue, UBC9, were both blunted by this PTM (Fig. 1a). Accordingly,
105 mutation of Cys139 resulted in accumulation of SUMO1/2 conjugates (Fig. 1b), disabled
106 immune responses and increased pathogen susceptibility (Skelly *et al.*, 2019). Collectively,
107 these findings establish that *S*-nitrosation of SCE1 at Cys139 enables NO bioactivity to
108 promote immune activation by relieving SUMO1/2-mediated suppression. This discovery is
109 important because it suggests a new paradigm for the regulation of SUMOylation. The global
110 control of this PTM is predominantly thought to occur at the level of each substrate via complex
111 local machineries (Bossis & Melchior, 2006). In contrast, these new findings uncover a novel,
112 parallel and complementary mechanism by establishing that total SUMO conjugation is
113 additionally regulated directly by SNO formation at SCE1 Cys139. Significantly, this Cys

114 residue is evolutionary conserved and specifically *S*-nitrosated in human UBC9, implying this
115 immune-related regulatory process might be conserved across phylogenetic kingdoms (Skelly
116 *et al.*, 2019). Thus, NO bioactivity conveyed through *S*-nitrosation is a key regulator of
117 SUMOylation, a ubiquitous eukaryotic PTM.
118
119 **Phosphorylation**
120
121 The emerging data suggests that NO is also a major regulator of phosphorylation-dependent
122 signalling cascades. NO accumulation can trigger the activation of protein kinases (PKs) as
123 well as the phosphorylation of numerous proteins related to diverse cellular processes (Besson-
124 Bard *et al.*, 2008a; Frederickson Matika & Loake, 2014; Del Castello *et al.*, 2019). NO-
125 dependent PKs include $Ca^{2+}$-dependent PKs (CDPKs), sucrose non-fermenting 1-related PKs
126 (SnRKs), mitogen-activated PKs (MAPKs) and phosphoinositide-dependent PKs (PDKs).
127 However, the mechanism(s) by which NO modulates the activity of these target PKs remains
128 unclear.
129
130 NO is thought to mediate the activation of MAPKs and CDPKs indirectly through the
131 mobilization of cytosolic free $Ca^{2+}$ (Besson-Bard *et al.*, 2008b). Yet, the subtle mechanisms
132 underlying this process also remain to be determined. Direct S-nitrosation has not been
133 confirmed for SnRKs (Wawer *et al.*, 2010), nor reported for MAPKs or CDPKs. However, the
134 activity of the tomato cell-death regulator PDK1 was found to be inhibited by S-nitrosation of
135 a critical catalytic Cys residue. Additionally, the activity of MAPKs may be modulated by
136 tyrosine nitration as suggested by preliminary experiments (Ling *et al.*, 2012). Indeed, MAPKs
137 become activated by MAPK kinases (MAPKKs) through the dual phosphorylation of a Thr-X-
138 Tyr motif in the activation loop. It is therefore tempting to speculate that nitration of the Tyr
139 residue within the activation loop could interfere with its phosphorylation by MAPKKs and,
140 consequently, negatively modulate MAPK activity.
141
142 Finally, NO might modulate phosphorylated PK and, more generally, phosphorylated proteins
143 through the redox regulation of protein phosphatases (PPs). This process is well established in
144 animals and affects major phosphatases, including tyrosine phosphatases (Nakamura & Lipton,
145 2019). In this context, either activation, inhibition or a protective effect of the PP against
146 oxidation-induced inactivation have been observed, depending on the specific PP. However, to

147 date, no NO-dependent PP have been characterized in plants. So, this would be an interesting
148 area for future exploration.
149
150 More generally, it is tempting to speculate that the post-translational modification of residues
151 by NO or NO-derived compounds could trigger steric hindrance altering the interaction with
152 and phosphorylation by upstream kinases. For instance, S-nitrosation of the phosphotransfer
153 protein AHP1, involved in cytokinin signalling, suppresses its phosphorylation, repressing
154 cytokinin signalling (Feng *et al.*, 2013). The reciprocity of this mechanism could be also
155 possible: phosphorylation of a given protein could also impact its subsequent S-nitrosation.
156

157 **Histone acetylation and methylation**

158
159 Chromatin structure in eukaryotic organisms is very dynamic and is altered during growth and
160 development and in response to environmentally stimuli. Modification of histone proteins
161 induces chromatin remodelling to control transcription, replication, recombination and repair
162 (Bannister & Kouzarides, 2011). Adjustment of histone acetylation or methylation, catalyzed
163 by histone acetyltransferases / histone deacetylases (HDAs) and methyltransferases /
164 demethylases, respectively, are integral to these processes (Servet *et al.*, 2010; Shen *et al.*,
165 2015). Recently, it has been demonstrated that NO affects histone acetylation by targeting and
166 inhibiting histone deacetylase (HDA)-complexes (Mengel *et al.*, 2017). Genome-wide NO-
167 dependent H3K9/14ac profiling in *Arabidopsis* seedlings identified NO-regulated histone
168 acetylation of genes integral to immunity, abiotic stress and chloroplast function, suggesting
169 that NO bioactivity might regulate gene expression by the modulation of chromatin structure
170 (Mengel *et al.*, 2017). A direct effect of NO on enzymes catalyzing DNA or histone
171 methylation/de-methylation in plants has not been reported. However, genes encoding these
172 enzymes are induced by NO or differentially expressed in plants with impaired NO
173 homeostasis (Shi *et al.*, 2014; Hussain *et al.*, 2016; Kovacs *et al.*, 2016). Moreover, NO
174 accumulation has been shown to induce global DNA hypomethylation, resulting in altered
175 expression of chromatin remodeling enzymes (Ou *et al.*, 2015). This implies an indirect effect
176 of NO on chromatin methylation mechanisms in plants. In aggregate, the emerging data
177 suggests NO bioactivity might play important roles in the nucleus, however, the molecular
178 details still require further investigation.
179
180

181 **Crosstalk between NO, ROS and $H_2S$**
182
183 Nitro-fatty acids are reactive signaling mediators that are formed when unsaturated fatty acids,
184 typically oleic or olenic acid, react with NO or reactive nitrogen species (RNS) (Kelley *et al.*,
185 2008; Corpas *et al.*, 2013). Recently, nitro-oleic acid has been found to activate NADPH
186 oxidase (RBOH) altering reactive oxygen species (ROS) production (Arruebarrena *et al.*, 2020)
187 which implies a novel signal link between NO- and ROS-based signalling. It is already well
188 established that the isoenzyme RBOHD is S-nitrosated at Cys890 inhibiting the activity of this
189 enzyme and thus curbing the pathogen-triggered oxidative burst to limit the extent of HR
190 associated cell death (Yun et al., 2011). Additionally, the main enzymatic source of
191 peroxisomal hydrogen peroxide ($H_2O_2$), glycolate oxidase, is also inactivated by S-nitrosation
192 (Ortega-Galisteo *et al.*, 2012) and possibly also nitration (Lozano-Juste *et al.*, 2011),
193 suggesting dual NO-dependent regulation. NO-based PTMs may also affect several ROS
194 scavenging enzymes and some of these, for example, ascorbate peroxidase (APX) and
195 superoxide dismutase (SOD), were found to be inversely regulated by S-nitrosation and
196 nitration (Yang *et al*., 2015; Kolbert & Feigl, 2017). Thus, NO-related PTMs may act as an on-
197 off switch for antioxidant enzyme activities.
198
199 In addition to NO, hydrogen sulphide ($H_2S$) and $H_2O_2$ are also recognized as redox signal
200 molecules in both animal and plant cells. They can also affect protein function through their
201 redox interactions with critical thiols (-SH) on side groups of Cys residues, leading to PTMs.
202 $H_2O_2$ causes oxidation of cysteinyl thiols to sulphenic acid, also identified as S-sulfenylation
203 (Huang *et al*., 2019), whilst $H_2S$ results in persufidation (Hancock, 2019; Corpas *et al.*, 2019b).
204 Surprisingly, many of the targets for these molecules are key enzymes involved in ROS
205 metabolism (Table 1).
206
207 In aggregate, the emerging evidence suggests that NO-related PTMs modulate enzymes
208 involved in both ROS production and scavenging, suggesting that NO tightly regulates ROS
209 homeostasis. Beyond direct protein modifications, NO may also compete for direct targets of
210 both ROS and $H_2S$-based PTMS, indicating the possibility of multi-level regulation.
211
212 **NO regulation of the N-end rule protein degradation pathway**
213

214 Transcriptional responses to reduced oxygen (hypoxia) are achieved by oxygen-dependent
215 degradation by the ubiquitin proteasome system (UPS) of transcription factors mediated
216 through the N-end rule (Gibbs *et al.*, 2016; Dissmeyer *et al.*, 2018). This pathway of targeted
217 proteolysis relates the stability of a protein to the nature of its N-terminus. The Arginine (Arg)
218 branch of the N-end rule results in the exposure of Cys at the N-terminus which can undergo
219 *S*-nitrosation or oxidation to sulphenic or sulphonic acid, triggering arginylation of the target
220 protein by arginyl-tRNA transferases (ATEs). These enzymes transfer Arg from Arg-tRNA to
221 the Nt alpha-amino group of the Nt residue leading to N-recognin-mediated ubiquitination and
222 subsequent degradation (Varshavsky, 2011).
223
224 Group VII ethylene response factors (ERFs) are important regulators of oxygen sensing as they
225 become substrates of the N-end rule pathway. Significantly, group VII ERFs are also degraded
226 in the presence of NO and oxygen and may thus serve as NO and oxygen sensors regulating
227 NO function in a number of developmental processes (Gibbs *et al.*, 2014). Thus, oxygen
228 sensing during hypoxia (reduced oxygen levels), occurring, for example, in flooded roots, also
229 requires low levels of NO in order to stabilise group VII ERFs, which orchestrate cellular
230 responses ameliorating the impact of hypoxia. Under hypoxia, as the oxygen level decreases
231 typically NO levels increase (Gupta *et al.*, 2005), presenting a problem. It has recently been
232 shown that ethylene can enhance ERFVII stability prior to hypoxia by increasing the NO-
233 scavenger Phytoglobin1 (Hartman *et al.*, 2019). This ethylene-mediated NO depletion and
234 consequent ERFVII accumulation might enable pre-adaptation of plants prior to hypoxia. In
235 aggregate, the emerging findings suggest that NO-dependent modification of sentinel proteins
236 embedded within the N-end rule protein degradation pathway may under some circumstances
237 enable NO perception, while depletion of this molecule by Phytoglobin1 supports pre-
238 adaptation to hypoxia.
239

240 **NO regulation of methylation linked to pre-mRNA splicing**

241

242 Recently, a novel mechanism of NO crosstalk with protein arginine methylation, a common
243 post-translational modification that regulates multiple biological processes (Fuhrmann *et al*.,
244 2015) has been identified in plant stress responses (Hu *et al.*, 2017). Arginine
245 methyltransferases (PRMTs), utilize S-adenosyl-L-methionine as a donor of the methyl group

246 transferred to target arginine residues. PRMTs play wide roles in the biology of the cell
247 including pre-mRNA splicing and mRNA translation (Blanc & Richard, 2017). Plant PRMTs
248 are known to control key developmental processes including growth, flowering, the circadian
249 cycle and also response to salinity (Ahmad & Cao, 2012). Among nine PRMT families,
250 PRMT5 is localized to both the nucleus and cytoplasm and is one of the most highly conserved
251 and broadly expressed genes in multicellular eukaryotes. Recently, stress-induced NO-
252 dependent S-nitrosation of *Arabidopsis* PRMT5 at Cys125 has been demonstrated, which
253 increases the methyltransferase activity of this enzyme (Hu *et al.*, 2017). Enhanced S-
254 nitrosation of PRMT5 in plants with loss-of-function mutations in S-nitrosoglutathione
255 (GSNO) reductase (GSNOR) (Feechan *et al.*, 2005; Lee *et al.*, 2008b; Chen *et al.*, 2009),
256 suggests that this enzyme is indirectly regulated by GSNOR activity, which controls global
257 levels of GSNO, a natural NO donor. Importantly, through its effect on PRMT5 activity, NO
258 modulates pre-mRNA splicing during plant stress. This might represent a novel post-
259 transcriptional mechanism by which NO diversifies the stress-induced proteome through
260 regulation of functional transcripts and formation of new splice variants mediated by S-
261 nitrosation of PRMT5 (Frungillo & Spoel, 2017). Whether S-nitrosation of other PRMT Cys
262 residues, Cys260 and Cys425 (Hu *et al.*, 2017), is biologically relevant, requires further
263 investigation, in addition to how S-nitrosation might potentially affect other PRMT5 functions
264 in plants, i.e. control of circadian rhythms (Hong *et al.*, 2010). Interestingly, rat PRMT1 is also
265 under redox control through reversible oxidations of Cys residues to sulfenic acid by $H_2O_2$,
266 resulting in concentration-dependent inhibition of methyltransferase activity (Begara-Morales
267 *et al.*, 2015). Thus, in the wider context of redox signalling, it is intriguing to speculate that
268 plant PRMTs might also be modulated by ROS.

269

270 **Conclusions**

271

272 It is now becoming apparent that a major route for NO bioactivity is through the manipulation
273 of key PTMs, for example, SUMOylation, phosphorylation, persulfidation and acetylation
274 (Fig. 2). By targeting key Cys residues, which function as regulatory redox switches, for
275 oxidative modification, principally through *S*-nitrosation, NO is able to modulate the functions
276 of these ubiquitous and fundamental PTMs, tailoring cellular responses to diverse challenges.
277 The identification and subsequent characterisation of these strategically evolved redox
278 switches will present exciting future opportunities to shape protein function towards

279 advantageous outcomes. For example, redox switches could be designed and implemented by
280 emerging gene editing strategies to potentially control a plethora of key biological processes
281 underpinning a variety of important agricultural traits. The ability of NO to regulate the
282 regulator may be at the heart of these new technologies.
283

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286

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288

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457 **Table and figure legends**

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459 **Table 1.** Representative examples of enzyme involved in ROS metabolism whose activities
460 are regulated by both NO and $H_2S$.

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462 **Figure 1. NO regulates SUMOylation through S-nitrosation of SUMO conjugating**
463 **enzyme**. (a) In the absence of nitric oxide (NO), SUMO (small ubiquitin-like modifier)
464 conjugating enzyme (SCE1) SUMOylates key substrates with SUMO1/2 contributing to the
465 repression of salicylic acid (SA)-dependent genes and by extension, the suppression of
466 immunity in the absence of pathogens.

467 (b) Pathogen recognition triggers a nitrosative burst leading to NO accumulation, which results
468 in the S-nitrosation of SCE1 at Cysteine (Cys)139. This redox based post-translational
469 modification inhibits SCE1 activity blocking SUMO 1/2 SUMOylation. Consequently, this
470 enables the expression of SA-dependent genes and the subsequent activation of plant
471 immunity.

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473 **Figure 2. NO regulates a series of diverse post-translational modifications / signalling**
474 **systems**. Integrative schematic representation of cross-talk between NO and various post-
475 translational modification / signalling systems. A major route for NO bioactivity is through
476 protein S-nitrosation (SNO) to form S-nitrosated proteins. NO-modified regulators modulate
477 downstream processes through diverse chemical modification systems. Chemical
478 modifications include ubiquitinylation (Ub), SUMOylation (S), phosphorylation (Pi),
479 methylation (Me), acetylation (Ac), S-sulfenylation (SOH) and persulfidation (SSH). Plant
480 functions regulated by these processes are indicated at the periphery of the diagram. ERFVII,
481 group VII ethylene response factor; ERFVII-CR, arginylated ERFVII; HAD, histone
482 deacetylase; PKs, protein kinases; PRMT5, protein arginine methyltransferase 5; RBOH,
483 Respiratory burst oxidase homolog (NADPH oxidase); SCE1, SUMO E2 enzyme; SUMO,
484 small ubiquitin-like modifier.

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