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### Learning To Breathe

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1                   **Learning to breathe: developmental phase transitions in oxygen status**

2  
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20  
21 **Key words.** Oxygen tension, ROS/ RNS, N-end rule proteolysis, Redox, Development,  
22 Differentiation

25 **Abstract (100 words)**

26 Plants are developmentally disposed to considerable changes in oxygen availability, yet our  
27 understanding of the importance of hypoxia is almost entirely limited to stress biology.  
28 Differential patterns of the abundance of oxygen, nitric oxide (NO) and reactive oxygen  
29 species (ROS), and redox potential occur in organs and meristems, and examples are  
30 emerging in the literature of mechanistic relationships of these to development. Here, we  
31 describe the convergence of these cues in meristematic and reproductive tissues, and discuss  
32 the evidence for regulated hypoxic niches, within which oxygen-, ROS-, NO- and redox-  
33 dependent signalling curate developmental transitions in plants.

## 34 **The nature of developmental hypoxia and metabolism**

35 Molecular oxygen is essential for efficient production of ATP through oxidative  
36 phosphorylation, serving as the terminal electron acceptor for the mitochondrial electron  
37 transport chain. Oxygen and reduction-oxidation (redox) biochemistry pervades cellular  
38 metabolism and signalling in plants, as in all aerobic life forms. Yet even in optimal growth  
39 conditions, various higher plant tissues such as seeds, tubers and buds reside in a state of low  
40 oxygen status [1-3]. Internal oxygen concentrations in such organs range from 1 to 50  $\mu\text{M}$ ,  
41 compared with an air-saturated concentration of *ca.* 260  $\mu\text{M}$  (*cf.* 21 kPa  $\text{O}_2$  partial pressure at  
42 standard atmosphere and pressure), and this is reflected in the spatial patterns of metabolic  
43 control, energy status and gene expression, particularly anaerobic glycolysis [1, 4, 5]. Despite  
44 the fundamental metabolic importance of oxygen, our knowledge of oxygen as a curator of  
45 growth, differentiation and reproduction in plants is only beginning to emerge. Increasing  
46 evidence points to the presence of regulated hypoxic niches during plant development.

47

48 Until recently, oxygen signalling in plants was defined by the consequences of oxygen  
49 metabolism, such as changes in energy status, production of reactive oxygen and nitrogen  
50 species (ROS, RNS), or the accompanying dynamics of the redox network. By contrast, the  
51 basic mammalian hypoxia (low-oxygen) signalling and transduction pathways were defined  
52 over 20 years ago [6]. It is now widely accepted that local tissue hypoxia plays a central role  
53 in mammalian embryogenesis [7] and constitutes a key regulatory feature of adult stem cell  
54 niches [8]. The prevailing model applied to mammalian tissues and stem cells is that low  
55 oxygen provides a protective environment, conducive to quiescence, low ROS, and a  
56 relatively reduced redox state, all of which promote genome stability [9]. Regulated ROS  
57 synthesis in mammalian stem cells is central to the transition to proliferation and  
58 differentiation.

59

60 Parallel research programs in 2011 provided a step change in our understanding of oxygen  
61 signalling in plants, defining an oxygen-dependent N-end rule of proteolysis (discussed  
62 further below) [10, 11; see **Box 1**]. Nevertheless, research on N-end rule signalling in plants  
63 to date has been largely undertaken in the context of stress, particularly waterlogging and  
64 flooding [12, 13]. Thus the current state of the art of developmental oxygen signalling in  
65 plants is constrained by the ability to relate stress signalling via the N-end rule to the  
66 developmental understanding via redox and energy signalling (see Outstanding Questions).  
67 We discuss the roles of hypoxia in plant development and the nexus between oxygen, ROS,  
68 nitric oxide (NO) and redox cues. We consider the differential patterns of these cues within  
69 organs and meristems, and the evidence suggesting that hypoxic niches are central to  
70 meristem function and differentiation in plants. In this context we highlight particular  
71 examples from the recent literature on seeds, seedlings and anthers that illustrate functional  
72 roles for oxygen status in developmental transitions, in partnership with ROS, RNS and redox  
73 status.

74

#### 75 **Gradients in oxygen, ROS, NO and redox potential in organs and meristems**

76 During evolution, the formation of niches and gradients in oxygen and redox status were  
77 important forces shaping multicellular life [14]. Cell identity within multicellular organisms  
78 became a critical factor in determining sensitivity to cellular cues including ROS and RNS  
79 such as NO. The presence of pockets of cells with a low oxygen status is a prominent feature  
80 of many developing, reproductive and quiescent plant tissues (**Fig. 1**). These areas can form  
81 when oxygen diffusion fails to keep pace with aerobic respiration or when the oxygen supply  
82 is occluded by cell wall modifications, such as the deposition of callose. Within hypoxic

83 niches, ROS appear to function alongside NO, phytooglobins and plant hormones to regulate  
84 developmental events such as growth, flowering and wood formation [15].

85

86 Hypoxia may be defined as a condition in which the cellular availability of oxygen is  
87 insufficient to support oxidative phosphorylation at full capacity. Glycolytic activity is  
88 increased to supply ATP in cells experiencing low oxygen availability and fermentation is  
89 induced to recycle pyridine nucleotides, in a response known as the Pasteur Effect. Hypoxia  
90 is characterised by specific transcriptional programs that are induced and maintained in  
91 response to perception of reduced oxygen availability [12, 13]. Oxygen-limited metabolism  
92 triggers the expression of specific set of hypoxia-related genes, such as those encoding  
93 sucrose synthase and alcohol dehydrogenase, and leads to remobilisation of carbohydrates to  
94 meet the increased glycolytic demand. These conserved transcriptional and metabolic  
95 responses are seen across life forms [16]. Survival and release from hypoxia is  
96 developmentally programmed to enable effective phase transition from quiescence to active  
97 metabolism. By contrast, survival through stress-induced hypoxia thereafter is much less  
98 certain. For example, an auxin-induced oxidative state defines the root stem cell niche  
99 without risk of programmed cell death [17], while hypoxia resulting from abiotic stress sees a  
100 persistent increase in ROS production that is frequently associated with impaired cell  
101 function and death [18]. The parallel with mammalian stem cells is tempting to consider [19],  
102 where glycolysis predominates and ROS homeostasis defines the balance of quiescence,  
103 proliferation and differentiation. Mitochondria in mammalian stem cells appear to fulfil  
104 different roles in maintaining cell integrity [20]. It is interesting to consider how such  
105 findings may translate to plant development (see Outstanding Questions).

106

107 The patterns shown in **Box 2**, particularly tissue oxygen status, may be organ- and species-  
108 specific. In the root, oxygen profiles may be influenced by the cortical gas space, surface area  
109 to volume ratio, depth below the soil surface and experimental system, such as embedding  
110 within versus above agar, and the presence of light. The presence of surface water films and  
111 root hairs will likely reduce radial oxygen diffusion into the root, reinforcing the polar  
112 oxygen gradient. Species differences will also be significant [21]; for example, maize roots  
113 have significant amounts of cortical gas space, whereas pea and *Arabidopsis* roots have little.  
114 Nevertheless, current data point to a convergence of polar and radial oxygen gradients to a  
115 hypoxic condition in the cells of quiescent centre (QC) and stem cells of roots. Mugnai *et al.*  
116 [22] demonstrated considerable induction of alcohol dehydrogenase and pyruvate  
117 decarboxylase activities in whole *Arabidopsis* roots only when the meristem was exposed to  
118 hypoxia, and that respiratory demand was greatest at the proximal region of the meristem. It  
119 should be noted however, that there is no obvious signature of hypoxia in the transcripts  
120 enriched in the QC of *Arabidopsis* roots, with exception that one of the hypoxia-inducible  
121 Group VII ethylene response factors (ERFVIIIs), discussed below was enriched in the QC  
122 [23]. Patterns in ROS and  $\cdot\text{NO}$  in the root apical meristem appear to be highly specific to  
123 developmental state, as is also the case in a typical seed (**Fig. 2**). The known functions of  
124 ROS and  $\cdot\text{NO}$  in roots and seeds are discussed in subsequent sections. Meanwhile, the state in  
125 the shoot apical meristem is less clear, confounded by technical challenges identifying the  
126 meristem proper and combining this with available resolution of technologies (see  
127 Outstanding Questions) [24]. Hence, while current evidence suggest gradients in tissue  
128 oxygen status converge to a hypoxic state in the vital tissues such as the QC and stem cells of  
129 roots, more mechanistic evidence is required from other organs and in a range of conditions.  
130 Nevertheless, these features point to a potentially important role for oxygen-, ROS- and  $\cdot\text{NO}$ -  
131 dependent signalling during plant development.

132

### 133 **The N-end rule of proteolysis in a developmental context**

134 Responses to hypoxia in animals are mediated by the hypoxia-inducible factor (HIF1 $\alpha$ )  
135 transcription factor; oxygen-dependent modification of HIF1 $\alpha$  by prolyl hydroxylases  
136 initiates its degradation via the proteasome, whilst decreased oxygen levels lead to its  
137 accumulation and a concomitant induction of the hypoxic transcriptome [6]. A functionally  
138 analogous, but qualitatively different, protein degradation-based mechanism for sensing  
139 oxygen also exists in plants, where ERFVIIIs act as ‘master regulators’ of hypoxia responsive  
140 gene expression [13, 25]. Under normoxic conditions, ERFVIIIs are degraded in an oxygen-  
141 and NO-dependent manner via the N-end rule pathway of targeted proteolysis (see **Box 1**),  
142 whilst a small stable subpopulation localises to the plasma membrane [10, 11, 26, 27]. Under  
143 hypoxia, ERFVIIIs localise to nucleus, where newly synthesised ERFVIIIs also accumulate, to  
144 activate gene expression. These nuclear ERFVIIIs are then rapidly destroyed upon re-  
145 oxygenation, which quickly dampens the hypoxic transcriptional response, providing the cell  
146 with a sensitive mechanism for directly adjusting transcription relative to oxygen availability.  
147 ERFVIIIs regulate the expression of over half of the ‘core 49’ hypoxia induced genes that are  
148 activated and preferentially translated across cell types when oxygen is depleted [16, 26, 28,  
149 29]. These include genes associated with glycolysis and ethanol fermentation, various  
150 transcription factors, and genes coding for proteins of unknown function that likely contribute  
151 to cellular homeostasis under oxygen deficiency.

152

153 There is mounting evidence that oxygen- and NO-dependent ERFVII regulation by the N-  
154 end rule pathway is important for coordinating responses during developmentally-imposed  
155 hypoxia and the transition to oxygen-replete conditions, in addition to stress. The examples of  
156 seed dormancy, germination and photomorphogenesis are described in subsequent sections



157 and **Figure 2**. In addition, it has previously been shown that loss of function mutants for  
158 several enzymatic components for N-end rule pathway display aberrant phenotypes relating  
159 to leaf and shoot development and the timing of leaf senescence [30, 31]. This finding could  
160 implicate roles for oxygen and NO in the control of development and senescence processes.  
161 However, the oxygen/NO-dependent branch of the N-end rule pathway only provides these  
162 enzymes with a subset of their substrates, and the relevant targets need to be identified in  
163 order to establish a firm link to regulation by oxygen and/or NO levels. It is also interesting  
164 to consider that under hypoxia, several genes are induced that attenuate ERFVII activity,  
165 providing feedback mechanisms to fine tune the response [32, 33]. This includes the plant  
166 cysteine oxidases, which are critical for oxygen-dependent ERFVII destruction (**Box 1**), and  
167 the trihelix transcription factor HYPOXIA RESPONSIVE ATTENUATOR 1 (HRA1), which  
168 negatively regulates the activity of the ERFVII RAP2.12 through direct protein-protein  
169 binding [33]. Giuntoli *et al.* [33] demonstrated through histochemical staining that HRA1 is  
170 expressed in young growing leaves of the rosette and meristematic regions under non-stressed  
171 conditions, and the authors speculated that it may play a role in counterbalancing the extent  
172 of the hypoxic transcriptional response in developmental contexts where oxygen availability  
173 is reduced. Further analyses are required to confirm such a role for HRA1.

174

## 175 **Sources and roles for reactive oxygen and nitrogen species in development**

176 Cellular energy metabolism employs reductive anabolic reactions to store energy, and  
177 oxidative catabolic reactions to release energy. While oxygenic photosynthesis and  
178 respiration operate four-electron exchange mechanisms between oxygen and water, without  
179 release of partially reduced intermediates, many enzymes catalyse partial oxygen reduction  
180 producing superoxide ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ). Consequently, ROS levels are  
181 intrinsically linked to oxygen availability, and therefore constitute important components of

182 oxygen and hypoxia signalling. These and other redox signals have become integrated in  
183 every aspect of plant biology and are crucial regulators of pre- and post-translational gene  
184 expression, cell division and expansion, and cell defence, morphology, and fate [34]. Within  
185 this context, cellular antioxidants not only determine the extent of ROS accumulation in the  
186 different compartments of the plant cell but they also can act as signal transmitters. The  
187 intracellular compartments that are major ROS producers show substantial plasticity in  
188 organelle shape, with extensions such as stromules, peroxules and matrixules playing crucial  
189 roles in inter-organelle communication [35]. For example, ROS accumulation triggers direct  
190 stromule-nucleus communication that facilitates direct transfer of oxidants and proteins [36].  
191 The sensitivity of different tissues and organs to ROS accumulation, and to oxidation, is  
192 regulated to a large extent by the abundance and intracellular distribution of low molecular  
193 weight antioxidants such as glutathione and ascorbate [34]. Antioxidant enzymes and redox-  
194 sensitive proteins also calibrate tissue sensitivity to redox signalling appropriate to the  
195 conditions.

196

197 The major sites of intracellular ROS production in plants are the chloroplasts, mitochondria  
198 and peroxisomes [37]. Direct electron transfer to oxygen occurs during photosynthesis and  
199 respiration leading to  $O_2^-$  production [38].  $O_2^-$  is converted to  $H_2O_2$  by superoxide dismutase  
200 (SOD) [39]. In peroxisomes, ROS are produced by a number of different oxidases including  
201 glycolate oxidase and xanthine oxidase and through  $\beta$ -oxidation of fatty acids. In addition,  
202 ROS are produced in the apoplast by different enzymes including: the plasma membrane-  
203 bound NADPH-oxidases (RBOH); class III secretory plant peroxidases; amine oxidases such  
204 as polyamines oxidases (PAO); germin-like oxalate oxidases, and; quinone reductases [40].  
205 Of these, RBOH-mediated ROS production has been linked to signal transduction pathways  
206 that mediate plant cell growth and development [41]. For example, tip growth in pollen tubes

207 and root hairs is regulated by ROS-mediated cell wall loosening and stiffening [42]. PAO has  
208 also been associated with pollen tube extension by promoting  $\text{Ca}^{2+}$  influx followed by RBOH  
209 activation [42, 43]. Apoplastic  $\text{H}_2\text{O}_2$  also regulates cell division and expansion during leaf  
210 development, where a MYB-like transcription factor KUA1 represses peroxidase expression  
211 during cell expansion [44].

212

213 ROS production and redox homeostasis are considered to play key roles in root [45] and  
214 shoot [24] meristem development. A mechanistic relationship between ROS localisation and  
215 cell identity in the root was determined by Tsukagoshi *et al.* (see **Box 2**) [46]. There, the  
216 UPBEAT1 transcription factor, expressed in the extension and differentiation zones,  
217 represses peroxidase activity, moderating the balance of  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  in the differentiation  
218 and meristem zones, independent of the auxin gradient [46].  $\text{NO}$  also appears to be required  
219 to maintain root meristem cell identity, as dependent on the auxin gradient [47], and two  
220 recent studies pointed to the importance of mitochondrial ROS homeostasis in cell-specific  
221 signalling, determining the identity of the root distal stem cells [48], and the maintenance of  
222 the shoot apical meristem [49]. These conclusions are in line with the general consensus that  
223 redox regulation is involved in multiple processes related to self-renewal and differentiation.  
224 Nevertheless, caution must be used when interpreting some of these approaches [50]. There  
225 also remains debate on the oxidation state and ROS synthesis in the cells of the root QC. In  
226 maize, current data show the QC cells are maintained in a highly oxidised state, and where  
227 oxidation of the core redox buffers ascorbate and glutathione is functionally related to the  
228 polar auxin gradient, interacting with hormonal and transcriptional controls [17, 51-53]. More  
229 recently in *Arabidopsis*, the redox potential in the medial plane of the root was shown to be  
230 most reduced in the area of QC and stem cells [54]. These data are in line with the  
231 enrichment of genes encoding enzymes leading to or requiring glutathione in the QC of

232 *Arabidopsis* [23]. There is a need to resolve the basis of these differences, whether genetic,  
233 physiological or due to the experimental system. Moreover, no signal study to date has  
234 investigated each of the oxygen-dependent cues in one system.

235

### 236 **Mitochondrial plasticity in relation to oxygen availability**

237 It is implicit that considerable adjustment of mitochondrial metabolism is required to ensure  
238 that energy metabolism is sustained under hypoxia. Respiratory electron transport generates  
239 ROS as an inevitable consequence of oxidative phosphorylation, NO through participation in  
240 Hb-NO cyclic respiration (discussed further below), and regenerates pyridine nucleotides to  
241 enable continued cytosolic and organelle functions. The importance of mitochondrial ROS  
242 homeostasis in the identity and fate of the root- [48] and shoot- apical meristem [49] was  
243 introduced above. Accumulating evidence suggests that the availability of oxygen and the  
244 requirements of oxidative phosphorylation can alter the composition, numbers and structure  
245 of mitochondria. Mitochondrial biogenesis and interdependence with chloroplast during seed  
246 germination is illustrated in **Figure 2**. Rice seedlings germinated under anaerobic conditions  
247 initially develop a normal mitochondrial structure, but later the mitochondria showed  
248 degraded cristae with vesicles [55]. Even within 48h of anoxia, mitochondria had reduced  
249 protein levels of tricarboxylic acid cycle components and cytochrome-containing complexes  
250 of the respiratory chain, resulting in repressed respiratory functionality [56]. In other tissues,  
251 oxygen deprivation can lead to the generation of giant mitochondria, as in *Arabidopsis* leaves  
252 [57] and tobacco cells [58]. However, the response of mitochondrial structure to hypoxia may  
253 depend on whether cells are in a quiescent or metabolically active state, or whether the  
254 experimental context is stress-acclimation or developmental (see Outstanding Questions).

255

256 The glutathione redox potentials of root mitochondria have been estimated using ro-GFP.  
257 Such measurements showed that root mitochondria were substantially more reduced (*ca.* -360  
258 mV) than the surrounding cytosol (*ca.* -320 mV) of the same tissues [59, 60]. Moreover,  
259 mitochondria were found to be much more able to buffer changes in redox state than the  
260 cytosol [59]. This is consistent with the observation that mitochondria accumulate more  
261 glutathione than any other compartment of plant cells [61]. In contrast to the other cell types  
262 in the maize root, the QC cells were found to have little or no glutathione, as discussed above  
263 [53]. The mitochondria within this oxidising environment look structurally similar to those in  
264 the cells surrounding the QC [62]. However, compared to mitochondria in the adjacent,  
265 rapidly dividing cells, the QC mitochondria have much lower tricarboxylic acid cycle enzyme  
266 activities, with a much reduced capacity to generate ATP and NADH [62]. A similar situation  
267 has been described for potato tuber mitochondria, which reside in very low oxygen  
268 environments [63]. Nevertheless, it is not known whether quiescent cells of meristems,  
269 including shoot meristems, are specifically hypoxic, and hence whether these features are a  
270 consequence of low oxygen or low metabolic requirements for quiescence (see Outstanding  
271 Questions).

272

### 273 **Phytoglobins and the haemoglobin-nitric oxide cycle under hypoxia**

274 Phytoglobins are also important in the survival in hypoxic conditions that arise during  
275 development, and are central to cell fate decisions during embryogenesis, as well as during  
276 seed germination, xylem formation, and lateral and adventitious root development [64, 65].  
277 *HAEMOGLOBIN (Hb)1* is a core hypoxia-responsive gene, which is induced by hypoxia  
278 alongside NO accumulation [66]. Heterologous expression of *Vitreoscilla Hb* in several plant  
279 species led to improved energy status and enhanced growth [67]. The overexpression of *Hb1*

280 in *Arabidopsis* led to enhanced shoot development [68], and to earlier bolting [69], while  
281 silencing of *Hb1* and *Hb2* proved to be lethal [70].

282

283 The Hb-NO cycle has been suggested to relieve mitochondrial transport chain inhibition by  
284 NO under hypoxia [71]. In the process of Hb-NO cyclic respiration or nitrate-NO  
285 respiration, nitrate is first reduced to nitrite by nitrate reductase. Nitrite is then transported  
286 from the cytosol to the mitochondria, where it is reduced to NO, via the mitochondrial  
287 electron transport chain. NO then diffuses from the mitochondrial matrix to the cytosol,  
288 where it is oxidised by Hb [66]. To complete the cycle MetHb is regenerated by a MetHb  
289 reductase [72]. In this way, NO accumulation in developmentally hypoxic tissues may be  
290 controlled by the non-symbiotic Hbs in an NADH-coupled reaction, while facilitating  
291 respiration and ATP production.

292

### 293 **Hypoxia and re-oxygenation during plant development**

294 Regulated hypoxia and re-oxygenation have recently been shown to play a critical role in  
295 non-stress-associated plant development. Here we highlight particular examples as case  
296 studies: seed germination and bud burst, photomorphogenesis and anther development, to  
297 illustrate roles for oxygen availability, and related ROS/ RNS levels, in the control of these  
298 processes.

299

### 300 **Seed germination and bud burst**

301 Seeds and latent buds are spatially complex organs, which transit from quiescence to  
302 extension and synthetic growth over a period of hours to days [2, 73]. Prior to germination or  
303 bud burst, the organ is hypoxic,  $<50 \mu\text{M} [\text{O}_2]$ , heterotrophic and desiccated, often  $<0.3 \text{ g}$   
304  $\text{H}_2\text{O.g DW}^{-1}$  (*cf.* up to  $260 \mu\text{M} [\text{O}_2]$ ,  $3\text{-}12 \text{ g H}_2\text{O.g DW}^{-1}$ ) [74]. Imbibition sees a rapid relief

305 from desiccation, and gradual relaxation of hypoxia, accompanied by spatiotemporal bursts  
306 of ROS and NO (**Fig. 2**). The biogenesis of mitochondria and chloroplast appears to be  
307 partially interdependent, with chloroplast metabolism being initially photoheterotrophic,  
308 relying on mitochondria to re-oxidise pyridine nucleotides and to sustain the cytosolic and  
309 plastid adenylate pools (described in **Fig. 2**) [4]. In the seed, hydration [75] and local  
310 oxidation [76, 77] occurs initially within the embryonic axis and peripheral tissues, with  
311 synthesis of ROS, principally  $O_2^-$  and  $H_2O_2$ , driven by apoplastic peroxidases and NADPH  
312 oxidases. During imbibition, ROS appear to function in cell wall elasticity ( $O_2^-$ ,  $\cdot OH$ ) and  
313 cross-linking ( $H_2O_2$ ), to enable extension growth of the radicle. Genetic analysis of the  
314 NADPH/ NADP-thioredoxin reductase/ thioredoxin system also indicates a role for redox  
315 regulation of hydrolytic proteins during imbibition and radicle extension, a feature that has  
316 been exploited in preventing precocious germination [78]. The rise in internal oxygen is  
317 augmented by the restriction of oxidative phosphorylation by partially-nitrite-dependent NO  
318 synthesis, which may inhibit complex IV, enabling photosynthetic oxygen to accumulate  
319 [79]. NO synthesis is prominent in the peripheral tissues of the seed during imbibition [80],  
320 associated with an increase in S-nitrosothiols in the embryo [81]. In the bud, hydration  
321 appears to be facilitated by  $O_2^-$ -mediated development of protoxylem [2], and degradation of  
322 callose occlusions of the plasmodesmata [81]. However, no spatial resolution of RNS in the  
323 bursting bud is yet known.

324

325 Mechanistic relationships between oxygen- and RNS-dependent signalling and germination  
326 have recently emerged, notably the role of NO in attenuating abscisic acid (ABA)-dependent  
327 repression of germination. The ERFVII transcription factors are positive regulators of the  
328 *ABA INSENSITIVE 5 (ABI5)* transcription factor, which acts downstream of ABA to repress  
329 germination [27]. The enhanced degradation of ERFVIIs during germination, as NO and

330 oxygen levels rise, attenuates the action of ABI5. Oxygen and NO appear both to be required  
331 for the destabilisation of the ERFVIIIs by the N-end rule pathway [27], while NO/ RNS  
332 appear to function to further attenuate ABI5 signalling without direct dependence on oxygen  
333 via two further mechanisms. Firstly, NO promotes the degradation of ABI5 during  
334 germination by the S-nitrosylation of cysteine-153 [82]. Secondly, tyrosine nitration acts  
335 upstream of ABI5 by inactivating the PYR/PYL/RCAR receptor [83], leading to the  
336 dephosphorylation of the SUCROSE NONFERMENTING-RELATED KINASE 2 (SnRK2),  
337 and thus preventing the activity of this positive regulator of ABI5. Hence by several modes,  
338 RNS-dependent modifications enable germination by attenuating ABA-dependent repression.  
339 At present, the only direct link to oxygen signalling is via the proteolysis of ERFVIIIs,  
340 however further dissection of these interactions are required.

341

#### 342 **Anther development**

343 Recent studies have shown that reproductive cell differentiation from pluripotent precursor  
344 cells is controlled by hypoxia in developing maize anthers. In contrast to animals, which  
345 sequester germ line cells during embryogenesis, the somatic-to-germinal switch in plants is  
346 regulated post-embryonically in response to endogenous and environmental cues. Maize  
347 anthers develop in tightly encased tassels that undergo short-term transient hypoxia (*ca.* 1.2-  
348 1.4 kPa  $pO_2$ , 15-30  $\mu M$   $[O_2]$ ) due to diffusion limitation and constraint by non-  
349 photosynthetic, rapidly growing leaves with a high metabolic demand [84]. Reduced oxygen  
350 availability in the anther lobe triggers the activity of the glutaredoxin MALE STERILE  
351 CONVERTED ANTHER 1 (MSCA1) in the central multipotent somatic cells, specifying  
352 them as germ initial (archesporial) cells that then enlarge and secrete MULTIPLE  
353 ARCHESPORIAL CELLS 1 (MAC1) protein, which represses proliferation and directs the  
354 development of surrounding supportive tissues [84, 85]. Analysis of microdissected



355 archesporial cells revealed gene expression patterns biased towards reduced ROS  
356 accumulation, enhanced reductive capacity, and alternative metabolism, indicating that these  
357 cells bypass the electron transport chain to limit potentially harmful ROS production and  
358 accommodate hypoxia [86]. Intriguingly, artificial manipulation of redox status in developing  
359 anthers (using hypoxia or hyperoxia treatments) revealed that every cell has the capacity to  
360 develop as a germ cell, suggesting that the natural hypoxic gradient that forms during the  
361 early development of this tissue is required for normal spatiotemporal reproductive cell  
362 differentiation [84]. Genetic studies in other species also highlight ROS management as an  
363 important component of fertility in plants. For example, the *Arabidopsis* glutaredoxin ROXY  
364 regulates floral organ and germline development [87], whilst mutants in the rice glutaredoxin  
365 MICROSPORELESS1 are male sterile similarly to maize *mcsal* mutants [88]. Thus, redox  
366 status and hypoxia may play a conserved role in the regulation of meiotic fate acquisition.

367

### 368 **Photomorphogenesis**

369 Following germination, newly emerged seedlings growing in the dark adopt a  
370 skotomorphogenic developmental program, characterised by a rapidly elongating hypocotyl,  
371 yellow folded cotyledons and an apical hook [89]. Once exposed to light,  
372 photomorphogenesis is induced, where cotyledons expand, hypocotyl growth ceases and  
373 mature chloroplasts develop. This growth transition coincides with the initiation of  
374 photosynthesis and a congruent production of ROS, which is potentially damaging to the  
375 plant. Long-term growth in the dark exacerbates photo-oxidative damage upon light  
376 perception, due to accumulation of the chlorophyll precursor protochlorophyllide [90].  
377 Recent work has shown that environmental hypoxia (which frequently occurs in soils) acts as  
378 a positive developmental cue for facilitating seedling survival during de-etiolation,  
379 particularly following extended darkness [91]. Under low oxygen conditions, stable ERFVIIIs

380 repress several photomorphogenic traits, restrict chlorophyll biosynthesis and limit  
381 protochlorophyllide abundance, which increases the capacity for seedling survival through  
382 limiting ROS production upon exposure to light. Accordingly, it was shown that *Arabidopsis*  
383 seedlings grown under hypoxia survived much longer periods of skotomorphogenesis than  
384 those grown in normoxia [91]. Following emergence, seedlings are typically exposed to  
385 atmospheric oxygen levels, and endogenous NO production also increases [92], which  
386 collectively would induce ERFVII destabilisation to relieve their repressive function and  
387 facilitate the light-induced transition to photomorphogenesis. Thus, hypoxia facilitates  
388 seedling survival by coordinating photomorphogenesis.

389

### 390 **Concluding statements**

391 Recent insights from root apical meristems, seeds, seedlings and anthers point to a  
392 mechanistic function for hypoxic niches and re-oxygenation events during plant  
393 development, where the roles of ROS, NO and redox-signalling become paramount in  
394 determining the balance of quiescence, proliferation and differentiation. Our summary of  
395 these concepts is presented in the diagram in **Figure 1B**. Importantly, it is clear that these  
396 cues rarely act in isolation. The combination of more deliberate attention with the use of more  
397 sensitive cellular technologies will improve our understanding of how these cues cooperate to  
398 effect developmental programming, and at the interface with environmental perception.

399

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407

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628

629

630 **Text boxes and Figure legends**

631

632 **Figure 1. Demonstrated and hypothetical gradients in tissue oxygen and redox status in**  
633 **plant developmental phase transitions. A.** Axillary bud burst and anther meiosis are  
634 developmentally augmented by oxygenation and hypoxia respectively [2, 84]. Cell identity  
635 and fate, and organ polarity of the root apical meristem (RAM) are governed by differential  
636 patterns in ROS, NO and gradients in oxygen status and redox potential (see **Box 2**). We  
637 hypothesise these cues are functionally relevant in the shoot apical meristem (SAM).  
638 Climacteric-type fruit ripen with a rapid burst of respiration, resulting in hypoxia-driven  
639 transcription [93]. Germination and the skoto-photomorphogenic transition are detailed in  
640 **Figure 2. B.** Accumulating evidence has highlighted the key functions of ROS and NO in  
641 defining the balance of plant cell proliferation and differentiation. In addition, recent  
642 evidence suggests hypoxia plays an important role in the maintenance of quiescence in plants,  
643 as it does in animals, by constraining oxidative metabolism and stabilising transcription  
644 factors [9, 12, 25, 74]. In the accompanying model we illustrate how tissue oxygen status  
645 might influence the balance between quiescence, proliferation and differentiation via  
646 regulated stabilisation/ destabilisation of N-end rule transcription factors, and influencing the  
647 cellular redox poise, and specifically through the differential generation of ROS species and  
648 NO. We consider that mitochondria and plasma membrane-bound NADPH-oxidases  
649 (RBOH), together with peroxidases (POX) are particularly important in regulating specific  
650 ROS expression and the cellular redox poise in this context. Rights for photographic images  
651 were purchased from [www.shutterstock.com](http://www.shutterstock.com).

652

653 **Figure 2. Typical spatiotemporal profiles of internal oxygen [O<sub>2</sub>], ROS and NO during**  
654 **seed imbibition and germination, and biogenesis of plastids and mitochondria during**



655 **imbibition through to de-etiolation.** Quiescent seeds are hypoxic, and plastids and  
656 mitochondria are prototypical, with poorly developed inner membranes [94-96]. During  
657 imbibition, hypoxia is gradually relieved, while ROS play a role in radicle extension, NO  
658 plays a role in activating hydrolytic activities in the endosperm. Plastids differentiate to  
659 etioplast, characterised by a prolamellar body (PLB) and prothylakoid membranes [Pth; 96].  
660 Mitochondria rapidly develop inner membranes (IMM) and cristae, protein import capacity  
661 and subsequently a functional electron transport chain [ETC; 94, 95]. Upon exposure to light,  
662 plastids have primordial thylakoid membranes (Th) and grana (Gr), and functional  
663 photosynthetic apparatus, which is co-dependent on mitochondria [photoheterotrophic; 4].  
664 Here, chloroplast provide oxygen and reducing power (NAD(P)H), which augments oxidative  
665 phosphorylation in the mitochondria via external NAD(P)H dehydrogenase (Ext NDH), ETC  
666 and ATP synthase (ATPase), which enables recycling of NAD(P)H and P<sub>i</sub> for continued  
667 photosynthesis [4, 97]. Nitrate-dependent NO serves to partially inhibit oxidative  
668 phosphorylation, augmented the increase in internal [O<sub>2</sub>] [4, 79]. The progressive switch from  
669 Ext NDH to the tricarboxylic acid cycle (TCA) is hypothetical. Absence of arrows between  
670 fully functional chloroplasts and mitochondria does not imply absence of relationships.

671

### 672 **Box 1. The N-end rule pathway**

673 The eukaryotic N-end rule pathway of proteolysis is a highly conserved branch of the  
674 ubiquitin proteasome system that targets proteins for degradation based on their N-terminus  
675 [27, 98]. Substrates of the pathway undergo a number of regulated N-terminal processing  
676 events to produce an 'N-degron' prior to ubiquitination and destruction. There are two known  
677 divisions of the pathway: the Ac/N-end rule targets proteins that have been N-terminally  
678 acetylated, whilst the Arg/N-end rule degrades proteins bearing specific unmodified (but  
679 post-transcriptionally exposed) hydrophobic or basic N-terminal amino acids [27, 99].

680 Primary residues of the Arg/N-end rule are directly recognised by specific E3 ligases (Arg/N-  
681 recognins), whereas secondary and tertiary residues (including Nt-Cys) must first undergo  
682 chemical modification followed by N-terminal arginylation before they are turned over. The  
683 Cysteine-branch of the Arg/N-end rule regulates oxygen and NO perception and  
684 transduction, through controlling the stability of proteins initiating with the residues Met-Cys  
685 [10, 11, 27, 100]. In mammals this includes several RGS proteins, which monitor oxygen  
686 availability to coordinate angiogenesis [100]. In plants, the group VII ERF transcription  
687 factors (ERFVIIs) – of which there are five in *Arabidopsis* - have a Met-Cys- N-terminus,  
688 embedded in a longer consensus sequence [13]. ERFVIIs are processed via the N-end rule  
689 pathway as such (**Fig. I**):

- 690 (i) Cytoplasmic methionine amino peptidases (MetAPs) cleave Nt-Met.  
691 (ii) Exposed tertiary Nt-Cys is oxidised to Cys-sulfenic or Cys-sulfonic acid in an oxygen-  
692 and NO-dependent manner. In plants this oxidation is catalysed by plant cysteine oxidases  
693 (PCOs), which use oxygen as a co-substrate [32]; functionally homologous enzymes in the  
694 animal Arg/N-end rule are yet to be identified.  
695 (iii) Oxidised Nt-Cys functions as a secondary residue of the pathway and likely targeted by  
696 Arginyl t-RNA transferase (ATE), which conjugates an arginine molecule to produce Nt-Arg-  
697 Cys.  
698 (iv) Nt-Arg, a primary destabilising residue, can be recognised by the Arg/N-recognin  
699 PROTEOLYSIS6 (PRT6), which leads to degradation by the 26S proteasome.

700 It is through this regulated, condition-dependent control of their stability that the ERFVIIs  
701 function as homeostatic sensors of oxygen and NO availability [10, 11, 27].

702

703 (FIGURE IN BOX)

704 **Figure I.** Schematic diagram of the major steps in the oxygen/  $\cdot$ NO branch of the N-end rule  
705 pathway of targeted proteolysis, as described in accompanying text.

706

707 **Box 2. Differential localisation of ROS and  $\cdot$ NO in root tissues with respect to oxygen**  
708 **and redox status.**

709 It is worthwhile considering the tissue patterning of the various oxygen-related cues in  
710 meristematic tissues. The root apical meristem is a convenient developmental model, for its  
711 relative polar and radial simplicity [51]. Even more-so in the context of oxygen and ROS  
712 metabolism, due to the lack of light. Oxygen enters the root by inward radial diffusion from  
713 the rhizosphere or cortical gas space diffusion from shoot system [101]. Armstrong and  
714 colleagues [101] measured and modelled polar and radial patterns of oxygen concentration in  
715 maize roots. In **Figure II**, two stylised profiles are shown, representing the modelled (upper)  
716 and measured (lower) transect through the proximal meristem [101]. Assuming these are  
717 reflective of the range, we see the steep radial gradient towards a minimum of <10% air-  
718 saturated  $[O_2]$ , *i.e.* <25  $\mu$ M  $[O_2]$  or 2 kPa  $O_2$  partial pressure in the vascular tissue. Although  
719 not shown here, data from Armstrong *et al.*, [101] clearly demonstrate a strong polar gradient  
720 also, whereby more proximal tissues are more oxygenated.

721 Studies of ROS and  $\cdot$ NO localisation have demonstrated rather discrete and differential  
722 patterns. Hydrogen peroxide ( $H_2O_2$ ) is concentrated towards the extension and differentiation  
723 zones, particularly the epidermis and vascular tissues, as well as the columella and lateral root  
724 cap [46, 102]. By contrast, superoxide ( $O_2^{\cdot-}$ ) is predominantly localised to the vascular and  
725 dermal tissues of the proximal meristem and elongation zone [46, 102]. Although not shown  
726 here, both  $H_2O_2$  and  $O_2^{\cdot-}$  were previously found to be more concentrated in the quiescent  
727 centre cells (QC) than the proximal meristem [53]. Meanwhile,  $\cdot$ NO localisation is  
728 concentrated towards the cortical and endodermal stem cells [47]. A recent study of redox

729 status demonstrated a relatively reduced cellular environment in the proximal meristem and  
730 columella, including the QC cells [54]. Although only polar data were presented [54], authors  
731 indicated there was no evidence of a radial gradient.

732 We consider variables affecting these findings, such as genetic and experimental conditions  
733 in the main text. To date, no single study has examined these data in one system.  
734 Nevertheless, taken together these data illustrate the importance of both polar and radial  
735 gradients in oxygen status and of tissue-specific localisation of ROS and NO, and potentially  
736 redox in the root apical meristem.

737

738 (FIGURE IN BOX)

739 **Figure II.** Differential tissue distributions of oxygen, ROS, NO and redox potential in a  
740 stylised root. Two alternative profiles of an oxygen transect through the proximal meristem  
741 (dashed line) are presented; the upper (yellow) line is the modelled profile, the lower (orange)  
742 is the measured profile, both interpreted from [101]. The redox profile through a longitudinal  
743 plane is interpreted from [54], H<sub>2</sub>O<sub>2</sub> (purple) and O<sub>2</sub><sup>-</sup> (blue) localisation from [46, 102] and  
744 NO localisation (green) from [47].

745