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Foyer, CH and Noctor, G (2016) Stress-triggered redox signalling: What's in pROspect?
Plant, Cell and Environment, 39 (5). pp. 951-964. ISSN: 0140-7791

<https://doi.org/10.1111/pce.12621>

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EDITORS' FORWARD LOOK

Stress-triggered redox signalling: What's in pROspect?

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Statistics

Abstract:198

Main text excluding references and figure legends:8023

Number of tables: 2

Number of figures: 6

1 **Abstract**

2

3 Reactive oxygen species (ROS) have a profound influence on almost every aspect of plant biology.
4 Here, we emphasize the fundamental, intimate relationships between light-driven reductant
5 formation, ROS, and oxidative stress, together with compartment-specific differences in redox
6 buffering and the perspectives for their analysis. Calculations of approximate H₂O₂ concentrations in
7 the peroxisomes are provided, and based on the likely values in other locations such as chloroplasts,
8 we conclude that much of the H₂O₂ detected in conventional *in vitro* assays is likely to be extracellular.
9 Within the context of scant information on ROS perception mechanisms, we consider current
10 knowledge, including possible parallels with emerging information on oxygen sensing. Although ROS
11 can sometimes be signals for cell death, we consider that an equally important role is to transmit
12 information from metabolism to allow appropriate cellular responses to developmental and
13 environmental changes. Our discussion speculates on novel sensing mechanisms by which this could
14 happen and how ROS could be counted by the cell, possibly as a means of monitoring metabolic flux.
15 Throughout, we place emphasis on the positive effects of ROS, predicting that in the coming decades
16 they will increasingly be defined as hallmarks of viability within a changing and challenging
17 environment.

18

19

20

21

22 **Introduction**

23

24 A simple perusal of the ISI scientific database reveals that more than 50 000 papers have been
25 published on oxidative stress in plants in the last five years. This bears testimony to the ongoing and
26 ever-growing interest in the multiple roles of this phenomenon in plant biology. While this and the
27 older literature include key advances that have led to a better understanding of certain aspects of
28 the field, there has been little clarification of other areas such that relatively few unifying concepts
29 emerge. Inherent difficulties arise from the nature of reactive oxygen species (ROS). Reactive equals
30 short-lived, meaning that the action of ROS is likely to be highly localized in many compartments.
31 However, the lifetime of ROS varies between different compartments, because of differences in
32 redox buffering, ie, the capacity of antioxidative systems to remove ROS. A set of discrete locations
33 defines specificity in redox signalling in different compartments of the plant cell. Independent control
34 and buffering permit redox-sensitive signal transduction to occur in locations such as the thylakoid
35 lumen and the apoplast/cell wall, and across the membranes that separate these compartments
36 from the highly redox-buffered spaces such as the stroma and cytosol. This concept underpins the
37 interpretation of data on how plants respond to stress. For example, a hormone-induced H₂O₂ burst
38 on the plasma membrane will not have immediate access to the same signalling routes as
39 H₂O₂ produced in the thylakoid membrane. Moreover, the lifetime is likely to be completely different.
40 While many researchers accept this notion, it is rarely considered in data analysis, because of the
41 absence of technologies that can explore compartment specificity. This forward look takes a back-to-
42 basics look at some of the fundamental concepts underlying redox relationships, explores current
43 concepts of compartmentalization, and presents our own personal perspective of future areas of
44 exploration in ROS-related redox research in plants.

45

46 **Context: understanding redox hierarchies and relationships between different redox couples**

47

48 Given that plant cells may produce copious amounts of ROS even in optimal conditions, more than
49 99% of amounts produced inside the cell are likely to be metabolized through dedicated antioxidant
50 systems. In the case of H₂O₂, the most stable ROS, increased production either accelerates
51 dismutation by catalase or causes increased engagement of metabolic pathways that produce
52 reductant to support peroxidases. Thus, unlike some other key signalling factors that are not
53 biochemically transformed (eg, calcium ions), ROS are directly integrated into metabolism, and the
54 associated redox reactions may cause profound changes in metabolite profiles (Noctor *et al.* 2015).
55 Understanding the operation of the key redox couples that primarily interact with ROS is
56 fundamental to any analysis of relevant signalling pathways in a given situation.

57

58 The chloroplast is a major sensor of environmental change and a driver of plant metabolism through
59 photosynthesis and other biosynthetic pathways. The electron transport chain in this organelle
60 transduces light energy to produce highly reducing compounds (ferredoxin, NADPH) that drive
61 metabolism as well as the reduction of ascorbate and glutathione. The same highly reducing
62 properties of the electron transport chain allow reduction of oxygen to superoxide and, thereafter,
63 production of H₂O₂. They also drive the reduction of various stromal thioredoxins, which either
64 contribute to antioxidant metabolism alongside ascorbate and glutathione or function as redox
65 regulators that activate metabolic enzymes or chloroplast gene expression (Buchanan & Balmer
66 2005; Dietz & Pfannschmidt 2011). Recent evidence suggests that thioredoxin-dependent
67 peroxiredoxins and thylakoid-bound ascorbate peroxidases have overlapping antioxidant functions in
68 the chloroplast (Awad *et al.* 2015).

69

70 A redox couple is composed of oxidized and reduced forms of a component that can be
71 interconverted by exchanging electrons with a second redox couple. Three examples of redox
72 couples are Cu²⁺/Cu⁺, plastoquinone/plastoquinol, and NAD⁺/NADH. For any given redox couple, its
73 midpoint potential is an intrinsic property (at standard conditions of pressure, temperature and pH)
74 that allows its relative potential reducing/oxidizing power to be defined. From it, one can calculate
75 the redox state of any compound at any given assumed actual redox potential. Table 1 shows the
76 redox states of ferredoxin, thioredoxin, glutathione, and NAD(P) at a theoretical equilibrium
77 potential of -320 mV, which is the midpoint potential of NADP(H). We emphasize that this is a
78 theoretical value. It is close to values often cited for the “cellular redox potential” in animal cells
79 (Schafer & Buettner 2001), but redox potentials in the soluble phase of plant cells may be more
80 variable, particularly in the chloroplast. Further, as we discuss below, the accuracy or usefulness of
81 the concept of a single cellular redox potential is not established.

82

83 From the most reducing to the least powerful, the approximate order in chloroplasts is ferredoxin >
84 thioredoxin/NADP(H) > glutathione > ascorbate, with a difference in the midpoint potentials of
85 ferredoxin and ascorbate of almost 500 mV (Table 1). This means that if the compounds had similar
86 *redox states* in planta (say, they were all 50% reduced), their *actual* redox potentials would be very
87 different and, except for glutathione, equal to their midpoint potentials. Such a situation favours net
88 redox flux, from more reducing compounds to less reducing ones. Inversely, if a single redox
89 potential prevails so that the components are all present at the same value in the cell, they must
90 have very different redox states (Table 1). In such a situation, the compounds are in thermodynamic
91 equilibrium and the net electron flux is zero.

92

93 At -320 mV, NADP(H) would be 50% reduced. Given its lower midpoint potential, ferredoxin would be
94 less than 5% reduced at -320 mV, whereas the less reducing antioxidant pools would have GSH:GSSG
95 and ascorbate:dehydroascorbate ratios of greater than 10^5 and 10^{12} , respectively (Table 1; Figure 1a).
96 These values may of course be overestimated if ascorbate and glutathione do not have an actual
97 redox potential of -320 mV in the stroma, as discussed below. Nevertheless, it is interesting to note
98 that measurements with redox-sensitive green fluorescent proteins (roGFPs), which monitor the
99 glutathione redox potential, give values that are close to -320 mV in many compartments
100 (Schwarzländer *et al.* 2008).

101

102 What are the implications for redox function and for the measured redox states of these components
103 in the chloroplast stroma? In the light, the photosynthetic electron transport chain drives electrons
104 into these pools of stromal redox carriers, ie, light promotes more reducing states (Figure 1b,
105 "PUSH"). The consequent increase in the ferredoxin reduction state in turn promotes electron
106 transfer into the thioredoxin and NADP(H) pools (Figure 1b). The increase in reduced thioredoxin
107 activates the thiol-regulated enzymes of carbon metabolism, a key factor allowing NADPH to be
108 consumed in the production of triose phosphate. However, because they are already so highly
109 reduced, this driving force alone should not greatly alter the reduction states of the glutathione or
110 ascorbate pools, at least in a way that can be measured by commonly used techniques. In other
111 words, the measured GSH:GSSG and ascorbate:dehydroascorbate ratios should be similar in the light
112 and the dark. Most evidence supports this concept. In part, this lack of effect is explained by a
113 significant difference in midpoint redox potentials between the different pools, such that the extent
114 of the "push" from the electron transport chain has relatively little influence on ascorbate and
115 glutathione redox states. Respiratory reactions in the dark are enough to keep these pools reduced in
116 the absence of stress.

117

118 Changes in the GSH:GSSG and ascorbate:dehydroascorbate ratios that are both physiologically
119 relevant and detectable could be generated through a rapid rise in superoxide and H_2O_2 production as
120 these species react preferentially with antioxidants (Figure 1c, "PULL"). If a rapid burst of ROS
121 production pulls electrons out of the ascorbate, glutathione, and thioredoxin pools faster than they
122 can be replenished from NADPH and ferredoxin, the pools move out (or further out) from
123 thermodynamic equilibrium and their oxidation would be observed. However, it is unclear how often
124 the redox states of ascorbate and glutathione in the chloroplast drop appreciably below highly
125 reduced values. The systems are highly integrated because most of the ROS within the stroma is
126 produced by oxidation of reduced ferredoxin and upstream photosystem I (PSI) components by

127 molecular O₂ (Figure 1d). In other words, production of oxidants such as ROS is in fact favoured by
128 excess of reductant. Thus, although this condition is often called “oxidative stress”, it could equally
129 be referred to as “reductive stress”.

130

131 The fact that both ROS production and antioxidant regeneration depend on reductant is an
132 important point. This produces a “push-pull” system that (1) can contribute to ensuring that oxidized
133 acceptors for the electron transport chain are always present (redox poising), thus avoiding
134 bottlenecks in electron flow; (2) ensures that ROS are not produced at high rates if reductant is not
135 available for their metabolism; (3) should promote high reduction states of ascorbate and
136 glutathione unless oxidants are imported from outside the chloroplast; and (4) enables high rates of
137 ROS production to occur in the light without necessarily causing oxidative stress. These principles
138 should hold true regardless of the enzymes and reductant systems used to metabolize peroxides in
139 the chloroplast (ascorbate peroxidases, peroxiredoxins, etc). In contrast, when catalase is decreased,
140 large increases in GSSG can be observed (Queval *et al.* 2007). While this may partly reflect
141 sequestration in the vacuole (see below), it is probably also because ROS production through
142 peroxisomal oxidases is less directly coupled to the production of excess reductant than when ROS
143 are generated by the photosynthetic electron transport chain.

144

145 In terms of signalling, we wish to emphasize two points with regard to the “push-pull” system shown
146 in Figure 1d. First, the *in vivo* redox potential of thioredoxins is a relatively flexible variable, allowing
147 light-driven redox signalling independent of changes in ROS and antioxidative systems. Second, any
148 increase in ROS (and related signalling) produced by photosynthetic electron transport will be short-
149 lived because the system is self-correcting. Adding to this stability is the operation of photosynthetic
150 control, which means that excessive flux from electron transport to the stromal redox pools will be
151 restricted by consumption of ATP and, therefore, the transthylakoid proton gradient (Foyer *et al.*
152 2012). While increases in the proton gradient might be expected to favour singlet oxygen formation
153 in PSII, the stimulation of non-photochemical quenching of excited chlorophyll states, also under the
154 control of the proton gradient, will tend to act to offset any such effect (Foyer *et al.* 2012; Ruban *et*
155 *al.* 2012).

156

157

158 **Compartmentation in redox signalling**

159

160 It is crucial to distinguish between the chloroplast and some of the other cell compartments in which
161 redox signalling is important. In contrast to the chloroplast, compartments such as the cytosol, which
162 are less directly affected by light, have greater redox stability. This is crucial for signalling involving
163 gene expression because the cytosolic redox state directly influences that of the nucleus. Concepts
164 developed in animals and yeast may be more relevant to cytosolic events, and cannot easily be
165 applied to redox regulation in the chloroplast. While reductant pools in the cytosol might be
166 influenced by light *via* redox shuttles (Scheibe *et al.* 2005; Foyer & Noctor 2009), ferredoxin is absent
167 and NADP(H) redox states are probably relatively stable. Nevertheless, several lines of evidence
168 suggest that changes in thiol status driven by enhanced cysteine and glutathione synthesis in the
169 chloroplast, which typically occur over a timescale of minutes to hours (rather than seconds to
170 minutes), appear to be crucial in regulating hormone signalling during biotic stress responses
171 (Vanacker *et al.* 2000; Ball *et al.* 2004; Koornneef *et al.* 2008; Han *et al.* 2013a,b; Park *et al.* 2013).
172 Such changes are likely to interface with specific thioredoxins implicated in redox regulation in the
173 cytosol (Tada *et al.* 2008), as there is clearly crosstalk between the cytosolic glutathione and
174 thioredoxin pools to a greater extent than in the chloroplast (Reichheld *et al.* 2007). Many other
175 intermediates, such as compounds involved in plastid terpenoid synthesis (Xiao *et al.* 2012), may also
176 act as metabolite signals from the chloroplast during stress. In most cases, however, the precise
177 nature of the interactions between signals from these metabolites and redox state or oxidative stress
178 is not yet clear.

179

180 *Redox gradients in plant cells*

181

182 The different compartments of plant cells are not all equally redox-buffered (Figure 2). While the
183 chloroplast stroma is highly reducing, notably due to the large amounts of ascorbate, glutathione and
184 other antioxidants, the lumen appears to have only limited antioxidant capacity. To date, no
185 transporters for ascorbate or glutathione have been identified on the thylakoid membrane and
186 therefore enzymes like violaxanthin de-epoxidase may be dependent on the diffusion of ascorbate
187 from the stroma. The enzymes of the lumen also include photosystem II (PSII) repair cycle
188 components and a number of redox-active cyclophilins (Järvi *et al.* 2015). One unresolved problem
189 concerning the chloroplast stroma is how proteins are oxidatively folded in a highly reducing
190 environment (Kieselbach 2013). The best characterized example of a stromal redox component
191 involved in folding is the cyclophilin 20-3, the only known cyclophilin in this compartment. This

192 protein is a thioredoxin-regulated foldase for the cysteine synthase complex in the stroma, thus
193 linking sulfur assimilation to redox regulation (Dominguez-Solis *et al.* 2008) and also oxylipin
194 signalling (Park *et al.* 2013). It is well established that the endoplasmic reticulum (ER) lumen is
195 relatively oxidized, with low GSH:GSSG ratios, presumably to allow protein disulfide bond formation.
196 Estimates with roGFP suggest that whereas the glutathione redox potential in the cytosol and other
197 compartments is lower than -300 mV, the ER value is around 100 mV higher (Birk *et al.* 2013).

198

199 The apoplast differs from some of the highly reduced spaces of the cytoplasm such as chloroplasts,
200 mitochondria, or the cytosol. This extracellular compartment is the site of oxidative bursts and also
201 contains many oxidant-requiring reactions, involving generation of hydroxyl radicals and other
202 equally strong oxidants at local sites within the cell wall (Pignocchi & Foyer 2003). Current evidence
203 suggests that ascorbate is the only reductant present at appreciable levels in the apoplast. However,
204 the presence of ascorbate oxidase means that apoplastic ascorbate contents are always more
205 oxidized than in the cytosol. Indeed, the apoplastic pool is depleted continuously by degradation,
206 starting from dehydroascorbate produced by ascorbate oxidase (Parsons & Fry 2012). Oxidative
207 bursts in the apoplast involve a shift of the redox status from very mildly reducing (compared to the
208 cytosol) to highly oxidizing. This may be necessary to allow activation of plasmamembrane-
209 associated proteins such as receptor-like kinases and calcium channels. The physiological significance
210 of ascorbate oxidase and many similar enzymes such as laccases remains unclear but may include
211 maintenance of a redox gradient across the plasmamembrane by removing reductant. Glutathione is
212 thought to be present at only very low levels in the apoplast but, similar to ascorbate, the apoplast is
213 probably one of the main compartments for its degradation (Ohkamu-Ohtsu *et al.* 2007).

214

215 While some parallels between the apoplast and the vacuole are clearly recognized, it is important to
216 note that they also share a number of similar redox characteristics. In addition to being major
217 locations of class III peroxidases, some of which may be ROS-producing rather than antioxidant in
218 function (Cosio & Dunand 2009; O'Brien *et al.* 2012), the vacuole also contains ascorbate but only
219 low levels of glutathione in most conditions (Rautenkranz *et al.* 1994; Queval *et al.* 2011). Both
220 compartments are also notable for their very low levels of reduced pyridine nucleotides and
221 associated reductant-generating enzymes. Similarly, there is no evidence that GSH or glutathione
222 reductase are present at appreciable levels at either location, although chemical oxidation of the
223 thiol group would be slow at vacuolar and apoplastic pH values. However, the vacuole can
224 accumulate significant amounts of GSSG when cells are subject to peroxisome-derived oxidative
225 stress (Queval *et al.* 2011), and also contains enzymes allowing glutathione degradation (Grzam *et al.*
226 2007). Accumulation of GSSG in the vacuole is probably the result of import from the cytosolic pool, a

227 process that may act to offset excessive oxidation of the cytosol during stress (Noctor *et al.* 2013).
228 Immunolocalization studies also suggest that changes in glutathione compartmentation are key to
229 many stress responses (Zechmann 2014).

230

231 While redox metabolites can only cross the inner membranes of the chloroplast and mitochondria at
232 high rates by virtue of transporters and/or metabolite shuttles, they can move much more easily
233 through the pores of the outer membranes of these organelles as well as through the
234 nuclearenvelope. Current concepts suggest that, like the mitochondrial matrix, the mitochondrial
235 intermembrane space is relativelyreducing. The less selective outer membrane allows movement of
236 reductant from the cytosol, for example, to support the activity of externally oriented NAD(P)H
237 dehydrogenases in the inner membrane (Rasmusson *et al.* 2004). The external face of the inner
238 membrane is also the site of the terminal step of ascorbate synthesis (Bartoli *et al.* 2000). Less
239 information is available on the redox characteristics of the intermembrane space of the chloroplast
240 envelope, which is defined by a double membrane whose permeability characteristics are similar to
241 the two mitochondrial membranes. Several stroma-exposed components of the translocon at the
242 inner envelope membrane of chloroplasts(TIC) are redox-sensitive. These components are found to
243 be enriched in chloroplasts compared to non-photosynthetic plastids, and may allow coordination of
244 protein trafficking with stromal NADP(H) redox state and/or the thioredoxin system (Kovács-Bogdán
245 *et al.* 2010).

246

247 As noted above, there is increasing evidence that cytosolic accumulation of GSH is necessary for
248 biotic stress signalling. The chloroquine resistance transporter-like transporters (CLTs) of the inner
249 chloroplast envelope membrane presumably play a key role, either directly by exporting GSH or by
250 transferring precursors for production of GSH in the cytosol. Indeed, mutants deficient in expression
251 of all three CLTs show impaired expression of pathogenesis-related genes (Maughan *et al.* 2010).
252 Transporters that import ascorbate into the chloroplast have long been sought, and very recently
253 a member of the phosphate transporter family was shown to import ascorbate into chloroplasts
254 (Miyaji *et al.* 2014). However, given the relatively mild phenotype of the corresponding mutants,
255 other routes of ascorbate transport into the chloroplast may await discovery (Foyer 2015).

256

257 Although pores regulate the passage of components between the cytosol and the nuclear interior,
258 smaller metabolites can probably diffuse, allowing the redox potentials of the two compartments to
259 be maintained at similar values (Diaz-Vivancos *et al.* 2015). This may be one reason why it is crucial to
260 regulate oxidative shifts in the cytosol extremely rigorously, in order to allow an appropriate adaptive
261 response to a given stimulus, as illustrated in Figure 4. Moreover, the nucleus contains a number of

262 specific redox regulators such as nuclear TRX, GRXC1, GRXC2, GRXS17, ROXY2 and ROXY19 (Marchal
263 *et al.* 2014; Belin *et al.* 2015). Even in yeast, where glutathione appears to be less crucial than in
264 plants, it has recently been shown that gene expression under oxidative stress is dependent on the
265 nuclear glutathione pool (Hatem *et al.* 2014).

266

267 While the concept of oxidative stress is widely accepted, less attention has been paid to reductive
268 stress. Reductive stress of oxidized compartments such as the ER and apoplast could be just
269 as challenging and signal-transducing as enhanced oxidation is in the more reduced compartments.
270 Treatment of leaves in the dark with an artificial reductant, dithiothreitol (DTT), caused many
271 metabolic changes that were interpreted in terms of reduction of thioredoxins (Kolbe *et al.* 2006),
272 which would normally be relatively oxidized in the dark, at least in the chloroplast (Figure 1).
273 However, few indicators of stress were observed, although this may have been related to the
274 relatively short duration of the treatment (1 h; Kolbe *et al.* 2006). Treatment with GSH or pyridine
275 nucleotides for somewhat longer times is sufficient to trigger biotic stress signalling (Gomez *et al.*
276 2004; Zhang & Mou 2009; Han *et al.* 2013a), even though the total tissue contents of these
277 endogenous redox compounds are often not greatly enhanced by the treatments. These effects may
278 be partly triggered by changes in apoplastic redox state, although oxidized pyridine nucleotides
279 (NAD^+ , NADP^+) were shown to be just as effective as the reduced forms (Zhang & Mou 2009). One
280 possibility is that the degradation products of these molecules may be perceived as signals (Noctor *et al.*
281 2015). In *Chlamydomonas*, DTT treatment induces some features of an autophagic response,
282 which was proposed to result from ER stress, consistent with an increased total cellular GSH:GSSG
283 ratio (Perez-Martin *et al.* 2014).

284

285 *H₂O₂ concentrations and compartmentation*

286

287 Its relative stability and potential for reactivity with specific thiol groups make H_2O_2 a major player in
288 oxidative signal transmission. Despite its acknowledged importance, we still do not know what
289 H_2O_2 concentrations are likely to occur *in vivo* in each compartment under either optimal or stress
290 conditions. This gap in our knowledge is an impediment to interpreting correctly the quantitative
291 processes and signalling mechanisms occurring during oxidative stress. It is important to note that
292 global measurements on homogenized tissue are highly variable (Queval *et al.* 2008). Although
293 numerous papers have reported on increases in H_2O_2 in stress conditions, plants deficient in catalase,
294 which might be considered a benchmark for H_2O_2 accumulation, show minor, transient or
295 undetectable changes in the extractable levels of this oxidant, even when many other indicators
296 show that the plants are oxidatively stressed (Noctor *et al.* 2015).

297

298 As most plant tissues are composed of around 90% water, 1g FW is equivalent to a volume of about 1
299 mL. Therefore, a value of 1 $\mu\text{mol. H}_2\text{O}_2 \text{ g}^{-1}$ fresh weight (FW), which is not uncommon in the
300 literature, implies that the average tissue concentration is around 1 mM (1 $\mu\text{mol/mL}$). Even though
301 the logic is debatable, as discussed below, interpretation in the literature often considers that
302 compartments with the highest capacity for H_2O_2 production, such as the chloroplast and
303 peroxisomes, are likely to contribute most to tissue H_2O_2 contents. Unfortunately, while probes are
304 under development for *in vivo* measurement of H_2O_2 (Costa *et al.* 2010; Esposito-Rodriguez *et*
305 *al.* 2012), their value as quantitative techniques has yet to be established and there is still no
306 consensus value for compartment-specific concentrations.

307

308 *Estimating in vivo H_2O_2 concentrations*

309

310 On the basis of simple modelling, we have previously drawn attention to the high rates of H_2O_2
311 production in the peroxisomes of C_3 photosynthetic cells (Noctor *et al.* 2002). Based on similar logic,
312 we have here attempted to derive a likely concentration of H_2O_2 in the peroxisomes during
313 moderately high rates of photosynthesis and associated photorespiration. Peroxisomes make a
314 relatively small contribution to total cell volume compared to the chloroplasts: <1% for peroxisomes
315 and about 12% for chloroplasts in *Arabidopsis mesophyll* cells (Queval *et al.* 2011). Despite this, they
316 are predicted to be a major site of H_2O_2 production, especially when photorespiration is significant
317 (Noctor *et al.* 2002). The predominant dismutation reaction of catalase does not involve substrates
318 other than H_2O_2 . This means that, together with knowledge of the relationship of photorespiration to
319 measured photosynthesis (Sharkey 1988), the approximate operating H_2O_2 concentration in the
320 peroxisomes in the light can be estimated from the kinetics of catalase, subject to certain
321 assumptions. One assumption is that the photorespiratory glycolate oxidase reaction is the major
322 producer of peroxisomal H_2O_2 , while others are discussed below.

323

324 Catalase has a very high capacity because (1) it is a highly expressed enzyme in C_3 leaves and (2) its
325 turnover rate is among the fastest known. However, its affinity for H_2O_2 is low: estimates of K_M
326 values can exceed 100 mM (Del Rio *et al.* 1977). In agreement with this, catalase activities in
327 *Arabidopsis* leaf extracts continue to accelerate as H_2O_2 concentrations increase up to at least 40 mM
328 with the dependence on substrate being almost linear below 20 mM (Figure 3a).

329

330 On the assumption that most of the catalase activity is found in the peroxisomes of photosynthetic
331 cells (the photorespiratory catalase accounts for almost 90% of total extractable activity in

332 Arabidopsis leaves; Queval *et al.* 2007), we can make an approximate estimate of the H₂O₂
333 concentrations that are required to drive catalase activity to cope with typical photorespiratory flux.
334 If the majority of photorespiratory glycolate is metabolized by glycolate oxidase, the rate of H₂O₂
335 production is approximately equal to the rate of RuBP oxygenation (Figure 3c). In Arabidopsis grown
336 in standard conditions, rates of net photosynthetic CO₂ assimilation in air do not exceed 10 μmol m⁻² s⁻¹
337 (eg, Veljovic-Jovanovic *et al.* 2001) and accompanying rates of RuBP oxygenation are probably
338 around 40% of this value (Sharkey 1988). In this context, the key question is: what peroxisomal
339 concentrations of H₂O₂ would be attained under these conditions of rapid photorespiration?

340

341 The leaf fresh mass:area ratio for Arabidopsis used to obtain the data shown in Figure 3 was
342 measured as 260 g m⁻². An RuBP oxygenation rate of 4 μmol m⁻² s⁻¹ therefore converts to slightly
343 below 60 μmol H₂O₂ g⁻¹ FW h⁻¹ (= 4 × 3600/260). The *in vitro* catalase activity in Arabidopsis leaf extracts
344 measured at 10 mM H₂O₂ is approximately 1000 times higher than this estimate of *in vivo*
345 production, and the high K_M value of the enzyme means that rates decrease linearly as H₂O₂
346 concentration falls below this value (Figure 3b). Standard catalase assays are not sensitive enough to
347 allow accurate measurement of the activity at sub-millimolar substrate concentrations. To derive an
348 *in vivo* concentration from the *in vitro* assays, we have to assume that the relationship between
349 catalase activity and H₂O₂ concentration is linear between zero and the lowest substrate
350 concentration measured (2 mM H₂O₂). If this is so, a catalase activity of 60 μmol g⁻¹ FW h⁻¹ would be
351 driven by an H₂O₂ concentration of 10 μM (Figure 3b,c).

352

353 These calculations illustrate that, despite its low affinity, catalase is able to maintain H₂O₂
354 concentrations at low values. Unless a substantial part of the catalase activity that can be measured
355 in extracts is inactive *in vivo*, even comparatively high rates of production of H₂O₂ through
356 photorespiration are unlikely to maintain global peroxisomal H₂O₂ concentrations much higher than
357 10 μM. Without inactivation or other forms of down-regulation, catalase would simply be too potent.
358 In this connection, it is very interesting that several mechanisms have been identified that may be
359 important in down-regulating catalase to drive phenomena such as biotic stress responses (Mhamdi
360 *et al.* 2010b), although their precise *in vivo* importance remains to be established.

361

362 These calculations are necessarily approximate. Apart from the assumptions discussed above, other
363 potentially complicating factors include the existence of catalase in a quasi-crystalline form in the
364 peroxisomes. It is possible that when these structures are disrupted by extraction into a dilute
365 solution, latent enzymes become exposed and able to contribute to the activity in the assay. This
366 could cause the extent to which H₂O₂ accumulates *in vivo* to be underestimated. Nevertheless, we

367 note that even our estimated concentration of 10 μM in the peroxisomes probably exceeds values in
368 other compartments inside the cell, where the main players in H_2O_2 metabolism are ascorbate- and
369 thiol-dependent peroxidases. The primary role of such antioxidative enzymes is to maintain H_2O_2 at
370 concentrations compatible with ongoing cell function, and they have K_M values for H_2O_2 of the order
371 of 5-50 μM (Mittler & Zilinskas 1991; König *et al.* 2002; Barranco-Medina *et al.* 2007). While the
372 above discussion of catalase shows that substrates can be much lower than K_M values, the reverse is
373 unlikely, especially for a potent stress signalling molecule. This is because if substrates routinely
374 accumulate in the absence of stress to values well above the K_M , then there is little direct and rapid
375 sensitivity to stress-induced changes in the concentration of the substrate. Thus, we infer that H_2O_2
376 concentrations in the chloroplast, cytosol, and mitochondria are unlikely to exceed those in the
377 peroxisomes. This is consistent with the high sensitivity of thiol-dependent metabolic enzymes to
378 oxidative inactivation by micromolar concentrations of H_2O_2 . It is also consistent with modelling that
379 suggests that “wave signalling” by H_2O_2 is only possible against a relatively low background cytosolic
380 concentration (Vestergaard *et al.* 2012).

381
382 If 10 μM is a typical H_2O_2 concentration inside the cell, intracellular pools would explain only a small
383 part of values that are typically measured in extracts. The total contribution of peroxisomes,
384 chloroplasts, mitochondria, and cytosol would not greatly exceed 1 $\text{nmol g}^{-1}\text{FW}$ (Table 2). In contrast,
385 most quantitative assays of leaf H_2O_2 in optimal conditions range from 100 $\text{nmol g}^{-1}\text{FW}$ upwards, ie,
386 about 100 times higher. This marked difference strongly suggests that currently used *in vitro* assays
387 are unlikely to provide relevant information on the *in vivo* H_2O_2 concentrations at these locations
388 (although they might still reflect changes in rates of production or removal inside the cell).

389
390 Discounting artefacts in H_2O_2 extraction and assay, a possibility that is not to be dismissed lightly
391 (Queval *et al.* 2008), the discrepancy between *in vivo* concentrations and *in vitro* estimates may be
392 resolved if there is a large apoplastic pool of H_2O_2 . However, if this is the only or main explanation,
393 then more than 90% of the extracted H_2O_2 would be outside the cell. Assuming the apoplast accounts
394 for 10% cell volume, then more than 90% of 100 nmol located in the apoplast of 1 g tissue would
395 equate to a concentration of about 1 mM. This may not be an unrealistic concentration for the
396 oxidative reactions required for cell wall biosynthesis and dynamics, although it would pose the
397 question of what concentrations can be reached during oxidative bursts. A key point could be the
398 proximity of ROS-generating enzymes such as peroxidases and NADPH oxidases to ROS-sensitive
399 targets at the plasmalemma. While relatively high amounts of H_2O_2 could also be localized in other
400 oxidized compartments such as the vacuole (Figure 2), there is relatively little information available
401 to address this question.

402

403 **ROS as life signals that drive acclimation through shifts in redox homeostasis**

404

405 The traditional notion that ROS are purely damaging compounds has become increasingly questioned
406 by studies that have appeared during the last twenty years or so. Growing evidence demonstrates
407 that ROS production linked to hormone action is required for a wide range of plant responses to
408 developmental and environmental cues, leading to growth and acclimation responses. Current
409 knowledge suggests that, rather than a negative influence that must be overcome, ROS are a major
410 driver for acclimation and adaptation in plants, in a similar manner to their role in the improvement of
411 physical fitness in athletes (Radak *et al.* 2005, 2008), where adaptive responses to oxidative stress
412 induce an anti-inflammatory phenotype, enhancing exercise salience and leading to greater fitness,
413 by “revving up the metabolic engine”(Nunn *et al.* 2010)., For example, ROS are required to
414 force mitochondrial biogenesis in the production of skeletal muscle (Gomez-Cabrera *et al.* 2009).
415 Within the context of the redox poisoning of the plant cell at values not too far from -300 mV (Figure 1),
416 enhanced ROS production is suggested to temporarily shift the redox potential to more oxidizing
417 values that will alter the operational controls of many redox-sensitive proteins. A relatively small
418 global shift in the glutathione redox potential to values of about -260 mV is associated with a very
419 large change in gene expression and plant development (Aller *et al.* 2013; Schnaubelt *et al.*
420 2015). Unfortunately, there are relatively few studies in which redox potentials have been
421 determined in conjunction with the regulation of cell metabolism or gene expression. This is a key
422 issue to be resolved in the future.

423

424 While the picture is far from clear, Figure 4 outlines our vision of the flexible control of plant growth
425 and development by dynamic changes in redox state. According to this scheme, ROS are the triggers
426 that drive acclimation by modulating redox state, either at a very local subcellular level or more
427 globally across cells and tissues. As by-products of energy metabolism, ROS are a hallmark of living
428 cells that drive the continued existence of organisms at the thermodynamic expense of the
429 environment. In this way, genetic and epigenetic processes might be directly linked to metabolism
430 through ROS production and its effects on cellular redox balance.

431

432 It has long been accepted that continuous oxidation inevitably leads to cell death, although this very
433 much depends on cell type. Moreover, the adverse effects of reductive stress are hardly discussed in
434 the plant literature, even though this syndrome must exist. In this view, redox couples such as
435 glutathione (GSH + GSSG) have a dual function in not only attenuating but also propagating ROS
436 signals (Han *et al.* 2013a). Essentially, flexible ROS production might prevent cells succumbing to the

437 adverse effects of stress by allowing the establishment of appropriate responses. Future work will
438 establish whether this concept of ROS as life signals is tenable.

439

440 **How are ROS perceived by the cell?**

441

442 Perhaps more than any other development, the observation of close parallels between the
443 symptoms of ozone exposure and the hypersensitive response to pathogens laid the foundations of
444 the concept that oxidative signals were key to the environmental responses of plants (Sandermann *et*
445 *al.* 1998; Vainonen & Kangasjärvi 2015). Numerous studies since have established that ROS interact
446 intimately with recognized signal transmission components, such as phytohormones, calcium ions,
447 and mitogen-activated protein kinase (MAPK) cascades (reviewed by Xia *et al.* 2015). Despite the
448 characterization of the involvement of these components, sources of ongoing frustration include our
449 ignorance of complete signalling pathways and especially of the initial processes of ROS perception.

450

451 Despite their inflammatory name, the chemical reactivity of the most abundant ROS is quite limited.
452 Hydrogen peroxide and superoxide are relatively poor oxidants. Even in the case of thiol groups, rate
453 constants for oxidation are quite low, unless the group is located in an environment that promotes
454 deprotonation to the thiolate anion. Free glutathione, whose thiol group has a pKa of about 9, reacts
455 only very slowly with H₂O₂ and is oxidized much more rapidly by chemical reaction with
456 dehydroascorbate (Rahantaniaina *et al.* 2013). The plethora of enzymes able to dismutate or reduce
457 H₂O₂ may in part be explained by the need to minimize production of the hydroxyl radical within the
458 cell through Fenton-type reactions catalyzed by redox-active metal ions (Møller *et al.* 2011). This
459 raises the question of whether ROS *per se* are signals or rather whether they give rise to signals
460 through their metabolism. Direct oxidation of transcription factors has been well documented, as
461 have effects of ROS on translation (Benina *et al.* 2015). In addition, as discussed above, ROS could
462 produce a global shift in the main cellular redox buffers such as glutathione or thioredoxins that is
463 then perceived by sensitive proteins that interact with these components. Specificity may be
464 conferred by specific peroxidases that transmit oxidative signals to sensitive proteins such as
465 transcription factors. One well-known example in yeast is Gpx3 that, once oxidized by peroxide, then
466 oxidizes and activates the transcription factor, Yap1 (Delauney *et al.* 2003). Based on oxidation of
467 roGFP, this has been proposed as a general mechanism of peroxide-based signalling (Gutscher *et al.*
468 2009). However, despite interest in this idea, there have been only very few reports of this kind of
469 signalling in plants (Miao *et al.* 2006). Until more evidence is available, it is difficult to conclude on
470 the importance of specific thiol-peroxidases as signal relays in plants, in which ROS orchestrate
471 numerous processes of growth and development.

472

473 As noted above, several studies have shown that pathogenesis responses are altered by the
474 availability of glutathione. For more than ten years, the main focus has been on regulation of the
475 protein nonexpressor of pathogenesis related genes1 (NPR1), which may be activated by
476 thioredoxins or glutathione (Mou *et al.* 2003; Gomez *et al.* 2004; Laloi *et al.* 2004; Tada *et al.* 2008;
477 Maughan *et al.* 2010). However, it seems that glutathione can also influence redox-driven
478 pathogenesis responses upstream of NPR1, at the level of salicylic acid(SA) synthesis (Mhamdi *et al.*
479 2010a; Han *et al.* 2013a). So far, there is no evidence that this effect is due to direct redox regulation
480 in the chloroplast, where key enzymes involved in SA synthesis, such as isochorismate synthase (ICS),
481 are located (Strawn *et al.* 2007). Rather, it may reflect glutathione status in the cytosol or nucleus,
482 consistent with the observation that SA contents and *ICS1* transcripts are similarly affected (Han *et*
483 *al.* 2013a).

484

485 The vast array of protein thiol groups, including those found in the battery of antioxidant thiol-
486 peroxidases, as well as the multiple reactions that can be undergone (disulfide formation, S-
487 glutathionylation, S-nitrosylation, etc) still makes these processes attractive candidates (Noctor *et al.*
488 2014; Dietz & Hell 2015). A further complication is that ROS signalling may include downstream
489 events involving reduction rather than oxidation, possibly involving ROS-induced responses in
490 reductant production. This further emphasizes the close relationship between over-oxidation and
491 over-reduction. By far the best known example of a reductant-activated protein triggered by initial
492 oxidative events is NPR1 (Mou *et al.* 2003; Tada *et al.* 2008), but many other thiol-containing proteins
493 may be involved in both the early and later events of ROS signal transmission. The likely complexity
494 of this network, as well as the difficulties currently inherent in quantitative redox proteomics *in vivo*,
495 makes the identification of these proteins and their physiological importance a rather daunting,
496 technically challenging but necessary task for the future.

497

498 In the case of singlet oxygen, which is more reactive than superoxide or H₂O₂, some aspects of
499 signalling have become clearer. However, much of the work has been done on a model system, the
500 *flu* mutant, in which singlet oxygen may not be produced at what is generally considered to be its
501 most physiologically important site (PSII). It has been shown that singlet oxygen can trigger
502 carotenoid breakdown, leading to secondary reactive organic compounds (Ramel *et al.* 2012). These
503 secondary compounds can themselves elicit similar changes in gene expression to those caused by
504 singlet oxygen (Ramel *et al.* 2012). Such signalling pathways reflect the reactivity of singlet oxygen,
505 which is unlikely to diffuse far before reacting with components that are in relatively close proximity.

506

507 It has long been acknowledged that a major effect of ROS is to trigger lipid peroxidation, which may
508 result in lipid peroxides and other reactive electrophiles such as aldehydes that may themselves have
509 signalling competence (Farmer & Mueller 2013). Even here, however, it is becoming clear that the
510 production of such compounds is dependent on the action of enzymes such as lipoxygenases (Farmer
511 & Mueller 2013). Protein breakdown products may also be important in signalling (Møller &
512 Sweetlove 2010), as well as carbohydrate fragments such as oligosaccharides that are produced in
513 the cell wall during pathogen attack. Other possibilities are ROS-induced changes in metabolite
514 profiles, as cellular function adjusts to altered redox state (Møller *et al.* 2007; Noctor *et al.* 2015).

515

516 **Oxidative signalling and hypoxia**

517

518 An alternative way to control cellular oxidation and related ROS signalling is by maintaining a hypoxic
519 environment. The most studied example is perhaps the legume root nodule where oxygen is made
520 available for respiration only *via* leghaemoglobins. In addition, many quiescent tissues such as seeds
521 and dormant buds are hypoxic (Meitha *et al.* 2015). Hypoxia is required to maintain the niche that
522 houses stem cells in the undifferentiated state in animals and plants (Mohyeldin *et al.*, 2010).
523 Moreover, low oxygen concentrations influence cell proliferation and cell-fate commitment
524 (Mohyeldin *et al.*, 2010). It is not surprising therefore that cell types such as the quiescent centre
525 cells in the root apical meristem express a hypoxia-related transcriptome and function continuously
526 at very low oxygen (Jiang & Feldman 2005). In dormant tissues, the transition to aerobic metabolism
527 and the metabolically active state requires a shift from energy metabolism based on glycolytic ATP
528 production to one driven by oxidative phosphorylation that encompasses an abrupt adjustment to
529 high ROS concentrations that accompany the onset of mitochondrial electron transport. These
530 observations underline the cell-specific nature of plant responses to oxygen. It is likely that oxygen
531 and ROS sensitivity are highly variable between different cell types. While some organs such as
532 expanded leaves are rarely subject to hypoxia, others can often suffer this condition, for example
533 roots during flooding. However, such organs appear to have only a limited capacity for survival,
534 unless they generate air pipes (aerenchyma).

535 Understanding why some cell types are able to survive low oxygen could throw much light on
536 improving stress tolerance in plants. Regulation of oxygen concentrations may be intimately linked to
537 NO metabolism and signalling. The operation of a hemoglobin-nitric oxide (NO) cycle in hypoxic cells
538 serves not only to conserve NO but also allows protected NO transport for physiological functions. It
539 thus links metabolic activity in mitochondria to S-nitrosylation reactions (Hebelstrup & Møller
540 2015). Oxygen sensing through the N-end rule of protein degradation has added a completely new

541 dimension to our understanding of oxidative signalling (Figure 5). In this mechanism, the removal of
542 the relatively stabilizing N-terminal methionine in certain transcription factors unmask a cysteine
543 residue that is sensitive to S-nitrosylation and oxidation to sulfenic and sulfonic acid groups. This
544 enhances the susceptibility of the transcription factor to proteolytic degradation, keeping levels low
545 and preventing activation of gene expression. At low oxygen concentrations, this continuous
546 stimulation of degradation is prevented and appropriate gene expression is triggered (Gibbs *et al.*
547 2014). Oxidation of the cysteine residue appears to be dependent on novel plantcysteine oxidases
548 that confer specificity. These enzymes rapidly reverse transcription factor accumulation when oxygen
549 becomes more plentiful by introducing two oxygen groups into an N-terminal thiol to produce
550 cysteine sulfinic acid groups that promote degradation (Weits *et al.* 2014). To our knowledge, no
551 such mechanism has been described in ROS signalling, but it is tempting to speculate that analogous
552 pathways could exist depending on non-enzymatic oxidation of cysteines. One striking observation
553 pointing to interactions at some level is that glutathione and other reductants can largely prevent the
554 effects of methionine aminopeptidase deficiency on Arabidopsis phenotypes and proteomes (Frottin
555 *et al.* 2009).

556

557 **ROS: a key to monitoring metabolic flux by cells?**

558

559 As the above discussion has emphasized, ROS production by cells may provide current status
560 information although we do not know precisely how these oxidizing molecules are monitored. While
561 components that respond to changes in ROS concentrations are no doubt involved, it is possible that
562 ROS may also contribute to the molecular memory of stress through durable accumulation of redox
563 information on metabolism. One may speculate on how ROS could form part of a molecular counting
564 system. Since ROS signalling involves their biochemical transformation to more stable species, a key
565 feature could be production of secondary states that have enough stability to act as information
566 stores. This notion is obviously speculative but it is interesting to imagine what kind of biochemical
567 system could act in molecular memory storage. Examples of information storage (or memory) exist in
568 biological systems. Simple examples in plants are enzymes that have a gated system such as the ATP
569 synthase that requires a certain number of protons to pass through the CF₀ channel to drive
570 conformational changes in CF₁ that are converted to condensation of ADP and phosphate to ATP.
571 While this is an example of very limited “counting”, other phenomena such as D1 turnover in
572 photosystem II provide examples of a more extensive counting system, allowing the PSII reaction
573 center to turn over after a more or less precise number of photochemical events.

574

575 At least in some situations, ROS might be indirectly reflecting metabolic flux and, therefore, could
576 have been recruited during evolution as metabolic flux counters. If so, the key issue would not simply
577 be increases in ROS concentrations but rather the rate of reaction of ROS with specific proteins that
578 may be coupled to “counting” components. A simple example would be a protein complex that must
579 accumulate a certain number of oxidation-driven turnovers before undergoing some conformational
580 change (Figure 6) or, perhaps, being degraded. Gearing could be possible through association of
581 sequential structural components, each of which requires a relatively well defined number of ROS-
582 dependent oxidation events before association: the hypothetical mechanism shown in Figure 6
583 would require 100 oxidation events prior to conformational changes allowing transition to a signaling
584 state. If the signaling complex required the association of ten oxidized proteins, 1000 events would
585 be required. The scheme envisages that each oxidation produces a stable configuration but if
586 reversion to the reduced state was possible, as is perhaps likely, this would create a threshold
587 intensity of ROS action whereby each oxidation must be sufficiently close in time for information to
588 be accumulated. In this way, the system would be dependent on the rate of ROS formation, the ROS
589 concentration, and competing antioxidant systems that remove ROS or that revert the oxidized
590 states. Developmental and environmental effects on the expression of such hypothetical counting
591 systems, as well as the influence of cellular antioxidant status on their sensitivity to reversion to the
592 initial state, could contribute to the conditionality of ROS sensitivity in plants.

593

594 The possibility of defined macromolecular complexes able to “count” in this manner obviously
595 remains speculative. However, such a phenomenon could also occur through a probabilistic model.
596 For example, if the probability of ROS escaping the antioxidative system to react with component A is
597 1 in 1000, and there is a 1 in 1000 chance that the oxidation of A leads to oxidation of B, then this
598 could serve to achieve a similar relay effect that ultimately causes integration of individual events
599 before some threshold value is reached, leading to changes in gene expression that underpin cellular
600 and developmental programmes.

601

602 **Conclusions and outlook**

603

604 The concept that ROS and reactive nitrogen species (RNS) are key signalling molecules that facilitate
605 a plethora of adaptive metabolic, molecular genetic and epigenetic responses is now established.
606 Although we acknowledge that much of ROS action may be in close association with RNS signalling,
607 for reasons of space we have focused specifically on redox biology linked to ROS. The notion that
608 cells exploit highly reactive and potentially harmful species to drive life-promoting changes in gene

609 expression still sits uneasily with earlier ideas such as oxidant-induced damage and cell death, as well
610 as the ever-popular free radical theory of ageing. However, our view is that the ubiquitous and
611 sustained production of ROS in metabolism, even in the absence of stress, makes them excellent
612 candidates to provide key information on cellular status in a changing environment. Hence, ROS
613 functions may include acting as conveyors of metabolic and nutritional information, as suggested
614 here, although further technological developments will be required to test and (in)validate some of
615 these ideas. Moreover, cellular oxygen levels are managed in order to provide hypoxic niches in
616 plants such as those required to sustain stem cells. Gaining a better understanding of how plant cells
617 manage the release from the hypoxic to the aerobic state once division is initiated will provide new
618 insights into how we might improve the survival of plants exposed to stresses such as flooding.

619
620 High reactivity, and hence characteristic instability, may be crucial to the success of ROS as versatile
621 signalling molecules. Successful signalling cascades may be fundamentally dependent on the high
622 abundance of reactive but relatively short-lived interacting molecules that can either directly or
623 indirectly (e.g. through thiol intermediates) alter protein structure or function *via* at least partially
624 reversible mechanisms. Technological challenges encountered in measuring redox modifications *in*
625 *vivo* undoubtedly remain a huge obstacle to be overcome in defining the circuitry of redox signalling.
626 While many catalogues of potential thiol-modified targets have been produced, relatively abundant
627 proteins such as enzymes involved in primary metabolism are heavily over-represented in these lists.
628 Both the exact nature of the modification and its extent and effect *in planta* have generally so far
629 proved difficult to determine. The interpretation of data on S-modified proteins remains fraught with
630 difficulty. Key obstacles are the identification of the modification occurring *in vivo* as well as its
631 quantification, ie, the proportion of the protein cysteines that undergo any given modification.
632 Ongoing developments in mass spectrometry will probably resolve some of these issues, but easy
633 analysis would also be greatly facilitated by the use of probes that are specific to the different
634 modifications.

635
636 A key question that is still outstanding is the role of different cell compartments during redox
637 signaling linked to different stresses. At present, interpretation often does not take into account
638 heterogeneity of the status of either ROS or antioxidants, which inevitably limits the insight
639 generated in many studies. The development of *in vivo* probes that are genetically encoded, and so
640 able to be precisely targeted to specific compartments, may underpin a step change in current
641 concepts. Technological breakthroughs are undoubtedly required. Nevertheless, our view is positive:
642 tremendous progress has been made over the last decade and we anticipate that the exciting new

643 developments on the horizon will certainly resolve the problems discussed in this review, perhaps
644 surprisingly quickly.

645

646 **Acknowledgments**

647

648 C.H.F. and G.N.wish to acknowledge financial support from, respectively, European Union FP7 grant
649 (KBBE-2012-6-311840: ECOSEED) and the French Agence Nationale de la Recherche grant (ANR12-
650 BSV6-0011: Cynthiol).

651

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Table 1. Relationship between redox state and redox potentials of major cellular redox actors. The table gives the midpoint redox potentials for each component (left) and their calculated redox states (right), assuming that all have an actual redox potential of -320 mV. This was done according to the Nernst equation, where actual redox potential = midpoint redox potential - $59.2/n \log$ [reduced form]/[oxidized form], where n = numbers of electrons involved in the reaction (1 for ferredoxin, 2 for the others). For thioredoxins, the midpoint potential reported for spinach thioredoxin *f* was taken (Hirasawa *et al.* 1999). Note that for glutathione, concentration must also be taken into account as well as the ratio of reduced and oxidized forms. Here, it is taken to be 5 mM. At this concentration, the midpoint redox potential of glutathione (-230 mV) is achieved at GSH:GSSG >100. Together with the 90 mV difference between the NAD(P) and glutathione midpoint potentials, this explains why glutathione would be so highly reduced at -320 mV. Ascorbate would be even more reduced at this potential, simply because its midpoint potential is 380 mV more positive than that of NAD(P). ASC, ascorbate; DHA, dehydroascorbate; Fd, ferredoxin; GSH, glutathione; GSSG, glutathione disulphide. Ox, oxidized. Red, reduced. TRX, thioredoxin.

Component	Midpoint potential, mV (pH 7)	Redox state at -320 mV (reduced form/oxidized form)
Ferredoxin	-430	0.014
Ox Fd + 1 e ⁻ → Red Fd		
NAD(P)	-320	1
NAD(P) ⁺ + 2 e ⁻ + H ⁺ → NAD(P)H		
Thioredoxin	-290	10
Ox TRX + 2 e ⁻ + 2H ⁺ → Red TRX		
Glutathione	-230	2.1 x 10 ⁵
GSSG + 2 e ⁻ + 2H ⁺ → 2 GSH		
Ascorbate	+ 60	6.9 x 10 ¹²
DHA + 2 e ⁻ + 2H ⁺ → ASC		

Table 2. Estimations of possible contributions of major H₂O₂-producing organelles to contents typically measured in extracts of leaves. A maximal compartmental concentration of 10 μM H₂O₂ is assumed (for explanation, see text). Intracellular volumes are derived from data for Arabidopsis leaf mesophyll cells (Queval *et al.* 2011). Even assuming a relatively low tissue content of 100 nmol g⁻¹FW, the main intracellular H₂O₂-producing compartments would contribute only 1.5 % of this value. Assuming that tissue contents are not largely artefactual, we tentatively attribute the major contribution to the apoplast, where ROS-producing and ROS-requiring oxidative functions are crucial.

	Volume μl.g ⁻¹ FW	Contribution nmoles.g ⁻¹ FW
A. Metabolically active highly reduced compartments		
Chloroplasts	95.0	0.95
Mitochondria	4.0	0.04
Peroxisomes	1.5	0.015
Cytosol	48	0.48
SUM	148.5	1.49
B. More oxidized compartments		
Apoplast	100	>90?
Vacuole	500	??

Figure legends

Figure 1. The push-pull model of redox hierarchy in the chloroplast stroma. (a) Cartoon of relationships between stromal redox couples and their reduction states. Note that the midpoint potential are standard values for each component that do not indicate the actual redox potential in the stroma (here, assumed to be -320 mV, see also Table 1). (b) “Push” effect of light-driven electron transport. In the light, the ferredoxin, NADP(H), and thioredoxin pools become more reduced, at least transiently. Because they are all already so highly reduced, the glutathione and ascorbate pools are less affected by light. (c) “Pull” effect of reactive oxygen species (ROS) on redox couples. ROS production preferentially oxidizes glutathione and ascorbate pools. (d) “Push-pull” model that emphasizes the dependence of ROS generation on reductants in the light. For simplicity, the source

of electrons for ROS is shown as ferredoxin but other components of the electron transport chain can also produce superoxide.

Figure 2. Simple scheme showing known or likely redox gradients across subcellular membranes. Compartments shown in blue contain powerful antioxidant systems that dictate short lifetimes for reactive oxygen species (ROS). In compartments shown in red, antioxidant capacity is lower and ROS can be more long-lived. ER, endoplasmic reticulum. IM, intermembrane.

Figure 3. Peroxisomal H_2O_2 concentrations derived from measured catalase kinetics and typical rates of photorespiration. Catalase activity was measured in two different extracts of Arabidopsis leaves as a function of H_2O_2 concentration in the assay, according to standard protocols (Mhamdi *et al.* 2010a). Plants were grown for three weeks under a 16h photoperiod at an irradiance at leaf level of $200 \mu\text{mol}\cdot\text{m}^{-2} \text{ s}^{-1}$. After centrifugation and desalting of leaf extracts, catalase activities were assayed by removal of H_2O_2 measured at 240nm in extract 1 (white circles) and extract 2 (black circles). Rates were calculated as the initial decrease in A_{240} over the first 30 seconds after addition of extract. (a) Curvilinear relationship at H_2O_2 concentrations between 2.5 and 40 mM. (b) Zoom into the linear relationship in the range 2.5 to 10 mM (red box in (a)). (c) Simplified scheme of the first part of the photorespiratory pathway showing how typical rates of RuBP oxygenation compare to catalase (CAT) activity. In the steady-state, assuming that all photorespiratory glycolate is metabolized by glycolate oxidase and all the resulting H_2O_2 is removed by catalase, the rate of RuBP oxygenation equals the rate of H_2O_2 removal by catalase, ie, $60 \mu\text{moles}\cdot\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$ in this case. In (b) and (c) the units are in bold to emphasize that one is in mmoles and the other in μmoles .

Figure 4.

Schematic model showing how plant cells use reactive oxygen species (ROS) in signal transduction pathways leading to growth and acclimation responses. Hormesis theory considers that all biological systems show a range of different responses to potentially harmful toxins and chemicals including ROS. For example, the beneficial effects of regular exercise in animals are considered to accrue from the ROS-generating capability of exercise, which stimulates gene expression leading to a decrease in oxidative stress-related diseases and delay of the ageing process (Radak *et al.*, 2005, 2008). By analogy therefore we propose that ROS provide a sufficient redox stimulus to modulate growth and adaptation effects in plants that can essentially be described by the hormesis curve. In the same way that ingesting high doses of antioxidants on a regular basis might actually hamper the performance of athletes, the plant antioxidant system is designed to temper ROS accumulation rather than

prevent it. This feature is particularly important for processes that require a stronger redox stimulus, for example ROS-mediated activation of cell suicide programs, as occurs for example in the formation of aerenchyma or during the hypersensitive response, which involve regulated decreases in antioxidant capacity.

Figure 5. Oxidative signalling through the N-end rule. Low oxygen tensions are signalled by decreased degradation of group VII ethylene-responsive transcription factors (ERFs; red ovals). Inhibited oxidation of N-terminal cysteines unmasked by methionine aminopeptidase (MAP) allows ERFs to accumulate and activate appropriate gene expression.

Figure 6. The final countdown: flux-mediated ROS signalling? The scheme shows a hypothetical protein or protein complex that, in undergoing successive ROS-dependent oxidations, is progressively converted through relatively stable states to a final conformation that allows interaction with other similarly modified proteins to drive changes in cellular functions that accompany growth and development.

(a)

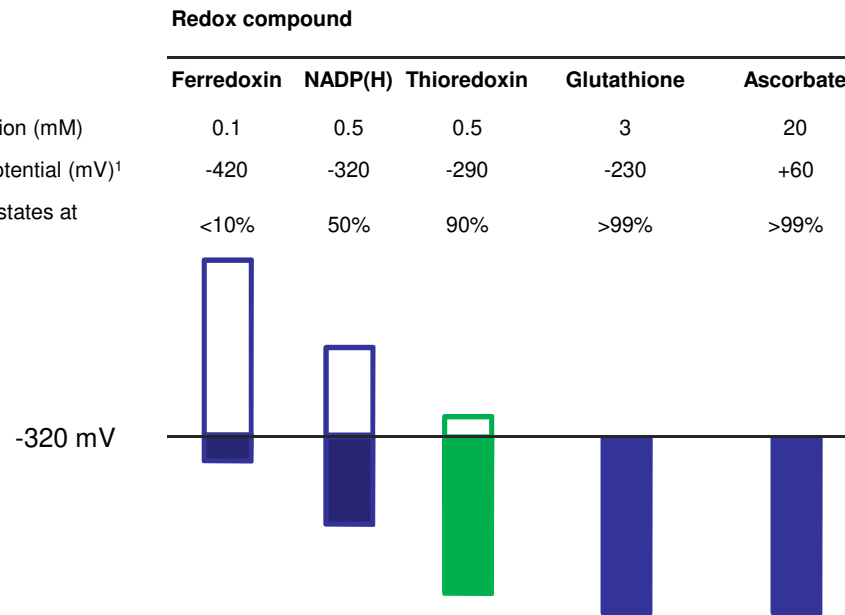


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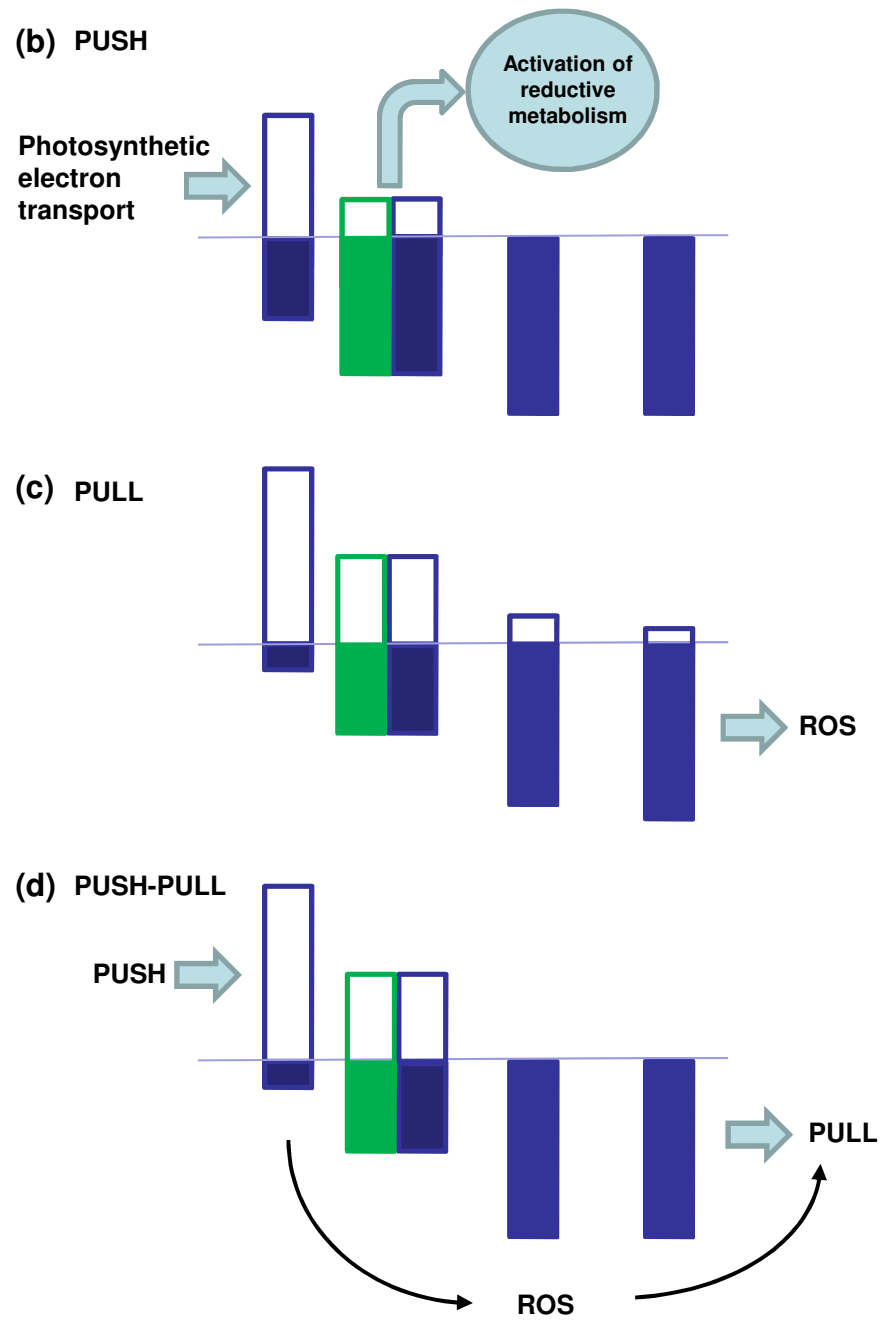


Figure 1 (continued)

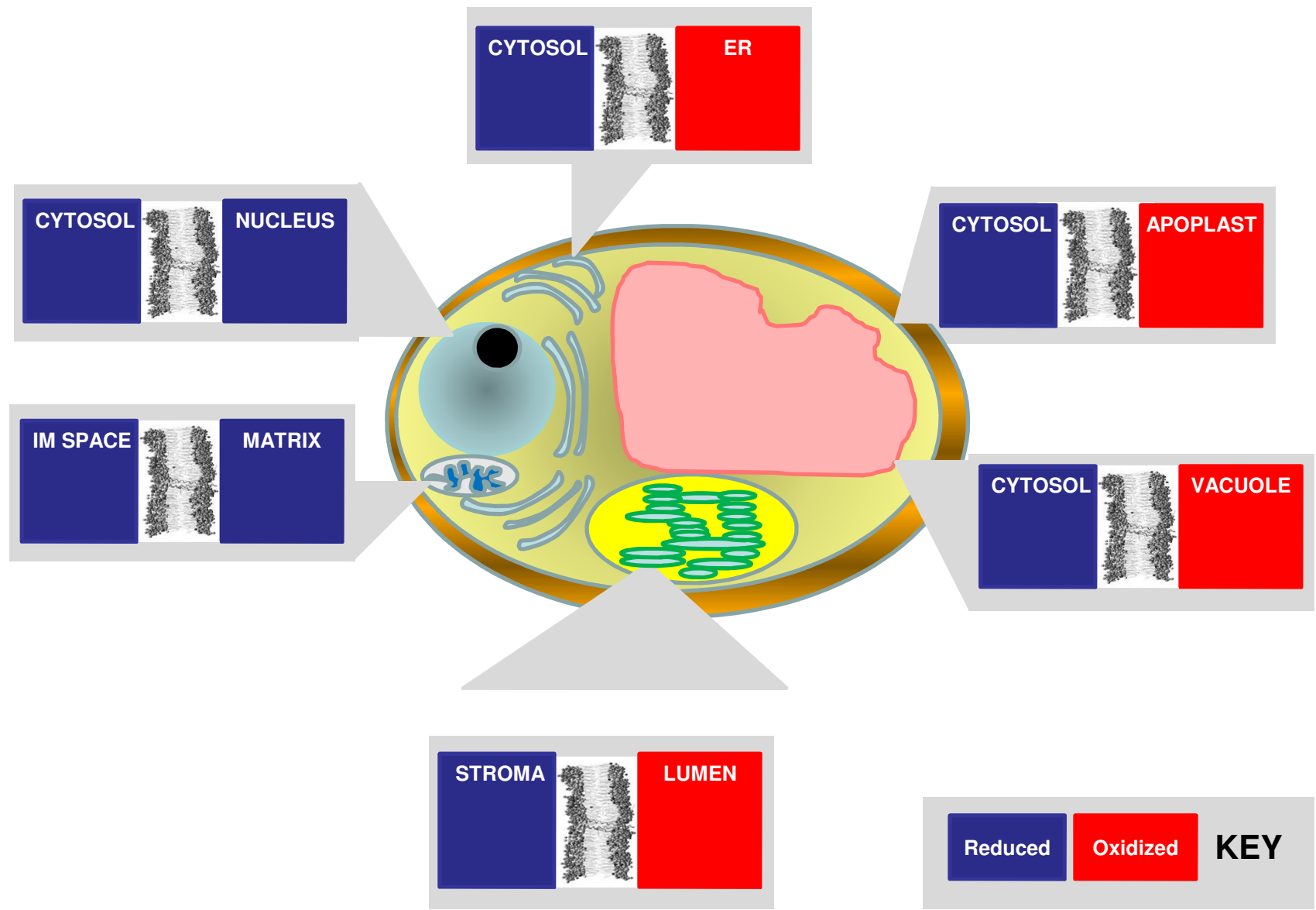


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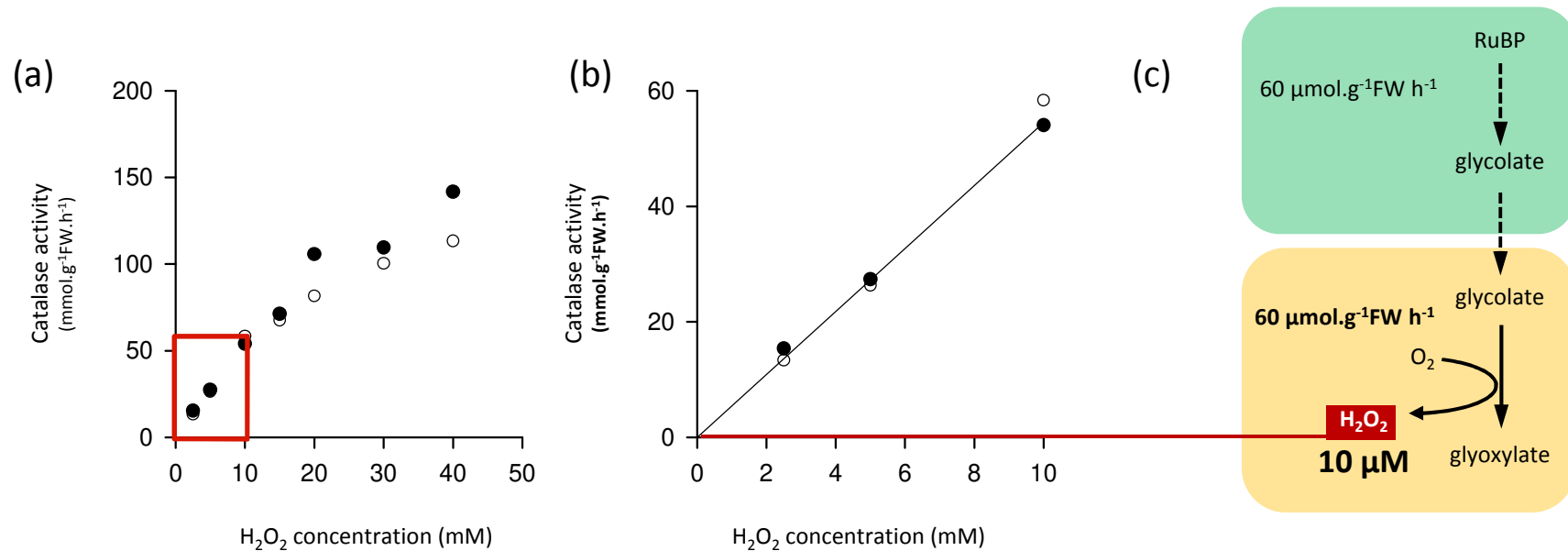


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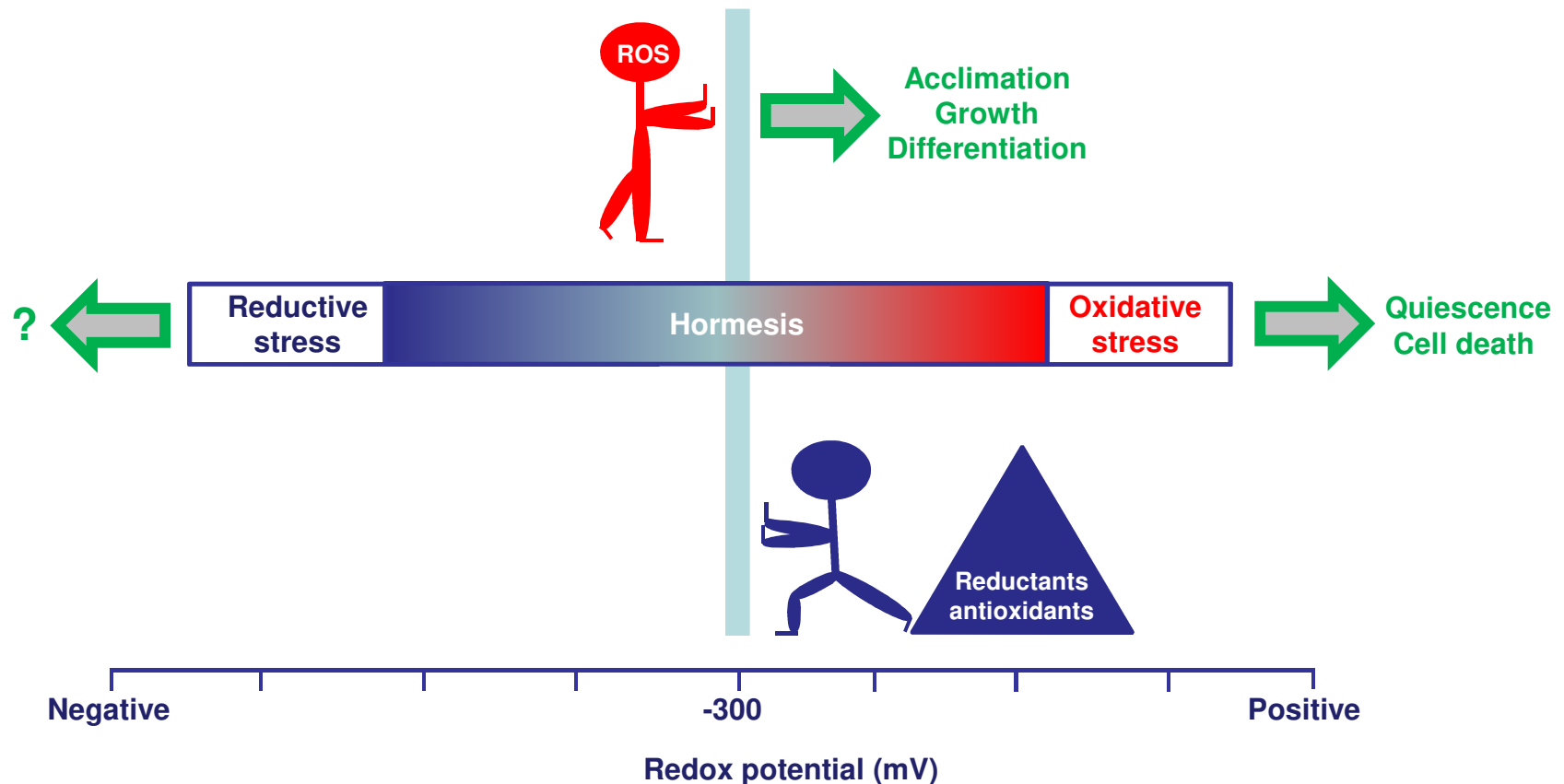


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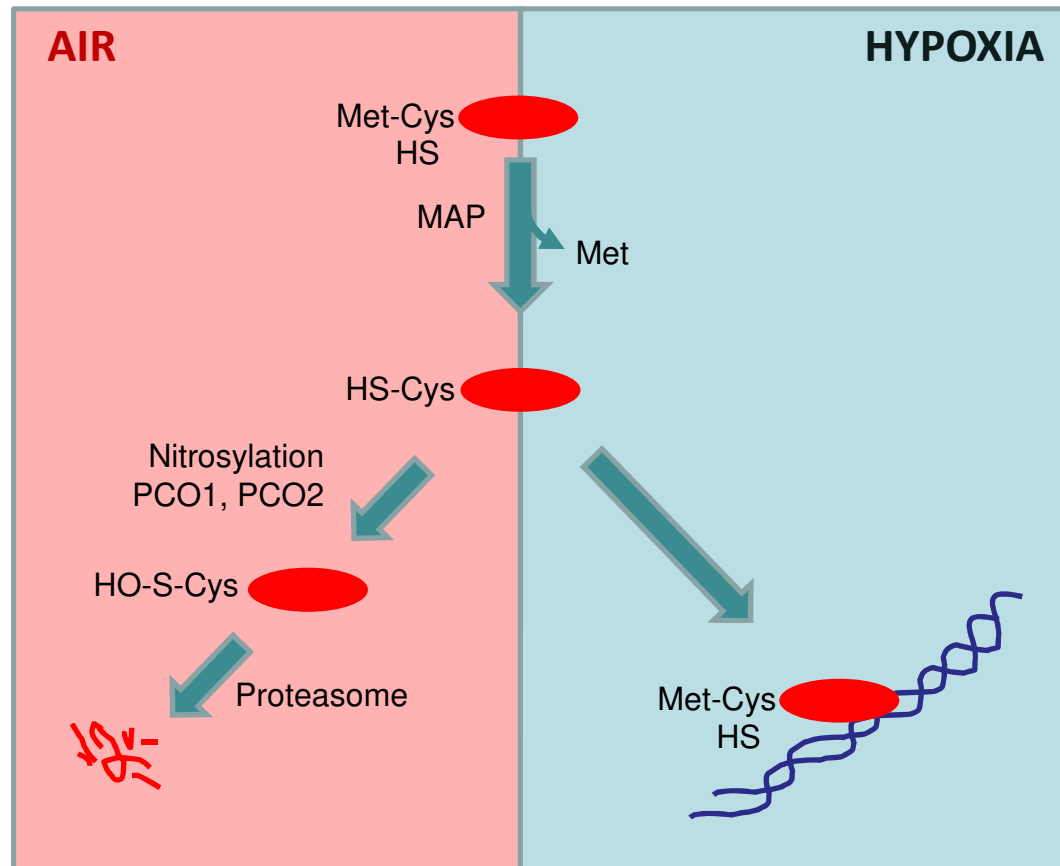


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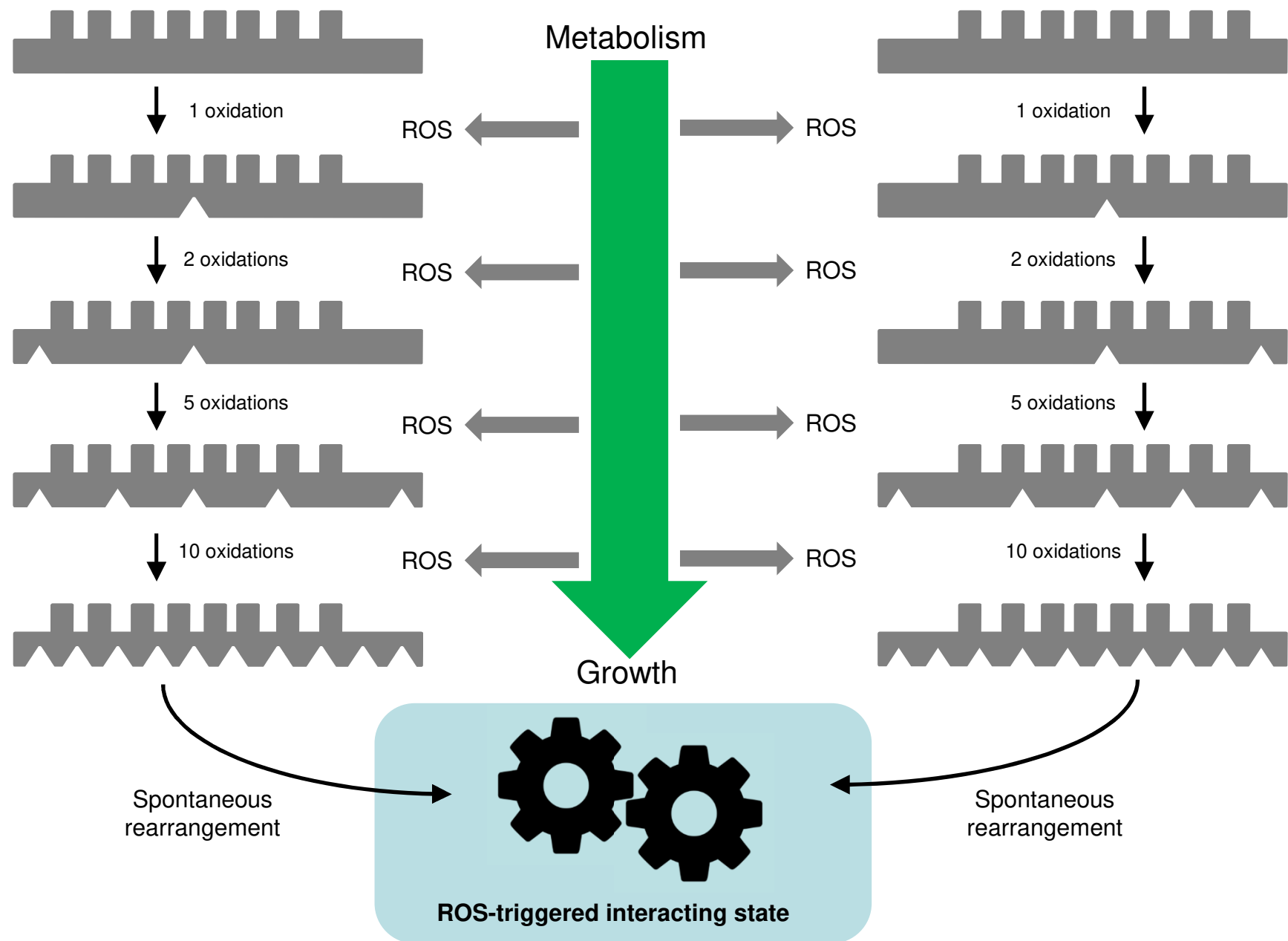


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