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EDITORS' FORWARD LOOK

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

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Abstract

Reactive oxygen species (ROS) have a profound influence on almost every aspect of plant biology. Here, we emphasize the fundamental, intimate relationships between light-driven reductant formation, ROS, and oxidative stress, together with compartment-specificdifferences in redox buffering and the perspectives for their analysis. Calculations of approximate H_2O_2 concentrations in the peroxisomes are provided, and based on the likely values in other locations such as chloroplasts, we concludethat much of the H_2O_2detected in conventional *in vitro* assays is likely to be extracellular. Within the context of scant information on ROS perception mechanisms, we consider current knowledge, including possible parallels with emerging information on oxygen sensing. Although ROS can sometimes be signals for cell death, we consider that an equally important role is to transmit information from metabolism to allow appropriate cellular responses to developmental and environmental changes. Our discussion speculates on novel sensing mechanisms by which this could happen and how ROS could be counted by the cell, possibly as a means of monitoring metabolic flux. Throughout, we place emphasis on the positive effects of ROS, predicting that in the coming decades they will increasingly be defined as hallmarks of viabilitywithin a changing and challenging environment.

Introduction

A simple perusal of the ISI scientific database reveals that more than 50 000 papers have been published on oxidative stress in plants in the last five years. This bears testimony to the ongoing and ever-growing interest in the multiple roles of this phenomenon in plant biology. While this and the older literature include key advances that have led to a better understanding of certain aspects of the field, there has been little clarification of other areas such that relatively few unifying concepts emerge. Inherent difficulties arise from the nature of reactive oxygen species (ROS). Reactive equals short-lived, meaning that the action of ROS is likely to be highly localized in many compartments. However, the lifetime of ROS varies between different compartments, because of differences in redox buffering, ie, the capacity of antioxidative systems to remove ROS. A set of discrete locations defines specificity in redox signalling in different compartments of the plant cell. Independent control and buffering permit redox-sensitive signal transduction to occur in locations such as the thylakoid lumen and the apoplast/cell wall, and across the membranes that separate these compartments from the highly redox-buffered spaces such as the stroma and cytosol. This concept underpins the interpretation of data on how plants respond to stress. For example, a hormone-induced H_2O_2 burst on the plasma membrane will not have immediate access to the same signalling routes as H_2O_2produced in the thylakoid membrane. Moreover, the lifetime is likely to be completely different. While many researchers accept this notion, it is rarely considered in data analysis, because of the absence of technologies that can explore compartment specificity. This forward look takes a back-to-basics look at some of the fundamental concepts underlying redox relationships, explores current concepts of compartmentalization, and presents our own personal perspective of future areas of exploration in ROS-related redox research in plants.

Context: understanding redox hierarchies and relationships between different redox couples

Given that plant cells may produce copious amounts of ROS even in optimal conditions, more than 99% of amounts produced inside the cell are likely to be metabolized through dedicated antioxidant systems. In the case of H_2O_2, the most stable ROS, increased production either accelerates dismutation by catalase or causes increased engagement of metabolic pathways that produce reductant to support peroxidases. Thus, unlike some other key signalling factors that are not biochemically transformed (eg, calcium ions), ROS are directly integrated into metabolism, and the associated redox reactions may cause profound changes in metabolite profiles (Noctor *et al.* 2015). Understanding the operation of the key redox couples that primarily interact with ROS is fundamental to any analysis of relevant signalling pathways in a given situation.

The chloroplast is a major sensor of environmental change and a driver of plant metabolism through photosynthesis and other biosynthetic pathways. The electron transport chain in this organelle transduces light energy to produce highly reducing compounds (ferredoxin, NADPH) that drive metabolism as well as the reduction of ascorbate and glutathione. The same highly reducing properties of the electron transport chain allow reduction of oxygen to superoxide and, thereafter, production of H_2O_2. They also drive the reduction of various stromal thioredoxins, which either contribute to antioxidant metabolism alongside ascorbate and glutathione or function as redox regulators that activate metabolic enzymes or chloroplast gene expression (Buchanan & Balmer 2005; Dietz & Pfannschmidt 2011). Recent evidence suggests that thioredoxin-dependent peroxiredoxins and thylakoid-bound ascorbate peroxidases have overlapping antioxidant functions in the chloroplast (Awad *et al.* 2015).

A redox couple is composed of oxidized and reduced forms of a component that can be interconverted by exchanging electrons with a second redox couple. Three examples of redox couples are Cu^{2+}/Cu^{+}, plastoquinone/plastoquinol, and NAD^{+}/NADH. For any givenredox couple, its midpoint potential is an intrinsic property (at standard conditions of pressure, temperature and pH) that allows its relative potential reducing/oxidizing power to be defined. From it, one can calculate the redox state of any compound at any given assumed actual redox potential. Table 1 shows the redox states of ferredoxin, thioredoxin, glutathione, and NAD(P) at a theoretical equilibrium potential of -320 mV, which is the midpoint potential of NADP(H). We emphasize that this is a theoretical value. It is close to values often cited for the “cellular redox potential” in animal cells (Schafer & Buettner 2001), but redox potentials in the soluble phase of plant cells may be more variable, particularly in the chloroplast. Further, as we discuss below, the accuracy or usefulness of the concept of a single cellular redox potential is not established.

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

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

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

Changes in the GSH:GSSG and ascorbate:dehydroascorbate ratios that are both physiologically relevant and detectable could be generated through a rapid rise in superoxideand H_2O_2production as these species react preferentially with antioxidants (Figure 1c, "PULL"). If a rapid burst of ROS production pulls electrons out of the ascorbate, glutathione, and thioredoxin pools faster than they can be replenished from NADPH and ferredoxin, the pools move out (or further out) from thermodynamic equilibrium and their oxidation would be observed. However, it is unclear how often the redox states of ascorbate and glutathione in the chloroplast drop appreciably below highly reduced values. The systems are highly integrated because most of the ROS within the stroma is produced by oxidation of reduced ferredoxin and upstream photosystem I (PSI) components by

molecular O_2 (Figure 1d).In other words, production of oxidants such as ROS is in fact favoured by excess of reductant. Thus, although this condition is often called “oxidative stress”, it could equally be referred to as “reductive stress”.

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

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

Compartmentationin redox signalling

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

Redox gradients in plant cells

The different compartments of plant cells are not all equally redox-buffered (Figure 2). While the chloroplast stroma is highly reducing, notably due to the large amounts of ascorbate, glutathione and other antioxidants, the lumen appears to have only limited antioxidant capacity. To date, no transporters for ascorbate or glutathione have been identified on the thylakoid membrane and therefore enzymes like violaxanthin de-epoxidase may be dependent on the diffusion of ascorbate from the stroma. The enzymes of the lumen also include photosystem II (PSII) repair cycle components and a number of redox-active cyclophilins(Järvi *et al.* 2015). One unresolved problem concerning the chloroplast stroma is how proteins are oxidatively folded in a highly reducing environment (Kieselbach2013**).** The best characterized example of a stromal redox component involved in folding is the cyclophilin 20-3, the only known cyclophilin in this compartment. This

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

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

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

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

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

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

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

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

While the concept of oxidative stress is widely accepted, less attention has been paid to reductive stress. Reductive stress of oxidized compartments such as the ER and apoplast could be just aschallenging and signal-transducing as enhanced oxidation is in the more reduced compartments. Treatment of leaves in the dark with an artificial reductant, dithiothreitol (DTT), caused many metabolic changes that were interpreted in terms of reduction of thioredoxins (Kolbe *et al.* 2006), which would normally be relatively oxidized in the dark, at least in the chloroplast (Figure 1). However, few indicators of stress were observed, although this may have been related to the relatively short duration of the treatment (1 h; Kolbe *et al.* 2006). Treatment with GSH or pyridine nucleotides for somewhat longer times is sufficient to trigger biotic stress signalling (Gomez*et al.* 2004; Zhang & Mou2009; Han *et al.*2013a), even though the total tissue contents of these endogenous redox compounds are often not greatly enhanced by the treatments. These effects may be partly triggered by changes in apoplastic redox state, although oxidized pyridine nucleotides (NAD^+, $NADP^+$) were shown to be just as effective as the reduced forms (Zhang & Mou 2009). One possibility is that the degradation products of these molecules may be perceived as signals (Noctor *et al*. 2015). In*Chlamydomonas*, DTT treatment induces some features of an autophagic response, which was proposed to result from ER stress, consistent with an increased total cellular GSH:GSSG ratio (Perez-Martin *et al.* 2014).

H_2O_2concentrations and compartmentation

Its relative stability and potential for reactivity with specific thiol groups make H_2O_2 a majorplayerin oxidative signal transmission. Despite its acknowledged importance, we still do not know what H_2O_2concentrations are likely to occur *in vivo* in each compartment under either optimal or stress conditions. This gap in our knowledge is an impediment to interpreting correctly the quantitative processes and signalling mechanisms occurring during oxidative stress. It is important to note that global measurements on homogenized tissue are highly variable(Queval *et al.* 2008). Although numerous papers have reported on increases in H_2O_2 in stress conditions, plants deficient in catalase, which might be considered a benchmark for H_2O_2accumulation, show minor, transient or undetectable changes in the extractable levels of this oxidant, even when many other indicators show that the plants are oxidatively stressed(Noctor *et al.*2015).

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

Estimating in vivo*H_2O_2concentrations*

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

Catalase has a very high capacity because (1) it is a highly expressed enzyme in C_3leaves and (2) its turnover rate is among the fastest known. However, its affinity for H_2O_2 is low : estimates of K_M values can exceed 100 mM (Del Rio *et al.* 1977). In agreement with this, catalase activities in Arabidopsis leaf extracts continue to accelerate as H_2O_2 concentrations increase up to at least 40 mM with the dependence on substratebeing almost linear below 20 mM (Figure 3a).

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

Arabidopsis leaves; Queval *et al.* 2007), we can make an approximate estimate of the H_2O_2 concentrations that are required to drive catalase activity to cope with typical photorespiratory flux. If the majority of photorespiratoryglycolate is metabolized by glycolate oxidase, the rate of H_2O_2 production is approximately equal to the rate of RuBP oxygenation (Figure 3c). In Arabidopsis grown in standard conditions, rates of net photosynthetic CO_2 assimilation in air do not exceed 10 $\mu molm^{-2}s^{-1}$ (eg, Veljovic-Jovanovic *et al.* 2001) and accompanying rates of RuBP oxygenation are probably around 40% of this value (Sharkey 1988). In this context, the key question is: what peroxisomal concentrations of H_2O_2 would be attained under these conditions of rapid photorespiration?

The leaf fresh mass:area ratio for Arabidopsis used to obtain the data shown in Figure 3 was measured as 260 g m^{-2}. AnRuBP oxygenation rate of 4 $\mu molm^{-2}$ s^{-1}therefore converts to slightly below60 $\mu molH_2O_2g^{-1}$ FW h^{-1} (= 4 x 3600/260). The *in vitro*catalase activity in Arabidopsis leaf extracts measured at 10 mM $H_2O_2$is approximately 1000 times higher than this estimate of *in vivo* production, and the high K_M value of the enzyme means that rates decrease linearly as H_2O_2 concentration falls below this value (Figure 3b). Standard catalase assays are not sensitive enough to allow accurate measurement of the activity at sub-millimolar substrateconcentrations. To derive an *in vivo* concentration from the *in vitro* assays, we have to assume that the relationshipbetween catalase activity and H_2O_2 concentration is linear between zeroand the lowest substrate concentration measured (2 mM H_2O_2). If this is so, a catalase activity of 60 $\mu molg^{-1}$ FW h^{-1} would be driven by an H_2O_2 concentration of 10 µM (Figure 3b,c).

These calculations illustrate that, despite its low affinity, catalase is able to maintain H_2O_2 concentrations at low values. Unless a substantial part of the catalase activity that can be measured in extracts is inactive *in vivo*, even comparatively high rates of production of H_2O_2 through photorespiration are unlikely to maintain global peroxisomal H_2O_2 concentrations much higher than 10 µM. Without inactivation or other forms of down-regulation, catalase would simply be too potent. In this connection, it is very interesting that several mechanisms have been identified that may be important in down-regulating catalase to drive phenomena such as biotic stress responses (Mhamdi *et al.* 2010b), although their precise *in vivo* importance remains to be established.

These calculations are necessarily approximate. Apart from the assumptions discussed above, other potentially complicating factors include the existence of catalase in a quasi-crystalline form in the peroxisomes. It is possible that when these structures are disrupted by extraction into a dilute solution, latent enzymes become exposed and able to contribute to the activity in the assay. This could cause the extent to which H_2O_2accumulates*in vivo* to be underestimated.Nevertheless, we

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

If 10 µM is a typical H_2O_2concentration inside the cell, intracellular pools would explain only a small part of values that are typically measured in extracts.The total contribution of peroxisomes, chloroplasts, mitochondria, and cytosolwould not greatly exceed 1 $nmol g^{-1}$FW (Table 2). In contrast, most quantitative assays of leaf H_2O_2in optimal conditions range from 100 $nmol g^{-1}$FW upwards, ie, about 100 times higher.Thismarked difference strongly suggests that currently used *in vitro* assays are unlikely to provide relevant information on the *in vivo*H_2O_2concentrations at these locations (although they might still reflect changes in rates of production or removal inside the cell).

Discounting artefacts in H_2O_2 extraction and assay, a possibility that is not to be dismissed lightly (Queval *et al*. 2008), the discrepancy between *in vivo*concentrations and *in vitro* estimates may be resolved if there is a large apoplastic pool of H_2O_2. However, if this is the only or main explanation, then more than 90% of the extracted H_2O_2 would be outside the cell. Assuming the apoplast accounts for 10% cell volume, then more than 90% of 100 nmol located in the apoplast of 1 g tissue would equate to a concentration of about 1 mM. This may not be an unrealistic concentration for the oxidative reactions required for cell wall biosynthesis and dynamics, although it would pose the question of what concentrations can be reached during oxidative bursts. A key point could be the proximity of ROS-generating enzymes such as peroxidases and NADPH oxidases to ROS-sensitive targets at the plasmalemma. While relatively high amounts of H_2O_2could also be localized in other oxidized compartments such as the vacuole (Figure 2), there is relatively little information available to address this question.

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

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

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

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

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

How are ROS perceived by the cell?

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

Despite their inflammatory name, the chemical reactivity of the most abundant ROS is quite limited. Hydrogen peroxide and superoxide are relatively poor oxidants. Even in the case of thiol groups, rate constants for oxidation are quite low, unless the group is located in an environment that promotes deprotonation to the thiolate anion. Free glutathione, whose thiol group has a pKa of about 9, reacts only very slowly with H_2O_2 and is oxidized much more rapidly by chemical reaction with dehydroascorbate (Rahantaniaina *et al.* 2013). The plethora of enzymes able to dismutate or reduce H_2O_2may in part be explained by the need to minimize production of the hydroxyl radical within the cell through Fenton-type reactions catalyzed by redox-active metal ions (Møller *et al*. 2011). This raises the question of whether ROS *per se* are signals or rather whether they give rise to signals through their metabolism. Direct oxidation of transcription factors has been well documented, as have effects of ROS on translation (Benina *et al.* 2015). In addition, as discussed above, ROS could produce a global shift in the main cellular redox buffers such as glutathione or thioredoxins that is then perceived by sensitive proteins that interact with these components. Specificity may be conferred by specific peroxidases that transmit oxidative signals to sensitive proteins such as transcription factors. One well-known example in yeast is Gpx3 that, once oxidized by peroxide, then oxidizes and activates the transcription factor, Yap1 (Delauney *et al.* 2003). Based on oxidation of roGFP, this has been proposed as a general mechanism of peroxide-based signalling (Gutscher *et al.* 2009). However, despite interest in this idea, there have been only very few reports of this kind of signalling in plants (Miao *et al.* 2006). Until more evidence is available, it is difficult to conclude on the importance of specific thiol-peroxidases as signal relays in plants, in which ROS orchestrate numerous processes of growth and development.

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

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

In the case of singlet oxygen, which is more reactive than superoxide or H_2O_2, some aspects of signalling have become clearer. However, much of the work has been done on a model system, the *flu* mutant, in which singlet oxygen may not be produced at what is generally considered to be its most physiologically important site (PSII). It has been shown that singlet oxygen can trigger carotenoid breakdown, leading to secondary reactive organic compounds (Ramel *et al.* 2012). These secondary compounds can themselves elicit similar changes in gene expressionto those caused by singlet oxygen (Ramel *et al.* 2012).Such signalling pathways reflect the reactivity of singlet oxygen, whichis unlikely to diffuse far before reacting with components that are in relatively close proximity.

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

Oxidative signalling and hypoxia

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

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

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

ROS: a key to monitoring metabolic flux by cells?

As the above discussion has emphasized, ROS production by cells may provide current status information although we do not know precisely how these oxidizing molecules are monitored. While components that respond to changes in ROS concentrations are no doubt involved, it is possible that ROS may also contribute to the molecular memory of stress through durable accumulation of redox information on metabolism. One may speculate on how ROS could form part of a molecular counting system. Since ROS signalling involves their biochemical transformation to more stable species, a key feature could be production of secondary states that have enough stability to act as information stores. This notion is obviously speculative but it is interesting to imagine what kind of biochemical system could act in molecular memory storage. Examples of information storage (or memory) exist in biological systems. Simple examples in plants are enzymes that have a gated system such as the ATP synthase that requires a certain number of protonsto pass through the CF_0 channel to drive conformational changes in CF_1 that are converted to condensation of ADP and phosphate to ATP. While this is an example of very limited “counting”, other phenomena such as D1 turnover in photosystem II provide examples of a more extensive counting system, allowing the PSII reaction center to turn over after a more or less precise number of photochemical events.

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

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

Conclusions and outlook

The concept that ROS and reactive nitrogen species (RNS) are key signalling molecules that facilitate a plethora of adaptive metabolic, molecular genetic and epigenetic responses is now established. Although we acknowledge that much of ROS action may be in close association with RNS signalling, for reasons of space we have focused specifically on redox biology linked to ROS. The notion that 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
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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
$NAD(P)^+ + 2\ e^- + H^+ \rightarrow NAD(P)H$		
Thioredoxin	-290	10
$Ox\ TRX + 2\ e^- + 2H^+ \rightarrow Red\ TRX$		
Glutathione	-230	2.1×10^5
$GSSG + 2\ e^- + 2H^+ \rightarrow 2\ GSH$		
Ascorbate	+ 60	6.9×10^{12}
$DHA + 2\ e^- + 2H^+ \rightarrow ASC$		

Table 2. Estimations of possible contributions of major H_2O_2-producing organelles to contents typically measured in extracts of leaves. A maximal compartmental concentration of 10 µM H_2O_2 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^{-1}FW, the main intracellular H_2O_2-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 $\mu mol.m^{-2}\ s^{-1}$. 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 $\mu moles.g^{-1}FWh^{-1}$ 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.

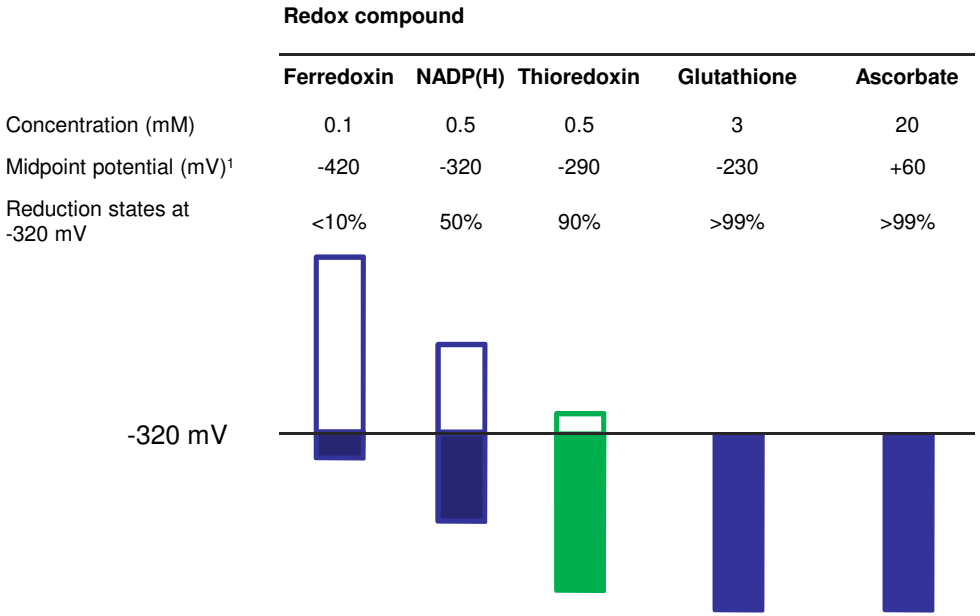
Schematic model showing how plant cells use reactive oxygen species (ROS) in signal transduction pathways leading to growth and acclimation responses. Hormesis theory considers that all biological systems show a range of different responses to potentially harmful toxins and chemicals including ROS. For 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 decreases 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 stimulusto 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 exampleROS-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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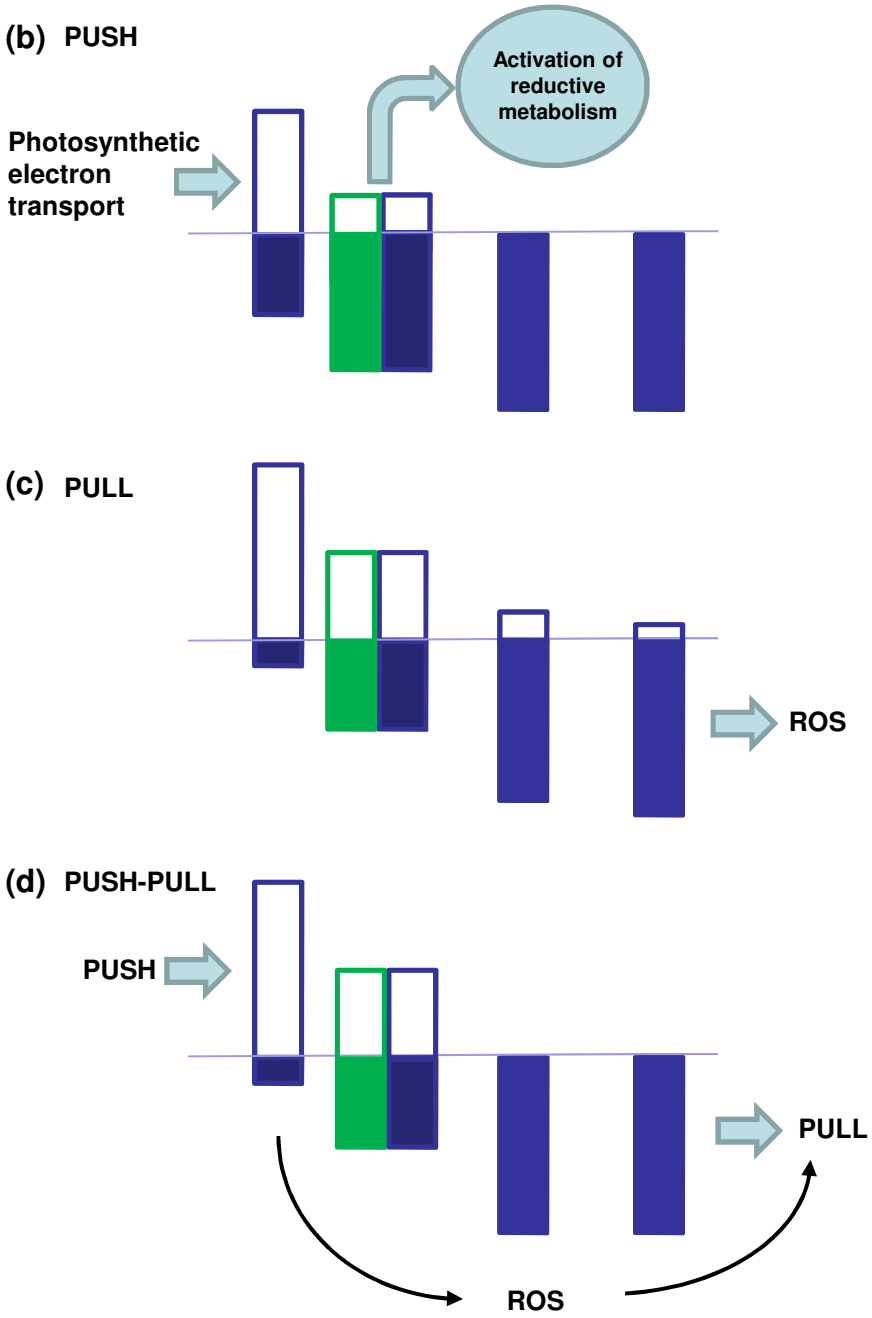
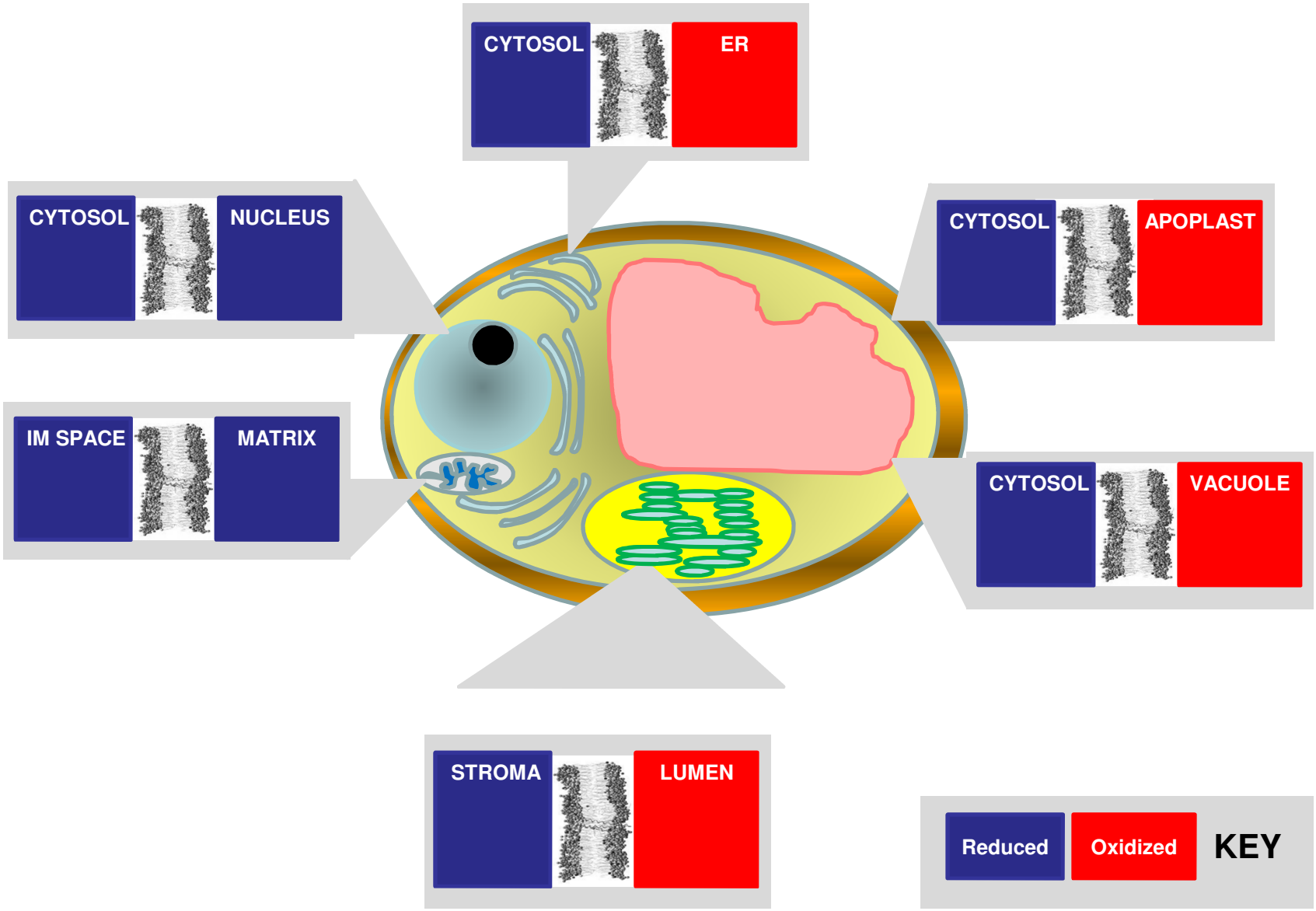


Figure 1 (continued)



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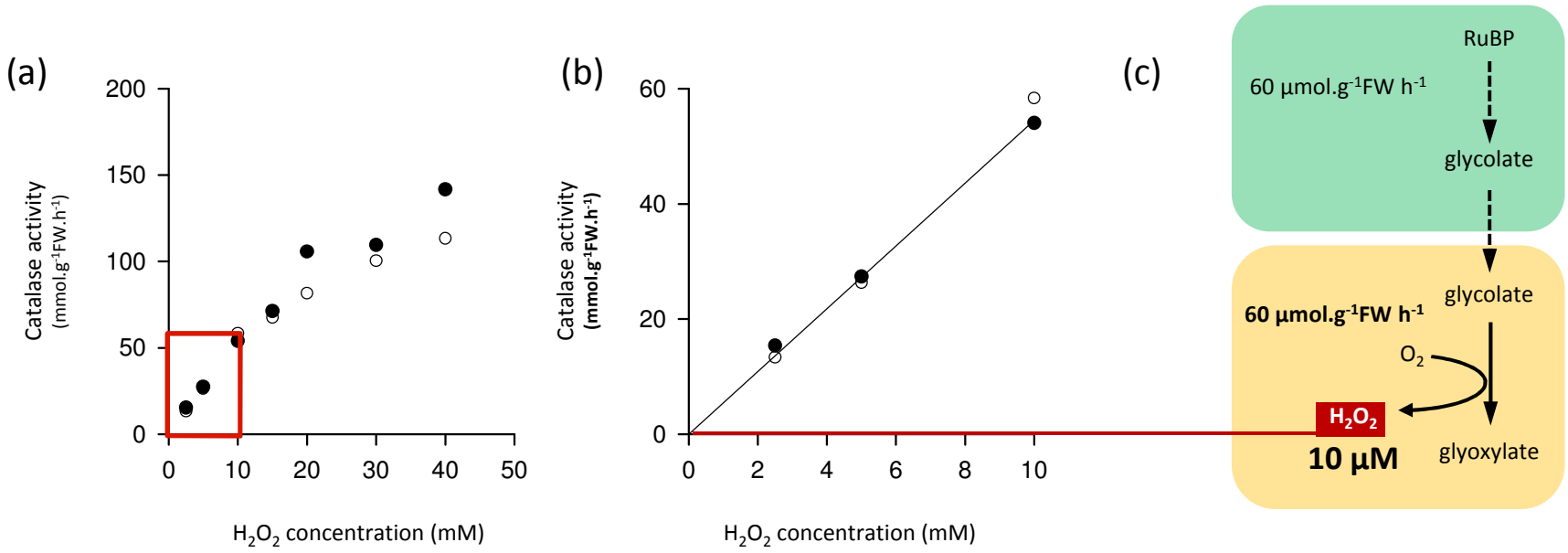

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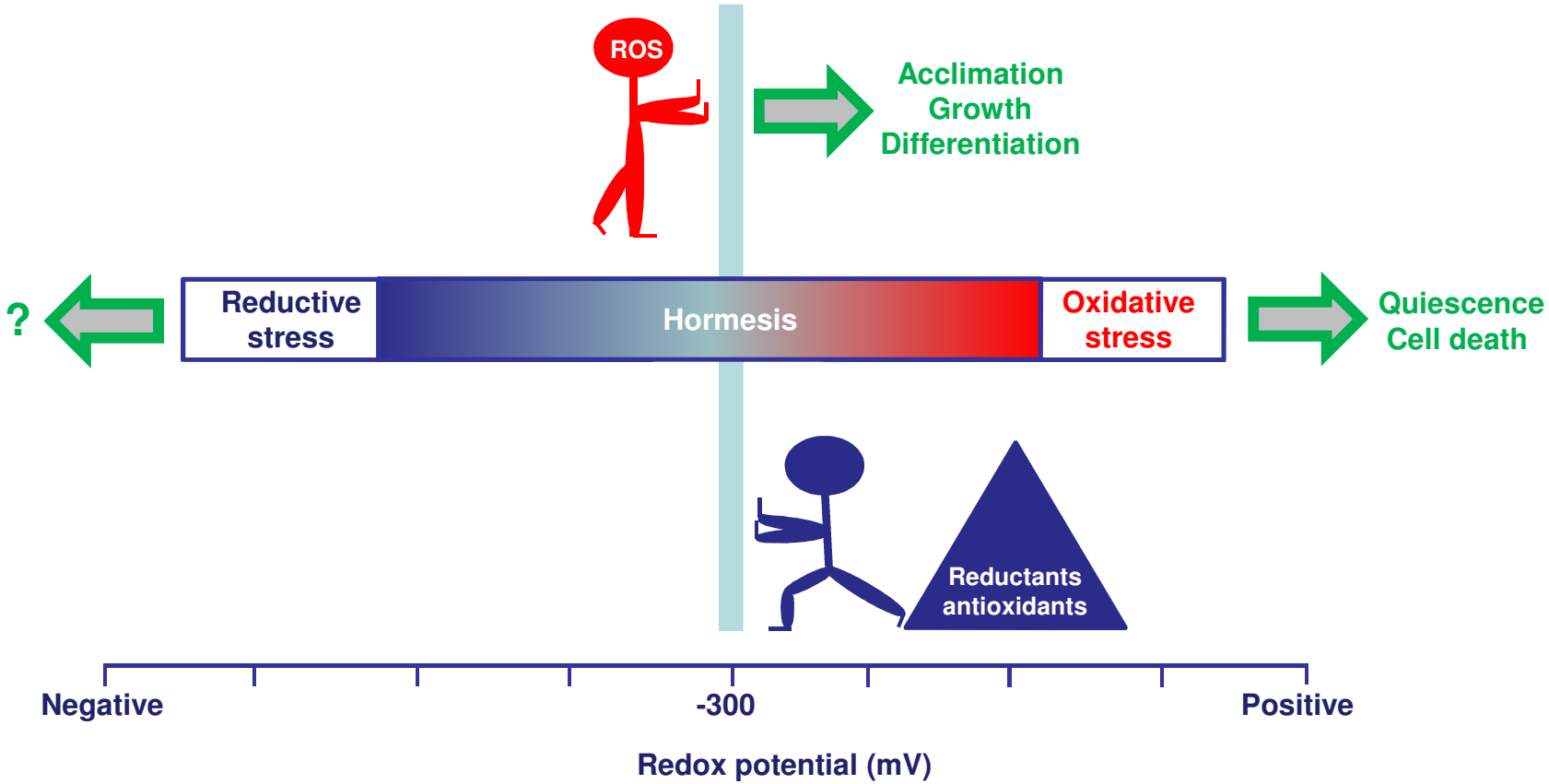

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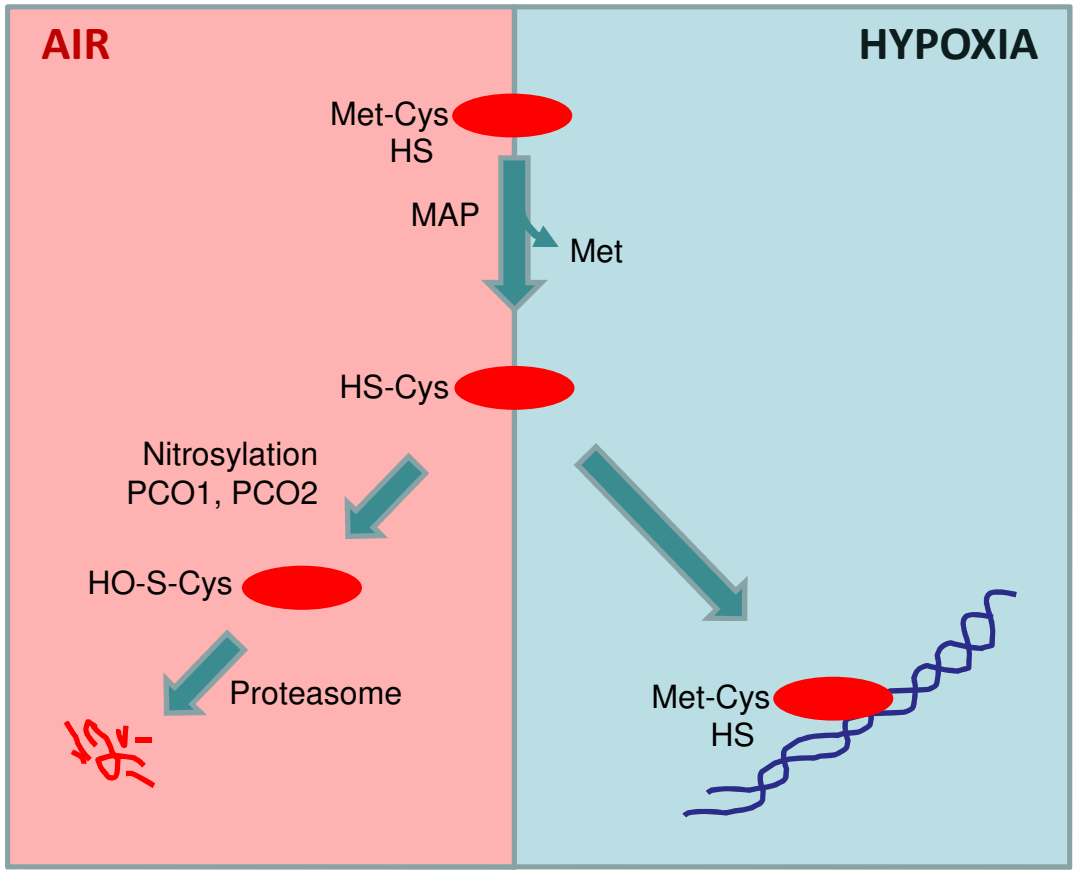

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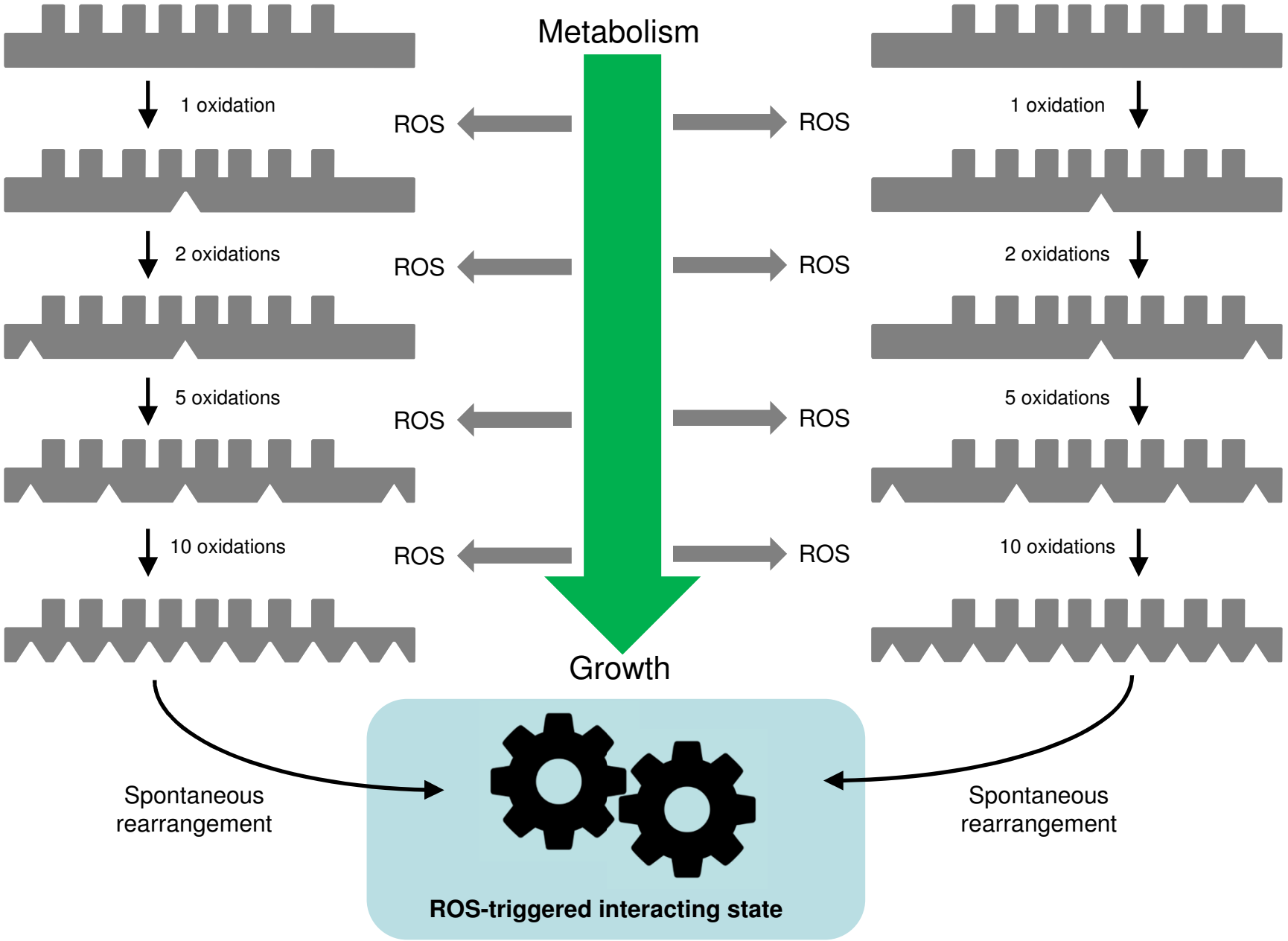

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