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# **Intracellular redox compartmentation and ROS-related communication in regulation and signaling<sup>[1][W]</sup>**

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## Footnotes

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## **Abstract**

Recent years have witnessed enormous progress in understanding redox signaling related to reactive oxygen species (ROS) in plants. The consensus view is that such signaling is intrinsic to many developmental processes and responses to the environment. ROS-related redox signaling is tightly wedded to compartmentation. Because membranes function as barriers, highly redox-active powerhouses such as chloroplasts, peroxisomes, and mitochondria may elicit specific signaling responses. However, transporter functions allow membranes to also act as bridges between compartments, and so regulated capacity to transmit redox changes across membranes influences the outcome of triggers produced at different locations. As well as ROS and other oxidizing species, antioxidants are key players that determine the extent of ROS accumulation at different sites and that may themselves act as signal transmitters. Like ROS, antioxidants can be transported across membranes. In addition, the intracellular distribution of antioxidative enzymes may be modulated to regulate or facilitate redox signaling appropriate to the conditions. Finally, there is substantial plasticity in organelar shape, with extensions such as stromules, peroxules and matrixules playing potentially crucial roles in organelle-organelle communication. We provide an overview of advances on subcellular compartmentation, identifying the gaps in our knowledge and discussing future developments in the area.

## **One-sentence summary**

We discuss the key roles of subcellular compartmentation and spatial redox transfer in signaling related to reactive oxygen and antioxidants.

**Key words:** Reactive oxygen species; antioxidants; photosynthesis, chloroplast; peroxisome; mitochondria; retrograde signaling; metabolite transport; signal transduction

## **Introduction**

Compartmentation in organelles is the key feature of eukaryotic cells, and is essential for appropriate partitioning of metabolism and other biological functions (Sweetlove and Fernie, 2013). Among other things, compartmentation allows differences in metabolite concentrations, because organelles are surrounded by one or more membranes that act as a barrier to passive diffusion. However, membranes can also act as bridges between the compartments they separate if they contain porins or transporters able to facilitate the regulated passage of metabolites or proteins. These basic principles are crucial to our understanding of cellular redox homeostasis.

Chloroplasts and mitochondria have unique energy-transducing functions leading to the generation and use of reducing power and production of ATP. Because the processes of photosynthetic and respiratory electron transport generally occur in an oxygen-rich environment, transfer of electrons or energy to oxygen is inevitable, leading to the formation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide ( $H_2O_2$ ), the hydroxyl radical, and singlet oxygen. In acting as an electron acceptor, oxygen has a regulatory function in alleviating electron pressure (over-reduction) in the chain, particularly during stress (Noctor et al., 2014). Together with peroxisomes, which generate superoxide and  $H_2O_2$  through multiple reactions, chloroplasts and mitochondria are the metabolic ROS powerhouses of plant cells (Foyer and Noctor, 2003). Because of their high capacity for ROS generation, it is often assumed that these organelles can accumulate high ROS levels. It is sometimes overlooked that, if this does occur, the resulting oxidative events will prohibit the classical functions of these organelles. Maintenance of metabolic functions is only possible if operating levels of ROS are kept at concentrations low enough to be compatible with processes such as carbon and nitrogen assimilation. This is achieved by regulation that ensures the smooth running of energy and electron flows in a fluctuating environment and by the presence of a battery of antioxidant systems. Maintenance of low ROS levels inside the cell is also crucial to allow regulated ROS-driven redox changes to be used for signaling purposes.

The functions of the plasmalemma and apoplast cell wall compartment are linked to their position as a dynamic interface between the cell and the outside world, with all its threats, challenges and opportunities. It is now clear that ROS are involved in systemic long-distance intercellular signaling (Miller et al., 2009). However, many basic processes involved in cell wall growth and dynamics require a highly oxidizing environment. Unlike the cell interior, the wall requires generation of strong oxidants such as the hydroxyl radical (Müller et al., 2009). Consequently, the apoplast has evolved a relatively low capacity for antioxidant accumulation, together with enzymes that actively remove these compounds (Pignocchi and

Foyer, 2003; Ohkamu-Ohtsu et al., 2007; Parsons and Fry, 2012). This means that the lifetime of ROS in the apoplast is much longer than inside the cell.

Our aim in this update is to provide a concise overview of current knowledge surrounding ROS-related redox compartmentation and its consequences for signaling in plant cells. We emphasize key recent advances in the light of current concepts. We also discuss data regarding oxidant and antioxidant concentrations and, where unambiguous information is not yet available, we propose likely values based on a consideration of indirect evidence.

### **Subcellular redox transport and compartmentation**

As outlined above, chloroplasts, peroxisomes, and mitochondria are the key redox-active compartments within plant cells. Cellular functions such as carbon assimilation, respiration, photorespiration, and gene expression in chloroplasts and mitochondria are made possible by a battery of enzymes that process ROS to keep their steady-state concentrations low. In chloroplasts, ascorbate peroxidases (APX) and 2-cys peroxiredoxins are the frontline defenses against H<sub>2</sub>O<sub>2</sub> accumulation (Dietz, 2011; Awad et al., 2015). Other compartments such as the mitochondria, peroxisomes, and cytosol contain APX. The reducing substrates required by these antioxidative peroxidases are supplied by several systems that depend on ferredoxin, NAD(P)H and glutathione (Foyer and Noctor, 2016). As well as these enzymes, the peroxisomes house catalases, which are required to avoid oxidative stress caused by high rates of H<sub>2</sub>O<sub>2</sub> generation linked to processes such as photorespiration (Mhamdi et al., 2012; Sandalio and Romero-Puertas, 2015).

In addition to keeping ROS levels low, antioxidants could play roles in transmitting ROS signaling. This is possible based on first principles according to which an antioxidant is defined as a compound that outcompetes others in reacting with ROS to give a relatively stable oxidized product. It is also supported by direct analysis of the roles of thiol compounds such as glutathione in signaling triggered by H<sub>2</sub>O<sub>2</sub> (Han et al., 2013). Other thiol-based antioxidants such as peroxiredoxins, thioredoxins, and glutaredoxins are likely to be involved in ROS signaling cascades (Dietz and Hell, 2015), perhaps in a similar way to processes occurring in yeast (Delauney et al., 2002). Differences in the composition and complement of such redox-active antioxidant components may be among the factors contributing to the specificity of signaling at each location (Fig. 1). In this way, cells could have the capacity to modulate the tightness of coupling between H<sub>2</sub>O<sub>2</sub> and signaling by modifying the metabolic pathway through which this ROS is metabolized (Fig. 1). Together with genetic and/or functional redundancy, this may be one reason why it has proved so difficult to identify generic ROS sensors and define discrete ROS signaling pathways.

Our knowledge of subcellular movement of redox-active metabolites such as H<sub>2</sub>O<sub>2</sub>, glutathione, and ascorbate is very incomplete. Despite intense interest in ROS-related redox signaling in plants, there has been only a relatively modest focus on the systems that could allow transport of these molecules across membranes. Nevertheless, work over recent decades has demonstrated uptake of ascorbate and glutathione into cells or subcellular organelles at rates well in excess of diffusion. Because of space limitations, we refer the reader to studies cited in other recent papers (Maughan et al., 2010; Szarka et al., 2013; Noctor et al., 2013; Fernie and Toth, 2015; Foyer, 2015). With the exceptions noted below, many of the transporters remain to be characterized at the molecular level.

Chloroplast and mitochondrial outer membranes are permeable to most metabolites, although attention has been drawn to possible selectivity even at this level (Bolter and Soll, 2001). The inner membranes are a significant barrier to metabolite movement, and transporters are therefore required (Fig. 2). Chloroplast inner envelope membrane transporters for glutathione (CLT-like) and ascorbate (AtPHT4.4) have been described in Arabidopsis at the molecular level (Maughan et al., 2010; Miyaji et al., 2014). Based on uptake studies on purified organelles, transporters for these metabolites presumably also exist on the mitochondrial inner membrane. Indeed, the mitochondria are rich in glutathione (Zechmann et al., 2008) despite little evidence that they can produce this compound. Although mitochondria are the site of the final step of ascorbate synthesis, the compound is produced in the intermembrane space, meaning that import into the matrix is required (Szarka et al., 2013). Several lines of evidence suggest that ascorbate may cross the inner mitochondrial membrane as the oxidized form, dehydroascorbate (DHA; Szarka et al., 2013). DHA can also be transported across the plasma membrane, while ascorbate is exported from the cell interior to replenish the pool present in the apoplast (Fig. 2). For glutathione, an unresolved issue concerns which transporters may be involved in determining redistribution of the disulfide form (GSSG) during oxidative stress. Furthermore, systems have not yet been characterized that ensure ascorbate transport across the thylakoid membrane into the lumen to support violaxanthin de-epoxidase activity and other biochemical functions.

Pyridine nucleotide pools in different compartments are linked by NAD transporters (Palmieri et al., 2009). Chloroplasts and mitochondria also house systems that can transfer NAD(P)-linked metabolites to ensure the exchange of redox equivalents. These systems allow indirect transfer of redox equivalents across membranes at much more rapid rates than can occur through direct transport of pyridine nucleotides, which are relatively large and highly charged metabolites. Dicarboxylate transporters can exchange redox equivalents as malate and oxaloacetate (Kinoshita et al., 2011). Such shuttles have long been considered to

play key roles in several areas of redox homeostasis. The redox-regulated chloroplast NADP-malate dehydrogenase is thought to function together with chloroplast envelope dicarboxylate transporters to avoid over-reduction in the stroma by transferring reducing equivalents to the cytosol (Scheibe et al., 2005). Functional analysis of *nadp-mdh* knockout mutants uncovered some evidence in support of this role though marked effects on phenotypes were not apparent (Hebbelmann et al., 2012). Another potentially important redox shuttle is made possible by the chloroplast envelope phosphate translocator, which can exchange triose phosphate for phosphate as a chloroplast-cytosol carbon exporter or triose phosphate for 3-phosphoglycerate as a reductant (and ATP) exporter. Based on observations on loss-of-function mutants, this transporter has been implicated in chloroplast-nucleus signaling (Vogel et al., 2014).

Although they are best known as channels enabling water transport across biological membranes, aquaporins have been shown to facilitate the movement of a wide range of metabolites, including H<sub>2</sub>O<sub>2</sub> (Henzler and Steudle, 2000; Bienert et al., 2007; Gomes et al., 2009). The emerging view is that, like water, H<sub>2</sub>O<sub>2</sub> movement between compartments requires the aid of a transport system (Bienert and Chaumont, 2014). As well as the plasma membrane and the tonoplast, aquaporins appear to be present on chloroplast and mitochondria membranes (Bienert and Chaumont, 2014). In the case of chloroplasts, these aquaporins were shown to be crucial to the movement of CO<sub>2</sub> (Uehlein et al., 2008).

Plant aquaporins present on the plasma membrane and tonoplast have been shown to be competent in H<sub>2</sub>O<sub>2</sub> movement, where they are assumed to play roles in H<sub>2</sub>O<sub>2</sub> signaling or regulation (Bienert and Chaumont, 2014). However, these studies have mainly been done in heterologous systems, and a key outstanding question is the direction of net movement within plant cells. In terms of the intracellular movement of H<sub>2</sub>O<sub>2</sub>, this is a difficult issue to resolve. It has been discussed in terms of the balance between H<sub>2</sub>O<sub>2</sub> production rates and sink strength (Henzler and Steudle, 2000). Based on the ROS-producing capacities of chloroplasts, peroxisomes and mitochondria, one might expect them to influence ROS movement as net exporters. However, these organelles also house highly active antioxidative enzymes, meaning that they could also function as ROS sinks. The relative importance of these two opposing functions could be dependent on conditions that alter organellar composition.

### **Subcellular distribution of H<sub>2</sub>O<sub>2</sub>, antioxidants, and pyridine nucleotides**

The short lifetimes of superoxide, the hydroxyl radical, and singlet oxygen make them unlikely candidates to diffuse over appreciable distances within the cell. In contrast, H<sub>2</sub>O<sub>2</sub> is more stable and attempts to quantify this molecule in extracts of plant tissues often produce quite high values (100-200 nmol.g<sup>-1</sup> FW,

although substantially higher values can also be found in the recent literature). Accurate determination of subcellular metabolite concentrations is a challenging task, notably because disruption of organelles or transporter activities may alter metabolite distribution during sample preparation. For redox metabolites, these problems are potentially compounded by instability (particularly of redox states) following tissue disruption. Non-aqueous extraction of organelles from tissue or very rapid fractionation of protoplasts (within seconds) can minimize these problems. More recent approaches have involved *in situ* labelling with specific antibodies for ascorbate and glutathione (Zechmann et al., 2008, 2010).

Based on information obtained using these approaches, Table 1 presents values for ascorbate, glutathione and pyridine nucleotide concentrations and reduction states (Heineke et al., 2001; Igamberdiev and Gardeström, 2003; Szal et al., 2008; Zechmann et al., 2008, 2010; Smirnoff, 2011; Queval et al., 2011). Notable features are: (1) ascorbate is highly reduced in all compartments apart from the vacuole and apoplast, where DHA can accumulate either because of ascorbate oxidase or because of the lack of regeneration systems; (2) like ascorbate, glutathione in extracts is highly reduced in the absence of stress; indeed, *in situ* analysis suggests that the reduction state is very high in many compartments (Meyer et al., 2007), with most of the GSSG present in the vacuole or apoplast; (3) pyridine nucleotide pools are poised at more reduced states in the mitochondria than in the cytosol and chloroplast, which contain more oxidized pools. Relatively oxidized pools of NAD and NADP are probably required for the operation of glycolysis in the cytosol and the photosynthetic electron transport chain in the chloroplast.

In the case of H<sub>2</sub>O<sub>2</sub>, there is little reliable information available in the literature on concentrations in different compartments. Table 1 provides approximate estimates of likely concentrations in the absence of stress, based on the following reasoning. As noted above, measured H<sub>2</sub>O<sub>2</sub> contents in plant extracts are rarely lower than 100 nmol.g<sup>-1</sup>FW, translating to a global concentration of around 100 μM if H<sub>2</sub>O<sub>2</sub> is uniformly distributed. This is well above accepted concentrations in organisms such as animals and yeast. Where is all the H<sub>2</sub>O<sub>2</sub> in plant cells? For the intracellular compartments other than the vacuole, the estimates in Table 1 are based on (1) the affinities of the major H<sub>2</sub>O<sub>2</sub>-removing peroxidases localized in these compartments; (2) the known inhibition of important enzymes by H<sub>2</sub>O<sub>2</sub> in the low micromolar range; and (3) inferred concentrations in the peroxisome, which is commonly considered to be a compartment relatively rich in H<sub>2</sub>O<sub>2</sub> (Foyer and Noctor, 2016). The vacuolar concentration is estimated assuming that H<sub>2</sub>O<sub>2</sub> will mainly result from movement from the cytosol without active transport, tending to equilibrium on both sides of the tonoplast. We infer the high concentrations in the apoplast based on the presence of numerous ROS-producing systems, a relatively weak antioxidative system, and the need to account for reported values of H<sub>2</sub>O<sub>2</sub>. A ceiling concentration of 10 μM inside the cell (under unstressed conditions)

would account for less than 10% and 1%, respectively, of measured tissue contents of  $100 \text{ nmol.g}^{-1}$  FW and  $1 \mu\text{mol.g}^{-1}$  FW.

We emphasize that the  $\text{H}_2\text{O}_2$  concentrations shown in Table 1 are speculative and that, as we discuss further below, accumulation in specialized structures such as vesicles should also be taken into account. It should also be noted that Table 1 refers largely to mesophyll cells and does not consider tissues such as the vasculature, where  $\text{H}_2\text{O}_2$  concentrations remain poorly characterised. Changes in the apparent  $\text{H}_2\text{O}_2$  concentrations of vascular tissues can be substantial during development or responses to stress. For example, a substantial increase in leaf  $\text{H}_2\text{O}_2$  contents following acclimation of the Mediterranean shrub, *Cistus albidus* L. to summer drought was ascribed mainly to the accumulation of this molecule in mesophyll cell walls, xylem vessels, and developing sclerenchyma cells (Jubany-Mari et al., 2009). Possible compartmentation of this type needs to be taken into account in assessing the relevance of  $\text{H}_2\text{O}_2$  contents measured in whole tissue extracts to  $\text{H}_2\text{O}_2$  concentrations in specific subcellular compartments, eg, photosynthesizing chloroplasts.

### **The cytosol as a key site of redox signal integration**

Organelles, together with ribosomes and the cytoskeleton, are embedded in the cytosol. Redox reactions taking place in this compartment are essential for the maintenance of the metabolic competence of the cell and for the regulation of translation (Benina et al., 2015). The cytosol is therefore much more than a buffer zone between the organelles and the nucleus, particularly since post-translational modifications of the translational apparatus allow very fast and highly effective control of protein synthesis (Moore et al., 2015). Regardless of effects on transcription rates, oxidative signaling can be mediated via the regulation of translation and hence protein production (Branco-Price et al., 2008).

Few sources of ROS in the cytosol itself are very well characterized yet this compartment contains a complete ascorbate-glutathione pathway as well as other enzymes that could play antioxidative roles. Where does cytosolic  $\text{H}_2\text{O}_2$  come from? This question cannot as yet be answered unequivocally but it may largely originate from organelles such as chloroplasts, peroxisomes, and mitochondria, as well as import from the apoplast. For instance, in *cat2* mutants deficient in catalase, considered to be mainly or exclusively a peroxisomal enzyme, cytosolic antioxidative systems are the most strongly induced at the level of transcripts (Rahantiania et al., 2013). Studies of the effects of mutations for cytosolic antioxidative enzymes in the wild-type and *cat2* background point to a key role for this compartment in dealing with excess  $\text{H}_2\text{O}_2$  and the resulting signaling (Davletova et al., 2005; Vanderauwera et al., 2009;

Mhamdi et al., 2010). Measurements with redox-sensitive green fluorescent proteins (roGFP), which are thought to detect the glutathione redox potential, show that stresses such as wounding or drought produce a more oxidized cytosol (Meyer et al., 2007; Jubany-Mari et al., 2010). These changes in the cytosolic redox potential may play some role in oxidative signaling but the mechanisms remain to be elucidated. It is clear that thiol-related redox changes drive key cytosol-nucleus signaling in some stresses, such as in pathogenesis-related (PR) signaling (Tada et al., 2008). It may be that stress-induced changes in glutathione redox potential accompany an integrated cellular response involving multiple related components such as thioredoxins, glutaredoxins, and nitric oxide. Glutathione S-transferases, which are encoded by a relatively large gene family in plants, may also be important, possibly through as yet undescribed functions related to signaling (Dixon and Edwards, 2010).

### **Organellar redox signaling: reaching out to the nucleus**

Chloroplasts and mitochondria have retained a small but essential part of their ancestral bacterial genomes. Because most of the proteins within these organelles are now encoded by nuclear genes, there has to be intimate communication to ensure coordinated expression of components involved in photosynthesis, respiration, and other processes. This notably involves the modification of nuclear gene expression by signals originating in organelles, a process referred to as retrograde signaling (Kleine and Leister, 2016).

In the case of the chloroplasts, there is direct regulation of gene expression within the organelle by the reduction state of photosynthetic electron transport chain components, notably plastoquinone and the cytochrome b<sub>6</sub>f complex. This involves regulation of protein kinases located in the thylakoid membrane and in the stroma that, together with thioredoxin z, control the function of the plastid polymerase complex (Allen, 2015). It is less clear how the redox state of the photosynthetic electron transport chain influences the expression of nuclear genes, even though it has been clear for many years that this occurs (Karpinski et al., 1997).

Among the possible signals, the roles of ROS have been much discussed (Galvez-Valdivieso and Mullineaux, 2010). Other signals such as heme or its breakdown products are probably also important. Breakdown products such as red chlorophyll catabolite have also been implicated. ACCELERATED CELL DEATH2 (ACD2) is involved in chlorophyll catabolism in chloroplasts but is also localized to mitochondria (Pattanayak et al., 2012). Specific targeting of the ACD2 protein to the mitochondria decreases tissue amounts of toxic intermediates produced in chloroplasts, pointing to roles for these compounds in interorganellar dialog

during cell death responses (Pattanayak et al., 2012). Retrograde signaling can also be transmitted by the modification of transmembrane proteins bound to organelle outer membranes. An example of this mode of signaling is the PTM protein, a plant homeodomain transcription factor whose N-terminal domain can move to the nucleus following proteolytic cleavage (Sun et al., 2011).

Analysis of multiple datasets led to the conclusion that different types of ROS produced at different subcellular sites trigger distinct transcriptomic signatures (Gadjev et al., 2006). However, the influence of other factors related to growth conditions could affect, to some extent, the transcriptomic signature that is produced (discussed further below).

### **How are ROS signals perceived and transmitted to the nucleus?**

One possibility is that redox changes within organelles are transmitted to the external membrane, where a receptor protein relays the signal to cytosolic pathways (Fig. 3). As yet, little is known about such putative receptors in ROS-related organelle-nucleus signaling, but precedents do exist. For example, in abscisic acid (ABA) signaling, receptor proteins have been reported on the chloroplast envelope amongst other places (Shen et al., 2006). Once ROS-dependent signals reach the cytosol, they may largely be transmitted by mitogen-activated protein kinase (MAPK) cascades that are well known to be important in stress responses (Fig. 3). Indeed, MPK6, a known player in biotic stress responses, has recently been implicated in chloroplast-to-nucleus retrograde signaling (Vogel et al., 2014). MAPK signaling modules may also be important in transmitting ROS-related signals originating from mitochondria (Fig.3).

Another possibility is that redox metabolites such as ROS are transported from organelles (Fig. 2). As noted above, technical issues make this a difficult question to resolve. Given the short lifetime of ROS within the cell, it is unclear to what extent this could function as a signal-transducing mechanism. Cytosolic *apx1* mutants show oxidative stress and decreased photosynthetic performance under high light conditions (Davletova et al., 2005), pointing to some link between chloroplastically produced ROS and cytosolic pools of H<sub>2</sub>O<sub>2</sub>. However, it is remarkable that 2-cys PRX in the same compartment, rather than APXs in other compartments, compensate for loss of thyakoid APX function (Awad et al., 2015). This observation points to discrete pools of H<sub>2</sub>O<sub>2</sub> within the cell, within only limited exchange between them. Nonetheless, the concept of flow from organelles or the apoplast into the cytosol remains an important tenet in the field. In the case of the apoplast, the details of events responding to ROS produced in this compartment are becoming increasingly well defined (Wrzaczek et al., 2013). Redox-sensitive soluble and membrane-bound receptors are likely to transmit the redox signal to the cell interior. However, although the details of the

signaling pathways are being elucidated (Wrzaczek et al., 2015), information on the identity of the initial targets that are affected by ROS is still relatively scarce. These targets, which are presumably subject to ROS-induced oxidation, may include lipids or sensitive Cys residues on proteins such as ion channels (Garcia-Mata et al., 2010).

Although such ROS-sensing mechanisms exist at the cell surface, it is still assumed that some of the H<sub>2</sub>O<sub>2</sub> produced in the apoplast signals via its movement to the cytosol through aquaporins and subsequent effects on organelles such as the chloroplast. As yet, it is not clear how this happens but studies of mutants lacking both catalase and NADPH oxidase functions (AtRbohD or AtRbohF) clearly suggest that processes such as salicylic acid (SA) and related defense signaling can be regulated by interplay between apoplastic and intracellular ROS (Chaouch et al., 2012). A recent modelling analysis suggests that high-frequency signaling through waves of H<sub>2</sub>O<sub>2</sub> traveling over micrometer distances in the cytosol is possible. The authors also reported that the interplay between H<sub>2</sub>O<sub>2</sub> production and its removal by the antioxidative system is crucial to set signal amplitude and frequency (Vestergaard et al., 2012). In this analysis, the source of H<sub>2</sub>O<sub>2</sub> was assumed to be the plasmalemma, presumably due to apoplastic ROS production and the activity of aquaporins.

Many phytohormones and signaling compounds whose synthesis occurs at least partly in the chloroplast (eg, ABA, SA, jasmonates, lipid peroxides, strigolactones) are intimately linked to ROS-dependent signaling. It should be noted, however, that in most of these cases, activation of phytohormone synthesis has to be preceded by ROS signal transduction to the nucleus to up-regulate the expression of the appropriate synthesis pathway genes. One notable exception is lipid peroxidation products, which can be generated in a singlet oxygen-dependent fashion during stresses such as excess light (Triantaphylidès et al., 2008). Further, singlet oxygen may modify gene expression through carotenoid breakdown products, some of which are volatile (Ramel et al., 2012). Although its exact relationship to ROS status is not clear, one example of a redox-related factor that is transported across the chloroplast envelope is the metabolite 3'-phosphoadenosine 5'-phosphate (PAP), a by-product of sulfur assimilation in the plastids (Estavillo et al., 2011). Stress-induced increases in PAP leads to its movement to the cytosol through the PAPS translocator, allowing it to somehow modify nuclear gene expression (Estavillo et al., 2011). Given that photosynthetic carbon metabolism is generally working orders of magnitude faster than sulfur assimilation in the light, export of malate and triose phosphate from the chloroplast may influence cytosolic (and nuclear) redox state on more rapid timescales than PAP (Scheibe et al., 2005; Vogel et al., 2014).

A number of ANAC and WRKY transcription factors have been implicated in both chloroplast and mitochondrial retrograde signaling (Kleine and Leister, 2016). One example is the role of ANAC013 in mitochondrial retrograde signaling, although the association of this protein with the mitochondria is not fully established and ANAC013 may also play roles in transmitting signals generated at other locations (De Clercq et al., 2013). The mechanistic links between mitochondrial ROS or other redox factors and nuclear gene expression remain elusive. Interestingly, mitochondrially located Cys-containing proteins that may contribute to retrograde signaling have recently been identified (Wang et al., 2016).

### **Organellar extensions: a hot line to the nucleus?**

As noted above, transport of ROS or related compounds from organelles (Fig. 4A) could theoretically transmit signals to the exterior to affect translation or transcription (or both). While the short lifetimes of ROS may be considered to limit the effectiveness of such mechanisms, chloroplasts, mitochondria and peroxisomes are dynamic rather than fixed structures. All three organelles can produce extensions in the form of fluid-filled tubules that contain the soluble components of the compartments. These structures are known as stromules (chloroplasts), matrixules (mitochondria), and peroxules (peroxisomes; Scott et al., 2007; Foyer and Noctor, 2007).

It has been known for some years that peroxule formation is stimulated by oxidative stress (Sinclair et al., 2009). Recently, it has been reported that the application of H<sub>2</sub>O<sub>2</sub> or SA to tobacco leaves stimulates rapid generation of stromules (within 1h; Caplan et al., 2015). Moreover, formation of stromules is regulated by a specific protein localized on the chloroplast outer envelope membrane, named CHUP1 (chloroplast unusual positioning 1). In tobacco knockdown or Arabidopsis knockout lines for CHUP1, spontaneous formation of stromules was favored, leading to enhanced PR responses and associated pathogen-induced cell death (Caplan et al., 2015). This study reported transfer of signaling proteins and metabolites, such as H<sub>2</sub>O<sub>2</sub>, to the nucleus directly from the chloroplast (Caplan et al., 2015).

It is worth noting that all kinds of plastids, not just chloroplasts, can form stromules, and that they can associate closely with the plasma membrane and mitochondria, as well as the nucleus. This suggests that direct transfer of proteins and metabolites between these organelles and the apoplast can occur. This offers a route for passage of redox components that is alternative to transmembrane transport systems (Fig. 4B). As noted in the introduction, massive ROS accumulation is not compatible with classical organellar functions. It is often assumed that organelles such as chloroplasts form a homogeneous population within cells or even tissues. This is clearly not the case because analysis of chloroplast composition at different

positions in the leaf reveals considerable heterogeneity that has been attributed to light piping effects (Vogelmann et al., 1996). Thus, different chloroplasts, even within the same cell, may have different functions. Chloroplasts that are more susceptible to photoinhibition, or that possess down-regulated antioxidant systems, could be primed to function mainly as ROS-producing organelles, rather than as photosynthetic ones.

In addition to stromules, chloroplasts produce a number of different types of vesicles that enter the trafficking system. These have been implicated in the degradation of stromal proteins but they could also carry metabolites such as ROS or other redox-active compounds like chlorophyll catabolites. Moreover, the formation of ROS-containing vesicles has been documented at the plasma membrane during biotic stress and in the vesicular-trafficking system during stresses such as high salt (An et al., 2006; Leshem et al., 2006).

### **Longer-term signaling downstream of ROS**

The idea that ROS act as signals at low concentrations but cause damage at higher concentrations is still pervasive in the literature. However, at least some types of ROS-induced cell death are mediated by signaling processes, rather than accumulated damage, and specific proteins are involved in cell death execution (Wrzaczek et al., 2013). In view of this, we would suggest that signaling is commonplace but that accumulated damage is quite rare. One reason is that the cell houses a plethora of policing systems that scour the horizon for oxidatively modified proteins and either repair them or remove them. The autophagy pathway is a major route for the removal of oxidized proteins. Autophagosomes assemble in the cytosol to remove oxidatively modified structures as large as organelles. Interestingly, impaired chloroplast antioxidant function is sufficient to trigger this pathway (Cheng et al., 2016).

Spatial range and temporal scale are likely to be key factors that distinguish different types of signaling. While there is ever-increasing focus on rapid signaling, both within and between cells (Miller et al., 2009; Verstergaard et al., 2013), many ROS-dependent events occur on a longer timescale. In this respect, we can distinguish rapid ROS signaling that does not involve oxidative stress, perhaps occurring over seconds to minutes, and longer term signaling (hours to days) in which sustained ROS production is necessary to reach some threshold perturbation of cell redox state that activates signal pathways (Foyer and Noctor, 2016). One of the best studied of the latter processes is in PR signaling, involving salicylic acid and other phytohormones. While this involves initial oxidative events, it also requires adjustment of cell redox state to allow subsequent reductive activation of signaling (Tada et al., 2008).

Ascorbate and glutathione transporters are likely to catalyze exchange at significantly lower rates than high-capacity metabolite transporters such as the phosphate translocator. However, they can still play important roles because redox signaling is clearly not restricted to short timescales. In plant responses to certain pathogens, changes in intracellular thiols that are associated with activation of NPR1 occur over a period of hours (Vanacker et al., 2000). In addition to activation of NPR1, changes in thiol status may be required for up-regulation of SA synthesis itself. Mutants with impaired function of a chloroplast enzyme involved in glutathione synthesis no longer accumulated this compound in response to intracellular oxidative stress. As a result, the mutants were less able to accumulate SA and to induce resistance-related mechanisms (Han et al., 2013). Interactions between a specific cyclophilin and a sulfur assimilation enzyme may be an important part of the signaling that allows the chloroplast to export thiol compounds to the cytosol (Park et al., 2013). Indeed, it has been shown that chloroplast glutathione transporters are required to allow the cytosol to achieve a redox environment that is appropriate for optimal activation of NPR1 (Maughan et al., 2010).

Key chloroplastic steps of cysteine and glutathione synthesis are up-regulated by oxidative stress at both transcriptional and post-transcriptional levels (Hicks et al., 2007; Gromes et al., 2008; Queval et al., 2009). In *cat2* mutants, where the initial oxidative stress trigger is peroxisomal, activation of glutathione synthesis is linked to GSSG accumulation (Queval et al., 2009). Much of the GSSG accumulated in the *cat2* mutant is localized in the vacuole but chloroplastic contents are also increased, suggesting that increased oxidation of the chloroplast glutathione pool may be one factor that activates the neosynthesis of this key redox buffer (Queval et al., 2011). This could be explained by transmembrane movement of oxidants such as H<sub>2</sub>O<sub>2</sub> into the chloroplast. However, movement of DHA and GSSG across the envelope may also occur in such contexts, because these compounds can be taken up at significant rates by isolated chloroplasts (Anderson et al., 1983).

### Concluding remarks

ROS-related signaling does not occur in a vacuum. Numerous studies have highlighted the highly integrated nature of many signal transduction pathways. In the case of ROS, interacting factors extend far beyond recognized interactors such as NO and calcium to encompass compounds such as phytohormones and sugars. This underscores the central positioning of ROS signaling at the interface between metabolism and developmental or environmental responses (Foyer and Noctor, 2005). It also implies that even the same

ROS produced at the same location may not always trigger a uniform and predictable response. One good example showing that ROS signaling is highly dependent on context comes from analysis of the effects of ROS produced in photorespiration. Excess ROS production in the peroxisomes triggers oxidative stress responses that are dependent on growth day length (Chaouch et al., 2010). Growth in long days allows peroxisomal H<sub>2</sub>O<sub>2</sub> to trigger PR responses that are not apparent in plants grown in short days, an effect that is linked to protein phosphorylation status (Li et al., 2014). Long day contexts also favor development of lesions in response to equal-time exposure of *Arabidopsis* to ozone, a condition that initially triggers an initial ROS burst and oxidative stress in the apoplast (Dghim et al., 2013).

The search for ROS sensors goes on, with much work focusing on the sulfenome and other thiol-related pathways (Waszczak et al., 2014). These pathways will undoubtably turn out to be influential. However, by analogy to what is now known about signaling through phytohormones such as ABA, a multiplicity of ROS receptors is likely to exist in numerous compartments (apoplast, cytosol, organelles). This would circumvent the need for reactive molecules to move great distances and could explain observations such as transcriptomes that are specific to different types or ROS or to the same type of ROS produced at different locations (Gadjev et al., 2006). It would also allow plasticity of responses in terms of strength and kinetics, and perhaps allow the plant to distinguish patterns of ROS production generated during different conditions, as illustrated in Figure 5. A key point is that this may allow the directionality of oxidative stress to be perceived. By monitoring the relative intensity of redox stimuli at different locations, the cell may be able to decode stress-specific ROS-dependent signatures, and this could be part of the activation of stress-appropriate responses.

Uncertainties remain surrounding H<sub>2</sub>O<sub>2</sub> concentrations in plants and the distribution of this key oxidant between different compartments. Unequivocal information is needed on this point to inform investigations of redox signaling, and to allow proper evaluation of the physiological significance of *in vivo* and *in vitro* data. Such information will also be required for modelling analyses that are likely to become increasingly important in unravelling the complexity of regulatory networks. The development of new *in situ* probes such as HyPer (Esposito-Rodriguez et al., 2013) should add crucial information on this point. Nonetheless, even the concept of a single ROS concentration specific to a given compartment may be too simplistic, because of heterogeneity in organellar composition and function. In the case of the chloroplast, for example, photosynthesis involves interconnected chains of highly energetic and inter-dependent reactions. While such chains are working smoothly, ROS accumulate (and indeed must accumulate) to relatively low concentrations. However, relatively simple changes in a subset of chloroplasts would be sufficient to interrupt the smooth flow of energy and electrons, leading to massive increases in ROS accumulation at

these sites. Numerous mechanisms can be imagined by which this could happen. Just some examples are: inactivation of the water-splitting system; inhibition of electron transport by endogenously produced inhibitors or secreted effectors; switching off non-photochemical quenching of excitation energy; down-regulation of Rubisco and other Benson-Calvin enzymes; and inhibition or withdrawal of antioxidative systems. Programmed disruption of redox and energy flows could transform metabolic factories into killer organelles, with high concentrations of ROS reaching other compartments through transporters or stromules. Such changes may be crucial in allowing chloroplasts or other ROS-generating organelles to play key executor functions in processes like programmed cell death and senescence. While recent reports have focused on ROS, organellar extensions could also transfer other compounds such as hormones or reductants. For instance, changes in nuclear glutathione status that occur during the cell cycle (Diaz-Vivancos et al., 2010) may at least partly depend on stromule formation since this redox-active compound is produced through enzymes that are mainly localized in the chloroplast.

**Table I.** Estimated or measured concentrations of H<sub>2</sub>O<sub>2</sub> and major soluble redox couples in different subcellular compartments. Estimated values for H<sub>2</sub>O<sub>2</sub> are in italics. Typical leaf contents are given for Arabidopsis growing in moderate light and otherwise standard conditions. Figures in parentheses indicate approximate redox states (% reduced form). Sources are given below the table.

	H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	Ascorbate <sup>b</sup>	Glutathione <sup>c</sup>	NADP(H) <sup>d</sup>	NAD(H) <sup>d</sup>
Typical leaf content nmol.gFW (% reduced)	200	3000 (90)	300 (95)	20 (50)	30 (20)
<b>Concentrations (mM)</b>					
Cytosol	≤ 0.01	21 (≥95)	3.5 (≥99)	0.3 (50)	0.7 (10)
Chloroplast	≤ 0.01	10 (≥95)	0.9 (≥99)	0.9 (40)	0.2 (<10)
Mitochondrion	≤ 0.01	10 (≥95)	6.5 (≥99)	0.3 (80)	2 (70)
Peroxisome	≤ 0.01	23 (≥95)	3.6 (≥99)	n.m.	n.m.
Nucleus	≤ 0.01	16 (≥95)	4.9 (≥99)	n.m.	n.m.
Vacuole	≤ 0.01	2 (20)	0.03 (≤10)	n.m.	n.m.
Apoplast	1?	n.m. (20)	n.m. (≤10)	n.m.	n.m.

<sup>a</sup>Estimated approximate values. See text for discussion.

<sup>b</sup>Immunolocalization (Zechmann et al., 2010)

<sup>c</sup>Immunolocalization (Queval et al., 2011)

<sup>d</sup>Non-aqueous or rapid fractionation (Heineke et al., 1991 ; Igamberdiev and Gardeström, 2003 ; Szal et al., 2008)

n.m., not measured.

## Figure legends

**Figure 1.** Compartment-specific signals due to the presence or absence of an antioxidant signaling component that interacts with H<sub>2</sub>O<sub>2</sub>. The cartoon shows a hypothetical example in which the compartment on the left has two modes of H<sub>2</sub>O<sub>2</sub> removal, one of which does not lead to signaling (heme-based enzyme such as APX or catalase). The other mode involves a thiol-based enzyme in which oxidation alters the association kinetics with its interactor (yellow box) so that the component is free to bind to a different interactor. The compartment on the right lacks this signaling mechanism. Such specificities could explain differences in ROS sensitivity between different compartments or between the same compartment under different conditions. The figure shows a simple example based on a thiol-disulfide exchange mechanism but other reactions may be important, and the difference between compartments could be quantitative rather than all-or-nothing.

**Figure 2.** Transporters linked to ascorbate, glutathione and redox exchange in the chloroplast and mitochondria and on the plasma membrane. Yellow, ascorbate transporters. Gray, aquaporin or other H<sub>2</sub>O<sub>2</sub> transporter. Blue, glutathione and glutathione precursor transporter. Orange, dicarboxylate transporter. Question marks indicate transporters that are as yet uncharacterized at the molecular level. ASC, ascorbate. DHA, dehydroascorbate.  $\gamma$ -EC,  $\gamma$ -glutamylcysteine. Mal, malate. OAA, oxaloacetate.

**Figure 3.** Organellar redox signaling via in situ sensing and signal export through specific pathways linked to activation of receptors located on the outer organelle membranes. MAPK, mitogen-activated protein kinase.

**Figure 4.** The cytosol: integrator hub or bypassed onlooker? A, Classical transporter functions allow chloroplast, peroxisomes and mitochondria to feed information into cytosolic hubs that transmit the signal to the nucleus. B, Feeding information to the nucleus through organellar extensions. The scheme shows a stromule relaying stromal status to the nucleus but analogous extensions are possible from the peroxisomes (peroxules) and mitochondria (matrixules).

**Figure 5.** Multiple receptors located at different sites allow identification and integration of signals. The blue barrel shapes indicate the various ROS receptors that may be quite diverse in chemical nature and that may each give rise to a characteristic signaling cascade. The figure shows one receptor at each of the following locations: soluble apoplast, plasma membrane, cytosol, chloroplast, chloroplast envelope, peroxisome, mitochondrial, mitochondrial membrane. However, each location may have more than one

type of receptor and there may be intricate interplay between them. In this way, the ROS language could be rich in vocabulary with a well-defined grammar.

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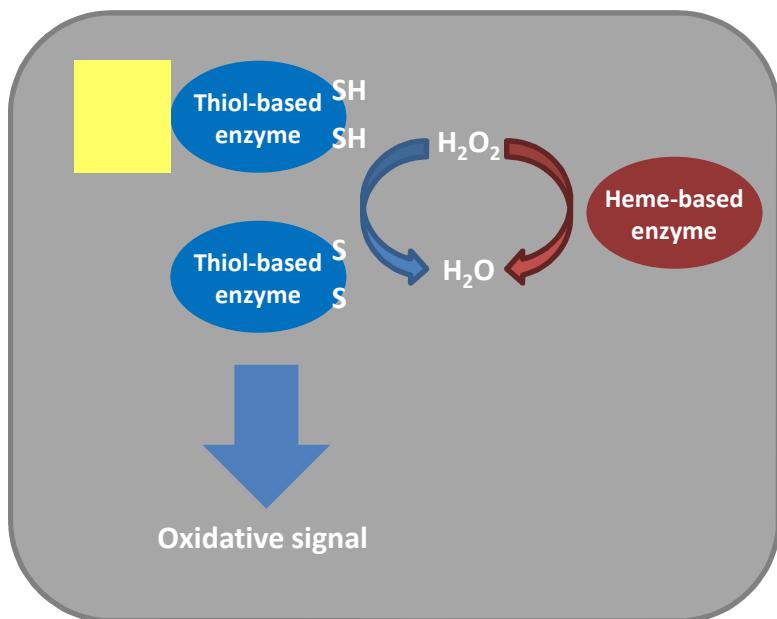
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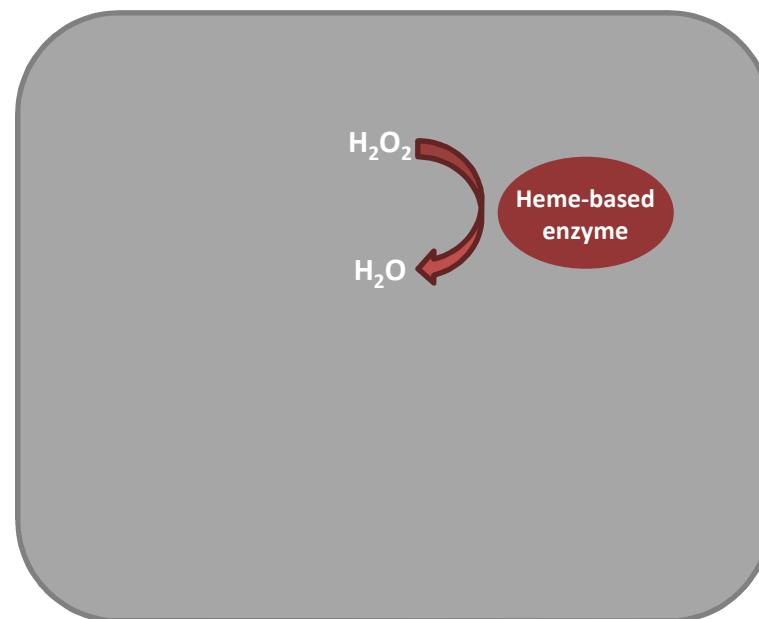
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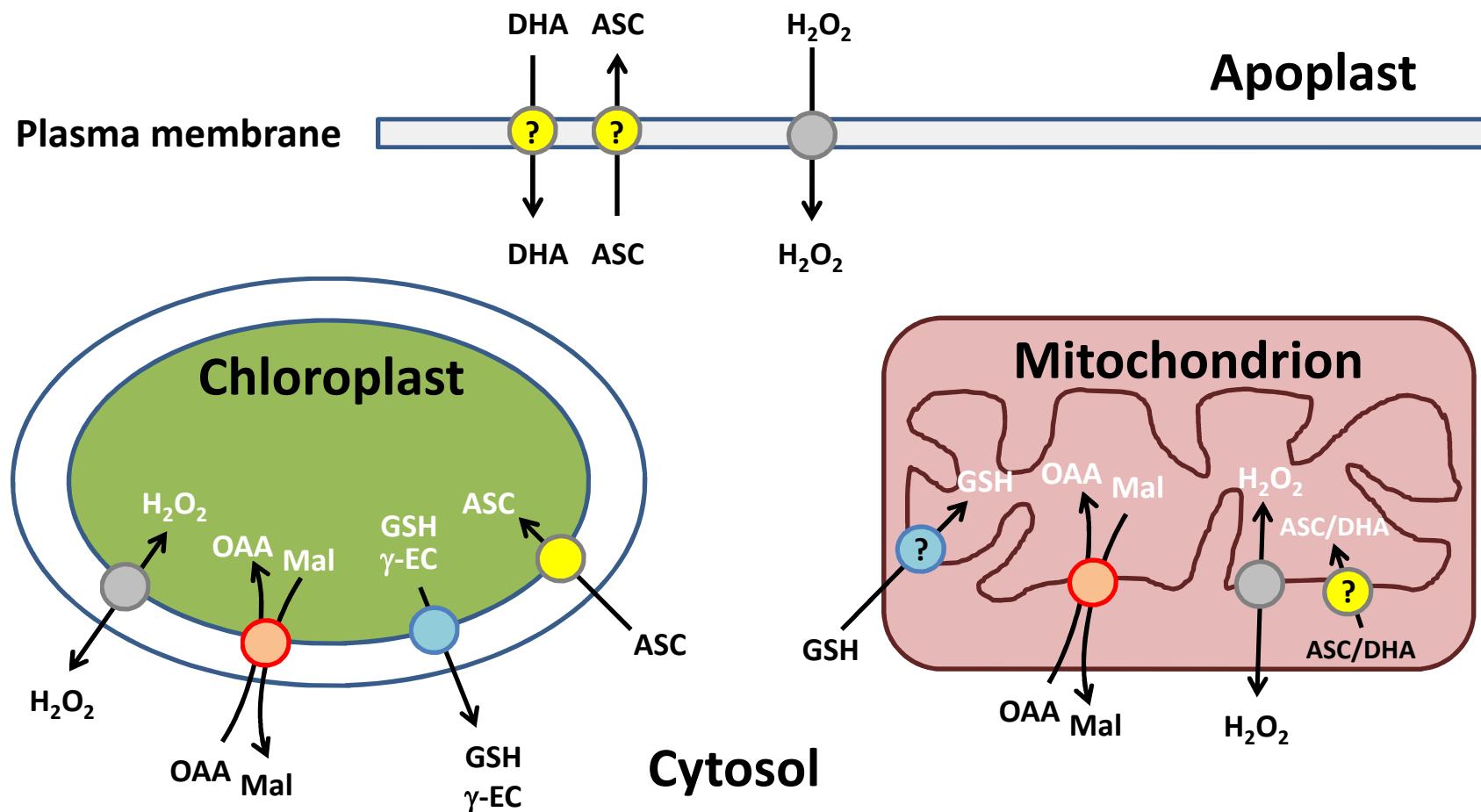
### Signal-coupled H<sub>2</sub>O<sub>2</sub> metabolism



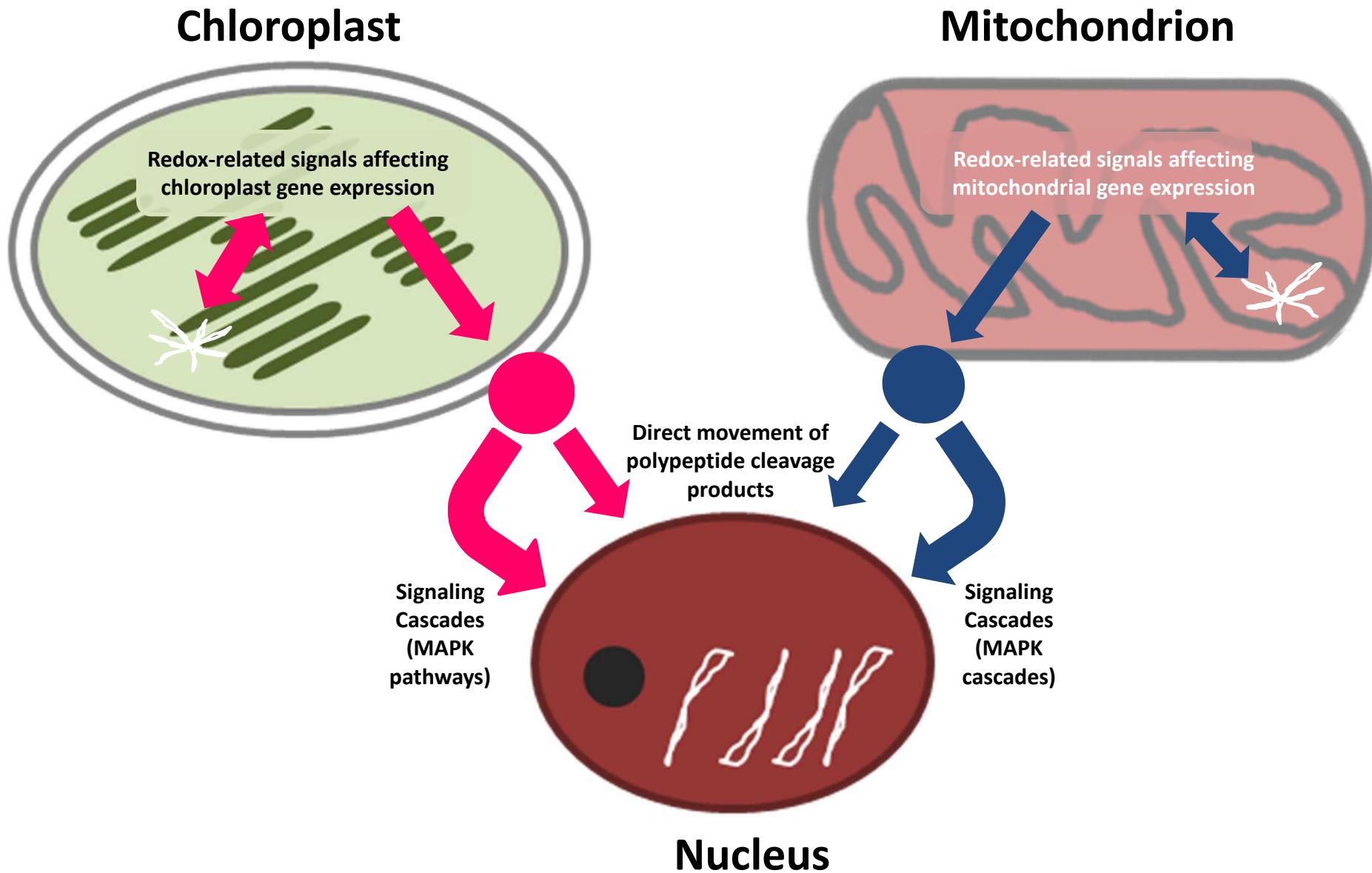
### Signal-uncoupled H<sub>2</sub>O<sub>2</sub> metabolism



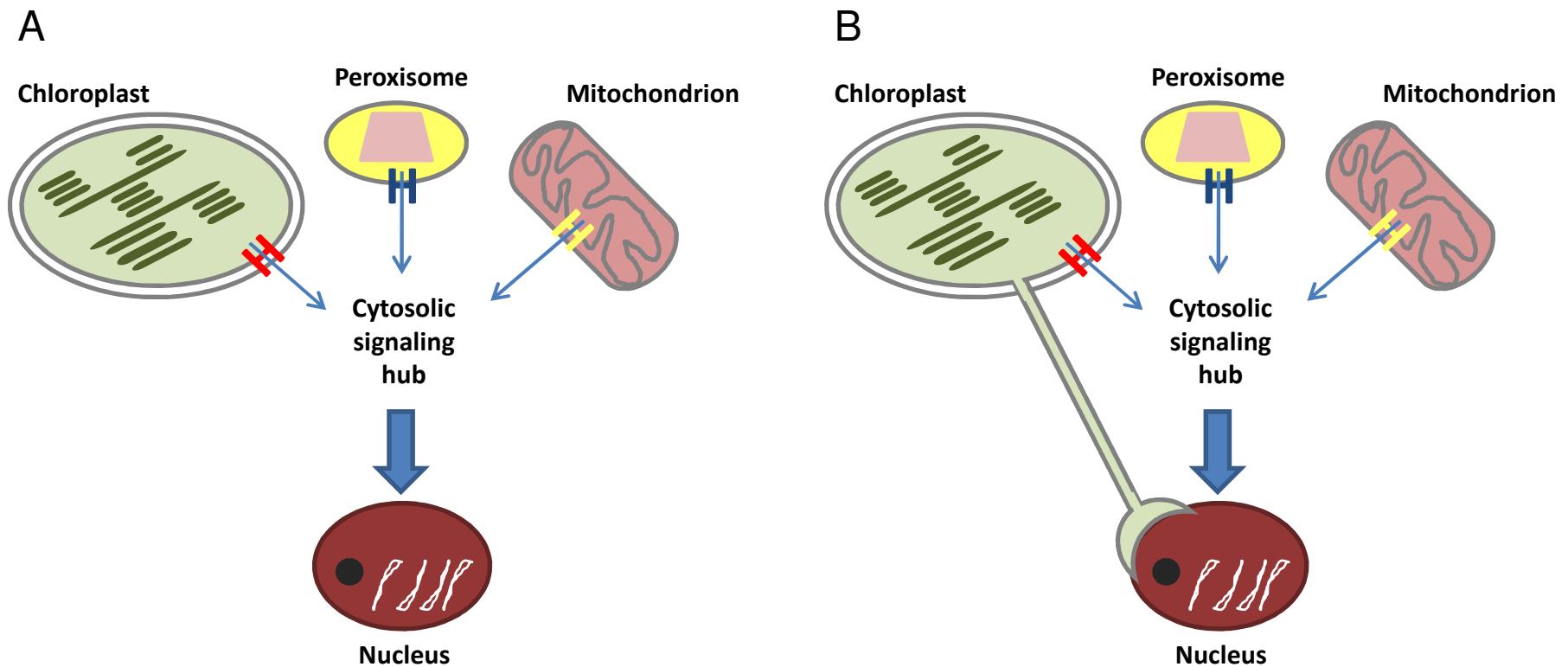
**Figure 1.** Compartment-specific signals due to the presence or absence of an antioxidant signaling component that interacts with H<sub>2</sub>O<sub>2</sub>. The cartoon shows a hypothetical example in which the compartment on the left has two modes of H<sub>2</sub>O<sub>2</sub> removal, one of which does not lead to signaling (heme-based enzyme such as APX or catalase). The other mode involves a thiol-based enzyme in which oxidation alters the association kinetics with its interactor (yellow box) so that the component is free to bind to a different interactor. The compartment on the right lacks this signaling mechanism. Such specificities could explain differences in ROS sensitivity between different compartments or between the same compartment under different conditions. The figure shows a simple example based on a thiol-disulfide exchange mechanism but other reactions may be important, and the difference between compartments could be quantitative rather than all-or-nothing.



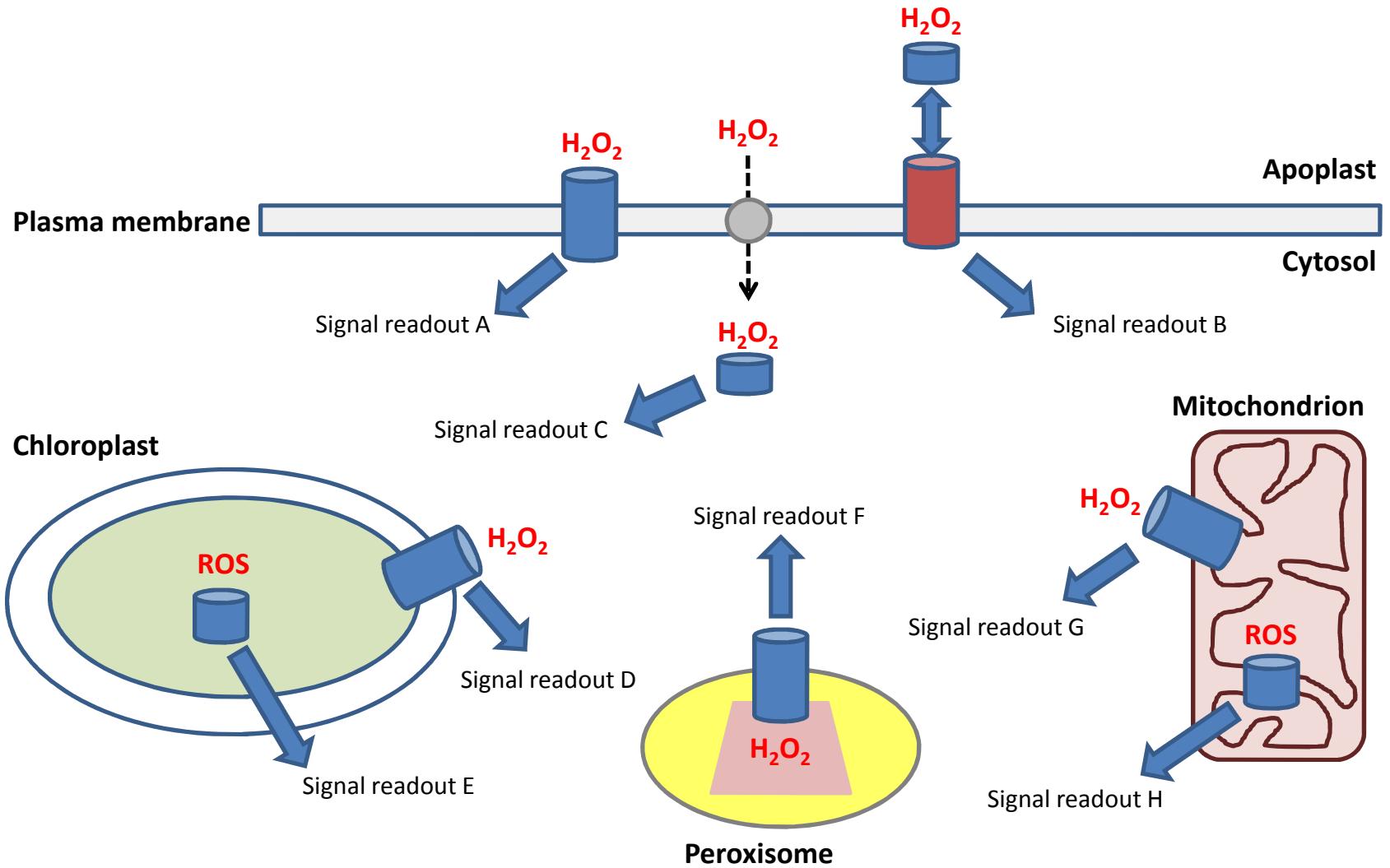
**Figure 2.** Transporters linked to ascorbate, glutathione and redox exchange in the chloroplast and mitochondria and on the plasma membrane. Yellow, ascorbate transporters. Gray, aquaporin or other  $\text{H}_2\text{O}_2$  transporter. Blue, glutathione and glutathione precursor transporter. Orange, dicarboxylate transporter. Question marks indicate transporters that are as yet uncharacterized at the molecular level. ASC, ascorbate. DHA, dehydroascorbate.  $\gamma$ -EC,  $\gamma$ -glutamylcysteine. Mal, malate. OAA, oxaloacetate.



**Figure 3.** Organellar redox signaling via *in situ* sensing and signal export through specific pathways linked to activation of receptors located on the outer organelle membranes. MAPK, mitogen-activated protein kinase.



**Figure 4.** The cytosol: integrator hub or bypassed onlooker? A, Classical transporter functions allow chloroplast, peroxisomes and mitochondria to feed information into cytosolic hubs that transmit the signal to the nucleus. B, Feeding information to the nucleus through organellar extensions. The scheme shows a stromule relaying stromal status to the nucleus but analogous extensions are possible from the peroxisomes (peroxules) and mitochondria (matrixules).



**Figure 5.** Multiple receptors located at different sites allow identification and integration of signals. The blue barrel shapes indicate the various ROS receptors that may be quite diverse in chemical nature and that may each give rise to a characteristic signaling cascade. The figure shows one receptor at each of the following locations: soluble apoplast, plasma membrane, cytosol, chloroplast envelope, peroxisome, mitochondrial, mitochondrial membrane. However, each location may have more than one type of receptor and there may be intricate interplay between them. In this way, the ROS language could be rich in vocabulary with a well-defined grammar.