

The role of nitrite and nitric oxide under low oxygen conditions in plants

Kapuganti Jagadis Gupta^{1*}, Luis A.J. Mur², Aakanksha Wany¹, Aprajita Kumari¹, Alisdair R. Fernie³, R. George Ratcliffe^{4*}

¹National Institute of Plant Genome Research, Aruna Asaf Ali Marg, 110067, New Delhi, Delhi, India.

²Institute of Environmental and Rural Science, Aberystwyth University, Edward Llwyd Building, Aberystwyth SY23 3DA, UK

³Max-Planck-Institute of Molecular Plant Physiology, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

⁴Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

*To whom correspondence should be addressed

Email: jgk@nipgr.ac.in

Tel: +91-11-26735111

Twitter @DrJagadisNIPGR

ORCID Id: <http://orcid.org/0000-0002-7090-5097>

Email: george.ratcliffe@plants.ox.ac.uk

ORCID Id: 0000-0001-8394-157

Tel: +44 (0) 1865 275001

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27 **Summary**

28 Plant tissues, particularly roots, can be subjected to periods of hypoxia due to
29 environmental circumstances. Plants have developed various adaptations in
30 response to hypoxic stress and these have been extensively described. Less well-
31 appreciated is the body of evidence demonstrating that scavenging of nitric oxide
32 (NO) and the reduction of nitrate/nitrite regulate important mechanisms that
33 contribute to tolerance to hypoxia. Whilst ethylene controls hyponasty and
34 aerenchyma formation, NO production apparently regulates hypoxic ethylene
35 biosynthesis. In the hypoxic mitochondrion, cytochrome c oxidase, which is a major
36 source of NO, is also inhibited by NO, thereby reducing the respiratory rate and
37 enhancing local oxygen concentrations. Nitrite can maintain ATP generation under
38 hypoxia by coupling its reduction to the translocation of protons from the inner side of
39 mitochondria and generating an electrochemical gradient. This reaction can be
40 further coupled to a reaction whereby non-symbiotic haemoglobin oxidizes NO to
41 nitrate. In addition to these functions, nitrite has been reported to influence
42 mitochondrial structure and supercomplex formation, as well as playing a role in
43 oxygen sensing via the N-end rule pathway. These studies establish that nitrite and
44 NO perform multiple functions during plant hypoxia and suggest that further research
45 into the underlying mechanisms is warranted.

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50 **Keywords:** cytochrome c oxidase, hyponasty, hypoxia, mitochondria, nitric oxide,
51 nitrite.

52 Introduction

53 Molecular oxygen facilitates the efficient production of ATP in all aerobic eukaryotic
54 organisms by providing the terminal electron acceptor for the mitochondrial electron
55 transport chain. Oxygen deprivation, leading to a state of hypoxia (low oxygen) or
56 anoxia (no oxygen), compromises the process of oxidative phosphorylation. For
57 plants, this problem typically arises during flooding (Bailey-Serres *et al.*, 2012) as a
58 result of the 10⁴-fold reduction in gaseous diffusion in flood water (Armstrong, 1979).
59 Moreover, even under optimal growth conditions, certain dense tissues such as
60 seeds (Borisjuk *et al.*, 2007) and tubers (Geigenberger *et al.*, 2000) are hypoxic, with
61 O₂ concentrations in the range 1 - 50 µM. While the latter observations indicate that
62 plants can cope with low levels of O₂ during normal development, hypoxia inevitably
63 restricts the availability of oxygen for oxidative phosphorylation and increases the
64 importance of fermentation as a source of ATP (Ricard *et al.*, 1994). As a result,
65 plant metabolism must adapt to lower ATP production, including the induction of
66 energy-conserving pathways of sucrose degradation that lead to improved plant
67 performance under hypoxia (Geigenberger *et al.*, 2000; Bologa *et al.*, 2003).

68 As well as metabolic adaptations to hypoxia, plants have also developed anatomical
69 and morphological adaptations, including the formation of aerenchyma, aerial
70 adventitious roots, and leaf gas films (Bailey-Serres *et al.*, 2012). Responses to
71 hypoxia have been extensively studied in plants and many transcriptional, post-
72 translational and metabolic events that regulate these responses have been
73 identified (Geigenberger *et al.*, 2000; Licausi *et al.*, 2011; Narsai *et al.*, 2017; Fukao
74 *et al.*, 2019). One emerging theme is the involvement of reactive oxygen species
75 (ROS) and nitric oxide (NO) signalling under low oxygen in plants (Pucciariello and
76 Perata, 2017), and in this update the intention is to focus on the multiple roles of NO
77 and nitrite in the hypoxic response.

78 Hypoxic synthesis and turnover of NO

79 NO is a free radical signalling molecule that is produced by several oxidative and
80 reductive pathways (Gupta *et al.*, 2011; Astier *et al.*, 2017). The reductive pathways
81 are active under hypoxic conditions, with mitochondria playing a major role in NO
82 production through the action of cytochrome oxidase (COX) and other

83 deoxyhemeproteins (Figure 1). Isolated plant mitochondria typically produce NO at a
84 rate of 1-20 nmol.mg protein⁻¹.h⁻¹ within a few minutes of adding NADH and nitrite to
85 a hypoxic incubation medium, and the K_i for oxygen, which inhibits the process, is
86 0.05% (Gupta *et al.*, 2005). High levels of NO lead to cell death (Wang *et al.* 2013),
87 so if NO is to have other signalling functions under hypoxia it is necessary to have
88 mechanisms for preventing its excessive accumulation.

89 NO production is countered by NO degradation, with several haem proteins such as
90 flavohaemoglobin, haemoglobin (Hb), myoglobin and their associated reductases
91 fulfilling this role in animal cells (Gardner, 2005) and flavoglobin scavenging NO in
92 yeast (Liu *et al.*, 2000; Cassanova *et al.*, 2005). In plants, Class 1 phytooglobins (Pgb)
93 are efficient NO scavengers (Hebelstrup *et al.*, 2008) and their very high affinity for
94 oxygen ($K_m \sim 2$ nM) allows them to function under hypoxia (Figure 1).
95 Overexpression of the Pgb gene in barley decreased NO release under hypoxia,
96 while knockdown of the gene increased it, confirming that Pgb makes a significant
97 contribution to the regulation of NO levels (Cochrane *et al.*, 2017). The inhibition of
98 COX by NO (Brown and Cooper, 1994; Cleeter *et al.*, 1994) also facilitates the
99 operation of the oxygen-requiring Pgb-NO cycle under hypoxia by inhibiting
100 respiration at low oxygen concentrations. Thus under hypoxia oxygenated Pgb
101 converts NO to nitrate, and the resulting metphytooglobin is converted back to Pgb by
102 monodehydroascorbate reductase-mediated ascorbate reduction (Igamberdiev *et al.*,
103 2006; Gupta and Igamberdiev, 2011). The Pgb-NO cycle also regenerates NAD⁺ and
104 may be considered an alternative to the usual pathways of fermentation
105 (Igamberdiev and Hill, 2004), although like lactate fermentation, the Pgb-NO cycle is
106 acidifying (Libourel *et al.*, 2006) and thus a potential contributor to acidosis under
107 hypoxia.

108 S-nitrosogluthathione reductase (GSNOR) is another enzyme that contributes to the
109 regulation of NO levels in plants (Leterrier *et al.*, 2011). GSNOR converts the NO
110 derivative S-nitrosogluthathione (GSNO) to oxidised glutathione (GSSG) and
111 ammonia, and it was recently shown that the inhibitory NO-dependent S-nitrosation
112 of GSNOR1 (Frunzillo *et al.*, 2014) leads to degradation of the enzyme by selective
113 autophagy (Zhan *et al.*, 2018). Elimination of the S-nitrosation site in GSNOR
114 abolished the positive effect of NO on the hypoxic germination of Arabidopsis seeds,

115 indicating that the NO-dependent post-translational modification of GSNOR is a
116 physiologically relevant process that contributes to the hypoxic response (Zhan *et*
117 *al.*, 2018). Elevated GSNO was shown to increase the expression of both alcohol
118 dehydrogenase and pyruvate decarboxylase in germinating Arabidopsis seeds (Zhan
119 *et al.*, 2018) emphasising the importance of GSNOR regulation by NO under
120 hypoxia.

121 NDB-type dehydrogenases also play a role in NO degradation by forming superoxide
122 anions that convert NO to peroxynitrite (ONOO⁻) (de Oliveira *et al.*, 2008). This route
123 of NO degradation is stimulated by calcium, and abolished by superoxide dismutase
124 and complete anoxia. These observations indicate that NDB dehydrogenases
125 actively generate superoxide, and are involved in superoxide-dependent NO
126 degradation. NDB-type dehydrogenases were also found to be induced in transgenic
127 Arabidopsis plants with downregulated expression of GSNOR (Frunghillo *et al.*, 2013),
128 providing further correlative evidence for their role in NO homeostasis.

129 The net result of these biosynthetic and degradative pathways is a marked increase
130 in NO production in response to hypoxia. This increase is in turn increasingly
131 implicated in a range of adaptive responses to oxygen deprivation, including
132 hyponasty, aerenchyma formation, oxygen homeostasis, mitochondrial activity and
133 oxygen sensing

134 **Role of NO in hyponasty under hypoxia**

135 The hyponastic response is a highly effective escape strategy employed by most
136 plant species following submergence, shade or elevated ambient temperatures. It is
137 accompanied by a strong directional growth mediated by reversible turgor reactions
138 and changes in the osmotic state of the cells. It mainly depends on the unequal
139 growth rates of two anatomically different sides of the organ in question. Hyponasty
140 can be defined as a type of asymmetric growth, whereby abaxial tissue displays a
141 higher growth rate than the adaxial cells. It is a common feature in leaf blades and
142 petioles of many monocots and dicots (Polko *et al.*, 2011).

143 During flooding, ethylene plays a major role in the hyponastic response due to its
144 reduced diffusion in submerged tissues (Voeselek *et al.*, 1993). Vreeburg *et al.*
145 (2005) showed that ethylene-mediated hyponastic signaling is characterized by

146 acidification of the apoplast and a higher expression of expansin proteins, both of
147 which play important roles in modifying cell wall structure. Ultimately, ethylene
148 responsive factors (ERFs) mediate hyponasty, however, the function of these
149 proteins is gibberellin (GA) dependent (Polko *et al.*, 2011). Other hormones are also
150 implicated in the process, with auxins such as indole-3-acetic acid (IAA) positively
151 regulating stage-specific submergence-induced hyponasty, whilst abscisic acid
152 (ABA) acts as a negative regulator of this process (Cox *et al.*, 2006).

153 NO is also involved in the complex signalling network leading to hyponasty.
154 Arabidopsis seedlings produce increased ethylene and NO under hypoxia, and Pgb
155 gene expression (*AtGLB1*) correlated with hyponastic growth (Hebelstrup *et al.*,
156 2012). These observations led to the proposal that NO likely acts as a regulator of
157 hyponasty via induction of ethylene synthesis. Plants also experience shading and
158 reduced light levels during flooding, and since shading alone can induce an
159 ethylene-dependent hyponastic response (Pierik *et al.*, 2009) it is possible that
160 shade also has an impact on NO metabolism. It is known that light-dark dynamics
161 can influence both NO and nitrite levels (Planchet *et al.*, 2005), but the relevance of
162 this observation under more natural conditions remains to be evaluated.

163 **Role of NO in the formation of ethylene-induced aerenchyma**

164 Another adaptive response of plants to hypoxia is the formation of aerenchyma, the
165 gas-filled tissue that allows the exchange of gases between shoot and root under
166 conditions of flooding and waterlogging (Drew *et al.*, 2000). Schizogenous
167 aerenchyma is formed by a process of cell separation at the middle lamella during
168 cell development, whilst lysigenous aerenchyma is formed as a consequence of the
169 random death of cortical cells. It was previously shown that hypoxia is an inducer of
170 aerenchyma formation (Drew *et al.*, 2000), and that ethylene plays a role in cortical
171 cell death (Yamauchi *et al.* 2014). NO also plays a role in programmed cell death
172 (Delledonne *et al.*, 1998; Chen *et al.*, 2009; Wang *et al.*, 2013), and so the possibility
173 arises that NO could play a role in aerenchyma formation in hypoxic tissues given
174 that NO production takes place within a few minutes of the onset of hypoxia (Gupta
175 *et al.*, 2005).

176 Recently Wany *et al.* (2017) investigated whether ethylene-induced aerenchyma
177 formation in wheat roots required hypoxia-induced NO. Wheat roots produced NO
178 under hypoxia as expected, and scavenging of NO by 2-(4-carboxyphenyl)-4,4,5,5-
179 tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) led to a marked reduction in
180 aerenchyma formation following 24 or 48 hours of hypoxia. Interestingly, it was found
181 that hypoxically-induced NO was important for the induction of the genes encoding
182 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, both of
183 which are required for ethylene biosynthesis, as well as the ethylene-responsive
184 genes *ERF1* and *PDF13*. Cell death events such as increased electrolyte leakage,
185 increased cellulase activity, DNA fragmentation, and cytoplasmic streaming were all
186 inhibited under hypoxia in the presence of the NO scavenger, reinforcing the
187 conclusion that NO is essential for the development of ethylene-induced aerenchyma
188 (Wany *et al.*, 2017). Moreover, ethylene inhibitors and NO scavengers, either alone
189 or in combination, suppressed the genes involved in signal transduction leading to
190 aerenchyma development. These findings suggest that NO plays a role in
191 aerenchyma formation in wheat, acting either upstream of ethylene, or in parallel
192 with it. The involvement of NO in aerenchyma formation was also indicated by the
193 observation that the *respiratory burst oxidative homolog/NADPH oxidase*
194 (*RBOH/NOX*) gene, which is known to have a role in aerenchyma formation (Yun *et*
195 *al.*, 2011), is also induced by NO (Wany *et al.*, 2017). *RBOH/NOX* plays a role in
196 superoxide production, hence the induction of *RBOH/NOX* by NO, correlated with
197 the generation of ROS in wheat cortical cells. Since superoxide reacts with NO to
198 form peroxynitrite, a strong nitrating agent, increased tyrosine nitration was observed
199 during aerenchyma formation. It will be interesting to see whether these results can
200 be replicated under conditions more akin to natural flooding events, since ethylene is
201 known to accumulate to saturating levels rapidly and the possibility of induction by
202 NO has not been considered hitherto (Sasidharan *et al.*, 2018).

203 Plants are well equipped with detoxification systems to counter the deleterious
204 effects of ROS and NO. In particular, there are many regulatory mechanisms that
205 influence the levels of ROS and NO, thus protecting plants from severe damage
206 under stress conditions. However, during processes such as programmed cell death
207 and aerenchyma formation, plant cells need to maintain high ROS levels in the
208 tissues where limited cell death is advantageous. One strategy to achieve this is to

209 lower antioxidant gene expression (Liu *et al.*, 2018). Wany and Gupta (2018) found
210 an inverse correlation between antioxidant gene expression and increased ROS
211 following 24 h of hypoxia in wheat roots during aerenchyma formation. NO is known
212 to increase antioxidant gene expression during stress (Tossi *et al.*, 2011), but during
213 cell death it seems that suppression of the antioxidant mechanism is required. A
214 prominent example is the inhibition of glycine decarboxylase by S-nitrosation, which
215 alters cellular redox status and promotes cell death (Palmieri *et al.*, 2010). A survey
216 of S-nitrosylated proteins in cells undergoing aerenchyma formation could potentially
217 afford new insights into the role of NO in the promotion or limitation of cell
218 progression. Such insights could be based on transgenic manipulation of root NO
219 levels, for example by over-expression of Pgb, during aerenchyma formation,
220 although this approach might also affect oxygen homeostasis and root development
221 (Gupta *et al.*, 2014). An alternative approach might be to test the relationship
222 between the nitrogen status of the soil and aerenchyma formation, given that the
223 synthesis of NO ultimately depends on the availability of nitrate (Planchet *et al.*,
224 2005; Gupta *et al.*, 2013). Preliminary evidence suggests that nitrate nutrition, as
225 opposed to ammonium nutrition, favours aerenchyma formation and this needs
226 further investigation (Wany and Gupta, 2018).

227 While the recent evidence suggests that NO is essential for the development of
228 aerenchyma via cortical cell death under flooding stress (Wany *et al.*, 2017), it is
229 necessary to regulate the levels of NO in other root zones to avoid cell death in
230 tissues required for continued growth (Mira *et al.*, 2016a). The root apical meristem
231 (RAM) contains stem cells which are important for root growth and it has been
232 shown that transgenic suppression of the hypoxically-induced phytoglobins
233 ZmPgb1.1 or ZmPgb1.2. led to structural abnormalities in RAM (Mira *et al.*, 2016b).
234 Suppression of Pgb also enhanced expression of ethylene biosynthetic and
235 responsive genes, providing further support for the role of Pgb in regulating NO
236 levels under hypoxia. In contrast, overexpression of Pgb improved hypoxic root
237 growth by alleviating apical meristem cell death again emphasising the role of Pgb
238 as NO scavengers. These observations highlight the need for differential regulation
239 of Pgb expression in different root zones to ensure that hypoxically-induced NO can
240 promote cortical cell death and aerenchyma formation without damage to the stem
241 cells in the RAM. The mechanism by which this is achieved is not fully understood,

242 but it is relevant that exposure of the root apex to hypoxia has been shown to lead to
243 increased hypoxic acclimation of the entire root (Mugnai *et al.*, 2012), emphasising
244 the existence of systemic signalling pathways that coordinate cell-type specific
245 responses to hypoxia.

246 **Role of NO in oxygen homeostasis**

247 Oxygen homeostasis is important for maintaining an appropriate internal oxygen
248 level in tissues during normal development. This phenomenon is crucial when
249 environmental effects, such as flooding and waterlogging, reduce the oxygen supply
250 and drive the tissues towards anoxia. Recently it was shown that NO has a potential
251 role in oxygen homeostasis under normoxia via the regulation of respiration (Gupta
252 *et al.*, 2014). It is well known that NO inhibits respiration by inhibiting cytochrome c
253 oxidase in isolated mitochondria (Millar and Day, 1998). NO binds to the Fe²⁺-haem
254 group at the O₂-binding site of the binuclear centre Fe_{a3}Cu_B in COX (Cleeter *et al.*,
255 1994) and this provides the basis for an autoregulatory mechanism in which
256 increasing NO under hypoxia reduces oxygen consumption. The relevance of this for
257 oxygen homeostasis has been demonstrated in normoxic barley roots, where
258 overexpression of Pgb promoted NO scavenging, increased the respiration rate, and
259 decreased the internal oxygen level (Gupta *et al.*, 2014). Overexpression of Pbg also
260 affected the normoxic NO signalling pathways in barley (Cochrane *et al.*, 2017). The
261 physiological significance of this effect has been shown in both seeds (Borisjuk *et al.*,
262 2007) and isolated mitochondria (Benemar *et al.*, 2008), where it was shown that
263 nitrite reduction at complex III reversibly inhibited COX, and thus contributed to the
264 maintenance of a steady state level of oxygen in the mitochondria.

265 The role of NO in oxygen homeostasis is also important in seed germination. This
266 process is associated with the production of NO and a decrease in ABA via
267 regulation of *CYP707A2* transcription and (+)-abscisic acid 8'-hydroxylase (Liu *et al.*,
268 2009). Gibbs *et al.* (2014b) reported that both NO and oxygen availability promote
269 degradation of ERF VII transcription factors during the metabolically active state of
270 seed development, leading to down-regulation of AB15 in the endosperm and the
271 promotion of germination.

272 **Role of NO in mitochondrial activity under hypoxia**

273 Oxygen deprivation can have a marked effect on plant mitochondrial structure, and
274 the observed changes correlate to some extent with the ability of the plant to survive
275 periods of hypoxia or anoxia (Vartapetian *et al.*, 2003; Shingaki-Wells *et al.*, 2014).
276 Nitrate has been shown to have a protective effect on mitochondrial ultrastructure
277 under these conditions (Vartapetian *et al.*, 2003), but recent evidence suggests that
278 nitrite confers similar protection, and that the reduction of nitrite to NO under hypoxia
279 is important for the maintenance of some level of mitochondrial activity (Gupta *et al.*,
280 2017). Thus incubating hypoxic pea root mitochondria with 0.5 mM nitrite resulted in
281 increased NO production, improved mitochondrial integrity, improved energization of
282 the inner mitochondrial membrane, increased ATP synthesis, lower levels of reactive
283 oxygen species, and decreased lipid peroxidation. Nitrite also increased the activities
284 of complex I and the supercomplex I + III₂ under hypoxia. These observations
285 highlight the far-reaching effects of nitrite on the hypoxic mitochondrion (Gupta *et al.*,
286 2017).

287 The effect of nitrite on the activities of complex I and the supercomplex I + III₂ under
288 hypoxia (Gupta *et al.*, 2017) may well be important in promoting the reduction of
289 nitrite to NO through the maintenance of a fully functional electron transport chain. In
290 tobacco plants deficient in complex I, reduced electron flow in the mitochondrial ETC
291 led to lower NO production (Shah *et al.*, 2013); while supercomplex formation is
292 considered to increase the efficiency of electron transport (Cogliati *et al.*, 2016). The
293 fact that nitrite treatment under hypoxia increases the activities of complex I and the
294 supercomplex I + III₂ hints at a regulatory role for either nitrite or NO under these
295 conditions.

296 Interpretation of all the effects of nitrite on mitochondrial function under hypoxia is
297 complicated by the potential regulatory effects of the hypoxically-generated NO. For
298 example, COX, the major site for the production of hypoxically-generated NO, is
299 inhibited by NO (Cleeter *et al.*, 1994) and the interaction with NO can increase the
300 efficiency of oxidative phosphorylation (Clerc *et al.*, 2007). These factors are seen in
301 the nitrite-stimulated increase in ATP synthesis in hypoxic pea root mitochondria
302 (Gupta *et al.*, 2017), but at the same time it should be noted that NO scavenging by
303 mitochondria, or via cytosolic scavenging systems, is considerable, with tobacco root

304 mitochondria, for example, consuming 87% of the NO applied within two minutes
305 (Kumari *et al.*, 2016).

306 Hypoxically-produced NO may also alter mitochondrial activity through changes in
307 AOX activity. There are established links between AOX and NO, with AOX
308 preventing excess NO production in tobacco leaves (Cvetkovska and Vanlerberghe,
309 2012), and NO inducing AOX under hypoxia (Gupta *et al.*, 2012) and phosphate
310 deficiency (Royo *et al.*, 2015). Recently, Vishwakarma *et al.* (2018) demonstrated
311 that AOX prevents excess production of NO, peroxynitrite and tyrosine nitration
312 under normoxia. However, it was also found that AOX can generate NO under
313 hypoxia, and that the NO was oxidized via the Pgb-NO cycle (Vishwakarma *et al.*,
314 2018). Inhibiting AOX under hypoxia led to lower ATP, but AOX overexpressing lines
315 produced more ATP. These data suggested that AOX-mediated NO production plays
316 a role in the production of ATP under hypoxia by supporting proton translocation
317 through complex I. Interestingly, in contrast to normoxia, it was shown that excess
318 NO generated under hypoxia did not lead to the formation of peroxynitrite and
319 tyrosine nitration. Thus, the link between AOX and NO differs between normoxia and
320 hypoxia.

321 The phenomena of nitrite-driven ATP synthesis and mitochondrial protection are
322 important in specialized structures such as nodules (Berger *et al.*, 2018). *Medicago*
323 *truncatula* nodules have been shown to increase their production of NO when
324 submitted to hypoxic conditions (Horchani *et al.*, 2011). The nodule oxygen
325 concentration in the cytosol of the host plant cells is typically in the range 5-60 nM
326 due to diffusion resistance and the respiration of the bacteroids. Under these
327 conditions AOX does not contribute to respiration due to its higher K_m , but COX with
328 a K_m value of 50 nM (Millar *et al.*, 1995) is expected to be functional. However,
329 whether the amount of oxygen supplied by leghemoglobin (K_m for oxygen binding 2
330 nM) to the mitochondria is sufficient for energy production remains an open question
331 (Horchani *et al.*, 2011).

332 As both Lb and Pgb have the capacity to oxidize NO or nitrate, this may allow the
333 Pgb-NO cycle to operate generating a limited amount of ATP to sustain nodule
334 development and function. The study by Horchani *et al.* (2011) provided evidence
335 that in N_2 -fixing nodules of *M. truncatula*, the energy status of the nodules depends

336 largely on NR functioning under normoxic, or hypoxic conditions. Thus, the Pgb-NO
337 cycle can increase energy efficiency in specialised hypoxic organs such as nodules.

338 **Role of NO in oxygen sensing under hypoxia**

339 The precision and specificity of the control of molecular and physiological responses
340 to low oxygen stress (Geigenberger *et al.*, 2000) suggests that plants possess
341 sensitive oxygen-sensing mechanisms to initiate hypoxic responses. Direct and
342 indirect sensors help in the development of these responses. Direct sensors are
343 specific proteins such as transcriptional activators or repressors that sense oxygen.
344 Prominent examples are the transcription factor hypoxia-inducible factor-1-alpha
345 (HIF-1 α) in animals (Brahimi-Horn *et al.*, 2005), and a heme-binding protein kinase,
346 FixL in rhizobial bacteria (Akimoto *et al.*, 2003). In contrast, indirect sensing relies on
347 hypoxically-induced changes in such properties as calcium levels, energy status and
348 redox status to trigger regulatory mechanisms (Bailey-Serres and Chang, 2005).

349 Recently, the direct oxygen sensing mechanism known as the N-end pathway has
350 been shown to initiate the response of plants to hypoxia (Licausi *et al.*, 2011; Gibbs
351 *et al.*, 2011). This is an evolutionarily conserved pathway for protein degradation
352 whereby the stability of a protein is determined by the identity of its N-terminal
353 residues (Varshavsky, 2011; Gibbs *et al.* 2014a; 2015). Specifically, the presence of
354 N-degrons and N-terminal destabilizing residues determines whether a protein will
355 be degraded by the proteasome (Graciet *et al.*, 2009; Holman *et al.*, 2009). Group
356 VII ethylene response factors (ERFs), which have been shown to be important
357 regulators of the response to low oxygen (Hinz *et al.*, 2010; Licausi *et al.*, 2010,
358 2011; Gibbs *et al.*, 2011, 2015), are oxygen-dependent substrates of the N-end rule
359 pathway: these proteins are destabilised in the presence of oxygen and NO and
360 stabilised in their absence. More recently, the polycomb repressive complex 2
361 component VRN2 has also been identified as an O₂/NO regulated target of the N-
362 end rule pathway, suggesting a potential link between low oxygen/NO and the
363 epigenetic control of gene expression (Gibbs *et al.*, 2018).

364 The N-terminal (Nt) MCGGAIL/L domain of the ERFVII transcription factors is the
365 target for the N-end rule degradation pathway. Under aerobic conditions, methionine
366 amino peptidase cleaves the Nt-Met to reveal an Nt-Cys, which is then oxidized by

367 plant cysteine oxidases (PCOs) to produce Nt-Cys sulfinic acid. The oxidized Nt-Cys is
368 arginylated by an arginyl transferase, creating a substrate for an E3 ligase which
369 leads to polyubiquitination and proteasomal degradation of the ERFVII protein
370 (Figure 2). The role of the PCOs in controlling hypoxic gene expression has been
371 confirmed by genetic studies (Weits *et al.*, 2014) and the molecular mechanism of
372 the oxidation and arginylation steps has been characterized *in vitro* (White *et al.*,
373 2017).

374 The extent to which NO influences the oxygen sensing system in plants is unclear.
375 In animal systems, it has been shown that the *in vivo* oxidation of Nt-Cys before
376 arginylation requires NO (Hu *et al.*, 2005) and the hydrolysis of S-nitrosothiols can
377 produce sulfenic acids as the first step in the formation of sulfinic acids (Reddie and
378 Carroll, 2008). In agreement with this, there is some evidence that NO, as well as
379 oxygen, may be required for the degradation of ERFVII. Gibbs *et al.* (2014b)
380 showed that ERFVII are destabilized in the presence of NO, and stabilized in their
381 absence. In particular, the stability of two ERFVII, RAP2.3 and HRE2, was
382 increased in Arabidopsis seedlings in the presence of NO scavengers, and also in
383 the nitrate reductase-deficient *nia1nia2* mutant, which has greatly reduced levels of
384 NO. More recently, Vicente *et al.* (2017) demonstrated that down-regulation of nitrate
385 reductase in Arabidopsis led to lower NO levels and increased stability of ERFVII,
386 an effect which was implicated in abiotic stress sensing under normoxia. While it
387 remains the case that low oxygen is the primary determinant of ERFVII stability, the
388 sensitivity of ERFVII to NO raises the possibility that the change in NO levels under
389 hypoxia could modulate the oxygen sensing role of the ERFVII.

390

391 Another consideration, which needs further investigation, is the potential impact of
392 ROS generation under low oxygen (Vergara *et al.*, 2012). In principle, this could
393 oxidise the Cys residues of ERFVII and thus work against their stabilization under
394 hypoxia. At the same time, hypoxia-induced NO generation by mitochondria could
395 play a role in removing excess ROS to ensure the stability of ERFVII under hypoxia,
396 but the extent to which this is important has yet to be established and could well
397 differ between nitrate- and ammonium-grown plants (Wany *et al.*, 2019). More
398 generally the sensitivity of mitochondrial activity to oxygen availability might suggest

399 that mitochondrially-derived NO and ROS could contribute to retrograde signalling in
400 the hypoxic state, but this also remains to be established.

401

402 **Concluding remarks**

403 It is now clear that nitrite and NO play important and multi-faceted roles in the
404 response of plants to hypoxia. These include classical morphological changes such
405 as hyponasty, the protection of mitochondrial structure, ATP generation and ROS
406 scavenging. However, the role of hypoxically produced NO in various other plant
407 anatomical adaptive responses to flooding, such as aerial lateral root formation, stem
408 elongation, suberin and lignin accumulation needs more investigation. Cross talk
409 between NO and growth hormones such as ethylene, auxin, and ABA during these
410 adaptive responses to hypoxia would also merit further investigation. Another area of
411 interest is the potential effect of the nitrogen supply on hypoxic tolerance given that
412 both nitrite and NO are derived from nitrate. For example, lines with differing nitrogen
413 use efficiency could improve the availability of nitrate and hence affect tolerance to
414 hypoxia. Other targets for further analysis of the role of nitrite and NO under hypoxia
415 include germinating seeds, which experience varying degrees of hypoxia during
416 development, and bulky tissues, where the availability of nitrite might be key to the
417 maintenance of metabolism through its protective effect on the mitochondria. Finally,
418 soil microbes can produce high levels of NO during hypoxia which raises the
419 important question of whether plants are able to distinguish soil-derived NO from that
420 produced endogenously.

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428 .

429 **Figure Legends:**

430 **Figure 1:** Operation of the ~~(p~~Phytoglobin/nitric oxide (Pgb/NO) cycle under hypoxic
431 conditions. The reduction of nitrite to NO occurs at complex III (*bc₁*), complex IV
432 (cytochrome c oxidase), (COX) and the alternative oxidase (AOX). The NO diffuses
433 to the cytosol where it is converted to nitrate (NO₃⁻) by the hypoxia-induced class 1
434 phytoglobin (PgbO₂), which leads to the formation of metphytoglobin (MetPgb),
435 which is ~~then~~ reduced by metphytoglobin reductase (MetPgbR). Nitrate is then
436 reduced by nitrate reductase (NR) to nitrite, which is imported into mitochondria by
437 either a putative nitrite transporter (NT) or passive diffusion. NAD(P)H generated in
438 the cytosol is oxidized by the externally facing calcium-dependent mitochondrial
439 dehydrogenases (ND), or, after import into the mitochondria as reducing equivalents,
440 by complex I. ~~Q, ubiquinone; and Cyt c, cytochrome c (Cyt c); In the figure IMM, is~~
441 inner mitochondrial membrane; and IMS, is inner mitochondrial intermembrane space
442 space. UQ is ubiquinone pool; UQ, ubiquinone.

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444 **Figure 2:** A. The role of NO and oxygen in the control of group VII- ethylene
445 response factors (ERF) stability under normoxia. Methionine amino peptidase
446 (MetAP) cleaves the Nt-Met to reveal an Nt-Cys, which is then oxidized by plant
447 cysteine oxidases (PCOs) to produce Nt-Cys sulfinic acid (shaded yellow symbol). NO
448 potentially facilitates this oxidation. The oxidized Nt-Cys is arginylated by an arginyl-
449 tRNA protein transferase (ATE1/2), creating a substrate for the N-end rule E3 ligase
450 (PRT6) which leads to polyubiquitination and proteasomal degradation of ~~the~~ERFVII.
451 It has been shown that NO is also required for degradation of ERFVIIIs, and that
452 stress induced reductions in endogenous NO levels can lead to enhanced stability of
453 ERFVIIIs even under normoxia (Vicente *et al.*, 2017), thus identifying NO as a signal
454 controlling the accumulation of these proteins in response to stress.

455 B. Under hypoxia, high levels of NO are produced via increased activity of nitrate
456 reductase and mitochondrial nitrite reduction. However, because ERFVII degradation
457 also requires oxygen, these increased levels of NO are not able to degrade ERFVIIIs.
458 The NO generated under hypoxia can help in plant survival via aerenchyma
459 formation, protection of mitochondria, hyponasty, regulation of reactive oxygen

460 [species](#) (ROS), the Pgb-NO cycle, and the limited production of ATP. Some of these
461 adaptive responses are mediated by NO, and some are mediated by stabilization of
462 ERFVIs or both.

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